Function-Structure Relationships of Acetylated Pea Starches

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Function-Structure Relationships of Acetylated Pea Starches

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To my family

ABSTRACT

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Starches from cowpea and chickpea seeds were isolated and their properties were compared with those of commercial yellow pea starch. Pullulanase digestion released two distinct populations from their amylopectins, representing short (DP 6-50) and long (DP 50-80) side chains. The higher gelatinization temperature, the greater pasting peak viscosity and a slight difference in crystalline structure found for cowpea starch compared to chickpea and yellow pea starches correlated with the larger proportion of long side chains found for cowpea amylopectin.

Furthermore, yellow pea, cowpea and chickpea starches were modified with acetic anhydride and vinyl acetate followed by sieving into different size fractions to study the effect of granule size and reagent type on the properties of acetylated starches. The degree of substitution (DS) was found to depend on the granule size for modification with rapidly reacting acetic anhydride, whereas the DS was granule size independent for modification with slowly reacting vinyl acetate. Modification with vinyl acetate resulted in only slight different DS values while significant higher peak viscosities were observed compared to modification with acetic anhydride.

Therefore, the effect of reagent type on the distribution pattern of acetyl groups over the starch molecules was studied for cowpea starch by enzymatic degradation combined with chromatographic and mass spectrometric analyses of the fragments obtained. Pronounced differences in the mass spectra of α-amylase hydrolysates revealed that the acetyl groups along the amylose and amylopectin chains were more clustered for modification with vinyl acetate as compared to modification with acetic anhydride.

In addition, yellow pea starch was acetylated by using two procedures: one including starch sieving into different size fractions and then acetylation, the other one including starch acetylation and then sieving into different size fractions. For acetylation with acetic anhydride, these two procedures resulted in different DS values for small size granules. However, their pasting viscosities were quite similar. No clear differences were found for both DS and pasting behaviour when the two procedures were applied for modification with vinyl acetate. The substituent distribution was found to depend on the type of reagent. It is postulated that acetylation occurs more homogeneously throughout the granule when vinyl acetate is used as reagent, while the reaction with acetic anhydride takes place to a large extent in the outer lamellae of the granule.

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Chapter 1

General introduction

Aim of this research

The purpose of the research is to relate the physicochemical properties of three legume starches, i.e. cowpea, chickpea and yellow pea starches to the chain length profile of their amylopectin populations. Information on structure-function relationships of these legume starches is the basis for predicting their functionality and application. So far, the information for cowpea and chickpea starches does not cover the relationship between molecular structure of amylopectin and starch properties. In our study, the characteristics of starches isolated from cowpea and chickpea seeds were compared with those of commercial yellow pea starch.

Acetylation is a common form of starch modification and acetylated starch is widely used as an additive in many food products. Acetic anhydride and vinyl acetate are both used for producing acetylated starch at industrial scale. These two reagents are quite different in reactivity but little attention has been paid to the influence of reagent type on the properties and structure of acetylated starch. Another aim of this study was to obtain understanding of the effect of reagent type (acetic anhydride vs. vinyl acetate) on the properties and chemical fine structure of acetylated legume starches.

General introduction

Starch is deposited as granules in almost all green plants and in various types of plant tissues and organs, e.g., leaves, roots, shoots, fruits, grains, and stems (Wurzburg, 1986; Preiss, 2004). In storage organs like fruit or seed, synthesis of starch occurs during the development and maturation of the tissue. At the time of sprouting or germination of the seed or tuber, or ripening of the fruit, starch degradation in these tissues then occurs and the derived metabolites are used as a source both for carbon and energy (Preiss, 2004). Being one of the most abundant carbohydrates in the biosphere, starch serves as the most important energy source for human consumption. Moreover, it is a “renewable” substance; a new supply of starch is grown annually. Being a biodegradable polymer with well-defined chemical properties, it has a huge potential as a versatile renewable resource for various material applications both in food and non-food areas.

Sources of starch

A vast range of native starches with highly different functionalities are already on the market (Mayes, 1993; Preiss, 2004; Blennow, 2004). Each starch is named according to its plant source, e.g. potato starch, maize starch, cassava starch. The sources of starch can be divided into five groups (Table 1). The first group comprises the tuber (potato) and root (cassava, sweet potato, taro, tannia, yam and arrowroot) starches. The second group comprises the common cereal (maize, wheat, rice and sorghum) starches. These two groups are distinctly different from each other with respect to chemical composition and physical properties (Swinkels, 1985). The third group comprises legume (chickpea, cowpea, smooth pea, mung bean, horse gram, lima bean) starches. The fourth group comprises tree crops (sago, mango, plantain, jackfruit, breadfruit, screw pine) starches (Moorthy, 2004). The fifth group comprises the waxy (waxy maize, waxy sorghum, waxy rice, waxy wheat and waxy potato) starches (Swinkels, 1985) and high amylose mutant (amylomaize) starch.

Table 1 Different sources of starch (from Moorthy (2004) and Swinkels (1985))

Tube/root crops	Cereals	Legumes	Tree crops	Mutants
Potato	Maize	Smooth pea	Sago palm	Waxy maize
Cassava	Wheat	Chickpea	Mango	Amylomaize
Sweet potato	Rice	Cowpea	Plantain	Waxy sorghum
Taro	Sorghum	Mung bean	Jackfruit	Waxy rice
Tannia		Horse gram	Breadfruit	Waxy wheat
Yam		Lima bean	Screw pine	Waxy potato
Arrowroot				

Although starch is distributed widely in green plants, there are only a limited number of plants utilized extensively for the production of commercial starches. Maize and potato are the major sources of most of the starches produced. Over 95% of the starch produced in the U.S. is from maize or a special variety of maize starch (i.e. waxy maize or amylomaize) (Wurzburg, 1986). Compared to the situation in the U.S., the role of potatoes in starch production is much greater in Europe, where potatoes are grown specifically for starch production in France, Holland, Germany, Poland, and Sweden (Wurzburg, 1986). Wheat starch is also produced in many countries because of the abundance of the wheat crop and the importance of industrial wheat gluten-starch separation process. Cassava starch and sago starch are produced in the more tropical areas such as South China, East Indies and Brazil. Small amounts of starches from other sources are produced in certain

regions, like sweet potato and mung bean starches in East Asia (Chen, Schols, & Voragen, 2003a); smooth pea starch in Europe and Canada (Barron, Bouchet, Della Valle, Gallant, & Planchot, 2001; Li & Vasanthan, 2003), arrowroot starch in Africa and in the Caribbean (Wurzburg, 1986).

Starch structure

Amylose and amylopectin

Starch, a homopolymer yielding only glucose on hydrolysis, is called a glucosan or glucan. While the detailed chemical fine structure has not been fully elucidated, it has been established that starch is a heterogeneous material consisting of two major types of polymers—essentially linear amylose and highly branched amylopectin (Wurzburg, 1986; Seib, 1997; Bertoft, 2004).

Amylose

The amylose content of most starches is 20-30%. However, certain mutant plants, commonly called waxy because of the waxy appearance of the seed endosperm, have a much lower amylose content, or even lack the amylose component completely. Other types of mutant plants possess an increased amylose content (Seib, 1997; Bertoft, 2004). Amylose is mainly found as linear chains of about 2000 to 12,000 units of α-glucopyranosyl residues linked by α-D-(1,4) glucosidic linkages (molecular weight around 0.3 to 2.0 $\times 10^6$ Da). Its molecular size varies with plant species, stage of development and processing conditions employed in extracting the starch. Some of the amylose molecules are branched to a small extent at O-6 (α-1,6-D glucopyranose; one per 170 to 500 glucosyl units). About 0.3-0.5% of the total linkages are branch points (α-1,6) (Wurzburg, 1986; Seib, 1997; Blennow, 2004; Preiss, 2004; Vermeylen, Goderis, Reynaers, & Delcour, 2004).

At one end of the polymeric glucan chain, the anhydroglucose unit contains one primary and two secondary hydroxyls as well as an aldehydic reducing group in the form of an inner hemiacetal. This is called the reducing end of the molecule. The opposite end is called the nonreducing end (Fig. 1). The abundance of hydroxyl groups imparts hydrophilic properties to the polymer, giving it an affinity for moisture and dispersibility in water. However, because of their linearity, mobility, and the presence of many hydroxyl groups, amylose polymers have a tendency to orient themselves in a parallel fashion and approach each other closely enough to permit hydrogen bonding between hydroxyl groups of adjacent polymers. As a result, the affinity of the polymer for water is reduced

and the sol becomes opaque. In dilute solutions, the aggregate size of the associated polymers may increase to a point where precipitation may occur. At higher concentrations, steric hindrance may interfere, so only partial orientation between segments of the polymers may occur, producing a gel consisting of a three-dimensional network held together by hydrogen bonding at those sections where close alignment has occurred (Wurzburg, 1986).

Amylopectin

The detailed chemical fine structure of amylopectin is still subject to debate. However, it is sufficient to look upon amylopectin as a large branched molecule (Wurzburg, 1986). Amylopectin molecules are highly branched with about 4-5% of the glucosidic linkages being α-1,6, and are constructed of thousands of short α-1,4 linked D-glucose chains linked together by α-1,6 bonds. The average molecular weight of amylopectin (0.4-35 $\times 10^6$ glucose units) is 0.6-56 $\times 10^8$ Da, about 100 to 1000 times that of amylose. The short chains in amylopectin are arranged in clusters attached to the long chains. Most of the chains in amylopectin contain 6-75 glucose units (Seib, 1997; Blennow, 2004; Vermeylen et al., 2004)

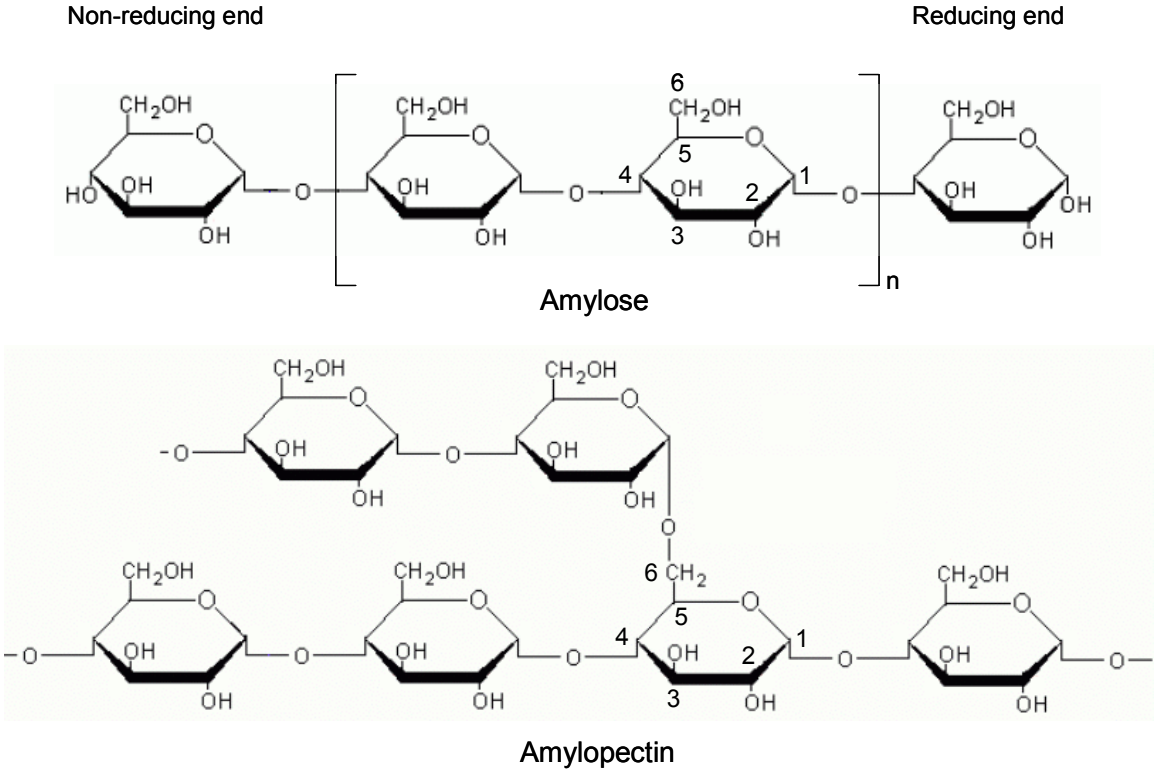

Fig. 1. Amylose and amylopectin chains.

In the cluster model, 80-90% of the chains are short and are connected to one cluster. Another 10% are twice as long as the shortest chains, and 1-3% are 3-4 times as long (Seib, 1997). A-chains are defined as unsubstituted, whereas B-chains are substituted by other chains. The macromolecule also contains a single C-chain that carries the sole reducing end group (Bertoft, 2004). However this chain is not distinguished from the B-chains in most experiments. The B-chains are further subdivided according to their positions in the cluster structure model proposed by Hizukuri (1996). Thus, B1-chains are short chains, being components of a cluster, whereas B2 are long chains that span over two clusters, thereby interconnecting them (Fig. 2). The chains are also simply classified into short and long chains, but there is no exact definition of their lengths. Note also that the definition can be very different for amylose compared to amylopectin (Bertoft, 2004). The chains are further divided into characteristic segments. An external chain is the part of a chain that extends from the outmost branch point to the non-reducing end (Fig. 2). Thus, all A-chains are external, whereas a part of the B-chains is external. The rest of a B-chains are called the total internal chain and include all the glucosyl residues involved in branch points (Seib, 1997; Bertoft, 2004).

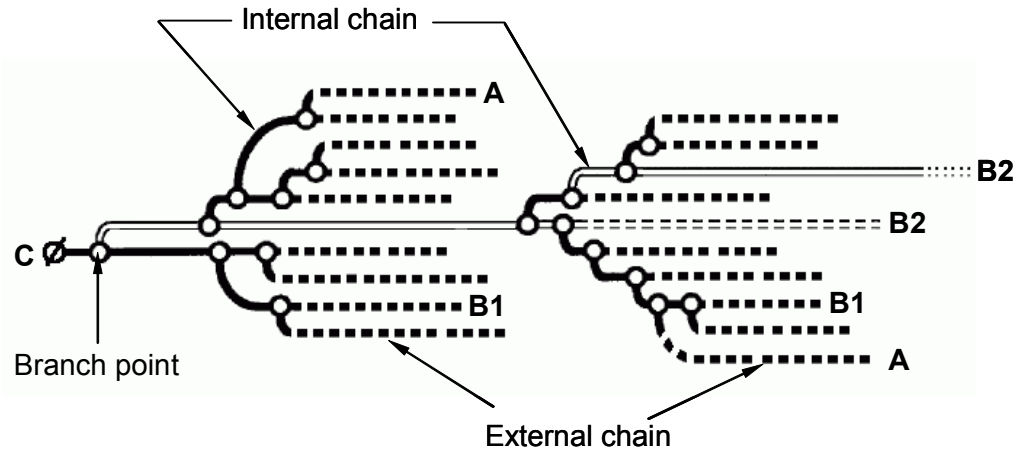

Fig. 2. The cluster model of amylopectin schematically redrawn on the basis of Thompson (2000), Hizukuri (1996) and Bertoft (2004).

The large size and branched nature of amylopectin reduce the mobility of the polymers and interfere with any tendency for them to become oriented closely enough to permit significant levels of hydrogen bonding. As a result, aqueous sols of amylopectin are characterized by clarity and

stability as measured by resistance to gelling on aging. Amylopectin sols do not form as strong and flexible films as linear amylose (Wurzburg, 1986).

Starch Granules

Granule structure

Starch is unique among carbohydrates since it naturally occurs as tiny granules. The amylose and amylopectin fractions are considered to be oriented in the starch granule in radical fashion (Wurzburg, 1986). Hydrogen bonds between adjacent linear segments of the amylopectin or amylose molecules segments may form micellar crystallites which are responsible for the granule integrity and for imparting birefringent properties which are manifested by polarization crosses. These polarization crosses are characteristic for intact starch granules (Wurzburg, 1986). In a cluster of amylopectin, two neighbouring chains intertwine into a double helix. The linear arrays of the double helices then form crystallites. The alternating zones of different densities of amylopectin account for the crystalline and amorphous phases of starch. Amylose occurs predominantly in the amorphous phase (Seib, 1997). It is now widely accepted that the amylopectin is predominantly responsible for granule crystallinity. Three different types of crystal structures have been identified and classified based on X-ray diffraction patterns (type A, B or C). The differences between A- and B-types relate to the packing of double helices in the crystal unit cell and the quantity of water molecules stabilizing these double helices (Tang, Mitsunaga, & Kawamura, 2006). C-type starches actually consist of a mixture of A-type and B-type (Donald, 2004).

Research on the architecture of starch granules has made substantial progress during the last decades. This is due to the result of new microscopic techniques and the progress in solid state NMR and crystallographic techniques, which made it possible to analyse the conformation of amylopectin inside the granules (Bertoft, 2004).

Crystalline (hard) and semi-crystalline (soft) shells are alternatively arranged in starch granule. Although the thickness and hardness of the shells differ with the plant starch origin, the thickness tends to decrease toward the edges of the granules, while the hardness tends to increase (Gallant, Bouchet, & Baldwin, 1997; Tang et al., 2006). The architecture of starch granules and the structure of blocklet are proposed by Tang et al. (2006). The blocklet is a semi-crystalline ultrastructure. It is hypothesized that there are two types of blocklet, ‘normal’ and ‘defect’ in the same starch granule. A normal blocket is mainly constructed by the crystalline and amorphous lamellae that are formed with the clusters of amylopectin molecule(s). The participation of lower branching molecules such

as amylose may result in a defective blocklet. The normal blocklets construct the hard shells, while the defective blocklets construct the soft shells. It is believed that the surface layer of starch granules consists of the hard shell (Fig. 3). The brittle gathering of defective blocklets forms the pore in the surface of starch granules. A normal blocklet generally consists of several amylopectin molecules. It is thought that the reducing terminal of amylopectin molecules in the blocklets is toward the hilum of the granules. Amylose may be localized among the blocklets, and contribute to the strength and flexibility of the starch granule. The phosphates are believed to exist on the surface of the blocklet to increase the hydrophilicity of the blocklets. Lipids are believed to exist either in an amylose complex or in free form in the granule. The lipids may contribute to the stability of the helical structure and blocklet structure (Tang et al., 2006)

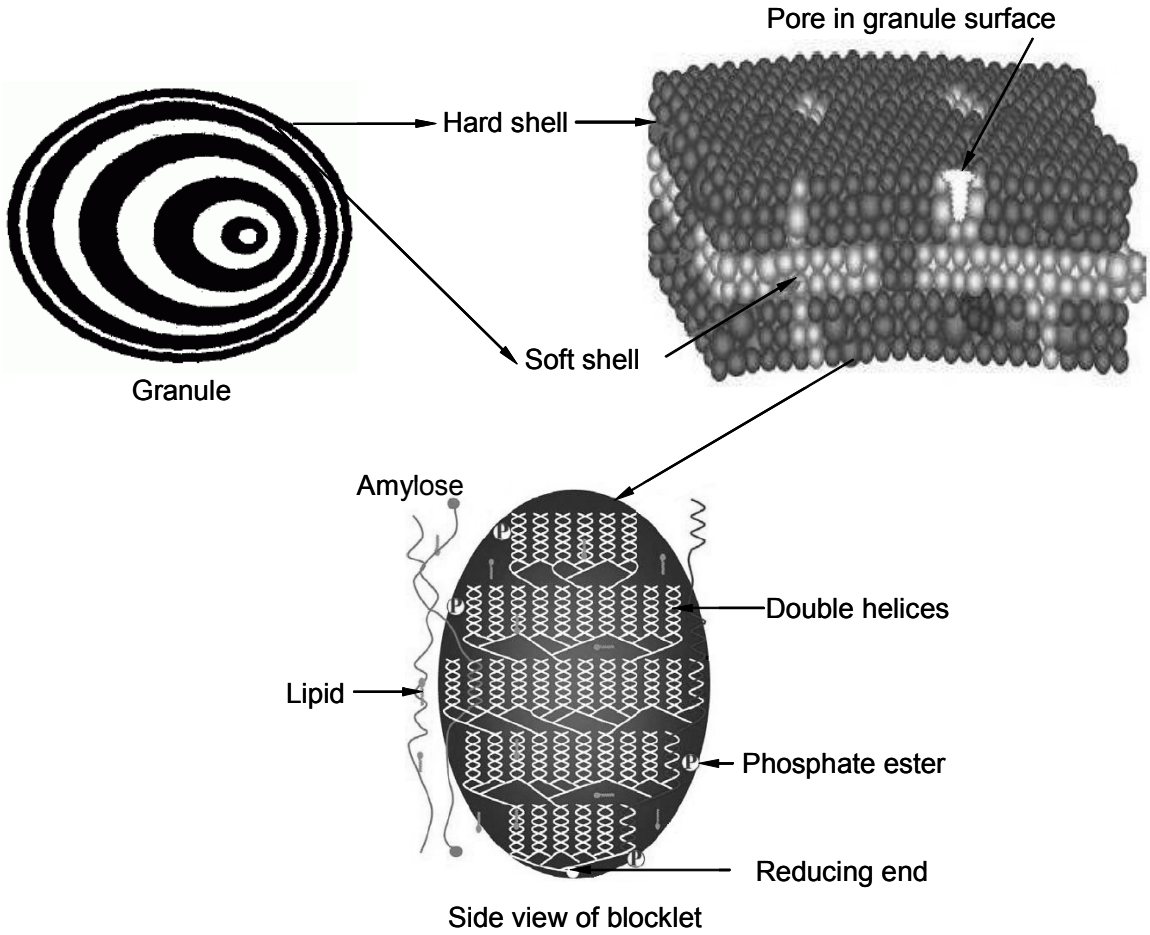

Fig. 3. Schematic view of starch granule (based on Tang et al., 2006).

The organization of amylose and amylopectin within the starch granule has also been characterized by using a chemical gelatinization method. Potato and normal maize starches were treated with aqueous $CaCl_2$ (4 M) and LiCl (13 M) solutions, respectively, and were chemically gelatinized at the periphery of the granules. The analyses showed that amylose was more concentrated at the periphery than at the core of the granule, and amylopectin had longer branch chains at the core than at the periphery of the granule (Jane & Shen, 1993; Pan & Jane, 2000).

Although the knowledge that starch is composed of two major macromolecules is more than 60 years old, knowledge concerning the details of the starch components is still limited. These details have to be resolved in order to understand the structure and functionality of starch (Bertoft, 2004). Starch structure-function models are not yet developed to the level where functionality can be predicted from structure only. Clearly, multidisciplinary efforts at the genetic, biosynthetic, chemical and physical levels are required to complete the starch system (Blennow, 2004).

Granule size

Starch granules in storage tissues can vary in shape, size and composition. The shape and size of the granules depends on the source, which allows one to identify the botanical source of the starch by microscopic examination (Swinkels, 1985; Mayes, 1993; Preiss, 2004). Table 2 shows the size and

Table 2 Size and shape of starch granules (from Swinkels (1985)).

Starch	Type	Size (diameter) range (μm)	Size (diameter) number average (μm)	Shape
Potato	Tuber	5-100	33	Oval, spherical
Cassava	Root	4-35	20	Oval, truncated
Arrowroot	Root	5-70	30	Oval, truncated
Sweet potato	Root	5-25	15	Polygonal
Maize	Cereal	3-26	15	Round, polygonal
Wheat	Cereal	2-35	15	Round, lenticular
Waxy maize	Cereal	3-26	15	Round, polygonal
Sorghum	Cereal	3-26	15	Round, polygonal
Rice	Cereal	3-8	5	Polygonal, angular
Amylomaize	Cereal	3-24	12	Round, deformed
Sago	Pith	5-65	30	Oval, truncated

shape of starch granules from different sources. The size of granule varies from the tiny granules in rice to the large granules in potato starch. In general, starches from cereals contain smaller granules than starches from tuber and root (Swinkels, 1985).

Because of the broad range of particle size distribution in starch, the effect of granule size on the composition, properties, structure and applications of starch have received quite some attention. Small size granule fractions of potato, sweet potato and normal maize starches showed lower amylose contents compared to their large size granule fractions (Jane & Shen, 1993; Chen, Schols, & Voragen, 2003b; Pan & Jane, 2000). The phosphorus content in potato starch decreases with an increase in the granule size (Jane & Shen, 1993; Chen et al., 2003b).

Investigation of large, medium and small size granule fractions from normal and waxy barley starches revealed that small granules and large granules exhibited differences in enthalpy, swelling power, relative crystallinity, susceptibility to enzymes, retrogradation, the number-average degree of polymerisation (DP_n) and chain-length distribution of amylopectin (Tang, Ando, Watanabe, Takeda, & Mitsunaga, 2000, 2001a; Tang, Ando, Watanabe, Takeda, & Mitsunaga, 2001b; Tang, Watanabe, & Mitsunaga, 2002; Tang, Mitsunaga, & Kawamura, 2004). Waxy maize starch granules with a smaller diameter showed greater enzymatic hydrolysis than larger ones, but there was no difference in the chemical fine structure of starch residues obtained from enzymatic hydrolysis between the two size fractions (Franco, Rreto, Ciacco, & Tavares, 1998).

The gelatinization temperatures of different size fraction of potato starch were quite similar. The swelling power of small size granule fraction was higher than that of large size fraction. (Chen et al., 2003b). The amylopectin of small size granule fraction separated from normal maize starch was found to consist of longer branch chains, whereas amylopectin in the large size fractions had shorter branch chains (Pan & Jane, 2000). The application of differently sized potato and sweet potato starches in starch noodles was investigated by Chen et al. (2003b). The small size granule fractions had better processibility in starch noodle making and produced better quality product compared to large size granule fractions.

The reactivity of different size granule fraction with chemical reagent has also been investigated. Maize, wheat, and potato starches were hydroxypropylated with a slowly reacting reagent, propylene oxide, then fractionated into large and small granule fractions. The molar substitution values of the different size fractions within each starch were similar (Stapley & BeMiller, 2003). In contrast, granule size has been reported to affect the degree of molar substitution values for acetylated starches using acetic anhydride as reagent for potato and sweet potato starches: small size granule fractions showed higher DS values than the large size granule fractions (Chen, Schols, & Voragen, 2004).

Starch-degrading enzymes

Starch-degrading enzymes, such as amylases, debranching enzymes, and phosphorylase, are widely used for the production of glucose, maltose, various oligosaccharides, and modified starches, in addition they are very useful for the analysis of the chemical fine structures of starch molecules (Bertoft, 2004). In this section four enzymes, commonly used for structural analysis, are briefly described with respect to their specificities.

Alpha-amylases (EC 3.2.1.1) hydrolyze starch endowise at inner α-(1,4)-linkages and rapidly reduce viscosity and iodine coloration (blue value) with gradual increase in reducing value. They are unable to split α-(1,6)-linkages and some neighbouring α-(1,4)-linkages. All the branch linkages remain in the branched oligosaccharides (dextrins) after α-amylase hydrolysis (Fig. 4) (Butler, van der Maarel, & Steeneken, 2004; Bertoft, 2004).

Amylose and amylopectin are hydrolyzed exowise by ß-amylase (EC 3.2.1.2) at the second α-(1,4)-linkages from the nonreducing terminal residues until near the branch linkages, and maltose and ß-limit dextrin (ß-LD) are produced. It has been suggested that the ß-LD may consist of two or three glucosyl residues on A chains and two or one glucosyl residues on B chains, depending on the even or odd number of glucosyl residues on the external chains. ß-Amylase is a useful tool for analyzing the branched nature of amylopectin and amylose because all the branch linkages remain intact in the ß-LD after degradation (Bertoft, 2004).

Amyloglucosidase (EC 3.2.1.3) is capable to hydrolyze completely both α-1,4- and α-(1,6)-linkages in starch and glycogen exowise from the nonreducing terminal residues producing ß-D-glucose. The α-(1,6)-linkage is hydrolyzed only after the main outer chain is completely removed (Hizukuri, 1996). The branched glucosyl residues are not hydrolyzed easily as in α-(1,4)-linked residues; therefore, hydrolysis of α-(1,6)-linkage is the rate-limiting step. Since amylopectin molecules contain large amounts of α-(1,6)-linkages, it is difficult to hydrolyze starch completely with sole amyloglucosidase in practice. The combination of α-amylase with amyloglucosidase were reported to hydrolyze the starch completely, due to more nonreducing terminal residues released by α-amylase (Hizukuri, 1996).

Pullulanase (EC 3.2.1.41) hydrolyzes the α-(1,6)-glucosidic linkages of amylopectin in an exo-wise action and appears to produce A chains at the initial stage. The unit chain profile of amylopectin can be analyzed with pullulanase digestion. Amylopectin and amylose with branch linkages were

able to be hydrolyzed completely into maltose (and some glucose and maltotriose) with the cooperative action of pullulanase and ß-amylase (Hizukuri, 1996; Butler et al., 2004; Bertoft, 2004).

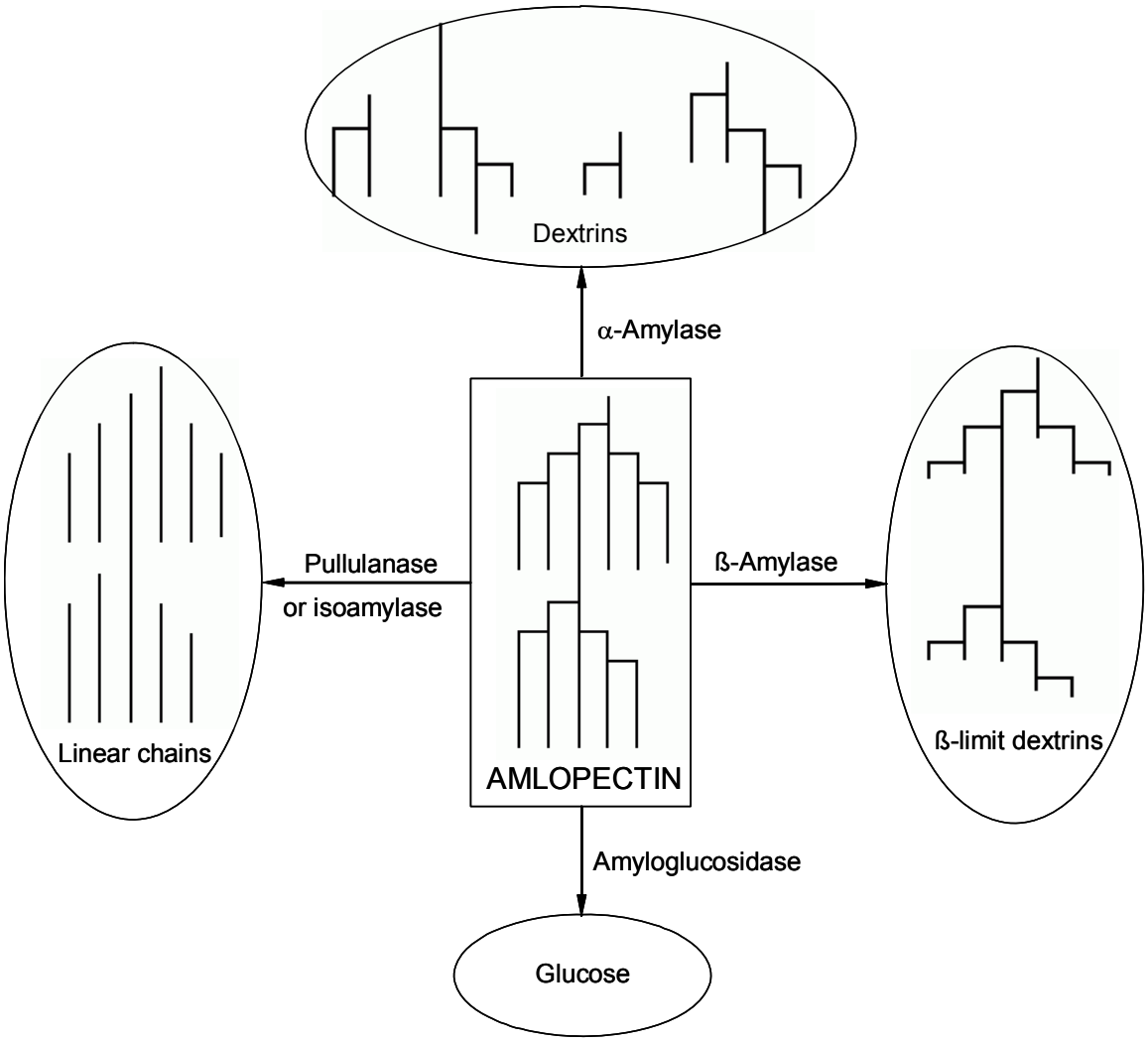

Fig. 4. Schematic view of the degradation of native amylopectin by starch-degrading enzymes (based on Bertoft, 2004).

Starch modification

Starch modification is a way to alter the structure and affect the hydrogen bonding in a controllable manner to enhance and extend their applications (Fleche, 1985; Taggart, 2004). Many reactions have been described since the first starch modification was discovered in 1821. Figure 5 presents

some chemical and biochemical modifications. The reaction(s) chosen for starch modification usually exert a major change on a desirable physical property. Nevertheless, low levels of modification already dramatically alter the physical properties of starch, such as paste viscosity, gelling, syneresis, clarity, adhension, and emulsifying properties (Fleche, 1985). Cross-linked starches offer acid, heat and shear stability over their parent native starches. The lipophilic substitution is particularly useful for stabilizing interactions between materials such as oil and water. For example, starch octenylsuccinates can stabilize the oil-water interface of an emulsion (Taggart, 2004).

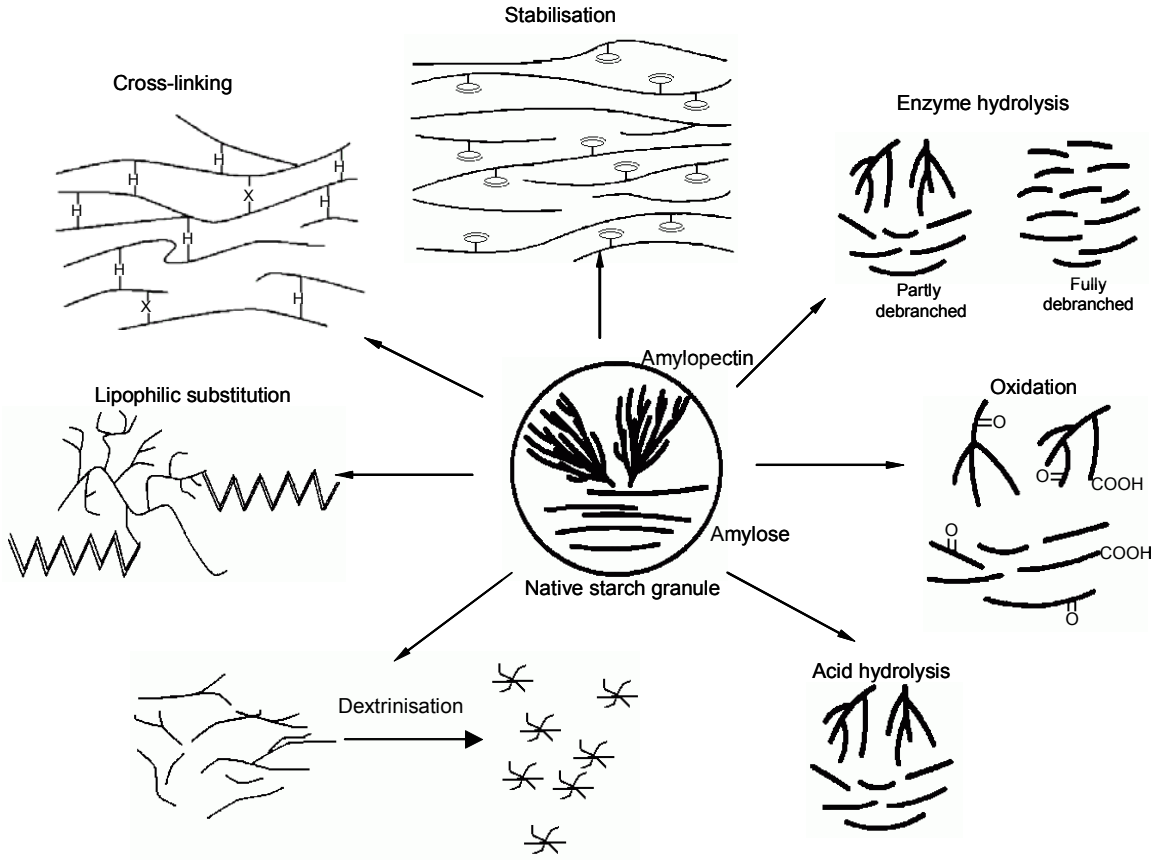

Fig. 5. Chemical and biochemical modifications of starch (from Taggart, 2004)

Dextrinisation is a partial depolymerisation (depolymerisation and transglycosylation) achieved through hydrolysis. Depending on the range of viscosity, cold-water solubility, color, reducing sugar and stability, the dextrins are typically classified as white dextrins, yellow dextrins or British

gums. Acid hydrolysis is conducted in aqueous conditions, which is different from dextrinisation. Acid-hydrolyzed starch offers a lower hot viscosity and a stronger gel develops on cooling compared to the native parent starch. Carboxyl (COOH) and carbonyl (C=O) groups are introduced by oxidation. Oxidised starches exhibit a significantly reduced hot viscosity and gel strength (Taggart, 2004). Stabilisation of starch is aimed at preventing retrogradation by introducing substituent groups. The interactions of glucan chains in the granule are weakened by the introduction of the substituents and, consequently, hydration and gelatinisation of starch by cooking is achievable at lower temperatures. Esterificaiton or etherification provides stabilization.

The effectiveness of stabilisation depends upon the number and nature of the substituted group. Acetylation and hydroxylpropylation are the primary food-approved types of stabilization, and those with a degree of substitution (DS) below 0.2 are of commercial importance (Fleche, 1985; Taggart, 2004). The amount of substituents introduced is the basic parameter for chemically substituted starch. DS is defined as the average number of hydroxyl groups on the D-glucosyl units that have been substituted. The maximum value of DS is three, that is when all possible (three) hydroxyl groups are substituted on each glucose residue. Another term used is the molar substitution (MS), which is the average number of moles of substituent per D-glucosyl residue. Commonly, MS equals DS, like in acetylated starch, but if more than one substituent can be attached at the same position on the residues, as in hydroxypropylated starch, MS can exceed three (Seib, 1997; Bertoft, 2004).

In addition to DS, the positions of the substituted residues within the polymers, and their distribution between the starch components, are of interest. To establish these characteristics, enzymatic methods have been used. The introduced substituents act as barriers to hydrolytic attack in a similar way as the branches stop the exo-acting enzymes and restrict the action of endo-amylase (Bertoft, 2004).

Analysis of chemically modified starches with enzymatic method

Pure and well characterized starch-degrading enzymes have been used to analyze the chemical fine structure of modified starches for many years. Steeneken and Woortman (1994) hydrolyzed methylated potato starch consecutively with α-amylase and glucoamylase. Larger amounts of glucose and highly substituted high molecular weight dextrins were obtained from the granular form than from the non-granular counterpart with similar DS. It is concluded that the granular form resulted in a heterogeneous distribution of the methyl groups within the starch polymers, whereas

non-granular starch was homogeneously substituted. Kavitha and BeMiller (1998) investigated the isolated amylose fraction from hydroxypropylated potato starch with the combination of α-amylase and amyloglucosidase and found heterogeneous substitution within this component. Van der Burgt et al. (2000) used a combination of α-amylase and ß-amylase to investigate the distribution pattern in methylated potato and amylopectin potato starches. The methyl groups were found to be located close to the branch points. Zhu and Bertoft (1997) analysed oxidised potato starches (MS 0.04-0.05) by size-exclusion chromatography before and after ß-amylase hydrolysis. The ß-limit value of sodium hypochlorite oxidised starch was lower (44%) than that of native starch (66%). However, hydrogen peroxide oxidised starch had only slightly decreased ß-limit value (60%). Thus, the method used for the oxidation influenced the distribution pattern of oxidised carbons. Kavitha and BeMiller (1998) also reported that the ß-limit value of isolated amylopectin fraction from hydroxypropylated potato starch was lower than the value of amylopectin from native starch and concluded that some substituents were present near the non-reducing ends of amylopectin branch chains.

Methyl amyloses prepared under various conditions were exhaustively digested by means of α-amylase and amyloglucosidase (Mischnick, 2001). The portion of glucose that could be liberated by the enzymes decreased with increasing DS. But at the same DS, the amount of released glucose was considerably higher for heterogeneously prepared amylose ethers than for those prepared under homogeneous conditions. Further investigation of the degradation products by mass spectrometry gave evidence for different oligomer patterns and average values of degree of substituent/degree of polymerisation (DS/DP) in dependence on the methylation conditions applied (Mischnick, 2001). More detailed analysis of the O-methylated positions of the oligosaccharides showed that the reducing glucose end group was usually unsubstituted, while the non-reducing glucosyl residues could be 2-, 6- or even 2,6-di-O-substituted. In contrast, all 3-O-methyl groups were located in the inner 1,4-linked glucosyl units, indicating inhibition of both enzymes by the substituents (Mischnick, 2001).

Acetylated starches

Among a great variety of starch esters, starch acetate is the one that is actively marketed (Fleche, 1985). The maximum level of 2.5% acetyl for food use corresponds to a DS value of 0.1 (Seib, 1997). The steric disturbance of the acetyl group will make starch solubilization easier by lowering the energy level of the network created by the hydrogen bonds and will slow down the

retrogradation (Bergthaller, 2004; Taggart, 2004). The starch-starch interactions in the granule are weakened by the introduction of acetyl groups and, consequently, hydration and gelatinization by cooking is achievable at lower temperatures. Such starches benefit from easy cooking and are particularly useful in low-moisture environments and in applications where the moisture level is restricted by competition from co-ingredients, e.g. in extruded and coated snacks, frozen fish and cooked meat products, flour based noodles, bakery products and in various frozen or cold stored ready-to-eat menus (Bergthaller, 2004). The acetyl group also generates a hydrophobic type of structure suitable for certain applications, including acid pH-resistant binders in the food industry, and adhesive and grease resistance in paper sizing (Fleche, 1985). Starch acetates are readily prepared by reacting the starch with acetic anhydride in the presence of diluted sodium hydroxide (Fig. 6). Alternatively vinyl acetate can be used for acetylation in aqueous suspension in the presence of sodium carbonate as catalyst (Bergthaller, 2004; Cornell, 2004).

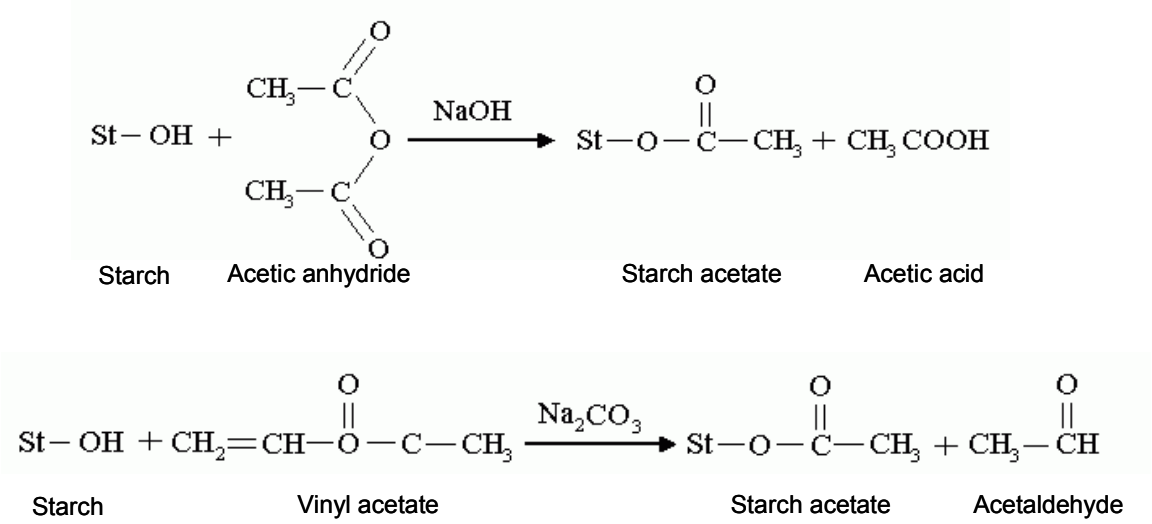

Fig. 6. Reaction of starch with acetic anhydride and vinyl acetate to produce acetylated starch.

Analysis of acetylated starches

Acetylation has been investigated from several aspects, i.e. the effects of catalyst, amylose content and granule size on the properties of acetylated starch. Liu, Ramsden, and Corke (1999) reported that rice starches with different amylose content differed in their response to acetylation with vinyl acetate. For example, acetylation increased the swelling power and freeze-thaw stability of non-

waxy starch, but decreased these properties in waxy rice. The influence of starch hybrids on the properties of acetylated starch was evaluated. Starches were isolated from eleven waxy maize and nineteen dent maize hybrids and modified with acetic anhydride. Variability was found in reaction efficiency and pasting properties among hybrids (Wilkins, Wang, Xu, Niu, Tumbleson, & Rausch, 2003a, 2003b).

Wang and Wang (2002) studied the effects of the catalyst on the chemical and physicochemical properties of acetylated starch. Waxy maize starch was acetylated with acetic anhydride by using sodium hydroxide (NaOH), potassium hydroxide (KOH), and calcium hydroxide ($Ca(OH)_2$) as catalyst. As compared to the acetylated starch prepared under NaOH or KOH catalysis, the $Ca(OH)_2$-catalyzed acetylated starch showed different pasting temperature, ß-amylolysis limit, and carbohydrate profile. This phenomenon was explained by intermolecular cross-linking which might be induced by calcium. The effect of granule size toward derivatisation reactions was reported for acetylated potato and sweet potato starches by Chen et al. (2004). Potato and sweet potato starches were fractionated by sieving into different size granule fractions after modification with acetic anhydride. Small size granule fractions showed higher DS values than the large size granule fractions.

The chemical fine structure of acetylated starch has also been studied. An acetylated distarch phosphate derivative of smooth pea starch (DS 0.06) was debranched with pullulanase before and after ß-amylase hydrolysis, and compared with a commercial hydroxypropyl distarch phosphate derivative of waxy maize starch (Biliaderis, 1982). The size exclusion chromatograms showed that the chain length profile of the acetylated derivative nearly matched that of the unmodified pea starch, whereas the profile of the hydroxypropyl waxy maize starch did not match that of its blank. Those results led Biliaderis (1982) to conclude that the acetylation occurred exclusively in certain part of the granule, whereas hydroxypropylation was more uniform throughout the starch granule.

The chemical fine structure of the isolated acetylated amylose fraction was analyzed by Chen et al. (2004) with respect to the degradability by α-amylase, ß-amylase and amyloglucosidase. The amylose populations isolated from small size granule fractions were less susceptible to all the enzyme digestions than the amylose originating from the large granule fractions, even though the DS was similar. The acetyl groups were found to be more heterogeneously distributed over the amylose molecules and more closely located to the non-reducing ends for amylose originating from small size granule fractions when compared to amylose from large sized granules.

Legume starches

Legumes are important ingredients of a balanced human diet in many parts of the world due to their high protein (15–40%) and starch (35–60%) content. They have been consumed traditionally as whole seeds or as ground flour after dehulling. The rapidly growing food industry constantly demands new ingredients, which has drawn the attention of researchers to legume components i.e. starch and protein obtained by the wet-fractionation process (Schoch & Maywald, 1968; Czuchajowska, Otto, Paszczynska, & Baik, 1998). The legumes have been subjected to protein extraction studies (Arora & Das, 1976; Longe, 1980; Soetrisnot & Holmes, 1992; Clemente, Sanchez-Vioque, Vioque, Bautistab, & Millan, 1998; Jirapa, Normah, Zamaliah, Asmah, & Mohamad, 2001; Rangel, Domont, Pedrosa, & Ferreira, 2003). The production of legume protein can be of economic value only if their starch component is made profitable simultaneously. Legume starches have been recognized as a potential food ingredient (Czuchajowska et al., 1998). Containing a relatively high proportion of amylose when compared to cereal starches, legume starches are characterized by a high gelatinization temperature, a high resistance to shear thinning, fast retrogradation, and a high elasticity of the gel (Schoch & Maywald, 1968; Gujska, Reinhard, & Khan, 1994). A fast retrogradation and high elasticity of a starch gel is necessary for food products like sausages, paté-type meat products, and gluten-free oriental noodles. Resistance of starch paste to shear thinning at high temperature is important for canned foods and extruded snacks (Czuchajowska et al., 1998).

Yellow pea, cowpea and chickpea starches

Starches from three legumes are the major materials used in this research. Yellow pea (*pisum sativum*), which is also known as field pea, garden pea and smooth pea (Li & Vasanthan, 2003; Boyer, 1981; Gernat, Radosta, Damaschun, & Schierbaum, 1990), is a yellow seeded cultivar of *pisum sativum.* It has been consumed as a healthy vegetable food since centuries. Yellow pea is an annual legume with rapid growth, very common in Northern Europe. Cowpea (*Vigna unguiculata*) has a number of commonly used names, including southern pea, black-eyed pea and crowder pea. Native to Asia and Africa, it is one of the most ancient crops known to man and the most widely cultivated among these three legumes (Prinyawiwatkul, McWatters, Beuchat, & Phillips, 1997). Chickpea (*Cicer arietinum*), also known as garbanzo (Schoch & Maywald, 1968) and Bengal gram,

is cultivated in the Mediterranean Basin, North America and Asia. It is the third most commonly consumed legume in the world.

Yellow pea starch

The physicochemical properties and chemical fine structure of field pea starch obtained from four cultivars have been investigated (Ratnayake, Hoover, Shahidi, Perera, & Jane, 2001). The total amylose content was about 49%, of which 11–12% was complexed by lipid. The degree of polymerization (DP) of amyloses ranged from 1300 to 1350. The chain length distributions of debranched amylopectins of the starches were analyzed by using HPAEC (high performance anion-exchange chromatography). The proportion of short branch chains (DP 6-12) ranged from 16% to 19%. The average amylopectin branch chain length was around 23 (Ratnayake et al., 2001).

Vasanthan and Bhatty (1998) improved the resistant starch content in native field pea starch by retrogradation. The effect of hypochlorite oxidation on the pasting properties of field pea starch and the suitability of native and oxidized starch for noodle making by extrusion cooking were investigated by Li and Vasanthan (2003). The peak viscosity of oxidized starch was found to decrease with increasing degree of oxidation. The cooking quality attributes of noodles prepared from native field pea starches were acceptable but were negatively influenced by hypochlorite oxidation (Li & Vasanthan, 2003).

Biliaderis (1982) investigated the pasting properties, enzymatic digestibility, and chemical fine structure of native and acetylated smooth pea starch. Acetylation of the starch with acetic anhydride decreased the pasting temperature and decreased the susceptibility to α-amylase, ß-amylase and pullulanase actions. Structural characterization of acetylated smooth pea starch was carried out by combining enzymatic degradation with chromatographic analysis. The results suggested that an assessment of the substituent distribution is more meaningful than a DS value in characterizing chemically modified starches (Biliaderis, 1982).

Cowpea starch

The functional properties of cowpea starch were investigated by Chung, Cho, Chung, Shin, Son, and Lim (1998). The cowpea starch gel displayed exceptionally high values for hardness, chewiness and gumminess. The chemical fine structure of the starch was studied by debranching the whole starch followed by size exclusion chromatography. Cowpea amylose showed an average DP value of 1611. Amylopectin chains were divided into 2 fractions, short and long chains (Chung et al., 1998).

The influence of granule size and size distribution on the flow behaviour was measured by heating cowpea starch dispersion for various time intervals above its gelatinization temperature (Okechukwu & Rao, 1996; Rao, Okechukwu, Da Silva, & Oliveira, 1997; Rao, & Tattiyakul, 1999). The rheological behaviour of the starch dispersions was strongly influenced by the granules. The standard deviation of the granules size described the transition of flow behaviour from shear thickening in the early stages of gelatinization to shear thinning in the latter stages and influenced the critical shear rate for the onset of shear thickening in starch dispersions. The granules swelled to a maximum of about 3.5 times the raw starch granule mean diameter (Rao et al., 1997).

Won, Choi, Lim, Cho, and Lim (2000) reported that cowpea starch was capable of forming exceptionally strong and elastic gels with good storage stability at 4°C in comparison with acorn, maize, and potato starches. The freeze-thaw stability of cowpea starch gel was significantly improved by incorporating surfactants with hydrophilic-lipophilic balance 1.8–11.0 (Mwasaru & Muhammad, 2001).

Chickpea starch

The physicochemical properties of chick pea starch from different cultivars were examined by Hoover and Ratnayake (2002) and Singh, Sandhu and Kaur (2004). Variability was found in physicochemical, thermal, morphological and rheological properties of starches among chickpea cultivars.

Chickpea and finger millet starches were submitted to structural studies by using ß-amylase and pullulanase to explain at a molecular level the differences in the digestibility of legume (chickpea) and cereal (finger millet) starches (Madhusudhan & Tharanathan, 1996). The lower digestibility of chickpea starch compared to finger millet starch was explained by higher amylose content and longer branch chains of amylopectin in chickpea starch (Madhusudhan & Tharanathan, 1996).

The conditions for producing granular cold water gelling starch with native chickpea starch by treatment with liquid ammonia at low temperature and atmospheric pressure have been studied by Jackowski, Czuchajowska and Baik (2002). Chickpea starch has also been submitted to chemical modification studies. Compared to native starch, hydroxypropylated chickpea starch showed increased clarity and better freeze-thaw stability, whereas hot paste viscosity remained constant (Rege & Pai, 1996).

Aim and outline of the thesis

The aim of this thesis was to broaden our knowledge on the effect of reagent type (acetic anhydride vs. vinyl acetate) on the properties and chemical fine structure of acetylated starch. Starches from three legumes, commercial yellow pea starch, and starches isolated from cowpea and chickpea seeds were investigated first on their physicochemical properties: amylose contents, volume mean diameter of granules, X-ray diffraction pattern and swelling volume. The chain length profiles of their amylopectins were determined as well (Chapter 2). Based on the information obtained from the unmodified starches, the effect of reagent type on the properties of acetylated starches was studied for yellow pea, cowpea and chickpea starches after modification with acetic anhydride and vinyl acetate, with respect to swelling volume, peak viscosity and the DS in Chapter 3. The effect of granule size was also studied by sieving the starches into small and large size granule fractions after acetylation. Due to the distinguished difference on pasting viscosities of the same starch obtained from different reagents, the chemical fine structure of acetylated amylose and amylopectin was investigated on cowpea starch in Chapter 4. Amylose and amylopectin populations were isolated and the DS values were evaluated. The acetyl substitution patterns were analyzed by enzymatic degradation followed by characterization of the obtained fragments using chromatographic and mass spectrometric techniques. The effect of reagent type based on different size granule fractions was further investigated on two modification procedures on yellow pea starch: the first procedure included fractionation of the starch into small and large size granule fractions and then acetylation of these fractions (acetylation after sieving), and the second procedure included acetylation of the starch and then fractionation into small and large size fractions (acetylation before sieving). The degree of substitution and pasting viscosity were analyzed. The location and distribution of acetyl groups were investigated by analyzing the α-amylase hydrolysates of isolated amylose and amylopectin population with chromatography and mass spectrometry (Chapter 5). A model for the different distribution patterns of acetyl groups at the granular level was proposed for yellow pea starch modified with acetic anhydride and vinyl acetate. Finally, an overview of the research work and further discussions are given in Chapter 6.

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Chapter 2

Physicochemical properties and amylopectin chain profiles of cowpea, chickpea and yellow pea starches

Abstract

Starches from cowpea and chickpea seeds were isolated and their properties were compared with those of commercial yellow pea starch. Amylose contents were 25.8%, 27.2%, and 31.2%, and the volume mean diameter of granules determined in dry state were 15.5μm, 17.9μm, and 33.8μm for cowpea, chickpea and yellow pea starches, respectively. All three legume starches showed a C-type X-ray diffraction pattern and two-stage swelling pattern. Amylopectin populations were isolated and the unit chain profiles were analyzed by HPLC after debranching with pullulanase. The degree of polymerization (DP) of short chain populations was about 6 to 50 and the populations of long chain had a DP of 50 to 80. Cowpea showed a lower weight ratio of short:long chains than chickpea and yellow pea starches. The larger portion of long side chains in cowpea amylopectin can be correlated with a higher gelatinization temperature, greater pasting peak and a slight difference in crystalline structure found for cowpea starch. Chickpea and yellow pea starches exhibited similarity in unit chain profile of amylopectin as well as in gelatinization temperature and pasting profile, while they differed in amylose content, particle size and syneresis. It is assumed that the chain length distribution of amylopectin has a large influence on starch properties.

Keywords: Pea starches; Physicochemical properties; Pullulanase; HPSEC; HPAEC

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Introduction

Legumes are important ingredients of a balanced human diet in many parts of the world due to their high protein and starch content (Czuchajowska, Otto, Paszczynska, & Baik, 1998). Cowpea (*Vigna unguiculata*), native to Asia and Africa, has a number of commonly used names, like southern pea, black-eyed pea and crowder pea (Prinyawiwatkul, McWatters, Beuchat, & Phillips, 1997). Chickpea (*Cicer arietinum*), also known as garbanzo (Schoch & Maywald, 1968) and Bengal gram, is cultivated in the Mediterranean Basin, North America and Asia. Yellow pea (*Pisum sativum*), which is also known as field pea (Sosulski, Hoover, Tyler, Murray, & Arntfield, 1985), garden pea and smooth pea (Li & Vasanthan, 2003; Boyer, 1981; Gernat, Radosta, Damaschun, & Schierbaum, 1990), is a yellow seeded cultivar of *Pisum sativum*, very common in Northern Europe.

Cowpea, chickpea and yellow pea have been historically consumed as whole seeds or ground flour. The utilization of their components as new ingredients in food industry has drawn the attention of researchers. The protein contents of cowpea, chickpea and yellow pea are in the range of 15%-35% (Longe, 1980; Arora & Das, 1976; Sosulski et al., 1985; Rangel, Domont, Pedrosa, & Ferreira, 2003; Jirapa, Normah, Zamaliah, Asmah, & Mohamad, 2001; Clemente, Sanchez-Vioque, Vioque, Bautistab, & Millan, 1998; Soetrisnot & Holmes, 1992). These values are much higher than the levels found in cereal grains and root crops. Consequently cowpea, chickpea and yellow pea have been subjected to protein studies (Okechukwu & Rao, 1997; Rangel et al., 2003; Sanchez-Vioque, Clemente, Vioque, Bautista, & Millan, 1999; Soetrisnot et al., 1992) to satisfy the demand of new sources of plant proteins. Protein production can only be economic when the other major component – starch is utilized simultaneously.

Information on structure-property relationships of these legume starches is essential to predict their functionality and subsequent end-use in foods. There are some studies on gelatinization, gel and rheological behaviours of cowpea starch, but few systematic studies on its phycochemical properties and amylopectin structure. There is more information for chickpea starch, but this does not cover the relationship between molecular structure of amylopectin and starch properties. An attempt was made to relate the properties of cowpea and chickpea starches to the chain profiles of their amylopectin fractions. Characteristics of chickpea starch exhibit considerable variability in literature, so do those of yellow pea starch. Therefore, the physicochemical properties and the chain length distributions of amylopectin populations of chickpea and yellow pea starches were investigated together with cowpea starch in this study.

Materials and Methods

Materials

Cowpea and chickpea starches were prepared in the laboratory from cowpea and chickpea seeds kindly supplied by AVEBE Food Innovation Centre, Asia Pacific (Shanghai, China). Yellow pea starch was a gift from COSUCRA (Warcoing, Belgium). Pullulanase (EC 3.2.1.41) (M2, from Bacillus licheniformis, 400 U/mL) was purchased from Megazyme (Ireland).

Starch isolation from cowpea and chickpea

Starches were isolated from cowpea and chickpea according to the method of Schoch et al. (1968) with some modifications. Peas were steeped in deionized water at 4°C for 24h (cowpea) and 48h (chickpea). The steep water was decanted, and the softened legumes were ground in a blender (Waring, New Hartford, USA) for 3 min in deionized water (4°C) at low speed. The ground slurry was sieved through a 0.450 mm sieve on a AS200 digit shaker (Retsch GmbH & Co., Haan, Germany). The residual pulp was again ground for 3min in the blender with fresh water, and sieved again. The combined starch suspension was then sieved through a set of sieves (0.250 and 0.063 mm). The starch was allowed to settle overnight at 4°C. The supernatant was drained off, and the upper non-white layer was removed. The starch layer was resuspended in cold 0.2% NaOH and kept at 4°C for 17h (cowpea) and 2h (chickpea). Then starch slurries were neutralized with 2 mol/L HCl to pH 6 and centrifuged at 100 × g (cowpea) and 1500 × g (chickpea) at 4°C. The starch layer was suspended in deionized water and centrifuged 6–7 times, until the settled starch gave a firm, dense deposit on the bottom and was substantially free of fine fiber (as examined by microscopy). The final sediment was suspended in cold deionized water and screened through a 0.032 mm sieve. The starch was recovered by filtration, drying at 40°C for 72h and ground into powder using a blender.

Chemical characteristics of materials

Cowpea and chickpea were milled with a ultra centrifugal mill (ZM200, Retsch GmbH & Co., Haan, Germany) into fine powder. The moisture contents and lipid contents of two raw materials and three starch samples were determined according to Chen, Schols, & Voragen (2003a). The starch contents

of cowpea, chickpea seeds and three starches were determined using the enzymatic Roche starch test kit (Boehringer Mannheim, Darmstadt, Germany). The amylose contents of cowpea, chickpea and yellow pea starches were tested using the enzymatic amylose/amylopectin assay kit (Megazyme International Ireland Ltd., Co. Wicklow, Ireland). Nitrogen contents were determined according to Chen, Schols, & Voragen (2003b). Phosphorus contents of the starches were measured according to the method of Rameau & Have (1951).

Physical properties of starch

Particle size distribution of starch was measured both in dry state and in water with a laser diffraction system (H1140, Sympatec Inc., New Jersey, USA). The analysis of the crystalline structure of the starches was carried out using a Philips diffractometer (PW 1830, Almelo, the Netherlands). Degree of relative crystallinity was calculated based on the method of van Soest, Tournois, de Wit, & Vliegenthart (1995) with the equation: Relative Crystallinity (%) = Ac / At × 100, where Ac is the area of crystalline peak and At is total area measured from the basline, which was a straight line from 7° 2-theta in the X-ray diffractogram as shown in Fig. 1.

The temperature range of gelatinization was measured using a Differential Scanning Calorimeter (Perkin Elmer DSC-7, Norwalk, Connecticut, USA). Starches (10mg) and 40 - 50 mg deionized water were weighed, sealed and hold for 5 min at 10°C, then heated from 10°C to 150°C at a rate of 10°C/min. The PE Pyris Series – DSC7 software was used for data handling. The enthalpy of the endothermic peak was expressed on the basis of dry material (J/g dry starch).

Swelling volume was determined by the method of Collado & Corke (1999). The sample (0.20 g, dry substance) was mixed with 10 ml deionized water, equilibrated at 25°C for 30 min and heated for 30 min at 10°C intervals between 50°C and 90°C with continuous mixing. The sample was cooled down to 25°C and then centrifuged at 1000 × g for 15 min. For less than 2 ml gel volume, the volume was adjusted to 10ml. The supernatant was removed gently and measured with a graduated cylinder. The gel volume was then calculated as: ml of gel = 10ml- ml of supernatant (Prinyawiwatkul et al., 1997). Swelling volume was calculated as gel volume to the dry weight of the sample.

Characteristics of starch pastes and gels

The pasting behaviours of the starches were measured using a Rapid Visco Analyzer (RVA-4, Newport Scientific Pty. Ltd., Warriewood NSW, Australia) in a defined program: 28g of 6% (w/w) starch suspensions were stirred with a paddle speed of 160 rpm/min and heated from 30°C to 90°C at 15°C/min, held at 90°C for 5 min, cooled to 30°C at −15°C/min and held at 30°C for 7 min.

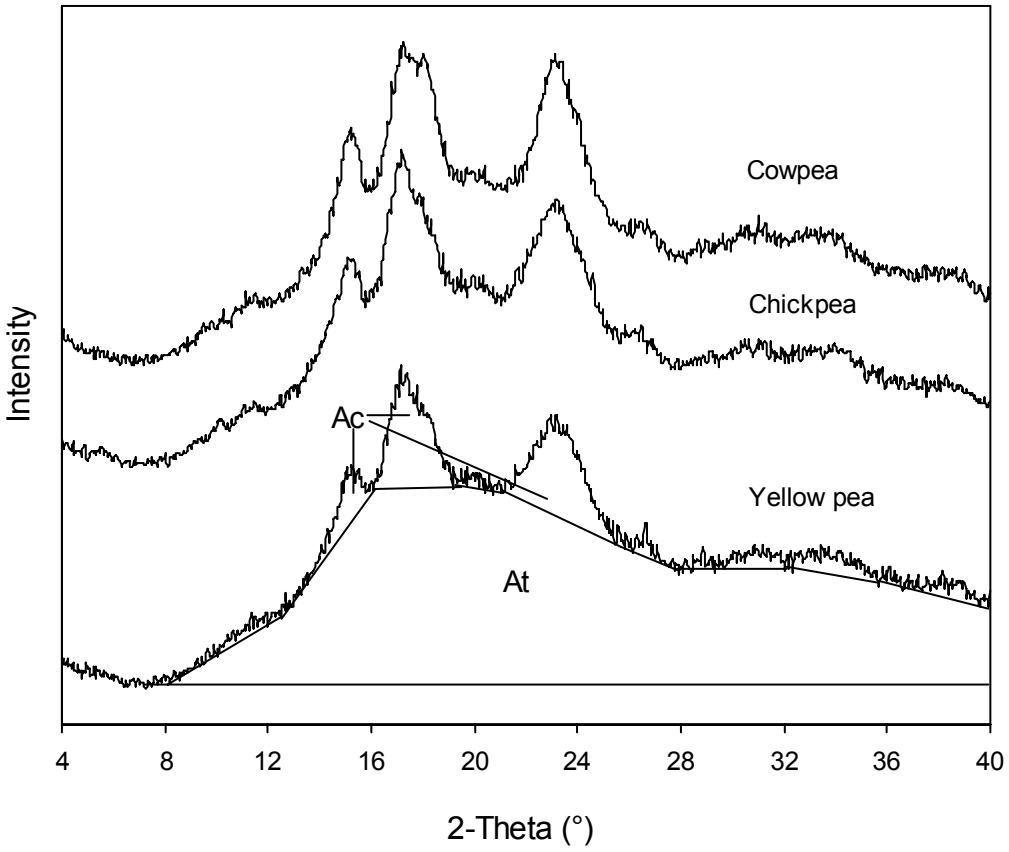

Fig. 1. X-ray diffractograms of cowpea, chickpea and yellow pea starches. Ac is the area of crystalline peak and At is total area measured from the basline.

Freeze-thaw stability was tested according to the method of Yuan & Thompson (1998) with some modifications. Starch suspensions of 5% (w/v) were mixed and equilibrated at 25°C for 30 min, then 1ml starch slurry was dispensed into 2ml tubes with vigorous stirring. After heating in a boiling water bath for 30min while stirring, the samples were kept at -20°C for 24h and thawed at 30°C for 1.5h. Free water was removed by putting the gel on 16 layers tissue paper for 5min. Five

freeze-thaw cycles were considered. The extent of syneresis was calculated as the ratio of exuded water weight to the original paste weight. The syneresis of the starch gels without freeze-thaw treatments were measured by storing at 2°C for 5 days. Every 24h the exuded water was measured after the starch gel was centrifuged at 1000 × g for 10 min.

Amylopectin Structure

The separation of amylopectin and the digestion of pullulanase were conducted according to Chen, Schols, & Voragen (2004). The purity of the separated amylopectin was checked by HPSEC (High-performance size-exclusion chromatography) after pullulanase treatment according to Kobayashi, Schwartz, & Lineback (1985). The unit chain profile was analyzed by HPSEC and HPAEC (high-performance anion-exchange chromatography). HPSEC and HPAEC were performed as described in Chen, Huang, Suurs, Schols, & Voragen (2005), except that a CarboPac PA1 column (2 × 250 mm) with guard column (Dionex, USA) was operated at a flow rate (0.3 mL/min) in HPAEC analysis.

Results and Discussion

Chemical composition of raw materials and starches

Cowpea and chickpea starches were isolated from commercial cowpea and chickpea seeds. Analyses on these two raw materials showed that starch was the most abundant component (Table 1) as found by others (Longe, 1980; Arora et al., 1976; Sosulski et al., 1985; Oluwatosin, 1998; Ereifej, Al-Karaki, & Hammouri, 2001). Their protein contents were similar, while the lipid level in

Table1 The chemical composition of cowpea and chickpea seeds (w/w, %)

Source	Moisture	Starch content (db)[b]	Protein(db)	Lipid (db)
Cowpea	11.0±0.0[a]	49.6±0.1	23.1±0.16	1.3±0.26
Chickpea	10.6±0.0	50.4±1.3	23.0±0.23	5.8±0.09

[a] Standard deviation of triplicate.

[b] Dry basis.

cowpea was around a quarter of that in chickpea. The chemical characteristics of isolated starches from cowpea, chickpea and the commercial yellow pea starch are summarized in Table 2. It is

Table 2 Composition (w/w, %) of cowpea, chickpea and yellow pea starches

Starch	Moisture	Starch(db)	Amylose(db)[b]	Protein(db)	Lipid(db)	Phosphorus(db)
Cowpea	11.5±0.42[a]	93.1±0.48	25.8	0.49±0.03	0.15±0.05	0.022
Chickpea	11.9±0.23	94.0±0.39	27.2	0.57±0.01	0.10±0.01	0.012
Yellow pea	11.3±0.12	92.3±0.56	31.2	0.52±0.02	0.07±0.01	0.007

[a] Standard deviation of triplicate.

[b] Dry basis.

rather difficult to obtain pure starches from legumes, due to the high protein content (Schoch et al., 1968; Moorthy, 2004). The isolation process of cowpea starch is one of the most difficult ones because of the fine fiber, slowing down the sedimentation and co-settling with the starch to give a light, loose deposit. The purities of isolated cowpea and chickpea starches were higher than 93%. The lipid contents in chickpea, cowpea and yellow pea starches were low and in the same level as in tuber starches and much lower than in cereal starches (1%, Eliasson & Wahlgren, 2004). Their phosphorus contents were lower than that in potato starch (0.08%, Chen et al., 2003a). Legume starches have been characterized by high amylose contents (Czuchajowska et al., 1998; Singh, Sandhu, & Kaur, 2004). The amylose levels of three starches were within the ranges in literature (20.9% - 48.7% for cowpea; 20.7% - 42.2% for chickpea; and 22.0% - 49.6% for yellow pea starches) (Arora et al., 1976; Won, Choi, Lim, Cho, & Lim, 2000; Saini & Knights, 1984; Hoover & Ratnayake, 2002; Ratnayake, Hoover, Shahidi, Perera, & Jane, 2001).

Physical properties of starch

Size distribution

The granule size distributions of cowpea and chickpea starches were unimodal, while of yellow pea starch there was a slight shoulder at high granule diameters (Fig. 2). In dry state, the VMD (volume

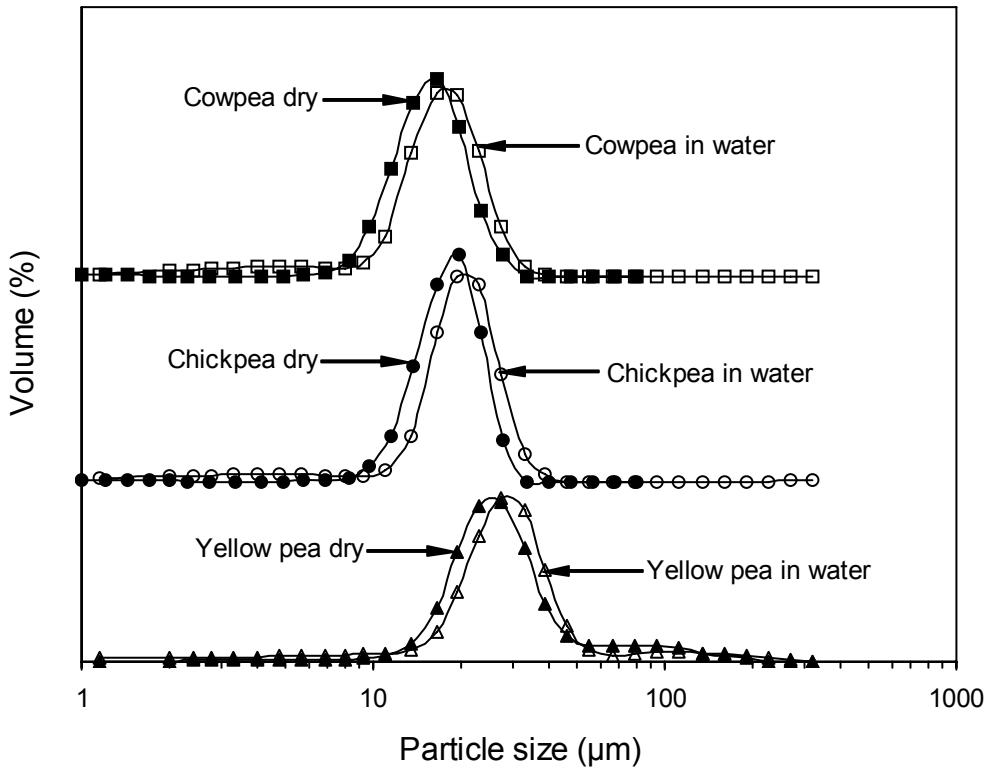

Fig. 2. Particle size distributions of cowpea, chickpea and yellow pea starches measured in dry state and in water.

mean diameter) were 15.5 µm, 17.9 µm and 33.8 µm for cowpea, chickpea and yellow pea starches, respectively. When measured in water, the starches had a shift toward larger granule sizes, indicating slight swelling of the granules in cold water. The size of cowpea starch granules was smaller than reported by Okechukwu & Rao (1996a, 1996b) (range, 3µm-64µm; mean, 19µm). Similar result for chickpea starch (6µm-31µm) using microscopy has been observed by Hoover et al. (2002) and Singh et al. (2004). Hoover et al. (2002) reported that the starch granule size ranged from 14µm-37µm for smooth pea.

Crystalline structure

Three different types of crystal structures have been identified and classified by Katz and Itallie in 1930. C-type starches actually consist of a mixture of A-type and B-type (Donald, 2004). Crystals in cowpea, chickpea, and yellow pea starches determined by X-ray diffraction were all C-type (Fig.

1), which is known as the characteristic pattern of legume starches (Donald, 2004). All three starches showed peaks at 15.2º, 17.2º and 23.2º 2-theta, corresponding to d-spacings of 0.58 nm, 0.52 nm and 0.38 nm, respectively. Cowpea starch showed an extra peak at 18.0º 2-theta (d-spacing 0.49 nm). This indicates that the crystalline structure in cowpea starch is slightly different from that in chickpea and yellow pea starches. The relative crystallinity of yellow pea starch was lower than that of cowpea and chickpea starches (Table3). This may be explained by the fact that yellow pea starch had the highest amylose content among three starches. The double helical content decreases with increasing amylose content (Cheetham & Tao, 1997). C-type X-ray pattern has been reported for chickpea and smooth pea starches (Hoover et al., 2002; Gernat et al., 1990; Davydova, Leont'ev, Genin, Sasov, & Bogracheva, 1995). However El Faki, Desikachar, Paramahans, & Tharanathan (1983) reported cowpea starch as A-type and chickpea starch as B-type. The X-ray diffraction pattern may depend on the starch origin as well as the environmental growth conditions.

Table 3 Properties of cowpea, chickpea and yellow pea starches

Starch	Gelatinization Temperature °C (DSC)[a]				Enthalpy (J/g dry starch) (DSC)[a]	Relative crystallinity % (X-ray)
	T_o[b]	T_p	T_c	ΔT		
Cowpea	70.5	75.4	81.0	10.5	15.2	26
Chickpea	57.9	63.5	70.4	12.5	17.6	26
Yellow pea	58.2	65.1	70.4	12.2	16.1	21

[a] Differential Scanning Calorimeter.

[b] T_o = onset temperature, T_p = peak temperature, T_c = completion temperature, $\Delta T = T_c - T_o$.

Thermal properties

DSC was used to study the thermal properties of the starches. Chickpea and yellow pea starch showed similar onset and completion temperatures of gelatinization, which were much lower than that of cowpea starch (Table 3). The gelatinization temperature ranges and enthalpies of chickpea and yellow pea starches were higher than that of cowpea starch. The higher gelatinization temperature was an indication of more perfect crystals (van Soest, Bezemer, de Wit, & Vliegenthart,

1996; Sasaki & Matsuki, 1998) or a higher co-operative unit, that is, longer chains in the crystal or a larger crystal size (Matveev et al., 2001). Chickpea and yellow pea starches showed single endothermic peaks, while cowpea starch showed a slightly double peak (results not shown). This confirms the findings from the X-ray measurement that the crystalline structure in cowpea starch is different from that of the other two legume starches. Double peaks represented two transitions, the melting of B polymorphs and the melting of A polymorphs (Bogracheva, Morris, Ring, & Hedley, 1998).

The onset gelatinization temperatures 65°C - 71°C, 59.4°C - 66°C and 60.8°C – 64°C have been reported for cowpea, chickpea and yellow pea starches, respectively (Okechukwu et al., 1996a, 1997; Sosulski et al., 1985; Kerr, Ward, McWatters, & Resurreccion, 2000; El Faki et al., 1983; Hoover et al., 2002; Czuchajowska et al., 1998; Singh et al., 2004; Ratnayake et al., 2001; Schoch et al., 1968). The gelatinization temperature seems to be influenced by the molecular architecture of the crystalline region rather than the amylose content (proportion of crystalline region) of starch (Bao & Bergman, 2004). In addition, the isolation procedures may have an impact on the value.

Swelling volume

When starch is heated in enough water, hydrogen bonds stabilizing the structure of the double helices in crystallites are broken and replaced by hydrogen bonds with water (Tester & Karkalas, 1996), the starch granule swells and its volume increases. The swelling volume of cowpea starch increased slightly from 50°C to 70°C (Fig. 3). At 80°C the value was about 10 times as much as the amount at 70°C, indicating that only after the temperature reaches the onset gelatinization point the starch granule undergoes rapid swelling. Similar relationships between swelling behaviour and gelatinization temperature were found for chickpea and yellow pea starches. A two-stage swelling pattern has been reported by Agunbiade & Longe (1999) for cowpea starch and Gujska, Reinhard, & Khan (1994) for field pea starch, and considered to be the typical swelling pattern of legume starches (Oates, 1991; Ratnayake et al., 2001). The higher swelling volume at 90 °C indicates that amylopectin chains within crystalline regions are more strongly associated in cowpea starch than in the other two legume starches as suggested by Hoover, Li, Hynes, & Senanayake (1997) for mung bean starch. Swelling volume of starch was affected by amylose content and the structure of amylopectin (Sasaki et al., 1998).

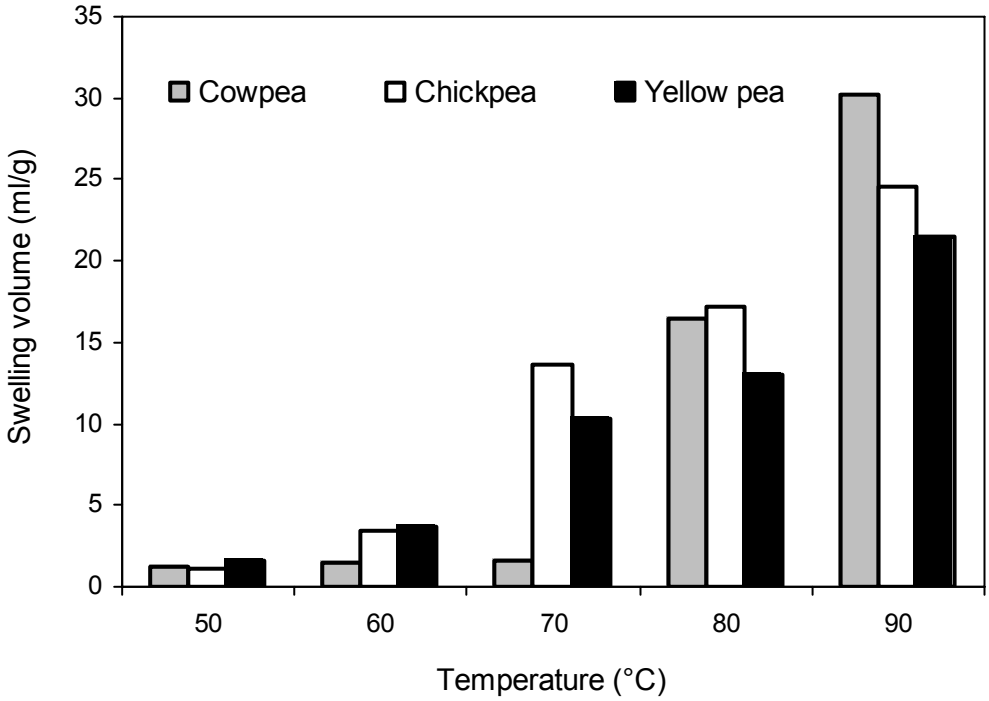

Fig. 3. Swelling volume of cowpea, chickpea and yellow pea starches influenced by temperature. Swelling volume was calculated as gel volume to the dry weight of starch.

Characteristics of starch pastes and gels

Pasting behaviour

Pasting temperature is a measure of the temperature at which a starch starts to thicken. In a RVA curve, the point at which viscosity starts to increase is considered to be the pasting temperature. Cowpea starch showed much higher pasting temperature (80.7 °C) than chickpea (70.9 °C) and yellow pea (70.5 °C) starches. The results were in accordance with the gelatinization temperatures obtained with DSC (Table 3). According to the classification of Schoch et al. (1968), chickpea and yellow pea starches showed type C pasting profiles (Fig. 4), presenting no pasting peak, the viscosity remained constant during cooking and increased during cooling down. The maximum value at 90 °C was reported as peak viscosity (Agunbiade et al., 1999). Cowpea starch had a type B viscosity, with a peak viscosity (PV) of 1440 cP and setback (SB = final viscosity – peak viscosity)

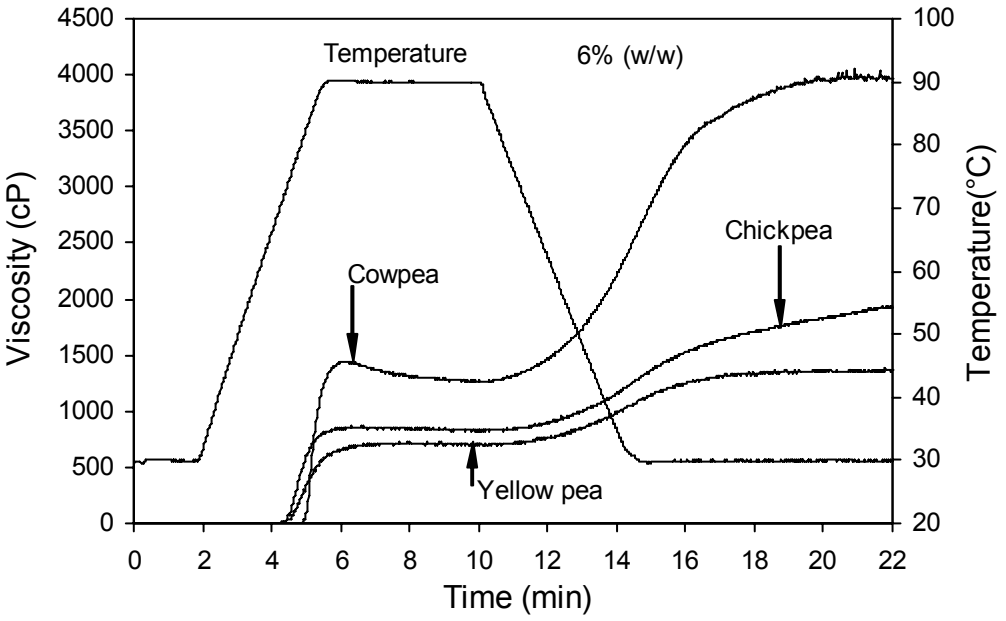

Fig. 4. RVA pasting curves of cowpea, chickpea and yellow pea starches. RVA: rapid visco analyzer. cP: centipoises.

of 2535 cP, which were much higher than those of chickpea (PV, 871 cP; SB, 1071 cP) and yellow pea (PV, 724 cP; SB, 643 cP) starches. The fast retrogradation tendency of cowpea starch, indicated by its high setback value, is favourable for food products like gluten-free oriental noodles (Czuchajowska et al., 1998). Type B viscosity pattern for cowpea starch and Type C viscosity pattern for chickpea and field pea starches have been observed (Tolmasquim, Correa, & Tolmasquim, 1971; El Faki et al., 1983; Agunbiade et al., 1999; Singh et al., 2004; Li et al., 2003). Pasting properties of chickpea, cowpea and yellow pea starches were influenced by granule swelling as pointed out by Ratnayake et al. (2001). Cowpea starch showed highest swelling volume at 90 °C, and the highest viscosity during cooking at 90 °C among the starches.

Syneresis

Freeze-thaw stability of gelatinized starch pastes is a desired property for the use of starch by the food industry (Jobling, Westcott, Tayal, Jeffcoat, & Schwall, 2002). The syneresis occurred rapidly in the first two cycles, slowed down in the next three cycles, it did not increase steadily with

increasing number of the freeze-thaw cycle (Fig. 5). Similar findings for waxy maize, amaranth, wheat, maize, rice and potato starches have been reported (Yuan et al., 1998; Baker & Rayas-Duarte, 1998; Jobling et al., 2002). Our results confirm the finding of Yuan et al. (1998) that the estimation of freeze-thaw stability of starch pastes should be based on data obtained from several freeze-thaw cycles. The lowest rate of syneresis observed for cowpea starch suggested that it could be more suitable for use in products that are stored frozen and thawed for consumption. As the procedure to determine freeze-thaw stability of starch has not been standardized (Karim, Norziah, & Seow, 2000), the syneresis values obtained by others were quite variable, namely 33.2% after three freeze-thaw cycles, 50% and 30% after five freeze-thaw cycles, with amylose contents of 27.9%, 21.2% and 43.7% for cowpea, chickpea and field pea starches, respectively (Chung, Cho, Chung, Shin, Son, & Lim, 1998; Hoover et al., 2002; Ratnayake et al., 2001).

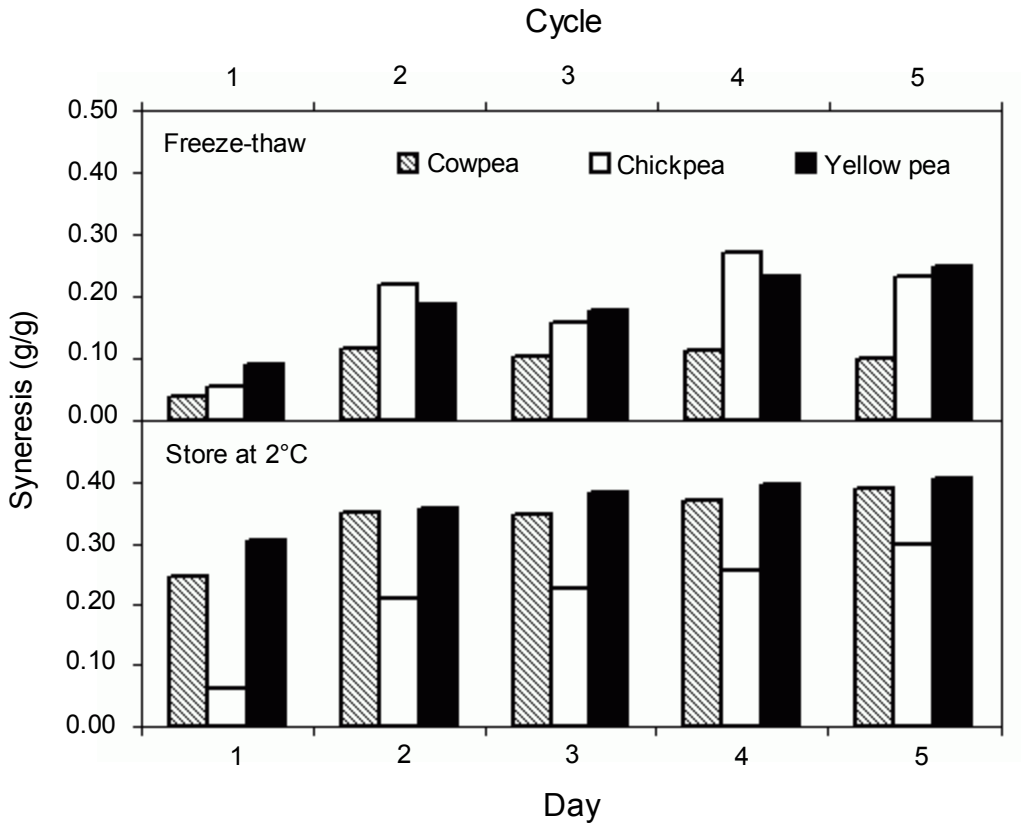

Fig. 5. Syneresis of cowpea, chickpea and yellow pea starches during freeze-thaw cycles and stored at 2°C. The extent of syneresis was calculated as the weight ratio of exuded water to the original paste.

When stored at 2 °C for five days, yellow pea starch showed the highest syneresis (Fig. 5). The separation of phases caused by α-glucan chain re-association is a sign of starch retrogradation (Jobling et al., 2002). The retrogradation tendencies measured by the syneresis with and without freeze-thaw treatment were not in agreement with each other, as reported by Chen et al. (2003a). The difference in the rates of syneresis for all the three starches between two types of treatments may be explained by the fact that as water freezes it increases in volume. As the water freezes and expands, the gel network may be broken by ice crystals. The weaker the starch gel, the stronger physical damage appeared to the gel network, and the higher the rate of syneresis. Chickpea and yellow pea starches had weaker gels and the networks were destroyed more by freeze-thaw treatment as compared with cowpea starch. Singh et al. (2004) reported that the syneresis of chickpea starch, with amylose content 34.3%, at 2% starch concentration, was 18.5% after storage at 4 °C for 120 h.

Chain length distribution of amylopectin

Properties of starch depend on the molecular structure of its components. Amylopectin predominated in cowpea, chickpea and yellow pea starches and played an important role in their properties. Biliaderis, Grant, & Vose (1981) and Chung et al. (1998) found that studies on isolated amylopectin can give more precise chain length information than using the whole starch sample. Structural investigation on amylopectin using pullulanase was carried out to explain the differences in the properties of the cowpea, chickpea and yellow pea starches at a molecular level.

After treatment with pullulanase, only smaller polysaccharide fragments appeared and no other peaks were detected in HPSEC chromatograms. Therefore, the amylopectin populations isolated from cowpea, chickpea and yellow pea starches were believed to be pure.

All three amylopectin samples displayed two populations: long chains (fraction I) and short chains (fraction II) in HPSEC chromatogram (Fig. 6). The bimodal chain distribution profiles have been reported for cowpea amylopectin based on the study on the whole starch sample, and for isolated chickpea and smooth pea amylopectin (Chung et al., 1998; Biliaderis et al., 1981). The weight ratio of short:long chains of chickpea (4.4:1) was close to that of yellow pea (4.2:1) amylopectin. The results were in agreement with data found by Biliaderis et al. (1981) on the molar ratio of short:long chains of chickpea (8.0) and smooth pea (7.7) amylopectin. Cowpea amylopectin had a lower weight ratio of short:long chains (3.1:1), which was the evidence of the higher amount of long chains that accounted for higher gelatinization temperature, greater pasting peak, and better stability

in freeze-thaw cycles of cowpea starch than those of chickpea and yellow pea starches. It was evident that the chickpea and yellow pea starches were very similar in some properties as well as the chain length profiles. The shortest chain in all three samples was DP 6 as revealed by HPAEC (Fig. 7), which is also reported by Ratnayake et al. (2001) for field pea starch and mentioned to be a general feature of all amylopectin described so far (Bertoft, 2004). Individual chains up to DP 40 were recognized in the HPAEC pattern. Considering the data sets from HPSEC and HPACE together, the DP (degree of polymerization) of short chains was about 6 to 50 and of long chains about 50 to 80.

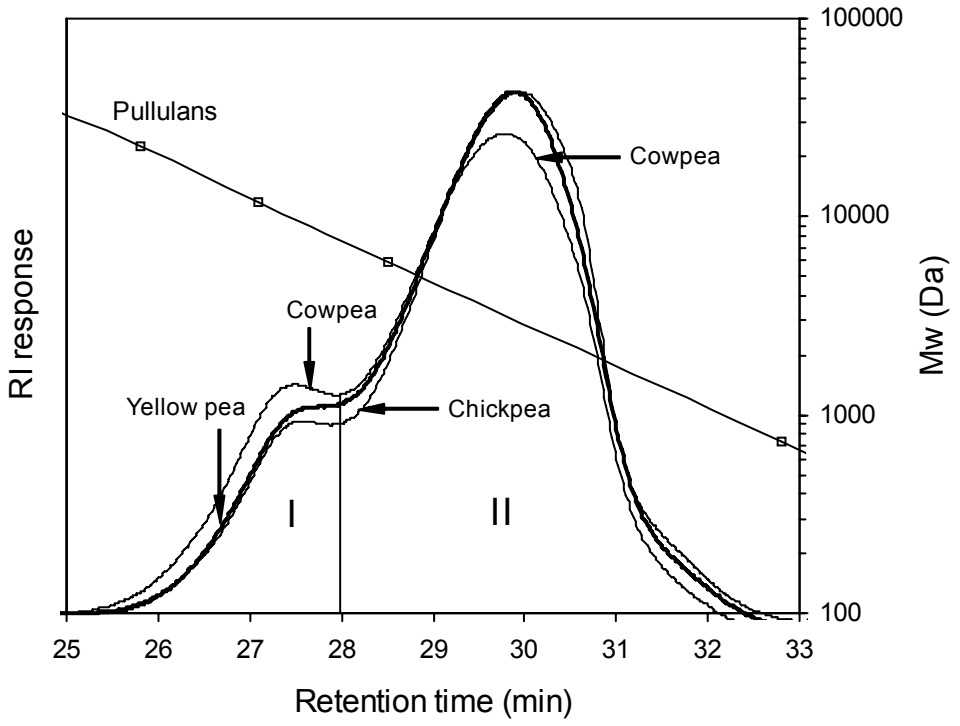

Fig. 6. HPSEC elution profiles of the pullulanase hydrolysates of the amylopectin populations isolated from cowpea, chickpea and yellow pea starches. RI: refractive index.

Conclusions

From our results of the physicochemical properties and the unit chain profiles of cowpea, chickpea and yellow pea starches, it can be proposed that the higher gelatinization temperature, slight difference in crystalline structure and higher peak viscosity of cowpea starch, is partly provided by

its higher amount of long chains in amylopectin molecules as compared with those of chickpea and yellow pea starches. Similarity in chain length distribution between chickpea and yellow pea starches correlated to the nearly identical gelatinization temperatures, similar X-ray diffraction patterns and RVA profiles. Their different swelling and syneresis behaviours can be explained by

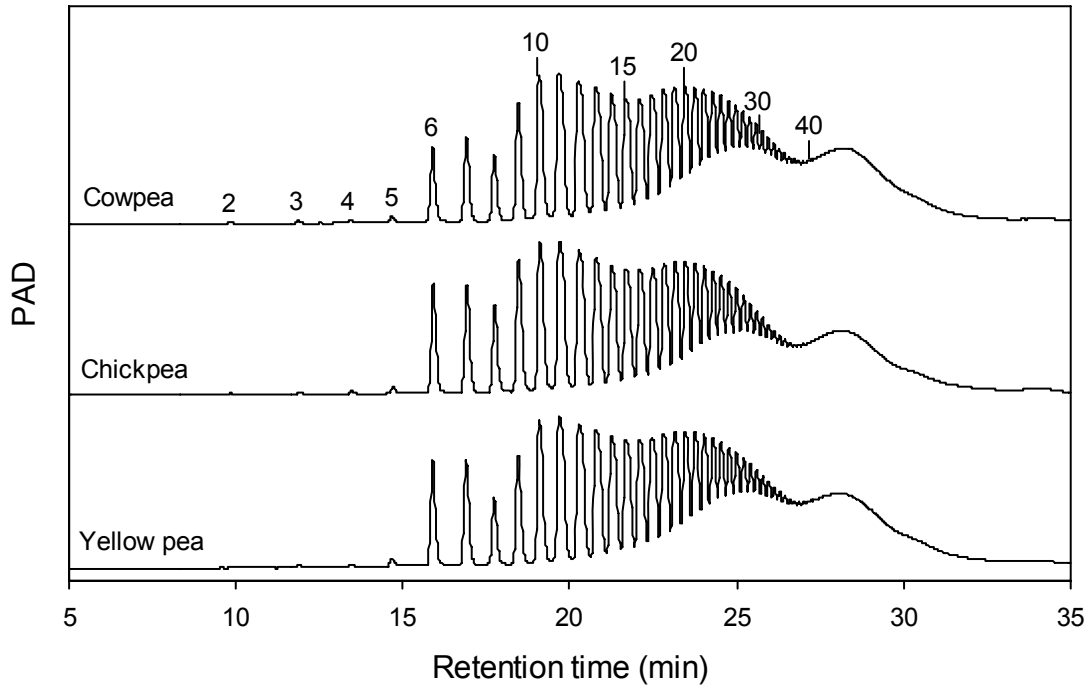

Fig. 7. HPAEC elution profiles of the pullulanase hydrolysates of the amylopectin populations isolated from cowpea, chickpea and yellow pea starches. Numbers indicate degree of polymerization. PAD: pulsed amperometric detection.

the difference in amylose content and particle size distribution. Smaller granule size, lower amylose level and larger degree of swelling at 90°C suggest that the crystallites in cowpea starch are of a higher order of stability and that amylopectin chains within crystalline regions were more strongly associated than in chickpea and yellow pea starches. The significantly higher setback value for cowpea starch indicates that this starch may be suitable for food products where fast retrogradation is necessary, like glass noodle.

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Chapter 3

Characterization of Differently Sized Granule Fractions of Yellow pea, Cowpea and Chickpea Starches after Modification with Acetic Anhydride and Vinyl Acetate

Abstract

The effect of reagent type on the properties of acetylated starches was studied for yellow pea, cowpea and chickpea starches after modification with acetic anhydride and vinyl acetate. Samples modified with vinyl acetate showed higher swelling volume and peak viscosity than those acetylated with acetic anhydride for the same starch. In addition, the reagents reacted differently towards granules having different sizes as present in un-fractionated starch. After sieving of the acetylated starches, the Degree of Substitution (DS) differed for the differently sized starch granules acetylated by the rapidly reacting acetic anhydride but not for the size fractions obtained from the starches acetylated by the slowly reacting vinyl acetate. Smaller size granule fractions exhibited larger swelling volume and higher peak viscosity as compared with the corresponding larger size fractions. The reagent type and granule size are important factors for pasting and swelling behaviours of acetylated granular starches.

Key words: Acetylation; Pea starches; Granule size; Acetic anhydride; Vinyl acetate

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Introduction

Irrespective of the source, all starches occur in nature as minute granules. The shape of the starch granule depends on the botanical source, and many different forms are found. Also the size varies, from the tiny granules in rice and oat to the large ones in potato and banana starches. Particle size ranges 3-8 μm, 3-24 μm, 2-35 μm, 5-70 μm and 5-100 μm have been reported for rice, amylomaize, wheat, arrowroot and potato starches, respectively (Swinkels, 1985). Because of the broad range of particle size distribution in starch, the effect of granule size on the properties and applications of starch have received attention. Other factors affecting starch functionality are composition (amylose-to-amylopectin ratio, contents of non-starch components), crystallinity, and amylopectin structure. Waxy maize starch granules with a smaller diameter showed greater enzymatic hydrolysis than larger ones, but there was no difference in the fine structure of starch residues obtained from enzymatic hydrolysis between the two size fractions (Franco, Rreto, Ciacco, & Tavares, 1998). Investigation of large, medium and small size granule fractions from barley starch revealed that small granules and large granules exhibited differences in enthalpy, swelling power, relative crystallinity, susceptibility to enzymes, retrogradation, the number-average degree of polymerisation (DP_n) and chain-length distribution of amylopectin (Tang, Ando, Watanabe, Takeda, & Mitsunaga, 2000, 2001a; Tang, Ando, Watanabe, Takeda, & Mitsunaga, 2001b; Tang, Watanabe, & Mitsunaga, 2002; Tang, Mitsunaga, & Kawamura, 2004). The content of phosphorus, pasting properties, and digestibility by amylase, varied greatly according to the granule size of potato starch (Noda et al., 2005). Chen, Schols, & Voragen (2003b) reported that starch noodles made from small size granule fractions of potato and sweet potato starches had better processibility and better quality than those made from large size granule fractions.

Modified starch is defined as starch whose hydroxyl group has been altered by a chemical reaction (esterification, etherification, or oxidation) or by enzymatic modification, as in the case of dextrins (Fleche, 1985). The reaction(s) chosen for starch modification usually exert a major change on the desirable physical properties. Nevertheless, low levels of modification already dramatically alter the physical properties of starch, such as paste viscosity, gelling, syneresis, clarity, and adhesion properties. The most typical starch ester is acetylated starch, which is actively marketed due to the specific properties arising from the substitution groups.

Low degree of substitution (DS < 0.1) starch acetate has been applied in both food industry and industrial areas (Fleche, 1985) for many years. Acetic anhydride is usually used as acetylating agent, while an alternative reagent like vinyl acetate can also be used to prepare starch acetate. Acetic

anhydride and vinyl acetate are reagents of different nature and little information is available about the effect of reagent type on the properties of acetylated starch. This research was designed to make a direct comparison on the properties of starch modified with acetic anhydride and vinyl acetate, respectively. Acetylated starches are prepared most often under reaction conditions that preserve granular structure. In previous work, we observed increased degree of substitution (DS) with decreasing granule size for small and large size granule fractions of potato and sweet potato starches modified with acetic anhydride (Chen, Schols, & Voragen, 2004). In this study, we investigated the effect of granule size on modification with vinyl acetate and acetic anhydride as acetylating reagents.

Yellow pea (*Pisum sativum*), cowpea (*Vigna unguiculata*) and chickpea (*Cicer arietinum*) starches, with typical legume starch properties (Huang, Schols, van Soest, Jin, Sulmann, & Voragen, 2007), are of industrial interest to be utilized in their acetylated form as well. Hence, investigation on these three starches was undertaken to obtain information on the effects of reagent type (acetic anhydride vs. vinyl acetate) and granule size on the properties of acetylated starches.

Materials and Methods

Materials

Yellow pea starch was a gift from COSUCRA (Warcoing, Belgium). Cowpea and chickpea starches were prepared in the laboratory as reported previously (Huang et al., 2007). Two types of acetylated yellow pea, cowpea and chickpea starches were prepared by AVEBE Food Innovation Centre (Veendam, The Netherlands). Acetylated starch was prepared by treating aqueous starch suspensions (38-40%) at pH 7.5-9.0 for acetic anhydride and pH 9-10 for vinyl acetate at 20-25 °C for about 1-2 h. Sodium carbonate is used as catalyst and buffer in reactions with vinyl acetate. For all the starches, per mole glucose 0.088 moles of reagent were added.

Separation of different size granule fractions

Native and acetylated starch samples were fractionated by sieving through test sieves (32 μm and 20 μm) on a Retsch AS200 digit shaker (Retsch GmbH & Co., Haan, Germany) with deionized water, and then air-dried at 40°C. Native and acetylated yellow pea starch was separated into three

fractions: larger than 32 μm, 20-32 μm, smaller than 20 μm; native and acetylated cowpea and chickpea starches were separated into two fractions: larger than 20 μm and smaller than 20 μm, respectively.

Analytical methods

Moisture content was determined according to Chen, Schols, & Voragen (2003a). Amylose content was tested using the enzymatic amylose/amylopectin assay kit (Megazyme, Ireland). Particle size distribution was measured both in dry state and in water with a laser diffraction system (H1140, Sympatec Inc., USA). The pasting behaviours of the starches were measured using a Rapid Visco Analyzer (RVA-4, Newport Scientific Pty. Ltd., Australia). Crystalline structure was analyzed using a Philips diffractometer (PW 1830, the Netherlands). Gelatinization temperature was measured using a Differential Scanning Calorimeter (Perkin Elmer DSC-7, USA). These analyses and the determination of swelling volume were carried out as described in our previous paper (Huang et al., 2007). The content of acetyl group was determined using the titration method according to Miladinov & Hanna (2001) except that the sample was saponified at room temperature for 2-3h. The degree of substitution (DS) was expressed as moles of substituent per mole of D-glucose residue.

Statistical analysis

SPSS10.0 for Windows was used for statistical analysis. Differences between samples of different fractions and different modification types were tested using the general linear model univariate test. A significance level of $p<0.05$ was used throughout the study.

Results and Discussion

Characterization of different size granule fractions of native starch samples

Particle size distribution

Starches from yellow pea, cowpea and chickpea as obtained and characterized before (Huang et al., 2007) were further fractionated according to their granule size by sieving. The particle size distributions of different size granule fractions separated from native yellow pea starch are

presented in Fig. 1. The granule size range of >32 μm fraction was broader than that of 20-32 μm and <20 μm fractions and an overlap between the fractions could be seen. According to the software used, in each fraction, 50%-60% of all granules represented the given size. Similar results were obtained for cowpea and chickpea starches with respect to overlap between large and small size granule fractions. The extent of variation in VMD (volume mean diameter) between granule size fractions was in the order yellow pea > chickpea > cowpea (Table 1). The >32 μm fraction of yellow pea starch when measured in water had a greater shift toward larger granule sizes when compared to the 20-32 μm and <20 μm fractions, indicating a greater swelling of the granules. In particle size distribution measurement, the reproducibility of the >32 μm fraction was relatively bad, may be due to the presence of fiber-like impurities as observed by microscopy. This fraction also showed some agglomeration (hard to obtain a homogeneous powder), a bit brownish colour and lower purity. Therefore, the >32 μm fraction is not included in the following discussion about behaviour of different size granule fractions. Nevertheless, its properties are presented for reference.

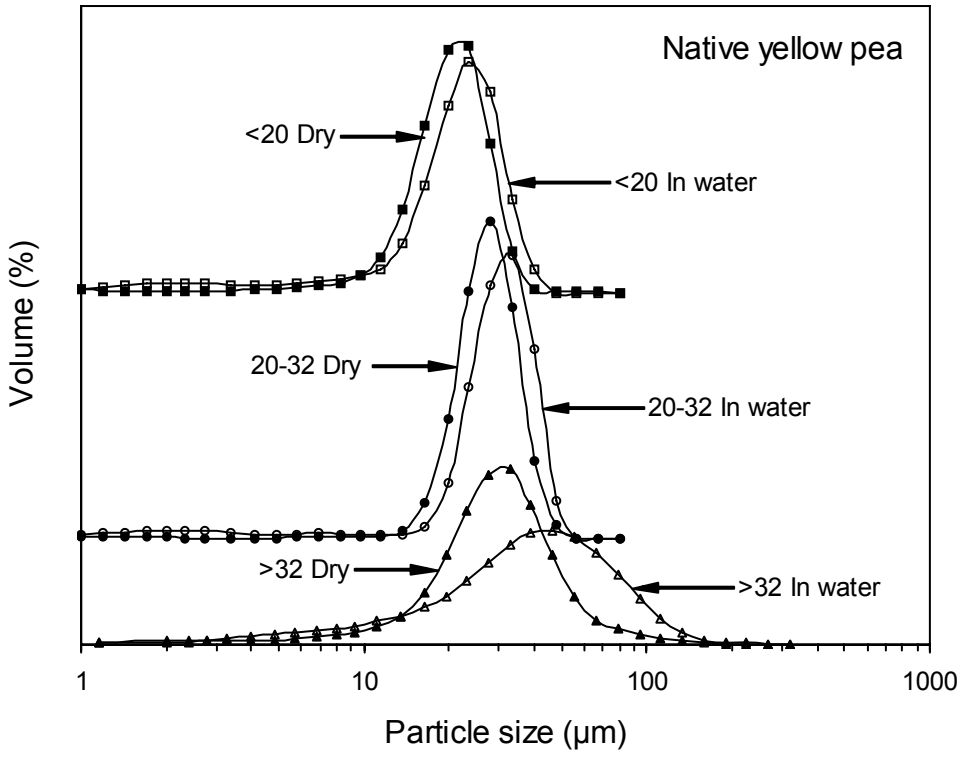

Fig. 1. Particle size distributions of different granule size fractions separated from native yellow pea starch. <20 Dry: <20μm granule fraction, measured in dry state. 20-32 In water: 20-32μm granule fraction, measured in water.

Amylose content and swelling volume

For yellow pea starch, <20 μm fraction showed similar amylose level as that of 20-32 μm fraction. For cowpea starch, a higher amylose content was found for <20 μm fraction as compared with >20 μm fraction, while the opposite result was obtained for chickpea starch. No clear relationship between the amylose content and granule size was also observed for potato starch. Chen et al. (2003b) found a lower amylose level in the smaller-size potato starch granule fraction, while Noda et al. (2005) reported that there was no difference in amylose content among the differently sized potato starch granule fractions. Thus it is hard to predict the amylose content of different size fractions from the result of un-fractionated starch.

Table 1 Characterization of different size granule fractions separated from native yellow pea, cowpea and chickpea starches

Starch	Yield (%)[a]	Moisture (%)	VMD[b] (μm)		Starch (%)[a]	Amylose (%)[a]	Swelling volume (ml/g)[a]
			Dry	In water			
Yellow pea <20μm	25	11.6	20.2	21.0	96.5a	28.4a	22.0a
Yellow pea 20-32μm	61	12.3	27.3	29.2	94.0b	27.9a	19.5b
Yellow pea >32μm	14	9.6	32.1	43.8	72.5c	25.8b	20.4ba
Cowpea <20μm	91	12.9	15.7	16.0	94.0	25.0	33.9
Cowpea >20μm	9	10.4	16.1	16.9	92.5	22.5	25.3
Chickpea <20μm	73	10.1	17.2	18.2	94.4	25.6	28.6
Chickpea >20μm	27	8.8	20.4	21.2	93.0	30.2	24.7

[a] Values are based on dry matter and means of triplicate.

[b] Volume mean diameter. Values are means of duplicate.

Values with different letters in the same column of the same variety are significant different at $p<0.05$.

The extent of swelling, which provides evidence of non-covalent bonding between starch molecules (Moorthy, 2004), was determined at 90°C and expressed as swelling volume. For yellow pea, chickpea and cowpea starches, higher swelling volume values were observed for smaller size granule fractions as also reported for waxy and normal barley, potato, and sweet potato starches

(Singh & Kaur, 2004; Tang et al., 2004; Tang et al., 2002; Chen et al., 2003b). Other factors like chain length and molecular weight distribution, degree/length of branching and conformation of amylopectin as well as the amylose content also contribute to the swelling behaviour of starches (Moorthy, 2004).

Pasting behavior

Besides some cases in which starch is used in its natural granular shape—starches used for molding in confectionery, powdering of surgical gloves, dilution of insecticides or herbicides—most of the time starch must be subjected to hydrothermal dispersion (Fleche, 1985). For the application of starch in a process, the pasting behaviour during different temperatures is of interest. It is apparent that there was little difference in pasting temperature among the granule size fractions separated from yellow pea starch (Fig. 2). Similar results were observed for chickpea and cowpea starches (results not shown). This is in agreement with the findings for sweet potato and potato starches (Chen et al., 2003b; Noda et al., 2005).

The smaller the granule size, the higher the peak viscosity was found for yellow pea and chickpea starches. For cowpea starch, the lack of substantial difference in peak viscosity between <20 μm and >20 μm fractions may be explained by the small differences in their VMD. This is an indirect evidence for the statement that the peak viscosity varies according to the granule size. However, in literature, findings on potato starch were not in agreement with each other. Chen et al. (2003b) reported similar peak viscosity of the different size granule fractions. In contrast, Noda et al. (2005) found decreased peak viscosity as the granule size decreasing.

Characterization of starch samples modified with acetic anhydride and vinyl acetate, respectively

Degree of molar substitution and swelling volume

The reaction level, determined by the analysis of the introduced chemical group, is usually indicated by the term “degree of substitution ” (DS). The same moles of acetic anhydride and vinyl acetate were added in the derivatisation of yellow pea, cowpea and chickpea starches. However, a higher DS was obtained from modification with vinyl acetate than with acetic anhydride for all three starches (Table 2), indicating a higher reaction efficiency for the reaction with vinyl acetate. This may be due to the fact that acetic anhydride is a highly reactive reagent, and its side reaction with water may result in a lesser reaction efficiency.

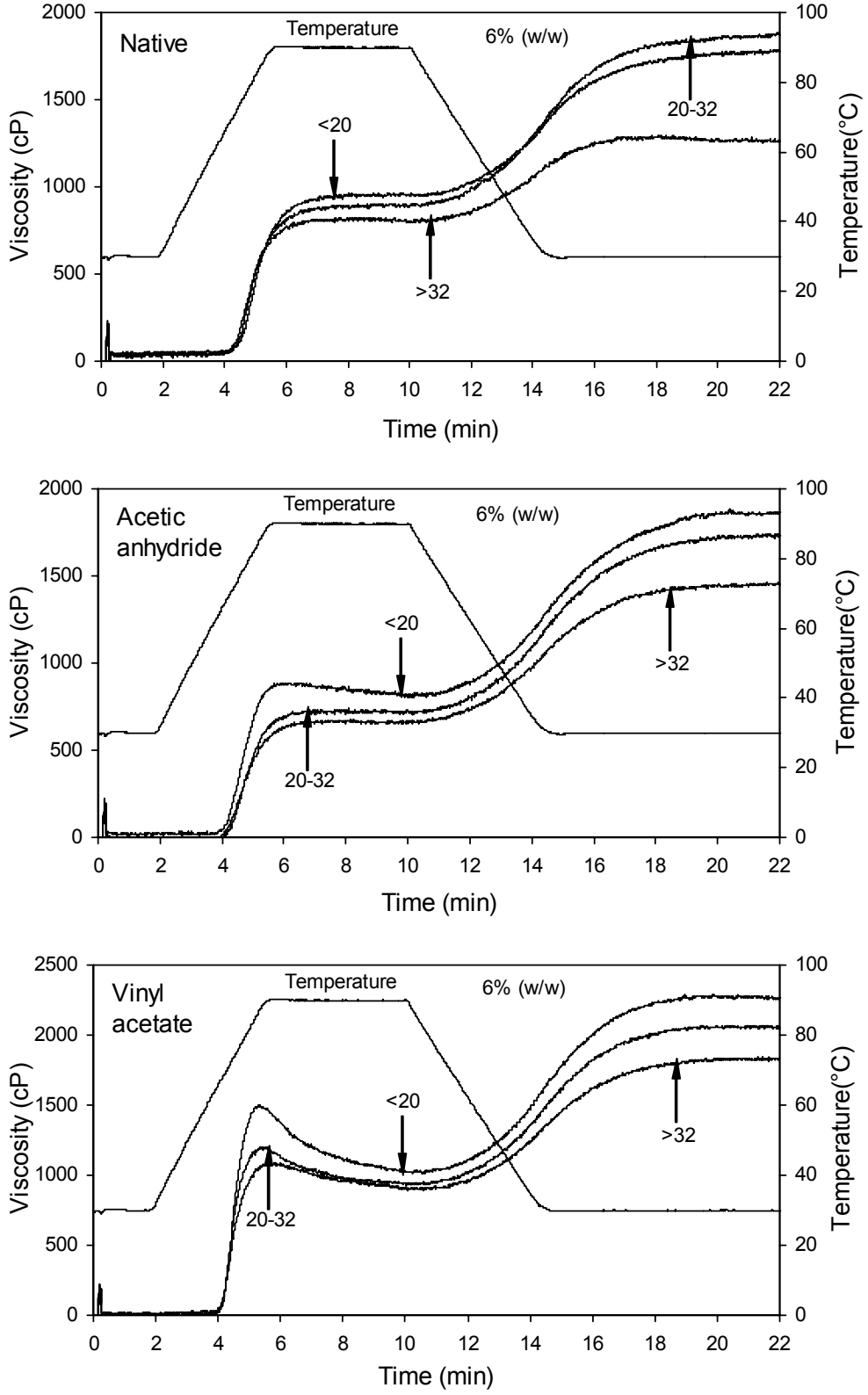

Fig. 2. RVA pasting curves of different granule size fractions separated from native and acetylated yellow pea starch modified with acetic anhydride and vinyl acetate, respectively. <20: <20μm granule fraction. 20-32: 20-32μm granule fraction. RVA: rapid visco analyzer.

Table 2 Properties of native and acetylated yellow pea, cowpea and chickpea starches modified with acetic anhydride and vinyl acetate, respectively

Starch	Moisture (%)	VMD[bg] (μm)		DS[cde]	Amylose (%)[de]	Swelling volume (ml/g)[de]	T_o [fg] (°C)	Enthalpy (J/g)[dg]
		Dry	In water					
Yellow pea								
Native [a]	11.3	33.8	34.0	-	31.2a	19.8b	58.2	16.1
Acetic anhydride	12.8	26.9	30.8	0.066	30.3a	21.5b	54.2	13.7
Vinyl acetate	12.7	25.4	31.3	0.071	29.3a	29.4a	55.3	14.2
Cowpea								
Native [a]	11.5	15.5	16.5	-	25.8a	31.4b	70.5	15.2
Acetic anhydride	12.7	15.6	16.4	0.059	25.2a	26.0c	65.5	15.6
Vinyl acetate	12.4	15.8	16.4	0.064	26.1a	40.9a	64.8	20.0
Chickpea								
Native [a]	11.9	17.9	19.8	-	27.2b	26.8b	57.9	17.6
Acetic anhydride	12.9	18.0	19.5	0.057	33.3a	25.3b	55.2	15.8
Vinyl acetate	12.7	18.7	19.6	0.068	33.6a	40.0a	54.7	17.2

[a] From Huang et al., 2007.

[b] Volume mean diameter.

[c] Degree of molar substitution.

[d] Values are based on dry matter.

[e] Values are means of triplicate.

[f] Onset gelatinization temperature.

[g] Values are means of duplicate.

Values with different letters in the same column of the same variety are significant different at $p<0.05$.

The particle size distributions of acetylated cowpea and chickpea starches showed similar results as their corresponding native starches. The acetylated yellow pea starch showed a unimodal distribution of particle size, and a lower VMD compared to the native form. This might be due to the washing step during preparation of the acetylated starch, which can remove some fiber-like

impurity that is responsible for the shoulder peak at high granule diameters exhibited in native yellow pea starch (Huang et al., 2007).

For yellow pea and cowpea starches, there were no significant differences in amylose content between the native and acetylated samples. After acetylation a higher amylose level was exhibited for chickpea starch. Reduced amylose levels for cocoyam, jack bean, maize, and rice starches (Lawal, 2004a, 2004b; Lawal & Adebowale, 2005; González & Pérez, 2002), and increased values for jack bean starch (Betancur, Chel, & Canizares, 1997) after modification with acetic anhydride have been reported.

For yellow pea, cowpea and chickpea starches, the swelling volume values all increased after acetylation with vinyl acetate. After modification with acetic anhydride, the values were similar for yellow pea and chickpea, and lower for cowpea starch as compared with those of the native starches. It is obvious that modification with vinyl acetate resulted in higher swelling volumes than modification with acetic anhydride. This could be explained by the fact that vinyl acetate is a slowly reacting agent, and can diffuse deep into the granule matrix, which makes acetyl groups present on the surface of crystals in both interior and exterior lamella of the granules. However, the reaction with acetic anhydride occurs in the outer lamella of crystalline regions (Chen et al., 2004).

Thermal properties and crystal structure

Differential scanning calorimetry (DSC) was used to study the thermal characteristics of un-fractionated native and acetylated starch samples. The onset temperatures and the enthalpy of thermal transition are presented in Table 2. Yellow pea, cowpea and chickpea starches all showed lower gelatinization temperatures after acetylation as also observed for maize, cocoyam, jack bean, potato and rice starches modified with acetic anhydride or vinyl acetate (Liu, Ramsden, & Corke, 1997, 1999; Lawal, 2004a, 2004b; Wang & Wang, 2002; Lawal et al., 2005; Singh, Kaur, & Singh, 2004). The introduction of acetyl groups interrupts the ordered structure of native starch, leading to decreased gelatinization temperature (Wang et al., 2002). There was little difference in gelatinization temperature between the two types of acetylation within each starch.

After modification with acetic anhydride, the enthalpy was reduced for yellow pea and chickpea starches, and remained similar for cowpea starch. In the case of vinyl acetate, the value decreased for yellow pea, remained similar for chickpea, and increased for cowpea starch. Similarity in enthalpy was found between two types of acetylation for yellow pea starch, while acetylation with vinyl acetate resulted in higher enthalpy than modification with acetic anhydride for cowpea and chickpea starches. Decreased enthalpy after modification with acetic anhydride or vinyl acetate was

reported for maize, potato, jack bean, and cocoyam starches (Liu et al., 1997; Lawal, 2004a, 2004b; Singh et al., 2004b; Lawal et al., 2005). Since enthalpy is the latent heat absorbed by melting of crystallites in the granules, it depends on a number of factors like crystallinity, intermolecular bonding, rate of heating of the starch suspension, presence of other chemicals, etc. (Moorthy, 2004). Variations in enthalpy of acetylated starches were indications of structural divergence: molecular structure (amylose and amylopectin fine structures, distribution pattern of acetyl groups), and composition (amylose-to-amylopectin ratio, DS).

X-ray diffraction patterns and the relative crystallinity did not show substantial changes after reaction with acetic anhydride and vinyl acetate for yellow pea (Fig. 3), cowpea and chickpea starches. This information confirms the findings of others (Wang et al., 2002; Chen et al., 2004; Lawal, 2004a) for waxy maize, sweet potato, potato and cocoyam starches that at low level of acetylation, hydroxyl groups react in the amorphous region and on the surface of crystals (Seib, 1997), no substantial change occurred in crystal structures of granular starch.

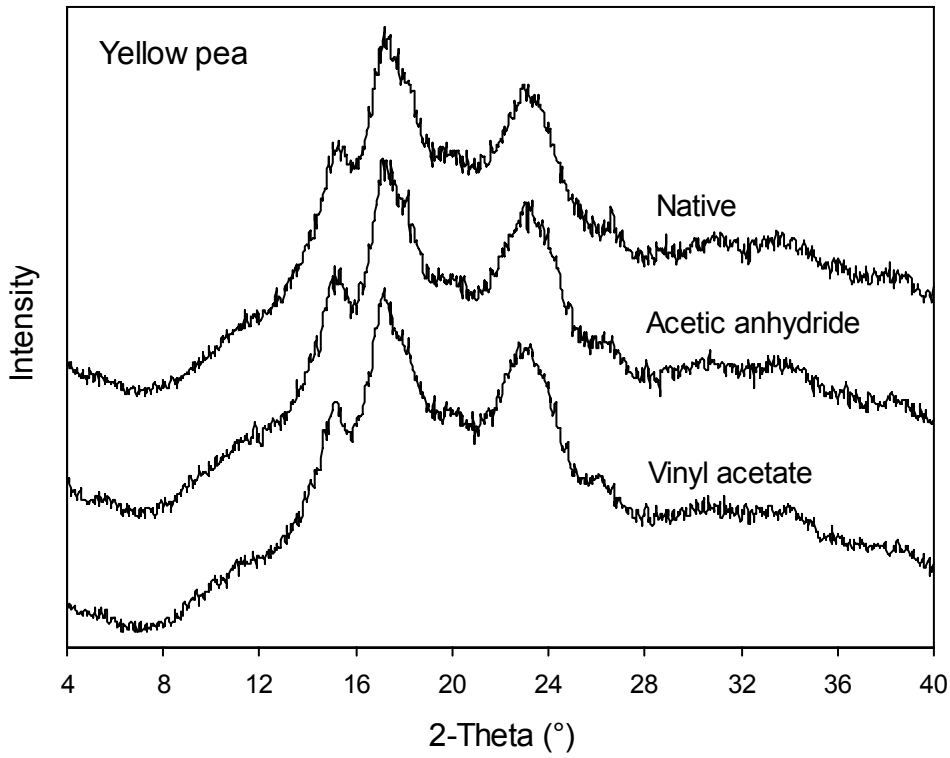

Fig. 3. X-ray diffractogram of native and acetylated yellow pea starch modified with acetic anhydride and vinyl acetate, respectively.

Pasting behavior

The pasting viscosity during heating starch in excess water was recorded using a Rapid Visco Analyzer (RVA). Yellow pea, cowpea and chickpea starches all showed lower pasting temperatures after acetylation, consistent with the gelatinization temperature results determined by DSC. The starch-starch interactions in the granules are weakened by the introduction of acetyl groups (Taggart, 2004). For yellow pea and cowpea starches, pasting temperatures were similar for both types of acetylation. However, for chickpea starch, modification with acetic anhydride resulted in a higher pasting temperature than vinyl acetate treatment. The reason is not clear.

As described previously (Huang et al., 2007), native yellow pea and chickpea starches showed type C pasting profiles, and cowpea starch showed a type B viscosity according to the classification of Schoch & Maywald (1968). The pasting profile of yellow pea and chickpea starches remained type C after acetylation with acetic anhydride, but changed to type B after modification with vinyl acetate (Fig. 4). For cowpea starch, both acetylated samples exhibited the same pasting profiles as the native starch. The peak viscosity was increased after modification with vinyl acetate for all three starches. With regard to acetylation with acetic anhydride, peak viscosity remained similar for yellow pea, decreased for cowpea and chickpea starches as compared with those of their native counterparts. Increases in peak viscosity were observed for waxy maize, dent maize, jack bean, rice, smooth pea and sweet potato starches after acetylation with acetic anhydride (Wilkins, Wang, Xu, Niu, Tumbleson, & Rausch, 2003a, 2003b; Liu et al., 1997; Betancur et al., 1997; González et al., 2002; Biliaderis, 1982; Chen, Schols, & Voragen, 2003c), and for waxy rice starch after acetylation with vinyl acetate (Liu et al., 1999). Contradicting results were mentioned for maize, canna, cocoyam and potato starches which showed a lower peak viscosity after acetylation with acetic anhydride (Lawal, 2004a, 2004b; Saartrat, Puttanlek, Rungsardthong, & Uttapap, 2005; Chen et al., 2003c).

A noticeable relationship between swelling volume and peak viscosity was observed. Acetylation with vinyl acetate resulted in both a larger degree of swelling and a higher peak viscosity than modification with acetic anhydride. The setback (= final viscosity – peak viscosity) values of yellow pea starch following modification with acetic anhydride and vinyl acetate were 960cP and 889cP, respectively, both higher than that of native starch (643cP, Huang et al., 2007). For cowpea starch, the setback values were lower after both types of acetylation, while reaction with acetic anhydride and vinyl acetate induced only slight change in setback value for chickpea starch. Following modification with acetic anhydride, different starch samples were found to exhibit variability in setback value. Reductions were observed for rice, cocoyam, maize, and canna starches

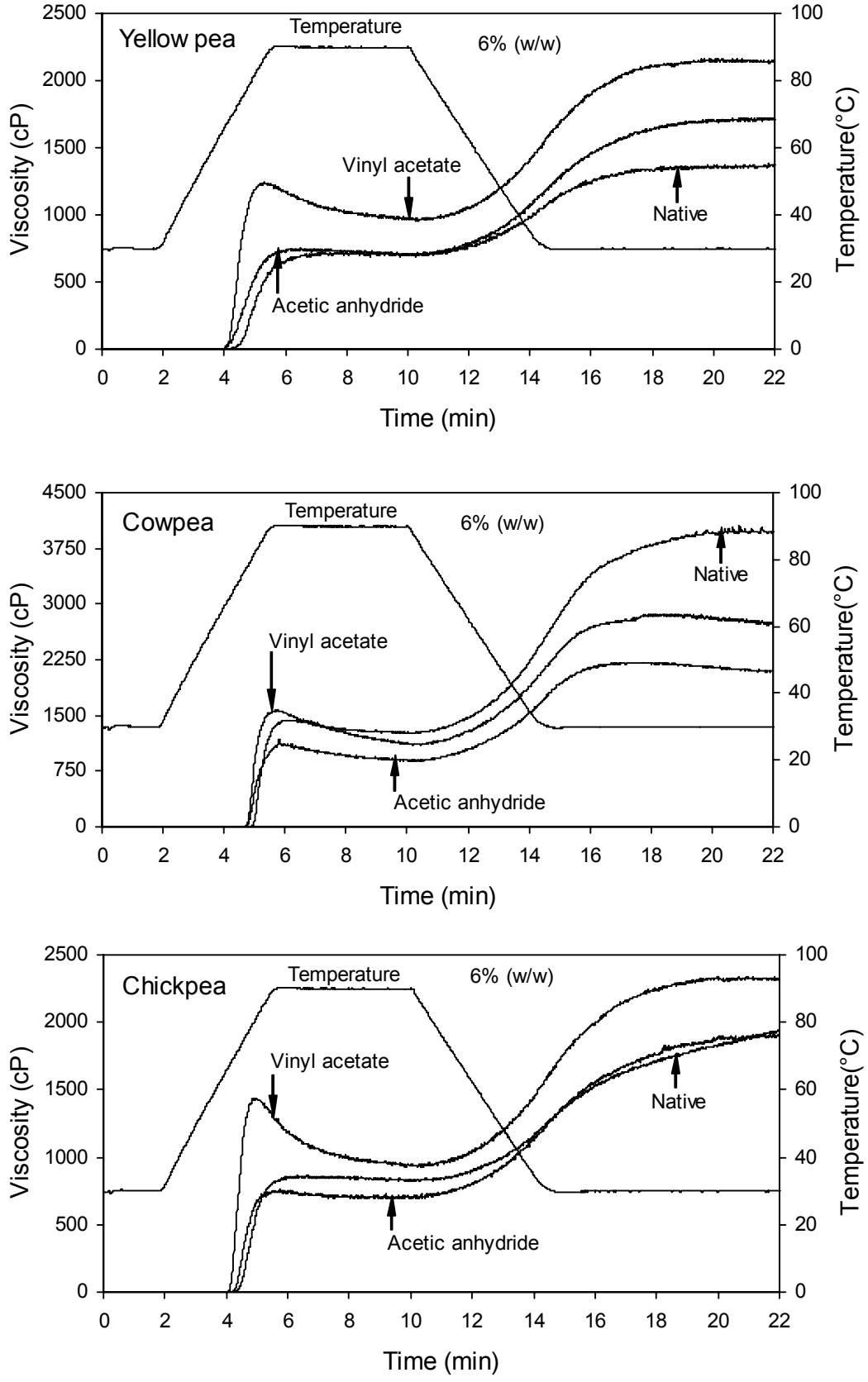

Fig. 4. RVA pasting curves of native and acetylated yellow pea, cowpea and chickpea starches modified with acetic anhydride and vinyl acetate, respectively. RVA: rapid visco analyzer.

(González et al., 2002; Lawal, 2004a, 2004b; Saartrat et al., 2005); and increases for waxy and high-amylose maize, and dent maize starches (Liu et al., 1997; Wilkins et al., 2003b); similarities for normal maize and waxy maize starches (Liu et al., 1997; Wilkins et al., 2003a). Our findings and those reported by others suggest that the change in viscosity after acetylation strongly depends on the starch source and the nature of acetylating reagent.

Characterization of different size granule fractions of acetylated starch samples

Degree of molar substitution

Yellow pea, cowpea and chickpea starches were fractionated by size *after* reaction with acetic anhydride and vinyl acetate. The VMD of granule size fractions separated from acetylated starches (Table 3) were identical with those of their native counterparts.

When modified with acetic anhydride, smaller size (<20 μm) granule fractions of yellow pea, cowpea and chickpea starches had a higher DS as compared with larger size granule fractions (yellow pea 20-32 μm; cowpea and chickpea >20 μm). This could be explained by the fact that smaller size granule fractions have larger specific surface area (Chen et al., 2004). For acetylated starches prepared with vinyl acetate, smaller size (<20 μm) granule fractions of yellow pea, cowpea and chickpea starches were acetylated at the same level as larger size granule fractions (yellow pea 20-32 μm; cowpea and chickpea >20 μm). This phenomenon and the DS results of un-fractionated starch samples revealed that differences between reagent types lead to variations in the levels of acetylation. Granule size had a greater effect on DS values when modified with the rapidly reacting agent acetic anhydride than with the slowly reacting reagent vinyl acetate. Stapley & BeMiller (2003) pointed out that highly reactive reagents like phosphoryl chloride react to a large extent at granule surfaces compared to slowly reacting reagents like propylene oxide. When using the slowly reacting reagents, having time to migrate deeper into the granule matrix, the specific surface areas have less effect on the modification. The >32 μm fraction of yellow pea starch was an exception, showing the highest level of derivation in both acetylation types. The most likely explanation was the difference in granule swelling. This fraction showed the largest degree of granule swelling among all native and acetylated starch samples as revealed by particle size distribution measurements. The extent of granule reaction is a function of the magnitude of granule swelling (Huber & BeMiller, 2001).

Table 3 Characterization of different size granule fractions separated from yellow pea, cowpea and chickpea starches modified with acetic anhydride and vinyl acetate, respectively

Starch	Yield (%)[c]	Moisture (%)	VMD[d] (μm)		DS[ec]	Amylose (%)[c]	Swelling volume (ml/g)[c]
			Dry	In water			
Yellow pea							
AAY <20 μm [a]	23	10.7	20.2	22.2	0.074b	30.2b	25.1c
AAY 20-32 μm	63	11.9	26.5	28.7	0.058d	28.9b	19.7e
AAY >32 μm	14	11.2	30.3	40.6	0.081a	31.9a	19.8e
VAY <20 μm	24	11.5	20.0	20.8	0.068cb	29.3b	30.6a
VAY 20-32 μm [b]	60	8.5	26.5	29.6	0.067c	29.4b	27.7b
VAY >32 μm	16	12.8	29.0	46.7	0.084a	30.2b	21.1d
Cowpea							
AAC <20 μm	89	8.7	15.3	16.3	0.058b	25.2ba	28.7c
AAC >20 μm	11	9.8	15.9	17.2	0.053c	24.0ba	23.7d
VAC <20 μm	93	12.8	15.8	16.1	0.062ab	27.0a	42.2a
VAC >20 μm	7	9.8	17.5	18.1	0.063a	23.6b	36.1b
Chickpea							
AACH <20 μm	68	10.5	17.4	18.3	0.057b	32.5a	25.3c
AACH >20 μm	32	10.2	19.8	21.3	0.053c	29.4b	22.7d
VACH <20 μm	78	8.5	17.7	18.9	0.066a	31.6a	40.6a
VACH >20 μm	22	9.1	20.7	22.8	0.067a	29.5b	36.3b

[a] Yellow pea starch modified with acetic anhydride (< 20μm granule fraction).

[b] Yellow pea starch modified with vinyl acetate (20-32μm granule fraction).

[c] Values are based on dry matter and means of triplicate.

[d] Volume mean diameter. Values are means of duplicate.

[e] Degree of molar substitution.

Values with different letters in the same column of the same variety are significant different at $p<0.05$.

Pasting behavior

Different size granule fractions separated from acetylated yellow pea starch showed similar pasting temperatures (Fig. 2). The same results were obtained with cowpea and chickpea starches

(viscoamylograph not given). The smaller the granule size, the higher peak viscosity values were found for yellow pea and chickpea starches of both native and acetylated samples, while cowpea starch exhibited little difference in peak viscosity between large and small size granule fractions. All granule size fraction samples modified with vinyl acetate exhibited higher swelling volume values and peak viscosities than did their counterparts produced with acetic anhydride. This is in accordance with the findings for un-fractionated starch samples. The <20 μm fraction separated from yellow pea starch acetylated with vinyl acetate showed lower DS but higher peak viscosity and swelling volume than the corresponding fraction obtained from reaction with acetic anhydride. These observations confirm that acetylation with vinyl acetate results in higher peak viscosity and swelling volume than obtained with acetic anhydride and suggests that the difference in starch properties might be due to the different distribution pattern of acetyl groups rather than the DS.

Conclusions

The fact that acetylation did not affect the X-ray diffraction patterns is an indication that neither acetic anhydride nor vinyl acetate reacted in the crystalline region of the granules when the starches were weakly substituted (DS < 0.1). The smaller the granule size fraction, the higher the DS obtained when modified with acetic anhydride, which is due to the larger specific surface area of the smaller size granule fractions. While for reaction with vinyl acetate, different size granule fractions showed the same level of acetylation. Thus granule size had a greater effect on reactivity when modified with acetic anhydride (a rapidly reacting reagent) than with vinyl acetate (a slowly reacting reagent).

For yellow pea, cowpea and chickpea starches and their granule size fractions, modification with vinyl acetate resulted in a larger degree of granule swelling and higher peak viscosity than acetic anhydride. However, the differences in DS values between the two types of acetylation were minor. These results suggest that the distribution pattern of acetyl groups rather than the DS determines the variation in physical properties of acetylated starch.

Acetylated cowpea starch showed higher gelatinization temperature and peak viscosity than yellow pea and chickpea starches, which was in accordance with the behaviour found for the native starches in our previous work. Therefore, besides the origin of starch, factors that influence the properties of acetylated granular starches are reagent type and granule size. Further investigation is needed to understand differences between two types of acetylated starches at a molecular level by assessment of the substituent distribution on starch components (amylose and amylopectin).

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Chapter 4

Acetyl Substitution Patterns of Amylose and Amylopectin Populations in Cowpea Starch Modified with Acetic Anhydride and Vinyl Acetate

Abstract

To study the effect of reagent type on the distribution pattern of acetyl groups in acetylated cowpea starch, amylose and amylopectin populations were isolated from the starch granules after modification to a low degree of substitution (DS < 0.1) with acetic anhydride and vinyl acetate, respectively. Slowly reacting reagent vinyl acetate resulted in higher DS values for the amylopectin populations when compared to the rapidly reacting reagent acetic anhydride. The two reagents had similar effects on the acetylation level of amylose, suggesting that the amorphous regions of granules were easily accessible for both reagents. The acetyl substitution patterns were analyzed by enzymatic degradation followed by characterization of the obtained fragments using chromatographic and mass spectrometric techniques. The distributions of acetyl groups along the amylose and amylopectin chains were more clustered for modification with vinyl acetate as compared with modification with acetic anhydride. Between the two acetylation types, pronounced differences in the acetyl substitution patterns were observed for the large fragments obtained after α-amylase digestion; only slight differences were exhibited for the small fragments obtained by exhaustive enzymatic digestion of amylose and amylopectin populations.

Key words: Cowpea starch; Amylose; Amylopectin; Acetic anhydride; Vinyl acetate

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Introduction

It has been almost a hundred years since commercial production of starch for food and industrial applications was initiated. Two major polymeric components—amylose and amylopectin, play important roles in the structure, characteristics and properties of the different starch sources (Luallen, 2004). Amylose molecules are essentially linear and are comprised predominately of α-1,4 linked D-glucose units with a limited number of α-1,6 branching points (Seib, 1997; Bertoft, 2004). Amylose was considered to exist mainly in the amorphous region of starch granules (Luallen, 2004). Differing significantly from amylose, and having an average molecular weight about 100 to 1000 times that of amylose (Seib, 1997; Vermeylen, Goderis, Reynaers, & Delcour, 2004), amylopectin molecules are highly branched and are constructed of a large number of short α-1,4 linked D-glucose chains, arranged in clusters and linked by α-1,6 bonds to longer chains which transverse two or more clusters (Hizukuri, 1996; Seib, 1997; Thompson, 2000; Bertoft, 2004; Vermeylen et al., 2004). The linear arrays of double helices, formed by two neighboring chains in a cluster, alternate with clusters of branch points in the radial direction of the granule (Seib, 1997). These alternating zones of differing densities of amylopectin (Seib, 1997) make the granules both firm and flexible, which might be essential for being an energy reserve in plants.

Starch modifications are a means of altering the structure and affecting the hydrogen bonding of amylose and amylopectin in a controllable manner to enhance and extend starch application. When low levels of alterations take place in the molecules, only slight or no change can be observed in the superficial appearance of the granule (Taggart, 2004). Following cross-linking, esterification and etherification are the second most important modifications in the starch industry (Taggart, 2004). Acetylated starches are the most typical starch ester in the market (Fleche, 1985) and they are used in many food products, such as bakery, frozen, canned foods and white salted noodles (Chen, Schols, & Voragen, 2004), to improve texture, stability and appearance. Starch acetates are also used as adhesives, and acid pH-resistant binders in the food industry, and as sizing agent in paper manufacture or textiles (Fleche, 1985).

The reagents used for preparation of starch acetate are normally acetic anhydride or vinyl acetate (Seib, 1997). Our previous research (Huang, Schols, Jin, Sulmann, & Voragen, 2006) on the effect of reagent type on the properties of acetylated granular starches showed that the degree of substitution (DS) differed for the differently sized starch granule fractions when the starch had been acetylated by the rapidly reacting acetic anhydride. With the slowly reacting vinyl acetate, no

difference in DS of the differently sized granule fractions was observed. Modification with vinyl acetate resulted in higher peak viscosity and swelling volume compared to acetic anhydride. However, the differences in DS values between the two types of acetylation were minor. Thus the substitution pattern was believed to be more important on the properties of acetylated starch.
In this study, amylose and amylopectin populations were isolated from two types of acetylated cowpea starch samples, and enzymatic digestion in combination with chromatographic and mass spectrometric techniques were used to study the substitution pattern at molecular level to understand the effect of reagent type (acetic anhydride vs. vinyl acetate).

Materials and Methods

Materials

Cowpea starch was prepared in the laboratory as reported previously (Huang, Schols, van Soest, Jin, Sulmann, & Voragen, 2007). Two types of acetylated cowpea starch samples were prepared by AVEBE Food Innovation Centre (Veendam, The Netherlands). Acetylated starch was prepared in granular form by reaction of starch in aqueous suspension with acetic anhydride and vinyl acetate, respectively. Both reagents were added in 0.088 moles per mole glucose residue of starch. The acetylated starches were fractionated into two granule size fractions: one larger than 20µm and one smaller than 20µm (Huang et al., 2006). Since there were only slight differences in the volume mean diameters and pasting behaviours between two fractions and the amount of the large sized fractions are not sufficient for further investigations, only the smaller than 20µm fractions with DS of 0.058 and 0.062 for modification with acetic anhydride and vinyl acetate (Huang et al., 2006) were used for this study.
α-Amylase (EC 3.2.1.1) (product number 10069, from *Bacillus subtilis*, 393 U/mg) and ß-amylase (EC 3.2.1.2) (product number 10100, from *barley*, 22 U/mg) were purchased from Fluka (Switzerland). Pullulanase (EC 3.2.1.41) (M2, from *Bacillus licheniformis*, 400 U/ml) was obtained from Megazyme (Ireland). Amyloglucidase (EC 3.2.1.3) (A9268, from *Aspergillus oryzae*, 1400 U/ml) was purchased from Sigma (USA).
α-Amylase was dissolved in millipore water, ß-amylase was dissolved in sodium acetate buffer (0.01mol/L, pH4.8), pullulanase was diluted in sodium acetate buffer (0.01mol/L, pH5.0) and amyloglucosidase was diluted in sodium acetate buffer (0.01mol/L, pH 4.5), to make solutions containing 0.38 U/µl, 0.22 U/µl, 0.22 U/µl and 0.14 U/µl, respectively.

Isolation of amylose and amylopectin

Amylose and amylopectin populations were isolated from acetylated cowpea starch using the aqueous leaching method according to Chen et al. (2004). The purity of isolated amylose and amylopectin was checked with high-performance size-exclusion chromatography (HPSEC) after pullulanase digestion according to Kobayashi, Schwartz, & Lineback (1985).

Determination of degree of substitution

Four milligrams of samples were saponified in 150 µl of 0.1 mol/L NaOH for 2h at room temperature and neutralized with 150 µl of 0.1 mol/L citric acid. The amount of released acetate was determined using the EnzyPlus Acetic Acid test kit (Diffchamb, Sweden). The DS is calculated as molar substitution (mole acetate/mole glucose).

Enzymatic digestion

Five milligrams of acetylated amylose or amylopectin samples were saponified with 150 µL 0.02mol/L NaOH for 2h at room temperature and neutralized with 150 µL 0.02mol/L acetic acid. Amylose samples were submitted to α-amylase, ß-amylase, or combined α-amylase and amyloglucidase digestion according to Chen et al. (2004). Amylopectin samples were submitted to α-amylase, ß-amylase, pullulanase, or combined pullulanase, α-amylase and amyloglucidase digestion according to Chen, Huang, Suurs, Schols, & Voragen (2005). The ß-limit value, defined as the relative amount of maltose formed during ß-amylase digestion, was calculated from HPAEC peak and the sample concentration, using pure maltose as external standard.

HPSEC, HPAEC and MALDI-TOF-MS

HPSEC (high-performance size-exclusion chromatography) was performed on a ThermoFinnigan (USA) HPLC, with three TSK gel columns (7.8 mm ID × 30 cm per column) in series (G4000PW_{XL}, G3000 PW_{XL}, G2500PW_{XL}; Tosohaas, Japan), in combination with a PW_{XL}-guard column (Tosohaas, Japan). Elution was at 30°C using 0.2 mol/L sodium nitrate at a flow rate of 0.8 mL/min. The elution was monitored using a Shodex SE-61 Refractive Index detector. Calibration was

performed using pullulans (Polymer laboratories, UK). The data were processed using ChromQuest (ThermoFinnigan, USA).

HPAEC (high-performance anion-exchange chromatography) was performed on a Dionex (USA) HPLC system. The system was equipped with a quaternary gradient pump, an autosampler completed with a helium degassing unit and an EC detector in the PAD mode. A CarboPac PA1 column (2 × 250 mm) (Dionex, USA) with a CarboPac PA1 guard column (2× 50 mm) (Dionex, USA) was operated at a flow rate of 0.3 mL/min at 20 °C. The gradient was obtained by mixing solutions of 0.1 mol/L NaOH and 1 mol/L NaOAc in 0.1 mol/L NaOH. After 15 min equilibration with 0.1 mol/L NaOH, 20μL of the sample was injected and a linear gradient to 0.50 mol/L NaOAc in 0.1 mol/L NaOH within 30 min was followed by a linear gradient in 5 min to 1 mol/L NaOAc in 0.1 mol/L NaOH. Finally, the column was washed for 5 min with 1 mol/L NaOAc in 0.1 mol/L NaOH. The data were processed using Chromeleon (Dionex, USA) software.

MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry) was carried out using an Ultraflex workstation (Bruker Daltonics GmbH, Germany) equipped with a nitrogen laser of 337 nm. The mass spectrometer was selected for positive ions. After a delayed extraction time of 100ns, the ions were accelerated to a kinetic energy of 20 kV. Hereafter, the ions were detected in the reflector mode. The lowest laser power required to obtain good spectra was used. The mixture of 1μL sample and 1μL of matrix was dried on a sample plate. The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzoic acid in a 1mL mixture of acetonitrile:water (300μL:700μL). External calibration was performed using a mixture of maltodextrins (Mw range 400-3500Da).

Results and Discussion

Degree of molar substitution of amylose and amylopectin populations isolated from cowpea starch modified with acetic anhydride and vinyl acetate, respectively

It is of primary importance to determine the amount of substituents because this affects the functional properties of the starch considerably. The degree of substitution (DS) is the average number of hydroxyl groups on the D-glucosyl units that have been substituted (Bertoft, 2004). Amylose and amylopectin fractions were isolated from two types of acetylated cowpea starch samples using acetic anhydride and vinyl acetate as reagents resulting in DS values of 0.058 and

0.062, respectively (Huang et al., 2006). The purities of these fractions were checked enzymatically. After treatment with pullulanase, the elution profiles of amylose populations were similar as the un-treated counterparts. All amylopectin populations were degraded to smaller fragments by pullulanase digestion and no other peaks were detected in the HPSEC elution profiles. Therefore, the amylose and amylopectin populations isolated from two types of acetylated cowpea starch were considered to be pure and used for further studies.

For both acetylation types, the DS values of the amylose populations were much higher than those of the amylopectin populations (Table 1), indicating that the amorphous phase was more accessible for chemical reaction than the crystalline phase. This is in agreement with previous results obtained for potato and sweet potato starches modified with acetic anhydride (Chen et al., 2004). Similar findings have been reported for starch ether. The molar substitution (MS) of the amorphous domains was higher than that of the crystalline parts (van der Burgt, Bergsma, Bleeker, Mijland, Kamerling, & Vliegenthart, 2000a; van der Burgt et al., 1999), and a higher substitution of the amylose fraction was also observed in methylated granular starch (Steeneken & Woortman, 1994; van der Burgt et al., 2000b).

Table 1 Degree of molar substitution and β-limit values of amylose and amylopectin populations isolated from cowpea starch modified with acetic anhydride and vinyl acetate, respectively

Sample	Degree of molar substitution		β-limit value (%)	
	Amylose	Amylopectin	Amylose	Amylopectin
Acetic anhydride	0.099	0.029	40	55
Vinyl acetate	0.092	0.039	37	47
Saponified	-	-	86	63

Modification with vinyl acetate resulted in higher DS than with acetic anhydride when both reagents were added in the same mole amount per glucose residue of starch (Huang et al., 2006). Different acetylation levels were also exhibited in the amylose and amylopectin populations. The DS of amylose isolated from cowpea starch modified with acetic anhydride was higher than that of amylose isolated from starch acetylated with vinyl acetate. The opposite result was obtained for the corresponding amylopectin populations. The difference in DS between the two types of acetylated amylopectin was found to be more pronounced than that between the two types of acetylated

amylose: it is the higher acetylation level in amylopectin that explains the higher DS of the parental starch modified with vinyl acetate. Since vinyl acetate reacts more slowly it can penetrate further in the starch granule and react with more hydroxyl groups of the glucosyl residues in amylopectin molecules than the rapidly reacting acetic anhydride. Higher acetylation levels in amylopectin population may contribute to the higher swelling volume and peak viscosity of the parent starch acetate modified with vinyl acetate as compared with modification with acetic anhydride (Huang et al., 2006).

Smaller effects of reagent types on the acetylation levels were found for the amylose populations. Glucose hydroxyl groups in the amorphous domains of granules had similar relative reactivity as hydroxyl groups in dissolved starch solution as has been reported for highly methylated potato starch with DS up to 0.8 (Steeneken & Woortman, 1994).

Both types of acetylation resulted in a higher substitution level in amylose than in amylopectin. However, the distribution of the amount of acetyl groups over amylose and amylopectin was not the same: for cowpea starch composed of 25.8% of amylose (Huang et al., 2007), around 55% of the total acetyl groups was present in the amylose population when modified with acetic anhydride; while about 45% of all acetyl groups was present in the amylose population when acetylated with vinyl acetate.

Distribution of acetyl groups over amylose populations isolated from cowpea starch modified with acetic anhydride and vinyl acetate, respectively

α-Amylase digestion

In addition to the DS, the substitution pattern on the D-glucosyl residues within the starch components (amylose and amylopetin) is of interest. The introduced acetyl groups act as barriers to amylase attack (Chen et al., 2004), and pure and well characterized enzymes can be used to investigate the distribution pattern of acetyl groups. α-Amylase is an *endo*-hydrolase which cleaves α-1,4-glucosidic linkages in a random fashion (Mischnick, 2001). From the HPSEC analyses of the degradation products obtained after α-amylase hydrolysis (Fig. 1A), it is obvious that the degradability of the amylose sample isolated from cowpea starch modified with acetic anhydride (AA AM) was lower than that of the vinyl acetate counterpart (VA AM). This can be partly explained by the fact that slightly more acetyl groups were present in AA AM than in VA AM.

The structural characterization of the fragments was carried out using MALDI-TOF-MS. Only fractions smaller than DP 17 were observed by MALDI-TOF-MS oligomer-analysis. In figure 2

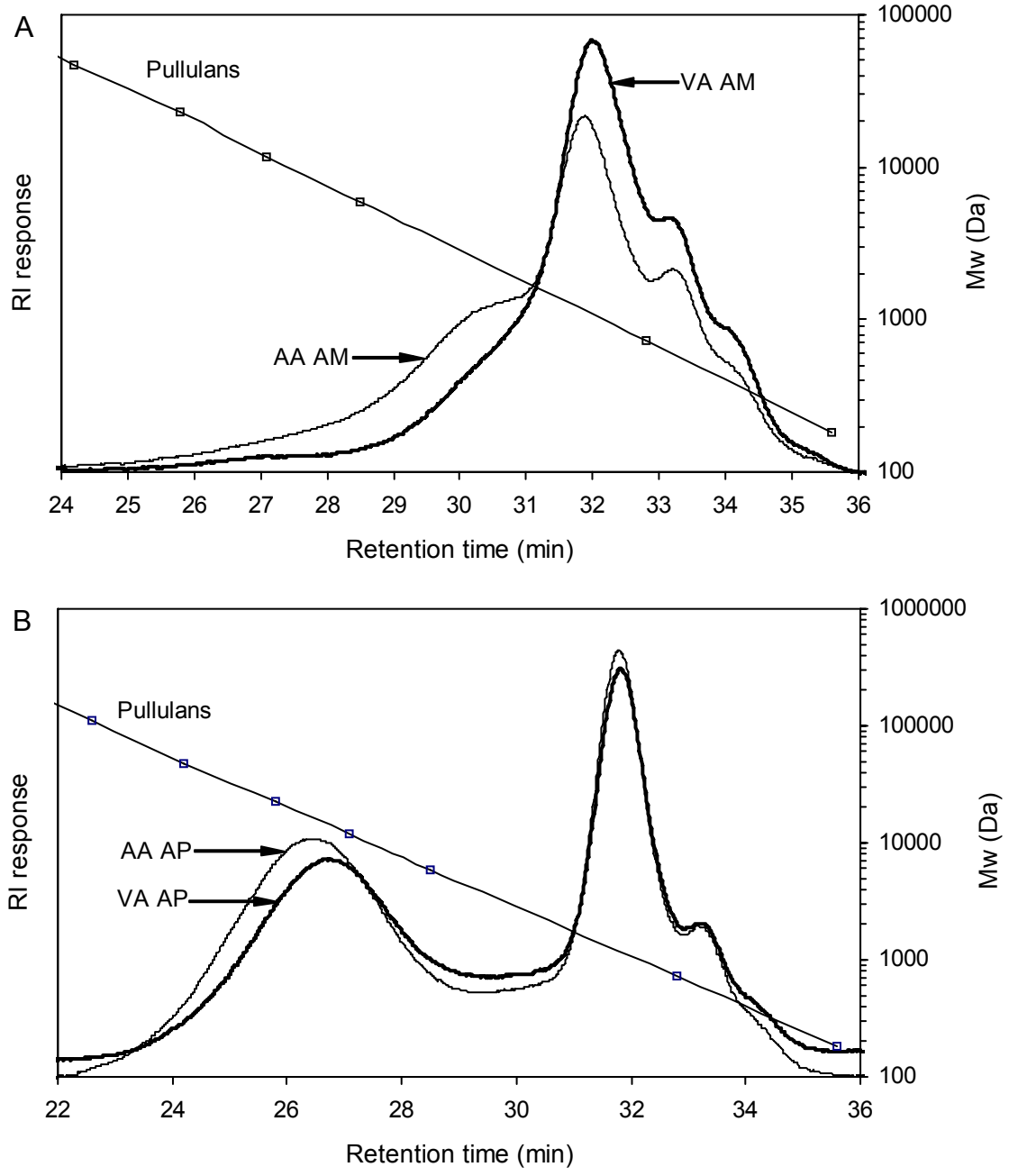

Fig. 1. HPSEC elution profiles of the α-amylase hydrolysates of (A) amylose (AM) and (B) amylopectin (AP) isolated from cowpea starch modified with acetic anhydride (AA) and vinyl acetate (VA), respectively. RI: refractive index.

fractions of a certain DP are normalized to 100% according to Mischnick (2001). Although the peak intensity may not completely correlate to the concentration for oligomers with different DP, the relative ratios within one DP are only slightly distorted in MALDI-TOF-MS (Mischnick, 2001).

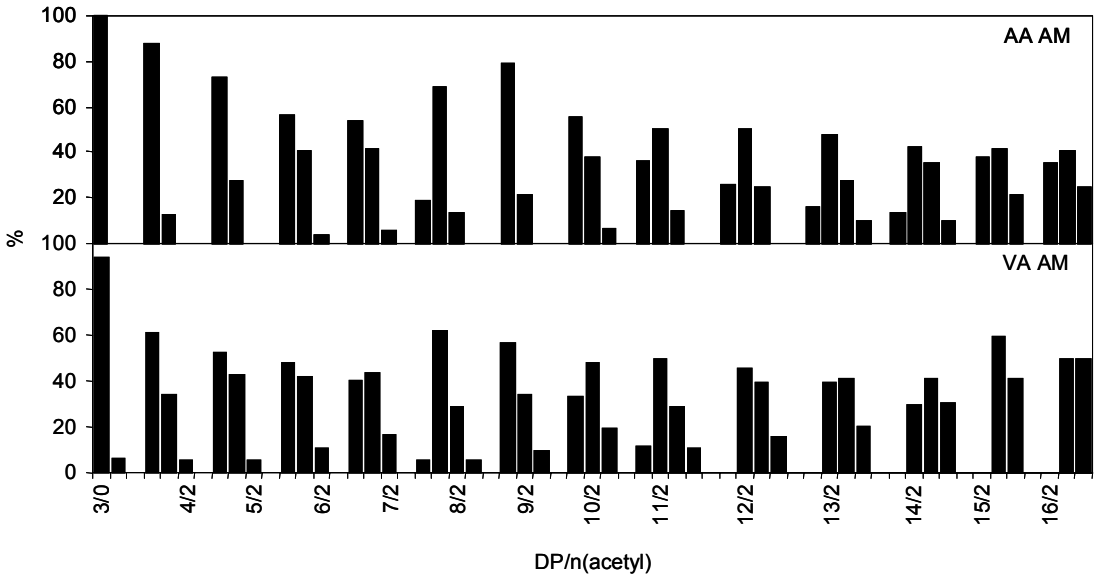

Fig. 2. Distribution of acetyl groups in the oligosaccharide fractions in the α-amylase hydrolysates of amylose (AM) isolated from cowpea starch modified with acetic anhydride (AA) and vinyl acetate (VA), respectively. The total signal intensities of the oligomers in a certain DP are normalized to 100%. DP: degree of polymerization. 3/0: unsubstituted trimer. 4/2: disubstituted tetramer.

Clear differences in the distribution of acetyl groups in the oligosaccharide fractions between AA AM and VA AM samples can be observed. The largest unsubstituted oligomer was DP 8 for both AA AM and VA AM; the smallest substituted unit was DP4 with 1 acetyl group for AA AM, and DP3 with 1 acetyl group for VA AM. The highest substituted fractions were DP6 with 2 acetyl (DS 0.33), and DP4 with 2 acetyl (DS 0.5) for AA AM and VA AM, respectively. More higher substituted fragments in VA AM hydrolysates suggests that the distribution of acetyl group along the chains of VA AM was more blockwised. Besides the degree of substitution, the substitution pattern was a very important factor in the degradation by α-amylase has been reported for methylated starch by Heins, Kulicke, Käuper, & Thielking (1998).

β-Amylase digestion

β-Amylase splits of maltose from α-1,4-glucans starting from the non-reducing ends of the chains, but can not attack α-1,6-linkages, neither by-pass them (Butler, van der Maarel, & Steeneken, 2004). The β-limit value, defined as the relative amount of maltose formed during β-amylase digestion (Bertoft, 2004), can be used to estimate substituents along the amylose chains. The results are

summerized in Table 1. It can be seen that the saponified amylose sample was not totally converted to maltose, which might be due to the non-linear nature of the amylose population from cowpea starch, as is also known for amylose from other starches (Bertoft, 2004).
As expected, the β-limit value decreased after acetyl group has been introduced into the amylose molecules. Although the DS of VA AM was lower, the degradability by β-amylase was lower, indicating that acetyl groups were located closer to the non-reducing end of VA AM chains compared to those of AA AM.

Combined digestion with α-amylase and amyloglucosidase

To further investigate the acetyl distribution patterns in AA AM and VA AM, the samples were submitted to combined enzymatic digestion with α-amylase and amyloglucosidase. Amyloglucosidase is an *exo*-enzyme and release glucose from the non-reducing end of the glucan chains by attacking both α-1,4 and α-1,6-glucosidic linkages (Mischnick, 2001). From Figure 3A, it can be seen that the saponified amylose sample was converted to a single product—glucose. The oligomers present in the digests of acetylated amylose samples after combined α-amylase and amyloglucosidase attack arose from incomplete degradation of amylose due to the presence of acetyl groups. The unsubstituted oligomers up to DP8 and substituted fractions present in the α-amylase hydrolysates could be further degraded by amyloglucosidase as revealed by MALDI-TOF-MS (Fig. 4). The fragments remaining were DP3 to DP9 with 1 to 4 acetyl groups, and DP3 to DP8 with 1 to 3 acetyl groups for AA AM and VA AM, respectively. The highest substituted fragment was DP3 with 2 acetyl groups (DS 0.67) for both AA AM and VA AM. The DS values of the enzymes resistant fragments were higher than the DS values of the parent amylose samples, suggesting uneven distribution of the acetyl groups along the amylose chains.
The differences in the substitution pattern between AA AM and VA AM revealed by the mass spectrum of the digest of combined enzymatic hydrolysis was not so pronounced as in the mass spectrum obtained from the sole α-amylase digest. The further degradation of the oligosaccharides by amyloglucosidase after the α-amylase attack reduced the difference of the fragments in the hydrolysates. This suggests that the effect of reagent types on the substitution pattern over the amylose chains could be better demonstrated at the level of fragments with broader chain length range obtained from α-amylase hydrolysis than at the level of the much smaller oligomers obtained from combined digestion with α-amylase and amyloglucosidase.

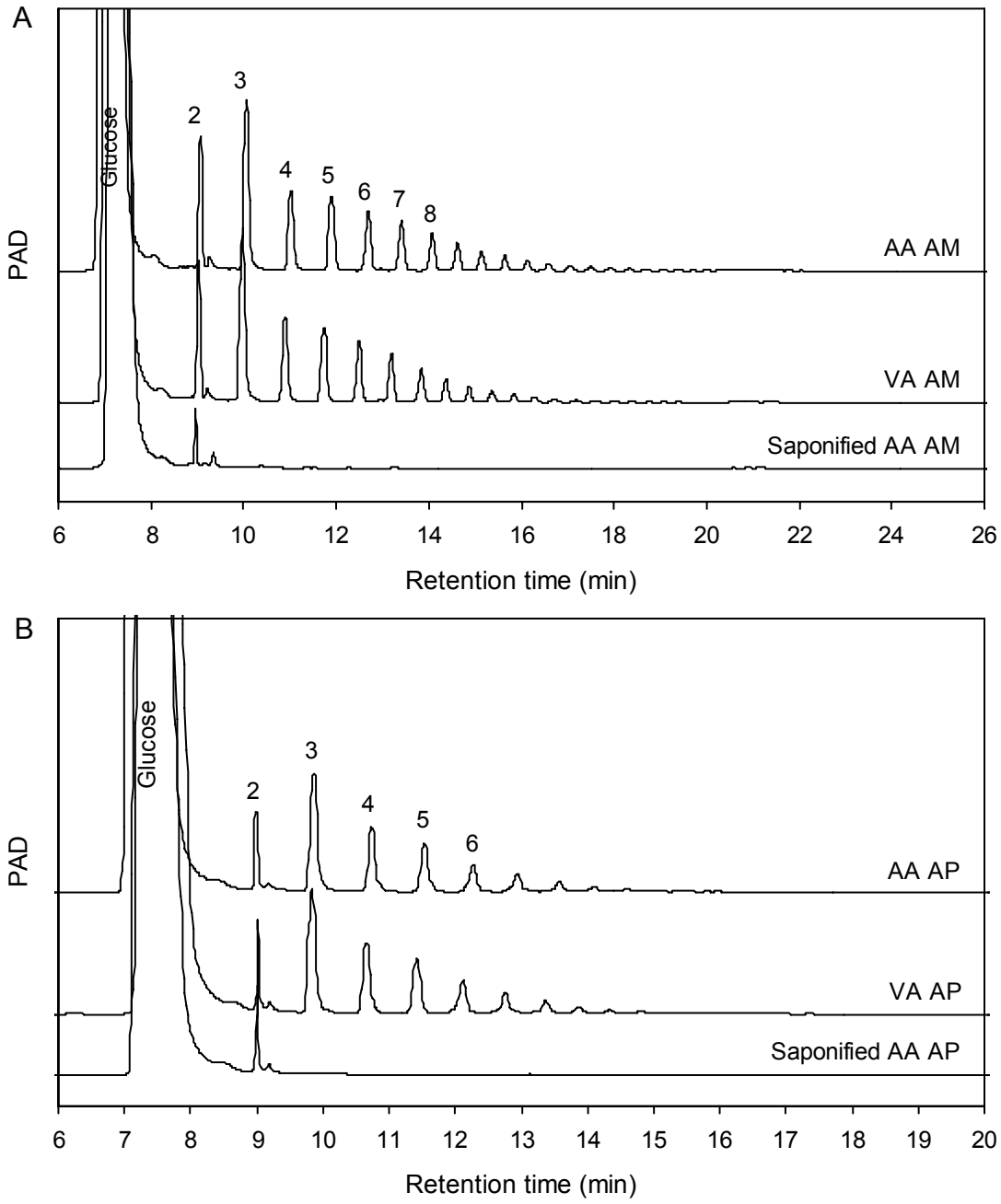

Fig. 3. HPAEC elution profile of (A) the α-amylase and amyloglucosidase hydrolysates of amylose (AM) and (B) the pullulanase, α-amylase and amyloglucosidase hydrolysates of amylopectin (AP) isolated from cowpea starch modified with acetic anhydride (AA) and vinyl acetate (VA), respectively. Numbers indicate degree of polymerization. PAD: pulsed amperometric detection.

Distribution of acetyl groups over amylopectin populations isolated from cowpea starch modified with acetic anhydride and vinyl acetate, respectively

Enzymatic digestion by α-amylase, β-amylase and pullulanase

Similar approaches as discussed above were used to explore the substitution pattern of the amylopectin component. The difference in the degradability by α-amylase (Fig. 1B) between two amylopectin samples isolated from cowpea starch modified with acetic anhydride (AA AP) and the vinyl acetate counterpart (VA AP) was not so pronounced as their difference in DS. The acetyl groups in AA AP exhibited more hinder to the attack of α-amylase than those in VA AP, although the DS of AA AP was lower, suggesting a more dense distribution of acetyl groups over the internal chains of AA AP. The DP of unsubsitituted oligomers in the hydrolysates was higher for

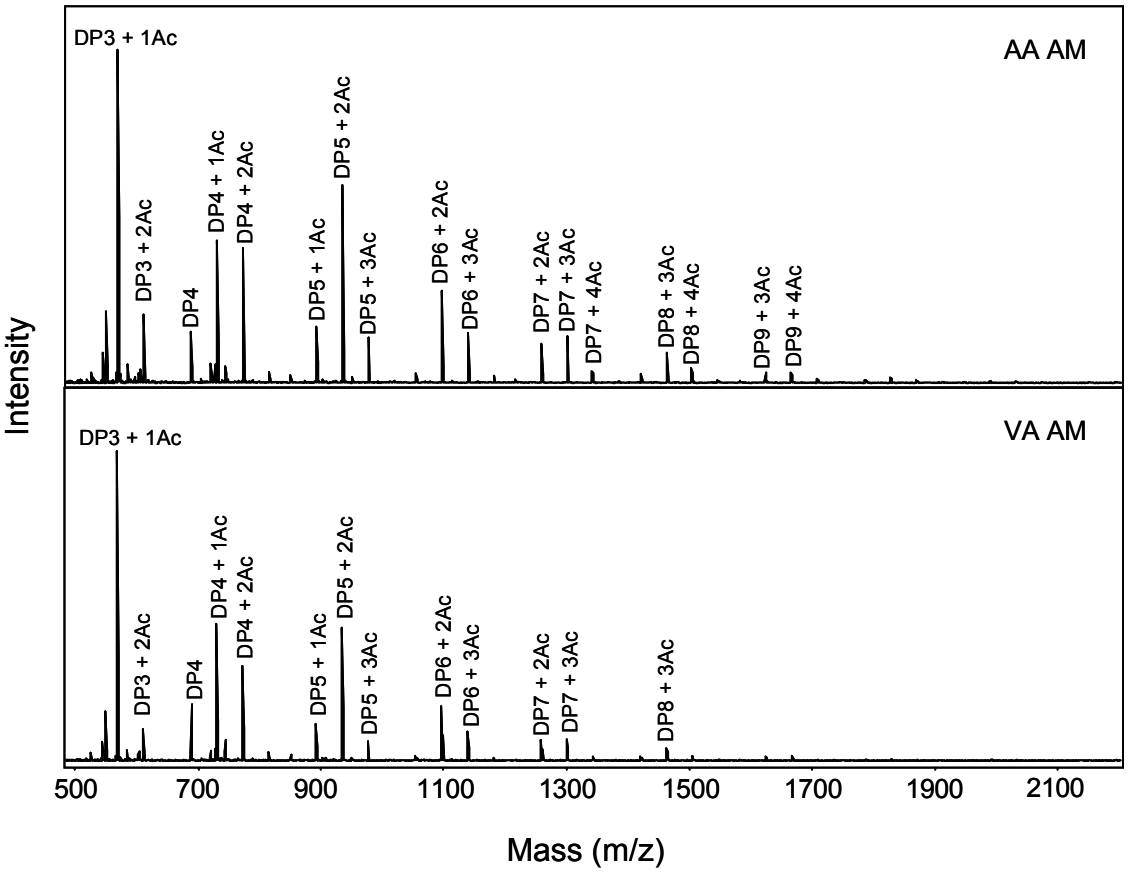

Fig. 4. MALDI-TOF mass spectrum of the α-amylase and amyloglucosidase hydrolysates of amylose (AM) isolated from cowpea starch modified with acetic anhydride (AA) and vinyl acetate (VA), respectively. DP: degree of polymerization. Ac: acetyl group.

amylopectin (Fig. 5) than for amylose, due to the fact that the DS of amylopectin was much lower and the fact that branch points act as barriers to α-amylase attack. For AA AP and VA AP, the smallest substituted unit was DP6 with 1 acetyl group and DP5 with 1 acetyl group, respectively. Of oligomers of DP10 and higher, fragments with 2 acetyl groups were present in VA AP, but not in AA AP, indicating that the substitution pattern along the chains of VA AP was more clustered and that more acetyl groups were located near the branch points.

As was found for the acetylated amylose samples also the acetylated amylopectin samples showed lower β-limit values than the saponified amylopectin sample due to the presence of acetyl groups (Table 1). More maltose released from AA AP is evidence for less acetyl groups being distributed along the external chains of AA AP. This result is in agreement with the observation from α-amylase digestion that more acetyl groups presented in the internal chains of AA AP.

The HPSEC elution profiles of the pullulanase hydrolysates suggest that the acetyl groups in VA AP showed more inhibition than those in AA AP (Fig. 6). This indicates more substituents in the vicinity of the branch points of VA AP. Also van der Burgt et al. (2000a) reported that methylation takes place preferably at the branched regions of amylopectin.

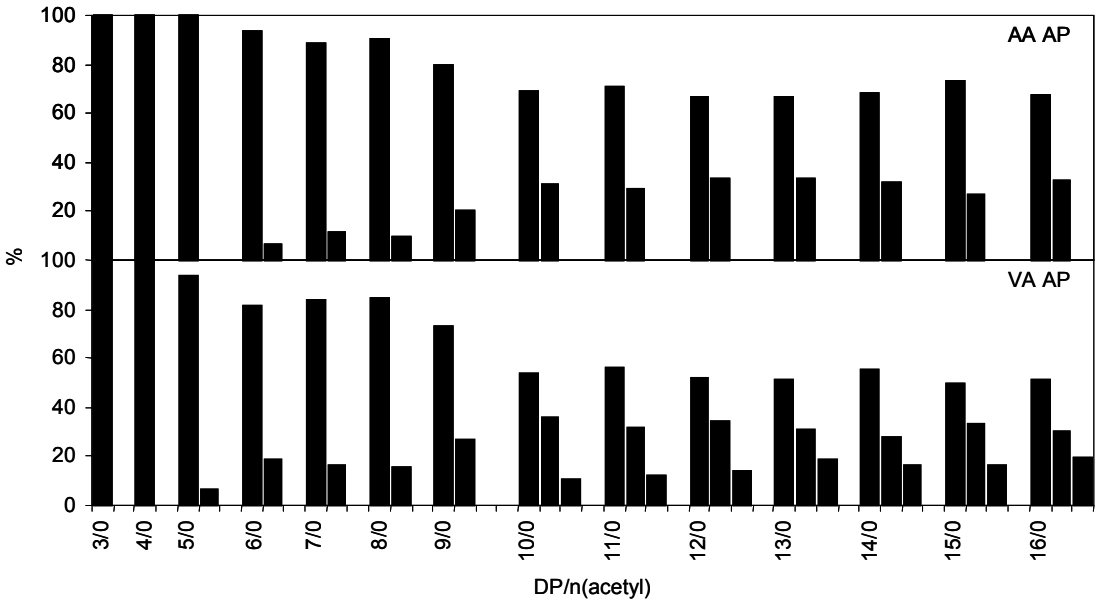

Fig. 5. Distribution of acetyl groups in the oligosaccharide fractions in the α-amylase hydrolysates of amylopectin (AP) isolated from cowpea starch modified with acetic anhydride (AA) and vinyl acetate (VA), respectively. The total signal intensities of the oligomers in a certain DP are normalized to 100%. DP: degree of polymerization. 3/0: unsubstituted trimer.

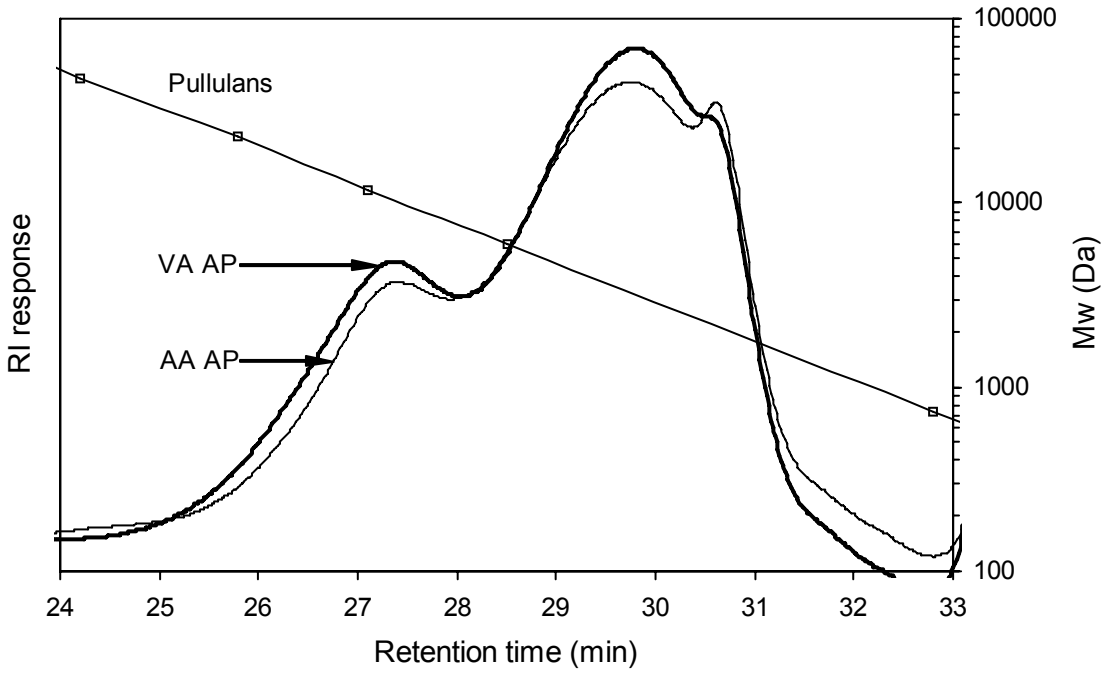

Fig. 6. HPSEC elution profile of the pullulanase hydrolysates of amylopectin (AP) isolated from cowpea starch modified with acetic anhydride (AA) and vinyl acetate (VA), respectively. RI: refractive index.

Combined digestion with pullulanase, α-amylase and amyloglucosidase

AA AP and VA AP were hydrolyzed with a combination of pullulanase, α-amylase, and amyloglucosidase. Saponified amylopectin sample produced almost exclusively glucose after combined enzymatic degradation (Fig. 3B). The amounts of enzyme resistant oligomers were much less for acetylated amylopectin samples than those of the amylose counterparts, due to the lower substitution level of amylopectin populations. The enzyme resistant fractions contained fragments of DP3-8 and DP3-9 with 1 to 3 acetyl groups for AA AP and VA AP, respectively (Fig. 7). The highest DS fragment was DP5 with 3 acetyl groups (DS 0.60) for both AA AP and VA AP. It can therefore be concluded that the acetyl groups were unevenly distributed over both the amylose and amylopectin chains of the two types of acetylated cowpea starch similar as reported for potato and sweet potato starch modified with acetic anhydride (Chen et al., 2004; Chen et al., 2005). It is the organized nature of starch granules that obstructs the acetyl groups in being regularly distributed along the polymer chains. A similar phenomenon has been observed for methylated granular starch (Steeneken & Woortman, 1994; van der Burgt et al., 2000a).

The occurrence of unsubstituted tetramer in the combined enzymatic hydrolysates for all the acetylated samples was puzzling. It can only be stated that the presence of this fraction was due to the presence of acetyl groups in the parent amylose and amylopectin samples, since there were no oligomers presented in the hydrolysates of the corresponding saponified samples.

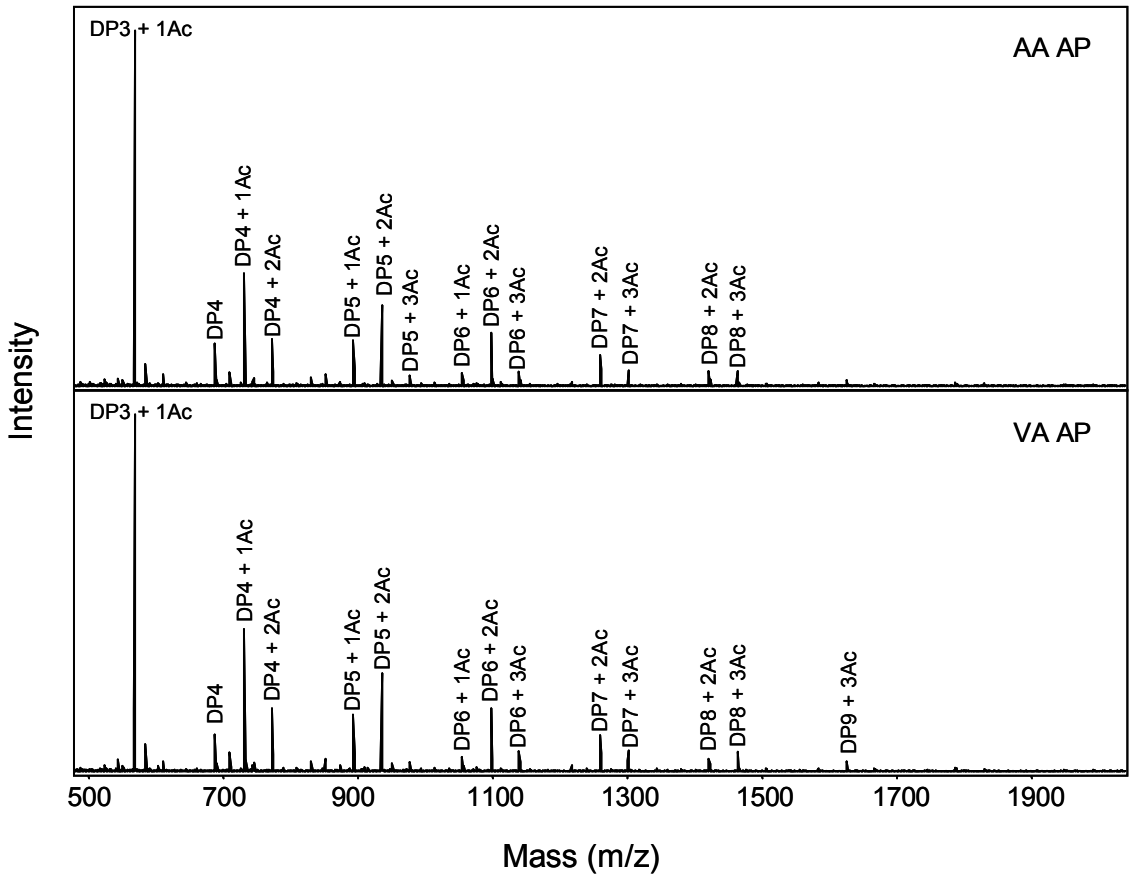

Fig. 7. MALDI-TOF mass spectrum of the pullulanase, α-amylase and amyloglucosidase hydrolysates of amylopectin (AP) isolated from cowpea starch modified with acetic anhydride (AA) and vinyl acetate (VA), respectively. DP: degree of polymerization. Ac: acetyl group.

Enzyme resistant oligomers obtained from AA AP were similar to those from VA AP as exhibited in the MALDI-TOF mass spectrum. For both amylose and amylopectin samples, combined enzymatic degradation reduced the potential to distinguish between differently substituted starch components compared to α-amylase degradation. Thus a rough estimation of the difference in acetyl substitution pattern between two types of acetylation can be made simply by analysing the fragments from α-amylase degradation.

Conclusions

For both acetylation types, amylose populations showed a much higher level of acetylation than amylopectin populations. Modification with slowly reacting vinyl acetate resulted in higher DS values for amylopectin and smaller DS difference between amylose and amylopectin compared to the modification with rapidly reacting acetic anhydride.

For both amylose and amylopectin, the oligomers in α-amylase hydrolysates showed clear differences between the two acetylation types as exhibited in the MALDI-TOF mass spectra. The presence of more high DS fragments in digests of vinyl acetate modified starch suggests that acetyl groups were more clustered along the polymer chains modified with vinyl acetate than of those modified with acetic anhydride.

The results of α-amylase, β-amylase, and pullulanase digestion reveals that there were more acetyl groups present in the external chains and in the vicinity of branch points of amylopectin isolated from cowpea starch after acetylation with vinyl acetate than the counterpart obtained from cowpea starch after acetylation with acetic anhydride.

Between two types of acetylation, the difference in acetyl substitution pattern can be demonstrated at the level of fragments with broader chain length range obtained from α-amylase digestion.

However, such information could not be observed at the level of too small oligomers obtained from exhaustive enzymatic digestion using α-amylase and amyloglucosidase for amylose, and α-amylase, pullulanase and amyloglucosidase for amylopectin.

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Chapter 5

Pasting properties and (chemical) fine structure of acetylated yellow pea starch is affected by acetylation reagent type and granule size

Abstract

Yellow pea starch was fractionated into small and large size granule fractions and then modified with acetic anhydride and vinyl acetate (acetylation after sieving) or first acetylated in the same way and then fractionated into small and large size fractions (acetylation before sieving). Acetylation with different procedures (acetylation *before* or *after* sieving) resulted in different degrees of substitution for small size granule fractions when acetic anhydride was used as reagent. However, little impact of these different DS values was found on the pasting viscosities. In the case of acetylation using vinyl acetate, similar acetylation levels and pasting viscosities were found for both the small and large size fractions obtained either before or after sieving.

The location and distribution of acetyl groups was investigated by analyzing the α-amylase hydrolysates of isolated amylose and amylopectin with chromatography and mass spectrometry. Mass spectra showed that the substituent distribution mainly depended on the type of reagent and was not affected by the granule size. Neither amylose nor amylopectin was uniformly modified and the reactions took place in different regions of the granule. It is postulated that acetylation occurs more homogeneously throughout the granule when vinyl acetate is used as reagent, while the reaction with acetic anhydride to a large extent takes place in the outer lamellae of granule.

Key words: Yellow pea starch; Amylose; Amylopectin; Acetic anhydride; Vinyl acetate

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Introduction

Starches from various sources exhibit different granule morphologly (size, shape), molecular structure (amylose and amylopectin fine structures) and composition (amylose content, non-starch components) (Huber & BeMiller, 2001). Granules from a given starch type also consist of a range of sizes. Chemically substituted granular starches play important roles in many industrial applications. The granular structure, preserved throughout the course of derivatization reactions, influences greatly the substitution pattern of the starch components (Bertoft, 2004). Stapley and BeMiller (2003) separated maize, wheat and potato starches into granule subpopulations by sedimentation *after* hydroxypropylation with propylene oxide and found similar degrees of molar substitution for the different size fractions within each starch. In contrast, granule size has been reported to affect the degree of molar substitution values for acetylated potato and sweet potato starches using acetic anhydride as reagent (Chen, Schols, & Voragen, 2004): potato and sweet potato starches were fractionated by sieving into different size granule fractions after modification with acetic anhydride and it was found that small size granule fractions showed higher DS values than the large size granule fractions. In previous work (Huang, Schols, Jin, Sulmann, & Voragen, 2006a) we established the effect of reagent type on acetylated starches by sieving the starch after the modification process for yellow pea, cowpea and chickpea starches. The DS values differed for the differently sized starch granules acetylated by the rapidly-reacting acetic anhydride, which confirmed the findings of Chen et al. (2004) for potato and sweet potato starches. It was for the first time reported that differently sized granule fractions showed similar DS values when slowly-reacting vinyl acetate was used. In all these cases, the starches were acetylated before fractionation by size. Furthermore, there is no report on the substitution pattern of size fractions for acetylated starch modified with vinyl acetate. In this study two acetylation procedures were employed to determine how reagent type (acetic anhydride vs. vinyl acetate) and differently size granule fractions affect properties and fine structures of acetylated yellow pea starch: the first procedure included fractionation of the starch into small and large size granule fractions and then acetylation of these fractions (acetylation after sieving), and the second procedure included acetylation of the starch and then fractionation into small and large size fractions (acetylation before sieving) (Fig. 1).

Materials and Methods

Materials

Yellow pea starch was a gift from COSUCRA (Warcoing, Belgium). Since the purities of both this starting material and its >32 μm granule size fraction were rather low (92.3% and 72.5%, respectively; Huang et al., 2006a), the starch was further purified in the laboratory by sieving through a series of test sieves (0.250 mm, 125 μm and 71 μm) on a Retsch AS200 digit shaker (Retsch GmbH & Co., Haan, Germany) with deionized water and then dried at 40°C. The starch contents of the purified starch samples were determined by using the enzymatic Roche starch test kit (Boehringer Mannheim, Darmstadt, Germany). α-Amylase (EC 3.2.1.1) (product number 10069, from *Bacillus subtilis*, 393 U/mg), purchased from Fluka (Switzerland), was dissolved in millipore water to make a solution containing 0.38 U/μl of enzyme.

Acetylation and separation of differently sized granule fractions

Two procedures were used to prepare acetylated yellow pea starch (Fig. 1). One procedure included acetylation *after* sieving, while in the other procedure acetylation was performed *before* sieving. Yellow pea starch samples were separated by sieving into three fractions: smaller than 20 μm, 20-32 μm and larger than 32 μm. The small (<20 μm) and large (>32 μm) size granule fractions were used in this study. Acetylated yellow pea starch samples were prepared by reacting with acetic anhydride and vinyl acetate, respectively, by AVEBE Food Innovation Centre (Veendam, The Netherlands), 0.088 moles of reagent per mole glucose residue of starch were added. Sieving and acetylation were conducted as described previously (Huang et al., 2006a).

Particle size distribution and pasting behavior

Particle size distribution was measured in water using a laser diffraction system (H1140, Sympatec Inc., USA). The pasting behaviors of the starches were measured using a Rapid Visco Analyzer (RVA-4, Newport Scientific Pty. Ltd., Australia). These analyses were carried out as described by Huang, Schols, van Soest, Jin, Sulmann, & Voragen (2007).

Isolation of amylose and amylopectin

Amylose and amylopectin populations were isolated from acetylated yellow pea starch using the aqueous leaching method according to Chen et al. (2004). The purity of isolated amylose and amylopectin was checked with high-performance size-exclusion chromatography (HPSEC) after pullulanase digestion according to Kobayashi, Schwartz, and Lineback (1985).

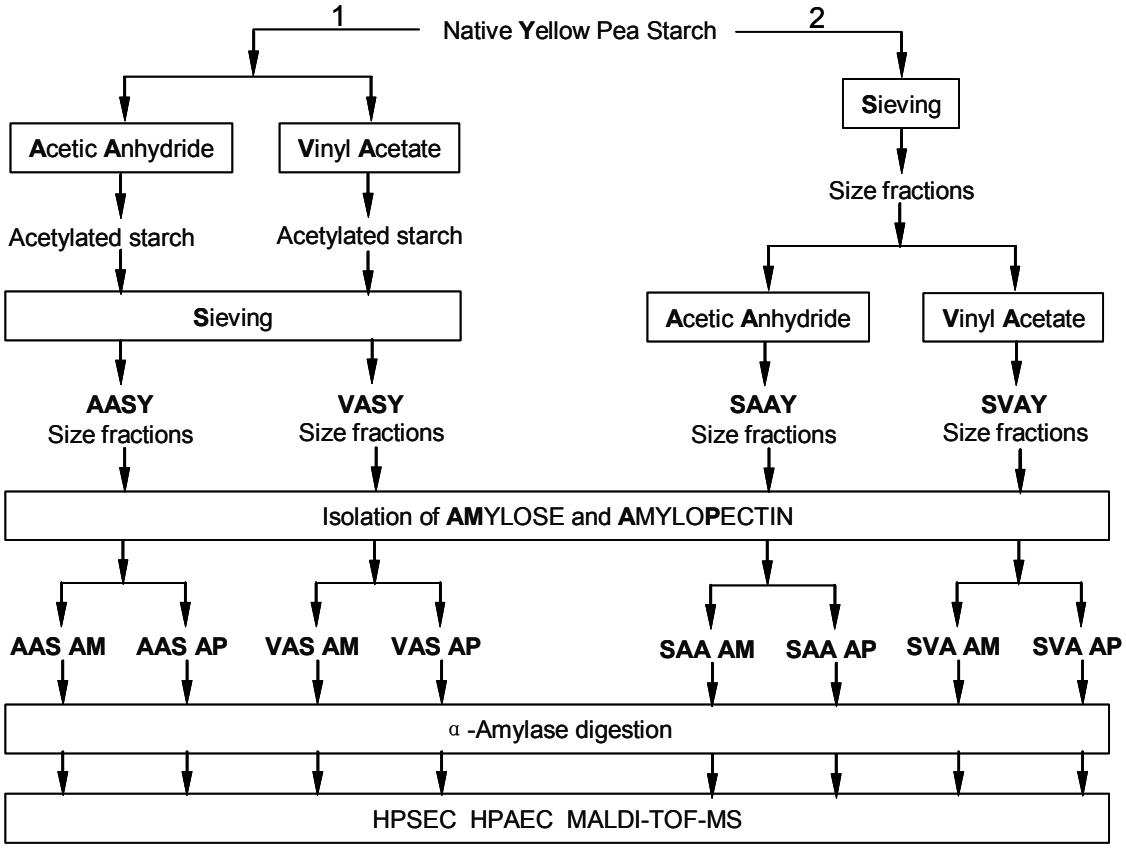

Fig. 1. Schematic overview of the approach followed to reveal the impact of reagent type and granule size on the fine structure of acetylated yellow pea starch.

Determination of degree of substitution and α-amylase digestion

The degrees of molar substitution (DS) of whole starch samples were determined using the titration method according to Huang et al. (2006a). The DS values of amylose and amylopectin samples

were determined using the EnzyPlus Acetic Acid test kit (Diffchamb, Sweden) according to Huang, Schols, Klaver, Jin, & Voragen (2006b).

Five milligrams of acetylated amylose or amylopectin samples were submitted to α-amylase digestion according to Chen et al. (2004).

HPSEC, HPAEC and MALDI-TOF-MS

HPSEC (high-performance size-exclusion chromatography) was performed on a ThermoFinnigan (USA) HPLC, with three TSK gel columns (7.8 mm ID × 30 cm per column) in series (G4000PW_{XL}, G3000 PW_{XL}, G2500PW_{XL}; Tosohaas, Japan), in combination with a PW_{XL}-guard column (Tosohaas, Japan). Elution was at 30°C using 0.2 mol/L sodium nitrate at a flow rate of 0.8 mL/min. The elution was monitored using a Shodex SE-61 Refractive Index detector. Calibration was performed using pullulans (Polymer laboratories, UK).

HPAEC (high-performance anion-exchange chromatography) was performed on a Dionex (USA) HPLC system. The system was equipped with a quaternary gradient pump, an autosampler, a helium degassing unit and an electrochemical detector in the PAD mode. A CarboPac PA1 column (2 × 250 mm) (Dionex, USA) with a CarboPac PA1 guard column (2× 50 mm) (Dionex, USA) was operated at a flow rate of 0.3 mL/min at 20 °C. The gradient was obtained by mixing solutions of 0.1 mol/L NaOH and 1 mol/L NaOAc in 0.1 mol/L NaOH. After 15 min equilibration with 0.1 mol/L NaOH, 20μL of the sample was injected and a linear gradient to 0.50 mol/L NaOAc in 0.1 mol/L NaOH within 30 min was followed by a linear gradient in 5 min to 1 mol/L NaOAc in 0.1 mol/L NaOH. Finally, the column was washed for 5 min with 1 mol/L NaOAc in 0.1 mol/L NaOH. The data of HPSEC and HPAEC were processed using Chromeleon software (Dionex, USA).

MALDI-TOF-MS (Matrix-Assisted Laser Desorption/Ionisation Time-Of-Flight Mass Spectrometry) was carried out using an Ultraflex workstation (Bruker Daltonics GmbH, Germany) equipped with a nitrogen laser of 337 nm. The mass spectrometer was selected for positive ions. After a delayed extraction time of 100ns, the ions were accelerated to a kinetic energy of 20 kV. Hereafter, the ions were detected in the reflector mode. The lowest laser power required to obtain good spectra was used. The mixture of 1μL sample and 1μL of matrix was dried on a sample plate. The matrix solution was prepared by dissolving 9 mg of 2,5-dihydroxybenzonic acid in a 1mL mixture of acetonitrile:water (300μL:700μL). External calibration was performed using a mixture of maltodextrins (Mw range 400-3500Da).

Results and Discussion

Characterization of small and large granule fractions of acetylated yellow pea starch samples

Starting materials

Since the purity of >32 μm granule fraction separated from yellow pea starch as studied before was quite low (72.5%; Huang et al., 2006a), the commercial starch as well as this fraction were further purified in the laboratory by sieving to a purity of 94.8% and 92.5%, respectively. The purity of another starting material, <20 μm granule fraction of purified yellow pea starch, was 95.9%. Eight samples were obtained from two procedures (Fig. 1): SAAY<20; SAAY>32; AASY<20; AASY>32; SVAY<20; SVAY>32; VASY<20 and VASY>32.

Particle size distribution and degree of molar substitution

The particle size distributions of the eight acetylated yellow pea starch samples are presented in Fig. 2. The four <20μm granule fractions all showed similar size distribution patterns and their volume mean diameters (VMD) measured in water were all in the range of 19.1-20.8 μm. The granule size distributions of SAAY>32 and SVAY>32 were unimodal with VMD of 30.1 and 31.2 μm, while for AASY>32 and VASY>32 there was a slight shoulder at high granule diameters with higher VMD values (46.9 and 45.0 μm). The shoulder may be due to the presence of fiber-like impurities as observed by microscopy. Due to the purification, the >32 μm granule fraction did not show a much broader peak than <20 μm granule fraction as found in our previous work (Huang et al., 2006a).

For chemically substituted starches, the degree of molar substitution (DS) is a basic parameter to get information about reaction efficiency and the amount of substituents introduced. Acetylation with acetic anhydride using different procedures (acetylation *before* and *after* sieving) resulted in different degrees of substation for the <20 μm granule fractions (Table 1). In the case of acetylation using vinyl acetate, similar acetylation levels were found for all size fractions obtained either before or after sieving. In our previous work, the DS values were also found to differ for differently sized granule fractions acetylated by acetic anhydride but not for the size fractions acetylated by vinyl acetate (Huang et al., 2006a). It should be noted that, for modification with acetic anhydride, the <20 μm and >32 μm granule fractions that were obtained from the first procedure (acetylation *after* sieving) showed less difference in DS levels compared to the <20 μm and >32 μm fractions obtained from the second procedure (acetylation *before* sieving).

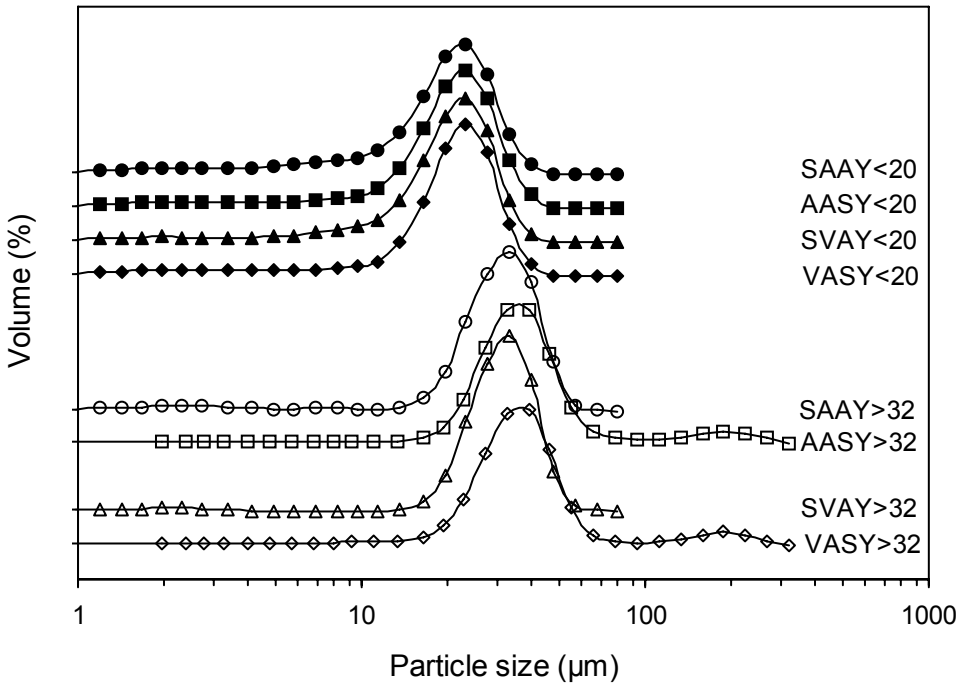

Fig. 2. Particle size distributions of small (<20µm) and large (>32µm) granule size fractions of yellow pea starch (Y) modified with acetic anhydride (AA) and vinyl acetate (VA) before and after sieving (S), respectively.

Table 1 Degree of substitution of amylose and amylopectin isolated from small (<20µm) and large (>32µm) size granule fractions of yellow pea starch (Y) modified with acetic anhydride (AA) and vinyl acetate (VA) before and after sieving (S), respectively.

	Starch	Degree of molar substitution[a]		
		Starch[b]	Amylose	Amylopectin
Procedure 1	SAAY <20 µm	0.067c	0.092b	0.042c
Acetylation	SAAY >32 µm	0.061d	0.082d	0.038d
after	SVAY <20 µm	0.067c	0.093b	0.043c
Sieving	SVAY >32 µm	0.066c	0.089c	0.042c
Procedure 2	AASY <20 µm	0.079a	0.097a	0.057a
Acetylation	AASY >32 µm	0.064cd	0.082d	0.041cd
before	VASY <20 µm	0.068cb	0.089c	0.046bc
Sieving	VASY >32 µm	0.071b	0.088c	0.045bc

[a] Values are and means of triplicate.

[b] Values are based on dry matter.

Values with different letters in the same column are significant different at $p<0.05$.

When samples were fractionated after modification, rather than fractionated before modification, small and larger size granule fractions compete for the rapidly reacting reagent as mentioned by Stapley et al. (2003) for hydroxypropylated starches. It was not surprising that the extent of difference in reaction efficiency between the two size fractions was larger when there was a competition for acetic anhydride than when there was no competition. The lower reaction efficiency of the >32 μm fraction than that of the <20 μm fraction even when they reacted separately with acetic anhydride may be due to the difference in reagent distribution over the granule surface during the reaction: a thin-layer around the <20 μm granules and a thicker layer around the >32 μm granules. Since the reaction with acetic anhydride took place preferably on and near the outside granular surface (Chen et al., 2004), small size granule fractions with more surface hydroxyl groups reacted with more reagent due to larger specific surface areas compared to large size fractions. When vinyl acetate was used, which had time to penetrate deeply into granule before reacting, the reaction efficiency was independently from the specific surface area of the granules. Thus, especially for acetylation with acetic anhydride, sieving prior to reaction resulted in greater level of difference in the acetylation level of the differently sized granule fractions which may consequently have impact on the physical properties.

Pasting behavior

The pasting behaviors of acetylated yellow pea starch samples were determined by RVA (Rapid Visco Analyzer). The small size granule fractions exhibited the same pasting temperature, while higher pasting viscosity compared to the corresponding large size granule fractions (Fig. 3). Small and large size granule fractions modified with vinyl acetate exhibited greater peak viscosities than the corresponding size fractions modified with acetic anhydride. Therefore, a higher pasting viscosity of acetylated starch could be obtained by using vinyl acetate instead of acetic anhydride as the reagent and/or choosing small size granule fractions.

The DS value of SVAY<20μm was the same as that of SAAY<20μm. While SVAY<20μm showed higher peak viscosity than SAAY<20μm. These findings confirm that the difference in pasting behavior between two acetylation types is not due to a different DS value (Huang et al., 2006a). One possible explanation is that acetylation with acetic anhydride was limited to the outer lamellae of the granule (Chen et al., 2004), thus a large proportion of the inner granule remained unmodified which contributed to the apparent pasting behavior of the starch to such an extent that it was similar to that of native yellow pea starch (Huang et al., 2006a). When vinyl acetate was used, reactions were located throughout the granule (from outer to inner lamellae), the more homogeneously

modified granules showed significantly different pasting viscosity than did native granules (Huang et al., 2006a). Biliaderis (1982) already postulated this explanation based on enzymatic debranching studies of two modified starches: an acetylated (DS 0.06) smooth pea starch obtained after reaction with acetic anhydride and a commercial hydroxypropyl (DS 0.09) waxy maize starch. The size exclusion patterns showed that the chain length profile of the acetylated derivative nearly matched that of the unmodified pea starch, whereas the profile of the hydroxypropyl waxy maize starch did not match that of its blank. Those results led Biliaderis (1982) to conclude that the acetylation occurred exclusively in certain part of the granule, whereas hydroxypropylation was more uniform throughout the starch granule.

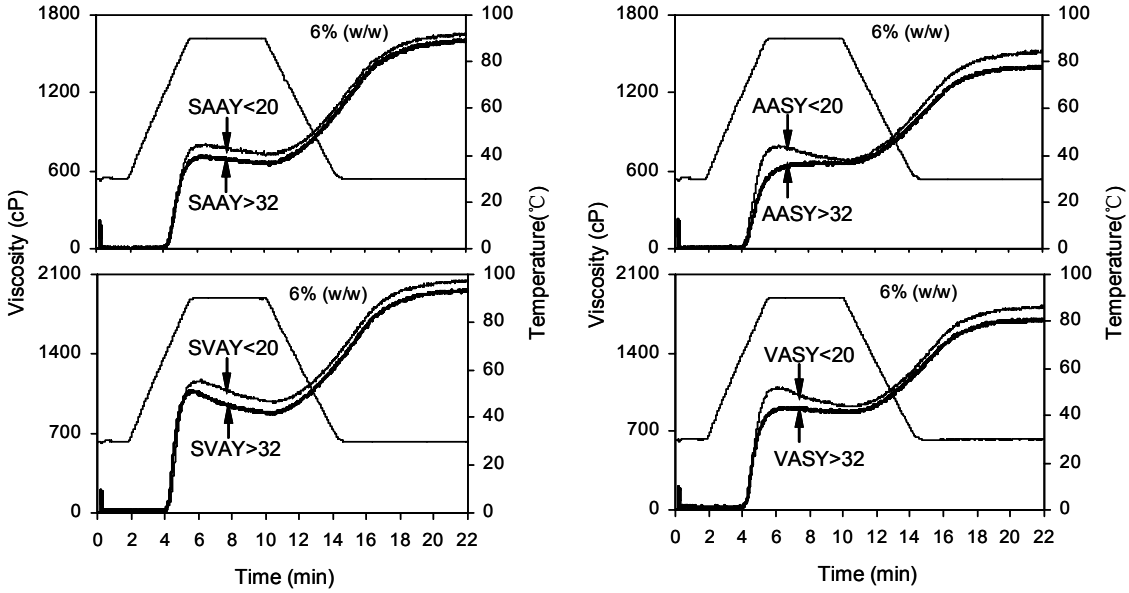

Fig. 3. RVA pasting curves of the small (<20µm) and large (>32µm) granule size fractions of yellow pea starch modified with acetic anhydride (AA) and vinyl acetate (VA) before and after sieving (S), respectively. RVA: rapid visco analyzer

For modification with acetic anhydride, the peak viscosities of AASY<20µm and SAAY<20µm were similar in spite of the fact that the acetylation level of AASY<20µm was much higher than that of SAAY<20µm. The results suggest that although different modification procedures may induce differences in the acetylation level for the same size fractions, the influence on the pasting behavior was only minor. The peak viscosity of the >32µm fraction obtained from the first

procedure (acetylation *after* sieving) was higher than that of the >32µm fraction obtained from the second procedure (acetylation *before* sieving) within one acetylating type. This might be due to the lower volume mean diameter values obtained from the first procedure than from the second procedure of the >32µm fractions.

It may be concluded that for the low acetylation level (DS<0.1) used in our study the DS value obtained for modification with acetic anhydride was not so important for the pasting behavior of the starch as was the case for modification with vinyl acetate. Thus, it is essential to mention the reagent used when discussing properties of acetylated starch.

Characterization of amylose and amylopectin isolated from acetylated yellow pea starch samples

Degree of molar substitution of amylose and amylopectin populations

The purities of isolated amylose and amylopectin were checked by treating them with pullulanase. The elution profiles of acetylated amylose samples were similar as the untreated counterparts. The acetylated amylopectin samples exhibited smaller fragments and no high molecular weight material was found to be present. Therefore, the isolated amylose and amylopectin samples were considered to be pure and used for further studies.

For all acetylated yellow pea starch samples, the DS of amylose was much higher than that of amylopectin (Table 1). Similar observations have also been reported for potato and sweet potato starches modified with acetic anhydride (Chen et al., 2004), methylated potato starch (Steeneken & Woortman, 1994; van der Burgt et al., 2000), and hydroxypropylated potato starch (Kavitha & BeMiller, 1998). This suggests a difference in reactivity of amorphous and crystalline regions. It is likely that granule derivatization starts in the more amorphous regions and proceeds to the more crystalline regions of the granule (Gray & BeMiller, 2004).

The acetylation level of amylose was 1.7-2.2 times that of amylopectin, but it should be realized that due to the higher weight fraction of amylopectin (Huang et al., 2006a) 52-58% of the total acetyl groups were present in the amylopectin population of the yellow pea starch.

For modification with acetic anhydride, amylose and amylopectin that were obtained from small size granule fractions showed higher DS than obtained from large size fractions. The DS ratios of <20 : >32 µm fractions for amylose were 1.1 and 1.2 for acetylation after and before sieving, respectively. The DS ratios of <20 : >32 µm fractions for amylopectin were 1.1 (acetylation after sieving) and 1.4 (acetylation before sieving). The reactivity of amylopectin increased to a larger extent than that of amylose in small size fraction when there was a competition for acetic anhydride

between two size fractions (acetylation before sieving) compared to when there was no competition (acetylation after sieving). The clusters of amylopectin chains arrange in the radial direction of the granule (Seib, 1997). More amylopectin chain ends may therefore locate on the surface of small size granule fraction than on the surface of large size fraction.

When yellow pea starch samples were modified with vinyl acetate, there were only small difference between two size fractions and two procedures. The DS of amylose from the <20 μm fraction was slightly higher than that of amylose from the >32 μm fractions for acetylation after sieving. Similarity in DS values between the two size fractions were found for amylose obtained from acetylation before sieving and for amylopectin from both procedures. The observations were in accordance with those of whole starch samples.

Chen et al. (2004) separated potato and sweet potato starch size fractions after modification with acetic anhydride. DS values determined for isolated amylose and amylopectin showed that the acetylation level of amylose was constant for differently sized granule fractions, while that of amylopectin was increased with decreasing granule size. This may be due to the different architecture of granules from different sources.

Distribution of acetyl groups over amylose populations

Investigation on the substitution patterns on amylose and amylopectin isolated from cowpea starch samples modified with acetic anhydride and vinyl acetate has revealed that the difference in acetyl substitution pattern between two types of acetylation can be demonstrated better by α-amylase degradation than by combined enzymatic (α-amylase and amyloglucosidase for amylose samples, α-amylase, pullulanase and amyloglucosidase for amylopectin samples) degradation (Huang et al., 2006b). In this study, the distribution and location of acetyl groups on amylose and amylopectin were determined by analyzing the degradation products after α-amylase hydrolysis.

α-Amylase is an endo acting enzyme which hydrolyzes α-(1,4)-D-glucosidic linkages in starch in a random fashion. Its action might be hindered by acetyl substitution (Biliaderis, 1982; Chen et al., 2004). Acetic anhydride modified amylose that was isolated from small size fractions exhibited apparent differences in susceptibility to α-amylase degradation between two modification procedures (acetylation before and after sieving). There were more high molecular weight fragments in the digests of AAS AM<20 than in the digests of SAA AM<20 as revealed by HPSEC elution profiles (Fig. 4). HPAEC analysis showed that much less glucose was produced from AAS AM<20 than from SAA AM<20 (results not shown). Although this might be partly explained by the

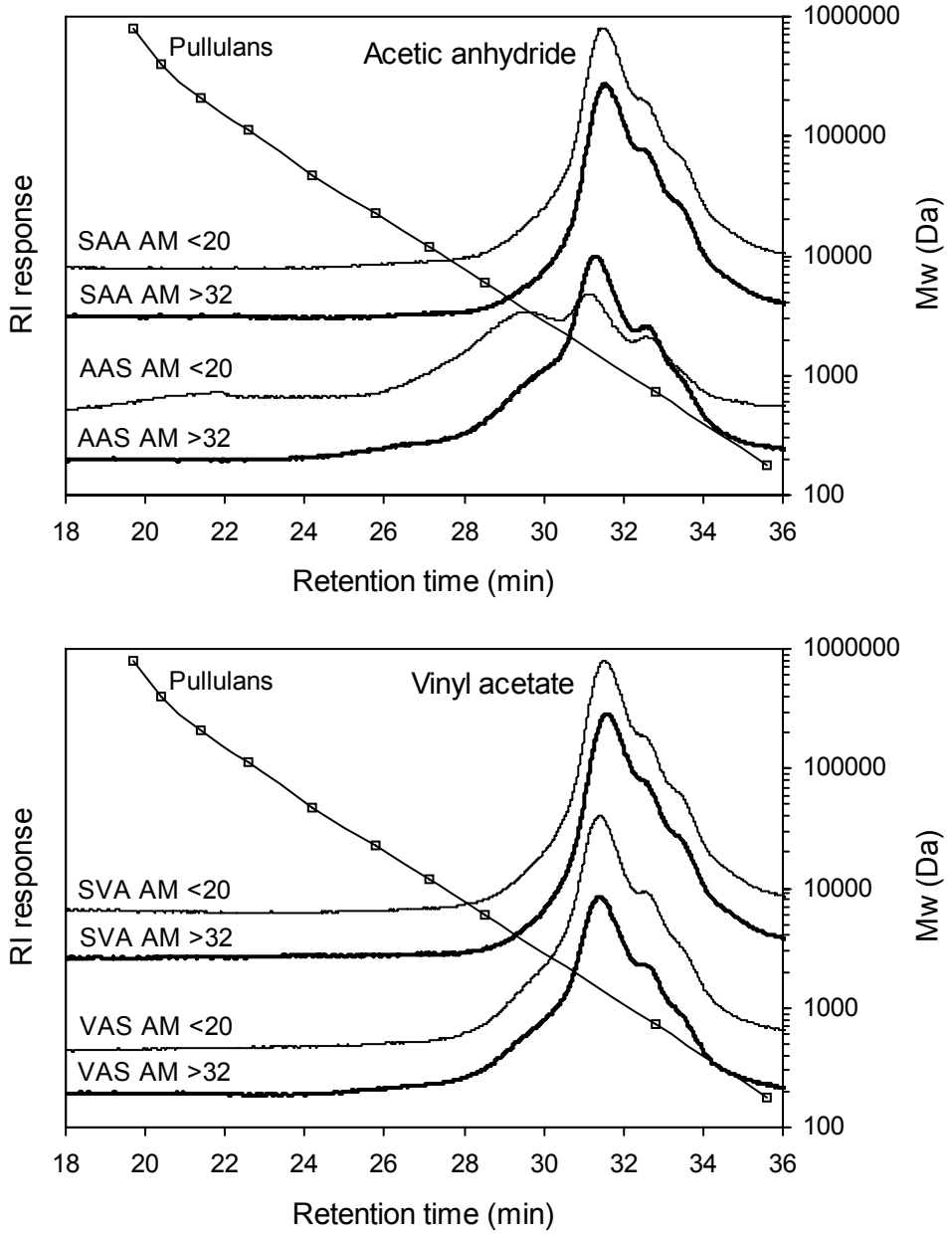

Fig. 4. HPSEC elution profiles of the α-amylase hydrolysates of amylose (AM) isolated from small (<20μm) and large (>32μm) granule size fractions of yellow pea starch modified with acetic anhydride (AA) and vinyl acetate (VA) before and after sieving (S), respectively. RI: refractive index.

fact that more acetyl groups were present in AAS AM<20 than in SAA AM<20, the DS difference (0.097 vs. 0.092) was only minor when compared to the difference in the degradation profiles

obtained after α-amylase treatment of these two samples. The DS values of SAA AM<20 and AAS AM<20 and were 1.1 and 1.2 times those of SAA AM>32 and AAS AM>32, respectively. The susceptibility to α-amylase degradation was similar for SAA AM<20 and SAA AM>32, but obviously different for AAS AM<20 and AAS AM>32 as shown in the HPSEC elution profiles. Similar phenomena have been found by Chen et al. (2004) for both acetylated potato and sweet potato starches, although the DS of amylose samples obtained from different size granule fractions were quite similar. Therefore, it is the substitution pattern rather than the DS that induces the different degradability by α-amylase of acetic anhydride acetylated amylose populations obtained by the two procedures. For modification with vinyl acetate, the four amylose samples showed similarity in the HPSEC and HPAEC (not shown) elution profiles, which was consistent with their DS results.

The location of acetyl groups on amylose molecules was determined by using MALDI-TOF-MS after α-amylase digestion. When modified with acetic anhydride, large size granule fractions (SAA AM>32 and AAS AM>32) showed similar spectra as the corresponding small size fractions (SAA AM<20 and AAS AM<20), although the DS values of small size fractions were higher. The comparison of SAA AM<20 and AAS AM<20 was given as an example to show differences between the two procedures (Fig. 5). Fragments not larger than DP14 were observed by MALDI-TOF-MS oligomer-analysis. Fragments of DP5-6, DP8-10 and DP11-13 with 2, 3 and 4 acetyl groups, respectively, were present in SAA AM<20, but not in AAS AM<20. On the other hand, the fragments of unsubstituted DP8-9 and of DP11-13 with 1 acetyl group were present in AAS AM<20, but not in SAA AM<20. The highest substituted fragments were DP5 with 2 acetyl (DS 0.40), and DP6 with 2 acetyl (DS 0.33) for SAA AM<20 and AAS AM<20, respectively. The observation of lower amount of higher substituted fragments in AAS AM<20 hydrolysates suggests that the acetyl groups were distributed in a more uniform manner along the polymer chain of AAS AM<20 compared to SAA AM<20. In addition, the acetylation level of AAS AM<20 was higher, therefore, acetyl groups seemed to be present on more chains of amylose, which resulted in much less glucose released from AAS AM<20 than from SAA AM<20. There was more glucose present in the α-amylase hydrolysates of SAA AM>32 as revealed by HPAEC analysis (not shown) since its DS was lower than that of SAA AM<20.

For modification with vinyl acetate, there was little difference in the distribution pattern between large size granule fractions (SVA AM>32 and VAS AM>32) and the corresponding small size fractions (SVA AM<20 and VAS AM<20) (spectra not shown). The largest unsubstituted oligomer was DP7, and the smallest substituted unit was DP4 with 1 acetyl group for all four samples, similar

to the results for cowpea amylose (DS 0.092), the corresponding oligomers of DP8 and DP3 with 1 acetyl group were found to be present in the α-amylase hydrolysates (Huang et al., 2006b). Only slight differences were found between the two procedures, for example, DP6 with 3 acetyl groups and DP10 with 4 acetyl groups were present in SVA AM<20 and SVA AM>32, but not in VAS AM<20 and VAS AM>32. α-Amylase degradation revealed that amylose was not homogeneously modified as indicated by the presence of unsubstituted oligomers and oligomers with different substitution levels in the digests of acetylated amylose.

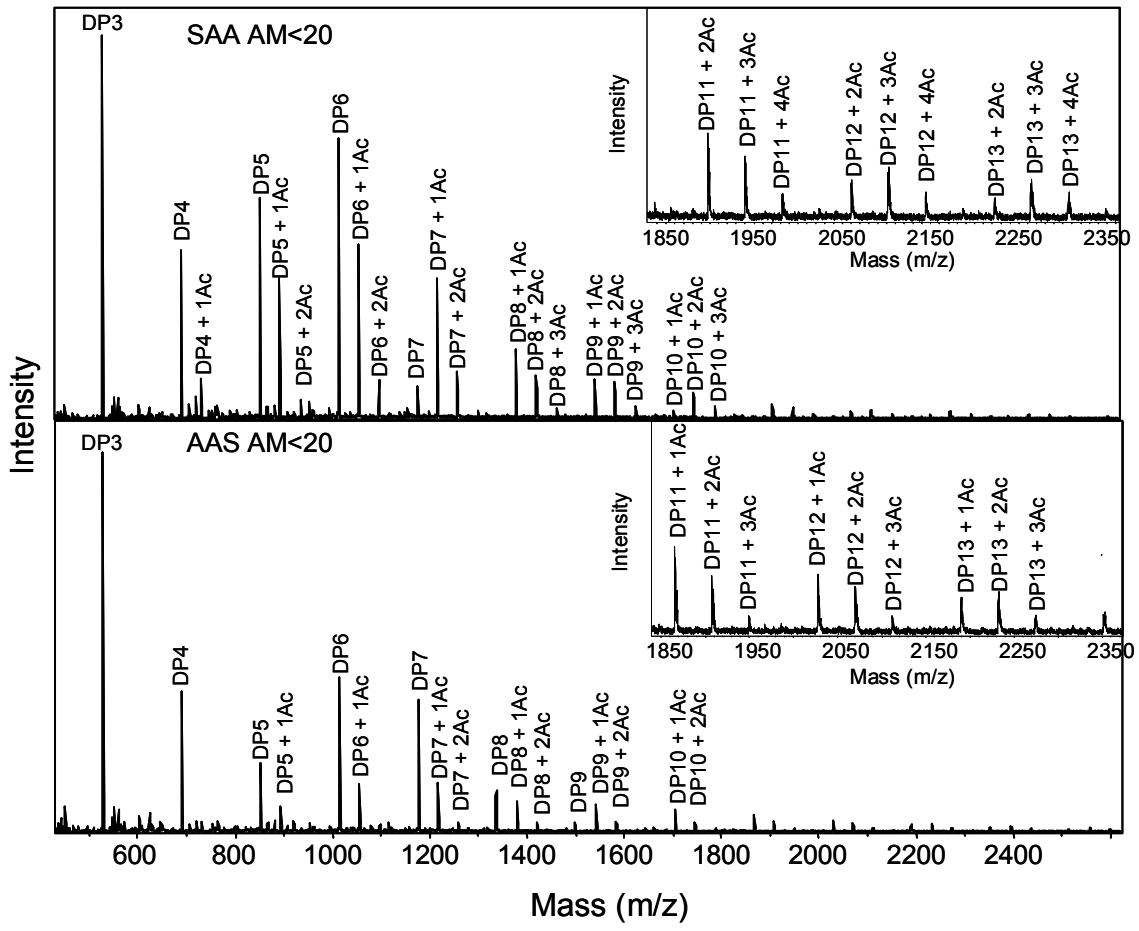

Fig. 5. MALDI-TOF mass spectra of the α-amylase hydrolysates of amylose (AM) isolated from small (<20μm) granule size fractions of yellow pea starch modified with acetic anhydride (AA) before and after sieving (S), respectively. DP: degree of polymerization. Ac: acetyl group. A zoom of DP11-13 is inserted.

Distribution of acetyl groups over amylopectin populations

Amylopectin samples isolated from small size fractions showed different degradation pattern by α-amylase for the two modification procedures when modified with acetic anhydride. HPSEC elution profiles suggested that α-amylase was hindered to a larger extent by the presence of acetyl groups in AAS AP<20 than by those in SAA AP<20 (Fig. 6). Less maltose was released from AAS AP<20 than from SAA AP<20 as determined by HPAEC (results not shown). This is in accordance with the higher acetylation level of AAS AP<20 (0.057) compared to the DS of SAA AP<20 (0.042). However the distribution pattern on AAS AP<20 was similar to that on SAA AP<20 as shown in the MALDI-TOF mass spectra (Fig. 7). For the first modification procedure, SAA AP<20 showed similar degradability by α-amylase as SAA AP>32 and a similar substitution pattern despite the slight DS difference (0.042 vs. 0.038). For the second procedure, AAS AP<20 was less degraded by α-amylase than AAS AP>32 confirming the results found for acetylated potato and sweet potato starches (Chen, Huang, Suurs, Schols, & Voragen, 2005). In all four amylopectin samples modified with acetic anhydride, unsubstituted fragments of DP3 to 14, and substituted fragments of DP5 to 14 with 1 acetyl group were observed, which was similar to the results for amylopectin of acetylated cowpea starch (Huang et al., 2006b).

The DP of unsubsitituted oligomers in the hydrolysates was higher in the amylopectin than the amylose fraction, and more high molecular weight fragments were present in the digests of amylopectin than in that of amylose (Fig. 6 vs. Fig. 4). This was due to the fact that the DS of amylopectin was lower and the fact that branch points act as barriers to α-amylase attack (Huang et al., 2006b).

With low reagent levels, hydroxyl groups in granular starch selectively react in the amorphous region and on the surface of crystals (Seib, 1997). Our observation suggests that the acetylation reaction was limited to hydroxyl groups in certain parts of amylopectin molecules (like branch points) and confirms the finding of Biliaderis (1982) that only particular regions of the amylopectin molecule were highly acetylated.

For modification with vinyl acetate, there was little, if any, difference between small size granule fractions (SVA AP<20 and VAS AP<20) and large size fractions (SVA AP>32 and VAS AP>32) with respect to degradability by α-amylase and substitution pattern. Fragments of DP9 and higher with 2 acetyl groups were present in the hydrolysates of amylopectin obtained from modification with vinyl acetate but not in that of the counterparts obtained after modification with acetic anhydride. For both amylose and amylopectin of yellow pea starch, modification with vinyl acetate

resulted in a more blockwise distribution of acetyl groups compared to modification with acetic anhydride, which is in agreement with the observation for cowpea starch (Huang et al., 2006b).

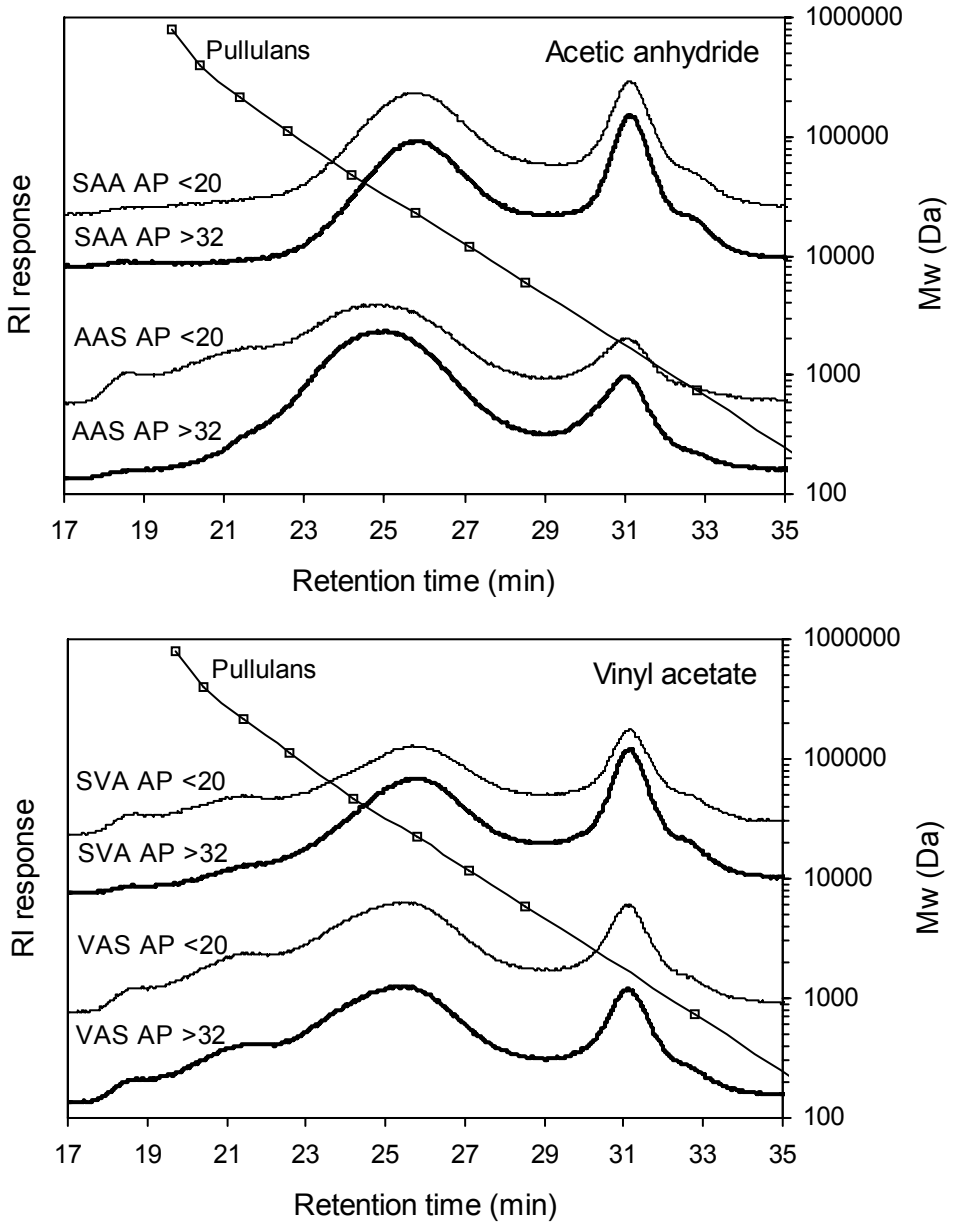

Fig. 6. HPSEC elution profiles of the α-amylase hydrolysates of amylopectin (AP) isolated from small (<20μm) and large (>32μm) granule size fractions of yellow pea starch modified with acetic anhydride (AA) and vinyl acetate (VA) before and after sieving (S), respectively. RI: refractive index.

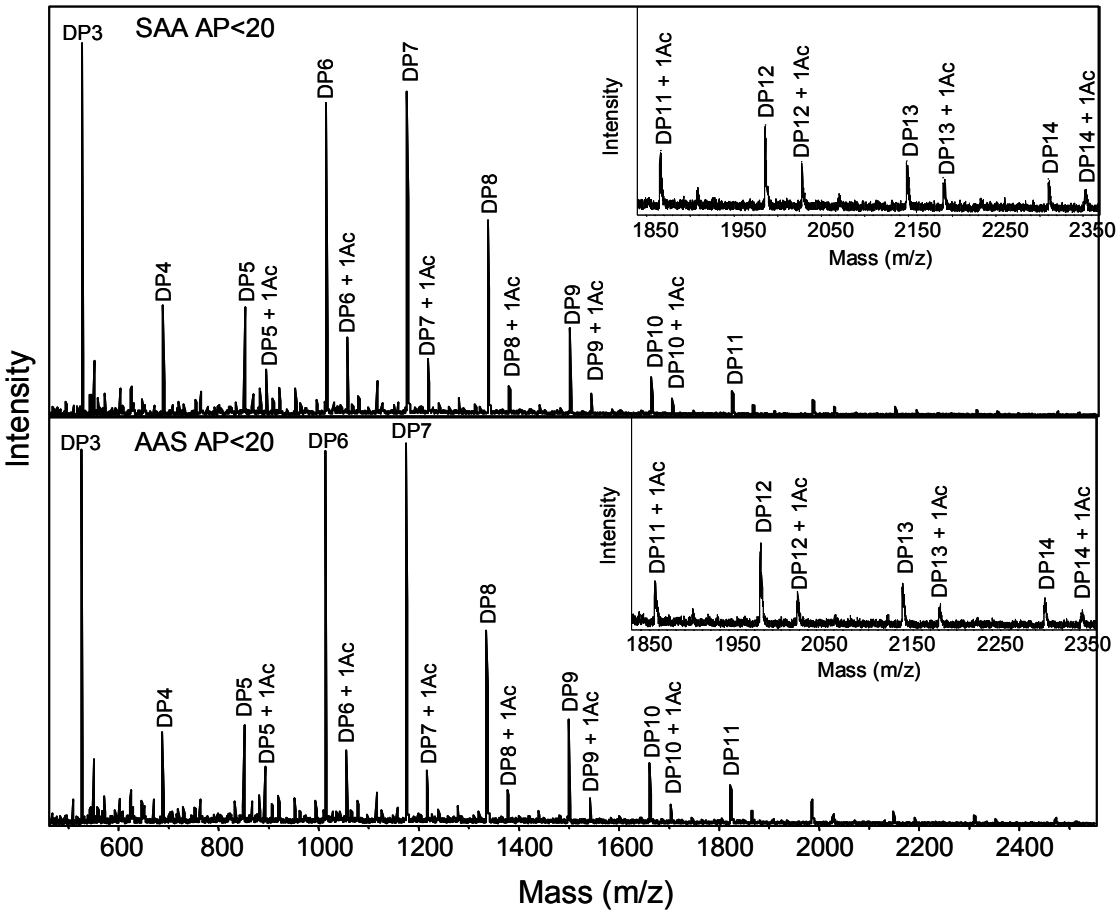

Fig. 7. MALDI-TOF mass spectra of the α-amylase hydrolysates of amylopectin (AP) isolated from small (<20μm) granule size fractions of yellow pea starch modified with acetic anhydride (AA) before and after sieving (S), respectively. DP: degree of polymerization. Ac: acetyl group. A zoom of DP11-14 is inserted.

These findings, combined with our previously results (Huang et al., 2006b), led us to believe that the distribution of acetyl groups differed not only at a molecular level but also at the granular level between modification with acetic anhydride and with vinyl acetate. It is proposed that, for acetylated granular yellow pea starch, acetyl groups are more intensely distributed on and near the granule surfaces when modified with acetic anhydride, while they are more uniformly distributed throughout the granule when modified with vinyl acetate (Fig. 8). Reaction sites within starch granules have been located by utilizing microscopy (Huber et al., 2001; Gray et al., 2004). Investigations on phosphorylated potato starch and hydroxypropyl waxy maize starch revealed that

phosphoryl chloride (highly reactive) appeared to react most prominently on peripheral surfaces of potato granules, while reaction with propylene oxide analog (less reactive) appeared to occur throughout the granule matrix of waxy maize starch in a more uniform manner. Findings from this study suggest that when acetyl groups distributed only in certain regions of granule, the amount of acetyl groups introduced has little impact on the pasting properties and that the reagent type is an important fact for the functional properties of acetylated starch.

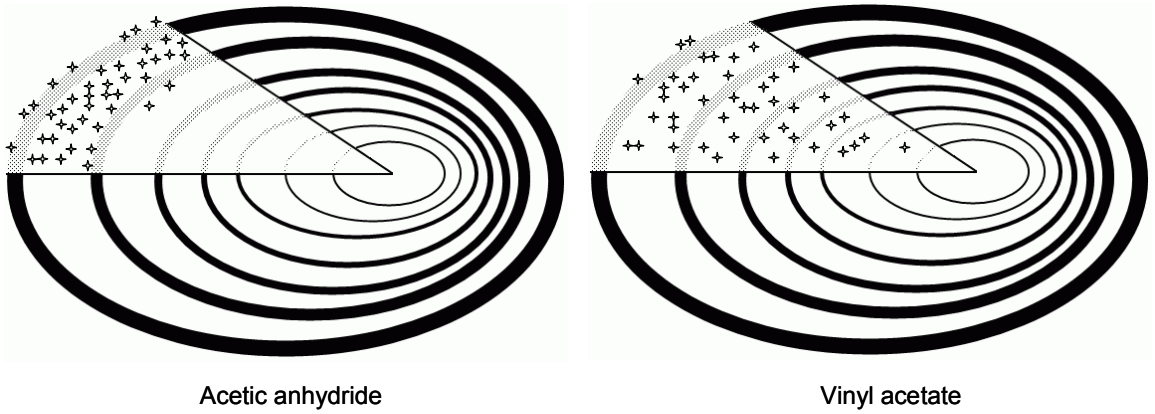

Fig. 8. Proposed model structures for acetylated granular yellow pea starch modified with acetic anhydride and vinyl acetate, respectively. The alternating crystalline (dark) and amorphous (light) lamellae are shown.

Conclusions

When modified with the rapidly reacting acetic anhydride, acetylation before and after sieving resulted in rather different DS values but similar pasting behaviors for small size granule fractions. This suggests that when the distribution of acetyl groups was limited to certain (outer) regions of the granule, the amount of acetyl groups introduced had little impact on the functional properties of starch. When the slowly reacting vinyl acetate was used, the acetylation level and the pasting behavior of the same size fractions were similar for the two modification procedures. The analysis of the α-amylase hydrolysates by mass spectrometry showed that the acetyl distribution pattern was similar between small and large size fractions, but different between two acetylation types for both amylose and amylopectin. Both pasting behavior and fine structure of acetylated starch were found

to depend on the type of reagent. Therefore, reagent type is an important factor for the functional properties of acetylated starch. Consequently, it is essential to mention the reagent used when discussing properties of acetylated starch. The distribution of acetyl groups differed not only at molecular level but also at granular level between modification with acetic anhydride and vinyl acetate. It is proposed that with acetic anhydride only hydroxyl groups in certain parts of the granule, preferably those in the outer lamellae will react, while acetylation occurs throughout the whole granule when the starch reacts with vinyl acetate.

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Chapter 6

General discussion

Introduction

Nowadays a large number of legumes are grown world wide and are mainly used for food due to their high protein and starch content (Moorthy, 2004). The starch component from legumes, representing 35–60% of seed dry weight, has been identified as a new ingredient for food applications (Schoch & Maywald, 1968; Otto, Baik, & Czuchajowska, 1997; Czuchajowska, Otto, Paszczynska, & Baik, 1998). Yellow pea (*pisum sativum*) is very common in Northern Europe, cowpea (*Vigna unguiculata*) and chickpea (*Cicer arietinum*) are cultivated in Asia, Africa, Mediterranean Basin and North America. Until now, the application of these three legume starches has been limited due to limited knowledge of their structure-function relationships, which is especially the case for cowpea and chickpea starches. In our research, the characteristics of starches isolated from cowpea and chickpea seeds were compared with those of commercial yellow pea starch. Furthermore, the legume starches were converted to starch acetates, a common type of modified starches which are produced on an industrial scale. Two reagents, acetic anhydride and vinyl acetate, knowing to have different chemical reactivities (Seib, 1997; Bergthaller, 2004), have been used for acetylation and the acetylated starches were compared for their chemical and physical properties.

Characteristics of yellow pea, cowpea and chickpea starches

Chemical composition of cowpea, chickpea and yellow pea starches

The chemical composition of three legume starches were determined and compared with the characteristics of starches from various plant sources (Table 1). Cowpea and chickpea starches were found to have similar amylose levels as maize and wheat starches, although a high amylose content was mentioned to be one of the characteristics of legume starches (Czuchajowska et al., 1998; Singh, Sandhu, & Kaur, 2004). The amylose content of yellow pea starch, which was close to that of mung bean starch, was higher than that of tuber, root and cereal starches (17-28%). Certain starches such as genetic modifications of maize, namely waxy maize and amylomaize, depart significantly from this range.

Cowpea, chickpea and yellow pea starches were found to have rather high protein contents. The lipid contents of these three legume starches were in the same level as in tuber and root starches. Their phosphorus contents were much lower than that of potato starch which is well known for its

high phosphorus content which has a high impact on the properties of potato starch (Chen, Schols, & Voragen, 2003). The chemical composition of cowpea, chickpea and yellow pea starches were different from those of cereal, tuber and root starches, which may introduce these legume starches in new applications.

Table 1 Chemical composition (w/w, % of dry matter) of starches from different sources

Starch	Type	Amylose	Lipids	Proteins	Phosphorus	Reference
Cowpea	Legume	26	0.15	0.49	0.022	Chapter 2
Chickpea	Legume	27	0.10	0.57	0.012	Chapter 2
Yellow pea	Legume	31	0.07	0.52	0.007	Chapter 2
Mung bean	Legume	32	0.27	0.3	--	Chen et al. (2003)
Potato	Tuber	22	0.1	0.1	0.08	Chen et al. (2003)
Cassava	Root	17	0.1	0.1	0.01	Swinkels (1985)
Sweet potato	Root	20	0.17	0.18	0.019	Chen et al. (2003)
Maize	Cereal	28	0.6	0.35	0.015	Swinkels (1985)
Wheat	Cereal	28	0.8	0.4	0.06	Swinkels (1985)
Waxy maize	Cereal	0	0.2	0.25	0.007	Swinkels (1985)
Amylomaize	Cereal	50-80	0.4	--	0.07	Swinkels (1985)

Properties of cowpea, chickpea and yellow pea starches

Some properties of legume starches, root and cereal starches like average size, X-ray type, gelatinization temperature, swelling power and peak viscosity are summarized in Table 2.
The average granule size of cowpea, chickpea, and yellow pea starches were in the middle of the granule size range (5-42μm) of the starches presented. The gelatinization temperatures of chickpea and yellow pea starches were lower than those of other starches. Only cowpea starch, showing the highest onset gelatinization temperature, is in line with the statement that legume starches are characterized by a high gelatinization temperature (Schoch & Maywald, 1968; Gujska, Reinhard, & Khan, 1994). Pasting properties of cowpea, chickpea, and yellow pea starches were found to be influenced by granule swelling at 90 °C, the higher the swelling power, the higher the peak viscosity. The same tendency was found for potato, cassava, sweet potato, maize and rice starches (Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005b). The C-type X-ray diffraction pattern of these three legume starches was similar as found for mung bean starch by Hoover, Li, Hynes, and Senanayake (1997), and is in agreement with the statement that the C-type is typical for legume starches (Donald, 2004). Potato starch displayed the typical ‘B’ type, and cassava, sweet potato,

maize and rice starches exhibited the 'A' type pattern (Srichuwong, Sunarti, Mishima, Isono, & Hisamatsu, 2005a). A- and B-type were ascribed to a different crystalline packing of double helices, with A-type crystallites being denser and less hydrated, the C-type was attributed to the joint presence of A- and B-crystallites (Gallant, Bouchet, & Baldwin, 1997; Vermeylen, Goderis, Reynaers, & Delcour, 2004).

Table 2 Properties of starches from different origin

Starch	Average size (μm)	X-ray type	Gelatinization temperature(°C)[a]	Swelling power (90 °C)	Peak viscosity (cP)	Reference
Cowpea	16	C	71-81	30	1440	Chapter 2
Chickpea	18	C	58-64	25	870	Chapter 2
Yellow pea	34	C	58-65	21	720	Chapter 2
Mung bean	15	C	65-74	12	900	Chen et al. (2003); Hoover et al. (1997)
Potato	42	B	61-78	42	6000	Srichuwong et al. (2005a, 2005b)
Cassava	23	A	59-80	27	900	Srichuwong et al. (2005a, 2005b)
Sweet potato	23	A	67-87	36	1440	Srichuwong et al. (2005a, 2005b)
Maize	21	A	63-81	21	840	Srichuwong et al. (2005a, 2005b)
Rice	5	A	62-80	30	960	Srichuwong et al. (2005a, 2005b)

[a] Onset and completion gelatinization temperatures.

The properties of cowpea starch were found to be significant different from those of chickpea and yellow pea starches: in addition to the highest gelatinization temperature, cowpea starch also exhibited the largest extent of swelling at 90 °C, the highest pasting viscosity and best freeze-thaw stability among these three starches. The properties of starch can often be attributed to the presence of minor components and the fine structural features of starch. The amylose contents, and levels of other minor components could not be fully related to the properties of cowpea, chickpea and yellow pea starches. Therefore, we continued to characterize the major component amylopectin, which represents 69–74% of these three legume starches.

Relationship between chemical and physical properties of starch

The chain length profiles of amylopectins isolated from cowpea, chickpea and yellow pea starches were analyzed by determining the degradation products after pullulanase hydrolysis with HPSEC (high-performance size-exclusion chromatography) and HPAEC (high-performance anion-exchange chromatography) (Chapter 2).

For all three legume starches, pullulanase digestion released two distinct populations, representing short (DP 6-50) and long (DP 50-80) side chains. The relative proportions of these two populations differed and cowpea amylopectin showed to have the highest level of long chains. The weight proportion of long chains in cowpea amylopectin was both about 1.4 times that of chickpea and yellow pea amylopectins. It is known that longer chains would make longer helices and strengthen hydrogen bonds between chains, whereas shorter chains would form shorter and therefore weaker double helices. Consequently, crystalline regions packed by high proportions of longer chains are more stable, which could retard gelatinization (Srichuwong et al., 2005a)

The onset gelatinization temperature, swelling volume at 90 °C, and RVA peak viscosity of cowpea starch were about 1.2 and 1.2, 1.2 and 1.4, 1.7 and 2.0 times when compared to the corresponding values of chickpea and yellow pea starches. It should be noted that cowpea starch had the highest amount of amylopectin and smallest granule size among the three tested starches. The higher gelatinization temperature was an indication of more perfect crystals (van Soest, Bezemer, de Wit, & Vliegenthart, 1996; Sasaki & Matsuki, 1998) or a higher co-operative unit, which means longer chains in the crystal or a larger crystal size (Matveev et al., 2001). The swelling volume of starch was affected by amylose content and the structure of amylopectin (Sasaki & Matsuki, 1998). The higher swelling volume implied more strongly associated amylopectin chains within the crystalline regions (Hoover et al., 1997). We propose that higher gelatinization temperature, greater pasting peak, and better freeze-thaw stability found for cowpea starch compared to chickpea and yellow pea starches can be (partly) explained by the interactions between the long chains in cowpea amylopectin molecules.

Two recent reports (Srichuwong et al., 2005a, 2005b) came to a similar conclusion that the distribution of unit-chains of amylopectin significantly correlated with functional properties of the starches based on statistic analysis on fifteen starches from different botanical sources. This confirms the idea that the molecular structure of amylopectin is a critical factor in determining physicochemical properties of starch.

Effect of reagent type and granule size on the properties of acetylated starches

Effect of reagent type on the properties of acetylated yellow pea, cowpea and chickpea starches

The effect of reagent type on the properties of acetylated starches was studied for yellow pea, cowpea and chickpea starches after modification with acetic anhydride and vinyl acetate to DS values of 0.06-0.07 which are commonly used in food applications. The X-ray diffraction patterns and the relative crystallinity did not show substantial changes after reaction with acetic anhydride and vinyl acetate for all three legume starches. For low levels of acetylation, the reactions seem to predominantly occur in the amorphous region and on the surface of the crystals, which would explain why no substantial change occurred in crystal structures of granular starch. This was also indicated before by Seib (1997).

Yellow pea, cowpea and chickpea starches showed lower gelatinization temperatures compared to the corresponding native starches. This is due to the insertion of acetyl groups into the starch molecules, especially into the amorphous region, which obstructs the formation of H-bonds and this results in decreased integrity (Saartrat, Puttanlek, Rungsardthong & Uttapap, 2005). Lowering of the gelatinization temperature is one of the many advantages achieved by acetylation (Bergthaller, 2004; Saartrat et al., 2005). This is particular advantageous for application in food products in which heat-labile components are to be incorporated, like in various frozen or cold stored ready-to-eat menus (Moorthy, 2004; Bergthaller, 2004).

Significant differences between the two acetylation types in the pasting and swelling properties of the starches were found. Modification with vinyl acetate resulted in both higher swelling volumes and higher peak viscosities than modification with acetic anhydride for the same starch (Chapter 3). This phenomenon could not be explained by the level of acetylation, since the differences in DS for the two acetylation types were only minor. We suggest that due to the difference in reactivity, the slowly reacting vinyl acetate had time to diffuse more deeply into the granule matrix before reacting with the hydroxyl groups of starch, whereas the rapidly reacting acetic anhydride reacted only with the hydroxyl groups on and near the surface of starch granule. It is proposed that the different pasting and swelling behaviours induced by modification with two reagents is reflecting different distribution patterns of acetyl groups over the starch molecules.

Effect of granule size on properties of native and acetylated yellow pea, cowpea and chickpea starches

There is a broad range of particle sizes present within one starch. The influence of granule size on the properties of native starches and of acetylated starches which have been obtained from modification with either acetic anhydride or vinyl acetate was evaluated (Table 3).

Table 3 Some phycochemical properties of different size fractions of native and acetylated yellow pea, cowpea and chickpea starches modified with acetic anhydride and vinyl acetate

Starch	Average size (µm)	Amylose (%)[a]	Swelling volume (ml/g)[a]	Pasting temperature (°C)	Peak viscosity (cP)	DS[a]
Native						
Yellow <20µm	20.2	28.4	22	68	960	-
Yellow 20-32µm	27.3	27.9	20	69	900	-
Cow <20µm	15.7	25.0	34	80	1420	-
Cow >20µm	16.1	22.5	25	80	1410	-
Chick <20µm	17.2	25.6	29	69	1020	-
Chick >20µm	20.4	30.2	25	70	910	-
Acetic anhydride						
Yellow <20µm	20.2	30.2	25	66	880	0.074
Yellow 20-32µm	26.5	28.9	20	67	740	0.058
Cow <20µm	15.3	25.2	29	76	1110	0.058
Cow >20µm	15.9	24.0	24	77	1130	0.053
Chick <20µm	17.4	32.5	25	67	970	0.057
Chick >20µm	19.8	29.4	23	68	830	0.053
Vinyl acetate						
Yellow <20µm	20.0	29.3	31	66	1500	0.068
Yellow 20-32µm	26.5	29.4	28	67	1200	0.067
Cow <20µm	15.8	27.0	42	76	1570	0.062
Cow >20µm	17.5	23.6	36	76	1530	0.063
Chick <20µm	17.7	31.6	41	66	1530	0.066
Chick >20µm	20.7	29.5	36	66	1390	0.067

[a] Values are based on dry matter.

Effect of granule size on properties of native starches

The granule size showed no influence on the pasting temperature but did influence the swelling volume and peak viscosity (Chapter 3): the smaller the granule size, the higher the swelling volume

and the higher the peak viscosity values found for native yellow pea and chickpea starches. An exception found was that the <20 μm and >20 μm fractions of cowpea starch samples showed similar peak viscosities. This is probably due to the small differences in average size of the two fractions, and this is considered to be indirect evidence for the statement that the peak viscosity relates to the granule size.

The different properties between small and large size fractions could not be related to the amylose content. The small size fractions of native yellow pea starch showed similar amylose contents but higher peak viscosities compared to the large size fractions (Table 3). The chemical fine structure of the small size granule fraction might be different from that of the large size fraction. Tang, Ando, Watanabe, Takeda, and Mitsunaga (2001) reported that the number-average degree of polymerisation (DPn) of amylopectin decreased when granule size decreased for waxy barley starch.

Effect of granule size on the properties of acetylated starches

Yellow pea, cowpea and chickpea starches were fractionated by size after acetylation. Overall, our results showed that the degree of substitution (DS) varied according to the granule size, small size fractions had higher DS than the corresponding large size fractions when acetic anhydride was used as reagent. The DS values were similar, irrespectively of the granule size when the starch was modified with vinyl acetate. The difference in reaction efficiency of differently sized granule fractions resulting in different DS values is due to the fact that acetic anhydride is a rapidly reacting reagent and vinyl acetate is a slowly reacting reagent. Our observations are in accordance with findings by others. Chen, Schols and Voragen (2004) found that granule size affected the DS of acetylated potato and sweet potato starches using acetic anhydride as reagent. Stapley and BeMiller (2003) modified maize, wheat, and potato starches with propylene oxide (a slowly reacting reagent) and found similar degrees of molar substitution for large and small granule fractions of the same starch. Therefore for the reaction efficiency, the influence of granule size should be considered when a highly reactive reagent is used, whereas granule size is not an important factor when starch reacts with a slowly reactive reagent.

Similar as found for the native starches, the swelling volume and pasting viscosity of acetylated starches depended on granule size. These differences in properties between small and large size fractions could not be related to the DS. For example, the small size fractions had similar DS values but higher swelling volume and peak viscosity compared to the large size fractions for chickpea starch modified with vinyl acetate.

The <20 μm fractions of native and acetylated cowpea starch showed a higher gelatinization temperature and peak viscosity than the <20 μm fractions of native and acetylated yellow pea and chickpea starches, which is in agreement with the behaviour of the unfractionated starches. Therefore, discussions on the effect of granule size on the properties of starch are only meaningful when they are limited to one starch type.

Effect of reagent type on the chemical fine structure of acetylated cowpea starch

Analytic methods used for the determination of the chemical fine structure of acetylated cowpea starch

It was found that modification with acetic anhydride and vinyl acetate resulted in different pasting properties of acetylated starches for yellow pea, cowpea and chickpea starches, but no correlation was found between the DS and the physical properties. We therefore further studied the effect of reagent type on the distribution pattern of acetyl groups in cowpea starch. The chemical fine structures of isolated amylose and amylopectin populations were analyzed by enzymatic degradation followed by characterization of the obtained fragments using chromatographic and mass spectrometric techniques (Fig. 1).

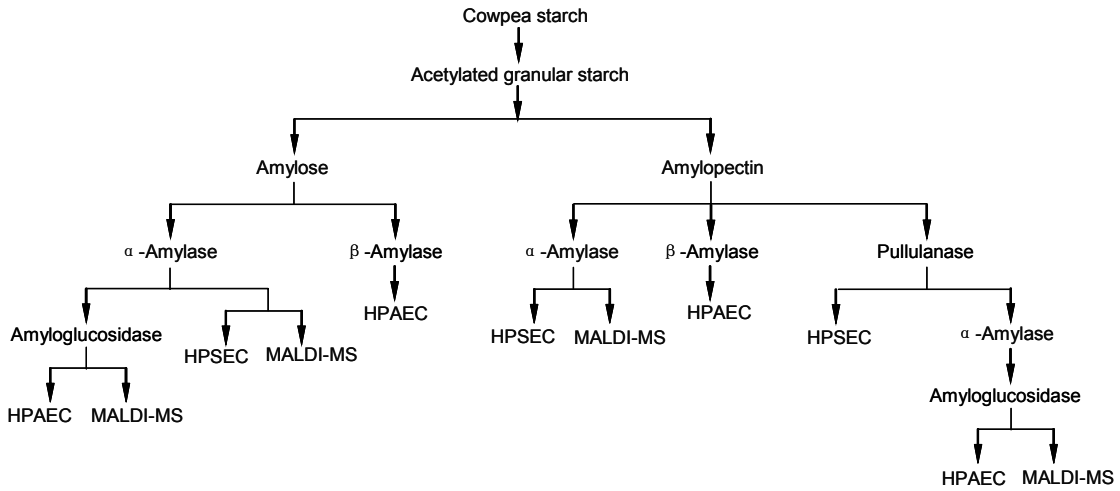

Fig. 1. Analytical scheme used to study the fine structure of acetylated cowpea starch.

Acetyl groups hinder the action of starch hydrolyzing enzymes (Biliaderis, 1982; Chen et al., 2004), and because of that hindrance pure and well characterized enzymes can be used to investigate the distribution pattern of acetyl groups. ß-amylase was used for the estimation of acetyl groups along the external chains, pullulanase for the information of acetyl groups near the branch points of amylopectin. Amylose and amylopectin were exhaustively degraded by the combination of α-amylase and amyloglucosidase, and the combination of pullulanase, α-amylase, and amyloglucosidase, respectively. The oligomers present in the digests of acetylated amylose and amylopectin samples after combined action of the enzymes were enzyme resistant oligomers.

The information on the acetyl distribution was obtained by analyzing both the high Mw fragments by HPSEC and the oligomeric fragments by HPAEC and MALDI-TOF-MS (matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry). HPSEC elution profiles give an overview of the Mw range of the non-degradable fragments. HPAEC analysis gives information on the size and amount of enzyme resistant oligomers. MALDI-TOF mass spectrometry enables determination of the acetyl groups in oligomers after enzymatic degradation, providing additional insight into the chemical fine structure.

A similar approach has been successfully employed for understanding the acetyl distribution pattern in amylopectin molecules obtained from differently sized fractions of potato and sweet potato starches modified with acetic anhydride (Chen, Huang, Suurs, Schols, & Voragen, 2005) and for the chemical fine structure analysis of waxy maize and amylomaize starch ethers like 1-allyloxy-3-methoxy-propan-2-ol starches (Huijbrechts et al., 2006).

In literature, various combinations of enzymatic degradation and chromatography and/or mass spectrometry analyses have been used for investigating the chemical fine structure of acetylated, hydroxypropylated, and methylated starches (Biliaderis, 1982; Steeneken & Woortman, 1994; Kavitha & BeMiller, 1998; van der Burgt, Bergsma, Bleeker, Mijland, Kamerling, & Vliegenthart, 2000b; van der Burgt et al., 2000c; Chen et al., 2004). We were the first to use α-amylase digestion followed by analyzing the digests with MALDI-TOF mass spectrometry. This turned out to be an effective method for determining the distribution of acetyl groups along amylose and amylopectin chains, which will be described in the next section. This method can be applied for structural analysis of other starch esters and starch ethers as well.

Effect of reagent type on the chemical fine structure of acetylated cowpea starch

Since there were only slight differences in the volume mean diameters and pasting behaviours between two size fractions and the amount of the large sized fractions are not sufficient for further investigations, only the small size fractions with DS of 0.058 and 0.062 obtained by modification with acetic anhydride and vinyl acetate were used for the study on chemical fine structure of acetylated cowpea starch.

The analyses of the degradation products obtained after enzymatic hydrolysis by using HPSEC revealed that for both the amylose and amylopectin populations, the susceptibility to α-amylase and β-amylase was different for starches modified with acetic anhydride or vinyl acetate. Clear differences between the two acetylation types were exhibited in the MALDI-TOF mass spectra of the digests obtained from α-amylase hydrolysis indicating that acetyl groups were more block wise distributed along the polymer chains obtained by modification with vinyl acetate compared to modification with acetic anhydride. When amylose and amylopectin were exhaustively degraded with a combination of enzymes, the mass spectra showed that the oligomers in the hydrolysates were similar for the two acetylation types. Therefore, no additional information is obtained by digestion with a combination of enzymes compared to digestion with α-amylase alone on the acetyl substitution pattern between two types of acetylation (Chapter 4).

The granule structure is preserved throughout the course of acetylation. The organization of granules influences the substitution pattern since reagents with different reactivity penetrate into the granule with different efficiencies (Bertoft, 2004).

Modifications with reagents with different reactivity have been compared between different modification types and/or between different starch sources in literature. Biliaderis (1982) compared acetylated smooth pea starch with hydroxypropylated waxy maize starch and suggested that acetylation occurred exclusively in certain part of the granule, whereas hydroxypropylation was more uniform throughout the starch granule. In studies toward the reaction sites within phosphorylated potato starch and hydroxypropyl waxy maize starch at granular level, Huber and BeMiller (2001) and Gray and BeMiller (2004) used microscopy. Highly reactive phosphoryl chloride appeared to react most prominently on peripheral surfaces of potato granules, while reaction with a less reactive propylene oxide analog appeared to occur throughout the granule matrix of waxy maize starch in a more uniform manner. In all these cases, the investigations were performed with respect to the fine structural of the modified starches.

Combined with our findings that the rapidly- and slowly- acetylating reagents resulted in different chemical fine structures as well as different pasting viscosities of acetylated starch, it may be predicted that in other types of chemically modified starch, reagents with different reactivity might result not only in different fine structures but also in different properties of the starch within one modification type.

Acetylation as a potential tool for elucidating the detailed structure of amylose and amylopectin in starch granules

A similar approach has been used to investigate the chemical fine structure of acetylated potato and sweet potato starches (Chen et al., 2004, 2005). However, differences were found between our results obtained from cowpea starch and those from potato and sweet potato starches. HPAEC analysis showed that significant amounts of dimer were present in the digests of acetylated cowpea amylose and amylopectin after combined enzymatic hydrolysis. A similar finding was observed for amylopectins isolated from acetylated potato and sweet potato starches (Chen et al., 2005), but there was no dimer present in the digests of the corresponding amyloses (Chen et al., 2004). This suggests that the acetyl group distribution on amylose may be different according to the starch source, while this is not the case for amylopectin. The results might be indicative for the roles of amylose and amylopectin in starch granule: amylose is the more flexible component, whereas amylopectin is the structural component forming crystallites in the granule.

Further study could be carried out on the individual molecules to identify the glucosyl moiety within the oligomer substituted by the acetyl group, to determine the linkage type (1,4 vs. 1,6 linkage) and the precise location of the acetyl groups on the glucosyl unit, that is, the position of O-acetylation. In the investigation of the specificity of enzymes on hydrolysis of methyl amylose, Mischnick (2001) found that the position of O-methylation was an important structural feature for the action of the α-amylase and amyloglucosidase, showing inhibition in the following order: 3-O-methylation > 2-O-methylation > 6-O-methylation. Establishing the specificity of enzymes on degradation of acetylated amylose and amylopectin will be helpful for a better understanding of the fine structure of acetylated starch. In addition, by comparing the enzyme degradation products obtained from different acetylated starches, acetylation can be used as a tool to elucidate the detailed structure of amylose and amylopectin in starch granules.

Effect of reagent type and granule size on the chemical fine structure of acetylated yellow pea starch

Effect of reagent type on the chemical fine structure of acetylated yellow pea starch based on granule size

The effect of reagent types on the properties of acetylated yellow pea, cowpea and chickpea starches has been investigated by first acetylating and then sieving into different size fractions, and the chemical fine structure of acetylated cowpea was studied for one size fraction. Further investigation on acetylated yellow pea starch was carried out by using two modification procedures: the first procedure included fractionation into small and large granule fractions and then acetylation of these fractions (acetylation after sieving), the second procedure included acetylation and then fractionation of acetylated starch into small and large granule fractions (acetylation before sieving) (Chapter 5).

When using acetylation after sieving, the <20μm fraction modified with acetic anhydride had the same size and the same DS as the <20μm fraction modified with vinyl acetate, however the former fraction showed a lower peak viscosity than the later one. These findings confirm that it is not the different DS but the different acetyl distribution which determines the different pasting behaviours of starches obtained from two acetylation types (Chapter 3).

The extent of difference in reaction efficiency between two size fractions was larger when there was a competition for rapidly reacting acetic anhydride (acetylation before sieving) than when there was no competition (acetylation after sieving). The lower reaction efficiency of the large (>32 μm) granule fraction than that of the small (<20 μm) fraction even when they reacted separately with acetic anhydride (acetylation after sieving) could be explained by the difference in specific surface area of these two fractions. Assuming that the granule is a sphere and that the densities of small and large granules are uniform, it can be approximated that the diameter of a >32 μm granule is about 1.5 times that of a <20 μm granule, which means that the specific surface area of the <20 μm fraction is about 1.5 times that of the >32 μm fraction. When reacting with same amount of acetic anhydride, the distribution of the reagent over the granule surface is different between these two fractions: a thin layer around small granules and thicker layer around large granules (Fig. 2). The thin layer of acetic anhydride around small granules may increase the chance of reaction with starch and thereby reduce the chance of its side reaction with water and consequently result in a higher reaction efficiency.

The distribution pattern of acetyl groups over amylose and amylopectin molecules was determined by α-amylase degradation followed by HPSEC and HPAEC analyses. When acetic anhydride was used as acetylating reagent, the α-amylase degradation pattern of the small and large size fractions was significant different for both amylose and amylopectin when starch was obtained from acetylation before sieving, whereas only slight differences were found when acetylation was obtained after sieving. Such phenomena did not appear when starch was modified with vinyl acetate: similar α-amylase degradation pattern of amylose was found for the small and large size fractions for both modification procedures, and it was the same case for amylopectin.

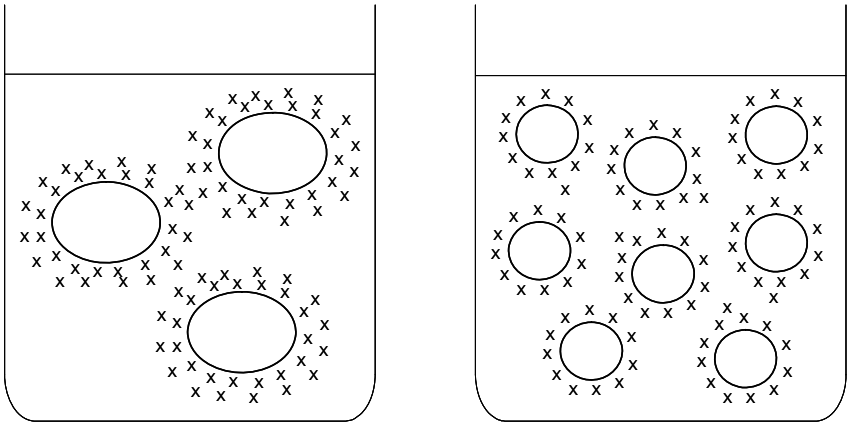

Fig. 2. Schematic view of the reactions of small and large size granule fractions with acetic anhydride, respectively (acetylation after sieving).

The information about the location of acetyl groups on amylose and amylopectin molecules was obtained by characterizing the oligomers in the α-amylase hydrolysates with MALDI-TOF mass spectrometry. For yellow pea starch modified with acetic anhydride, the distribution of acetyl groups over amylose molecules appeared to differ for the two acetylation procedures: acetylation before sieving resulted in less higher substituted fragments in the α-amylase hydrolysates, whereas acetylation after sieving resulted in more higher substituted fragments. However, only slight differences in the distribution of acetyl groups were found for the amylopectin samples between the two acetylation procedures. This indicates that only hydroxyl groups in certain region (like branch points) of the amylopectin are accessible for chemical reagents due to their presence in the amorphous parts, and that amylose has flexibility in the organization of the starch granule as mentioned in the former section.

For modification with vinyl acetate, there was only a slight difference in the distribution pattern of acetyl groups as initiated by the two acetylation procedures. In general, our findings show that the reagent type is an important factor for both functional properties and chemical fine structure of acetylated granular starches, and that the different distribution of acetyl groups obtained by modification with the two reagents (acetic anhydride vs. vinyl acetate) can be demonstrated at various structural levels of starch granule: at the level of crystalline and amorphous regions, and at the molecular level of amylose and amylopectin.

Topics for future investigation--acetyl distribution pattern at granular level

Our studies provide evidence for differences between modification with acetic anhydride and with vinyl acetate only at molecular level. Since different substitution patterns found in amylose and amylopectin molecules for two acetylation types could not fully explain the different pasting behaviour, a difference at granular level was proposed for yellow pea starch: acetyl groups were distributed more intensely on and near the granule surfaces when modification was performed with acetic anhydride, while the distribution of acetyl groups was more uniform throughout the granule in the case that vinyl acetate was used (Chapter 5).

For cowpea and chickpea starches, which showed decreasing peak viscosity after modification with acetic anhydride, investigation of the properties of the acetylated starch with a series of DS may be needed for the further proof of the proposed model.

The distribution of acetyl groups at granular level can be further explored by separating the modified and unmodified fractions from acetylated starch modified with acetic anhydride. The chemical gelatinization method developed by Jane and Shen (1993) maybe suitable for this purpose. The starch was chemically gelatinized at the periphery of the granules at room temperature. The gelatinized and thus diffused outside layer of starch granules was isolated from the remaining granules mechanically. The method has been used for yielding a series of “pealed” remaining methylated potato and amylopectin potato starch granules (van der Burgt, Bergsma, Bleeker, Mijland, Kamerling, & Vliegenthart, 2000a) and the similar molar substitution of all remaining granules indicates that the methyl substituents are distributed equally within each starch granule.

In general, it can be stated that our study contributes to the knowledge of functional properties and its relationship with the chemical fine structure of acetylated pea starches based on reagent type used. The information is also practical for producing acetylated starch on a commercial scale. The

reagent type is a very important factor for pasting properties of acetylated starch. Modification with vinyl acetate results in much higher peak viscosity than modification with acetic anhydride with the same DS and same granule size. For modification with acetic anhydride, the DS varies on granule size, whereas for modification with vinyl acetate, the DS is independent on granule size. However, the DS value for starches obtained by modification with acetic anhydride was not so important for the pasting behaviour of the starch as was the case for modification with vinyl acetate. Granule size affects the peak viscosity of both native and acetylated starches. The importance of the factors for the properties of acetylated starch is in the order of: reagent type> granule size > acetylation level. The acetyl distribution on amylose and amylopectin molecules depends mainly on the type of reagent and is not affected by the granule size.

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SUMMARY

Yellow pea (*Pisum sativum*), cowpea (*Vigna unguiculata*) and chickpea (*Cicer arietinum*) have been consumed traditionally as whole seeds or as ground flour. Their main component—starch, has been identified as a new ingredient for food application in addition to the more commonly used starches from tuber, root and cereal. Due to the limited knowledge on the structure-function relationships of these three legume starches, their applications have not been well developed, as is especially the case for cowpea and chickpea starches. Therefore, the aim of this thesis was to improve the basic knowledge of cowpea, chickpea and yellow pea starches, in particular the relationships between the molecular structure and the properties of starch.

Acetylated starch, one of the common forms of modified starches, has been widely used in food and non-food industries. Acetic anhydride and vinyl acetate, which are quite different in chemical reactivity, are both used for producing commercial acetylated starch. But little information is available for the effect of the reagent type on the properties and structure of acetylated starch. So, the second aim of this thesis was to obtain understanding of the effect of reagent type (acetic anhydride vs. vinyl acetate) on the physical properties and chemical fine structure of acetylated legume starches.

The general description of starch sources, starch structure, modified starches especially acetylated starch, together with general information on yellow, cowpea, and chickpea starches are summarized in Chapter 1, while also different approaches and methods to analyze the chemical fine structure of starch molecules is discussed.

The chemical composition, properties and amylopectin chain length profiles of starches isolated from cowpea and chickpea seeds were determined and compared with the characteristics of commercial yellow pea starch (Chapter 2). The amylose content of yellow pea starch was higher than that of cowpea and chickpea starches. The average granule size of cowpea, chickpea, and yellow pea starches were around 16, 18, and 34 μm, respectively. All three legume starches showed a C-type X-ray diffraction pattern and a two-stage swelling pattern. Cowpea starch exhibited the highest gelatinization temperature, the largest extent of swelling at 90 °C, the highest pasting viscosity and the best freeze-thaw stability among the three starches. These phenomena could not be explained by the amylose contents or granule sizes. Therefore, the unit chain profiles of isolated amylopectin molecules from the three legume starches were investigated by analyzing the degradation products after pullulanase hydrolysis using HPSEC (high-performance size-exclusion

chromatography) and HPAEC (high-performance anion-exchange chromatography). For all three legume starches, the unit chains of amylopectin can be divided into two populations, representing short (DP 6-50) and long (DP 50-80) chains. The proportion of long chains in cowpea amylopectin was found to be significant higher than those in chickpea and yellow pea amylopectins. It is proposed that the higher gelatinization temperature, the greater pasting peak viscosity, and better freeze-thaw stability found for cowpea starch compared to chickpea and yellow pea starches can be attributed to the interactions between the long chains in cowpea amylopectin molecules.

Yellow pea, cowpea and chickpea starches were modified with acetic anhydride and vinyl acetate to study the effect of reagent type on the properties of acetylated starches (Chapter 3). The X-ray diffraction patterns and the relative crystallinity of the starches did not show substantial changes upon derivatisation, while the gelatinization temperatures decreased after acetylation for all three starches. Yellow pea, cowpea and chickpea starches were fractionated by size after acetylation and the degree of substitution (DS) was found to vary according to the granule size when the rapidly reacting acetic anhydride was used as reagent. The DS values were independent on the granule size when the starch was modified with the slowly reacting vinyl acetate. Modification with vinyl acetate resulted in both a higher swelling volume and a higher peak viscosity when compared with modification by acetic anhydride. It is proposed that the different properties were induced by a different distribution of acetyl groups over the starch molecules for the two acetylation types.

In Chapter 4, the effect of reagent type on the chemical fine structure of acetylated starch was studied for cowpea starch using enzymatic degradation followed by characterization of the obtained fragments by chromatographic and mass spectrometric techniques. For both acetylation types, the amylose population showed a much higher level of acetylation than amylopectin populations, indicating that hydroxyl groups of glucose moieties present in the amorphous region are more accessible for substitution than glucose hydroxyls present in the crystalline regions of starch granules. The results of α-amylase, β-amylase, and pullulanase digestion reveal that more acetyl groups were present in the external chains and in the vicinity of branch points of amylopectin isolated from cowpea starch acetylated with vinyl acetate than in the external chains and in the vicinity of branch points of amylopectin from cowpea starch acetylated with acetic anhydride. For both amylose and amylopectin, the oligomers in α-amylase digests showed clear differences between the two acetylation types as exhibited in the MALDI-TOF (matrix-assisted laser desorption/ionisation time-of-flight) mass spectra. The presence of more highly acetylated fragments in digests of vinyl acetate modified starch suggests that acetyl groups were present more clustered along the polymer chains compared to modification with acetic anhydride. When amylose

and amylopectin were exhaustively degraded with a combination of enzymes, the mass spectra showed that the oligomers in the hydrolysates were similar for the two acetylation types. It is recommended that the difference in acetyl substitution pattern between two types of acetylation can be simply analyzed by α-amylase degradation.

Further investigations on acetylated yellow pea starch was carried out by using two modification procedures: the first procedure included fractionation into small and large granule fractions and then acetylation of these fractions (acetylation after sieving), the second procedure included acetylation and then fractionation of acetylated starch into small and large granule fractions (acetylation before sieving) (Chapter 5). When modified with acetic anhydride, the two acetylation procedures resulted in rather different DS values but similar pasting behaviors for small size granule fractions. When vinyl acetate was used, the two acetylation procedures had little impact on the acetylation level and the pasting behavior of the same size fractions. This suggests that DS may be less important for the pasting properties of acetylated starches obtained after modification with acetic anhydride. The substitution pattern over amylose and amylopectin molecules was determined by α-amylase degradation followed by HPSEC and HPAEC analyses. When acetic anhydride was used as acetylating reagent, the α-amylase degradation pattern was found to be depend on the acetylation procedure for both amylose and amylopectin. Acetylation before sieving resulted in significant different degradation patterns of the digests from the small and large size fractions, whereas only slight differences in the degradation patterns of the two size fractions were found when starch was acetylated after sieving. On the contrary, the α-amylase degradation pattern was independent from the acetylation procedure for starch modification with vinyl acetate.

The location of acetyl groups on amylose and amylopectin molecules was determined by characterizing the oligomers in the α-amylase hydrolysates with MALDI-TOF mass spectrometry. For yellow pea starch modified with acetic anhydride, the location of acetyl groups over amylose molecules appeared to differ for the two acetylation procedures: acetylation before sieving resulted in less higher-substituted fragments in the α-amylase hydrolysates, whereas acetylation after sieving resulted in more higher-substituted fragments. However, the oligomers in the hydrolysates of the amylopectin samples were similar for the two acetylation procedures. For modification with vinyl acetate, only slight difference in the acetyl distribution was found for the two acetylation procedures. In general, our findings show that the reagent type is an important factor for both functional properties and chemical fine structure of acetylated granular starches.

Since the different substitution pattern in starch molecules found for the two acetylation types could not fully explain the difference in pasting behaviour, the different substitution pattern at granular level was proposed for yellow pea starch: acetyl groups distributed more intensely on and near the granule surfaces when modified with acetic anhydride, while more uniformly throughout the granule when vinyl acetate was used.

In the final chapter (Chapter 6), the main results of this study are summarized and discussed in more detail. Reagent type (acetic anhydride vs. vinyl acetate) was found to have influence on both substituent distribution in amylose and amylopectin molecules and the pasting properties of acetylated legume starches. Strategies which could be useful for further characterization of the substitution patterns of acetylated starches at granular level are discussed.

SAMENVATTING

De erwt (*Pisum sativum*), cowpea (*Vigna unguiculata*) en de kikkererwt (*Cicer arietinum*) worden zowel als hele zaden en als meel geconsumeerd. Hun voornaamste bestandsdeel, zetmeel, wordt gebruikt als een nieuw ingrediënt in levensmiddelen, naast de meer algemeen toegepaste zetmelen uit knol, wortel en graan. Als gevolg van de beperkte kennis van de structuur - functie relatie van deze drie peulvrucht zetmelen, is hun toepassing nog niet goed ontwikkeld. Dit geldt zeker voor zetmeel afkomstig van de cowpea en de kikkererwt. Het doel van dit proefschrift is daarom de basiskennis van zetmeel afkomstig van cowpea, kikkererwt en erwt, in het bijzonder de relatie tussen de moleculaire structuur en de eigenschappen van zetmeel, te verbeteren.

Geacetyleerd zetmeel, een veel voorkomende vorm van gemodificeerde zetmelen, wordt veelvuldig gebruikt door zowel binnen als buiten de levensmiddelensector. Azijnzuuranhydride en vinylacetaat, die een verschillende chemische reactiviteit bezitten, worden beiden gebruikt voor het produceren van commercieel geacetyleerde zetmelen. Er is weinig informatie beschikbaar over het effect van het type reagens op de eigenschappen en structuur van de geacetyleerde zetmelen. Daarom is het tweede doel van dit proefschrift om kennis te vergaren over het effect van het type reagens op de fysische eigenschappen en chemische fijn structuur van geacetyleerde peulvruchtzetmeel.

De algemene beschrijving van zetmeelbronnen, zetmeelstructuur, gemodificeerde (geacetyleerd) zetmeel, samen met algemene informatie over erwt-, cowpea- en kikkererwtzetmeel is samengevat in hoofdstuk 1. Dit hoofdstuk bevat tevens een discussie over de verschillende benaderingen en methoden om de chemische fijnstructuur van zetmeel te analyseren.

De chemische opbouw, eigenschappen en de lengte van amylopectine ketens van zetmeel geïsoleerd uit cowpea en kikkererwt werden bepaald en vergeleken met de karakteristieken van commercieel erwtenzetmeel (Hoofdstuk 2). Het amylose gehalte van erwtenzetmeel was hoger dan die van cowpea- en kikkererwtzetmeel. De gemiddelde korrelgrootte van cowpea-, kikkererwt- en erwtenzetmeel was respectievelijk 16, 18 en 34 μm.

Alle drie peulvruchtzetmelen vertoonden een C-type röntgendiffractie en een twee-staps zwellinggedrag. Het cowpea zetmeel vertoonde de hoogste verstijfselingstemperatuur, met de grootste mate van zwelling bij 90°C, de grootste piekviscositeit en de beste vries-dooi stabiliteit van de drie zetmelen. Deze verschijnsels konden niet worden verklaard door het zetmeelgehalte en de korrelgrootte. Daarom werd de lengte van de zijketens van geïsoleerd amylopectine uit de drie peulvruchtzetmelen bestudeerd d.m.v. de analyse van de pullulanase-afbraakproducten m.b.v. HPSEC (high-performance size-exclusion chromatography) en HPAEC (high-performance anion-exchange

chromatography). Voor alle drie peulvruchtzetmelen kunnen de amylopectine zijketens worden verdeeld in 2 populaties: korte zijketens (DP6-50) en lange zijketens (DP 50-80). Het aandeel van lange zijketens in cowpea amylopectine was significant hoger dan die in kikkererwt- en erwtenamylopectine. Er wordt gepostuleerd dat de hogere verstijfselingstemperatuur, de grotere piekviscositeit, en de betere vries-dooi stabiliteit van cowpea zetmeel in vergelijking met kikkererwt- en erwtenzetmeel, wordt veroorzaakt door de interactie tussen de lange zijketens in de cowpea amylopectine.

Erwten-, cowpea en kikkererwtzetmeel werd gemodificeerd met azijnzuuranhydride en vinylacetaat om het effect van het type reagens op de eigenschappen van geacetyleerd zetmeel te bestuderen (Hoofdstuk 3). De röntgendiffractiepatronen en de relatieve kristalliniteit van de zetmelen vertoonde geen substantiële verschillen na derivatisering, terwijl de verstijfselingstemperatuur afnam na acetylering voor alle drie zetmelen.

Erwten-, cowpea- en kikkererwtzetmeel werd gefractioneerd op grootte na acetylering met het snel reagerende azijnzuuranhydride en de mate van substitutie (DS) bleek te variëren met de korrelgrootte. De DS waarden waren onafhankelijk van de korrelgrootte wanneer zetmeel werd gemodificeerd met het langzaam reagerende vinylacetaat. Modificaties met vinylacetaat resulteerde in een groter volume na zwelling en in een hogere piekviscositeit, wanneer dit werd vergeleken met de modificatie door azijnzuuranhydride. Er wordt gesteld dat de afwijkende eigenschappen werden veroorzaakt door een verschillende distributie van acetyl groepen over het zetmeelmolecuul voor de twee acetyleringsreagentia.

In hoofdstuk 4 wordt het effect van het type reagents op de chemische fijnstructuur van geacetyleerd zetmeel voor cowpea zetmeel bestudeerd, gebruikmakend van enzymatische afbraak gevolgd door karakterisering van de verkregen fragmenten met chromatografische en massa spectrometrische technieken. Voor beiden acetyleringsreagentia vertoonde de amylose populatie een veel hogere acetyleringsgraad dan de amylopectine populatie. Dit duidt erop dat de hydroxylgroepen van de glucose-eenheden aanwezig in de amorfe delen van de zetmeelkorrel beter toegankelijk zijn voor substitutie dan hydroxylgroepen die aanwezig zijn in de kristallijne regionen. De resultaten van α-amylase, β-amylase en pullulanase afbraak toonden aan dat er meer acetyl-groepen aanwezig zijn in de externe ketens en in de nabije omgeving van vertakkingspunten van amylopectine geïsoleerd uit cowpea zetmeel na acetylering met vinylacetaat in vergelijking met cowpea zetmeel na acetylering met azijnzuuranhydride. Zowel voor amylose als voor amylopectine is er een verschillend oligomerenpatroon te zien m.b.v. MALDI-TOF (matrix-assisted laser desorption/ionisation time-of-flight) massa spectrometrie in de α-amylase digesten van de

verschillend geacetyleerde zetmelen. De aanwezigheid van fragmenten met meerdere acetylgroepen in digesten van vinylacetaat-gemodificeerd zetmeel duidt erop dat acetylgroepen meer geclusterd binnen de polymeerketen voorkomen wanneer vergeleken met zetmeelketens gemodificeerd met azijnzuuranhydride. Wanneer amylose en amylopectine volledig werden afgebroken met een combinatie van enzymen, bleek uit de massa spectra dat de oligomeren in de hydrolysaten vergelijkbaar waren voor de twee acetylerings typen. Het wordt aanbevolen om het verschil in acetylsubstitutiepatroon tussen twee acetyleringstypen te analyseren met alleen maar een α-amylase afbraak.

Vervolgonderzoek naar geacetyleerd erwtenzetmeel werd uitgevoerd door twee modificatie methoden te vergelijken: de eerste procedure omvatte fractionering in kleine en grote korrelfracties en vervolgens acetylering van deze fracties (acetylering *na* het zeven); de tweede procedure omvatte acetylering en vervolgens fractionering van geacetyleerd zetmeel in kleine en grote korrelfracties (acetylering *voor* het zeven)(hoofdstuk 5). Wanneer werd gemodificeerd met azijnzuuranhydride, resulteerde de twee procedures in verschillende DS-waarden, maar vergelijkbaar verstijfselingsgedrag voor de kleine korrelfracties. Wanneer vinylacetaat werd gebruikt, was er nauwelijks invloed van de twee procedures op de mate van acetylering en het verstijfselingsgedrag op fracties met dezelfde grootte. Dit wijst erop dat de DS mogelijk van minder belang is op de verstijfselingseigenschappen van zetmelen, geacetyleerd met azijnzuuranhydride. Het substitutiepatroon van amylose en amylopectine moleculen werd bepaald na α-amylase afbraak en HPSEC- en HPAEC-analyse. Wanneer azijnzuuranhydride werd gebruikt als acetyleringsreagens, bleek voor zowel amylose als amylopectine dat het α-amylase afbraakpatroon afhankelijk was van de acetyleringsprocedure. Acetylering *voor* het zeven resulteerde in duidelijk verschillend afbraakpatroon voor de digesten van de kleine en grote zetmeelkorrels, terwijl slechts marginale verschillen in het afbraakpatroon van de beide korrelgroottes werd gevonden, wanneer zetmeel werd geacetyleerd *na* het zeven. Voor zetmeel dat werd gemodificeerd met vinylacetaat werd juist het tegendeel gevonden: het α-amylase afbraakpatroon was onafhankelijk van de acetylerings procedure.

De locatie van de acetylgroepen binnen amylose- en amylopectinemoleculen werd bepaald door middel van het karakteriseren van oligomeren in de α-amylase hydrolysaten met behulp van MALDI-TOF massa spectrometrie.

Voor erwtenzetmeel gemodificeerd met azijnzuuranhydride, lijkt de locatie van acetylgroepen over amylosemoleculen te verschillen voor de twee acetyleringsprocedures: acetylering *voor* het zeven

resulteert in minder hoog-gesubtitueerde fragmenten in de α-amylase hydrolysaten dan voor amylose verkregen d.m.v. acetylering *na* het zeven. Voor de hydrolysaten van amylopectine verkregen met beide acetyleringsprocedures werd een gelijk oligomerenpatroon gevonden. Voor modificatie met vinylacetaat, werden voor de twee acetylerings procedures alleen kleine verschillen gevonden in de verdeling van de acetylgroepen. In het algemeen tonen onze bevindingen aan dat het type reagens een belangrijke factor is voor de chemische fijnstructuur en de functionele eigenschappen van geacetyleerde zetmeelkorrels.

Gevonden wordt dat de verschillen in de distributiepatronen voor zetmelen geacetyleerd met beide reagentia niet helemaal het verschil in verstijfselingsgedrag kunnen verklaren. Daarom wordt voor erwtenzetmeel het verschil in substitutiepatroon op korrelniveau als volgt voorgesteld: acetylgroepen zijn vooral op en dichtbij het korreloppervlak gesubstitueerd in het geval van modificatie met azijnzuuranhydride, terwijl een meer uniforme verdeling over de korrel gevonden wordt in het geval van modificatie met vinylacetaat.

In het laatste hoofdstuk (hoofdstuk 6), worden de voornaamste resultaten van dit onderzoek samengevat en bediscussieerd. Het type reagens (azijnzuuranhydride t.o.v. vinylacetaat) bleek invloed te hebben op de substituentverdeling in amylose- en amylopectinemoleculen en op de verstijfselingseigenschappen van geacetyleerd peulvruchtzetmeel. Strategieën worden besproken voor een verdere karakterisering van substitutiepatronen van geacetyleerd zetmeel op korrelniveau.

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Junrong Huang

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Curriculum vitae

Junrong Huang was born on November 19, 1971 in Zhenjiang city, Jiangsu province, China. She graduated from Wuxi Institute of Light Industry in 1993 and received a Bachelor's degree in Food Science. After graduation, she worked as a technician at Quanzhou Non-Staple Food Factory. She obtained her Master's degree in Food Engineering from South China University of Technology in 1998. After working at R&D in Global Chicken Company for half a year, she worked as a teaching assistant, and then as a lecturer in the Department of Food Engineering in Shaanxi University of Science & Technology. From September 2002, she started her PhD project in Southern Yangtze University. From May 2004, she worked on the project of "The properties and fine structure of acetylated starches" in Food Chemistry Group, Wageningen University and Research Centre. She will return to China after the graduation.

List of Publications

Chen, Z., Huang, J., Suurs, P., Schols, H. A., & Voragen, A. G. J. (2005). Granule size affects the acetyl substitution on amylopectin populations in potato and sweet potato starches. *Cabohydrate Polymers*, 62, 333-337.

Huang, J., Schols, H. A., van Soest, J. J. G., Jin, Z., Sulmann, E., & Voragen, A. G. J. (2007). Physicochemical properties and amylopectin chain profiles of chickpea, cowpea and yellow pea starches. *Food Chemistry*, 101, 1355–1362.

Huang, J., Schols, H. A., Jin, Z., Sulmann, E., & Voragen, A. G. J. (2006). Characterization of differently sized granule fractions of yellow pea, cowpea and chickpea starches after modification with acetic anhydride and vinyl acetate. *Cabohydrate Polymers,* In press.

Huang, J., Schols, H. A., Jin, Z., Klaver, R., & Voragen, A. G. J. (2006). Acetyl substitution patterns of amylose and amylopectin populations in cowpea starch modified with acetic anhydride and vinyl acetate. *Cabohydrate Polymers,* In press.

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Huijbrechts, A. A. M. L., Huang, J., Schols, H. A., van Lagen, B., Visser, G. M., Boeriu, C. G., Sudhölter, E. J.R. (2006). 1-Allyloxy-3-methoxy-propan-2-ol-starch: Synthesis and characterization. *Biomacromolecules,* Submitted.

Addendum

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Overview of completed training activities

Discipline specific activities

Courses:

Advances in Carbohydrate Chemistry (Southern Yangtze University, Wuxi, China, 2002-2003)

Mathematical Modeling (Southern Yangtze University, Wuxi, China, 2002-2003)

Macromolecular Physics (Southern Yangtze University, Wuxi, China, 2002-2003)

Experiments of Modern Instrumentation (Southern Yangtze University, Wuxi, China, 2002-2003)

VLAG Summer Course Glycosciences (Wageningen, June 2004)

Conferences:

5th International Conference of Food Science and Technology (Wuxi, China, October 2003)

The 57th Starch Convention (Detmold, Germany, April 2006)

General courses:

Study on Information Sources (Southern Yangtze University, Wuxi, China, 2003)

Food Chemistry PhD trip (Japan, December 2004)

Additional activities:

Food Chemistry Seminars (Wageningen, 2004-2006)

Food Chemistry Colloquia (Wageningen, 2004-2006)

Food Chemistry Carbohydrate and Enzyme meetings (Wageningen, 2004-2006)

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