

COMPARATIVE BIOCHEMISTRY OF INSECT EXO- β -N-ACETYLGLUCOSAMINIDASES: CHARACTERIZATION OF A THIRD ENZYME FROM PUPAL HEMOLYMPH OF THE TOBACCO HORNWORM, *MANDUCA SEXTA* L.*

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Abstract—1. A third exo- β -N-acetylglucosaminidase (III) was purified from pupal hemolymph of the tobacco hornworm, *Manduca sexta* by ammonium sulfate fractionation, anion-exchange chromatography, hydroxylapatite chromatography and gel filtration.

2. Two forms of the enzyme were separated by polyacrylamide gel electrophoresis, one composed of a single polypeptide chain with $MW_{app} = 6.1 \times 10^4$ and the other composed of two chains with $MW_{app} = 5.3 \times 10^4$ and 1×10^4 .

3. The K_m and k_{cat} values for *pNp* β GlcNAc were 265 μ M and 374 sec^{-1} , respectively.

4. The pH rate profile suggested that two groups with $pK_a = 8.1$ and 3.7 participate in catalysis.

5. N-Acetylglucosamine and 1-O-methyl derivatives inhibited III in a competitive fashion with K_i values = 3–10 mM.

6. III was related immunologically to exo- β -N-acetylglucosaminidases I and II which are also present in pupal hemolymph.

7. Only I was detected in pharate pupal molting fluid and integument.

INTRODUCTION

In preceding papers (Dziadik-Turner *et al.*, 1981; Koga *et al.*, 1982), we showed that two exo- β -N-acetylglucosaminidases are present in the tobacco hornworm, *Manduca sexta* L. One of these (EI), found in larval and pharate pupal molting fluid, integument and pupal hemolymph, probably catabolizes chitin *in vivo*. The other (EII) is present only in hemolymph, both larval and pupal, and probably hydrolyzes N-acetylglucosamine containing substrates other than chitin. When the purification procedure for these enzymes was scaled-up, a third enzyme (EIII) was also detected in pupal hemolymph. We report here a comparison of the biochemical properties of EIII with those of glucosaminidases I and II.

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MATERIALS AND METHODS

Insects and hemolymph collection

Eggs of *M. sexta* were a gift from the Metabolism and Radiation Laboratory, Agricultural Research Service, US Department of Agriculture, Fargo, North Dakota. Larvae were reared on a standard diet (Bell & Joachim, 1976) and kept at 28°C and 60% relative humidity during a 16 hr light–8 hr dark photoperiod. Hemolymph from 2 day old pupae was collected by cutting off the proboscis and expressing the fluid into a beaker chilled in dry ice. The samples were lyophilized and stored at –20°C.

Enzyme purification, electrophoresis and assay

The enzymes were purified by a series of chromatographic steps as described by Dziadik-Turner *et al.* (1981). Homogeneity was determined by polyacrylamide gel electrophoresis under nondenaturing conditions at pH 8.5 according to Davis (1964) and under denaturing conditions according to Weber *et al.* (1972). The enzyme was assayed for *pNp* β GlcNAc hydrolytic activity by monitoring *pNp* production at 337 nm and for β GlcNAc oligosaccharide hydrolytic activity by high performance liquid chromatography (Koga *et al.*, 1982).

Hydrolysis of proteins and amino acid analysis

Purified enzymes were hydrolyzed in 6 N HCl containing 0.1% phenol according to Moore & Stein (1963). The amino acid composition was determined by the method of Hill *et al.* (1979) where amino acids were separated as o-phthaldialdehyde derivatives by high performance liquid chromatography on a Waters reverse phase C18 column.

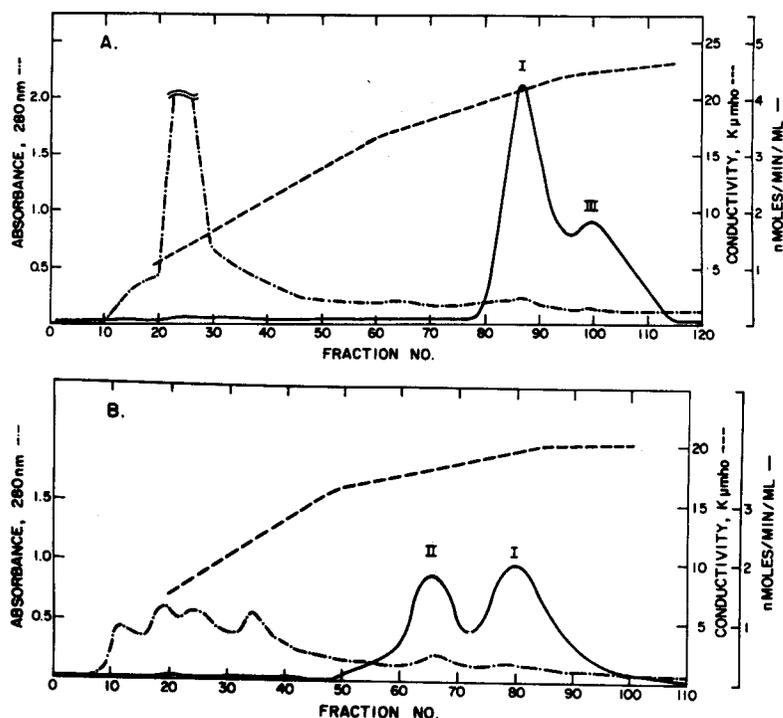


Fig. 1. Hydroxylapatite chromatography of $pNp\beta\text{GlcNAc}$ hydrolytic fractions from DEAE chromatography. A. Fraction eluting from DEAE chromatography at 0.14 M NaCl. B. Fraction eluting at 0.2 M NaCl. Stock solutions were applied to a hydroxylapatite column (3×22 cm) equilibrated with 5 mM sodium phosphate, pH 7.5 at room temperature. Protein was eluted with a linear sodium phosphate gradient. Enzyme activity was assayed by monitoring the continuous production of pNp from $pNp\beta\text{GlcNAc}$ at 337 nm. Symbols: absorbance at 280 nm (---); conductivity (---); $pNp\beta\text{GlcNAc}$ hydrolytic activity (—).

Carbohydrate analysis

Neutral and amino monosaccharides were quantitated after acid hydrolysis (2 N HCl at 100°C for 2 hr *in vacuo*) on an anion-exchange column in 0.5 M borate, pH 8.6 as tritium-labelled alditols after reduction with NaB^3H_4 (Barr & Nordin, 1980). Retention times were compared with those of standard alditols. Neutral sugars were also assayed by using the anthrone reagent (Roe, 1955).

Preparation of antisera

Antisera were prepared by inoculating 2.3 kg New Zealand white albino rabbits intramuscularly in four sites with a total of 0.5 mg enzyme suspended in complete Freund's adjuvant. After 4 weeks the rabbits were reinoculated with another 0.5 mg dose subcutaneously in incomplete Freund's adjuvant. The animals were bled at the end of 1 week and each subsequent week thereafter. Serum was prepared as described previously (Campbell *et al.*, 1970).

Ouchterlony assay

Ouchterlony gels were prepared in 1% agarose, 50 mM sodium phosphate, pH 7.0 containing 0.85% sodium chloride and 0.01% thimerosal. Antigen and antiserum were placed in wells and allowed to incubate overnight at room temperature. Precipitation bands were visible within 12 hr.

RESULTS

Purification of *exo*- β -N-acetylglucosaminidase III from pupal hemolymph

The purification steps for III were essentially the same as those reported previously for two other β -N-

acetylglucosaminidases except that an ammonium sulfate fractionation step and additional hydroxylapatite chromatographic steps were added in order to scale-up the procedure (Dziadik-Turner *et al.*, 1981). As expected the anion-exchange DEAE chromatography distinguished two peaks of $pNp\beta\text{GlcNAc}$ hydrolytic activity with the first and second eluting at 0.14 M (EI) and 0.2 M (EII) salt concentrations respectively at pH 8.5 (data not shown). When the first peak was fractionated further by hydroxylapatite chromatography, EI and a new enzyme species (EIII) which had not been detected before were observed (Fig. 1A). The second DEAE peak was separated into EI and EII by hydroxylapatite chromatography (Fig. 1B). EI, EII and EIII were eluted at concentrations of 0.16, 0.15 and 0.19 M sodium phosphate, respectively, from hydroxylapatite. The hydroxylapatite chromatography was repeated with individual enzyme pools to ensure isolation from each other. The final purifications were approx 350, 130 and 390-fold with overall yields of 8, 2 and 2% (Table 1). The specific activities of EI and EIII were about three times larger than that of EII, indicating that the former two enzymes are more active catalytically and possibly related. When prepharate pupal molting fluid and integument were examined by chromatographic methods for the presence of β -glucosaminidase, only enzyme I was detected.

Homogeneity and molecular weight

Gel filtration of *exo*- β -N-acetylglucosaminidase III

Table 1. Purification of three *exo-β-N*-acetylglucosaminidases from *M. sexta* pupal hemolymph

Step	Total units*			Total protein			Specific activity			Overall yield†		
	III	I	II	III	I	II	III	I	II	III	I	II
Starting material	4520			15600			0.29			100 (1)		
Ammonium sulfate precipitation	3080			7720			0.40			68 (1.4)		
Anion-exchange chromatography	663	499		177	376		3.7	1.3		15 (12.8)	11 (4.5)	
Hydroxylapatite chromatography	98	398	105	0.88	4.44	2.82	112	90	37	2.2 (386)	8.8 (310)	2.3 (127)
Gel filtration	81	360	94	0.72	3.58	2.47	112	101	38	1.8 (386)	8.0 (348)	2.1 (131)

* Unit = $\mu\text{mol}/\text{min}$.

† Overall purification listed in parentheses.

on Sephacryl S-200 or Sephadex G-100 at pH 8.5 yielded a single symmetrical peak of activity coincident with absorbance at 280 nm (data not shown). Based on elution volumes of standard proteins, the apparent molecular weight was estimated to be 6.1×10^4 , the same as the other glucosaminidases. EIII migrated as a single protein during electrophoresis at pH 8.5 under nondenaturing conditions with a relative mobility intermediate between those of EI and EII on 9% polyacrylamide gels. Electrophoresis of the three enzyme forms in the presence of SDS and 6 M urea and in the absence of 2-mercaptoethanol revealed a single Coomassie blue stained band in each case whose mobility corresponded to that of a protein with an apparent molecular weight of 6.1×10^4 . When the sulfhydryl compound was added to the sample, the mobility of EI remained unchanged, but EII and EIII exhibited two and three bands, respectively. As reported previously (Dziadik-Turner *et al.*, 1981), EII is composed of two peptide chains with apparent molecular weights of 5×10^4 and 1×10^4 that are held together by one or more disulfide bridges. EIII displayed three polypeptide chains with apparent molecular weights of 6.1×10^4 , 5.3×10^4 and 1×10^4 . These results indicated that two forms of exochitinase III were present in about equal amounts, one a single polypeptide and the other composed of two disulfide-linked chains which result from proteolytic cleavage of the intact form.

Amino acid analysis

The amino acid composition of *N*-acetylglucosaminidase III is given in Table 2, together with those of enzymes I and II for comparison. Aspartic acid (or asparagine), glutamic acid (or glutamine) and leucine were the three most abundant residues in all of the enzymes. The least comparable residues were histidine, methionine, alanine and tyrosine. Although each enzyme had a unique amino acid composition, all were composed of approx 525 residues and had a mol. wt of 6.1×10^4 .

Carbohydrate composition

Previously glucosaminidase II was tentatively identified as a glycoprotein because of its reaction with the periodic acid-Schiff's reagent (PAS) after

electrophoresis on polyacrylamide (Dziadik-Turner *et al.*, 1981). Enzyme I did not react likewise. We have carried out a quantitative analysis of carbohydrate content for all three homogeneous enzymes. No neutral or amino sugars were detected by the radio-labelled alditol procedure or by the anthrone reaction (<0.5% carbohydrate by weight), demonstrating that none of these enzymes are glycoproteins.

Kinetic parameters and inhibitors

Analysis of initial velocity experiments with *pNpβGlcNAc* as substrate yielded the following kinetic parameters for glucosaminidase III: $K_m = 265 \pm 44 \mu\text{M}$, $k_{cat} = 374 \pm 56 \text{ sec}^{-1}$ and $k_{cat}/K_m = 1.348 \times 10^6 \text{ sec}^{-1} \text{ M}^{-1}$, values essentially identical to those of EI (Koga *et al.*, 1982). EIII exhibited a nearly identical pH rate profile to that of enzyme I which suggested that two ionizable groups with $pK_a = 8.1$ and 3.7 participate in catalysis. The reactivity of EIII toward βGlcNAc oligosaccharides was also comparable to that of EI and much greater than that of EII, indicating that EIII is an exochitinase. The shorter oligosaccharides were cleaved more rapidly than the longer ones. For example, the $\beta(1 \rightarrow 4)$ linked dimer of *N*-acetylglucosamine was hydrolyzed twice as fast as the hexasaccharide.

Several compounds were characterized in terms of their ability to inhibit glucosaminidase III (Table 3). For comparison the inhibition constants for EI and EII are also listed. EIII exhibited parameters indistinguishable from those of EI with inhibition constants in the low millimolar range. As expected, the most effective inhibitor of the three tested was the β -methyl acetal of *N*-acetylglucosamine.

Immunological characterization

The antigenicity of the *M. sexta* *exo-β-N*-acetylglucosaminidases was examined in rabbits by means of the Ouchterlony double-diffusion procedure. We utilized antibodies to enzymes I and II. Due to a lack of an adequate quantity of homogeneous sample, we could not elicit an antibody to EIII. Antiserum collected four and six weeks after inoculation reacted more specifically than antiserum collected thereafter. Six-week collected EI antiserum reacted with EI and EIII, but not EII (Fig. 2A). The similar precipitin

Table 2. Amino acid composition of *exo-β-N*-acetylglucosaminidases I, II and III from *M. sexta*

Amino acid	Residues per molecule* (mol/%)		
	III	I	II
Asx	63.7 ± 3.7 (12.1)	68.3 ± 3.2 (13.0)	66.1 ± 4.5 (12.6)
Glx	44.6 ± 5.9 (8.4)	52.4 ± 2.9 (9.9)	46.2 ± 2.4 (8.8)
Ser	28.3 ± 0.9 (5.4)	27.7 ± 0.8 (5.3)	27.9 ± 0.8 (5.3)
His	19.5 ± 0.8 (3.7)	8.6 ± 0.4 (1.6)	16.8 ± 1.0 (3.2)
Thr	22.9 ± 1.4 (4.3)	22.3 ± 1.3 (4.2)	24.4 ± 1.4 (4.7)
Gly	25.3 ± 0.9 (4.8)	24.1 ± 1.4 (4.6)	27.8 ± 0.4 (5.3)
Ala	39.3 ± 3.5 (7.4)	38.5 ± 3.4 (7.3)	27.5 ± 2.4 (5.2)
Tyr	25.1 ± 0.6 (4.8)	24.7 ± 0.6 (4.7)	31.2 ± 0.9 (6.0)
Arg	27.8 ± 0.7 (5.3)	27.5 ± 0.8 (5.2)	27.6 ± 0.5 (5.3)
Val	33.9 ± 0.8 (6.4)	33.2 ± 0.8 (6.3)	35.9 ± 1.2 (6.8)
Met	4.7 ± 0.2 (0.9)	13.6 ± 0.4 (2.6)	18.2 ± 0.4 (3.5)
Ile	30.0 ± 0.6 (5.7)	28.5 ± 0.8 (5.4)	27.1 ± 0.9 (5.2)
Leu	39.5 ± 1.9 (7.5)	39.2 ± 2.0 (7.4)	38.6 ± 2.2 (7.4)
Phe	20.8 ± 0.3 (3.9)	20.6 ± 0.4 (3.9)	17.6 ± 0.5 (3.4)
Lys	35.5 ± 6.2 (6.7)	31.1 ± 6.7 (5.9)	27.4 ± 4.4 (5.2)
Trp	20 (3.8)	20 (3.8)	17 (3.2)
Pro	29 (5.5)	29 (5.5)	29 (5.5)
Cys	18 (3.4)	18 (3.4)	18 (3.4)
Total residues	528	527	524
Molecular weight	61000	61000	61000

* Mean values for three analyses of protein hydrolyzed for 24 hr *in vacuo* in 6N HCl containing 1% phenol. Proline not quantitated by *o*-PHTH method. Cysteine and tryptophan destroyed during hydrolysis. Tryptophan estimated from molar extinction coefficients at 280 nm and quantities of tyrosine and phenylalanine determined from amino acid analysis. Proline and cysteine values assumed from average percentage composition of amino acids in 108 proteins (Dayhoff & Hunt, 1972).

lines, together with their fused pattern suggested that I and III are closely related antigenically. Six week EII antiserum reacted only with EII (Fig. 2b). One microgram of enzyme could be detected with antisera diluted about 200 fold. However, higher titer antisera that were collected 7–20 weeks post-inoculation cross-reacted with the other enzymes. These results indicated that all of the glucosaminidases are related immunologically but that EI and EIII are the most similar. Neither antisera crossreacted with *Streptomyces* chitinase (Calbiochem) or hen's egg white lysozyme.

DISCUSSION

Three separate *exo-β-N*-acetylglucosaminidases have been isolated from tissues of the tobacco horn-

worm. The third enzyme to be identified EIII was detected only in pupal hemolymph. EI was found in pharate pupal molting fluid, integument and hemolymph while EII occurred in larval and pupal hemolymphs (Dziadik-Turner *et al.*, 1981). EIII resembled EI more closely than EII in kinetic and immunological properties. EI is a single polypeptide enzyme. EII contains two polypeptides connected by disulfide bridges and EIII is composed of two related enzymes, one a single chain and the other a two chain form. Previously we suggested that a post-translational modification such as proteolysis may change the kinetic behavior of the *β-N*-acetylglucosaminidases (Dziadik-Turner *et al.*, 1981). However, this does not appear to be the case for III which is composed of both a single and two chain forms. No evidence was

Table 3. Inhibition constants for *exo-β-N*-acetylglucosaminidases from *M. sexta*

Inhibitor	K_i (mM)*		
	III	I	II
<i>N</i> -Acetylglucosamine	11.1 ± 0.8	9.7 ± 1.1	4.2 ± 0.1
α -1-0-Methyl- <i>N</i> -acetylglucosamine	8.1 ± 1.3	8.2 ± 0.8	28.0 ± 0.4
β -1-0-Methyl- <i>N</i> -acetylglucosamine	3.1 ± 0.3	2.9 ± 0.1	2.4 ± 0.3

* Competitive inhibition patterns were observed.

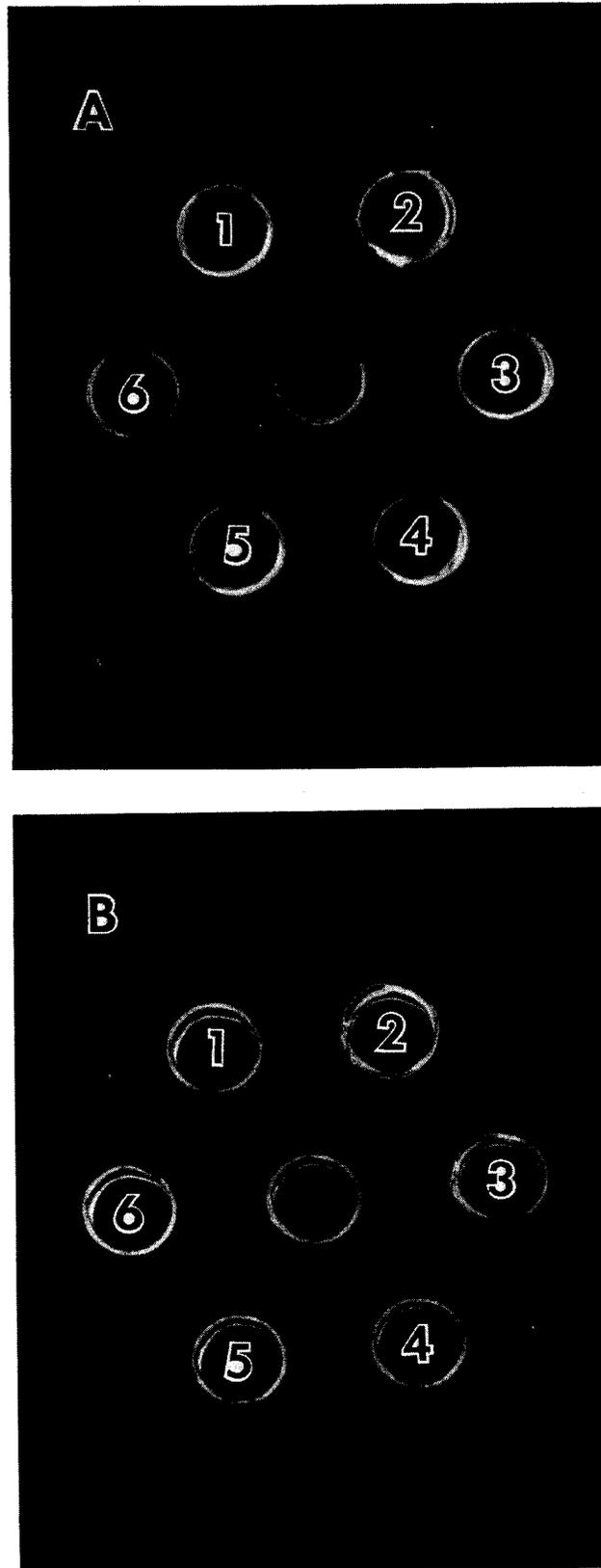


Fig. 2. Ouchterlony double-diffusion immunological analysis of *M. sexta* *exo-β-N*-acetylglucosaminidases. A. Center well contains 6 week EI antiserum; wells 1-6 contain 1.4 μg EI, EI, EII, EII, EIII, EIII, respectively. B. Center well contains 6 week EII antiserum; wells 1-6 contain same as described in A.

obtained that indicated any difference in kinetic properties.

A distinguishing feature of one of these enzymes that was suggested previously on the basis of periodic acid-Schiff's reagent staining was that EII (and not EI or EIII) was a glycoprotein (Dziadik-Turner *et al.*, 1981). We have now determined using the ³H-alditol and anthrone procedures that glucosaminidase II does not contain carbohydrate, even though it gives a positive reaction with the periodic acid-Schiff's reagent. We do not know why EII stains in this fashion.

The silkworm, *Bombyx mori* L., contains two exo- β -N-acetylglucosaminidases that are similar to those described here (Kimura, 1974, 1976, 1977). One of the enzymes was found in molting fluid and the other in larval hemolymph. The former appears to have properties very similar to *M. sexta* EI and EIII while the latter is much like *M. sexta* EII. These classes of enzymes may be common to other insects and they may occur in analogous tissues as well.

On the basis of immunodiffusion experiments with antiserum collected 6 weeks after the first injection of the *B. mori* larval enzyme into rabbits, Kimura (1977) concluded that the molting fluid enzyme was completely distinct from the hemolymph enzyme. We obtained a similar result with *M. sexta* enzymes if antiserum was recovered 4-6 weeks after injection. However, antisera collected subsequently exhibited cross-reactivities which suggested that the *M. sexta* glucosaminidases are all related, at least at one or more antigenic sites.

Based on the properties and tissue localization of EI, we propose that it is an exochitinase that digests endocuticular chitin prior to larval-pupal ecdysis. The functions of EII and EIII remain less clear. The sera of higher animals contain important lysosomal β -N-acetylglucosaminidases whose deficiency leads to cellular pathology and clinical disease (Neufeld *et al.*, 1975). Presumably the hornworm serum enzymes are related to those and metabolize amino sugar-containing substances such as glucosaminoglycans, mucins, glycoproteins and glycolipids.

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