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PURIFICATION AND PROPERTIES OF A β-*N*-ACETYLAMINOGLUCOHYDROLASE FROM MALTED BARLEY*

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Abstract—β-*N*-Acetylaminoglucohydrolase (β-2-acetylamino-2-deoxy-D-glucoside acetylaminodeoxyglucohydrolase, EC 3.2.1.30) was extracted from malted barley and purified. The partially purified preparation was free from α- and β-glucosidase, α- and β-galactosidase, α-mannosidase and β-mannosidase. This preparation was free from α-mannosidase only after affinity chromatography with *p*-amino-*N*-acetyl-β-D-glucosaminidine coupled to Sepharose. The enzyme was active between pH 3 and 6.5 and had a pH optimum at pH 5. A MW of 92000 was obtained by sodium dodecyl sulfate-acrylamide gel electrophoresis and a sedimentation coefficient of 4.65 was obtained from sedimentation velocity experiments. β-*N*-Acetylaminoglucohydrolase had a K_m of 2.5×10^{-4} M using the p-nitrophenyl *N*-acetyl β-D-glucosaminidine as the substrate.

INTRODUCTION

β-*N*-Acetylaminoglucohydrolase (AAG-hydrolase) has been purified from mammalian tissue [1–3] from plants [4] and from microorganisms [5–6]. Previously [7,8] we have reported the partial purification of α-mannosidase and β-mannosidase from malted barley. We now report the isolation and purification of AAG-hydrolase. Furthermore, the physical properties and kinetics of the hydrolysis of *p*-nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranoside (NPADG) by this enzyme are described.

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RESULTS AND DISCUSSION

A 200 fold purification of AAG-hydrolase was achieved by the 5-step procedure as outlined in Table 1 with the details in the Experimental section. The enzyme was stable in lyophilized form at each step in the purification scheme. Instability occurred only when the enzyme solution was stored at 4°. The elution from a Bio-Gel P-100 column (Step 3) and the elution from a carboxymethyl cellulose column (Step 4) gave a 100 fold purification. The use of DEAE cellulose chromatography yielded protein with lower sp act than the crude extract. Since we lost activity and could account for all of the protein that was applied to the DEAE cellulose column, it was decided to eliminate a DEAE step in the purification scheme.

When partially purified AAG-hydrolase was applied to the *p*-aminophenyl 2-acetamido-2-deoxy β-D-glucopyranoside affinity column (Step 5) in 50 mM acetate buffer

Table 1. Purification of β-*N*-acetylaminoglucohydrolase from crude extract of barley

Procedure	Volume (ml)	Concn (nkat/ml)	Total units nkat	Protein (mg/ml)	Specific activity nkat/kg ($\times 10^{-6}$)	Yield (%)	Purification (fold)
1. Crude extract	6000	1.60	9600	34	0.047	100	1.0
2. Ammonium sulfate ppt. (20–70%)	300	6.21	1860	34	0.182	19	3.8
3. Bio-Gel P-100	205	2.05	420	1.59	1.29	4	27.0
4. CM-52 Column	20	1.45	29	0.29	5.0	0.30	106
5. Affinity chromatography	3	0.64	1.92	0.045	14.2	0.01	241

pH 5, the majority of the protein was eluted, while enzyme activity was eluted at a higher ionic strength. α-Mannosidase activity was present in the partially purified enzyme through the carboxymethyl cellulose column and was eluted from the affinity column. Careful examination of the final preparation did not show any protease activity as tested by the use of Hide Powder Azure [9], nor did it show any other glucosidase activity.

Na dodecyl sulfate-polyacrylamide gel (SDS gel) electrophoresis of the protein obtained from the carboxymethyl cellulose column showed several bands; however, SDS gel electrophoresis of the material prepared from the affinity column showed a single band. Because some proteins exhibit anomalous behavior in SDS gel electrophoresis [10–12] we determined the MW in gels with various concentrations of acrylamide. An apparent MW of 92000 was obtained and there appears to be no anomalous behaviour of this protein in SDS gel electrophoresis. Sedimentation velocity experiments using the purified protein showed a single symmetrical peak and a sedimentation coefficient was calculated to be 4.64 sec. This also indicates that the 92000 MW estimation is not unrealistic.

The effect of pH on the activity of the enzyme was investigated by using NPADG as the substrate and acetate–citrate buffers of pH 3.6–6.5. The pH activity profile shows a pH optimum of 4.6 and half maximal activity pH values of 4 and 6. The effect of substrate concentration of the velocity of the enzyme reaction was studied with NPADG as the substrate. The enzyme followed simple Michaelis–Menten kinetics, and the apparent K_m value of 2.5×10^{-4} M was calculated.

The kinetics and properties thus far indicate that the enzyme from malted barley is similar to the enzyme from other sources [1–6]. Because of the wide availability of barley, it is expected that the isolation and purification of glycosidase enzymes from this source will have extensive application in the study of polysaccharides and glycoproteins.

EXPERIMENTAL

Malted barley was the gift of Kurth Malting Company, Milwaukee. All enzyme purifications were carried out at 0–5°.

Enzyme assay. *p*-Nitrophenylglycosides were purchased from Sigma Chemical, St. Louis. AAG-hydrolase was assayed by measuring *p*-nitrophenol described in ref. [7]. 10 mM NaOAc (pH 5.5), 1.5 μmol NPADG, and diluted enzyme in 2 ml are incubated for 15 min at 30° and the reaction terminated by adding 3 ml of 5% Na_2CO_3. The liberated phenol is determined at 420 nm. One unit of enzyme activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol/sec and sp act is expressed as nkat of enzyme per kg of protein. Protein was determined by the method of ref. [13] with BSA as standard.

Affinity chromatography. Derivatives for the affinity chromatography purification of AAG-hydrolase were prepared by coupling glucosidic substrates to the Sepharose 4β by means of an extension arm according to the procedures of ref. [14]. The *p*-aminophenyl 2-acetamido-2-deoxy β-D-glucopyranoside and *p*-amino α-mannoside derivative were prepared using the succinyl-diaminopropylamine extension of agarose. In typical expts, the Sepharose derivatives were incubated with enzyme in a small beaker with stirring. Suspensions were poured into Pasteur pipettes fitted with glass wool. The column was eluted with 5 ml additions of 5 mM NaOAc buffer (pH 5) with 0.05 M NaCl, 0.1 M NaCl, 0.12 M NaCl and 0.14 M NaCl, respectively and 0.1 ml fractions were collected. The elution pattern

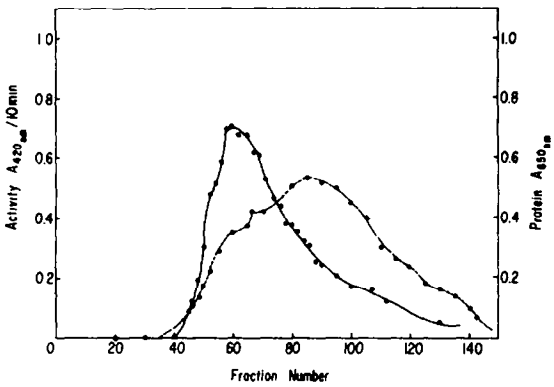

Fig. 1. Elution profile of β-*N*-acetylaminoglucohydrolase from malted barley on Bio-Gel P-100 column (5 × 60 cm) O——O, enzyme activity, ●——● protein measurement.

was obtained by monitoring the fractions for AAG-hydrolase activity.

Analytical methods. Polyacrylamide gel electrophoresis was conducted in the system described in ref. [15] and SDS gel electrophoresis MW determination as described in ref. [16]. Sedimentation velocity expts were conducted at 59700 rpm at 20 ± 0.1°. Enzyme solns were dialyzed against 50 mM NaOAc buffer, pH 5 with 0.1 M KCl.

Purification of enzyme. Step 1: *extraction.* Ground malted barley (1.44 kg) was suspended in 6 l. of 10 mM NaOAc buffer pH 5.5 and stirred for 6 hr at 4°. The suspension was centrifuged at 14000 g for 20 min and the residue discarded. Step 2: *$(NH_4)_2SO_4$ fractionation.* To the above supernatant, solid $(NH_4)_2SO_4$ (176 g/l.) was added with stirring over a period of 20 min, to yield 30% satn. The resulting mixture was left for 1 hr and then centrifuged at 14000 *g* for 20 min. The ppt. was discarded and the supernatant was again treated with $(NH_4)_2SO_4$ (273 g/l.) to 70% satn followed by centrifugation at 14000 *g* for 20 min. The supernatant was discarded and the pellet resuspended in 100 ml of 10 mM NaOAc pH 5.5. This material was dialyzed against the same buffer, lyophilized and stored at 0°. Step 3: *column chromatography on Bio-Gel P-100.* A 20-ml sample (50 mg/ml) was placed on a 0.5 × 60 cm Bio-Gel P-100 column that had been equilibrated with 10 mm NaOAc pH 5.5. Fractions (3 ml) were collected and tubes 50–85 (Fig. 1) were pooled for further AAG-hydrolase purification. Step 4: *column chromatography on carboxymethyl cellulose.* Pooled fractions from the Bio-Gel column were concentrated by a Diaflo membrane apparatus and a 2 ml aliquot (20 mg/ml) was subjected to chromatography on a column (0.5 × 45 cm) of carboxymethyl cellulose-CM-52. The column was equilibrated with 10 mM NaOAc pH 6 and fractions 14–18 were collected after elution with the equilibration buffer. These fractions were tested for α- and β-glucosidases, α- and β-mannosidases, α- and β-galactosidase and β-fucosidase activities. The fractions containing β-*N*-acetylaminoglucohydrolase activity were stored frozen.

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