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ISOLATION AND PROPERTIES OF TRYPSIN INHIBITORS FROM THE SEEDS OF *Lupinus luteus* AND *L. polyphyllus*

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*From dormant seeds of European yellow lupin (*Lupinus luteus*) and Washington lupin* (*Lupinus polyphyllus*) inhibitors have been isolated that effectively suppress the activity of trypsin and interact nonstochiometrically with chymotrypsin. From their physicochemical properties and aminoacid compositions, the inhibitors from the species of lupin investigated belong to different types.*

Protein inhibitors of proteinases are widely distributed in animal and plant tissues [1]. The highest level of these proteins has been found among representatives of the families *Leguminosae*, *Graminae*,and *Solanaceae*. Proteinase inhibitors have been isolated in the pure form from many plants of the family *Leguminosae*. One type of inhibitors from the *Leguminosae* has a low molecular mass (about 1000 Da), and another a higher one (2000 Da). As examples of the first type may be given inhibitors isolated from soybean seeds (the Bowman-Birk inhibitor) [2], garden beans [3], and alfalfa (lucerne) leaves [4], and of the second type inhibitors from soybean seeds (the Kunitz inhibitor) [5] and gleditsin [6]. An investigation of the distribution of proteinase inhibiors of the Bowman-Birk and the Kunitz types among leguminous plants led to the conclusion that the inhibitors of the Bowman-Birk type are younger from the evolutionary point of view [7]. The inhibitors of each type may be represented by several molecular forms the appearnce of which may be the result of posttranslational modification of the protein [8], or the mechanism of their formation may be based a multiplicity of alleles of a single gene locus [9].

In Belarus, one of the most widespread leguminous crops is alfalfa, which is in second place after soybeans with respect to its protein content. But, in contrast to soybeans, the level of proteinase-inhibiting proteins in this crop is quite insignificant [10-12], although perennial (wild) species of alfalfa are richer in them. There is no information in the literature available to us on the isolation of a trypsin inhibitor in the pure form from lupin seeds and its physicochemical properties. There are only some of our own findings [13].

The aim of the present work was to investigate trypsin inhibitors from the seeds of *Lupinus luteus* and *L. polyphyllus* and to investigate some of their physicochemical properties.

The conditions for isolating the trypsin inhibitors are described in the Experimental part. The main purification steps are given in Table 1. As can be seen from this table, an increase in activity was observed at all stages of purification, but the main purifiaction of the inhibitors was achieved after the affinity chromatography of the protein. Electrophoresis of the proteins in polyacrylamide gel under native conditions showed that both types of lupin possessed two main components, with relative electrophoretic mobilities of 1.0 and 2.0, Electrophoresis in polyacrylamide gel under denaturing conditions showed that the inhibitor from the *L. luteus* seeds migrated in the form of three bands with molecular masses of 25, 19 and 15 kDa, while those from *L. polyphyllus* had molecular masses of 24, 16.2, and 10.4 kDa.

For further purification, the total inhibitor fraction after affinity chromatography was passed through a column of CM-Sephadex C-25. As the results showed, the inactive part of the protein was eluted with the initial 0.005 M acetate buffer, pH 4.0. Slight activity was detected in a fraction eluted by 0.2 M acetate buffer, pH 4.0. Considerable activity was found in a second peak, eluted by 0.2 M acetate buffer, pH 7.0. This fraction was used for the investigations. Electrophoresis in

*The Russian name given here actually corresponds to *L. perennis*, the sundial lupin. It has been assumed that the Washington lupin is meant throughout the paper.

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TABLE 1. Purification of the Trypsin Inhibitors from the Seeds of *Lupinus luteus* (I) and *L. polyphyllus* (II)

Stage of purification	Total protein		Total activity, IU		Specific activity, IU/mg of protein		Yield, %		Degreee of purification	
	I	II	I	II	I	II	I	II	I	II
Initial extract	120300	95300	102500	152480	0.85	1.6	100	100	1	1
Extract after precipitation of ballast proteins	49500	48000	86850	101120	1.75	2.1	84.7	66.0	2.0	1.3
Affinity chromatography	4.0	7.0	1397	8510	349.2	1215.7	1.4	5.6	410.8	759.8
Chromatography on CM-Sephadex C-25	1.8	3.0	780	3620	433.3	1206.0	0.8	2.4	509.8	753.7

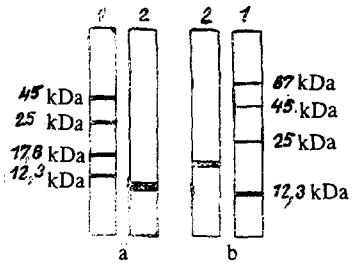

Fig. 1

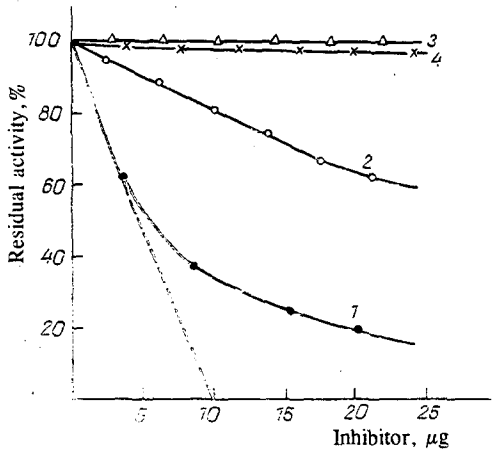

Fig. 2

Fig. 1. Disk electrophoresis of trypsin inhibitors from *Lupinus polyphyllus* (a) and *L. luteus* (b) in 15% PAAG in the presence of Na-DS and β-mercaptoethanol: 1) marker proteins; 2) isolated inhibitor.

Fig. 2. Suppression of the activity of trypsin (1), chymotrypsin (2), papain (3), and subtilisin by the inhibitor from *Lupinus luteus* seeds.

polyacrylamide gel in the presence of 0.1% Na-DS showed that the inhibitor from *Lupinus luteus* seeds was homogeneous and had a molecular mass of 18.6 kDa, while that from *L. polyphyllus* had one of about 10 kDa. Some diffusion of the band from *l. polyphyllus* must be mentioned (Fig. 1). Isofocussing of the trypsin inhibitor proteins in polyacrylamide gel showed isoelectric points of 5.2 for the *L. luteus* inhibitor and 4.9 for that from *L. polyphyllus*.

The results of a study of the actions of the preparations obtained on enzymes are given in Figs. 2 and 3. As can be seen from the Figures (curves 1) the relationship between the amount of the inhibitor from *Lupinus luteus* seeds that was added and the degree of suppression of the activity of trypsin was linear until 50% inhibition had been reached, while for *L. polyphyllus* the correponding level was 80% inhibition. Extrapolation of the relationships obtained to zero enzymatic activity showed that complete inhibition of trypsin by the *L. luteus* inhibitor should have been reached at 10 mg, and by the *L. polyphyllus* inhibitor at 0.87 mg or at weight ratios of 1 μg of inhibitor to 1 μg of enzyme and of 0.087 μg of inhibitor to 1 μg of enzyme respectively. Thus, the inhibitor from *L. polyphyllus* seeds may be one of the most active inhibitors of plant origin. As the results of the investigations (Figs. 2 and 3, curves 2) showed, in the case of interactions of the inhibitors with

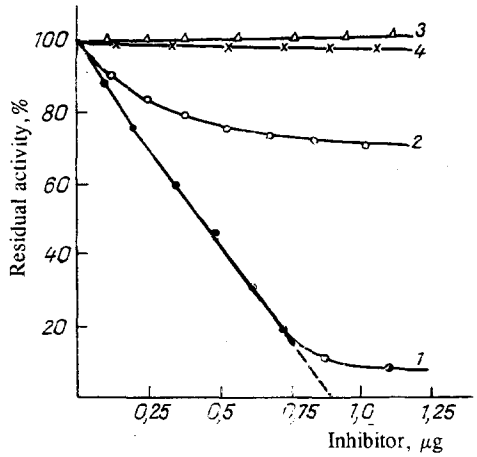

Fig. 3. Suppression of the activities of trypsin (1), chymotrypsin (2), papain (3), and subtilisin (4) by the inhibitor from *Lupinus polyphyllus* seeds.

chymotrypsin, the relationship between the degree of inhibition of the enzyme and the amount of inhibitor added was no longer linear; i.e., the inhibitors formed less stable compounds with chymotrypsin. The higher capacity of the inhibitor from *L. polyphyllus* for inhibiting the activity of chymotrypsin must be mentioned. Neither protein was active in relation to subtilisin and papain.

Amino acid analysis of the trypsin inhibitors showed that the largest numbers of amino acid residues in their molecules were those of aspartic acid, glutamic acid, threonine, serine, and arginine (6-12% of the sum of the amino acids). We must also mention the fairly high content of semicystine (about 16%) for the *L. polyphyllus* inhibitor, while the *L. luteus* inhibitor contained about 6% of it. Characteristic for the inhibitors from the lupins, as for inhibitors from other legumes, is the complete absence of tryptophan and the comparatively small amount of other aromatic amino acids.

The results of our investigations also showed that the trypsin inhibitors are fairly resistant to denaturing actions, that from *L. polyphyllus* being so to a considerably greater degree. Thus, its activity on trypsin did not change appreciable after boiling for 15 minutes, and after 30 and 45 minutes its activity had fallen by 10 and 25%, respectively. On being boiled for 15 minutes, the activity of the *L. luteus* inhibitor fell by 34%, and after 30 and 45 minutes by 52 and 64%, respectively. The *L. polyphyllus* inhibitor possessed greater stability in the pH range of 2-12. Both inhibitors were resistant to the action of pepsin at pH 2 and 25°C for 2 h in a ratio of 1:1 (by weight). The carbohydrate (hexoses and pentoses) contents in the inhibitors from both species of lupin were about 3%.

Modification of the lysine and arginine residues showed that the activities of the trypsin inhibitors were retained under the action of maleic anhydride but fell considerably under the action of cyclohexane-1,2-dione. Consequently the inhibitors from *L. luteus* and *L. polyphylla* are of the arginine type; i.e., they contain an arginine residue in the active center.

Thus, our investigations have shown that with respect to molecular mass and amino acid composition the inhibitor from *L. luteus* seeds is close to the Kunitz trypsin inhibitor from soybeans, while that from *L. polyphyllus* seeds is close to the Bowman-Birk inhibitor.

EXPERIMENTAL

The trypsin inhibitors were isolated from ripe seeds of *Lupinus luteus* of the Kastrichnik variety and from seeds of *L. polyphyllus*. The flour was extracted with 0.2 M NaCl in a ratio of 1:5 (weight/volume). The precipitate was separated off by centrifugation (6000 × g, 30 min). The supernatant liquid was acidified to pH 4.0 in order to precipitate ballast proteins. After centrifugation, the proteins were precipitated from the extract with with ammonium sulfate at 85% saturation. The inhibitor was bound with immobilized trypsin on CNBr-activated Sepharose 4B. The column with the sorbent was washed with 0.05 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl until protein was absent from the eluate. The inhibitor protein was eluted from the column with 0.2 M KCl in 0.01 M HCl (pH 2.0).

The fractions containing the inhibitor were dialyzed against water, centrifuged, and concentrated. Then the protein was passed through a column of CM-Sephadex C-25, which was first washed with the initial 0.005 M acetate buffer, pH 4.0, and then with 0.2 M acetate buffer, pH 4.0, and 0.2 M acetate buffer, pH 7.0. The inhibitor fractions were combined, dialyzed, and freeze-dried.

The activities of the inhibitors were evaluated from the degrees of suppression of the activities of enzymes. The activity of trypsin was determined by the use of N_{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPA) as described by Gofman and Vaisblai [14]. The proteolytic activities of chymotrypsin, subtilisin, and papain were determined with azocasein as substrate.

The disk electrophoresis of the native proteins was conducted by Davis's method [15]. The molecular masses of the trypsin inhibitors were determined in 15% polyacrylamide gel in Tris-glycine buffer, pH 8.3, in the presence of 0.1% Na-DS. The gel was fixed with 20% TCA and was stained with a 0.1% solution of Coomassie R-250 in ethanol–acetic acid–water (25:5:70). The excess of dye was washed out with 7% acetic acid. As marker proteins we used bovine albumin (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), myoglobin (17.8 kDa), and cytochrome C (12.3 kDa).

The isofocusing of the trypsin inhibitors was carried out in a horizontal thin layer of polyacrylamide gel on a Multiphor 5 instrument (LKB, Sweden). Standard LKB gel plates with a pH gradient of 3.5-9.5 were used.

The amino acid compositions of the proteins were determined after hydrolysis with 5.7 N HCl at 110°C for 24 h on a T 339 analyzer (Czechoslovakia). The tryptophan contents were determined after alkaline hydrolysis [16] and on a fluorimeter. The cysteine and cystine contents were detrmined in a separate sample after oxidation with performic acid [17]. Lysine residues were modified by treating the inhibitors with maleic anhydride [18], and arginine residues by treatment with cycohexane-1,2-dione [19]. Carbohydrates were determined by Dische's method [20]and protein contents by Lowry's method in Hartree's modification.

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