Carbohydrate Polymers 83 (2011) 1843–1849

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Development of innovative technologies to decrease the environmental burdens associated with using chitin as a biomass resource: Mechanochemical grinding and enzymatic degradation

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ARTICLE INFO

Article history:
Received 2 September 2010
Received in revised form 21 October 2010
Accepted 21 October 2010
Available online 29 October 2010

Keywords:
Converge mill
Mechanochemical effect
Chitin
Enzymatic degradation
N-acetylglucosamine

ABSTRACT

The production of *N*-acetylglucosamine (GlcNAc, chitin monosaccharide) from crab or shrimp shells generally requires numerous steps and the use of deleterious substances. One goal for researchers is the direct production of GlcNAc and *NN′*-diacetylchitobiose [$(GlcNAc)_2$, chitin structural dimeric unit] from both chitin and crab shells. The present study reports the development of an intensive ball "converge" mill for the rapid mechanochemical conversion of chitin or crab shells into amorphous, chitinase-sensitive microparticles. Optimal crab shell grinding parameters were determined, and close to 100% direct degradation of chitin from crab shell to GlcNAc was achieved. This is the first report of using a mechanochemical process with enzymatic degradation to decrease the environmental burdens associated with GlcNAc production from chitin.

1. Introduction

Chitin is a highly abundant biomass present in crab, shrimp, and insect shells, as well as fungal cell walls, and is synthesized in nature at a rate of 10^{11} tons per year. Chitin is a weakly cation polysaccharide composed of GlcNAc units covalently connected by β-1,4 linkages. Native chitins in crustacean shells are highly crystalline with strong hydrogen bonding. Chitins have attracted particular interest for their potential conversion to GlcNAc and oligomers, which possess versatile functional properties as skin moisturizers, joint-pain relievers, and antitumoral and antimicrobial agents (Liang, Chen, Yen, & Wang, 2007; Suzuki et al., 1986; Wang, Lin, Yen, Liao, & Chen, 2006; Wang et al., 2008). Compared to glucosamine (GlcN), GlcNAc is chemically stable and has a refreshing, sweet taste. GlcNAc therefore has tremendous potential as a functional food or usage for cosmetics.

The chitin product *NN′*-diacetylchitobiose [$(GlcNAc)_2$] has potentially milder or other physiological activities compared to the monosaccharide. $(GlcNAc)_2$ is especially useful when it was used as a donor substrate of transglycosylation by chitinolytic enzyme such as lysozyme for synthesis of chitin oligomers that have higher degree of polymerization or synthesis of novel glycosides (Usui, Matsui, & Isobe, 1990). Chitin oligomers are known for their elicitor activities in plants and their immunoenhancing, intestinal regulating, and bifidobacteria growth-stimulating activities. It is expected that chitin oligomers could also be used in many applications, including biopesticides or foods (Hirano, 2004).

In light of the difficulties associated with traditional GlcNAc production processes, environmentally compatible and reproducible enzymatic degradation alternatives are desired. If chitin could be completely degraded by enzymes, it would not be necessary to use deleterious substances or to produce excessive amounts of waste water, allowing the establishment of an environmentally conscious process. However, crustacean shells are not soluble in general medium and their crystallinity is potentially too high to be degraded enzymatically. Furthermore, enzymes are expensive. To solve these problems, shellfish waste pretreatment to loose the chitin crystal structure is widely considered to be important. The direct degradation and separation of α-chitin from crab or shrimp shells are a significant challenge, due to the difficulty in separating GlcNAc from the crude degradation products derived from shell proteins.

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doi:10.1016/j.carbpol.2010.10.050

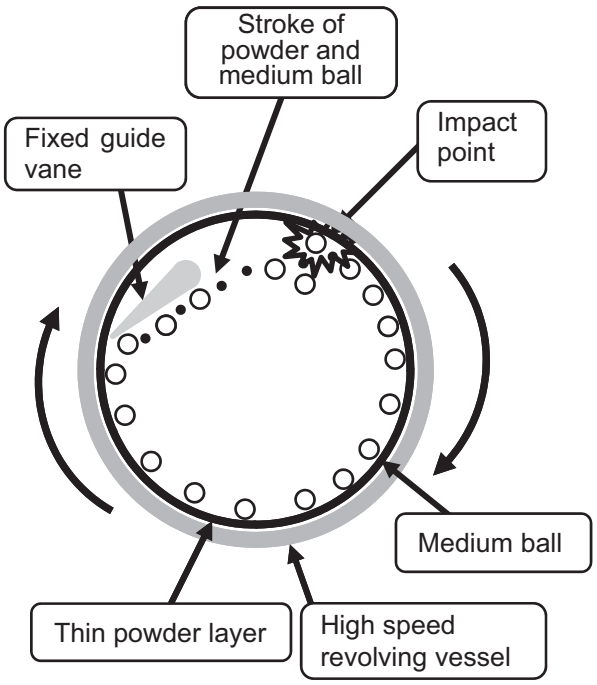

Fig. 1. Simplified schematic of converge mill. The converge mill has larger amount of grinding capacity than the planetary ball mill (Tanno et al., 2006; Sato et al., 2006). Rounding revolving vessel (outer circle) and fixed guide vane made thin powder layer of substances (inner black circle) onto the inner wall. Fixed guide vane change the direction of medium balls (small open circles) and crystal structure of substance is destroyed at a defined focal point as compared to general tumbling ball mill.

Recently, we developed a novel "converge" mill (Sato, Asada, Takeda, & Tanno, 2006; Tanno, Sato, Maruyama, Yamaya, & Fujitaka, 2006) that is a derivative of the medium ball mill. A powder layer forms on the inner wall of a revolving vessel, which undergoes intensive impact from a medium ball (Fig. 1). The target materials are most impacted by the medium ball at a defined focal point, and the generated mechanochemical effect changes the crystal structure and properties of the materials. This causes the crushed materials to become amorphous or potentially activated (Takeda et al., 2009; Van Craeyveld, Delcour, & Courtin, 2008; Van Craeyveld et al., 2009; Venkataraman & Narayanan, 1998). There are several types of ball mills using medium balls, such as the conventional tumbling mill or planetary ball mill. However, the milling time is much shorter and the amount of sample loaded is larger in the converge mill.

Fujimoto et al. (2008) and Takeda et al. (2009) previously developed a successful degradation process combining mechanochemical pretreatment and enzymatic degradation. We also greatly enhanced the degradation of cider sawdust by grinding using a converge mill with cellulase. In this study, suitable conditions for the mechanochemical treatment of chitin were investigated, resulting in the successful direct degradation of crustacean shells. Using our process, the steps to product GlcNAc decreased to less than 1/3 (Fig. 2).

2. Materials and methods

2.1. Materials and enzymes

Crab chitin, crab shell, and shrimp chitosan (Yaizu suisankagaku industry) were used for materials. For the enzymatic degradation, crude enzyme from *Streptomyces griseus* (Eikon CHL from Rakuto kasei industrial, enzyme E) and commercially available enzyme preparation from *Corynebacterium* sp. (Yatalase from Takara Bio, enzyme Y) were used as chitinase, and commercially available enzyme preparation from *Bacillus subtilis* (Umamizyme from Amano enzyme, peptidase U) which is known hyperactive chitobiase was used. For degradation each substrate, we used 90 U of enzyme E and 108 U of enzyme Y. Enzyme Y has high chitobiase activity (703 U/g) and produces monosaccharide from chitin, enzyme E produces $(GlcNAc)_2$ as a major product from chitin, and peptidase U produces GlcNAc from $(GlcNAc)_2$ (140 U/g). To obtain monosaccharide after degradation with Enzyme E, we used peptidase U.

2.2. Mechanochemical grinding

The grinding equipment comprised a converge mill made by Makabe Giken (internal capacity, 1000 cm^3). The mill structure, made of two flat zirconian drums, contains a fixed guide vane within it (Fig. 2). Twenty grams of the three materials and 271 g of zirconian balls (10 mm dia.) were ground at 500–800 rpm for 30–120 min (Table 1).

2.3. Determination of average particle size

The average particle size (median size D_{50}) was determined by a particle size distributer (Nikkiso, HRA [X-100]). For disperse media, methanol was used.

2.4. Crystallinity determination by X-ray diffraction (XRD)

Equatorial diffraction profiles were obtained with Cu-Kα from a powder X-ray generator (Japan Electronic Organization Co. Ltd., JDX-3530) operating at 30 kV and 30 mA. The crystallinity index was calculated from normalized diffractograms according to equation below. The intensities of the peaks at [1 1 0] lattice (I_{110}, at $2\theta = 20$ corresponding to the maximum intensity of chitin) and I_{am} at $2\theta = 16$ (amorphous diffraction) were used to calculate ICR (Lavall, Assis, & Campana-Filho, 2007).

$$\text{Crystallinity index}\,(\%) = \frac{I_{110} - I_{am}}{I_{110}} \times 100$$

2.5. Scanning electron microscopy (SEM)

The morphology of the fine particles of the ground chitin samples was observed using SEM (Keyence VE-7800).

2.6. Assay for hydrolytic activity

Chitinase activity was measured as follows. Reaction mixture was composed of 0.05% ethylene glycol chitin, 10 mM sodium phosphate buffer (pH 6.0) and enzyme solution in total volume of 1.1 ml.

Table 1
Grinding conditions and average particle size of each material.

Materials	Grinding		Average particle size (D50) (μm)
	rpm	Time (min)	
Crab shell	500	30	15.1
		60	12.0
	700	30	10.1
		60	10.7
Crab chitin	500	30	30.5
		60	23.0
	700	30	22.9
		60	19.5
Shrimp chitin	500	30	–
		60	90.1
	700	30	99.8
		60	36.4

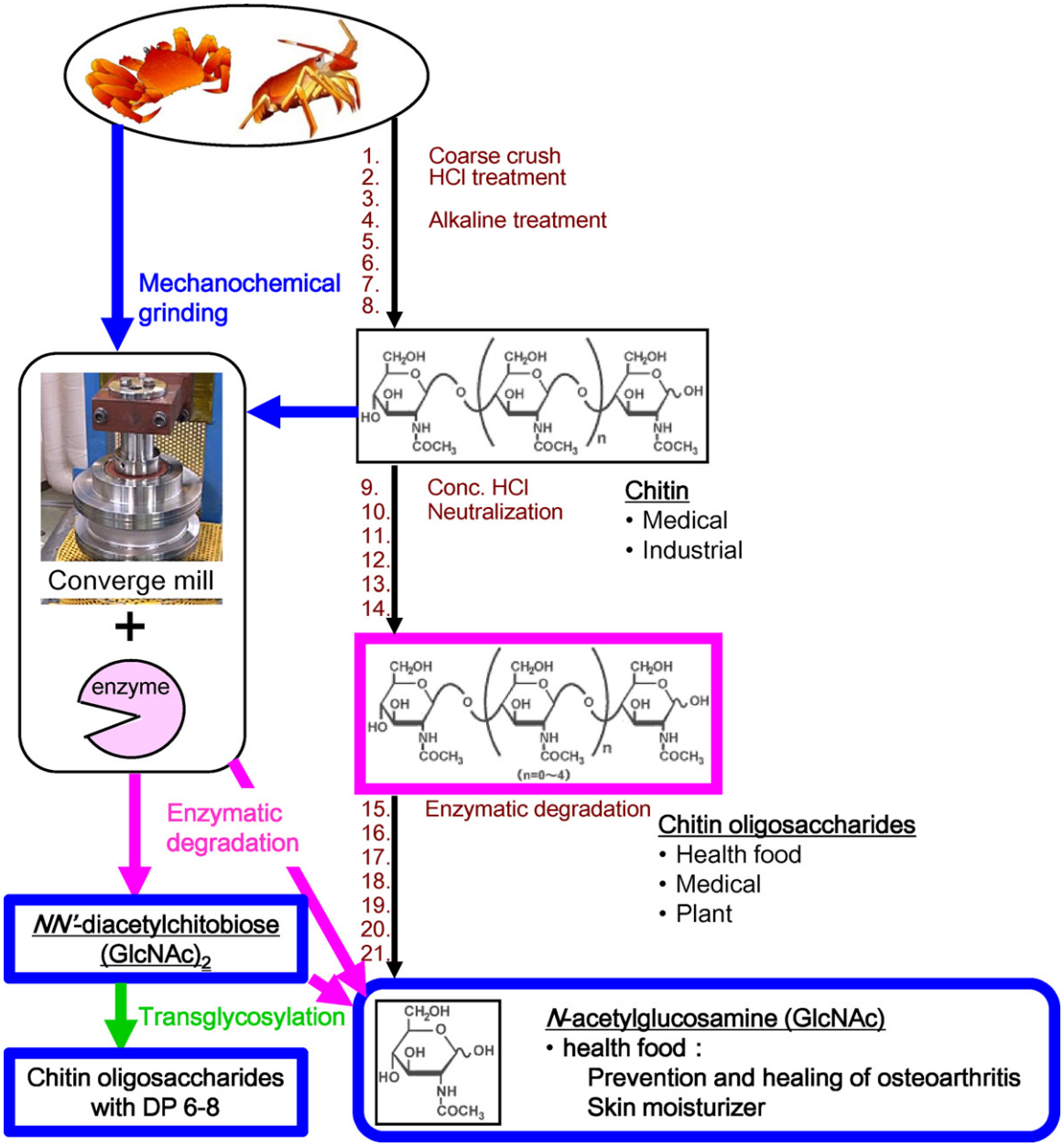

Fig. 2. The traditional method of manufacturing *N*-acetylglucosamine and decrease the environmental burdens by the innovative technology Left side shows industrially applied process to make chitin, chitin oligomers and GlcNAc from crustacean shells. Using converge mill for pre-treatment, it is possible to decrease 2/3 steps from traditional method.

After incubation at 40 °C for 30 min, the reaction of the enzyme was terminated by heating at 100 °C for 10 min and the amount of reducing sugar was determined by the method of Imoto and Yagishita (1971) using *N*-acetylglucosamine as a reference compound. One unit of the enzyme activity was defined as the amount of enzyme required to release 1 μmol of *N*-acetylglucosamine from ethylene glycol chitin solution in 1 min at 40 °C, pH 6.0.

Chitobiase activity was measured by determining *p*-nitrophenol released from *p*-nitropheny-*N*-acetyl-β-D-glucosaminide calorimetrically at 405 nm. One unit of the enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol from 2 mM substrate solution in 1 min at 37 °C, pH 6.0.

2.7. Enzymatic degradation of chitin

The reaction mixture was prepared as follows. Twenty micrograms of each substrate (1% of final concentration) was mixed with 1.8 ml of 10 mM phosphate buffer (pH 4.0–8.0) and 0.2 ml of 10 mg/ml enzyme diluted with 10 mM phosphate buffer (0.1% of final concentration, about 100 U). The reaction mixture was shaken at 1400 rpm at 25–60 °C, and 0.4 ml of the mixture was harvested at the appropriate time.

2.8. High-performance liquid chromatography (HPLC)

The harvested reaction solution was filtered (pore size 0.45 μm, Advantec) after boiling for 10 min, and centrifuged. The HPLC system consisted of an SPD-10A UV detector (Shimadzu), RI-101 RI detector (Shodex), and LC-10Avp system (Shimadzu). Sugars were separated on a Shodex SUGAR KS-802 column (Ø 0.8 × 30 cm) using milliQ water (Millipore) as the mobile phase, at a flow rate of 0.6 ml min^{-1} at 60 °C. GlcNAc and $(GlcNAc)_2$ were detected by monitoring the absorbance at 210 nm. Degradation ratio was calculated from the square of the peak of the enzymatic reaction mixture and

Table 2
Yields of GlcNAc and $(GlcNAc)_2$ using activated charcoal–celite column chromatography.

Substrates	Enzymes	GlcNAc (mg)	$(GlcNAc)_2$ (mg)	Total yield (mg)	Yield constant (%)	Degradation ratio (%)
Chitin (1%, 1 g)	E	38	380	418	42	43
	E + P	291	–	291	29	37
Crab shell (1%, 1 g)	E	10	180	190	84[a]	97[a]

[a]Calculated by chitin content of crab shell tested: 22.5%.

two standards of mono- and disaccharides. The product ratio was divided by the substrate concentration and reported as a percent.

2.9. Nuclear magnetic resonance (NMR) analysis

Digested products were pretreated as for HPLC, and the monosaccaride and $(GlcNAc)_2$ peaks were isolated. These fractions were vacuum concentrated, freeze-dried, and analyzed by ^1H or ^{13}C NMR (400-MR, Varian Inc.) in D_2O at 25 °C.

2.10. Isolation and purification of enzymatically degraded products

The total volume of the enzymatic degradation was scaled to 100 ml (50×), and used the following combination of substrate and enzymes: (1) chitin and enzyme E, (2) chitin, enzyme E, and peptidase U, (3) crab shell and enzyme E, and (4) crab shell, enzyme E, and peptidase U. The weight ratio of enzyme E and peptidase U in combination 2 was 1:2.

Chromatography on an activated charcoal–celite column (Ø 2 × 30 cm, charcoal: celite = 1:1, Wako Pure Chemical Industries) was performed to purify the digested products. The sample was loaded onto the column, which had been washed with 50% ethanol, equilibrated with milliQ water, and eluted with a 0–50% linear gradient of ethanol. The product fractions were vacuum-concentrated and freeze-dried. Products were analyzed by HPLC to determine the purity and yield (%) = [(dry weight of product)/(weight of chitin) × 100]. The extracted chitin content of this crab shell is 22.5%.

3. Results

3.1. Determination of suitable conditions for degradation of chitin materials

All three enzymes could degrade substrates under mildly acidic (pH 4.0–5.0) conditions. When the substrate concentration was fixed at 1% (w/v), the enzyme E concentration achieved 50% degradation over a 24-h reaction period. A 0.1% (w/v) enzyme E (around 100 U in 2 ml) was also necessary for degradation. We used an equal unit of the enzyme Y. The Peptidase U was used combined with enzyme E and more than 70 U was necessary to obtain GlcNAc from the degraded product of enzyme E. Since the sodium acetate buffer (pH 5.8) affected HPLC analysis, chitinases with sodium phosphate buffer at pH 6.0 and 40 °C were used for enzymatic degradation.

3.2. Properties of the grinding materials and enzymatic degradation ratio

Table 1 displays the grinding conditions and median diameters of the grinding materials. The best combination for grinding α-chitin was determined when chromium balls and a stainless mill were used compared to zirconia (data not shown). The median diameters were decreased after grinding with the converge mill (Fig. 1). The particle diameter was immediately decreased by grinding, and crab shell showed the smallest median diameter (10.1 μm) (Table 1). Compared to the particle diameter, crystallinity gradually but obviously decreased with an increasing number of rotations or grinding time (Fig. 3(a and d)).

The XRD patterns of untreated and mechanochemical-ground crab chitin and crab shell are shown in Fig. 3(a and d), respectively. The untreated crab chitin and shell had the same two specific peaks at $2\theta = 9.4°$ and 19.2°. Peaks in the XRD patterns of materials ground with the converge mill became smaller and smoother, until eventually no obvious peaks were apparent, especially under severe conditions. The SEM images confirmed these results (Fig. 3(c and f)). These ground materials were digested as substrates by the enzymes.

3.3. Analysis of the degraded products

The products of enzymatic digestion were analyzed by HPLC. Fig. 3(b and e) displays the degradation ratios calculated with the total concentrations of GlcNAc and $(GlcNAc)_2$. The degradation ratio of ground crab chitin using the converge mill was 10 times greater than that of untreated substrate (Fig. 3(b)). Increased vessel revolving speeds and grinding times gave higher degradation ratios. Crab shell showed the same trend (i.e., more severe grinding conditions increased the degradation ratio) (Fig. 3(e)), although excessive grinding reduced the degradation ratio due to microparticle aggregation (data not shown).

The HPLC chromatograms from crab chitin and crab shell degraded by different enzymes are shown in Fig. 4. All enzymes showed two major peaks (Fig. 4(c–h)), one of which had the same pattern as the GlcNAc (Fig. 4(a)) or $(GlcNAc)_2$ (Fig. 4(b)) standard. Enzyme Y mainly produced GlcNAc (Fig. 4(c–d)), enzyme E produced $(GlcNAc)_2$ (Fig. 4(e–f)), and peptidase U degraded $(GlcNAc)_2$ to GlcNAc (Fig. 4(g–h)). These results indicate that mono- and disaccharides were successfully obtained by enzymatic degradation from ground chitin or crab shells. The corresponding peaks for mono- and disaccharides were then isolated and analyzed by NMR. The spectra of both sugars corresponded with the standard preparations of GlcNAc and $(GlcNAc)_2$, which were confirmed as the degradation products of chitin and crab shell, respectively (data not shown).

3.4. Isolation and purification of the enzymatically degraded products

GlcNAc resulting from the enzymatic degradation of chitin or crab shells was isolated from the flow-through of the activated carbon and celite mixed column. $(GlcNAc)_2$ was isolated from the binding fraction eluted by a linear ethanol concentration gradient (Fig. 5(a and c)). The product from fraction 2 (F-2 in Fig. 5(a and c)) was confirmed by HPLC (Fig. 5(b and d)). Table 2 shows the yields and purity by HPLC analysis of GlcNAc and $(GlcNAc)_2$ from chitin and crab shell. We successfully purified 190 mg of GlcNAc and $(GlcNAc)_2$ from 1 g of crab shell.

4. Discussion

4.1. Degradation and mechanochemical effects

This is the first report of a chitinolytic system utilizing combinations of mechanochemical grinding and enzymatic degradation.

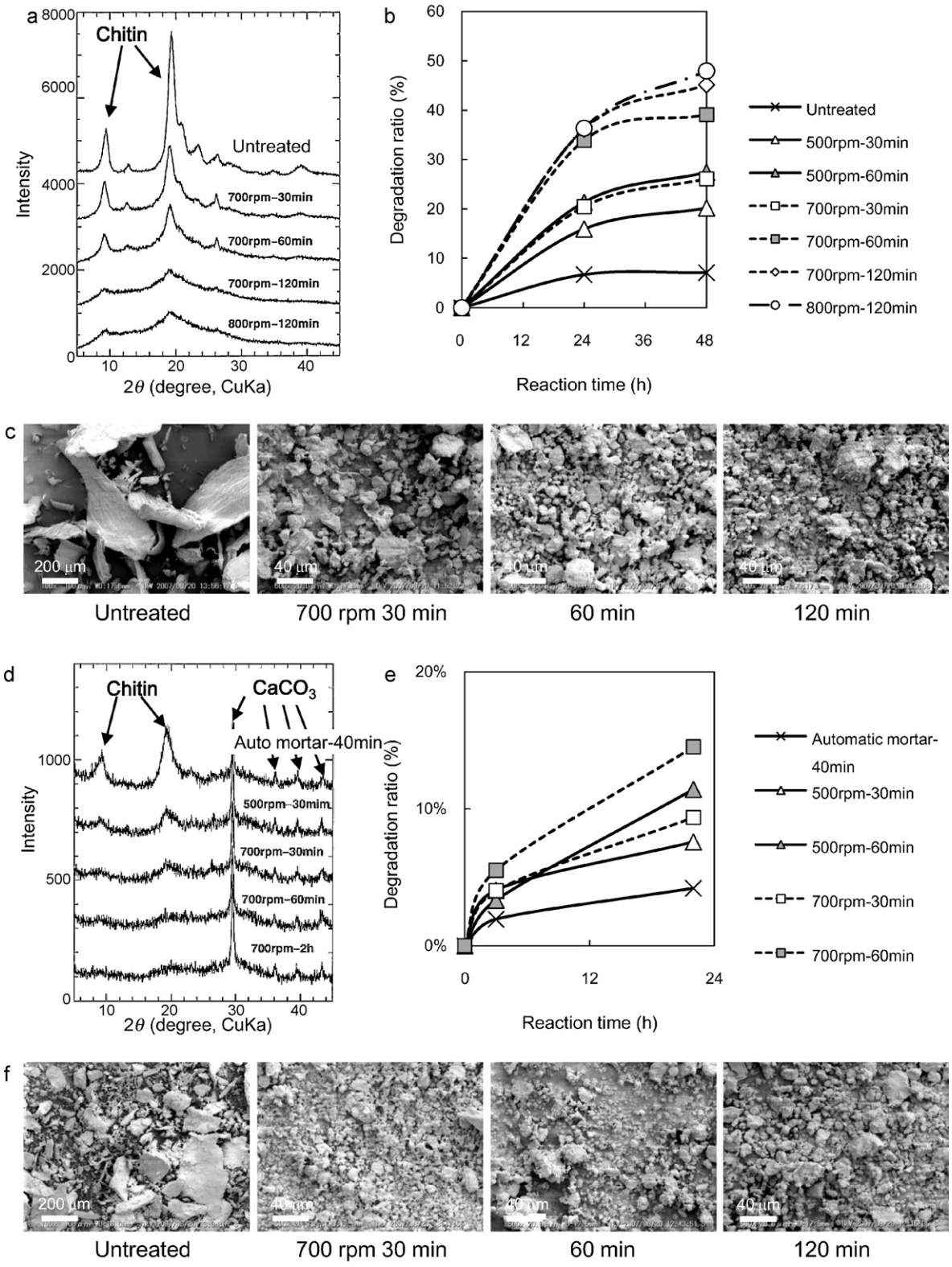

Fig. 3. Relationship between grinding parameters and degradation ratio of crab chitin or shell. (a–c) Represented crab chitin and (d–f) crab shell. (a) Peaks in the XRD patterns of chitin ground with different milling. 20 g of crab chitin was mixed with 271 g of 10 mm dia. zirconia ball in 1-l size zirconia pot, and ground at 500–800 rpm for 30–120 min. (b) Degradation ratio of crab chitin with enzyme E under various grinding parameters. (c) SEM images of micro-particle crab shell chitin grinding with converge mill at 700 rpm. White bar in untreated material represented 200 μm and the other bars represented 40 μm. (d) Peaks in the XRD patterns of crab shell ground for different milling condition. 20 g of crab shell was mixed with 271 g of 10 mm dia. zirconia ball in 1-l size zirconia pot, and ground at 500–700 rpm for 30–120 min. (e) Degradation ratio of crab shell with enzyme E under various grinding parameters. The ratio of crab shell is weight percent of GlcNAc and $(GlcNAc)_2$ toward not chitin but crab shell which contains chitin in 23%. (f) SEM of micro-particle crab shell grinding with converge mill at 700 rpm. Automatic mortar-40 min corresponds to "Untreated". White bar in untreated material represented 200 μm and the other bars represented 40 μm.

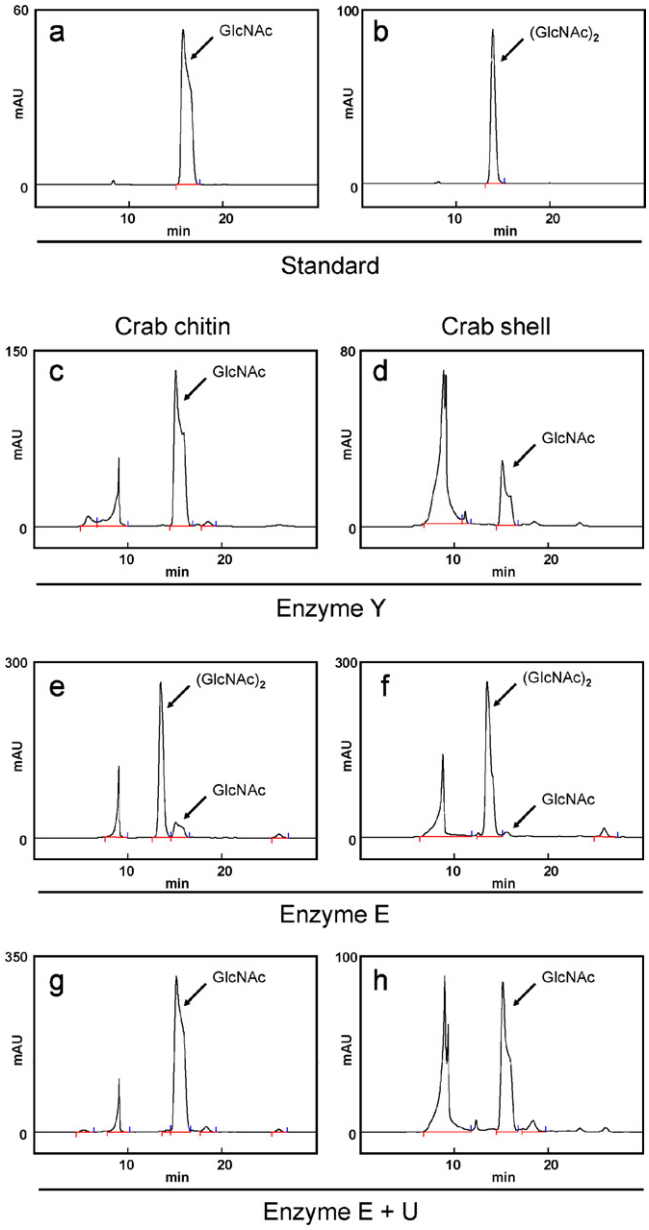

Fig. 4. HPLC chromatogram of enzymatic degraded product from each substrate. (a) GlcNAc and (b) $(GlcNAc)_2$ are the chromatogram of standard. From (c) to (h), left panels represented patterns of crab chitin and right panels represented crab shell. (c and d) Enzyme Y, (e) and (f) enzyme E and (g and h) enzyme E + peptidase U were used for degradation.

Breaking down the chitin crystal structure reduced the crystallinity and improved enzymatic degradation (Fig. 3(a, b, d, and e)), allowing the enzymes to easily access and exert the catalytic action. Wu and Miao (2008) previously showed that mechanochemical treatment markedly increases the glucose yield from enzymatic corn flour hydrolysis. Similar results were observed by Fujimoto et al. (2008) for lignocellulosic biomass and Van Craeyveld et al. (2008) showed improvement of extractability and affects molecular properties of Psyllium seed husk arabinoxylan by ball milling. Crystallinity not only affects the enzymatic degradation, but also serves as one component of the degradation ratio.

Mechanochemical grinding with the converge mill was extremely effective for pretreatment of chitin and crab shell before enzymatic digestion same as plant cell walls (Takeda et al., 2009). The direct enzymatic degradation ratios of untreated crab chitin (7.1%) and crab shell (4.2%) were 7-fold and 3.5-fold increased, respectively, by mechanochemical grinding (Fig. 3(b and e)). After mechanochemical grinding, the crab shell showed the smallest median particle diameter (10.1 μm, Table 1) due to its hard calciferous materials. Hard materials were ground easily, while soft and fluffy materials were unsuitable to be made small by the converge mill. We also enzymatically degraded chitosan by enzyme Y to allow comparison with the other materials. Chitosan is originally soluble and grinding had no effect on this material (data not shown). From these results, it can be concluded that the converge mill is suitable for grinding hard crystal materials, such as chitin and crab shell.

4.2. Direct degradation of chitin and crab shell

The degradation ratio was calculated using 100% for the weight of the crab shell (Fig. 3(e)). The direct degradation ratio of the chitin in crab shell was ~100%, since the chitin content of the used crab shell was 22.5%. Direct degradation of both ground chitin and crab shell to GlcNAc or $(GlcNAc)_2$ was enabled by mechanochemical grinding. Ilankovan, Hein, Ng, Trung, and Stevens (2006) produced $(GlcNAc)_2$ from 0.5 vol.% of chitin using 0.1% of commercial enzymes (pepsin) and the yield was around 10%. Thus, grinding with the converge mill is very effective for the pretreatment of chitin or crab shell before enzymatic treatment.

4.3. Degradation products

The degradation products were analyzed by HPLC and NMR, revealing that the produced monosaccharide and biose were

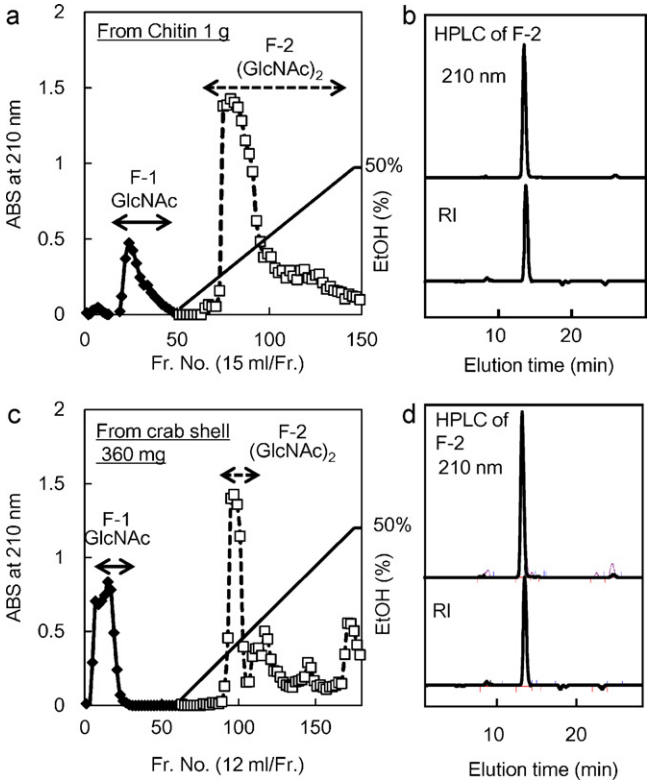

Fig. 5. Spectrum of charcoal–celite column chromatography and HPLC analysis. (a) Elution pattern of enzymatic degraded product of crab chitin from the column. Absorbance (ABS) was observed at 210 nm. (b) HPLC analysis of the product from crab chitin that separated products at 210 nm and by RI. (c) Elution pattern of crab shell. (d) HPLC analysis of the product from crab shell.

GlcNAc and $(GlcNAc)_2$, respectively. These products were easily purified using activated charcoal–celite column chromatography in one step, with a 40% yield and 95% purity. Using hydrochloric acid, it is hard to control the length of the generated oligomers and to avoid deacetylation from the C-2 amide residue.

5. Conclusion

We have clearly demonstrated for, to the extent of our knowledge, the first time that the mechanochemical pretreatment of chitin and crustacean shell carries with it significant advantages for their direct enzymatic degradation. Ground chitin and crab shell showed significant reduction of crystallinity. With mechanochemical grinding, direct degradation ratio of crab shell became about 100%. The products from enzymatic degradation were determined as GlcNAc and $(GlcNAc)_2$. We successfully purified 190 mg of GlcNAc and $(GlcNAc)_2$ from 1 g of crab shell using activated carbon and celite mixed column by one-step. To dissolve many problems such as low oligosaccharide yields, environmental pollution with massive amounts of discharged water and avoid complicated production processes result in an expensive cost of GlcNAc product from the traditional method, our novel method is effective and implementable.

Acknowledgements

This work was supported by grants in 2007 and 2008 from the Japan Science and Technology Agency (JST), and was partly supported by the Program for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry.

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