

PURIFICATION OF AN *N*-ACETYLGLUCOSAMINIDASE FROM THE LIMPET *PATELLA VULGATA* (L.)

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1. Introduction

N-Acetylglucosaminidases are useful tools for the study of the structure of glycolipids, glycoproteins and polysaccharides. β -*N*-Acetylglucosaminidase (EC 3.2.1.30) has been partially purified from various sources and has been characterised electrophoretically from jack bean meal [1], *Aspergillus oryzae* [2], *Staphylococcus aureus* [3], hen oviduct [4], beef spleen [5] and *Bacillus subtilis* [6]. α -*N*-Acetylglucosaminidase has been partially purified only from pig liver [7] and *Turbo cornutus* liver [8].

In this paper we describe the purification to a state of apparent homogeneity of an *N*-acetylglucosaminidase from the digestive gland of the limpet *Patella vulgata* (L.) which hydrolyses both the α - and β -glycosides of *N*-acetylglucosamine. Limpet digestive gland had previously been noted to possess α - and β -*N*-acetylglucosaminidase activity [9].

2. Materials and methods

2.1. Materials

Limpets of the species *Patella vulgata* (L.) were obtained from the Marine Biological Laboratory at Plymouth in batches of 60 dozen. The digestive gland was dissected from each limpet within 24 hr of the animals being removed from the water and stored as a 20% homogenate in distilled water at -15° until required.

2.2. Purification

The frozen homogenate was thawed at room temp., filtered through gauze and centrifuged at 30,000 *g* max for 30 min. The supernatant was adjusted with rapid

stirring to pH 4.0 with 1 M citric acid. The solution was left standing for 36 hr and then centrifuged at 30,000 *g* max for 30 min. The supernatant fluid was then applied to a column (2.5 \times 30 cm) of Whatman CM-cellulose, type CM-52 (B.D.H. Ltd) equilibrated with 0.02 M citrate buffer, pH 4.0. The column was washed with 1 l of the equilibrating buffer and then eluted with stepwise increases in sodium chloride concentration in the equilibrating buffer. 10 ml fractions were collected at a flow rate of 40 ml/hr.

The active eluate obtained from the CM-52 column was dialysed against 0.02 M citrate buffer, pH 4.0, concentrated by ultrafiltration and applied to a Sephadex G-150 column, 2.5 \times 75 cm, equilibrated with 0.02 M citrate buffer, pH 4.0. The column was eluted with the same buffer and 5 ml fractions were collected at a flow rate of 15 ml/hr.

Fractions from the Sephadex G-150 column containing enzyme activity were pooled and concentrated by ultrafiltration. The concentrated enzyme solution was applied to a Sepharose 4B column 2.5 \times 80 cm, equilibrated with 0.02 M citrate buffer, pH 4.0. The column was eluted with the equilibrating buffer and 5 ml fractions were collected at a flow rate of 15 ml/hr.

Unless otherwise stated all the purification steps were carried out at 4° and buffers were prepared using cold distilled water.

2.3. Enzyme assays

α -*N*-Acetylglucosaminidase and β -*N*-acetylglucosaminidase activities were assayed using *p*-nitrophenyl- α -*N*-acetylglucosaminide and *p*-nitrophenyl- β -*N*-acetylglucosaminide, respectively. Both substrates were the products of Koch-Light Laboratories Ltd. The assay for both enzymes was carried out by adding 0.1 ml of enzyme to 0.9 ml of a solution containing 2.22 mM

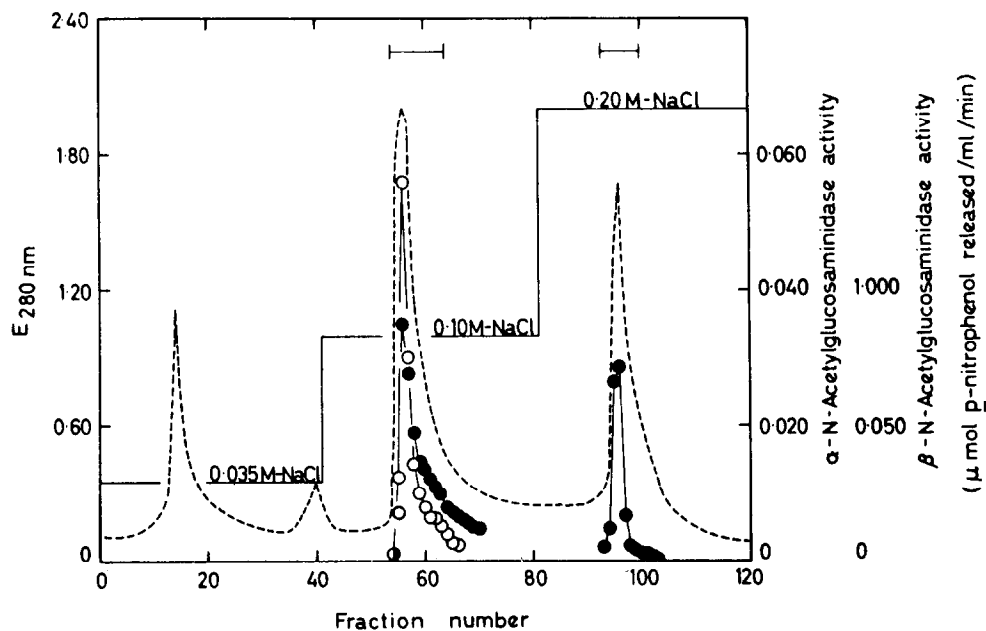


Fig. 1. Carboxymethyl-cellulose chromatography of limpet digestive gland *N*-acetylglucosaminidases. $A_{280\text{nm}}$ (---), α -*N*-acetylglucosaminidase activity (\circ — \circ — \circ), β -*N*-acetylglucosaminidase activity (\bullet — \bullet — \bullet).

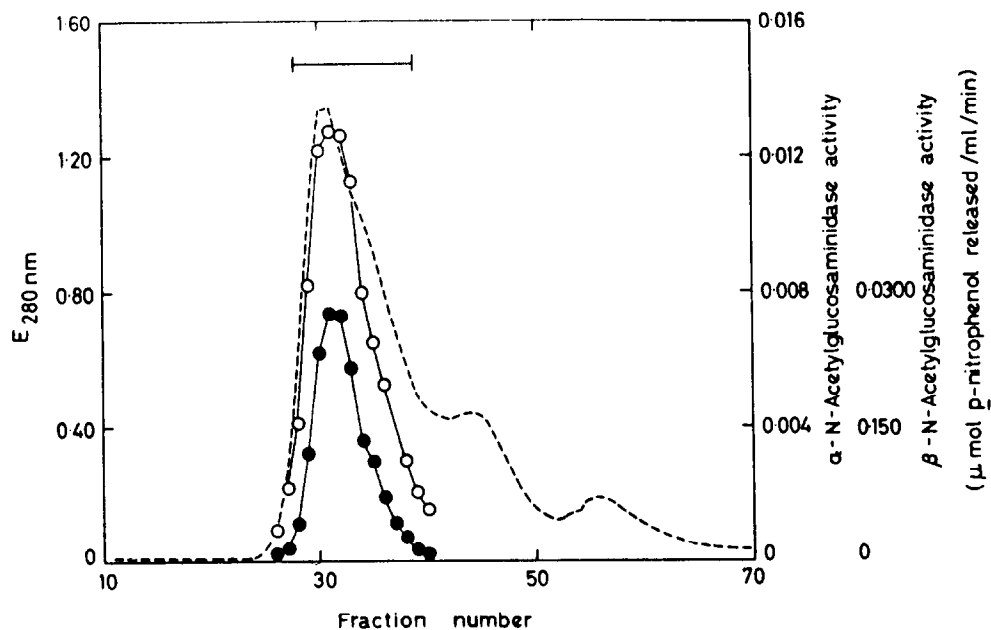


Fig. 2. Elution pattern of 0.1 M NaCl eluate on Sephadex G-150 chromatography. $A_{280\text{nm}}$ (---), α -*N*-acetylglucosaminidase activity (\circ — \circ — \circ), β -*N*-acetylglucosaminidase activity (\bullet — \bullet — \bullet).

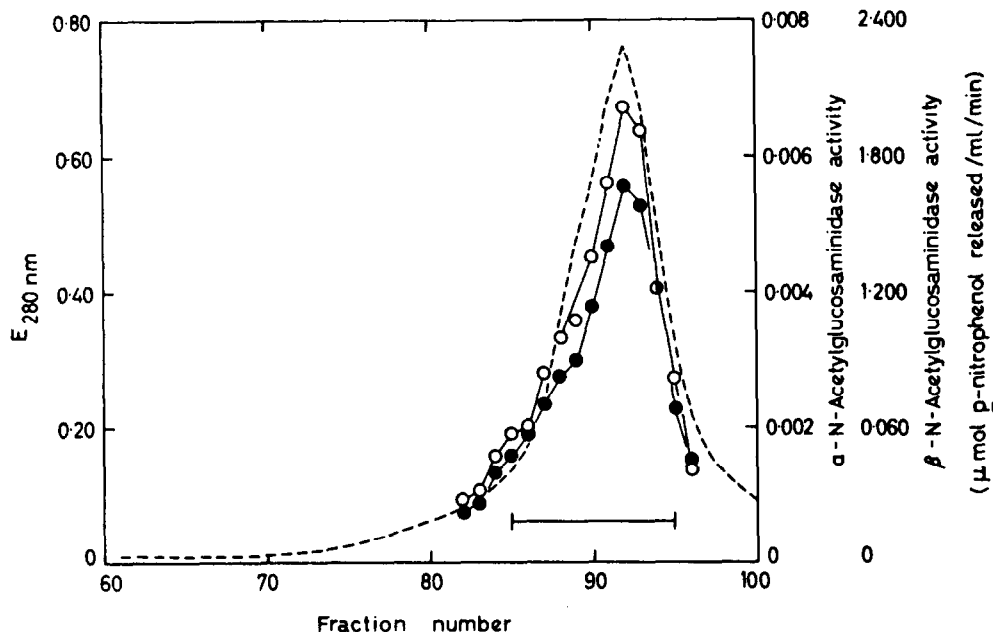


Fig. 3. Elution pattern of α , β -N-acetylglucosaminidase on Sepharose 4B. $A_{280\text{nm}}$ (---), α -N-acetylglucosaminidase activity (\circ — \circ — \circ), β -N-acetylglucosaminidase activity (\bullet — \bullet — \bullet).

of the corresponding substrate dissolved in 0.1 M citrate buffer, pH 4.0. The mixture was incubated at 37° for the estimation of α -N-acetylglucosaminidase activity and 3 min for the estimation of β -N-acetylglucosaminidase activity. Hydrolysis was linear with incubation time for both enzymes. The incubation was terminated by the addition of 2 ml of 0.25 M sodium carbonate [10] and the liberated *p*-nitrophenol was estimated at 400 nm using a Pye-Unicam SP 3000 spectrophotometer.

2.4. Analytical methods

Protein was estimated by the method of Lowry et al. [11] using crystalline bovine serum albumin as standard. Analytical gel electrophoresis was performed on gels of 4% strength obtained by diluting stock solution of 30% (w/v) recrystallised acrylamide and 1% (w/v) methylenebisacrylamide. Electrophoresis was carried out in 0.3 M β -alanine-acetic acid buffer, pH 4.0 [12] for 2 hr. The gels were stained with Coomassie Blue [13] and destained in 5% trichloroacetic acid. Enzyme activity was detected on gels run at 4° using the *p*-nitrophenyl substrates.

Isoelectric focussing was performed according to

Vesterberg and Svensson [14] in an LKB-8101 column of 110 ml capacity using 1% (w/v) solution of carrier ampholine (pI range 3–10). The pH gradient was stabilised with a linear sucrose gradient 0–50% (w/v).

Analytical ultracentrifugation was carried out on a Beckman Model E ultracentrifuge and the molecular weight was determined by the meniscus depletion method [15, 16].

3. Results and discussion

Limpet digestive gland contains two β -N-acetylglucosaminidases which can be separated on CM-cellulose chromatography at pH 4.0 (fig. 1). The β -N-acetylglucosaminidase eluted with 0.1 M sodium chloride also possesses α -N-acetylglucosaminidase activity and is referred to below as α , β -N-acetylglucosaminidase. The β -N-acetylglucosaminidase eluted with 0.2 M sodium chloride (fig. 1) was not further purified for the purpose of this investigation. The behaviour of the α , β -enzyme on column chromatography on Sephadex G-150 and Sepharose 4B is shown in figs. 2 and 3 and the yields and specific activities at various stages of the

Table 1
Purification of α , β -*N*-Acetylglucosaminidase from *P. vulgata* (L.).

Fraction	Protein (mg)	Total activity ¹ (α)	Total activity ¹ (β)	Specific activity ² (α)	Specific activity ² (β)	Yield (%) α - β	Ratio α - β
Acid treatment	314	4.9	14.2	0.016	0.45	100-100	29
Cm-cellulose	29	0.94	23.4	0.032	0.90	19-17	25
Sephadex G-150	7.6	0.35	8.6	0.045	1.26	7.2-6.0	24
Sepharose 4B	3.3	0.16	4.0	0.048	1.35	3.3-2.8	25

¹One unit of enzyme activity is defined as the number of μ moles of *p*-nitrophenol released per ml per min.

²Specific activity is units of activity per mg. of protein.

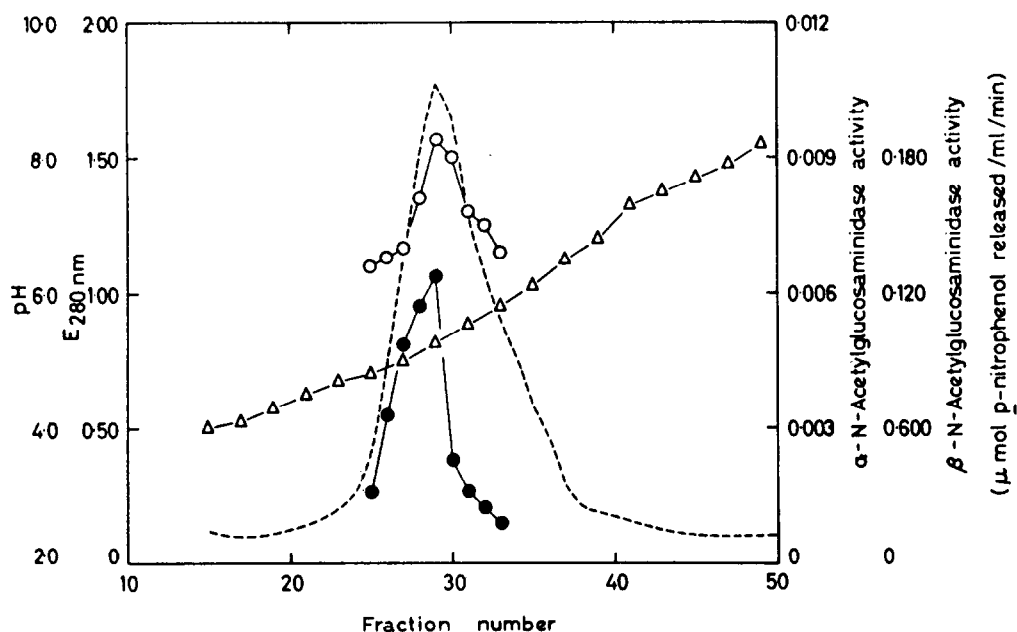


Fig. 4. Isoelectric focussing of α , β -*N*-acetylglucosaminidase. $A_{280\text{nm}}$ (---), α -*N*-acetylglucosaminidase activity (\circ — \circ — \circ), β -*N*-acetylglucosaminidase activity (\bullet — \bullet — \bullet), pH (\triangle — \triangle — \triangle).

purification are given in table 1. It can be seen that after separation from the β -enzyme, the ratio of hydrolysis of the substrates of the α , β -enzyme remains constant. However, in the different preparations of the enzyme the values of the ratio varied. This may be related to the time of year at which the limpets were collected.

Gel electrophoresis of the α , β -enzyme after Sepharose 4B chromatography gave a single band which

stained for both activities. Isoelectric focussing also revealed a single protein which possesses both activities (fig. 4) and it moved as a single component in sedimentation equilibrium experiments. A plot of the natural logarithm of the concentration (fringe displacement) against radial distance squared resulted in a straight line. This gave an average molecular weight of 217,000.

The results suggest that the purified α , β -*N*-acetyl-

glucosaminidase may be a single protein. Further studies are in progress to establish whether the enzyme possesses a single site capable of hydrolysing both substrates or whether it possesses active sites specific for each anomer. Isozymes of β -*N*-acetylglucosaminidase have been isolated [17, 18, 4, 5] and α -*N*-acetylglucosaminidase from pig liver [7] and *Turbo cornutus* liver [8] is apparently specific for α substrates. The data presented is the first evidence for an enzyme possessing both α - and β -*N*-acetylglucosaminidase activity.

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