A novel subtilase from common bean leaves

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Abstract **We describe the isolation of a protease from common bean leaves grown in the field. On the basis of its biochemical properties it was classified as serine proteinase belonging to the subtilisin clan. Isoelectric focusing resulted in a single band at pH 4.6, and SDS–PAGE in a single band corresponding to M_r 72 kDa. The proteinase activity is maximal at pH 9.9 and shows high stability in the alkaline region. The relative activities of the proteinase for eight different synthetic substrates were determined. The requirement for Arg in the P1 position appeared obligatory. k_{cat}/K_m values indicate that, for highest catalytic efficiency, a basic amino acid is also required in the P2 position, presenting a motif typical of the cleavage site for the kexin family of subtilases. The sequence of the 17 N-terminal amino acids of this proteinase shows similarity to those of other plant subtilases, sharing the highest number of identical amino acids with proteinase C1 from soybean seedling cotyledons and a cucumisin-like proteinase from white gourd (*Benincasa hispida*).**

Key words: Leaf; Subtilase; Kexin family; Enzyme purification; *Phaseolus vulgaris*

1. Introduction

Barrett and Rawlings [1] grouped serine proteinases into six clans; the second largest is the subtilisin clan. The majority of subtilases are synthesized as prepro-enzymes and subsequently processed to the active form [2]. Siezen and Leunissen [3] aligned over 170 amino acid sequences of subtilases on the basis of their structurally conserved regions which include the conserved catalytic residues Asp32, His64 and Ser221, and the oxyanion-hole residue N155 (subtilisin BPN' numbering), all of which are located in the N-terminal domains of the mature enzymes. Based on sequence similarity, six families were proposed but only two, the pyrolysin and kexin families, include proteinases from higher organisms. All the plant subtilases so far known are members of the pyrolysin family, which combines enzymes of varied origin and low sequence homology (mostly $<37\%$). The most striking characteristic of plant subtilases is the presence of long insertions of up to 169 amino acids in the central region of the catalytic domain, causing a shift of the Ser of the catalytic triad towards the C-terminus [3].

All characterized members of the subtilisin clan are tripeptidylpeptidases or endoproteinases. In lower eukaryotes the endoproteinases are responsible for degrading different proteins based on their broad specificity. In higher eukaryotes some subtilisins have retained their degradative function while others, typically those of the kexins [4] which cleave at the carboxy-terminal side of a pair of basic residues and some members of the pyrolysin family [5,6], have developed into highly specialized enzymes involved in activating polypeptide precursors by specific proteolysis.

The first plant subtilase to be described was cucumisin from the sarcocarp of melon fruit (*Cucumis melo*) [7,8]. Subsequently, more cucumisin-like subtilases were isolated from other plants [9–14], but it was cDNA cloning that revealed the high diversity of subtilisins in the plant kingdom [8,15–31]. Knowledge about their function, however, was mainly inferred from gene sequences.

Little direct evidence is available about the proteolytic activities of plant subtilases, except for those with broad specificity such as cucumisin [32–34] and cucumisin-like proteases [9,10–14]. One of the pathogen-inducible P69 proteases from tomato is capable of post-translational processing of LRP cell wall protein [35]. Another tomato subtilase, encoded by *Le*SBT1 and obtained by overexpression in insect cells, exhibited significant specificity for cleaving the peptide bond carboxyl-terminal to glutamine residues [36]. The protease involved in initiating the degradation of storage proteins in soybean seeds, recently recognized as subtilisin-like [30], is specific for sites containing long strings of acidic amino acid residues [37,38]. In addition to these findings, circumstantial evidence indicates the existence of plant proteases with processing activity typical of the kexin family of subtilases. In tomato, a 50 kDa systemin-binding protein has been identified, having similar properties to the kex2 protease from yeast [39]. Further, within the secretory pathway of tobacco cells, KP6 killer toxin is processed by a proteinase, later shown to have the substrate specificity of a kex2 protease [40,41].

Plant subtilases appear to be involved in degradative processes and in processing of pre-proteins, although direct evidence based on the specificity of purified enzymes is limited. Our search for proteolytic enzymes in common bean leaves grown in the field resulted in the isolation of a new plant subtilase named KLSP. Here, we describe some of its physical properties, as well as its specificity and kinetics towards different synthetic substrates.

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Abbreviations: Boc, *t*-butyloxycarbonyl; E-64, L-3-carboxy-2,3-*trans*-epoxypropionyl-leucylamido(4-guanidino)butane; MCA, 4-methyl-7-coumarylamide; PMSF, phenylmethylsulfonyl fluoride; Z, benzyloxycarbonyl

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2. Materials and methods

2.1. Plant material

Seeds of common bean (*Phaseolus vulgaris* L. cv. Češnjevec, Semenarna Ljubljana, Slovenia) were planted in the field. At the stage just preceding the flowering, mature leaves were harvested, stored at −20°C and used for protein purification.

2.2. Purification of KLSP

Protein concentrate from common bean leaves was obtained by a modification of the method of Denisson [42]. Frozen leaves were ground in liquid nitrogen, rinsed twice with cold 80% (v/v) solution of acetone in water, and separated from the solvent by centrifugation. 0.05 M sodium ascorbate in 0.1 M sodium–potassium phosphate buffer, pH 5.0, containing 2 mM EDTA was added to the pellet. When the pellet thawed, the suspension was centrifuged for 20 min at 4200×*g* in a Sorvall centrifuge. Solid ammonium sulfate was added to the supernatant to a final concentration of 2 M and *t*-butanol to 30% (v/v) final concentration. The solution was thoroughly mixed and stood for 1 h. Three phases were separated with the proteins concentrated in the middle phase, which was collected. The extract obtained from 1 kg of leaves was dialyzed against 0.05 M sodium–potassium phosphate buffer, pH 6.9, containing 1 mM EDTA, and applied to a column of DEAE-Sephacel (3×43 cm), equilibrated with the same buffer. Fractions active towards Z-Phe-Arg-MCA were eluted in two partially resolved peaks by a gradient of NaCl concentration (0–0.9 M). The first active peak was concentrated and dialyzed against 0.02 M sodium acetate buffer, pH 5.2 containing 1 mM EDTA and further purified on a CM-Sephadex C-50 column (2.4×40 cm), equilibrated with the same buffer. Active material, which eluted in the column flow-through peak, was concentrated by ultrafiltration through an Amicon YM-10 membrane and applied to a calibrated Sephacryl S-200 column (1.8×110 cm) at a flow rate of 13.8 ml/h. The column was calibrated with blue dextran, bovine serum albumin, egg albumin and chymotrypsinogen. The whole purification procedure was carried out at 4°C.

2.3. Protein determination

Protein concentrations in unpurified samples were determined by Bio-Rad Protein Assay [43] using bovine serum albumin as a standard. Protein concentrations in eluted fractions and in purified samples were determined by measuring absorbance at 280 nm. Specific absorbance at 280 nm in a 1 cm cuvette ($A^{1\%}$) of 1% KLSP solution was taken as 0.87, which is the average value of $A^{1\%}$ for cucumisin [8], proteinase C1 [30] and *SCS1* protease [25] as calculated from their amino acid contents [44].

2.4. SDS–PAGE and isoelectric focusing (IEF)

Electrophoresis in the presence of 5% (w/v) SDS was carried out in 8–25% polyacrylamide gradient gels in Tris-acetate buffer, pH 6.4, on a Pharmacia PhastSystem apparatus, as recommended by the manufacturer. Gel was calibrated with a Pharmacia Low Mass calibration kit and stained either with 0.1% (w/v) Coomassie brilliant blue R-250 or with 0.2% (w/v) silver nitrate as indicated by the manufacturer. The same apparatus was used for analytical IEF on a commercial precast pH 3–9 gradient gel following the instructions provided. The p*I* value was determined using the Pharmacia broad-p*I* calibration kit (p*I* range 3.65–9.30). Staining was performed as for SDS–PAGE.

2.5. Amino acid sequencing

Prior to N-terminal sequencing, KLSP was run on high-performance liquid chromatography on a C4 Chromspher column, using an acetonitrile gradient (0–80%) in 0.1% trifluoroacetic acid. The main peak, still active against Z-Phe-Arg-MCA substrate, was vacuum-dried and applied to an Applied Biosystems liquid pulse sequencer 475 A connected on-line to a model 120 A phenylthiohydantoin analyzer from the same manufacturer.

2.6. Enzyme assays

During the isolation procedure enzyme activities were followed fluorimetrically using 10 μM Z-Arg-Arg-MCA or Z-Phe-Arg-MCA as substrates in the assay buffer (0.1 M Tris–HCl, pH 9.0). When testing for cysteine proteinases 10 mM cysteine and 1 mM EDTA were present. After 15 min incubation at 37°C, 2 ml of 10 mM iodoacetic acid was added to stop the reaction. The product of hydrolysis was monitored on a Perkin Elmer fluorimeter LS 30 at 370 nm excitation and 460 nm emission wavelengths [45]. The activity of the enzyme was tested on different 7-methylcoumarylamide substrates (Bachem), at a final concentration of 100 μM.

2.7. Influence of inhibitors

The influence of different inhibitors on enzyme activity was tested by incubating each inhibitor at the indicated concentration in the assay buffer with the enzyme for 15 min prior to the addition of the substrate. The inhibitors E-64, PMSF, Pefablock SC, pepstatin, aprotinin, antipain were from Boehringer Mannheim, while Bowman Birk inhibitor and soybean trypsin inhibitor were from Sigma. All other protein inhibitors were isolated in our laboratory: clitocypin [46], egg white cystatin [47], PCPI 8.6 [48] and PCPI 6.6 [48].

2.8. pH optimum

Dependence of the enzyme activity on pH was determined using the following buffers in 0.1 M concentration: sodium acetate for pH 2.5–5.5, sodium–potassium phosphate for pH 5.5–7.5, Tris–HCl buffer for pH 7.5–9 and glycine/NaOH buffer for pH 9–12.

2.9. pH stability

10 μl of KLSP was incubated in 0.5 ml samples of buffer with various pH values for 28 h at 25°C. The buffers were the same as for pH optimum determination. Aliquots of the incubation mixture of negligible volume were transferred to 0.5 ml of the assay buffer (pH 9.9). 10 μl of 1 mM Z-Arg-Arg-MCA was added and the activity measured as described above.

2.10. K_m determination

Enzyme kinetic measurements with fluorogenic substrates were performed in continuous assays followed by a fluorescence system, Photon Technology International. Michaelis constants K_m were calculated by non-linear regression analysis using the equation:

$$v_o = V_{max}/(1 + K_m/[S])$$

where v_o is initial rate of product formation, V_{max} is maximal rate and [S] is substrate concentration.

2.11. Determination of relative k_{cat} values

The initial rate v_o of hydrolysis of different substrates with the same quantity of the enzyme was determined. The substrate concentrations were at least seven times lower than their K_m. k_{cat} values of different substrates were then compared on the basis of the following equation

$$E_o k_{cat} = v_o K_m/[S]$$

2.12. Determination of inhibition constant K_i

KLSP was incubated with different amounts of aprotinin to obtain a non-linear dose-response curve of residual activity. The exact concentration of aprotinin was determined by titration with trypsin,

Table 1
Quantitative course of purification of KLSP from 1 kg of common bean leaves

Purification step	Protein (mg)	Total activity[a] (nmol min^{-1})	Specific activity (nmol min^{-1} mg^{-1})	Purification (-fold)	Yield (%)
Protein extract	3505	296	0.09	1	100
DEAE-Sephacel	251	149	0.33	7	50
CM-Sephadex C-50	15	92	6.1	67	31
Sephacryl S-200	0.1	0.7	6.7	79	0.2

[a]The substrate used for activity measurements was Z-Phe-Arg-MCA.

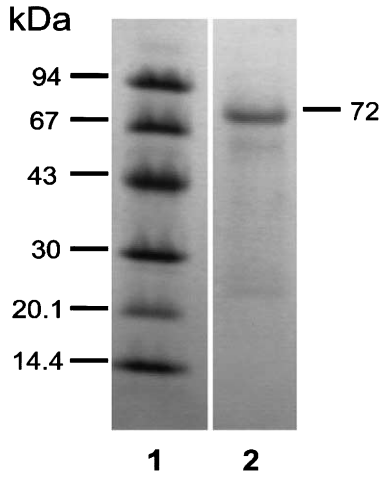

Fig. 1. SDS–PAGE of KLSP from common bean leaves, stained with Coomassie brilliant Blue R-250. Lane 1, standard proteins; lane 2, KLSP.

which had been previously titrated with *p*-nitrophenyl *p*′-guanidinobenzoate (Sigma) [49]. The enzyme and inhibitor were incubated for 15 min at room temperature in 0.1 M Tris–HCl buffer, pH 9.9. Then 5 μl of Boc-Gly-Arg-Lys-MCA was added to give a final concentration of 10 μM and incubated for 10 min at 37°C. The reaction was stopped by adding iodoacetic acid to 10 mM. The released 7-methylcoumarylamine was measured fluorimetrically. The apparent inhibition constant $K_{i,app}$ was obtained graphically using the linear equation derived by Henderson [50]. The influence of substrate on the inhibition constant was eliminated by the equation:

$$K_i = K_{i,app}/(1 + [S]/K_m)$$

where [S] is the initial substrate concentration and K_m the Michaelis constant.

3. Results

The quantification of the purification of KLSP from 1 kg of common bean leaves is presented in Table 1. The method includes fractionation of plant material with *t*-butanol [42], further purification by two ion exchange chromatography steps and finally by gel filtration on a calibrated Sephacryl S-200 column, resulting in 100 μg of purified KLSP. The elution volume of KLSP corresponded to an apparent molecular mass of 66 kDa.

SDS–PAGE and IEF (Figs. 1 and 2) both gave a single band for KLSP after staining with Coomassie blue or with silver nitrate. An apparent molecular mass of 72 kDa was determined by SDS–PAGE (Fig. 1). In IEF KLSP focused at pH 4.6 (Fig. 2). Localization of KLSP by Z-Arg-Arg-MCA after IEF led to a single bright band under UV light (not shown).

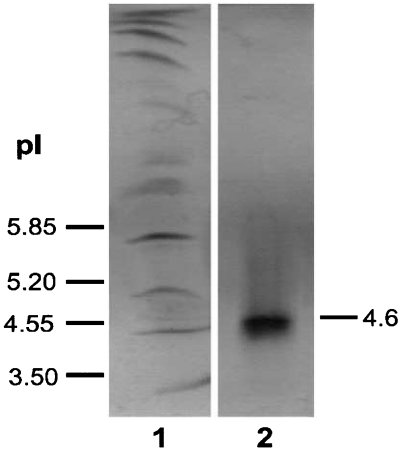

Fig. 2. IEF of KLSP from common bean leaves visualized by silver staining. Lane 1, standard proteins; lane 2, KLSP.

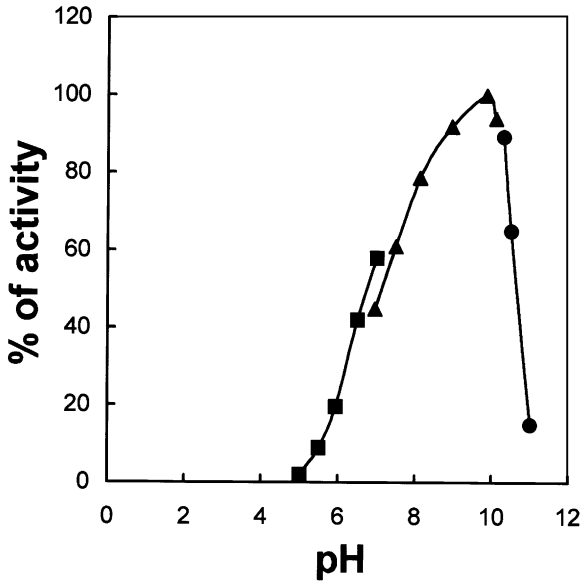

Fig. 3. Effect of pH on the activity of KLSP from common bean leaves. Enzyme activities were determined towards Z-Arg-Arg-MCA at different pH values (squares, sodium phosphate buffer; triangles, Tris–HCl buffer; circles, glycine/NaOH buffer) as described in Section 2.

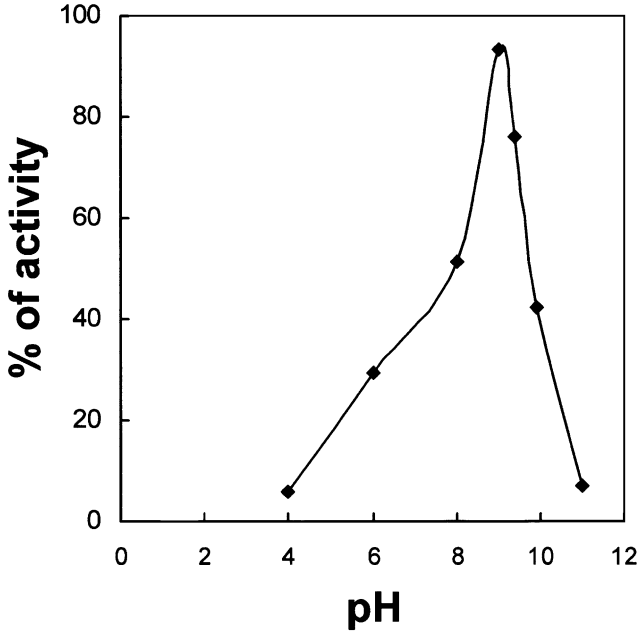

Fig. 4. The stability of KLSP from common bean leaves as a function of pH. Residual activities of the proteinase after 28 h of incubation at different pH values at 25°C were determined with Z-Arg-Arg-MCA as substrate at pH 9.9. The activity of the enzyme at pH 9.9 without preincubation was considered as 100% activity.

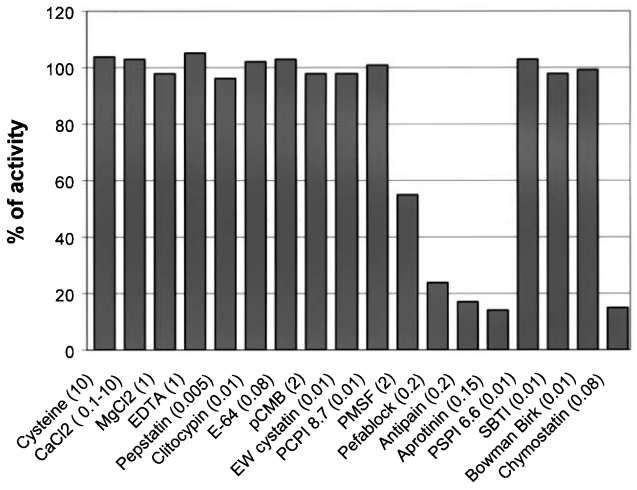

Fig. 5. The influence of various compounds on the activity of KLSP from common bean leaves. The enzyme was preincubated in the assay buffer for 15 min with each compound at the mM concentration indicated in the brackets. The enzyme was then assayed against Z-Arg-Arg-MCA as described in Section 2. The activity of the enzyme without the addition of any compound was considered as 100%.

The activity of KLSP versus pH exhibits a bell-shaped curve with a maximum at pH 9.9; between pH 8.0 and 10.5 it exhibited more than 50% of the maximum activity (Fig. 3).

The stability of KLSP at different pH values is shown in Fig. 4. The enzyme retained more than 90% of its initial activity after 28 h of incubation at 25°C in Tris–HCl buffer, pH 8.9, but only about 50% of its activity retained after incubation at pH 7 or 10.

The influence of different substances on the activity of KLSP is summarized in Fig. 5. Inhibitors and effectors of cysteine, aspartic and metalloproteinases had no influence on the enzyme activity. Two inhibitors of trypsin-like proteinases, SBTI and Bowman Birk inhibitor, also had no effect. Other serine proteinase inhibitors however strongly inhibited the enzyme. The strongest inhibition was observed with antipain, aprotinin and chymostatin, while inhibition with PMSF and Pefablock SC was somewhat weaker. The K_i value for the inhibition of the enzyme with aprotinin was 50 nM.

The relative activities of KLSP for different synthetic substrates with the 7-methylcoumarylamine leaving group are presented in Table 2. Kinetic constants for the reactions of KLSP with five different substrates with Arg in the P1 position are listed in Table 3. K_m values for substrates with RR, GRR and GKR motifs at the N-terminal sides of the cleavage sites were determined to be below 10 nM. Significantly higher

Table 2
Relative activities of the plant subtilase KLSP from common bean leaves against different substrates

Substrate[a]	Activity (%)
Z-Phe-Arg-MCA	100
Boc-Gly-Lys-Arg-MCA	90
Boc-Val-Gly-Arg-MCA	86
Z-Arg-Arg-MCA	54
Boc-Gly-Arg-Arg-MCA	43
Suc-Ala-Ala-Pro-Phe-MCA	0.21
Suc-Leu-Leu-Val-Tyr-MCA	0.15
Suc-Ala-Ala-Ala-MCA	0.03

[a]The substrate concentrations in the assay mixtures were 100 μM.

Fig. 6. Amino acid sequence alignment. The sequence of the 17 N-terminal amino acids of KLSP from common bean leaves is aligned with N-terminal sequences or deduced amino acid sequences (D) of other plant subtilases and kex2 proteinase from yeast: Prot C1, protease C1 from soybean seedling cotyledons [30]; Cucum, cucumisin-like protease from white gourd [11]; *SCS1*, subtilisin-like proteinase from soybean seed coat [25]; P69B, pathogen-induced protease from tomato [21]; *KEX2*, kex2 proteinase from yeast [51]. Black background indicates residues identical to KLSP and gray background indicates conserved and semi-conserved substitutions as defined by CLUSTAL W.

K_m values were determined for substrates with Gly or Phe in the P2 position. Because of lack of an appropriate titrating agent for the determination of the exact concentration of the active KLSP in the isolated sample, only relative k_{cat} values could be determined. The highest k_{cat} value was exhibited by Z-Phe-Arg-MCA as substrate. Relative values of k_{cat}/K_m showed that the enzyme exhibited the highest specificity for Boc-Gly-Arg-Arg-MCA.

The sequence of the 17 N-terminal amino acids of KLSP (Fig. 6) is compared with determined or cDNA-deduced N-terminal sequences of other plant subtilases and kex2 protease from yeast [51]. The highest number of identical amino acid residues is shared with protease C1 from soybean seedling cotyledon [30].

4. Discussion

The isolation and biochemical characteristics of a new plant subtilase, KLSP, from common bean leaves grown in the field are described. The isolation of KLSP used the modified procedure that led to the isolation of cysteine proteinases [52]. The introduction of Denisson's method [42] for preparing the starting material proved to be efficient in extracting colored compounds from the protein fraction, which simplified further purification of the proteinase and its activity determination. The first chromatographic step on Sephacryl S-200 of the previously described method [52] could be omitted without any reduction in yield or purity of KLSP. After purification on two ion exchange chromatographic steps and final gel filtration, single bands on SDS–PAGE and IEF were obtained. Determination of the position of KLSP in the gel after IEF by Z-Phe-Arg-MCA staining confirmed the identity of the purified protein.

Typical inhibitors of cysteine, aspartic and metalloproteinases did not affect KLSP, while inhibitors of serine proteinases PMSF, Pefablock SC, antipain, aprotinin and chymostatin inhibited it effectively. This inhibition profile, together with absence of inhibition of KLSP by trypsin inhibitors, SBTI Bowman Birk inhibitor and PSPI 6.6, classifies KLSP as a member of the subtilisin-like clan of proteinases. The absence of stabilizing or activating effects of Ca^{2+} ions for KLSP is, however, rather atypical for this class of proteinases, although there are a few reports on similar activities in plants which are

Table 3
Kinetic constants for the hydrolysis of the synthetic substrates with KLSP from common bean leaves

Substrate	K_m (nM)	k_{cat}rel (%)	(k_{cat}/K_m)rel (%)
Boc-Gly-Arg-Arg-MCA	6.0	38	100
Z-Arg-Arg-MCA	9.5	39	65
Boc-Gly-Lys-Arg-MCA	78.5	97	20
Z-Phe-Arg-MCA	512.5	100	3
Boc-Val-Gly-Arg-MCA	351.5	62	3

Relative values for k_{cat} and k_{cat}/K_m are presented since the exact active concentration of the enzyme was not determined (see text).

independent of the presence of Ca^{2+} ions [53–55]. The apparent inhibition constants of KLSP with aprotinin at different substrate concentrations indicate the competitive nature of the inhibition and the strong affinity for this inhibitor. Preliminary measurements predict the same order of magnitude of K_i for chymostatin and antipain, but exact values could not be determined, since the exact concentrations of inhibitors were not known. The pH optimum of KLSP in the alkaline region is typical for the subtilisin-like nature of proteinases together with its high stability [56].

Further biochemical characterization of KLSP using different synthetic substrates indicated the requirement for Arg in the P1 position for cleavage of the peptide bond. K_m values for substrates with RR, GRR and GKR motifs at the N-terminal ends of the cleavage sites were much lower than is general for degradative proteinases of broad specificity, and similar to that determined for trypsin-like protease from soybean seeds [53]. In addition to Arg in the P1 position, all three substrates have a basic amino acid in the P2 position. Significantly higher K_m values were determined for substrates with Gly or Phe in the P2 position. Of the substrates tested, Z-Phe-Arg-MCA exhibited the highest k_{cat} value, which indicates, together with determination of relative k_{cat} for other substrates, that the nature of the P2 residue does not greatly affect k_{cat}. The relative values of the specificity constant k_{cat}/K_m showed that the enzyme exhibited the highest specificity for Boc-Gly-Arg-Arg-MCA substrate. It can be concluded that, for high catalytic efficiency of the enzyme, a substrate with Arg in the P1 and P2 positions is preferred, although high specificity is also achieved if a substrate has another basic amino acid, Lys, in the P2 position. These requirements for maximal activity relate KLSP to kexin-like proteinases [6]. In contrast to most other plant subtilases, therefore, KLSP is not likely to play a degradative function but may be involved in selective protein processing, as are mammalian kexins [6].

N-terminal sequencing undoubtedly classified KLSP as a member of the subtilisin clan of proteinases [3]. It showed the high similarity of KLSP to subtilases from soybean [30,25] and to cucumisin-like proteinases [8], even though the KLSP sequence does not have Thr in the second place as do all other known plant subtilases. The enzyme shares much less sequence homology with kex2 proteinase from yeast [51], but on the other hand its specificity for different substrates resembles that of kex2 proteinase [57], leading to the conclusion that the 17 N-terminal residues do not determine the specificity of the enzyme.

In conclusion, the biochemical characterization of KLSP reported here is the first step to understanding the function in vivo of this new member of the plant subtilases. Further studies will elucidate tissue localization of the proteinase and address the search for the physiological substrates. Determination of the nature of the expression of the proteinase as a consequence of developmental or environmental cues would give more insight in the function of KLSP.

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