SIGNIFICANCE OF PHOSPHORYLASE ON STARCH SYNTHESIS OF POTATO TUBERIZATION

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Introduction

The view expressed by ARTSCHWAGER[1] is that potato tuber is a modified stem with a shortened axis with poorly developed leaves. The first sign of the tuber formation is a swelling of the sub-apical region of stolon tip and such an early tuber development is primarily attributable to enlargement of the pith cells, although cell division probably rapidly follows[3].

As to a massive deposit of starch in the tuber, its accumulation started in a localized sub-apical region of the stolon prior to any visible sign of tuberization. Most promising current line of investigation seems to be a shift in carbohydrate metabolism during the course of the tuberization. A critical evaluation on hormone-induced modification of several enzymes activities involved in starch synthesis would provide a valuable insight into the mechanism of starch accumulation at the initiation of tuberization, citing the fact that some growth substances as gibberellin and cytokinin are closely related to the regulation of potato tuber formation[12,15]. In an earlier work[4], the starch synthesis in the living bark of tree was outlined and suggested no major role of phosphorylase in relation to the starch synthesis. However recently extensive studies of this line have alluded to an important possibility that phosphorylase would implicate, to a greater or lesser extent, in the determination of metabolic pattern of starch at the time of potato tuberization[7,10,11,17]. GERBRANDY and VERLEUR[6] reported that several isozymes of phosphorylase were found in potato tuber and some of them were associated with amyloplast. According to an opinion expressed by HAWKER *et al*[7], a massive accumulation of starch in the tuber seems to be partially controlled by the activities of phosphorlyase as well as UDP glucose pyrophosphorylase. Despite starch metabolism in the growing or mature tubers

has been receiving considerable attention from deverse angles, relatively little work has in fact done on the modification of starch metabolic pattern, which causes or results in switch over from the stolon elongation to its thickening growth being accompanied with starch deposition in their cells. It seems to be, therefore, fundamental to a proper understanding on physiological significance of phosphorlyase in growing potato stolons and tubers. *In-vitro* culture of one-node stem segments cut from potato etiolated shoots has been proved to be a potentially useful system for the physiological study of their tuberization[2,10,13].

In the present investigation, an attempt was made to study this problem further by means of the stem segment culture of potato shoots *in vitro*.

Materials and Methods

Plant material:

The experiments were carried out on potato (*Solanum tuberosum* L.) tubers cv. Irish Cobbler supplied by the Central Foundation of Seed Potato Farm in Hokkaido, in 1981 harvested. The etiolated potato shoots were raised from the seed pieces of the tubers and the preparation of one-node stem segments and the procedure of *in-vitro* culture were conducted in essentially the same manners as previously described[14]. In the present study, the cultures were performed on the medium with or without gibberellic acid (GA) at 0.1 mg/l in order to directly regulate the tuberization.

Preparation of phosphorylase:

A bunch of growing tips, 5 mm in length, were collected from the apical part of elongating stolons or juvenile tubers, and measured their fresh weights. These samples were homogenized in cold Tris-HCl buffer (50 mM, pH 7.5) with 0.25 M sucrose, 0.1% cysteine and 10^{-3} M EDTA. The homogenate was centrifuged at 15,000 g for 10 min, and the supernatant was brought to 60% saturation with solid $(NH_4)_2SO_4$, allowed to stand for 2 hr and centrifuged again 15,000 g for 10 min. The precipitate was dissolved in the same buffer as described above, and applied to Sephadex G-200 column (1.5 × 25 cm). Finally the phosphorylase was eluted with the buffer solution supplied with 0.1 M NaCl. All operations were performed at ca. 4°C.

Assay of phosphorylase:

According to the manner of HEDRICK and FISCHER[8], phosphorylase activity was determined by measuring the liberation of phosphate from glucose-1-phosphate with following solution: 50 mM glucose-1-phosphate in 0.15 M sodium citrate buffer solution (pH 6.0) and 0.2% soluble starch. The

reaction was started by the addition of 0.1 ml enzyme solution to 0.1 ml of the above mixture. After the incubation for 10 min at 30°C, the reaction was stopped by 3 ml of 0.7 M H_2SO_4 and the liberated phosphate was assayed[5]. Amount of the enzyme was converted to a activity unit which is equivalent to liberating one μ mole of phosphate per min.

Fractionation and determination of starch:

The crude homogenate of potato tissues was loaded on the top of a Sephadex G-200 column (1.5×25 cm) to separate different sized starch granules and eluted with the same buffer as using for its extraction. One group of starch granules was remained on the top of the column and the other was passed through the column. The former was designated as L-starch (10–25 μ in diameter) and the latter was S-starch (2–5 μ in diameter). Both types of starch were digested separately with amylase. The resulting soluble sugar was determined by means of the method of anthrone-H_2SO_4[19] and represented as glucose molar equivalent.

Absorption spectrum of iodine-starch complex:

To evaluate the structural difference between several types of starch, absorption spectra of starch complexed with iodine were recorded with a two-wavelength spectrophotometer (type 356, Hitachi). Iodine-starch complex was prepared as described by KRISMAN[9].

Results

External appearance of culture:

There appeared to be a outstanding differences in external appearance between growing stolons cultured with and without GA. The control cultures spontaneously started to induce tuberization within 10 days after inoculation and its thickening growth proceeded until reaching small but mature tuber. On the contrary, the GA-treated cultures continued their linear growth of the stolons without any sign of tuberization over the entire period of the culture. However, when the stolon tips grew against the side wall of culture container, the stolon hooks collapsed by producing necrosis and eventually stopped their growth leading to start outgrowth of several laterals. Therefore it was no longer possible to determine the starch content and the enzyme activities in the stolon tips.

Changes in soluble phosphorylase activity in relation to the accumulation of different sized starch granules:

Although a slight accumulation of starch in the stolon tips was detected

as the S-starch granules alone, contents of both types starch were simultaneously rised upto almost equal level at the time of the tuberization (Fig. 1). Subsequently L-starch content continued to increase keeping pace with lowering the S-starch content correspondingly, by a modification of starch metabolic pattern caused by tuberization. On the other hand, the stolons cultured with GA stored exclusively the S-starch at a quite lower but significant level as compared with that in the control culture, and its level consistently rised in parallel with progressive stolon elongation. Of interesting was the fact that there was no detection of L-starch throughout the culture period.

As to the changes in the soluble phosphorylase activity of the stolon tips during the culture, the tuber forming culture maintained a relative higher

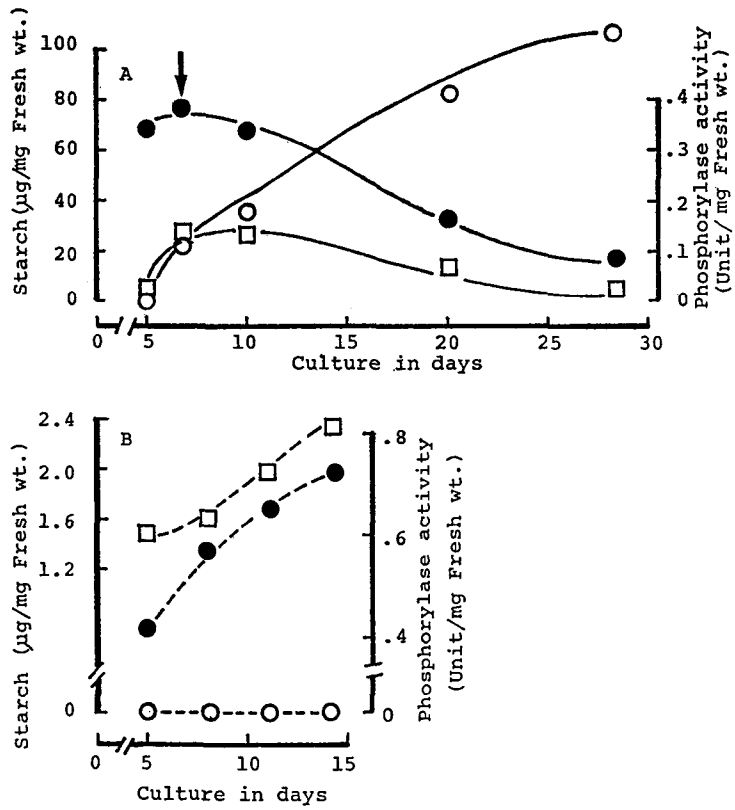

Fig. 1. Time-course of changes in starch content (S-starch □, L-starch ○) and soluble phosphorylase (●) in the stolon tips and juvenile tubers cultured in the medium with or without gibberellin. Arrow indicates time of stolon swelling. A; without GA (—), B; with GA (---).

activity of the enzyme before tuberization, and a transient increase occurred in the activity during the first 7 days of culture and reached maximal level when the tuberization initiated. Subsequently the increasing trend reversed to fall with the advance of the tuber development. When cultured with GA, the phosphorylase activity persisted at the initial high level throughout the culture and showed a similar tendency as the increasing pattern of S-starch contents.

Availability of different types of starch for glucose acceptor:

In the experiment using purified preparation of potato phosphorylase, the affinities of various sized starch to the phosphorylase enzyme were examined. As listed in Table 1, the phosphorylase was capable of utilizing soluble starch best, followed by S- and L-starch in that order. By comparing the availability of L-starch for the phosphorlyase action, one and half fold increase of that of S-starch was ascertained.

TABLE 1. Availability of different types of starch for phosphorylase acceptor. Each value is means of ten replication with standard error

Glucose acceptor	"soluble starch"	S-starch	L-starch
Phosphorylase activity (Unit)	.417±.020	.321±.0.16	.214±.008

"soluble starch": a product of Wako pure Chemicals Ind. Co.

Absorption spectra of iodine-starch complex of different sized starch:

According to the fact reported by KRISMAN[9], each iodine-complex of amylose and amylopection was found to represent a characteristic profile of their absorption spectra which showed two difinite peaks at 535 μm and 645 μm and corresponded to amylopectin and amylose, respectively. This evidence prompted us to investigate the characteristics of different starches and distinguish between soluble, S- and L-starches, individually. From the data presented in Fig. 2, it is evident that these profiles of iodine-starch complexes showed somewhat different in spite of the similarity in their basic pattern which indicated to comprise amylopectin and amylose. From the inspection of these spectra, much more amount of amylopectin conteined in the potato endogenous starch as compared to the soluble starch. The spectra of the S- and L-starches indicated a prominent participation of amylopectin as the potato starch components, and those were resembled fundamentally but somewhat different each other. It proved to be important, therefore, that the feature of the endogenous starch in potato plant is char-

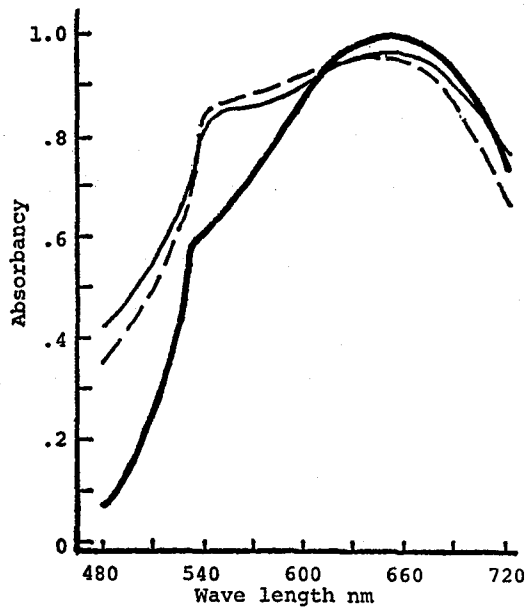

Fig. 2. Comparison of absorption spectrum between iodine complexes with several type of strach. Iodine complexes of amylopectin and amylose represent maximum absorbancy at 530–540 nm and 640–650 nm, respectively. Each curve means as follows: soluble starch (—), S-starch (—), L-starch (- -).

acterized by comprising much more level of amylopectin than soluble starch.

Comparative chromatography of soluble and S-starch bound phosphorylases:

Using the crude homogenate of the parenchymatous cells of the mature tubers, the chromatographs were performed to separate the preparations of the phosphorylases which was soluble in cytoplasm and released from S-starch granules, as described in Methods. Elution patterns of the chromatographic steps were shown in Fig. 3. During the preparation of the soluble phosphorylase, a pronounced single peak of the activity was readily separated as a major component. Subsequently, S-starch granules were separated by passing through the chromatographic column of Sephadex G-200 and each fraction of eluted S-starch granules was divided. A most predominant yielding in the phosphorylase was found as a single peak released from the S-starch complex, which occurred in the chromatography nearly after flowing off the void volume of eluate firstly, and thereafter the activities gradually declined being accompanied with decreasing in the eluting amount of S-starch. This fact implicates that the amyloplast including the smaller S-

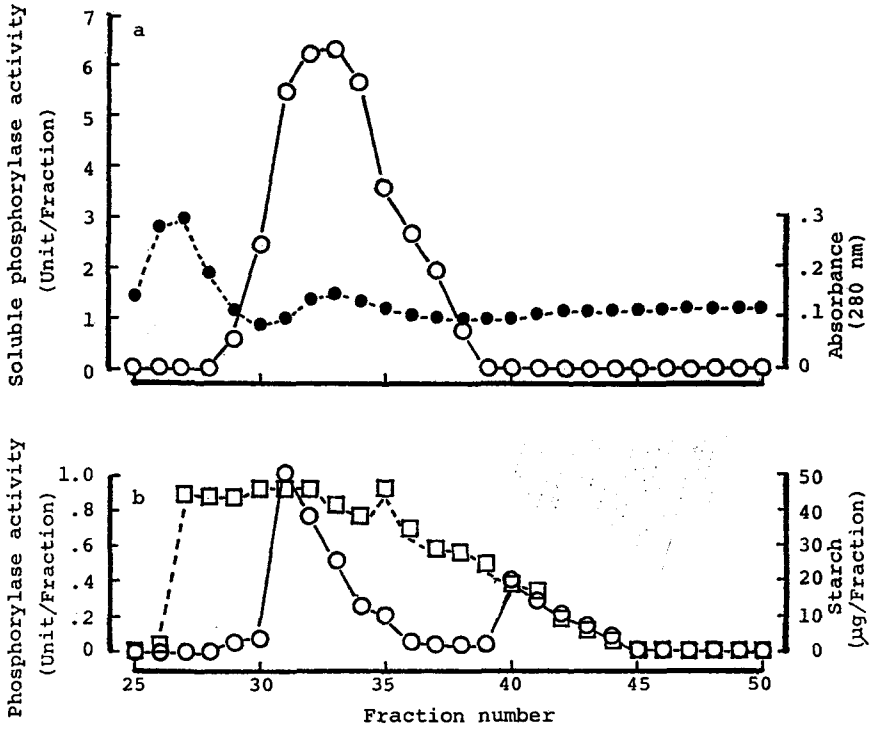

Fig. 3. Elution profiles of the soluble (a) and S-starch bound phosphorylase (b) activities and S-starch contens (—□—), chromatographed by Sephadex G-200. Absorbance at 280 nm is shown (····●····).

starch granule may bind much more active phosphorylase protein as compared with the large particle of starch which eluted later.

Comparison between activities of phosphorylase bound with S- and L-starch granules:

The parenchymatous cells of mature tubers verified to contain much less amount of S-starch granules than that of L-starch ones, whereas the activity of phosphorlyase derived from S-starch was extremely higher than that from L-starch (Table 2). These data may indicate that the affinity of phosphorylase for the membrane of amyloplast including S-starch would be superior to that of the L-starch. Alternatively, the degradation of amyloplast would take place with increasing the size of starch granules leading to disappear the activity.

TABLE 2. Comparison between the activities of phosphorylase bound with S- and L-starch granules

Fraction	Phosphorylase activity (Unit)	Starch (mg)	Specific activity (Unit/mg starch)
S-starch	5.78	.72	8.03
L-starch	.27	103.4	.0026

Discussion

From the data of the present investigation using *in-vitro* cultures of potato one-node stem segments, an accumulation pattern of different sized starch granules in the stolon tips was ascertained to be characteristically modified at the time of tuberization. A most interesting aspect of this results is that the situation of starch accumulating pattern was reversed from small granules to large ones, in conformity with the commencement of decrease in the soluble phosphorylase activity at the tuberization. Contrarily there was no similar modification of starch metabolism in the GA-treated cultures which continued the elongation of the stolon throughout the incubation period. In the light of the data presented by MINGO-CASTEL *et al.*[10] with kinetin-induced *in-vitro* tuberization, starch was confirmed to be synthesized initially by the aid of phosphorylase. In addition, PREISS and LEVI[16] assumed that marked changes in enzyme activity during plant organogenesis were primarily caused via regulation of the synthesis of the starch biosynthesic enzymes. Bearing in mind that the supply of sugars may be a factor regulating stolon elongation and both enzyme synthesis and activity[7], the decline of soluble phosphorylase activity may be reflection of the lowering concentration of soluble sugar. In this context, an earlier finding by TAGAWA and OKAZAWA[18] that a sharp decline of the reducing sugar content in growing potato stolon occurred immediately after the onset of tuberization seems to not contradict but support this idea.

We can expect a bulk of enzymes to be contained in amyloplast, since starch is generally accepted to synthesize in a special compartment as amyloplast in plant storage tissues. In fact, there is an evidence to support this idea, and ultrastructual studies revealed that there is an intracellular localization of phosphorylase in the amyloplasts during starch synthesis[17]. This finding is also much strengthened by the results presented in Table 2 and Fig. 3 that an appreciable high activity of phosphorylase was released from starch granules, and suggests that much more amount of enzyme protein was bound with membrane of amyloplast containing small starch granules

in comparison with that of large ones.

Another interesting point arising from this study is the fact as summarized in Table 1 that S-starch is superior to L-starch as the acceptor of glucose molecular unit for starch synthesis by phosphorylase. Taking the data of Fig. 2 into account, this might be attributable to the different amount of amylopectin containing in starch molecule. Additionally, a parallelism between phosphorylase activity and S-starch content in the stolon tips during their elongating growth also may be interpreted to depend greatly on the good affinity between this enzyme and S-starch.

An inevitable question still remains as important problem, however, that L-starch granules instead of S-starch ones started to deposit vigorously in the juvenile tubers even at the declined level of the soluble phosphorylase activity. Assuming that both soluble and starch-bound phosphorylases no longer participate in the starch synthesis after the tuberization, some other enzymes would have to be elicited their activities prior to the incerease in amount of L-starch. Although a completely convincing documentation of this point does not be available, it can only be said at the moment that several workers have alluded to the possible involvement of some other enzymes as starch synthetase and UDP glucose pyrophosphorylase. It seems to not contradict but support our assumption, however, we are unable to state which enzyme does behave directly for starch synthesis because of insufficient data here. More detailed studies are necessary to answer this question.

In conclusion, it can be said with certainty that a transition in the biochemical behavior of phosphorylase may be occurred as a consequence of the tuber initiation leading to the modification of starch synthetic system in the potato stolons.

Summary

The changes in the activity of phosphorylase involved in the starch accumulation of potato plant (*Solanum tuberosum* L.) were measured periodically during the stolon elongation and the tuberization of the one-node stem segments cultured *in vitro*. An occurrence of small sized starch granules (S-starch) was revealed predominantly during the course of stolon elongation, and subsequently large sized starch ones (L-starch) increased consistently with time after tuberization in place of S-starch. A close parallelism between S-starch content and soluble phosphorylase activity was also observed. The S-starch as an acceptor of glucose molecular unit for starch synthesis was more dominant than the L-strach, and much more activity of phosphorylase was found to be released from S-starch granules than that from L-starch

ones. At the time of tuberization, a modification of starch anabolic pattern may take place at the morphological alteration from the elongation growth of stolon to the thickening growth of the tubers.

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