Starch synthesis in potato tubers

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CHAPTER 3:

SOLUBLE POTEINS RELATED TO STARCH SYNTHESIS IN POTATO TUBERS (*SOLANUM TUBEROSUM* L.).

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ABSTRACT.

Potato tubers were shown to contain primed and unprimed soluble starch synthase activity, which co-eluted in a single peak from DEAE-cellulose, aminobutyl-Agarose, Superose-12 (FPLC) and cyclohexa-amylose-Sepharose, suggesting that potato tubers contain only one soluble starch synthase. However, small differences in the elution profile of primed and unprimed soluble starch synthase activity, and two protein bands coinciding with enzyme activity after the final purification step point to the existence of two isozymes of soluble starch synthase activity. The native molecular weights of both proteins were approximately 220 kDa, the denatured molecular weights 78 and 85 kDa.

Neutralization tests with antiserum raised against SSS-I from maize (MacDonald and Preiss 1985) also suggested the existence of two soluble isozymes. The antibodies neutralized unprimed and primed soluble starch synthase activity catalyzed by SSS-I from maize but failed to inhibit primed soluble starch synthase activity catalyzed by SSS-II from maize. In potato extracts a complete inhibition of unprimed and a 30% inhibition of primed soluble starch synthase activity was observed, which can be explained by the existence of multiple soluble isozymes in potato.

Furthermore, a comparison was made between the soluble and granule-bound starch synthases from potato. Since (*i*) the denatured molecular weights of the starch synthases clearly differed (60 versus 78 and 85 kDa), and (*ii*) antibodies raised against the denatured granule-bound starch synthase

were not able to neutralize soluble starch synthase activity we conclude that soluble and granule-bound starch synthase activities are catalyzed by different proteins.

INTRODUCTION.

The synthesis of the α-1,4 glucan linkages which are present in both amylose and amylopectin molecules is catalyzed by starch synthases (EC 2.4.1.21). A substantial portion of the starch synthase activity in non-photosynthetic plant cells is tightly associated with the starch granules (Leloir et al. 1961, Perdon et al. 1975, Shure et al. 1983, Konishi et al. 1985, MacDonald and Preiss 1985, Villareal and Juliano 1986, Vos-Scheperkeuter et al. 1986, Imam 1989). This granule-bound starch synthase utilizes both UDP-Glc and ADP-Glc as its *in vitro* substrate (Leloir et al. 1961). In potato tubers the major 60 kDa protein present in protein extracts prepared from potato starch granules, was identified as the granule-bound starch synthase (Vos-Scheperkeuter et al. 1986).

The remainder of starch synthase activity in non-photosynthetic plant cells is soluble and exclusively utilizes ADP-Glc as the substrate (Frydman and Cardini 1964, Frydman et al. 1966, Hawker et al. 1972). The soluble starch synthase has never been purified to homogeneity from any plant species, and the molecular weight of the denatured enzyme is consequently unknown.

In maize endosperm, both the granule-bound and the soluble starch synthases appear to consist of two types. In order of their elution from DEAE-cellulose they are called type I and type II starch synthases (MacDonald and Preiss 1985). The type I isozymes show activity without the addition of primer molecules, provided that sodium citrate is present (Boyer and Preiss 1979, MacDonald and Preiss 1985). The type II isozymes do not show "unprimed" activity. Both types show primed soluble starch synthase activity although the relative priming efficiency of various primers differs (MacDonald and Preiss 1985).

Ever since the demonstration of granule-bound and soluble starch synthase activities there has been speculation about their relationship: do these proteins differ at the amino acid level, or do they differ only in the extent of post-translational modification and/or in their intracellular localization? The partitioning of granule-bound and soluble starch synthase activity in potato, as a function of the extraction medium, (Hawker et al. 1979) favors the idea that at least in potato, granule-bound and soluble starch synthases are different states of the same protein. However, in maize differences are found in native

molecular weights and immunological reactions (MacDonald and Preiss 1985) indicating that soluble and granule-bound starch synthases are different proteins.

The aim of the present work was to gain more insight in the enzymes catalyzing starch synthase activity in potato tuber. Our results favor the notion that, as in maize, granule-bound and soluble starch synthases are different proteins, and that possibly multiple soluble proteins are present.

MATERIALS AND METHODS.

Materials.

All chemicals were of analytical grade.

Preparation of a crude potato extract.

Potato tubers cv. Producent, Promesse and Hooglandster were bought locally and stored at 4 to 10°C before use. All further operations were carried out at 4°C. Potatoes (500 g) were peeled, washed with cold water and homogenized in a Waring Blender (4-6 periods for 15 seconds, separated by one min intervals), in 250 mL 100 mM Tris-HCl pH 7.5, containing 10 mM EDTA, 2.5 mM DTT and 0.1 % (w/v) sodium dithionite. The homogenate was filtered through synthetic cloth and starch granules were collected by centrifugation (10 min, 10.000g) and further purified as described by Vos-Scheperkeuter et al. (1986). Ammonium sulphate was added to the supernatant to give a final concentration of 40 % saturation. Insoluble proteins were collected by centrifugation (30 min, 20.000g) and solubilized in 50 mM Tris-HCl pH 7.5 containing 5 mM EDTA and 2.5 mM DTT (TED buffer) and dialyzed against the same solution for 16 h.

DEAE-cellulose fractionation.

The crude extracts from Producent, Promesse and Hooglandster potatoes were applied to a 50 mL column of DEAE-cellulose (Whatmann) equilibrated in TED buffer. The column was washed with TED buffer (300 mL) and eluted with a linear gradient of 0 to 0.5 M NaCl in buffer (300 mL). Fractions of 8 mL each were collected and assayed for citrate stimulated and glycogen primed starch synthase activity. Fractions showing enzyme activity (fractions 36 to 48 in Figure 1) were pooled and concentrated by ammonium sulphate precipitation (0 to 50 % saturation). This preparation is further referred to as the DEAE/SSS pool.

Aminobutyl-Agarose chromatography.

The DEAE/SSS pool obtained from Producent potatoes (Figure 1) was dissolved in TED buffer containing 10 % (v/v) ethylene glycol and dialyzed against the same buffer for 16 h. The protein solution (624 mg in 55 mL) was divided into 7 portions which were applied to a 1.5 x 16.5 cm column of aminobutyl-Agarose (P.-L. Biochemicals Inc. Milwaukee, USA), equilibrated in the former buffer. Several runs were performed to avoid overloading. Fractions of 5 mL were collected at a flow rate of 6 mL/hour and assayed for primed and unprimed soluble starch synthase activity. Soluble starch synthase activity was loosely bound to the column. The addition of 1 M maltose to the buffer overcame the weak binding (Figure 2A and Figure 5 lanes D and E).

Ten µL of every second fraction was used to assay for primed and unprimed soluble starch synthase activity. Active fractions (fractions 13 to 33 in Figure 2A) were pooled (ABA/SSS pool), concentrated by ammonium sulphate precipitation (0 to 50 % saturation) and dialyzed against either TED containing 100 mM NaCl or 25 mM Bis-Tris pH 7.1, containing 2.5 mM DTT.

FPLC Superose-12 chromatography.

Gelfiltration chromatography was performed with a FPLC Superose-12 column (Pharmacia, Sweden) equilibrated in TED buffer (10 mM EDTA) containing 100 mM NaCl. The column was calibrated with 200 µL of each of the following solutions: 1.3 mg/mL β-amylase (206 kDa), 2 mg/mL alcohol dehydrogenase (150 kDa) and 4 mg/mL BSA (68 kDa). Fractions (0.5 mL) were collected at a flow rate of 0.4 mL per min.

Part of the ABA/SSS pool (200 µL containing 2 mg protein) was dialyzed against the above buffer, and applied to the FPLC gelfiltration column. Several runs were performed and fractions were monitored for primed and unprimed soluble starch synthase activity. The addition of salt to the above buffer (up to 500 mM NaCl) did not change the elution pattern. The fractions showing soluble starch synthase activity fractions 12 to 15 in Figure 3) were pooled (Sup-12/SSS pool).

Cyclohexa-amylose-Sepharose affinity chromatography.

Cyclohexa-amylose (= α-cyclodextrin)-Sepharose was prepared according to Vretblad et al. (1975). The column (1.5 mL) was equilibrated in 50 mM Tris-HCl pH 7.5, containing 10 mM EDTA, 0.5 M sodium citrate and 2.5 mM DTT.

Samples (either part of the DEAE/SSS pool from Producent potatoes, containing approximately 50 mg protein, or the Sup-12/SSS pool containing

approximately 4 mg protein) were pre-equilibrated in the same buffer by slow addition of half the volume of 1.5 M sodium citrate in the above buffer over a period of 30 min. Precipitated material was collected by centrifugation (20 min, 30.000g) and the supernatant fraction was applied to the column with a maximal flow rate of 5 mL per h. The column was washed with 10 to 15 mL of the above buffer, and proteins were eluted with a decreasing gradient (30 mL) of 0.5 to 0.0 M sodium citrate in buffer. Fractions (1 mL each) were collected and monitored for enzyme activity. Part of all the fractions was dialyzed against water before gelelectrophoresis was performed.

Mono Q ion-exchange chromatography.

Part of the DEAE/SSS pool obtained from Promesse potatoes (12.8 mg protein in 2 mL 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and 1 mM DTT) was applied to a FPLC Mono Q column (Pharmacia, Sweden). The ion- exchange column was washed with 9 mL of the above buffer. Fractions (0.5 mL) were eluted with a 0.1 to 0.4 M NaCl gradient (16 mL) in buffer. Twenty-five µL of each fraction was used to measure the amount of primed soluble starch synthase activity per fraction.

Chromatofocusing.

Mono P (Pharmacia, Sweden) chromatography was performed with a small amount (2 mg, in 0.6 mL) of the ABA/SSS pool, dialyzed against 25 mM Bis-Tris pH 7.1 containing 2.5 mM DTT. Proteins were eluted with Polybuffer 74 (Pharmacia, Sweden) pH 4, containing 2.5 mM DTT. Fractions (1 mL) were collected and screened for protein content. Since soluble starch synthase activity was labile in the Polybuffer used to elute the proteins (data not shown), 1 mL of a saturated ammonium sulphate solution was added to each fraction immediately following collection. The insoluble proteins were collected by centrifugation (40 min, Eppendorf centrifuge) and solubilized in 125 µL TED buffer containing 10 rather than 5 mM EDTA. Twenty-five µL of each fraction was assayed for primed soluble starch synthase activity.

Isolation of the soluble starch synthases from maize.

Both soluble starch synthases from maize were isolated from *waxy* maize kernels and partly purified by ammnonium sulphate precipitation and DEAE-cellulose chromatography as described by Pollock and Preiss (1980).

SDS-gelelectrophoresis and immunoblotting.

Protein samples were boiled in denaturation buffer (5 min) and analyzed on 8 or 10 % SDS-polyacrylamide gels (Laemmli 1970). The gels were either stained (Wray et al. 1981) or screened for immunological cross-reactions by

Western blot analysis (Hovenkamp-Hermelink et al. 1987), with antiserum raised against the potato granule-bound starch synthase (Vos-Scheperkeuter et al. 1986).

Gelelectrophoresis under non-denaturing conditions followed by activity staining.

Analysis under non-denaturing conditions was performed essentially like SDS-gelelectrophoresis, except that SDS was replaced by 1 mM EDTA and 1 mM β-mercapto-ethanol in all buffers. Furthermore, glycogen was added to the resolving gel to give a final concentration of 0.08 % (w/v). Gels were prerun for 30 min at 30 mA and 4°C, loaded with protein (10 to 20 µL containing 5 to 10 mU primed soluble starch synthase activity) and run at 20 mA and 4°C. Once gel-electrophoresis was completed relevant lanes were excised from the gel and incubated separately in 2 mL assay mixture (as below, except that glycogen and radio-active ADP-Glc were omitted) at 30°C for 16 to 20 h. The gels were soaked afterwards in 7 % (v/v) acetic acid and stained for carbohydrate with iodine (Schiefer et al. 1973).

Neutralization and immunoadsorption experiments.

IgG was isolated from both the preimmune serum and the anti-GBSS serum as described by Vos-Scheperkeuter et al. (1989). The procedure yielded 1.1 mg IgG per mL preimmune and 6.4 mg IgG per mL antiserum. The preimmune- and antiserum against SSS-I from maize were obtained from Dr. Jack Preiss and were previously specified (MacDonald and Preiss 1985).

For neutralization experiments with anti-SSS-I serum, the DEAE-cellulose fraction containing the peak amount of maize SSS-I activity was used (per incubation approximately 0.25 mU primed soluble starch synthase activity). For the potato, fractions 14 (Figure 3) and 33 (Figure 4) were used (containing 0.15 mU primed soluble starch synthase activity). The protein samples were incubated with an increasing amount of antiserum IgG (and a decreasing amount of preimmune IgG so that 25 µL was added in each case) for 30 min at room temperature. Immediately following incubation duplicate samples (10 µL) were assayed for soluble starch synthase activity. The activities were expressed as a percentage of the original amount of soluble starch synthase activity.

For neutralization experiments with anti-GBSS serum 8 µL of a crude potato extract (containing 0.21 mU primed soluble starch synthase activity) was incubated with an increasing amount of antiserum IgG (diluted with preimmune IgG so that 12 µL was added in each case) for 30 min at room temperature. Duplicate samples (8 µL) were subjected to the assay for soluble

starch synthase activity.

Immunoadsorption experiments were performed with anti-GBSS serum as described previously (Vos-Scheperkeuter et al. 1989), followed by measurements of the residual soluble starch synthase activity in the supernatant. The activity found with antiserum was expressed as a percentage of the activity found with pre-immune coated SaCI cells.

Assay conditions.

Assays for unprimed and primed soluble starch synthase activity and granule-bound starch synthase activity were performed as described before (Ponstein et al. 1990).

Protein determinations.

The amount of protein was determined by absorption measurements at 280 nm or according to the method described by Lowry (1951) using BSA as the standard.

RESULTS.

Ratio of soluble to granule-bound starch synthase activity as a function of the homogenization medium.

Potato tubers were homogenized in different buffers to study the effect of sodium dithionite, DTT and β-mercapto-ethanol (β-ME) on the distribution of soluble and granule-bound starch synthase activity. DTT and β-ME were added to all buffers used throughout the isolation procedure. Sodium dithionite was present only during tissue homogenization and further purification of the starch granules, and was not added to the soluble fraction after ammonium sulphate precipitation (in these experiments 0 to 30 % saturation).

The activities of the soluble and the granule-bound starch synthases were determined and are summarized in Table I. The amount of granule-bound starch synthase activity is roughly constant but the amount of soluble starch synthase activity depends on the extraction medium used. The results indicate that soluble starch synthase activity is destroyed in the absence of sodium dithionite. DTT serves as a better sulfhydryl reagent than β-ME. The distribution of granule-bound and soluble starch synthase activity seemed to be independent on the homogenization buffer used.

Table I: Soluble and granule-bound starch synthase activities after homogenization of Promesse potato tubers in several buffers. Potato tubers (50 g) were homogenized in different buffers to study the effect of several buffer components on the distribution of soluble and granule-bound starch synthase activity. Starch granules (1.5 to 2 mg) were assayed for granule-bound starch synthase activity and the soluble protein samples were assayed for glycogen primed starch synthase (SSS-A) activity. Soluble proteins were precipitated from solution by the addition of solid ammonium sulphate to give a final concentration of 30 % saturation. All other operations were carried out as described for the preparation of a crude potato extract.

homogenization buffer	GBSS (μU/ mg starch)	[protein] (mg/mL)	SSS-A (mU/ mg protein)
$T_{50}E_{10}D_1$ pH 8.5	43.3	9.9	< 0.1
$T_{50}E_{10}D_1$ pH 8.5 + $Na_2S_2O_5$	61.8	5.3	4.2
$T_{50}E_{10}\beta$-ME_2 pH 8.5 + $Na_2S_2O_5$	58.1	2.3	2.8

Purification of the soluble starch synthase from potato tubers.

A crude protein extract from Producent potatoes was applied to a DEAE-cellulose column to separate the soluble starch synthases (Hawker et al. 1972). Soluble starch and branching enzyme activities were found to elute separately (Figure 1). The single peak of primed soluble starch synthase coincided with unprimed soluble starch synthase activity (Figure 1). Similar results were found for Promesse and Hooglandster potatoes (data not shown).

The pool of soluble starch synthase activity obtained by DEAE-cellulose chromatography was further purified by hydrophobic interaction chromatography over an aminobutyl-Agarose column (Shaltiel and Er-El 1973, Pollock and Preiss 1980). The potato protein was loosely bound to the column (Figure 2A), but a 10-fold increase in specific activity was achieved by the employment of a low flow of buffer. The primed and unprimed soluble starch synthase activities eluted slightly different over this column. Branching enzyme activity was bound and eluted in the presence of 1M maltose. The fractions containing soluble starch synthase activity (fractions 13 to 33 in Figure 2A) were pooled and further purified by gelfiltration over Superose-12

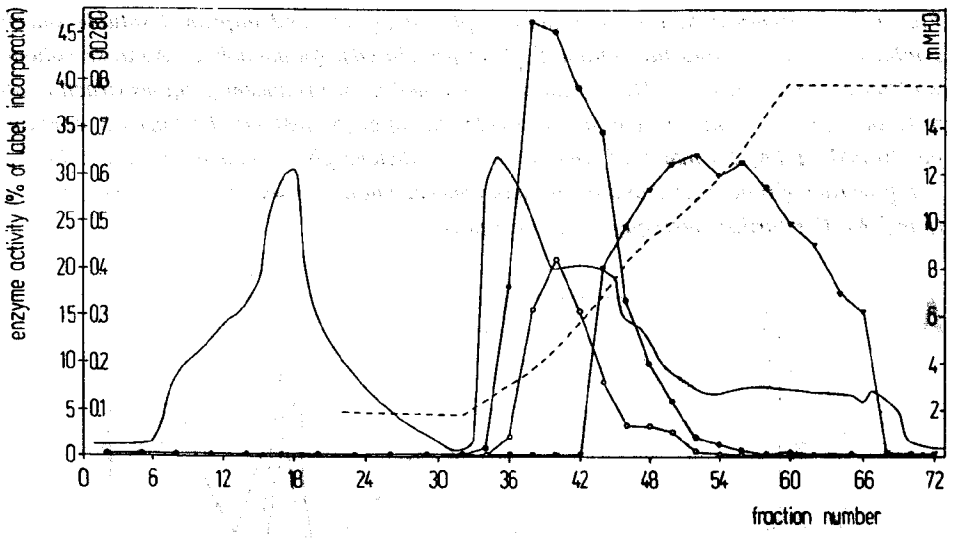


Figure 1: DEAE-cellulose chromatography of starch synthesizing enzymes from Producent potatoes. A crude potato extract was prepared and the 0 to 40 % ammonium sulphate precipitate (78 mgr) was dissolved in TED buffer, and loaded onto a DEAE-cellulose column. The column was washed with 300 mL TED buffer (50 mM Tris-HCl, pH 7.5, containing 5 mM EDTA and 2.5 mM DTT) and eluted with a linear salt gradient (0 to 0.5 M NaCl in buffer). The protein concentration (A_{280}, —) and the conductivity (---) of the fractions were determined and 1 µL of each fraction was used to assay for primed (●) and unprimed (o) soluble starch synthase activity. Ten µL of each fraction was used to measure branching enzyme activity (▾).

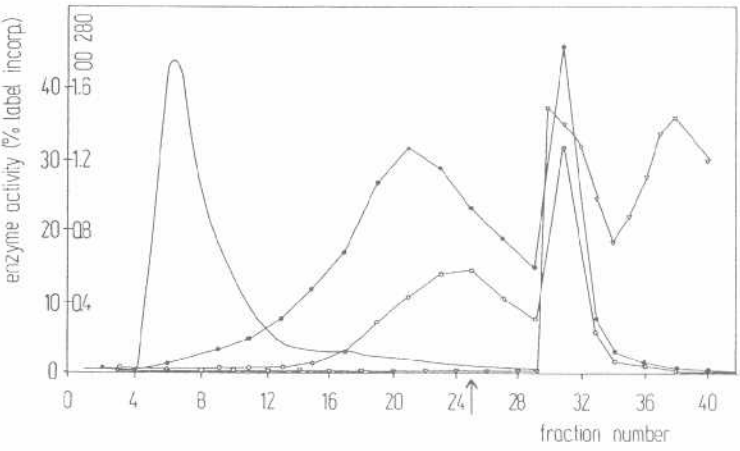
(FPLC). Both primed and unprimed soluble starch synthase activity eluted as single, a rather sharp peak (Figure 3) with an estimated molecular weight of 200 to 220 kDa.

After gelfiltration chromatography relevant fractions were analyzed by SDS-PAGE. No protein band was found coinciding with soluble starch synthase activity (Figure 3).

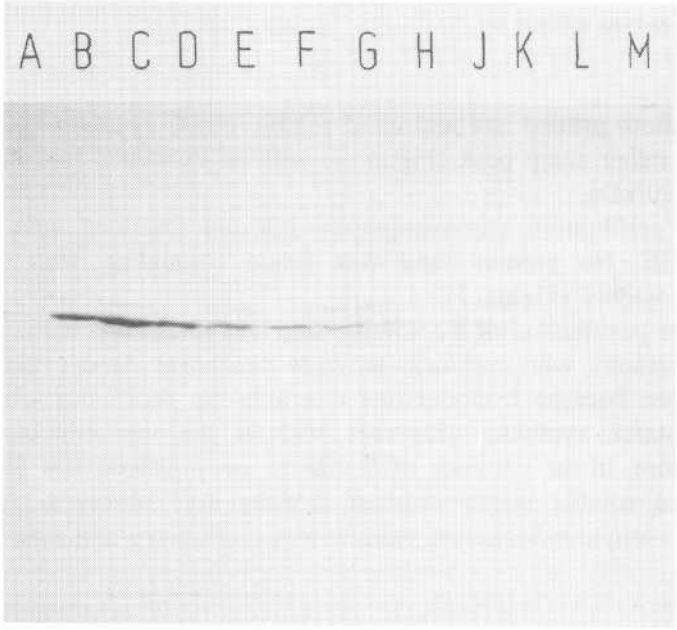
Further purification of the soluble starch synthase was achieved by affinity chromatography, with cyclohexa-amylose-Sepharose. An α-cyclodextrin matrix was chosen because cyclodextrins resemble the molecules which prime the soluble starch synthases (however without the non-reducing end group). Furthermore, in the presence of 5 mM α- or γ-cyclodextrin 50 % inhibition of primed soluble starch synthase activity was observed. Accordingly, a cyclohexa-amylose-Sepharose column was synthesized and tested as an affinity

Figure 2: Aminobutyl-Agarose chromatography of primed and unprimed soluble starch synthase activity (A) and the soluble 60 kDa protein (B). *An aminobutyl-Agarose column was loaded with part of the DEAE/SSS pool obtained from Producent potatoes (fraction 36 to 48 in Figure 1; 90 mg protein) and washed with buffer (50 mM Tris-HCl pH 7.5, containing 10 mM EDTA, 2.5 mM DTT and 10 % (v/v) ethylene glycol. Fractions were collected at a flow rate of 6 mL per h, and the protein concentration (—) was determined per fraction (panel A). For further description, see next page.*

A



B



Ten µL of every second fraction was assayed for primed (•) and unprimed (o) soluble starch synthase activity as well as branching enzyme activity (▽), as shown in panel A. The arrow indicates when 1 M maltose was added to the elution buffer.

An immunoblot of every second fraction (from fraction 2 to 24 shown in panel A in lanes A to M respectively) demonstrated the presence of a soluble 60 kDa protein cross-reacting with antiserum against the granule-bound starch synthase (panel B, opposite page).

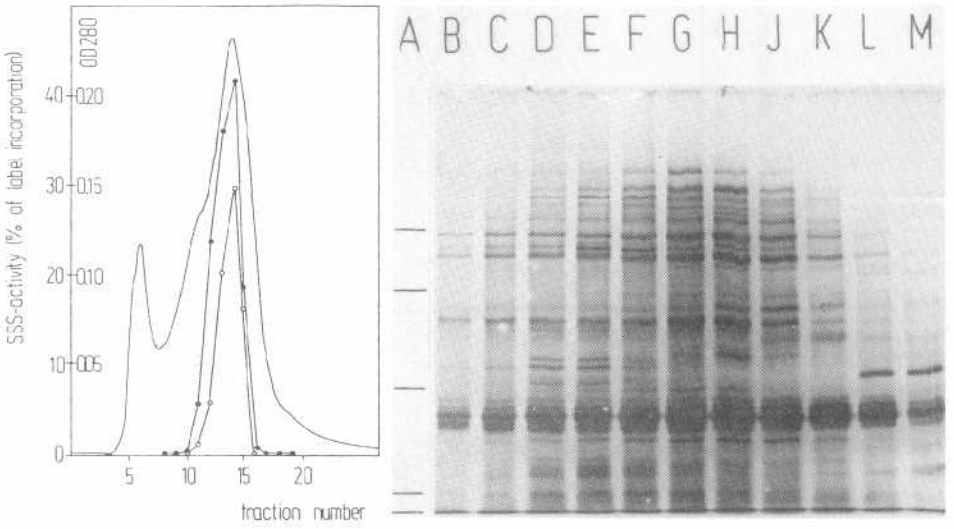
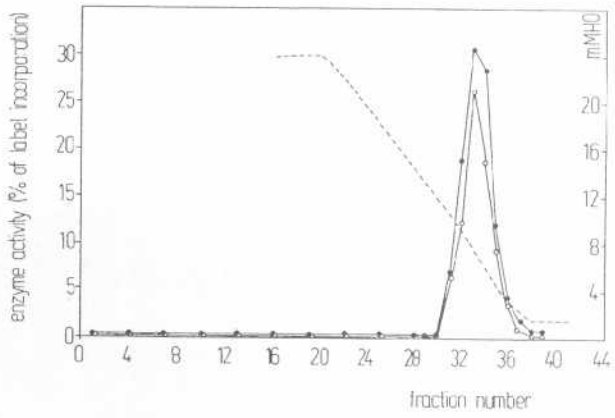


Figure 3: Gelfiltration chromatography of primed and unprimed soluble starch synthase activity. *The soluble starch synthase pool obtained after hydrophobic interaction chromatography (fractions 13 to 33 in Figure 2A) was partly (2 mg) applied to a FPLC Superose-12 column, equilibrated in TED buffer and calibrated with β-amylase (206 kDa), alcohol dehydrogenase (150 kDa) and BSA (68 kDa) in the same buffer. Fractions (0.5 mL) were collected and monitored for protein content (A_{280}, —), and assayed for primed (•) and unprimed (o) soluble starch synthase activity (left panel). Five µL of each fraction was used in each assay.*

Twenty µL of each of the fractions 7 to 17 was separated on a 8 % SDS-PAA gel (right panel lanes B to M). The proteins were visualized by silver staining. The lines in lane A correspond with phosphorylase b (94 kDa), albumin (68 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa).

matrix. It appeared that soluble starch synthase activity bound to the matrix in 50 mM Tris-HCl, pH 7.5 containing 10 mM EDTA, 2.5 mm DTT and 0.5 M sodium citrate. This binding was specific since an unmodified Sepharosecolumn did not retard soluble starch synthase activity under the same conditions (data not shown). The soluble starch synthase was eluted with a reversed citrate gradient (Figure 4). A solution of glycogen (20 mg/mL) in the citrate containing buffer was not effective as an eluent (data not shown).

A



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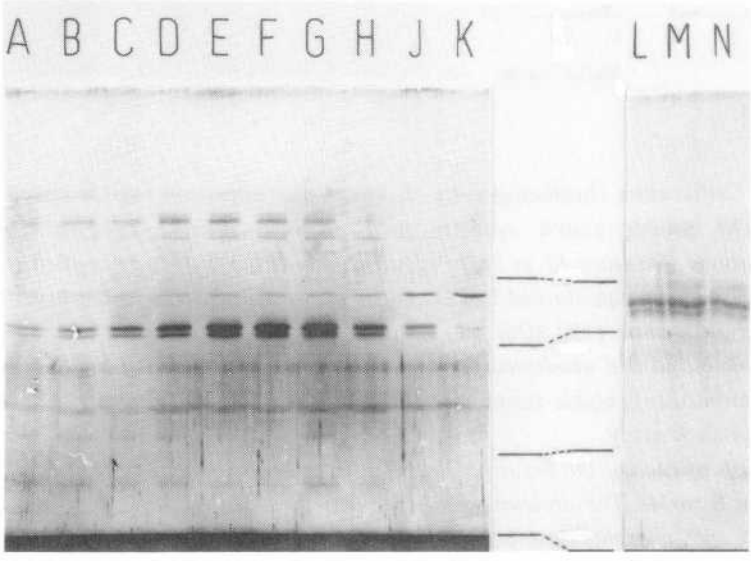


Table II: Purification scheme of soluble starch synthase activity from Producent potatoes. The purification of the soluble starch synthase from Producent potato tubers was followed by assays for glycogen primed (SSS-A) and unprimed, citrate stimulated (SSS-B) starch synthase activity.

fraction	Volume (mL)	Protein (mg)	SSS-A (mU/mg)	SSS-B (mU/mg)	SSS-A/SSS-B
0-40 % $(NH_4)_2SO_4$	105	2,010	32.8	3.7	8.9
DEAE-cellulose	55	624	45.2	10.6	4.3
aminobutyl-Agarose	1.8	16.3	327	188	1.7
		17.2*)	310*)	178*)	
peak fraction Superose-12	2.0	0.4*)	1348*)	909*)	1.5

*) expressed based on OD_{280} measurements (and taking that the absorption coefficient to be 1 per cm per mg protein per mL (Scopes 1987)).

Figure 4 (opposite page): Affinity chromatography of soluble starch synthase activity. The protein pool (fractions 12, 13, 14, and 15 in Figure 3; approximately 4 mg protein) obtained after several runs of gelfiltration, was applied to a cyclohexa-amylose-Sepharose column. The column was eluted with a reversed citrate gradient in TED buffer, and 10 µL of each fraction (1 mL) was assayed for primed (●) and unprimed (o) soluble starch synthase activity (upper panel). The fractions 32, 33, and 34 were concentrated 10 times and 50 µL was applied to a 8 % SDS-PAA gel (lanes L, M, and N respectively). Proteins were visualized by silver staining.

Part of the protein pool obtained after DEAE-cellulose chromatography (fraction 36 to 48 in Figure 1; approximately 50 mg protein) was also applied to a cyclohexa-amylose-Sepharose column. The column was eluted and 15 µL of the fractions showing soluble starch synthase activity was applied to a 8 % SDS-PAA gel, which was silver stained. The corresponding label incorporations in the assay for primed soluble starch synthase were 3.6 % (lane A), 12.6 % (lane C), 18.8 % (lane E), 30.9 % (lane F), 28.0 % (lane G), 10.5 % (lane H), 7.2 % (lane J) and 3.3 % (lane K) per 2.5 µL. The lanes L and M are loaded with a reference mixture consisting of phosphorylase b (94 kDa), albumin (68 kDa), ovalbumin (45 kDa) and carbonic anhydrase (30 kDa, and running on the front).

The first steps used to purify the soluble starch synthase are summarized in Table II. After passage through Superose-12 the specific activity of primed and unprimed soluble starch synthase activity increased 41 and 246 fold respectively. However, the recovery of enzyme activity was low (less than 2 and 10 % respectively), so that the actual purification factor may be higher.

Passage of the Sup-12 pool (fractions 12 to 15 in Figure 3) through cyclohexa-amylose-Sepharose, followed by SDS-PAGE of the fractions showing starch synthase activity revealed two major protein bands of 78 and 85 kDa coinciding with enzyme activity. A large amount of enzyme activity (80 to 90 %) was lost during the addition of sodium citrate. Passage of the DEAE/SSS pool from Producent potatoes through cyclohexa-amylose-Sepharose gave somewhat more contamination with other proteins (Figure 4), but the 78 and 85 kDa proteins were easily recognized and appeared to be more resistant to inactivation.

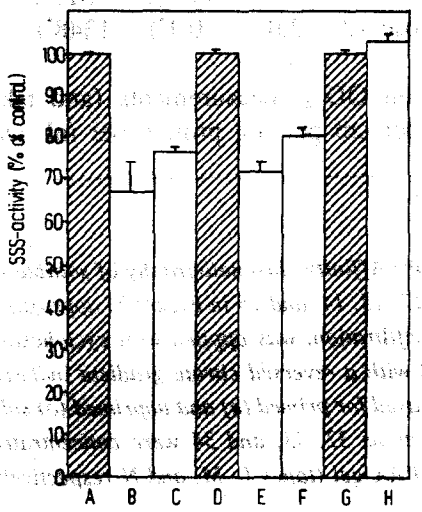


Figure 5: Neutralization and immunoadsorption of primed and unprimed soluble starch synthase activity by antiserum against the granule-bound starch synthase. *Crude potato extracts obtained after ammonium sulphate precipitation (containing 0.21 mU primed soluble starch synthase activity) were incubated at room temperature without IgG (lanes A and D), with preimmune IgG (lanes B, E and G) or with specific IgG against the granule-bound starch synthase from potato tubers (lanes C, F and H). The amount of glycogen primed (lanes A, B, C, G and H) and unprimed (lanes D, E and F) starch synthase activity was recorded immediately (lanes A to F) or after the removal of antibody-antigen complexes by adsorption to protein A (lanes G and H).*

Immunological comparison of the soluble and the granule-bound starch synthases from potatoes.

Antibodies raised against the denatured granule-bound starch synthase (Vos-Scheperkeuter et al. 1986) were tested for their ability to recognize the soluble starch synthases. First, they were used to neutralize soluble starch synthase activity in *in vitro* assays. Primed soluble starch synthase activity was not neutralized by the antiserum (Figure 5A, 5B and 5C). Furthermore, no immunoadsorption of the protein occurred (Figure 5G and 5H), indicating that the native soluble starch synthase was not recognized by polyclonal antiserum against the denatured granule-bound starch synthase. Comparable results were found for unprimed soluble starch synthase activity (Figure 5D, 5E and 5F).

Fractions obtained by DEAE-cellulose chromatography were used to test for recognition between antiserum against the denatured granule-bound starch synthase and the denatured soluble starch synthase. Both the Promesse fractions and the Producent fractions were separated on 10 % SDS-PAA gels, transferred to nitrocellulose and screened for cross reacting proteins. The fractions exhibiting soluble starch synthase activity contained a 60 kDa protein cross-reacting with the antiserum, in both cases (data not shown).

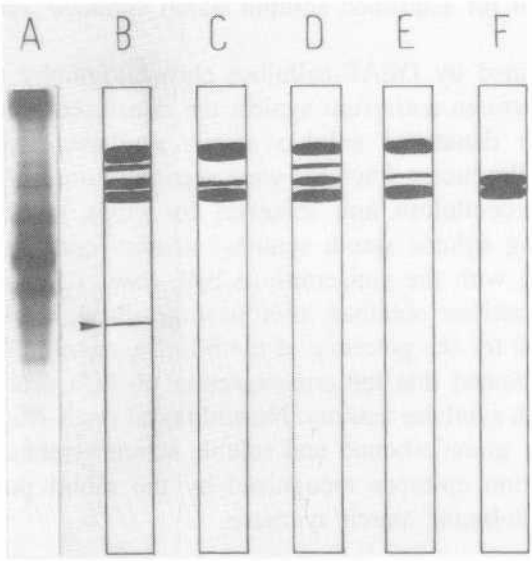
The protein fractions obtained after passage through aminobutyl-Agarose were also screened for the presence of the 60 kDa protein. The results (shown in Figure 2B) indicated that the cross-reacting 60 kDa protein was separated from soluble starch synthase activity. No additional cross-reactions were found, indicating that the granule-bound and soluble starch synthases from potato do not contain common epitopes recognized by the rabbit polyclonal antibody against the granule-bound starch synthase.

Attempts to separate primed soluble starch synthase activity.

Crude potato extracts contained several distinct protein bands catalyzing primed soluble starch synthase activity, as shown by native gelelectrophoresis followed by activity staining (Figure 6, lane B).

It was investigated whether these proteins could be separated due to differences in their affinity for DEAE-cellulose, as in maize (Boyer and Preiss 1979). Primed soluble starch synthase activity eluted in a single peak from DEAE-cellulose (Figure 1) and all the protein bands staining for starch synthase activity after native gelelectrophoresis were recovered (compare lanes B and C in Figure 6) except for the smallest and weakest one (indicated by an arrow in lane B). Subsequently, no separation took place during aminobutyl-Agarose chromatography (compare lanes C, D and E). Since comparable activity patterns were found after eluting enzyme activity from the

Figure 6: Zymogram patterns of fractions containing soluble starch synthase activity at several stages of the purification procedure. *Fractions from Producent potatoes (10 to 20 µL containing 5 mU primed soluble starch synthase activity) were loaded on 8 % PAA gels, and stained for protein (lane A) or primed soluble starch synthase activity (lanes B to F). The protein and soluble starch synthase activity pattern of the ammonium sulphate fraction is shown in lane A and B respectively, and the activity pattern obtained after passage through DEAE-cellulose is shown in lane C. Further purification by aminobutyl-Agarose gives the patterns shown in lane D (retardation) and E (elution with 1 M maltose in buffer). After Superose-12 chromatography the pattern shown in lane F was found.*



column, with buffer and buffer containing 1 M maltose, all active fractions were pooled and subjected to affinity chromatography.

We tried to enhance the separation of the primed soluble starch synthases by employing higher resolution techniques, like FPLC ion-exchange chromatography and chromatofocusing. Therefore, part of the DEAE/SSS pool obtained from Promesse potatoes was applied to a FPLC Mono Q column. However, primed soluble starch synthase activity eluted in a single peak from the column (Figure 7). Furthermore, no separation of primed soluble starch synthase activity was observed after the application of part of the ABA/SSS pool from Promesse potatoes to a FPLC Mono P column (Figure 8). Thus, other parameters had to be employed to investigate whether potato tubers contain multiple soluble starch synthases, like other higher plants.

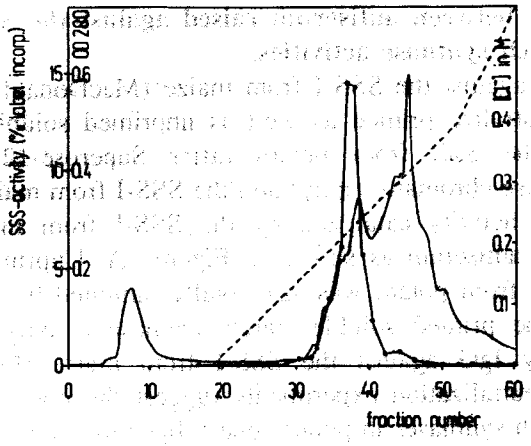


Figure 7: Mono Q chromatography of primed soluble starch synthase activity. *Part of the soluble starch synthase pool obtained after DEAE-cellulose chromatography of Promesse potatoes (corresponding to fractions 3 to 48 in Figure 1 and containing 12.8 mg protein) was applied to a FPLC ion-exchange column. Protein concentration (—) and conductivity (---) were determined. Soluble starch synthase activity was assayed (25 µL of the fractions) with glycogen as the primer molecule (●).*

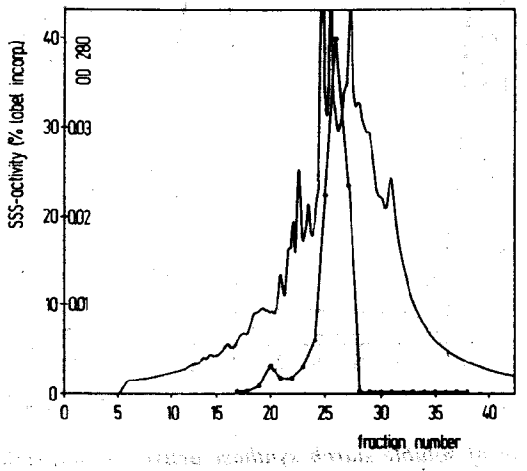


Figure 8: Chromatofocusing of primed soluble starch synthase activity after passage through aminobutyl-Agarose. *Mono P chromatography was performed with a small amount (2 mg) of the SSS pool obtained after aminobutyl-Agarose chromatography of Promesse potatoes (fractions 13 to 33 in Figure 2A). Fractions (1 mL) were collected and protein content (A_{280}, —) and primed soluble starch synthase activity (●) were measured.*

Cross-reactivity between antiserum raised against the SSS-I from maize and potato starch synthase activities.

Purified IgG against the SSS-I from maize (MacDonald and Preiss 1985) was used to neutralize primed as well as unprimed soluble starch synthase activity from the SSS from potato (after Superose-12 and cyclohexa-amylose-Sepharose chromatography) and the SSS-I from maize. The unprimed and the primed activity catalyzed by the SSS-I from maize were totally inhibited by the antiserum as shown in Figure 8A. Unprimed soluble starch synthase activity from potato was also totally inhibited by adding antiserum, but 70 % of the primed soluble starch synthase activity was resistant to neutralization by IgG against the SSS-I from maize (Figure 8B). These differences in neutralization experiments suggest the involvement of at least two soluble starch synthases in potato: one subject to neutralization, catalyzing both primed and unprimed starch synthase activity and one resistant to neutralization, catalyzing only primed soluble starch synthase activity.

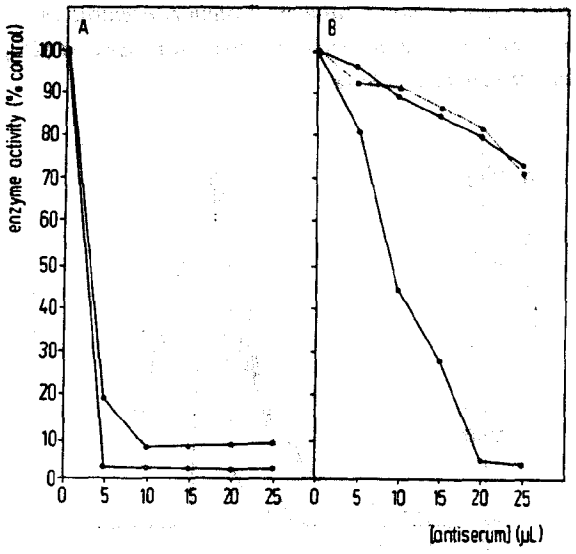


Figure 8: Inactivation of soluble starch synthase activity by antiserum against the SSS-I from maize. The DEAE purified SSS-I from maize (panel A) and the Superose-12 (solid line) and cyclohexa-amylose- Sepharose (dotted line) purified soluble starch synthases from potato (panel B) (exhibiting 0.25, 0.15 and 0.15 mU primed soluble starch synthase activity respectively) were incubated with increasing amounts of IgG against the SSS-I from maize. The extent of neutralization was determined using the glycogen primed assay (•) as well as the citrate stimulated assay (o).

DISCUSSION.

The soluble starch synthases from potato.

In this paper we show that the unprimed and primed soluble starch synthase activities from potato co-migrated on DEAE-cellulose (Figure 1), aminobutyl-Agarose (Figure 2A), and during gelfiltration on Superose-12 (Figure 3). The enzyme activities also co-eluted from cyclohexa-amylose-Sepharose (Figure 4). The above data might indicate that potato tubers contain only one soluble starch synthase. However, we think that potato tubers contain at least two soluble starch synthases, since (*i*) two protein bands corresponded with enzyme activity after passage through cyclohexa-amylose-Sepharose (Figure 4) and (*ii*) the primed and unprimed soluble starch synthases reacted differently in neutralization experiments with antiserum raised against SSS-I from maize (Figure 8B), whereas both the primed and unprimed activities of the SSS-I from maize were completely neutralized (Figure 8A) and the primed starch synthase activity of SSS-II from maize was resistant to inhibition (MacDonald and Preiss 1985).

Additional evidence comes from the changing ratio of primed versus unprimed soluble starch synthase activity during the purification procedure (Table II) and the different elution profiles of the primed and unprimed activities after passage through aminobutyl-Agarose (Figure 2A). However, these changes may also be influenced by a changing amount of degradative enzymes and/or a changing ratio of endogenous primer molecules versus soluble starch synthases.

Based on the results presented in Figure 8 we think that both protein bands visible after cyclohexa-amylose-Sepharose (Figure 4) exhibit primed, whereas only one band exhibits unprimed starch synthase activity. Since unprimed soluble starch synthase activity passed somewhat slower through Superose-12 than primed starch synthase activity (Figure 3) we conclude that the 78 kDa protein represents the SSS-I and the 85 kDa the SSS-II from potato tubers. The molecular weights of the spinach soluble starch synthases differed more (70 and 94 kDa), but in this case SSS-I was also smaller than SSS-II (MacDonald and Preiss 1985).

From the differences in the native (200 to 220 kDa) and the denatured (78 and 85 kDa) molecular weights of the soluble starch synthases we suggest that both enzymes are multimers as in the case of the *E.coli* glycogen synthase (Fox et al. 1976). In fact, the fastest moving protein in Figure 6, lane B (indicated by an arrow) may result from a monomeric soluble starch synthase.

Multiple protein bands were observed after native gelelectrophoresis and activity staining (Figure 6), indicating the existence of multiple proteins or

protein complezes catalyzing primed soluble starch synthase activity in potato tubers. Since the major activitiy bands remained present throughout the purification procedure, we assume that the dramatic loss of enzyme activity (Table II) is not caused by loss of one of the major soluble starch synthases but by enzyme lability and/or separation from stimulating enzyme activities (like the branching enzyme). As mentioned above no separation of primed soluble starch synthase activity was observed after DEAE-cellulose as in other plants (Boyer and Preiss 1981, Hawker et al. 1974, Matters and Boyer 1981, Boyer and Fisher 1984, Boyer 1985). Even higher resolution techniques like Mono Q (FPLC) and Mono P (FPLC) chromatography revealed single peaks of primed soluble starch synthase activity (Figures 7 and 8). Hawker et al. (1972) reported the separation of primed soluble starch synthase activity from potato tubers after DEAE-cellulose. Indeed, we occasionally found two peaks of primed soluble starch synthase activity after DEAE-cellulose chromatography. However, further analysis in these cases showed that the separation was artificial and caused by some kind of inhibiting agent, since a single starch synthase peak remained after reassaying diluted fractions, intermediate between the previous ones.

How our results relate to the primer dependent soluble starch synthase which was partly purified by Baba et al. (1990) is not clear at present, since these authors did not use DEAE-cellulose chromatography as the initial purification step.

Relationship between the soluble and the granule-bound starch synthases from potato tubers.

It was previously demonstrated that the distribution of soluble and granule-bound starch synthase activity changes as a function of the extraction medium used (Hawker and Downton 1979). This was used as evidence that soluble and granule-bound starch synthases are identical proteins which merely differ in their cellular localization. We were not able to reproduce these results (Table I) and sought for other parameters to examine the relationship of both the starch synthases. We found that (*i*) the molecular weights of the denatured soluble starch synthases (78 and 85 kDa) differed from the molecular weight of the denatured granule-bound starch synthase (60 kDa; Vos-Scheperkeuter et al. 1986); (*ii*) an antiserum against the denatured granule-bound starch synthase did not neutralize nor adsorb soluble starch synthase activity (Figure 5); (*iii*) the cross-reaction between the antiserum and a denatured soluble 60 kDa protein did not coincide with soluble starch synthase activity (Figure 2), and (*iv*) that this cross-reaction was not present in potato tubers, successfully transformed with anti GBSS sequences while the level of

primed and unprimed enzyme activities was unaffected (Chapter 2). These results indicate a total lack of common sequences between granule-bound and soluble starch synthases and suggest that both enzymes are unrelated, as in maize (MacDonald and Preiss 1985).

The soluble 60 kDa protein seems to be related to the granule-bound starch synthase (Chapter 2). The enzyme failed to elongate glycogen (5 mg/mL) as well as endogenous primer molecules in the presence of 0.5 M sodium citrate (Figure 2A). Amylopectin (5 mg/mL), amylose (DP 200; 0.1 % (w/v)) and maltotriose (250 mM) were also ineffective as primers (data not shown). However, the enzyme is shown to be catalytically active when solubilized from starch granules in the case of maize and suspension cultured soy bean cells (MacDonald and Preiss 1985, Miyamoto et al. 1989). The lack of enzyme activity in our case may be caused by proteolytic breakdown of the protein, since the active site of the enzyme is probably located at the N-terminus (Chapter 2). Alternatively, other factors crucial for enzyme activity (for instance the association with primer molecules), might be missing.

Conclusions.

Generally, two or even more isoforms of soluble starch synthase activity are present in higher plants, including maize endosperm (Boyer and Preiss 1981), spinach leaves (Hawker et al. 1974), teosinte (Boyer and Fisher 1984), pea (Matters and Boyer 1981) and sorghum (Boyer 1985), which can be easily separated due to their different affinities for DEAE-cellulose. The only exception to this rule is the castor bean in which one peak of soluble starch synthase activity was found after ion-exchange chromatography (Goldner and Beevers 1989).

Here, we describe the latter situation for the soluble starch synthases from potato tuber. However, further analysis demonstrates that potato tubers possibly contain two (or more?) different isozymes. Both isozymes immunologically differ from the granule-bound starch synthase (Figures 2, 5).

The purification of soluble starch synthase activity, as described in this paper, will open up the possiblities to study the roles of the soluble starch synthases in starch biosynthesis, in more detail.