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Identification of protease enzyme in salep orchid tubers, and investigation of the usability of the enzyme in casein and gluten hydrolysis

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Abstract

In recent years, due to many diseases transmitted from animals to humans (coronavirus disease, severe acute respiratory syndrome, mad cow, and bird and swine flu), consumers are concerned about the use of protease enzymes derived from animal sources in the production of food products. These concerns have increased the demand for protease enzymes of plant origin. The fact that very few of the protease enzymes used in the production of foodstuffs are produced from plant sources has led researchers to seek a new source of plant-based protease. In the present work, the protease enzyme was isolated from the tubers of the salep orchid (*Dactylorhiza osmanica*) by ammonium sulphate precipitation and size exclusion chromatography. The isolated protease had an optimal pH of 6.5 and an optimal temperature of 48°C. The K_m value was 8.22 µM. The molecular mass of the enzyme was 31 kDa. The enzyme retained its 100% activity up to 21 h at 40°C. At 50°C, the enzyme maintained its 100% activity for up to 4 h. The isolated protease acquired from the salep orchid tubers hydrolysed α-, β-, and κ-casein, and formed new peptides larger than 15 kDa. The isolated enzyme is known to be effective in milk clotting, which is the first step of cheese making, and might also contribute to the production of cheese with specific flavours. However, the protease extracted from the salep orchid tubers cannot hydrolyse gluten at the same level.

Introduction

Proteases (proteolytic enzymes, peptidases) are enzymes that catalyse the cleavage of peptide bonds found in proteins. Protease enzymes are divided into two classes: exopeptidases that cleave peptide bonds from regions at the end portions of the peptide chain (EC 3.4.11-19), and endopeptidases that cleave internal peptide bonds (EC 3.4.21-25). Endopeptidases are further divided into five groups according to the amino acids and cofactor involved in the reaction itself in the active site of the enzyme. Regarding the amino acids that carry out the reaction, proteases can be selected from the group consisting of serine proteases (EC 3.4.21), cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23), and threonine proteases (EC 3.4.25). The last group is metalloproteases (EC 3.4.24) that use metal ions as a cofactor. Protease enzymes are found in almost all living things, including plants, because they catalyse a vital reaction such as seed germination, proteolytic activation of proenzymes, and senescence (Beynon and Bond, 2000; Wang *et al.,* 2013; Buono *et al.,* 2019).

Protease enzymes are widely used in food, detergent, pharmaceutical, and leather industries (Hamada *et al.,* 2013; Liang *et al.,* 2014; Taga and Hattori 2017). These enzymes are used in many different areas of the food industry such as in the preparation of protein hydrolysates, cheese making, meat softening, and preparation of bakery products such as biscuit, bread, and pizza (Demir *et al.,* 2005; Kong *et al.,* 2007; Guan *et al.,* 2009; Olanca and Özay, 2010). In recent years, there has been an increase in certain diseases transmitted from animals to humans such as coronavirus disease, severe acute respiratory syndrome, mad cow, and bird and swine flu. The use of animal proteases especially in food products causes concern about the spread of these diseases. Therefore, there is increased demand for products from plant sources. Only a small proportion of commercially available enzymes are herbaceous, which encourages researchers to seek for novel plant-based protease sources to be commercially used (Dubey and Jagannadham, 2003).

The main purpose of using proteolytic enzymes in bakery products such as bread, biscuit, pastry, cookie, and pizza is to break peptides/amino acids into basic protein, gluten. In the bakery industry, gluten in the wheat is hydrolysed by the protease enzyme for different reasons such as reducing the

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consistency of the paste, regulating the gluten strength in the bread, and increasing the homogeneity of the dough (Goesaert *et al.,* 2005; Miguel *et al.,* 2013). In the production of biscuits and cakes, wheat gluten is hydrolysed by proteases to reduce elasticity. As a result of this process, the shrinkage of dough or paste is reduced after moulding and layering.

In the world's enzyme market, protease enzymes are in the first place with a share of 60%. In addition, according to the market research report on commercial enzymes used globally, proteases have a share of six billion dollars, and commercial proteases are produced from plants, animals, and microorganisms (Rodarte *et al.,* 2011).

The salep orchid (family Orchidaceae) is a perennial and lumpy plant which grows in the Mediterranean, Southeast Anatolia, and Eastern Anatolia regions in Turkey, and has many endemic species (Sandal and Söğüt, 2010). Salep, obtained by milling the dried tubers of wild orchids, is a food ingredient used for the traditional production of the salep drink, Kahramanmaraş-type ice cream, and medicine (Karaman and Kayacier, 2010).

In the present work, the protease enzyme was first isolated from *Dactylorhiza osmanica* tubers, and their characteristic features were determined. The enzyme was then used for the first stage of cheese making; i.*e.,* clotting. In addition, the degree of enzymatic hydrolysis of wheat flour and wheat protein gluten was also investigated.

Materials and methods

Materials

Gluten (80% purity) was purchased from Makeks Mak, Gıda Industry Limited Company (İstanbul, Turkey). PageRuler™ Plus Prestained Protein Ladder 10 - 250 kDa was purchased from ThermoFisher Scientific. All other chemicals were of analytical grade.

Plant materials

Dactylorhiza osmanica was collected during the flowering season (beginning of June) from Aşkale district, Turkey, and identified by Dr. İbrahim Gümüş, a biologist from Atatürk University according to routine procedures described by Sezik (1984). The orchids were cleaned by washing, and stored at -20°C until purification.

Isolation of protease enzyme

The orchid plants were collected with their flowers, leaves, and tubers. The protease activity was measured in the homogenates of flowers, leaves, and tubers. The highest level of protease activity was detected in the tuber homogenate; therefore, protease was purified from the tubers using ammonium sulphate precipitation and gel filtration chromatography.

Fresh *D. osmanica* tubers (58.6 g) were homogenised in a Waring blender with 600 mL distilled water. The obtained homogenate was centrifuged at 9,000 *g* for 60 min at 4°C. Then, the protein was precipitated from the obtained supernatant with 60% ammonium sulphate. The precipitated protein (PP) was collected by centrifugation, dissolved in 50 mM phosphate buffer (pH 7.0), and the resulting solution, the PP homogenate, was loaded onto a Sephacryl S-200 HR column (1.5 × 58 cm) pre-equilibrated with the same phosphate buffer. Fractions of 1 mL were eluted with equilibration buffer at a flow rate of 0.5 mL/min. Protein determination was performed by reading absorbance at 280 nm in the eluates. The protease activity was measured, and the eluates exhibiting activity were collected and stored at -20°C for characterisation studies.

Protease activity assay and protein determination

Protease activity was determined with the casein digestion method as described by Fadýloğlu (2001). A casein solution was prepared by dissolving 0.7 g of casein in 100 mL of 100 mM phosphate buffer (pH 6.5). Under magnetic stirring, the suspension was heated for 10 min to 80 - 85°C. The stock solution of casein was stored at 4°C until use. This solution would be stable for about 10 d. Protease activity was measured by adding 125 µL enzyme solution to 250 µL casein solution. The reaction mixture was incubated at 48°C for 60 min. Following incubation, 375 µL of 3% trichloroacetic acid (TCA) was added to stop the reaction. Non-hydrolysed casein was allowed to precipitate for 30 min. Then, the mixture was centrifuged at 10,000 *g* for 15 min at 4°C. A reaction blank was prepared by adding 375 µL TCA solution to the substrate solution, which was also incubated at 48°C for 60 min immediately after the enzyme solution was added. The precipitated proteins were separated from the supernatant by centrifugation at 10,000 *g* for 15 min at 4°C. The amount of degraded proteins based on protease remaining in the supernatant was determined by reading the absorbance at 280 nm. One unit of protease activity is the amount of enzyme that causes an increase of 0.001 abs at 280 nm in 1 min. All experiments were performed in triplicate.

In the fractions of gel filtration chromatography, the protein content was determined by measuring

the absorbance of the eluates at 280 nm. The protein concentration was determined by the method described by Bradford (1976) using bovine serum albumin as a standard.

Effect of pH and temperature on enzyme activity

The optimum pH of the protease obtained from the *D. osmanica* tubers was also determined by assaying protease activity using three different buffers: acetate (pH 5.0 to 6.0), phosphate (pH 6.0 to 8.0), and carbonate (pH 9.0 to 11.0). The optimum temperature of the isolated protease was determined by assaying protease activity in 100 mM phosphate buffer (pH 6.5) at a temperature range from 0 to 90°C at an interval of 5°C (from 44 to 50°C at 2°C intervals). The maximum activity was considered as 100%.

Determination of K_m

The Michaelis-Menten constant (K_m) of the protease isolated from *D. osmanica* was calculated using natural substrate, casein. The protease activity was determined by increasing the casein concentration from 0.78 µM to 87.5 µM under optimum pH and temperature. K_m was calculated by using the Lineweaver-Burk plot.

Determination of the molecular mass

The molecular mass of the protease isolated from *D. osmanica* tubers was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (1970). Stacking and resolving gels were prepared at concentrations of 4 and 15% acrylamide, respectively. Protease samples were prepared by adding 50 µL of enzyme solution to 50 µL of electrophoresis sample buffer containing 2% SDS and 5% β-mercaptoethanol (β-ME). This mixture was incubated at 95°C for 10 min. Following incubation, the samples were loaded onto SDS-PAGE gel; then, the gel was run at 50 mA and 200 V for 40 min in SDS-PAGE buffer. Following electrophoresis, the protein bands in the gel were stained using Coomassie Brilliant Blue R-250. The molecular mass, determined using marker proteins, ranged from 10 to 250 kDa.

The molecular mass of the protease isolated from the *D. osmanica* tubers was also determined by gel filtration chromatography, and compared with the SDS-PAGE results to determine the oligomeric structure of the purified protease. The following standard proteins were used for the calibration of gel filtration column: cytochrome c (12.4 kDa), albumin (66 kDa), alcohol dehydrogenase (150 kDa), and β-amylase (200 kDa).

Effect of various inhibitors and metal ions on enzyme activity

The effects of SDS, dithiothreitol (DTT), protease inhibitors (pepstatin, E-64, EDTA, and PMSF), and some chlorinated salts of metal ions ($CaCl_2$, $MgCl_2$, $FeCl_3$, $CoCl_2$, and $ZnCl_2$) on the activity of the protease enzyme obtained from *D. osmanica* tubers were investigated. The chemicals were added to the enzyme solution, and then incubated for 1 h at room temperature. The control experiment was carried out by adding buffer solution instead of effectors. The activity of control experiment (without effectors) was considered as 100%.

Determination of stability

The thermal stability of the protease enzyme isolated from *D. osmanica* tubers was determined after the incubation of the enzyme at 40, 50, and 60°C for 21 h. The enzyme aliquots were taken every 3 h to be tested for protease activity.

SDS-PAGE analyses of casein hydrolysis

The casein cleavage by the PP homogenate obtained from *D. osmanica* tubers was also checked by SDS-PAGE (Laemmli, 1970). Stacking gel and resolving gel were prepared at 4 and 15% acrylamide concentrations, respectively. 1 mL of substrate was added to 0.5 mL of PP homogenate, and incubated at 48°C. A sample (50 µL) was taken every 3 h over 21 h. Electrophoresis sample buffer (50 µL) containing 5% β-mercaptoethanol was added to the samples without any delay, and the samples were prepared for electrophoresis by heating to 95°C for 10 min. To prepare the control sample, 1 mL of substrate was incubated for 21 h at 48°C without enzyme addition, and after 21 h, 0.5 mL of enzyme was added, followed by the same treatment as in the other samples. Each gel was run at 50 mA for about 40 min by loading 50 µL (20 µg protein) of samples and 10 µL of the ladder. The gel was stained for 2 h with the staining solution (0.1 g Coomassie Brilliant Blue R-250, 10% acetic acid, 40% methanol, and 50% pure water). Then, the gel was destained with solution (10% acetic acid, 40% methanol, and 50% pure water) until the background was clear.

Clotting of milk with protease

In order to determine whether the protease enzyme obtained from *D. osmanica* tubers could be used in cheese making, it was investigated whether the enzyme could clot milk. For this purpose, the Berridge method was applied in modified form (Berridge, 1945). A 10% solution of the milk powder

was prepared with distilled water. To one portion, $CaCl_2$ was added to a concentration of 10 mM. Next, 1 mL of the enzyme solution (PP homogenate) was added to each 5 mL of the prepared solutions, and incubated at 48°C. For control purposes, 1 mL of buffer solution was added to each 5 mL of the solution with and without $CaCl_2$, and kept under the same conditions. How long it took the enzyme to clot the milk was noted. Milk clotting activity (MCA) was expressed as the mL of the substrate that was clotted by 1 mL of enzyme within 180 min. MCA was calculated using Eq. 1:

$$\text{MCA U} = (180 \times V_1 \times n) / (t \times V_2) \quad \text{Eq. 1}$$

where, V_1 = substrate volume (mL), n = ratio of the dilution of the PP homogenate, t = milk clotting time (min), and V_2 = volume of the PP homogenate (mL).

Hydrolysis of wheat flour and gluten

There are water-soluble and insoluble high-molecular proteins in the composition of flour. The present work also investigated whether these proteins could be hydrolysed by the PP homogenate obtained from *D. osmanica* tubers, and if so, which protein were hydrolysed. For this purpose, 14 mg of flour was kept in 1 mL of the enzyme solution (PP homogenate) for 20 h at 48°C. In the same conditions, 14 mg of flour was added to 1 mL of buffer solution for control purposes. Both samples were then extracted twice with 1 mL of 1-propanol for 30 min at room temperature and centrifuged at 10,000 *g* for 10 min. The supernatants were combined to form 50% 1-propanol soluble proteins (50% PS). These proteins form monomeric proteins and soluble gluten. The precipitate composed of insoluble glutenin and non-prolamin proteins was considered as 50% PI. This precipitate was extracted twice with 50% 1-propanol containing 1 mL of 1 mM DTT for 30 min at 60°C. The supernatants containing insoluble gluten were combined, and the precipitate was the insoluble residue. The amount of cleavage was monitored by SDS-PAGE (Sapirstein and Fu, 1998; Suchy *et al.,* 2007).

Flour and gluten were hydrolysed with the PP homogenate. The experiments were carried out at two different pH values (6.5 and 8.5). For this purpose, 14 mg of flour and gluten was kept in 1 mL of the enzyme solution (PP) for 20 h at 48°C. For the blank sample, 14 mg of flour and gluten was added to 1 mL of buffer solution under the same conditions. A sample was taken every hour for 20 h. Non-hydrolysed protein was precipitated by TCA, and removed after centrifugation. Protein hydrolysed by protease remains in the supernatant. The amount of protein degraded by the protease enzyme in flour and gluten (80% pure) was determined as described by Lowry *et al.* (1951).

Results and discussion

Preparation of homogenate and purification of D. osmanica tubers protease

The protease enzyme isolated from *D. osmanica* tubers was 10.31-fold purified using ammonium sulphate precipitation and gel filtration chromatography (Table 1). The homogenate was prepared using a blender. The shredding process was performed for a maximum of 20 - 30 s. The tubers of *D. osmanica* contain glucomannan, a carbohydrate that was used as a stabiliser in the present work. Glucomannan has a molecular mass of up to 2,000,000 Da, and causes the homogenate to have high viscosity (Kurt and Kahyaoglu, 2017). The viscosity of the homogenate would reduce the efficacy of the purification column. For this reason, the homogenisation time was kept as short as possible to prevent the increase in the homogenate viscosity. The homogenate was diluted with two-fold distilled water to reduce its viscosity prior to centrifugation. Then, the homogenate was centrifuged to remove insoluble impurity and glucomannan. Glucomannan was identified to precipitate at 65% ammonium sulphate. Therefore, a maximum of 60% ammonium sulphate was used to separate glucomannan from the homogenate. The protein in the homogenate was separated by precipitation with ammonium sulphate at 0 - 20, 20 - 40, and 40 - 60%. After centrifugation with 0 - 20% ammonium sulphate, no precipitate was obtained. Protease activity was found in the

Table 1. Purification of protease from *Dactylorhiza osmanica* tubers.

Step	Activity (EU/mL)	Total activity (EU)	Protein (mg/mL)	Total protein (mg)	Specific activity (EU/mg)	Purification (Fold)	Yield (%)
Crude extract	0.77	115.00	0.134	20.19	5.75	-	100
60% $(NH_4)_2SO_4$	2.67	66.75	0.072	1.80	36.39	6.33	58
Sephacryl S-200	2.89	14.45	0.049	0.25	58.97	10.31	13

precipitates obtained with 20 - 40 and 40 - 60% ammonium sulphate. The specific activities were calculated in two precipitates, and found to be close to each other. Thus, the protein in homogenate was precipitated with 60% ammonium sulphate, and approximately 90% of glucomannan was separated from the precipitate. The precipitate was dissolved in 0.01 M phosphate buffer at pH 7.0 and was considered as the PP homogenate. The slightly viscous solution was stored at 4°C for about a week, centrifuged again, and the resulting red-brown precipitate was discarded. The viscosity of the homogenate was reduced without dilution. Next, 4 mL homogenate was loaded onto a Sephacryl S-200 HR column. The eluates with activity were collected. The characterisation of the enzyme was performed.

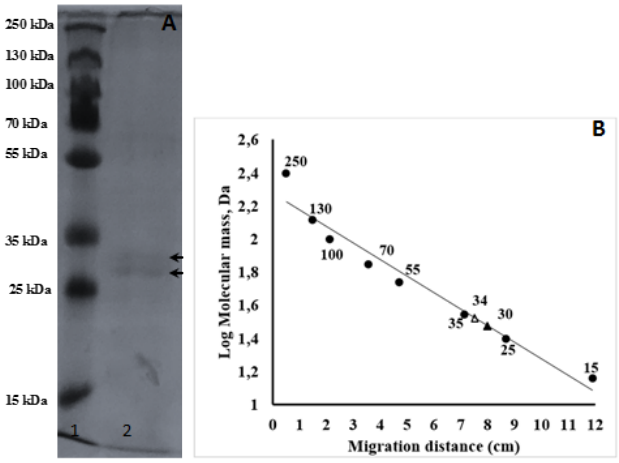

Figure 1. (A) SDS-PAGE of isolated protease from *Dactylorhiza osmanica* tubers. Lane 1: Ladder (15, 25, 35, 55, 70, 100, 130, and 250 kDa), Lane 2: Protease isolated by gel filtration chromatography. (B) Molecular mass (M) determination of the isolated protease from *D. osmanica* tubers. Calibration curve for the determination of protease molecular mass by SDS-PAGE. The filled triangle indicates the protease enzyme.

Optimum pH, temperature, and Km of protease isolated from D. osmanica tubers

The purity of the enzyme was checked (Figure 1a), and the molecular mass of the protease enzyme obtained from *D. osmanica* tubers (Figure 1b) was calculated using SDS-PAGE. This analysis showed that there were two bands close each to other: one with a mass of 34 kDa, and the other with a mass of 30 kDa (Figure 1b). The molecular mass of the enzyme was also determined as 31 kDa by gel filtration chromatography. These results suggested that the protease enzyme was a monomer with a mass of 30 to 31 kDa. However, it was concluded that the band of 34 kDa was impure, indicating that the protease isolated from *D. osmanica* tubers was not sufficiently purified. In the literature, the molecular masses of proteases isolated from different sources were determined as 64 kDa for streblin obtained from the latex of *Steblus asper* (Tripathi *et al.,* 2011), 62 and 120 kDa for sunflower (*Helianthus annus*) (Nasr *et al.,* 2016), 31 kDa for ginger (Huang *et al.,* 2011), 35 kDa for tubers of *Crocus biflorus* (Yıldırım Çelik, 2018), 54 kDa for latex of *Euphorbia amygdaloides* (Demir *et al.,* 2005), and 46 kDa for capparin obtained from the capsules of *Capparis spinosa* (Demir *et al.,* 2008).

The optimum pH and temperature of the partly isolated protease were found to be 6.5 and 48°C, respectively. The optimal reaction temperature of proteases from different sources were reported as 30°C for hieronymain obtained from the unripe fruit of *Bromelia hieronymi* (Bruno *et al.,* 2010), 65°C for streblin from the latex of *Steblus asper* (Tripathi *et al.,* 2011), and 60°C for capparin from the capsules of *Capparis spinosa* (Demir *et al.,* 2008). The optimum pH of the protease isolated from *D. osmanica* tubers (pH 6.5) was higher as compared to capparin (pH 5) and streblin (pH 2 - 4), and was the same as that of hieronymain. It was also evident that the enzyme maintains a minimum of 40% of its activity at pH 5 - 9 and in the temperature range of 0 - 60°C. The K_m value calculated from the Lineweaver-Burk plot was 8.22 µM. The K_m values of proteases from different sources were reported as 50 µm for protease obtained from the latex of *Wrightia tinctoria* (Tomar *et al.,* 2008), 27 µm for streblin from the latex of *Steblus asper* (Tripathi *et al.,* 2011), and 33.3 µm for milin from the latex of *Euphorbia milii* (Yadav *et al.,* 2006). The comparison of these results to our findings shows that the protease from *D. osmanica* tubers exhibited a lower K_m value for casein, thus indicating its higher affinity for this protein.

Effects of pH, temperature, and effectors of protease

The maximum pH and temperature and temperature stability of the protease enzyme in the PP homogenate obtained from *D. osmanica* tubers were also determined. The maximum pH of the protease enzyme from the PP homogenate was 6.5, and the optimum temperature was 48°C. Previous studies showed that the pH of milk was acidic (ranging from 6.5 to 6.7) and milk clotting was usually displayed at pH 5.5 and 6.5 during the initial period of cheese manufacturing (Huang *et al.,* 2011; Zhang *et al.,* 2019). The isolated protease exhibited maximum activity at acidic pH, thus indicating that this enzyme may be suitable for processing dairy products.

Temperature stability was monitored at the temperatures which the PP homogenate showed maximum activity (40, 50, and 60°C). The isolated enzyme was observed to retain its activity for up to 21 h at 40°C and 4 h at 50°C, but after this time, the activity was lost. At 60°C, the enzyme appeared to have only 49% activity for up to 1 h. It was evident from this experiment that the isolated enzyme can be used in long processes taking up to 4 h at 50°C and up to 21 h at 40°C. In a study conducted by Hayaloglu *et al.* (2014), the thermal stability of the protease enzymes obtained from *Rhizomucor miehei* and calf rennet, which are sold commercially for cheese making, were examined. During the 10-min incubation, the protease from *R. miehei* retained 35% of its activity at 80°C, while calf rennet was completely inactive at 62°C. The protease from the *D. osmanica* tubers had higher thermal stability than calf rennet, maintaining 49% activity in 1-h incubation. In a study by Hayaloglu *et al.* (2014), it was stated that protease enzymes with high temperature stability could be more suitable for the manufacturing of cheese that scalds at high temperature, such as Halloumi (Hayaloglu *et al.,* 2014) and traditional Erzurum Civil cheese (Çelik and Uysal, 2009; Yildiz *et al.,* 2010). The products formed through protease enzyme activities during cheese ripening are known to contribute to the flavour and aroma (McSweeney *et al.,* 2006). Therefore, protease enzymes must remain active during ripening in types of cheese produced by scalding at high temperatures.

When the effects of metal ions (Ca^{2+}, Mg^{+2}, Fe^{3+}, Co^{2+}, and Zn^{2+}), specific protease inhibitors (pepstatin, E-64, EDTA, and PMSF), DTT, and SDS on the activity of the PP homogenate were investigated (Figures 2 and 3), none of the metal ions (1 mM) showed significant inhibition. Fe^{3+} acted as an activator, and almost doubled the activity of the enzyme (182%). A detergent, SDS, completely inhibited the enzyme at a concentration of 1 mM. Cysteine protease inhibitor E-64 (50 µM) reduced the enzyme activity by only 8%, while DTT, which is expected to activate cysteine proteases, increased the activity by only 10% at 1 mM concentration. Based on these results, it was concluded that there was no cysteine protease in the PP homogenate. Although only 20% inhibition of the protease enzyme by the metalloprotease inhibitor EDTA confirmed that the enzyme could not be a metalloprotease, 182% activation of Fe^{3+} proved that there could be metalloproteases present in the enzyme mixture. The enzyme inhibition by the aspartyl protease inhibitor pepstatin (50 µM) and the serine protease inhibitor PMSF (1 mM) was 72 and 66%, respectively. The

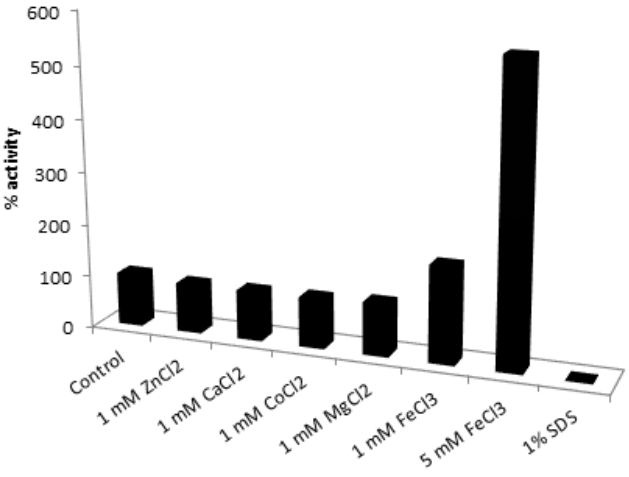

Figure 2. The effect of Ca^{2+}, Mg^{2+}, Fe^{3+}, Co^{2+}, Zn^{2+} ions and SDS on activity of protease enzymes from *Dactylorhiza osmanica* tubers.

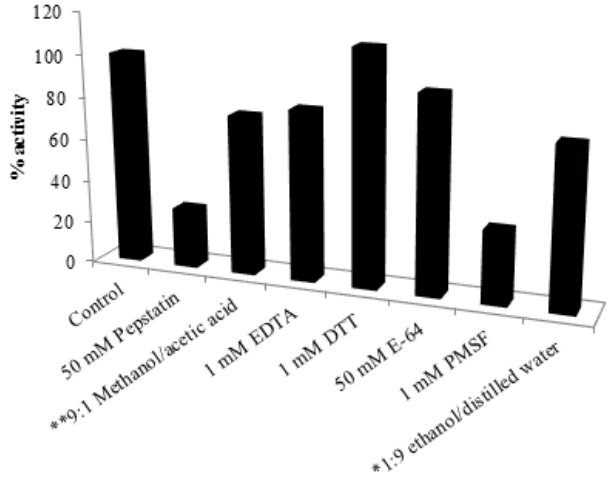

Figure 3. The effect of several inhibitors (pepstatin, E-64, EDTA, PMSF, and DTT) on the activity of protease enzymes from *Dactylorhiza osmanica* tubers. *PMSF was dissolved in 1:9 ethanol/distilled water, and **Pepstatin was dissolved in 9:1 methanol/acetic acid.

solvents, in which pepstatin and PMSF were dissolved, were determined to inhibit 25% (9:1, methanol/acetic acid) and 31% of the enzyme (1:9, ethanol/distilled water), respectively. A similar inhibition effect of the serine (PMSF) and aspartyl protease (pepstatin) inhibitors raised doubt as to the type of this protease enzyme. It was thus suggested that the PP homogenate obtained from *D. osmanica* tubers may be a mix of aspartyl protease (E.C. 3.4.23), serine protease (EC 3.4.21), and metalloprotease (EC 3.4.24).

Analysis of casein hydrolysate

The hydrolysis of casein by the PP homogenate obtained from *D. osmanica* tubers was

checked by SDS-PAGE (Figure 4). The hydrolysis process was carried out at 48°C, and a sample was taken every 3 h and boiled for 5 min with sample buffer containing β-mercaptoethanol, and stored for electrophoresis. The casein hydrolysis continued from the 3rd hour until the 21st hour, and the casein bands gradually decreased. Comparing the sample (3rd hour of hydrolysis) to the control, the SDS-PAGE analysis revealed that more than 50% of the casein was hydrolysed. There are three types of bovine milk casein: α-, β-, and κ-casein. The mass of these caseins was reported as 22.0 - 23.7 kDa for α-s1, 25.2 kDa for α-s2, 23.9 - 24.0 kDa for β-, and 19 kDa for κ-casein (Modler, 1985). κ-casein increased the solubility of casein by wrapping α- and β-caseins that are hydrophobic. In cheese making, hydrophobic casein was formed by the hydrolysis of κ-casein, and clotting of milk was provided (Creamer *et al.,* 1998). In the SDS-PAGE gel, the casein band with the smallest molecular size (κ-casein = 19 kDa) was almost absent by the 3rd hour. New peptides heavier than 15 kDa appeared after the 3rd hour (Figure 4), thus suggesting that the isolated enzyme may be successful in milk clotting, which is the first step of cheese making.

In a study conducted by Zhang *et al.* (2019), it was found that calf rennet and *R. miehei*, which are used in cheese making, hydrolysed the bonds found in α- and β-caseins, and formed new peptides in the range of 14 - 24 kDa. In the present work, the density of the band formed by the new peptide was too large to be caused by κ-casein alone (Figure 4). In addition, the bands formed by α- and β-casein significantly decreased by the 3rd hour. Based on these results, it was concluded that the PP homogenate began to hydrolyse α- and β-caseins by the 3rd hour, and formed new peptides larger than 15 kDa. In previous studies, the protease obtained from glutinous rice wine almost completely hydrolysed α-casein, whereas κ- and β-casein were partially degraded by the 12th h (Jiang *et al.,* 2007). κ-casein was also hydrolysed at a higher level than α- and β-caseins by the protease obtained from ginger (Huang *et al.,* 2011). The hydrolysis capability of the isolated protease from ginger rhizome was higher for α-casein, followed by β- and κ-casein (Hashim *et al.,* 2011). The proteases extracted from the sunflower and albizia seeds exhibited proteolytic activity towards α-, β-, and κ-caseins (Egito *et al.,* 2007). New protease enzymes can cause different cleavage sites in α-, β-, and κ-caseins, and produce different peptides in cheese making. These peptides might lead to the production of cheese with specific flavours (Zhang *et al.,* 2019). It was hypothesised that the PP homogenate obtained from *D. osmanica* tubers can be used in the production of cheese with specific flavours, since it could form new peptides of different sizes as a result of casein hydrolysis.

MCA

The MCA of reconstituted milk by the PP homogenate obtained from *D. osmanica* tubers was also investigated. For this purpose, the experiments were carried out in two different media: 10% reconstituted milk containing 10 mM $CaCl_2$, and 10% reconstituted milk. The samples were incubated at 48°C where the protease enzyme activity was maximum. The MCA were 365 MCA/mg (in 10% reconstituted milk containing 10 mM $CaCl_2$), and 180 MCA/mg (in 10% reconstituted milk).

Analyses of flour and gluten hydrolysis

To investigate whether the PP homogenate

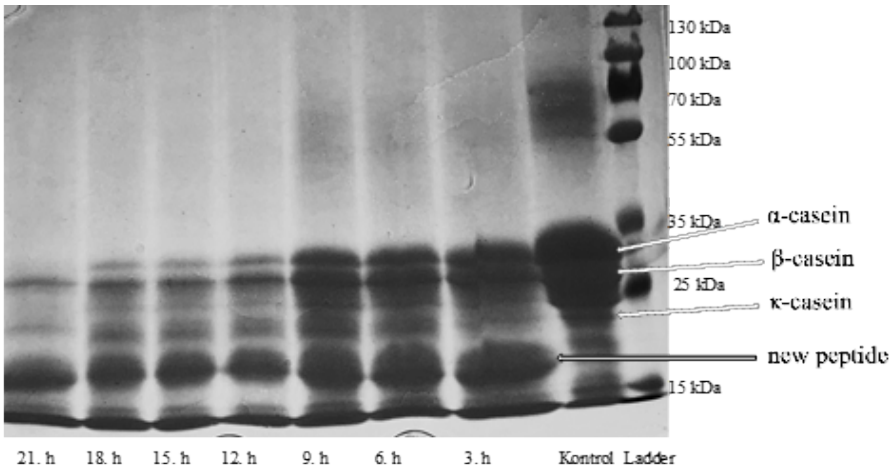

Figure 4. SDS-PAGE pattern of casein hydrolysis. Casein preparation was hydrolysed with protease enzyme from *Dactylorhiza osmanica* tubers for 21 h. Every 3 h, the degree of casein hydrolysis was checked by SDS-PAGE.

obtained from *D. osmanica* tubers could be used in the production of flour products, SDS-PAGE was conducted to determine the hydrolysis of flour protein, if any. In addition, the amount of protein degraded by the protease enzyme in flour and gluten (80% pure) was determined as described by Lowry *et al.* (1951). Flour proteins were found to be hydrolysed at an amount of 14% at pH 6.5 and 3% at pH 8.5 after 4 h. Gluten was hydrolysed at an amount of 0.27% at pH 6.5 and 0.13% at pH 8.5 after 15 h. It was observed that the PP homogenate obtained from *D. osmanica* tubers was more active at pH 6.5. Based on previous studies, 80 - 85% of the protein in wheat composed of glutens, and 15 - 20% composed of albumins and globulins. Half of the gluten proteins were gliadins responsible for the elasticity and extensibility of dough, and the other were glutenins responsible for the strength of dough (Suchy *et al.,* 2007). In the present work, it was observed that the PP homogenate obtained from *D. osmanica* tubers hydrolysed flour more than pure gluten. This result shows that the enzyme hydrolysed albumin and globulin proteins, but did not hydrolyse gluten in flour. The proteolytic hydrolysis of 50% of 1-propanol (PS) and 50% of 1-propanol insoluble (PI) proteins of flour and gluten was checked by SDS-PAGE as compared to the blank test. There was not a very clear difference in the SDS-PAGE images.

Conclusion

In the present work, the protease enzyme was isolated from the tubers of the salep orchid (*Dactylorhiza osmanica*). The optimum pH was determined to be 6.5 for the partially isolated protease. The fact that the pH of milk is acidic (range 6.5 to 6.7), and milk clotting is usually seen at pH 5.5 and 6.5 during the initial period of cheese manufacturing (Huang et al., 2011; Zhang et al., 2019) indicates that the isolated enzyme can be used in the production of cheese and dairy products. The isolated enzyme retained its activity at 100% for up to 21 h at 40°C. The enzyme proceeded to function at 100% for up to 4 h at 50°C. The isolated protease with high temperature stability are more suitable for the manufacturing of cheese that scalds at high temperature, such as Halloumi and traditional Erzurum Civil cheese. The isolated protease obtained from the salep orchid tubers hydrolysed α-, β-, and κ-casein, and formed new peptides larger than 15 kDa. The isolated protease can also be used in the production of cheese with specific flavours.

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