Studies on D-Glucose-isomerizing Enzyme from *Bacillus coagulans*, Strain HN-68

Part IV Purification, Crystallization and Some Physicochemical Properties

By Gen-ichi DANNO

*Department of Agricultural Chemistry, Faculty of Agriculture,
Kobe University, Nada-ku, Kobe, Japan*

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D-Glucose-isomerizing enzyme was purified in a crystalline form with a good yield from the cells of *Bacillus coagulans*, strain HN-68, and some physicochemical properties were investigated.

The purified enzyme was homogeneous on both ultracentrifugal and disc-electrophoretic analyses. The molecular weight of the enzyme was determined to be 175,000 and 160,000 from the sedimentation-viscosity method and the gel filtration method, respectively.

The sedimentation coefficient ($s_{20,w}^0$), partial specific volume, $E_{1\text{cm}}^{1\%}$ at 280 m μ , and the nitrogen content of the enzyme were determined to be 10.2×10^{-13} sec., $0.705 \text{ cm}^3 \text{ g}^{-1}$, 10.6 and 16.2%, respectively. The integral numbers of amino acid residues per molecule calculated on the basis of 160,000 were as follows; Lys₁₂₀, His₄₉, Arg₆₁, Asp₁₈₂, Thr₈₇, Ser₇₀, Glu₁₃₆, Pro₄₄, Gly₁₀₆, Ala₁₄₀, Half-Cys₀, Val₅₃, Met₂₇, Ileu₆₁, Leu₁₃₄, Tyr₅₈, Phe₉₆, Try₁₃, and amide-ammonia₈₀.

In the previous paper,¹⁻³⁾ the author has reported that D-glucose-isomerizing enzyme from *Bacillus coagulans*, strain HN-68, newly isolated from soil, is D-xylose-dependent inducible enzyme and its enzymatic properties were also reported on the partially purified enzyme using D-glucose as a substrate. D-Glucose-isomerizing enzyme was activated by Co^{2+} and was capable of isomerizing not only D-glucose but also D-xylose. However, the ratio of D-glucose- to D-xylose-isomerizing activities varied during the purification process. Recently it was found that D-xylose-isomerizing activity of partially purified preparation was

strongly activated by Mn^{2+} , which could not activate D-glucose-isomerizing activity in the same preparation. These facts seem to suggest the some differences between D-glucose- and D-xylose-isomerizing activities in the enzyme preparation. In order to elucidate more comprehensive characters of D-glucose-isomerizing enzyme from *B. coagulans*, strain HN-68, further purification of the enzyme was attempted.

The present paper describes the procedures of purification and crystallization, and some physicochemical properties of this D-glucose-isomerizing enzyme.

1) S. Yoshimura, G. Danno and M. Nataka, *Agr. Biol. Chem.*, **30**, 1015 (1966).

2) G. Danno, S. Yoshimura and M. Nataka, *ibid.*, **31**, 284 (1967).

3) G. Danno, *ibid.*, **34**, 1658 (1970).

MATERIALS AND METHODS

Organism, culture conditions and preparation of cell free extract. *Bacillus coagulans*, strain HN-68 was used in

this study. The growth medium contained: D-glucose, 20 g; yeast extract, 3.0 g; NH_4Cl , 3.0 g; K_2HPO_4 , 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.05 g and CaCO_3 , 10 g in 1 liter of water. When the organism was grown at 40°C for 16 hr in a 20-liter jar-fermenter, the cells were harvested by continuous centrifuge and were washed with sterilized water. The washed cells were suspended at a concentration of 20 mg (dry basis) per ml in a following induction medium: D-xylose, 20 g; polypeptone (Daigo-Eiyo Co.), 30 g; KH_2PO_4 , 0.08 g. K_2HPO_4 , 0.02 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g in one liter. After the suspension was adjusted to pH 5.5–6.0 with 0.1 N NaOH or HCl, 100 ml each of the suspension were taken into 500-ml shaking flasks, and the flasks were incubated for 5 to 6 hr at 40°C with reciprocal shaking. The cells were again harvested by centrifugation, washed three times with 0.05 M phosphate buffer, pH 7.0, and resuspended in 3 liters of 0.05 M phosphate buffer containing 0.005 M EDTA (pH 7.0). To extract the enzyme from cells, the cell suspension containing 60 g of cells (dry basis) was mixed with 50 ml of toluene and 100 mg of crystalline lysozyme and incubated at 20°C for 20 hr. Cell debris was removed by centrifugation for 30 min at $15,000 \times g$. Resulting supernatant solution was used following experiment as crude enzyme preparation.

Sedimentation analysis. Ultracentrifugal analysis was performed with a Hitachi UCA-1 in Faculty of Science, Kobe University. All runs were made at 55,430 rpm at 20°C. The protein was dissolved in 0.01 M phosphate buffer (pH 7.0) containing 0.1 M sodium chloride. Sedimentation coefficient was corrected to zero protein concentration, at 20°C, and in water as described by Svedberg.⁴⁾

Determination of protein concentration. Protein concentration was determined by the method of Lowry *et al.*⁵⁾ with crystalline bovine serum albumin as a standard. For the determination of protein concentration in the eluate in column chromatography, optical density at 280 m μ was measured.

Assay of D-glucose-isomerizing activity. The incubation mixture containing 1 ml of enzyme solution and 1 ml of 0.03 M CoCl_2 solution was preincubated for 20 min at 40°C, and then 1 ml of 0.3 M D-glucose

solution in 0.02 M barbital buffer (pH 7.0) was added to this mixture. After incubation at 40°C for 30 min, the reaction was stopped by adding 5 ml of 0.3 M perchloric acid, and D-fructose formed was assayed by cysteine-carbazole method.⁶⁾

Assay of D-xylose-isomerizing activity. The incubation mixture containing 1 ml of enzyme solution and 1 ml of 0.03 M MnCl_2 solution was preincubated for 20 min at 40°C, and then 1 ml of 0.03 M D-xylose solution in 0.3 M borate buffer (pH 7.0) was added to this mixture. After incubation at 40°C for 30 min, the reaction was stopped by adding 1 ml of 1.0 M perchloric acid, and ketose formed was assayed by cysteine-carbazole method. Color was developed at 35°C for 20 min.⁷⁾

Assay of D-ribose-isomerizing activity. The incubation mixture containing 1 ml of enzyme solution and 1 ml of 0.03 M CoCl_2 solution was preincubated for 20 min at 40°C, and then 1 ml of 0.06 M D-ribose solution in 0.02 M barbital buffer (pH 7.0) was added to this mixture. After incubation at 40°C for 30 min, the reaction was stopped by adding 1 ml of 1.0 M perchloric acid, and ketose formed was assayed by cysteine-carbazole method. Color was developed at 20°C for 20 min.

The linear relationship between enzyme activity and amount of protein were obtained as shown in Fig. 1. One unit of these enzymatic activities was defined as the enzyme amount which produces 1 μ mole of ketose per one minute under the above assay conditions.

Disc-electrophoresis. Analytical gel electrophoresis was performed in 7.5% polyacrylamide gels using Tris-glycine buffer, pH 8.9, by the method of David.⁸⁾ About 200 μ g of proteins in 15% sucrose solution was layered on the gel and samples were run at 2.5 mA per tube for 2 hr, after electrophoresis the gels were stained with Amide black 10 B.

Viscosity determination. Viscosity determinations were carried out with an Ostwald's viscometer immersed in a constant temperature water bath at 20°C. A volume of 5 ml was used for these determinations.

4) T. Svedberg and K. O. Pedersen, "The Ultracentrifuge," Oxford Univ. Press, 1940.

5) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **93**, 265 (1951).

6) Z. Dishe and E. Borenfreund, *J. Biol. Chem.*, **192**, 583 (1951).

7) K. Yamanaka, a private letter.

8) Y. Nagai, "Buturi-kagaku Jikken-ho," 3, Kyoritu, 1967, p. 3.

FIG. 1. Linear Relationship between Activity and Enzyme Protein.

The conditions are described in the text.

A: D-Glucose-isomerizing activity (550 mμ)

B: D-Xylose-isomerizing activity (530 mμ)

C: D-Ribose-isomerizing activity (530 mμ)

Protein was dissolved in 0.01 M sodium acetate of pH 6.0 containing 0.1 M sodium chloride.

Partial specific volume. The partial specific volume of the enzyme was determined with 5-ml pycnometer at 25°C. The samples at 0.4, 0.6 and 1.0% protein concentration yielded a value of 0.705 for the partial specific volume.

Molecular weight determination by gel filtration. The method of Andrews⁹⁾ was applied to the enzyme. A column (2.5×45 cm) of Sephadex G-200 was equilibrated with 0.01 M phosphate buffer of pH 7.0 containing 0.1 M sodium chloride. To measure the void volume (V_0) of the column, blue dextran 2000 was used. Four standards were used: pepsin (mol. wt. 35,000), bovine serum albumin (mol. wt. 67,000), yeast alcohol dehydrogenase (mol. wt. 150,000) and yeast catalase (mol. wt. 250,000). Ten milligram of each protein was separately dissolved in 1.0 ml of the buffer with blue dextran and developed with the same buffer. The enzyme of *B. coagulans* was treated in the same manner. Each sample was run twice, and an average value for V/V_0 was calculated from V_0 and the elution volume (V). The same column was repeatedly used after washing with the same buffer as used in each run.

Amino acid analysis. About 5 mg of the enzyme

was hydrolyzed with 1 ml of twice distilled 6 N HCl in a sealed, evacuated tube for 24, 48 and 72 hr at $110 \pm 1^\circ\text{C}$. The hydrolysates were cooled, evaporated to dryness under vacuum, and then dissolved in appropriate buffers. The amino acid analysis was performed at Faculty of Medicine, Kobe University.

Nitrogen analysis. The micro-Kjeldahl method was used for the determination of the nitrogen content of the enzyme.

Amide content. The Conway diffusion method¹⁰⁾ was used to determine the amide content. For this determination, several 10-mg samples of enzyme were dissolved in 1 ml of 1 N NaOH in the outer compartment. The inner well contained 1 ml of 2% boric acid. After standing for 24, 48 and 72 hr, the inner well was titrated with 0.01 N HCl with bromphenol blue as an indicator. The value extrapolated to zero time was adopted as amide ammonia.

Tryptophan content. For the determination of the tryptophan content in the enzyme, the spectrophotometric method of Goodwin and Morton,¹¹⁾ and the *p*-dimethylaminobenzaldehyde method¹²⁾ were used.

10) D. Tsuru, J. D. McConn and K. T. Yasunobu, *J. Biol. Chem.*, **240**, 2415 (1965).

11) T. W. Goodwin and R. A. Morton, *Biochem. J.*, **40**, 628 (1946).

12) J. R. Spies and D. C. Chambers, *Anal. Chem.*, **21**, 1249 (1949).

9) P. Andrews, *Biochem. J.*, **91**, 222 (1964); **96**, 395 (1965).

Phosphorus and carbohydrate content. The phosphorus content was determined by the methods of Allen and Nakamura.¹³⁾ The tryptophan-sulfuric acid method¹⁴⁾ the phenol-sulfuric acid method,¹⁵⁾ and the orcinic-sulfuric acid method¹⁶⁾ were used for determining the total carbohydrate and pentose contents.

RESULTS AND DISCUSSION

I. Purification of D-glucose-isomerizing enzyme

All operations were conducted at 2~5°C, except for otherwise noted.

Treatment with manganese sulfate

To the crude cell-free extract, 1 M manganese sulfate solution was added slowly with mechanical stirring up to 0.1 M of the final concentration of manganese sulfate. A pH of about 7 was maintained during this operation by periodic dropwise addition of 0.5 N sodium hydroxide. The solution was stirred for an additional 10 min after the addition of manganese sulfate followed by standing for 30 min. It was then centrifuged for 10 min at 10,000×*g* and the precipitate was discarded. The floating materials in the supernatant fluid was filtered off with filter paper.

Ammonium sulfate fractionation

To the above supernatant fluid, powdered ammonium sulfate was added slowly under mechanical stirring to give a 0.5 saturation. The solution was then adjusted to pH 4.5~4.7 by dropwise addition of 10% acetic acid. The solution was stirred for 10 min and allowed to stand for overnight before being centrifuged for 30 min at 15,000×*g*. The precipitate obtained was dissolved in 150 ml of 0.05 M potassium

phosphate buffer (pH 7.0), and dialyzed against distilled water for 3 days. The resulted precipitate was centrifuged and discarded.

DEAE-Sephadex A-50 chromatography

One molar concentration of acetate buffer (pH 6.0) was added to the dialyzed enzyme up to 0.05 M acetate concentration. The enzyme solution was applied to a column (2.5×30 cm) of DEAE-Sephadex A-50 which had been previously equilibrated with 0.05 M sodium acetate buffer (pH 6.0). Then, 500 ml of 0.05 M sodium acetate buffer (pH 6.0) was passed through the column. The enzyme was then eluted from the DEAE-Sephadex column by linear gradient elution with 1500 ml of 0.05 M sodium acetate buffer (pH 6.0) in the mixing chamber and 1500 ml of 0.3 M sodium acetate buffer (pH 6.0) in the reservoir. Figure 2 presents a

FIG. 2. Column Chromatography of D-Glucose-isomerizing Enzyme on DEAE-Sephadex A-50 Column (pH 6.0).

The enzyme solution (250 ml) was applied to a DEAE-Sephadex A-50 column (2.5×30 cm) which was previously equilibrated with 0.05 M acetate buffer (pH 6.0), and effluent was collected in 15 ml per fraction. ---, Optical density at 280 mμ; ○, D-glucose-isomerizing activity; ●, D-xylose-isomerizing activity (×0.5); △, D-ribose-isomerizing activity; ---, concentration of sodium acetate (M).

13) M. Nakamura, *Nippon Nogeikagaku Kaishi*, **23**, 1 (1950).

14) J. Badin, J. Clifford and M. Schubert, *Proc. Exptl. Biol.*, **84**, 288 (1953).

15) M. Dubois, K. A. Gisses, J. K. Hamilton, P. A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956).

16) J. B. Sumner, *J. Am. Chem. Soc.*, **45**, 2378 (1923).

typical elution profile showing protein concentration and enzyme activities. Fractions with a high specific activity were pooled and dialyzed against 0.01 M phosphate buffer containing 0.1 M sodium chloride (pH 8.6).

Rechromatography on DEAE-Sephadex A-50

The enzyme solution from the preceding step was applied to a column (2×30 cm) of DEAE-Sephadex A-50 previously equilibrated with 0.01 M phosphate buffer (pH 8.6) containing 0.05 M sodium chloride. The enzyme was eluted with 0.05 M to 0.2 M sodium chloride in 0.01 M phosphate buffer (pH 8.6), by linear gradient elution. As shown in Fig. 3, the enzyme was eluted as a single peak and this preparation was homogeneous in disc electrophoresis.

FIG. 3. Rechromatography of D-Glucose-isomerizing Enzyme on DEAE-Sephadex A-50 Column (pH 8.6).

The solution obtained from the previous step was dialyzed against 0.01 M phosphate buffer (pH 8.6) containing 0.05 M NaCl and applied to a DEAE-Sephadex A-50 column (2.0×30 cm) equilibrated with the same buffer. ---, Optical density; ○, D-glucose-isomerizing activity; ●, D-xylose-isomerizing activity (×0.5); △, D-ribose-isomerizing activity; ---, concentration of NaCl (M).

Crystallization using ammonium sulfate

The enzyme fractions (tube No. 62~79) from second DEAE-Sephadex A-50 column were combined and dialyzed against deionized water for 2 days. The dialyzed solution was concentrated by ultrafiltration method with collodion bag (Sartorius-Membranfilter GmbH). This enzyme solution (about 3% concentration) was transferred into a cellophane dialysis tubing (φ 6.4 mm) and dialyzed against 500 ml of 0.1 M sodium acetate buffer solution containing 0.5 saturated ammonium sulfate (pH 6.0) for 24 hr in a cold room. Saturated ammonium sulfate solution was added dropwise into outer

FIG. 4. Photomicrograph of Crystalline D-Glucose-isomerizing Enzyme from *B. coagulans*, Strain HN-68.

A: Crystallization with 0.6 sat. ammonium sulfate (pH 6.0) ca. ×300, B: Crystallization with aqueous acetone ca. ×600.

solution of the tubing to reach 0.6 saturation, crystals of the enzyme appeared after a days and were collected by centrifugation. The crystals were dissolved in a small amount of 0.1 M sodium acetate buffer of pH 6.0 and recrystallized by the same manner as above. Microphotograph of crystalline enzyme preparation is shown in Fig. 4a. The final suspension of crystals was stored at 2°C. The crystalline enzyme, when stored as a suspension in 0.6 saturate ammonium sulfate, was quite stable for several months. Samples were centrifuged and dissolved in water before use.

Crystallization in aqueous acetone

The enzyme solution obtained from the re-chromatography on DEAE-Sephadex A-50 column was dialyzed against distilled water. To the dialyzed solution cooled in ice bath was added equal volume of acetone which was cooled to -20°C beforehand. The addition of acetone was performed with mechanical stirring to keep a temperature of 0~2°C. The precipitate thus formed was rapidly collected by centrifugation (8000×g) and then dissolved in a small volume of 0.1 M sodium acetate (pH 6.0). The insoluble materials were removed by centrifugation and discarded. To the supernatant solution cold acetone was added until permanent turbidity was observed. The turbid solution was placed at 5°C and

crystallization began after 2 days. The crystals were collected by centrifugation and dissolved in 0.1 M sodium acetate and crystallization was repeated as above. The crystals are shown in Fig. 4b. As the crystalline enzyme in aqueous acetone was somewhat unstable, the crystals were collected by centrifugation and dissolved in distilled water, and then stored at 3~5°C.

The results of the overall purification procedure are summarized in Table I. The ratios of D-xylose- and D-ribose-isomerizing activities to D-glucose-isomerizing activity were practically constant through the whole purification procedure, when Co^{2+} , Mn^{2+} and Co^{2+} were added in the metal free enzyme solution for D-glucose-, D-xylose- and D-ribose-isomerizing reaction, respectively. This result suggests that three activities are not due to individual enzymes. D-Glucose-isomerizing enzyme was purified about 44-fold over the crude extract with an overall yield of 46%.

The purified enzyme solution, when it was stored in a refrigerator at 2~5°C, was stable for two monthes. However, when the enzyme solution was freezed and lyophilized, the activity decrease was accompanied with irreversible coagulation of the enzyme.

Homogeneity

The homogeneity of the purified enzyme

TABLE I. COMPARISON OF ISOMERIZING ACTIVITIES ON D-GLUCOSE, D-XYLOSE AND D-RIBOSE DURING PURIFICATION OF D-GLUCOSE-ISOMERIZING ENZYME FROM *B. coagulans*.

	Total volume (ml)	Total protein (mg)	Total activity (unit) ^{a)}			Ratio of	
			D-glucose	D-xylose	D-ribose	X/G	R/G
Crude extract	3,000	28,000	930	4,200	175	4.5	0.19
Mn-treated supernatant	3,260	17,000	850	3,900	157	4.6	0.18
Ammonium sulfate fraction	250	1,900	700	3,200	130	4.6	0.19
DEAE-Sephadex column (pH 6.0) chromatography, fractions 81~100	300	430	610	2,750	115	4.6	0.19
DEAE-Sephadex column (pH 8.6) chromatography, fractions 62~78	300	390	560	2,550	103	4.6	0.18
Crystals, from 0.6 sat. ammonium sulfate		295	430	1,950	79	4.5	0.18
Crystals, from aqueous acetone ^{b)}		240	340	1,600	63		

^{a)} The enzyme solution was dialyzed against 0.01 M EDTA, then against distilled water before assay.

^{b)} Another lot.

FIG. 5. Sedimentation Patterns of Purified D-Glucose-isomerizing Enzyme from *B. coagulans*, Strain HN-68.

The enzyme at a concentration of 10 mg per ml in 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium chloride, was centrifuged at 55,430 rpm at 20°C. Photographs were taken at every 10 min interval after reaching maximum speed.

FIG. 6. Purity at Various Stage in the Purification of D-Glucose-isomerizing Enzyme as Monitored by Polyacrylamide Gel Electrophoresis.

Disc electrophoresis was carried out as described in the text using 7% polyacryl amide gel (runing at pH 8.9).

1: ammonium sulfate precipitate, 2: fraction from DEAE-Sephadex column (pH 6.0), 3: fraction DEAE-Sephadex column (pH 8.6), 4: crystals with ammonium sulfate.

was determined by the following two methods: ultracentrifugation and disc-electrophoresis.

In the analytical ultracentrifuge, the purified enzyme was homogeneous with a single sedimentation peak (Fig. 5).

The homogeneity of the purified enzyme was also observed by disc-electrophoresis with acrylamide gel at pH 8.9. Both enzyme preparations from rechromatography on DEAE-Sephadex column and crystalline enzyme were homogeneous with a single band (Fig. 6).

II. Physicochemical properties

Ultraviolet absorption spectra

The ultraviolet absorption spectra of the enzyme in 0.1 N NaOH, and in 0.1 M sodium acetate buffer (pH 6.0) are presented in Fig. 7. The native enzyme (in 0.1 M sodium acetate buffer) shows a single absorption peak having a maximum at 278 m μ and minimum at 251 m μ . The extinction coefficient ($E_{1\text{cm}}^{1\%}$) value of the native enzyme at 280 m μ was 10.6. The ratio of absorption at 280 m μ to that at 260 m μ was 1.9, indicating the absence of significant amount of nucleic acid component. The purified D-glucose-isomerizing enzyme did not contain any carbohydrate and phosphorus.

Isoelectric point

Enzyme solution spotted at center of the

FIG. 7. Absorption Spectrum of Purified D-Glucose-isomerizing Enzyme from *B. coagulans*, Strain HN-68.

A: in 0.1 M acetate buffer (pH 6.0)

B: in 0.1 M sodium hydroxide

The protein concentration was 0.942 mg per ml.

FIG. 8. Plots of Moving Distance as a Function of pH on Cellulose Acetate Electrophoresis.

cellulose acetate film (2×10 cm) bufferized with acetate buffer (ionic strength, 0.1) over a pH range of 4.5~5.3. The film was sandwiched between slide glasses coated with silicone grease and then run at 5°C for a hour.

The protein was detected by staining with amido black 10 B. Figure 8 shows a plot of moving distance as a function of pH. The apparent isoelectric point of this enzyme was determined to lie around pH 4.9

Sedimentation coefficient, intrinsic viscosity and partial specific volume

The $s_{20,w}$ values of the protein over the concentration range of 0.25 to 1.0% fell on a straight line as shown in Fig. 9. The least squares method gave a value of 10.4 S. Viscosity measurement using an Ostwald's visco-

FIG. 9. Sedimentation Coefficient Observed at Various Concentrations of Enzyme.

The enzyme was dissolved in 0.01 M phosphate buffer containing 0.1 M sodium chloride (pH 7.0).

FIG. 10. Reduced Viscosity (η_{sp}/c) Observed at Various Concentration of Enzyme.

Viscosity measurement were made in 0.01 M phosphate buffer containing 0.1 M sodium chloride (pH 7.0). The intrinsic viscosity obtained $[\eta] = 0.035$ dl/ml.

meter were performed over the concentration range of 0.25 to 1.5%. Plot of reduced viscosity (η_{sp}/c) of the enzyme against enzyme concentration is shown in Fig. 10. The intrinsic viscosity $[\eta]$ was determined to be 0.035 dl/g. The partial specific volume (v) was 0.705 ml/g.

Estimation of molecular weight

The molecular weight of the enzyme was determined by the following two methods: the sedimentation-viscosity method and the gel filtration method with Sephadex G-200 column.

a. *Molecular weight by the sedimentation-viscosity method.* The molecular weight of the D-glucose-isomerizing enzyme of *B. coagulans*, strain HN-68 was calculated from the formula according to Sheraga and Mandelkern¹⁷⁾;

$$M = \frac{4700 (s_{20,w}^0)^{3/2} [\eta]^{1/2}}{(1 - v\rho)^{3/2}}$$

where, $s_{20,w}^0 = 10.2$, $[\eta] = 0.035$, and $v = 0.705$.

FIG. 11. Relationship between Elution Volume and Molecular Weight.

A column (2.5×45 cm) of Sephadex G-200 was used. V/V_0 is scaled in the ordinate.

1. pepsin; 2, bovine serum albumine; 3, yeast alcohol dehydrogenase; 4, yeast catalase; 5, D-glucose-isomerizing enzyme from *B. coagulans*, strain HN-68.

17) H. A. Sheraga and L. Mandelkern, *J. Am. Chem. Soc.*, **75**, 179 (1953).

From these data, the molecular weight was calculated to be 175,000.

b. *Molecular weight by gel filtration.* The estimation of the molecular weight was performed according to the method of Andrews with a Sephadex G-200 column which was previously equilibrated with 0.01 M phosphate buffer (pH 7.0) containing 0.1 M sodium chloride. As seen in Fig. 11, the standard proteins showed a linear relation between log value of molecular weight and a ratio of V/V_0 (see Materials and Methods). The enzyme of *B. coagulans*, strain HN-68 was eluted just before yeast alcohol dehydrogenase and gave a molecular weight of approximately 160,000. The molecular weight of the enzyme from *L. brevis*¹⁸⁾ and *Streptomyces* sp.¹⁹⁾ were reported to be 195,000 and 157,000, respectively. Therefore, it appears that the molecular weight of D-glucose-isomerizing enzyme from these strains is similar. The physicochemical properties of the enzyme are summarized in Table II.

TABLE II. SUMMARIZED PHYSICOCHEMICAL PROPERTIES OF D-GLUCOSE-ISOMERIZING ENZYME FROM *B. coagulans*, STRAIN HN-68

1. $E_{1cm}^{1\%}$ at 280 $m\mu$	10.6
2. Partial specific volume (V)	
experimental	7.05 cm ³ /g
3. Sedimentation coefficient ($s_{20,w}^0$)	10.2×10^{-13} sec
4. Apparent isoelectric point (pI_{app})	
cellulose acetate electrophoresis	pH 4.9
5. Molecular weight (M)	
$s_{20,w}^0$	175,000
gel filtration Andrews' method	160,000
6. Intrinsic viscosity ($[\eta]$)	0.035 dl/g
7. Frictional ratio (f/f_0)	1.14 ^{a)}
8. Axial ratio (a/b)	3.4 ^{b)}
9. Stokes radius	35.4 Å ^{c)}

a) Calculated from the data of $s_{20,w}^0$, M and V.

b) Calculated from frictional ratio.

c) Supposed not to be hydrated.

18) K. Yamanaka, *Abstracts of Papers, Annual meeting of Agr. Chem. Soc. Japan*, Nagoya, April, 1968, p. 234.

19) Y. Takasaki, Y. Kosugi and A. Kanbayashi, *Agr. Biol. Chem.*, **33**, 1527 (1969).

TABLE III. AMINO ACID COMPOSITION OF D-GLUCOSE-ISOMERIZING ENZYME FROM *B. coagulans*, STRAIN HN-68

Amino acid	Amino acid residues g/100 g protein ^{a)}	Residues to nearest integer ^{b)}
Aspartic acid	13.1	182
Threonine	5.50 ^{c)}	87
Serine	3.85 ^{c)}	70
Glutamic acid	11.1	136
Proline	2.66	44
Glycine	3.80	106
Alanine	5.68	140
Half-cystine	0	0
Methionine	2.25	27
Valine	3.32 ^{d)}	53
Isoleucine	3.61 ^{d)}	51
Leucine	9.55	135
Tyrosine	5.94	58
Phenylalanine	8.86	96
Tryptophan	1.55 ^{e)}	13
Hystidine	4.20	49
Lysine	8.80	120
Arginine	5.92	61
Amide-ammonia	1.25 ^{f)}	80

^{a)} Average values from the 24-, 48-, and 72-hr hydrolysates.

^{b)} Based on a molecular weight of 160,000.

^{c)} Values extrapolated to zero hydrolysis time.

^{d)} Maximum values adopted.

^{e)} Value obtained by the spectrophotometric procedure.

^{f)} Value obtained by the Conway method.

Amino acid composition

Results of amino acid analysis are shown in Table III. Serine and threonine were decomposed with increased time of hydrolysis,

therefore, the values for those amino acids were obtained by extrapolating to zero hydrolysis time. The values for valine and isoleucine were maximum values obtained from 72-hr hydrolysis. Half-cystine was not detected in the hydrolyzate. Therefore, in another experiment, the enzyme was oxidized with performic acid as described by Moore and Crestifield²⁰⁾ and hydrolyzed for 24-hr at 110°C with 6N hydrochloric acid. No cysteic acid was detected in this hydrolyzate. Yamanaka¹⁸⁾ also reported that D-xylose ketol-isomerase from *L. brevis* had no half-cysteine.

Total nitrogen content of the enzyme, estimated by the Micro-Kjeldahl method, was 16.2%. The amide content was determined by extrapolating the ammonia content of the hydrolyzates to zero time as well as by the Conway microdiffusion method and calculated to be 1.25%. The tryptophan content was estimated as 1.8 and 1.72% by the spectrophotometric and the *p*-methylaminobenzaldehyde method, respectively. Total weight of amino acid residues was 99.59 g per 100 g protein. Integral numbers of amino acid residues per molecule were calculated on the basis of molecular weight of 160,000 (Table III).

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20) S. Moore and A. Crestifield, *J. Biol. Chem.*, **238**, 235 (1963).