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Depolymerization of β-chitin to mono- and disaccharides by the serum fraction from the *para* rubber tree, *Hevea brasiliensis*

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Abstract—The serum fraction of latex from *Hevea brasiliensis*, the para rubber tree, is known to contain an *endo*-chitinolytic enzyme, hevamine. Herein the activity of the rubber serum towards β-chitin is investigated. The serum contained 6 mg/mL of protein and a chitinolytic activity of 18 mU per mg of protein. The optimum ratio of enzyme to chitin was 0.22 mU/mg, and the optimum substrate concentration was 60 mg/mL. The optimum pH range was pH 2–4, and the optimum temperature was 45 °C. At these conditions both $(GlcNAc)_2$ and GlcNAc were produced in a molar ratio of approximately 2:1. The hydrolysis of 300 mg of chitin with 64 mU of the rubber serum for 8 days under the optimum conditions gave 39 mg of GlcNAc and 108 mg of $(GlcNAc)_2$ as determined by HPLC. Mixing the rubber serum preparation with an *Aspergillus niger* pectinase preparation containing β-*N*-acetylhexosaminidase can be used to produce almost exclusively the GlcNAc monomer in about 50% yield.

Keywords: *Hevea brasiliensis*; Chitin; Hevamine; D-Glucosamine

1. Introduction

Chitin [a polymer of (1→4)-2-acetamido-2-deoxy-β-D-glucose] is a polysaccharide abundant in invertebrate exoskeletons including crustacean shells and fungal cell walls.[1,2] A gigantic amount of chitin is processed as solid waste from seafood processing industry.[2,3] The natural degradation of chitin presents an important feature, not only in the global recycling of carbon and nitrogen sources, but also in the production of useful chemical reagents.[4] *N*-Acetyl-D-glucosamine (GlcNAc) and glucosamine (GlcN), the monomer of chitin and chitosan, are therapeutic agents for osteoarthritis,[5] inflammatory bowel diseases[6] and gastritis.[7] Chitinous oligomers also have been reported as having antitumor, antifungal and antimicrobial activities,[8,9] as well as being chemical building blocks for biologically important glycolipids and glycoproteins.[10]

In nature, chitin-degrading enzymes are widely distributed among bacteria, arthropods, protozoans and plants.[11,12] Nevertheless, commercial preparations of chitinases are inevitably expensive, especially, the purified chitinases. Hence, the use of crude enzymes has become an interesting alternative in the preparation of GlcNAc and chito-oligomers.[13–15]

Hevea brasiliensis, the *para* rubber tree, is known to produce an endochitinase called hevamine, which belongs to glycoside hydrolase family no. 18. The enzyme was isolated from the lutoid body of the rubber latex.[16] A few reports have described chitinolytic activities and structures of hevamine.[17–19] In the present work, we illustrate here that the rubber serum fraction from *H. brasiliensis* contained interestingly different chitinolytic activities from hevamine in certain aspects. The rubber serum can potentially be an excellent source of low-cost

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chitinolytic enzymes, as it is regarded as a massive waste product that must be disposed of from rubber-processing plants.

2. Results and discussion

The hydrolysis of β-chitin with enzyme in the rubber serum gave two major chitinolysis products, $(GlcNAc)_2$ and GlcNAc. The production yield of $(GlcNAc)_2$ and GlcNAc increased proportionally with the increment of enzyme:chitin ratio from 0.11 to 1.08 mU/mg (Fig. 1). The enzyme:chitin ratio of 0.22 mU/mg was thus selected for further condition optimization as this enzyme:chitin ratio also produced appropriate concentration of the products to be effectively analyzed by HPLC throughout the course of the hydrolysis studied.

The most convenient means to increase the product yields and concentration is to raise the concentration of both chitin substrate and the enzyme while maintaining the enzyme:chitin ratio. In the next experiment, the concentration of both chitin substrate and the enzyme were increased while the enzyme:chitin ratio was kept constant at 0.22 mU/mg. As expected, the yield of both saccharides went up with the increasing concentration of chitin (Fig. 2) up to 60 mg/mL where the stirring became ineffective due to the high content of solid chitin.

A pH-dependence study of chitinolytic activity of the enzyme in the rubber serum revealed that the chitinolytic enzyme in the serum was acidophilic with an optimum pH range of pH 2–4 (Fig. 3). The results found here indicated that the enzyme complexes in the serum respond to pH rather differently from hevamine, the reported major chitinase found in the rubber serum. Hevamine was reported to show the optimum pH in the range of pH 4.0–6.0.[17] This difference may be attributed to some chitin-binding proteins and other cooperative enzymes present in the serum.[20,21]

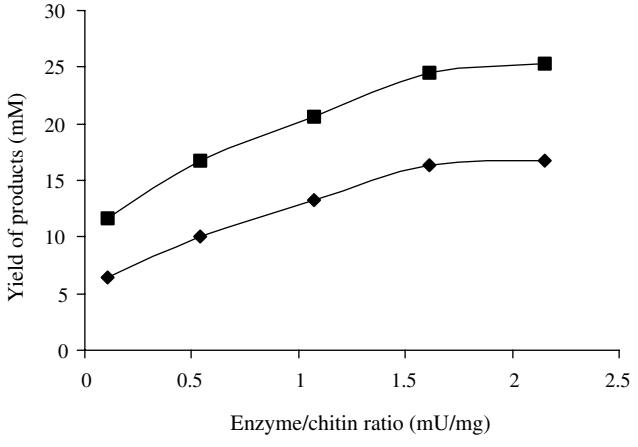

Figure 1. The effect of the enzyme:chitin ratio on the chitinolysis of β-chitin with rubber serum. (◆) GlcNAc, (■) $(GlcNAc)_2$, [chitin] = 20 mg/mL, pH 4.0 (0.05 M NaOAc), 37 °C; 8 days.

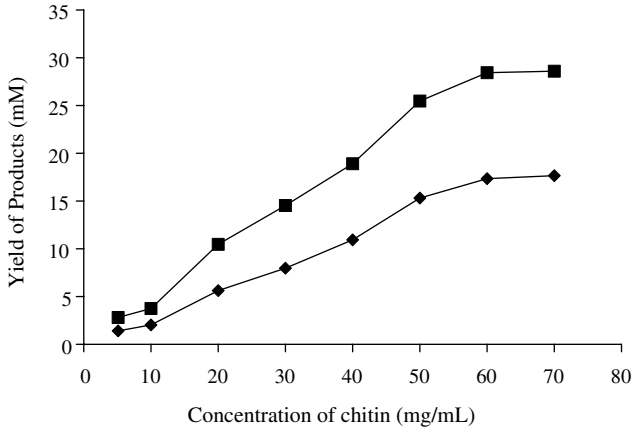

Figure 2. The effect of concentration of chitin on the chitinolysis of β-chitin with rubber serum. (◆) GlcNAc, (■) $(GlcNAc)_2$, enzyme: chitin = 0.22 mU/mg, pH 4.0 (0.05 M NaOAc), 37 °C, 8 days.

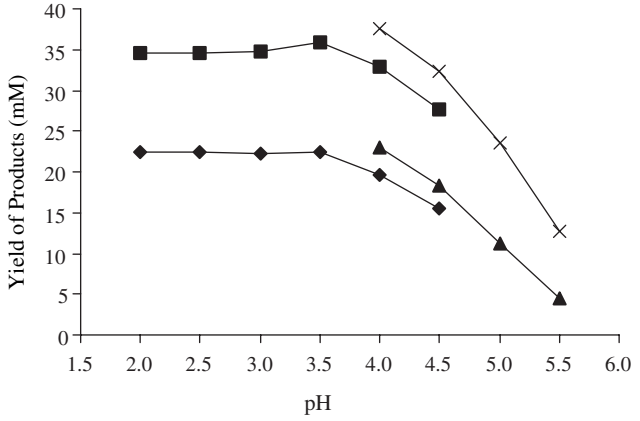

Figure 3. The effect of pH on the chitinolysis of β-chitin with rubber serum. pH 2–4.5 (0.05 M citrate–phosphate buffer): (◆) GlcNAc, (■) $(GlcNAc)_2$, pH 4.0–5.5 (0.05 M NaOAc): (▲) GlcNAc, (×) $(GlcNAc)_2$, [chitin] = 60 mg/mL, enzyme:chitin = 0.22 mU/mg, 37 °C, 8 days.

The optimum temperature for chitinases has been reported to vary with the sources and generally lies between 30 and 50 °C. The optimum temperature for hevamine was reported to be 45 °C.[17] In this particular study, the enzyme in the rubber serum displayed the highest chitinolytic activity at 45 °C (Fig. 4), which is the same as that for hevamine. At 37 °C and at 55 °C the activity dropped from that at the optimum temperature by approximately 25–30%.

Subroto et al. also reported that the chitinolytic activity of hevamine depended on the concentration of salts in the reaction media.[17] In this study, we found that the chitinolytic activity of the enzyme in the rubber serum depended on the concentration of buffer up to 0.1 M NaOAc (Fig. 5). At higher concentration of the buffer, the activity was relatively constant. The buffer concen-

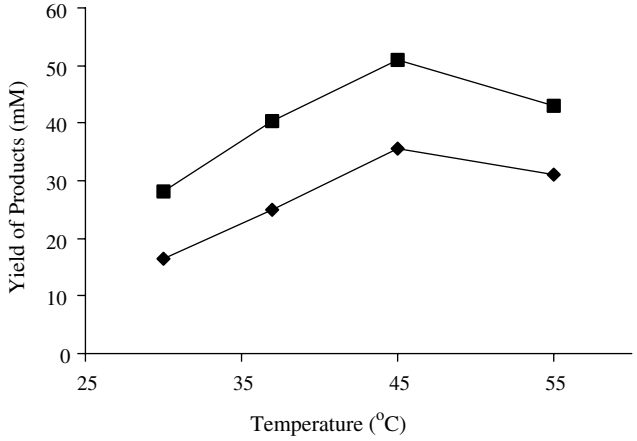

Figure 4. The effect of temperature on the chitinolysis of β-chitin with rubber serum. (◆) GlcNAc, (■) $(GlcNAc)_2$, [chitin] = 60 mg/mL, enzyme:chitin ratio = 0.22 mU/mg, pH 4.0 (0.1 M NaOAc), 8 days.

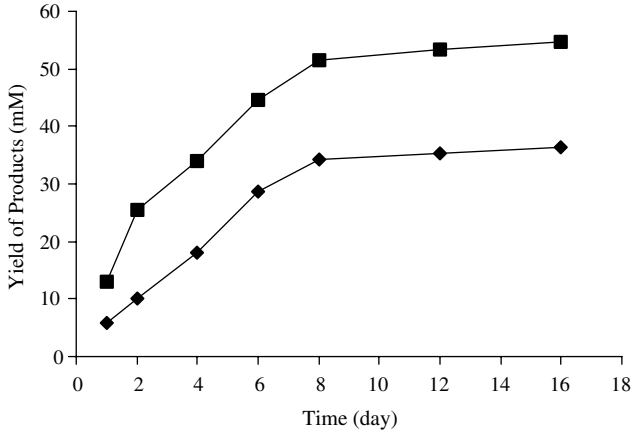

Figure 6. The time course of the hydrolytic products from chitinolysis of β-chitin with rubber serum. (◆) GlcNAc, (■) $(GlcNAc)_2$, [chitin] = 60 mg/mL enzyme:chitin ratio = 0.22 mU/mg, pH 4.0 (0.1 M NaOAc), 45 °C.

tration of 0.1 M was thus the minimum concentration required for maintaining the effective activity level of the enzyme throughout the course of the hydrolysis.

After 8 days of incubation, the HPLC yields of GlcNAc and $(GlcNAc)_2$ from the hydrolysis of β-chitin (300 mg) with the serum at the optimum conditions were 38 and 109 mg, respectively, representing 12% and 36% of the starting chitin. The total conversion of chitin to GlcNAc and $(GlcNAc)_2$ was thus around 48%, and the reaction was virtually terminated after 8 days (Fig. 6). We first speculated that the enzymatic hydrolysis reactions stopped at only 50% conversion rather than going to completion due to either enzyme denaturation or product inhibition. Various enzyme:chitin ratios 0.22, 0.44, 0.66 and 0.88 mU/mg were used in the hydrolysis. The production of both GlcNAc and $(GlcNAc)_2$ during the first four days tended to increase with the enzyme:chitin ratio, but the final production yields after 8 days were virtually the same at the total yield around 50% (Fig. 7). Furthermore, addition of more rubber serum after 8 days gave no additional yields. These results indicated that enzyme denaturation was unlikely to be the major cause for limiting the final yields; otherwise the final yields should increase with the increase of the serum.

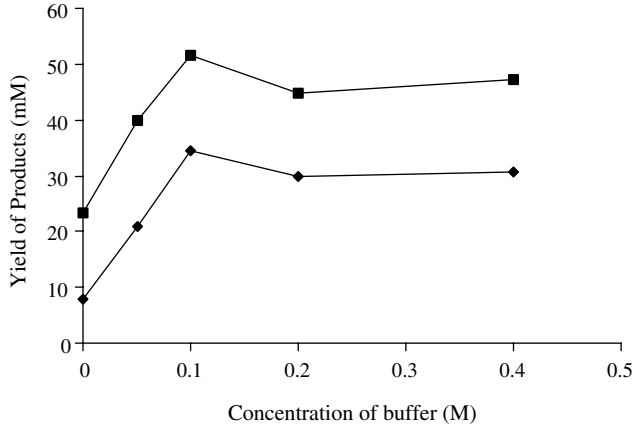

Figure 5. The effect of concentration of buffer on the chitinolysis of β-chitin with rubber serum. (◆) GlcNAc, (■) $(GlcNAc)_2$, [chitin] = 60 mg/mL, enzyme:chitin ratio = 0.22 mU/mg, pH 4.0 (NaOAc), 45 °C, 8 days.

GlcNAc product inhibition was investigated by carrying out the reaction in the presence of GlcNAc intentionally added in various concentrations at the beginning of the hydrolysis. The results showed that high initial concentration of GlcNAc did not inhibit the reaction (Table 1). We also found evidence, which will be described later, that $(GlcNac)_2$ was not the cause for the hydrolysis to be halted.

Since neither denaturation of enzyme nor the product inhibition could explain the self-termination of the hydrolysis, we speculated that the hydrolysis of chitin stopped halfway to completion because the chitin starting material remained in the reaction in an unsuitable form for further degradation by the hevamine in the serum. This *endo*-type chitinase, known to be the major chitinolytic enzyme in *para* rubber trees, has a (−4, −3, −2, −1, +1, +2) binding cleft that hydrolyzes chitooligosaccharides containing a GlcNAc unit longer than $(GlcNAc)_4$.[18,19] The remaining unhydrolyzable chitin particles may contain GlcNAc units densely packed in a crystalline nucleus surrounded by a loose polymeric shell enriched with GlcN units that prevent the attack by hevamine (Fig. 8).

From the data in all previous experiments, the major products from the enzymatic hydrolysis of chitin were $(GlcNAc)_2$ and GlcNAc with $(GlcNAc)_2$:GlcNAc molar ratio of approximately 2:1. It is known that GlcNAc can be produced from the hydrolysis of $(GlcNAc)_2$ by β-*N*-acetylhexosaminidase. The β-*N*-acetylhexosaminidase

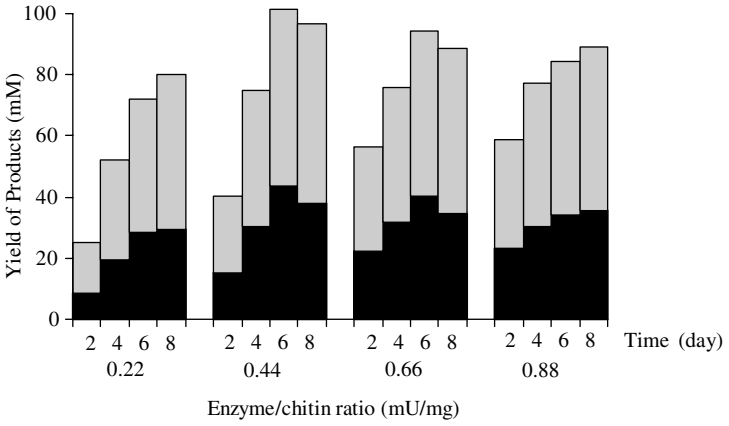

Figure 7. The yields of GlcNAc and $(GlcNAc)_2$ in the chitinolysis of β-chitin with rubber serum at various enzyme:chitin ratios. (■) GlcNAc, (▩) $(GlcNAc)_2$, [chitin] = 60 mg/mL, pH 4.0 (0.1 M NaOAc), 45 °C, enzyme:chitin ratio = 0.22, 0.44, 0.66, 0.88 mU/mg.

Table 1. The effect of GlcNAc concentration in the product inhibition study

Initial concentration of GlcNAc (mM)	Yield of products (mM)	
	GlcNAc	$(GlcNAc)_2$
0.00	32.4	45.0
9.31	31.5	44.4
18.17	29.9	43.5
28.21	32.5	46.4
36.31	33.2	47.3
47.19	31.0	45.2

[chitin] = 60 mg/mL, enzyme:chitin ratio = 0.22 mU/mg, pH 4.0 (0.1 M NaOAc), 45 °C, 6 days.

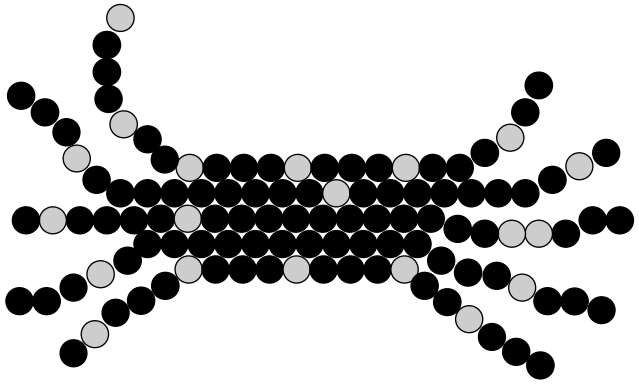

Figure 8. The proposed structure of the unhydrolyzable chitin particles in the chitinolysis of β-chitin with rubber serum. (●) GlcNAc and (◉) GlcN.

activity in the rubber serum was thus evaluated by incubating $(GlcNAc)_2$ with the serum. No GlcNAc from the mixture of $(GlcNAc)_2$ and the serum was detected even after 24 h of incubation. These results clearly indicated the absence of β-*N*-acetylhexosaminidase activity in the serum. These results also agreed well with a relatively constant $(GlcNAc)_2$:(GlcNAc) molar ratio of ~2 throughout the hydrolysis time course. However, to account for the significant amount of GlcNAc observed and the absence of higher oligosaccharides in the hydrolysis of chitin with the serum, the serum must contain at least one more chitinolytic enzyme besides hevamine. We would like to propose here that the major oligosaccharide produced by the hydrolysis of chitin with hevamine was likely to be $(GlcNAc)_5$, and another enzyme presented in the serum was chitobiosidase, an exochitinase that usually hydrolyzes chitin and oligosaccharides from the non-reducing end to release $(GlcNAc)_2$. The products obtained from the hydrolysis of $(GlcNAc)_5$ with chitobiosidase would be $(GlcNAc)_2$ and (GlcNAc) with a $(GlcNAc)_2$:(GlcNAc) molar ratio of 2:1, which corresponds to the experimental observations.

Although the rubber serum exhibited reasonably high chitinolytic activity, it produced two major hydrolytic products, GlcNAc and $(GlcNAc)_2$. We were interested in producing GlcNAc from a one-step hydrolysis of chitin with the rubber serum. To achieve this goal, the serum must contain both chitinase (EC 3.2.1.14) and β-*N*-acetylhexosaminidase (EC 3.2.1.52) activities. The chitinase is responsible for hydrolyzing polymeric chitin chains into smaller chitooligosaccharides, which are in turn further hydrolyzed by β-*N*-acetylhexosaminidase to GlcNAc. Unfortunately, the serum contained no β-*N*-acetylhexosaminidase activity for this purpose. Commercial pectinase from *Aspergillus niger* (*An*) was reported to contain high β-*N*-acetylhexosaminidase activity.[13] It should thus be interesting to mix the rubber serum with pectinase *An* to produce a low-cost enzyme complex for production of GlcNAc from the direct hydrolysis of chitin.

In order to find a suitable mixing ratio between pectinase *An* and the rubber serum, the pectinase *An* (β-*N*-acetylhexosaminidase activity = 21.35 mU/mg) and the rubber serum (chitinase activity = 108 mU/mL) were mixed at various activity ratios in the chitinolysis of β-chitin. When the activity ratio of pectinase *An* to the

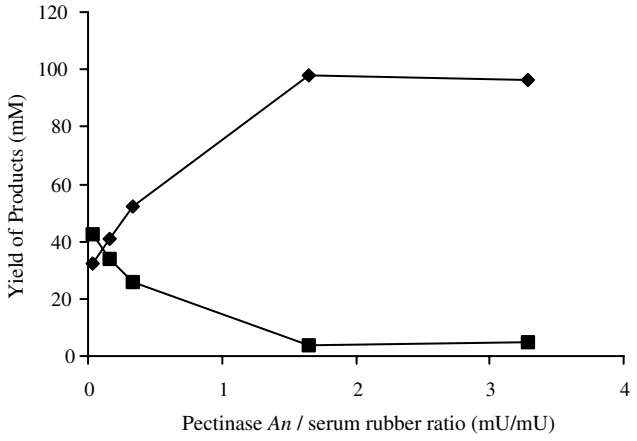

Figure 9. The yields of GlcNAc and $(GlcNAc)_2$ obtained from the chitinolysis of β-chitin with the mixed pectinase *An*/rubber serum at various activity mixing ratios. (◆) GlcNAc; (■) (GlcNAc)2, [chitin] = 60 mg/mL, 0.1 M NaOAc pH 4.0, 45 °C, 2 days.

rubber serum was higher than 1.5, $(GlcNAc)_2$ was quantitatively converted to GlcNAc (Fig. 9).

It is also noteworthy to point out here that the hydrolysis of β-chitin by the mixed enzyme preparation also stopped at 50% yield of GlcNAc (Fig. 10), which is in line with the results observed in the hydrolysis solely with the serum described previously. These results suggested that $(GlcNAc)_2$ product inhibition was not a cause for ending the hydrolysis at this low level of conversion.

In conclusion, we have demonstrated herein that the serum fraction from the latex of *para* rubber tree (*H. brasiliensis*) possesses interesting chitinolytic activities that included both endochitinase, hevamine, and exochitinase, chitobiosidase activities. The enzyme complex in the serum cooperatively hydrolyzes chitin to produce $(GlcNAc)_2$ and GlcNAc with 2:1 molar ratio. The hydrolysis, however, gave a relatively low conversion

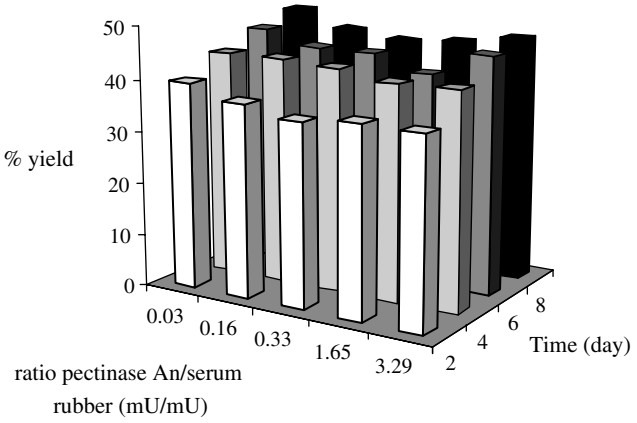

Figure 10. The conversion of chitin to GlcNAc and $(GlcNAc)_2$ obtained from the chitinolysis of β-chitin with mixed pectinase *An* and rubber serum at various activity mixing ratios. [chitin] = 60 mg/mL; 0.1 M NaOAc pH 4.0; 45 °C; 2–8 days.

of chitin around 50%. This limited conversion should be a result of hevamine present as a main enzyme in the serum capable of hydrolyzing only the amorphous part of chitin with at least four connecting GlcNAc units. Mixing the serum with an enzyme preparation containing β-*N*-acetylhexosaminidase is used for converting $(GlcNAc)_2$ to GlcNAc in situ. The final yield of GlcNAc was nevertheless 50%. Further investigation in mixing the serum with another low-cost enzyme containing different types of endochitinase to improve the conversion is currently under investigation.

3. Experimental

3.1. Materials

β-Chitin from squid pen was purchased from Ta-Ming Enterprises (Samutsakorn, Thailand) and was ground in an ultracentrifugal mill (Recter 970) to give puffy fibrous chitin about 50 μm in diameter and 100 μm in length. Rubber serum was obtained from Pan-Asia Biotechnology Co., Ltd. (Thailand) and analyzed for its protein content by settling protein with the ISO/FDIS procedure[22] following Lowry's method.[23] The analysis showed that the serum contained 6.0 mg protein per 1 mL of the serum solution. The rubber serum possessed chitinolytic activity of 18 mU/mg protein determined according to the modified Schale's method.[24]

3.2. General procedures

A typical enzymatic hydrolysis was carried out by incubating a mixture of the rubber serum and the chitin substrate in a buffer solution in the presence of NaN_3 (500 ppm) as a preservative. At each time point, a portion of the reaction mixture was sampled out, diluted with water, then mixed with CH_3CN at the ratio of 30:70 (v/v), filtered, and analyzed by HPLC (controller: Water controller 600, column: Shodex Asahipak NH2P-50, flow rate: 1 mL/min, mobile phase: 30:70 CH_3CN–water, detector: Water 991 photodiode array at 210 nm). The amounts of GlcNAc and $(GlcNAc)_2$ in the reaction mixture were determined from the calibration lines of the corresponding compounds. The percentage yields were calculated based on the number of moles of GlcNAc or $(GlcNAc)_2$ produced against the number of moles of the corresponding repeating units in the starting chitin.

The unit (U) of *endo*-type chitinolytic activity was determined based on the number of reducing end groups of the sugars produced from the hydrolysis of colloidal chitin at pH 4.0 and 37 °C using a modified Schale's method (1 U = the amount of enzyme capable for producing 1 μmol of reducing end of sugar per minute).[22]

The activity of β-*N*-acetylhexosaminidase (chitobiase) was determined by HPLC using $(GlcNAc)_2$ as a substrate. One unit of β-*N*-acetylhexosaminidase activity was defined as the amount of an enzyme able to hydrolyze 1 μmol of $(GlcNAc)_2$ to 2 μmol of GlcNAc per minute.

Acknowledgements

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