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Food uses of enzyme-modified starches

PhD Thesis by
Michael Riis Hansen

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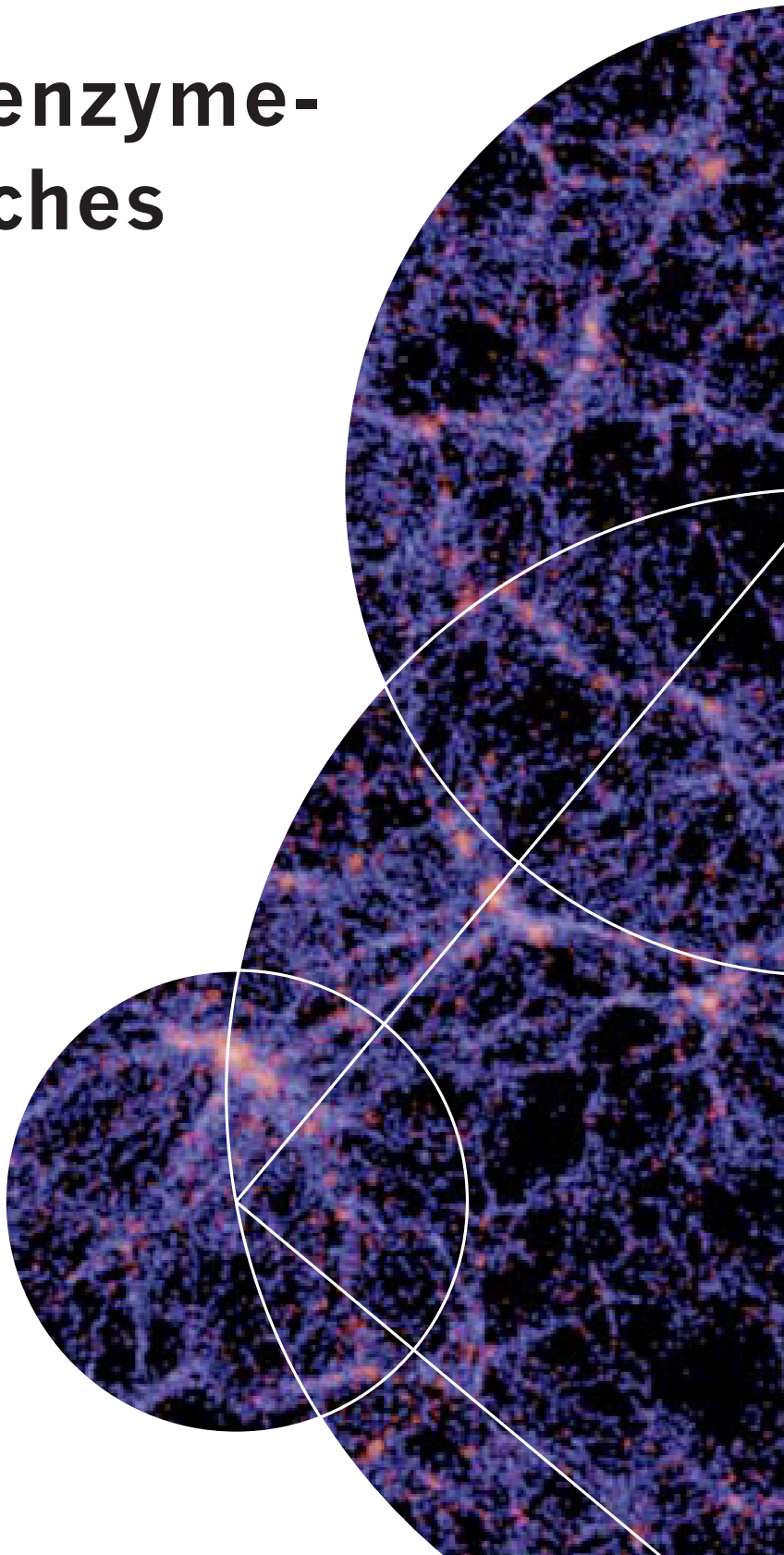

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Preface

This thesis is part of the requirement for obtaining a PhD degree at University of Copenhagen. It summarizes my work carried out at Novozymes, VKR Research Center Pro-Active Plants and Quality & Technology, Department of Food Science at the Faculty of Life Sciences, University of Copenhagen under the supervision of Andreas Blennow, Sven Pedersen and Søren B. Engelsen. The majority of the work has been carried out at the Faculty of Life Sciences.

The study was funded by the FOOD Graduate School, The Royal Veterinary and Agricultural University (now University of Copenhagen) and Novozymes A/S, each contributing ⅓ of the PhD scholarship.

I would like to thank Andreas Blennow, Sven Pedersen and Søren B. Engelsen for excellent supervision, as well as Niels, Gilda and Lars Nørgaard and the Q&T and Novozymes people for their support.

Michael Riis Hansen
November 2008

Summary

Replacing gelatin (and other unwanted/hazardous gel formers such as pectin and gum arabic) with a starch component is a long sought-after prize. In many food applications, native starch has an inadequate number of uses (e.g. thickener or binder). The most common way to improve starch functionality today is by chemical modification, which is widely used in the food industry. Improving starch functionalities by *in planta* modification certainly has great potential, but lacks the ability to produce similar or higher starch yield than the equivalent crop producing normal starch. Today's food market demands more natural and public appealing food components that involve avoiding chemical treatment, genetically modified crops and potential health-risk components.

The aim of this PhD study was to generate gelatin-like starch gel functionality by enzyme modification. Starches from various botanical sources with different functionnality were selected: potato, high-amylose potato (HAP), maize, waxy maize, wheat and pea starch. A chemically oxidized potato starch (Gelamyl 120) was also included. In total, 51 enzyme-modified starches were produced using amylomaltase (AM) (4-α-glucanotransferase; E.C. 2.4.1.25) from the 7 parent starches. AM modification of starch results in gels with acquired thermoreversible character (van der Maarel, Capron, Euverink, Bos, Kaper, Binnema, and Steeneken, 2005), which is an important property in many food products. In addition to AM, branching enzyme (BE) (1,4-α-D-glucan branching enzyme; EC 2.4.1.18) was used in combination with AM in one time-course modification. BE has potential use in the baking industry as an anti-staling agent (Spendler and Jørgensen, 1997; Fuertes and Petitjean, 2006). Today, the majority of currently used industrial starch-acting enzymes are hydrolases (Kirk, Borchert, and Fuglsang, 2002; van der Maarel, van der Veen, Uitdehaag, Leemhuis, and Dijkhuizen, 2002). Common to AM and BE is that they are transferases, which when modifying starch results in chain transfer and thereby retaining a relatively high molecular weight. AM catalyzes the transfer of a α-1,4-glucan segment to a new 4-position in a disproportionation reaction, whereas BE transfers a segment of α-1,4 glucan to a 6-position.

Amylopectin chain length (CL) distribution, textural properties, molecular mobility and melting behavior of gels were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), texture analyzer, low-field ^{1}H nuclear magnetic resonance (LF NMR) and differential scanning calorimetry (DSC), respectively, and compared with gelatin gels (Papers I, II and III). Modifying starch with AM caused broadening of the amylopectin CL distribution, creating a unimodal distribution with an increased fraction of long chains (degree of polymerization (DP) > 35) and very short chains (DP 4-5) and decreased proportion of short chains (DP 13-23) (Paper I). The broadening effect was independent of the starch type being AM-modified. Extended AM treatments reduced the apparent molecular weight (Mw) significantly, but had minor effect on the amylopectin CL distribution. A considerable decrease in long chains and increase in short chains was found for pea starches being subjected to combined AM/BE modification (Paper I).

Using exploratory principal component analysis (PCA) and multivariate curve resolution (MCR) data analysis of the entire amylopectin CL distribution dataset revealed that the data was composed of two and three components explaining 94.2% and 94.0% of the total variation, respectively. Three types of amylopectin CL profiles were identified using MCR: parent, AM-modified and combined AM/BE starch system. Parent starches and each time-course modification were separated by PCA (Paper I). The final amylopectin mean CL depends on the starch material being subject to AM modification, but irrespective of the amylose content.

AM modification of starch resulted in products having increased gel texture and gel peak temperature (T_p), and decreased spin-spin relaxation time (T_2) constants, whereas combined AM/BE modification of pea starch had the opposite effect (Papers I, II and III). The effects were all found to be attributed to the increase of long chains (DP 60-80) in amylopectin. A weak correlation ($r^2 = 0.66$) was found between sum of DP 60-80 and the gel texture. A high correlations ($r^2 = 0.82$ and $r^2 = 0.85$) were found between sum of DP 60-80 and the LF NMR relaxation curves of Day 10 and the gel peak temperature (T_p), respectively.

During initial 1 sec gel compression, 5% (w/w) AM-modified HAP and potato starch gels were comparable to 5% (w/w) gelatin gel. However, the texture profiles (8.4 sec compression and 2.6 sec retraction) of all AM-modified starch gels were clearly very different from gelatin gels. Gelatin made highly elastic transparent gels, whereas AM-modified starch gels made opaque gels and fractured after 1-2 sec compression. AM modification of starch resulted, in addition to improved gel texture, in significantly altered gel-melting behavior and molecular mobility that were very different from gelatin (Papers I, II and III). E.g. modification of potato starch decreased T_2 from 461 to 88 ms after Day 10 of storage and increased T_p from 65 to 74ºC, which is in contrast to gelatin (T_2 = 420 ms and T_p = 27ºC). Combined AM/BE modification of pea starch resulted in products with increased T_2 times and decreased T_p, but also resulted in pastes unable to form gel network at a concentration of 12% (w/w) due to the decreased fraction of long chains in amylopectin (Papers I, II and III).

Generating an enzyme-modified starch with AM that matches the complete functional spectrum of gelatin is currently not feasable. AM-modified starches could be used in certain food applications as texturizer and where transparency is not required.

Sammendrag

Erstatning af gelatine (og andre uønskede hydrokolloider som pektin og gum arabic) med en stivelseskomponent har længe været efterspurgt. I mange fødevare applikationer er anvendelse af naturlig stivelse utilstrækkelig (f.eks. som fortykningsmiddel eller binder). Den mest anvendte måde at forbedre funktionaliteten af stivelse i dag er ved kemisk modificering, som er meget udbredt i fødevareindustrien. Forbedring af funktionaliteten af stivelse ved *in planta* modificering har stort potentiale, men GMO-afgrøder evner ikke at give lignende eller højere stivelsesudbytte i forhold til den naturlige afgrøde. I dag efterspørges naturlige og 'forbruger indbydende' komponenter i fødevareindustrien, hvilket indebærer at undgå kemiske behandlinger, genetisk modificerede afgrøder og potentielle sundheds-farlige komponenter.

Formålet med dette ph.d. studium var at producere en stivelse med gelatine-lignende funktionaliteter vha. enzymatisk modificering. Stivelser med en række forskellige funktionaliteter blev udvalgt: kartoffel-, høj-amylose kartoffel- (HAP), majs-, waxy majs-, hvede- og ærtestivelse. En kemisk oxideret kartoffelstivelse (Gelamyl 120) blev også inkluderet. I alt blev der produceret 51 enzym-modificeret stivelser med amylomaltase (AM) (4-α-glucanotransferase; E.C. 2.4.1.25) ud fra de 7 naturlige (parent) stivelser. Modificering af stivelse med AM producerer geler med thermoreversible egenskaber (van der Maarel *et al.*, 2005), som er vigtige for mange fødevareprodukter. Ud over AM, blev et forgreningsenzym, BE (1,4-α-D-glucan branching enzyme; EC 2.4.1.18), anvendt i kombination med AM i en time-course modificering. BE har et potentiale i bage industrien som et anti-staling middel (Spendler *et al.*, 1997; Fuertes *et al.*, 2006). I dag er det hovedsageligt hydrolase enzymer som anvendes i stivelsesindustrien (Kirk *et al.*, 2002; van der Maarel *et al.*, 2002). Fælles for AM og BE er, at de er transferaser, som ved modificering af stivelse resulterer i kædeoverførelse, som derved bevarer en relativ høj molekylevægt. AM katalyserer overførelsen af α-1,4-glucan kæder til en ny 4-position ved en disproportionation reaktion, mens BE overfører kæder af α-1,4-glucan til en 6-position.

Amylopektin kædelængde (CL) distribution, geltekstur egenskaber, molekylær mobilitet og smelte egenskaber af geler blev analyseret henholdsvis ved high performance anion exchange chromatography med pulsed amperometric detection (HPAEC-PAD), texture analyzer, low-field ^{1}H nuclear magnetic resonance (LF NMR) og differential scanning calorimetry (DSC), og sammenlignet med gelatine geler (Publikationer I, II og III). Modificering af stivelse med AM forårsagede en bredere amylopektin CL distribution, og resulterede i en unimodal distribution med større andel af lange kæder (DP > 35) og meget korte kæder (DP 4-5) og mindre andel af korte kæder (DP 13-23) (Publikation I). Denne effekt var uafhængigt af stivelsestype. Længerevarende AM-modificering reducerede den tilsyneladende molekylevægt betydeligt, men havde kun mindre påvirkning på amylopektin CL distributionen. Kombineret AM/BE-modificering af ærtestivelse resulterede i en betragtelig reduktion af lange kæder og større andel af korte kæder (Publikation I).

Ved eksplorativ principal component analysis (PCA) og multivariate curve resolution (MCR) dataanalyse af amylopektin CL distribution for alle prøverne viste det sig, at data var sammensat af henholdsvis to og tre komponenter, som forklarede 94.2% og 94.0% af den totale variation. Tre typer amylopektin CL profiler blev identificeret ved MCR: 1) parent, 2) AM-modificeret og 3) kombineret AM/BE-modificeret stivelse. Parent stivelser og hver enkelt time-course modifikation blev separeret ved PCA (Publikation I). Den resulterende gennemsnitlige amylopektin CL er afhængig af stivelsestype som AM-modificeres, men uafhængigt af amylose indhold.

AM-modificering af stivelse resulterede i produkter som har forøget geltekstur og gel peak temperatur (T_p) og lavere spin-spin relaksationtidskonstanter (T_2), mens kombineret AM/BE-modificering af ærtestivelse havde den fuldstændig modsatte effekt (Publikationer I, II og III). Dette kunne i alle tilfælde tillægges den forøgede fraktion af lange kæder (DP 60-80) i amylopektin. En svag korrelation ($r^2 = 0.66$) blev fundet mellem DP 60-80 og gelteksturen. Gode korrelationer ($r^2 = 0.82$ og $r^2 = 0.85$) blev fundet mellem DP 60-80 og henholdsvis LF NMR relaksationkurverne fra Dag 10 og gel peak temperatur (T_p).

Geler af 5% (w/w) AM-modificeret HAP- og kartoffelstivelse havde sammenlignelig tekstur med 5% (w/w) gelatine inden for det første sekund gelkompression. Teksturprofilerne (8.4 sek kompression og 2.6 sek tilbagetrækning) for alle AM-modificerede stivelsesgeler var markant forskellige fra gelatinegeler. Gelatine lavede meget elastiske klare geler, mens AM-modificerede stivelsegeler alle lavede uklare geler, der brudte efter 1-2 sek kompression. AM-modificering af stivelse resulterede i, ud over en forbedret geltekstur, en betydelig ændret gel-smeltning profil og molekylær mobilitet, som var meget forskellige fra gelatine (Publikationer I, II og III). F.eks. modifikation af kartoffelstivelse reducerede T_2 fra 461 til 88 msek efter Dag 10 opbevaring og forøget T_p fra 65 til 74ºC, som er i modsætning til gelatine ($T_2 = 420$ msek og $T_p = 27$ºC). Kombineret AM/BE-modificering af ærtestivelse resulterede i produkter med forøget T_2 tider og reduceret T_p, men resulterer også i manglende evne til at danne geler ved 12% (w/w), hvilket skyldes den reducerede fraktion af lange kæder i amylopektin (Publikationer I, II og III).

Frembringelse af en enzym-modificeret stivelse med AM, som har tilegnet sig alle gelatines funktionaliteter er pt ikke muligt. AM-modificerede stivelser kan bruges i bestemte fødevare applikationer som 'texturizer', hvor gennemsigtighed ikke er påkrævet.

Publications

Paper I
Hansen, M.R., Blennow, A., Pedersen, S., Nørgaard, L., Engelsen, S.B., 2008.
Gel texture and chain structure of amylomaltase-modified starches compared to gelatin.
Food Hydrocolloids 22, 1551-1566.

Paper II
Hansen, M.R., Blennow, A., Pedersen, S., Nørgaard, L., Engelsen, S.B.
Comparative NMR relaxometry of gels of amylomaltase-modified starch and gelatin.
Submitted.

Paper III
Hansen, M.R., Blennow, A., Pedersen, S., Engelsen, S.B.
Enzyme modification of starch with amylomaltase results in increasing gel melting point.
Submitted.

Abbreviations

AM	Amylomaltase
BE	Branching enzyme
BSE	Bovine spongiform encephalopathy
CGTase	Cyclodextrin glycosyltransferase
CL	Chain length
CPMG	Carr-Purcell-Meiboom-Gill
DBE	Debranching enzyme
DE	Dextrose equivalent
DSC	Differential scanning calorimetry
ΔH	Enthalpy of transition
D-enzyme	Disproportionating enzyme
DP	Degree of polymerization
DSC	Differential scanning calorimetry
EC number	Enzyme commission number
GBSS	Granule-bound starch synthase
Gelamyl 120	Oxidized potato starch
GH	Glycoside hydrolase
GMO	Genetically modified organism
GWD	α-glucan water dikinase
HAP	High-amylose potato
HPAEC	High-performance anion exchange chromatography
LF NMR	Low-field ^{1}H nuclear magnetic resonance
MALLS	Multi angle laser light scattering
MCR	Multivariate curve resolution
Mw	Molecular weight
PAD	Pulsed amperometric detection
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PLS	Partial least squares
PWD	Phosphoglucan water dikinase
RVA	Rapid visco analyzer
RMSECV	Root mean square error of cross-validation
RI	Refractive index
SEC	Size exclusion chromatography
T_2	Spin-spin relaxation time constant
T_p	Gel peak temperature
TA	Texture analyzer

Table of Contents

1. Introduction

Starch is a major component of the human diet, and is after cellulose the most abundant polysaccharide. It is an α-glucose polymer composed of linear amylose and highly branched amylopectin generally present in the ratio of 1:4, respectively (Tester, Karkalas, and Qi, 2004a). Starch contributes significantly as a texturizer, thickener and stabilizer of processed foods. Native starches have several distinctive properties, but lack the flexibility of today's food industry that requires starch to have improved viscosity, temperature stability, freeze-thaw and storage stabilities, improved gelling properties, reduced (or increased) digestibility, and to mimic properties of gelatin and fats. Improving and introducing new functionality in starch have traditionally been attained by means of physical and chemical modifications.

One of the first starch modification reports dates back to 1811, when the German scientist Kirchhoff published a method of acid hydrolysis in starch producing sweet-tasting syrup. Today, the production of starch syrup by the acid hydrolysis method is largely replaced by starch-hydrolyzing enzymes (such as α-amylase, glucoamylase, pullulanase and β-amylase), because they perform more specific reactions with fewer by-products and give higher product yields. This is just one application in which enzymes have 'marched into' the food industry and replaced the use of chemicals. In general, the use of enzymes in a range of diverse industries has been growing, thanks to recombinant gene and protein design/redesign technology (Kirk *et al.*, 2002; Jackel, Kast, and Hilvert, 2008).

Recently, it has been shown that modifying potato starch with amylomaltase (AM) (4-α-glucanotransferase; E.C. 2.4.1.25) results in starches acquiring thermoreversible character (van der Maarel *et al.*, 2005) and for that reason has potential applications in the food industry as a substitute for gelatin (Euverink and Binnema, 2005). Gelatin, an animal protein, is one of many hydrocolloids used in the food industry. Starch too is classified as a hydrocolloid, which is a colloidal substance where the material is dispersed in water. Additional known hydrocolloids used in the food industry are carrageenan (isolated from red seaweed), pectin (obtained from citrus peel) and gum arabic (extracted from tree gum). There is a huge industrial interest in substituting these more expensive and potentially hazardous hydrocolloids with a starch component.

1.1. Project description

The objective of the present PhD project was to test whether it is possible to emulate the functionalities of gelatin by enzyme modifying various starches such as potato, high-amylose potato, maize, wheat and pea starch. Two enzymes were used in this project: AM from *Thermus thermophilus* for production of thermoreversible gels (van der Maarel *et al.*, 2005; Euverink *et al.*, 2005) and to some extent branching enzyme (BE) (1,4-α-D-glucan branching enzyme; EC 2.4.1.18) from *Rhodothermus obamensis* for production of starches with high solubility (Shinohara, Ihara, Abo, Hashida, Takagi,

and Beck, 2001; Imanaka, Terada, Takaha, Yanase, Okada, Takata, Nakamura, and Fujii, 2001). Modification of starch with these two enzymes will result in starches with new or improved functionality as an effect of altered starch structure. Understanding the relationship between starch structure and functionally of the enzyme-modified starches will in addition be examined. Chemometric analysis was expected to be a key tool in data exploration and elucidating possible correlations.

2. Starch

2.1. Introduction

Starch is, after cellulose, the most abundant carbohydrate in nature. It is an important energy reserve in plants and is the most important energy source in the human diet. Besides being widely used in the food industry, starch is also used in the pharmaceutical, textile, paper and renewable energy industries.

Starch is readily isolated from cereal seeds (e.g. maize, wheat, rice, barley), tuberous root (e.g. potato, cassava) and legumes (e.g. pea, mung bean). The world production of starch in 2005 has been estimated to be nearly 60 million tons (Fig. 1). Maize is the most important source of starch isolation constituting about 81% of the world production, whereas wheat, rice, potato, and tapioca make up a minor portion of the total. Most of the maize starch is produced in the USA, while Europe is the major producer of starch from wheat and potato.

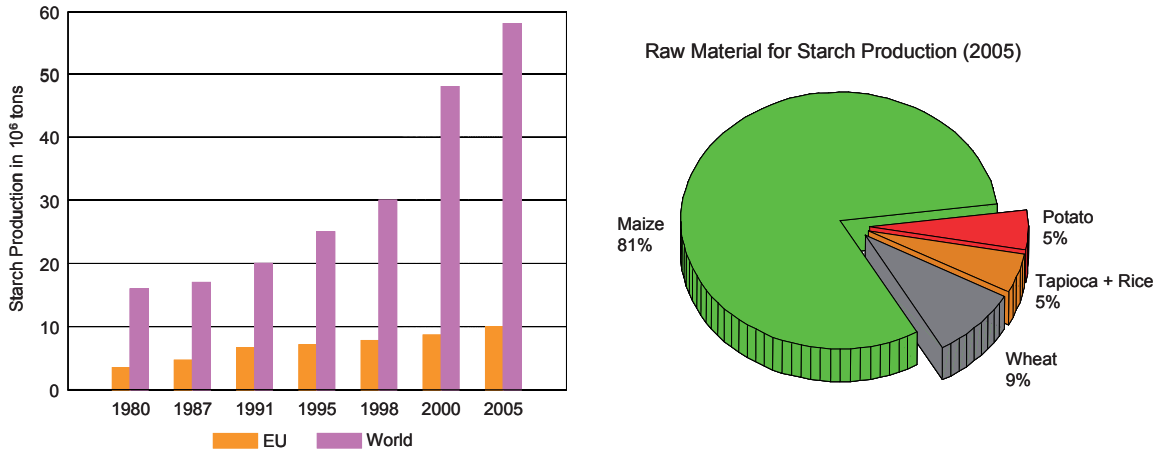


Fig. 1. Starch production. Left: Starch production in the EU and world. Right: The world production of starch by botanical origin in 2005. Source: Food and Agriculture Organization of the United Nations.

Starch is deposited as semicrystalline granules that are cold-water insoluble (Fig. 2). Variation in the granule size, shape and chemical composition reflect the botanical origin (Gallant, Bouchet, and Baldwin, 1997).

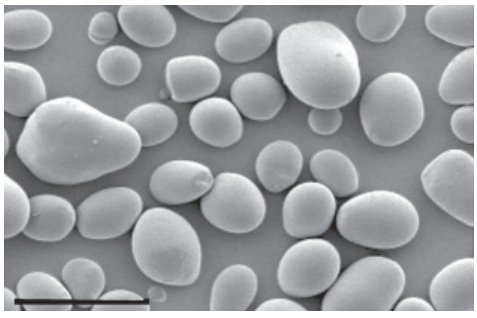


Fig. 2. Starch granules from potato. Scale bar is two µm. From Bustos, Fahy, Hylton, Seale, Nebane, Edwards, Martin, and Smith, 2004.

2.2. Starch composition and structure

2.2.1. Amylopectin and amylose

Starch granule consists of two α-glucan polymers, amylose and amylopectin. Both polymers are based on chains of α-1,4 linked glucose (Fig. 3). Amylose is essentially a linear molecule with typical molecular weight (Mw) in the range of 10^5 to 10^6 Dalton, whereas amylopectin in addition to linear chains of α-1,4 linked glucose also contains α-1,6 linked branch points for every 20-25 straight chain residues. The Mw of amylopectin is in the region of 10^8 Dalton (Parker and Ring, 2001).

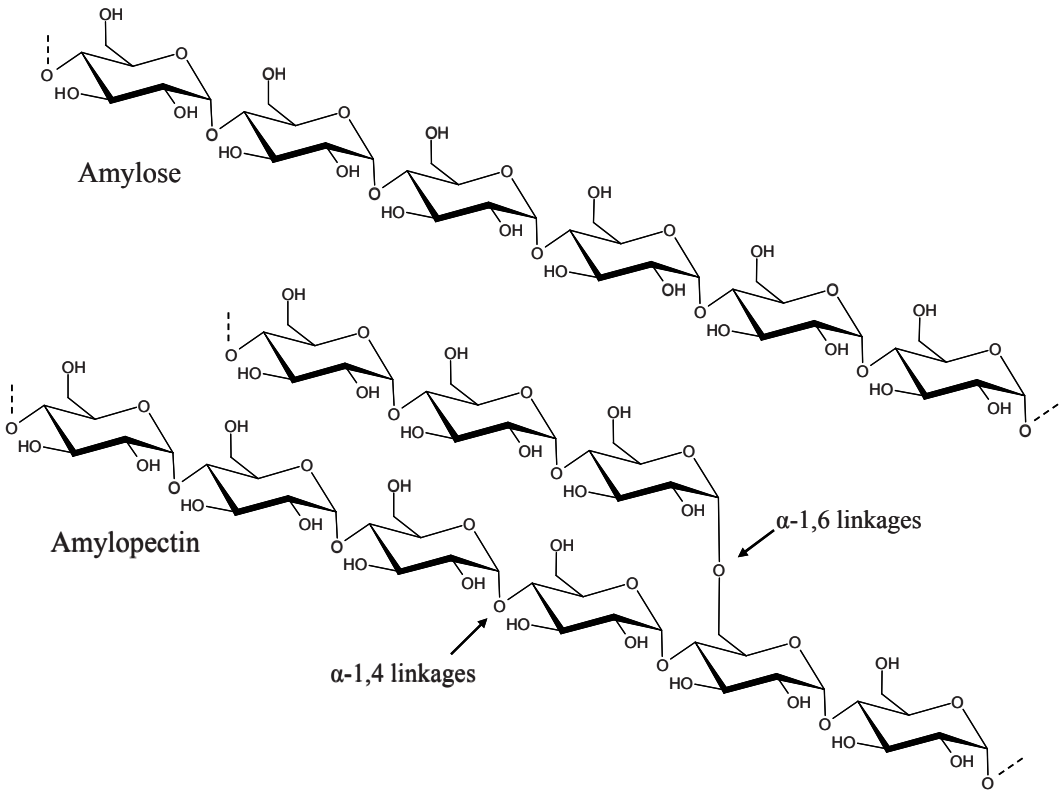


Fig. 3. Structure of amylose and amylopectin.

The molecular structure of amylopectin and amylose is related to starch functionality (Fredriksson, Silverio, Andersson, Eliasson, and Aman, 1998). The amylose/amylopectin ratio is a major factor controlling starch functionalities such as viscosity, shear resistance, gelatinization, textures, solubility, gel stability, cold swelling and retrogradation (Fredriksson *et al.*, 1998).

Most natural starches contain about 20-30% amylose, but vary according to the botanical origin (Fredriksson *et al.*, 1998; Jane, Chen, Lee, McPherson, Wong, Radosavljevic, and Kasemsuwan, 1999). In a genetic variety of maize where granule-bound starch synthase (GBSS) activity is deficient, starch granules contain essentially 100% amylopectin and are for that reason amylose-free (Shure, Wessler, and Fedoroff, 1983). Amylose is synthesized by GBSS. The mutant maize is referred to as *waxy* starch, because of its waxy appearance of sliced maize kernel. Waxy maize starch

forms clear paste upon gelatinization, which is in contrast to normal maize starch (contains ~25% amylose) that forms opaque paste (Ellis, Cochrane, Dale, Duffus, Lynn, Morrison, Prentice, Swanston, and Tiller, 1998). The lack of amylose in waxy starches has certain advantages for the food industry, as they have improved freeze-thaw stability compared to normal starches (Zheng and Sosulski, 1998; Jobling, Westcott, Tayal, Jeffcoat, and Schwall, 2002). Today, several waxy mutants from various botanical origins such as potato (Visser, Somhorst, Kuipers, Ruys, Feenstra, and Jacobsen, 1991) and wheat (Nakamura, Yamamori, Hirano, Hidaka, and Nagamine, 1995) are known, but only waxy maize is grown on a commercial scale. In the food industry, waxy starches are used as stabilizers, thickeners and emulsifiers.

In contrast to waxy starches, which are amylose-free, it is a considerably more complicated task to produce amylopectin-free starches, because the synthesis of amylopectin requires the combined action of numerous enzymes such as soluble starch synthase (SSS), branching enzyme (BE) and debranching enzyme (DBE), each of which has multiple isoforms (Ball and Morell, 2003). However, simultaneous inhibition of two starch-branching enzymes, SBE1 and SBE2, in potato results in a so-called high-amylose potato starch with amylose contents above 60% (Schwall, Safford, Westcott, Jeffcoat, Tayal, Shi, Gidley, and Jobling, 2000). The increased amylose content makes them useful in gelled products. High-amylose starches are more resistant to enzyme digestion in the small intestine and can therefore be classified as dietary fibers, which have both health and nutritional benefits (Sajilata, Singhal, and Kulkarni, 2006). Drawbacks included higher process temperature and considerably lower yield compared to the corresponding normal starch. Starches completely lacking amylopectin have not yet been genetically produced, only high-amylose starches that contains higher amylose/amylopectin ratio.

Amylose is depicted as a straight chain structure in Fig. 3. In fact, amylose is often helical. The stability and clarity of dissolved amylose is low due to association of linear molecules through H-bonding formation, resulting in firm and opaque gels upon cooling. Amylose is capable of forming complexes with certain components such as iodine and some alcohols. The formation of amylose-iodine complex causes an intense blue color, which is in contrast to amylopectin that gives a reddish-purple color. This enables the determination of the apparent amylose content spectrophotometrically in starch, and it has been used for many years (know as iodometric method) (Hovenkamp-Hermelink, Devries, Adamse, Jacobsen, Witholt, and Feenstra, 1988). In addition to the iodometric method, amylose content can be estimated by size exclusion chromatography with refractive index (SEC/RI) detection (Blennow, Bay-Smidt, and Bauer, 2001;Chen and Bergman, 2007). Amylose is also well known for forming inclusion complexes with fatty acids due to its hydrophobic inner core of the helical structure (Biliaderis, Page, Slade, and Sirett, 1985). The amylose-lipid formation influences starch functionality such as reduced swelling and lower retrogradation (Singh, Singh, Kaur, Sodhi, and Gill, 2003). The determination of amylose content by iodometric

method is highly influenced by this amylose-lipid complex, and starches need extensive washing prior to analysis.

2.2.2. Minor constituents of the starch granule

Isolated starch contains in addition to amylose and amylopectin, about 10-20% moisture, small quantities of protein (0.05-0.5%), lipid (0.1-1.7%), non-carbohydrates (0.5-2%) and ash (0.1-0.3%). The content of these components varies considerably with the botanical source of the starch. Cereal starches in general have more lipid and protein than potato starch. Both have the potential to moderate starch functionality (Appelqvist and Debet, 1997; Singh *et al.*, 2003).

Starch is slightly phosphorylated, which is believed to play a central role in starch metabolism (Blennow, Engelsen, Nielsen, Baunsgaard, and Mikkelsen, 2002). It is the only naturally occurring covalent modification of starch that is catalyzed by the ATP-dependent α-glucan water dikinase (GWD) (Ritte, Lloyd, Eckermann, Rottmann, Kossmann, and Steup, 2002) and phosphoglucan water dikinase (PWD) (Kotting, Pusch, Tiessen, Geigenberger, Steup, and Ritte, 2005). Phosphate esters substituted mostly at C-6 and C-3 positions of the glycosyl unit of the amylopectin component. However, about 70% of the esterified phosphate content is found at the C-6 position (Tabata and Hizukuri, 1971). Amylose is virtually free of phosphate. The amount of starch-bound phosphate is basically undetectable or present in only little amounts in cereal starches, whereas starch from potato tubers is highly phosphorylated (0.2-0.5% w/w) (Blennow *et al.*, 2002). Even though only attached to 1 in every 215-560 glucose units, it has been shown that thermal and rheological properties of potato starch are related to the phosphorus content (Kim, Wiesenborn, Orr, and Grant, 1995; Hopkins and Gormley, 2000). Antisense inhibition of the GDW enzyme resulted in a starch with severely reduced phosphate content and changed amylopectin structure (Blennow, Hansen, Schulz, Jorgensen, Donald, and Sanderson, 2003). A high correlation between the phosphate content and the distribution of chain length (CL) in amylopectin has been found to exist (Blennow, Bay-Smidt, Wischmann, Olsen, and Moller, 1998; Blennow, Engelsen, Munck, and Moller, 2000; Blennow, Wischmann, Houborg, Ahmt, Jorgensen, Engelsen, Bandsholm, and Poulsen, 2005). Short chain with the degree of polymerization (DP) of 12-13 and medium chain with 28-42 was found to be negatively and positively correlated to phosphate content, respectively (Blennow *et al.*, 2005).

Some of the properties of amylose and amylopectin are summarized in Table 1.

Table 1. Summary of some amylose and amylopectin properties.

Property	**Amylose**	**Amylopectin**
Molecular structure	Essential linear	Branched (~5%)
Linkage	α-1,4	α-1,4 and α-1,6

Mw	10^5 to 10^6 Dalton	10^8 Dalton
Dilute aqueous solution	Unstable	Stable
Gels	Firm	Soft
Solution clarity	Opaque	Clear
Complex formation	Yes	Poor
Iodine color	Blue	Reddish-purple
Phosphorylated	No	Yes (mainly tuber and root starch)

2.3. Amylopectin structure

In amylopectin, short chains are clustered together, and the units of clusters are interconnected by longer chains (Manners and Matheson, 1981; Hizukuri, 1986). The model of amylopectin structure is illustrated in Fig. 4. The individual chains can be classified in terms of *A*, *B* and *C* chains, based on their CL and how the chains are linked to the rest of the molecule. According to Peat *et al*., *C* chain carries the only reducing end of the amylopectin molecule (Peat, Whelan, and Thomas, 1952). *A* chains are unsubstituted, whereas *B* chains are substituted by other *B* chains or *A* chains. Both *A* and *B* chains can be linked to a *C* chain.

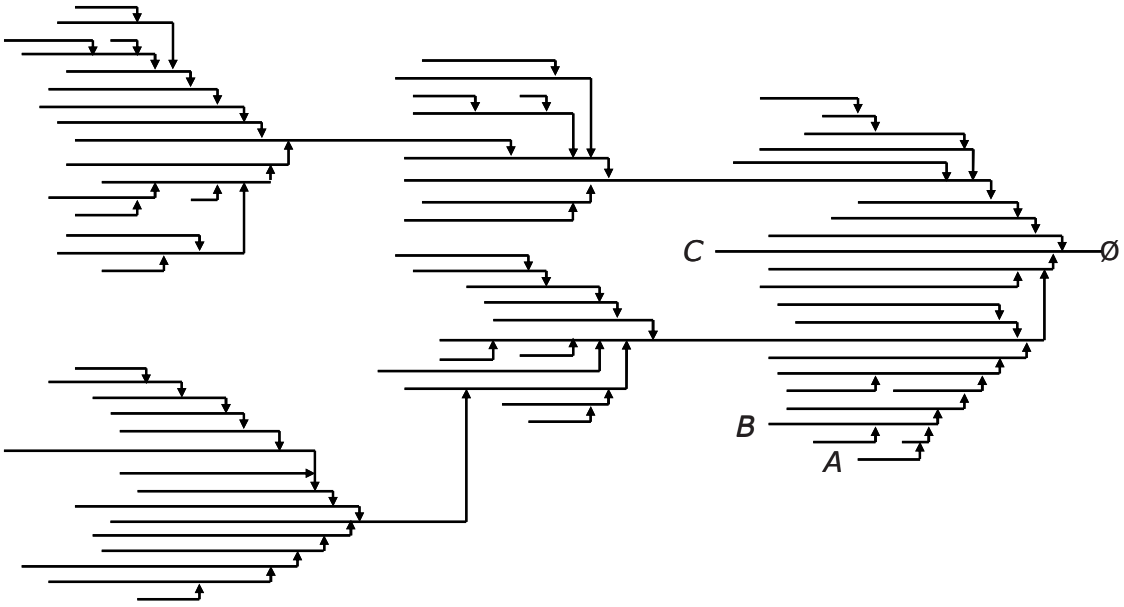


Fig. 4. Cluster model of amylopectin. The linear lines indicate α-1,4-glucan segments, and vertical arrows within the structure indicate α-1,6 glycosidic linkage. Ø: non-reducing end; *A*, *B* and *C* chain are indicated. The cluster model is adapted from Hizukuri, 1986.

The branch point in amylopectin are not randomly distributed (Thompson, 2000). Amylopectin chains are moderately short compared to amylose molecules, having a broad distribution profile. The structure is dependent on botanical origin of the starch. On average, amylopectin chains are 25-35 units long.

A distribution of the amylopectin CL can be obtained by debranching a starch sample with isoamylase followed by anion exchange chromatography (This method will be discussed in more detail in Section 5.1). Most native starch shows a bimodal amylopectin CL distribution usually having the main peak at approximately DP 15-20 with a shoulder around DP 20-25 and a minor peak at around DP 40-50. Examples of this are illustrated in Fig. 5 showing the amylopectin CL distribution up to DP of 80 for maize, wheat, potato and high-amylose potato (HAP) starches. The overall picture is that short chains (DP 10-25) are the most abundant fraction. Wheat starch contains considerably higher fraction of short chains, but lower fraction of long chains (DP 60-80) compared to potato starch. For the genetically engineered HAP starch (Blennow *et al.*, 2005), the bimodal appearance was almost lost, and contains higher fraction of long chains compared to its parent potato starch (Fig. 5 right). The altered amylopectin structure in HAP starch was accomplished by simultaneous antisense of SBE1 and SBE2 isoforms. The inhibition of both isoforms is important, as silencing of just SBE1 in potato has no effect on the amylopectin CL distribution (Safford, Jobling, Sidebottom, Westcott, Cooke, Tober, Strongitharm, Russell, and Gidley, 1998). The higher fraction of long chains for high-amylose starches compared to their parent starch is common (Yoshimoto, Tashiro, Takenouchi, and Takeda, 2000).

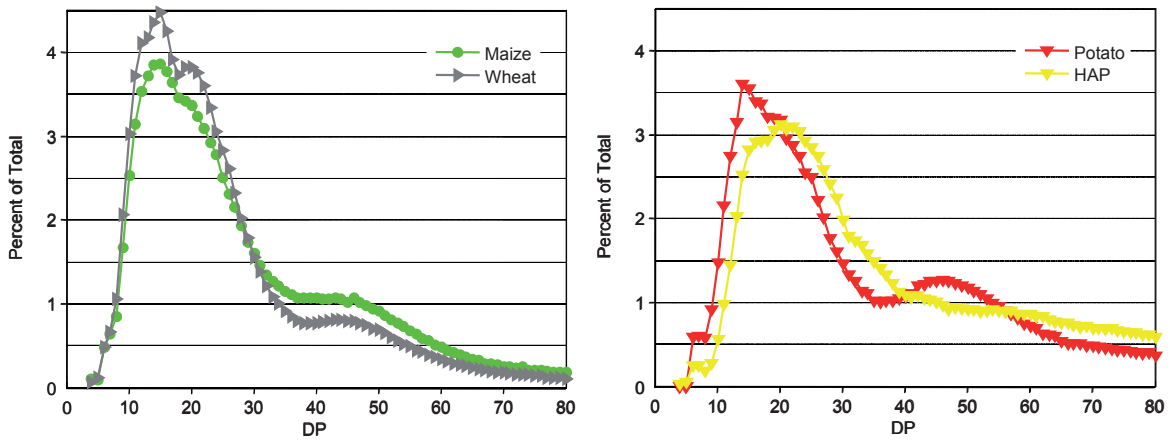


Fig. 5. Amylopectin CL distribution of parent maize and wheat starch (left) and parent potato and HAP starch (right). Presented in Paper I.

2.4. Starch granule and crystallinity

Starch granules are organized into semicrystalline and amorphous regions, which make starch insoluble in cold water. This is illustrated in Fig. 6. The semicrystalline regions are exclusively composed of amylopectin, whereas amylose is believed to be localized in both the amorphous and crystalline lamellae (Jacobs and Delcour, 1998; Koroteeva, Kiseleva, Krivandin, Shatalova, Blaszczak, Bertoft, Piyachomkwan, and Yuryev, 2007). The amorphous lamellae are believed to contain the branching linkages of amylopectin. The semicrystalline region consists of ordered regions composed of double helices formed by short amylopectin branches, most of which are further or-

dered into crystalline structures (the crystalline lamellae) (Imberty, Buleon, Tran, and Perez, 1991). The double helices are mainly formed by short chains (DP 10-25) that depend on the botanical source (Hizukuri, 1985; Li, Vasanthan, Rossnagel, and Hoover, 2001; Franco, Wong, Yoo, and Jane, 2002).

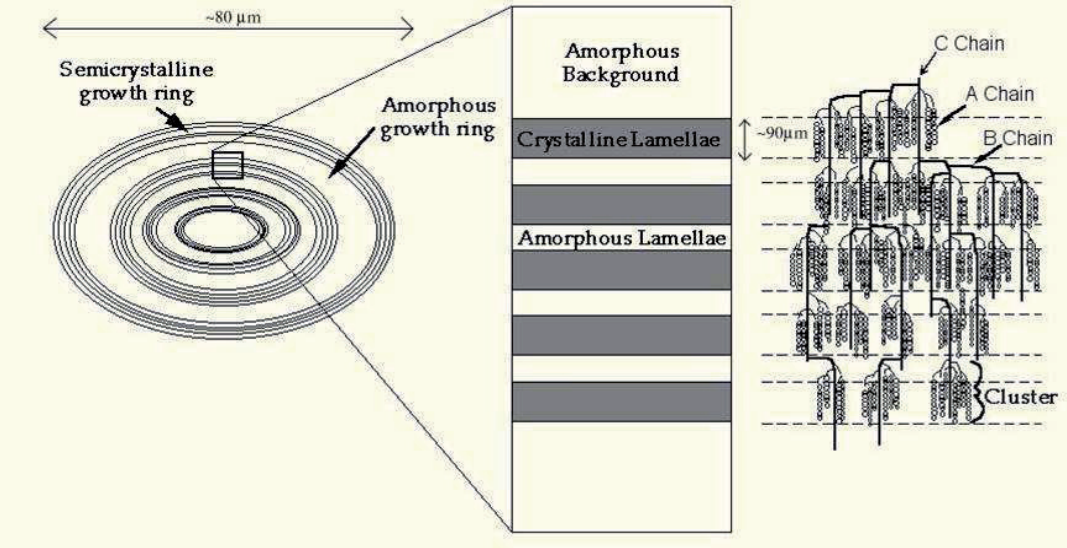


Fig. 6. The molecular organization of the starch granule. Left in the figure shows a single granule from Fig. 2 with alternating amorphous and crystalline lamellae forming 'growth rings'. Each crystalline lamellae is roughly 90 µm constituting the cluster structure of amylopectin. Adapted from Jenkins and Donald, 1995.

Three different semicrystalline structures were identified in native starch granule using x-ray analysis: A-, B- and C-type (Zobel, 1988). The A-type structure consists of closely packed double helices, whereas a more open helical packing containing water molecules is found for the B-type structure (Imberty, Chanzy, Perez, Buleon, and Tran, 1988). The A-type is characteristic of the cereal starches while the B-type is mostly found in tuberous starches. The C-type is a mixture of the A- and B-types and is found in various bean starches. In addition, amylose in gelatinized cereal starches form a V-type complex with the granule fatty acid (Zobel, 1988).

2.5. Gelatinization and pasting

The native granule of starch is usually not used in food application. As mentioned in previously Sections, starch granules are insoluble in cold water. However, when an aqueous suspension of starch is heated above a specific temperature, the granules undergo an irreversible swelling, resulting in melting of crystalline regions and double-helix unwinding, as described by Donald and co-workers using the "side-chain liquid-crystalline model" (Cooke and Gidley, 1992; Waigh, Gidley, Komanshek, and Donald, 2000). This process is termed gelatinization. After gelatinization, follow granule swelling many times its original size, loss of crystalline order, solubilization and development of viscosity. This process is termed pasting.

Rapid visco analyzer (RVA) is widely used for the analysis of starch gelatinization and pasting. The pasting profiles of a few selected starches are shown in Fig. 7. Most native starch granules are insoluble in water below 50ºC. In the case of potato starch, swelling begins around 65ºC, and this is referred to as the pasting temperature (indicated in Fig. 7). At peak viscosity, the majority of the granules are completely swollen, and during the temperature hold-phase at 95ºC, granules rupture, resulting in decreased viscosity. Upon cooling (95 → 50ºC), solubilization of amylose and amylopectin molecules decreases and reassociation of the molecules begins. The final viscosity gives an indication of the stability of the cooled, cooked paste under low shear. Gelamyl 120 (oxidized potato starch) shows the same pasting temperature as normal potato starch, but after solubilization completely lacks viscosity. Maize and waxy maize starches show higher pasting temperature. Waxy maize has the lowest final viscosity due to lack of amylose. Pea starch, containing around 40% amylose, has the highest final viscosity. As clearly illustrated in Fig. 7, pasting profiles are highly interconnected to the botanical origin from which the starch is isolated.

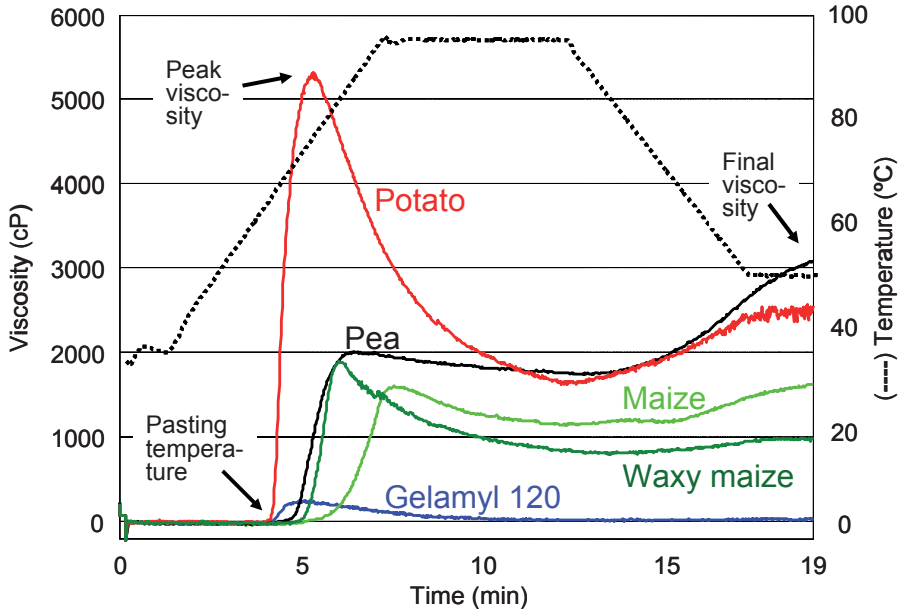


Fig. 7. Pasting profiles of normal potato, maize, waxy maize starch and Gelamyl 120 as measured by a RVA model 4 (Newport Scientific, Australia). The starch concentration was 8% starch (w/w) in suspension of 25 mM K_xPO_4, pH 7. The dotted black line depicts the temperature profile.

The length of double helices and molecular reorganization within the granules is proposed to be a determinant of pasting temperature (Moates, Noel, Parker, and Ring, 1997; Tester, Debon, Davies, and Gidley, 1999). In a potato starch system, the pasting temperature was shown to have a negative correlation with DP 6 and positive correlation with DP 15–17 (Singh, Isono, Srichuwong, Noda, and Nishinari, 2008). Furthermore, water fraction, salts, sugars and chemical modification have strong influences on

the pasting temperature, swelling and gelation (Donovan, 1979; Ahmad and Williams, 1999a; Ahmad and Williams, 1999b).

2.6. Retrogradation and gelation

Gelatinized starch paste frequently undergoes reassociation of starch molecules to an ordered structure upon cooling. This is referred to as retrogradation (Hoover, 1995), and results in the formation of crystalline aggregates that become opaque and form a gelled texture. The retrogradation of starch is important in most food applications, because it strongly influences many qualities such as texture, flavor release, resistance to enzymatic hydrolysis and shelf-life of starch-containing food products (Biliaderis, 1991). Often retrogradation is unwanted in starch-containing products such as staling of breads (Kulp and Ponte, 1981). Retrogradation can also be a desirable property in certain starch-based products in which starch has retrograded to such an extent that digestion in the small intestine is greatly diminished and for that reason is called resistant starch (Sajilata *et al.*, 2006). Resistant starch apparently has beneficial nutritional effects on humans. Several techniques can be applied to study starch retrogradation (reviewed by Abd Karim, Norziah, and Seow, 2000).

When gelatinized starch is allowed to form a gel network, amylose is a key player in the initial development because of the strong and quick tendency to form double-helix that associate to give connected aggregates (Miles, Morris, and Ring, 1985; Gidley, 1989). Particularly low Mw amylose containing a DP of 100 to 200 residues is important for this process (Gidley and Bulpin, 1989). A minimum of DP 10 is required for double-helix formation, although chains as short as DP 6 can co-crystallize (Gidley and Bulpin, 1987; Pfannemuller, 1987). Amylopectin retrogradation, in contrast to amylose, is a much slower process ranging from days to weeks and involving inter- and intramolecular chain associations (Goodfellow and Wilson, 1990). Amylopectin with high fraction of long chain, retrograded more easily than amylopectin with shorter CL (Kalichevsky, Orford, and Ring, 1990; Shi and Seib, 1995). A positive correlation between the amylopectin mean CL and retrogradation was found (Fredriksson *et al.*, 1998). Besides being time-dependent, starch retrogradation is also influenced by the ratio of amylose to amylopectin (Kitahara, Hamasuna, Nozuma, Otani, Hamada, Shimada, Fujita, and Suganuma, 2007), storage temperature (Farhat, Blanshard, and Mitchell, 2000), starch concentration (Lionetto, Maffezzoli, Ottenhof, Farhat, and Mitchell, 2005) and chemical modification (Tharanathan, 2005).

For the gel to actually form, a certain degree of non-covalent cross-linking requires to develop a 3D polymer network (Gordon and Ross-Murphy, 1975). Starch gel can be considered as a ‘physical gel’, where chains between starch molecules in a liquid medium entangle to form permanent cross-links. The cross-linking in hydrocolloid gels typically involve ordered segments from two or more chains, which form ordered ‘junction zones’ (Rees, Moris, Thom, and Madden, 1982). Several definitions of polymer gel have been given (Renard, van de Velde, and Visschers, 2006; Rogovina, Va-

sil'ev, and Braudo, 2008). X-ray diffraction experiments suggest that the junction zone nature of amylose gels is a side-by-side double-helical packing (Gidley, 1989). Factors affecting the gel strength are polymer concentration, the nature of the junction zones and the quantity of junction zones per molecule. The gel network of starch gels is considerably more complex in nature when compared to other hydrocolloid gels such as gelatin.

2.7. Applications

Starch is readily isolated from various plant sources and used in a wealth of different applications in the food industry, such as in baked products (Mondal and Datta, 2008), snack foods, confectionery, dairy products, salad dressings (Sikora, Badrie, Deisingh, and Kowalski, 2008) and used as resistant starch (Sajilata *et al.*, 2006) and in the production of a range of syrups (van der Maarel *et al.*, 2002). Various applications of genetically modified starches have already been discussed throughout Sections 2. The use of starch hydrolysis products is mentioned in Section 3.2 (Enzymes used in the starch industry).

In addition to the food industry, starch is also used in the paper, adhesives, textiles, renewable, cosmetic and pharmaceutical industries (Ellis *et al.*, 1998; Gray, Zhao, and Emptage, 2006).

3. Enzymes used in starch modification

3.1. Introduction

Native starch has numerous disadvantages for food applications such as high tendency toward retrogradation, low solubility, low freeze-thaw stability of pastes, low thickener and binder ability. These 'undesirable characteristics' are often overcome by physical and chemical modifications in order to improve its performance and to introduce new functionalities (Jacobs *et al.*, 1998).

Physical modification involves pregelatinization, heat-moisture treatment and extrusion (Bello-Perez, Romero-Manilla, and Paredes-Lopez, 2000). Chemical modification, on the other hand, relies upon hydroxyl group chemistry on starch molecules. Chemically modified starches (Singh, Kaur, and McCarthy, 2007) are used extensively in the food industry and are today the most important way to modify, improve and introduce new functionalities in starch. The chemical modifications involve treatment of granular starch in a suspension of water and a chemical agent at a specific temperature and incubation time until the desired property is obtained. The chemical modification alters the functionality by changing the hydrogen bonding, hydrophobic character and charge interactions and thereby changing the nature of the interactions between the α-glucan chains resulting in altered gelatinization, pasting and retrogradation behavior (Perera, Hoover, and Martin, 1997; Liu, Ramsden, and Corke, 1999; Choi and Kerr, 2003). The most common chemical modification of starch includes oxidation, acid-catalyzed hydrolysis, esterification and etherification. E.g. oxidation of starch results in low viscosity, high stability and increased ability to form a gel (Veelaert, deWit, Gotlieb, and Verhe, 1997). In the confectionery industry, oxidized potato starch is used especially in jelly, fruit gums and liquorice pastilles as a complete or partial substitute for gelatin or gum arabic (KMC, www.kmc.dk; AVEBE, www.avebe.com).

An alternative route to modify functionality is by *in planta* modification through the use of genetic engineering (Davis, Supatcharee, Khandelwal, and Chibbar, 2003; Blennow, 2004) either by suppressing or by introducing new enzyme activities into the plant source of interest. Examples of this were given in Sections 2.2 and 2.3. Major concerns about the safety of genetically modified organisms (GMO) within the scientific community and the public have been increasing over the past years (Baker and Burnham, 2001; van den Eede, Aarts, Buhk, Corthier, Flint, Hammes, Jacobsen, Midtvedt, van der Vossen, von Wright, Wackernagel, and Wilcks, 2004).

A fourth route of improving and introducing new functionalities is by enzymatic modification of starch. Enzymes are biological catalysts that increase the velocity of a chemical reaction. Today, enzymes are increasingly replacing chemical catalysts, because they perform specific reactions, reduce unwanted by-product and operate under mild conditions. The use of enzymes in various industries is a growing market because of the emergence of new technologies such as recombinant gene technology, protein design by directed molecular evolution and protein redesign (Jackel *et al.*, 2008).

The value of the worldwide use of industrial enzymes is expected to reach nearly $5.1 billion in 2009 (The Freedonia Group, www.freedoniagroup.com). Technical enzymes implicated in the detergent, textile and bioethanol industries account for the major consumption of industrial enzymes, whereas enzymes used in the food industry account for one quarter.

With respect to food applications, physically treated starches are labeled "starch". Chemically modified starches are regarded as a food additives belonging to the 'additional chemicals' category and labeled with "E numbers" ranging from E1000 to E1999. Enzymatically modified starches used in food applications are labeled as "starch" or "hydrolyzed starch" and are not considered as a food additives unless nutritional properties are claimed.

3.2. Enzymes used in the starch industry

Enzymes are divided into six classes on the basis on the chemical reactions they catalyze and on their substrate-specificity (IUBMB, www.chem.qmul.ac.uk/iubmb):
EC 1: Oxidoreductase
EC 2: Transferase
EC 3: Hydrolase
EC 4: Lyase
EC 5: Isomerase
EC 6: Ligase

Starch-acting enzymes belong primarily to the glycosyl transferase (EC 2.4.x.y) and glycosyl hydrolase (EC 3.2.1.x) group. Transferases catalyze the transfer of functional groups from one molecule to another, and hydrolases catalyze the reactions between a substrate and water molecule (hydrolysis).

The majority of starch-acting enzymes are involved in the hydrolysis of starch and includes α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), glucoamylase (EC 3.2.1.3) and pullulanase (EC 3.2.1.41) (see Fig. 8) (reviewed by van der Maarel *et al.*, 2002). The conversion of starch involves a liquefaction and saccharification step. In the liquefaction step, a suspension of 30-40% starch is gelatinized and liquefied by jet-cooking at 105ºC for 5-10 min in the presence of a highly thermostable α-amylase. An often used α-amylase in the industry is Termamyl, which is a bacterial enzyme from *Bacillus licheniformis* (Fitter, Herrmann, Dencher, Blume, and Hauss, 2001). It is an endo-acting enzyme, hydrolyzing non-randomly at α-1,4 linkages in the starch molecules producing maltodextrins and it results in reduced viscosity. Maltodextrins have considerable applications in the convenience and processed food products, because they have virtually no sweetness and do not mask other flavors. They are also used as thickeners, pastes and stabilizers. Maltodextrins often have a dextrose equivalent (DE) in the range of 15-25. DE is a measure of the degree of hydrolysis and designates the quantity of

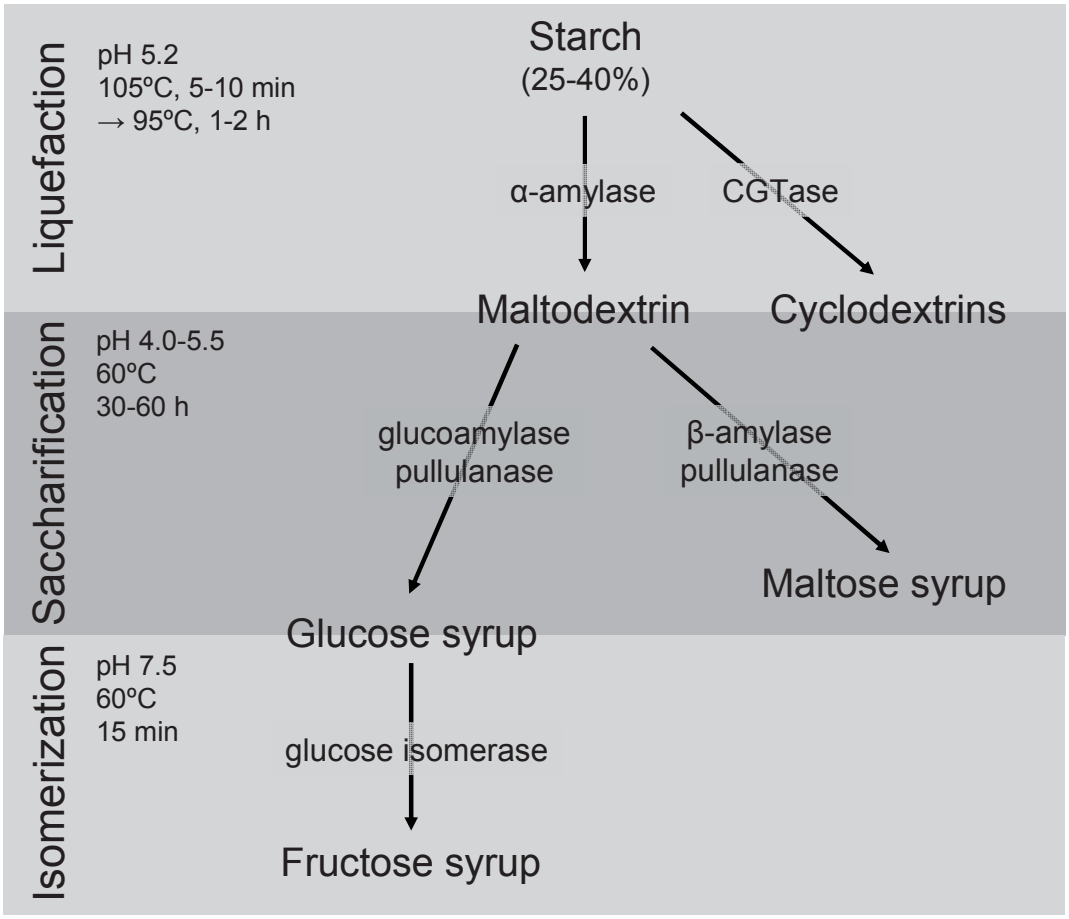


Fig. 8. Enzymatic conversion of starch.

reducing equivalents expressed as dextrose (traditional name for glucose) per unit dry weight. Starch has a DE of ~0 and glucose of 100. A major drawback of the α-amylase from *Bacillus licheniformis* is its calcium dependents, which must be added to the process to stabilize the enzyme. New α-amylases with improved thermal stability, acid tolerance and calcium-independency have been developed by protein engineering. Calcium independent, acid and temperature stable α-amylases were successful developed by site-directed mutagenesis for starch liquefaction and detergent application (Bisgaard-Frantzen, Svendsen, Norman, Pedersen, Kjærulff, Outtrup, and Borchert, 1999;Hashida and Bisgaard-Frantzen, 2000).

Further hydrolysis of maltodextrin into simpler sugar with higher DE is termed saccharification and leads to various syrups. One of these is maltose syrup, which requires the combined actions of β-amylase and pullulanase on maltodextrin (Fig. 8). Pullulanase catalyzes the hydrolysis of α-1,6 linkages in amylopectin (and pullulan). β-amylase is an exo-acting enzyme hydrolyzing from the non-reducing ends of dextrin liberating maltose. Maltose syrups with DE around 43-52 controls the texture and moisture in soft confections, and softness and freezing characteristics in ice cream.

For complete hydrolysis to glucose (glucose syrup), maltodextrin is further converted by glucoamylase that cleaves both α-1,4 and α-1,6 linkages from the non-reducing end, releasing α-glucose (Fig. 8). The exo-acting enzyme is usually from *Aspergillus niger*. As glucoamylase is less efficient at cleaving α-1,6 than α-1,4 linkages, pullulanase is used in combination with glucoamylase in order to use less glucoamylase dosage that prevents accumulation of branched oligosaccharides (e.g. isomaltose). The outcome is higher DE values and glucose contents, and higher substrate concentrations may be treated. Glucose syrups are used in the confectionery products as a replacement of sucrose. Most glucose syrup is used for production of fructose syrup using immobilized glucose isomerase (EC 5.3.1.5) (Fig. 8) (Bhosale, Rao, and Deshpande, 1996).

In Section 2.6, it was mentioned that retrogradation of starch in bread is highly unwanted. During storage, several undesirable changes such as lost flavor, texture and moisture in the bread takes place due to the retrogradation of starch molecules (Kulp *et al.*, 1981). These changes are altogether termed staling. Over the last 40 years, fungal α-amylases have been added to the dough to delay staling in bread (Pritchard, 1992). The minor degradation of starch molecules produces branched maltodextrins that prevent retrogradation after gelatinization. The amount of α-amylase used is critical, as an overdose results in sticky bread. In addition to having anti-staling effect, α-amylases are also supplemented to increase fermentable compounds utilizable by yeast. There are several other starch-acting enzymes such as maltogenic α-amylase (EC 3.2.1.133), β-amylase and DBE (EC 3.2.1.68), which are either commercial available or have been reported to have anti-staling effect in bread (Wursch and Gumy, 1994; Morgan, Gerrard, Every, Ross, and Gilpin, 1997; Gupta, Gigras, Mohapatra, Goswami, and Chauhan, 2003). A maltogenic α-amylase from *Bacillus stearothermophilus* called Novamyl (Diderichsen and Christiansen, 1988), has been commercially available since 1991. The native enzyme has certain drawbacks such as low stability in food application with low pH, e.g. sour and rye dough. By direct evolution using error-prone PCR (polymerase chain reaction) followed by DNA shuffling, a mutant Novamyl with increased heat and low-pH stability was produced (Jones, Lamsa, Frandsen, Spendler, Harris, Sloma, Xu, Nielsen, and Cherry, 2008).

Until now in this Section, only starch hydrolyzing enzymes (EC 3.2.1.x) has been discussed. One starch-acting transferase used in the food industry is cyclodextrin glycosyltransferase (CGTase) (EC 2.4.1.19). This enzyme is used in the production of cyclodextrins (cyclic α-1,4 glucan) (Fig. 8) that are capable of forming complexes with various guest molecules, such as hydrophobic flavors, resulting in increased solubility and stabilization of the flavor ligand (Hedges, 1998). The cavity is highly hydrophobic, since both the primary and secondary hydroxyl groups ‘stick out’, making the outer surface hydrophilic. Commercial cyclodextrin with DP of 6, 7 and 8 (α-, β- and γ-cyclodextrin, respectively) are the main products of CGTase action (Larsen, 2002). Fig. 9 shows the structure of α-cyclodextrin.

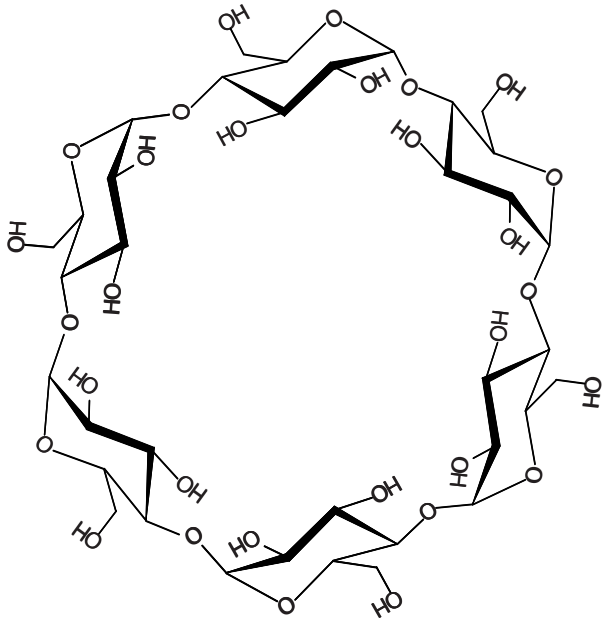


Fig. 9. Structure of α-cyclodextrin. The dimension of the cavity is 5.7 Å x 7.8 Å (diameter x height).

In addition to CGTases, two other starch-acting transferases (EC 2.4.x.y) expected to find application in the food industry are branching enzyme and amylomaltase (Takata, Takaha, Nakamura, Fujii, Okada, Takagi, and Imanaka, 1997; Spendler *et al.*, 1997; Fujii, Takata, Yanase, Terada, Ohdan, Takaha, Okada, and Kuriki, 2003; Euverink *et al.*, 2005). These two enzymes will be addressed in the following two Sections: 3.3 and 3.4.

3.3. Amylomaltase - produces thermoreversible starch gel

Amylomaltase (AM) (4-α-glucanotransferase; E.C. 2.4.1.25) is found in numerous microorganisms and plants. In the thermophilic bacteria *Aquifex aeolicus*, AM is presumed to be involved in glycogen synthesis (von Mering, Huynen, Jaeggi, Schmidt, Bork, and Snel, 2003). In *Escherichia coli*, it is involved in malto-oligosaccharide utilization (Boos and Shuman, 1998). The role of *E. coli* AM is to convert malto-oligosaccharides into longer chains following phosphorolytic cleavage to glucose-1-phosphate by maltodextrin phosphorylase (Fig. 10).

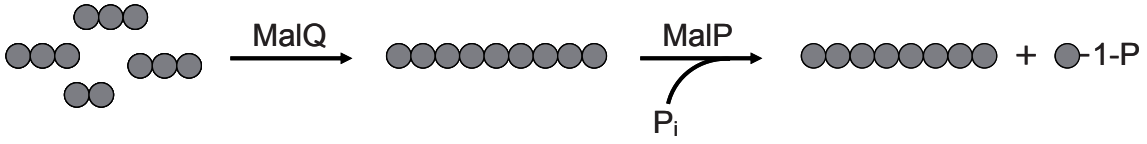


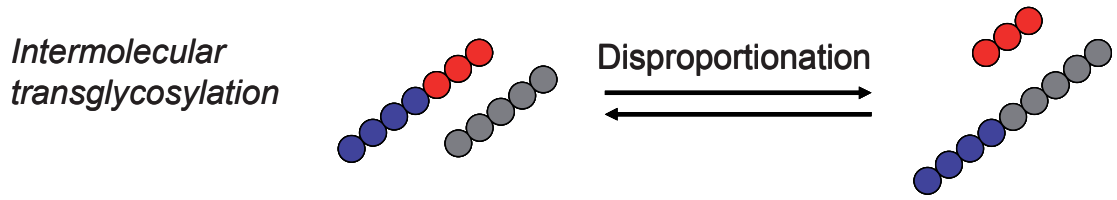
Fig. 10. Physiological function of AM in *E. coli*. MalQ: AM. MalP: maltodextrin phosphorylase. P_i: orthophosphate. ●-1-P: glucose-1-phosphate.

In plants, AM is known as the disproportionating enzyme (D-enzyme) and has been proposed to play a role in starch metabolism (Smith, Zeeman, Thorneycroft, and Smith,

2003). In potato with reduced D-enzyme activity, it was found to accumulate maltose in leaves, whereas the starch metabolism in tubers and synthesis of starch in leaves was not altered (Lloyd, Blennow, Burhenne, and Kossmann, 2004). Recently, it was found that D-enzyme is essential for maltose metabolism in plant leaves (Steichen, Petty, and Sharkey, 2008).

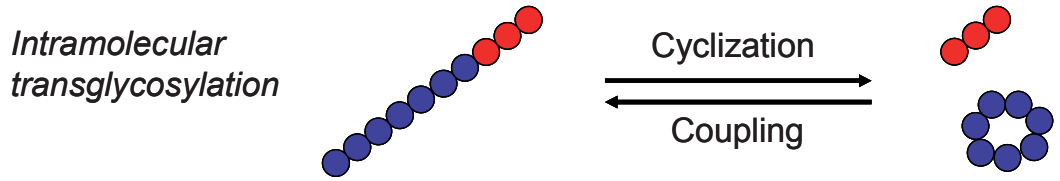
Based on amino acid sequence homology, AM has been assigned to the glycoside hydrolase family 77 (GH 77), which belongs the α-amylase superfamily (Henrissat, 1991; MacGregor, Janecek, and Svensson, 2001).

AM possesses three activities. It catalyzes the reversible intermolecular transfer of a α-glucan chain to a new 4-position in another α-1,4-glucan segment in a disproportionation reaction:



$$(\alpha\text{-}1,4\text{-glucan})_m + (\alpha\text{-}1,4\text{-glucan})_n \leftrightarrow (\alpha\text{-}1,4\text{-glucan})_{m-x} + (\alpha\text{-}1,4\text{-glucan})_{n+x} \quad (\mathbf{1})$$

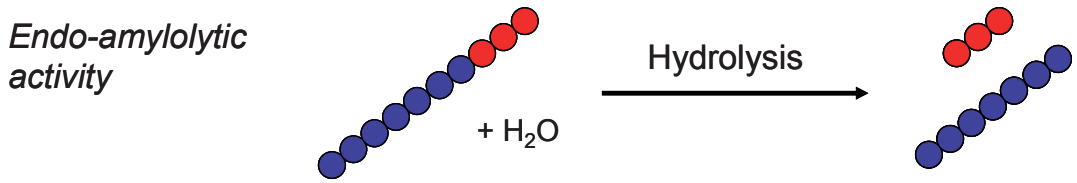
The disproportionation reaction is a variation of the α-retaining mechanism (Davies and Henrissat, 1995). AM also catalyzes the intramolecular transglycosylation reaction from linear α-1,4-glucan to produce cyclodextrin (Takata, Takaha, Okada, Hizukuri, Takagi, and Imanaka, 1996; Uitdehaag, Mosi, Kalk, van der Veen, Dijkhuizen, Withers, and Dijkstra, 1999):



$$(\alpha\text{-}1,4\text{-glucan})_m \leftrightarrow \text{cyclic}(\alpha\text{-}1,4\text{-glucan})_x + (\alpha\text{-}1,4\text{-glucan})_{m-x} \quad (\mathbf{2})$$

The reverse reaction is termed ‘coupling’. For simplicity, the formation of β-cyclodextrin is depicted. AM produces larger cyclodextrins with DP of 17 or more (Terada, Fujii, Takaha, and Okada, 1999; Takaha and Smith, 1999). AM from *Thermus aquaticus*, which is 99% identical to the *Thermus thermophilus* enzyme, converts 84% of the total amylose to cyclodextrin having a minimum DP of 22 (Terada *et al.*, 1999). Production of cyclodextrin mainly proceeds in the presence of linear amylose molecule and requires a relatively high amount of enzyme (van der Maarel *et al.*, 2002; Larsen, 2002).

A third activity of AM is a minor endo-amylolytic activity:



$$(\alpha\text{-}1,4\text{-glucan})_m + H_2O \rightarrow (\alpha\text{-}1,4\text{-glucan})_x + (\alpha\text{-}1,4\text{-glucan})_{m-x} \quad (\mathbf{3})$$

However, of the three activities the disproportionation reaction is by far the dominating activity for the *T. thermophilus* enzyme (van der Maarel *et al.*, 2005; Kaper, Leemhuis, Uitdehaag, van der Veen, Dijkstra, van der Maarel, and Dijkhuizen, 2007). For the transglycosylation reactions (reactions 1 and 2), no additional α-1,4-glycosidic bond is introduced or removed.

Treating gelatinized starch with AM result in the disappearance of the amylose fraction and a product with broadened amylopectin CL distribution (Kaper, Talik, Ettema, Bos, van der Maarel, and Dijkhuizen, 2005; van der Maarel *et al.*, 2005). This suggests a complete transfer of amylose chains to amylopectin molecules (Fig. 11).

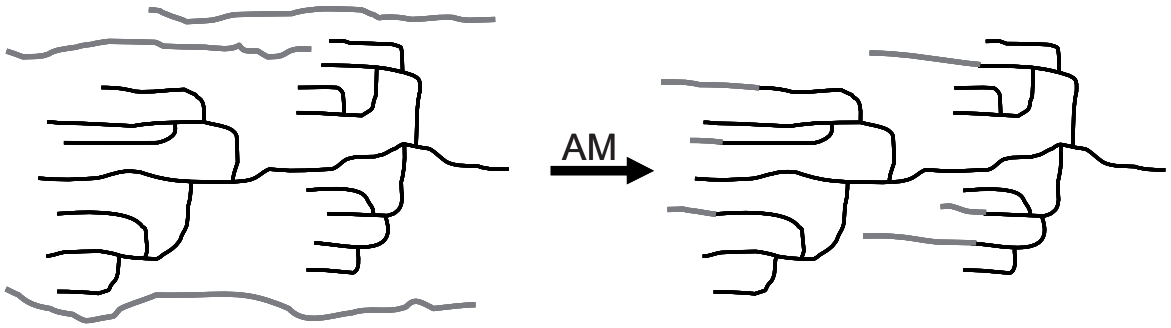


Fig. 11. Starch modified with AM. The black and grey lines represent amylopectin and amylose, respectively.

Potato starch modified with AM from *T. thermophilus* results in a product with increased gel texture compared to the corresponding parent starch (van der Maarel *et al.*, 2005). It was found to have comparable texture to gelatin gels at 8% (w/w). In general, AM modification of starch results in products that have acquired thermoreversible gelation character (Kaper *et al.*, 2005; van der Maarel *et al.*, 2005; Lee, Kim, Park, and Lee, 2006; Oh, Choi, Lee, Kim, and Moon, 2008), and for that reason has potential applications in replacing expensive or otherwise unwanted hydrocolloids such as gelatin in the food industry (Euverink *et al.*, 2005). Rice starch modified with AM from *Thermus scotoductus* was reported to melt between 40 to 70ºC, being independent of starch concentration (Lee, Kim, Park, and Lee, 2008). No specific gel-melting temperature was given for potato starch modified with AM from *T. thermophilus* other than gels melts below 70ºC (van der Maarel *et al.*, 2005). Gel of potato starch modified with AM from *Pyrobaculum aerophilum* was 50% melted at 37ºC and completely melted at 60ºC (Kaper *et al.*, 2005). This is interesting, because gelatin gels melt around human body

temperature. The most important factors determining the mouthfeel-perception of gelled food products are flavor release (which is affected by melting) and texture (Renard *et al.*, 2006). Freeze-thaw stability of food products is also an important property in certain applications. AM-modified rice starch has shown to have increased freeze-thaw stability (Lee *et al.*, 2006). In 2007, AVEBE launched a AM-modified potato starch, which is used as fat replacer and enhancer of creaminess in yoghurt (Alting, van de Velde, Kanning, Burgering, Mulleners, Sein, and Buwalda, 2009).

A recent study reports the modification of granular starch using AM from *Thermotoga maritime* (Oh *et al.*, 2008). Although granular starch is insoluble in cold water, enzyme hydrolysis is often relatively slow and requires gelatinization for optimal degradation (Tester, Karkalas, and Qi, 2004b). Enzyme modification with AM from *Thermotoga maritime* was carried out in a suspension of maize starch and water at 65ºC. At this temperature, maize starch is not gelatinized. The modification resulted in highly changed granular structure, disappearance of amylose, only slightly altered amylopectin CL distribution, higher solubility and paste clarity as well as gels with acquired thermoreversibility (Oh *et al.*, 2008). This is remarkable, because the granular structure remained after AM modification, which could be subject to further modification (e.g. chemical modification) in order to improve the functionality (e.g. lower the gel-melting point). Furthermore, working with granular starch is much more convenient than gelatinized starch. Granular starch-acting enzymes are of particular interest in the production of bioethanol, as they eliminate jet cooking and use less water + energy (Robertson, Wong, Lee, Wagschal, Smith, and Orts, 2006). The best result is obtained in combined hydrolysis with α-amylase and glucoamylase (Abe, Nakajima, Nagano, Hizukuri, and Obata, 1988).

Additional potential applications describing the use of AM in the food industry are: 1) in the production of large cyclic α-1,4-glucans (Hedges, 1998; Takaha *et al.*, 1999; Larsen, 2002), which was discussed in Section 3.2. And 2) in the production of 'short chain amylose' as so-called resistance starch that can be used as a replacement for the more expensive high-amylose starch (Norman, Pedersen, Stanley, Stanley, and Richmond, 2007).

3.4. Branching enzyme - produces slowly digestible starches

Branching enzyme (BE) (1,4-α-D-glucan branching enzyme; EC 2.4.1.18) is widely distributed in animal tissues, microorganisms and plants, and is involved in the metabolism of glycogen and starch (Smith, Denyer, and Martin, 1997). BE also belongs to the α-amylase superfamily, and has been classified to the glycoside hydrolase family 13 (GH 13) (Henrissat, 1991).

BE transfers a segment of α-1,4 glucan to a primary hydroxyl group in another α-glucan chain, resulting in the formation of a new α-1,6 glycosidic bond (Fig. 12). Besides having inter- and intrachain branching activity, thermostable branching enzymes

have also been reported to produce cyclic α-glucans (Takata *et al.*, 1996). The BE from *Rhodothermus obamensis* used in this project has higher activity toward amylose than amylopectin (Shinohara *et al.*, 2001).

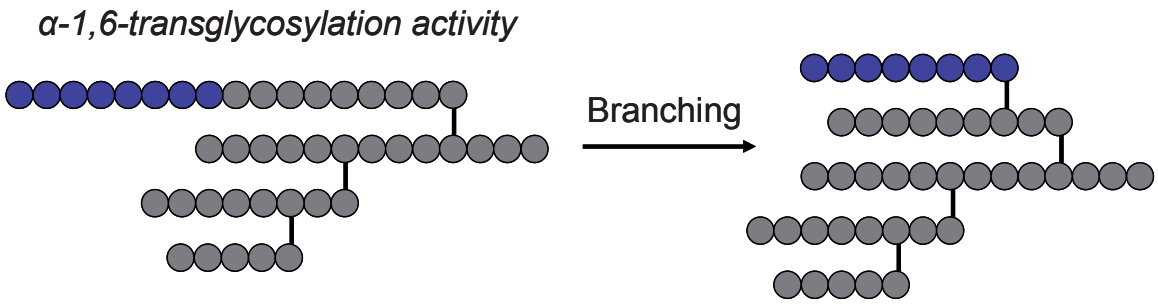


Fig. 12. Intramolecular reaction catalyzed by BE on amylopectin. Circles along with horizontal lines indicate α-1,4 glucan chains. Vertical lines indicate α-1,6 branch points.

Treating gelatinized starch with BE obviously results in increase branching and shortening of amylopectin chains (van der Maarel, Vos, Sanders, and Dijkhuizen, 2003). BE has potential use in producing slowly digestible carbohydrates that is highly branched and have least 10 % of DP 5-7 (van der Maarel, Binnema, Semeijn, Buwalda, and Sanders, 2008), and producing highly branched amylopectin cluster as well as highly branched amylose claimed to have anti-diabetes and anti-obesity effects (Park, Song, and Lee, 2008). BE also has potential use in the baking industry as an anti-staling agent and for increasing loaf volume (Spendler *et al.*, 1997; Fuertes *et al.*, 2006).

Some main characteristics about AM and BE are summarized in Table 2.

Table 2. Summary of the two enzymes used in this PhD project.

	***T. thermophilus* AM**	***R. obamensis* BE**
EC	2.4.1.25	2.4.1.18
GH family	77	13
Temperature optimum	85ºC	65ºC
pH optimum	5.5	6.0-6.5
Transfer activity	α-1,4-glucan segment → a new 4-position	α-1,4-glucan segment → 6-position
Industrial interest	- thermoreversible gels - large cyclic α-1,4 glucan - short chain 'amylose' - fat replacer	- slowly digestible carbohydrates - increase shelf life in baked goods

3.5. Experimental design

In this thesis, I have used AM and BE to modify starches prepared from the various botanical origins having a broad range of molecular structures. They were selected in order to broaden the variation in functionality and include potato, genetically engineered HAP, maize, waxy maize, pea and wheat starch. Maize starch is widely used in the food industry. Potato starch contains similar amylose content to maize starch, but is unique in having high phosphorous content (Blennow *et al.*, 2002). Waxy maize starch is amylose-free and is also widely used in the food industry. HAP starch (Blennow *et al.*, 2005) and pea starch can both be considered high-amylose starches. Starch isolated from pea is a relatively cheap source compared to starch isolated from maize and potato, but rarely used in food applications due to its poor functional properties (Ratnayake, Hoover, and Warkentin, 2002). Wheat starch has low amylopectin mean CL compared to the other starches. An oxidized potato starch (Gelamyl 120) was also included in the dataset, since it is used as a gelatin replacer in the confectionery industry (KMC, www.kmc.dk). Gelamyl 120 has reduced viscosity and improved textural properties.

Each starch was modified in a time-course, resulting in 7 non-enzyme-modified samples (parent starches) and 51 enzyme-modified starch samples. An overview of the samples and modification conditions is given in Table 3. Papers I-III are based on this dataset.

Table 3. Overview of starch and gelatin samples analyzed in this thesis.

Sample #	**Enzyme activity** (units/g starch)	**Incubation temp & time** (ºC)	(hours)	**Amylopectin CL** [a)] Mean CL (DP)	DP13-23 (%)	DP60-80 (%)
Potato						
1	Parent starch	-	-	32.0	35.1	10.3
2	2 units AM	85	1½	35.0	26.3	12.7
3	── "" ──	85	3½	37.1	22.0	15.1
4	── "" ──	85	5½	37.6	21.1	15.4
5	── "" ──	85	22	36.8	21.8	14.7
6	10 units AM	85	¼	34.8	25.8	12.9
7	── "" ──	85	¾	36.0	22.4	14.5
8	── "" ──	85	1½	37.0	20.1	14.6
9	── "" ──	85	2½	37.7	20.7	15.9
10	── "" ──	85	3½	37.6	20.6	15.8
11	── "" ──	85	20	36.4	22.4	14.9
12	100 units AM	85	1½	36.2	22.7	14.2
13	─── "" ───	85	3½	37.8	19.9	16.1
14	─── "" ───	85	5½	37.8	20.2	16.1

15	—— "" ——	85	22	37.0	21.0	15.4
16	10 units AM	70	¼	33.9	28.6	11.7
17	—— "" ——	70	¾	35.6	23.9	13.7
18	—— "" ——	70	1½	35.8	23.5	13.5
19	—— "" ——	70	3½	38.0	20.3	15.8
20	—— "" ——	70	5½	37.6	20.8	15.9
21	—— "" ——	70	22	37.0	21.4	15.2
Gelamyl 120						
22	Parent starch	-	-	33.3	30.8	11.1
23	10 units AM	85	1½	34.7	26.8	12.7
24	—— "" ——	85	3½	34.5	27.9	12.6
25	—— "" ——	85	5½	34.3	28.1	12.5
26	—— "" ——	85	22	34.6	25.9	12.4
HAP						
27	Parent starch	-	-	35.0	31.4	14.8
28	2 units AM	85	1½	37.9	22.7	17.5
29	—— "" ——	85	3½	40.6	17.7	20.2
30	—— "" ——	85	5½	41.2	17.2	21.6
31	—— "" ——	85	22	39.5	19.0	19.6
Maize						
32	Parent starch	-	-	28.1	38.3	5.8
33	10 units AM	85	¼	32.3	30.6	9.2
34	—— "" ——	85	¾	33.3	27.9	10.2
35	—— "" ——	85	1½	33.5	27.1	10.5
36	—— "" ——	85	3½	34.5	25.1	11.1
37	—— "" ——	85	5½	34.1	25.8	10.6
38	—— "" ——	85	22	34.5	24.4	11.4
Waxy Maize						
39	Parent starch	-	-	26.5	42.8	4.0
40	2 units AM	85	1½	29.4	34.4	6.7
41	—— "" ——	85	3½	30.1	32.2	7.1
42	—— "" ——	85	5½	31.3	30.4	7.9
43	—— "" ——	85	22	31.3	30.0	7.7
Pea						
44	Parent starch	-	-	28.6	39.8	6.2
45	10 units AM	85	1½	37.4	21.1	15.7
46	—— "" ——	85	3½	37.9	20.5	16.4
47	—— "" ——	85	5½	37.7	20.6	16.1
48	—— "" ——	85	22	36.6	21.9	14.8
49 [b)]	Combined AM/BE [c)]	60	0	37.4	20.8	15.1
50	—— "" ——	60	1½	34.3	24.7	11.2
51	—— "" ——	60	3½	29.5	31.4	6.8

52	── "" ──	60	5½	27.3	34.0	4.2
53	── "" ──	60	22	22.0	41.8	0.7
Wheat						
54	Parent starch	-	-	25.4	43.1	4.0
55	2 units AM	85	1½	28.6	34.4	5.9
56	── "" ──	85	3½	29.9	31.9	6.8
57	── "" ──	85	5½	30.7	29.9	7.3
58	── "" ──	85	22	30.2	30.0	6.3
Gelatin						
59	Gelatin bloom 120	-	-	-	-	-
60	Gelatin bloom 300	-	-	-	-	-

[a)] Data from Paper I.
[b)] Modified with 10 units AM/g starch for 2 h at 85ºC, then cooled to 60ºC and used to produce # 50–53.
[c)] Combined modified with 1000 units BE/g starch plus the original 10 units AM/g starch.

4. Gelatin – the reference hydrocolloid

4.1. Introduction

Gelatin is a multifunctional animal protein widely used in the food industry. Several reviews have been published (Djagny, Wang, and Xu, 2001; Wasswa, Tang, and Gu, 2007). Gelatin is generally isolated from the connective tissue in the skin, bone and tendon of pigs, cattle, poultry and fish of by alkaline or acidic treatment. The world production of gelatin has been gradually increasing since 2000, and was in 2007 around 0.3 million tons (Fig. 13).

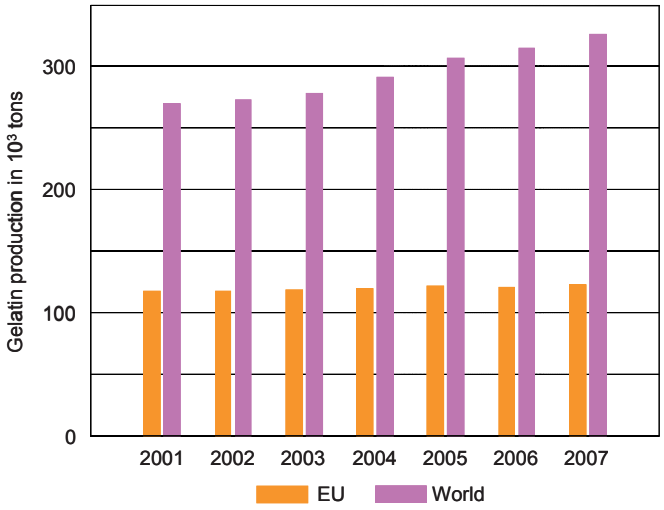


Fig. 13. Gelatin production – EU and for worldwide. Source: GME (the Gelatine Manufacturers of Europe).

Gelatin is a relatively expensive product compared to starch. It is widely used in the food industry due to its multifunctional properties as a gelling agent (primarily), thickener, emulsifier, stabilizer and foaming agent. A gelatin food product melts in the mouth around 35-37ºC, a unique property among the hydrocolloids, making gelatin an essential flavor-releasing food ingredient (Renard *et al.*, 2006). Lately, public concerns have been raised against the wide use of gelatin based on possible transmission of bovine spongiform encephalopathy (BSE) to humans (Bruce, Will, Ironside, McConnell, Drummond, Suttie, McCardle, Chree, Hope, Birkett, Cousens, Fraser, and Bostock, 1997) as well as social, ethnical and religious concerns related to vegetarian and pork-free diets. However, a recent study has shown that BSE-causing agents (prion proteins) are destroyed during the manufacturing process of bovine bone gelatin (Grobben, Steele, Somerville, and Taylor, 2004). Several gelatin replacers for the food industry have recently been reviewed by Karim and Rajeev, 2008.

Gelatin in food products was previously labeled E441, but is today considered an ingredient and labeled ‘Gelatin’. Information on origin can only be obtained from the producer.

4.2. Gelling

The purified gelatin is a white water-soluble powder. It is about 84-90% pure and, in addition to protein, contains 1-2% mineral salt and the rest consists of water. Gelatin dissolves readily in warm water and makes transparent thermoreversible elastic gels on cooling. A gel is said to be thermoreversible, when it is capable of undergoing physical change from solid-gel state to a liquid after heating, and subsequently forming a solid-gel again upon cooling.

The gelation of gelatin is a result of conformational transition of the chains from flexible coils to rigid triple-helices (disordered → ordered transition) (Stainsby, 1977). It has been suggested that one gelatin molecule in the triple-helix structure contributes two strands, whereas the third strand comes from another molecule (Busnel, Morris, and Rossmurphy, 1989). The gel network is predominantly composed by hydrogen-bonded junction zones. Joly-Duhamel *et al*. proposed a gel network composed of entangled rigid rods (Joly-Duhamel, Hellio, Ajdari, and Djabourov, 2002), which is in contrast to the cross-linking network in starch gels. Fig. 14 shows the schematic illustration of gelatin gel network. The triple helices form the rigid rods, which are connected by flexible links. The rigid strand can withstand large deformations without gel-fracture because of the flexible links (Joly-Duhamel *et al.*, 2002).

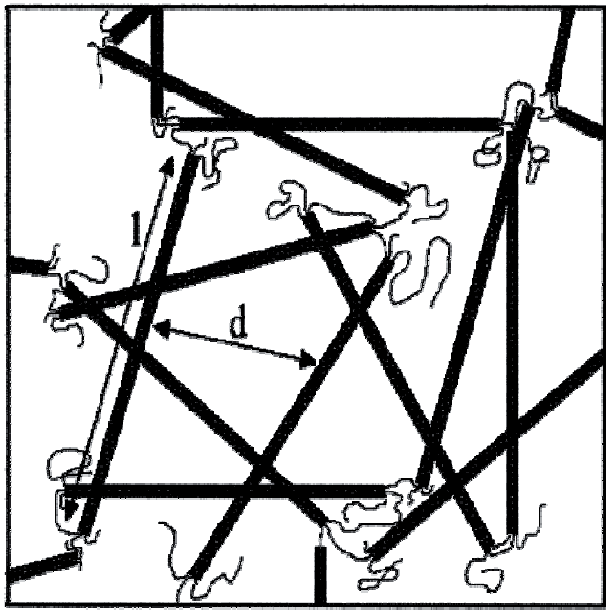


Fig. 14. Schematic illustration of gelatin gel network. l: length of the rods; d: distance between the rods. Reproduced from Joly-Duhamel *et al.*, 2002.

The strength of gelatin gels has been shown *not* to depend on the Mw, but exclusively on helix content (Joly-Duhamel, Hellio, and Djabourov, 2002; Joly-Duhamel *et al.*, 2002). The elasticity is correlated with the helix concentration. The formation of helices from coils is a slow time- and temperature-dependent process (Djabourov, Leblond, and Papon, 1988), unlike most biopolymers. Hence, upon cooling the conversion of flexible coil chains to helices is accelerated, resulting in increasing gel-strength.

The helix concentration is obviously related to the gelatin concentration, which is linearly correlated to the gel-strength. This is in contrast to starch gel that is not linearly correlated to the dry weight concentration.

Additional examples of thermoreversible cold-setting gelling agents are agar, methylcellulose, carrageenan, low-methoxyl pectin and gellan gum. Except for agar and methylcellulose, gel formation of these hydrocolloids requires the presence of potassium, calcium salt or divalent cation.

4.3. Applications

In the food industry, gelatin is used in a range of applications. It is used as a stabilizing agent in many bakery products such as pies and cakes. The elasticity of gelatin is exploited in wine gums, liquorices and marshmallows. Gelatin is also used as texturizer in ice cream, jams and yoghurt. In some applications, gelatin is used to obtain clearness of drinks and beverages by forming complex with hazy elements, resulting in flocculation or sedimentation that subsequently can be separated from the drink.

In addition to the food industry, gelatin is also used the pharmaceutical industry in applications such as tablets for protecting drugs against light and keep them active, and in vitamin preparations for shielding specific vitamins against oxygen and light.

5. Starch analysis and data methods

The chemical composition of the starch molecule is simple compared to other macromolecules such as proteins and DNA, which are polymers of 20 different kinds of α-amino acids and 4 different kinds of deoxyribonucleotides, respectively. Starch is composed of one kind of monomer residue: α-glucose. Nevertheless, the analysis of starch structure is relatively complicated, since it is impossible to organize α-glucose into a meaningful primary sequence. The Mw and structure of amylose and amylopectin molecules determine starch functionality.

In this Section, I will address some of the techniques that were used to study the structure of the enzyme-modified starches and their functionalities.

5.1. High-performance anion exchange chromatography (HPAEC)

HPAEC with pulsed amperometric detection (PAD) is a excellent technique for characterization of native and enzyme-modified starches for structural studies on amylopectins, since CL distribution is an important parameter for characterizing the molecular structure (Hizukuri, 1986; Tester *et al.*, 2004a). The technique has been reviewed extensively (Hardy, Townsend, and Lee, 1988; Townsend, Hardy, Hindsgaul, and Lee, 1988; Lee, 1990).

HPAEC takes advantage of the weak acidic nature of α-glucose polymers. The ionized formation occurs above pH 12, allowing highly selective separations by ion exchange, which is based on the relative affinity of the analyte ion (e.g. linear α-1,4-glucan) competing with the eluent ion (e.g. acetate) for the same exchange sites (Fig. 15).

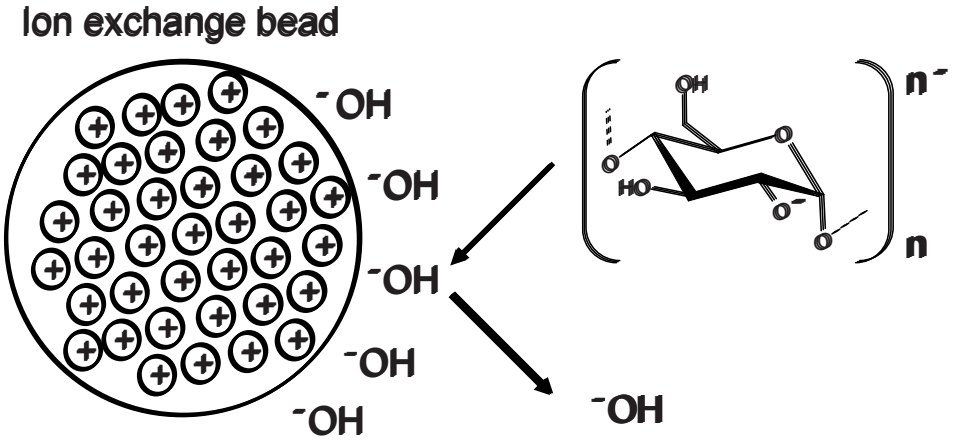


Fig. 15. The principle in ion exchange separations at high pH of linear α-1,4 glucose polymers in its oxyanion form ($[\alpha\text{-1,4 glucose}]_n^{n-}$). The ion exchange resin is composed of non-porous beads allowing mass transfer, resulting in high-resolution chromatography and quick re-equilibration.

Larger DP gives greater affinity of the ion to the exchange column, resulting in longer retention time. HPAEC coupled with pulsed amperometric detection allows direct quantification of the analyte, making the HPAEC-PAD technique highly sensitive even at low picomole levels.

When studying the molecular structure of starch, the sample is analyzed by HPAEC-PAD with gradient elution (e.g. sodium acetate gradient in 100 mM NaOH), which has a resolution of chains up to the order of DP 70-90 when separated on a CarboPac PA-200 column (Dionex, Sunnyvale, USA). For that reason, only the amylopectin CL distribution can be analyzed, since amylose chains typically contain more than 1000 α-glucose residues (Tester *et al.*, 2004a). Fig. 16 shows the CL distribution up to a DP of 80 for debranched wheat starch.

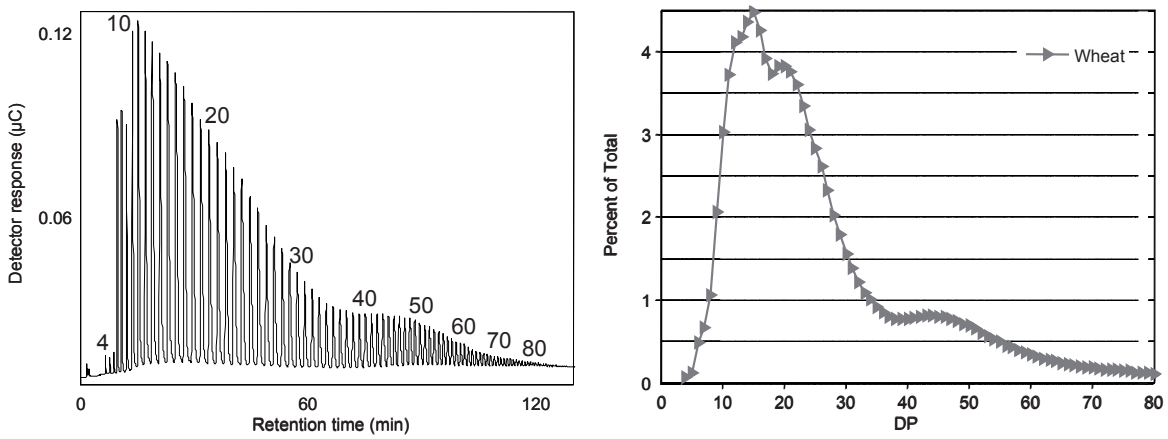


Fig. 16. Amylopectin CL distribution of parent wheat starch. Left: The raw chromatograms showing the separation of debranched parent wheat starch by HPAEC-PAD with gradient elution. Peak labels indicate the DP. Sample of 5 μg/ml was separated on a CarboPac PA-200 column (Dionex, Sunnyvale, USA) using 1 ml/min flow rate, 150 mM isocratic NaOH following a sodium acetate gradient. Right: Single peaks between the DP 4-80 from the chromatograms were integrated, corrected for detector response factor and normalized to 100% as previously described Blennow *et al.*, 1998.

5.2. Size exclusion chromatography (SEC)

The Mw of starch is known to have specific and important impacts on the functional properties of starches in solution and in the gel state (Blennow *et al.*, 2001). A useful method to analyze the Mw distribution of starch is by SEC, where the molecules are separated by their size. In contrast to HPAEC, which provides information about the relative amount of individual linear chains, SEC is capable of analyzing a more complete starch molecule profile.

Fig. 17 (Frames 1-4) illustrates the principle of SEC of an ideal mixture of two molecules of different size. A SEC column is composed of a mobile and a stationary

phase. The particles of the stationary phase (e.g. silica-based packing) are porous with a defined pore size. Smaller molecules are able to penetrate the pores during elution through the column (Frame 2). The smaller the molecules are, the longer the elution time. Larger molecules penetrate less easily and therefore flow more rapidly through the column (Frames 2-3).

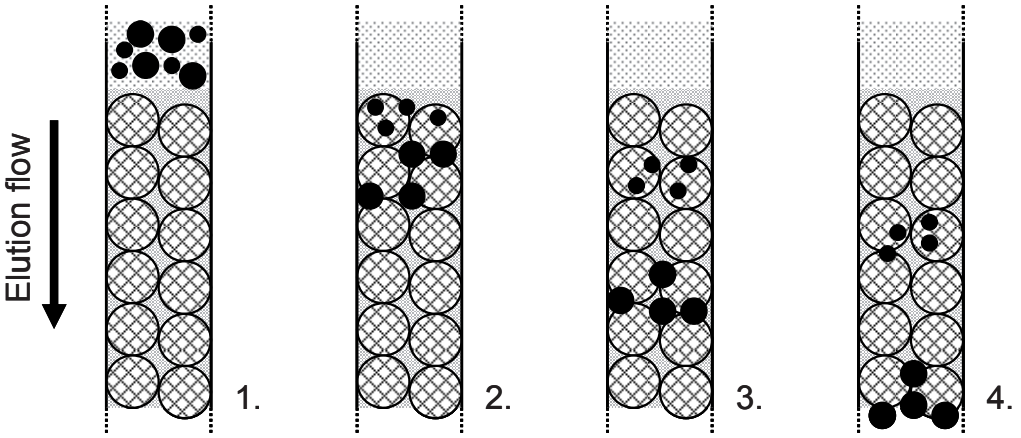


Fig. 17. The principle of SEC shows here the separation of two molecules of different molecular size. Frame 1: Sample mixture before entering the column packing. Frames 2-3: Separation of molecules occurs. Frame 4: Complete separation.

Fig. 17 shows the separation of an ideal mixture. However, starch molecules are not globular, but linear and highly branched, so their behavior differs significantly from that of proteins under native conditions on SEC. Instead of measuring the difference in molar mass, classical SEC actually measures the differences in hydrodynamic size (volume), which is not correlated with the Mw of amylose and amylopectin. E.g. amylopectin molecules with identical molar masses can have dissimilar hydrodynamic volume due to difference in branching degree and CL distribution.

The graphical data typically display a linear detector response as a function of the chromatographic elution volume. SEC monitored using a RI detector does not determine the absolute Mw value, because it requires calibration using carbohydrate standards of known Mw. Often pullulan or dextran is used. Although both are α-glucan polymers, their molecular structures are highly different compared to amylose and amylopectin molecules. Pullulan is a linear chain of maltotriose units. Dextran is based on chains of α-1,6 linked glucose units containing branched α-1,3, α-1,4 and to some extent α-1,2 linkages. For that reason, the Mw of starch using SEC/RI is determined from an extrapolation from pullulan standards and should be considered ‘apparent’.

A much more reliable method is SEC coupled with multi-angle laser light scattering (MALLS) (You and Lim, 2000; Chen *et al.*, 2007) that measures both the absolute Mw and molecular size of starch molecules without any column calibration. In SEC-MALLS, light is more scattered by large molecules than by small molecules. The out-

put data of the light scattering detector is proportional to the concentration and molar mass of starch molecules.

5.3. Low-field ^1H nuclear magnetic resonance (LF NMR)

The molecular mobility of hydrocolloids and water is important in many food products because of its influence on the storage stability as well as textural and functional properties. The proton relaxation can be studied by Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence (Meiboom and Gill, 1958), which is a quick and accurate LF NMR method. CPMG is derived from the Hahn spin-echo sequence (Hahn, 1950).

The basic principles of NMR are: Nuclei that possess a spin are applicable in NMR. One of the most abundant nuclei is the proton (^1H) that has a spin of ½. When it is introduced into a magnetic field B_0, the nuclei magnetic moment will orient in two orientations: ½ and -½. Upon applying a second magnetic field B_1 orthogonal to B_0, the net magnetization is rotated to such an extent that depends on the duration of the pulse. The delay between the 90º and 180º pulse is termed tau (τ). After each radio frequency pulse, the protons relax in the direction of equilibrium state. This process is known as spin-spin or transverse relaxation.

CMPG consists of a radio frequency 90 pulse, followed by N radio frequency 180 pulses. The CPMG pulse sequence is illustrated in Fig. 18. The resulting CPMG decay is a time-domain relaxation curve, often composed by one or more exponential. Depending on the number of components, one or more transverse or spin-spin relaxation time constants (T_2) can be extracted.

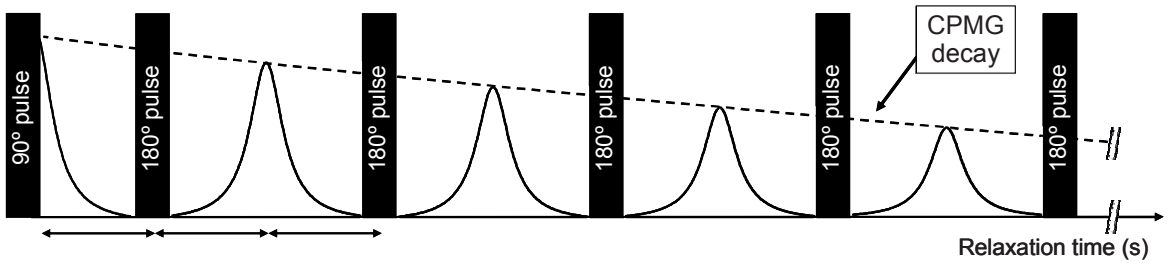


Fig. 18. CPMG pulse sequence. The first four echoes are shown. τ: The pulse spacing (tau).

The output data of CPMG analysis is time-domain relaxation curves (see e.g. in Fig. 19). Each curve relaxes exponentially and can be described by one or more exponential T_2 relaxation time constants. In a starch gel system (water and amylose/amylopectin), the T_2 describes the fast exchange between hydroxyl groups in water and starch molecules (Hills, Wright, and Belton, 1989). Changes in the mobility or conformation (e.g. aggregation) of starch molecules alters the accessibility of the hydroxyl groups and thereby alter the fraction of hydroxyl groups available in exchanging protons with water (Hills, Cano, and Belton, 1991).

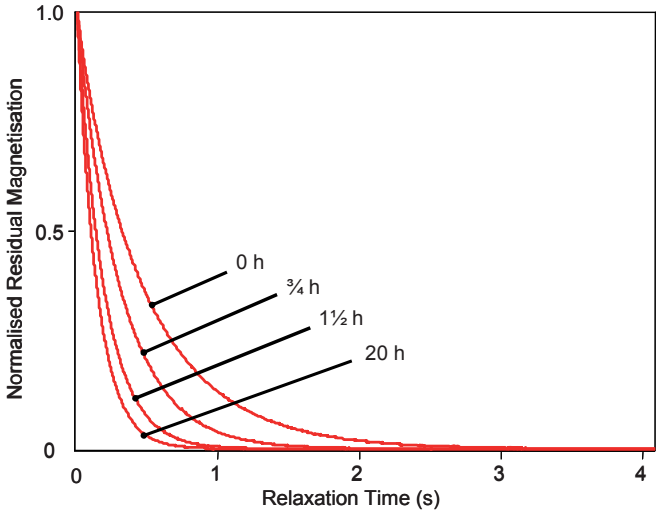


Fig. 19. Maximum-normalized LF NMR relaxation curves recorded after 1-day storage at 20°C of 12.0% (w/w) potato starch modified with 10 units AM/g starch for 0, ¾, 1½ and 20 h as indicated. Data was obtained using a 23 MHz 1H NMR Maran instrument from Resonance Instruments (Witney, Oxfordshire, UK). Presented in Paper II.

In the example given in Fig. 19, the AM modification of potato starch resulted in products with faster relaxation times (decreasing T_2 values), as more polysaccharide hydroxyl protons become accessible to the water.

5.4. Texture Analyzer (TA)

After flavor, gel texture is one of the most important perception qualities of gelled food products (Moskowitz and Krieger, 1995; Renard *et al.*, 2006). Texture profile analysis (Bourne, 1978; Pons and Fiszman, 1996) has been widely adapted to the study of the texture of starch gel systems (Edwards, Berrios, Mossman, Takeoka, Wood, and Mackey, 1998; Cayot, Lafarge, Arvisenet, and Taisant, 2000).

In a single uniaxial compression test (Fig. 20 left), a sample of specific dimensions is compressed. During the experiment, compressive force is recorded as a function of distance or time. Fig. 20 right shows the texture profile for a AM-modified potato starch gel. In the beginning of compression, a build-up of force occurs followed by a fracture of the gel that is termed *fracturability*. Compression continues after *fracturability*, resulting in accumulation of force until probe retraction, which in this example is the maximal force and is termed *hardness*. The area of compression is termed *force of deformation* (expressed in N sec). During retraction, the gel stick to the probe resulted in positive and negative retraction areas termed *adhesion I* and *adhesion II* , respectively.

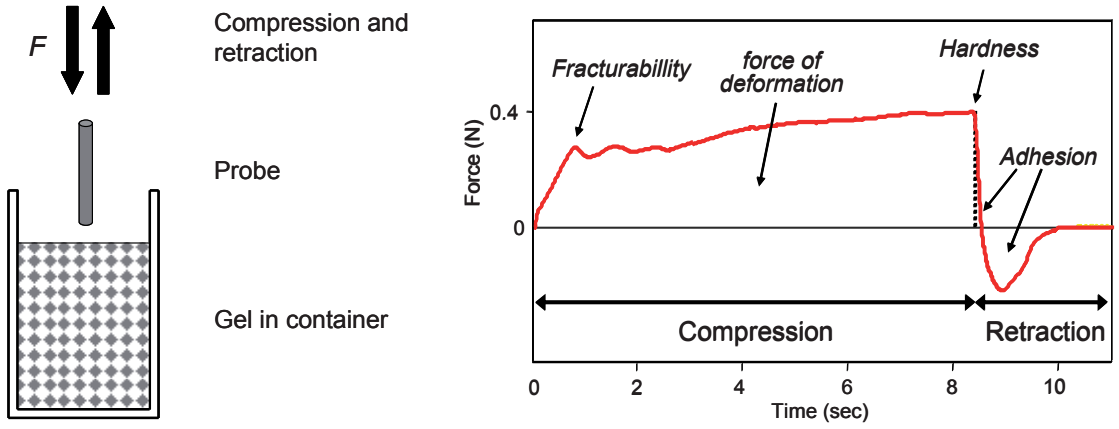


Fig. 20. Left: Principle of single uniaxial compression. Right: Texture profile of 12% (w/w) AM-modified potato starch (# 11) gel obtained using a TA-XT2i texture analyzer (Stable Micro Systems, Godalming, UK). The sample was compressed with a test speed of 0.9 mm/sec. Compression: 0-8.4 sec. Retraction: 8.4-11.0 sec. The area of compression is termed *force of deformation*. The positive and negative areas of retraction are termed *adhesion I* and *adhesion II*, respectively.

For the starch gels used in this thesis, springiness (elasticity), cohesiveness, gumminess and chewiness were not determined, since the sample lacked the ability to regain its original 'height' due to loss of internal gel structure. It must be emphasized that the parameters obtained from a texture profile are highly influenced by the sample size (gel geometry), concentration, probe used and compression depth. Furthermore, a linear correlation between concentration and starch gel texture does not exist (Ortega-Ojeda, Larsson, and Eliasson, 2004).

The gel-strength of gelatin is often indicated by a 'bloom value', which corresponds to the force required to press a plunger a specific distance into a 6.67% (w/w) gelatin gel at 10ºC. High Bloom value indicate greater the gelling strength of the gelatin. In gelatin food products, the bloom value is typically between 50 and 250.

5.5. Differential scanning calorimetry (DSC)

DSC is a widely used method for measuring the thermal properties of a material (Watson, Justin, Brenner, and Oneill, 1964). During heating or cooling of a material, its structure and/or composition often changes. If a phase transition occurs, heat flows into or out of the samples. DSC measures these heat exchanges of a sample during a controlled change temperature. Some examples of phase transitions are gelatinization of starch granules and melting of gels.

There are two types of DSC systems in common use: power-compensation DSC and heat-flux DSC. In power-compensation DSC (Fig. 21), the sample and the reference pan are placed in independent furnaces, where two individual temperature sensors are used to monitor the individual heating rates of the individual ovens. A system

rapidly controls the temperature difference between the sample and reference pan. In the event of a temperature difference in the sample material compared to the reference pan, differential thermal power is applied or removed to maintain the temperature at the program value. The energy required to maintain system equilibrium is a direct measure of the enthalpy changes occurring in the sample relative to the reference. When an exothermic or endothermic reaction occurs, the instrument delivers the compensation energy in order to maintain equal temperature in both pans.

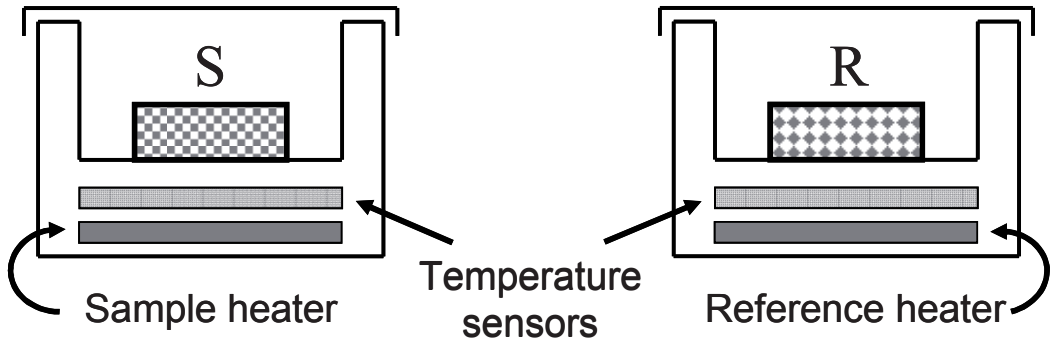


Fig. 21. Power-compensation DSC showing the two independent furnaces. S indicates pan containing the sample. R indicates the reference pan.

In heat-flux DSC, the sample and reference pan are placed in a single furnace. With the heat-flux DSC systems, the temperature differential is converted to energy flow via a mathematical equation and is a more indirect approach compared to the 'pure' energy flow measurements obtained via the Pyris 1 power-compensated DSC. The power-compensation DSC used in Paper III, is superior to the heat-flux DSC, because the sample mass required is much lower. Sample material as low as ~1-50 mg can be analyzed, making power-compensation DSC a rapid and highly sensitive technique capable of detecting even weak transition of low energy.

The results from the power-compensation DSC scan experiment are displayed as a thermal analysis profile, where heat flow is plotted vs. sample temperature. Fig. 22 shows the thermal profile of a 20% (w/w) gelatin gel. In this figure, the peak represents an exothermic event, where heat is released due to melting transition (going from solid-gel to liquid). For this experiment, gelatin starts melting at ~16ºC, completely melted at ~50ºC, and has a peak temperature (melting point) of ~30ºC. The low melting point is one of many unique abilities of gelatin gels (Djagny *et al.*, 2001). From the thermal profile, gel peak temperature (T_p) (the temperature at maximum enthalpy) and the enthalpy of transition (ΔH) (determined as the area under the peak) can be extracted. The ΔH indicates how much energy is required for disrupting (melting) H-bonding of the junction zones, and the T_p indicates the loss of double-helical segments within the gel network (Cooke *et al.*, 1992; Lazaridou and Biliaderis, 2004). In the case of starch gel, a very broad gel-to-solution transition phase is observed (Fredriksson *et al.*, 1998;

Blennow *et al.*, 2005; Lionetto *et al.*, 2005), indicating multiple distributions of recrystallized regions.

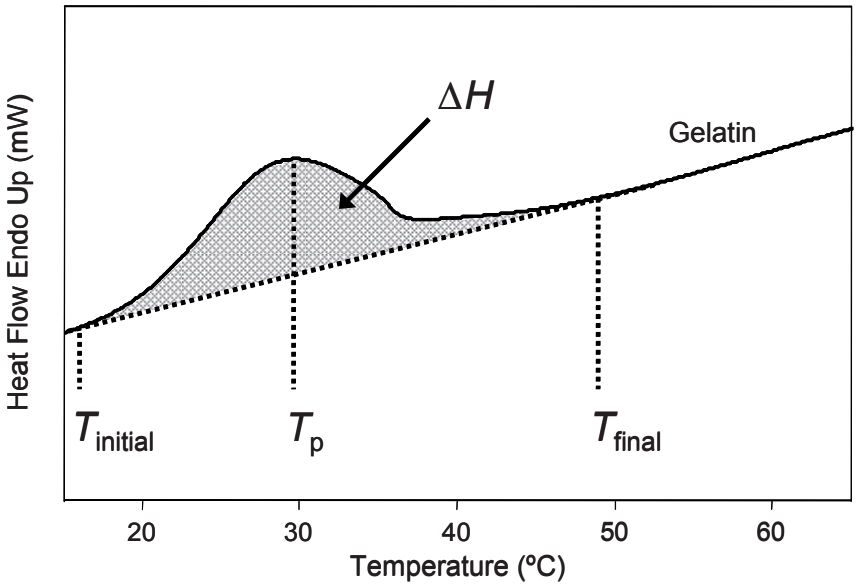


Fig. 22. Thermal profile of 20% (w/w) gelatin examined by DSC using a Pyris Diamond DSC instrument (PerkinElmer, Norwalk, USA). The sample was scanned at 10°C/min. T_p: gel peak temperature, ΔH: enthalpy of transition, $T_{initial}$ and T_{final} are the initial and final temperatures of the peak, respectively. Data presented in Paper III.

5.6. Chemometrics

Obtaining a meaningful overview of a huge amount and complex data can indeed be a complicated task. Most often, a property of interest depends not on one, but on several variables. When handling complex data, it is rarely obvious which variables carry important information and those with no information related to a given property. For example, what variables are important for starch functionality? Although starch in build up of only one kind of residue connected with two different kinds of α-glycosidic linkage, understanding the relationship between structure and function is not straightforward. Several variables important for starch functionality are: the ratio of amylose/amylopectin, branching degree, Mw, the length and quantity of chains in amylopectin, the degree of phophorylation, distance between each branching, etc. Starch science is certainly very multivariate, and it is for that reason advantageous to use chemometric techniques for data exploration, modeling and elucidating indirect correlations that were not anticipated. Two of the most used chemometric techniques are principal component analysis (PCA) and partial least squares (PLS) regression. Both techniques have proven very useful in all three Papers. In addition, I have also used a third technique called multivariate curve resolution (MCR) to further aid in interpretation of the hidden phenomena observed in PCA of amylopectin CL distribution data (see Section 6.1.2).

5.6.1. Principal component analysis (PCA)

PCA (Hotelling, 1933; Wold, Esbensen, and Geladi, 1987) is a useful unsupervised technique to analyze large amounts of data with the aim of extracting important information and compression of data into a few components (factors). Additional goals of PCA could be outlier detection, classification and prediction of samples. The principle of PCA is given by this equation:
X = **TP'** + **E** (' means transposed)
where the dataset **X** (with the dimension *samples* x *variable*) is decomposed into a score matrix (**T**) and a loading matrix (**P**) plus the residual matrix (**E**) containing the unsystematic variation. The score and loading matrices capture the systematic variation of the data in **X**, and their outer products (**TP'**) are called *principal components* (PC's). The first PC (PC 1) always describes the greatest amount of variation in the data as possible, and the second PC (PC 2) captures the second largest variation, etc. Although, a huge dataset can be decomposed into many PCs (PC 1, PC 2, PC 3 and ... PC k), it is in general sufficient to describe the data with just a few PC's.

PCA is graphically depicted by a score and a loading plot. The score plot contains information on how the samples relate to each other, while the loading plot contains information on how the variables relate to each other. Samples in the score plot that cluster share some underlying physical phenomena and are said to be correlated to each other (as variables in the loading plot). Outlying samples are easily identified in the score plot. The presence of outliers in the dataset does not necessarily arise because of experimental error, but could be due to the unique property of a sample, and can therefore be considered as an extreme compared to the rest of the samples in the dataset.

Understanding the structure of starch molecules and starch functionality has been explored by PCA in several studies (Blennow *et al.*, 2000; Thygesen, Blennow, and Engelsen, 2003; Wischmann, Blennow, Madsen, Jorgensen, Poulsen, and Bandsholm, 2005; Sanderson, Daniels, Donald, Blennow, and Engelsen, 2006).

5.6.2. Multivariate curve resolution (MCR)

MCR with non-negative constraints (Tauler, 1995) is another chemometric technique for multivariate data analysis and the principle of the method is similar to PCA. MCR decomposes the data matrix **X** (*samples* × *variables*) into a concentration profile matrix (**C**, comparable to the score matrix) and a matrix containing the pure component spectra (**S**, comparable to the loading matrix) plus a residual matrix **E**, where **E** is minimized in a least squares sense:
X = **CS'** + **E**

The goal of MCR is to extract concentration profiles and pure component spectra in order to acquire physically meaningful **C** and **S**, which cannot be obtained directly

from PCA decomposition of **X**. This implies that the vectors of **C** and **S** will not be pair wise orthogonal, respectively, and that the variance explained for the extracted MCR components does not necessarily decrease for the next component as is seen in PCA.

MCR has proven useful in discriminating a mixture of 231 simple alcohol into pure concentrations and NMR spectra (Winning, Larsen, Bro, and Engelsen, 2008), and to identify a second zein protein in mature maize kernel resolved from FT-IR microspectroscopic image (Budevska, Sum, and Jones, 2003).

5.6.3. Partial least squares (PLS) regression

PLS regression (Geladi and Kowalski, 1986) is, like PCA, a widely used chemometric method. PLS determines the relations between predictor variables **X** (e.g. amylopectin CL distribution) and the property of interest **y** (e.g. gel-melting point). PLS is a linear multivariate model that decomposes **X** and **y** in such a approach that the information in the **y** vector is directly used to direct the decomposition of **X**. The regression model is defined as:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{E}$$

where b is the vector of regression coefficients and E is the residuals. The robustness of the model is evaluated by cross-validated correlation coefficient (r^2) that describes the correlation between the measured Y and the predicted Y, prediction error RMSECV (root mean square error of cross-validation) (Næs, Isaksson, Fearn, and Davies, 2002).

PLS is used today in several different scientific fields. Two nice examples where PLS was applied are in the determination of dioxin in fishmeal samples and fish oil. Determination of dioxin in fish product is expensive and time-consuming. A direct correlation of dioxin in fish oils by fluorescence screening was established (Pedersen, Munck, and Engelsen, 2002). This is a direct determination of dioxin. In fishmeal samples, an indirect determination of dioxin was found by analyzing the fatty acid composition (Bassompierre, Munck, Bro, and Engelsen, 2004). A dioxin prediction model was developed based on 32 fatty acids determined by gas chromatography, which is a much more time- and cost-effective method compared to commercial dioxin determination. Depending on the data, obtained PLS regression models can further be optimized using interval PLS (iPLS), where only the variables carrying the most important information are selected and used in regression modeling (Norgaard, Saudland, Wagner, Nielsen, Munck, and Engelsen, 2000).

6. Amylomaltase-modified starches (result and discussion)

A unique property of gelatin is its ability to make firm transparent elastic gels. When heated to around human body temperature, the gels melt and form a gel network again after cooling. Gelatin is said to make thermoreversible gels. Firm texture and gel-melting properties are two important qualities of a food product, because they have great influence on the perception and mouthfeel by consumers (Renard *et al.*, 2006).

An important tool used to emulate gelatin functionality is enzyme modification of starch with AM. Modifying starch with AM has shown in several studies to produce gels with thermoreversible character (Kaper *et al.*, 2005; van der Maarel *et al.*, 2005; Lee *et al.*, 2006; Oh *et al.*, 2008) and to increase the gel texture (Kaper *et al.*, 2005). For this reason, AM-modified starch is expected to find application in the food industry as a replacement for gelatin (Euverink *et al.*, 2005). Several gelatin alternatives have been reviewed by Karim *et al.*, 2008. AM-modified starch has recently shown to be effective as fat replacer in yoghurts (Alting *et al.*, 2009).

In this thesis, I have used AM and BE to modify starches from various botanical origins. Both enzymes have been highlighted as important in producing starches with new functionalities, which are expected to find specific applications in the food industry (Fujii *et al.*, 2003). Additional industrial interests of these two enzymes were discussed in Section 3.3 and 3.4. The dataset (see Table 3) consists of 51 enzyme-modified and 7 non-enzyme-modified starches (parent samples), and have formed the basis of Papers I-III.

6.1. The effect of amylomaltase modification on amylopectin structure

The amylopectin CL distribution in the range of DP 4 to 80 was determined for all starch samples in Paper I. AM catalyzes the transfer of an α-glucan chain to a new 4-position in another α-1,4-glucan segment (or water). When acting on starch, AM has been proposed to transfer segments of amylose to amylopectin (Kaper, van der Maarel, Euverink, and Dijkhuizen, 2004). AM modification of potato starch results in the disappearance of amylose (Kaper *et al.*, 2005; van der Maarel *et al.*, 2005; Oh *et al.*, 2008), and it is therefore highly feasible that the amylose fraction is transferred to amylopectin. Nevertheless, multiple reactions must be taken in consideration, because normal starch is composed of two α-glucan molecules, structure of amylopectin is highly complex and, thirdly, because AM possess three activities. In Paper I, a model was proposed for AM actions on starch. Amylose is considered an essential linear α-glucan, but is in fact slightly branched (roughly one α-1,6-linkages per 1000 glucose residues) (Takeda, Hizukuri, and Juliano, 1986). Especially for the highly branched amylopectin molecules, multiple ‘substrate sites’ exist. Except for the genetically engineered HAP starch, the normal starch shows a bimodal amylopectin CL distribution having the main peak at ~DP 15-20 and a minor peak at around DP 40-50. Each chain in amylopectin can be considered a ‘substrate’, either acting as a donor or acceptor segment. The

dominating actions of AM on starch are probably a combination of amylose transfer to amylopectin and re-organization of chains in amylopectin molecules. Cyclization of amylopectin is possible in time-course modification using a relatively high amount of AM (van der Maarel *et al.*, 2002; Larsen, 2002). This was not investigated for any enzyme treated-starches.

Exactly what happens during AM modification of starch is difficult to say. One of things we know is that AM modification of starch results in disappearance of amylose (Kaper *et al.*, 2005; van der Maarel *et al.*, 2005; Oh *et al.*, 2008) and broadens amylopectin CL distribution (Kaper *et al.*, 2005; van der Maarel *et al.*, 2005). The broadened CL profiles were also shown for all starches in Paper I. All parent starches changed from a bimodal to a unimodal distribution of side chains in amylopectin, irrespective of the starch type being AM-modified (Fig. 23). The broadening resulted simultaneously in a decrease in short chain (DP 13-23) and an increase in long chains (DP 60-80). This increased the amylopectin mean CL, e.g. potato and maize starch goes from a mean CL from 32 → ~37 DP and 28 → 35 DP, respectively, following AM treatment (Fig. 23 left). Despite the lack of amylose in waxy maize starch, AM-modification of parent waxy maize starch also resulted in a broadening of the CL distribution (Fig. 23 right). This indicates the broadening effect is amylose-independent and must be an effect of intra-amylopectin chain transfer. Barely visible in Fig. 23 is a small increase in very short chains (below DP 6) following AM modification of starch. This can only be attributed to shortening of longer chains.

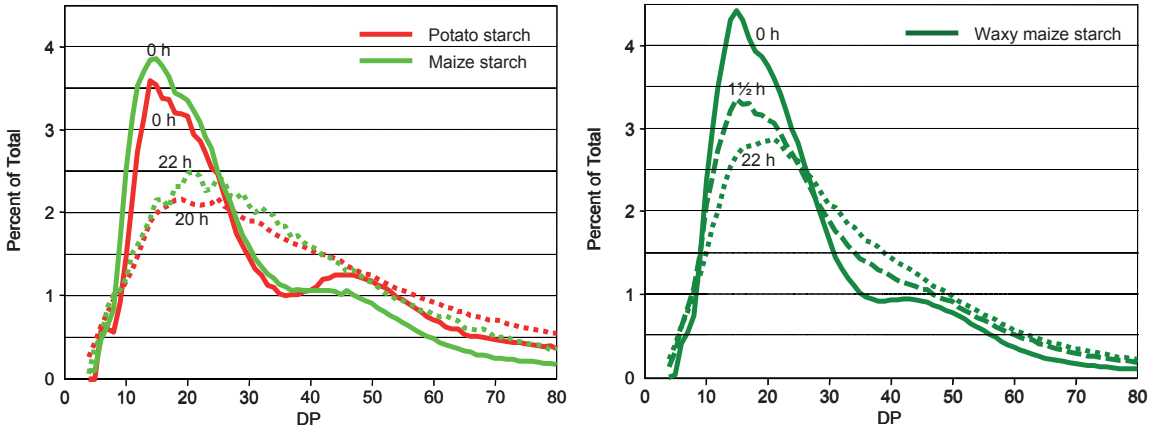


Fig. 23. The amylopectin CL distribution of potato and maize starch modified with 10 units AM/g starch (left) and waxy maize starch modified with 2 units AM/g starch (right). Presented in Paper I.

Extended AM-modification did not result in additional broadening of the amylopectin CL distribution. E.g. modifying potato starch comprehensively (20-22 h) with 2 units AM/g starch compared to 5-fold higher specific activity resulted in products with almost identical CL distributions, which indicates a limit to the degree of chain transfer that is possible. However, as noted in Paper I, within each time-course modification, extended AM modification resulted in a minor decrease in long chains.

Although only small changes were detected in the amylopectin CL distribution after extended enzyme treatment, clear changes in the structure of amylopectin were visualized when examining the Mw distribution by SEC/RI. When modifying potato starch for 15 min with 10 units AM/g starch the hydrodynamic size initially increases, indicating that the apparent Mw increases (Fig. 24). This is in good agreement with the suggested model in Paper I where amylose chains are transferred to amylopectin molecules (Kaper *et al.*, 2004). After 3½ h enzyme treatment, the hydrodynamic size decreases. Further modification (for 20 h) did not alter the amylopectin CL distribution (Paper I), but resulted in a product (# 11) having even more decreased hydrodynamic size. Potato starch modified for 22 h with 100 units AM/g starch eluted around 40 min and had a more homogeneous Mw distribution compared to # 11 (data not shown). The decrease in the absolute Mw was also reported elsewhere (van der Maarel *et al.*, 2005; Park, Kim, Kim, Cha, Kim, Kim, Kim, and Park, 2007). Both authors attributed the reduction of Mw as a result of minor hydrolytic activity of cluster segments. During catalysis, AM can use water molecules as an acceptor instead of a new α-glucan segment. The transglycosylation activity is about 5000-fold higher than the hydrolytic activity of the AM from *Thermus thermophilus* (Kaper *et al.*, 2007).

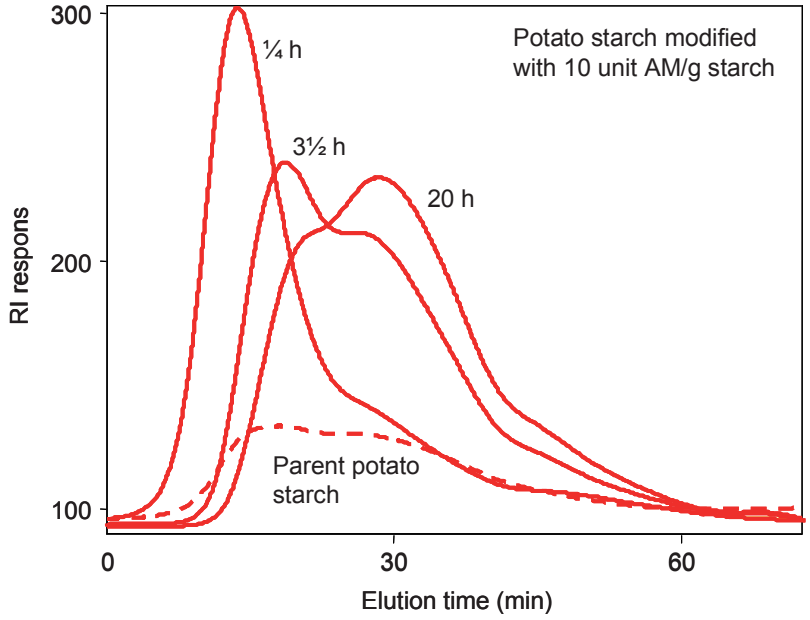


Fig. 24. SEC profile of the time-course AM-modified potato starch. The eluting fractions were monitored using a RI detector (Waters, USA). The apparent Mw values of the starches were determined from an extrapolation from pullulan standards.

6.1.1. Analysis of amylopectin CL distribution by PCA

The entire starch data set was evaluated by PCA in Paper I. The two most important PC's explain 94.2% of the total variance (Fig. 25). A quick examination of the score plot reveals that parent starches (located in lower right corner) are separated from the rest of the enzyme-modified starches. Each time-course modification as well as the botanical origin are distinguished (the modification direction is indicated by arrows). Hence, even after AM modification, the botanical origin is reflected in the amylopectin

CL distribution in the range of DP 4 to 80. Only AM-modified pea and potato starches cannot be distinguished (located middle left in the score plot). They have similar PC 1 and 2 score values, illustrating that these starches have almost identical amylopectin CL distribution in the range of DP 4 to 80.

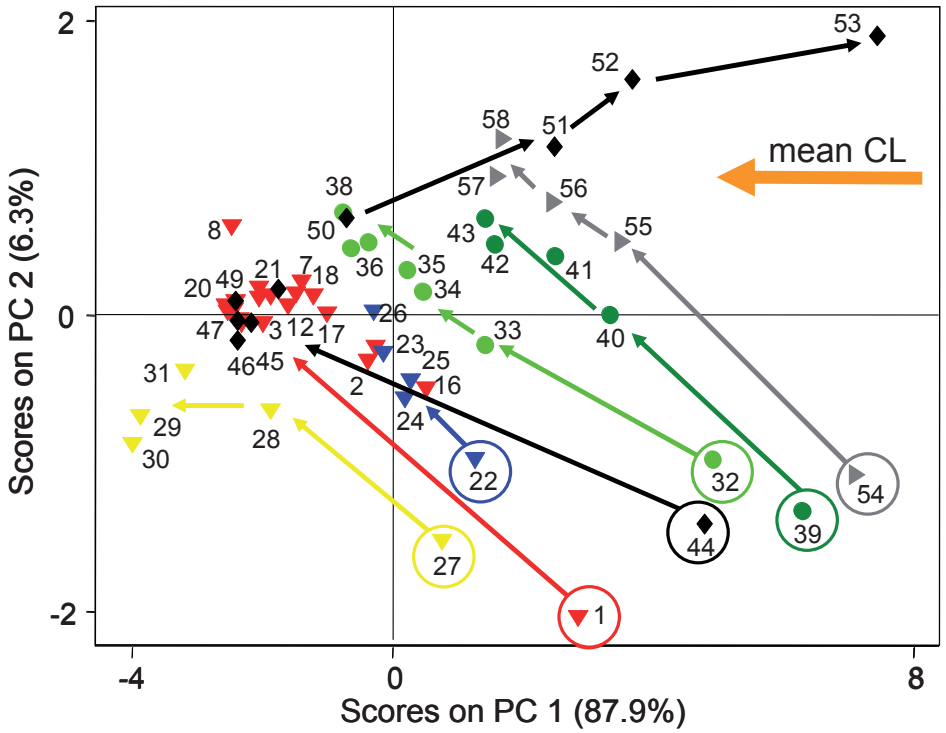


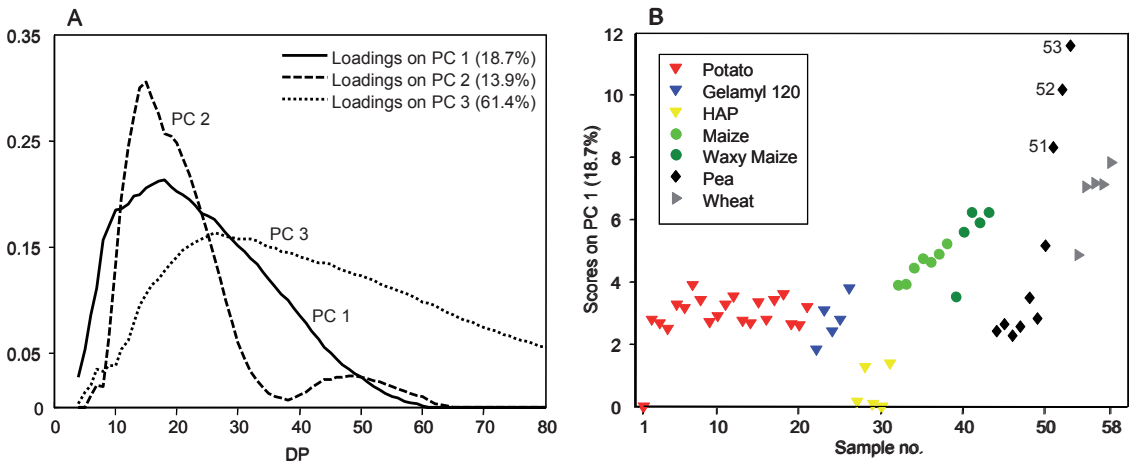
Fig. 25. PCA score plot of all amylopectin CL distribution for PC 1 vs. 2. The two PCs explain 94.3% of the variation. Data were mean-centered (Bro and Smilde, 2003) prior to PCA calculation. The arrows indicate the incubation time direction of the enzyme modification from the parent samples (circle). Starch samples and arrows are colored according to botanical origin: potato (red ▼), Gelamyl 120 (blue ▼), HAP (yellow ▼), maize (light green ●), waxy maize (dark green ●), pea (black ♦) and wheat (gray ►). Orange arrow indicates the levels of amylopectin mean CL. Sample numbers are indicated as in Table 3. Presented in Paper I.

PC 1 explains most of the total variance in the PCA model with 87.9%, and describes the amylopectin mean CL as indicated by the orange arrow. The second PC accounts only for 6.3% and describes very short chains. AM modification of the starches resulted in products having lower PC 1 and higher PC 2 score values compared to the parent starches. From the score plot it is evident that parent maize, waxy maize, pea and wheat starches have low mean CL (scoring high PC 1 value), and AM-modified HAP starches are those having highest mean CL (scoring lowest PC 1 value). The starting point seems to be important for the final amylopectin mean CL value following AM modification. That is: AM modification can only 'displace' to a certain degree, depending on the 'starting' material. The higher the starting mean CL of a parent starch, the higher the mean CL is after AM treatment, e.g. compare the modification of parent wheat and potato starch in Fig. 25. Modifying Gelamyl 120 with AM

resulted only in slight changes in the amylopectin CL distribution, which is illustrated by the small displacement in the score plot. Clearly, these structures are improper substrates for AM, probably because the oxidized groups on the starch interfere with binding to the active site or preventing enzyme catalysis. The effect of BE was investigated in combination with AM. BE from *Rhodothermus obamensis* has 6-fold higher activity toward amylose than amylopectin (Shinohara *et al.*, 2001). For that reason, pea starch was initially treated with AM for 2 h to allow disappearance of amylose, resulting in # 49 (located middle left in the score plot). The combined modification of # 49 with AM and BE as expected resulted in a decrease in long chains (DP 60-80) and increase in short chains while keeping the unimodal CL profile (for more details, see Paper I). The modification direction is easily identified on the PCA score plot where AM/BE-modified starches score high PC 1 and PC 2 values simultaneously and display orthogonal behavior to the AM modifications.

6.1.2. Analysis of amylopectin CL distribution by MCR

To further aid in interpretation of the hidden phenomena observed in PCA the method of MCR was applied. MCR is similar to PCA, but instead of describing major trends in the data according to variance, MCR extracts concentration profiles and pure component spectra instead of scores and loadings, respectively. Fig. 26 shows the concentration profiles plot and pure component spectra plot for the first three PC's of the MCR of the amylopectin CL data. Three PC's were found to give an appropriate description of the data. Each individual PC is shown for the scores in Fig. 26B-D. The three MCR PC's explain 94.0% of the total variation, which is about 2% lower than for the first PC of PCA in Fig. 25. Although PCA captures variance most efficiently, MCR is capable of obtaining physically meaningful concentration profiles and pure component spectra.



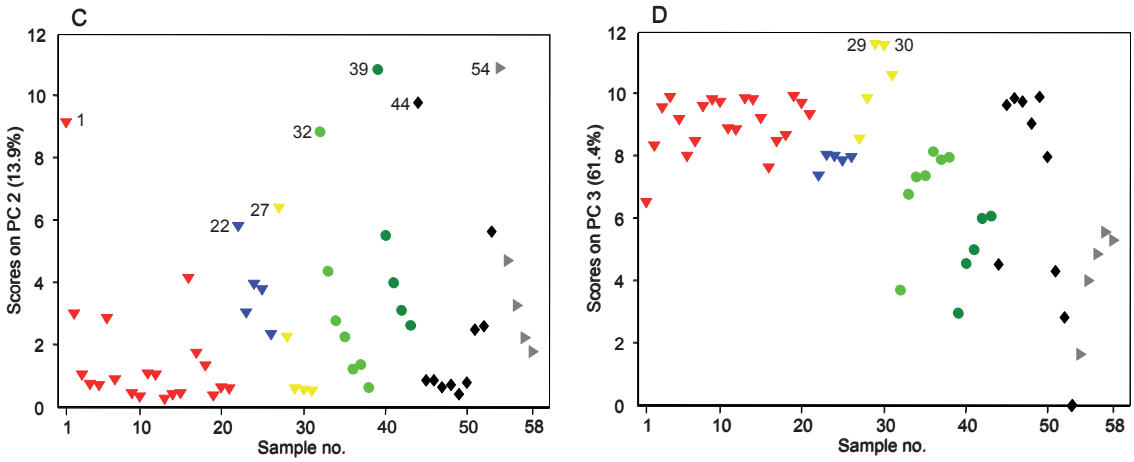


Fig. 26. MCR model of amylopectin CL data of all starch samples showing the loading plot (A), score plot for PC 1 (B), PC 2 (C) and PC 3 (D). The three PC's explain 94.0% of the total variation. Data was not preprocessed prior to MCR computation. Numbers in parenthesis represent explained data variance. Sample numbers are indicated as in Table 3.

The MCR model generated three major PC's, with PC 3 responsible for explaining 61.4% of the total variation. This PC describes the majority of the starches modified with AM. Unlike PCA, MCR does not necessarily describe the greatest variation in the first PC, as observed in this case. AM-modified HAP starches (# 29-30) scored the highest values (Fig. 26D) and had the most similar profile to PC 3 describing AM-modified starches that have large fraction of long chains and peaking at DP 25 (Fig. 26A). The BE-modified pea starches (# 51-53) were mostly described by PC 1, explaining 18.7% of the total variation (Fig. 26A and B). PC 1 describes samples that comprise a unimodal profile with few long chains, many short chains (DP < 10), and peaking at DP 15. PC 2 explains 13.9% of the total variation and describes the bimodal CL distribution of parent starches (Fig. 26A and C). Hence, the three types of profiles in the dataset are characteristic for the AM-modified, combined AM/BE-modified and parent starch systems.

6.2. The effect of modification on texture, starch mobility in gels & gel-melting

The effect of AM modification on starch gel functionalities was analyzed in Papers I-III.

6.2.1. Gel texture

The influence of AM modification on gel texture was analyzed in Paper I. In general, AM modification resulted in starches with increased gel texture compared to the parent sample at a concentration of 12% (w/w). Extended enzyme treatment, however, had a negative impact on gel texture. E.g. modifying potato starch for 22 h using 2 and 100 units AM/g starch resulted in gels with a 2½-fold reduction in *force of deformation* (3.9 → 1.5 Ns). AM modification of parent maize starch for 22 h increased the *force of deformation* more than 5-fold, whereas AM-treatment of parent HAP only slightly in-

creased (Fig. 27 left). AM-modified HAP starch gel (# 31) was twice as hard as gelatin gel (# 59). AM-modified potato starches made gels with comparable *force of deformation* compared to gelatin. The texture profile of gelatin was clearly very different from all the starch gels (Fig. 27 left). Gelatin makes much more elastic gels that during the 8 sec compression withstood gel fracture. An interesting thing about AM-modified maize starch gels is that they fracture latter from AM-modified potato starch gels, indicating stronger internal texture for longer time.

Combined AM/BE modification of pea starch had a dramatic negative effect on the gel texture (Fig. 27 right). After 1½ h modification of sample # 49, the *force of deformation* was reduced by 2½-fold. Modification for 5½ h and longer resulted in starches that were not capable of forming a gel network at a concentration of 12%. BE modification produced starches with a reduced fraction of long chains (DP 60-80) and an increased fraction of shorter chains (DP 4-25), which demonstrate the importance of long chains for the formation of a gel network.

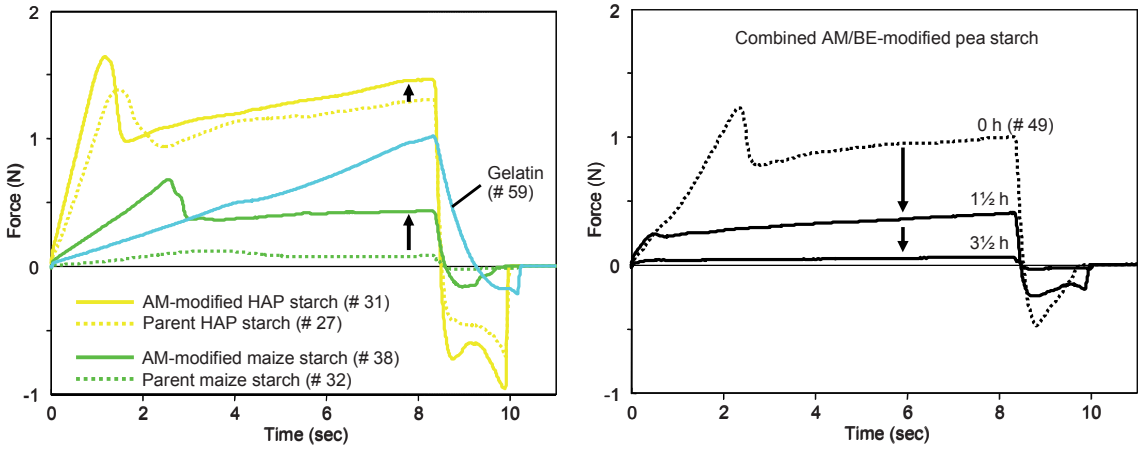


Fig. 27. Texture profiles of 12.0% (w/w) gels. Left: gelatin; parent and AM-modified HAP starch; parent and AM-modified maize starch. Right: Combined AM/BE-modified pea starch. Gels were stored at 4ºC for 24 h prior to texture analysis. Presented in Paper I.

In food application, lower amount of gelatin are usually used from that described in Paper I. Several of the starches in the dataset were unable to form gel network at lower concentration, and for direct comparison between the AM-modified starches a concentration of 12% was used. AM-modified HAP starch gel (# 31) was twice as hard as gelatin gel (# 59) at a concentration of 12%, but at lower starch concentration, gels of # 31 were less comparable to gelatin. Fig. 28 shows the gels # 31 at different concentrations compared to 5% gelatin gel. The non-linear relationship between starch concentration and gel strength is clearly apparent. The gel fracture was independent of starch concentration, fracturing after ~1 sec. A minimum concentration required for gel formation was approximately 5%. For extended AM-modified potato (# 5) and maize starch (# 38) the minimum concentration was approximately 5% and 7%, respectively. Compared to 5% gelatin gels, about 7½% # 31 was required to make a comparable gel texture (*force of deformation*). However, during the first second of compression, the gel

texture of 5% gel was comparable to 5% gelatin (Fig. 28). This was also found for 5% gel of extended AM-modified potato starch (# 5) (data not shown).

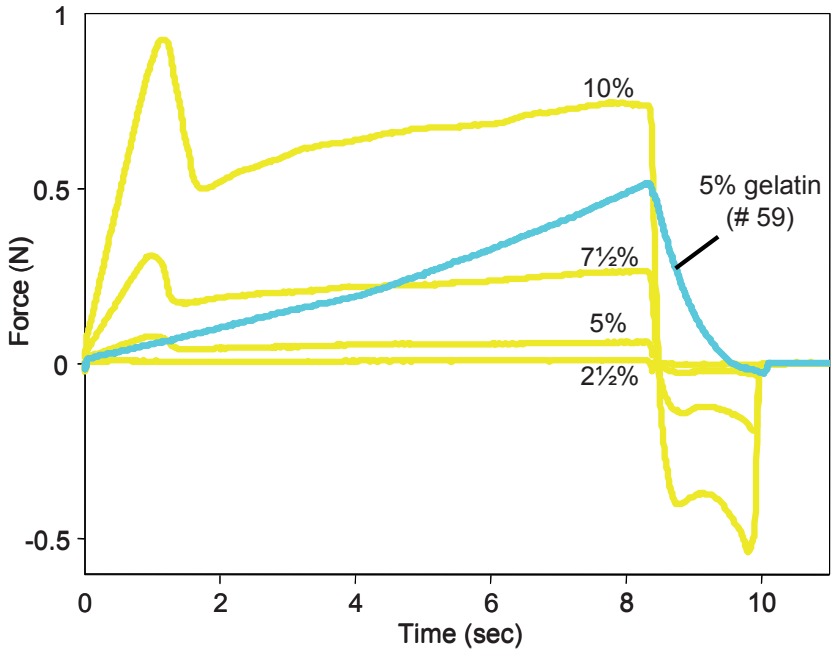


Fig. 28. Texture profiles of 2½, 5, 7½ and 10% (w/w) AM-modified HAP (# 31) gels compared to 5% (w/w) gelatin gel. Note that the force scale is different compared to Fig. 27.

Van der Maarel (2005) reported that 8% AM-modified potato starch gel had comparable gel strength to 6% gelatin gel (van der Maarel *et al.*, 2005). They measured the gel strength using a rheometer that performs a small deformation test in a non-destructive approach. This technique is suitable for studying the structural features and gelling mechanisms of starch gels. Texture profile analysis is a large deformation test that imitates the action (stroke) of the human jaw by compressing the gel sample. Textural parameters correlate very well with sensory evaluation parameters. The texture profiles in Fig. 27 and Fig. 28 can be considered as a 'one-bite-test'. However, 8.4 sec 'bite'/compression is rather unrealistic; the texture profiles clearly show great textural differences between AM-modified starch and gelatin gels. These differences have a great influence on the mouthfeel when chewing food products where gelatin has been replaced with an AM-modified starch.

6.2.2. Starch mobility

In Paper II, the changes/differences in polysaccharide mobility (or possibly exchange rate or hydroxyl accessibility) in the modified starch gels were evaluated by LF NMR. Water proton relaxation in 12.0% (w/w) starch and gelatin gels was determined after 1 and 10 days of storage at 20.0ºC. All starch and gelatin gels were characterized by a one-water component system as shown by discrete and distributed exponential analysis of the CPMG relaxation data. AM modification of potato starches (potato, Gelamyl 120 and HAP) resulted in products with decreased and narrower T_2 distribution compared to the parent sample, whereas AM-treatment of wheat, maize and waxy

maize starch resulted in similar or slightly increased T_2 time constant. This is illustrated in Fig. 29 showing the change in T_2 relaxation for each sample from Day 1 to Day 10. The ageing (gelation and/or retrogradation) of gelatin only slightly changes from Day 1 to Day 10, indicating minor changes in the polymer mobility. Except for parent Gelamyl 120, the parent starches were virtually unaffected after storage, as indicated in Fig. 29. All AM-modified HAP and AM-modified pea starch gels made harder or comparable gels to gelatin gels, but settled to a common T_2 value of ~100 ms after Day 10, which is about 260-330 ms lower compared to gelatin gels. The common low T_2 value shows that these starches have reached a limit of recrystallization. Hence, additional solidification of the polymers is not possible.

The negative effect on gel texture of combined AM/BE-modified pea starch gels, had a positive effect on polymer mobility, making gels with gelatin-like T_2 spectrum. Combined AM/BE-treatment resulted in increased T_2 relaxation. Gels of combined AM/BE-modified pea starch for 3½ (# 51), 5½ (# 52) and 22 h (# 53) resulted in T_2 going from 285, 392 and 477 ms, respectively, after 1 day of storage (Fig. 29). The parent potato starch and pea starch extended combined AM/BE-modified (# 53) had T_2 relaxations comparable to gelatin 1 sample (# 59) during both Day 1 and 10 Days of storage. However, these two starch samples were unable to form gel network at 12% (w/w).

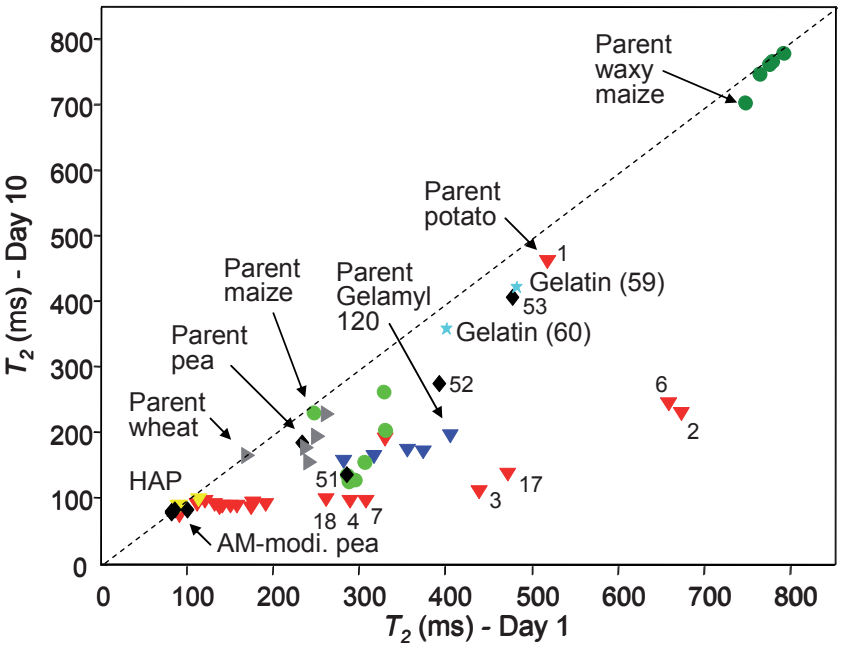


Fig. 29. T_2 relaxation time constants of Day 1 vs. Day 10 obtained by discrete exponential fitting of the raw CPMG relaxation curves. Presented in Paper II.

6.2.3. Gel-melting

A third functionality investigated in this thesis was the melting properties of the enzyme-modified starch gels (Paper III). AM modification of all starches resulted in increased gel peak temperature, T_p, compared to their corresponding parent starch. E.g.

parent starch (# 44) modified with 10 units AM/g starch for 2 h increased T_p from 62.9 to 79.4ºC (Fig. 30). The melting range of parent pea starch (from 40 to 80ºC), decreased following AM-treatment (range: 65 to 90ºC) indicating a more homogeneous distribution of crystal in the AM-modified pea starch gel. In general, the T_p of recrystallized amylopectin of all the starches ranged from 49.0 to 85.9ºC, and the enthalpy of transition (ΔH) ranged from 0.17 to 4.81 J/g starch. AM-modified HAP starches making comparable gels to gelatin were those with the highest T_p (82-85ºC). The increasing and relatively high T_p following AM modification of starch was unwanted, as gelatin gels had a melting point at 27-28ºC. Both gelatin gels were nearly completely melted at 37ºC (for more details, see Paper III). Combined modification of AM-modified pea starch (# 49) with BE for 1½ h resulted in decrease of T_p from 79.4 to 74.7ºC (Fig. 30). After 5½ h of combined AM/BE-treatment, T_p decreased by nearly 20ºC from 79.4 to 61.0ºC, and further treatment resulted in a product (# 52) that had no detectable transition even at 20% (w/w) due to its failure to form a gel network. Hence, BE modification suppresses the negative effect that AM modification had on gel T_p.

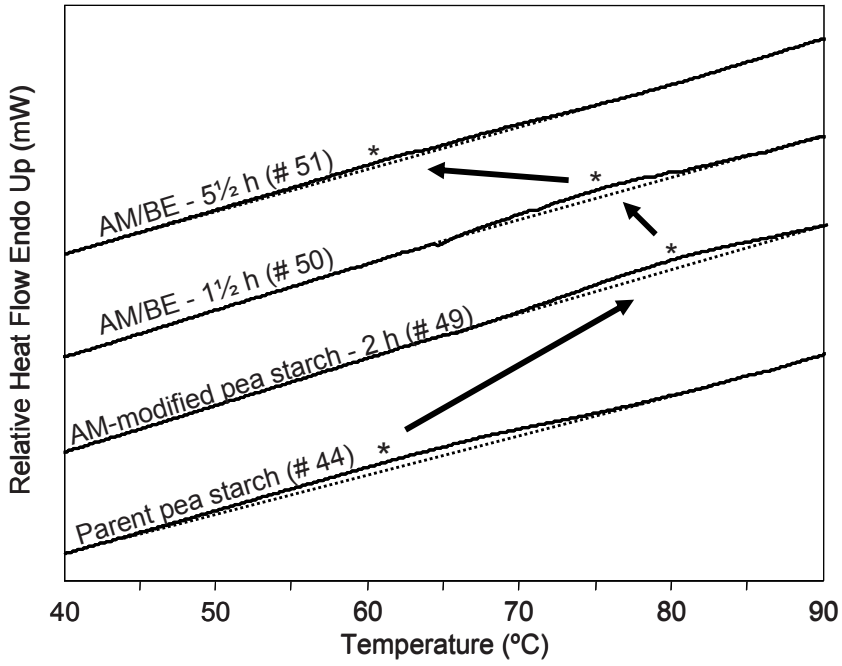


Fig. 30. DSC traces showing the thermal profiles of 20% (w/w) gels of pea starch modified with 10 units AM/g starch for 2 h following combined BE-treatment with 1000 units BE/g starch for additional. Stars indicate T_p. Scan rate: 10ºC/min. Presented in Paper III.

6.3. Relationship between amylopectin CL data and starch functionalities

Several interesting correlations between amylopectin CL distribution and starch functionalities were found in Papers I-III.

Numerous studies have emphasized the importance of amylopectin CL to functionnality or structure. It has been suggested that the CL is an important determinant of crystalline polymorph (Hizukuri, 1985). PCA of the amylopectin CL distribution from 44 plant species, starches of A-, B- and C-type crystalline polymorphs were separated in a

score plot (Blennow *et al.*, 2000), indicating that the CL data 'carries' information about the crystalline polymorph. Gelatinization properties of potato and wheat starch was found to be independent of amylose content, and related to the amylopectin CL distribution, especially short chains below DP 17 (Noda, Takahata, Sato, Suda, Morishita, Ishiguro, and Yamakawa, 1998; Singh *et al.*, 2008). A high correlation between the degree of phosphorylation and the distribution of CL in amylopectin has been established in several studies (Blennow *et al.*, 1998; Blennow *et al.*, 2000; Blennow *et al.*, 2005). More precisely, short chains (DP 12-13) and medium chains (DP 28-42) were found to be negatively and positively correlated to phosphate content, respectively (Blennow *et al.*, 2005). This correlation is actually indirect. A recent study has shown that the activity of GWD (and therefore the degree of phosphorylation) depends on the molecular order of α-glucan helices and is not correlated to the DP (Hejazi, Fettke, Haebel, Edner, Paris, Frohberg, Steup, and Ritte, 2008). However, it is very reasonable that the molecular order is correlated to the amylopectin CL distribution.

In Paper I, a correlation between CL data and gel texture was discussed. The possible correlation was also indicated in PCA models of descriptors in Papers II and III. A weak correlation was found between the gel texture and the sum of DP 60-80. A three-component PLS model gave a correlation of $r^2 = 0.66$ (RMSECV = 2.5%) between actual sum of DP 60-80 and sum of DP 60-80 predicted from all the starch gel texture. During AM-modification the fraction of DP 60-80 increases, generating amylopectin molecules having 'amylose-like' chains that are important forming formation of associated and connected aggregates forming strong gels (Miles *et al.*, 1985; Gidley, 1989).

The entire amylopectin CL distribution was found to be correlated to the gel (melting) peak temperature in Paper III. A five-component PLS model gave a correlation of $r^2 = 0.87$ (RMSECV = 2.5ºC) between actual T_p and T_p predicted from the CL data in the range of DP 4 to 80. When examining the relationship of each individual chain to T_p, it was found that short chains (DP 11-22) were negatively correlated to T_p, whereas long chains above DP 60 were positively correlated to T_p (Fig. 31). Accordingly, the effect of increased mean CL in amylopectin causes an increase in T_p in gels. This was rather unwanted, because AM-treatment of starch resulted in less-gelatin-like gels. Although acquiring thermoreversible gelling properties, it has little interest in the food industry, if the gels do not melt in the mouth. AM-treatment not only results in increase in long chains, but also decrease in short chains – both parameters cause elevated T_p. The correlation explains the decreased T_p of combined AM/BE-modified pea starch gels. The disadvantage of BE modification was the decreased gel texture.

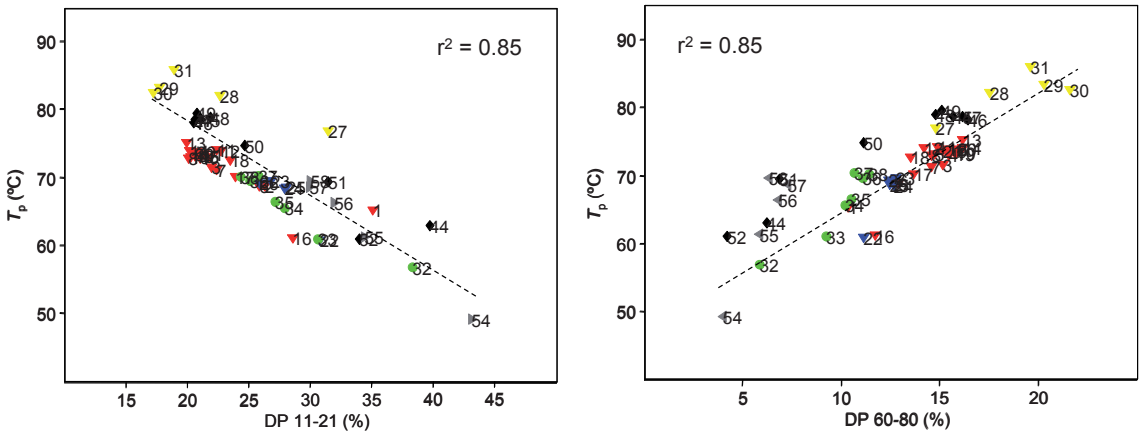


Fig. 31. Scatter plot of the sum of DP 11-21 vs. T_p (left) and the sum of DP 60-80 vs. T_p (right). Waxy maize starches (# 39-43) and pea starch modified with AM/BE for 22 h (# 53) were excluded. Starch samples are colored according to botanical origin: potato (red ▼); Gelamyl 120 (blue ▼); HAP (yellow ▼); maize (light green ●); pea (black ♦); wheat (gray ►).

In Paper II, a correlation between proton relaxation data and amylopectin mean CL data was found. Better prediction was also obtained using Day 10 relaxation data than using Day 1 data. The importance of both short and long chains was also evident for this functionality. A six-component PLS model predicting short chains (DP 13-23) and long chains (DP 60-80) using Day 10 relaxation data gave a leave-one-out validated correlation of $r^2 = 0.66$ (RMSECV = 3.76%) and $r^2 = 0.82$ (RMSECV = 1.79%), respectively (Fig. 32). The weaker correlation found between short chains and proton relaxation Day 10 data is attributed to the 'deviating' parent samples (# 1, 27, 32, 39, 44 and 54).

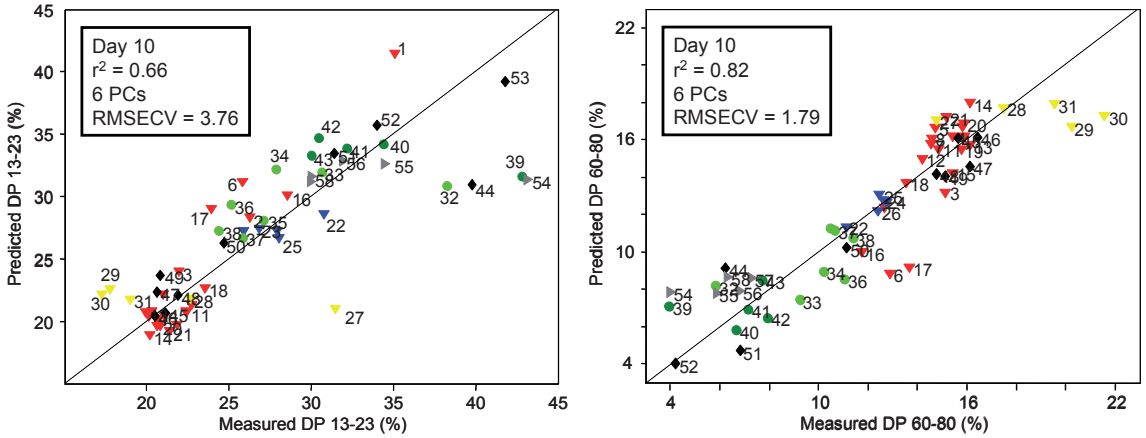


Fig. 32. Prediction of sum of DP 13-23 (left) and sum of DP 60-80 (right) from Day 10 proton relaxation data. Prior to PLS modeling, the data was preprocessed by mean-centering (Bro *et al.*, 2003). In the PLS regression model to the right, samples # 49 and 53 were excluded. Starch samples are colored according to botanical origin: potato (red ▼); Gelamyl 120 (blue ▼); HAP (yellow ▼); maize (light green ●); waxy maize (dark green ●); pea (black ♦); wheat (gray ►).

The high correlation between proton relaxation Day 10 data and amylopectin CL distribution data was exploited to estimate a 'gelatin-amylopectin' CL distribution using PLS2 regression (Geladi *et al.*, 1986). Fig. 33 shows the amylopectin CL distribution of all starch samples overlaid with the two *in silico* estimated CL distributions modeled from gelatin proton relaxation Day 10 data. Compared to most of the AM-modified potato, AM-modified HAP and AM-modified pea starches, the gelatin estimated CL distributions have a higher fraction of short chains (DP 10-25) and a lower fraction of both medium chains (DP 30-50) and long chains (DP 60-80). The amylopectin mean CL and the sum of DP 60-80 for the estimated gelatins are between 32-33 DP and 11-13%, respectively, and show distributions similar to parent Gelamyl 120 and HAP starches.

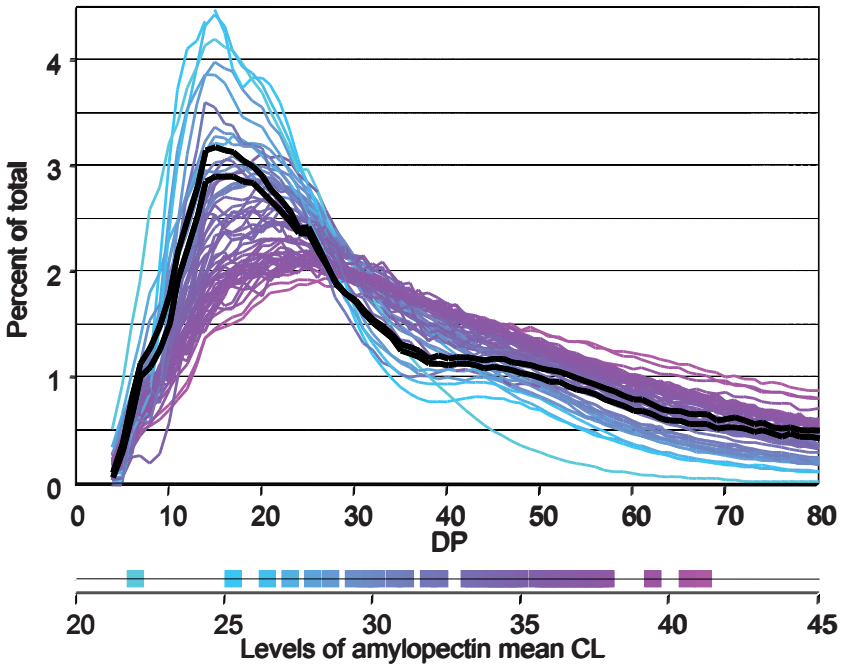


Fig. 33. The estimated 'gelatin-amylopectin' (black) and amylopectin CL distribution of all starch samples colored according to the levels of amylopectin mean CL. The 'gelatin-amylopectin' CL distributions were estimated using PLS2 regression using proton relaxation data in the X-matrix (58 starch samples × 4094 variables) and the amylopectin CL data in the Y-matrix (58 samples × 77 variables). In contrast to PLS1, PLS2 can handle multivariate properties of interest in the y-matrix. Presented in Paper II.

Chemometrics may provide some insight into a number of structural requirements for generating starch with gelatin-like functionality. It is interesting that the gelatins have comparable CL profile to Gelamyl 120, because the parent oxidized potato starch had similar T_2 relaxation time constant after 1 day of storage at 20.0ºC (Fig. 29). However, not accounted for in the modeling is the oxidized nature of Gelamyl 120 that has great influence on starch functionality, and can therefore not be used as a guideline for gelatin-like CL structure. Parent HAP starch also had comparable CL structure to gelatin, but had T_2 distribution very different from gelatin for both Day 1 and Day 10 of

storage (Fig. 29). Furthermore, applying the estimated gelatin CL data to the calculated gel texture and T_p PLS regression models previously mentioned in this Section predict gel-melting point to be 64 and 66ºC for gelatin 1 and 2, respectively (measured by DSC to be 27 and 28ºC) and predict gel texture to be 0.95 and 1.96 Ns for gelatin 1 and 2, respectively (measured by TA to be 4.16 and 6.16 Ns).

Hence, estimating starch structure having gelatin-like functionalities might only be achievable for one functionality at a time, and cannot cover the complete functional spectrum of gelatin through enzyme treatment of starch with AM or in combination with BE.

Despite of the 'misfiring' estimate of 'gelatin-amylopectin' CL distribution with regards to functionality, Papers I-III have clearly revealed interesting relationships between CL data and starch functionalities. It might be that the CL data is the most determinant factor controlling starch functionality. E.g. potato starch modified with 2 units AM/g starch for 5½ h (# 4) has almost identical amylopectin CL distribution to pea starch modified with 10 units AM/g starch for 5½h (# 47) (Fig. 34). Gels prepared from these starches showed similar gel texture, melting behavior and proton relaxation time constants at Day 10 in spite of a 4-fold apparent Mw difference. However, gels prepared from potato starch modified with 2 and 100 units AM/g starch for 22 h (# 5 and 15, respectively), which had similar CL distribution and differed by a factor 880 higher in the apparent Mw, showed about 2½-fold decrease in gel texture. This effect, however, can very well be attributed to the molecular size.

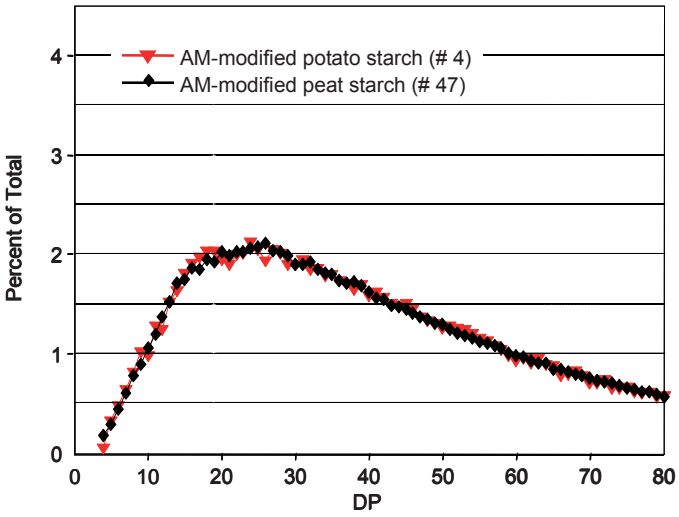


Fig. 34. Amylopectin CL distribution of AM-modified maize starch combined AM/BE-modified pea starch.

6.4. Overall picture

An overview of the correlations found in Papers I-III is illustrated in a PCA model calculated using descriptors from gel texture, apparent Mw, amylopectin CL, T_2 relaxation time constants for Day 1 and Day 10, and DSC (Fig. 35 left). Fig. 35 shows the loading and score plots for the two most important PC's. Pea starch modified with AM/BE for 22 h (# 53) and all waxy maize starches (# 39-43) were excluded in the

model, as the T_p and ΔH could not be determined. Gelatin samples were also excluded, as they are extremes concerning gel-melting and gel texture when compared to the starch samples. PC 1 describes the DP 60-80, amylopectin mean CL, gel texture and molecular mobility, whereas PC 2 mainly describes the ΔH variable. Four groups could be identified: Groups 1, 2 and 3 consisted exclusively of AM-modified potato, pea and HAP samples, whereas the rest of the samples comprised the fourth group (Fig. 35 right). Parent potato starch (# 1) located in the lower left corner was characterized by having low amylopectin mean CL, gel texture and T_p, but had comparable T_2 relaxation times (Days 1 and 10) to gelatin. Both AM-modified potato and pea starch gels had comparable gel texture to gelatin at a concentration of 12% (w/w), and had low T_2 relaxation times and higher mean CL, T_p and ΔH values. The ΔH reflects the energy required for disrupting the ordered junction zones, and was very low for AM-modified HAP starch gels, which were twice as hard as gelatin gels at 12% (w/w) (located in lower right corner). However, HAP gels also had the highest T_p (83-85ºC) and very low T_2 times (88-91 ms), all due to the high fraction of DP 60-80 (~20%).

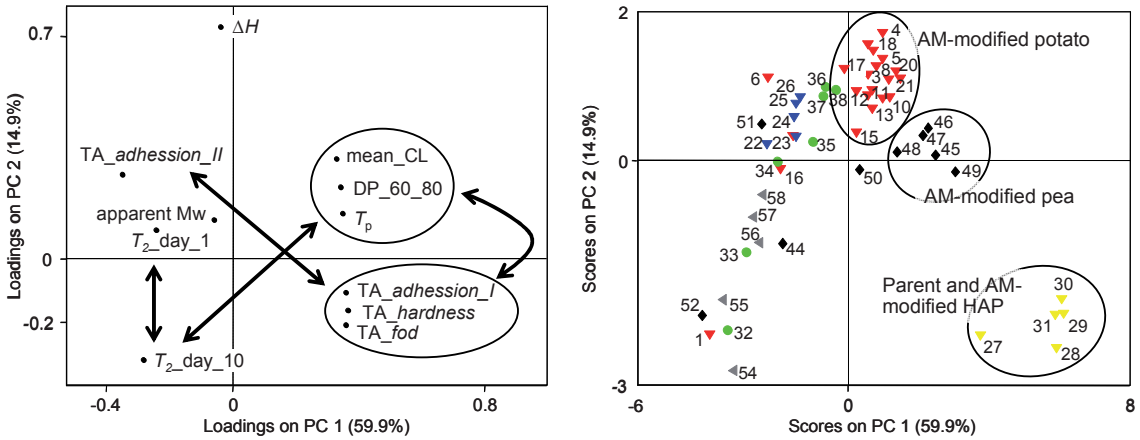


Fig. 35. PCA model of all descriptors of all starches. Loading plot (left) and score plot (right) for PC 1 vs. 2. The two PC's explain 74.8% of the total variation. Prior to modeling, descriptors were preprocessed by autoscaling (Bro *et al.*, 2003). Gelatin samples, waxy maize starches (# 39-43) and pea starch modified with AM/BE for 22 h (# 53) were excluded. Starch samples are colored according to botanical origin: potato (red ▼); Gelamyl 120 (blue ▼); HAP (yellow ▼); maize (light green ●); pea (black ♦); wheat (gray ►). Black arrows in the loading plot indicate the correlations between the descriptors. Presented in Paper III.

A measure of the thermoreversibility of the enzyme-modified starch gels would be an interesting supplement to the PCA model in Fig. 35. This could be accomplished by measuring the loss of storage modulus (G') as a function of temperature of AM-modified starch gels using a rheometer. Compared to gelatin gels, AM-modified starches do not form 'true' thermoreversible gels in a sense that they melt completely from solid to liquid upon heating. The AM-modified starch gels do lose storage modulus upon heating, but not completely, whereas the parent starch gels have unchanged storage modulus (Lee *et al.*, 2006; Oh *et al.*, 2008; Alting *et al.*, 2009) (and

unpublished observation). Furthermore, a clear difference in the melting behavior between AM-modified and the corresponding parent starch gels was evident (Paper III).

What makes AM-modified starches capable of forming thermoreversible gels has been proposed by Lee *et al.*, 2006 to be a combination of disappearance of amylose and a re-organized side chain distribution of amylopectin molecules. A recent study where maize starch was granular modified with AM from *Thermotoga maritima* resulted in consumption of amylose, an almost unaltered amylopectin CL distribution and gels with thermoreversible gelling property (Oh *et al.*, 2008). This shows that the main factor for acquired thermoreversible gelling property is the disappearance or consumption of amylose, and *not* the broadening of amylopectin CL distribution. However, the consumption of amylose without an increase in long chains in amylopectin results in similar gel texture to the parent starch (Oh *et al.*, 2008), whereas the broadening of amylopectin CL distribution result in gels with increased gel texture (Kaper *et al.*, 2005; van der Maarel *et al.*, 2005) (and Paper I). It is advantageous to use maize starch compared to amylose-free waxy maize starch, because normal maize starch contains a higher fraction of long chains, which in Paper I was implied to be important for gel formation. Modification of granular starch is of great interest in the food industry, because it bypasses the gelatinization and the precipitation step, and the granular structure remains intact after enzyme treatment that could be subject to further modification (e.g. chemical modification).

A problem with AM modification of starch is the increased gel-melting temperature (Paper III). Modifying potato starch with AM from *Pyrobaculum aerophilum* apparently result in gels having gel-melting point at 37ºC and the authors claim that the gel was completely melted at 60ºC as evaluated by DSC (Kaper *et al.*, 2005). The enzyme modification clearly caused a broadened amylopectin CL distribution and therefore resulted in a product (PyTSP) with increased mean CL. The low gel-melting point of PyTSP contradicts the Paper III result saying that increased CL in amylopectin result in elevated gel-melting point. Other studies have reported gel-melting of AM-modified starch gels to begin around ~45ºC (Lee *et al.*, 2006; Lee *et al.*, 2008; Alting *et al.*, 2009).

Although AM-modified starch gels have elevated melting property compared to gelatin gels, it does not have to be a problem in certain food products. Saliva contains α-amylase that readily degrades starch in the mouth. Perception of a starch-containing food product is affected by the presence of α-amylase activity in saliva. Alting *et al*. have shown that the addition of α-amylase led to an quicker decrease of firmness of AM-modified starch gels than without α-amylase activity (Alting *et al.*, 2009). The effect of both temperature and α-amylase activity comparable with human saliva on AM-modified starch gel was shown to have higher ‘melting’ performance compared to gelatin (Alting *et al.*, 2009).

7. General conclusions and perspectives

The overall conclusion of this thesis is that:

Structure:
- Modifying starch with AM caused broadening of the amylopectin CL distribution, creating a unimodal distribution having an increased fraction of long chains (DP 60-80) and decreased fraction of short chains (DP 13-23) irrespective of the starch type being modified.

- The broadening was independent of amylose content, as shown for the AM modification of waxy maize starch.

- The final fraction of DP 60-80 depends on the starch material being subject to AM modification. AM-modified potato and pea starch contains about 15% DP 60-80, which is about 5% more than AM-modified maize starch. AM-modified HAP starch contains about 20% DP 60-80.

- Extended enzyme treatment treatments reduced the apparent Mw and causing only small changes in the amylopectin CL distribution.

- Combined AM/BE modification resulted in starches with significantly increased short chains and reduced fraction of long chains.

Functionalities:
- The long chains were found to be positively correlated to gel texture and gel peak temperature (T_p), and negatively correlated to T_2 relaxation time (Day 1 and Day 10). Hence, AM modification of starch caused an increased fraction of long chains that resulted in improved gel texture, elevated $T_{p,}$ and reduced T_2 relaxation time. Combined AM/BE modification had the opposite effects.

- Extended enzyme treatment treatments reduced the gel texture without changing the T_p and T_2 times.

- AM-modified potato, pea and HAP starch gels made higher or comparable gel texture to gelatin at a concentration of 12% (w/w). They also made, to some extent, comparable gel texture to gelatin at 5% (w/w). However, the texture profile of all AM-modified starch gels was clearly very different to gelatin, which makes highly elastic gels.

- AM-modified starch gels had T_p ranging from 60 to 85ºC, which is in contrast to gelatin gels having T_p of 27-28ºC. Furthermore, the molecular mobility of AM-modified starch gels was significantly different to gelatin at Day 10 of storage. Combined AM/BE modification of pea starch resulted in reduced T_p and increased T_2 relaxation time, but because of reduced fraction of long chain, paste was unable to form gel network at a concentration of 12%(w/w).

Requirements for generating gelatin-like starch:

- The completely functional spectrum of gelatin cannot be obtained through enzymatic modification of starch with AM or in combination with BE. Some of these include transparency of gels, high gel-elasticity and cold water solubility.

- A unique property of gelatin is its ability to form thermoreversible gels. This property is mainly acquired through disappearance or consumption of amylose (Oh *et al.*, 2008).

- Increase of long chains is an important requirement as it result in improved gel texture. The increased T_p was demonstrated by Alting *et al.*, 2009 to be less important due to the presence of α-amylase in saliva, which improves gel-melting performance.

- Producing a starch with extra long chains can be accomplished by AM modification of starch supplemented with linear maltodextrin or debranched starch.

In future work, analysis of freeze-thaw stability, film forming properties, resistant starch parameters, solubility and how thermoreversible the gels are should be investigated. Additional structural analysis of the starches is required. AM modification of starch in combination with other starch-acting enzymes such as maltogenic α-amylase and β-amylase should also be investigated.

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Paper I

Gel texture and chain structure of amylomaltase-modified starches compared to gelatin

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Gel texture and chain structure of amylomaltase-modified starches compared to gelatin

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bstract

Amylomaltase (AM) (4-α-D-glucanotransferase; E.C. 2.4.1.25) from *Thermus thermophilus* was used to modify starches from various otanical sources including potato, high amylose potato (HAP), maize, waxy maize, wheat and pea, as well as a chemical oxidized potato arch (Gelamyl 120). Amylopectin chain length distribution, textural properties of gels and molecular weight of 51 enzyme and 7 non-zyme-modified starches (parent samples) were analyzed. Textural data were compared with the textural properties of gelatin gels. odifying starch with AM caused broadening of the amylopectin chain length distribution, creating a unimodal distribution. The crease in longer chains was supposedly a combined effect of amylose to amylopectin chain transfer and transfer of cluster units within e amylopectin molecules.

Exploratory principal component analysis (PCA) data analysis revealed that the data were composed of two components explaining .2% of the total variation. Parent starches formed a cluster separated from that of the AM-modified starches.

Extended AM treatments reduced the apparent molecular weight and the gel texture without changing the amylopectin chain length stribution. However, the gel texture was typically increased as compared to the parent starch. AM-modified HAP gels were about twice hard as gelatin gels at identical concentration, whereas gels of pea starch were comparable to gelatin gels. Modifying Gelamyl 120 and axy maize with AM did not change the textural properties. Branching enzyme (BE) (1,4-α-D-glucan branching enzyme; EC 2.4.1.18) om *Rhodothermus obamensis* was used in just one modification and in combination with AM. The combined AM/BE modification of ea starch resulted in starches with shorter amylopectin chains and pastes unable to form gel network even at concentration as high as .0% (w/w). The PCA model of all gel texture data gave suggestive evidence for starch structural features being important for nerating a gelatin-like texture.

ywords: Starch structure; Gelatin; Amylopectin chain length distribution; Gel texture; Amylomaltase; Branching enzyme; Chemometrics

Introduction

Starch is the most important energy source in human ets. Besides being an energy resource starch is an nportant ingredient widely used in the food industry as functional texturizer. Starch is stored in granules and und in almost every kind of plant cell, but cereal seeds .g. maize, wheat, rice, barley) and tubers/roots (e.g. potato, tapioca) are especially rich in starch. Starch is generally composed of two different types of α-glucan polymers: (1) amylose, a linear molecule consisting almost exclusively of α-1,4-linked glucose residues; (2) amylopectin, which, in addition to linear chains of α-1,4-linked glucose, also contains α-1,6-linked branch points. When heated in water gelatinization occurs, generating a highly viscous paste. Upon cooling, the gelatinized starch forms a textural gel network provided that the starch concentration has reached a critical concentration. For the development of the initial gel network amylose is a key player, having a

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tendency to quickly form connected double-helix aggregates (Gidley, 1989; Miles, Morris, & Ring, 1985). Amylopectin gelation, on the other hand, is a slower process involving weaker reversible chain associations.

Starch is a major contributor for food texture. However, native starch has several limitations, including low textural properties, high tendency towards retrogradation ('recrystallization'), low solubility in cold water, low shear and thermal resistance, poor flavor release properties, and a generally high viscosity. To improve performance, chemical or physical modification is required (Jacobs & Delcour, 1998). Alternatively, improved functionality can be achieved by genetic approaches directly in the crop (Jobling, 2004) or post-harvest enzymatic modification (Kaper, van der Maarel, Euverink, & Dijkhuizen, 2004; Lee, Kim, Park, & Lee, 2006). The advantage of using enzymes technology as compared to chemical modification is the specificity of enzymatic reactions, higher yields, fewer by-products and consumer safety.

In this study, starches of different botanical origin were modified using amylomaltase (AM) (4-α-D-glucanotransferase; E.C. 2.4.1.25) isolated from the hyperthermophilic bacterium *Thermus thermophilus* (Kaper et al., 2004; van der Maarel et al., 2005) and branching enzyme (BE) (1,4-α-D-glucan branching enzyme; EC 2.4.1.18) isolated from the thermophilic bacterium *Rhodothermus obamensis* (Shinohara et al., 2001).

AM has three activities. It catalyzes the reversible intermolecular transfer of a segment of a α-1,4-D-glucan to a new 4-position in another α-1,4-D-glucan or glucose in a disproportionation reaction:

$$(\alpha\text{-}1,4\text{-D-glucan})_m + (\alpha\text{-}1,4\text{-D-glucan})_n \leftrightarrow (\alpha\text{-}1,4\text{-D-glucan})_{m-x} + (\alpha\text{-}1,4\text{-D-glucan})_{n+x}. \quad (1)$$

This reaction is a variation of the α-retaining mechanism (Davies & Henrissat, 1995). AM also catalyzes intramolecular transglycosylation reaction from linear α-1,4-D-glucan as substrates, resulting in circular α-glucans (termed cycloamylose) with a DP of 16 or more (Takata et al., 1996; Uitdehaag et al., 1999):

$$(\alpha\text{-}1,4\text{-D-glucan})_{\mathrm{m}} \leftrightarrow \mathrm{cyclic}(\alpha\text{-}1,4\text{-D-glucan})_x + (\alpha\text{-}1,4\text{-D-glucan})_{m-x}. \quad (2)$$

This reaction is also reversible and the reverse reaction is termed "coupling". For the transglycosylation reactions (reactions (1) (2)), no new α-1,4-glycosidic bond is introduced or removed. In addition, the AMs also have minor endoamylolytic activity (van der Maarel et al., 2005):

$$(\alpha\text{-}1,4\text{-D-glucan})_m + \mathrm{H_2O} \rightarrow (\alpha\text{-}1,4\text{-D-glucan})_x + (\alpha\text{-}1,4\text{-D-glucan})_{m-x}. \quad (3)$$

However, of the three activities, the disproportionation reaction is by far the dominating activity for the *Thermus thermophilus* enzyme (van der Maarel et al., 2005). The resulting starch gels acquire thermoreversible characteristics (van der Maarel et al., 2005) and therefore AM-modified starches are expected to find application in the foo industry as a replacement of gelatin—a protein produ derived from animal collagen (Euverink & Binnema 200 Gelatin is a relatively expensive product having the abili of forming firm, thermoreversible, nearly tasteless tran parent gels—properties which normal starch lacks (Djagn Wang, & Xu, 2001). However, the reactions taking place different treatments and type of starches as well as t resulting functionalities of the resulting modified starch have not been studied in detail. To date, AMs fro different organisms have been investigated, includi potato (Takaha, Yanase, Takata, Okada, & Smith, 199 *Thermus thermophilus* (van der Maarel et al., 200 *Pyrobaculum aerophilum* (Kaper et al., 2005); *Therm scotoductus* (Lee et al., 2006); *Thermus aquaticus* (Przyl et al., 2000; Terada, Fujii, Takaha, & Okada, 1999); a *Aquifex aeolicus* (Bhuiyan, Kitaoka, & Hayashi, 200 Terada et al., 1999). However, only modification of pota and rice starch has been reported.

The second enzyme used in combination with AM is B It catalyzes the inter- or intramolecular transglycosylatio of a segment of a α-1,4-D-glucan chain to form a new α-1, glycosidic branching linkage in the glucan. BE can al catalyze intramolecular reactions in amylose and amyl pectin, resulting in the formation of cyclic structur (Takata et al., 1997, 1996). The increase of α-1,6 branchi linkages results in the reduction of amylopectin cha length, thereby increasing solubility and reducing retr gradation (Spendler and Jørgensen, 1997; Takata et a 1997).

Both AM and BE have recently been highlighted important in producing starches with new functionalitie which are expected to find specific applications in the foo industry (Fujii et al., 2003).

In this work, we have used AM to modify potat oxidized potato (Gelamyl 120), high amylose pota (HAP), maize, waxy maize, pea and wheat starch. Th starches were selected to provide a wide range of differe structures and physical characteristics. We report here th structural and textural properties of 58 modified starche The textural properties were compared with two gelat types. Exploratory data analysis and extraction of releva information from the data were carried out using princip component analysis (PCA). The potential for usi enzyme-modified starch systems to generate gelatin-li textures is discussed.

2. Materials and methods

2.1. Materials

Potato, maize and waxy maize starch were fro Cerestar-AKV I/S, Cargill (www.cerestar.com). Oxidize potato starch (Gelamyl 120) and pea starch were provide from KMC, Denmark (www.kmc.dk). Pajbjerg Found tion and Bente Wischmann DTU (Technical University Denmark) kindly provided wheat starch. Genetical

ngineered high amylose potato (HAP) starch was gener-ed as described (Blennow et al., 2005). Gelatin bloom 120 ample no. 59) and bloom 300 (no. 60) were from gma-Aldrich (www.sigmaaldrich.com). Purified amylo-altase (AM) from *Thermus thermophilus* (van der Maarel al., 2005) and branching enzyme (BE) from *Rhodother-us obamensis* (Shinohara et al., 2001) were provided by ovozymes A/S, Denmark (www.novozymes.com). One nit of AM activity is defined as the release of 1 μmol of ucose per minute at pH 6.5, 60 °C with maltotriose as bstrate, and one unit of BE is defined as the amount of zyme that decreases A_{660} of the amylose–iodine complex 1% per minute at 60 °C, pH 7.0.

2. Production of enzyme-modified starch

AM and BE were used to modify starches of different otanical origin in a time-course series ranging from 0 to 2 h. Native, chemically modified and genetically engi-eered starch were enzymatically modified with AM at 85 70 °C, pH 5.5 and BE at 60 °C, pH 6.5. Starch was spersed at a concentration of 15.0% (w/w) in 1.5 l eionized water, pH was adjusted to the appropriate value, nd gelatinization carried out by autoclaving (multicontrol utoclave, CertoClav, www.certoclav.com) for 30 min at 40 °C. After cooling to appropriate temperature, pH was adjusted to 5.5 or 6.5. AM was added to a final specific ctivity of 2, 10 or 100 unit/g starch, whereas BE was added a final activity of 1000 unit/g starch. After incubation for , $3\frac{1}{2}$, $5\frac{1}{2}$ and 22 h, the enzyme reaction was terminated by oiling for 10 min. Selected samples were incubated for 5 min (nos. 6, 16 and 33) and 45 min (nos. 7, 17 and 34) nd others for $2\frac{1}{2}$ h (no. 9) and 20 h (no. 11). Starch was urified by precipitation in 90% ethanol, blended, filtered, ashed and dried at 22 °C for 4–5 days. Native starches, ere not enzyme treated, and were also autoclaved, urified and dried under the same conditions as for the zyme-modified samples. These samples (nos. 1, 22, 27, 2, 39, 44 and 54) are from here on mentioned as parent mples. An overview of all samples is given in Table 1.

3. Starch analyses

Several physico-chemical and functional properties of e starch samples were analyzed. All data are average of uplicate measurements.

3.1. Starch phosphate

The phosphate covalently esterified to the starch was etermined enzymatically as the content of glucose-6-hosphate after complete acid hydrolysis, as described reviously (Blennow, Bay-Smidt, Wischmann, Olsen, & oller, 1998).

3.2. Amylose content

The amylose content was determined by iodine com-exation as described by Hovenkamp-Hermelink et al. (1988). Instead of using purified amylose and amylopectin as standard, starches with verified amylose:amylopectin ratios as determined by size exclusion chromatography (Blennow, Bay-Smidt, & Bauer, 2001) were used as standards, avoiding problems with non-pure amylopectin and amylose.

2.3.3. Amylopectin chain length distribution

Starch samples (5 mg) were debranched using 1.4 unit isoamylase (Megazyme) in 50 mM $NaCH_3COO$ (pH 4.0) followed by incubation for 1 h at 40 °C. The resulting linear glucan fragments were analyzed by high-performance anion-exchange chromatography with pulsed ampero-metric detection (HPAEC–PAD) carried out using the Dionex DX 500 system. Samples of 10 μl (50 μg of linear α-glucan) were separated on a CarboPac PA-200 column using 1 ml/min flow rate, 150 mM isocratic NaOH and the following NaOAc gradient profile: 0–5 min, 0–110 mM linear gradient, 5–130 min, 110–350 mM convex gradient. Single peaks between the degree of polymerization (DP) 4–80 were integrated and corrected for detector response factor as previously described (Blennow et al., 1998).

2.3.4. Textural properties of gels

Texture profile analysis (Bourne, 1978) was performed by a single compression using a TA-XT2i texture analyzer (Stable Micro Systems). Starch and gelatin pastes (12.0% w/w) were prepared in an RVA canister by dispersion of 3.0 g sample with 22.0 ml standard buffer (50 mM K_xP_i buffer, pH 7.0, 0.02% NaN_3) at 95 °C for 17 min under constant stirring. The dissolved sample was allowed to form a gel in the RVA canister (gel height: 2.5 cm and diameter: 3.5 cm) after storage at 4 °C for 24 h prior to analysis. The samples were compressed with a cylindrical probe (part no. P/4, Stable Micro Systems) at a pre-test speed of 5 mm/s, test speed of 0.9 mm/s and post-test speed of 5 mm/s. The data were evaluated as follows: the force built up during compression generated a gel fracture that often resulted in the maximal force and was hence termed *max force* (N). Following *max force*, compression continued until probe retraction. The area of compression (0–8.4 s) is termed *force of deformation* (*fod*; N s). During retraction (8.4–11.0 s), the negative force generated as the gel adheres to the probe resulted in positive and negative retraction areas termed *adhesion I* (N s) and *adhesion II* (N s), respectively. We are well aware that using a concentration of 12.0% for gelatin is an unrealistic high value. However, some of the AM-modified starches are unable to form gel network at lower concentration, since the experimental design requries that all samples form a gel network even at the lowest concentration.

2.3.5. Molecular weight distribution

The apparent molecular weight averages (Mw) of starch samples were determined by using size exclusion chromatography. Filtered and degassed ammonium for-mate buffer (50 mM, pH 3.4) was pumped at a flow rate of

Table 1
Physico-chemical characteristics and enzyme modification conditions of starch samples and gelatin

Sample no.	Botanical origin	Starch modification		Amylopectin mean chain length (DP)	Starch phosphate (nmol G6P/mg starch)	Amylose conten (%)
		Enzyme activity (unit AM/g starch)	Incubation temperature (°C)			
1[a]	Potato	–	–	32.0	23.2	17.2
2–5[b]	Potato	2	85	35.0–37.6[c]		
6–11[d]	Potato	10	85	34.8–37.7		
12–15[b]	Potato	100	85	36.2–37.8		
16–21[e]	Potato	10	70	33.9–37.8		
22[a]	Gelamyl 120	–	–	33.3	–	ND
23–26[b]	Gelamyl 120	10	85	34.3–34.7		
27[a]	HAP	–	–	35.0	60.0	38.5
28–31[b]	HAP	2	85	37.9–41.2		
32[a]	Maize	–	–	28.1	0.1	28.2
33–38[e]	Maize	10	85	32.2–34.5		
39[a]	Waxy maize	–	–	26.5	0.1	0.1
40–43[b]	Waxy maize	2	85	29.4–31.3		
44[a]	Pea	–	–	28.6	0.5	39.4
45–48[b]	Pea	10	85	36.6–37.9		
49[f]	Pea	10	85	37.4		
50–53[b,g]	Pea	10	60	22.0–34.3		
54[a]	Wheat	–	–	25.4	0.0	35.2
55–58[b]	Wheat	2	85	28.6–30.7		
59–60[h]	Gelatin	–	–	–	–	–

AM: amylomaltase. G6P: glucose-6-phosphate. Gelamyl 120: oxidized potato starch. HAP: high-amylose potato. ND: not detectable.
[a]Not enzyme-modified starches (parent samples), but autoclaved and purified by ethanol precipitation.
[b]Enzyme modified for $1\frac{1}{2}$, $3\frac{1}{2}$, $5\frac{1}{2}$ and 22 h, respectively.
[c]Only the lowest and highest values given.
[d]Enzyme modified for 15 min, 45 min, $1\frac{1}{2}$, $2\frac{1}{2}$, $3\frac{1}{2}$ and 20 h, respectively.
[e]Enzyme modified for 15 min, 45 min, $1\frac{1}{2}$, $3\frac{1}{2}$, $5\frac{1}{2}$ and 22 h, respectively.
[f]Enzyme modified for 2 h where after cooled to 60 °C and used to produce nos. 50–53.
[g]Combined modified with 1000 unit BE/g starch.
[h]Not modified.

0.8 ml/min through a column system thermostated at 40 °C consisting of TSK-G2500-PWXL, TSK-G3000-PWXL, TSK-G4000-PWXL and TSK-G6000-PWXL analytical columns protected by a guard column (TSK gel PWXL) (Sigma-Aldrich, www.sigmaaldrich.com). Each starch sample was dissolved in Milli-Q water at a concentration of 0.5% (w/w) in a boiling water bath for 10 min or until a clear sample was achieved. The sample was filtered through a Minisart plus (0.45 μm) filter and 100 μl was injected onto the columns at ambient temperature. PL Polysaccharide Std. Kit SAC-10 (Polymer Laboratories, www.polymerlabs.com) was used as standard. The eluting fractions were monitored using an RI detector from Waters, USA. The apparent Mw values of the starches were determined from pullulan standards. It should be noted that there are certain limits to the method as the apparent Mw values are determined from an extrapolation from the standards.

2.4. Data analysis

Two chemometric techniques were chosen for data compression, extraction and visualization. Principal component analysis (PCA) (Hotelling, 1933; Wold, Esbense & Geladi, 1987) was carried out for describing systemat variations in the complex data structures. Partial leas squares (PLS) regression (Geladi & Kowalski, 1986) w used to enable prediction of a property of interest (**y** dat from a measured amylopectin chain length or gel textu data (**X** data).

2.4.1. Principal component analysis (PCA)

PCA (Hotelling, 1933; Wold et al., 1987) is a usef technique for data compression and extraction of inform tion. This technique describes major trends in the data b the extracted principal components (PC). Mathematicall it relies upon an eigenvector decomposition of th covariance matrix of the data. PCA decomposes the da matrix **X** (samples × variables) into a score matrix (**T**) an a loading matrix (**P**) plus an error/noise or residual matr (**E**): $\mathbf{X} = \mathbf{TP}' + \mathbf{E}$ (apostrophe means transposed). Th score values contain information on how the sampl relate to each other, whereas the loading values conta information on how the variables relate to each other. Th outer product of the first score vector $\mathbf{t_1}$ and th

rresponding loading vector $\mathbf{p_1}$ gives the first PC, which escribes the largest variation in the data matrix **X**. The cond PC is constrained to be orthogonal to the first one oth for score and for loading vectors) and describes the cond largest variation, etc. (each PC is constrained to be rthogonal to all previous PCs). The PCA result is sualized in scores and loading plots. The loading values n be considered as hidden composite profiles that are mmon to all the measured samples, and the score values e the concentrations of the profiles for each sample.

4.2. Partial least-squares (PLS) regression

Relationships between predictor variables **X** (e.g. a atrix of gel texture) and the property of interest **y** (e.g. m of DP 60–80) were modeled using PLS regression Geladi and Kowalski, 1986). PLS estimate components, hich simultaneously maximize the covariance between ores in **X** and scores in **y** and describe the variance in **X**. LS is a linear multivariate model that decomposes **X** and in such a way that the information in the **y** vector is rectly used to direct the decomposition of **X**. The linear gression model is defined as: $\mathbf{y} = \mathbf{Xb} + \mathbf{f}$, where **b** is the ctor of regression coefficients and **f** is the residuals. The odel performance is evaluated by the cross-validated rrelation coefficient (r^2), which is the correlation between e measured **y** and the predicted **y**, and by the root mean-quare error of cross-validation (RMSECV) (Næs, Isaks-n, Fearn, & Davies, 2002).

4.3. Validation

PCA and PLS models are all mean centered and the MSECV was used to determine the optimal number of LS components. All models are leave-one-out cross-alidated, where each sample is left out of the model and redicted once (Stone, 1974; Wold, 1978).

4.4. Programs

PCA and PLS regression techniques were carried out sing MatLab 7.2 (The Mathworks, www.mathworks.com) stalled with the PLS Toolbox ver. 3.5.3 (Eigenvector esearch, www.eigenvector.com). Fig. 5A was created sing LatentiX Ver 1.00 (Latent5, www.latentix.com).

Results

Starches prepared from the various botanical origins were nzymatically modified using amylomaltase (AM) from *hermus thermophilus*. Starches with a wide range of olecular structures were selected in order to broaden the ariation in functionality. Potato, oxidized potato (Gelamyl 20), high amylose potato (HAP), maize, waxy maize, pea nd wheat starches were all modified with AM, whereas pea arch was subjected to a combined modification by AM rther modified with branching enzyme (BE) from *Rho-othermus obamensis*. Each starch was modified in a time-ourse, resulting in 51 enzyme-modified starch samples, hich were compared to their corresponding parent starch (7 non-enzyme-modified samples). See Table 1 for an overview of the samples and modification conditions.

3.1. Physico-chemical properties

3.1.1. Phosphate and amylose content of the parental starches

The phosphate contents of maize, waxy maize, pea and wheat starch (nos. 27, 32, 39, 44 and 54) were essentially phosphate-free (below 0.5 nmol G6P/mg starch). Potato starch (no. 1) had a G6P content of 23.2 nmol/mg starch (Table 1), equivalent to one phosphate group for every 200–300 glucose units. Transgenically, HAP starch contained 3-fold higher phosphate content compared to parent potato starch. The high phosphorus content for potato is a unique property among natural starches.

Potato and maize starch had amylose contents of 17.2% and 28.2%, respectively, whereas waxy maize starch was essentially amylose free (Table 1). Amylose was not detectable in Gelamyl 120, supposedly as an effect of amylose leakage and interference with oxidized groups. Pea starch had the highest amylose content (39.4%), and HAP starch had about 2-fold higher amylose content as compared to the parent potato starch.

3.1.2. Molecular weight

Starch molecular weight is known to have specific and important impacts on the functional properties of starches in solution and in the gel state (Blennow et al., 2001). The apparent weight-average molecular mass (Mw) of all starch samples is given in Fig. 1.

In the early stage of AM modification ($1\frac{1}{2}$ h and below), the Mw of starch generally increased. This is in good agreement with the suggested model in which amylose chains are transferred to amylopectin (Kaper et al., 2004). For the waxy maize starch and Gelamyl 120, both of which virtually lack amylose, no increase in Mw was detected. Parent Gelamyl 120 had an Mw of 0.5×10^6 Da, which is about 13 and 2 times lower than parent potato and HAP starch, respectively (Fig. 1A). Parent waxy maize starch displayed the highest Mw of 51×10^6 Da (Fig. 1B).

At later stages of AM modification (after $1\frac{1}{2}$ h), we typically observed clear reduction in Mw dependent upon the amount of specific enzyme activity used. A 50-fold increase in AM concentration (100 units AM/g starch) significantly increased the rate of product formation but not the final Mw, demonstrating that the enzyme is stable, that the reaction has reached an end-point and that no severe side-reactions took place (Fig. 1A). Modification of HAP and maize starch were the only starches for which no decrease in Mw was observed even after 22 h incubation (Fig. 1A and B). Instead, these starches increased their Mw 3.6- and 6.5-fold, respectively.

3.2. Amylopectin chain length distribution

The distribution of amylopectin chain length in the range of degree of polymerization (DP) 4–80 were determined for

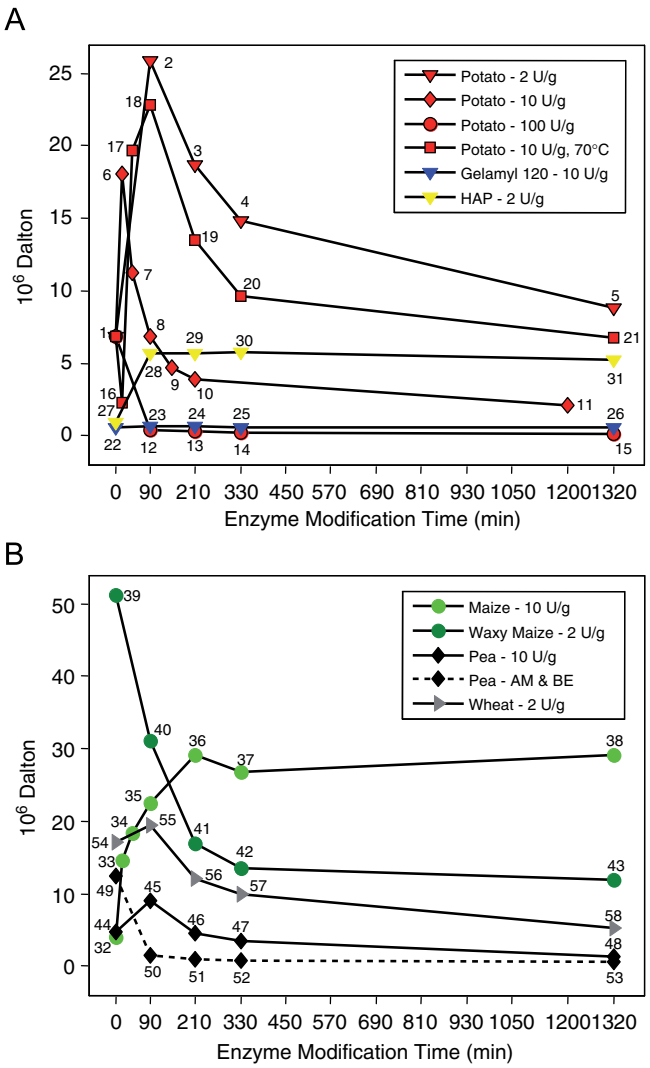


Fig. 1. The time-course progress of molecular weight (Mw) for all starch samples (given in 10^6 Da). (A) Potato, Gelamyl 120 and HAP samples. (B) Maize, waxy maize, pea and wheat samples. All samples were modified with AM. Samples 50–53 were in combination with AM further modified with BE. Sample nos. are as in Table 1.

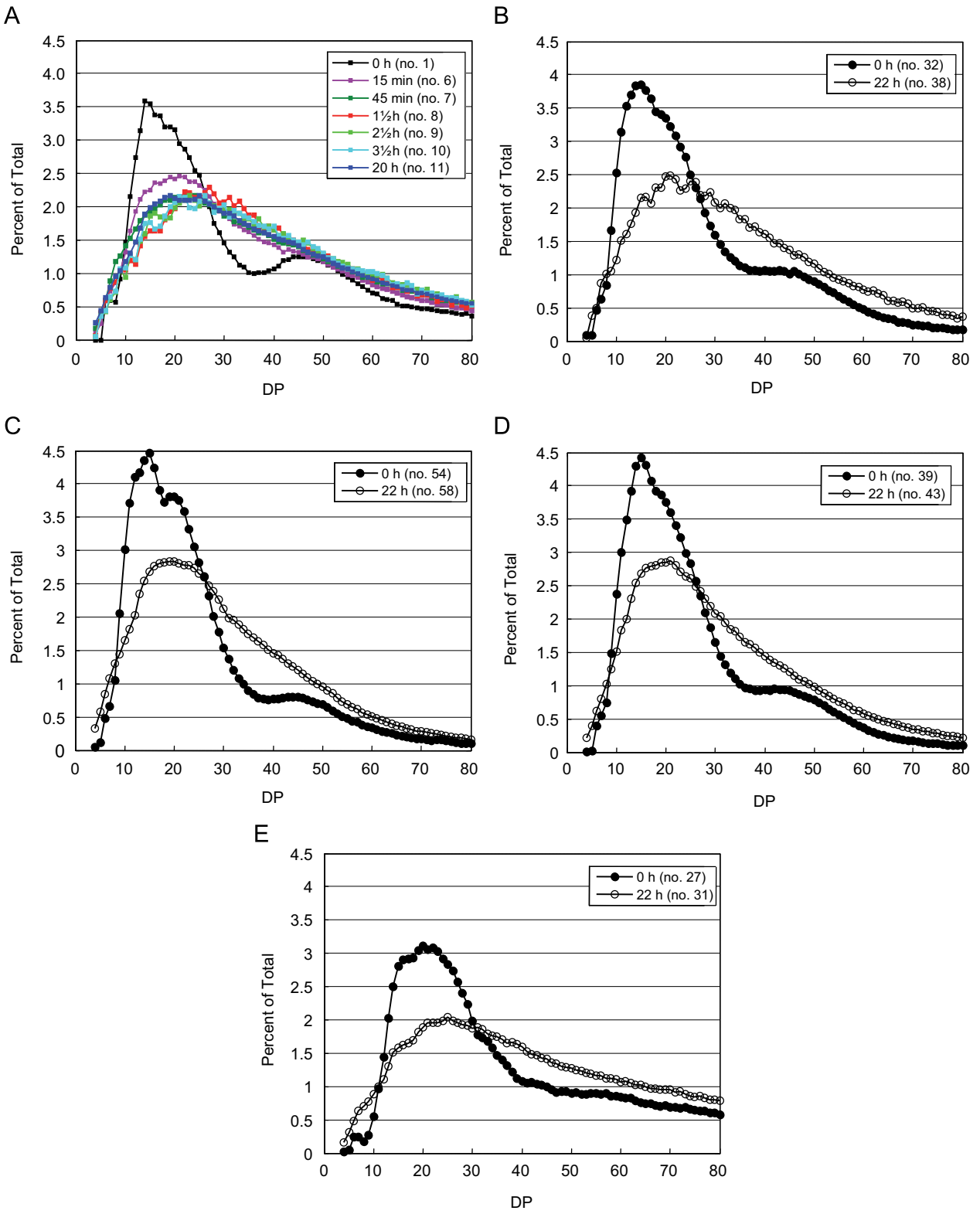
all starch samples. All parent starches showed a typical bimodal profile having the main peak at approximate DP 15–20 with a shoulder around DP 20–25 and a minor peak at DP 40–50 (Fig. 2). However, for HAP (no. 27), the bimodal appearance was virtually lost and an increase of chains larger than DP 40 appeared (Fig. 2E). Moreover, HAP had a significantly higher amount of DP 60–80 compared to the other parent starches. It accounted for 15% of the total DP, which is about $1\frac{1}{2}$-fold higher as compared to potato and Gelamyl 120, 3-fold higher than maize and pea, and 4-fold higher than waxy maize and wheat parent starches.

In general, starch modification with AM resulted in broadening of the chain length, giving a unimodal distribution of side chain in amylopectin irrespectively of the starch type being modified (Fig. 2). Most of the parent starches had very low levels of chains below DP
However, after AM modification, the amount of suc
chains increased. The time-course modification of pota
shows that the broadening effect continues at ve
extensive enzyme treatments (Fig. 2A). The main broa
ening occurred within the first 30 min of modification usi
10 units AM/g potato starch; subsequently, only min
changes was observed. After 20 h of modification t
amount of DP 60–80 decreased slightly, which was
general phenomenon for all the starches (Fig. 3). Th
could indicate minor hydrolytic activity of AM. Howeve
for the potato starches this effect was very minor, and we
independent on the enzyme activity used (Fig. 3).

Many reasons for the broadened side chain compositio
can be suggested. Modifying potato starch with AM resul
in the disappearance of the amylose fraction (van d
Maarel et al., 2005), indicating a virtually complete transf
of amylose chains to amylopectin. Modifying maize, whe
and HAP starches (containing 28.2%, 35.2% and 38.5%
amylose, respectively) with AM for 22 h resulted
products with twice as much of the DP 60–80 fractio
for the maize starch (5.8→11.4%), but only $1\frac{1}{2}$-fo
increase for wheat (4.0→6.3%) and HAP (14.7→19.6%
starches (Fig. 2B, C and E, respectively, and Fig. 3). Und
the assumption of a unidirectional transfer of chai
from amylose to amylopectin, this result is unexpecte
since the wheat and HAP both starches have high
amylose contents as compared to the parent maize. O
the other hand, AM modification of parent waxy mai
also resulted in a broadening of the amylopectin cha
length distribution, giving an increased amount
longer chains despite the lack of amylose (Fig. 2D). Th
indicates the presence of amylose-independent intra-am
lopectin chain transfer by AM taking place possib
simultaneously with the amylose-to-amylopectin cha
transfer.

The oxidized potato starch, Gelamyl 120, was the on
starch which after AM modification virtually did not resu
in changed amylopectin chain length distribution (data n
shown). There was basically no increase in the amount
DP 60–80 after modification (Fig. 3) and, at the same tim
the reduction of the Mw was very low (Fig. 1A
Obviously, these structures are improper substrates f
the enzyme, probably because the oxidized groups on th
starch interfere with binding to AM or preventing enzym
catalysis.

The influence on the amylopectin chain length distrib
tion and textural properties of pea starch modified wi
combined AM/BE was investigated, since starches mod
fied with BE are claimed to have increased solubility an
reduced retrogradation (Takata et al., 1997; Spendl
and Jørgensen, 1997). Pea starch was modified wi
10 unit AM/g starch for 2 h in order to 'remove' amylos
resulting in sample no. 49. Meanwhile, maintainin
the AM activity, no. 49 was further modified wi
1000 unit BE/g starch for $1\frac{1}{2}$–22 h at 60 °C (resulting
nos. 50–53). Modification with BE expectedly resulted in



g. 2. Amylopectin chain length distribution of potato (A), maize (B), wheat (C), waxy maize (D) and HAP (E) starches modified with AM. Potato and aize starch were modified with 10 unit AM/g starch, and wheat, waxy maize and HAP were modified with 2 unit AM/g starch. Modification time and mple nos. are as indicated in Table 1.

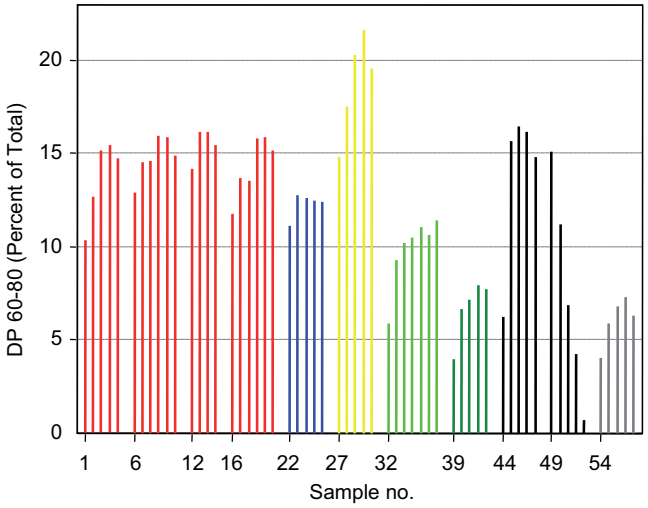


Fig. 3. The fraction of DP 60–80 of the total amylopectin chain length distribution (DP 4–80) for all starch samples colored according to botanical origin: potato (red); Gelamyl 120 (blue); HAP (yellow); maize (light green), waxy maize (dark green); pea (black); wheat (gray). Each modification time-course has been grouped. Parent sample nos. are 1, 22, 27, 32, 39, 44 and 54.

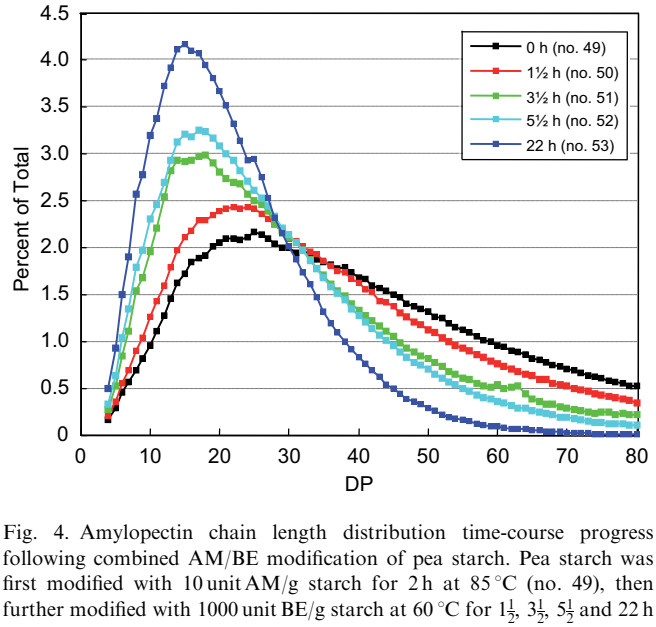


Fig. 4. Amylopectin chain length distribution time-course progress following combined AM/BE modification of pea starch. Pea starch was first modified with 10 unit AM/g starch for 2 h at 85 °C (no. 49), then further modified with 1000 unit BE/g starch at 60 °C for $1\frac{1}{2}$, $3\frac{1}{2}$, $5\frac{1}{2}$ and 22 h (nos. 50–53, respectively). Modification time is indicated. Sample nos. are as in Table 1.

decrease in long chains and increase of small chain (Fig. 4). The unimodal profile of the AM treatment was kept, but the maximum peak was shifted from DP 25 to DP 15. After a further 22 h modification with BE, the DP 60–80 merely constituted 0.7% of the chains (Fig. 3).

Hence, a series of different starch structures were produced by a combination of enzyme treatments. The resulting dynamic system of molecular branch structures and texture profiles of the resulting gels will be addressed in the coming sections.

3.3. PCA on amylopectin chain length distribution data

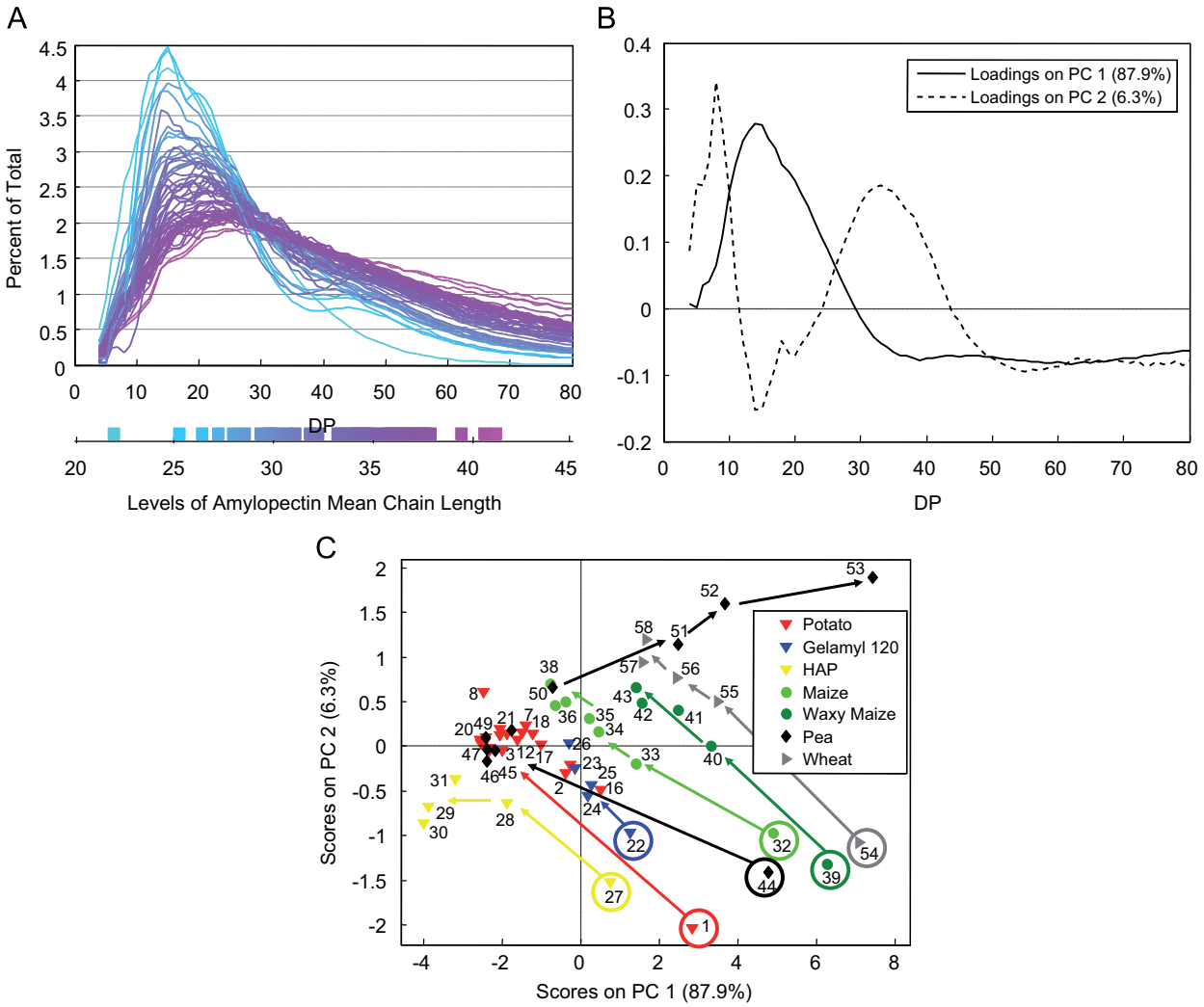
In order to reveal possible common and underlyi phenomena in the amylopectin chain length distribution all 58 starch samples (Fig. 5A), the entire data set w subjected to PCA (Blennow, Engelsen, Munck, and Molle 2000). Figs. 5B and C shows the loadings and scores plo respectively, for the first two PCs explaining 94.2% of t total variance. Enzyme-modified and parent starches for separate clusters when plotting the scores of PC 1 vs. PC (Fig. 5C). Parent starch samples (in circles) cluster wi high PC 1 and low PC 2 score values. The PCA score pl clearly illustrates the effect of the AM modificatio direction (indicated by arrows), and that structur modification is dependent on the parent samples.

The long amylopectin chains dominated the mode accounting for 87.9% by the 1st PC (Fig. 5B and C). As a effect, the PC1, PC2 scores plot readily discriminat parent potato starch, Gelamyl 120 and HAP starc samples (nos. 1, 22 and 27, respectively) from the pare maize, waxy maize, pea and wheat samples (nos. 32, 39, 4 and 54, respectively). PC 2 only explains 6.3% of the tot variance but gives an interesting contribution to t broadening effect due to enzyme treatment, i.e. t production of short and very long chains at the expen of medium-length chains. Hence, AM modification of t starches resulted in samples having lower PC 1 and high PC 2 scores values as compared to the parent starches. Th AM-catalyzed reaction for specific starches can be readi followed with the arrows in Fig. 5C. The starting point important for the final location of the enzyme-modifi starch. Modification of Gelamyl 120 samples only result in little displacement in agreement with the low degree chain length broadening, whereas most other samples sho substantial, but similar, displacements. The combined AM BE-modified pea starch (no. 49) resulted in samples wi low amount of long chains and increase of small chai (DP < 10), easily identified on the PCA scores plot whe these samples score high PC 1 and PC 2 values and displa orthogonal behavior to the other modifications. Moreove most of the modified potato starches cluster with AM modified pea samples (nos. 45–48), illustrating that the starches have very similar molecular structures and beha similarly in the reaction.

3.4. Gel texture

In order to study the influence of the enzyme modific tion on gel texture, gels of the enzyme-modified starch were compared with gels from the parent starches. Gelat samples were included for comparison. Texture analys was performed with 12.0% (w/w) gels by a sing compression after storage at 4 °C for 24 h.

In general, AM modification resulted in starches wi increased gel texture as compared to the parent sampl Fig. 6A shows the texture profiles of potato starch modifi with AM during the 0–20 h time-course. After 15 m



g. 5. PCA model of amylopectin chain length data. Raw data (A), PCA loadings plot (B) and scores plot for PC 1 vs. 2 (C). The two PCs explain 94.3% the variation. Data were mean centered prior to PCA calculation. Numbers in parentheses represent explained data variance. The amylopectin chain ngth data are colored according to the levels of amylopectin mean chain length. Loadings and starch symbols indicated and sample nos. are as in Table 1. he arrows indicate the incubation time direction of the enzyme modification from the parent samples (circle).

zyme modification, the potato starch generated a gel hich increased the *force of deformation* (*fod*; the area of ompression) from ~0 to 1.2 N s as compared the parent arch (see also Fig. 7A). Further modification for 30 min creased *fod* to 4.1 N s, from which point it decreased to 7 N s after 20 h modification. However, not only extended cubation time but also high enzyme activity had a gative impact on gel texture. Modifying potato starch for 2 h using 2 and 100 unit AM/g starch, respectively, sulted in gels with a $2\frac{1}{2}$-fold reduction in *fod* (3.9→ 5 N s) (see also Fig. 7A). During AM modification the mount of longer chains increased, which resulted in creased gel *fod* of the corresponding starch samples. loreover, when modified for more than 20 h there was a nall decrease in longer chains, which again was identified reduced gel *fod*. Modified potato starch also resulted in roducts with longer chains (Fig. 3). However, both *fod* nd Mw were reduced for this starch (Fig. 1A), indicating the importance of retaining a high molecular weight for generation of high *fod* gels. We also modified potato starch at 70 °C with 10 unit AM/g starch generating nos. 17–21. AM-catalyzed reaction temperature had no significant effect on any of the measured parameters as demonstrated for the potato starch modified with 2 unit AM/g starch at 85 °C (Figs. 1A, 3 and 7A).

The textural profiles of HAP and pea starches modified with AM constitute an interesting system when compared with the texture of a gelatin gel at identical concentration in Fig. 6B. Modification of parent HAP and parent pea starch with AM for $1\frac{1}{2}$ h resulted in 1.3- and 2-fold increase in *fod*, respectively. The modified HAP sample had the highest *fod* (11.5 N s) of all the samples, which was almost twice as high compared to gelatin (no. 60) and AM-modified pea starch (nos. 45 and 49, Fig. 7A). However, the textural properties of the gelatin samples (nos. 59 and 60) were very different as compared to most of the

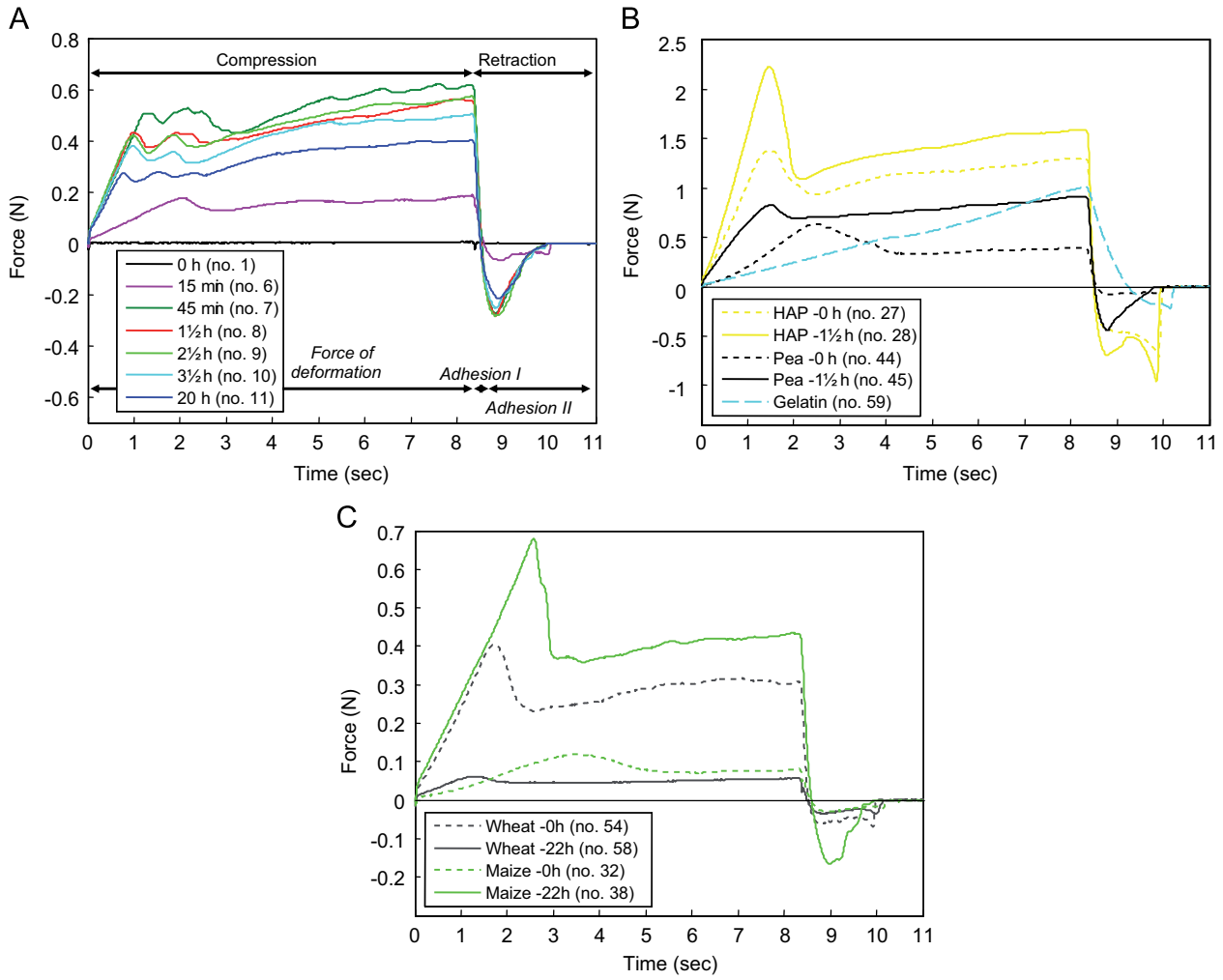


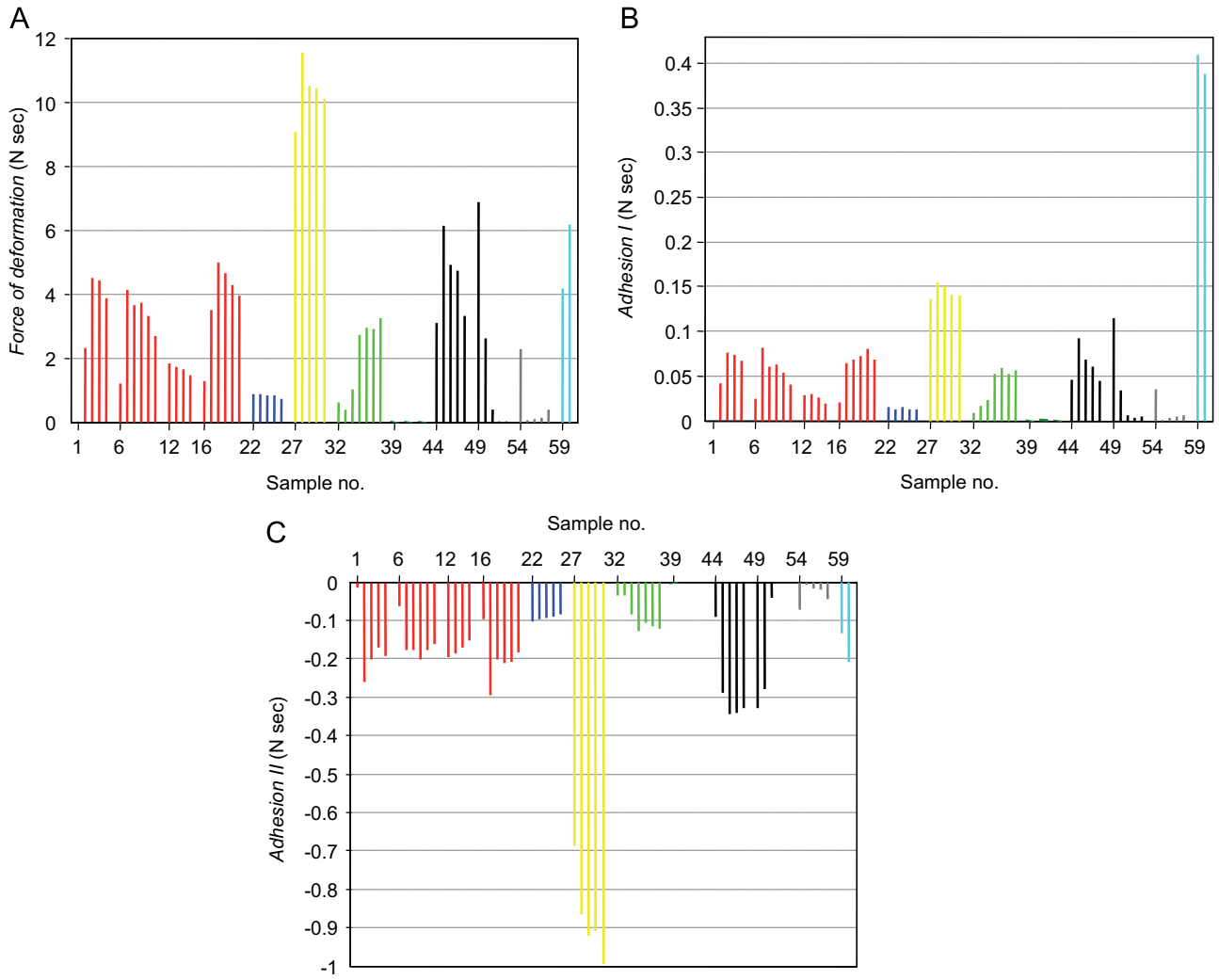
Fig. 6. Texture profiles of 12.0% (w/w) gelatin and starch gels. (A) Potato starch modified with 10 unit AM/g starch. (B) Parent and AM-modified HA (yellow) and pea (black) starch compared to gelatin (cyan). HAP and pea were modified with 2 and 10 unit AM/g starch for 1½ h, respectively. (C) Ma (light green) and wheat (gray) was modified with 10 and 2 unit AM/g starch for 1½ h, respectively. Modification time and sample nos. are as in Table

modified starch samples. The gelatin gels showed no or only slight fractures (Fig. 6B) and the gelatin samples adhered more firmly to the probe during probe retraction, resulting in a 2½-fold higher *adhesion I* than the HAPs (Fig. 7B). Note from Fig. 7 that an increased *fod* correlated with both *adhesion I* and *II*.

AM-modified wheat starch was the only product that resulted in extensive decreased gel texture as compared to the parent starch (Fig. 6C). After 1½ h modification, gel texture was virtually lost, but slightly increased after further modification. After 22 h, the *fod* was reduced 5½ fold (Fig. 7A). This was not anticipated as the amounts of long chain increased by AM modification (Fig. 3). Parent wheat starch made firm gel, although it contains about 5% DP 60–80, which is equal to parent waxy maize (Fig. 7A and 3). However, the parent wheat starch had an amylose content of 35.2%, whereas waxy maize practically is amylose free (Table 1). This indicates the presence of unknown structural parameters important for gel network formation.

The parent maize starch (no. 32) made a much weak gel as compared to parent HAP and pea samples (Figs. 6 and 7A). However, extended AM modification of mai starch for 22 h increased the *fod* more than 5-fold. T increase in gel *fod* was greatest for potato and maize starc modification, whereas only a slight increase in *fod* for t HAP and pea-modified starches was observed. Pare maize and pea starch had similar amount of long chai (~6 DP 60–80%), but pea had 2-fold higher amylo content than maize, which can explain the good gelation this starch. Parent HAP not only had higher amylo content than the parent potato starch, but also had high amount of longer DP 60–80 chains (10% and 15% respectively), which is in good agreement with the fa that HAPs had higher gel strength.

Waxy maize starch (no. 39) was not capable of making measurable gel network (Fig. 7A). Although AM mo ification broadens the amylopectin chain length di tribution, no textural changes were detected at the giv concentration for any of the resulting samples (nos. 40–43



g. 7. Texture of gelatin, parent and modified starch. *Force of deformation* (A), *adhesion I* (B) and *adhesion II* (C) were calculated. Starch samples are lored according to botanical origin: potato (red); Gelamyl 120 (blue); HAP (yellow); maize (light green), waxy maize (dark green); pea (black); wheat ray). Gelatin samples are colored cyan. Each modification time-course has been grouped. Parent sample nos. are 1, 22, 27, 32, 39, 44 and 54.

ote from Fig. 1B that waxy AM modified for 22 h (no. 43) tains a very high Mw of 12×10^6 Da, about 3×10^6 Da gher compared to AM-modified potato starch (no. 5), hich was capable of making a firm gel (Figs. 1B and 7A). owever, the modified potato starch contains twice as uch DP 60–80, again demonstrating the importance of ng amylopectin chains for gel formation. Modification of elamyl 120 (no. 22) resulted in slightly decreased textural roperties of the resulting samples (see nos. 23–26, Fig. 7). ll Gelamyl 120 samples made very soft gels (Fig. 7A).

Fig. 8 shows the enzyme treatment time courses of the xture profile of pea starch modified with AM combined ith BE. Modifying pea starch (no. 49) using BE had a ramatic effect on the gel texture. After $1\frac{1}{2}$ h modification, e *fod* was reduced by $2\frac{1}{2}$-fold. Modification for $5\frac{1}{2}$ h and nger resulted in starches that were not capable of forming els at 12.0% (w/w) concentration. Again, this can be nderstood by considering the chain length data (Fig. 4). BE modification produced starches with a reduced fraction of longer chains (DP 60–80) and an increased fraction of shorter chains (DP 4–25), clearly establishing the importance of longer chain for the formation of a gel network.

3.5. PCA on texture profile data

A PCA model was generated from the textural data of all 60 samples (see Fig. 9A). The loadings and scores plot of PC 1 vs. PC 2 explained 97.7% of the total variance (Fig. 9B and C). Three groups could be identified: groups 1 and 2 consisted exclusively of gelatin and HAP samples, respectively, whereas the rest of the samples comprised the third group.

PC 1 captured the majority of the total variance with 94.9%. From the loading plot, it is apparent that the first PC mainly describes *fod* and *max force*, which are positively correlated (see loadings on PC 1, Fig. 9B). PC

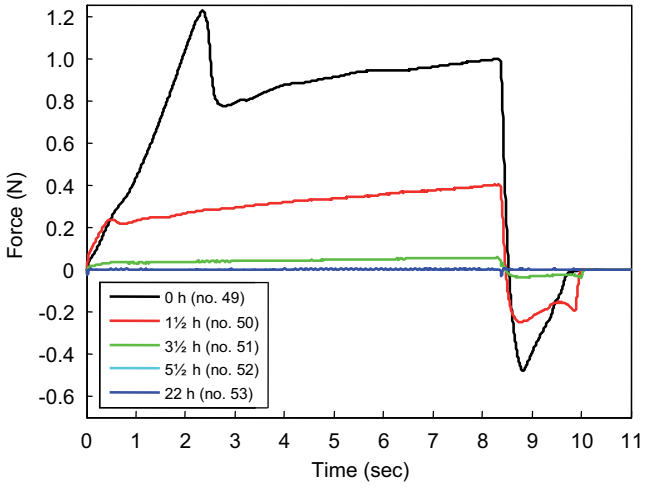


Fig. 8. Enzyme treatment time courses of the texture profiles of pea enzyme modifications. Pea starch was first modified with 10 unit AM/g starch for 2 h, then further modified with 1000 unit BE/g starch. Modification time and sample nos. are as in Table 1.

2 explains 2.8% of the variance and describes *adhesion I*, *adhesion II* and the time point of gel fracture (see loadings on PC 2, Fig. 9B). Fracturability, i.e. the force at which the gel fractures during compression, is to some extent also described by PC 1. *Adhesion I* is the main value that separates group 1 (gelatins) from group 3 (rest of the samples).

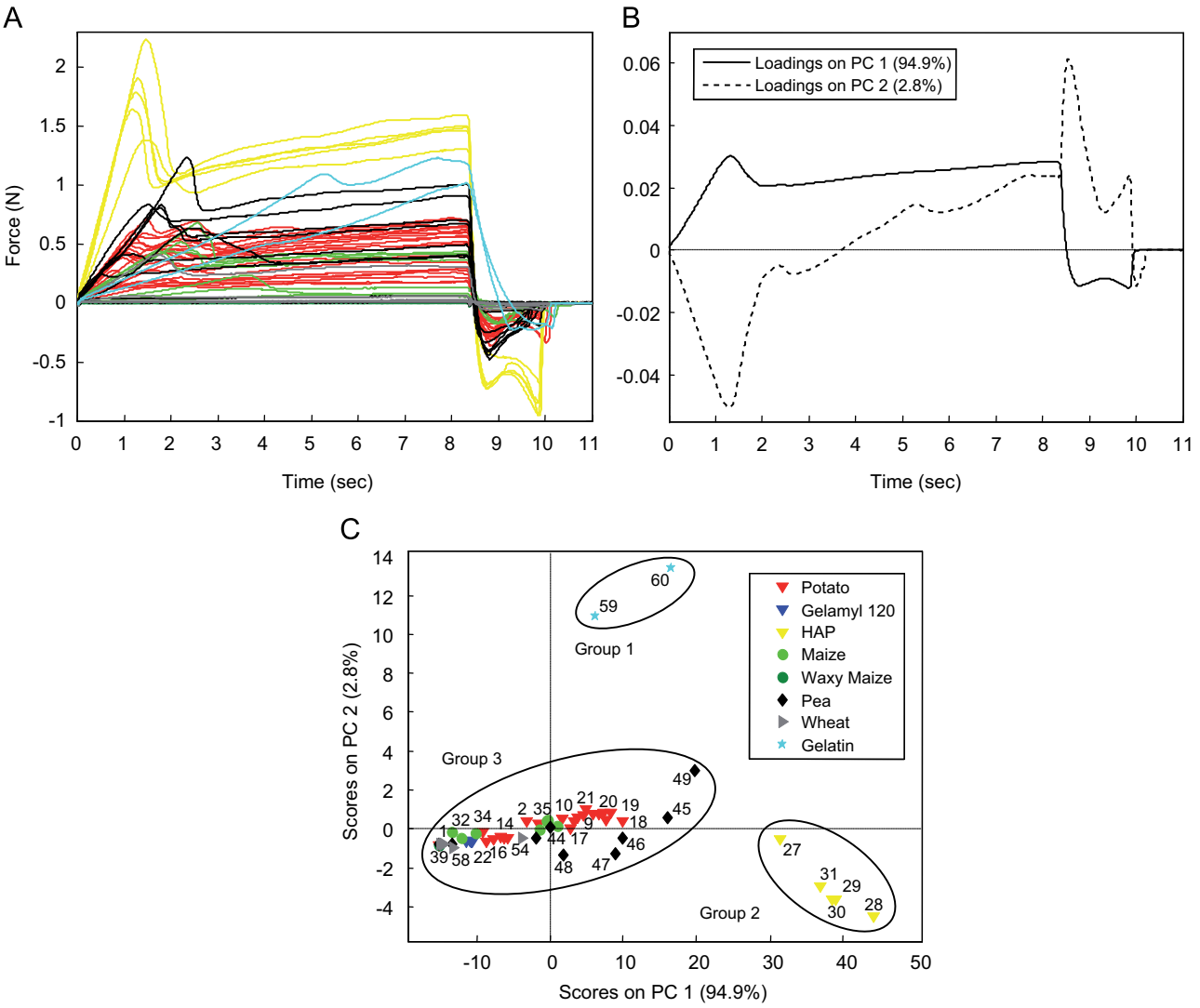
The scores plot shows that all the HAP samples had high gel *fod* and *adhesion II* values, scoring high in PC 1 (Fig. 9C). Modification of HAP (no. 27) with AM further increased the gel *fod*. The scores plot shows that HAP no. 28, modified with 2 unit AM/g starch for $1\frac{1}{2}$ h, had the highest *fod* value. Gelatin (group 1) and enzyme-modified HAP samples (nos. 28–31) separated in the PC 2 direction because of *adhesion I* and *II*. Gelatins had approximately 5-fold higher *adhesion I* values as compared to the starch-based samples, whereas HAP nos. 28–31 had about 3–5 higher *adhesion II* value. BE-modified pea starch, Gelamyl 120, waxy maize and wheat samples did not form gels (*fod* < 1 N s), and therefore cluster to the left in the scores plot having low PC 1 scores values. As an effect of enzymatic modification, *fod* of the gels as indicated by PC1 increase in group 3. Hence, the PCA model clearly describes the general features of the starch gel systems as compared to gelatin gels.

4. Discussion

There is a large industrial interest in replacing expensive or otherwise unwanted gel formers such as gum arabic, pectin and gelatin with starch in food products. Starch is an alternative, but amongst the known problems of native starch are sub-optimal textural properties. In this study, we used AM from *Thermus thermophilus* and BE from *Rhodothermus obamensis* to modify a range of differe starches such as potato, HAP, maize, waxy maize, whe and pea and chemically oxidized potato starch (Gelam 120) in order to improve and investigate the resulting g texture. Both enzymes are expected to find application the food industry as important tools in producing starch with new functionalities (Takata et al., 1997; Spendler ar Jørgensen, 1997; van der Maarel et al., 2005). Here, v have analyzed the Mw, amylopectin chain length distrib tion and gel texture of 51 enzyme-modified and 7 pare starch samples. The texture profiles of 2 gelatin sampl were compared to the starches.

Modification of starch using AM causes the disappea ance the amylose, broadens of amylopectin chain leng distribution and improves the gel texture (Figs. 2, 6 and 7 Amylose is important to form associated and connecte aggregates forming strong gels (Gidley, 1989; Miles et a 1985). Upon AM-catalyzed chain transfer, gelation prope ties are generated from amylopectin having 'amylose-lik longer chains that can interconnect. The influence of suc chains was investigated using PLS regression techniqu A weak correlation was found between the gel texture ra data and the sum of DP 60–80. A three-component PL model gave a correlation of $r^2 = 0.66$ between actual su of DP 60–80 and sum of DP 60–80 predicted from all th starch gel texture, and gave a root mean square error cross validation (RMSECV) of 2.5% (data not shown This is a rather good prediction when taking into accou that the data is in the range from 4 to 22%. A similar resu was obtained when predicting the *fod* from the amylopect chain length distribution data, although using four com onents instead of three ($r^2 = 0.66$, RMSECV = 1.7 N s This demonstrates a relationship between the texture starch gels and the amylopectin chain length distributio Intriguingly, modification of waxy maize and wheat starc did not improve gel texture despite the increase in long chains (Figs. 3 and 7A), indicating that a critical fraction DP 60–80 or amylose content is required for gel formatio The wheat starch was the only starch which did not ga improved texture as compared to the parent sample. This peculiar as the amount of longer chains increased after A modification. The wheat starch was selected based on i large fraction of small chains as compared to long chain This can be the reason for the reduced gel texture upo AM treatment.

Modification of parent HAP using AM resulted starches having the highest amount of longer chains. Th amount of DP 60–80 increased by 5% after 22 h modific tion, which resulted in slightly increased gel texture (Figs. and 7A). Parent and modified HAPs made very firm gels 12.0% (w/w), being twice as hard compared to the gelat samples, whereas AM-modified pea starches, and to son extent potato starch, were comparable to gelatin (Fig. 7A AM modification not only generally improves texture, b also has a profound effect on thermoreversible gelation ar freeze–thaw stability (Kaper et al., 2004; Lee et al., 2006 Furthermore, the viscosity significantly decreased ar



g. 9. PCA model of the textural data for all starch and gelatin gels. Raw texture profiles (A), PCA loadings (B) and scores plot for PC 1 vs. 2 (C). The st two PCs explain 97.7% of the variation. Data were mean centered prior to PCA computation. Numbers in parentheses represent explained data riance. Loadings and starch symbols indicated and sample no. are as in Table 1. Starch samples are colored according to botanical origin: potato (red); elamyl 120 (blue); HAP (yellow); maize (light green), waxy maize (dark green); pea (black); wheat (gray). Gelatin samples are colored cyan.

olubility increased after treatment with both AM and BE ata not shown). Regardless of improved texture, the xtural profile of starch gels was very different from elatin (Fig. 9A). The elasticity of gelatin gels was much gher, making them more firm than starch gels. The arches fractured within the first 1–3 s, whereas gelatin o. 59) did not fracture at all during compression ig. 9A). In addition, AM-modified starches formed paque gels, while gelatin gels were fully transparent. etrogradation and flavor release are major problems with e application of starch in the food industry. These effects ust be further investigated. Increasing α-1,6 linkages by E action reduces the retrogradation of starch in solutions pendler and Jørgensen, 1997; Takata et al., 1997), but as a negative effect on the gel texture (Fig. 8). AM-odified starches could be used in certain food applica-ons but it cannot match the complete functional spectrum of gelatin. Several combinations of enzymatic and/or chemical modification will be needed. Despite its low gel-forming properties, Gelamyl 120 is a chemically modified potato starch presently used as a gelatin replacer in confectionery industry. Improving the gel texture of this starch by AM was however not successful, probably because the oxidized starch was an improper substrate for the enzyme.

The broadening of amylopectin side-chain distribution following AM modification of potato starch is not merely a result of amylose transfer to amylopectin as proposed by van der Maarel et al. (Kaper et al., 2004), as waxy maize shows substantial broadening from a bi- to a unimodal amylopectin chain length distribution (Fig. 2D). The apparent Mw of waxy maize starch was reduced from 50 to 10×10^6 Da after 22 h AM modification (Fig. 1B). Since hydrolytic activity of AM is not significant (van der Maarel

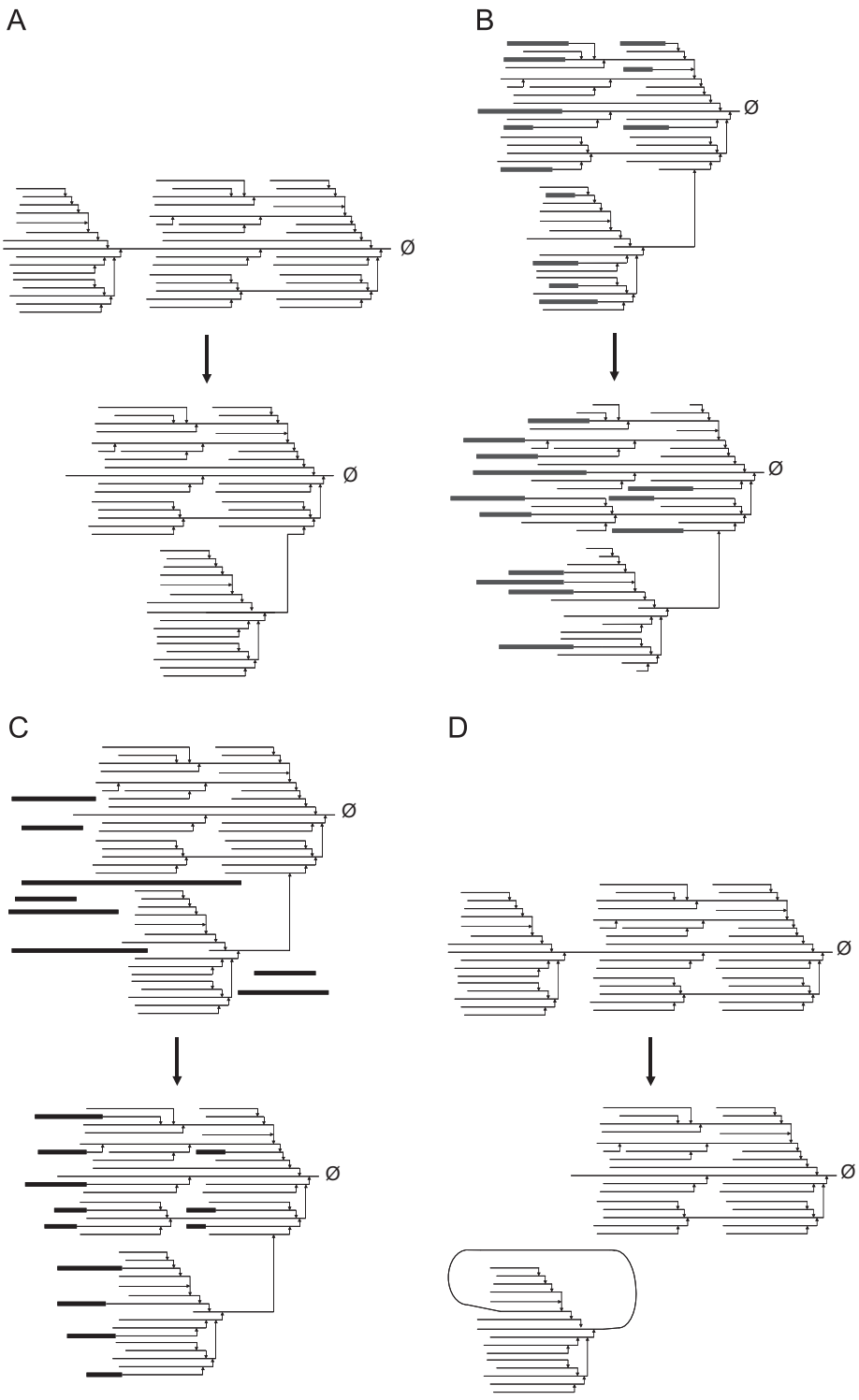


Fig. 10. Schematic action for amylomaltase on starch. Disproportionation of amylopectin cluster (A), disproportionation of α-glucan chains with amylopectin (B), disproportionation of α-glucan segments from amylose to amylopectin (C) and cyclization of amylopectin (D). Gray bars in B illustrate glucan chains being transferred. Black bars in C illustrate amylose molecules. The linear lines indicate α-1,4-D-glucan segments, whereas vertical arro within the structure indicate α-1,6-glycosidic linkage. Ø: non-reducing end. The cluster model of amylopectin is adapted from Hizukuri (1986).

et al., 2005), this shows that the decrease in Mw is a result of molecular reorganization in the amylopectin molecules generating more compact structures having less hydrodynamic radii, and not because of hydrolytic activity. Such structures can be formed through disproportionation entire amylopectin cluster units or reorganization clusters (Fig. 10A) and/or transfer of long chains on th same molecule (Fig. 10B) as proposed by Takaha, Yanas

akata, Okada, & Smith (1998). This is in good agreement ith the increase in DP 4–6, which can only be a result of ortening of longer chains i.e. these chains were residual gments from the donor chains. We therefore suggest that e increase in longer chains is a result of initial transfer of mylosic segments to amylopectin (Fig. 10C), and in a cond phase cluster units and long chains in amylopectin ere transferred. Given that AM also is capable of talyzing intramolecular transglycosylation, the formation cyclo-amylopectin clusters could also occur (Fig. 10D) as roposed by Takaha et al. (1998). This would result in arches with reduced Mw and increased longer chains ithout introducing additional reducing ends.

Conclusion

It can be concluded that all starches showed broadened mylopectin chain length profiles following AM treatment. otato, HAP, maize and pea starch resulted in improved el texture. On the contrary, modified wheat starch showed ecreased texture as compared to the corresponding parent mple despite of an increase in longer chains and roadening of the amylopectin chain length profile. roadening was not dependent on the amylose content as dicated by the broadening of amylose-free waxy maize arch following AM treatment. Extended AM treatments nly slightly changed the amylopectin chain length stribution but significantly reduced the apparent mole- ular weight resulting in reduced the gel texture. Chemo- etrics provided insight into some main structural quirements towards generating gelatin-like textures spiring further studies in this area.

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Paper II

Comparative NMR relaxometry of gels of amylomaltase-modified starch and gelatin

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Submitted

Comparative NMR relaxometry of gels of amylomaltase-modified starch and gelatin

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Keywords: Enzyme-modified starch; proton relaxation; low-field ^{1}H NMR; amylomaltase; branching enzyme; chemometrics.

Abstract

With the aim at generating gelatin-like starch gel functionality, starches extracted from normal potato, high amylose potato, maize, waxy maize, wheat and pea and oxidized potato starch were modified with amylomaltase (AM) (4-α-glucanotransferase; E.C. 2.4.1.25) from *Thermus thermophilus*. Gel characteristics after storage for 1 and 10 days at 20°C of 12.0% gels was assessed by monitoring proton relaxation for the resulting 51 enzyme-modified starches and 2 gelatins using low-field ^{1}H nuclear magnetic resonance (LF NMR) relaxometry. Discrete and distributed exponential analysis of the Carr-Purcell-Meiboom-Gill (CPMG) LF NMR relaxation data revealed that the pastes and gels contained one water component and that the spin-spin relaxation time constants (T_2) and distributions differed with respect to starch type and enzyme modification. Typically, AM modification resulted in starches with decreased T_2 relaxation time and a more narrow T_2 distribution indicating a more homogeneous water population. In contrast, treatment with branching enzyme (BE) (EC 2.4.1.18) combined with AM increased T_2 relaxation time and a broadened T_2 distribution. As evaluated by principal component analysis (PCA), long chains of amylopectin generated hard gels and decreased T_2 relaxation time at both day 1 and day 10. Especially at day 10, T_2 relaxation time could be predicted from the amylopectin chain length (CL) distribution. Reconstructed amylopectin CL distribution required to emulate gelatin LF NMR data suggest the importance of combined fractions of long (DP 60-80) and short (DP 10-25) amylopectin chains.

1. Introduction

The physical state of water is of tremendous importance in foods because of its influence on storage stability, textural, and functional properties. As a plasticizer, water influences the molecular mobility of starch molecules providing a fluid environment in which dissolvation, aggregation, gelation and water retention can take place.

Starch is one of the most used hydrocolloids in the food industry. It exhibits a wide range of functional properties, and is widely used as a gelling and thickening agent in many food products. Starch is composed of two types of α-glucan polymers: amylose and amylopectin. Amylose, an essentially linear molecule consisting of α-1,4 linked glucose residues, and amylopectin, which in addition to linear chains of α-1,4 linked glucose, also contains α-1,6 linked branch points (Hizukuri, 1996;Tester, Karkalas, and Qi, 2004).

Starch gelation is a phenomenon occurring when cooling starch paste from its gelatinized state e.g. from 95°C to 20°C. Typically, starch makes a firm gel network upon cooling which is dominated by double helix formation between amylosidic polymers. However, normal starch lacks the ability of forming thermoreversible transparent gels. Most starch types are subjected to retrogradation, which is an irreversible recrystallization process (Hoover, 1995). This process is a result of changes in the molecular mobility between starch and water and is of great concern in the food industry since starch retrogradation contribute extensively to many functional properties like staling or firming of bread, sensory properties like texture and flavor release, and nutritional properties such as resistance to enzymatic hydrolysis (Hermansson and Svegmark, 1996). Gel formation and retrogradation of starch depend on a variety of factors including the botanical source of the starch (Zaidul, Absar, Kim, Suzuki, Karim, Yamauchi, and Noda, 2008), the ratio of amylose to amylopectin (Kitahara, Hamasuna, Nozuma, Otani, Hamada, Shimada, Fujita, and Suganuma, 2007), long amylopectin chains (Wischmann, Blennow, Madsen, Jorgensen, Poulsen, and Bandsholm, 2005), degree of phosphorylation (Blennow, Wischmann, Houborg, Ahmt, Jorgensen, Engelsen, Bandsholm, and Poulsen, 2005), storage temperature (Farhat, Blanshard, and Mitchell, 2000), starch concentration (Lionetto, Maffezzoli, Ottenhof, Farhat, and Mitchell, 2005) and chemical modification (Tharanathan, 2005). In the initial development of the gel structure, amylose readily retrogrades. Especially amylose with low molecular weight containing a degree of polymerization (DP) of 100-200 glucose units is important for this process (Gidley and Bulpin, 1989). Because of its branched structure and high DP, amylopectin retrogrades slowly and reversibly during long-term storage (Goodfellow and Wilson, 1990). However,

amylopectin molecules with high mean chain length (CL) can promote starch retrogradation (Jane and Chen, 1992).

Low-field ^{1}H nuclear magnetic resonance (LF NMR) relaxometry has proven useful to study proton relaxation in starch gels, since all protons in a sample contribute to the LF NMR signal, which means that the state and distribution of water can be probed (Teo and Seow, 1992;Farhat *et al.*, 2000;Thygesen, Blennow, and Engelsen, 2003). The method is non-destructive and fast. The relation of proton relaxation to the gelation properties of hydrocolloid systems makes LF NMR a powerful tool for monitoring their gelation and texture processes. Especially the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence is an accurate LF NMR method useful to study the proton relaxation that is primarily driven by exchange with the polysaccharide hydroxyl protons and thus depend upon the accessibility on the polysaccharide hydroxyl concentration in liquids and partly to the water compartmentalization in gels (Hills, Cano, and Belton, 1991). The output data is composed of time-domain relaxation curves, which can be characterized by one or more exponential time constants. The T_2, also called the transverse or spin-spin relaxation time, is an excellent probe of proton exchange in gels, since the majority the water molecules giving rise to this relaxation are likely to move free. Hills *et al*., 1989 have shown that chemical exchange of protons between water and carbohydrate hydroxyl groups plays a major role in determining the T_2 relaxation behavior of water-rich polysaccharide systems (Hills, Wright, and Belton, 1989). The relaxation pattern of the protons of water in a sample depends on the environment of the protons, and other factors as diffusive exchange of water protons between separated regions and chemical exchange of protons in narrow regions may also influence the T_2 constant (Hills, Takacs, and Belton, 1990). In dilute carbohydrate systems, 'bound water' (associated with short T_2) has no significant part to play compared to the proton exchange mechanism (Hills *et al.*, 1991). Solid materials typically have short relaxing T_2, whereas liquid materials have long relaxing T_2 time constants. In food samples, T_2 varies between a few hundred ms to three s. Several studies have reported water proton relaxation data in native and in chemical modified starch systems, but to our knowledge, no systematic studies are available on enzyme-modified starches.

In this study, we have used amylomaltase (AM) (4-α-glucanotransferase; E.C. 2.4.1.25) and branching enzyme (BE) (1,4-α-D-glucan branching enzyme; EC 2.4.1.18) for modification of a variety of different starches previously described (Hansen, Blennow, Pedersen, Norgaard, and Engelsen, 2008). Modifying potato starch with AM results in

starches having a thermoreversible character (van der Maarel, Capron, Euverink, Bos, Kaper, Binnema, and Steeneken, 2005), and therefore has potential applications in the food industry as a replacement of gelatin (Euverink G.J.W. and Binnema D.J., 2005), mainly in products where thermoreversibility is an important property. Gelatin is a collagen derived animal protein having the ability of forming thermoreversible, nearly tasteless transparent gels, properties which normal starch lacks (Djagny, Wang, and Xu, 2001;Karim and Rajeev, 2008). In addition to make thermoreversible gels at relatively low temperatures, the use of AM-modified starches also provide products having increased texture (van der Maarel *et al.*, 2005;Hansen *et al.*, 2008), increased freeze-thaw stability (Lee, Kim, Park, and Lee, 2006) and increased solubility. The general effects can be attributed to lower amylose content, broadened amylopectin chain distribution (Kaper, Talik, Ettema, Bos, van der Maarel, and Dijkhuizen, 2005;van der Maarel *et al.*, 2005;Hansen *et al.*, 2008), and decreased molecular weight (Lee *et al.*, 2006;Hansen *et al.*, 2008).

AM isolated from the hyperthermophilic bacterium *Thermus thermophilus* (Kaper, van der Maarel, Euverink, and Dijkhuizen, 2004;van der Maarel *et al.*, 2005) has three activities. AM catalyzes the intermolecular transfer of a segment of a α-1,4-D-glucan to a new 4-position in another α-1,4-D-glucan or glucose in a disproportionation reaction. The catalytic mechanism is believed to be a variation of the α-retaining mechanism (Davies and Henrissat, 1995). AM also catalyzes intramolecular transglycosylation reaction from linear α-1,4-glucan as substrates resulting in cycloamylose with a DP of 16 or more (Terada, Fujii, Takaha, and Okada, 1999;Takaha and Smith, 1999). In addition, AM also has minor endo-amylolytic activity (van der Maarel *et al.*, 2005). However, of the three activities, the disproportionation reaction is by far the dominating activity (van der Maarel *et al.*, 2005). Modification of starch with BE isolated from the thermophilic bacterium *Rhodothermus obamensis* (Shinohara, Ihara, Abo, Hashida, Takagi, and Beck, 2001) also gives some interesting structural and functional improvements. It catalyzes the inter- and intramolecular transglycosylation of a segment of a α-1,4-D-glucan chain to form a new α-1,6-D-glycosidic branching linkage in the α-glucan. The resulting product shows increased solubility and reduced retrogradation in water (Takata, Takaha, Nakamura, Fujii, Okada, Takagi, and Imanaka, 1997;Spendler and Jørgensen, 1997).

Enzymatic modification of structurally different starches obtained from selected starch crop genotypes provides a strategy for further extension of starch functionalities. The aim of this study was to relate water proton relaxation and texture of aqueous 12.0% (w/w) AM-modified starch gels and compare to the results of gelatin gels. Principal

component analysis (PCA) (Hotelling, 1933;Wold, Esbensen, and Geladi, 1987) combined with discrete and distributed exponential fitting were used to combine and extract relevant structural and physical characteristics from the LF NMR data. Partial least squares (PLS) regression (Wold, Martens, and Wold, 1983) models was used to correlate the amylopectin CL distribution to gel texture and LF NMR relaxation data providing a direct link between starch chain structure and gelatin-like characteristics.

2. Materials and Methods

2.1. Materials and samples

Potato, Gelamyl 120 (a chemical oxidized potato starch), high amylose potato (HAP), maize, waxy maize, wheat and pea starch were enzyme-modified using mainly amylomaltase (AM) (4-α-D-glucanotransferase; E.C. 2.4.1.25) isolated from the hyperthermophilic bacterium *Thermus thermophilus* (Kaper *et al.*, 2004;van der Maarel *et al.*, 2005) and branching enzyme (BE) (1,4-α-D-glucan branching enzyme; EC 2.4.1.18) isolated from the thermophilic bacterium *Rhodothermus obamensis* (Shinohara *et al.*, 2001) as previously described (Hansen *et al.*, 2008). Gelatin 1 (bloom 120) and gelatin 2 (bloom 300) were from Sigma-Aldrich (www.sigmaaldrich.com). An overview of the samples is given in Table 1.

2.2. Preparation of starch and gelatin pastes

The 51 enzyme-modified, the seven normal starches (parent samples) and the two gelatin samples were used to prepare 12.0% (w/w) pastes by dissolving 3.0 gram polymer with 22.0 ml buffer (50 mM K_xP_i buffer, pH 7.0, 0.02% NaN_3) at 95ºC for 17 min with constant stirring. After stirring a 15 ml sample of the paste was immediately transferred to an NMR glass tube, sealed and stored for 1 and 10 days at 20.0°C.

2.3. Low-field ^1H NMR analysis

A 23 MHz ^1H NMR Maran instrument from Resonance Instruments (Witney, Oxfordshire, UK, www.resonance.co.uk) was used to obtain Carr-Purcell-Meiboom-Gill (CPMG) (Meiboom and Gill, 1958) relaxation curves of the starch and the gelatin gels at 20.0°C. Sixteen scans (acquisitions) were accumulated to increase the signal-to-noise ratio. The recycle delay between the scans was 10 sec to allow full spin relaxation. The pulse spacing (τ) was set to 250 μs between the 90° and 180° pulses and the number of echoes was 8190. Each sample was measured twice. Data was collected using software RINMR 4

(Resonance Instruments Inc., www.resonanceinstruments.com).

2.4. Analysis of LF NMR relaxation data

The LF NMR data were maximum-normalized and analyzed by discrete and distributed exponential fitting, principal component analysis (PCA) and partial least squares (PLS) regression using MatLab 7.2 (The Mathworks Inc., USA, www.mathworks.com) installed with the PLS toolbox version 3.5.3 (Eigenvector Research, www.eigenvector.com). Fig. 5A was created using LatentiX Ver 1.00 (Latent5, Copenhagen, Denmark, www.latentix.com).

2.4.1. Discrete exponential fitting

The LF NMR relaxation data were individually decomposed into five exponential components using the LF NMR toolbox (Pedersen, Bro, and Engelsen, 2002), which performs non-linear curve-fitting according to the equation:

$$R_{mag}(t) = \sum_{n=1}^{N} M_{2n} \quad \exp\left(\frac{t}{T_{2n}}\right) + E,$$

(eq. 1)

where R_{mag} is the residual magnetization at a given time t after application of the first radio frequency pulse, N the number of exponential functions or components in the sample. Fitting result in spin-spin relaxation time constants (T_2), the concentrations of proton population (M_2) plus a residual error (E). The optimal number of components fitted to the relaxation data was validated from the decrease in residual variance (non-explained NMR signal). If the residual ratio (root mean square error – the ratio between the residual variance when fitting one- and two-components) increased more than a factor three, an additional component was accepted.

2.4.2. Distributed exponential fitting

Distribution analysis is a simplification of the oligo-exponential analysis where the relaxation data was fitted to a continuous distribution of characteristic relaxation times (Butler, Reeds, and Dawson, 1981) using in house DXP software (Mortensen, Andersen, Engelsen, and Bertram, 2006). Data were fitted simultaneously to a larger number of exponentials resulting in a distribution of intensities (M_2) found for a large number of T_2 values. A total of 256 distributed relaxation times from 30 to 1000 ms were fitted.

2.4.3. Principal Component Analysis (PCA)

PCA (Hotelling, 1933;Wold *et al.*, 1987) was carried out for graphical description of systematic variations in the generated complex data structures. PCA is a useful technique for data compression and for extraction of possible underlying phenomena. PCA describes major trends in the data by the principal components (PC 1, PC 2, etc.). Mathematically, it relies upon an eigenvector decomposition of the covariance matrix. PCA decomposes the data matrix **X** (samples × variables) into a score matrix (**T**) and a loading matrix (**P**) plus a residual matrix (**E**):

$$\mathbf{X} = \mathbf{TP}' + \mathbf{E},$$

(eq. 2)

(' means transposed). The PCA model is visualized through score and loading plots. The score values contain information on how the samples relate to each other, whereas the loading values contain information on how the variables relate to each other. Samples or variables containing similar score or loading values, respectively, are correlated to each other. Prior to PCA calculation the data was pre-processed by mean centering or autoscaling (Bro and Smilde, 2003).

2.4.4. Partial Least Squares Regression (PLS)

Relationships between predictor variables **X** (e.g. a matrix of LF NMR relaxation data) and the property of interest **y** (e.g. sum of DP 60-80) were modeled using PLS regression (Wold *et al.*, 1983). PLS estimates components, which simultaneously maximize the covariance between scores in **X** and **y** and describe the variance in **X**. PLS is a linear multivariate model that decomposes **X** and **y** in such a way that the information in the **y** vector directs the decomposition of **X**. The linear regression model is defined as $\mathbf{y} = \mathbf{Xb} + \mathbf{f}$, where **b** is the vector of regression coefficients and **f** is the residuals. The model performance is evaluated by the cross-validated correlation coefficient (r^2), which is the correlation between the measured **y** (reference value) and the predicted **y**, and by the root mean square error of cross-validation (RMSECV) (Næs, Isaksson, Fearn, and Davies, 2002) both having the same unit as the property of interest. Prior to PLS modeling the **X** and **y** data sets are preprocessed by mean centering or autoscaling (Bro *et al.*, 2003).

The above presented method is designated a PLS1 regression if only one property of interest is modeled. The method can be extended to handle multivariate properties of interest, i.e., including a **y** matrix in the modeling. This method is called a PLS2 regression (Geladi and Kowalski, 1986).

3. Results

Water proton relaxation in 12.0% (w/w) starch and gelatin gels was determined after 1 and 10 days storage using 23 MHz ^{1}H low-field nuclear magnetic resonance (LF NMR). The spin-spin relaxation time constant (T_2) and the concentrations of proton population (M_2) were determined for all 60 samples by discrete exponential fitting. An overview of the starch samples and their key properties is given in Table 1. A more detailed structural analysis of the starches is described elsewhere (Hansen *et al.*, 2008).

3.1. T_2 relaxation measurements for day 1

LF NMR relaxation data recorded for selected starch and gelatin gels stored for 1 day at 20°C are shown in Fig. 1A-D. Potato starches modified with amylomaltase (AM) were found to relax faster as function of modification time (Fig. 1A), whereas the opposite was found when modifying pea starch with combined amylomaltase/branching enzyme (AM/BE) (Fig. 1B). Little change in the relaxation rate was found when modifying high amylose potato (HAP), Gelamyl 120 (oxidized potato starch), maize and waxy maize starch with AM (Fig. 1C-D). Gelatin samples show similar relaxation profile as parent potato starch, parent Gelamyl 120 and pea starch combined AM/BE-modified for 5½ and 22 h. The LF NMR relaxation data was further analyzed by discrete (Fig. 1E-H) and distributed (Fig. 1I-L) exponential fitting.

3.1.1. Number of components

In the time domain, the relaxation data for each sample is assumed to be the sum of one or a few pure mono-exponential curves, which carry information about the state of the main water populations, e.g. different water compartmentalization near to the starch macromolecules. From discrete exponential fitting, it was determined that most of the samples could be adequately described by one water component (Fig. 1I-L). For those samples (marked by star in Table 1) having a residual ratio above 3.0 were considered as being two-component systems in mathematically terms. In those cases the additional minor component (below 0.07 M_2 population) was either very fast (14-65 ms) or very slowly relaxing (570-1700 ms) only the primary and dominating exponential component is listed. However, the parent potato (no. 1) and maize (no. 32) and to some extent parent Gelamyl 120 (no. 22) and pea (no. 44) starches having more dominating M_2 populations, were considered as bimodal systems having a second small distribution peak (Fig. 1I-L). Even

though, both the discrete and distributed exponential fitting of the relaxation data, in mathematically terms, shows that some samples in the dataset could be considered as having two water components. However, it is most likely that they should be considered as an one-component system due to the dominant proton exchange mechanism.

3.1.2. Discrete exponential fitting

The T_2 values obtained from the discrete exponential fitting of the starch samples were in the range 82–791 ms, whereas gelatins displayed characteristic T_2 decays of 481 and 401 ms (Table 1). Generally, AM treatment resulted in a dramatic decrease in T_2 times. There was a clear difference in proton relaxation of potato starch when modified using 2, 10 and 100 unit AM/g starch. Increasing enzyme activity reduced the T_2 relaxation time. A clear example is nos. 3, 10 and 13, which were modified with 2, 10 and 100 unit AM/g starch for 3½ h showing decrease of T_2 relaxation times from 439 to 151 and finally down to 111 ms, respectively (Table 1). AM modification of potato starch resulted in reduced T_2 times caused by increased proton exchange between water and carbohydrate hydroxyl groups presumably due to more hydroxyl groups being exposed to water. Moreover, extended AM modification of pea, Gelamyl 120 and high amylose potato (HAP) starch also produced starches with faster relaxation time (Fig. 1F-G). T_2 relaxation was particular fast in AM-modified pea and HAP starches with T_2 of 82-86 and 89-91 ms, respectively (Fig. 1F-G), whereas relaxation was slow in waxy maize starches with T_2 of 763-791 ms (Fig. 1H). These starches can be considered as rather extreme when compared to gelatin having T_2 of 481 and 401 ms. As well as for parent HAP, AM modification of maize and waxy maize starch only had minor effect on the proton relaxation (Fig. 1G-H). AM modifications of maize and waxy maize starches were the only samples that resulted in slower relaxation compared to their corresponding parent starch even after comprehensive enzyme treatment (T_2 going from 247 → 287 ms and 747 → 791 ms, respectively) (Fig. 1H). This indicates that AM modification of maize starches had the opposite effect, as compared to AM-modified potato starches, by decreasing the accessibility between water and carbohydrate hydroxyl groups. Moreover, the potato starch modified for short time (15 min with 10 unit AM/g starch) transiently with low modification degree resulted in gels having slower T_2 relaxation time (Fig. 1E). The reason for this effect is not clear.

In order to assess the effect of branching in more detail, pea starch modified with a combination of AM and BE was also investigated. First, pea starch was modified with 10 unit AM/g starch for 2 h in order to transfer amylose to amylopectin (results in sample no.

49). While maintaining the AM activity, no. 49 was further modified with 1000 unit BE/g starch for up to 22 h resulting in nos. 50-53. Expectedly, AM modification of parent pea starch reduced T_2 from 234 to 82 ms (Fig. 1F). Additional enzyme treatment with BE for 1½ h slightly increased T_2 to 101 ms. Extended enzyme treatment for 5½ h and 22 h generated highly branched starch that resulted in pastes with comparable proton relaxation to that of gelatin.

3.1.3. Distributed exponential fitting

In order to get a better understanding of the data, distributed exponential fitting was applied on the LF NMR day 1 data by assuming a continuous distribution of T_2 relaxation time constants rather than a single well defined T_2 constant. When comparing discrete and distributed exponential fitting for each individual sample it was found that the T_2 time constant as calculated in the discrete analysis corresponds well with the peaks resulting from distributed exponential analysis (compare Fig. 1E-H with I-L). However, the distribution of T_2 differed within each modification time-course. AM-modified starches resulted in a more narrow distribution of T_2 as a function of enzyme incubation. Especially in the case of AM modification of potato starch, major changes in the T_2 distribution were obtained (Fig. 1I). This was true also for pea (Fig. 1J), Gelamyl 120 (Fig. 1K) and maize (Fig. 1L) starch, whereas waxy maize starches remained unchanged (Fig. 1L). However, for the combined AM/BE-modified samples the water distribution was broader and the calculated relaxation time was increased displaying T_2 changes from around 70 to 490 ms after 22 h enzyme treatment, which correspond well to the T_2 found from discrete fitting (82 → 477 ms).

The narrowing of the T_2 distribution indicates that the water population gets more homogenous as an effect of AM modification, possibly due to smaller compartments in the gel network structure providing more rapid exchange spin energy with surrounding water molecules. Such compartments in starch systems are formed as an effect of 'junction zones' of the gel network i.e. ordered chains linked together throughout non-covalently linked chain segments (Rees, Moris, Thom, and Madden, 1982). Depending on the origin of starch and the type of enzyme modification, the arrangement of junction zones is expected to cause different water cavity sizes resulting in dissimilar water proton relaxations. This effect was also seen for starch types not forming visible gels e.g. the waxy maize starches, combined AM/BE pea starch modified more than 5½ h, wheat starch modified more than 1½ h and parent potato starch (Table 1). This indicates the presence of

a well dispersed polymer solution with micro gel segments.

3.2. Gelation and retrogradation

The effects of starch gelation and retrogradation (which cannot be discriminated using the LF NMR method) were analyzed by principal component analysis (PCA) using both day 1 and day 10 LF NMR relaxation data. The scores of the two most important principal components (PC 1 and 2), explain together 99.7% of the variation (Fig. 2A). The lines connecting the day 1 and day 10 samples (displacement) indicates the assumption-free measure of ageing, comprising the combined gelation/retrogradation process of the hydrocolloid system (Thygesen *et al.*, 2003). Hence, a long line indicates large change in the proton relaxation from day 1 and day 10 gels. PC 1 describes most of the variation explaining 91.6% of the total variance, and describes mainly the relaxation rate. Samples scoring high PC 1 values relax slowly, and samples scoring low PC 1 values relax fast. The score plot show that all waxy maize starches (grouped in Fig. 2A) relax clearly slower (702-790 ms) compared to the rest of the samples (78-516 ms). As visualized by the PCA plot in Fig. 2A, all the samples 'migrate' to a more ordered state from day 1 to day 10, and are all shifted in a similar direction during ageing as indicated by the gray arrow. AM-modified HAP, pea and most of the potato starches having fast T_2 relaxation times are located in the lower left side of the scores plot.

The ageing of the samples depended on botanical origin, enzyme activity and type of enzyme used in starch modification. Fig. 2B lists the displacement of all the samples caused by the ageing over 10 days. It must be emphasized that gelatin does not retrograde wherefore the gelatin lines represent the reproducibility of the experiment and perhaps a weak syneresis effect. Compared to parent potato (no. 1) and gelatin samples (nos. 59-60), potato starch modified with 2 unit AM/g starch for 1½ h (no. 2) retrograde to 10-fold higher ageing level (Fig. 2B). Further AM modification of no. 2 results in starches with less ageing. After 22 h AM modification, the ageing was reduced three-fold (compare nos. 2 with 5). Modifying potato starch extensively with 50 times more AM activity, resulted in starches having comparable ageing as gelatin (compare nos. 12-15 with 59-60). Modification of Gelamyl 120 resulted in slightly reduced ageing. However, this sample had approximately 5-times higher ageing as compared to parent potato. HAP, waxy maize and pea starch modified with AM all show ageing levels comparable to the change in gelatin ageing was slightly reduced compared to their parent samples (nos. 27-31, 39-43 and 44-48, respectively). Modification of maize and wheat starch on the other hand

resulted in increased ageing, even after extended enzyme treatment showing five- and two-fold higher ageing comparable to the change in gelatins, respectively. BE-modified starches are generally expected to generate gels with low tendency for gelation and ageing (Takata et al., 1997;Spendler et al., 1997). However, a combined AM/BE is not readily predicted. Hence, we investigated in the combined AM/BE modification using pea starch as model starch. In the beginning ageing increases. Hereafter ageing decreases after prolonged BE enzyme treatment to a level, which is comparable to the changes in the gelatins (Fig. 2B). Hence, AM-catalyzed transfer of amylose segments to amylopectin is important for increased gel formation and this process can be readily reversed by BE resulting in a branched starch resulting in reducing gelation.

3.3. T_2 values from discrete exponential fitting of day 1 and 10 LF NMR relaxation data

The demonstrated changes in proton relaxation data of the gels during storage and its dependence on the biopolymer used (Fig. 2A) were addressed in more detail by employing discrete exponential fitting to the data set. Fig. 3 illustrates the change in proton relaxation for each sample showing T_2 (day 1) plotted against T_2 (day 10). From this, it is observed that in most cases the T_2 relaxation time is either reduced or unchanged. The parent HAP, pea, potato, wheat, maize and waxy maize starch plus the AM-modified HAP, pea and waxy maize starches and the extensively enzyme treated potato starches were virtually unaffected after storage (Fig. 3). Potato starch extensively modified using 2, 10 and 100 unit AM/g starch all settled to a common T_2 value around 100 ms after day 10, which is equally to AM-modified HAP and pea starches indicating a lower limit of T_2 relaxation time in starch gels irrespectively of the structure of the starch. This shows that these starches have reached a maximum of recrystallization, and thus further solidification of the polymers is not possible. Although AM-modified HAP and waxy maize starches displayed very little ageing, they showed completely different relaxation behavior when compared to gelatin relaxation. The water is rapidly exchanging in the HAP starches while they appear much more slowly relaxation moving in the waxy maize starches. The parent potato (no. 1) and pea starch combined AM/BE-modified (no. 53) had a T_2 relaxation comparable to gelatin samples (nos. 59-60) during the 10 days of storage.

3.4. Linking molecular structure to gel properties

With the aim of finding relationships between molecular data, and the relaxation data as well as gel textural data provided from a previous study (Hansen *et al.*, 2008), a

PCA model (Fig. 4) was built using the relaxation data, amylopectin chain length (CL), apparent molecular weight (Mw) and gel texture data for all samples. Obviously, for gelatin, no CL data can be obtained and hence, a PCA model capable of handling missing values (Walczak and Massart, 2001) was applied for the calculations. Fig. 4A and 4B shows the scores and loadings plot, respectively, for the first two PCs explaining 70.1% of the total variance. Four clusters are identified in the PCA score plot: cluster one, two and three contain exclusively the waxy maize starches, gelatin samples and HAP starches, respectively. Cluster four includes the rest of the samples. PC 1 describes amylopectin CL, gel texture and T_2 relaxation times, whereas PC 2 mainly describes the apparent Mw.

The PCA loading plot reveals a clear clustering between the textural parameters *force of deformation*, *hardness* and *adhesion I* (Fig. 4B). This cluster was negatively correlated to *adhesion II* localized in the opposite corner in the plot (indicated by black arrow). The loading plot also reveals a positive correlation between the gel texture and the amylopectin CL showing similar PC 1 score values. This correlation verify the previous study (Hansen *et al.*, 2008) in which a similar correlation with a three-component PLS model was computed. Furthermore, T_2 (day 1) and T_2 (day 10) are highly positively correlated (which is also observed from Fig. 3), demonstrating that proton relaxation in the gels after 10 days storage is strongly dependent on the reference proton relaxation in the gels after just one day storage at 20ºC. The PCA loading plot also shows that T_2 (day 1 and 10) is negatively correlated to long amylopectin CL (sum of DP 60-80). A seven-component PLS model gave a leave-one-out validated correlation of $r^2 = 0.69$ between measured and predicted DP 60-80 from the day 1 relaxation data and gave an RMSECV = 2.4% (DP 60-80 range from 4.0 to 21.6%). However, when using day 10 relaxation data instead of day 1, a six-component PLS model predicting DP 60-80 resulted in improved prediction was found ($r^2 = 0.82$, RMSECV = 1.8%). The improved prediction using day 10 relaxation data is explained by the fact that the amylopectin in the gels have retrograded to a certain point after 10 days of storage, of which is 'native' to the functionality expressed by the amylopectin CL. A slightly better prediction was obtained when using the DP 60-80 parameter as compared to the amylopectin mean CL. This confirms the importance for specific long amylopectin chains for gel texture and gel formation as monitored by the proton relaxation of the system. In addition, a weak negative correlation between the T_2 relaxation time and gel texture was also revealed. A seven-component PLS model predicting the gel texture (*force of deformation*) from the day 10 relaxation data with a correlation of $r^2 = 0.78$ and RMSECV = 1.3 N s (the *force of deformation* range from 0.0 to

11.5 N s). In addition, a better prediction was also obtained using day 10 relaxation data than using day 1 data (not shown). The correlation between the apparent Mw and proton relaxation as indicated in the loading plot in Fig. 4B could not be confirmed using PLS.

3.5. Construction of a starch-based gelatin

The correlation between the LF NMR relaxation at day 10 and the amylopectin CL data for the starch-based samples was exploited to construct a starch system with gelatin-like gel properties. This backward approach can provide the molecular chain length requirements of a starch system to emulate gelatin functionality. The estimate was produced by a PLS2 model with the LF NMR relaxation data in the **X** matrix (58 non-gelatin samples × 4094 variables) and the amylopectin CL data in the **y** matrix (58 samples × 77 variables). The model was cross-validated and the optimal number of PLS-components was estimated to be five. The LF NMR relaxation curves for the two gelatin samples was then used as input to the developed model giving rise to a model amylopectin CL distribution closely resembling gelatin water binding.

Fig. 5A shows the amylopectin CL distribution of all starch samples colored according to the levels of amylopectin mean CL together with the two *in silico* calculated CL distributions marked in black as modeled from gelatin LF NMR data. The calculated CL distributions show complex profiles having the main peak at approximate DP 15–18 with a broad smaller shoulder around DP 38-48. Compared to most of the AM-modified potato, Gelamyl 120, HAP, maize and pea starches, the gelatin estimated CL distributions have higher fraction of short chains (DP 10-25), lower fraction of medium chains (DP 30-50) but almost similar fraction of longer chains (DP 60-80).

Fig. 5B shows the PCA score plot of all starch and calculated amylopectin CL data for the first two PCs. PC 1 mainly describes the amylopectin mean CL, and PC 2 describes very short chains (below DP 10) and the position of the main peak. Consequently, samples scoring lower PC 1 values have higher amylopectin mean CL. Starch samples located in close proximity to the gelatin-like generating CL distributions (nos. 59-60) are parent Gelamyl 120 (no. 22) and limit AM-modified potato (no. 16) and maize (no. 33). These samples have an amylopectin CL distribution that generate gelatin-like proton relaxation. Parent HAP starch (no. 27) is also rather similar but its distribution is slightly shifted a few DP's. It is interesting that parent Gelamyl 120 is similar to the model distributions, as it is fully or in part used to replace gelatin (and gum arabic) primarily for the confectionery industry (especially for jelly, wine/fruit gums and liquorice pastilles). However, the main

contribution to Gelamyl 120 functionality is its oxidation, and its amylopectin CL distribution can therefore not readily be used as a guideline for gelatin-like CL structure. The potato, Gelamyl 120 and maize samples however make rather weak gels and T_2 relaxation is faster compared to the gelatin (Table 1). Parent HAP starch on the other hand makes gels twice as strong, but has much lower T_2 relaxation times compared to the gelatins.

4. Discussion

In this study, the proton relaxation of amylomaltase- (AM) and branching enzyme- (BE) modified starch gels compared to gelatin gels were analyzed by LF NMR. We found that following enzyme modification the spin-spin relaxation time constant (T_2) distribution of gels prepared from the modified starches gets narrower (more homogeneous) and the apparent proton exchange increase. Previous investigations have demonstrated that modification of the starches with AM results in disappearance of amylose, broadening of the amylopectin chain length (CL) distribution and improved gel texture (van der Maarel *et al.*, 2005;Hansen *et al.*, 2008). We have now further demonstrated that the CL distribution of the starch samples and especially a pool of long chains (DP 60-80), correlates with gel texture as well as proton relaxation of the 12.0% (w/w) gels. Hence, very specific lengths of the amylopectin chains have a tremendous effect on physico-chemical properties of starch gels. It has been suggested that the CLs are important determinant of crystalline polymorph (Hizukuri, 1985), the degree of phosphorylation (Blennow, Bay-Smidt, Wischmann, Olsen, and Moller, 1998) and melting properties (Wischmann *et al.*, 2005). Interestingly, most of the AM-modified potato and pea starches have very similar amylopectin CL distributions (see no. 38 and 50 in upper left corner in Fig. 5B), and yet have diverse functionalities such as gel texture and proton relaxation (Table 1). We address this as an effect of that even small changes in the CL distribution can have a significant effect on the functionality of the starch. However, other factors as branch point positioning in the starch and its apparent molecular weight (Mw) does also likely influence functionality. No correlation was found between the apparent Mw and T_2 relaxation times or gel texture, demonstrating that lower scale molecular dimensions are more important. However, for selected samples molecular size seems to be important. Potato starch modified with 2 and 100 unit AM/g starch for 22 h (nos. 5 and 15, respectively), had very similar CL distribution, but differed by a factor 880 higher in the apparent Mw. Gels prepared from with these starches showed about 2½-fold increase in gel texture and

significantly reduced proton relaxation after 1 day storage (Table 1). This effect can very well be attributed to the molecular size. Introducing more α-1,6-glycosidic branching linkage has a positive effect on the proton relaxation of the starch gels as demonstrated by combined AM/BE modification of pea starch.

Our study demonstrates that the understanding between starch physico-chemical characteristics and functionalities is challenging and that multivariate, chemometric strategies are necessary to extract the relevant parameters. By applying PCA and PLS chemometric tools it was found that a specific pool of long chains in amylopectin are negatively correlated to proton relaxation in starch gels. Hence, specific increase of CLs in the starch producing crop or with enzyme technology would result in starches with reduced proton relaxation and gelatin-like functionality.

A biopolymer-based gel such as amylopectin can be considered as an entangled polymer solution in which the entanglements between different polymer chains are more than just transient, and can be classified as permanent cross-links. In hydrocolloid gels the cross-links are usually composed of ordered 'junction zones', in which chain segments from different polymer chains are packed in an ordered array of non-covalently linked chain segment. Both gelatin and starch gel forms junction zones based on helical structures, which involve that one or more chain segments in a helical structure aggregate via hydrogen bonds and thus 'sacrifices' numerous hydroxyls from being available for the dynamic chemical exchange with the water protons. The junction zones of the gelatin gel network have a triple-helical-ordered conformation (Stainsby, 1977), whereas starch gelation involves double-helical junction zones (Gidley, 1989). While structuring of the biopolymer in the gel, polymer hydroxyls are 'lost' as relaxation sinks and compartmentalization of the water populations may be created, which explains how proton relaxation depend on the amylopectin CL distribution.

5. Conclusion

In this work, the proton relaxation of a set of parent and amylomaltase- (AM) modified starch gels with a range of different amylopectin chain length (CL) distributions, apparent molecular weight (Mw), textural data were analyzed using low-field ^{1}H nuclear magnetic resonance (LF NMR) relaxometry. It can be concluded that multiexponential fitting and distributed exponential fitting confirmed that the spin-spin relaxation time constant (T_2) in starch gels was characterized by one water component system. AM-

modified starches resulted in reduced proton relaxation to around 90 ms and got a more narrow distribution of T_2 as a function of enzyme modification time, whereas the combined amylomaltase/branching enzyme- (AM/BE-) modified samples was found to get a broader water distribution and increased proton relaxation from around 80 to 480 ms after 22 h of enzyme treatment. Highly branched starch generated by combined AM/BE enzyme treatment and parent potato starch resulted in gels with proton relaxation comparable to that of gelatin. No correlation was found between storage displacement and the apparent Mw. Hence, enzyme-modified starches apparently behave differently as compared to native starches. The long chains in amylopectin (DP 60-80) are not only important for efficient gel formation, but also for the proton relaxation for 12.0% (w/w) gels after 1 and 10 days storage at 20ºC. A hypothetical 'amylopectin CL distribution' required for enzymatically modified starches to emulate gelatin functionality was established. This calculation showed that limit AM-modified potato (no. 16) and maize (no. 33) and parent HAP starches (no. 27) have an amylopectin CL distribution similar to the gelatin-model distribution.

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Figure Caption

Fig. 1. Maximum-normalized LF NMR relaxation curves recorded after 1 day storage at 20°C showing selected starch and gelatin samples (A-D). The LF NMR data was analyzed by discrete (E-H) and distributed (I-L) exponential fitting. Potato starch modified with 10 unit AM/g starch for 0, ¼, ¾, 1½ and 20 h (red); gelatin (cyan); pea starch combined modified with AM/BE for 0-22 h (black); HAP starch modified with 2 unit AM/g starch for 0 and 22 h (yellow); Gelamyl 120 modified with 10 unit AM/g starch for 0 and 22 h (blue); maize starch modified with 10 unit AM/g starch for 0, ¼, and 22 h (light green); waxy maize starch modified with 2 unit AM/g starch for 0 and 22 h (dark green).

Fig. 2. The ageing (gelation and retrogradation) process. (A) PCA score plot for principal components (PCs) 1 vs. 2 of both day 1 (●) and day 10 (●) relaxation curve data. The two PCs explain 99.7% of the variation. The lines connect days 1 and 10 for each sample. The length of the line (the displacement) is used as an assumption-free measure of starch paste retrogradation. Numbers in parenthesis represent explained data variance. Data was mean centered and max-normalized prior to PCA calculation. Gray arrow indicate the direction during ageing from day 1 to day 10. (B) The day 1 to day 10 displacement. Starch samples are colored according to botanical origin: potato (red); Gelamyl 120 (blue); HAP (yellow); maize (light green), waxy maize (dark green); pea (black); wheat (gray). Gelatin (cyan). Each modification time-course has been grouped. Parent samples are nos. 1, 22, 27, 32, 39, 44 and 54.

Fig. 3. Relaxation time constants (T_2) of day 1 vs. day 10 obtained by discrete exponential fitting of the LF NMR relaxation curves. Starch samples are colored according to botanical origin: potato (red ▼); Gelamyl 120 (blue ▼); HAP (yellow ▼); maize (light green ●), waxy maize (dark green ●); pea (black ♦); wheat (gray ►). Gelatin (cyan).

Fig. 4. PCA model of the autoscaled descriptors: T_2, ageing (displacement), gel texture (*force of deformation*, *hardness*, *adhesion I* and *II*), amylopectin CL data (sum of DP 60-80 and amylopectin mean CL) and apparent Mw for all the starch and gelatin samples. (A) Score plot for PC 1 vs. 2 and (B) corresponding loading plot. The two PCs explain 70.1% of the total variation. Correlations between the clusters are indicated by black arrows. Starch samples are colored according to botanical origin: potato (red ▼); Gelamyl 120

(blue ▼); HAP (yellow ▼); maize (light green ●), waxy maize (dark green ●); pea (black ♦); wheat (gray ►). Gelatin (cyan). Texture, amylopectin CL and apparent Mw data is from (Hansen *et al.*, 2008).

Fig. 5. (A) The amylopectin CL distribution of all starch samples colored according to the levels of amylopectin mean CL. The estimated gelatin samples are marked in black. (B) PCA score plot for PC 1 vs. 2 of the mean centered amylopectin CL distribution. The two PCs explain 93.5% of the total variation. Starch samples are colored according to botanical origin: potato (red ▼); Gelamyl 120 (blue ▼); HAP (yellow ▼); maize (light green ●), waxy maize (dark green ●); pea (black ♦); wheat (gray ►). Estimated gelatin (cyan).

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Tables

Table 1. An overview of the samples amylopectin chain length, gel texture, apparent molecular weight (app. Mw) and proton relaxation data. Relaxation time constants (T_2) and corresponding concentrations (M_2) obtained by discrete mono-exponential fitting of the Carr-Purcell-Meiboom-Gill (CPMG) curves obtained at 4°C after 1 day and 10 days. Samples marked with black star can only in mathematically terms can be considered as having two water components.

Sample no.	Enzyme modification[a)]		Chain length[b)c)]	Gel texture[d)c)]	App. Mw[c)]	Discrete exponential fitting			
	(Enzyme activity, incubation time)		(% of total)	(N s)	(10^6 Da)	Day 1		Day 10	
						T_2 (ms)	M_2 (%)	T_2 (ms)	M_2 (%)
Potato									
1	No enzyme	0 h	10.3	0.02	6.8	516.3*	84.6*	461.0*	82.0*
2	2 unit AM/g starch	1½ h	12.7	2.32	25.9	672.0	94.4	229.5	96.0
3	" "	3½ h	15.1	4.51	18.7	438.5*	94.7*	111.7*	93.6*
4	" "	5½ h	15.4	4.43	14.8	289.0	95.7	95.4*	92.5*
5	" "	22 h	14.7	3.88	8.8	173.5	96.8	87.5*	93.7*
6	10 unit AM/g starch	15 min	12.9	1.20	18.0	657.7	93.7	245.2	95.0
7	" "	45 min	14.5	4.14	11.2	307.3	95.8	95.6*	94.4*
8	" "	1½ h	14.6	3.65	6.8	190.4	96.4	91.5*	93.7*
9	" "	2½ h	15.9	3.73	4.7	158.5	97.0	89.5*	93.9*
10	" "	3½ h	15.8	3.33	3.9	150.7	96.8	88.4*	93.6*
11	" "	20 h	14.9	2.68	2.1	141.0	97.1	89.8*	95.0*
12	100 unit AM/g starch	1½ h	14.2	1.84	0.4	132.4	97.1	91.7*	95.7*
13	" "	3½ h	16.1	1.72	0.3	111.0*	96.1*	91.3*	94.8*
14	" "	5½ h	16.1	1.65	0.2	90.9*	96.6*	75.5*	95.8*
15	" "	22 h	15.4	1.46	0.1	100.8	97.6	86.1*	96.6*
16[e)]	10 unit AM/g starch	15 min	11.7	1.29	2.3	329.9	94.3	190.9	95.0
17[e)]	" "	45 min	13.7	3.50	19.7	471.2	94.2	137.1	96.0
18[e)]	" "	1½ h	13.5	4.98	22.8	260.0	96.0	98.6*	94.8*

19[e)]	" "	3½ h	15.8	4.63	13.4	176.1	96.8	92.7*	93.0*
20[e)]	" "	5½ h	15.9	4.30	9.6	138.3	96.5	88.9*	92.5*
21[e)]	" "	22 h	15.2	3.96	6.7	137.5	97.2	87.5*	94.1*
Gelamyl 120									
22	No enzyme	0 h	11.1	0.87	0.5	404.8*	90.9*	196.7	95.2
23	10 unit AM/g starch	1½ h	12.7	0.86	0.6	373.9	95.0	172.2	96.7
24	" "	3½ h	12.6	0.84	0.6	354.2*	94.0*	174.8	96.5
25	" "	5½ h	12.5	0.84	0.6	315.6	95.6	164.9	96.7
26	" "	22 h	12.4	0.72	0.6	281.5	95.6	155.9	96.8
HAP									
27	No enzyme	0 h	14.8	9.05	1.0	113.4*	92.4*	98.0*	91.5*
28	2 unit AM/g starch	1½ h	17.5	11.52	5.7	90.6*	89.9*	88.0*	89.9*
29	" "	3½ h	20.2	10.51	5.7	90.8*	91.3*	89.5*	91.3*
30	" "	5½ h	21.6	10.43	5.8	90.2*	90.3*	89.2*	90.4*
31	" "	22 h	19.6	10.09	5.2	88.8*	90.8*	88.3*	90.7*
Maize									
32	No enzyme	0 h	5.8	0.62	3.9	246.7*	86.7*	229.0*	87.1*
33	10 unit AM/g starch	15 min	9.2	0.37	14.6	327.9	93.9	262.2	94.6
34	" "	45 min	10.2	1.02	18.3	329.1	93.9	203.8	95.0
35	" "	1½ h	10.5	2.72	22.4	304.4	95.2	153.8	96.6
36	" "	3½ h	11.1	2.96	29.2	284.2	94.3	134.3	95.3
37	" "	5½ h	10.6	2.91	26.8	294.7	95.3	127.7	96.7
38	" "	22 h	11.4	3.26	29.2	286.6	95.2	125.5*	94.4*
Waxy Maize									
39	No enzyme	0 h	4.0	0.01	51.2	746.5	94.2	702.8	94.4
40	2 unit AM/g starch	1½ h	6.7	0.00	31.4	762.7	93.6	747.1	93.5
41	" "	3½ h	7.1	0.01	17.0	774.1	93.5	760.6	93.5
42	" "	5½ h	7.9	0.00	13.6	776.8	93.4	765.0	93.3
43	" "	22 h	7.7	0.01	11.9	790.7	93.7	778.9	93.6
Pea									
44	No enzyme	0 h	6.2	3.08	4.7	233.7	93.0	183.2	93.2
45	10 unit AM/g starch	1½ h	15.7	6.15	9.0	82.1*	91.2*	77.6*	91.1*
46	" "	3½ h	16.4	4.93	4.6	82.0*	92.6*	77.5*	92.0*
47	" "	5½ h	16.1	4.71	3.4	82.0*	92.2*	77.8*	91.7*
48	" "	22 h	14.8	3.30	1.3	85.8*	95.9*	80.4*	95.8*

49[f)]	AM/BE combined	0 h	15.1	6.87	12.5	82.4*	90.0*	78.9*	88.2*
50[f)]	" "	1½ h	11.2	2.61	1.5	101.4	96.9	81.1*	93.7*
51[f)]	" "	3½ h	6.8	0.37	1.0	285.1	94.2	135.7	94.4
52[f)]	" "	5½ h	4.2	0.02	0.8	392.3	93.6	274.9	92.6
53[f)]	" "	22 h	0.7	0.02	0.6	476.8	93.2	405.2	92.2
Wheat									
54	No enzyme	0 h	4.0	2.27	17.1	168.5	95.1	164.6	94.4
55	2 unit AM/g starch	1½ h	5.9	0.05	19.4	260.7	94.6	227.1	93.8
56	" "	3½ h	6.8	0.09	12.1	249.9	94.1	193.8	93.3
57	" "	5½ h	7.3	0.15	10.0	236.8	95.2	176.3	94.3
58	" "	22 h	6.3	0.40	5.3	239.9	93.5	155.8	93.9
Gelatin									
59	-	-	-	4.16	-	481.3	96.2	420.4	96.3
60	-	-	-	6.16	-	400.8	96.6	355.8	96.8

One unit of AM activity is defined as the release of 1 µmol of glucose per minute at pH 6.5, 60°C with maltotriose as substrate, and one unit of BE is defined as the amount of enzyme that can decrease A_{660} of the amylo-iodine complex by 1% per minute at 60°C pH 7.0. AM: amylomaltase; BE: branching enzyme.

a) After autoclavation for 30 min at 140ºC, starches were modified at 85°C.

b) Chain length indicates the sum of amylopectin chains from the degree of polymerization (DP) from 60 to 80 given in percent of total.

c) From (Hansen, Blennow, Pedersen, Norgaard, and Engelsen, 2008).

d) Gel texture indicates the *force of deformation*, which is the area of compression during texture analysis of 12% gels (w/w) after storage at 4°C for 24 h.

e) Starches were modified at 70°C.

f) Pea starch was modified with 10 unit AM/g starch for 2 h in order to 'remove' amylose, resulting in sample no. 49. Meanwhile maintaining the AM activity, no. 49 was further modified with 1000 unit BE/g starch for 1½-22 h at 60°C (resulting in nos. 50-53).

Fig. 1

Note:
Intended for color reproduction on the Web and in print.

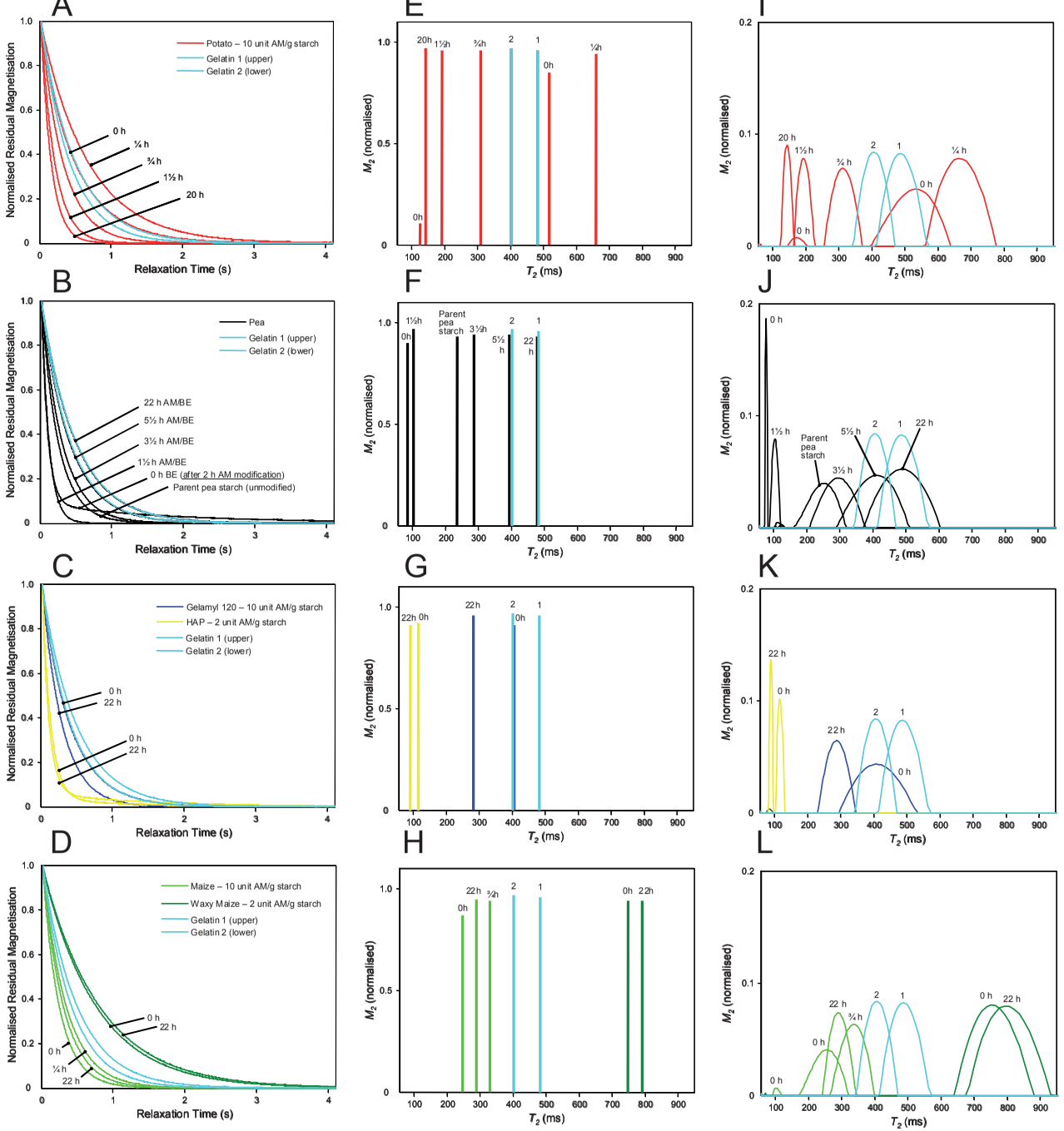

Fig. 2

Note:
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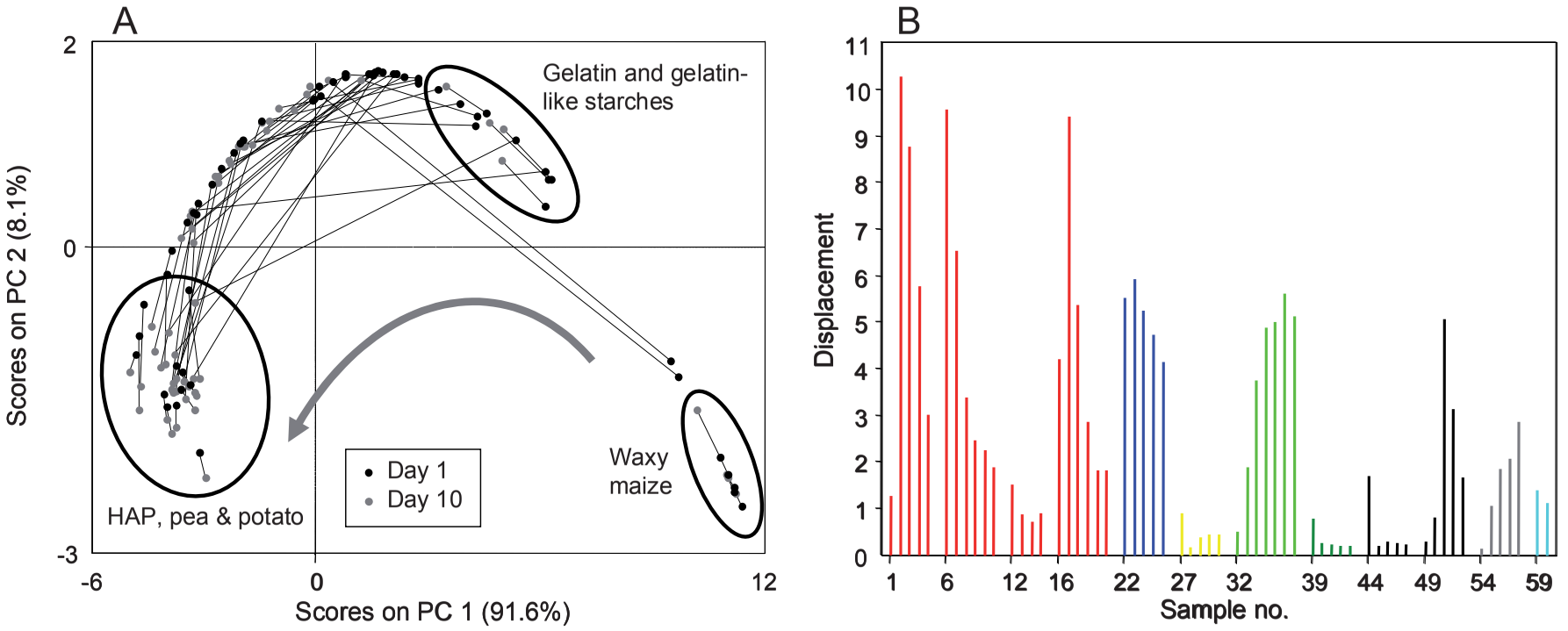

Fig. 3

Note:
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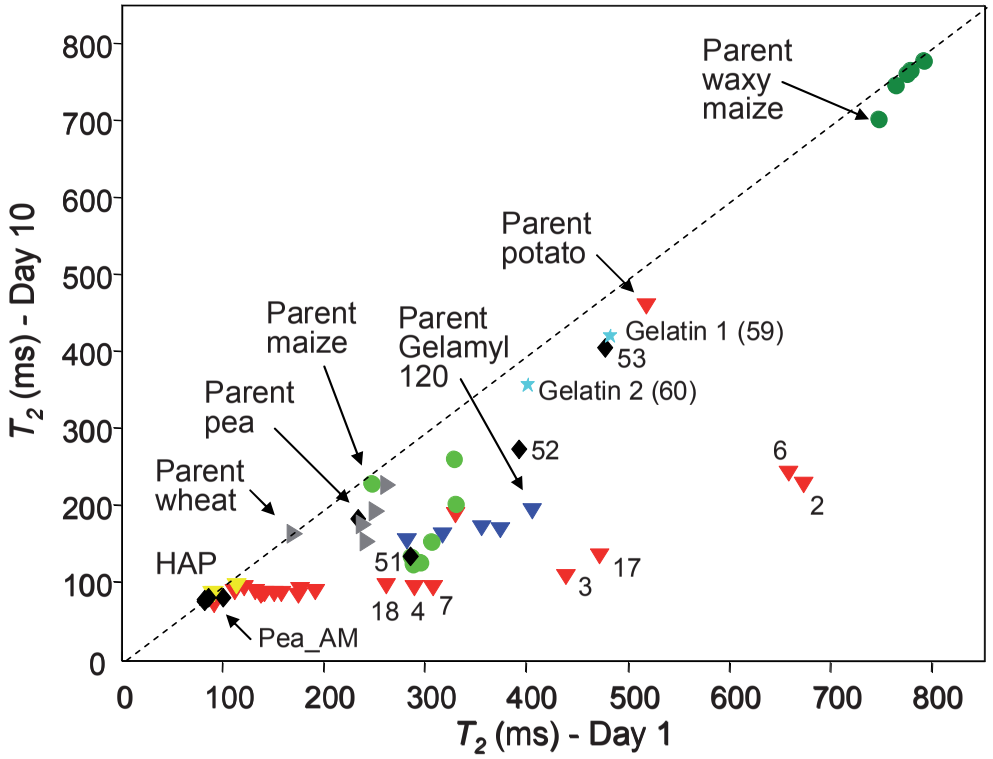

Fig. 4

Note:
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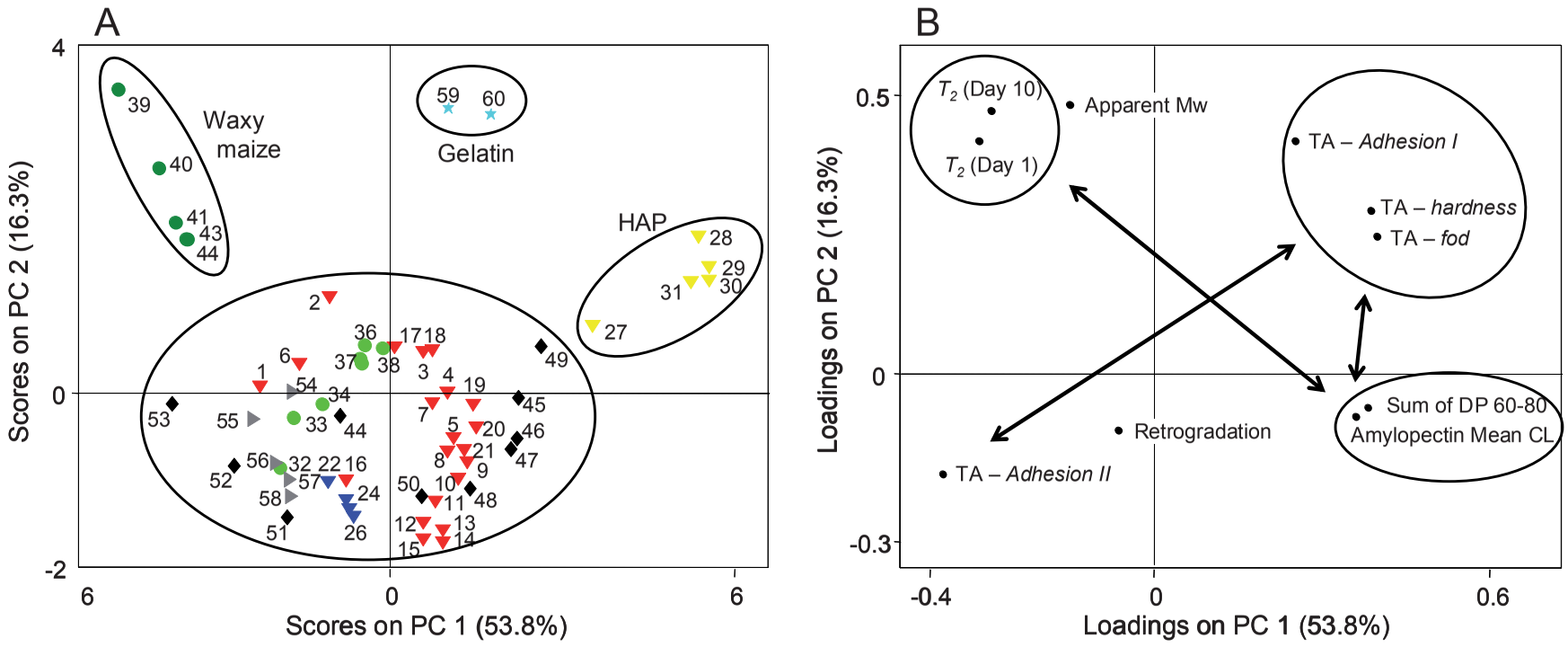

Fig. 5

Note:
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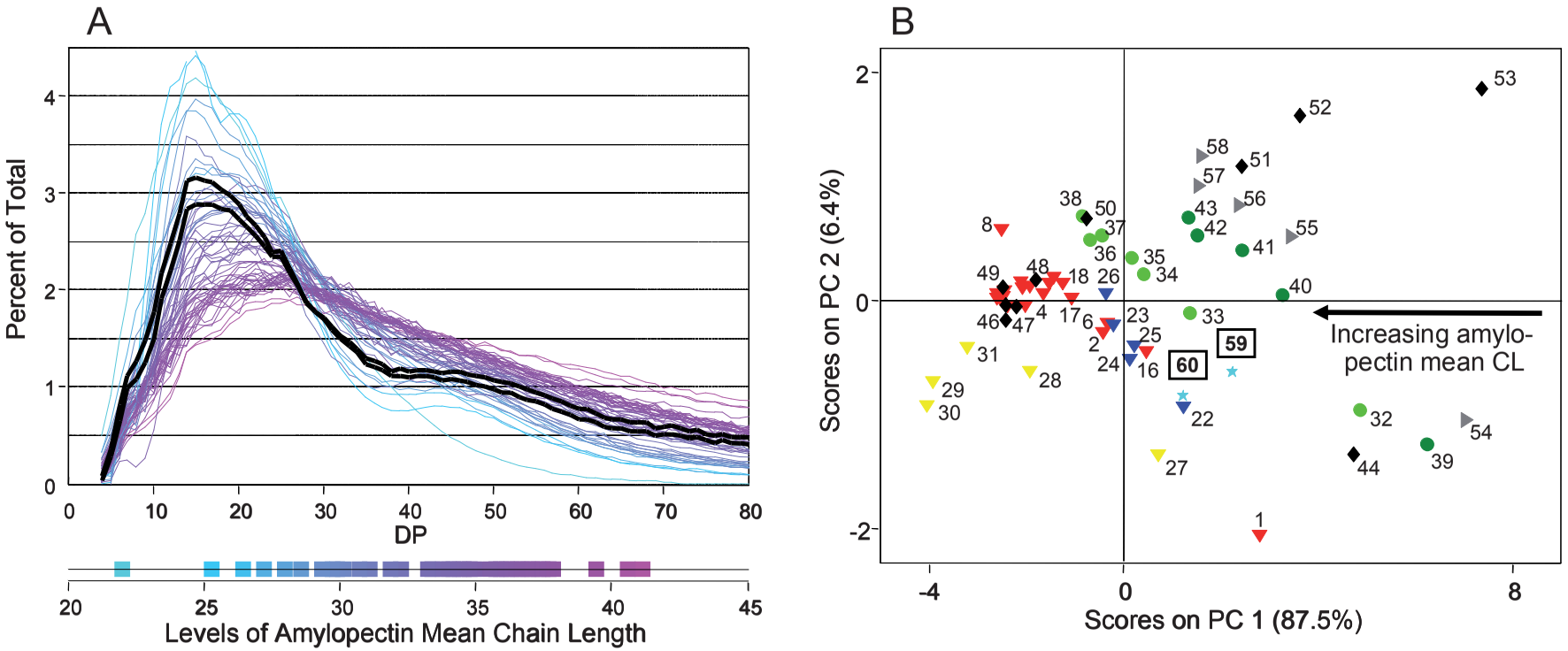

Paper III

Enzyme modification of starch with amylomaltase results in increasing gel melting point

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Submitted

Enzyme modification of starch with amylomaltase result in increasing gel melting point

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Abstract

Melting properties of gelatin-based gels are fundamental for their functionality. With the aim at generating gelatin-like starch-based systems, thermodynamic properties of 20% (w/w) gels of 51 amylomaltase- (AM) (4-α-glucanotransferase; E.C. 2.4.1.25) modified starches, 7 non-enzyme-modified starches and 2 gelatins were investigated using differential scanning calorimetry (DSC). AM modification generally increased gel peak temperature (T_p) and enthalpy of transition (ΔH). The increase in T_p for the potato starches was from 65ºC to 74ºC, whereas for the maize starches it was elevated from 57ºC to 70ºC. Only for the combined AM and branching enzyme (BE) modified pea starches decreased T_p (from 79ºC to 61ºC) was obtained. This effect was followed by a decreased gel formation and hence a fully gelatin comparable gel was not obtained. A two-component principal component analysis (PCA) model of the entire DSC dataset revealed the gross features indicating the ΔH information. The T_p was highly correlated to the amylopectin chain length distribution. In particular, T_p was found to be negatively correlated to short chains (DP 11-21) and positively correlated to long chains (DP 60-80).

Keywords: Enzyme modified starch; amylomaltase; branching enzyme; differential scanning calorimetry; chemometrics.

1. Introduction

Starch is widely used in the food industry. The majority of the industrial starch is extracted from maize, tapioca, potato, and wheat sources. Most starches can form gels, but lack the property of reversible phase transition of a defined gel state to a liquid (solution) upon heating. A property termed thermoreversibility. A classic example of a thermoreversible polymer widely used in the food industry is gelatin that is an animal protein derived from skin, connective tissue and bones (Djagny *et al.*, 2001). A unique ability of gelatin gels is its melting around human body temperature making aroma release optimal and accountable for the appetizing mouth feeling of food products containing gelatin (Renard *et al.*, 2006).

A plant and chemical-free alternative to gelatin is amylomaltase- (AM) (4-α-glucanotransferase; E.C. 2.4.1.25) modified starches that is expected to find application in the food industry (Euverink G.J.W. and Binnema D.J., 2005). AVEBE launched in 2007 an AM-modified potato starch that is used as fat replacer and enhancer of creaminess in yoghurt (Alting *et al.*, 2009). The search for a gelatin-replacer has been ongoing for many years, and several potential polysaccharide-based alternatives for the food industry have been reviewed (Karim and Rajeev, 2008). Modifying potato and rice starch with AM from *Thermus thermophilus*, *Pyrobaculum aerophilum* and *Thermus scotoductus* results in products with acquired thermoreversibility gel characteristics (Kaper *et al.*, 2005;van der Maarel *et al.*, 2005;Lee *et al.*, 2006). Gels of potato starch modified with AM from *Pyrobaculum aerophilum* was 50% melted at 37ºC and completely melted at 60ºC (Kaper *et al.*, 2005). AM catalyzes the intermolecular transfer of a segment of a α-1,4-D-glucan to a new 4-position in another α-1,4-D-glucan in a disproportionation reaction. The basis behind the gained thermoreversibility and increased gel-texture is supposedly a direct affect of the quite dramatic molecular changes catalyzed by AM including the disappearance of free amylose, broadened amylopectin chain distribution and decreased molecular weight (Kaper *et al.*, 2005;van der Maarel *et al.*, 2005;Lee *et al.*, 2006;Hansen *et al.*, 2008a). In a recent study, maize starch was granular modified with AM from *Thermotoga maritima* causing amylose consumption, an almost unaltered amylopectin chain length distribution and products capable of forming gels with thermoreversible gelling property (Oh *et al.*, 2008).

DSC is a useful technique for monitoring thermal transitions of solids or gels. In a

typical DSC experiment, the difference in energy input into the sample and reference is measured as a function of temperature (Watson *et al.*, 1964). For most gel systems, the phase transition is endothermic and the endotherm, ΔH, is recorded as the peak area and peak temperature (T_p) is recorded as the temperature at maximum enthalpy. For starch, both these parameters are typically interpreted "crystallinity" (ΔH) and molecular perfection or order (T_p) (Kozlov *et al.*, 2007). Native starches show complex thermal profiles that depends on their botanical origin. Moreover, the ratio between the amylopectin and amylose fractions affects the melting behavior considerably and the onset temperature of recrystallized amylopectin and amylose is above 40 and 90ºC, respectively (Sievert and Pomeranz, 1990;Creek *et al.*, 2007). For starch gels, the main phase transitions originate mostly from disentanglement of double helical junction zones formed by adjacent chains (Gidley, 1989).

The purpose of this investigation was to study the thermal properties of gels prepared from starches of various botanical origin after being subject to enzyme modification with AM from *Thermus thermophilus* (Kaper *et al.*, 2004;van der Maarel *et al.*, 2005). Some starches were also subjected to combined modification with branching enzyme (BE) (1,4-α-D-glucan branching enzyme; EC 2.4.1.18) from *Rhodothermus obamensis* (Shinohara *et al.*, 2001). Data was primarily analyzed using chemometrics (Daszykowski *et al.*, 2007) permitting extraction of applicable and/or hidden information in the data set, and providing relationships with data from previous studies (Hansen *et al.*, 2008a;Hansen *et al.*, 2008b).

2. Materials and Methods

2.1. Materials

Potato, high amylose potato (HAP), maize, waxy maize, wheat and pea starch and Gelamyl 120 (chemical oxidized potato starch) were enzyme-modified using mainly amylomaltase (AM) isolated from the hyperthermophilic bacterium *Thermus thermophilus* (Kaper *et al.*, 2004;van der Maarel *et al.*, 2005). In one time-course experiment branching enzyme (BE) isolated from the thermophilic bacterium *Rhodothermus obamensis* (Shinohara *et al.*, 2001) was used in a combined modification with AM. Gelatin 1 (bloom 120) and gelatin 2 (bloom 300) were from Sigma-Aldrich (www.sigmaaldrich.com). An overview of the

samples is given in Table 1.

2.2. Differential scanning calorimetry (DSC)

Gel melting temperatures were examined by DSC using a Pyris Diamond DSC instrument (PerkinElmer, www.perkinelmer.com). Approximately 10 mg of a sample (dry weight basis) was weighed in high-pressure stainless steel pans (24 atmospheres; 60 µl; operating in the range from minus 40 to plus 300ºC; Perkin-Elmer). Phosphate buffer (50 mM, pH 7.0) was added to make a suspension of 20% (w/w). The pans were sealed and heated for 15 min at 120°C in the instrument, and subsequently stored at 4ºC for 24 h allowing formation of a gel network. The samples were scanned from 1 to 150°C at 10°C/min. Experiments were performed in duplicates. An empty stainless steel pan was used as reference. The amylose melting information in the 90-150ºC region is very dependent on the presence of lipids as helical amylose-lipid inclusion complexes having higher endothermic transition than free amylose (Creek *et al.*, 2007). However, no data was recorded in this region, and hence only melting of amylopectin segments was recorded. Thermal profiles were analyzed using Pyris Software version 7.0 software (PerkinElmer, www.perkinelmer.com) and the peak temperature (T_p) and the enthalpy of transition (ΔH) were calculated (se Table 1).

2.3. Data mining

Principal component analysis (PCA) (Hotelling, 1933;Wold *et al.*, 1987) and partial least squares (PLS) regression (Geladi and Kowalski, 1986) was carried out by use of MatLab 7.2 (The Mathworks Inc., USA, www.mathworks.com) installed with the PLS toolbox version 3.5.3 (Eigenvector Research, www.eigenvector.com). Data was either mean centered or autoscaled (Bro and Smilde, 2003) prior to building PCA models. All PLS models were mean centered and leave-one-out cross validated (Stone, 1974;Wold, 1978). The optimal number of PLS components was determined from the root mean square error of cross-validation (RMSECV). Figures 2 and 5 were created using LatentiX (Latent5, Copenhagen, Denmark, www.latentix.com).

2.3.1. Principal Component Analysis (PCA)

PCA is a technique for data compression, extraction of the main variation

(information) and data visualization (Hotelling, 1933;Wold *et al.*, 1987). The systematic variation is described by the principal components (PC 1, PC 2 etc.). In PCA, the original data **X** (samples x variables) is decomposed into a score matrix (**T**) and a loading matrix (**P**): $\mathbf{X} = \mathbf{TP}^T$. The PCA is visualized with score plot (describes the relationship between samples) and loading plot (describes the relationship between variables). Samples and variables located very close to each other in the score and loading plot, respectively, are highly correlated.

2.3.2. Partial least squares (PLS) regression

PLS regression (Geladi *et al.*, 1986) is used to model the relationship between two data sets: the predictor matrix **X** (e.g. amylopectin chain length distribution) and a property of interest **Y** (e.g. gel melting point). PLS performs a simultaneous decomposition of **X** and **Y** in such a way that the information in the **Y** is directly used as a guide for the decomposition of **X**. The linear regression model is defined as $\mathbf{Y} = \mathbf{X}b + E$, where b is the regression coefficient and E is the residuals (model errors). The model performance is given by the cross-validated correlation factor (R^2), which describes the correlation between the measured **Y** and the predicted **Y**, and by the prediction error RMSECV.

3. Results

3.1 Thermal data for 20% (w/w) gels

The endothermic peaks for melting of recrystallized amylopectin appeared over a broad temperature range. The widths of the melting transition as deduced from the difference between the onset and conclusion temperature of both AM-modified potato (Figure 1A) and maize (Figure 1B) starches appeared over a ~45ºC range (from about 40 to 85ºC), whereas melting of the AM-modified pea starches was narrower (~25ºC range spanning from 65 to 90ºC) (Figure 1C). Likewise, the melting range for gels of AM-modified HAP starches was 75 - 90ºC (data not shown). The general broad range of melting suggests the presence of a wide distribution of recrystallized chain segments in amylopectin molecules having varying stability. The narrower melting range of the high amylose starches HAP and pea indicates a more homogeneous crystal distribution for these type of starches

The peak temperature (T_p) of recrystallized amylopectin of all the starches ranged from 49.0 to 85.9ºC, and the enthalpy of transition (ΔH) ranged from 0.17 to 4.81 J/g starch. For the waxy maize starches (# 39-43) and extended combined AM/BE-modified pea starch (# 53), the T_p and ΔH were not detectable even at a starch concentration as high as 20% (w/w). The ΔH, which is a measure of the overall crystallinity of the amylopectin molecules, was 3-80 times lower compared to the granule/native starch (Blennow *et al.*, 2005;Karlsson *et al.*, 2007). Hence, only a fraction of the original molecular order of the starch granules can be recovered after enzyme treatment and re-crystallization (Cooke and Gidley, 1992).

The effects of AM modification caused a general increase in both T_p and ΔH as compared to its corresponding parent starch gels. For example, modification of parent potato starch (# 1) using 2 unit AM/g for 1½ h starch increased T_p from 65.2 to 68.7ºC. Additional treatment increased T_p to 73ºC and resulted in a 19-fold increase in ΔH (Figure 1A). Extensive modification did not significantly change the T_p (Table 1). Comparable result was found for the maize system, although the parent maize starch (# 32) had a lower T_p of 56.8ºC as compared to the potato system. Modified maize starch also gradually increased both T_p and ΔH with enzyme treatment (Figure 1B). Extended treatment of maize starch increased T_p from 56.8 to 70.1ºC and ΔH nearly 8 times. This is seemingly the upper limit for T_p, which is in good agreement with the previously finding that the amylopectin chain length (CL) can only be increased up to a certain level as a result of AM modification (Hansen *et al.*, 2008a). Similarly, for the pea starch system, products with T_p ranging from 62.9 to 78.8ºC were generated after extended enzyme treatment (Figure 1C). Only for combined modification of pea starch with AM and BE generating shorter chains, it was possible to suppress T_p as function of enzyme treatment (Figure 1D). After 5½ h combined AM/BE treatment, T_p decreased by nearly 20ºC from 79.4 to 61.0ºC, and additional treatment result in a product with no detectable transition even at 20% (w/w). Hence, the CL of the amylopectin is clearly important for the generation of crystalline segments in the gels.

As compared to the starch samples the two gelatins had extreme transitions about 22-64ºC lower T_p (26.8-27.6ºC) and 5-100 times higher ΔH (19.6-23.0 J/g gelatin) (Figure 1E and Table 1). The low melting point is one of many unique ability of gelatin gels. In gelatin gels the molecules resume an ordered triple helix conformation upon cooling by

forming a cross-linked network held together predominantly by hydrogen bonded junction zones (Stainsby, 1977). The much higher melting enthalpy of the gelatin gels is due to the stronger interaction and higher content of triple helix structures as compared to the presence of dispersed double helical α-glucan junction zones in starch (Gidley, 1989).

3.2 Data mining

Analysis of systematic variations in the raw DSC dataset of all starches was performed using PCA modeling (Hotelling, 1933;Wold *et al.*, 1987). Gelatin data were excluded as they are extremes with regards of having very low T_p and high ΔH compared to the starches. A PCA score plot for a model based on the first two principle components (PCs) describing 95.9% of the total variation clearly demonstrates the thermal relation between the samples (Figure 2). Samples that cluster have comparable thermal properties, whereas disperse samples are dissimilar. The PC 1 in the PCA score plot describes well the variation in ΔH. The PCA also demonstrates the similar thermal properties between the majorities of the AM-modified potato starches. The entire AM-modified pea and all the HAP starches group in separate clusters. The discrimination of the AM-modified potato starches located in upper right corner from the samples located in the lower right corner , both having comparable ΔH, is the slightly higher T_p (~5ºC), which partly described by PC 2.

To examine possible correlation between the thermal data and previously obtained data (Hansen *et al.*, 2008a;Hansen *et al.*, 2008b), a PCA model was calculated using DSC descriptors (ΔH and T_p), gel texture descriptors (*adhesion I-II*, *hardness* and *force of deformation*), amylopectin mean CL, apparent molecular weight (Mw) and spin-spin relaxation time constants (T_2) day 1 and day 10 data (Figure 3). Data for waxy maize starches (# 39-43) and pea starch modified with AM/BE for 22 h (# 53) were excluded in the model as the T_p and ΔH could not be determined for these starches. The first two PCs in this model explain 74.1% of the total variation. Also in this score plot, AM-modified potato, pea and HAP starches separate from the rest of the samples (Figure 3A). As evident from the close clustering in the loading plot of the HAP starches scoring high in PC 2 and low in PC 1 these starch types are characterized by having high gel texture, high amylopectin mean CL and high T_p. (Figure 3B). Moreover, they have low ΔH and low T_2 as judged from the clear discrimination in the plot. The PCA loading plot reveals multiple

relations between the variables. Correlations between amylopectin CL and gel texture and T_2 relaxation data have previously been discussed (Hansen *et al.*, 2008a;Hansen *et al.*, 2008b) . These effects are again well visualized (indicated by black arrows) in the loading plot. The ΔH variable is described by PC 2 and does not show any correlation to the rest of the variables. A high positive correlation between T_p and amylopectin mean CL was found, as they are located in close proximity to each other. Consequently, the effect of increased average chain length in amylopectin molecules cause an increase in T_p in gels. The correlation is linear having a correlation factor (R^2) of 0.85, which is depicted in the scatter plot of amylopectin mean CL vs. T_p (Figure 4A). The correlation between amylopectin CL and T_p was further analyzed by partial least squares (PLS) regression (Geladi *et al.*, 1986). Instead of using an average chain length number, PLS is able to analyze correlation between the entire dataset of amylopectin CL distribution to T_p. A five-component PLS model gave a correlation of $R^2 = 0.87$ between actual T_p and T_p predicted from all the amylopectin CL distribution data, and gave a root mean square error of cross validation (RMSECV) of 2.5ºC (Figure 4B). Although using 5 components (latent variables), a slightly improved model was obtained using PLS. Using this model, it was possible to predict T_p of waxy maize starches (# 39-43) and pea starch modified with AM/BE for 22 h (# 53), which was not included in the model. The parent waxy maize starch is predicted to have a T_p of ~56ºC, and extended AM modification for 22 h is predicted to increase T_p to ~68ºC. Extended combined AM/BE-modified pea starch is predicted to have T_p to ~57ºC, which seem reasonable when comparing to the rest of the starches from the enzyme modification time-course (compare # 53 with # 49-52 in Table 1).

Figure 4 shows that the whole distribution of chain in amylopectin molecules is correlated to the T_p. With the aim of exploring the relationship of individual chains in the amylopectin molecule, a correlation factor distribution was calculated. Figure 5 shows the amylopectin CL distribution of all starches overlaid with the R^2 distribution (in red) for each DP going from 4 to 80. High correlation was found for chains with a specific length. Fig. 5 shows that T_p was highly correlated to short chains (DP 11-21) as well as to long chains (DP 60-80) both having R^2 around 0.81-0.85. The short chains are found to be negatively correlated to T_p, whereas long chains (DP 60-80) are positively correlated. No relationship between T_p and very short chains (below DP 9) and middle length chains (DP 26-35).

4. Discussion

In this study, we have used DSC to analyze and compare the thermal profiles of 20% (w/w) gels prepared from amylomaltase- (AM) modified starches from various botanical origins and gelatins. AM-modified starches are expected to find application in the food industry because products obtain thermoreversible character with gelatin-like properties (Euverink G.J.W. *et al.*, 2005). There are several factors important for the perception of gelled food products – the two most important are gel texture and flavor release (Renard *et al.*, 2006). A previous study demonstrated that modification of potato and maize starch with AM result in products with increasing gel texture as a result of increasing longer chains (DP 60-80) in amylopectin molecules (Hansen *et al.*, 2008a). Even though AM-modified starches acquire thermoreversible character, the melting temperatures obtained in this study are too high for applications of gelled food product which should be around (or below) body temperature in order to obtain optimal organoleptical properties and aroma release in the mouth.

In this study it was found that modifying potato and maize starch with AM resulted in products with increasing peak temperature (T_p) during enzyme treatment (Figure 1A-B). Typically, AM-modification of parent starches resulted in products with increasing T_p (Table 1), which actually is in the opposite direction of what is preferable to achieve an enzyme-modified starch with gelatin-like melting point. The high amylose potato (HAP) starches had the highest T_p around 90ºC and at the same time they had the lowest enthalpy of transition (ΔH) (below 0.4 J/g starch), whereas parent wheat starch (not enzyme-modified) had the lowest T_p at 49ºC (Table 1). These transition temperatures are 30-70ºC higher than the gelatin samples, which melts at 27-28ºC (Figure 1E). However, pea starch modified with a combined AM and branching enzyme (BE) strategy resulted in products with lower T_p (Figure 1D). As an effect of the combined AM/BE modification, ΔH and as well as gel-texture decreased and after extended modification, the resulting product was not able to form a gel even at 20% (w/w) (Table 1). Hence, combined modification of starch with AM and BE is certainly ambiguous since it results in the acquired thermoreversibility and decreased T_p, but the decreased gel texture is certainly undesirable. As a result, in food application, AM-modified starches will need additional modification

e.g. chemical modification to overcome the drawback of high melting point.

The reason for the increased T_p of AM-modified starches was revealed by PCA (Figure 3B) which demonstrates that T_p is highly correlated to amylopectin mean chain length (CL). Correlation is linear (Figure 4A) and interestingly, when examining the individually chains, it was found that short chains around DP 11-22 were negatively correlated to T_p, whereas long chains above DP 60 were positively correlated to T_p (Figure 5). The opposite correlation between the short and the long chains demonstrates that during AM modification, the sum of long chains increase on the expense of a specific pool of short chains. The chain transfer is amylose-independent as shown previously for AM-modified waxy maize starch (Hansen *et al.*, 2008a) demonstrating that chains are transferred within the amylopectin molecules. The data cannot show whether T_p increases as a result of increase in long chains or decrease in short chains. However, short chains can disturb crystallization of the longer chains so the effect can very well be combined. Combined AM/BE-modified pea starches, which was the only treatment which resulted in decreasing T_p, cannot help to clarify this as long chains is converted into short chains (Hansen *et al.*, 2008a). Given that AM modification of starches results in disappearance of amylose, broadened amylopectin CL distribution from a bimodal to an unimodal long chain profile (Hansen *et al.*, 2008a), and given that recrystallized amylose melts in the region of 90 to 150ºC (Creek *et al.*, 2007), the most reasonable explanation for the increasing T_p during AM-treatment is that the newly formed longer chains in amylopectin molecules exhibit an amylose-like behavior. The increase in longer chains results in a higher content of longer double helices formation becoming more heat resistant. This is in good agreement with the decrease in T_p during combined AM/BE-modified pea starches.

In contrast to our results, Kaper *et al*. (2005) reported that gels made of potato starch modified with AM from *Pyrobaculum aerophilum* had melting temperature around 37ºC and that the gel was completely melted at 60ºC (Kaper *et al.*, 2005). No analysis of the thermal profile of the parent potato starch was performed, but we conclude that the T_p must have decreased during this treatment, which is opposite of what we observed and about 30ºC lower than our AM-modified potato starches. They also reported that modifying potato starch with the *Pyrobaculum aerophilum* AM resulted in broadened amylopectin CL distribution that is in accordance with our data. The difference cannot readily be explained from existing data. Comparing the two enzymes: The *Pyrobaculum*

aerophilum enzyme shares 43% amino acid identity with the *Thermus thermophilus* enzyme is 30 amino acids smaller and has higher temperature optimum. They both share the four conserved short motifs comprising the catalytic residues, including two aspartate and one glutamate (Takaha and Smith, 1999), which is highly conserved in the α-amylase superfamily (MacGregor *et al.*, 2001). The production of enzyme-modified potato starches using the AM from *Pyrobaculum aerophilum* only slightly differed from our study (Kaper *et al.*, 2005;Hansen *et al.*, 2008a) and the preparation of the gels prior to DSC analysis was the same. This indicates that very minor differences in the molecular structure of the glucan products are detrimental for gel formation and more detailed studies of the products at the molecular level e.g. the positioning of branch points and existence of longer chains than analyzed here, are required.

5. Conclusion

The effect of modifying starch with amylomaltase (AM) resulted in products generating aqueous gels with increasing peak temperature (T_p) and enthalpy of transition (ΔH) as compared to their corresponding parent samples. Starches modified with combined AM and BE catalysis had the opposite effect i.e. reduced T_p and ΔH. Using PCA, detailed phenomena of the produced gel systems was provided. The T_p was negatively correlated to the short (DP 11-22) and positively correlated to long (DP 60-80) amylopectin chains. Thermoreversible AM-modified starches are expected to have potential for many applications in the food industry. However, to serve as true plant-derived alternatives to gelatin, further suppression of the melting point has to be achieved.

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Figure Captions

Figure 1. Differential scanning calorimetry (DSC) traces showing the thermal profiles of potato starch modified with 2 unit AM/g starch for 0-22 h (A), maize starch modified with 10 unit AM/g starch for 0-22 h (B), pea starch modified with 10 unit AM/g starch for 0-22 h (C), pea starch combined AM (10 unit/g starch) and BE (1000 unit/g starch) modified (D) and gelatins (E) at 20% (w/w). The gels were prepared directly in the DSC pans by heating for 15 min at 120ºC. After 24 h storage at 4ºC, the samples were scanned from 1 to 150°C at a scan rate of 10ºC/min. Notice that temperature scale in E is different as compared to A-D.

Figure 2. PCA score plot colored according to levels of ΔH for the first two PCs of the raw DSC data from 40-90ºC of all starches. Prior to PCA calculation, data was detrended (Barnes *et al.*, 1989) then mean centered, in order to get a better representation of the data. The two PCs explain 95.9% of the total variation. Waxy maize starches (# 39-43) and pea starch modified with combined AM/BE treatment for 22 h (# 53) having no detectable ΔH are marked with black dots.

Figure 3. PCA model of the autoscaled descriptors: T_2 relaxation times (day 1 and 10), gel texture (*force of deformation*, *hardness*, *adhesion I-II*), amylopectin mean CL, apparent Mw and DSC data (ΔH and T_p) for all the starches except for the high AM/BE-modified pea starch (# 53) and waxy maize starches (# 39-43). Score plot (A) for PC 1 vs. PC 2 and corresponding loading plot (B). The two PCs explain 74.1% of the total variation. Starch samples are colored according to botanical origin: potato (red ▼); Gelamyl 120 (blue ▼); HAP (yellow ▼); maize (light green ●); pea (black ♦); wheat (gray ►). Black arrows indicate the correlations between the descriptors.

Figure 4. Correlations between amylopectin CL and T_p data. Scatter plot of amylopectin mean CL vs. T_p (A). Five-component PLS model predicting the T_p from the amylopectin CL distribution profiles (B). Waxy maize starches (# 39-43) and pea starch modified with AM/BE for 22 h (# 53) were excluded. Starch samples are colored according to botanical origin: potato (red ▼); Gelamyl 120 (blue ▼); HAP (yellow ▼); maize (light green ●); waxy maize (dark green ●); pea (black ♦); wheat (gray ►).

Figure 5. The amylopectin CL distribution from DP 4 to 80 of all starch samples colored according to the levels of amylopectin mean CL. Red line indicate the correlation factor (R^2) between T_p and each individual chain length in amylopectin. Waxy maize starches (# 39-43) and pea starch modified with AM/BE for 22 h (# 53) were not included in the CL distribution and in the excluded in the calculation of R^2.

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Tables

Table 1. An overview of the starch and gelatin samples. Parent starches are marked in grey.

Sample no.	Enzyme modification [a]		Mean CL [b)c)]	Gel texture [d)c)]	App. Mw [c)]	Spin-spin relaxation times [e)]		Thermal data (this study)	
	(Enzyme activity, incubation time)		(DP)	(N s)	(10^6 Da)	Day 1	Day 10	T_p	ΔH
						T_2 (ms)	T_2 (ms)	(ºC)	(J/g starch)
Potato									
1	No enzyme	0 h	32.0	0.02	6.8	516.3	461.0	65.2	0.23
2	2 unit AM/g starch	1½ h	35.0	2.32	25.9	672.0	229.5	68.7	2.94
3	" "	3½ h	37.1	4.51	18.7	438.5	111.7	71.5	3.77
4	" "	5½ h	37.6	4.43	14.8	289.0	95.4	72.9	4.72
5	" "	22 h	36.8	3.88	8.8	173.5	87.5	73.0	4.43
6	10 unit AM/g starch	15 min	34.8	1.20	18.0	657.7	245.2	68.8	4.06
7	" "	45 min	36.0	4.14	11.2	307.3	95.6	71.2	3.79
8	" "	1½ h	37.0	3.65	6.8	190.4	91.5	72.9	4.16
9	" "	2½ h	37.7	3.73	4.7	158.5	89.5	73.4	3.27
10	" "	3½ h	37.6	3.33	3.9	150.7	88.4	73.2	3.10
11	" "	20 h	36.4	2.68	2.1	141.0	89.8	74.0	3.26
12	100 unit AM/g starch	1½ h	36.2	1.84	0.4	132.4	91.7	74.0	3.42
13	" "	3½ h	37.8	1.72	0.3	111.0	91.3	75.2	2.41
14	" "	5½ h	37.8	1.65	0.2	90.9	75.5	73.9	3.93
15	" "	22 h	37.0	1.46	0.1	100.8	86.1	73.2	3.38
16[f)]	10 unit AM/g starch	15 min	33.9	1.29	2.3	329.9	190.9	61.0	2.73
17[f)]	" "	45 min	35.6	3.50	19.7	471.2	137.1	70.2	4.46
18[f)]	" "	1½ h	35.8	4.98	22.8	260.0	98.6	72.5	4.81
19[f)]	" "	3½ h	38.0	4.63	13.4	176.1	92.7	73.1	3.95
20[f)]	" "	5½ h	37.6	4.30	9.6	138.3	88.9	73.7	3.83
21[f)]	" "	22 h	37.0	3.96	6.7	137.5	87.5	73.5	3.88
Gelamyl 120									
22	No enzyme	0 h	33.3	0.87	0.5	404.8	196.7	60.7	3.49

23	10 unit AM/g starch	1½ h	34.7	0.86	0.6	373.9	172.2	69.6	2.71
24	" "	3½ h	34.5	0.84	0.6	354.2	174.8	68.5	3.34
25	" "	5½ h	34.3	0.84	0.6	315.6	164.9	68.6	3.69
26	" "	22 h	34.6	0.72	0.6	281.5	155.9	69.2	3.70
HAP									
27	No enzyme	0 h	35.0	9.05	1.0	113.4	98.0	76.9	0.49
28	2 unit AM/g starch	1½ h	37.9	11.52	5.7	90.6	88.0	82.0	0.39
29	" "	3½ h	40.6	10.51	5.7	90.8	89.5	83.3	0.18
30	" "	5½ h	41.2	10.43	5.8	90.2	89.2	82.6	0.17
31	" "	22 h	39.5	10.09	5.2	88.8	88.3	85.9	0.17
Maize									
32	No enzyme	0 h	28.1	0.62	3.9	246.7	229.0	56.8	0.53
33	10 unit AM/g starch	15 min	32.3	0.37	14.6	327.9	262.2	61.0	1.11
34	" "	45 min	33.3	1.02	18.3	329.1	203.8	65.5	2.70
35	" "	1½ h	33.5	2.72	22.4	304.4	153.8	66.4	3.24
36	" "	3½ h	34.5	2.96	29.2	284.2	134.3	69.5	3.90
37	" "	5½ h	34.1	2.91	26.8	294.7	127.7	70.3	3.74
38	" "	22 h	34.5	3.26	29.2	286.6	125.5	70.1	4.03
Waxy Maize									
39	No enzyme	0 h	26.5	0.01	51.2	746.5	702.8	n.d.	n.d.
40	2 unit AM/g starch	1½ h	29.4	0.00	31.4	762.7	747.1	n.d.	n.d.
41	" "	3½ h	30.1	0.01	17.0	774.1	760.6	n.d.	n.d.
42	" "	5½ h	31.3	0.00	13.6	776.8	765.0	n.d.	n.d.
43	" "	22 h	31.3	0.01	11.9	790.7	778.9	n.d.	n.d.
Pea									
44	No enzyme	0 h	28.6	3.08	4.7	233.7	183.2	62.9	2.94
45	10 unit AM/g starch	1½ h	37.4	6.15	9.0	82.1	77.6	78.6	2.38
46	" "	3½ h	37.9	4.93	4.6	82.0	77.5	78.1	2.85
47	" "	5½ h	37.7	4.71	3.4	82.0	77.8	78.5	2.67
48	" "	22 h	36.6	3.30	1.3	85.8	80.4	78.8	2.13
49[g)]	AM/BE combined	0 h	37.4	6.87	12.5	82.4	78.9	79.4	2.42
50[g)]	" "	1½ h	34.3	2.61	1.5	101.4	81.1	74.7	2.31
51[g)]	" "	3½ h	29.5	0.37	1.0	285.1	135.7	69.4	4.11
52[g)]	" "	5½ h	27.3	0.02	0.8	392.3	274.9	61.0	1.10
53[g)]	" "	22 h	22.0	0.02	0.6	476.8	405.2	n.d.	n.d.

Wheat									
54	No enzyme	0 h	25.4	2.27	17.1	168.5	164.6	49.0	0.28
55	2 unit AM/g starch	1½ h	28.6	0.05	19.4	260.7	227.1	61.3	0.31
56	" "	3½ h	29.9	0.09	12.1	249.9	193.8	66.3	1.51
57	" "	5½ h	30.7	0.15	10.0	236.8	176.3	68.6	0.91
58	" "	22 h	30.2	0.40	5.3	239.9	155.8	69.4	2.34
Gelatin									
59	-	-	-	4.16	-	481.3	420.4	26.8	19.6
60	-	-	-	6.16	-	400.8	355.8	27.6	23.0

One unit of AM activity is defined as the release of 1 μmol of glucose per minute at pH 6.5, 60°C with maltotriose as substrate, and one unit of BE is defined as the amount of enzyme that can decrease A_{660} of the amylo-iodine complex by 1% per minute at 60°C pH 7.0. AM: amylomaltase; BE: branching enzyme; T_p: peak temperature; ΔH: enthalpy of transition; n.d.: not detected.

a) After autoclavation for 30 min at 140ºC, starches were modified at 85°C.

b) Amylopectin mean chain length (CL).

c) From (Hansen *et al.*, 2008a).

d) Gel texture indicates the *force of deformation*, which is the area of compression during texture analysis of 12% gels (w/w) after storage at 4°C for 24 h.

e) Measured by LF NMR. From (Hansen *et al.*, 2008b).

f) Starches were modified at 70°C.

g) Pea starch was modified with 10 unit AM/g starch for 2 h in order to 'remove' amylose, resulting in sample # 49. Meanwhile maintaining the AM activity, # 49 was further modified with 1000 unit BE/g starch for 1½-22 h at 60°C (resulting in # 50-53).

Figure 1.
Michael Riis Hansen
2008

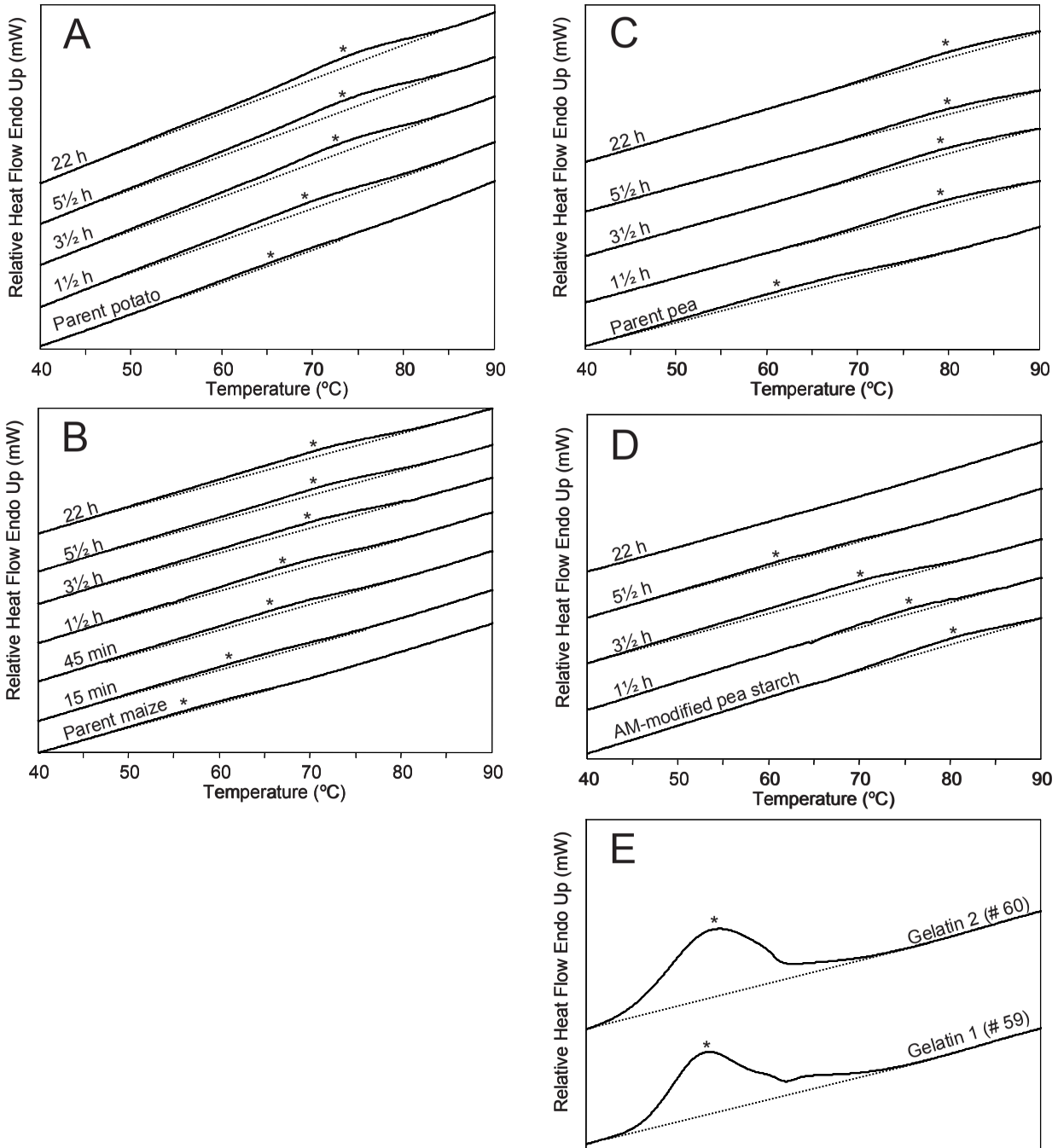

Figure 2.
Michael Riis Hansen
2008

Note:
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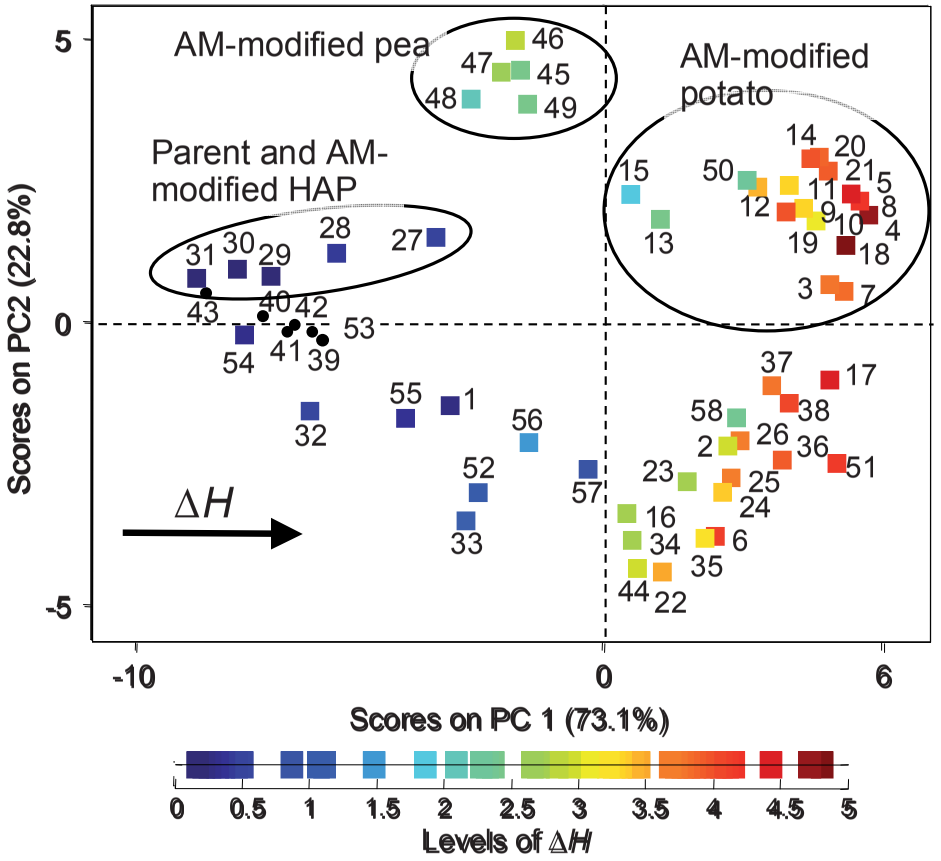

Figure 3.
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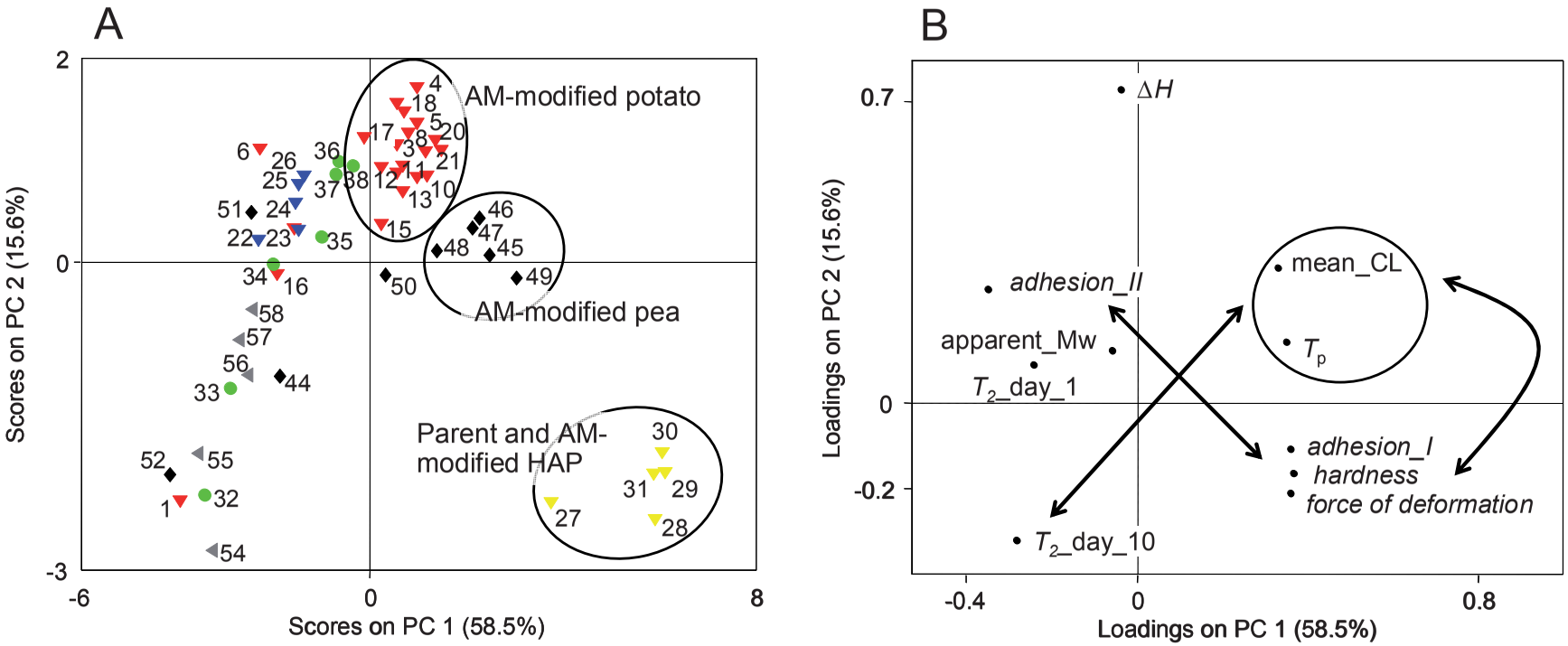

Figure 4.
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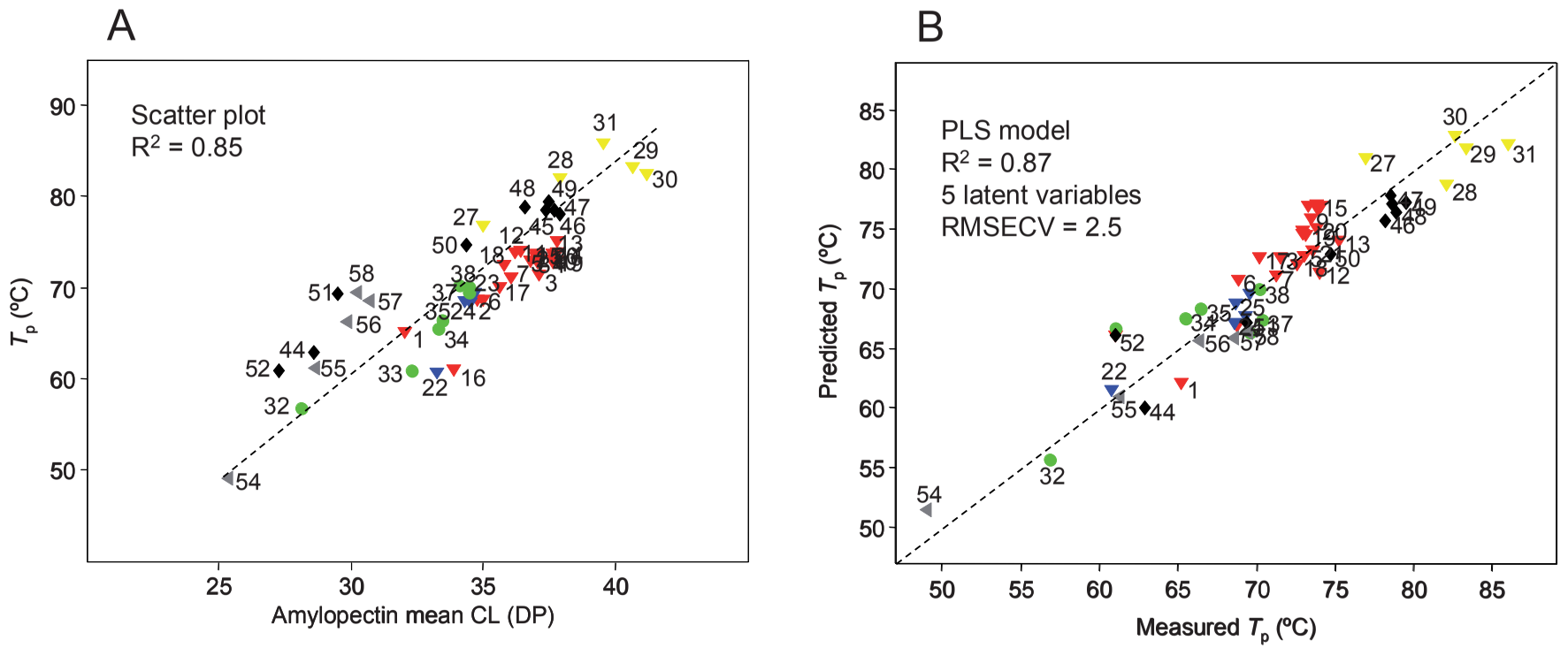

Figure 5.
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