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PURIFICATION AND CHARACTERISATION OF β-*N*-ACETYLHEXOSAMINIDASE FROM THE BUTTER CLAM, *Saxidomus purpuratus*

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ABSTRACT

A β-*N*-acetylhexosaminidase [EC 3.2.1.30] has been purified ~98-fold from an extract of the digestive organs of *Saxidomus purpuratus* by using ammonium sulfate fractionation, and chromatography on Toyopearl HW-50, CM-cellulose, and Sepharose 4B. The purified enzyme, the molecular weight of which was estimated to be ~66,000 by gel filtration, was composed of two sub-units of molecular weight 30,000 as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The purified enzyme had a pH optimum of 3.8 and an optimum temperature of 55°, and its activity was enhanced ~2-fold in the presence of 0.1M sodium chloride. The Michaelis constants toward *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucoside and -galactoside were 1.2×10^{-4} and 1.3×10^{-4}M, respectively.

INTRODUCTION

Glycosidases from many origins[1–7] have been purified and are useful tools in the study of the structure and function of the carbohydrate components of glycoproteins. Glycosidases from shellfishes have been widely investigated and some are commercially available, for example, β-*N*-acetylhexosaminidase[8,9], α-L-fucosidase[7], α-galactosidase, and α-mannosidase[11–13] from *Turbo cornutus*. We have described[14] the purification and characterisation of a lectin from *Saxidomus purpuratus* which has an affinity for GlcNAc and GalNAc. A β-*N*-acetylhexosaminidase is also present in this shellfish, and the lectin and enzyme appear to have an affinity for the same sugars.

We now describe the purification and characterisation of the β-*N*-acetylhexosaminidase [EC 3.2.1.30].

EXPERIMENTAL

Materials. — Shellfish, *Saxidomus purpuratus*, were purchased from a local fish-shop. Toyopearl HW-50 (for gel-permeation chromatography) was obtained from Toyo Soda Manufacture Co. (Japan).

Methods. — Enzyme activity was measured by a modification of the method

described by Borooah *et al.*[15], using the appropriate *p*-nitrophenyl glycosides. Incubation was carried out at 37° for 20 min in a medium containing substrate (mM), buffer (0.01M acetate, pH 4.5), and enzyme (0.1 mL) in a final volume of 0.5 mL. One unit of enzyme activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol/min under the assay conditions. Specific activity is expressed as units/mg of protein. The protein content was determined by the method of Lowry *et al.*[16], using bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis was performed at pH 9.0 using a 7.5% gel[17]. SDS (sodium dodecyl sulphate)-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn[18]; standards: bovine serum albumin (mol. wt. 67,000), hen-egg albumin (45,000), chymotrypsinogen A (25,000), and cytochrome C (12,500).

The molecular weight of the enzyme was determined by gel filtration on a column (1.0 × 80 cm) of Bio-Gel P-200, which was equilibrated with 0.15M NaCl–10mM Tris-HCl buffer (pH 7.4) and eluted at 2 mL/h at 20°. The molecular weight of the enzyme was estimated by comparison of its distribution coefficient (K_d) with those of the standards.

Purified enzyme was hydrolysed with 6M HCl at 110° for 24 h. The amino acid contents of the hydrolysate were determined with a Durrum D-500 amino acid analyzer.

Analytical isoelectric focusing was carried out, as described by Doerr and Chrambach[19], in 5% polyacrylamide gels containing 2% of Ampholine (pH 3–10, LKB Chemical). Protein samples were stained with Coomassie Brilliant Blue R (Sigma).

Purification of the enzyme. — The digestive organs (~250 g) from the shellfish were homogenised with 500 mL of saline for 5 min. After centrifugation at 10,000*g* for 40 min, the precipitate and the fatty substance which accumulated on the surface were discarded. Ammonium sulfate was added gradually to the supernatant solution, and the fraction which precipitated at 0.4–0.8 saturation was collected by centrifugation at 9,000*g* for 20 min. A solution of the precipitate in 10mM acetate buffer (pH 4.5) was dialysed against the same buffer overnight. The dialysate was centrifuged at 10,000*g* for 20 min, applied to a column (3.0 × 60 cm) of Toyopearl HW-50 previously equilibrated with 10mM acetate buffer (pH 4.5), and eluted with the same buffer. The active fractions were combined and applied to a column (3.8 × 48 cm) of CM-cellulose (CM-52, Whatman) previously equilibrated with 10mM acetate buffer and washed with the same buffer. Stepwise elution was performed with 0.15, 0.3, and 0.5M NaCl in 10mM acetate buffer (pH 4.5). The active fraction was concentrated by ultrafiltration (using an Amicon UM-20 membrane filter), applied to a column (2.2 × 80 cm) of Sepharose 4B previously equilibrated with 10mM acetate buffer containing 0.15M NaCl, and eluted with the same buffer. The fraction containing the purified enzyme was dialysed against water and lyophilised.

RESULTS

Purification of β-N-*acetylhexosaminidase from* Saxidomus purpuratus. — The enzyme was precipitated by ammonium sulfate at 0.4–0.8 saturation and subjected to chromatography on columns of Toyopearl HW-50, CM-cellulose, and Sepharose 4B (see Table I). As shown in Fig. 1, the activities of β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase were found in the same fractions for chromatography on Toyopearl HW-50, and the lectin was eluted later. Fig. 2 shows the elution pattern on CM-cellulose, and two peaks for glycosidase activity were found. Since the specific activity associated with the second peak was lower than that of the first, the protein in the first peak was purified. As shown in Fig. 3, β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities were found in the same fraction on chromatography of Sepharose 4B. Table II shows the relative activities of the glycosidases in the ammonium sulfate fraction and the purified enzyme. The purified enzyme showed mainly β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities. The purified enzyme was a homogeneous protein (Fig. 4).

Properties of purified β-N-*acetylhexosaminidase.* — The isoelectric point of the purified enzyme, estimated by isoelectric focusing, was 5.4, and the most notable

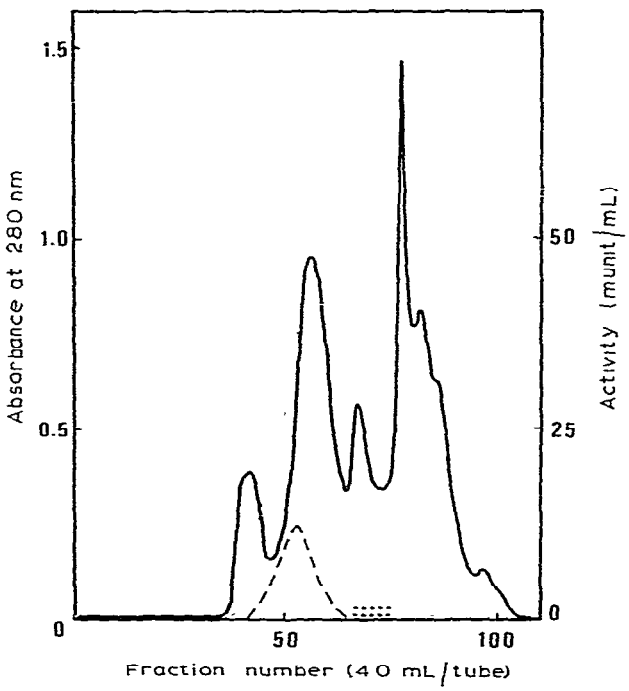


Fig. 1. Gel filtration of β-*N*-acetylhexosaminidase of Toyopearl HW-50: ———, protein concentration measured at 280 nm; — — — —, β-*N*-acetylglucosaminidase activity; —·—·—·—, β-*N*-acetylgalactosaminidase activity; : : : , hemagglutination activity for A erythrocytes.

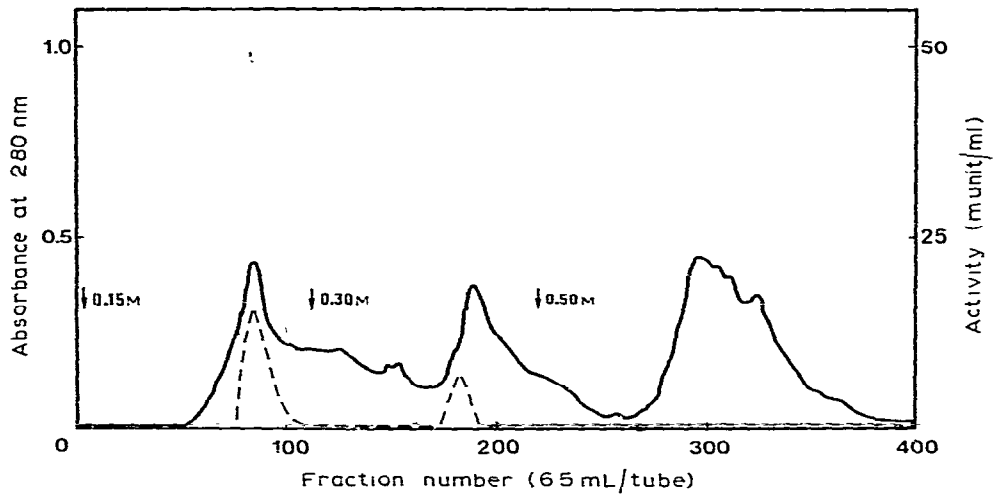
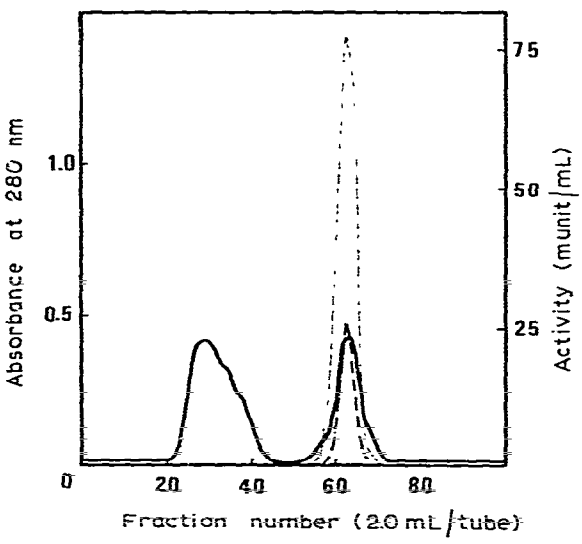


Fig. 2. Chromatography of β-*N*-acetylhexosaminidase on CM-cellulose. Arrows indicate start of elution each for concentration of NaCl; see Fig. 1, for key.



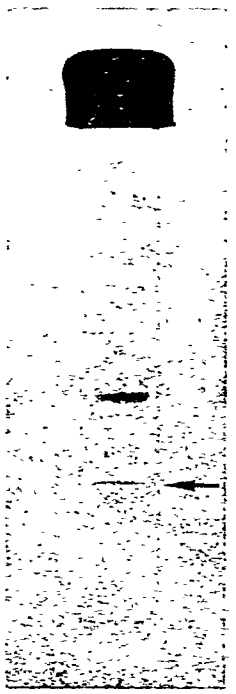


Fig. 3. Gel filtration of β-*N*-acetylhexosaminidase on Sepharose 4B; see Fig. 1, for key.

Fig. 4. Polyacrylamide gel electrophoresis of purified β-*N*-acetylhexosaminidase; arrow indicates Bromophenol Blue.

TABLE I

PURIFICATION OF β-*N*-ACETYLHEXOSAMINIDASE FROM THE DIGESTIVE ORGANS OF *Saxidomus purpuratus*

	Total protein (mg)	*Specific activity (units/mg of protein)*	*Yield (%)*
Crude extract	3680	0.015	100
40–80% $(NH_4)_2SO_4$ fraction	1060	0.034	66
Toyopearl HW-50	251	0.068	31
CM-Cellulose	38	0.183	13
Sepharose 4B	3.0	1.47	8.7

TABLE II

RELATIVE REMAINING ACTIVITIES OF OTHER GLYCOSIDASES IN THE AMMONIUM SULFATE FRACTION AND THE PURIFIED β-*N*-ACETYLHEXOSAMINIDASE

	Relative activity (%)	
	Ammonium sulfate fraction	*Purified enzyme*
β-*N*-Acetylglucosaminidase	100	100
β-*N*-Acetylgalactosaminidase	35	33
α-*N*-Acetylglucosaminidase	4.2	N.d.[a]
α-Glucosidase	10	N.d.
β-Glucosidase	270	N.d.
α-Galactosidase	17	3.2
β-Galactosidase	38	1.1
α-L-Fucosidase	20	N.d.
α-Mannosidase	154	N.d.

[a]N.d., not detected

TABLE III

AMINO ACID COMPOSITION OF THE β-*N*-ACETYLHEXOSAMINIDASE FROM THE DIGESTIVE ORGANS OF *Saxidomus purpuratus*

	Amino acid residues (residue/molecule[a])		*Amino acid residues (residue/molecule[a])*
Aspartic acid	51.4	Isoleucine	18.9
Threonine	31.3	Leucine	26.4
Serine	89.6	Tyrosine	13.6
Glutamic acid	84.6	Phenylalanine	15.0
Proline	26.2	Histidine	12.7
Glycine	107	Lysine	19.9
Alanine	46.1	Arginine	13.4
Valine	91.0		
Methionine	1.1	Total	648.2

[a]The molecular weight of the β-*N*-acetylhexosaminidase was assumed to be 65,000.

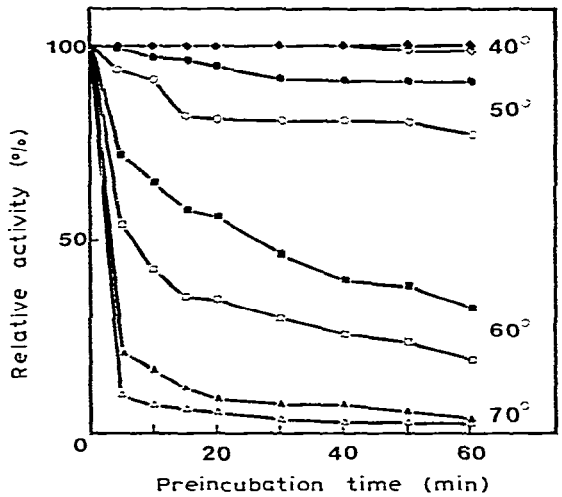


Fig. 5. Heat stability of the enzyme. Solid symbols indicate the presence of 5mM $CaCl_2$ in the incubation mixture, and the open symbols indicate its absence.

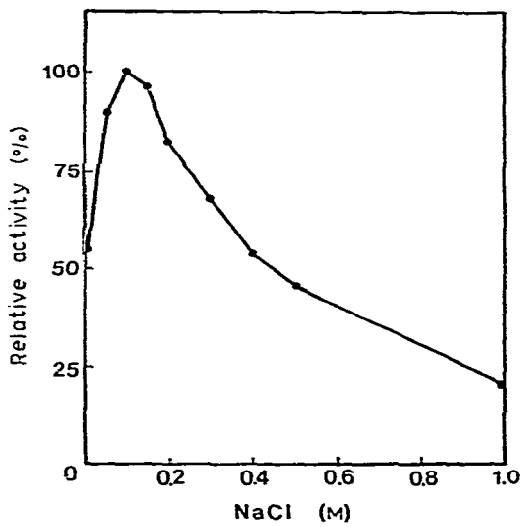


Fig. 6. Effect of sodium chloride on the activity of β-*N*-acetylhexosaminidase against 10mM *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside.

feature of the amino acid composition was the high content of glycine, valine, serine, and glutamic acid (Table III).

The molecular weight was estimated to be 66,000 by gel filtration on Bio-Gel P-200, and 64,000 by polyacrylamide gel electrophoresis from the slope–molecular weight relationship for standard proteins. When the β-*N*-acetylhexosaminidase was treated with 1% of 2-mercaptoethanol and subjected to SDS-polyacrylamide gel

electrophoresis, a single protein band having molecular weight 30,000 was obtained, indicating two sub-units.

The optimal temperature of hydrolysis of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside by the purified enzyme was 55° when incubated for 20 min in 10mM acetate buffer (pH 3.8). The thermostability data shown in Fig. 5 indicated that the purified enzyme was stable at 40° or 50°, but not at 60° or 70°.

The optimum pH for the hydrolysis of *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside and -galactopyranoside by the purified enzyme in 50mM acetate buffer was ~3.8. When 0.1M sodium chloride was added to an incubate containing *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside in 10mM acetate buffer (pH 3.8) at 55°, the activity of β-*N*-acetylglucosaminidase was increased 2-fold (Fig. 6).

The K_m values of the purified enzyme were estimated to be 1.2×10^{-4}M for *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, and 1.3×10^{-4}M for the *galacto* isomer, by using Lineweaver–Burk plots.

DISCUSSION

Many glycosidases have been found in mammalian and invertebrate organs, and purified enzymes from some shellfishes are widely available for the study of carbohydrate moieties of glycoproteins and cell surfaces[7–13]. We have purified an enzyme from the shellfish *Saxidomus purpuratus*, and shown it to have a molecular weight of 66,000 by analytical gel-filtration and both β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities. The homogeneous protein was shown to contain two sub-units of 30,000 molecular weight by SDS-polyacrylamide gel electrophoresis. Thus, the purified enzyme was a β-*N*-acetylhexosaminidase.

We have also shown[14] that the molecular weight of a lectin purified from the same shellfish was 40,000. The isoelectric point of the enzyme was 5.4 and that of the lectin was 4.1–4.5. These data indicate that the enzyme and the lectin are not identical.

The pH optimum of β-*N*-acetylhexosaminidase from *Saxidomus purpuratus* was 3.8, whereas those of the enzyme isolated from other shellfishes are between 3.8 and 5.2. α-Mannosidase from *Charonia lampas*[12], α- and β-mannosidases[20], and α-*N*-acetylhexosaminidase[8] from *Turbo cornutus* are activated by sodium chloride, and the β-*N*-acetylhexosaminidase from *Saxidomus purpuratus* also showed this property. The activation of the glycosidases of marine gastropods or pelecypoda by sodium chloride may be related to the high ionic strength of sea water.

The relative remaining activities of other glycosidases in the purified β-*N*-acetylhexosaminidase were below 3.2%.

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