Note

β -N-Acetyl-D-glucosaminidases
from Integument of the Silkworm,
Bombyx mori: Comparative
Biochemistry with the Pupal
Alimentary Canal Enzyme

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There are two types of β -N-acetyl-D-glucosaminidase in insects. One is an exo-type chitinolytic enzyme which is called chitobiase¹⁾ or exochitinase,²⁾ and is involved in insect ecdysis together with endo-type chitinolytic enzyme.³⁾ The other may serve for carbohydrate metabolism other than chitin degradation.^{1,4)} The former is different from the latter in substrate specificity and immunological determinants.^{1,4,5)} The exo-type chitinolytic enzyme (β -N-acetyl-D-glucosaminidase) has been purified from the pupal alimentary canal of the silkworm.⁶⁾ The enzyme is a heterodimer consisting of two subunits, A and B, having molecular weights of 67,500 and 57,500, respectively. In this paper, we investigated the existence of the exo-type chitinolytic enzyme in the larval integument which should be degraded during ecdysis, and compared the integumental enzymes with the pupal alimentary canal enzyme.

The silkworms, *Bombyx mori* (Kohaku $\times$ Doci), were reared on mulberry leaves. Integuments were collected from the fifth-instar larvae at the SP3 stage (one day before the ecdysis from larvae to pupae) after dissection, rinsed twice with 50 mM sodium phosphate buffer, pH 7.8, containing 20% sucrose, 1 mM diisopropylphosphofluoridate and 0.01 mM phenylthiourea in an ice bath, and kept frozen until the use. 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, Co. pNp- β -GlcNAc and pNp- β -GalNAc were purchased from Nakarai Chemicals, Ltd. N-Acetylchitooligosaccharides

were prepared from crab chitin by the method of Raftery *et al.*⁷⁾ β -N-acetyl-D-glucosaminidase activity was measured in 50 mM sodium phosphate buffer, pH 6.0, at 25°C using pNp- β -GlcNAc and N-acetylchitooligosaccharides (β -GlcNAc_n, n = 2 to 6) as the substrate as described in our previous study.⁶⁾ Kinetic analyses of the enzymatic reactions with the former and latter substrates were done spectrophotometrically at 337 nm and by HPLC with a Toyo Soda TSK G2000 PW apparatus (0.75 $\times$ 60 cm) with a TSK PWL Guard column as a first column, respectively.⁶⁾ The reaction of pNp- β -GalNAc was done in the same way as that of pNp- β -GlcNAc. Kinetic parameters were calculated by the non-linear optimization method of Koga *et al.*⁴⁾ Protein was measured by the method of Lowry *et al.*⁸⁾ with bovine serum albumin as the standard. Disc-PAGE was done at 4°C the method of Davis⁹⁾ with 7% acrylamide gel at pH 9.5, and the method of Williams and Reisfeld¹⁰⁾ with 7% acrylamide gel at pH 8.0. SDS-PAGE was done by the method of Weber *et al.*¹¹⁾ with 10% acrylamide gels containing 0.1% SDS. Samples were first boiled for 2 min or incubated for 12 hr with 1% SDS and 10% glycerol with or without 1% 2-mercaptoethanol. Protein bands were stained with Coomassie brilliant blue R-250. The pH optimum and stability, temperature optimum and stability, and inhibition by divalent cations were measured as described in our previous study.⁶⁾ The antiserum against the pupal alimentary canal β -N-acetyl-D-glucosaminidase was obtained from male Japanese white rabbits by the method of Koga *et al.*¹²⁾ The antisera against the fifth-instar larval haemolymph exo- β -N-acetylglucosaminidase and the moulting fluid chitobiase were obtained by the method of Kimura.¹⁾ 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.

The enzymes were purified by the method of Koga *et al.*⁶⁾ from 311 insects by ammonium sulfate fractionation and the column chromatographies. The enzyme was eluted as two broad peaks (tentatively named Ex1 and Ex2) on a hydroxylapatite column at the concentration of sodium phosphate buffer of 120 and 180 mM, respectively. Each enzyme was again chromatographed with hydroxylapatite, followed by DEAE Bio-Gel A, chromatofocusing, and Sephacryl S-200. The enzymes, Ex1 and Ex2, were purified about 3,000-fold with a recovery of 5.2%, and 3,200-fold with that of 7.7%, respectively. Their purities were confirmed by SDS-PAGE for Ex1 and disc-PAGE for Ex2.

The properties of Ex1, Ex2, and the pupal alimentary canal β -N-acetyl-D-glucosaminidase are summarized in Table I. Ex2 showed two bands on SDS-PAGE similarly to the alimentary canal enzyme. On the other hand, Ex1

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Abbreviations: β -GlcNAc_n, β (1-4) linked oligosaccharide of GlcNAc (N-acetylchitooligosaccharide); DEAE, diethylaminoethyl; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranoside; pNp- β -GalNAc, p-nitrophenyl 2-acetamido-2-deoxy- β -D-glactopyranoside; pNp- β -GlcNAc, p-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis.

TABLE I. PROPERTIES OF β -N-ACETYL-D-GLUCOSAMINIDASES FROM *Bombyx mori*

Property	Integumental enzyme		Alimentary canal ^a enzyme
	Ex1	Ex2	
Molecular weight			
SDS-PAGE	67,500	67,500 + 57,500	67,500 + 57,500
Sephacryl S-200	$> 1.2 \times 10^5$	1.2×10^5	1.2×10^5
pI	5.00	4.84	4.86
Optimum pH	5.0 ~ 6.0	5.0 ~ 6.0	5.0 ~ 6.0
pH stability	6.0 ~ 8.0	5.0 ~ 9.0	5.0 ~ 8.5
Optimum temperature	55°C	n.d.	45°C
Temperature stability	< 48°C 62°C (50%)	n.d.	< 40°C 58°C (50%)
Inhibitor	HgCl ₂	HgCl ₂	HgCl ₂
Reaction with antibody against			
alimentary canal enzyme	+	+	+
moulting fluid chitinase	+	+	+
larval haemolymph exo- β -N-acetylglucosaminidase	—	—	—
Estimated enzyme form ^b	A-A	A-B	A-B

^a From ref. 6.^b A, subunit A (MW 67,500); B, subunit B (MW 57,500).
n.d., not determined.TABLE II. KINETIC PARAMETERS FOR INTEGUMENTAL β -N-ACETYL-D-GLUCOSAMINIDASES OF *Bombyx mori*

Substrate	k_{cat} (sec ⁻¹)		K_m (mM)		k_{cat}/K_m (sec ⁻¹ mM ⁻¹)		V_0^* (μ mol/min/mg)	
	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2	Ex1	Ex2
pNp- β -GlcNAc	890 ± 210	720 ± 190	0.49 ± 0.15	0.42 ± 0.15	1810	1700	65.0 ± 1.2	71.4 ± 0.6
pNP- β -GalNAc	570	320 ± 30	0.64	0.31 ± 0.03	890	1050	53.9	44.2 ± 2.4
β -GlcNAc ₂	620 ± 270	580 ± 260	0.67 ± 0.29	0.64 ± 0.31	930	910	36.1 ± 0.7	39.1 ± 2.6
β -GlcNAc ₃	130 ± 25	82 ± 10	0.87 ± 0.04	0.05 ± 0.02	1450	1710	36.1 ± 3.6	26.0 ± 0.7
β -GlcNAc ₄	110 ± 11	42 ± 2	0.16 ± 0.03	0.03 ± 0.01	710	1400	19.4 ± 0.9	15.5 ± 0.2
β -GlcNAc ₅	57 ± 5	76 ± 12	0.07 ± 0.03	0.09 ± 0.03	860	830	14.8 ± 2.8	19.1 ± 0.2
β -GlcNAc ₆	55 ± 4	29 ± 2	0.10 ± 0.02	0.06 ± 0.01	530	500	11.0 ± 1.6	9.0 ± 9.0

* The final concentration of the substrate was 0.1 mM.

showed only one band, which corresponds to the large subunit of Ex2 and the pupal alimentary canal enzyme. The molecular weights of Ex1 and Ex2 were also estimated by gel-filtration of Sephacryl S-200. Ex2 was eluted at the almost same position as the alimentary canal enzyme. Ex1 was eluted earlier than Ex2. Since the molecular weight of

the alimentary canal enzyme was 125,000,⁶⁾ the molecular weights of Ex1 and Ex2 were estimated to be 135,000 and 125,000, respectively. Ex1 is a homodimer, and Ex2 is a heterodimer similarly to the alimentary canal enzyme. The other properties of Ex2 were similar to those of the pupal alimentary canal enzyme with respect to eluting position in

the column chromatography, pH optimum, pH stability, and pI value. Ex1 was a little more basic (pI 5.00) than Ex2 (pI 4.84) and the pupal alimentary canal enzyme (pI 4.86), and eluted earlier than the other enzymes in DEAE Bio-Gel A column chromatography. Although Ex1 was similar to the other enzymes in pH optimum, Ex1 was more stable at the high temperature, but less stable to pH than the other enzymes. All of these enzymes were inhibited by HgCl_2 . The antigenicities of Ex1 and Ex2 were investigated using the antisera raised against the pupal alimentary canal enzyme, the moulting fluid chitinase, and the fifth-instar larval haemolymph exo- β -N-acetylglucosaminidase. Both Ex1 and Ex2 showed a single precipitation band with the pupal alimentary canal enzyme-antiserum, and with the chitinase-antiserum, but not with the exo- β -N-acetylglucosaminidase-antiserum. The results are also summarized in Table I. From the results, it is suggested that both the subunit of Ex1 and the large subunit of Ex2 are the same as the large subunit of the alimentary canal enzyme. Ex1 is A-A type. Both Ex2 and the alimentary canal enzyme are A-B type. All of these enzymes are immunologically similar to the moulting fluid enzyme, chitinase, but not the larval haemolymph enzyme. The molecular weight of the chitinase was estimated to be about 150,000 by Kimura.¹³⁾ The chitinase from moulting fluid might be the same as the integumental enzyme, Ex1.

To investigate the type of enzyme reaction, kinetic analysis was done. Substrate inhibition was also observed at high substrate concentrations, above 0.2 mM, as previously reported in the pupal alimentary canal enzyme.⁶⁾ Kinetic parameters were obtained at substrate concentrations from 0.05 mM to 0.2 mM, and summarized in Table II. All substrates were cleaved in a typical exo-enzyme pattern by both Ex1 and Ex2. Although individual values are different, the substrate specificities of Ex1 and Ex2 are similar. They are also similar to that of the alimentary canal enzyme.⁶⁾ Therefore, it is suggested that the integumental enzymes, Ex1 and Ex2, are exo-type chitino-

lytic enzymes, and involved in insect ecdysis.

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REFERENCES

- 1) S. Kimura, *Insect Biochem.*, **7**, 237 (1977).
- 2) C. Dziadik-Turner, D. Koga, M. S. Mai and K. J. Kramer, *Arch. Biochem. Biophys.*, **212**, 546 (1981).
- 3) K. J. Kramer and D. Koga, *Insect Biochem.*, **16**, 851 (1986).
- 4) D. Koga, M. S. Mai, C. Dziadik-Turner and K. J. Kramer, *Insect Biochem.*, **12**, 493 (1982).
- 5) D. Koga, M. S. Mai and K. J. Kramer, *Comp. Biochem. Physiol.*, **74B**, 515 (1983).
- 6) D. Koga, M. Nakashima, T. Matsukura, S. Kimura and A. Ide, *Agric. Biol. Chem.*, **50**, 2357 (1986).
- 7) M. A. Raftery, T. Rand-Meir, F. W. Dahlquist, S. M. Parsons, C. L. Borders, R. G. Wolcott, W. Beranek and L. Jao, *Anal. Biochem.*, **30**, 427 (1969).
- 8) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
- 9) B. J. Davis, *Ann. N. Y. Acad. Sci.*, **121**, 404 (1964).
- 10) D. E. Williams and R. A. Reisfeld, *Ann. N. Y. Acad. Sci.*, **121**, 373 (1964).
- 11) K. Weber, J. R. Pringle and M. Osborn, "Methods in Enzymology," Vol 26, ed. by C. H. Hirs and S. N. Timascheff, Academic Press Inc., New York, 1972, p. 3.
- 12) D. Koga, J. Jilka, R. D. Speirs and K. J. Kramer, *Insect Biochem.*, **13**, 407 (1983).
- 13) S. Kimura, *Comp. Biochem. Physiol.*, **49B**, 345 (1974).