Purification and Properties of β -N-Acetyl-D-glucosaminidase from Alimentary Canal of the Silkworm, *Bombyx mori*

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β -N-Acetyl-D-glucosaminidase (EC 3.2.1.30) was purified from the alimentary canal of the silkworm, *Bombyx mori*, by ammonium sulfate fractionation and chromatography with hydroxylapatite, DEAE Bio-Gel A, chromatofocusing, and Sephacryl S-200. The purified enzyme was a single band on disc-PAGE. The molecular weight was 119,000 by gel filtration and 125,000 by SDS-PAGE. The enzyme was separated into two peptides whose apparent molecular weights were 67,500 and 57,500 by SDS-PAGE. The pI was 4.86 by chromatofocusing. The optimum pH was 5.5 to 6.0 and the optimum temperature, 45°C, using pNp- β -GlcNAc as the substrate. The enzyme was stable from pH 5.5 to 8.5 and below 30°C. It was strongly inhibited by HgCl₂. Small N-acetylchitooligomers were as good substrates as pNp- β -GlcNAc, and the enzyme cleaved colloidal chitin to GlcNAc, even though the relative velocity was slow. Smaller N-acetylchitooligomers were preferred substrates with *K_m* 0.787 to 0.056 mM, *k_{cat}* 1013 to 52 sec⁻¹, and *k_{cat}/K_m* 1690 to 754 mm⁻¹ sec⁻¹. The enzyme precipitated in as band with moulting fluid chitinase antiserum, but not with haemolymph exo- β -N-acetylglucosaminidase antiserum. The results suggest that this enzyme is an exo-type enzyme which is involved in the degradation of chitin to GlcNAc.

Chitinolytic enzymes are widely distributed in nature. In plants the enzymes are produced for self defense against chitin-containing invaders^{1,2)} and also in some fungi for cell division and budding.^{3,4)} For degradation of chitin-containing foods, hunting spiders⁵⁾ and snails,⁶⁾ and some bacteria^{7,8)} secrete the enzyme into digestive juice and out of the cell, respectively.

Insect must shed the old cuticle on every moulting cycle for growing and developing. Endo- and exo-type chitinolytic enzymes have been isolated and purified from integuments and haemolymph at certain growing stages,

and it was proposed that both enzymes act synergistically for degradation of chitin to GlcNAc.^{9~13)} Chitinolytic enzymes are also found in the alimentary canal of the silkworm just before the beginning of spinning, and the pre- and postportions of the alimentary canal are chitinous tissue.^{14,15)} In connection with the development to the adult, the production of GlcNAc from discarded chitin would be physiologically important for the imaginal disc formation. The β -N-acetyl-D-glucosaminidase which was found in the alimentary canal at the larval-pupal transformation stage is expected to be involved in the degradation of chitin.

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Abbreviations: β -GlcNAc, β (1 $\rightarrow$ 4) linked oligosaccharide of GlcNAc; BPB, bromophenol blue; DEAE, diethylaminoethyl; DFP, diisopropylphosphorfluoridate; EDTA, ethylenediaminetetraacetic acid; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranoside; HPLC, high performance liquid chromatography; pNp- β -GalNAc, *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-galactopyranoside; pNp- β -GlcNAc, *p*-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside; PAGE, polyacrylamide gel electrophoresis; PAS, periodic acid-Schiff reagent; PTU, phenylthiourea; SDS, sodium dodecyl sulfate.

Furthermore, there are two types of β -*N*-acetyl-D-glucosaminidase which occur in the insect.^{9,11)} Both enzymes are similarly active on a pseudo-substrate, pNp- β -GlcNAc. However, there is a large difference in the activity toward *N*-acetylchitooligomers.

This study describes the purification of the enzyme from alimentary canal of *Bombyx mori* at the pupal stage. Also some of the physical, chemical, and kinetic properties of the purified β -*N*-acetyl-D-glucosaminidase were measured and compared with those of similar enzymes from other tissues of the silkworm and other insects.

MATERIALS AND METHODS

Materials.

Insect and tissue collection. The silkworms, *Bombyx mori* (N 124 $\times$ C 124, hybrid) were reared on mulberry leaves. Alimentary canals were collected from about 5-day old pupae by dissection and then lyophilized.

Chemicals. Hydroxylapatite and DEAE Bio-Gel A were obtained from Bio Rad Laboratories. Polybuffer Exchanger, Polybuffer-74, and Sephacryl S-200 (superfine) were obtained from Pharmacia Fine Chemicals. pNp- β -GlcNAc was purchased from Nakarai Chemicals. β -*N*-Acetylchitooligomers were prepared from crab chitin by the method of Raftery *et al.*¹⁶⁾ Colloidal chitin was prepared from shrimp chitin by the method of Boden *et al.*¹⁷⁾

Enzyme assay. β -*N*-Acetyl-D-glucosaminidase activity was measured by monitoring the release of *p*-nitrophenol from pNp- β -GlcNAc.⁹⁾ The enzyme (5 to 50 μ l) was added to 2 ml of 0.1 mM pNp- β -GlcNAc dissolved in 50 mM sodium phosphate buffer, pH 6.0, at 25°C and the changes in the difference of absorbance were monitored at 337 nm with a Hitachi Spectrophotometer 124. The reaction of pNp- β -GalNAc was followed in the same way as that of pNp- β -GlcNAc.

The enzymatic reaction was also analyzed by HPLC. Enzyme solution (10 or 25 μ l) was incubated with 0.1 or 2 ml of β -*N*-acetylchitooligomer dissolved in 50 mM sodium phosphate buffer, pH 6.0, at 25°C. The reaction mixture or a sample (200 μ l) was boiled for 3 min at an appropriate time to stop the enzymatic reaction and then was kept frozen until HPLC analysis. The sample (10 μ l) was injected into a Shimadzu PNH₂-10 column (0.4 $\times$ 25 cm) and eluted with 89% ethanol at 25°C, or into a Toyo Soda TSK Gel G2000PW column (0.75 $\times$ 60 cm) with a TSK PWL Guard column as a precolumn and eluted with 50 mM sodium phosphate buffer, pH 6.0. The β -*N*-acetylchitooligomers were monitored at 210 nm

with a Shimadzu spectrophotometric detector SPD-2A. Kinetic parameters were calculated by the non-linear optimization method of Koga *et al.*¹⁰⁾

Protein concentration. Protein was measured by the method of Lowry *et al.*¹⁸⁾ with bovine serum albumin as the standard.

Amino acid analysis. Purified enzyme was hydrolyzed in 6 N HCl at 110°C for 20 hr. The amino acid composition was found with an amino acid analyzer (Toyo Soda HLC-805). Tryptophan was measured spectrophotometrically by the method of Edelhoch.¹⁹⁾

Polyacrylamide gel electrophoresis. Disc-PAGE was done at 4°C by the method of Davis²⁰⁾ with 7% acrylamide in Tris-HCl buffer, pH 8.3. An enzyme-active band was stained by immersing the gel into 0.1 mM pNp- β -GlcNAc dissolved in 50 mM sodium phosphate buffer, pH 6.0. Protein and glycoprotein bands were stained with Coomassie Brilliant Blue R-250 and with PAS by the method of Zacharius *et al.*,²¹⁾ respectively. The bands stained with pNp- β -GlcNAc and Coomassie Brilliant Blue R-250 were measured with a Digital Densitrol DMO-33C densitometer (Toyo Kagaku Sangyo Co., Ltd.) at 440 and 660 nm, respectively.

Measurement of molecular weight.

i) *Gel filtration on Sephacryl S-200.* Gel filtration was done on Sephacryl S-200 (2 $\times$ 134 cm) with 50 mM sodium phosphate buffer, pH 6.0, containing 0.1 M NaCl.

ii) *SDS-PAGE.* SDS-PAGE was done by the method of Weber *et al.*²²⁾ with 10 and 12% acrylamide containing 0.1% SDS. Samples were boiled for 2 min or incubated for 12 hr with 1% SDS and 10% glycerol in the presence or absence of 1% 2-mercaptoethanol. Bromophenol blue was used as a front marker.

Immunological study. The antisera against exo- β -*N*-acetylglucosaminidase and chitinase purified from fifth-instar larval haemolymph and moulting fluid were obtained from male rabbits by the method of Kimura²³⁾ then lyophilized for storage. Just before use, the lyophilized antisera were reconstituted in 50 mM sodium phosphate buffer, pH 7.0, containing 0.85% NaCl. Ouchterlony double diffusion was done in 1% agarose gel in 50 mM sodium phosphate buffer, pH 7.0, containing 0.85% NaCl and 0.01% thimerosal. Antigen and antiserum were placed in wells and incubated overnight at room temperature. Precipitation bands were visible within 12 hr.

*Purification of β -*N*-acetyl-D-glucosaminidase.* All operations were done at 4°C except for the hydroxylapatite column. The buffers used were all degassed. The concentrations of NaCl and the buffer solutions were measured by their conductivity with a Conduct Meter CM-7B (Toa Denpa Kogyo Co., Ltd.).

Extraction and ammonium sulfate fractionation. The lyophilized alimentary canal (15 or 30 g) was reconstituted in 3 volumes of 20 mM sodium phosphate buffer, pH 7.8, containing 20% sucrose, 1 mM DFP, and 0.01 mM PTU, and homogenized with a Polytron homogenizer (Kinematica). The homogenate was centrifuged at $11,000 \times g$ for 30 min. The precipitate was homogenized and centrifuged again. The combined supernatants, the crude extract, were fractionated with ammonium sulfate. The fraction between 30 and 70% saturated ammonium sulfate was collected by centrifugation, dissolved in a small amount of 50 mM sodium phosphate buffer, pH 6.8, and then dialyzed against 5 mM sodium phosphate buffer, pH 6.8.

Hydroxylapatite column chromatography. The dialyzed solution was put on a hydroxylapatite column (2×20 cm) equilibrated with 10 mM sodium phosphate buffer, pH 6.8. The enzyme was eluted with a linear gradient of sodium phosphate buffer, pH 6.8, from 10 to 500 mM at a flow rate of 50 ml/hr. The active fraction eluting at 120 mM (10 mmho) was rechromatographed with a shallow gradient from 25 to 300 mM sodium phosphate buffer, pH 6.8.

DEAE Bio-Gel A column chromatography. The active fraction was concentrated to about 30 ml by ultrafiltration on a UK-10 membrane filter (Toyo Kagaku Sangyo Co., Ltd) and dialyzed against 20 mM sodium phosphate buffer, pH 6.0. The dialyzed solution was put on a DEAE Bio-Gel A column (2.5×25 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of NaCl from 0 to 0.3 M in the same buffer at a flow rate of 30 ml/hr.

Chromatofocusing. The active fraction obtained from DEAE Bio-Gel A was concentrated with a Collodion bag (Sartorius) and dialyzed against 25 mM imidazole-HCl buffer, pH 7.4. The dialyzed solution was put on a Polybuffer exchange column (1×20 cm). The enzyme was eluted with 8-fold diluted Polybuffer-74, pH 3.0, at a flow rate of 10 ml/hr.

Gel filtration. The active fraction was concentrated with a Collodion bag to about 2 ml and put on a Sephacryl S-200 column (1.8×150 cm) equilibrated with 50 mM sodium phosphate buffer, pH 6.0, containing 0.1 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 20 ml/hr.

RESULTS

Purification of β -N-acetyl-D-glucosaminidase

The enzyme was recovered in the 30 to 70% saturated ammonium sulfate fraction, and separated into two peaks on hydroxylapatite at the concentration of sodium phosphate buffer of 20 mM (5 mmho) and 120 mM (10 mmho) (Fig. 1). The enzyme eluting at 120 mM was chromatographed on DEAE Bio-Gel A and eluted at 80 mM NaCl (10 mmho) (Fig. 2). The elution pH was 4.86 during chromatofocusing (Fig. 3). A single symmetrical peak was obtained after gel filtration on Sephacryl S-200 (Fig. 4). The purification procedure is sum-

FIG. 1. Chromatography of *Bombyx mori* β -N-Acetyl-D-glucosaminidase on Hydroxylapatite.

See MATERIALS AND METHODS for details. The arrows indicate the starting positions of the gradient (G) and the washing with 0.5 M sodium phosphate buffer, pH 6.8 (W). The fraction indicated by the bar was used for the next purification.

FIG. 2. Anion Exchange Chromatography of β -N-Acetyl-D-glucosaminidase on DEAE Bio-Gel A. See MATERIALS AND METHODS. The arrows indicate the starting positions of the gradient (G) and the washing with the buffer containing 0.5 M NaCl (W). The fraction indicated by the bar was used for the next purification.

FIG. 3. Chromatofocusing of β -N-Acetyl-D-glucosaminidase.

See MATERIALS AND METHODS. The arrow indicates the starting position of the washing with 1 M NaCl. The fraction indicated by the bar was used for the next purification.

marized in Table I. The enzyme was purified about 64-fold with a recovery of 3.77%.

Homogeneity

The purified enzyme was confirmed to be homogeneous by disc-PAGE as shown in Fig.

5A. The protein band and the enzyme-active band were observed as single bands, respectively, and their R_f values agreed on the gel at 0.39. No PAS-stained band was observed.

FIG. 4. Gel Filtration of β -N-Acetyl-D-glucosaminidase.
See MATERIALS AND METHODS.

TABLE I. PURIFICATION OF β -N-ACETYL-D-GLUCOSAMINIDASE FROM *Bombyx mori*

Step	Total protein (mg)	Total activity (μ mol/min)	Specific activity (μ mol/min/mg)	Overall yield (%)	Overall purification
Crude extract	14270.4	13393.0	0.94	100	1
Ammonium sulfate	4479.9	5516.6	1.24	41.1	1.32
Hydroxylapatite (1)	996.7	3331.2	3.34	24.9	3.35
Hydroxylapatite (2)	419.1	3030.4	7.24	22.6	7.70
DEAE Bio-Gel A	94.1	2297.8	24.42	17.2	25.98
Chromatofocusing	13.1*	532.0	40.6	3.97	43.19
Sephacryl S-200	8.3	505.0	60.6	3.77	64.47

* Estimated from molar extinction coefficient of 96,000 at 280 nm.

Molecular weight

The molecular weight was estimated by gel filtration on Sephacryl S-200 and SDS-PAGE. The one peak which was obtained by gel filtration was estimated to be 119,000 (Fig. 6A). On the other hand, the purified enzyme which showed a single band on disc-PAGE separated into two bands on SDS-PAGE (Fig. 5B). The separation was observed either in the presence or absence of 2-mercaptoethanol. Those molecular weights were estimated to be 67,500 and 57,500 (Fig. 6B). A faint band on the front marker was sometimes observed on 10% gel. However, no such small peptide was obtained on 12% gel. Therefore, the enzyme

was thought to be a heterodimer with subunit molecular weights of 67,500 and 57,500.

Isoelectric point

The isoelectric point was 4.86 at 4°C by chromatofocusing (Fig. 3).

Amino acid composition

The amino acid composition is presented in Table II. The number of amino acids was calculated to be about 1078, using a molecular weight of 125,000. Since cysteine is partly destroyed under the conditions used, the number was assumed from the average percentage of amino acid composition in 108 proteins.²⁴⁾

A

B

FIG. 6. Measurement of Molecular Weight by Gel Filtration on Sephacryl S-200 (A) and SDS-PAGE (B). Standard proteins used: symbols, a to e, are the same as in Fig. 5-B; f, yeast alcohol dehydrogenase (MW 140,000); g, lipoxygenase (MW 102,000). The arrows indicate the enzyme.

TABLE II. AMINO ACID COMPOSITION OF β -N-ACETYL-D-GLUCOSAMINIDASE FROM *Bombyx mori*

Amino acid	No. of residues ^a per molecule (n = 3)	Mole percent (%)
Asx	109 $\pm$ 2.3	10.11
Thr	58 $\pm$ 2.0	5.38
Ser	68 $\pm$ 0.6	6.31
Glx	116 $\pm$ 2.9	10.76
Pro	44 $\pm$ 1.2	4.08
Gly	68 $\pm$ 1.1	6.31
Ala	72 $\pm$ 2.8	6.68
Val	56 $\pm$ 2.3	5.19
Met	18 $\pm$ 7.9	1.67
Ile	51 $\pm$ 6.4	4.73
Leu	94 $\pm$ 1.9	8.72
Tyr	49 $\pm$ 1.9	4.55
Phe	40 $\pm$ 1.6	3.71
His	22 $\pm$ 1.1	2.04
Lys	58 $\pm$ 1.4	5.38
Arg	69 $\pm$ 7.5	6.40
Trp ^b	49 $\pm$ 1.9	4.55
Cys ^c	37	3.43

^a Calculated based on the molecular weight of 125,000.

^b Measured by UV spectrophotometry.

^c Assumed from average percentage of amino acid composition in 108 proteins.²⁴⁾

Effects of pH on the enzyme activity and stability

The optimum pH for pNp- β -GlcNAc was 5.5 and 6.0 in 50 mM sodium phosphate buffer and Britton-Robinson buffer,²⁵⁾ respectively (Fig. 7). As shown in Fig. 8, the enzyme was stable in the range of pH 5.5 to 8.5.

Effects of temperature on the enzyme activity and stability

The enzyme had its maximum activity at 45°C in 50 mM sodium phosphate buffer, pH 6.0 (Fig. 9). As shown in Fig. 10, the enzyme

was unstable above 30°C after incubation for 10 min.

Effects of divalent cations on the enzyme activity

The effects of divalent cations are summarized in Table III. The enzyme was strongly inhibited by HgCl₂. CaCl₂ and MgSO₄ also caused partial inhibition. EDTA treatment increased the enzyme activity by approximately 11%. However, no stimulation by divalent cations was observed.

FIG. 5. Polyacrylamide Gel Electrophoresis of β -N-Acetyl-D-glucosaminidase.

A) Disc-PAGE. The purified enzyme (20 μ g) was put on a 7% gel. Protein (1) and the enzyme (2) were stained with Coomassie Brilliant Blue R-250 and pNp- β -GlcNAc, respectively.

B) SDS-PAGE. The purified enzyme (5 μ g) was treated with SDS and 2-mercaptoethanol at 100°C for 2 min. The protein was stained with Coomassie Brilliant Blue R-250. The standard proteins (2 μ g each) used: a, phosphorylase A (MW 92,000); b, BSA (MW 66,200); c, ovalbumin (MW 45,000); d, carbonic anhydrase (MW 31,000); e, soybean trypsin inhibitor (MW 21,500). Lane (1), standard proteins; lane (2), the purified enzyme.

FIG. 7. Effects of pH on β -N-Acetyl-D-glucosaminidase Activity.

The enzyme activity was assayed with pNp- β -GlcNAc at 25°C in 50 mM sodium phosphate buffer (O) and Britton-Robinson buffer (●) at the various pHs under the standard conditions described in MATERIALS AND METHODS. The final concentration of the enzyme was 4.28 nM.

FIG. 8. Effects of pH on *Bombyx mori* β -N-Acetyl-D-glucosaminidase Stability.

The enzyme (0.42 μ M) was incubated for 24 hr at 4°C in 50 mM sodium phosphate buffer (O) and Britton-Robinson buffer (●) of various pHs, and then the remaining activity was assayed with pNp- β -GlcNAc under the standard conditions described in MATERIALS AND METHODS. The final concentration of the enzyme was 2.1 nM.

FIG. 9. Effects of Temperature on *Bombyx mori* β -N-Acetyl-D-glucosaminidase Activity.

The enzyme was assayed with pNp- β -GlcNAc in 50 mM sodium phosphate buffer, pH 6.0, at various temperatures under the standard conditions described in MATERIALS AND METHODS. The final concentration of the enzyme was 2.3 nM.

Cleavage pattern

To find the cleavage pattern of a substrate, the degradation of β -GlcNAc₄ was followed. As shown in Fig. 11, the monomer and trimer initially increased together with the decrease in β -GlcNAc₄, and then the dimer increased with a time-lag. The monomer, GlcNAc, increased in a linear fashion over the course of the reaction. The results indicated an exo-type enzyme cleavage pattern.

Substrate specificity

Since inhibition was observed at high substrate concentrations (>0.2 mM), the kinetic parameters for β -N-acetyl-D-glucosaminidase were obtained at substrate concentrations from 0.05 to 0.2 mM. All substrates were cleaved in a typical exo-enzyme pattern, producing GlcNAc. The kinetic parameters are summarized in Table IV. The experiments were done using the different preparations of the purified enzyme. The values of *k*_{cat} and

FIG. 10. Effects of Temperature on *Bombyx mori* β -N-Acetyl-D-glucosaminidase.

The enzyme (0.85 μ M) was incubated in 50 mM sodium phosphate buffer, pH 6.0, at various temperatures for 10 min, and then the remaining activity was assayed with pNp- β -GlcNAc under the standard conditions described in MATERIALS AND METHODS. The final concentration of the enzyme was 4.28 nM.

K_m decreased moderately as the substrate chain length increased. The smallest K_m was obtained from β -GlcNAc₆. The result suggests that the binding site consists of several binding subunits, perhaps more than six. The pseudo-substrates pNp- β -GlcNAc and pNp- β -GalNAc were also good substrates. The enzyme cleaved colloidal chitin to release GlcNAc, even though the velocity was slow, about 1/500 that of β -GlcNAc₂. From the

TABLE III. EFFECTS OF DIVALENT CATIONS ON *Bombyx mori* β -N-ACETYL-D-GLUCOSAMINIDASE ACTIVITY

The enzyme was treated with 2.5 mM EDTA and 10 mM divalent cation. Then the enzyme was assayed with pNp- β -GlcNAc under the standard conditions described in MATERIALS AND METHODS. The final concentrations of the enzyme, EDTA, and divalent cation in the enzyme reaction were 4.28 nM, 7.5 μ M, and 30 μ M, respectively.

Compound	Relative activity (%)
None	100
EDTA alone	111.0 $\pm$ 10.0
+ HgCl ₂	7.1 $\pm$ 7.1
+ CaCl ₂	42.9 $\pm$ 7.8
+ MgSO ₄	71.4 $\pm$ 0.5
+ ZnCl ₂	88.6 $\pm$ 2.4
+ CuSO ₄	104.3 $\pm$ 4.3

FIG. 11. Course of Degradation of β -GlcNAc₄ by *Bombyx mori* β -N-Acetyl-D-glucosaminidase.

The enzyme (46.69 nM) was incubated with 1.53 mM β -GlcNAc₄ in 50 mM sodium phosphate buffer, pH 6.0, at 25°C. For the quantitative analysis, HPLC was done with a Shimadzu PNH₂-10 column as described in MATERIALS AND METHODS. Symbols: $\square$, $\blacksquare$, $\circ$, and $\bullet$ represent β -GlcNAc_n n = 1, 2, 3, and 4, respectively.

TABLE IV. KINETIC PARAMETERS FOR *Bombyx mori* β -N-ACETYL-D-GLUCOSAMINIDASE

The enzyme (1.4 to 4.2 nm) was incubated with the substrate (0.05 to 0.2 mM) in 50 mM sodium phosphate buffer, pH 6.0, at 25°C. The quantitative analyses were done by spectrophotometry and HPLC as described in MATERIALS AND METHODS.

Substrate	k_{cat} (sec^{-1})	K_m (mM)	k_{cat}/K_m ($\text{sec}^{-1} \text{mM}^{-1}$)	v_0^a ($\mu\text{mol}/\text{min}/\text{mg}$)
pNp- β -GalNAc	820.1	0.397	2065.6	
pNp- β -GlcNAc	1148.5 ± 169.0	0.433 ± 0.039	2639.0 ± 178.9	99.5 ± 3.1
β -GlcNAc ₂	1013.4 ± 376.4	0.787 ± 0.431	1460.5 ± 401.9	51.6 ± 4.1
β -GlcNAc ₃	209.4 ± 60.5	0.122 ± 0.016	1688.9 ± 327.0	40.6 ± 0.4
β -GlcNAc ₄	129.1 ± 75.0	0.118 ± 0.073	1114.5 ± 68.4	23.0 ± 2.2
β -GlcNAc ₅	75.6 ± 11.6	0.090 ± 0.028	753.8 ± 12.4	23.3 ± 0.5
β -GlcNAc ₆	51.9 ± 1.7	0.056 ± 0.009	946.8 ± 122.1	17.3 ± 6.1
Colloidal chitin				0.1

^a The final concentration of the substrate was 0.1 mM except colloidal chitin (5 mg/ml).

FIG. 12. Ouchterlony Double Diffusion Immunological Analysis.

The center wells (A and B) contained 10 μl of the purified enzyme (1.5 μg). The outer wells contained 10 μl of either *Bombyx mori* chitobiase-antiserum (A) or *exo*- β -N-acetylglucosaminidase-antiserum (B). The concentrations of the antisera: 1, 5-fold concentrated; 2, non-diluted; 3, 2-fold diluted; 4, 2.5-fold concentrated.

data of initial velocity and the reaction-turnover, k_{cat}/K_m , it is also suggested that the enzyme preferred the smaller substrates.

Immunological study

As shown in Fig. 12, the purified enzyme precipitated in a band with *Bombyx mori* chitobiase-antiserum, but not with *Bombyx mori* *exo*- β -N-acetylglucosaminidase-anti-

serum. The precipitation bands extended to the outer wells with the dilution of the antiserum. The result indicates that this enzyme is antigenically similar to the chitobiase purified from the moulting fluid of fifth-instar larval *Bombyx mori* by Kimura.²³⁾

Enzyme stability during storage

The enzyme was not stable even at 4°C. The activity did not change for the first 12 days and then rapidly decreased. After storage at -20°C for 27 days, the remaining activity was 87.1%. Lyophilization reduced the activity to 34.5%.

DISCUSSION

The purified enzyme was a single band on disc-PAGE and two bands on SDS-PAGE (Fig. 5). Two such bands were also observed without 2-mercaptoethanol treatment during SDS-PAGE. The results suggest that the enzyme is a heterodimer and consists of two subunits with MW 67,500 and 57,500, associating by bonds other than S-S linkages.

The enzyme was not stable even at 4°C. A rapid decrease in the activity was observed after 12 days. Lyophilization is also not a good method for storage.

As we can see from the cleavage pattern and substrate specificity (Fig. 11 and Table IV), the enzyme purified from the pupal alimentary

canal of *Bombyx mori* is a typical exo-type enzyme, very active toward N-acetylchitooligomers. Since a chitinase can be isolated from the same tissues (unpublished data and Kimura¹⁴⁾), we propose that the enzyme participates in the complete degradation of chitin to GlcNAc together with chitinase. That is, the exo-type enzyme degrades N-acetylchitooligomers which are produced from chitin by chitinase. A bienzyme system has been identified in *Manduca sexta*.^{10,12,13,26)}

Two types of β -N-acetyl-D-glucosaminidase (EC 3.2.1.30) are known. One type has been purified from larval integument, moulting fluid, and pupal haemolymph of several insects and is very active toward β -N-acetylchitooligomers as well as pNp- β -GlcNAc.^{9~11,14,23,27)} This type of enzyme is called chitobiase²³⁾ or exochitinase.¹⁰⁾ Another enzyme has been purified from larval and pupal haemolymph, and is not very active toward β -N-acetylchitooligomers.^{9~11,23,27,28)} Both types of the enzymes in *Bombyx mori*²³⁾ and *Manduca sexta*^{11,29)} seem to have immunologically different protein structures. As shown in Fig. 12, the enzyme purified from the pupal alimentary canal reveals a similar protein structure to moulting fluid chitobiase which exhibited no precipitation band with exo- β -N-acetylglucosaminidase-antiserum.²³⁾ The enzyme we studied has kinetic properties comparable to those of the chitobiase from the moulting fluid of *Bombyx mori* and the exochitinase from the pupal haemolymph of *Manduca sexta*. This enzyme can degrade even colloidal chitin to liberate GlcNAc, although the reactivity is low (Table IV). Such a low reactivity seems to come from the low hydration state of substrate, because chitin is insoluble in aqueous solutions. The reactivity of colloidal chitin was affected by the conditions of preparation (data not shown). Chitobiase (EC 3.2.1.29) has been deleted from the enzyme classification nomenclature and now is included with β -N-acetyl-D-glucosaminidase (EC 3.2.1.30). Therefore, a more appropriate enzyme name such as N-acetylchitooligomerase is now required to distin-

guish it from the other β -N-acetyl-D-glucosaminidases.

There are few reports which clarify the substrate specificity of β -N-acetyl-D-glucosaminidase. However, some enzymes have been reported to have similar substrate specificity and high molecular weight from microorganisms,^{30,31)} the snail,⁶⁾ the spider,⁵⁾ the fruit fly,³²⁾ and the moulting fluid of *Bombyx mori*.³³⁾ However, these enzymes are all single peptides. A low molecular weight enzyme has been purified from pupal haemolymph of *Manduca sexta*, having MW 61,000.⁹⁾ We isolated such a small enzyme from fifth-larval integument of *Bombyx mori* whose molecular weight is 67,500 daltons, the same as the larger subunit of the enzyme from the alimentary canal (unpublished data). It is physiologically interesting to investigate the relationship among the enzymes from the integument, moulting fluid, and alimentary canal. The peritrophic membrane is shed just before the beginning of spinning behavior and the titer of the enzyme we studied is maximum at the beginning of pupation.¹⁴⁾ Therefore, the enzyme of alimentary canal might be involved in degradation of discarded chitin in a synergistic action together with chitinase to liberate GlcNAc which could then be reused for development from pupae to adult, perhaps in developing imaginal discs.

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