STUDIES ON GLUCOHYDROLASES OF BOVINE SPLEEN

By Ken Fujimori

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Glucohydrolases [EC 3.2.1.3; EC 3.2.1.20] of bovine spleen have been separated into two fractions by gel filtration on Sephadex G-200. One fraction contained only acid glucohydrolase which was finally isolated in crystalline state. The crystalline acid enzyme showed apparent homogeneity upon ultracentrifugation and polyacrylamide gel electrophoresis. The sedimentation coefficient was 3.85 S, and the molecular weight 43,000. This enzyme catalyzed the liberation of glucose from maltose and glycogen as well. Glycogen was hydrolyzed almost completely to glucose.

The results of heat treatment and of inhibition by turanose demonstrated that the other fraction obtained by the gel filtration contained both acid and neutral glucohydrolases. The neutral enzyme was further separated into four different fractions by preparative polyacrylamide gel electrophoresis. All of these neutral enzymes showed the similar catalytic properties, and hydrolyzed maltose much more rapidly than glycogen. The acid enzyme in this fraction was chromatographically different from the one obtained in crystalline form, but not further purified.

Glycogen in mammalian tissues is metabolized through either phosphorolytic or hydrolytic pathway. The hydrolytic pathway is catalyzed by α-amylase [EC 3.2.1.1, α-1,4-glucan 4-glucanohydrolase] and glucohydrolase [EC 3.2.1.3, α-1,4-glucan glucohydrolase; EC 3.2.1.20, α-D-glucoside glucohydrolase].* The existence of glucohydrolase in various mammalian tissues has been reported by various investigators (1-6). Rat liver contains at least two glucohydrolases which displayed differences in their intracellular localization as well as their catalytic properties including the optimal pH (1). Although these enzymes were called acid and neutral glucohydrolases, respectively, according to their optimal pH, the both enzymes showed no remarkable difference in their properties, and liberate glucose directly from maltose and glycogen (1,2). The neutral enzyme of human liver, however, appeared to be specific for maltose and maltotriaose having no capacity to hydrolyze glycogen (4). It was also known (7) that the tissues of patients with Type II glucogenosis lack glucohydrolase which is active at pH 4.5. For the purpose of elucidating the mechanism of the hydrolytic pathway of glycogen, we started to isolate and examine of glucohydrolase from bovine spleen. This tissue was chosen as the starting material because of the high specific activity of glucohydrolase in the crude extract (3).

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* see next page

* This enzyme has been called maltase or α-glucosidase [EC 3.2.1.20, α-D-glucoside glucohydrolase]. However, as the present report shows, this enzyme also possesses the similar enzymatic properties to glucoamylase [EC 3.2.1.3, α-1,4-glucan glucohydrolase], which would be significant in glycogen metabolism. The name of glucohydrolase is used in this paper.

In the previous paper (6), we reported purification and some properties of acid glucohydrolase from bovine spleen. The enzyme was purified through differential precipitations and chromatographies on Phospho-cellulose and DEAE- Sephadex, and isolated in crystalline state. The crystalline enzyme possessed the specific activity 2,500 times larger than that of the crude extract. However, this preparation still contained more than two glucohydrolases, as judged from the sensitivities against heat inactivation and from the behavior upon the inhibitor. In fact, two chromatographically and enzymatically distinct forms were separated from this crystalline preparation by the Sephadex G-200 gel filtration.**

The present paper describes the results of further experiments on glucohydrolases of bovine spleen. This includes the isolation of crystalline acid glucohydrolase which showed apparent homogeneity upon ultracentrifugation and polyacrylamide electrophoresis. Some properties of other glucohydrolases separated from the same tissue are also described.

MATERIALS AND METHODS

Chemicals and Enzymes

Sephadex G-200 was obtained from Pharmacia Fine Chemicals. Maltose and glycogen were purchased from Wako Pure Chemical Industries. Glucose oxidase and peroxidase were purchased from Boehringer and Soehne Company. Turanose and β-amylase were the products of Sigma Chemical Company. Isomaltose was supplied by Hayashibara Company.

** K. Fujimori, unpublished experiments.

Preparation of Partially Purified Enzyme

Bovine spleen obtained at a slaughter house was cut into pieces, and kept in a deep freezer until used. Unless otherwise stated, following operations were all carried out at 2-5°C, and centrifugation was at 15,000 x g for 10 min. One kg of frozen spleen was minced by a knife, and suspended in 4 liters of 0.05 M acetate buffer (pH 4.8). After homogenizing in an electric homogenizer for two min, the slurry was centrifuged. The resulting supernatant fluid was used as crude extract. Solid ammonium sulfate was added slowly to the crude extract until 33 % saturation was reached. After removing the precipitate by centrifugation, the solution was brought to 55 % saturation by the addition of solid ammonium sulfate. After 30 min, the precipitate was collected by centrifugation, and dissolved in 400 ml of 0.05 M acetate buffer (pH 4.8). To this solution was added isopropanol to 33 % (v/v). After removing the precipitate by centrifugation, isopropanol concentration was brought up to 60 % (v/v). The precipitate formed was collected by centrifugation, and dissolved in 80 ml of 0.05 M acetate buffer (pH 4.8). This was used as partially purified enzyme. Specific activity of this fraction was 18.45 units/mg protein which was 45 times higher than that of the crude extract, and the recovery of total activity was 53.6 % of the crude extract (6).

Assay of Glucohydrolase Activity

Glucohydrolase activity was measured by the amount of glucose liberated from maltose. The reaction mixture contained 0.05 ml of 0.25 % maltose in 0.05 M acetate buffer (pH4.8) or 0.05 M phosphate-citrate buffer (pH 6.5) and 0.05 ml of the enzyme solution in a total volume of 0.1 ml. After 30 min incubation at 37°C was stopped the reaction by heating in boiling water bath for one min or by adding each 0.2 ml of $Ba(OH)_2$ and $ZnSO_4$ (8). After removing the precipitate formed by centrifugation, amount of glucose in the clear supernate was determined by the glucose oxidase method (9) with some minor modifications. Units of the enzyme activity are expressed as micromoles of maltose hydrolyzed for 30 min under the above conditions. Protein was measured by the method of LOWRY et al. (10) using bovine serum albumin as standard. Specific activity is expressed as units of enzyme activity per mg protein. The reactions at the various pH values were followed by using acetate buffer in the range from pH 3.2 to pH 6.2, and phosphate-citrate buffer in the range from pH 6.4 to 8.0. For determining substrate specificity, maltose was substituted by various other carbohydrates.

Heat Treatment

Heat treatment of the enzyme fraction was carried out in a test tubes by keeping at 45°C in a water bath for 90 min. At the end of this period, the tubes were rapidly cooled in an ice-water bath. After removing the precipitate formed by

centrifugation, a portion of the supernatant fluid was taken for assaying the activity. Acetate and phosphate-citrate buffers were used at pH 4.0 and pH 7.4, respectively.

Hydrolysis of Glycogen

The enzyme solution (0.5 ml) containing either 350 units of the acid glucohydrolase or 27.5 units of the neutral enzyme was incubated with 0.5 ml of 2.5 % glycogen in 0.05 M acetate buffer, pH 4.8 or phosphate-citrate buffer, pH 6.5 at 37°C. At various time intervals, aliquots of the reaction mixtures were taken, and the amount of glucose liberated was determined as described above.

Ultracentrifugal Analysis

Ultracentrifugal studies were carried out with a Hitachi model UCA-1 analytical ultracentrifuge.

Analytical Polyacrylamide Gel Electrophoresis

Analytical polyacrylamide gel electrophoresis was carried out by the procedure described by DAVIS (11).

Preparative Polyacrylamide Gel Electrophoresis

Preparative electrophoresis on polyacrylamide gel was carried out by the method of ISHIBASHI and KAWAI (12), in a glass cylinder (2.4 x 25 cm) under the following condition; 7.5 % gel, 25 mA, 280 V, 2-5°C for 36 hr. After the electrophoresis, the gel was cut lengthwise into four thin slices by a gel cutter, a frame with taut stainless steel wires. Two slices were each stained in bromophenol blue and toluidine

blue in order to locate proteins. The remainders were used for extraction of enzyme proteins. The unstained gel slices were cut into nine blocks corresponding to each protein band. The proteins were eluted separately from each block with 5 ml of 0.05 M acetate buffer, pH 4.8 in a Potter-type glass homogenizer equipped with a teflon pestle. The homogenate was centrifuged to remove the gel at 10,000 x g for 10 min. After dialysis against 0.05 M acetate buffer (pH 4.8), the supernatant fluid was examined for glucohydrolase activity.

RESULTS

Separation on Sephadex Column

Fifteen ml of the partially purified enzyme of bovine spleen containing 263 mg protein were charged on the Sephadex G-200 column (2.6 x 90 cm) which had been equilibrated with 0.05 M acetate buffer, pH 4.8. The protein was eluted with the same buffer at a flow rate of 30 ml per hr. Fractions of 10 ml were collected at 2-5°C.

Fig. 1

Fig. 1 (a) shows the elution patterns of protein and of glucohydrolase activities determined at two different pH values. Glucohydrolase activities were appeared in two separate peaks. Although the first peak contained both neutral

and acid enzyme activities, only acid enzyme activity was detected in the second peak which was eluted after the volume of eluate exceeded a total volume of the column. Fractions 35-45 and fractions 63-75 were separatly pooled, and concentrated in collodion bags. These two fractions were called Fractions A and B, respectively. Upon rechromatographing Fractions A and B separately on the same column, they were eluted in the corresponding original positions, as shown in Fig. 1 (b,c).

Fig. 2

As a comparison, the crude extract prepared in 0.1 M sodium bicarbonate (pH 8.3) in place of 0.05 M acetate buffer (pH 4.8) was partially purified, and chromatographed in the same manner. The elution patterns are given in Fig. 2. Glucohydrolase activities were separated into two peaks. However, the first peak contained less acidic enzyme activity as compared to the coresponding value of the enzyme prepared at pH 4.8. The neutral enzyme activity in the first peak and the acidic enzyme activity in the second peak were similar to those of the enzyme extracted at the acidic pH value.

Effect of pH

The pH dependence of the hydrolysis of maltose by Fractions A and B is shown by broken lines in Fig. 3. The highest acti-

vities were attained around pH 4.8 for both enzyme fractions. However, the activities of Fraction B declined at higher pH values more sharply than those of Fraction A.

Fig. 3

Heat Treatment

Upon heat treatment at the different pH values, the pH-activity profiles showed some characteristic changes, as shown by the continuous lines in Fig. 3. Although treating Fraction B at pH 4.0 and 45°C for 90 min caused no loss of the activity in the whole pH range, some losses of the activity of Fractions A were observed after the same treatment only when assayed at higher pH values. Treating at the neutral pH, pH 7.4, resulted in the marked declines of the activities of Fraction A assayed at lower pH values, but only slight change assayed at higher pH values. Thus, the treatment resulted in shifting the optimal pH of Fraction A from the original pH 4.8 to pH 6.5. Fraction B, upon the same treatment, lost most of the activities in the whole pH range. These results might suggest the presence of the both acid and neutral glucohydrolases in Fraction A, and of only the acid glucohydrolase in Fraction B.

Inhibition by Turanose

Turanose was known to be a specific inhibitor for the

lysosomal acid maltase of rat liver (1).

Fig. 4

The continous lines in Fig. 4 shows the pH-activity profile of Fractions A and B when assayed in the presence of 30 mM turanose. The activities of Fraction A at lower pH values were more inhibited than at higher pH values. Remarkable inhibitions were observed on the activities of Fraction B in the whole pH range. These results were compatible with the assumption that Fraction A contained both the acid and neutral glucohydrolases, and Fraction B only the acid glucohydrolase, and further only the acid enzyme was sensitive to turanose inhibition.

Crystallization and Properties of Fraction B

Since the above results showed that Fraction B contained the acid glucohydrolase of high purity, this fraction was used for crystallization of the acid glucohydrolase of bovine spleen. Fraction B (120 ml) containing 4250 units of maltose-hydrolyzing activity and 36 mg protein was concentrated by a collodion bag to about 10 ml. The fraction precipitated between 0.33 and 0.55 saturation of ammonium sulfate was obtained from this solution. After centrifugation, the residues were dissolved in small volume of 0.05 M acetate buffer, pH 4.8. The insoluble materials were removed by centrifugation after standing for

about three hr with occasional stirring at 2-5°C. To the clear supernatant fluid was added acetone at -20°C dropwise with stirring. The addition of acetone proceeded gradually until the solution became slightly turbid. If no crystals appeared in 30 min, more acetone can be added to the solution. Thin plate, fragile crystals appeared while cooling in ice-salt mixture.

Fig. 5

Fig. 6

Fig. 7

The microscopic appearance of the crystalline materials is shown in Fig. 5, which is very similar to the crystalline acid glucohydrolase we have previously obtained from the same material by the different method (6). The electrophoretic pattern on polyacrylamide gel is shown in Fig. 6. The crystalline protein migrated as a single component. Upon analytical ultracentrifugation, it exhibited a single boundary (Fig. 7). The sedimentation coefficient ($s20,_w$) was calculated as 3.85 S. The molecular weight determined by the method of ARCHIBALD (13) was 43,000.

Electrophoresis of Fraction A

As described above, Fraction A might contain both the acid and neutral enzymes, which were not separated on Sephadex G-200 gel filtration. Preparative polyacrylamide gel electrophoresis was applied to this fraction. The result is schematically shown in Fig. 8. Unstained slices of the gel were cut into several blocks corresponding to the protein bands, and the proteins were extracted separately out of each block. Glucohydrolase activities were examined in these extracts. Of nine protein bands, only four slow moving ones contained glucohydrolase activities. Total and specific activities of each fractions were shown in Table I.

Fig. 8

TABLE I

TABLE II

Total activities recovered in these four fractions were much lower than the value originally applied to the gel. This could be partly understandable from the results, as shown in Table II, that all of these four fractions contained only the neutral glucohydrolases. The acid glucohydrolase would be inactivated during the electrophoresis in alkaline pH.

As already shown, the acid enzyme in Fraction A was labile on heating at higher pH values. Attempts to separate the components in Fraction A in acidic pH were all unsuccessful. No pronounced difference was found in other properties of these four glucohydrolases, including the ratio of the activity for maltose to that for glycogen. Frigidity of all the enzymes to turanose inhibition agreed with the supposition that these were all the neutral glucohydrolases.

Substrate Specificity

Various carbohydrates were tested for the substrate of the acid and neutral glucohydrolases of bovine spleen. The crystalline enzyme isolated from Fraction B was used for the acid enzyme, and Fraction A treated at pH 7.4 and 45°C for 90 min for the neutral enzyme. Relative activities were calculated by taking those for maltose as 100, and shown in Table III.

TABLE III

Sucrose was not detectably hydrolyzed by the both enzymes. Isomaltose and methy α-D-glucoside were poor substrates but still hydrolyzable by the both enzymes. Although glycogen was almost as good substrate as maltose for the acid enzyme, it is a poor substrate for the neutral enzyme. This was one of the most characteristic differences between the acid and neutral enzymes of bovine spleen.

Hydrolysis of Glycogen

Extent of the hydrolysis of glycogen was examined for the both acid and neutral glucohydrolases. The enzymes used were the same as in the experiments on substrate specificity.

Fig. 9

The both acid and neutral glucohydrolases of bovine spleen could hydrolyze glycogen almost completely to glucose.

D I S C U S S I O N

The experimental results described above showed that glucohydrolases of bovine spleen might exist in several different species; at least two acid and four neutral enzymes which exhibited the highest activities at acid and neutral pH, respectively.

An acid glucohydrolase was separated from other glucohydrolases upon gel filtration by Sephadex G-200. This enzyme protein, Fraction B, was eluted after the volume of eluate exceeded a total volume of the column. Retardation of this fraction on the gel filration might be caused by the interaction between the enzyme protein and dextran gel. The similar phenomena have recently been reported on rat liver acid α-glucosidase (5), rabbit intestinal sucrase-isomaltase (14), and rat intestinal sucrase (15). The acid glucohydrolase of bovine spleen separated by this procedure was finally isolated in crystalline state, and the crystalline material showed apparent homogeneity upon ultracentrifugation and polyacrylamide gel electrophoresis.

AURICCHIO et al. (16) reported very recently that acid α-glucosidase has been isolated as a homogeneous protein from rat liver, but did not succeed in crystallizing the enzyme. The crystalline glucohydrolase of bovine spleen catalyzes the liberation of glucose from maltose and glycogen as well. Glycogen can be almost completely hydrolyzed to glucose by the action of this enzyme. Isomaltose is also splitted into glucose. The acid glucohydrolase of bovine spleen would

probably catalyze the stepwise degradation of glycogen from the non-reducing ends through hydrolyzing both α-1,4 and α-1,6 glucosidic bonds.

Results of the heat treatment and of the inhibition by turanose demonstrated that the first peak upon Sephadex G-200 gel filtration, Fraction A, contained acid glucohydrolase besides the neutral enzyme. Separate rechromatographies of Fractions A and B suggested that these two fractions contained the different entities. Extensive purification of acid glucohydrolase of Fraction A has not yet performed. The existence of two separable acid α-glucocidase was also suggested in rat liver (16).

The neutral glucohydrolases of bovine spleen were separated into four different bands by preparative polyacrylamide gel electrophoresis. The neutral enzymes showed the similar catalytic properties each other, and catalyzed the liberation of glucose much more rapidly from maltose than from glycogen. However, glycogen could be finally hydrolyzed almost completely to glucose.

HERS (7), showed that the tissues of the patients with Type II glucogenosis lack α-glucosidase active at acidic pH. However, the physiological role of glucohydrolase in the catabolism of glycogen should be further investigated with the consideration that this enzyme would exist in multiple forms, as presuming from the present results on bovine spleen and the results by AURICCHIO et al. (16) on rat liver.

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REFERENCES

(1) N. Lejeune, D. Thinies-Sempoux, and H. G. Hers, Biochem. J., 86, 16 (1963)

(2) H. N. Torres and J. M. Olararria, J. Biol. Chem., 239, 2427 (1964)

(3) E. L. Rosenfeld, "Ciba Foundation Symposium. Control of Glycogen Metabolism," P. 176, Jand A Churchill (1965)

(4) B. I. Brown, and D. H. Brown, Biochim. Biophys. Acta, 110, 124 (1965)

(5) F. Auricchio, and A. B. Bruni, Biochem. J. 105, 35 (1967)

(6) K. Fujimori, S. Hizukuri, and Z. Nikuni, Biochem. Biophys. Res. Comm., in press

(7) H. G. Hers, Biochem. J. 86, 11 (1963)

(8) T. E. Weichselbaum, and M. Somogyi, J. Biol. Chem., 140, 5 (1941)

(9) A. Dahlqvist, Biochem. J., 80, 547 (1961)

(10) O. H. Lowry, N. J. Rosebrough, A. L. Farr. and R. J. Randall, J. Biol. Chem., 193, 265 (1951)

(11) B. J. Davis, Ann. N. Y. Acad. Sci., 121, 404 (1964)

(12) F. Ishibashi, and K. Kawai, Memoires of Osaka Kyoiku University. Vol. 17. Natural Science and Applied Science. III. NO. I (1968)

(13) W. J. Archibald, J. Phys. and Colloid Chem., 51, 1204 (1947)

(14) C. Semenza and J. Kolnska, "Protides of the Biological Fluides," ed. by H. Peeters, Elsevier Publishing Company, Amsterdam, P. 581 (1968)

(15) T. Kashiwagi, Y. Takesue, T. Yoshida, The Journal of Japanese Biochemical Society (in Japanese), 39, 223 (1967)

(16) F. Auricchio, C. B., Bruni and V. Sica, Biochem. J., 108, 161 (1968)

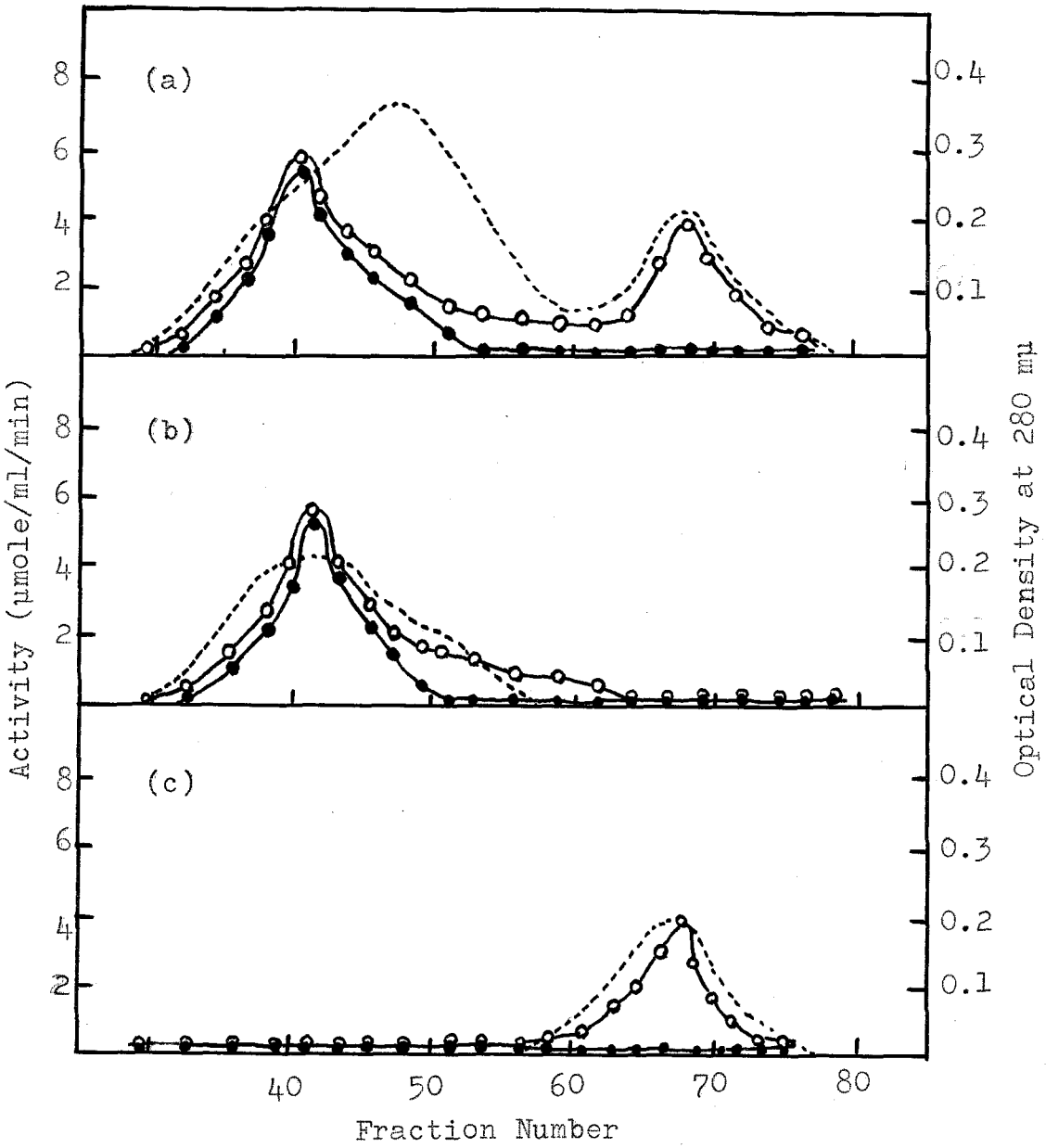

Fig. 1 (a) Gel filtration of the partially purified glucohydrolase extracted at pH 4.8 on a Sephadex G-200 column.

(b) Gel filtration of Fraction A on a Sephadex G-200 column.

(c) Gel filtration of Fraction B on a Sephadex G-200 column.

Fig. 1 (a) Gel filtration of the partially purified glucohydrolase extracted at pH 4.8 on a Sephadex G-200 column.

The enzyme was extracted with 0.05 M acetate buffer (pH 4.8), and fractionated by precipitaions with ammonium sulfate and isopropanol. The sample (15 ml) containing 17.5 mg protein/ml was applied to the column (2.6 x 90 cm). The protein was eluted with 0.05 M acetate buffer (pH 4.8) at the flow rate of 30 ml/hr. The fractions of 10 ml each were collected and analyzed for absorption at 280 mμ and for glucohydrolase activities at pH 4.8 and pH 6.5, by using maltose as substrate.

(b) Gel filtration of Fraction A on a Sephadex G-200 column.

The fractions 35-45 of Fig. 1 (a), Fraction A, were pooled, concentrated in a collodion bag, and rechromatographed under the same conditions.

(c) Gel filtration of Fraction B on a Sephadex G-200 column.

The fractions 63-75 of Fig. 1 (a), Fraction B, were pooled, concentrated in a collodion bag, and rechromatographed under the same conditions.

------- : Optical density at 280 mμ

—o—o— : Glucohydrolase activity at pH 4.8

—●—● : Glucohydrolase activity at pH 6.5

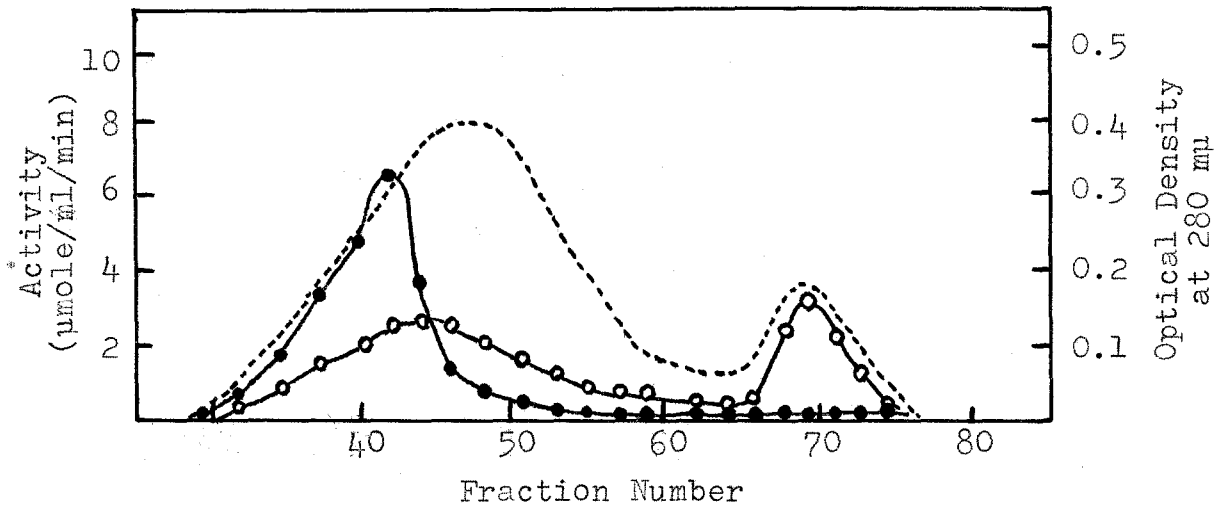

Fig.2 Gel filtration of the partially purified glucohydrolase extracted at pH 8.3 on a Sephadex G-200 column.

Fig. 2 Gel filtration of the partially purified glucohydrolase extracted at pH 8.3 on a Sephadex G-200 column.

The enzyme was extracted with 0.1 M sodium bicarbonate (pH 8.3), fractionated, and chromatographed in the similar way to the experiment shown in Fig.1 (a).

------- : Optical density at 280 mμ

—o—o— : Glucohydrolase activity at pH 4.8

—●—●— : Glucohydrolase activity at pH 6.5

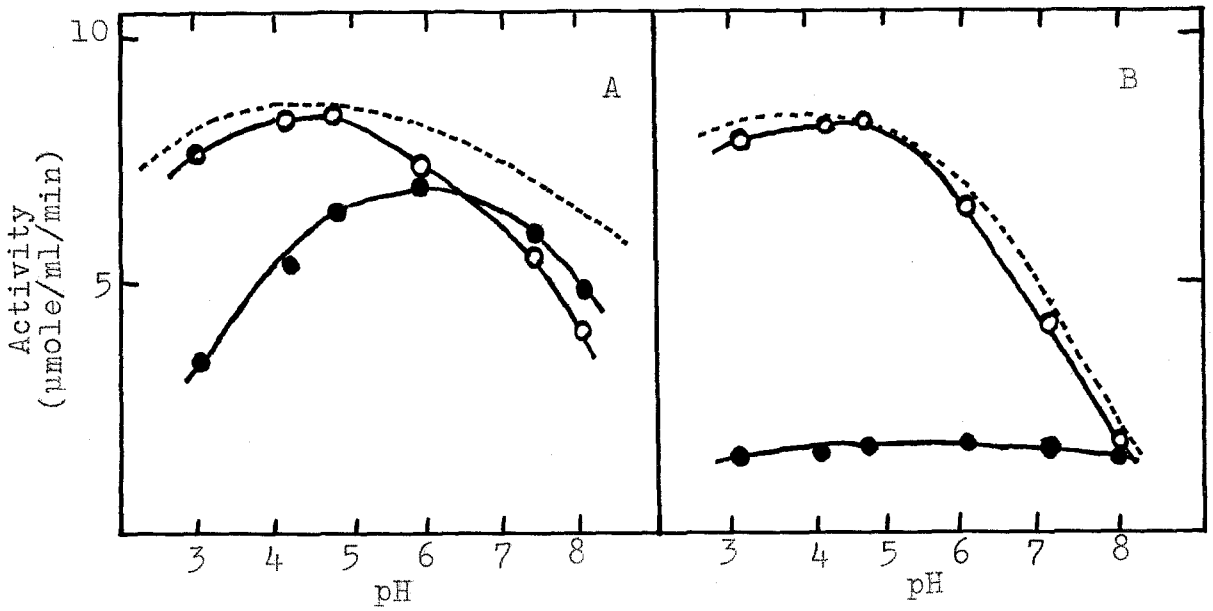

Fig. 3 The pH-activity profiles of Fractions A and B before and after heat treatment.

Fig. 3 The pH-activity profiles of Fractions A and B before and after heat treatment.

Fractions A and B were obtained by gel filtration on Sephadex G-200 of partially purified enzyme of bovine spleen. Activity was measured by using maltose as substrate in 0.05 M acetate buffer (pH 3.2-6.2) and phosphate-citrate buffer (pH 6.4-8.0). The heat treatment was carried out by keeping the enzyme solution at 45°C for 90 min at two different pH values ; pH 4.0 in acetate buffer and pH 7.4 in phosphate-citrate buffer.

-------- : no treatment

—o—o— : treated at pH 4.0

—●—● : treated at pH 7.4

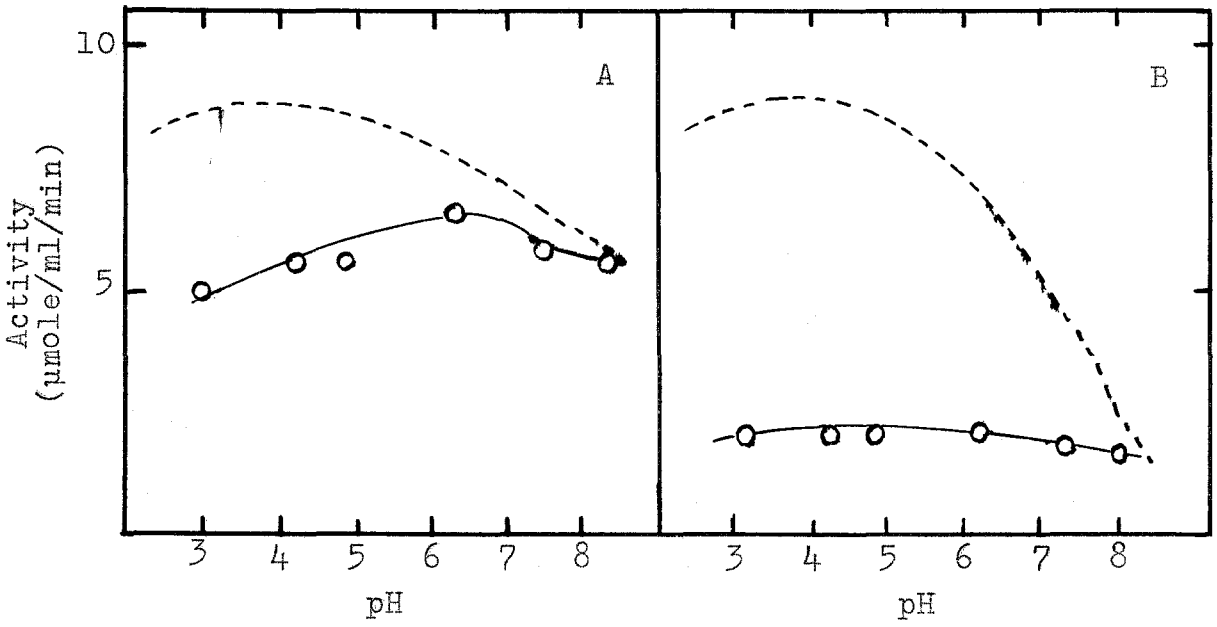

Fig. 4 The pH-activity profiles of Fractions A and B in the presence of turanose.

Fig. 4 The pH-activity profiles of Fractions A and B in the presence of turanose.

The enzyme solutions were the same as in Fig 3. The activity was measured in the presence of 30 mM turanose, by using maltose as substrate.

------ : without turanose

—o—o— : with turanose

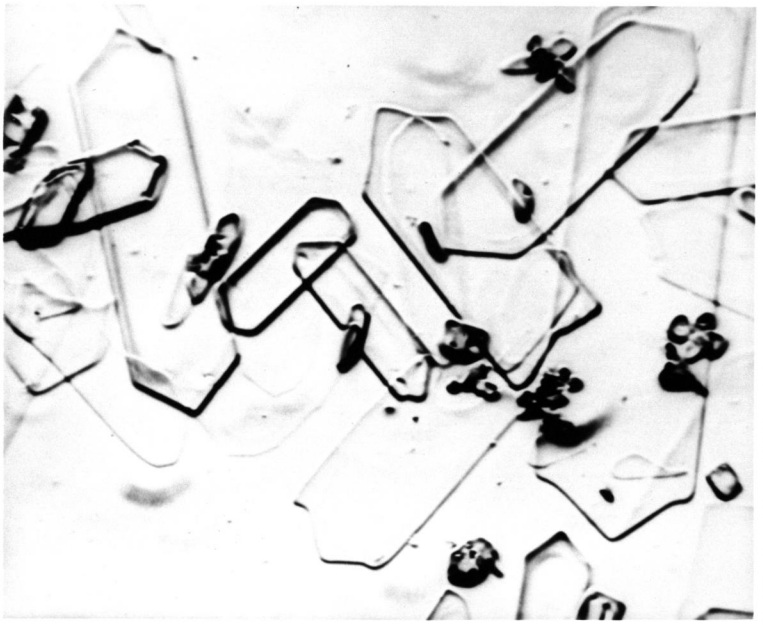

Fig. 5 Crystalline acid glucohydrolase obtained from Fraction B of bovine spleen.
X 150

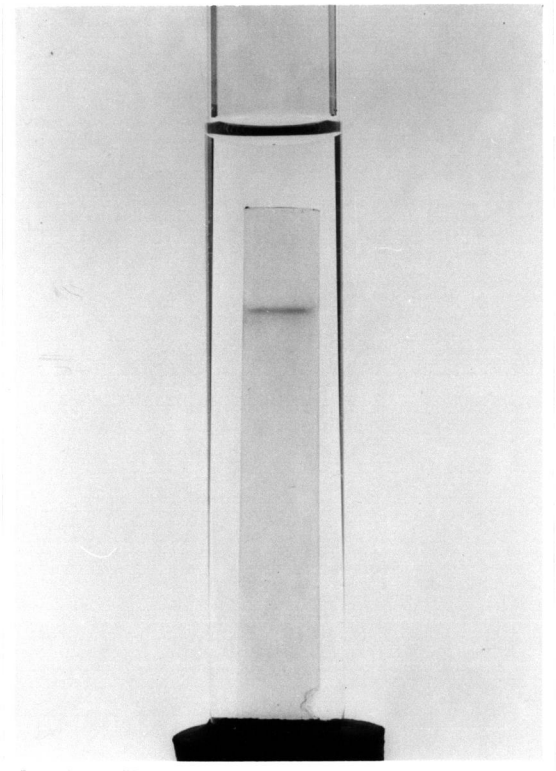

Fig. 6 Polyacrylamide gel electrophoretic pattern of crystalline acid glucohydrolase obtained from Fraction B of bovine spleen.

Fig. 6 Polyacrylamide gel electrophoretic pattern of crystalline acid glucohydrolase obtained from Fraction B of bovine spleen.

The sample (0.5 ml) contained 0.12 mg protein/ml was applied to the gel (8.2 %). Electrophoresis was carried out at pH 8.9, 180 V, 5 mA for 2 hr. The gel was stained with amide black.

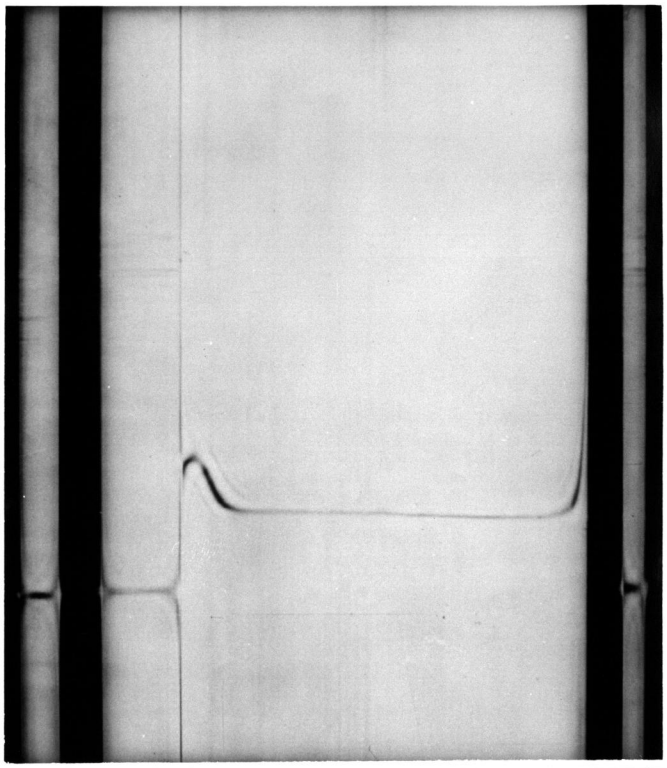

Fig. 7 Sedimentation pattern of crystalline and glucohydrolase obtained from Fraction B of bovine spleen.

Fig. 7 Sedimentation pattern on crystalline acid glucohydrolase obtained from Fraction B of bovine spleen.

Crystalline enzyme (0.3 %) in 0.05 M acetate buffer (pH 4.8) containing 0.1 M NaCl. The photograph was taken 16 min after the rotor reached 54,100 rpm at 24.7°C.

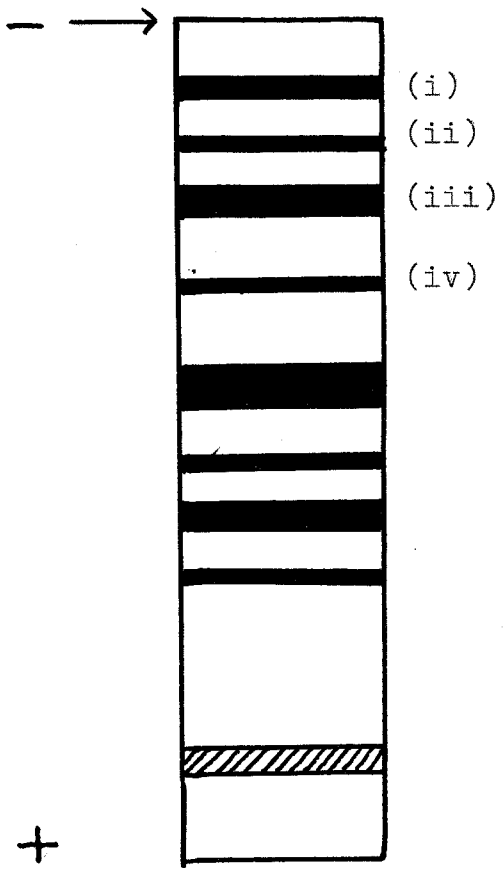

Fig. 8 Schematic representation of polyacrylamide gel electrophoresis of Fraction A.

Fig. 8 Schematic representation of polyacrylamide gel electrophoresis of Fraction A.

Fraction A was obtained by gel filtration on Sephadex G-200 of the partially purified enzyme of bovine spleen. The sample (5 ml) containing 90 mg protein/ml was applied to the gel (7.5 %) in a glass cylinder (2.4 x 25 cm). The electrophoresis was carried out at 280 V, 25 mA for 36 hr. The gel was then cut lengthwise into four thin slices, two of which were stained each in bromophenol and toluidine blue. An arrow in the figure shows the starting point. Closed areas indicate the bands stained in bromophenol blue and hatched area in toluidine blue.

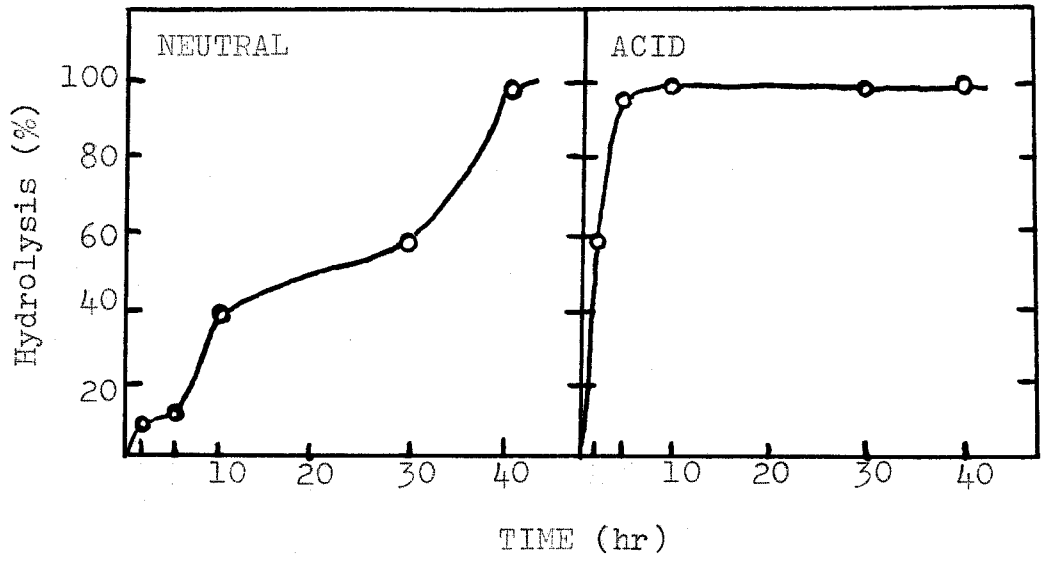

Fig. 9 Hydrolysis of glycogen by the acid and neutral glucohydrolases of bovine spleen.

Fig. 9 Hydrolysis of glycogen by the acid and neutral glucohydrolases of bovine spleen.

The enzymes used were the same as in Table III. The enzyme solution (0.5 ml) containing either 350 units of the acid enzyme or 27.5 units of the neutral enzyme was incubated with 0.5 ml of 2.5 % glycogen, in 0.05 M acetate buffer, pH 4.8 or phosphate-citrate buffer, pH 6.5 at 37°C. At the time intervals shown, aliquots of the reaction mixtures were taken, and the amount of glucose liberated was determined by the glucose oxidase method.

TABLE I. Glucohydrolase activities of the fractions obtained through polyacrylamide gel electrophoresis of Fraction A.

Unstained slices prepared in the experiment of Fig.8 were cut into nine blocks corresponding to each protein band. The proteins were extracted with 0.05M acetate buffer, pH 4.8, dialyzed against the same buffer and assayed for glucohydrolase activity at pH 6.5, and for amount of protein. Only the results of four active fractions, (i)—(iv), were shown in this table. The fraction numbers are corresponded to the ones indicated in Fig. 8, which is ordered from cathode to anode.

Fraction	Total Activity (unit)	Protein (mg)	Specific Activity (unit/mg protein)
(i)	57.8	2.1	27.5
(ii)	62.4	3.2	19.5
(iii)	226	7.2	31.5
(iv)	26.7	1.2	22.3

TABLE II. Enzymatic properties of the fractions obtained through polyacrylamide gel electrophoresis of Fraction A.

The fractions used were the same as in Table I. All the activities were assayed at pH 6.5 in phosphate-citrate buffer. The inhibition by turanose was measured in the assay system in the presence of 30 mM turanose.

Fraction	Optimal pH	$\frac{\text{Activity for maltose}}{\text{Activity for glycogen}}$	% Inhibition by turanose
(i)	6.5	25	14
(ii)	6.7	28	12
(iii)	6.5	53	10
(iv)	6.0	26	15

TABLE III. Substrate specifcity of the acid and neutral glucohydrolases of bovine spleen.

For the acid glucohydrolase was used the crystalline acid enzyme prepared from Fraction B. Fraction A treated at pH 7.4 and 45°C for 90 min was used for the neutral enzyme. Activities were determined in the assay system in which maltose was replaced by other carbohydrates, and calculated by taking those for maltose as 100.

Substrate	Relative activity	
	acid enzyme	neutral enzyme
Sucrose	0	0
Maltose	100	100
Isomaltose	1.5	2.5
Methyl α-D-glucoside	1.2	1.2
Glycogen	85	2.0