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Identification and characterization of a Bowman–Birk inhibitor active towards trypsin but not chymotrypsin in *Lupinus albus* seeds

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ABSTRACT

The paper describes the purification, structural characterization and inhibitory properties of a trypsin inhibitor from *Lupinus albus* L., a leguminous plant believed to be devoid of any protease inhibitor. The protein has been isolated by a newly set-up procedure and characterized by direct amino acid sequencing, MALDI-TOF mass spectroscopy and circular dichroism. Inhibitory properties toward bovine trypsin and chymotrypsin, as well as its thermal and pH stabilities, have been also assessed. The inhibitor is 63 amino acid long (M_r 6858; pI 8.22) and it is capable to inhibit two trypsin molecules simultaneously, with a *Kd* of 4.2 ± 0.4 nM, but not chymotrypsin. BLAST search against UniProtKB/TrEMBL database indicates that the inhibitor belongs to the Bowman–Birk inhibitor (BBI) family. The interest in these serine-protease inhibitors arises from the ability to prevent or suppress carcinogen-induced transformation, as shown in various *in vitro* and *in vivo* model systems.

1. Introduction

Plant seeds contain, apart from the storage proteins, several biologically active polypeptides which play various specialized functions, mostly related to defence mechanisms against pathogens. Among these molecules, enzyme inhibitors, lectins, and ribosome inactivating proteins are the most represented (Domoney, 1999). The class of the enzyme inhibitors comprises various protein types, including amylase and protease inhibitors (PIs). The accumulation of PIs is elicited by various biotic and abiotic stresses, including mechanical wounding, insect and pathogen attack, and various signal molecules, such as plant hormones, fungal cell wall oligomers, and the fatty acid amino acid conjugates found in larval oral secretions (Ryan, 1990; O'Donnell et al., 1996; Koiwa et al., 1997; Korth and Dixon, 1997; Balestrazzi et al., 2004; Roda et al., 2004).

Legume seeds contain several PIs, classified into various families, such as the trypsin and chymotrypsin inhibitor of the Bowman–Birk class (BBIs) (Laskowsky and Kato, 1980; Mosolov and Valueva, 2005; Qi et al., 2005).

BBI was first discovered and described in soybean seeds (Bowman, 1948) and since then it has been found in monocotyledonous and dicotyledonous plants. In legume seeds BBIs are present at higher concentrations in comparison with other plant families and tissues. The BBIs from legume seeds are single chain small proteins with a molecular weight of approximately 7–8 kDa and show a conserved and characteristic pattern of 14 cysteine residues, all forming intra-chain disulphide bridges (Prakash et al., 1996; Qi et al., 2005). This conserved array of seven disulphide bridges plays a main role in the stabilisation of BBI the double headed structure (Odani and Ikenaka, 1973; Qi et al., 2005). The two heads are located at the opposite sides of the molecule and make it possible the simultaneous and independent interaction with two, not necessarily identical, molecules of serine proteases. The specificity of each reactive site is determined by the identity of the amino acid in position P_1 of the reactive site, as defined according to the convention of Schechter and Berger (1967).

Because of their ability to inhibit the enzymes involved in the digestive processes of humans and animals, PIs have been referred to as "antinutritional compounds" so far. Some of them have now been favorably reconsidered in view of the potential exploitation of their biological behaviour in pharmacological and medical applications (Scarafoni et al., 2007). The interest for BBIs arises from their ability to prevent or suppress carcinogen-induced transformation, as detected in various *in vitro* and *in vivo* model systems and human trials (Kennedy, 1998; Clemente and Domoney, 2006). The potentiality of BBIs as therapeutic agents outlines the necessity to elucidate the intimate mechanisms at the base of their bioactivity (Scarafoni et al., 2007). Thus, more research is needed to either search novel BBI forms, such as the case of sunflower cyclic BBI

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(Qi et al., 2005), and to fully characterize the structural and functional properties of these proteins, such as the recent case of a BBI from lentil (*Lens culinaris*) seeds (Ragg et al., 2006).

White lupin (*Lupinus albus* L.) is a leguminous grain crop which represents an important source of proteins for human and animal nutrition, since its seed is one of the richest in protein content (up to 44% on dry weight basis), with a biological value of 91% relative to egg proteins (Egaña et al., 1992). The bulk of lupin proteins belongs to the vast families of 11S (also referred to as α-conglutins or as "legumin-like") and 7S (also referred to as β-conglutins or as "vicilin-like") globulins (Magni et al., 2007). In addition to these main protein families, lupin seeds contain significant amounts (5–6%) of γ-conglutin, a peculiar basic 7S protein (Duranti et al., 1981) and a low M_r size protein similar to other 2S albumins, named δ-conglutin (Duranti et al., 1981; Salmanowicz and Weder, 1997).

Information about PIs in *Lupinus* spp. is very limited. Up to now, only one PI has been described. It is a partially purified trypsin inhibitor with a M_r of about 21 kDa similar, for its physico-chemical properties, to a Kunitz trypsin inhibitor (Domash, 1991). Apart from this reference, white lupin seeds are since long considered completely lacking of any PI activity (Gallardo et al., 1974; Hove, 1974). This fact sounds quite strange, since it has been postulated that PIs play key roles in important cellular mechanisms, including for example the regulation of the activity of endogenous proteases both in plants and other organisms (Koiwa et al., 1997; Domoney, 1999; Mosolov and Valueva, 2005).

The present work has been carried out to unveil the presence of serine-protease inhibitors also in lupin seeds. The paper describes the purification, structural characterization and inhibitory properties of a trypsin inhibitor, which represents the first BBI protein isolated from white lupin seeds.

2. Results and discussion

2.1. Purification and molecular characterization

It is generally accepted that all legume seeds contain considerable amounts of protease inhibitor (Domoney, 1999). *Lupinus* spp. would have represented an exception, since it was believed that PIs did not accumulate in *L. albus* cotyledons and were present only in traces in those of *L. angustifolius* and *L. luteus* (Gallardo et al., 1974; Birk, 1993). Our findings indicated instead that white lupin seeds contain a non-negligible amount of TIA (Table 1). In the aqueous extract 12,700 TIU/g seed have been found, a value which corresponds to about 29 TIU/mg protein in the dry seed, considering a seed protein content of 42% on the dry matter basis (Egaña et al., 1992). This is approximatively 10% of the TIUs found in soybean seed (Duranti et al., 2003) and three times those found in garden pea or common bean seeds (Hove and King, 1979; Birk, 1993; Muel et al., 1998).

The preparation of the crude extract (CE) took advantage of the thermal and pH stability of the BBIs. The procedure caused the loss of more than 99% of the proteins present in the aqueous extract, whereas the inhibitory activity yield was about 1.3%. The specific activity (SA) increased about three times. This suggests that most of the pH and heat labile inhibitor activity present in the aqueous extract is due to a different inhibitor, most likely belonging to the Kunitz family. The further purification of BBI was obtained by two chromatographic steps. The proteins recovered in the not bound fraction of the DEAE chromatography possessed 90% of total TIU loaded onto the column. The SA of the BBI in this fraction was 1029 TIU/mg and the purification fold (p.f.) achieved was nearly 15. The solution has been then loaded onto a trypsin-agarose affinity (TAC) column. In this case, the fraction retained by the matrix contained about 75% of the TIUs, whereas the remaining 25% were not bound. When the unbound fraction was loaded again onto a regenerated TAC column, only negligible amounts of proteins were retained (not shown). This fraction was not further analyzed. The bound protein was named LaBBI. SA and p.f. reached 28,370 TIU/mg and 411, respectively. The yield of LaBBI was nearly 4 μg/g of seed, which represent less than the 0.022% of the protein content on the dry seed matter basis.

The SDS-PAGE (Fig. 1) showed that LaBBI consisted of a single polypeptide with M_r of approximately 6 kDa.

MALDI-TOF mass spectrometry analysis of LaBBI revealed the presence of two molecular species, a major one with M_r 6771.04 and a minor one with M_r 6858.47 (Fig. 2). The two polypeptides thus differ for 87.43 Da.

2.2. Amino acid sequencing and sequence alignments

N-terminal sequence of the entire reduced and carbamidomethylated purified material revealed a single polypeptide sequence starting with Ser1up to residue 30. This suggests that the two peaks observed in MALDI-MS originated from partial proteolysis at the C-terminus of the same polypeptide, most likely due to removal of a single Ser residue. Thus, no purification of the two forms was performed prior to the following analyses. The complete sequence

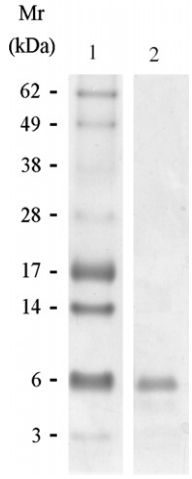

Fig. 1. Electrophoretic analysis (15% SDS-PAGE, under reducing conditions) of LaBBI after trypsin-agarose affinity chromatography (TAC). The gel was stained with Coomassie blue dye. Two micrograms of protein was loaded in each lane. Lane 1: standard proteins; lane 2: purified LaBBI.

Table 1
Purification table of LaBBI from lupin seeds

Purification step	μg prot/g seed	TIU/g seed	TIU/mg prot	Purification fold	Protein yield (%)	Activity yield (%)
Aqueous extract	183,100	12,705	69	1	100.00	100.00
Crude extract	910	169	186	2.7	0.50	1.32
DEAE-cellulose	148	150	1029	14.9	0.076	1.18
TAC	4.0	112	28,370	411.1	0.022	0.88

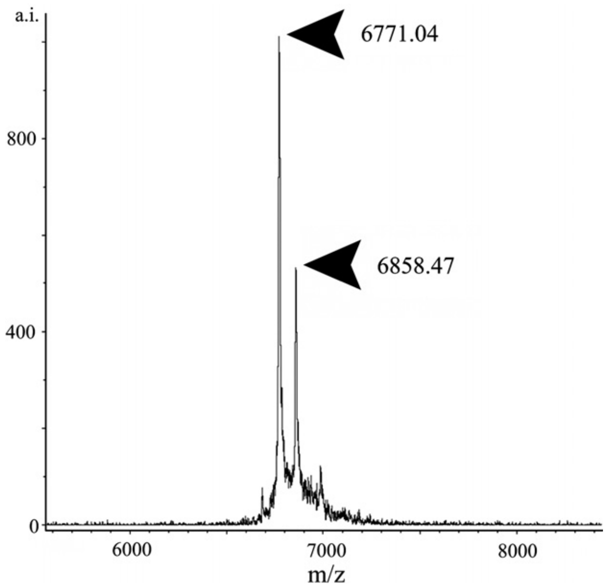

Fig. 2. MALDI-TOF mass spectrum of LaBBI. See text for details.

was obtained by N-terminal sequence and MALDI-MS analysis of all the peptides produced by trypsin digestion and separated by RP-HPLC. Alignment of the peptides was based on the sequence of the entire protein and on similarities with the sequence of other BBIs. The complete primary structure is reported as first sequence in Fig. 3 and it has been deposited in the UniProtKB/Swiss-Prot sequence databank under the accession number IBB1_LUPAL (P85172). The protein consists of 63 amino acids and the calculated M_r of 6858.96 (assuming 7 disulfide bonds) is in good agreement with the value of 6858.47 determined by mass spectrometry under non-reducing conditions, confirming that also in LaBBI all 14 Cys are involved in disulfide bridges. As expected, two different peptides corresponding to the C-terminal portion with the same amino acid sequence CSDITHFCYKPCTSS except for the final Ser residue and with M_r of 1862.72 (calculated 1862.75) and 1775.69 (calculated 1775.72), thus differing for 87.03, where identified. This confirms that the protein is purified as a mixture of two forms partially proteolysed at the C-terminus. The theoretical pI of 8.22 coincided with that assessed by 2D IEF/SDS-PAGE (not shown).

The BLAST search (Altschul et al., 1997) against UniProtKB/TrEMBL database (Release 37.3) indicated that LaBBI is clearly structurally related to the Bowman–Birk inhibitor family. The 14 cysteine residues are in the conserved position (Fig. 3) previously described for other BBIs (Qi et al., 2005) and the residue at the P_1 position (Schechter and Berger, 1967) of the N-terminally located reactive site is arginine (Arg15), conferring the specificity for the trypsin inhibition (Laskowsky and Kato, 1980; Qi et al., 2005). As far as the LaBBI second reactive site is concerned, the residue at the P_1 position was again an arginine (Arg41). This makes LaBBI a double headed trypsin inhibitor. The two reactive loops of LaBBI show an identical amino acid sequence, with the only difference of the residues in position P'_2 (Ile and Phe, respectively). Ile is the amino acid most frequently found at position P'_2 in BBIs trypsin site from legumes (Odani and Ikenaka, 1978; Piergiovanni and Galasso, 2004).

Database search also revealed very few database entries regarding double headed trypsin inhibitors (less than 40 entries out of more than 280,000). The amino acid sequence of the majority of them are inferred from nucleotide sequence and only four proteins have been experimentally evidenced in plant tissues, although not all have fully been characterized at molecular level. Interestingly, all these proteins have been identified in seeds. For that reason, only these sequences have been considered to produce the multiple alignment with ClustalW (Higgins et al., 1994) shown in Fig. 3. It can be observed an overall limited number of conserved amino acid residues, mostly concentrated in the nearby of the two reactive sites. However, when considering the alignment of LaBBI one by one with any of the selected sequences, the amino acid identity ranges from 74% (snail medic) to about 69% (soybean).

2.3. Kinetics of inhibition and inhibition constant determination

The inhibitory activity of LaBBI was determined by monitoring the hydrolysis of the chromogenic substrates BApNA and GPpNA in the presence of bovine trypsin and α-chymotrypsin, respectively, and increasing concentrations of LaBBI. The experimental data (white circles) obtained for trypsin inhibition (213 μM BApNA) are plotted in Fig. 4. The best fitting curve was traced by mean of the GraFit software using Eqs. (1) and (2), as reported in Section 4. In order to take in consideration the binding stoichiometry in Eq. (1), the correction factor (x) for the inhibitor concentration has been set to 2. This value is the x-intercept value of the traced tangent line of the best fitting curve, which clearly indicates an enzyme/inhibitor stoichiometry of 0.5 (Brauer et al., 2002). The resulting dissociation constant (Kd) was 4.2 ± 0.4 nM.

When the same experimental setting has been followed for assessing the bovine chymotrypsin activity in the presence of LaBBI no measurable inhibitory activity was observed in all the conditions tested, including increasing inhibitor concentration and extending the incubation time up to 40 min.

2.4. Thermal and pH stability

The thermal stability of LaBBI solutions have been assessed by progressive heating and subsequent cooling back. The specific activities of the solutions have been determined before and after heat treatment. As shown in Table 2, no significant variations of the inhibitory activity have been detected. No protein precipitation following the heat treatment was observed (not shown).

```
                           1        10        20        30        40        50        60
                           |--------|---------|---------|---------|---------|---------|---
IBB1_LUPAL      -----------SLASKPCCDSCLCTRSIPPQCRCTDIG-ETCHSACKSCICTRSFPPQCRCSDITHFCYKPCTSS----- 63
IBB_MEDSC       ----------TKSTTTACCDFCPCTRSIPPQCQCTDVR-EKCHSACKSCLCTRSFPPQCRCYDITDFCYPSCS------- 62
IBB2_PHAAN      SVHHQDSSDEPSESSHPCCDLCLCTKSIPPQCQCADIRLDSCHSACKSCMCTRSMPGQCRCLDTHDFCHKPCKSRDKD-- 78
IBB_PHAAU       ------SHDEPSESSEPCCDSCDCTKSIPPECHCANIRLNSCHSACKSCICTRSMPGKCRCLDTDDFCYKPCESMDKD-- 72
IBBD2_SOYBN     --SDQSSSYDDDEYSKPCCDLCMCTRSMPPQCSCEDIRLNSCHSDCKSCMCTRSQPGQCRCLDTNDFCYKPCKSRDD--- 75
                                 *** * ** * ** * *       *** *  * **** *  *** *   **   *
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Fig. 3. Amino acid sequence of LaBBI and alignment with double headed BBIs specific against trypsin only, which have previously been isolated and characterized experimentally. Accession numbers are from Swiss-Prot database and refer to the following proteins and plant species, respectively: IBB1_LUPAL (LaBBI, *Lupinus albus*, this work), IBB_MEDSC (MSTI, *Medicago scutellata*) IBB2_PHAAN (I-A, *Phaseolus angularis*), IBB_PHAAU (MBILF, *Phaseolus aureus*), IBBD2_SOYBN (D-II, *Glycine max*). Residue numbers on the top of the alignment refer to IBB1_LUPAL sequence. White letters on black background indicate the two reactive sites. Asterisks indicate identical residues in all sequences. White asterisks on black background indicate the conserved cysteine residues. Gaps (-) have been inserted to maximize the global alignment.

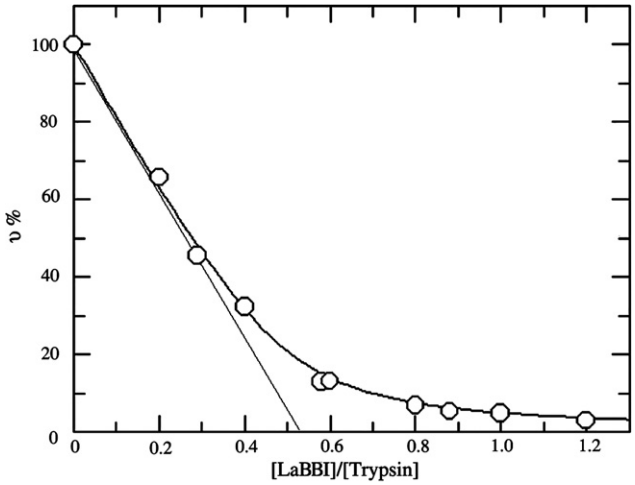

Fig. 4. Titration curve of trypsin inhibition by LaBBI. Increasing concentration of inhibitor were added to a fixed concentration of enzyme (0.1 μM). Residual enzyme activity are expressed as percentage of the control (*v*%) and was determined by using 213 μM BApNA as substrate. White circles represent the experimental data. See text for further experimental details. The *x*-intercept value of the traced tangent line indicates an enzyme/inhibitor stoichiometry of 0.5. Each point is the mean of three assays.

Table 2
Effects of thermal treatment and pH variations on the specific activity of LaBBI

	Thermal treatment	
	–	+
pH 3.0	28,030	n.d.
pH 8.0	28,120	27,990

n.d.: not determined.
Values are expressed as TIU/mg and are the mean of two assays. For experimental details see the text.

Analysis of the temperature effects on the LaBBI secondary and tertiary structures have been assessed by circular dichroism (CD) spectral analysis. Although the buffer system used to dissolve the protein did not allowed to record spectra below 200 nm (Kelly et al., 2005), it was however possible to get enough information to evaluate the conformational changes of the protein under the tested conditions. Far-UV CD spectra (Fig. 5A) of the protein showed that the protein secondary structure appeared almost completely restored after cooling. As far as tertiary structure modifications is concerned, near-UV CD spectra (Fig. 5B) indicated that the heat treatment induced only modest not completely reversible structural changes.

The structural variations of the protein have been also assessed after the pH of the solution was brought to 3.0 with HCl. Fig. 5C shows spectra recorded at pH 8.0 and pH 3.0. The two CD spectra are coincident, indicating that the two tested pH conditions did not perturb the overall folding of the protein. After spectra have been recorded at pH 3.0, the pH solution has been adjusted to pH 8.0 with 1 M Tris-base and the inhibitory activity assessed. Again, no differences in activity were observed (Table 2).

3. Conclusions

The present work unveils the presence of a Bowman–Birk serine-protease inhibitor in the mature seeds of white lupin, a plant previously described as almost completely lacking of any protease inhibitor activity.

Functionally, LaBBI is unusual in that it stoichiometrically inhibits trypsin in a molar ratio of 1:2. Two forms of LaBBI, differing only for a single amino acid at the C-terminal, simultaneously coexists in the seeds. This has already been observed for other BBIs (Wilson, 1988; Ragg et al., 2006) and processing at both N- and

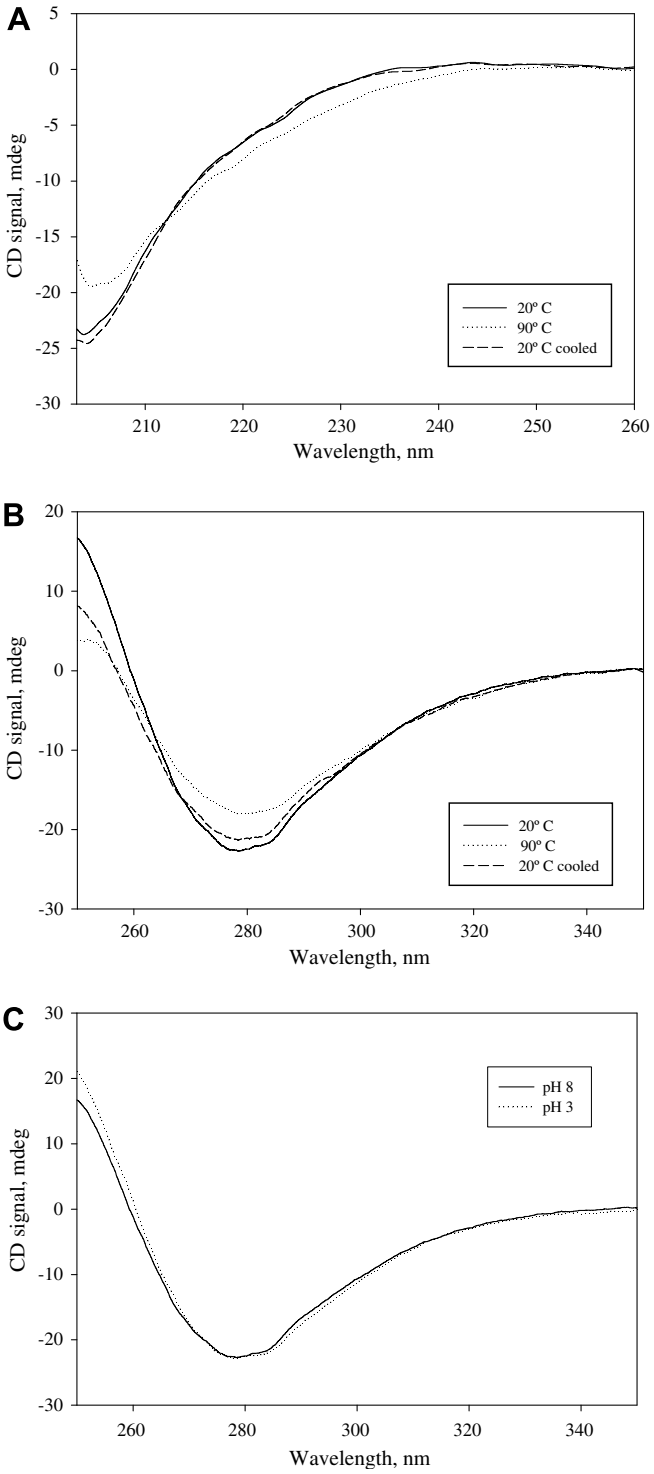

Fig. 5. CD spectra of LaBBI. Temperature effects on the LaBBI secondary and tertiary structures have been assessed by far-UV (A) and near-UV (B) spectral analyses, respectively. Stability at pH 3.0 was assessed in the near-UV (C). In the near-UV spectra (B and C) the protein concentration was 0.23 mg ml^{-1}, whereas in the far-UV (A) experiment was 0.058 mg ml^{-1}. The protein was dissolved in 75 mM Tris/HCl, 5 mM $CaCl_2$, pH 8.0. Time progressive heating of protein solutions was carried out at 0.5 °C/min from 20 to 90 °C, while continuously monitoring ellipticity changes. The samples were then cooled back to 20 °C at 5 °C/min and the spectrum recorded.

C-terminal ends of pea protease inhibitors during seed desiccation *in vivo* has been described (Domoney, 1999). Furthermore, LaBBI could not be the sole BBI present in lupin seed. As other seed proteins, BBIs have a multigene origin, which is responsible for the presence of different isoforms in legume seeds (Mello et al., 2003).

4. Experimental

4.1. General

Lupinus albus L. seeds of the sweet Multitalia variety were kindly provided by Prof. M. Fagnano (University of Naples, Italy).

The affinity chromatography matrix (agarose) with immobilized trypsin, *N*-benzoyl-L-arginine-*p*-nitroanilide (BApNA), *N*-glutaryl-L-phenylalanine *p*-nitroanilide (GPpNA), trypsin (TPCK-treated from bovine pancreas), α-chymotrypsin (TLCK-treated from bovine pancreas), and all other reagent grade chemicals were from Sigma–Aldrich (Milano, Italy) if not otherwise indicated.

4.2. Purification

Dry lupin seeds were ground to a meal with a coffee grinder. The flour was defatted in a Soxhlet apparatus by extraction with *n*-pentane at 37 °C for 4 h and it was sieved through a 60 mesh metal sieve. The defatted flour (1600 g) was suspended (1:5, w/v) in 100 mM sodium acetate buffer, pH 4.5, at 4 °C overnight, under mild stirring. The suspension was then centrifuged at 10,000*g* for 30 min at 4 °C. The supernatant, which was named aqueous extract, was heated in a water bath at 80 °C for 3 min, cooled on ice and centrifuged as described. The proteins contained in the supernatant were precipitated with ammonium sulphate (70% saturation) and centrifuged as described. The pellet was dissolved with distilled water (1000 ml) and dialyzed against milli Q water at 4 °C, overnight. The solution was brought to pH 4.0 by adding 0.2 N HCl in drops under stirring and centrifuged. The buffer of the supernatant was exchanged to 50 mM Tris/HCl buffer, pH 8.0, using an Amicon ultrafiltration (membrane cut-off 3000 MW) apparatus (Millipore, Billerica, MA, USA), final volume 1000 ml. This solution represented the crude extract (CE). The solution was kept frozen at −20 °C until use or immediately applied to a DEAE-cellulose (Whatman, Maidstone, UK) chromatographic column (4.5 × 25 cm) equilibrated with 50 mM Tris/HCl buffer, pH 8.0. The unbound fraction was concentrated to 50 ml by ultrafiltration (as before described) and loaded to a trypsin-agarose affinity chromatography (TAC). The column (1.5 cm × 8 cm) was equilibrated with 20 mM Tris/HCl buffer, pH 7.2 and the elution of the bound proteins was obtained by using a 3 mM HCl solution. The eluate was immediately neutralized with 1 M Tris/HCl buffer, pH 8.0. After extensive dialysis against water, the protein was lyophilized.

4.3. Electrophoretic techniques

The SDS-PAGE was performed in NuPAGE Novex Bis-Tris 10% gels using a XCell SureLock Mini-Cell (Invitrogen, Milan, Italy). NuPAGE MES SDS Running Buffer and SeeBlue Plus2 Prestained Standard (Invitrogen, Milan, Italy) were used. The gels were stained with SimplyBlue SafeStain (Invitrogen, Milan, Italy).

4.4. Protein determination

Protein concentration of all the fractions from chromatographic steps was determined by the Coomassie blue dye binding method (Bradford, 1976) using BSA as a standard protein. LaBBI concentrations in enzyme inhibitory assays and CD spectroscopy analyses were determined by UV absorbance at 280 nm, using a molar extinction coefficient value of 1490 M^{-1} cm^{-1}, calculated on the basis of the amino acid sequence assuming that no cystein residues appear as half cystines with the ProtParam tool available on line at www.expasy.org (Gasteiger et al., 2005).

4.5. Enzyme inhibition activity

Solutions of BApNA and GPpNA were freshly prepared by dissolving suitable amounts of the chromogenic substrate in Milli Q water, 150 mM Tris/HCl, 1 mM $CaCl_2$, pH 8.0. Concentrations were checked by absorbance measurements on an aliquot of substrate solution after complete enzyme catalysed hydrolysis (*p*-nitroaniline: $\lambda = 410$ nm, $\varepsilon = 8800$ M^{-1} cm^{-1}). The reaction solutions contained BApNA at concentrations between 100 μM and 300 μM or GPpNA at concentrations between 200 μM and 400 μM in 75 mM Tris/HCl, 5 mM $CaCl_2$, pH 8.0. Enzymes and LaBBI were dissolved in the same buffer: trypsin at concentrations varying between 0.05 μM and 0.5 μM, chymotrypsin between 200 μM and 400 μM and LaBBI between 0.5 μM and 2 μM. Enzymes have been employed with the assumption they were 100% active. Hydrolysis of the chromogenic substrates was monitored at 410 nm, sampling the absorbance every 10 min. The UV–visible spectrophotometer was a Perkin–Elmer Lambda 25 (Milano, Italy), equipped with a thermostatted cell. Temperature was set at 37 °C.

One trypsin inhibitor unit (TIU) was defined as the amount of inhibitor that decrease the activity of one trypsin units by 50%, where one trypsin unit hydrolyze 1.0 mmol of BApNA per minute at pH 8.0 at 37 °C.

The nonlinear regression GraFit software (Leatherbarrow, 1992) was used to analyse inhibition data. The rate of enzymatic substrate hydrolysis (v) was expressed as a function of the uninhibited rate (v_o), the apparent equilibrium dissociation constant (Kd^*), the total enzyme concentration ($[E_o]$) and the total inhibitor concentration ($[I_o]$). A factor x for the inhibitor concentration was introduced to determine the apparent binding stoichiometry (Brauer et al., 2002):

$$v = v_0\{[E_0] - [Kd^* + [E_0] + x[I_0] - \sqrt{(Kd^* + [E_0] + x[I_0]) - 4[E_0]x[I_0])}/(2[E_0])\} \quad (1)$$

The following equation was used to calculate the equilibrium dissociation constant (Kd) by correction for substrate competition, where $[S_o]$ and K_M are the total substrate concentration and Michaelis–Menten constant, respectively:

$$Kd = Kd^* \frac{1}{1 + [S_0]/K_M} \quad (2)$$

4.6. Mass spectrometry

Matrix assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometric analyses were performed by using a Bruker Daltonics Reflex IV instrument (Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm) and operated in linear or in reflectron mode with a matrix of sinapinic acid or α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA/CH_3CN 2/1, respectively. External standards were used for calibration (Bruker protein calibration standard) ranging from 5 to 16 kDa.

4.7. Amino acid sequencing

N-terminal sequence of polypeptides was determined using a pulse liquid sequencer (Procise mod. 491, Applied Biosystems, Foster City, USA). Carbamidomethylation was performed by the following procedure: the protein (0.2 mg) was dissolved in 8 M

urea, 50 mM dithiothreitol, 100 mm Tris/ HCl, pH 8.6, and the mixture was deoxygenated under vacuum and incubated overnight at 37 °C. The reduced peptides were treated with iodoacetamide (0.1 ml of a 0.625 M solution in 100 mM Tris/HCl, pH 8.6) in the dark for 45 min. The carbamidomethylated LaBBI was purified from the reaction mixture on a HPLC mod. 510, equipped with a Symmetry C18 column (Waters, Milano, Italy). The two buffers system consisted of 0.1% trifluoroacetic acid in Milli-Q water (buffer A) and the same buffer containing 80% acetonitrile (buffer B). After elution with buffer A for 5 min at a flow rate of 0.8 ml min^{-1}, a linear gradient to 75% of buffer B in 75 min was applied. An aliquot of the material (200 pmol) was used to determine the N-terminal sequence of the entire polypeptide, allowing the identification of the first 30 residues. The remaining part was digested with sequence grade trypsyn at a molar [*E*]/[*S*] ratio of 1:100 in 25 mM Tris/HCl buffer, 1 mM EDTA, pH 8.5, at 37 °C for 18 h. The peptides were separated on a Symmetry C18 column under the same conditions described above. The recovered peptides were vacuum-dried and submitted to amino acid sequencing and MALDI-MS. Alignment of the peptides was based on the N-terminal sequences of the entire protein and of homologous BBIs.

4.8. Circular dichroism studies

Circular dichroism (CD) spectra were recorded on a J-810 Jasco spectropolarimeter, equipped with a Peltier-based computer-driven temperature control, and analyzed by means of Jasco software. The cell path was 1 cm for measurements above 250 nm (Near-UV), and 0.1 cm for measurements in the 200–250 nm region (Far-UV). Time progressive heating of protein solutions was carried out at 0.5 °C/min from 20 to 90 °C, while monitoring continuously ellipticity changes. The sample were then cooled back to 20 °C at 5 °C/min and spectra recorded to assess the reversibility of temperature-induced spectral modifications. The protein was dissolved in 75 mM Tris/HCl, 5 mM $CaCl_2$, pH 8.0, which is the same buffer used for the kinetics of inhibition studies.

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