Crystal Structure of a Papain-fold Protein Without the Catalytic Residue: A Novel Member in the Cysteine Proteinase Family

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A 31 kDa cysteine protease, SPE31, was isolated from the seeds of a legume plant, Pachyrhizus erosus. The protein was purified, crystallized and the 3D structure solved using molecular replacement. The cDNA was obtained by RT PCR followed by amplification using mRNA isolated from the seeds of the legume plant as a template. Analysis of the cDNA sequence and the 3D structure indicated the protein to belong to the papain family. Detailed analysis of the structure revealed an unusual replacement of the conserved catalytic Cys with Gly. Replacement of another conserved residue Ala/Gly by a Phe sterically blocks the access of the substrate to the active site. A polyethyleneglycol molecule and a natural peptide fragment were bound to the surface of the active site. Asn159 was found to be glycosylated. The SPE31 cDNA sequence shares several features with P34, a protein found in soybeans, that is implicated in plant defense mechanisms as an elicitor receptor binding to syringolide. P34 has also been shown to interact with vegetative storage proteins and NADH-dependent hydroxypyruvate reductase. These roles suggest that SPE31 and P34 form a unique subfamily within the papain family. The crystal structure of SPE31 complexed with a natural peptide ligand reveals a unique active site architecture. In addition, the clear evidence of glycosylated Asn159 provides useful information towards understanding the functional mechanism of SPE31/P34.

Keywords: crystal structure; elicitor receptor; plant defense; papain glycoprotein

Introduction

Cysteine proteases constitute an important class of proteolytic enzymes found in prokaryotes as well as eukaryotes like plants and animals. Structurally they belong to the papain family where individual members accomplish catalysis employing a common mechanism involving the highly conserved catalytic triad (Cys-His-Asn) residues.¹⁻⁵ Although the overall topological features and mechanism of catalysis are conserved, unique active site residues define substrate specificities for individual members.

Protein SPE31 isolated from the seeds of the legume Pachyrhizus erosus was designated a papain-like protein based on N-terminal amino acid sequencing results.⁶ Analysis of the sequence alignments for SPE31 identified P34, a protein commonly found in soybeans, as sharing 78% sequence identity. A striking feature of the catalytic site common to both SPE31 and P34, a glycine replacing the highly conserved catalytic cysteine, makes both these proteins unique members of the papain family.⁷,⁸ It was suggested that another cysteine residue close to the active site of P34 participates in the catalysis by mounting a nucleophilic attack upon deprotonation.³ However, so far, no enzymatic activity has been reported for P34. P34 was also recognized as a major soybean allergen protein accounting for IgE binding.³ Binding of P34...
Figure 1. (a) Multiple-sequence alignment of SPE31 and P34 against cysteine proteases with known crystal structures. The sequences of SPE31 from *Pachyrhizus erosus*, P34 (Swiss-Prot accession number: P22895) from soybean, papain, GP-12, actinidin, ervataminB, and ervataminC are compared with cysteine proteases of known crystal structures.
to syringolide and interaction with VSP and a NADH-dependent hydroxypyruvate reductase implicated P34 as playing an important role in plant defenses against bacteria.\textsuperscript{10–12} These evidences suggest SPE31 and P34 may represent a new subfamily of cysteine proteinases.

The novel replacement of conserved catalytic cysteine by a glycine prompted us to undertake a 3D structural investigation of the active site architecture. Here we report the crystal structures of SPE31 bound with a polyethylene glycol (PEG) molecule and a natural peptide. The structures are named SPE31_PEG and SPE31_PEP, respectively. Detailed analysis of the structures clearly shows the absence of cysteine residues around the active site. The ligand-bound structure solutions suggest possible sites for the interaction of P34 and syringolide or other proteins.

Results

The cDNA sequence of SPE31

The cDNA encoding SPE31 is 951 base-pairs in length with a poly(A)\textsuperscript{7} tail. The open reading frame consists of 738 base-pairs encoding 246 amino acid residues and is predicted to express a 27 kDa protein with two N-glycosylation sites (Asn118 and Asn159). The SPE31 amino acid sequence alignment results indicated the protein to be papain-like (Figure 1(a)). SPE31 retains most of the conserved residues found in and around the active site of cysteine proteases. One striking feature that sets SPE31 apart from other cysteine proteases is the replacement of catalytic Cys25 with Gly26. The conserved catalytic triad Cys25-His159-Asn175 (papain numbering) found in normal cysteine proteases is replaced by Gly26-His168-Asn188 in SPE31. This modified catalytic triad has previously been reported for another cysteine protease, P34.

Overall structure of SPE31

The crystal structures of SPE31_PEG and SPE31_PEP were refined with good crystallographic values (Table 1). The final model for both the structures contains one monomer per crystallographic asymmetric unit. There are no obvious differences in the fold between the two structures with a root mean square (r.m.s) difference of 0.23 Å for all C\textsuperscript{α} atom pairs. The overall fold is identical to those previously reported for papain-like proteins. SPE31 is made up of two domains (R domain and L domain) with a cleft between them (Figure 1(b)). The C-terminal region seems disordered with no clear electron density for residues 227–246 in SPE31_PEG and for residues 228–246 in SPE31_PEP. This suggests that the C terminus could be very flexible. The three disulfide bonds (Cys23–Cys64, Cys57–Cys97, Cys160–Cys213) conserved in the papain family are also seen in SPE31. In comparison to other members of the papain family, there is a seven residue insertion between β2 and α4 in SPE31 and P34 (Figure 1(a)). However, the insertion has little effect on the overall fold and is not involved in the formation of the active site.

Oligosaccharide chains of SPE31

SPE31 was reported as a glycoprotein previously.\textsuperscript{6} Two N-glycosylation sites, Asn118 and Asn159, were predicted using NetNGlyc\textsuperscript{†}. Electron density for three glycosyl residues (N-GlcNAc, GlcNAc, Fuc) bound to Asn159 was observed (Figure 2). Similar glycosylation has been reported for cysteine protease GP-II; however, the site of glycosylation is Asn156. Asn159 in SPE31 and
Asn156 in GP-II are both located in similar regions, but are not correspondent to each other structurally. In addition, the glycosyl chain in GP-II was shown to participate in the crystal lattice arrangement, while no such involvement of the carbohydrate in intermolecular bond formation was observed for SPE31. While comparing the glycosylation of other papain family members, the sequence N159C(S/T)SPYG165 seems unique to SPE31 and P34 (Figure 1(a)), which indicates that P34 may have the same glycosylation site as SPE31. The physiological role of this oligosaccharide needs further investigation.

Although Asn118 is also predicted to be a glycosylation site, the electron density for the carbohydrate is not well defined to identify the exact nature of the glycosyl structure (Figure 2). Another reason for the poor density could be that the oligosaccharide is disordered. The corresponding residue in P34 is not conserved, with an aspartic acid replacing the asparagine.

The active cleft of SPE31

In SPE31, Gly26 of the L domain replaces the conserved catalytic cysteine (Figure 1(d)). The substitution has no effect on the position of the remaining two residues (His168 and Asn188) of the catalytic triad (Figure 1(d)). Detailed analysis of the active site failed to locate any cysteine, serine or aspartic acid, in close proximity that could act as a potential nucleophile. In addition, Phe169 of SPE31 (also found in P34) replaces the highly conserved Ala/Gly found in papain and normal cysteine proteases (Figure 1(a)). The aromatic side-chain of Phe169 extends into the active cleft and obstructs substrate binding (Figure 1(d)). The absence of catalytic cysteine and the substitution of conserved Ala/Gly with Phe169, prevents SPE31 from exhibiting a normal proteinase activity.

Table 1. Data collection and structure refinement statistics

<table>
<thead>
<tr>
<th>Data collection</th>
<th>SPE31_PEG</th>
<th>SPE31_PEP</th>
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<tr>
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<td>SPE31_PEP</td>
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<td>Additional allowed (%)</td>
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Numbers in parentheses are for the highest resolution shell.

$^a$ $R_{\text{merge}} = \Sigma |I_i − I_m|/\Sigma I_i$, where $I_i$ is the intensity of the measured reflection and $I_m$ is the mean intensity of all symmetry-related reflections.

$^b$ $R_{\text{cryst}} = \Sigma ||F_{\text{obs}}|-|F_{\text{calc}}||/\Sigma |F_{\text{obs}}|$, where $F_{\text{obs}}$ and $F_{\text{calc}}$ are observed and calculated structure factors. $R_{\text{free}} = \Sigma |F_{\text{obs}}|-|F_{\text{calc}}|/\Sigma |F_{\text{obs}}|$, where $T$ is a test data set of about 5% of the total reflections randomly chosen and set aside prior to refinement.

Figure 2. The N-linked oligosaccharide chains of SPE31. The electron density ($2F_o-F_c$ maps contoured at 1$\sigma$, blue) and the difference Fourier maps ($F_o-F_c$ maps contoured at 3$\sigma$, purple) surrounding the oligosaccharide chain linked to Asn159 and the possible disordered oligosaccharide chain linked to Asn118 are shown in (a) and (b), respectively. The structure of the oligosaccharide chain linked to Asn159 is depicted as stick-and-ball model.
Figure 3. Omit maps of ligands bound to SPE31 and the mode of binding of the ligands to SPE31 and papain. The $F_0 - F_c$ omit map was constructed by omitting the PEG molecule (a) and the peptide fragment (b) and was contoured at 3σ. (a) The stereo view of the PEG molecule binding to SPE31. (b) The stereo view of the peptide fragment binding to SPE31. B1, B2, B3, B4, and B5 indicate the five residues (KASVG) of the peptide fragment. The hydrogen bonds between the peptide fragment and SPE31 are indicated by broken lines and bond lengths (Å) are labeled. (c)–(e) Binding surfaces of the PEG, the peptide fragment in SPE31 and leupeptin (N-acetyl-l-leucyl-l-leucyl-l-argininal) in papain (PDB id: 1POP) are shown from left to right. The increase in the binding surface of the peptide fragment compared to that of the PEG molecule is indicated by a red circle, which is caused by the double-conformation of the side-chain of His148.
Binding of the ligands to the active cleft of SPE31

The structure of SPE31_PEG showed a PEG molecule bound to the surface of the “active cleft” (Figure 3(a)). A much longer electron density was observed for the structure of SPE31_PEP at the same location, when the crystals were grown in the absence of PEG. The density was later identified as a peptide fragment bound to the active cleft (Figure 3(b)). The intensity of the density of the peptide fragment was comparable to that of the protein, suggesting a 1:1 binding of the peptide to SPE31. Since no ligands were added during the purification or crystallization of the protein, the bound peptide seems to have originated from the seeds from where the protein was isolated. On the other hand, high concentrations of PEG used for crystallization of SPE31_PEG seem to have displaced the peptide from the active cleft. The peptide fragment was identified as KASVG at 2.4 Å resolution with the C-terminal glycine adopting a double-conformation (Figure 3(b)).

In case of typical cysteine proteinases, the substrate or the peptide-like inhibitor binds deeply inside the active cleft and has direct interaction with the catalytic cysteine. However, the SPE31 structure shows the PEG and the peptide, both bound at the surface and not able to stretch into the cleft (Figure 3(c)–(e)). Phe169 with a large side-chain in the substrate-binding pocket seems to sterically hinder the access of the ligands to the active site cleft. (Figure 1(d)). The binding of the peptide fragment to SPE31 was mediated primarily through hydrogen bonding and hydrophobic interactions (Figure 3(b)). In addition, the side-chain of His148 adopts a double-conformation in the SPE31_PEP structure. One conformation is similar to that found in SPE31_PEG, while in another conformation, a hydrogen bond is formed between the N\textsuperscript{e} atom of the imidazole ring of the histidine and the oxygen atom of Val44 in the peptide fragment (Figure 3(b) and (d)). This interaction seems to be crucial for the tight binding of the peptide fragment. However, no hydrogen bonding was found between the PEG molecule and SPE31. The PEG bound to SPE31 through Van der Waals interactions.

Discussion

Comparison with P34

SPE31 shares 78% amino acid sequence identity with P34, a protein commonly found in the seeds of soybeans. In both the proteins the catalytic cysteine is replaced by a glycine. SPE31 and P34 are seed proteins of leguminous plants. Besides the unusual substitution of the crucial cysteine, additional differences in the primary amino acid sequence sets SPE31 and P34 apart from the other members of the papain family. The fact that the SPE31 cDNA could only be isolated from the mid maturation seeds and not mature seeds or seedlings is consistent with similar observations reported for P34, which is also synthesized primarily during seed maturation. This expression pattern is different from that of other cysteine proteinases of plant seed origin that are usually synthesized during seedling growth. In addition, SPE31 co-exists in seeds with another protein, SPE32, which is 11 amino acid residues longer at the N terminus when compared to SPE31, while P34 co-exists in soybeans with P32 which is 11 amino acid residues shorter than P34. Both the 11 amino acid residue sequences are very similar, with seven residues being identical. These unique features shared by SPE31 and P34 and not found amongst other cysteine proteinases suggest that they form a separate group distinct from the other members of the papain family.

In P34, the catalytic cysteine is replaced by a glycine (Gly26). A neighboring cysteine residue, Cys23, was assumed to play the catalytic role, since no other Cys residue was found nearby that could form a disulfide linkage with Cys23. In SPE31, Cys23 forms a disulfide bridge with Cys64 as in papain; while in P34, the position 64 is occupied by a serine. This difference could possibly be attributed to an error in the cDNA sequencing results for P34. The coding triplets for a cysteine are UGU or UGC, while UCX codes for a serine. A misinterpretation of the second base of the coding triplet could have likely resulted in serine being reported for cysteine. In case of protein Bd 30k (the same protein as P34), the sequence reported by another group (T. Takano, M. Y. Yamada, Swiss-Prot accession number: O64458), shows position 64 to be occupied by a cysteine. The X-ray crystal structure of SPE31 rules out the possibility of the role of Cys23 as a nucleophile.

Binding sites for syringolide elicitor or other proteins

Syringolides are elicitors produced by pathogens, which upon contact elicit a hypersensitive response specifically in soybean plants carrying the *Rpg4* disease resistance gene. P34 shows specific syringolide binding activity and could very well function as a syringolide receptor in the signal perception of plant defenses. P34 could also associate with VSP proteins in the presence of syringolides and interact with NADH-dependent hydroxypyruvate reductase (HPR), which could be a secondary messenger to invoke defense response. HPR is an important enzyme of the plant photorepiration system, which catalyzes the conversion of hydroxypyruvate to glycerate. It is assumed that the hypersensitive response results from the inhibition of the HPR activity due to interaction with syringolide-P34, but the mechanism of the signal recognition and transmission is still unknown. Since P34 and SPE31 are highly homologous, the structure of SPE31 can be used to predict the possible binding sites for syringolide.
elicitor or other proteins for the unique group composed by SPE31 and P34. The structure of SPE31 provides a means to identify the possible binding sites for syringolide elicitors or other proteins. The results of sequence alignment and comparison of the structure suggest that SPE31 and P34 both have retained the original papain-fold but lost the catalytic activity by replacing the catalytic cysteine with glycine. The substitution, however, seems to have no effect on the protein binding activity, which has been retained during the course of evolution. Thus, the first site for binding of other proteins appears to be the surface on the cleft where the peptide was seen bound. The second binding site might be the conserved Asn159, which is glycosylated in SPE31, and may also be glycosylated in P34. Oligosaccharides are commonly involved in protein–protein or protein–ligand recognition. Structurally syringolides resemble a tri-ribose-like head enzyme–protein or protein–ligand recognition. Structurally syringolides resemble a tri-ribose-like head.

Materials and Methods
cDNA cloning and sequence analysis of SPE31
Total RNA was isolated from the mid maturation seeds of Pachyrhizus erosus. The seeds were ground to powder in liquid nitrogen and the total RNA was extracted using RNeasy Plant Mini Kit (QIAGEN). The sequence of the forward primer S'-gat(c) gcl ccI gag(a) tc(g)I tgg gat(c) tgg-3' was designed according to the N-terminal amino acid sequence ΔAPESWDW previously obtained. Oligo(dT) primers were used as reverse primer. 25 μl RT reaction mixtures consisted of 5 μl total RNA, 4 μl oligo(dT) (20 μM), 2000 units of M-MLV reverse transcriptase, 1.5 μl 2.5 mM dNTP, 1 μl RNase inhibitor (40 units/μl) and 5× buffer. After completion of the RT reaction, 8 μl of the product was used for PCR amplification in 50 μl reaction mixtures containing 10× PCR buffer, five units of Taq DNA polymerase, 0.25 mM dNTP, and 0.8 mM each of forward and reverse primers. The PCR program consisted of an initial cycle of denaturation (95°C, 3 min), annealing (52°C, 1 min) and prolonged extension (72°C, 15 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (52°C, 40 s) and extension (72°C, 1 min). A final 10 min extension step at 72°C ended the amplification. The RT-PCR product was gel purified and subcloned into the pGEM T vector (TaKaRa Biotechnology Co., Ltd). Positive cDNA clones were identified by subjecting the plasmids extracted from transformants to PCR amplification using the conditions described above. The positive clones were further verified by sequencing (TaKaRa Biotechnology Co., Ltd).

The similarity searches for the amino acid sequence were performed using BLAST.14 NetNGlyc 1.0 Server1 was used for the prediction of N-glycosylation sites of SPE31.

Purification, crystallization and data collection
Protein purification and crystallization of SPE31_PEG were carried out as described before. SPE31_PEG crystals were grown at 281 K using the hanging-drop vapor-diffusion method. 2 μl hanging drops consisted of 1 μl of 15 mg/ml protein solution and 1 μl of reservoir solution (2.0 M ammonium sulfate, 5% isopropyl alcohol). Diffraction data were collected at 100 K with MarResearch (for SPE31_PEG) and Rigaku R-AXIS IV++ (for SPE31_PEP) imaging-plate detectors. Data were processed using DENZO and SCALEPACK.15 Data collection statistics are listed in Table 1.

† http://www.cbs.dtu.dk/services/NetNGlyc/

Conclusions
We have determined the crystal structure of SPE31, which provides the first structural details for a novel group of proteins that posses a papain fold but lack the catalytic cysteine in the active site. Although SPE31 shows considerable homology both in sequence and structure to other members of the papain family, the structural differences are quite remarkable. Besides the substitution of the catalytic cysteine by a glycine, the substrate-binding cleft occupied by an aromatic residue (Phe169) interferes with the enzyme activity. By analyzing the natural peptide fragment bound to the surface of SPE31 and its comparison with P34, we suggest the role of SPE31/P34 in HR and provide some plausible modes of binding of SPE31/P34 with other proteins and small molecules (especially syringolides).
Structure determination

The SPE31_PEG structure was solved by molecular replacement (AMoRe) using papain (PDB id: 1POP) as a search model. The program O was used to rebuild the initial model. The model was refined at 50–2.0 Å resolution range by using CNS. PEG, oligosaccharide initial model. The model was refined at 50–2.0 Å resolution range by using CNS. PEG, oligosaccharide initial model. The model was refined at 50–2.0 Å resolution range by using CNS. The refined structure of SPE31_PEG was used as the initial model for the SPE31_PEP structure refinement. The refined structure of SPE31_PEG was used as the initial model for the SPE31_PEP structure refinement. A five residue peptide fragment (KASVG) was added to the model and water molecules were added to the model and individual atomic B-factors were refined at latter stage of refinement.

References


GenBank and Protein Data Bank accession codes

The cDNA sequence for SPE31 has been deposited in the GenBank database and assigned GenBank accession number DQ152924. The atomic coordinates of SPE31_PEG (PDB id: 2B1M) and SPE31_PEP (PDB id: 2B1N) are available at the RCSB Protein Data Bank.

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† http://www.rcsb.org/


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