Phytaspase is a member of the plant subtilase family

Phytaspase, a plant subtilisin-like serine protease, has recently been reported to be involved in the programmed cell death of plants, including tobacco, rice and tomato. In spite of their structural dissimilarities, phytaspases are functionally homologous to animal caspases, as they both hydrolyze multiple synthetic caspase substrates. Phytaspases are highly specific in cleaving these substrates after aspartic acid residues.

Subtilisin-like serine proteases (S8 family), commonly termed subtilases, are the second most ubiquitous serine proteases and can be found in bacteria, eukaryotes and even viruses. Subtilases possess the same catalytic triad found in chymotrypsin, differing only in arrangement; Asp-His-Ser is found in subtilases, and His-Asp-Ser is found in chymotrypsin. Approximately 200 subtilases have been discovered, and they have been categorized into 6 families based on sequence homology: subtilisin, thermitase, proteinase K, lantibiotic peptidase, kexin and pyrolysin. Unlike kexin, which belongs to the S8B subfamily, plant subtilases are members of the S8A subfamily and, like bacterial subtilisin, they have broad substrate specificity. However, our current knowledge about plant subtilases is limited and demands further investigation.

Subtilases are synthesized as zymogens. They contain an N-terminal signal peptide followed by a pro-domain and a mature protease catalytic domain. The zymogen undergoes maturation to the mature protease by autocatalytically cleaving the peptide bond between the pro-domain and the mature protease domain. This self-processing is widely adapted by bacterial, mammalian and plant subtilases.

Heterologous expression of plant subtilases

The heterologous expression of plant subtilases in E. coli, though potentially biomedically significant, is challenging. Researchers have failed to obtain large amounts of heterologously expressed Arabidopsis subtilase AtSBT1. Even though expression occurs, the majority of proteins are expressed as inclusion bodies (IBs). High levels of expression of barley subtilases BAJ93208 have been achieved in E. coli, but only using dual tags such as an N-terminal His(6)-tag and a C-terminal strep-tag. The difficulties associated with heterologous expression could be due to the formation of cysteine bridges or the cytotoxicity to the host.
expression system following the autocatalytic activation of these proteases.\textsuperscript{10}

Regarding the expression of plant subtilases, experts have always tried to answer the question: \textit{is it important to express the protease along with its pro-domain?} Many reports state that the pro-domain is important as an intramolecular chaperone for proper folding of the protease.\textsuperscript{11,12} On the other hand, there are a significant number of reports describing the auto-inhibition of the mature protease caused by complex formation with the pro-domain via non-covalent binding.\textsuperscript{13-15} The issues of pro-domain cytotoxicity have been successfully overcome by expressing only the mature catalytic domain of the protease, which retains its catalytic activity. The bacterially expressed \textit{Thermococcus kodakarensis} recombinant mature protease MP-TK1689 was reported to be catalytically active with no host cytotoxicity.\textsuperscript{16} Another group of researchers have demonstrated that the mature \textit{Thermococcus kodakarensis} subtilisin, when expressed in \textit{E. coli}, showed activity without co-expression of the pro-domain, indicating that the pro-domain was not crucial for folding of the mature protease.\textsuperscript{17}

**Expression of pre-prophytaspase in a bacterial system**

Phytaspase, like other plant subtilases, is synthesized as inactive proenzyme (pre-prophytaspase) containing an N-terminal signal peptide followed by a pro-domain and a mature protease catalytic domain. Pre-prophytaspase (82 kDa), containing 763 amino acid residues, constitutively and autocatalytically processes itself, releasing the pro-domain and the active mature phytaspase enzyme (646 amino acid residues, 69 kDa). We have recently demonstrated the successful expression of mature phytaspase (without the pro-domain) from tobacco in a bacterial system.\textsuperscript{18} Despite our success and other reports of the effective expression of active recombinant mature subtilases without the pro-domain, we cannot reject the possibility that more active phytaspase can be obtained when expressed with the pro-domain. Therefore, we focus on the bacterial expression of pre-prophytaspase in the present communication. Based on previous results, we chose His-tag and GST-tag fusion systems for the purification.\textsuperscript{10,14,19,20}

We cloned the tobacco pre-prophytaspase gene into the pGEM-T Easy vector as described in a previous study.\textsuperscript{18} The pGEM-T Easy-pre-prophytaspase construct was used as a template for the further amplification of pre-prophytaspase for subcloning into the pET-28a and pGEX-4T2 vectors (Fig. 1). The pET-28a-pre-prophytaspase, pET-28a-mature phytaspase and pGEX-4T2-pre-prophytaspase constructs were transformed into \textit{E. coli} BL21. Transformed \textit{E. coli} BL21 were grown in LB broth containing kanamycin (50 \(\mu\)g/ml, for pET-28a constructs) or ampicillin (100 \(\mu\)g/ml, for pGEX-4T2 construct). The expression of pre-prophytaspase or mature phytaspase was induced by adding 0.5 mM IPTG (at 28°C for 8–10 h). Finally, the induced cells were pelleted

**Figure 1.** (A) Subcloning of pre-prophytaspase into the pET-28a vector using forward primer 5' AGAAGCTTTGATGGCCAATTGTATTACC-3' and reverse primer 5' TGCTCGAGTCACAGGGATCCAC-3' with overhangs for HindIII and XhoI, respectively. Lane 1: Digested pET-28a-pre-prophytaspase construct and lane 2: undigested pET-28a-pre-prophytaspase. (B) Subcloning of mature phytaspase into the pET-28a vector using forward primer 5' ACAAGCTTTGACGACACACGTCTC-3' and reverse primer 5' TGCTCGAGTCACAGGGATCCAC-3' with overhangs for HindIII and XhoI, respectively. Lane 1: Digested pET-28a-mature phytaspase construct and lane 2: undigested pET-28a-mature phytaspase. (C) Subcloning of pre-prophytaspase into the pGEX-4T2 vector using forward primer 5' ATGTCGACGATGGCCAATTGTATTACC-3' and reverse primer 5' TTCGGCCGTTCACAGGGATCCAC-3' with overhangs for SalI and EagI, respectively. Lane 1: Digested pGEX-4T2-pre-prophytaspase construct and lane 2: undigested pGEX-4T2-pre-prophytaspase. Lane M: DNA marker (10 kbp-200 bp). PCR conditions used for amplification of the gene were: 94°C initial denaturation for 5 min, 54°C/annealing for 1 min and 72°C/final extension for 8 min. Finally, the amplified gene was sub-cloned into both expression vectors.
down, lysed in lysis solution (10 mM HEPES pH 7.4, 150 mM NaCl) and spun at 12,100 rpm at 4°C for 20 min. The supernatant and pellet were run separately in 12% SDS PAGE. The *E. coli* BL21 containing the His-tag fusion showed no traces of the expression of pre-pro-phytaspase, even after several attempts with varied parameters. However, His-tagged mature phytaspase was expressed in IBs (data not shown). The GST-tag fusion system resulted in expression of pre-pro-phytaspase and mature phytaspase, however both were expressed in IBs (Fig. 2a).18

**Purification and autoprocessing of GST-pre-pro-phytaspase**

After the optimization of various induction parameters, recombinant GST-pre-pro-phytaspase was successfully expressed in soluble form. The protein was subsequently purified by affinity chromatography using glutathione-agarose beads as described previously.18 Consistent with our results expressing GST-mature phytaspase in our recent study, we were successful in obtaining GST-pre-pro-phytaspase in a soluble fraction, though with very low expression (Fig. 2b). Surprisingly, we observed a protein band corresponding to the size of the mature phytaspase (69 kDa) in the ‘wash’ fraction (Lane 5, Fig. 2b). Similar bands could also be visualized in all the eluted fractions, where we observed a mixture of 3 bands: GST-pre-phytaspase (109 kDa), mature-phytaspase (69 kDa) and a third, lower molecular weight (LMW) band of approximately 58 kDa. The presence of the LMW band could be due to the degradation of the protease or a C-terminal trimming of mature phytaspase, as is common in most plant subtilases, though this has not been confirmed in the present study.8,10,20,21 The bands equivalent to 26 kDa in the wash and eluted fractions corresponded to free GST tag. The appearance of the ~69 kDa bands in the ‘wash’ as well as the eluted fractions may have occurred due to autocatalytic cleavage of the ‘pro’ domain from pre-phytaspase releasing mature phytaspase. To confirm the successful purification of GST-pre-phytaspase, we performed a Western blot analysis using an anti-GST antibody (produced in rat, Sigma). The appearance of a band in the lane with the eluted protein fraction (Fig. 2c) confirmed the presence of purified GST-pre-pro-phytaspase. However, the absence of any other band below GST-pre-pro-phytaspase supported our hypothesis of conversion of mature phytaspase. However, amino acid sequencing is necessary to verify the autocatalytic processing of pre-phytaspase, which is an aim of our future studies.

**Concluding remarks**

Our attempt to heterologously express tobacco phytaspase has led us to clone pre-pro-phytaspase as well as mature phytaspase (catalytic domain only) with an N-terminal GST tag in *E. coli* BL21. Our recent study on GST-mature phytaspase has demonstrated that the mature phytaspase without any pro-domain maintains its catalytic activity once the GST-tag is removed.18 We have also been successful in expressing pre-pro-phytaspase in a bacterial system and have made preliminary observations of the recombinant pre-phytaspase. However, like many other research

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**Figure 2.** (A) Induction of GST-pre-pro-phytaspase in *E. coli* BL21. Lane *M*: protein marker (180 kDa-10 kDa), lanes 1 and 2: cell pellet and supernatant from induced *E. coli* BL21 containing pGEX-4T2-pre-pro-phytaspase. The band in lane 1 indicated by an arrow corresponds to GST-pre-pro-phytaspase (109 kDa). (B) Purification of GST-pre-pro-phytaspase by affinity chromatography using glutathione-agarose beads packed in a purification column. Lane *M*: protein marker (180 kDa-10 kDa), lane 1–4: eluted serial fractions from the column, lane 5: last wash before eluting the protein and lane 6: flow through. (C) Western blot showing the presence of GST-pre-pro-phytaspase in the eluted fraction after affinity chromatography. The absence of any band in the lower part of the lane indicates the self-processing of GST-pre-pro-phytaspase into mature phytaspase.
groups working on plant subtilases, we also have failed to obtain this precursor phytaspase in high enough amounts to carry out further investigations. Nonetheless, important observations made in our investigation include the following:

1) A GST tag stabilizes the expression of GST-pre-phytaspase, promoting the expression of soluble protein.

2) The inability to detect any expression of His-pre-phytaspase in the host system may be due to the fact that the protease self-processes at a higher rate due to the smaller size of the tag.

3) Successful subtilase expression with C-terminal fusion tags has been reported; however, we found that the presence of an N-terminal GST tag did not hinder the processing of pre-pro-phytaspases into their mature form.

4) It has been reported that the pro-domain is needed for protecting the mature enzyme from degradation. However, we found this to not necessarily be true, as we could obtain stably expressed His-tagged and GST-tagged mature phytaspase.

5) Although these preliminary results demonstrate the production of mature phytaspase from bacterially expressed pre-phytaspase, future studies, including amino acid sequencing and mutant phytaspase expression, are needed to support our hypothesis of the autocatalytic processing of pre-phytaspase in a bacterial expression system.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Funding

Financial support from the Department of Biotechnology Program Support, (BT/PR13560/COE/34/44/2015) and the Department of Electronics and Information Technology (No. 5 (9)/2012-NANO (Vol. II)), Government of India, are acknowledged. P.S. acknowledges financial support from the Department of Science and Technology, Government of India (SB/FTP/ETA-0122/2014). The authors thank the Center for Nanotechnology and the Central Instruments Facility (CIF), IIT Guwahati for providing the instrument facility.

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