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Synergic Effects among Endo-xylanase, β-Xylosidase, and α-L-Arabinofuranosidase from *Bacillus stearothermophilus*

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Synergism among endo-xylanase, β-xylosidase, and α-L-arabinofuranosidase from *Bacillus stearothermophilus* upon xylan hydrolysis was investigated by using birchwood, oat spelt, and arabinoxylan as substrates. Endo-xylanase and β-xylosidase showed the cooperative action on all three substrates tested, revealing the fact that β-xylosidase assists endo-xylanase action in xylan hydrolysis by relieving the end-product inhibition upon endo-xylanase conferred by xylooligomers. α-L-Arabinofuranosidase also exhibited synergic effects with endo-xylanase and β-xylosidase on oat spelt and arabinoxylan, which contained significant amounts of arabinose side chains, whereas no synergism was detected on birchwood xylan which had only trace amounts of the side chain. Thus, the hydrolysis of xylan containing arabinose side chains required α-L-arabinofuranosidase as well as endo-xylanase and β-xylosidase for the better hydrolysis of the substrates, and these enzymes work cooperatively in order to maximize the extent and rate of xylan hydrolysis.

Xylan is the second most abundant polysaccharide next to cellulose (2, 7). It is composed of a xylan backbone, which is made up of a long chain of xylose subunits joined by β-1,4 linkage, with various side chains such as arabinose, acetic acid, and 4-*O*-methylglucuronic acid. The single chain of xylan is also attached to lignin in the cell wall matrix by phenolic acids (16). Thus, complete digestion of xylan requires not only the major xylanolytic enzymes like endo-xylanase and β-xylosidase but also the side chain-removing emzymes like α-L-arabinofuranosidase, acetyl xylan esterase, and α-glucuronidase (2, 4, 7, 16). It is therefore very desirable for these enzymes to work cooperatively in xylan hydrolysis. Numerous studies have been reported revealing the synergic effects among xylanolytic enzymes from various microorganisms (1, 8, 11, 12, 16, 21).

In this lab, a bacterium possessing the xylanolytic enzyme system was isolated from soil and identified as *Bacillus stearothermophilus* (22). Since then, the bacterial genes responsible for endo-xylanase (5), β-xylosidase (20), acetyl xylan esterase (13, 15), and α-L-arabinofuranosidase (9) were isolated and cloned into *Escherichia coli*. Furthermore, the nucleotide sequences of the endo-xylanase (6) and the β-xylosidase (19) were determined, and all of the cloned gene products were purified and characterized (10, 14, 18).

In this study, synergism among endo-xylanase, β-xylosidase, and α-L-arabinofuranosidase from *B. stearothermophilus* was examined and the possible mechanism of the synergic action was also discussed.

MATERIALS AND METHODS

Chemicals

Birchwood xylan, oat spelt xylan, *p*-nitrophenol-β-D-xylopyranoside (pNPX), and *p*-nitrophenol-α-L-arabinofuranoside (pNPAf) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Arabinoxylan was obtained from Megazyme Pty. Ltd. (Sidney, Austrailia). Other materials were of analytical grade.

Enzyme Production

Endo-xylanase was produced by culturing *E. coli* HB 101/pMG12 (5) containing *B. stearothermophilus* endo-xylanase gene (*xyn*A) at 37°C for 25 h in LB medium supplemented with 50 μg/ml ampicillin. The extracellular culture broth was obtained as the crude enzyme solution by centrifugating at 5 K for 20 min.

For β-Xylosidase production, *E. coli* HB101/pMG1 (20) containing *B. stearothermophilus* β-xylosidase gene (*xyl*A) incubated at 37°C for 10 h in the same medium as above. The cultured cells were harvested by centrufugating at 5 K for 20 min and then the intracellular cell extract was obtained as the enzyme solution by soni-

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cation.

α-L-Arabilofuranosidase was prepared by culturing *E. coli* HB101/pKMG7 (9) harboring the *B. stearothermophilus* acetyl xylan esterase gene (estII) at 37°C for 19 h in the same medium as above, followed by sonicating the cells to obtain crude cell extract as the enzyme solution.

Enzyme Assays

Endo-xylanase activity was measured by incubating 0.5 ml of birchwood xylan (2%) with the same volume of the enzyme solution for 20 min at 45°C and the produced reducing sugar was determined by the DNS method (17). One unit of enzyme was defined as the amount of enzyme required to release 1 μmol of xylose equivalents per min.

β-Xylosidase and α-L-arabinofuranosidase activities were assayed by monitoring the liberation of *p*-nitrophenol from pNPX (10 mM) and pNPAf (10 mM), respectively (9, 23). The enzyme solution 0.1 ml was incubated at 45°C for 20 min with the same amount of the substrate and the reaction was terminated by adding 2 ml of 0.4 M Na_2CO_3. The released *p*-nitrophenol was determined by reading the absorbance at 405 nm. One unit of enzyme was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per min.

Measurement of Synergic Effects

Endo-xylanase and β-xylosidase. Synergism between endo-xylanase and β-xylosidase in the hydrolysis of xylan was examined by using 2% oat spelt xylan as the substrate. The reaction mixture contained 0.2 ml of the substrate and the same amount of either endo-xylanase or β-xylosidase alone, or both enzymes simultaneously. Incubation was done at 45°C for 30, 60, 90, and 120 min. Measurement of the reducing sugar released was done by the DNS method (17).

Endo-xylanase and α-L-arabinofuranosidase. Arabinoxylan (1%) was used as a substrate to study synergic effects between endo-xylanase and α-L-arabinofuranosidase. Assay was done by mixing 0.2 ml of the substrate with the same volume of either endo-xylanase or α-L-arabinofuranosidase individually, or both enzymes at the same time. Reaction was performed at 45°C for 60, 90, 120, and 150 min, and the reducing sugar released was determined by the DNS method (17).

β-Xylosidase and α-L-arabinofuranosidase. Synergism between β-xylosidase and α-L-arabinofuranosidase was examined as described above except that the incubation was done at 45°C for 20, 40, 60, and 80 min.

Endo-xylanase, β-xylosidase, and α-L-arabinofuranosidase. Cooperativity among endo-xylanase, β-xylosidase, and α-L-arabinofuranosidase was inspected by mixing 2% birchwood xylan, 2% oat spelt xylan, or 1% arabinoxylan with the enzymes either individually or in the various combinations as shown in Table 1 at 45°C for 30 min. Released sugar content was measured by the DNS method (17). For arabinoxylan, enzyme assay was performed additionally for 60, 90, 120 min.

Table 1. Cooperative action of endo-xylanase, β-xylosidase, and α-L-arabinofuranosidase on birchwood, oat spelt, and arabinoxylan.

	Reducing sugar yields (μg of xylose equivalents)		
	Birchwood[a]	Oat spelts[b]	Arabinoxylan[c]
Xylanase	161.2	158.7	136.7
Xylosidase	224.4	ND*	ND
Arabinofuranosidase	ND	9.9	ND
Arabinofuranosidase & xylanase			
expected	161.2	168.6	136.7
observed	162.9	209.0	155.7
Arabinofuranosidase & xylosidase			
expected	224.4	9.9	0.0
observed	240.9	135.2	157.1
Xylosidase & xylanase			
expected	385.6	158.7	136.7
observed	446.2	399.7	283.2
Arabinofuranosidase, xylosidase, & xylanase			
expected	446.2	409.6	283.2
observed	425.4	458.5	460.8

All reactions were done at 45°C for 20 min. Reducing sugar release was quantitated by the DNS method (17). Reaction mixture contained 0.2 ml of xylan substrate and the same volume of either endo-xylanase, β-xylosidase or α-L-arabinofuranosidase alone, or in various combination.

[a]Enzymes used were endo-xylanase (0.015 U), β-xylosidase (1.6 U), α-L-arabinofuranosdase (11.4 U). 2% birchwood xylan was used as the substrate.

[b]Enzymes used were endo-xylanase (0.03 U), β-xylosidase (31.6 U), α-L-arabinofuranosidase (11.4 U). 2% oat spelt xylan was used as the substrate.

[c]Enzymes used were endo-xylanase (0.01 U), β-xylosidase (4.8 U), α-L-arabinofuranosidase (173 U). 1% arabinoxylan was used as the substrate.

* ND: Not Detected.

RESULTS AND DISCUSSION

Endo-xylanase and β-Xylosidase

The effect of β-xylosidase on the hydrolysis of oat spelt xylan by endo-xylanase was examined as shown in Fig. 1. When endo-xylanase and β-xylosidase were added simultaneously to 2% oat spelt xylan, the extent of liberation of reducing sugar was increased by 30 fold in a time dependent manner, while β-xylosidase alone produced only a negligible amount of reducing sugar. The result indicated that β-xylosidase increased reducing sugar production by hydrolyzing the xylooligomers,

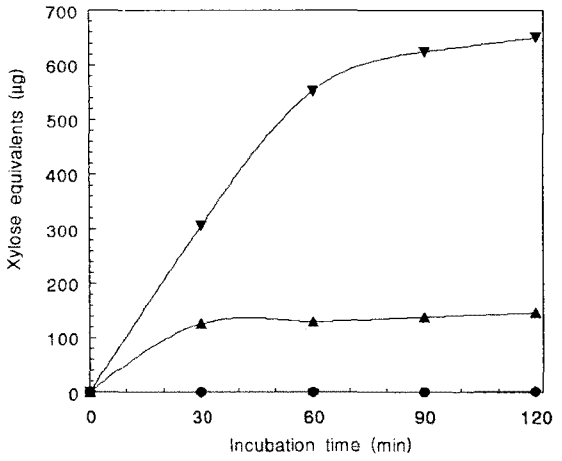

Fig. 1. Synergic effects of β-xylosidase in the hydrolysis of oat spelt xylan by endo-xylanase.

Reaction mixture contained 0.2 ml of 2% oat spelt xylan and the same volume of either endo-xylanase (0.06 U) or β-xylosidase (4.8 U) alone, or both enzymes simultaneously. Reaction was done at 45°C for 30, 60, 90, and 120 min. At each time point reducing sugar release was quantitated by the DNS method (17), and the values were plotted against the incubation time. ●—●, β-xylosidase; ▲—▲, endo-xylanase; ▼—▼, endo-xylanase & β-xylsodiase.

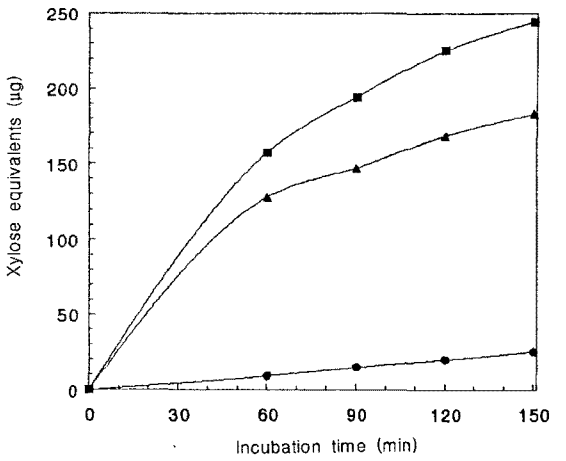

Fig. 2. Synergism between endo-xylanase and α-L-arabinofuranosidase in the hydrolysis of arabinoxylan.

Reaction mixture contained 0.2 ml of 1% arabinoxylan and the same volume of either endo-xylanase (0.009 U) or α-L-arabinofuranosidase (172.9 U) alone, or both enzymes simultaneously. Reaction was done at 45°C for 60, 90, 120, and 150 min. At each time point reducing sugar release was quantitated by the DNS method (17), and the values were plotted against the incubation time. ●—●, α-L-arabinofuranosidase; ▲—▲, endo-xylanase; ▼—▼, endo-xylanase & α-L-arbinofuranosidase.

which were the products of endo-xylanase activity, and this possibly relieved the end-product inhibition imposed upon endo-xylanase. Similar results have been reported previously by others as well (1, 8).

Endo-xylanase and α-L-Arabinofuranosidase

Arabinoxylan (1%) which contained 46.2% arabinose residue was used as a substrate to detect the synergism between endo-xylanase and α-L-arabinofuranosidase (Fig. 2). When α-L-arabinofuranosidase was added at the same time with endo-xylanase, the reducing sugar yield was increased by 15-20% compared to the level produced by endo-xylanase alone. The result obtained corresponds well to the one reported by Bachmann *et al.* (1) and can be explained by the fact that α-L-arabinofuranosidase removes arabinose side chains, which hinders sterically the action of endo-xylanase, exposing more sites at which endo-xylanase can act (11, 12).

β-Xylosidase and α-L-Arabinofuranosidase

β-Xylosidase and α-L-arabinofuranosidase were added to 1% arabinoxylan either respectively or in combination. As shown in Fig. 3, β-xylosidase could not act upon arabinoxylan in the absence of α-L-arabinofuranosidase. The very large increase in xylan hydrolyzing activity could be observed when two enzymes were present at the same time. β-Xylosidase of *B. stearothermophilus* has been reported to function as exo-xylanase in addition to β-xylosidase activity, which confers the uniqueness to this enzyme (18). Thus, exo-acting xylosdiase hydrolyzes xylan from the end of the sugar backbone by

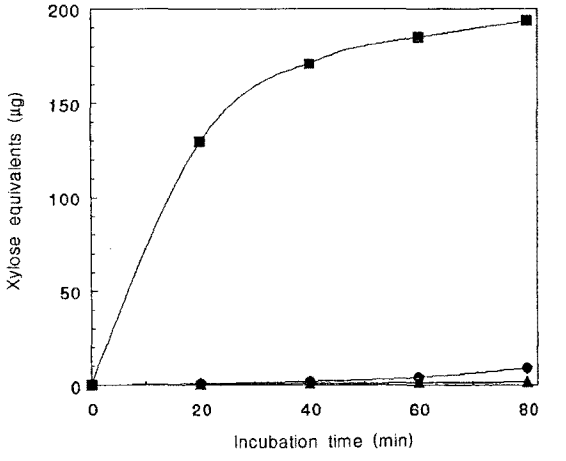

Fig. 3. Synergism between β-xylosidase and α-L-arabinofuranosidase in the hydrolysis of arabinoxylan.

Reaction mixture contained 0.2 ml of 1% arabinoxylan and the same volume of either β-xylosidase (4.8 U) or α-L-arabinofuranosidase (172.9 U) alone, or both enzymes simultaneously. Reaction was done at 45°C for 20, 40, 60, and 80 min. At each time point reducing sugar release was quantitated by the DNS method (17), and the values were plotted against the incubation time. ●—●, β-xylosidase; ▲—▲, α-L-arabinofuranosidase; ■—■, β-xylosidase & α-L-arbinofuranosidase.

cutting one xylose residue at a time. When the enzyme encounters any side chain attached to a xylan backbone, it can no longer proceed further but either pauses or leaves the substrate looking for other substrates. With the aid of α-L-arabinofuranosidase, which facilitates β-

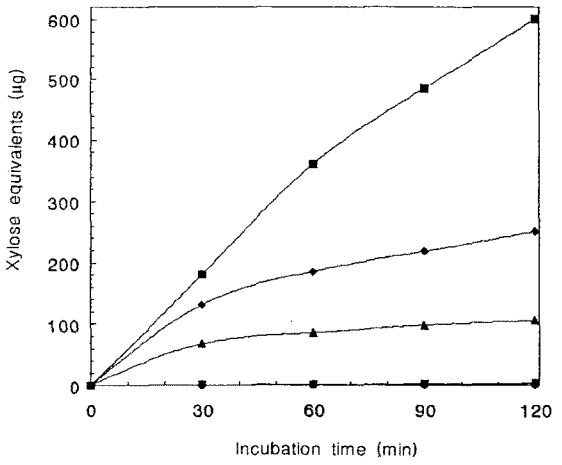

Fig. 4. Cooperative action of endo-xylanase, β-xylosidase, and α-L-arabinofuranosidase in the hydrolysis of arabinoxylan.
Reaction mixture contained 0.2 ml of 1% arabinoxylan and the same volume of either endo-xylanase (0.006 U), β-xylosidase (0.12 U) or α-L-arabinofuranosidase (86.4 U) alone, or in various combinations. Reaction was done at 45°C for 30, 60, 90, and 120 min. At each time point reducing sugar release was quantitated by the DNS method (17), and the values were plotted against the incubation time. ●—●, α-L-arabinofuranosidase; ▼—▼, β-xylosidase; ▲—▲, endo-xylanase; ◆—◆, endo-xylanase & β-xylosidase; ■—■, endo-xylanase, β-xylosidase & α-L-arbinofuranosidase.

xylosidase by removing the arabinose side chain, β-xylosidase now can proceed to hydrolyze more xylose residues from the arabinose-removed xylan substrate. This presumptive model for the exo-acting xylosdiase, therefore, can explain the synergism with α-L-arabinofuranosidase upon arabinoxylan hydrolysis.

Endo-xylanase, β-Xylosidase, and α-L-Arabinofuranosidase

In order to reveal the integrative synergic action among endo-xylanase, β-xylosdiase, and α-L-arabinofuranosidase based on substrate specificity, three enzymes were added to birchwood, oat spelt, and arabinoxylan either individually or in various combinations with one another. Table 1 summarizes the results obtained. When birchwood xylan was used as the substrate, synergism could not be detected except the case between endo-xylanase and β-xylosidase, which resulted in a 15% increase in reducing sugar yield. On the contrary, synergism between α-L-arabinofuranosidase and any of the major xylanolytic enzymes was not observed, implying that α-L-arabinofuranosidase was not needed in the hydrolysis of birchwood xylan which contained only few arabinose side chains.

When oat spelt xylan was used as the substrate, an unexpected result was obtained. Apparent synergic effects were shown not only between xylanase and α-L-arabinofuranosidase but also between β-xylosidase and α-L-arabinofuranosidase. This unexpected result could well be explained by the fact that oat spelt xylan from Sigma co. contained 10% arabinose residue and this residual amount of sugar side chain was large enough to require the presence of α-L-arabinofuranosidase for the better hydrolysis of the substrate by endo-xylanase and β-xylosidase.

Synergic effects among the enzymes upon arabinoxylan was studied further according to time courses. As shown in Fig. 4, the reducing sugar release was increased as the incubation time increased and when the three enzymes were present at the same time, the extent of the increase was at its maximum level.

In conclusion, the hydrolysis of xylan containing arabinose side chains by endo-xylanase and β-xylosdiase can be enhanced by adding α-L-arabinofuranosidase which releases the steric hindrance of the side chain, and this results in the exposure of additional sites at which endo-xylanase and β-xylosidase can act. Therefore, the hydrolysis of natural xylan would require not only the major xylanolytic enzymes such as endo-xylanase and β-xylosidase but also the presence of the arabinose side chain removing-enzymes, α-L-arabinofuranosidase.

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