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RESEARCH ARTICLE

A novel serine metalloprotease from a newly isolated *Lactococcus lactis subsp. Lactis* BR16 : Purification and characterization

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Abstract

An extracellular metalloprotease producing strain was isolated from Algerian traditional fermented food and identified as *Lactococcocus lactis subsp. Lactis* BR16 on the basis of the16SrRNA gene sequencing with accession number AB618806.1 and biochemical properties. The BR16 metalloprotease was purified from the culture supernatant to homogeneity using ultrafiltration, SP-Sepharose ion exchange chromatography Sephadex G-50 gel filtration, with a 5.83-fold increase in specific activity and 47% recovery. The molecular weight of the purified enzyme was estimated to be 38 kDa by SDS-PAGE and gel filtration. The enzyme was highly active over a wide range of pH from 5.0 to 8.0, with an optimum at pH 6.5. The relative activities at pH 6.0 and 7.5 were about 94% and 90% of that obtained at pH 6.5. The enzyme was extremely stable in the pH range of 5.0-9.0. It exhibited maximal activity at 55 °C. The activity of the enzyme was totally lost in the presence of phenantroline, suggesting that the purified enzyme is a metalloprotease. Reversed-phase high performance liquid chromatography (RP-HPLC) of commercial casein digests showed a more-complex peptide pattern produced by the proteinase of *Lactococcus lactis subsp lactis* BR16.
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Introduction

The core lactic acid bacteria (LAB) genera Lactobacillus, Lactococcus, Leuconostoc, Pediococcus and Streptococcus share a long history of safe usage in the processing of fermented foods. The antimicrobial effects and safety of LAB in food preservation is widely accepted (De Vuyst and Leroy, 2007; Sit and Vederas, 2008).

Today, the diverse group of LAB includes species that are among the best-studied microorganisms and proteolysis is one of the particular physiological traits of LAB of which detailed knowledge was obtained. The proteolytic system involved in casein utilization provides cells with essential amino acids during growth in milk and is also of industrial importance due to its contribution to the development of the organoleptic properties of fermented milk products (Savijoki et al., 2006).

For the most extensively studied LAB, *Lactococcus lactis*, a model for casein proteolysis, transport, peptidolysis, and regulation thereof is now established. Comparative genomics reveals some differences between the proteolytic systems of LAB; differences that are thought to reflect the various environmental niches these bacteria occupy (Boekhorst et al., 2004).

To date, the most extensively studied proteolytic enzymes of LAB are cell envelope-associated (CEP) proteinases and intracellular aminopeptidases. Oligopeptidases (PepO, PepF) which do not hydrolyze casein are also quite well characterized (Monnet et al., 1994; Fox et al., 1995; Law and Haandrikman, 1996). 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., 1990; Akuzawa and Okitani, 1995; Stepaniak et al., 1996) showed differences in general properties indicating an intracellular metalloprotease in lactococci. However, the data on the extracellular especially metalloproteases from lactococci are not signalled.

In the present study, we report the purification and characterization of extracellular metalloprotease produced by *lactococcus lactis subsp lactis* isolated from traditional fermented food and provides information about its main biochemical characteristics.

2. Materials and Methods

2.1. Screening for proteolysis activity

Collection of sixty five strains from *Laboratoire de Bio-ressources Naturelles Chlef* (Algeria) was tested. Sample was plated onto skim-milk agar plates containing (g/L): peptone 5, yeast extract 3, bacteriological agar 12 and skim-milk 250 mL. Plates were incubated 24- 48h at 37°C. A clear zone of skim-milk hydrolysis gave an indication of protease-producing strains. Among 08 strains isolated in the laboratory and showed proteolytic activity, BR16 strain was selected.

2.2. Bacterial strain identification

The isolate was identified as *Lactococcus lactis subsp. lactis* on the basis of the biochemical characteristics (API 50 CHL) and the 16S ribosomal DNA (rDNA) sequence analysis. Sequence comparison with the databases was performed using the program Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information (NCBI), National Institutes of Health, USA).

2.3. Kinetic of protease production

The Kinetic of protease production was conducted as follow: Sterile flask containing a working volume of 500 mL of commercial sterilized skim milk (UHT) was inoculated with an overnight pre-cultured BR16 strain (at a level of 5% v/v), then incubated for 48h at 37°C with rotatory shaker (150 rpm). Periodically each three hours, an aliquot volume of 10 mL was collected, centrifuged at 10.000 rpm for 15 min at 4 °C, and the cell-free supernatants were used for estimation of proteolytic activity and pH measurement. In parallel, colonies count was conducted on Elliker agar.

2.4. Assay of protease activity

Protease activity was measured by the modified method of Beynon (1989) using the chromogenic substrate, azocasein (sigma), as a substrate. Briefly, a 0.15 mL aliquot of the purified enzyme, suitably diluted, was mixed with 0.25 mL of 100 mM sodium phosphate buffer (pH 6.5) containing 2% (w/v) azocasein, and incubated for 60 min at 55°C. The reaction was stopped by addition of 1.2 mL of trichloroacetic acid 10% (w/v). The mixture was allowed to stand at room temperature for 15 min and then centrifuged at 10,000 g for 5 min to remove the precipitate. 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 protease activity was defined as the amount of the enzyme that results in an increase of 0.01absorbance per hour at 440 nm under the experimental conditions.

2.5. Protease purification

2.5.1. Ultrafiltration

The culture supernatant containing the extracellular enzyme was first concentrated by ultrafiltration cell (Sartorius vivacell 250, 10000 MWCO). Thirty millilitres was recovered and checked for protease activity with azocasein as the substrate.

2.5.2. SP-Sepharose cation-exchange chromatography

The ultrafiltration fraction was applied to a SP-Sepharose column (2.6 cm × 15 cm) (GE Healthcare Bio-Sciences AB) equilibrated with buffer A (50 mM Tris-HCl, pH 8.0). After being washed with the same buffer, bound proteins were eluted with a linear gradient of sodium chloride in the range of 0-1M in the equilibrating buffer. Fractions (4 mL each) were collected at a flow rate of 2 mL/min and analyzed for protease activity and protein concentration.

2.5.3. Sephadex G-50 gel filtration

Fractions from SP-Sepharose column hydrolysing azocasein were concentrated by 10-kDa ultrafiltration cell (Sartorius vivacell 250), then applied to a Sephadex G-50 column (1.2 cm × 120 cm) (Pharmacia-Amersham, Uppsala, Sweden) pre-equilibrated with buffer C (50 mM Tris-HCl buffer (pH 7.0), NaCl 0.15 M). Fractions of 4 mL each were collected with the same buffer at a flow rate of 0.5 mL/min and analysed for protein content and protease activity, using azocasein, as a substrate. Active fractions were pooled and stored at 4°C for further analysis. All the purification steps were conducted at temperatures not exceeding 4 °C.

2.5.5. Polyacrylamide gel electrophoresis and casein zymography

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the determination of purity and molecular weight of the purified protease as described by Laemmli (1970), using a 5 % (w/v) stacking gel and a 15 % (w/v) separating gel. Samples were prepared by mixing the purified enzyme at 1:5 (v/v) ratio with distilled water containing 10 mM Tris-HCl pH 8.0, 2.5% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.002% bromophenol blue. Samples were heated at 95 °C for 5 min before electrophoresis. After electrophoresis, the gel was stained with 0.25% Coomassie Brilliant Blue R-250 in 45% ethanol, 10% acetic acid and destained with 5% ethanol and 7.5% acetic acid. The molecular weight of the enzyme was estimated using a molecular weight calibration kit as markers (fermentas PageRuler, prestained Protein Ladder).

Native-PAGE was performed according to the procedure of Laemmli (1970), except that the sample was not heated and SDS and reducing agent were left out.

Zymography is a sensitive and rapid assay method for analysing protease activity. Casein zymography was performed on native-PAGE. Briefly, after electrophoresis, the gel was submerged in 1% casein in 100 mM Tris-HCl buffer, pH 8.0, and incubated at 37 °C for 90 min. After washing, the gel was stained with Coomassie Brilliant Blue R-250 for zymography analysis. A clear zone on the blue background indicated the presence of protease.

2.5.6. Protein determination

Protein concentration was determined by the method of Bradford (Bradford, 1976) using the Bio-Rad assay reagent (Bio-Rad, Munich, Germany) and bovine serum albumin (BSA) as the standard. During the course of purification, the protein concentration was determined by measuring the absorbance at 280 nm.

2.6. Molecular weight determination

To determine the molecular mass of the native enzyme, the purified protease was loaded on size exclusion Sephacryl G-100 column (1.6 x 60 cm) equilibrated in 50 mM Tris-HCl buffer (pH 7.0). Elution was performed with same buffer at 0.6 mL/min. The molecular weight of the enzyme was estimated using a low molecular weight calibration kit (Sigma). The molecular mass markers used are: bovine serum albumin (67,000 Da); ovalbumin (43,000 Da); ribonuclease (25,000 Da) and chymotrypsinogene A (13,700 Da). The apparent molecular weight of the enzyme was calculated by comparison with the relative elution volumes of protein standards.

2.7. Biochemical characterization of the purified protease

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

The optimum pH of the purified enzyme was studied over a pH range of 3.0-11.0. For the study of pH stability, the enzyme was incubated for 1 h at 55 °C in different buffers and the residual proteolytic activity was determined under standard assay conditions. The following buffer systems were used: 100 mM glycine–HCl buffer, pH 3; 100 mM sodium acetate buffer, pH 4.0-6.0; 100 mM phosphate buffer, pH 7.0; 100 mM Tris-HCl buffer, pH 8.0; 100 mM glycine-NaOH, pH 9.0-11.0.

2.7.2. Effect of temperature on activity and stability of the enzyme

To investigate the effect of temperature, protease activity was assayed at different temperatures ranging from 20 to 70 °C for 60 min at pH 7. For thermal stability, the enzyme was incubated at different temperatures for 60 min and immediately cooled in ice water. Thereafter, the residual activity was determined at pH 7 and 55 °C for 60 min. The non-heated enzyme was considered as the control (100%).

2.7.3. Effects of enzyme inhibitors

The effects of enzyme inhibitors on protease activity were studied using phenylmethylsulfonyl fluoride (PMSF), aprotinine, phenantroline, pepstatin A, iodoacetamid, β-mercaptoethanol and ethylene-diaminetetraacetic acid (EDTA). The purified enzyme was preincubated with inhibitors for 30 min at 25 °C and then the remaining enzyme activity was estimated using azocasein as a substrate (pH 7.0 at 55 °C). The activity of the enzyme assayed in the absence of inhibitors was taken as 100%.

2.7.4. 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+}or Al^{3+}) to the reaction mixture. The activity of the enzyme without metallic ions was considered as 100% control.

2.8. Digestion of Caseins with purified BR16 protease

Aqueous solution of Bovine sodium caseinate (sigma) 5% (w/v) in 100 mM Tris-HCl buffer (pH 7.0) and digested with 5% (v/v) of purified BR16 protease at 55°C for 20 h. Proteolytic digestions were terminated by heating at 80 °C for 15 min then cooled and any resultant insoluble material removed, as described by McCann et al. (2005).

2.9. Analytical HPLC

Reversed-Phase High-performance Liquid Chromatography (RP-HPLC) was performed with an automated HPLC system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millennium software was used to plot, acquire and analyze chromatographic data. An analytical XBridge C-18 column, (5 µm, 4.6 × 250 mm) (Waters, Ireland) was used with a flow rate of 1ml/min. Gradient elution was carried out with a mixture of solvant A (0.1 %, trifluoroacetic acid (TFA), v/v) in deionized water and solvent B (0.1% TFA in acetonitrile, v/v). Proteins and peptides were eluted as follows; 0-80 min, 40% B; 80-85 min, 40- 100% B; 85-90 min, 100% B; 90-100 min, 100-0% B and the HPLC system was equilibrated for 10 min with 100% A. Elution was performed at room temperature. Samples were filtered through 0.2 µm filters. On-line UV absorbance scans were performed between 200 and 300 nm at a rate of one spectrum per second with a resolution of 1.2 nm. Chromatographic analyses were completed with Millennium software.

3. Results and Discussion

3.1. Bacterial strain identification

In order to identify BR16 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 accession number AB618806.1. The isolate was identified as *Lactococcus lactis subsp. lactis*.

3.2. Kinetic of protease production

The amount of proteolytic activity during the various growth phases was determined in sterilized skim milk in order to obtain high yield for purification. The time courses of protease production and growth of *Lc. Lactis subsp lactis* BR16 in milk medium are shown in Fig. 1. The results revealed that protease synthesis is mainly associated with the exponential growth. The maximum amount of enzyme was produced at 18h of incubation reaching 3.93 (U/mL) corresponding to the end of exponential phase. Thus, the evolution of pH and proteolytic activity show that the strain developed a highly proteolytic and weakly acidifying character.

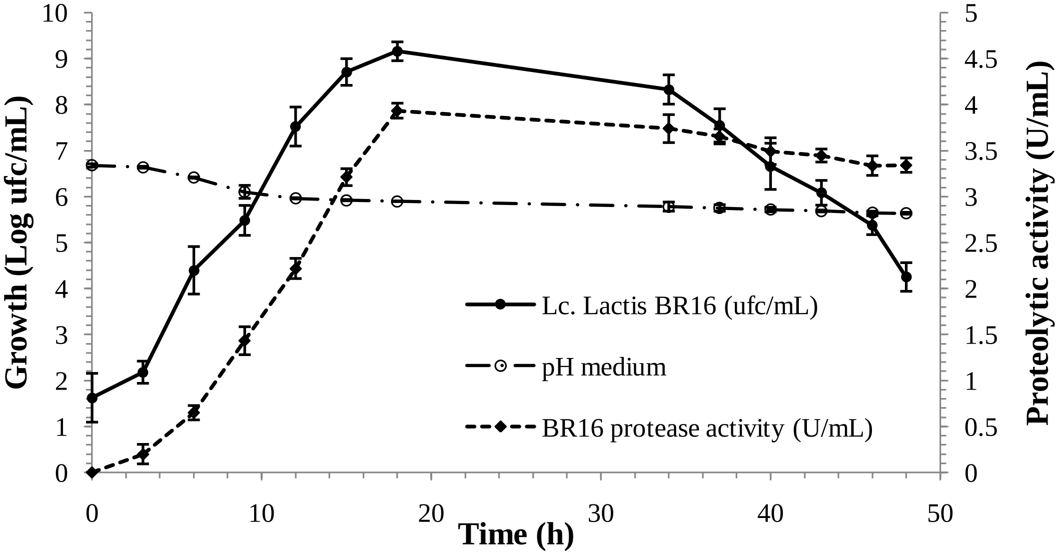


Fig.1. Growth kinetics and protease production of *Lactococcus lactis subsp. lactis* BR16. Samples were withdrawn at 3 h interval for determination of cell growth (Log CFU/ mL) and protease activity as described in section3. Results are mean ± SD of triplicates.

According to Helinck et al., (1997) and Flambard et al., (1998) showed that the type of protease influence the growth of *Lc. Lactis* in milk. However, many reports have reported the maximum protease production was at the end of the exponential phase and in the early stationary phase (Nissen-Meyer and Sletten ,1991; Meijer et al., 1996)

3.3. Purification of BR16 protease

The BR16 protease from *Lactococcus lactis subsp. lactis* was purified successively by the three-step procedure described in Section 2. In the first step, the crude extract was concentrated with ultrafiltration cell using 10-kDa cut-off membrane. The concentrated enzyme was subjected to chromatography on a SP-Sepharose column. Binding proteins were eluted with a linear gradient of NaCl from 0 to 1 M. Protease activity appeared in a second peak together with adsorbed fractions (Fig. 2). Fractions containing protease activity were pooled, and then loaded to gel filtration on a Sephadex G-50 column pre-equilibrated with buffer B. This procedure yielded a single peak of protease activity (Fig. 3).

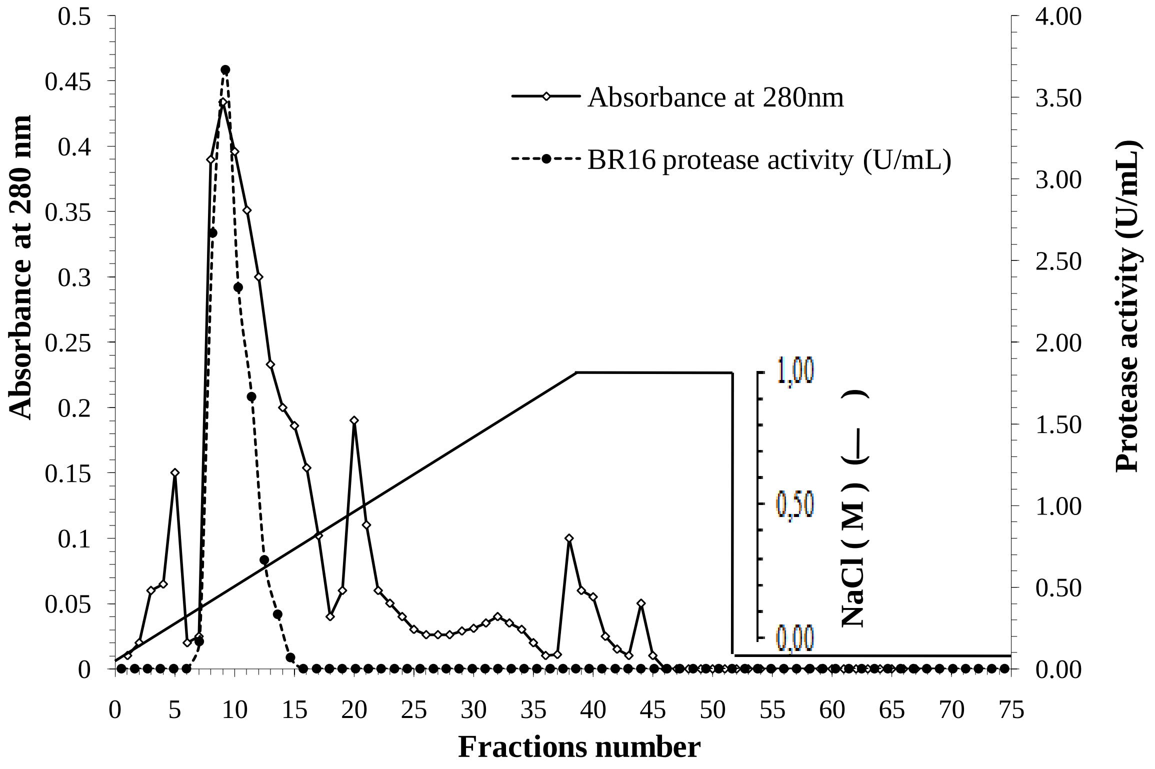


Fig. 2. Elution profile of *Lactococcus lactis subsp. lactis* BR16 protease from a SP-Sepharose column. The enzyme was eluted with a linear gradient of NaCl (0-1 M) in 50 mM Tris-HCl buffer (pH 8.0) at a flow rate of 120 mL/h. Fractions (4 ml each) collected from the column were assayed for proteins content at 280 nm and protease activity as described in Section 2.

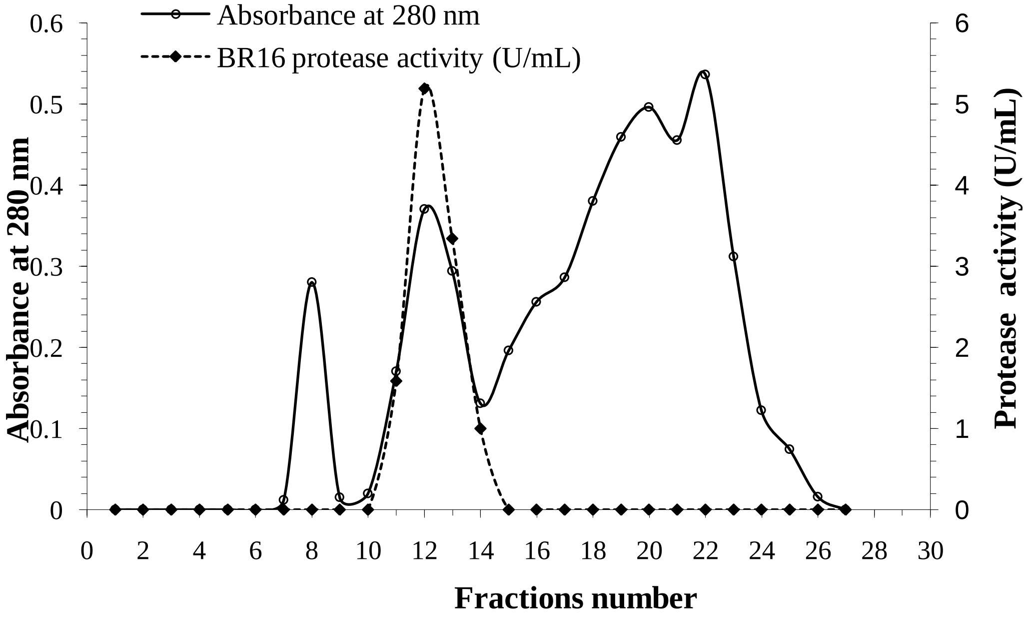


Fig. 3. Purification profile of the BR16 protease from *Lactococcus lactis subsp. lactis* by gel filtration on Sephadex G-50 column. The active fractions were applied to a 1.2 cm × 120 cm column, equilibrated and eluted with buffer C. Fractions (4 mL each) collected from the column were assayed for proteins content at 280 nm and protease activity as described in Section 2. Flow rate = 30 mL/h.

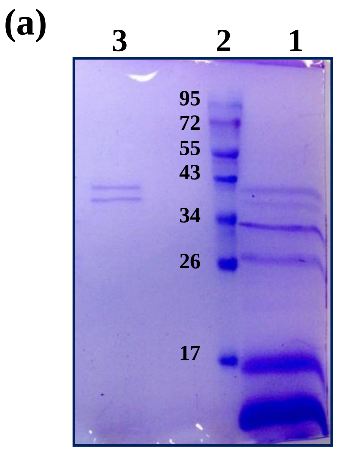
After the final purification step, the enzyme was purified 5.83-fold with a recovery of 47 % and a specific activity of 2054.95 U/mg of protein. The results of the purification procedure are summarized in Table 1.

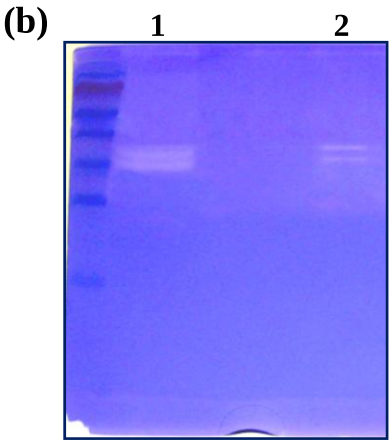
Table 1. Summary of the purification of *Lactococcus lactis subsp. Lactis* BR16 protease

Purification steps	**Volume (mL)**	**Total activity (U)**	**Total protein (mg)**	**Specific activity (U/mg)**	**Recovery (%)**	**Purity (fold)**
Crude extract	100	6100	70.15	86.96	100	1
Ultrafiltration	50	5853	38.12	153.54	96	1.77
SP-Sepharose	25	3996	11.33	352.69	68	2.3
Sephadex G-50	10	1870	0.91	2054.95	47	5.83

The purified protease was homogenous on SDS-PAGE and its molecular weight was estimated to be 38 kDa (Fig. 4a), corresponding with that determined by gel filtration (Fig. 4c). Purity of the enzyme was also evaluated by zymogram activity staining. As shown in Fig. 4b, two clear band of proteolytic activity of casein hydrolysis was observed in the gel, indicating possibly the autoproteolytic cleavage. Indeed, Nissen-Meyer and Sletten (1991) were reported the presence of the autoporteolytic degradation of the protease isolated from *Lactococcus lactis subsp. cremoris*

Generally, the molecular weights of proteases from lactococci range between 12 and 180 kDa. The molecular weight of the purified metalloprotease from *Lactococcus lactis subsp. lactis* BR16 was lower than those of *Lc. lactis ssp. Cremoris* H61 (140 kDa) (Ohmiya and Sato, 1975) and *Lc. lactis subsp. cremoris* Wg2 (165 kDa) (Laan and Konings, 1991) and *Lc. lactis subsp. cremoris* H2 (180 kDa) (Coolebear et al., 1992). In addition, the purified protease from *Lc.lactis ssp. lactis* NCDO 763 had a molecular mass of 93 kDa (Muset et al., 1989). On the other hand, the molecular weight of BR16 protease was higher than those from *Lc. lactis ssp. lactis* IAM 1198 (12 kDa) (Akuzawa and Okitani, 1995).





(c)

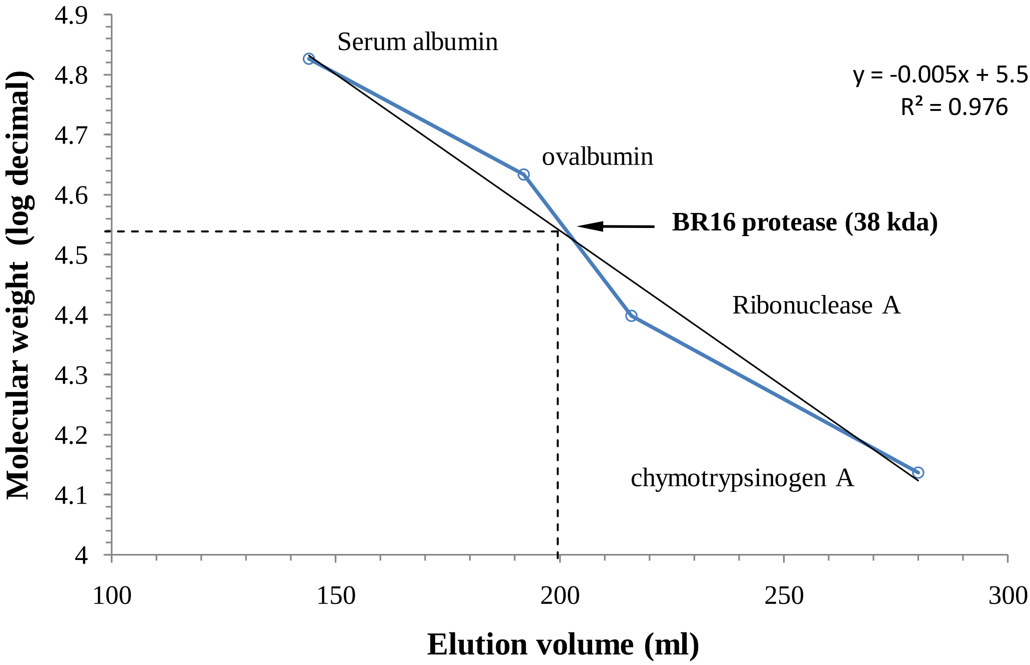


Fig. 4. a) SDS-PAGE of the purified metalloprotease from *Lactococcus lactis subsp. lactis* BR16. Lane 1: crude enzyme extract; lane 2: molecular mass markers; lane 3: purified enzyme. b) Zymogram activity staining of the crude supernatant of *Lactococcus lactis subsp. lactis* (lane 1) and the purified enzyme (lane 2). c) Molecular weight estimation of the BR1 protease.

3.3. Effect of pH on protease activity and stability

The pH activity profile of the purified BR16 protease was determined using different buffers of varying pH values. Interestingly, BR16 protease was highly active in the pH range of 6.0-7.5, with an optimum around pH 6.5 (Fig. 5a). The relative activities at pH 6.0 and pH 7.5 were about 90 %, of that measured at pH 6.5. However, BR16 protease activity decreased significantly above pH 9.0 and was only 4.80% at pH 10.5. These findings are in accordance with several earlier reports showing pH optima between 5.5 and 7.5 for proteases from *Lc. lactis ssp. lactis* IAM 1198 (5.5-6.0) (Akuzawa and Okitani, 1995), *Lc. lactis ssp. lactis* IAM 1198 (6.0-6.5) (Akuzawa et al., 1994), *Lc. lactis subsp lactis* MG 1363 (pH 7-7.5) (Stepaniak et al., 1996).

The pH stability profile showed that the purified protease is highly stable in a broad pH range 6.0-8.0 maintaining more than 90% of the initial activity after 1 h incubation at 55 °C (Fig. 5b).

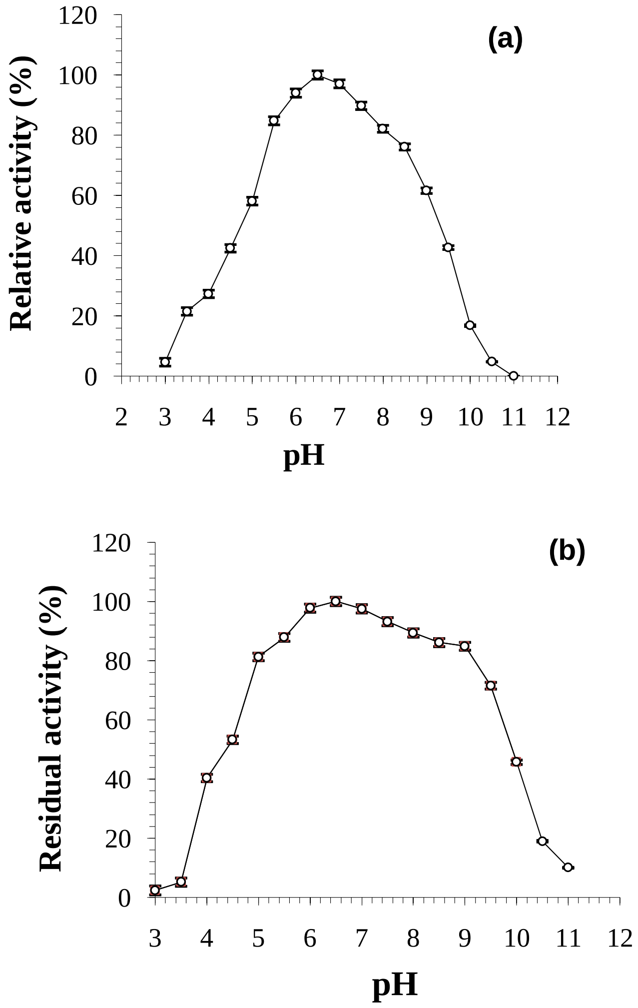
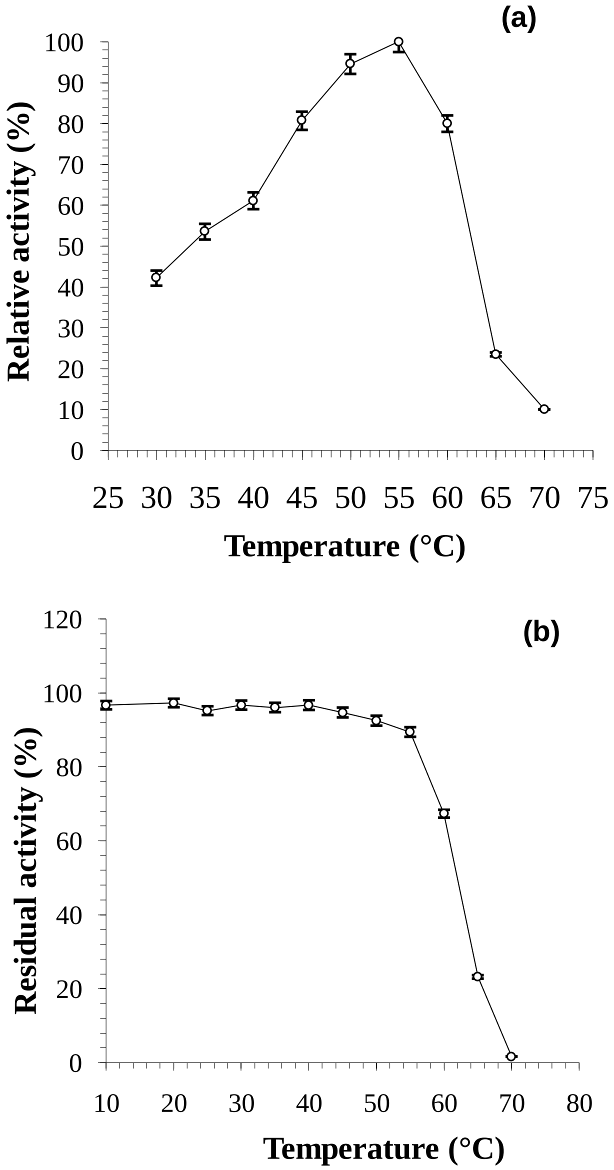


Fig.5. Effect of pH on activity (a) and stability (b) of the purified BR16 protease. The protease activity was assayed in the pH range of 5.0-11.0 using buffers of different pH values at 55°C. The maximum activity obtained at pH 7.0 was considered as 100% activity. The pH stability of the enzyme was determined by incubating the enzyme in different buffers for 1 h at 40°C and the residual activity was measured at pH 7.0 and 55°C. The activity of the enzyme before incubation was taken as 100%. Buffer solutions used for pH activity and stability are presented in Section 2. Results are mean ± SD of triplicates.

3.4. Effect of temperature on the activity and stability

The effect of temperature on *Lactococcus lactis subsp. lactis* BR16 protease activity was examined at various temperatures. The BR16 enzyme was active between 30 and 70°C with an optimum around 55°C (Fig. 6a). The relative activities at 45, 50 and 60°C were about 81, 94 and 80 %, respectively. Temperature optima of BR16 protease is higher than the lactococci proteases reported in the literature such as from *Lc. lactis ssp. cremoris* H61 (30°C) (Ohmiya and Sato, 1975), *Lc. lactis subsp. lactis* NCDO (45°C) (Muset et al., 1989), Lc.lactis subsp. cremoris Wg2 (30°C) (Laan and Konings, 1991), *Lc. lactis subsp. cremoris* H2 (37°C)(Coolebear et al., 1992) and *Lc. lactis ssp. lactis* IAM 1198 (30°C) (Akuzawa and Okitani, 1995).

For thermal stability, residual activity of *Lactococcus lactis subsp. lactis* BR16 protease was evaluated after heat treatment for 60 min at various temperatures and results are shown in Fig. 6b. The thermal stability profile of the purified protease showed that the enzyme was highly stable at temperatures below 60 °C but was inactivated at higher temperatures. The enzyme retained more than 96.7 %, 94.7% and 92.5 % of its initial activity at 40, 45 and 50°C respectively. The thermal stability of BR16 protease is higher than the protease from *Lactococcus lactis subsp. lactis* IAM 1198 reported by Akuzawa and Okitani (1995), which loosed totally of its initial activity after 1 h incubation at 55°C.



3.5. Effect of various inhibitors on protease activity

Proteases can be classified by their sensitivity to various inhibitors (North, 1982). The effects of various class-specific protease inhibitors on the purified protease were investigated (Table 2). The enzyme was completely inhibited by the specific metalloenzyme inhibitor phenantroline (1 mM) indicating that the purified enzyme is a metalloprotease. The enzyme was not affected by serine-protease inhibitors (PMSF and aprotinin), aspartic protease inhibitor (pepstatine) and cysteine protease inhibitor (iodoacetmid).

The enzyme was slightly inhibited by the chelating agent EDTA (5 mM), with 47 % of its original activity being lost, indicating the importance of metal ions in enzyme stabilization. These findings are in line with several earlier reports showing that active structure of some proteases contains Ca^{2+} binding site(s) and the removal of Ca^{2+} from the strong binding site is associated to a significant reduction in thermal stability (Vieille and Zeikus, 2001).

Table 2. Effect of inhibitors on the BR16 protease activity

Inhibitors	**Concentration (mM)**	**Residual activity (%)**
Control	-	100
EDTA	5	53 ± 1.6
β-mercaptoethanol	5	100

Aprotinin	5	100
Phenantroline	1	0
Pepstatine	5	100
PMSF	5	97 ± 1.3
Iodoacetamid	5	100

Purified enzyme was pre-incubated with various enzyme inhibitors for 30 min at 25°C and the residual activity was determined at pH 6.5 and 55°C. Enzyme activity measured in the absence of any inhibitor was taken as 100%. Results are mean ± SD of triplicates.

3.6. Effect of metal ions

The effects of some metal ions, at a concentration of 5 mM, on the activity of BR16 protease were studied at pH 7.0 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). As shown in Table 3. The enzyme activity was not affected by monavalent (Na^+ and K^+) ions. However, the purified enzyme was partially inhibited by Mn^{2+}, $Fe^{2+}$and Zn^{2+} and strongly affected by and Cu^{2+}, suggesting the adverse effect of heavy metal ions on BR16 protease.

Table 3. Effect of various metal ions (5 mM) on BR16 protease activity

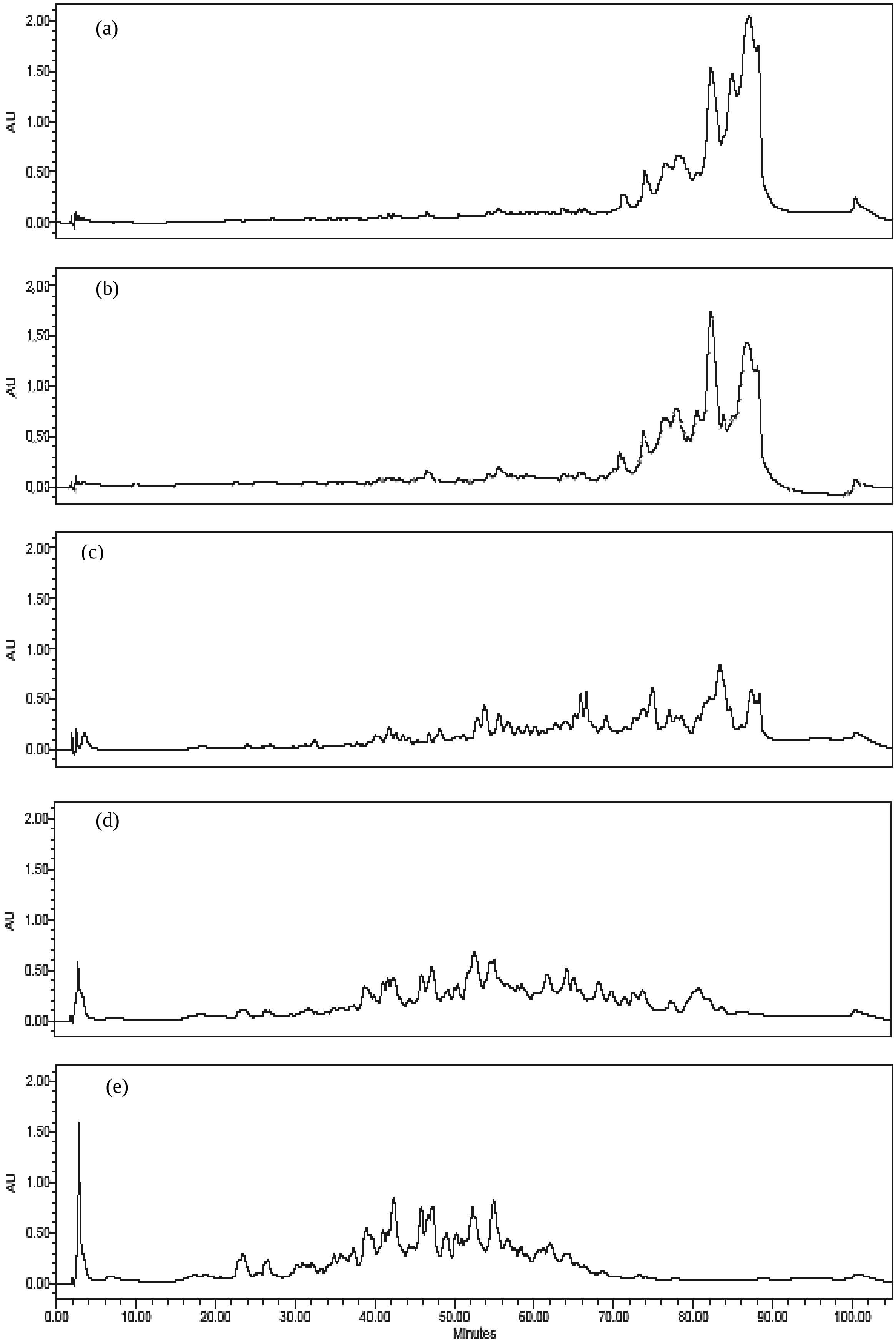
Metal ions (5 mM)	**Relative activity (%)**
Control	100
Na^+	100
K^+	99.10 ± 2.5
Cs^+	96.12 ± 2.9
Ca^{2+}	96.00 ± 1.6
Zn^{+2}	65.00 ± 0.6
Cu^{+2}	55.00 ± 1.1
Mg^{+2}	100
Fe^{+2}	76.00 ± 2.9
Mn^{+2}	86.99 ± 1.2
Al^{+3}	100

The activity of the protease was determined by incubating the enzyme in the presence of various metal ions (5 mM) for 1 h at 55°C and pH 6.5. Results are mean ± SD of triplicates.

3.7. Peptic Hydrolysis of Bovine Casein

Bovine casein was hydrolyzed by BR16 protease at pH 6.5; 55° C and kinetics studies were followed at 10, 20, 30 min and from twenty hours of hydrolysis. As shown in Fig.7, the obtained chromatograms of peptic hydrolysates of bovine casein indicates the occurrence, in the initial stage of hydrolysis, of several large fragments, with elution times close to those of the substrate. As the evolution of the hydrolysis, the rate of the initial components decreases in favor of those of smaller peptides, which appear in small amounts to the top of the chromatograms and continued to increase during the hydrolysis. This suggests that BR16 protease acts on casein as a mechanism namely one by one which is characterizing enzymatic reactions when the reaction mixture was composed of intact substrate and final product of small size. This means that the protease cleaves one molecule of protein substrate at a time (Srividhya et al., 2006).

According to Morales et al. (2001), analysis by RP-HPLC profiles of peptide derived from the hydrolysis of the casein by a protease collection lactococcal strains (AM1, AM2, HP, Wg2 and SK11), showed the presence of two groups of peptides, the hydrophilic and hydrophobic peptides.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60.00
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80.00
90.00
100.00
Minutes

Fig. 7. Reverse-phase HPLC profiles of bovine casein hydrolysates obtained at different times of hydrolysis (a) t = 0 min ; (b) t = 5 min ; (c) t = 90 min ; (d) t = 6h and (c) t = 20h .

4. Conclusion

In this study, we report the purification and characterization of a new metalloprotease from *Lactococcus lactis subsp. lactis* BR16. The purification to homogeneity of the enzyme was achieved by ultrafiltration, ion exchange chromatography on SP-Sepharose and gel filtration through Sephadex G-50. After the final purification step, the enzyme was purified 5.83-fold with a specific activity of 2054.95 U/mg and 47% recovery. The purified enzyme was homogenous on SDS-PAGE and its molecular weight was estimated to be 38 kDa. One of the most important features of the present protease from *Lactococcus lactis subsp. lactis* BR16 its high activity and stability in a broad pH range. The enzyme maintains more than 80% of its original activity between pH 5.0 and 9.0 after 1 h incubation at 55 °C. The optimum temperature was 55 °C.

Therefore, more research on the specific molecular characteristics of this interesting enzyme and the determination of the properties of this protease as a possible biotechnological tool in the food industries will be done.

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