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Environmental chitinous materials as adsorbents for one-step purification of protease and chitosanase

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Abstract We describe the use of chitinous materials as adsorbents for purification of protease and chitosanase from bromelain solution and chitosanase from culture supernatants of three bacterial strains: *Serratia marcescens* TKU011, *Bacillus cereus* TKU022 and *Acinetobacter calcoaceticus* TKU024. The best adsorption results were observed when crude shrimp shell chitin (CSSC) and 750-nm chitin nanoparticles (CNP) were used. The optimum temperatures for protease adsorption from bromelain solution (22.9 mg/mL) by CSSC (0.1 g) and by 750-nm CNP (0.1 g) were 4 and 25 °C, respectively. The purification folds of bromelain by CSSC and 750-nm CNP were 5.2 and 4.5, respectively. For purification of protease from culture supernatants of TKU011, 750-nm CNP was 4.0-fold better than CSSC. However, CSSC exhibited purification folds of 2.9 and 3.3 for the chitosanases from TKU022 and TKU024, respectively. The adsorbed chitinolytic enzymes TKU015 chitinase (30 kDa) and TKU024 chitosanase (27 kDa) exhibited high purity by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Thus,

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CSSC and 750-nm CNP indicate potential for use as tools for one-step purification of bacterial chitinolytic enzymes from culture supernatants.

Keywords Chitin · Chitosanase · Nanoparticle · Adsorption · Purification · Bromelain

Introduction

Chitin and chitosan are polymers of interest to researchers for removal of undesirable materials [1–4] and delivery of bioactive compounds [5, 6] due to the adsorption effect of these polymers and their derivatives. Chitin, a homopolymer of *N*-acetyl-D-glucosamine residues linked by β-1-4 bonds, is one of the most abundant, easily obtained and renewable natural polymers [7]. Chitin is commonly produced from shrimp shells (SS), crab shells and squid pens. Chitosan, a poly-(D)-glucosamine, is prepared from chitin by deacetylating its amido groups. Chitin and its derivatives are characterised not only as abundant biomass resources but also as specialised biopolymers for preparing advanced functional materials [8]. These materials exhibit great economic value because of their versatile biological activities and agrochemical applications [9–11].

Chitinolytic enzymes, chitinases and chitosanases, can be produced by a number of organisms, including bacteria, fungi, yeasts and plants, and they are capable of catalysing hydrolysis of chitinous materials into smaller chitooligomers [7, 11]. The enzymes have potential as antagonists for plant pathogens, and chitinolytic enzymes can be used as biocontrol agents in agricultural applications [9–12]. Consequently, it is potentially beneficial to isolate and purify chitinolytic enzymes from fermentation broths for industrial applications [13, 14]. There are several methods for chitinase and chitosanase purification, including, for example, gel filtration, ion exchange and chitin affinity chromatography [15, 16]. Among these methods, chitin affinity chromatography has been demonstrated to be a promising approach for purification of chitinase due to its high specificity and capacity [15].

In this study, instead of direct use of chitin [15] or chemically modified chitin [17] in chitin affinity chromatography, various chitin-containing materials were used as affinity media for purification of enzymes from culture supernatants of chitinolytic enzyme-producing bacteria. For comparison, the effect of size on protein adsorption was also evaluated using chitin nanoparticles (CNP) of various sizes.

Experimental

Chemicals

Crude shrimp shell chitin (CSSC), crude crab shell chitin (CCSC), crude squid pen chitin (CSPC) and SS were all purchased from Shinma Frozen Food Company, Ilan, Taiwan. Pure chitin powder was obtained from C&B Chenli Company, Taipei,

Taiwan. Magnetic chitosan/Fe_3O_4 nanocomposite particles (CNP) with different sizes (400, 750, 1200 and 1400 nm) were gifts from Prof. Y.-K. Twu of Dayeh University [18]. Activated charcoal (AC) was purchased from Choneye Pure Chemicals, Taipei, Taiwan. Bromelain was obtained from Chyanen Biotech Company, Taichuon. *Serratia marcescens* TKU011 [19, 20], *Bacillus cereus* TKU022 [21] and *Acinetobacter calcoaceticus* TKU024 [22] were stocked strains isolated in our laboratory.

Production of chitosanase and/or protease

Production of chitosanase and/or protease from *S. marcescens* TKU011 [19], *B. cereus* TKU022 [21] and *A. calcoaceticus* TKU024 [22] was performed under previously described culture conditions.

Protein concentration determination

Protein content was determined using the Bradford method with Bio-Rad dye reagent concentrate and bovine serum albumin as standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm [21].

Preparation of bromelain solution

One gram of bromelain was dissolved in 100 mL of 50 mM sodium phosphate buffer (pH 7) and dialysed against the same buffer (NPB) at 4 °C overnight. The dialysed bromelain preparation was then diluted with the appropriate amount of NPB to prepare different concentrations of bromelain samples for the adsorption test.

Measurement of protease activity

To measure the protease activity, a diluted enzyme solution (0.2 mL) was mixed with 1.25 mL of 1.25 % casein in 50 mM NPB and incubated for 30 min at 37 °C. The reaction was terminated by adding 5 mL of 0.19 M trichloroacetic acid (TCA). The reaction mixture was centrifuged, and the soluble peptide in the supernatant fraction was measured using the method described by Todd with tyrosine as reference compound. One unit of protease activity was defined as the amount of enzyme required to release 1 μmol tyrosine/min [23].

Measurement of chitosanase activity

The chitosanase activity of the enzyme was measured by incubating 0.2 mL of the enzyme solution with 1 mL of 0.3 % (w/v) water-soluble chitosan (Kiotec Co., Hsinchu, Taiwan; with 60 % deacetylation) in 50 mM NPB at 37 °C for 30 min. The reaction was stopped by heating the reaction mixture to 100 °C for 15 min. The amount of reducing sugar produced was measured using the method described in our previous report [21].

Determination of molecular mass

The molecular masses of the purified enzymes were determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [23] with 12.5 % acrylamide and 2.67 % methylene bis-acrylamide in 0.375 M Tris-HCl buffer (pH 8.8) with 0.1 % (w/v) SDS.

Studies of sorption and desorption

For protein sorption studies, an appropriate amount of sorbent was brought into contact with 2 mL of protein (22.9 mg/mL) containing 50 mM NPB at various temperatures. After centrifugation, the concentration of unadsorbed protein in the filtrate was analysed. For desorption studies, the adsorbed protein in the precipitate was eluted by washing twice with 2 mL of 1 M NaCl solution. The desorption solution was then dialysed against 50 mM NPB overnight at 4 °C, and the dialysate was used to analyse the adsorbed protein content.

Results

Comparison of the adsorption effect of chitinous materials on bromelain protease

The adsorbents included powders prepared from SS, pure chitin (PC), CSSC, CCSC, CSPC, activated charcoal (AC) and magnetic chitosan/Fe_3O_4 nanocomposite particles (CNP) of various sizes (400, 750, 1200 and 1400 nm). Mixtures of 2 mL of bromelain solution (22.9 mg/mL) containing 0.1 g of the tested adsorbents were incubated at 4 °C for 30 min. As shown in Table 1, the CNP exhibited the best results, followed by CSSC. The specific activity of the bromelain protease adsorbed by CSSC was 0.033 U/mg. Regarding the CNP of different sizes, the best specific activity of the recovered protein was found for the 750-nm CNP (0.034 U/mg). Although the weight of the recovered protein increased as the CNP particle size decreased, the specific activity was observed in the group of greater CNP size. This finding may be observed because the smaller CNP adsorbed more protein but exhibited no protease activity and thus yielded lower specific activity. AC has been widely used as an adsorbent in many fields; therefore, we also tested the adsorption effect of AC on protein. There was no protein released from AC (data not shown). This may be caused by tight adsorption between AC and the adsorbed protein. The 750-nm CNP and the cheaper adsorbent CSSC were therefore selected for further evaluation.

Effect of adsorbent concentration on bromelain adsorption using 750-nm CNP and CSSC

Various amounts of 750-nm CNP (0.05, 0.1, 0.125, 0.15 and 0.2 g) and CSSC (0.05, 0.1, 0.125, 0.15 and 0.2 g) were added to 2 mL of bromelain solution (22.9 mg/mL), and the mixture was then incubated at 4 °C for 30 min. As shown in Table 2, the

Table 1 Adsorption effect of various adsorbents for protease purification from bromelain

Adsorbent	Protease activity (U)	Protein weight (mg)	Spec. act. (U/mg)
SS	0.034	3.948	0.009
PC	0.038	3.121	0.012
CSSC	0.313	9.630	0.033
CCSC	0.144	9.503	0.015
CSPC	0.201	7.097	0.028
CNP (450 nm)	0.354	11.620	0.030
CNP (750 nm)	0.295	8.616	0.034
CNP (1,200 nm)	0.251	7.615	0.033
CNP (1,400 nm)	0.228	7.479	0.030

Table 2 Effect of 750-nm CNP concentration on bromelain adsorption

Adsorbent weight (g)	Protease activity (U)	Protein weight (mg)	Spec. act. (U/mg)
0.05	0.188	5.324	0.035
0.1	0.295	8.616	0.034
0.125	0.361	13.640	0.026
0.15	0.353	16.649	0.021
0.2	0.342	20.648	0.017

Table 3 Adsorption effect of CSSC and 750-nm CNP on bromelain and enzymes from three bacterial strains

Enzyme (45.88 g)	Adsorbent weight (g)	Spec. act. (U/mg) (before/after)	Purifying rate (fold)
Bromelain protease	0.1 g CNP	0.006/0.027	4.5
TKU011 protease	0.1 g CNP	0.006/0.024	4.0
TKU022 chitosanase	0.15 g CSSC	0.019/0.056	2.9
TKU024 chitosanase	0.15 g CSSC	0.033/0.108	3.3

amount of protease adsorbed increased with the amount of CNP added, but the specific activity decreased. Among these amounts, the best specific activity was found for 0.05 and 0.1 g of CNP with values of 0.035 and 0.034 U/mg, respectively. Considering cost and efficiency, 0.1 g CNP was used for further analysis. With the CSSC, the best specific activity was found using 0.1 g CSSC with a value of 0.033 U/mg (data not shown).

Effect of temperature on bromelain adsorption using 750-nm CNP and CSSC

The adsorption processes using 750-nm CNP and CSSC as adsorbents were conducted at pH 7 at various temperatures (4, 25 and 37 °C). The protein adsorbed

by 750-nm CNP and CSSC showed the highest specific activities at 25 and 4 °C, respectively (data not shown). The purification folds of the bromelain protease by 750-nm CNP and CSSC were 4.5 and 5.2, respectively.

Adsorption effect of CSSC and CNP on enzymes from three bacterial strains

Crude enzyme preparations containing 45.8 mg of protein were mixed with 0.1 g of 750-nm CNP or CSSC. As shown in Table 3, the 750-nm CNP showed better results for the adsorption of bromelain and TKU011 protease, with purifying rates of 4.5- and 4.0-fold, respectively. CSSC showed better adsorption results for the TKU022 and TKU024 chitosanases, with purifying rates of 2.9- and 3.3-fold, respectively.

Discussion

In this study, we determined that CSSC was a potential adsorbent for purification of chitinolytic enzymes from culture supernatants of *B. cereus* TKU022 and *A. calcoaceticus* TKU024. The 750-nm CNP exhibited potential for purification of proteases from bromelain and culture supernatant of *S. marcescens* TKU011. When compared with the specific activities obtained by SS, PC, CCSC, CSPC and AC, CSSC and CNP exhibited the highest specificity for chitosanase and protease, respectively.

There have been rare reports of using chitinous materials to purify proteases and chitinolytic enzymes from bacterial culture supernatants. Kao et al. [16] reported that a bacterial processing method was used to modify chitin, and the chitin obtained possessed chitinase adsorption capacity in chitinase affinity purification. They found that colloidal chitin adsorbed the highest amount of proteins, and the adsorbed proteins exhibited the lowest chitinase activity. In contrast, the chitin obtained by pre-fermentation with *Paenibacillus* sp. CHE-N1 adsorbed almost the same amount of protein as that of crab shell chitin powder. In this study, CNP showed a higher specific activity for the chitosanase at a lower temperature (4 °C). This result was different from those reported in the literature [16] that have shown that the affinity between chitin and chitinase was significantly reduced at low temperature of 5 °C.

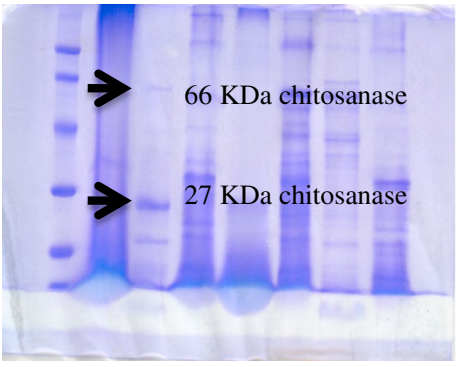

Fig. 1 SDS-PAGE of Acinetobacter calcoaceticus chitosanases TKU024. The lanes from left to right are the marker, the crude enzyme, the protein adsorbed by CSSC (0.1 g), the protein nonadsorbed by CSSC (0.1 g), the protein adsorbed by CSSC (0.15 g), the protein nonadsorbed by CSSC (0.15 g), the protein adsorbed by CNP (0.1 g) and the protein nonadsorbed by CNP (0.1 g)

Trimukhe et al. [17] reported the use of crosslinked chitosan as a matrix for selective adsorption and purification of a lipase of *Aspergillus niger* NCIM 1207. They found that trimellitic anhydride-crosslinked deacetylated chitin adsorbed lipase activity, yielding an approximate fivefold purification of the crude lipase. In this study, the 750-nm CNP showed better results for adsorption of bromelain and TKU011 protease with 4.5- and 4.0-fold purification, respectively. CSSC showed better adsorption results for the chitosanases produced by TKU022 and TKU024 with fold purifications of 2.95 and 3.27, respectively. The higher purification could also be observed in the SDS-PAGE results (Fig. 1). Thus, CCSS and 750-nm CNP may have potential for use in one-step purification of chitinolytic enzymes from culture supernatants of these enzyme-producing bacteria.

Conclusions

One-step purification of bacterial protease and chitosanase with high purifying fold was achieved through use of CNP and a powder composed of crude SS chitin. In addition to enzyme purification, these chitinous adsorbents may be useful in many other applications.

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