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

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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\(^\ast\))\(_6\)-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\(^\ast\))\(_6\)-BAJ93208-strep protease with an anti-strep-tag antibody. Finally, an additional alanine was removed either at the N-terminus (Ala\(^{765}\)) or at the C-terminus (Ala\(^{764}\)).

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**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 subtilins 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 prosprotein convertases which convert inactive prosproteins 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 methyl-esterase by cleaving after dibasic sites like RRLL within the proprotein [10]. The Arabidopsis growth factor AtPSK4 that is synthesized as a prosprotein is processed by the subtilase AtSBT1.1. In this case the enzyme cleaves at the C-terminal side of the amino acid sequence RSSLVL [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 muclase 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 preproteins 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 signal peptide. A modified version of the plasmid pMAA-RED [21] 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 and BamHI to produce overhanging ends and purified. The previously amplified (His)_6-tagged 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 and BamHI to produce overhanging ends 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 was shown in Fig. S1 and was confirmed by sequencing (LGCGenomics, 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 amplimers. The first amplimer was amplified with the primers BAJ93208forNde2 and BAJ93208StreprevBam. This amplified fragment was cut from the gel, extracted with the QIAquick™ Gel Extraction Kit, and the resulting DNA was purified with PCR purification Kit and ligated into the vector pMAA-RED digested with NdeI and BamHI for 16 h at 37°C. The digested vector was used to transform chemically competent DH10β cells. Ampicillin resistant clones harboring the newly generated plasmid pJOEHisBAJstrepS556A 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 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 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 0.2 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 5000g. 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 15,000g 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 dia- lyzed against protease storage buffer (25 mM MES, 100 mM NaCl, 10 mM CaCl₂, 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 15,000g 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...
three times in 10% acetic for 1 h each time and results were documented with a digital camera.

**Western blotting**

Pre-stained protein marker (Thermo Scientific) and protein samples were resolved on glycine SDS polyacrylamide gels as described above. Proteins were transferred to an Immobilon-PSQ PVDF (0.2 µm) transfer membrane (Millipore) by using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad). The gel, the membrane and the extra-thick filter paper were equilibrated in transfer buffer (Bjerrum and Schäfer-Nielsen buffer: 48 mM Tris, 39 mM glycine, 20% (vol/vol) methanol, pH 9.2) for 15 min [27]. The gel–membrane sandwich was prepared with extra-thick filter paper at the anode side, followed by the membrane, the gel and an extra-thick filter paper at the top. Air bubbles were rolled out from all the layers before blotting. After protein transfer for 25 min at 15 Volts, the membrane was air-dried and blocked with PBS (4 mM KH2PO4, pH 7.4, 16 mM Na2HPO4, 115 mM NaCl) blocking buffer (PBS buffer with 2.5% skim milk and 0.5% (vol/vol) Tween-20) for 1 h at room temperature.

Primary antibodies (see Supplementary Table 2) were diluted in 15 ml PBS blocking buffer and incubated together with the membrane for 1 h at room temperature. Subsequently, the membrane was washed three times for 5 min each time with 50 ml PBS-Tween buffer (PBS buffer with 0.1% (vol/vol) Tween-20) at room temperature with gentle shaking. Secondary antibodies were diluted in 15 ml PBS blocking buffer as listed in Supplementary Table 2 and incubated together with the membrane for 30 min at room temperature. After incubation the membrane was washed three times for 5 min each time with 50 ml PBS-Tween buffer at room temperature with gentle shaking. Residual Tween-20 was removed by an additional 1 min wash step in PBS buffer without Tween-20. The membrane was left to dry and fluorescent signals were detected at 700 nm and 800 nm with an Odyssey® Classic Infrared Imaging System (LI-COR).

**Protease assay**

A fluorogenic protease assay was used to measure the proteolytic activity of BAJ93208. The quenched fluorogenic peptide (5-amino-2-nitrobenzoyl-S-D-Y-P-K-L-N-L-L-P-K-7-methoxycoumarinyl-4-acetyl) was synthesized by JPT Peptide Technologies GmbH (Berlin, Germany). In a standard reaction, 10 µl of protease containing sample was added to a solution containing a final concentration of 2 µM fluorogenic peptide, 25 mM MES, pH 6.5, supplemented with 150 mM NaCl and 10 mM CaCl2. Kinetic assays were performed at 25 °C and formation of the MCA – labeled cleavage product was followed at 330 nm excitation and 420 nm emission using black 96 well microtiter plates (Greiner, Germany) in a FLUOstar Omega spectrophotometer (BMG Labtech).

Specific activity was determined using 10 µM fluorogenic peptide and 0.1 µg of enzyme in 25 mM MES, pH 6.5 in a 200 µl reaction with or without 5 mM DTT. Serial dilutions of 7-amino-4-methylcoumarin (AMC, Peptanova, Germany) ranging from 0 to 150 pM per well in 25 mM MES at pH 6.5 were prepared and relative fluorescence units per pm free MCA were measured. The value was corrected by the difference between background fluorescence and expected maximal fluorescence and used to calculate the specific activity of the protease.

**Mass spectrometry**

The purified enzyme was analyzed by liquid chromatography mass spectrometry (LC–ESI–MS) using a Dionex Ultimate 3000 capillary LC and a Bruker maXis 4G Q-TOF MS. Separation of the different truncated versions was performed using a reverse phase Supelco Discovery Bio Wide Pore C5 column (50 * 0.32 mm, 3 µm packing) with elution achieved by using a gradient of 55 min from 5% acetonitrile (solvent A: 0.1% formic acid) to 80% acetonitrile.

Data generated by mass spectrometry was processed using Data Analysis 4 (Bruker Daltonics) and MASCOT [28]. Deconvolution was performed using MaxEnt software (Micromass, Manchester, UK).

**Results**

**Expression of BAJ93208 in E. coli**

In order to characterize the barley protease BAJ93208 [29] we decided to express the recombinant enzyme in E. coli. Secretion into the periplasmic space was attempted because the protein contained 10 Cys residues, but it was not possible to obtain the protein in adequate amounts (data not shown). Since we were able to express thionin proproteins which contained 14 cysteine residues in the E. coli cytoplasm [23], we tried to also express the subtilase in the cytoplasm. The coding sequence without the signal peptide was cloned and expressed in the E. coli expression vector pJOE-SP-MCS [23] (see Materials and methods). To facilitate the purification and detection of the protein, a N-terminal (His)6-tag and a C-terminal strep-tag were included (see Fig. 3). The final plasmid (pJOEHisBAJstrep) coding for His-BAJ93208-strep was transformed into the E. coli strain SHuffle C3030 [30] to express the protease. After induction with rhamnose an additional protein was found in the soluble protein fraction (Fig. 1A). This protein of 80 kD could be purified using the strep-tag (Fig. 1A). Western blots with antibodies against the (His)6-tag and the strep-tag (Fig. 2B), respectively, showed a single band containing the (His)6-tagged protein (80 kD) and two bands for strep-tagged protein (80 and 70 kD), indicating that the inhibitor was partially removed during the purification procedure. The BAJ93208 protease was further purified using an anion exchange column. Protease activity was found in fractions A6–A11 (Fig. 2A) and these fractions were loaded on an SDS–PAGE gel (Fig. 2B). One single protein band at 74 kDa could be detected in these fractions. The active fractions were pooled and dialyzed against protease storage buffer. Specific activity of the protease towards the fluorogenic peptide was determined as 1.08 µmol min⁻¹ mg⁻¹ without DTT and as 1.41 µmol min⁻¹ mg⁻¹ with 5 mM DTT as a reducing agent. A Western blot using anti-strep antibodies was performed to detect the C-terminus of the protease after the ion exchange chromatography. Surprisingly, no strep-tag signal could be detected at this point (results not shown), indicating that the BAJ93208 protease was processed at the C-terminus.

**MS analysis of the self-processing of His-BAJ93208-strep protease**

Purified (His)6-BAJ93208-strep protease was analyzed by LC–ESI–MS. The measurement revealed four distinctive masses which could be assigned to four differently processed versions of the protease. Fig. 3A shows the MS scan with the masses found which corresponded to the fragments shown in Fig. 3B together with the calculated average masses. At the N-terminus, one cleavage removed the endogenous inhibitor and a second cleavage step confirmed the calculated average masses. At the N-terminus, one cleavage removed the endogenous inhibitor and a second cleavage step confirmed the calculated average masses. At the N-terminus, one cleavage removed the endogenous inhibitor and a second cleavage step confirmed the calculated average masses. At the N-terminus, one cleavage removed the endogenous inhibitor and a second cleavage step confirmed the calculated average masses. At the N-terminus, one cleavage removed the endogenous inhibitor and a second cleavage step confirmed the calculated average masses. At the N-terminus, one cleavage removed the endogenous inhibitor and a second cleavage step confirmed the calculated average masses.
or at the C-terminus (Ala$^{764}$). This could not be decided because both products would have exactly the same mass.

The BAJ93208$^{556}$Ala mutant is inactive

The processing of BAJ93208 in E. coli could either be an autocatalytic process or the result of a digestion by endogenous E. coli proteases. To clarify this question, we constructed and expressed an inactive BAJ93208 mutant. The active site of subtilases is characterized by a catalytic triad which is found in the BAJ93208 sequence as amino acids His$^{227}$, Asn$^{331}$, and Ser$^{556}$ (compare Fig. 3C). To produce an inactive mutant and to prove that Ser$^{556}$ is indeed involved in the catalytic process, we produced a mutated BAJ93208 version by replacing Ser$^{556}$ with Ala. This mutant was expressed in E. coli in the same way as the active enzyme and purified using the strep-tag. In contrast to the wild-type enzyme, the mutant gave only a single band of 80 kDa on a Western blot of the purified enzyme using either anti-(His)$_6$ or anti-strep antibodies (Fig. 4A) while the wild type protease showed two bands with strep-tag. No protease activity of the Ser$^{556}$Ala mutant was found in the assay using a fluorescent peptide (Fig. 4B). This experiment proved that Ser$^{556}$ is part of the catalytic site of the protease and that the processing of the BAJ93208 protease after expression in E. coli was an autocatalytic process.
Discussion

To our knowledge, heterologous expression of plant subtilases in *E. coli* has not been reported before. The tomato subtilase LeSBT1 has been expressed in insect cells [31] while LeSBT3 was expressed in a tomato suspension culture system [32]. Expression in *E. coli*, to our knowledge, has not been reported so far. The reason might be toxicity of the proteases to the host or the inefficient formation of cysteine bridges in the cytoplasm. Other authors have mentioned that they were unable to produce the Arabidopsis subtilase AtSBT1.1 in heterologous systems in large amounts [11]. We report here the successful production of the barley subtilase BAJ93208 (GI:326526063) in *E. coli*. After several unsuccessful approaches, we succeeded by using the *E. coli* SHuffle expression system and the expression vector pJOE-SP-MCS [23] without the MalE signal peptide. The rhamnose-inducible promoter in the pJOE vectors [22] is very tightly regulated and might have been the reason for success. It has been reported that SOC medium improved the cloning of a cDNA for *S. americanum* by suppressing the lac promoter of the cloning vector [33] thus avoiding expression of the protease during cloning. Furthermore, since the protein contains a total of 10 cysteine residues, the yield of functional protease should be enhanced in the *E. coli* expression host SHuffle C3030. This strain provides a more oxidizing cytoplasm due to the *trxB*gor mutations and provides cytoplasmic expression of disulfide bond isomerase (DsbC) [30]. A (His)₆-tag at the N-terminus was included to track the intact precursor including the inhibitor. The BAJ93208 subtilase contains a putative internal inhibitor which might also be involved in the folding of the mature protease. Cleavage of the inhibitor domain in the cytoplasm of *E. coli* was not observed. A Western blot detecting the C-terminal strep-tag and the N-terminal (His)₆-tag in induced *E. coli* cells showed only one single band. This might be attributed to the relatively high pH of between 7.4 and 7.8 of the *E. coli* cytoplasm [34] and also to the low induction temperature of 16 °C. However, after the strep-tag purification at room temperature a partial processing was detected as a second smaller band appeared in the Western blot using an anti-strep-tag antibody. This band did not bind any anti-(His)₆-tag antibodies indicating a N-terminal processing of the protease.

For purification of the mature protease a C-terminal strep-tag was attached. However, mass spectrometry analysis of the recombinant protease revealed that the protein was processed autocatalytically not only N-terminal, removing the inhibitor, but at additional sites, including removal of the C-terminal strep-tag. Subtilases are known to undergo several post-translational modifications in plant cells; pH-triggered activation by degradation of the inhibitor domain and sometimes C-terminal trimming [31,32,35]. This unintended removal of the strep-tag explained why we faced problems to purify the active protease with the help of the strep-tag (data not shown) and had to use anion exchange chromatography to purify the mature protease. C-terminal processing is not unusual for subtilases and has been observed for SDD in Arabidopsis [36] and for cucumisin [37], both at different sites. However, the C-terminal trimming which we observed might be triggered by the strep-tag extension and it is possible that in the wild type enzyme no trimming occurs. To test this, it would be necessary to produce the BAJ93208 subtilase without strep-tag. The selfprocessing of the BAJ93208 subtilase during purification could have been prevented by using inhibitors. However, these inhibitors must be removed for activity assays of the protease. Furthermore, it was possible to purify the active protease without tags using chromatographic purification methods.

While removal of the strep-tag was unsuspected, this activity of the BAJ93208 subtilase could perhaps be useful for...
biotechnological applications. The strep-tag was removed completely behind two alanine residues, one of which was also partly cleaved off. Thus, this protease could be used to remove C-terminal strep-tags quite precisely. However, further work is needed to characterise this activity in detail because it is not known if the information for cleavage is contained in the amino acid sequence of the strep-tag, in the amino acid sequence before the strep-tag, or in both.

Mass spectrometry also revealed that at the N-terminus not only the inhibitor was removed but an additional 19 amino acids. The inhibitor domain was found to be cleaved off between His\textsuperscript{99} and Thr\textsuperscript{100}. This cleavage site is known from many other plant subtilases including cucumisin\textsuperscript{37}, SBT1\textsuperscript{31}, and SBT3\textsuperscript{32}. Cleavage of the additional 19 N-terminal amino acids probably leads to the major active form of the protease but this needs to be tested with additional experiments. A similar processing was found for SBT1\textsuperscript{31} where after cleavage of the inhibitor a second cleavage takes place at acidic pH which removes an additional peptide consisting of 21 amino acids from the N-terminus leading to a highly active protease.

To exclude that the observed processing steps was due to the activity of contaminating\textit{E. coli} proteases, we produced a mutated BAJ93208 enzyme by converting the serine in the catalytic domain to alanine. This protein did not show any of the processing steps that we observed for the wild type enzyme, proving that all observed processing steps were autocatalytic processes.

Our work has shown that the protease BAJ93208 and its mutant could be produced to apparent homogeneity. It might therefore be a candidate for crystallographic studies. Up to now, the structures of only two plant subtilases have been determined and these had to be purified from the original source\textsuperscript{35} or from a tomato cell culture\textsuperscript{38}. Having an\textit{E. coli} expression system would not only allow to produce the active enzyme for crystallographic studies. It would also be possible to produce mutants of the enzyme as we have shown here and to produce the precursors by including inhibitors during the purification procedure.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.pep.2014.05.014.

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Fig. 4. (A) SDS–PAGE and Multiplex Western blot detecting the N-terminal (His)6-tag and the C-terminal strep-tag. IN: IPTG induced E. coli cells expressing the mutated (His)6-BAJ93208-strep (indicated by a star), F3: strep-tag purified mutated (His)6-BAJ93208-strep sample. The blot shows that the mutated protease is not processed during the purification process. (B) Activity analysis of BJ93208-strep and mutated (His)6-BJ93208-strep proteases using the fluorogenic peptide as substrate. The mutated version of the protease shows no activity.
