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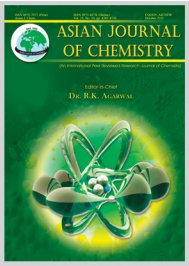

# Cysteine Protease from Primrose (*Primula vulgaris*)

YASAR DEMIR[1], NAZAN DEMIR[1,*], ELIFE KAYA[2] and BAHAR AYDIN[2]

[1]Department of Chemistry, Faculty of Sciences, Mugla University, Mugla, Turkey
[2]Chemistry Department, College of Education, Atatürk University, 25240 Erzurum, Turkey

*Corresponding author: Fax: +90 252 2360948; Tel: +90 252 2314439; E-mail: demirn@yahoo.com

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Proteases are enzymes that perform important functions in organisms and are used for a variety of objectives *in vitro*. In recent years, proteases have been used for clinical, pharmaceutical (alimentary digestion, antiinflammatory, *etc*.) and industrial applications (cheese production, meat tenderizing, leather tanning). In this research, a protease has been purified from primrose (*Primula vulgaris*) and characterized. *Primula vulgaris* has been used for medicine since ancient times. The plant grows abundantly in certain regions of Turkey. Ammonium sulphate fractionation and a CM Sephadex column were used for purification of the enzyme. The purification enzyme has an optimum pH = 6.0 and its optimum temperature was 60 ºC. The $v_{max}$ and Km values determined by Lineweaver-Burk graphics were 6.6 mg/L dak and 0.19 mg/mL respectively. The purification degree and the molecular mass of the enzyme (30 kDα) were determined by SDS-PAGE and gel filtration chromatography. It was investigated whether the purified and characterized protease could cause milk to congeal or digest chicken and cow meat. The results show that protease can be used for industrial production.

**Key Words: Protease, Primrose (*Primula vulgaris*), Food production.**

## INTRODUCTION

The existence of two forms of flower in species of *Primula* has long been documented; Darwin's paper to the Linnean society in 1862[1] and his subsequent book. The different forms of flowers in plants of the same species[2], describe his observations on the structure and breeding behaviour of pin and thrum flowers of *Primula* species including the primrose, *Primula vulgaris*. Pin flowers are characterized by a long style with a stigmatic surface presented at the corolla mouth. In these flowers, the anthers are attached to the petals by short filaments half way down the corolla tube. By contrast, the stamens in thrum-form flowers are presented at the mouth of the flower with the stigma midway down the corolla[1,2].

Enzymes that hydrolyze peptide bonds, peptides or proteins are classified as proteases. Proteases are also called proteolytic enzymes. They are considered to have a protective role against plant parasites and herbivores. Nowadays, seven catalytic types of proteases (peptidases) are recognized in which serine, cysteine, aspartic acid, glutamic acid, threonin, metalloprotease or peptidase of unknown catalytic type are involved in catalysis. Cysteine proteases have an essential cysteine residue at the active site[3,4].

Proteases have important physiological roles. They are used in different branches of industry, mainly in leather, pharmaceutical, detergent and food industries[4]. The coagulation of milk is the main step for producing cheese and is generally done by using the rennin enzyme. This protease enzyme is obtained from the stomach of calves[5]. Our aim is to explore an alternative enzyme of vegetal origin for use in the production of cheese, which could be cheaper and more useful than rennin, so we have chosen to purify and characterize a protease from primrose (*Primula vulgaris*). In the present paper, purification, characterizations, substrate specificity, along with other properties of this protease, which was named primrosen, are reported. Later studies will determine whether this characterized enzyme can be used in the production of cheese, predigested meat and sauces used in the preparation of meat.

## EXPERIMENTAL

**Purification of protease:** Primrose (*Primula vulgaris*) were collected from Tarsus, southern Turkey in July, 2008. They were kept in deep freeze until they were used. Capsules (25 g) of the plant were ground in liquid $N_2$ and then homogenized in a blender with 50 mL of distilled water and centrifuged at 5000 × g for 1 h. The supernatant was used for the enzyme purification procedure.

Protease was purified from the supernatant. It was partially purified by precipitation with $(NH_4)_2SO_4$ followed by ion exchange chromatography on CM Sephadex. Solid $(NH_4)_2SO_4$ was added to the supernatant containing 50 % $(NH_4)_2SO_4$ to

obtain 70 % fraction. After it was mixed in an ice-bath for 1 h with magnetic stirring, it was centrifuged (10 000 × g for 0.5 h at 4 ºC). The supernatant was discarded and the precipitate was dissolved in 0.01 M acetate buffer (pH = 5.0) and dialyzed against the same buffer.

The dialyzed solution (0.01 M acetate buffer, pH = 5.0) was subjected to cation exchange chromatography using a CM Sephadex fast flow column. The bound proteins were eluted with a 0.01 M acetate buffer (pH = 5.0) using a linear gradient of 0 to 1 M NaCl. Protein elution was monitored spectrophotometrically by measuring the absorbance at 280 nm. Activity was measured using casein as the assay substrate. After the active eluents were collected, they were dialyzed against 10 Mm *Tris*-HCl (pH = 7). The dialyzate was used for the characterization procedures. The protein concentration was determined according to Bradford's method using bovine serum albumin as a standard[6].

**Determination of protease enzyme activity:** Proteolitic activity was determined by the casein digestion method in the presence of 1 % casein. The stock substrate solution was prepared by dissolving casein (1.0 g) in 99 mL of phosphate buffer (0.1 M, pH = 7.6). This solution was aged for 0.5 h in a hot water bath and the durability of thus prepared solution was about one week.

Purified enzyme solution (0.5 mL) was added to 1 mL of casein solution to start the reaction. The reaction mixture was incubated at 40 ºC for 20 min and the reaction was stopped by adding trichloroacetic acid (3 mL, 5 % by mass per volume). After 1 h, proteins that were not digested were separated by centrifugation (10 000 × g for 5 min). The supernatant was filtered and 0.1 mL of the sample was taken for protein determination as mentioned above[6]. One enzyme unit (U) is expressed as the amount of protein digested by the enzyme per min[7].

Kinetic parameters for the protease activity, $V_{max}$ and $K_m$ values, were determined by using different substrates (gelatin, azocasein, casein, hemoglobin, azoalbumin and bovine serum albumin).

**SDS polyacrylamide gel electrophoresis (SDS-PAGE):** SDS-PAGE was performed for molecular mass determination and control of enzyme purity after its purification. It was carried out in 3 and 10 % acrylamide concentrations for the stacking and running gels, respectively, each containing 0.1 % SDS according to Laemmli[8]. The sample (20 mg) was applied to the electrophoresis medium. Bromothymol blue was used as tracking dye. Gels were stained for 1.5 h with 0.1 % Coomassie Brilliant Blue R-250 in 50 % methanol, 10 % acetic acid and 40 % distilled water. It was destained by washing several times with 50 % methanol, 10 % acetic acid and 40 % distilled water[8]. The electrophoretic pattern was photographed.

**Molecular mass determination by gel filtration:** A column (3 cm × 70 cm) of Sephacryl S-100 was prepared. The column was equilibrated for 4 h with the buffer (0.05 M $Na_3PO_4$, 1 mM dithioerythretol, pH = 7) until the absorbance at 280 nm was zero. The standard protein solution (egg ovalbumine, 66 000 Da; bovine albumin, 45 kDa; pepsin, 34.7 kDa; trypsinogen, 24 kDa; lysozyme, 14.3 kDa) was added to the column. The purified protease enzyme was added into the column separately and then eluted under the same conditions. The flow rate through the column was 20 mL/h. The elution volume was compared with standard proteins[9].

**Carbohydrate content:** Carbohydrate content of primrosen was determined by the phenol-sulphuric acid method[10]. Different concentrations of purified capparin (0.1 to 1 mg in a volume of 10 mL of buffer) and 25 mL of 4 % aqueous phenol were added to each tube. After 5 min, 200 mL of concentrated $H_2SO_4$ were added and the increase in absorbance was measured at 492 nm. Carbohydrate content of the enzyme was determined by composing absorbance with a galactose standard.

**Effect of various metal ions on the protease activity of primrosen:** The effect of various metal ions (10 mM $Ca^{2+}$, $Mg^{2+}$, $Hg^{2+}$, $Co^{2+}$ and $Zn^{2+}$) on the protease activity of primrosen was determined by incubating 0.5 mL of enzyme in the presence of increasing concentrations of metal ions in 1.5 mL of the final volume of 0.05 M *Tris*-HCl buffer, pH = 7 at 40 ºC for 20 min and assayed with casein as substrate. A control assay of the enzyme activity was done without inhibitors and the resulting activity was taken as 100 %.

**Effect of compounds on the protease activity of primrosen:** The effect of various compounds on the activity of primrosen was determined using thiol specific inhibitors, activators and non-specific compounds. A volume of 0.5 mL of the enzyme was incubated in the presence of increasing concentrations of thiol reagents in 1.5 mL of the final volume of 0.05 M *Tris*-HCl buffer, pH = 7, at 40 ºC and assayed with casein as substrate. The compounds used were phenylmethanesulphonyl fluoride (PMSF), diisopropyl fluorophosphate (DIPF), β-mercaptoethanol, sodium dodecyl sulphate (SDS), phenanthrene (PHT), ethylenediaminetetra-acetic acid (EDTA) and iodoacetamide at concentrations of 10, 1 and 0.1 mM. A control assay of the enzyme activity was done without inhibitors and the resulting activity was taken as 100 %.

**Coagulation of milk using the purified protease enzyme:** The modified Berridge method[11] was used in the determination of the coagulation of milk, which was determined against the blank sample used as control, by changing the parameters of temperature (5, 20, 40 and 60 ºC) and time (1 to 24 h). Milk (10 mL) was put into each of the two tubes, with 1 mL of buffer (10 mM *Tris*--HCl, pH = 7.0) added into the control tube and 1 mL of the purified enzyme solution added into the other tube. Control and enzyme tubes were stirred and kept at various temperatures from 5 to 60 ºC, for 1 to 24 h. At the end of 24 h, no coagulation was observed in the control tube. The time was recorded when the protease enzyme caused coagulation in the test tube[7].

**Predigestion of chicken and cow meat using the purified protease:** A mass of 1 g of chicken or cow meat was put into each of the two tubes, along with 1 mL of buffer (10 mM *Tris*-HCl, pH = 7.0) added to the control tube and 1 mL of purified enzyme solution was added to the test tube. Both of the tubes were stirred and kept at different temperatures from 5 to 60 ºC for 20 min. At the end of the experiment, the amount of protein in the supernatant was determined by the Bradford method[6,12].

TABLE-1
PURIFICATION OF PROTEASE FROM PRIMROSE *(Primula vulgaris)*

| Enzyme fraction | Volume (V/mL) | Activity (U/mL) | Total activity | | Protein (mg/mL) | Specific activity (EU/mg) | Purification fold |
|---|---|---|---|---|---|---|---|
| | | | U | % | | | |
| Crude extract | 15 | 0.064 | 0.96 | 100 | 0.261 | 0.24 | - |
| CM Sephadex | 5 | 1.17 | 5.85 | 609.3 | 0.033 | 35.4 | 147.5 |

## RESULTS AND DISCUSSION

News of diseases passing over from animals to humans (*e.g*. mad cow) is so prevalent in the press that suspicion of contamination in food production has increased. The cost of animal origin enzymes used in food production is high and it does not meet increasing processing demands. We have searched for an alternative enzyme that is cheap and can be obtained easily from primrose (*Primula vulgaris*). For this purpose, the activity of protease (primrosen) in primrose was investigated, characterized and used in the coagulation of milk.

The main purpose of choosing primrose (*Primula vulgaris*) for this study is that this plant contains an abundance of proteases, while on the other hand, primrose can be grown readily in the rural regions of Turkey. In addition, primroses are used pharmaceutically and are known to be a medicinal plant for public use and have no toxic effect.

Primrosen was purified by precipitation with $(NH_4)_2SO_4$ followed by cation-exchange chromatography. Casein was used as a substrate in the determination of protease activity in the protein eluted from the CM Sephadex column as shown (Fig. 1). The degree of enzyme purification for each step is shown in Table-1.

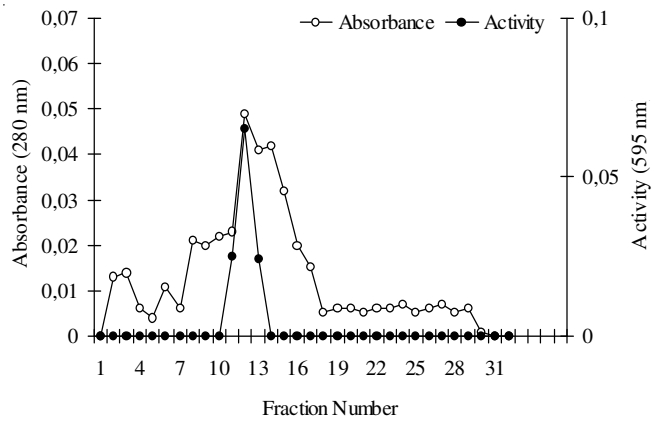


Fig. 1. CM Sephadex ion-exchange chromatography of protease from primrose (*Primula vulgaris*)

The purified protease was examined by SDS-PAGE (Fig. 2). The molecular mass of the enzyme was determined as 30 kDa by using the gel filtration chromatography and comparing it with the standard.

An enzyme showing some selectivity for bonds adjacent to leucine residues has been isolated as a homogeneous protein of molecular mass of 43 kDa from the latex of *Euphorbia lathyris* and is called euphorbain 1[13,14]. The molecular mass of the plant protease isolated from *Calotropis procera* (family Asclepiadaceae) is 28.8 kDa.

The hydrolysis of azoalbumin by the enzyme from the plant *Calotropis procera* was optimal at 55-60 ºC[14]. The activity of protease (primrosen) from primrose (*Primula vulgaris*) was determined in the temperature range of 0-90 ºC. The temperature was increased by increments of 10 ºC from 0 to 90 ºC and the optimal temperature was found to be 60 ºC (Fig. 3). The high temperature optimum of primrosen shows thermal stability of this enzyme, which makes it an excellent choice enzyme for the food industry[5,13].

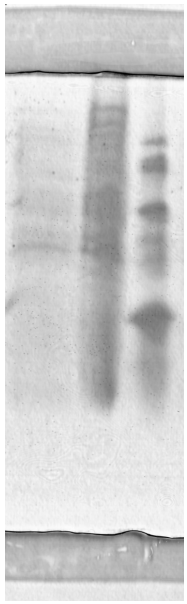

Fig. 2. Electrophoretic pattern of standart proteins 66.0, 45.0, 34.7, 24.0, 18.4, 14.0 kDa (right) and protease from primrose (*Primula vulgaris*) (left).

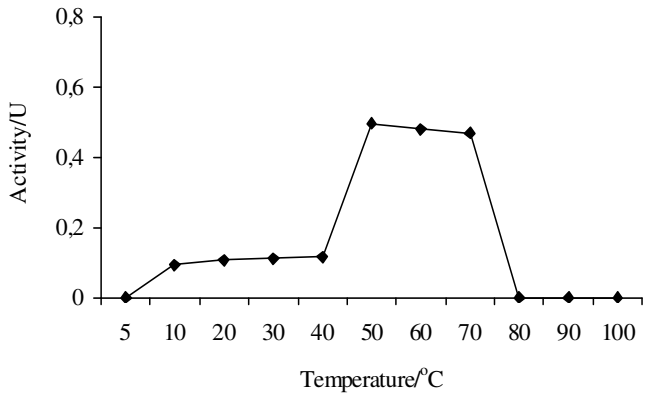


Fig. 3. Effect of temperature on the activity of purified protease from primrose (*Primula vulgaris*)

$K_m$ and $\nu_{max}$ values at optimum pH = 7 and 40 ºC were determined by means of the Linewaver-Burk graph. $K_m$ and $\nu_{max}$ values for six different substrates are shown in Table-2. The protease exhibited the greatest activity with azocasain (0.173 U/mg) and no activity toward gelatin.

TABLE-2
DETERMINATION OF $K_m$ AND $v_{max}$ FOR DIFFERENT SUBSTRATES

| Substrate | Specific activity (U/mg) | Km (μg/L) | $\nu_{max}$ (μg/L min) |
|---|---|---|---|
| Azocasaine | 0.173 | 0.86 | 1.23 |
| Haemoglobin | 0.092 | 0.75 | 1.012 |
| Azoalbumin | 0.143 | 1.03 | 0.75 |
| Gelatin | nd | nd | nd |
| Casaine | 0.047 | 0.066 | 1.136 |

nd: not detected

The maximum protease activity for euphorbain 1 was found by Lynn and Clevette-Radford[13] to be pH = 7-7.5. The hydrolysis of azoalbumin by the enzyme from Calotropis procera was optimum in the range of pH = 7.0-9.0. The activity of capparin was determined over the pH range of 1-10 by increments of 1 pH. The optimal pH value was found to be 7 (Fig. 4). It was observed that the enzyme kept its activity at low pH. Durability of the enzyme is an advantage in the production of food.

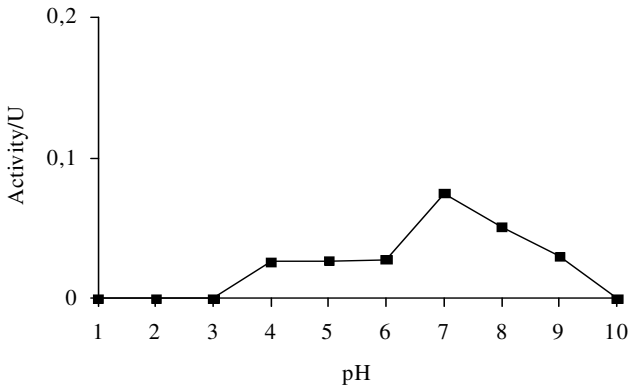


Fig. 4. Effect of pH on the activity of purified protease from primrose (*Primula vulgaris)*

Many proteases have been isolated from fruits and seeds and most of them belong to cysteine superfamily (1). When the effect of metal ions on enzyme activity was measured, all $Hg^{2+}$ concentrations inhibited protease activity (Table-3)[15]. $Ca^{2+}$ and $Fe^{2+}$ activated the enzyme, especially at lower concentrations, while $Co^{2+}$ inhibited the enzyme, although less than $Hg^{2+}$, at high concentrations (Table-3).

Table-4 shows results obtained with some chemical compounds, most of them are inhibitors of some type of proteases, as serine-(DIPF = DIP and PMSF), metallo-(1,10-phenanthroline, EDTA) and cysteine protease (iodoacetamide and some of them by PMSF). Inhibition was observed with all the tested compounds, which made the classification by catalytic type of this protease difficult. The inhibition by iodoacetamide indicates thiol groups from cysteine, but there are many serine and metallo-type peptidases that show significant thiol dependence[16].

## ACKNOWLEDGEMENTS

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TABLE-3
EFFECT OF SOME METAL IONS ON PROTEASE ACTIVITY

| Metal ions | C/mM | Protease activity (%) |
|---|---|---|
| Kontrol | - | 100 |
| $CaCl_2$ | 0.1 | 121 |
| | 1 | 69 |
| | 10 | 50 |
| $MgCl_2$ | 0.1 | 73.8 |
| | 1 | 85.7 |
| | 10 | 92.8 |
| $HgCl_2$ | 0.1 | 78.57 |
| | 1 | 78.57 |
| | 10 | 78.57 |
| $ZnCl_2$ | 0.1 | 52.3 |
| | 1 | 73.8 |
| | 10 | 78.5 |
| $CoCl_2$ | 0.1 | 61.9 |
| | 1 | 50.0 |
| | 10 | 35.7 |
| $FeCl_2$ | 0.1 | 411 |
| | 1 | 311 |
| | 10 | 415 |

TABLE-4
EFFECT OF SOME CHEMICAL COMPOUNDS ON PROTEASE ACTIVITY

| Chemical compounds | C/mM | Protease Activity (%) |
|---|---|---|
| Control | - | 100 |
| β-Mercaptoethanol | 0.1 | 383.23 |
| | 1 | 347.56 |
| | 10 | 357.0 |
| SDS | 0.1 | 288.12 |
| | 1 | 378.56 |
| | 10 | 409.17 |
| EDTA | 0.1 | 326.02 |
| | 1 | 376.32 |
| | 10 | 469.45 |
| Iodoacetamide | 0.1 | 89.80 |
| | 1 | 51.58 |
| | 10 | 25.24 |
| Phenanthroline | 0.1 | 139.18 |
| | 1 | 61.67 |
| | 10 | 23.22 |
| Phenylmethane-sulfonylfluoride | 0.1 | 158.61 |
| | 1 | 78.50 |
| | 10 | 0 |

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