APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1399–1407 Vol. 61, No. 4
0099-2240/95/$04.00+0

Purification of *Thermotoga maritima* Enzymes for the Degradation of Cellulosic Materials

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Received 26 September 1994/Accepted 7 February 1995

A separation procedure for the analysis of the enzyme components of the hyperthermophilic bacterium *Thermotoga maritima* involved in cellulose and xylan degradation was developed. Resolution of the enzymes was achieved by a combination of fast protein liquid chromatography anion exchange and hydrophobic interaction chromatography. Enzyme fractions were assayed for hydrolysis of Avicel, carboxymethylcellulose (CMC), β-glucan, laminarin, xylan, *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-xyloside, *p*-nitrophenyl-α-L-arabinofuranoside, and 4-*O*-*methyl*-glucuronosyl-xylotriose. The activities of two cellulases, one laminarinase, one xylanase, two putative β-D-xylosidases, α-D-glucuronidase, and α-L-arabinosidase were identified. Because of their selective retardation on a Superdex gel filtration column, the two cellulases could be purified to homogeneity. According to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, molecular masses of 27 and 29 kDa, respectively, were determined for cellulase I and cellulase II. Maximal activities of both enzymes were observed at 95°C between pH 6.0 and 7.5. In the presence of 2.5 M NaCl the purified enzymes retained about 90% of their initial activities after a 6-h incubation at 80°C. On the basis of its activity towards CMC, cellulase I was classified as endo-β-1,4-glucanase. Cellulase II was able to attack Avicel in addition to CMC, β-glucan, and *p*-nitrophenyl-β-D-cellobioside. It releases cellobiose and cellotriose from Avicel. The latter product is further cleaved into glucose and cellobiose. Cellulase II may therefore be classified as exo-β-1,4-glucanase.

Microbial degradation of cellulose and hemicelluloses has enormous economic potential for the conversion of plant biomass into fuels and chemicals. Cellulose consists of linear chains of β-1,4-linked D-glucose residues. Its enzymatic hydrolysis is considered to require the action of both endoglucanases (1,4-β-D-glucan glucanohydrolase [EC 3.2.1.4]) and exoglucanases (1,4-β-D-glucan cellobiohydrolase [EC 3.2.1.91]). A synergistic interaction of these enzymes is necessary for the complete hydrolysis of crystalline cellulose (8). Xylans, major components of the hemicelluloses, are branched heteroglycans with a backbone of β-1,4-linked D-xylopyranosyl residues. Branches consist of α-1,3-linked L-arabinofuranosyl and α-1,2-linked 4-*O*-*methyl*-glucuronic acid residues. Enzymatic hydrolysis of the xylan backbone involves endo-β-1,4-xylanases (1,4-β-D-xylan xylanohydrolase [EC 3.2.1.8]), β-D-xylosidases (1,4-β-D-xyloside xylohydrolase [EC 3.2.1.37]), and possibly, exo-β-1,4-xylanases (1,4-β-D-xylan xylohydrolase). Removal of side groups is catalyzed by α-L-arabinofuranosidases (EC 3.2.1.55) and α-D-glucuronidases (EC 3.2.1). Esterase activities are responsible for the liberation of acetyl, coumaryl, and feruloyl substituents (9).

Thermophilic bacteria have received considerable attention as sources of highly active and thermostable cellulolytic and xylanolytic enzymes (6, 22, 34). Recently, the most extremely thermophilic organotrophic eubacteria presently known have been isolated and characterized. These bacteria, which belong to the genus *Thermotoga*, are fermentative microorganisms metabolizing a variety of carbohydrates (16). It seems reasonable to look for polysaccharide depolymerizing enzymes in these organisms in order to compare them with their counterparts from less thermophilic organisms that have been studied previously. The enzymes isolated to date from *Thermotoga maritima* all proved to be extremely thermostable (1, 7, 12, 19, 28, 38, 39). Reports concerning cellulolytic and xylanolytic enzymes are available for *Thermotoga neapolitana* (11) and for an isolate from New Zealand, *Thermotoga* sp. strain FjSS3-B.1 (23, 24, 33). Information on polysaccharide-hydrolyzing enzymes from *T. maritima* is limited to one report on amylolytic enzymes (29). Recently, β-galactoside- and β-glucoside-cleaving enzymes have been described for the latter organism (12, 13).

This article reports the resolution, purification, and preliminary characterization of various enzymes of *T. maritima* involved in the breakdown of cellulose and xylan.

MATERIALS AND METHODS

Preparation of the crude enzyme. *T. maritima* DSM 3109 was grown under anaerobic conditions at 80°C in the *Thermotoga* medium recommended by the DSM (German Collection of Microorganisms), in which starch was replaced by 0.5% xylose and 0.5% cellobiose. This medium contained (per liter) KH_2PO_4, 0.5 g; $NiCl_2 \cdot 6H_2O$, 2 mg; NaCl, 20 g; yeast extract (Difco), 0.5 g; Resazurin, 1 mg; $Na_2S \cdot 9H_2O$, 0.5 g; trace element solution, 15 ml; artificial seawater, 250 ml; and distilled water, 750 ml. The medium was adjusted to pH 6.5. The trace element solution contained (per liter) nitrilotriacetic acid, 1.5 g; $MgSO_4 \cdot 7H_2O$, 3 g; $MnSO_4 \cdot 2H_2O$, 0.5 g; NaCl, 1 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; $CoSO_4 \cdot 7H_2O$, 0.18 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 0.18 g; $CuSO_4 \cdot 5H_2O$, 0.01 g; $KAl(SO_4)_2 \cdot 12H_2O$, 0.02 g; H_3BO_3, 0.01 g; $Na_2MoO_4 \cdot 2H_2O$, 0.01 g; $NiCl_2 \cdot 6H_2O$, 0.025 g; and $Na_2SeO_3 \cdot 5H_2O$, 0.3 mg (pH 6.5). The artificial seawater contained (per liter) NaCl, 27.7 g; $MgSO_4 \cdot 7H_2O$, 7 g; $MgCl_2 \cdot 6H_2O$, 5.5 g; KCl, 0.65 g; NaBr, 0.1 g; H_3BO_3, 30 mg; $SrCl_2 \cdot 6H_2O$, 15 mg; citric acid, 10 mg; KI, 0.05 mg; and $CaCl_2 \cdot 2H_2O$, 2.25 g. The cells were harvested by centrifugation, washed twice with 20 mM Tris-HCl, pH 8.0, containing 1 M NaCl, and suspended in 20 mM Tris-HCl, pH 8.0. The cell extract was prepared by passage through an Aminco French pressure cell. Debris was removed by repeated centrifugation (45,000 × *g*, 40 min, 4°C), and the cleared cell extract was dialyzed against the equilibration buffer of the first column.

Chromatography. The chromatographic system consisted of a Pharmacia fast protein liquid chromatography (FPLC) apparatus. Anion exchange chromatog-

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TABLE 1. Enzyme activities related to cellulose and xylan degradation in the extract of *T. maritima* cells

Substrate[a]	Total activity (U)	Sp act (mU/mg)
pNP-β-D-glucoside	18.0	15.50
pNP-β-D-cellobioside	5.0	3.87
pNP-β-D-xyloside	47.0	40.70
pNP-α-L-arabinofuranoside	11.0	9.75
CMC	673.0	578.00
β-Glucan	4,230.0	3,640.00
Avicel	0.6	0.51
Xylan	585.0	229.00
Aldotetraouronic acid	9.6	8.24

[a] pNP, *p*-nitrophenyl.

raphy on Q Sepharose was carried out as described previously (3). Solid ammonium sulfate was added to the pooled fractions up to a concentration of 1.2 M. Hydrophobic interaction chromatography was performed on a Pharmacia Phenyl-Sepharose HP HiLoad 16/10 column as described in the legend to Fig. 2. After dialysis against equilibration buffer, pool I of the hydrophobic interaction chromatography (HIC) column was loaded on a Pharmacia Mono Q HR 5/5 column. FPLC anion exchange chromatography was carried out as described elsewhere (4). Chromatofocusing was performed on a Pharmacia Mono P HR 5/20 column equilibrated with 25 mM imidazol-HCl, pH 7.4. Pools II and III of the HIC column were diluted 1:3 with equilibration buffer before application to the column. Elution was performed with 50 ml of Pharmacia Polybuffer 74, diluted 1:10 and adjusted to pH 3.5 with HCl. Fractions (1 ml) were assayed for Avicelase, carboxymethylcellulase (CMCase), β-glucanase, β-D-glucosidase, β-D-cellobiosidase, β-D-xylosidase, and α-L-arabinofuranosidase. Gel filtration was carried out on a Pharmacia Superdex 200 prep grade HiLoad 16/60 column as described in the legend to Fig. 3. The pooled fractions of the Mono P and the Mono Q columns were applied without prior concentration or buffer exchange. Apparent molecular masses were estimated from the partition coefficients as described previously (4). Affinity chromatography was performed in a Pharmacia XK 26/10 column filled with 10 g of HBS-cellulose (Serva, Heidelberg, Germany) in 50 mM Tris-HCl, pH 7.5, containing 1 M NaCl. After addition of solid NaCl up to a concentration of 1 M, crude extract (140 mg of protein) was applied to the column. The column was washed with 50 mM Tris-HCl, pH 7.5, and elution of bound protein was effected with distilled water.

Enzyme assays. CMCase, β-glucanase, laminarinase, and xylanase were assayed by incubation for 15 min to 4 h at 80°C in 0.5% (wt/vol) solutions of the corresponding substrate in 0.1 M Tris-HCl, pH 7.5. Avicelase activity was assayed by incubation for 24 to 72 h in a 1% (wt/vol) suspension of Avicel in 0.1 M Tris-HCl, pH 7.5. After removal of solids by centrifugation, aliquots of the supernatants were assayed for the release of reducing sugars. One unit of enzyme corresponds to the release of 1 μmol of glucose or xylose eq/min.

β-D-Glucosidase, β-D-cellobiosidase, β-D-xylosidase, and α-L-arabinofuranosidase activities were determined by measuring the release of *p*-nitrophenol from the corresponding *p*-nitrophenyl glycosides at 60°C in 0.1 M Tris-HCl, pH 7.5, as described previously (2). One unit of activity is defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per min.

α-Glucuronidase activity was determined at 50°C with aldotetraouronic acid (4-*O*-*methyl*-glucuronosyl-xylotriose) as a substrate as described by Khandke et al. (18).

Analytical methods. Protein concentrations were measured by the method of Sedmark and Grossberg (31). Reducing sugars were determined with the 3,5-dinitrosalicylic acid reagent (37). Glucose was assayed with the glucose oxidase reagent from a glucose assay kit (Sigma Diagnostics no. 510). Thin-layer chromatography was carried out as described previously (5). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and activity staining were performed as described by Schwarz et al. (30). Cellodextrins were analyzed by high-pressure liquid chromatography (HPLC) on an Aminex HPX 42A column as described previously (3).

RESULTS

Fractionation of crude extract by anion exchange chromatography. The extract of *T. maritima* cells grown in a medium containing cellobiose and xylose exhibited enzyme activities necessary for the degradation of cellulose and xylan (Table 1). The cell extract was fractionated on a Q Sepharose Fast Flow column. Figure 1 shows the elution profile of the cellulolytic and xylanolytic enzymes expressed under the cultivation conditions used. Enzyme fractions were designated according to the substrate hydrolyzed in an attempt to avoid premature assignment of physiological functions in cellulose and xylan degradation.

Activity towards the insoluble microcrystalline cellulose substrate Avicel was eluted between 0.2 and 0.4 M NaCl. The activities towards barley β-glucan which contains alternating β-1,4- and β-1,3-glycosidic linkages and towards the soluble cellulose derivative carboxymethylcellulose (CMC) showed approximately the same elution pattern as the Avicelase activity. With the substrates *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-cellobioside two overlapping peaks of activity were found to elute around 0.28 M NaCl.

The *p*-nitrophenyl-β-D-xyloside and *p*-nitrophenyl-α-L-arabinofuranoside hydrolyzing activities were detected in the fractions with a slightly lower salt concentration (0.25 M NaCl). The elution profile of the α-L-arabinofuranosidase coincided completely with that of the β-xylosidase and partially with the *p*-nitrophenyl-β-D-glucoside and -cellobioside hydrolyzing activities. The major peak of xylanase activity which was eluted at about 0.2 M NaCl can be clearly separated from the enzymes exhibiting activity with Avicel, CMC, and barley β-glucan as well as from the β-xylosidase and the α-L-arabinofuranosidase activities.

The α-glucuronidase activity detected in the cell extract with the substrates aldotetraouronic acid and aldopentaouronic acid behaved rather strangely. A significant part of the activity did not bind at all to the Q Sepharose Fast Flow matrix at pH 8, whereas the bound fraction showed an extremely broad elution profile.

Separation of cellulolytic and xylanolytic enzymes by HIC. Further separation of the enzymes was achieved by HIC. As the primary interest of this study was the isolation of an Avicelase from *T. maritima*, the fractions from the Q Sepharose Fast Flow column exhibiting activity with CMC, β-glucan, and Avicel (fractions 50 to 79) were pooled and loaded onto a Phenyl-Sepharose HP column. Furthermore, this pool completely contained the *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-cellobioside hydrolyzing activities, while the α-L-arabinofuranosidase and the β-D-xylosidase activities were only partly included.

HIC (Fig. 2) clearly separated the CMC and β-glucan hydrolyzing activities into two peaks. The first peak eluted in the middle of the descending ammonium sulfate gradient showed an about 12-fold-higher activity with β-glucan than with CMC. The fractions of this peak were also able to hydrolyze *p*-nitrophenyl-β-D-cellobioside. The second peak began to be eluted at the end of the salt gradient. Its ratio of CMCase activity to β-glucanase activity was about 1:4 and, thus, differed from the first peak by a factor of 3. The Avicelase activity as well as a comparably weak activity towards *p*-nitrophenyl-β-D-cellobioside coeluted with the latter CMCase peak. Washing the HIC column with distilled water resulted in the elution of an additional Avicelase peak. The activities with CMC and β-glucan in these fractions were significantly lower than those of the first Avicelase peak.

The fractions of the Phenyl-Sepharose HP column were also assayed for enzymes showing specificity for β-1,3-glycosidic linkages. With the β-1,3-glucan laminarin as a substrate, one peak of activity overlapping partly with the first Avicelase peak could be detected. The fractions of this laminarinase peak expressed no activity towards the β-1,3-glycosidic linkages of the mixed-linkage substrate β-glucan.

The activities towards the aryl substrates were resolved into three peaks. Two of the peaks exhibited β-D-xylosidase and β-D-glucosidase activity, whereby the latter activity was about 1,000-fold lower than the former. The fractions of the peak eluted at 0.35 M ammonium sulfate also hydrolyzed *p*-nitro-

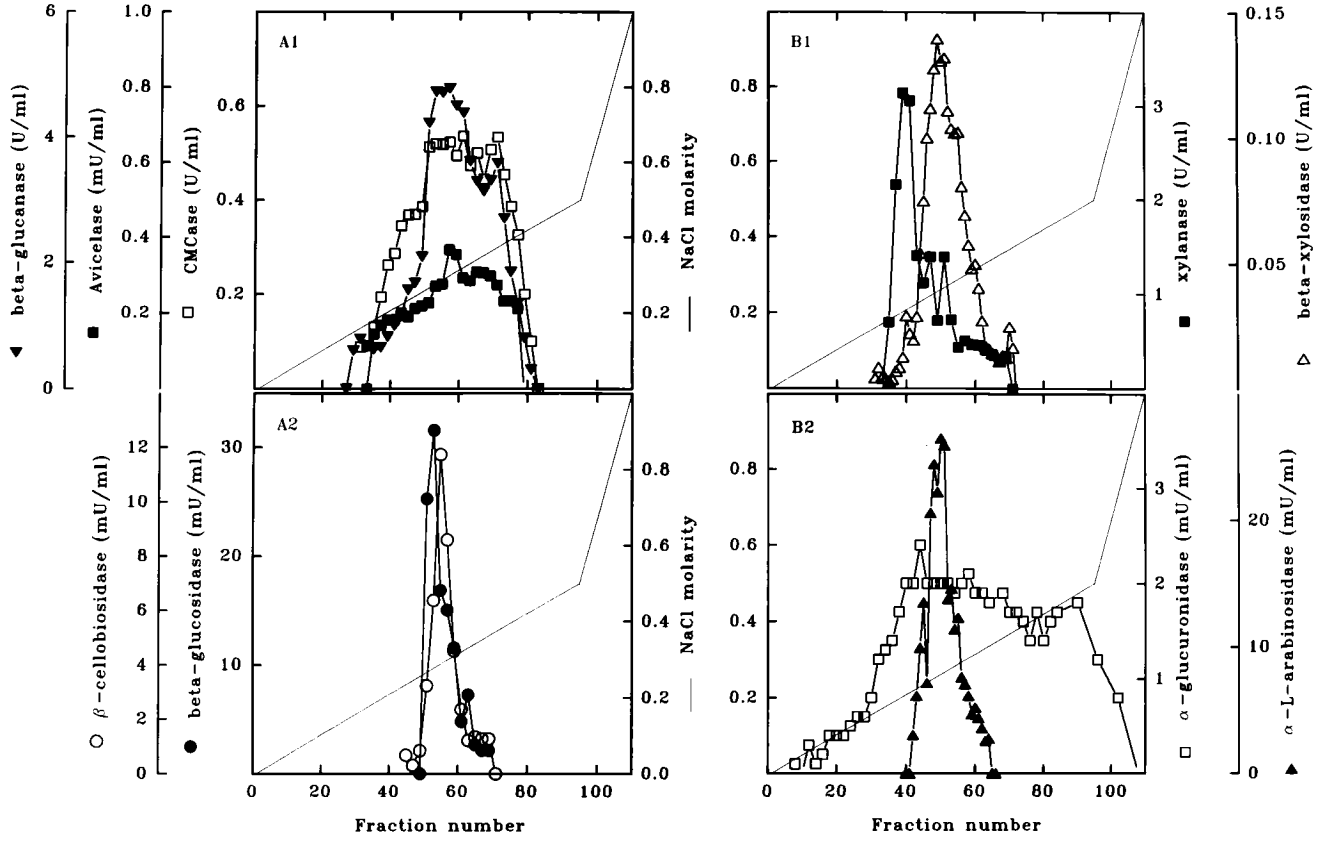

FIG. 1. Fractionation of *T. maritima* enzymes involved in the depolymerization of cellulose and xylan by Q Sepharose anion exchange chromatography. The extract of *T. maritima* cells (580 mg of protein) was applied to a Q Sepharose Fast Flow XK 26/10 column equilibrated with 20 mM Tris-HCl, pH 8.0. Elution was performed with a 940-ml linear gradient (0.0 to 0.5 M NaCl) in equilibration buffer at a flow rate of 5 ml/min. Fractions (10 ml) were collected and assayed for Avicelase, CMCase, and β-glucanase (A1); β-D-glucosidase and β-D-cellobiosidase (A2); xylanase and β-D-xylosidase (B1); and α-glucuronidase and α-L-arabinofuranosidase (B2).

phenyl-α-L-arabinofuranoside, while the fractions of the second peak cleaved *p*-nitrophenyl-β-D-cellobioside in addition to *p*-nitrophenyl-β-D-xyloside and *p*-nitrophenyl-β-D-glucoside. The third peak showed α-L-arabinofuranosidase activity exclusively.

The fractions from the gradient shown in Fig. 2 expressing cellulolytic activities were combined in three pools. Pool I (fractions 21 to 26) contained the activity with CMC and β-glucan as well as the *p*-nitrophenyl-β-D-cellobioside hydrolyzing activity. Pool II (fractions 46 to 53) included the Avicelase activity besides the CMC and β-glucan hydrolyzing activities. Pool III contained the Avicelase activity which had been eluted with distilled water. Furthermore, a fourth pool was formed with fractions 43 to 45, showing activity with laminarin as well as with *p*-nitrophenyl-β-D-xyloside, *p*-nitrophenyl-β-D-glucoside, and *p*-nitrophenyl-β-D-cellobioside. The aryl glycosidase eluting between the cellulase of pool I and the laminarinase of pool IV was included in pool V (fractions 32 to 38).

Separation of aryl glycosidases from cellulase II by FPLC chromatofocusing and gel filtration. Pools II and III of the HIC column containing Avicelase activity were subjected to chromatofocusing on a Mono P column with a pH gradient from 7.4 to 3.5. On this column the Avicelase, CMCase, and β-glucanase activities of both pools were eluted again as single but rather broad peaks in a pH interval from 4.2 to 3.7. Pool II also contained the α-L-arabinofuranosidase peak of the HIC column as well as residual activities with *p*-nitrophenyl-β-D-xyloside, *p*-nitrophenyl-β-D-glucoside, and *p*-nitrophenyl-β-D-cellobioside. The β-D-xylosidase activity was coeluted with the *p*-nitrophenyl-β-D-glucoside and *p*-nitrophenyl-β-D-cellobioside cleaving activities at pH 4.2, whereas the α-L-arabinofuranosidase activity was released from the column at pH 4.8. Thus, chromatofocusing with the broad pH gradient used did not result in a sufficient separation of the individual enzyme activities.

In order to determine the size of the enzyme, all fractions of the Mono P column displaying Avicelase activity were combined and loaded onto a Superdex 200 column. On this column the Avicelase activity was remarkably retarded because of interaction with the column matrix (Fig. 3). Both the CMCase activity and the β-glucanase activity were again coeluted with the former activity. Furthermore, the corresponding fractions also exhibited activity with *p*-nitrophenyl-β-D-cellobioside. Since SDS-PAGE revealed only one protein band in the Avicelase-positive fractions, we conclude that all four activities reside in a single protein. The protein band was detected at a position corresponding to a molecular mass of 29 kDa (Fig. 4). The contaminating activities with the aryl substrates *p*-nitrophenyl-β-D-glucoside, *p*-nitrophenyl-β-D-cellobioside, *p*-nitrophenyl-β-D-xyloside, and *p*-nitrophenyl-α-L-arabinofuranoside migrated at positions corresponding to molecular masses in the range of 83,000 to 30,000 Da. Thus, they could be clearly resolved from the cellulolytic activities.

Purification of cellulase I. The activities present in pool I of the HIC column were further fractionated by Mono Q anion exchange chromatography at pH 6 (Fig. 5). The *p*-nitrophenyl-

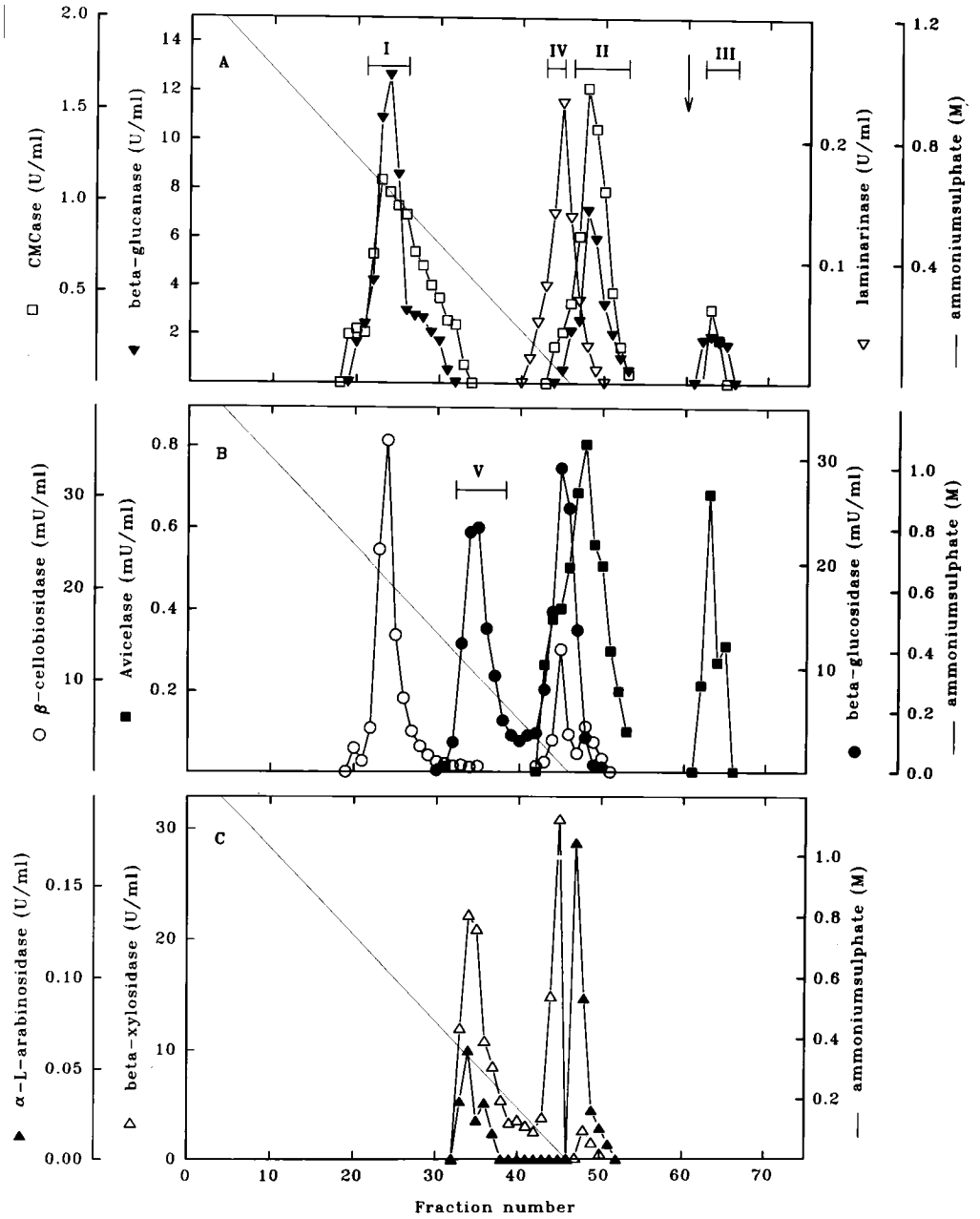

FIG. 2. Separation of cellulolytic and xylanolytic enzymes by hydrophobic interaction chromatography. The Avicelase pool of the Q Sepharose column (fractions 50 to 79; 176 mg of protein) was applied to a Phenyl-Sepharose HP HiLoad 16/10 column equilibrated with 50 mM Tris-HCl, pH 7.5, containing 1.2 M ammonium sulfate. Elution was performed with a 210-ml linear gradient (1.2 to 0.0 M ammonium sulfate) in 50 mM Tris-HCl, pH 7.5, then with 100 ml of 50 mM Tris-HCl, pH 7.5, and finally with 100 ml of distilled water at a flow rate of 2 ml/min. Fractions (5 ml) were assayed for CMCase, β-glucanase, and laminarinase (A); Avicelase, β-D-glucosidase, and β-D-cellobiosidase (B); or β-D-xylosidase and α-L-arabinofuranosidase (C).

β-D-cellobioside hydrolyzing activity again copurified with the CMCase and the β-glucanase activities, indicating that all three activities are properties of the same enzyme.

Like cellulase II, cellulase I was also subjected to gel filtration. Cellulase I was retarded even more strongly on the Superdex column than cellulase II. Again, the observed retardation resulted in an electrophoretically homogeneous protein with an apparent molecular mass of 27 kDa (Fig. 4). Identification of the single protein bands shown in Fig. 4 with cellulases I and II was confirmed by in situ activity staining for CMCase and β-glucanase activities.

Optimal conditions for cellulase action. Maximum activities of both enzymes towards β-glucan were observed between pH 6.0 and 7.5 (data not shown). Figure 6 shows the temperature activity profiles of the two cellulases. Both enzymes were highly thermoactive. By using a 20-min assay, maximum substrate hydrolysis rates were measured at about 95°C. In the absence of substrate, cellulase I exhibited a half-life of about 2 h at 95°C while cellulase II lost one half of its activity when incubated for 30 min at this temperature (Fig. 7). The thermostability of the purified cellulases at 80°C was increased by addition of NaCl. Cellulase I suffered merely a 10% loss of activity during a 6-h

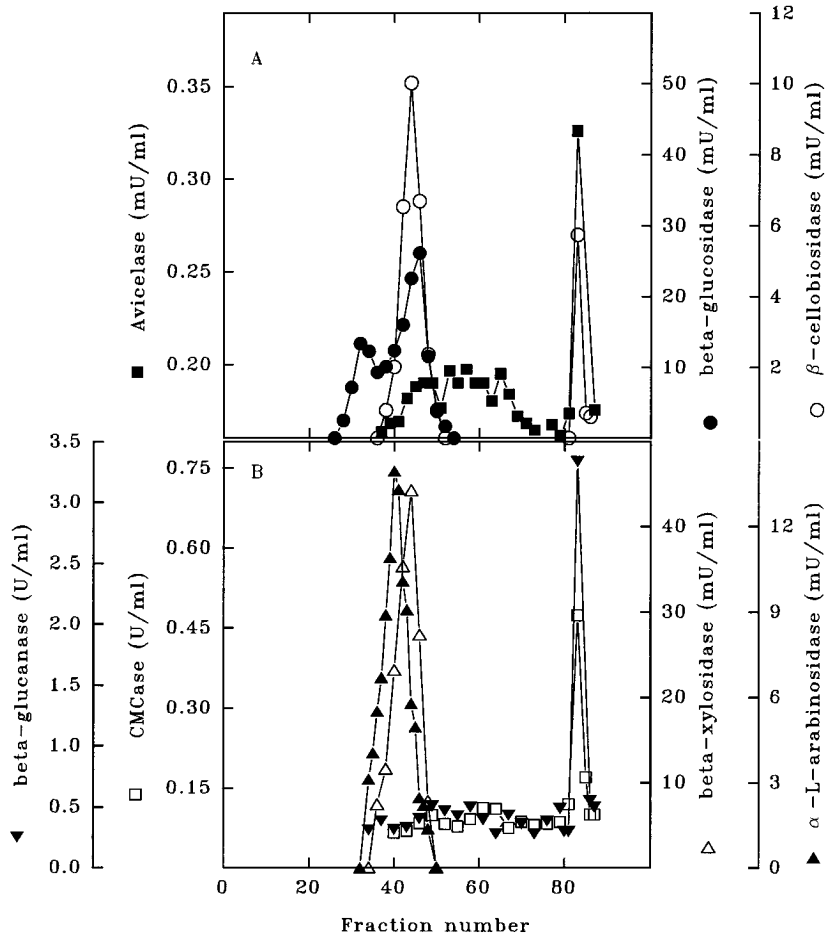

FIG. 3. FPLC gel filtration of cellulase II. Fractions 21 to 29 from the Mono P column (2.5 mg of protein) were applied to a Superdex 200 prep grade HiLoad 16/60 column equilibrated with 150 mM Tris-HCl, pH 7.0. The column was eluted with equilibration buffer at a flow rate of 1 ml/min. Fractions (1 ml) were assayed for Avicelase, β-D-glucosidase, and β-D-cellobiosidase (A) and CMCase, β-glucanase, β-D-xylosidase, and α-L-arabinofuranosidase (B).

period in the presence of 2.5 M NaCl, while cellulase II proved to be completely stable under these conditions.

Analysis of degradation products. The degradation products formed by cellulase II action on cellulosic substrates and cellodextrins were analyzed by HPLC. The results may be summarized as follows: (i) cellobiose and cellotriose were released from the cellodextrin chain, (ii) cellotriose was hydrolyzed at a low rate into cellobiose and glucose, (iii) the rate of hydrolysis increased with increasing chain length of the cellodextrin, and (iv) at high cellodextrin concentrations transglycosylation reactions occurred. In agreement with these results, cellobiose and glucose were found as end products formed by cellulase II action on Avicel (Fig. 8). Thus, cellulase II may be classified as an exoglucanase splitting off cellobiose and cellotriose from the end of the sugar chain.

DISCUSSION

The present study demonstrates the existence of cellulolytic as well as xylanolytic enzyme systems in *T. maritima* MSB 8. The enzyme fractions as resolved by the FPLC methods used are summarized in Table 2. The major portions of the enzyme activities studied were found associated with the cells. Localization of the enzymes in the toga (outer membrane) of this organism as discussed for amylases of *T. maritima* (29) and for the polysaccharide hydrolyzing enzymes of *Thermotoga* sp. strain FjSS3-B.1 (24) still remains to be demonstrated.

Concerning xylan depolymerization, both backbone hydrolyzing and debranching activities could be isolated. The first purification step (anion exchange chromatography) already separated a xylanase peak from the other hydrolytic enzymes. Recent results have demonstrated the presence of two distinct endoxylanases in *T. maritima* MSB 8 grown on xylose as the carbon source (36a). Hydrophobic interaction chromatography clearly resolved two enzyme fractions with high β-D-xylosidase activity. The activities of the enzyme contained in pool V of the Phenyl-Sepharose column with *p*-nitrophenyl-α-L-arabinofuranoside and *p*-nitrophenyl-β-D-glucoside amounted to only 0.3 and 0.1%, respectively, of the activity measured with *p*-nitrophenyl-β-D-xyloside. The *p*-nitrophenyl-β-D-xyloside hydrolyzing enzyme included in pool IV of the same column showed a comparably low activity with *p*-nitrophenyl-β-D-glucoside and an even lower activity with *p*-nitrophenyl-β-D-cellobioside. The laminarinase activity copurifying with the β-D-xylosidase activity of this pool might be attributed to a separate enzyme, but it is also possible that both activities are due to a single enzyme as in the case of the β-D-glucosidase purified from *Thermotoga* sp. strain FjSS3-B.1 (24). A more detailed characterization of the substrate specificity of the above-mentioned aryl glycosi-

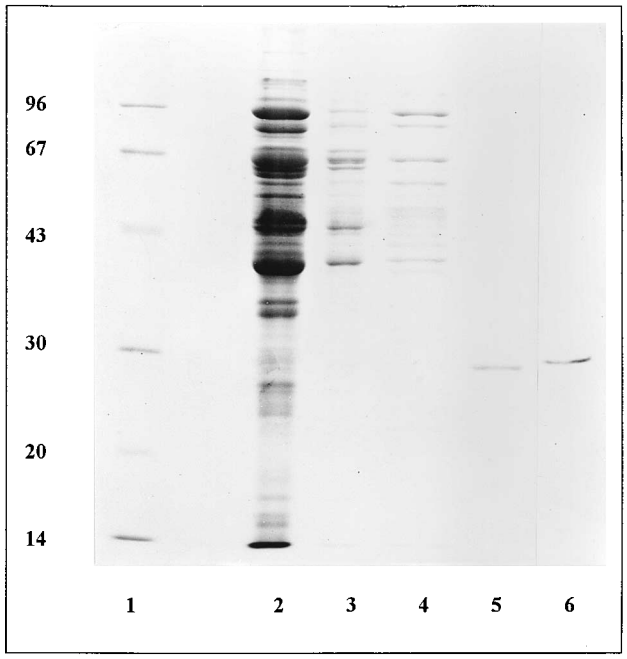

FIG. 4. SDS-PAGE of cellulase I and cellulase II fractions. Lane 1, molecular mass markers (in kilodaltons); lane 2, crude extract; lane 3, pooled fractions of the Q Sepharose column; lane 4, pooled cellulase I fractions of the Phenyl-Sepharose column; lane 5, purified cellulase I; lane 6, purified cellulase II.

dases is a prerequisite to an appropriate designation of the enzymes.

In the case of arabinoxylans, side groups are cleaved off by α-L-arabinofuranosidase. The latter enzyme is normally assayed with the aryl substrate *p*-nitrophenyl-α-L-arabinofuranoside. An enzyme displaying activity with *p*-nitrophenyl-α-L-arabinofuranoside but also with *p*-nitrophenyl-β-D-xyloside at a ratio of 2:1 is included in pool IV of the Phenyl-Sepharose column. Aryl glycosidases hydrolyzing *p*-nitrophenyl-α-L-arabinofuranoside as well as *p*-nitrophenyl-β-D-xyloside have been described frequently (25, 32, 35). The ability of the *T. maritima* enzyme to remove L-arabinose branches from xylan remains to be demonstrated. Enzymes necessary for removal of branches in glucuronoxylan also seem to be present in *T. maritima*. A broad peak of α-D-glucuronidase activity overlapped with the other xylanolytic enzyme activities. This peak contains at least two distinct enzymes which could be distinguished by their differential activities towards aldopentao- and aldotetraouronic acid (unpublished results).

The data reported in this study clearly demonstrate that *T. maritima* produces cellulolytic enzymes. By a combination of anion exchange and hydrophobic interaction chromatography two enzymes, designated cellulases I and II, which differ in their abilities to degrade microcrystalline cellulose (Avicel), have been resolved.

On the basis of its high activity towards the substituted cellulose derivative CMC, cellulase I may be classified as endo-β-1,4-glucanase. As observed for endoglucanases in general, the activity of cellulase I towards unsubstituted substrates like β-glucan is remarkably higher than that towards CMC. The *p*-nitrophenyl-β-D-cellobioside hydrolyzing activity present in the cellulase I pool was copurified with the CMCase and β-glucanase activities during three different chromatographic steps. An aryl-β-D-cellobiosidase activity has also been described for other purified endoglucanases (22).

Cellulase II is able to attack Avicel in addition to CMC, β-glucan, and *p*-nitrophenyl-β-D-cellobioside. The activities towards these substrates could not be separated by the different chromatographic purification methods used. In particular, their retardation during gel filtration confirmed the notion that all four activities reside in the same protein. In its substrate specificity, cellulase II resembles the Avicelase purified from *Streptomyces reticuli* (27) as well as Avicelase I from *Clostridium stercorarium* (5). These Avicelases were shown to exhibit an endo- or exoglucanolytic mode of action, depending on the substrate. Concerning the degradation products, cellulase II bears resemblance to the exoglucanase of *C. stercorarium* (Avicelase II) which has been characterized as a cellodextrinohydrolase (3). Presumably, cellulase II of *T. maritima* belongs to a new type of cellulolytic enzyme. The Avicelases mentioned above are rather large enzymes (109, 79, and 87 kDa) consisting of catalytic domains of approximately 50 kDa and addi-

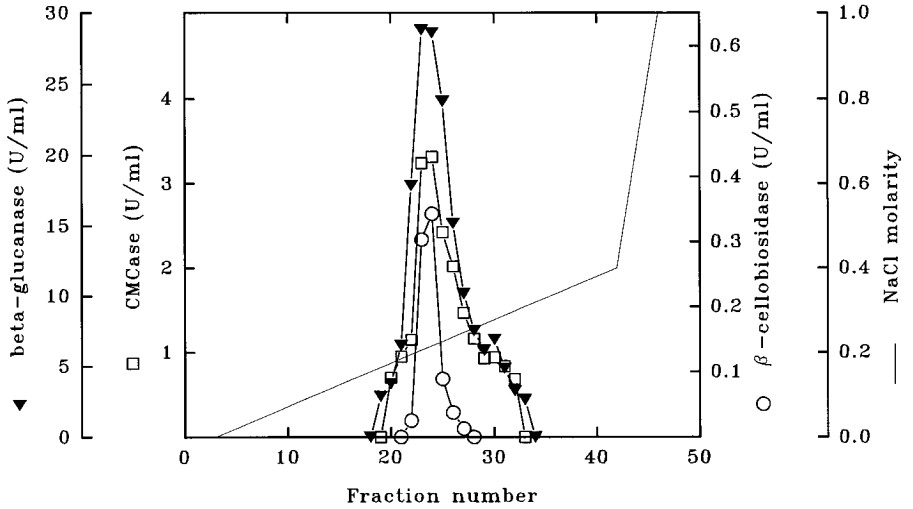

FIG. 5. Mono Q anion exchange chromatography of cellulase I. Pool I of the Phenyl-Sepharose column (fractions 21 to 26; 5.8 mg of protein) was applied to a Mono Q HR 5/5 column equilibrated with 20 mM Tris-HCl, pH 6.0. Elution was performed with a 20-ml linear gradient (0.0 to 0.4 M NaCl) in equilibration buffer at a flow rate of 1 ml/min. Fractions (0.5 ml) were assayed for CMCase, β-glucanase, and β-D-cellobiosidase.

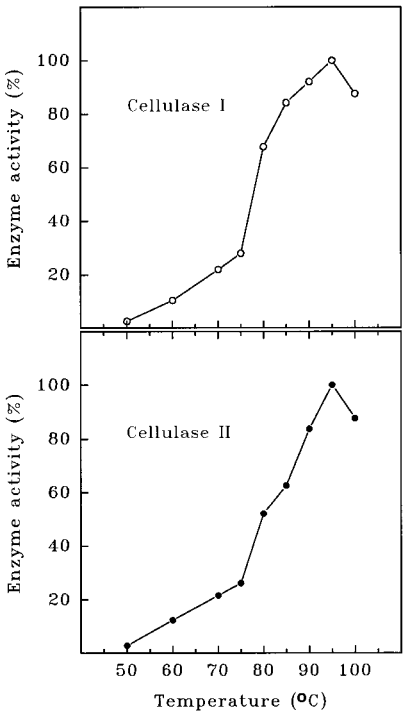

FIG. 6. Effect of temperature on activity. Incubations were carried out at the indicated temperatures under standard assay conditions with β-glucan as the substrate.

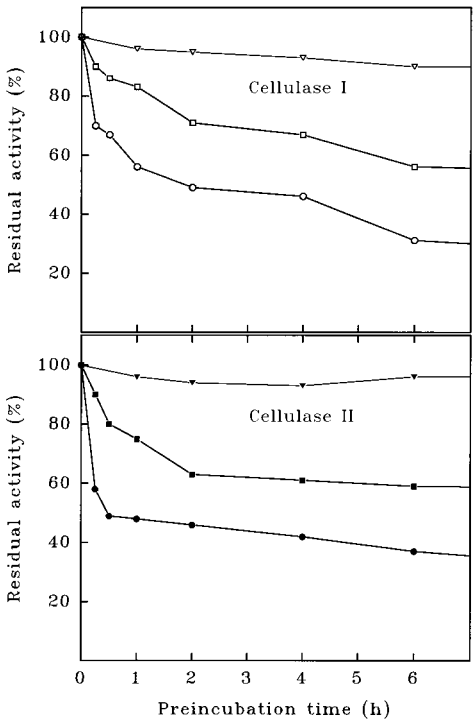

FIG. 7. Thermal stability of cellulase activity. Purified enzymes were incubated at 80 and 95°C in 50 mM Tris-HCl, pH 7.0. At the times indicated, samples were withdrawn for the determination of β-glucanase activity. Residual activity was expressed as the percentage of the activity for the untreated control. ■ and □, 80°C; ● and ○, 95°C; ▼ and ▽, 80°C in the presence of 1 M NaCl.

tional cellulose-binding domains (17, 28). Surprisingly, in denaturing SDS-PAGE the apparent molecular mass of the only protein band detectable in the cellulase II preparation was estimated to be 29 kDa, which is lower than expected for a multidomain cellulolytic enzyme. Because of interaction of the enzyme with the column matrix during gel filtration, its molecular weight under nondenaturing conditions could not be determined. Therefore, an oligomeric structure of the native enzyme cannot be excluded. A comparably low molecular mass of 36 kDa (SDS-PAGE) was determined for the cellobiohydrolase which has been purified from *Thermotoga* sp. strain FjSS3-B1 (23). Recently, a molecular mass of only 23 kDa (calculated from the amino acid sequence) has been reported for a new endoglucanase of *Trichoderma reesei* (26).

Cellulases have been shown to interact with their polysaccharide substrates by means of discrete cellulose-binding domains. Furthermore, adsorption to polysaccharide-based separation media has been observed for various cellulolytic enzymes (3, 10, 14, 36). It was proposed that the adsorption of cellulolytic enzymes to cellulose and other polysaccharides is due to hydrophobic interaction (14, 21). It is not known whether the *T. maritima* Avicelase possesses a cellulose-binding domain, but from its chromatographic behavior (strong binding to the Phenyl-Sepharose matrix and retardation on the Superdex column) it can be inferred that this Avicelase is a markedly hydrophobic protein. This is in accordance with the fact that the Avicelase could be isolated from the crude extract by an alternative procedure: affinity chromatography on a cellulose column (unpublished results).

Compared with the multienzyme cellulolytic complex termed cellulosome located at the cell surface of another thermophilic eubacterium, *Clostridium thermocellum*, the cellulolytic enzyme system of *T. maritima* seems to be rather simple. It resembles the low-complexity cellulase system produced by the thermophilic cellulolytic clostridium *C. stercorarium*, consisting only of an endoglucanase and an exoglucanase (6). *T. maritima* might produce a third cellulolytic enzyme besides cellulases I and II. The activities towards Avicel, CMC, and β-glucan which have been eluted from the Phenyl-Sepharose column with distilled water may be attributed either to residual cellulase II or to an additional, strongly hydrophobic enzyme. Strik-

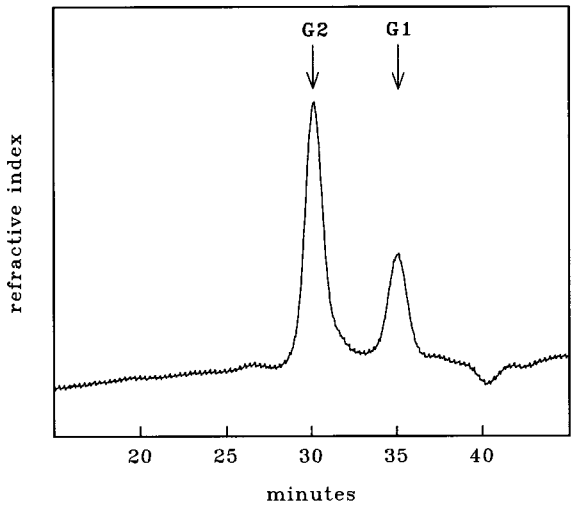

FIG. 8. HPLC analysis of degradation products released from Avicel by purified cellulase II. A standard reaction mixture containing a 1% (mass/vol) suspension of Avicel was incubated for 72 h at 80°C with fraction 83 from the Superdex column. G1, glucose; G2, cellobiose.

TABLE 2. Enzymes isolated from the cell extract of *T. maritima*[a]

Enzyme	Activity with:										Fraction
	Xylan	CMC	β-Glucan	Avicel	Laminarin	pNPG	pNPC	pNPX	pNPA	m-GA-X3	
Xylanase	+	−	−	−	−	−	−	−	−	−	QS
Cellulase I	−	+	+	−	−	−	+	−	−	−	PS, pool I
Cellulase II	−	+	+	+	−	−	+	−	−	−	PS, pool II
Laminarinase/β-D-xylosidase	−	−	−	−	+	+	+	+	−	−	PS, pool IV
β-D-Xylosidase	−	−	−	−	−	+	−	+	+	−	PS, pool V
α-D-Glucuronidase	−	−	−	−	−	−	−	−	−	+	
α-L-Arabinofuranosidase	−	−	−	−	−	−	−	+	+	−	

[a] pNPG, *p*-nitrophenyl-β-D-glucopyranoside; pNPC, *p*-nitrophenyl-β-D-cellobioside; pNPX, *p*-nitrophenyl-β-D-xylopyranoside; pNPA, *p*-nitrophenyl-α-L-arabinofuranoside; m-GA-X3, 4-*O-methyl*-glucuronosyl-xylotriose (aldotetraouronic acid); QS, Q Sepharose column; PS, Phenyl-Sepharose column.

ing is the lack of β-D-cellobiosidase activity as well as the higher ratio of Avicelase to CMCase and β-glucanase activities. However, the reaction products released from Avicel are the same for both activities (data not shown).

Recently, we were successful in isolating several *Escherichia coli* clones with similar recombinant plasmids expressing highly thermostable β-glucanase and CMCase activities from a gene library of *T. maritima* MSB8 constructed earlier (13). Work is in progress to determine if the recombinant enzyme which has an apparent molecular mass of about 28 kDa (data not shown) resembles cellulase I or II reported here.

ACKNOWLEDGMENTS

We thank W. Huber and K. Zacharias for growing the culture; J. Puls and H. Meissner for performing α-glucuronidase assays; and the students C. Banaschewski, D. Gibson, K. Saur, and S. Schilling for their engaged assistance throughout the chromatographic analysis.

This work was supported by the Deutsche Forschungsgemeinschaft (grant SFB 145/YW4 to W. L. Staudenbauer and grant SFB145/B9 to W. Liebl).

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