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Biochemistry

β-*N*-Acetylhexosaminidase involvement in α-conglutin mobilization in *Lupinus albus*

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ABSTRACT

Glycosylation is an important post-translational modification involved in the modulation of a wide variety of cellular processes. Because glycosydases are central, the aim of this study was to investigate the glycosyl activity present in the cotyledons of the seeds of an important crop legume, *Lupinus albus*, as well as potential natural substrates of the detected enzymes.

The glycosyl activity detected in the cotyledons beginning at seed imbibition and continuing until 9 days after, was due to a β-*N*-acetylhexosaminidase (β-NAHase), which was molecularly and biochemically characterized after purification. Two isoenzymes with molecular masses of 64 and 61 kDa were detected, each having five isoenzymes with p*I*s 5.3–5.6. The 64 and 61 kDa isoenzymes had the same protein core showing different degrees of glycosylation. The N-terminal sequence of the enzyme protein core was determined [VDSEDLI(EN)AFKIYVEDDNEHLQGSVD] and to our knowledge, is the first reported protein sequence from a plant β-NAHase.

L. albus β-NAHase had K_m values of 2.59 mM and 2.94 mM and V values of 18.40 μM min^{-1} and 2.73 μM min^{-1}, for *p*NP–GlcNAc and *p*NP–GalNAc, an optimum pH of 5.0 and 4.0 and temperature of 50 °C and 60 °C were detected toward *p*NP–GlcNAc and *p*NP–GalNAc. In the presence of $AgNO_3$, $CoCl_2$, $CuSO_4$, $FeCl_3$, $CdCl_2$ and $ZnCl_2$ the enzymatic activity decreased more than 50%, and when in the presence of sugars, an activity reduction of no more than 25% was observed.

A physiological role for β-NAHase in *L. albus* storage protein mobilization was investigated. β-NAHase has already been implicated in several biological processes, namely in glycoprotein processing during seed germination and seedling growth. However, the natural substrates used by this enzyme are not yet completely clarified.

By gathering *in vivo* and *in vitro* data for β-NAHase activity together with globulin degradation, we suggest that *L. albus* β-NAHase is involved in the mobilization of storage protein degradation, with α-conglutin being a potential natural substrate for this enzyme.

Introduction

Glycosylation, in eukaryotes, is an essential process involved in a number of cellular processes, including correct protein folding and protein protection against proteolytic degradation (Bernard, 2008; Aebi et al., 2009). In particular, *N*-acetylglucosamine (GlcNAc), an abundant hexose, is known to play multiple roles in cells either as a monomer or as part of macromolecules, especially as a residue of oligo- and polysaccharides and conjugated with lipids or proteins (Bernard, 2008).

A typical glycoside hydrolase involved in the cleavage of terminal GlcNAc, and also GalNAc, from the non-reducing end of oligosaccharides is β-*N*-acetylhexosaminidase (β-NAHase; EC 3.2.1.52). This enzyme has been shown to be universally distributed among most types of living organisms and, in contrast to the well studied mammalian, insect and fungal β-NAHases, only limited information is available on the corresponding plant enzymes (Slamova et al., 2010).

Three putative β-NAHases encoded by the *Arabidopsis thaliana* genome were cloned and analyzed with respect to their enzymatic

Abbreviations: DMS, dimethylsuberimidate; DW, dry weight; FW, fresh weight; β-ME, β-mercaptoethanol; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; β-NAHase, β-*N*-acetylhexosaminidase; *p*NP, *p*-nitrophenyl; *p*NP–GalNAc, *p*-nitrophenyl–β-D-*N*-acetylgalactosamine; *p*NP–GlcNAc, *p*-nitrophenyl–β-D-*N*-acetylglucosamine; *p*NP–$(GlcNAc)_2$, *p*-nitrophenyl–β-D-*N*,*N*′-diacetylchitobiose; *p*NP–$(GlcNAc)_3$, *p*-nitrophenyl–β-D-*N*,*N*′,*N*″-triacetylchitotriose; *p*NP–Glcp, *p*-nitrophenyl–α-D-glucopyranosyde.

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properties (Liebminger et al., 2011; Strasser et al., 2007). Since these enzymes were found to be located in different subcellular compartments, they were hypothesized to be involved in different cellular processes (Strasser et al., 2007).

In higher plants, β-NAHases are involved in the formation of paucimannosidic *N*-glycans, which are generated in late stages of the *N*-glycosylation pathway, by the removal of GlcNAc terminal residues of glycoproteins. Paucimannosidic *N*-glycans constitute the majority of glycans present on vacuolar glycoproteins, such as bean phaseolin (Sturm et al., 1987) and occur in smaller amounts on extracellular plant glycoproteins (Dirnberger et al., 2001; Takahashi et al., 1986) and cell wall bound proteins (Kotake et al., 2001). The presence of large amounts of truncated paucimannosidic *N*-glycans in plant glycoproteins indicates that the removal of terminal GlcNAc residues from *N*-glycans by β-NAHase, which presumably takes place in a post-Golgi compartment like vacuoles, plasma membrane, cell wall or apoplast (Strasser, 2009), have an important role in plant metabolism.

In addition to the posttranslational trimming of oligosaccharide chains of storage proteins and lectins deposited in the protein bodies during seed development (Vitale and Chrispeels, 1984), β-NAHase has been implicated in other biological processes, such as glycoprotein processing and turnover during seed germination (Harris and Chrispeels, 1975; Poulton et al., 1985). Together with other glycosydases, β-NAHase was proposed to be involved in *N*-glycan metabolism during fruit ripening (Jagadeesh and Prabha, 2002; Jagadeesh et al., 2004; Yong and Gross, 1994), softening of climacteric and non-climacteric fruits (Meli et al., 2010; Ghosh et al., 2011) and processing of *N*-glycans present in secretory glycoproteins of vacuoles and plasma membrane (Strasser et al., 2007). With endochitinase, β-NAHase was suggested to act in the plant defense system against fungal pathogens, by degrading chitin, or by inhibiting the spore germination and mycelial growth (Hodge et al., 1995; Bol et al., 1990; Broekaert et al., 1988; Schlumbaum et al., 1986).

However, the exact physiological roles of β-NAHase in plant *N*-glycan metabolism, as well as the natural substrates used by the enzyme, are not completely clarified.

In order to contribute to this area, the β-NAHase purified from *L. albus* seeds was molecularly and biochemically characterized. Furthermore, this enzyme activity was screened during *L. albus* seed germination and seedling growth in the different plantlet organs, and the purified enzyme incubated with one of the main storage proteins from *L. albus* seeds, α-conglutin, to evaluate whether this enzyme can use this storage protein as substrate.

Materials and methods

Chemicals

N-Acetylgalactosamine (GalNAc), *N*-acetylglucosamine (GlcNAc), *N*-acetylglucosamine–asparagine (GlcNAc–Asn), β-*N*-acetylhexosaminidase (β-NAHase), 4-chloro-1-naphtol, Concanavalin A (ConA), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), dichitobiose, *p*-dimethylaminobenzaldehyde, dimethylsuberimidate (DMS), fucose (Fuc), galactosamine (Gal), glucosamine (Glc), lactose (Lac), α-D-mannopyranoside (α-Man), 4-methylumbelliferyl-*N*-acetyl-β-D-glucosamine, *p*-nitrophenyl (*p*NP), *p*-nitrophenyl–β-D-*N*-acetylgalactosamine (*p*NP–GalNAc), *p*-nitrophenyl–β-D-*N*-acetylglucosamine (*p*NP–GlcNAc), *p*-nitrophenyl–β-D-*N*,*N*′-diacetylchitobiose (*p*NP–$(GlcNAc)_2$), *p*-nitrophenyl–β-D-*N*,*N*′,*N*″-triacetylchitotriose (*p*NP–$(GlcNAc)_3$), trifluoromethanesulfonic acid (TFMSA) were purchased from Sigma (St. Louis, MO). Concanavalin A Sepharose, Mono S HR 5/50, Superose 12 HR 10/300, Mono Q HR 5/50, Superose 6 HR 10/300, PD-10 and ampholytes (p*I* 3.5–10.0) were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). All other chemicals were of reagent grade or pure grade.

Plant material

Dry seeds of white lupin (*Lupinus albus* L. cv. Mizak) were surface sterilized with 1% (w/v) $HgCl_2$ and 0.02% (w/v) Tween-20 for 15 min and extensively washed with sterilized bi-distilled water. Germination was initiated by seed immersion in running tap water for 2 days, and seedling growth continued in pots filled with a mixture of sand and peat (1:1) for 7 more days. In the germinated seeds, the coats were removed and the intact cotyledons dissected from the embryos and axes and then stored at −80 °C until required.

Enzyme purification

Cotyledons were ground [13 mL $(g\,FW)^{-1}$] with an aqueous solution (10 mM $CaCl_2$ and 10 mM $MgCl_2$) at pH 8.0 and stirred for 4 h. After passing through two layers of cheesecloth, the extract was centrifuged at 30,000 × *g* for 1 h. The resulting pellet was used for total globulin extraction and the supernatant for total albumin extraction.

For globulin extraction, the pellet was suspended by stirring it in a solution containing 10% (w/v) NaCl, 10 mM EDTA and 10 mM EGTA [13 mL $(g\,FW)^{-1}$] for 12 h. The suspension was centrifuged for 1 h at 30,000 × *g* and the resulting globulin solution concentrated by ammonium sulphate (561 g L^{-1}) precipitation. The precipitated globulins were centrifuged at 30,000 × *g* for 20 min and resuspended in 20 mM Tris–HCl at pH 7.5 and stored at −20 °C until further use.

The supernatant, corresponding to the albumin fraction, was subjected to 30–70% (w/v) ammonium sulphate precipitation. The pellet obtained after the precipitation was resuspended [0.5 mL $(g\,FW)^{-1}$] in a buffer solution at pH 5.0 (50 mM $NaCH_3CO_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.5 M NaCl and 10 mM GlcNAc). All of the steps described were carried out at 4 °C (Santos et al., 2012; Franco et al., 1997).

The supernatant collected from the albumin fraction, after a centrifugation of 13,800 × *g* for 15 min, was subjected to affinity chromatography on a ConA-Sepharose column (eluted with 0.5 M α-Man in a buffer solution of 50 mM $NaCH_3CO_2$ at pH 5.0) at room temperature. Fractions containing β-NAHase activity were pooled and applied to a Mono S column in a FPLC system (GE Healthcare, Uppsala, Sweden) and eluted with an increasing gradient of NaCl (up to 1 M NaCl using the same buffer solution). Fractions containing higher β-NAHase activity were pooled and desalted by gel filtration in a PD-10 column eluted with 50 mM Tris–HCl at pH 7.5. The resulting fractions were subjected to anionic chromatography with a Mono Q column eluted with increasing gradient of NaCl (up to 1 M NaCl in 50 mM Tris–HCl at pH 7.5). Unless stated otherwise, all steps were carried out at 10 °C. Throughout the purification procedure, the enzyme activity was monitored by the release of *p*NP from *p*NP–GlcNAc as described below. Fractions having β-NAHase activity were stored at 4 °C until further use.

Protein determination

The protein concentration was determined according to Lowry's method (Lowry et al., 1951) using bovine serum albumin as standard.

Electrophoresis

SDS–PAGE carried out by the method of Laemmli (1970) was performed in 1.5 mm thick gels with 10% T resolving gels and 3%