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Isolation and Characterization of *p*-Coumaroyl Esterase from the Anaerobic Fungus *Neocallimastix* Strain MC-2

W. SCOTT BORNEMAN,[1,2]* LARS G. LJUNGDAHL,[2] ROY D. HARTLEY,[1] AND D. E. AKIN[1]

Richard B. Russell Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Athens, Georgia 30613,[1] and Department of Biochemistry, Center for Biological Resource Recovery, University of Georgia, Athens, Georgia 30602[2]

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An extracellular *p*-coumaroyl esterase produced by the anaerobic fungus *Neocallimastix* strain MC-2 released *p*-coumaroyl groups from *O*-[5-*O*-((*E*)-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (PAXX). The esterase was purified 121-fold from culture medium in successive steps involving ultrafiltration, column chromatography on S-Sepharose and hydroxylapatite, isoelectric focusing, and gel filtration. The native enzyme had an apparent mass of 11 kDa under nondenaturing conditions and a mass of 5.8 kDa under denaturing conditions, suggesting that the enzyme may exist as a dimer. The isoelectric point was 4.7, and the pH optimum was 7.2. The purified esterase had 100 times more activity towards PAXX than towards the analogous feruloyl ester (FAXX). The apparent K_m and V_{max} of the purified *p*-coumaroyl esterase for PAXX at pH 7.2 and 40°C were 19.4 μM and 5.1 μmol min^{-1} mg^{-1}, respectively. *p*-Coumaroyl tetrasaccharides isolated from plant cell walls were hydrolyzed at rates similar to that for PAXX, whereas a dimer of PAXX was hydrolyzed at a rate 20-fold lower, yielding 4,4′-dihydroxy-α-truxillic acid as an end product. Ethyl and methyl *p*-coumarates were hydrolyzed at very slow rates, if at all. The purified esterase released *p*-coumaroyl groups from finely, but not coarsely, ground plant cell walls, and this activity was enhanced by the addition of xylanase and other cell wall-degrading enzymes.

An understanding of relationships between plant cell wall constituents and wall biodegradation is important to the economics of animal production. Such an understanding is also important in elucidating the role of fiber in human nutrition, in the decomposition of organic matter, in the production of chemical feedstocks, and in bioremediation.

Various components of plant cell walls, including lignin, cellulose with highly ordered structure, and acetyl groups linked to hemicellulose have been suggested as possible factors limiting the rumen degradation of wall polysaccharides. Studies involving various methodologies have consistently indicated that phenolic constituents are among the factors most inhibitory to the biodegradability of plant cell wall polysaccharides (1, 12, 16). The xylans of graminaceous cell walls are often highly substituted with arabinosyl, *O*-acetyl, and uronyl residues; many of the arabinosyl residues are esterified with *p*-coumaroyl and feruloyl groups (18). The in vitro esterification of phenolic acid to plant walls significantly reduced the biodegradability of plant cell walls (5). Another possible mechanism for limiting wall biodegradation is the cross-linking of xylan chains through the dimerization of *p*-coumaroyl and feruloyl groups, a process shown to occur in vitro by sunlight (19).

Little is known about the enzymes capable of releasing aromatics that are covalently linked to xylans. An esterase that cleaves *p*-coumaroyl groups from corn-stover "lignocellulose" has been identified (14), and an esterase that cleaves feruloyl groups from arabinoxylans has been found in the rumen bacterium *Fibrobacter succinogenes* (25) and the aerobic fungus *Schizophyllum commune* (24). Borneman and Akin (6) reported that *F. succinogenes* and *Butyrivibrio fibrisolvens* had feruloyl esterase activity against *O*-[5-*O*-((E)-feruloyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (FAXX), but neither of these bacteria nor *Ruminococcus flavefaciens* released *p*-coumaroyl groups from *O*-[5-*O*-((E)-*p*-coumaroyl)-α-L-arabinofuranosyl]-(1→3)-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (PAXX). To date, no rumen bacterium has been reported to possess *p*-coumaroyl esterase activity.

The anaerobic rumen fungi are known to preferentially colonize and degrade lignified cell walls, which are the least biodegradable walls (2). Recently, these fungi were found to produce high levels of both *p*-coumaroyl and feruloyl esterase (6, 9). The present work reports the purification and characterization of an extracellular *p*-coumaroyl esterase from the anaerobic fungus *Neocallimastix* strain MC-2.

MATERIALS AND METHODS

Fungal culture conditions. The anaerobic rumen fungus *Neocallimastix* strain MC-2 was maintained as described previously (7). For enzyme production, the fungus was grown at 39°C for 5 days in 20-liter Pyrex carboys, each containing 14 liters of the basal medium of Caldwell and Bryant (11) and 50 g of coastal Bermuda grass [*Cynodon dactylon* (L.) Pers.] (CBG) cell walls (8). The medium was autoclaved 1 h, after which cysteine HCl (0.05%, wt/vol) was added. The medium was then cooled under a stream of CO_2. Oxytetracycline (15 μg ml^{-1}), penicillin (1,000 U ml^{-1}), and chloramphenicol (12.5 μg ml^{-1}) were filter sterilized (0.2-μm-pore-size Acrodisc; Gelman Science, Ann Arbor, Mich.) and added just prior to inoculation. The inoculum consisted of a 5% (vol/vol) 72-h culture which had been previously transferred three consecutive times in the basal broth medium containing CBG cell walls plus antibiotics.

Enzyme assays. One unit of enzyme activity was defined as the amount which released 1 μmol of product min^{-1}, and specific activity is given in units per milligram of protein. Protein was determined by the method of Bradford (10).

* Corresponding author.

Assays for *p*-coumaroyl esterase and feruloyl esterase were performed at 40°C at pH 7.2, using PAXX and FAXX obtained from CBG cell walls as substrates (8). A similar assay was used for determining activity towards the *p*-coumaroyl tetrasaccharides [5-*O*-((E)-*p*-coumaroyl) (*O*-β-D-xylopyranosyl-(1→2))-*O*-α-L-arabinofuranosyl-(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (PAX_3-I) and *O*-β-D-xylopyranosyl-(1→4)-*O*-[5-*O*-((E)-*p*-coumaroyl)-α-L-arabinofuranosyl(1→3)]-*O*-β-D-xylopyranosyl-(1→4)-D-xylopyranose (PAX_3-II) isolated from CBG cell walls (17). The structures of the *p*-coumaroyl tetrasaccharides are shown in Fig. 1a. Assays for ethyl and methyl *p*-coumaroyl esterases were performed as described previously (9). Cellulase, xylanase, xylosidase, arabinosidase, and acetyl esterase were assayed as described previously (7).

The ability of *p*-coumaroyl esterase alone and in combination with other cell wall-degrading enzymes to release *p*-coumaroyl and feruloyl groups was studied, using intact cell walls as substrate. CBG was ground to allow it to pass through a 80-μm-pore-size mesh screen in a centrifugal mill (Retsch; Brinkmann Instruments, Los Angeles, Calif.). Triplicate tubes containing CBG (10 mg), enzymes, sodium azide (0.02%, wt/vol) and 100 mM 1,3-bis[tris(hydroxymethyl)-methylamino]propane (BTP; pH 7.0) in 2.0 ml were incubated at 40°C for 5 h, after which the reactions were halted by freezing the tubes in liquid nitrogen. Control tubes were run by the same procedure, using autoclaved enzyme solutions. The enzymes were as follows: *p*-coumaroyl esterase (this paper), xylanase (catalog number X 3876; Sigma Chemical Co., St. Louis, Mo.), driselase (catalog number D 9515, Sigma; purified as previously described [8]), and *Neocallimastix* strain MC-2 culture supernatant (see "Enzyme purification"). The enzymes were assayed as previously described (7, 9). The driselase and *Neocallimastix* strain MC-2 culture supernatants, respectively, contained the following per milliliter: *p*-coumaroyl esterase, 0 and 1.9 mU; feruloyl esterase, 0 and 2.4 mU; exoglucanase, 22.5 and 7.5 mU; endoglucanase, 833 and 294 mU; β-glucosidase, 89 and 21 mU; xylanase, 1 and 1 U; and β-xylosidase, 19 and 69 mU. *trans*-*p*-Coumaroyl and *trans*-feruloyl moieties released from CBG cell walls were determined by high-pressure liquid chromatography (HPLC) (8). Reducing sugars released from CBG cell walls were analyzed by the tetrazolium blue method (23), with xylose as a reference.

Acetylxylan esterase was assayed by measuring the acetyl groups released from acetylated oat spelt xylan. Acetylated xylan was prepared by the method of Johnson et al. (22). The assay consisted of 475 μl of a 4% (wt/vol) suspension of acetylxylan in 50 mM BTP buffer and 25 μl of purified enzyme (1 μg of protein). The mixture was incubated 30 min at 40°C. The reaction was halted by the addition of 25 μl of 6 N HCl. The acetyl groups released as acetate were measured with a commercial enzyme kit (catalog number 148261; Boehringer Mannheim, Indianapolis, Ind.).

Activity towards dimerized PAXX (Fig. 1b) was determined by analyzing the reaction products formed by reverse-phase HPLC; PAXX was dimerized by the method of Hartley et al. (19). The assay consisted of 100 μg of dimerized PAXX dissolved in 500 μl of BTP buffer (pH 7.0) and 25 μl of *p*-coumaroyl esterase solution (1 μg of protein). The mixture was incubated for 4 h at 40°C. The reaction was halted by the addition of 50 μl of 20% (vol/vol) formic acid. The reaction products were separated by reverse-phase HPLC by a modification of a previously described method (8). The mobile phase contained a final methanol concentration of 18% (vol/vol), and the absorbance was measured at 280 nm. 4,4′-Dihydroxy-α-truxillic acid was prepared as described by Hartley et al. (20) and used as an assay standard.

Molecular weight determination. The molecular weight of the purified native enzyme was determined by fast protein liquid chromatography (FPLC) gel filtration on two BioSil TSK SEC-250 columns in series (0.75 by 60 cm each). The column was equilibrated with 50 mM BTP buffer (pH 7.0) containing 150 mM NaCl, 0.02% sodium azide, and 5% (wt/vol) betaine to prevent protein aggregation (25a). The enzymes were eluted at a flow rate of 0.4 ml min^{-1}. Standards used were sheep immunoglobulin G (IgG) (150 kDa), bovine serum albumin (66 kDa), Fab fragment from IgG (50 kDa), carbonic anhydrase (29 kDa), horse heart cytochrome *c* (12.5 kDa), and bovine lung aprotinin (6.5 kDa). The column was calibrated with the standards before and after the enzyme was chromatographed.

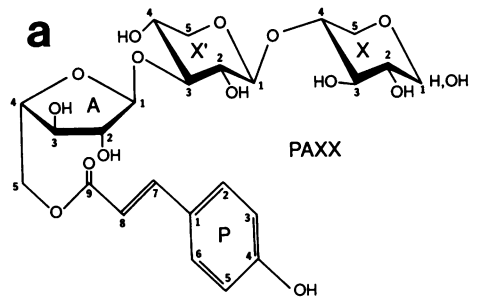
Agarose IEF. Analytical isoelectric focusing (IEF) was performed with a Bio-Rad model 1415 horizontal electrophoresis cell. The gel was a precast agarose electrofocusing gel (pH range, 3 to 7; FMC BioProducts, Rockland, Maine). The samples and standards were focused, fixed, and stained with Coomassie brilliant blue R-250 according to the manufacturer's procedure (14a). Isoelectric points were determined by using IEF protein markers (catalog number I 8012; Sigma).

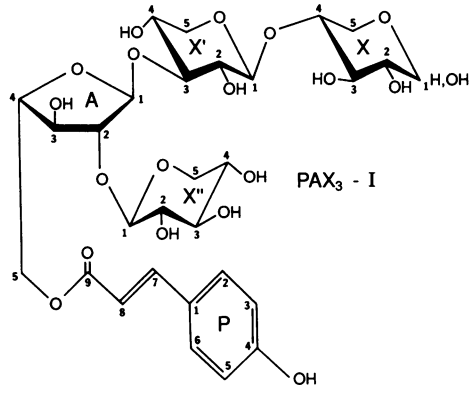
SDS-PAGE. Enzyme purity was checked and subunit composition was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was performed by the method of Schägger and von Jagow (26). This method utilizes tricine as a trailing ion to allow for the superior resolution of small proteins and peptides (1 to 20 kDa). Molecular weights were determined by using a low-range molecular weight marker kit (catalog number SE130001; Endoprotech, Hyde Park, Mass.) and molecular weight markers for peptides (catalog number MW-SDS-17S; Sigma). Protein bands were stained by a combined Coomassie blue-silver stain procedure (13).

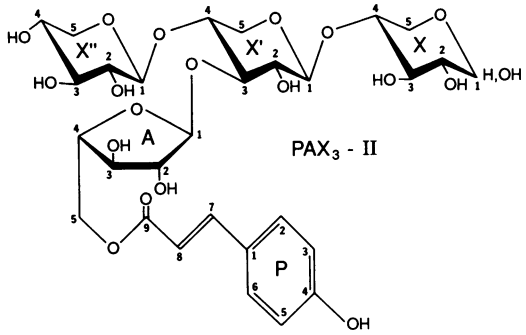
Other analytical procedures. Protein was measured by the method of Bradford (10) with a commercial protein assay kit (Pierce, Rockford, Ill.). Bovine serum albumin was used as the standard. The phenolic acid content of protein fractions containing nonprotein aromatics was determined by saponification with sodium hydroxide (1.0 M) (9). Glycoprotein stains of SDS-PAGE were performed as described by Gerard (15) by using the periodic acid-Schiff (PAS) base reagent. The carbohydrate content of the pure enzyme was further characterized by monosaccharide analysis using high pH anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) (Dionex BioLC carbohydrate system; Dionex, Sunnyvale, Calif.) as described by Weitzhandler and Hardy (29).

The pH optimum of the pure enzyme was determined at 40°C with PAXX as the substrate. Buffers (50 mM) used were sodium acetate (range, pH 4.0 to 5.5), sodium phosphate (pH 5.5 to 7.5), and tris(hydroxymethyl)-aminomethane HCl (Tris-HCl) (range, pH 7.0 to 9.0). The effect of substrate concentration on *p*-coumaroyl esterase activity was examined at 40°C in 50 mM BTP buffer (pH 7.2) by using appropriately diluted purified enzyme and PAXX at various concentrations between 0.1 and 100 μg ml^{-1}. K_m and V_{max} values were calculated by the double-reciprocal method of Lineweaver and Burk.

The effect of divalent cations was tested by using purified enzyme after it had been dialyzed against 50 mM BTP in a Centricon 10 microconcentrator (Amicon, Beverly, Mass.) to remove EDTA present in storage buffer. The chloride







salts of the cations were dissolved in 50 mM BTP buffer (pH 7.0) to give final cation concentrations of 1 and 5 mM. The enzyme was incubated for 15 min (4°C) with the various cations, and residual enzymatic activity was measured. The assay was carried out for 20 min at 40°C with PAXX as the substrate. The effects of NaCl and KCl (30 and 150 mM), EDTA (0.1 and 1.0 mM), dithiothreitol (1.0 and 10 mM), SDS (0.1%, wt/vol), betaine (5%, wt/vol), and glycerol (15%, vol/vol) on *p*-coumaroyl esterase activity were similarly examined. The enzyme was preincubated with the reagents dissolved in 50 mM BTP buffer for 1 h at 4°C and then assayed at 40°C.

Enzyme purification. The enzyme purification was performed at 4°C. After 5 days of growth, the culture was filtered (50 mesh nylon) to remove plant cell wall fragments and fungal biomass. The residual cell walls were washed with 1 liter of 50 mM histidine buffer (pH 6.0) and then with 1 liter of distilled water. Culture supernatant and cell wall washings were combined, concentrated 100-fold, and dialyzed against 25 liters of 20 mM histidine buffer (pH 6.0) containing 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.02% (wt/vol) sodium azide by using tangential flow ultrafiltration (Pellicon Cassette System with PTGC 10,000 NMWL cassette; Millipore, Bedford, Mass.). The final concentrate (155 ml) was first centrifuged (20,000 × *g*) for 20 min to remove precipitated material and then clarified by centrifugation at 100,000 × *g* for 1 h.

The concentrate (155 ml) was applied to a column (3.2 by 30 cm) of S-Sepharose Fast Flow (Pharmacia Fine Chemicals, Piscataway, N.J.). Elution was with 500 ml of 50 mM histidine buffer (pH 6.0) and then with 1 liter of NaCl gradient (0 to 1.0 M) in the same buffer at a flow rate of 5 ml min^{-1}. Fractions of 10 ml were collected.

Fractions containing *p*-coumaroyl esterase activity were combined and dialyzed for 24 h against 10 mM sodium phosphate buffer (three times, 4 liters each; pH 6.7) containing 1 mM EDTA and 1 mM PMSF. The dialysate was concentrated to 40 ml by ultrafiltration (UK 10,000; Micro Filtration Systems, Dublin, Calif.). Aliquots (8 ml) were applied to a column of hydroxylapatite (2 by 18 cm; HT, Bio-Rad, Richmond, Calif.) connected to an FPLC system (Pharmacia). Enzymes were eluted at a flow rate of 0.5 ml min^{-1} with 50 ml of 10 mM sodium phosphate buffer (pH 6.7), with 125 ml of the buffer (gradient, 0 to 300 mM), and

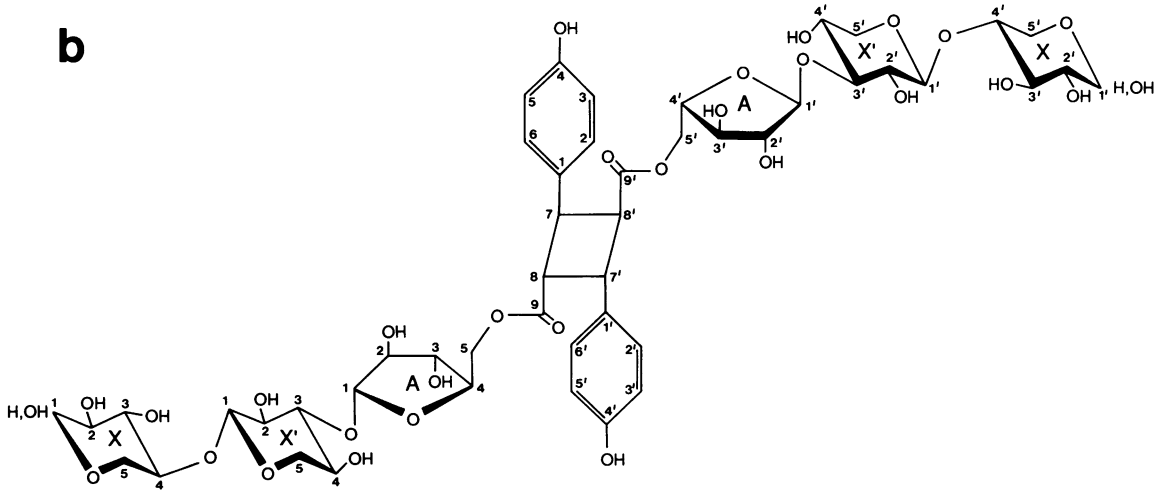


FIG. 1. Structures of the *p*-coumaroyl oligosaccharides (a) and dimerized PAXX (b) that are used as substrates for the purified *p*-coumaroyl esterase.

then with a further 25 ml of buffer (gradient, 300 to 500 mM). Fractions of 5 ml were collected.

Fractions with *p*-coumaroyl esterase from the hydroxylapatite column were combined and dialyzed for 24 h against 1 mM sodium phosphate buffer (three times, 4 liters each; pH 6.7). The dialysate was concentrated by ultrafiltration to 10 ml as described above. IEF was performed in a Rotofor preparative IEF cell (Bio-Rad). The following protocol was used to help prevent protein precipitation during IEF. Distilled water (40 ml) containing 2% (wt/vol) ampholytes (Biolyte pH 3 to 5, 1.0% [wt/vol]; Biolyte pH 5 to 8, 1.0% [wt/vol]; Bio-Rad), 3 M urea, and glycerol (10%, vol/vol) was prefocused in the Rotofor cell for 1 h at 12 W of constant power and 4°C. Prefocusing protected the enzyme solution from any abrupt pH fluctuations during the gradient formation of the ampholytes. The enzyme solution was applied to the center of the Rotofor cell, where the pH was close to the isoelectric point of the *p*-coumaroyl esterase. The solution was focused for 4 h at 12 W of constant power until the voltage and current remained constant (start conditions, 600 V and 18 mA; end conditions, 1,000 V and 11 mA). Fractions (2.5 ml) were collected, and the pH and *p*-coumaroyl esterase activity were determined.

The active fractions were combined and dialyzed against 50 mM BTP buffer (pH 7.0)–1 mM EDTA–1 mM PMSF (three times, 4 liters each). The dialysate was concentrated to 1.5 ml as described above. An aliquot (0.5 ml) was applied to two BioSil TSK SEC-250 columns (0.75 by 60 cm each; Bio-Rad) connected in series to an FPLC. The column was equilibrated with 50 mM BTP buffer (pH 7.0) containing 5% (wt/vol) betaine, 150 mM NaCl, 0.02% (wt/vol) sodium azide, 1 mM EDTA, and 1 mM PMSF. The enzymes were eluted at a flow rate of 0.4 ml min^{-1}, and fractions of 1 ml were collected. The fractions containing *p*-coumaroyl esterase activity were combined and dialyzed against 20 mM BTP buffer (pH 7.0)–1 mM EDTA–1 mM PMSF. Glycerol (15%, vol/vol) was added to the pure enzyme dialysate, and it was stored at −80°C.

RESULTS

Purification of *p*-coumaroyl esterase. A summary of the purification of *p*-coumaroyl esterase is presented in Table 1. Four separate purifications were performed, with similar results. A crude enzyme preparation was made by a 100-fold concentration of the culture supernatant by ultrafiltration (10,000-M_r cutoff). The retentate contained 98% of the original activity.

In the S-Sepharose chromatography step, *p*-coumaroyl esterase and a majority of other proteins did not bind to the column. This step, however, greatly improved the ensuing purification, as it prevented the formation of large protein aggregates that made further purification steps difficult.

Chromatography on hydroxylapatite resulted in a 4.9-fold increase in the specific activity of the enzyme, with a loss of 51% of the total activity (Table 1). A major part of *p*-coumaroyl esterase activity was bound to the column and eluted with 150 mM sodium phosphate (Fig. 2, fractions 22 to 24). Additional activity was eluted after the main peak, but it was of low specific activity and was not further investigated. Fractions 22 to 24 were combined, dialyzed, and concentrated as described above. Absorption spectra of the concentrate revealed a maximum at 280 nm (indicating protein aromatics) and a second maximum at 310 nm, suggesting that nonprotein aromatics were present. Saponification revealed that the pooled hydroxylapatite fraction containing *p*-coumaroyl esterase activity contained 790 ng of *p*-coumaroyl and 60 ng of feruloyl moieties per mg of protein.

TABLE 1. Purification of *p*-coumaroyl esterase from *Neocallimastix* strain MC-2[a]

Purification step	Total protein (mg)	Total enzyme (U)[b]	Sp act (U mg^{-1})	Yield (%)	Purification (fold)
1. Culture supernatant	4,420	177	0.041	100	1.0
2. Concentrate	3,930	173	0.043	98	1.1
3. S-Sepharose	2,750	154	0.056	87	1.4
4. Hydroxylapatite	406	78.7	0.27	45	4.9
5. IEF	4.67	7.83	1.67	4.4	42
6. Gel filtration	0.88	4.32	4.91	2.5	121

[a] The purification was from 20-liter carboy cultures.
[b] Units are defined as micromoles of *p*-coumaroyl groups released from PAXX per minute at 40°C and pH 7.2.

Further purification was achieved by preparative IEF. This procedure resulted in a 42-fold purification over the crude supernatant. The major part of the esterase activity focused within a pH range of 4.6 to 4.9. This technique was essential for the separation of the *p*-coumaroyl esterase from the protein fraction associated with phenolic compounds. During IEF, protein-phenolic complexes containing some of the *p*-coumaroyl esterase activity migrated to the anode, whereas the noncomplexed *p*-coumaroyl esterase migrated to its isoelectric point. This step resulted in a 10-fold loss of activity but was found to be necessary to separate the phenolic-enzyme complex from the noncomplexed enzyme. All purification steps that were attempted in which the phenolic moieties were associated with the enzyme resulted in aggregation, and upon fractionation, *p*-coumaroyl esterase activity was found in multiple fractions.

The final step involved gel filtration using two BioSil TSK SEC-250 columns in tandem (Fig. 3). It yielded an enzyme preparation (fractions 26 to 28) purified 121-fold, with an overall recovery of 2.5% (Table 1). When betaine was included in the eluant, the recovery of the *p*-coumaroyl esterase increased threefold. Absorption spectra of the pu-

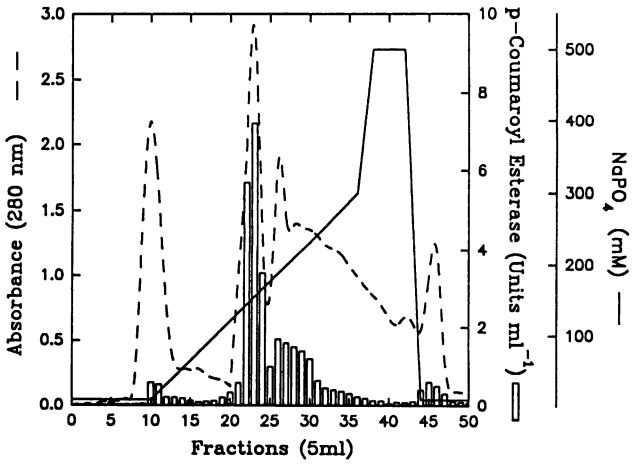


FIG. 2. Hydroxylapatite chromatography (Table 1, step 4) of *p*-coumaroyl esterase. The column was equilibrated with 10 mM sodium phosphate buffer (pH 6.7) at a flow rate of 0.5 ml min^{-1}. The enzyme was eluted with 50 ml of buffer, with a 125-ml buffer gradient (0 to 300 mM), and then with a 25-ml buffer gradient (300 to 500 mM).

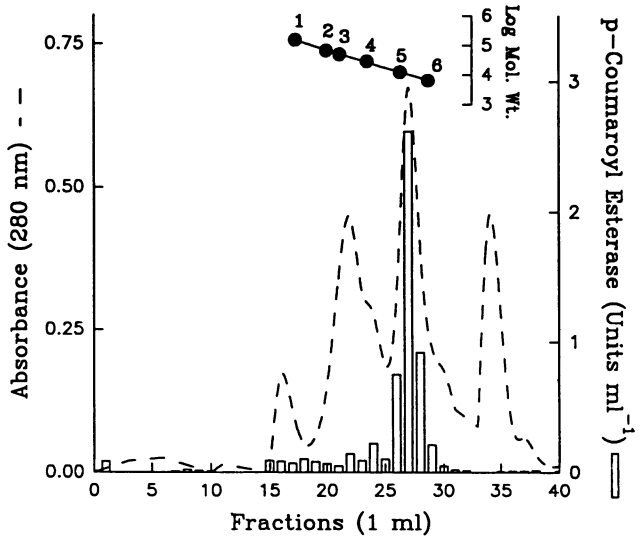


FIG. 3. BioSil TSK SEC-250 chromatography (Table 1, step 6) of *p*-coumaroyl esterase. The column was equilibrated with 50 mM BTP buffer (pH 7.0) containing 5% betaine (wt/vol), 150 mM NaCl, 0.02% (wt/vol) sodium azide, 1 mM EDTA, and 1 mM PMSF. The flow rate was 0.4 ml min^{-1}. The column was calibrated with the following, indicated by solid circles: 1, sheep IgG (150 kDa); 2, bovine serum albumin (66 kDa); 3, Fab fragment from IgG (50 kDa); 4, carbonic anhydrase (29 kDa); 5, horse heart cytochrome *c* (12.5 kDa); 6, bovine lung aprotinin (6.5 kDa).

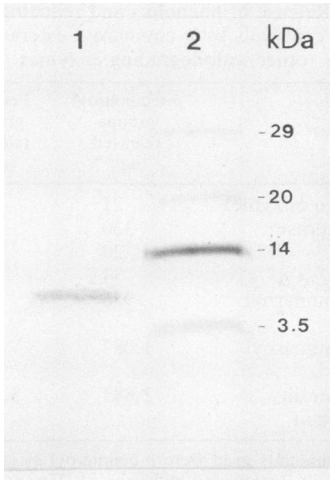


FIG. 4. SDS-PAGE of the purified *p*-coumaroyl esterase (4.25 μg) (lane 1) and molecular weight protein standards (lane 2).

rified enzyme revealed no absorbance at 310 nm, suggesting that no contaminating phenolic compounds were present. The purified enzyme was stored at −80°C in the elution buffer after the addition of glycerol (15%, vol/vol). The frozen enzyme lost 40% of its original activity after 1 month, compared with a 70% loss of activity with no added glycerol.

The enzyme was tested for purity by SDS-PAGE and gave a single protein band visualized by double staining with Coomassie and silver stain (Fig. 4). The polypeptide was estimated to have an M_r of 5,800. Chromatography with BioSil TSK SEC-250 was used to determine the M_r for the enzyme under nondenaturing conditions. As shown in Fig. 3, gel filtration indicated an M_r of about 11,000. PAS staining of SDS polyacrylamide gels initially suggested that the enzyme was glycosylated, but a more precise analysis of the monosaccharide content of the enzyme by HPAE-PAD demonstrated that the purified enzyme was not glycosylated.

Properties of the purified *p*-coumaroyl esterase. The enzyme had optimal activity at pH 7.2 and was stable from pH 5.0 to 8.5. *p*-Coumaroyl esterase obeyed Michaelis-Menten kinetics. The V_{max} and K_m values for PAXX were 5.1 μmol min^{-1} mg^{-1} and 19.4 μM, respectively, at pH 7.2 and 40°C. The purified enzyme maintained ≥91% activity for 6 h at 40°C in the presence of PAXX. The isoelectric point was determined to be 4.7 by analytical IEF.

The effect on the enzyme of divalent cations Zn^{2+}, Ca^{2+}, Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Fe^{2+}, and Hg^{2+} was tested at 1 and 5 mM concentrations. Hg^{2+} and Fe^{2+} at 1 mM concentration inhibited enzyme activity by 58 and 19%, respectively. At 5 mM concentration, Hg^{2+}, Fe^{2+}, Co^{2+}, and Cu^{2+} inhibited activity by 72, 30, 23, and 16%, respectively. When the esterase was assayed in the presence of some commonly used reagents in protein purification, its activity was inhibited 26% by 10 mM dithiothreitol, 85% by 0.1% (wt/vol) SDS, and 15% by 150 mM NaCl. Activity of the purified esterase was increased 12% in glycerol (15%, vol/vol). One millimolar EDTA, 4 M urea, 5% (wt/vol) betaine, and 150 mM KCl had little effect on activity.

Activity toward various substrates. The ability of the purified enzyme to hydrolyze other carbohydrates and phenolic-carbohydrate esters was examined (Table 2). In addition to releasing *p*-coumaroyl groups from PAXX, the enzyme released trace amounts of feruloyl groups from FAXX, but at a 100-fold lower rate. When activity against *p*-coumaroyl tetrasaccharides was compared with that for PAXX (Fig. 1a), the esterase exhibited a 22% increase in activity toward PAX_3-I and a 11% decrease in activity toward PAX_3-II. Dimerized PAXX was used (Fig. 1b) to examine the ability of the enzyme to hydrolyze a larger phenolic-carbohydrate complex which is probably present in plant cell walls (19). The enzyme was able to cleave both ester bonds, forming 4,4′-dihydroxy-α-truxillic acid as the end product. However,

TABLE 2. Substrate specificity of *p*-coumaroyl esterase[a]

Substrate	Activity (U mg^{-1})
PAXX[b]	4.91
PAX_3-I[b]	6.01
PAX_3-II[b]	4.34
Dimerized PAXX[b]	0.26
FAXX[c]	0.05
CBG cell walls (80 μm)	0.07
CBG cell walls (1 mm)	0
Ethyl *p*-coumarate	0.004
Methyl *p*-coumarate	0.003
Acetylxylan	0
Xylan	0
Avicel	0
Carboxymethyl cellulose	0
PNP-acetate[d]	0.002
PNP-glucopyranoside	0.001
PNP-xylopyranoside	0
PNP-arabinofuranoside	0

[a] Average of four assays in duplicate varied <10%; assays were performed as described in Materials and Methods.
[b] Structures are defined in Materials and Methods and are shown in Fig. 1.
[c] FAXX is analogous to PAXX, with a feruloyl group replacing the *p*-coumaroyl group.
[d] PNP, *p*-nitrophenyl.

TABLE 3. Release of phenolics and reducing sugars from CBG cell walls by *p*-coumaroyl esterase and other wall-degrading enzymes

Enzyme(s)[a]	*p*-Coumaroyl groups released (ng)[b]	Feruloyl groups released (ng)[b]	Reducing sugars released (μg)[b]
Buffer control (no enzyme)	21	4	5.2
p-Coumaroyl esterase	320	17	6.7
Xylanase	20	13	38
Driselase	34	12	87
Xylanase + *p*-coumaroyl esterase	956	49	44
Driselase + *p*-coumaroyl esterase	1,087	40	115
Neocallimastix strain MC-2 supernatant	1,883	3,124	178

[a] Enzymes and amounts used were *p*-coumaroyl esterase, 2.5 mU; xylanase, 1 U; driselase (containing xylanase, 1 U); xylanase, 1 U, plus *p*-coumaroyl esterase, 2.5 mU; driselase (containing xylanase, 1 U) plus *p*-coumaroyl esterase, 2.5 mU; *Neocallimastix* strain MC-2 supernatant (containing xylanase, 1 U) and *p*-coumaroyl esterase, 2.5 mU. Incubations were for 5 h at 40°C in 2 ml of 100 mM BTP, pH 7, containing 10 mg of CBG cell walls and 0.02% (wt/vol) NaN_3.

[b] Values are the means of triplicate analyses and varied <10%.

the specific activity toward dimerized PAXX was only 5% of that of the activity toward PAXX (Table 2), and the enzyme did not further hydrolyze the 4,4′-dihydroxy-α-truxillic acid even after extensive incubation periods. The purified *p*-coumaroyl esterase had little activity toward finely ground (approximately 80 μm) CBG walls (Table 2), the activity being only 1.4% of that of the enzyme toward PAXX. No free *p*-coumaroyl groups were released by the enzyme from CBG cell walls that were ground to allow them to pass through a 1-mm-mesh screen.

The enzyme did not release ester-linked acetyl groups from xylan, nor did it hydrolyze ethyl or methyl *p*-coumarate (Table 2). It did not hydrolyze any of the *p*-nitrophenyl esters tested, with the exception of trace amounts of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenylacetate. Also, the enzyme did not release reducing sugars from oat spelt xylan or from crystalline cellulose (Avicel) or amorphous carboxymethyl cellulose (Table 2).

The ability of the purified *p*-coumaroyl esterase, both alone and in combination with other cell wall-degrading enzymes, to release free *p*-coumaric and ferulic acids from finely ground CBG cell walls was examined (Table 3). Acting alone, the *p*-coumaroyl esterase released 320 ng of *p*-coumaroyl groups from 10 mg of CBG cell walls (80 μm). When *p*-coumaroyl esterase was combined with xylanase (lacking *p*-coumaroyl and feruloyl esterase) or driselase (containing exo- and endo-β-glucanases, xylanases, pectinases, and mannases but no phenolic esterase), the release of *p*-coumaroyl groups from 10 mg of cell walls increased to 956 and 1,087 ng, respectively, indicating interactions by the enzymes to release phenolic groups from the CBG cell walls. This enzyme interaction was further substantiated by using *Neocallimastix* strain MC-2 crude supernatant, which released 1,883 ng of *p*-coumaroyl groups, and by the effect of *p*-coumaroyl esterase on the release of reducing sugars by xylanase and driselase. The addition of 2.5 mU of *p*-coumaroyl esterase to xylanase and driselase increased the level of reducing sugars 16 and 32%, respectively.

DISCUSSION

Previous investigations have shown that colony development by ruminal fungi is preferentially initiated on phenolic-containing plant cell walls (2, 4). Phenolic-containing walls that are only partially degraded by rumen bacteria (e.g., sclerenchyma) are extensively and often completely solubilized by rumen fungi (2, 3). Other types of phenolic-containing tissue that are totally resistant to bacterial degradation (e.g., xylem) are partially degraded by rumen fungi. Evidence from ultrastructural studies suggests that rumen fungi possess enzymes that are different from those of bacteria, allowing the fungi to degrade these recalcitrant phenolic-containing walls. Enzymatic studies using PAXX and FAXX as substrates showed that three major fiber-degrading rumen bacteria (*F. succinogenes*, *B. fibrisolvens*, and *R. flavefaciens*) produced little feruloyl and no *p*-coumaroyl esterase activity (6). We propose that *p*-coumaroyl and feruloyl esterases may provide the anaerobic fungi with an advantage in the degradation of phenolic-containing plant cell walls and thus provide an ecological niche for the fungi in the rumen.

In the present work, *p*-coumaroyl esterase from the anaerobic fungus *Neocallimastix* strain MC-2 was purified by monitoring enzyme activity, which was determined by the release of *p*-coumaroyl groups from PAXX. Several steps in the purification protocol, although resulting in a low yield, were necessary. The steps which separated the enzyme from the enzyme-phenolic aggregates were particularly important. It is well established that phenolics may cause aggregation and possible precipitation of proteins (27). When phenolics and proteins are present together in solution, a hydrophobic surface layer is formed, involving complexes of phenolic groups with the protein surface and cross-linkages of protein molecules by the phenolics. When *p*-coumaroyl esterase is being isolated from culture filtrates of *Neocallimastix* strain MC-2, the problem of protein-phenol aggregation was intensified. Cell walls of CBG, which were used as the fungal substrate, contain high levels of phenolics (9); some of these phenolics were released by the fungi into the medium and subsequently associated with proteins, including *p*-coumaroyl esterase. The use of other non-phenolic-containing substrates (i.e., xylan and cellulose) instead of CBG cell walls for the growth of *Neocallimastix* strain MC-2 was less desirable because of a 15-fold-lower production of *p*-coumaroyl esterase.

The purified enzyme separated by SDS-PAGE gave a positive test for glycoprotein by the PAS procedure, but a more sensitive test (HPAE-PAD) (29) for monosaccharides gave negative results. In this latter method, the purified enzyme was hydrolyzed with acid and assayed for monosaccharide composition. Possibly, a false positive was observed with PAS staining because of the incomplete removal of SDS (15) or because of variations in oxidation conditions (29).

EDTA and PMSF inhibit proteolysis in cultures of *Neocallimastix frontalis* (28) and were subsequently used in all purification steps. The molecular mass as determined by HPLC gel filtration under nondenaturing conditions was 11.0 kDa. Under the denaturing conditions of SDS-PAGE, a single protein band was observed that corresponded to a molecular mass of 5.8 kDa, suggesting that the enzyme exists as a dimer. It is noteworthy that four esterases from *Aspergillus niger* which hydrolyzed methyl esters of short-chain fatty acids (21) also have relatively low molecular masses (12.7 to 29.5 kDa).

The purified *p*-coumaroyl esterase displayed the highest activity toward low-molecular-weight *p*-coumaroyl-oligosac-

charide esters. The activity of the purified enzyme was decreased 95% with dimerized PAXX as the substrate. The addition of a 1→4 linked xylose residue to the X′ of PAXX (i.e., PAX_3-II; Fig. 1) reduced the specific activity by 11% whereas, when a xylose residue was 1→2 linked to the arabinose of PAXX (i.e., PAX_3-I; Fig. 1), activity increased 22%. Possibly, the structure of the phenolic-oligosaccharide ester impedes accessibility of the enzyme because of steric hindrance.

The purified esterase had no detectable activity toward CBG cell walls that were ground to pass through a 1-mm-pore-size sieve but had low levels of activity toward very finely ground (80-μm) cell walls, suggesting that substrate size and/or accessibility impedes enzyme activity. Other researchers have shown that feruloyl esterase has a greater specificity for short xylo-oligomers and that synergism occurs between xylanase and feruloyl esterase in the degradation of large feruloyl-polysaccharides (24). This is supported by our finding that the action of *p*-coumaroyl esterase in combination with xylanase releases substantially more *p*-coumaroyl groups from intact CBG walls than the esterase alone. Even greater amounts of *p*-coumaroyl groups were released from CBG cell walls when the purified esterase was combined with a mixture of cell wall-degrading enzymes, such as those present in driselase. The crude supernatant of *Neocallimastix* strain MC-2 produced much greater amounts than these enzyme combinations did. Although the supernatant contained the same xylanase and *p*-coumaroyl esterase activities as the other enzyme mixtures, it released twice the amount of *p*-coumaroyl groups. This ability of the *Neocallimastix* strain MC-2 enzyme preparation to release considerably more *p*-coumaroyl groups from the finely ground CBG walls than driselase plus *p*-coumaroyl esterase requires careful interpretation. Driselase contained much higher levels of exo- and endoglucanase, as well as β-glucosidase, while the *Neocallimastix* strain MC-2 enzyme supernatant had substantially more β-xylosidase. This suggests that β-xylosidase may be particularly important in acting synergistically with *p*-coumaroyl esterase to release phenolics. Previous research has suggested a positive correlation between the levels of β-xylosidase and *p*-coumaroyl esterase in removing *p*-coumaroyl groups from intact plant cell walls (9). However, *Neocallimastix* strain MC-2 may produce other enzymes not assayed or as yet unknown that impart the superior ability to release *p*-coumaroyl groups from cell walls. A likely hypothesis is that the actions of xylanase, β-xylosidase, and possibly other wall-degrading enzymes produce a *p*-coumaroyl-oligosaccharide substrate which can be readily degraded by the *p*-coumaroyl esterase. The different levels of reducing sugars liberated from the CBG cell walls by enzyme treatments suggest that *p*-coumaroyl esterase acts to free carbohydrates which are ester linked to phenolics and are not otherwise available for hydrolysis by other enzymes.

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