Production and characterization of alkaline protease from hemoglobin-degrading *Bacillus pumilus* NJM4 to produce fermented blood meal

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Revised: 18 October 2010 / Accepted: 18 December 2010 / Published online: 20 January 2011
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Abstract The aim of the research was to isolate the hemoglobin-degrading bacterial strain to produce fermented blood meal and to characterize the protease produced by this strain. The strain NJM4, a kind of hemoglobin-degrading bacterial strain, was isolated by blood agar plates from slaughterhouse and identified as a *Bacillus pumilus* by physiological, biochemical, and morphological characteristics and by 16S rRNA gene sequencing. *Bacillus pumilus* NJM4 could degrade hemoglobin up to 85% in 36 h under the laboratory conditions. The optimal conditions for protease production was achieved at an initial pH level of 8.67, inoculum size of 4%, incubation temperature of 37 °C, and agitation rate 200 rpm. The optimum pH and temperature of hemoglobin-degrading proteases were at 9.0 and 50 °C, respectively. The protease activity was slightly decreased in presence of Ca^{2+} and DTT. It was significantly inhibited in the presence of PMSF and EDTA identifying it as alkaline serine-metalloproteinase. *Bacillus pumilus* NJM4 and hemoglobin-degrading proteases provide potential use for biotechnological process of fermentation and enzymolysis blood meal as animal feed supplement.

Keywords Hemoglobin · Degradation · *Bacillus pumilus* · Protease

Introduction

Increasing costs of conventional protein supplement, mainly from fish meal, have generated interest for new

and less expensive protein sources for animals. Blood meal, an important animal protein by-product, contains about 850 g protein kg^{-1} DM, 75 g total lysine kg^{-1} DM, and small amounts of ash and lipids (King' Ori et al. 1998). It is a good source of arginine, cystine, leucine, and valine, and is very rich in lysine, although, it contains low amount of isoleucine and less methionine than fish meal (Amy and Nick 2009). Recent studies have suggested that fish meal can be partially replaced by blood meal without adverse effects on daily weight gain, survival and feed conversion ratio (Donkoh et al. 1999; Millamena 2002; Odunsi 2003; Seifdavati et al. 2008). It has been reported that dried rotary steam and ring had been included up to 6% and 8.9% in pig diets, respectively without adversely affecting animal performance (King and Campbell 1978; Wahlstrom and Libal 1977). King' Ori et al. (1998) reported that small-scale farmers can apply both cooked dried blood meal and fermented blood meal, although the latter is superior to cooking because it has higher performance than former when they supplied equal N levels especially at higher levels of N supply. Hence fermented blood meal is the best protein supplement among blood meals. The aim of this study was to isolate hemoglobin-degrading bacterial strain and to characterize the alkaline protease produced by the strain.

Materials and methods

Isolation of hemoglobin-degrading bacterial strain

Sludge sample (1 g) collected from a slaughterhouse was suspended in 100 mL of the enrichment medium comprising (g/L) peptone-10, beef extract-3, and sodium chloride-5 in 1,000 mL distilled water (pH 7.5) and incubated at 37 °C,

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and 180 rpm for 24 h. The dilute culture (0.1 mL) was spread onto the blood agar plate. One colony named NJM4, which produced the maximum zone of hemoglobin hydrolysis, was chosen for further study.

Identification of hemoglobin-degrading strain

The isolate was identified according to Bergey's Manual of Determinative Bacteriology (Holt 1977). The template DNA for PCR amplification was prepared using TIANamp Bacteria DNA Kit (TIANGEN, China), according to the manufacturer's instructions. Bacterial 16S rRNA gene primers (Suzuki and Giovannoni 1996) 27F 5'-AGAGTTT-GATCTTGGCTCAG-3' and 1522R 5'-AAGGAGGT-GATCCATCCTCA-3' were prepared by Invitrogen (Shanghai, China) and used to amplify about 1.5 kb 16S rRNA gene fragment. The sequencing of PCR products was conducted by Invitrogen, using both the forward and reverse primers. Sequence alignments were performed using BLASTN, which is available on the NCBI server (<http://www.ncbi.nlm.nih.gov>).

Degradation of hemoglobin by NJM4

Reconstituted hemoglobin solution consisting (g/L) hemoglobin-10, NaCl-0.5, K₂HPO₄-0.3, KH₂PO₄-0.4, and MgSO₄·7H₂O-0.1 (pH 7.5) was digested by NJM4 (10⁵ CFU/mL) while incubating at 37 °C, and 180 rpm. During fermentation, hemoglobin solution was taken out at 0, 10, 18, 24 and 36 h. Loading sample buffer of 6×SDS-PAGE was immediately added to each hemoglobin solution, boiled for 10 min, and then stored at -20 °C. Hemoglobin solution not digested by NJM4 was also incubated concurrently as a negative control. Each sample (20 µl) was subsequently subjected to SDS-PAGE, which was conducted with the discontinuous buffer system of Laemmli (Sambrook and Russell 2001) using a 4% stacking gel and 15% separation gel. After separation, the protein bands were visualized with Coomassie Brilliant Blue. Hemoglobin degradation was measured by analyzing the band intensity and area with Gel Image System (Tanon Gis-2500, China).

Assay of protease activity

The culture medium was centrifuged at 8000 rpm for 15 min at 4 °C and the cell-free supernatant was used for estimation of protease activity. Protease activity was determined using casein as a substrate according to the method reported (Bakhtiar et al. 2005). The reaction mixture with a total volume of 2 mL was composed of 1 mL of 1% casein (sigma, USA) in 0.2 mol/L Tris-HCl pH 8.0 and 1 mL of the culture supernatant. The reaction mixture was incubated for 30 min in a water bath at 50 °C

and terminated by the addition of 2 mL trichloroacetic acid with subsequent centrifugation. For 1 mL supernatant 5 mL sodium carbonate was added followed by 1 mL 1 M Folin-Ciocalteu phenol reagent (sigma). The reaction mixture was allowed to stand for 20 min at 40 °C before the absorbance was measured at 680 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 µg tyrosine min⁻¹ under the assay conditions.

Factors affecting protease activity

Cultivations were conducted on a rotatory shaker (200 rpm) in 250 ml Erlenmeyer flasks with a working volume of 25 ml for 24 h at 37 °C. The factors studied included the initial pH of the medium (4.92, 6.24, 7.38, and 8.67), inoculum size (0.5%, 1%, 4%, 6%, and 10%), blood meal concentrations (1%, 2%, 3%, 6%, and 12%), carbon sources and nitrogen sources. In the investigation of nitrogen sources, cultivations were carried out with media that comprise of 20 g blood meal, 0.5 g NaCl, 0.3 g K₂HPO₄, 0.4 g KH₂PO₄, and 0.1 g MgSO₄·7H₂O in 1000 mL distilled water supplemented with different nitrogen and carbon sources at a concentration of 2.5 g L⁻¹.

Characterization of crude enzyme produced by *Bacillus pumilus* NJM4

The effect of temperature and pH on protease activity was assayed in pH range of 7–10 and temperature range of 40–80 °C. The effect of inhibitors, detergents, reducing agents and metal ions on protease activity was investigated. The protease inhibitors phenylmethanesulfonyl fluoride (PMSF), ethylene diamine tetraacetic acid (EDTA), the detergents sodium dodecyl sulfate (SDS) and triton X-100, the reducing

Table 1 Effect of metal ions and inhibitors on enzyme activity

Reagent	Concentration	Residual activity (%)
Control		100 ^a
PMSF	2.0 (mmol/L)	18.2±0.11 ^h
	5.0 (mmol/L)	5.1±0.08 ⁱ
EDTA	1.0 (mmol/L)	39.1±0.07 ^f
	5.0 (mmol/L)	20.0±0.74 ^g
Mg ²⁺	5.0 (mmol/L)	96.2±0.14 ^b
	10.0 (mmol/L)	101.1±1.4 ^a
Ca ²⁺	5.0 (mmol/L)	93.2±0.03 ^c
	10.0 (mmol/L)	77.0±0.13 ^e
DTT	10% (W/V)	85.0±0.05 ^d
Tween-80	10% (W/V)	93.2±0.04 ^c

(n=3); Values followed by different letters differ significantly from each other at P<0.05

Fig. 1 Transmission electron microscopy picture of NJM4 ($\times 6.0k$, 80kv)

agents dithiothreitol (DTT), metal ions Ca^{2+} , and Mg^{2+} were used at the concentration as shown in Table 1.

Statistical analysis

Experiments were carried out in triplicate. The analysis of variance (ANOVA) was performed using standard statistical package (SPSS 17.0) to examine whether any significant difference exists between different treatments and to draw figures. The level of confidence was determined at $P < 0.05$.

Results and discussion

Identification of hemoglobin-degrading strain

By comparing the zones of hemolysis in the blood agar plate, one strain showing the maximum hemoglobin-

degrading activity was purified and designed as NJM4. Phylogenetic analysis of the 16S rRNA gene sequence of NJM4 (EU234500) showed that the sequence exhibited a high level of homology (99.9%) with other *Bacillus pumilus*. The strain NJM4 was Gram-positive bacillus, 0.6–0.7 μm wide and 2.0–3.0 μm long (Fig. 1), aerobic, motile rods, oxidase-, catalase-, protease- and gelatinase-positive and amylase-, lecithinase- negative, citrate- positive and malonate- negative. Based on physiological and biochemical characteristics, morphological characteristics and the 16S rRNA gene sequence, the strain NJM4 was identified as a *Bacillus pumilus* strain.

Degradation of hemoglobin by NJM4

Products of hemoglobin degradation were analyzed by SDS-PAGE (Fig. 2a). The hemoglobin (13.0 KDa) was digested into low molecular weight fractions (8.1, 6.4, 5.0, and 3.8 KDa) in 10 h. There were only 3.8 KDa polypeptides in 36 h. The rate of degradation was up to 82% in 36 h by analyzing the band intensity and area with Gel Image System (Fig. 2b). Hemoglobin is a biomacromolecule and not hydrolyzed by common enzyme present in the animal digestive tract. Products of hemoglobin degradation of less than 4 KDa, were utilized more easily by animals.

Optimization fragmental conditions and medium composition for protease production

The effect of the initial pH value of the medium on protease activity is shown in Fig. 3(a). The optimum pH for protease production was 8.67 ($P < 0.05$). These results are in line with previous reports indicating that protease produced by *Bacillus* species could be classified as an alkaline protease and most active under neutral or basic conditions (El-Refai et al. 2005; Sudeepa et al. 2007; Werlang and Brandelli

Fig. 2 Time-dependent hemoglobin degradation by *Bacillus pumilus* NJM4 **a** SDS-PAGE analysis of products of hemoglobin degraded by NJM4. **b** Intensity analysis of band with Gel Image System

Fig. 3 Effect of the initial pH of the medium, blood meal concentration, inoculum size and nitrogen sources on protease activity ($n=3$). **a** blood meal 20%, inoculum size 2%; **b** pH 7.5, inoculum size 2%; **c** pH 7.5, blood meal 20%; **d** pH 8.67, blood meal 20%, inoculum size 4%

2005). The effect of different blood meal concentration on protease activity is shown in Fig. 3(b). The amount of protease activity depended on blood meal concentration. Protease activity increased as the amount of feather increased (0.5–2%); However when the concentration was raised to 6%, activity significantly decreased. The highest activity was obtained at 2% blood meal ($P<0.05$), showing that high blood meal concentrations may cause substrate inhibition or repression of protease production. The highest activity was obtained at 4% ($P<0.05$) inoculum size (Fig. 3(c)). The effect of the addition of various carbon sources and nitrogen sources on protease production was shown in Fig. 3(d). The results suggested that the addition of dextrin, beef extract, and casein hydrolysate could improve the protease production ($P<0.05$). The other carbon sources and nitrogen sources generally suppressed protease production. The optimal conditions for protease production was achieved at the initial pH value of 8.67, inoculum size of 4%, incubation temperature of 37 °C, agitation rate of 200 rpm, and the medium composed of 20 g blood meal, 2.5 g beef extract, 2.5 g dextrin, 0.5 g NaCl, 0.3 g K_2HPO_4 , 0.4 g KH_2PO_4 , and 0.1 g $MgSO_4 \cdot 7H_2O$ in 1,000 mL distilled water. The maximum protease activity attained was 35.437 U/mL (OD_{680nm} 1.256) using these optimized conditions.

Characterizations of the crude enzyme of NJM4

The effects of pH on protease activity are shown in Fig. 4. The optimum enzyme activity was at pH 9.0, indicating that protease of NJM4 may be an alkaline protease. Previous

Fig. 4 Effect of pH and temperature on protease activity Temperature ($n=3$). (□) 40 °C; (○) 50 °C; (△) 60 °C; (▽) 70 °C; (◇) 80 °C

studies suggested that most protease produced by *Bacillus pumilus* were alkaline protease (Jaouadi et al. 2008; Miyaji et al. 2006). Alkaline proteases from bacteria find numerous applications in various industrial sectors and different companies worldwide have successfully launched several products based on alkaline protease (Gupta et al. 2002).

The effect of protease activity on temperature was detected. As shown in Fig. 4, the optimum temperature range was at 50 °C, which is similar to some protease isolated from the *Bacillus* genus (Kim et al. 2001; Kumar 2002; Lin et al. 1992).

The effect of various metal ions and protease inhibitors on protease activity is presented in Table 1. Protease activity was strongly inhibited by PMSF and EDTA which are well-known inhibitors of serine-metalloproteinase. The Ca^{2+} (1 and 5 mmol L⁻¹) and Mg^{2+} (1 and 10 mmol L⁻¹) have no effect on protease activity because they were present in the fermenting broth. However, the high level of Ca^{2+} (10 mmol L⁻¹) has inhibiting effect (77.0%). These characterizations of the protease of NJM4, suggest that protease of NJM4 may be alkaline serine- metalloproteinase. Most of the microbial proteases produced by the *Bacillus* species reported to date belong to serine protease (Fakhfakh et al. 2009; Ghosh et al. 2008) and metalloproteinase (Allpress et al. 2002; Brandelli and Riffel 2005).

Conclusion

The quality of blood meal's protein is affected by the methods of preparation. Microbial degradation of hemoglobin may provide a viable alternative for improving blood utilization because it is ecologically safe, low-cost, and offers mild reaction conditions. *Bacillus pumilus* NJM4 showed a remarkable potential for the degradation of hemoglobin with associated production of protease. The optimal conditions for protease production was achieved at initial pH level of 8.67, inoculum size of 2%, incubation temperature of 37 °C and agitation rate of 200 rpm. The proteases produced by NJM4 may be alkaline serine-metalloproteinase. The conditions are interesting in terms of their application in industry and being useful for the further isolation and purification. *Bacillus pumilus* NJM4 and hemoglobin-degrading proteases provide potential use for biotechnological processes for hemoglobin-degrading.

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