PROTEOLYTIC ACTIVITY IN THE STEM CAMBIAL REGION OF *PINUS SYLVESTRIS* L. – A CONTRIBUTION TO THE SPECIFIC DIFFERENTIATION OF SECONDARY XYLEM AND PHLOEM

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ABSTRACT

Proteolytic activity was studied in the differentiating xylem and phloem of Scots pine (*Pinus sylvestris* L.) to determine the specificity of xylem and phloem differentiation. The activity of autolytic proteases was demonstrated in the differentiating xylem during spring, summer and autumn and it was not detectable during winter. It was initiated with the onset of cambial activity in spring and unchanged during subsequent stages of xylem differentiation. The same proteolytic activity was not detectable in the extract of fresh phloem tissue. It could be detected in phloem after removal of the inhibitor found in the extract. The same pH optimum was determined for proteases extracted from xylem and phloem. However, their identity remains uncertain because of different electrophoretic mobility. On the other hand the presence of protease inhibitor in phloem tissue can be an important factor in determining the specificity of xylem and phloem differentiation.

KEY WORDS: pine, secondary xylem, phloem differentiation, proteolytic system, acid protease inhibitor.

INTRODUCTION

Derivative of cambium, conducting cells of secondary xylem and phloem (tracheids and sieve cells) undergo destruction of vacuolar membranes at the final stage of differentiation (Engelman 1965, Wodzicki and Humphreys 1972, Wodzicki and Brown 1973). In both cases, cytoplasm comes into direct contact with vacuolar sap containing digestive enzymes (Novikoff and Essner 1962, Matile 1969a, 1969b, Berjak 1972). Differentiating tracheids rapidly digest protoplasts at the final stage of cell maturation period while the cytoplasm of sieve cells remains not autolysed. Sieve cells function in phloem transport at least for a few months, to be autolysed later on, simultaneously with termination of the cell function.

Differentiation of cambial derivatives takes various periods of time. The time lapse between start and termination of the tracheid maturation phase of scots pine trees is 10-20 days in spring and up to 60 days in late summer (Wodzicki 1971). Breakdown of the tonoplast terminates the maturation phase, determines the deposition of the secondary wall and mass of wood substance being produced (Wodzicki 1961, 1971, Wodzicki and Zajaczkowski 1974, Poradowski et al. 1982).

In the present paper we determine the proteolytic activity in differentiating xylem and phloem to examine the role of autolytic proteases during differentiation. We also examine the possibility of presence of the proteinase inhibitor in phloem tissue, responsible probably for protection of sieve cells against autolysis.

MATERIALS AND METHODS

Plant material and extraction.

Scots pine trees (7-12 year-old) were grown in the Experimental Forest Plantation of the Warsaw Agricultural University in Rogów (Central Poland). The differentiating xylem and phloem were collected by knife from 3 year-old stem segment end extracted fresh or frozen and stored at the temperature of dry ice. The separated tissues were homogenized with ice cold extraction medium containing 1% PVPP in 0.03 M phosphate-citrate buffer, pH 7.2. The homogenate was filtered and centrifuged at 8,000 g for 10 min at 0°C. The pellet was discarded and the supernatant was acidified to pH 4.1 with phosphate-citrate buffer and centrifuged once more at 9,500 g for 10 min at 0°C.

The supernatant obtained from the last centrifugation was incubated at 37°C, pH 4.1 (Wodzicki and Szkutnicka 1975) without substrate other than the proteins already present in the extract, or at the presence of dialysed 0.5% bovine hemoglobin as substrate at 37°C, pH 4.0. The reaction was stopped after 1 h by adding 10% TCA (1/1- v/v) and the amount of digested proteins or liberated amino acids and peptides were determined. Before and after incubation the amount of precipitated, with 5% TCA, proteins was determined using Lowry's, method, following the original technique described by Folin and Ciocalteu (1927). The amount of amino acids and peptides was determined before and after incubation by Anson's method (Singh and Kalinsky 1978).

Dialysis

For dialysis the 2.4 nm pore size membrane obtained from SERVA was used. The dialysis was conducted for 24 hours at 2°C twice distilled water.

Molecular filtration

For molecular filtration SEPHADEX G-150 column (2.5x95 cm) obtained from PHARMACIA was used. Seven ml of 5 times condensed and dialysed extract, obtained from acetone powder, was applied to Sephadex G-150 column, equilibrated and eluted with 0.03 M phosphate-citrate buffer pH 7.2 at 5°C. After filtration 10 ml fractions were collected and the protein content was determined by measuring of absorbance at 280 nm. Proteolytic activity in each fraction was determined at the presence of 0.5% hemoglobin as substrate. Before incubation at 37°C each fraction was acidified to pH 4.0 with phosphate-citrate buffer and centrifuged at 4,500 g for 20 min at 0°C. The supernatant obtained after this centrifugation was used for incubation. All the procedure was repeated 4 times.

The following protein standards obtained from SERVA were used for determination of molecular weight: cytochrome C (12.4 kD), chymotrypsinogen A (25 kD), bovine serum albumin (67 kD), rabbit aldolase (147 kD).

Electrophoresis

The electrophoresis was carried out using 5% polyacrylamide gel plates (20x20x0.25 cm) in 0.05 M tris-citrate-borate buffer, pH 8.7 at 5°C. 0.45 ml of dialysed and condensed phloem or xylem extract was applied to 40-mm long starting

line and electrophoresis was conducted for 5h. Bromophenyl blue was used for marking protein front. After electrophoresis gel was cut into 1 cm wide and 4 cm long segments and each was eluted separately in 5 ml of 0.4 M phosphate-citrate, pH 4.0 for 18 hours at 2°C. For determination of proteolytic activity eluates were incubated with 0.5% hemoglobin for 4 hours at pH 4.0 and 37°C. All the procedure was repeated 4 times.

Conventional fixing and staining for light microscopy

For anatomical analyses 1 cm long stem segments were collected and stored in 70% ethanol until sectioning. After staining with safranin and light green the transverse stem sections were mounted in Canada balsam and the radial number of cells in particular zones of differentiating and mature xylem was determined under the light microscope as described earlier (Wilson et al. 1966, Wodzicki and Zajaczkowski 1974).

RESULTS

Seasonal wood formation and autolytic proteases activity

Proteolytic activity in xylem extract of 7 year-old pine trees was detected at the beginning of cambial activity and ceased in October along with reduction of the zone of maturing tracheids (Fig. 1). It was not detectable from January until March, when cambium was dormant. A significant increment of proteolytic activity could be correlated with the increase of the radial number of differentiating tracheids. The determined specific activity remained constant during the whole season

Fig. 1. Proteolytic activity ($\mu\text{g prot. hydrolysed mg prot.}^{-1} \text{ h}^{-1}$) in differentiating xylem during winter and growth season. Cambial zone - (C), radially growing tracheids - (G), maturing tracheids - (D). Averages of 4 trees $\pm$ se.

Fig. 2. Proteolytic activity ($\mu\text{g prot. hydrolysed mg prot.}^{-1} \text{ h}^{-1}$) in differentiating xylem or phloem extract. Phloem – (Ph), cambial zone – (C), radially growing tracheids – (G), maturing tracheids- (D). Averages of 1-4 trees $\pm$ se.

except of a depression in April and June. It could be correlated with the depression of the cambial activity.

Localization of proteolytic activity in the cambial region

Proteolytic activity was determined separately in extracts of tissues collected successively, starting from the cambial zone towards xylem and phloem. The maximum of proteolytic activity was observed in differentiating xylem and decreased rapidly close to mature xylem (Fig. 2). Low activity could be

also detected in mature xylem of the last formed annual ring. Analogous proteolytic activity could not be detected in extracts prepared from fresh phloem tissue. However, it could be detected in extracts prepared from acetone powder at the presence of hemoglobin as substrate with pH optimum 4.0, (Fig. 3). The electrophoretical separation (Fig. 4), revealed one band of activity in the differentiating xylem and two separated bands in the phloem.

Fig. 3. Effect of pH upon proteolytic activity ($\text{mmol L-tyr. mg prot.}^{-1} \text{ h}^{-1}$) in differentiating xylem – (A) and phloem – (B) extract at the presence of bovine hemoglobin as substrate.

Fig. 4. Polyacrylamide gel electrophoresis of dialysed differentiating xylem – (A) and phloem – (B) extract. Proteolytic activity ($\text{mmol L-tyr. mg prot.}^{-1} \text{h}^{-1}$) determined at the presence of bovine hemoglobin as substrate. Averages of 4 replicates $\pm$ se.

Fig. 5. SEPHADEX G-150 molecular filtration of dialysed differentiating xylem extract. (A) proteolytic activity ($\text{mmol L-tyr. mg prot.}^{-1} \text{h}^{-1}$) determined at the presence of bovine hemoglobin as substrate. (B) protein content ($\mu\text{g g fr. wt.}^{-1}$). Molecular weight of standards marked by arrows. Averages of 4 replicates $\pm$ se.

Molecular weight determination of protease from differentiating xylem

Two peaks of protease activity could be detected at fractions after molecular filtration (SEPHADEX G-150) of acetone powder extract from differentiating xylem. One peak represents proteins of molecular weight about 67 kD. and another represents proteins of molecular weight about 25 kD. (Fig. 5).

Inhibitor of proteolytic activity extracted from phloem

The phloem extract revealed a strong inhibitory effect on proteolytic activity determined in xylem extract (Table 1). Phloem extract diluted 4 and 8 times resulted in lesser inhibitory effect after mixing with xylem extract.

Phloem extract, after dialysis, revealed same inhibitory effect in comparison with the non dialysed extract (Table 2).

TABLE 1. Inhibitory effect of phloem extract upon proteolytic activity in differentiating xylem extract.

Extract from		Phloem extract diluted (times)	Proteolytic activity (µg prot. hydrolysed mg prot. ⁻¹ h ⁻¹)		
Xylem	Phloem		July 27	Aug. 16	Sept. 13
+	-	-	500	800	278
+	+	8	333	432	222
+	+	4	333	214	71
+	+	2	0	0	0
+	+	0	0	0	0
-	+	0	0	0	0

(+) extract added
(-) extract not added

TABLE 2. Inhibitory effect of dialysed and not dialysed phloem extract upon proteolytic activity in differentiating xylem extract.

Extract from		Dialysed extract from	Proteolytic activity (µg prot. hydrolysed mg prot. ⁻¹ h ⁻¹)		
Xylem	Phloem	Phloem	July 27	Aug. 16	Sept. 13
+	-	-	454	704	154
+	+	+	0	0	0
+	+	-	0	0	0
-	+	+	0	0	0
-	+	-	0	0	0

(+) extract added
(-) extract not added

Molecular filtration using SEPHADEX G-150 column revealed one peak with a strong inhibitory effect, corresponding to proteins of molecular weight of about 150 kD (Fig. 6).

DISCUSSION

The activity of acid proteinases in the differentiating xylem has been shown earlier (Wodzicki and Szkutnicka 1975). Results of the present study revealed that proteolytic activity ob-

served in differentiating xylem during the growing season is not present during winter dormancy. Activity of autolytic proteases was observed during all subsequent stages of xylem differentiation. Analogous proteolytic activity was observed in acetone powder extract of phloem tissue but not in extract of fresh phloem. Acid proteases from differentiating xylem and phloem expressed similar optima of activity at pH 4.0. Results of preliminary electrophoretic identification suggest differences between autolytic proteases extracted from differentiating xylem and phloem. Proteases extracted from differentiating

Fig. 6. SEPHADEX G-150 molecular filtration of phloem extract. (A) proteolytic activity (µg prot. hydrolysed mg prot.⁻¹ h⁻¹) determined after mixing of successive fractions with differentiating xylem extract. (B) protein content (µg g fr. wt.⁻¹). Horizontal lines denote proteolytic activity and protein level in the differentiating xylem extract. Molecular weight of standards marked by arrows. Averages of 4 replicates ± se.

xylem formed a broad band of activity. Proteases extracted from phloem were segregated into two well separated peaks. However, the effect of molecular filtration revealed two separated peaks of activity in extract of differentiating xylem. It seems probable that both proteases extracted from differentiating xylem and phloem are similar.

The specificity of differentiation of the two types of cambial derivatives could be demonstrated, however, at the presence of inhibitor in the extract of phloem tissue. The inhibitor extracted from the phloem tissue appeared not to be specific in prevention of proteolytic activity of only phloem proteases, but it inhibit also the activity of autolytic proteases extracted from differentiating xylem. It is possible that repression of synthesis of this inhibitor in differentiating xylem may be specific for the developmental program of xylem cambial derivatives.

The molecular weight of the inhibitor (approx. 150 kD) is similar to that of P-protein of phloem (Kleinig et al, 1975, Kollmann 1980). This raises the question, whether it is not a complex of high molecular weight substrate protein-low molecular weight inhibitory protein, which has been isolated in the present study. Formation of such a complex was demonstrated in other studies (Baumgartner and Chispels 1976, Salmia 1980, Katunuma et al. 1983). It is probable especially because of the low molecular weight inhibitor of proteases found in Scots pine seeds by Salmia (1980).

It is also possible that this inhibitor plays a role in blocking of the foreign acid proteases introduced by pathogens, as suggested by Ryan (1968), Green and Ryan (1972, 1973), Mosolov et al. (1976), Wong et al. (1976) and Cleveland and Black (1982), who observed accumulation of the protease inhibitor after pathogen attack or after wounding.

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AKTYWNOŚĆ PROTEOLITYCZNA W REGIONIE KAMBIALNYM
PNIA *PINUS SYLVESTRIS* L. JAKO ELEMENT SPECYFIKI RÓŻNICOWANIA SIĘ
DREWNA WTÓRNEGO I ŁYKA

STRESZCZENIE

Wykazano występowanie aktywności proteolitycznej w ekstraktach z różnicującego się drewna wtórnego sosny pospolitej. Aktywność proteolityczna nie występowała w tkankach regionu kambialnego pnia w okresie zimowego spoczynku oraz uległa zmniejszeniu w czasie zahamowania aktywności kambialnej. Analogiczna aktywność kwaśnych proteaz mogła być zaobserwowana w ekstraktach z floemu, ale dopiero po przypuszczalnym rozpadzie kompleksu enzym-inhibitor. Proteazy ekstrahowane z różnicującego się drewna i łyka mają podobne optimum pH lecz różnią się pod względem ruchliwości elektroforetycznej. Wydaje się być prawdopodobne, że występowanie inhibitora kwaśnych proteaz może decydować o specyfice różnicowania się elementów przewodzących floemu oraz może być czynnikiem obronnym w czasie ataku organizmów patogenicznych.

SŁOWA KLUCZOWE: sosna, drewno wtórne, różnicowanie się łyka, system proteolityczny, inhibitor kwaśnych proteaz.