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Chapter

Determination of Substrate Specificity of the Purified Novel Plant Cysteine Protease Solanain From the Latex of *Vallaris solanacea*

Silpa Somavarapu

Abstract

Peptide synthesis refers to the production of peptides. Proteases are the enzymes that degrade proteins. They hold first place in the world market of enzymes, estimated at ~US$3 billion. Proteases are distributed widely in different parts of the biological sources. In occurrence of proteases, plant kingdom occupies the highest rank. The increasing demand for proteases and the need for economical production of commercially useful industrial proteases from novel sources are taken into consideration. A novel plant latex cysteine protease namely *Vallaris solanacea* was identified in Biodiversity Park, Visakhapatnam which showed maximum protease activity. It has been shown that the latex of *Vallaris solanacea* contains a high protease activity. The cysteine protease solanain was purified by fractionation with ammonium sulphate followed by DEAE-cellulose ion exchange and gel chromatography. Specificity studies towards synthetic peptide and ester substrates by the protease purified from the latex of *Vallaris solanacea* were performed. The purified solanain exhibited broad specificity similar to other cysteine proteases. However considerable differences were also noticed in the rate of hydrolysis and specificity towards simple peptide substrates.

Keywords: peptide synthesis , proteases, *Vallaris solanacea*, specificity studies, cysteine proteases, peptide substrates

1. Peptide synthesis

Peptide synthesis refers to the production of peptides. Peptides and proteins are linear polymers of amino acids linked by amide peptide bonds. Amino acids are primary amines that contain an anomeric carbon that is connected to a hydrogen atom (H), an amino (NH_3) group, a carboxyl group (COOH) and a variable side group (R) (**Figure 1**).

Peptides are chemically synthesized by the condensation reaction of the carboxyl group of one amino acid to the amino group of another. The formation of peptide bonds by linking amino acids together dates back to 100 years. The first peptides to be synthesized, including oxytocin and insulin took about 50–60 years

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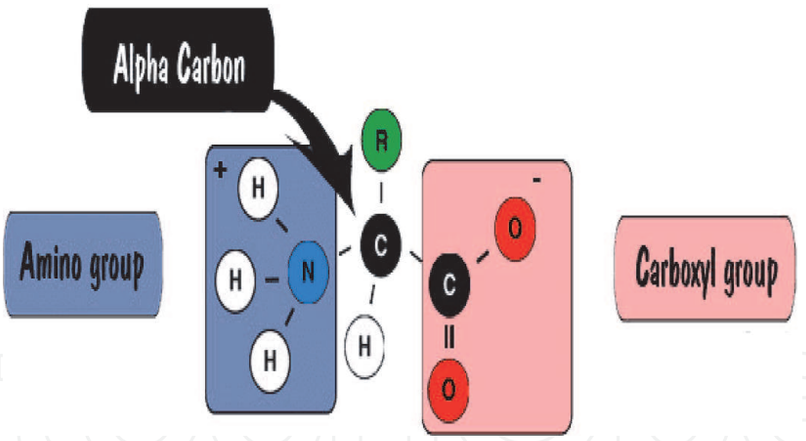


Figure 1.
Peptide structure.

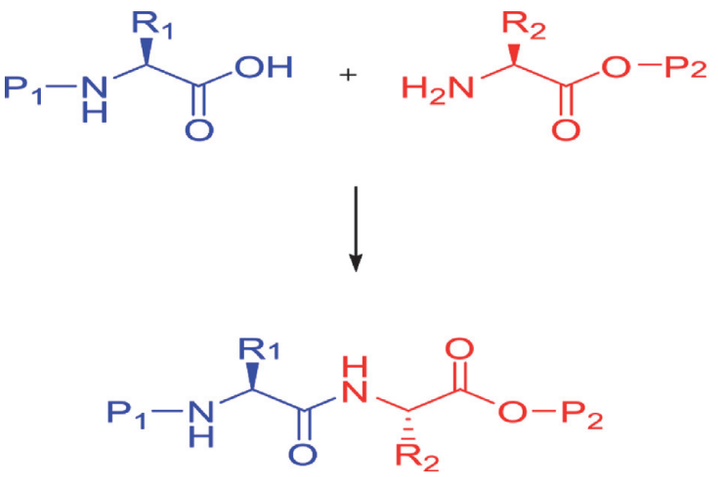


Figure 2.
Coupling of two amino acids in solution. The unprotected amine of one reacts with the unprotected carboxylic acid group of the other to form a peptide bond.

which clearly indicates chemical synthesis of peptides is a difficult task [1]. But advances in protein synthesis today made peptide synthesis easier today which has varied applications in high-throughput biological research and drug development [2] (**Figure 2**).

2. Peptide synthesis

They are two methods of peptide synthesis, namely

- Liquid-phase peptide synthesis
- Solid-phase peptide synthesis

Liquid-phase peptide synthesis is the classical method that is slow and labour intensive in which separate peptides are synthesized and then coupled together to create larger peptides. In this method chemical group is used to protect the C-terminus of the first amino acid.

Solid-phase peptide synthesis is the most common method where the C-terminus of the first amino acid is coupled to an activated solid support, such as polystyrene or polyacrylamide. This method is advantageous as the resin acts as the

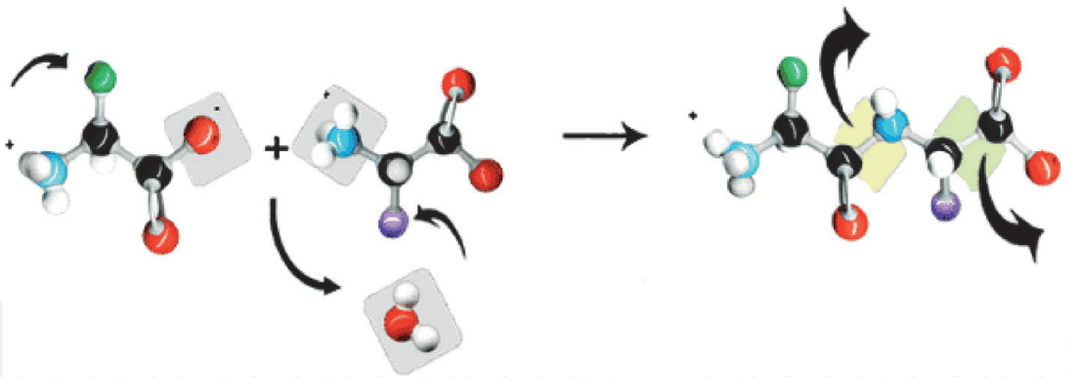


Figure 3.
Peptide bonds created with peptide coupling agents.

C-terminal protecting group and it is a rapid method to separate the growing peptide product from the different reaction mixtures during synthesis.

These two ways are combined in an exceedingly method known as native chemical tying. LifeTein's customary amide synthesis method uses the solid part. The liquid-phase approach is employed for the synthesis of short peptides, like di- and tripeptides, and C-terminally changed peptides, like accelerator substrates (**Figure 3**).

3. Solid phase synthesis

The important method for the synthesis of peptides in the lab is known as solid-phase peptide synthesis (SPPS) [3]. Merrifield [4] developed this method of SPPS which allows the rapid assembly of a peptide chain through successive reactions of amino acid derivatives on an insoluble porous support. Unlike ribosome protein synthesis, artificial synthesis builds peptides in the C to N direction. During solid-phase peptide synthesis, each peptide is anchored to an insoluble polymer at the C-terminus.

3.1 Solid support

Solid support in SPPS is Polystyrene, a styrene cross-linked with 1–2% divinylbenzene which is a popular carrier resin in SPPS. Other common gel-type supports include polyacrylamide and polyethylene glycol (PEG). Polystyrene is chemically inert under SPSS conditions. The solid support helps in anchoring the amino acids and formation of peptide.

3.2 Protecting groups

Amino acids contain side chains with different functional groups with different reactivity. Thus different protecting groups are required. A major problem in peptide synthesis is the side reactions due to multiple reactive groups in amino acids. In order to perform peptide formation with minimal side reactions or to protect the functional groups from non-specific reactions reactive groups in the amino acids need to be blocked or protected. For this many chemical groups have been developed that bind to the amino acids and protect it. They are two types of protecting groups, namely

- Temporary protecting groups
- Permanent protecting groups

3.2.1 Temporary protecting groups

N-terminal ends of amino acids are protected by groups called 'temporary' protecting groups as they are easily removed to allow peptide bond formation.

3.2.2 Permanent protecting groups

But Side chain protecting groups are known as permanent protecting groups as they remain at all the multiple cycles of chemical treatment during the synthesis phase and they can be removed only on treatment with strong acids after synthesis is complete (**Figure 4**).

Peptide synthesis involves coupling of carboxyl group and the incoming amino acid to the N-terminus of the growing peptide chain. It is a step wise method and also a cyclic process. It involves the following steps.

1. Blocking of amino group of amino acid by N-terminal protecting groups

Prior to protein synthesis, individual amino acids are reacted with these protecting groups. Two common N-terminal protecting groups are tert-butoxycarbonyl (Boc) and 9-fluorenylmethoxycarbonyl (Fmoc), and each group has distinct characteristics that determine their use (**Table 1**; **Figure 5**).

2. Blocking of carbonyl group of amino acid by C-terminal protecting groups

Similarly the carbonyl group of amino acid is protected by C-terminal protecting group. The C-terminal protecting group depends on the type of peptide

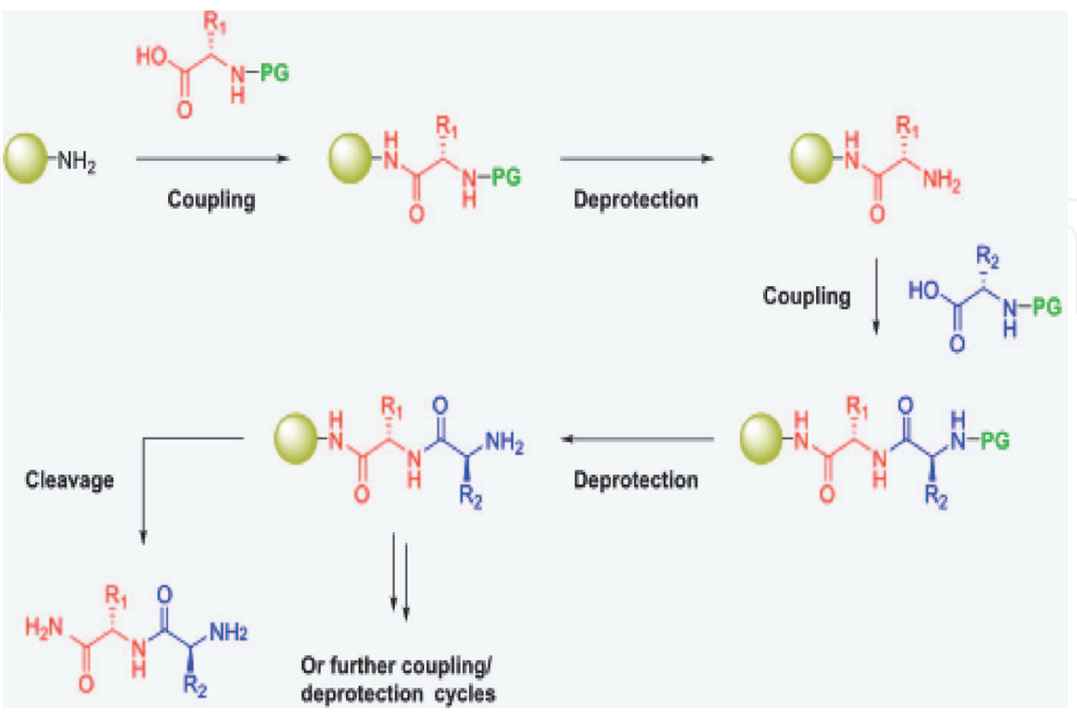


Figure 4.
Solid-phase synthesis of a dipeptide victimization associate degree (amine-functionalized) organic compound. The N-terminal protective cluster (PG) will be Fmoc or Boc, reckoning on the protective cluster theme used (see below). The amino alkanoic acid facet chains (R1, R2, etc.) are orthogonally protected (not shown).

S. No	Fmoc	Boc
1	Routine synthesis	Requires special equipment
2	Relatively safe	Potentially dangerous
3	Acid-sensitive peptides and derivatives	Base-labile peptides
4	Frequent aggregation	Moderate aggregation
5	TFA final deprotection	HF final deprotection

Table 1.
Difference between Fmoc and Boc.

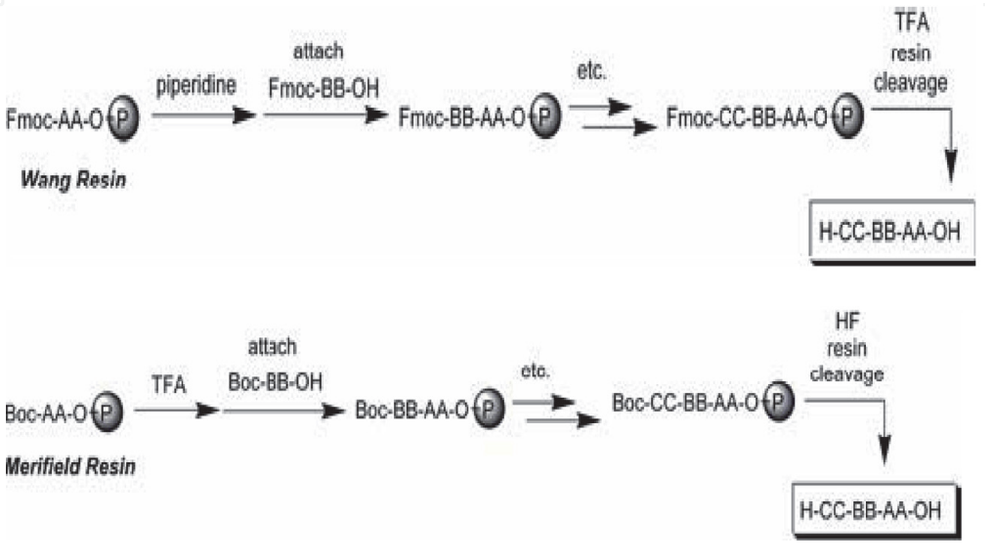


Figure 5.
Fmoc strategy (Wang resin) and Boc strategy (Merrifield resin).

synthesis used. For liquid-phase peptide synthesis C-terminus of the first amino acid is protected by chemical reagent while in solid-phase peptide synthesis solid support (resin) acts as the protecting group for the C-terminal amino acid (**Figure 6**).

3. Coupling of the protected amino acids to form a peptide

Once peptide synthesis is completed, all the protecting groups are removed from the nascent peptides. First C-terminal carboxylic acid is activated using carbodiimides like dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC). These coupling reagents respond with the carboxyl gathering to shape an exceptionally receptive O-acylisourea moderate that is immediately dislodged by nucleophilic assault from the deprotected essential amino gathering on the

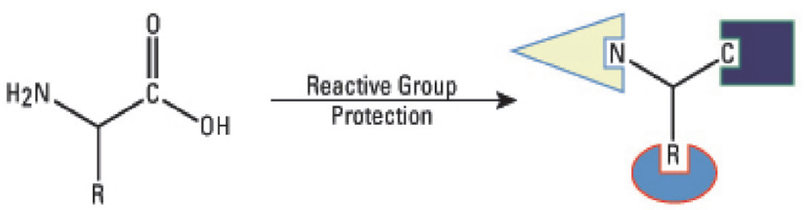


Figure 6.
Amino acid functional group protection.

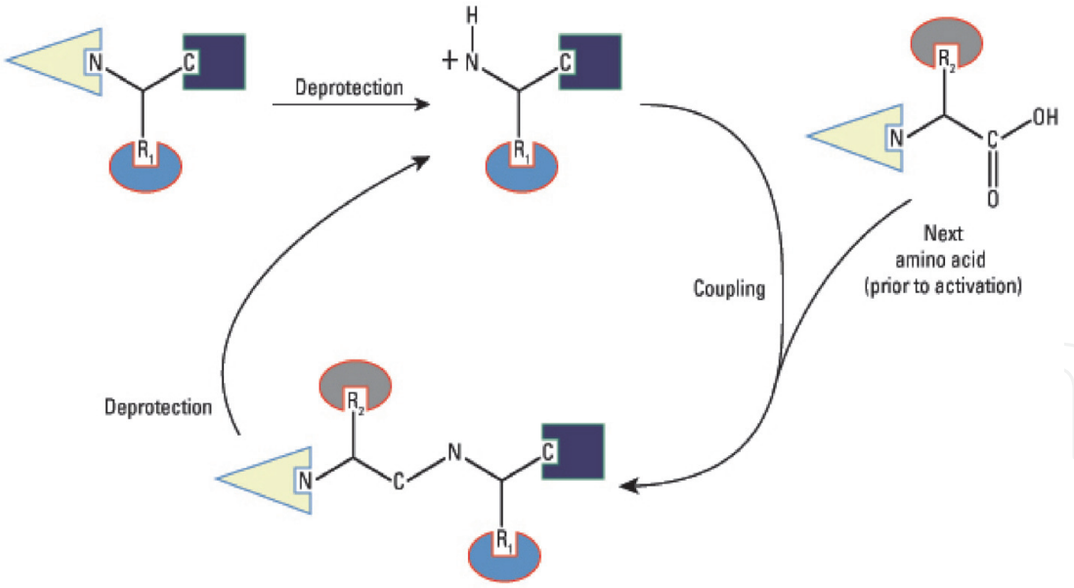


Figure 7.
Diagram of peptide synthesis. Peptide bond formation between the deprotected N-terminus of the first amino acid and the activated C-terminus of the incoming amino acid. This cycle of deprotection and coupling is repeated until the full-length peptide is formed.

N-end of the developing peptide chain to frame the beginning peptide security. To affirm total coupling a test is performed called Kaiser Test (**Figure 7**).

4. Deblocking the amino group of amino acid

Removal of specific protecting groups from the newly added amino acid immediately after coupling to allow the next incoming amino acid to bind to the growing peptide chain is called deprotection. Boc is removed using moderately strong acid such as trifluoroacetic acid (TFA) while Fmoc is a base-labile protecting group that is removed with a mild base such as piperidine.

4. Deblocking the carbonyl group of amino acid

The synthetic peptide purification is by compounds like water, anisol or thiol derivatives are added in excess during the deprotection step to react with any of

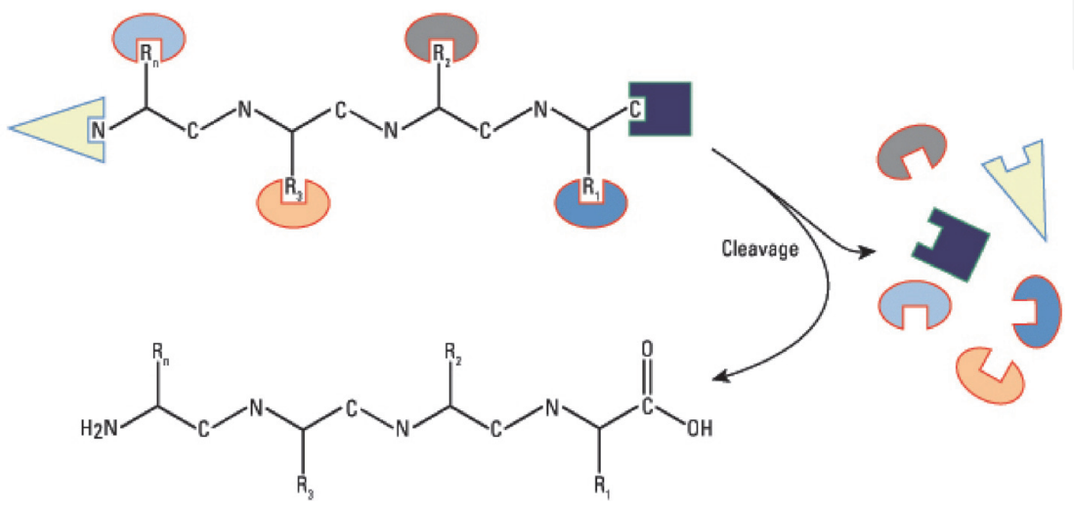


Figure 8.
Diagram of peptide cleavage after synthesis. The remaining N-terminal protecting groups, all side-chain protecting groups and the C-terminal protecting group or solid support are removed by strong acid treatment after peptide synthesis is completed.

these free reactive species. This cycle of deprotection and coupling is repeated. The remaining protecting groups are cleaved by acidolysis, using; strong acids such as hydrogen fluoride (HF), hydrogen bromide (HBr) or trifluoromethane sulphonic acid (TFMSA) (**Figure 8**).

5. Peptide purification

Peptides are purified by the following techniques namely Ion exchange chromatography (IEC), Partition chromatography, Size-exclusion chromatography, Reverse-phase chromatography (RPC) and High-performance liquid chromatography (HPLC).

6. Applications of synthetic peptides

The Peptide synthesis (**Figure 9**) holds varied applications including

- Synthetic peptides are used to study enzyme-substrate interactions within important enzyme classes such as kinases and proteases, which play a crucial role in cell signalling.
- The development of epitope-specific antibodies against pathogenic proteins.
- The study of protein functions and the identification and characterization of proteins.

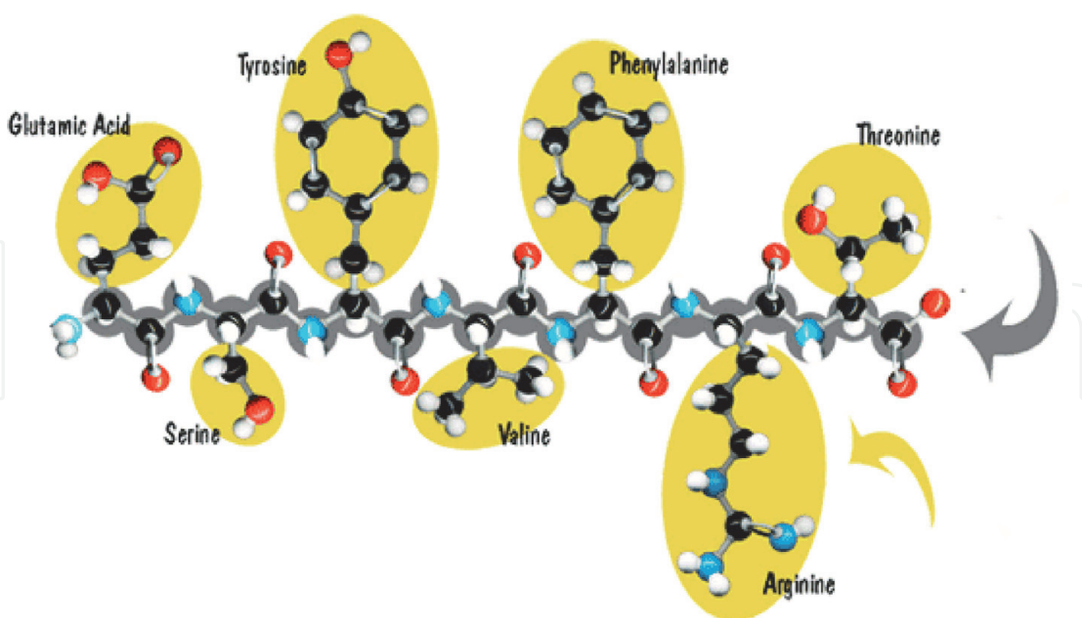


Figure 9.
Polypeptide formation.

7. Proteases

For about a century, the enzymes that play the central role in the degradation of proteins by hydrolysing peptide bonds have been known as 'proteases' and the term protease is therefore equivalent to 'peptide hydrolase'. They hold first place in the

world market of enzymes, estimated at ~US$3 billion [5]. Proteases are distributed widely in different parts of the biological sources. In occurrence of proteases, plant kingdom occupies the highest rank (43.85%) followed by bacteria (18.09%), fungi (15.08%), animals (11.15%), algae (7.42%) and viruses (4.41%). According to

Family, species	Name, EC-number	Reference(s)
In latices	Papain, 3.4.22.2	[9, 11, 19]
(I) Caricaceae	Chymopapain,	Kunimitsu and Yasunobu, 1970, Robinson, 1975;
1. *Carica papaya*	3.4.22.6	Khan and Polgar, 1983
2. *Pileus mexicanus*	Papayapeptidase-I	Lynn, 1979; Lynn and Yaguchi, 1979; Polgar, 1981;
(II) Moraceae	(A and B)	Baines and Brocklechurt, 1982
3. *Ficus carica*	Mexicain, 3.4.99.14	Castaneda-Agullo et al., 1942; Soriano et al., 1975
4. *Ficus racemosa*	Ficin, 3.4.22.3	Sgarbieri et al., 1964; Kramer and Whitaker, 1964;
(III) Asclepiadaceae	Protease	Sugiura and Sasaki, 1974
5. *Asclepias speciosa*	Asclepain, 3.4.22.7	Devaraj et al., 2008
6. *Funastrum clausum*	Funastrain c II	Greenberg and Winnick, 1940
(IV) Apocynaceae	Tabernamontanain,	Morcelle et al., 2004
7. *Tabernaemontana*	3.4.99.23	Jaffe, 1943
grandiflora	Indicain	Singh, 2008
(V) Urticaceae	Milin	Yadav et al., 2006
8. *Morus indica*	Taraxilisin	Rudenskaya et al., 1998
(VI) Euphorbiaceae	Fruit bromelain,	Cooreman et al., 1976; Murachi 1970; 1976
9. *Euphorbia milii*	3.4.22.4	Messing and Santoro, 1960
(VII) Asteraceae	Pinguinain,	Agundis et al., 1977
10. *Taraxacum*	3.4.99.18	Arcus, 1959; McDowall, 1973; Brocklehurst et al., 1981
officinale	Hemisphaericin	Murachi, 1970; 1976; Heinicke and Gortner, 1957
In fruits	Actindin, 3.4.22.14	[12]
(IX) Bromeliaceae	Stem bromelain,	Daley and Vines, 1978
30. *Ananas comosus*	3.4.22.4	Yamaguchi et al., 1982
31. *Bromelia penguin*	Ananain	Yamaguchi et al., 1982
32. *Bromelia hemisph*	Leaf proteases	
(X) Actinidiaceae	... ...	
33. *Actinidia chinensis*	... ...	
In vegetative organs		
(XI) Bromeliaceae		
34. *Ananas comosus*		
(XII) Zingiberaceae		
35. *Zingiber officinale*		
36. Miut		

Table 2.
List of plant cysteine proteases and their sources.

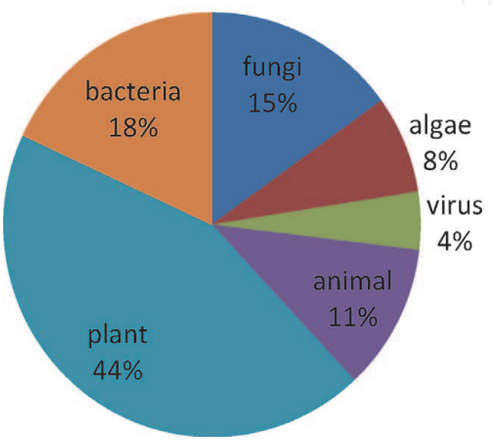


Figure 10.
Distribution of proteases.

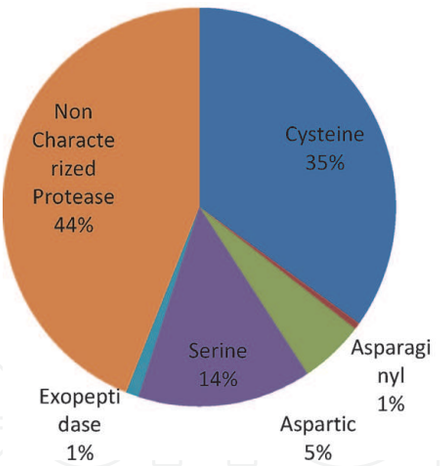


Figure 11.
Plant proteases.

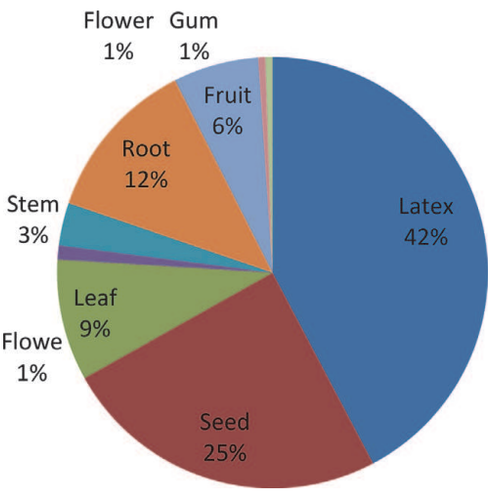


Figure 12.
Distribution of proteases in plant parts.

Actinidin	Asp-Ile-Lys-Ser-Gln-Gly-Glu-Cys-Gly-Gly-Cys-Trp
Papain	Pro-Val-Lys-Asn-Gln-Gly-Ser-Cys-Gly-Ser-Cys-Trp
Ficin	Pro-Ile-Arg-Gln-Gln-Gly-Gln-Cys- Gly-Ser-Cys-Trp
Stem-bromelain	Asn-Gln-Asp-Pro-Cys-Gly-Ala-Cys-Trp
Fruit –bromelain	Asn-Gln-Asn-Pro-Cys-Gly-Ala-Cys
Chymopapain	Lys-Arg-Val-Pro-Asp-Ser-Gly-Glu-Cys-Tyr

Papain	Val-Gly-Pro-Cys-Gly-Asn-Lys-Val-His-Ala-Val-Ala-Ala-Val-Gly-Tyr
Ficin	Thr-Pro-Cys-Gly-Thr-Ser-Leu-Asp-His-Ala-Val-Ala-Leu
Stem- bromelain	His-Ala-Val-Thr-Ala-Ile-Gly-Tyr
Actinidin	Pro-Cys-Gly-Thr-Ala-Val-Asp-His-Ala-Ile-Val-Ile-Val-Gly

Figure 13.
Amino acid sequence around the active site cysteine and histidine residues (in bold) of some plant cysteine proteases.

Barrett and McDonald [6], plant proteases are classified into serine, cysteine, aspartic and metalloproteases. Cysteine proteases (EC 3.4.2.2) are found in bacteria [7], eukaryotic microorganisms [8], plants [9] and animals. Cysteine proteases are represented by 70 families belonging to 12 different classes [10] (**Table 2**; **Figures 10–13**).

8. Screening of plant lattices for novel plant latex cysteine protease

Taking into consideration, the increasing demand for proteases and the need for economical production of commercially useful industrial proteases from novel sources. A number of plant lattices belonging to different plant families have been collected in and around Visakhapatnam and screened for protease activity. Their activities were assayed immediately and were stored in ice for further investigations. A novel plant latex cysteine protease namely *Vallaris solanacea* was identified in Biodiversity Park, Visakhapatnam which showed maximum protease activity. Preliminary studies on protease activity from the latex of *Vallaris solanacea* were carried out. The protease was purified and characterized. Specificity studies towards synthetic peptide and ester substrates by the protease purified solanain.

9. Substrate specificity of the purified solanain from the latex of *Vallaris solanacea*

Plant lattices are rich source of proteases. Latex of *Vallaris solanacea* also showed high protease activity. The cysteine protease solanain was purified by ammonium sulphate precipitation followed by DEAE-cellulose ion exchange and gel chromatography. The purpose of the present study is determination of specificity of purified solanain towards synthetic peptide and ester substrates. Extensive and more systematic studies [13–16] have been made on papain, ficin [17] and bromelain [18].

10. Materials and methods

10.1 Preparation of peptide solutions

First step is preparation of 10 mM peptide solution having tryptophan, tyrosine and phenylalanine. Peptides were solubilized using dilute acid, the assay mixture contained higher buffer concentration viz. 0.15 M.

10.1.1 Solanain action on peptides

Peptide was incubated by solanain in 0.05 M Phosphate buffer (pH 7.0), 10 mm β-mercaptoethanol, 2 mM EDTA, 2.5 mm amide and 0.5 mg to 1.0 mg/ml catalyst protein. Incubation was administered for 48 h at 30°C. The reaction was detected by sampling the digestible mixture on aid. For every digestion mixture, a bearing while not substrate or without catalyst was analysed. Skinny layer activity was performed as delineated by Renderath (1963).

10.1.2 Preparation of colloid silica gel G plates

About 25 weight unit of colloid Silica Gel G was mixed with 50 cm^3 of H_2O and stirred smartly during a closed round shape flask till completely distributed. The suspension was transferred into Stahl's mechanical spreader adjusted to 250 μ

thickness, victimization the spreader; layers were ready on 20 × 20 cm glass plates. The gel was allowed to dry for a couple of minutes and so activated by drying in associate degree kitchen appliance at 1100°C for half-hour.

10.1.3 Developing the chromatographic plate

About 5–10 μl of the sample was loaded. Solvent used was 4:1:1 butanol: carboxylic acid: water (v/v/v). After development the plates were removed, dried and detected by spraying with 0.2% ninhydrin in butanol: ethanoic acid (95, 5 v/v) mixture. Rf values of the spots were calculated. Amino acids were identified.

11. Results and discussion

Studies on substrate specificity were done and results were tabulated. Solanain was capable of hydrolysing peptide bonds involving the amino groups of hydrophilic amino acid residues (peptides 1 to 5) and incapable of peptide bonds involving the groups of deliquescent organic compound residues (peptides one to 5) and incapable of hydrolysing amide bonds wherever amino group was given by a

S. No	Peptides	Protease activity
Simple dipeptides		
1	Gly – Gly	++
2	Gly – L - α- Ala	+
3	Gly – L – Asn	+++
4	Gly – DL – Asn	+
5	Gly – D – Asn	+++
6	Gly – L – Leu	–
7	Gly – L - β- phe	–
8	Gly – L – Trp	–
9	L – Ala – L – Met	–
10	L – Leu– L – Met	+++
11	L – Trp – Gly	–
12	L – Trp – L – Tyr	–
13	L – Tyr – Gly	–
N-aryl-dipeptides		
14	N – Z – L – Glu – L – phe	+++
15	N – Z – L – Glu – L – Tyr	++
16	N – Z – L – Ileu – L – Met	–
17	N – Z – L – Met – Gly – OEt*	++++
18	Hippuryl – L – Arg	++
Tripeptides		
19	Gly – Gly – Gly	–
20	L-Glu-L-Val-L-Phe	–
21	L – Leu- Gly – Gly	+

**Both ester bond and the peptide bond of N – Z – L – Met – Gly – OEt were hydrolysed.*

Table 3.
Reaction of di- and tripeptides by refined Solanain.

Substrate		$10^8(E_0)(M)$	V_0/E_0 Moles/sec/mole enzyme	% Relative activity*
Amino acid side chain	p-Nitrophenyl ester			
Nil	Z-Gly-Onp	1.63	5.29	10
Unbranched	Z-L-Ala-Onp	3.27	16.94	320.00
Branched	Z-L-Val-ONP	16.30	0.48	9.07
	Z-L-Leu-Onp	3.27	2.14	10.45
	Z-L-lleu-Onp	16.30	0.21	3.97
Aromatic	Z-L-phe-Onp	3.27	1.02	09.28
	Z-L-Tyr-Onp	3.27	1.69	31.94
	Z-L-Trp-Onp	3.27	0.74	13.99
Imino acid	Z-L-pro-Onp	163.00	0.0	0.0
	N,N^1-di-Z-L Lys-Onp	163.00	0.0	0.0

**Calculated taking activity toward Z-Gly-ONp as 100%.*

Table 4.
Hydrolysis of various synthetic ester substrates with purified solanain.

hydrophobic residues (peptides half dozen to 9) with a large aspect chain e.g., leucine, essential amino acid, essential amino acid and essential amino acid. However, if the organic compound contributory the group of the bond was aromatic the bond was not hydrolysed although the C-terminal amino acid was deliquescent. Neither L-Trp-Gly nor L-Tyr-Gly was hydrolysed. It seems that the presence of a deliquescent organic compound at the N-terminal finish is not needed for the protein.

In **Table 3**, the results of the speed measurements given for 10 parts as V_o/E_o wherever V_o is initial rate and E_o is concentration of the protein in moles. The concentration of substrate used was one.56 × 10-4 M. If the metric linear unit values of the p-nitrophenyl esters of CBZ-amino acids were of constant magnitude as for different thiol proteases like papain [9], ficin [17], bromelain [18] and ananain [12] then V_o/E_o values obtained for Glycine and amino acid were love the Kcat values of the corresponding esters of different proteases.

The refined solanain of *Vallaris* family Solanaceae showed close to identical specificity towards all the substrates.

Solanain, differs from the amino alkanoic acid proteases in having low amidase activity with BAPNA and conjointly did not show any esterase activity with BAEE. This finding suggests considerably low specificity for essential amino acid residues. It differs from enzyme, ficin and bromelain during this facet. By showing broad specificity, Solanain resembled enzyme [11], ficin [14, 17] and bromelain [18] that change a spread of amide bonds. All these, however, showed a preference for basic amino acids. The solanain of *Vallaris* potato family hydrolysed leucyl bonds with efficiency and during this respect resembled ficin [17] and differed from bromelain [18] (**Table 4**).

12. Conclusion

Substrate specificity studies showed solanain exhibited broad specificity. It showed peptidase activity, amidase activity. The enzyme was capable of catalysing the hydrolysis of p-nitrophenyl esters of amino acids. It exhibited difference in specificity towards simple peptide substrates.

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