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Starch Phosphorylase Inhibitor Is β-Amylase[1]

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ABSTRACT

The proteinaceous noncompetitive inhibitor of starch phosphorylase isolated from the root of sweet potato (*Ipomoea batatas* [L.] Lam.) (TC Chang, JC Su 1986 Plant Physiol 80: 534–538) has been identified as a β-amylase. The starch phosphorylase inhibitor and β-amylase activities copurified to give a protein indistinguishable from commercial β-amylase by electrophoretic and immunological methods, and the two activities showed parallel responses in pH, temperature, and inhibitor sensitivity tests. The amylolytic pattern of the inhibitor corresponded to that of β-amylase and its inhibitory effect toward starch phosphorylase was due to neither deprivation of starch, the primer for the phosphorylase assay, nor the inhibitory effect of amylolytic products.

An SPI[3] was found in and purified from sweet potato (*Ipomoea batatas* [L.] Lam.) roots (2). The inhibitor is now proved to be an enzyme well known in sweet potato, BA. This paper describes the evidence in support of this conclusion.

MATERIALS AND METHODS

A new sample of electrophoretically pure SPI was prepared according to the procedure reported previously except both SPI and BA activities were monitored simultaneously during the purification (2). Anti-SPI serum used here was described in a previous work (2). A commercial sweet potato BA was purchased from Sigma (type I-B). The SPI was assayed by its inhibition of starch phosphorylase (SP) activity in the direction of glucan synthesis as previously reported (1, 2). The SPI assay mixture contained 1% soluble starch (Merck) and 10 mM G1P (Sigma) in 50 mM sodium acetate (pH 5.7). The BA assay mixture contained 1% soluble starch in 50 mM sodium acetate (pH 5). After addition of the enzyme, incubation was done at 37°C for 3 min, and the reaction terminated by heating the reaction mixture in a boiling water bath for 20 s. The reducing sugar was determined with a 3,5-dinitrosalicylic acid reagent (4).

In order to assess the identity of SPI as BA, and the inhibitory mechanism of SPI, the following experiments were performed. Electrophoretically pure SP and SPI preparations were used. (a) An SPI solution was heated at the designated temperature for 3 min and the residual BA and SPI activities were assayed under the standard conditions. (b) The SPI and BA activities were assayed under standard conditions except the incubation temperature varied. (c) SP was assayed with or without SPI in the reaction mixture of different pH. (d) SPI and SP were separately incubated at room temperature with *p*-chloromercuribenzoate at 4×10^{-5} M for 10 min, then the decreases in BA and SPI activities caused by the reagent were compared. (e) Disc PAGE patterns of SPI and a commercial BA were compared in a 7.5% gel with a running buffer of pH 8.3 (3). (f) Two μg of SPI and 0.5 ml of 2% potato starch in 16 mM sodium acetate (pH 5) were incubated at 56°C for 1.5 h. An aliquot of the reaction mixture was analyzed for reducing sugar by using maltose as the standard. A small amount of the reaction mixture was filtered through a 0.22 μm membrane before HPLC analysis. A Perkin-Elmer series 4 LC equipped with a 7125S injector, a LC-25 refractive index detector, and a 3600 data station were used. Sugars were separated on a Lichrosorb NH_2 column (250 × 4 mm i.d.; Merck, Darmstadt; Cat. 50231) with acetonitrile:water (80:20, 1 mL/min) as the mobile phase. (g) Neutralization of both BA and SPI activities of the SPI preparation was tested with the antiserum against SPI. (h) Ouchterlony immunodiffusion test (5) of anti-SPI serum against the commercial BA and our purified SPI was done. (i) The SP or SPI was incubated in the standard SP assay mixture at 37°C for 5 min. After heat inactivation at 100°C for 30 s, the reaction mixture was used directly as the substrate solution for SP, BA, or SPI assay under the same conditions of incubation (treatments 4–5 of Table III).

RESULTS AND DISCUSSION

In the DEAE-Sepharose CL 6B and Sephacryl S-200 chromatograms (not shown), SPI and BA activities coeluted. The heat inactivation profiles of SPI and BA activities were also similar (Fig. 1A). The temperature effects on the SPI and BA were parallel in the range from 20 to 50°C (Fig. 1B). Although the highest BA activity was observed at 60°C, no SPI activity could be shown because the SP activity was zero at this temperature. The pH optima of both SPI and BA were identical between 5.5 to 5.8 (Fig. 1C). In the range of pH 5.5 to 6.5, the pH effect on both activities was consistent. Discrepancies at higher and lower pH regions existed but no explanation could be offered. *p*-Chloromercuribenzoate is a known potent inhibitor of sweet potato BA. Data in Table I indicated that the sulfhydryl reagent at a concentration capable of completely inhibiting BA was also able to inhibit the sweet potato SP activity by 14%. This degree of inhibition could be considered as the maximum positive interference to the SPI test, which was performed under the condition where the sulfhydryl reagent pretreated BA was added to SP. Since this possible maximum reagent interference value (14%) is larger than the test value (6%) and both are much smaller than the original SPI activity (85%), it is safe to say that, similar to the BA activity, the SPI activity of the protein is largely

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[3] Abbreviations: SPI, starch phosphorylase inhibitor; BA, β-amylase; SP, starch phosphorylase; G1P, α-D-glucose 1-phosphate.

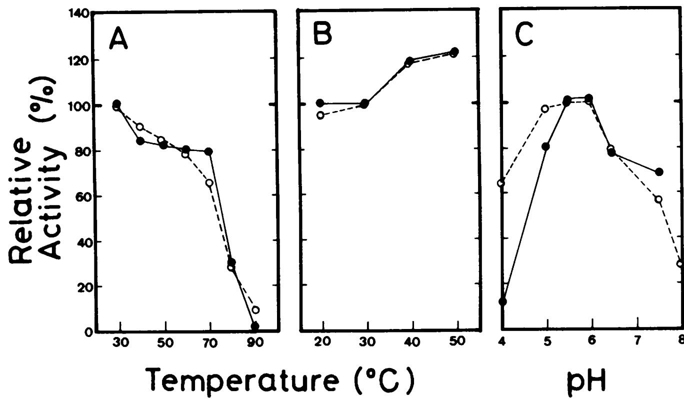

FIG. 1. Comparison of some physicochemical properties of the SPI and BA activities. See the text for conditions of treatment. SPI (●), BA (O). Each assay contained 24 μg SP and 6 μg SPI. Either activity measured at 30°C or pH 5.5 was taken as the full activity (100%). A, Thermal stability; B, temperature effect; C, pH effect.

Table I. *The Effect of p-Chloromercuribenzoate on SP, SPI, and BA Activities*

Treatment	Relative Activity		
	SP	SPI[a]	BA
		%	
SP	100		
SP + SPI	15	85	100
SP + (SPI)[b]	94	6	0
(SP)[b]	86		

[a] The absolute figure of the difference of relative activities of SP in the presence and absence of SPI. Each assay contained 45 μg of SP and 10 μg of SPI. [b] The enzyme was preincubated in 4×10^{-5} M *p*-chloromercuribenzoate at room temperature for 10 min.

FIG. 2. Disc PAGE patterns of purified SPI and a commercial BA in 7.5% gel (pH 8.3). Lanes 1 to 3 contained 1.5, 2.5, and 5 μg of purified SPI; lanes 4 to 7 contained 3, 5, 15, and 30 μg of Sigma BA, respectively, and were stained with Coomassie blue R.

Table II. *Neutralization of SPI and BA Activities by an Antiserum raised against SPI*

To the enzyme solution was added an equal volume of PBS (control) or the serum diluted with PBS. The mixtures were incubated at 37°C for 30 min. After centrifugation, the supernatant was used for either the BA or the SPI assay

Treatment	Relative Activity	
	BA	SPI
	%	
Control	(100)	(100)
Plus preimmune serum		
dilution 1/10	100	100
Plus anti-SPI serum		
dilution 1/100	28	100
1/75	15	91
1/50	9	56
1/10	2	32

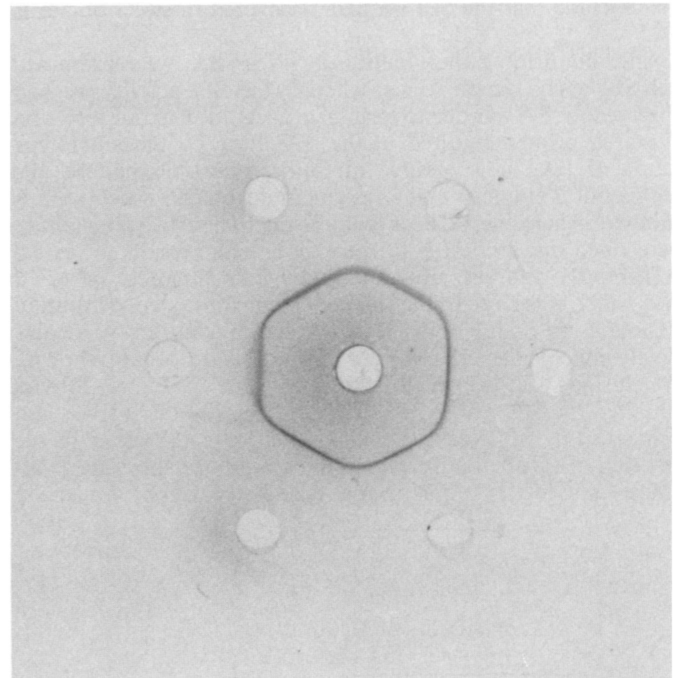

FIG. 3. Ouchterlony double diffusion pattern of anti-SPI serum against SPI and a commercial BA. The central well contained the anti-SPI rabbit serum (x1) and the surrounding wells contained alternately the SPI (2 μg/well) and a commercial BA (4 μg/well) from Sigma.

Table III. *Tests for Interactions Between SP and SPI*

Each assay contained 42 μg of SP and 7 μg of SPI.

Enzyme Treatment	SP Activity	BA Activity
	Pi μmol	*RS[a] μmol*
1. (SP)[b]	1.004	
2. (SPI)		4.112
3. (SP + SPI)	0.387	4.136
4. (SP) →[c] (SPI)		6.104
5. (SPI) → (SP)	1.007	
6. (SPI) → (SP + SPI)	0.337	6.152
7. (SP) → (SP + SPI)	1.024	6.138
8. (SP), No Primer	0.092	

[a] Reducing sugar. [b] ( ) denotes one step of incubation with the designated enzyme(s) at 37°C for 5 min. [c] → denotes heat treatment at 100°C for 30 s between two incubation steps.

if not completely inhibited by the mercurial reagent under these experimental conditions.

The electrophoregram of a commercial BA had a main band showing a mobility identical to that of our purified SPI (Fig. 2). After purifying by gel filtration, electrophoregrams of either native PAGE or SDS-PAGE of the commercial BA became completely identical to those of our SPI preparation. Although the conditions of PAGE used should be able to discern a contaminant protein band in excess of 0.01 μg (0.2% of total SPI in the sample), none could be seen in our SPI preparation. The main product of SPI acting on starch was found to be maltose by the HPLC method, and the degree of hydrolysis was estimated as about 50% (data not shown). Data shown in Table II demonstrated that the anti-SPI serum was able to neutralize both BA and SPI activities, with the former much highly. The double diffusion pattern in Figure 3 showed a completely fused circle, clearly indicating that the commercial BA and our purified SPI had identical antigenic determinants. When the specific amylolytic activities of SPI and the commercial BA were compared, the former was much higher. Based on the results described above, we conclude that the SPI we had discovered in sweet potato is BA.

After confirming the identity of SPI as BA, we reexamined our SPI assay system to see whether the SPI activity we had observed for BA was due to certain artifacts, such as the depletion of starch primer required in the SPI assay by the amylolytic action of BA, or a possible inhibitory effect of maltose, the product of β-amylosis and an α-glucoside, on SP, which utilizes another α-glucoside, G1P, as the substrate. These two possibilities were ruled out according to the experimental results presented in Table III, and also from the findings that maltose up to 10 mM, which was close to the theoretical maximum concentration attainable by β-amylolysis in a 1% starch solution, was completely inactive as an inhibitor toward SP (data not shown). Conclusions that may be drawn from Table III are: (a) SP was inhibited by SPI while the amylolytic activity of SPI was not influenced by SP (treatments 1, 2, and 3); (b) SP alone in the first step of incubation increased the amount of substrate available for amylolysis by SPI in the second step, but SP under the inhibitory influence of SPI was able to do so only insignificantly (treatments 2, 3, and 4); (c) SPI alone in the first step incubation neither affected the amount of primer available nor produced inhibitor for the SP assay in the second step (treatments 1 and 5); (d) amylolysis catalyzed by SPI in the first incubation step did not influence the SPI assay in the second step, although amylolysis proceeded more extensively because SPI was used in both steps (treatments 3, 5, and 6); (e) treatment 7 again revealed that use of SP alone in the first incubation step increased the degree of amylolysis in the second step where SPI was used, and coexistence of SP did not affect the amylolytic activity of SPI. Since the SP activity in treatment 7 was only slightly elevated than in treatment 1 although the former had one more SP using step than the latter, the inhibition of SP by SPI in the second incubation step of treatment 7 was apparent. We may conclude from all these findings that the SPI activity assay is not influenced by the amylolytic activity of SPI.

The data of neutralizing BA and SPI activities by the anti-SPI serum (Table II) have shown that, under certain conditions the SPI could be fully active while the BA was mostly inhibited, suggesting that the SPI activity of the protein is independent of its BA catalytic activity.

The physiological significance of the dual roles played by BA is intriguing because it is an inhibitor toward an enzyme with which it shares a common substrate, and in the sweet potato root at least, the inhibitor and the inhibited enzyme share the same loci of cellular distribution (1, 2).

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