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Trypsin inhibitors from the garden four o'clock (*Mirabilis jalapa*) and spinach (*Spinacia oleracea*) seeds: Isolation, characterization and chemical synthesis

Jolanta Kowalska [a], Katarzyna Pszczoła [a], Anna Wilimowska-Pelc [a], Irena Lorenc-Kubis [a], Ewa Zuziak [a], Mateusz Ługowski [a], Anna Łęgowska [b], Anna Kwiatkowska [b], Małgorzata Śleszyńska [b], Adam Lesner [b], Aleksandra Walewska [b], Ewa Zabłotna [b], Krzysztof Rolka [b], Tadeusz Wilusz [a,*]

[a] *Faculty of Biotechnology, University of Wrocław, Tamka 2, 50-137 Wrocław, Poland*
[b] *Faculty of Chemistry, University of Gdańsk, Sobieskiego 18, 80-952 Gdańsk, Poland*

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Abstract

Five serine proteinase inhibitors (*Mirabilis jalapa* trypsin inhibitors, MJTI I and II and *Spinacia oleracea* trypsin inhibitors, SOTI I, II, and III) from the garden four-o'clock (*M. jalapa*) and spinach (*S. oleracea*) seeds were isolated. The purification procedures included affinity chromatography on immobilized methylchymotrypsin in the presence of 5 M NaCl, ion exchange chromatography and/or preparative electrophoresis, and finally RP–HPLC on a C-18 column. The inhibitors, crosslinked by three disulfide bridges, are built of 35 to 37 amino-acid residues. Their primary structures differ from those of known trypsin inhibitors, but showed significant similarity to the antimicrobial peptides isolated from the seeds of *M. jalapa* (MJ-AMP1, MJ-AMP2), *Mesembryanthemum crystallinum* (AMP1), and *Phytolacca americana* (AMP-2 and PAFP-S) and from the hemolymph of *Acrocinus longimanus* (Alo-1, 2 and 3). The association equilibrium constants (K_a) with bovine β-trypsin for the inhibitors from *M. jalapa* (MJTI I and II) and *S. oleracea* (SOTI I–III) were found to be about 10^7 M^{-1}. Fully active MJTI I and SOTI I were obtained by solid-phase peptide synthesis. The disulfide bridge pattern in both inhibitors (Cys1–Cys4, Cys2–Cys5 and Cys3–Cys6) was established after their digestion with thermolysin and proteinase K followed by the MALDI-TOF analysis.

Keywords: *Mirabilis jalapa*; *Spinacia oleracea*; Purification; Chemical synthesis; Primary structure; Disulfide bonds topology; *Mirabilis* serine proteinase inhibitor family; Trypsin inhibitors

1. Introduction

Proteolytic enzymes are involved in numerous biochemical and physiological processes. They are responsible not only for cellular protein digestion, but also for intracellular protein turnover associated with defense mechanisms, elimination of misfolded proteins, signal propagation, the activation of proenzyme, regulatory proteins, and receptors, the release of hormone and biologically active peptide, assembling processes, cellular differentiation and ageing, seed development, mobilization of storage protein during seed germination or seedling growth, pathogen suppression, and pest proteinases (Ehrmann and Clausen, 2004; Antão and Malcata, 2005). All of these processes are under precise control, amongst which naturally occurring proteinase inhibitors play a very important function.

Plant seeds and storage organs have proven to be a very rich source of protein proteinase inhibitors, particularly the inhibitors of cysteine and serine proteinases. The last ones,

* Corresponding author. Tel.: +48 71 3752640; fax: +48 71 3752608.
E-mail address: Tadeusz.Wilusz@biotech.uni.wroc.pl (T. Wilusz).

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on the basis of amino-acid sequences, disulfide bridge topology, localization of the reactive sites, and mechanism of action, were grouped into at least seven well-characterized families: Kunitz, Bowman–Birk, Potato I, Potato II, Squash, Cereal, and Mustard (Laskowski and Kato, 1980; Birk, 2003; De Leo et al., 2002). Some of these inhibitors, especially the very small ones, such the inhibitors belonging to the squash family, built of barely 30 amino-acid residues (Kowalska et al., 2006), are of particular interest as therapeutics because they inhibit trypsin, plasmin, kallikrein, cathepsin G, and the blood clotting factors X_a and XII_a (Otlewski and Krowarsch, 1996). In addition, such small proteins are very attractive models for the design of molecules with simplified structure (Rolka et al., 1992) with selective activity against chymotrypsin, human leukocyte elastase (Rolka et al., 1991), or even against carboxypeptidase A (Le-Nguyen et al., 1989).

In this paper, we report the purification and characterization of five novel trypsin inhibitors: two from *Mirabilis jalapa* (MJTI I and II) and three other from *Spinacia oleracea* (SOTI I–III) seeds. For all of them besides MJTI II, their complete amino-acid sequences, and for MJTI I and SOTI I the disulfide bond topology were established. The isolated inhibitors showed no homology to any other inhibitor; instead they are homologous to a class of antimicrobial peptides from *M. jalapa* seeds (Cammue et al., 1992). Two of the inhibitors (MJTI I and SOTI I) were obtained by chemical synthesis.

2. Results and discussion

2.1. Purification of MJTIs

Two trypsin inhibitors (MJTI I and II) were isolated from the acidic extract (pH 4.0) of garden four-o'clock seed meal by precipitation with cold ethanol (96%) and affinity chromatography (in batch procedure) on immobilized methylchymotrypsin in the presence of 5 M NaCl. The adsorbed inhibitors were eluted with water. In this step of purification we used catalytically inactive enzyme, with His57 converted to 3-methylhistidine, although still capable of forming complexes with inhibitors (Ryan and Feeney, 1975). Employing affinity chromatography on this support, we isolated not only chymotrypsin-specific inhibitors (Wojtaszek et al., 2006) but also trypsin inhibitors when the procedure was carried out in a presence of 5 M NaCl (Polanowski et al., 2003). This procedure provides facilities for isolating the inhibitors in their virgin forms (reactive site peptide bond unsplit).

The next step of the purification procedure was preparative native-PAGE at pH 9.4 (Fig. 1). The inhibitory fractions with similar electrophoretic mobility were pooled, lyophilized, and then purified on a Nucleosil-100 C-18 column (Fig. 2). After rechromatography (not shown), two inhibitors (MJTI I and II) were obtained. From 1 kg of garden four-o'clock seeds, about 20 mg of both inhibitors were obtained.

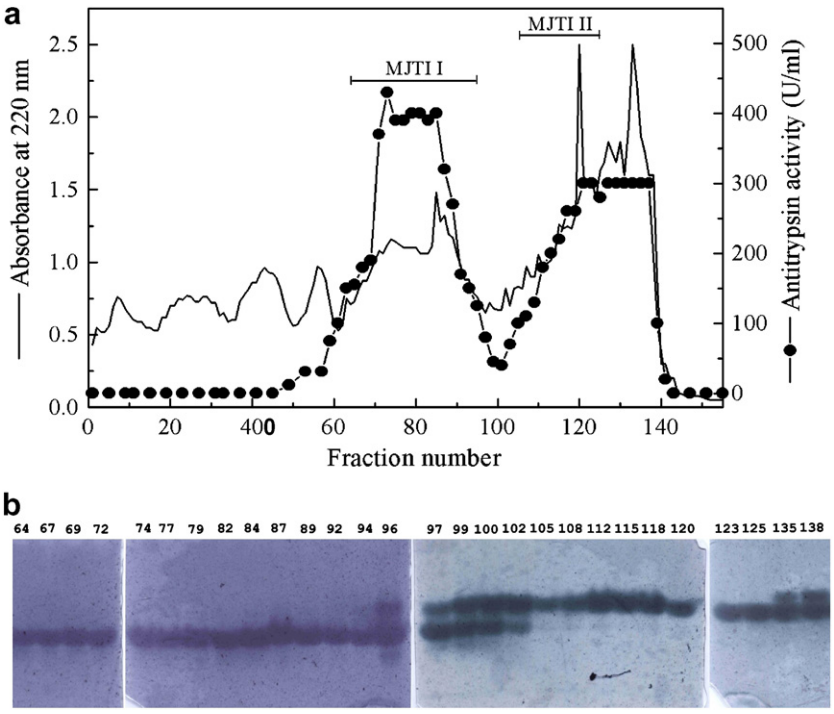

Fig. 1. Preparative electrophoresis at pH 9.4 under native conditions (a) and electrophoretic mobility of the obtained fractions (b). (a) Proteins (127.2 mg) were applied on 7.5% polyacryloamide gel. Electrophoresis was performed in a discontinuous system at a constant voltage of 450 V at 4 °C. Ten-ml fractions at a flow rate of 1 ml/min of water were collected. Fractions marked by "I–I" were pooled. (b) Inhibitors were separated in 7.5% gel copolymerized with 0.1% edestin.