Available online at www.sciencedirect.com
ScienceDirect
Bioresource Technology 99 (2008) 5436–5443
BIORESOURCE TECHNOLOGY

Bioconversion of squid pen by *Lactobacillus paracasei* subsp *paracasei* TKU010 for the production of proteases and lettuce growth enhancing biofertilizers

San-Lang Wang [a,*], Tsai-Yi Huang [a], Chun-Yuan Wang [a], Tzu-Wen Liang [a], Yue-Horng Yen [b], Yusuke Sakata [c]

[a] *Graduate Institute of Life Sciences, Tamkang University, Taipei 251, Taiwan*
[b] *Department of Bioindustry Technology, Da-Yeh University, Changhwa 515, Taiwan*
[c] *Agricultural Sciences and Natural Resources, Kagoshima University, Kagoshima 890, Japan*

Received 14 September 2007; received in revised form 3 November 2007; accepted 5 November 2007
Available online 9 January 2008

Abstract

A protease-producing bacterium, strain TKU010, was isolated from infant vomited milk and identified as *Lactobacillus paracasei* subsp. *paracasei*. A surfactant-stable protease, purified 64-fold from the third day culture supernatant to homogeneity in an overall yield of 11%, has a molecular weight of about 49,000. The enzyme degraded casein and gelatin, but did not degrade albumin, fibrin, and elastin. The enzyme activity was increased about 1.5-fold by the addition of 5 mM Ba^{2+}. However, Fe^{2+} and Cu^{2+} ions strongly inhibited the enzyme. The enzyme was maximally active at pH 10 and 60 °C and retained 94% and 71% activity in the presence of Tween 20 (2% w/v) and SDS (2 mM), respectively. The result of identification of TKU010 protease showed that nine tryptic peptides were identical to *Serratia* protease (serralysin) (GenBank accession number gi999638) with 35% sequence coverage. In comparison with the tryptic peptides of *L. paracasei* subsp. *paracasei* TKU012 protease, TKU010 protease possessed two additional peptides with sequences of AATTGYDAVDDLLHYHER and QTFTHEIGHALGLSHPGDYNAGEGNPTYR. The fourth day culture supernatant of TKU010 showed maximal activity of about 5-fold growth enhancing effect on lettuce weight, which was not shown with *L. paracasei* subsp *paracasei* TKU012.

Keywords: *Lactobacillus paracasei*; Extracellular protease; Squid pen; Biofertilizers; Chitin

1. Introduction

The application of probiotic bacteria in food products is increasing due to potential health benefits associated with the consumption of these bacteria (Ong et al., 2007). *Lactobacillus* is the predominant genus of lactic acid bacteria found in milk samples. The genus *Lactobacillus* has been associated with a proteolytic activity (Ong et al., 2007; Oneca et al., 2007; Morea et al., 2007; Jung et al., 2007; Tavaria and Malcata, 2003), however, data concerning of proteases from lactobacilli are more limited (Wang et al., in press; Degraeve and Martial-Gros, 2003).

Bioconversion of squid pen has been proposed as a waste treatment alternative to the disposal of chitin containing seafood processing wastes (Wang et al., in press; Wang et al., 2006; Lavall et al., 2007). Squid pen contains abundant protein and chitin and its mineral salts are much lower than those of shrimp or crab shell. Therefore, we have previously investigated microbial reclamation of squid pen for the production of lactobacilli protease, and published the first report on the production and purification of extracellular proteases from lactobacilli (Wang

* Corresponding author. Fax: +886 2 8631 1015.
E-mail address: sabulo@mail.tku.edu.tw (S.-L. Wang).

0960-8524/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2007.11.006

et al., in press). In this study, isolated from the same infant vomited split milk, we found *L. paracasei* subsp *paracasei* TKU010, which is the same species as *L. paracasei* subsp *paracasei* TKU012. When *L. paracasei* subsp *paracasei* TKU010 was cultured in a squid pen powder medium, besides the protease activity displayed, the culture supernatant possessed vegetable growth enhancing effect, which was not found with TKU012. Considering the potential of these enzymes to be used in biotechnological process and the fact that the vegetable growth enhancing effect of the culture supernatant of squid pen fermented by lactobacilli has not been reported, the current work presents the purification and characterization of a protease obtained from *L. paracasei* subsp *paracasei* TKU010, comparison with the peptide mass mapping of *L. paracasei* subsp *paracasei* TKU012 protease, and the vegetable growth enhancing effect of the culture supernatant of squid pen fermented by lactobacilli.

2. Methods

2.1. Materials

The squid pen powder (SPP), shrimp shell powder (SSP), shrimp and crab shell powder (SCSP) used in these experiments were prepared as described earlier (Wang et al.,in press). Squid pens, shrimp shells, crab shells, chitin flake of shrimp shells (CFSS), and chitin flake of crab shells (CFCS) were purchased from Shin-Ma Frozen Food Co. (I-Lan, Taiwan). Katsuobushi from mackerel (KM) and bonito (KB) were purchased from Yi-Fu Food Co. (I-Lan, Taiwan). In the preparation of the SPP, SSP, and SCSP, the squid pens, shrimp shells, and crab shells were washed thoroughly with tap water and then dried. Powdered chitin was purchased from Sigma Chemical Co. (St. Louis, MO). DEAE-Sepharose CL-6B and Sephacryl S-100 were purchased from Amersham Pharmacia Biotech AB (Uppsala, Sweden). All other reagents used were of the highest grade available.

2.2. Microorganism and taxonomic study

The novel protease producing strain L. paracasei subsp paracasei was isolated from infant vomited. The strain was identified according to the methods described in Bergey's Mannual of Systematic Bacteriology (Seneath et al., 1986), and on the basis of 16S ribosomal DNA sequence (Wang et al., in press). The stock culture was maintained in agar at 4 °C and as a glycerol stock at −20 °C.

2.3. Microorganism and enzyme production

Lactobacillus paracasei subsp *paracasei* TKU010 was isolated from infant vomited milk and maintained on MRS agar plates at 37 °C. For the production of protease, *L. paracasei* subsp *paracasei* TKU010 was grown in 100 mL of liquid medium in an Erlenmeyer flask (250 mL) containing 1% SPP, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$ (pH 9). Two milliliters of the seed culture was transferred into 100 mL of the same medium and grown in an orbital shaking incubator (150 rpm) for one day at 25 °C and pH 9. During all the experiments, the agitation speed was kept at 150 rpm. Growth was measured spectrophotometrically (SmartSpec™3000, Bio-Rad) at 660 nm in each case. The cells in the culture broth were harvested by centrifuging the culture at 12,000*g* for 20 min at 4 °C. The cell precipitate was discarded to obtain the supernatant, which was referred to as crude protease.

2.4. Purification of the protease

(i) DEAE-Sepharose CL-6B chromatography. To the culture supernatant (790 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 dialysate (36 mL) was loaded onto a DEAE-Sepharose CL-6B column (5 cm × 30 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7). The unadsorbed materials were washed from the column with the same eluting buffer, and the proteases were fractionated with a linear gradient of 0–1 M NaCl in 50 mM phosphate buffer (Fig. 1). The eluted fractions were combined and concentrated by ammonium sulfate precipitation. The resultant precipitate was collected by centrifugation and dissolved in 50 mM sodium phosphate buffer (pH 7).

(ii) Sephacryl S-100 chromatography. The resultant enzyme solution was loaded onto a Sephacryl S-100 gel filtration column (2.5 cm × 120 cm), which had been equilibrated with 50 mM sodium phosphate buffer (pH 7), then eluted with the same buffer. One peak exhibiting protease activity was obtained, combined, and used as a purified preparation.

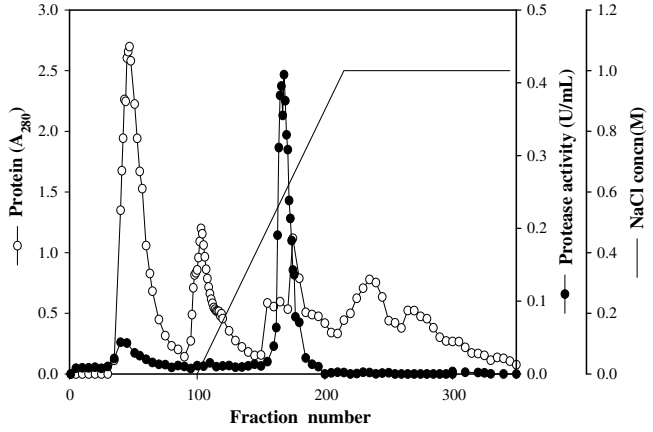

Fig. 1. Elution profile of TKU010 protease on DEAE-Sepharose CL-6B.