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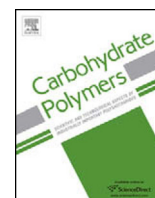
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The influence of amylose-LPC complex formation on the susceptibility of wheat starch to amylase



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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.

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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 South-east Asia (Cui & Oates, 1997). In this respect, the rate and extent of starch digestion is of great interest as it affects the glycemic response (Tufvesson, Skrabanja, Björck, Elmstahl, & Eliasson, 2001). 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 \rightarrow 4 D-glucose and amylopectin is a branched polymer of α -1 \rightarrow 4 D-glucose and α -1 \rightarrow 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) (Guraya, Kadan, & Champagne, 1997). The GI describes the level of the postprandial glucose rise in blood as compared to ingestion of a standard dose

of glucose (Zhang, Ao, & Hamaker, 2006; Zhang, Venkatachalam, & Hamaker, 2006). 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 (Singh, Dartois, & Kaur, 2010). Upon ingestion, starch is exposed to salivary α -amylase. Glucose absorption mainly occurs in the small intestine (Lehmann & Robin, 2007) where the pancreatic α -amylase hydrolyses amylose/amylopectin to maltose and larger oligosaccharides (maltose, maltotriose and maltotetraose) (Hasjim, Lavau, Gidley, & Gilbert, 2010). The α -amylases hydrolyze α -(1–4) glycosidic bonds (Singh et al., 2010). Maltose-glucoamylase and sucrose-isomaltase, two brush border enzymes, degrade oligosaccharides to glucose which then passes the blood stream (Gray, 1992).

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 (Englyst, Englyst, Hudson, Cole, & Cummings, 1999; Englyst, Kingman, & Cummings, 1992).

Generally, the digestion of starch is a complex process that is strongly dependent on the substrate, enzyme adsorption by the

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substrate and presence of other components like lipids and proteins (Lehmann & Robin, 2007).

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 (Kwasniewska-Karolak, Nebesny, & Rosicka-Kaczmarek, 2008) thus rendering the amylose less susceptible to amylolytic enzymes (Nebesny, Rosicka, & Tkaczyk, 2002; Zhang, Ao, et al., 2006; Zhang, Venkatachalam, et al., 2006). *In vivo* and *in vitro* digestibility studies on the effect of these components have shown that they can considerably slow down the enzymatic digestibility (Singh et al., 2010).

In our previous study (Ahmadi-Abhari, Woortman, Hamer, Oudhuis, & Loos, 2013), 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 (Putseys et al., 2010). 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 (Ahmadi-Abhari et al., 2013; Cui et al., 1997). Also, the poor digestibility of the amylose-lipid complexes has been demonstrated (Seneviratne & Biliaderis, 1991). 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 (Hasjim et al., 2010). The Englyst method (Englyst et al., 1992) 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.

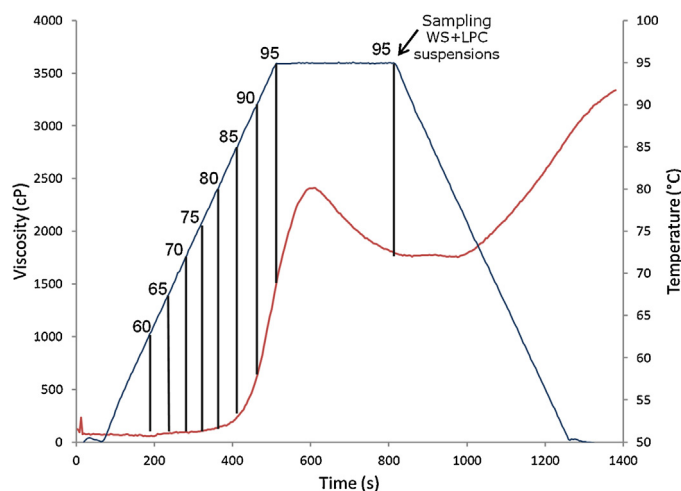


Fig. 1. RVA profile indicating the temperature-time profile and sampling point.

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 $6^{\circ}\text{C}/\text{min}$ 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 $6^{\circ}\text{C}/\text{min}$ (see Fig. 1).

2.3. Preparation of DNSA reagent

The DNSA solution was prepared by dissolving 1 g DNSA (3,5-dinitrosalicylic acid) in 20 mL 2 N 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, 0.025 M, 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 (Qian, Ajandouz, Payan, & Nahoum, 2005). 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, Langensfeld, 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 μ l 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 (Duedahl-Olesen, Pedersen, & Larsen, 2000). 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 = 28.45 J/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_0), 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 suspensions 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 (Ahmadi-Abhari et al., 2013) 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

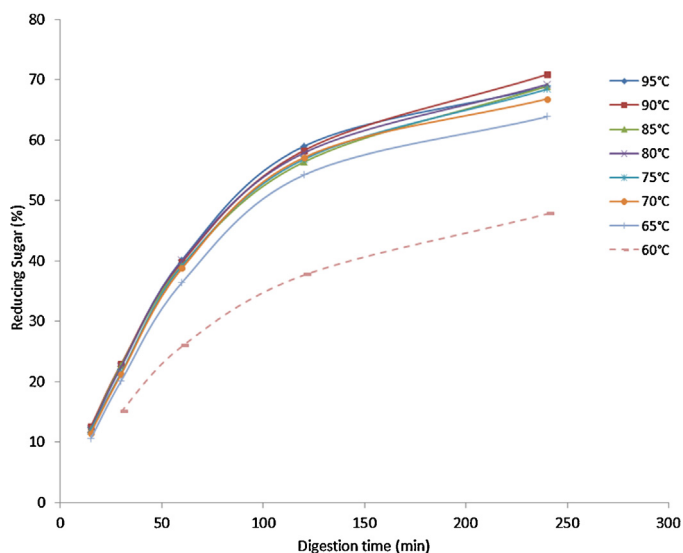


Fig. 2. Influence of the temperature of the heat treatment in RVA on the enzyme susceptibility of native wheat starch suspensions. Reducing sugar (%) is calculated as the amount of reducing sugars relative to the condition in which starch is completely digested. The result of 15 min digestion is not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

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 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 (Dona, Pages, Gilbert, & Kuchel, 2010). 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 (Dona et al., 2010; Zhang, Ao, et al., 2006; Zhang, Venkatachalam, et al., 2006). 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 (Chung, Lim, & Lim, 2006).

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.

The ratio of the reducing sugars generated in the LPC containing suspensions to the reference represents the “Reduction of reducing

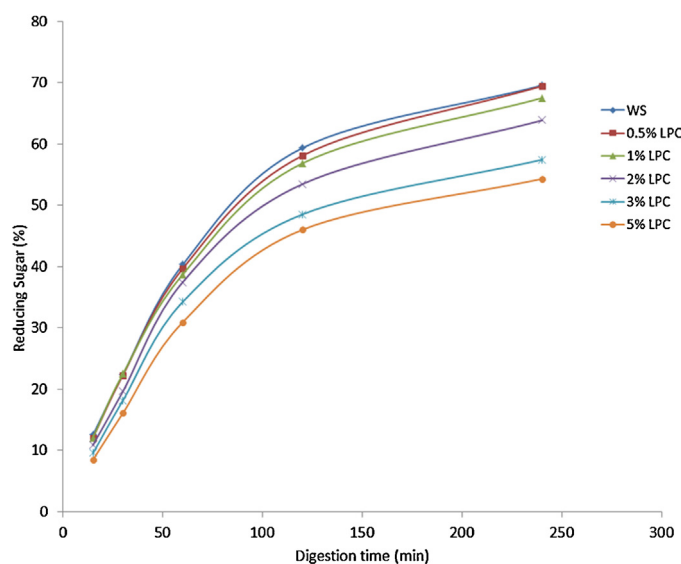


Fig. 3. Influence of LPC on the enzyme susceptibility of wheat starch after gelatinization in the RVA at 95 °C. WS stands for wheat starch.

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

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.

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; therefore only the most stable complexes survive during the digestion. In addition, the increase in peak

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_p (°C)	ΔH (J/g)	T_p (°C)	ΔH (J/g)
WS + 2%LPC	103.8	5.7	108	3.7
WS + 5%LPC	104.4	6.6	108	5.1

temperature observed after prolonged amylose digestion is another proof of the presence of stable complexes. The shift indicates a higher thermal stability. It is known that amylose inclusion complexation increases crystalline region, which increases the melting temperature (Godet, Tran, Colonna, & Buleon, 1995). 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 stable 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 ingress 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 (Ahmadi-Abhari et al., 2013).

Less swelling, due to the presence of LPC, reduces the accessibility of amylase to the starch molecules. In addition, Putseys et al. (2010) 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) confirmed this when they reported amylose-lysophosphatidylcholine 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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