Taraxalisin – a serine proteinase from dandelion *Taraxacum officinale* Webb s.l.

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Abstract Latex of dandelion roots contains a serine proteinase that hydrolyzes a chromogenic peptide substrate Glp-Ala-Ala-Leu-pNA optimally at pH 8.0. Maximal activity of the proteinase in the roots is attained in April, at the beginning of plant development after the winter period. The protease was isolated by ammonium sulfate precipitation of the root extract followed by affinity chromatography on a Sepharose-Ala-Ala-Leu-mrp and gel filtration on Superose 6R performed in FPLC regime. Pure serine proteinase named taraxalisin was inactivated by specific inhibitors of serine proteinases, diisopropylfluorophosphate (DFP) and phenylmethylsulfonylfluoride (PMSF). Its molecular mass is 67 kDa and p*I* 4.5. pH stability range is 6–9 in the presence of 2 mM Ca^{2+}, temperature optimum is at 40°C; K_m=0.37 ± 0.06 mM. The substrate specificity of taraxalisin towards synthetic peptides and insulin B-chain is comparable with that of two other subtilisin-like serine proteinases, cucumisin and macluralisin. The taraxalisin N-terminal sequence traced for 15 residues revealed 40% coinciding residues when aligned with that of subtilisin Carlsberg.

Key words: Serine proteinase; Plant subtilisin; Taraxalisin; *Taraxacum officinale* proteinase

1. Introduction

Proteinases play a prominent role in plant physiology, being the catalysts of important processes like hydrolysis of storage proteins during seed germination, activation of proenzymes, degradation of defective proteins, etc. Proteolytic enzymes of seeds and fruits are most studied, those from leaves to a much lesser degree. Root proteinases are almost unknown, except for a serine proteinase from maize roots [1,2] referred by the authors to elastase-like enzymes according to its inhibition pattern and substrate specificity.

It was observed that latexes of tropical plants *Hevea*, *Ficus*, *Euphorbia* and *Parthenium* are rich in proteolytic enzymes. Serine subtilisin-like proteinase present in the latex of *Maclura pomifera* from the *Moraceae* family was isolated earlier in this laboratory [3]. The *Compositae* (*Asteraceae*) family also includes a number of latex-forming plants. We have chosen dandelion (*Taraxacum officinale* Webb s.l.), a plant widely spread in Central Russia, as an object of further search for proteolytic enzymes. Preliminary experiments have shown that the latex from the roots of this plant contains a proteinase that hydrolyzed Glp-Ala-Ala-Leu-pNA, a typical substrate of serine proteinases of chymotrypsin or subtilisin families. Isolation of the serine proteinase from dandelion roots, named taraxalisin, and its molecular and functional characteristics are described in the present article.

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Abbreviations: DFP, diisopropylfluorophosphate; PMSF, phenylmethylsulfonylfluoride; Glp, pyroglutamyl; pNA, *p*-nitroanilide; mrp, morpholine residue; EGd, L-*trans*-epoxysuccinyl-leucylamido-(4-guanidino)butane

2. Materials and methods

2.1. Sorbents

Sepharose-Ala-Ala-Leu-mrp was prepared as described previously [4].

2.2. Substrates

Glp-Ala-Ala-Ala-pNA and other peptide *p*-nitroanilides were synthesized in this laboratory. Oxidized insulin B-chain and azocasein were purchased from Diagnosticum (Moscow).

2.3. Activity measurements

Glp-Ala-Ala-Leu-pNA solution in dimethylformamide (20 mg/ml) was used as substrate. The activity unit was defined as the amount of the enzyme capable to produce 1 μmol of *p*-nitroaniline (pNA) per min under the described conditions [5]. Activity against other peptide p-nitroanilides was measured analogously.

Proteolytic activity was determined towards azocasein from NPO Ferment (Lithuania) by modification of the method in [6] using 0.5% solution of azocasein in 0.05 M Tris-HCl buffer, pH 8.0. A unit (u) of the enzyme activity was defined as the amount of the enzyme increasing the absorbance at 440 nm by one optical density unit per min.

Proteinase activity was determined towards 0.5% bovine serum albumin from Diam (Russia), ovalbumin from Reakhim (Russia) and thrombin from NPO Ferment (Lithuania) solutions in 0.05 M Tris-HCl buffer, pH 8.0. One unit of proteinase activity was defined as the amount of the enzyme capable of hydrolyzing the substrates to products corresponding to one milliequivalent of tyrosine per min under experimental conditions.

2.4. Isolation of taraxalisin

Seventy grams of *Taraxacum officinale* Webb s.l. roots, harvested in Moscow in May, 1996, washed and preserved at −40°C, were chopped with a knife into 4–5-mm pieces, then homogenized in 100 mM Tris-HCl buffer, pH 8.0, with 5 mM $Ca(CH_3COO)_2$ using a Waring Blendor type homogenizer. The homogenate was centrifuged at 12 000 rpm for 1 h at 5°C, then 54.1 g of $(NH_4)_2SO_4$ (80% saturation) and 8 ml 1 M of Tris-HCl buffer, pH 8.0, were added to the supernatant. After 24 h the precipitate was collected by centrifugation at 15 000 rpm for 45 min, dissolved in 50 ml of 25 mM Tris-HCl buffer, pH 8.0, concentrated by ultrafiltration in an Amicon cell with YM-10 membrane and applied to Sepharose-Ala-Ala-Leu-mrp column (4.5×2.5 cm) equilibrated with the same buffer. The column was washed with this buffer, then eluted with 50 ml of 25% ethyl alcohol in the same buffer containing 1 M NaCl (Table 1). The eluate that contained the enzyme active against Glp-Ala-Ala-Leu-pNA was dialyzed against water and lyophilized. The residue was dissolved in minimal volume of 50 mM Tris-HCl buffer, pH 8.0, which contained

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Table 1
Isolation of taraxalisin from dandelion roots

Purification stage	Volume (ml)	Protein content (mg)	Activity		Purification (fold)	Yield (%)
			Total (units)	Specific (units/mg)		
Extract	103	175	4.7	0.027	1	100
Precipitation by $(NH_4)_2SO_4$ (80% saturation)	50	40.5	3.4	0.08	3	72
Ultrafiltration	50	30.5	2.0	0.065	2	42
Chromatography on Ala-Ala-Leu-mrp-Sepharose	50	1.6	1.8	1.11	41	38
Ultrafiltration	19	1.2	1.9	1.58	59	40
Gel filtration on Superose 6R	4	0.2	1.7	8.50	316	36

0.5 M NaCl and 5 mM $Ca(CH_3COO)_2$. The solution was applied to a Superose 6R column (30×1 cm) equilibrated with the same buffer (Fig. 2). The fraction containing the enzymes was desalted by dialysis against water and lyophilized.

2.5. Molecular mass assessment

Gel filtration on a Superose 6R column (30×1 cm) equilibrated with 0.5 M NaCl in 50 mM Tris-HCl buffer, pH 8.0, was used.

2.6. pI measurement

The p*I* of the proteinase was determined by isoelectrofocusing in 5% polyacrylamide gel in the presence of 2% ampholines Biolyte 3/10 with mini IEF Cell, Bio-Rad (USA). The calibration kits from Serva (Germany) were used for checking the pH.

2.7. Specificity of taraxalisin

The enzyme specificity was studied on a series of chromogenic peptide substrates under the conditions identical to those used for the activity measurement against Glp-Ala-Ala-Leu-pNA. Hydrolysis of bovine insulin B-chain was performed as follows. Six μg of the proteinase in 50 μl of triethylammonium-bicarbonate buffer were added to 0.7 mg of oxidized insulin B-chain in 200 μl of the same buffer, pH 8.2. After incubation for 3.5 h at 37°C the hydrolysis was stopped by deep freezing of the mixture. The hydrolysate was separated in an Altex HPLC instrument, Beckman (USA) using a Vydac Octyl column (200×4.6 mm). The column was equilibrated with 0.1% trifluoroacetic acid in water, then separation was achieved with a 0–8% gradient of isopropyl alcohol containing 0.1% trifluoroacetic acid at 0.8 ml/min, and 8%–20% at 0.3 ml/min. Elution of the peptides was monitored simultaneously at 215 and 280 nm. The fractions containing the peptides were collected, evaporated, hydrolyzed with 5.7 M HCl at 105°C for 24 h and subjected to amino acid analysis.

2.8. Taraxalisin inhibition

The following inhibitors were used: diisopropylfluorophosphate (DFP) and phenylmethylsulfonylfluoride (PMSF) from Serva (Germany), ovomucoid from Sigma Chemicals (USA). After incubating the enzyme with the inhibitors for 1 h at pH 8.0 and 20°C the residual activity was measured against Glp-Ala-Ala-Leu-pNA.

2.9. pH optimum of the protease

Activity of the proteinase was measured at 37°C against Glp-Ala-Ala-Leu-pNA in the following buffer solutions: 50 mM MES-buffer, pH 6.0; 50 mM Tris-HCl, pH 7.0, 7.5, 8.0, 8.5, 9.0, 9.6.

2.10. Enzyme stability as a function of pH

Ten μl of the enzyme solution (0.15 mg/ml) were added to 0.45 ml of the buffer solution (see Section 2.9). The aliquots of the mixture were taken to measure the activity under standard conditions immediately after the mixture preparation, after 4, 24 and 48 h. In another series of probes the buffer solutions contained 2 mM calcium acetate.

2.11. Enzyme activity as a function of temperature

The enzyme activity towards Glp-Ala-Ala-Leu-pNA in 50 mM Tris-HCl buffer, pH 8.0, was measured at 20–55°C.

2.12. K_m determination

Twenty-five μl of Glp-Ala-Ala-Leu-pNA solution (0.83–29.7 mg/ml) in dimethylsulfoxide were added to 2.5 ml of 50 mM Tris-HCl buffer, pH 8.0. The final concentration of the substrate in 1% dimethylsulfoxide varied from 13.55 μM to 0.16 μM. The initial reaction rate was determined by monitoring absorbance at 410 nm. K_m was calculated from 1/V against 1/[S] double reciprocal plot using the ENZFITTER program.

2.13. Amino acid composition

The enzyme was hydrolyzed in tubes sealed under reduced pressure with 5.7 M HCl for 24 h at 105°C, and the amino acid content was determined with a 835 amino acid analyzer (Hitachi, Japan). Half-cysteine and methionine contents were assayed after sample oxidation with performic acid, that of tryptophan after 24 h hydrolysis with 4 M methanesulfonic acid in the presence of 0.2% tryptamine.

2.14. N-Terminal sequence determination

The sample of the enzyme inactivated by DFP was purified by HPLC on an Agnopore column (100×4.5 mm) (Applied Biosystems, Germany) using a 15–60% gradient of acetonitrile containing 0.1% trifluoroacetic acid. The protein was collected, immobilized on Immobilon P and sequenced using a model 816 sequencer (Knauer, Germany).

3. Results and discussion

3.1. Isolation of taraxalisin

Dandelion, growing in Central Russia, belongs to biennial or perennial herbs, which form a rosette of leaves during the first year, and form flower buds when still under the snow. It blooms after melting of snow and ripens from the middle of May to early June. The proteolytic activity in dandelion roots against Glp-Ala-Ala-Leu-pNA, a typical substrate for subtilisin-like enzymes, as well as soluble protein present in root latex, depend on the phase of seasonal development.

Dandelion is a plant, capable to accumulate nutrients in its roots: inulin (up to 24%), rubber (3%), triterpenes, fatty acids and triglycerides [7]. All these substances hamper proteinase isolation and complicate its chromatography. Protein precipitation with ammonium sulfate was used to remove inulin and rubber (Table 1). Maintenance of the solution pH above 6.0–7.0 is an important condition, because the proteinase is vulnerable even to occasional pH lowering. Concentration and desalting of the enzyme at the intermediate steps was made by ultrafiltration. Affinity chromatography was used for further separation of the enzyme from pigments and other admixtures. A sorbent that contained Ala-Ala-Leu-mrp attached

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Taraxalisin	V P T E I G E L L I L X X – V F –
Subtilisin Carlsberg	A Q V P Y G I P – – L I K A D K V Q –

Fig. 1. N-terminal sequences of taraxalisin and subtilisin Carlsberg. The common residues are marked.

Table 2
Characteristics of plant subtilisin-like proteinases

Subtilisin-like proteinase from	Mol. mass (kDa)	Activity optimum		pH range of stability (25°C)
		pH	Temperature (°C)	
Taraxacum officinale (taraxalisin)	67	8.0	40	6.0–9.0
Maclura pomifera (macluralisin) [3]	65	8.5	58	7.0–9.0
Cucurbita ficifolia [9]	60	9.2	55	8.0–11.0
Benincasa cerifera [10]	50	9.2	70	4.5–9.5
Cucumis melo (cucumisin) [8]	67	10.5	70	4.0–12.0
Trichosantus cucumeroides A [11]	50	10.0	70	4.0–12.5

via *p*-benzoquinone to AH-Sepharose (Sepharose-AAL-mrp) [4] as a ligand has proved its efficiency. The proteinase from dandelion roots possessed high affinity to this sorbent, and no loss of enzyme was observed during chromatography. Final separation of a pigment was achieved by gel filtration using HPLC on a Superose 6R column.

The proteinase active against Glp-Ala-Ala-Leu-pNA appears in the peak that corresponds to a 67-kDa protein. The enzyme taraxalisin purified 316 times was obtained with a yield of 36%. The homogeneity of the proteinase was confirmed by detection of a sole N-terminal amino acid sequence by automated Edman degradation (Fig. 1). N-terminal sequence of taraxalisin traced for 15 amino acid residues revealed 40% identity with that of subtilisin Carlsberg.

Table 3
Amino acid composition of taraxalisin

Amino acid	mol/mol	% mol
Asx	50	7
Thr	35	5
Ser	108	16
Glx	105	16
Pro	23	3
Gly	119	18
Ala	61	9
Val	29	4
1/2Cys	17	3
Met	17	3
Ile	17	3
Leu	20	3
Tyr	15	2
Phe	12	2
Lys	17	3
His	15	2
Arg	6	1
Trp	0	0
Total	666	100

Table 4
Activity of taraxalisin against peptides and protein substrates

Substrate	Activity	
	Units/h/mg	Units/h/mg$\times 10^{-3}$
Glp-Ala-Ala-Leu-pNA	8.5	
Glp-Phe-Ala-pNA	12.6	
Glp-Ala-Ala-Phe-Leu-pNA	0	
Bz-Arg-pNA	0	
Ovalbumin		5.2
Bovine serum albumin		3.3
Azocasein		2.4
Thrombin		11.7

3.2. Enzyme characteristics

Basic molecular and enzymatic characteristics of taraxalisin are presented in Table 2. The molecular mass of the enzyme equal to 67 kDa was determined by gel filtration. It is comparable with the values found for other plant serine endopeptidases [8–13]. Isoelectric point of the enzyme is at pH 4.5. Amino acid composition of taraxalisin (Table 3) evades comparison with that of subtilisins from fruits of southern plants with substantially higher molecular masses. Amino acid composition data for serine proteinases from roots are absent in literature. The presence of six half-cystine residues, the absence of tryptophan and relatively low content of hydrophobic amino acids are the most characteristic of taraxalisin molecule.

It should be noted that the serine proteinase from dandelion roots was rapidly inactivated in solutions containing more than 2% of organic solvents, such as dimethylformamide and isopropyl alcohol. This circumstance made impossible application of hydrophobic chromogenic substrate Z-Ala-Ala-Leu-pNA commonly used for the activity assessment of bacterial subtilisins. More hydrophilic Glp-Ala-Ala-Leu-pNA that required 10 times less dimethylformamide for dissolution was successfully used for activity measurement of taraxalisin. This substrate turned to be one of the best among the substrates studied (Table 4). K_m equal to 0.37 ± 0.06 mM was one order of magnitude higher than that for subtilisin-like enzyme from *Maclura pomifera* fruits [3], but somewhat lower than that found for subtilisin BPN′.

Taraxalisin is capable to split, although relatively slowly, different protein and peptide substrates (Table 4). The true substrate of the serine proteinase from dandelion roots remains unknown. As shown by oxidized insulin B-chain hydrolysis (Fig. 2), taraxalisin has rather broad substrate specificity, splitting the peptide bonds formed by carboxyl groups of hydrophobic amino acids – Leu, Val, Tyr, Phe – which is characteristic of subtilisin-like plant proteinases, as well as of glutamic and cysteic acids. Serine proteinase from maize roots splits the peptide substrates only after alanine residues [2].

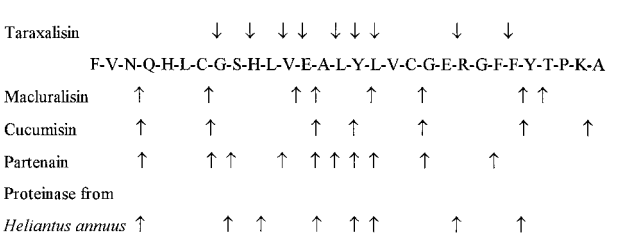

Fig. 2. Oxidized bovine insulin B chain hydrolysis by taraxalisin and other serine proteinases. The arrows indicate the cleaved peptide bonds.

Table 5
Inhibition of taraxalisin

Inhibitor	Inhibitor conc. (mM)	Enzyme/inhibitor molar ratio	Residual activity (%)
DFP	0.5	1:100	0
PMSF	1.0	1:100	24
$Hg(CH_3COO)_2$	0.3	1:100	100
Ovomucoid	0.2	1:70	83
Na_2EDTA	0.1	1:300	100
EGd	0.6	1:100	417

Table 5 demonstrates that taraxalisin is inhibited by specific inhibitors of serine proteinases DFP and PMSF, which confirms that the dandelion root proteinase belongs to the class of serine proteinases. The data presented in this paper indicate once more that subtilisin-like serine proteinases are present in various plant organs, where these enzymes may take part in regulation of physiological processes.

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