Journal of Microbiology and Biotechnology
Vol. 6. No. 6, 432-438, 1996

Enzymatic Hydrolysis of Crystalline Chitin in an Agitated Bead Reaction System and Its Reaction Characteristics

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Native crystalline chitin was hydrolyzed in an agitated bead reaction system using crude chitinase excreted from *Aspergillus fumigatus* JC-19. The reaction was enhanced significantly, and the concentration and yield of reducing sugar after 48 hours were measured to be 35.42 g/l (w/v) and 0.64, respectively, around 1.86 times higher than those of the conventional system that was carried out without glass beads. The effect of reaction conditions, such as the amounts of chitin, chitinase and glass beads, and the size of glass bead, were examined. Ball milled chitin was also hydrolyzed in the agitated bead reaction system, the conversion yield and reaction rate of ball milled chitin for 24 hours increased up to 0.87 and 48.02 g/l, respectively. Chitinase showed relatively high stability in the agitated bead reaction system, particularly in the presence of enzyme stabilizer, Ca^{++}, which played a critical role in preventing the deactivation of chitinase by the physical impact of glass beads. The variations of the structural features of chitin during the reaction were followed by SEM and X-ray diffraction, and the enhanced hydrolysis reaction was caused by both the fragmentation of chitin particles and the destruction of the crystalline structure owing to the synergic effects of the attrition of glass beads and the hydrolytic action of chitinase.

Chitin is a polysaccharide composed of *N*-acetyl-D-glucosamine (GlcNAc) residue by a β-1,4-glucosidic linkage, and is widely distributed in nature (15). Chitooligosaccharides produced by the acidic or enzymatic hydrolysis of chitin possess various functional properties, such as, blood cholesterol control, antitumor activity, acceleration of wound healing, antifungal activity, and acceleration of the growth of *Bifidobacterium* (1, 18, 21).

Enzymatic hydrolysis has distinct advantages compared to acid hydrolysis, especially for the production of chitooligosaccharides composed of various degrees of polymerization because the composition of chitooligosaccharides can be adjusted readily by changing either the amount and type of chitinolytic enzymes or controlling reaction conditions. The enzymatic hydrolysis of chitin is carried out by three chitinolytic enzymes, chitinase (EC 3.2.1.14), an endo-acting enzyme that cleaves chitin to chitobiose or higher soluble oligomers, chitobiase (*N,N'*-diacetylchitobiase; EC 3.2.1.30), that converts the above products to *N*-acetyl-D-glucosamine, and exo-type chitinase, which hydrolyzes chitin from the non-reducing ends to chitobiose (5).

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Key words: crystalline chitin, chitinase, enzymatic hydrolysis, agitated bead reaction system, *Aspergillus fumigatus* JC-19, *N*-acetyl-D-glucosamine

The enzymatic hydrolysis of crystalline chitin is a very slow process, therefore, to increase the enzyme reaction rate, various chemical pretreatment methods, such as partial hydrolysis of crystalline chitin to its colloidal state by hydrochloric acid (7) or methansulfonic acid (19), and swelling of crystalline chitin by phosphoric acid (14), have been used. However, the above methods have the following distinct disadvantages, such as the use of caustic chemicals, the requirement of neutralizing chemicals, and low recovery yields of pretreated chitin.

Insoluble substrates, such as crystalline cellulose and raw starch, have been effectively hydrolyzed in a heterogeneous enzyme reaction system using glass beads as the attrition milling media (8, 9, 10, 11). The enhanced hydrolysis rate and yield was caused by the structural modification due to the synergic effect of the physical impact of glass bead and enzymatic action (4, 16). Crystalline chitin has similar structural features with crystalline cellulose and raw starch, such as insolubility and crystallinity, that make crystalline chitin insusceptible to chitinase.

Therefore, it can be postulated that the efficiency of enzymatic hydrolysis of native crystalline chitin also can be improved significantly provide the agitated bead reaction system is employed. The above reaction system can also achieve the simultaneous effect of carrying physical

pretreatment and enzymatic hydrolysis together, which will simplify the process for enzymatic hydrolysis of native crystalline chitin.

The purpose of this work was to evaluate the efficiency of the agitated bead reaction system for enzymatic hydrolysis of native crystalline chitin. The effect of operational variables, such as the amounts of chitin, chitinase and glass bead, and the size of glass bead, were also examined to determine the optimum reaction condition. The stability of chitinase during reaction in the agitated bead reaction system was examined with or without the presence of Ca^{++} as an enzyme stabilizer. The changes in the structural features of residual chitin were followed by SEM and X-ray chromatogram to elucidate the enhancing mechanism of the enzymatic hydrolysis of chitin.

MATERIALS AND METHODS

Strain and Chitinase

Aspergillus fumigatus JC-19 was cultivated in colloidal chitin basal medium at 37°C for 4 days as previously described (6), and the culture broth was concentrated by vacuum evaporation and ultrafiltration to prepare crude chitinase. The chitinase activity was measured as follows, 1 ml of culture broth and 1 ml of colloidal chitin solution (5 mg of colloidal chitin/ml) were mixed with 1 ml of 50 mM phosphate buffer (pH 7.0), and then incubated at 37°C for 1 h, and then reducing sugar concentration was determined. 1 Unit of chitinase was defined as the amount of enzyme required to produce 1 μM of *N*-acetyl-D-glucosamine for 1 h.

Chitins

Crystalline chitin was powder type chitin(practical grade, Sigma Chemical Co., St. Louis, MO, U.S.A.), and it was milled by grinder to particle size less than 100 mesh before use.

Ball milled chitin was prepared using a ball-mill (Dong Yang Science Co., Inchon, Korea). 0 g of crystalline chitin and 1 kg of various size (12-26 mm) ceramic balls were mixed and ball-milled at 80 rpm for 4, 12, 24, and 72 h, respectively and the fractions below 150 mesh were used as substrates.

Enzyme Reaction in an Agitated Bead Reaction System

2.5 g of crystalline chitin and 750 units of chitinase (corresponding 300 unit/g of crystalline chitin) were suspended in 50 ml of 50 mM phosphate buffer (pH 7.0), and then 20 g of 3 mm of glass bead was added. The reaction was carried out in a shaker at 250 rpm, 50°C for 48 h. The amounts of enzyme, crystalline chitin, and the size and amount of glass bead were changed accordingly.

Stability of Chitinase

750 units of chitinase were suspended in 50 ml of 50 mM phosphate buffer (pH 7.0) with or without 20 g of glass beads, and then reacted at 250 rpm, 50°C for 24 h. The enzyme stabilizer, Ca^{++}, was added to 5 mM as a final concentration. The residual activity of crude chitinase was measured during reaction.

Analytical Methods

The reducing sugar concentration was determined by two methods; DNS method (12) and Morgan-Elson method (2) using *N*-acetyl-D-glucosamine as the standard. The composition of chitooligosaccharides was analyzed by HPLC (Model-305, Gilson Co., France); Cosmosil 5NH2 packed column (Nacalai Tesque, Kyoto, Japan), acetonitrile/H_2O (65/35), 1.0 ml/min, and RI detector. *N*-acetyl-D-glucosamine, chitobiose, chitotriose, chitotetraose, chitopentaose, chitohexaose (Seikagaku Kogyo, Tokyo, Japan) were used as the standard materials.

Scanning Electron Microscope (SEM)

The size and surface structure of crystalline chitin was observed by scanning electron microscope (S-570 SEM, Hitachi, Japan) during hydrolysis with or without agitated bead.

X-ray Diffraction Patterns

Crystallinity of native chitin was measured by X-ray diffractometer (Philips X'Pert, Philips), by the powder method using Ni-filter to produce Cu(Kα) radiation at 30 kV, 20 mA, time constant range 1,000 cps, scanning speed 2° 2θ/min, and diffraction intensity from the Bragg angles (2θ) 4° to 35°.

RESULTS AND DISCUSSION

Effectiveness of the Agitated Bead Reaction System on Enzymatic Hydrolysis of Crystalline Chitin

Fig. 1 compares the extent of the enzymatic hydrolysis of the native crystalline chitin hydrolyzed in the agitated bead reaction system and that in the conventional system carried out without agitated beads. For the conventional system the final total reducing sugar concentration and the conversion yield reached 19.2 g/l and 0.35, respectively. However, in the agitated bead reaction system, the final total reducing sugar concentration and conversion yield increased around 1.86 times above those of the conventional method at 35.6 g/l and 0.65, respectively. As expected, the agitated bead reaction system was found to be an effective reaction system for enzymatic hydrolysis of native crystalline chitin as with crystalline cellulose and raw starch.

Lee *et al.* (8, 10, 11), who studied the enzymatic hydrolysis of crystalline cellulose in the agitated bead reaction system, also achieved two-fold increased conversion yield after 24 h from 0.40 for the conventional system to 0.80. They reported that the enhanced hydrolysis of crystalline cellulose was caused by the enlarged accessible surface area due to the fragmentation of cellulose par-

ticles by the synergic actions of the physical impacts of glass beads and the hydrolytic action of cellulase (8, 17).

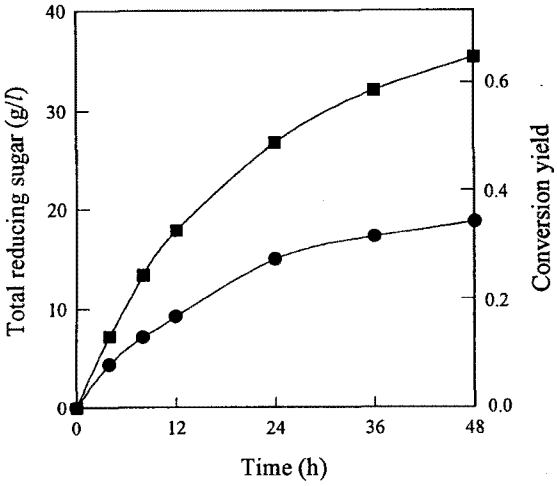

Fig. 1. Extent of enzymatic hydrolysis of crystalline chitin in the agitated bead reaction system.

●, without glass bead; ■, with glass bead. Reaction conditions: 50 g of crystalline chitin/l (w/v), 300 units chitinase/g crystalline chitin, 400 g of 3 mm glass bead/l, 50°C, pH 7.0, 250 rpm, and for 48 h.

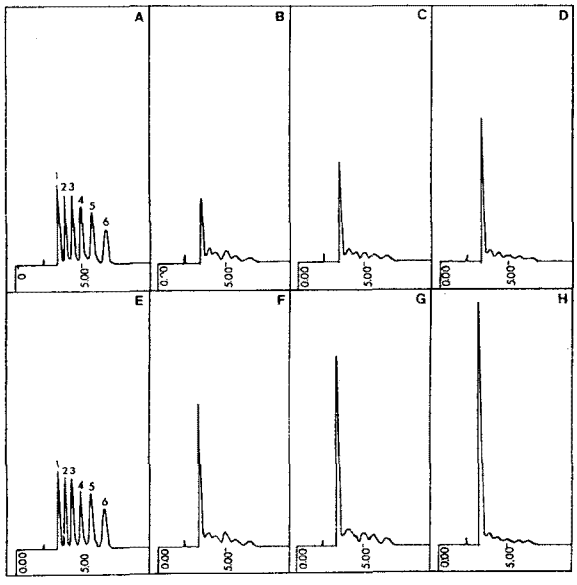

Fig. 2. Compositional changes of chitooligosaccharides during enzyme reactions.

(A), (E), chromatogram for standard materials; (B), (C), (D), chromatogram for products of enzymatic hydrolysis without glass beads after 12, 24, 48 h, respectively; (F), (G), (H), chromatogram for products of enzymatic hydrolysis with glass beads after 12, 24, 48 h, respectively. Reaction conditions: 50 g of crystalline chitin/l (w/v), 300 units chitinase/g crystalline chitin, 400 g of 3 mm glass bead/l, 50°C, pH 7.0, 250 rpm, and for 48 h. Standard materials: 1, *N*-acetyl-D-glucosamine; 2, chitobiose; 3, chitotriose; 4, chitotetraose; 5, chitopentaose; 6, chitohexaose.

Fig. 2 compares HPLC chromatogram which shows the compositional changes of chitooligosaccharide both hydrolyzed in the agitated bead reaction system and in conventional system. The sugar composition was not much differed each other throughout the reaction time, and *N*-acetyl-D-glucosamine was produced mainly from the initial stage of hydrolysis. This indicates that the physical impact of attrition media may not affect on mode of action of chitinase but only on the hydrolysis rate.

Optimal Reaction Conditions for Enzymatic Hydrolysis of Chitin in the Agitated Bead Reaction System

Amount of chitinase. Fig. 3 illustrates the effects of changing the chitinase concentrations from 2,500 to 25,000 unit/l where the chitin concentration of 50 g/l, calculated to be 50 to 500 unit of chitinase/g crystalline chitin. The total reducing sugar concentration increased proportionally as the amount of chitinase increased up to 300 units of chitinase/g crystalline chitin, however, it was not increased further after a certain level. This may be attributed to the limitation of accessible surface area for reaction of chitinase because of the insoluble and crystalline nature of native chitin.

Crystalline chitin concentration. Fig. 4 illustrates the effect of chitin concentrations on hydrolysis reactions carried out varying from 20 to 100 g/l but fixing chitinase applied to 300 unit of chitinase/g crystalline chitin. The extent of hydrolysis increased in accordance with chitin concentration up to 50 g/l, and the reducing sugar concentration and conversion yield after 24 h were at-

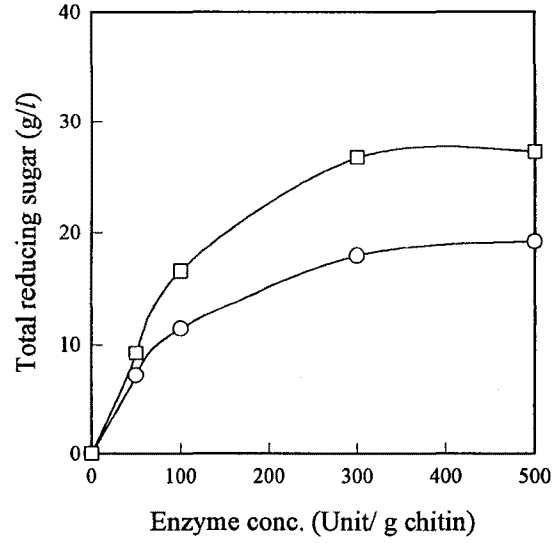

Fig. 3. Effect of the amount of chitinase on enzymatic hydrolysis of crystalline chitin in the agitated bead reaction system.

○, 12 h; □, 24 h. Reaction conditions: 50 g of crystalline chitin/l (w/v), 400 g of 3 mm glass bead/l, 0~500 units chitinase/g crystalline chitin, 50°C, pH 7.0, 250 rpm, and for 24 h.

tained to 24.6 g/l and 0.45, respectively. The optimal concentration for enzymatic hydrolysis of crystalline chitin in the agitated bead reaction system was determined to be 50 g/l, similar with that of crystalline cellulose (10, 11).

The hydrolysis showed only slight improvement over the above mentioned level, consequently, the conversion yield decreased. The reduced reaction can be explained by the decreased attrition effect of glass beads, arising from the loss of the fluidity of reaction mixture caused by the penetration of water molecules into the highly hygroscopic chitin particles.

Size and amount of glass bead. As shown in Table 1, the hydrolysis reaction showed the highest value of 26.8 g/l at 3 mm glass bead, and the enhancing effect was considerably reduced at diameter less than 3 mm. Small size glass beads can not give sufficient impact for destruction of the crystalline structure of chitin. Because chitinase can readily react with more decomposed structure, the hydrolysis of chitin with glass beads of smaller size shows lower value than that with glass beads of the proper size which may induce crystalline chitin to a more decomposed structure.

The maximum conversion yield can be obtained at the amount of 400 g/l, thereafter, the conversion yield was reduced beyond the above level. The excess amount of glass bead prevents the proper mixing of the reaction mixtures due to the limitation of volumetric space. The optimum conditions for hydrolysis of crystalline chitin in the agitated bead reaction system were determined to be 50 g of crystalline chitin/l, the amount of chitinase by the ratio of 300 unit of chitinase/1.0 g of chitin, and 400 g of 3 mm glass beads/l.

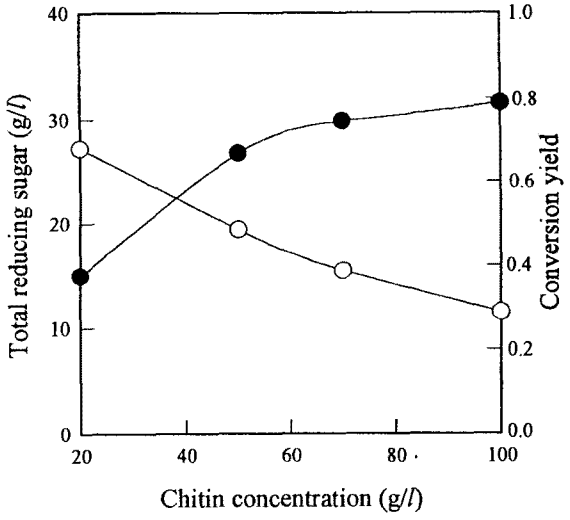

Fig. 4. Effect of chitin concentration on enzymatic hydrolysis of crystalline chitin in the agitated bead reaction system.
●, total reducing sugar; ○, conversion yield. Reaction conditions: 20~100 g of chitin/l (w/v), 400 g of 3 mm glass bead/l, 300 units chitinase/g crystalline chitin, 50°C, pH 7.0, 250 rpm, and for 24 h.

Table 1. Effect of the size and amount of glass bead on enzymatic hydrolysis of crystalline chitin in the agitated bead reaction system.

Amount of beads (g/l)	Size of bead (mm)		
	1.2	3.0	4.5
0	14.0*	14.4	14.3
200	15.8	22.0	21.2
400	16.5	26.8	23.5
600	15.6	25.7	25.2
1,000	13.4	24.1	24.8

*Total reducing sugar (g/l). Reaction condition: 50 g of crystalline chitin/l (w/v), 300 units chitinase/g crystalline chitin, 50°C, pH 7.0, 250 rpm and after 24 h.

Enzymatic Hydrolysis of Ball Milled Chitin in the Agitated Bead Reaction System

To examine the effect of the structural modification of chitin on hydrolysis reaction in the agitated bead reaction system, the crystalline chitin was ball milled for different periods of time. As shown in Fig. 5, the enzyme reaction of ball milled chitin increased substantially as the milling time was prolonged. The final reducing sugar and conversion yield increased remarkably up to 48.02 g/l and 0.87 for 48 h when 24 h pretreated chitin was used as substrate. This indicates that further increased degree of conversion in the agitated bead reaction system can be achieved through the modification of structure by means

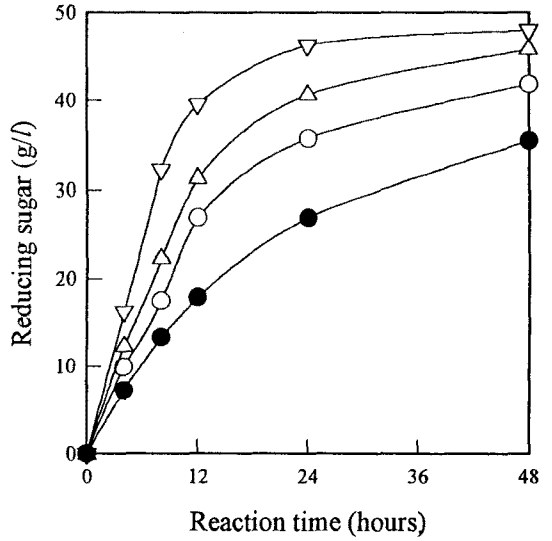

Fig. 5. Extent of hydrolysis of ball-milled chitin in the agitated bead reaction system.
●—●, crystalline chitin; ○—○, chitin ball milled for 4 h; △—△, chitin ball milled for 12 h; ▽—▽, chitin ball milled for 24 h. Reaction condition: 50 g of chitin/l (w/v), 300 units chitinase/g crystalline chitin, 400 g of 3 mm glass bead/l, 50°C, pH 7.0, 250 rpm, and for 48 h.

of physical and chemical methods, and an effective pretreatment method needs to be developed.

Stability of Chitinase in an Agitated Bead Reaction System

The agitated bead enzyme reaction system possesses the potential danger of the loss of activity of chitinase by the shearing action of attrition milling media. Table 2 compares the residual activities of chitinase maintaining both in the agitated bead reaction system and in the conventional system with or without addition of 5 mM of Ca^{++} as an enzyme stabilizer.

The chitinase showed relatively high stability even without the addition of Ca^{++}, and the residual chitinase activity was obtained around 63% in the agitated bead reaction system compared to 72% for the conventional system. The enzyme stabilizer played significant role in maintaining the stability of chitinase, and the residual chitinase activity after 24 h remained high at 89% for both reaction systems. This observation accords well with the results of previous works (16, 21) where it was reported that Ca^{++}, Mg^{++}, or Co^{++} plays as a part of stabilizer and activator of chitinase excreted from *Aspergillus* sp.

Structural Features of Chitin during Enzymatic Hydrolysis in the Agitated Bead Reaction System

Scanning electron microscope (SEM). The structural features of crystalline chitin in the agitated bead reaction system were observed to elucidate the enhancing mechanism of hydrolysis reaction. To examine the effect of physical attrition of glass beads on the structure of native chitin, the native chitin was wet-milled without addition of chitinase. Native crystalline chitin showed initially the sheet and flake-like structure as observed in Fig. 6A. After wet-milling for 24 and 48 h without the addition of chitinase, the structure changed and became not only fluffy but also partially swollen structure, and also only some portion of chitin was fragmented to smaller size (Fig. 6B, 6C).

Table 2. The stability of chitinase in the agitated bead reaction system.

addition	Ca^{++} (mM)	Time (h)				
		0	4	8	12	24
Without glass beads	0	1.00*	0.93	0.86	0.79	0.72
	5	1.12	1.05	0.97	0.93	0.89
With glass beads	0	1.00	0.93	0.82	0.74	0.63
	5	1.12	1.03	0.96	0.91	0.89

*Residual activity. Reaction condition: 15,000 units of chitinase/l, 400 g of 3 mm glass bead/l, 50°C, pH 7.0, and 250 rpm.

The microcrystalline structure of residual chitin after hydrolyzing it by chitinase only without the addition of glass beads was also observed as shown in Fig. 7A-7C. According to SEM photograms of the structure of chitin that was hydrolyzed simply by chitinase only but without glass beads, the cracks started to form in some portion of the surface of chitin particles from 12 h, and the chitin particles changed to a fluffy swollen structure as the reaction proceeded, and then only some portion of chitin fragmented.

Meanwhile, in enzyme reaction in the agitated bead reaction system (Fig. 7D-7F), the intensive structural modification, the fluffy and fragmented structure (Fig. 7D), started to occur from the initial 12 h. Thereafter, the structure of chitin changed more drastically to the extensively fragmented and fluffy swollen form (Fig. 7E, 7F). The structural modification of chitin either by wet-milling with glass beads or by the enzymatic reaction of chitinase only was not significant, meanwhile, it was carried out intensively provide the synergetic effect of physical impact of glass bead and hydrolytic action of chitinase imposed together. The remarkable increment of reaction rate in the agitated bead reaction system is due to the enlarged accessible surface area where the enzymatic reaction of chitinase can be occurred.

Fig. 6. Scanning electron microscope photograms of chitin wet-milled by glass beads without addition of chitinase. (A), native crystalline chitin; (B) and (C), wet-milled without chitinase for 24 and 48 h, respectively.

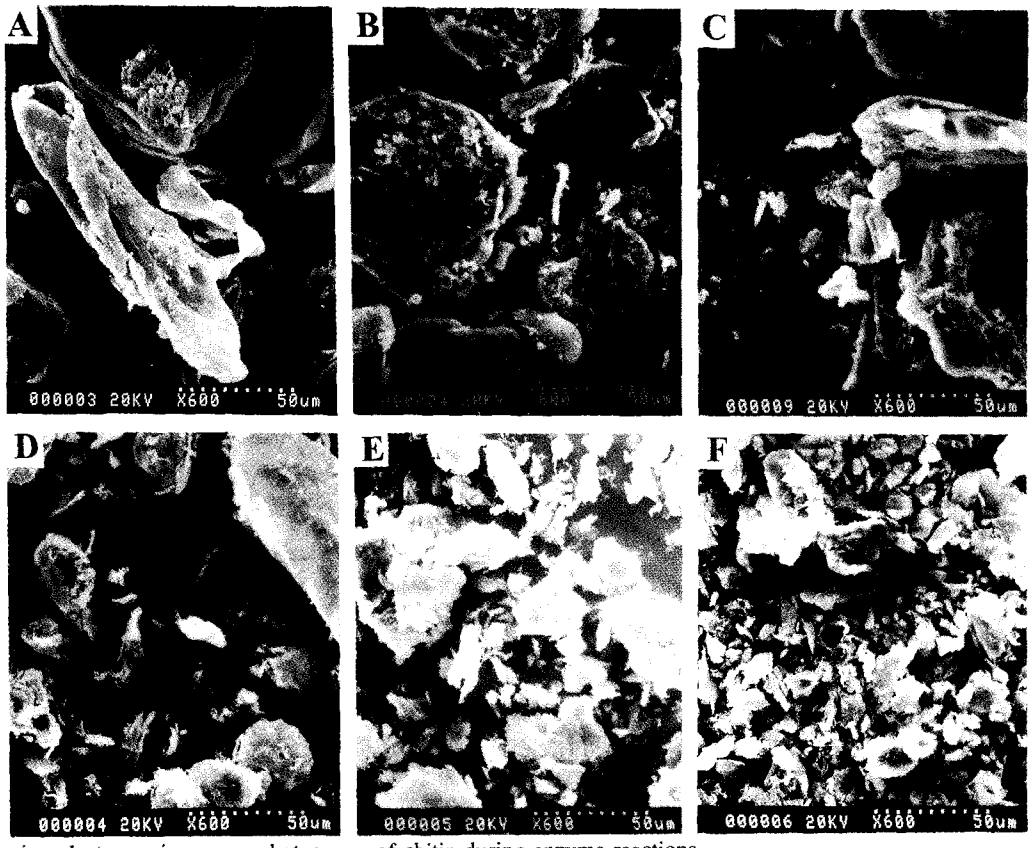

Fig. 7. Scanning electron microscope photograms of chitin during enzyme reactions.
(A)-(C), enzyme reaction without glass bead for 12, 24, and 48 h, respectively; (D)-(F), enzyme reaction with glass bead for 12, 24, and 48 h, respectively. Reaction conditions: 50 g of crystalline chitin/l (w/v), 300 units chitinase/g crystalline chitin, 400 g of 3 mm glass bead/l, 50°C, pH 7.0, 250 rpm, and for 48 h.

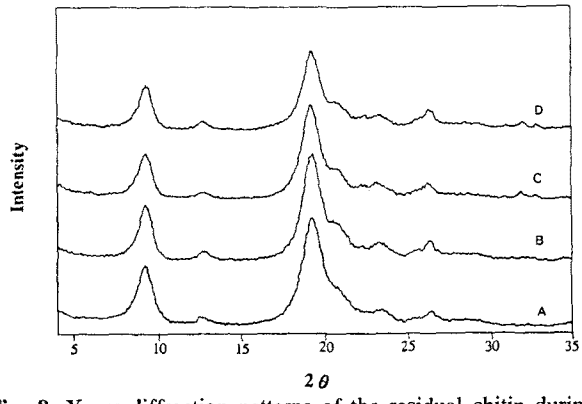

Fig. 8. X-ray diffraction patterns of the residual chitin during enzyme reaction in the agitated bead reaction system.
(A), native crystalline chitin; (B)-(D), enzyme reaction with glass bead for 12, 24, and 48 h, respectively. Reaction conditions: 50 g of crystalline chitin/l (w/v), 300 units chitinase/g crystalline chitin, 400 g of 3 mm glass bead/l, 50°C, pH 7.0, 250 rpm, and for 48 h.

X-ray diffraction patterns. The changes of crystallinity of residual chitin during enzymatic hydrolysis in the agitated bead reaction system was followed as shown in Fig. 8. The strong diffraction intensity of native crystalline chitin was detected at the Bragg angles (2θ) of 9.3, 19.2, and 23.4, respectively (Fig. 8A). The diffraction intensity at the Bragg angles of 19.2, which was mainly used for the estimation of crystallinity of chitin by Shimahara *et al.* (22), decreased gradually as the reaction proceeded. This result indicates that the crystalline nature of chitin is partially reduced during the enzymatic reaction in the agitated bead reaction system.

Acknowledgement

This paper was supported by 1995 NON DIRECTED RESEARCH FUND from the Korea Research Foundation.

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(Received September 17, 1996)