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Self-processing of a barley subtilase expressed in *E. coli*

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ABSTRACT

The barley protease BAJ93208 belongs to the subtilase family of serine proteases. We have expressed BAJ93208 in the cytoplasm of the *Escherichia coli* strain SHuffle C3030 using a rhamnose-inducible promoter. The expression construct included a $(His)_6$-tag at the N-terminus and a strep-tag at the C-terminus. Western blot analysis revealed that the protein was processed at the N- and C-terminus. To exclude that this processing was due to contaminating *E. coli* proteases, a mutated BAJ93208 protease was constructed. This inactive mutant was not processed, demonstrating that the processing was an autocatalytic process. To define the exact cleavage sites mass spectrometry was used which detected four differently processed versions of the protease. At the N-terminus, the self-processing removed the internal inhibitor and an additional 19 amino acids. At the C-terminus there was a cleavage site after Ala^{765} which also removed the *strep*-tag. This explained the inability to detect the purified $(His)_6$-BAJ93208-*strep* protease with an anti-*strep*-tag antibody. Finally, an additional alanine was removed either at the N-terminus (Ala^{119}) or at the C-terminus (Ala^{764}).

Introduction

Proteases or peptidases are enzymes which hydrolyse the amino acid bonds in proteins and peptides. According to the catalytic domain they are classified for instance as cysteine proteases and serine proteases. Serine proteases include a large number of families including the subtilase family which has been named after the subtilisin proteases produced by bacteria of the genus *Bacillus* [1].

Subtilases are widely distributed in all kingdoms of life as well as in viruses. According to the protease database MEROPS [2] they constitute the family S8 of serine proteases related to subtilisin having the catalytic triade Asp, His, and Ser. Kexin, encoded by the yeast gene Kex2 [3], and related enzymes from mammals are proprotein convertases which convert inactive proproteins into active molecules with a high substrate specificity by cleaving after two adjacent basic residues [4,5]. Kexins are separated from the majority of subtilases into subfamily S8B. All others are grouped into subfamily S8A (MEROPS). Subtilases are especially abundant in plants, with 63 genes known in the rice genome and 56 genes in Arabidopsis [6,7].

Some of the Arabidopsis subtilases are known to be involved in the processing of precursor proteins and their function has been characterized in some detail. The Arabidopsis protease AtSBT6.1 is a component of the signaling pathway that mediates salt stress responses. AtSBT6.1 releases a part of the ER membrane-localized b-ZIP transcription factor, AtbZIP17, which is subsequently translocated into the nucleus to promote the transcription of salt stress genes [8]. In addition, AtSBT6.1 is able to process the plant peptide hormone AtRALF23 at the recognition site RRIL↓ [9]. Moreover, AtSBT6.1 is likely to be involved in maturation of a pectin methylesterase by cleaving after dibasic sites like RRLL↓ within the proprotein [10]. The Arabidopsis growth factor AtPSK4 that is synthesized as a proprotein is processed by the subtilase AtSBT1.1. In this case the enzyme cleaves at the C-terminal side of the amino acid sequence RRSLVL↓ [11]. Additionally, subtilases take part in plant specific developmental processes, for example the Arabidopsis enzyme SDD1 (AtSBT1.2) is involved in the regulation of stomatal density. Indeed, a T-DNA knockout of the gene led to increased appearance of stomata [12]. The enzyme AtSBT1.7 is involved in mucilase release from Arabidopsis seed coats during seed germination [13]. Another subtilase, AtSBT5.4, appears to play a role in shoot meristem development by interacting with the clavata pathway [14].

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Thionins are plant antimicrobial peptides which are part of the plant immune system [15–17]. They are usually basic with approximately 45 amino acids and 6 or 8 cysteine residues which form 3 or 4 disulfide bridges. Thionins are produced as preproproteins with a N-terminal signal peptide and a C-terminal prodomain which is called acidic domain because it is usually basic [18]. Thionins have been repeatedly isolated from cereals, mistletoes and some other plants [19,20] but isolation of the acidic domain or the proprotein from plants has never been reported. In our work to characterize the processing of thionin proproteins we obtained a partial amino acid sequence for the barley protease BAJ93208, a member of the subtilisin class of proteases (Plattner and Bohlmann, unpublished results). Here we describe the expression of the protein in *Escherichia coli* and characterize the self-processing of the protease.

Materials and methods

Cloning

A *BAJ93208* cDNA from etiolated barley seedlings was synthesized by the 3′ UTR specific primer BAJ93208rev2. Gene specific primers BAJ93208forNcoI and BAJ93208StreprevBam were used to amplify the gene and to introduce the restriction sites NcoI and BamHI together with a sequence coding for a *strep*-tag at the 3′ end. The amplicon was purified and digested with the restriction enzymes NcoI and BamHI to produce overhanging ends and cloned into the vector pMAA-RED [21]. This clone was used as a template for further cloning. Primers BAJ93208HisNdeFor and BAJ93208StreprevBam were used to remove the signal peptide sequence and to introduce a sequence for an N-terminal $(His)_6$-tag. The PCR product was purified and digested with the restriction enzymes NdeI and BamHI to produce overhanging ends and purified. A modified version of the plasmid pJOE4905.1 was used as expression vector [22,23]. This vector, named pJOE-SP-MCS, contained the restriction sites NdeI, a MalE signal peptide and a small poly-linker harboring the restriction site BamHI. The plasmid was digested with NdeI and BamHI (this also removed the signal peptide) and purified. The previously amplified $(His)_6$-tagged and *strep*-tagged *BAJ93208* sequence was introduced into the digested and purified pJOE-SP-MCS vector. The final vector, named pJOEHisBAJstrep is shown in Fig. S1 and was confirmed by sequencing (LGC Genomics, Berlin, Germany) using the primers listed in Supplementary Table 1.

Site directed mutagenesis by overlap extension [24] was used to change the amino acid $Ser^{(556)}$ to alanine in the coding sequence of *BAJ93208*. The plasmid pJOE harboring the coding sequence for the double tagged pJOEHisBAJstrep was used as a template to amplify two overlapping amplicons. The first amplicon was amplified with the primers BAJ93208forNde2 and BAJ208MUTS556Arev. The second amplicon was amplified with the primers BAJ208MUTS556Afor and BAj93208revBam2. The two amplicons were used as a template for the fusion PCR with the outer primers BAJ93208forNde2 and BAj93208revBam2. The PCR product was analyzed on an agarose gel. The band corresponding to a size of 2200 bp was cut from the gel, extracted with the QIAquick® Gel Extraction Kit, and digested with NdeI and BamHI for 16 h at 37 °C. The digested DNA was purified with a PCR purification Kit and ligated into the vector pJOE-SP-MCS digested with the same enzymes. Ligation was performed for 4 h at room temperature and 5 µl were used to transform chemically competent *E. coli* DH10β cells. Ampicillin resistant clones harboring the newly generated plasmid pJOEHisBAJ*strep*S556A were tested for successful ligation and transformation by colony PCR using the two primers pJOEfor and pJOErev. Positive colonies were grown in LB medium containing 100 µg/ml ampicillin overnight at 37 °C and plasmids were extracted and sequenced to confirm successful mutation using the primers pJOEfor, HTPPEfor4nest, HTPPErev3, BJ208seqRev and pJOErev. One clone (pJOEHisBAJstrepS556A) containing a correct sequence of pJOEHisBAJstrep with the desired mutation in the catalytic triad at the amino acid position 556 (serine to alanine) was transformed into the *E. coli* expression strain SHuffle C3030 for protein expression.

Expression of BAJ93208 in E. coli

3 ml of overnight cultures of *E. coli* SHuffle C3030 harboring the plasmid pJOEHisBAJstrep were grown in LB medium containing 100 µg/ml ampicillin at 37 °C overnight and used to inoculate 500 ml TB medium supplemented with 100 µg/ml ampicillin. Baffled flasks were used to improve oxygen intake. Cells were grown at 37 °C with constant shaking until an OD_{600} of 0.5 was reached. The culture was then cooled down to 16 °C and expression was induced by adding rhamnose to a final concentration of 0.2% (wt/vol). After 20 h of growth at 16 °C with constant shaking the cells were harvested at 4 °C by centrifugation at 5000*g*. The pellet was resuspended in 50 ml *strep*-tactin Sepharose washing buffer (100 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0). Cells were disrupted by sonication (Sonifier W-250D, Branson Ultrasonics) on ice as described previously [25] with an amplitude of 65% for 2 min. Insoluble material was removed by centrifugation at 15,000*g* at 4 °C for 15 min. After centrifugation the supernatant was applied to a 1 ml *strep*-tactin Sepharose gravity flow column (IBA, Germany). The column was washed with 5 column volumes (CV) washing buffer. Bound proteins were eluted from the column with 4 CV elution buffer (2.5 mM desthiobiotin, 100 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, pH 8). Fractions were analyzed for protease activity and SDS PAGE and BCA protein quantification was performed. Active enzyme containing fractions were pooled, diluted in 40 mM Tris/HCl pH 8 and loaded onto a Mono Q HR 16/10 strong ion exchange column (GE Healthcare) previously equilibrated with 20 mM Tris/HCl, pH 8 (buffer A). The column was washed with 20 ml buffer A and bound proteins were eluted with a linear, 0–100% gradient of Buffer B (20 mM Tris/HCl, 1 M NaCl, pH 8) over 20 min. A constant flow rate of 1 ml/min was used and elution of proteins was monitored by detection of absorbance at 280 nm. 1 ml fractions were collected and protease activity was analyzed. Active enzyme containing fractions were pooled and dialyzed against protease storage buffer (25 mM MES, 100 mM NaCl, 10 mM $CaCl_2$, pH 6.5) with Amicon Ultra 10 K ultrafiltration centrifugal filters (Millipore) according to the recommendations of the manufacturer. The yield was 400 µg from 1 L of culture.

The mutant protease encoded by pJOEHisBAJstrepS556A was expressed and purified using the *strep*-tag purification method described above.

Gel electrophoresis

Proteins were resolved on T12.5/C1 SDS polyacrylamide gels [26]. The Mini-PROTEAN® Tetra cell system (Bio-Rad) was used to prepare and run hand cast gels at a constant voltage of 150 V. 10 µl desalted samples were mixed with 10 µl sample buffer, incubated at 95 °C for 10 min, centrifuged at 15,000*g* for 5 min, and loaded on the gel. Unstained protein ladders or pre-stained protein markers (Thermo Scientific) were run as molecular weight standards. Electrophoresis was stopped when the dye front reached the end of the gel. Gels were removed and proceeded to multiplex Western blotting or stained in Coomassie solution (Coomassie brilliant blue G-250, 10% acetic acid, 40% methanol) for 1 h at room temperature with constant shaking. Destaining was performed