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Characterisation of a β-*N*-acetylhexosaminidase from a commercial papaya latex preparation

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ABSTRACT

A β-*N*-acetylhexosaminidase (β-NAHA) (EC 3.2.1.52) with molecular mass of 64.1 kDa and isoelectric point of 5.5 was purified from a commercial papaya latex preparation. The optimum pH for *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*NP-β-GlcNAc) hydrolysis was five; the optimum temperature was 50 °C; the K_m was 0.18 mM, V_{max} was 37.6 μmol min^{-1} mg^{-1} and activation energy (E_a) was 10.3 kcal/mol. The enzyme was thermally stable after holding at 30–45 °C for 40 min, but its activity decreased significantly when the temperature exceeded 50 °C. Heavy metal ions, Ag^+ and Hg^{2+}, at a concentration of 0.25 mM and Zn^{2+} and Cu^{2+}, at a concentration of 0.5 mM, significantly inhibited enzyme activity. The β-NAHA had only one active site for binding both *p*NP-β-GlcNAc and *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (*p*NP-β-GalNAc). A prototropic group with p*Ka* value of about five on the enzyme may be involved in substrate binding and transformation, as examined by Dixon–Webb plots.

1. Introduction

β-*N*-acetylhexosaminidase (EC 3.2.1.52) is defined as an exo-type enzyme that hydrolyses *N*-acetylhexosaminides at the non-reducing end, whereby an *N*-acetylhexosamine, usually *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc), is released. The enzyme that catalyses the cleavage of β-*N*-acetylglucosaminide is known as β-*N*-acetylglucosaminidase, while an enzyme for β-*N*-acetylgalactosaminide has not been detected or has not been researched (Oikawa, Itoh, Ishihara, & Iwamura, 2003). In general, β-*N*-acetylhexosaminidases (β-NAHAs) hydrolyse the β-glycosides of *N*-acetylglucosaminide and *N*-acetylgalactosaminide; they are not specific for the aglycone group, although they prefer aryl substituents. These enzymes are widely distributed in nature and have now been detected in animal tissues, microorganisms, and plants (Conzelmann & Sandhoff, 1987). β-NAHAs are thought to participate in the processing and turnover of glycoprotein during germination in plants (Chen, Liu, Hsu, Lee, & Chen, 2004; Jin et al., 2002); they are considered to function in the metabolism of *N*-glycans during ripening in apple fruits (Choi & Gross, 1994). On the other hand, some plant β-NAHAs also degrade chitin and chitin oligomers (Barber & Ride, 1989; Li & Li, 1970). Therefore, their participation in the defence system, against chitinous pathogens in plants, has been suggested. However, since the natural substrate of β-NAHAs has not been identified in plants and substrate specificity has been examined only for a limited number of compounds, the physiological significance of the enzyme has yet to be elucidated.

Latex is the cytoplasm of highly specialised cells known as laticifers. Laticifers are anastomosed as a result of partial hydrolysis of adjacent walls and thus form a tube-like network or para circulatory system throughout the plant. It has been suggested that latex secretion provides a defence against wounds and/or predators such as insects and microorganisms. Latexes from different plants contain various defence-related proteins, such as chitinase, β-1,3-glucanase, hevamine and hevein (Broekaert, Lee, Kush, Chua, & Raikhel, 1990; Rozenboom, Budiani, Beintema, & Dijstra, 1990; Van Parijs, Broekaert, & Peumans, 1991). Papaya (*Carica papaya*) latex contains papain, multiple forms of chymopapain A, peroxidase, peptidase A and chitinase (Brockehurst & Salih, 1985; Huet et al., 2006; Park, Kim, Shin, & Noh, 1979).

Recently, we isolated a chitosanase from a commercial papaya latex preparation (Liao, Wu, Chiang, & Chang, 2006) and found that various glycosidases were present in the papaya latex preparation.

Abbreviations: CBR, Coomassie brilliant blue R-250; CHD, 1,2-cyclohexanedione; DEPC, diethyl pyrocarbonate; DNFB, 2,4-dinitro-1-fluorobenzene; EAM, ethyl acetamidate; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; IEF, isoelectric focusing electrophoresis; K_{av}, available distribution coefficient; K_m, Michaelis constant; β-NAHA, β-*N*-acetylhexosaminidase; *p*HMB, *p*-hydroxymercuribenzoate; p*I*, isoelectric point; PMSF, phenylmethanesulfonyl fluoride; *p*NP-β-GlcNAc, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide; *p*NP-β-GalNAc, *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide; Ve, elution volume; V_{max}, maximal velocity; Vo, void volume; Vt, total bed volume; WRK, *N*-ethyl-5-phenylisoazoline-3′-sulfonate (Woodward's reagent K).

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The highest level of glycosidase activity was exhibited by β-NAHAs. Here, we report on the purification and characterisation of a β-NAHA from a papaya latex preparation. Properties, including the molecular mass, isoelectric point, effectors and thermostability, of the purified β-NAHA and the effect of pH on the kinetic parameters and kinetics of competition for the enzyme with mixed substrates, were studied.

2. Materials and methods

2.1. Chemicals

Lyophilised papaya latex powder (P.3250), ethyl acetamidate (EAM), *p*-hydroxy-mercuribenzoate (sodium salt, *p*HMB), diethyl pyrocarbonate (DEPC), *N*-ethyl-5-phenylisoazoline-3′-sulfonate (Woodward's reagent K; WRK), phenylmethanesul-fonyl fluoride (PMSF), 1,2-cyclohexanedione (CHD), 2,4-dinitro-1-fluorobenzene (DNFB), *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*NP-β-GlcNAc), *p*-nitrophenyl-*N*-acetyl-α-D-glucosaminide (*p*NP-α-GlcNAc), *p*-nitrophenyl-*N*-acetyl-β-D-galactosaminide (*p*NP-β-GalNAc), *p*-nitrophenyl-*N*-acetyl-α-D-galactosaminide (*p*NP-α-GalNAc) and naphthol AS-BI *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were purchased from Sigma (St. Louis, MO). Sephacryl S-100 HR, Superose 12 HR 10/30, DEAE–Sephacel, PhastGel IEF 3–9, and isoelectric focusing markers (p*I* 3–10) were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). All other chemicals were of reagent grade or purer.

2.2. Measurement of β-NAHA activity

β-NAHA activity was determined by measuring the release of *p*-nitrophenol from *p*NP-β-GlcNAc according to the method previously described (Agrawal & Bahl, 1968). A mixture of 0.25 ml of 0.1 M sodium acetate buffer (pH 5.0), 0.5 ml of 5 mM *p*NP-β-GlcNAc, 0.2 ml of water and 0.05 ml of enzyme solution was incubated at 37 °C for 20 min, and the reaction was then stopped by the addition of 1 ml of 0.25 M Na_2CO_3. The amount of liberated *p*-nitrophenol was measured by determining the absorbance at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol per minute.

2.3. Preparation of crude enzyme

Five grammes of commercial lyophilised papaya latex were dissolved in 120 ml of 25 mM imidazole–HCl buffer (pH 9.0) containing 1.66 mM *p*HMB (Liao et al., 2006). The mixture was stirred for 20 min. The insoluble substances were removed by centrifugation (6000*g* for 20 min). The resulting supernatant was dialysed against 2 l of 0.14 M sodium phosphate buffer (pH 7.2) overnight at 4 °C. The precipitated material was removed by centrifugation (6000*g*, 20 min) and the resulting supernatant was designated as crude β-NAHA.

2.4. Ammonium sulfate fractionation

Crude β-NAHA was fractionated by the addition of $(NH_4)_2SO_4$ (Gulcin, Kufrevioglu, & Oktay, 2005). The precipitate formed between 50% and 80% saturation of $(NH_4)_2SO_4$ was collected by centrifugation (17,540*g*, 10 min) and dissolved in 10 ml of 0.14 M sodium phosphate buffer (pH 7.2).

2.5. Sephacryl S-100 HR gel filtration

The β-NAHA obtained from the above $(NH_4)_2SO_4$ fractionation was concentrated by ultrafiltration with a 10 kDa molecular weight cutoff (MWCO) membrane (Millipor, Corrigtwohill, Co., Cork, Ireland) and applied to a Sephacryl S-100 HR column (2.6 × 70 cm) pre-equilibrated with 0.14 M sodium phosphate buffer (pH 7.2) (Liao et al., 2006). Fractions containing β-NAHA were pooled.

2.6. DEAE–Sephacel ion-exchange chromatography

The β-NAHA obtained from the Sephacryl S-100 HR column was dialysed against 25 mM imidazole–HCl buffer (pH 7.4), concentrated by ultrafiltration with a 10 kDa molecular weight cutoff (MWCO) membrane (Millipor, Corrigtwohill, Co., Cork, Ireland), and then applied to a DEAE–Sephacel column (1.0 × 20 cm) pre-equilibrated with 25 mM imidazole–HCl (pH 7.4). After sample absorption, the column was washed with equilibrium buffer until most of the non-bound protein was eluted. The bound β-NAHA was eluted from the column with a linear NaCl gradient (0–0.5 M) in 25 mM imidazole–HCl (pH 7.4) at a flow rate of 30 ml/h, and the fractions (2 ml) containing the enzyme were pooled (Chang, Young, Chang, & Sung, 1998).

2.7. Determination of optimum pH and optimum temperature

The optimum pH for β-NAHA was identified from pH 2 to 10 using a universal buffer (Britton and Robinson type) (Dawson, Elliott, Elliott, & Jones, 1969) at 37 °C. The optimum temperature for β-NAHA was identified at pH 5 from 30 to 80 °C.

2.8. Determination of thermal stability

To assess thermal stability, the enzyme solutions were immersed in a thermostatic water bath for 1.25–40 min at various temperatures (from 30 to 80 °C). The remaining activities were measured as described in Section 2.2.

2.9. Determination of Michaelis constant, maximal velocity and Hill coefficient

The Michaelis constant (K_m) and maximal velocity (V_{max}) of *p*NP-β-GlcNAc hydrolysis were determined at substrate concentrations ranging from 0.25 to 5 mM at pH 5.0 and 37 °C (Sisecioglu, Cankaya, Guicin, & Ozdemir, 2009).

The K_m and V_{max} of β-NAHA for *p*NP-β-GlcNAc were calculated using the Lineweaver–Burk reciprocal plot. The Hill coefficient of β-NAHA for *p*NP-β-GlcNAc was calculated from the slope of the Hill plot, a plot of $\log V/(V_{max}-V)$ versus $\log (S)$, where V is the initial velocity and S is the substrate concentration.

2.10. Determination of activation energy

The relationship between the rate constant of a reaction, k, and the activation energy, E_a, is given by the Arrhenius equation as

$$k = k_0 \times e^{-E_a/RT}$$

$$\log k = \frac{-E_a}{2.303R}(1/T) + \log k_0$$

where k_0 is the frequency factor (per minute), E_a is the activation energy (kcal/mol or kJ/mol), R is the universal gas constant (1.987 cal/mol K or 8.314 J/mol K), and T is the absolute temperature (K = °C + 273.15).

A plot of $\log k$ versus $1/T$ is linear. In practise, a plot of $\log V_{max}$ versus $1/T$ yields the E_a for the catalytic step since the V_{max} of a given preparation is proportional to k ($V_{max}/[E]_t = k$).

The maximal velocity of β-NAHA for *p*NP-β-GlcNAc hydrolysis at various temperatures (30–50 °C) was determined. The activation