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Protein Trypsin Inhibitor from Potato Tubers

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Abstract—A protein of 22 kDa designated as PKTI-22 was isolated from potato tubers (*Solanum tuberosum* L., cv. Istrinskii) and purified to homogeneity using CM-Sepharose CL-6B ion-exchange chromatography. The protein efficiently suppressed the activity of trypsin, affected chymotrypsin less, and did not affect subtilisin Carlsberg. The N-terminal sequence of PKTI-22 (20 amino acid residues) was found to be highly homologous with the amino acid sequences of the potato Kunitz-type proteinase inhibitors of group B (PKPI-B) that were aligned from the corresponding gene sequences and was identical to the sequence (from the 2nd to the 20th residue) of the recombinant protein PKPI-B10. These data together with the observed similarity of the properties of two proteins indicate that the PKTI-22 protein is encoded by the *PKPI-B10* gene.

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cDNA and genes encoding protein Kunitz-type proteinase inhibitors designated as PKPI (potato Kunitz-type proteinase inhibitors) have been found and identified in different cultivars of potato (*Solanum tuberosum* L.) of Japanese, European, and Russian selection [1-7]. It is suggested that some of them are expressed during the growth and development of the plants and accumulate in the tubers, while other are expressed in response to attack by phytopathogenic microorganisms and injurious insects [1, 8]. However, the mechanism of their expression is little explored. Analysis of amino acid sequences obtained based of the nucleotide sequences of the encoding genes revealed conservative motifs within the N- and C-terminal regions. Based on these differences, PKPI proteins have been divided into three structural groups: PKPI-A, PKPI-B, and PKPI-C [1, 5]. The proteins from these groups exhibited different specificity towards proteinases [1]. Thus, PKPI-A proteins suppress the activity of trypsin and aspartic proteinases [4], PKPI-B affect only serine proteinases of the chymotrypsin-like clan (trypsin, chymotrypsin, and elastase) [9, 10], and PKPI-C proteins affect cysteine proteinases and subtilisin [5, 11].

Abbreviations: BAPA, N-benzoyl-L-arginine *p*-nitroanilide; HLE, human leukocyte elastase; N-Suc-GGF-pNa, N-succinyl-L-glycyl-L-glycyl-L-phenylalanine *p*-nitroanilide; Pth, phenylthiohydantoin; Z-AAL-pNa, N-carbobenzoxy-L-alanyl-L-alanyl-L-leucine *p*-nitroanilide.

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In our previous works [6-8], we demonstrated that the genome of the Istrinskii cv. potato contains multiple genes encoding proteins of all three groups. In particular, four genes encoding proteins of group B were identified: *PKPI-B1*, *PKPI-B2*, *PKPI-B9*, and *PKPI-B10* [6, 7]. Analysis of the amino acid sequence obtained from the nucleotide sequence of the gene *PKPI-B9* showed that it encodes the protein PSPI-21-6.3 isolated previously from potato tubers of the same cultivar and acting as a highly-efficient inhibitor of human leukocyte elastase (HLE), trypsin, and chymotrypsin [10]. The recombinant protein PKPI-B10 was obtained by heterologous expression of the gene *PKPI-B10* (AF536175) in *E. coli* cells [12]. This protein efficiently suppressed the activity of trypsin, much less affected α-chymotrypsin, and did not act on HLE, subtilisin Carlsberg, proteinase K, and papain [12]. The recombinant protein appeared to suppress the growth and development of the fungus *Fusarium culmorum*, a causative agent of one of the most dangerous potato diseases, so-called Fusarium blight [13]. Besides, it was shown that proteinase inhibitors including the protein PSPI-21-6.3 encoded by the gene *PKPI-B9* and a trypsin inhibitor of unknown structure accumulated in potato tubers infected by the oomycete *Phytophthora infestans* (Mont.) de Bary, as well as in tubers subjected to mechanical damage [8, 14]. These data suggest that the protein inhibitors of the PKPI-B group play a significant role in the defensive response of potato plants when attacked by pathogenic microorgan-

isms, this likely being due to the variability of the structure of these proteins [7].

The goal of this work was to isolate the protein trypsin inhibitor from potato tubers of Istrinskii cv., to investigate its properties, and to determine its N-terminal amino acid sequence. Comparison of the structure and properties of the protein with those of the recombinant protein PKPI-B10 will elucidate the expression mechanisms of proteinase inhibitor genes in potatoes.

MATERIALS AND METHODS

The inhibitor protein was isolated from potato tubers of the breed Istrinskii that were kept for 2-4 months in the dark at 4°C. The following enzymes were used in this work: trypsin (EC 3.4.21.4; Spofa, Czech Republic) recrystallized from magnesium sulfate, α-chymotrypsin (EC 3.4.21.1; Serva, Germany), subtilisin Carlsberg (EC 3.4.21.14; Sigma, USA), and papain (EC 3.4.22.1; Calbiochem, USA). The substrates were casein (NPO Biolar, Latvia) and *p*-nitroanilides of N-succinyl-L-glycyl-L-glycyl-L-phenylalanine (N-Suc-GGF-pNa), N-benzoyl-L-arginine (BAPA), and N-carbobenzoxy-L-alanyl-L-alanyl-L-leucine (Z-AAL-pNa) (Calbiochem). Sephadex G-75, CM-Sepharose CL-6B, and low molecular mass protein standards for SDS-PAGE were from Pharmacia (Sweden).

The potato sap was acidified with 5.7 M HCl to achieve pH of 4.5-5.0 and freed from starch by centrifugation at 3000*g*. The supernatant was supplemented with dry $(NH_4)_2SO_4$ to 75% saturation and left overnight at 4°C for precipitation. The precipitate was removed by centrifugation at 3000*g* and purified by gel chromatography as described previously [9, 10]. The fractions containing proteins of approximately 20 kDa were pooled and lyophilized.

A solution of the proteins (30 mg) was applied to a CM-Sepharose CL-6B column (0.5 × 15 cm) equilibrated with 0.04 M acetate buffer, pH 4.6. The column was washed with the starting buffer until no absorption at 280 nm was detected in the eluate. The adsorbed protein was eluted from the column with a linear 0-0.5 M NaCl gradient in 0.04 M acetate buffer, pH 4.6. The fractions exhibiting maximal trypsin inhibitor activity were pooled, desalted on a G-25 Sephadex column, and lyophilized.

Disc electrophoresis was run in 20% polyacrylamide gel in the presence of SDS and β-mercaptoethanol (SDS-PAGE) as described by Laemmli [15]. The gels were stained with 0.1% Coomassie R-250 solution in 25% ethanol containing 5% formaldehyde.

Protein concentration was determined by the modified Bradford's method [16] using BSA as the standard.

Proteins were sequenced using a 470A sequencer (Applied Biosystems, USA) with the standard program 02 RPTH [17]. Phenylthiohydantoin (Pth) derivatives of amino acids were identified by reversed-phase HPLC on a C18 column (Applied Biosystems) in 20% acetonitrile. Pth derivatives of amino acids were detected using a 757 flow absorption detector (Cratos, USA).

To determine the number of Cys residues, the protein was oxidized with performic acid and hydrolyzed in 5.7 M HCl under vacuum at 105°C for 24 h [18]. The content of cysteic acid was determined using an Hitachi 855 analyzer (Japan).

The activity of the inhibitor was evaluated by the extent of suppression of corresponding enzymes, whose activity was determined by the rate of hydrolysis of casein [19] or chromogenic substrates. The activity of trypsin and papain was determined in the presence of BAPA, the subtilisin activity was determined in the presence of Z-AAL-pNa, and the activity of chymotrypsin was determined in the presence of Suc-GGF-pNa [20-22]. Concentration of trypsin active sites was determined by titration with *p*-nitrophenyl-*p'*-guanidine benzoate [23].

The effect of the protein on the growth and development of macroconidia of the fungus *F. culmorum* was studied as described in [11]. The number of growing and lysed macroconidia and the length of the formed hyphae were determined using a microscope (Laboval, Germany) with a magnification ×120 (in triple replicates for each protein concentration). A suspension of macroconidia without the protein was used as the control.

RESULTS AND DISCUSSION

Protein inhibitors with molecular mass of 20-25 kDa were isolated using gel-filtration on Sephadex G-75 as described earlier [11]. These proteins were purified by ion-exchange chromatography on CM-Sepharose CL-6B at pH 4.6. The proteins bound to the sorbent were eluted with a linear 0-0.5 M NaCl gradient in the starting buffer (Fig. 1). Three protein fractions (I, II, and III) exhibited activity of proteinase inhibitors. Fractions I and II eluted with 0.1 and 0.2 M NaCl, respectively, efficiently suppressed the activity of subtilisin Carlsberg and chymotrypsin, but had much less they effect on trypsin. Fraction III eluted with 0.38 M NaCl efficiently inhibited trypsin, weakly affected chymotrypsin, and had virtually no effect on Carlsberg subtilisin.

From the data of SDS-PAGE in the presence of β-mercaptoethanol, fraction III contained the only protein component with molecular mass of 22 ± 1 kDa (Fig. 2). Thus, it can be concluded that fraction III contains a homogeneous protein, designated as PKTI-22 (potato Kunitz-type trypsin inhibitor), that is composed of a single polypeptide chain.

The homogeneity of PKTI-22 was confirmed by the determination of N-terminal amino acid sequence. After 19 steps of automatic Edman degradation, the first 20 amino acid residues were identified as LPSDATPVLD-

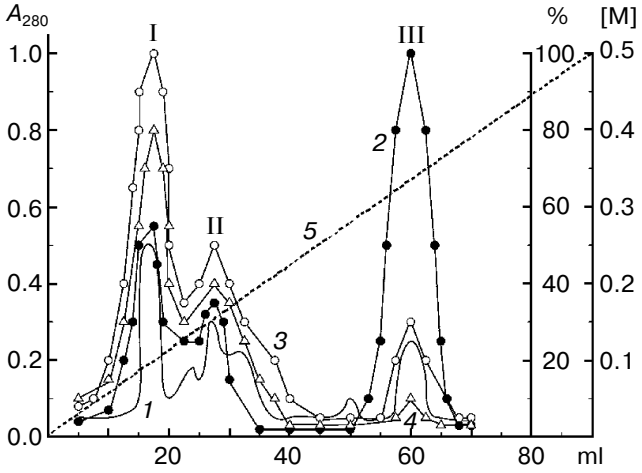

Fig. 1. CM-Sepharose CL-6B ion-exchange chromatography of 20-25 kDa proteins from potato tubers (I-III – protein fractions): *1*) A_{280}; *2-4*) suppression of trypsin, chymotrypsin, and subtilisin activity, respectively (percentage relative to original enzymatic activity towards casein); *5*) NaCl concentration (M).

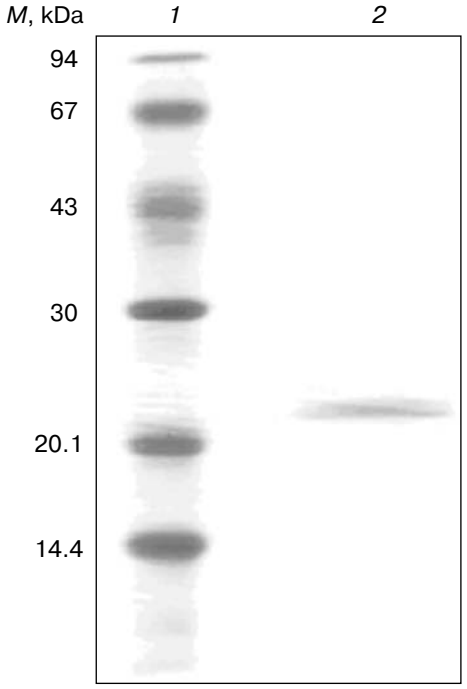

Fig. 2. SDS-PAGE of fraction III obtained after ion-exchange chromatography on CM-Sepharose CL-6B: *1*) protein standards (from top to bottom): phosphorylase b, BSA, ovalbumin, carboanhydrase, soybean Kunitz trypsin inhibitor, lactalbumin (kDa); *2*) the purified inhibitor (fraction III).

VTGKELDPRL-. Figure 3 presents comparative analysis of the first 20 residues of PKTI-22 and four proteins isolated from potato tubers of Istrinskii cv. that were obtained from the sequences of the corresponding *PKPI-B* genes. It is seen that the sequences are virtually identical except for the residue in position 18. The first 10 residues are conservative for the members of the PKPI-B group [6]. Their presence in the molecule of PKTI-22 indicates that the protein PKTI-22 belongs to this group. Besides, the N-terminal sequences of the recombinant protein PKPI-B10 and protein PKTI-22 are identical and contain the replacement Ser→Pro in position 18.

Amino acid analysis showed that PKTI-22 contains six cysteine residues. Since Ellman's reaction [24] did not reveal free SH-groups in the protein, we conclude that it contains three disulfide bonds. According to the data of Speranskaya et al. [12], the recombinant protein PKPI-B10 also contains three disulfide bonds. It should be noted that the proteins encoded by genes *PKPI-B1*, *PKPI-B2*, and *PKPI-B9* contain only two disulfide bonds [7, 10]. Therefore, this suggests that the proteins PKTI-22 and PKPI-B10 are encoded by the same gene, *PKPI-B10*.

The protein PKTI-22 had virtually no effect on subtilisin and papain. The effect of PKTI-22 on the activity of trypsin and chymotrypsin is demonstrated in Fig. 4. It is seen that the protein efficiently inhibits trypsin, but less affects chymotrypsin. In fact, the extent of the trypsin activity suppression (up to 90% inhibition) linearly depended on the amount of the added inhibitor (Fig. 4, curve *1*). The stoichiometry of the binding was found to be 1 mol inhibitor per mol trypsin. At the same time, the dependence of the extent of chymotrypsin inhibition on the amount of the added PKTI-22 exhibited non-stoichiometric character: 50% inhibition required more than 3-fold excess of the inhibitor (Fig. 4, curve *2*). The equilibrium dissociation constant ($K_i = 2.06 \pm 0.03$ nM) of the trypsin–PKTI-22 complex was calculated based on the experimental dependence of the trypsin activity suppression on the inhibitor concentration in the reaction of BAPA hydrolysis (trypsin and BAPA concentrations were 1.0 nM and 1.35 mM, respectively).

The influence of PKTI-22 on the growth and development of the phytopathogenic fungus *F. culmorum* was

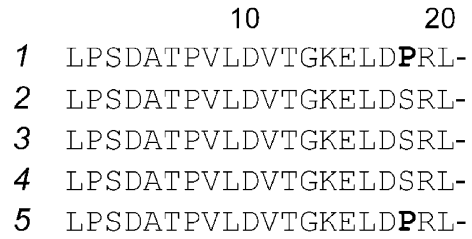

Fig. 3. Comparison of the N-terminal amino acid sequences of PKTI-22 and PKPI-B proteins: *1*, *2*) PKTI-22 and PSPI-21-6.3, respectively (isolated from potato tubers in the present work and [10]); *3-5*) proteins PKPI-1, PKPI-2, and PKPI-B10, respectively (obtained basing the sequences of the encoding genes [7]). Differing residues are shown bold.

investigated. As seen from Fig. 5, addition of 150 μg of PKTI-22 to the suspension of fungal macroconidia decreased the hypha length by 50% compared to the control, and the addition of 450 μg of PKTI-22 completely suppressed the growth of the hyphae (curve *1*), resulting in damage of all conidia (curve *2*). These data indicate that PKTI-22 exhibited a high toxicity towards the phytopathogen. It not only suppressed the growth of the hyphae, but also destroyed macroconidia of *F. culmorum*.

Comparison of the above-described properties of PKTI-22 with properties of the previously investigated recombinant protein PKPI-B10 [12] revealed virtually complete similarity of the proteins. This confirms the assumption mentioned above that PKTI-22 is encoded by the gene *PKPI-B10* that, according to Speranskaya et al. [6], was found in the genome of potato of the Istrinskii cv. It can be assumed that this gene is activated during the growth and development of the plant, and the product of its expression is accumulated in the tubers. Since PKTI-22 efficiently suppressed the growth and development of the phytopathogenic fungus *F. culmorum*, it can be assumed that this protein is a protein of the defense system of the potato. Infection with a pathogen presumably results in the induction of the *PKPI-B10* gene, leading to the accumulation of the expression product in the damage area of the tubers or leaves of the plant.

It should be noted that the PKTI-22 molecule is composed of one polypeptide chain. This indicates that, in contrast to the protein PSPI-21-6.3 encoded by the gene *PKPI-B9*, PKTI-22 is not subjected to posttranslational proteolysis at residues 135-136 (the site is located in the C-terminal region inside the loop formed by the disulfide bond Cys98–Cys165) [10]. It is suggested that in the molecules of Kunitz-type inhibitors composed two chains, this loop is located on their surface and easily accessible to the action of proteinases [25]. In the work of Speranskaya et al. [12], it was demonstrated that in the primary structure (obtained from the sequence of the encoding gene) of the recombinant PKPI-B10 and consequently in the structure of PKTI-22, the third disulfide bond is localized in the C-terminal region between residues Cys153-Cys156. In the molecule of PKTI-22 containing the additional short disulfide bond, the region between residues 135-165 is apparently not accessible to proteinases.

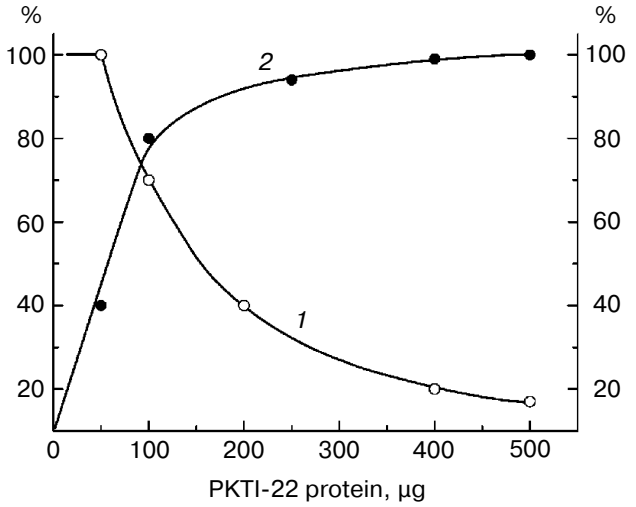

Fig. 5. Effect of PKTI-22 on the growth of hyphae and lysis of macroconidia of the fungus *F. culmorum*: *1*) hypha length; *2*) number of lysed conidia. Median values from three independent experiments are presented. The standard error was 2-10%.

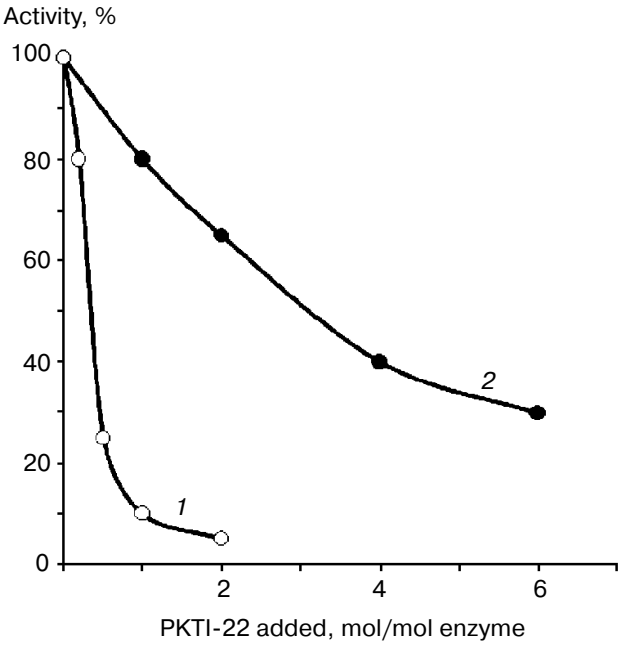

Fig. 4. Effect of PKTI-22 on the activity of trypsin (*1*) and chymotrypsin (*2*) determined in the presence of BAPA and Suc-GGF-pNa, respectively.

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REFERENCES

1. Ishikawa, A., Ohta, S., Matsuoka, K., Hattori T., and Nakamura, K. (1994) *Plant Cell Physiol.*, **35**, 303-312.
2. Herbers, K., Prat, S., and Willmitzer, L. (1994) *Plant Mol. Biol.*, **26**, 73-83.
3. Gruden, K., Strukelj, B., Ravnikar, M., Poljsak-Prijatej, M., Mavric, I., Brzin, J., Pungercar, L., and Kregar, I. (1997) *Plant. Mol. Biol.*, **34**, 317-323.
4. Pouvreau, L., Gruppen, H., van Koningsveld, G. A., van den Broek, L. A., and Voragen, A. G. (2003) *J. Agric. Food Chem.*, **51**, 5001-5005.
5. Heibges, A., Glaczinski, H., Ballovora, F., Salamini, F., and Gebhardt, C. (2003) *Mol. Gen. Genom.*, **269**, 526-534.

6. Speranskaya, A. S., Dorokhin, A. V., Novikova, S. I., Shevelev, A. B., and Valueva, T. A. (2003) *Genetika*, **39**, 1490-1497.
7. Speranskaya, A. S., Krinitsyna, A. A., Poltronieri, P., Fazano, L., Santino, A., Shevelev, A. B., and Valueva, T. A. (2005) *Biochemistry (Moscow)*, **70**, 292-299.
8. Valueva, T. A., Speranskaya, A. S., Revina, T. A., and Shevelev, A. B. (2008) *Bioorg. Khim.*, **34**, 344-359.
9. Valueva, T. A., Revina, T. A., Kladnitskaya, G. V., and Mosolov, V. V. (1998) *FEBS Lett.*, **426**, 131-134.
10. Valueva, T. A., Revina, N. A., Mosolov, V. V., and Mentele, R. (2000) *Biol. Chem. Hoppe-Seyler*, **381**, 1215-1221.
11. Revina, T. A., Speranskaya, A. S., Kladnitskaya, G. V., Shevelev, A. B., and Valueva, T. A. (2004) *Biochemistry (Moscow)*, **69**, 1092-1098.
12. Speranskaya, A. A., Krinitsyna, T. A., Revina, T. A., Gerasimova, N. G., Keruchen'ko, Ya. S., Shevelev, A. B., and Valueva, T. A. (2006) *Biochemistry (Moscow)*, **71**, 1176-1182.
13. Popkova, K. V., Schneider, Yu. I., Volovik, A. S., and Shmyglya, V. A. (1980) *Potato Deceases* [in Russian], Kolos, Moscow.
14. Valueva, T. A., Revina, T. A., Gvozdeva, E. L., Gerasimova, N. G., and Ozeretskovskaya, O. L. (2003) *Bioorg. Khim.*, **29**, 499-504.
15. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
16. Appenroth, K. J., and Angsten, H. (1987) *Biochem. Physiol. Pflanzen*, **182**, 85-89.
17. Levina, N. B., Slepak, V. Z., Kiseleva, O. G., Shemyakin, V. V., and Khokhlachev, A. A. (1989) *Bioorg. Khim.*, **15**, 24-31.
18. Hirs, C. H. W. (1956) *J. Biol. Chem.*, **219**, 611-621.
19. Northrop, D., Kunitz, M., and Heriott, R. (1950) in *Crystalline Enzymes* [Russian translation] (Orekhovich, V. N., ed.) IL, Moscow, pp. 303-306.
20. Kakade, M. L., Simons, N., and Liener, I. E. (1969) *Cereal Chem.*, **46**, 518-526.
21. Knight, C. G. (1986) in *Proteinase Inhibitors* (Barret, A. J., and Salvesen, G. R., eds.) Elsevier, Amsterdam, pp. 23-51.
22. Lyublinskaya, L. A., Yakusheva, L. D., and Stepanov, V. M. (1977) *Bioorg. Khim.*, **3**, 273-279.
23. Chase, T., and Shaw, E. (1970) *Meth. Enzymol.*, **19**, 20-27.
24. Ellman, G. L. (1959) *Arch. Biochem. Biophys.*, **82**, 70-77.
25. Bode, W., and Huber, R. (1992) *Eur. J. Biochem.*, **204**, 433-451.