Biomacromol. J. Vol. 1, No. 1, 93-103, July 2015.

Biomacromolecular Journal
www.bmmj.org

Microsciadin, a New Milk-Clotting Cysteine Protease from an Endemic Species, *Euphorbia microsciadia*

H. Rezanejad[a], H.R. Karbalaei-Heidari[a,b,]*, S. Rezaei[a] and R. Yousefi[b,c]

[a]*Molecular Biotechnology Laboratory, Department of Biology, Faculty of Science, Shiraz University, Shiraz 71454, Iran*

[b]*Institute of Biotechnology, Shiraz University, Shiraz, Iran*

[c]*Protein Chemistry Laboratory, Department of Biology, Faculty of Science, Shiraz University, Shiraz, Iran*

(Received 11 January 2015, Accepted 19 February 2015)

ABSTRACT

In the present work, a new branch of biotechnological advantage of the latex of an endemic perennial plant, *Euphorbia* microsciadia has been introduced. A novel cysteine protease, designated as microsciadin, was purified from the latex of *Euphorbia* microsciadia by a combination of sequential usage of SP-Sepharose Fast Flow column in two different pHs and a final gel filtration chromatography. Microsciadin is a monomeric protein with an apparent molecular mass of 60 kDa by SDS-PAGE. Although the enzyme was stable over a wide range of pH and temperatures, it displayed the maximum activity at 45 °C and pH of 4.5. The enzyme was strongly inhibited by Iodoacetamide, E-64 and Hg^{2+} ions indicated that it belongs to the cysteine protease family. Furthermore, the enzyme showed suitable stability in the presence of various denaturants and organic solvents. Moreover, primary studies on milk clotting activity of the enzyme revealed its high potential to dairy industry. The acidophilic feature of microsciadin in associated with its high milk-clotting activity and remarkable operational stability suggest its potential application in cheese industry, as well as other food and biotechnological fields.

Keywords: *Euphorbia* microsciadia, Cysteine protease, Dairy industry, Microsciadin

INTRODUCTION

Proteases are a highly complex group of enzymes that differ in their substrate specificity, catalytic mechanism and active site chemistry [1]. Proteolytic enzymes account for approximately 60% of all worldwide sales of enzymes [2], because of their various applications in food, medical, pharmaceutical, detergent, leather and extraction of silver from used x-ray films, as well as basic research studies [3,4]. The largest applications of proteases are in the most likely food industry and in laundry detergents. In food industries, proteases are used in cheese elaboration, brewing, and bread manufacturing, as well as for improvement of functional and nutritional properties of proteins, meat tenderization, hydrolysis of gelatin, soy protein, casein and whey proteins [5,6].

Several useful proteases have been obtained from various sources such as plants, animals and microorganisms. Proteolytic enzymes from the plant sources have received special attention because of their broad substrate specificity, well property of being active over a wide range of temperature and pH, as well as unique active site chemistry and safety of their usage. These properties enable them to use in some special biotechnological applications such as food and pharmaceutical industries [7]. Plant cysteine proteases such as papain and stem bromelain are extensively used for medicine, brewing wine, and food industry [8]. Recently, purification and biotechnological application of a novel cysteine protease from latex of *calotropis procera* was also reported [9,10]. Cysteine proteases (CPs) or thiol proteases are one of the largest groups of proteolytic enzymes involved in numerous regulatory processes in both prokaryotes and eukaryotes (*e.g.* bacteria, parasites, plants, invertebrates and vertebrates). The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine. In plants, they are widely spread among

*Corresponding author. E-mail: karbalaei@shirazu.ac.ir

different taxonomic groups and are found to be involved in a number of physiological processes such as development and ripening of fruits, nutritional reserve, degradation of storage protein in germinating seeds, activation of proenzymes, and degradation of defective proteins. CPs comprise a family of enzymes, consisting of papain and related plant proteases such as chymopapain, ficin, caricain, bromelain, actinidin, aleurain, and the mammalian lysosomal cathepsins B, H, L, S, C and K [11].

Euphorbia microsciadia Boiss., belonging to family Euphorbiaceae, is a perennial plant growing largely in the Middle East area particularly in Iran. Traditionally, the entire *Euphorbia* including the leaves, stem and roots is used to treat skin diseases, gonorrhea, migraines, intestinal parasites, and warts [12,13] inflammations, tumors, rheumatism, nerve discomfort, microbial infections and as a purgative [14,15]. Phytochemical studies have led to the isolation of nonacosane, cyclomyrsinol esters, and β-sitosterol from different parts of the plant [14,16].

On the other hand, dairy farmers in some parts of Iran use the latex of *E. microsciadia* to make white soft cheese using goat and sheep milk. Milk coagulation takes about 1 h and the curd is pressed to remove whey. However, the proteins and other biochemical constituents of the latex have not been investigated in detail. In the present paper, the isolation, partial purification and biochemical properties of a milk-coagulating cysteine protease from latex of this endemic plant, *E. microsciadia*, is reported.

MATERIALS AND METHODS

Materials

Aerial flowering parts of *Euphorbia microsciadia* (Euphorbiaceae) were collected from Kherame, Fars province (Iran). Plant material was identified by Dr. A. Khosravi, plant taxonomist and a voucher specimen deposited in the herbarium of the Faculty of Sciences, Shiraz University (Iran). Superficial incisions on the young stems of the plant *Euphorbia microsciadia* provided milky latex, which was received fresh. SP-Sepharose and Sephacryl S-200 were purchased from Pharmacia. BSA, 1,10-phenanthroline, EDTA, E-64, chymostatin, Pefabloc SC, phosphoramidon, pepstatin, leupeptin, bestatin or antipain-dihydrochloride, were from Roche (Germany). Coomassie brilliant blue R 250, Agarose, glycerol, β-mercaptoethanol, and BSA (Fraction V) were from Sigma Chemical Co. (USA). All other chemicals were of highest purity commercially available.

Purification

All purification steps were carried out at 4 °C unless stated otherwise.

Removal of gum. Latex was collected in 10 mM sodium acetate buffer (pH 4.5) by superficial incisions on stems of *E. microsciadia* plants, and then frozen at -20 °C for 24 h. Subsequently, the frozen latex thawed at room temperature and centrifuged at 20000 g for 15 min to remove insoluble gum and other debris. The clear supernatant was dialyzed against the same acetate buffer, and centrifuged as above again to remove any precipitant that resulted during dialysis. The clear supernatant thus obtained was loaded on a SP-Sepharose fast flow column.

Cation exchange chromatography on SP-Sepharose. The clear protein solution obtained in the above step was loaded on SP-Sepharose fast flow column (9 cm × 1.5 cm), pre-equilibrated by 10 mM sodium acetate buffer (pH 4.5), at a flow rate of 1 ml min^{-1}. The column was washed with the buffer till no proteins were detected in the effluent. The bound proteins were eluted with a linear gradient of 0.0-0.75 M NaCl in the same buffer at a flow rate of 1 ml min^{-1} and fractions of 3.0 ml volume were collected. Absorbances at 280 nm as well as the proteolytic activity of all fractions were measured (Fig. 1a). Then, the active fractions were pooled and dialyzed against 10 mM sodium acetate buffer (pH 5.5). The enzyme solution was subjected to another cation exchange chromatography on the SP-Sepharose fast flow column with new pH of 5.5. The column was washed thoroughly with 10 mM sodium acetate buffer (pH 5.5) until no protein was detected in the eluate. The bound proteins were eluted with the same buffer using a linear gradient of 0.0-0.75 M NaCl. Fractions of 3 ml volume were collected at a flow rate of 1 ml min^{-1}. Absorbance at 280 nm and proteolytic activity of all fractions was also measured. The elution profile showed three peaks (Fig. 1b). Out of these, fractions of peak I exhibited the maximum activity were pooled and dialyzed against 50 mM sodium acetate buffer (pH 5.0) containing 0.1 M NaCl. Further the protein solution was concentrated by the Amicon membrane

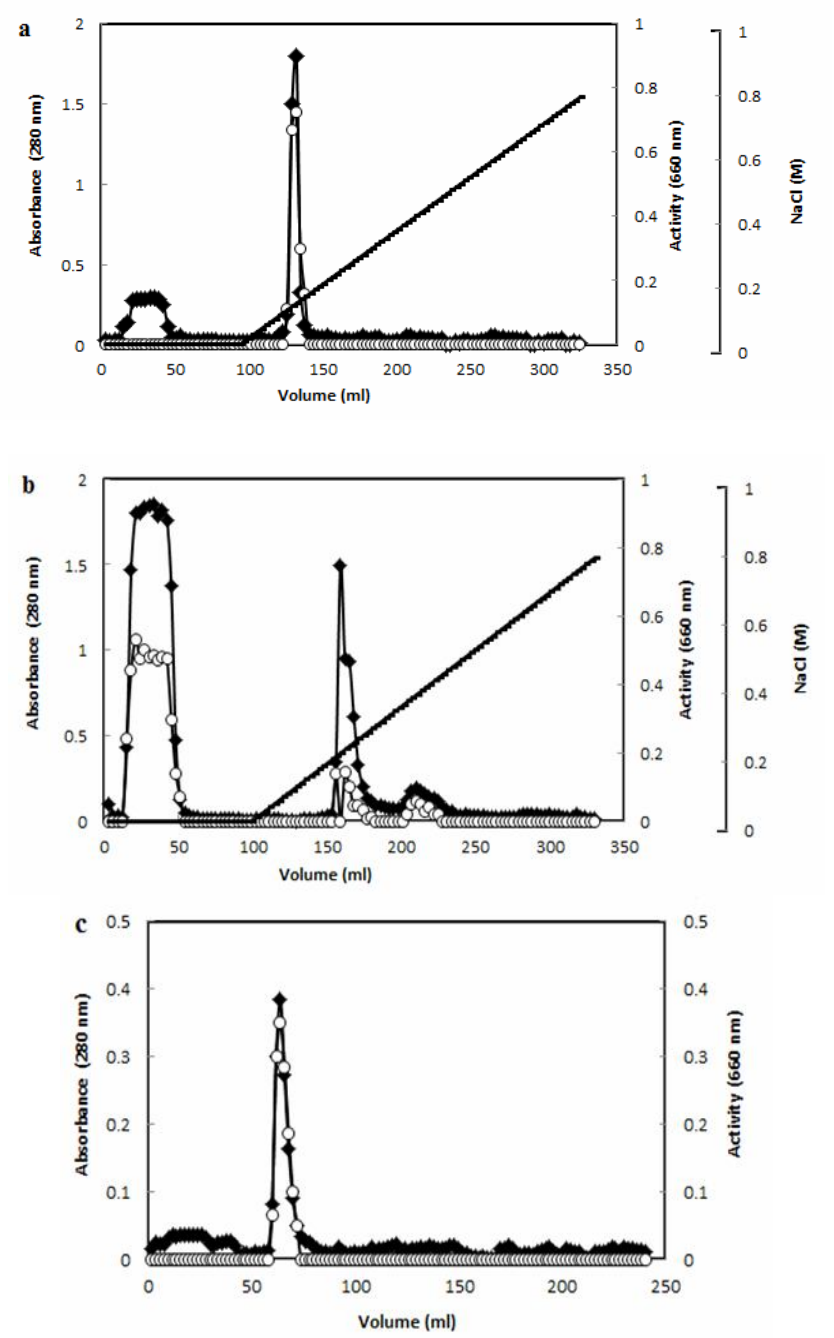

Fig. 1. Elution profile of crude latex after removal of gum (a) Cation exchange chromatography on the SP-Sepharose fast flow column pre-equilibrated with 10 mM sodium acetate (pH 4.5). (b) The peak II fractions (No. 43-46) from the SP-Sepharose column elution profile was pooled and dialyzed against 10 mM sodium acetate buffer (pH 5.5) and loaded on second SP-Sepharose column. The column was pre-equilibrated with 10 mM sodium acetate buffer (pH 5.5). (c) Gel filtration on Sephacryl S-200 column. The peak I fractions (No. 4-14) from the second SP-Sepharose column elution profile was concentrated and loaded on Sephacryl S-200 column and eluted with 50 mM sodium acetate buffer (pH 5.0) containing 100 mM NaCl, at a flow rate of 0.5 ml min^{-1}. All eluted fractions from the chromatography columns were assayed for activity (open circles) and protein content (closed squares).

concentrator with a 10 kDa cutoff. The clear protein solution was stored at 4 °C for the next step.

Size-exclusion Chromatography. The concentrated enzyme obtained from the above step was subjected to gel filtration on a Sephacryl S-200 column (70 cm × 1.5 cm), pre-equilibrated with 50 mM sodium acetate buffer (pH 5.0) containing 0.1 M NaCl, and the column was eluted with the same buffer, at a flow rate of 0.5 ml min^{-1}. All about fractions were analyzed as described above (Fig. 1c). The active and homogenous fractions were pooled, concentrated and stored at 4 °C for further use. The partial purified enzyme named microsciadin according to the nomenclature of plant proteases in other reports [22,25,31-33].

Protein Concentration

Protein concentrations were determined spectrophotometrically (absorbance at 280 nm) as well as by the Bradford assay [17]. BSA was used as standard to generate the calibration curve.

Activity Measurements

The hydrolyzing activity of the protease was monitored using denatured BSA, as described by Tognil [18]. For activity measurement, about 20 µl suitably diluted enzyme solution was added to 480 µl of 1% BSA in 50 mM sodium acetate (pH 4.5). The mixture was incubated at 45 °C for 20 min and the reaction was stopped by adding 500 µl of 5% TCA. Then the assay cocktail was incubated at room temperature for 10 min, and centrifuged at 10,000 g for 10 min to remove the precipitate. After precipitation of undigested substrate by trichloroacetic acid and centrifugation, soluble peptides remained in the supernatant were measured by the Lowry procedure [19]. 0.5 ml of the supernatant was mixed with 2.0 ml of the solution C of Lowry reagent and kept for 10 min at room temperature in a dark place. Then 750 µl mixed of 1/9 (v/v) folin and deionized water was added and kept for 30 min in dark place for color development. A blank was prepared in the same way, but with the addition of TCA to the enzyme solution before the substrate. The absorbance was read at 660 nm and based on a calibration curve for BSA protein, the peptide fragment concentration measured. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of peptide fragment per minute under the conditions described. The specific activity is expressed in units of enzyme activity/mg of protein.

Electrophoresis and Zymography

Assessment of the homogeneity of the enzyme at different stages of purification, as well as molecular mass determination of the purified enzyme was conducted using 12% SDS-PAGE under reducing and non-reducing conditions according to the method of Laemmli [20]. The proteins were stained with Coomassie brilliant blue R-250. For zymography, 0.1% gelatin was added in the 12% polyacrylamide gel. The gel was run at 20 mA for 2 h and soaked in 2% Triton X-100 to displace the SDS. Gels were then incubated in reaction buffer (50 mM sodium acetate at pH 4.5) for 1 h at 37 °C and later stained with Coomassie Brilliant Blue R-250. The unstained region of the gel reveals the gelatinolytic activity of the enzyme.

Effect of Temperature and pH on Microsciadin Activity and Stability

The optimum temperature for enzyme activity was determined by conducting the assay at various temperatures from 20 to 90 °C. The thermostability of the enzyme was measured after pre-incubation of the enzyme in 50 mM sodium-acetate buffer (pH 4.5) for 30 min at various temperatures. The effect of pH on protease activity was determined by incubating the reaction mixture at pH values ranging from 3.0 to 10.0, in the following mix buffer system: 50 mM sodium-acetate (pH 4.0-5.5), 25 mM sodium-phosphate (pH 6.0-7.5), 50 mM Tris-HCl (pH 8.0-9.0) and 50 mM glycine-HCl or -NaOH (pH 2.0-3.5 and 9.5-12.0). To check the pH stability, the enzyme solution (10 µl) was mixed with 190 µl of each buffer solution and after incubation for 1 h at 25 °C, protease activity was measured under standard assay conditions.

Effect of Various Inhibitors and Metal Ions on the Enzyme Activity

The effect of inhibitors on the protease activity was examined after the protease had been pre-incubated with inhibitor for 30 min at 37 °C, and the residual activity was determined by standard assay method. The concentrations of inhibitors (in the pre-incubation mixture) are listed in Table 2. For determining the influence of metal ions, the

same procedure was used. The level of inhibition was expressed as a percentage of the activity remaining (with inhibitor) of the control activity (without inhibitor). The ability of the enzyme to retain its activity in the presence of denaturants (Urea and GudHCl) and organic solvents (ethanol, methanol, butanol and acetonitrile) was also examined. The enzyme was incubated under specified conditions of chemical denaturant or organic solvent for 1 h at 37 °C, and the residual activity was determined. Reproducibility of the data presented was confirmed by repeating the experiments at least three-times.

Milk-Clotting Activity

Milk-clotting activity (MCA) was determined according to the methods described by Arima [21] with slight modification. 10% skim milk in 10 mM $CaCl_2$ was prepared and the pH was adjusted to 6.5. The substrate (2.0 ml) was pre-incubated for 5 min at 37 °C, and 0.2 ml of the suitably diluted enzyme was added, and the curd formation was observed while manually rotating the test tube occasionally. The end point was recorded when discrete particles were discernible. One milk-clotting unit was defined as the amount of enzyme that clots 10 ml of the substrate in 40 min. The milk-clotting activity/proteolytic activity (MCA/PA) ratio of the enzyme was also measured.

RESULTS AND DISCUSSION

Purification of Microsciadin

The first step to introduce a candidate enzyme for industrial applications is a serious concern to find suitable methods for its isolation. Afterward, the purified enzyme was used to evaluate its biochemical characterization which would be helpful to establish the usefulness, or otherwise, of the enzyme in such processes. This report describes the partial purification and characterization of a novel isoform of the cysteine protease from the latex of an endemic species, *Euphorbia microsciadia* which is called microsciadin. The crude enzyme obtained from the latex of the *Euphorbia microsciadia*, after degumming process was subjected to cation-exchange chromatography at two different pH. The elution profile from the SP-Sepharose column at pH~4.5 revealed two peaks, as shown in Fig. 1a. The first unbound eluted fractions did not show any proteolytic activity. The second peak contained the target enzyme which was purified 2.84-fold with 44.3% yield and a specific activity of 46.21 U/mg. To further purification, the active fractions of the second peak were pooled, dialyzed against 10 mM sodium acetate buffer (pH 5.5) and again loaded on the same cation exchange column. Upon loading the processed soluble protein on the column, followed by appropriate elution, the resulting profile resolved into three peaks (Fig. 1b), with peak I being the highest in magnitude both in terms of proteolytic activity and homogeneity. Besides, the majority of the total activity loaded on the column was eluted in this peak. Elution with linear gradient of 0.0-0.75 M NaCl, resulted in fractions II and III that exhibited some protein content with measurable proteolytic activity (Fig. 1b). Sequential loading on the SP-Sepharose column at pH of 4.5 and 5.5, purified the enzyme to 7.77-fold with a recovery of 21.7% and specific activity of 126.59 U/mg. Size exclusion chromatography was conducted to further purification of the target containing enzyme in the first peak of the second ion exchange column. Only one peak with protease activity was obtained when the partially purified enzyme was applied to Sephacryl S-200 column (See Fig. 1c). An overall, 11.6-fold purity was achieved with a yield of 20.7% and specific activity of 189 U/mg. The 11.6-fold purity of microsciadin suggested that the target enzyme is a highly abundant constituent of the *Euphorbia* latex. The purification results are summarized in Table 1.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed a partially purified band with an apparent molecular mass of ca. 60 kDa, and gelationolytic activity was detected by Zymogram (Fig. 2). The molecular weight of microsciadin is higher than other plant cysteine proteases reported so far. The molecular weight of cysteine proteases isolated from germinating cotyledons of *Horse gram* [22], *Curcuma longa* [23], *Euphorbia nivulia* [24] and Ginger rhizome [25] varied between 30-45 kDa. Albeit, two serine proteases from the latex of *Euphorbia supina* and *Euphorbia neriifolia* have been reported with apparent molecular masses of 80 and 94 kDa, respectively [26,27].

Effect of pH and Temperature on Microsciadin Activity

The proteolytic activity of microsciadin was monitored

at different pH values from 3.0 to 7.0 and various temperatures in the range of 20-90 °C, using BSA as substrate. The temperature and pH optima for the activity of enzyme were 45 °C and 4.5, respectively (Fig. 3). The temperature profile of the protease revealed mesophilic feature and showed that microsciadin retained 75% of its maximum activity at temperature ranges of 30-60 °C. The stability of enzymes is one of the most important factors that limit their industrial application. Since a long time assay applied to evaluate the temperature dependency of microsciadin activity, the temperature stability curve showed the almost similar pattern with its temperature profile (Fig. 3a). Nevertheless, the optimum temperature for cheese ripening is about 37 °C, and since added biocatalysts should be inactivated above 60 °C to prevent further proteolysis and cheese bitterness, microsciadin indicates suitable potential for using in the dairy industry. Based on pH profile data, the acidophilic characteristic of the enzyme exhibits it is appropriate to use as cheese ripening additive. Moreover, evaluating the pH stability profile of the enzyme revealed that more than 80% of its proteolytic activity was observed in the pH range of 4.0-8.0 (Fig. 3b).

The enzyme obeyed Michaelis-Menten kinetics when using BSA as substrate in standard assay conditions. The

Table 1. Summary of Purification Steps of Microsciadin from *Euphorbia Microsciadia* Latex

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude latex	242.34	3945.6	16.28	100	1
Ammonium Sulfate	153.85	3855.4	25.06	97.7	1.54
SP-Sepharose (pH~5.5)	37.8	1746.7	46.21	44.3	2.84
SP-Sepharose (pH~4.5)	6.75	854.5	126.59	21.7	7.77
Sephacryl S-200	4.32	816.5	189.0	20.7	11.60

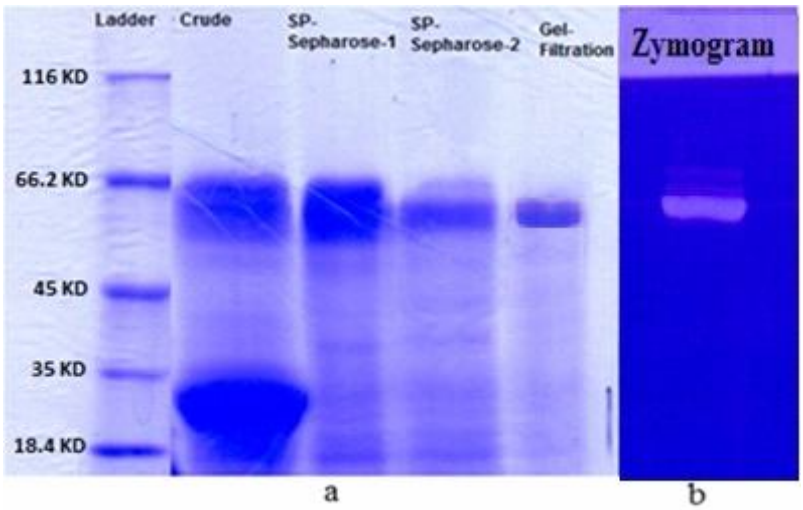

Fig. 2. (a) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), including purification steps, stained with Coomassie blue R-250. Molecular mass standards: β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35 kDa), β-lactoglobolin (18.4 kDa). (b) Zymogram of 10 μg of partially purified enzyme, showing the proteolytic activity of the microsciadin.

effect of increasing substrate concentration on the reaction velocity follows typical Michaelis-Menten equation with BSA. The nature of the kinetics with respect to the substrate is typically hyperbolic, and at higher concentrations of the substrate, microsciadin activity attains saturation. K_m and V_{max} values of microsciadin for BSA using the Lineweaver-Burk plot were calculated 0.106 mM and 0.234 U, respectively.

Effect of Various Inhibitors on the Protease Activity of Microsciadin

Effect of inhibitors on the activity of microsciadin is

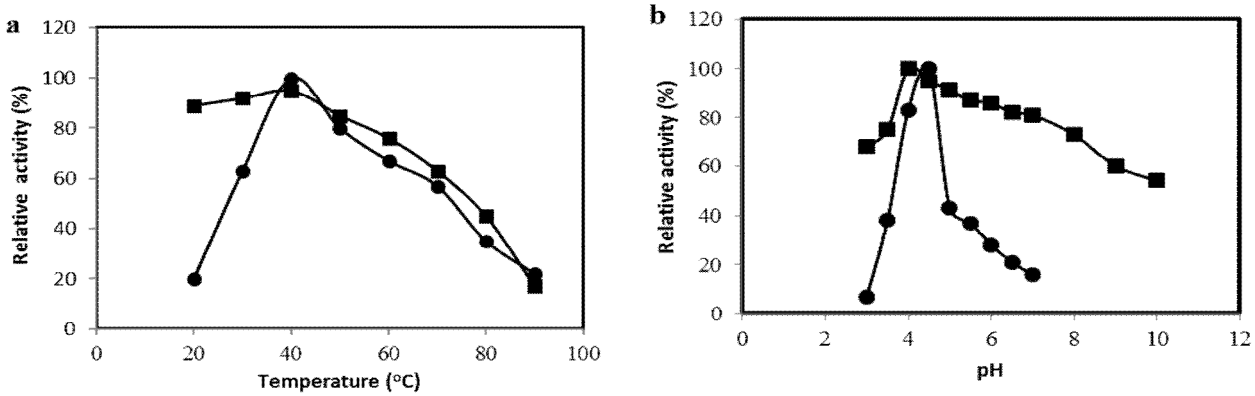

Fig. 3. (a) Effect of temperature, on activity (●) and stability (■) of microsciadin. For optimum temperature, activity was determined at 20-90 °C as described in Materials and methods. For determining thermostability, the protease was incubated for 30 min at different temperatures and cooled for 30 min on ice before assay. (b) Effects of pH, on activity (●) and stability (■) of microsciadin. For pH optima, activity was determined at pH 3.0-7.0 as described in Materials and methods. For pH stability profile, the enzyme was incubated at different pH for 1 h at 37 °C and the remaining activity was determined under standard assay conditions.

Table 2. Effect of Inhibitors on Activity of Microsciadin

Inhibitor type	Inhibitor name	Concentration (mM)	Residual activity (%)[a]
Control	-	-	100
Serine/Cysteine protease	Leupeptin	1	87.00 ± 0.05
Serine protease	Aprotinin	0.3	101.06 ± 0.75
	Chymostatin	1	88.96 ± 0.95
	PMSF	5	69.00 ± 0.61
	Pefabloc SC	4	96.00 ± 0.88
Cysteine protease	E-64	0.1	28.00 ± 0.79
	Iodoacetamide	1	12.12 ± 0.95
	$HgCl_2$	0.1	2.00 ± 0.89
Aspartic protease	Pepstatin	1	97.10 ± 0.87
Metalloprotease	EDTA-Na_2	5	103.00 ± 1.15
	1-10-Phenanthroline	5	97.12 ± 0.89
	Phosphoramidon	1	101.00 ± 0.91

[a]Residual activities are shown as the mean ± SD (n = 3).

Table 3. Biochemical Comparison of some Proteases Isolated from *Euphorbia* Genus

Euphorbia species	Protease type	Designated as	MW (kDa)[a]	pH Optimum[a]	Temp. Optima[a]	Ref.
Euphorbia cotinifolia	metalloprotease	Cotinifolin	79.76	7.0-8.0	50	[33]
Euphorbia hirta	Serine protease	Hirtin	34	7.2	50	[34]
Euphorbia lathyris	Serine protease	euphorbain 1	43	7.0-7.5	N	[30]
Euphorbia microsciadia	Cysteine protease	**Microsciadin**	60	4.5	45	This study
Euphorbia milii	Serine/Cysteine protease	Eumiliin	29.81	8.0	40	[32]
Euphorbia milii	Serine protease	Miliin	N	9.0	35	[35]
Euphorbia neriifolia	Serine protease	Neriifolin	35.24	8.5	55	[27]
Euphorbia nivulia	Cysteine protease	Nivulian	43.67	6.6	45	[24]
Euphorbia pulcherrima	Serine protease	euphorbain p	N	7.0	N	[31]
Euphorbia supina	Serine protease	Protease B	80	8.0	60	[26]

[a]N: not determined.

Table 4. Effect of Various Agents on Microsciadin Activity

Agents	Concentration[b]	Relative activity (%)[a]
None	-	100
$CuCl_2$	5 mM	96.00 ± 0.21
$CaCl_2$	5 mM	110.00 ± 0.12
$MgCl_2$	5 mM	106.00 ± 0.15
$MnCl_2$	5 mM	115.00 ± 0.16
H_3Bo_3	5 mM	105.00 ± 0.16
$CdCl_2$	5 mM	95.00 ± 0.15
$CoCl_2$	5 mM	80.00 ± 0.13
$NiCl_2$	5 mM	86.00 ± 0.12
$FeCl_2$	5 mM	82.00 ± 0.13
$ZnCl_2$	5 mM	89.00 ± 0.14
$HgCl_2$	5 mM	2.00 ± 0.12
Urea	7.0 M	100.00 ± 0.14
GndCl	3.0 M	100.00 ± 0.17
NaCl	2.0 M	100.00 ± 0.16
Methanol	40%	100.00 ± 0.12
Ethanol	70%	100.00 ± 0.13
Butanol	30%	75.00 ± 0.21
Acetonitrile	30%	86.00 ± 0.17
DMSO	50%	83.00 ± 0.12
Triton X-100	0.8%	99.00 ± 0.18
H_2O_2	3.0%	70.00 ± 0.11

[a]Relative activities are shown as the mean ± SD (n = 3). [b]The dimension of solvents and other aqueous compounds concentration is (V/V).

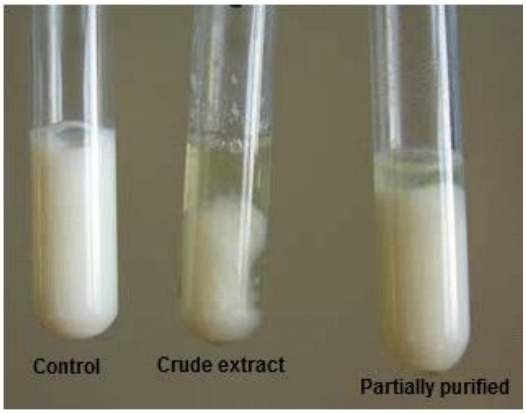

Fig. 4. Coagulation of skim-milk by crude extract and purified microsciadin from *Euphorbia microsciadia* latex.

shown in Table 2. Inhibitors of metalloproteases, aspartic proteases, and to lesser extent serine proteases do not affect the enzyme activity. Phenylmethylsulfonyl fluoride (PMSF) as a serine inhibitor affects the enzyme by 31% inhibition. In spite of optimum pH of 4.5, microsciadin could not be an acid protease because it is not inhibited by Pepstatin. Moreover, resistance to inhibition by Chymostatin, Pefabloc and aprotinin as well as slight inhibition by PMSF indicates that the enzyme is not a serine protease. Lack of inhibition by EDTA, 1,10-phenanthroline and Phosphoramidon suggests that metal independent character of the enzyme. However, inhibition of the enzyme by E-64, iodoacetamide and $HgCl_2$ indicated that microsciadin is a thiol protease belongs to cysteine protease family (See Table 2). Similar kind of inhibition profile was also reported for some plant cysteine proteases, such as papain [28], ervatamin [3], P1, P2 ginger proteases [29] and cysteine protease from ginger rhizome [25]. Until now, most reported proteases from other *Euphorbia* species are in the serine protease family and show different characteristics compared to microsciadin. Table 3 exhibits biochemical properties of various proteases which have been isolated from *Euphorbia* genus so far [24,26,27,30-35]. As shown in Table 3, most reported proteolytic activities classified in the serine protease family and only a cysteine protease isolated from *E. nivulia* with a molecular mass of 43.67 kDa which exhibited neutral properties and its optimum pH was 6.6 [24]. So, microsciadin could be introduced as a new cysteine protease which is isolated from the latex of an endemic *Euphorbia* species, *E. micriosciadia* Boiss.

Effect of Different Reagents on Microsciadin Stability

Influence of metallic salts, chaotropic agents and organic solvents on the protease activity is depicted in Table 4. Using 5 mM final concentration of each metal ions, the partially purified protease was slightly inhibited by Co^{2+}, Ni^{2+}, Fe^{3+} and Zn^{2+} ions and significantly inhibited by Hg^{2+} ions. However, other divalent metal ions like Ca^{2+} ,Cu^{2+}, Mg^{2+}, Mn^{2+} and Cd^{2+} did not show any inhibitory impact on the proteolytic activity of the enzyme. The weak inhibition of Co^{2+}, Ni^{2+}, Fe^{3+} and Zn^{2+} ions might be due to the formation of bridges with active site residues of the enzyme such as His and Ser [36]. The inhibition of microsciadin by Hg^{2+} suggested the existence of a -SH group containing amino acids in or near the active site of the enzyme.

Complete retention of proteolytic activity was observed in 7.0 M urea, 3.0 M GudHCl (guanidine hydrochloride), 2.0 M NaCl, 40% methanol, and 70% ethanol after 1 h incubation. Moreover, the enzyme retained more than 75% of its activity when incubated for 1 h in 30% butanol, 30% acetonitrile, and 50% DMSO (Table 4). In spite of the general opinion in regard to cysteine proteases which are readily inhibited by metal ions [27], the microsciadin reveals a fairly stable enzyme toward most metal ions and even chemical denaturants. Suitable operational stability in the presence of chaotropic agents and some toxic organic solvents exhibited the biotechnological impact of the microsciadin as a biocatalyst. The observed high stability of the protease under various conditions such as strong denaturants, organic solvents, and various metallic salts could facilitate exploring the utilization of the enzyme in industrial and biotechnological applications.

Divalent metal ions are involved in enzyme catalysis in a variety of ways which include activation of electrophiles or nucleophiles and bridging an enzyme with substrate together by means of coordinate bonds as well as holding reacting groups in the required three dimensional orientations [37]. Ca^{2+}, Mg^{2+} and Mn^{2+} increased the enzyme activity and considering the relative abundance of

these cations in industrial processes, which suggests that the enzyme can easily be activated during the course of an industrial process. The observed effects of other divalent cations (analyzed herein), may suggest a probable modification of the enzyme protein conformation after binding in a different pattern [8] and hence causing a decrease in enzyme activity.

Milk Coagulation

The microsciadin strongly coagulated skimmed milk and formed a white and firm curd. Furthermore, the ratio of milk-clotting activity to proteolytic activity of the enzyme was determined and compared with Calf rennet. It was found that the ratio of milk-clotting activity to the proteolytic activity of microsciadin was 20.14 comparable to Calf rennet with 26.39 MCA/PA [38]. As shown in Fig. 4, the partially purified enzyme greatly coagulated the skim milk compared to crude extract and the control sample. Moreover, to the best of our knowledge, this is the first report of a milk-coagulating cysteine protease from *Euphorbia microsciadia* Lin. Although the most popular proteases which are used in cheese making are from aspartic proteases, but some other types of proteases have been introduced to be suitable in cheese processing [39,40].

CONCLUSIONS

Application of orthologous enzymes such as papain and bromelain in food industries [41] suggest that the microsciadin could be a suitable candidate for using in meat tenderization and other food industrial field. Besides, most of the proteolytic enzymes clot milk, but the ratio of the milk-clotting activity and proteolytic activity is a useful indicator of the protease efficiency when used as a coagulant for cheese making. The capacity of this protease to produce milk curds, beside its high ratio of milk-clotting to proteolytic activity, could make it useful as a potential cheese making enzyme from a vegetable source in the cheese industry. Despite the extensive research on several aspects of proteases from ancient times, there are several gaps in our knowledge of these enzymes, and there is tremendous scope for improving their properties to suit projected applications. The next line of development would include analyzing the structure-function relationship, using genetic approaches to design recombinant systems for overproduction of the plant enzyme and also elucidating the tissue localization of the protease. In addition, further purification is required to get a high pure enzyme to analyze its structure spectroscopically and more studies necessary to investigate the quality of both the milk curds and the cheese formed to confirm microsciadin's efficacy in the dairy industry.

ACKNOWLEDGEMENTS

This work was funded by the research affairs of Shiraz University, Shiraz, Iran.

REFERENCES

[1] A. Sumantha, C. Larroche, A. Pandey, Food Technol. Biotechnol. 44 (2006) 211.

[2] M.B. Rao, A.M. Tanksale, M.S. Ghatge, V.V. Deshpande, Microbiol. Mol. Biol. Rev. 62 (1998) 597.

[3] S. Nallamsetty, S. Kundu, M.V. Jagannadham, J. Protein Chem. 22 (2003) 1.

[4] R. Sathya, B. Pradeep, J. Angayarkanni, M. Palaniswamy, Biotechnol. Bioprocess Eng. 14 (2009) 788.

[5] R. Tomar, R. Kumar, M. Jagannadham, J. Agric. Food Chem. 56 (2008) 1479.

[6] I.A.M. Ahmed, I. Morishima, E.E. Babiker, N. Mori, Food Chem. 116 (2009) 395.

[7] R.P. Yadav, A.K. Patel, M. Jagannadham, Process Biochem. 46 (2011) 1654.

[8] M. Ibrahim, J. Olatominwa, A. Aliyu, M. Bashir, A. Sallau, Int. J. Biol. 4 (2012) 79.

[9] A.N. Singh, A.K. Shukla, M. Jagannadham, V.K. Dubey, Process Biochem. 45 (2010) 399.

[10] A.N. Singh, V.K. Dubey, Appl. Biochem. Biotechnol. 164 (2011) 573.

[11] M. Grudkowska, B. Zagdanska, Acta Biochim. Pol. (2004) 609.

[12] M.M. Cowan, Clin. Microbiol. Rev. 12 (1999) 564.

[13] S.M. Ghanadian, A.M. Ayatollahi, S. Afsharypuor, S.H. Javanmard, N. Dana, J. Nat. Med. 67 (2013) 327.

[14] S.A. Ayatollahi, S.A.R. Mortazavi, Feyz J. Kashan Uni. Med. Sci. 8 (2004) 39.
[15] A.R. Jassbi, Phytochem. 67 (2006) 1977.
[16] S.M. Ghanadian, A.M. Ayatollahi, S. Afsharypour, S. Hareem, O.M. Abdalla, *et al.*, Iranian J. Pharm. l Res. 11 (2012) 925.
[17] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[18] G. Togni, D. Sanglard, R. Falchetto, M. Monod, FEBS Lett. 286 (1991) 181.
[19] O.H. Lowry, N.J. Rosebrough, A. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[20] U.K. Laemmli, Nature 227 (1970) 680.
[21] K. Arima, J. Yu, S. Iwasaki, Methods Enzymol. 19 (1970) 446.
[22] R. Jinka, V. Ramakrishna, S.K. Rao, R.P. Rao, BMC Biochem. 10 (2009) 28.
[23] R. Nagarathnam, A. Rengasamy, R. Balasubramanian, J. Sci. Food Agric. 90 (2010) 97.
[24] S.B. Badgujar, R.T. Mahajan, Indian J. Nat. Prod. Resour. 3 (2012) 152.
[25] M.M. Hashim, D. Mingsheng, M.F. Iqbal, C. Xiaohong, Phytochem. 72 (2011) 458.
[26] K. Arima, T. Uchikoba, H. Yonezawa, M. Shimada, M. Kaneda, Phytochem. 53 (2000) 639.
[27] R.P. Yadav, A.K. Patel, M. Jagannadham, Food Chem. 132 (2012) 1296.
[28] C. Ryan, M. Walker-Simmons, Proteins Nucleic Acids 6 (1981) 321.
[29] H.P. Su, M.J. Huang, H.T. Wang, J. Sci. Food Agric. 89 (2009) 1178.
[30] K. Lynn, N. Clevette-Radford, Phytochem. 23 (1984) 682.
[31] K. Lynn, N. Clevette-Radford, Biochim. Biophys. Acta 746 (1983) 154.
[32] K. Fonseca, N. Morais, M. Queiroz, M. Silva, M. Gomes, *et al.*, Phytochem. 71 (2010) 708.
[33] R. Kumar, K.A. Singh, R. Tomar, M.V. Jagannadham, Plant Physiol. Biochem. 49 (2011) 721.
[34] G.K. Patel, A.A. Kawale, A.K. Sharma, Plant Physiol. Biochem. 52 (2012) 104.
[35] L. Moro, H. Cabral, D. Okamoto, I. Hirata, M. Juliano, *et al.*, Process Biochem. 48 (2013) 633.
[36] K.S. Larsen, D.S. Auld, Biochemistry (Mosc) 30 (1991) 2613.
[37] S. Advani, P. Mishra, S. Dubey, S. Thakur, Biochem. Biophys. Res. Commun. 402 (2010) 177.
[38] M.A. Mazorra-Manzano, T.C. Perea-Gutiérrez, M.E. Lugo-Sánchez, J.C. Ramirez-Suarez, M.J. Torres-Llanez, *et al.*, Food Chem. 141 (2013) 1902.
[39] A.R. Lo Piero, I. Puglisi, G. Petrone, J. Agric. Food Chem. 50 (2002) 2439.
[40] J. Ageitos, J. Vallejo, A. Sestelo, M. Poza, T. Villa, J. Appl. Microbiol. 103 (2007) 2205.
[41] V.K. Dubey, M. Pande, B.K. Singh, M.V. Jagannadham, African J. Biotechnol. 6 (2007) 1077.