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Synergism among Endo-xylanase, β-Xylosidase, and Acetyl Xylan Esterase from *Bacillus stearothermophilus*

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Synergic effects among endo-xylanase, β-xylosidase, and acetyl xylan esterase of *Bacillus stearothermophilus* in the hydrolysis of xylan were studied by using birchwood, oat spelt, and acetylated xylan as substrates. Synergism between endo-xylanase and β-xylosidase was observed on all three substrates tested, indicating that β-xylosidase enhanced the production of xylose by relieving the end-product inhibition upon endo-xylanase conferred by xylooligomers. Endo-xylanase and β-xylosidase also showed synergism with acetyl xylan esterase in the hydrolysis of birchwood and acetylated xylan, while no synergic effect was detected in oat spelt xylan hydrolysis. Thus, the hydrolysis of xylan containing acetic acid side chains required the action of acetyl xylan esterase, which eliminated the steric hindrance of the side chains, leading to the better hydrolysis by endo-xylanase and β-xylosidase , and the acetyl xylan esterase activity was also enhanced by endo-xylanase and β-xylosidase for the latter enzymes provided acetyl xylan esterase with shorter xylan oligomers, the better substrate for the enzyme.

Xylan, next to cellulose, is the most abundant polysaccharide in nature. It is a major hemicellulose component of plant cell walls and is located primarily in the secondary cell walls of angiosperms and gymnosperms (8, 16). Xylan is composed of β-1,4,-linked xylose units forming a xylan backbone and of side chains connected to it. Various side chains are composed of arabinose, acetic acid, and 4-*O*-methylglucuronic acid, and their compositions in xylan differ depending on the plant origin (2, 4).

Enzymatic hydrolysis of xylan to its monomers thus requires the presence of several enzymes with different functions (2, 4). The major xylanolytic enzymes include endo-xylanase and β-xylosidase, both of which cleave unsubstituted xylan substrate into its xylose subunits. But the presence of side chains still requires additional enzymes such as α-L-arabinofuranosidase, acetyl xylan esterase, and α-glucuronidase, which remove arabinose, acetic acid, and 4-*O*-methylglucuronic acid, respectively. Thus, it is reasonable to assume that these enzymes would work cooperatively with one another in xylan hydrolysis. In fact several studies have been done regarding the synergism among xylanolytic enzymes (1, 3, 9, 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 (10) 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 (11, 14, 18).

The present article reports the synergic actions among endo-xylanase, β-xylosidase, and acetyl xylan esterase from *B. stearothermophilus* in the hydrolysis of xylan, and the possible mechanism of the synergism is also discussed.

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MATERIALS AND METHODS

Chemicals

Birchwood xylan, oat apelt xylan, and *p*-nitrophenol-β-D-xylopyranoside (pNPX) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methyl-acetylsalycylate (MAS) was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan), and Test-Combination Kit for Acetic Acid was from Boehringer Mannheim (Mannheim, Germany). Other materials were of analytical grade.

Acetylated Xylan Preparation

Birchwood xylan was chemically acetylated according to the method of Johnson *et al.* (12). Shortly, 20 grams

of xylan was added to 400 ml of dimethyl sulfoxide at 60°C and then 5 g of dimethylaminopyridine was added as a catalyst. Acetic anhydride was then added dropwise and the solution was dialyzed. Insoluble materials were obtained by filtration, dried completely at 30°C, and used as acetylated xylan substrate.

Enzyme Production

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

β-Xylosidase was produced by culturing *E. coli* HB 101/pMG1 (20) containing *B. stearothemophilus* xylosidase gene (*xyl*A) at 37°C for 10 h in the same medium as above. Cells were harvested by centrufugating at 5 K for 20 min and then the cell extract was obtained as the enzyme solution by sonication.

For acetyl xylan esterase production, *E. coli* HB101/pKMG7 (13) harboring *B. stearothermophilus* acetyl xylan esterase gene (*est*II) was incubated at 37°C for 19 h in the same medium as above. The intracellular cell extract was prepared by sonication and used as the crude enzyme solution.

Enzyme Assays

Endo-xylanase activity was measured by incubating 0.2 ml of birchwood xylan (2%) with the same volume of the enzyme solution for 20 min at 45°C. Released sugar content was quantitated 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 activity was determined by reacting 0.1 ml of the enzyme solution with the same volume of pNPX (10 mM) at 45°C for 20 min (23). Released *p*-nitrophenol was quantitated 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.

Acetyl xylan esterase activity was assayed by adding 0.05 ml of the enzyme solution to the reacting solution containing 3 ml of MAS (10 mM), 0.5 ml of 0.025 M sodium phosphate buffer (pH 6.5), and incubating at 45°C for 20 min, followed by reading the absorbance at 300 nm (15). One unit of enzyme was defined as the amount of enzyme required to produce 1 μmol of methylsalicylate per min.

Measurement of Synergic Effects

Endo-xylanase and β-xylosidase. Synergism between endo-xylanase and β-xylosidase was examined by using 2% birchwood 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 at the same time. Reaction was done at 45°C for 30, 40, 50, and 60 min. Released sugar content was determined by the DNS method (17) and plotted against the incubation time.

Endo-xylanase and acetyl xylan esterase. Synergic effects between endo-xylanase and acetyl xylan esterase both in reducing sugar production and acetic acid liberation was determined by using 10% acetylated xylan as the substrate. For reducing sugar measurement, 0.3 ml of the substrate solution was incubated with the same volume of either xylanase or esterase alone, or both enzymes at the same time at 45°C for 1, 2, 3, and 4 h. Reaction was stopped by placing in ice and centrifugated at 12 K for 2 min to remove insoluble materials. The upper portion was used to determine released reducing sugar by the DNS method (17).

For acetic acid measurment, a reaction mixture containing the substrate and the enzymes in the same volume as above was incubated at 45°C for 10, 20, and 30 h, and the liberated acetic acid was quantitated by the Test-Combination Kit for Acetic Acid from Borhinger Mannheim (Mannheim, Germany) accordining to the manufacturer's instruction.

β-Xylosidase and acetyl xylan esterase. Synergism between β-xylosidase and acetyl xylan esterase was examined as described above. Reducing sugar was measured by the DNS method (17) and acetic acid liberation was also quantitated.

Endo-xylanase, β-xylosidase, and acetyl xylan esterase. Cooperativity among endo-xylanase, β-xylosidase, and acetyl xylan esterase was inspected by mixing 2% birchwood xylan, 2% oat spelt xylan, or 10% acetylated xylan with the enzymes either individually or in various combinations as in Table 5 at 45°C for 1 h. Released sugar content was measured by the DNS method (17). For acetylated xylan, incubation was done additionally for 2, 3, and 4 h.

RESULTS AND DISCUSSION

Endo-xylanase and β-Xylosidase

When 2% birchwood xylan was used to detect the effect of β-xylosidase on xylanase activity, the reducing sugar release was increased in a time dependent manner (Fig. 1) when two enzymes were present at the same time, compared to the results obtained when two enzymes existed individually. The result indicated that β-xylosidase relieved the end-product accumulation by degrading xylooligomers produced by xylanase, which blocked further xylanase activity (1, 9).

Endo-xylanase and Acetyl Xylan Esterase

Acetylated xylan (10%), which was chemically prepared (12), was used to determine the synergism between endo-xylanase and acetyl xylan esterase. The

synergic effect of acetyl xylan esterase on endo-xylanase activity was shown in Fig. 2, revealing the fact that acetyl xylan esterase helped endo-xylanase activity by removing acetic acid side chains exposing more sites for endo-xylanase action. Endo-xylanase also contributed to the increase of acetic acid release by supplying shorter xylan substrates on which acetyl xylan esterase could act more efficiently (Table 1) (1, 3, 21).

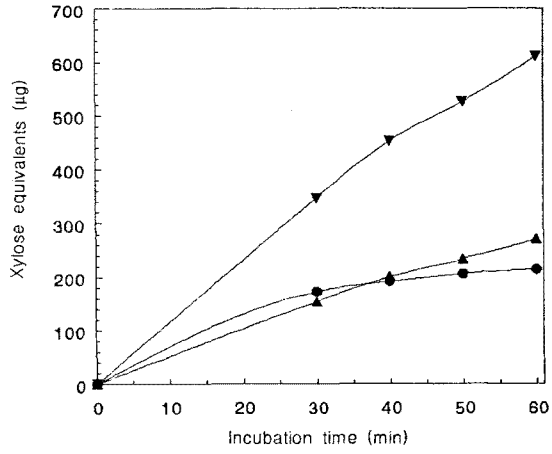

Fig. 1. Synergic effects of β-xylosidase in the hydrolysis of birchwood xylan by endo-xylanase.
Reaction mixture contained 0.2 ml of 2% birchwood xylan and the same volume of either endo-xylanase (0.01 U) or β-xylosidase (0.97 U) alone, or both enzymes simultaneously. Reaction was done at 45°C for 30, 40, 50, and 60 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-xylosidase.

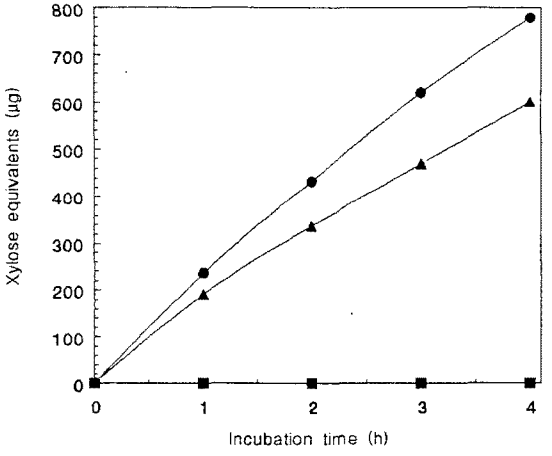

Fig. 2. Synergism between endo-xylanase and acetyl xylan esterase in the hydrolysis of acetylated xylan.
Reaction mixture contained 0.3 ml of 10% acetylated xylan and the same volume of either endo-xylanase (3.7 U) or acetyl xylan esterase (1623.5 U) alone, or both enzymes simultaneously. Reaction was done at 45°C for 1, 2, 3, and 4 h. At each time point reducing sugar release was quantitated by the DNS method (17), and the values were plotted against the incubation time. ▲—▲, endo-xylanase; ■—■, acetyl xylan esterase; ●—●, endo-xylanase & acetyl xylan esterase.

β-Xylosidase and Acetyl Xylan Esterase

Reducing sugar release was increased substantially when acetyl xylan esterase coexisted with β-xylosidase. As shown in Table 2, β-xylosidase alone could not act upon the substrate but supplementation with acetyl xylan esterase resulted in a large amount of reducing sugar release. β-Xylosidase from *B. stearothermophilus* was reported not only as β-xylosidase but also as exo-xylanase (18). Exo-xylanase cuts one xylose unit at a time starting from the end of the xylan chain and it can be assumed that the acetic acid side chain would block the forward movement of exo-xylanase. Acetyl xylan esterase which removes the acetic acid side chain from acetylated xylan substrate, therefore, facilitates the exo-acting xylanase by eliminating the acetic acid barrier. β-Xylosidase in turn was found to increase the acetic acid release by acetyl xylan esterase as shown in Table 3. This can be explained by assuming that β-xylosidase places the acetic acid side chain at the very end of the xylan backbone and this would make it easier for acetyl xylan esterase to cleave the side chain compared to the case in which acetyl xylan esterase acts on internally situated acetic acid side chains. Removal of acetic acid then again made it possible for exo-xylanase to act further upon the substrate

Table 1. Synergic effects of endo-xylanase on acetyl xylan esterase activity.

	Acetic acid liberated (µg)		
	10 h	20 h	30 h
Xylanase	ND*	ND	ND
Esterase	291.5	416.5	595.0
Xylanase & esterase	388.0	571.0	840.0

Endo-xylanase (1.26 U) and acetyl xylan esterase (1897.1 U) were added to 10% acetylated xylan either individually or at the same time at 45°C for 10, 20, and 30 h, and then acetic acid liberation was quantitated by Test Combinations for Acetic Acid from Boehringer Mannheim (Mannheim, Germany). *ND: Not Detected.

Table 2. Synergic effects of acetyl xylan esterase on β-xylosidase activity.

	Reducing sugar yields (µg of xylose equivalents)			
	1 h	2 h	3 h	4 h
Xylosidase	ND*	ND	ND	ND
Esterase	ND	ND	ND	ND
Xylosidase & esterase	103.0	113.0	171.5	269.0

β-Xylosidase (563.8 U) and acetyl xylan esterase (1623.5 U) were added to 10% acetylated xylan either individually or at the same time at 45°C for 1, 2, 3, and 4 h, and then reducing sugar releeased was quantitated by the DNS method (17). *ND: Not Detected.

till the next acetic acid chain is encountered, and this cycle would be repeated until the complete digestion of the substrate is achieved.

Endo-xylanase, β-Xylosidase, and Acetyl Xylan Esterase

Substrate specificity regarding the synergism among endo-xylanase, β-xylosdase, and acetyl xylan esterase was examined by using 2% birchwood xylan, 2% oat spelt xylan and 1% acetylated xylan. As summarized in Table 5, no synergic effects were observed when oat spelt xylan was used except the case between endo-xylanase and β-xylosidase, in which 90% of increase was shown. Thus, the hydrolysis of the substrate such as oat spelt xylan which contains only the trace amount of acetic acid side chains would not require the action of acetyl xylan esterase.

On the contrary, synergic effects among the enzymes were detected when birchwood xylan and acetylated xylan were tested. Even though the synergic effect upon birchwood xylan was smaller than that of acetylated xylan, significant amounts of acetic acid present in birchwood xylan still required the presence of acetyl xylan esterase for the better hydrolysis of the substrate by endo-xylanase and β-xylosidase (21).

Endo-xylanase and β-xylosidase in turn facilitated the removal of acetic acid by supplying acetyl xylan esterase with shorter xylan substrates on which acetyl xylan esterase can act more efficiently (Table 4).

The extent of synergic effects among the three enzymes was at the maximum level when acetylated xylan was used, and theses effects proceeded in a time-dependent manner as shown in Fig. 3.

In conclusion, the hydrolysis of xylan substrates containing acetic acid side chains required the presence of acetyl xylan esterase for better results. Acetyl xylan esterase removes the side chains exposing more sites for the endo-xylanase and β-xylosidase action, and the hydrolysis by endo-xylanase and β-xylosidase also provides the acetyl xylan esterase with the better substrate with shorter length. Thus, endo-xylanase, β-xylsodase, and acetyl xylan esterase may work cooperatively in the

Table 3. Synergic effects of β-xylosidase on acetyl xylan esterase activity.

	Acetic acid liberated (μg)		
	10 h	20 h	30 h
Xylosidase	9.8	11.4	13.0
Esterase	116.6	166.6	238.0
Xylosidase & esterase			
expected	126.4	178.0	251.0
observed	333.4	487.8	646.2

β-Xylosidase (86.1 U) and acetyl xylan esterase (758.8 U) were added to 10% acetylated xylan either individually or at the same time at 45°C for 10, 20, and 30 h, and then acetic acid liberation was quantitated by Test Combinations for Acetic Acid from Boehringer Mannheim (Mannheim, Germany).

Table 4. Synergic effects of endo-xylanase and β-xylosidase on acetyl xylan esterase activity.

	Acetic acid liberated (μg)		
	10 h	20 h	30 h
Xylanase	ND*	ND	ND
Xylosidase	4.9	5.7	6.5
Esterase	58.3	83.3	119.0
Xylanase, xylosidase, & esterase			
expected	63.2	89.0	125.5
observed	196.8	391.6	583.2

Endo-xylanase (0.25 U), β-xylosidase (43.0 U), and acetyl xylan esterase (379.4 U) were added to 10% acetylated xylan either individually or in various combination at 45°C for 10, 20, and 30 h, and then acetic acid liberation was quantitated by Test Combinations for Acetic Acid from Boehringer Mannheim (Mannheim, Germany). *ND: Not Detected.

Table 5. Cooperative action of endo-xylanase, β-xylosidase, and acetyl xylan esterase on birchwood, oat spelt, and acetylated xylan.

	Reducing sugar yields (μg of xylose equivalents)		
	Birchwood[a]	Oat spelts[b]	Acetylated xylan[c]
Xylanase	250.6	181.5	114.9
Xylosidase	154.5	18.3	ND*
Esterase	7.4	19.9	ND
Xylanase & esterase			
expected	258.0	201.4	114.9
observed	296.9	183.0	141.6
Xylosidase & esterase			
expected	161.9	38.2	0.0
observed	227.3	43.3	61.8
Xylanase & xylosidase			
expected	405.1	199.8	114.9
observed	467.4	386.2	201.6
Xylanase, xylosidase, & esterase			
expected	474.8	406.1	201.6
observed	522.4	407.4	294.9

All reactions were done at 45°C for 1 h. Reducing sugar release was quantitated by the DNS method (17). Reaction mixture contained 0.3 ml of xylan substrate and the same volume of either xylanase, xylosidase or acetyl xylan eaterase alone, or in various combination.
[a]Enzymes used were endo-xylanase (0.002 U), β-xylosidase (0.25 U), acetyl xylan esterase (324.7 U). 2% birchwood xylan was used as the substrate. [b]Enzymes used were endo-xylanase (0.02 U), β-xylosidase (63.5 U), acetyl xylan esterase (324.7 U). 2% oat spelt xylan was used as the substrate. [c]Enzymes used were endo-xylanase (0.73 U), β-xylosidase (112.8 U), acetyl xylan esterase (324.7 U). 10% acetylated xylan was used as the substrate. *ND: Not Detected.

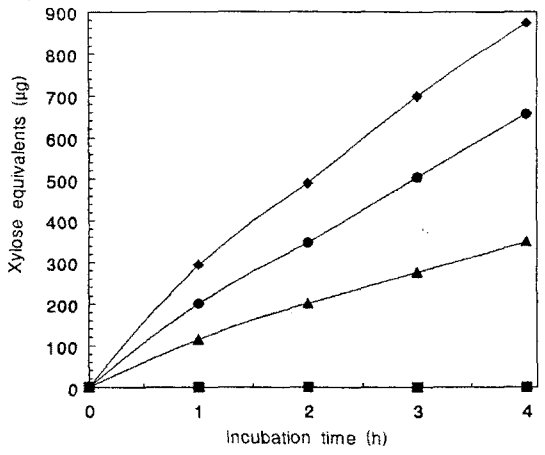

Fig. 3. Cooperative action of endo-xylanase, β-xylosidase, and acetyl xylan esterase in the hydrolysis of acetylated xylan. Reaction mixture contained 0.3 ml of 10% acetylated xylan and the same volume of either endo-xylanase (2.2 U), β-xylosidase (338.3 U) or acetyl xylan esterase (974.1 U) alone, or in the various combinations. Reaction was done at 45°C for 1, 2, 3, and 4 h. At each time point reducing sugar release was quantitated by the DNS method (17), and the values were plotted against the incubation time. ▲—▲, endo-xylanase; ■ —■, acetyl xylan esterase; ●—●, endo-xylanase & β-xylosidase; ◆— ◆, endo-xylanase, β-xylosidase & acetyl xylan esterase.

hydrolysis of natural xylan containing a significant amount of acetic acid side chains.

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