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Production of barley endoprotease B2 in *Pichia pastoris* and its proteolytic activity against native and recombinant hordeins

Anne Lind Rosenkilde, Giuseppe Dionisio, Preben B. Holm, Henrik Brinch-Pedersen *

Aarhus University, Faculty of Science and Technology, Dept. of Molecular Biology and Genetics, Research Centre Flakkebjerg, DK-4200 Slagelse, Denmark

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ABSTRACT

Barley (*Hordeum vulgare* L.) cysteine proteases are of fundamental biological importance during germination but may also have a large potential as commercial enzyme. Barley cysteine endoprotease B2 (HvEPB2) was expressed in *Pichia pastoris* from a pPICZαA based construct encoding a HvEPB2 C-terminal truncated version (HvEPB2ΔC) and a proteolytic resistant His_6 tag. Maximum yield was obtained after 4 days of induction. Recombinant HvEPB2ΔC (r-HvEPB2ΔC) was purified using a single step of Ni^{2+}-affinity chromatography. Purified protein was evaluated by SDS–PAGE, Western blotting and activity assays. A purification yield of 4.26 mg r-HvEPB2ΔC per L supernatant was obtained. r-HvEPB2ΔC follows first order kinetics (K_m = 12.37 μM) for the substrate Z-Phe-Arg-pNA and the activity was significantly inhibited by the cysteine protease specific inhibitors E64 and leupeptin. The temperature optimum for r-HvEPB2ΔC was 60 °C, thermal stability T_{50} value was 44 °C and the pH optimum was 4.5. r-HvEPB2ΔC was incubated with native purified barley seed storage proteins for up to 48 h. After 12 h, r-HvEPB2ΔC efficiently reduced the C and D hordeins almost completely, as evaluated by SDS–PAGE. The intensities of the B and γ hordein bands decreased continuously over the 48 h. No degradation occurred in the presence of E64. Recombinant hordeins (B1, B3 and γ1) were expressed in *Escherichia coli*. After 2 h of incubation with r-HvEPB2ΔC, an almost complete degradation of γ1 and partial digests of hordein B1 and B3 were observed.

1. Introduction

The proteolysis of barley (*Hordeum vulgare* L.) grain hordein storage proteins is of fundamental biological importance as it ensures regular progression of germination by supplying nitrogen required for the development of a plantlet from the embryo. However also from an industrial perspective, proteases are of major importance. Recently, proteases were estimated to represent ~60% of all commercialized enzymes in the world (Feijoo-Siota and Villa, 2011). Their fields of application are very diverse, including improving the tenderness of meat, proteases in detergents, for treatment of diseases (such as celiac disease), production of fish protein hydrolysates, in antibody preparation and in brewing (Feijoo-Siota and Villa, 2011).

Several barley proteases have been identified. However, during germination the cysteine proteases account for more than 90% of the total proteolytic activity in the endosperm (Enari and Sopanen, 1986). A range of barley cysteine proteases have been identified and one of the key enzymes is the endoprotease B2 (HvEPB2) (E.C. 3.4.22) (Martinez et al., 2003; Rogers et al., 1985; Whittier et al., 1987; Zhang and Jones, 1996).

The HvEPB cysteine endoproteases (covering both isomers, HvEPB2 and HvEPB1) were purified in 1990 from gibberellic acid-induced aleurone barley tissue (Koehler and Ho, 1990b). The two isoforms have high homology (99% protein identity) and constitute an intronless gene family of proteases (Mikkonen et al., 1996). The protein exists both with a pro-domain and in a mature form where the pro-domain is cleaved off. The active, mature form of the purified enzyme has a molecular mass of 30 kDa and by sequence analysis of a cDNA clone, the inactive immature form was shown to have a molecular mass of 42.5 kDa (Koehler and Ho, 1990a,b). Functionality of the active form was confirmed using recombinant hordein C and the primary cleavage sites in hordein C (FR-QQ, FQ-QP, VQ-QP, LQ-QP and LQ-SP) were mainly found near the N and C terminus. Removal of primary sites in C hordein by site directed mutagenesis reduced the rate of degradation and indicated that HvEPB is an initiator of the C hordein degradation during germination (Davy et al., 2000). In addition to degradation of C hordeins, it has been demonstrated that a recombinant HvEPB purified from inclusion bodies of *E. coli* also degrades D and B

Abbreviations: HvEPB1, *Hordeum vulgare* endoprotease B1; HvEPB2, *Hordeum vulgare* endoproteaseB2; DTT, dithiothreitol; Endo H, endo-β-*N*-acetylglucosaminidase H; Trx, thioredoxin.

* Corresponding author. Tel.: +45 87158268; fax: +45 87156082.
E-mail address: Henrik.BrinchPedersen@agrsci.dk (H. Brinch-Pedersen).

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hordeins (Martinez et al., 2009). The degradation of γ hordeins has not been studied. In SDS–PAGE γ hordeins are often masked by the large amounts of B hordein. However, as a major cross-reactant with the major wheat allergen omega-5 gliadin, an assessment of γ hordein degradations by HvEPB is highly relevant (Palosuo et al., 2001).

Proteases can be promiscuous in their choice substrate and handling of active enzymes requires the utmost attention. For applied purposes and for controlled and detailed studies using proteases like HvEPB, a pure preparation (HvEPB1 or B2), easily prepared (i.e. after secretion into a media), that can be activated under controlled conditions is highly desirable. From barley tissues it has only been possible to purify the active mature form of mixed HvEPB1 and HvEPB2 (Davy et al., 1998; Koehler and Ho, 1990a,b).

Recombinant expression of HvEPB2 has been attempted in *Trichoderma reesi* and *E. coli*. In *Trichoderma reesi*, r-HvEPB (r, recombinant) was successfully secreted but the mature form had only ~25% activity of HvEPB obtained from barley tissues (Nykanen et al., 2002). The low activity was due to incomplete processing of the pro-domain in the fungal host. In the first processing step, removal of an N-terminal signal peptide occurred followed by a step-wise but incomplete removal of the pro-domain. The incomplete removal of the pro-domain caused an incomplete activation of r-HvEPB and a reduced activity (Nykanen et al., 2002). r-HvEPB2 was further expressed in *E. coli* for structural studies by X-ray crystallography (Bethune et al., 2006). In this investigation, the N-terminal signal peptide was removed creating a truncated version of HvEPB2. His6-tags were placed both N- and C-terminal of the truncated r-HvEPB2. The r-HvEPB2 had to be purified from inclusion bodies and refolded to create the disulfide bridges necessary for the activity. r-HvEPB2 was found to be autocatalytic like other related cysteine proteases and capable of removing the pro-domain when the disulfide bridges were reduced and the pH was low (Bethune et al., 2006; Mach et al., 1994; Vernet et al., 1995). However, despite that both T. reesi and *E. coli* were capable of expressing r-HvEPB2, the heterologous protein was either not fully active or not secreted.

Expression in the methyltrophic yeast *Pichia pastoris* is based on the ability of the yeast to utilize methanol through activation of alcohol oxidase promoters (AOX1 and AOX2) (Cregg et al., 1989). Slow growing Muts strains have in numerous examples been reported as efficient hosts for the production of foreign proteins (Cereghino and Cregg, 2000). Previously, the expression of plant enzymes in *P.* pastoris has provided the right posttranslational modifications and has with success been used for heterologous protease expression (Brömme et al., 2004; Mazorra-Manzano and Yada, 2008; Pechan et al., 2004).

In the present work, a C-terminal truncated version of HvEPB2 (HvEPB2ΔC) was cloned into a modified *P. pastoris* expression vector that contained a new proteolytic resistant tag, developed for protecting the terminal His6-tag. Expression of recombinant HvEPB2ΔC (r-HvEPB2ΔC) in the Muts *P. pastoris* strain KM71H during alkaline fermentation was achieved and for the first time the HvEPB2 including its pro-domain was secreted into the induction media. From the harvested growth medium, purification of inactive r-HvEPB2ΔC was achieved via a single step of Ni^{2+}-affinity chromatography. Fully active r-HvEPB2ΔC was obtained simply by activation via exposure to acidic pH and reducing agents. The production of recombinant endoproteases such as HvEPB2 in *P. pastoris* using this approach represents hence a first report for a plant cysteine endoprotease of class C1A. Cysteine protease activity was confirmed using specific inhibitors. Moreover, in addition to studying the activity of activated r-HvEPB2ΔC on native purified C-, D- and B-hordeins, recombinant barley hordein B1, B3 and γ hordein-1 were included to separate the degradation of B and γ hordeins. A very efficient degradation of γ hordein-1 by HvEPB2 is revealed.

2. Results and discussion

2.1. Cloning and expression of r-HvEPB2ΔC

pPiczαA-Δ*cMyc*:*HvEPB2ΔC* was electro transformed into Mut^s *P. pastoris* strain KM71H. Eleven colonies were randomly selected for growth in liquid cultures. Each culture was screened for protein concentration and for endoprotease activity in the growth media. The substrate used for activity screenings was endoprotease-specific, containing Phe in position P_2 (Z-Phe-Arg-pNA), thereby biasing the r-HvEPB2ΔC activity (Davy et al., 2000). In preliminary induction experiments, a construct containing the c-Myc epitope and an unmodified His_6-tag was used. Heterologous protein expression in *P. pastoris* was achieved but all attempts to isolate r-HvEPB2ΔC from the harvested supernatant by nickel affinity chromatography failed. Activity assaying of fractions from the purification with the endoprotease-specific substrate revealed that all endoprotease activity was found in the unbound protein fraction. Western blots using antibodies against c-Myc or penta-His showed no signal from the His_6 or c-Myc epitopes. Several *P. pastoris* proteases are secreted during growth of the yeast cells and removal of the His_6-tag can occur via the activity of *P. pastoris* proteases (Zhang et al., 2007). The proteases have a preference for C-terminal cleavage sites with a dibasic motif (Lys/Arg-Arg) and a typical target is the linker region between the tag and recombinant protein (Bourbonnais et al., 1993; Cooper and Bussey, 1989; Mizuno et al., 1989). A new proteolytic resistant tag (-Pro-X-Pro-) has been designed by our group (PCT/DK2012/050505). The tag consists of three amino acids in a sequence that was found not to be cleaved by the yeast proteases. By combining the proteolytic resistant tag with the His_6-tag, the unwanted proteolysis was prevented and nickel affinity chromatography was possible.

In a small scale induction experiment, already during the first 24 h after switching to glycerol-containing medium, increases in protein concentration and enzyme activity were observed (Supplementary Fig. S1). For the majority (nine) of the clones, the endoprotease activity reached a constant level after 24 h whereas the protein concentration continued to increase during the following 72 h. One clone (deriving from colony 10) showed a continuously increasing activity until 96 h. The reason for why the protease activity in most of the clones did not increase from 24 to 72 h is not clear. Auto-proteolysis equivalent to or exceeding the rate of functional r-HVEPB2ΔC synthesis may be an explanation, although this needs to be confirmed. Besides the negative controls, supernatant protein concentration increased during the growth period, though much slower and with no accompanying increase in enzyme activity. From one transformed line (deriving from colony 3) only enzymatic inactive protein was secreted into the media. The background for the production of proteolytic inactive protein in line 3 was not pursued and the line was excluded from further expression experiments.

In order to generate a sufficient protein for nickel affinity purification, the high producing colony 10 was selected for methanol-grown shaking-flask cultures. Time samples determining protein and activity levels in shaking-flask cultures resembled what was already observed for the small scale inductions.

2.2. Purification of r-HvEPB2ΔC

For the first time reported, the r-HvEPB2 pro-enzyme was secreted from the host cell into the induction media. The secreted enzyme was purified by nickel affinity chromatography and the elution profile showed after endoprotease-specific substrate screenings, a distinct activity peak (Supplementary Fig. S2). The purification fold was ~75-fold and the yield was 4–6 mg L^{-1}

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