An alkaline protease production from *Brevibacillus brevis* and its application in the chitin extraction from shrimp shells

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ABSTRACT

Production of chitin is of great interest because of its biological activities as well as its industrial and biomedical applications. In this study, an extracellular protease but no chitinolytic enzyme-producing strain, *Brevibacillus brevis* TKU046, has been isolated and analyzed for deproteinization test of shrimp heads by liquid fermentation. The optimized condition for protease production (8.02 U/mL) was in a medium containing 1% shrimp head powder (SHP), 0.1% K_2HPO_4, and 0.05% $MgSO_4$. The protease was purified in a three-step procedure involving ethanol precipitation, Macro-Prep DEAE ionic exchange chromatography, and KW 802.5 HPLC. The molecular mass of this monomeric protease was determined as 32 kDa and 34 kDa, respectively, by SDS-PAGE and HPLC. The enzyme showed activities against casein, bovine serum albumin, and keratin. The protease exhibited optimum activity at 50℃ and pH 6-7 and was stable at temperature under 60℃ and pH 6-10. The enzyme was completely inhibited by EDTA suggested that it belongs to the metallic protease family. Deproteinization assays of shrimp heads with this microbe showed protein removal of 95% after 4 days fermentation. The culture supernatant released after fermentation greatly exhibited prebiotic activity on *Lactobacillus rhamnosus* growth. Overall, the findings provide strong support for the potential candidacy of this enzyme as an effective and eco-friendly alternative to the conventional chemicals used for the deproteinization of shrimp heads in the chitin processing industry as well as the production of prebiotics to be used in the nutraceutical industry.

INTRODUCTION

After cellulose, chitin, a homopolymer of *N*-acetyl-D-glucosamine (Glc-NAc) residues linked by ß-1-4 bonds, is the most abundant renewable natural resource. Chitin and its derivatives hold great economic value because of their versatile biological activities [1,2]. Fishery processing by-products, shrimp shells, crab shells, and squid pens, contain chitin, protein, and inorganic compounds such as calcium carbonate. Conventionally, preparation of chitin from these marine waste materials involves demineralization and deproteinization with the use of strong acids or bases [4-7]. However the use of these chemicals may cause a partial deacetylation of chitin and hydrolysis of the polymer, resulting in a final inconsistent physiological properties [14]. These chemical treatments also create waste disposal problems, because neutralization and detoxification of the discharged waste water may be necessary. Furthermore, the value of the deproteinization liquid is diminished due to the presence of sodium hydroxide [7]. To overcome the shortage of the chemical treatments, studies have been conducted using microorganisms or proteolytic enzymes for deproteinization of marine crustacean wastes [3-12].

Proteases are by far the most important group of enzymes produced commercially and are used in many areas of applications, such as detergent, brewing, meat, photographic, leather, and dairy industries [15,16]. In recent years, ample successes in degradation of proteinaceous waste into useful biomass by proteases have also been demonstrated [17-21]. *Brevibacillus brevis* was reclassified the genus from Bacillus into *Brevibacillus* in 1996 [22]. Proteases produced by *Bacillus* sp., including *Bacillus* sp. [8], *B. subtilis* [9], *B. firmus* [13], are by far the most important group of enzymes being exploited. However, the production, characterization, and application of *B. brevis* proteases for the deproteinization of

marine processing by-products is rarely seen. This study demonstrates that protease-producing *B. brevis* can be used for deproteinization of shrimp heads. The culture conditions for maximal protease productivity were studied and the protease produced in shrimp head powder-containing medium was purified and partially characterized.

RESULTS (Purification of TKU046 Protease)

In order to investigate the enzyme characterization and compare to other researches, the protease was purified from the culture supernatant of *B. brevis* TKU046 by ethanol precipitation and ion exchange chromatography of Macro-Prep DEAE Cartridge. As shown in Figure 3, only one protease was eluted with a linear gradient of 0-1 M NaCl in the same buffer. The protease were pooled for further purification by HPLC gel filtration on KW802.5 column. The purification results of TKU046 protease were summarized in Table 3. TKU046 protease were purified from the culture supernatant with the weight recovery 5.13 mg, respectively. The final specific activity and recovery yields of TKU046 protease were 29.14 U/mg and 13.36 %, respectively (Table 3).

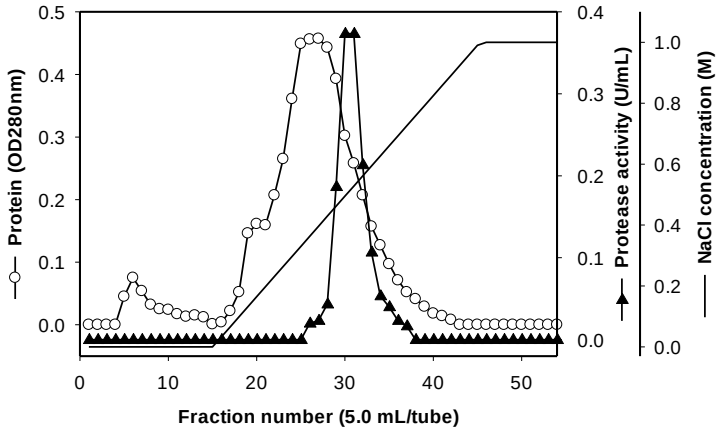

Figure 3. A typical elution profile and protease on Macro-Prep DEAE.

Table 3. Purification of the protease from TKU046.

Step	Total protein (mg)	Total activity (U)	Specific Activity (U/mg)	Recovery (%)	Purification (fold)
Culture supernatant	2827.02	1118.00	0.40	100.00	1.00
Ethanol precipitation	359.93	837.33	2.33	74.90	5.88
Macro-Prep DEAE	114.15	597.33	5.23	53.43	13.23
KW 802.5 column	5.13	149.33	29.14	13.36	73.68

HPLC gel filtration chromatography by a KW802.5 column analysis showed that the pooled fraction after purification displayed only one peak with a retention time of 7.9 mins, corresponding to a protein of nearly 34 kDa. In the addition, SDS-PAGE analysis also showed that the pooled fractions displayed one band with the molecular weight approximately about 32 kDa. There results strongly confirmed that the enzyme purification was success with high purity and TKU046 protease was a monomeric protein similar to those from other *Brevibacillus* strains [17,32]. In the comparison, the molecular weight of TKU046 protease differed from other reports, including *B. brevis* US575 keratinase (29 kDa) [17], *B. parabrevis* CGMCC 10798 keratinase (28 kDa) [28], *B. laterosporus* G4 keratinase (30 kDa) [31], *Brevibacillus* sp. OA30 protease (64.6 kDa) [29], *B. brevis* FF02B brevithrombolase (55 kDa) [32], *Brevibacillus* sp. KH3 protease (40 kDa) [30].

CONCLUSIONS

This study reports using shrimp heads as the sole carbon/nitrogen source for the production of a novel protease, prebiotics, and chitin from shrimp heads by microbial deproteinization. The molecular mass of the purified *B. brevis* protease (32 kDa by SDS-PAGE analysis and 34 kDa by gel filtration) differed from that of the other *Brevibacillus* strains. Conversion shrimp head to bioactive materials production by fermentation via *B. brevis* TKU046 were achieved not only raw chitin with high deproteinization rate but also the supernatant with greatly prebiotic activity. Taking together, shrimp head is a potential marine-processing by-products for the production of biomaterials including prebiotics and chitin through conversion by *B. brevis* TKU046. This bioconversion process provide an acceptable products which have potential to be applied in medicine and food industries.