

## IMMUNOLOGICAL RELATIONSHIPS BETWEEN $\beta$ -N-ACETYLGLUCOSAMINIDASES FROM THE TOBACCO HORNWORM, *MANDUCA SEXTA* (L.)\*

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**Abstract**—Antisera were raised in rabbits directed against three purified  $\beta$ -N-acetylglucosaminidases from the tobacco hornworm, *Manduca sexta* (L.). Gamma globulin fractions were prepared by ammonium sulphate precipitation and ion-exchange chromatography. Ouchterlony diffusion and quantitative precipitation reactions demonstrated that the antibodies recognized specific types of  $\beta$ -N-acetylglucosaminidases: one precipitated exochitinase, another *exo*- $\beta$ -N-acetylglucosaminidase and the third endochitinase. These results are in accord with the enzyme classifications proposed previously based upon substrate specificity and kinetic properties.

**Key Word Index:** Antisera, gamma globulin,  $\beta$ -N-acetylglucosaminidases, exochitinase, endochitinase, tobacco hornworm, *Manduca sexta*

### INTRODUCTION

TWO GENERAL classes of  $\beta$ -N-acetylglucosaminidases from the tobacco hornworm, *Manduca sexta*, have been characterized physically, chemically and kinetically (DZIADIK-TURNER *et al.*, 1981; KOGA *et al.*, 1982, 1983a,b). One class hydrolyzes N-acetylglucosamine-containing substrates with an *exo* cleavage mechanism while the other follows an *endo* cleavage mechanism. On the basis of substrate specificity, the former class has been further subdivided into an exochitinase and an *exo*- $\beta$ -N-acetylglucosaminidase.

In order to obtain more information on possible structural interrelationships between these carbohydrases, their immunochemical properties have been examined by producing and utilizing three antisera, one raised against endochitinase, another against exochitinase and the third against *exo*- $\beta$ -N-acetylglucosaminidase.

### MATERIALS AND METHODS

#### *Insects and fluid collection*

Eggs of *M. sexta* were a gift from the Metabolism and Radiation Laboratory, Agricultural Research Ser-

vice, U.S. Department of Agriculture, Fargo, North Dakota. Larvae were reared on a standard diet (BELL and JOACHIM, 1976) and kept at 28°C and 60% relative humidity during a 16 hr light-8 hr dark photoperiod. Haemolymph, moulting fluid and integument were obtained as described previously (DZIADIK-TURNER *et al.*, 1981; KOGA *et al.*, 1982).

#### *Enzyme purification*

The exochitinases, *exo*- $\beta$ -N-acetylglucosaminidase and endochitinases were purified by a series of chromatographic steps as described by DZIADIK-TURNER *et al.* (1981), KOGA *et al.* (1982), and KOGA *et al.* (1983b). Homogeneity was determined by polyacrylamide gel electrophoresis under non-denaturing conditions at pH 8.5 according to DAVIS (1964) and under denaturing conditions according to WEBER *et al.* (1972).

#### *Enzyme assay*

*Exo*- $\beta$ -N-acetylglucosaminidase was assayed for *p*-nitrophenyl-2-acetamido-2-deoxy- $\beta$ -D-glucopyranoside (pNp $\beta$ GlcNAc) hydrolytic activity by monitoring pNp production at 337 nm (FORD *et al.*, 1973). *Endo*- $\beta$ -N-acetylglucosaminidase was measured using glycol chitin as substrate by following the production of reducing end groups colorimetrically with the potassium ferrirocyanide reagent at 420 nm (IMOTO and YAGISHITA, 1971).

#### *Preparation of gamma immunoglobulins*

Antisera were prepared by immunization of 2.3 kg New Zealand white albino rabbits with multisite intramuscular injections using a total of 0.5 mg enzyme suspended in complete Freund's adjuvant. The enzymes used included exochitinase (E-I), *exo*- $\beta$ -N-acetylglucosaminidase (E-II) and endochitinase (E-VI). A second injection was administered subcu-

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taneously in incomplete Freund's adjuvant after four weeks. The rabbits were bled one week after the second injection and each subsequent week thereafter for six weeks. Serum was prepared from whole blood by coagulation, separation from the clot and clarification as described previously (CAMPBELL *et al.*, 1970a). Gamma globulin was purified by repeated ammonium sulphate precipitation at a final concentration of one-third saturation (KERWICK, 1940; CAMPBELL *et al.*, 1970b) followed by DEAE cellulose chromatography using 10 mM potassium phosphate buffer, pH 7.5 (SOBER *et al.*, 1956; CAMPBELL *et al.*, 1970c).

#### Immunological procedures

Ouchterlony diffusion was performed in 1% agarose gel in 50 mM sodium phosphate-0.85 sodium chloride-0.01% merthiolate buffer pH 7.0. Antigen and antiserum were placed in wells and incubated overnight at 27°C. Precipitation bands were visible within 12 hr.

The quantitative precipitation reaction was conducted by incubating various doses of  $\gamma$ -globulin with the enzymes in 0.1 M Tris buffer pH 7.5. Controls were included in which immunoglobulin was replaced by Tris buffer. After incubation for 12 hr at 4°C, solutions were centrifuged at 5000 *g* for 10 min at 4°C and the supernatants were assayed for the unprecipitated enzyme activity.

### RESULTS AND DISCUSSION

Six hydrolytic enzymes that cleave N-acetylglucosamine containing substrates have been purified from tissues of the tobacco hornworm (DZIADIK-TURNER *et al.*, 1981; KOGA *et al.*, 1982, 1983a,b). On the basis of their substrate specificity and kinetic properties, they have been denoted as either an exochitinase (E-I and E-III), *exo*- $\beta$ -N-acetylglucosaminidase (E-II) or endochitinase (E-IV, E-V and E-VI). Antisera were obtained from rabbits that had been immunized with homogeneous preparations of exochitinase I (Ab-I), *exo*- $\beta$ -N-acetylglucosaminidase II (Ab-II) and endochitinase VI (Ab-VI). Antisera to the other enzymes were not prepared because adequate quantities of homogeneous protein were unavailable.

Antibody titre was monitored by the technique of Ouchterlony double diffusion. Ab-I that was collected

32 days after inoculation with E-I gave precipitation lines with 0.5  $\mu$ g of E-I and E-III but not with E-II. However, Ab-I collected on day 47 and thereafter also reacted with E-II. Ab-II collected 47 days after injection of E-II precipitated only that enzyme, but 61-day Ab-II also precipitated E-I and E-III. These results showed that the higher titre antisera are not specific for their respective antigens. However, dilution of these higher titre antisera by a factor of three prevented cross-reactions in double diffusion experiments.

Ouchterlony assays using Ab-VI yielded precipitation lines with 0.5  $\mu$ g of endoenzymes E-V and E-VI, but not with E-IV. The endoenzyme antiserum did not precipitate any of the exoenzymes while the exoenzyme antisera did not cross react with the endochitinases. None of the three antisera reacted with commercial chitinases from *Streptomyces* (Calbiochem, La Jolla, CA) or with hen's egg white lysozyme (Worthington Biochemical Corp., Freehold, NJ).

Whole body extracts of *M. sexta* larvae and pupae were assayed using the Ouchterlony diffusion method for proteins that reacted with the various antisera. Single precipitation lines were obtained in all cases, except for the pupal extract which exhibited a second slower moving line with Ab-I and -II but not with Ab-VI. Apparently there are multiple proteins present in the pupal stage of development that are related to exochitinase and *exo*- $\beta$ -N-acetylglucosaminidase.

Immuntitrations were performed with purified gamma globulin preparations in order to obtain a more quantitative comparison of the six enzymes. Figure 1 shows the titrations of E-I, E-II and E-III with IgG-I (panel A) and IgG-II (panel B). The apparent dissociation constants of E-I, E-II and E-III for IgG-I were  $1.05 \times 10^{-6}$ ,  $>10^{-4}$  and  $2.75 \times 10^{-6}$  M with relative binding affinities of 1,  $<0.01$  and 0.38, respectively. These results demonstrated that the exochitinases E-I and E-III are closely related immunologically. The apparent dissociation constants for IgG-II were  $>10^{-5}$ ,  $2.75 \times 10^{-7}$  and  $>10^{-5}$  M for E-I, E-II and E-III, respectively. IgG-II bound the *exo*- $\beta$ -N-acetylglucosaminidase, E-II, at least 35 times tighter than either of the exochitinases, again indicating that E-II is a unique enzyme.

The IgG fraction obtained from Ab-VI was used to distinguish the three endochitinases into two anti-

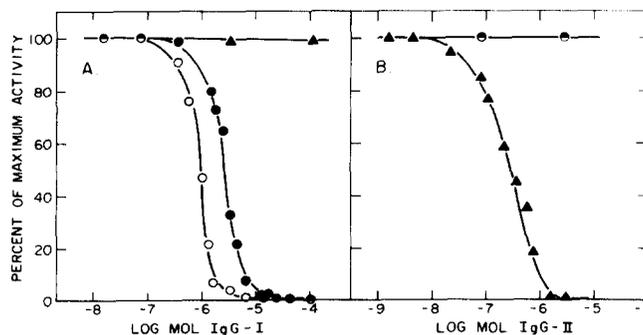


Fig. 1. Neutralization of *M. sexta* *exo*- $\beta$ -glucosaminidases with IgG-I and -II. Enzyme concentrations =  $3-9 \times 10^{-8}$  M. (A) Titration with IgG-I. (B) Titration with IgG-II. Symbols E-I,  $\circ$ : E-II,  $\blacktriangle$ : E-III,  $\bullet$ . Typical data from individual enzyme preparations are presented with S.E.M.  $\pm 15\%$ .

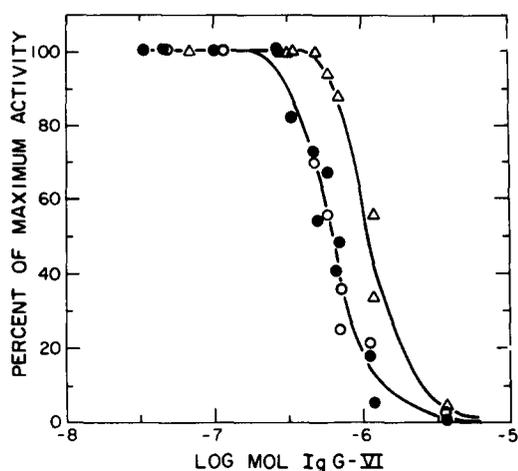


Fig. 2. Neutralization of *M. sexta* endo- $\beta$ -glucosaminidases with IgG-VI. Enzyme concentration =  $3\text{--}27 \times 10^{-8}$  M. Symbols: E-IV,  $\Delta$ ; E-V,  $\circ$ ; E-VI,  $\bullet$ . Typical data from individual enzyme preparations are presented with S.E.M.  $< \pm 15\%$ .

genic classes. Figure 2 shows the immunotitrations of the enzymes with IgG-VI. The apparent dissociation constants for E-V and E-VI were essentially identical,  $6.5 \times 10^{-7}$  M while that of E-IV was  $1.07 \times 10^{-6}$ . The affinity of IgG-VI for E-IV was only 60% of that for the other two endochitinases.

Each of the purified IgG fractions was tested for toxicity to *M. sexta* larvae. Fourth stadium larvae (~1 g) were injected with IgG-I (0.8 mg), IgG-II (0.2 mg) and IgG-VI (0.02 mg), and observed twice daily thereafter. Controls were injected with an equivalent amount of bovine serum albumin or rabbit serum IgG. The most toxic immunoglobulin was IgG-VI. All of the IgG-VI treated animals were unable to commence ecdysis to the fifth stadium. More than half of the larvae administered the other

immunoglobulin fractions were able to complete ecdysis to the fifth stadium. These results suggested that inhibition of the  $\beta$ -N-acetylglucosaminidases *in vivo*, in particular endochitinase, is lethal to insects.

A summary of the physical, chemical, kinetic and immunological properties of the *M. sexta*  $\beta$ -N-acetylglucosaminidases is given in Table 1. The results of the immunological study are in accord with the three classifications of exochitinase, exo- $\beta$ -N-acetylglucosaminidase and endochitinase that have been proposed earlier based on other properties (KOGA *et al.*, 1982, 1983a,b). All of these enzymes are rather large proteins ranging in molecular weight from  $5 \times 10^4$  to  $7.5 \times 10^4$ . They are distinguishable in several different ways. The endochitinases hydrolyze polymeric and oligomeric substrates but the exoenzymes cleave only the smaller saccharides. Two of the exoenzymes degrade chitin oligosaccharides much more efficiently than the third exoenzyme. The endochitinases are glycoproteins while none of the exoenzymes are glycosylated. Apparently each of these  $\beta$ -N-acetylglucosaminidases may serve a separate physiological function and may be coded for by unique structural genes.

The  $\gamma$ -globulin fractions described here recognize a specific type of  $\beta$ -N-acetylglucosaminidase. IgG-I neutralized only the exochitinases, IgG-II the exo- $\beta$ -N-acetylglucosaminidase, and IgG-VI the endochitinases. These antibodies should prove useful in determining the titres of individual enzymes during development of *M. sexta* and also in detecting related enzymes present in other organisms.

It is noteworthy that factors possessing anti- $\beta$ -N-acetylglucosaminidase activity such as the specific antibody fractions described in this study are detrimental to insect development. The immunoglobulins probably inactivated enzymes in haemolymph and perhaps in other tissues as well. We found that IgG-VI which is specific for endochitinase was the most toxic fraction. This result was somewhat unexpected because endochitinase occurs in integument or

Table 1. Properties of  $\beta$ -N-acetylglucosaminidases from *M. sexta*

Property	Exochitinase*		Exo- $\beta$ -N-acetylglucosaminidase*	Endochitinase†		
	E-I	E-III	E-II	E-IV	E-V	E-VI
Molecular weight ( $\times 10^{-4}$ )	6.1	6.1	6.1	7.5	6.2	5.0
$k_{cat}/K_m$						
pNp $\beta$ GlcNAc	1.58	1.35	2.05	—	—	—
$\beta$ GlcNAc <sub>2</sub>	2.07	+	0.01	—	—	—
Glycol chitin	—	—	—	8.7	13.4	7.3
Glycoprotein	—	—	—	+	+	+
Tissue distribution	Moulting fluid, integument, pupal haemolymph		Larval and pupal haemolymph	Moulting fluid and integument		
Number of polypeptides	1	2	2	1	1	1
Reactive with						
IgG-I	+	+	—	—	—	—
IgG-II	—	—	+	—	—	—
IgG-VI	—	—	—	+	+	+

\* From this study and DZIADIK-TURNER *et al.*, 1981, KOGA *et al.*, 1982, 1983b. Kinetic Units =  $\text{sec}^{-1} \text{M}^{-1} \times 10^6$  at pH 5.6.

† From this study and KOGA *et al.*, 1983a. Kinetic units =  $\text{ml sec}^{-1} \text{mg}^{-1}$  at pH 9.0.

moulting fluid and not in haemolymph. The observation that the insect is sensitive to endochitinase antiserum suggests that endochitinase is more critical for moulting. This enzyme rather than exochitinase probably initiates the process of chitin catabolism.

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