Purification and Some Properties of the *Chalara* Enzyme†

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A new raw starch-digesting amylase was obtained from a strain of mold, identified as *Chalara paradoxa*. This enzyme was purified by starch adsorption, DEAE-Cellulose column chromatography and Toyopearl HW-55 gel filtration to 60 fold of the activity of the original culture liquor. It gave a single band on disc gel electrophoresis, and the molecular weight estimated by SDS disc gel electrophoresis was 82,000. In isoelectric focusing, the enzyme showed six bands, at isoelectric points (pI) of 3.20, 3.26, 3.34, 3.41, 3.49 and 3.52. This amylase showed maximum activity at 45–50°C and pH 5.0. The pH-stability range was relatively wide. The enzyme retained more than 80% of its initial activity in the range of pH 4.5 to 7.5. It was stable below 50°C, and thermostability was improved by the addition of calcium ion. The relative reaction rates of the enzyme on various metal ions and inhibitors were also studied. The anomeric configuration of the product obtained from starch by the enzyme was beta-configuration. The ratio of raw starch-digesting activity to gelatinized starch-saccharifying activity of the enzyme was 31.5%.

INTRODUCTION

In the previous papers,^{1,2)} we reported the discovery of a new raw starch-digesting amylase from *Chalara paradoxa* and the studies of the optimum culture conditions of *Chalara paradoxa* and degradation of various raw starches by the amylase. This paper summarizes our

studies of the purification and characterization of the amylase.

MATERIALS AND METHODS

Materials. Raw starch was sterilized by gamma rays at the level of 3 mega rad. Heat-moisture treated starch was obtained by heating potato starch at 121°C for 20 min at the moisture content of 18%. Corn steep liquor was donated by Nihon Shokuhin Kako Co., Japan. Carrier Ampholyte (23% solution, pH 2.5–5.0) for the isoelectric focusing was purchased from Pharmacia Fine Chemicals AB, Uppsala.

Purification of the enzyme. *Chalara paradoxa* was cultured on a rotary shaker (200 rpm) using 1-liter liquid medium (pH 4.5) containing 6.5% corn steep liquor, 4% raw sago starch, 0.7% beef extract (Difco), 0.3% NaCl and 0.08% FeSO₄·7H₂O in 5-liter Erlenmeyer flask for 7 days at 30°C. The cells were removed by

† Studies on the Novel Raw Starch-Digesting Amylase Obtained from *Chalara paradoxa* (Part 3).

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Symbols and abbreviations: IU, one International Unit (IU) as defined according to the International Commission on Enzymes is the amount of enzyme that hydrolyzes one micromole of glycosidic bonds per min under optimal conditions.

centrifugation at 7500 rpm for 30 min (4°C), and was obtained approximately 2 liters of culture liquor from several culture flasks. Then, starch adsorption was carried out as follows. Sixteen g of heat-moisture treated starch was added into 400 ml of culture liquor in 500-ml Erlenmeyer flask and the suspension was stirred for 15 hr at 4°C. At the end of this period, the starch adsorbed enzyme was collected by centrifugation and washed with cold water. The washed starch was suspended in distilled water with stirring at 40°C for 20 min to desorb the enzyme. The adsorption and desorption process were repeated twice to increase the yield of the enzyme from culture supernatant. Approximately 2.5 liters of the extract solution was applied to a DEAE-Cellulose (DE 52, Whatman Co.) column (2.64×53 cm), equilibrated in 5 mM phosphate buffer (pH 8.0) and eluted by a gradient of 0 to 0.3 M sodium chloride solution. The enzyme fraction was rechromatographed on a smaller DEAE-Cellulose column (1.5×36 cm) using the same gradient system. The resulting enzyme fraction was further purified by gel-filtration chromatography on a Toyopearl HW-55 (Toyosoda Co.) column (1.90×90 cm).

Analytical methods. For the determination of the reducing sugar, the Somogyi-Nelson method³⁾ was employed with glucose as the standard. Raw starch-digesting activity was assayed using a reaction mixture consisting of 20 mg of corn starch, 0.2 ml of 0.1 M acetate buffer solution (pH 4.5) and 1.6 ml of distilled water. After preincubation at 40°C for 3 min, 0.2 ml of enzyme solution was added and incubated at 40°C in a shaker for 30 min.

Gelatinized starch-saccharifying activity was assayed using substrate solution consisting of 0.25 ml of 2% soluble starch solution and 0.25 ml of 0.1 M acetate buffer solution (pH 4.5). After preincubation, 0.5 ml of suitably diluted enzyme solution was added, then the mixture was incubated at 40°C for 30 min.

Cleavage of one μ mol of glucosidic bond per min under the conditions was expressed as one International Unit (IU) of enzyme activity.

Protein concentration was measured by modified Hartree method⁴⁾ with bovine serum albumin as the standard.

Gel electrophoresis. In gel electrophoresis, polyacrylamide gel mixtures in tubes were overlaid with distilled water and photopolymerized by exposure to a fluorescent light for 30 min. The gels were stained for protein with 0.25% Coomassie brilliant blue R-250 (Nakarai Kagaku Co., Japan) solution. Bromophenol blue (Nakarai Kagaku Co.) was used as the dye marker. For developing the zymogram, the unstained gel was incubated for 10 min at 40°C in 2.0% soluble starch solution. After staining with 0.2% iodine-2% potassium iodide solution and washing with water, the location of amylase activity appeared as a clear band on a blue background.

Polyacrylamide disc gel electrophoresis (7.5%, pH 8.9) was carried out to test the homogeneity of the purified enzyme as described by Davis.⁵⁾ Samples were run on 5×70 mm gels for 2 hr at 4°C using 2 mA/gel.

SDS polyacrylamide disc gel electrophoresis was performed at pH 7.2 by the method of Weber and Osborn⁶⁾ using thermostable RNA polymerase B (SDS-Page marker I, Seikagaku Kogyo Co., Japan) as a standard protein, which had five subunits (Z , α , X , β , β'). Isoelectric focusing in 4% polyacrylamide gel was performed to examine the isoelectric point of the enzyme as described by Miyazaki *et al.*⁷⁾ As electrode solutions, 0.1 M H_2SO_4 solution (+) and 0.2 M L-histidine solution (−) were used (pH 2.5–5).

Samples were run on 5×90 mm gel tubes for 15 hr at 4°C using 200 V. Zymogram was carried out by the same procedure as in 7.5% polyacrylamide disc gel electrophoresis.

Optimum reaction conditions, enzyme stability and effects of various metal ions and inhibitors. The reducing sugar formed by enzyme action was determined by the Somogyi-Nelson method.³⁾

Amylase assays were performed to distinguish raw starch-digesting activity and gelatinized starch-saccharifying activity from each other. Raw starch-digesting activity was determined, in most case, by following method. 0.1 M acetate buffer (pH 5.0) and raw corn starch suspension (20 mg/ml) were used as buffer solution and substrate, respectively. The optimum pH of the reaction was determined as relative activity

after incubation for 30 min at 40°C in a shaker. The reaction mixture consisted of 0.1 ml of enzyme solution (0.03 IU as raw starch-digesting activity), 0.4 ml of 0.1 M buffer solution of different pH value and 0.5 ml of the substrate suspension (10 mg corn starch). To determine the pH-stability, 0.4 ml of the enzyme solution was treated with 0.2 ml of 0.1 M buffer solution of various pH values for 60 min at 40°C. Then the remaining enzyme activity was determined. The thermostability of the enzyme was determined by treating 0.4 ml of the enzyme solution for 15 min with 0.2 ml of 0.1 M acetate buffer (pH 5.0) at various temperatures. Then, the remaining activity was determined at 40°C. The optimum temperature of the reaction was determined by incubating 0.1 ml of the enzyme solution (0.03 IU) with 0.4 ml of 0.1 M acetate buffer (pH 5.0) and 0.5 ml of the substrate suspension (10 mg) at various temperatures in the shaker for 30 min. The effect of calcium ion on the thermostability was examined using the enzyme dialyzed against deionized-distilled water for 8 hr. The dialyzed enzyme was incubated for 60 min at various temperatures in the presence and absence of 5 mM calcium chloride, and the remaining activity was determined. The effects of various metal ions and inhibitors were determined by incubating 0.1 ml of the dialyzed enzyme solution (0.03 IU) with 0.3 ml of 0.1 M acetate buffer solution, 0.1 ml of 10 mM metal ion- or inhibitor solution and 0.5 ml of substrate suspension at 40°C in the shaker for 30 min. Gelatinized starch-saccharifying activity was assayed using 2% soluble starch solution instead of raw starch granule. Principles of the determination was essentially as by the methods mentioned above.

Determination of anomeric configuration. Anomeric configuration of the product formed by the action of the enzyme was determined according to the method of Robyt and French⁸⁾ using digital polarimeter DIP-181 (Nihon Bunko Co., Japan). Raw corn starch (180 mg) was suspended in 18 ml of 0.01 M acetate buffer (pH 5.0). 8.7 IU of enzyme was added to the starch suspension. Optical rotation measurements were made at various intervals using a 5 cm tube. 7 mg of anhydrous sodium carbonate was added per 6 ml of digest. This brought the

pH to 10.1 and accelerated the mutarotation.

Ascending paper chromatography was carried out by the same method as reported in the previous paper.²⁾

RESULTS AND DISCUSSION

1. Purification of the enzyme

Culture liquor of *Chalara paradoxa* PNS-80 was purified by several steps starting from starch adsorption, 1st and 2nd DEAE-Cellulose column chromatography and Toyopearl HW-55 gel filtration chromatography. As shown in Table 1, 54% of the initial raw starch-digesting activity was recovered by starch adsorption and the specific activity of the enzyme increased 12 fold by this step. The black color of the culture liquor was removed to a great extent by this step. Desorbed solution from starch was further purified by DEAE-Cellulose column chromatography using a gradient elution system of sodium chloride (0-0.3 M) in 5 mM phosphate buffer (pH 8.0). By this step, most of α -amylase activity was removed. The active fractions were combined and rechromatographed using smaller DEAE-Cellulose column. Figure 1 shows the elution profile of the rechromatographed enzyme. The peak of the enzyme activity almost corresponded to that of protein. The active fractions were collected, concentrated in a collodion bag, then applied to a gel filtration on Toyopearl HW-55 (Fig. 2). The specific activity of each fraction was determined and the fractions of higher specific activity (>8.0 IU/mg protein) were collected for the determination of optimum conditions of the enzyme reaction and stability. The collected fractions, shown as shadow in Fig. 2, showed a single band on

Table 1. Purification scheme for *Chalara paradoxa* amylase.

Purification step	Total activity (IU)	Specific activity (IU/mg)	Recovery (%)
Culture sup.	2830	0.22	100
Starch adsorption	1524	2.62	54
DEAE-Cellulose (1st)	303	5.14	11
DEAE-Cellulose (2nd)	289	7.27	10
Gel filtration	88	13.30	3

Fig. 1. DEAE-Cellulose column rechromatography of the partially purified enzyme.

Active enzyme fractions (fraction numbers 46 to 60) from the first DEAE-Cellulose column were pooled and rechromatographed on a column (1.5×36 cm) using the same gradient system. Activity indicates raw starch-digesting activity.

Fig. 2. Toyopearl HW-55 gel filtration of the partially purified enzyme.

Active enzyme fractions (fraction numbers 39 to 58) from the second DEAE-Cellulose column (Fig. 1) was pooled and chromatographed on a Toyopearl HW-55 column (1.9×90 cm). The column was equilibrated with 0.02 M phosphate buffer (pH 6.5) containing 0.2 M sodium chloride. The elution (18 ml/hr) was performed with the same buffer. Activity indicated raw starch-digesting activity. Shaded fractions (Fr. 23-38) are pooled and used as purified enzyme.

7.5% polyacrylamide disc gel electrophoresis and zymogram as shown in Fig. 3. The purification scheme of the enzyme (Table 1)

Fig. 3. Polyacrylamide gel electrophoresis. (1) Polyacrylamide disc gel electrophoresis (7.5%), (2) Zymogram.

Fig. 4. pH-activity curve of the enzyme.

○—○, raw starch-digesting activity; ●—●, gelatinized starch-saccharifying activity.

pH 3-4, $\text{CH}_3\text{COONa-HCl}$ buffer; pH 4-6.5, $\text{CH}_3\text{COONa-CH}_3\text{COOH}$ buffer; pH 6.5-8.5, $\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ buffer; pH 8.5-10, $\text{NaHCO}_3\text{-Na}_2\text{CO}_3$ buffer.

shows that the specific activity of the enzyme is 13.3 IU/mg-protein for raw starch-digesting activity and 42.4 IU/mg-protein for gelatinized starch-saccharifying activity. The ratio of raw starch-digesting activity to gelatinized starch-saccharifying activity was 31.5%. The puri-

Fig. 5. pH-Stability curve of the enzyme.
Various buffers were used as in Fig. 4.

Fig. 6. Optimum reaction temperature of the enzyme.

○—○, raw starch-digesting activity;
●—●, gelatinized starch-saccharifying activity.

fication factor of this step is 60 fold over the original culture liquor.

2. Properties of the *Chalara paradoxa* PNS-80 raw starch-digesting amylase

pH-activity and pH-stability. Figures 4 and 5 show pH-activity and pH-stability of the enzyme. The optimum pH of the enzyme was 5.0 and the enzyme kept higher than 80% of the maximum activity between 4.5 and 6.0 in both raw starch-digesting activity and gelatinized starch-saccharifying activity. After being kept at various pH values for 60 min at 40°C in the absence of substrate, the enzyme retained about 80% of its activity between pH 4.5 and 7.5,

Fig. 7. Thermostability of the enzyme.

Fig. 8. SDS polyacrylamide gel electrophoresis.

SDS polyacrylamide (5%) gel electrophoresis was carried out using thermostable RNA polymerase B as a standard, which had five subunits. MW: Z, 39,000; α , 42,000; X, 100,000; β , 140,000; β' , 180,000. St, thermostable RNA polymerase B; S, *Chalara paradoxa* amylase.

in both starch-digesting activities. At a pH above 9.0, most of the activity was lost.

Optimum temperature and thermostability. Figures 6 and 7 show the optimum reaction temperature and thermostability of the enzyme. The optimum temperature of raw starch-digesting activity was 45°C and that of gelatinized starch-digesting activity was 50°C.

Fig. 9. Isoelectric focusing of the enzyme.

(1) Isoelectric focusing (4% polyacrylamide gel), pI: A₁, 3.52; A₂, 3.49; A₃, 3.41; A₄, 3.34; A₅, 3.26; A₆, 3.20. (2) Zymogram (isoelectric focusing).

Fig. 10. Optical rotation study of the action of the *Chalara paradoxa* raw starch-digesting amylase with raw corn starch.

The triangular symbol indicates the optical rotation after the addition of alkali to the digests.

Table 2. Effects of various metal ions and inhibitors on the activity of *Chalara paradoxa* amylase.

	Gelatinized	Raw
Metal ion		
CoCl ₂	103	100
BaCl ₂	103	98
Li ₂ SO ₄	100	100
NiCl ₂	100	98
MgSO ₄	100	97
CaCl ₂	99	100
ZnSO ₄	98	97
FeSO ₄	97	98
CuSO ₄	90	87
MnSO ₄	87	85
AgNO ₃	88	73
Al ₂ (SO ₄) ₃	79	18
FeCl ₃	79	7
HgCl ₂	43	21
SrCl ₂	113	99
Inhibitor		
Iodoacetamide	99	84
PCMB*	99	78
None	100	100
(Cysteine)	115	140

* *p*-Chloromercuribenzoate.

The concentration of metal ions and inhibitors were 1 mM.

Fig. 11. Paper chromatogram of hydrolysis product of raw corn starch by the *Chalara paradoxa* raw starch-digesting amylase.

Corn starch 50 mg, enzyme 0.46 IU, total volume 5 ml, temperature 40°C. St, standards.

The enzyme was extremely unstable above 50°C, and all activity was lost after 15 min at 60°C. The discrepancy between the optimum temperature and the thermostability of the enzyme apparently results from the protective effect of the substrate on the enzyme stability. The thermostability of the both activities was improved by 5°C by addition of 5 mM CaCl₂.

SDS polyacrylamide gel electrophoresis and isoelectric focusing. Figure 8 shows the result of SDS polyacrylamide gel electrophoresis. The molecular weight of the enzyme was 82,000 by this method. Figure 9 shows the result of the isoelectric focusing in 4% polyacrylamide gel. The enzyme showed six protein bands (A₁, A₂, A₃, A₄, A₅ and A₆), and all of those bands had amylase activities on the zymogram. The isoelectric points of A₁ to A₆ were 3.52, 3.49, 3.41, 3.34, 3.26 and 3.20, respectively. At this moment, we are not aware whether these bands are isozymes.

Effect of metal ions and inhibitors. In Table 2, the results of the effects of various metal ions and inhibitors on the enzyme activity are shown. At the level of 1 mM concentration, Hg²⁺, Fe³⁺, Al³⁺ and Ag⁺ strongly inhibited both activities. The inhibition of these metal ions was stronger in raw starch-digesting activity compared with gelatinized starch-saccharifying activity. Iodoacetamide and *p*-chloromercuribenzoic acid slightly inhibited only raw starch-digesting activity.

Anomeric configuration of the product. The anomeric configuration of the reaction products by the enzyme action on raw starch was observed by the method of Robyt *et al.* Sampled reaction mixture was centrifuged to remove unreacted starch granules and clear supernatant was obtained. The optical rotation of the solution was determined before and after addition of anhydrous sodium carbonate to adjust the pH of the solution which accelerate the mutarotation. As shown in Fig. 10, the addition of alkali to a digest resulted in an increase in the optical rotation, which means that the products of the enzyme action were in beta-configuration. Figure 11 shows a paper chromatogram of the enzyme reaction on raw starch granule. The chromatogram clearly shows that the product is only glucose. From these

two experiments, *Chalara paradoxa* raw starch-digesting amylase should be classified as a glucoamylase.

CONCLUSION

The *Chalara paradoxa* raw starch-digesting amylase was specified as glucoamylase from the experimental data of the reaction product and its anomeric configuration. The purified enzyme showed a single band on polyacrylamide disc gel electrophoresis and its molecular weight was 82,000. The raw starch-digesting activity of the enzyme was 31.5% of the gelatinized starch-saccharifying activity, which is extremely high compared with the conventionally studied raw starch-digesting glucoamylase. There are some difference in optimum reaction condition and stability between raw starch-digesting activity and gelatinized starch-saccharifying activity. Further study will be required to clarify that these two activities are from one single enzyme or two independent enzymes of very close protein chemical properties.

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Chalara paradoxa が生成する新しい
生澱粉分解酵素に関する研究 (第3報)

酵素の精製と性質

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本酵素は、カビの一種である *Chalara paradoxa* が
生産する新しい生澱粉分解酵素である。澱粉吸着、

DEAE-セルロースカラムクロマトグラフィー 2 回、
Toyopearl HW-55 によるゲル濾過によって、培養液上
澄の60倍の比活性で、電気泳動的に単一なバンドまで精
製した。SDS-ポリアクリルアミドゲル電気泳動による
酵素の分子量は、82,000 であった。この酵素は至適温
度 45~50°C, 至適 pH 5.0, また、pH 安定性は pH
4.5~7.5 の範囲であり、温度安定性は 50°C 以下であ
った。さらに、カルシウムイオンの存在下で耐熱性は約
5°C 上昇した。本酵素をコーンスターチに作用させた場
合の生産物は、グルコースのみであり、そのアノマー型
は β 型であった。この酵素の糊化澱粉分解活性に対する
生澱粉分解活性の比は、31.5%であった。