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Purification and Properties of Cyclodextrinase from *Bacillus stearothermophilus* HY-1

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ABSTRACT

A thermostable cyclodextrinase (EC 3.2.1.54) from *Bacillus stearothermophilus* HY-1 was purified to homogeneity by disc-electrophoresis after sonication disruption, ammonium sulfate fractionation, DEAE-cellulose(DE32) column chromatography, hydroxyapatite chromatography, Sephadex G150 gel-filtration, and α-cyclodextrin-AH-Sepharose 4B affinity chromatography. The enzyme was purified 230-fold with 21.2% of activity recovery. The optimal substrates of the enzyme were α-, β-, and γ-cyclodextrins and linear maltooligosaccharides, and the final product was mainly maltose. The enzyme could hydrolyze pullulan to produce panose. It could also hydrolyze soluble starch, amylose, and amylopectin, but not glycogen. The Km and Vmax for α-, β-, and γ-cyclodextrins were 1.79, 1.67, and 2.50 mg/mL, and 336, 185, and 208 μmol/mg/min, respectively. The molecular weight of the enzyme was 61,000 by SDS-gel-electrophoresis. The isoelectric point was pH 5.0. The enzyme was most active at pH 6.2 and 55°C, and it was strongly inhibited by Cu^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, and slightly by Fe^{3+}. The effect of some protein modification reagents on the activity of the enzyme suggested that tryptophan and histidine residue(s) may be located at the active site. The amino acid composition of the enzyme was also determined.

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Index Entries: Thermostable cyclodextrinase; *Bacillus stearothermophilus HY-1;* purification; properties.

Abbreviations: CDase, cyclodextrinase; CDs, cyclodextrins; G1–G6, glucose, maltose, maltotriose, maltotetraose, maltohexaose; IAA, iodo acetamide; NEM, N-ethylmaleimide; NAI, N-acetylimidazole; DEPC, diethylpyrocarbonate; NBS, N-bromosuccinimide.

INTRODUCTION

Cyclodextrinase (EC 3.2.1.54) (CDase) hydrolyzes cyclic dextrins and linear maltodextrins. Cyclodextrins (CDs) are cyclic oligosaccharides containing six or more glucose residues linked by 1,4-linkages (*1*). CDase's have been isolated from *Bacillus macerans* (*2*), *Bacillus coagulans* (*3*), *Bacillus sphaericus* (*4*), Alkalophilic *Bacillus* sp. (*5*), and *Clostridium thermohydrosulfuricum* (*6*). It has also been reported that the amylases from *Pseudomonas* sp. (*7*) and *Flavobacterium* sp. (*8*) show activity toward cyclodextrins in addition to amylose and amylopectin.

Recently, we isolated a *Bacillus stearothermophilus* HY-1 from soil in southern China that produced cyclodextrinase. The enzyme hydrolyzes CDs to produce oligosaccharides in which maltose is the main component. It could also hydrolyze pullulan to panose. This paper describes the purification and properties of this enzyme.

MATERIALS AND METHODS

Microorganism and Cultivation

The bacterial strain of *B. stearothermophilus* HY-1 was used as the CDase producer throughout this investigation. The bacterium was grown in 4.0 L of the medium at 65°C, 200 rpm for 24 h. The medium was composed of 1% soluble starch, 1% polypepton, 0.5% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$, 0.1% $K_2HPO_4 \cdot 3H_2O$, 0.1% maltose, adjusted to pH 7.0–7.2.

Enzyme Purification

Cells of *B. stearothermophilus* HY-1 were harvested from the culture liquor by centrifugation at 4000 rpm for 30 min. The cells were washed with 200 mL of 0.1*M* acetate buffer (pH 5.8) containing 10 m*M* mercaptoethanol and then suspended in the same buffer. The cells were disrupted by sonication for 4 min at 300W with a Labsonic 2000 Ultrasonic Oscillator in an ice-water bath. The cell debris was removed by centrifugation at 4°C, 15,000 rpm for 20 min and the supernatant was used as the crude enzyme. Solid ammonium sulfate was slowly added to the supernatant in an ice-water bath to 25% saturation; the mixture was kept for 2 h at 4°C. The precipitate was removed by centrifugation at 12,000 rpm, 4°C, for 30

min; additional ammonium sulfate was added to the supernatant give a final concentration of 45% saturation. The mixture was left overnight at 4°C. The formed precipitate was collected by centrifugation and dissolved in 20 m*M* of phosphate buffer (pH 5.8). The solution was dialyzed overnight against the same buffer.

The dialyzed enzyme solution was applied to a DEAE-cellulose (DE32) column (4 × 32 cm) pre-equilibrated with 20 m*M* phosphate buffer (pH 5.8) containing 10 m*M* mercaptoethanol. The column was washed with the same buffer and the enzyme was eluted with a 2000-mL linear gradient of NaCl (0.15–0.4*M*) in the same buffer at a flowrate of 45 mL/h. The active CDase peak fractions were pooled, concentrated, and dialyzed against the same buffer. The dialyzed enzyme solution was applied to a column of hydroxyapatite pre-equilibrated with the same buffer. The column was washed with buffer and the enzyme was eluted with a 600-mL linear gradient of phosphate buffer (20–400 m*M*) at a flowrate of 20 mL/h. The active protein peak fractions were pooled and concentrated. The enzyme solution was applied to a column of Sephadex G150 (2 × 100 cm) pre-equilibrated with a 20-m*M* phosphate buffer (pH 5.8) containing 0.2*M* NaCl and 10 m*M* mercaptoethanol, eluted with the same buffer at a flowrate of 12 mL/h. The active CDase peak fractions were pooled, concentrated, and dialyzed with the initial buffer for affinity chromatography. The affinity matrix of α-cyclodextrin-AH-Sepharose 4B was prepared by the method of Enevoldson (*9*). The concentrated and dialyzed gel-filtration enzyme solution was applied to an affinity column (1 × 8 cm) pre-equilibrated with the 30-m*M* citrate–phosphate buffer (pH 5.8). The same buffer containing 2*M* NaCl was applied to remove any nonbiospecifically adsorbed materials. Washing was continued until protein no longer was released from the column. Up to this point, the CDase activity could not be detected in the elute. Deabsorption of CDase from the affinity column was performed using 2% of α-CD in the 30-m*M* citrate–phosphate buffer (pH 5.8) containing 10 m*M* mercaptoethanol and 0.5*M* NaCl. The enzyme protein was released from the affinity column and emerged as a narrow peak in a volume of 15 mL. The elute was concentrated to 5 mL passed through a Sephadex G25 column (2 × 42 cm), then eluted with 20-m*M* phosphate buffer (pH 5.8) containing 10 m*M* mercaptoethanol. CDase was excluded by the gel and was separated from the α-CD and NaCl.

Enzyme Assay

The standard activity assay procedure used for measurement of CDase activity entailed incubating 1.0 mL of reaction mixture that consisted of 0.5 mL of 2% α-CD, 0.1 mL of 0.1*M*, pH 5.8 acetate buffer, 0.3 mL distilled water, and 0.1 mL enzyme solution at 50°C for 20 min. The reducing sugars formed were determined by the dinitrosalicylic acid method (*10*). One unit of the enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar per min at 50°C, with glucose as standard.

Table 1
Purification of Cyclodextrinase from *Bacillus stearothermophilu* HY-1

Step	Total volume, mL	Total activity, U	Total protein, mg	Specific activity, U/mg	Total Purification, fold
Cell free extract	400	620	4400	0.140	1
$(NH_4)_2SO_4$ fractionation	56	347	1260	0.276	1.97
DEAE-cellulose	320	253	294.4	0.855	6.11
Hydroxyapetite	103	163	67.0	2.43	17.4
Sephadex G150	28	141	32.2	4.39	31.4
Affinity chromatography	16	93.6	2.8	33.4	239

Other Methods

Protein was determined by the method of Lowry (*11*) with bovine serum albumin as the standard. Disk-electrophoresis was carried out by the method of Davis (*12*). SDS-polyacylamide-gel electrophoresis was performed by the method of Weber (*13*). pI was determined by the method of Vesterberg (*14*). The amino acid composition was determined on a Beckman 121 MB amino acid analyzer.

The enzyme reaction products were analyzed by Silica thin-layer chromatography and paper chromatography (Whatman No. 3 paper) with a solvent system of *n*-butanol-pyridine-water (6:4:3 v/v). Sugars on the paper were detected by an aniline reagent. Quantitative analysis of products was performed by HPLC (Water 244).

Chemicals

α-, β-, and γ-CD, pullulan were purchased from Hayashibara. G1–G6, amylose, amylopectin, panose, isopanose were from Sigma. AH-Sepharose 4B, Sephadex, Amphilin (pH3-10), calibrations kits for molecular weight and pI determination were from Phamacia Fine Chemicals.

RESULTS AND DISCUSSION

Purification of Enzyme

Cyclodextrinase of *Bacillus stearothermophilus* HY-1 was purified by the following procedures: disruption by ultrasonication, fractionation with ammonium sulfate, column chromatography on DEAE-cellulose and hydroxyapatite, gel filtration on Sephadex G150, and affinity chromatography on α-cyclodextrin-AH-Sepharose 4B. The results are summarized in Table 1 and Fig. 1. The enzyme was purified 230-fold with 21.1% recovery of activity. The purified enzyme was homogeneous by PAGE (Fig. 2).

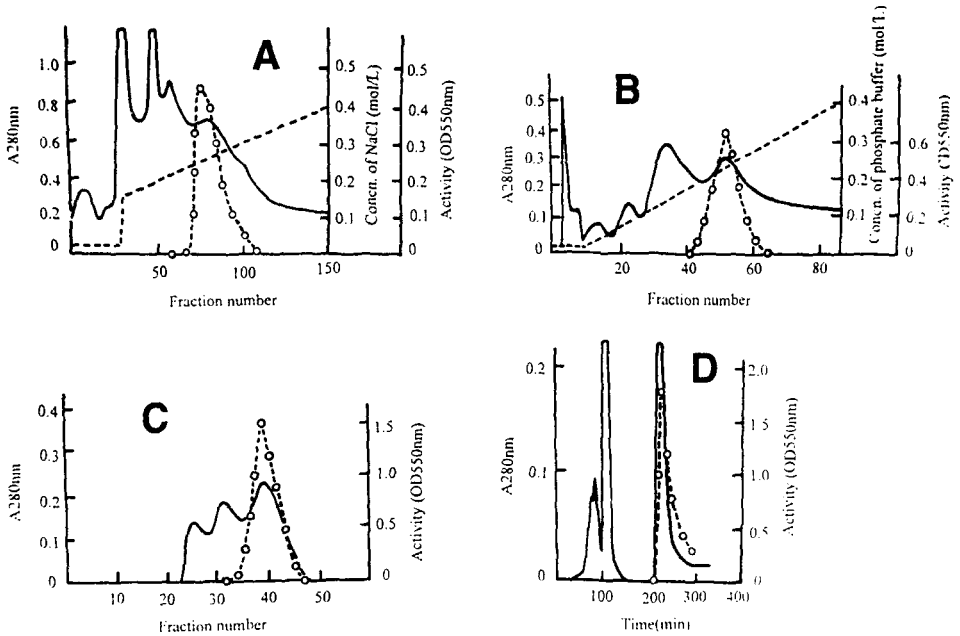

Fig. 1. Purification of cyclodextrinase by column chromatography. (**A**) DEAE-cellulose (DE32); (**B**) Hydroxyapatite; (**C**) Sephadex G150; (**D**) α-cyclodextrin-AH-Sepharose 4B affinity chromatography. ____, A280 nm; ○---○, activity; -----, concentration gradient.

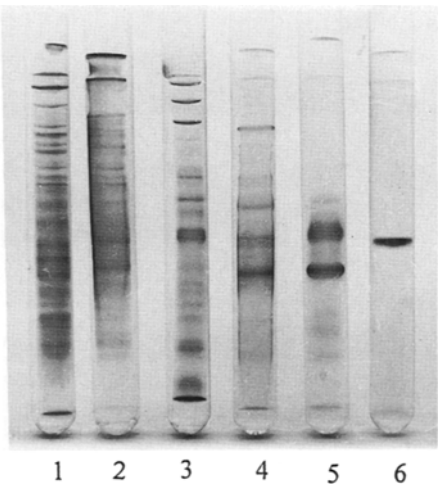

Fig. 2. Polyacrylamide gel electrophoresis of CDase. 1, Cell free extract; 2, $(NH_4)_2SO_4$ fractionation; 3, DEAE-cellulose; 4, Hydroxyapatite; 5, Sephadex G150; 6, α-CD-AH-Sepharose 4B.

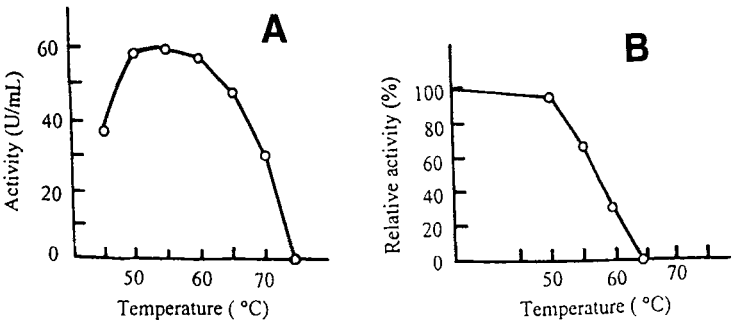

Fig. 3. Effect of temperature on the activity and stability of CDase. (**A**) Effect of heat on activity. The enzyme activity was assayed at various temperature by the standard assay method. (**B**) Thermal stability. The enzyme was placed in 50 m*M* acetate buffer (pH 5.8) and preincubated at various temperatures for 30 min, and then residual activity was determined.

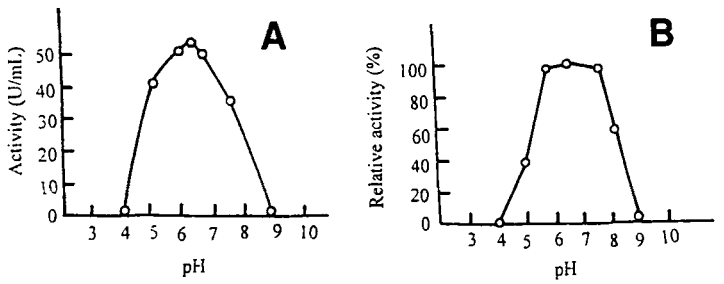

Fig. 4. Effect of pH on the activity and stability of CDase. (**A**) Effect of pH on the activity. The enzyme activity was determined by changing the buffer to obtain the desired pH. (**B**) Effect of pH on the stability. The enzyme was incubated in various pH values of buffer at 30°C for 1 h. After adjustment of pH, the residual activity was determined.

Enzymatic Properties

Effect of Temperature on Activity and Stability

The enzyme activity was assayed at various temperatures (Fig. 3A). The optimum temperature of the enzyme was 55°C. To check the thermal stability, the enzyme was placed in 50 m*M* acetate buffer (pH 5.8) and preincubated at various temperatures for 30 min. The remaining activity was determined (Fig. 3B). The enzyme was stable at temperature below 50°C.

Effect of pH on Activity and Stability

The enzyme activity was measured in different pH values of buffer. The results are shown in Fig. 4A. The optimum activity of the enzyme was at pH 6.2. To check the pH stability, the enzyme solution was incubated in various pH values of buffer at 30°C for 1 h. After adjustment of pH, the residual activity was determined (Fig. 4B). The enzyme was stable at pH 5.8–7.6.

Table 2
Effect of Metallic Ions on the Activity

Ions	Residual activity, %
None	100
Mg^{2+}	102
Mn^{2+}	98
Co^{2+}	97
Ba^{2+}	93
Fe^{3+}	33
Cu^{2+}	1.6
Hg^{2+}	1.6
Zn^{2+}	1.5

Table 3
Relative Hydrolyzing Velocity
of the Enzyme on Various Substrates

Substrate	Relative activity, %
α-CD	100
β-CD	56.6
γ-CD	62.3
Soluble starch	10.0
Amylose	15.7
Amylopectin	10.8
Pullulan	10.2
Glycogen	0

Effects of Various Metal Ions on Activity

The various metal ions were added to the standard enzyme activity assay system to make its final concentration at 1.0 m*M*. The remaining activity was determined. The results are shown in Table 2. The enzyme was strongly inhibited by Cu^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, and slightly by Fe^{3+}.

Substrate Specificity and Products Analysis

The rates of the reaction of the enzyme with various substrates were measured quantitatively by the standard enzyme assay method. The results are shown in Table 3. The CDase could hydrolyze α-CD much faster than the β- and γ-CD. The enzyme had lower activity on starch, amylose, amylopectin, and pullulan, and no activity on glycogen. The standard reaction mixture was incubated overnight; a 10-μL sample of solution was applied to a gelate TLC plate (Fig. 5). The final products of the enzyme reaction on starch, amylose, and amylopectin were maltose and glucose. Panose was the final product on pullulan. The enzyme was reacted with α-CD and pullulan at 50°C overnight; a 10-μL sample of solution was taken for

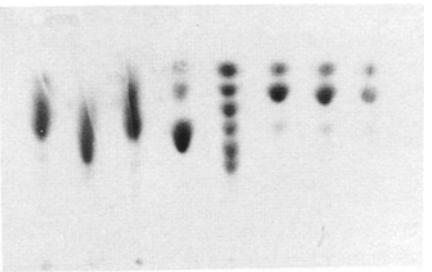

Fig. 5. Final product analysis of the enzyme on various substrates by the silica thin-layer chromatography. 1, soluble starch; 2, amylose; 3, amylopectin; 4, pullulan; 5, G1-G6; 6, α-CD; 7, β-CD; 8, γ-CD.

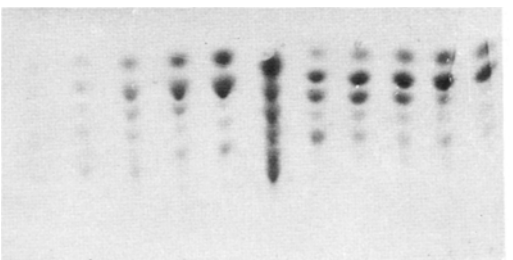

Fig. 6. Action pattern of the enzyme on α-CD and maltohexaose. 1-5, α-CD at 20, 40, 80, 160 min, and 14 h; 6, G1-G6; 7-11, maltohexaose 20, 40, 80, 160 min, and 14 h. The substrates were hydrolyzed in standard assay method system.

thin-layer chromatography (Fig. 6). The results indicated that the intermediate products include G1-G6 oligosaccharides and the reaction of the α-CDase with maltohexaose was complete at 50°C for 20 min, but with α-CD about 80 min. The enzyme hydrolyzed maltohexaose much faster, showing that cleavage of the pyronese ring is the velociting limiting step for α-CD hydrolysis.

In order to determine the product of the enyzme on pullulan, 4.0 mL of standard reaction mixture were incubated at 50°C overnight, concentrated in a boiling water bath, and applied to a Whatman No. 3 filter-paper. The paper chromatogram was developed twice and dried. The triosesaccharide fraction was cut out and dipped in 10 mL of water. After centrifugation, the supernatant was concentrated by boiling water bath and an appropriate fraction was mixed with panose, isopanose, and maltotriose and applied to thin-layer chromatography. The results are shown in Fig. 7. It indicated that panose was its final product.

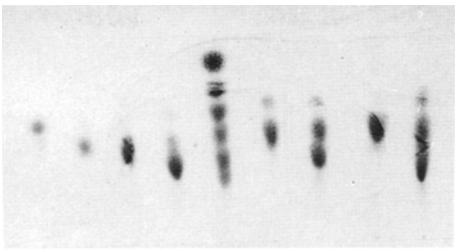

Fig. 7. Analysis of product from pullulan hydrolysis by the enzyme. 1, G3; 2, panose; 3, product; 4, isopanose; 5, G1–G6; 6, product + G3; 7, product + isopanose; 8, product + panose; 9, product + G3 + isopanose.

Table 4
HPLC Quality Analysis of the Final Products
of α-CD and Pullulan Hydrolysis by the CDase

	Products, g/mL						
Substrate	Pullulan	α-CD	Maltotriose	Panose	Maltose	Glucose	Total
Pullulan	0.246	—	—	0.961	0.211	0.094	1.511
α-CD	—	0.284	0.08	—	1.811	0.041	2.220

The quantitative analysis of the final products for α-CD and pullulan was peformed by HPLC (Waters 244, equipped with a Sugar Par I column, a Refractive-Index detector: Model RI:4x, the flow phase was water, flow rate was 0.7 mL/min, the column temperature was 90°C). The results are summarized in Table 4. The final products of the enzyme for α-CD were maltose and a little glucose, whereas those for pullulan were panose, maltose, and glucose (3:1:1) (Table 4). The above results indicate that the enzyme hydrolyzed starch, amylose, and amylopectin to maltose and glucose; it could also hydrolyze pullulan to panose, maltose, and glucose (3:1:1), resembling the activity of neopullulanase (*15*). It hydrolyzed α-, β-, and γ-CDs to produce maltose, a small amount of glucose and a very small amount of maltotriose and maltotetraose. It appears that the CDase initially opens the corresponding number of glucose units (slow) and then degrades the linear molecules into lower malto-oligosaccharides in an exofashion by preferential cleavage of maltose units from noneduced ends (fast).

K_m and V_{max}

Initial velocities of the enzyme reaction were determined under the standard assay conditions and were expressed as μmols of reducing groups per mg protein (Table 5). The α-CD was the optimal substrate of the enzyme.

Table 5
Kinetic Values of the Enzyme for Various Substrates

Substrate	Km, mg/mL	Vmax, μmol/mg/min
α-CD	1.79	336
β-CD	1.67	185
γ-CD	2.50	208
Pullulan	8.33	63.4

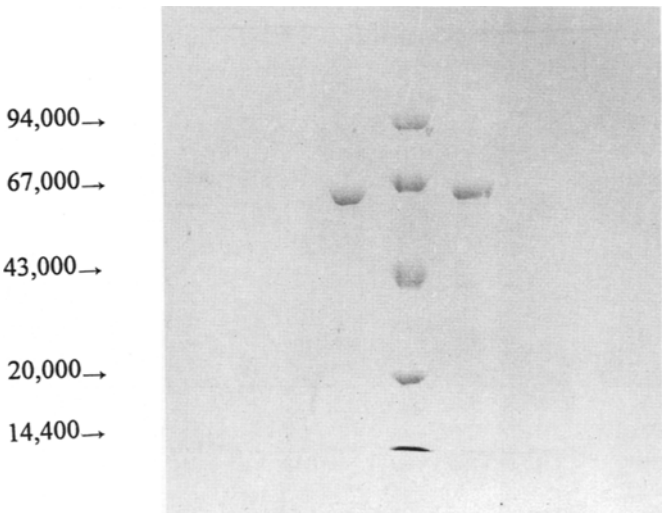

Fig. 8. Determination of molecular weight of the enzyme by SDS-PAGE.

Molecular Properties

The molecular weight of the enzyme was estimated to be 61,000 Daltons by SDS-polyacrylamide-gel electrophoresis (Fig. 8). The isoelectric point of the enzyme was pH 5.4 (Fig. 9). The amino acid composition of the enzyme is shown in Table 6.

Chemical Modification

The enzyme was preincubated with various chemical modification reagents at 25°C for 45 min. The reaction mixture consisted of 50 μL appropriate enzyme solution, 50 μL buffer, 100 μL distilled water, and 50 μL modification reagents solution. The reaction was stopped by adding 750

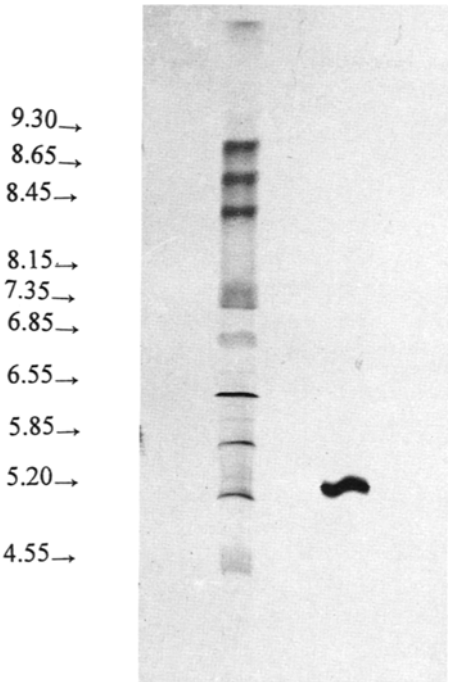

Fig. 9. Determination of pI by PAGE-IEF.

Table 6
Amino Acid Composition of the Cyclodextrinase

Amino acid	Mol percent, %	Amino acid residues/mole
Asx	13.30	72
Thr	5.42	29
Ser	3.20	17
Glx	9.34	50
Pro	5.25	28
Gly	9.94	54
Ala	11.60	63
Val	6.33	34
Met	0.48	3
Ile	4.43	24
Leu	9.44	51
Tyr	2.37	13
Phe	4.04	22
Lys	5.51	30
His	3.21	17
Arg	6.24	34
Cys	Nd	—

Asp, Asn and Glu, Gln were determined as Asx and Glx.

Table 7
Effect of Chemical Modification Reagents on the Activity

Reagent	Concn, m*M*	Buffer, 50 m*M*	pH	Relative activity, %
None	—	—	—	100
NEM	10	phosphate buffer	7.0	92.6
IAA	1	phosphate buffer	7.0	95.0
AII	1	phosphate buffer	8.0	107
DEPC	1	phosphate buffer	6.0	83.6
	10	phosphate buffer	6.0	2.3
NBS	0.01	acetate buffer	4.6	100
	0.1	acetate buffer	4.6	81.1
	1.0	acetate buffer	4.6	4.6

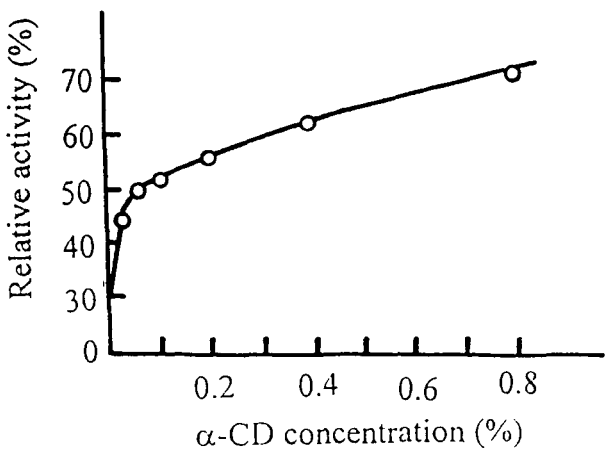

Fig. 10. Protection of CDase activity from NBS treatment by various concentration α-CD.

μL of 0.1*M* acetate buffer (pH 5.8). The results are shown in Table 7. *N*-ethylmaleimide, iodoacetamide, and *N*-acetylimidazole had little effect on the activity; this suggests that -SH groups and tyrosine residues are not essential for the enzyme activity. The enzyme was strongly inhibited by diethylpyrocarbonate and *N*-bromosuccinimide. After the enzyme was incubated with 10 m*M* diethylpyrocarbonate at 25°C for 45 min, 250 mL of 0.5*M* hydrolylamine chloride solution were added and incubated for another 45 min. The enzyme activity recovered to 52.4%. This indicated that the histidyl residue(s) is essential for activity. The various concentrations of α-CD were added to the enzyme solution 3 min before adding *N*-bromosuccinimide. The mixture was incubated under the same conditions as previously described. The enzyme activity was protected by substrate (Fig. 10). After protection and NBS modification, the enyzme solutions were applied to a Model 850 Spectrophotometer to measure the fluorescence spectra. The results are shown in Fig. 11. After modification by NBS, the 335-nm emitting peak of the enzyme decreased, whereas the

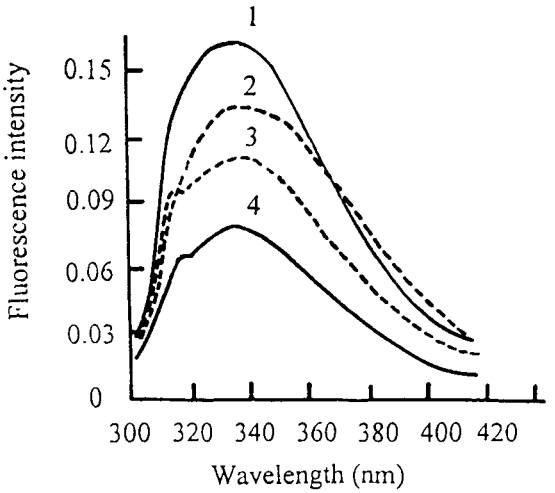

Fig. 11. Fluorescence spectra of CDase after NBS treatment and α-CD protection. 1, 0 m*M* NBS, 0% substrate conc.; 2, 10 m*M* NBS, 0.8% substrate conc.; 3, 10 m*M* NBS, 0.2% substrate conc.; 4, 10 m*M* NBS, 0% substrate conc.

peak was present with the protected sample. These results proved that the tryptophyl residue(s) is located at or near the active site of the enzyme.

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