# A Protease from *Monascus purpureus*

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## ABSTRACT

*Monascus purpureus* CCRC31499 produced a protease when it was grown in a medium containing shrimp and crab shell powder (SCSP) of marine wastes. An extracellular protease was purified from the culture supernatant to homology. The protease had a molecular weight of 40,000 and a p*I* of 7.9. The optimal pH, optimum temperature, and pH stability of the protease were pH 9, 50℃, and pH 7-12, respectively. In addition to protease activity, CCRC 31499 also exhibited activity of enhancing vegetable growth in culture supernatant. This is also the first report of isolation of a protease from *Monascus* species.

## KEYWORDS

protease, shrimp and crab shell, *Monascus*, chitin

## INTRODUCTION

Chitin, a homopolymer of N-acetyl-D-glucosamine (Glc-NAc) residues linked by $\beta$-1-4 bonds, is a common constituent of insect exoskeletons, shells of crustaceans and fungal cell walls. Chitin bioconversion has been proposed as a waste treatment alternative to the disposal of shellfish waste [Wang *et al* 2002a; Wang *et al* 1999; Oh *et al* 2000; Wang *et al* 2002b; Yeh *et al* 2002]. To further enhance the utilization of chitin-containing marine crustacean waste, we have recently investigated the bioconversion of shrimp and crab shell powder (SCSP) for chitinase production [Wang and Hwang 2001; Wang *et al* 2002c; Wang *et al* 2002d].

We have shown that *Monascus purpureus* CCRC31499, one of the safe and widely used traditional food microorganisms, is a chitinase-producing strain in a SCSP medium [Wang *et al* 2002b]. The purification and characterization of an antimicrobial chitinase extracellularly produced by this fungus in SCSP medium has also been described [Wang *et al* 2002d].

Among fungi, only the proteases from *Aspergillus niger* Bo-1 [Aalbaek *et al* 2002], *Mucor bacilliformis* [Fernindez *et al* 1995], *Neosartorya fischeri* var. *spinosa* IBT 4872 [Wu *et al* 2000], *Penicillium roqueforti* P2 [Durand-Poussereau *et al* 1996], *Pleurotus ostreatus* [Palmieri *et al* 2001], and *Rhizopus hangchow* [Ichishima et al 1995] have been characterized in detail. In the present work, we further found that *M. purpureus* CCRC31499, cultured in an SCSP medium, displayed protease activities and the ability of enhancing the

growth of rape. The purification and characterization of the protease thus produced was also investigated.

## EXPERIMENTAL METHODS

### Materials

The shrimp and crab shell powder (SCSP) used in these experiments was prepared in our laboratory. In the preparation of the SCSP, the shrimp and crab shells collected from the marine food processing industry were washed thoroughly with tap water and then steamed. The solid material obtained was dried, milled, and sieved to powder with diameters of $< 0.053$ mm. DEAE-Sepharose CL-6B, Sephacryl S-200, and PBE 94 were purchased from Pharmacia. Powdered chitin was purchased from Sigma Chemical Co., St. Louis, Mo. All other reagents used were of the highest grade available.

### Microorganism

*Monascus purpureus* CCRC31499 was purchased from the Culture Collection and Research Center (CCRC), Taiwan. The plant-pathogenic fungi used in this study were *Fusarium oxysporum* CCRC35100 (from the Culture Collection and Research Center, Taiwan) and *F. solani* (kindly supplied by Dr. Chaur-Tseuen Lo, Department of Plant Pathology, Taiwan Agricultural Research Institute, Taichung, Taiwan).

### Shrimp and crab processing waste

Five preparations of the same type of shrimp and crab processing wastes were used in this study, namely:

*i) Untreated shrimp and crab shell powder (SCSP):* The SCSP used in these experiments was prepared as described earlier [Wang *et al* 2002c; Wang *et al* 2002d]. In the preparation of the SCSP, the shrimp and crab shells collected from a marine food processing factory were washed thoroughly with tap water and followed by steaming. The solid material obtained was dried, milled, and sieved to powder with diameters <0.053 mm.

*ii) SCSP treated with HCl (HCl-SCSP):* In this process, the SCSP was treated with 2N HCl at room temperature for 2 days. The ratio of SCSP to solvent was 1:8 (w:v). This is identical to the demineralization method for the preparation of crustacean chitin [Oh *et al* 2000]. The demineralized material was recovered by filtration, thoroughly rinsed with deionized water, and dried at 65℃. This product is referred to as HCl-SCSP.

*iii) The filtrate from SCSP treated with HCl (HCl-extract):* This extract was the filtrate obtained from the HCl-SCSP preparing process as described above. The filtrate was adjusted to pH 7 by NaOH solution. This product is referred to as HCl-extract.

*iv) SCSP treated with NaOH (NaOH-SCSP):* In this process, SCSP was treated

with 2N NaOH at 100℃ for 30 min. The ratio of SCSP to solvent, 3:40 (w:v), was referred from the deproteinization method for the preparation of crustacean chitin [Oh *et al* 2000]. The deproteinized material was recovered by means of filtration, thoroughly rinsed with deionized water, and dried at 65℃.

*v) The filtrate from SCSP treated with NaOH (NaOH-extract):* This extract was the filtrate obtained from the NaOH-SCSP preparation process described above. The extract was adjusted to pH 7 using HCl solution. This preparation is referred to as NaOH-extract.

*vi) SCSP treated with HCl/NaOH (HCl/NaOH-SCSP):* In this process, SCSP was first treated with HCl washed and dried, and then treated with NaOH. The methods used were the demineralization and deproteinization methods described above. This preparation is is referred to as HCl/NaOH-SCSP.

*vii) The filtrate from SCSP treated with HCl/NaOH (HCl/NaOH-extract):* This extract is the NaOH-treated filtrate obtained from the HCl/NaOH-SCSP preparation process described above. The filtrate was adjusted to pH 7 using HCl. This preparation is referred to as HCl/NaOH-extract.

The concentrations of the four preparations other than the raw SCSP are expressed as the weight percentage of raw SCSP used before treatment. For example, X g of raw SCSP, treated with acid or alkali, produced X weight percent of SCSP in the acidic/alkaline solution (w/v).

**Fermentation**

*M. purpureus* CCRC 31499 was maintained on potato dextrose agar plates at 25℃. For maximum production of the enzyme, we checked protease activity in the culture supernatant at different stages of growth of *M. purpureus* CCRC31499. For the production of protease, *M. purpureus* CCRC31499 was grown aerobically in 100ml of liquid medium in an Erlenmeyer flask (250 mL) containing 2% shrimp and crab shell powder, 0.1%yeast extract, 0.15% polypeptone, 0.1% $K_2HPO_4$, and 0.05% $MgSO_4 \bullet 7H_2O$, pH 7. Two mililiters of the seed culture ($10^5$ spores per ml) was transferred into 100 ml of the same medium and grown in an orbital shaking incubator for 4 days at 25℃. The supernatant containing the crude protease was used for the assessment of its growth enhancing effects on rape and amaranth as well as for the recovery of pure protease.

**Effect of pH and temperature**

The pH stability of the protease was determined by measuring the residual activity at pH 7 as described above after dialyzing the samples against a 50mM buffer solution of various pHs (pH 3-13) in seamless cellulose tubing (Sankyo, Japan). The buffer systems used were glycine-HCl (50mM, pH 3), $CH_3COOH$-$CH_3COONa$ (50mM, pH 4, 5), $Na_2HPO_4$-$NaH_2PO_4$ (50mM, pH 6, 7), Tris-HCl (50mM, pH 8, 9), glycine-NaOH (50 mM, pH 10, 11), KCl-NaOH (50 mM, pH 12, 13). The thermal stability of the protease was studied by

heating the samples at 100℃ for various time periods. The residual activity was measured as described above.

**Purification of the protease**

***(i). Production of the protease.*** For the production of the protease, *M. purpureus* CCRC31499 was grown in 100mL of liquid medium in an Erlenmeyer flask (250 mL) containing 2% shrimp and crab shell powder, 0.1%yeast extract, 0.15% polypepton, 0.1% $K_2HPO_4$, and 0.05% $MgSO_4 \bullet 7H_2O$, pH 7. Two milliliters of the seed culture was transferred into 100 mL of the same medium and grown in an shaking incubator for 4 days at 25℃ and pH 7. The culture broth was centrifuged (4℃ and 12,000 x *g* for 20 min.), and the supernatant was used for further purification by chromatography.

***(ii). DEAE-Sepharose CL-6B chromatography.*** To the cell-free culture broth (1173 mL), ammonium sulfate was added (608 g/L). The resultant mixture was kept at 4 ℃ overnight and the precipitate formed was collected by centrifugation at 4℃ for 20 min at 12,000 x *g*. The precipitate was dissolved in a small amount of 50 mM sodium phosphate buffer (pH 7), and dialyzed against the buffer. The resultant dialysate (30 mL) was loaded onto a DEAE-Sepharose CL-6B column (5 by 30 cm) pre-equilibrated with 50 mM sodium phosphate eluting buffer (pH 7). The unadsorbed materials with protease activity were washed from the column with the same eluting buffer, and the adsorbed materials were fractionated with a linear gradient of 0 to 1 M NaCl in 50 mM phosphate buffer (Figure 1). The eluted fractions (355 mL) were concentrated by ammonium precipitation as described above.

***(iii). Sephacryl S-200 chromatography.*** The resultant dialysate (11 mL) was loaded onto a Sephacryl S-200 gel filtration column (2.5 x 120 cm, which had been equilibrated with 50 mM phosphate buffer (pH 7), and then eluted with the same buffer at a flow rate of 20 mL/h. Fractions (3 mL each) were collected and assayed for protease activity.

**Measurement of enzyme activity**

For measuring protease activity, a diluted solution of purified enzyme (0.2 ml) was mixed with 2.5 ml of 1% casein in phosphate buffer pH 7 and incubated for 10 min at 37℃. 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.

**Determination of molecular weight and isoelectric point**

The molecular weights of the purified enzymes were determined by sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn. Before electrophoresis, proteins were exposed overnight to 10mM phosphate buffer (pH 7) containing 2-mercaptoethanol. The gels (12.5%) were stained with Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (5:1:5, v/v), and decolorized in 7% acetic acid. The isoelectric point of the antifungal enzyme was estimated by chromatofocusing. The enzyme soultion (1 mL) was loaded onto a

chromatofocusing PBE 94 column (0.9 by 27 cm) equilibrated with 50mM Tris-HCl buffer (pH 6), and the elution was done with Polybufer 74-Tris-HCl (pH 6) as described in the manufacturer's manual (Pharmicia).

**Protein determination**

Protein content was determined by the method of Bradford using Bio-Rad protein dye reagent concentrate and bovine serum albumin as the standard.

**Enhancing effect on the growth of rape and amaranth seedlings**

Culture soil without nutrients added was placed in nursery plates. After evenly inseminating certain amount of rape and amaranth seeds and watering, the plates were placed at sunny and well circulated place for 7 days. Seedlings of uniform size were transplanted to pots for further study.

For rape and amaranth seedling growth enhancing test, the seedlings of the same size were divided into four groups, A, B, C, and D with 9 seedlings each. In group A, 10 mL of culture supernatant were added into the pot everyday. In group B, 100 mL of the protease-containing culture supernatant reacted with 20 g of SCSP at 37℃ for 6hrs, after the reaction, 10 mL of the supernatant of the product mixture was added into the pot everyday. In group C, the same supernatant in group B was autoclaved to inactivate the enzymes, and then 10 mL of the autoclaved solution was added into the pot everyday. In group D, only 10 ml of water was added everyday. After 3 weeks of culture, the weight and the height of the whole plant including above and beneath the earth, were measured.

## RESULTS & DISCUSSION

**Comparison of the Effects of SCSP with Other Related Preparations**

Shrimp and crab shells powder (SCSP) contain chitin, protein, and inorganic compounds such as calcium carbonate. Conventionally, preparation of chitin from marine waste material involves demineralization and deproteinization with strong acids or bases. However, the chemical treatments create waste disposal problems, because neutralization and detoxification of the discharged waste water are necessary [Gagne and Simpson 1993]. In order to investigate the feasibility of utilizing such processing waste by microbial bioconversion, series of experiments were carried out to study the effects of the seven preparations on protease production. The protease production with *M. purpureus* CCRC31499 is shown in Table 1. Maximum protease activity was obtained when the strain was grown in the medium containing HCl-SCSP (21.8 U/ml), SCSP (19.1 U/ml), HCl/NaOH-SCSP (16.5 U/ml ), NaOH-extract (11.6 U/ml), HCl/NaOH extract (6.9 U/ml), NaOH-SCSP (5.7 U/ml), and HCl-extract (4.4 U/ml). The result showed that protein-containing substrate was suitable for being the inducer for the production of protease by *M. purpureus* CCRC31499. For example, HCl-SCSP showed better inducing effect since only protein and chitin were left after demineralization by HCl. As for NaOH-SCSP, it was less suitable for the substrate for protease production since almost all of its original proteins had removed by NaOH. The results showed that strain CCRC31499 can be applied to acid and/or alkali liquid waste in the chitin production process.

**Table 1. Summary of the optimal culture conditions for protease production by *M. purpureus* CCRC31499 grown on the seven preparations**

| Type of SCSP | Concentration (%) | Maximum protease yield (U/mL) | Day of maximum yield (day) |
|---|---|---|---|
| SCSP | 2 | 19.1 | 3-4 |
| HCl-SCSP | 2 | 21.8 | 3-4 |
| HCl-extract | 2 | 4.4 | 3-4 |
| NaOH-SCSP | 1 | 5.7 | 3-4 |
| NaOH-extract | 3 | 11.6 | 4-5 |
| HCl/NaOH-SCSP | 1 | 16.5 | 4-5 |
| HCl/NaOH-extract | 2 | 6.9 | 4-5 |

**Purification of CCRC31499 Protease**

In the presence of SCSP as a major carbon source, *Monascus purpureus* CCRC31499 released protease into the culture fluid. The activities were highest at 4 days. The purification of the antifungal chitinase from the culture supernatant (1173 mL) was described in Materials and Methods. The purification procedures are summarized in Table 2. The purification steps were very effective and combined to give overall purification of 25-fold. The overall activity yield of the purified protease was 6 %, with specific protease activities of 92.3 U/mg. The final amount of this protease obtained was 16 mg. The purified enzyme was also confirmed to be homogeneous by SDS-PAGE and chromatofocusing.

**Table 2. Purification of protease from *M. purpureus* CCRC31499**

| Step | Volume (mL) | Protein (mg) | Activity (U) | Specific activity (U/mg) | Purification fold | Activity yield (%) |
|---|---|---|---|---|---|---|
| Culture supernatant | 1173 | 7039 | 26134 | 3.7 | 1 | 100 |
| $(NH_4)_2SO_4$ precipitate | 30 | 2682 | 15275 | 5.7 | 1.5 | 58 |
| DEAE-Sepharose | 355 | 183 | 9780 | 53.4 | 14.4 | 37 |
| $(NH_4)_2SO_4$ precipitate | 11 | 57 | 3520 | 61.7 | 16.7 | 13 |
| Sephacryl S-200 | 75 | 16 | 1545 | 92.3 | 24.9 | 6 |

**Molecular weight and p*I***

The protease of *M. purpureus* CCRC31499 (40-kDa) was obviously different, in molecular weight, from the antifungal chitinase (81-kDa) produced by *M. purpureus* CCRC31499 itself and also different from the fungal proteases from *Aspergillus niger* Bo-1 (91, 73, 59-kDa), *Neosartorya fischeri* var. *spinosa* IBT4872 (45-kDa), *Penicillium roqueforti* P2 (43-kDa), *Pleurotus ostreatus* (75-kDa), and *Rhizopus hangchow* (37 k-Da).

**pH-activity and pH-stability profiles**

The optimal pH (9) of CCRC31499 protease was different from those of protease from *A. niger* Bo-1 (3.5), *Neosartorya fischeri* var. *spinosa* IBT4872 (2.5), *Penicillium* sp. LPB-5 (6.5), *R. hangchow* (3), and *R. oligosporus* ACM 145F (2). The pH stability (7-12) of CCRC31499 protease was also different from those of protease from *A. niger* Bo-1 (3-5), *Mucor bacilliformis* (3.0-5.8), *Penicillium* sp. LPB-5 (6-9), *R. hangchow* (3-4), and *R. oligosporus* ACM 145F (1.5-5). Both the optimal pH and pH stability range of CCRC31499 protease were alkali and obviously different from the other acidic fungal proteases.

**Effect of temperature on activity and stability**

The optimum temperature (50℃) of CCRC31499 protease was different from those of proteases from *A. niger* Bo-1 (32℃), *Mucor miehei* (37℃), *Neosartorya fischeri* var. *spinosa* IBT4872 (30℃), *Penicillium roqueforti* P2 (25℃), *Penicillium* sp. LPB-5 (45℃), *R. hangchow* (45℃), and *R. oligosporus* ACM 145F (60℃). Comparing with other fungal proteases, the optimum temperature of CCRC 31499 protease was more thermal.

**Enhancing effect on the growth of rape and amaranth seedlings**

The result in this research was more than twice better than our previous study [Wang *et al* 2002b]. In our previous study, culture medium (1% SCSP, 0.1% yeast extract, 0.1% polypeptone, 0.1% $K_2HPO_4$, 0.05% $MgSO_4 \bullet 7H_2O$, 0.3% $NaNO_3$, and 0.05% KCl), which is more suitable for the production of chitinase, was used for fermentation by CRC 31499 for 4 days. However, the culture medium (containing 2% SCSP) in this research was more suitable for the production of protease. The major difference was the content of SCSP. Consequently, if CCRC 31499 would be used to produce biofertilizer with vegetable growth enhancing effect, the SCSP content in the culture medium had better be 2%. The vegetable enhancing activities in the culture supernatant could be related to the protease activity, i.e., vegetable enhancing compounds could be amino acids and peptides from the hydrolysis of the SCSP proteins by proteases. This requires further investigation.

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