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Purification and Characterization of Chitinase from a New Species Strain, *Pseudomonas* sp. TKU008

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The chitinase-producing strain TKU008 was isolated from soil in Taiwan, and it was identified as a new species of *Pseudomonas*. The culture condition suitable for production of chitinase was found to be shaking at 30°C for 4 days in 100 ml of medium containing 1% shrimp and crab shell powder, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7). The TKU008 chitinase was suppressed by the simultaneously existing protease, which also showed the maximum activity at the fourth day of incubation. The molecular mass of the chitinase was estimated to be 40 kDa by SDS–PAGE. The optimum pH, optimum temperature, pH stability, and thermal stability of the chitinase were pH 7, 50°C, pH 6–7, and <50°C, respectively. The chitinase was completely inhibited by Mn^{2+} and Cu^{2+}. The results of peptide mass mapping showed that 11 tryptic peptides of the chitinase were identical to the chitinase CW from *Bacillus cereus* (GenBank Accession No. gi 45827175) with a 32% sequence coverage.

Keywords: *Pseudomonas*, chitinase, chitin, shrimp and crab shell wastes

Shrimp and crab shell (SCS) contain chitin, protein, and inorganic compounds, which are mainly composed of calcium carbonate. The production of chitin and its hydrolyzates, such as *N*-acetylglucosamine and chitooligosaccharides, from waste of the shellfish industry has been limited because of the high cost of chitinase and the SCS pretreating process. The oligosaccharides of chitin, preparaed by hydrolyzing chitin with chitinase, have various potential applications in the food, agricultural, and pharmaceutical industries [9].

Chitinase has been found in microorganisms, plants, and animals. The major source of chitinase is microorganisms. Since chitin represents a vast renewable fermentation feedstock of both carbohydrate and nitrogen sources, enzymes capable of bioconverting chitin to low-molecular-weight fermentable products potentially have significant commercial value. Almost all of the chitinase-producing strains use chitin, colloidal chitin, or other chitin derivatives as major carbon sources for chitinase production. However, the preparation of chitin involves demineralization and deproteinization of shellfish waste with the use of strong acids or bases. The utilization of shrimp and crab shell powder (SCSP) directly as the sole carbon/nitrogen source to produce chitinase not only solves environmental problems but also decreases the production costs of microbial chitinases [6, 7].

Reports about the production of chitinases by *Pseudomonas* species seem to be scarce [3, 4, 8, 9]. In this study, we attempted to optimize the culture conditions of *Pseudomonas* sp. TKU008 for maximal chitinase productivity by using the cheap carbon/nitrogen source of shrimp and crab shell powder. In addition, the chitinase from the new species of *Pseudomonas* was also purified, characterized, and compared with chitinases isolated from other bacterial sources.

Materials and Methods

Materials

The squid pen powder (SPP), shrimp shell powder (SSP), and shrimp and crab shell powder (SCSP) used in these experiments were prepared as described earlier [7]. Squid pens, shrimp shells, and crab shells were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). In the preparation of the SPP, SSP, and SCSP, the squid

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pens, shrimp shells, and crab shells were washed thoroughly with tap water and then dried. The dried materials obtained were milled to powders for use as the carbon source for chitinase production. Chitin flakes from shrimp shell (CFSS) and crab shell (CFCS) were purchased from Biotech Co. (Kau-shyuon, Taiwan). Water soluble chitosan (82% deacetylation) was obtained from Tenwell Biotech Co. (Tau-yuen, Taiwan). Powdered chitin was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). DEAE-Sepharose CL-6B, Phenyl Sepharose, and Sephacryl S-100 were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). All other reagents used were of the highest grade available.

TKU008 was isolated from soil in Taiwan. It is a Gram-negative and nonspore-forming bacillus, with catalase oxidase and mobility, which grows in both aerobic and anaerobic environments. According to 16S rDNA partial base sequencing, TKU008 is most closely related to the species of *Pseudomonas* with a similarity of more than 97%. On the basis of glucose metabolic patterns, TKU008 is an aerobic organism. Further identification found that TKU008 belongs to a new species strain, whose GenBank Accession Number is EU103629 (*Pseudomonas* sp. BCRC 17751) [5]. The identification of strain TKU008 was carried out by the Bioresource Collection and Research Center (Shin-Chu, Taiwan).

Microorganism and Enzyme Production

Pseudomonas sp. TKU008, a chitinase-producing strain, was isolated from soil in Taiwan and maintained on nutrient agar plates at 5°C. In the investigation of the culture condition, growth was carried out in a basal medium containing 0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7), and supplemented with 0.5–2% (w/v) of various chitin-containing seafood processing wastes as the sole carbon/nitrogen (C/N) sources to be investigated. The chitin-containing wastes investigated included shrimp shells powder (SSP), shrimp and crab shell powder (SCSP), and squid pen powder (SPP). One hundred ml of the resultant medium in a 250-ml Erlenmeyer flask was aerobically cultured at 30°C for 1–5 days on a rotary shaker (150 rpm). After centrifugation (12,000 ×*g*, 4°C, for 20 min), the supernatants were collected for measurement of chitinase activity.

Purification of the Chitinase

Production of chitinase. For the production of chitinase, *Pseudomonas* sp. TKU008 was grown in 100 ml of liquid medium in an Erlenmeyer flask (250 ml) containing 1% SCSP, 0.1% K_2HPO_4, and 0.05% $MgSO_4 \cdot 7H_2O$ (pH 7). Two ml of the seed culture was transferred into 100 ml of the same medium and grown in an orbital shaking incubator for one day at 30°C and pH 7 (the pH after autoclaving was 7.5). After incubation, the culture broth was centrifuged (4°C and 12,000 ×*g* for 20 min), and the supernatant was used for further purification by chromatography.

DEAE-Sepharose CL-6B chromatography. To the culture supernatant (800 ml), ammonium sulfate was added (608 g/l). The resultant mixture was kept at 4°C overnight and the precipitate formed was collected by centrifugation at 4°C for 20 min at 12,000 ×*g*. The precipitate was then dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7) and dialyzed against the buffer. The resultant dialyzate was loaded onto a DEAE-Sepharose CL-6B column (5 cm× 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). As shown in Fig. 1, the chitinase was washed from the column with the same buffer.

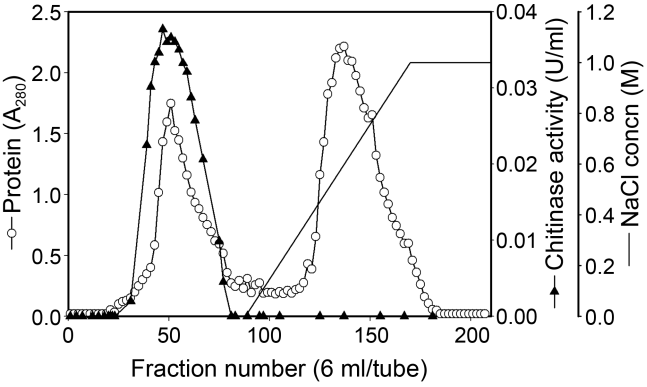

Fig. 1. Elution profile of TKU008 chitinase on DEAE-Sepharose CL-6B: (○) absorbance at 280 nm; (▲) chitinase activity (U/ml).

Phenyl Sepharose chromatography. The unadsorbed chitinase fractions obtained from above step were combined and ammonium sulfate was added to the final concentration of 1 M, and the mixture was then chromatographed on a column of Phenyl Sepharose (1.6 cm ×10 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH) containing 1 M ammonium sulfate. The chitinase was unadsorbed and eluted with the 1 M ammonium-sulfate-containing buffer. The fractions of the peak showing high chitinase activity were combined and concentrated by ammonium sulfate precipitation. The resultant precipitates were collected by centrifugation and dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7).

Sephacryl S-100 chromatography. The resultant enzyme solution (4 ml) obtained from the above step was loaded onto a Sephacryl S-100 gel filtration column (2.5 cm×100 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7), and then eluted with the same buffer. As shown in Fig. 2, one peak exhibiting chitinase activity was obtained, combined, and used as purified chitinase.

Protein Determination

Protein content was determined by the method of Bradford [1] using Bio-Rad dye reagent concentrate and bovine serum albumin as the

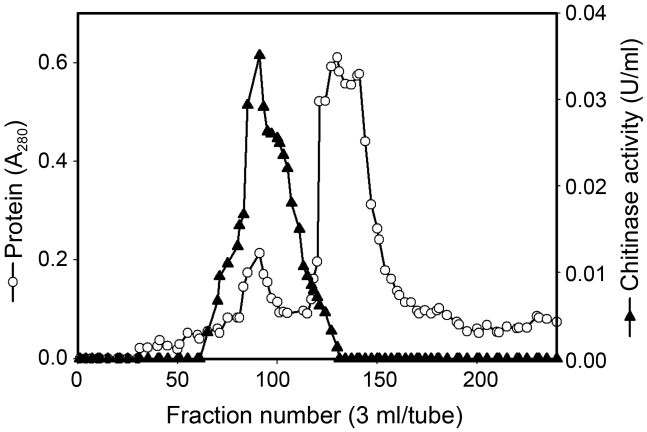

Fig. 2. Elution profile of TKU008 chitinase on Sephacryl S-100: (○) absorbance at 280 nm; (▲) chitinase activity (U/ml).

standard. After column chromatography, the protein concentration was estimated by measuring the absorbance at 280 nm.

Measurement of Enzyme Activity

Chitinase activity was measured with colloidal chitin as a substrate. Enzyme solution (0.5 ml) was added to 1.0 ml of substrate solution, which contained a 1.3% suspension of colloidal chitin in a phosphate buffer (50 mM, pH 7). The mixture was incubated at 37°C for 30 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Imoto and Yagishita with *N*-acetylglucosamine as a reference compound. One unit of chitinase activity was defined as the amount of enzyme that produced 1 μmole of reducing sugars per minute [7].

For measuring protease activity, a diluted enzyme solution (0.2 ml) was mixed with 1.25 ml of 1.25% casein in pH 7 phosphate buffer 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 by the method of Todd with tyrosine as the reference compound. One unit of protease activity was defined as the amount of enzyme required to release 1 μmole of tyrosine per minute [7].

Determination of Molecular Mass

The molecular mass of the purified chitinase was determined by sodium dodecyl sulfate–polyacryamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli [2]. The standard proteins used for calibration were Prestained Protein Marker. Before electrophoresis, proteins were exposed overnight to 10 mM phosphate buffer (pH 7) containing β-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue R-250 in methanol:acetic acid:water [5:1:5 (v/v)], and decolorized in 7% acetic acid.

Effects of pH and Temperature on the Chitinase Activity

The optimum pH of TKU008 chitinase was studied by assaying the samples at different pH values. The pH stability of the chitinase was determined by measuring the residual activity at pH 7, as described above, after the sample had been dialyzed against a 50 mM buffer solution of various pH values (pH 3–11) in seamless cellulose tubing (Sankyo). The buffer systems used were glycine·HCl (50 mM, pH 3), acetate (50 mM, pH 4–5), phosphate (50 mM, pH 6–8), and Na_2CO_3–$NaHCO_3$ (50 mM, pH 9–11). To determine the optimum temperature for the chitinase, the activity values of the samples were measured at various temperatures (25–90°C). The thermal stability of the chitinase was studied by incubating the samples at various temperatures for 30 min. The residual activity was measured as described above.

Effects of Various Chemicals

The effects of various chemicals on the chitinase activity were investigated by preincubating the chitinase with chemicals in 50 mM phosphate buffer solution (pH 7) for 10 min at 37°C followed by measuring the residual chitinase activity.

Effects of Various Surfactants

The enzyme solutions (250 μl) were incubated, in the absence or presence of 0.25 ml of surfactant solutions, at 25°C for 30 min. The residual activities were estimated by the assay procedure described above.

RESULTS AND DISCUSSION

Culture Conditions and Enzyme Production

In our preliminary experiments, we found that 100 ml of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7) containing 1% SCSP was better for the production of chitinase by strain TKU008 at 30°C. However, at the same time, we also found protease activity in the culture supernatant. To study the effect of carbon/nitrogen sources on the production of chitinases and proteases, growth was carried out at 30°C for 1–5 days in 100 ml of basal medium (0.1% K_2HPO_4 and 0.05% $MgSO_4 \cdot 7H_2O$, pH 7) containing additional carbon/nitrogen sources of 0.5–2% (w/v) SCSP, SSP, SPP, CFSS, CFCS, or casein, respectively. The results showed that 1% SCSP was the most suitable as an inducer for the production of chitinase and protease than the others (data not shown). As shown in Fig. 3, the maximum activities of chitinase and protease both appeared on the fourth day of incubation, and thereafter the activity of chitinase decreased remarkably with the appearance of protease activity. That is, incubating in 100 ml of 1% SCSP-containing medium (pH 7) at 30°C for four days was a better condition for the production of chitinase (0.04 U/ml) and protease (0.28 U/ml) by strain TKU008.

In comparison of the medium used for chitinase-producing strains of *Pseudomonas*, *Pseudomonas* sp. YHS-A2 produced chitinase by using colloidal chitin as the major carbon source [3], but TKU008 used 1% (w/v) shrimp and crab shell powder as the sole carbon/nitrogen source for chitinase production. Consequently, the medium for TKU008 is obviously much simpler and cheaper. Regarding the utilization of chitin-containing marine crustacean waste, we have also investigated the bioconversion of shellfish chitin wastes for the production of chitinase from another strain of *Pseudomonas* sp., TKU015 [8]. In the same reaction

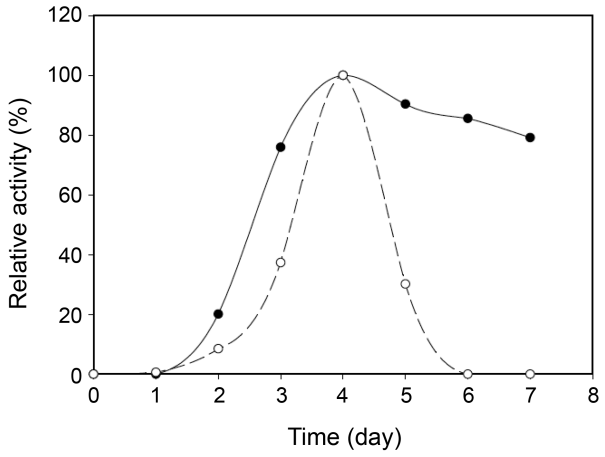

Fig. 3. Effect of cultivation time on the production of chitinase (○) and protease (▲).

Table 1. Purification of a chitinase from *Pseudomonas* sp. TKU008.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Culture supernatant	4,116	29.89	0.007	1.0	100
$(NH_4)_2SO_4$ ppt.	864	17.54	0.020	2.9	59
DEAE-Sepharose	263	5.55	0.021	3.0	19
Phenyl Sepharose	39	3.60	0.092	13.1	12
Sephacryl S-100	15	2.63	0.175	25.0	9

system, the maximum activity of TKU015 chitinase was 0.01 U/ml [8], a value much lower than that for TKU008 (0.04 U/ml). Therefore, shellfish chitin waste is very effective for chitinase production by *Pseudomonas* sp. TKU008.

Isolation and Purification

The purification of the TKU008 chitinase from the culture supernatant (800 ml) is described under Materials and Methods. As shown in Table 1, fractions from the purification steps were combined to give an overall purification of 25.0-fold. The overall activity yield of the purified chitinase was 9%, with specific chitinase activities of 0.175 U/mg. The final amount of TKU008 chitinase obtained was 15 mg. The purified enzyme was also confirmed to be homogeneous by SDS–PAGE (Fig. 4). The molecular mass of TKU008 chitinase was 40 kDa as determined by SDS–PAGE.

The molecular mass of TKU008 chitinase (40 kDa) was obviously different from other *Pseudomonas* chitinases, such as those of *Pseudomonas* sp. TKU015 (68 kDa) [8], *Pseudomonas* sp. YHS-A2 (67 kDa) [3], *P. aeruginosa* 385 (58 kDa) [4], and *P. aeruginosa* K-187 (30 kDa, 32 kDa) [9].

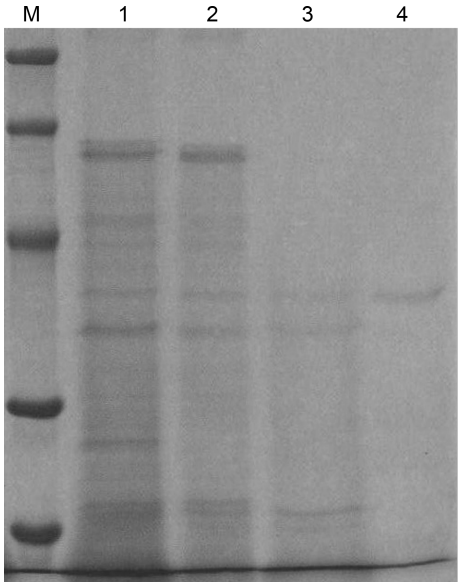

Fig. 4. SDS–PAGE of the protein bands from the various purification steps.
Lane M, molecular mass markers (97.4, 66.2, 45.0, 29.0, 20.1 kDa); lane 1, concentrated culture supernatant; lane 2, purified by ion-exchange chromatography with DEAE-Sepharose CL-6B; lane 3, purified by hydrophobic interaction chromatography with Phenyl Sepharose; lane 4, the purified TKU008 chitinase.

Effects of pH and Temperature

The effect of pH on the catalytic activity was studied by using colloidal chitin as a substrate under the standard assay conditions. The pH activity profile of the chitinase showed a maximum value at pH 7. Compared with other *Pseudomonas* chitinases, similar optimal pHs were obtained; pH 6.7 for *P. aeruginosa* strain 385 [4] and pH 7 for *P. aeruginosa* K-187 [9].

The pH stability profile of the chitinase activity was determined by the measurement of the residual activity at pH 7 after incubation at various pH values at 37°C for 60 min. The chitinase activity was stable at pH 6–7. The pH stability profile of TKU008 chitinase was narrower than the other *Pseudomonas* chitinases, such as between pH 5 and 10 for *P. aeruginosa* strain 385 [4] and *P. aeruginosa* K-187 [9].

The effect of temperature on the activity of chitinase was studied with colloidal chitin as a substrate. The optimum temperature for TKU008 chitinase was 50°C, similar to most of the other *Pseudomonas* chitinases [3, 4, 8, 9]. To examine the heat stability of TKU008 chitinase, the enzyme solution in 50 mM phosphate buffer (pH 7) was allowed to stand for 60 min at various temperatures, and then the residual activity was measured. TKU008 chitinase maintained its initial activity from 25 to 50°C and had only 20% of its activity at 60°C but was completely inactivated at 70°C. Similar thermostability was obtained below 50°C for the other *Pseudomonas* chitinases [3, 4, 8, 9].

Effects of Various Chemicals

To further characterize the *Pseudomonas* sp. TKU008 chitinase, we next examined the effects of some known enzyme inhibitors and divalent metals on its activity. The effects of various chemicals on the enzyme activity were investigated by preincubating the enzyme with chemicals in 50 mM phosphate buffer (pH 7) for 10 min at 37°C and then measuring the residual chitinase activity by using colloidal chitin as a substrate. The results showed that TKU008 chitinase was completely inactivated by Mn^{2+} and Cu^{2+} at 5 mM concentration (data not shown). The chitinases of *P. aeruginosa* TKU015 [8] and *P. aeruginosa* K-187 [9] were also inhibited by Mn^{2+}.

Effects of Various Surfactants

Enzymes are usually inactivated by the addition of surfactants to the reaction solution. The effects of different surfactants [2% (w/v)] on the stability of the purified TKU008 chitinase were also studied. The chitinase preparation was incubated with surfactants at 25°C for 60 min and the remaining enzymatic activity was determined under normal assay conditions. The chitinase activity of the sample without any surfactants (control) was taken as 100%. It was found that, even in the presence of 2 mM SDS (anionic surfactant), or 2% of Tween 20, Tween 40, and Triton X-100 (nonionic surfactants), the activity of TKU008 chitinase retained 54%, 72%, 64%, and 71% of its original activity, respectively. The chitinase activity was increased to 125% in the presence of 0.5 mM SDS (data not shown).

Identification of TKU008 Chitinase by LC–MS/MS Analysis

To identify the chitinase appearing as a prominent 40 kDa band on SDS–PAGE gel, the band was excised and analyzed after tryptic digestion. The 40 kDa band from the SDS–PAGE gel was subjected to electrospray tandem mass spectrometry analysis. The fragment spectra were evaluated and submitted to the Bacteria subset of the NCBInr database of GenBank. The spectra matched 11 tryptic peptides (Table 2) that could be correlated to the protein of chitinase CW (GenBank Accession No. gi 45827175) from *Bacillus cereus* with 32% sequence coverage. The sequence of the *B. cereus* chitinase CW gave a calculated nominal mass of 74 kDa, different to the experimental value obtained with the TKU008 chitinase (40 kDa).

Unlike most of the other reports regarding chitinase-producing strains of *Pseudomonas* sp., this research aimed for the microbial reclamation of shrimp and crab processing wastes. Shrimp and crab shells were used as the sole carbon/nitrogen source to screen the chitinase-producing bacteria. Consequently, although TKU008 belongs to *Pseudomonas* species, the same as the reported chitinase-producing strains of TKU015 [8], YHS-A2 [3], and strain 385 [4], some properties (such as molecular mass and pH stability) of the chitinase produced were different. Unlike most of the other bacterial chitinases, which used chitin or colloidal chitin as the major carbon source, TKU008 used 1% (w/v) shrimp and crab shell powder as the sole carbon/nitrogen source for chitinase production. Therefore, the medium for TKU008 is obviously much simpler and cheaper. Considering the production cost and the reutilization of bioresources, utilizing TKU008 on the microbial reclamation of food processing wastes such as shrimp and crab shell wastes for the production of chitinase seems to provide a promising approach.

Table 2. Identification of TKU008 chitinase by LC–MS/MS[a].

Peptide sequence
IVGYFPSWGVYGRNYQVADIDASKLTHLNYAFADICWNGK
EVPNGTLVLGEPWADVTKSYPGSGTTWEDCDK
TIISVGGWTWSNR
KVFAESTVAFLR
QNFTLLQDVR
AGAEGKQYLLTIASGASQR
LVLGYPFYGR
GTWDDYSTGDTGVYDYGDLAANYVNK
VPYLYNATTGTFISYDDNESMK
GLSGAMFWELSGDCR
LLDTLVKELLGGPISQK

[a]Peptide fragments were identified by LC–MS/MS and by database searching.

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