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PURIFICATION OF AN ACIDIC CONIFERIN-HYDROLYSING β-GLUCOSIDASE FROM DEVELOPING XYLEM OF *PINUS BANKSIANA*

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Abstract—Two cytosolic, acidic (p*I* 3.8) glycoproteins (M_r 110 000 and 90 000) from lignifying xylem of *Pinus banksiana* were electrophoretically isolated and confirmed by combined gas chromatography-mass spectrometry to be capable of hydrolysing *E*-coniferin to *trans*-coniferyl alcohol. These isoenzymes yielded identical N-terminal sequences having negligible homology with documented glucosidases, suggesting that they constitute a novel form of β-glucosidase.

INTRODUCTION

E-Coniferin (coniferin, 4-*O*-β-D-glucopyranoside of *trans*-coniferyl alcohol) is abundantly present in the cambium of conifers during wood formation [1–3]. When coniferin is supplied as an exogenous compound to plant tissues, lignification of cell walls is a predictable response [4–8]. Hence, although coniferin itself is not oxidized by the peroxidases and oxidases catalysing the final step of lignification [9, 10, Savidge, unpublished data], coniferin has long been considered to be a storage metabolite in support of lignification. For utilization during lignification, coniferin evidently must be hydrolysed by a β-glucosidase, followed by oxidation and polymerization of coniferyl alcohol into lignin [11, 12].

Following incubation of *Araucaria excelsa* stem tissues in indican (indoxyl β-D-glucoside) solution, the existence of a coniferin-hydrolysing β-glucosidase in developing xylem was advocated by Freudenberg [11] based on the appearance of a blue (indigo) oxidation product. Upon incubating stem tissue of seedling *Picea abies* in indican solution, however, Marcinowski *et al.* [13] were unable to repeat the histochemical observations of Freudenberg [11]. Moreover, when *Araucaria* stem tissues were re-examined, it was observed that indigo production was restricted to cell walls of mature (fully lignified) xylem, no histochemical response being detected in the cambium or its differentiating derivatives [Savidge, unpublished data].

Marcinowski *et al.* [13] noted that even if there was a positive indican reaction in developing xylem of conifer stems this would not necessarily be evidence for a β-glucosidase capable of hydrolysing coniferin. Numerous cell-wall-bound enzymes having β-glucosidase activity, but with varied substrate specificities, have been documented in higher plants. In fact, coniferin-hydrolysing β-glucosidase activity could not be found in the active cambium of *P. abies* stems, rather only in hypocotyls and roots of recently germinated seedlings [14], the enzyme apparently being most concentrated in the inner secondary cell-wall layer of cells making up the hypocotyl epidermis [13]. In other words, despite coniferin being the dominant glucoside in conifer stems there is as yet no definitive evidence for coniferin-hydrolysing β-glucosidase activity in relation to lignification in conifers. Here we provide evidence that a coniferin-hydrolysing β-glucosidase is in fact present, but at low abundance, during wood formation in the conifer *Pinus banksiana*.

RESULTS AND DISCUSSION

Following buffer extraction of developing xylem (~250 g fr. wt) isolated from mature stem regions of *P. banksiana* trees, the possibility of coniferin-hydrolysing activity was investigated sequentially beginning with ammonium sulphate fractionation. Recognizing that degradatory enzymes undoubtedly exist in developing xylem (a tissue that undergoes protoplasmic autolysis [15]), use of protease inhibitors (e.g. aminocaproic acid), low temperatures, and rapid processing to eliminate degradative enzymes were adopted as routine procedures. By UV spectroscopic examination of the shift in absorption maxima (coniferin 258 nm, coniferyl alcohol 264 nm), no activity was detected in the protein fraction precipitating at ≤40% $(NH_4)_2SO_4$ saturation. Weak activity was detected, however, in precipitate obtained at 40–85% $(NH_4)_2SO_4$ saturation (Table 1). Preparative IEF (pH 3.5–9.5) of the 40–85% $(NH_4)_2SO_4$ fraction yielded a zone between pH 3 and 4 capable of hydrolysing coniferin

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Table 1. Purification of coniferin-hydrolysing β-glucosidase from 280 g of developing xylem of *P. banksiana*

Purification stage	Volume (ml)	Activity (total)*	Protein (mg)	Specific activity†	Yield (%)	Purification factor
Crude extract	2804	0.41	338.5	0.0012	100.0	1.0
40–85% $(NH_4)_2SO_4$ saturation	208	0.38	117.9	0.0032	92.7	2.6
Preparative IEF (pH 4.0–6.5)	2.2	0.09	2.7	0.0333	21.9	21.9
HPLC-CEC Fraction	1.4	0.02	0.51	0.0392	4.9	30.1

*Decrease in 258 nm absorbance per min.
†Total activity divided by total protein.

(Fig. 1, Lane 1), the production of coniferyl alcohol being confirmed by combined gas chromatography-mass spectrometry (GC-MS).

Coniferin-hydrolysing activity was found to be exclusively acidic and could be readily separated from non-coniferin hydrolysing β-glucosidases by preparative isoelectric focusing (IEF, pH 4.0–6.5). Attempts to fractionate the enzyme by anion-exchange chromatography were unsuccessful, apparently due to irreversible binding. When the p*I* 4 zone was subjected to high performance liquid chromatographic (HPLC) cation-exchange chromatography (CEC), coniferin-hydrolysing β-glucosidase eluted in the void volume and by this step was determined to be 30-fold purified (Table 1). Analytical IEF (pH 3.0–9.0) of the acidic β-glucosidase following HPLC-CEC fractionation yielded a singular protein band of p*I* 3.8, the most acidic band in the preparation (Fig. 1, Lane 3).

As analysed by non-denaturing polyacrylamide-gel electrophoresis (native PAGE, 8–25%), the acidic β-glucosidase consisted of four protein bands (Fig. 2). Following incubation in buffered coniferin solution, each band was confirmed by GC-MS to be active in production of coniferyl alcohol. Following $(NH_4)_2SO_4$ precipitation and two successive IEF runs (pH 4.0–6.5, pH 2.5–4.5), a single band of coniferin-hydrolysing β-glucosidase activity was obtained that separated into four constituent bands by sodium dodecyl sulphate-PAGE (SDS-PAGE). These four bands were electro-transferred to a polyvinylidene difluoride membrane, subjected to Edman-degradation and N-terminal sequenced as phenylthiohydantoin amino acid derivatives. Useful sequence data were obtained only with the two large proteins (M_r 110 000 and 90 000), both of which yielded similar sequences (Table 2). SwissPrott, PIR and GenBank databank searches (February 1994) indicated that the N-terminal sequences of the 110 000 and 90 000 proteins were not homologous to known proteins; however, full sequence information for any higher plant β-glucosidase evidently remains to be reported.

In developing xylem of *P. banksiana*, coniferin-hydrolysing β-glucosidase is a very scarce enzyme. The low activity of this β-glucosidase may be at least part of the explanation for coniferin accumulating rather than being utilized immediately in support of lignification [2, 3]. It previously was suggested that the rate of O_2 supply to coniferyl-alcohol oxidase could be a limiting factor in coniferyl alcohol dehydrogenation and polymerization [10], but our findings with β-glucosidase suggest that the

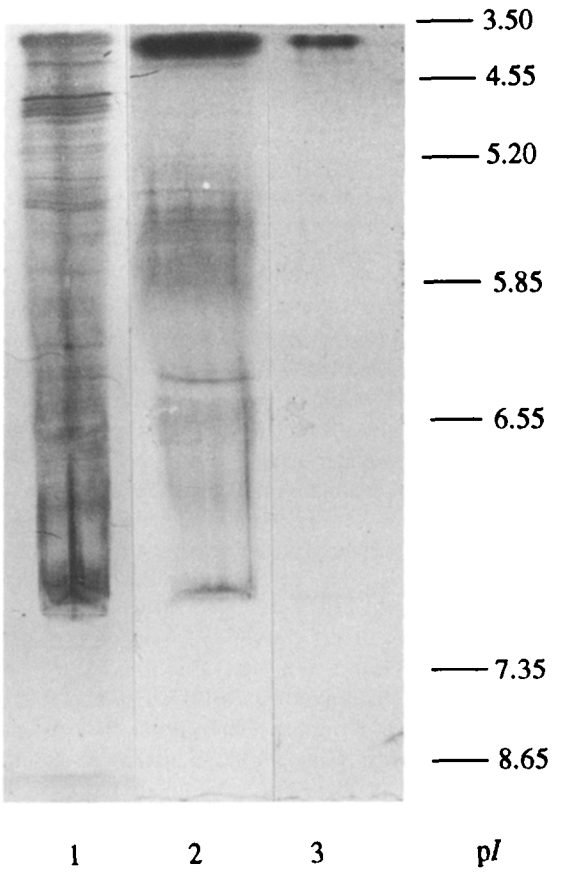

Fig. 1. Analytical IEF (pH 3.0–9.0, cathodal loading) of protein (silver-stained) from developing xylem of *Pinus banksiana*. Lane 1: dialysed concentrated precipitate (~5 μg protein) following 40–85% $(NH_4)_2SO_4$ saturation. Lane 2: pH 4.0–4.2 coniferin-hydrolysing β-glucosidase fraction (~2 μg protein) from preparative IEF (pH 4.0–6.5). Lane 3: unbound coniferin-hydrolysing β-glucosidase fraction eluting from HPLC-CEC column (~1 μg protein). Markers: broad p*I* calibration kit (Pharmacia).

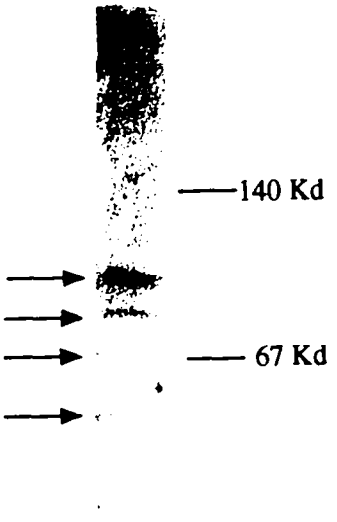

Fig. 2. Native PAGE (8–25%) of unbound coniferin-hydrolysing β-glucosidase fraction eluting from HPLC-CEC column. The lane was loaded with ~0.5 μg protein. Each of the four indicated bands (silver-stained, arrowed) was confirmed by GC-MS to hydrolyse coniferin. Positions of M_r markers are shown on the right.

activity of this enzyme could be a factor limiting the rate of lignification during xylem development [2, 16]. *In vitro* investigations with the cambium of *P. strobus* indicated that coniferin-hydrolysing β-glucosidase activity could be under auxin-cytokinin regulation [2], but how these phytohormones promote activity remains to be elucidated.

Several other, less acidic β-glucosidases in developing xylem of *P. banksiana* were detected histochemically on analytical IEF gels. They yielded indigo from indican and could hydrolyse 4-methylumbelliferyl β-D-glucoside, *p*-nitrophenyl 4-*O*-β-D-glucoside and other β-D-glucosides. However, none of these glucosidases was able to hydrolyse coniferin. Indican-active bands were detected at p*I* 4.5 and higher, the acidic coniferin-hydrolysing β-glucosidase not utilizing indican as substrate. 4-Methylumbelliferyl β-D-glucoside and *p*-nitrophenyl 4-*O*-β-D-glucoside hydrolysing activities were detected in bands at p*I*s 4.8, 5.2 and 7.5, as well as in the p*I* 3.8 band hydrolysing coniferin. Thus, the non-coniferin-hydrolysing β-glucosidase activities were spatially separated and functionally distinct from the more acidic coniferin-hydrolysing band.

In contrast to the β-glucosidases present in developing xylem of conifers, commercial β-glucosidase preparations obtained from angiosperms readily hydrolyse coniferin [Savidge, unpublished data]. β-Glucosidases with coniferin-hydrolysing activity have been characterized in tissues of at least two angiosperm species [9, 17–19]. In addition, Hösel *et al.* [20] noted evidence for coniferin-hydrolysing activity in cell-cultures of various angiosperms. Plausibly, coniferin does not accumulate in angiosperm tissues to the same extent that it does in coniferin-enriched developing xylem of conifers because of the apparent differences in coniferin-hydrolysing β-glucosidase activity.

In summary, this research has confirmed the long-suspected existence of a coniferin-hydrolysing β-glucosidase as an active but scarce enzyme in lignifying tissue of conifers. Coniferin-hydrolysing β-glucosidase in developing xylem of *P. banksiana* appears to be due to at least two glycoproteins of M_r near 100 000 having similar if not identical N-terminal sequences. Being of higher M_r and fully soluble, the *P. banksiana* enzyme evidently is distinct from the cell-wall bound enzyme reported by Marcinowski and co-workers [13, 14] in *P. abies*.

EXPERIMENTAL

Reagents. *E*-Coniferin was crystallized in house from *Pinus strobus* L. cambial extracts as previously described [2]. All other compounds, of highest available purity, were purchased from chemical suppliers.

Trees. Healthy *Pinus banksiana* Lamb. trees between 10 and 20-years-old growing in the University of New Brunswick Forest, Fredericton, NB, Canada, were harvested beginning in late spring and throughout the period of cambial growth. After felling and branch removal, the main stems were transported to the laboratory and immediately processed.

Extraction of protein from cambial tissues. After peeling and discarding the bark, a clean knife was used to scrape developing xylem from mature wood directly into liquid N_2 as previously described [10]. The frozen tissue was pulverized to a fine powder using mortar and pestle, weighed and extracted for 1 hr at 4° with pre-chilled pH 5.8 Na acetate (0.1 M)–NaCl (0.05 M)–Triton-X 100 (0.1%) in a 10:1 (v/w) ratio. The extract was filtered through 4 layers of cheese cloth and squeezed to damp dryness.

The filtrate was centrifuged (10 000 *g*, 4°, 20 min) and after decanting, aminocaproic acid (2 mg ml^{-1}) was added to the supernatant to suppress protease activity. The pellet was discarded.

Ammonium sulphate fractionation. Solid $(NH_4)_2SO_4$ was added slowly to 40% saturation of the supernatant

Table 2. N-Terminal sequences of two coniferin-hydrolysing β-glucosidase proteins purified from developing xylem of *Pinus banksiana* and electroblotted on to polyvinylidene difluoride membrane

110 000 band:													
Ala	?	Ser	**Phe**	**Pro**	Ser	**Asp**	**Pro**	Ser	**Thr**	**Gly**	**Leu**	?	Ser	?
90 000 band:													
Ala	Ser	Ser	**Phe**	**Pro**	?	**Asp**	**Pro**	**Ser**	**Thr**	**Gly**	**Leu**	**Ser**	?	?

Bold letters: real signal, other letters: weak signal, ?: no definitive signal.

using gentle stirring. After 1 hr at 4°, the solution was centrifuged (16000 *g*, 4°, 30 min). Preliminary investigations having shown that no coniferin-hydrolysing activity was present in the 40% $(NH_4)_2SO_4$-saturated precipitate, the pellet was discarded. The supernatant was adjusted to 85% $(NH_4)_2SO_4$ -saturation and left overnight at 4°. Following centrifugation (16000 *g*, 4°, 30 min) and discarding of the supernatant, the pellet was dissolved in pH 5.8 NaOAc (10 mM)–NaCl (5.0 mM).

Dialysis of the 85% $(NH_4)_2SO_4$ *precipitate.* Using Spectrapor cellulose tubing (cutoff M_r 12000–14000, Spectrum Medical Ind.), dialysis was done in buffer [pH 5.8 NaOAc (10 mM)–NaCl (5.0 mM), 4°, 4 l, five changes over 48 hr]. The retentate was clarified by centrifugation (23000 *g*, 4°, 30 min), discarding the inactive pellet. The supernatant was decanted to Centriprep 10 (Amicon) tubes and centrifuged (2455 *g*, 4°, 30 min) and the concentrated retentate ($M_r > 10000$) containing β-glucosidase activity was transferred to sterile cryovials (Nalgene, 5000-0020), capped and stored at −18°.

Preparative isoelectric focusing (IEF). Ultrodex granulated gel (4 g, Pharmacia) was added over 1 hr to 100 ml of 2% ampholine solution (pH 3.5–9.5, pH 4.0–6.5, or pH 2.5–4.5, Pharmacia), poured into a 10 × 20 cm gel tray and evaporated to 32% moisture (2 mm gel thickness). Enzyme solution (2 ml ≡ 33.2 mg protein) was mixed with a portion of gel removed from behind the cathode and the mixture returned to the same location, smoothing the sample-application zone with a clean spatula. IEF on a 2117 Multiphor II unit (LKB) was performed at 10° under described conditions (LKB Application note 318) for 20 hr.

Non-denaturing polyacrylamide gel electrophoresis (native PAGE). Native PAGE was done on minigels (43 × 50 × 0.45 mm PhastGel, gradient 8-25, Pharmacia) using a PhastSystem (Pharmacia) according to manufacturer's specifications. Protein solutions (5 μl) were loaded on to gels and separated under conditions described (Separation Technique no. 120, Table 1, PhastSystem User's Manual, Pharmacia), using thyroglobulin (M_r 669000), ferritin (M_r 440000), catalase (M_r 232000), lactate dehydrogenase (M_r 140000), and albumin (M_r 67000) as markers (HMW calibration kit, Pharmacia). Following electrophoresis, half of the gel was silver stained as described above. Corresponding protein bands on the non-stained half were scraped off by razor blade, transferred to 1.5 ml vials, and incubated with coniferin solution for subsequent coniferyl alcohol analysis (see 'Enzyme Assays' below).

Determination of isoelectric points. Analytical IEF (pH 3–9 PhastGel minigels, 43 × 50 × 0.45 mm, Pharmacia) was done on a PhastSystem (Pharmacia) according to manufacturer's specifications (Separation Technique no. 100), using basic lentil lectin (p*I* 8.65), middle lentil lectin (p*I* 8.45), acidic lentil lectin (p*I* 8.15), basic horse myoglobin (p*I* 7.35), acidic horse myoglobin (p*I* 6.85), human carbonic anhydrase B (p*I* 6.55), bovine carbonic anhydrase B (p*I* 5.85), β-lactoglobulin A (p*I* 5.20), soybean trypsin inhibitor (p*I* 4.55), and *Aspergillus niger* amyloglucosidase (p*I* 3.50) as markers (broad p*I* calibration kit, Pharmacia). Protein solutions (5 μl) were loaded on to gels and separated at 15°. Silver staining (1 ng protein-detection limit) of minigels was done in a PhastSystem Development Unit (Pharmacia, PhastSystem Development Technique no. 210, protocol of Table 1). β-Glucosidase p*I*s were calculated from a calibration curve.

Glycoprotein determination. Following preparative IEF (pH 4.0–6.5), the acidic band was eluted, analysed on minigels (analytical IEF, pH 3–9, Pharmacia), and stained with Alcian blue using described procedures [21]; however, this yielded no detectable glycoprotein at acidic p*I*s. Using the periodic acid–Schiff reagent [22], a singular band corresponding to that of coniferin-hydrolysing β-glucosidase was glycoprotein positive.

Cation exchange high performance liquid chromatography (HPLC-CEC). Following elution of protein from gel fractions obtained by preparative IEF (pH 4.0–6.5), the solutions were filtered (0.2 μm) and aliquots (100 μl ≡ 100 μg protein) were injected (Rheodyne 7125) on to a wide-pore CBX cationic exchange column (5 μm particles, 7.75 × 100 mm, JT Baker) pre-equilibrated with K-Pi buffer (pH 4.6, 10 mM) flowing at 0.5 ml min^{-1} (LDC constametric III pumps controlled by a LDC Gradient Master, Milton Roy). Elution was isocratic, changing after 20 ml of pH 4.6 buffer to pH 9.0 K-Pi (500 mM) and monitoring at 280 nm (LDC Spectromonitor 3100, Milton Roy). Fractions corresponding to 280 nm peaks were collected, concentrated and dialysed by Centriprep 10 to 250 μl, and tested for activity. Active fractions were pooled and stored in cryovials at −18°.

Protein estimation. Soluble protein was estimated by a modified Bradford method [23] using bovine serum albumin as standard.

Enzyme assays. To visualize β-glucosidase activity following native PAGE or analytical IEF, minigels were incubated in 4-methylumbelliferyl β-D-glucoside solution (10 mM in 0.1 M citrate buffer, pH 5.0, 30°), observing 4-methylumbelliferone fluorescence by UV light. To detect indoxyl formation following analytical IEF, the surfaces of minigels were wetted with indican (10 mM in 0.1 M citrate buffer, pH 5.0, 30°) and left in air (ambient temp., 4 hr).

Following preparative IEF and native PAGE, gel beds were divided into equal sections. The gel from each section was scraped into a 5 ml plastic syringe having glass wool at the outlets. Protein was eluted by addition of 4 ml CO_2-free all-glass double-distilled H_2O. The pH of each eluate was determined, then adjusted to pH 5.8. Aliquots of each eluate were incubated with coniferin (4 hr, 30°) and analysed by GC-MS (see below). Fractions containing coniferin-hydrolysing β-glucosidase activity were transferred to Centriprep 10 tubes, concentrated, transferred to cryovials, capped and stored at −18°.

Coniferin hydrolysis was assayed spectrophotometrically as follows. NaOAc buffer (pH 5.8, 0.1 M, 1.35 ml) and soluble protein (100 μl, 10–50 μg) were combined and equilibrated at 30°. Coniferin (50 μl ≡ 50 μg) was added and the mixture was incubated (30°), monitoring the change in absorbance from 258 nm to 264 nm (HP 8452 UV/vis spectrophotometer, 2 nm photodiodes, Hewlett Packard) with time.

Gas chromatography-mass spectrometry. Coniferin (1.5 mg) in NaOAc buffer (pH 5.8, 100 mM, 1.5ml) with or without enzyme (i.e. gel eluate) additions was incubated (30°, 5 hr) as triple replicates. Incubations were terminated by extracting solutions with equal volumes of EtOAc (3 × 1.5 ml), combining the three EtOAc extracts and drying under N_2. To the dry residue was added *N,O*-bis-(trimethylsilyl)trifluoroacetamide (100 μl, Pierce). After 1 hr at 70°, splitless injections (1.0 μl) were separated by gas chromatography (0.18 mm i.d. × 14 m cross-linked methyl silicone, 0.4 μm thickness, DB-1, J&W Sci.). After 1 min at 40°, temperature programming at 16° min^{-1} to 300° followed. Carrier gas (He) flowed at 0.55 ml min^{-1} (36 cm sec^{-1}). Electron impact (70 eV) full-scan spectra were collected from 8 to 16 min using a mass spectrometer (HP5970B hyperbolic quadrupole mass filter with *m/z* 0.1 steps, Hewlett Packard). Data were stored, analysed and used to generate hard-copy reports using a HP59970C ChemStation (Hewlett Packard). Coniferyl alcohol production from coniferin was estimated from relative abundances of *m/z* 324, 309, 293, 204 at the retention time of coniferyl alcohol (11.5 min), using known masses of synthetic coniferyl alcohol (Fluka) as reference standard.

Protein sequencing. The concentrated pH 4.0–4.2 zones obtained from the pH 4.0–6.5 prep. IEF step were pooled and subjected to a second prep. IEF (pH 2.0–4.5) run, yielding a singular protein band of p*I* 3.8 by analytical IEF (pH 3–9, PhastSystem minigel, Pharmacia). Following elution from the pH 2.0–4.5 gel, the protein was lyophillized, reconstituted in H_2O, mixed with an equal volume of SDS reducing buffer (4.0 ml H_2O, 1.0 ml Tris–HCl (pH 6.8, 1.0 M), 1.6 ml glycerol, 1.6 ml 20% (w/v) sodium dodecyl sulphate, 0.8 ml 2,β-mercaptoethanol, 0.2 ml 0.1% (w/v) bromophenol blue), heated at 100° for 4 min, and loaded on to a 10% acrylamide minigel (83 × 52 × 0.75 mm). SDS-PAGE was done on a Mini-Protean II unit (Bio-Rad) according to the manufacturer's specifications (Mini-Protean II Dual Slab Cell Instruction Manual, Bio-Rad). Following SDS-PAGE, minigels were soaked in transfer buffer (10 mM 3-(cyclohexylamino)-1-propanesulphonic acid–10% methanol, pH 11.0, 5 min). Proteins were electro-transferred (250 mA, 15 min) on to polyvinylidene difluoride membrane (PVDF Problot, Applied Biosystems) using a mini trans-blot electrophoretic transfer cell (Bio-Rad) [24].

PVDF Problot membranes were washed in double-distilled H_2O (5 changes, 5 min, ambient temp.), stained with 0.1% Coomassie Blue R-250 in 50% MeOH (5min, ambient temp.), and then destained in 50% MeOH–10% HOAc (5 min, ambient temp.). The membranes were finally rinsed in double-distilled H_2O (5 min, ambient temp.), air-dried and stored at −18° between sheets of blotting paper.

N-Terminal sequencing of protein on PVDF Problot membranes was done according to general protocol [25] by Ms France Dumas (NRC-Biotechnology Research Institute, Montreal, Canada) using an automated Edman-degradation system (Model 470A gas-phase sequencer equipped with on-line model 120A phenylthiohydantoin analyser (Applied Biosystems). Blotted protein (25–50 pmol) was loaded on to the sequencer with a glass-fibre filter disk (trifluoroacetic acid pretreated). A standard sequencing program (03RPTH, Applied Biosystems) was employed.

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