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Production of tailor made short chain amylose–lipid complexes using varying reaction conditions

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ABSTRACT

When amylose was synthesized using potato phosphorylase in the presence of amylose complexing lipids, monodisperse populations of amylose–lipid complexes were formed. Enzyme dosage and glucose-1-phosphate (glc-1-P)/primer ratio influenced the reaction rate of the enzymic synthesis, presumably by changing the balance between amylose synthesis and amylose–lipid complexation and precipitation, and impacted the molecular weight of the complexes. Lipid characteristics affected the dissociation properties and amylose chain lengths of the amylose–lipid complexes presumably by determining the minimal amylose chain length necessary for complexation and precipitation. Tailor made short chain amylose–lipid complexes can hence be produced by choosing the appropriate reaction conditions. We propose a synthesis mechanism in which the primer is elongated until an amylose chain is obtained which is of sufficient length to complex a first lipid. Further chain extension then occurs, together with subsequent complexation until the complex becomes insoluble and precipitates.

1. Introduction

Amylose, the essentially linear glucose polymer of starch, can form inclusion complexes with suitable ligands such as emulsifying lipids. Crystalline amylose–lipid complexes, have a V-type X-ray diffraction pattern, and can be endogenously present in starch granules (Morrison, 1988) or formed upon heating of starch suspensions in the presence of either endogenous or exogenous lipids (Morrison, Law, & Snape, 1993). The functionality of emulsifiers in starch containing systems is often related to complex formation with amylose, and explains the importance of the resultant complexes (Batres & White, 1986; Eliasson & Ljunger, 1988; Goesaert et al., 2005; Gudmundsson, 1992; Gudmundsson & Eliasson, 1990; Kulp & Ponte, 1981; Takeo, Tokumura, & Kuge, 1973).

Most laboratory methods for synthesizing amylose–lipid complexes (further referred to as classical synthesis methods) involve mixing amylose and lipid solutions at elevated temperature. Incubation at 60 °C yields amorphous type I amylose–lipid complexes, while, at 90 °C, semi-crystalline type II amylose–lipid complexes are formed (Biliaderis, Page, Slade, & Sirett, 1985; Eliasson & Krog, 1985; Galloway, Biliaderis, & Stanley, 1989; Gelders, Vanderstukken, Goesaert, & Delcour, 2004; Godet, Bizot, & Buléon, 1995a; Godet, Tran, Colonna, Buléon, & Pezolet, 1995b; Karkalas, Ma, Morrison, & Pethrick, 1995). Such procedures generally result in rather polydisperse complexes (Gelders et al., 2004).

An alternative approach for synthesizing amylose–lipid complexes is the so-called semi-enzymic method developed by Gelders and coworkers (2005). In this procedure, amylose chains are synthesized *de novo* in a reaction of primer (a short chain of α-(1 → 4)-bound glucose units, $[\alpha\text{-}(1 \rightarrow 4)\text{-glc}]_n$) and glucose-1-phosphate (glc-1-P) catalysed by potato phosphorylase (EC 2.4.1.1). Phosphate (P_i) is formed as a co-product (Pfannemuller & Burchard, 1969).

$$\text{Glc-1-P} + [\alpha\text{-}(1 \rightarrow 4)\text{-glc}]_n \leftrightarrow P_i + [\alpha\text{-}(1 \rightarrow 4)\text{-glc}]_{n+1}$$

In the presence of amylose complexing lipids, the reaction forms monodisperse short chain amylose–lipid complexes (Gelders et al., 2005).

Gelders and coworkers (2005) described the use of potato phosphorylase in the synthesis of amylose–lipid complexes only for specified enzyme, substrate and lipid [glyceryl monostearate and behenic fatty acid] conditions. Gelders, Goesaert, and Delcour (2006) showed that semi-enzymically synthesized monodisperse short chain amylose–lipid complexes can function as controlled release agents of lipids in starch containing systems. However, it is still unclear to what extent the reaction conditions determine the properties and functionality of semi-enzymically synthesized amylose–lipid complexes and in what way lipids impact the phosphorylase catalysed polymerization of the amylose chain. Literature dealing with both the classical as well as the semi-enzymic

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synthesis methods is rather vague on whether, and, if so to what extent, lipids interact with very short amylose chains.

We therefore evaluated the impact of different reaction variables (enzyme dosage and ratio of substrate to primer; lipid type, chain length and degree of unsaturation) on both the progress of the synthesis reaction (*i.e.* conversion of glc-1-P) as well as the characteristics of the obtained complexes (chain length of the complexing amylose, dissociation properties). By doing so, we gained insight in the underlying reasons for the resulting properties of the produced short chained amylose–lipid complexes and developed an understanding of the different steps occurring in this semi-enzymic synthesis method.

2. Materials and methods

2.1. Materials

All chemicals and reagents, including lipids, α-D-glc-1-P disodium salt tetrahydrate and oyster glycogen, were of at least analytical grade and from Sigma–Aldrich (Bornem, Belgium) unless specified otherwise. *Pseudomonas sp.* isoamylase was from Megazyme (Bray, Ireland). Dimodan (distilled monoacyl glycerols; predominantly glyceryl monostearate and glyceryl monopalmitate) was from Danisco (Brabrand, Denmark). Potato phosphorylase was isolated from locally purchased Nicola potatoes (*Solanum tuberosum cv. Nicola*) according to Roger and coworkers (2000) with some minor adaptations made by Gelders and coworkers (2005). The procedure involves peeling and liquefying the potatoes, followed by filtration and centrifugation of the crude extract. After α-amylase inactivation by incubation at 56 °C and subsequent centrifugation, the enzyme was recovered by ammonium sulfate precipitation. The α-amylase activity of the final phosphorylase preparation was negligible when assayed with the Amylazyme method (Megazyme, Bray, Ireland).

2.2. Methods

2.2.1. Phosphorylase activity and phosphate determination

The phosphorylase activity of the enzyme suspension was determined by quantifying the concentration of liberated P_i according to Saheki, Takeda, and Shimazu (1985). One enzyme unit (EU) of phosphorylase activity was the amount of enzyme (in ml suspension) releasing 1.0 μmol of P_i per minute at 37 °C and pH 6.2 (0.10 M sodium citrate buffer).

The procedure for determination of P_i contents developed by Saheki and coworkers (1985) involves addition of 2.0 ml molybdate reagent (15 mM ammonium molybdate, 100 mM zinc acetate; adjusted to pH 5.0 with 1.0 M HCl) and 0.5 ml of buffered ascorbic acid (10.0% ascorbic acid adjusted to pH 5.0 with 40.0% w/v sodium hydroxide) to 0.2 ml of sample. After incubating the mixture for 15 min at 30 °C, the extinction at 850 nm was measured.

2.2.2. Synthesis of the primer by debranching glycogen

Oyster glycogen (5.0 g) was dispersed in 250 ml sodium acetate buffer (0.05 N, pH 3.8) and incubated at 37 °C. After addition of isoamylase (25 EU, units according to supplier), the mixture was stirred for 48 h at this temperature. The enzyme was inactivated by keeping the mixture at 100 °C for 10 min. The average chain length of the primer, calculated as the ratio of the total glucose content, according to the phenol–sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), to the total reducing sugar content, determined with the Somogyi–Nelson method (Nelson, 1944; Somogyi, 1945), was 10.

2.2.3. Synthesis of amylose–lipid complexes

The required amount of glc-1-P (for the different molar ratios glc-1-P/primer) was dissolved in 10.0 ml deionised water. The pH of the solution was adjusted to 6.0 with 1.0 M acetic acid. Sodium citrate buffer (4.0 ml, 0.10 M, pH 6.0) and 1.5 ml debranched glycogen solution (containing 16.4 μmol primer) were added and the mixture was kept at 45 °C. Excess lipid (0.1 mmol), solubilised in 1.0 ml of hot ethanol (±70 °C) was added dropwise under continuous stirring (250 rpm). The required dosage of phosphorylase (see below) was added to the mixture and the volume was adjusted to 100 ml.

To monitor the primer elongation as a function of time, aliquots were removed from the mixtures at selected time intervals. They were immediately heated for 10 min at 100 °C (to inactivate the enzyme) and centrifuged (10,000*g*, 5 min). The degree of elongation of the primer was estimated from the concentration of P_i in the supernatant. The curve (concentration of P_i as a function of time) was fitted with a Levenberg–Marquardt non-linear least square equation. The concentration of P_i was used to calculate the average degree of polymerization of the amylose (DP_{calc}) in the obtained complexes.

The synthesis reaction was stopped after 24 h by centrifugation (10,000*g*, 30 min, 6 °C). The pellet containing precipitated amylose–lipid complexes was removed from the reaction medium. It was washed for 30 min with deionised water at room temperature and recovered by centrifugation (10,000*g*, 15 min, 6 °C). Finally, the crude complexes were suspended in water and lyophilised.

The control reaction conditions for all experiments were 10 EU of phosphorylase per 100 ml, a glc-1-P to primer molar ratio of 100 and Dimodan as lipid.

In our experimental setup, one variable was changed at a time, while the others were the same as those of the control conditions: (i) enzyme dosages were 0.1, 0.5, 2.5, 10 or 20 EU per 100 ml; (ii) ratios of glc-1-P/primer were 25, 50, 100, 200 or 400 mol/mol; (iii) both fatty acids (FA) and monoacyl lipids (MAG) were used; (iv) lipids varying in aliphatic chain lengths (*i.e.* chains of 12, 14, 16 or 18 carbon atoms) and the number of double bonds (*i.e.* 0, 1, 2 or 3 double bonds in a 18 carbon atom lipid chain). Each synthesis experiment was performed at least in duplicate.

2.2.4. Removal of excess lipids

Uncomplexed lipids were removed from the crude complexes by suspending them in chloroform (60 ml $CHCl_3$/g complex). The suspensions were washed for 30 min, and filtered over a sintered glass filter (porosity 4). The defatted complexes were air-dried overnight.

2.2.5. Differential Scanning Calorimetry

The dissociation temperatures and enthalpies of the defatted complexes were determined by Differential Scanning Calorimetry (DSC Q1000, TA instruments, New Castle, DE, USA), calibrated with indium. Amylose–lipid complexes [1.5–4.0 mg dry matter (dm)] were accurately weighed into coated aluminium pans (Perkin-Elmer, Waltham, MA, USA). Water was added to obtain a final dm content of 25.0%, after which the pans were hermetically sealed. The sample pans and an empty reference pan were equilibrated at 0 °C and heated to 140 °C at a heating rate of 4 °C/min. Onset (T_o), peak (T_p), conclusion (T_c) temperatures, dissociation temperature range (T_c–T_o), all expressed in °C, and dissociation enthalpies (ΔH) of the synthesized amylose–lipid complexes, expressed in J/g defatted complex, were determined using Universal Analysis 2000 software (TA instruments). Results shown are averages of at least three measurements.

2.2.6. High Performance Size Exclusion Chromatography

The synthesized amylose–lipid complexes were analyzed by Size Exclusion Chromatography (SEC) on a Biologic Duo Flow Core System (Bio-Rad Laboratories, Hercules, CA, USA) with a Signal Import Module-HR (SIM-HR) and a Refractive Index Detector (RID-10A, Shimadzu, Kyoto, Japan). Amylose–lipid complexes (15 mg) were solubilised in 1.0 M potassium hydroxide (KOH) by stirring gently overnight. The solutions were diluted to 0.10 M KOH by adding deionised water. They were then filtered (cellulose syringe filter, 0.45 μm) and loaded (100 μl) onto a Superose^{TM} 12 column (1 × 30 cm, Amersham Biosciences, Uppsala, Sweden). The samples were eluted at room temperature with 0.10 M KOH (containing 0.02% sodium azide) at a flow rate of 0.5 ml/min. Shodex-82 pullulan standards (Showa Denko K.K, Tokyo, Japan), maltoheptaose and glucose were used as standards. Under the used elution conditions, pullulan standards can replace linear amylose standards for chromatographic calibration purposes (Roger et al., 2000).

A second-order polynomial correlation between the logarithm of the molecular weights of the standards and the elution volume was used to determine the values of peak degree of polymerization (DP_{peak}) and polydispersity (P). The P-value is the ratio of number to weight degree of polymerization and was calculated according to Gelders and coworkers (2004). It provides an indication of the distribution of individual molecular weights in a batch of polymers.

2.2.7. Statistical analyses

DSC-results were evaluated statistically using the t-test (PROC ANOVA) with a significance level $P < 0.05$. Statistical analyses were conducted using the Statistical Analysis System software 8.1 (SAS Institute, Cary, NC, USA).

3. Results

3.1. Enzyme dosage

Fig. 1 shows the influence of enzyme dosage (0.1, 0.5, 2.5, 10 and 20 EU per 100 ml) on the semi-enzymic synthesis reaction. The reaction was performed at pH 6.0 and 45 °C with a molar ratio glc-1-P/primer of 100 and Dimodan as the complexing lipid. The initial reaction rate was strongly influenced by the enzyme concentration. At 0.1 EU/100 ml, P_i concentration increased linearly during the first 7 h without reaching a maximum. In the case of 0.5 EU/100 ml, the glc-1-P conversion was initially faster, but the reaction later slowed down until the P_i concentration reached a final plateau value around 7 mM after 7.5 h. The reaction with 2.5 EU/100 ml reached such maximum after 1 h. For higher enzyme dosages (10 and 20 EU per 100 ml), the reaction almost immediately reached its final conversion value.

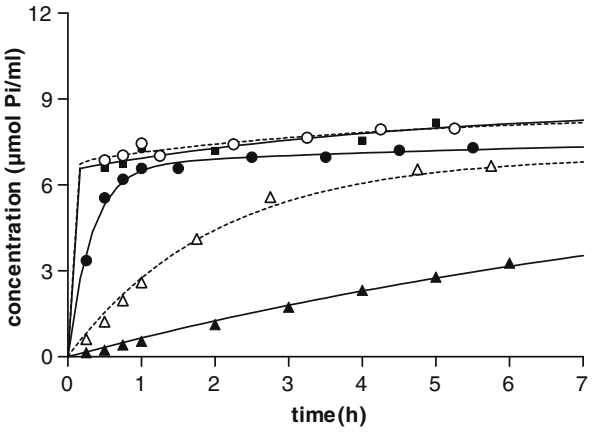

Fig. 1. Progression of the semi-enzymic synthesis reaction during the first 7 h using different enzyme dosages: 0.1 EU (▲), 0.5 EU (△, dotted line), 2.5 EU (●), 10 EU (○, dotted line) and 20 EU (■).

Table 1 lists different parameters of the complex formation and the obtained amylose–lipid complexes. Glc-1-P conversion values (% conv) at the end of the reaction (after 24 h) and the DP_{calc} of the obtained complexes, which were based on the P_i levels liberated during the synthesis reaction, increased with enzyme dosage. Similarly, SEC showed that higher enzyme dosages resulted in complexes with higher DP_{peak}. The synthesized complexes were of low polydispersity ($1.11 < P < 1.65$), irrespective of the enzyme dosage used. Their dissociation enthalpies were not significantly influenced by the enzyme dosages used for their synthesis (Table 1). However, the dissociation temperatures ranges increased with enzyme dosage.

3.2. Ratio glc-1-P/primer

Fig. 2 shows that the progress of the reaction was affected by the molar ratio of glc-1-P to primer (25, 50, 100, 200 and 400). The reaction was performed at pH 6.0 and 45 °C, with an enzyme dosage of 10 EU/100 ml and Dimodan as the complexing lipid. In all cases, amylose synthesis occurred very rapidly and P_i concentration reached a plateau value within 60 min. Although the maximum concentration of liberated P_i increased with increasing glc-1-P to primer ratio (going from about 3 mM for ratio 25 to 10 mM for ratio 400), the final conversion value was decreased (Table 1). This indicated a less efficient percentagewise conversion to free P_i in the presence of excess glc-1-P. DP_{calc} increased with increasing ratio of glc-1-P/primer. This was also reflected in the DP_{peak} values obtained by SEC, which rose with the glc-1-P concentration. The polydispersity of the synthesized complexes was not related to the molar ratio glc-1-P/primer, and remained rather low. In addition, when synthesizing the complexes with higher glc-1-P concentrations, their dissociation and conclusion temperatures increased significantly (Table 1). The temperature ranges and enthalpies, on the other hand, remained constant for the different ratios.

3.3. Lipid type and chain length

Fig. 3a shows the progress of the reaction when adding fatty acids (FA) with increasing lipid chain length (from 12 to 18 carbons) to the medium. The reactions were performed at pH 6.0 and 45 °C with an enzyme dosage of 10 EU per 100 ml and a molar ratio glc-1-P/primer of 100. The largest increase in free P_i occurred during the first 15 min (as could also be seen for varying molar ratios and higher enzyme dosages) and the initial reaction rate seemed unaffected by the lipid chain length. Increasing P_i concentration plateau values were obtained when synthesizing amylose in the presence of lipids of increasing chain lengths [except for lauric acid (C12:0)], going from about 6 mM for myristic acid (C14:0) to about 10 mM for stearic acid (C18:0). The latter was also reflected in the conversion values (% conv) of the reaction and the DP_{calc} of the complexes synthesized with different lipids (Table 1). DSC dissociation characteristics indicated that the peak and conclusion temperatures and the temperature ranges increased significantly with increasing FA chain length. Similar observations were made for the progress of the reaction and the characteristics of the complexes when synthesizing amylose in the presence of monoacyl glycerols (MAG) (Fig. 3b, Table 1). However, the conversion values and subsequent DP_{calc} and DP_{peak} of these lipids were higher than those of their FA counterparts. Polydispersity values were not related to the lipid chain lengths, but remained low for all lipids tested ($1.13 < P < 1.73$).

Table 1
Properties of complexes synthesized using different experimental conditions [enzyme dosages; ratios glc-1-P/primer; lipid types (FA, fatty acids; MAG, monoacyl glycerols) and chain lengths (from 12 to 18 carbon atoms); and lipids with different degree of unsaturation] in an otherwise standardised procedure. The percentages of final conversion (% conv) were deduced from the phosphate determination after a 24 h reaction, and were used to calculate the theoretical DP (DP_{calc}). The peak degrees of polymerization (DP_{peak}) and the polydispersity values (P) were obtained by SEC. Onset (T_o), peak (T_p) and conclusion (T_c) temperatures, dissociation temperature range (T_c–T_o) and dissociation enthalpies (ΔH), obtained by DSC, are shown with standard deviations between brackets.

Variable		% Conv	DP_{calc}	DP_{peak}	P	T_o (°C)	T_p (°C)	T_c (°C)	T_c–T_o (°C)	ΔH (J/g)
Enzyme dosage	0.1 EU	34.66	45.9	59.6	1.11	77.0 (0.8) *b*	96.2 (0.2) *a*	104.0 (0.3) *a*	27.1 (1.1) *a*	25.5 (1.5) *a*
	0.5 EU	39.06	50.5	64.0	1.50	78.3 (0.4) *c*	98.7 (0.4) *c*	106.8 (0.6) *b*	28.5 (1.0) *a*	26.6 (0.7) *a*
	2.5 EU	44.05	55.7	73.5	1.65	78.3 (0.3) *c*	98.5 (0.3) *c*	106.8 (0.5) *b*	28.5 (0.8) *a*	24.8 (2.1) *a*
	10 EU	49.22	61.0	84.2	1.34	75.5 (0.4) *a*	98.0 (0.1) *bc*	107.1 (0.3) *b*	31.7 (0.7) *b*	24.7 (0.4) *a*
	20 EU	51.75	63.7	90.0	1.26	75.0 (0.4) *a*	97.7 (0.2) *b*	107.6 (0.2) *b*	32.6 (0.6) *b*	23.8 (1.3) *a*
Ratio glc-1-P/primer	25	76.41	29.8	76.4	1.26	72.3 (0.2) *a*	92.4 (0.1) *a*	102.0 (0.2) *a*	29.6 (0.4) *b*	22.0 (0.2) *a*
	50	76.86	49.9	75.8	1.65	79.6 (0.2) *b*	97.3 (0.1) *b*	105.6 (0.3) *b*	26.0 (0.5) *a*	24.4 (0.7) *a*
	100	47.88	59.7	84.9	1.10	79.2 (0.5) *b*	99.3 (0.3) *c*	108.3 (0.4) *c*	29.1 (0.9) *b*	25.7 (0.7) *a*
	200	26.83	65.7	85.1	1.49	80.3 (1.2) *b*	99.5 (0.3) *c*	108.6 (0.6) *c*	28.2 (1.8) *ab*	24.7 (2.8) *a*
	400	17.16	81.2	91.8	1.67	80.9 (0.5) *b*	100.5 (0.2) *d*	109.0 (0.2) *d*	28.2 (0.7) *b*	23.6 (0.4) *a*
Lipid type and chain length	FA 12	59.63	71.8	83.5	1.43	79.9 (0.7) *a*	93.9 (0.3) *a*	101.0 (0.9) *a*	21.1 (1.6) *a*	19.8 (2.3) *a*
	FA 14	48.53	60.3	72.9	1.13	79.8 (0.3) *a*	95.5 (0.3) *b*	103.9 (0.4) *b*	24.1 (0.7) *b*	21.2 (0.2) *ab*
	FA 16	50.63	62.5	74.4	1.56	78.9 (0.5) *a*	97.7 (0.2) *c*	107.0 (0.6) *c*	28.1 (1.1) *c*	24.8 (0.5) *c*
	FA 18	69.91	82.2	106.8	1.49	79.0 (0.3) *a*	99.5 (0.5) *d*	109.8 (0.6) *d*	30.8 (0.9) *d*	24.1 (1.6) *bc*
	MAG 12	71.73	84.4	92.3	1.22	80.5 (0.2) *b*	94.3 (0.2) *a*	102.1 (0.3) *a*	21.6 (0.5) *a*	20.8 (0.5) *a*
	MAG 14	55.70	67.8	72.2	1.43	80.0 (0.4) *a*	95.6 (0.2) *b*	104.1 (0.1) *b*	24.1 (0.4) *b*	21.3 (1.4) *ab*
	MAG 16	60.85	73.1	82.5	1.34	81.4 (0.3) *c*	99.0 (0.2) *c*	107.6 (0.1) *c*	26.2 (0.4) *c*	24.2 (0.8) *c*
	MAG 18	73.04	85.7	113.2	1.73	79.2 (0.3) *a*	96.0 (0.2) *b*	109.4 (0.3) *d*	30.2 (0.6) *d*	23.7 (1.3) *bc*
Lipid unsaturation	18:0	66.67	79.1	111.8	1.42	78.8 (1.0) *d*	98.5 (0.6) *d*	109.7 (0.4) *a*	30.9 (1.4) *a*	25.5 (4.4) *a*
	18:1	51.19	63.1	79.0	1.26	72.9 (0.6) *c*	95.0 (0.8) *c*	105.5 (0.8) *b*	32.5 (1.4) *b*	23.5 (0.3) *a*
	18:2	71.65	84.3	107.3	1.14	63.7 (0.0) *b*	90.4 (0.2) *b*	101.0 (0.2) *c*	37.3 (0.2) *c*	24.1 (0.3) *a*
	18:3	76.21	89.0	117.0	1.72	59.5 (0.1) *a*	87.2 (0.3) *a*	98.4 (0.2) *d*	38.9 (0.3) *d*	22.9 (0.5) *a*

a, *ab*, *b*, *bc*, *c*, *d*: Tukey groups for the dissociation characteristics with P-value < 0.05.

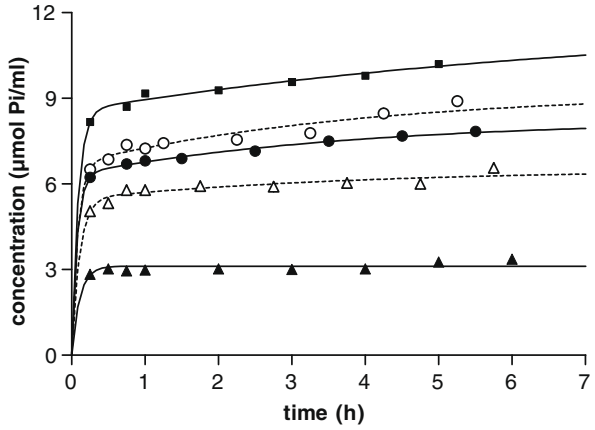

Fig. 2. Progression of the semi-enzymic synthesis reaction using different molar ratios of glc-1-P to primer: glc-1-P/primer = 25 (▲), glc-1-P/primer = 50 (△, dotted line), glc-1-P/primer = 100 (●), glc-1-P/primer = 200 (○, dotted line) and glc-1-P/primer = 400 (■).

3.4. Lipid unsaturation

Fig. 4 demonstrates that the glc-1-P conversion was related to the number of unsaturated bonds present in lipids with the same lipid chain length (18 carbon atoms). The reaction was performed at pH 6.0 and 45 °C with an enzyme dosage of 10 EU/100 ml and a molar ratio glc-1-P/primer of 100. In the first 15 min, the initial glc-1-P conversion rate was not affected by the number of double bonds. For all lipids, the maximum concentration of liberated P_i was obtained after 60 min and increased with increasing number of double bonds [except for oleic acid (C18:1)]. Similar trends were observed for the conversion values and the accompanying DP_{calc} (Table 1). Again, SEC data confirmed longer amylose chain lengths (higher DP_{peak}) for complexes with these polyunsaturated lipids. The synthesized complexes had low polydispersity. The higher the level of unsaturation of the lipid being complexed by the synthesized amylose, the lower the dissociation temperature of the resulting complex was, and the broader its dissociation temperature ranges. Significant differences were obtained only with respect to their dissociation temperatures. However, little if any differences were found for the dissociation enthalpies of the complexes.

4. Discussion

In classical complexation methods, both amylose and lipid interact at high temperatures. Precipitation happens during cooling of the solution. Several studies have shown that the amylose chain length necessary for amylose–lipid complex formation and precipitation is one which accommodates at least four lipid molecules in the helix (Gelders et al., 2005; Godet, Bouchet, Colonna, Gallant, & Buléon, 1996).

We here discuss our insights on how the semi-enzymic amylose–lipid complex synthesis reaction proceeds, particularly to what extent phosphorylase in a productive enzyme-glc-1-P-primer complex limits the lipid complexation capacity of the growing amylose chain and to what extent the presence of the lipid influences the polymerization process catalysed by the phosphorylase enzyme. To this end, we distinguish three subprocesses in the semi-enzymic amylose–lipid complex synthesis, *i.e.* (i) enzymic synthesis of the amylose chain, (ii) complexation with the lipid, and (iii) precipitation of the amylose–lipid complex. The latter will most probably inhibit further enzyme activity on the prolonged primer.

4.1. Enzyme and substrate concentration

Our results show that varying concentrations of enzyme and glc-1-P predominantly (or even solely) influence the enzymic syn-

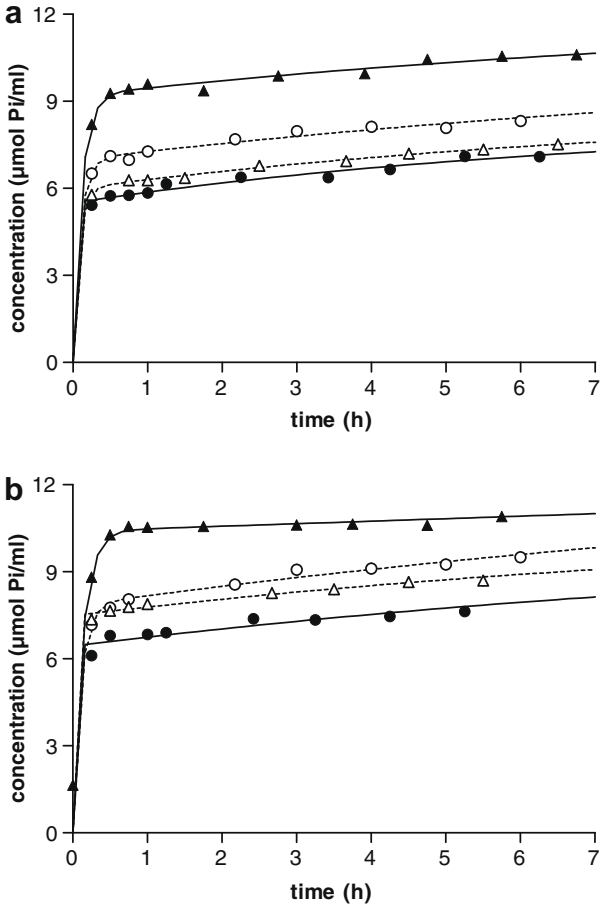

Fig. 3. (a) Progression of the semi-enzymic synthesis reaction using fatty acids with different chain lengths: stearic acid (▲), palmitic acid (△, dotted line), myristic acid (●), lauric acid (○, dotted line). (b) Progression of the semi-enzymic synthesis reaction using monoacyl glycerols with different chain lengths: glyceryl monostearate (▲), glyceryl monopalmitate (△, dotted line), glyceryl monomyristate (●), glyceryl monolaurate (○, dotted line).

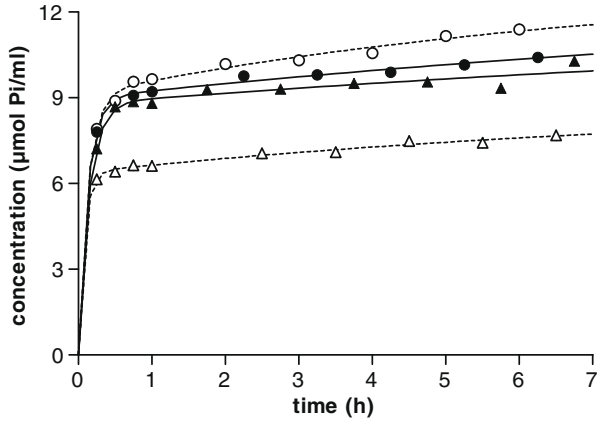

Fig. 4. Progression of the semi-enzymic synthesis reaction during the first 7 h using fatty acids with different degree of unsaturation: stearic acid (▲), oleic acid (△, dotted line), linoleic acid (●), linolenic acid (○, dotted line).

thesis step in the semi-enzymic amylose–lipid complex formation process. Potato phosphorylase acts in a 'random bi bi' reaction mechanism (Gold, Johnson, & Sanchez, 1971). In such reaction, two (bi) substrates are needed/used (*i.e.* primer and glc-1-P) and two (bi) products are formed (*i.e.* elongated primer and P_i). As a result of this reaction mechanism, it matters neither which substrate binds first to the enzyme nor which of the two products is released first. The transfer reaction resulting in chain elongation occurs between the ternary complexes (Leskovac, 2003). When varying only one parameter at a time, however, the reaction mechanism can be described using an apparent Michaelis–Menten kinetic model (Cornish-Bowden, 2004). In the present case, this held true when varying enzyme dosages or ratios glc-1-P/primer. When either glc-1-P or enzyme concentrations were plotted against the initial reaction rates derived from the linear relation of P_i concentrations in function of time at the start of the reaction, data could be described by an apparent Michaelis–Menten curve (R^2 values of 0.984 and 0.985 for enzyme and glc-1-P concentrations, respectively). In line with Michaelis–Menten kinetics, with increasing enzyme or substrate concentration x, the reaction initially progresses faster, according to:

$$V_{app} = V_{max} * x/(K_m + x)$$

with V_{app} the apparent initial reaction rate, V_{max} the maximal reaction rate and K_m the apparent Michaelis constant.

The products of the reaction, in particular the short amylose chains, are formed faster with increasing concentrations of either enzyme or glc-1-P. Amylose chains with the minimum chain length necessary to complex and precipitate with the ligand will thus be obtained sooner, enabling the final steps of the complex formation and/or precipitation to proceed more rapidly as well. Our data can thus be explained on the basis of the changed enzyme/substrate ratio in the reaction mixture. The variation in the final conversion values with varying enzyme dosages can then be ascribed to differences in kinetic constants of the subprocesses involved: at the given reaction conditions, the enzymic synthesis, on the one hand, and the complexation and/or precipitation, on the other, presumably all progress with a different rate which itself depends on the amount of enzyme or glc-1-P added.

Increasing enzyme concentrations resulted in the final conversion value being reached sooner due to the higher initial reaction rate. In the reactions with 0.1, 0.5 or 2.5 EU/100 ml, the enzyme dosage was the limiting factor in the reaction. These reaction conditions resulted in slow synthesis of amylose. This implies that complexation occurred as soon as a certain chain length is obtained, resulting in a homogeneous population of complexes as reflected in the small range of dissociation temperatures. In the presence of excess enzyme (10 or 20 EU per 100 ml), polymerization was fast, as could have been expected for this type of bimolecular reaction (Whelan & Bailey, 1954). In our view, fast enzymic synthesis can lead to amylose chains longer than strictly necessary for complexation with lipids. As a result, a heterogeneous amylose–lipid complex population (higher values of T_c–T_o, Table 1) is formed.

With increasing molar ratio of glc-1-P/primer, the average amylose chain length (DP_{peak}, Table 1) and T_p of the complexes increased. Since debranched glycogen chains consist on average of ca. 10–12 glucose units, theoretical DP values (in the absence of lipids) of about 35 and 60 were expected for molar substrate/primer ratios of 25 or 50, respectively. However, the DP_{peak} of complexes obtained with such ratio of 25 was much higher than the theoretical value and (almost) equal to that obtained with molar ratio 50. This discrepancy between the theoretically calculated and experimentally obtained chain lengths in amylose–lipid complexes can possibly be attributed to the impact of the lipid on the enzyme catalysed amylose synthesis. When the same experiment was performed without lipids (data not shown), amylose chain lengths of 52, 80 and 285 were obtained for syntheses with a molar ratio of 25, 50 or 200, respectively. This suggests that lipids reduce the chain length in case of high relative glc-1-P concentration through complex formation and precipitation. The latter inhibits

further elongation of the amylose chain. When the substrate is present in low concentrations, another driving force seems to be in play, resulting in higher chain lengths than were theoretically expected or obtained in the absence of ligand.

Furthermore, the use of debranched glycogen can also influence these chain lengths. As opposed to maltohexaose used in the method described by Gelders et al. (2005), our primer is an inhomogeneous mixture of short chains, so calculation based on liberated phosphate most probably leads to less accurate results (Whelan & Bailey, 1954).

In literature, the action pattern of the potato phosphorylase enzyme in the synthesis of amylose has been described as a multichain action. This conclusion was based on iodine staining throughout the polymerization reaction (Whelan & Bailey, 1954). From maltotetraose onwards, oligosaccharides are about equally reactive as primers in the reaction (Suganuma, Kitazono, Yoshinaga, Fujimoto, & Nagahama, 1991). This indicates that the different oligosaccharides present in the debranched glycogen mixture would be elongated to the same extent. Knowing that all synthesis reactions started from the same primer population, this would mean that polymerization in the presence of a lower relative amount of glc-1-P would result in much shorter amylose chains produced. Our results, particularly those with glc-1-P/primer ratios of 25 and 50, however, apparently do not point to such multichain mechanism. The chain lengths of complexes formed at low ratios of glc-1-P/primer led us to hypothesize that, instead of elongating all primers at once, it is possible that, for limiting substrate conditions (and in the presence of lipids), primers are elongated one at a time (single chain mechanism). This way, a primer is extended until the chain is long enough to complex the necessary lipids to precipitate, before another primer is attacked by the enzyme.

4.2. Lipid characteristics

The characteristics of the added lipid (lipid chain length and/or unsaturation) greatly impact the complexation and precipitation subprocesses in the synthesis of the complexes. Indeed, under otherwise standardised conditions, the observed differences in conversion values result from the different properties of the emulsifiers. In general (in the classical method), the minimum length of the amylose chain, needed to form a complex and precipitate, is determined by the length of the fatty acid chain. Karkalas and Raphaelides (1986) proposed a model for calculating the minimal amylose chain length necessary to complex a fatty acid or monoacyl glycerol with a given lipid chain length. They showed that, in complex formation and precipitation, a longer lipid needs a longer amylose chain to form a complex. For the semi-enzymic synthesis, this would imply that the complexation/precipitation step of the reaction only takes place after the enzymic amylose synthesis [subprocess (i)] has sufficiently progressed and, hence, after adequate levels of glucose, from glc-1-P, have been incorporated.

In the present case, dissociation temperatures and their ranges increased with lipid chain length. The longer the lipid chain, the longer the amylose chain needs to be to complex the lipid and the stronger the interactions between the lipid and the amylose in the complex (Whittam et al., 1989). This is in agreement with the findings for amylose–lipid complexes synthesized with the classical method (Biliaderis & Galloway, 1989; Eliasson & Krog, 1985; Gelders et al., 2004, 1995a; Karkalas et al., 1995; Tufvesson, Wahlgren, & Eliasson, 2003a,b; Whittam et al., 1989).

Also, longer lipid chain lengths are more hydrophobic and less soluble in water (Tufvesson et al., 2003b). Hence, they have a stronger preference for residing within the hydrophobic cavity of the amylose single helix instead of remaining in the surrounding hydrophilic aqueous environment (Fanta, Shogren, & Salch, 1999). However, lauric acid (C12:0) consistently yielded higher values than myristic (C14:0) or palmitic (C16:0) acid for reaction progression and DP. Although it needs a longer amylose polymer, the binding of the lipid is weaker than in the case of C14:0 and C16:0. Similar results have been reported by other authors (Godet et al., 1995a,b; Lebail, Buléon, Shiftan, & Marchessault, 2000) in their study of varying lipid properties in the classical synthesis method.

Furthermore, also in agreement with earlier findings based on the classical synthesis methods, our results show that monoacyl lipid complexes have higher DP_{peak} values than those of their respective fatty acids. A longer amylose linker is required to bypass the more voluminous head of the monoacyl lipid to associate with the next lipid molecule, resulting in amylose molecules of longer chain length, *i.e.* higher DP_{peak} in the amylose–lipid complex. Irrespective of the type of polar head, the interaction between the amylose and the lipid chains seem similar for both fatty acids and monoacyl lipids, as reflected in their respective T_ps.

Our results for complexes with unsaturated lipids are also in accordance with findings for such complexes synthesized by the classical method (Eliasson & Krog, 1985; Karkalas et al., 1995; Ozcan & Jackson, 2002; Raphaelides & Karkalas, 1988; Tufvesson et al., 2003b). According to Karkalas et al. (1995), each additional double bond leads to a (further) decrease in dissociation temperature. These authors suggested that the non-linear structure of a lipid with *cis* double bonds makes insertion in the linear helix cavity more difficult. However, the carbon atoms adjacent to the double bond might be able to rotate freely, leading to a rather linear structure. This implies that complexation could still occur, but that a wider helix cavity would be required, consisting of more than the usual six glucose units per turn. The results for the complexes with linoleic (C18:2) and linolenic (C18:3) acids are in agreement with this view. For oleic acid (C18:1), on the other hand, a shorter amylose chain was obtained (lower DP_{calc} and DP_{peak}) during the synthesis. In this context, Yamada, Kato, Tamaki, Teranishi, and Hisamatsu (1998) presumed that unsaturated lipids are only partially included in the helix.

Our results hence indicate that tailor made amylose–lipid complexes can be produced. By changing the reaction variables, complexes from a hetero- or homogeneous population with a defined chain length and with desired dissociation temperature can be obtained.

4.3. Interaction between growing amylose chain and lipid

As indicated above, in the semi-enzymic synthesis method, it is unclear how and to what extent the lipid interacts with the very short amylose chains, when they are being enzymically elongated.

In general, several interactions between the growing amylose chains and the lipids can be envisaged. First, enzymic synthesis and complexation/precipitation may occur rather independently, thus resembling the classical synthesis (Fig. 5A). Complexation and precipitation would only take place after a longer amylose chain has been synthesized. Our results for synthesis in the presence of excess enzyme or substrate and for the impact of lipid properties on the characteristics of the synthesized short chain amylose–lipid complexes show, indeed, good resemblance to findings reported with the classical synthesis. This would imply that little if any interaction occurs between the lipid and the very short amylose chains. Unfortunately, literature relating to the classical synthesis method with different amylose populations does not discuss this aspect of amylose–lipid complexation and/or precipitation.

However, the influence of either the presence of the lipid on polymerization or the presence of the enzyme on complexation/precipitation suggests a more sophisticated interaction between the different subprocesses of the semi-enzymic synthesis. In this

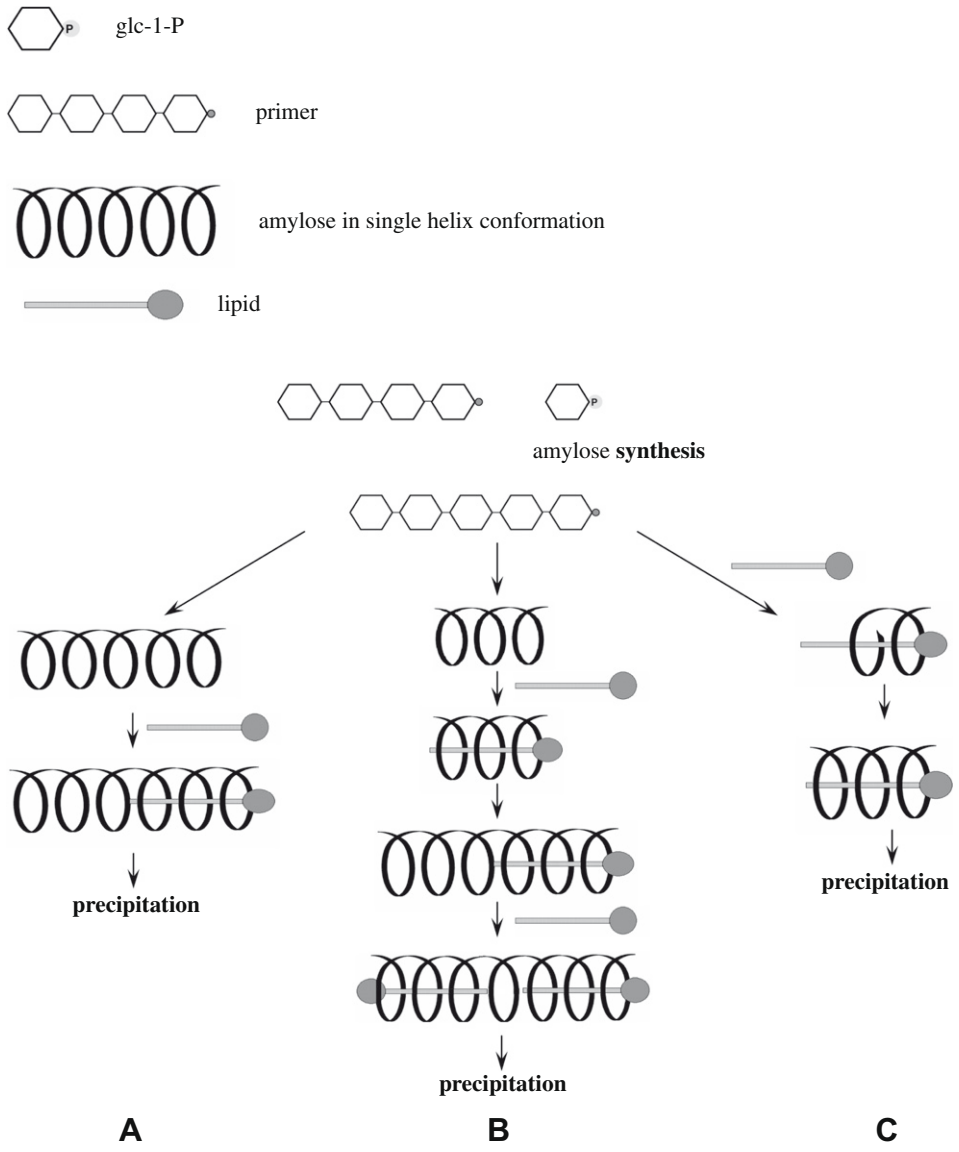

Fig. 5. Possible mechanisms of the semi-enzymic synthesis of amylose–lipid complexes. (A) Complexation and precipitation only when amylose chain is long enough; (B) complexation after a small amylose chain is formed, further elongation of the amylose and subsequent complexation, precipitation occurs when a sufficient number of lipids are complexed; (C) 'vine-twining': amylose synthesis around lipid, with precipitation when a sufficient number of lipid molecules are complexed. Our data point to mechanism B.

respect, it is also possible that amylose is synthesized around the lipid present, resulting in immediate complexation, while precipitation only occurs when a sufficient number of lipids are incorporated in the amylose helix (as presented in Fig. 5C). Such so-called vine-twining complexation method has already been described for complexation between amylose and polyesters (Kadokawa, Nakaya, Kaneko, & Tagaya, 2003). However, amylose–lipid complexes consist of multiple lipids complexed in one amylose chain (Gelders et al., 2005), as opposed to the multiple amylose chains necessary to complex one polymeric ligand as described by Kadokawa and coworkers (2003). It is hence difficult to envisage how the growing amylose chain would continue its vine-twining reaction with subsequent lipids after completely surrounding a first lipid molecule.

We believe an intermediate between these two to be the way in which amylose–lipid complexes are synthesized semi-enzymically (Fig. 5B): elongation of the primer occurs until an amylose chain is obtained that is sufficiently long to complex at least one lipid molecule. This complex then stays in solution and the enzymic chain extension of the amylose chain continues, together with the subsequent complexation, until the formed amylose–lipid complex becomes insoluble and precipitates. Indeed, amylose–lipid complexes only precipitate when a sufficient number of lipids [e.g. four lipid molecules, as described by Gelders and coworkers (2005)] have been included in the helix and the complex as a whole becomes insoluble. This model, however, is hypothetical, and more research is still needed to confirm its validity.

5. Conclusions

Enzyme dosage, molar ratio glc-1-P/primer and lipid characteristics influence both the progress of the reaction [the extent and (initial) rate of conversion of the substrate] and the characteristics of the obtained amylose–lipid complexes (dissociation temperature and range, DP). Enzyme dosages and substrate concentrations presumably influence the properties of amylose–lipid complexes

in a more or less indirect way. They mainly affect the kinetic properties of the enzymic synthesis reaction of amylose. The obtained differences in the properties of the complexes could be attributed to a shift in the balance of the rate of the enzymic amylose polymerization on the one hand and the rate of amylose–lipid complex formation and precipitation on the other hand. Lipid properties, however, mainly impact the complexation and precipitation steps in the enzymic formation of amylose–lipid complexes by determining the minimal amylose length necessary to accommodate the lipids and for precipitation of the resulting amylose–lipid complex. These results enabled us to speculate about the order in which different subprocesses occur during the semi-enzymic synthesis of amylose–lipid complexes. In this hypothetical mechanism, the phosphorylase elongates the primer, producing an amylose chain with a minimal chain length necessary to complex a first lipid molecule. After this initial complexation, enzymic chain extension continues, with subsequent complexation. This way, the amylose–lipid complex grows until it becomes insoluble and precipitates. We believe the synthesis to be an intermediate between the classical synthesis of amylose–lipid complexes on the one hand, and the vine-twining method on the other.

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