Botanical Studies (2011) 52: 23-34.

Biochemical characterization of a β-*N*-acetylhexosaminidase from fig latex

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(Received April 16, 2010; Accepted July 8, 2010)

ABSTRACT. A major isoform of β-*N*-acetylhexosaminidase (β-NAHA) (EC 3.2.1.52) was purified from fig latex in three chromatography steps, affinity chromatography on *p*APMA-Sepharose CL-4B to remove cysteine proteases, Sephacryl S-100 HR gel filtration, and DEAE-Sephacel ion-exchange chromatography. The purified β-NAHA appeared almost homogeneous on SDS-polyacrylamide gel electrophoresis and enzyme-activity staining. The purified enzyme catalyzed the hydrolysis of both *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*NP-β-GlcNAc) and *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (*p*NP-β-GalNAc). The optimum pH for the *p*NP-β-GlcNAc hydrolysis was 4.5, the optimum temperature was 60°C, the K_m was 1.3 mM, the V_{max} was 6.0 μmol min^{-1} mg^{-1} and the activation energy was 8.93 kcal/mol. The molecular mass of the enzyme was 13.7 kDa, as estimated by gel filtration. The isoelectric point of the enzyme was 3.5, as estimated by isoelectric focusing electrophoresis and activity staining. The enzyme was thermally stable after 60 min at 30-50°C, but its activity decreased significantly at temperatures greater than 55°C. Both the heavy metal ion Hg^{2+} (0.25 mM) and the chemical modification reagent diethyl pyrocarbonate (2.5 mM) significantly inhibited enzyme activity. Substrate specificity and competition kinetics analysis indicated that the activity of the purified β-NAHA was specific for the β-glycosidic linkage, and the enzyme had only one active site for substrates, *p*NP-β-GlcNAc and *p*NP-β-GalNAc.

Keywords: Characterization; Fig (*Ficus carica*) latex; β-*N*-acetylhexosaminidase; Purification.

INTRODUCTION

β-*N*-acetylglucosaminidase catalyzes the release of *N*-acetylglucosaminyl residues from the non-reducing terminus of oligosaccharides. It is also referred to as β-*N*-acetylhexosaminidase (β-NAHA) because it is capable of cleaving terminal *N*-acetyl-β-D-galactosaminyl residues from oligosaccharides as well (Dey and Campillo, 1984; Conzelmann and Sandhoff, 1987; Webb, 1992). This enzyme is widely distributed in nature and has been detected in animal tissues, microorganisms, and plants (Conzelmann and Sandhoff, 1987). In plants, β-NAHA is thought to participate in the processing and turnover of glycoprotein during germination (Neely and Beevers, 1980; Yi, 1981; Vitale and Chrispeels, 1984) and the metabolism of *N*-glycans during ripening in apples (Choi and Gross, 1994). However, β-NAHA and chitinase activites were also seen in the whole tissues of individual seeds and seedlings (Hodge et al., 1996) and in rubber tree latex (Martin, 1991; Giordani et al., 1992). Some plant β-NAHAs also degrade chitin and chitin oligomers (Li and Li, 1970; Yi, 1981; Barber and Ride, 1989), suggesting that the enzyme plays a role in the defense system in plants against chitinous pathogens. However, the physiological signification of this enzyme in plants has yet to be fully elucidated.

Latex is the cytoplasmic fluid, containing the usual plant organelles in laticifer cells. Laticifers are anastomosed as a result of partial hydrolysis of adjacent walls, and thus form a tube-like network, or paracirculatory system, through-out the plant (Esau, 1967). When laticifers are injured, latex flows from the wound site. The latex exuded from laticifers is known to contain a variety of defense-related proteins such as chitinase, β-1,3-glucanase, hevamine and hevein (Broekaert et al., 1990; Rozeboom et al, 1990; Van Parijs et al., 1991). It has been suggested that latex secretion provides a defense against wounds and/or predators such as insects and microorganisms. Fig tree latex contains proteolytic enzymes (ficin) (Robbins and Lamson, 1934; William, 1958; Whitaker, 1959), chitinolytic enzymes such as plant lysozyme (Glazer et al.,

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1969), and cDNA that encodes basic class I chitinase (Kim et al., 2003). Commercial crude ficin prepared from fig latex, was found to possess chitosanolytic activity (Yalpani and Pantaleone, 1994).

Recently, we isolated a chitosanase from a commercial ficin preparation, and found that β-NAHA and several other glycosidases were present in fig latex in addition to proteases and chitinolytic enzymes (Chiang et al., 2005; Chiang and Chang, 2006). Here we report on the purification and characterization of a predominant form of β-NAHA in fig latex. Enzymatic properties of the purified β-NAHA including molecular mass, thermal stability, effectors and substrate specificity, as well as the kinetics of competition with mixed substrates were studied.

MATERIALS AND METHODS

Latex Material

Latex was collected directly from the unripe fruits of fig trees (*Ficus carica*) grown on a farm in Taichung, Taiwan. After lyophilization, the latex sample was stored at -20°C.

Chemicals

N-Acetylimidazole (NAI), 1, 2-cyclohexanedione (CHD), *p*-hydroxymercuribenzoate (sodium salt, *p*HMB), diethyl pyrocarbonate (DEPC), 2,4-dinitro-1-fluorobenzene (DNFB), ethyl acetimidate (EAM), phenylmethanesulfonyl fluoride (PMSF), *N*-ethyl-5-phenylisoazoline-3′-sulfonate (Woodward's reagent K; WRK), *N*-acetyl-D-glucosamine (GlcNAc), D-glucosamine (GlcN), *p*-nitrophenyl-α-D-galactopyranoside (*p*NP-α-Gal), *p*-nitrophenyl- *N*-acetyl-β-D-glucosaminide (*p*NP-β-GlcNAc), *p*-nitrophenyl-β-D-galactopyranoside (*p*NP-β-Gal), *p*-nitrophenyl-β-D-glucopyranoside (*p*NP-β-Glc), *p*-nitrophenyl-α-D-mannopyranoside (*p*NP-α-Man), *p*-nitrophenyl-β-D-xylopyranoside (*p*NP-β-Xyl) and *p*-aminophenylmercuric acetate (*p*APMA) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). PhastGel IEF 3-9 gels, Sepharose CL-4B, Sephacryl S-100 HR, DEAE-Sephacel, Superose 6 HR 10/30 columns, Superdex 75 HR 10/30 columns, and HiLoad Superdex 75 HR 16/60 columns were obtained from GE Healthcare Bio-Sciences Corp. (MA, USA).

Preparation of *p*APMA-Sepharose CL-4B affinity adsorbent

Sepharose CL-4B, 100 mL packed, was activated with CNBr at pH 11 as described by Cuatrecasas (1970). At the end of the reaction the Sepharose-CL-4B was washed in a Buchner funnel with 1.5 L of 0.1 M $NaHCO_3$, pH 9.0, and resuspended in 200 mL of 10% dimethyl sulfoxide at 4°C. To this suspension, 1.3 g of *p*APMA dissolved in 20 mL of dimethyl sulfoxide was added slowly. The suspension was stirred slowly at 4°C for 20 h then warmed to 30°C and filtered. The substituted Sepharose CL-4B was resuspended in 130 mL of 20% dimethyl sulfoxide for 5 min then filtered. This treatment was repeated 3 times. The gel was packed into a column and slowly washed with 400 mL of 20% dimethyl sulfoxide, followed by 200 mL of 0.1 M sodium phosphate buffer containing 0.1 M 2-mercaptoethanol, pH 8.0 (Sluyterman and Wijdennes, 1970).

Measurement of β-NAHA activity

A mixture of 0.5 mL of 5 mM *p*NP-β-GlcNAc in 0.1 M sodium acetate buffer, pH 4.5 and 0.05 mL of enzyme solution was incubated at 40°C for 20 min. The reaction was stopped by the addition of 1 mL of 0.25 M Na_2CO_3. The amount of *p*-nitrophenol formed was measured by determining the absorbance at 420 nm. One unit of enzyme was defined as the amount of enzyme that liberated 1 micromole of *p*-nitrophenol per minute.

Measurement of exo-glycosidase activity

The activities of exo-glycosidases, including β-*N*-acetylglucosaminidase, β-galactosidase, α-galactosidase, β-glucosidase, α-glucosidase, β-mannosidase, α-mannosidase and β-xylosidase were determined by measuring the liberation of *p*-nitrophenol (as the phenolate anion) from their respective glycosides. In the standard assay, 0.05 mL of enzyme solution was incubated with 0.5 mL of 4 mM *p*-nitrophenyl glycoside in 20 mM sodium citrate buffer pH 4.0, at 50°C for 10 min, and the *p*-nitrophenol formed was measured as described above. One unit of exo-glycosidase was defined as the amount of enzyme that librated 1 micromole of *p*-nitrophenol per minute.

Measurement of chitinase and chitosanase activities

A mixture of 0.15 mL of 0.1 M sodium acetate buffer, pH 4.5, 0.1 mL of 1% ethylene glycol chitin or 0.5% chitosan in the same buffer, and 0.1 mL of H_2O was equilibrated at 37°C for 5 min. After addition of 50 μL of an appropriate dilution of enzyme, the reaction was allowed to proceed at 37°C for 30 min. The reducing sugar produced was measured colorimetrically as described (Dygert et al., 1965). One chitinase or chitosanase unit was defined as the amount of enzyme that produced 1 micromole of *N*-acetyl-D-glucosamine or D-glucosamine per minute.

Measurement of protease activity

Protease activity was measured using casein as a substrate according to the method of Anson (1939) with some modifications. A mixture of 5 mL of 0.6% casein (in 0.05 M sodium phosphate buffer, pH 7.0) and 1 mL of an appropriate dilution of enzyme was incubated at 37°C for 10 min as described previously (Chiang et al., 2005).

Purification of β-NAHA

Lyophilized fig latex, 300 mg, was dissolved in 6 mL of 50 mM sodium acetate buffer containing 0.5 M NaCl, pH 4.5. After centrifugation (20 min at 10,000 *g*), the supernatant was applied to a *p*APMA-Sepharose CL-4B column (1.6 × 10 cm) pre-equilibrated with 50 mM sodium acetate

buffer containing 0.5 M NaCl, pH 4.5, for affinity adsorption of cysteine proteases. After sample application, the column was washed with the same buffer at a flow rate of 30 mL/h to elute the non-bound β-NAHA, and then with 0.15 M 2-mercaptoethanol in the equilibrium buffter to elute the bound proteases; 1 mL fractions were collected. Each fraction was monitored for absorbance at 280 nm as well as β-NAHA and protease activities. Fractions containing β-NAHA were pooled, concentrated by ultrafiltration on an Amicon Centricon Plus-20 (NMWC 5,000) filter. The concentrated enzyme solution was applied to a Sephacryl S-100 HR column pre-equilibrated with 0.025 M imidazole-HCl buffer, pH 7.4 and eluted with the equilibrium buffer at a flow rate of 30 mL/h; 10 mL fractions were collected. The β-NAHA was eluted in minor (P1) and major (P2) activity peaks. Fractions containing major peak of β-NAHA were pooled, concentrated by ultrafiltration and applied to a DEAE-Sephacel column pre-equilibrated with 0.025 M imidazole-HCl buffer, pH 7.4. β-NAHA adsorbed on the column was eluted with a linear NaCl gradient (0-0.5 M) in equilibrium buffer at a flow rate of 30 mL/h; 2 mL fractions were collected. Each fractions was monitored for absorbance at 280 nm and β-NAHA activity. Fractions containing β-NAHA were pooled, concentrated by ultrafiltration and desalted by gel filtration on a Superose 6 HR 10/30 column using 0.025 M imidazole-HCl buffer as elution buffer. The purified enzyme was stored at -10°C until use.

Determination of optimum pH

The optimum pH for the purified β-NAHA was assayed in universal buffer (Britton and Robinson type; Dawson et al., 1969) at pH values ranging from 3 to 10 at 40°C.

Determination of optimum temperature and thermal stability

The optimum temperature for the purified β-NAHA was assayed at temperatures ranging from 30 to 80°C at pH 4.5. To assay thermal stability, aliquots of enzyme solutions were incubated in a thermostatic water bath set to temperatures ranging from 30 to 60°C for 5 to 60 min. The enzymatic activity remaining in each sample was then measured at 40°C, pH 4.5, as described above. The β-NAHA activity in the unheated enzyme solution represented the initial activity (100%) from which the log proportion (percent) of the activity remaining after heat treatment was calculated.

Determination of Michaelis constant and Hill coefficient

The Michaelis constant (K_m) and maximum velocities (V_{max}) of *p*NP-β-GlcNAc hydrolysis were determined at substrate concentrations ranging from 0.5 to 5 mM at pH 4.5 and 40°C. The K_m and V_{max} of β-NAHA for *p*NP-β-GlcNAc were calculated using the Lineweaver-Burk reciprocal plot method (Lineweaver and Burk, 1934). The Hill coefficient of β-NAHA for *p*NP-β-GlcNAc was calculated from the slope of Hill plot ($\log[V/(V_{max} - V)]$ against log (S), where V is the initial velocity and S is substrate concentration).

Determination of activation energy

The activation energy of β-NAHA for *p*NP-β-GlcNAc hydrolysis was determined by plotting log V_{max} versus 1/T (Segal, 1976).

Determination of active sites

The number of active sites in the purified β-NAHA was determined using an equimolar mixture of the two substrates, *p*NP-β-GlcNAc and *p*NP-β-GalNAc, at concentrations from 0.5 mM to 5 mM for each substrate. The experimental maximum velocity of the enzyme incubated with the mixed substrates was calculated from the Lineweaver-Burk plots (Lineweaver and Burk, 1934). The theoretical maximum velocity of β-NAHA incubated with the mixed substrates, V_{max}^{A+B} was calculated by the following equation (Calvo et al., 1978):

$$\frac{K_m^A}{\alpha \cdot K_m^B} = \frac{V_{max}^A - V_{max}^{A+B}}{V_{max}^{A+B} - V_{max}^B}$$

where K_m^A, K_m^B, V_{max}^A, V_{max}^B are the Michaelis constants and maximum velocities for each substrate, as determined from separate reactions of β-NAHA with each of the substrates, A (*p*NP-β-GlcNAc) and B (*p*NP-β-GalNAc). In this case, α, the ratio of the concentration of substrates = 1.

Determination of native molecular mass

The native molecular mass of the purified β-NAHA was determined by gel filtration (Andrews, 1965) on an FPLC system (Amersham Pharmacia Biotech AB, Uppsa, Sweden) with a Superdex 75 HR 10/30 column using Pharmacia low molecular weight gel filtration calibration kit (Uppsa, Sweden) as marker proteins. The native molecular mass of β-NAHA was calculated from the calibration curve. The native molecular mass of minor peak of β-NAHA was determined on an FPLC system with a Superpose 6 HR 10/30 column using Pharmacia high molecular weight gel filtration calibration kit (Uppsala, Sweden) as marker proteins.

Isoelectric focusing electrophoresis (IEF) and gel activity staining

The isoelectric point (p*I*) of β-NAHA was determined by IEF using PhastGel IEF 3-9 gel and gel activity staining. A detailed procedure for IEF is outlined in the Pharmacia PhastSystem Separation Technique File No. 100. After electrophoresis, the gels were stained for protein with Coomassie Brilliant Blue R-250 (CBR) or for β-NAHA activity with naphthol AS-BI-β-*N*-acetyl-D-glucosaminide as a substrate and new fuchsin as a diazo color reagent (Hayashi, 1965; Loida et al., 1979). An aliquot of the standard p*I* marker proteins mixture from the Pharmacia p*I* calibration kit was run beside the β-NAHA

sample. The R_f values measured directly from the gel were used to construct the p*I* calibration curve. The R_f value of the β-NAHA band was used to estimate the p*I* value of β-NAHA from the calibration curve.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and gel activity staining

SDS-PAGE was carried out with a PhastGel gradient 8-25% using the Pharmacia PhastSystem flat bed apparatus (Amersham Pharmacia Biotech AB; Uppsala, Sweden). The detailed procedure is outlined in the Pharmacia PhastSystem Separation Technique File No. 110. Before being loaded onto the gel, the enzyme solution was treated with an equal volume of sample buffer containing only 5% SDS followed by heating at 50°C for 30 min. After electrophoresis, renaturation of enzyme activity was carried out in 0.1 M citric acid-sodium phosphate buffer pH 6.0, containing 1% (v/v) Triton X-100 for 30 min at room temperature (25°C). β-NAHA activity was detected as described above (Hayashi, 1965). Proteins were stained with CBR.

Effect of metal ions and other compounds

Aliquots of purified β-NAHA (25 μL) were incubated at 25°C for 30 min with 25 μL of deionized water as control or 25 μL of one of the following compounds at concentrations of 0.05 to 10 mM: $HgCl_2$, $AgNO_3$, $CuCl_2$, $CaCl_2$, $MgCl_2$, $ZnCl_2$, NaCl, KCl, EDTA, SDS and BSA. Following treatment, enzyme activity in each sample was measured and expressed as a relative activity percentage calculated from the ratio of the specific activity of the treated β-NAHA to that of the untreated sample.

Effect of chemical modification reagents

Purified β-NAHA was concentrated by ultrafiltration on an Amicon Centricon Plus-20 (NMWC 5000) filter. After concentration, 0.5 mL aliquots of the enzyme solution were incubated at 30°C for 30 min with 0.5 mL of corresponding reaction buffer as the control or 0.5 mL of chemical modification reagent. After dialysis against 0.05 M sodium acetate buffer, pH 5.0, the enzyme activity in each sample was measured and expressed as the relative activity percentage calculated from the ratio of the specific activity of β-NAHA treated with chemical modification reagents to that of the non-treated sample.

Data analysis

For analysis of effects of metal ions and chemical modification reagents on enzyme activity, analytic measurements were performed in triplicate. Other analytic measurements were performed in duplicate.

RESULTS AND DISCUSSION

Glycosidase activities in fig latex

As shown in Table 1, fig latex was shown to contain a variety of glycosidase activities in addition to proteases, chitinase and chitosanase activities. The highest level of glycosidase activity was seen for α-galactosidase, followed by activities for β-NAHA, β-galactosidase, β-glucosidase, α-mannosidase, and β-xylosidase. No activities for α-glucosidase, β-mannosidase and β-fucosidase were detected. Plant glycosidases that catalyze the hydrolysis of aryl and alkyl glycosides have been found to be involved in a wide variety of biological processes (Dey and Campillo, 1984). In plant latex, glycosidases may be involved in the cell-wall degradation process during differentiation of articulated laticifers (Gioradani and Noat, 1988). Li and Li (1972) isolated α-galactosidase from fig latex and found that this enzyme is involved in cell-wall degradation, and is useful for the study of the sequence and anomeric configuration of α-galactosyl residues in the oligosaccharide chains of various complex carbohydrates.

Table 1. Protease, chitinase, chitosanase and glycosidase activities in fig latex.

Enzyme	Substrate	Activity[a] (mU/g powder)[b]
Protease	Casein	$6{,}140\times10^3$
Chitinase	Ethylene glycol chitin	35,925
Chitosanase	Chitosan	81,840
α-Galactosidase	*p*NP-α-Gal	19,775
β-*N*-acetylhexosaminidase	*p*NP-β-GlcNAc	8,866
β-Galactosidase	*p*NP-β-Gal	4,956
β-Glucosidase	*p*NP-β-Glc	4,932
α-Mannosidase	*p*NP-α-Man	2,391
β-Xylosidase	*p*NP-β-Xyl	596

[a]One unit of protease is defined as the amount of enzyme needed to produce 1 μg of tyrosine per minute from casein at 37°C. One unit of chitinase is defined as the amount of enzyme needed to produce 1 micromole of GlcNAc per minute from ethylene glycol chitin at 37°C. One unit of chitosanase is defined as the amount of enzyme needed to produce 1 micromole of GlcN per minute from chitosan at 37°C. Units of glycosidases for hydrolysis of various *p*-nitrophenylglycoside substrates are defined as the amount of enzyme needed to produce 1 micromole of *p*-nitrophenol per minute at 50°C at pH 4.0.

[b]One mU is 10^{-3} unit.

Table 2. Purification of β-*N*-acetylhexosaminidase from fig latex[a].

Step	Total volume (mL)	Total activity (mU)[b]	Total protein (mg)	Specific activity (mU/mg)	Purification (fold)	Yield (%)
Crude extract	4.4	1,339	66.4	20.2	1	100
*p*APMA-Sepharose affinity removal of thiol proteases	13	1,151	35.8	32.2	1.6	86
Sephacryl S-100 gel filtration						
P1 (minor)	10	145.4	0.08	1,818	90	11
P2 (major)	20	819.7	7.75	105.8	5.2	62
P2 Purification						
DEAE-Sephacel	16.2	758.3	0.012	61,525	3,046	57

[a]Data were obtained from 300 mg of lyophilized fig latex. [b]One mU is 1×10^{-3} unit.

Purification of β-NAHA

Purification of β-NAHA using the protocols summarized in Table 2 enabled us to remove proteolytic enzymes and isolate a predominant form of β-NAHA from fig latex. The β-NAHA was purified 3,046 fold, and the total yield was 57%. Affinity chromatography on a *p*APMA-Sepharose CL-4B column was initially used for the adsorption of thiol proteases. Neither β-NAHA nor chitinolytic enzymes were adsorbed and were eluted directly by washing the column with equilibrium buffer. Thiol proteases were adsorbed on the column and were eluted by equilibrium buffer containing 0.15 M 2-mercaptoethanol (Figure 1A).

After affinity chromatography, the β-NAHA was further purified by gel filtration on a Sephacryl S-100 HR column. As shown in Figure 1B, the β-NAHA was resolved into minor and major peaks. The minor peak was eluted in fractions 23-24 (indicated as P1). The predominant form was eluted in the major peak in fractions 47-51 (indicated as P2). This predominant isoform was further purified on a DEAE-Sephacel column. β-NAHA was bound to the column at pH 7.4 and eluted with a linear NaCl gradient (0-0.5 M) in equilibrium buffer (Figure 1C). Most of the inactive proteins were removed by this step. After being desalted on Superose 6 HR, the purified β-NAHA was concentrated and subjected to SDS-PAGE and gel activity staining. As shown in Figure 2A and B, both CBR and activity staining revealed a single band with a molecular mass of 12.4 kDa. These results indicated that the purified enzyme was almost homogeneous.

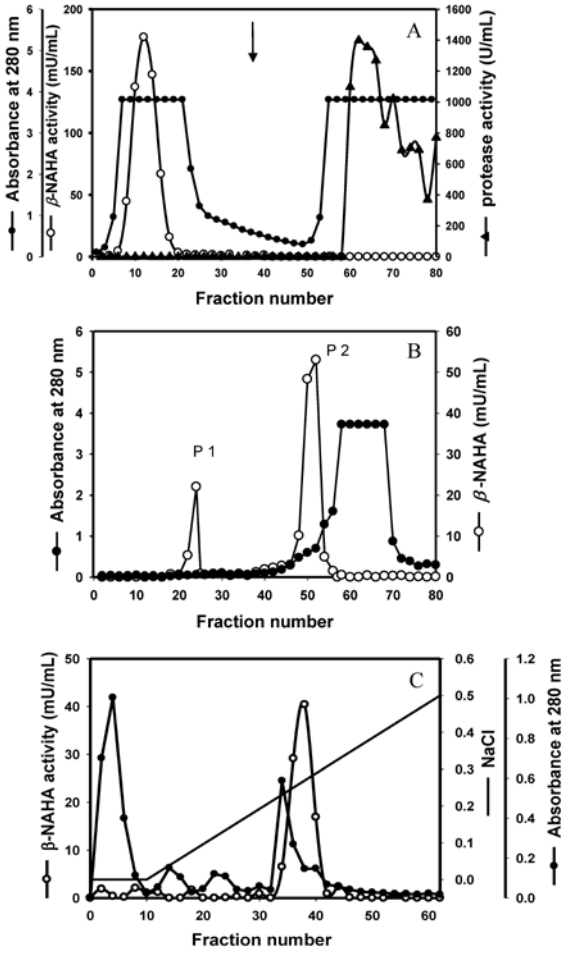

Figure 1. Purification of β-N-acetylhexosaminidase from fig latex in three chromatography steps, affinity chromatography on *p*APMA-Sepharose CL-4B (A), Sephacryl S-100 HR gel filtration (B), and DEAE-Sephacel ion-exchange chromatography (C). The *p*APMA-Sepharose CL-4B column (1.6 × 10 cm) was equilibrated with 50 mM sodium acetate buffer containing 0.5 M NaCl, pH 4.5, after which the crude extract from fig latex was applied. Unbound proteins were eluted with equilibrium buffer; bound proteases were eluted with equilibrium buffer containing 0.15 M 2-mercaptoethanol (arrow indicates beginning of elution) at a flow rate of 30 mL/h; 1 mL fractions were collected. The Sephacryl S-100 HR column (2.6 × 70 cm) was equilibrated with 0.025 M imidazole-HCl buffer, pH 7.4, after which the β-NAHA recovered from the *p*APMA-Sepharose CL-4B column was applied. The enzyme was eluted with the same buffer at a flow rate of 30 mL/h; 10 mL fractions were collected. The DEAE-Sephacel column (1.6 × 10 cm) was equilibrated with 0.025 M imidazole-HCl buffer, pH 7.4, after which the predominant peak of β-NAHA (P2) from the Sephacryl S-100 HR column was applied. Bound proteins were eluted with a linear NaCl gradient (0-0.5M) in equilibrium buffer at a flow rate of 30 mL/h; 2 mL fractions were collected.

Optimum pH, optimum temperature and thermal stability

As shown in Figure 3A, the purified β-NAHA retained 60-100% activity between pH 3 and 6, but was rapidly inactivated above pH 6. The optimum pH of the enzyme was 4.5. This is in the range of the values reported for β-NAHA from other sources, from pH 4.0 in cabbage (Chang et al., 1998) to pH 6.0 in fenugreek seeds and apple (Bouquelet and Spik, 1976; Choi and Gross, 1994).

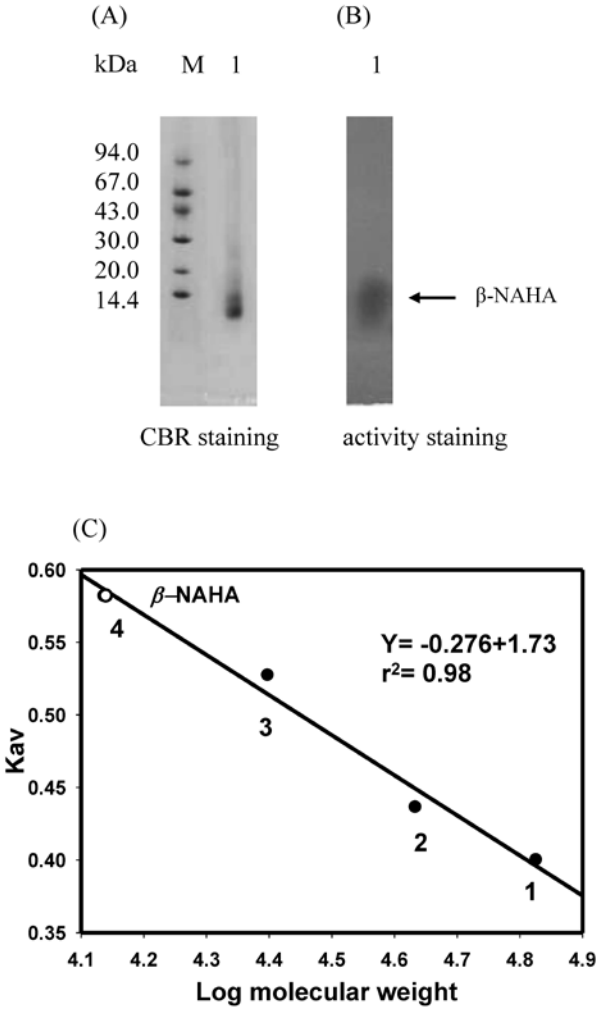

Figure 2. SDS-PAGE and determination of native molecular mass of β-NAHA by gel filtration. SDS-PAGE was performed with a PhastGel gradient 8-25% and PhastGel SDS buffer strips. After electrophoresis, the gels were subjected to coomassie blue staining (A) or β-NAHA activity staining (B) Lane 1 contained the purified β-NAHA. Lane M contained molecular mass marker proteins, including phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), chicken egg albumin (45.0 kDa), carbonic anhydrase (29.0 kDa), trypsin inhibitor (20.1 kDa), and lysozyme (14.4 kDa). The native molecular mass of the purified β-NAHA was estimated by gel filtration on a HiLoad Superdex 75 HR 16/60 column (C). The numbers 1 to 4 on the line of the molecular weight calibration curve indicate low molecular weight standard proteins bovine serum albumin (MW = 67 kDa), ovalbumin (MW = 43 kDa), chymotrypsinogen A (MW = 25 kDa), and ribonuclease A (MW = 13.7 kDa), respectively.

As shown in Figure 3B, β-NAHA exhibited 80-100% of its maximum activity, over a temperature range of 50-60°C, with a sharp decrease at temperatures above 60°C. The optimum temperature for enzyme activity was approximately 60°C.

To examine the thermal stability of β-NAHA, the enzyme was incubated at various temperatures (from 30 to 60°C) for 5 to 60 min and measured the residual activity under standard assay conditions. As shown in Figure 3C, the enzyme was fairly stable between 30 and 50. There was a relatively modest loss of activity at 55°C. At 60°C the enzyme was rapidly inactivated.

Molecular mass

The molecular mass of the predominate form of the β-NAHA was determined by gel filtration on Superdex 75 HR to be 13.7 kDa (Figure 2C). This value is close to the value of 12.4 kDa estimated from SDS-PAGE and gel activity staining of β-NAHA (Figure 2B), indicating the β-NAHA was a monomeric enzyme. The molecular mass of β-NAHA found in plants varies from 40 kDa in lupin seeds (McFarlane et al., 1984) to 236 kDa in apple cortical tissue (Choi and Gross, 1994). Different oligomeric structures of β-NAHA have also been described. The β-NAHA from apples was reported to be a homo-octamer (Choi and Gross, 1994). The β-NAHA from cabbage was reported to be a hetero-trimer with a molecular mass of 150 kDa (Chang et al., 1988). β-NAHA isolated from germinating fenugreek seeds was found to exist in 4 molecular forms, two trimers, a pentamer and a hexamer, each composed of identical subunits (Bouquelet and Spik, 1976; 1978). The β-NAHA from pinto bean was found to be a homo-dimer with a molecular mass of 112 kDa (Agrawal and Bahl, 1972). The β-NAHAs from lupin, rice seeds and maize seedlings were monomers with molecular masses of 40, 52 and 72 kDa, respectively (McFarlane et al., 1984; Jin et al., 2002; Oikawa et al., 2003). The molecular mass of the predominant form of β-NAHA isolated from fig latex was much lower than these previously reported values. However, the molecular mass of the minor form of β-NAHA (232 kDa; data not shown) is similar to the values reported for pea seeds (210 kDa; Harley and Beevers, 1987) and apples (236 kDa; Chio and Gross, 1994).

Isoelectric point

As shown in Figure 4, CBR staining of the IEF gel revealed a major protein band (Figure 4A) which exhibited β-NAHA activity on gel activity staining (Figure 4B). The isoelectric point of the purified β-NAHA was found to be 3.5 as determined by comparing the relative mobility (R_f) of β-NAHA with that of the p*I* markers developed in the same IEF gel (Figure 4C). This value is lower than published data for other β-NAHAs, for which, most of the p*I* values reported for plant β-NAHAs are in the range of 4.65 to 6.78 (Bouquelet and Spik, 1978; Carratu et al., 1985; Barber and Ride, 1989; Oikawa et al., 2003).

Effect of metal ions and various compounds

As shown in Table 3, the purified β-NAHA was inhibited by 0.25 mM Ag^{+} and Hg^{2+} and slightly inhibited by 5 mM Cu^{2+}. Similar inhibitory effects of Ag^{+} and Hg^{2+} were previously seen on β-NAHA from jack bean meal (Li and Li, 1970), the four molecular forms of β-NAHA from fenugreek seeds (Bouquelet and Spik, 1978) and three isoforms of β-NAHA from mungbean seedlings (Chen et al., 2004). Other metal ions and compounds studied had no significant effect on the activity of the enzyme.

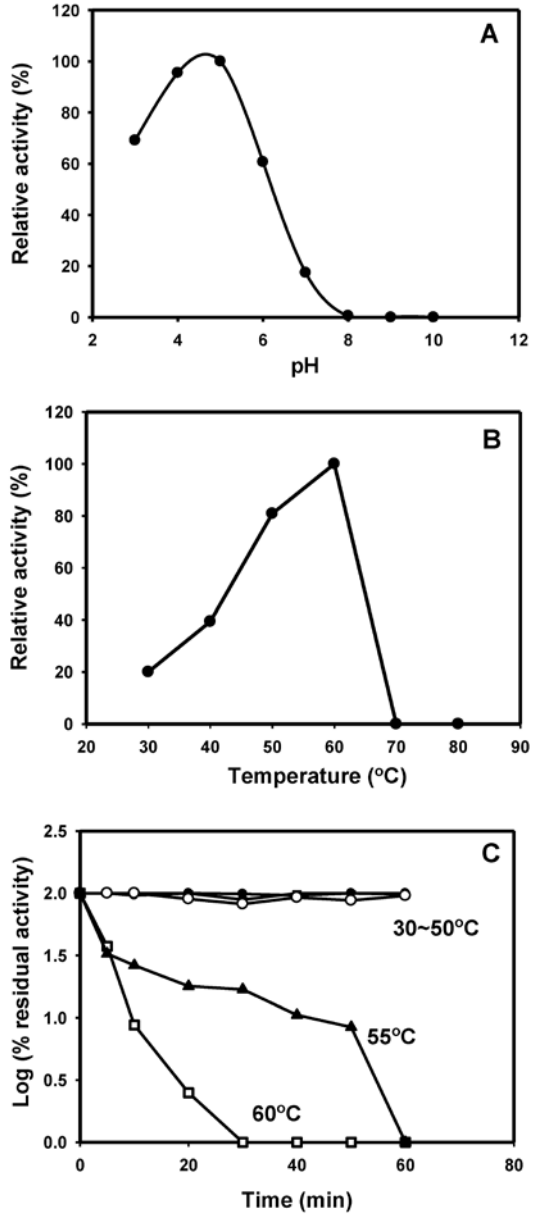

Figure 3. Optimum of pH (A), optimum temperature (B) and thermal stability (C) of β-NAHA. For determination of optimum pH, β-NAHA activity was assayed in a universal buffer, at pH 3 to 10. For determination of optimum temperature, β-NAHA activity was assayed at pH 4.5 from 30 to 80°C. Thermal stability was measured by incubating the enzyme for 0-60 min at 30, 40, 50, 55 or 60°C prior to assaying.

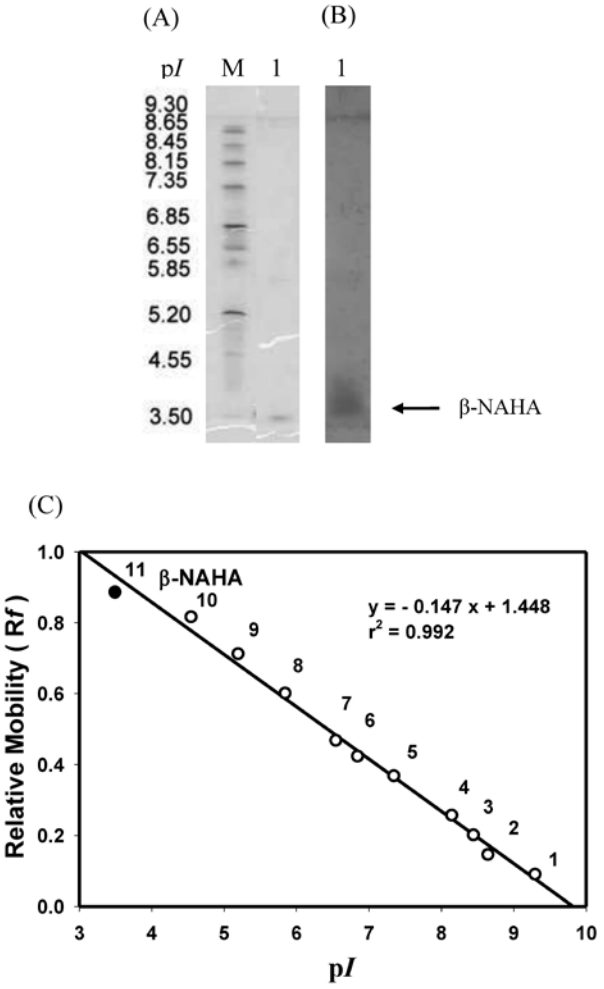

Figure 4. Determination of p*I* of β-NAHA by isoelectric focusing electrophoresis: (A) CBR staining, (B) gel activity staining, (C) p*I* calibration curve. Electrophoresis was performed in a PhastGel IEF 3-9 gel. Lane 1 contained purified β-NAHA. Lane M contained p*I* marker proteins from a p*I* calibration kit. The numbers 1 to 11 on the line of p*I* calibration curve including p*I* marker proteins trypsinogen (p*I* = 9.3), lentil lectin-basic band (p*I* = 8.65), lentil lectin-middle band (p*I* = 8.45), lentil lectin-acidic band (p*I* = 8.15), myoglobin-basic band (p*I* = 7.35), myoglobin-acidic band (p*I* = 6.85), human carbonic anhydrase B (p*I* = 6.55), bovine carbonic anhydrase B (p*I* = 5.85), β-lactoglobulin A (p*I* = 5.20), soybean trypsin inhibitor (p*I* = 4.55), and amyloglucosidase (p*I* = 3.55), respectively.

Effect of chemical modification reagents

It is generally believed that an enzyme will be inhibited or inactivated if the amino acid side chain involved in catalytic activity is chemically modified. In this study, a number of reagents were used to modify the side chain of β-NAHA (Table 4). Among the chemical modification reagents tested, 2.5 mM DEPC, a reagent that usually acts on the histidine residues in proteins, completely inhibited enzyme activity. Therefore, the imidazole group (histidine side chain) appears to be essential for the catalytic activity of the enzyme. However, it should be noted that the

activity of this enzyme was also inhibited from 15.9% to 26.9% by DNFB, PMSF, *p*HMB and CHD. DNFB is a chemical modification reagent that usually acts selectively on the lysine residues in proteins, whereas PMSF, *p*HMB and CHD act on serine, cysteine and arginine residues, respectively. Therefore, lysine, serine, cysteine and arginine residues may also be related to enzyme activity. These residues may not be located at the active site of the enzyme, but instead may be involved in maintenance of conformation of the enzyme. Not much information has been reported on enzyme inhibition by chemical modifications. Fenugreek β-NAHAs was inhibited by 1 mM *p*HMB (Bouquelet and Spik, 1978). Mung bean β-NAHA III was inhibited by 5-5′-dithiobis (2-nitrobenzoic acid), a reagent for the selective chemical modification of cysteine residues (Chen et al., 2004). Rice β-NAHA was inhibited by 1 mM chloramine T (Jin et al., 2002), a potential chemical modifier of tryptophan residues in proteins.

Activation energy

An Arrhenius plot obtained by plotting V_{max} versus 1/T, was used to determine the activation energy of the enzyme for catalysis of *p*NP-β-GlcNAc hydrolysis. The Arrhenius plot for β-NAHA showed a straight line. The activation energy (Ea) calculated from the line was 8.93 kcal/mol. Not much information is available on the activation energy of plant β-NAHA-catalyzed reactions. The activation energy of β-NAHA from pinto beans (Agrawal and Bahl, 1972) and the latex of *Ficus glavrata* (Orlacchio et al., 1985) for the catalysis of *p*NP-β-GlcNAc hydrolysis was found to be 9.8 and 13.2 kcal/mol, respectively.

Kinetic properties

To determine the kinetic properties of β-NAHA purified from fig latex, the enzymatic activity was assayed using *p*NP-β-GlcNAc, *p*NP-β-GalNAc, and an equimolar mixture of the two at various concentrations. The kinetic parameters, K_m and V_{max}, of β-NAHA obtained from Lineweaver-Burk plots are summarized in Table 5. The K_m values for *p*NP-β-GlcNAc and *p*NP-β-GalNAc were 1.3 and 0.7 mM, respectively, whereas the V_{max} values were 6.0 and 1.8 μmol min^{-1} mg^{-1}, respectively. These results indicate that the purified β-NAHA had a higher specific constant (V_{max}/K_m) for *p*NP-β-GlcNAc than for *p*NP-β-GalNAc. Therefore, *p*NP-β-GlcNAc appears to be the preferred substrate for the enzyme. The kinetics of competition was investigated by using a mixed substrates to determine whether the β-NAHA had a single active site for both substrates or 2 separate active sites, one for each substrate. The experimental V_{max} value for β-NAHA determined from the Lineweaver-Burk plot was the same as the theoretical value (Table 5, V_{max}^{A+B}). This finding indicates that both substrates used a single active site. If an enzyme catalyzes the hydrolysis of two substrates at the same active site, the total rate of reaction will be less than the sum of the rates of the separate reactions with each substrate (Dixon and Webb, 1964). Because one active site is used, there is a competition between the substrates. As shown in

Table 3. Effect of metal ions and other compounds on the activity of β-NAHA.

Reagent[a]	Final concentration (mM)	Relative activity (%)[b]
None	--	100
$HgCl_2$	0.25	0 ± 0.5
$AgNO_3$	0.25	69.4 ± 5.0
$AgNO_3$	1	69.1 ± 2.7
$CuCl_2$	5	82.0 ± 1.5
$CaCl_2$	5	94.5 ± 1.0
$MgCl_2$	5	107.7 ± 0.2
$ZnCl_2$	5	96.0 ± 1.1
NaCl	5	99.6 ± 5.1
KCl	5	96.7 ± 4.6
EDTA	5	106.0 ± 0.9
BSA	0.1	103.6 ± 3.5
SDS	0.5	105.6 ± 2.9
SDS	0.1	107.0 ± 1.2

[a]BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; EDTA, ethylenediamine tetraacetic acid.
[b]Data are represented as mean ± standard deviation (n = 3).

Table 4. Effect of various chemical modification reagents on the activity of β-NAHA.

Reagent[a]	Final concentration (mM)	Relative activity (%)[b]
None	--	100
NBS	0.5	100.9±1.2
NBS	5	102.6±0.3
EAM	250	104.2±6.5
NAI	2.5	101.4±1.1
DNFB	2.5	84.1±0.8
PMSF	2.5	73.1±7.7
DEPC	2.5	0±0.3
WRK	50	106.3±5.2
pHMB	0.5	75.9±0.6
CHD	1.25	75.1±2.6

[a]NBS, in 50 mM sodium acetate buffer, pH 4.0 (Viswanatha and Lanwson, 1961); EAM, in 0.2 M sodium phosphate buffer, pH 8.0 (Shaw and Chang, 1988); NAI, in 0.1 M sodium phosphate buffer, pH 8.0 (Yamasaki et al., 1989); DNFB, in 0.1 M sodium phosphate buffer, pH 8.0 (Welches and Baldwin, 1981); PMSF, in 0.2 M sodium phosphate buffer, pH 8.0 (Gold and Fahrney, 1964); DEPC, in 0.1 M sodium phosphate buffer, pH 6.0 (Miles, 1977); WRK, in 0.25 M sodium phosphate buffer, pH 6.0 (Vangrysperre et al., 1989); *p*HMB, in 50 mM sodium phosphate buffer, pH 7.0 (Liu and Speer, 1977); CHD, in 0.1M sodium phosphate buffer, pH 8.0 (Liu et al, 1968).
[b]Data are represented as mean ± standard deviation (n = 3).

Table 5. Kinetic constants for the study of the competition between *p*NP-β-GlcNAc and *p*NP-β-GalNAc for the active sites of β-NAHA.

Substrate	K_m (mM)	V_{max} (μmol min^{-1} mg^{-1})	$V^A_{max} + V^B_{max}$ (μmol min^{-1} mg^{-1})	V^{A+B}_{max} (μmol min^{-1} mg^{-1})[a]		V_{max} / K_m (μmol min^{-1} mg^{-1})×(mM)$^{-1}$
				Theoretical	Experimental	
*p*NP-β-GlcNAc (A)	1.3	6.0	7.8	3.3	3.3	4.6
*p*NP-β-GalNAc (B)	0.7	1.8				2.6

[a]Theoretical V_{max} determined according to the formula: $K^A_m / \alpha \cdot K^B_m = (V^A_{max} - V^{A+B}_{max}) / (V^{A+B}_{max} - V^B_{max})$, where $\alpha = 1$.

Table 5, the rate of the hydrolysis of the mixed substrates (V^{A+B}_{max} = 3.3 μmol min^{-1} mg^{-1}) was less than the sum of the rates obtained with the separate substrates ($V^A_{max} + V^B_{max}$ = 7.8 μmol min^{-1} mg^{-1}). The total rate of the reaction was 3.3 μmol min^{-1} mg^{-1}, which is less than the sum of the individual reactions, 6.0 + 1.8 = 7.8 μmol min^{-1} mg^{-1}. From our kinetic assays, it may be deduced that the purified β-NAHA had a single active site for both substrates. These results agree with those for the enzyme isolated from jack bean meal (Li and Li, 1970), Golden Delicious-apples (Choi and Gross, 1991) and rice seeds (Jin et al., 2002).

Substrate specificity

As shown in Table 6, the purified β-NAHA showed specificity for β-glycosidic linkages. The ratio of the hydrolysis of *p*NP-β-GlcNAc to that of *p*NP-β-GalNAc was 2.2. This value is in the range of previously reported values: 1.25 to 18.0 for the 4 isozymes of β-NAHA from fenugreek seeds (Bouquelet and Spik, 1978), 1.9 to 4.8 for the three isozymes of β-NAHA from lupin seeds (McFarlane et al., 1984), 4.0 for the β-NAHA from rice seeds (Jin et al., 2002), and 3.1 to 3.6 for the 3 isoforms of β-NAHA from mungbean seedlings (Chen et al., 2004). Some plant β-NAHAs also degrade chitin and chitin oligomers. For instance, cotton seed β-NAHA liberates GlcNAc from chitin, ovalbumin and pronase-digested wheat germ lectin (Yi, 1981), wheat leaf β-NAHA (Barber and Ride, 1989) liberates GlcNAc from chitin oligomers, and maize β-NAHA (Oikawa et al., 2003) releases GlcNAc from chitin and chitin oligomers. Therefore, their participation in the process of chitin-elicited lignifications (Barber et al., 1989) and in the system of defense against chitinous pathogens (Hodge et al., 1996) have been suggested. In fenugreek seeds, four isoforms of β-NAHAs have been isolated (Bouwuel and Spok, 1978), and form 1 showed higher activity towards $GlcNAc_2$ compared to *p*NP-β-GlcNAc, while the other three forms had little activity for hydrolyzing $GlcNAc_2$. The β-NAHA purified from maize (Oikawa et al., 2003) also showed much higher activity for hydrolyzing $GlcNAc_2$ than for hydrolyzing *p*NP-β-GlcNAc. Therefore, there are at least two types of β-NAHAs in plant with respect to substrate specificity ultizing these two classes of substrate, *p*NP-β-GlcNAc and $GlcNAc_2$.

In conclusion, the β-NAHA isolated from fig latex is very similar in most properties to β-NAHA isolated from other plants. However, the molecular mass of the enzyme was much small than those of other plant β-NAHAs. From substrate specificity and competition kinetic studies, we found that the enzyme is specific for glycosidic bonds of β configuration and has a single active site for binding both *p*NP-β-GlcNAc and *p*NP-β-GalNAc substrates. The activity of this enzyme in the hydrolysis of chitin oligomers remains to be investigated to clarify whether this β-NAHA is involved in chitin degradation and participated in the defense system in fig latex.

Table 6. Substrate specificity of β-NAHA.

Substrate	Relative activity (%)
*p*NP-β-GlcNAc	100
*p*NP-β-GalNAc	45.5
*p*NP-α-GlcNAc	0
*p*NP-α-GalNAc	0

Acknowledgements. Financial support provided by the National Science Council (NSC-96-2313-B-126-003-MY3) of the Republic of China for this study is greatly appreciated.

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無花果乳汁 β-*N*-乙醯胺基六碳糖苷酶之生化性質研究

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無花果 (*Ficus carica* Linn.) 乳汁所含 β-N- 乙醯胺基六碳糖苷酶 (β-NAHA) 經由 *p*APMA-Sepharose CL-4B 管柱親和性吸附去除硫醇型蛋白酶、Sephacryl S-100 HR 膠體過濾層析和 DEAE-Sephacel 離子交換層析等三管柱層析步驟純化，可得一純化之主要 β-NAHA，以 SDS-PAGE 及 β-NAHA 活性染色分析顯示純化之 β-NAHA 幾乎已達均質純度。純化之 β-NAHA 具水解 *p*-nitrophenyl-N-acetyl-β-D-glucosaminide (*p*NP-β-GlcNAc) 及 *p*-nitrophenyl-N-acetyl-β-D-galactosaminide (*p*NP-β-GalNAc) 活性，其水解 *p*NP-β-GlcNAc 之最適 pH 為 4.5，最適溫度為 60°C，K_m 值為 1.3 mM 而 V_{max} 為 6.0 μmol min^{-1} mg^{-1}。以膠體過濾法測得酵素分子量為 13.7 kDa，以等電焦集電泳及活性染色測得酵素等電點為 pH 3.5, 熱穩定性分析顯示酵素於 30 至 50°C，保溫 60 分鐘幾無活性損失，頗為穩定，但 55°C 以上則顯著失去活性，重金屬離子 Hg^{2+} (0.25 mM) 及化學修飾劑 diethyl pyrocarbonate (2.5 mM) 顯著抑制酵素活性，以基質專一性及混合基質競爭動力學分析顯示純化之 β-NAHA 對 β 組態醣苷鍵具專一性，而催化水解 *p*NP-β-GlcNAc 和 *p*NP-β-GalNAc 二基質係屬同一活性中心。

關鍵詞：無花果 (*Ficus carica* Linn.) 乳汁 ; β-N- 乙醯胺基六碳糖苷酶 ; 純化 ; 性質。