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Author(s)	AZUMA, Jun-ichi; KOSHIJIMA, Tetsuo
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Enzymatic Saccharification of Woody Plants

II. Synergistic Effects on Enzymatic Saccharification

Jun-ichi AZUMA* and Tetsuo KOSHIJIMA*

Abstract——Four different lignocellulosic materials including a softwood (AKAMATSU, *Pinus densiflora* Sieb. et Zucc.), a hardwood (BUNA, *Fagus crenata* Blume), a gramineous plant (bamboo, *Phyllostachis edulis* A. & Ribiere) and an agricultural waste (rice straw) were subjected to ball-milling and microwave heating pretreatments, and enzymatically saccharified using commercially available cellulases together with a cellulase preparation extracted from worker termites of *Coptotermes formosanus* Shiraki. Since the use of termite cellulase brought about only a lower yield of reducing sugar in saccharification owing to its significantly low cellulolytic activity. However, synergistic effects induced by using mixed enzyme preparations occurred to result in a good yield of reducing sugars. Similar synergistic effects were also shown to occur in enzymatic saccharification of organosolve-delignified pulps of a softwood (AKAEZOMATSU, *Picea glehnii* Mast.) and a hardwood (MIZUNARA, *Quercus mongolica* Fish. *var. grosseserrata* Rehd. et Wils.). Present results suggest that the synergistic effects could widely be applicable to enzymatic saccharification of lignocellulosic materials.

1. Introduction

An intense interest has been aroused in the use of lignocellulosic materials as fuel, chemicals and feedstuffs since oil-crisis in 1973. Enzymatic saccharification of lignocellulosics is an intitial step for realisation of these aims. Since the accessibility of cellulolytic enzyme to cellulose in the native lignocellulosics is low because of its crystallinity in combination with lignin, pretreatment prior to enzymatic saccharification is indispensable to achieve satisfied degree of saccharification[1]. So far numerous pertreatments have been tried. Recent developments in this field provide a number of pretreatments which permit effective enzymatic saccharification, *e.g.* ball-milling[2~9], roll-milling[10~12], steaming[13~16], explosion[17~19] and organosolve delignification[20~22]. Previously we examined the effects of expanded softening on enzymatic saccharification[23] and developed a new pretreatment method for heating lignocellulosics by irradiation with microwave[24]. On the other hand, a considerable interest has been payed on the improvement of cellulolytic activity of cellulases. Previously, we examined applicability of eleven commercially available cellulase preparations[23], and isolated a new cellualse from termite, *Coptotermes formosanus* Shiraki[25]. Since degradation of cellulose is performed by co-operative action between endoglucanases and exoglucanases, synergism should be taken into account for improve-

* Research Section of Wood Chemistry.

ment of enzymatic saccharification. Pioneering works by Toyama[26] and Wood[27~29] on solubilization of cellulosic materials established synergistic effects between several different cellulases, and indicated that the synergistic effects varied considerably depending upon differences in the modes of action of cellulsaes used. Recently, the synergistic effect has been shown to be applicable to saccharification of lignocellulosics[2,10,30].

In this study, we investigated the synergism between various enzymes in saccharification of various lignocellulosics pretreated with ball-milling, organosolve delignification and microwave heating.

2. Materials and methods

2.1 Materials and general methods

Four different lignocellulosic materials including a softwood (AKAMATSU, *Pinus densiflora* Sieb. et Zucc.), a hardwood (BUNA, *Fagus crenata* Blume), a gramineous plant (bamboo, *Phyllostachis edulis* A. & Ribiere) and an agricultural waste (rice straw) were extracted with ethanol-benzene (1:2, v/v) and ball-milled for 24 hr under nirtrogen atmosphere. These extractive free lignocellulosics were separately milled to 60–80 mesh and subjected to microwave heating at 230°C (AKAMATSU), 228°C (BUNA), 228°C (bamboo) and 235°C (rice straw), respectively, following the procedure previously described[24]. Wood tips of MIZUNARA (*Quercus mongolica* Fish. *var. grosseserrata* Rehd. et Wils.) and AKAEZOMATSU (*Picea glehnii* Mast.) were subjected to organosolve delignification with crezol-water (1:1, v/v) for 30 min at 180°C and 2 hr at 190°C (solvent to the wood tip ratio, 4:1), respectively. These organosolve-delignified pulps were kindly supplied by Prof. A. Sakakibara, University of Hokkaido. These organosolve-delignified pulps were thoroughly washed with acetone and distilled water until free from crezol prior to enzymatic saccharification. Lignin contents of MIZUNARA and AKAEZOMATSU pulps were 1.8% and 14%, respectively. Unless otherwise specified, other materials and methods were the same as those previously described[23~25].

2.2 Isolation of cellulase preparation from termites

Worker termites were collected from colonies of *Coptotermes formosanus* Shiraki maintained with AKAMATSU for about ten years at 26°C. Fifty milliliters of 0.05M sodium acetate buffer (SAB), pH 4.8, was added to 10 g of worker termites and sonicated at 5°C for 6 × 30 sec. The homogenate was centrifuged at 15,000 × g for 20 min at 5°C to give a crude enzyme solution. In the previous study we recovered carbohydrases by ammonium sulfate fractionation followed by gel filtration on Sephadex G-50[25] This procedure, however, needs throublesome and time consuming operations for preparation of a large amount of enzyme. In this study, therefore, we intended to

Table 1. Activities of Carbohydrolases of Enzymes from Termites*

Enzymes	Activities**	Enzymes	Activities***
α-Galactosidase	6.24	Avicelase	4.65
β-Galactosidase	4.59	CMCase	39.01
α-Glucosidase	5.25	Xylanase	46.60
β-Glucosidase	12.80	Galactanase	5.02
α-Mannosidase	11.27	Mannanase	37.27
β-Mannosidase	5.69	Arabanase	9.26
α-Xylosidase	0.22	Amylase	27.08
β-Xylosidase	1.20	Pectinase	18.59
α-Arabinosidase	0.66	Dextranase	0.40

* Values are expressed as unites/mg protein.

** One unit of aryl-glycosidase activity is defined as the amount of enzyme which liberates 1 μmol of *p*-nitrophenol/min under the condition described prviously[23].

*** One unit of polysaccharase activity is defined as the amount of enzyme which liberates 1 μmol of monosaccharide under the condition pescribed previously[23].

develop a simple method for preparation of a large amount of enzyme from termites. The crude enzyme solution was treated with 5 vol. of ethanol. The precipitate formed was recovered by centrifugation at 5°C as above, washed 4 times with acetone and dried in a vacuum desiccator to obtain an enzyme preparation which was stable for more than 3 months at 5°C. The activities of carbohydrolases of the present enzyme preparation are shown in Table 1. When beeing compared with the previously reported result[23], activities of Avicelase and Carboxymethylcellulase (CMCase) were respectively 1.5 fold and 1.9 fold higher but activities of β-glycosidases were lower than those of the enzyme preparation obtained by gel-filtration on Sphadex G-50.

3. Results and Discussion

3.1 Effect of ball-milling on synergistic effect

Since ball-milling pretreatment increased accessibility of enzyme[2~9], and the synergistic effect between Cellulosin AC and Onozuka R-10 was prominent with ball-milled pine wood[2], we firstly selected ball-milling to further examine the synergistic effect.

The termite enzyme preparation gives only a low yield of reducing sugars from lignocellulosics as shown in Table 2. This could be ascribed to that the activities of carbohydrolases of termite cellulase preparation shown in Table 1 were substantially lower than those of the commercial cellulases[23]. The amount of reducing sugars liberated was, however, enhanced to 59.2% from 6.0% when AKAMATSU previously ball-milled for 24 hr was hydrolyzed with 1:1 enzyme mixture (Table 2). The ratio of mixing was chosen as 1:1, based on the previously published results[2].

Table 2-(a). Effects of Ball-Milling on Synergistic Action*

Substrates	Termites**		Onozuka R-100**	
	Weight loss (%)	Reducing sugar content (%)	Weight loss (%)	Reducing sugar content (%)
AKAMATSU	16.8	5.5	46.0	36.5
BUNA	14.6	6.7	38.9	39.3
Bamboo	29.2	23.6	51.4	41.5
Rice straw	29.0	17.2	54.4	50.5
Substrates	Celluase (MERK)**		Dricelase**	
	Weight loss (%)	Reducing sugar content (%)	Weight loss (%)	Reducing sugar content (%)
AKAMATSU	49.6	38.3	39.7	39.4
BUNA	40.7	35.8	32.4	38.0
Bamboo	50.5	49.0	45.4	48.2
Rice straw	52.9	47.3	51.7	45.8

* Substrate concentration is 5.0%.
** Enzyme concentration is 0.25%.

Table 2-(b). Effects of Ball-Milling on Synergistic Action*

Substrates	Termites+Onozuka R-10		Termites+Dricelase	
	Weight loss (%)	Reducing sugar content (%)	Weight loss (%)	Reducing sugar content (%)
AKAMATSU	57.3	59.2	48.7	51.4
BUNA	51.1	58.3	44.3	48.6
Bamboo	68.9	83.1	38.0	50.0
Rice straw	74.1	75.2	68.1	67.0
Substrates	Termites+Cellulase (MERK)		Dricelase+Cellulase (MERK)	
	Weight loss (%)	Reducing sugar content (%)	Weight loss (%)	Reducing sugar content (%)
AKAMATSU	55.2	55.9	51.8	51.4
BUNA	51.0	51.4	51.0	51.2
Bamboo	65.8	73.9	63.1	68.5
Rice straw	69.0	69.6	68.5	64.6

* Substrate concentration and each enzyme concentration were 5.0% and 0.25%, respectively.

Since only 36.5% of reducing sugars was liberated by saccharification with Onozuka R-10 as a sole enzyme, the increased yield of reducing sugars is considered to be due to synergistic effect. Similar synergistic effects coming from the mixing with other cellulase preparations are also shown in Table 2. The origins of the cellulase preparations used in the present study are different from each other such as Onozuka from *Trichoderma* sp., Merk Cellulase from *Oxiporus* sp., Dricelase from *Irpex* sp. and Cellulosin from *Aspergillus* sp. Although mixed enzyme systems consistently gave

high synergistic effects, the greatest one was obtained with termite cellulase and Onozuka R-10. The differences in synergistic effect may occur by the differences in properties of constituent enzyme molecules. Wood reported that the mechanism of synergistic effect is difficult to elucidate because in the cellulase system both enzyme-enzyme and enzyme-substrate interactions are involved[29].

3.2 Effect of organosolve delignification on synergistic effect

Organosolve delignification has been interested in utilization of both pulps and degraded lignin. We secondly examined the organosolve delignification on synergistic effect using commercial cellulase preparations. As shown in Fig. 1 for

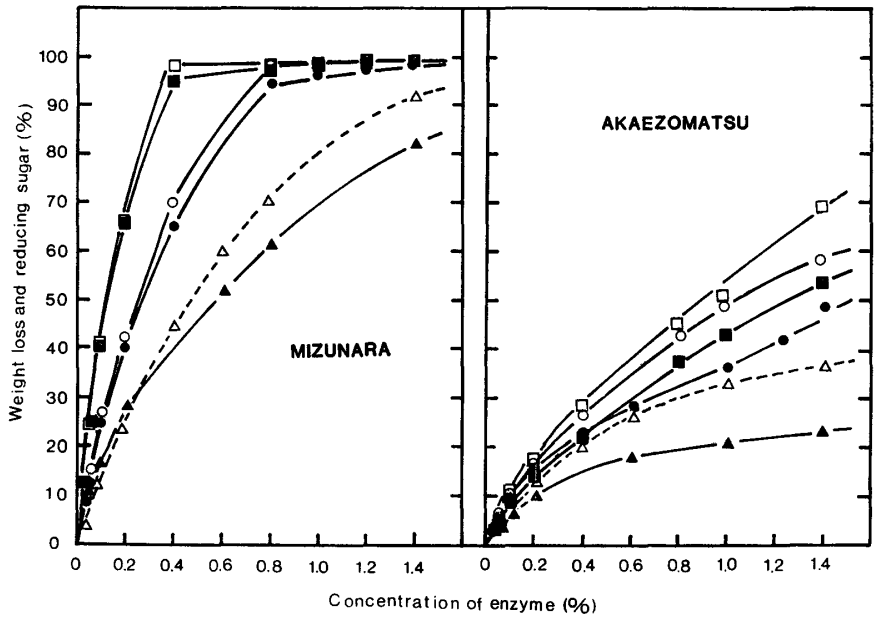

Fig. 1. Synergistic enzymatic degradation of organosolve pulps with Cellulosin AP and Onozuka R-10. Symbols indicate weight loss (—▲—) and reducing sugar production (---△---) with sole Cellulosin AP (0.25%), weight loss (—●—) and reducing sugar production (—○—) with sole Onozuka R-10 (0.25%), and weight loss (—■—) and reducing sugar production (—□—) with co-reaction with Cellulosin AP (0.25%) and Onozuka R-10 (0.25%), substrate concentration being 5.0%.

MIZUNARA pulp, Cellulosin AP alone could not give a plateau in hydrolytic activity even at 1.4% of enzyme concentration, whereas more than 90% of the pulp was saccharified by Onozuka R-10. Note that more than 0.8% of Onozuka R-10 was necessary to achiev effective saccharification. In contrast, a high rate of saccharification was attained when 1 : 1 mixture of Cellulosin AP and Onozuka R-10 was used as in the case of ball-milling[2]. Each enzyme preparation of 0.25% was enough to achiev 95-98% saccharification, indicating the occurence of high synergistic effects. These results may be useful for the investigation of the feasibility of utilization of

Table 3. Enzymatic Degradation of Organosolve-Delignified Pulps by Cellulase from Termites*

Substrates		Termites		Termites+Onozuka R-10	
		Weight loss (%)	Reducing sugar content (%)	Weight loss (%)	Reducing sugar content (%)
MIZUNARA	(wet)	17.5	12.7	47.3	47.0
	(dried)	7.1	9.3	23.1	35.5
AKAEZOMATSU	(wet)	11.5	9.1	12.2	23.2
	(dried)	1.5	4.5	3.4	5.7

* Substrate concentration and each enzyme concentration were 5.0% and 0.25%, respectively.

lignocellulosic materials. It was found that organosolve delignification of hardwood is more effective for enzymatic saccharification than ball-milling. We, therefore, tried to saccharify MIZUNARA pulp with the enzyme preparation from termites. As shown in Table 3, only 12.7% of reducing sugar was produced. The amount of reducing sugar was, however, enhanced to 47.0% with 1:1 mixture of termite enzyme and Onozuka R-10, indicating the operation of a high synergistic effect.

As for softwood (AKAMATSU), although synergistic effects occurred, the degree of saccharification was low even if 1:1 mixture of Cellulosin AP and Onozuka R-10 was used, and did not attain its maximum even at 1.4% of enzyme concentration (Fig. 1). The same was true in utilization of the termite enzyme preparation, since the yield of reducing sugar produced with termite enzyme alone was less than a half of that produced with 1:1 mixture of the termite enzyme and Onozuka R-10. The reason of the discrepancy in enzyme susceptibility between MIZUNARA and AKAEZOMATSU may be ascribed to lignin remained after organosolve delignification: it is known that softwood is resistant to enzymatic attack[9,14~17]. Further delingification is desired to attain a high enzymatic hydrolysis susceptibility[31]. It was noted that the degree of saccharification was markedly decreased if the substrate was completely dried-up at 105°C before use. This may be ascribed to reformation of hydrogen bonds which induced possible recrystallization of cellulose. The same was observed in microwave heating followed by enzymatic saccharification[25]. Thus care should be taken not to dry the samples up prior to enzymatic treatment.

3.3 Effect of microwave heating on synergistic effect

Recently we developed a new microwave-heating preteratment system which permits effective saccharification of woody plants in the presence of lignin[25]. We now examined the possibility of co-operative effect of two different enzyme preparations. As shown in Table 4, Onozuka R-10 weakly co-operated with termites enzyme preparation indicating that synergistic effect occured in a wide variety of substrates treated with various pretreatments. Generally ball-milling is suitable for enzymatic saccharification of softwood, and gives a higher amount of reducing

Table 4. Effects of Microwave Irradiation on Enzymatic Saccharification (%)*

Substrates	Termites		Termites+Onozuka R-10		Temperature (°C)
	Weight loss (%)	Reducing sugar content (%)	Weight loss (%)	Reducing sugar content (%)	
AKAMATSU	38.8	31.7	52.4	55.9	230
BUNA	28.7	27.9	54.9	61.5	228
Bamboo	53.2	47.4	66.4	59.4	228
Rice straw	52.3	44.2	67.7	54.2	235

Substrates	Onozuka R-10		Temperature (°C)
	Weight loss (%)	Reducing sugar content (%)	
AKAMATSU	42.9	40.7	230
BUNA	46.0	45.5	228
Bamboo	48.4	50.0	228
Rice straw	54.2	46.8	235

* Substrate concentration and each enzyme concentration were 5.0% and 0.25%, respectively.

sugars than the other physical preteratments. Therefore, it is noteworthy that microwave-heating preteratement was as much effective as ball-milling as shown in Tables 2-(a) and 4. The yield of reducing sugars is expected to become higher in microwave preteratment than in others because of less degradation of sugars released by effective heating from interior part of lignocellulosic materials and by shortness of heating time[25].

In conclusion, we demonstrated the occurrence of synergistic effects on cellulose hydrolysis between commercially available enzymes which differed in origin and cellulase isolated from termites using substrates pretreated by use of three different methods, *i.e.* ball-milling, organosolve delignification and microwave heating. It seems preferable to enhance the degradation rate by use of synergistic effect in order to investigate the feasibility of utilization of lignocellulosic materials.

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