DOES THE OXIDATION OF METHIONINE RESIDUE PRECEDE THE INACTIVATION OF THE TRYPSIN INHIBITOR (LUTI) IN GERMINATING SEEDS OF COMMON FLAX (*LINUM USITATISSIMUM*)?

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ABSTRACT

Antitrypsin activity in germinating common seeds of flax (*Linum usitatissimum*) was investigated. At the early stage of germination an increase in antitrypsin activity was observed, followed by its decrease during the development of the seedlings. From 6-day-old seedlings a trypsin inhibitor (gerLUTI) was purified. The purification procedure involved fractionation of proteins from seedling homogenate with alcohol and successive chromatography on CM-Sephadex C-25 on immobilised methylchymotrypsin in the presence of 5 M NaCl, and finally on a C18 column in RP-HPLC.

The gerLUTI migrated in SDS PAGE as a single band, but in mass spectroscopy analysis it exhibited the presence of at least three forms with molecular masses of 7654 ± 3 Da, 7668/7670 ± 3 Da, and 7687 ± 3 Da. The preparation of LUTI isolated from resting seeds contained only one form, with a molecular mass of 7655 ± 3 Da. LUTI and gerLUTI differed also in methionine contents. LUTI contained two methionine residues, whereas in gerLUTI only a trace of methionine was detected. The obtained results might suggest that during flax seeds germination the inhibitor molecules undergo selective modification, e.g. oxidation at methionine residues, before being degraded by proteolytic enzymes.

KEY WORDS: *Linum usitatissimum* seedlings, trypsin inhibitor, protein, germination, immobilised methylchymotrypsin, mass spectra.

INTRODUCTION

Plants and seeds contain considerable amounts of proteins and peptides inhibiting serine proteinase activity. The role of these inhibitors in the physiology of the plant is not fully understood. They may be involved in the regulation of endogenous proteinases during seed dormancy, in the mechanism of reserve protein mobilisation (McGrain et al. 1992), or they may protect plants against herbivorous pests and pathogens (Wolfson and Murdock 1995).

Most of serine protease inhibitors of plant origin are small proteins of molecular weight rarely exceeding 20 kDa. They were isolated from the seeds of Leguminosea, Gramineae, Solanaceae (Richardson 1991), Cruciferaea (Volpicelleae 2000), *Cucurbitaceae* (Wieczorek et al. 1985), Alismaceae (Luo et al. 1997), Amaranthaceae (Hejgaard et al. 1994), Compositae (Luckett et al. 1999) and Linaceae (Lorenc-Kubis et al. 2001).

In a previous paper (Lorenc-Kubis et al. 2001) we reported on the purification of a trypsin inhibitor (LUTI) from the resting seeds of common flax (*Linum usitatissimum*). The inhibitor consists of a single polypeptide chain of 69 residues with one disulfide bridge; its NMR solution structure has been determined (Cierpicki and Otlewski 2000). LUTI shows a high level of sequence similarity to the potato I inhibitor family, and it is the first serine proteinase inhibitor isolated from Linaceae.

Seed germination and growth of young seedlings are characterised by the breakdown of seed reserve proteins. The breakdown is accompanied both by an enhancement of proteolytic activity, and by a decrease in the activity of protease inhibitors (Chrispeels and Baumgartner 1978), but still we do not know the details of these processes.

The following is an account of our study of the changes in antitrypsin activity in germinating flax seeds, and of the purification and partial characterisation of the trypsin inhibitor (gerLUTI – ***L****inum* ***u****sitatissimum* **t**rypsin inhibitor from germinating seeds) from the seeds germinated for 6 days.

MATERIALS

The seeds of *Linum usitatissimum* were supplied by Plant Seeds Corporation PPHU „ROLMAK" from Staszów, Poland. Bovine trypsin (EC 3.4.21.4) was prepared according to Wilimowska-Pelc and Mejbaum-Katzenellenbogen (1978). Bovine a-chymotrypsin (EC 3.4.21.1), *N*-α-benzoyl-DL-Arg *p*-nitroanilide (BAPNA), Coomassie Brilliant Blue R-250, bovine serum albumin (BSA), divinyl sulfone, molecular weight markers for SDS-PAGE were from Sigma (St. Louis, MO, USA). Methylchymotrypsin was obtained by the method of Nagawa and Bender (1970). Sepharose 4B and CM-Sephadex C-25 were from Pharmacia LKB Biotechnology (Uppsala, Sweden); acetonitrile (HPLC grade), trifluoroacetic acid (TFA), reagents for PAGE and edestin were from ICN Biomedicals (Costa Mesa, CA, USA); YM 3 membrane filters were purchased from Amicon (Danvers, MA, USA). All other reagents were of analytical grade. A reverse phase Delta PAK C_{18} column was from Waters Division of Millipore (USA).

METHODS

Germination of the seeds

The study was carried out on seeds and seedlings of common flax. The seeds were rinsed with water for 24 hours, sown in a moist germination bed of filter paper and allowed to germinate at 24°C for 10 days with 10-12 hours of daylight. The seedlings were harvested after 1, 2, 3, 4, 5, 6, 8, 11 and 13 days. No fungal contamination was observed on the germinating seeds

Extraction of proteins

Ground seeds (4 g) were homogenised with 10 volumes and seedlings with 5 volumes (w/v) of 0.1 M sodium acetate buffer, pH 5.1. The extraction was performed at 4°C for 1 hour with constant mechanical stirring. After centrifugation (for 20 min at 12 000 × g) the pellet was discarded and the supernatant was used for the analysis of inhibitory activity.

ANALYTICAL METHODS

Measurement of enzyme and inhibitor activities

Enzyme activities were measured spectrophotometrically at 410 nm after 10 min. incubation at 25°C in 0.05 M Tris-HCl buffer, pH 8.0, with 20 mM $CaCl_2$, using BAPNA as a substrate for trypsin (Erlanger et al. 1961). Enzymatic reactions were terminated by the addition of 0.5 ml of 30% acetic acid. One unit of inhibitory activity was defined as the amount of protein required to reduce the activity of 2 µg of an enzyme to 50% of the original value.

Protein determination

Protein was estimated by either microbiuret method of Goa (1953) using bovine albumin as a standard, or by spectrophotometric measurement of the absorbance at 215 (A_{215}) and 225 (A_{225}) nm, and calculation of the concentration *c* by using the following equation (Wolf 1983):

$$c\ [\mu g\ mL^{-1}] = 144\ (A_{215}-A_{225})$$

Immobilisation of methylchymotrypsin

Methylchymotrypsin (1 g) was immobilised on divinylsulfone-activated Sepharose 4B (70 ml) according to Pepper (1992).

Polyacrylamide gel electrophoresis (PAGE)

Samples of protein were subjected to electrophoresis on 16.5% gels in the presence of SDS according to Schägger and von Jagow (1987).

Amino acid analysis

Samples containing 1-5 µg of protein were hydrolysed by vapour-phase HCl hydrolysis at 112°C for 20 hours in a protein hydrolyser (Knauer, Germany). The hydrolysis was followed by manual derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Cohen and Michaud 1993). The AQC-derivatives of amino acids were separated by HPLC on an AccQ. Tag column (160 × 3.9 mm i.d.; Waters, USA).

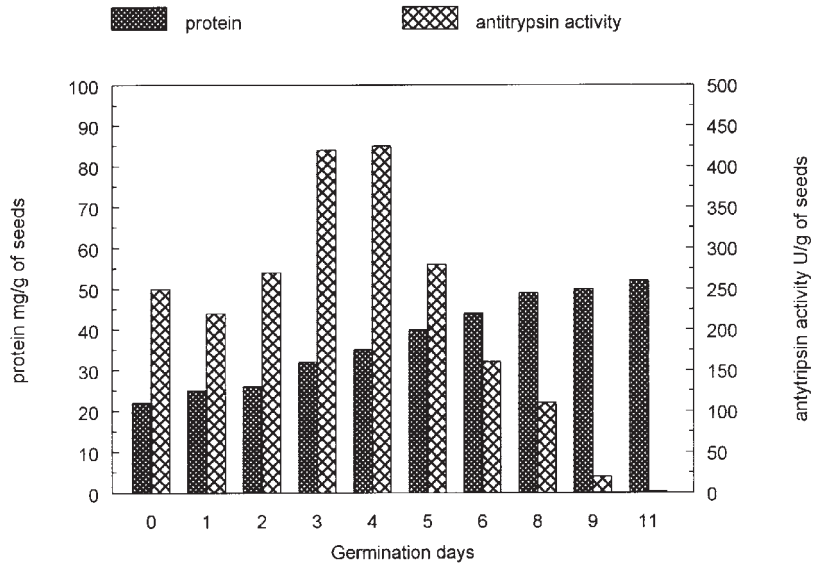

Fig. 1. Changes in protein content and antitrypsin activity in seedlings at different development stages. Data are the mean of four replications. Protein content and inhibitory activity were calculated per 75 seedlings, which corresponds to 1 g of dry resting seeds.

Mass spectrometry

Mass spectra were recorded on a Finnagan MAT TSQ 700 triple-stage quadrupole mass spectrometer equipped with an electrospray ion source (ESI-MS). Samples were dissolved in methanol/water/acetic acid (50:45:5, v/v/v) and introduced into the electrospray needle by mechanical infusion through a microsyringe at a flow rate of 2 µl min^{-1}. A potential difference of 4.5 kV was applied between the electrospray needles. Nitrogen gas (99.9% pure) was used to evaporate the solvent from the charged droplets. At least twenty scans were averaged to obtain each spectrum. Transformations of the resulting spectra were performed with the *BioWorks* software package (Finnigan MAT).

RESULTS AND DISCUSSION

Antitrypsin activity of the developing seedlings of common flax

Proteins from resting and imbibed seeds and developing seedlings (1-10 days old) were extracted with 0.1 M acetate buffer, pH 5.1, and antitrypsin activity was monitored during seeds germination and seedlings development (Fig. 1). During the first two days of germination an increase in antitrypsin activity was observed. It was probably due to the more efficient extraction of the inhibitor after the imbibition of the seeds On the 6th day of germination the amount of the inhibitor markedly decreased and, finally, on the 13th day no traces of the activity were detected. Until the 8th day of germination the amount of protein soluble at pH 5.1 gradually increased.

Isolation of the inhibitor from the seedlings

From 320 g of 6-day-old seedlings we isolated the trypsin inhibitor following the previously published procedure (Lorenc-Kubis et al. 2001). The seedlings were homogenised with 5 volumes (w/v) of 0.1 M sodium acetate buffer, pH 5.1, stirred for 1 hour at 4°C, then clarified by centrifugation. Proteins were precipitated with 80% ethanol and collected by centrifugation, solubilized in distilled water, dialysed against 0.1 M acetate buffer, pH 5.5, and applied to a CM-Sephadex C-25 column. The inhibitor from seedlings, contrary to the preparation obtained from resting seeds (Lorenc-Kubis et al. 2001), was not very well separated from inactive proteins (Fig. 2). Active fractions were pooled, adjusted to pH 7.5 with 2 M Tris, and NaCl was added to the final concentration of 5 M. The solution was applied to a column with immobilised methylchymotrypsin, equilibrated with 0.05 M Tris-HCl buffer, pH 7.5, containing 5 M NaCl. In the presence of high NaCl concentration all antitrypsin activity was adsorbed onto the affinity column and, alike the inhibitor from the resting flaxseeds, it was eluted in two peaks: first with water, and second with 0.01 M HCl (Fig. 3).

In the case of the inhibitor of the resting seeds (LUTI) (Lorenc-Kubis et al. 2001), both inhibitory peaks I and II turned out to be identical proteins, that is why we focused our further study on seedlings inhibitor I (gerLUTI I). The inhibitor preparation was desalted on a YM 3 membrane filter and chromatographed by reverse-phase HPLC. On a C_{18} column gerLUTI I separated into 3 peaks, designated as gerLUTI Ia, gerLUTI Ib and gerLUTI Ic (Fig. 4). The first two fractions were not well separated from each other

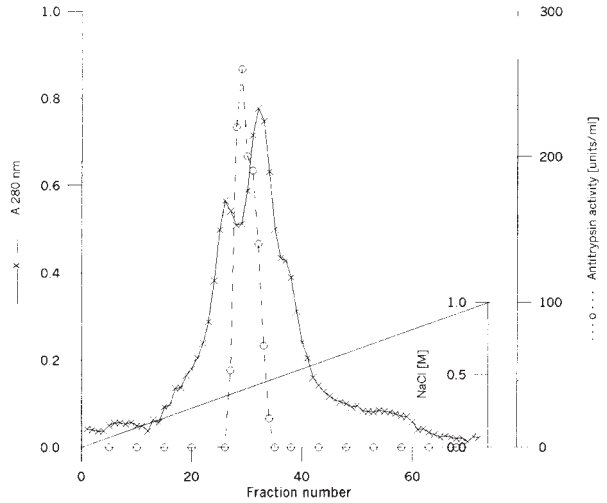

Fig. 2. CM-Sephadex C 25 chromatography of ethanol-precipitated protein from 5-day-old seedlings. Protein precipitated with 80% ethanol and resolved in 0.1 M acetate buffer, pH 5.5, was loaded on a column (22 × 170 mm), equilibrated with 0.1 M acetate buffer, pH 5.5. Elution was developed with NaCl gradient (0.0 to 1.0 M). Fractions (5 ml) were collected and analysed for protein and antitryptic activity. Fractions from 28 to 34 were pooled and chromatographed on immobilised metylchymotrypsin.

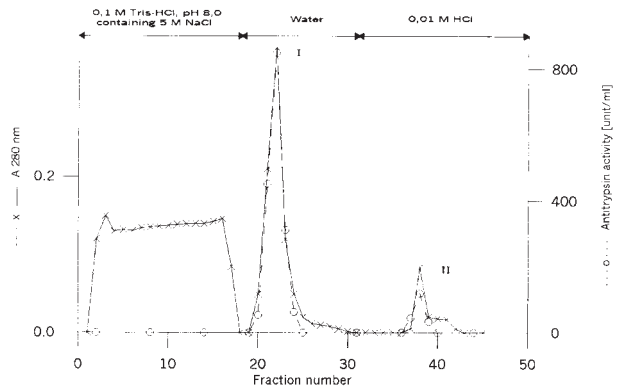

Fig. 3. Affinity chromatography of trypsin inhibitor from flax seedlings on immobilised methylchymotrypsin. A sample of 30 ml (500 mg of protein) containing 5 M NaCl in 0.1 M Tris-HCl buffer, pH 8.0, was loaded on a methylchymotrypsin-Sepharose 4B column (8 × 120 mm) equilibrated with a starting buffer containing 5 M NaCl. The column was intensively equilibrated with the buffer containing 5 M NaCl until the A_{280} dropped below 0.02. Finally, the inhibitor was eluted first with water (peak I), followed by 0.01 M HCl (peak II). Fractions of 4 ml were collected at a flow rate of 140 ml/h^{-1}.

therefore they were rechromatographed under the same conditions (not shown).

Characterisation of the inhibitor

The amino acid compositions of gerLUTI Ia, Ib and Ic were compared with the resting seed inhibitor (LUTI). The only difference concerned the methionine residues. LUTI contained 2 methionine residues (positions 20 and 43 in Fig. 7), whereas the inhibitor preparations from germinating seeds were essentially deprived of methionine content (Table 1).

The U.V. absorption spectra of gerLUTI Ia, Ib, and Ic were very similar to each other with the maximum at 275 nm (not shown).

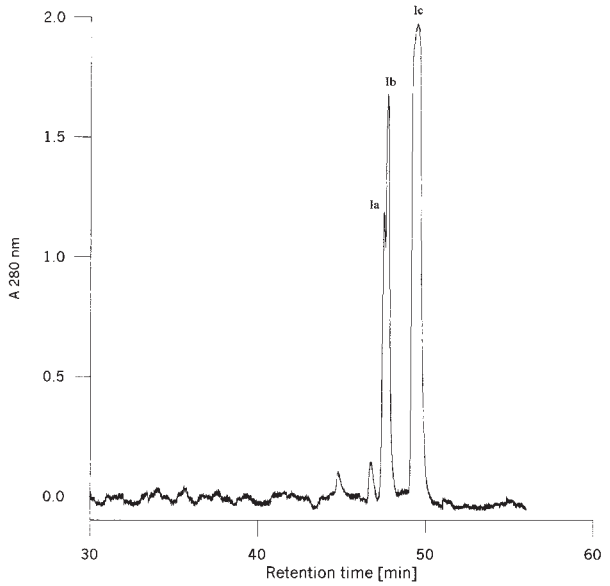

Fig. 4. RP-HPLC of the inhibitor (80 µg from peak I after affinity chromatography) on a Nucleosil 100 C_{18} column (10 µm; 250 × 8.0 mm i.d.). The proteins were eluted with acetonitrile (linear gradient in 0.1% TFA).

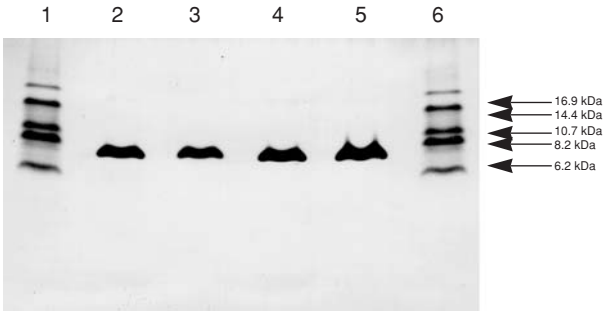

Fig. 5. SDS-PAGE of the trypsin inhibitors from resting (LUTI) and germinating flax seeds (gerLUTI I-peak I after affinity chromatography). Lanes 1 and 6: molecular weight markers; lane 2: LUTI (15 µg); lane 3: gerLUTI Ia (10 µg); lane 4: gerLUTI Ib (10 µg); and line 5: gerLUTI Ic (10 µg).

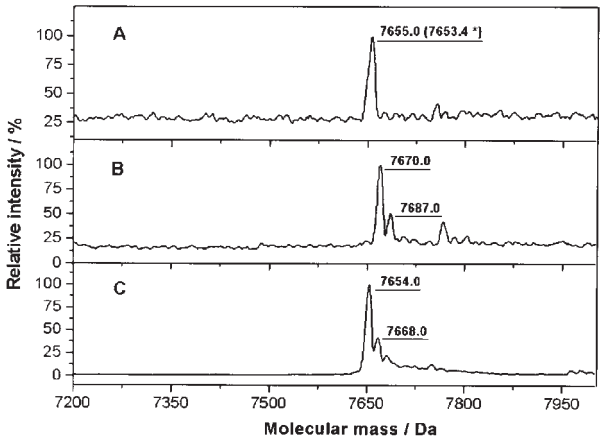

Fig. 6. Mass spectra (electrospray ionisation) of HPLC purified: (A) LUTI from resting seeds; (B) gerLUTI I b; (C) gerLUTI Ic. *Molecular weight calculated according to amino acid sequence.

During electrophoresis in the presence of sodium dodecyl sulfate under reducing conditions all three preparations: gerLUTI Ia, Ib and Ic, migrated as a single band and had a mobility similar to LUTI from the resting seeds (Fig. 5); calculated molecular masses were about 7600 Da.

To better characterise preparations of the two main peaks: gerLUTI Ib and Ic, mass spectrometric analysis was performed. The mass spectra of gerLUTI Ib and Ic showed the presence of several closely related proteins slightly differing in molecular masses. The gerLUTI Ic preparation, besides the protein with a molecular mass of 7654 ± 3 Da, which might have corresponded to LUTI from the resting seeds (7653.4 Da calculated from amino acid sequence analisis) Lorenc-Kubis I. et al. 2001), contained a distinctly „heavier" peak with a molecular weight of 7668 ± 3 Da (plus 14.6 ± 3 Da). The gerLUTI Ib did not contain an inhibitor with a molecular mass corresponding to LUTI from the resting seeds at all, instead, in the preparation dominated a protein with a molecular mass of 7670 ± 3 Da (plus

TABLE 1. Amino acid composition of LUTI from resting seeds and gerLUTI Ia, Ib and Ic from germinating seed. In the parentheses the nearest integer values are given.

Amino acid	LUTI I (Lorenc-Kubis et al. 2001)		gerLUTI					
			Ia		Ib		Ic	
Asp	7.77	(8)	7.7	(8)	7.9	(8)	8.1	(8)
Ser	3.82	(4)	4.3	(4)	4.1	(4)	3.7	(4)
Glu	4.48	(4)	4.6	(4)	4.2	(4)	4.0	(4)
Gly	5.76	(5)	6.0	(6)	5.5	(5)	5.2	(5)
His	2.44	(3)	2.6	(3)	2.6	(3)	2.7	(3)
Arg	5.90	(6)	6.9	(7)	7.1	(7)	7.5	(7)
Thr	4.25	(4)	4.0	(4)	4.2	(4)	4.0	(4)
Ala	6.33	(6)	6.0	(6)	6.3	(6)	6.0	(6)
Pro	3.58	(3)	3.2	(3)	3.2	(3)	3.1	(3)
Tyr	0.00	(0)	0.0	(0)	0.0	(0)	0.0	(0)
Val	7.51	(10)	7.3	(7)	7.6	(8)	8.2	(8)
Met	1.47	(2)	0.2	(0)	0.5	(0)	0.5	(0)
Lys	3.84	(4)	3.8	(4)	3.9	(4)	4.0	(4)
Ile	2.60	(3)	2.2	(2)	2.3	(2)	2.1	(2)
Leu	2.23	(2)	2.3	(2)	2.3	(2)	2.1	(2)
Phe	1.09	(1)	1.0	(1)	1.0	(1)	1.0	(1)
Cys	2.15	(2)	nd	nd	nd	nd	nd	nd
Trp	2.13	(2)	nd	nd	nd	nd	nd	nd

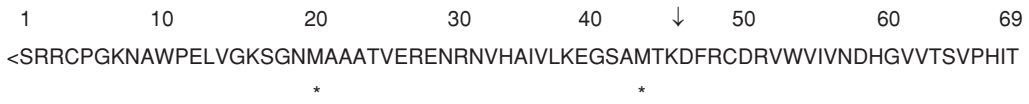

Fig. 7. Amino acid sequence of LUTI (Lorenc-Kubis et al. 2001). The P_1 residue of the reactive site and methionine residues as a possible (potential) place of oxidation are indicated by an arrowhead and stars, respectively.

16.8 ± 3 Da), and a few minor peaks, e.g. with molecular masses of 7687 (plus 33.6 ± 3 Da) (Fig. 6).

The 14.6 to 33.6 ± 3 Da increase in the molecular masses of LUTI indicate that during flax seeds germination some selective modification of inhibitor molecules took place, e.g. oxidation of one or two methionine residues. This observation correlated with the decrease in the methionine contents of the gerLUTI Ib and Ic (Table 1). The oxidation of the inhibitor molecule during germination might precede its proteolytic breakdown and might be a crucial process in the proteolytic degradation pathway at least for some storage proteins.

In the preparation of gerLUTI Ib (Fig. 6) one could observe several additional small peaks with gradually increasing molecular masses, as compared to LUTI from the resting seeds This might indicate that the catabolism of the inhibitor occurs in a more complicated way, finally yielding the modified peptide products with a definite, by not yet known, function, connected, for example, with the regulation of the germination process.

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CZY UTLENIENIE RESZT METIONINY POPRZEDZA INAKTYWACJĘ INHIBITORA TRYPSYNY (LUTI) W KIEŁKUJĄCYCH NASIONACH LNU ZWYCZAJNEGO (*LINUM USITATISSIMUM*)?

STRESZCZENIE

Badano aktywność antytrypsynową w kiełkujących nasionach lnu (*Linum usitatissimum*). W początkowym stadium kiełkowania obserwowano wzrost, a w dalszym etapie rozwoju siewek, spadek aktywności antytrypsynowej. Z sześciodniowych siewek wydzielono inhibitor trypsyny (gerLUTI). W procesie oczyszczania zastosowano frakcjonowanie białek homogenatu alkoholem oraz chromatografię na CM-Sephadex C-25, immobilizowanej metylochymotrypsynie w obecności 5 M NaCl i w końcowym etapie na kolumnie C_{18} w RP-HPLC.

GerLUTI w SDS-PAGE wędruje w postaci pojedynczego pasma, ale w spektrometrii masowej wykazuje obecność co najmniej trzech form o masie cząsteczkowej 7654 ± 3 Da, 7668/7670 ± 3 Da, oraz 7687 ± 3 Da. Preparat LUTI uzyskany z nasion spoczynkowych posiada tylko jedną formę o masie cząsteczkowej 7655 ± 3 Da. Preparaty LUTI i gerLUTI różnią się również zawartością metioniny. LUTI posiada 2 reszty metioniny, podczas gdy w gerLUTI wykazano zaledwie ślad metioniny. Uzyskane wyniki sugerują, że w procesie kiełkowania nasion lnu cząsteczka inhibitora ulega selektywnej modyfikacji np. poprzez utlenienie reszt metioniny, a proces ten poprzedza proteolityczną degradację tego białka.

SŁOWA KLUCZOWE: Siewki lnu zwyczajnego (*Linum usitatissimum*), inhibitory trypsyny, białka, kiełkowanie, immobilizowana metylowana chymotrypsyna, spektrometria masowa.