BBA 66501

PURIFICATION AND PROPERTIES OF 4-L-ASPARTYLGLYCOSYLAMINE AMIDOHYDROLASE FROM HOG KIDNEY

MICHIAKI KOHNO AND IKUO YAMASHINA

Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto (Japan)

(Received August 30th, 1971)

SUMMARY

1. An enzyme that hydrolyzes the aspartyglycosylamine linkage of synthetic 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glycopyranosylamine (4-L-aspartylglycosylamine) or of glycopeptides was extracted from hog kidney and purified about 20 000-fold over the crude extract. The most purified preparation was homogeneous on disc and sodium dodecyl sulphate disc electrophoreses and on ultracentrifugation. The molecular weight determined by means of gel filtration and sodium dodecyl sulphate disc electrophoresis was about 70 000. The presence of two other forms of the enzyme was demonstrated.

2. The enzyme had a pH optimum at 5.5, but was considerably active at alkaline pH values. From analysis of the products of enzymatic degradation of the aspartylglycosylamine at an alkaline pH, the formation of 1-amino-*N*-acetylglucosamine was demonstrated indicating that the enzyme is an amidase.

3. Rates of enzymatic hydrolysis of the aspartylglycosylamine linkage of the glycopeptides from ovalbumin and Taka-amylase were about the same as that for synthetic aspartylglycosylamine. Michaelis constants of the enzyme for ovalbumin glycopeptide and the synthetic substrate were also about the same. The enzyme was unusual in that the Michaelis constant varied depending on the enzyme concentration.

INTRODUCTION

In many glycoproteins, carbohydrate is linked to protein through the aspartylglycosylamine linkage[1]. Enzymatic cleavage of this linkage was shown using preparations from various sources[2–5], but no experimental proof on its mode of action had been presented before work from this laboratory demonstrated a specific enzyme in serum that hydrolyzed the amide bond of the linkage[6]. This enzyme was found to be widespread in mammalian tissues[7–9], and the lysosomal localization was demonstrated[7,8]. This enzyme is believed to work in glycoprotein catabolism. In fact, it was observed that a defect in this enzyme results in disorder of glycoprotein catabolism

which leads to excretion of a large amount of aspartylglycosylamine in urine[10,11]. The enzyme has been partially purified from various sources, *i.e.* hog serum[6,12], rat liver and kidney[7,9] and hen oviduct[13]. Some of its properties have also been investigated.

We isolated this enzyme in a homogeneous state from hog kidney and studied of its chemical and physical characteristics and its kinetic properties using synthetic aspartylglycosylamine and glycopeptides isolated from glycoproteins.

The enzyme should be named "4-L-aspartylglycosylamine amidohydrolase", but in this paper it is referred to as amidase for convenience.

MATERIALS

Syntheses of 2-acetamido-1-*N*-(4-L-aspartyl)-2-deoxy-β-D-glycopyranosylamine (Asn-GlcNAc) and 1-β-amino-*N*-acetylglucosamine were performed according to methods described previously[12,14].

Ovalbumin glycopeptide was prepared by the method of YAMASHINA AND MAKINO[15], and Taka-amylase glycopeptide was generously given by Prof. Y. Matsushima of Osaka University. The molar ratios of the constituents of these glycopeptides determined by amino acid analyzer after hydrolysis in 6 M HCl at 100° for 16 h were as follows:

Ovalbumin glycopeptide: aspartic acid, 1.00; glucosamine, 2.89.

Taka-amylase glycopeptide: aspartic acid, 1.00; serine, 0.03; glucosamine, 1.87.

The average molecular weights of these glycopeptides were calculated from the following average molecular structures:

Ovalbumin glycopeptide: aspartic acid, 1.0; *N*-acetylglucosamine, 3.0; mannose, 5.0[16].

Taka-amylase glycopeptide: aspartic acid, 1.0; *N*-acetyglucosamine, 2.0; mannose, 6.0[17].

Hydroxylapatite was prepared according to the method of SIEGELMAN *et al.*[18]. DEAE-Sephadex and Sephadex were obtained from Pharmacia, Sweden, and DEAE-cellulose from Brown and Co., U.S.A.

Bovine serum albumin, trypsin inhibitor (soy bean), cytochrome *c* (equine heart) and ribonuclease A (bovine pancreas) were obtained from Sigma Chemical Co., U.S.A. Lysozyme (egg white), trypsin and carboxypeptidase A were from Worthington Biochem. Corp., U.S.A. Ovalbumin was prepared according to KECKWICK AND CANNAN[19]. Ceruloplasmin of human serum was supplied from Karolinska Institutet, Stockholm, Sweden, through the courtesy of Prof. B. Blombäck. Yeast alcohol dehydrogenase was generously given by Dr. T. Yamada of Tokyo University.

METHODS

Enzyme assay and analytical methods

Amidase was assayed in a mixture consisting of 0.05 M sodium phosphate–citrate buffer (pH 5.50), 5 mM Asn-GlcNAc and enzyme preparation, unless otherwise stated. After incubation for appropriate times at 37°, the reaction was stopped by heating at 100° for 5 min, and the *N*-acetylglucosamine released was determined by the Morgan–Elson reaction according to LEVVY AND MCALLAN[20]. When the reaction

was carried out at alkaline pH values or glycopeptides were used as substrate, the reaction was stopped by addition of an equal volume of 10% trichloroacetic acid. The mixture, after being kept for another 60 min to ensure decomposition of a reaction intermediate (1-amino-*N*-acetylglucosamine from Asn-GlcNAc or 1-amino-oligosaccharide from glycopeptides) to *N*-acetylglucosamine or oligosaccharide and ammonia, was then neutralized with 0.6 M NaOH and used for the Morgan–Elson reaction or for ammonia determination according to LUBOCHINSKY AND ZALTA[21]. To determine the effect of pH on enzyme activity, a universal buffer composed of phosphoric acid, acetic acid, boric acid and NaOH according to BRITTON AND ROBINSON[22] was used.

One unit of enzyme activity is defined as the amount which liberates 1 μmole of *N*-acetylglucosamine from Asn-GlcNAc per min at pH 5.50 and 37°.

Assays for α- and β-*N*-acetylglucosaminidases, α- and β-mannosidases, α- and β-galactosidases and α-fucosidase were performed according to CONCHIE *et al.*[23].

Protein was determined according to LOWRY *et al.*[24] with ovalbumin as the standard.

To identify a reaction intermediate (1-amino-*N*-acetylglucosamine), paper chromatography using ethylacetate–1-propanol–water–aq. ammonia (s.g. = 0.90) (1:7:2:0.1, by vol.) and paper electrophoresis in 0.05 M barbital buffer (pH 9.0) were carried out. For the latter, a voltage of 50 V/cm was applied for a period of 60 min with cooling in *n*-hexane. Reducing sugars were detected on paper using an alkaline $AgNO_3$ reagent[25].

Amino acid analysis of the purified enzyme preparation was performed using a Hitachi KLA-3B amino acid analyzer after hydrolysis of the sample in 6 M HCl at 105° for 24 h in an evacuated tube. Sialic acid determination was carried out according to the method of WARREN[26] after hydrolysis of the sample in 2.5% trichloroacetic acid at 80° for 60 min.

Polyacrylamide gel electrophoresis

The purified enzyme preparation was subjected to polyacrylamide gel electrophoresis (disc electrophoresis) in the system described by DAVIS[27] using 7.5% gel of pH 9.4 prepared in Davis's Tris–glycinate buffer. A sample of about 50 μg protein was applied to one gel, and electrophoresis was carried out at 4° for 60 min at 5 mA per gel tube. Gels were stained with Amido black 10B. Amidase activity on the gel was localized according to the method of GABRIEL AND WANG[28] after gels had been incubated with 2 mM Asn-GlcNAc in 0.1 M sodium phosphate–citrate buffer (pH 5.50) for 30 min at 37°.

Electrophoresis in the presence of sodium dodecyl sulphate and mercaptoethanol (sodium dodecyl sulphate disc electrophoresis) was performed according to the directions of WEBER AND OSBORN[29] using 10% gel with the normal amount of cross-linker. A sample of about 30 μg protein was applied to one gel, and mobility was expressed as that relative to trypsin. By comparing the mobility of amidase to the mobilities of proteins of known molecular weight, molecular weight of amidase was estimated.

Molecular weight determination using gel filtration on Sephadex G-150

Proteins of known molecular weight were used to calibrate a column of Sepha-

dex G-150 (2 cm × 100 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl. The purified amidase preparation, about 30 μg protein dissolved in 1.0 ml of the buffer, was placed on the column and eluted at a flow rate of 7–8 ml/h at 4°. Fractions of 2.5 ml were collected and aliquots were used for enzyme assays. Molecular weight of the amidase was calculated according to the directions of ANDREWS[30].

To check if molecular weight of the amidase varied depending on pH, gel filtration at pH 5.50 was also carried out. For this, the gel column was equilibrated with 0.05 M sodium phosphate–citrate buffer (pH 5.50); the buffer that is used for amidase assay. Flow rate of elution was doubled, *i.e.* 15 ml/h, to avoid inactivation of the enzyme during the gel filtration.

Ultracentrifugal analysis

A sedimentation velocity experiment was performed on a 0.3% solution of the purified amidase preparation in phosphate buffer, pH 7.0 and $I = 0.1$, using a Hitachi model UCA-I analytical ultracentrifuge. Rotor speed was 50 696 rev./min at 20°, and the sedimentation coefficient was calculated by the method of SCHACHMAN[31].

Determination of isoelectric points

Isoelectric points of the amidase preparations were determined by isoelectric focusing using a carrier ampholyte giving a pH gradient between pH 4 and 6 (LKB-Produkter AB, Sweden) at a mean concentration of 0.5% according to the directions of VESTERBERG AND SVENSSON[32]. Focusing was carried out at 4° in a special vertical electrolysis column (1.8 cm × 32 cm). Stabilization against convection was achieved using a density gradient prepared from 50% sucrose and water. After focusing the column was drained and 1.5-ml fractions were collected, aliquots of which were used for enzyme assays. Measurement of pH was made at 20°.

TABLE I

AMIDASE ACTIVITIES EXTRACTED FROM DIFFERENT HOG TISSUES WITH VARIOUS SOLVENTS

Each tissue was homogenized with 5 vol. of each solvent for 10 min in a Waring Blendor. The homogenate was centrifuged at 10 000 × *g* for 20 min, and the supernatant was assayed for amidase activity.

Solvent	*Kidney*		*Spleen*		*Liver*		*Pancreas*	
	Specific activity (units/mg protein × 10⁵)	*Total activity (units/g tissue × 10³)*	*Specific activity (units/mg protein × 10⁵)*	*Total activity (units/g tissue × 10³)*	*Specific activity (units/mg protein × 10⁵)*	*Total activity (units/g tissue × 10³)*	*Specific activity (units/mg protein × 10⁵)*	*Total activity (units/g tissue × 10³)*
Water	3.25	2.73	2.98	1.75	1.23	1.19	0.80	0.89
0.9% NaCl	3.50	2.71	2.56	1.71	1.30	1.66	0.76	0.91
0.05 M Citrate buffer, pH 5.0	4.74	2.61	3.82	1.85	1.70	1.10	0.78	1.03
0.05 M Phosphate buffer, pH 7.5	3.28	3.20	2.85	2.20	1.22	1.38	1.08	1.15

RESULTS

Purification of amidase

Amidase was assayed in hog tissues, *i.e.* kidney, spleen, liver and pancreas with the results shown in Table I. Since kidney contained the largest amount of amidase, consistent with the results of OHGUSHI AND YAMASHINA[8] for rat tissues, and since amidase could be effectively extracted with 0.05 M citrate buffer (pH 5.0), the extract was used to isolate the enzyme. Subsequent operations were carried out at 2–4° unless otherwise stated.

Step 1. Extraction and $(NH_4)_2SO_4$ fractionation. A 1 kg portion of minced kidney was homogenized with 2 l of 0.05 M citrate buffer (pH 5.0) in a Waring Blendor for 20 min, and the homogenate was centrifuged at 8000 × *g* for 20 min. The residue was reextracted with 1 l of the buffer. $(NH_4)_2SO_4$ was added to the combined supernatant to 35% saturation, and the mixture was centrifugated at 10 000 × *g* for 20 min. The supernatant was then brought to 60% $(NH_4)_2SO_4$ saturation, and the resulting precipitate was collected by centrifugation. The precipitate was dissolved in water and dialyzed. An inactive deposit formed during dialysis was removed by centrifugation.

Step 2. Heat treatment. The supernatant from the previous step was diluted with water to give a protein concentration of about 1%, then it was heated at 60° for 15 min. After centrifugation to remove insoluble materials, the supernatant was brought to 80% $(NH_4)_2SO_4$ saturation, and the resulting precipitate was collected by centrifugation. The pellet was dissolved in water, dialyzed and lyophilized.

Step 3. Acetone fractionation. Material from the previous step was dissolved in a solution containing 0.04 M $(NH_4)_2SO_4$ and 0.01 M zinc acetate to give about 1% protein concentration, the pH being adjusted to 6.1 with acetic acid. Cold 50% acetone made up of an equal volume of acetone and 0.01 M zinc acetate buffer (pH 5.9) was added with vigorous stirring to give 30% acetone concentration. The mixture was kept at −5 to −10° for 20 min, then centrifuged at −10°. The supernatant was dialyzed and concentrated to about one-thirtieth its original volume by ultrafiltration using a membrane filter (Diaflo Ultrafiltration, Model 200, with membrane PM-10, Amicon Corp., U.S.A.). The concentrate was then dialyzed to equilibrium against 0.02 M potassium phosphate buffer (pH 7.0).

Step 4. DEAE-cellulose chromatography. The dialysate from the previous step, corresponding to about 1.7 g protein, was applied to a column of DEAE-cellulose (4 cm × 50 cm) equilibrated with 0.02 M potassium phosphate buffer (pH 7.0). After eluting inactive proteins with the same buffer, amidase was eluted with 0.02 M buffer containing 0.1 M NaCl. Fractions containing the peak of amidase activity were pooled and concentrated to about 50 ml by ultrafiltration, then dialyzed to equilibrium against 0.01 M potassium phosphate buffer (pH 7.0).

Step 5. Hydroxylapatite chromatography. The dialysate from the previous step was applied to a column of hydroxylapatite (4.5 cm × 16 cm) equilibrated with 0.01 M potassium phosphate buffer (pH 7.0). Elution was carried out with potassium phosphate buffer (pH 7.0) in stepwise increasing concentrations, *i.e.* 0.03 M (640 ml), 0.06 M (1360 ml) and 0.15 M (1170 ml). The flow rate was 35 ml/h, and 16-ml fractions were collected. Two peaks of amidase activity were obtained on elution with 0.06 and 0.15 M buffer, which were designated as Fraction H-1 and Fraction H-2, respec-

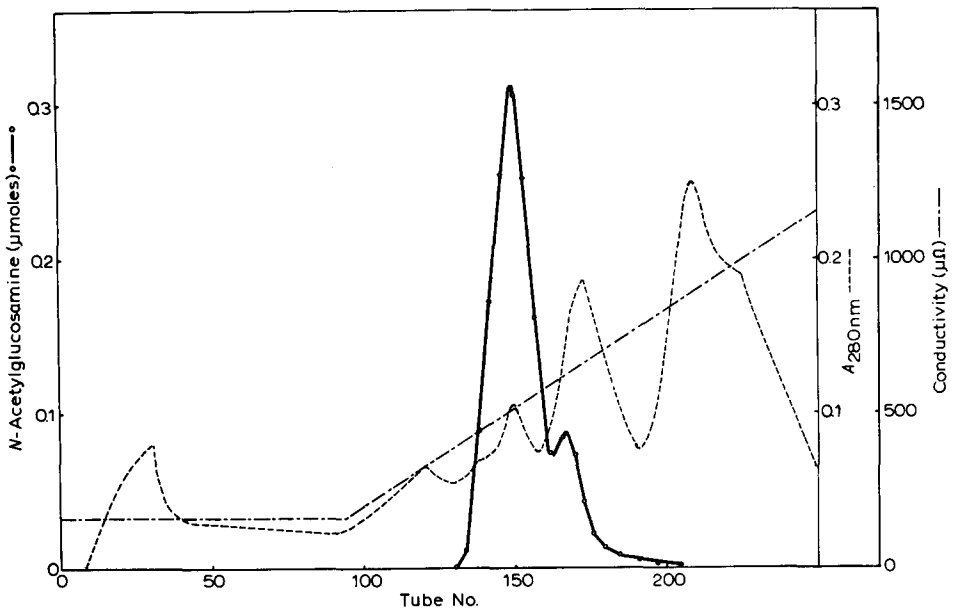

Fig. 1. Chromatography of amidase (Fraction H-1) on DEAE-Sephadex A-50. A 50-ml solution of Fraction H-1 corresponding to 190 mg protein in 0.02 M potassium phosphate buffer (pH 7.0) was applied to a column of DEAE-Sephadex A-50 (2.4 cm × 28 cm) equilibrated with the same buffer. Elution was carried out with a linear gradient from zero to 0.3 M NaCl in the buffer at a flow rate of 27 ml/h. Fractions of 9 ml were collected, of which 0.15 ml was used for enzyme assay in a reaction mixture of 0.3 ml. The enzyme activity was expressed as the number of μmoles of the released *N*-acetylglucosamine per 0.3 ml of the reaction mixture after incubation for 30 min. Fractions in Tubes 133–160 and 162–176 were pooled and designated as Fraction H-1-A-1 and Fraction H-1-A-2, respectively.

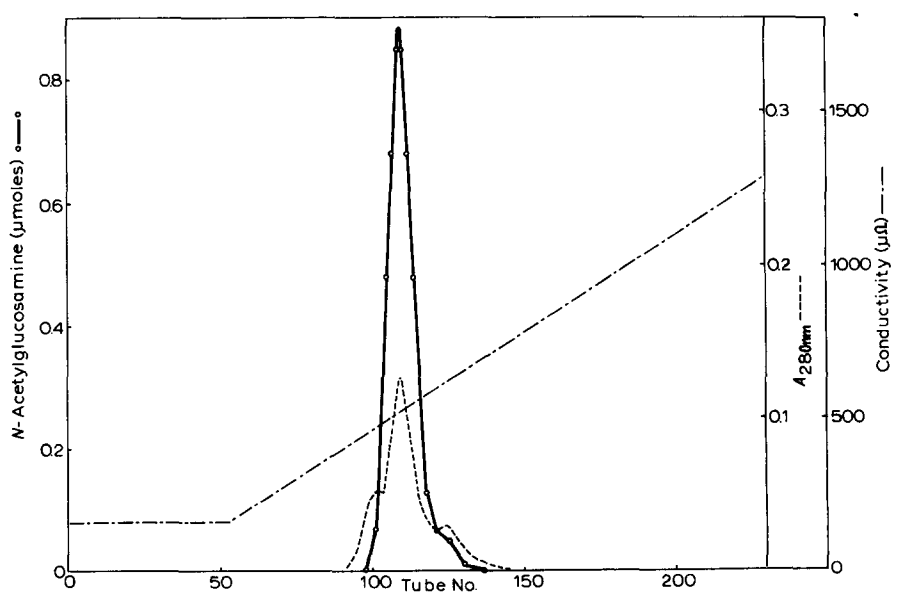

Fig. 2. Rechromatography of Fraction H-1-A-1 on DEAE-Sephadex A-50. Fraction H-1-A-1 from the previous step (Fig. 1) was concentrated to about 50 ml, then dialyzed against 0.02 M potassium phosphate buffer (pH 7.0). The dialysate was chromatographed on a DEAE-Sephadex A-50 column (1.5 cm × 56 cm) under conditions similar to those for Fig. 1 at a flow rate of 18 ml/h. Fractions of 5.6 ml were collected, of which 0.15 ml was used for enzyme assay. The enzyme activity was expressed as in Fig. 1. Fractions in Tubes 107–113 were pooled and designated as amidase-1.

tively. Total recovery of activity was about 70%, of which two thirds were accounted for by Fraction H-1. Each fraction was further purified.

Step 6. DEAE-Sephadex A-50 chromatography. Fraction H-1 was concentrated to about 50 ml by ultrafiltration, then dialyzed against 0.02 M potassium phosphate buffer (pH 7.0). The dialysate was applied to a column of DEAE-Sephadex A-50. Amidase activity was eluted in two fractions (Fractions H-1–A-1 and H-1–A-2), as shown in Fig. 1. Fractions in Tubes 133–160 were pooled, concentrated and dialyzed against 0.02 M buffer. The dialysate was rechromatographed on a column of DEAE-Sephadex A-50 under conditions similar to those of the initial chromatography with the results shown in Fig. 2. Fractions with constant specific activity (Tubes 107–113) were pooled. This pooled fraction, designated as amidase-1, had a specific activity of 1.55 units/mg protein; 20 000-fold purification over the original extract, and appeared homogeneous as is described later.

Upon chromatography of Fraction H-2 on a column of DEAE-Sephadex A-50

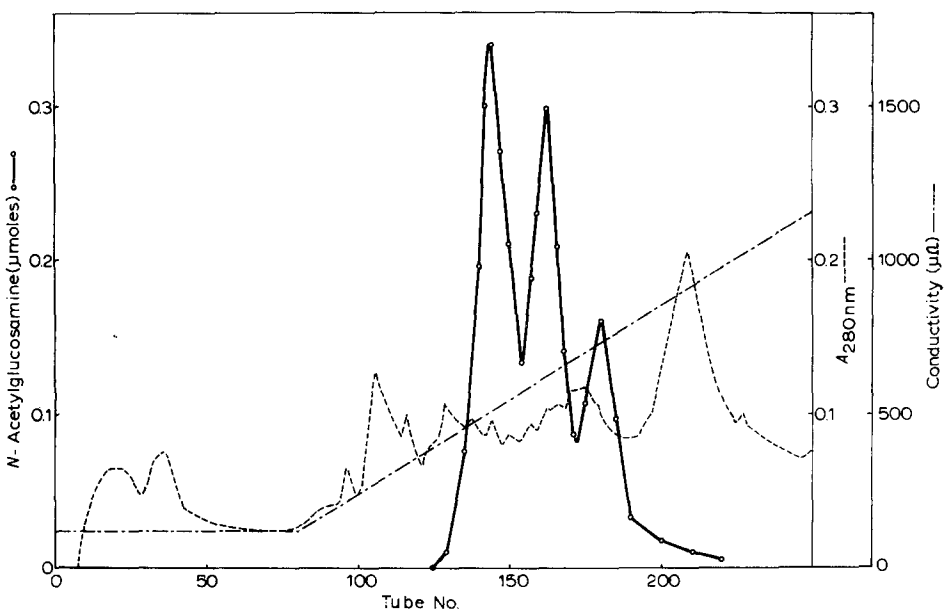

Fig. 3. Chromatography of Fraction H-2 on DEAE-Sephadex A-50. A 30-ml solution of Fraction H-2 corresponding to 90 mg protein in 0.02 M potassium phosphate buffer (pH 7.0) was chromatographed on a DEAE-Sephadex A-50 column (1.6 cm × 49 cm) under conditions similar to those for Fig. 1 at a flow rate of 20 ml/h. Fractions of 6.9 ml were collected, of which 0.15 ml was used for enzyme assay. The enzyme activity was expressed as in Fig. 1. Fractions in Tubes 140–149, 156–167 and 175–185 were pooled and desginated as Fraction H-2-A-1, Fraction H-2-A-2 and Fraction H-2-A-3, respectively.

under conditions similar to those used for Fraction H-1, three peaks of amidase activity (Fractions H-2-A-1, H-2-A-2 and H-2-A-3) were obtained as shown in Fig. 3. Fraction H-2-A-1 was indistinguishable from Fraction H-1-A-1 in cochromatography on DEAE-Sephadex A-50. Similarly, Fraction H-2-A-2 was indistinguishable from Fraction H-1-A-2, and the combined fraction was designated as amidase-2. Fraction

TABLE II

SUMMARY OF PURIFICATION OF AMIDASE-1 FROM HOG KIDNEY

Fraction from	*Protein (mg)*	*Total activity (units)*	*Specific activity (munits/mg protein)*	*Yield (%)*
0.05 M citrate buffer extract	1250 000	101.0	0.081	100
$(NH_4)_2SO_4$ fraction	178 000	93.7	0.526	92.4
Heat treatment	43 200	77.9	1.80	76.8
Acetone fractionation	6 050	80.6*	13.3	79.5*
DEAE-cellulose chromatography	780	64.7	82.9	63.7
Hydroxylapatite chromatography (Fraction H-1)	191	30.7	161	30.2
DEAE-Sephadex A-50 chromatography (Fraction H-1-A-1)	18	18.5	1 030	18.3
DEAE-Sephadex A-50 rechromatography	9	14.0	1 550	13.8

* Slight increases in total activity from the previous step may be due to removal of substances interfering with amidase activity.

H-2-A-3, which differed from amidases-1 and -2 on DEAE-Sephadex A-50 chromatography, was designated as amidase-3.

Purification procedures for amidase-1 are summarized in Table II.

Properties of the purified amidases

Homogeneity. Amidase-1 was homogeneous on disc as well as on sodium dodecyl sulphate disc electrophoreses, as shown in Fig. 4. On disc electrophoresis, the band of protein coincided with that of amidase activity.

Ultracentrifugal analysis of amidase-1 also showed a single symmetric peak with a sedimentation coefficient of 4.60 S.

Amidase-1 showed none of the following glycosidase activities, *i.e.* α- and β-*N*-acetylglycosaminidases, α- and β-mannosidases, α- and β-galactosidases and α-fucosidase, when each substrate (synthetic glycosides) was incubated with 300 munits/ml of amidase-1 for 5 h under optimal conditions.

Amidases-2 and -3 were heterogeneous, and further purification was not possible due to limited amounts of the materials.

Stability of amidase. Amidase-1 was invariably stable if stored in 0.02 M phosphate buffer (pH 7.0) at −20°. Repeated freezing and thawing did not affect the enzyme activity. The stability of amidase-1 varied depending on the pH of the solution. At a pH above 6, amidase was invariably stable, but inactivation occurred at lower pH values; 50 and 67% of the activities being lost at pH values, 5.5 and 5.0, respectively, after standing at 37° for 2 h. However, the enzyme appeared to be stabilized by its substrate, Asn-GlcNAc, since the enzyme reaction proceeded linearly with time (up to 100 min) at pH 5.50 as described below.

Amidases-2 and -3 were as stable as amidase-1.

Effect of duration of the enzyme reaction. The release of *N*-acetylglucosamine from Asn-GlcNAc was linear for at least 100 min as shown in Fig. 5, when 3.75, 2.9 and 1.05 munits/ml of amidases-1, -2 and -3, respectively, were used. The release of

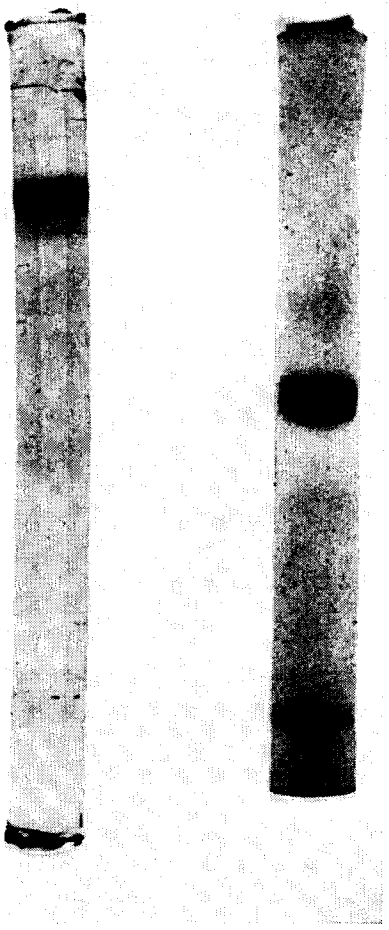

Fig. 4. Disc and sodium dodecyl sulphate disc electrophoreses of amidase-1. Disc electrophoresis (right) was performed in 7.5% gel (pH 9.4 gel) for 60 min using a current of 5 mA per tube. About 50 μg of protein was applied to each gel. Migration was from top (cathode) to bottom. Sodium dodecyl sulphate disc electrophoresis (left) was performed in 10% gel with the normal amount of cross-linker containing sodium dodecyl sulphate and mercaptoethanol for 4 h using a current of 8 mA per tube. About 30 μg of protein was applied to each gel. Migration was from top (cathode) to bottom.

ammonia was in equimolar relationship to that of *N*-acetylglucosamine all the time. After completion of the reaction, products were equimolar amounts of ammonia, aspartic acid (determined by amino acid analyzer) and *N*-acetylglucosamine.

Effect of varying enzyme concentration. The curve obtained by plotting the rate of release of *N*-acetylglucosamine from Asn-GlcNAc *versus* the enzyme concentration was slightly concave upwards, as shown in Fig. 6. This was not observed for crude amidase preparations (before *Step 4*) or amidases-2 (shown in the figure) and -3. This

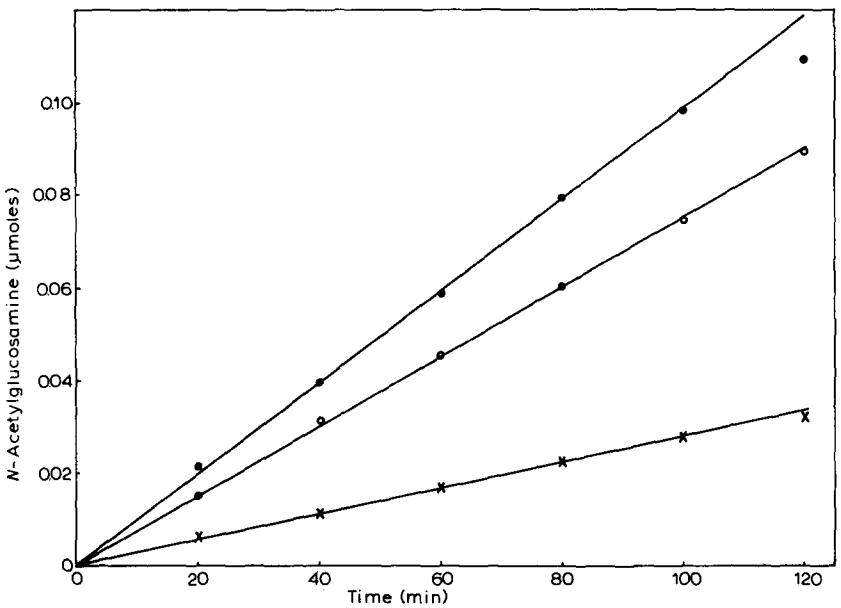

Fig. 5. Effect of duration of the amidase reaction. Enzyme concentrations were 3.75, 2.90 and 1.05 munits/ml for amidases-1, -2 and -3, respectively, and the enzyme activity was expressed as the number of μmoles of the released *N*-acetylglucosamine per 0.3 ml of the reaction mixture. ●—●, amidase-1; ○—○, amidase-2; ×—×, amidase-3.

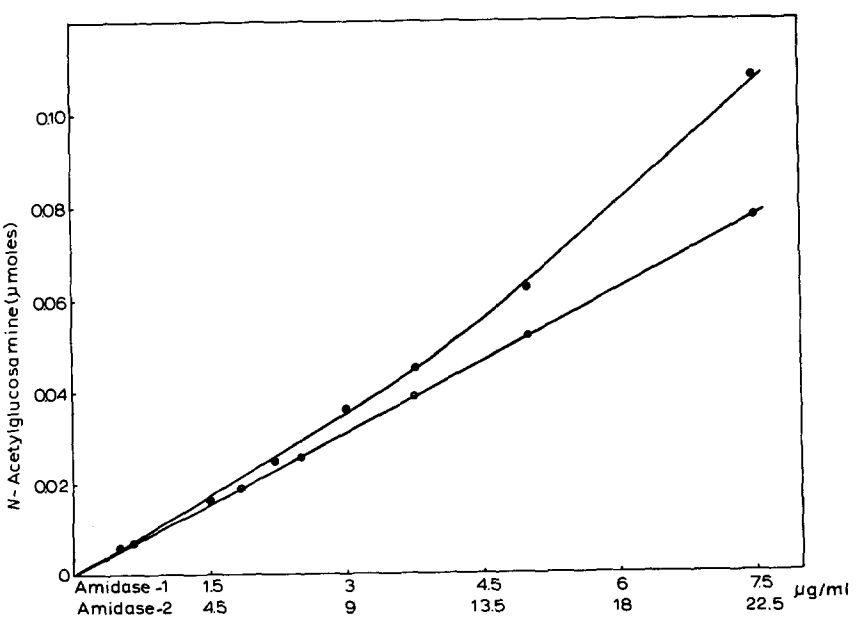

Fig. 6. Effect of varying amidase concentration. Incubation time was 30 min, and the enzyme activity was expressed as the number of μmoles of the released *N*-acetylglucosamine per 0.3 ml of the reaction mixture. ●—●, amidase-1; ○—○, amidase-2.

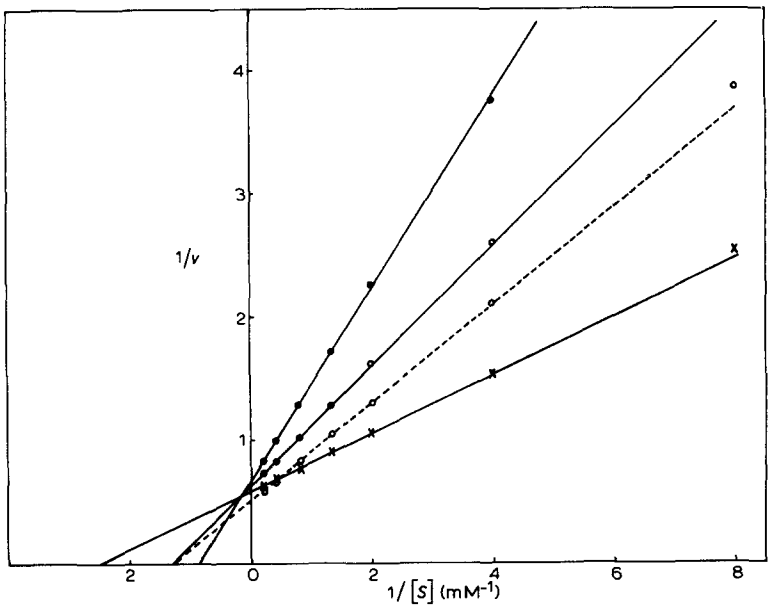

Fig. 7. Effect of substrate concentration on amidase-1 (Lineweaver–Burk plot). Incubation time was 30 min for enzyme concentrations of 5 and 7.5 μg/ml, and 110 min for 2.5 μg/ml. For ovalbumin glycopeptide, enzyme concentration was 5 μg/ml. Reaction velocity (v) was expressed as the number of μmoles of either *N*-acetylglucosamine (for Asn-GlcNAc) or ammonia (for ovalbumin glycopeptide) released per min per mg protein. ●—●, reaction of Asn-GlcNAc with 2.5 μg/ml of enzyme; ○—○, 5 μg/ml; ×—×, 7.5 μg/ml; ○---○, reaction of ovalbumin glycopeptide with 5 μg/ml enzyme.

effect was more apparent at lower substrate concentrations as illustrated in the Lineweaver–Burk plot (see Fig. 7). It should be noted that the specific activity of amidase-1 was determined at a protein concentration of about 4 μg/ml.

Action on amidase of glycopeptides and the effect of varying the substrate concentration. Reaction rates and Michaelis constants (K_m) of amidase-1 towards Asn-GlcNAc and glycopeptides from ovalbumin and Taka-amylase were compared.

In the reaction with glycopeptides, 5 mM of each glycopeptide was incubated with 6 μg/ml amidase-1 for the indicated times, and released ammonia was determined after stopping the reaction by acidification with trichloroacetic acid. The rate of hydrolysis of Taka-amylase glycopeptide was about the same as that of Asn-GlcNAc, and that of ovalbumin glycopeptide was even higher.

Using 5 μg/ml amidase-1, K_m values with Asn-GlcNAc and ovalbumin glycopeptide were the same (0.77 mM) as shown in Fig. 7. However, it was remarkable that the K_m value of amidase with Asn-GlcNAc varied depending on the enzyme concentration; a linear relationship being obtained by plotting the K_m value *versus* the enzyme concentration over the range used in the experiments, as shown in Figs. 7 and 8. This results in the marked dependency of the reaction rate, expressed in terms of activity per mg protein, on the enzyme concentration at lower substrate concentrations. Maximum velocity (specific activity) was also dependent slightly on enzyme concentration.

K_m values for amidases-2 and -3 determined using enzyme concentrations

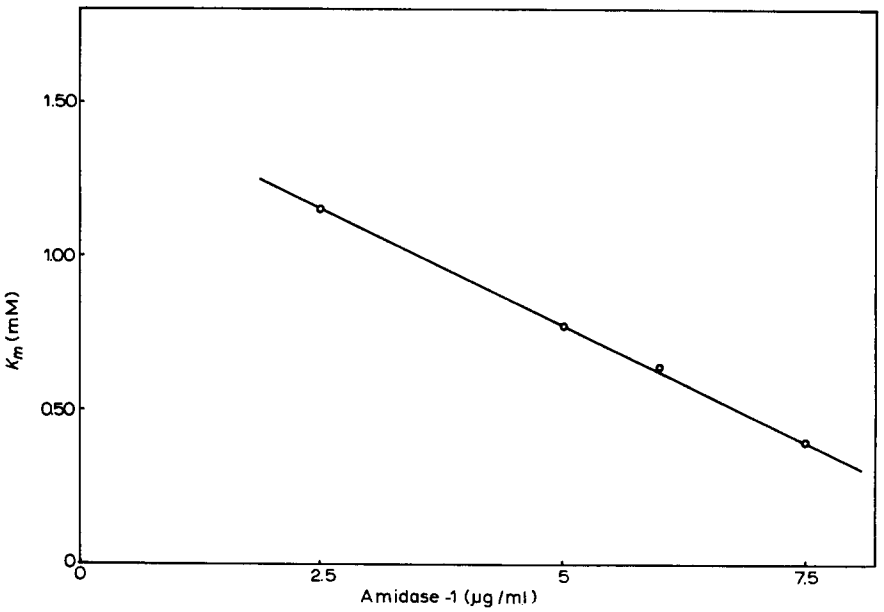

Fig. 8. Variation of Km values with amidase-1 concentration. K_m values obtained from Fig. 7 were plotted against enzyme concentration.

corresponding to 6 μg/ml amidase-1 were nearly the same as that for amidase-1, 0.64 mM and 0.40 mM, respectively, as compared to 0.64 mM for amidase-1.

Variation of activity with pH. The pH profile of amidase activity varied depending on conditions used to terminate the reaction. When the enzyme reaction was terminated by adding 10% trichloroacetic acid, a sharp peak was observed at a pH of about 5.5, but activity was still considerable at higher pH values with a broad peak at around pH 8. The same pH profile was obtained irrespective of the assay methods, *i.e.* the Morgan–Elson reaction or ammonia determination. However, when the enzyme reaction was terminated by applying ammonia determination directly to the reaction mixture, it was found that the formation of ammonia was much less than that of the amino sugar at alkaline pH values. This finding was similar to that previously reported by MAKINO *et al.*[6,12] for hog serum amidase.

Amidases-2 and -3 were similar to amidase-1 in the pH profile.

Effect of various compounds on activity. Activity (amidase-1) was only slightly affected by *p*-chloromercuribenzoate. Cu^{2+}, Ni^{2+}, Zn^{2+} and Mn^{2+} at 1 mM inhibited the enzyme 30–40%. These results are similar to those for amidase from rat kidney[7]. Some metal ion may participate in the activity since 1 mM EDTA inhibited the enzyme 50%, but no detailed study of this phenomenon has been performed. Of the reaction products, aspartic acid at 1 mM inhibited the enzyme 40%, but neither *N*-acetylglucosamine nor ammonia at 1 mM inhibited the enzyme.

Mechanism of the enzyme action. MAKINO *et al.*[6,12] presented convincing evidence for the following action mechanism of hog serum amidase.

Asn–GlcNAc + H_2O → 1-amino-*N*-acetylglucosamine + aspartate (1)
1-Amino-*N*-acetylglucosamine + H_2O → NH_3 + *N*-acetylglucosamine (2)

To prove this reaction sequence for hog kidney amidase, 1.0 mg of Asn-GlcNAc

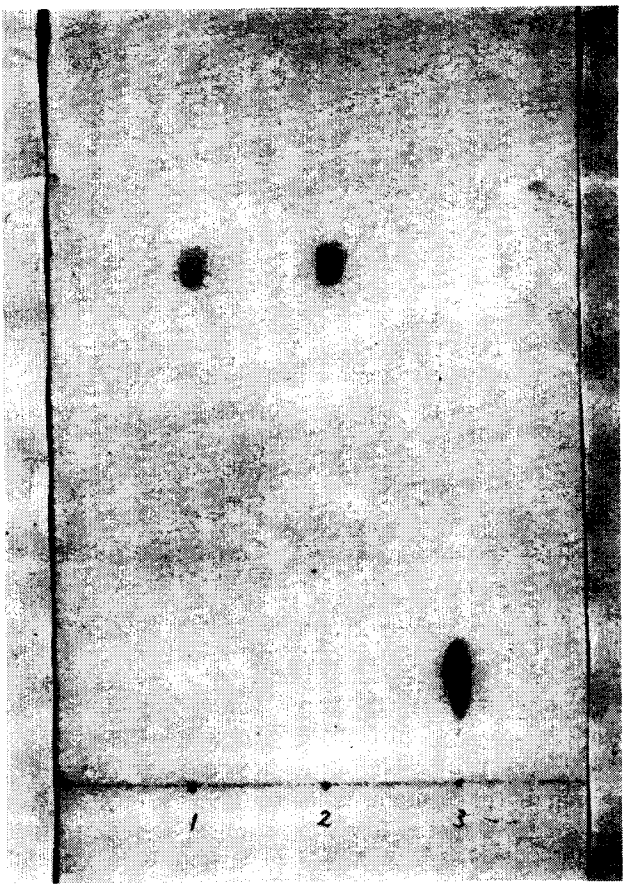

Fig. 9. Identification of a reaction product from the reaction of amidase-1 with Asn-GlcNAc at pH 9.0. Aliquots of the reaction mixture were directly applied to paper, and electrophoresis was performed at pH 9.0 for 60 min at 50 V/cm, cooled in *n*-hexane. Reducing sugars were stained with the silver nitrate reagent. 1, 1-amino-*N*-acetylglucosamine; 2, the reaction product; 3, *N*-acetylglucosamine. Other details are described in the text.

and 120 μg of amidase-1 were incubated in 0.5 ml of 0.05 M barbital buffer (pH 9.0). After the reaction had been completed as judged by the Morgan–Elson reaction, aliquots of the reaction mixture were submitted to paper electrophoresis and paper chromatography. The electrophoretogram (shown in Fig. 9) and chromatogram showed that the major reducing sugar in the reaction mixture was 1-amino-*N*-acetylglucosamine. This is consistent with the pH profile, which showed that the ammonia formation was less than 10% of the amino sugar formation at pH 9.0.

It should be noted that no acceleration in hydrolysis of 1-amino-*N*-acetylglucosamine was observed when this amino sugar was incubated with the hog kidney homogenate at pH values of 6.0 and 8.0.

Molecular weight of amidase. The apparent molecular weights of amidases were estimated on Sephadex G-150 columns. Fig. 10 presents a plot of the elution volume of several well characterized proteins as a function of the logarithms of their molecular

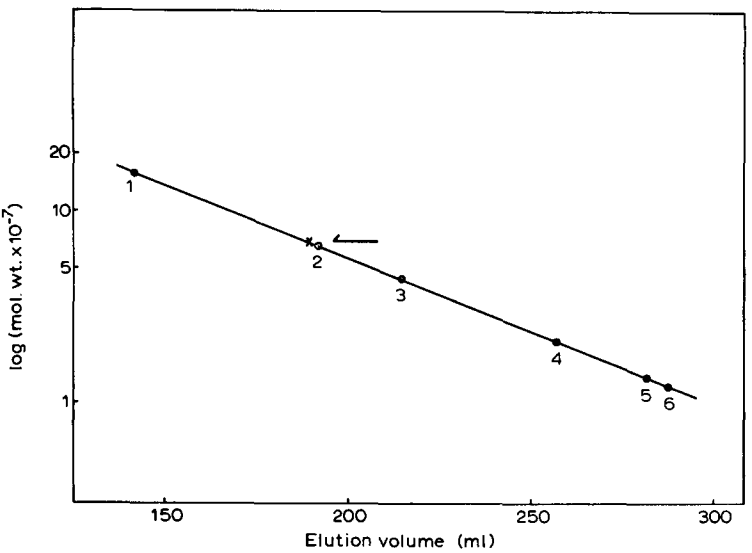

Fig. 10. Estimation of the molecular weight of amidase-1 by gel filtration on Sephadex G-150. Logarithms of molecular weights of reference proteins were plotted against elution volume. Conditions for gel filtration are described in the text. 1, ceruloplasmin; 2, bovine serum albumin; 3, ovalbumin; 4, trypsin inhibitor (soy bean); 5, ribonuclease A; 6, cytochrome *c*.

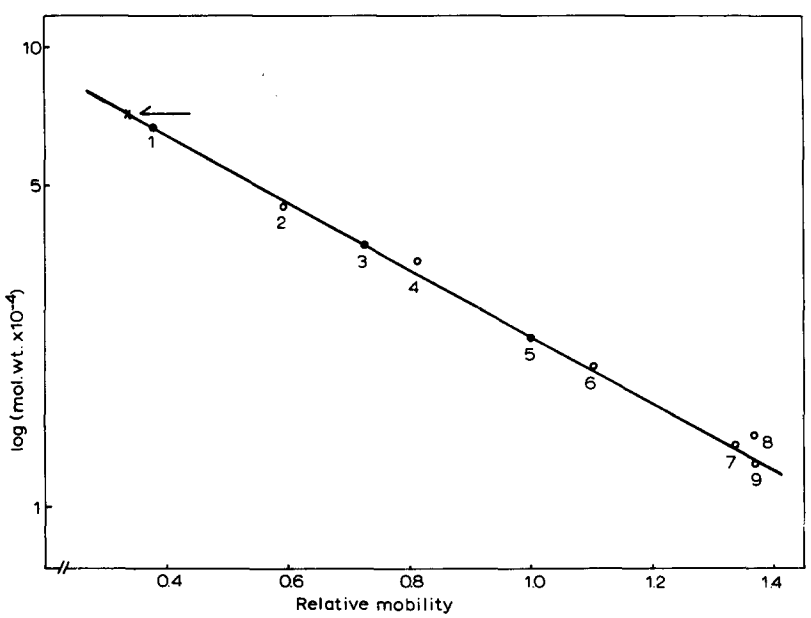

Fig. 11. Estimation of the moelcular weight of amidase-1 by sodium dodecyl sulphate disc electrophoresis. Logarithms of molecular weights of reference proteins were plotted against mobility relative to trypsin. Conditions for electrophoresis are described in the text. 1, bovine serum albumin; 2, ovalbumin; 3, alcohol dehydrogenase (yeast); 4, carboxypeptidase A; 5, trypsin; 6, trypsin inhibitor (soy bean); 7, ribonuclease A; 8, lysozyme (egg white); 9, cytochrome *c*.

weights. As shown, the elution volume of amidase-1, obtained by assaying the enzyme activity corresponds to a molecular weight of 70 000 ± 3000. Similar values were obtained irrespective of the amount of amidase-1 (30 or 15 μg) or of the solvent (0.01 M potassium phosphate buffer, pH 7.0, containing 0.1 M NaCl or 0.05 M sodium phosphate–citrate buffer, pH 5.5) used for the gel filtration. In every case, the enzyme activity was quantitatively recovered.

The values were also similar for amidases-2 and -3.

Molecular weight of amidase-1 was also determined by sodium dodecyl sulphate disc electrophoresis. Fig. 11 presents a plot of the electrophoretic mobilities of several well characterized proteins as a function of the logarithms of their molecular weights. The electrophoretic mobility of amidase-1 exceeded slightly the range of reference proteins, but by extrapolating the line the molecular weight of amidase-1 appeared to be about 70 000.

The sedimentation coefficient of amidase-1, 4.60 S, appeared to be consistent with a molecular weight of about 70 000 if the molecule is assumed to be nearly spherical.

This led us to conclude that amidase-1 consists of a single polypeptide chain with a molecular weight of about 70 000 and that amidases-2 and -3 are very similar in molecular weight to amidase-1.

Isoelectric points of amidases. From a pattern of isoelectric focusing obtained, using 40 munits for amidase-1, 20 munits for amidase-2 and 8 munits for amidase-3,

TABLE III

AMINO ACID COMPOSITION OF AMIDASE-1

Amidase-1 (0.2 mg) was hydrolyzed in 6 M HCl at 105° for 24 h. No correction was made for losses during hydrolysis. Tryptophan was not determined.

Amino acid	*Number of residues per 100 residues*	*Amino acid*	*Number of residues per 100 residues*
Lysine	4.7	Methionine	2.4
Histidine	1.8	Glycine	10.9
Arginine	4.1	Alanine	11.3
Aspartic acid	10.3	Valine	5.8
Glutamic acid	7.1	Isoleucine	5.3
Threonine	8.4	Leucine	6.3
Serine	7.9	Tyrosine	3.3
Proline	3.9	Phenylalanine	4.1
Half-cystine	2.1	(Glucosamine	0.5)

the observed values, 5.12, 4.85 and 4.70 were assigned to amidases-1, -2 and -3, respectively, since the activities were quantitatively recovered, and the isoelectric point of amidase-1 had been determined in a separate experiment. Thus, all the amidase proteins are rather acidic.

Amino acid composition of amidase-1. Using 0.2 mg of amidase-1, the amino acid composition was determined with the results shown in Table III. Tryptophan was not determined. Amidase-1 contained glucosamine. Sialic acid was not detected when 0.5 mg of amidase-1 was used for the analysis.

DISCUSSION

Amidase is widely distributed in mammalian tissues. Hog kidney was used to extract and purify the enzyme since this tissue has relatively high enzyme activity (though the content of the enzyme in tissues was very low) and can be handled on a large scale. We succeeded in purifying amidase in a substantial yield.

Our purified amidase from hog kidney was quite similar in its enzymatic properties to the enzyme from hog serum reported previously by MAKINO *et al.*[6,12]. Convincing evidence presented by MAKINO *et al.*[6,12] using the serum enzyme which showed that the enzyme is an amidase was confirmed by MAHADEVAN AND TAPPEL[7], TARENTINO AND MALEY[13] and CONCHIE AND STRACHAN[9] using the enzymes from rat liver and kidney, hen oviduct and rat liver, respectively. By analyzing the products of the reaction of our kidney amidase with Asn-GlcNAc at pH 9.0, we found that 1-amino-*N*-acetylglucosamine is a major reducing sugar. Hence, we concluded that the kidney amidase was the same as serum amidase in its mode of enzyme action.

We noticed that even at pH 5.50, the optimum pH, enzymatic release of 1-amino-*N*-acetylglucosamine from Asn-GlcNAc, when it is too rapidly catalyzed by potent amidase, can not immediately be followed by non-enzymatic deamination to produce *N*-acetylglucosamine and ammonia. This makes the assay, using the Morgan–Elson reaction applied directly to the reaction mixture, inaccurate since the colour yield of 1-amino-*N*-acetylglucosamine in the Morgan–Elson reaction is different from that of *N*-acetylglucosamine[6]. For an accurate and convenient assay, therefore, the reaction mixture should be either heated or further acidified to quantitatively convert 1-amino-*N*-acetylglucosamine to *N*-acetylglucosamine and ammonia prior to applying the Morgan–Elson reaction or ammonia determination.

The pH profile of the activity of our kidney amidase was somewhat different from those of enzymes from rat liver and kidney[7,9]. This may be due either to differences in the enzyme sources or to differences in assay conditions.

Our enzyme was very stable at alkaline pH values, but was rather unstable at acid pH values. In this regard hog kidney amidase is similar to the enzymes from hog serum[6] and hen oviduct[13], but differs from those from rat liver and kidney[7,9].

The maximum velocity of amidase-1 as determined using Asn-GlcNAc increased slightly with an increase in enzyme concentration. The change in K_m value was more marked, and a linear relationship was observed between the K_m value and enzyme concentration. Dependency of these kinetic enzyme parameters on enzyme concentration may be related to the association–dissociation nature of the enzyme protein. However, amidase-1 behaved as a homogeneous protein with a molecular weight of about 70 000 in all the systems used for molecular weight determination. The protein concentration was very low in gel filtration (initially 30 μg/ml, but about 2 μg/ml or less in the eluate at the top of the peak) and very high (initially 0.3%) in ultracentrifugal analysis. In sodium dodecyl sulphate disc electrophoresis amidase-1 was reduced and denatured. Thus, dependency of the kinetic parameters on enzyme concentration can not be ascribed to the association–dissociation nature of the enzyme protein. The enzyme preparation might contain minute amounts of some substance which causes decrease in Michaelis constant and increase in maximum velocity as enzyme concentration increases.

The molecular weight of hog kidney amidase differed from that of amidases

from other sources. Values of 31 000 and 110 000 have been reported for enzymes from rat kidney[7] and hen oviduct[13], respectively.

The specific activity of amidase-1 was rather low (only 1.55 units per mg protein when Asn-GlcNAc was used as substrate) as compared to glycosidases acting on glycoproteins or glycopeptides. For example, the specific activity of α-mannosidase prepared from hog kidney in a nearly homogeneous state in our laboratory[33] was about 20 units per mg protein when assayed using p-nitrophenyl-α-mannoside. However, this glycosidase released mannose from ovalbumin glycopeptide at a rate only 1/50 that from the synthetic substrate. This contrasts with the property of amidase, since amidase is capable of hydrolyzing glycopeptides at rates comparable to the rate for Asn-GlcNAc. Thus, amidase may be comparable to α-mannosidase in its activity towards glycopeptides. This also implies that size and structure of the carbohydrate moiety are not important factors for substrates to undergo the amidase action. Further specificity studies of amidase are in progress in our laboratory.

Since the amount of amidase-1 obtained was rather limited, detailed chemical studies of the enzyme protein could not be carried out. However, the presence of 3–4 moles of glycosamine per mole of amidase-1 could be demonstrated, indicating that amidase-1 is a glycoprotein.

At least two other forms of amidase were found in addition to amidase-1. They differ from each other in their behaviours on ion-exchange chromatography and on isoelectric focusing. It might be possible that the presence of multiple forms is due to different carbohydrate moieties attached to the same polypeptide chain. Thus, it is worth mentioning that GOLDSTONE *et al.*[34] have recently claimed that essentially all the enzyme proteins of rat kidney or liver lysosomes are glycoproteins, and that the occurrence of multiple forms is due to differences in sialic acid content.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Ministry of Education, Japan. The authors wish to thank Dr. K. Kakiuchi of the Protein Research Institute, Osaka University, for performing the sedimentation analysis.

Once of us (M.K.) was Postgraduate Fellow of California Foundation for Biochemical Research.

REFERENCES

1 A. NEUBERGER, A. GOTTSCHALK AND R. D. MARSHALL, in A. GOTTSCHALK, *Glycoproteins*, Elsevier, Amsterdam, 1966, p. 273.
2 C. P. J. ROSTON, J. C. CAYGILL AND F. R. JEVONS, *Biochem. J.*, 97 (1965) 43 P.
3 M. MURAKAMI AND E. H. EYLAR, *J. Biol. Chem.*, 240 (1965) PC 556.
4 E. D. KAVERZNEVA, *Khim. Prir. Soedin. Akad. Nauk U.S.S.R.*, 2 (1966) 130; *Chem. Abstr.*, 65 (1966) 9257F.
5 J. R. CLAMP, G. DAWSON, L. HOUGH AND M. Y. KHAN, *Carbohydr. Res.*, 3 (1966) 254.
6 M. MAKINO, T. KOJIMA AND I. YAMASHINA, *Biochem. Biophys. Res. Commun.*, 24 (1966) 961.
7 S. MAHADEVAN AND A. L. TAPPEL, *J. Biol. Chem.*, 242 (1967) 4568.
8 T. OHGUSHI AND I. YAMASHINA, *Biochim. Biophys. Acta*, 156 (1968) 417.
9 J. CONCHIE AND I. STRACHAN, *Biochem. J.*, 115 (1969) 709.
10 F. A. JENNER AND R. J. POLLITT, *Biochem. J.*, 103 (1967) 48 P.
11 R. J. POLLITT, F. A. JENNER AND H. MERSKEY, *Lancet*, (1968) 253.
12 M. MAKINO, T. KOJIMA, T. OHGUSHI AND I. YAMASHINA, *J. Biochem. Tokyo*, 63 (1968) 186.

13 A. L. TARENTINO AND F. MALEY, *Arch. Biochem. Biophys.*, 130 (1969) 295.
14 I. YAMASHINA, M. MAKINO, K. BAN-I AND T. KOJIMA, *J. Biochem. Tokyo*, 58 (1965) 168.
15 I. YAMASHINA AND M. MAKINO, *J. Biochem. Tokyo*, 51 (1962) 359.
16 M. MAKINO AND I. YAMASHINA, *J. Biochem. Tokyo*, 60 (1966) 262.
17 H. YAMAGUCHI, T. IKENAKA AND Y. MATSUSHIMA, *J. Biochem. Tokyo*, 68 (1970) 843.
18 H. W. SIEGELMAN, G. A. WIECZOREK AND B. C. TURNER, *Anal. Biochem.*, 13 (1965) 402.
19 R. A. KECKWICK AND R. K. CANNAN, *Biochem. J.*, 30 (1936) 227.
20 G. A. LEVVY AND A. MCALLAN, *Biochem. J.*, 73 (1959) 127.
21 B. LUBOCHINSKY AND J. ZALTA, *Bull. Soc. Chim. Biol.*, 36 (1954) 1363.
22 T. S. BRITTON AND R. A. ROBINSON, *J. Chem. Soc.*, (1931) 1456.
23 J. CONCHIE, J. FINDLAY AND G. A. LEVVY, *Biochem. J.*, 71 (1959) 318.
24 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
25 W. E. TREVELYAN, D. P. PROCTER AND J. S. HARRISON, *Nature*, 166 (1950) 444.
26 L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971.
27 B. J. DAVIS, *Ann. N. Y. Acad. Sci.*, 121 (1964) 404.
28 P. GABRIEL AND S. WANG, *Anal. Biochem.*, 27 (1969) 545.
29 K. WEBER AND M. OSBORN, *J. Biol. Chem.*, 244 (1969) 4406.
30 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
31 H. K. SCHACHMAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. **2**, Academic Press, New York, 1957, p. 32.
32 O. VESTERBERG AND H. SVENSSON, *Acta Chem. Scand.*, 20 (1966) 820.
33 T. OKUMURA AND I. YAMASHINA, *J. Biochem. Tokyo*, 68 (1970) 561.
34 A. GOLDSTONE, P. KONECNY AND H. KOENIG, *FEBS Lett.*, 13 (1971) 68.