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SIMULTANEOUS PURIFICATION OF α- AND β-AMYLASE FROM GERMINATED RICE SEEDS AND SOME FACTORS AFFECTING ACTIVITIES OF THE PURIFIED ENZYMES[1,2]

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Abstract

α- and β-Amylase from germinated rice seeds were purified simultaneously by a two-step procedure involving glycogen precipitation and DEAE-Sephadex A-50 column chromatography. Both enzymes were apparently homogeneous as they showed only one band respectively on each Disc-PAGE gel. The α-amylase was relatively insensitive to Schardinger dextrins. It was strongly inhibited by cobalt and cupric ions and moderately inhibited by lithium ion. In contrast, the β-amylase can only hydrolyze soluble starch but not β-limit amylopectins and was competitively inhibited by Schardinger α-dextrin with inhibition contant(Ki) of 0.14 mM. It was strongly inhibited by mercurous and cupric ions, only moderately inhibited by potassium and ammonium ions and was stimulated by sodium and cobalt ions.

Key words: *Oryza sativa*; α-amylase; β-amylase; germinated rice seeds; purification; cations; Schardinger dextrins.

Introductions

α-Amylase (α-1, 4-glucan 4-glucanohydrolase, EC 3.2.1.1) and β-amylase (β-1, 4-glucan maltohydrolase, EC 3.2.1.2) catalyze the hydrolysis of 1-4 linked glucose polymer such as starch in plant. Cereal amylases have been the subjects of great research interest because of their importance in plant starch metabolism and food processing. (Kulp, 1975; Thoma *et al.*, 1971; Preiss and Levi, 1980). In germinated

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rice seeds, both α- and β-amylase are present in significant amount. In our previous paper (Shaw and Chuang, 1982), we have purified α-amylase from germinated rice seeds (*Oryza sativa* L. var. Kaohsiung 1). Okamoto and Akazawa (1978) also separately purified α- and β-amylases from *japonica* rice (var. Kimmaze) by different procedures. However, these procedures all seemed to be quite tedious. In the present report, we described a simplified scheme which would purify both α- and β-amylase from germinated rice seeds. Some properties of the purified α- and β-amylase were also investigated.

Materials and Methods

Chemicals

Starch soluble acc. to Zulkowsky was obtained from E. Merk Co. β-Limited amylopectin was prepared according to Greenwood *et al.* (1965). Crystalline sweet potato β-amylase, amylopectin, glycogen and Schardinger dextrins were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

Enzyme Assay

Amylase activities were deterimined either by Nelson's method (Nelson, 1944) or starch-iodine method (Chrispeels Varner, 1967) as indicated in each experiment. In Nelson's method, one enzyme unit was defined as the enzyme amount which produced one mg maltose equivalent of reducing sugar per min at pH 5.5 and 25°C. In starch-iodine assay, one enzyme unit was defined as the enzyme amount that caused 10% absorbance decrease at 620 nm during 8 min reaction at pH 5.5 and 25°C.

Enzyme Purification

Rice seeds (*Oryza sativa* L. var. Taichung 3) were germinated at 27°C in the dark for eight days as described previously (Shaw and Chuang, 1982). The germinated rice samples were homogenized with 20 mM potassium phosphate buffer (pH 6.5) containing 1 mM calcium chloride and 10 mM 2-mercaptoethanol by a polytron homogenizer. After cenntrifuging at 25,000 g for 15 min, the supernatant was subjected to glycogen precipitation as described previously (Shaw and Chuang, 1982) The glycogen precipitated enzymes were collected by centrifugation (25,000g, 20 min), dissolved in a minimum volume of the same phosphate buffer. The sample was applied to a DEAE-Sephadex A-50 column (1 × 30 cm) and eluted with linear gradient of the same phosphae buffer containing NaCl (0.01–0.5 M). The α- and β-amylase can thus be purified simultaneously.

Measurement of Protein

The protein content was determined by the method of Lowry *et al.* (1951)

Assessment of Purity

Disc gel electrophoresis on polyacrylamide gel (13%) at pH 8.9 was carried out as described by Gabriel (1971) to monitor the enzyme purity.

Kinetic Analysis

The inhibition of β-amylases by cyclohexaamylose was analyzed by Dixon method (1953). A plot of 1/v versus inhibitor concentration (I) at two different substrate concentrations was made. If the two lines intersect above the (I) axis, the inhibitor is competitive and projection of this point on the (I) axis is −Ki. If the lines intersect on the (I) axis, the inhibitor is noncompetitive.

Results and Discussion

Rice α- and β-amylase can be purified simultaneously by a two-step procedure, i.e. glycogen precipitation and DEAE-Sephadex A-50 chromatography (Table 1, Fig. 1). Both enzymes were purified to apparent homogeneity as the disc gel electrophoresis showed only one band in each gel (Fig. 2). In DEAE-Sephadex A-50 elution profile (Fig. 1) the first peak amylase can not hydrolyze β-limited dextrin but hydrolyze soluble starch and thus it is consistent with β-amylase. The second peak amylase can use both α-limited dextrin and soluble starch as substrate, therefore, it is consistent with α-amylase, The β-amylase was eluted between 0.80 M to 0.20 M sodium chloride gradient and the α-amylase was eluted between 0.20 M to 0.30 M sodium chloride gradient. Therefore, these two amylases could be purified in large amount using batchwise operation.

Table 1. *Purification of α- and β-amylase from germinated rice seeds*

Fifty grams of rice seeds (Taichung 3) were germinated in the dark for eight days. Enzyme activities were assayed by starch-iodine method.

Step of purification	Volume (ml)	Amylase activity (units/ml)	Total activity (units)	Protein conc. (mg/ml)	Specific activity (units/mg)	Recovery (%)
1. Crude extract	700	15.8	11,060	6.07	2.6	100
2. Glycogen complex formation	30	95.1	2,853	3.69	25.8	26
3. DEAE-Sephadex A-50						
α-Amylase	34	18.1	615	0.26	69.6	5.6
β-Amylase	47	28.7	1,349	0.52	55.2	12.2

Table 2 shows the differential effect of some cations on the rice α- and β-amylase activities. At 27 mM ion concentration, α-amylase was relatively insensi-

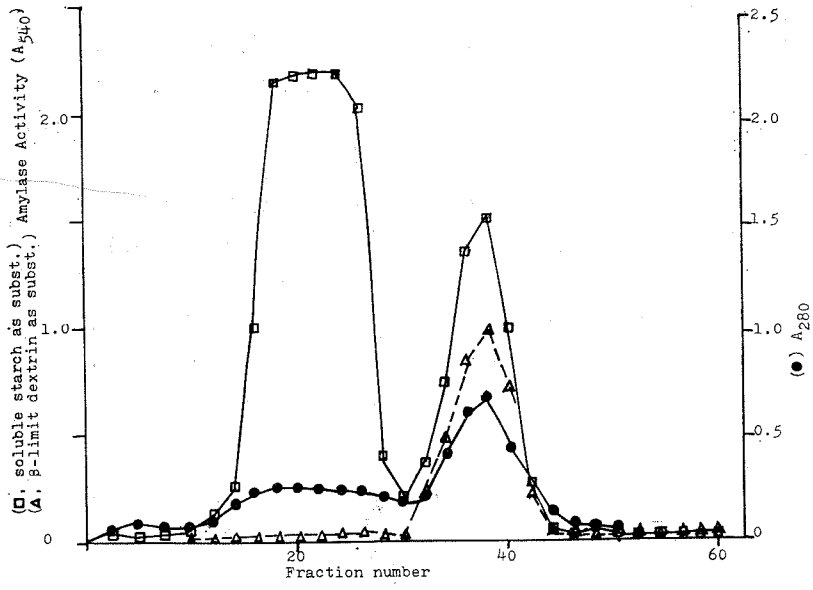

Fig. 1. Elution pattern of α- and β-amylase in DEAE-Sephadex A-50 column chromatography. Glycogen precipitated amylase (21 ml) was applied to DEAE-Sephadex A-50 column (1 × 30 cm). After washing with 100 ml phosphate buffer (pH 6.5), the enzyme was eluted with linear gradient with the same buffer containing NaCl (0.01-0.5 M, 100 ml each) and 3 ml per fraction was collected. The enzyme activities were assayed by Nelson's method.

Fig. 2. Disc gel electrophoresis patterns of purified rice amylase. The purity of α-amylase (a) and β-amylase (b) after DEAE-Sephadex G-50 chromatography was evaluated by Disc-PAGE (13% polyacrylamide) at pH 8.9 according to the method of Gabriel (1971).

tive to monovalent cations and only lithium ion produced moderately inhibitory effect, whereas β-amylase was stimulated by sodium ion and moderately inhibited by potassium, ammonium and cesium ion. As for divalent cations tested, cupric ion completely inhibited both α- and β-amylase activities, cobalt ion strongly inhibited α-amylase but stimulated β-amylase activities and zinc ion strongly inhibited α-amylase but had no effect on β-amylase. Since α-amylase has absolute requirement of caclium ion for its activity (Chuang and Shaw, 1982), the inhibitory effect of these divalent cations might be due to competing calcium binding site and monovalent cations and magnesium ion could be poor competitors with calcium binding. The biological significance of the stimulatory effect of sodium and cobalt ions and the completely inhibitory effect of cupric ion on β-amylase activity are unknown at present, however they appear to be due to specific binding effect rather than nonspecific effect of ionic strength.

Table 2. *Effect of cations on purified rice α- and β-amylase*

Enzymes were preincubated with salt solutions at room temperature for 30 min and then assayed by Nelson's method. The salt concentnation used was 27 mM, except that Hg_2Cl_2 was 1 μM.

Salts	Relative activities (% of control)	
	α-Amylase	β-Amylase
none	100	100
LiCl	55	90
NaCl	100	151
KCl	81	58
NH_4Cl	93	58
CsCl	88	69
$MgCl_2$	74	74
$CoCl_2$	17	198
$CuCl_2$	0	0
$ZnCl_2$	22	100
Hg_2Cl_2	70	9

Mercurous chloride effectively inhibited β-amylase activity at low concentration (1 μM). Since mercurous ion are always in equilibrium with free mercury and mercuric ions: $Hg_2^{+2} \rightleftarrows Hg^0 + Hg^{+2}$ (King, 1959), it might be possible that the inhibition is due to the reaction of mercuric ion with sulfhydryl groups of the enzyme (Okamoto and Akazawa, 1978).

The binding of Schardinger α-dextrin (cyclohexaamylose) and β-dextrin (cyclo-

Table 3. *Effect of Schardinger dextrins on the activities of purified α- and β-amylase from germinating rice seed*

Enzyme activities were assayed by Nelson's method.

Effectors	Relative activity	
	α-Amylase	β-Amylase
None	100	100
Cycloheptaamylose, 3.2 mM	103	89
Cyclohexaamylose, 3.2 mM	75	25

heptaamylose) to sweet potato β-amylase have been used by Thoma and Koshland (1960) as the support for the induced-fit theory of specificity. Sepharose 6B immobilized Schardinger dextrins have been used for the purification of α- and β-amylases (Vretblad, 1974; Silvanovich and Hill, 1976). We did try to purify the rice α-amylase by Sepharose 6B immobilized Schardinger β-dextrin, however, the yield was very low. A recent report (Jacobsen and Higgins, 1982) indicated that cycloheptaamylose-Sepharose affinity chromatography was not able to differentiate α-amylase isozymes. These prompted us to investigate the effect of Schardinger dextrins on the rice α- and β-amylase activities. As shown in Table 3, cycloheptaamylose had little effect on both α- and β-amylase, while cyclohexaamylose at 3.2 mM inhibited α-amylase and β-amylase by 25% and 75%, respectively. This might account for the weak binding of rice α-amylase to Sepharose 6B immobilized cycloheptaamylose. In the Dixon type plot (Fig. 3), cyclohexaamylose showed competitive inhibition of rice β-amylase. Since the inhibitor constant Ki for rice

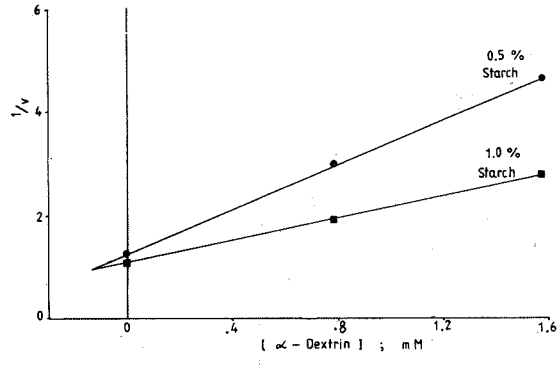

Fig. 3. Dixon plot for the evaluation of cyclohexaamylose inhibition of rice α-amylase. Enzyme activity was assayed by Nelson's method at pH 6.5 (20 mM potassium phosphate buffer) and 30°C. Velocity (v) was expressed as OD_{540} in 8 min reaction.

β-amylase inhibition (0.14 mM) was much lower than the Ki for sweet potato β-amylase inhibition (0.5 mM), cyclohexaamylose should have higher affinity for rice β-amylase.

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發芽水稻 α- 及 β-澱粉酶之同時純化及一些影響酵素活性之因素

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發芽水稻之 α- 及 β-澱粉酶可以肝醣沉澱及 DEAE-Sephadex A-50 管柱層析兩步驟達到同時純化。該二酵素在聚丙烯胺電泳膠中均各顯示其爲單一純度酵素。此 α- 澱粉酶可同時分解溶性澱粉及 β-限制膠質澱粉而 Shardinger 糊精對其活性影響不大。它受鈷及銅離子强抑制但只受鋰離子中等程度抑制。另一方面，β-澱粉酶只能水解溶性澱粉而不水解 β-限制膠質澱粉。它也受 Shardingger α-糊精之競爭性抑制（抑制常數爲 0.14 mM）。亞汞及銅離子爲强抑制劑而鉀及胺離子爲中程度抑制劑。鈉及鈷離子則反而促進 β-澱粉酶活性。