

Effect of Some Inhibitors on Protease Extracted from Caper (*Capparis spinosa* L.)

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Abstract

Caper protease showed maximum activity at pH 6.0 when it was determined at various pH's while it was no activity in pH 9.0 and 9.5. No effect of chelating agent (EDTA) on activity where as reducing reagent (Cysteine) showed perceptible effect typically at 0.7 mM. Protease was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation, CM Cellulose ion exchange and Sephadex G100 size exclusion. Purification fold for three steps were 1.33, 1.96 and 4.26, respectively. The inhibitor E64 gave complete inhibition compared with other inhibitors. Finally activation energy of thiol protease to convert and denaturation were 37.24 kJ mole⁻¹ and 129.47 respectively.

خلاصة:

وجدت اعلى فعالية للبروتيز المستخلص من نبات الكبر (*Capparis spinosa* L.) باستخدام مدى متغير من الارقام الهيدروجينية عند الرقم 6 بينما فقدت الفعالية تماما عند الارقام 9 و 9.5. لم يلاحظ تغير في الفعالية بوجود العامل الكلايبي (EDTA) في حين وجد تاثير واضح للعامل المختزل سستائين بتركيز 7 ملي مولر. نقي البروتيز بالتركيز بواسطة كبريتات الامونيوم و المبادل الايوني الموجب كاربوكسي مثيل سليلوز والترشيح الهلامي بهلام سيفادكس G100 اذ كان عدد مرات التنقية 1.33، 1.96 و 4.26 على التوالي. فقدت الفعالية كليا باستخدام المثبط E64 مقارنة مع المثبطات الاخرى. قدرت طاقة تنشيط البروتيز للتحويل والدنترة اذ كانت 37.24 و 129.47 كيلو جول / مول على التوالي.

Introduction

Thiol protease contain a cysteine residue at its active center is usually identified depending on the effect of inhibitors (iodoacetate, iodoacetamide and E64) and activation of the enzymes by thiol compounds (1). Most of these proteases show acidic pH optima (2) Thiol protease has two important functions: contribute in synthesis of storage proteins during seed maturation and its degradation during germination also in programmed cell death (3). Thiol proteases were isolated and purified from different plants such as *Carica papaya* (4), *Bromelia plumier* (5) and *Ananas comosus* (6). In addition thiol protease extracted from another organisms including fungi, such as *Penicillium* sp (7) protozoan parasites *Giardia lamblia* (8) and bacteria, *S. aureus* (9). Proteases isolated from plants are natural substances can easily extracted by aqueous solutions with low cost and no legal barriers for utilizing in food manufacturing and medical fields (10), led us to find new source of proteases in our present work.

Materials and Methods

Fresh flower buds of *Capparis spinosa* were collected from wild plants located in AL- Qasim district. Buffers were prepared according to (11). Gelatin, EDTA and Cysteine were from BDH. Tyrosine, Pepstatin, E64 and PMSF were from Sigma. CM cellulose and Sephadex G-100 from LKB.

Enzyme Extraction

The flower buds (15g) were ground in a pre-chilled mortar with pre-chilled 0.05 M Phosphate buffer pH 7.2. The extract was filtered and centrifuged at 3000 rpm for 15 min (12). The supernatant was used for the enzyme activity and protein estimation.

Estimation of Enzymes Activity

The enzyme was assayed following the method as described by (13) using gelatin as substrate. One unit of enzyme activity is defined as the amount of enzyme producing 1 μ M tyrosine per minute at 45°C.

Measurement of Proteins

Protein concentration in the flower buds extract was measured according to the method described by (14).

Purification Techniques

Crude solutions of protease were fractioned by ammonium sulfate 20, 30, 40, 50, 60, 70 80 and 90% saturation. Participate was suspended with 20 mM citrate buffer pH5. The active fraction was dialyzed against the same buffer to remove ammonium sulfate salts. For additional purification dialyzed solution was passed through CM Cellulose column (1 \times 38) cm previously equilibrated with 20 mM citrate buffer pH5 (15). The fractions of active peak were pooled and dialyzed against 10mM Phosphate buffer pH7.2 then subjected on the 2 \times 42 cm of Sephadex G100 column pre equilibrated with the same buffer. The fractions of active peak were pooled and stored at 4°C for next experiments.

Effect of Inhibitors

Traditional inhibitors of proteases were used at varied concentrations according to instructions of manufactured company included E64 (1.00, 10 μ M), pepstatin (1.00, 10 μ M), PMSF (0.1, 1.00mM) and EDTA (1.00, 10.00mM). Same volumes of each inhibitors and enzyme were mixed, incubated separately and residual activity was estimated (13).

Activation Energy Determination

Activation energy was determined according to following equation mentioned by (16) $\ln k_2/ k_1 = E_a/ R (T_2-T_1/ T_1 T_2)$.

Results and Discussion

For initial investigation of proteases types in plant material, assay of activity with different range of pH were used. Results presented in table 1 showed the high level of specific activity was located in acidic pH compared with neutral and alkaline buffers.

Most of thiol protease was active at low pH (1) and required reducing agent such as β -Mercaptoethanol, Cysteine and Dithiothreitol to enhance their activity (17). Aspartic proteases, are the endopeptidases have aspartate amino acid at its active site. Most aspartic proteases also show higher activity at acidic pH (18). Serine proteases contain of a serine residue in active site, this type of protease was active at neutral and alkaline pH especially between pH 7-11 (19). Metalloproteases are needed for a divalent metal ion for its activity such as zinc atom which can be replaced in some cases by another metal such as cobalt or nickel without loss of the activity (20). According to above results we need further modifications in assay of enzyme activity for detection of major type of protease.

Using of reducing and chelating agents gave us obvious image for detection of major type of protease. Chelating factor (EDTA) with different concentrations (0.05-10 mM) has no effect on enzyme activity (data not shown), this indicates the absence of metals in catalytic site and enzyme does not belong to metalloproteases group. Ability to readily recognize of this type of protease from the other proteases by treatment with metal chelating agents such as ethylene diamine tetra-acetic acid (EDTA) or 1,10-phenanthroline leading to the removal of the metal ion cofactor and inactivation (21). The results illustrate in figure 1 indicated the increase of activity directly with the increase of reducing factor until activity is stable at higher concentration of cysteine, that reflex saturated the active site (thiol group) by reducing reagent. Increase of activity in the presence of cysteine refer to the protease extracted in this study may be belong to thiol protease. Generally, activity of thiol proteases reaches to maxima in the presence of reducing agents like HCN or cysteine. Cysteine residue at active site plays important role in enzyme activity and sulfhydryl group of this single amino acid is very susceptible to oxidation (17).

Table1. Initial detection of proteases types in flower buds of *Capparis spinosa*

Buffers pH	Activity (unit ml ⁻¹)	Protein (mg ml ⁻¹)	Specific activity (unit mg ⁻¹) protein
0.05M pH 4.5	1.40	0.60	2.30
0.05M pH 5.0	1.70	0.60	2.83
0.05M pH 5.5	2.00	0.60	3.33
0.05M pH 6.0	2.70	0.60	4.50
0.05M pH 6.5	1.50	0.60	2.50
0.05M pH 7.0	0.50	0.60	0.83
0.05M pH 7.5	0.30	0.60	0.50
0.05M pH 8.0	0.20	0.60	0.33
0.05M pH 8.5	0.08	0.60	0.13
0.05M pH 9.0	0.00	N.D.	0.00
0.05M pH 9.5	0.00	N.D.	0.00

N.D. Not determined

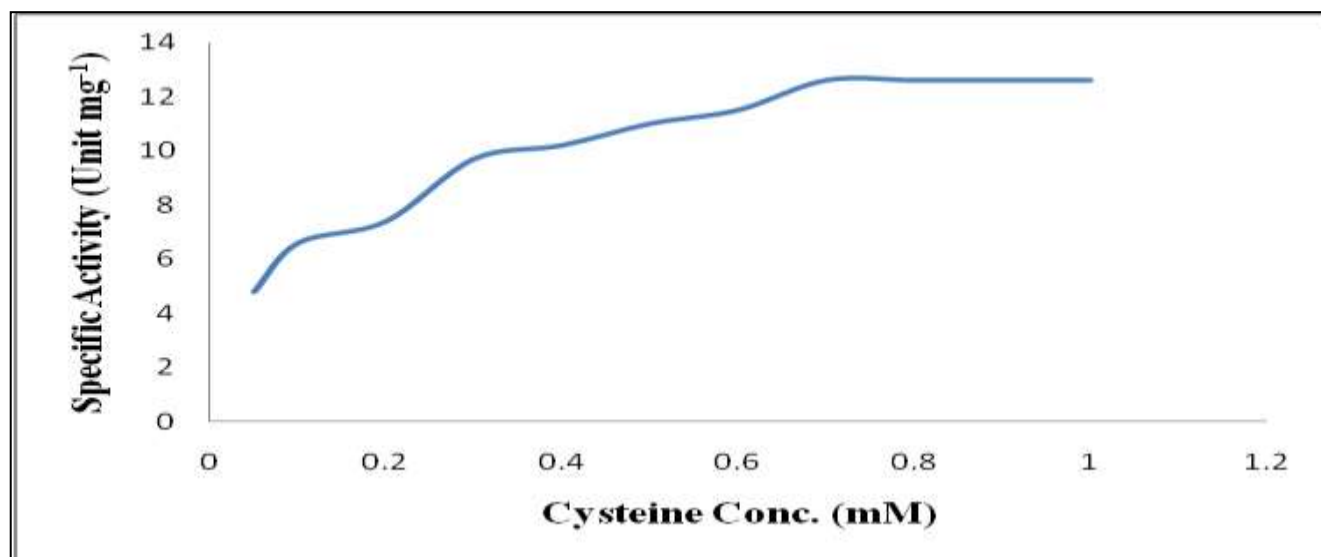


Fig1. Effect of reducing agent (Cysteine) on protease specific activity in flower buds of *Capparis spinosa*

Inhibitors for proteases, as serine (DIPF=DIP and PMSF), metallo (1,10-phenanthroline, EDTA), and thiol protease (iodoacetamide and some of them by PMSF) were used by (15) and they cannot precisely detect type of protease. Protease was inhibited with these chemical compounds in different concentrations result in difficult detection of protease type.

The crude extract was precipitated by ammonium sulfate in saturation ratio 60% yielded 57.38 % recovery and 1.33 fold purification factor (Table 2). In similar study thiol protease was precipitated from germinating cotyledons of *Macrotyloma uniflorum* with (30-60%) ammonium sulfate (22). The concentrated protease solution loaded on CM-Cellulose column showed presence of two peaks of proteins in washed fractions absent to enzyme activity. Eluted fractions revealed three peaks of proteins have protease activity only in first peak contained 53.11% from yield and 1.96 purification fold (Figure 2 and Table 2).

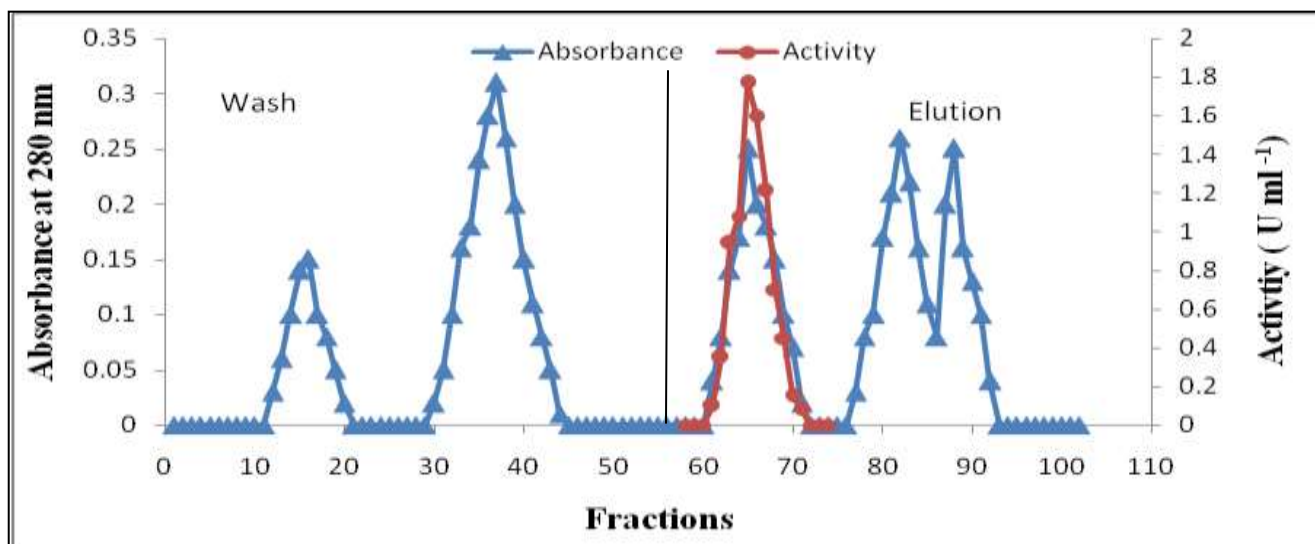


Fig2. Wash and elution of protease extracted from *Capparis spinosa* by cation exchanger column (1×38 cm) equilibrated with 20 mM citrate buffer pH5. Washing achieved by 20 mM citrate buffer pH5 while elution carried out by 20 mM citrate buffer pH5 have linear gradient (0-1 M) of NaCl. Fractions were collected (3 ml) at flow rate approximately 25 ml/h.

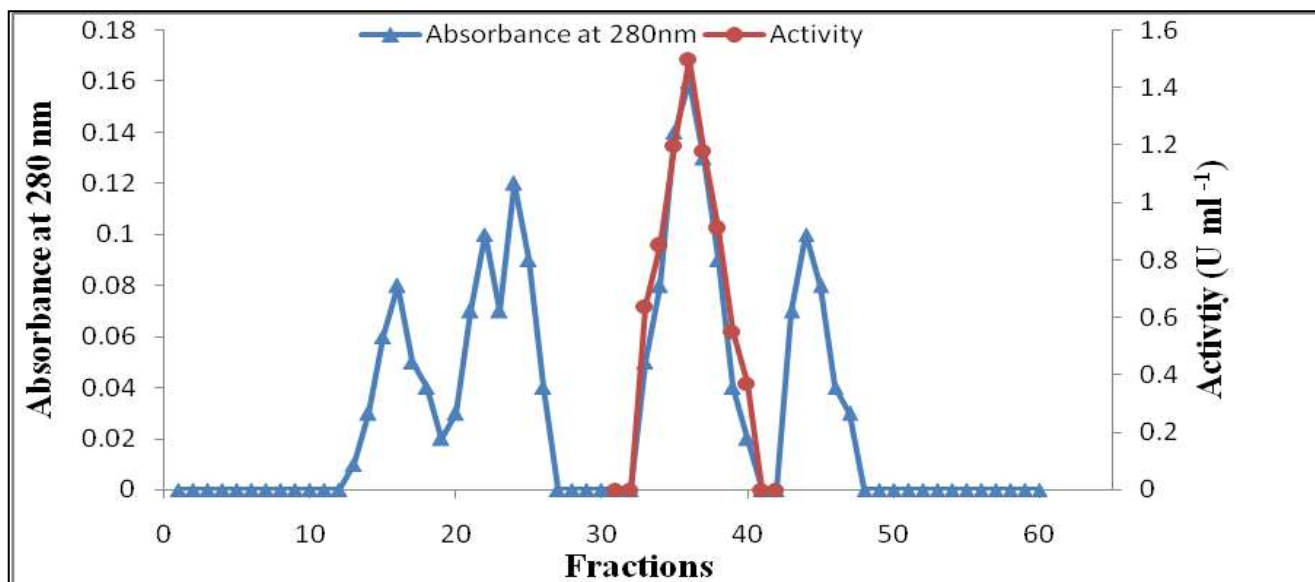


Fig3. Gel filtration of protease extracted from *Capparis spinosa* by Sephadex G100 column (2×42) cm pre equilibrated 10mM Phosphate buffer pH7.2. Fractions of 3 ml were collected at flow rate equal to 25 ml/h.

Cation and anion exchangers were used for protease clarification, one of thiol protease found in potato leaves purified by using of cation exchanger (2). The recovery and fold of purification was 33.56% and 4.26 respectively, the in fourth of five protein peaks that present when using Sephadex G-100 as last stage of clarification (Figure 3 and Table 2). Jack fruit (*Artocarpus integrifolis*) regarded as source of thiol protease purified by series of purification including gel filtration on Sephadex G-100 (23).

Table2. Purification steps of protease from *Capparis spinosa*

Step	Activity (U)	Protein (mg)	Specific activity (U mg ⁻¹) protein	Purification Fold	Recovery (%)
Raw proteases	75.80	6.10	12.42	1.00	100.00
60% (NH ₄) ₂ SO ₄	43.50	2.70	16.11	1.29	57.38
CM Cellulose	40.26	1.65	24.40	1.96	53.11
Sephadex G100	25.44	0.48	53.00	4.26	33.56

For precise detection of protease group, influences of different types of proteases inhibitors were used. Data in table 3 showed total inhibition of protease by thiol proteases inhibitor (E64). Other inhibitors (PMSF, Pepstatin and EDTA) which inhibit serine, aspartic and metallo-proteases respectively have no clear effect on activity that means the protease belong to thiol proteases. E64 irreversible inhibitor inhibits thiol proteases, does not affect cysteine residues in other enzymes or react with low molecular weight thiols such as β-mercaptoethanol and very specific to active site (24).

Table3. Effect of inhibitors on enzyme activity of protease purified from *Capparis spinosa*.

Inhibitor	Concentration	Residual Activity %
E64	1.00 μM	0.00 %
	10.00μM	0.00%
Pepstatin	1.00μM	100.00%
	10.00μM	97.00%
PMSF	0.100mM	100.00%
	1.00mM	96.00%
EDTA	1.00mM	100.00%
	10.00mM	100.00%

Determination ability of enzyme in decrease of activation energy to convert substrates into products required experimental detection of heat effect on thiol protease velocity (*k*) at different absolute temperatures presented figure 4. The value of *E_a* to transformation was 37.24 kJ mole⁻¹ while to denaturation was 129.47 kJ mole⁻¹. The *E_a* obtained in this study is closely similar to range of most *E_a* which located between 40-50 and 84-837 kJ mole⁻¹ respectively and can be calculated from the slope of the Arrhenius plot or assaying *v* at two different temperatures *T*₁ and *T*₂ according equation as above (25, 26).

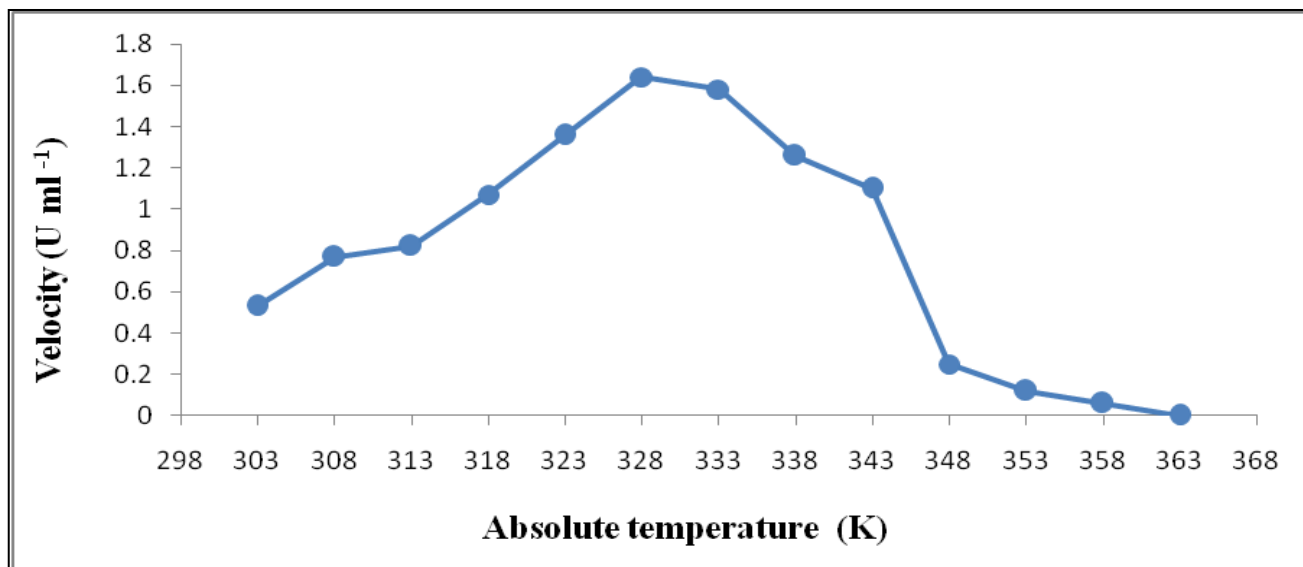


Fig4. Influence of temperatures on protease activity purified from *Capparis spinosa*

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