In search of plant sources for serine protease inhibitors: I. Detection of serine protease inhibitors in callus cultures induced from somatic explants of flax (*Linum usitatissimum* L.)

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ANA ROSU*, MIHAELA-CARMEN EREMIA**, MARIA SPIRIDON**, SILVANA GUIDEA*, IRINA LUPESCU***, STEFANA JURCOANE*

*Center of Microbial Biotechnology, 59, Bd. Marasti, 011464 Bucharest, Romania. E-mail: anabiotech@yahoo.com
**National Institute for Chemical Pharmaceutical Research and Development, 112 Calea Vitan, 031299, Bucharest, Romania

Abstract

A study was conducted in order to generate callus cultures in somatic explants of flax (*Linum usitatissimum* L.) for their possible exploitation as sources for production of useful serine protease inhibitors. The effect of plant growth regulators on callus induction in cotyledonary explants and apical nodes prelevated from 10 days old flax seedlings was studied in order to produce rapidly growing callus biomass. The callus growth / culture vessel was recorded before the transfers performed at 3 weeks intervals. The most efficient culture formulation for callogenesis consisted in MS basal medium supplemented by NAA, TDZ and CH. The biochemical totipotency of the callus cells was evaluated by the presence of serine-protease inhibitors in callus biomass compared to seeds, assessed by analytical determinations of enzymatic dosing toward trypsine.

Key words: auxins, cytokinins, in vitro cultures, callogenesis, trypsine, trypstase inhibitors.

Introduction

The plant kingdom has a tremendous potential in natural product synthesis, its biosynthetic repertoire including compounds which can be harnessed for commercial applications as dyes, pharmaceuticals, fragrances, insecticides and other phytochemicals of practical interest. Among these compounds, plant-derived medicines constitute a substantial component of present day human healthcare systems worldwide, because many irreplaceable medicinal products are derived from plants. Over 25% of the new drugs approved in the last 30 years are based on a molecule of plant origin, and about 50% of the top selling chemicals derive from knowledge on plant secondary metabolism (1, 2, 4,16). Less exploited up to now are some protease inhibitors, ubiquitously abundant not only in animals and microorganisms, but also in some plant species, with important uses in medicine and biotechnology.

Proteases are proteolytic enzymes that catalyste 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 (5,12).

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. These enzymes are also involved in many diseases, therefore they are often targets for therapeutic interventions (11).
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Despite their key role in many biological processes, proteases may be potentially damaging when over-expressed or present in higher concentrations. The process is carefully controlled, for not being hazardous to the organism itself, by a large number of naturally occurring protease inhibitors described in animals, microorganisms and plants (5).

Plant PIs are generally small proteins that have mainly been described as occurring in storage tissues, such as tubers and seeds, but they have also been found in the aerial parts of plants, often induced in response to injury or attack by insects or pathogens (14).

Among compounds able to selectively abrogate serine protease actions, the serine protease inhibitors are able to form less active or fully inactive complexes with their cognate enzymes and therefore they can be used either as drugs by themselves or may serve as templates for the design and development of highly specific drugs (7). Consequently much work has been done to identify new sources for these compounds and to develop effective methods for purification of both proteolytic enzymes and their inhibitors.

Among the serious diseases in which some serine proteases are involved, allergies, including asthma, have high clinical and economic impact. B – Tryptase is a serine protease involved in the pathogenesis of asthma and in other allergic and inflammatory disorders that are resistant to all known endogenous protease inhibitors (9). Therefore much attention is focused on the development of highly potent and selective tryptase inhibitors, including those of plant origin.

In the range of plant species important as sources of tryptase 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 (3). 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 (8).

The present researches aimed both to experiment the production of new sources of serine protease inhibitors, consisting in callus cell biomass induced from somatic explants of flax and to develop effective methods for purification of proteolytic enzymes and their inhibitors.

**Materials and methods**

*Explant sources:* experiments were carried out using as explants sources 10 days old plantlets obtained by germinating flax seeds purchased from local market in laboratory conditions, in a 2:1 mixture of compost and peat;

*Explant types:* cotyledon fragments and cotyledon nodes with fully expanded cotyledons;

*Surface sterilization for callus culture establishment:* 3 minutes soaked in 10% w/v commercial bleach (Domestos), followed by 3 rinses (5 minutes each) in sterile distilled water; the external sterilization, inoculations and the subsequent transfers were carried out under aseptic conditions, in a laminar flow cabinet;

*Culture conditions for callus cultures induction and establishment:* sterilized explants were placed on the surface of the culture media (Variants 1 and 2) distributed in 5 cm ø Petri plates (containing 5 mL of sterile autoclaved culture medium variants solidified with 8 g/l agar) and the incubation was performed in the growth chamber at 25± 2 °C, under a 16/8 h photoperiod, with a light intensity of 3000 lux. The periodical transfers on fresh culture media were performed at 3 week intervals.
**Culture media:** full-strength Murashige and Skoog (1962) basal medium (MS), with the addition of 3% sucrose, 0.8% agar and with different concentrations and combinations of plant growth regulators (PGRs) - Table 1; plant growth regulator supplements were added prior to the media autoclaving, which was carried out for 20 min at 121° C and the pH was adjusted to 5.8 – 6, prior to autoclaving. The evaluation of the best media formulations for callus biomass development was performed by determining the average of fresh weight increase of the cell biomass /culture vessel during the 3 week intervals between transfers on each of the media variants (Table 2).

**Table 1.** Variants of PGRs in culture medium for callus induction in somatic explants of flax (*Linum usitatissimum*) and for transfers

<table>
<thead>
<tr>
<th>Variant</th>
<th>Auxins – mg/l</th>
<th>Cytokinins – mg/l</th>
<th>CH mg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NAA</td>
<td>2,4-D</td>
<td>Kin</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>-</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Legend: PGRs = Plant growth regulators; NAA = α-naphthalene-acetic acid; 2,4-D = 2,4-dichlorophenoxyacetic acid; Kin = Kinetin; TDZ = Thidiazuron (N-phenyl-N-1,2,3-thiadiazol-5-yl urea); CH = Casein enzymatic hydrolysate

**Isolation and purification of protease inhibitor.**

The extraction of plant proteins with serine-protease inhibiting activity from flax callus biomass compared to the flax seeds was conducted according to the following protocol:

1 – defatting of the pulverized seeds and of the minced callus cell biomass by stirring for 2 hours at room temperature with 5 volumes (w/v) of acetone. The precipitate was separated by centrifugation and dried under vacuum;

2 – the dry defatted powder is submitted to an aqueous extraction with an optimal volume of 0.1 M acetate buffer, at pH 5.0, with stirring at 4° C, for 1h;

3 – the resulted suspension was clarified by centrifugation at 4000rpm for 30 min, the pellet was discarded and the supernatant rich in proteins was separated;

4 – the aqueous supernatant contains besides proteins some other compound, such as polysaccharides, which were fractionally precipitated with ethanol in 2 stages: a) treatment of the supernatant with an equal volume of ethanol for 1 h at room temperature and removing the precipitate by filtration; b) the clear supernatant is treated with 4 volumes of ethanol at low temperature (-20°C) and maintained in the refrigerator at 4° C for 24 hours;

5- the crude protein precipitate was collected by centrifugation at 4000 rpm and dried under vacuum and the resulted powder was dissolved in distilled water and clarified by filtration;

6 – in the case of flax seed extract, the protein solution containing serine protease inhibitor was brought to pH 7.5 with 2m Tris and applied to a column packed with immobilized trypsin in chitosan gel by crosslinking glutaraldehyde and equilibrated with 0.05 m Tris-HCl buffer, pH 7.5. The adsorbed inhibitor was eluted with 0.1M glycine HCl buffer pH 2.8.

**Measurement of enzyme inhibitory activity and protein assays.** The kinetic of the enzymatic reactions was followed by using a rapid method based on BAPA (N-benzoyl-DL-arginin-4-nitroaniline), the inhibitory activity being determined as the difference between the activity of a trypsin solution and the residual activity of the same solution after adding the inhibitor. The ability of various trypsin inhibitors (trypptase is serine protease like trypsin) to
prevent trypsin hydrolysis of BAPNA is measured spectrometrically (405 nm, \( \varepsilon = 9.96 \text{ cm}^2 \mu\text{mol}^{-1} \) at 25°C, with a UV-Vis spectrometer by time course measurement of \( \Delta \text{Abs} \). One trypsin unit hydrolyzes 1.0 \( \mu \text{mole of BAPNA per minute at pH 7.8 and } 25^\circ \text{C and one trypsin inhibitor unit (TIU) will decrease the activity of 2 trypsin units by 50\%.} \) Protein concentration was determined by Lowry method.

**Results and discussions**

Though the common flax ( *Linum usitatissimum* L.) enjoy a special place in the range of plant species important as sources of tryptase inhibitors and despite that it was proven that biotechnology based on cell and tissue cultures represents a reliable alternative method for producing a wide range of active compounds, the researches focused up to now only on purification and characterization of serine protease inhibitors from flax seeds (8, 11). As regards the *in vitro* approaches, the efforts focused on regeneration strategies by applications of the techniques of somatic embryogenesis, protoplast isolation and fusion, obtaining of haploids, gene transfer and expression (10). Therefore, to our knowledge, there are no data in the scientific literature regarding developing and exploiting flax cell and tissue cultures as valuable sources of serine-protease inhibitors.

It is well known that where possible, plantlets obtained from seeds germinated in aseptic conditions represent an optimal source of explants, with a pronounced callogenetic and morphogenetic capacity. Our attempts to efficiently surface sterilizing flax seeds without affecting the germination, was not successful. Consequently, our source of explants consisted of 10 days old plantlets, with fully expanded cotyledons, obtained by seed germination in laboratory conditions (Fig. 1).
propensity to develop roots (Fig. 2, Fig. 3). The transfers were performed on fresh culture media at 3 weeks intervals, and the increase of the callus biomass on every culture media variant was recorded, by weighing the callus biomass from every culture vessel after 3 weeks of culture, compared with the initial weight of the transferred callus piece. The average increase of the callus biomass/Petri plate after the 3 week/intervals between transfers on fresh transfer media are presented in Table 2.

**Figure 2.** Developing calli induced from cotyledon and cotyledonary node explants after 2 weeks since inoculation on Variant 1.

**Figure 3.** Developing calli induced from cotyledon and cotyledonary node explants after 2 weeks since inoculation on Variant 2

**Figure 4.** Actively growing callus after transfer on Variant 5
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The transfer media consisted of the 2 variants used for callus initiation (V1 and V2) and on the variants V3 and V4, improved by the addition of 200 mg/l of casein hydrolysate. This ingredient represents an organic complex that stimulates the callogenesis by contributing to the culture medium composition with amino-acids, vitamins and growth regulators.

**Table 2.** Average of callus biomass increase/culture vessel during a 3 week interval between transfers.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Average of callus biomass increase/culture vessel (g)</th>
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<tr>
<td>3</td>
<td>3.82</td>
</tr>
<tr>
<td>4</td>
<td>3.59</td>
</tr>
<tr>
<td>5</td>
<td>5.10</td>
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</table>

By transferring the *in vitro* callus cultures on Variants 3 and 4, the increase of the callus biomass on a three weeks of culture registered closed values – 3.82 g/culture vessel and respectively 3.59 g/vessel. The highest average increase of the callus biomass (5.10 g/culture vessel) was recorded on the Variant 5, in which the basal culture medium MS was supplemented with the auxin NAA, in combination with TDZ, a potent cytokinin for promoting callus formation in many species. On this variant the calli were green (Fig.4) with no rhizogenic capacity but with occasionally developed buds.

In order to achieve the extraction of the plant proteins displaying serine-protease inhibitory activity, the *Linum usitatissimum* seeds and the *in vitro* grown callus cell biomass were grounded and defatted with acetone, and the precipitate was separated by centrifugation and dried under vacuum. The dry powder was extracted with ethanol and from the protein solution containing the serine protease inhibitor a precipitate was obtained by centrifugation and drying under vacuum. The analytical determinations of the total protein content and of the trypsin inhibitory activity, as well as the extraction efficiency are presented in Table 3.

**Table 3.** Biochemical characteristics of the protein extracts from flax seeds and callus cell biomass.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Defatted powder (g)</th>
<th>Powder precipitated with ETOH (g)</th>
<th>Assays</th>
<th>Extraction efficiency* (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeds</td>
<td>55</td>
<td>1.1</td>
<td>176</td>
<td>105,652</td>
</tr>
<tr>
<td>Callus biomass</td>
<td>20</td>
<td>0.94</td>
<td>90.2</td>
<td>196,540</td>
</tr>
</tbody>
</table>

*Extraction efficiency was expressed in amount of precipitate (g) obtained from 100 g defatted powder.

The obtained results show that as regards the extraction efficiency, following the ethanol extraction from 100g of defatted powder, a 2 times higher quantity of precipitate can be obtained from callus biomass compared to seeds, having in the same time a superior serine-protease inhibiting activity.

The present preliminary studies focused both on defining the conditions for obtaining dependable *in vitro* flax cell biomass as well as on isolation and purification of plant proteins displaying specific inhibitory activities.

Based on the obtained results we may conclude that after the *in vitro* induction and establishment of the callus cultures on the variants 1 and 2 of the culture media, the variant 5, with a hormonal supplement consisting in 1 mg/l NAA, 0.5 mg/l TDZ and 200 mg/l CH proved to be more suitable for achieving a reliable callus biomass increase (5.10 g/Petri plate), during a relative short period of time (3 weeks). Therefore, our experimental data
reveal the potential of somatic explants of *Linum usitatissimum* to develop in vitro long-term, continuous callus cultures, under the effect of optimal hormone concentrations.

The studies regarding the presence of serine protease inhibitors in callus biomass compared to seeds revealed a specific activity expressed as UI/mg protein of 600 in seed extracts and of 2184 in callus biomass, following the purification of the biologic material by precipitation with ethanol. The higher quantity of the protein precipitate /100 g defatted material and with a better specific activity in the case of cell culture biomass compared to the seeds represents an important argument that flax cell and tissue cultures may represent a reliable alternative method for producing active compounds with important uses in medicine.

**Acknowledgments**

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**References**