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Starch Phosphorylase and its Inhibitor from Sweet Potato Root

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Abstract—Based on a tracer study, starch phosphorylase was implicated as an agent in the starch synthesis in sweet potato roots. The enzyme was purified from the tissue as a cluster of isozymes with an average mw of 205K (fresh roots) or 159K (roots stored for 3 mon.). On SDS polyacrylamide gel electrophoresis, one large subunit of 98K mw and several small ones of 47~57K mw were observed. From the mw data and the results of peptide mapping and immunoelectrophoretic blotting using mono- and polyclonal antibodies, it was deduced that a large part of the large subunit was cleaved at the middle part of the peptide chain to give rise to the small subunits, and on storage, the enzyme molecules were further modified by proteolysis. During the course of phosphorylase purification, a proteinaceous inhibitor of the enzyme was isolated. It had a mw of 250K and was composed of 5 identical subunits of 51K mw. In the direction of starch synthesis, the inhibitor showed a noncompetitive kinetics with a Ki of 1.3×10^{-6} M. By immunohistochemical methods, both the enzyme and the inhibitor were located on the cell wall and amyloplast. Crossreacting materials of the inhibitor were present in spinach leaf, potato tuber and rice grain. These findings indicate the wide occurrence of the inhibitor and also imply its possible participation in regulating starch phosphorylase activity *in vivo*.

Keywords—Sucrose-starch transformation · starch phosphorylase · phosphorylase inhibitor · immunohistochemistry of proteins

The biochemical pathway of sucrose-starch transformation in starch accumulaing organs of higher plants has been the main interest of research in our laboratory for the past decade[1]. The approach to the problem was firstly to perform a tracer feeding experiment in which a double-labeled sucrose, e.g. ^{14}C-glucosyl-^{3}H-fructoside is used, and to analyze the distribution of the two tracers in intermediates as well as the final product of the pathway, secondly to identify or deduce the key enzyme(s) or enzyme (s) of interest of the pathway based on the tracer study data, thirdly to purify and characterize the enzyme(s) thus identified, and finally to evaluate the originally proposed pathway in the light of the enzyme data. In this type of work, an equimolar mixture of glucose and fructose containing different kinds of radioatoms in their molecules (usually a hydrolysate of double-labeled sucrose) is used to perform the

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Table I. Radioactivity ratio of $^{14}C/^{3}H$ in starch and sugar nucleotides recovered from sweet patato slices fed with radioactive saccharides

Substrate	Product	^{14}C (cpm)	^{3}H (cpm)	$^{14}C/^{3}H$
Sucrose[a]	Starch[c]	3882	2543	1.52
	UDPGlucose	689	298	2.30
	ADPGlucose	713	907	0.78
Glucose + Fructose[b]	Starch[c]	6927	1712	4.05
	UDPGlucose	3757	1847	2.03
	ADPGlucose	2861	2023	1.40

[a]A mixture of sucrose ([6(n)-^{3}H]fructose) (ca. 0.3 nmole, $^{3}H=6.6 \times 10^{5}$ cpm) and sucrose ([U-^{14}C]glucose) (ca. 1.5 nmole, $^{14}C=6.6 \times 10^{5}$ cpm).
[b]The same amount as in a, but hydrolyzed with 1 N HCl.
[c]Hydrolyzed with a mixture of alpha-amylase and amyloglucosidase before scintillation counting.

control experiment.

By this kind of approach, sucrose synthetase was identified as the enzyme which provided sugar nucleotide precursor(s) to starch synthesis in the maturing rice grain. A similar experiment performed with sweet potato root, although also a nonphotosynthetic organ as the rice grain, however, gave results quite contrary to those of the latter. In the sweet potato root, when an equimolar mixture of glucose and fructose was fed, the ratio of glucose and fructose contributing to the starch synthesis was about 4, while when a double-labeled sucrose was fed, the ratio was about 1.5 (Table I). The poor rate of sugar nucleotide formation in the sucrose-fed experiment and the much lower glucose/fructose contribution ratio in sugar nuclotides than in starch in the monosaccharides-fed experiment disfavored both sugar nucleotide pyrophosphorylase and sucrose synthetase as the agents in providing precursors to the starch synthesizing system in sweet potato roots. The possible candidate for catalyzing starch synthesis in this system is starch phosphorylase although it is generally regarded as a starch degrading enzyme.

Nevertheless, we purified the enzyme from the mature roots of sweet potato to homogeneity, and during the course of the work, the total enzyme activity increased 3 fold in an ion exchange step, suggesting the presence of a phosphoyrlase inhibitor in the enzyme extract. Thus an assay procedure for the inhibitor was set up and the inhibitor was also purified to homogeneity. This paper gives a brief account of the purification and characterization of the enzyme and its inhibitor.

Materials and Methods

All the purification steps were carried out at 0～4° unless indicated otherwise. Roots of sweet potato (*Ipomoea batatas* (L.) Lam. cv Tainon 65) were homogenized with an equal volume (w/v) of 50 mM imidazole-HCl buffer pH 6.4, containing 2 mM dithiothreitol, 0.1 mM sodium ethylenediamine tetraacetate and 10% (w/v) sucrose (buffer A). After clarification by centrifugation, the precipitate salted out from the supernatant with ammonium sulfate at 25～45% saturation was collected and ion-exchange chromatographed through a column of DEAE-Sephacel (buffer A with a linear gradient of 0 to 0.4 M NaCl). The inhibitor emerged at about 0.15 M salt while the enzyme was eluted at about 0.3 M. From this step on, the inhibitor could be purified to homogeneity by a single step of gel filtration

using a molecular sieve with pore size larger than that of Sephadex G-100. For the enzyme, a second step of DEAE-Sephacel was necessary after gel filtration purification with Sepharose CL-6B.

The enzyme was assayed in the direction of glucan synthesis using glucose 1-phosphate as the substrate and soluble starch as the primer. The reaction was monitored by the liberation of inorganic phosphate in the reaction system. The inhibitor was assayed by the decrease in the activity of added sweet potato phosphorylase[2]. Protein was assayed by the Folin's phenol reagent using bovine serum albumin as the standard[3].

Proteins were characterized by electrophoretic and immunological methods. Polyacrylamide gel electrophoresis (PAGE) was performed with 0.8 mm thick gels. For molecular weight determination of native proteins, gradient gels were used[4], and for that of denatured subunits, sodium dodecylsulfate(SDS)-PAGE[5]. Analytical isoelectric focusing (IEF) was run in 0.5mm thick gels containing 1% Ampholine[6]. After electrophoresis, gels were stained with Coomassie blue and dried by the sandwich method[7]. Activity stain was performed by incubating the gel with shaking in 50 mM Mes buffer pH 5.9, containing 10 mM glucose 1-phosphate and 0.3% soluble starch, followed by iodine stain[8]. Antisera against the enzyme and the inhibitor were raised in New Zealand white rabbits. For characterizing the enzyme, several monoclonal antibodies were also prepared by fusing spleen cells from the immunized BALB/c mouse and FO cells in the presence of polyethylene glycol. The antibody secreting hybridoma cells were screened by an ELISA, expanded and cloned by limiting dilution method[9] using BALB/c thymocytes as feeder layers. Ascites fluid obtained by innoculating hybridoma cells to BALB/c mice was used as the source of monoclonal antibody. A DEAE ion-exchange chromatographic procedure was employed in purifying immunoglobulins from the rabbit antisera[10].

Ouchterlony double diffusion, immunoelectrophoresis and Western blotting of electrophoretically separated proteins were the three methods of choice in characterizing the protein by antibodies.

Results and Discussion

The results of starch phosphorylase and its inhibitor purifications are given in Tables II and III, respectively. The homogeneity of the final products were ascertained by electrophoretic and immunological tests. The inhibitor gave one sharp band in IEF, gradient PAGE, SDS-PAGE, immunoelectrophoresis or double immunodiffusion. In case of the enzyme, after PAGE, a group of poorly separated proteins comprising of 6 bands were revealed in the dye staining, and all of them showed positive phosphorylase activity. In IEF analysis, they cannot be focused in one band; instead they were separated into 3 diffused bands. The precipitin lines of Ouchterlony double diffusion seemed to contain several components which coalesced, and the precipitin arch of immunoelectrophoresis was really composed of several small arches. As the crude extract in which protease inhibitors were included also showed identical zymogram, the possibility of proteolytic modification during the isolation process to cause the heterogeneity was low. However, when stored sweet potato roots were used as the enzyme source, the average molecular weight of the group of enzymes isolated was smaller than that from the fresh roots, indicating the enzyme was susceptible to proteolytic modification. The members in the group were all simultaneously probed by monoclonal antibodies indicating that they possessed common antigenic determinants. SDS-PAGE revealed a large subunit together with 4 small ones, and the large

Table II. Purification of sweet potato phosphorylase.

Fraction	Protein	Total activity	Specific activity	Purification	Recovery
	mg	units	units/mg	fold	%
Crude extract	2000.2	497.2	0.25	(1)	(100)
25～45% $(NH_4)_2SO_4$	952	307.3	0.32	1.3	61.8
lst DEAE-Sephacel	73	917.4	12.57	50.7	184.5
Sepharose CL-6B	16.9	795.5	47.07	189.8	160
2nd DEAE-Sephacel	13.5	758.4	56.18	226.5	152.5

The results were obtained with 300 g of sweet potato root.

Table III. Purification of phosphorylase inhibitor from sweet root.

Purification steps	Protein(mg)	Total activity(IU)	Sepcific activity (IU/mg)	Purification (fold)	Recovery(%)
1. 25～45% ammonium sulfate	952	1536	1.6	(1)	(100)
2. DEAE-Sephacel	31.7	1343.8	42.4	26.3	87.5
3. Sephadex G-100	4.5	1014.9	225.5	140.1	66.1

The results were based on 300 g of sweet potato.

one had a molecular weight approximately twice the average of the small ones. Immunoblotting of the zymogram with monoclonal antibodies revealed that two of the small ones shared the common antigenic determinants with the large one. When the large and the cluster of small subunits were separately digested with chymotrypsin and the products separated by PAGE, the peptide bands obtained with the cluster of small subunits could also be almost all found in the digest of the large one. These results are taken together to indicate that the small subunits are the proteolytic products of the large one. From the molecular size data, it is proposed that the enzyme has a tertiary structure in which the middle region of the polypeptide is susceptible to enzymic hydrolysis without significant loss of activity. It is thus postulated that the enzyme is originally a dimer of the large subunit (L), and as the degradation of the large subunit to the small subnunits (S) progresses, there would be a population of enzyme molecules with the quaternary stuructures of L_2, LS_2 and S_4. The molecular weights, isoelectric points, etc., of the enzyme and its inhibitor are summarized in Table IV.

In contrast to the animal phosphorylase which is well established to catalyze the phosphorolytic degradation of glycogen and known for hormone mediated allosteric control of action, the metabolic role of plant phosphorylase has not been settled definitely and nothing has been known about its regulatory mechanism. In this connection, the finding of a proteinaceous inhibitor of the enzyme in sweet potato is of great interest because it could be an agent of regulating the enzyme activity by an unknown mechanism. In order to understand whether the enzyme and its inhibitor are physically associated in the plant tissue or not, their subcellular localization was studied by the immunohistochemical techniques. The light microscopic method utilizing fluorescein isothiocyanate probe located both of them in the cell wall and amyloplast. Since the localization of starch phosphorylase in the cell wall has never been reported before, it was further proved by electron microscopic method utilizing ferritin and gold particle probes. A kinetic study of the inhibitor in the direction of glucan synthesis using glucose 1-phosphate as the

Table IV. Properties of sweet potato phosphorylase and its inhibitor

		Phosphorylase from fresh or stored root		Inhibitor
Molecular native weight		190K～220K	140K～160K	250K
Subunit	Large	98K	Similar to the fresh root.	51K
	Small	57K		
		55K		
		50K		
		47K		
Quaternary structure		Di-Tri-& Tetramer		Pentamer
Isoelectric point		4.60×4.64 4.84～4.88 4.92		4.63
		Residue %		
Amino acid composition*	Asp	12.05	10.85	14.49
	Thr	4.85	4.96	4.35
	Ser	6.22	6.16	5.81
	Glu	13.86	15.49	10.18
	Pro	5.18	5.32	6.46
	Gly	7.54	6.59	9.65
	Ala	9.03	7.67	9.19
	Val	4.60	5.60	5.52
	Met	1.81	1.47	3.02
	Ile	4.57	5.93	3.87
	Leu	8.81	8.90	7.72
	Tyr	4.18	3.70	4.14
	Phe	4.45	4.53	4.45
	His	1.52	1.66	1.69
	Lys	7.02	6.40	5.32
	Arg	4.31	4.78	4.14

* Acid hydrolysates were analyzed in an amino acid autoanalyzer.

variable substrate indicated that it was a noncompetitive inhibitor toward the enzyme. The significance of these findings is completely unknown for the time being but intriguing.

In order to further understand the importance of the inhibitor, its presence in three arbitrarily selected plant mateieals, viz. potato tuber, rice grain and spinach leaf was demonstrated. The crude extracts of these materials were brought to 65% saturation of ammonium sulfate and the precipitates were taken up in smallest possible amount of phosphate buffered saline for testing by Ouchterlony double diffusion against rabbit antiphosphorylase inhibitor of sweet potato. The results showed that cross-reacting materials with the sweet potato inhibitor were present in all of them, indicating that the inhibitor could be of universal occurrence in higher plants (in mono- and dicots and in grain, root, tuber or stem and leaf).

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