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PROPERTIES OF THE RAW-STARCH DIGESTING AMYLASE OF *Aspergillus* SP. K-27: A SYNERGISTIC ACTION OF GLUCOAMYLASE AND ALPHA-AMYLASE

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ABSTRACT

Glucoamylase and alpha-amylase have been purified from a crude enzyme preparation of *Aspergillus* sp. K-27. The former was thermostable and seemed to have a "starch-binding site", judging from the results of a kinetic study, and the latter synergistically enhanced the degradation of starch granules with glucoamylase.

INTRODUCTION

Conditions have been reported[1,2] for the production of raw-starch digesting amylase by *Aspergillus* sp. K-27, which was isolated from soil using the water-insoluble, lipid–amylose helical complex of wheat starch as the sole carbon source. The enzyme hydrolysed not only cereal starches but also potato starch, and consisted of two enzymes, glucoamylase and alpha-amylase. We now describe the purification, properties, and actions on raw starches of these two enzymes.

EXPERIMENTAL

The fungus, *Aspergillus* sp. K-27, was cultivated as previously described[2].

Glucoamylases from *Rhizopus delemar*[3] and *Aspergillus niger*[4] were purified as described. Purified *A. oryzae* alpha-amylase (Taka-amylase)[5] and rabbit-liver glycogen were generous gifts from Drs. Y. Takeda and M. Nakagawa (Kagoshima University), respectively. *Bacillus subtilis* liquefying- and saccharifying-type alpha-amylases were provided from Daiwa Kasei Co. and Seikagaku Kogyo Co., respectively. Potato starch was fractionated under nitrogen into amylopectin and amylose, using an aqueous mixture of 1-butanol and 3-methyl-1-butanol[6]. Corn

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starch was donated by Sanwa Denpun Kogyo Co. Cyclomalto-hexaose (cG_6), -heptaose (cG_7), and -octaose (cG_8), and maltose and maltotriose (the highest grade) were donated by Nihon Shokuhinkako Co. and Hayashibara Biochemical Lab.

Enzyme assays. — *(a) Method* A. A mixture containing enzyme solution (100 μL) and 1.11% of soluble starch in 50mM sodium acetate buffer (900 μL, pH 5.5) containing 2mM $CaCl_2$ was incubated at 45°. The reaction was stopped by adding 1 mL of Somogyi reagent[7]. The resulting reducing sugar was determined colorimetrically by the method of Nelson[8], except that the heating time was extended to 30 min in order to obtain the same reducing value per mole of malto-oligosaccharides[9].

(b) Method B. An aliquot (50 μL) of the enzyme solution was incubated with 2% of soluble starch in water (200 μL) for 15 min at 45°. The reaction was stopped by heating for 1 min at ~100° and the resulting D-glucose was measured by the D-glucose oxidase–peroxidase method[10]. The enzyme solution was diluted with 50mM sodium acetate buffer containing 0.02% of bovine serum albumin to prevent inactivation by high dilution[11].

One unit (U) of activity is defined as the amount of enzyme which produces 1 μmol of glucose or reducing sugar equivalent to glucose per min under the conditions.

Total carbohydrate was measured by the anthrone–sulfuric acid method[12].

RESULTS AND DISCUSSION

Isolation and purification of glucoamylase and alpha-amylase. — The culture filtrate was adjusted to pH 5.5 and concentrated to one-fifth volume, and

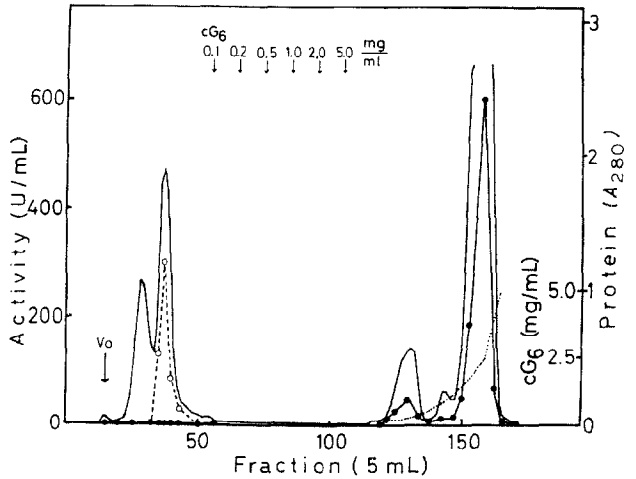

Fig. 1. Chromatography (5.6-ml fractions at 34 mL/h) of the crude enzyme preparation of *Aspergillus* sp. K-27 on a column (1.8 × 40 cm) of cG_6-coupled Sepharose 6B: ——, protein (A_{280}); --○--, activity determined by method *A*; —●—, activity determined by method *B*; ·····, cG_6 concentration.

ammonium sulfate was added to 60% saturation. The precipitate was collected, dissolved in 50mM sodium acetate buffer (pH 5.5), and freed from insoluble material, and 2-propanol at −20° was added to 50% to the solution in an ice bath. The precipitate was collected quickly, and a solution in 50mM sodium acetate buffer (pH 5.5) was applied to a column of cG_6-coupled Sepharose 6B[3]. Two protein peaks were obtained on elution with the buffer, and one large and two small peaks on stepwise elution with the buffer containing increasing amounts of cG_6 (Fig. 1).

The activity associated with the large protein peak (fractions 24–49) was measurable only by method *A*, and thus was identified as alpha-amylase. Fractions 24–49 were combined, concentrated, applied to a column of Sephadex G-100, and eluted by 50mM sodium acetate buffer (pH 5.5). The active fractions were combined and applied to a column of DEAE-Toyopearl 650S. As shown in Fig. 2, alpha-amylase activity was found as two peaks. Disc electrophoresis gave a single band for the first peak, fractions 54–59 (Fig. 3A), but the second peak was a mixture of the enzyme in the first peak and another one. Since the latter enzyme could not be separated by re-chromatography on this column, fractions 54–59 were combined and purified by re-chromatography on the same column as an alpha-amylase fraction.

The two activities (fractions 122–137 and 147–163 in Fig. 1) eluted from the cG_6-coupled Sepharose 6B column were due to glucoamylase because the activities determined by methods *A* and *B* coincided. A major peak (fractions 151–166) was collected, and then purified by chromatography on a column of DEAE-Toyopearl 650M to be homogeneous in disc gel electrophoresis (Fig. 3B).

The purifications of alpha-amylase and glucoamylase are summarised in Tables I and II. Alpha-amylase did not hydrolyse maltose, and glucoamylase showed no contamination by alpha-amylase in the sensitive assay[3] using partially hydroxypropylated corn starch as substrate. The glucoamylase contaminated with

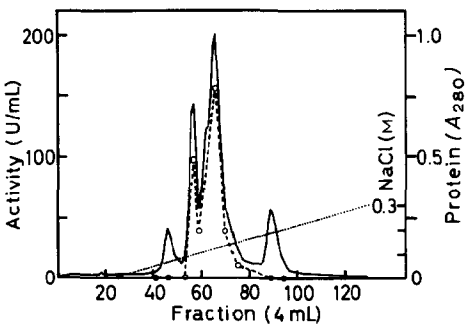

Fig. 2. Chromatography (5-mL fractions at 34 mL/h) of the alpha-amylase fraction on a column (0.9 × 27 cm) of DEAE-Toyopearl 650S: ——, protein (A_{280}); --○--, activity determined by method *A*; ·····, NaCl concentration.

Fig. 3. Disc-gel electrophoresis of purified alpha-amylase (**A**) and glucoamylase (**B**) in 7.5% polyacrylamide gels at pH 9.5. The proteins were stained with Coomassie Brilliant Blue R-250.

TABLE I

PURIFICATION OF THE ALPHA-AMYLASE OF *Aspergillus* SP. K-27

	Protein (mg)	*Activity[a] (U)*	*Specific activity (U/mg)*	*Recovery (%)*
Culture filtrate	1570	24000	15.3	100
Ammonium sulfate precipitation	393	20100	51.1	83.8
2-Propanol precipitation	251	18500	73.7	77.1
cG_6-Sepharose 6B column	18.8	2780	149	11.6 (100)[b]
Sephadex G-100 column	15.4	2310	150	9.6 (83.1)
DEAE-Toyopearl 650S column (1)	5.2	855	163	3.6 (30.8)
DEAE-Toyopearl 650S column (2)	4.7	770	164	3.2 (27.7)

[a]Determined by method *A* (see Experimental). [b]At this step, the enzyme prepration was free from glucoamylase; therefore, the recovery was taken as 100%.

TABLE II

PURIFICATION OF THE GLUCOAMYLASE OF *Aspergillus* SP. K-27

	Protein (mg)	*Activity[a] (U)*	*Specific activity (U/mg)*	*Recovery (%)*
Culture filtrate	1570	16700	10.6	100
Ammonium sulfate precipitation	393	15500	39.4	92.8
2-Propanol precipitation	251	14200	56.6	85.0
cG_6-Sepharose 6B column	149	13300	89.3	79.6
DEAE-Toyopearl 650M column	112	10100	90.2	60.5

[a]Determined by method *B*.

alpha-amylase is capable of hydrolysing the substrate rapidly beyond a limit, but hydrolysis by the pure enzyme stops at the limit. The preparations of glucoamylase and alpha-amylase were each free from phosphatase.

Properties and actions of glucoamylase. — Glucoamylase showed a single band in SDS-gel electrophoresis, and its molecular weight was estimated to be 76,000. The enzyme was shown to be a glycoprotein on staining of the disc gel by the method of Zacharius *et al.*[13]. The optimum pH values for soluble and raw starches were 4.5–7.1 and 4.2–5.9, respectively, and it was stable between pH 4.0 and 7.1 (30 min, 55°). The enzyme was fairly thermostable, *i.e.*, it retained 100% and 90% of its activity after treatment for 4 h at 55° and for 1 h at 60°, respectively, but lost 80% of its activity on incubation for 1 h at 65°. The activity was not affected by 5mM Al^{3+}, Ba^{2+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Pb^{2+}, and Zn^{2+} (as chlorides), but 15% of the activity was lost with Hg^{2+}.

The enzyme (1 U/mg of substrate) hydrolysed rabbit-liver glycogen and potato amylopectin to the extents of 97.8% and 80.5%, respectively, *i.e.*, to the same extents as with the glucoamylases of *Rhizopus delemar*[3] and *A. niger*[4]. Hydrolysis did not proceed further after incubation for 24 h, although the enzyme was still active. Therefore, the phosphate esters in the substrate appear to block the action of the microbial glucoamylases.

The kinetic constants for maltose and maltotriose as low-molecular-weight substrates and for potato amylopectin and rabbit-liver glycogen as high-molecular-weight substrates are listed in Table III. Comparison of these values with those (see Tables III and IV of ref. 3) for *R. delemar* and *A. niger* showed that (*a*) the K_m values for low-molecular-weight substrates were almost the same as those for *A. niger* and were lower than those for all the components of *R. delemar,* and that the V_{max} values for K-27 were slightly greater than those for *A. niger* and GIII of *R. delemar* and were slightly smaller than those for GI and GII of *R. delemar*; (*b*) the K_m values for amylopectin and glycogen were the same and half the value of those for GIII, and 1/1300–1/1400 and 1/1200–1/2700 of those for *R. delemar* GI and GII and *A. niger,* respectively, and the V_{max} values for these high-molecular-weight substrates were double those for *A. niger* and slightly larger and smaller than those for GIII, and GI and GII, respectively. Thus, the glucoamylase of K-27 showed high affinity not only for high- but also for low-molecular-weight substrates. The V_{max}/K_m values indicate that the activity of this enzyme is superior to those of other similar enzymes. The above data suggest that the enzyme has a similar "starch-binding site" to that indicated for *R. delemar* glucoamylase GIII[3].

Properties and actions of alpha-amylase. — The molecular weight of the alpha-amylase was estimated to be 65,000 by SDS-gel electrophoresis. This enzyme also was found to be a glycoprotein. The optimum pH for soluble starch was 5.5, and it was stable at ~45° (pH 5.5, 30 min) and between pH 5.5 and 6.5 (30 min, 45°).

TABLE III

KINETIC CONSTANTS OF GLUCOAMYLASES

Substrate[a]	K_m (μM)[b]	V_{max} (U/mg)[c]	V_{max}/K_m (U/mg/μM)
Maltose	1100	7.9	7.2×10^{-3}
	(1300)[d]	(11)	(8.5×10^{-3})
Maltotriose	350	37	1.1×10^{-1}
	(440)	(51)	(1.2×10^{-1})
Amylopectin (Potato)	0.2	64	3.2×10^{2}
	(0.2)	(68)	(3.4×10^{2})
Glycogen (Rabbot liver)	0.3	57	1.9×10^{2}
	(0.3)	(87)	(2.9×10^{2})

[a]The D-glucose oxidase method was used when maltose and maltotriose were the substrates, and Park–Johnson's method[16] when they were amylopectin and glycogen. [b]Concentration of non-reducing, terminal residues. [c]Reaction conditions: 40°, pH 4.5. [d]The values in parentheses were obtained at 45°.

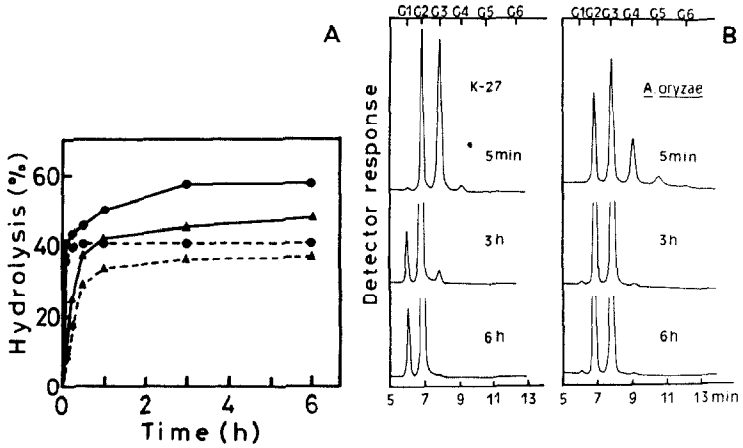

Fig. 4. Actions of alpha-amylases on potato amylose. Each reaction mixture contained 5 mg of amylose and 5 U (●) or 0.5 U (▲) of the enzymes (——, K-27; ----, *A. oryzae*) in 2 mL of 50mM sodium acetate buffer (pH 5.5). Hydrolysates were analysed essentially by method *A* (A) or by h.p.l.c. on TSK gel NH_2-60 using aqueous 50% acetonitrile as the eluant (B).

The K-27 alpha-amylase and Taka-amylase (1 U/mg of substrate) hydrolysed potato amylose to extents of 60% and ~40%, respectively, on incubation for 6 h (Fig. 4A). The results of h.p.l.c. analyses showed that maltotriose accumulated in the initial stage with each enzyme, and then it was hydrolysed into maltose and glucose with the K-27 enzyme but remained with Taka-amylase (Fig. 4B).

The relative activities for various substrates of the two enzymes (Table IV) indicated that the K-27 alpha-amylase showed higher activity toward amylose than toward amylopectin and soluble starch, but Taka-amylase showed almost the same activities toward these substrates. In addition, the K-27 enzyme showed higher relative activities toward cG_7 and cG_8 than Taka-amylase. This finding suggests that the alpha-amylase of *Aspergillus* sp. K-27 shows higher activity for helical and circular substrates than Taka-amylase, because amylose has a partial helical structure in solution[14].

TABLE IV

RELATIVE ACTIVITIES OF THE ALPHA-AMYLASES OF *Aspergillus* SP. K-27 AND *Aspergillus oryzae*

Substrate (0.5%)	*K-27*	A. oryzae
Maltose	>0.01	>0.01
Maltotriose	6.5	0.3
cG_6	1.9	1.9
cG_7	19	4.6
cG_8	72	65
Glycogen (oyster)	57	68
Amylopectin (potato)	101	95
Amylose (potato)	121	102
Soluble starch	100	100

Synergistic action of glucoamylase and alpha-amylases during the digestion of raw starch. — Twenty U of glucoamylase digested 250 mg of raw corn-starch rapidly, 40% of the starch being hydrolysed on incubation for 9 h. The addition of 5 U of alpha-amylase to the glucoamylase increased the hydrolysis of the raw starch 2.9- and 1.7-fold on incubation for 3 and 9 h, respectively, although the alpha-amylase hydrolysed only 2 and 15% of the starch, respectively (Fig. 5A). Furthermore, the addition of only 0.2 U of alpha-amylase led to a 1.8-fold increase in the initial reaction rate, and was sufficient for almost complete hydrolysis within 24 h under the conditions employed. Thus, the glucoamylase played a major role in the hydrolysis of raw starch and the alpha-amylase greatly synergised the action of glucoamylase. Similarly, the two enzymes together digested synergistically raw potato starch but at a somewhat lower rate than for corn starch (Fig. 5B), *i.e.*, the addition of 0.2 U of alpha-amylase increased the degradation of the starch almost 2-fold. Another alpha-amylase fraction (fractions 60–70 in Fig. 2), although a mixture, showed the same properties as those mentioned above (data not shown). In considering the mode of action of glucoamylase, particular attention should be paid to the purity of the enzyme, because contamination with a small amount of alpha-amylase has a marked synergistic effect on the action of the glucoamylase and may lead to misunderstanding about the action of the enzyme.

Alpha-amylase also enhanced the hydrolysis of raw starch with *Rhizopus delemar* glucoamylase GIII, which has a "starch-binding site", but did not show the synergistic effect with glucoamylase GII, which does not have the site. Thus, 20 U

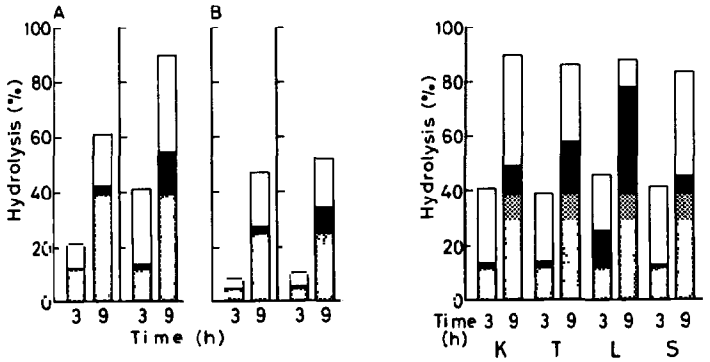

Fig. 5. Hydrolyses of raw corn starch (**A**) and potato starch (**B**) with *Aspergillus* sp. K-27 glucoamylase and the synergistic effect. Each reaction mixture contained 250 mg of starch, 20 U of glucoamylase, and 0.2 U (left) and/or 5 U (right) of alpha-amylase in 5 mL of 50mM sodium acetate buffer (pH 5.5) containing 2mM $CaCl_2$ and was incubated at 45° with mild stirring. The dotted and filled, and open bars show hydrolysis with the individual actions of glucoamylase and alpha-amylase and the increment with the concomitant actions of the two enzymes (synergistic effect), respectively.

Fig. 6. Synergistic actions with *Aspergillus* sp. K-27 glucoamylase and alpha-amylases of various origins on raw corn starch: 5% raw corn starch was digested with 20 U of glucoamylase and/or 5 U of alpha-amylase at 45°; **K**, K-27 alpha-amylase; **T**, Taka-amylase; **L**, *B. subtilis* liquefying-type; **S**, *B. subtilis* saccharifying-type; for the other symbols, see Fig. 5.

of *R. delemar* glucoamylase GIII and GII hydrolysed 32.4% and 4.8% of raw corn starch, respectively, on incubation for 9 h, and the hydrolysis increased to 74.7% (1.6-fold) and 18.5% (0.99-fold), respectively, on the addition of the K-27 alpha-amylase (5 U) which hydrolysed 15.0% of the starch. This aspect is being studied further.

Several alpha-amylases of microbial origin also synergised the digestion of corn starch with glucoamylase of *Aspergillus* sp. K-27 (Fig. 6). The ratios of [hydrolysis involving the concomitant actions of the two enzymes] to [sum of the hydrolyses by the individual enzymes] on the addition of Taka-amylase, and *Bacillus* liquefying and saccharifying type alpha-amylases, were 2.7 (1.5), and 1.8 (1.1) and 3.1 (1.8), respectively, on incubation for 3 h (9 h). Thus, the synergism of alpha-amylase with glucoamylase is not a phenomenon specific to the K-27 amylase system. The alpha-amylases exhibiting lower activities for raw starch are more synergistic with glucoamylase, and the synergism is more obvious in the early stage of hydrolysis.

Ueda and Kano[15] reported that Taka-amylase and *Rhizopus* alpha-amylase could enhance the raw-starch degradation by *Rhizopus* glucoamylase, and it was assumed that this was due to the hydrolysis of amylose with alpha-amylase. *Aspergillus* sp. K-27 was isolated on a medium containing an amylose–lipid complex as the sole carbon source[1], and the alpha-amylase appears to act well on this complex which is an obstacle in the hydrolysis with glucoamylase. This may be one of the keys for the degradation of raw starch.

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