

Extraction, Precipitation and Characterization of Urease from *Vicia Faba L.*

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ABSTRACT

This study aimed to extract urease enzyme from available plant source which was broad beans (*Vicia faba L.*) using aqueous solution in a ratio 1:3(w:v). The crude extract appeared enzyme activity 33.3 U/ml. Results of this study revealed the possibility to precipitate urease enzyme after extraction using different precipitation methods consisted of Acetone precipitation; Alcohol precipitation and Ammonium sulfate precipitation, these ways of urease precipitation gave enzyme activities 74.8; 43.6 and 82.2 U/ml respectively. The enzyme precipitation using 60% saturation ratio of Ammonium sulfate gave the maximum activity of urease precipitation among other precipitation methods. Enzyme characterization appeared the optimum pH of urease activity was 8.0 and gave enzyme activity 93.8U/ml, while the optimum pH of urease stability was 6.0; the enzyme maintains its activity. On the other side the optimum temperature of precipitated urease activity was 50°C and the enzyme activity reached 99.3U/ml while the optimal temperature of urease stability was 40°C, the precipitated urease maintains 100% of activity. The effect of other factors on urease action were studied also, these are consisted of the storage time of enzyme, urease maintain its activity for 8days and the activity begun to decline after that time; the effect of time reusability was also revealed that the enzyme could be used for six times and maintain its activity. The influence of different mineral salts on precipitated urease were also recorded; these salts were CuSO₄, MgSO₄, MgCl₂, KCl, CaCl₂ and FeSO₄, results appeared that magnesium and calcium salts were activator for the precipitated urease while copper, potassium and ferrous salts were inhibitor of the precipitated urease. Urea concentration as an enzyme substrate were also examined and results appeared the optimal substrate concentration was 30mM; enzyme activity reached to 125.2U/ml.

KEYWORDS: Urease, Extraction, Precipitation, Characterization, *Vicia faba L.*

الخلاصة

تهدف هذه الدراسة الى استخلاص انزيم اليوريز من مصدر نباتي متوفر وهو نبات الباقلاء المحلي باستخدام محلول مائي بنسبة 1:3 حجم: وزن. حيث اعطى المستخلص الخام للانزيم فعالية انزيمية 33,3 وحدة/مل. وكشفت نتائج هذه الدراسة الى امكانية ترسيب انزيم اليوريز بعد الاستخلاص باستخدام طرق الترسيب المختلفة والتي تضمنت الترسيب بالاسيتون, الترسيب الكحولي والترسيب بكبريتات الامونيوم, هذه الطرق لترسيب اليوريز اعطت فعاليات انزيمية مختلفة وكانت 74,8, 43,6, 82,2 وحدة/مل على التوالي. ان ترسيب انزيم اليوريز باستخدام كبريتات الامونيوم ونسبة اشباع 60% اظهرت اقصى فعالية للترسيب بين طرق الترسيب الاخرى. وكشفت توصيف الانزيم ان الاس الهيدروجيني الامثل لفعالية اليوريز كان 8,0 و اظهر فعالية انزيمية 93,8 وحدة/مل بينما كان الاس الهيدروجيني الامثل لثباتية اليوريز 6,0 حيث احتفظ الانزيم بالفعالية بشكل كامل. من جانب اخر ان درجة الحرارة المثلى لفعالية انزيم اليوريز المرسب كانت 50 درجة سيليزية بفعالية انزيمية 99,3 وحدة/مل بينما درجة الحرارة المثلى لثباتية اليوريز كانت 40 درجة سيليزية, حيث احتفظ اليوريز المرسب 100% من الفعالية الانزيمية, ودرست تأثير عوامل اخرى على عمل اليوريز وتضمنت زمن الخزن للانزيم حيث احتفظ الانزيم بالفعالية الانزيمية بعد فترة خزن 8 ايام وبعد ذلك بدأت الفعالية الانزيمية بالتناقص. وكشفت الدراسة امكانية اعادة استخدام الانزيم حيث احتفظ الانزيم بالفعالية بعد الاستخدام السادس. كما سجل تأثير الاملاح المعدنية المختلفة على الفعالية الانزيمية وهذه الاملاح تضمنت CaCl₂, KCl, MgCl₂, MgSO₄, CuSO₄ و FeSO₄ و اظهرت النتائج ان املاح المغنيسيوم والكالسيوم كانت محفزة لفعالية اليوريز المرسب بينما املاح الحديد والبوتاسيوم والنحاس كانت مثبطة لعمل اليوريز المرسب. وتم تحديد تركيز اليوريا كمادة اساس للانزيم و اظهرت النتائج ان التركيز الامثل للمادة الاساس هو 30 مللار حيث وصلت الفعالية الانزيمية 125,2 وحدة/مل.

INTRODUCTION

Ureasases enzymes (EC 3.5.1.5) are a nickel depending metallo enzymes that responsible for

urea hydrolysis into Ammonia and CO₂ [1], these enzymes are found in plants, algae, yeasts and filamentous fungi. Fungal and plant ureases are composed of identical repetition of protein. While

bacterial ureases are consist of different repetitions of 2-3 subunits of protein [2]. Ureases have been used in many fields such as a biosensors for determining urea in human blood; diagnosis kit for urea measuring and used as a urea reducing agent in alcoholic beverages [3], [4], microbial and plant ureases appeared other biological functions as in blood platelets activation in addition to insecticidal and antifungal activity ; this confirm that urease enzyme participate in a mechanism of plant cells defense [5].

Ureases extracted from jack beans were the first enzymes to be crystallized in laboratory and they were stilled the best characterized ureases from plants [6]. Other researchers isolated urease from *Cajanus cajan* [7] and it could be isolated from seeds of water melon [8].

The objective of this study was to extract and precipitate urease enzyme from broad beans (*Vicia faba L.*) and to characterize its properties after precipitation that consisted of its optimal pH of activity and stability, thermal activity and stability, enzyme reusability, influence of urea concentration and the storage time of precipitated urease, in addition to study the effect of mineral salt on enzyme activity.

MATERIALS AND METHODS

1. Broad beans *Vicia faba L.* were collected from local Iraqi market.
2. Urea, Ammonia, Acetone, KH_2PO_4 , K_2HPO_4 and Ammonium sulfate were obtained from SIGMA co.
3. CuSO_4 , MgSO_4 , MgCl_2 , KCl , CaCl_2 and FeSO_4 were from GCC(U.K)company.

Preparation of aqueous extract

According to method described in [9], 500gram of local broad beans *Vicia faba L.* seeds were collected from local market and the peel were removed, the seeds were cut into small pieces and added to a cool distilled water in a ratio 1:3(w:v) and the mixture crushed using a blender for 5 minutes then it was filtered using gauze layers and isolated using cooling centrifuge at 4 °C for 15 min, then the enzyme activity was determined according to indophenol method described in [10] and the standard curve of enzyme activity was drawn using a relation between Ammonia concentration and absorbance at 625nm.

Different methods of enzyme precipitation were used in order to estimate the best way that maintains the highest urease enzyme activity, these are consisted of:

• Acetone precipitation

A crude extract was saturated to 50% by acetone solution with stirring. The precipitate had been collected; centrifuged and dissolved in 5ml phosphate buffer 0.05M. The extract was centrifuged for 5 min and the activity of clear suspension would be determined [11]

• Alcohol precipitation

Two milliliter of crude extract was added to 2ml of ethanol 60% and left for 6 hours, the precipitation solution was separated by centrifuge 3000 r/min for 10 minutes, then it was dissolved in 0.05 M of sodium acetate buffer pH 5 and the enzyme activity was determined [12]

• Ammonium sulfate precipitation

The crude extract of broad beans was isolated and saturated using ammonium sulfate in order to estimate urease precipitation in a ratio ranged between 20-80%, the protein molecules would be precipitated in a solution, the precipitate was washed against 0.02 M of phosphate buffer [13]

According to method described in [13] with slight modification, ureases enzymes activities were estimated .The precipitated enzyme and the reaction mixture consisted of 0.02M urea; 0.002 M CaCl_2 and 0.1 M of Tris-Hcl pH 8.0 were mixed in a ratio 1:1 (v/v) then incubated for 30 minutes at 30°C, then the reaction was stopped using 0.5 ml of H_2SO_4 (1 N). Urease activity was estimated by determining the amount of ammonia resulted from urea hydrolysis.

In order to investigate the pH effect on urease activity, the precipitated enzyme was incubated with urea solution in a different pH values ranged between (5.0-9.0) and the enzyme activity was determined. The optimum pH of precipitated urease stability was determined by incubating the extracted enzyme in different buffers pH 5.0-9.0 for 30 min at 4°C, then the stability pH was determined in the presence of urea solution [14]

In order to estimate the optimal temperature of precipitated urease activity, the precipitated enzyme was incubated with urea solution and the enzyme activity was estimated in a different temperature values ranged between (30-90) °C. On

the other hand the optimal temperature of precipitated enzyme stability was estimated by incubation the extracted enzyme in different temperature value ranged between (30-70)°C and determining the enzyme activity in the presence of urea solution [15].

The activity of precipitated urease was estimated after enzyme storage at 4°C in refrigerator for a regular time ranged between (1-10 days). Urease activity was determined daily till tenth day [14]

In order to estimate number of times using the precipitated urease, the enzyme activity was checked at different time of use, after each step of using the enzyme it washed with 100 mM of Tris acetate buffer and stored until the next use [16].

Different salts were experimented to estimate their effect on the activity of precipitated urease, these are consisted of CuSO₄, MgSO₄, MgCl₂, KCl, CaCl₂ and FeSO₄; they were added to reaction solution in a different concentration 10⁻³ and 10⁻⁵M as described in [17] with a slight modification.

Different concentrations of urea substrate were experimented in order to detect their effect on precipitated urease activity from broad beans *Vicia faba L.*, these are ranged between (10-80) mM as described by [18].

RESULTS AND DISCUSSION

Urease extraction

Urease enzyme was extracted from broad beans *Vicia faba L.* using Aqueous extract as a crude enzyme to determine enzyme activity in the presence of ammonia standard curve and slope of reaction, the enzyme activity of crude extract was 33.3 U/ml (Table1).

Urease enzyme was precipitated using acetone 50% and the clear suspension of precipitated enzyme was collected and determined it's enzyme activity which reached to 74.8U/ml in order to compare with other precipitation method (Table 1). Others [9] precipitated urease enzyme using acetone and found the enzyme specific activity 120 U/mg while others extracted urease from germinating *Pisum Sativum L.* seeds and precipitated the enzyme using acetone and gave enzyme activity 190 U/g [11].

Alcohol Precipitation is an important method to concentrate proteins especially enzymes, the

precipitation using ethanol has a low-cost. Results in Table 1 appeared the precipitation of extracted urease using ethanol alcohol and the enzyme activity recorded 43.6 U/ml. Others precipitated urease enzyme using ethanol after extracting the enzyme from *Lactobacillus reuteri* and the enzyme specific activity reached to 30.3 U/mg [19].

The activity of extracted urease was assayed at different saturation values of ammonium sulfate, which were 20%, 30%, 40%, 50%, 60%, 70% and 80% and those gave enzyme activities 22.2; 40.1; 44.4; 63.3; 82.2; 78.9 and 73.3U/ml respectively, these results appeared 60% saturation value of ammonium sulfate more suitable for urease precipitation from broad bean (Table 1).

Table 1. Extraction and precipitation of urease from *Vicia faba L.*

No.	Fraction	Enzyme activity U/ml
1	A crude extract	33.3
2	Acetone precipitation of enzyme extract	74.8
3	Alcohol precipitation (Ethanol Precipitation) of enzyme extract	43.6
4	Ammonium sulfate precipitation (60% saturation value) of enzyme extract	82.2

Characterization of precipitated urease:

Effect of pH

The effect of pH on precipitated urease activity was studied using different values ranged between (5.0-9.0), results in Figure 1 appeared the optimum pH of precipitated urease activity was 8.0 and gave the enzyme activity 93.8U/ml, Other studies appeared the optimal pH of free and immobilized urease extracted from Citrus Seeds *Citrus colocythis* were 7.5 and 8.0 respectively [15].

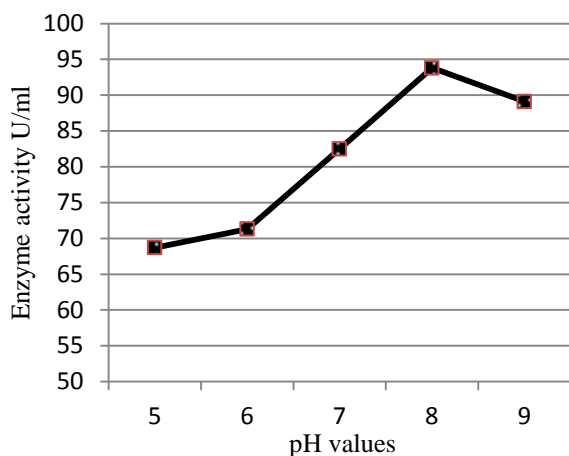


Figure 1. Optimum pH of urease activity.

On the other side the optimal pH of precipitated urease stability was estimated using pH values ranged from 5.0 to 9.0, result in Figure 2 appeared the optimum pH of precipitated enzyme stability was 6.0; the enzyme maintains 100% of its activity then the remaining activity begun to decline with increasing the pH values. Other study [9] showed the optimal pH of stability urease was 8.0.

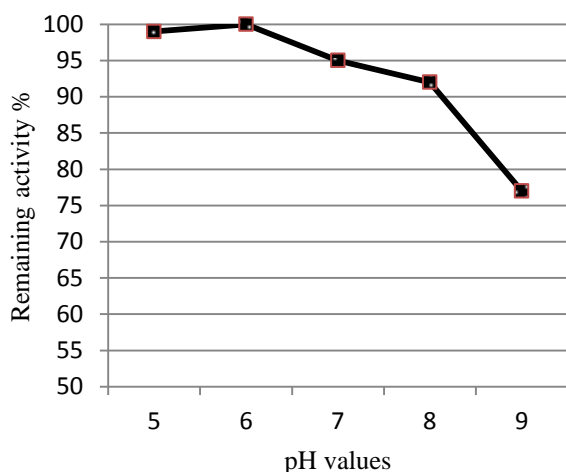


Figure 2. Optimum pH of urease stability.

Effect of temperature

The optimum temperature of precipitated urease was detected, results in Figure 3 appeared the optimum temperature of enzyme activity was 50°C and the enzyme activity was 99.3U/ml. Other researchers [14] mentioned the optimum temperature of free urease activity was 40°C while the optimum temperature of immobilized urease activity ranged between 40-50°C.

The optimum temperature of urease stability was 40°C. the precipitated urease maintain 100% of activity as in Figure 4. Other researchers studied

the optimal temperature of free and immobilized urease stability and estimated the optimum temperature of free and immobilized urease stability were 40°C [15].

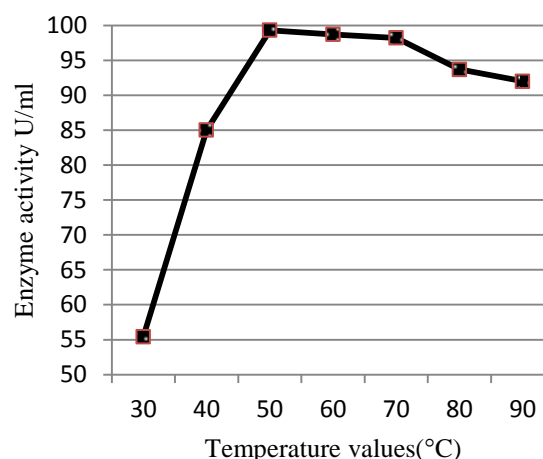


Figure 3. Optimum temperature of urease activity.

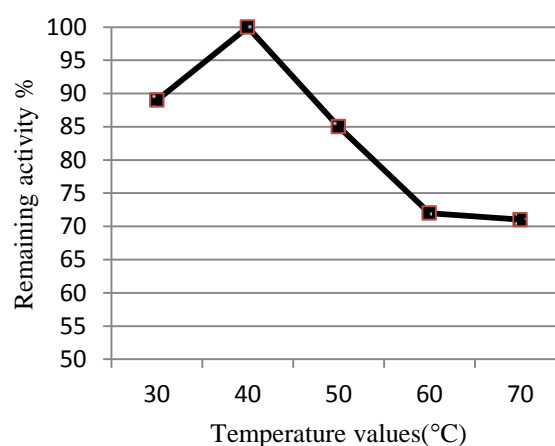


Figure 4. Optimum temperature of urease stability.

Effect of storage time

In order to illustrate the effect of storage period on precipitated urease action, the precipitated enzyme was preserved in refrigerator for 10 days and the enzyme activity was determined every day to estimate the effect of cooling on enzyme activity, results in Figure 5 appeared the enzyme maintain its activity for 8 days and after that the enzyme begun to decline its activity. Others mentioned that urease enzyme preserved 70% of its activity when it was stored at 4°C for 30 days after enzyme extraction from broad beans [9].

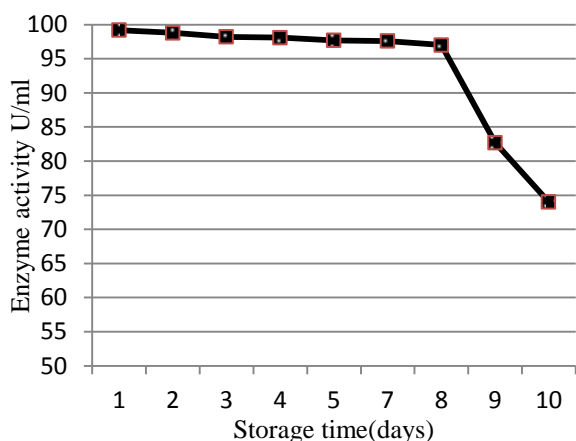


Figure 5. Effect of storage time on urease activity.

The number of time using of precipitated urease

Figure 6 illustrated the number of time using precipitated urease isolated form *Vicia faba L.*, results appeared the extracted enzyme could be used for six times and maintain its activity, others [20] noticed that 30 times was the possibility of using the extracted urease from pea seeds.

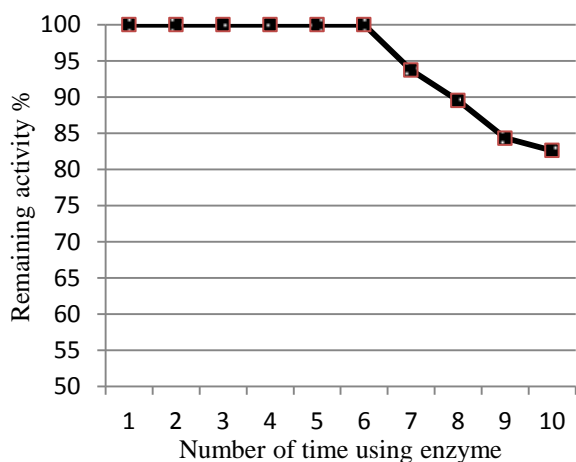


Figure 6. Number of times of enzyme reusability.

Effect of mineral salt

In order to estimate the effect of different mineral salts on precipitated urease activity, Different types of salts were examined and added to reaction solution in a different concentration consisted of 10^{-3} and 10^{-5} M, these salts were CuSO_4 , MgSO_4 , MgCl_2 , KCl , CaCl_2 and FeSO_4 , results in Table 2 appeared that magnesium and calcium salts were activator for the precipitated urease while copper, potassium and ferrous salts were inhibitor of the precipitated urease. Others studied the effect of metallic salt on purified urease extracted from *Rhizopus oryzae* and

recorded that zinc ,copper and potassium salts were inhibitor for enzyme activity while calcium, magnesium and manganese salts were activator for enzyme activity [17]

Table 2. Effect of different mineral salts on precipitated urease activity.

Mineral salts	Enzyme activity U/ml	
	10^{-3} M	10^{-5} M
CuSO_4	37	52
MgSO_4	104	117
MgCl_2	100	109
KCl	26	48
CaCl_2	113	121
Fe SO_4	53	67

Effect of substrate concentration on enzyme activity

Different concentration of urea were examined which were ranged between (10-80) mM, results in Figure 7 appeared the optimal substrate concentration was 30mM ; enzyme activity reached to125.2U/ml and considered the greatest activity in contrast to the activities values in the presence of other concentration , other researchers Studied the effect of urea concentration on urease action extracted from Soybeans and found the optimal concentration was 36mM [18].

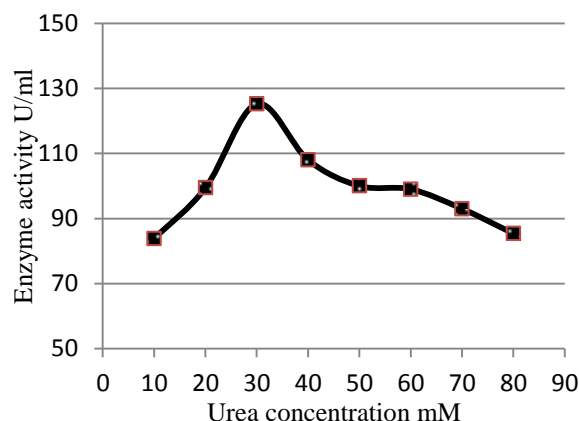


Figure 7. Effect of substrate concentration on precipitated urease activity.

CONCLUSIONS

It is possible to extract and precipitate urease enzyme from available cheap source of broad beans, the enzyme was characterized also. In future studies, it must be concentrated on purify urease enzyme partially in addition to using immobilization technique in order to get more enzyme action.

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