Isolation and properties of a metalloproteinase from buckwheat (*Fagopyrum esculentum*) seeds

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A homogeneous preparation of metalloproteinase, purified 1000-fold, was obtained from buckwheat (*Fagopyrum esculentum*) seeds. The M_r of the enzyme, determined by SDS/PAGE, was 34000 (it was 39000 by gel chromatography). Its pH optimum was 8.0–8.2 with 13 S globulin, from buckwheat seeds, as substrate. Atomic-absorption spectroscopy revealed the presence of one Zn^{2+} ion per enzyme molecule. The enzyme was completely inhibited by EDTA (1 mM), zincone (1 mM) and 1, 10-phenanthroline (1 mM). The metalloproteinase performed limited proteolysis of the following seed storage proteins: 13 S globulin from buckwheat seeds and 11 S globulin from soybean (*Glycine max*) seeds. It hydrolysed three peptide bonds formed by the amino groups of Leu¹⁵, Tyr¹⁶ and Phe²⁵ in the oxidized B-chain of insulin. In its main properties the enzyme is similar to metalloproteinases of animal and bacterial origin.

INTRODUCTION

Metalloproteinases (EC 3.4.24.-) are a group of less-well-studied plant proteolytic enzymes. So far, not a single homogeneous metalloproteinase has been obtained from plant tissues, and there are only few references to the presence of these enzymes in the seeds of pumpkin (*Cucurbita pepo*) (Hara & Matsubara, 1980), soybeans (*Glycine max*) (Bond & Bowles, 1983) and jackbeans (*Canavalia ensiformis*) (Dalkin *et al.*, 1983). Metalloproteinases from animals and micro-organisms have been studied much more extensively, and the best known are collagenases, thermolysin from *Bacillus thermoproteolyticus*, the three-dimensional structure of which has been studied (Matthews *et al.*, 1972), and endopeptidase-24.11, the major mammalian membrane endopeptidase (Kerr & Kenny, 1974a,b; Fulcher *et al.*, 1982).

The past few years have seen an increased interest in metalloproteinases and their significant role in the regulation of various biochemical events (Bond & Beynon, 1985). This must be relevant to plants too. Thus it was found that proteolysis of the main storage protein of buckwheat (*Fagopyrum esculentum*) seeds, 13 S globulin, during germination can be initiated by a metalloproteinase present in the dry seeds (Dunaevsky & Belozersky, 1989). The aim of the present study was to purify this enzyme and to study its physico-chemical properties and substrate specificity.

MATERIALS AND METHODS

Chemicals

CM-Sephadex C-25 and Sepharose 6B were from Pharmacia, Uppsala, Sweden. DEAE-Toyopearl 650 M was from Toyo Soda Co., Tokyo, Japan. Ampholines and Ultrogel AcA 54 were from LKB, Stockholm, Sweden. BSA, dansyl chloride (DnsCl), 1,10-phenanthroline, benzoyl (Bz)-Gly-Lys, benzoyloxycarbonyl (Z)-Glu-Tyr, Z-Gly-Phe, Z-Gly-Gly, EDTA and Servalits were from Serva, Heidelberg, Germany. Di-isopropyl fluorophosphate (iPr₂P-F) was from Fluka, Buchs, Switzerland. *p*-Chloromercuribenzoate (PCMB), Coomassie Blue R-250 and G-250, Hepes, pepstatin and zincone were from Sigma, St. Louis, MO, U.S.A. Dinitrophenyl (Dnp)-Gly-Gly-Met-Arg, Dnp-Gly-Gly-Ile-Arg

and Dnp-Gly-Gly-Val-Arg were a gift from Dr. G. N. Rudenskaya (Department of Chemistry, Moscow State University, Moscow, U.S.S.R.); 11 S globulin from soybeans was a gift from Dr. A. D. Shutov (Department of Biology, Kishinev State University, Kishinev, U.S.S.R.); 13 S globulin was isolated from buckwheat seeds as described previously (Belozersky, 1975).

Plant material

Dry buckwheat (*Fagopyrum esculentum* Moench, cv. Shatilovskaya 5) seeds were used in the present study.

Enzyme assays

Proteinase activity was determined by the method of Habeeb (1966). Substrates used, as 0.2 % solutions of non-denatured and denatured proteins, were: BSA, haemoglobin, 11 S globulin from soybeans and 13 S globulin from buckwheat seeds. The proteins were denatured by heating the samples in boiling water for 10 min. The reaction mixture (100 μ l) contained 20 μ g of the enzyme and 200 μ g of the substrate in 10 mM-sodium phosphate, pH 8.0, and was incubated at 37 °C. One unit of enzyme activity corresponded to the formation of 1 nmol of amino groups under the incubation conditions for 1 h, using glycine as a standard.

Carboxypeptidase activity was measured by trinitrophenylation (Habeeb, 1966) with 1 mM solutions of Z-Gly-Tyr, Z-Gly-Phe, Z-Gly-Gly, Bz-Gly-Lys used as substrates in 10 mM-sodium phosphate, pH 8.0.

Amino-peptidase activity towards L-leucine β -naphthylamide (1 mM solution in 10 mM-sodium phosphate, pH 8.0) was determined spectrophotometrically at 335 nm (Lee *et al.*, 1971).

Proteolytic activity towards the synthetic Dnp-tetrapeptides Dnp-Gly-Gly-Met-Arg, Dnp-Gly-Gly-Ile-Arg and Dnp-Gly-Gly-Val-Arg was determined as described by Lyublinskaya *et al.* (1977). The reaction mixture, containing 50 μ g of the enzyme and 250 μ g of the substrate, was incubated at 37 °C for 18 h and the reaction was stopped by the addition of an equal volume of 50 % acetic acid. The sample was applied to a CM-Sephadex C-25 micro-column (0.5 ml) equilibrated with 500 mM-acetic acid, and elution was performed with 500 mM-acetic acid. The absorbance of the eluate was measured at 360 nm.

Abbreviations used: a.a.s., atomic-absorption spectroscopy; Bz, benzoyl; iPr₂P-F, di-isopropyl fluorophosphate; Dnp, dinitrophenyl; DnsCl, dansyl chloride; PCMB, *p*-chloromercuribenzoate; Z, benzyloxycarbonyl.

Hydrolysis of the oxidized insulin B-chain was performed in 10 mM-sodium phosphate, pH 8.0, at 37 °C for 16 h. The incubation mixture (300 µl) contained 2 µg of the enzyme preparation and 500 µg of the insulin B-chain. The peptides obtained were separated by h.p.l.c. on a reversed-phase Ultrasphere-Octyl (Altex, Berkeley, CA, U.S.A.) column (15 cm × 0.46 cm), using the Beckman (Vienna, Austria) 344 chromatography system. The peptides were applied to the column, equilibrated with buffer A, containing 2% (v/v) propan-2-ol, 0.1% trifluoroacetic acid and 0.33% *N*-methylmorpholine. To elute the peptides from the column, a gradient (0–50%) of buffer B (60% propan-2-ol/0.1% trifluoroacetic acid/0.33% *N*-methylmorpholine) was used for 25 min at a flow rate of 0.75 ml·min⁻¹, and finally the column was washed with 100% buffer B for 15 min. The fractions (1.0 ml) were collected and the peptides were detected at 215 nm. The peptide fractions were concentrated by freeze-drying and the peptides were hydrolysed with HClO₄/trifluoroacetic acid (2:1, v/v) at 155 °C for 1 h. The amino acid composition of the peptides was determined on a Hitachi (Tokyo, Japan)-835 amino acid analyser.

Purification of the proteinase

The freshly ground buckwheat seeds were extracted overnight with 10 mM-sodium phosphate, pH 6.8 (1:3, w/v), containing 1% NaCl at 4 °C. The extract was centrifuged (18 000 *g*, 40 min) and the supernatant was adjusted to 80% saturation with (NH₄)₂SO₄. The precipitate was separated by centrifugation (18 000 *g*, 30 min), dissolved in 10 mM-sodium phosphate, pH 6.8, and dialysed overnight at 4 °C against the same buffer.

The dialysis residue was run through a Sepharose 6B column (4 cm × 85 cm) in 10 mM-sodium phosphate, pH 6.8. The active fractions were pooled, concentrated, and passed through a DEAE-Toyopearl 650 M column (1.5 cm × 10 cm) in 10 mM-sodium phosphate, pH 6.8. Active fractions, which under these conditions were not adsorbed on the column, were pooled and concentrated. The sample was run through an Ultrogel AcA 54 gel-filtration column (1.6 cm × 95 cm) in 50 mM-sodium phosphate, pH 5.8.

Fig. 1. Chromatography of the metalloproteinase preparation on an Ultrogel AcA 54 column

The marked fractions were pooled and used for purification of the enzyme. For details, see the Materials and methods section. ○, *A*₂₈₀; ■, proteinase activity.

After 90 ml of buffer had been run through the column, fractions (2.4 ml each) were collected (Fig. 1). The EDTA-sensitive proteolytic activity was determined by using denatured 13 S globulin as substrate. The active fractions were pooled and concentrated.

For further purification, f.p.l.c. on an ion-exchange column (Mono S HR 5/5; Pharmacia) was used. The sample (7 mg of protein) was applied to the column in 10 mM-sodium phosphate, pH 5.8. The column was washed with the same buffer (10 ml) and the protein was eluted with linear gradients of NaCl (20 ml of 0–300 mM; 10 ml of 300–400 mM). Finally, the column was washed with 1 M-NaCl (2 ml). Fractions (1.0 ml each) with maximal EDTA-sensitive proteolytic activity measured by using

Fig. 2. F.p.l.c. of the proteinase preparation on a Mono S HR 5/5 column at pH 8.15 in a linear NaCl gradient

For details, see the Materials and methods section. —, *A*₂₈₀; ■, proteinase activity; — —, NaCl gradient.

denatured 13 S globulin as substrate were pooled, concentrated, and rechromatographed on a Mono S HR 5/5 column in 25 mM-Hepes, pH 8.15 (Fig. 2). The sample (300 µg) was dialysed against 25 mM-Hepes, pH 8.15, and applied to the column equilibrated with the same buffer. The column was washed with the equilibration buffer (10 ml) and eluted with 30 ml of a linear NaCl gradient (0–500 mM). The column was then washed with 2 ml of 100 mM-NaCl. Two fractions of the gradient with maximal activity (Fig. 2) were pooled, concentrated and used for further study.

PAGE and isoelectric focusing

Electrophoresis was performed in 7.5 % (w/v) polyacrylamide gels at pH 8.9 (Davis, 1964) for 1 h at 4 mA per gel tube. Gels were stained with 0.02 % solution of Coomassie Blue G-250.

Electrophoresis in a 12.5 % gel in the presence of SDS was carried out for 3 h at 25 mA, as described by Laemmli (1970). After electrophoresis, the gel was fixed in 10 % (w/v) trichloroacetic acid and stained with Coomassie Blue R-250.

Isoelectric focusing of proteins was performed in a 110 ml focusing column (LKB) at 4 °C. Ampholines and Servalts were used for obtaining pH gradients of 3–10, 8–10 and 2–11.

M_r determination

The M_r of the enzyme was determined by gel filtration on a Sephadex G-150 column (1.6 cm × 80 cm) equilibrated with 10 mM-phosphate buffer, pH 6.8. The following were used as marker proteins: cytochrome *c* (M_r 12400), papain (20700), soybean trypsin inhibitor (21500), chymotrypsin (22000), pepsin (34200), ovalbumin (45000) and BSA (68000).

The M_r of proteins was also determined by SDS/PAGE as described by Laemmli (1970). A kit of marker proteins with M_r values ranging from 12300 to 78000 (LKB) was used for calibration: ovotransferrin (78000), BSA (66250), ovalbumin (45000), carbonic anhydrase (30000), myoglobin (17200) and cytochrome *c* (12300).

Atomic-absorption spectrophotometry (a.a.s.)

Metals in the enzyme preparation were assayed by a.a.s. on a Textron (Varian, Mulgrove, Australia) AA-4 spectrophotometer at the following wavelengths: Fe, 248.3 nm; Co, 240.7 nm; Ni, 232.0 nm; Mn, 279.4 nm; Mg, 285.2 nm; Ca, 422.6 nm; and Zn, 213.8 nm. The amount of metal in the proteinase preparation was calculated from a calibration curve.

Protein determination and concentration

The protein concentration in the solutions was measured spectrophotometrically at 280 nm in the experiments involving chromatography of proteinase preparations. In all other cases it was determined as described by Lowry *et al.* (1951), with BSA as standard.

Protein solutions were concentrated by ultrafiltration on UM-10 and XM-30 membranes (Amicon, Oosterhout, Netherlands).

RESULTS

The results of the purification of the metalloproteinase from buckwheat seeds are summarized in Table 1.

At each purification step the enzyme activity with denatured 13 S globulin was determined by the trinitrophenylation technique as described above. The activity of all the enzyme preparations was measured twice: in the presence and in the absence of EDTA (10 mM). The difference between these two values was assumed to be the activity of the metalloproteinase. As Table 1 shows, after ion-exchange chromatography on DEAE-Toyopearl there is a drastic rise in the total activity of the enzyme as compared with the level of its activity at the preceding steps of purification. That may be due to the removal, on the ion-exchanger, of an inhibitor of the enzyme, the presence of which in dry buckwheat seeds was reported previously (Belozersky *et al.*, 1982).

Taking into account that the highest total enzyme activity was determined after this purification step, the yield of activity after ion-exchange chromatography on DEAE-Toyopearl was assumed to be 100 %.

The final purification of the enzyme was carried out by two-step ion-exchange of f.p.l.c. The first step was performed at pH 5.8 to remove most of the acidic and neutral proteins, and the second at pH 8.15 (according to preliminary titration-curve analysis) to achieve the best separation of the remaining proteins.

This purification procedure made it possible for one to obtain a homogeneous (according to electrophoretic data) enzyme preparation (Fig. 3), purified 1000-fold with a yield of 0.1 % (Table 1).

Fig. 3. SDS/PAGE of the purified metalloproteinase from buckwheat seeds

For details, see the Materials and methods section. (a) Position of the M_r standard proteins; (b) metalloproteinase preparation.

Table 1. Purification of the metalloproteinase from buckwheat seeds

Purification step	Protein* (mg)	Activity (units)	Specific activity (units · mg ⁻¹)	Yield (%)	Purification (fold)
Extraction and precipitation with (NH ₄) ₂ SO ₄	10500	31500	3.0		1.0
Gel filtration on Sepharose 6B	420	13440	32.0		10.7
Chromatography on DEAE-Toyopearl	210	87360	416	100	138.7
Gel filtration on Ultrogel AcA 54	7	3150	450	3.6	150
F.p.l.c. and rechromatography on Mono S	0.03	90	3000	0.1	1000.0

* Protein was measured by the method of Lowry *et al.* (1951), with BSA as standard.

Table 2. Effect of inhibitors on the activity of the metalloproteinase from buckwheat seeds

Enzyme, 20 μ g in 100 μ l of 100 mM-sodium phosphate, pH 7.5, was pretreated with each of the inhibitors used for 2 h at 20 °C and the activity was measured as described in the Materials and methods section.

Reagent	Concn. (mM)	Inhibition (%)
EDTA	1.0	100
1,10-Phenanthroline	1.0	100
Zincon	1.0	100
iPr ₂ P-F	0.9	0
PCMB	0.2	20
Pepstatin	0.16	0

The M_r of the enzyme, according to SDS/PAGE, was 34000 (Fig. 3). The molecular weight of the enzyme, determined by gel filtration on a Sephadex G-150 column, was equal to 39000. This indicates that the enzyme is a monomer.

The pI of the buckwheat seed metalloproteinase was higher than 9.6.

An inhibition analysis showed that the enzyme was completely inhibited by the inhibitors of metalloenzymes (EDTA, 1, 10-phenanthroline and zincon), whereas the inhibitors of cysteine proteinases (PCMB), serine proteinases (iPr₂P-F) and aspartic proteinases (pepstatin) exerted little, if any, inhibitory effect on the activity of the enzyme (Table 2). The data are consistent with the enzyme being a metalloproteinase.

This conclusion was confirmed by an a.a.s. assay of the metal in the investigated enzyme so as to determine the presence of metals which occur most frequently in proteins of various origins. The results obtained showed that the enzyme contained only Zn²⁺ ion (1.2 g-atom) per molecule of the proteinase.

Furthermore, the a.a.s. technique was used to study the action of EDTA and some metal ions on metalloproteinase activity. An a.a.s. analysis of a metalloproteinase preparation completely inactivated with EDTA (1 mM) and intensively dialysed to remove this reagent revealed that inhibition of the enzyme is not accompanied by a release of Zn²⁺ from its molecule.

Fig. 4. Hydrolysis of the denatured 13 S globulin from buckwheat by metalloproteinase

Symbols: Δ , in the presence of 10 mM-Mg²⁺; $\circ$, EDTA-inactivated enzyme in the presence of 10 mM-Mg²⁺; $\blacktriangle$, in the presence of 0.01 mM-Zn²⁺; $\bullet$, EDTA-inactivated enzyme in the presence of 0.01 mM-Zn²⁺; $\square$, in the absence of additives; $\blacksquare$, EDTA-inactivated enzyme.

Table 3. Action of the metalloproteinase on protein substrates

For details, see the Materials and methods section. Abbreviations used: N, non-denatured; D, denatured.

Substrate	Amino groups released (nmol · mg ⁻¹)		Bonds hydrolysed (% · mol ⁻¹)	
	N	D	N	D
BSA	10	120	0.12	1.4
Haemoglobin	15	110	0.16	1.2
Soybean 11 S globulin	85	580	1.0	6.8
Buckwheat 13 S globulin	135	340	1.5	3.8

The addition of excess Zn²⁺, Co²⁺ and Mg²⁺ to the EDTA-treated enzyme resulted in complete restoration of activity (Fig. 4). For this purpose the EDTA-inactivated enzyme preparation was dialysed against 10 mM-sodium phosphate, pH 6.8, containing 1 mM-MgCl₂, 1 mM-CoCl₂ or 0.1 mM-ZnCl₂. At the same time, the addition of an excess of these and Ca²⁺ ions to the active enzyme preparation did not increase its activity (Fig. 4). It is possible that Mg²⁺, Co²⁺ and Zn²⁺ activate the inactive metalloproteinase by competing with Zn²⁺, which is present in the enzyme molecule, to bind to EDTA.

A study of the pH-dependence of enzyme activity showed that, with the non-denatured 13 S globulin, the maximal activity was observed at pH 8.0–8.2, with that for the denatured 13 S globulin occurring at pH 7.6.

The enzyme exhibited no carboxypeptidase and aminopeptidase activities.

We also studied the metalloproteinase's action on a number

Fig. 5. SDS/PAGE analysis of the products of hydrolysis of 13 S globulin from buckwheat (a) and 11 S globulin from soybean (b) by metalloproteinase

Lanes: (a)1 and (b)1, non-hydrolysed proteins; (a)2 and (b)2, hydrolysed non-denatured proteins; (a)3 and (b)3, hydrolysed denatured proteins. The positions of the M_r standard proteins are indicated on the left-hand side.

Table 4. Amino acid compositions of peptides obtained from a 16 h hydrolysate of oxidized insulin B-chain by buckwheat seed metalloproteinase

The number of amino acid residues in the proposed segment is given in parentheses.

Amino acid	Peptide... B-chain segment...	Composition (residues/molecule)				
		1 25-30	2 1-14	3 16-24	4 15-30	5 1-15
Asp		—	0.96 (1)	—	—	1.02 (1)
Thr		0.84 (1)	—	—	0.78 (1)	—
Ser		—	0.88 (1)	—	—	0.95 (1)
Glx		—	2.26 (2)	1.22 (1)	1.37 (1)	2.25 (2)
Pro		0.93 (1)	—	—	0.99 (1)	—
Gly		—	1.04 (1)	1.76 (2)	2.0 (2)	1.17 (1)
Ala		1.0 (1)	1.0 (1)	—	1.04 (1)	1.0 (1)
Val		—	1.69 (2)	0.96 (1)	1.10 (1)	1.72 (2)
Leu		—	2.12 (2)	1.0 (1)	1.23 (1)	3.18 (3)
Tyr		0.98 (1)	—	0.79 (1)	1.58 (2)	—
Phe		0.92 (1)	0.69 (1)	0.88 (1)	1.50 (2)	0.74 (1)
Lys		0.91 (1)	—	—	0.88 (1)	—
His		—	1.64 (2)	—	—	1.74 (2)
Arg		—	—	0.81 (1)	0.80 (1)	—
Cys		—	0.41 (1)	0.35 (1)	0.42 (1)	0.40 (1)

of other proteins, including both typical seed storage proteins (13 S globulin from buckwheat seeds and 11 S globulin from soybeans) and exogenous proteins (BSA and haemoglobin). The degree of hydrolysis of non-denatured 11 S and 13 S globulins was similar, and insignificant (1 % of the peptide bonds were cleaved per 11 S globulin molecule and 1.5 % per 13 S globulin molecule) (Table 3). Electrophoresis patterns of the products of hydrolysis of 11 S and 13 S globulins after SDS/PAGE showed that, in both cases, high- M_r subunits disappeared and lower- M_r components appeared (Fig. 5). When the above-mentioned proteins were denatured before hydrolysis with

metalloproteinase, a noticeable increase in the extent of their hydrolysis was observed (Table 3). In contrast with the proteolysis of non-denatured storage proteins, when high- M_r subunits are cleaved, the hydrolysis of denatured 11 S and 13 S globulins did not show this phenomenon, and all of the subunits of both proteins were hydrolysed (Fig. 5).

To find out which peptide bonds are hydrolysed by the isolated metalloproteinase, we studied the action of this enzyme on the oxidized insulin B-chain. The amino acid compositions of the five main peptides, obtained after hydrolysis, are given in Table 4. Their comparison with the amino acid sequence of the insulin

Fig. 6. Cleavage sites of insulin B-chain by buckwheat seed metalloproteinase in comparison with other metalloproteinases

B-chain shows that peptide 1 represents the part of the B-chain sequence from Phe²⁵ to Ala³⁰, peptide 2 that from Phe¹ to Ala¹⁴, peptide 3 that from Tyr¹⁶ to Phe²⁴, peptide 4 that from Leu¹⁵ to Ala³⁰, and peptide 5 that from Phe¹ to Leu¹⁵. These peptides could arise from the B-chain after the cleavage of the following peptide bonds: Ala¹⁴–Leu¹⁵, Leu¹⁵–Tyr¹⁶ and Phe²⁴–Phe²⁵ (Fig. 6). The presence of small amounts (slightly larger than the base level) of a few other peptides among the products of B-chain hydrolysis with metalloproteinase may indicate that some other peptide bonds in the B-chain are also cleaved, but at significantly lower rates.

Next, the action of the investigated enzyme on a few Dnp-tetrapeptides (Dnp-Gly-Gly-Met-Arg, Dnp-Gly-Gly-Val-Arg and Dnp-Gly-Gly-Ile-Arg), often used as substrates for animal and bacterial metalloproteinases, was studied. The buckwheat seed metalloproteinase hydrolysed only one of the three Dnp-tetrapeptides, namely Dnp-Gly-Gly-Met-Arg, cleaving the bond formed by an amino group of methionine (–Gly–Met–). These data and those on the hydrolysis of insulin B-chain indicate that the buckwheat seed metalloproteinase, like other enzymes of this class of proteinases, hydrolyses peptide bonds formed by amino groups of aromatic and hydrophobic amino acids. However, the absence of hydrolysis by the enzyme of the other two DNP-tetrapeptides may indicate that not every peptide bond formed by the amino groups of hydrophobic or aromatic amino acids is hydrolysed by the metalloproteinase. This may be due to a narrow specificity of the investigated enzyme.

DISCUSSION

The proteolytic enzyme isolated from buckwheat seeds is apparently the first highly purified metalloproteinase obtained from the plant material.

The low yield of the active enzyme (0.1%) is mainly due to a significant loss of activity during gel filtration on Ultrogel AcA 54, where only a small part of the peak of activity was taken for further purification (Fig. 1; Table 1). However, we found it necessary to use this approach for enzyme purification because it enabled us to remove the bulk of low- M_r proteins co-purifying with the metalloproteinase.

Most of the previously studied proteolytic enzymes hydrolysing storage proteins in the seeds are cysteine proteinases (EC 3.5.22.-), synthesized *de novo* in the course of seed germination (Shutov & Vaintraub, 1987). Seed metalloproteinases, capable of hydrolysing endogenous storage proteins, have so far been found only in dry seeds of soybean and pumpkin (Bond & Bowles, 1983; Hara & Matsubara, 1980). It should be mentioned that pumpkin and soybean proteinases were not homogeneous and their properties were not studied in detail.

In a number of properties, metalloproteinase from buckwheat seeds reveals a noticeable similarity to analogous enzymes of bacterial and animal origin. The M_r values of known metalloproteinases have a wide range (from 1700 to 800000), though most frequently these enzymes have M_r values ranging from 30000 to 50000 (Bond & Beynon, 1985). Their activity optima lie in neutral or weakly basic pH ranges and their pI values are often basic as well (McConn *et al.*, 1964; Ohta *et al.*, 1966; Harper, 1980; Sagara *et al.*, 1984; Bond & Beynon, 1985; Diermayr *et al.*, 1987). Most metalloproteinases contain Zn²⁺ ions (McConn *et al.*, 1964; Ohta *et al.*, 1966; Kerr & Kenny, 1974b; Harper, 1980; Bond & Beynon, 1985; Evans & Mason, 1986). Some bacterial and animal metalloproteinases contain, along with Zn²⁺, also Ca²⁺ ions, which stabilize the enzyme molecule (Feder *et al.*, 1971; Evans & Mason, 1986; Diermayr *et al.*, 1987).

Since there are no plant metalloproteinases with known specificity, the specificity of the studied enzyme can be compared only with the specificities of animal and bacterial metalloproteinases (Fig. 6). Such a comparison with metalloproteinases from *Pseudomonas aeruginosa* (Moriwaka & Tsuzuki, 1970), *Serratia sp.* E-15 (Miyata *et al.*, 1970), *Bacillus thermoproteolyticus* (Matsubara *et al.*, 1966), *Legionella pneumophila* (Gulnik *et al.*, 1987) and endopeptidase 24.11 (Kerr & Kenny, 1974a) showed that the studied plant enzyme possessed the most narrow specificity. It hydrolysed mainly three peptide bonds in the insulin B-chain, whereas the other metalloproteinases hydrolysed from six to 14 bonds in the insulin B-chain. Significantly, all of the metalloproteinases compared, including buckwheat seed metalloproteinase, digest the peptide bond Ala¹⁴–Leu¹⁵, whereas the bond Leu¹⁵–Tyr¹⁶ is not hydrolysed by the enzymes of micro-organisms.

On the whole, the investigated metalloproteinase from buckwheat seeds has much in common with metalloproteinases of animals and micro-organisms, despite some differences in physicochemical properties, sensitivity to inhibitors and substrate specificity.

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