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Partial Purification of Metalloprotease from *Acacia farnesiana*

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Abstract

Not previously studied metalloprotease was tested in four species of leguminosae, purified and characterized from *Acacia farnesiana* which have maximum specific activity 25.01 U/mg. Precipitation by 50% ammonium sulfate referred to increase of specific activity (35.84) U/mg while loading on DEAE-Cellulose exchanger pointed out elevation of specific activity and purification fold, 51.39 unit/mg and 2.06 respectively. Molecular mass, pH and temperature optima of purified metalloprotease were 55.11kDa, 7.5 and 50^{o}C respectively. All metal ions were decreased enzyme activity except the zinc showed the increasing about 16%. EDTA was inhibiting the activity in otherwise other inhibitor not affected on enzyme activity. Optimum substrate for activity was BSA and has K_m and V_{max} 1.11mM and 625 mM/ min respectively.

Keyword: Metalloprotease, *Acacia farnesiana*,

Introduction

Proteases are famous enzymes which speed up the hydrolytic reactions of protein molecules to degrade into peptides and amino acids. Several groups of proteases differ in its features include specificity to substrate, active site, catalytic mechanism, optimum activity, stability to pH and temperature [1]. One of these groups are metalloproteases which present in bacteria, fungi and animals [2], and also reported in plant which involved the purification and characterization. [3]. Metalloproteases recognized from the rest proteases by contain, zinc atom or replaced by another metal such as cobalt or manganese in their active site. Exo- and endopeptidases metalloproteases in many different sub-cellular locations has ability to digestion or highly specific processing function [4]. According to few available articles about plants were obtained, we can mentioned the physiochemical properties of these type of protease which reviewed by [5] from plants and other organisms as follow: Molecular weights, pHs and temperatures optima and isoelectric points were located between 35-79.7 kDa, 5.5-8, 20-60^{o}C and 3.3-7.7 respectively.

Methods

Protease Extraction

50 gram fresh young leaves of 4 species of leguminosae (*Vigna radiate, Medicago sativa, Acacia farnesiana and Vicia faba*) were ground in blender with 250 ml of 50 mM phosphate buffer pH7.6 for 15 min at 25^{o}C. The mixtures were filtered by whatman No.4 filter paper to isolate and remove of all precipitations [6].

Protease Activity

Activity of protease was estimated according to Fadiloglu (2001). Digestion reaction start by adding of protease extract (0.5ml) to 1ml of 1% w/v casein previously dissolved in 100 ml of mM of phosphate buffer pH 7.6. Solution was incubated at 40 $^{\circ}$C for 20 min then reaction arrested by 3 ml of 5% (w/v) trichloroacetic acid (TCA). The non-digested protein in mixture after 60 min was isolated by centrifugation at 3000 rpm for 10 min and supernatant was measured at 280 nm for protease activity. Preparation of blank has similar steps mentioned as above but TCA was added prior

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the protease extract. One enzyme unit (U) is represented the amount of proteins were digested by the enzyme per min.

Proteins Concentration

Concentrations of proteins in the extractions were determined according to the [8] using BSA.

Purification Procedures

Traditional first step of clarification is sedimentation of protease in raw solution of extraction by subjected to solid ammonium sulfate added to 15 ml of the extract separately to reach final concentrations namely 30- 80% at 10 intervals. The sediment was collected by centrifugation (15 min at 10000 rpm and 4^{o}C) then suspended in 4 ml of 50 mM phosphate buffer pH 7.6 and dialyzed for 12 h at 4^{o}C against 1000 ml of the buffer. Second step included preparation of DEAE-Cellulose exchanger by packaging it in column with dimension 2.5×15 cm. The column was equilibrated by 50 mM phosphate buffer pH 7.6 for 24 h at flow rate 30ml/h. Dialyzed solution passed through column then washed by 50 mM phosphate buffer pH 7.6 and fractions collected (2ml for each fraction). Absorbance and activity were measured in wash fractions to detect the fractions have protease activity whereas elution of bounded proteins obtained by liner gradient NaCl (0-1M) dissolved 50 mM phosphate buffer pH 7.6. Collection, absorbance and activity were determined as in washing fractions.

Characterization

Molecular Mass

Molecular mass of protease was determined by size exclusion on Sephacyle S-200 column (1×42 cm) previously equilibrated by 200 mM phosphate buffer pH 7.6 using different marker proteins (Ferritin, BSA, Trypsin and Lysozyme). Flow rate of equilibrium and elution for marker proteins and protease were 30ml/h while volume of each fraction was 3ml.

Effect of pH

Protease activity was estimated at varied pH values ranging from 3-10 to detect the optimum pH of activity using appropriate buffers.

Effect of Temperature

The optimum temperature activity of protease was assayed at different temperatures (30-80 ºC).

Effect of Metals Ions

, $^{++}$, Hg^{2+}, $Ba^{2}Mn^{2+}$, , Mg^{2++}, Ca^{2}The effect of many metal ions included 1mM of Co^{2} $^{+}$ on protease activity were determined. Protease (0.5 ml) was $^{+}$, Ni^{2+}, $Zn^{2}Cu^{2}$ 50 mM phosphate buffer pH 7.6 incubated with metal ion have final volume 1.5 ml of at 45 ^{o}C for 20 min. Control was prepared as above except absence of metal ion.

Effect of Inhibitors

Same procedure in effect of the metal ions on protease was used but replaced by specific inhibitor with its appropriate concentration. Iodoacetamide (cysteine protease inhibitor), Pestatin A (aspartic protease inhibitor), PMSF (serine protease inhibitor) and EDTA (metalloprotease inhibitor).

Effect of different substrates

In addition of casein, BSA, hemoglobin and gelatin were used to determine of protease activity under optimum conditions.

***K_m* and *V_{max}* Studies**

The determination of kinetic parameters depending on double reciprocal plot for BSA using different concentrations included 0.5, 1, 2, 4, 6, 8 and 10 mM.

Results and Discussion

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The results in showed that all plant varies in enzyme activity, *Acacia farnesiana* leaves liberate optimum (25.01U/mg) compared with other plants followed by *Vigna radiate, Vicia faba and Medicago sativa* (table1). Type of protease in these legumes may be belong to metallo-enzyme of [9] who pointed out present of mealloprotease in leaves of *Glycine max* (leguminosae).

Table 1 Protease activity in selected legumes

Species	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)
Vigna radiata	3.62	0.24	15.08
Medicago sativa	12.70	3.30	3.84
Acacia farnesiana	64.55	2.58	25.01
Vicia faba	10.32	0.81	12.7

For investigation and characterization of major type of protease in plant have maximum specific activity (*Acacia farnesiana*), purification techniques should be accomplished. Precipitation by ammonium sulfate at 50% concentrate appeared increase of proteins content compared with crude extract (2.62mg/ml), but increase of the activity (93.92U/ml) subsequently gave higher specific activity (35.84 mg/ml) and led to increase in purification index (table2). Usually proteins have very high molecular weight precipitate below 25% ammonium sulfate saturation [10], that indicate protease in the present study not involved to this group of proteins (see below). Final purification index and recovery were 2.06 and 39.45% respectively at the peaks in 61-65 fractions of the DEAE-Cellulose exchanger (Figure 1 and table 3). Low volume of target protease in protein solution (10 ml) may be belong to the often enzymes are sticky, adhering to surfaces such as glass or the packing substance in the ion exchange column chromatography [11].

Table 2 Activity of *Acacia farnesiana* protease at various concentration % of ammonium sulfate

Conc. of ammonium sulfate (%)	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification index	Recovery (%)
Raw protease	15.00	64.52	2.59	24.91	967.8	1	100
30%	4.00	22.08	3.20	6.90	88.32	0.27	9.12
40%	4.00	48.14	2.05	23.48	192.56	0.92	74.61
50%	4.00	93.92	2.62	35.84	375.68	1.43	38.81
60%	4.00	36.07	1.82	19.81	144.28	0.79	14.90
70%	4.00	76.04	4.08	18.63	304.16	0.74	31.42
80%	4.00	51.60	3.00	17.20	206.4	0.69	21.32

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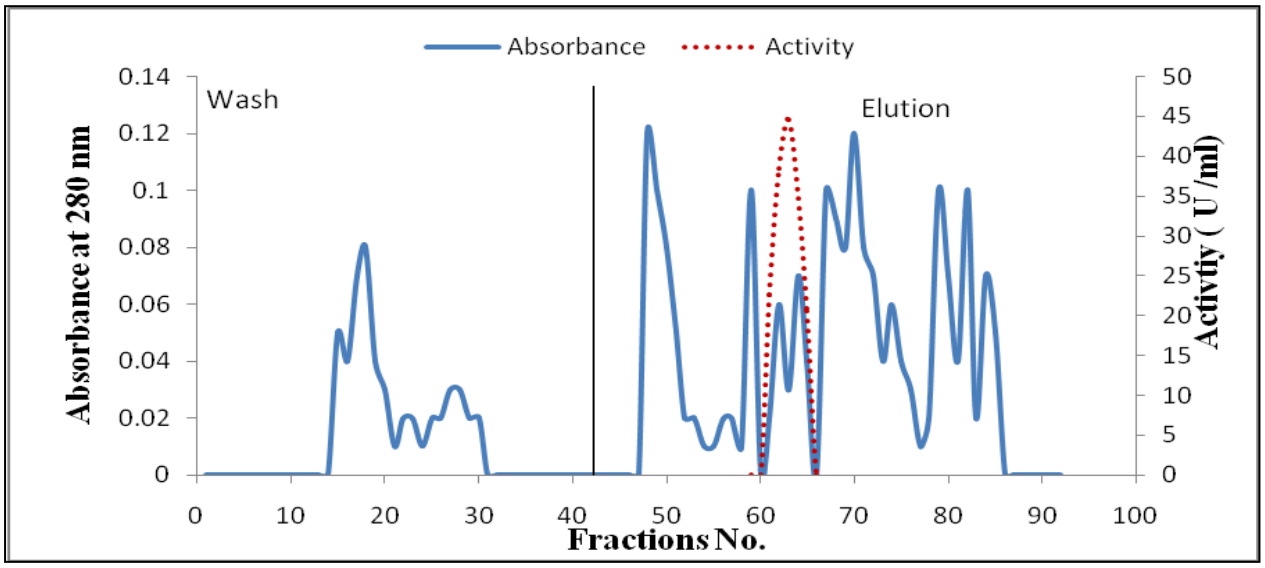

Figure 1 Ion exchange of *Acacia farnesiana* protease on DEAE-Cellulose exchanger

Molecular mass of protease was 55.11 kDa (Figure 2), the value with the elution of soy beans metalloprotease on a calibrated gel filtration was 15 kDa (Graham *et al*., 1991). The same type of protease was 79.7 kDa on SDS-PAGE under reducing condition when purified from *Euphorbia cotinifolia* [5] while *Nicotiana tabacum* have two forms with apparent molecular masses of ~30 and 55 kDa [12].

Table 3 Purification steps of *Acacia farnesiana* protease

Step	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification index	Recovery (%)
Raw protease	15.00	64.52	2.59	24.91	967.8	1.00	100
50% $(NH4)_2SO_4$	4.50	92.90	2.54	36.57	418.05	1.46	43.19
DEAE-Cellulose	10.00	38.03	0.74	51.39	380.30	2.06	39.45

The maximum activity for casein degradation was 38.02 at pH 7.5 (Figure 3), at this pH the ionizable groups in active site located in suitable ionic interaction for three dimensional binding between enzyme and substrate. These enzymes have a pH optimum near neutral values; hence some times the metalloproteases called neutral proteases [13].

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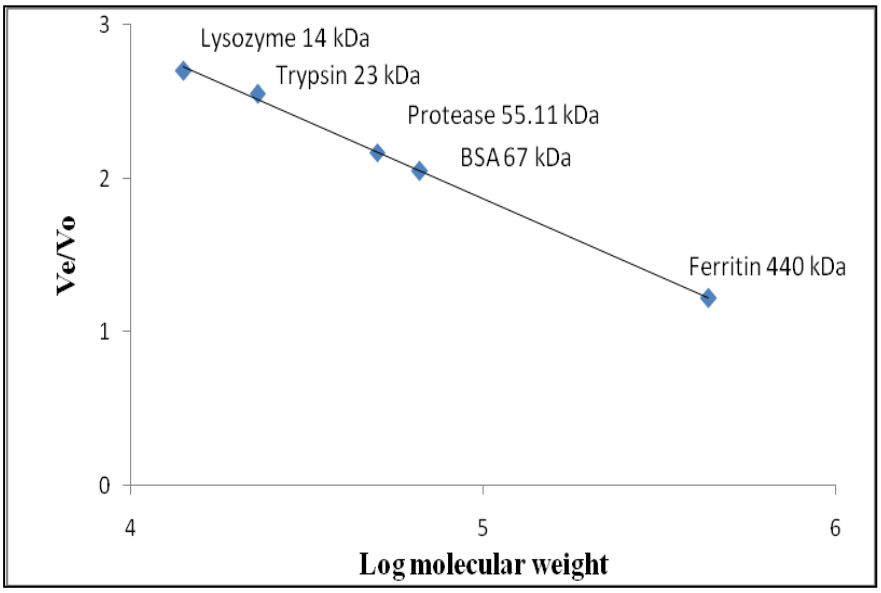

Figure 2 Molecular mass of *Acacia farnesiana* protease

Effect of temperature in protease activity was the best at 45°C which produce activity 42.62 (Figure 4), very less studies about optimum temperature on plants metalloprotease. Sorghum malt variety KSV8-1 and *Euphorbia cotinifolia* have temperature optima 50 and 60 °C respectively [13; 5].

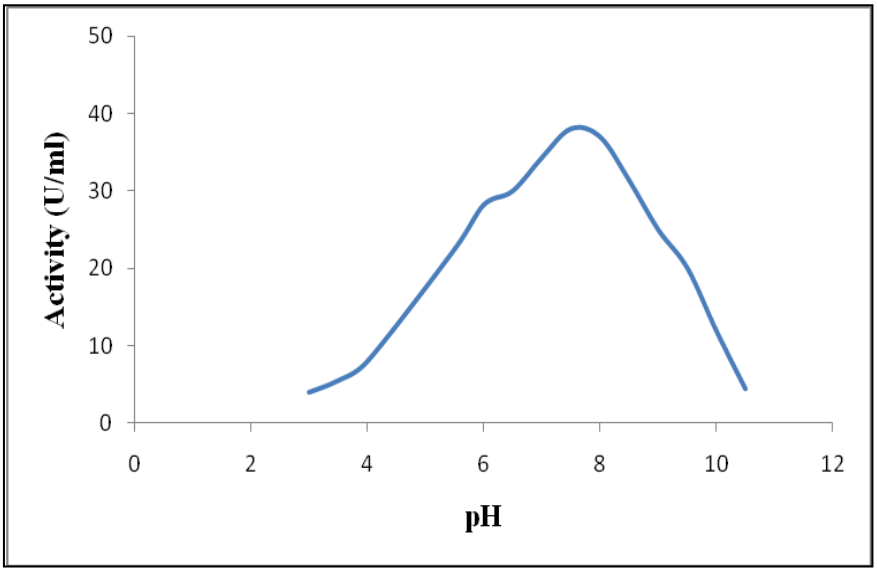

Figure 3 Effect of pH on protease activity of *Acacia farnesiana*

The obvious effect of selective ions on enzyme activity were cobalt followed by mercury and copper while other ions showed low to moderate inhibition except zinc which raised of the activity about 16% (table 4). Some metal ions and cofactors may be necessary to maintain the native structure and biological activity of the enzyme [14].

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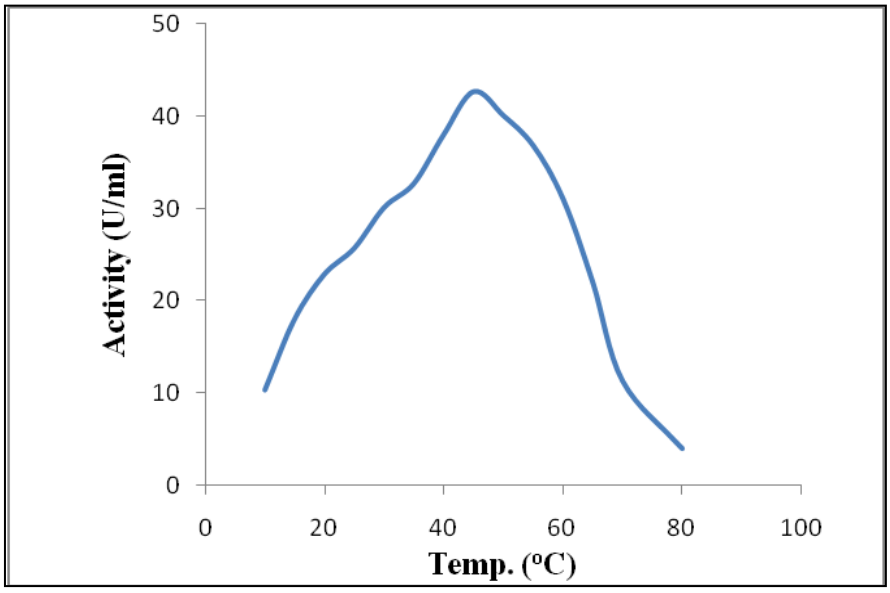

Figure 4 Effect of temperature on protease activity of *Acacia farnesiana*

About 53% and 100% of enzyme activity was lost at 1 and 10 mM of EDTA inhibitor respectively, where as no significant influences of other inhibitors at its concentration (table 5). Protease extracted purified, characterize and strongly inhibited by EDTA from *Acacia farnesiana* belong to metalloprotease [15].

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Table 4 Effect of metal ions on protease of *Acacia farnesiana*

Ion	Conc. mM	Remaining activity (%)
None	0	100
Co^{2+}	1	10
Ca^{2+}	1	90
Mg^{2+}	1	82
Mn^{2+}	1	86
Hg^{2+}	1	20
Ba^{2+}	1	90
Cu^{2+}	1	54
Ni^{2+}	1	95
Zn^{2+}	1	116

Table 5 Effect of inhibitors on protease activity of *Acacia farnesiana*

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Inhibitor	Concentration	Remaining Activity %
None	0	100
EDTA	1 mM	47 %
	10 mM	0%
Pepstatin	1 µM	100%
	10 µM	96%
PMSF	0.1mM	100%
	1 mM	94%
Iodoacetamide	1 mM	100%
	10 mM	98%

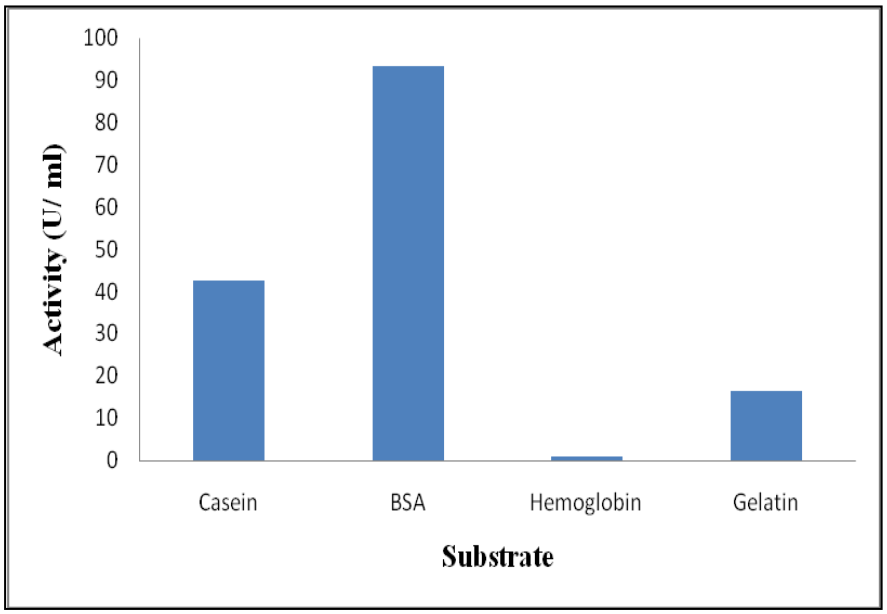

Figure 5 Effect of different substrates on protease activity of *Acacia farnesiana*

The kinetics constants K_m and V_{max} were 1.11mM and 625 mM/ min respectively (Figure 6). Matelloprotease can obtained from straight line generated from1/[S] is plotted against 1/v. The exact crossing of the line with the x and y axis produce $-1/K_m$ and $1/V_{max}$ respectively. This type of plot is very obvious, but not suitable in practical purposes for estimation of V_{max} and K_m [17].

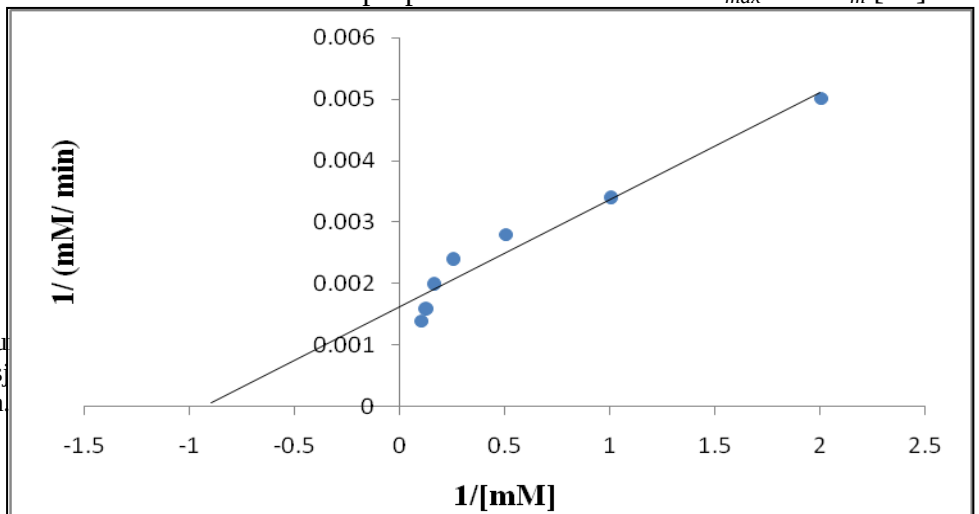

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Figure 6 Kinetics parameters of *Acacia farnesiana* metalloprotease

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تنقية جزئية لأنزيم البروتيز المعدني المستخلص من نبات شوك الشام *Acacia farnesiana*

نزار عبدالأمير حمزة و مهند محمد صاحب/ جامعة القادسية/ كلية العلوم/ قسم علوم الحياة

الخلاصة
تم التحري عن بروتيزات معدنية لم تشخص سابقا في اربعة انواع من نباتات العائلة البقولية، حيث نقيت ووصفت في نبات شوك الشام والذي امتلك اعلى فعالية نوعية (25.01) وحدة/ملغم، والتي ارتفعت لتصل الى35.84 وحدة/ملغم عند الترسيب ب50% كبريتات الامونيوم.استمر ارتفاع الفعالية النوعية وعدد مرات التنقية اذ كانت 51.39 وحدة/ملغم و 2.06 على التوالي عند استخدام المبادل الايوني السالب ثنائي اثيل امينو اثيل سليلوز. قدر الوزن الجزيئي (55.11 كيلودالتون) والاس الهدروجيني والحرارة المثلى للانزيم اذ كانت و 7.5 و 50 درجة مئوية على التوالي. انخفضت الفعالية عند الحضن مع الايونات المعدنية عدا ايون الزنك اذ ارتفعت بمقدار 16%. ثبط الانزيم كليا عند تركيز 10ملي مولر بواسطة اثلين داي امين تترا استك اسد مقارنة مع التراكيز المختلفة للمثبطات الاخرى. درس ثابت مكالس منتن و السرعة القصوى(1.11 ملي مولر و625 ملي مولر/دقيقة) بوجود البومين المصل البقري كمادة اساس.
الكلمات المفتاحية: البروتيزات المعدنية،شوك الشام