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RESEARCH ARTICLE

Purification of Protease Enzyme from Freesia (*Freesia refracta*) Flowers and Investigation of Its Use in Cosmetics

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ABSTRACT

This study aims to research and analyze a newly identified plant-based protease for potential applications within the cosmetic industry. The research focuses on isolating and characterizing a protease enzyme derived from Freesia refracta flowers. The purification method involves employing ammonium sulfate precipitation and ion exchange chromatography. Additionally, the protease's substrate specificity will be examined, particularly its reactions with azoalbumin and serum albumin. Enzymatic kinetics, inclusive of determining Vmax and Km values using Lineweaver-Burk plots, yielded readings of 0.784 mg/L.min and 0.317 mM, respectively. Furthermore, SDS-PAGE and gel filtration chromatography were utilized to determine the enzyme's degree of purification and molecular weight, indicating a molecular weight of 24 kDa. These outcomes identify the specific protease obtained from Freesia flowers, suggesting its potential suitability for cosmetic applications. Moreover, investigations into the impact of various compounds SDS, DIPF, EDTA, phenanthraline, iodoacetamide, Ca^{2+}, Zn^{2+}, Hg^{2+}, and Co^{2+} at a 10 mM concentration significantly enhanced the enzymatic activity of the purified protease. The research findings affirm the successful utilization of the protease extracted from Freesia flowers (*Freesia refracta*) in cosmetic formulations, showcasing its practical suitability for cosmetic production.

Contents

1. Introduction 66
2. Materials and Methods 66
   2.1. Purification of Protease enzyme 66
   2.2. Determination of Protease Enzyme Activity 66
   2.3. SDS-PAGE Electrophoresis 67
   2.4. Determining Kinetic Properties of the Purified Enzyme 67
   2.5. Assessing the Impact of Cations on Protease Enzyme Activity 67
3. Results and Discussion 67
   3.1. Isolation and Purification of Protease Enzyme 67
   3.2. Analysis of SDS-PAGE Electrophoresis 67
   3.3. pH and Sıcaklık Optimum 68
   3.4. Determination of Tyrosine and Tryptophan Amount 68
4. Conclusion 69
Acknowledgment 69
Funding 69

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Conflict of Interest 69
References 69

1. Introduction

Originating in South Africa, the Freesia plant has gained acclaim for its visual allure and captivating fragrance. Through hybridization, enthusiasts now enjoy a vast spectrum of sizes and colors. These hybrids, resulting from crosses between Freesia refracta and Freesia hybrida, have significantly expanded the array of yellow Freesia shades. Typically, the plant flaunts its vibrant flowers for around two weeks, but by staggering planting, a continuous display can be achieved.

Traditional propagation of Freesias involves corms and, due to their vulnerability to frost, indoor planting in autumn is advised. A mix of peat moss, light sand, and garden soil, in equal parts and slightly moistened, should be readied before placing in pots or deep troughs. Five to six corms fit comfortably in a 5-inch pot. Corms must be completely dried before planting, spaced about 2 inches apart, and embedded at least 1/2 inch below the soil surface

Containers housing planted corms should be kept in shaded areas at temperatures between 45 and 55 degrees Fahrenheit. Rapid root growth typically follows planting. As shoots emerge, ample sunlight exposure, especially from a south-facing window, is recommended. Moderate watering to maintain soil moisture, without complete drying out, is advisable [1, 2].

Proteases, also known as peptidases or proteinases, are pivotal enzymes catalyzing peptide bond hydrolysis in proteins. Their involvement spans various physiological processes, encompassing cellular metabolism, growth, and development. Industrially and biotechnologically, proteases have gained prominence for their diverse functionalities and potential commercial value.

Microbial proteases have been extensively studied concerning molecular and biotechnological aspects, focusing on gene cloning and sequencing from bacteria, fungi, and viruses. This research aims to facilitate overproduction, elucidate pathogenic roles, and modify properties for commercial applications [3]. Microbial proteases have surged in interest due to the inadequacy of plant and animal proteases in meeting global enzyme demands, especially in multiple industries [4].

Food and beverage sectors acknowledge microbial proteases, including those from plants, for their significant role in cheese-making. Nevertheless, further studies are warranted to enhance their application in food processing. Additionally, insights into the three-dimensional structure of aspartyl protease from HIV-1 have shed light on autoproteolytic release and virus maturation control [5]. Proteases hold industrial importance in producing bioactive peptides, meat tenderization, detergent manufacture, and bioremediation processes. A substantial portion of global enzyme production comprises proteases, widely used in pharmaceuticals, food, and leather industries [6, 7]. Their potential role in bioremediation accentuates their versatility and environmental relevance [8].In therapeutic realms, proteases' inhibitory effects on viral proteases, like HCV NS3 protease, have been explored. Understanding their characteristics and mechanisms is pivotal for developing inhibitors to control pathological protease activity [9, 10]. Moreover, grasping proteases' structural and dynamic properties aids in inhibitor design [11].

Proteases, a diverse enzyme class, hold vast implications in biotechnology, medicine, and industries. Ongoing research underscores their potential to meet global enzyme demands, develop therapeutics, and advance industrial applications.

This study aims to investigate and characterize the protease enzyme from Freesia (*Freesia refracta*) flowers in Turkey for potential utilization in the cosmetic industry

2. Materials and Methods

2.1. Purification of Protease enzyme

Freesia (*Freesia refracta*) flowers were sourced from the Çamlıyayla district in Mersin province, collected between May and July, and stored at -20 °C until analysis.

15 grams of Freesia (*Freesia refracta*) flowers were finely ground in liquid nitrogen, then suspended in 100 mL of 0.1 M phosphate buffer (pH: 6.0), agitated at 300 rpm for 30 minutes, followed by centrifugation at 5000xg for 15 minutes. The resulting supernatant was collected and utilized for protease enzyme production [12, 13].

In the purification process of the protease enzyme from Freesia (Freesia refracta) flowers, initial ammonium sulfate precipitation was performed within the range of 10% to 100%. Protein determination and enzyme activity were assessed at each stage [14].

Upon identifying the optimal range showcasing the highest protease enzyme activity, further purification involved CM-Cellulose ion exchange chromatography. The protein solution obtained from ammonium sulfate precipitation was introduced into a CM-Cellulose ion exchange column pre-equilibrated with 0.05 M acetate buffer (pH: 6.0), followed by washing with the same buffer solution. Subsequently, an ionic gradient of 0-1.5 M NaCl in 300 mL of 0.05 M acetate buffer (pH: 6.0) was employed to elute the protease enzyme from the ion exchange column [12]. Activity and protein content were assessed across all fractions, and fractions displaying activity were merged for further analysis.

2.2. Determination of Protease Enzyme Activity

Proteolytic activity was assessed using the casein digestion method involving a 1% casein solution. In this process, 0.5 mL of enzyme solution was combined with 1 mL of 1% casein, thoroughly mixed, and incubated at 40 °C for 20 minutes. Subsequently, 3 mL of 5% TCA (Trichloroacetic acid) was added and incubated for an hour. After centrifugation at 5,000 xg for 10 minutes, the resulting supernatant was filtered, and the concentration of cleaved protein was determined using the biuret method. The proteolytic activity (EU) was calculated as mg protein/mL degraded per minute by the enzyme [15].

2.3. SDS-PAGE Electrophoresis

Following enzyme purification, the structural analysis of the purified enzyme will be conducted using 3-10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, according to the Laemmli method [16].

2.4. Determining Kinetic Properties of the Purified Enzyme

The investigation sought to determine the purified protease enzyme's optimal pH and temperature obtained from Freesia (*Freesia refracta*) flowers. The optimal pH range was determined by measuring enzyme activity across pH 4.5-8.0 buffer solutions.

The optimal temperature was assessed by measuring proteolytic activity at intervals of 5 °C within the 0-90 °C range.

2.5. Assessing the Impact of Cations on Protease Enzyme Activity

The study explored the impact of various chemicals, including DIPF (diisopropyl fluorophosphate), SDS (sodium dodecyl sulfate), PHT (phenanthrene), EDTA (ethylenediaminetetetraacetic acid), iodoacetamide, and specific metal ions such as Ca^{2+}, Co^{2+}, Hg^{2+}, and Zn^{2+}, on the activity of the purified protease enzyme derived from Freesia (*Freesia refracta*) flowers.

3. Results and Discussion

3.1. Isolation and Purification of Protease Enzyme

This study aimed to purify the protease enzyme extracted from freesia flowers using ammonium sulfate fractionation and CM Sepharose ion exchange chromatography methods while exploring its kinetic properties. Initially, the highest quantity of protease enzyme was obtained by precipitating proteins in the crude extract with 30-50% saturation of ammonium sulfate, a widely practiced fractionation technique. Subsequently, the precipitated protease enzyme underwent purification through CM Sepharose ion exchange chromatography.

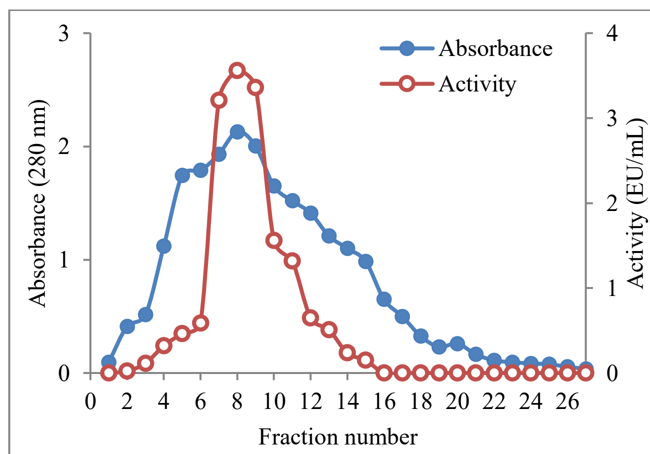

Figure 1 The electrophoretic pattern of standard proteins 66.0, 45.0, 34.7, 29.0, 18.4, 14.3 kDa (1, 2) and protease from freesia (*Freesia refracta*) (3, 4).

During the ammonium sulfate fractionation, a comprehensive analysis (as detailed in Table 1) summarized the purification process of the protease enzyme extracted from homogenized Freesia flowers. Optimal purification was achieved at 30-50% saturation, resulting in a 28.5-fold purification and a yield of 35.6%, surpassing other ammonium sulfate concentrations. The concentrated protease fraction obtained was then introduced into a CM Sepharose column. The enzyme was eluted from the column using an NaCl concentration gradient ranging from 0 to 1.5 M (depicted in Figure 1).

Consequently, the purification process yielded a 176.6-fold purified protease enzyme, exhibiting a yield of 72.05%. The specific activity of the purified enzyme was calculated at 4617.9 EU/mg protein (as outlined in Table 1) sourced from Freesia (*Freesia refracta*) flowers.

3.2. Analysis of SDS-PAGE Electrophoresis

The assessment of purified protease subunits involved SDS-PAGE analysis, visually presented in Figure 2. Comparative analysis with standard references, conducted through gel filtration chromatography, determined the enzyme's molecular mass to be 24 kDa.

Figure 2 The electrophoretic pattern of standart proteins 66.0,45.0,34.7,29.0,18.4,14.3 kDa (1, 2) and protease from freesia (Freesia refracta) (3, 4).

Previously, research isolated Euphorbain 1, an enzyme showcasing a distinct affinity for bonds adjacent to leucine residues, as a homogenous protein with a molecular mass of 43 kDa from the latex of *Euphorbia lathyris* [17, 18] Moreover, investigations into the plant protease isolated from *Calotropis procera* (family Asclepiadaceae) revealed a molecular mass of 28.8 kDa.

Table 1 Purification of Protease from flowers of Freesia (*Freesia refracta*)

Purification steps	Volume (mL)	Activity (EU/mL)	Total Activity EU	%	Protein (mg protein /mL)	Spesific activity (EU/mg protein)	Purification fold
Homogenate	100	16.3±0.11	1632	100	0.624±0.01	26.2	-
(30-50%) $(NH_4)_2SO_4$	15	38.7±1.33	580.5	35.6	0.052±0.18	744.6	28.5
CM Sepharose	20	58.8±2.16	1176	72.05	0.028±0.01	4617.9	176.6

3.3. pH and Sıcaklık Optimum

The impact of pH levels ranging from pH 4.0 to 11.0 on the activity of the protease enzyme derived from Freesia (*Freesia refracta*) flowers was examined, and the findings have been illustrated in Figure 3. The investigation revealed that the protease enzyme exhibited its peak activity at pH 8.0.

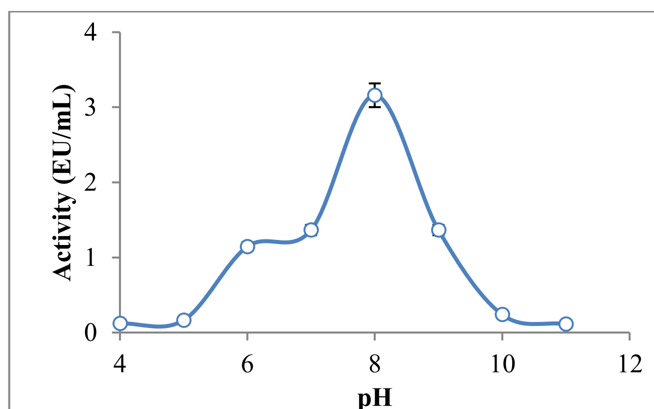

Figure 3 Effect of pH on purified protease activity

The impact of temperature variations ranging from 0 to 90 °C on the activity of the protease enzyme extracted from Freesia (*Freesia refracta*) flowers was assessed, with the outcomes illustrated in Figure 4. It was observed that the protease enzyme displayed its maximum activity at 40 °C

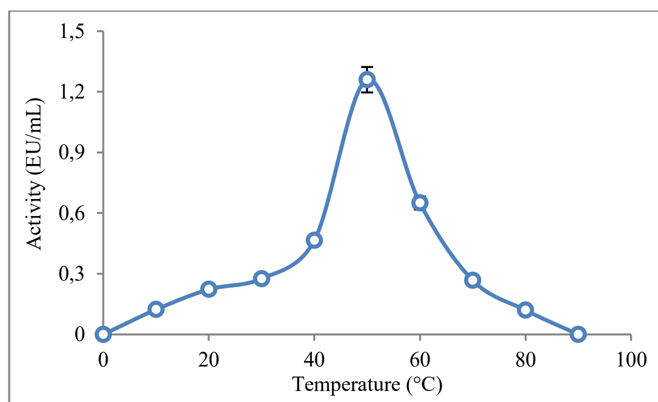

Figure 4 Effect of temperature on purified protease activity

Akhtaruzzaman et al. [19] identified the optimal pH for the protease enzyme sourced from leguminous seeds at pH 7.5, while the optimum temperature was recorded at 37 °C in their study. Similarly, Amid et al. [20] reported the optimum pH for the protease enzyme extracted from pitaya (*Hylocereus polyrhizus*) waste to be pH 8.0. Additionally, Tomar et al. (2008) [21]. determined the optimal pF range for the protease isolated from the Latex of the Plant Wrightia tinctoria to fall between 7.5 and 10. These referenced studies corroborate and support our own findings.

3.4. Determination of Tyrosine and Tryptophan Amount

The amount of tyrosine and tryptophan of the protease enzyme was calculated using Equation (1):

$$w = \frac{(A_{280} - x\varepsilon_y)}{(\varepsilon_w - \varepsilon_y)} \quad (1)$$

w= Tryptophan concentration in moles per litre

The molar extinction coefficient of tyrosine and tryptophan at 280 nm in ε_w and ε_y=0.1 N NaOH is (ε_y=1576 and ε_w=5225 at 280 nm at 280 nm in ε_w and $\varepsilon_y = 0.1\ N$ NaOH is ($\varepsilon_y = 1576\ and\ \varepsilon_w = 5225$) at 280 nm.

The total tyrosine and tryptophan concentrations in the protease enzyme were calculated using ε_294.4=2375 .x [22]. The total tryptophan content of the purified protease enzyme was calculated as 2.27×10^{-5} M and the total tyrosine content as 2.2×10^{-5} M.

Table 2 Effect of some chemicals and metal ions on protease activity: protease activator / inhibitor activity

Chemical (10 mM)	Activity (%)	Metal ions (10 mM)	Activity (%)
Control	100	Control	100.0
SDS	10	Zn2+	109.2
DIPF	15.71	Ca2+	486.7
EDTA	18.9	Co2+	-
Phenanthroline	36.6	Hg2+	-
Iodoacetamide	84.1		

The impact of various inhibitors and divalent metal ions, including SDS, DIPF, EDTA, iodoacetamide, and phenanthroline, was evaluated at a concentration of 10 mM on the protease enzyme derived from Freesia (Freesia refracta) flowers, resulting in activity reductions of 10%, 15.71%, 18.9%, 36.6%, and 84.1%, respectively, compared to the control.

Additionally, at the same 10 mM concentration, Zn2+ and Ca2+ demonstrated activities of 109.2% and 486.7%, correspondingly, while Co2+ and Hg2+ completely inhibited the protease enzyme sourced from Freesia (*Freesia refracta*) flowers.

Anigboro et al. [23] investigated the impact of certain chemicals and metal ions on the protease enzyme obtained from Moringa oleifera leaves, indicating effective inhibition by Zn2+, Mg2+, SDS, dithiothreitol, and β-mercaptoethanol. The findings from their study align with and support our own outcomes.

4. Conclusion

In summary, this study focused on purifying and characterizing the protease enzyme extracted from Freesia plant flowers. The purification process involved ammonium sulphate precipitation followed by ion exchange column chromatography, while the enzyme's activity was assessed using the casein digestion method. The findings underscored the robust activity of the protease enzyme sourced from Freesia flowers, showcasing resilience against various chemicals. Furthermore, the purified enzyme exhibits potential applicability in cosmetic formulations.

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Conflict of Interest

The authors declared no conflict of interest.

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