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Production of N-acetyl chitobiose from various chitin substrates using commercial enzymes

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Abstract

N-acetyl chitobiose was produced by partial hydrolysis of the marine biopolymer chitin using industrially bulk-produced enzyme preparations. Pepsin showed the highest chitinolytic activity. Pepsin treatment of chitin at pH 5.4 at 44 °C yielded 71.5% of chitobiose, 19% of *N*-acetyl glucosamine and 9.5% chitotriose after incubation during 24 h. Suspensions of amorphous chitin and phosphoric decrystallized chitin were found to be the best substrates. From kinetic studies, it was concluded that the amount of enzyme used is in excess. The substrate is present in excess as well, because only a small part of the substrate is converted. The results suggest that only a small fraction of the glycosidic bonds in decrystallized chitin is available for enzymatic attack. This suggestion is supported by additional enzymatic hydrolysis observed after dissolution and precipitation of already used substrate.

Keywords: Chitobiose; Decrystallized chitin; Enzymatic hydrolysis of chitin; Pepsin

1. Introduction

The marine biopolymer chitin converted to its deacetylated product chitosan has many industrial and medical applications (Hirano, 1996). The hydrolytic products of chitin have gained special interest in agriculture and food industry. Oligo-chitins have been proposed as anti-microbial agents, promoters of plant growth, elicitors of plant resistance, enhancers of the immune response and agents against malignant growth. The monomers, *N*-acetyl-D-glucosamine and D-glucosamine, are candidates as food supplements and for the treatment of osteoarthritis (Sashiwa, Yamano, Fujishima, Muraki, Kawasaki and Nakayama, 2001).

Chitin can be hydrolyzed into oligomers and monomers by acid hydrolysis. However, an enzymatic process is preferable in order to apply a mild reaction condition, to avoid the use of mineral acid and to control the extent of hydrolysis and the consistency of the product. An extracellular Bacillus sp. WY22 chitinase produces chitotriose as a major product from colloidal chitin and glycol chitin (Woo & Park, 2003). Tanaka, Fujiwara, Nishikori, Fukui, Takagi and Imanaka (1999) reported an extracellular chitinase from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1, producing chitobiose as the major end product. Chitinase from *Bacillus licheniformis* SK-1 hydrolyzes chitin completely into *N*-acetyl glucosamine (Pichyangkura, Kudan, Kuttiyawong, Sukwattanasinitt, & Aiba, 2002). However, these enzymes are not yet available for large-scale commercial application.

Commercially available non-chitinase enzymes have been reported to be able to hydrolyze chitin and chitosan. Lysozyme, lipase, papain, pectinase, tannase and several glycanases can hydrolyze chitosan moderately in acidic media (Lin, Wang, Xue, & Ye, 2002; Muzzarelli, 1997; Muzzarelli, Tomasetti, & Ilari, 1994; Pantaleone, Yalpani, & Scollar, 1992). Recently, Sashiwa, Fujishima, Yamano, Kawasaki, Nakayama and Muraki (2003) have reported the non-specific action of cellulase crude enzymes of *Trichoderma viride* and *Acremonium cellulolyticus* in the production of *N*-acetyl glucosamine from β-chitin.

The data presented in this paper describe the use of commercially available enzymes for the hydrolysis of natural chitin and its decrystallized forms. Among them, bovine pepsin shows the ability to hydrolyze chitin and to produce chitobiose as a major chitinolytic product.

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2. Materials and methods

2.1. Substrate preparation

α-Chitin from shrimp waste was in-house produced in the Bioprocess Technology Laboratory, Asian Institute of Technology, Thailand (Stevens, 2001). The following decrystallized products of chitin were prepared from chitin powder of 100–200 mesh.

2.1.1. Chitin precipitated from phosphoric acid solution (PA-CT)

Chitin was stirred in 85% phosphoric acid at room temperature for 40 min. The viscous chitin solution was filtered through cheesecloth to remove insoluble particles. The chitin was precipitated by adding 6 M NaOH to pH 8.0 The precipitate was washed several times with distilled water until the washing water reached the conductivity of original distilled water. Finally, the chitin was freeze–dried (Vincendon, 1997).

2.1.2. Chitin precipitated from hydrochloric acid solution (HCl-CT)

The sample was prepared in a similar way as in the PA-CT preparation. Hydrochloric acid (37%) was used to dissolve chitin instead of phosphoric acid.

2.1.3. Amorphous chitin (Am-CT)

A cold solution (4 °C) of sodium hydroxide (40 ml, 40%, w/w) containing 0.2% sodium dodecylsulfate (SDS) was added to shrimp chitin (10 g). The chitin matrix was allowed to swell for 1 h at 4 °C. The chitin slurry was kept at −20 °C overnight (Tokura, Nishi, Yoshida, & Hiraoki, 1982) and subsequently neutralized with 6 N HCl. The precipitate was collected by filtration and freeze–dried after successive washing with ethanol, water, ethanol, and finally with acetone.

2.1.4. Super fine chitin (SF-CT)

Chitin (0.1 g) was dissolved for 24 h in 100 ml of methanol containing 83% calcium chloride dihydrate (w/v) and 2% glucose (w/v). Then, the chitin was reprecipitated from the solution by adding methanol slowly. After decantation, centrifugation and washing, a 1% superfine chitin suspension in water was obtained. The reprecipitated chitin was suspended again in 18% formic acid for 2 h at room temperature. Then, the solution was adjusted to pH 5.4 with NaOH before enzymatic hydrolysis (Win & Stevens, 2001).

2.2. Substrate characterization

X-ray diffractograms were recorded by a Rigaku MINIFLEX X-ray diffractometer using Cu Kα radiation at 30 kV and 10 mA with a scanning speed at 2° 2θ/min. The degree of deacetylation was analyzed using the acid hydrolysis-HPLC method (Ng, Hein, Chandrkrachang, & Stevens, in press).

2.3. Enzymatic hydrolysis

The following commercial enzymes were used:

1 Bovine pepsin from Carlo Erba (Italy)
2 Papain from Carica Papaya and lysozyme from chicken egg from Sigma (USA)
3 Cellulast 1.5 L (cellulase) from Trichoderma reesei, cellulase T from Trichoderma viride and lipase from Aspergillus niger kindly provided by Nordisk Company, Denmark and Amano Pharmaceutical Company, Japan.

To assay chitinolytic activity of commercial enzyme preparations, a known amount of enzyme (10–20 mg) and 100 mg of chitin substrate were mixed in 20 ml 0.01 M sodium acetate buffer (pH 5.4), followed by incubation in a stoppered conical flask shaken at 150 rpm and 44 °C for 24 h. The enzymatic hydrolysis was terminated by heating the mixture at 90 °C for 10 min. The hydrolysate was filtered through Whatman No. 1 filter paper and 0.45 μm cellulose nitrate membrane.

2.4. Characterization of enzyme hydrolysate

The oligomer composition of 40 μl hydrolysate was analyzed using a Waters HPLC system equipped with a GPC column (PL Hi-Plex Na). Column and detector temperature were maintained at 60 and 40 °C, respectively. Ultra-pure distilled water was used as mobile phase at a flow rate of 0.4 ml/min. The *N*-acetyl glucosamine (ICN Biochemicals, USA), *N*-acetyl chitodimer and *N*-acetyl-chito trimer (Seikagaku, Japan) were run as chito-oligomer standards. The peak areas were used to plot the calibration curve for the determination of the amount of oligomer in the sample hydrolysate. The HPLC data were analyzed by a pre-set program (Millenium Software, Waters). For the integration of fused peak or small peak, force to drop line method was used (Fig. 1b).

Reducing sugar concentration was determined by the Scale method (Imoto & Yagishita, 1971). Reducing sugars reduce the colored ferric iron to a colorless ferrous complex. The reduction of the color was measured at 420 nm. Residual chitin was removed by filtration before the absorbance was measured. The percentage reducing sugar is expressed as g/100 g chitin in terms of *N*-acetyl glucosamine.

All experiments were done in triplicate. Data are presented as the mean of three determinations with its standard deviation.

3. Results and discussion

3.1. Chitinolytic activity of commercial enzymes

Among the chitinolytic activity of the commercial enzymes investigated with amorphous chitin (Am-CT) as substrate, the bovine pepsin showed the highest chitinolytic activity (Fig. 1a) . It mainly produced *N*-acetyl chitobiose (Fig. 1b), that was apparently resistant to further hydrolysis, because its yield did

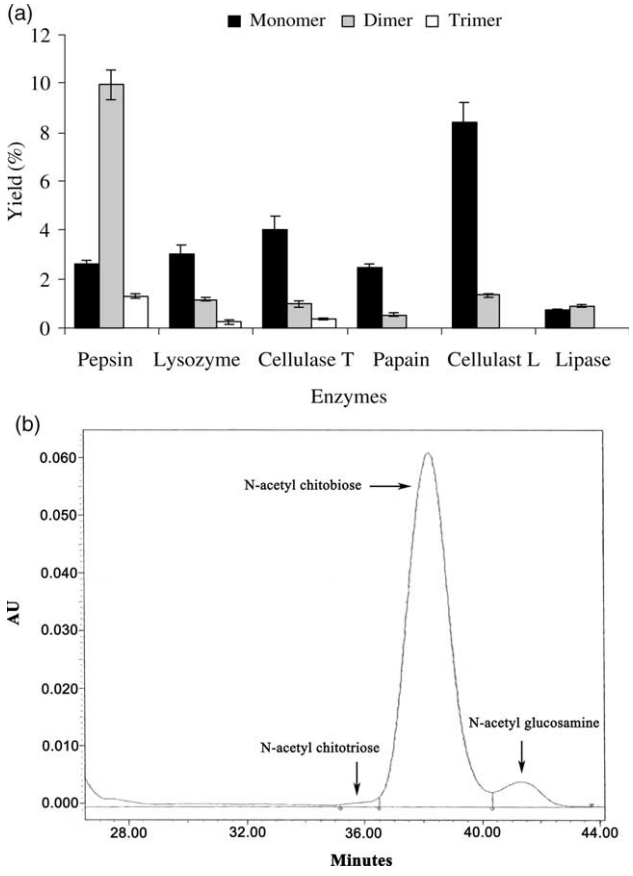

Fig. 1. (a) Yield of *N*-acetyl chito-oligomer expressed as weight% of original chitin. *Hydrolysis conditions:* Amorphous chitin (100 mg) and 20 mg enzyme were incubated in 20 ml acetate buffer pH 5.4 at 44 °C for 24 h and 150 rpm. (b) A typical HPLC profile of the hydrolysate of the amorphous chitin using pepsin. AU: arbitrary units (composition of hydrolysate: 71.5% chitobiose, 19% *N*-acetyl glucosamine and 9.5% chitotriose).

not change upon further incubation (Fig. 2). The optimum for the chitinolytic activity of pepsin was found to be 44 °C and pH 5.4 (data not shown). The choice of buffer is crucial. Phosphate buffer (0.05 M, pH 5.4) was shown to inhibit the chitin hydrolysis by pepsin completely. Acetate buffer (0.05 M, pH 5.4) was used throughout the study.

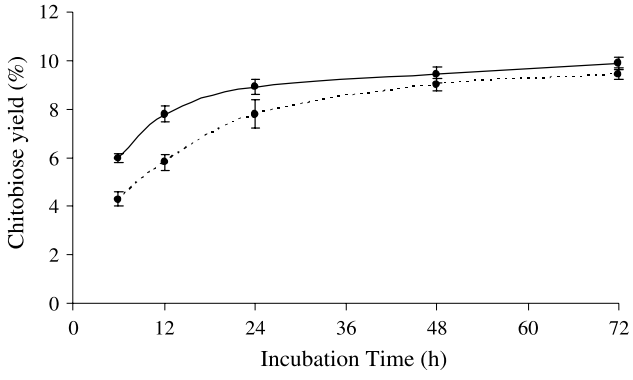

Fig. 2. Kinetics of enzymatic hydrolysis of amorphous chitin using different amounts of bovine pepsin (Carlo Erba). *Hydrolysis conditions:* Amorphous chitin (100 mg) and 10 mg of pepsin (dotted line), 20 mg pepsin (solid line) were incubated in 20 ml acetate buffer pH 5.4 at 44 °C and 150 rpm for 24 h.

The formation of oligochitins using 0.05% bovine pepsin was studied for 72 h. To prevent microbial spoilage of the reaction mixture, sodium azide (0.001 M) was added during long incubation periods. A rapid production of dimer was observed in the first 24 h. Thereafter, a zero-order rate of enzymatic reaction was observed (Fig. 2). The final yield of chitobiose was not significantly increased even when the amount of the pepsin was increased from 0.05 to 0.1%. The enzymatic reaction was studied in the presence of *N*-acetyl glucosamine (0.01 and 0.02%). The final product of enzymatic hydrolysis in these concentrations did not affect the chitinolysis since no significant change in the net yield of chitobiose was found (data not shown).

Muzzarelli et al. (1994) demonstrated a high chitinolytic activity of papain and lipase against chitosan, but these enzyme preparations were found to have only limited activity on chitin (Fig. 1a). Sashiwa et al. (2001) found that cellulase T from *Trichoderma viride* produced mainly *N*-acetyl glucosamine when squid chitin was used as a substrate. In our study, cellulast 1.5 L produced a higher amount of *N*-acetyl glucosamine than cellulase T from *Trichoderma viride* when amorphous chitin was used as a substrate at the optimized hydrolysis conditions (Fig. 1a).

3.2. Decrystallized chitin substrates

The limited enzymatic hydrolysis of natural chitin may be attributed to a limited accessibility of the β-glycosidic bonds in the interior of the chitin crystal particles. The suitability of chitin to act as substrate for enzymatic hydrolysis might be improved by treatments that lead to its decrystallization. Therefore, natural shrimp chitin was treated to reduce its crystalline structure mainly by dissolution–precipitation procedures. Four preparations of decrystallized chitins were used as substrate for the bovine pepsin hydrolysis: chitin decrystallized by phosphoric acid (PA-CT), by HCl (HCl-CT), by alkaline treatment (Am-CT) and by methanol $CaCl_2 \cdot 2H_2O$ (SF-CT). The results showed that the decrystallized chitins were more susceptible to enzymatic hydrolysis than the natural chitin (Figs. 3 and 4).

The extent of enzymatic chitinolytic activity was also investigated for two different physical forms (solid and suspension) of two decrystallized chitin substrates (PA-CT and Am-CT). The suspensions of both chitins forms were freeze dried to get the solid form. The chitobiose yield was reduced to about 50% when the substrates were converted from suspension into the solid form (Fig. 4). Probably, the freeze–drying results in re-establishment of crystalline regions by dehydration.

Under the conditions specified, the total yield of oligochitins was, for Am-CT, PA-CT and SF-CT almost equal, 12.3, 14.2, and 12.8%, respectively, while HCl-CT produced 6.5% and natural shrimp chitin only 3.1% (Fig. 3). The difference in chitinolytic activity may be caused by differences in the crystalline properties of the chitin substrates. To validate this hypothesis, X-ray diffractograms of different chitin substrates were taken (Fig. 5). The diffractogram of HCl-CT (Fig. 5c)

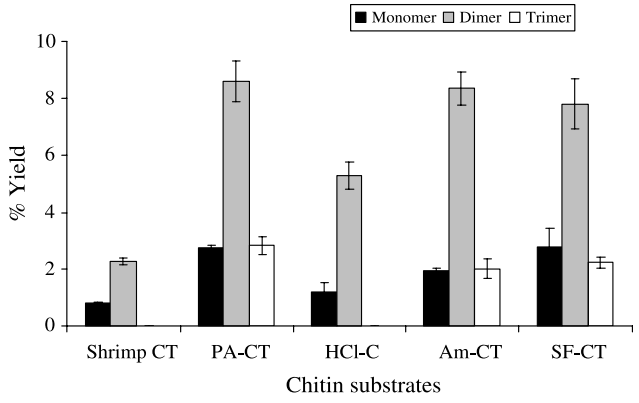

Fig. 3. Production of *N*-acetyl glucosamine, *N*-acetyl chitobi- and triose from decrystallized chitin preparations: phosphoric acid regenerated chitin (PA-CT), HCl regenerated chitin (HCl-CT), amorphous chitin (Am-CT) and superfine chitin (SF-CT). *Control:* Natural shrimp chitin (Shrimp CT) not treated for decrystallization. *Hydrolysis conditions:* Chitin preparation 100 mg) and 20 mg pepsin were incubated in 20 ml acetate buffer pH 5.4 at 44 °C and 150 rpm for 24 h.

showed a close similarity to that of natural shrimp chitin (Fig. 5e). Crystalline peaks from natural chitin were present in HCl-CT except that the intensity of the 020 reflection at 9.4 2θ degree in HCl-CT is lower than that of natural chitin.

Diffractograms of Am-CT, PA-CT and SF-CT showed more amorphous nature with much lower intensities of the 110 reflection around 20.0 2θ degree. The intensity at the 020 reflection around 9.4 2θ degree was lowest in Am-CT. Apparently, in the decrystallization process, a group of chitin preparations was produced with lower crystallinity (Am-CT, PA-CT, SF-CT), whereas the HCl-CT reclaimed the high crystallinity, close to that of the natural chitin.

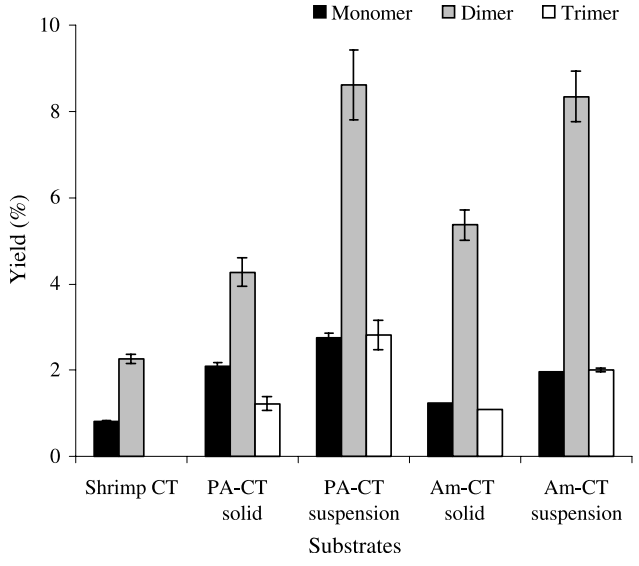

Fig. 4. Susceptibility of solid and suspension forms of two decrystallized chitins PA-CT and Am-CT for bovine pepsin. *Control:* Natural shrimp chitin (Shrimp CT) not treated for decrystallization. *Hydrolysis conditions:* Decrystallized chitin (100 mg) and 20 mg pepsin were incubated in 20 ml acetate buffer pH 5.4 at 44 °C for 24 h and 150 rpm.

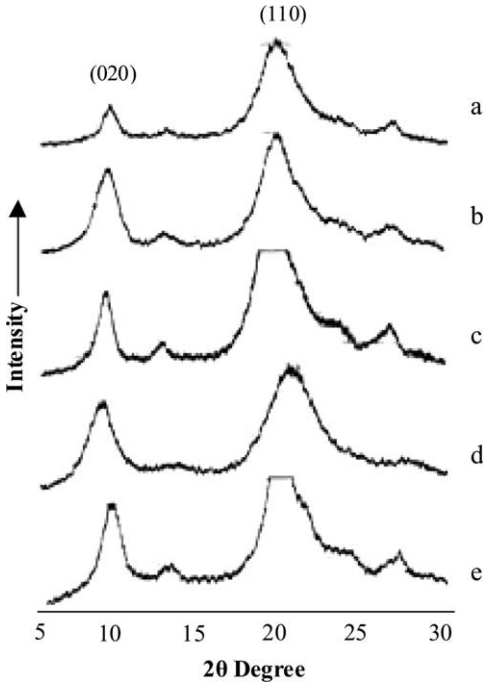

Fig. 5. X-ray diffractograms of different chitin substrates: (a) amorphous chitin; Am-CT, (b) phosphoric acid regenerated chitin; PA-CT, (c) HCl regenerated chitin HCl-CT (d) superfine chitin; SF-CT and (e) natural shrimp chitin.

During HCl treatment, considerable hydrolysis of chitin occurs (Vårum, Ottøy, & Smidsrød, 2001). The resulting smaller molecular weight chitin chains may arrange in a more ordered form during the precipitation of chitin from HCl solution. Ogawa, Yui, and Miya (1992) also found that films prepared from low molecular weight chitosan contribute to higher crystallinity. This could be the reason of higher crystallinity in HCl-CT compared with the other decrystallized counterparts. However, the rearrangement of chitin chains in HCl-CT might not precisely result in the level of crystallinity of the natural chitin. This could explain the slightly less crystallinity of HCl-CT compared with natural CT. These findings support the interpretation of the results of the enzymatic hydrolysis that showed that the substrates of the lower crystalline group are more amenable for enzymatic attack.

Studies using FT-IR spectroscopy demonstrated that all chitomaterials, both de-crystallized and non-decrystallized chitins are chemically chitin in nature (data not shown). Also, the degree of deacetylation (%DD) of all chitins determined by acid hydrolysis-HPLC method revealed that the %DD of Am-CT, PA-CT, SF-CT and HCl-CT were similar: 11.2, 9.3, 10.4 and 10.1%, respectively. Natural chitin had a DD of 8–10%. The data imply that the process of de-crystallization did not affect the degree of deacetylation.

The differences in enzymatic chitinolysis by bovine pepsin observed for various decrystallized chitins gives insight into the structure of these compounds. Chitin dissolves completely

in phosphoric acid and methanol saturated with $CaCl_2 \cdot 2H_2O$. Upon addition of base and water, respectively, the chitin chains precipitate randomly and form a loose network, more susceptible to enzymatic attack. Because of the strong hydrolytic power of HCl towards chitin and the chain rearrangement during the precipitation process, the HCl-CT is likely highly crystalline and less suitable for enzymatic hydrolysis.

3.3. Limiting factors in pepsin chitinolysis

Sashiwa et al. (2001) treated β-chitin with cellulase T for 12 days for the production of *N*-acetyl glucosamine. In the research presented here, however, most of the chitobiose produced in 72 h was already produced in the first 24 h (Fig. 2). No noticeable production took place in the subsequent 48 h. This could not be caused by depletion of chitin since the substrate was only converted for 12–14%. The absence of further enzymatic hydrolysis after 24 h might be due to immobilization of the enzyme by binding to the substrate. It is well known that chitin can adsorb protein efficiently. It was found that about 30% of the enzyme co-precipitated with substrate (data not shown). To check adsorption to the substrate as a cause of the fall in hydrolytic activity after 24 h, fresh enzyme was added after 24 h of enzymatic hydrolysis. The result shows only a slight increment in the production of chitobiose. Apparently, the lack of sustained enzymatic hydrolysis is not caused by absence of active free enzyme either.

A third possibility is that nearly all sites of the crystalline chitin particle that can be reached by the enzyme have been hydrolyzed. This hypothesis is supported by experiments in which chitin treated with enzyme for 24 h, was recovered, dissolved, reprecipitated and re-used. The chitin was separated from the pepsin buffer solution by filtration, dried, dissolved in the solvent (methanol:$CaCl_2 \cdot 2H_2O$; 81:19, w/w), precipitated by addition of water, collected and washed by filtration and added again to the saved pepsin buffer reaction mixture already used for 24 h. A new burst of chitinolytic activity was observed (Fig. 6, comparison of T1 and T3). The dissolution and precipitation of the already used chitin apparently resulted in exposure of previously non-accessible sites for chitinolytic action of the enzyme. A similar explanation has been given for the complete deacetylation of chitin by a fungal deacetylase. Only after decrystallization by dissolution and precipitation, resulting in the exposure of the internally located acetylated groups, complete enzymatic deacetylation of chitin was achieved (Win & Stevens, 2001).

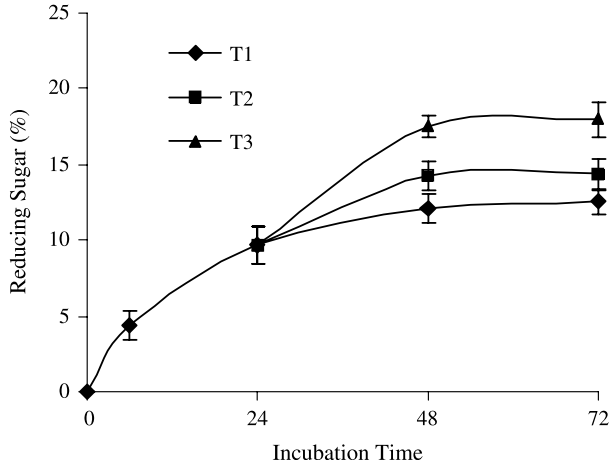

Fig. 6. Effect of extra addition of bovine pepsin to amorphous chitin which was previously subjected to 24 h pepsin treatment. T1: control, no extra addition of enzyme after 24 h of incubation. T2: Addition of another 20 mg fresh enzyme after 24 h of incubation. T3: Chitin remaining after 24 h of incubation was dissolved, precipitated and readded to the previous hydrolysis mixture without adding of fresh enzyme.

4. Conclusion

N-acetyl chitobiose can be produced by the hydrolysis of decrystallized chitin using the commercial bovine pepsin with a yield of 10%. This enzyme has not been reported for oligochitin production earlier. The optimum conditions for the hydrolysis were 44 °C, pH 5.4 and 150 rpm shaking. Enzyme was added in an enzyme to substrate ratio of 1% (w/w). The more decrystallized form of chitin, the higher yield of chitobiose. Amorphous chitin and phosphoric acid treated chitin were found to be the most suitable substrates.

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