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Purification and characterization of a new xylanase (xylanase B) produced by *Streptomyces lividans* 66

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A new extracellular xylanase produced by *Streptomyces lividans* 66 was isolated from a genetically engineered clone of that strain. This enzyme, named xylanase B, has an M_r of 31000 and acts specifically on xylan as an endo-type xylanase producing short-chain xylo-oligosaccharides. The activity is optimal at pH 6.5 and at a temperature of 55 °C, which is similar to that of the previously characterized xylanase A. Xylanase B is glycosylated and has a pI of 8.4; its K_m and $V_{max.}$ values are 3.71 mg/ml and 1.96 mmol/mg of enzyme respectively. Specific antibodies raised against xylanase A show no cross-reaction with xylanase B; however, the anti-(xylanase B) antibodies react slightly with xylanase A. A comparison of the hydrolysis products obtained from oat-spelts xylan with both enzymes show that xylanase A preferentially degrades short-chain oligo-xylosides, whereas xylanase B acts on the longer, water-insoluble, molecules.

INTRODUCTION

Microbial xylanases have received considerable attention over the last years owing to a multitude of possible applications. These enzymes have potential in the biodegradation of lignocellulosic biomass to fuels and chemicals and in improving rumen digestion (Wong *et al.*, 1988). More recently the use of xylanases as bleaching agents in the pulp and paper industry has been suggested to replace of some of the chemicals presently used for this purpose (Paice *et al.* 1988). Such applications could have an important positive impact on the environment.

Complete xylan degradation requires generally the combined action of endo-xylanases as well as that of a β-xylosidase and less frequently exo-xylanases (Reilly, 1981). Such enzyme systems have been found in many of the micro-organisms investigated to date, showing a wide range of substrate specificities and multiple functions on xylan which serve well in the biodegradation of this rather complex and often heterogeneous polymer (Lee *et al.*, 1987). Over the last few years we have studied and reported the production of xylanases in different *Streptomyces* species (Ishaque & Kluepfel, 1981; Kluepfel *et al.*, 1986). Since then we have purified a xylanase from *Streptomyces lividans* 66 (Morosoli *et al.*, 1986) and homologously cloned, by functional complementation, the gene coding for this xylanase A in a xylanase- and cellulase-negative mutant of this wild-type strain (Mondou *et al.*, 1986). Recently, after screening the previously prepared gene bank for additional enzymes, we were able to select clones encoding for an additional xylanase activity which we named xylanase B.

In the present paper we report the purification and characterization of xylanase B isolated from the cellulase-negative clone *S. lividans* IAF42 as well as a comparison of its xylan-hydrolysis patterns with those of the previously purified xylanase A.

EXPERIMENTAL

Preparative methods

Micro-organisms and culture conditions. *Streptomyces lividans* IAF42, a xylanase-producing clone, was obtained by cloning the *xlnB* gene of *S. lividans* 66 (strain 1326) by functional complementation in *S. lividans* 10–164, a xylanase- and cellulase-negative mutant (Mondou *et al.*, 1986; Vats-Mehta *et al.*, 1990). The strain was maintained on 7-day-old agar slant cultures containing a modified yeast-extract malt-extract medium (Pridham *et al.*, 1956/57), in which 0.4% maltose substituted for glucose. When needed, solid or liquid culture media contained respectively 50 μg or 5 μg of thiostrepton (a gift from Squibb Canada)/ml. Spore suspensions prepared from such slants were used as inocula for vegetative cultures in trypticase soy broth (TSB; Frappier Diagnostique, Laval, Qué., Canada). Incubation was carried out at 34 °C on a rotary shaker at 240 rev./min for a period of 24–30 h. Enzyme production was carried out either in 500 ml Erlenmeyer flasks containing 100 ml of M-13 medium, previously described by Bertrand *et al.* (1989), using 1% of D-xylose as main carbon source, or in a 16-litre fermenter containing 10 litres of the same medium. The flask cultures were incubated at 34 °C on a rotary shaker at 240 rev./min. The same temperature was used in the fermenter, and the agitation and aeration rates were set at 500 rev./min and 0.5% (v/v)/min respectively. The inoculum size was, in each case, 4% of the vegetative TSB culture, and the incubation time was set at 65 h.

Enzyme recovery. The crude enzyme was recovered by centrifugation of the fermentation broth in a Beckman J2-21 centrifuge at 11000 *g*. From this supernatant the xylanase was precipitated with 3 vol. of cold ethanol. After being allowed to settle overnight, the precipitate was recovered by decanting the clear supernatant and filtering the remainder on a Buchner funnel. This precipitate was washed thoroughly with acetone and dried under vacuum overnight. The dried crude xylanase preparations obtained in this manner could be stored without loss of activity at 4 °C for several months.

Enzyme purification. For purification of the xylanase, 200 mg of the crude enzyme preparation were dissolved in 10 ml of 20 mM-Tris/HCl buffer, pH 8.5. After cooling on ice for 2 h, the solution was centrifuged at 3000 *g* for 10 min and then filtered through an Acro 0.2 μm disposable filter assembly (Gelman Sciences, Ann Arbor, MI, U.S.A.). The filtrate was adsorbed on to an anion-exchange DEAE Protein Pak 5 PW semi-preparatory

Abbreviations used: RBB, Remazol Brilliant Blue; DNS, dinitrosalicylic acid.

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h.p.l.c. column (Waters–Millipore, St. Laurent, Qué., Canada) that had been equilibrated with the same buffer. The elution was carried out using a linear gradient of 0.5 M-NaCl and was monitored with a u.v. recorder set at 280 nm. Fractions were collected and the enzyme activity detected by the Remazol Brilliant Blue (RBB) method described below under 'Enzyme assays'. The active fractions were pooled, then concentrated by ultrafiltration in an Amicon cell (Amicon Corp., Danvers, MA, U.S.A.) using a PM 10 membrane washed with 0.1 M-sodium phosphate buffer, pH 7.0. Final purification was achieved by gel chromatography on two Waters PW 300 h.p.l.c. columns which had been equilibrated with 0.1 M-sodium phosphate buffer, pH 7.0. The active fractions were again pooled, dialysed for 65 h against distilled water at 4 °C and then freeze-dried.

Enzymic hydrolysis. The substrate used for the studies of enzymic hydrolysis was generally xylan from oat spelts (Sigma Chemical Co.). In one experiment larchwood xylan from Aldrich Chemical Co. was used. Whereas the latter was completely soluble in water, the former contained some insoluble material (insoluble xylan), which was recovered by centrifugation after boiling a suspension of 10 g of xylan in 200 ml of water for 30 min. The supernatant was freeze-dried and used as soluble xylan for the enzyme assays and some of the hydrolysis studies. These were carried out with 500 μg of pure xylanase A or B, dissolved in 0.5 ml of 0.1 M McIlvaine buffer, pH 6, to which was added 9.5 ml of a 1% solution (or suspension) of xylan in the same buffer. The mixture was incubated at 60 °C and samples were withdrawn at intervals of 0, 10, 20, 40, 80, 160 and 1260 min. the reaction was stopped by heating for 10 min in a boiling-water bath, after which the samples were filtered through an Acro filter (0.45 μm pore size) and analysed by h.p.l.c.

Studies on the sequential action of the two xylanases were carried out in the same manner, a 1% suspension of complete oat spelts xylan being incubated for 20 h first with 50 μg of one enzyme/ml, stopping the reaction by boiling, and then re-incubating this preparation with the second xylanase for another 20 h, followed by another inactivation step. The hydrolysis products were analysed after each reaction by h.p.l.c. as described above.

Chemical hydrolysis. Chemical hydrolysis of xylan, which was carried out on oat-spelts xylan (Sigma Chemical Co.), as well as the separation of the xylo-oligosaccharides, was performed by the method described by Lee *et al.* (1987).

Analytical methods

Enzyme assays. Endo-1,4β-xylanase (β-1,4-D-xylan xylanohydrolase; EC 3.2.1.8) and cellulase [1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase; EC 3.2.1.4] activities were determined by the dinitrosalicylic acid (DNS) method. *p*-Nitrophenyl xyloside (Sigma Chemical Co.) was used as substrate to determine the activity of xylan 1,4β-xylosidase (EC 3.2.1.37). Details of these assays were given previously by Morosoli *et al.* (1986).

Routine determinations of xylanase activity in fermentation broths or in fractions during the purification steps were carried out by the RBB–xylan plate assay described by Kluepfel (1988). The substrate was prepared by covalently binding the dye RBB (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) to oat-spelts xylan (Sigma Chem. Co., St. Louis, MO, U.S.A.) by the method of Biely *et al.* (1985). Test solutions in 50 μl portions were placed into 5 mm wells dug in the plates with a cork borer and incubated overnight at 40 °C enclosed in plastic bags to avoid excessive evaporation of the medium. Xylanase activity was revealed by the appearance of clear or diffuse zones of substrate hydrolysis.

Enzyme activities. All enzyme activities were expressed in EC units, where 1 unit is defined as the amount of enzyme that releases 1 μmol of reducing sugars (expressed as xylose) in 1 min.

All results reported are averages for at least two independent experiments.

Protein. The protein contents of solutions or enzyme preparations were determined as described by Lowry *et al.* (1951), with bovine serum albumin as standard.

Determination of isoelectric point. Analytical isoelectric focusing was carried out on PhastGel containing Ampholines in the pH range 3–10 using the Phastsystem (Pharmacia, Baie d'Urfé, Qué., Canada). The gels were stained with Coomassie Blue after the run.

Determination of M_r. The M_r of the purified xylanase was estimated by SDS/PAGE as described by Laemmli (1970). The gels were stained either with Coomassie Blue or with Schiff's reagent for the detection of glycoproteins (Glossmann & Neville, 1971).

Antibodies and Western blotting. Rabbit antibodies were raised against the purified xylanase, and Western blotting were carried out using anti-(xylanase A) antibodies coupled to ^{125}I-Protein A (Amersham International) as previously reported by Mondou *et al.* (1986).

Influence of metal ions. The influence of metal ions on the xylanase activity was investigated by including different metal salts at a final concentration of 1 mM in the reaction mixture used for the determination of the enzyme activity as described under 'Enzyme assays'.

Analysis of oligosaccharides. The end products of both the chemical and enzymic hydrolysis of xylan were analysed by h.p.l.c. The samples were centrifuged in an Eppendorf centrifuge at maximum speed for 30 s in order to remove any solids and the supernatants were filtered through 0.45 μm-pore-size Acro filters. These filtrates containing the xylo-oligosaccharides were analysed by injecting a 10 μl portion on to a Sugar Pak I h.p.l.c. column (Waters–Millipore) heated at a constant 90 °C and separated by using water as eluant at a flow rate of 0.5 ml/min. The hydrolysis products were analysed with a Waters 410 differential refractometer and the retention times and elution patterns of the sugars registered on a Water 740 data module. For identification purposes xylobiose and xylose were used as standards.

RESULTS

The enzyme production was carried out with *S. lividans* IAF42, chosen among the three clones coding for xylanase B since it produced the highest enzyme levels (Vats-Mehta *et al.*, 1990).

Submerged cultures in M13 medium with either 1% xylan or 1% D-xylose as main carbon source yielded respectively 275 and 390 units after 72 h of incubation. Large-scale productions were carried out with D-xylose medium, since higher enzyme yields could be obtained after 65 h of fermentation. The low recovery of xylanase in the supernatant of xylan-grown cells as compared with that of xylose-grown cells was due to the formation of an enzyme–substrate complex, which was centrifuged down in the first step of enzyme purification. The enzyme was released from the remaining substrate after incubation at 60 °C for 2 h and was detected in the solubilized fraction by h.p.l.c. gel chromatography.

All xylanase B was found in the culture medium, and cell

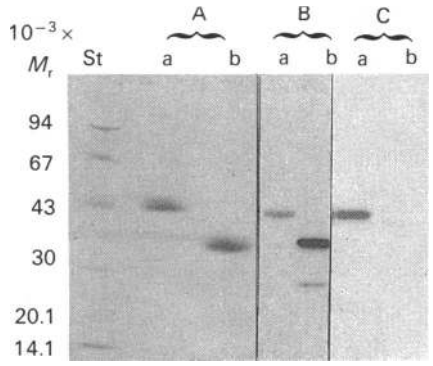

Fig. 1. (A) SDS/PAGE of xylanases A and B purified by h.p.l.c., (B) Western blot of SDS/polyacrylamide gel with anti-(xylanase B) antibodies and (C) Western blot of SDS/polyacrylamide gel with anti-(xylanase A) antibodies

Track 'St' contained M_r marker proteins: phosphorylase (M_r 94000), bovine serum albumin (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soya-bean trypsin inhibitor (M_r 20100) and α-lactalbumin (M_r 14400). Tracks 'a' contained purified xylanase A (10 μg). Tracks 'b' contained the purified xylanase B (10 μg).

extracts prepared by sonication of the mycelium showed only trace amounts of enzyme, probably due to artefacts of the manipulation or transient enzyme before secretion.

Crude xylanase B was obtained from the supernatants by precipitation with ethanol, and stable enzyme powders were recovered after washing the precipitates with acetone, this constituting a significant first purification step. The dry powders were easily resolubilized in 20 mM-Tris/HCl buffer, pH 8.5, and the concentrated enzyme solutions were separated by DEAE h.p.l.c. Xylanase B was eluted at a 10 mM-NaCl, whereas xylanase A, produced in small quantities, was eluted at 300 mM-NaCl (Morosoli *et al.*, 1986). The purification steps are summarized in Table 1. The purity of xylanase B was verified by h.p.l.c. on a cation-exchange column using 20 mM McIlvaine buffer, pH 4.5, with a 0 M–0.5 M linear NaCl gradient. The enzyme was eluted as a sharp well-defined peak after 9.44 min at a salt concentration of 45 mM. Analysis of the purified enzyme by SDS/PAGE revealed a single protein band, as shown in Fig. 1(*a*), with an apparent M_r of 31000 calculated from a standard curve. The enzyme stained positive with Schiff's reagent, indicating that it was glycosylated (results not shown). To determine whether xylanases A and B were immunorelated, SDS/PAGE of the two enzymes was followed by Western transfer on to nitrocellulose membranes and probed with anti-(xylanase A and B) antibodies (Figs. 1*b* and 1*c*). Anti-(xylanase B) antibodies reacted slightly with xylanase A, but no reaction was observed between xylanase B and anti-(xylanase A) antibodies.

Analytical isoelectric focusing of the enzyme on a pH gradient of 3.0–10 showed one major band with a pI of 8.4 and minor bands at pI of 7.3 and 9.0 respectively (Fig. 2). Since the purified xylanase B gave only one band on SDS/PAGE and one regular peak in the h.p.l.c. chromatograms, the secondary bands, which were also active, could be due to differences in glycosylation.

The stability of xylanase B was determined by incubating the enzyme aseptically without substrate in 50 mM McIlvaine buffer, pH 6.0 at various temperatures over a period of 24 h, withdrawing samples at appropriate intervals. These samples were analysed by the standard assay. The enzyme showed good stability at 30 °C, whereas above 37 °C the stability decreased gradually. Fig. 3 shows the stability of xylanase B at temperatures of 37–60 °C for 24 h.

The temperature and pH optima for xylanase B activity were

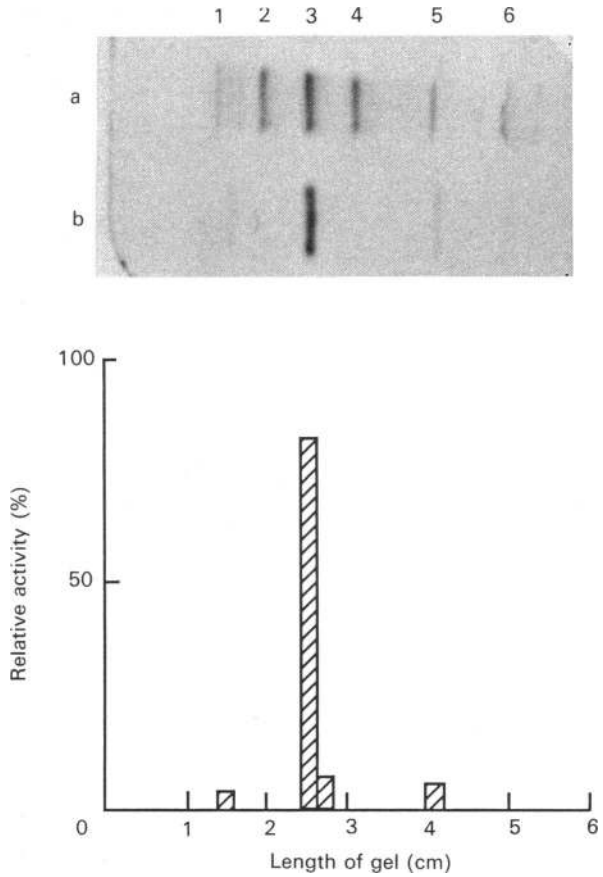

Fig. 2. Analytical electrofocusing of xylanase B purified by h.p.l.c.

Track 'a' contained pI marker proteins: (1) trypsinogen (pI 9.30), (2) lentil lectin (pI 8.65), (3) lentil lectin (pI 8.45), (4) lentil lectin (pI 8.15), (5) horse myoglobin (pI 7.35) and horse myoglobin (pI 6.85). Track 'b' contained purified xylanase B (4 μg). One part of the gel was stained with Coomassie Blue (top) and the other was sliced. The individual slices were assayed for xylanase activity (bottom).

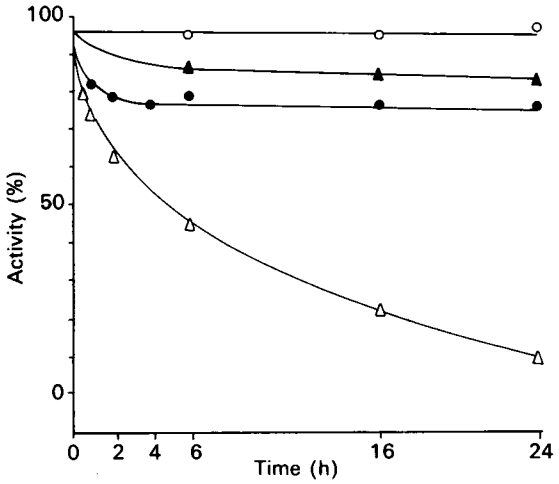

Fig. 3. Effect of temperature on xylanase B activity

Purified xylanase B at a concentration of 4 μg/ml in 100 mM McIlvaine buffer, pH 6.0, was incubated without substrate at 30 °C (○), 37 °C (▲), 50 °C (●) and 60 °C (△). The enzyme activity was determined at intervals as described in the Experimental section.

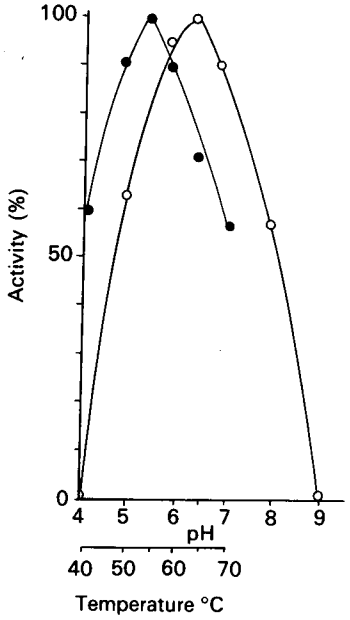

Fig. 4. Effect of temperature (●) and pH (○) on xylanase B activity

Purified xylanase B (1.5 μg) was incubated for 10 min at the temperatures or pH values indicated and the activity was measured by the DNS method as described in the Experimental section.

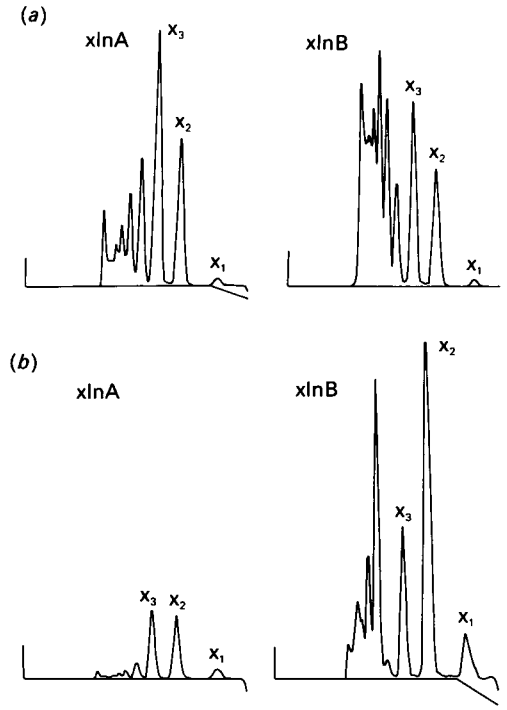

Fig. 5. Comparison of hydrolysis patterns obtained with xylanases A and B (xlnA and xlnB) on (*a*) soluble xylan and (*b*) insoluble xylan (x_1, xylose; x_2, xylobiose; x_3, xylotriose)

Soluble and insoluble xylan at a concentration of 10 mg/ml in 0.1 M McIlvaine buffer, pH 6, were hydrolysed with 50 μg of xylanase A or xylanase B for 10 min at 60 °C. Then 10 μl portions of filtrates were analysed by h.p.l.c. as described in the Experimental section.

55 °C and pH 6.5 respectively (Fig. 4) and were similar to those found for xylanase A (Morosoli *et al.*, 1986). The Michaelis–Menten constant was determined with soluble oat-spelts xylan at concentrations ranging from 0.44 to 15 mg/ml. From a

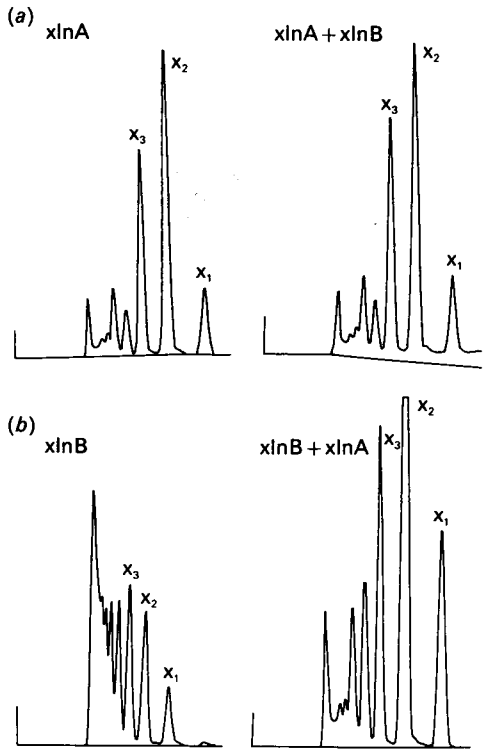

Fig. 6. Sequential hydrolysis of total oat-spelts xylan [*a*, xylanase A (xlnA) followed by xylanase B (xlnB); *b*, xylanase B followed by xylanase A (x_1, xylose; x_2, xylobiose; x_3, xylotriose)]

Suspensions of 1% xylan in 0.1 M McIlvaine buffer, pH 6, were incubated at 60 °C for 20 h with 50 μg of one enzyme/ml and, after stopping the reaction in boiling water, the other enzyme was added at the same concentration and the incubation continued for another 20 h. Portions (10 μl) of filtrates were analysed by h.p.l.c. as described in the Experimental section.

Lineweaver–Burk plot the K_m was determined to be 3.71 mg/ml and the $V_{max.}$ to be 1.96 mmol/mg of xylanase B. The enzyme showed neither β-xylosidase nor endoglucanase activities when tested with methylumbelliferyl β-xyloside and CM-cellulose respectively. The activity of xylanases A and B was similarly affected by certain metal ions. At 1 mM, both Hg^{2+} or Pb^{2+} inhibited both enzyme activities, by 70 and 15% respectively. Fe^{2+}, Ca^{2+}, Mg^{2+} and Zn^{2+} had no significant influence, whereas Cu^{2+} and Co^{2+} increased the xylanolysis by 10 and 20% respectively. The hydrolysis studies of xylanases A and B were carried out on soluble and insoluble fractions of xylan from oat spelts, the results of which are shown in Figs. 5(*a*) and 5(*b*) respectively. The soluble substrate, consisting of the shorter xyloside chain fragments, was degraded rapidly by both enzymes to oligosaccharides. Xylanase A yielded predominantly xylotriose and xylobiose, whereas xylanase B produced a variety of xylosides consisting of five or more sugar moieties (Fig. 5*a*). The hydrolysis pattern of the water-insoluble xylan showed very little action, if any, by xylanase A, giving mainly small quantities of xylotriose and xylobiose. On the other hand, xylanase B yielded a large amount of oligoxylosides ranging in size from one to eight xylose molecules (Fig. 5*b*).

In Fig. 6 the sequential action of xylanases A and B on total oat spelts xylan is shown. The hydrolysis pattern obtained by reaction with xylanase A remained essentially unchanged when followed by an incubation with xylanase B (Fig. 6*a*), showing that this enzyme was unable to carry out a further breakdown of the substrate. When reaction order was reversed, the hydrolysis

of the xylan proceeded considerably further, accumulating large quantities of xylotriose, xylobiose and xylose as end products (Fig. 6*b*).

DISCUSSION

The cloning of the xylanases A and B genes from *S. lividans* by functional complementation of the xylanase- and cellulase-negative mutant strain 10–164 (Mondou *et al.*, 1986; Vats-Mehta *et al.*, 1990) established that each coded for a specific enzyme. Xylanase A had been purified previously and characterized biochemically (Morosoli *et al.*, 1986). The xylanase B described in the present paper had not been detected when the enzymes of the wild-type strain had been investigated (Kluepfel *et al.*, 1986). Its presence was discovered during the screening of the xylanase-positive clones obtained by the abovementioned 'shotgun' cloning experiments. The substrate clearing zones on the RBB–xylan plates resulting from the enzyme action were characteristic for each xylanase: xylanase A giving clear well-defined zones, whereas xylanase B produced larger, but diffused, halos.

The enzyme was produced in submerged cultures on media containing either xylan or xylose as main carbon source. The latter substrate gave higher yields due to the absence of enzyme–substrate formation which was observed with xylan. As had been shown for xylanase A (Bertrand *et al.*, 1989) and also for this xylanase B (Vats-Mehta *et al.*, 1990), xylose did not repress enzyme synthesis, as is the case in *S. lividans* 1326 (wild-type). This is likely to be due to a gene-dosage effect of pIAF42, which was constructed with the multicopy vector pIJ702 (Katz *et al.*, 1983). Furthermore, the xylanase B produced by the clone IAF42 represented about 80% of the total extracellular proteins as estimated on SDS/PAGE after staining with Coomassie Blue. However, the enzyme activity in the culture filtrates, shown in Table 1, was likely to be underestimated, since they contained, besides xylanase B, also some xylanase A (Vats-Mehta *et al.*, 1990). As demonstrated in Fig. 6(*a*), hydrolysis products of the latter inhibited the activity of the former. A comparison of the biochemical and physical characteristics of xylanase B from clone *S. lividans* IAF42 with those of other xylanases from prokaryotes and eukaryotes shows many similarities in M_r and pI values. The pattern of a high-M_r/acidic and a low-M_r/basic enzyme multiplicity proposed by Wong *et al.* (1988) can be seen also in the xylanases A and B from *S. lividans*. The K_m values using oat-spelts xylan as substrate are similar for the two enzymes, whereas the $V_{max.}$ of xylanase B at 1.96 mmol of reducing sugars/min per mg of protein is about a third of that of xylanase A (5.57 mmol/min per mg). Furthermore, the enzyme stability at various temperatures and the activity optima both for pH and temperature are very similar for the two xylanases. They compare closely with the values reported for the enzymes from other *Streptomyces* strains (Wong *et al.*, 1988). The stimulation of activity achieved for both xylanases by metal ions such as Cu^{2+} or Co^{2+} will have to be investigated further, since it could be possible that the ions react or complex with the substrate, rendering it more accessible for the enzyme molecules. Both xylanases isolated from their respective clones *S. lividans* IAF18 and IAF42 were found to be glycosylated. This glycosylation might explain the two minor active bands observed in the isoelectric focusing of xylanase B (Fig. 2). The contradiction with the results we obtained for xylanase A previously is possibly due to the fact that the enzyme was isolated from another cellulase-negative mutant strain IAF8-7 (Morosoli *et al.*, 1986). Thus it is possible that this post-translational modification be related to the overexpression of the enzymes by these clones.

As shown in Figs. 5(*a*) and 5(*b*), xylanase A hydrolysed efficiently the water-soluble short-chain oligoxylosides, but had no or little action on the longer, water-insoluble, fragments. Its reaction products were xylobiose and xylotriose, the latter being hydrolysed to xylobiose and xylose upon further incubation,. Xylanase B acted on both substrate fractions, but appeared to cleave preferentially the long-chain oligoxylosides. According to Reilly (1981), both enzymes should be classified as endoxylanases.

The results of the studies on the sequential action of xylanases A and B, carried out on whole oat-spelts xylan, gave some insight into the manner in which the two enzymes acted on this substrate. Thus hydrolysis by xylanase B followed by that of xylanase A produced a rapid and efficient degradation of xylan and resulted in the accumulation of xylotriose, xylobiose and xylose (Fig. 6*b*). When the order of the two enzymes was reversed, xylanase A, as expected, hydrolysed rapidly all the soluble xylan. With the addition of xylanase B we expected a degradation of the water-insoluble substrate, as had been seen in Fig. 5(*b*). This was not the case, since the hydrolysis pattern remained unchanged (Fig. 6*a*), indicating that the degradation products of xylanase A (xylobiose or xylotriose) could inhibit xylanase B.

Further studies will be necessary to establish this inhibition of xylanase B as well as the relationships involved in sequential or concomitant action of the two enzymes.

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Table 1. Purification of xylanase B from *S. lividans* IAF42

Purification step	Total quantity (ml)	Protein content (mg/ml)	Activity (units/ml)	Specific activity (units/mg of protein)	Yield (%)
Culture filtrate	300	1.66	211	127	100.0
DEAE h.p.l.c.	25	0.60	486	835	19.6
Gel h.p.l.c.	4	0.68	699	1034	4.4

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