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Characterization and stability of proteases from *Penicillium* sp. produced by solid-state fermentation

Sandro Germano [a,b], Ashok Pandey [c], Clarice A. Osaku [d], Saul N. Rocha [a], Carlos R. Soccol [a,*]

[a] *Laboratório de Processos Biotecnológicos, Departamento de Engenharia Química, Universidade Federal do Paraná (UFPR), 81531-970 Curitiba, PR, Brazil*
[b] *TUIUTI, Facudade de Farmácia and FEPAR, Faculdade Evangélica do Paraná, Universidade Tuiuti do Paraná, Curitiba, PR, Brazil*
[c] *Biotechnology Division, Regional Research Laboratory, CSIR, Trivandrum 695019, India*
[d] *UNIOESTE, Faculdade de Farmácia, Cascavel, PR, Brazil*

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Abstract

Investigations were carried out to characterize the protease produced by a wild strain of *Penicillium* sp. in solid-state fermentation (SSF). Defatted soybean cake was used as carbon and nitrogen source and solid matrix for SSF. The enzyme was produced at 28 °C using defatted soybean cake supplemented with 0.2 mol/l citrate–phosphate buffer and with an initial pH and substrate moisture of 5.0 and 55% (w/w), respectively. Optimum temperature for enzyme activity in the crude extract was 45 °C at a slightly acidic pH (6.5). The studies on pH stability showed that the enzyme was stable in a range of pH 6.0–9.0 and the effect of the inhibitors showed it to be possibly a serine protease. Stability studies revealed temperatures around 35–45 °C. The activity was reduced in the presence of Co^{2+}, Mg^{2+} and Zn^{2+} ions, while the presence of Ca^{2+} and Na^{+} resulted in a discreet increase in proteolytic activity. The enzyme presented good stability towards oxidizing agent. The crude enzyme preparation was compatible with commercial detergents, retaining their 50–60% activities.

The results demonstrated the importance of solid-state fermentation for the production of protease using defatted soybean cake as substrate, which offer significance benefit due to cheaper cost and abundant availability.

Keywords: Protease; Filamentous fungi; *Penicillium* sp.; Solid-state fermentation; Defatted soybean cake

1. Introduction

Proteolytic enzymes account for nearly 60% of the industrial enzyme market and are widely used in food industry for cheese ripening, meat tendering, the production of protein hydrolysate and bread making [1]. The use of proteases as detergent additives in the 1960s stimulated their commercial development and led to a considerable expansion of fundamental research into these enzymes [2,3]. Since then, there has been renewed interest in the discovery of proteases with novel properties. Fungi as enzymes producers have many advantages, considering that the produced enzymes are normally extra-cellular, making easier its recuperation from the fermentation broth [19]. Besides, the use of fungi as enzyme producer is safer than the use of bacteria, since they are normally recognised as GRAS (generally regarded as safe) [4,5].

As part of this search for new enzymes, some workers have turned their attention to alternative production systems, such as solid-state fermentation (SSF). SSF systems are in many ways advantageous over submerged liquid fermentation systems for production of fungal enzymes [6–13]. SSF processes are usually simpler and can use wastes or agro-industrial substrates, such as defatted soybean cake, wheat and rice bran for enzyme production. The minimal amount of water allows the production of metabolites in a more concentrated form, making the downstream processing less time consuming and less expensive [8,12]. In addition, the conditions in SSF, especially the low moisture content in the system, lead to several potential advantages for the production of fungal enzymes. Firstly, these conditions favour the growth of filamentous fungi, which typically grow in nature on solid substrates, such as pieces of wood,

* Corresponding author. Tel.: +55-41-361-3191; fax: +55-41-266-0222.
E-mail address: soccol@ufpr.br (C.R. Soccol).

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plants leaves and roots and other organic natural materials [7,11]. Secondly, the low moisture content can minimize problems with bacterial contamination during the fermentation. Finally, the environmental conditions in solid-state fermentation can stimulate the microbe to produce enzymes with different properties than those of enzymes produced by the same organism under the conditions experienced in submerged fermentation [9,10,12].

Previously we reported the production of a proteolytic enzyme from a newly isolated strain of *Penicillium* sp. in our laboratory, which appeared to be good proteolytic enzyme producer [6]. The objective of this study was to investigate the characteristics of the protease produced by *Penicillium* sp. in solid-state fermentation.

2. Materials and methods

2.1. Microorganism and inoculum

A fungal strain of *Penicillium* sp. LPB-5, isolated from defatted soybean cake was used in this study. The culture was maintained on potato–dextrose–agar (PDA) plates and stored at 4 °C. Inoculum was prepared on PDA medium, which was sterilised (121 °C, 20 min). After cooling, 10 ml were poured into Petri dishes, which after cooling were inoculated using an actively growing culture of *Penicillium* sp. and incubated at 28 °C for 6 days. Spores were suspended in 10 ml of a Tween-80 solution (0.1%) by adding this to the dishes. The spores were counted in a Malassez chamber.

2.2. Chemicals

Azocasein was purchased from Sigma (St. Louis, MO, USA). Culture media components were obtained from DIFCO. All the chemicals were of analytical grade and used without further purification. Defatted soybean cake used as substrate was obtained from a local soy oil industry (Paranaguá, PR, Brazil).

2.3. Substrate preparation

Defatted soybean cake was washed thoroughly with distilled water and dried in a hot air oven at 60 °C for 24 h. The dry materials was then ground in a grinder and sieved to obtain the fraction retained between 0.8 and 1.2 mm sieves.

2.4. Solid-state fermentation

SSF was performed in 250-ml Erlenmeyer flasks by taking the moist substrate (55%) with citrate–phosphate buffer containing (g/l): KNO_3—2.0, $MgSO_4{\cdot}7H_2O$—0.5, K_2HPO_4—1.0, $ZnSO_4{\cdot}7H_2O$—0.439, $FeSO_4{\cdot}7H_2O$—1.116, $MnSO_4{\cdot}7H_2O$—0.203, pH 5.0. After autoclaving (121 °C, 20 min) the substrate was inoculated with a spore suspension (10^8 spores/g dry substrate) and cultivated at 28 °C for 48 h [6].

2.5. Enzyme extraction

The crude enzyme extract was obtained by adding 50 ml of 1% NaCl solution to the fermented matter. The flasks were mixed for 20 min at room temperature (28 °C). Solids were removed firstly by filtering through muslin cloth and then by centrifuging at 5000 rpm for 5 min (CR21E, Hitachi, Japan). The supernatant so obtained was used as crude enzyme extract.

2.6. Protease activity measurement

The protease activity in the crude extract was measured using sulphanilamide azocasein substrate according to the method of Leighton et al. [14], in initial conditions for the test (37 °C, buffer phosphate 0.2 M, pH 7.2). The assay was optimized and carried out at 45 °C with 250 μl 1% (w/v) azocasein in 0.2 M citrate–phosphate buffer, pH 6.5. The reaction was started by the addition of 150 μl of the crude extract (dialyzed in water for 12 h at 4 °C). After incubation for 1 h, the enzyme was inactivated by addition of 1.2 ml trichloroacetic solution (10%, v/v) and the solution was neutralized using a NaOH 1 M solution. The absorbance was read against a blank of the inactivated crude extract (100 °C for 10 min) at 440 nm. One unit of proteolytic enzyme activity was defined as the amount of enzyme that produced absorbance difference during 1-h incubation at 45 °C for millilitre of solution of crude extract.

2.7. Protein determination

The protein concentration of the samples was determined by Bradford method [15]. BSA was used as a standard.

2.8. Effect of protease inhibitors

The effect of protease inhibitors was investigated following the manufacturer's guide of the inhibitors. The inhibitors used were 3 mM phenyl-methanosulfonatesodium; 1.0 mM *trans*-epoxysuccinyl-L-leucylamido (4-guanidino)-butane (E-64); 5.0 mM, *p*-chloromercuribenzoate (pCMB); 5.0 mM ethylenediaminetetraacetic acid (EDTA); 5 mM ethyleneglicol-bis(β-aminoethyleter) *N*′,*N*′-tetraacetic acid (EGTA); 1.3 mM pepstatin A. After the crude extract incubation at 24 °C for 15 min with the inhibitors, the protease activity was determined as above.

2.9. Determination of the kinetic parameters and stability of the enzyme

2.9.1. Effect of substrate

Three different substrates were chosen for determination of the specificity (azocoll, azoalbumin and azocasein) at 1%

concentration in phosphate buffer 0.2 M (pH 6.5) at 45 °C (optimized conditions). Apparent K_m value was determined using azocasein at different concentrations (0–25 mg/ml) of the substrate and at different times (0–60 min). ENZIFIT programme was used for analysing the results.

2.9.2. Effect of pH on the activity and stability of the enzyme

The crude extract was diluted (1:10) in four different 0.2 M buffers: sodium–phosphate (pH 5.7, 6.0, 6.5, 7.0, 7.2, 8.0), citrate–phosphate (pH 5.0, 5.7, 6.0, 6.5, 7.0), sodium bicarbonate (pH 9.1, 10.2) and Tris–HCl (pH 7.2). For the stability experiments, the crude extract was incubated in initial conditions for 1 h at 37 °C and the residual proteolytic activity was determined according to the standard conditions cited above. The buffers used in this study were: acetate (pH 4.0, 4.5, 4.8, 5.0), glycine–HCl (3.2), phosphate (pH 6.0, 7.0, 8.0), citrate (pH 3.2, 4.0, 4.5, 4.8, 5.0, 5.8), glycine–NaOH (10.6, 12.0).

2.9.3. Effect of temperature on the activity and stability

For analysing the effect of temperature, the crude extract was incubated for 1 h at different temperatures using azocasein in phosphate buffer (0.2 M, pH 7.2). After the incubation, the crude extract of the enzymes was cooled and protease activity was determined.

The enzyme's thermal stability was assayed by incubating the crude extract at different temperatures in the range of 0–70 °C at different times using phosphate buffer (0.2 M, pH 6.5). To analyse the relationship between the temperature and pH, a 3^{2-0} experimental design was adopted using the computer program "Statistic". The values were pH 6.0, 7.0 and 8.0 (0.2 M phosphate buffer) and temperature (42, 45 and 48 °C).

2.9.4. Effect of ions and oxidizing agents

The crude extract was incubated with different ionic solutions: $CaCl_2$, $MnSO_4$, $ZnSO_4$, $CoCl_2$ and NaCl at 0.1 mM.

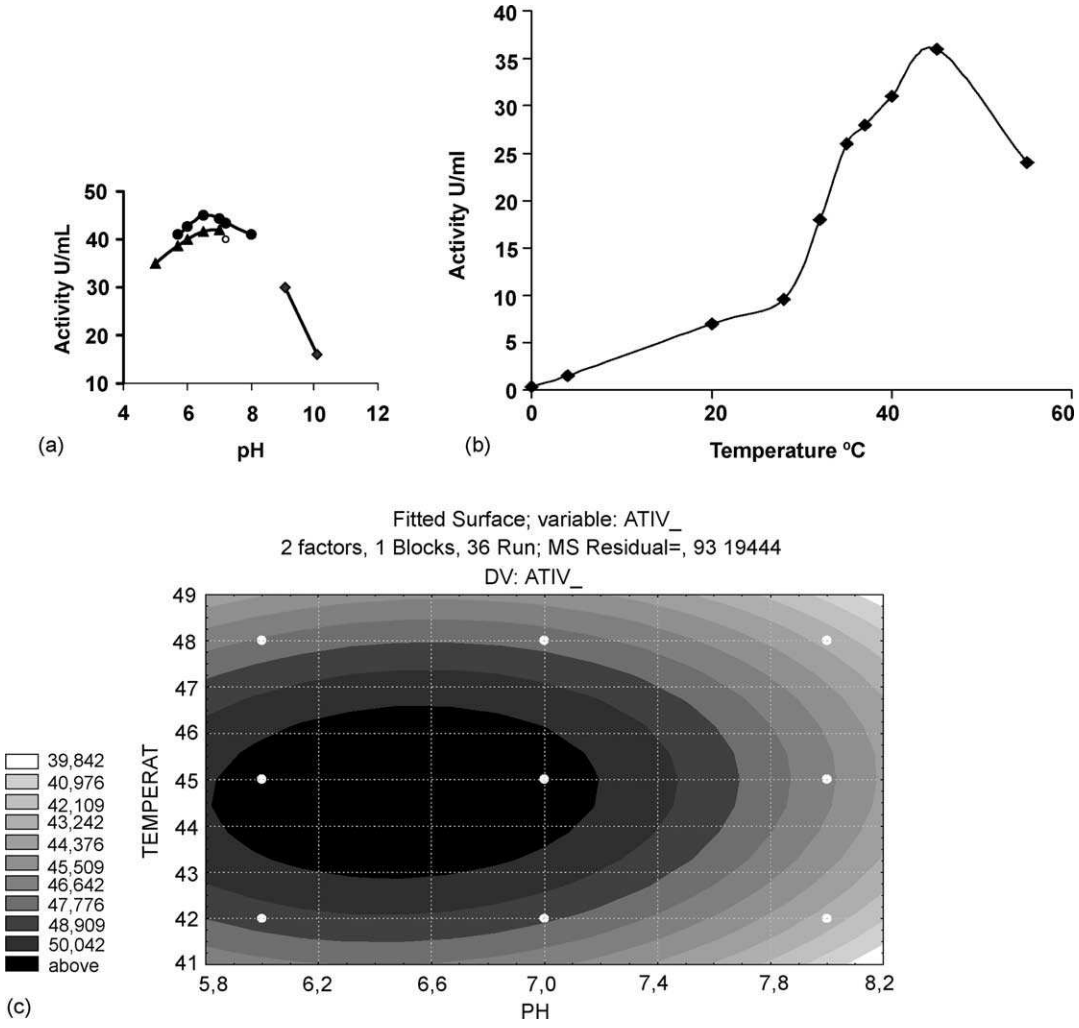

Fig. 1. Effect of (a) pH and (b) temperature on activity of the protease produced by *Penicillium* sp. in solid-state fermentation. Assay conditions: azocasein as the substrate, 37 °C. Buffers: (●) sodium–phosphate (5.7, 6, 6.5, 7, 7.2, 8); (▲) citrate–phosphate (5, 5.7, 6, 6.5, 7); (◆) sodium bicarbonate (9.1, 10.2); (○) control: Tris–HCl (7.2). Temperatures: 0–60 °C. (c) Interaction between the pH and temperature to result the proteolytic activity.

For the study on oxidizing agents, H_2O_2 (20%, v/v) was employed in different concentrations (5, 10 and 15%, v/v of commercially obtained product). Residual proteolytic activity was determined according to the standard conditions cited above.

2.9.5. Compatibility studies with commercial detergents

Two commercial detergents were used in this experiment OMO® Máquina (A) and OMO® Multi-Ação (B). The detergents were diluted in water and the final concentration was 7 mg/ml. The enzyme present in detergent was inactivated by heating at 100 °C for 10 min. After this, the crude enzyme extract was added (0.15 mg/ml) in solution and incubated at 28 °C for 1 h with constant agitation using a magnetic mixer. Aliquots were taken at different time intervals and residual enzyme activity was measured.

3. Results and discussion

The crude extract in salt solution (NaCl 1%) of the protease *Penicillium* sp. showed high activities in pH range from 6.0 to 9.0 and pH 6.5 was found as the optimum, which showed highest activity (43 U/m; Fig. 1a). The best temperature was 45 °C (Fig. 1b). This was supported by the results shown in Fig. 1c showing interactive impact of pH and temperature. Regarding the effect of the buffers themselves, differences were observed in the activity with different buffers. Based on the results on inhibition by PMSF, we found this protease of *Penicillium* sp. as a possible serine protease (Table 1). There is some overlap between this mechanistically classification of proteases and that based on pH dependence [16–18].

The results showed the azocasein, a globular protein, resulted maximum protease production by the *Penicillium* sp. The results showed what appeared to be a fairly typical curve for Michaelis–Menten kinetics using azocasein as substrate. However, when the double reciprocal plot was drawn, it could be seen that the kinetics deviated from Michaelis–Menten kinetics at high substrate concentrations. The maximum activity obtained in the assays was lower than that which was expected based on the activities at lower substrate concentrations (i.e. 0–25 mg/ml azocasein).

Table 1
Effect of various inhibitors on *Penicillium* sp. protease

Inhibitor	Inhibition (%)
Control	0
PMSF (3 mM)	93
E-64 (1 mM)	16
EDTA (5 mM)	23
EGTA (5 mM)	20
PCMB (1 mM)	37
Pepstatin (1.3 mM)	1.6

Conditions of assay—temperature: 45 °C and pH 6.5.

Table 2
Effect of metallic ions (0.1 mM) on enzymatic activity

Cation	Activity (U/ml)
Control	51.6
Mg^{2+}	22.7
Na^{+}	53.0
Ca^{2+}	55.0
Co^{2+}	37.4
Zn^{2+}	2.2

Conditions of assay—temperature: 45 °C and pH 6.5.

The apparent K_m value obtained from this analysis was 2.6 mg/ml.

3.1. Effect of metallic ions on enzyme activity

The crude extract was incubated with different solution of salts (final concentration of 0.1 mM) at 28 °C for 1 h. Results as recorded in Table 2 showed that the activity was reduced in presence of Co^{2+}, Mg^{2+} and Zn^{2+} ions, while the presence of Ca^{2+} and Na^{+} resulted in discreet increase of proteolytic activity.

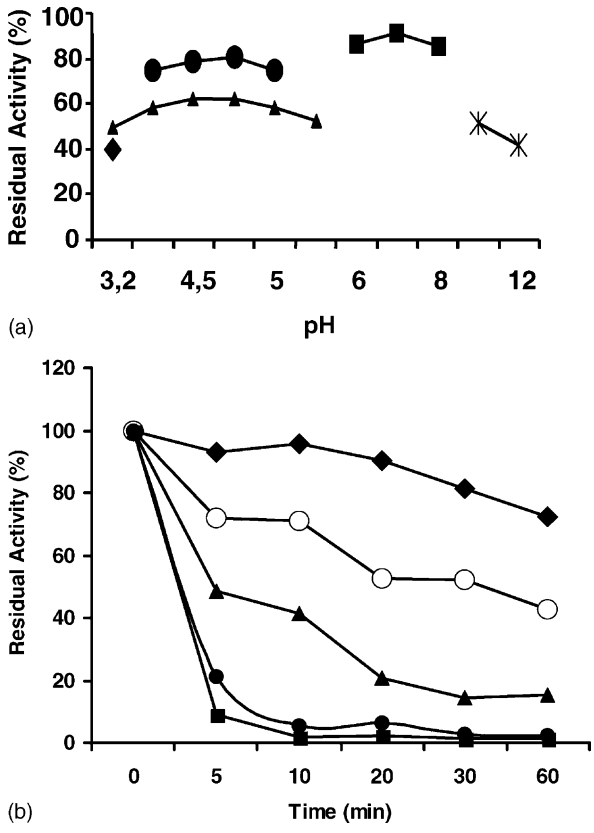

Fig. 2. Effect of (a) pH on the stability of the protease produced by *Penicillium* sp. in solid-state fermentation. Assay conditions: azocasein as the substrate, 37 °C. Buffers: (●) acetate (pH 4.0, 4.5, 4.8, 5.0), (◆) glycine–HCl (pH 3.2), (■) phosphate (pH 6.0, 7.0, 8.0), (▲) citrate (pH 3.2, 4.0, 4.5, 4.8, 5.0, 5.8), (∗) glycine–NaOH (pH 10.6, 12.0). (b) Effect of temperature on the stability of the enzyme. Temperatures: (◆) 35 °C, (○) 45 °C, (▲) 50 °C, (●) 60 °C, (■) 70 °C.

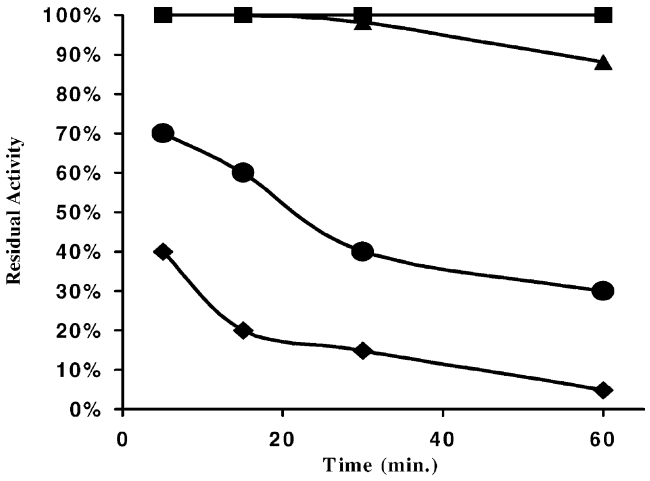

Fig. 3. Effect of one oxidant agent (H_2O_2) in the activity of the enzyme. Concentrations: (■) none (control); (▲) 5%; (●) 10%; (◆) 15%.

3.2. Effect of pH and temperature on stability of the enzyme

Fig. 2a showed that the shape of the curve of pH stability was similar with that of the optimal pH values for the highest activity, as expected. The enzyme was quite stable when incubated in the range of pH from acidic to basic, which could be associated with the effect of the incubation conditions (24 h, 4 °C). The results showed that the enzyme was quite stable at pH 6.0–8.0.

Fig. 2b showed that at temperatures higher than 50 °C, the residual activity was decreased significantly, but at 35 °C, the residual activity was maintained nearly 90%. At 45 °C, the enzyme retained 60% of its activity. At 28 °C, 100% activity was retained after 3 days and at the temperature of 4 °C, 98% of the residual activity remained for 30 days.

3.3. Studies about the interaction with oxidants agents and compatibility with commercial detergents

Fig. 3 shows the stability of the crude extract in presence of hydrogen peroxide. The results obtained showed good stability in peroxide solution in a concentration of 5% (v/v) for 1 h of incubation and at a temperature of 28 °C, retaining 80% of its activity. At high concentrations, a decrease in activity was observed. Commercial detergents generally contain 10% (v/v) hydrogen peroxide [20].

In the studies on compatibility with commercial detergents (Fig. 4), two of these were tested, showing compatibility with both. The results obtained in these studies were compatible with the results showed with an alkaline protease of *Conidiobolus coranatus* [3].

4. Conclusions

Partial characterisation of the crude extract, without any purification, suggested the presence of a neutral serine proteases produced by *Penicillium* sp. because it was inhibited in about 93% of its activity by PMSF and the crude extract showed that the optimum pH was 6.5 in a 0.2 M buffer, the optimum temperature was 45 °C. It was stable in the pH range from 6.0 to 9.0. With respect to temperature, the studies revealed that the enzyme was quite stable in the temperatures of 35 and 45 °C. The activity increased in the presence of metallic ions such as Ca^{2+} and Na^{+}. Enzyme produced was compatible with commercial detergents and oxidants agents.

The results presented in this study suggested the possibility to produced this enzyme by solid-state fermentation using a cheaper substrate (defatted soybean cake) and its industrially usage with commercial detergents.

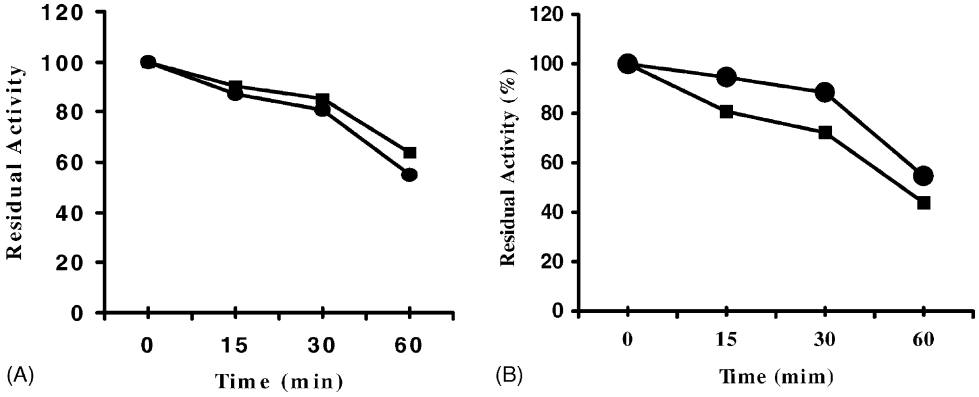

Fig. 4. (A) Graph that shows the compatibility of the crude extract with one type of commercial detergent; (●) shows the residual activity of the pure crude extract and (■) shows the residual activity of the enzyme with the commercial detergent A in solution. (B) The same relation with the commercial detergent B. Condition of assay: temperature 28 °C, detergent in concentration of 7 mg/ml (0.05 mg/ml protein) and crude extract in final concentration of protein 0.15 mg/ml.

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