Purification and characterization of cysteine protease from miswak *Salvadora persica*

Wesam H. Abdulaal

**Abstract**

**Background:** Generally, proteases in medicinal plants had different therapeutic effects such as anti-inflammatory effect; modulate the immune response and inhibitory effect toward tumor growth. In this study, protease was purified and characterized from miswak roots, as medicinal plant and natural toothbrush.

**Results:** Physical and chemical characterization of cysteine protease P1 were studied such as pH optimum (6.5), optimum temperature (50 °C), thermal stability (50 °C) and Km (3.3 mg azocasein/ml). The enzyme digested some proteins in the order of caseine > haemoglobin > egg albumin >gelatin > bovine serum albumin. Hg^{2+} had strong inhibitory effect on enzyme activity compared with other metal ions. Kinetic of inhibition for determination the type of protease was studied. Iodoactamide and p-Hydroximercuribenzoic acid (p-HMB) caused strong inhibitory effect on enzyme activity indicating the enzyme is cysteine protease.

**Conclusions:** The biochemical characterization of this enzyme will be display the suitable conditions for using of this enzyme in toothpaste in the future and the enzyme may be used in other applications.

**Keywords:** Miswak, *Salvadora persica*, Cysteine protease, Purification, Characterization

**Background**

Proteases has been characterized from plants such as pea roots [1], ginger [2], *Euphorbia microscidia* [3] and sweet potato [4]. Plant Proteases had medicinal properties such as bromelain proteases which treated anticancer and osteoarthritis [5–8]. Bromelain has also been effective in the treatment of cardiovascular diseases, where it inhibited the aggregation of blood platelet and minimized the risk of arterial thrombosis [9]. In human intestine, bromelain was absorbed without its degrading and losing its biological activity [10, 11]. Other proteases from malian medicinal plants treated schistosomiasis [12]. Papaya proteases had pharmaceutical applications such as antitumorals, anti-inflammatory, wound healing and digestive disorder [13]. The partially purified protease from *B. subtilis* substantially dehaired cow skin [14].

Chemical compositions of miswak (*Salvadora persica*), natural toothbrush, included salvadoside and salvadora-side [15–17]. Silica removed stains from tooth surfaces [17, 18]. Miswak had several biological activities such as oral hygiene, antibacterial and antifungal [17–21].

Previously, we published article on purification and characterization of α-amylase from miswak [22]. In the present study, proteases from miswak has been purified and characterized.

**Methods**

**Miswak**

Miswak root was purchased from local market of Jeddah, Saudi Arabia.

**Measurement of protease used azocasein**

Proteolytic activity was measured by Dominguez and Cejudo [23]. One unit of protease was defined as μg azocasein hydrolyzed/ml/h under standard assay conditions.

**Ninhydrin assay**

α-Amino nitrogen was determined by Moore [24] for the substrates gelatin, casein, egg albumin, bovine serum albumin and hemoglobin.

**Measurement of protein**

Protein was measured by Bradford [25].
Purification procedure of miswak protease
Protease was purified from miswak root by using ion exchange and gel filtration chromatography techniques. By ion exchange, DEAE-Sepharose column (10 × 1.6 cm. i.d.) was used and the elution buffer was Tris-HCl pH 7.2 in presence of gradient of NaCl ranged from 0.0 to 0.3 M. The same buffer was used for gel filtration (Sephacryl S-200 column, 90 × 1.6 cm. i.d.) in absence of NaCl.

Molecular mass
The molecular mass of purified protease was determined by two techniques, gel filtration and sodium dodecyl sulphate (SDS-PAGE) [26].

Characterization
Physical and chemical characterization of protease with respect to pH optimum (pH 4–9), optimum temperature (30–80 °C), thermal stability (30–80 °C), substrate specificity (caseine, hemoglobin, egg albumin, gelatin and bovine serum albumin) and effect of metal ions (Ca²⁺, Ni²⁺, Pb²⁺, Hg²⁺, Cu²⁺ and Zn²⁺) were studied. Kinetics of inhibition (phenylmethylsulfonyl fluoride (PMSF), 1,10 phenanthroline, ethylenediaminetetraacetic acid (EDTA), p-HMB and iodoacetamide) for determination the types of protease were carried out.

Determination of km
The km values were determined from Lineweaver–Burk plots by using different concentrations of azocasein ranged from 1.5–4.5 mg/ml [27].

Results
Two chromatography columns were used for purification of protease from miswak. By DEAE-Sepharose column, three isoforms of proteases (P1, P2 and P3) were eluted by gradient steps of NaCl at 0.0, 0.1 and 0.2 M, respectively (Fig. 1). Protease P1 possessed the most activity (412 units) and the highest specific activity (89.5 units/mg protein) compared with the other isoforms (Table 1). Protease P1 was chromatographed on Sephacryl S-200 column (Fig. 2), where the specific activity and fold purification raised to 355 units/mg protein and 9.1 fold, respectively. The homogeneity of protease P1 was detected by SDS-PAGE (Fig. 3). The molecular mass of protease P1 was found to be 42 kDa by using Sephacryl S-200 and SDS-PAGE.

The substrate specificity of protease P1 was detected by using different proteins (Table 2). The protease P1 digested proteins in the order of caseine > haemoglobin > egg albumin > gelatin > bovine serum albumin with 100, 95, 72, 68 and 53% residual activity, respectively. The kinetic of protease P1 was detected by determining its km. The km of protease P1 was found to be 3.3 mg azocasein/ml by using reciprocal of Lineweaver-Burk plot (Fig. 4).

The effect of pH on the activity of protease P1 was determined (Fig. 5). The pH optimum of protease P1 was detected at pH 6.5. The enzyme acts on acidic and alkaline sides of pH profile, where its residual activity % was 45 and 38 at pH 4 and 9, respectively. The temperature optimum of the protease P1 was determined from temperature profile (Fig. 6). The protease P1 had temperature optimum at 50 °C. The enzyme retained 40% of its activity at 80 °C.! In the same manner, the protease P1 was thermal stable up to 50 °C and the enzyme lost 50% of its activity at 80 °C after incubation for 1 h (Fig. 7).

The effect of metal ions (Ca²⁺, Ni²⁺, Pb²⁺, Co²⁺, Cu²⁺ and Zn²⁺) on the activity of the protease P1 was detected (Table 3). All metal ions caused partial inhibitory effect on the protease P1 except of Hg²⁺.

![Fig. 1](image-url) Chromatography of miswak protease on DEAE-Sepharose column. (—) Absorbant at 280 nm, (x — x) units/fraction.
which caused strong inhibitory effect. The study of protease inhibitors on the activity protease P1 was evaluated (Table 4). PMSF, 1,10 phenanthroline and EDTA caused slightly inhibitory effect on the activity protease P1, while p-HMB and iodoacetamide caused strong inhibitory effect.

### Discussion

In this study, protease was purified and characterized from miswak roots. After two steps of chromatography, the homogeneity of protease P1 was detected by SDS-PAGE. The molecular mass of protease P1 was found to be 42 kDa. Different molecular weights (30–45

| Steps                                      | T. units | T. Protein mg | SA* | Fold purification | Recovery 100% |
|--------------------------------------------|----------|---------------|-----|-------------------|---------------|
| Crude extract                              | 545      | 14            | 39  | 1                 | 100           |
| Chromatography on DEAE-Sepharose           |          |               |     |                   |               |
| 0.0 M NaCl (P1)                            | 412      | 4.6           | 89.5| 2.29              | 75            |
| 0.1 M NaCl (P2)                            | 40       | 2.2           | 18  | 0.46              | 7.3           |
| 0.2 M NaCl (P3)                            | 60       | 6.5           | 9.2 | 0.23              | 11            |
| Sephacryl S-200 P1                         | 355      | 1.0           | 355 | 9.1               | 65            |

*S.A Specific activity (units/mg protein)*

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**Fig. 2 a** Chromatography of miswak protease P1 DEAE-Sepharose fraction on Sephacryl S-200 column. (— — —) Absorbant at 280 nm, (x — — x) units/fraction. **b** Molecular weight value for miswak protease P1 was calculated from calibration curve of Sephacryl S-200 column. Standard proteins from 1 to 5 were 1) Cytochrome C (12,400 Da); 2) Carbonic anhydrase (29,000 Da); 3) Bovine albumin (66,000 Da); 4) Alcohol dehydrogenase (150,000 Da); 5) β-Amylase (200,000 Da). Void volume was determined with Dextran blue (2000, 000 Da).
kDa) were detected for cysteine proteases from horse gram [28], *Curcuma longa* [29], *Euphorbia nivulia* [30] and ginger rhizome [31].

The substrate specificity of protease P1 showed that the enzyme acted on caseine, haemoglobin, egg albumin, gelatin and bovine serum albumin. Similar digestion was detected for horse gram cysteine protease toward the substrates [28]. The Km of protease P1 was found to be 3.3 mg azocasein/ml. Similar Km (2.8 mg azocasein/ml) was detected in germinated wheat cysteine protease [32]. The high value of Km (6.74 mg azocasein/ml) was detected for onion [33].

The maximum activity of protease P1 was detected at pH 6.5. However, horse gram cysteine protease showed higher activity at pH 5.5 [28]. The acidic pH optimum of germinated wheat cysteine protease was detected at 4.0 [32]. The protease P1 had temperature optimum at 50 °C and thermal stability up to 50 °C. The maximum activity of cysteine proteases from horse gram and *Euphorbia microscadia* was detected at 40 °C and 45 °C, respectively [3, 28].

All metal examined caused partial inhibitory effect on the protease P1. However, the inhibitory effect of Hg$^{2+}$

| Table 2 | Substrate specificity of miswak protease P1. Each value represents the mean of three experiments ± S.E |
|---------|--------------------------------------------------|
| Substrate | Units/mg protein | Relative activity % |
| Casein   | 370 ± 18.5 | 100 ± 5.0 |
| haemoglobin | 350 ± 16.9 | 95 ± 4.6 |
| Egg albumin | 264 ± 13.9 | 72 ± 3.8 |
| Gelatin  | 348 ± 20.4 | 68 ± 4.0 |
| Bovine serum albumin | 197 ± 10.4 | 53 ± 2.8 |

*Fig. 3* SDS-PAGE for miswak protease P1. 1, molecular markers, 2, crude extract, 3, DEAE-Sepharose P1, 4, Sephacryl S-200 P1

*Fig. 4* Reciprocal of Lineweaver-Burk plot relating miswak protease P1 reaction velocities to azocasein concentrations (1.5–4.5 mg). Km was calculated as mg azocasein/ml

*Fig. 5* pH optimum of miswak protease P1. The reaction mixture contained in 1.0 ml: 3% azocasein, 100 µl of enzyme and 50 mM sodium acetate buffer (pH 4.0–6.5), and 50 mM Tris-HCl buffer (pH 7.0–9.0) and adjusted to 1 ml with distilled water. Each point represents the mean of three experiments ± S.E.
was strong indicated that the protease P1 is cystein protease. This agreed with cysteine proteases from *Euphorbia microscadia* and horse gram which inhibited by Hg^{2+} [3, 28]. The inhibition of the activity of protease P1 by protease inhibitors determined the types of protease. The slightly inhibitory effect of PMSF, 1,10 phenanthroline and EDTA on the activity of protease P1 showed that the enzyme did not serine or metalloprotease. *p*-HMB and iodoacetamide caused strong inhibitory effect on the activity of the protease P1 indicating the enzyme is cysteine protease. Similar inhibitory effects were detected for cysteine proteases from *Euphorbia microscadia* [3], horse gram [28] and ginger rhizome [31].

From the above findings the miswak protease P1, as cysteine protease, could be used in toothpaste for oral hygiene. However, Pleszczyńska et al. [34] studied the potential applications of enzymes in the treatment and prevention of oral diseases. Proteases of plant origin have been tested for removal of tooth stains and calculus [35]. A papain, as cysteine protease, gel has been used for removal of caries, which eliminates infected dentin and simultaneously preserves a healthy dental structure [36].

**Conclusions**

The study indicated that the purified miswak protease P1 is cysteine protease depending on the study of the inhibition by cysteine protease inhibitors and Hg^{2+}. Depending on cysteine proteases such as papain are used in oral gel, miswak protease P1 may be digested the protein residues in the oral when the miswak is used as natural toothbrush. The biochemical characterization of this enzyme will be display the suitable conditions for using this enzyme in toothpaste in the future and the enzyme may be used in other applications.

| Table 3 | Influence of metal ions at 5 mM on miswak protease P1. Each value represents the mean of three experiments ± S.E |
|---------|--------------------------------------------------------|
| Metal cations | Units/mg protein | Relative activity % |
| Control | 355 ± 12.4 | 100 ± 3.3 |
| Ca^{2+} | 286 ± 15.0 | 80 ± 4.2 |
| Ni^{2+} | 293 ± 13.5 | 82 ± 3.8 |
| Pb^{2+} | 347 ± 16.4 | 97 ± 4.6 |
| Co^{2+} | 286 ± 11.7 | 80 ± 3.3 |
| Hg^{2+} | 164 ± 9.2 | 46 ± 2.6 |
| Cu^{2+} | 268 ± 14.6 | 75 ± 4.1 |
| Zn^{2+} | 272 ± 12.8 | 76 ± 3.6 |

| Table 4 | Effect of protease inhibitors at 2 mM on miswak protease P1. Each value represents the mean of three experiments ± S.E |
|---------|--------------------------------------------------------|
| Substrate | Units/mg protein | Relative activity % |
| Control | 321 ± 17.3 | 100 ± 5.4 |
| PMSF | 289 ± 13.4 | 90 ± 4.2 |
| 1,10 Phenanthroline | 304 ± 13.1 | 95 ± 4.1 |
| EDTA | 293 ± 12.5 | 91 ± 3.9 |
| p-HMB | 71 ± 3.8 | 22 ± 1.2 |
| Iodoacetamide | 118 ± 5.1 | 35 ± 1.5 |
Abbreviations
EDTA: Ethylenediaminetetraacetic acid; p-HMB: p-Hydroxymercuribenzoic acid; PMSF: Phenylmethylsulfonyl fluoride; SDS-PAGE: Sodium dodecyl sulphate

Acknowledgements
Not Applicable.

Funding
This project was funded by the Deanship of Scientific Research (DSR) at King Abdulaziz University, Jeddah, under grant No. (G-204-130-438). The author, therefore, acknowledge with thanks DSR for technical and financial support.

Availability of data and materials
All data generated or analyzed during this study are included in this published article or available from the corresponding author on reasonable request.

Authors’ contributions
WA designed the experiments, analyzed the data and approved the final version of the manuscript.

Ethics approval and consent to participate
Not Applicable.

Consent for publication
Not Applicable.

Competing interests
The author declares that he has no competing interests.

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Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 9 September 2018 Accepted: 22 November 2018
Published online: 03 December 2018

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