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Purification and Characterization of β-N-Acetylglucosaminidase from Haemolymph of the Cabbage Butterfly, *Pieris rapae*.

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배추흰나비의 혈림프內 β-N-Acetylglucosaminidase의 精製 및 特性

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要 約

배추흰나비(*Pieris rapae*)의 前蛹期 혈림프에서 β-N-acetylglucosaminidase를 DEAE-cellulose ion exchange 및 Sephadex G-200 column chromatography 법을 이용하여 精製하였다. 정제된 효소는 7% acrylamide gel 電氣泳動에서 단일 band를 나타내었으며, 糖을 함유한 복합단백질이었다. 최적 pH는 5.0, 최적반응온도는 60°C이었으며, 각 온도에서 10분간 incubation한 熱安定性에 있어서는 60°C까지 비교적 높은 活性을 나타내었지만 70°C 이후에는 거의 활성을 나타내지 않았다. 또한 Hg^{2+} 處理는 심한 효소활성의 沮害 현상을 보였지만, Mn^{2+}, Mg^{2+} 등은 활성의 증가를 나타내었으며, Km 값은 6.67×10^{-3} M, pI 값은 5.8, 분자량은 4.1×10^{5} daltons 이었다.

INTRODUCTION

Chitin, polymer of N-acetylglucosamine was known to be a major constituent of cuticle and lining of fore and hindguts, peritrophic membrane, and trachea of insect and other arthropoda (Waterhouse *et al.*, 1961). It was also reported that in several insects including *Rhodnius* endocuticle was reabsorbed when insect was starved but deposited when fed, demonstrating that chitin is a kind of reserve nutrients (Locke, 1964). It was known that during the moult over 90% of chitin was hydrolyzed to monomer by chitinase and chitobiase and absorbed for reuse and these enzyme activity was present in haemolymph, integument, skin cast, and testis as well as moulting fluid (Waterhouse and McKellar, 1961; Kimura, 1977).

Among the chitinolytic system β-N-acetylglucosaminidase has been studied by Spindler

(1976) for *D. hydei* Zielkowski and Spindler (1978) for *L. migratoria* Kimura (1973a, 1974, 1976a, b, 1977) for *B. mori* Dziadik-Turner *et al.* (1981) for *M. sexta* Koga *et al.* (1981, 1982) for *M. sexta* and Fukamizo and Kramer (1985a, b) but the study for haemolymph β-N-acetylglucosaminidase has been greatly limited.

Chitinolytic enzymes in integument of last larval and pupal *Pieris rapae* has been reported previously by present authors (1985). Present study is to purify β-N-acetylglucosaminidase from prepupal haemolymph of *Pieris rapae* and also to determine the characterization of this enzyme.

MATERIALS and METHODS

Insects

Pieris rapae larvae were reared on Kail in vinyl house and transferred to laboratory and used for present work. Haemolymph was collected from prepupae washed in 0.85% KCl solution and small amount of phenylthiourea was added to inhibit melanization and centrifuged at 12,000 g for 20 min to remove haemocyte and tissue debris and the supernatant was stored at −70°C for use.

Measurement of enzyme activity

The enzyme activity was measured according to the method of Mega *et al.* (1970). 0.1 ml enzyme solution, 0.1 ml of 0.05 M citrate phosphate buffer (pH 5.5) and 0.1 ml of 0.3% p-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma) as substrate were mixed and incubated at 37°C for 5 min and then 0.01 N NaOH was added to stop reaction. The amount of p-nitrophenol formed was determined using Shimadzu UV-360 spectrophotometer at 410 nm. The concentration of protein was determined according to the method of Lowry *et al.* (1951) and also solution eluted through column was measured at 280 nm.

Purification of enzyme

20 ml of haemolymph collected from prepupae was saturated to 20% through 80% with ammonium sulfate while being stirred for 30 min at 4°C. The fractions (40~70%) with high enzyme activity were pooled and dialyzed against Tris-HCl buffer (pH 7.2) for 40 hrs at 4°C and concentrated for 10 hrs with freeze dryer and same buffer was added to give the final volume of 2 ml.

Concentrated enzyme solution was applied to DEAE-cellulose column (1.6 × 30 cm) equilibrated with Tris-HCl buffer (pH 7.2) and eluted at 8 ml per fraction using 0 to 0.2 M linear NaCl gradient at flow rate of 25 ml/hr. The fractions with high enzyme activity were pooled from DEAE-cellulose column and subjected to gel filtration through Sephadex G-200 column (1.6 × 50 cm) using LKB 2070 ultro Rac II. Elution was conducted with 4 ml of fraction size at flow rate of 12 ml/hr using 0.05 M acetate buffer(pH 5.0).

Electrophoresis

Electrophoresis was conducted in 7% acrylamide gel with Tris-glycine buffer (pH 8.3) at

the current of 3 mA/gel according to the method of Davis (1964). After electrophoresis gel was stained with Coomassie brilliant blue R 250(0.25%) for general protein and PAS reagent for glycoprotein (Caldwell and Pigmann, 1965).

Isoelectric focusing

6% polyacrylamide gels were used according to the method of Davis (1964). The concentration of ampholine was 1% and there was approximately 0.3 ml of space at one end of gel. After polymerization empty space at one end of gel was filled with protective solution (1.5% ampholine and 10% glycerol) and then connected to electrophoretic chamber. Buffer used was 0.1% H_3PO_4 in upper chamber and 0.1 N NaOH in lower chamber. Gels were pre-runned for 30 min at 200 V and then sample solution (purified enzyme solution dissolved in the solution containing 0.1 ml of 1.5% ampholine and 20% sucrose) was gently placed at the end of gel and runned for 20 hrs (Wrigly, 1968). After electropheresis, gel without sample was sliced 0.5 cm thick and each block was stored in 1.0 ml of second distilled water for 24 hrs and then pH of each extracted solution was measured.

Determination of molecular weight

The molecular weight of β-N-acetylglucosaminidase was determinated under native conditions according to the method of Hedrick and Smith (1968) using standard proteins of known molecular weights: α-lactalbumin (14, 200), carbonic anhydrase (29, 000), chicken egg albumin (45, 000), and bovine serum albumin (monomer; 66, 000, dimer; 132, 000).

RESULTS

Purification of enzyme

When 20 ml haemolymph collected from prepupae was saturated to 20 to 80% with ammonium sulfate, fractions between 40 to 70% showed high activity. These fractions were pooled and dialyzed against Tris-HCl buffer (pH 7.2) for 40 hrs and concentrated to 2 ml and applied to DEAE-cellulose column chromatography. As shown in Fig. 1, there

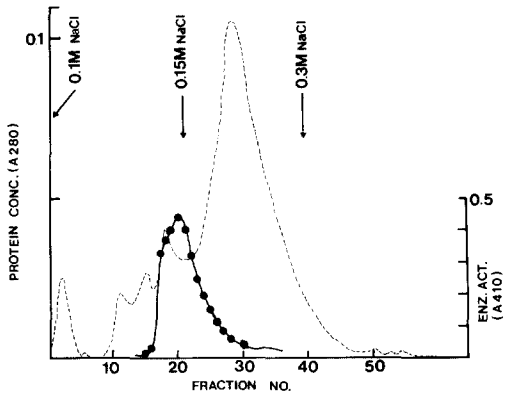

Fig. 1. Chromatography of β-N-acetylglucosaminidase on a DEAE-cellulose column.
·····, protein concentration measured at 280 nm;
•—•, enzyme activity.

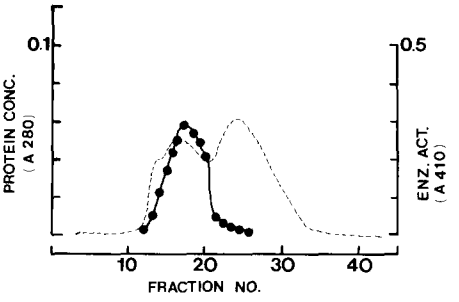

Fig. 2. Chromatography of β-N-acetylglucosaminidase on a Sephadex G-200 column.
·····, protein concentration measured at 280 nm;
•—•, enzyme activity.

was a single peak of high enzyme activity (fraction No. 15-25) around 0.15 M NaCl in the elution through 0-0.3 M linear NaCl gradient. Fractions No. 17 to 20 were pooled and concentrated to 3 ml using Amicon (PM-10 membrane). This solution was subjected to gel filtration using Sephadex G-200 column. Fig. 2 showed that there was the peak of high activity between fractions No. 16 to 20 in the elution curve for protein concentration and enzyme activity.

Confirmation of purification by electrophoresis

Enzyme solution from each step of purification procedure was applied to 7% acrylamide disc electrophoresis. Compared with those of crude prepupae haemolymph, precipitate through 40% to 70% saturation of ammonium sulfate showed at least 5 bands but sample solution through DEAE-cellulose and Sephadex G-200 (purified enzyme, No. 17) represented 3 bands and single band respectively (Fig. 3). Purified enzyme was placed between No. 3

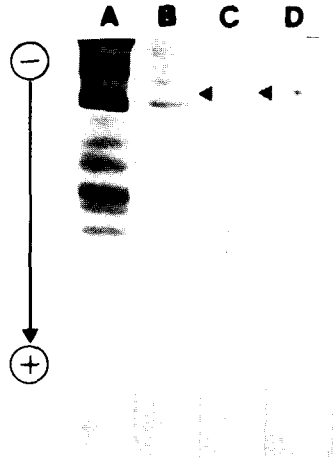

Fig. 3. 7% acrylamide disc electrophoresis of β-N-acetylglucosaminidase. A, prepupa haemolymph preparation; B, 0.4~0.7 saturated $(NH_4)_2SO_4$ preparation; C, DEAE-cellulose preparation; D, Sephadex G-200 preparation (purified enzyme).
▲, β-N-acetylglucosaminidase.

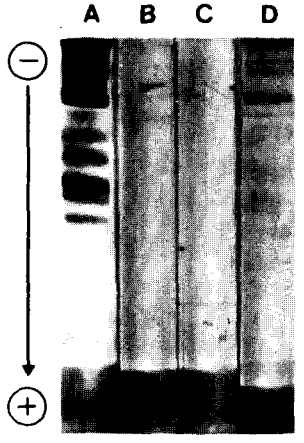

Fig. 4. 7% acrylamide disc electrophoresis. Prepupa haemolymph and purified enzyme stained with Coomassie blue (A, B) and with PAS reagent (C, D). A, D, Prepupa haemolymph preparation; B, C, Sephadex G-200 preparation (purified enzyme); △, positive reaction with PAS reagent.

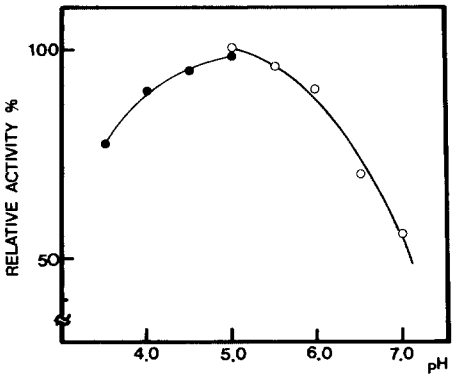

Fig. 5. Effect of the pH on the activity of β-N-acetylglucosaminidase using p-nitrophenyl NAcGm as substrate. The incubation time for the enzyme assay was 5min.
•—•, 0.05 M citrate buffer;
○—○, 0.05 M phosphate buffer.

and 4 major bands beginning from upper end and has the Rm value of 0.14 and stained with PAS reagent, demonstrating that this enzyme is a conjugated protein containing carbohydrate (Fig. 4).

Characterization of purified enzyme

Optimum pH and temperature. Citrate buffer, 0.05 M (pH 3-5) and 0.05 M phosphate buffer (pH 5-8) were used for pH optimum. Results showed that there is highest activity at pH 5.0 and also at 60°C (Figs. 5, 6).

Heat stability. Enzyme solution was incubated for 10 min at different temperature and heat stability for enzyme activity was measured. As shown in Fig. 7, the enzyme maintains

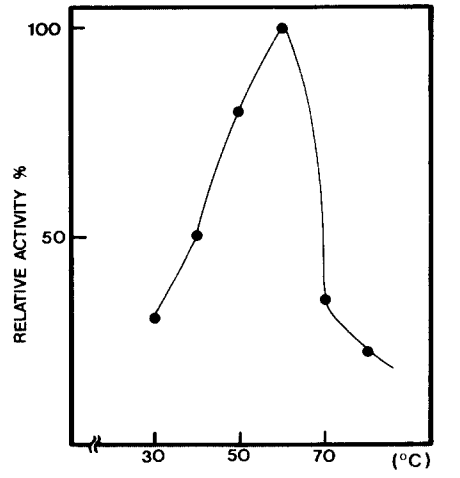

Fig. 6. Effect of temperature on the activity of β-N-acetylglucosaminidase.

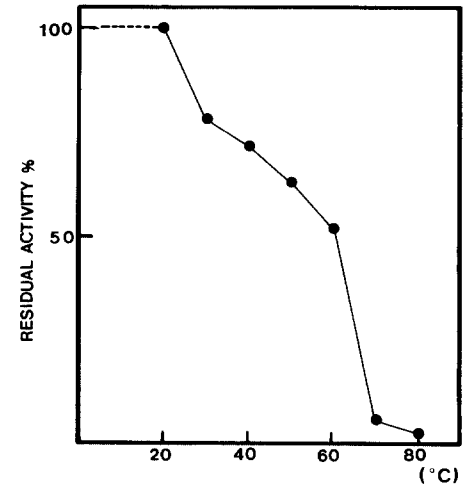

Fig. 7. Heat stability of β-N-acetylglucosaminidase. The enzyme was incubated at various temperatures for 10 min.

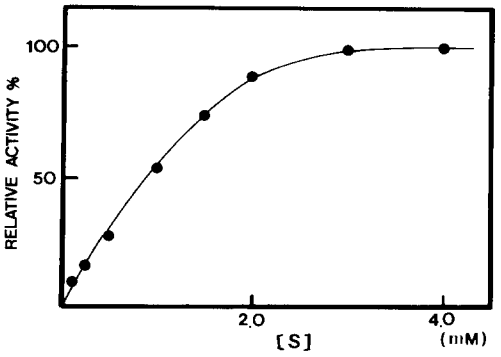

Fig. 8. Effect of the concentration of substrate (p-nitrophenyl NacGm) on the activity of β-N-acetylglucosaminidase.

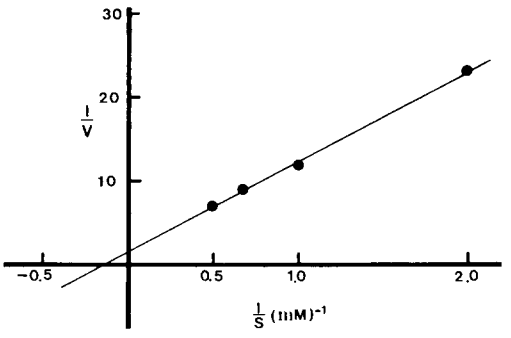

Fig. 9. Lineweaver-Burk plots of purified β-N-acetylglucosaminidase with various concentrations of p-nitrophenyl NAcGm.

relatively high activity until 60°C but shows the drastic decrease above 70°C.

The effect of substrate concentration and Km value. The effect of substrate concentration on enzyme activity was shown in Fig. 8. There is apparent increasing trend until 2.3×10^{-3} M and constant value above 3.0×10^{-3} M. Km value also was found to be 6.67×10^{-3} M according to Lineweaver-Burk plots (Fig. 9).

The effect of metal ions. Enzyme solution was treated with 10 mM various metal ion solutions. Results showed that Cu^{2+}, Mg^{2+}, Mn^{2+}, and K^{+} increase enzyme activity with 1.4 fold increase for Mn^{2+} but Hg^{2+} represents severe inhibition phenomenon

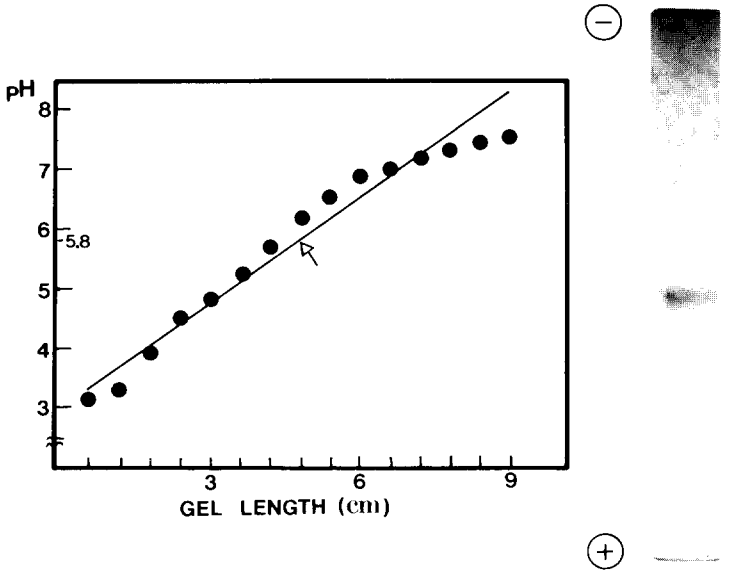

Fig. 10. Determination of isoelectric point of β-N-acetylglucosaminidase purified from haemolymph by the method of Wrigley (1968).

Table 1. Effects of cations on β-N-acetylglucosaminidase activity.

Ion (10^{-2} M)	Percentage activity (%)
Cu^{2+}	105
Mg^{2+}	110
Mn^{2+}	142
Ca^{2+}	92
Fe^{2+}	93
K^{+}	108
Hg^{2+}	20
None	100
EDTA*	83

(Table 1).

Determination of PI value and molecular weight. Isoelectric point of purified enzyme was determined to be 5.8 and molecular weight was estimated to be 4.1×10^{5} daltons (Figs. 10, 11).

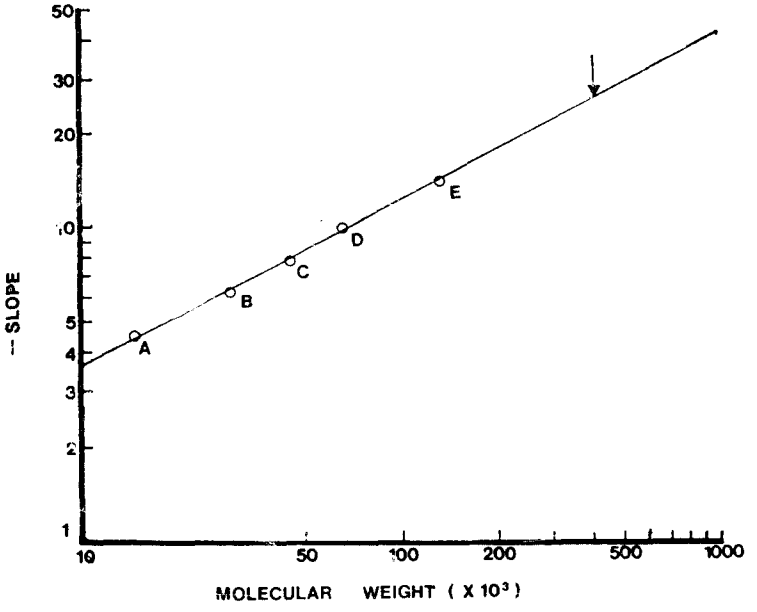

Fig. 11. Determination of mol. wt. for β-N-acetylglucosaminidase under native conditions according to the method of Hedrick and Smith (1968). 100[log($R_f\times100$)] values (ordinate) are plotted against the gel concentration as persent (abscissa) on standard graph paper for each protein. The negative slopes from these graphs (ordinate) are plotted against the known molecular weights of the standards (abscissa) on 2 cycle log-log paper. Marker proteins used were (A) α-Lactalbumin, 14,200; (B) Carbonic anhydrase, 29,000; (C) Chicken egg albumin, 45,000; (D) Bovine serum albumin, monomer, 66,000; (E) Bovine serum albumin, dimer, 132,000.

DISCUSSION

Chitinase and chitobiase involved in chitin degradation during moult were known to be present in more than one form respectively (Powning and Irzykiewicz, 1964; Winicur and Mitchell, 1974; Kimura, 1973a; Bade, 1974) and these enzyme activities also were reported to be controlled by molting hormone (Kimura, 1973b).

β-N-acetylglucosaminidase (or chitobiase) is mostly present in molting fluid, integument, and haemolymph. Both haemolymph enzyme and molting fluid enzyme were reported to degrade aryl compound (Berger and Reynolds, 1958; Kimura, 1973a). In silkworm haemolymph enzyme has greater affinity to aryl glycoside than molting fluid enzyme but molting fluid enzyme shows greater affinity than haemolymph enzyme for natural substrate (chitobiose). Molting fluid enzyme was immunologically identical to that in integument and posterior silkgland which is in accord with the appearance of chitinase activity (Kimura, 1977). Especially it is notable that β-N-acetylglucosaminidase was reported to have the same function as chitobiase (Powning and Irzykiewicz, 1964) while molting fluid enzyme is termed chitobiase whereas haemolymph enzyme named β-N-acetylglucosaminidase (Kimura, 1974, 1976, 1977).

Haemolymph enzyme of *Pieris rapae* has the optimum pH of 5.0 which is similar to those of *B. mori* and *M. sexta* and also optimum temperature of 60°C which was not reported in other insects. But *D. hydei* (Spindler, 1976) and *L. migratoria* (Zielkowski and Spindler, 1978) show the optimum temperature of 50°C (opt. pH $5.5 \sim 6.2$) and 70°C (opt. pH $4 \sim 5$) for integument enzyme respectively. Also as *p*-nitrophenyl-N-acetyl-β-D-glucosaminidase was used as substrate, Km value of haemolymph enzyme of *Pieris rapae* is 6.67 mM which is a little higher than those of *B. mori* and *M. sexta*. For the effect of metal ion on enzyme activity $HgCl_2$ shows inhibiting effect for *B. mori* but 1mM $HgCl_2$ and 1mM $CuSO_4$ represents no inhibiting effect for *M. sexta*. In the present work 10 mM $HgCl_2$ represent severe inhibiting effect but same concentration of Cu^{2+}, Mg^{2+}, Mn^{2+}, and K^+ stimulates the activity with 1.4 fold increase for Mn^{2+}.

Purified enzyme of *Pieris rapae* was found to have the PI value of 5.8 which is similar to that of *M. sexta* and also have the molecular weight of 4.1×10^5 which is higher than those of *M. sexta* and *B. mori*. Haemolymph β-N-acetylglucosaminidase of *Pieris rapae* proved to be glycoprotein like those of silkworm and hornworm.

On the while according to the work by Dziadik-Turner *et al.* (1981) and Koga *et al.* (1982) with *M.sexta* EI in molting fluid and integument shows the function of exochitinase (or chitobiase) whereas the function of pupal haemolymph EI was known to be used for internal tissue processing and restructuring. Also, larval and pupal haemolymph EII was reported to represent low activity for chitin substrate and are lacking in molting fluid, suggesting that at least *in vivo* function is not chitinolytic but rather plays a some role in