Purification and Some Properties of 1-Aspartamido- β -N-acetylglucosamine Amidohydrolase from Human Liver

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Human liver 1-aspartamido- β -N-acetylglucosamine amidohydrolase (aspartylglucosylaminase, EC 3.5.1.26) was purified 17 500-fold to apparent homogeneity as judged from polyacrylamide-gel disc electrophoresis. A pH optimum of 7.7–9.0 was found. The K_m value was pH- and temperature-dependent. At 37°C and pH 7.7, K_m was 0.16 mM and it increased to 0.29 at pH 6.0 and 0.23 at pH 9.0. At 25°C and pH 7.7, a K_m value of 0.99 mM was obtained. When the substrate concentration was varied, apparent Michaelis–Menten kinetics were obtained. *p*-Hydroxymercuribenzoate, glutathione or cysteine had no effect on the enzyme activity; 5 mM-*N*-acetylcysteine inhibited about 47% of the total enzyme activity. Apart from Cu^{2+} , other bivalent ions were virtually ineffective at 1 mM. The kinetic study differentiates this enzyme from aspartylglucosylaminase from other sources.

The enzyme involved in the splitting of the *N*-aspartylglycosylamine linkage was described by Murakami & Eylar (1965). The first indication of the mode of this reaction, catalysed by 1-aspartamido- β -N-acetylglucosamine amidohydrolase (aspartylglucosylaminase, EC 3.5.1.26) was presented by Makino *et al.* (1966). It is considered to be a two-step reaction, in which the second step is non-enzymic:

The interest in this enzyme system grew rapidly since Jenner & Pollitt (1967) described aspartylglucosaminuria, an uncommon and incurable inborn error of metabolism which may be caused by a deficiency of aspartylglucosylaminase (Pollitt *et al.*, 1968; Pollitt & Jenner, 1969; Aula *et al.*, 1973, 1976). Patients with this disease excrete high amounts of 2-acetamido-1-(β -L-aspartamido)-1,2-dideoxy- β -D-glucose (aspartyl-*N*-acetylglucosamine). They are mentally retarded (Pollitt *et al.*, 1968; Pollitt & Jenner, 1969; Autio, 1972).

Studies with the partially purified enzyme from different sources with different specific activities have been carried out (Murakami & Eylar, 1965; Makino *et al.*, 1966, 1968; Mahadevan & Tappel, 1967;

Roston *et al.*, 1965; Clamp *et al.*, 1966; Eylar & Murakami, 1966; Conchie & Strachan, 1969; Tarentino & Maley, 1969), but hardly any attention has been paid to the purification of the enzyme from human organs. Since the previous results (Dugal, 1977a) differentiated human liver aspartylglucosylaminase (hereafter termed 'the enzyme') from that from other species, we decided to purify this enzyme. In the present paper a purification is described in which the enzyme has been purified to apparent homogeneity as judged by polyacrylamide-gel disc electrophoresis. The kinetic studies presented differentiate the human liver enzyme from that obtained from sheep epididymis (Murakami & Eylar, 1965), ram epididymis (Eylar & Murakami, 1966), boar epididymis (Clamp *et al.*, 1966), rat liver (Mahadevan & Tappel, 1967; Conchie & Strachan, 1969), rat kidney (Mahadevan & Tappel, 1967), pig plasma (Makino *et al.*, 1968), ram testis (Roston *et al.*, 1965), hen oviduct (Tarentino & Maley, 1969) and human seminal fluid (Pollitt & Jenner, 1969).

Materials and Methods

Materials

Materials used here were as described previously (Dugal, 1977a). In addition, *p*-hydroxymercuribenzoate, 2-acetamido-1-(β -L-aspartamido)-1,2-dideoxy-D-glucose, reduced glutathione and calcium phosphate gel were purchased from Sigma, St. Louis, MO, U.S.A., acetone was from E. Merck, Darmstadt, Germany, Sephadex G-200 was from Pharmacia, Uppsala, Sweden, and DEAE-cellulose from Whatman, Maidstone, Kent, U.K. All the other

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reagents were of the highest purity grade commercially obtainable. Morphologically normal liver specimens were removed by autopsy 14–24 h after death.

General methods

The enzyme assay was carried out as described previously (Dugal, 1977a), and protein was measured by the method of Lowry *et al.* (1951) with bovine albumin as standard. Polyacrylamide-gel disc electrophoresis was carried out as described by Maurer (1968).

Purification of the enzyme

Preparation of enzyme of high specific activity from human liver requires rapidity and special care. The isolation procedure is therefore described in detail. All operations were carried out between 0 and 4°C, and all solutions and vessels were precooled to this temperature. The intracellular localization of this enzyme (Mahadevan & Tappel, 1967; Oghushi & Yamashina, 1968) indicated that enzyme activity, although mainly associated with the lysosomes, also occurs in mitochondria. Therefore the whole liver was used for the purification of the enzyme.

Extraction of the enzyme. For optimal extraction, phosphate/EDTA/Triton buffer was used (Dugal, 1977a). Human liver (400 g) was mixed with 1800 ml of this buffer, pH 7.7, in the ratio of about 1:5 (w/v) and homogenized for 75 s, with regular cooling, in a Waring Blendor at top speed. It was further homogenized in a Potter-Elvehjem homogenizer as described by Dugal (1977a). The homogenate was incubated at 37°C for 60 min in a water bath. This crude homogenate (2150 ml) was centrifuged for 45 min at 21 500 g in a Sorvall GSA rotor. The pH of the supernatant was adjusted with 1 M-K₂HPO₄ to pH 7.7 and subjected to (NH₄)₂SO₄ fractionation.

First (NH₄)₂SO₄ fractionation. The red supernatant (1790 ml) was fractionated (35–65%) with solid (NH₄)₂SO₄ as described previously (Dugal, 1977a).

Second (NH₄)₂SO₄ precipitation. The pellet obtained from this first (NH₄)₂SO₄ precipitation (35–65% satn.) was dissolved in 0.1 M-K₂HPO₄/KH₂PO₄ buffer, pH 7.7. To this protein solution (370 ml) solid (NH₄)₂SO₄ was added slowly to give 45% saturation (25.6 g/100 ml of enzyme solution). The mixture was kept at –25°C for 30 min and centrifuged at 48 000 g for 15 min (Sorvall SS 34 rotor). This pellet was discarded. The supernatant was adjusted to 75% saturation with solid (NH₄)₂SO₄ (19 g/100 ml of enzyme solution), kept at –25°C for 60 min and centrifuged for 20 min at 48 000 g (Sorvall SS 34 rotor). The supernatant with no activity was discarded, and the pellet obtained with 45–75% total (NH₄)₂SO₄ saturation was extracted as follows.

Extraction with saturated (NH₄)₂SO₄ solution. The

pellet obtained with 45–75% (NH₄)₂SO₄ saturation was extracted with (NH₄)₂SO₄ solution in decreasing concentration as described in detail elsewhere (Dugal & Louis, 1974). Each fraction obtained by extraction with 80, 60, 57, 55, 55, 52, 50 and 45%-saturated (NH₄)₂SO₄ solution had a total volume of 50 ml. As Table 1 shows, the highest activity was obtained between 55 and 45% saturation of (NH₄)₂SO₄. The remainder after 45% (NH₄)₂SO₄ saturation was discarded. The fractions between 55 and 45% of (NH₄)₂SO₄ saturation were dialysed against 4 × 10 litres of 0.01 M-K₂HPO₄/KH₂PO₄ buffer, pH 7.7, for 18 h. After the dialysis these fractions were pooled (390 ml) and subjected to calcium phosphate-gel adsorption for further purification.

Calcium phosphate-gel treatment. The dialysed fractions (2953 mg of protein/390 ml) were treated with calcium phosphate gel; 0.55 mg of calcium phosphate gel/mg of enzyme protein was added slowly to the protein solution with continuous stirring over about 8 min. The solution was stirred further for 15 min and then centrifuged for 10 min at 48 000 g (Sorvall SS 34 rotor). The pellet was discarded and the supernatant (390 ml) was purified further by heat treatment.

Heat treatment. The supernatant from the calcium phosphate-gel treatment was subjected to heat treatment in three batches. Supernatant (130 ml) was poured into 1-litre prewarmed vessels (about 60°C) and heated for 10 min at 60°C, cooled rapidly in an ice/salt mixture and centrifuged for 10 min at 27 000 g. The pellet was discarded and the supernatant was collected in a precooled vessel. The other two fractions, each 130 ml, were also treated in the same manner. The pooled supernatants (360 ml) were further fractionated with acetone.

Acetone fractionation and dialysis. Precooled (overnight at –25°C) acetone (264 ml) was added slowly to the enzyme solution in an ice/salt mixture with continuous stirring. When the enzyme solution just started to become turbid (acetone/enzyme solution = 3:1, v/v) it was taken out from the ice bath and kept at –25°C for 20 min, and centrifuged at 37 000 g for 20 min. It is essential here to add enough acetone to precipitate inactive proteins that produce the turbidity, but not so much as to precipitate the enzyme. The pellet was discarded, and 150 ml of the precooled acetone was added to the supernatant, as mentioned above. This turbid solution was kept at –25°C for 60 min and the pellet was collected by centrifugation at 37 000 g for 20 min. The supernatant with no activity was discarded. The pellet was dissolved in water (102 ml) and dialysed for 18 h against 4 × 10 litres of water. The dialysed fraction was then centrifuged at 37 000 g for 10 min and the brown pellet was discarded. The supernatant (110 ml) was chromatographed on a DEAE-cellulose column for further purification.

DEAE-cellulose chromatography. The dialysed fraction was applied to a column (2.5cm×25cm) of DEAE-cellulose that had been equilibrated previously with 0.01M-K₂HPO₄/KH₂PO₄ buffer, pH7.7. After the enzyme was adsorbed on the top of the column as a brown ring, the column was washed with 0.01M-K₂HPO₄/KH₂PO₄ buffer, pH7.7, until the *A*₂₈₀ of the effluent was decreased to its starting value (zero). It was followed by elution with 0.03M-NaCl solution in 0.01M-K₂HPO₄/KH₂PO₄ buffer until the *A*₂₈₀ was again decreased approximately to its original value (zero). Then 0.06M-NaCl in 0.01M-K₂HPO₄/KH₂PO₄ buffer, pH7.7, was applied to the column to elute much of the enzyme activity, and 8.5ml fractions of the effluent were collected. Then another protein peak containing enzyme activity was obtained with 0.08M-NaCl solution in 0.01M-K₂HPO₄/KH₂PO₄ buffer, pH7.7. This second peak had very low enzyme specific activity (230nmol/h per mg). It showed at least six to eight bands on the polyacrylamide-gel disc electrophoresis, and was not used for further purification. The fractions with high specific activity (eluted with 0.06M-NaCl) were pooled together (135ml) and concentrated with a

Amicon XM 50 membrane. This concentrate was washed with 50ml of water and the final concentrate (15ml) after centrifugation (10min, 48000g) was subjected to gel filtration on a column of Sephadex G-200.

Gel filtration. A column (2.5cm×80cm) of Sephadex G-200 was equilibrated with 0.01M-K₂HPO₄/KH₂PO₄ buffer, pH7.7, at a flow rate of 30ml/h. The enzyme fraction (15ml) was loaded on to this column and 9ml fractions were collected with the same buffer. The enzyme was eluted in a broad peak. The fractions were mixed together according to their specific activities. The highest specific activity thus obtained was 12780nmol/h per mg (Table 1).

Second heat treatment. The active fractions [see Table 1, fractions (a), (b) and (c)] were heated to 60°C for 10min as described above. The most active enzyme fraction (e.g. 17500-fold purification, 43334nmol of glucosamine/h per mg) thus obtained showed apparent homogeneity (with 18μg of enzyme protein) by moving towards anode on the polyacrylamide-gel disc electrophoresis with an *R_F* value of 0.58. The results of the purification procedure are summarized

Table 1. *Purification of 1-aspartamido-β-N-acetylglucosamine amidohydrolase from 44g of human liver*
The assay was carried out in 0.32ml at pH7.7 as described by Dugal (1977a). Each purification step is fully described in the Materials and Methods section. Abbreviation: GlcNAc, *N*-acetylglucosamine.

Purification steps and fractions	Vol. (ml)	Total activity (nmol of GlcNAc/h)	Total protein (mg)	Sp. activity (nmol of GlcNAc/h per mg)	Yield (%)
Crude homogenate	2150	48160	60630	2.48	10.0
25400g supernatant	1790	58998	37053	4.9	12.2
35–65%-satd. (NH ₄) ₂ SO ₄ fraction	379	26699	9879	8.5	55.4
Extraction of 45–75%-satd. (NH ₄) ₂ SO ₄ precipitate with following saturations of (NH ₄) ₂ SO ₄ solution:					
80%	90	—	92	—	—
60%	90	90	395	0.7	0.16
57%	84	1560	831	5.8	3.2
55%	88	6055	809	23.4	12.6
55%	88	4506	572	24.6	9.4
52%	86	4555	817	17.4	9.5
50%	45	2355	477	15.6	4.9
45%	82	1575	278	17.6	3.2
Calcium phosphate treatment	390	12917	1989	20.3	26.8
Heat treatment	360	12499	612	63.8	26.0
Acetone treatment	102	10233	204	156	21.3
Dialysis	110	10618	187	178	22.0
DEAE-cellulose column	135	5573	7.0	2580	11.6
Sephadex G-200 column (active fractions)					
(a)	34	1306	0.408	10000	2.7
(b)	21	773	0.189	12777	1.6
(c)	78	724	0.406	5673	1.5
Heat treatment (active fractions)					
(a)	34	1415	0.104	43334	2.95
(b)	21	773	0.063	40000	1.6
(c)	78	812	0.156	16250	1.7

in Table 1. The purified enzyme fraction was used for kinetic studies.

Results and Discussion

The activity of aspartylglucosylaminase is pH-dependent (Fig. 1). The pH-activity curve is distorted, probably due to the increased stability of 1-amino-*N*-acetylglucosamine at high pH (Makino *et al.*, 1966). The pH optimum is in the range 7.7–9.0. It is similar to that of the enzyme from sheep epididymis (about pH 7.7), although the latter shows an activity near the maximum from pH 6 to 8 (Eylar & Murakami, 1966). With our 17500-fold-purified enzyme we could not demonstrate the pH-activity curve obtained by Savolainen (1976) for 228-fold purified enzyme from human liver. Further, at pH 6.0 only about 20% of the total activity was obtained and not 92% as reported by Savolainen (1976).

With increasing concentration of substrate, Michaelis-Menten kinetics (one K_m value) were obtained (Fig. 2a), which were different from the co-operative kinetics (two K_m values) obtained for some other enzyme systems (Dugal, 1976, 1977b). Fig. 2(b) shows the pH-dependence of the K_m and V_{max} values. At pH 7.7 and 8.0, a K_m of 0.16 mM was obtained. The K_m varies with pH, being 0.29 mM at pH 6.0 and 0.23 mM at pH 9.0. The pH value has no effect on the Michaelis-Menten kinetics of this enzyme (Michaelis-Menten kinetics still hold despite pH changes), in contrast with what has been demonstrated for some other enzyme systems (Malik &

Stracher, 1977; Dugal, 1973). The K_m is also temperature-dependent, e.g. at pH 7.7 and 25°C a much higher value (0.99 mM) was obtained.

The aspartylglucosylaminase activity is decreased

Fig. 1. Effect of pH on the enzyme activity (formation of *N*-acetylglucosamine)

The enzyme assay was carried out as described previously (Dugal, 1977a). For this experiment, freshly prepared 1 M- K_2HPO_4/KH_2PO_4 buffer was used. The values between pH 8.5–9.5 were also repeated with Tris/HCl buffer.

Fig. 2. (a) Lineweaver-Burk plot showing the effect of substrate concentration on the enzyme activity (colour yield) at pH 6.0 (●) and at pH 8.0 (○), and (b) effect of pH on enzyme-substrate affinity (K_m) and on the value of V_{max} .

In (a) assay conditions were the same as described for Fig. 1. In (b) the K_m (○) and V_{max} (Δ) values were calculated from (a).

in the presence of *N*-acetylcysteine. Increasing the preincubation time with this component from 5 to 30 min did not change the percentage inhibition. Glutathione and cysteine (both 4 mM) had no inhibitory effect on the enzyme activity, and *p*-hydroxymercuribenzoate, when present at 4 mM for 24 h, did not change the activity. Cu^{2+} , Zn^{2+} , Ni^{2+} and Mn^{2+} , all at 1 mM, inhibited the enzyme activity 37, 16, 13 and 13% respectively. Other metal ions (Fe^{2+} , Mg^{2+} , Ca^{2+}) tested showed no significant effect (at 1 mM) on activity. These inhibitory studies differentiate the enzyme from human liver and that from rat kidney (Mahadevan & Tappel, 1967).

For optimal enzyme extraction liver had to be homogenized in the presence of 1% Triton X-100 in a Waring Blendor. This is in spite of the fact that the enzyme, in contrast with acid phosphatase and β -*N*-acetylglucosaminidase, is supposed to be loosely bound to the lysosomal membrane. Repeated extraction of the pellet from the crude homogenate did not show any increase of the enzyme activity in the supernatant. The extraction procedure applied is described in detail elsewhere (Dugal, 1974, 1977b).

The first treatment of the crude homogenate with solid $(\text{NH}_4)_2\text{SO}_4$ resulted in 45–50% loss of enzyme activity (Table 1). This instability of the enzyme activity of the human liver crude homogenate towards $(\text{NH}_4)_2\text{SO}_4$ was found repeatedly. However, the enzyme activity not destroyed was highly resistant to further $(\text{NH}_4)_2\text{SO}_4$ treatment (Table 1). Apparently there are two fractions present, one stable and another labile to this treatment. On the DEAE-cellulose column the activity eluted in two distinct peaks. The first peak, containing the high specific activity, eluted with 0.6 M-NaCl solution, was used for further purification. The second peak, with low specific activity, eluted with 0.08 M-NaCl, was discarded.

One can therefore suggest the presence of isoenzymes in the human body. This is further supported by the different values for pH optima (Fig. 1) and K_m (Fig. 2a) for the enzymes from human liver and human seminal fluid (Pollitt & Jenner, 1969). Such different behaviour of the enzymes from the same species has also been discussed for other enzymes (Dugal, 1977b). The enzyme from human liver differs with respect to pH optimum from that isolated from sheep epididymis (pH 7.7) (Murakami & Eylar, 1965), boar epididymis (pH 5.0–7.7) (Clamp *et al.*, 1966), pig serum (pH 5.5) (Makino *et al.*, 1966), human seminal fluid (pH 5.6) (Pollitt & Jenner, 1969), rat liver (pH 7.6 and 7.0) (Mahadevan & Tappel, 1967; Conchie & Strachan, 1969), the limpet *Patella vulgata* (pH 7.0) (Conchie & Strachan, 1969), rat epididymis (pH 8.0) (Conchie & Strachan, 1969), rat kidney (pH 7.6) (Mahadevan & Tappel, 1967), hen oviduct (pH 5.1) (Tarentino & Maley, 1969) and hog kidney (pH 5.5) (Yamashina, 1972).

With our 17500-fold-purified enzyme, we could not reproduce the results reported by Savolainen (1976) with 228-fold-purified enzyme from human liver. Further, Savolainen (1976) reported only minor contamination on polyacrylamide-gel disc electrophoresis with the enzyme from human liver (sp. activity 205.2 nmol/h per mg). Our impure enzyme (sp. activity 230 nmol/h per mg) revealed about eight bands; Tarentino & Maley (1969) claim about six bands with the partially purified enzyme (sp. activity 12943 nmol/h per mg) on polyacrylamide-gel disc electrophoresis.

Differences in the enzyme from human liver and from other sources were also found with respect to the K_m values under optimal conditions. Human liver enzyme shows an apparent K_m value of 0.16 mM at pH 7.7 and 8.0, which is different from the apparent K_m value obtained at 37°C for human seminal fluid (0.76 mM) (Pollitt & Jenner, 1969), rat liver (0.58 mM) and rat epididymis (0.65 mM) (Conchie & Strachan, 1969), rat kidney (0.59 mM) (Mahadevan & Tappel, 1967), hen oviduct (0.1 mM) (Tarentino & Maley, 1969), pig serum (1.0 mM) (Makino *et al.*, 1966), and hog kidney (0.39 mM) (Yamashina, 1972). The enzyme-substrate affinity of aspartylglucosylaminase from human liver was found to be pH-dependent.

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