Purification And Characterisation of Metalloproteases From *Lactococcus Lactis* Isolated From Traditional Fermented Algerian Food

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Abstract

An extracellular metalloproteases from lactococcus lactis isolated from traditional fermented Algerian food. The enzyme was purified to homogeneity by ultrafiltration, Sepharose SP ion exchange, Sephadex G-75 gel filtration and finally by a second Sephadex G-50 gel filtration at 46% recovery. The enzyme was estimated to be 44 kDa on SDS-PAGE. The enzyme exhibited maximal activity at pH 7 and 55°C.the activity was totally lost in the presence of 1M phenantroline, suggesting that the purified enzymes was metalloproease.
Key words: metalloprotease, lactococcus, fermented food, prurification

Introduction

Microbial proteases are important industrial enzymes among hydrolytic enzymes and account for over a half of the total enzyme sales in the world market. Many industrial microbial proteases are BEMPs, These proteases are widely used in the industries of food, medicine, brewing, leather, film, baking and some BEMPs for laboratory**.**

Many strains of lactic acid bacteria contain proteolytic systems that allow them to liberate essential and growth stimulatory amino acids and small peptides from the protein-rich substrates such as milk, meat and vegetables in which they are primarily found. Centuries of selection on these and other properties of lactic acid bacteria have resulted in the availability of strains very well suited for the production of fermented foods and feed.

Some metalloproteases function in the cell or on the membrane. Other metalloproteases that are secreted to the periplasm or outside the cell are called extracellular metalloproteases. Most of the bacterial extracellular proteases (BEMPs) that have been studied are various serine proteases and metalloproteases. Some BEMPs are important virulence factors of pathogenic bacteria (Goguen et al. 1995), and some BEMPs have been applied in biotechnology (Adekoya and Sylte 2009).

In recent years, a series of new BEMPs have been explored, and important progress has been made on the characterization and application of BEMPs.

To date, Some of lactococcal proteinases which have been characterized to various degrees (Westhoff et al, 1971; Ohmiya and Sato, 1975; Muset et al, 1989; Akuzawa et al, 1994; Akuzawa and Okitani, 1995) showed differences in general properties indicating an intracellular metalloprotease in lactococci. However, the data on the extracellular metalloproteases from lactococci are not signalled.

1. Bacterial Strain

The strain used in this study was isolated from an Algerian traditional fermented food.
Sample collected was plated into skim-milk agar plates. Plates were incubated 24 –48 h at 30°C. A clear zone of skim-milk hydrolysis gave an indication of protease-producing strains. Individual colonies were purified through repeated streaking on fresh agar plates. Among 08 strains, isolated in the laboratory and screened for proteolytic activity, BK2 strain was selected.
It was identified as *Lactococcocus lactis sub*sp. *Lactis* BK2 based on its morphological and physiological characteristics and by API 50 CHL and 16S rRNA sequence analysis. The nucleotide sequence of 16S rRNA has been submitted to the GenBank database and using BLAST program and assigned accession

2. Preparation of Cell Free Extracts

Cultivations were performed as follows : Purified strain BK2 was incubated an overnight in Elliker Broth at 30°C, 2,5ml of medium was added in 500ml flasks contains 250ml sterilized skim milk and incubated 48h at 30°C. The supernatants were collected by centrifugation10000 rpm at 4°C during 15 min. The supernatant (250ml) was used for purification and estimation proteinase activity. The growth of the microorganism was determined by measuring absorbance at 600 nm.

3. Proteinase Assay

The proteinase activity conducted in replicate assays was measured using the chromogenic substrate, azocasein, according to the modified method of Beynon (1992).
A portion (250 μl) of 2% (wt/vol) azocasein in 100 m*M* sodium phosphate buffer (pH 7.0) was mixed with 150 μl of enzyme in an Eppendorf tube. The mixture was incubated at 50°C for 1 h. The reaction was terminated by the addition of 1,2 ml of 10% (wt/vol) TCA solution and mixed thoroughly. After 15 min at room temperature, the mixture was centrifuged at 10000 *g* for 5 min. After, 1,2 ml of supernatant was added to 2,4 ml of 1.0 *M* NaOH, the absorbance was measured at 440 nm against a blank without enzyme. One unit of proteinase activity was defined as the amount of
the enzyme that results in an increase of 0,01 absorbance per hour at 440 nm.

4.Protein Determination

Protein concentration was determined by the method of Bradford using bovine serum albumin as a standard.

5.Protease Purification

5.1.Ultrafiltration

The supernatant (250 ml) was concentrated by ultrafiltration cell (Sartorius vivacell 250, 10000 MWCO).Thirty millilitres was recovered and checked for proteinase activity with azocasein as the substrate.

5.2.Sephadex G-75 Gel Filtration

The culture supernatant containing the extracellular enzyme were applied to gel filtration on a Sephadex G-75 column (2.5 cm x 90 cm) (Pharmacia-Amersham, Uppsala, Sweden) pre-equilibrated with 50mM Tris-Hcl pH 7 and eluted at a flow rate of 30 ml/h with the same buffer. Protein content (Abs 280 nm) and caseinolytic activity were measured (fig.1-a)

5.3.SP-Sepharose Cation-Exchange Chromatography

Fractions from the Sephadex G-75 showing protease activities were pooled and applied to a SP-Sepharose column (2,6 cm x 15 cm) (GE Healthcare Bio-Sciences AB) previously equilibrated with buffer (50 mM Tris-Hcl, pH 8). Proteins were eluted from three steps: Firstly with 100% 50mM Tris-Hcl pH 8 (Sol A) at flow rate 1ml/min; secondly with100 % 50mM Tris-Hcl + 1M Nacl at flow rate 2ml/min pH 8 Sol B) ; finally with 100% solution A. Fractions (4 ml) were collected and checked for their casein hydrolysis activity; those which were active were pooled and concentrated using a 10-kDa ultrafiltration cell (Sartorius vivacell 250) for further purification (fig1-b) .

5.4.Sephadex G-50 Gel Filtration

The last purification step was performed on a Sephadex G-50 column (Pharmacia-Amersham, Uppsala, Sweden) with 50 mM Bis-Tris buffer, pH 7 containing 0,15 M NaCl. The concentrated sample was applied to the column and eluted at a flow rate of 0. ml min21, and 0.6-ml fractions were collected. The column was calibrated with thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.35 kDa) (fig.1.c).

6. Biochemical Characterization of Proteinase.

6.1. Effect of Temperature On Activity and Stability of the Enzyme

Enzyme activity was assayed at different temperatures ranging from 20 to 70°C, using azocasein as a substrate. The assay was conducted at pH 7.0 for 1 h. For thermal stability, the enzyme was incubated at different temperatures for 60 min. The residual activity was assayed using azocasein as a substrate at pH 7.0 and 50°C for 1 h. The non-heated enzyme was considered as the control (100%).

6.2. Effects of Enzyme Inhibitors

The effects of enzyme inhibitors on trypsin activity were studied using phenylmethylsulfonyl fluoride (PMSF), aprotinine, phenantroline, pepstatin A, β-mercaptoethanol, iodoacetamid and ethylene-diaminetetraacetic acid (EDTA). The purified enzyme was pre- incubated with inhibitors for 30 min at 50°C and then the remaining enzyme activity was estimated using azocasein as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as 100%.

6.3. Effect of Metal Ions

The effects of various metal ions (5 mM) on enzyme activity were investigated by adding monovalent (Na^+ and K^+) and di-trivalent metal ions (Ca^{2+} , Mn^{2+}, Zn^{2+}, Fe^{2+}, Cu^{2+}, Mg^{2+}, Al^{3+}) to the reaction mixture. The activity of the enzyme in the absence of any additives was taken as 100%.

7. Results and Discussion

7.1. Isolation and Identification of the Microorganism

The strain used in this study was isolated from traditional fermented food. Samples collected were plated onto skim-milk agar plates. Plates were incubated 24–48 h at 30°C. Clear halos around colonies on skim-milk agar gave an indication of protease producing strains. Individual colonies were purified through repeated streaking on fresh agar plates. Among more than 20 strains, isolated in the laboratory and screened for proteolytic activity, K2 strain was selected.

In order to identify K2 strain, the internal transcribed spacer region of the 16S rDNA (1484 bp) was amplified and sequenced. The nucleotide sequence was analyzed with the GenBank database using BLAST program and the isolate was identified as *Lactococccocus lactis sub*sp. *Lactis* .

7.2. Purification of K2 Krotease

Results of the purification are summarized in Table 1. The cell-free supernatant was concentrated by ultrafiltration. The specific activity at this step was ***104,9*** U/mg. This preparation was successively subjected to SP Sepharose ion exchange chromatography, Sephadex G-75 gel filtration, and finally to a gel filtration on Sephadex G-50. After the final purification step, the enzyme was purified six-fold with a recovery of 46% and a specific activity of *1561,34* U/mg of protein.

Table 1 : Summary of the purification of *Lactococccocus lactis sub*sp. *Lactis* K2 protease

Purification steps	**Volume (ml)**	**Total activity (U)**	**Total protein (mg)**	**Specific activity (U/mg)**	**Recovery (%)**	**Purity (fold)**
Crude extract	*50*	*4021*	*58,8*	*68,39*	*100*	*1*
Ultrafiltration	*50*	*3810*	*36,32*	*104,9*	*95*	*1,53*
SP Sepharose	*25*	*2441*	*12,68*	*192,54*	*64*	*1,84*
Sephadex G-75	*15*	*1390*	*3,5*	*397,7*	*57*	*2,07*
Sephadex G-50	*10*	*642*	*0,41*	*1561,34*	*46*	*3,93*

7.3. Characterization of the Proteolytic Enzymes.

a- Effect Of Ph on Protease Activity and Stability

The relative activity values at various pH from 5,0 to 11,0 are shown in Fig.2a. The enzyme was highly active between pH 6,0 and 8,0 with an optimum at pH 7,0 when incubated for 1h at 55°C. The relative activities at pH6,0 and 8,0 were about 97,43% and 95,83%, respectively. Activity decreased above pH 8,0 and was19,33% of the maximum activity at pH 10,0.

The pH stability profile of the purified enzyme showed that the enzyme is highly stable between pH 5,0 and 9,0 (Fig.2.b). The enzyme retained about 5,23% and 11,04% of its initial activity at pH 4.0 and 11.0, respectively.

b- Optimum Temperature and Thermostability

The K2 protease was active at temperatures from 30 to 70°C, with an optimum around 55°C. The relative activities at 50 and 60°C were about 92,18% and 79,38% respectively.
The thermal stability profile of the purified K2 protease showed that the enzyme was highly stable at temperatures below 50°C but was inactivated above 60°C (Fig.3.a and b). The enzyme retained 92,03 and 87,51% of its initial activity after 60 min of incubation between 20 and 50°C respectively, than above 50°C the residual activity shutdown.

c- Effect of Various Inhibitors on Protease Activity

The effect of a variety of enzyme inhibitors, such as chelating agent and specific group reagents on the activity of K2 protease was investigated. Aprotinine, PMSF, pepstatine,

iodoacetmid (That are specific for serine, aspartic and cysteine protease respectively) were practically without influence on the activity of the enzyme.
However, metalloenzyme inhibitor (1 mM Phenantroline) caused 100% inactivation of the enzyme and partially inhibited by the chelating agent (5 mM EDTA).This suggests that the enzyme is a metalloprotease (Table 2).

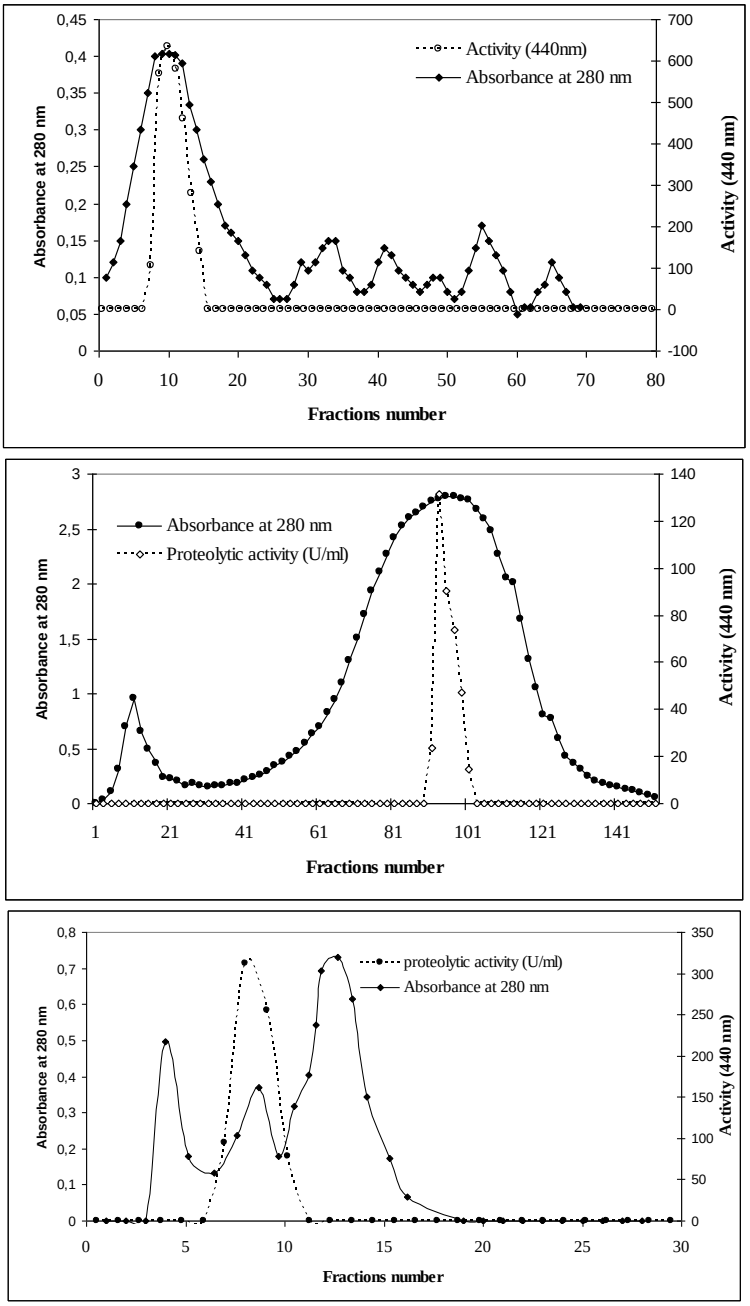

Fig.1 a- Purification profile of metalloprotease from L. lactis by gel filtration on Sephadex G-75 column. b- Purification profile of metalloprotease from L. lactis by ion exchange on SP Sepharose column c- Purification profile of metalloprotease from L. lactis by gel filtration on Sephadex G-50 column

Table2 : Effect of various enzyme inhibitors on the activity of the purified K2 protease from *lactococcus lactis subsp lactis*

Inhibitors	**Concentration (mM)**	**Residual activity (%)**
Control	**-**	**100**
EDTA	**5**	**68**

β-mercaptoethanol	**5**	**100**
Aprotinine	**5**	**100**
Phenantroline	**0,1** **1**	**11** **0**
Pepstatine	**5**	**100**
PMSF	**5**	**85**
Iodoacetamid	**5**	**100**

Purified enzyme was pre-incubated with various enzyme inhibitors for 1h at 25°C and the residual activity was determined at pH 7.0 and 40°C. Enzyme activity measured in the absence of any inhibitor was taken as 100%.

d- Effect of Metal Ions

The effect of some metal ions at a concentration of 5 mM on the activity of K2 protease L lactis sibsp lactis was studied at pH7.0 and 40°C by adding monovalent ions (Na^{+}, K^{+} and Cs^{+}) and divalent ions (Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+} ,Fe^{2+}, Mg^{2+}) and trivalent ion (Al^{3+}) to the reaction mixture (Table 3). Na+ showed no influence on the activity. The protease activity was slightly affected by K^{+}, Cs^{+}, Ca^{2+}, Mn^{2+}, Mg^{2+} and Al^{3+}. However, the addition of Cu^{2+}, Fe^{2+} and Zn^{2+} decreased the activity by 48,5%, 32% and 24,5%, respectively.

Table 3 Effect of various metal ions (5 mM) on K2 protease activity

Ions (5 mM)	**Relative activity (%)**
Na^{+}	100
K^{+}	95,77
Cs^{+}	92,78
Ca^{2+}	90,70
Zn^{+2}	75,55
Cu^{+2}	51,49
Mg^{+2}	99,08
Fe^{+2}	68,07
Mn^{+2}	88,49
Al^{+3}	91,45

The activity of the protease was determined by incubating the enzyme in the presence of various metal ions (5 mM) for 1h at 40°C and pH 7.0.

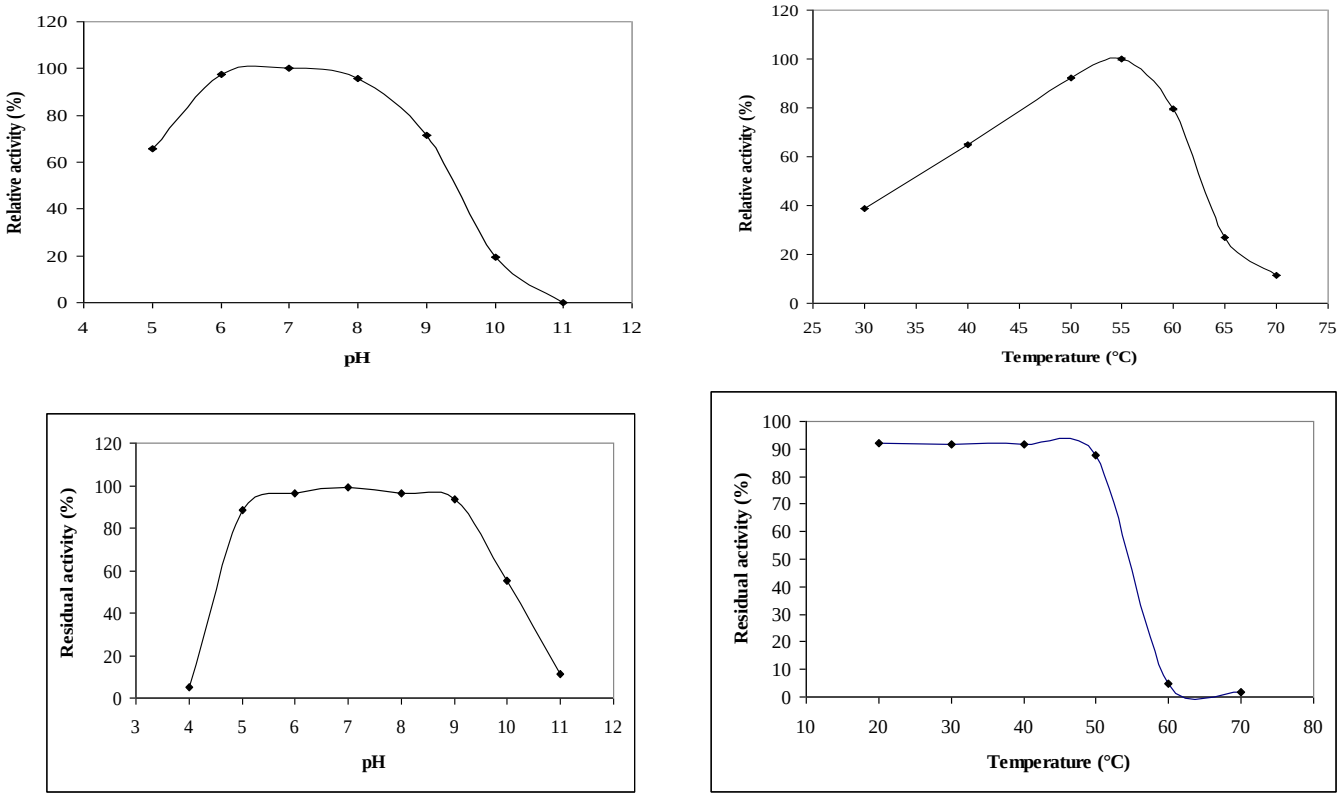

Fig. 2.a. Effect of pH on enzyme activity**. b.** Effect of pH on protease stability. **Fig.3.a.** . Effect of temperature on enzyme activity. **b.** Effect of temeperature on protease stability

8. Conclusion

In the present study, a K2 protease from L. lactis subsp lactis was purified and characterised. The purification to homogeneity of the K2 protease was achieved by ultrafiltration, gel filtration through Sephadex G-75, cation-exchange chromatography on SP-Sepharose columns and gel filtration through Sephadex G-50. After the final purification step, the enzyme was purified 3,93-fold with a specific activity of *1561,34* U/mg and 46% recovery. The molecular weight was of purified K2 protease was estimated to be 44,000 Da. The enzyme showed an optimum temperature at 55°C and optimum pH of 7.0. The enzyme was stable at a pH range of 5.0–9.0 and had a high thermostability, with 92,03 and 87,51% activity retained at 20 and 50°C respectively, after 60 min of incubation.

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