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RESEARCH ARTICLE

Purification and Characterization of Protease Enzyme from Oleander (*Nerium oleander*) Flowers of Different Colors

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HIGHLIGHTS

> Oleander (*Nerium oleander*) flowers collected from Mugla countryside in June-September period
> Protease enzyme was purified and characterized from oleander (*Nerium oleander*) flowers
> The protease enzyme from oleander flowers (white, pink and red) was obtained in order of high efficiency

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ABSTRACT

In this study, protease enzyme was purified and characterized from oleander (*Nerium oleander*) flowers collected from Mugla countryside in June-September period.
Protease catalyzes the hydrolysis of proteins to peptides and amino acids. It is one of the most important enzyme groups in both industrial and biochemical applications. Using the TPP method, the protease enzyme from oleander flowers (white, pink and red) was obtained in order of high efficiency.
Optimum pH and optimum temperature for enzyme, KM and Vmax values for casein substrates were determined. SDS-PAGE was used to control the purity of the protease enzyme purified from oleander flowers. The molecular weight of protease enzyme purified from oleander flowers was determined by gel filtration chromatography to be white oleander 40,537 kea pink oleander 21,386 kDa and 22,516 kDa.

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1. Introduction

Enzymes are the most studied subjects in the field of biochemistry. Enzymes are widely used in agriculture, food and chemical industries. Cheese, yogurt, wine, bread making are made from enzyme catalyzed reactions. In recent years, the prevalence of enzymes in the diagnosis and treatment of disease has increased steadily [1].

Food, detergent and starch industries constitute 75% of the world enzyme market. Hydrolases such as proteases, amylases, lipases, pectinases and celluloses are commonly used enzyme groups.

Proteases are widely used in food industry in bakery products, fermented products, maturing of birds, cheese industry, meat ripening, detergents, biomedical applications in the leather industry and similar fields [2].

Proteases (E.C. 4.3.1.1), also included in the main group of hydrolases, are also known as proteolytic enzymes. These catalyze the cleavage of peptide bonds by water. It is an important group of enzymes because of industrial use potentials and it has 60% of the enzyme market in the world [3].

Proteases can be obtained from plants, animals and microbial sources. There have been doubts about the use of animal proteases in the construction of food materials, especially due to the presence of some diseases in humans (SARS, mad cow, avian flu, etc.), which has prompted researchers to search for new herbal protease sources [4].

Proteases are divided into exopeptidases and endopeptidases according to their active regions. According to the reaction mechanisms, serine proteases, cysteine proteases, aspartic proteases and metalloproteases are separated in the same way. While cool and cysteine proteases attack nucleophilic substrata, aspartic and metalloproteases activate peptide molecules by activating water molecules [5].

Cysteine proteases include peptide hydrolase families, papain family, and caspase family, which combine with cysteine residues such as nucleophilic catalysts. Papain, a plant-derived enzyme, is the most thoroughly studied papain in terms of mechanics. Although Papain is an enzyme of plant origin, many enzymes like papain are also found in animals [5].

Peptidases include cathepsins, bleomycin hydrolase and ubiquitin C-terminal hydrolases belonging to the papain family [5]. The caspase family is calcium-dependent cysteine proteases, separated by the presence of functional aspartate sites from other proteases, and is still being studied because of its important role on apoptosis. Cysteine proteases have become an important target in the pharmaceutical industry [6]. The mechanism of action of all cysteine proteases seems to be similar, and intensive studies on papain are an important example for proteases.

In this study; The oleander (Nerium oleander) plant protease enzyme has been purified and characterized.

2. Materials and Method

2.1. Collection of samples

Oleander flowers were collected from Mugla countryside in June-September and kept in deep freezing at -80 °C until used in our work.

2.2. Purification of Protease Enzymes from Oleander Flowers by Using Three Phase Partitioning Method

2.2.1. *Preparation of the protease homogenate*

10 g of oleander flowers were weighed and thoroughly air-kneaded and thoroughly homogenized by adding 150 ml of sodium phosphate (pH: 7.0,05 M) buffer. A -80 °C cooler was placed in a container and after a few hours it was expected to be unpacked. This was done three times. The homogenate, which was solved by removing it from -80 °C, was separated from the pulp by filtration. Centrifuged at 6,000 rpm. Protein content determinations were made in the supernatant after centrifugation [7].

2.2.2. *Determination of homogenate t-butanol ratio for protease enzyme*

The TPP of the protease extract was performed in a 50 mL glass reactor using a mechanical stirrer. The homogenate was studied in the ratios (v / v) of t-butanol (1.0: 0.5, 1.0: 1.0, 1.0: 1.5 and 1.0: 2.0). The highest activity rate was determined. The process was stirred in a glass beaker with 4 g ammonium sulfate (40% w / v) in a 10 mL extract at room temperature (30 °C) and then 10 mL t-butanol was added. The reaction mixture was stirred at 200 rpm for 100 minutes and then the mixture was centrifuged at 6000 rpm for 25 minutes to facilitate separation of the phases. The mixture was then allowed to stand for one hour at room temperature until the phases were separated in a separatory funnel. Three different phases were observed after separation in the separation funnel. The protein precipitate in the middle and the lower aqueous phase were carefully separated to work later. The protein precipitate in the middle was dissolved in a minimum amount of 100 mM phosphate buffer (pH: 7).

The upper, middle, and lower franchises of TPP were first dialyzed to distilled t-butanol against purified water. It was then dialyzed against 100 mM phosphate buffer (pH: 7) for 12 hours and analyzed for protein content and enzyme activity. The purification rate of the middle phase and the activity gain were higher when compared to the upper and lower phases [7].

2.2.3. *Determination of ammonium sulfate concentration for protease enzyme*

After determining the ratio of T-butanol, the appropriate concentration of ammonium sulphate was determined. $(NH_4) S_2O_4$ were added separately at concentrations of 20, 30, 40 and 50 (w / v) on the determined crude homogenate: t-butanol ratio. The highest activity of ammonium sulphate con-

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centration was determined. The phases obtained from the determined concentration were carefully separated from each other. The protein precipitate was dissolved in the minimum amount of buffer prepared in the homogenate. Three phases were dialyzed against the same buffer for 12 hours. After dialysis, protein amounts and protease activity determinations were performed in the fractions. It is expected that the activity is high in the middle phase or the bottom phase. Between phases, purification coefficient and activity yield were compared. Optimization studies were carried out in phases with the highest activity. The effect of pH and temperature and kinetic parameters on the purity and yield of protease was investigated [7].

2.2.4. *Activity Determination for protease enzyme*

The proteolytic activity of protease enzyme purified from oleander flowers was determined according to the method of casein digestion in the presence of 1% casein. To measure proteolytic activity, 1 mL substrate (casein), 0.5 mL enzyme solution was added and the total volume was 2.5 mL with buffer solution. The tube containing the enzyme was incubated for 20 minutes in a water bath at 40 °C. The reaction was then stopped by the addition of 3 mL of 5% TCA. It was waited for 30 minutes for the decay to take place completely, and this was then centrifuged for 20 minutes at 6000 rpm. After the supernatant was filtered, the amount of the degraded products in the supernatant was determined by the Bradford method. Proteolytic activity was calculated as micrograms per minute protein / mL of enzyme [8].

2.2.5. *Control of enzyme purity by SDS-PAGE and determination of subunits*

It was checked whether the protease enzyme purified by Laemmli was purified and subunit by SDS-PAGE [9].

2.2.6. *Determination of optimum pH*

Proteolytic activity against casein substrate was utilized to determine the optimum pH of protease enzyme purified from oleander flowers. Acetate buffer for pH: 4-5, phosphate buffer for pH: 6-7, Tris-HCl buffer for pH: 8-9, and borate buffer for pH: 10 were used at optimum pH. To each sample tube was added 1 mL casein solution, 500 µL enzyme solution and 1 mL buffer solution at the appropriate pH. One blind solution was prepared as a blind for each sample, buffer was used instead of enzyme in the blind solution.

2.2.7. *Determination of optimum temperature*

To determine the optimum temperature of protease enzyme purified from oleander flowers, the activity was determined by increasing 10 °C at 10-90 °C. Blind tube and sample; 10 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C and 90 °C for 20 minutes. The reaction was then stopped by the addition of 3 mL of 5% TCA. It was waited for 30 minutes for the precipitation to take place completely, and this was then centrifuged for 15 minutes at 6,000 rpm. After the supernatant was filtered, the amount of the degraded products in the supernatant was determined by the Bradford Method [10].

2.2.8. *Determination of enzyme Vmax and K_M values for casein substrate*

Proteolytic activity of the enzyme was utilized to determine the Vmax and KM values of protease enzyme purified from oleander flowers. 100 µL, 200 µL, 400 µL, 600 µL, 800 µL of each tube was added to the solution containing 1 g of casein per 100 mL of solution. The tubes were supplemented with 500 µL enzyme solution and a final volume of 2.5 mL buffer solution. The tubes were incubated at 40 °C for 20 minutes at which time 3 mL of 5% TCA solution was added to the tubes. After 30 minutes, the precipitated proteins were centrifuged at 6,000 rpm for 20 minutes and then filtered, and the amount of protein in the supernatant was determined by the Bradford method. Blind same substrate concentrations were also prepared without enzyme, using buffer solution instead of enzyme.

1 / V versus 1 / [S] Lineweaver Burk graph was drawn. Vmax and KM values were calculated from the results obtained.

2.2.9. *Determination of molecular weight by gel filtration chromatography*

After protease enzyme purification from oleander flowers of different colors, molecular weight was determined using gel filtration chromatography. BSA (66 kDa), Albumin EGG (45 kDa) ß-Amylase (200 kDa) and ß-lactalbumin (18,4 kDa) Lysozyme (18 kDa) was first stabilized with 0.05 M Na_3PO_4 / 1 mM DTT (14.3 kDa) protein standards were loaded and standard graphics were generated by eluting with the same buffer. The purified protease enzyme was then loaded onto the column and eluted with 0.05 M Na3PO4 / 1 mM DTT (pH: 7) buffer. The results were compared to the standard graph. The molecular weight of the enzyme was determined with the help of the standard graphic created.

3. Results and Discussion

As a result of our literature research [11] for this study, it has been seen that the protease enzyme has a better efficiency with the triple phase method which is a new method. For this reason, the TPP method was used to purify the protease enzyme from the flowers of the oleander plant flower

It was observed that the amount of protease enzyme activity and protein purified from oleander flowers was good with the triple phase method. White, pink and red oleander flowers yielded 169.9%, 181.5% and 82.58%, respectively. Table 1 gives the purification results.

Table 1 The enzyme unit in protease enzyme homogenate obtained from flowers of oleander flower by triple phase method, specific activity and enzyme unit in homogenate purified protease enzyme, specific activity and purification results.

	Activity (EU/ml)	Total activity (U)	Protein (µg/mL)	Specific Activity (EU/mg)	Purification (folds)	Yield (%)
White oleander flower						
Homogenate	0.141	14.1	14	1.007	0	100
Medium Phase	0.234	23.4	25.25	0.926	0.919	165.9
Pink oleander flower						
Homogenate	0.15	15	15	1	1	100
Medium Phase	0.272	27.2	30	0.906	0.906	181.5
Red oleander flower						
Homogenate	0.155	15.5	15.375	1.008	1	100
Medium Phase	0.128	12.8	12	1.066	1.057	82.58

The interaction between t-butanol and ammonium sulphate was exploited in the TPP system to efficiently capture the enzymes at the interface. To determine the appropriate t-bu-

tanol ratio, 1.0: 0.5, 1.0: 1.0, 1.0: 1.5 and 1.0: 2.0 homogenate: t-butanol (v / v) in the presence of 30% ammonium sulphate were studied. The protease activity and protein content were determined at each middle and lower phase obtained from the homogenate: t-butanol ratio, and the phase with the highest rate of purification and purification was determined. The enzyme prefered to remain in a predominant medium phase while the ammonium sulfate saturation was 30% (w / v) and the homogenate: t-butanol ratio was 1: 1.5 in this TPP system (Figure 1).

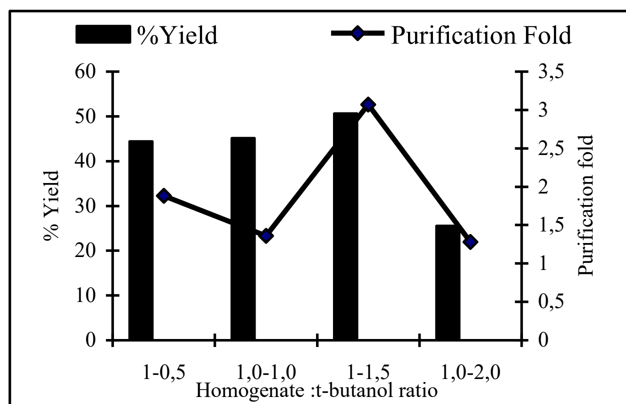

Figure 1 Effect of homogenate: t-butanol ratio on the% 30 ammonium sulphate saturation, purification coefficient on the purification of protease enzyme of oleander grass and activity yield

The precipitation of ammonium sulphate is based on the principle of salting out. The salting-out efficiency of proteins is dependent on the sulfate concentration and the net charge of the proteins. For this reason, saturation of ammonium sulphate is an important parameter and should be optimized. The most suitable homogenate: t-butanol ratio was found to be 1: 1.5. This fraction was reached in 30% ammonium sulfate saturation and purification was carried out separately at concentrations of 20%, 40% and 50% ammonium sulphate. Protease activity and protein content were determined in each medium and sub-phase obtained from ammonium sulfate concentrations and the phase with the highest dissolution and purification coefficients was determined (Figure 2).

In this system, while the ammonium sulfate saturation was 30% (w / v) and the homogenate: t-butanol ratio was 1: 1.5, the enzyme prefers to remain predominantly in the middle phase. Therefore, characterization was carried out using these ratios.

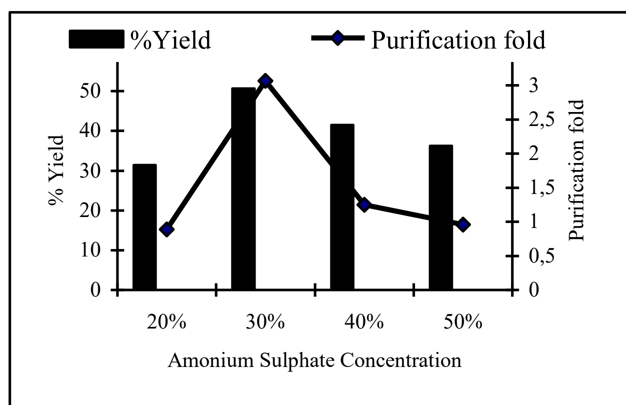

Figure 2 Effect of ammonium sulfate concentration at homogenate: t-butanol ratio on purification efficiency and activity yield of purifying protease enzyme of oleander grass

To determine the optimal pH value of protease enzyme purified from oleander flowers, activity measurements were made at a pH of 4-10 and the activity-pH graph was plotted. The amount of proteolytic activity versus pH change is given in Figure 3. It has been determined that the optimum pH of the enzyme for each species is in the range of 5-6 and the activity is in the range of pH: 4-9 [12].

Skin pH is between 4.0 and 6.0. When this result is taken into consideration, it is observed that the pH of oleander flower is suitable for the skin.

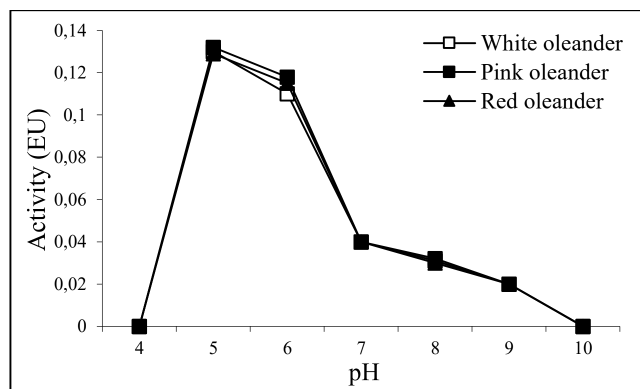

Figure 3 The effect of pH on the activity of protease enzyme purified from oleander flowers

In order to determine the optimum temperature value of protease enzyme purified from oleander flowers, enzyme activity was measured at 0-100 °C and optimum temperature was 30 °C and enzyme activity was 20-60 °C. The amount of proteolytic activity versus temperature change is shown in Figure 4. Enzymes lose activity above 40 °C [11]. The optimum temperature of the protease enzyme purified from the oleander flower is at a temperature suitable for the enzymes.

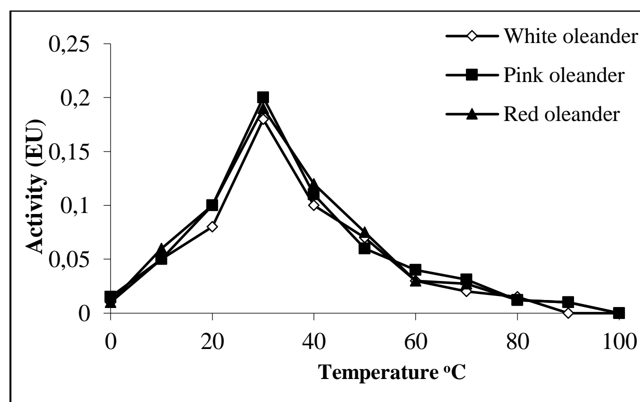

Figure 4 The effect of temperature on the activity of protease enzyme purified from oleander flowers

Measurements of standard proteins eluted with gel filtration chromatography for molecular weight determination were made at 280 nm. Ve / Vo and LnMW values were calculated by determining which tubers were based on the molecular weights of the standard proteins. It is given in Table 2.

From the data calculated in Table 2, a standard protein graph to be used in molecular weight calculation was generated. Is given in Figure 5.

Gel filtration chromatography of protease enzyme purified from oleander flowers obtained absorbance measurements at 280 nm of the eluted proteins. The enzyme activity was determined in the tubules with the presence of the protein, and it was determined which of the enzymes of the protease was present. The molecular weights of the proteins in the tubes were calculated using the standard protein graph (Figure 5). This process was done separately for White, Pink and red colored oleander flowers. The calculations related to the molecular weight are given in Table 3, Table 4 and Table 5.

Table 2 Calculations made for the standard protein graph

Standard protein mixture	MW	Tube sequence	Ve/Vo	LnMW
Lysozyme	14,300	44	13,681	9,568
ß-Lactoglobuline	18,400	35	10,882	9,820
Albumin, EGG	45,000	23	7,151	10,714
Albumine, Bovine	66,000	18	5,597	11,097
ß-Amylase	200,000	13	4,042	12,206

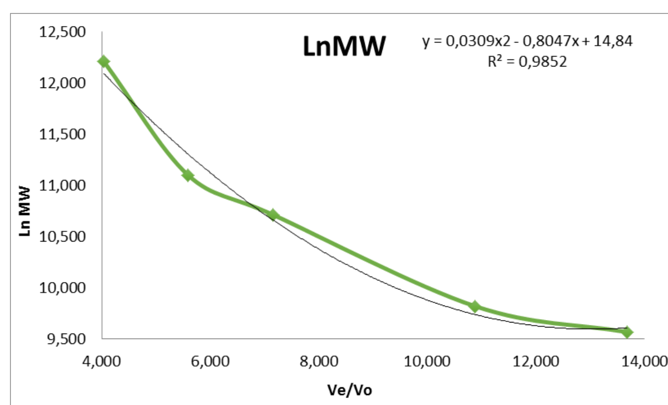

Figure 5 Gel filtration chromatography standard curve (Sepharose 4B, BSA (66 kDa), Albumin EGG (45 kDa), β-Amylase (200 kDa), β-lactalbumin (18,4 kDa), Lysozyme (14,3 kDa)

The molecular weight of protease enzyme purified from oleander flowers was determined by gel filtration chromatography to be white oleander 40,537 kea pink oleander 21,386 kDa and 22,516 kDa. It was observed that molecular weights of oleander herb had a molecular weight close to that of other plants [13, 14].

Table 3 Calculation of the molecular weight of protease enzyme purified from white oleander flowers by gel filtration chromatography.

Proteins	MW (Dalton)	Tube sequence	Ve/Vo	LnMW
1. Protein	691300	6	1,8656	13,446
2. Protein	81164	18	5,5967	11,304
3. Protein	22527	30	9,3278	10,022
4. Protein	17790	34	10,5715	9,786
5. Protein	15868	37	11,5043	9,672
6. Protein	14813	41	12,7480	9,603
7. Protein	**21386**	**53**	**16,4791**	**9,970**
8. Protein	24719	55	17,1009	10,115
9. Protein	113474	68	21,1430	11,639
10. Protein	156906	70	21,7648	11,963

Table 4 Calculation of the molecular weight of protease enzyme purified from pink oleander flowers by gel filtration chromatography.

Proteins	MW (Dalton)	Tube sequence	Ve/Vo	LnMW
1. Protein	70584	19	5,9076	11,165
2. Protein	**40537**	**23,5**	**7,3068**	**10,610**
3. Protein	23358	29,5	9,1723	10,059
4. Protein	18703	33	10,2606	9,836
5. Protein	14936	40	12,4370	9,612

Table 5 Calculation of the molecular weight of protease enzyme purified from red oleander flowers by gel filtration chromatography.

Proteins	MW (Dalton)	Tube sequence	Ve/Vo	LnMW
1. Protein	55160	12	3,731	10,918
2. Protein	12789	15	4,664	11,759
3. Protein	48089	22	6,840	10,781
4. Protein	26265	28	8,706	10,176
5. Protein	24245	29	9,017	10,096
6. Protein	**22516**	**30**	**9,3278**	**10,022**
7. Protein	21036	31	9,6387	9,954
8. Protein	21633	32	9,9496	9,892
9. Protein	14779	42	13,0589	9,601
10. Protein	17997	50	15,5463	9,798
11. Protein	18958	51	15,8572	9,850
12. Protein	20070	52	16,1681	9,907
13. Protein	26822	56	17,4119	10,197

1 / V versus 1 / [S] Linewear-Burk plots were drawn for the casein substrate of protease enzyme purified from oleander flowers and KM and Vmax values were calculated separately (Table 6).

Table 6 Results of casein substrate specificity of protease enzyme purified from oleander flowers

Substrate	KM (µM)	Vmax (µg/mL.min)
White oleander casein	1.187	1.303
Pink oleander casein	1.230	1.315
Red oleander casein	1.229	1.309

The KM and Vmax values were calculated as 1.187 µM white oleander and 1.303 µg / mL * minute pink oleander respectively at 1,230 µM and 1.315 µg / mL * min, red oleander at 1.229 µM and 1.309 µg / mL * min, respectively.

4. Conclusions

In this study, protease enzymes from flowers of oleander plant flora were purified using a novel method, the triple phase method. In order to efficiently collect the enzymes in the TPP system, the ammonium sulfate saturation at the interface was determined to be 30% (w / v) and the homogenate: t-butanol ratio was 1: 1.5 t-butanol. Therefore, characterization procedures were performed using these ratios. The enzyme was found to have an optimum temperature of 30 °C and an optimum pH of 5-6. The fact that the pH of oleander plant protease is suitable for the skin has led to the idea that this flower can be used in cosmetic studies and therefore the research is being done in this respect. The results obtained in

this study will shed light on the future studies to be focused on this aspect.

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