EXTRACTION AND IDENTIFICATION OF A SERINE PROTEASE INHIBITOR FROM FLAX SEED (*Linum usitatissimum* L.)

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**Abstract**

The trypsin inhibitor from common flax seeds (*Linum usitatissimum trypsin inhibitor, abbreviate LUTI*) is a member of the potato inhibitor I family. This was isolated and purified by ethanol fractionation, ion exchange chromatography and affinity chromatography. To determine the molecular mass of protease inhibitor we utilized gel electrophoresis in denatured conditions (SDS-PAGE). LUTI is the first serine protease-type inhibitor isolated from a plant of Linaceae family, and has a molecular mass 7000D.

**Key words**: serine protease inhibitors, plant protease inhibitors, Linaceae family, trypsin inhibitor.

**INTRODUCTION**

Protease inhibitors have a particular role in control and regulation of protease activity-enzymes participating in metabolic processes in organisms. Proteases are proteolytic enzymes that catalyse the hydrolytic cleavage of specific peptide bonds in target proteins, thus being indispensable to the maintenance and survival of their host organism. Proteases are mostly classified according to the main catalytic amino acid residue in their active site in several groups, such as: serine proteases, cysteine proteases, aspartic proteases and metalloproteases, the latter having a metallic ion in their active site (Habib H., Fazili K.M., 2007; Pouvreau L., 2004).

The serine proteases are by far the largest class, comprising about 1/3 of the total proteases, being recognized as important factors in the control of multiple pathways associated with coagulation, fibrinolysis, connective tissue turnover, homeostasis, fertilization, complement activation and inflammatory reactions.

Progress accomplished in knowledge at molecular level of intra- and extra-cell interactions have led to the validation of proteases as therapeutically targets due to their role in major diseases, evolution having different biological functions in human organism (Lorenc et al., 2001). This is the reason for the continuous development of using proteases inhibitors in medicine and biotechnology. Until recent times, the action of proteases was believed to be restricted to digestive purposes, extracellular modeling and/or remodeling of tissues, mainly through proteolytic activity on interstitial molecules, occurring throughout homeostasis and development or, in aberrant maladaptive circumstances, during disease pathogenesis.

This view has clearly become untenable as proteases are clearly involved in a myriad of homeostatic as well as pathological processes. Similarly, several novel physiological functions have been attributed to endogenous antiproteases including antimicrobial and immunomodulatory activities (Manoury et al., 2011).

Plant protease inhibitors continue to catch the attention of researchers because of their increasing use in medicine and biotechnology. They were classified into families according to Laskowski on the basis of their primary structure, topology, of disulfide bridges, and
The known plant protease inhibitors belong to at least nine families: Kunitz (STI) (Hejgaard et al., 1983), Bowman-Birk (BBI) (Ikenaka et al., 1986), squash (Wieczorek et al., 1985), cereal (Strobl et al., 1995), rape seed (Ascenzi et al., 1999), arrowhead (Xie et al., 1997), potato I (Graham et al., 1985), potato II (Graham et al., 1985), and barley (Mahoney et al., 1984).

Some of them seem to be restricted to only one botanical family, for example, inhibitors of the squash family (Cucurbitaceae). Representatives of other families, for example the potato inhibitors I, were isolated from Solanaceae (Graham et al., 1985) Gramineae (Svendsen et al., 1982), Leguminosae (Nozawa et al., 1989), Amaranthaceae (Valdes-Rodriguez et al., 1993), and Cucurbitaceae (Krishnamoorthi et al., 1990). In the range of plant species important as sources of trypsin inhibitors, the common flax (Linum usitatissimum) enjoy a special place, due to the well-known effect of the flax seeds to alleviate digestion disorders, stabilize blood sugar levels, improve health of the skin and inhibit the development of some forms of cancer (Chen et al., 2006). Some of these benefic effects are due to protease inhibitors.

The first serine proteinase type inhibitor isolated from seeds of the Linaceae family is known as a molecule consisting in 69 amino-acid residues and a molecular mass of 7655 Da, containing a single di-sulfidic link and two Cys residues (Lorenc - Kubis et al., 2001).

In this paper, we report the purification and the characterization of the protease inhibitor isolated from common flax seeds (Linum usitatissimum trypsin inhibitor), abbreviated as LUTI, a member of the Linaceae family (Rawlings et al., 2004). The inhibitor was isolated and purified by ethanol fractionation, ion exchange chromatography on CM-Sephadex C-25 and affinity chromatography (Polanowski et al., 2003; Eremia et al., 2013).

For determination molecular mass of protease inhibitor we utilized gel electrophoresis in denatured conditions (SDS-PAGE).

**MATERIALS AND METHODS**

The reagents (organic solvents, analytical reagents and mineral salts) used for research were purchased from Merck and Sigma-Aldrich.

**Preparation of trypsin inhibitor**

The powder of flax seeds was defatted by stirring with 5 volumes (w/v) of acetone for 24 h. The extraction was repeated two times. The particles of precipitate were separated by centrifugation and dried under vacuum. The dry powder was extracted at 40°C for 1h with 0.1 M acetate buffer pH 5.0, the suspension was clarified by centrifugation at 9000 g for 30 min, and the pellet was discarded. Mucilage from the supernatant was precipitated at room temperature with an equal volume of ethanol and removed by filtration. To the clear supernatant 4 volumes of cold ethanol (-20°C) were added, left for 24 h at 4°C, and then crude protein precipitate was collected by centrifugation and dried under vacuum. The resulting powder was solubilized in distilled water and clarified by filtration.

To the protein solution an equal volume of 0.1 M acetate buffer, pH 5.5, was added, and the mixture was purified on a CM-Sephadex C25 column, preequilibrated with 0.05 M acetate buffer, pH 5.5. The proteins were eluted with a linear gradient of NaCl (0.0-0.6 M) in the same buffer at a flow rate of 60 mlh-1. Fractions containing trypsin inhibitor activity were pooled, the pH adjusted to 7.5 with 2M Tris and the solution subjected to affinity chromatography using a column packed with trypsin immobilized in polyacrylamide gel and equilibrated with 0.05 M Tris-HCl buffer. The adsorbed inhibitor was eluted with 0.1 M glycine HCl buffer pH 2.8.

**Measurement of enzyme and inhibitors activities**

**Trypsin inhibition:** The ability of the various trypsin inhibitors (LUTI) to prevent trypsin hydrolysis of BAPNA is measured spectrometrically (405 nm, ε = 9.96 cm2μmol-1) at 25°C, with a Jasco UV-Vis spectrometer by time course measurement of ΔAbs. One trypsin unit hydrolyzes 1.0 μmole of N-a-benzoyl-DL-arginine p-nitroanilide (BAPNA) per minute at...
pH 7.8 and 25°C and one Trypsin Inhibitor Unit (TIU) will decrease the activity of 2 trypsin units by 50%.

**Protein assay**: Protein concentration was determined by Lowry method.

**Characterization of protease inhibitor** has been effectuated from determination molecular mass of protease inhibitor with method gel electrophoresis in denatured conditions (SDS-PAGE), according with protocol Laemmli’s (1970), used a electrophoresis miniapparatus, BIO-RAD, for vertical gels (10x20 cm).

### RESULTS AND DISCUSSIONS

**Isolation and purification protease inhibitor**
The purification procedure described resulted in the isolation and purification of the homogenous protease inhibitor from common flax seeds. The removal of the seed coats rich in mucilage at the beginning of the purification procedure improved the preparation and increased the concentration of extracted proteins. The protease inhibitor was isolated from the ethanol-precipitated proteins by CM-Sephadex C-25 chromatography followed by affinity chromatography on immobilized trypsin. The protease inhibitor was eluted as a single, symmetrical peak by ion exchange chromatography (Figure 1).

The mixture of proteins subjected to affinity purification is applied onto the column under slightly basic conditions and elution of an inhibitor is accomplished at a pH below 3.0. Employing this method we purified trypsin inhibitor from flax seeds. Also, we experimented adsorption capacity of immobilized trypsin and we determined optimal desorption yield of the protease inhibitor.

The results indicate a higher selectivity of affine support for plant protease inhibitor, giving a higher purification factor (20), a high purification yield (62.6%) and an increase in specific activity from 327.7 UI/mg proteins to 6.552 UI/mg proteins (Figure 2).

![Figure 1. Desorption of protease inhibitor on CM-Sephadex C-25 column, eluant NaCl 0-0.6 N, flow 1 ml/min](image1)

![Figure 2. Graph of desorption of protease inhibitor from immobilized trypsin on polyacrylamide gel](image2)
Characterization of protease inhibitor

To determine the purity of proteins extracted from flax seeds, biochemical analysis were made to determine the inhibitor specific activity on the raw extract and also on the products purified by ion exchange chromatography and affinity chromatography. The results are presented in Table 2.

Determination of molecular masses

The calibration kit used, made by Invitrogen, is a mixture of 10 pure proteins with known molecular masses: myosine 188 KDa, phosphorilase b 98 KDa, BSA 62 KDa, glutamic dehydrogenase 49 KDa, ADH 38KDa, carbonic anhydrase 28 KDa, myoglobin 17 KDa, lysozym 14 KDa, aprotinine 6 KDa and insulin 3 KDa (chain B).

Samples:
Sample 1 - concentration 2.22 μg/μl
Sample 2 had an initial concentration of 0.45 mg/ml and it compressed in vacuum reaching a final concentration of 8.4375 μg/μl
Sample 3 had an initial concentration of 0.0125 μg/μl in vacuum reaching a final concentration of 0.975 μg/μl
Sample 4 had an initial concentration of 0.0094 μg/μl in vacuum reaching a final concentration of 8.4375 μg/μl

On godeu we applied 10 μg/μl totally vegetal protease extract.

Following the migration of electrophoresis in PAA 15% gel of sample 1 resulted from ionic exchange chromatography, resulted 10 tapes, if the migration has been made in tampon with β-mercaptoethanol (godeul 2). If the same sample is migrated in similar conditions but loaded with SDS tampon, we can observe that some tapes disappear (godeul 6). Having electrophoresis profiles as a base, we can conclude that the protease inhibitor from sample 1 contains disulphric bridges that divide in the treatment with β-mercaptoethanol, which explains the big number of corresponding tapes to a bigger number of consistent units.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sample</th>
<th>Protein composition (mg/ml)</th>
<th>Activity Inhibitor U/ml</th>
<th>Specific U/mg x protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Protein extract (precipitated with ETOH)</td>
<td>2.22</td>
<td>433</td>
<td>194.8</td>
</tr>
<tr>
<td>2</td>
<td>Protein solution (ionic exchange chromatography)</td>
<td>0.45</td>
<td>700.3</td>
<td>1547.97</td>
</tr>
<tr>
<td>3</td>
<td>Protease inhibitor purified affine I</td>
<td>0.0125</td>
<td>32.5</td>
<td>2624</td>
</tr>
<tr>
<td>4</td>
<td>Protease inhibitor purified affine II</td>
<td>0.0094</td>
<td>35.8</td>
<td>3808.5</td>
</tr>
</tbody>
</table>

On sample 2, 3 and 4 we can observe an electrophoresis behavior similar to sample 1.

Following the migration of electrophoresis in PAA 15% gel of sample 2 obtained from affinity chromatography, pH 5.5, resulted 11 tapes, if the migration has been made in tampon with β-mercaptoethanol (godeul 3). On the sample loaded with SDS tampon we observed that some tapes disappear and a diminuation in there intensity (godeul 7).
Following the migration of electrophoresis in PAA 15% gel of sample 3 (purified I) and 4 (purified II) obtained by affinity chromatography, pH 2.8, the number of the tapes decreases signifiy, in the case of migration with β-mercaptoetanol (godeurs 4 and 5). If the migration has been made in SDS tampon the intensity of the tapes is much reduced resulting extinction (goderuls 8 and 9).

The inhibitor was isolated and purified by ethanol fractionation, ion exchange chromatography on CM-Sephadex C-25 and affinity chromatography (purification factor was 20 and purification yield 62.6%, specific activity increased from 327.7 UI/mg proteins to 6,552 UI/mg proteins).

REFERENCES


Polanowski Antoni, Anna Wilimowska-Pelec, Jolanta Kowalska, Joanna Grybe, Monika Zelazko Şi

CONCLUSIONS

We obtained for the first time in literature, a serine protein inhibitor from flax seeds (Linaceae family). It has been purified till electrophoresis homogeneousness by ethanol precipitation and chromatographic methods, which are accessible in biotechnological practice. By SDS-PAGE we determined a molecular mass of 7000 D.

We obtained a protease inhibitor from seed of common flax (Linum usitatissimum trypsin inhibitor).
INTRODUCTION

Lactic acid bacteria (LAB) constitute a large group of microorganisms that have been isolated from a variety of environments, including soil, plants, animals (milk, meat, fish, etc.) or plants (vegetables, wine, olives, sourdough etc.) (Ganzle, 1997; Doyle and Beuchat, 2007; Wood, 1997; Stitles, 1996). LAB are also well known for their health-related implications and therefore, they have attracted much attention from both the scientific community and the public. LAB are used as starter cultures in food industry due to their probiotic properties (Tadeusz Wilusz, 2003). Non-conventional affinity chromatography of serine proteinases and their inhibitors. Acta Biochimica Polonica, Vol. 50, No. 3, p. 765-773.


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