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Purification and Characterization of α-Amylase from Irpex lacteus (Polyporus tulipiferae)

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Summary

The major amylolytic enzyme present in Driselase, a commercial crude cellulase preparation from *Irpex lacteus (Polyporus tulipiferae)*, was purified by fractionation with ammonium sulfate and successive column chromatographies on DEAE-Sephadex and Bio-gel P-100 to homogeneity on polyacrylamide gel electrophoresis and characterized as an α-amylase [E. C. 3. 2. 1. 1].

Some properties of the purified α-amylase were investigated. The molecular weight was estimated to be 40,000 by gel filtration on Bio-gel P-100. The activity of the enzyme was significantly inhibited by Ag^{+}, Cu^{++}, Pb^{++}, or Cd^{++} at the concentration of ion used. The enzyme was completely inhibited by Hg^{++}. The optimum pH and temperature for the activity of the enzyme were pH 6.0 and 50°C. The enzyme was stable over the range pH 5.0 - 8.0 at 30°C and was completely inactivated by heating at 60°C for 30 min.

This α-amylase attacked soluble starch, amylose, dextrin, amylopectin, and glycogen, although the relative initial rates of hydrolysis on these glucans were different from one another owing to the structural peculiarities in the glucans, but the enzyme did not act on pullulan. Paper chromatography demonstrated that the α-amylase produced an appreciable amount of G_4 with small amounts of other oligosaccharides without formation of glucose in the initial reaction of hydrolysis when soluble starch was used as substrate. The enzyme also easily hydrolyzed a homologous series of malto-oligosaccharides, having higher molecular sizes than G_4, G_5H, and $PNPG_4$ to produce various oligosaccharides with

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Abbreviations : G_1, glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose; G_5, maltopentaose; G_6, maltohexaose; G_7, maltoheptaose; G_8, maltooctaose; G_2H, maltitol; G_3H, maltotritol; G_4H, maltotetraitol; G_5H, maltopentaitol; $PNPG_1$, *p*-nitrophenyl α-D-glucoside; $PNPG_2$, *p*-nitrophenyl α-D-maltoside; $PNPG_3$, *p*-nitrophenyl α-D-maltotrioside; $PNPG_4$, *p*-nitrophenyl α-D-maltotetraoside; CMC, sodium carboxymethyl cellulose; DP, average degree of polymerization; DS, degree of substitution.

maltosyl transfer action without release of glucose under the assay conditions. The highest frequency of cleavage by the enzyme of these malto-oligosaccharides tested in the earlier stage seemed to be at the second α-1, 4-glucosidic linkage from the nonreducing end of a substrate molecule, although the major cleavage point might be shifted from the linkage to others as the molecular size of oligosaccharide increases. Therefore, these findings may be taken to suggest that the hydrolytic action of α-amylase from this fungus on various substrates proceeded in an endo-fashion with incomplete random cleavage.

Introduction

α-Amylase [E. C. 3. 2. 1. 1] is generally known as the enzyme which catalyzes the hydrolysis of α-1, 4-glucosidic linkage of various α-1, 4-glucans by an endo mechanism producing glucose and various oligosaccharides of the α-anomeric form. Numerous studies on α-amylases[1] from different biological sources have hitherto been made by many investigators.

Occurrence of some amylolytic enzyme components was already revealed by starch zone electrophoresis[2] and column chromatography[3] with several cellulase components in a culture filtrate of *Irpex lacteus (Polyporus tulipiferae)* grown on a synthetic medium. However, no studies have hitherto been carried out on enzymatic properties of α-amylase from *Irpex lacteus* in its highly purified state, although β-glucanases[4–7] from this fungus have been investigated in detail.

In the course of studies on cellulases obtained from the fungus using Driselase, a commercial enzyme preparation from *Irpex lacteus (Polyporus tulipiferae)*, one of the authors found that the preparation of this fungus contained a potent amylolytic enzyme, consisting of at least three main components which showed different migrations on ion exchange chromatography. In a preliminary experiment it was derived that the responsible enzyme of this fungus might be composed of two minor glucoamylase and one major α-amylase components, from an inspection of products obtained when hydrolysates of soluble starch by these three components were chromatographed.

The present paper deals with a purification procedure for the major α-amylase component in preparation of *Irpex lacteus*, and also with general enzymatic properties and action patterns on various substrates of the purified α-amylase.

Materials and Methods

Enzyme Source. Driselase, a commercial enzyme preparation from *Irpex lacteus*, kindly given by Kyowa Hakko Co., Ltd., was used as the starting material for purification of α-amylase.

Substrates. a) Soluble starch, amylopectin, oyster glycogen, amylose A (M. W. 2900, DP=18), amylose B (M. W. 16000, DP=99), and pullulan were pur-

chased from Nakarai Chemical Co., and dextrin from Sigma Chemical Co., Ltd. (St. Louis, Mo., U. S. A.).

b) Sodium carboxymethyl cellulose (CMC, DS=0.63) was obtained from Daiichi Industrial and Pharmaceutical Co., Ltd.

c) *p*-Nitrophenyl α-D-glucoside and *p*-nitrophenyl α-D-maltoside were obtained from Nakarai Chemical Co., and *p*-nitrophenyl α-D-maltotrioside and *p*-nitrophenyl α-D-maltotetraoside from Calbiochem-Behring Corp. (Calif., U.S.A.).

d) Malto-oligosaccharides were supplied by Nihon Food Industry Co., Ltd.

e) DEAE-Sephadex A-50 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), and Bio-gel P-100 from Bio-Rad Laboratories (Calif., U.S.A.).

f) Pachyman was prepared from a Chinese medicine air-dried *Bukuryo (Poria cocos)* by the method described by Saito *et al*.[8]

g) Maltotetraitol and maltopentaitol (reduced malto-oligosaccharides) were prepared from maltotetraose and maltopentaose by the method of French and Abdullah,[9] respectively.

The all other chemicals used were of certified reagent grade, commercially available.

Enzyme Assays and Analytical Methods. *a) Soluble Starch Saccharifying Activity*. A reaction mixture (2 ml), consisting of 0.25% soluble starch, 0.05M sodium acetate buffer (pH 5.0), and enzyme, was incubated at 30°C for an appropriate period. The reducing sugar formed in the reaction mixture was estimated from the absorbance at 660 nm by the method of Somogyi[10] and Nelson.[11] One unit of saccharifying activity is defined as the amount of enzyme which produces a reducing power from the substrate equivalent to 1 μmol of glucose per min under the conditions described above.

b) CMC–and Pachyman–Saccharifying Activities. Assays were carried out under the same conditions as used for the soluble starch-saccharifying activity, except for the use of 1% CMC or 0.5% pachyman in place of 1% soluble starch.

c) β–Glucosidase Activity. The reaction mixture used contained 0.1 ml of 0.048 M $PNPG_1$, 0.2 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.1 ml of enzyme solution. After incubation at 30°C for 10 min, 3 ml of 1% Na_2CO_3 was added to the mixture, and the *p*-nitrophenol liberated was estimated from the absorbance at 420 nm.

d) Measurement of Blue Value. A reaction mixture containing 0.1 ml of 0.1 % soluble starch, 0.2 ml of 0.02 M sodium acetate buffer (pH 5.0), and 0.1 ml of enzyme solution (0.88 μM) was incubated at 30°C for 10 min. The starch-iodine color formed on adding 0.1 ml of the reaction mixture to 10 ml of 0.005 % iodine solution was measured at 660 nm spectrophotometrically.[12]

e) Determination of Protein. The protein in an enzyme solution was determined according to the method of Lowry *et al*.[13] Elution patterns of protein on column chromatography were estimated from the absorbance at 280 nm, using crystalline bovine serum albumin (from Miles Laboratories, Inc.) as a standard.

f) Disc Electrophoresis on Polyacrylamide Gel. To examine the homogeneity of purified enzyme preparation, polyacrylamide gel disc electrophoresis was carried out in glycine-Tris buffer at pH 8.3 as determined according to the procedure described by Ornstein[14] and Davis.[15] A sample containing about 10 μg of protein was applied on the top of gel and then subjected to an electrophoresis at 3.0 mA/gel at room temperature. Gels were stained for enzyme protein with 0.05 % coomassie brilliant blue R250.

g) Paper Chromatography. Enzymatic hydrolysis products from various substrates were detected by means of paper chromatography. After an appropriate period of incubation, aliquots of enzymatic hydrolysate of substrates and solution of authentic sugars used as the standard were spotted individually on a Whatman No. 3 MM filter paper and chromatograms were obtained after development by the ascending technique with *n*-butanol : pyridine : water (6 : 4 : 3, v/v) at room temperature for an appropriate period. The chromatograms were dried and dipped in silver nitrate solution[16] for development after glucoamylase treatment by the method of Kainuma and French.[17] The approximate relative amounts of hydrolysis products on the chromatograms were estimated on the basis of the color intensity and area of the spots.

h) Thin-layer Chromatography. Thin-layer chromatography[18] was performed with silica gel sheet (silica gel 60 Merk Art 5553) as absorbent which had been coated on a glass plate in 0.25 mm thickness and activated at 105°C for 60 min. For the ascent, a solvent system of methyl ethyl ketone, acetic acid, and water (3 : 1 : 1, v/v) was used. The sugars were located by spraying 10 % sulfuric acid and by heating the plate at 120°C for 30 min.

Results

Purification of α-Amylase Component.

Step 1. DEAE-Sephadex A-50 Column Chromatography. A crude enzyme solution obtained from Driselase powder (50 g) was applied on a DEAE-Sephadex A-50 column (acetate form) previously equilibrated with 0.02 M sodium acetate buffer (pH 5.0). Elution was carried out stepwise with 0.02, 0.1, and 0.2 M sodium acetate buffer at the same pH. Each fraction was tested for α-amylase, CMCase, β-1, 3-glucanase, and β-glucosidase activities and protein content. The elution pattern obtained is shown in Fig. 1. Three peak fractions (A-1, -2, and -3) having soluble starch-saccharifying activities were obtained, which roughly overlapped the main peaks of protein.

Two (A-1 and -2) of these three peak fractions each showed a glucoamylase activity and the other (A-3) showed an α-amylase activity on inspection of hydrolysis products formed from soluble starch by them.

Peak fraction A-3 (Tube Nos. 320-350 in Fig. 1), which has the highest saccharifying activity toward soluble starch, was collected and subjected to further

purification.

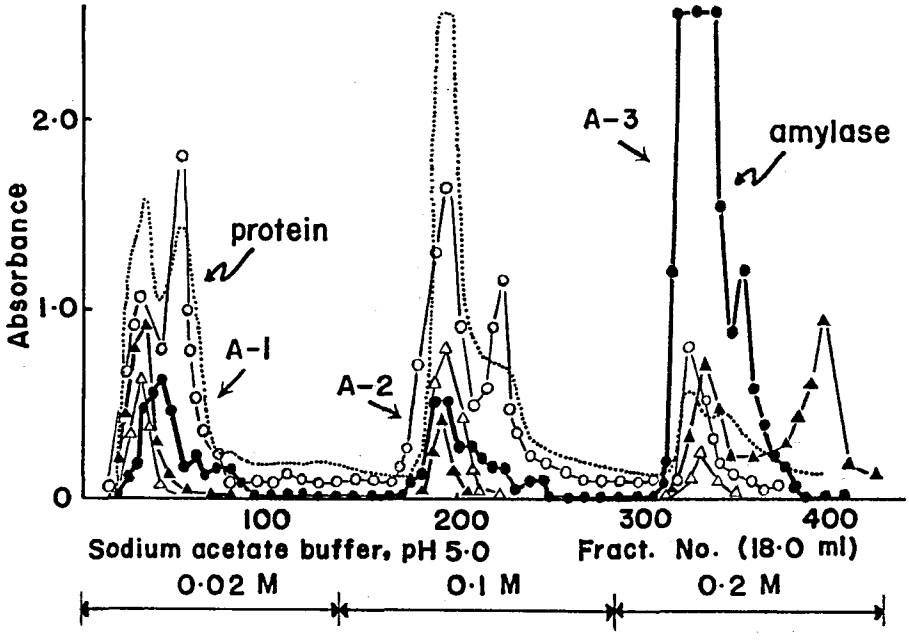

Fig. 1. Elution pattern of the crude enzyme preparation from a DEAE-Sephadex A-50 column.
—●—, starch-saccharifying activity (30 min incubation, A_{660}) of eluates diluted 10-fold; —○— , CMC-saccharifying activity (10 min incubation, A_{660}) of eluates diluted 10-fold; —▲—, pachyman-saccharifying activity (10 min incubation, A_{660}) of eluates diluted 10-fold; —△—, β-glucosidase activity (10 min incubation, A_{420}) of eluates diluted 2-fold; ---------, protein (A_{280}). Column, 5.0 x 40 cm; flow rate, 60 ml/h; fraction size, 18 ml.

Step 2. Ammonium Sulfate Fractionation. The fraction (A-3) was gradually brought to 80% saturation by addition of solid ammonium sulfate with continuous stirring at room temperature. It was allowed to stand at room temperature overnight, and the resulting precipitate of A-3 was collected by centrifugation, dissolved in a small volume of water, dialyzed overnight against running tap water with a membrane bag, and lyophilized.

Step 3. First Bio-gel P-100 Gel Filtration. An aqueous solution of fraction (A-3) obtained above was subjected to a gel filtration with a Bio-gel P-100 column, previously equilibrated with 0.1 M sodium acetate buffer (pH 5.0),

and eluted with the same buffer. As shown in Fig. 2, a single peak fraction with a soluble starch-saccharifying activity, contaminated with an activity of CMC-ase, was obtained. This fraction was concentrated in a collodion bag with suction and then lyophilized.

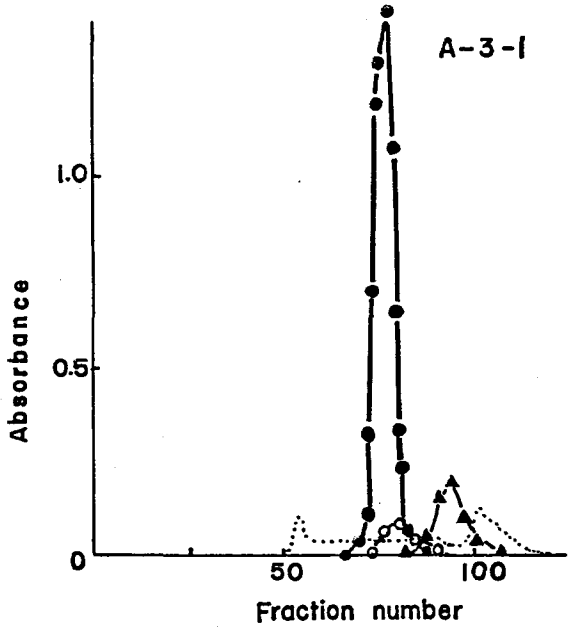

Fig. 2. Elution pattern of the ammonium sulfate precipitated enzyme preparation (A-3) on a Bio-gel P-100 column. —●—, soluble starch-saccharifying activity (10 min incubation, A_{660}) of eluates diluted 10-fold; —○—, CMC-saccharifying activity (10 min incubation, A_{660}) of eluates diluted 10-fold; —▲—, pachyman-saccharifying activity (10 min incubation, A_{660}) of eluates diluted 10-fold; ------, protein (A_{280}). Column, 3.0 x 100 cm; flow rate, 6.0 ml/h; fraction size, 3.0 ml.

Step 4. Second Bio-gel P-100 Gel Filtration. In an attempt to remove the accompanying CMCase, the resultant enzyme fraction (A-3-1) was again subjected to a gel filtration under the same conditions with the first Bio-gel P-100 gel filtration, except for the column size. Fig. 3 shows the profile of the enzyme elution from the column. A single peak fraction (A-3-1-1) with a soluble starch-saccharifying activity coincided fully with the protein peak, verifying its homogeneity. The active portion was collected and dialyzed against running tap water. After concentration using a collodion bag, it was lyophilized, and named A-III.

The final enzyme preparation (A-III) gave a single protein band on disc gel

electrophoresis of polyacrylamide at pH 8.3 (Fig. 3). Table 1 summarizes the results obtained on the specific activity and yield of the amylase fraction during the purification course from the starting solution. The specific activity of the purified amylase preparation (A-III) toward soluble starch was increased approximately 880-fold over that of the initial crude enzyme preparation. The purified enzyme (A-III) was used in the following experiments.

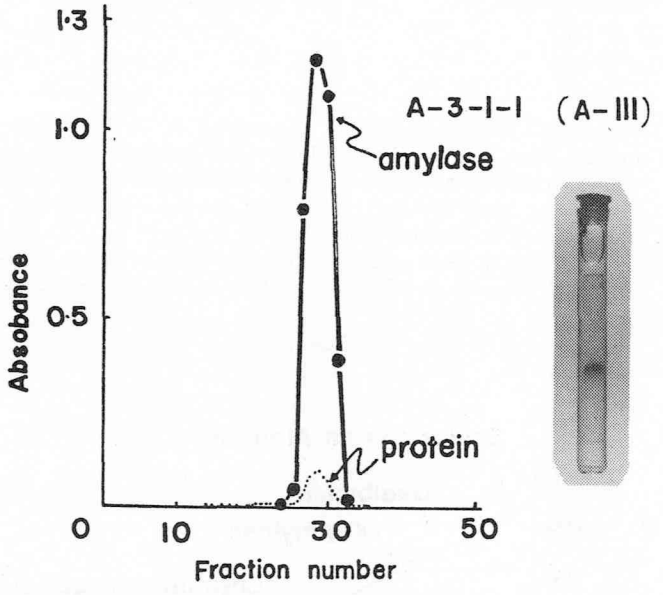

Fig. 3. Elution pattern of A-3-1 on a Bio-gel P-100 column and disc electrophoresis of A-3-1-1 (A-III) on polyacrylamide gel. —●—, soluble starch-saccharifying activity (10 min incubation, A_{660}), of eluates diluted 10-fold, -----, protein (A_{280}). Column, 1.0 x 150 cm; flow rate, 3 ml/h; fraction size, 3 ml. The disc electrophoresis of the purified enzyme (A-III) was carried out by the method of Ornstein[14] and Davis,[15] described in the "Materials and Methods" section.

Molecular Weight by Gel Filtration. The molecular weight of the purified enzyme preparation was estimated by the gel filtration method. A-III was applied on a Bio-gel P-100 gel column equilibrated with 0.1 M sodium acetate buffer (pH 5.0). Elution was carried out with the same buffer. Bovine serum albumin (M. W. 67,000), ovalbumin (M. W. 43,000), chymotrypsinogen A (M. W. 25,000),

and ribonuclease A (M. W. 13,700) were used as standards. The void volume was determined using blue dextran 2,000. The results are shown in Fig. 4. The molecular weight of A-III was estimated to be 40,000.

Table 1. Recoveries and activities of α-amylase during the purification procedure.

purification step	Volume ml	Protein mg	Total units	Specific activity[a] units/mg	Yield[b] %
starting solution	262	22083	1240	0.056	100
DEAE-Sephadex	384	71.8	384	5.3	0.32
$(NH_4)_2SO_4$ fractionation	44	39.6	232	5.8	0.179
1st. Bio-Gel P-100	34	3.4	152	44.7	0.015
2nd. Bio-Gel P-100	18	2.8	139	49.6	0.013

a) Specific activity is defined as activity units/mg of enzyme protein. One unit is the soluble starch-saccharifying activity which produces a reducing power equivalent to 1.0 μmol of glucose in one minute under the reaction conditions employed in the present work.
b) The yield in each purification step is expressed in terms of weight percentage to the dry matter of the starting solution.

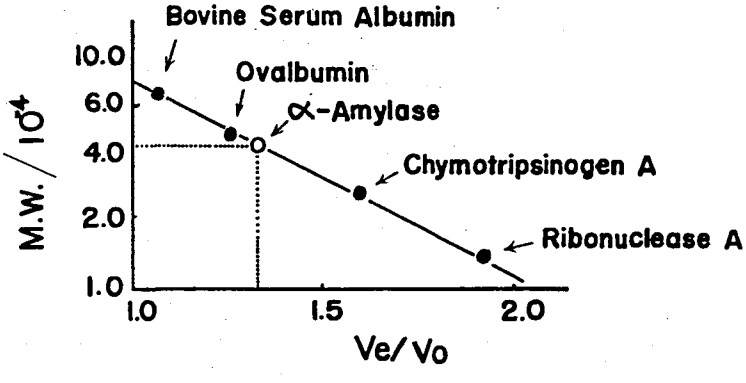

Fig. 4. Estimation of the molecular weight of the purified enzyme (A-III) by Bio-gel P-100 gel filtration.
Ve and Vo are the elution volume and void volume, respectively.
Column, 1.0 x 125 cm; flow rate, 3 ml/h; fraction size, 3 ml; elution buffer, 0.1 M sodium acetate buffer, pH 5.0.

Effects of pH and Temperature on Enzyme Activity and Stability. The pH dependence of the purified enzyme activity was studied under the standard assay conditions using sodium acetate buffer (0.05 M) at pH 3.2 – 5.9 and phosphate

buffer (0.05 M) at pH 6.29-8.25. As shown in Fig. 5, the optimum pH is around

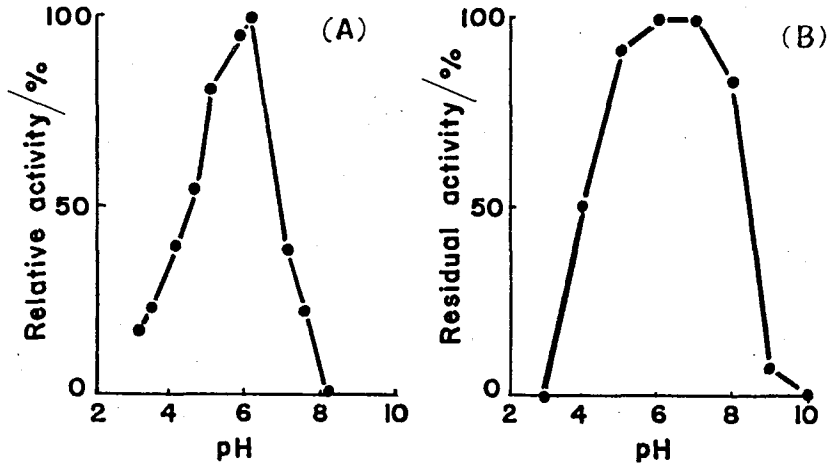

Fig. 5. Optimum pH and pH-stability curves for the soluble starch-saccharifying activity of the purified enzyme.
(A), optimum pH; (B), pH-stability.

6.0. The effect of pH on the stability of the enzyme was examined after preincubation for 24 hr at 30°C at various pH values. Remaining activities were then assayed at pH 5.0. The enzyme activity was stable in the narrow pH range from 5.0 to 8.0. The effect of temperature on the stability was studied by heating the enzyme at various temperatures for 30 min in acetate buffer (pH 5.0), cooling them quickly in ice water, and then determining remaining activities at 30°C for 10 min by measuring the increase in the amount of reducing sugar. Fig. 6 shows the optimum temperature and heat stability of the enzyme activity. The enzyme activity showed a maximum at 50°C and was stable at temperatures lower than 40°C but was completely lost by heating 60°C, under the conditions employed.

Effect of Metal Ions. Effects of metal ions on the enzyme activity were examined at a concentration of 2 mM of various metal compounds under the standard assay conditions. Results are expressed as percentage residual activity compared to a control containing substrate only. As shown in Table 2, no stimulation effect on the enzyme activity was observed. Metal ions such as Ag^{+}, Cu^{++}, Pb^{++}, and Cd^{++}, showed a significantly inhibitory effect. The en-

zyme was completely inhibited by Hg^{++}.

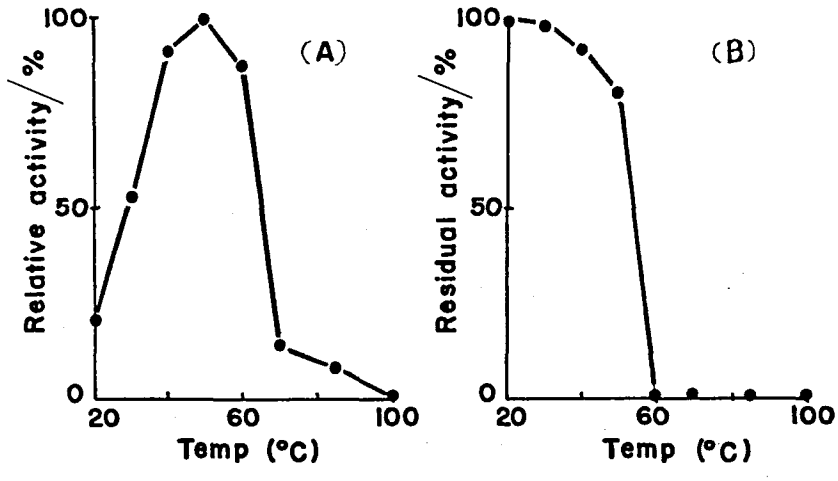

Fig. 6. Optimum temperature and thermal-stability curves for the soluble starch-saccharifying activity of the purified enzyme. (A), optimum temperature; (B), thermal-stability.

Table 2. Effect of metal ions on activity of the purified α-amylase.

Metal ion	Conc. mM	Residual activity/ %[a)]
None	—	100
$CaCl_2$	2.0	87.5
$BaCl_2$	2.0	87.5
$AgNO_3$	2.0	39.8
$CuSO_4$	2.0	20.9
NaCl	2.0	84.7
$CoCl_2$	2.0	71.1
KCl	2.0	84.3
$MgSO_4$	2.0	78.7
$FeSO_4$	2.0	71.5
$HgCl_2$	2.0	2.0
$Pb(AcO)_2$	2.0	21.8
$CdCl_2$	2.0	40.1

a) Enzyme activity was determined as the soluble starch-saccharifying activity.

Actions on Various Substrates: Hydrolysis of various substrates by α-amylase (A-III) was carried out under the standard assay conditions. Chromatographies of the products formed in reaction mixtures were done by the ascending technique described in the "Materials and Methods" section.

a) Hydrolysis of α-Glucans. Rates of hydrolysis of α-glucans, such as soluble starch, amylose, amylopectin, and glycogen, by the purified enzyme are compared in Table 3, in terms of the relative initial rate. The relative rates of hydrolysis for amylopectin and glycogen by the enzyme were much lower than those for other α-glucans tested. The enzyme did not show any activity on pullulan.

Table 3. Relative initial rates of hydrolysis of several polysaccharides by the purified α-amylase.

Substrates	Relative rate of hydrolysis / % [A)]
Soluble starch	100
Amylose (M.W≒2900)	137.8
Amylose (M.W≒16000)	92.7
Amylopectin	81.8
Oyster glycogen	68.8
Dextrin	101.6
Pullulan	0

The reaction mixture used consisted of 0.1 ml of 1% polysaccharide, 0.2 ml of 0.1 M sodium acetate buffer (pH 5.0), and 0.1 ml of enzyme solution (0.02 u). After incubation at 30°C for 10 min, the reducing sugars produced in the reaction mixture were determined by the method of Somogyi[10] and Nelson.[11]
A) The rate for soluble starch was arbitrarily set as 100.

b) Hydrolysis of Soluble Starch. Degradation products in the course of soluble starch hydrolysis with the enzyme were detected by paper chromatography. The results are shown in Fig. 7. G_4 with very small amounts of malto-oligosaccharides and higher dextrins was produced as the predominant product in the early stage of the enzyme reaction. As hydrolysis proceeded, G_2, G_3, G_5, and other larger oligosaccharides gradually increased with G_4. However, no detectable glucose was released under the experimental conditions. In later stages, smaller oligosaccharides (G_2–G_5) other than glucose were accumulated in the hydrolysis of soluble starch. The time courses of iodine color reaction (blue value) and reducing sugar formation in the hydrolysis of soluble starch by the enzyme are shown in Fig. 8. As reaction proceeded, the reducing power of the hydrolysate gradually increased, whereas the iodine staining power of

the hydrolysate rapidly decreased and finally almost disappeared.

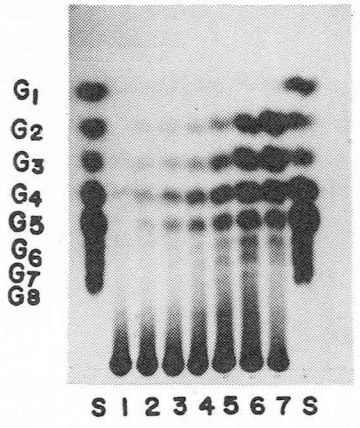

Fig. 7. Paper chromatogram showing products formed in course of hydrolysis of soluble starch by the purified α-amylase.
The reaction mixture (0.8 ml) containing enzyme solution (0.22 μM), 0.25% soluble starch and 0.05 M acetate buffer (pH 5.0) was incubated at 30°C. Paper chromatography was carried out by the same procedure as described in the "Materials and Methods" section.
S, standard of malto-oligosaccharides; G_1, glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose; G_5, maltopentaose; G_6, maltohexaose; G_7, maltoheptaose; G_8, maltooctaose.
Incubation times: 1, 0 min; 2, 3 min; 3, 5 min; 4, 10 min; 5, 20 min; 6, 60 min; 7, 120 min.
Development: twice by the ascending technique for 24 hr.

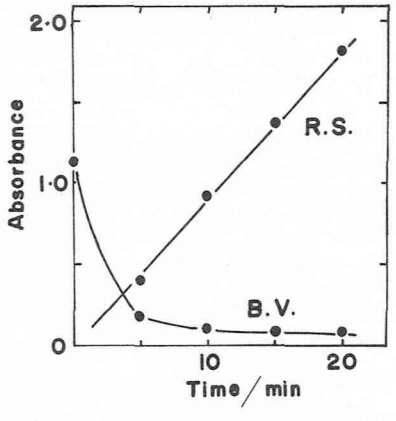

Fig. 8. Time courses of blue value and reducing sugar formation in hydrolysis of soluble starch by the purified enzyme.
Absorbance, 660 nm; RS, reducing sugar; B. V., blue value. Experimental details are described in the "Materials and Methods" section.

c) Hydrolysis of Malto-oligosaccharides. A reaction mixture composed of 0.1 ml of approximately 30 mM malto-oligosaccharide, 0.2 ml of 0.05 M acetate buffer (pH 5.0), and 0.1 ml of enzyme solution (0.88 μM) was incubated for an appropriate period at 30°C. Aliquots (0.1 ml) of the enzymatic hydrolysates were withdrawn and heated on a boiling water bath for 5 min. The hydrolysis products were identified by paper chromatography. Neither G_2 nor G_3 was attacked even after a prolonged incubation by the enzyme. Fig. 9 shows the paper chromatograms of the products formed in course of hydrolysis of malto-oligosaccharides (G_4–G_6) by the enzyme. The α-amylase hydrolyzed malto-oligosaccharides larger than G_4 to produce various oligosaccharides, but no formation of glucose was observed in the entire course of hydrolysis. During the reaction with G_4 by the enzyme, a mixture of $G_2 > G_6 > G_3$ in the earlier stage of hydrolysis was produced and as reaction proceeded, G_2, G_3, and G_5 gradually increased but G_6 disappeared at the terminal stage of the reaction (Fig. 10). G_5 gave a mixture of $G_3 = G_4 > G_2$ with small amounts of unknown oligosaccharides located under the spot of G_5 in the earlier stage. From G_6, four products, $G_4 = G_3 > G_2 > G_5$, were formed with small amounts of unknown oligosaccharides larger than G_6 in the earlier stage of digestion.

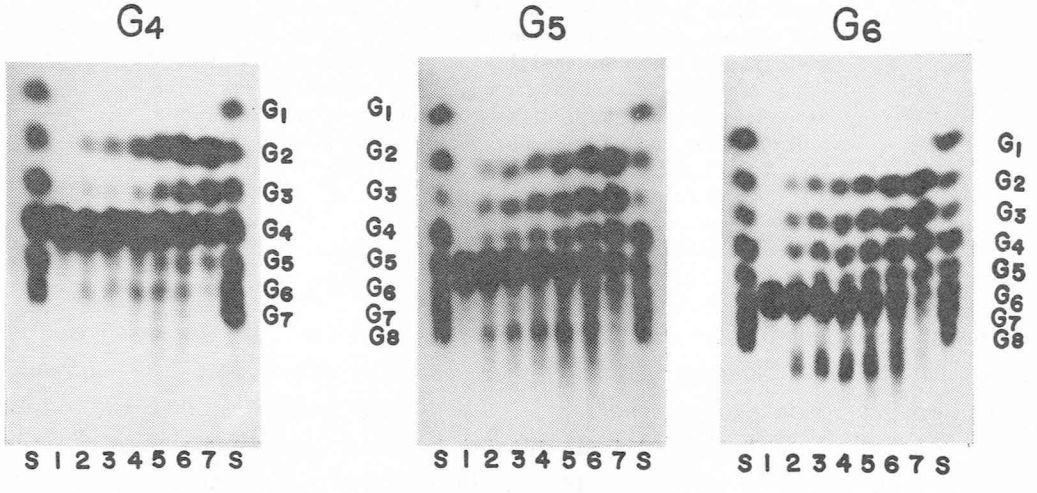

Fig. 9. Paper chromatograms showing products produced in course of hydrolysis of malto-oligosaccharides by the purified α-amylase.
Experimental details are given in the text.
Incubation times: 1, 0 min; 2, 10 min; 3, 20 min; 4, 30 min; 5, 60 min; 6, 120 min; 7, 240 min.
The symbols are the same as in Fig. 7.
Development: twice by the ascending technique for 24 hr.

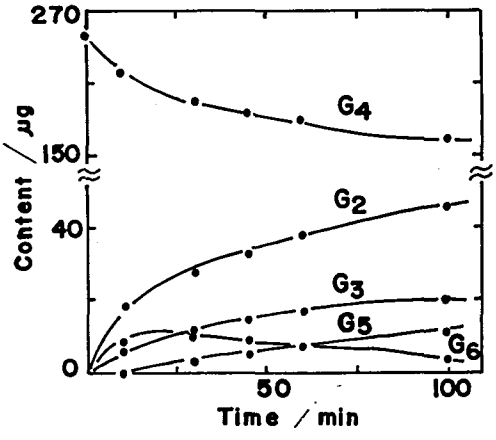

Fig. 10. Time course of hydrolysis of maltotetraose by the purified α-amylase.
Paper chromatography is described in the "Materials and Methods" section.
The quantitative amounts of hydrolysis products on the chromatograms were determined according to the procedures described by Saito and Horiuchi.[23] To check the position of each carbohydrate on the paper, controls of the chromatogram were monitered by the silver nitrate dip-method.[15] Each section of carbohydrate on the chromatogram was excised, extracted with distilled water in a test tube in a boiling water bath for 15 min, cooled, and filtered; the carbohydrate content of the filtrate was determined by the method of Somogyi[10] and Nelson.[11]

d) Hydrolysis of Maltotetraitol and Maltopentaitol. As the formation by the enzyme of unexpected oligosaccharides besides expected hydrolysates might be related to its possible transglycosylation activity, actions of the enzyme on malto-oligosaccharides (G_4H and G_5H), which had been obtained by reduction at C-1 of the reducing glucose ends of the original oligosaccharides, were also examined under the same conditions as in the hydrolysis of the malto-oligosaccharides. G_4H showed considerable resistance to attack by the enzyme, while G_5H was attacked at a rate of hydrolysis somewhat lower than that of the original G_5 under the same conditions (data not shown). Fig. 11 shows paper chromatograms of the products formed during the courses of hydrolysis of G_4H and G_5H. G_4H was hydrolyzed to produce very small amounts of G_2 and G_2H in the terminal stage of reaction. From G_5H, G_2 and G_3H were produced in the early stage, and small amounts of G_3 and G_4H, besides a trace of unknown oligosaccharide larger than G_5H, formed in the later stage of hydrolysis.

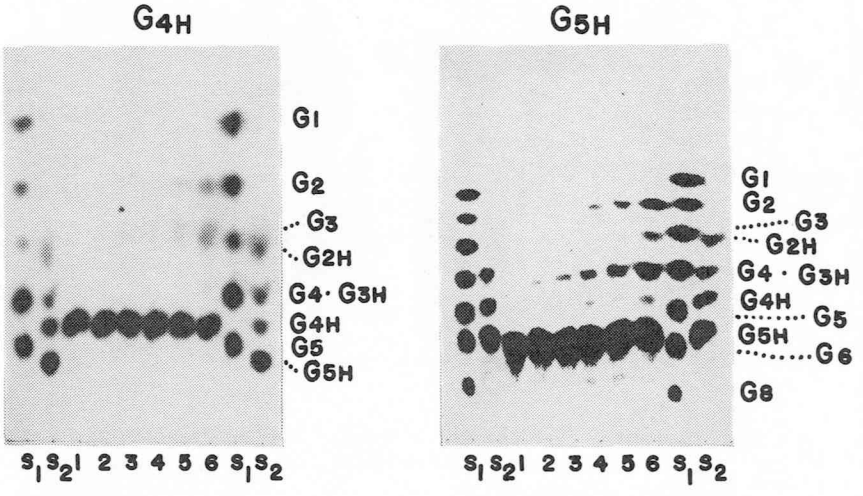

Fig. 11. Paper chromatograms of the hydrolysis products formed from reduced malto-oligosaccharides by the purified α-amylase.
Experimental details are given in the text.
Final enzyme concentration, 0.22 μM; final substrate concentration, 5 mM.
S_1, standard of malto-oligosaccharides; G_1, glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose; G_5, maltopentaose; G_6, maltohexaose.
S_2, standard of reduced malto-oligosaccharides; G_2H, maltitol; G_3H; maltotritol; G_4H, maltotetraitol; G_5H, maltopentaitol.
Incubation times: 1, 0 min; 2, 10 min; 3, 20 min; 4, 30 min; 5, 60 min; 6, 120 min.
Development: twice by the ascending technique for 24 hr.

e) Hydrolysis of p-Nitrophenyl Malto-oligosaccharides. To clarify further the action pattern of the enzyme, the same experimental investigation as above was also performed with $PNPG_3$ and $PNPG_4$. $PNPG_3$ or $PNPG_4$ (2.5 mM) was incubated with α-amylase (0.22 μM) at 30°C for a suitable time, and the products formed were identified by thin-layer chromatography. Fig. 12 shows thin-layer chromatograms of products produced during the course of hydrolysis of $PNPG_3$ or $PNPG_4$. $PNPG_3$ was not appreciably hydrolyzed by the enzyme even after prolonged incubation. But slight amounts of $PNPG_1$, $PNPG_2$, and G_3 together with G_2 were produced in the final reaction stage. From $PNPG_4$, $PNPG_2$ and G_2 as major products were formed in the early stage of the enzyme reaction, and in addition to them $PNPG_3$ and G_3 unexpectedly as minor products in the later stage of reaction. However, no detectable glucose was either seen under the experimental conditions in the hydrolysis of these oligosaccharide derivatives.

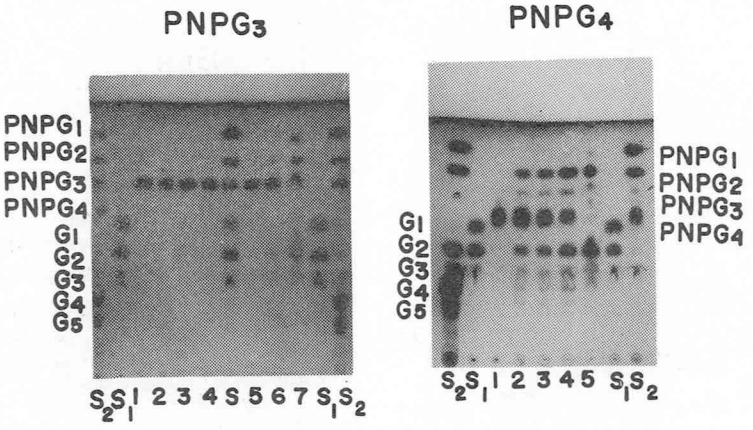

Fig. 12. Thin-layer chromatograms of hydrolysis products formed from *p*-nitrophenyl *α*-maltotrioside and *α*-maltotetraoside by the purified *α*-amylase.

The method is described in the "Materials and Methods" section and the text.

S_1, standard of malto-oligosaccharides; glucose, maltose, and maltotriose from the top.

S_2, standards of *p*-nitrophenyl *α*-malto-oligosaccharides and malto-oligosaccharides; $PNPG_1$, *p*-nitrophenyl *α*-glucoside; $PNPG_2$, *p*-nitrophenyl *α*-maltoside; $PNPG_3$, *p*-nitrophenyl *α*-maltotrioside; $PNPG_4$, *p*-nitrophenyl *α*-maltotetraoside; G_1, glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose; G_5, maltopentaose.

Incubation times: 1, 0 min; 2, 15 min; 3, 30 min; 4, 60 min; 5, 120 min; 6, 240 min; 7, 24 hr.

Development: once by the ascending technique with methyl ethyl ketone : acetic acid : water (3 : 1 : 1, v/v) for 2 hr.

Discussion

An *α*-amylase (A-III) was separated from Driselase, a commercial crude cellulase preparation of *Irpex lacteus (Polyporus tulipiferae)*, through several steps of purification such as salting out with ammonium sulfate, column chromatography on Sephadex ion exchanger, and gel filtration. The specific activity of the *α*-amylase was increased approximately 880-fold relative to that in the initial preparation. The purified *α*-amylase preparation showed a single protein band on polyacrylamide gel disc electrophoresis, and was completely free from contaminating cellulase and *β*-glucosidase. The approximate average molecular weight of this enzyme was estimated to be 40,000 by the gel filtration method. This value is lower than those, approximately 50,000–60,000, of other fungal *α*-amylases. No remarkable differences were observed with respect to enzymatic properties in pH and thermal stability, and those in pH and thermal optima for starch hydrolysis of this enzyme, as compared with other fungal *α*-amylases.

The purified *α*-amylase readily hydrolyzes the amylo-glucans such as short

chain amylose, soluble starch and dextrin containing α-1, 4 glucosidic linkages, although the initial rates of hydrolysis of these glucans are different from one another. Nevertheless, amylopectin and oyster glycogen containing the α-1, 6-linkage showed somewhat resistance to attack by the enzyme under the same conditions. This amylase had no activity on pullulan, unlike α-amylase from *Thermoactinomyces vulgaris*[19] (Table 3). The purified α-amylase of this fungus causes a rapid decrease in the starch-iodine color formed, along with an increase in the production of reducing power during the hydrolysis of soluble starch. Consequently, the enzyme is indicated, from the results of its action, to hydrolyze α-glucans in a random fashion.

Degradation products in the course of starch hydrolysis with this α-amylase were detected by paper chromatography. The amylase produced preferentially G_4 together with very small amounts of oligosaccharides and higher dextrins from soluble starch during the initial reaction of hydrolysis, and as reaction proceeded, smaller oligosaccharides (G_2-G_5) gradually increased. However, no glucose appeared in the entire course of hydrolysis. It was suggested that this α-amylase had no ability to remove glucosyl residues directly from the glucosidic linkages of starch and its produced oligosaccharides.[20] Thus these observations indicate that α-amylase of this fungus may be classified into a kind of endo-amylase (α-1, 4-glucan 4-glucanohydrolase) [E. C. 3. 2. 1. 1] which produces appreciably G_4, besides small amounts of smaller oligosaccharides without formation of glucose during the initial reaction.

Furthermore, in order to establish the action pattern of the α-amylase, the hydrolysis products by the amylase of a homologous series of malto-oligosaccharides were examined by paper chromatography. Neither of G_2 and G_3 were attacked by this enzyme, even after prolonged incubation, but the other oligosaccharides (G_4-G_6) tested were hydrolyzed to produce various saccharides by the same enzyme (Fig. 9). From G_4, G_2 and unexpected G_3 were produced by the amylase. G_5 was hydrolyzed into G_2 and G_3 forming unexpected G_4, and from G_6, three possible products (G_2-G_4) besides unexpected G_5 except G_1 were formed, although the amounts of products produced from the substrates by the enzyme were appreciably different from one another in the earlier stage of hydrolysis. Moreover, small amounts of some unknown oligosaccharides located under the spots of G_4, G_5, and G_6 were observed during the reaction of G_4, G_5, and G_6 with the enzyme under the standard assay conditions, respectively. These findings indicate that the α-amylase from *Irpex lacteus* has a glycosyl transfer action, like enzymes from the other fungi. In addition, the formations of these unexpected oligosaccharides (G_3, G_4, and G_5 from G_4, G_5, and G_6 as substrate, respectively) during the hydrolysis of the substrates without any detectable formation of glucose as seen in Fig. 9, are considered to have been caused by the two-step enzymatic reaction, "transglycosylation and subsequent hydrolysis," between donor and acceptor.

To obtain further information on the action pattern of the enzyme, the products of hydrolysis by the enzyme of reduced or *p*-nitrophenylated malto-oligosaccharides at C-1 of the reducing glucosyl residue, were also examined by paper or thin-layer chromatography, respectively. The enzyme hardly attacked G_4H, but hydrolyzed the major part of G_5H into G_3H and G_2 during the initial stage of hydrolysis, although the facility of hydrolysis is rather low compared to that of original G_5. At the final stage of reaction, small amounts of G_3 and G_4H, unexpectedly from G_5H, were produced without formation of glucose. The above results suggest that, first, the α-amylase in its initial attack on G_5H removes a maltosyl residue from the nonreducing end, resulting in the appearance of G_2 and G_3H, and that, next in a transfer process, the intermediate (G_2-enzyme complex) may be transferred to the nonreducing end of another molecule of G_5H as an acceptor to produce G_7H which is immediately degraded into G_3 and G_4H without accumulation by subsequent hydrolytic reaction.[21]

Furthermore, quite similar results as with G_4H and G_5H were obtained with such derivatives of malto-oligosaccharide as $PNPG_3$ and $PNPG_4$, respectively. $PNPG_3$ showed considerable resistance to attack by the amylase under the conditions examined, although it has the same three α-1, 4-glucosidic linkages as original G_4. $PNPG_4$ was split by the α-amylase into almost equal amounts of G_2 and $PNPG_2$ together with formation of slight amounts of G_3 and $PNPG_3$. This may also be explained on the assumption that the major part of $PNPG_4$ was split by the α-amylase into G_2 and $PNPG_2$, and then had its maltosyl residue transferred to $PNPG_4$ to produce $PNPG_6$, from which G_3 and $PNPG_3$ were formed by the action of the same enzyme.

Thus, the α-amylase from *Irpex lacteus* readily hydrolyzes a homologous series of α-1, 4-malto-oligosaccharides having degrees of polymerization higher than those of G_4, G_5H, and $PNPG_4$ with subsequent maltosyl transfer action, although evidence for the preferential transfer, without production of glucose is incomplete at present. Consequently, the fourth glucopyranosyl residue of α-1, 4-glucosidic linkage from the nonreducing end of the oligosaccharides appears necessary for the action of the α-amylase. From these patterns of hydrolysis products formed by the α-amylase under the assay conditions described, it seems that a major linkage cleaved in its initial attack on these malto-oligosaccharides tested is the second α-1, 4-glucosidic linkage from the nonreducing end of a substrate molecule, although the point of highest frequency of attack might be shifted from the linkage to others as the molecular size of malto-oligosaccharide increases.[22] However, quantitative determinations of the bond-cleavage frequencies for these malto-oligosaccharides and the transglycosylation reaction by the α-amylase using oligosaccharides labelled at the reducing end with radioactive glucose will be published elsewhere in detail in the near future.※

※ K. Wakabayashi and H. Daichō, in preparation.

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