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The Role of Amylose-LPC Inclusion Complexation on the Functional Properties and Digestibility of Wheat Starch

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The scope of the thesis

Public awareness of the effects of diet on human health has increased in the past decades with considerable emphasis on the glycemic index (GI) which describes the level of the postprandial glucose rise in blood comparing to glucose. Food intake with high GI is associated with obesity, diabetes and chronic and cardiovascular diseases. Starchy foods, derived from different sources, have an important role in increasing the GI; therefore starch is frequently correlated with the amount of rapidly digestible starch (RDS). Based on this, rendering starch digestion from fast to slow helps to reduce the GI of starch-based foods.

It is known that amylose is able to develop inclusion complexes with hydrophobic ligands, such as fatty acids and phospholipids. We used this to reduce the digestibility of starch, since the formation of amylose inclusion complexes renders amylose less accessible to amylase. However, this complex formation also widely influences the functional properties of starch. Therefore, this thesis consists of two parts. The first part investigates the fundamentals of the formation of inclusion complexes including the effect on the functional properties of wheat starch; and the second part assesses the effect of this complexation on the digestibility of starch granules.

Since wheat starch is a basic ingredient of foods and LPC (Lysophosphatidylcholine) is a prominent phospholipid naturally present in wheat starch, both components formed the core of the present study.

Contents

To my family

Chapter 1

General introduction

1. Starch

1.1. Introduction

Starch is the main source of carbohydrates and the second largest biomass, after cellulose, on earth. It is the major component of many plants like grains (e.g. wheat and rice), legumes (e.g. peas and beans), tubers (e.g. potato and cassava) and fruits (e.g. banana). Starch is a widely used macro-constituent to provide functional and technological properties in foods, related to the texturing ability. It is considered as the major source of energy in human nutrition, supplying more than 50% of the caloric energy. The nutritional properties of starch strongly depend on the processing and physical state of starch.

Starch granules range in size (1-100 μm) and shape (polygonal, spherical and lenticular)[7, 28]. Native starch granules have a semi-crystalline structure which varies between starch sources. Starch is a homopolymer of glucose units that consists of two anhydroglucose polymers: amylose and amylopectin, which are assembled in a cluster structure. The amylose-amylopectin proportion as well as the pectin, protein and lipids contents define the type and granule architecture of native starches. For instance, rice and wheat contain a higher amount of proteins and lipids compared to other sources of starch. Also, in wheat starch, the granules are ranged in size: A and B granules. "A" granules are bigger (15-40 μm) than "B" granules which are 1-10 μm; however are fewer in number[7]. In addition, "A" granules contain amylopectin with longer chains and less short chains[1].

Starch granules can also display different X-ray diffraction spectra (XRD), depending on the botanical

source. The difference relates to the moisture content and the organization of the double helices in the unit cell of the crystalline region[12]. A-type is found in the cereal crops and has shorter chains comparing with the B-type while B-type is formed in tubers and has a hydrate core[27]. The C-type, which is an intermediate between A and B, is found in legumes[25]. In general, amylopectin molecules in A-type starches have higher amount of short chain fractions; therefore the crystalline region in the A-type is more susceptible to enzyme attack[21]. The weight-average chain lengths of amylopectin from A-, B- and C-type starches are in the range of 23-29, 30-44 and 26-29, respectively[25].

Starch granules are inert toward chemical interactions and exhibit resistance to digestion. Amylase hydrolysis of native wheat and corn starches indicates that some areas of the surface are more susceptible to be attacked[53]. Therefore, the surface characteristics of starches are of great importance in starch studies; however the surface characteristics of starch granules are still not very well known.

A conceptual model of the starch granule (hairy billiard ball model) was first proposed by *Lineback 1984*[35] (see Fig. 1). In this model, the surface is not smooth and presents amylose partly as an amorphous (uncomplexed) structure and partly as a helical complex with lipids naturally present in cereal starches. The proposed surface is characterized by the reducing ends of amylose chains and the branches of amylopectin (hair) which are the start of the next growth layer[40]. In addition, the tight packing of the amylopectin chains at the surface results in an impenetrable surface to large molecules, such as amylase. Recent findings extended this so called Lineback model[35] by the facts that (i) amylose is present within the amylopectin regions, (ii) amylose is more concentrated at the periphery layers of granules and (iii)

the longer chains of amylopectin are centered towards the inner layers. The concentric pattern of the crystalline layers is responsible for the birefringence of native starch granules confirmed by Maltese cross under polarizing light microscopy[3].
The porous structure of the surface allows small molecules (not larger than 1000 Da) to penetrate through the granule; therefore the entry of enzymes is restricted and only possible after water ingression. The diameter of such pores, depending on the source of starch, varies between 0.1-0.3 μm[40]. The pores allow exogenous materials to the channels which run through the center of starch granule[40].

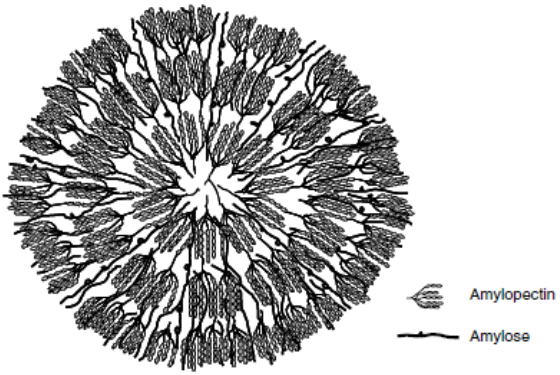

Fig. 1. Lineback's model of a starch granule[35]

The long amylopectin chains (more than 10 glucose units) form double helices that are arranged into A and B crystalline forms (see above). These double helices are associated by hydrogen and Van der Waals bonds and occur either between adjacent branches in the same amylopectin branch cluster or between adjacent clusters[40].

1.2. Amylopectin

Amylopectin is a branched polysaccharide (see Fig. 2,b) with a molecular weight of ca. 10^8 Da. Glucose units are linked via α-1→4 D-glycosidic bonds that form the linear parts and α-1→6 D-glycosidic bonds at the branching points which occurs every 24 to 30 glucose units. The branched structure renders amylopectin better soluble than amylose due to the several end points[25].
Amylopectin contents may vary depending on the source of starch. It is, therefore, found in higher amount in the waxy and in the lower amount in the amylo-starches. Waxy starches have a lower tendency to retrogradation after dissolving, heating, cooling and storage; therefore are widely used as stabilizer[22].
Within amylopectin three types of branches can be distinguished: the 'A chain' is unbranched and is joined to B and C chains through an α-1→6 bond at the reducing sugar; the 'B chain' that is branched and connected to the A chain via α-1→6 bond (A and B chains present in almost equal proportions), and the 'C chain' that is the backbone of amylopectin has the sole reducing group (see Fig. 3,c)[40]. The tight packing of these chains creates the crystalline order of starch granules.

1.3. Amylose

Amylose is a predominantly linear polysaccharide (see Fig. 2,a), built from glucose monomers, with a molecular weight of ca. 10^6 Da, depending on the source of starch[25]. The carbons of each glucose unit is numbered, starting at the aldehyde. Carbon-1 on one glucose is linked to carbon-4 of the next via an oxygen, resulting α-1→4 glycosidic bonds[3]. Amylose chains can form three main types of conformations: (i) random coil conformation which is mainly amorphous; (ii) a six-fold

left-handed single helical conformation: this can be formed in an aqueous environments while twisting around a hydrophobic guest molecule, such as iodine, fatty acid and aromatic compounds; and (iii) a double helical conformation: this is the amylose-amylose interaction (retrogradation) resulting in hardening and stalling in starchy foods during storage. In addition, amylose molecules with long chains can also participate in the formation of double helices with amylopectin[40]. Depending on the source of starch, the amylose content varies from 0 to 85%. Amylose bundles are interspersed among the amylopectin clusters; therefore have more liberty to move after the loss of crystallinity[41]. Amylose is more concentrated on the periphery than in the core of the starch granules[27]; however some studies report longer amylose chains in the core and shorter ones more towards the surface. This has been mostly observed in potato starch[40]. The proportions and dispersion of short and long amylose chains through a starch granules is not specifically reported.

High molar mass amylose is insoluble in water at room temperature; however it is an important thickener, water binder, emulsion stabilizer and gelling agent during heat treatment and processing of foods. Amylose loses its water holding ability after crystallization resulting in syneresis. The water binding ability of amylose is widely used in food industries to improve the texture of food and can be possibly used as a fat replacement.

Amylose rapidly forms inclusion complexes with iodine; therefore iodine can be used as a marker for non-complexed starch. The extent of complexation is determined by spectrophotometry, based on the developed blue color, and the maximum absorbance varies with degree of polymerization (DP)[36]. This technique is able to detect trace amounts of starch, as

little as 1 μg/mL[2]. Since the extent of the blue color corresponds to the DP of amylose, amylose-iodine binding can also be used to assess amylose chain length.

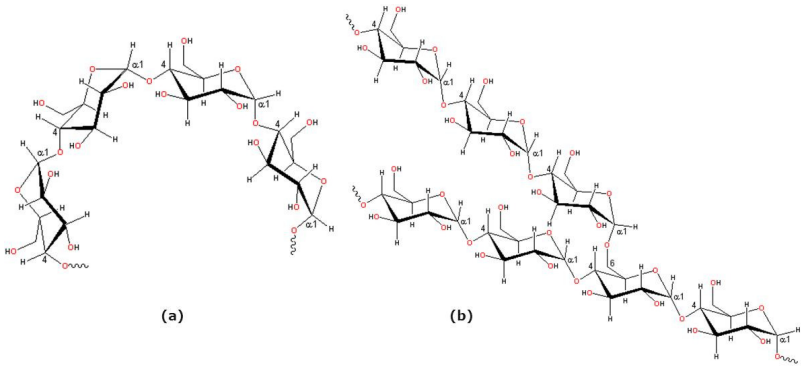

Fig. 2. Linear and branched structure of starch polymers: amylose (a) and amylopectin (b)

The formation of amylose inclusion complexes promotes the crystallinity of starch which can be shown by XRD and differential scanning calorimetery (DSC). Therefore, both are suitable methods for assessing amylose inclusion complexation with a ligand[12, 14, 41].
Studying starch complexation in DSC results in a lower enthalpy of the gelatinization endotherm and a higher enthalpy of the melting endotherm of the amylose inclusion complexes, as compared with native starch. The melting endotherm is a direct indication that inclusion complexes with amylose are formed. As the inclusion complexation is an exothermic transition, this results in a lower enthalpy of the gelatinization endotherm[4].

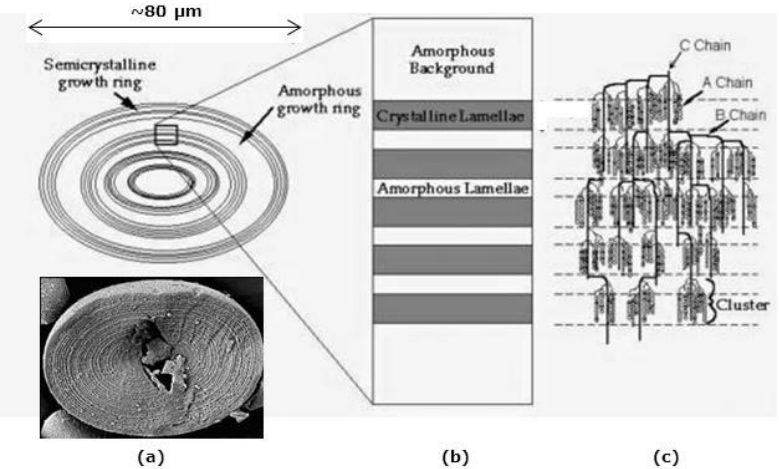

Fig. 3. Radial structure of a starch granule, amorphous and crystalline region. (a) granular rings containing amorphous and crystalline lamellae, shown by transmission electron microscopy (TEM); (b) amorphous and crystalline lamellae; (c) chains of amylopectin arranged in a cluster structure[30].

1.4. Starch biosynthesis

Starch, a natural polymer from glucose, is an indirect product of photosynthesis[34]. Starch synthesis occurs in plastids, including chloroplasts in photosynthetic tissues and amyloplasts in non-photosynthetic tissues like seeds, roots and tubers. Amyloplasts are non-pigmented organelles while chloroplasts are pigmented. However both are closely related and amyloplasts can turn into chloroplasts when the cells are exposed to light[50].

Hexose (a monosaccharide with six carbon atoms) is used in the cytosol (the liquid inside cell) to synthesize sucrose; however the majority of hexose is converted to

triose phosphate (an intermediate compound in the metabolic pathway) which is transported to the cytosol for sucrose synthesis. Subsequently, the sucrose is transported to amyloplasts for starch synthesis. The starting point of starch biosynthesis in amyloplast (non-pigmented organelles responsible for synthesis and storage of starch granules) is glucose-1-phosphate which is a product of sucrose degradation[37, 46].
Starch synthesis in the cereal occurs in the endosperm, which is a tissue inside the seeds[17].

The constituents of starch are densely packed resulting in the insoluble starch granules[30]. Biosynthetic events control the formation and structure of starch which depends on the growth condition of amylose-amylopectin layers, so-called growth rings extending from center to the surface, observed by light microscopy in large starch granules (potato and wheat) and by electron microscopy in small starch granules (barley and rice)[5] (see Fig. 3,a). The growth rings are about 120-400 nm in thickness[7] and consist of crystalline and amorphous regions alternating with higher and lower density, respectively (see Fig. 3,b). The first growth layer is initiated at the center which contains large proportion of the reducing ends and is usually less organized. The newly synthesized rings are deposited on the surface and increase the size of the granules[39]. Amylose forms the low density growth layers and is dispersed between the amylopectin lattice that constitutes the crystalline layers with higher density[28]. The non-reducing ends of amylose and amylopectin which are towards the surface of the granules, allow further glucose residue to add. The layer formation, the ratio of amylose to amylopectin and the structuring properties of starch determine the final product, the processing conditions and the digestion rate.

1.5. Thermal transitions of starch

Starch granules, depending on their botanical sources (grains, tubers and roots), are synthesized as insoluble crystalline structure[7].
Starches consumed by humans generally have undergone some processing typically involving heating in the presence of moisture under shear. This treatment strongly improves the digestibility of starch (see section 2.2).
Two events occur during conventional time-temperature processing of starch: swelling and gelatinization. Both are the results of starch-water interactions[26] as starch molecules are associated by hydrogen bonding. Native starch is insoluble in cold water; therefore creating a suspension that can be mechanically dispersed in water. Upon heating in excess water (ca. 55°C), the suspension changes to a homogenous starch-water mixture which is called pasting (water ingression and amylose leakage). During heat treatment, the crystalline lattice of the starch granules melts (ca. 60-65°C) and amylose molecules are released into the solution[6, 45] (see Fig. 4). The melting temperature highly depends on the water content, as water penetration into the starch granules is driven by differences in osmotic pressure. In the more amorphous areas, in which the molecules are not as closely associated, progressive hydration and swelling will occur more rapidly.
In the second stage (ca. 90-95°C), the first large increase in viscosity is observed when gelatinization occurs. Gelatinization is an irreversible physical change when native starch is heated in the presence of sufficient moisture[7]. At this stage, amylose is leached from the granules and enters the aqueous phase[7, 47].

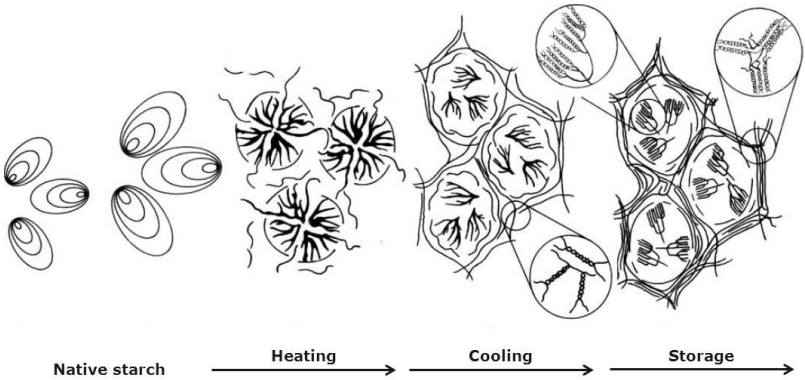

Fig. 4. Schematic representation of changes that occur in starch-water mixtures during heating, cooling and storage[15].

On cooling, the starch chains (mainly amylose) in the gelatinized paste tend to associate, leading to the formation of a more ordered structure which is termed retrogradation[25]. This results in a viscosity increase due to the formation of a firm gel, depending on the source of starch. Amylose retrogrades within minutes to hours and amylopectin over hours to days[7]. Therefore, the duration of retrogradation depends on the amylose and amylopectin content. Higher molecular weight amylose promotes faster retrogradation. In addition, the chain length distribution of amylopectin (mainly the proportion of A chains) has an influencing role[12].
In processed foods, amylose retrogradation is used to induce stickiness, water absorbance and lowering digestibility[34]; while the amylopectin retrogradation results in the staling of breads and cakes[25].

Starch behavior during these steps can be for instance monitored by a Rapid Visco Analyser (RVA). RVA measures the resistance of the sample to shear as

function of time and temperature. In the viscosity profile, shown by RVA, the viscosity peak (as a function of time and temperature) is at the highest level of water ingression[26].

1.6. V-amylose

During the early stages of starch gelation, the leached out amylose in solution becomes turbid. This is due to the conformation change of amylose from an expanded to a random coil[49]. This changes the solution to a two phase system of polymer-rich and polymer-deficient which increases the turbidity[25].
Amylose undergoes a conformation change from coil to a single and left-handed helix in presence of ligands, such as fatty acids and phospholipids, and forms inclusion complexes[4, 32]. These complexes are formed between amylose and the aliphatic chain of lipids/phospholipids (see Fig. 5) and are known as 'type I amylose'. This form is called 'V-amylose', named by *Katz 1937*[32], because it occurs upon gelatinization of starch in presence of a ligand (in German: Verkleisterter Stärke=gelatinized starch).

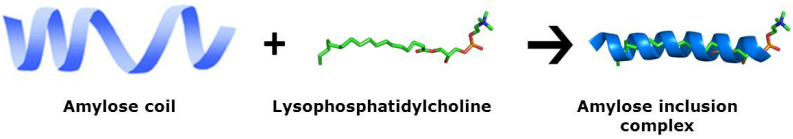

Fig. 5. The formation of a left-handed single amylose helix with aliphatic carbon chain of lysophosphatidylcholine (LPC). The polar head remains outside the helix.
The pitch is the distance between a sequential turns.

Depending on the dimension and position of the guest molecule, several forms of amylose inclusion complexation are defined.

- V_h-amylose or V_{6I}-amylose is the hydrated form of amylose (type I and II), which accommodates the ligand only inside the cavity. It is formed in presence of lipids and linear alcohols and develops an orthorhombic structure[42].

- V_a-amylose is the anhydrous form of V_h obtained after drying; therefore it is smaller than V_h[41].

- $V_{butanol}$ or V_{6II}-amylose is formed in presence of short chain alcohols, such as butanol and ethanol. The ligand is accommodated not only inside the helix but also between the helices; therefore the unit cell dimension is larger than V_h and V_a forms.

- $V_{isopropanol}$ or V_{6III}-amylose: the ligand resides inside the helix and between the helices; however the helix dimension is larger than $V_{butanol}$. V_{6II} and V_{6III} are converted to V_{6I} upon drying[20].

- V_7 and V_8-amylose are larger than V_{6III} while the helix accommodates more voluminous ligands, such as menthone and fenchone. V_7 is formed with seven glucose residue per turn and V_8 with eight glucose units per turn[38]. V_7 is converted to V_{6I} upon drying but this is not the case for V_8.

During starch gelatinization, amylose develops inclusion complexes with the lipids that are naturally present in starch granules (0.5-1% lipid)[41].

With heating, amylose twists around the hydrophobic carbon tail of the present lipid and develops intramolecular interactions, such as van der Waals forces and hydrophobic interactions, between amylose and ligand and hydrogen bonding between the turns along the helix[31]. The polar head (carboxyl group) of the lipid remains outside, due to steric hindrance, static and electrostatic repulsion. The tendency of amylose to minimize its interaction with water, results in the formation of inclusion complexes[19]. The helix has a hydrophobic cavity, lined with methylene groups and glycosidic oxygens, while the outer remains hydrophilic. The result is a water insoluble complex.
Although amylose inclusion complexes are very stable, the complexation is a reversible process, shown by a melting endotherm and an exotherm during heating and cooling in DSC, respectively[4, 8].

The chain length of amylose also plays a considerable role in the formation of amylose inclusion complexes. 18-24 glucose units (three turns: three pitch with six glucose units per pitch) are required to form an inclusion complex[41]. Long amylose chains provide more opportunities for complexation with more ligands; therefore the crystalline region develops more and the melting temperature increases[13, 14].
A number of reports also stated that long branches of amylopectin might contribute in inclusion complexation[19]. However, *Godet et al. 1995*[14] stated that complexation with amylopectin branches is almost impossible due to intramolecular crowding.

The aliphatic chain of the ligand is another key factor in the formation of the amylose inclusion complexes. *Putseys et al. 2010*[41] stated that a chain length of at least ten aliphatic carbon atoms is essential to induce amylose inclusion complexation. This shows that longer

chains develop more inclusion complexes with amylose molecules. This is attributed to (i) higher hydrophobicity of the longer fatty acids and subsequently development of more hydrophobic interactions[41]; (ii) providing more bindings inside the cavity[14]; and (iii) less solubility in the aqueous media results in better accommodation inside the helix[48].
In the case of unsaturated lipids, the number of double bonds is also an influencing factor in the formation of inclusion complexes. A high degree of unsaturation results in a partial inclusion complexation; however the trans-unsaturated fatty acids form complexes with amylose rather well[31] because they are more linearly oriented compared to the cis-unsaturated and therefore require less space inside the helix[31, 41].

The amount of amylose inclusion complexes depends on the concentration of lipid. At high concentrations, the uncomplexed lipids are trapped between the helices and participate in the less specific bindings.
In addition, the inclusion complexation also depends on water solubility and critical micellar concentration of the lipid[41].

Proteins also have an effect on the properties of starch-lipid complexes. Such multiple interactions are likely to be common in cooked starchy foods and may influence their functionality[52].
Amylose-lipid complexation reduces starch swelling and solubility, increases gelatinization temperature, alters thermal transition, varies paste properties[4] and decreases enzyme hydrolysis.

2. Nutrition and digestion of starch

2.1. Nutrition

Nutrition supplies the maintenance of life (metabolism). Some materials are used for the formation of body tissues (anabolism) and some for energy production (catabolism)[43]. Due to this, foods are classified as energy and growth (carbohydrates, fats and proteins) and non-energy (minerals, vitamins and water) foods.
The nutritional value of a diet is determined by not only the energy it provides, but also the ability of the body to digest and absorb it. In addition, the physical condition of food (liquid vs. semi-solid and solid) has an important role in its digestibility.
Food energy is estimated by the heat produced during complete combustion in a calorimeter. Kilocalorie is the unit to measure energy intake by foods. 1 kilocalorie (kcal) is defined as the amount of heat required to raise the temperature of 1 kg water by 1°C.
Carbohydrates (saccharides) are a good source of energy for the human body and starch is the most common polysaccharide in human diet. For instance, a serving size of 100 g wheat flour or rice provides 339 and 380 kcal energy, respectively. Carbohydrates should comprise 45-65% of the daily calorie intake that means 225-325 g carbohydrates if 2,000 kcal is required. An average man needs around 2,500 kcal a day to maintain his weight. For an average woman, that figure is around 2,000 kcal. These values can vary depending on age and physical activities. Therefore, carbohydrates have a prominent role in the human diet. Due to this, starch in staple foods (a food that is eaten routinely, supplying a large fraction of the daily needs and varies place to place) has been implicated in complications related to obesity which is a risk factor for several physiological disorders. Especially,

the rate of enzymatic digestion of starch is considered important. Hence, public awareness on the relation between human health and nutrition has increased. With respect to this, a particular focus has been given to starch and starchy foods.

2.2. Enzyme hydrolysis of starch

For nutritional purposes, starch in foods may be classified into RDS (rapidly digestible starch – starch that is digested to glucose after 20 min), SDS (slowly digestible starch – starch that is digested to glucose between 20 and 120 min) and RS (resistant starch – starch that cannot be digested but is fermented in the large intestine). RS may be further divided into three categories according to the reason for resistance to digestion. This characterization is based on the rate and duration of the glycemic response[9, 10]. A fast rate leads to a rapid increase in postprandial blood glucose levels which is considered a risk factor and a slow rate is recognized positive since this leads to lower metabolic stress. Based on this, Glycemic Index (GI) describes the level of glucose rise in blood stream as compared to ingestion of a standard dose of glucose[29]. In this respect, the source, amount and form of consumed carbohydrates are important[16].
In the human body, starch is hydrolyzed to glucose by enzymes through several steps[44]. Upon ingestion, starch is exposed to salivary α-amylase; however glucose absorption mainly occurs in the small intestine[33]. There are several hydrolytic enzymes within the digestive tract of the human body to break down starchy foods. In mammals, there are two types of enzymes for the digestion of carbohydrates:

(i) Endo-hydrolases – cleave accessible α-1→4D-glycosidase bonds[44, 51] and hydrolyse amylose/amylopectin to maltose and larger oligosaccharides (maltotriose and maltotetraose)[18]: the action of α-amylase.

(ii) Exo-hydrolases – release monosaccharide or disaccharide from non-reducing ends: the action of mucosal (brush-border) α-glucosidases of which there are four (commonly called maltase, glucoamylase, sucrose and isomaltase)[11].

The kinetics of enzymatic starch digestion depends on three factors: (i) the molecular and physicochemical characteristics of starch which are formed during the synthesis in the grain[1], (ii) the physical conformation of starch granules in aqueous solution[30] and (iii) the type and concentration of the enzyme. These factors alter the availability of starch polymers to the digestive enzymes.

Interestingly, a number of studies have shown that the formation of amylose inclusion complexes leads to a lower digestibility of starch[24, 41]. The complexation decreases the accessibility of starch granules to the enzymes due to the restriction of granule swelling. In addition, it can be explained by the low solubility of the complexes and the steric hindrance they exert[23]. The structural characteristics of the complexes also influences their degradability.

3. The aim of the thesis

Due to the important role of starch in daily life, as the major source of energy in human nutrition and as a leading structuring component in food industry, the aim of this thesis was to evaluate the influence of amylose complexation with LPC on the digestibility and functional properties of wheat starch.

Starch generally supplies more than 50% of the daily caloric energy; therefore its digestibility has a big impact on human health. Rapid postprandial glucose increase in the blood stream, due to the rapid digestibility, is considered as a risk factor that causes obesity and diabetes type II; while a slow digestion rate can prevent metabolic disorders. Starch digestion is a complex process that depends on several factors, such as the source of starch, the degree of gelatinization and presence of other components. Since wheat starch is a basic ingredient of several foods and LPC (Lysophosphatidylcholine) is a prominent phospholipid in wheat starch, both components formed the core of the present study. Therefore, we studied the influence of LPC, under time-temperature-shear condition, on wheat starch to get an insight into the mechanisms that occur during processing and result in alterations in the physical and functional properties of starch, leading also to the formation of SDS.

The main analytical tools employed in this study are Rapid Visco Analyser (RVA), Dynamic Scanning Calorimetry (DSC), Size-Exclusion Chromatography (SEC), Confocal Laser Scanning Microscopy (CLSM) and Spectrophotometry (UV-VIS).

Chapter 2 assesses the influence of LPC on the functional properties of starch while preserving starch functionality. This part describes the formation of amylose inclusion complexes with LPC while LPC is added at the starting point of the process, allowing the complex formation at each possible point of time and temperature which also helps the applicability of the study in the practical fields. In this part, the focus is on the temperature and time as well as the induced changes in starch and how amylose inclusion complexation alters the functionalities of starch.

Chapter 3 focuses in more detail on the influence of incubation time and temperature on inducing the formation of amylose inclusion complexes with LPC. This chapter investigates the extent of complex formation at several temperatures and times.

Chapter 4 aims to understand the digestion of wheat starch and the influence of LPC on hindering enzyme hydrolysis. In this part, an alternative *in vitro* method is established, under controlled time-temperature-shear conditions in a diluted suspension. This method revealed the difference between the degradation of starch in complex with LPC vs. native starch in releasing reducing sugars.

Chapter 5 obtains new structural insights into the digestibility of wheat starch after complex formation with LPC. SEC was employed to study the molecular size and structure of starch molecules collected from each digestion period compared to the native starch. The presented results provide information about the digestibility of starch, the molar mass distribution of carbohydrates and the role of LPC in the development of slow starch.

The digesta, resulting from the *in vitro* method of chapter 4, were exposed to iodine to investigate the extent of amylose degradation in **Chapter 6**. The preservation of amylose due to the inclusion complexation with LPC, before and after defatting, was shown by spectrophotometry after iodine complexation, accordingly.

Finally, the main results of the amylose inclusion complexation with LPC are discussed in **chapter 7**.

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Chapter 2

Influence of lysophosphatidylcholine on the gelation of diluted wheat starch suspensions

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Abstract

Starch is an omnipresent constituent which is used for its nutritional and structuring properties. Recently concerns have been raised since starch is a source of rapidly available glucose (RAG) which is tightly correlated with diabetes type II and obesity. For this reason, the possibilities for modulating the digestibility of starch while preserving its functional properties were investigated; therefore the focus of this paper is on starch gelatinization and the effect of Lysophosphatidylcholine (LPC) on the structuring properties of wheat starch. The effect of LPC on thermal properties and viscosity behavior of starch suspensions was studied using DSC and RVA respectively. The influence on granular structure was observed by light microscopy. The RVA profile demonstrated no viscosity increase at high LPC concentrations which proves intact granular structure after gelatinization. LPC in intermediate concentrations resulted in a notable delay of pasting; however the peak and end viscosities were influenced as well. Lower LPC concentrations demonstrated a higher peak viscosity as compared with pure starch suspensions. DSC results imply that inclusion complexes of amylose-LPC might be formed during pasting time. Since the viscosity profiles are changed by LPC addition, swelling power and solubility of starch granules are influenced as well. LPC hinders swelling power and solubility of starch granules which are stimulated by heating.

1. Introduction

Starch is the largest source of carbohydrates in human food. Starch is a key component of staple foods, such as wheat, rice and potato. Starch and starchy food products can be classified according to their digestibility, which is generally characterized by the rate and the duration of glycemic response[19]. The starch in staple foods has been implicated in the complications related to obesity and type II diabetes. It is specially the rate of enzymatic digestion of starch that is considered important. A fast rate leads to a rapid increase in postprandial blood glucose levels which is considered negative and a slow rate is recognized positive since this leads to lower metabolic stress. Predicting and controlling postprandial blood glucose levels is therefore of great interest in the context of worldwide health concerns.
Guraya et al. 1997[8] showed the higher resistance of amylose-lipid complexes to breakdown by human α-amylase. They were able to reduce digestibility by 41.6% after amylose-emulsifier complexation in non-waxy starch. Within another study by *Holm et al. 1983*[10], the complexed amylose with lysolecithin was exposed to pancreatic α-amylase that displayed a substantially reduced susceptibility to α-amylase *in-vitro* digestion. Their *in-vivo* study demonstrated slower rate of amylose digestion after inclusion complexation. At the same time, starch is widely used in food products for its structure forming properties. *Putseys et al. 2010*[14] demonstrated the impact of different concentrations of emulsifiers on pasting and gelation of starch. They assume that emulsifiers are absorbed by starch granules at the surface and water ingression is suppressed which results in less viscosity growth. This prompted us to study if starch digestibility can be decoupled from its structure forming properties. This study represents a first step to investigate if and how functional and structuring

properties of wheat starch can be combined with a slower digestibility after amylose inclusion complexation.
Three events occur during conventional time-temperature processing of starch: swelling, gelatinization as well as retrogradation which the last occurs after processing. All are results of starch-water interactions[12]. As starch molecules are associated by hydrogen bonding. Water penetrates inside the starch granules while heating, driven by differences in osmotic pressure, leading to disruption of the intra-chain and inter-chain hydrogen bonds. In the more amorphous areas, in which the molecules are not as closely associated, progressive hydration and swelling will occur more rapidly. In addition, linear amylose molecules are released into solution[3]. Hydrogen bonding forces in wheat starch granules weaken at two stages of swelling. The first stage occurs at 55-77°C. At 55°C pasting starts and between 60-65°C the granules lose their crystallinity so that they swell more. In the second stage, the first large increase in viscosity is observed when gelatinization occurs. Gelatinization is an irreversible physical change. At this stage, amylose is leached from the granules and enters the aqueous phase[23]. On cooling, the starch chains (mainly amylose) in the gelatinized paste tend to associate, leading to the formation of a more ordered structure which is termed retrogradation[11].

Amylose in a helical conformation has the ability to form inclusion complexes with components like fatty acids and phospholipids[15]. This so called V-complex is formed between the aliphatic chains of lipids and the amylose.
Lysophosphatidylcholine (LPC) is widely used in food products as surfactant to improve the functional properties of foods; e.g. in starch containing foods it complexes with the amylose helix and retards retrogradation. The formation of an amylose-LPC inclusion complex causes a transition in the amylose

molecular structure from coil to helix which results in an increase in the order of the molecular structure of amylose (visible as the V-type X-ray diffraction pattern) as well as less amylose leakage during processing[24]. The length of the fatty acid chains of LPC is an influencing factor on amylose inclusion complexation. Shorter fatty acid chains suppress amylose leaching more effectively, due to better accommodation into the amylose helix[20].
In addition, the complexed amylose with LPC is hardly hydrolyzed by α-amylase[21]. *Frei et al. 2003*[7] reported the lower glycemic response of high amylose rice cultivars after addition of phospholipids that was attributed to reduced enzyme susceptibility after the formation of complexes between amylose and phospholipids upon heating.

Previous studies have demonstrated the complex formation of LPC and amylose[24], although the effect on the functional properties of starch has not been adequately discussed. In addition, inclusion of LPC into the amylose helix can delay enzymatic degradation. The current study evaluates the influence of LPC on the structuring properties of wheat starch and aims to benefit from the amylose-LPC complexation while preserving these properties. For this reason, several methodologies were employed to relate the functionality of LPC in several concentrations to the alteration of structuring properties of the wheat starch. This is a precise look to figure out the formation of amylose inclusion complexes with LPC, while LPC is added at the starting point of the process, to allow the complexation at each possible point of time and thermal condition. That propels the applicability of the study in the practical fields. In this paper, the focus is on the temperature that induces changes in starch and how amylose-LPC inclusion complexation influences the physical and technologically relevant functionality of wheat starch such as viscosity.

2. Materials and methods

2.1. Materials

Egg yolk L-α-Lysophosphatidylcholine (LPC), type XVI-E, lyophilized powder, purity >99% and fatty acid content of 16:0 69%, 18:0 27% and 18:1 3%, from Sigma Chemical Company (St Louis, Missouri, USA) was used.
Unmodified wheat starch with a purity of 99%, a moisture content of 12.98%, a total lipid content of 0.4% and 2.8% damaged granules was obtained from Sigma Chemical Company as well.
LPC was kept at -20°C and wheat starch at room temperature under dark and dry conditions.
Lugol, as iodine solution to stain starch granules was purchased from Sigma Chemical Company.
GOPOD (Glucose Oxidase Peroxidase) was purchased from Megazyme. The kit includes reagent buffer (potassium phosphate buffer, p-hydroxybenzoic acid and sodium azide), reagent enzyme (glucose Oxidase plus Peroxidase and 4-aminoantipyrine) and D-Glucose standard solution (in benzoic acid).
All other used reagents were of analytical grade or better.

2.2. Viscosity measurement

A RVA-4 Newport Scientific (NSW, Australia) Rapid Visco Analyzer was employed to study the temperature-viscosity profile of the starch suspensions used in this study.
A series of 9% (w/w) wheat starch suspensions in deionized water was prepared by mixing starch with 0.1%, 0.3%, 0.5%, 1% and 5% LPC (based on dry matter (DM) wheat starch). The suspensions were kept 10 min at room temperature to equilibrate. The

temperature of each suspension was first equilibrated at 50°c for 60 s, increased to 95°C at a rate of 6°C/min, and held at 95°C for 300 s, decreased to 50°C at the same rate and finally held at 50°C for 120 s. The reference (pure starch) was subjected to the same temperature gradient.

2.3. Light microscopy observation

0.1%, 0.3%, 0.5% and 1% LPC (based on DM wheat starch) was added to 9% (w/w) wheat starch suspension in deionized water. Each suspension was processed by RVA to create the same temperature profile as described earlier. At 50°C, 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C as well as at the end of the temperature profile (50°C) samples were taken, diluted with distilled water to obtain 0.5% suspension and stained with 50 µl iodine solution. Starch granules were observed under bright-field illumination with a Nikon light microscope (Nikon, Eclipse 400, NY, USA) using 10x objective lens. Images were captured with a high resolution color camera (Nikon, COOLPIX 4500, MDC Lens, Japan).
Any changes in starch crystallinity at 50°C, 60°C and 65°C were observed by light microscopy under polarized light.

2.4. Swelling power

Swelling power was determined in duplicate (according to *Steeneken et al. 2009*[22] with some modifications) using 0.5%, 1%, 2%, 3% and 5% LPC (based on DM starch) in diluted starch suspensions. A series of 8 mL wheat starch suspensions in deionized water (3-8%, w/w, depending on starch weight and LPC concentration) were prepared and heated at 70°C, 80°C, 90°C and 95°C in a ventilation

oven for 45 min while rotating. Then the mixture was separated by 15 min centrifugation at 1000 rpm. The supernatant height was measured in mm where the Q (Swelling Power based on the volume of precipitated particles) was determined.

2.5. Soluble starch measurement

During swelling and gelatinization, especially linear amylose becomes soluble and may leak from the granules. This was followed by measuring the amount of soluble starch (SS). SS was determined (Megazyme Resistant Starch Assay Procedure, K-RSTAR 08/05, based on AACC Method 32-40) in duplicate using 0.5%, 1%, 2%, 3% and 5% LPC (based on DM starch) in diluted starch suspensions. A series of 8 mL wheat starch suspensions in deionized water (1.5-4%, w/w, depending on starch weight and LPC concentration) were prepared and heated at 70°C, 80°C, 90°C and 95°C in a ventilation oven for 45 min while rotating. The mixture was separated into the supernatant and the precipitate by centrifugation at 1000 rpm for 15 min. The supernatant was centrifuged for 15 min at 16,000 rpm. 1 mL diluted Amyloglucosidase (AMG) was added to 1 mL of the supernatant to convert amylose into glucose. After 16 h of incubation at 55°C, the enzyme was inactivated by heating the mixture to 100°C for 30 min. Then, 3 mL Glucose Oxidase Peroxidase (GOPOD) was added to 100 µl of sample and incubated for 20 min in a water bath at 50°C to stain the glucose. The absorbance was read at 510 nm using a Spectramax spectrophotometer (Spectramax M2 Dual Mode C, Molecular Devices, Virginia, USA). 100 µl D-glucose standard was mixed with 3 mL GOPOD and used as a reference. A diluted wheat starch suspension without LPC was taken as a blank sample. Absorbance values were corrected for the

absorbance of the enzyme solution. Finally, solubility was calculated from the carbohydrate concentration of the known amount of the supernatant solution, as measured by the swelling power method with GOPOD, after centrifugation at 16,000 rpm. Soluble starch is expressed as a weight fraction on a dry basis.

2.6. Thermal analysis

A series of 20% (w/w) wheat starch suspensions in deionized water was prepared by mixing starch with 0%, 0.5%, 1%, 2%, 3% and 5% LPC (based on DM wheat starch). Samples were rotated an hour at 50 rpm at ambient temperature. The suspension was pipetted into stainless steel pans (Perkin Elmer, Norwalk, CT, USA) which were sealed afterwards. Samples were analyzed by Perkin Elmer Pyris 1 DSC (Norwalk, CT, USA) previously calibrated with Indium (melting temperature= 156.6°C, melting heat= 28.45J/g). The baseline from 20°C to 120°C was obtained with an empty pan as reference as well as sample pan. The heating rate was 10°C/min. The onset (T_o), peak (T_p) and ending (T_e) temperatures for the different transitions were determined and calculated by DSC software. Enthalpy (ΔH, J/g of sample) for the different transitions was calculated based on the endothermic peaks. The samples were compared with a wheat starch reference suspension. All samples were measured in duplicate.

3. Results

3.1. Effect of LPC on pasting time and viscosity

The RVA measurements show that LPC alters the viscosity behavior of starch suspensions depending on its concentrations. At 5% LPC, the viscosity profile is linear and no increase was observed (see Fig. 1).

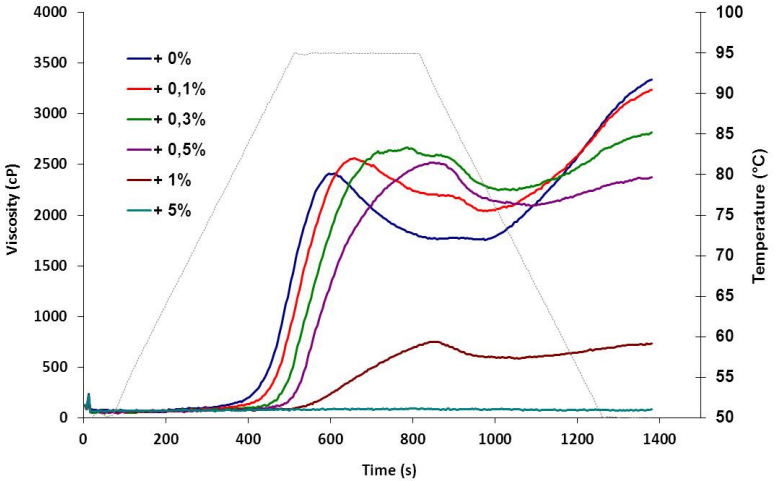

Fig. 1. Effect of different concentrations of LPC on pasting time and viscosity behavior of 9% wheat starch suspension.
Viscosity is plotted on the left axis against time. The temperature profile is represented by the dotted line. Different colors represent different LPC additions (see insert).

At lower LPC additions, the effect is less pronounced. At 1% LPC, viscosity increases slightly and at 0.5% and lower, swelling is just delayed but not hindered as at the higher concentrations. Pasting temperatures were

registered at 350 s, 400 s and 450 s for 0.1%, 0.3% and 0.5% LPC concentration respectively, comparing with the reference at 300 s. Starch suspensions with 1% LPC demonstrated an onset temperature at 500 s and a very low peak viscosity (750 cP), compared to the lower concentrations.

Addition of LPC resulted in different concentrations also resulted in markedly lower end viscosities, in comparison with the reference.

3.2. Swelling power

The swelling power of starch depends on the water-holding capacity of starch molecules by hydrogen bonding[18].

Swelling power of 9% starch suspensions were measured after addition of 0.5, 1, 2, 3 and 5% LPC in starch suspensions heated to 70°C, 80°C, 90°C and 95°C.
Without addition of LPC, starch swelling clearly increases with temperature (see Fig. 2). It becomes obvious that addition of LPC limits swelling. Lower values were observed at higher concentrations of LPC but in none of the studied LPC concentrations swelling is inhibited completely. The influence of LPC is clearly depended to the added amount. No further increase in inhibition at concentration higher than 3% was found.

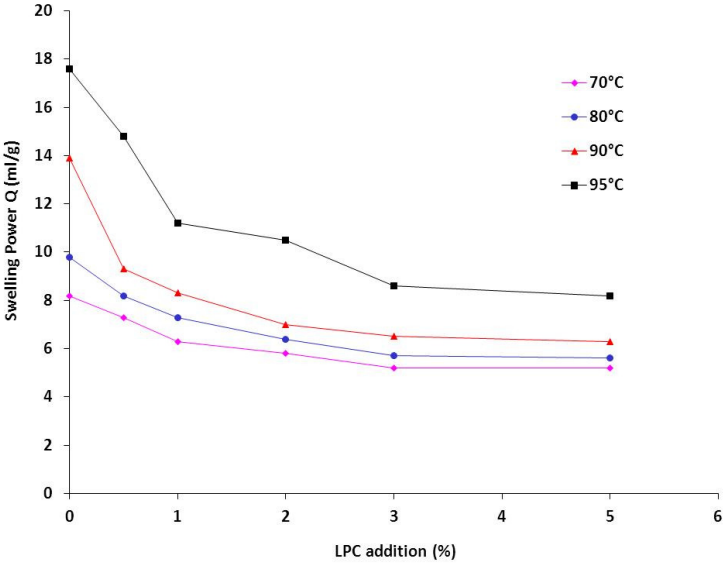

Fig. 2. Swelling power alteration of wheat starch at 70-95°C while increasing LPC concentration. Q represents swelling power that is plotted on the left axis. Different colors represent different temperatures.

3.3. Soluble starch measurement

The amount of water soluble starch, expressed as the amount of leached amylose, is an index to indicate the solubility of the macromolecular starch components. The influence of temperature and the addition of 0.5%, 1%, 2%, 3% and 5% LPC on the solubility index of wheat starch at 70°C up to 95°C were studied. The results were compared with the reference.

Both amylose and amylopectin are insoluble in cold water. Fig. 3 clearly shows that in the absence of LPC the solubility increases when starch suspensions are heated. It becomes clear that the leakage is suppressed by

addition of LPC although starch suspensions were subjected to high temperatures. The highest effect was observed while addition of highest concentration of LPC. As in the case of swelling power (see above), addition of 3% and 5% LPC resulted in about the same effect; indicating that the maximal effect is reached at 3%.

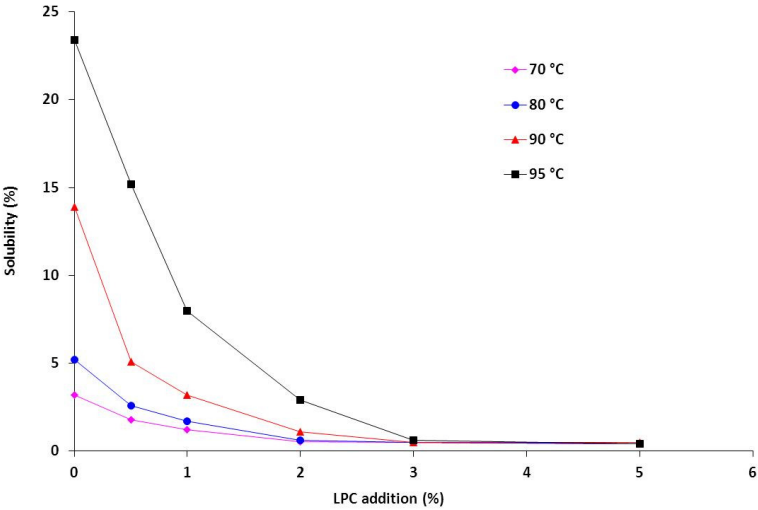

Fig. 3. Solubility values in wheat starch suspension at 70-95°C while increasing LPC concentration. Solubility is plotted on the left axis based on percentage. Different colors represent different temperatures.

3.4. Light microscopy

3.4.1. Iodine staining

Starch granules typically swell upon heating in the presence of water, leading to a loss of granule integrity. The effect of LPC addition on granular shape at different

temperatures was studied. The starch granules were observed by light microscopy after iodine staining.
Light microscopy images of wheat starch granules after addition of 0.3%, 0.5% and 1% LPC heated at 50-95°C as well as 50°C (the end point of RVA profile) are depicted in Fig. 4.

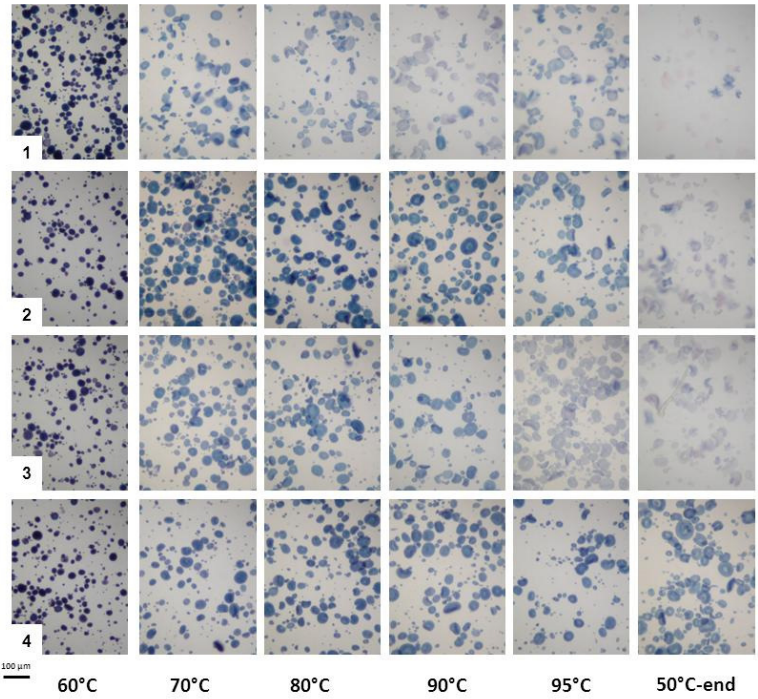

Fig. 4. Stained wheat starch (WS) granules with iodine under light microscope at temperatures between 60°C and 95°C as well as 50°C at end of viscosity profile. Row "1": WS (reference), Row "2": WS+0.3%LPC, Row "3": WS+0.5%LPC, Row "4": WS+1%LPC.

Images at 50°C are not shown since the granules have quite similar appearance and in all cases appear unchanged compared to the control.
In pure starch suspension, gelatinization starts at 60°C (see Fig. 4). At about 70°C, amylose begins to leach out, therefore some starch fragments, due to amylose leaching, are observed. Blue color, as an indicator, mainly appears due to amylose-iodine complexation. Less blue color indicates a high amount of leached amylose. The process continues with swelling and change of granular shape at 80°C. The granules clearly become fragmented above 90°C in which blue color is rarely seen.
The influence of LPC on granular shape is very much pronounced at high concentrations. After addition of 1% LPC, intact granules can be observed even at the end of RVA profile (see Fig. 4 fourth row). Fig. 4 at second and third rows demonstrates the influence of LPC after addition at moderate concentrations which results in low granular collapse and limited rupture. The influence of LPC on granular shape is not very pronounced at lower concentrations (0.3% and 0.5%) as amylose leakage was to some extent hindered. It can be seen also by the results which were presented above in swelling power measurement.

3.4.2. Loss of birefringence

Starch granules display birefringence upon exposure to polarized light, thus indicating the presence of crystalline regions within the starch granular matrix. Upon heating birefringence is lost due to the melting of these regions[17].
To study the effect of LPC on the crystalline region of starch granules, we therefore studied the birefringence loss of starch granules by polarized light (light microscopy).

Fig. 5 shows the light microscopic images of starch granules without and with 0.5% and 2% LPC demonstrating the effect of LPC on changes in starch crystallinity. It is clearly shown that the birefringence has disappeared at 65°C. Interestingly this behavior is not affected by the addition of LPC.

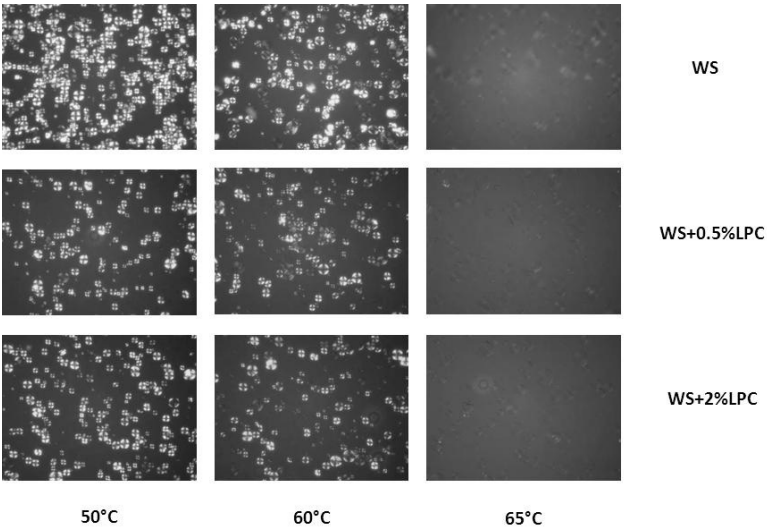

Fig. 5. Polarized light microscope images showing loss of birefringence at 50°C to 65°C in 9% wheat starch (WS) suspension. First row presents the reference, second: WS+0.5%LPC, third: WS+2%LPC.

3.5. Effect of LPC on thermal transition of starch

Heating starch suspension in DSC leads to two transitions (see Fig. 6); the first is related to the loss of the internal starch structure and the second is related to the presence and melting of amylose-LPC complexes (existing in starch

granules or formed by the added LPC: 0.5%, 1%, 2%, 3% and 5%).
For pure starch, the first transition starts at 60°C (see Fig. 6) and results in an enthalpy of 17.6 J/g and the second transition starts at 100°C and results in an enthalpy of 1.8 J/g.

Table 1. Thermal analysis of 20% wheat starch-LPC suspension with different LPC concentrations (based on starch) comparing with the reference. WS stands for Wheat Starch and LPC for lysophosphatidylcholine.

Samples	Starch			Amylose-LPC	
	Onset (°C)	**Peak (°C)**	**ΔH (J/g)**	**Peak (°C)**	**ΔH (J/g)**
WS+0%LPC	59.7	64.6	17.6 *(0.17)*	100.0	1.8 *(0.38)*
WS+0.5%LPC	60.3	65.4	16.7 *(0.4)*	101.9	3.2 *(0.63)*
WS+1%LPC	59.7	64.6	16.4 *(0.8)*	102.6	4.3 *(0.11)*
WS+2%LPC	60.0	64.8	14.0 *(0.22)*	103.8	5.7 *(0.16)*
WS+3%LPC	59.7	64.4	13.6 *(0.12)*	104.5	6.4 *(0.7)*
WS+5%LPC	59.8	64.4	12.2 *(0.34)*	104.4	6.6 *(0.2)*

WS= Wheat starch
LPC= Lysophosphatidylcholine
The values within the parentheses represent the SDs (n=2).

Addition of LPC does not affect the initial transition temperatures, however the peak height and enthalpy of the second transition (melting of the amylose-LPC inclusion complex) increases with addition of LPC (see Fig. 6 and Table 1). Higher amounts of LPC lead to lower enthalpy of the first transition and higher peak height and enthalpy of the second transition.

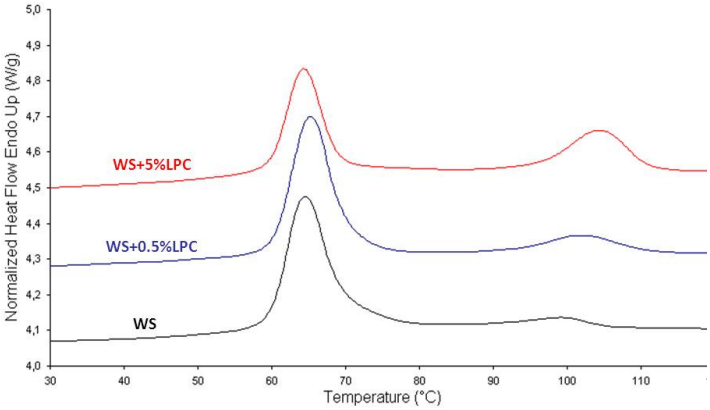

Fig. 6. DSC thermograms showing thermal properties of 20% wheat starch suspension and effect of 0.5% and 5% LPC (based on wheat starch) on the thermal transition. Heat Flow is plotted on the left axis. Different colors represent the wheat starch suspensions with different LPC addition comparing to the reference. WS stands for Wheat Starch and LPC for lysophosphatidylcholine.

4. Discussion

Starch is omnipresent in foods for two reasons. Starch is a main source of carbohydrates and is also an efficient structure builder. From a nutritional point of view, it is of interest to slow down the rate of starch digestion. This can be achieved by the addition of LPC, forming amylose V complexes that are reported to be more difficult to digest[8, 10]. It is not known however, to what extent this can be used without harming the structuring properties of starch. For this reason, the focus of this research was to systematically study the effect of LPC on the thermal transition of starch and resulting properties. To this end, different levels of LPC for complex with amylose were

induced. The results show that the complex formation is dependent on LPC concentration and leads to alteration of viscosity profile in the RVA, less swelling and better preservation of granule integrity due to less amylose leakage.
Studying the influence of LPC at different thermal transitions of starch gives an insight into the mechanisms that occur during processing and result in alterations in the physical and functional properties of starch.

LPC addition at high concentration prevents viscosity increase during RVA measurement, indicating limitation of water absorption by starch granules and therefore swelling is restricted. When water absorption is limited, granular dimensions remain almost with no change; therefore a viscosity increase is not observed.
At lower LPC concentrations, water ingression is less influenced by LPC. 1% LPC blocks only part of the starch to swell. At 0.5% LPC and lower, water absorption and swelling accordingly are delayed but not hindered as was observed at higher concentrations.
Addition of LPC at different concentrations also resulted in lower final viscosity compared with the reference. More LPC leads to lower end viscosity. This is in agreement with the results obtained by *Putseys et al. 2010*[14]. They reported that the presence of emulsifier resulted in a weaker and less structured network in the early cooling phase that is less shear resistant than the control. Moreover, the leached amylose chains are involved in complex formation with emulsifier and only small amylose fractions can form amylose double helices. Therefore, network formation occurs to a lesser extent than in the absence of emulsifier. *Conde-Petit et al. 1995*[4] also reported the formation of junction zones (as physical cross-link) in a network between granules due to leached amylose inclusion complexation with emulsifiers.

During swelling, amylose leaches from the granules. This is one of the processes involved in gelatinization[1]. Upon further heating, water uptake and swelling continue, the viscosity of starch suspension further increases until a maximum of viscosity (T_p). At this point granules rupture and break down into starch fragments which results in a viscosity decrease. The shear forces throughout the RVA process disrupt the formed starch gel. In the third phase, viscosity increases again upon cooling which marks the beginning of amylose retrogradation. Therefore, our results show that LPC is absorbed by the granules before reaching the gelatinization temperature and forms rather stable inclusion complexes with amylose inside the granule. This complexation successively hinders water uptake, represses amylose leaching and therefore swelling is limited. Hence, alteration in viscosity behavior depends on the degree of inclusion complex formation that correlates with the ratio of ligand to the existing amylose helices.
We think that starch granules become too rigid to swell when LPC is present at high concentration in the suspension and the amylose leakage is less accordingly, as was already discussed by *Putseys et al. 2010*[14].

The efficient role of LPC in preventing swelling was also illustrated by our microscopy images. This effect is more prominent at higher LPC concentrations. We observed no rupture after addition of 1% LPC, even at 60°C.
Richardson et al. 2003[16] observed a non-stained bright area around some of the granules by CLSM while addition of emulsifiers at high concentrations and low temperatures. The layer acts as a protective layer around the granules to slow down water transportation. In addition, *Putseys et al. 2010*[14] reported that the lipids form a layer surrounding the granules which results in less amylose leakage. *Eliasson et al. 1981*[6] also observed a lower degree of disruption of starch granules

when the amount of lipids present on the surface increases.

We observed no alteration in the temperature of birefringence loss. LPC even at high concentrations does not prevent the crystallinity loss. It becomes obvious that LPC does not interfere with the change in crystallinity order within the starch granules. The DSC results also show that the crystalline regions of the starch granules are not affected by the addition of LPC; as no difference in the onset of the first endotherm in comparison with the reference was observed.

LPC addition resulted in a lower enthalpy of the first endotherm and a higher enthalpy of the second endotherm in DSC scan. The second endotherm is a direct indication that inclusion complexes with amylose are formed. A higher enthalpy of the second endotherm upon LPC addition clearly proves that more inclusion complexes are formed with a higher amount of LPC. As the complex formation is an exothermic transition, this also results in a lower enthalpy of the first endotherm. Our results are in accordance with *Biliaderis et al. 1991*[2] who have observed the reduction of the enthalpy in the first endotherm due to complex formation of amylose with LPC as well. They have also stated that not only amylose but also the longer linear chains of amylopectin interact with LPC. These endothermic transitions were furthermore reported by *Yamashita et al. 2001*[25] in complexation of wheat starch and lysophospholipid and by *Siswoyo et al. 2003*[20] in complexation of defatted wheat starch with mono and diacyl-*sn*-glycerophosphatidylcholine as well.
Water content and the amount of present ligand influence the rate of amylose inclusion complexation. *Eliasson et al. 1980*[5] stated that the onset temperature and enthalpy of the first endotherm vary with water content

and later on, *Eliasson et al. 1981*[6] reported that at low water content, the gelatinization enthalpy and the gelatinization temperature are not influenced by the presence of lipids.
Jovanovich et al. 1992[13] demonstrated that at water content of 36-64%, lower moisture results in lower enthalpy and higher onset temperature of the first endotherm; therefore amylose-lipid inclusion complexation forms later.
Our starch suspensions were scanned with different amount of LPC at high water content (above 80%). We did not observe any shift in onset temperature of the first endotherm influenced by the amount of LPC at high water content.
We suppose that LPC has no influence on water ingression before the crystallinity loss but later in the process, amylose helices have more liberty to move and contribute in the complex formation with LPC. A difference in osmotic pressure leads to water ingression which is a function of the number of soluble molecules. This number would decrease when amylose-LPC complexes are formed. We hypothesize that a reduction in osmotic pressure is driven by this phenomenon and consequently that reduction of water uptake suppresses amylose leakage and swelling. *Hernandez-Hernandez et al. 2011*[9] stated that during the starch gelatinization, amylose tends to leach as consequence of osmotic pressure and water ingression; therefore approaches the external layers of the granule that there meets LPC that exists in the water phase. A protective barrier is constituted by the formed complexes which prevents rapid granule hydration. They believed that this barrier increases thermal stability of the granules and allows the granular morphology to remain intact even above gelatinization temperatures. Earlier study[14] also reported a layer formation around the granules, after amylose-lipid inclusion complexation that diminishes the

entry of water. However, in situ complexation between amylose and lipids, either at the surface or inside the granules, lessens amylose leaching[15].

The swelling power of starch granules and the amount of water soluble starch were substantially inhibited by addition of LPC. Heating enhances water penetration into the granules and amylose leakage into the solution accordingly. The dynamic role of temperature is well-known in weakening the intragranular binding forces of starch to accelerate leaching of amylose, which leads to an increase in solubility. By addition of LPC to starch suspensions and formation of amylose-LPC inclusion complexes, amylose does not easily leach out. In addition the crystalline arrangement of amylopectin prevents the easy penetration of water into the granules by contributing its longer chains in inclusion complexation[14]. These two phenomena limit solubility due to less amylose leakage and swelling, as a consequence of less water absorption by amylopectin. The observed decreased swelling and solubility by a higher amount of LPC is therefore again a direct indication for the enhanced amylose-LPC complex formation.

Fig. 7 combines the discussed results on the influence of LPC on granular properties of wheat starch at 90°C. It is clear that higher amounts of LPC results in less solubility and swelling power and a higher enthalpy of the second endotherm (melting of the amylose-LPC inclusion complexes). These results suggest that with a higher amount of LPC more amylose–LPC inclusion complexes are formed.

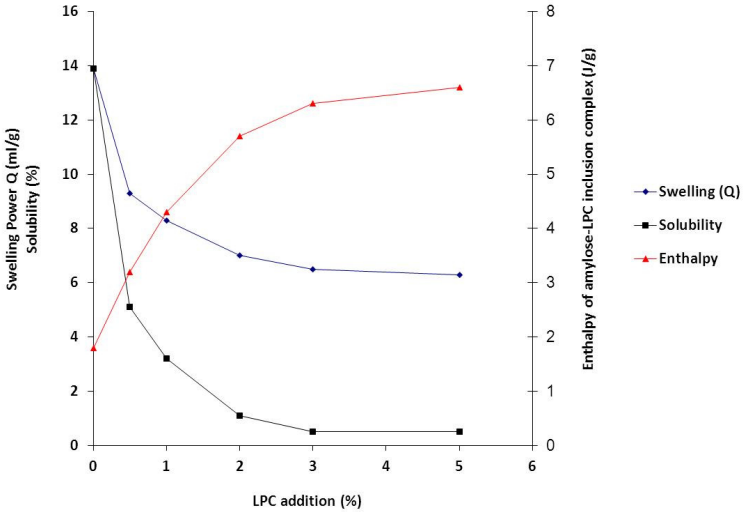

Fig. 7. Swelling power and solubility alteration at 90°C while enthalpy increase in the second transition (due to amylose-LPC inclusion complexes). Swelling power and solubility are plotted on the left axis against enthalpy. Different points represent different LPC additions.

Our results clearly indicate the influence of LPC on swelling power and solubility based on amylose-LPC complex formation. LPC slips into the amylose helices and forms inclusion complexation. Furthermore, amylose-LPC inclusion complexation on the granule surface reduces water mobility inside granule hence results in swelling reduction. The reduction of swelling, that at 5% LPC results in no viscosity formation, seems more likely due to the layers of amylose-LPC complexed materials in the concentric amorphous zones of the starch granule. This work also gives good support to the idea that amylose is only found in the concentric amorphous regions of starch granule.

5. Conclusion

This study describes the effects of LPC at different concentrations on pasting time, gelatinization, granular structure, amylose leakage and thermal transition of wheat starch.

Amylose-LPC complexation has an extensive influence on structuring properties of wheat starch. No viscosity increase due to less swelling, reduced rupture and thus limited amylose leakage was reported as consequences of LPC addition at high concentrations to a diluted wheat starch suspension.

At lower concentrations, its influences are moderate while retaining the starch functionalities.

From the structuring point of view, concentrations higher than 1% are unacceptable. At these levels, starch can no longer be used as a structure builder. However at LPC concentrations of around 0.5%, amylose-LPC complexes form and amylose leakage is largely prevented leading to less granular rupture.

Low digestibility of amylose-LPC complexes by enzymes comparing with amorphous amylose can be regarded as slow starch; however the effect on amylopectin is still unclear. The effect on starch digestibility will be subject of our future study.

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Chapter 3

The effect of temperature and time on the formation of amylose-lysophosphatidylcholine inclusion complexes

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Abstract

The formation of amylose inclusion complexes could help to decrease the susceptibility of starch granules against amylase digestion. We studied the formation of amylose-lysophosphatidylcholine (LPC) inclusion complexes at temperatures at and below the gelatinization temperature of starch, using Differential Scanning Calorimetry (DSC), Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM). At temperatures below 50°C, only low amounts of complexes were formed; even upon prolonged incubation time (16 h). However, our results show that heating at 50-60°C, for prolonged times, leads to a nearly complete formation of inclusion complexes. Our study therefore indicates that gelatinization is not a prerequisite for inclusion complex formation. Also, the thermal characteristics of the gelatinization peak (onset temperature and enthalpy) are not affected by amylose-LPC complex formation.

1. Introduction

Amylose is primarily a linear polysaccharide of α-1→4D-glucose units that is thought to be located primarily in amorphous region of the starch granule[3]. Amylose twists around the hydrophobic chain of several ligands (such as free fatty acids and surfactants) in aqueous media[7, 16] and forms a helical configuration which is called the V-complex[9]. The V-complex is visible as the V-type X-ray diffraction (XRD) pattern[4]. These complexations are of great interest in food systems since they modify both the structural and functional properties of starch[10]. XRD has indicated six, seven or eight D-glucose residue per turn (depending on the ligand) after amylose inclusion

complexation[9]. The interaction between amylose and a guest molecule generally reduces the solubility of starch in water and increases its melting temperature[1, 3]. Several methods can be used to study the interaction of amylose with ligands; including iodine absorption, enzymatic analysis, XRD analysis, thermal analysis such as DSC and rheological methods such as Rapid Visco-Analyser (RVA). DSC and RVA are the methods that have contributed the most to our understanding of the formation of amylose inclusion complexes[1, 4]. With DSC, the amylose inclusion complexes are characterized by an endothermic transition at temperatures around and above 100°C at which the complexes melt. Enthalpy (ΔH) is the parameter most often used to measure the total required heat. Enthalpy as well as transition temperature are attributed to the crystallite melting (amylopectin lattice), amylose-ligand crystallization (coil to helix transition) and the disintegration of the amylose complexes (helix to coil transition)[2, 4].

The formation of amylose-ligand complexes in starch suspensions is influenced by temperature, water activity, DP of amylose and chain length of ligand (aliphatic tail) as well as the extent of amylose saturation. These factors are further explained as follows:

(i) Temperature – Amylose has a low complexation tendency with present ligands at ambient temperature; however it is highly dependent on the type and size of the ligand. When the temperature reaches the gelatinization temperature, the amylose helix can be well formed around the ligand[2]. The ligand is stabilized inside the helix by intra-molecular bonds (van der Waals interactions and hydrogen bonding) between the turns along a helix[10]. DSC analysis of the complexed amylose shows an

endotherm during heating (95-115°C) and a subsequent exotherm during cooling (75-85°C)[9]. The formation of V-amylose is a thermoreversible process, proven by the exothermic transition in the cooling scan.

(ii) Water activity – At a water content above 40% w/w, the amorphous regions of starch plasticize[9]. Water influences both the glass transition (T_g) and the melting temperature (T_m)[4]. Water helps melting the crystallites at lower temperatures and facilitates chain mobility[2]. Amylose molecules disperse more freely in the aqueous phase in excess water; therefore their availability to form complexes increase[6].

(iii) DP of amylose and chain length of ligand (aliphatic tail) – 18-24 glucose units are required for the formation of an amylose inclusion complex[11]. In addition, *Godet et al. 1995*[5] stated that the amount of complexes formed increases with amylose chain length. A number of reports also stated that long branches of amylopectin could also participate in inclusion complexation[12]. However, *Godet et al. 1995*[5] stated that complexation with amylopectin branches is almost impossible due to intramolecular crowding.
The type of ligand is another key factor in extending the amylose inclusion complexes. *Siswoyo et al. 2002*[13] studied the formation of amylose inclusion complexes with glycerophosphatidylcholine (GPC) and observed lower enthalpy due to increasing the chain length of the ligand. In another study, a chain length of at least ten aliphatic carbon atoms is reported essential to induce amylose inclusion complexation[11]; however it is likely that the

minimum chain length of alkyl chain depends on the hydrophilicity of the head group.

(iv) Degree of amylose saturation – This parameter relates to the proportion of amylose helices interacting with ligand. Depending on the molar ratio of amylose and ligand and also the incubation conditions, it is reasonable to assume that a varying degree of amylose helices will not be involved in the complex formation. In addition, accessibility of amylose molecules will also play a role, the ones that are captured within the crystallite part of starch will not be able to form complexes[4].

In food systems, the formation of amylose inclusion complexes occurs around the temperature of gelatinization[15]. Most cereal starches contain small quantities (0.5% based on starch) of naturally occurring lipids (exclusively lysophospholipid) that appear as inclusion complexes with a fraction of amylose in native starch granules[6, 10].

It is generally accepted that gelatinization is a prerequisite for the formation of amylose inclusion complexes; however recent studies have established a high complexing ability of amylose and lysophosphatidylcholine (LPC) in wheat starch in the granular state[15]. This prompts the question if such complexes can also be formed below the gelatinization temperature.

The rate of amylose-LPC complex formation depends on factors outlined above. Recent studies have revealed the thermodynamic and kinetic characteristics of inclusion complexations[13]. Also, this complexation alters the conventional time-temperature behavior of starch when heated in water[15]. Our earlier study showed that amylose-LPC inclusion complexation mostly occurs

around the gelatinization temperature based on the RVA viscosity profile. RVA results proved that the viscosity increase, that is usually related to gelatinization, shifts to higher temperatures by addition of LPC. This is interpreted by LPC limiting water ingression and with that less water activity inside the granules. In addition, the onset temperature increases and a decrease in the viscosity peak is observed. Furthermore, the DSC results revealed a clear decrease of enthalpy in the gelatinization endotherm (as an exothermic transition). A clear increase in the second endotherm demonstrated the formation of inclusion complexes[1].

In the present study we set out to try and separate complex formation and gelatinization, understanding the optimum condition for more complex formation. Our previous study reported the complex formation during continuous heating passing the transition temperature for gelatinization. In this study, we aimed to unravel the extent of complex formation when incubating starch with LPC at temperatures below the gelatinization temperature for prolonged times.

2. Materials and methods

2.1. Materials

Native wheat starch with a purity of 99% and a total lipid content of 0.4% was obtained from Sigma Chemical Company. Moisture content (12.63%) was measured by a moisture analyzer (Sartorius MA35M, Sartorius AG, Germany). Damaged granules (2.8%) and 23.5% amylose content (wheat starch not defatted) were reported by Eurofins Food B.V.

Egg yolk L-α-Lysophosphatidylcholine (LPC), type XVI-E, lyophilized powder, purity >99% and fatty acid content of 16:0 69%, 18:0 27% and 18:1 3%, from Sigma Chemical Company (St Louis, Missouri, USA) was used.
Potato amylose M1551, with a molar mass of 180,000 g/mol, was a gift from AVEBE. 12.10% moisture content was measured by a moisture analyzer (Sartorius MA35M, Sartorius AG, Germany).
3,3'-dioctadecyl-5,5'-di(4-sulfophenyl)oxacarbocyanine ($C_{65}H_{91}N_2NaO_8S_2$) with a MW of 1115.5 g/mol was purchased from Molecular Probes (Grand Island, New York, USA).
Dimethylformamide (DMF) was obtained from Thermo Fisher Scientific Inc. (Rockford, USA).

2.2. Temperature-time effect

A series of 9% w/w wheat starch suspensions in deionized water was prepared by mixing starch with 2% LPC (based on dry matter wheat starch). The samples were rotated 15 min at 50 rpm at ambient temperature. The suspensions were then heated while rotating in a ventilation oven (Thermo Scientific Heraeus 6000, Langenselbold, Germany) at 20, 30, 40, 50 and 60°C for 1, 2, 4, 8 and 16 h and then slowly cooled down to room temperature. Ca. 55 µl of the suspension was pipetted into DSC stainless steel pan (Perkin Elmer, Norwalk, CT, USA) which was sealed afterwards (unwashed samples). The rest of the suspension was washed with 3 mL water followed by centrifugation for 15 min at 2000 rpm. The supernatant after 1 h heating was decanted in a separate tube, for the further assessment (Section 2.3), and the residues were washed with 8 mL water two additional times under similar conditions. Water was added to the residue to prepare a suspension of 5 g in total weight which afterwards was pipetted into DSC stainless steel

pan and sealed (washed samples). The washed and unwashed samples were analyzed (see Fig. 1) with a Perkin Elmer Pyris 1 DSC (Norwalk, CT, USA) which was previously calibrated with Indium (melting temperature= 156.6°C, melting heat= 28.45J/g). The baseline was obtained with two empty pans during a heating-cooling–reheating sequence (20-120°C). The onset (T_o), peak (T_p) and ending (T_e) temperatures (°C) as well as the enthalpy (ΔH, J/g) for the different transitions were determined and calculated by DSC software for each washed and unwashed sample. The heating rate was 10°C/min.

Finally, the washed samples were compared with the unwashed ones to calculate the yield of amylose inclusion complexation with LPC as function of temperature and time:

$$\text{Yield of amylose-LPC complexation (\%)} = \frac{\Delta H \text{ washed} - 1.5}{\Delta H \text{ unwashed} - 1.5} \text{ x } (100)$$

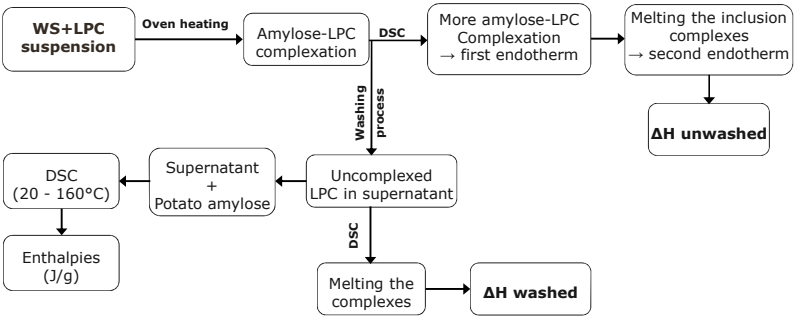

Fig. 1. Experimental design of the study.

The enthalpy due to the naturally present LPC in wheat starch (1.5 J/g, experimentally assessed) was excluded from the total enthalpy values to evaluate the formation of amylose inclusion complexes with the exogenous LPC.
All samples were measured in duplicate and the enthalpies of the amylose-LPC recrystallization (cooling scan) were considered for the calculation of the yield of amylose-LPC complex formation.

2.3. The role of temperature on the amount of complexed LPC

Uncomplexed LPC was measured as follows: 1 mL of the supernatant, resulting from Section 2.2, was freeze dried and subsequently dissolved in 200 µL deionized water. The solution was mixed with potato amylose to obtain an amylose suspension with a final concentration of 0.5% w/w (see Fig. 1).
The suspensions were directly prepared in DSC stainless steel pans (Perkin Elmer, Norwalk, CT, USA) which were sealed and kept 1 h at room temperature to equilibrate. Samples were analyzed by a Perkin Elmer Pyris 1 DSC (Norwalk, CT, USA). The heating rate was 10°C/min and the samples were heated from 20 to 160°C. A baseline was obtained with two empty pans during a heating-cooling–reheating sequence (20-160°C). The enthalpies (ΔH, J/g of sample) of potato amylose-LPC complexes were calculated by DSC software based on the endothermic peak, depending on the LPC concentration. More potato amylose-LPC complexation indicates less LPC complexation with wheat starch.
To calculate the amount of uncomplexed LPC, a calibration line was prepared using a series of LPC concentrations that were incubated with a fixed amount of potato amylose. The resulting calibration line had an

R^2 of 0.99. This allows us to accurately measure the amount of non-complexed LPC.

2.4. CLSM imaging

9% w/w wheat starch suspensions in deionized water were prepared with the addition of either 0%, 0.5% or 2% LPC (based on dry matter wheat starch). The suspensions were rotated 15 min at 50 rpm at room temperature and were subsequently heated in a ventilation oven (Thermo Scientific Heraeus 6000, Langenselbold, Germany) while rotating at 20, 50 and 60°C for 1 hour. After this, the samples were cooled down to room temperature.
A 0.2% dye solution was prepared (0.005% based on dry matter wheat starch). Sodium salt of 3,3'-dioctadecyl-5,5'-di(4-sulfophenyl)oxacarbocyanine (SP-$DiOC_{18}$(3)), as the fluorescence dye, was used. Dimethylformamide (DMF) was employed as stock solvent.

1 mL of the prepared starch-LPC suspension was stained by 20 µL of the dye for 30 min at room temperature. Imaging was performed on a watch glass using a Leica TCS SP5 Confocal Laser Scanning Microscope (CLSM) at room temperature. The set-up was configured with an inverted microscope (Leica DM16000) and with a set of four visible light lasers (Leica Microsystem CMS GmbH, Mannheim, Germany). An argon laser was used to excite the dye. HC PL APO 20x/0.70 IMM/CORR CS was used as the objective lens. A pixel resolution of 1024 x 1024 was used for the digital images.

2.5. SEM imaging

9% w/w wheat starch suspensions were prepared with 0%, 2% and 5% LPC (based on dry matter wheat starch) in deionized water. The suspensions were rotated 15 min at 50 rpm at ambient temperature and heated subsequently in a ventilation oven (Thermo Scientific Heraeus 6000, Langenselbold, Germany) while rotating at 30 and 60°C for an hour. The suspensions were cooled down to room temperature and subsequently centrifuged for 15 min at 2000 rpm. The supernatant was decanted and the residue was freeze dried in a laboratory freeze dryer (Zirbus technology, VaCo 2, Germany). The freeze-dried samples were coated with 3 nm Platinum/Palladium (80/20) alloy. The measurements were performed on a JEOL 6320F Field Emission Microscope operating at 3 kV with a beam current of $1x10^{-10}$ A.

3. Results

3.1. Temperature-time effect

To study the formation of amylose inclusion complexes with LPC, we chose short (1 h) and long (16 h) heating times at several temperatures (20, 30, 40, 50 and 60°C). As thermal analysis by DSC demonstrated, the complex formation highly depends on the incubation temperature and time. The results showed that the complexation takes place even at temperatures below the gelatinization temperature of starch; however the amount of complexes formed at 20-40°C is small. Complex formation is strongly increased when the incubation temperature is increased to 50°C (see Fig. 2). One hour heating at 60°C, just below the temperature of gelatinization, resulted in

maximal complex formation, which implies that all amylose is in complex with LPC. As shown in Fig. 2, heating at 60°C, during an hour, results in a slight increase above 100% (about 3%) which is due to the small enthalpy deviation between the references.

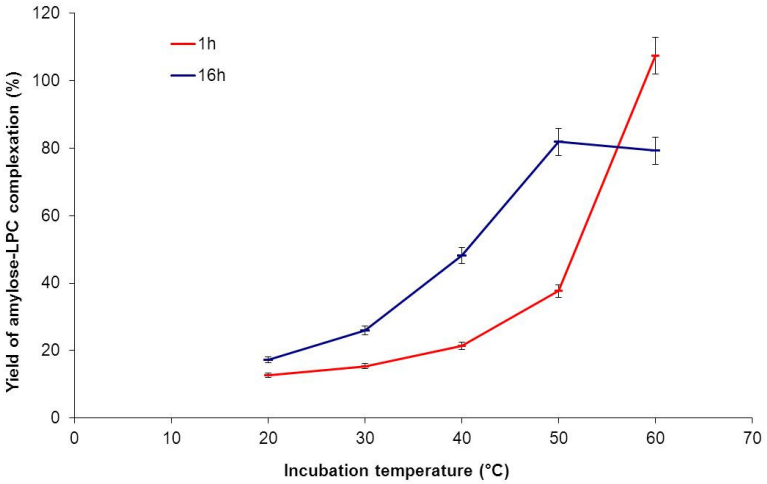

Fig. 2. Influence of temperature on amylose inclusion complexation with LPC during short and long heating times. The enthalpy ratio of washed and unwashed samples at each temperature point, excluding the 1.5 J/g due to the naturally present lipids in wheat starch, results in the yield of inclusion complexation.

Depending on the incubation temperature, the effect of incubation time was more pronounced: the formation of complexes was slow at low temperatures; while complexation increased by a factor of two at temperatures >40°C. At 60°C complex formation was fast, with no effect of further increasing the incubation time (see Fig. 2). Also, we observed that the thermal

analysis of the "unwashed sample", heated at 60°C for 16 h, resulted in an enthalpy value of 5.3 J/g (amylose-LPC dissociation endotherm) which is equivalent to the enthalpy value obtained for the "washed sample" after 1 h heating at 60°C (see Table 1). It is not unlikely that after 16 h at 60°C part of the complexes, formed at the peripheral layers, have detached from the surface of starch. These complexes are removed during the subsequent washing process.

This study also shows the influence of heating time on amylose inclusion complexation with LPC. Fig. 3 clearly shows in more detail that the complex formation increases with time. At 20°C only a slight increase was observed (see Fig. 3). However an almost double increase of complexation was observed upon 4 h heating at 50°C, comparing with 1 h. No further increase in complex formation was observed after 8 h heating at 50°C.
Table 1 presents the enthalpy values of amylose-LPC dissociation endotherms, as a function of time and temperature, before and after the washing process. These enthalpy values are a measure of the amount of formed inclusion complexes.

With the unwashed samples, considerable amounts of inclusion complex are formed, independent of temperature and incubation time. This is probably due to adhering LPC able to form complexes during the DSC analysis. The washed samples therefore present a more reliable assessment of the amount of formed complexes, again showing a clear dependency on both incubation time and temperature.

Interestingly, the formation of inclusion complexes at 50°C and below has no significant effect on the enthalpy of the starch gelatinization endotherm (see Table 2).

Since crystallinity is not affected when heating at 40 and 50°C[1], almost a normal gelatinization endotherm was observed in DSC (enthalpy of the gelatinization endotherm was measured 17 J/g for native wheat starch).

Heating at 60°C partly melts the crystalline region and therefore leads to a lower gelatinization endotherm, measured by DSC (see Table 2). Therefore, combining the results of Tables 1 and 2 leads to the observation that amylose inclusion complexation and gelatinization are not strictly related.

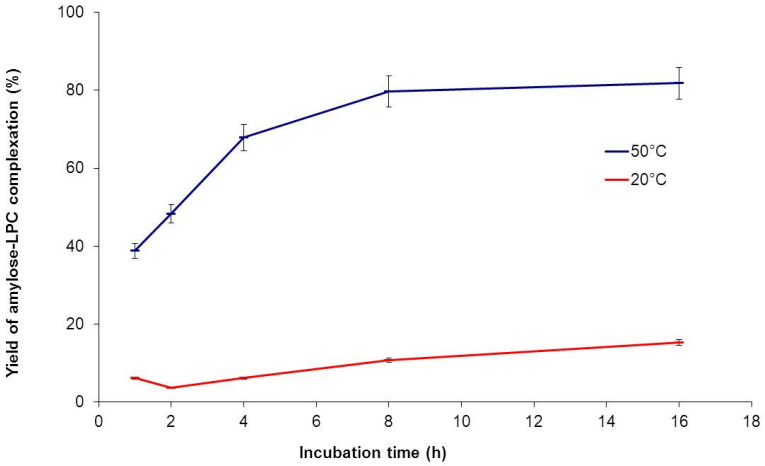

Fig. 3. Influence of heating time on the amylose inclusion complexation with LPC.

Table 1. Enthalpy values (before and after washing) of 9% wheat starch suspensions with addition of 2% LPC. The results present the average enthalpies of the amylose-LPC dissociation endotherm.

	ΔH (J/g) unwashed [a]					ΔH (J/g) washed [b]				
T (°C)	**1h**	**2h**	**4h**	**8h**	**16h**	**1h**	**2h**	**4h**	**8h**	**16h**
20	5.5 *(0.12)*	5.2 *(0.13)*	5.4 *(0.11)*	5.2 *(0.2)*	5.8 *(0.12)*	1.7 *(0.16)*	1.6 *(0.1)*	1.7 *(0.2)*	1.9 *(0.34)*	2.1 *(0.33)*
30	5.7 *(0.34)*				5.5 *(0.17)*	2.1 *(0.52)*				2.5 *(0.22)*
40	5.7 *(0.21)*				5.5 *(0.4)*	2.4 *(0.12)*				3.4 *(0.44)*
50	5.7 *(0.38)*	5.9 *(0.35)*	5.8 *(0.33)*	5.4 *(0.5)*	5.3 *(0.51)*	3.1 *(0.2)*	3.6 *(0.3)*	4.4 *(0.43)*	4.6 *(0.42)*	4.6 *(0.7)*
60	5.1 *(0.78)*				5.3 *(0.8)*	5.3 *(0.42)*				4.5 *(0.61)*

[ab] *Enthalpy (ΔH, J/g)*
The values within the parentheses represent the SDs (n=2).

Table 2. Enthalpy values (J/g) of the gelatinization endotherms after 1 and 16 h incubation (unwashed samples). The suspensions contain 9% wheat starch and 2% LPC (based on DM wheat starch). The values were compared with the enthalpy of the gelatinization endotherm of native wheat starch (17 J/g).

Temperature (°C)	**ΔH_{1h} (J/g) [a]**	**ΔH_{16h} (J/g) [b]**
40	15 *(0.45)*	15.5 *(0.51)*
50	14 *(0.7)*	13.2 *(0.66)*
60	4.5 *(0.85)*	3.4 *(0.74)*

[ab] *Enthalpy (ΔH, J/g)*
The values within the parentheses represent the SDs (n=2).

3.2. The role of temperature on the amount of complexed LPC

Table 3 presents the amount of complexed LPC during 1 h incubation of wheat starch with 2% LPC at 20, 30, 40, 50 and 60°C. Potato amylose was employed as an indicator to develop inclusion complexes with the uncomplexed LPC (present in the supernatant) resulting from the heating of wheat starch with LPC in a diluted suspensions. Therefore, more potato amylose-LPC complexation, the less wheat starch-LPC complex formation.

Table 3. The amount of complexed LPC (%) with wheat starch after one hour heating at 20, 30, 40, 50 and 60°C. The amounts are determined by the extent of potato amylose complexation with free LPC after washing. Amounts are expressed as % of the amount measured at 60°C.

Temperature (°C)	Complexed LPC (%)
20	18.7 *(0.4)*
30	21.6 *(0.32)*
40	32.9 *(0.46)*
50	48.8 *(0.59)*
60	100 *(0)*

The values within the parentheses represent the SDs (n=2). They are based on the melting enthalpies of potato amylose-LPC complexes.

As expected, we observed that a higher incubation temperature leads to more LPC complexation with wheat starch; hence less uncomplexed LPC in the supernatant.

This results in a lower melting enthalpy of potato amylose-LPC complexes in the next step. The effect of temperature is more pronounced between 50 and 60°C. This is in excellent agreement with our previous findings (see Figs 2 and 3).

3.3. CLSM imaging

Heating in the presence of water allows the starch granules to swell and results in loss of crystalline structure at 60°C and ultimately granular rupture at higher temperatures. We studied the influence of LPC on temperature-induced changes in the starch shape and structure. The starch granules were observed by CLSM after 1 h heating, cooling to room temperature and subsequent staining. Fig. 4 shows the CLSM images of wheat starch granules with 0%, 0.5% and 2% LPC each incubated at 20, 50 and 60°C.
In the absence of LPC, significant changes were observed at 60°C (see Fig. 4, first row). At 60°C and 0% LPC, the starch granules begin to swell, amylose molecules leach out and therefore some ruptures were observed.
Fig. 4, second row, shows the influence of LPC at 0.5% with less swelling and limited rupture compared to 0% LPC. The influence of LPC on the granular shape is most clear at 2% LPC concentration. Fig. 4, third row, clearly depicts intact starch granules even after 1 h heating at 60°C.

The images show that at 20°C, most of the fluorescence is in the continuous phase. Heating at 60°C shows that the fluorescence has disappeared from the continuous phase, indicating the formation of inclusion complexes.

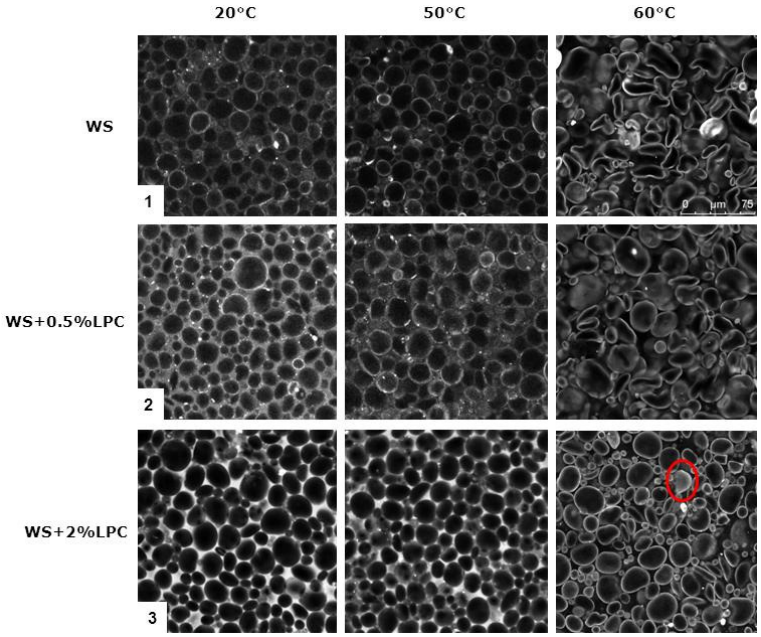

Fig. 4. CLSM images of wheat starch granules after addition of 0.5 and 2% LPC heated for 1 h at 20, 50 and 60°C, comparing with the reference. The bright areas are the regions rich in fluorescent label. WS denominates wheat starch and LPC Lysophosphatidylcholine.

3.4. SEM imaging

The influence of LPC, in different concentrations, on the morphology of the granules was also observed by SEM. Fig. 5 presents the SEM images of wheat starch granules, incubated at different temperatures, after addition of 0%, 2% and 5% LPC.

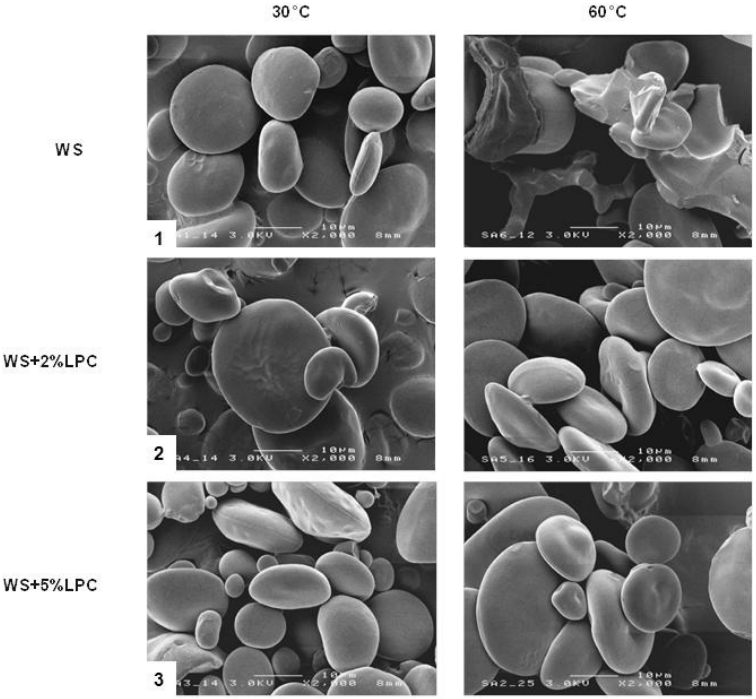

Fig. 5. SEM images of wheat starch after addition of 2% and 5% LPC heated for one hour at 30°C and 60°C, compared to the native wheat starch.
WS denominates wheat starch and LPC Lysophosphatidylcholine.

In the absence of LPC, the starch granules remained intact at 30°C; but fully ruptured after 1 h heating at 60°C (see Fig. 5, first row). At 2% LPC, we observed the granular shape after 1 h heating at 60°C (see Fig. 5, second row). Increasing the LPC concentration to 5% resulted just in a slight change compared with 2% LPC (see Fig. 5, third row).

4. Discussion

Amylose-LPC complexes are of interest in decreasing the susceptibility of starch to amylase. This study describes the influence of incubation temperature and time on the formation of amylose-LPC inclusion complexes. Earlier studies have clearly demonstrated the formation of the complexes at temperatures at or higher than the gelatinization temperature[4, 15]. In this study we specifically focused on studying the formation of inclusion complexes at temperatures lower than gelatinization onset temperature.
As expected, time and temperature are the most important factors that contribute to the formation of amylose inclusion complexes. LPC (2%) was employed in this study since we showed earlier that 2% LPC results in a rather high melting enthalpy[1]. Therefore, DSC and microscopy techniques were used to study the effect of incubation temperature and time on the formation of amylose inclusion complexes with LPC and the subsequent effect on the integrity of starch granules.

Studying the complex formation at temperatures lower than 50°C, clearly shows the formation of amylose inclusion complexes with LPC; however in lower amounts. Long term incubation (16 h) improved the complex formation at low temperatures, resulting from the fact that the water ingression is low and consequently little swelling occurs. At 60°C, starch granules start to absorb more water, due to the osmotic pressure, which results in the absorption of LPC by the starch granules. Water absorption plasticizes the amorphous region[14] and loss of birefringence occurs[1] which enhances amylose mobility and inclusion complex formation.
A significant increase of complex formation between 50 and 60°C (see Table 3) clearly shows that at temperatures closer to the start of gelatinization (65°C –

complete loss of crystallinity[1]), inclusion complex formation can more easily occur due to enhanced access to amylose molecules and also a better movement of LPC through the granule, while the crystalline region still exists even after 16 h incubation (see Table 2). This revealed: (i) the possibility of complex formation at temperatures well below gelatinization in presence of crystalline lattice in case of longer incubation time; and (ii) starch gelatinization is fully related to amylopectin melting.
The extent of inclusion complexation formed by potato amylose with free LPC – resulting from the washing process – proved our earlier findings. Our observation showed that a higher temperature propels amylose to form more inclusion complexes with LPC; therefore results in less uncomplexed LPC to develop inclusion complexes with potato amylose in the next step (see Table 3).

The CLSM images show bright rim areas around the starch granules in all samples and temperatures; again showing that inclusion complexes can form at temperatures well below the gelatinization temperature. Significant differences can be observed between native wheat starch and the samples containing LPC – rupture of the granules can be clearly seen at 60°C in native starch while less and no rupture can be observed at LPC concentrations of 0.5% and 2%, respectively. This is in excellent agreement with our earlier study in which we demonstrated an effect of LPC on starch solubility of 4% and 1% at 70°C in the presence of 0.5% and 2% LPC, respectively; while 6% was reported for native starch[1].
The difference in fluorescence between core and outer rim of the starch granules (the rim is significantly brighter) supports our assumption and previous results by *Jane et al. 1993*[8] that LPC forms inclusion complexes with the peripheral amylose molecules first which in

addition can prevents further LPC absorption. Various damaged granules observable in Fig. 4 (third row – one example is highlighted), which shows fluorescence all over the granule, support this as due to the damaged protective layer of these granules that the dye can diffuse all the way in.
The CLSM images with 2% LPC (Fig. 4, third row) showed that the bulk solution is brighter at low temperatures while at the fluorescence signal is located mainly at the outer layer of the granules at 60°C. This again proves that more inclusion complexes form at temperatures between 50 and 60°C. This is in good agreement with Fig. 2 and Table 3 and also our earlier results obtained by DSC[1].

The SEM observations are in excellent agreement with CLSM images. In presence of LPC, the structural integrity of starch granules is clearly preserved. Not much differences can be observed between the concentrations of 2% and 5% LPC which is in contradiction to our previous findings with DSC as well as the solubility and swelling power measurements[1]. This shows that SEM is not a technique to detect the spatial resolution of LPC rendering CLSM the superior method. This can be due to the dehydration step that the starch granules bear to be prepared for SEM imaging which is not a need for CLSM imaging.

5. Conclusion

This study demonstrates the influence of incubation temperature and time on the formation of amylose inclusion complexes. It clearly shows the formation of amylose-LPC complexes at temperatures well below

gelatinization temperatures. The extent of complex formation is increased at temperatures above 50°C due to starting loss of structural integrity (crystallinity). Complexation at lower temperatures is increased during longer complexation time. The different yields of complexation as the result of different incubation temperatures and times propel the applicability of this study in the cereal based products with a lower cooking temperatures.

We also demonstrated that the formation of amylose inclusion complexes with LPC has no influence on the gelatinization enthalpy of wheat starch; nevertheless the melting of amylopectin promotes amylose-LPC complexation at short incubation times.

The effect of LPC on the hydration of starch granules was also visualized by CLSM and SEM. The formation of inclusion complexes leads to a clear preservation of structural integrity of the starch granules at 60°C, as shown by SEM.

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Chapter 4

The influence of amylose-LPC complex formation on the susceptibility of wheat starch to amylase

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Abstract

This study was aimed to assess the role of lysophosphatidylcholine (LPC) in the development of Slowly Digestible Starch (SDS). The influence of LPC, on the enzymatic degradation of diluted 9% wheat starch suspensions (w/w) was investigated, using an *in vitro* digestion method. Wheat starch suspensions containing 0.5-5% LPC (based on starch) were heated in a Rapid Visco Analyser (RVA) till 95°C and subjected to enzyme hydrolysis by porcine pancreatic α-amylase at 37°C for several digestion periods. *In vitro* digestion measurements demonstrated that complexing starch with 5% LPC leads to a 22% decrease in rate of reducing sugar compared to the reference while the samples containing 0.5% LPC showed an equal digestibility comparable to the control. A clear decrease in the formation of reducing sugars was observed in presence of 2-5% LPC; since the results after 15 min digestion imply the formation of SDS due to the formation of amylose-LPC inclusion complexes. The DSC measurements proved the presence of amylose-LPC inclusion complexes even after 240 min digestion demonstrating the low susceptibility of amylose-V complexes to amylase.

1. Introduction

Public awareness on the relation between human health and nutrition has increased with the increased attention for obesity and diabetes type II. With respect to the latter, particularly a focus has been given to starch and starchy foods. Starch is the largest source of carbohydrates in the human diet. In the West, it constitutes 27% of the total food energy sources and it

reaches to above 50% in Southeast Asia[3]. In this respect, the rate and extent of starch digestion is of great interest as it affects the glycemic response[20]. Starch is a homopolymer of glucose units that consists of two fractions, amylose and amylopectin, assembled in a cluster structure. Amylose is a linear polysaccharide of α-1→4 D-glucose and amylopectin is a branched polymer of α-1→4 D-glucose and α-1→6 at the branching points.
The source, amount and form of consumed carbohydrates determine the digestibility and subsequently the rate of glucose release to the blood stream, called Glycemic Index (GI)[10]. The GI describes the level of the postprandial glucose rise in blood as compared to ingestion of a standard dose of glucose[21, 22]. High peaks in blood glucose are considered a risk factor in diabetes type II. Hence, there is an increased interest in controlling the rate of release of glucose from starch.

In the human body, starch is hydrolyzed to glucose by enzymes through several steps[19]. Upon ingestion, starch is exposed to salivary α-amylase. Glucose absorption mainly occurs in the small intestine[14] where the pancreatic α-amylase hydrolyses amylose and amylopectin to maltose and larger linear oligosaccharides (maltotriose and maltotetraose)[11] and also the branched α-dextrins which make up around 25% of the hydrolysate product. The α-amylases hydrolyze α-(1-4) glycosidic bonds[19]. Maltase-glucoamylase and sucrase-isomaltase, two brush border enzymes, degrade oligosaccharides to glucose which then passes the blood stream[9].
Starch, based on its digestibility, can be classified into three categories: RDS (Rapidly Digestible Starch – starch that is digested to glucose after 20 min), SDS (Slowly Digestible Starch – starch that is digested to glucose between 20 and 120 min) and RS (Resistant Starch – starch that cannot be digested but is fermented in the

large intestine) which are characterized by the rate and duration of the glycemic response[6, 7].

Generally, the digestion of starch is a complex process that is strongly dependent on the substrate, enzyme adsorption by the substrate and presence of other components like lipids and proteins[10, 14].

It is possible to increase the resistance of starch components to enzyme hydrolysis. For instance, endogenous lipids and phospholipids in cereal starches have the ability of complexation with amylose[13] thus rendering the amylose less susceptible to amylolytic enzymes[15, 22]. *In vivo* and *in vitro* digestibility studies on the effect of these components have shown that they can considerably slow down the enzymatic digestibility[19].

In our previous study[1], we have evaluated the influence of LPC on the structuring properties of wheat starch and have shown that it is possible to form considerable quantities of amylose-LPC complexes or V-complexes while maintaining part of the thickening function of starch.

Recent studies have reported the low digestibility of V-complexes[16]. V-complexes are characterized by a specific X-ray diffraction pattern and are formed between the aliphatic chains of lipids and the amylose molecules. Lysophosphatidylcholine (LPC) is a complexing agent that has shown high complexing ability with amylose, as indicated by DSC[1, 3]. Also, the poor digestibility of the amylose-lipid complexes has been demonstrated[18]. However it is not clear if this complexation only affects the digestibility of complexed amylose chains or also the overall rate of digestion of starch. Therefore, the purpose of this study was to establish an understanding of the digestion of wheat starch and the influence of LPC on hampering enzyme hydrolysis; revealing additionally the difference between an overall effect of LPC on starch digestibility *versus* the degradation of the amylose inclusion complexes.

Various *in vitro* starch digestion methods exist which are designed to simulate starch digestion in the human body[10, 11]. The Englyst method[7] is a widely used method for *in vitro* hydrolysis of starchy foods. Most studies have found a good correlation between the results of the Englyst method and *in vivo* results. The Englyst method is designed to assess whole meals, while in this study we work with a purified system and need to analyze the rate of starch digestion in considerable detail. This requires a slightly different setup. Therefore, in this study we demonstrate an alternative method that is established based on the optimum conditions to investigate the digestibility of well-defined starch-LPC mixtures under controlled time-temperature-shear conditions in a diluted suspension.

2. Materials and methods

2.1. Materials

Native wheat starch with a purity of 99% and a total lipid content of 0.4% was obtained from Sigma Chemical Company. 12.63% moisture content was measured by a moisture analyzer (Sartorius MA35M, Sartorius AG, Germany) and 2.8% damaged granules and 23.5% amylose content (wheat starch not defatted) were reported by Eurofins Food B.V.
Egg yolk L-α-Lysophosphatidylcholine (LPC), type XVI-E, lyophilized powder, purity >99% and fatty acid content of 16:0 69%, 18:0 27% and 18:1 3%, from Sigma Chemical Company (St Louis, Missouri, USA) was used.
The α-amylase from Porcine Pancreatic (150,000 U/g), free flowing powder, partially purified, from Megazyme International Ireland (Wicklow, Ireland) was employed.

LPC and α-amylase were kept at -20°C and wheat starch at room temperature under dark and dry conditions.
Monosodium phosphate monohydrate, sodium phosphate dibasic, sodium chloride, sodium hydroxide, 3,5-Dinitrosalicylic acid (DNSA), potassium sodium tartrate and maltose monohydrate purchased from Sigma were of analytical grade or better.

2.2. Starch gelatinization / complexation

A RVA-4 Newport Scientific (NSW, Australia) Rapid Visco Analyzer was employed to prepare samples for the enzymatic hydrolysis. A series of 9% (w/w) wheat starch suspensions in deionized water was prepared by mixing starch with 0%, 0.5%, 1%, 2%, 3% and 5% LPC (based on starch dry matter content), previously dissolved in deionized water. The suspensions were kept 10 min at room temperature to equilibrate. The RVA was programmed in three steps. The temperature of the suspensions was first equilibrated at 50°C for 60 s, increased to 95°C at a rate of 6°C/min and held at 95°C for 300 s. The reference (pure starch) was subjected to the same temperature gradient.

Another series of 9% (w/w) wheat starch suspensions, without LPC, were prepared in deionized water and after 10 min equilibration at room temperature, heated in the RVA first at 50°C for 60 s. Then the temperature increased to 60°C, 65°C, 70°C, 75°C, 80°C, 85°C, 90°C, 95°C at the same rate of 6°C/min (see Fig. 1).

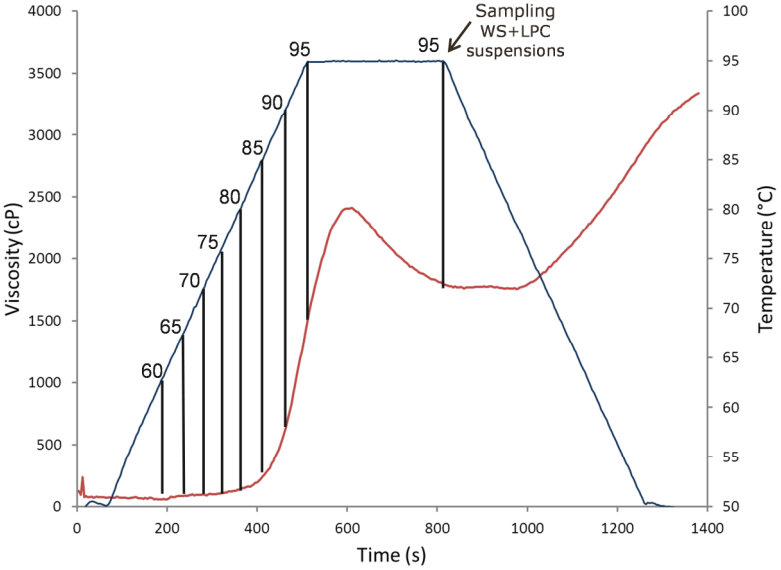

Fig. 1. RVA profile which indicates temperature-time profile and sampling point.

2.3. Preparation of DNSA reagent

The DNSA solution was prepared by dissolving 1 g DNSA (3,5-Dinitrosalycylic acid) in 20 mL 2N sodium hydroxide solution and 50 mL, 30% (w/v), potassium sodium tartrate solution. The solution was stirred while gently heating until a clear solution was obtained. This solution was diluted with deionized water to 100 mL. The DNSA solution was flushed with N_2 and stored in a dark place until use.

2.4. In vitro enzymatic digestion

5 g of each sample from the RVA was diluted with phosphate buffer (17 g, 0.025M, pH 6.9) to achieve a 2% (w/v) suspension. The phosphate buffer contained 6 mM sodium chloride to preserve the activity of the enzyme[17]. The suspensions were equilibrated at 37°C in a water bath to simulate body temperature. 0.5 mL of the enzyme solution (0.004% w/v, freshly prepared each day), was added to each suspension. Subsequently, the suspensions were incubated while rotating in a modified ventilation oven (Thermo Scientific Heraeus 6000, Langenselbold, Germany) at 37°C for 15, 30, 60, 120 and 240 min. At each digestion time, 5 mL of the incubation solution was pipetted into another test tube and heated immediately in a boiling water bath for 5 min to inactivate the enzyme. After cooling, 100 µl of the hydrolysate was mixed with 2 mL deionized water and vortexed. Then, 1 mL DNSA reagent was added, vortexed and followed by 5 min incubation at 100°C. The final solution was diluted with 1 mL deionized water after cooling and vortexing. The reaction product of reducing sugars-DNSA was measured using a Spectramax spectrophotometer (Spectramax M2 Dual Mode C, Molecular Devices, Virginia, USA) at 540 nm. Several concentrations of maltose solution were used as the standards to establish a calibration curve of maltose *versus* the absorbance for the reducing sugars determination[5]. A solution containing deionized water was prepared as a blank sample.
The samplings were done in duplicate.

2.5. Thermal analysis

Samples from the amylase digestion experiment, taken at the various digestion periods, were freeze dried in a

laboratory freeze dryer (Zirbus technology, VaCo 2, Germany). A series of 20% (w/w) freeze dried suspensions in deionized water was prepared in stainless steel pans (Perkin Elmer, Norwalk, CT, USA) which were sealed afterwards. The suspensions were kept one hour at room temperature to equilibrate. Samples were analyzed by a Perkin Elmer Pyris 1 DSC (Norwalk, CT, USA) previously calibrated with Indium (melting temperature= 156.6°C, melting heat= 28.45J/g). The heating rate was 10°C/min and the samples were heated during a heating-cooling-reheating sequence (20-120°C). The onset (T_o), peak (T_p) and ending (T_e) temperatures were calculated by DSC software. Enthalpy (ΔH, J/g of sample) was calculated based on the endothermic peak. The samples were compared to an undegraded wheat starch reference suspension.
All samples were measured in duplicate.

3. Results and discussion

3.1. Effect of temperature

A set of digestibility tests was performed on native wheat starch-LPC suspension subjected to different temperatures and digestion times to achieve a better insight into the effect of amylose-LPC complex formation on the amylase susceptibility of wheat starch. The rate of increase in the total amount of reducing sugars provided information on the susceptibility of starch to amylase. The key question studied was if LPC not only leads to a lower digestibility of amylose-V complexes but also to a lower total starch digestibility.

Fig. 2 clearly demonstrate the important role of temperature on enzyme susceptibility of the starch. We observed that at 60°C the action of the amylase is much slower than at higher temperatures; therefore a lower amount of reducing sugars are formed even after 240 min digestion. This is in agreement with our previous study[1] in which we reported the loss of starch crystallinity at 60°C. This is a prerequisite to the development of swelling and the viscosity increase of starch suspensions which do not occur below 60°C.
In Fig. 2 the result of 15 min digestion is not presented since no digestion could be observed; however 5°C temperature increase to 65°C strongly increased the amount of reducing sugars as a function of digestion time. More than 60% reducing sugars was observed after 240 min enzyme hydrolysis at 65°C. This sharp temperature effect corresponds to the change in the crystalline structure of starch granules. With heating to temperatures exceeding the gelatinization temperature of starch, the digestion rate increases because the crystalline phase melts, water ingresses and the accessibility for the enzyme increases. That leads to a sharp increase in reducing sugars.
When the starch is heated to 90-95°C (see Fig. 2), the amount of reducing sugars increases further to more than 70%. Interestingly, this increase is relatively small compared to the effect of crystallinity loss.

Cereal starches have susceptible zones that are attacked by the enzymes and form the surface channels[4]. Hydrolysis starts with the enlargement of these surface channels during heating allowing the enzyme to penetrate the core. The hydrolysis starts from the interior parts of the granules - a so-called inside-out digestion[4, 21]. Hence, starch in its crystalline structure is resistant to enzyme hydrolysis. Heating disrupts the bindings and with starch gelatinization the susceptibility of

polysaccharide chains to the digestive enzymes increases[2].

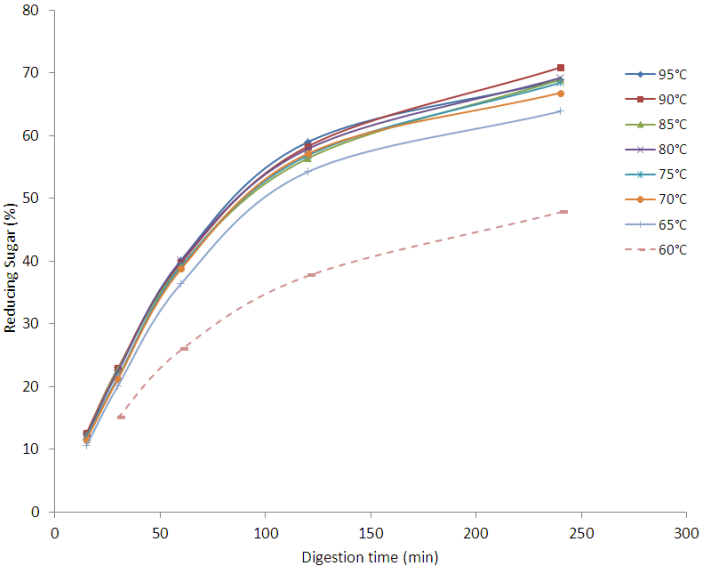

Fig. 2. Influence of the temperature of the heat treatment in RVA on the enzyme susceptibility of native wheat starch suspensions. Reducing sugar (%) is calculated as the amount of reducing sugars relative to a condition where starch is completely digested.
The result of 15 min digestion is not presented since no digestion could be observed.

3.2. Effect of LPC

Fig. 3 shows the amylase susceptibility of the starch granules after the formation of amylose-LPC complexes and gelatinization in the RVA at 95°C - a temperature at which native starch granules break down.

In the absence of LPC, the amount of reducing sugars increases as a function of digestion time (compare Figs 2 and 3). It becomes obvious that the susceptibility of starch to the enzyme decreases in presence of LPC even when the starch suspensions are subjected to a high temperature (95°C). The most pronounced effect was observed when high concentrations of LPC (3% and 5%) were added. LPC at high concentrations decreases the enzyme susceptibility of starch granules and results in less reducing sugars compared to the reference (see Fig. 3). The results indicate the presence of an undigested portion even after a long digestion time. This points to the formation of RS.

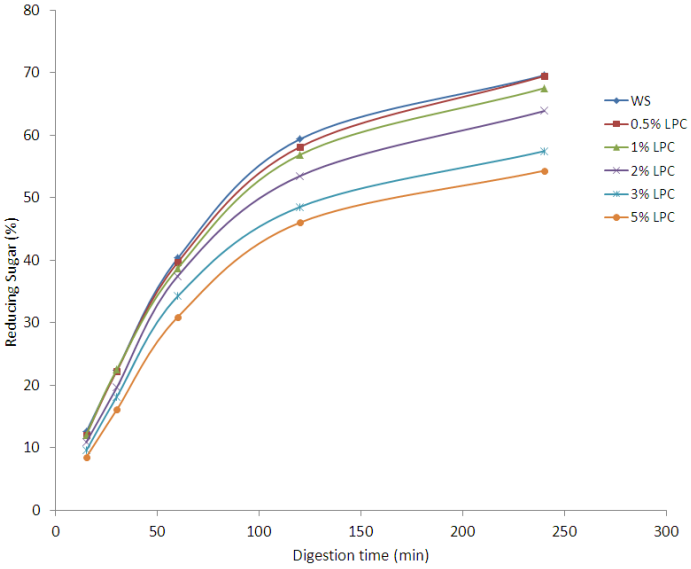

Fig. 3. Influence of LPC on the enzyme susceptibility of wheat starch after gelatinization in the RVA at 95°C. WS stands for wheat starch.

The ratio of the reducing sugars generated in the LPC containing suspensions to the reference represents the "Reduction of reducing sugars" as a function of digestion time. Table 1 shows that higher amounts of LPC lead to a significant reduction in the amount of reducing sugars.

Table 1. Reduction in the amount of reducing sugars due to the addition of LPC. This is the ratio of the reducing sugars in the presence of LPC to the reference, as a function of digestion time.

LPC (%)	Reduction in the amount of reducing Sugars (%)				
	15 min	30 min	60 min	120 min	240 min
0.5	1.8	0.7	1.5	2.0	0.1
1	1.2	0.6	3.7	4.0	3.4
2	11.0	12	6.7	10.1	8.6
3	22.0	18.7	14.9	18.1	17.2
5	31.2	28.1	23.1	23.2	22.4

In principle, the formation of inclusion complexes results in less starch available for digestion. It is not however clear, if this is due to the fact that inclusion complexes are slowly digested or if the formation of these complexes has an overall effect on starch digestibility.
We therefore first analyzed if amylose-LPC inclusion complexes still remain after enzyme hydrolysis. Heating the suspensions, prepared from the freeze dried digested samples (240 min digestion time), resulted in an endothermic transition at 108°C which is related to the presence and melting of amylose-LPC inclusion complexes. As for the undegraded samples, a higher amount of LPC leads to a higher enthalpy of the amylose-LPC endotherm. The enthalpy values of the enzyme incubated samples demonstrate the presence of inclusion complexes; however the enthalpies were lower than the undegraded samples (see Table 2). The loss of some complexes during the digestion explains the difference because not all complexes are stable (less crystalline);

therefore only the most stable complexes (well crystalline complexes) survive during the digestion. In addition, the increase in peak temperature observed after prolonged amylose digestion is another proof of the presence of stable (crystalline) complexes. The shift indicates a higher thermal stability. It is known that amylose inclusion complexation increases crystalline region, which increases the melting temperature[8]. We suppose that peak temperature is a resultant of the melting temperatures of heat stable and unstable complexes; therefore the peak temperature slightly increased after the degradation since only the most stable complexes remained in the digesta. This leads to a higher resultant in peak temperature which shows the digestion may remove less crystalline amylose-LPC complexes.
The DSC results clearly demonstrate the decrease of enzyme accessible starch due to the formation of amylose inclusion complexes. We also suppose that LPC at higher concentrations (5%) not only saturates more amylose molecules but also results in the formation of inclusion complexes with higher stability. A sharp enthalpy increase in presence of 5% LPC compared to 3%, after 240 min enzyme hydrolysis, supports this (see Table 2).

Amylose-LPC complex formation leads to less water ingression during hydration. In our previous study, we reported more than 50% decrease of swelling power, comparing to the reference, at 95°C - the temperature at which the samples were prepared for the present *in vitro* digestion method - when 3% and 5% LPC were employed; whereas 40% reduction was observed in presence of 2% LPC[1].

Table 2. Thermal analysis of 20% wheat starch-LPC suspension (freeze dried) with moderate and high LPC concentrations before and after 240 min degradation time (amylose-LPC endotherm).
WS stands for wheat starch and LPC for lysophosphatidylcholine.

Samples	Before degradation		After degradation	
	T_{Peak} (°C)	ΔH (J/g)	T_{Peak} (°C)	ΔH (J/g)
WS+2%LPC	103.8	5.7 *(0.59)*	108	3.7 *(0.4)*
WS+5%LPC	104.4	6.6 *(0.61)*	108	5.1 *(0.5)*

The values within the parentheses represent the SDs (n=2).

Less swelling, due to the presence of LPC, reduces the accessibility of amylase to the starch molecules. In addition, *Putseys et al. 2010*[16] proposed that the induced steric hindrance due to the presence of ligands diminishes the degradation of amylose inclusion complexes by enzymes. *Holm et al. 1983*[12] confirmed this when they reported amylose-lysolecithin complexes were hardly hydrolyzed after enzyme treatment as a result of a random coil to helix transition of the amylose molecules.

Comparing to the reference, ca. 25% reduction in the amount of reducing sugars was observed when 5% LPC was employed (see Fig. 3 and Table 1). This amount corresponds to the relative amount of amylose in the wheat starch which clearly indicates the complexation with nearly all available amylose molecules.

In answering the question if also the rate of amylopectin digestion is reduced as a consequence, we are aimed to

perform a detailed analysis of the relative rate of starch digestion. We assume that amylose complexation with LPC leads to a lower amount of starch available for digestion; however the overall effect will be the subject of our future study.

4. Conclusion

This study describes the influence of LPC at different concentrations on the enzyme susceptibility of wheat starch in detail. The results demonstrate that the complexation of amylose with LPC decreases the susceptibility of wheat starch granules to α-amylase, compared to the pure wheat starch that is rapidly degradable. Depending on the LPC concentration, amylose molecules develop inclusion complexation and are rendered less degradable. The difference in digestion between the samples containing LPC and the reference, based on the amount of reducing sugars, describes the lower accessibility of inclusion complexes to the enzyme. The difference is more pronounced after 240 min digestion.

A conformational hindrance to enzymatic attack due to the new V-helix form, explains the decrease in α-amylolysis. Complex formation hinders the digestive enzyme to access the glycosidic bonds throughout the helices. Depending on the stability of the complexes, this even leads to a full resistance of the amylose-LPC complexes against amylolysis. The current study is in agreement with our previous observations gained on the alteration of physical properties of starch granules as the consequence of amylose complexation with LPC.

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Chapter 5

Assessment of the influence of amylose-LPC complexation on the extent of wheat starch digestibility by Size-Exclusion Chromatography

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Abstract

Amylose forms inclusion complexes with lysophosphatidylcholine (LPC), that decrease the susceptibility of amylose to amylase degradation. This study on the influence of complexation on starch susceptibility to amylase explains the nature of this protective effect. Wheat starch suspensions (9% w/w) containing 0.5-5% LPC were subjected to hydrolysis by porcine pancreatic α-amylase at 37°C for several digestion times. The digesta were analyzed by Size-Exclusion Chromatography (SEC). The molar mass distribution was closely dependent on the digestion time and the amount of LPC. This study precisely demonstrates the alteration of the digestion profile of starch on a molecular level, influenced by amylose-LPC complexation; however the effect depends on the digestion time. During 15 and 30 min digestion, inclusion complexes not only protect amylopectin in the initial hydrolysis stage, but also demonstrate lower susceptibility of the molecular amylose complexes to amylase hydrolysis. Digestion for 240 min resulted in a lower oligosaccharide peak concentration, in the presence of a high LPC concentration, which is related to less degradation of complexed amylose fraction.

1. Introduction

Starch is a widely used component to provide functional properties to food and is considered as the major source of energy in human nutrition, supplying more than 50% of the caloric energy. It is a major component of many food plants like wheat, potato, maize and rice[4]. Starch is composed of two polymers: amylose and amylopectin.

Amylose is linear and has a molecular weight of ca. 10^6 Da and amylopectin is branched with a molecular weight of about ca. 10^8 Da. Enzymes are able to hydrolyze the α-1→4 and the α-1→6 glycosidic bonds, resulting in maltodextrins, maltose and glucose. The source of starch and the amylose: amylopectin ratio determine not only the functional properties but also the digestibility of starch and subsequently the amount of glucose release into the blood stream, that is related to the concept of glycemic index (GI)[10].

Starch, based on its digestibility, can be classified into three categories: RDS (Rapidly Digestible Starch – starch that is digested within 20 min), SDS (Slowly Digestible Starch – starch that is digested between 20 and 120 min) and RS (Resistant Starch – starch that cannot be digested but is fermented in the large intestine) which are characterized by the rate and duration of the glycemic response[7, 8]. Starch digestibility has a big impact on human health. Rapid postprandial glucose increase in the blood stream, due to the rapid digestible starches, is considered as a risk factor which may cause obesity and type II diabetes; while slow and resistant starches are suggested to help in preventing metabolic disorders and colon cancer[11].

Starch digestion is a complex process that is highly dependent on several factors, such as the source of starch, enzyme activity and presence of other components like lipids and proteins. In our previous studies, we followed the formation of amylose inclusion complexes with lysophosphatidylcholine (LPC)[1, 3]. The formation of inclusion complexes is reported to decrease water ingression into the starch granules at the temperature of gelatinization[9, 14].

In a previous paper, we demonstrated the effect of inclusion complex formation on the physical properties of starch, such as viscosity and swelling power[1]. Also, we evaluated the influence of inclusion complexation on the

degradability of starch with an *in vitro* digestion method[2]. We were able to show that amylose-LPC complexation decreases the susceptibility of starch to α-amylase. The influence is stronger at higher LPC concentrations and best observed during shorter digestion times. At longer incubation times (>60 min), the effect is notably less.

Our previous study provided reproducible results showing the overall influence of the formation of amylose inclusion complexes on the degradability of starch; however more insight into the molar mass and size distribution of starch polymers after each digestion time (in relation to the influence of amylose inclusion complexation with LPC) was required. We therefore, in this study, used Size-Exclusion Chromatography (SEC) to determine the molar mass (MM) of the starch polymers after enzyme treatment during different incubation times. Hence, the combination of our *in vitro* method and SEC allowed us to study the influence of LPC on the whole samples after each digestion time. While standard methods only assess the amount of reducing sugars after digestion; we are able to gain more detailed information on the nature of the protective effect of inclusion complex formation, assessing both the amount of reducing sugars and the molar mass of amylopectin, amylose and low molar mass sugars after amylase hydrolysis.

2. Materials and methods

2.1. Materials

Native wheat starch with a purity of 99% and a total lipid content of 0.4% was obtained from Sigma Chemical

Company. 12.63% moisture content was measured by a moisture analyzer (Sartorius MA35M, Sartorius AG, Goettingen, Germany). 2.8% damaged granules and 23.5% amylose content (wheat starch not defatted) were reported by Eurofins Food B.V.
Egg yolk L-α-lysophosphatidylcholine (LPC), type XVI-E, lyophilized powder, purity >99% and fatty acid content of 16:0 69%, 18:0 27% and 18:1 3%, from Sigma Chemical Company (St Louis, Missouri, USA) was used.
Porcine pancreatic (150,000 U/g, free flowing powder, partially purified) α-amylase was employed from Megazyme International (Wicklow, Ireland).
LPC and α-amylase were kept at -20°C and wheat starch at room temperature under dark and dry conditions.
Maltose and maltotriose (DP2 and DP3 respectively), monosodium phosphate monohydrate, sodium phosphate dibasic, sodium chloride and dimethylsulfoxide (DMSO) (CHROMASOLV Plus, HPLC grade, ≥99.7%) purchased from Sigma Chemical Company (St Louis, Missouri, USA) were of analytical grade or better. Anhydrous lithium bromide (99%) and pullulan (P 0.3-800) molar mass standards were obtained from Fisher Scientific and PSS (Polymer Standard Service, Mainz, Germany) respectively.
Maltoheptaose (DP7) was previously synthesized as a standard[18].

2.2. Sample preparation

A RVA-4 Newport Scientific (NSW, Australia) Rapid Visco Analyzer was employed to prepare samples for the enzymatic hydrolysis. A series of 9% w/w wheat starch suspensions in deionized water was prepared by mixing starch with 0%, 0.5%, 1%, 2%, 3% and 5% LPC (based on starch dry matter content). The suspensions were kept 10 min at room temperature to equilibrate. The RVA

was programmed in three steps. The temperature of all samples was first equilibrated at 50°C for 60 s, increased to 95°C at a rate of 6°C/min and they were ultimately held at 95°C for 300 s. The samples were cooled down to 37°C in a water bath.

2.3. Amylase hydrolysis

5 g of each sample from the RVA were diluted with phosphate buffer (17 g, 0.025M, pH 6.9) to achieve a 2% (w/v) suspension. The phosphate buffer contained 6 mM sodium chloride to preserve the activity of the enzyme[15]. The suspensions were equilibrated at 37°C in a water bath to simulate body temperature. 0.5 mL of the enzyme solution (0.004% w/v), freshly prepared, was added to each suspension. Subsequently, the suspensions were incubated while rotating in a modified ventilation oven (Thermo Scientific Heraeus 6000, Langenselbold, Germany) at 37°C for 15, 30, 60 and 240 min. After each digestion time, the amylase hydrolyzed sample was heated immediately in a boiling water bath for 5 min, to inactivate the enzyme, and freeze dried in a laboratory freeze dryer (Zirbus technology, VaCo 2, Germany).

2.4. Size-Exclusion Chromatography

LiBr in DMSO (50 mM) was stirred at room temperature for 2 h. The solution was degassed for 5 min by an ultrasonic cleaner (Branson 2510, Branson, Danbury, CT, USA). The freeze dried samples were dissolved in DMSO-LiBr at a concentration of 2 g/L while overnight rotation at ambient temperature was followed by 2 h rotation in a modified ventilation oven (Thermo Scientific Heraeus 6000, Langenselbold, Germany) at 80°C, obtaining clear

sample solutions. The samples were allowed to cool down slowly to room temperature and filtered through 5 µm Millex PTFE membrane (Millipore Corporation, Billerica, MA, USA) and 1 µm PTFE membrane (Pall Corporation, Port Washington, NY, USA) for the short (15 and 30 min) and long (60 and 240 min) degradation times, respectively. Each sample was made in duplicate.

Size-Exclusion Chromatography equipment (SEC, often termed Gel-permeation Chromatography, GPC) from PSS (Polymer Standard Service, Mainz, Germany) was used to analyze the molecular size distribution of the starch molecules after digestion, according to *Ciric et al. 2012*[6]. The system was equipped with an isocratic pump and an online degasser. DMSO-LiBr was employed as the eluent. The samples were injected with a flow rate of 0.5 mL per min by an autosampler to a PFG guard column and the separation was carried out by three PFG-SEC columns with porosity 100, 300 and 4000 Å, purchased from PSS. The columns were held at 80°C. The SEC setup consisted of a refractive index detector (G1362A 1260 RID Agilent Technologies,Santa Clara, CA) and a MALLS detector (SLD 7000 PSS, Mainz). The refractive index detector was thermostatted at 45°C.

The MALLS signal was used to assess the molar masses up to the elution volume of 21 mL (a refractive index increment dn/dc of 0.072 was used). In the low molar mass region, in which light scattering becomes unreliable, pullulan calibration standards (PSS, Mainz, Germany; molecular weights of 342, 1320, 6000, 10000, 21700, 48800, 113000, 210000, 366000 and 805,000 Da for P0.3, P1, P5, P10, P20, P50, P100, P200, P400 and P800, respectively) were used. All samples were filtered through a 0.45 µm PTFE membrane from VWR (Radnor, PA, USA), before injection.

The resulting SEC chromatograms were analyzed using WinGPC Unity software (PSS, Mainz, Germany).

3. Results

3.1. Relationship between molecular size and elution volume

Fig. 1 shows the SEC chromatograms of maltose, maltotriose, maltoheptaose and their mixture. In SEC, the elution volume is directly related to the hydrodynamic volume of the linear and branched molecules which leads to the size distribution of a sample. The hydrodynamic volume is the volume occupied by an equivalent sphere in the eluent. It is important to recall that SEC separates by hydrodynamic volume[5].

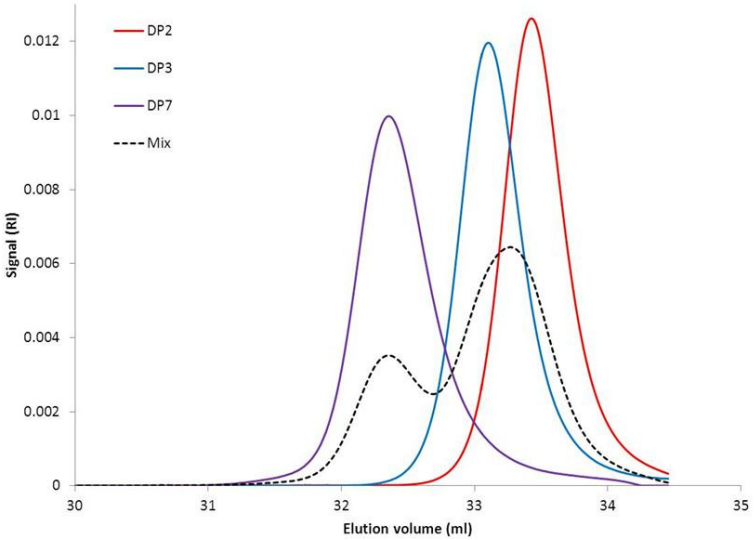

Fig. 1. SEC chromatograms of maltose (DP2), maltotriose (DP3), maltoheptaose (DP7) and a mixture of DP2, DP3 and DP7 thereof (1:1:1). All samples were used in a 2 g/L concentration.

Fig. 1 demonstrates that the used low molar mass maltodextrins can be separated and analyzed at the employed conditions. Since the amount of low molar mass sugars represents the extent of starch digestion, DP2, DP3 and Dp7 maltodextrins were employed as references to demonstrate the size distribution of the oligomers and soluble sugars after starch degradation of starch by α-amylase.

3.2. Molar mass distribution of starch molecules after enzyme hydrolysis

Figs 2, 3 and 4 present the distribution of starch molecules in the digesta, influenced by LPC, after amylase hydrolysis at different digestion periods.
We defined 15 and 30 min as short and also 60 and 240 min as moderate and long digestion times, respectively.

The observed difference between the LS signal and the pullulan standards (see Fig. 2) at an elution volume of ca. 19 mL is due to the branches of amylopectin and also to some small branches on the amylose molecules, that is 2-8 branches of DP 4-100[13]. This results in an upward shift of the molar mass *versus* elution volume curve. This is in agreement with *Roger et al. 1993*[16] and *Hizukuri 1984*[12].

After 15 min digestion, a considerable amount of high molar mass carbohydrates eluted at lower elution volumes. This was observed for the pure starch and the samples containing LPC; however the area under each curve is different (see Fig. 2).

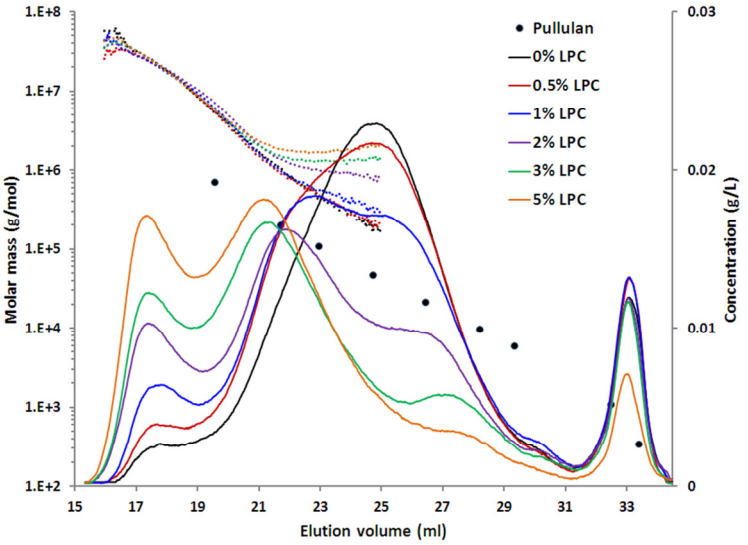

Fig. 2. Molar mass distribution and concentration of wheat starch suspensions containing different amounts of LPC after 15 min amylase hydrolysis.
The solid lines represent the concentration distribution of the digesta.
The dotted lines represent the molar mass of the digesta from LS signals.
● Pullulan standards

The total amount of high molar mass starch polymers in the digesta increases when increasing the amount of LPC. At added LPC levels of 3% and 5%, a bimodal RI-trace was observed with peaks at elution volumes around 17 mL and 21 mL which corresponds to relatively intact amylopectin and amylose. However, at lower LPC levels the latter peak gradually shifts to 25 mL. This led to a molar mass above 10^6 g/mol for the pure starch; while it increases to 10^7 g/mol in the presence of 5% LPC, representing the presence of amylopectin, due to the strong protecting action by LPC.

The low molar mass sugars, mainly the oligosaccharides, elute at higher elution volumes (peak at 33 mL); however the concentration was strongly reduced when more LPC was present at the concentration of 5%, as shown by refractive index (see Fig. 2). This shows the profound effect of the formation of amylose inclusion complexes on diminishing the enzyme hydrolysis.

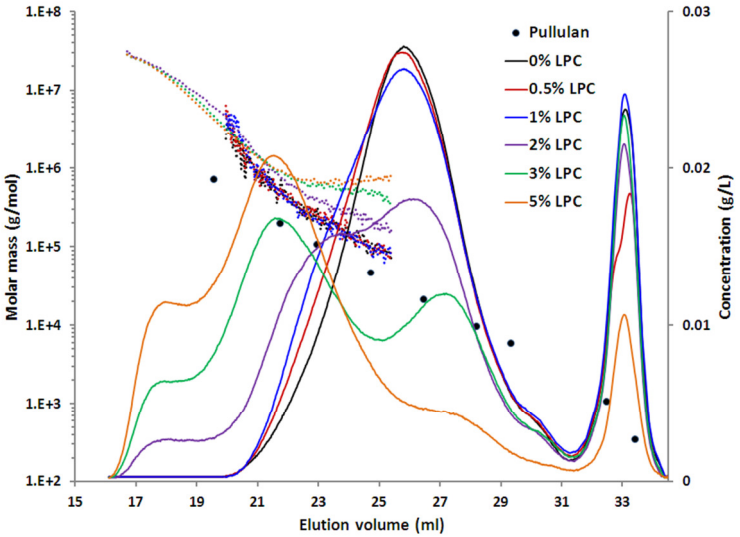

Fig. 3. Molar mass distribution and concentration of wheat starch suspensions containing different amounts of LPC after 30 min amylase hydrolysis.
The solid lines represent the concentration distribution of the digesta.
The dotted lines represent the molar mass of the digesta from LS signals.
● Pullulan standards

30 min digestion also resulted in a significant amount of high molar mass carbohydrates (above 10^7 g/mol) eluted

at lower elution volumes (see Fig. 3). A bimodal peak eluted at 18 mL and around 22 mL when the LPC concentration increased to 3% and 5%; while a broad single peak, eluting at 25 mL, was observed in the presence of 2% LPC. In the absence of LPC, the peak shifts slightly to higher elution volume, compared with the shorter digestion time, and appears at 26 mL. The low molar mass sugars elute at the same elution volume of 33 mL; however in distinctly higher concentrations. A significant decrease was observed in the presence of 5% LPC at the peak eluting at 33 mL.

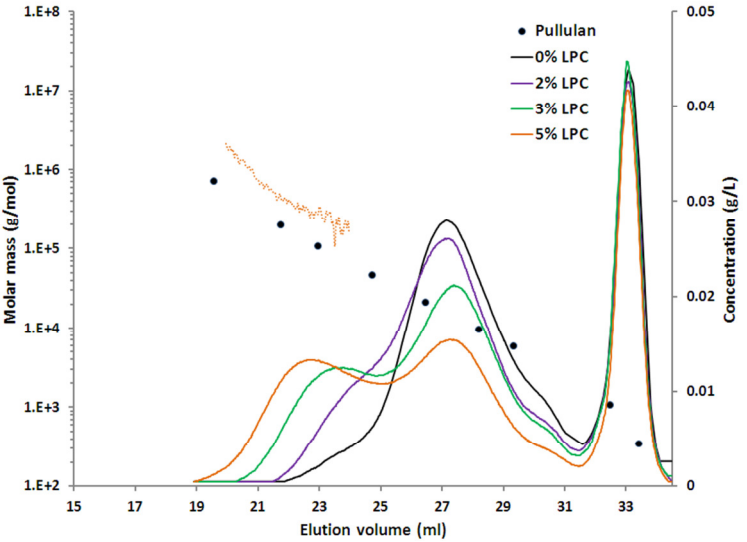

Fig. 4. Molar mass distribution and concentration of wheat starch suspensions containing different amounts of LPC after 60 min amylase hydrolysis.
The solid lines represent the concentration distribution of the digesta.
The dotted lines represent the molar mass of the digesta from LS signals.
● Pullulan standards

Increasing the digestion time to 60 min resulted in a pronounced difference in size distribution (see Fig. 4). After 60 min degradation, no peak appeared before 19 mL. Moderate digestion time resulted in a lower amount of high molar mass starch molecules indicated by the shift to higher elution volumes. A bimodal peak is observable for the samples containing 3% and 5% LPC at elution volumes of around 23 mL and 27 mL which still shows the strong protective action of LPC; however pure starch eluted at 27 mL as a single peak. Further to the higher elution volumes, the peak of the low molar mass sugars (elution volume at around 33 mL) evenly eluted for all the samples. Surprisingly, no obvious difference was observed in the amount of oligosaccharides (see Fig. 4).

The results after long time digestion, 240 min, were highly different compared to the 15, 30 and 60 min digestion (see Fig. 5). It can be clearly seen that no peaks appear before 23 mL which implies loss of amylose and amylopectin molecules having a molar mass higher than $3x10^5$ g/mol.
The first peak elutes at around 25 mL (molar mass of higher than 10^4 g/mol) and belongs to undegraded amylose due to the formation of amylose inclusion complexes with LPC, depending on the concentration of LPC. The difference in peak heights of the samples containing LPC and pure starch is a clear proof of this. A peak elutes at 29 mL which is LPC-independent, suggesting that it originates from amylase resistant branching zones in amylopectin.

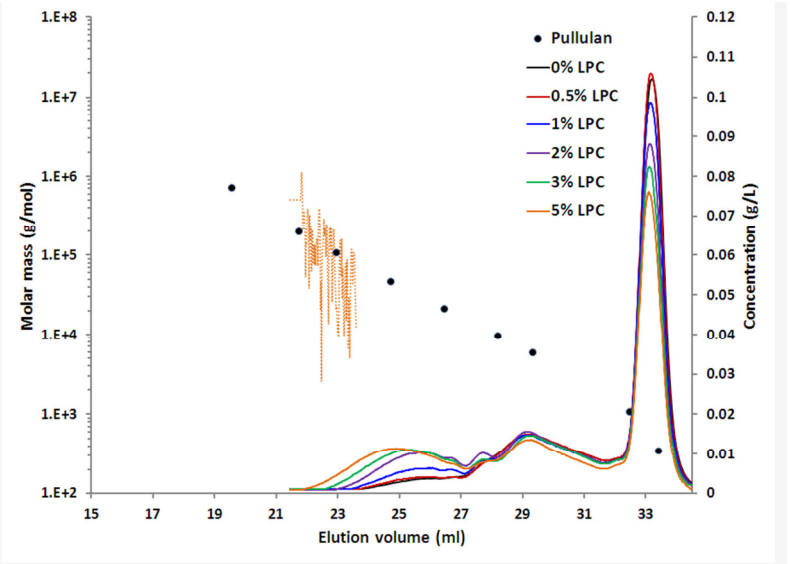

Fig. 5. Molar mass distribution and concentration of wheat starch suspensions containing different amounts of LPC after 240 min amylase hydrolysis.
The solid lines represent the concentration distribution of the digesta.
The dotted lines represent the molar mass of the digesta from LS signals.
● Pullulan standards

A closer look at the lower mass region again showed an effect of inclusion complexes: the higher the LPC level, the lower the concentration of maltodextrins compared to the non-complexed starch (at the elution volume of around 33 mL). This peak is a factor twice more abundant than the similar peak after 60 min digestion, due to the long degradation time.
Fig. 6 provides more detail. The peaks belonging to the pure starch and the sample containing 5% LPC (elution volumes at 33.2 mL and 33.1 mL) correspond to the peaks of DP2 and DP3, respectively (see Fig. 1).

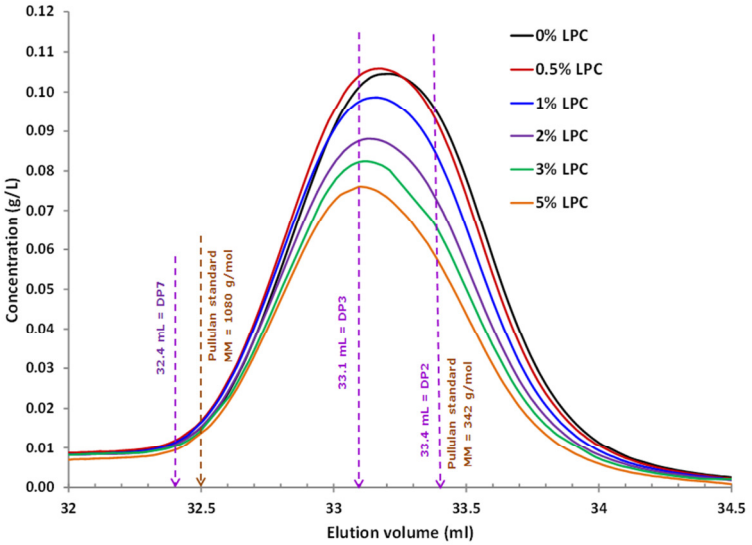

Fig. 6. Molar mass distribution and concentration of low molar mass sugars after 240 min amylase hydrolysis. The positions of pullulan standards and DP2, DP3 and DP7 represent the molar mass of the oligosaccharides in the digesta after 240 min digestion.

Fig. 7 presents the cumulative concentration in the digesta of the sample containing 5% LPC, at each possible point of the elution volume. It helps to understand the protective effect of LPC at 5% concentration on hindering the degradation of starch to low molar mass sugars, during several digestion times, which elute at 32.5-34.5 mL (see Figs 2, 3, 4, 5 and 6). Depending on the digestion time, the concentration of oligosaccharides is demonstrated in the cumulative plots (see Fig. 7). 15 min digestion of the native wheat starch resulted in 8% low molar mass sugars; while the amount increased to 15%, 27% and 58% after 30, 60 and 240 min digestion, respectively. Based on this comparison,

5% low molar mass sugars was observed during 15 min digestion with the addition of 5% LPC; however this amount decreases to 8%, 26% and 42% after 30, 60 and 240 min digestion times, respectively (see Fig. 7).

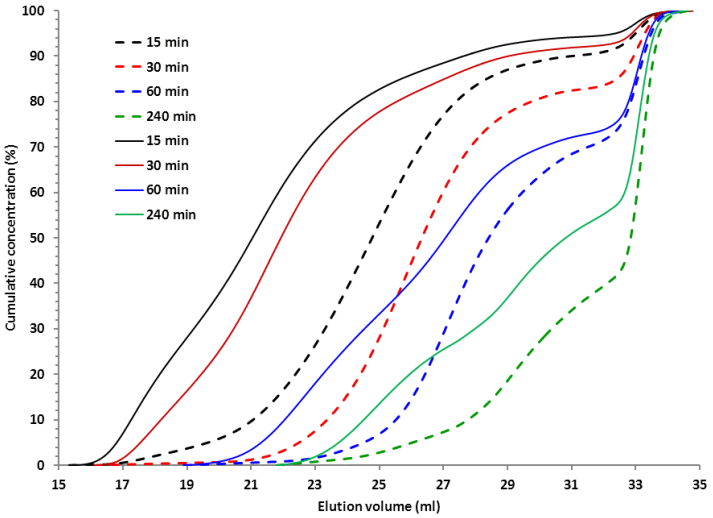

Fig. 7. Cumulative plots of wheat starch digesta as affected by precomplexation with 0% (dotted lines) and 5% (solid lines) LPC.

4. Discussion

Starch granules are composed of amylose and amylopectin in a layered fashion[4]. Amylopectin is a branched molecule with higher molecular weight (ca. 10^8 Da) than amylose (ca. 10^6 Da). The rate of their digestion depends on several factors including the type of digestion enzyme, the degradation time and presence of a ligand able to develop inclusion complexes with

amylose[19]. Information regarding starch digestibility is of the utmost importance for human diet disorders. *In vitro* digestion methods are very common to collect this information; since they are reproducible and practically applicable. The standard colorimetric *in vitro* methods, like the Englyst method, specifically determine the amount of sugars soluble in ethanol as the total amount of glucose but fall short assessing the exact molecular composition of digesta. In this study, we demonstrate a new *in vitro* method that provides information about not only the amount of soluble sugars, but also the molecular composition of the soluble and insoluble sugars as well.

This method is developed for gelatinized starch suspensions to evaluate the influence of amylose-LPC inclusion complexation on the enzyme susceptibility of wheat starch and the effect on the starch molecular size distribution as a function of digestion time (15, 30, 60 and 240 min). Temperature is the most important factor that not only contributes to the formation of amylose inclusion complexes but also determines the extent of starch susceptibility to the hydrolytic enzymes.
In our earlier study, we demonstrated that LPC at high concentrations results in a lower amount of reducing sugars[2]. The SEC results reported here (see Figs 2, 3, 4 and 5) clearly show that increasing the amount of LPC results in less molecular degradation after enzyme hydrolysis during the respective digestion time. With this, we have shown that the formation of inclusion complexes not only clearly decreases the enzyme susceptibility of amylose and amylopectin molecules, but also the digestion of the inclusion complexes itself is extensively inhibited.

The explicit bimodal peaks of the samples containing 3% and 5% LPC after short digestion times (15 and 30 min) showed the presence of mainly larger starch polymers

(molar mass higher than ca. 10^6 g/mol) indicating the preservation of amylopectin against degradation as a consequence of amylose-LPC inclusion complexation (see Figs 2 and 3). As we reported in our earlier study[1], the starch granules remain intact in the presence of 5% LPC. Enzyme penetration is hampered in the presence of LPC and results in less hydrolysis and slower release of reducing sugars accordingly. This is in a good agreement with our previous findings[2]. *Tester et al. 1990*[17] also reported less surface corrosion due to the formation of amylose inclusion complexes.

The molecular size distributions of the digesta after 60 min presented a shift to the region of carbohydrates with lower molar mass. The pure starch presented one peak while the samples containing 3% and 5% LPC appeared as bimodal peaks (see Fig. 4). This implies that hydrolysis of amylopectin is almost complete after 60 min digestion; however a large population of amylose molecules remains in the digesta in presence of LPC. The peak of the low molar mass sugars (elution volume of 33 mL), after 60 min digestion, appeared ca. three times higher compared to the similar peak after 15 min digestion (see Figs 2 and 4).
It is significant that the samples containing high amounts of LPC presented almost the same amount of low molar mass sugars as was observed for the reference, after 60 min digestion (see Fig. 4). This is not entirely in agreement with our previous study[2]; however it should be considered that the DNSA (3,5-dinitrosalicylic acid) assay demonstrates the amount of reducing sugars, including maltose, maltotriose, etc. This shows that the amount of oligosaccharides in the digesta after 60 min degradation is almost independent of the amount of LPC added, which suggests that the contribution of hydrolyzed amylose is not yet very significant at this stage. This would mean that the constraint on swelling

caused by addition of LPC decreases after 60 min digestion. However, this is not to say that all amylopectin has been fully hydrolyzed as the oligosaccharide peak concentration approximately doubles between 60 and 240 min digestion (see Figs 4 and 5). This doubling cannot be caused by amylose alone. In addition, the effect of LPC again becomes obvious at the elution volume of 29-31 mL which also belongs to the region of oligosaccharides (see Fig. 4).

Disappearance of high molar mass molecules, with the molar mass of above 10^6 g/mol, was observed after 240 min degradation time in the absence of LPC; however the samples containing LPC presented a larger area of high molar mass polysaccharides (see Fig. 5). This clearly indicates the significant effect of LPC even after 240 min degradation, as compared with pure starch.

240 min digestion clearly resulted in three residual population: (i) a LPC-dependent population, eluting at 25 mL, that represents complexed inaccessible amylose; (ii) a LPC-dependent population, eluting at 29 mL, that probably originates from residual branching zones which are susceptible to α-amylase; and (iii) a LPC-dependent oligosaccharide population, eluting at 33 mL, whose abundance is very probably related to the complexed amylose fractions.

In Fig. 5, a lower concentration of low molar mass sugars in the presence of 1%, 2%, 3% and 5% LPC, is clearly in agreement with our earlier study that the amount of reducing sugars showed a distinct decrease after 240 min degradation due to the addition of LPC, shown by the DNSA reagent. This is considered as the resistant part of starch as the consequence of amylose-LPC inclusion complexation. This peak of low molar mass sugars not only indicated a lower concentration in the presence of LPC (see Fig. 5) but also a slight peak deviation was observed (see Fig. 6). The significant shift observed for the sample containing 5% LPC suggests the absence of

maltose as a result of 5% LPC addition (see Figs 1 and 6).
The lower concentration of low molar mass sugars in the presence of 5% LPC, compared with the native starch, revealed the fact that high amount of LPC leads to the formation of more inclusion complexes (see Fig. 7). As the molar mass of amylopectin is ca. 100 times larger than that of amylose, a single intact amylopectin molecule has a far larger chance of undergoing a single scission than an amylose molecule, at least in the initial stage of the hydrolysis. Because of the predominance of amylopectin in wheat starch (ca. 75%) and the protection of the amylose fraction by complexation, it is therefore highly probable that the changes in the initial stages of hydrolysis are mainly due to amylopectin. This is clearly suggested by Fig. 7 which shows the largest relative reduction in the range of highest molar mass in the early stages of the hydrolysis.

This study shows the particular feature of our method compared with the standard calorimetric *in vitro* methods. This method provides information on the soluble and insoluble sugars of the whole sample; while the standard methods present specifically the soluble and reducing sugars. This study presents the LPC efficiency in restricting the starch swelling and digestibility, demonstrating different starch molecules separately in the digesta according to their molar masses, from high to low. Amylose-LPC complexation primarily affects the hydrolysis of amylopectin during the short digestion times (15 and 30 min). During the longer digestion times, the molecular amylose complexes demonstrate their lower digestibility.

5. Conclusion

A method to evaluate the influence of amylose-LPC inclusion complexation on the enzyme susceptibility of gelatinized wheat starch and the effect on the starch molecular size distribution as a function of digestion time was developed. In contrast to established methods, such as the Englyst assay, the method used here assesses both the amount of reducing sugars as well as the molar mass distribution of amylopectin, amylose and low molar mass sugars, simultaneously.

The developed method monitors the digestion profile of starch on a molecular level in detail and was successfully applied to study the effect of LPC on amylase digestion of starch. This method provides information on the molecular size distribution of the digesta after several degradation times. It shows that amylose-LPC inclusion complexation primarily protects amylopectin in the initial hydrolysis stage and in the final stage, the molecular amylose complexes are less susceptible to amylolytic enzymes. Digestion for 15 and 30 min resulted in a low amount of oligomers and soluble sugars in the presence of LPC; which are due to the reduced degradation of amylose and amylopectin. However, the resistant part of starch largely remained after 240 min degradation which results in significantly low amount of low molar mass sugars in the presence of LPC.

It can therefore be concluded that LPC delays starch digestion due to the formation of inclusion complexes with amylose. Our observation proved the significant effect of LPC concentration; suggesting that LPC at high concentrations results in not only more inclusion complexes but also more stable complexes.

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Chapter 6

Assessing the susceptibility of amylose-lysophosphatidylcholine complexes to amylase by the use of iodine

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Abstract

The formation of amylose-lysophosphatidylcholine (LPC) inclusion complexes renders amylose less susceptible to amylase digestion. In order to better understand this phenomenon on a structural level, the complexation of 9% wheat starch suspensions with 0%, 2%, 3% and 5% exogenous LPC was developed in RVA. Amylose-LPC inclusion complexes were isolated after 15, 30, 60, 120 and 240 minutes in vitro digestion of wheat starch suspensions containing 2-5% LPC. Remaining inclusion complexes were dissolved in Dimethylsulfoxide (DMSO) containing 0.5% LiBr. The resulting solutions were exposed to iodine to quantify the amount of non-complexed amylose by spectrophotometry. In addition, parts of the digesta were defatted and subjected to the same procedure to expose the total amount of amylose that remained undigested. In this way, more insight was obtained into the protective effect of amylose-LPC complex formation. This study confirms that the amylose susceptibility to amylolysis decreases in the presence of LPC. Higher LPC concentrations not only induced the formation of more amylose inclusion complexes but also resulted more stable complexes remained undigested as well as longer amylose chains after enzyme hydrolysis, due to the presence of LPC inside the amylose helix. In addition, a higher melting enthalpy of the amylose-LPC complexes in the digesta demonstrates the protective effect of LPC during enzyme hydrolysis.

1. Introduction

Amylose is a predominantly linear polysaccharide, consisting of α-1→4 D-glycosidic bonds, with a molecular

weight of ca. 10^6 Da[7]. There are two main conformations of amylose: random coil with an amorphous conformation, and a six-fold left-handed single helical conformation in aqueous solutions with the possibility to include hydrophobic guest molecules; such as iodine, fatty acids and aromatic compounds[25].
The ability of iodine to complex with amylose has been widely used to explain the structure of amylose. This complexation is demonstrated by several means:

(i) Iodine complexation with amylose leads to a blue color. Spectrophotometric analysis has shown that the observed maximum wavelength varies with the degree of polymerization (DP) of amylose[6, 10, 15, 18]. As the length of the chains increases, the number of iodine molecules, ready to form a complex, also increases. Therefore, the color changes from brown (DP=21-24) to red (DP=25-29), red-violet (DP=30-38), blue-violet (DP=39-46) and blue (DP>47)[23]. This relatively rapid technique is able to measures the content and DP of amylose[2].

(ii) X-ray diffraction spectra (XRD)[19] and Differential Scanning Calorimetery (DSC)[8, 16] assess the development of amylose-iodine complexes.

(iii) Potentiometric titration of iodine is a method that directly measures the amount of bound iodine independent of the DP of amylose.
A combination of spectrophotometric measurement and potentiometric titration can help to understand the molecular structure and helix capacity of amylose molecules[18].

In principle, six or eight glucose residue per turn (three turns) are required to support complex formation with a suitable ligand[12, 20]. *Banks et al 1971*[6] reported 19-22% iodine binding capacity for pure amylose; however *Knutson et al. 1982*[17] found it to be about 30% of the weight of amylose. Characteristic peak wavelengths (λ_{max}) have been reported to range from 642 nm[6] to 650 nm[15].

We previously reported the influence of amylose inclusion complexation with LPC on the functional properties[1] and thermal transitions[2] of wheat starch. In addition, we were also able to prove a lower susceptibility to amylase digestion due to amylose-LPC complexation[3, 4]. Enzyme hydrolysis of starch has been discussed by several studies, employing mostly α-amylase[11, 13, 26]. We have also used α-amylase in our previous work to study the efficiency of the enzyme in presence of LPC; however it is still unknown if the ligand is able to remain inside the helix during enzyme hydrolysis.
It is necessary to assess to what extent amylose molecules are preserved after enzymatic degradation to obtain a more complete picture. Accordingly, in this paper we quantify the amount of LPC inclusion complexes formed with amylose before and after digestion using spectrophotometric assessment of the amylose-iodine complexes.

2. Materials and methods

2.1. Materials

Native wheat starch with a purity of 99% and a total lipid content of 0.4% was obtained from Sigma Chemical

Company. 12.63% moisture content was measured by a moisture analyzer (Sartorius MA35M, Sartorius AG, Germany). 2.8% damaged granules and 23.5% amylose content (wheat starch not defatted) were reported by Eurofins Food B.V.
Egg yolk L-α-Lysophosphatidylcholine (LPC), type XVI-E, lyophilized powder, purity >99% and fatty acid content of 16:0 69%, 18:0 27% and 18:1 3%, from Sigma Chemical Company (St Louis, Missouri, USA) was used.
α-amylase from Porcine Pancreatic (150,000 Ceralpha U/g), free flowing powder, partially purified, from Megazyme International Ireland (Wicklow, Ireland) was employed.
LPC and α-amylase were kept at -20°C and wheat starch at room temperature under dark and dry conditions.
Monosodium phosphate monohydrate, sodium phosphate dibasic, sodium chloride, purchased from Sigma were of analytical grade or better.
Lugol as a iodine/Potassium iodide solution (containing 5%, 10% and 85% iodine, Potassium iodide and water, respectively) and Dimethylsulfoxide (CHROMASOLV Plus, HPLC grade, ≥99.7%) were purchased from Sigma Chemical Company (St Louis, Missouri, USA). Anhydrous Lithium Bromide (99%) was obtained from Fisher Scientific and ethanol 96% was purchased from Interchema Gmbh (Munich, Germany).
Potato amylose M1551, with a molar mass of 180,000 g/mol, was obtained from AVEBE. 12.10% moisture content was measured by a moisture analyzer (Sartorius MA35M, Sartorius AG, Germany).

2.2. Starch gelatinization / complexation

A Rapid Visco Analyzer (RVA-4 Newport Scientific; NSW, Australia) was employed to prepare samples for the enzymatic hydrolysis. A series of 9% w/w wheat starch

suspensions in deionized water was prepared by mixing starch with 0%, 2%, 3% and 5% LPC (based on starch dry matter content). The suspensions were kept 10 min at room temperature to equilibrate. The RVA was programmed in three steps. The temperature of the suspensions was first equilibrated at 50°C for 60 s, increased to 95°C at a rate of 6°C/min and held at 95°C for 300 s.

2.3. Amylase hydrolysis

5 g of each sample from the RVA was diluted with phosphate buffer (17 g, 0.025*M*, pH 6.9) to achieve a 2% (w/v) suspension. The phosphate buffer contained 6mM sodium chloride to preserve the activity of the enzyme[21]. The suspensions were equilibrated at 37°C in a water bath.
40 mg of the Pancreatic α-amylase was dissolved in 10 g of the phosphate buffer to prepare a transparent solution. The enzyme solution was subsequently centrifuged for 10 min at 2000 rpm. 100 µL of the supernatant was diluted in 9.9 g of the phosphate buffer, resulting in 0.004% w/v enzyme solution. 0.5 mL of this enzyme solution (freshly prepared each day), was added to each suspension. Subsequently, the suspensions were incubated while rotating in a modified ventilation oven (Thermo Scientific Heraeus 6000, Langenselbold, Germany) at 37°C for 15, 30, 60, 120 and 240 min. At each digestion time, 5 mL of the incubation solution was pipetted into another test tube and heated immediately in a boiling water bath for 5 min to inactivate the enzyme. After cooling, the samples were freeze dried in a laboratory freeze dryer (Zirbus technology, VaCo 2, Germany). The samplings were done in duplicate.

2.4. Sample preparation for iodine complexation with amylose

Since the freeze dried digesta are not readily water soluble, they were dissolved in DMSO containing 50 mM LiBr. Even though this has the advantage of completely dissolving the starch digesta, iodine complex formation is hampered at these concentrations of DMSO. In order to remove this obstacle, we subsequently added water to the samples to reach a final concentration of 60% (v/v). At this concentration, iodine complexation is complete[9].

2.4.1. Preparation of freeze dried solution

LiBr increases the ability of DMSO to dissolve starch by suppressing interactions of the carboxyl groups in starch that lead to aggregation[27]. We therefore included 50 mM LiBr in the DMSO solvent. The solution was stirred at room temperature for 2 h. The solution was degassed for 5 min in an ultrasonic bath (Branson 2510. CT, USA). Freeze dried samples were mixed into the DMSO-LiBr solution at a concentration of 2 g/L, followed overnight rotation at ambient temperature and subsequently 2 h rotation in a modified ventilation oven (Thermo Scientific Heraeus 6000, Langenselbold, Germany) at 80°C to obtain clear sample solutions. The samples were allowed to cool down slowly to room temperature. 0.5 mL of the solutions were separately mixed with 4.45 mL distilled water. 50 µL Lugol solution was added to all samples and they were promptly scanned from 250 to 800 nm by using a Spectramax spectrophotometer (Spectramax M2 Dual Mode C, Molecular Devices, Virginia, USA), since iodine-starch complexes are relatively light sensitive.

2.4.2. Defatting process

Freeze dried samples were mixed with ethanol (96%) at a concentration of 2 g/L in Pyrex screw-cap tubes and rotated for 60 min in a modified ventilation oven (Thermo Scientific Heraeus 6000, Langenselbold, Germany) at 100°C. The suspensions were centrifuged for 5 min at 2000 rpm. The supernatant was decanted and the residue was washed with ethanol one additional time under similar conditions. The residue was dried in an oven at 100ºC and subsequently dissolved in the DMSO-LiBr solution to prepare a concentration of 2 g/L. The solutions were rotated overnight at ambient temperature and 2 h in a modified ventilation oven (Thermo Scientific Heraeus 6000, Langenselbold, Germany) at 80°C to obtain clear sample solutions. After cooling down to room temperature, 0.5 mL of the solutions were mixed with 4.45 mL distilled water and 50 µL Lugol solution was added. The samples were directly scanned from 250 to 800 nm by using a Spectramax spectrophotometer (Spectramax M2 Dual Mode C, Molecular Devices, Virginia, USA).

2.5. Thermal analysis

A part of the freeze dried samples was used to prepare a series of 20% w/w suspensions in deionized water. The suspensions were directly prepared in DSC stainless steel pans (Perkin Elmer, Norwalk, CT, USA) which were sealed afterwards. The suspensions were kept one hour at room temperature to equilibrate. Samples were analyzed by a Perkin Elmer Pyris 1 DSC (Norwalk, CT, USA) previously calibrated with Indium (melting temperature= 156.6°C,

melting heat= 28.45J/g). The heating rate was 10°C/min and the samples were heated from 20°C to 120°C. The onset (T_o), peak (T_p) and ending (T_c) temperatures were calculated by DSC software. Enthalpy (ΔH, J/g of sample) was calculated based on the endothermic peak.
All samples were measured in duplicate.

3. Results and discussion

3.1. Iodine complexation with amylose-LPC inclusion complexes

A set of iodine-amylose complexations experiments was performed on native wheat starch-LPC digesta. Initial starch-LPC mixtures were subjected to α-amylase using different digestion times, to achieve a better insight into the protective effect of LPC on the amylose molecules. The key question studied was if LPC stays inside the amylose-V complexes during the various digestion times. This study also helps us to understand the protective effect of LPC on a molecular level, following our previous studies that discussed this effect at the level of the starch granule and molecular composition. We used UV-VIS spectra to observe the change in maximum wavelength, which depends on the level of helix occupation by LPC. This provides information on the susceptibility of amylose to amylase in presence of LPC. We, therefore, employed spectrophotometry since the UV-VIS spectra of amylose-iodine complexes can provide rapid information on free space of amylose helices and therefore on the quality of amylose and its degree of polymerization (see below).
The UV spectra of amylose-iodine complexes in the digesta of native wheat starch clearly showed the effect of digestion time on the amylose chain length. Fig. 1

presents the UV absorbance spectra of the iodine-amylose complexes present in the respective digesta of native wheat starch (exposed 15, 30, 60, 120 and 240 min to amylase hydrolysis and compared with the undigested sample). Although starch occurs naturally as water-insoluble granules, amylose can be molecularly dispersed in water once the granular structure has been destroyed (thermally, enzymatically or chemically), opening up the possibility to form complexes with iodine[22].

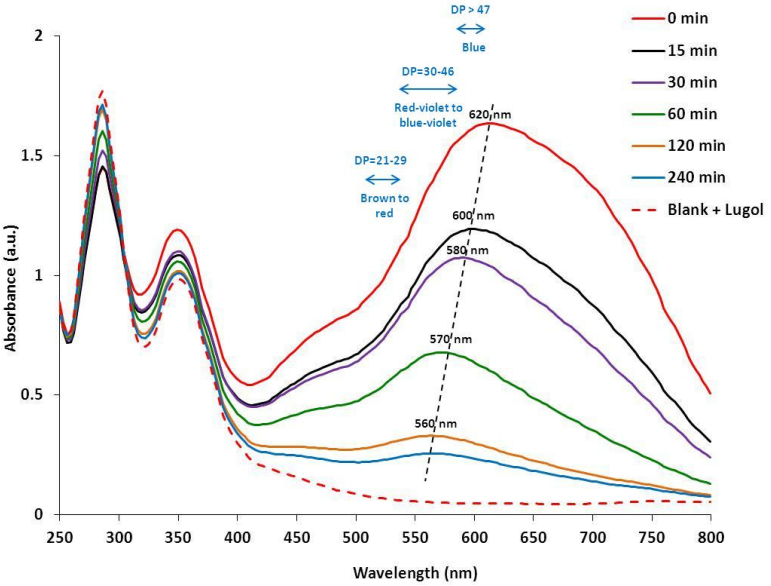

Fig. 1. UV spectra of amylose-iodine complexes in the digesta of native wheat starch after 0, 15, 30, 60, 120 and 240 min digestion.

As Fig. 1 shows, a wide iodine complexation peak was observed with the undigested wheat starch for which the peak was observed at λ_{max} of 620 nm. Digestion for 15 min led to significantly less absorption and a lower λ_{max} of 600 nm. Intermediate digestion times (30 and 60 min) caused even lesser absorption and a further decrease in λ_{max}. Almost no peak were observed after long digestion times of 120 and 240 min (see Fig. 1). Apparently, at these digestion times, the DP of the remaining amylose is too low. Furthermore, a sharp peak was observed at ca. 283 nm, for all samples, representing free iodine ions (see Fig. 1). That means amylose with higher DP results in more complexation with iodine which leads to less free iodine observable at the wavelength of 283 nm.
The results clearly show that during the course of the enzymatic degradation of native wheat starch, less amylose becomes available for complexation with iodine over time as indicated by the lower absorbance and also the explicit shift of the maximum wavelength of the UV spectra.

The presence of LPC resulted in a pronounced effect on the UV spectra, in comparison to native wheat starch, leading to a lower absorbance and a maximum wavelength of 550 nm (see Fig. 2); which suggests a lack of free space in the amylose helices with high molecular weight. This reveals the protective effect of LPC on decreasing the degradability of amylose. Our results are in a good agreement with *Liu et al. 2009*[18].

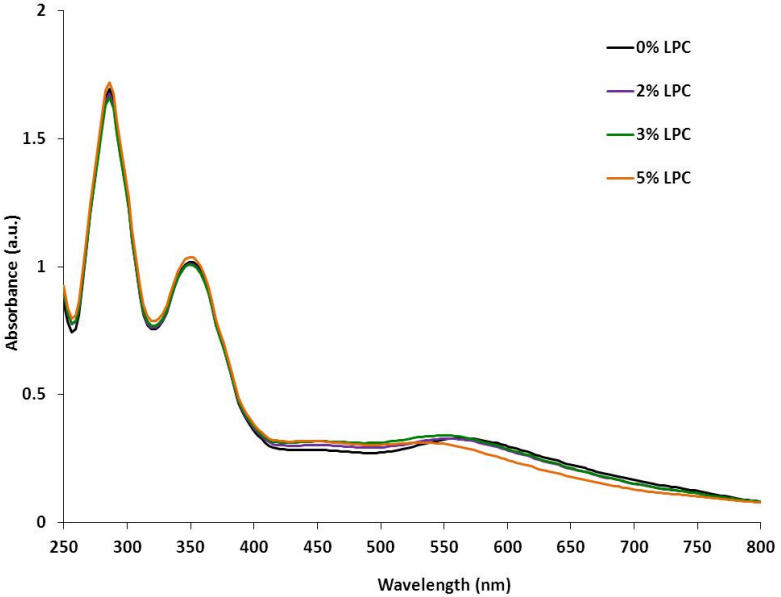

Fig. 2. UV spectra of amylose-iodine complexes after 120 min digestion in the presence of 0, 2, 3 and 5% LPC.

3.2. The effect of defatting on the amylose-iodine complexation

To prove that the results above are not caused by a complete lack of amylose, the digesta were defatted with ethanol to remove LPC from the amylose helices. Fig. 3 presents the UV spectra of amylose-iodine complexes in the digesta, in presence of 0%, 2%, 3% and 5% LPC, after 120 min enzyme hydrolysis and defatting accordingly.

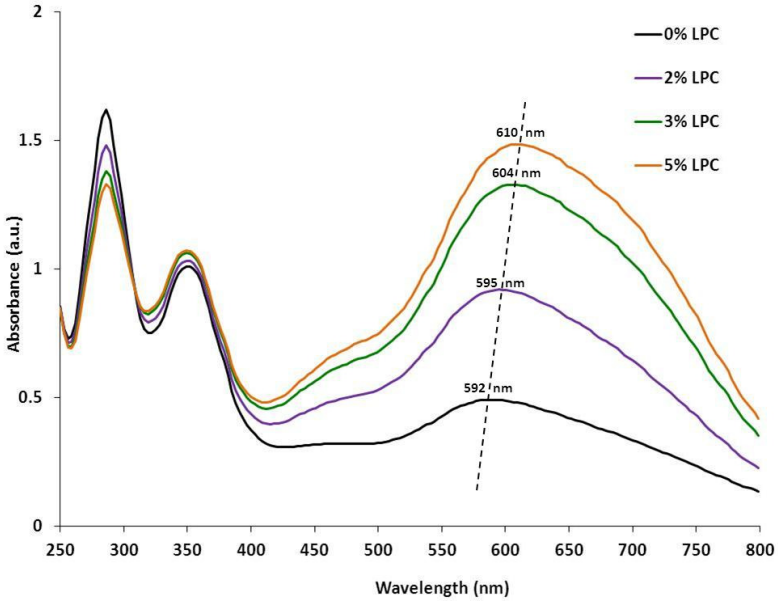

Fig. 3. UV spectra of amylose-iodine complexes after 120 min digestion in the presence of 0%, 2%, 3% and 5% LPC, after defatting.

It becomes obvious that the sample containing 2% LPC demonstrated higher absorbance and a slight shift of maximum wavelength compared to the native starch which shows more amylose-iodine complexation after defatting, representing a longer amylose chain after 120 min digestion. λ_{max} increased to 600 nm and 610 nm in presence of 3% and 5% LPC, respectively (see Fig. 3). It is noted that the maximum absorbance of the samples with 3% and 5% LPC after 120 min digestion is even higher than the native starch after 15 min degradation and comparable with undegraded wheat starch (see Figs 1 and 3). Also, the peaks at the wavelength of 283 nm,

representing free iodine, appeared at the lower amounts in presence of 3% and 5% LPC.
The obtained results clearly prove a significant protective effect of LPC during 120 min digestion, that clearly increases with increasing LPC concentrations.

Assuming that we have achieved 100% defatting, we can now assess the amount of undigested amylose. A series of potato amylose concentrations (g/L) were incubated with a fixed amount of iodine. The UV spectra of the complexes demonstrated the maximum wavelength at 619 nm. Based on this, a calibration line was prepared at 619 nm with an R^2 of 0.99. This allows us to measure the amount of amylose protected by LPC during 120 min digestion (see Table 1):

$$Amylose\ (\%) = \frac{\left(\text{Amylose concentration}[\frac{g}{L}]\text{\_defatted}\right) - \left(\text{Amylose concentration}[\frac{g}{L}]\text{\_non-defatted}\right)}{2\ (Concentration[\frac{g}{L}])}\ X\ 100$$

Table 1. Protected amylose helices (%) by 0%, 2%, 3% and 5% LPC and their approximate DP (according to *Bailey et al. 1960*[2]) after 120 min amylase hydrolysis.

LPC (%)	Abs. at 619 nm (Defatted)	Abs. at 619 nm (Non-defatted)	λ_{max} (nm) (Defatted)	Amylose (%)	DP
0	0.468	0.272	592	3.5	60
2	0.893	0.256	595	11.4	65
3	1.317	0.260	604	18.9	75
5	1.478	0.220	610	22.5	81

Based on this, we can calculate the amount of amylose remaining in the digesta after 120 min digestion. Comparing the absorbance before and after the defatting reveals the amount of intact amylose during the digestion

due to the complexation with LPC. As Table 1 shows, 22.5% of the amylose molecules are protected by 5% (w/w) LPC during 120 min amylase hydrolysis which clearly proves an efficient resistance to amylase hydrolysis.
The spectra were also analyzed to assess the DP of amylose helices after 120 min digestion. According to the λ_{max} of the synthesized amylose chains reported by *Bailey et al. 1960*[2], we estimated the chain lengths of the amylose helices after 120 min amylase hydrolysis, demonstrating a clear relation between LPC concentration and DP (see Table 1).
These results clearly show the protective effect of LPC against amylase degradation. As Table 1 shows, DP of 81 is estimated for amylose in presence of 5% LPC after 120 min digestion which is attributed to the formation of more helical arrangement and, therefore, precluding the enzyme hydrolysis. This is in agreement with our previous study. We demonstrated a range of amylose molecules with molar mass of ca. 10^5 g/mol after 240 min amylase hydrolysis[4].

3.3. Thermal analysis

To evaluate if amylose-LPC inclusion complexes are still stable after enzyme hydrolysis, the digesta were measured by DSC. Heating the freeze dried digested samples resulted in an endothermic transition which is related to the presence and melting of amylose-LPC inclusion complexes[1, 24]. The enthalpy values of the degraded samples demonstrate the presence and stability of the inclusion complexes after several digestion times (see table 2). A higher amount of LPC leads to a higher enthalpy of the amylose-LPC endotherm even after several digestion times, demonstrating the presence of inclusion complexes after amylase hydrolysis.

Table 2. Thermal analysis of 20% wheat starch-LPC suspension (freeze dried) with 2% and 5% LPC concentrations after 0, 15, 30, 60, 120 and 240 min degradation (amylose-LPC endotherm).
WS stands for wheat starch and LPC for lysophosphatidylcholine.

	WS+2%LPC	**WS+5%LPC**
Digestion times (min)	**ΔH ($J\ g^{-1}$)**	**ΔH ($J\ g^{-1}$)**
0	5.7 *(0.11)*	6.6 *(0.24)*
15	4.8 *(0.36)*	6.6 *(0.8)*
30	4.7 *(0.6)*	6.4 *(0.74)*
60	4.5 *(0.31)*	6.4 *(0.35)*
120	3.7 *(0.29)*	6.1 *(0.82)*
240	3.7 *(0.4)*	5.1 *(0.5)*

The values within the parentheses represent the SDs (n=2).

The DSC results further prove the decrease of enzyme accessible amylose due to the formation of inclusion complexes with LPC, confirmed by the appearance of a melting enthalpy in DSC measurements. This is in agreement with *Holm et al. 1983*[14] and *Putseys et al. 2010*[20]. *Holm et al. 1983*[14] reported the hardly hydrolysis of amylose-lysolecithin complexes after enzyme treatment, as a result of a coil to helix transition of the amylose molecules.

Furthermore, a slight decrease of enthalpy after 120 and 240 min digestion in presence of 5% LPC, compared with the undegraded sample, shows the extra protective effect occurring at high LPC concentrations (see Table 2).

4. Conclusion

As we previously reported, the amylose-LPC complex formation reduces starch susceptibility to enzyme hydrolysis[3, 4]. This study demonstrates a rapid method allowing a quick screen of the extent of amylose degradation rate after several digestion times.
In the present study we show that the combination of iodine complexation and defatting is an excellent tool to demonstrate the protection of amylose towards enzymatic hydrolysis by inclusion complexation with LPC. In addition, this method investigates the stability of LPC inside the amylose helices during the degradation, which was not demonstrated in our previous studies.
The iodine-amylose complexation substantially depends on the DP of amylose and the extent of complexation with another ligand (LPC). This reveals additional information about the formed complexes. Based on this, the defatting process helped us to get more insight into the protection of amylose by LPC. We show that LPC at higher concentrations leads to larger amylose molecules remaining after subsequent amylase digestion, shown by higher maximum wavelength, which is due to more LPC molecules complexed per amylose chain. These findings have practical significance and can be partly attributed to the formation of Slowly Digestible Starch (SDS) and Resistant Starch (RS).

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Chapter 7

Summary

Samenvatting

Summary

Starch is a widely used component that provides functional properties in foods. Starch is considered as the major source of energy in human nutrition; however its digestibility has a large impact on human health. Rapid postprandial glucose increase in the blood stream, due to the rapid digestibility, is considered as a risk factor that causes obesity and diabetes type II; while a slow digestion rate can prevent metabolic disorders. Starch digestion is a complex process that depends on several factors; such as the source of starch and presence of other components like fatty acids that promote the formation of amylose inclusion complexes.

Therefore, the aim of this thesis was to systematically study the formation of amylose inclusion complexes with LPC (lysophosphatidylcholine) and to evaluate the influence of this complexation on not only the structuring properties but also the digestibility of wheat starch. This approach reveals the key parameters contributing to the formation of inclusion complexes; such as the ligand concentration, heating time and temperature and the molecular composition of the digesta, which were not utterly discussed before. These findings could provide more details into the benefit of amylose inclusion complexation (slow digestible starch and the anti-stalling effect) while controlling the structural properties of starchy products.

This research showed how the formation of amylose-LPC complexes influences the physical and technological properties of wheat starch; such as pasting time, gelatinization, granular structure, thermal transition, swelling and solubility (Chapter 2). LPC at high concentrations resulted in less swelling and therefore reduced rupture of the starch granules which leads to a lower viscosity increase. In high moisture systems, at the levels above 1% LPC (based on starch), starch can no

longer be used as a structure builder. At lower LPC concentrations, amylose leakage is partly prevented leading to less granular rupture which results in retaining the starch functionalities.
Amylose inclusion complexation is enhanced at the temperatures of gelatinization due to the loss of structural crystallinity. However, studying the influence of incubation time and temperature revealed the possibility of amylose-LPC complexation at temperatures well below gelatinization temperatures during long incubation times (Chapter 3). We observed a double complexation upon 8 h heating at 50°C compared with the shorter times; however no further increase was observed after 8 h.
In addition, it was shown that the starch gelatinization endotherm is not affected by amylose inclusion complexation with LPC at high moisture contents. CLSM and SEM observations showed that the formation of inclusion complexes leads to a clear preservation of structural integrity of the starch granules at 60°C, in presence of 2% LPC.

Public awareness on the relation between human health and nutrition has increased with the increased attention for obesity and diabetes type II. With respect to the latter, a particular focus has been given to starch and starchy foods. Therefore, in the next step, our research was aimed to evaluate the influence of amylose-LPC complex formation on the digestibility of wheat starch (Chapter 4). The Englyst method is a widely used method for the *in vitro* digestion of starchy foods. However, it is designed to assess whole meals, while in this study we work with a purified system, which requires a slightly different setup. Therefore, an alternative method was established to investigate the digestibility of well-defined starch-LPC mixtures under controlled time-temperature-shear conditions in a diluted suspension.

The results demonstrated a lower susceptibility of wheat starch granules, protected by LPC, to α-amylase as compared to the pure wheat starch that is rapidly degradable. The effect depends on the LPC concentration. The difference in the amount of reducing sugars revealed a lower enzyme susceptibility of the samples containing more amylose-LPC complexes.

More insight into the molar mass and size distribution of starch polymers after each digestion time (in relation to the influence of amylose-LPC inclusion complexation) was gained, using Size-Exclusion Chromatography (Chapter 5). Therefore, the combination of our *in vitro* method (Chapter 4) and SEC allowed us to study the influence of LPC on the whole samples after each digestion time, based on molar mass of the starch polymers.

15 and 30 min digestion resulted in a low amount of starch oligomers and soluble sugars in presence of LPC; which is due to the reduced degradation of amylose and amylopectin. Additionally, a resistant part after 240 min degradation shows a significantly lower amount of low molar mass sugars in presence of LPC.

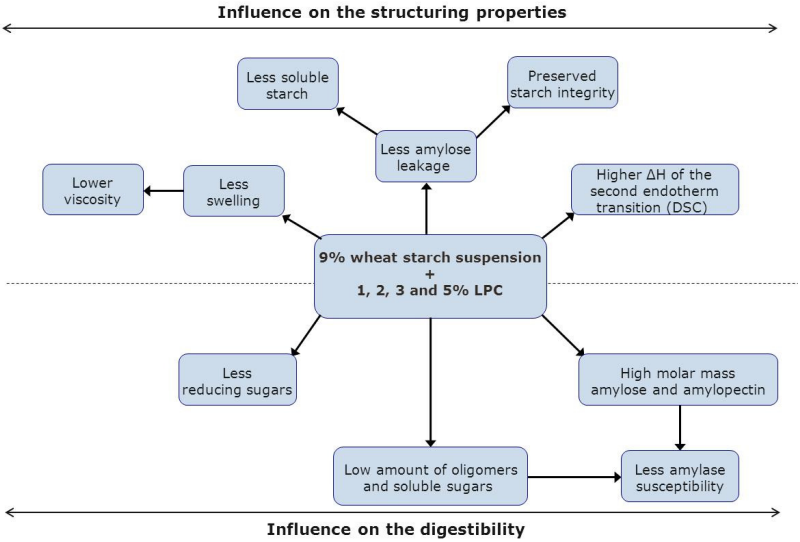
In contrast to established methods, such as the Englyst assay, our method assesses the molar mass distribution of amylopectin, amylose and low molar mass sugars, simultaneously.

To what extent the amylose helices are preserved after degradation was assessed in Chapter 6. We used iodine to quantify the inclusion complex formation with amylose before and after digestion, with and without LPC. This clearly revealed the important role of LPC in amylose protection during enzyme hydrolysis since iodine binding with amylose substantially depends on the DP of amylose and the extent of complexation with another ligand.

Depending on the LPC concentration and digestion time, the presence of LPC inside the amylose helices results in less iodine binding, accordingly. The defatting process helped us to approximately calculate the DP of amylose

after 120 min digestion. In addition, the enthalpy values of the degraded samples demonstrate the presence of inclusion complexes.

In conclusion, this study showed that not only the presence of LPC changes the structuring and functional properties of starch suspension but also that the formation of amylose inclusion complexes delays the starch digestion (see the following chart). Our observation proved the significant effect of LPC concentration; suggesting that LPC at high concentrations results in not only more inclusion complexes but also more stable complexes during enzyme hydrolysis.
Implicating this research work to some practical applications, in which viscosity increase is not substantial (e.g. biscuits and crackers), can show the significance of this study. How to obtain certain target viscosity and digestion properties can be regarded as the further investigations in line with this study.



Samenvatting

Zetmeel is een veel gebruikt bestanddeel dat functionele eigenschappen verschaft in levensmiddelen. Zetmeel wordt beschouwd als de belangrijkste energie bron van menselijke voeding; echter de verteerbaarheid is van grote invloed op de gezondheid van de mens. Snelle toename van postprandiale glucose in de bloedbaan ten gevolge van een snelle verteerbaarheid, wordt beschouwd als een risico factor welke obesitas en diabetes type II kan veroorzaken; terwijl een langzame vertering stofwisselingsstoornissen kan voorkomen. De vertering van zetmeel is een complex proces, welke afhankelijk is van verschillende factoren; zoals de oorsprong van het zetmeel en de aanwezigheid van andere componenten zoals vetzuren die de vorming van amylose inclusie complexen teweeg kunnen brengen.
Het doel van dit proefschrift was dan ook, om systematisch de vorming van amylose inclusie complexen met LPC (lysophosphatidylcholine) te bestuderen en de invloed van deze complexen niet alleen op de structuur eigenschappen, maar ook op de verteerbaarheid van tarwe zetmeel te evalueren. Deze aanpak onthult de belangrijkste parameters welke bijdragen aan de vorming van inclusie complexen; zoals de ligand concentratie, verhitting tijd en temperatuur en de moleculaire samenstelling van de digesta, welke niet eerder volkomen besproken zijn. Deze bevindingen konden meer details verschaffen ten voordele van amylose inclusie complexering (langzaam verteerbaar zetmeel en het anti staling effect) terwijl de structuur eigenschappen van de zetmeelproducten gecontroleerd werden.
Dit onderzoek heeft aangetoond hoe de vorming van amylose-LPC inclusie complexen de fysische en technologische eigenschappen van tarwe zetmeel beïnvloed; zoals het begin van de verstijfselingstemperatuur en de mate van gelering,

korrel structuur, thermische overgangen, zwelling en oplosbaarheid (Hoofdstuk 2). Een hoge concentratie aan LPC resulteerde in geringere zwelling en daardoor minder afbraak van de zetmeelkorrels wat leidde tot een lagere viscositeitstoename. In systemen met een hoog vochtgehalte, kan bij toevoeging van meer dan 1% LPC (op basis van zetmeel), zetmeel niet meer gebruikt worden als structuur builder. Bij lagere LPC concentraties wordt de amylose leaching gedeeltelijk voorkomen wat leidt tot een beperkte korrel afbraak met behoud van de zetmeel functionaliteiten.
De vorming van amylose inclusie complexen neemt toe tijdens de verstijfselingstemperatuur door de afname van de kristalliniteit. Echter, onderzoek naar de invloed van de incubatie tijd en temperatuur op de vorming van amylose-LPC complexen bracht de mogelijkheid aan het licht van amylose-LPC complex vorming na lange incubatie tijd onder de verstijfselingstemperatuur (Hoofdstuk 3). Na 8 uur verhitten bij 50 °C werd een verdubbeling van de complexering vastgesteld ten opzichte van kortere tijden; echter na 8 uur werd geen verdere toename waargenomen.
Daarnaast werd aangetoond, dat de zetmeel verstijfselingsendotherm bij hoge vochtgehaltes niet wijzigt door amylose inclusie complex vorming met LPC. CLSM en SEM waarnemingen lieten zien, dat dat de vorming van inclusie complexen leiden tot een duidelijk behoud van de integriteit van de zetmeelkorrel structuur bij 60 °C, in de aanwezigheid van 2% LPC.
Publieke bewustwording naar het verband tussen de volksgezondheid en voeding is gestegen met de toegenomen aandacht voor obesitas en diabetes type II. Met betrekking tot laatstgenoemde, bijzondere aandacht is gegeven aan zetmeel en zetmeel rijke voedingsmiddelen. Daarom was in de volgende stap, ons onderzoek er op gericht om de invloed van de vorming van amylose-LPC complexen op de verteerbaarheid van

tarwe zetmeel te evalueren (Hoofdstuk 4). De Englyst methode is een wijd gebruikte methode voor de *in vitro* vertering van zetmeelrijke voedingsmiddelen. Het is echter bedoeld om gehele maaltijden te beoordelen, terwijl we in dit onderzoek werken met een gezuiverd systeem, welke een enigszins afwijkende setup vereist. Daarom werd een alternatieve methode vastgesteld, om de verteerbaarheid van goed gedefinieerde zetmeel-LPC mengsels onder gecontroleerde tijd-temperatuur-shear condities in een verdund systeem te onderzoeken. De resultaten lieten een geringere gevoeligheid zien van tarwe zetmeelkorrels beschermd door LPC tot α-amylase ten opzichte van het onbehandelde zetmeel, dat snel wordt afgebroken. Het effect hangt af van de LPC concentratie. Het verschil in de hoeveelheid reducerende suikers toonde aan dat de enzym gevoeligheid lager is als de monsters meer amylose-LPC complexen bevatten.

Size-Exclusion Chromatography (SEC) is gebruikt om meer inzicht te verkrijgen in de molmassa en grootteverdeling van het zetmeel (met betrekking tot de invloed van amylose inclusie complexen met LPC) na iedere verteringstijd (Hoofdstuk 5). Met de combinatie van onze *in vitro* methode (Hoofdstuk 4) en de SEC, konden we de invloed van LPC na iedere verteringstijd aan het gehele monster op basis van de molmassa van het zetmeel polymeer bestuderen.

Een verteringstijd van 15 en 30 minuten in de aanwezigheid van LPC, resulteerde in een geringe hoeveelheid oligomeren en oplosbare suikers; wat het gevolg is van een verminderde afbraak van amylose en amylopectine. Daarnaast werd na 240 minuten degradatie in de aanwezigheid van LPC, een resistant deel waargenomen en minder laag moleculaire suikers.

In tegenstelling tot de gevestigde methoden, zoals de Englyst test, wordt met onze werkwijze de molmassa verdeling van amylopectine, amylose en laag moleculaire suikers gelijktijdig geëvalueerd.

In hoeverre de amylose helices behouden zijn na afbraak, werd beoordeeld in Hoofdstuk 6. Jodium is gebruikt om de inclusie complexvorming met amylose voor en na vertering, met en zonder LPC, te kwantificeren. Hierdoor wordt de belangrijke rol van LPC duidelijk in de bescherming van amylose tijdens de enzymhydrolyse, daar jodium binding met amylose afhankelijk is van de DP van de amylose en de mate van complexering met een ander ligand. Afhankelijk van de LPC concentratie en de verteringstijd, resulteerde de aanwezigheid van LPC in de amylose helices in dienovereenkomstig minder jodium binding. Een ontvettingsproces maakte het ons mogelijk, om na 120 minuten vertering de DP van amylose bij benadering te berekenen. Daarnaast toonden de enthalpie waarden van de afgebroken monsters, de aanwezigheid van inclusie complexen aan.

Concluderend, liet dit onderzoek zien dat de aanwezigheid van LPC niet alleen de structuur eigenschappen van een zetmeel suspensie wijzigt, maar ook dat de vorming van amylose inclusie complexen de verteerbaarheid van zetmeel vertraagt (zie het volgende schema). Onze waarneming heeft het significante effect van de LPC concentratie aangetoond; wat er op wijst dat LPC bij hoge concentratie niet alleen resulteert in meer complexen, maar dat de complexen ook stabieler zijn tijdens de enzymhydrolyse.
Het toepassen van dit onderzoek in een aantal praktische applicaties, waarbij de viscositeitstoename niet substantieel is (bijvoorbeeld koekjes en crackers) kan het belang van dit onderzoek aantonen. Hoe een zekere gewenste viscositeit en verteringseigenschappen te verkrijgen, kan worden beschouwd als een vervolg in lijn met dit onderzoek .

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List of publications

- Influence of Lysophosphatidylcholine on the Gelation of Diluted Wheat Starch Suspensions.
  Ahmadi-Abhari, S., Woortman, A. J. J., Hamer, R. J., Oudhuis, A. A. C. M., & Loos, K. (2013). Carbohydrate Polymers, 93, 224-231.

- The Influence of Amylose-LPC Complex Formation on the Susceptibility of Wheat Starch to Amylase.
  Ahmadi-Abhari, S., Woortman, A. J. J., Oudhuis, A. A. C. M., Hamer, R. J., & Loos, K. (2013). Carbohydrate Polymers, 97, 436-440.

- The effect of temperature and time on the formation of amylose-lysophosphatidylcholine inclusion complexes.
  Ahmadi-Abhari, S., Woortman, A. J. J., Oudhuis, A. A. C. M., Hamer, R. J., & Loos, K. (2013). Starch/Stärke, 65, 1-9.

- Assessment of the influence of amylose-LPC complexation on the extent of wheat starch digestibility by Size-Exclusion Chromatography.
  Ahmadi-Abhari, S., Woortman, A. J. J., Hamer, R. J., & Loos, K. (2013). Food Chemistry, 141, 4318-4323.

- Assessing the susceptibility of amylose-lysophosphatidylcholine complexes to amylase by the use of iodine.
  Ahmadi-Abhari, S., Woortman, A. J. J., Hamer, R. J., & Loos, K. (2013). Starch/Stärke, DOI: 10.1002/star.201300205

- Rheological and digestion properties of wheat starch influenced by amylose-lysophosphatidylcholine complexation at different gelation phases.
  Ahmadi-Abhari, S., Woortman, A. J. J., Hamer, R. J., & Loos, K. (2013). To be submitted

Oral presentations

- Cereals & Europe Spring Meeting 2013, Leuven – Belgium, 29 -31 May 2013
  The Influence of Amylose-LPC Complex Formation on the Susceptibility of Wheat Starch to Amylase

- Dutch Polymer Days, Lunteren – Netherlands, 18 – 19 March 2013
  The Influence of Amylose-LPC Complex Formation on the Susceptibility of Wheat Starch to Amylase

- AACC Annual Meeting, Hollywood – USA, 30 Sep – 3 Oct 2012
  Kinetics of the Formation of Amylose-LPC Inclusion Complexes and Their Influence on Enzymatic Digestibility of Wheat Starch Suspensions

- Starch Convention, Detmold – Germany, 17–18 April 2012
  Influence of Time and Temperature on Amylose-LPC Inclusion Complexation in Diluted Wheat Starch Suspensions

- Dutch Polymer Days, Lunteren – Netherlands, 12–13 March 2012
  Kinetics of Amylose-LPC Inclusion Complexation in Diluted Wheat Starch Suspensions

- AACC Annual Meeting, Palm Springs – USA, 16–19 Oct 2011
  Influence of Lysophosphatidylcholine (LPC) on the Gelation and Functional Properties of Diluted Wheat Starch Suspensions

- Polysaccharide Symposium, Wageningen – Netherlands, 28 Aug–2 Sep 2011
  Influence of Lysophosphatidylcholine (LPC) on Gelation of Diluted Wheat Starch Suspensions and Its Effect on Starch Digestibility

- Starch Convention, Detmold – Germany, 13–14 April 2011
  Influence of Lysophosphatidylcholine (LPC) on Gelation of Diluted Wheat Starch Suspensions and Its Effect on Starch Digestibility

Poster presentations

- AACC Annual Meeting, Albuquerque – USA, 29 Sep – 2 Oct 2013
  Assessment of the Influence of Amylose-LPC Complexation on the Extent of Wheat Starch Digestibility by Size-Exclusion Chromatography

- Dutch Polymer Days, Lunteren – Belgium, 18-19 March 2013
  The effect of Temperature and Time on the Formation of Amylose-LPC Inclusion Complexes

- CHAINS 2011, Utrecht – Netherland, 28–30 Nov 2011
  Influence of Lysophosphatidylcholine (LPC) on the Gelation and Functional Properties of Diluted Wheat Starch Suspension

- AACC Annual Meeting, Palm Springs – USA, 16–19 Oct 2011
  Influence of Lysophosphatidylcholine (LPC) on the Gelation and Functional Properties of Diluted Wheat Starch Suspensions

- Bi-annual Zernike Meeting, Vlieland – Netherlands, 15–17 May 2011
  Influence of Lysophosphatidylcholine (LPC) on Gelation of Diluted Starch Suspension

- Advanced Food Analysis, Wageningen – Netherlands, 15–19 Nov 2010
  The Influence of Lysophosphatidylcholine (LPC) on Viscosity Behavior and Granular Structure of Wheat Starch

- Glycoscience, Wageningen – Netherlands, 17–20 May 2010
  The Effect of Water Soluble and Water Insoluble Proteins on the Gelatinization of Wheat Starch

- Dutch Polymer Days, Veldhoven – Netherlands, 15-16 Feb 2010
  The Effect of Water Soluble and Water Insoluble Proteins on the Gelatinization of Wheat Starch

Workshops and courses

- Z-FEL by RUG and KVI, Wageningen – Netherlands, 25 Nov 2011
- Dynamic Presentation by RUG, Groningen – Netherlands, 22-23 Nov 2010
- Cellulose by BMBF, Bomlitz – Germany, 30 Aug- 1 Sep 2010
- How to write a scientific paper by RUG, Groningen – Netherlands, 28-29 Aug 2010
- Food Rheology by AACC International, Ghent – Belgium, 28-29 April 2010
- Food Rheology by Anton Paar, Lokeren – Belgium, 7-8 Oct 2009