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PURIFICATION AND CHARACTERIZATION OF AN ENDOPROTEASE FROM ALFALFA SENESCENT LEAVES

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Key Word Index—*Medicago sativa*; Leguminosae; alfalfa; purification; characterization; endoprotease; azocasein; leaves; senescence.

Abstract—We purified and characterized an endoprotease, whose activity increased during growth and senescence of alfalfa (*Medicago sativa* L.) leaf. The purification and characterization of proteolytic enzymes is a helpful step to understand their function and involvement in the process of protein degradation during foliar senescence. One of the two proteases, revealed after affinity chromatography on haemoglobin-agarose, was characterized. Physical and biochemical properties of the purified protease were identified and the effects of various inhibitors on enzyme activity were tested. The protease activity, with a pH optimum at 5.0, was affected by metallo-protease inhibitors and enhanced by organomercurial compounds. The purified protease was tested on ribulose-1,5-bisphosphate carboxylase/oxygenase, and the electrophoretic analysis of the reaction mixture showed that the large subunit decreases during the incubation, indicating that the purified protease is capable of degrading the large subunit *in vitro*. A possible involvement of such protease in the process of protein degradation during leaf senescence can be considered.

INTRODUCTION

Leaf senescence is associated with protein degradation [1] and the mobilization of the nitrogen reserves to other parts of the plant. Therefore, the importance of endoproteases in leaves is correlated with the role played both in normal cell function (protein turnover and protein maturation) and in senescing tissues as terminal degraders of protein [2, 3]. The amino acids produced by this degradation may be translocated to other vegetative tissues or to developing seeds. Since ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) represents most of the leaf cell protein [4, 5], understanding the mechanism involved in its degradation is extremely important. A correlation between the loss of Rubisco and the increase in proteolytic activity has been found in dark-senescing detached leaves, but no senescence-specific protease has been reported, although changes in protease activities during senescence were observed in leaves of several plants [6–9]. In some cases, the increase of proteolytic activities was not correlated with the onset of protein decrease [10–12]. However misleading results may be due to the use of artificial substrates to test the protease activities and to the fact that proteases and their natural substrates are probably located in different cell compartments, which may be regulatory [13].

Recently we have shown evidence of a possible correlation between leaf senescence and some protease activities assayed on artificial substrates [14–16]. However, the purification and characterization of proteolytic enzymes is needed to assess their involvement both in the general process of leaf protein degradation and particularly in the loss of Rubisco taking place during foliar senescence. Leaf proteases have usually been studied using crude extracts or slightly purified preparations: rarely leaf proteases of Leguminosae have been purified to homogeneity, while some endoproteases have been isolated in cereals [3, 17]. We purified and characterized an endoprotease from alfalfa senescent leaves, which is capable of hydrolysing the Rubisco (EC 4.1.1.39) large subunit (LSU) *in vitro*.

RESULTS AND DISCUSSION

Protease purification

Figure 1 shows the endoprotease activity during three representative stages of life-cycle of alfalfa leaves: proteolytic activity tested against azocasein

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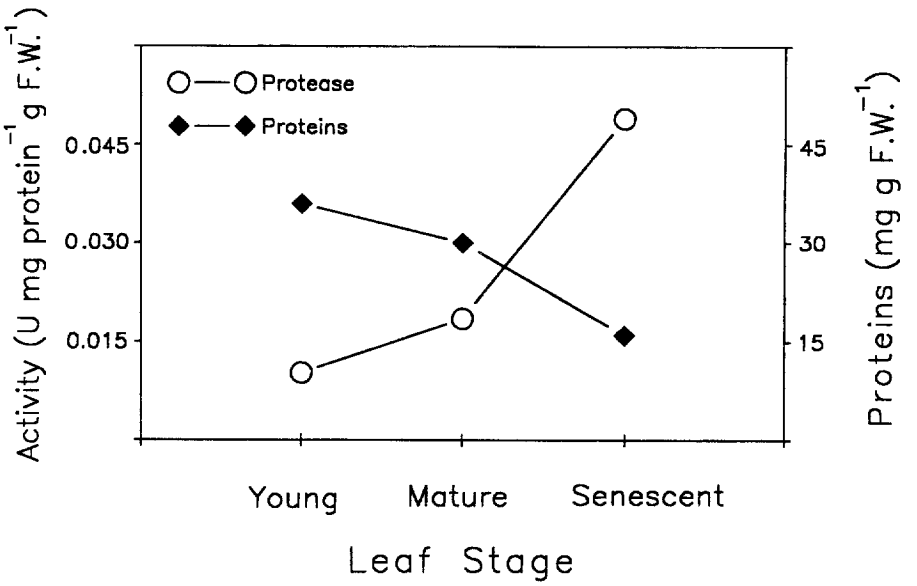

Fig. 1. Endoprotease specific activity during life-cycle of alfalfa leaves. Alfalfa leaves were homogenized in K–Pi buffer, pH 7.0, supplemented with 10 mM $Na_2S_2O_5$. After centrifugation and filtration, aliquots of extract were assayed on 0.5% (w/v) azocasein as substrate, in 50 mM NaOAc, pH 5.0. One unit (U) of enzyme activity is defined as the increase in A_{440} of 0.001 unit over 1 hr incubation period under the assay conditions. The values represent means of results from × 3 experiments and the s.e. did not exceed 15% of each value.

increases with leaf age, while protein content decreases. Similarly in *Lolium temulentum*, it has been shown that an endoprotease, tested on azocasein as substrate and maximally active at pH 5.0, increased with leaf age [18]. However, a correlation between the increase in protease activities and protein loss during foliar senescence has been rarely reported and assessed in Leguminosae. In order to characterize such activity, purification chromatography steps were performed employing senescent leaves. The results of a typical purification procedure are summarized in Table 1. The overall yield was low, *ca* 1.8%, but it was possible to repeat the procedure several times to get a larger amount of protease. The supernatant obtained after $(NH_4)_2SO_4$ fractionation was directly subjected to hydrophobic interaction chromatography (HIC) and the protease activity was eluted by reducing the ionic strength of the buffer (with *ca* 1.3-fold enrichment). The active fractions of the peak were pooled and after desalting, the sample was loaded on an anionic exchange matrix (DE52) column and a single peak of protease activity was eluted on increasing the concentration of NaCl in the buffer. This step gave *ca* 1.2-fold enrichment. Then we employed a haemoglobin-agarose matrix column. The affinity chromatography separated two different activities on azocasein: endoprotease activity was collected both before the starting of the linear gradient of NaCl (void volume), and within such gradient. When both peaks of activity (the void volume active fractions and the bound protein active fractions) were rechromatographed, they reappeared in the same position as in the original chromatogram, which is strong evidence that they are separate identities because rechromatography is con-

Table 1. Purification of the endoprotease from 30 g fr. wt senescent alfalfa leaves. Proteolytic activity was detected during enzyme purification using azocasein as substrate. One unit of proteolytic activity (U) corresponds to an increment in A_{440} of 0.001 over 1 hr incubation period under the assay conditions. The values represent means of results from × 3 experiments and the s.e. did not exceed 15% of each value

Purification steps	Total Proteins (mg)	Total activity (U)	Specific activity (U mg^{-1})	Recovery (%)	Purification (fold)
Crude extract	218	11.2	0.05	100	—
$(NH_4)_2SO_4$ precipitation	145	11	0.07	98	1.4
HIC	58	5.7	0.09	50	1.8
DE52	19	2.2	0.11	20	2.2
Haemoglobin-agarose	1.2	1.7	1.41	15	28
Superose 12	0.05	0.2	4.00	1.8	80

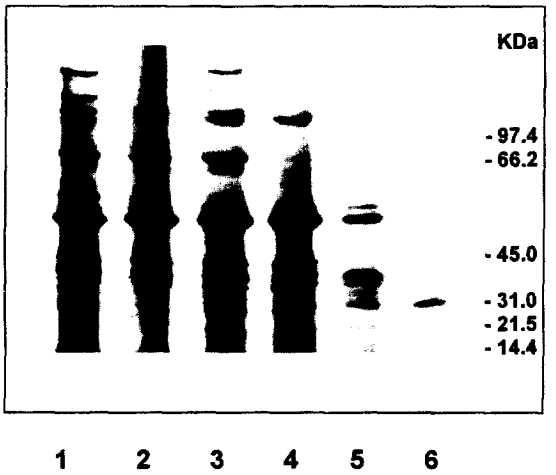

Fig. 2. Electrophoretic analysis of the polypeptides fractionated during the purification of the protease. Aliquots of samples obtained in each purification step listed in Table 1 were subjected to SDS–PAGE (on a gradient 10–15% polyacrylamide gel) and silver-stained. Lane 1, crude extract; lane 2, 40% $(NH_4)_2SO_4$ fraction; lane 3, pooled fractions from HIC; lane 4, pooled fractions from DE52; lane 5, pooled fractions from haemoglobin column; lane 6, purified protease (pooled fractions from Superose 12). Phosphorylase *b* (97400), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), trypsin inhibitor (21500), lysozyme (14400) were used as M_r marker proteins and their positions are indicated on the right.

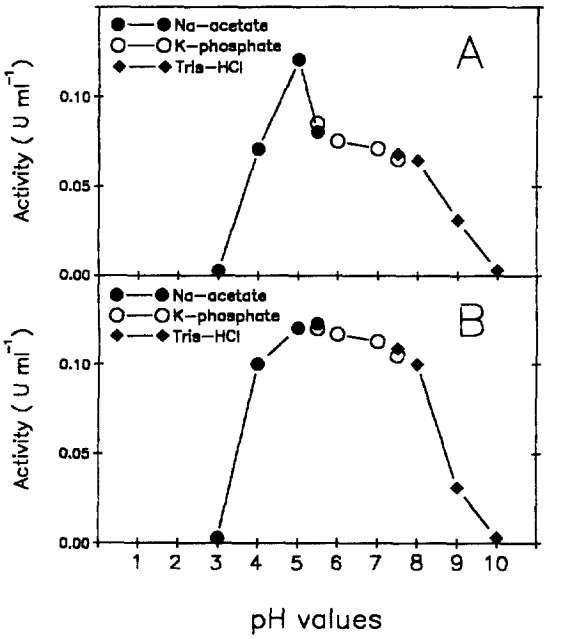

Fig. 3. Effect of pH variations on azocaseolytic activity and pH stability of the purified endoprotease: (A) the effects of pH on azocaseolytic activity were studied 50 mM buffers containing 0.5% (w/v) azocasein within pH range 3–10; (B) to test the pH stability of the enzyme, aliquots of purified enzymes were pre-incubated for 48 hr at 4° in the same 50 mM buffers indicated above, then an aliquot was tested for activity against azocasein under the assay conditions. One unit (U) of enzyme activity is defined as the increase in A_{440} of 0.001 unit over 1 hr incubation period under the assay conditions. The values represent means of results from ×3 experiments and the s.e. did not exceed 15% of each value.

sidered to be an important criterion in establishing heterogeneity [2]. The protease present in the void volume was the most abundant (about 90% of total azocaseolytic activity) and it underwent the further purification process. Hence, the purification procedure showed that two protease activities, capable of hydrolysing azocasein, are present in alfalfa senescent leaves. The presence of two different endoproteases, with acidic pH optima, active on azocasein was also reported in senescing barley leaves [17] and in green wheat leaves [2, 3]. The active fractions from haemoglobin-agarose were pooled and loaded onto a Superose 12 HR 10/30 gel column. This step enriched activity *ca* 3-fold. At the end of the purification procedure, the protease was purified *ca* 80-fold to yield a specific activity of 4 U mg protein^{-1}. The purification factor that was obtained is fully comparable with others obtained in oat leaves [19] and pea ovaries [20]. Figure 2 illustrates the electrophoretic analysis (SDS–PAGE) of the polypeptides fractionated during the purification of the protease, considering the peak fractions of each purification step. The electrophoretic pattern showed that the protease was purified to homogeneity, with M_r of *ca* 30 000, which confirmed the elution position obtained after molecular exclusion chromatography. The M_r is similar to that of purified proteases from mung bean cotyledons [21], French bean pods [22], pea chloroplasts [23] and ovaries [20]. Activity staining performed on the protein obtained at the end of the purification process, showed that it really was an azocaseolytic activity, developing an activity band with a mobility of Rf 0.25 (data not shown).

Protease characterization

Purified protease was active on azocasein over an acidic pH range, with a maximal activity at 5.0, but it retained some activity in neutral and slightly alkaline range of pH [Fig. 3(a)]. Qualitatively similar results were obtained using Rubisco as substrate (data not shown). Enzyme stability was rather broad, over a pH range of 5–8 [Fig. 3(b)].

The purified protease showed a pH optimum at 5.0, suggesting that it might be vacuolar, although the broad pH range of activity and stability indicates that it would be active also in the cytoplasm. To enrich the characterization of the enzyme, we report that the K_m for azocasein at optimal pH was 0.5 (% w/v).

To assay the specific endoproteolytic nature of the purified enzyme typical carboxypeptidase and aminopeptidase/iminopeptidase chromogenic substrates (Ala-Phe-carbobenzoxydipeptide and Phe-, Ala- or

Table 2. Inhibitors, final concentration, target protease-class and effect on the activity of the purified protease on azocasein. Proteolytic activities are expressed as percentage of the control in which inhibitors were not added. The incubation buffer was 50 mM NaOAc, pH 5.0. The values represent means of results from × 3 experiments and the s.e. did not exceed 15% of each value

Inhibitors	Concentration (mM)	Target protease class	Residual activity (%)
None	—	—	100
TLCK	0.1	Serine	82
EDTA	0.1	Metallo	90
EDTA	1.0	Metallo	61
Pepstatin	0.001	Aspartic	91
Bathocuproine	5.0	Metallo	28
Leupeptin	0.1	Serine-Cysteine	88
PMSF	1.0	Serine	79
PMSF	5.0	Serine	71
pCMB	1.0	Serine-Cysteine	150
1,10-Phenanthroline	1.0	Metallo	85
E-64	0.1	Cysteine	90
Iodoacetic acid	1.0	Cysteine	96

Pro-βnaphthylamides respectively) were tested and no hydrolysis of such compounds was detected.

Inhibition of protease activity

Specific inhibitors to each class of proteolytic enzyme activity [24] were used to characterize the activity of purified protease (Table 2). Some metallo-protease inhibitors, such as EDTA and especially bathocuproine, had marked negative effects on the protease activity. No apparent effect on proteolytic activity was observed when the reaction mixture was supplemented with serine-protease inhibitors (TLCK, PMSF), cysteine protease inhibitors (IOAc, E-64), serine-cysteine protease inhibitors (leupeptin), aspartic protease inhibitors (pepstatin) or different metallo-protease inhibitors (1,10-phenanthroline). Interestingly, pCMB, which is commonly considered as a non-specific serine-cysteine inhibitor, had marked positive effects on the enzyme, enhancing the protease activity. The use of protease inhibitors in our experiments has shown that the purified protease is probably a metallo-protease, since EDTA and especially bathocuproine have been the most effective inhibitors. A proteolytic activity, with acidic optimal pH, inhibited by EDTA has been found in orange leaves [25], but in barley and wheat some endoproteolytic activities involved in LSU degradation are inhibited by PMSF [26, 27]. In pea, some stromal endopeptidases, inhibited respectively by EDTA and PMSF, have been shown [28] and recently Brushnell *et al.* [23] detected a metallo-protease, requiring Zn^{2+} and the presence of Mg^{2+} or Ca^{2+} to be active, which is able to hydrolyse LSU. Concerning the increased proteolytic effect induced by pCMB and other organo-mercurial compounds, it has been shown that these compounds cause a conformational alteration of tertiary structure of Rubisco, blocking SH groups involved in its stability [29] and allowing proteolytic attack [30]. A similar effect on azocasein by pCMB may be hypothesised.

Effects of purified protease on Rubisco

Proteolytic activity on Rubisco was performed at different reaction pH (data not shown). The electrophoretic patterns of Rubisco incubated with the purified protease showed a clear proteolytic action at pH 5.0, since the protein band with M_r of *ca* 54000, corresponding to the LSU, decreased: densitometric analysis of this band indicated a relative intensity of *ca* 68.5% after 2 hr and 58.6% after 5 hr compared with to the starting time of incubation with the protease (Figure 4). The proteolytic attack on Rubisco and the most remarkable action at pH 5.0 were also confirmed quantitatively by the presence of TCA-soluble amino acids, as a result of its hydrolysis, indicated by the colorimetric determination with the ninhydrin method (data not shown). In our previous work we showed that the LSU degradation in autodigestion experiments is more intense at acidic pH, LSU band disappearing in *ca* 5 hr, but presumably an alkaline serine-class protease is involved, too [14–16]. In senescent oat leaves Van Loon *et al.* [31] have noted that there are two main endoproteolytic activities with pH optimum at 4.2 and 8.0 respectively: the optimal acidic pH of the former enzyme coincides with the pH value at which alfalfa Rubisco degradation is more intense. Similar results have been reported in autodigestion experiment carried out in soybean [10], oat [32] and orange leaves [25]. Interestingly, in *Lolium temulentum*, a shifting from a pH 5 endoprotease to a pH 8 endoprotease, associated with an evident decrease in M_r (from 60 000 to 30 000), has been described, thus indicating that the alkaline endoprotease is derived

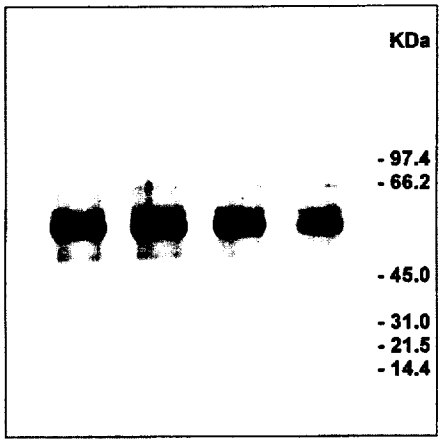

Fig. 4. Activity of the purified protease on the large subunit of Rubisco. Purified protease was incubated with 500 μg Rubisco in NaOAc, pH 5.0, at 30° for different times. The reaction mixture was analysed by SDS–PAGE followed by Coomassie Brilliant Blue staining. The lanes were loaded with *ca* 1 μl aliquot taken from each of the reaction mixtures. Lane 1, purified Rubisco after 5 hr of incubation; lane 2, reaction mixture at the starting time of incubation (t_0); lane 3, reaction mixture at 2 hr-incubation time (t_2); lane 4, reaction mixture at 5 hr-incubation time (t_5). M_r markers positions are indicated on the right.

from the acid endoprotease, perhaps by limited autolysis in protein at an advanced stage of senescence [18]. Yoshida and Minamikawa have recently reported evidence that Rubisco LSU degradation in French bean leaves is related to two types of vacuolar preoteases (serine-class and cysteine-class respectively), each catalysing one proteolytic step by removal of a small amino terminal peptide [33]. Therefore, although a role for exopeptidase activities can not be ruled out, our data support the conclusion that Rubisco degradation is mainly due to an acidic endoproteolytic attack.

CONCLUSIONS

We showed an endoprotease specific activity increased during alfalfa leaf aging, indicating that it was probably correlated with protein degradation characterizing leaf senescence, when Rubisco, one of the most abundant leaf protein, decreases. The purification procedure showed that two protease activities, capable of hydrolysing azocasein, are present in alfalfa senescent leaves, one being *ca* 90% of total azocaseolytic activity. Our data suggest that such most abundant protease is involved in Rubisco LSU degradation. However caution must be used when trying to use information directly from the *in vitro* hydrolysis of protein substrates to ascertain the role of the proteases *in vivo*, because of differential compartmentation within the cell. Nevertheless, since during *in vivo* senescence, sub-cellular organelles lose their integrity, Rubisco may become more accessible for proteolytic attack by several proteases from different organelles. The electrophoresis analysis of the reaction mixture containing purified protease from alfalfa senescent leaves and Rubisco showed that such acidic protease is capable of degrading Rubisco *in vitro*. Therefore it might be involved in the degradation of Rubisco *in vivo*. Although we can not establish that such 'acidic' protease is Rubisco-specific, in our experiments it is shown at least that this protein may be one of the targets of the purified protease.

EXPERIMENTAL

Plant material

Plant material (*Medicago sativa* L., cl. Iside) was supplied by 'Istituto Sperimentale per le Colture Foraggere' (Lodi). Young (blade length 0.5 cm) and mature leaves (petiole length 4.5 cm) were collected from actively growing alfalfa plants cultivated in a greenhouse. The senescent stage was obtained as described in refs. [14–16].

Protein extraction

3 g of leaves at the different stages of development were homogenized with 4 ml g fr. wt^{-1} of ice-cold 50 mM K–Pi buffer (pH 7.0) containing 10 mM $Na_2S_2O_5$. The homogenate was filtered through a nylon layer and then centrifuged at 20 000 *g* for 30 min. The supernatant was filtered through 0.2 μm filter and used for enzyme assays on crude extract. Each extraction was replicated × 3 and carried out at 0–4°.

Enzyme purification

30 g of dark-senescent detached leaves were homogenized as described above. The supernatant was brought to 40% satn with $(NH_4)_2SO_4$ and stirred for 30 min; the ppt was removed by centrifugation at 12 000 *g* for 30 min. The supernatant was further processed by mean of a FPLC apparatus. The sample was loaded onto a TSK Phenyl Toyopearl 650 M column (16 × 120 mm), equilibrated with 1.7 M $(NH_4)_2SO_4$ in 50 mM K–Pi buffer, pH 7.0. Proteins were eluted with a linear gradient of 1.7 to 0 M $(NH_4)_2SO_4$. Active fractions were pooled and desalted using commercially available desalting columns, equilibrated with 25 mM Tris, pH 8.0; the same buffer was used to elute proteins, and the pooled active fractions were loaded onto a DEAE-cellulose column (16 × 100 mm), equilibrated with 25 mM Tris, pH 8.0, and eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. The active fractions from anion exchange chromatography were pooled and desalted as described above against 10 mM K–Pi, pH 6.0, and loaded onto a haemoglobin-agarose column (10 × 10 mm) equi-

librated with the same buffer and eluted with a linear gradient of 0 to 1 M NaCl in the above buffer. Active fractions were pooled and concd using a microconcentrator and loaded on a Superose 12 (HR 10/30) column. The column equilibration buffer was 50 mM K–Pi containing 150 mM NaCl, pH 7.0.

Analytical methods

Quantitative endoproteolytic activity assays were performed using azocasein as substrate. Aliquots of extracts were added to 50 mM NaOAc, pH 5.0, containing 0.5% (w/v) azocasein and incubated for 4 hr at 30°. To stop the reaction, 1.2 ml 10% (w/v) TCA was added and the mixtures were incubated at 4° for 30 min and centrifugated for 10 min at 8000 *g*. TCA-soluble peptides generated during the reaction were estimated by recording the A_{440} of the supernatant. A blank containing boiled enzyme was used as control. Activity assays on commercially available Rubisco (EC 4.1.1.39, Sigma) were performed by mixing an aliquot of purified enzyme with 1% (w/v) Rubisco dissolved in 50 mM reaction buffer and incubating at 30° for different times. Proteolytic activity on Rubisco was analysed both qualitatively, considering the loss of intensity of the band with M_r of *ca* 54000, corresponding to the large subunit of Rubisco after SDS–PAGE analysis of the reaction mixture [34], and quantitatively as TCA-soluble amino acids detected by the ninhydrin method [35]. As control, Rubisco was incubated alone in the same reaction mixture. Carboxypeptidase (EC 3.4.12) and aminopeptidase (EC 3.4.11) activity was tested employing model substrates (carbobenzoxy-phe-ala; ala-, phe- and pro-β-naphthylamides) as decribed previously [15]. Proteins present in the fractions eluted from column chromatographies were estimated monitoring the A_{280}; protein contents were determined using a protein assay kit, with bovine serum albumin as standard. Protein bands after electrophoresis were analysed using the densitometric scanner programme for 'Microscan 2', UVP-Gel Base/Gel Blot.

Characterization of enzyme activity

The effects of pH were studied at 30° with 50 mM buffers containing 0.5% (w/v) azocasein (NaOAc at pH range 3–5.8; K–Pi at pH range 5.8–7.5; Tris at pH range 7.5–10). To test the pH stability of the enzyme, aliquots of purified enzymes were pre-incubated for 48 hr at 4° in the same 50 mM buffers indicated above. An aliquot was then removed and the residual activity was measured against azocasein. K_m for azocasein was determined varying the concn (w/v) of this substrate in the reaction mixture under the assay conditions.

Inhibition of enzyme activity

Inhibitors were added to the reaction mixture and after incubation the reaction was stopped as indicated above. PMSF, TLCK and 1,10-phenanthroline were dissolved in MeOH; E-64 and IOAc were dissolved in 50 mM Na–Pi buffer (pH 7.0); pCMB and EDTA were dissolved in 0.33 M KOH. As control, incubation was performed in buffer containing the specific solvent of each inhibitor.

Polyacrylamide-gel electrophoresis (PAGE)

Denaturing electrophoretic analysis (SDS–PAGE) during purification steps was performed at 15° using a Phast System apparatus with the appropriate gels and buffer strips; gels were ag-stained according to the instructions of the manufacturer (Pharmacia). To test the protease activity on Rubisco, SDS–PAGE analysis of proteins was carried out by loading denatured incubation mixure aliquots onto 10% (w/v) polyacrylamide slab gel discontinuous system, performed as described in refs. [14–16]. The gels were incubated at 25° for 15 min in 0.25% Coomassie Brilliant Blue R-250, 50% MeOH, 10% HOAc and destained for 2 hr in a soln containing 5% MeOH and 7.5% HOAc. Native PAGE was performed according the discontinuous system cited above, loading aliquots of sample onto 10% (w/v) polyacrylamide slab gel containing 0.5% azocasein in the separation gel. *Ca* the same volume of crude extract and purified protease was loaded on each lane (corresponding to *ca* 0.196 U ml^{-1} and 0.003 U ml^{-1}, respectively). Enzyme activity developed as a clear zone against an orange background, after incubation in NaOAc, pH 5.0, at 30°.

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