PROTEASE-PRODUCING MICROORGANISMS INHABITING SALTED FISH (MOLOHA) WITH SPECIAL REFERENCE TO PROTEASE ACTIVITY OF *BACILLUS SUBTILIS*

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ABSTRACT

The investigation was designed to isolate and identify the proteolytic microorganisms inhabiting salted fish. *Bacillus subtilis* was chosen as the most promising protease producer. Some properties of the crude protease are presented, the effect of metal ions on protease production has been studied. It was shown that Ca^{2+} and Mg^{2+} stimulated, while Co^{2+} , Zn^{2+} and Cu^{2+} inhibited the enzyme production. The effect of temperature and pH and salt tolerance have also been studied. Protease activity was stable in 25% NaCl. The favourable characteristics of the enzyme might have extensive application in laundry detergents and in tanning industry.

KEY WORDS: bacteria, fungi, extracellular proteases, *Bacillus subtilis*, proteolytic activity.

INTRODUCTION

Twenty-five isolates of protease-producing bacteria and fungi were isolated from salted fish and their proteolytic activity was determined after 4 days cultivation at 37°C on media containing 10% NaCl. Eight isolates belonged to *Bacillus subtilis* and two of them were recognized as *Bacillus licheniformis*. Fifteen isolates of fungi of two different genera comprised five species, among them three isolates of *Penicillium chrysogenum*, two isolates of *P. nigricans*, five isolates of *P. brevicompactum*, two isolates of *P. citrinum* and three isolates of *Aspergillus terreus*. One isolate of *Bacillus subtilis* was chosen as the most promising protease producer. Optimal conditions for enzyme production by *B. subtilis* A05 were pH 8.0, 37°C, 4% (w/v) starch as carbon source, 2% (w/v) casein as nitrogen source and after 24 h incubation. Ca^{2+} and Mg^{2+} stimulated enzyme synthesis. Maximal activity of the crude enzyme was at pH 8 and the optimal temperature was 60°C. The best substrate for enzyme activity was casein at 0.5 % (w/v). The protease retained 63% of its original activity after 40 min at 80°C. Protease was stable in NaCl with 62% of the original activity being retained in 25% (W/V) Na Cl.

Moloha is one of the best known salt-cured fish products in Egypt. It is traditionally made by mixing fish and sodium chloride in the ratio of 3:1 (W/W) and allowing the mixture to naturally ferment for 250 days. The fish product is hydrolysed by joint action of fish and microbial proteases.

Eleven species of bacteria, one of yeast and three of filamentous fungi were found to occur in salted fish (Crisan and Sands 1975, Ho et al. 1984, Zuberi et al. 1988). These microbes are responsible for considerable deteriorative changes in fishes including the loss in essential chemical constituents and proteins.

The present investigation was designed to isolate and identify the proteolytic microorganisms inhabiting salted fish. Some properties of the crude protease were also presented.

MATERIALS AND METHODS

Isolation and identification of protease-producing microbes

Five gram samples of Moloha were blended with 45 ml 10% (W/V) NaCl and after thorough mixing, diluted serially in 10% NaCl. The samples were spread onto nutrient agar containing (g/l): Tryptone, 10; yeast extract, 5; NaCl, 100, casein, 5; Agar, 15. The initial pH of the medium was adjusted to 7.3. After 4 days at 37°C, all colonies exhibiting clear haloes were isolated. The bacteria and fungi were identified according to Bergey's Manual of Systematic Bacteriology (Seneath et al. 1986) and to the key of *Penicillium* and *Aspergillus* (Pitt 1985, Raper and Fennell 1965), respectively.

Cultivation for enzyme production

The medium for liquid cultures consisted of (g/l): Tryptone, 10; yeast extract, 5; NaCl, 50; with the pH adjusted to 7.3 with 0.1M NaOH. The cultivation was carried out in 250 ml Erlenmeyer flasks containing 50 ml of sterile medium which contained various other substrates, as sources of carbon or nitrogen. Various metal ions were also added as indicated and various pH medium were used. The media were inoculated with, 1 ml of spore suspension (5×10^6). After 4 days incubation the cultures were taken, centrifuged (8000 X g, 10 min) and the supernatant was passed through a 0.45 µm filter to produce a cell-free enzyme solution.

Protease assay

The protease activity was measured by using a reaction mixture of 2 ml containing 10 mg of Hammarsten casein, 7.5 µmol $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.2, and an enzyme source. The mixture was incubated for 60 min at 37°C and then the reaction was stopped by adding 3 ml of 5% trichloroacetic acid and the acid soluble material was estimated at 280 nm after removing the precipitate by filtration through Whatman No. 1 filter paper (Hagihara et al. 1958). A blank was

prepared by adding 3 ml of precipitating agent to 2 ml of the casein solution before the addition of the enzyme solution. One protease unit is defined as the amount of enzyme which yields the equivalent of 1 μ g of tyrosine per min at 280 nm under control conditions. Protein concentration in enzyme solution was determined by the method of Lowry et al. (1951).

Substrate specificity of protease

Substrate specificity of crude enzyme was determined by using gelatine, bovine serum albumin, egg albumin and casein at 0.5 (w/v).

Effect of pH and temperature on enzyme activity

The protease activity was measured over pH range of 5.0 to 9.0. The buffer systems used for assay were: 50 mM sodium acetate/acetic acid for pH 5.0, 50 mM sodium potassium phosphate for pH 6.0 to 7.0 and 50 mM Tris/HCl for pH 8 to 9. The enzyme activity was performed at 35, 40, 50, 55, 60, 65, 70, 75 and 80°C in a thermostatic water bath.

pH stability

The stability of the protease preparation was studied in buffer solution of various pH. The mixture was incubated for 24 h at 4°C. The residual activity was measured at optimum conditions.

TABLE 1. Proteolytic enzyme activity in culture filtrates of different microbes recovered in salted fish.

Organism	Total protein	Total activity	specific activity
	(mg/ml)	(U/ml)	(U/mg protein)
<i>Bacillus subtilis</i>			
A01	0.21	6	28.6
A02	0.25	7	28
A03	0.23	8	34.8
A04	0.31	12	38.7
A05	0.30	13	43.3
A06	0.27	10	37.0
A07	0.23	8	34.8
A08	0.22	9	40.9
<i>Bacillus licheniformis</i>			
A09	0.30	6	20
A010	0.35	5	14.3
<i>Penicillium nigricans</i>			
MS1	0.27	7	25.9
MS2	0.32	9	28.1
<i>P. chrysogenum</i>			
MS3	0.25	6	24
MS4	0.34	9	26.4
MS5	0.36	8	22.2
<i>P. brevicompactum</i>			
MS6	0.41	10	24.4
MS7	0.37	11	29.7
MS8	0.25	8	32
MS9	0.38	7	18.4
MS10	0.31	6	19.4
<i>P. citrinum</i>			
MS11	0.36	8	22.2
MS12	0.29	6	20.7
<i>Aspergillus terreus</i>			
MS13	0.28	4	14.3
MS14	0.23	5	21.7
MS15	0.26	6	23.0

Thermal stability

The crude enzyme was incubated at various temperatures for 40 min, then immediately cooled in ice and its activity was assayed.

Salt tolerance test

The enzyme was incubated at 5.0 mM Tris/HCl buffer, pH 8, containing various NaCl concentrations for 24 h and residual activity was determined.

RESULTS AND DISCUSSION

Isolation and identification

Twenty-five isolates of protease-producing bacteria and fungi were isolated from salted fish. Ten isolates were identified by standard biochemical and bacteriological test as *Bacillus* strains. Eight strains were identified as *Bacillus subtilis* and two strains were identified as *Bacillus licheniformis*. Fifteen isolates of fungi including two different genera with five different species were identified as *Penicillium nigricans*, *P. chrysogenum*, *P. brevicompactum*, *P. citrinum* and *Aspergillus terreus* (Table 1). Eleven species of bacteria belonging to genus *Bacillus*, three of filamentous fungi and one of yeast were found in salted fish sauce (Crisan and Sands, 1975; Zuberi et al. 1988).

Protease activity in culture filtrates of 25 isolates were determined (Table 1). *Bacillus subtilis* A05 was the best producer and its activity (13 U/ml) was greater than that reported previously for *Bacillus subtilis* MT-2 (Fujii et al. 1983).

Our results have shown that the isolated microbes were capable of producing extracellular proteases. The rate of production varied among the species. Protease production seemed to be related to the loss in protein of salted fish, leading to deterioration and spoilage of the products.

Protease production by *Bacillus subtilis* A05

The cultural conditions were examined to obtain the highest production of proteolytic enzyme and the optimal conditions. The optimum pH, temperature and cultivation time for the maximum production of the enzyme was 8, 37°C and 24 h, in accord with the results of Loginova et al. (1980) and Disler (1982). However, Kang et al. (1987) reported maximal production of proteinase by *Saccharomycopsis* (*Yarrowia*) lipolytic at the initial pH of the medium 9.0.

Effect of carbon source

Utilization of starch at different levels (0.5 to 5%, w/v) were evaluated and a concentration of 4% found to be best for synthesis of enzyme. Substitution of starch in the basal medium by glucose, fructose or sucrose resulted in a decrease in enzyme production. Starch has been found to stimulate the production of proteolytic enzymes from several microbes (Novikova et al. 1986, Valdimirova and Kornienko 1987).

Effect of nitrogen source

Among the concentrations of the various organic nitrogen sources tested, 2% casein or 4% soybean flour, when added to the basal medium, stimulated enzyme production. Hydrolysed proteins, such as Bacto-peptone, Tryptone and casamino acid, did not exhibit any enhancing effect. Thus *Bacillus subtilis* A05 requires a complex nitrogen source with large molecular weight protein fragments for stimulation of enzyme production. Similarly, an exogenously supplied protein was found to increase production of proteinase by *Neurospora crassa*

(Drucker 1972, Cohen et al. 1975) fivefold when added to the medium. The role of proteins, or their large molecular weight fragments, in stimulating proteinase production was also demonstrated in *Erwinia chrysanthemi*. (Wandersman et al. 1986) and *Aspergillus oryzae* (Fukushima et al. 1989).

Effect of metal ions

The effect of addition of metal ions to culture medium was examined. The results obtained are shown in Table 2. Ca^{2+} , Mg^{2+} stimulated enzyme production, Co^{2+} , Zn^{2+} , Cu^{2+} completely stopped the growth and Fe^{2+} and Mn^{2+} decreased the enzyme activity.

TABLE 2. Effect of metal ions on protease production by *Bacillus subtilis* A05.

Metal ion concentration (0.1% W/V)	Protease activity of 24-h culture (U/ml)
Control (basal medium)	11.3
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	12.7
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	15.2
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	3.5
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	4.3
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0

Properties of protease

Optimum temperature for enzyme activity was 60°C (Fig. 1). The enzyme was stable at up to 75°C for 40 min, but at 80°C only 63% of the original activity was retained after this time.

The neutral proteases of *Bacillus stearothermophilus* and *Bacillus subtilis* retained about 80% of the initial activity after treatment at 65°C for 30 min (Fujii et al. 1983) and 20 min (Uehara et al. 1979), respectively. In terms of thermal stability, the enzyme is superior to proteolytic enzymes from *Ba-*

Fig. 1. Effect of temperature on protease activity (○) and stability (Δ).

cillus alvei (Novikova et al. 1986) and *Bacillus subtilis* (Treitskii et al. 1987).

Fig. 2. Effect of pH on protease activity (o) and stability (Δ).

Optimum pH of the enzyme was 8. The enzyme was stable over the pH range 5 to 9, but at more acidic or alkaline pHs it lost activity rapidly (Fig. 2). Most of *Bacillus* proteases were stable between pH 6 to 8 (Fujii et al. 1983). The best substrate for enzyme activity was casein followed by egg albumin, bovine serum albumin and gelatine at 0.5 % (w/v). Salt tolerance of the enzyme was monitored by incubating the enzyme solution in buffer containing various concentrations of NaCl up to 25% (W/V) for 24 h at 30°C and measuring the residual activity. Protease activity was stable in NaCl with 62% of the original activity being retained in 25% NaCl (Fig. 3). A literature survey has indicated that this is probably the first report on salt-tolerance proteases from *Bacillus subtilis*. The favourable characteristics of the enzyme could have extensive application in laundry detergents and in the tanning industries.

Fig. 3. Effect of NaCl on protease activity.

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MIKROORGANIZMY PROTEOLITYCZNE SOLONYCH RYB (MOLOHA)
ZE SZCZEGÓLNYM UWZGLĘDNIENIEM AKTYWNOŚCI PROTEOLITYCZNEJ *BACILLUS SUBTILIS*

STRESZCZENIE

Badania przeprowadzone na solonych rybach wykazały obecność w nich szeregu bakterii i grzybów wydzielających proteazy. Spośród zbadanych 25 gatunków bakterii i grzybów stwierdzono, że najwięcej proteaz pochodziło z *Bacillus subtilis*. Przebadano niektóre właściwości tych enzymów i wykazano, że są one odporne na działanie wysokich stężeń soli, a aktywność ich stymulowana jest przez jony Ca^{2+} i Mg^{2+} oraz hamowana przez Co^{2+} , Zn^{2+} i Cu^{2+} . Dane dotyczące tolerancji proteaz na zasolenie mogą mieć praktyczne zastosowanie.

SŁOWA KLUCZOWE: bakterie, grzyby, proteazy pozakomórkowe, *Bacillus subtilis*, aktywność proteolityczna.