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Proteolytic activity in relation to seasonal cambial growth and xylogenesis in *Pinus banksiana*

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Abstract

Proteolytic activity in the cambial zone and developing xylem of *Pinus banksiana* Lamb. was investigated over an annual cycle of growth and dormancy. Highest proteolytic activity was associated with the most active period of primary-wall radial expansion of cambial derivatives, in early spring, before protoplasmic autolysis was initiated in developing earlywood. Three pH maxima of proteolytic activity, near pH 3.0, 6.5 and 9.5, were observed at that time. In general, activities measured at pH values below 7.0 were greater than those determined above pH 7.0 at all stages in the annual cycle, in both cambial zone and developing xylem, although elevated activity at alkaline pH was also observed during springtime growth. Polyvinylpolypyrollidone (PVP) treatment markedly enhanced pH 7.5 but not pH 4.0 proteolytic activity in the cambial zone, but not in developing xylem, indicating the presence of PVP-binding proteinase regulators in the cambium. By fractionation and effector studies total proteolysis was determined to comprise interactions between serine, cysteine, aspartate and metallo-proteases having MWs, by gel chromatography, between 10 and 100 kDa. The observations point to a complex regulatory mechanism controlling the presence and catalytic rates of the distinct types of proteases in the cambial region throughout an annual cycle of growth and dormancy.

Keywords: *Pinus banksiana*; Pinaceae; Cambium; Cellular differentiation; Proteolytic activity; Proteases; Protoplasmic autolysis; Xylogenesis

1. Introduction

The vascular cambium is by far the largest meristem of perennial plants, responsible for producing more terrestrial biomass and stored chemical energy than any other biological system. As the primary producer of wood and the chief agent responsible for sequestering atmospheric CO_2 into permanently stabilized forms of carbon, the cambium is both economically and environmentally an immensely important biological system, being the most cost-effective source of renewable energy on earth (Landsberg & Gower, 1997). The factors regulating seasonal cambial growth and biomass accumulation nevertheless remain very inadequately understood (Savidge, 1996).

In all biological systems that have been well investigated, the activity of proteinases has emerged as a fundamental mechanism controlling when and where growth and tissue differentiation occur. In addition to digesting proteins into constituent amino acids, proteases modify protein structure as they cleave peptide bonds (Neurath, 1986). Through these structural modifications, proteases control both gene expression and rates of catalysis of life processes. Proteinases are especially interesting and challenging enzymes not only because of their highly varied specificities and pH optima but also because, in addition to occurring in the free form, they commonly occur as multicatalytic complexes known as proteasomes (Saitoh, Yokosawa, & Ishii, 1989; Avila, 1997). Growth and cellular differentiation are precisely the mechanistic events responsible for wood formation in trees (Savidge, 1996); thus, it seems reasonable to hypothesize that proteinases have important roles in regulating seasonal cambial growth.

Although both proteolytic enzymes (Tonecki, 1975a, 1975b; Salmia, Nyman, & Mikola, 1978; Szczotka & Tomaszewska, 1979; Salmia & Mikola, 1980; Salmia, 1981a, 1981b; Nassar & Newbury, 1987; Kovac & Kregar, 1989; Bourgeois & Malek, 1991a, 1991b; Otani, Iwagaki, & Hosono, 1991; Clarke, Gustafsson, & Lidholm, 1994; Tranbarger & Misra, 1995) and inhibitors of proteinase activity (Salmia, 1980; Weder, 1985; Otlewski, Zbyryt, Krokoszynska, & Wilusz, 1990;

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Klopfenstein et al., 1991; Seldal, Dybwad, Andersen, & Hogstedt, 1994; Hollick & Gordon, 1995; Kimura, Ikeda, Fukumote, Yamasaki, & Yonekura, 1995; Saarikoski, Clapham, von Arnold, & von Arnold, 1996) have been well studied in seeds, leaves and other parts of perennial plants, very little is yet known about proteases in relation to the regulation of seasonal cambial growth. In early work, Kuprevich (1949) noted the presence of proteolytic activity in the cambial region of several conifers, as well as in other tree species, and reported that activity was present only during growth. Sheldrake and Northcote (1968) reported proteolytic activity in xylem sap and considered that it could have a role in the regulation of xylogenesis. Sudachkova, Kozhevnikova, and Lyubarskaya (1981) provided evidence for proteolytic activity in both cambial zone and developing xylem of *Pinus sylvestris* in Siberia during the growing season, i.e. May, June and July. Zakrzewski and Rakowski (1987) found that physical separation of *P. sylvestris* cambial zone from developing xylem prevented autolysis but had little effect on activity of proteases in developing xylem. Rakowski and Wodzicki (1994) reported constant proteolytic activity in developing xylem of *P. sylvestris* in Poland during growth and, in agreement with Kuprevich (1949), no activity during dormancy. Rakowski and Wodzicki (1995) further reported that following removal of the extending shoots from *P. sylvestris*, proteolytic activity in the cambial region was reduced to zero within 2 weeks and that application of exogenous auxin in place of the removed shoot prevented the loss in activity.

In general, the research cited above has assumed that the principal role of proteinases in the cambial region must be in the catalysis of protoplasmic autolysis during xylogenesis. Protoplasmic autolysis of otherwise fully differentiated tracheary elements is nature's most common form of programmed cell death (Savidge, 1998), although other types of apoptosis also occur in plants (Wang, Li, Bostock, & Gilchrist, 1996; Beers, 1997; Pennell & Lamb, 1997). Because conversion of mature but still living tracheary elements into dead pipe-like members of the plant's water-conducting system involves total protoplasmic breakdown, a broad range of hydrolases undoubtedly contributes to the hydrolysis of the diverse macromolecular constituents of protoplasm (Wodzicki & Humphreys, 1972, 1973; Wodzicki & Brown, 1973; Gahan, 1981; Thelen & Northcote, 1989; Savidge & Barnett, 1993; Mittler & Lam, 1995; Fukuda, 1997a, 1997b; Savidge, 1998). Structural changes in tracheary elements during degradation of cellular contents are preceded by increased vacuolation (Savidge & Barnett, 1993) followed eventually by vacuolar collapse (Wodzicki & Humphreys, 1972, 1973; Wodzicki & Brown, 1973; Fukuda, 1997b; Groover, Dewitt, Heidel, & Jones, 1997), with some organelles being far more resistant than others to degradation (Wodzicki & Humphreys, 1972, 1973; Wodzicki & Brown, 1973; Savidge & Barnett, 1993; Fukuda, 1997b; Groover et al., 1997). Using *Zinnia* mesophyll cells induced to undergo synchronized tracheary element differentiation in vitro as a model system, advances in understanding the role of proteinases in xylogenic protoplasmic autolysis of angiosperms have been made (Wodzicki & Humphreys, 1972, 1973; Wodzicki & Brown, 1973; Kobayashi & Fukuda, 1994; Minami & Fukuda, 1995; Ye & Varner, 1996; Beers, 1997; Beers & Freeman, 1997; Fukuda, 1997a, 1997b; Groover et al., 1997; McCann, 1997; Roberts, Donovan, & Haigler, 1997; Yamamoto, Demura, & Fukuda, 1997). Thus, a growing body of knowledge on protoplasmic autolysis leading to maturation of functional tracheary elements now exists.

Seasonal cambial growth in perennial woody plants comprises many phenomena in addition to autolysis, however (Savidge, 1993, 1996, 1998). Through an annual cycle of growth and dormancy, several discrete developmental zones become sequentially distinguishable (by microscopy) in the cambial region, and essentially homogeneous tissues can be obtained in good quantities from each of those distinct zones by paying attention to tree phenology. When it becomes possible first to peel the bark from the wood in spring, the cambial zone ('CZ') can be obtained as an incompletely re-hydrated and meristematically inactive (i.e. dormant) tissue adhering to the wood surface. Later in spring, after fusiform cambial cells have become fully vacuolated and commenced cell-division activity, the CZ is obtained as an active meristem, now adhering to the phloem surface (i.e. inner bark) (Savidge, Heald, & Wareing, 1982; Savidge, 1993; Savidge, Udagama-Randeniya, Xu, Leinhos, & Forster, 1998). Following CZ reactivation, but before initiation of tracheary element differentiation per se, cambial derivatives on the inner side of the CZ cease dividing and become active in the process of primary-wall radial enlargement ('RE zone'). The RE zone can be scraped from the wood surface as an essentially pure tissue, free from the CZ adhering to the inner bark face. Because the pattern of springtime resumption of xylogenesis proceeds basipetally over the tree stem, from foliated toward non-foliated regions, whereas resumption of CZ activity and development of the RE zone begin first near the base of the live crown (Savidge & Wareing, 1984; Savidge & Udagama-Randeniya, 1992), enlarged cambial derivatives undergoing secondary-wall formation and lignification ('SL' zone) can be obtained together with RE tissue from the upper, younger parts concomitantly with pure RE tissue from more basal regions of the same tree (using conifers more than a few metres tall). It is possible to gather SL tissue at developmental stages both prior to initiation of protoplasmic autolysis in the SL zone (late spring) and during protoplasmic autolysis from early summer until autumn (Savidge & Wareing, 1984; Savidge et al., 1998).

The CZ gives rise to the RE zone which differentiates

into the SL zone and finally into mature xylem. Whether RE only, RE+SL together, or SL only, the tissue is referred to as 'developing xylem' (Savidge et al., 1982, 1998; Savidge & Wareing, 1984; Savidge & Udagama-Randeniya, 1992; Savidge, 1993). Developing xylem is a complex tissue comprising cells actively differentiating into parenchyma, which typically remain alive in the sapwood for many years, in addition to cells that will undergo protoplasmic autolysis once differentiated into tracheary elements or other types of prosenchyma (Savidge, 1996, 1998). By volume, tracheary elements predominate the xylem; however, by cell number there are approximately ten times more living parenchyma cells than dead tracheary elements present in sapwood of conifers. Therefore, past research data interpreted in terms of the contributions of proteolytic activity to protoplasmic autolysis might equally well have been considered in terms of maintaining the living state in parenchyma and other xylem cell types.

Here, we report our estimates of seasonal proteolytic activity in cambial zone and developing xylem of *Pinus banksiana* at different stages of seasonal cambial growth, together with preliminary data aimed at fully characterizing and understanding the regulatory roles of the proteases associated with the several zones of the cambial region.

2. Results and discussion

The total amount of extractable high-M_r compounds in the cambial region (CZ, and also developing xylem when present) of *P. banksiana* varied markedly over the seasons, being most abundant during the period of growth (Fig. 1). Kjeldahl nitrogen protein content, as a fraction of the total high-M_r material, varied similarly Fig. 1. During dormancy (Fig. 1, March and November),

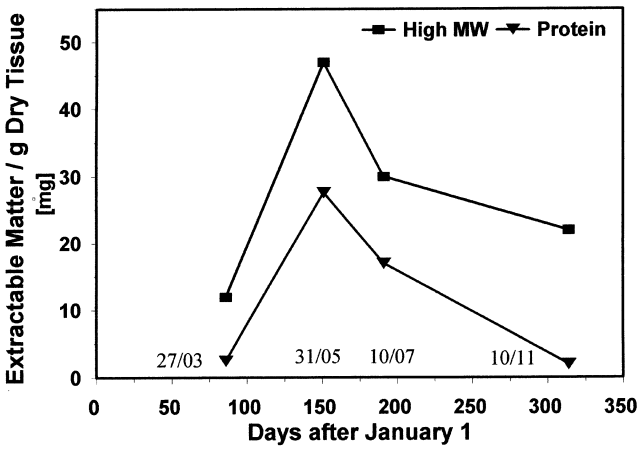

Fig. 1. Extractable high-M_r compounds and protein content per gram dry wt of tissue in the cambial region of *P. banksiana* at four dates over an annual cycle (March 27=dormant CZ; May 31=RE+SL; July 10=RE+SL; November 10=CZ+SL).

protein content was less than 20% and during active growth more than 50% of the high-M_r fraction (Fig. 1, late May and mid-July). Of the dates sampled, the maxima for both protein and high-M_r compounds were associated with the late May springtime exponential surge in cell-division activity ('grand period' of cambial activity) when the RE zone was also widening (in terms of cell number per radial file); the SL zone was only beginning to form at that time, hence protoplasmic autolysis was not underway. When the SL zone was well developed (Fig. 1, July), the contents of both protein and high-M_r compounds had decreased, possibly a consequence of advancing protoplasmic autolysis in association with maturation of earlywood tracheids. Although the cambium was dormant in both early-November and March, in November the SL zone contained three to four non-autolyzed tracheids per radial file, whereas in March there were no cambial derivatives remaining to differentiate into mature tracheids. Therefore, the higher content of high-M_r compounds present in November appeared to be associated with ongoing protoplasmic autolysis (Fig. 1). It should be noted, however, that the cambium is in the quiescent stage of dormancy in March, whereas it is in the resting stage in November (Savidge, 1993). Thus, the changing stages of dormancy could also have bearing on how much high-M_r extractable material is present in the cambial zone.

The proteolytic activity present in CZ and developing xylem, as separated tissues, was further investigated (Figs 2 and 3). Fig. 2(A) presents pH 4.0 activity, and Fig. 2(B) pH 7.5 activity, both in CZ only. Proteolytic activity was certainly present in dormant CZ of *P. banksiana*, in contrast to what was previously reported for *P. sylvestris* (Kuprevich, 1949; Rakowski & Wodzicki, 1994) but in agreement with the observation of activity in dormant apple shoots (Kang & Titus, 1980). Compared to dormant CZ, pH 7.5 proteolytic activity was lower during active growth, evidently being suppressed at that time (Fig. 2B). However, CZ proteolytic activity at pH 4.0 (Fig. 2A) was highest in May, when cell division was the principal activity and protoplasmic autolysis remained to be initiated in the developing annual layer of xylem (Fig. 2B). Two maxima of pH 4.0 activity were observed, on May 25th and August 6th, corresponding to the well known occurrence of two periods of shoot elongation and cambial growth in *P. banksiana* within an annual cycle.

Polyvinylpyrrolidone (PVP) forms complexes with flavonoids and tannins (Loomis & Battaile, 1966) which combine with proteins by hydrogen, ionic or covalent bonds to inhibit enzyme activity or, according to their structure, also activate some enzymes (Gortner & Kent, 1958). Addition of PVP serves to neutralize or precipitate such compounds, enabling them to be removed by centrifugation (Loomis & Battaile, 1966; Loomis, 1969). As shown in Fig. 2, PVP (M_r 360,000, 1% (w/v)) treatment

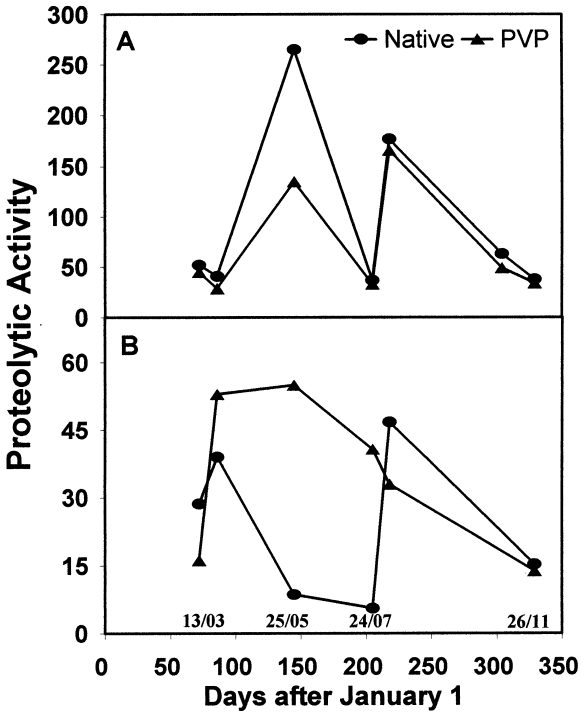

Fig. 2. Seasonal proteolytic activity in isolated cambial zone of *P. banksiana* on six dates over an annual cycle (March 13, 27 = dormant CZ; May 25, July 24, August 6 = dividing CZ; November 26 = dormant CZ. (A) pH 4.0 activity; (B) pH 7.5 activity.

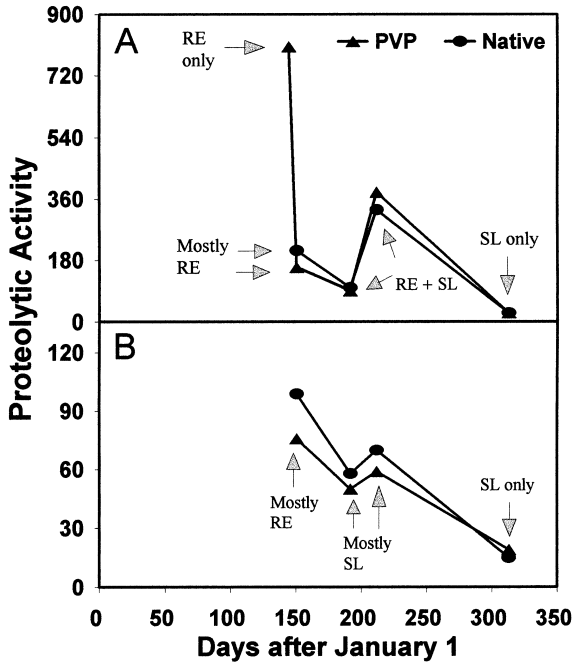

Fig. 3. Seasonal proteolytic activity in isolated developing xylem of *P. banksiana* on five dates over an annual cycle (May 25 = RE only; May 31 = RE + SL (non-autolyzing); July 10, July 24 = RE + SL (autolyzing); November 10 = SL (autolyzing). (A) pH 4.0 activity; (B) pH 7.5 activity.

decreased pH 4.0 proteolytic activity of CZ strongly in May and had a negligible effect on activities measured at that pH on other sampling dates (Fig. 2A). Proteolytic activity of the CZ at pH 7.5 was markedly increased in the presence of PVP during the active period and marginally decreased during the dormant stages (Fig. 2B). The pronounced effects of PVP on CZ proteolytic activity indicate the probable presence of endogenous regulators which not only can inhibit but also can stimulate proteolytic enzymes in the CZ. The identity of those PVP-inactivated compounds, and their seasonal fluctuations in relation to growth and proteolytic activity, remain to be investigated.

Proteolytic activity in developing xylem was higher than that in CZ at both pH values (cf. Figs. 2–3), and was higher at pH 4.0 than at pH 7.5 (cf. Fig. 3A and B, respectively). Highest activity at pH 4.0 occurred in early May, soon after formation of the RE zone (Fig. 3A), before initiation of protoplasmic autolysis. At this time, cambial derivatives are highly turgid and undergo more radial expansion than at any other time during the growth season. The pH 4.0 activity sharply decreased with the appearance on May 31 of the SL zone (Fig. 3A), indicating a possible role for one or more components of the acid protease complex in regulating (i.e. preventing) the onset of xylogenesis. At pH 7.5, proteolytic activity decreased during the transition of developing xylem from a tissue containing RE, only, to a tissue containing RE + SL (Fig. 3B). In contrast to the CZ, the influence of PVP on proteolytic activity in developing xylem was very weak to negligible at both pH values.

At both pH 4.0 and 7.5, the proteolytic activity maxima of both cambial zone (Fig. 2) and developing xylem (Fig. 3), whether treated with PVP or analyzed in the native state, clearly were not associated with protoplasmic autolytic events leading to terminal cellular differentiation, rather principally with primary and secondary cellular differentiation activities (Savidge, 1996). The data indicate that gross proteolytic activity declines as stem phenological stages proceed from occurrence of exclusively primary cellular differentiation events in early spring toward the onset of terminal events in late spring. Thus, in terms of seasonal diameter growth overall, proteolytic activity in the cambial region appears to be more important to growth than to xylogenesis, hardly an expected finding in relation to previous work focused on proteolytic activity as the principal agent of autolysis. It should also be noted that during autolysis of tracheary elements, the protoplasma of the adjoining ray cells for the most part remain non-autolyzed. In this light, it is difficult to know what proportion of the total proteolytic activity measured on the three dates (Fig. 3, July 10, 24, November 10) when autolysis was occurring is attributable to autolysis vis-a-vis the maintenance of xylem ray cells in a state of secondary cellular differentiation.

The proteases present in the extractable high-M_r frac-

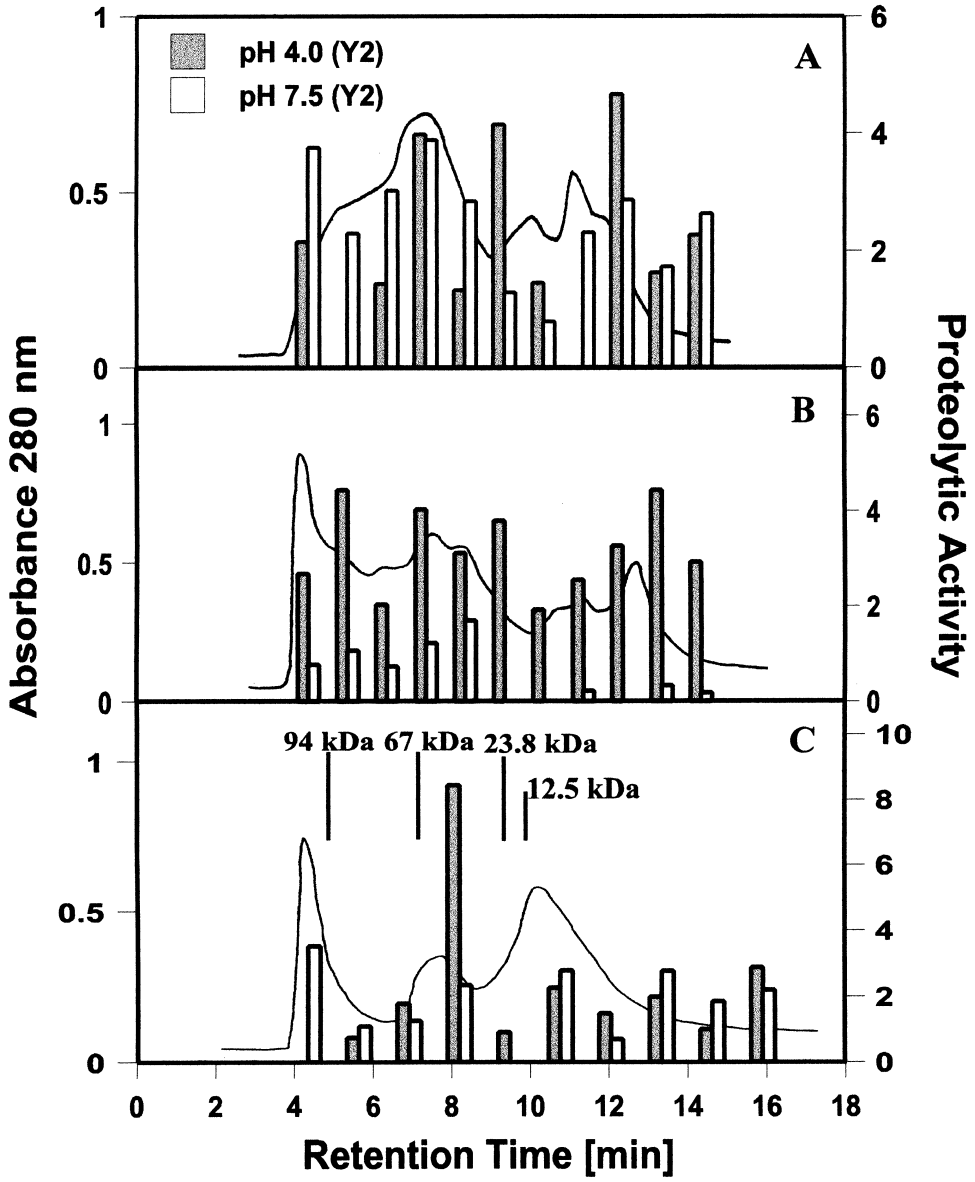

Fig. 4. Gel chromatography elution profiles and proteolytic activity distribution of lyophilized samples from the cambial region of *P. banksiana*: (A) May 31 = RE + SL; (B) July 10 = RE + SL; (C) November 10 = CZ + SL. The left-hand *Y* axis is absorbance units measured during chromatography at 280 nm; absorbances are shown as linear profiles. The right-hand (Y2) *Y* axis is proteolytic activity (bars) measured at pH 4.0 (shaded) and pH 7.5 (open). The standard MW markers used were: cytochrome c, 12.5 kDa; trypsin, 23.8 kDa; BSA, 67 kDa; phosphorylase b, 94 kDa.

tion of *P. banksiana* (Fig. 1) were fractionated by gel chromatography as shown in Fig. 4. The May (Fig. 4A) sample contained RE + SL; at that time development of the SL zone had only begun, and the RE zone was highly active. The July sample (Fig. 4B) also contained RE + SL; however, at this time RE activity was reduced, and SL activity heightened in comparison to the May harvest date. Protoplasmic autolysis was occurring in July but not in May. The bark still peeled in November; at that time the xylem surface contained fully differentiated but still autolyzing SL with associated dormant CZ lacking any RE derivatives (Fig. 4C). The elution profiles of Fig. 4 indicate that a complicated and changing mixture of proteases is involved in seasonal cambial activity and wood formation. Proteases with M_r from 10 to 100+ kDa were present at all stages of the annual cycle. In agreement with the data of Figs. 2–3, pH 4.0 proteolytic activity was abundantly present, particularly in the developing xylem of July (Fig. 4B). Fractionation of the preparations revealed that pH 7.5 activity could be of similar magnitude to pH 4.0 activity during May (Fig. 4A) and November (Fig. 4C), in contrast to what was found with crude protein preparations (Figs. 2–3).

When analyzed separately, CZ (Fig. 2) yielded lower

activities than developing xylem (Fig. 3), but pH 4.0 activity was stronger than pH 7.5 activity in both tissues. When the proteases in developing xylem were separated (Fig. 4), the difference between pH 4.0 and 7.5 activities was less apparent. It appears, therefore, that proteases of developing xylem must normally be compartmentalized and that when combined in vitro they work to counterbalance one another's activity.

Fig. 4 indicates that the protease component active at acidic pH probably functions in xylogenic protoplasmic autolysis, as well as in regulating CZ activity as indicated by Fig. 2. Activity at pH 4.0 was most evident in each of the two samples (Fig. 4B, 4C) having active autolysis. In November (Fig. 4C), when the cambium was dormant and protoplasmic autolysis of the last produced latewood tracheids was, presumably, the primary metabolic activity still occurring, the pH 4.0 proteolytic activity in the 8-min fraction had clearly become dominant. In May (Fig. 4A) when the SL zone had only just formed and no earlywood tracheids had yet begun to autolyze, activity in the 8 min fraction was low. In July (Fig. 4B), when protoplasmic autolysis was well underway, pH 4.0 activity was stronger in the 8-min fraction. Thus, the protease(s) eluting at 8 min appears to be important to the autolytic process; however, this hypothesis remains to be tested in detail and it is not clear whether the perceived significance relates to the promotion of autolysis in maturing tracheary elements or the inhibition of autolysis in xylem ray cells.

The pH 7.5 proteolytic activity in the November sample (Fig. 4C) was re-measured at the same pH in the presence of leupeptin and EDTA (Fig. 5). The changes in the observed activity in the presence of serine/cysteine and metallo protease inhibitors, respectively, served to confirm the existence of more than one protease as well as the presence of distinct kinds of proteases. In addition, it provides evidence that the several types are interacting (cf. Figs 4C and 5). Ye and Varner (1996) found two cysteine proteases and a serine protease associated with induced in vitro tracheary element differentiation of *Zinnia elegans*.

Proteolytic activity in the cambial region was observed at selected pH values, from pH 2 to 10.5, using lyophilized protein preparations from May and November harvests (Fig. 6). Three pH maxima, near pH 3.0, 6.5 and 9.5 were observed in both. The existence of activities at such extreme ends of the pH spectrum may appear to be of no physiological significance; however, it is in fact in good agreement with the 1930 histochemical observations made by Bailey and Conway (1930) on fusiform cambial cells. Using dyes, they reported pH to vary from strongly acidic to strongly alkaline in different vacuoles. Although the cell sap as expressed from the cambial region is buffered strongly near pH 6 during active growth, the cambial zone has been observed to give pH values below 3 during dormancy (Savidge, unpublished data). Conceivably, the varied pH optima of proteases in the cambial region is related to the observation that cell expansion and tracheary element differentiation are affected by changing pH (Roberts & Haigler, 1994). The fact that pH maxima for proteolytic activity in November, when the activity was lowest, and in May when it was highest, were the same (Fig. 6) is an indication that the kinds of proteases present may be similar at different phenological stages,

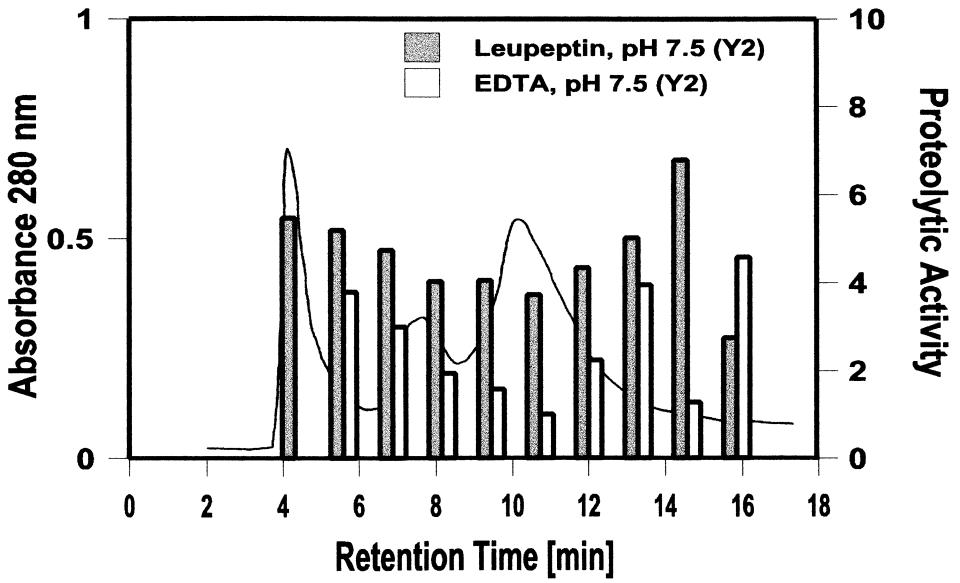

Fig. 5. Gel chromatography elution profile (280 nm) and proteolytic activity (bars) in the presence of protease inhibitors. The November 10th sample, containing CZ + SL, was investigated.

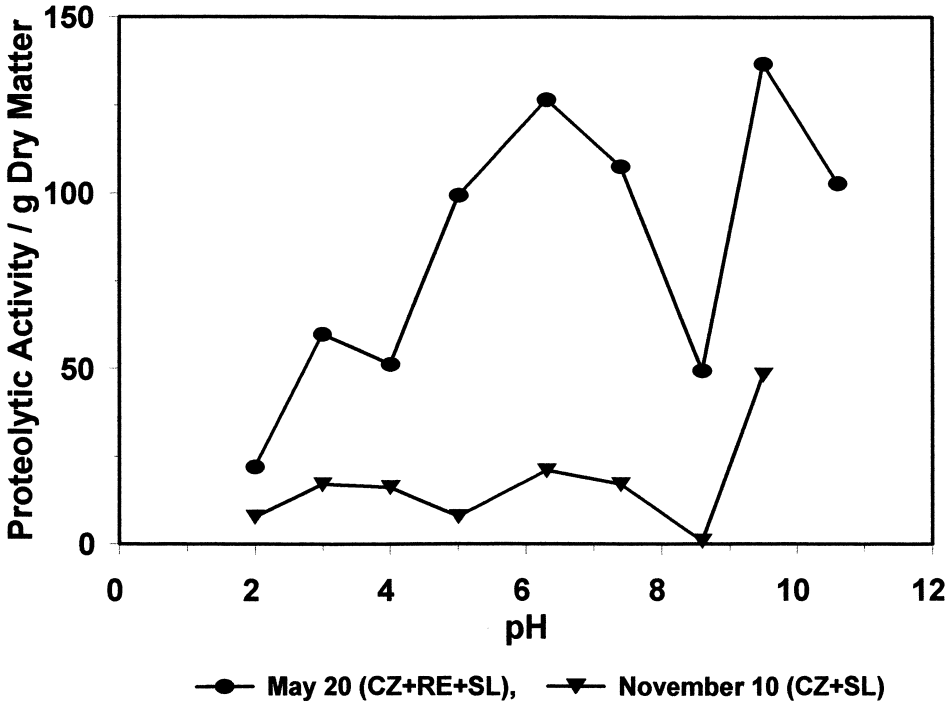

Fig. 6. Proteolytic activity as a function of pH in cambial zone with developing xylem harvested when the CZ was actively dividing (May 20) and dormant (November 10). Before assaying, these preparations were lyophilized. Proteolytic activity is expressed in terms of the corresponding tissue dry weights.

although their molarities and activities are clearly different.

3. Experimental

3.1. Reagents

Bovine haemoglobin, leupeptin, polyvinylpyrrolidone (M_r 360,000), trichloroacetic acid (TCA) and all salts were from Sigma Chemicals. EDTA was from BDH.

3.2. Trees

P. banksiana trees growing in the University of New Brunswick Forest, NB, Canada, were harvested from March to November at different stages of growth of the cambium and developing xylem. After felling and branch removal, the main stems were transported to the laboratory examined by microscopy and immediately processed. The bark was peeled and cambium and developing xylem were scraped into liquid N_2, snap frozen and ground to a fine powder in liquid N_2 using mortal and pestle.

3.3. Extraction

Frozen powdered samples were weighed and extracted with 0.05 M Tris–HCl buffer (pH 8.0) containing 5% glycerol for 1 h at 4°C under continuous stirring. The insoluble part was separated by centrifugation ($10\,000 \times g$, 2°C, 20 min). The supernatant was concentrated using a Centriprep 10 (10 kDa cut-off membrane, Amicon) at $2500 \times g$ and 2°C to yield the soluble enzyme preparation, which was used directly and after adding 1% (w/v) PVP to determine protease activity (Figs. 2–3). Centriprep 10 preparations were further desalted using Sephadex G-25 columns (PD-10, Pharmacia, Sweden) when the samples were subjected to freeze drying.

3.4. Protein content

Protein content (Figs. 1 and 4) in desalted freeze dried samples were determined using Kjeldahl N.

3.5. Dry weight

Dry weights were determined using the weight before and after 4 h heating at 110°C, using unextracted tissue.

3.6. Proteolytic activity assay

Proteolytic activity was defined as μg tyrosine liberated min^{-1} during the hydrolyses of 1.2% haemoglobin-containing 0.1 M NaCl at 37°C. An aliquot of each enzyme preparation was added to 5 ml haemoglobin/NaCl soln

and incubated at 37°C, stopping the reaction by adding 5 ml 5% TCA. The samples were filtered through Whatman 1 and the absorbances of the filtrates were read at 280 nm. The conditions for control samples were exactly the same except that TCA was added immediately after addition of enzyme. Two replicates were analyzed.

3.7. Protease inhibitors

Leupeptin (5 μg/ml) and EDTA (0.55 mg/ml) were investigated.

3.8. Gel chromatography

Lyophilized enzyme preparations (0.8 mg for each of the May and July and 2.6 mg of the November sample) dissolved in Tris–HCl buffer (pH 8.0, 0.05 M) were applied onto a Protein Pak 300 SW column (Millipore, 7.8×300 mm) equilibrated with 0.1 M potassium phosphate: pH 7.5, containing 0.1 M KCl. Fractions eluted (1 ml/min, 280 nm) isocratically with the same buffer were collected.

Acknowledgements

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