APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1995, p. 1666–1668 Vol. 61, No. 4
0099-2240/95/$04.00+0

Xylanase IV, an Exoxylanase of *Aeromonas caviae* ME-1 Which Produces Xylotetraose as the Only Low-Molecular-Weight Oligosaccharide from Xylan

BRUNO KILUNGA KUBATA,[1]* KAZUHIRO TAKAMIZAWA,[2] KEIICHI KAWAI,[2]
TOHRU SUZUKI,[2] AND HIROYUKI HORITSU[2]†

The United Graduate School of Agricultural Science[1] and Department of Biotechnology, Faculty of Agriculture,[2] Gifu University, Gifu 501-11, Japan

Received 8 September 1994/Accepted 24 January 1995

A novel xylanase (xylanase IV) which produces xylotetraose as the only low-molecular-weight oligosaccharide from oat spelt xylan was isolated from the culture medium of *Aeromonas caviae* ME-1. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the xylanase IV molecular weight was 41,000. Xylanase IV catalyzed the hydrolysis of oat spelt xylan, producing exclusively xylotetraose. The acid hydrolysate of the product gave D-xylose. The enzyme did not hydrolyze either *p*-nitrophenyl-β-D-xyloside, small oligosaccharides (xylobiose and xylotetraose), or polysaccharides, such as starch, cellulose, carboxymethyl cellulose, laminarin, and β-1,3-xylan.

Xylan is made up of a backbone containing β-1,4-linked xylosyl residues and side chains, including arabinosyl, glucuronosyl, 4-*O*-methyl glucuronosyl, and acetyl residues, which are attached to the xylan backbone. The xylan backbone is degraded by β-1,4-xylanases and β-xylosidases.

Available information about xylan backbone hydrolysis is derived from two enzymes, xylanases and β-xylosidases. Xylanolytic enzymes have been purified from many microorganisms, and their hydrolytic properties have been thoroughly investigated. However, little is known about specific exoxylanases which are able to produce only a single product from xylan.

The xylanases (β-1,4-xylanases) known to date are endo-type enzymes which usually show preference for internal glycosidic linkages in xylans or xylooligosaccharides and which act by a random attack mechanism (1–3, 7, 12, 14–16). Some other xylanases, such as those of *Bacillus polymyxa* (13) and *Chaetomium thermophile* (4), are known to hydrolyze xylans and to produce mainly xylobiose and traces of xylose and/or xylotriose by an exo-type mechanism. The enzyme of *Trichoderma viride* (17) was described as producing xylobiose from xylan without a concomitant production of D-xylose, although this enzyme was classified as an exocellulase because of its high affinity for cellulose rather than for xylans.

We have been studying a xylan-degrading bacterium, *Aeromonas caviae* ME-1 (8, 9, 18), which was isolated from the intestine of a herbivorous insect. Previously, we reported that this strain produced multiple xylanases. Xylanase I was found to be an endoenzyme (8). Recently, we have characterized an unusual xylanase (xylanase V) from the same strain (10). Xylanase V produced xylobiose as the only low-molecular-weight oligosaccharide from xylans by an exo-type mechanism.

In this paper, we report the purification and hydrolytic properties of another exoxylanase (xylanase IV) from *A. caviae* ME-1. Xylanase IV, which produces xylotetraose as the only low-molecular-weight oligosaccharide from xylan, has not been reported before.

The *A. caviae* ME-1 strain used in this investigation was isolated from the intestine of the lepidopteran larva *Samia cynthia pryeri* Butler in our laboratory. This insect is economically important in Japan and Taiwan, where it produces silk of commercial value (5), and its larval life cycle takes place on two plant hosts, *Picrasma quassioides* Benn. "Nigaki" and *Phedodendron amurense* Rupr. "Kihada" (larch wood), on which it feeds only on the leaves (6).

Unless otherwise specified, the other materials, methods, and instruments used in this study were the same as those previously described (8, 10).

Xylanase IV was purified from 4,000 ml of a culture supernatant. Ammonium sulfate was added to give 95% saturation. After being stirred at 4°C overnight, the precipitates formed were collected by centrifugation at 8,000 × *g* for 20 min and then were dissolved in 538 ml of 20 mM potassium phosphate buffer (pH 6.8). The enzyme solution was freeze-dried and dissolved in 60 ml of the phosphate buffer and then loaded onto a Sephadex G-75 column (84 by 2.6 cm). The column was equilibrated and eluted with 0.2 M NaCl in 20 mM potassium phosphate buffer (pH 6.8) at a flow rate of 30 ml/h, and 7 ml of the fractions was collected. Concentrated active fractions (36 ml) were further separated on a Toyo pearl HW-55 column (18 by 4 cm) which was equilibrated and eluted as described above. The active fractions were freeze-dried and applied to a high-performance liquid chromatography (HPLC) Mono Q HR 5/5 column (Pharmacia Co., Uppsala, Sweden). The column was equilibrated with 10 mM Tris-HCl buffer (pH 8.6) and eluted with a gradient of 0 to 1 M NaCl in the same buffer at a flow rate of 0.8 ml/min. The active peak was collected, concentrated, and reapplied to the same column.

Enzymatic hydrolysis products were obtained by incubation of a reaction mixture which contained 10 ml of 1% oat spelt xylan in 20 mM potassium phosphate buffer (pH 6.8) and 1 ml of diluted enzyme solution at 40°C for 36 h. The reaction mixture was then centrifuged at 3,000 × *g* for 10 min to remove insoluble materials and freeze-dried. In order to eliminate tailing, dried samples were acetylated conventionally with pyridine and acetic anhydride, spotted on thin-layer chromatogra-

* Corresponding author. Present address: Mori Institute of Ecology Co., Ltd., 3-2-7, Uchikyuhoji-cho, Osaka 540, Japan. Phone: 81-6-765-9321. Fax: 81-6-765-9310.

† Present address: Chukyo Women's University, Ohbu 474, Japan.

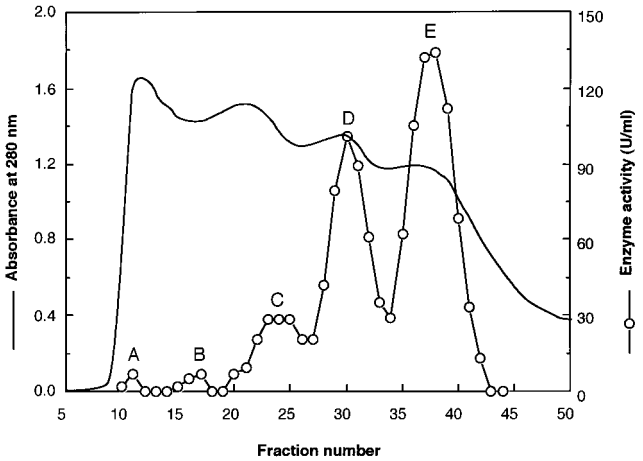

FIG. 1. Gel filtration of *A. caviae* ME-1 xylanases on Sephadex G-75. The column (84 by 2.6 cm) was equilibrated and eluted with 0.2 M NaCl in 20 mM potassium phosphate buffer (pH 6.8) at a flow rate of 30 ml/h, and 7 ml of the fractions was collected. Peaks A, B, C, D, and E correspond to xylanases V, IV, III, II, and I, respectively. —, A_{280}; ○, xylanase activity.

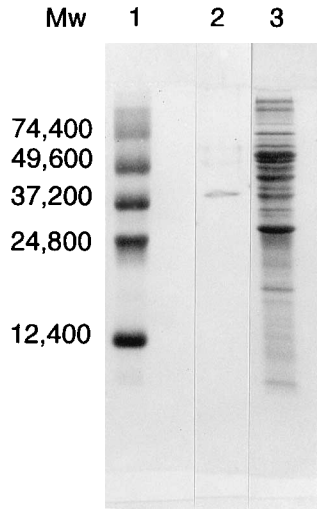

FIG. 2. SDS-PAGE of xylanase IV. Lanes: 1, cross-linked cytochrome *c* (10 μg); 2, 5 μg of purified xylanase IV; 3, 25 μg of crude xylanase. Mw, molecular weight.

phy plates (aluminum sheet silica gel 60 F_{254}; Merck, Darmstadt, Germany) and then developed with solvent system of ethyl acetate-hexane (5:3 [vol/vol]). The resolved sugars were detected by heating the plates after spraying them with ethanol-sulfuric acid (18:2 [vol/vol]). Authentic samples from xylose to xylopentaose were used as standards.

One percent of each polysaccharide (oat spelt xylan, larch wood xylan, carboxymethyl cellulose, cellulose powder, starch, and laminarin) or 0.5% of β-1,3-xylan from *Caulerpa racemosa* (Kumamoto Institute of Technology, Kumamoto, Japan) was incubated with 1 ml of diluted xylanase IV solution in 10 ml of 20 mM potassium phosphate buffer (pH 6.8). The reaction was carried out at 40°C for 30 min. β-Xylosidase activity was determined according to the method of Matsuo and Yasui (11), with *p*-nitrophenyl-β-D-xyloside from Sigma Co. (St. Louis, Mo.) as the substrate.

On Sephadex G-75, the crude xylanase gave five active peaks as shown in Fig. 1. Active fractions corresponding to peak B (from fractions 15 to 18) were concentrated and applied to the Toyo pearl HW-55 column. Active fractions from the Toyo pearl HW-55 column (from fractions 20 to 33) were further purified on the HPLC Mono Q column. The purification steps are summarized in Table 1. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2), purified xylanase gave a single protein band, indicating that it was electrophoretically homogeneous. Its molecular weight, as estimated from the relative mobilities of various standard proteins on SDS-PAGE (Fig. 2), was 41,000.

Thin-layer chromatographic analysis of enzymatic hydrolysis products (Fig. 3) shows that xylanase IV produced exclusively a product with an R_f value corresponding to xylotetraose. The acid hydrolysate of the product yielded only D-xylose (data not shown). Xylanase IV produced xylotetraose as the only low-molecular-weight oligosaccharide from xylan without a concomitant production of transient xylooligosaccharides during an incubation period from 0.5 to 36 h (data not shown). This hydrolytic pattern might lead to the suspicion that xylanase IV is a side-chain-releasing enzyme. However, the fact that its action on xylan led to the destaining of the Congo red of the zymogram (data not shown) indicates that this enzyme is involved in the hydrolysis of the backbone, because the dye Congo red is known to interact only with intact (1,3- and 1,4-) β-D-glucans (19). As mentioned previously, endo-type enzymes attack internal glycosidic linkages in xylans or xylooligosaccharides and act by a random attack mechanism, which often results in a mixture of xylooligosaccharides of various degrees of polymerization and composition. As a consequence, the average molecular mass of the substrate will gradually decrease as the enzymatic reaction proceeds. For a substrate like xylan, with heterogeneous side chains known for their ability to hamper the action of hydrolases, the production of a solo product strongly suggests that xylanase IV attacks from one of the ends of the polymer which does not contain side chains and therefore acts by an exo-type mechanism.

The xylanase was active on xylans from oat spelts (100% of activity) and larch wood (69%) and was inactive on β-1,3-xylan and other carbohydrates, such as cellulose, starch, and laminarin. These results suggest that xylanase IV is a true xylanase. In addition, it did not hydrolyze *p*-nitrophenyl-β-D-xyloside, xylobiose, or xylotetraose, indicating that it does not possess β-xylosidase activity.

TABLE 1. Purification of xylanase IV from *A. caviae* ME-1

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Yield (%)
Supernatant	1,168	9,547	8	1	100
Ammonium sulfate precipitation (0–95%)	642	7,623	12	1.5	80
Sephadex G-75	103	6,444	63	8	67
Toyo Pearl HW-55 column	43	3,807	89	11	40
HPLC Mono Q column	0.9	180	200	25	1.9

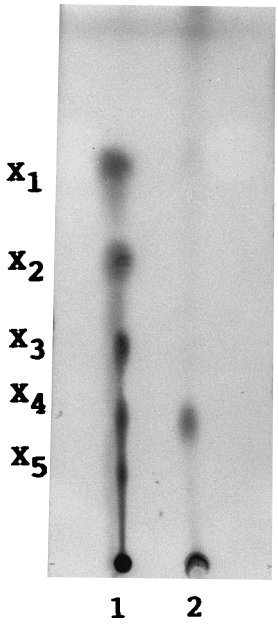

FIG. 3. Thin-layer chromatogram of hydrolysis products from oat spelt xylan by purified xylanase IV. The solvent system was ethyl acetate-hexane (5:3), with detection with ethanol-sulfuric acid (18:2). Lanes: 1, authentic samples of xylose (X_1), xylobiose (X_2), xylotriose (X_3), xylotetraose (X_4), and xylopentaose (X_5); 2, products of enzymatic hydrolysis after 36 h of incubation.

This is the first description of a xylanase which produces xylotetraose by an exo-type mechanism. Hitherto, the exoxylanase has been overlooked in the xylanase investigations, probably because of its relatively low level of activity in ordinary enzyme assays, such as the Somogyi-Nelson method. We suspect that exoxylanases may be wide spread among xylanolytic microorganisms and that they participate in the hydrolysis of hemicellulosic materials.

We are grateful to Saori Ito for help in identifying bacteria.

REFERENCES

1. **Bérenger, J. F., C. Frixon, J. Bigliardi, and N. Creuzet.** 1985. Production, purification, and characterization of thermostable xylanase from *Clostridium stercorarium*. Can. J. Microbiol. **31:**635–643.
2. **Biely, P.** 1985. Microbial xylanolytic systems. Trends Biotechnol. **3:**286–290.
3. **Dekker, R. F. H., and G. N. Richards.** 1975. Purification, properties, and mode of action of hemicellulase I produced by *Ceratocystis paradoxa*. Carbohydr. Res. **39:**97–114.
4. **Ganju, R. K., P. J. Vithayathil, and S. K. Murthy.** 1989. Purification and characterization of two xylanases from *Chaetomium thermophile* var. *coprophile*. Can. J. Microbiol. **35:**836–842.
5. **Hiroshi, I., S. Shigero, K. Hiroshi, M. Shigeru, and K. Atsushi.** 1982. Moths of Japan, vol. 1. Kodansha Co., Tokyo.
6. **Ito, S., T. Okutani, and K. Nishikawa.** 1977. Genshyoku Nihon Konchuzukan (Gekan), p. 385. Hoikushya, Osaka, Japan.
7. **John, M., B. Schmidt, and J. Schmidt.** 1979. Purification and some properties of five endo-1,4-β-D-xylanases and a β-D-xylosidase produced by a strain of *Aspergillus niger*. Can. J. Biochem. **57:**125–134.
8. **Kubata, B. K., H. Horitsu, K. Kawai, K. Takamizawa, and T. Suzuki.** 1992. Xylanase I of *Aeromonas caviae* ME-1 isolated from the intestine of a herbivorous insect (*Samia cynthia pryeri*). Biosci. Biotechnol. Biochem. **56:** 1463–1464.
9. **Kubata, B. K., H. Horitsu, K. Kawai, K. Takamizawa, and T. Suzuki.** 1994. Agricultural waste peanut shells, a raw material for the production of xylose and xylooligosaccharides. J. Jpn. Soc. Waste Management Experts **5:**29–35.
10. **Kubata, B. K., T. Suzuki, H. Horitsu, K. Kawai, and K. Takamizawa.** 1994. Purification and characterization of *Aeromonas caviae* ME-1 xylanase V, which produces exclusively xylobiose from xylan. Appl. Environ. Microbiol. **60:**531–535.
11. **Matsuo, M., and T. Yasui.** 1981. Hydrolysis of *para*-substituted phenyl-β-D-xylosides by β-xylosidase from *Malbranchea pulchella* var. *sulfurea* no 48. Agric. Biol. Chem. **45:**1603–1608.
12. **Mitsuishi, Y., T. Yamanobe, and M. Yagisawa.** 1988. The mode of three xylanases from mesophilic fungus strain Y-94 on xylooligosaccharides. Agric. Biol. Chem. **52:**921–927.
13. **Morales, P., A. Madarro, J. A. Pérez-González, J. M. Sendra, F. Piñaga, and A. Flors.** 1993. Purification and characterization of alkaline xylanases from *Bacillus polymyxa*. Appl. Environ. Microbiol. **59:**1376–1382.
14. **Panbangred, W., A. Shinmyo, S. Kinoshita, and H. Okada.** 1983. Purification and properties of endoxylanase produced by *Bacillus pumilus*. Agric. Biol. Chem. **47:**957–963.
15. **Puls, J., and K. Poutanen.** 1989. Mechanisms of enzymatic hydrolysis of hemicelluloses (xylans) and procedures for determination of the enzyme activities involved, p. 151–165. *In* M. P. Coughlan (ed.), Enzyme systems for lignocellulosic degradation. Elsevier, London.
16. **Sakka, K., Y. Maeda, Y. Hakamada, N. Takahashi, and K. Shimada.** 1991. Purification and some properties of xylanase from *Clostridium stercorarium* strain HX-1. Agric. Biol. Chem. **55:**247–248.
17. **Shikata, S., and K. Nisizawa.** 1975. Purification and properties of an exocellulase component of novel type from *Trichoderma viride*. J. Biochem. **78:**499–512.
18. **Suzuki, T., Y. Itoh, H. Naito, B. K. Kubata, H. Horitsu, K. Takamizawa, and K. Kawai.** Unpublished data.
19. **Teather, R. M., and P. J. Wood.** 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. **43:**777–780.