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The effects of culture conditions on neutral protease (NPRC10) in a recombinant *Escherichia coli* BL21 (DE3)

Nguyen Hoang Loc*, Hoang Tan Quang, Bui Thi Hong Lam, Dao Thi Thuy Trang

Institute of Resources, Environment and Biotechnology, Hue University, Hue, Vietnam

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ABSTRACT

The coding region of nprC10 gene corresponding to mature neutral protease (NPRC10) from Bacillus subtilis C10 was heterologously expressed in Escherichia coli BL21 (DE3) by using pET200/D-TOPO expression system. In this work, the effect of some factors such as cell density (OD_{600}) before induction, inducer (IPTG) concentration, induction time, incubation temperature after induction, soluble starch and Ca^{2+} ion concentrations on extracellular NPRC10 expression and stability were investigated. Our results showed that the crude enzyme exhibited a maximum specific activity of 331.97 unit/mg protein after induction by 0.1 mM IPTG for 19 h at 20°C, an OD_{600} of 2, a soluble starch concentration of 1.5%, and a Ca^{2+} ion concentration of 10 mM. NPRC10 belongs to metalloprotease family and it also displays a non-specific hydrolytic acitivity for starch. The enzyme has optimal temperature and pH for activity at around 55°C and 6.5, respectively.

Keywords: *Bacillus subtilis* C10, *Escherichia coli* BL21 (DE3), extracellular neutral protease, *nprC10*

INTRODUCTION

The protease (proteinase, EC 3.4.21.19) is a large group of enzymes that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain [11]. Proteases occur naturally in all organisms and are the most important industrial enzymes [19].

Many species of the genus *Bacillus* produce a variety of thermostable extracellular enzymes, some of which are industrially important. Among these enzymes, the neutral proteases have been

extensively studied not only for industrial production but also for the elucidation of mechanisms involved in thermostability of enzymes.

Several results on cloning and expression of protease gene in *E. coli* were reported such as intracellular protease from *Pyrococcus furiosus* [9], cysteine protease from *Streptococcus pyogenes* [7], extracellular endoprotease from *Aeromonas caviae* T-64 [20], cysteine protease from *Porphyromonas gingivalis* [18], heat-stable alkaline protease from *B. stearothermophilus* F1 [6], neutral protease from *B. nematocida* [21], and zinc-metalloprotease from *Salinivibrio* sp. AF-2004 [12]. The *B. subtilis* neutral protease gene (*npr*) were also cloned and expressed in *Lactococcus lacfis* spp. *lactis* JF254 [23].

In this study, we investigated the some characteristics of neutral protease (NPRC10) from *B. subtilis* C10 secreted by recombinant *E. coli* B21 (DE3) and the influences of culture conditions on its activity.

MATERIALS AND METHODS

Bacterial strain and plasmid

A strain of *E. coli* BL21 [F^- *ompT hsdS*B (rB^-mB^-)] carrying a lambda derivative (DE3) in the chromosome (Invitrogen) was used. For expression of the recombinant neutral protease (NPRC10), a plasmid vector was constructed by inserting the neutral protease gene (*npr*C10) from *Bacillus subtilis* C10 (Accession number: FJ822054) into the pET200/D-TOPO vector (Invitrogen). The *npr*C10 gene contains the signal peptide-like sequence from nucleotides 161 to 250 and the mature peptide sequence from nucleotides 824 to 1723 [17].

Batch culture medium and condition

A YJ medium (per liter: 20 g glycerol, 15 g tryptone, 20 g yeast extract, 2.5 g $K_2HPO_4.12H_2O$, 0.16 g KH_2PO_4, 0.5 g NaCl, 0.25 g $MgSO_4.7H_2O$, and 50 mg kanamycin, pH 7.0) [25] was used for batch culture. Cultures were carried out in 100-ml Erlenmeyer flask containing 10 ml medium, on a shaker with rotation speed of 200 rpm, at 37^{o}C. The cell density was determined by reading the optical density (OD) at 600 nm.

Assay of protease activity

The *E. coli* BL21 (DE3) containing vector harboring *npr*C10 gene was harvested by centrifugation at 6,000 rpm/4^{o}C for 10 min. The supernatant of fermentation broth was then collected and filtered to determine the activity of enzyme.

Enzyme activity was determined spectrophotometrically by the method of Anson with slight modifications [22]. The substrate was 1% (w/v) casein in 0.05 M Tris buffer (pH 7.4). Enzymatic hydrolysis was initiated by addition of 1 ml properly diluted enzyme solution in 2 ml substrate. After 10 min of incubation at 50°C, 5 ml of 5% (w/v) trichloroacetic acid was pipetted into the solution to stop the reaction. The mixture was incubated at 4^{o}C for 10 min to precipitate the residue substrate. After centrifugation at 14,000 rpm/4^{o}C for 10 min, the supernatant was mixed with 2.5 ml 0.55 M sodium carbonate and 0.5 ml Folin-hydroxybenzene agent followed by the incubation at 50^{o}C for another 10 min. The enzyme activity was measured with a

spectrophotometer at 750 nm. A calibration curve using L-tyrosine as a standard was completed to determine emission of L-tyrosine in the reaction.

One unit of protease activity is defined as the amount of enzyme required to release 1 µg of tyrosine per 1 ml per min under the assay conditions. Total soluble protein concentration was determined by the method of Bradford [4] using bovine serum albumin as a standard (Protein assay kit, Biorad). The protease specific activity is obtained by dividing units of enzyme by total protein soluble concentration.

Improvement of protease expression

When the fermentation broth reached a given OD_{600} value of 0.5-6, isopropyl-1-thio-β-D-galactopyranoside (IPTG) from 0.05 to 1 mM will be added to the fermentation broth to induce expression of recombinant protease. Soluble starch concentrations from 0.5 to 2.5% was used as a carbon sources for cell growth and enzyme production. The effect of Ca^{2+} ion on stability of enzyme activity was investigated at different concentrations from 5 to 100 mM. The cultures will be incubated at a range of temperatures from 14 to 25^{o}C. The activity of enzyme was determined from 4 to 24 h of induction.

Characterization of the crude protease

Optimal temperature assay was determined by performing a standard activity assay in a temperature ranging from 30 to 70^{o}C. To determine the thermo stability of the enzyme, experiments were conducted by measuring the residual activity (%) after incubation at different temperatures ranging from 20 to 80^{o}C for 30 min.

The effect of pH on the proteolytic activity of the crude protease was determined by assaying the enzyme activity at different pH values ranging from 5.0 to 8.0 at 55^{o}C using the following buffer system: 50 mM imidazole, 500 mM NaCl and 20 mM Tris. Two militers of crude enzyme was mixed with 2 ml of the buffer solution mentioned above and incubated at room temperature for 30 min. Afterwards, aliquots of the mixtures were taken to measure the residual protease activity (%) with respect to the control, under standard assay conditions.

The effect of inhibitors (EDTA and PMSF) on stability of enzyme activity was investigated to further characterize the enzyme. The crude enzyme was incubated with the above-mentioned chemicals for 30 min at room temperature, afterwards the residual activity (%) was tested by standard proteolytic activity assay.

Protease precipitation and dialysis

The culture filtrate was precipitated with ammonium sulfate to 60% saturation with stirrer, the mixture was kept at 4^{o}C for 2 h, and centrifugation was done at 15,000 rpm/4^{o}C for 10 min. The precipitate was resuspended in a minimum amount of lysis buffer (50 mM imidazole, 500 mM NaCl and 20 mM Tris pH 6.5). The solution was dialyzed against lysis buffer with Spectra/por® 4 Dialysis membrane (MW 12-14 kDa, Spectrum Laboratories Inc., USA) at 4^{o}C for 13 h, and then used for activity assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed at 4^{o}C according to the method of Heussen and Dowdle with slight modifications by using a Mini-PROTEAN III system (Biorad), 12% polyacrylamide gel, and 0.1% casein as a substrate. The gel was then shaken gently at room temperature for 30 min in 2.5% Triton X-100 to remove SDS. The gel slab was transferred to a bath containing 0.05 M Tris.HCl buffer (pH 6.7) and incubated at 37°C for 3 h to hydrolyse casein [10]. The gels were then fixed and stained in Coomassie Brillant Blue R250, and the image was analysed by Quality One software (ver 4.1, Biorad).

Statistical analysis

All experiments were repeated thrice. The data were analyzed in terms of means followed by comparisons of the mean via Duncan's tests at $p<0.05$ using SAS program.

RESULTS AND DISCUSSION

Cell culture

The initial culture of recombinant *E. coli* BL21 (DE3) cells was carried out at 37^{o}C from 1 to 24 h. The cell growth expressed a lag phase of 2 h, an exponential phase between hours 2 and 21, and a final, death phase. The phase of growth stabilization was quite short, and difficult to predict (data not shown). Cell density increased continuously from 2 to 21 h of culture, with a maximum OD_{600} value of 7.15. Our investigations on expression level of extracellular NPRC10 were designed based on the growth profile of recombinant *E. coli* cells.

Effect of incubation temperature

It was well-known that temperature affects recombinant protein expression significantly, especially for soluble proteins. In our study, the effect of the incubation temperatures on the proteolytic activity of NPRC10 was investigated by culturing recombinant *E. coli* cells in YJ medium at 14-25^{o}C for 20 h (Table 1). The culture was induced with 0.1 mM IPTG when OD_{600} (cell density) reached value of 1.

Table 1. Effect of incubation temperature on NPRC10 expression

Temperature (^{o}C)	Extracellular NPRC10		Intracellular NPRC10	
	Total activity (unit/ml)	Specific activity (unit/mg protein)	Total activity (unit/ml)	Specific activity (unit/mg protein)
14	18.12^{d}	45.30^{d}	5.07^{b}	5.98^{a}
16	25.37^{c}	56.17^{c}	6.89^{b}	6.52^{a}
18	34.43^{b}	68.86^{b}	7.07^{b}	4.35^{b}
20	45.30^{a}	79.73^{a}	10.33^{a}	6.34^{a}
22	30.80^{bc}	63.42^{b}	5.44^{b}	3.64^{c}
25	16.31^{e}	54.36^{c}	3.26^{c}	6.34^{a}

Different letters in the column indicate significantly different means using Duncan's test ($p<0.05$).

As results shown in table 1, NPRC10 was secreted from *E. coli* cells to the medium at all investigated temperatures. However, the maximum total and specific activities of extracellular enzyme were only found at 20^{o}C (45.30 unit/ml and 79.73 unit/mg protein, respectively). Those of intracellular enzyme were only 10.33 unit/ml and 6.34 unit/mg protein, respectively.

Unlike us, Xu et al (2006) reported the highest productivity of the human BD4 protein was achieved by cultivating recombinant *E. coli* cells at 34^{o}C [24], or at 25^{o}C for human GDP-L-fucose protein [5] and yeast YLR301W protein [1].

Effect of induction time

After adding the IPTG into the medium, the target protein begins to be synthesized and induction time is necessary for recombinant protein is secreted to free-cell medium. However, the studies indicated that the concentration of target protein is not proportional to the induction time [24]. In this work, the optimal induction time for expression was examined by analyzing samples taken in every 1 h, from 4 to 24 h, after induction with 0.1 mM IPTG (OD_{600} = 1) at 20^{o}C. The results were shown in table 2.

Table 2. Effect of induction time on extracellular NPRC10 expression

Induction time (h)	Total activity (unit/ml)	Specific activity (unit/mg protein)	Induction time (h)	Total activity (unit/ml)	Specific activity (unit/mg protein)
4	2.22^{de}	27.89^{c}	15	31.91^{b}	72.30^{b}
5	3.43^{de}	37.51^{d}	16	36.46^{b}	79.70^{b}
6	5.68^{de}	44.36^{cd}	17	38.51^{b}	79.60^{b}
7	8.79^{d}	47.11^{cd}	18	47.16^{ab}	85.99^{ab}
8	11.97^{d}	50.74^{c}	19	49.84^{a}	90.17^{a}
9	11.26^{d}	54.07^{c}	20	40.98^{b}	73.22^{b}
10	11.42^{d}	56.18^{c}	21	33.18^{b}	51.83^{c}
11	16.14^{cd}	59.67^{c}	22	30.35^{b}	45.37^{cd}
12	22.84^{c}	66.61^{bc}	23	25.43^{c}	38.29^{d}
13	26.76^{c}	72.28^{b}	24	19.39^{cd}	26.58^{e}
14	29.78^{bc}	72.06^{b}			

Different letters in the column indicate significantly different means using Duncan's test ($p<0.05$).

Generally, the total and specific activities of extracellular NPRC10 increased from 4 to 19 h, and reached maximum values of 49.84 unit/ml and 90.17 unit/mg protein, respectively. Both the activities of enzyme decreased after 19 h of induction.

There are different induction time for recombinant protease expression reported, e. g. the induction time for heat-stable alkaline protease from *B. stearothermophilus* is 72 h [6], or 6 h for human BD4 [24].

Effect of IPTG concentration

For the expression vector with a T7lac promoter, final IPTG concentration should be optimized because of its great contribution to recombinant protein expression and serious harm to cell growth [15]. We investigated the effect of IPTG at different concentrations from 0.05 to 1.0 mM on the NPRC10 production.

The enzyme activity was measured after 19 h of induction with IPTG (OD_{600} = 1) at 20^{o}C. The results showed the total and specific activities reached maximum values of 48.13 unit/ml and 97.16 unit/mg protein at 0.1 mM IPTG, respectively (Table 3).

Table 3. Effect of IPTG concentration on extracellular NPRC10 expression

IPTG concentration (mM)	Total activity (unit/ml)	Specific activity (unit/mg protein)
0.05	8.36^{c}	12.97^{d}
0.08	33.86^{b}	68.25^{b}
0.1	48.13^{a}	97.16^{a}
0.2	36.47^{b}	75.36^{b}
0.3	34.18^{b}	72.76^{b}
0.4	32.96^{b}	71.54^{b}
0.5	28.77^{b}	59.13^{bc}
1.0	28.69^{b}	57.72^{c}

Different letters in the column indicate significantly different means using Duncan's test ($p<0.05$).

The optimal IPTG concentration for NPRC10 expression is similar to that of chaperone [8] and GDP-L-fucose [5]. While optimal IPTG concentration for human BD4 is 0.4 mM [24], 0.04 mM for heat-stable alkaline protease from *B. stearothermophilus* F1 [6] and catalase from *Helicobacter pylori* [3], 0.02-0.2 mM for yeast YLR301W protein [1].

Effect of soluble starch concentration

According to Li et al (2007), several non-specific hydrolytic enzymes (e.g. neutral protease) were found to catalyze the cleavage of glycosidic bonds in chitosan [14]. Therefore, the aim of this experiment was to investigate the specificity of NPRC10 towards the starch, a carbohydrate consisting of a large number of glucose units joined together by glycosidic bonds. An understanding of degradation specificity of NPRC10 to starch is essential for better application.
The oligosaccharides were formed from degraded starch could infiltrate into the *E. coli* cells and to be used as a carbon sources for growth and enzyme production.

Rapid assay for an starch hydrolytic activity was performed by loading the extract of NPRC10 on assayed plate containing 0.8% agar and 0.5% soluble starch which was used as a substrate sources of enzyme. As shown in figure 1, the presence of clearing zone when incubated at 55^{o}C for overnight and stained by Lugol solution indicated the action of protease.

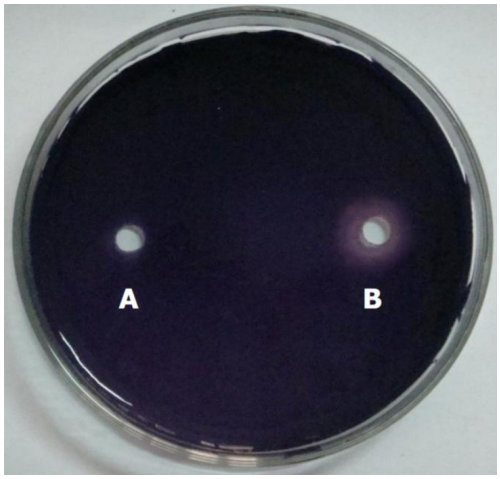

Figure 1. Assay for starch hydrolytic activity of NPRC10 on agar plate. A: Enzyme buffer solution (50 mM imidazole, 500 mM NaCl and 20 mM Tris.HCl, pH 6.5). B: NPRC10 enzyme.

The enzyme activity was measured after 19 h of induction with 0.1 mM IPTG (OD_{600} = 1) at 20^{o}C. Our findings indicate that extracellular protease activity is increased after the starch is added in medium. The optimum for the activities of NPRC10 was found to be 1.5% soluble starch concentration, 75.05 unit/ml for total activity and 134.04 unit/mg protein for specific activity (Table 4)

Table 4. Effect of starch concentration on extracellular NPRC10 expression

Starch concentration (%)	Total activity (unit/ml)	Specific activity (unit/mg protein)
0.5	46.13^{c}	76.57^{c}
1.0	56.04^{b}	81.80^{b}
1.5	75.05^{a}	134.04^{a}
2.0	70.23^{a}	124.13^{ab}
2.5	42.19^{c}	89.99^{b}

Different letters in the column indicate significantly different means using Duncan's test ($p<0.05$).

Effect of cell density

While larger biomass content before induction is necessary for enhanced expression of the recombinant protein, the cells overproducing recombinant protein should also be sufficiently active at the time of induction. Thus, optimizing the value of OD_{600} for induction plays a crucial role for maximizing expression of recombinant proteins [8].

The effect of cell density was evaluated by adding 0.1 mM IPTG and 1.5% soluble starch to the culture at different stages of growth (OD_{600} from 0.5 to 6). The hydrolytic activity of NPRC10 was analyzed after 19 h of induction at 20^{o}C. The results shown the maximum total and specific activities were observed when the cells induced at the mid-exponential phase, corresponding to the OD_{600} value of 2. The enzyme activity rapidly decreased when cells density reached an OD_{600} value of 3 or higher before induction (post-exponential phase) (Table 5). While Bai et al (2003) reported that the optimal induction time for catalase production of *Helicobacter pylori* is corresponding to an OD_{600} value of 0.6 [3].

Table 5. Effect of cell density on the extracellular NPRC10 expression

Cell density at OD_{600}	Total activity (unit/ml)	Specific activity (unit/mg protein)
0.5	32.85^{d}	65.59^{d}
1.0	68.02^{c}	152.57^{c}
1.5	78.28^{b}	169.62^{b}
2.0	91.76^{a}	184.11^{a}
2.5	87.75^{ab}	166.76^{b}
3.0	25.39^{d}	45.18^{e}
5.0	13.14^{e}	22.51^{f}
6.0	-	-

Different letters in the column indicate significantly different means using Duncan's test ($p<0.05$).

During the process of recombinant protein expression in *E. coli*, IPTG induction is the turning point between cell growth and recombinant protein synthesis. The addition of IPTG triggers the transcription of foreign gene in the plasmid and, consequently, brings great changes to the metabolism of host cells by initiating the translation of heterologous protein [24].

Effect of Ca^{2+} ion concentration

Ca^{2+} is an important ion for stability of protease activity and it is also the best metal ion for neutral protease from *B. nematocida*, 0.1 M Ca^{2+} enhanced enzyme by 60%, the other ions moderately reduced or significantly inhibited the enzyme activity [21].

In our study, when cell density reached an OD_{600} value of 2, various concentrations of Ca^{2+} ion was added to the culture to investigate the role of Ca^{2+} ion on stability of NPRC10. The hydrolytic activity of enzyme was analysed after 19 h of induction by 0.1 mM IPTG at 20^{o}C.
As data shown in table 6, the maximum total and specific activities obtained to be 154.95 unit/ml and 331.97 unit/mg protein at 10 mM Ca^{2+}, respectively. These results indicated that the medium supplemented with a lower Ca^{2+} concentration can enhance the NPRC10 activity while the higher concentrations will inhibit the enzyme.

Ayadi et al (2008) reported that Ca^{2+} ion concentrations ranging from 1 to 5 mM increased the activity of US105 type I pullulanase from *Geobacillus thermoleovorans* to 140% at room temperature [2]. In the present study, results shown that 10 mM Ca^{2+} ion enhanced NPRC10 activity to approximately 180%.

Table 6. Effect of Ca^{2+} ion concentration on the stability of NPRC10 activity

Ca^{2+} concentration (mM)	Total activity (unit/ml)	Specific activity (unit/mg protein)
5	126.56^{c}	281.84^{c}
8	156.90^{a}	301.67^{b}
10	154.95^{a}	331.97^{a}
20	141.46^{b}	295.42^{b}
50	146.77^{ab}	286.34^{bc}
100	44.67^{d}	140.23^{d}

Different letters in the column indicate significantly different means using Duncan's test ($p<0.05$).

Characterization of the crude protease

The optimum temperature of the NPRC10 was determined to be 55^{o}C. The enzyme activty was stable at 35-60^{o}C for 30 min incubation with more than 80% of activity was reained. The pH profile shown he optimal pH of NPRC10 was 6.5. More than 80% of maximum activity was retained in the pH range from 6.5 to 7 (Fig 2).

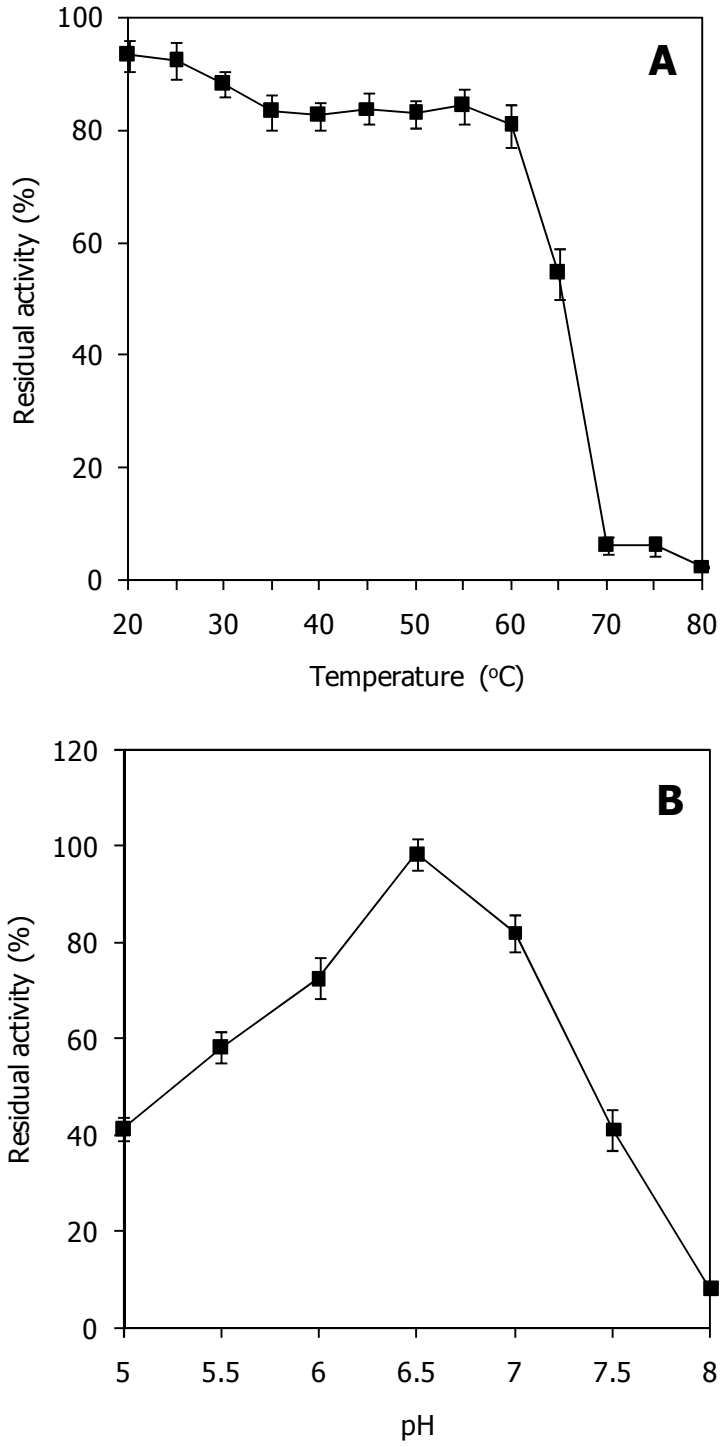

Figure 2. Effect of temperature (A) and pH (B) on NPRC10 activity

To further characterize NPRC10, enzyme was incubated with various inhibitors (EDTA and PMSF). As it is known EDTA is a specific inhibitor of metalloprotease and PMSF is known to sulphonate the essential serine residue in the active site of the protease, resulting in a total loss of

enzyme activity [13]. Results in table 7 showed that PMSF had no effect on the enzyme activity, but EDTA blocked the proteolytic activity. NPRC10 retained 93.36% of its activity after 1 h of incubation with 1 mM PMSF and was completely inhibited by 1 mM EDTA. This inhibition profile suggested that the NPRC10 belongs to the family of metalloproteases.

Table 7. Effect of inhibitors on the extracellular NPRC10 activity

Inhibitors	Residual activity (%)
Control	100.0
EDTA (0.1 mM)	14.0
EDTA (0.5 mM)	3.8
EDTA (1.0 mM)	0.0
PMSF (0.1 mM)	90.1
PMSF (0.5 mM)	92.3
PMSF (1.0 mM)	93.4

Partial purification of protease

The culture filtrate was initially purified by ammonium sulfate precipitation and dialysis. Purification factors and recoveries at each step were summaries in table 8. About 93.73% protease recovery was achieved with 1.28-fold purification (425.43 unit/mg) after precipitated by ammonium sulfate. Protease activity was observed in the fraction of dialysis with 1.41-fold purification (468.65 unit/mg).

Table 8. Initial purification of the extracellular NPRC10

NPRC10	Total activity (unit/ml)	Specific activity (unit/mg)	Purification factor	Recovery (%)
Culture filtrate	154.95	331.97	1.00	100.00
Ammonium sulfate precipitation	145.24	425.43	1.28	93.73
Dialysis	141.07	468.65	1.41	91.04

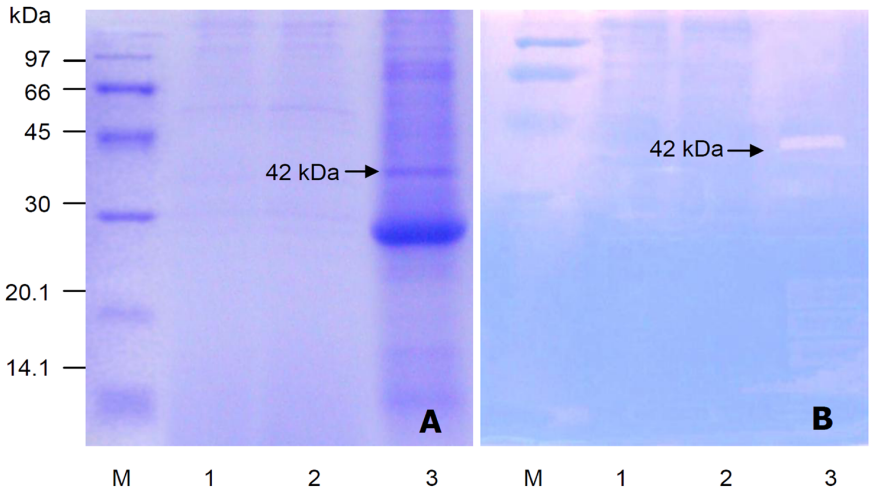

Figure 3. SDS-PAGE (12%) without (A) and with 0.1% casein (B). M: protein weight marker (97-14.4 kDa), lane 1: protein of IPTG-induced *E. coli* cells without pET200D/TOPO, lane 2: protein of IPTG-non-induced *E. coli* cells with pET200D/TOPO, lane 3: protein of IPTG-induced *E. coli* cells with pET200D/TOPO after ammonium precipitation and dialysis. Arrow showed a 42 kDa enzyme (NPRC10) expressed in the recombinant *E. coli* BL21 (DE3) cells.

The NPRC10 activity was also confirmed by SDS-PAGE with substrate. The molecular weight of the enzyme was estimated to be approximately 42 kDa (Fig 3). Thus, a 42 kDa band corresponds to mature peptide (33 kDa) plus peptidase-M4C region (9 kDa) [26].

CONCLUSION

The conditions of cell culture and induction obviously affected on the secretory level of extracellular protease from *E. coli* [12], [16], [20]. In this study, the expression level of recombinant NPRC10 was greatly improved when a relatively high-density culture was effectively induced at a suitable temperature. The NPRC10 from recombinant *E. coli* BL21 (DE3) cells was secreted and functioned in free-cell medium. Therefore, its application can be further exploited for large-scale production.

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