Extraction and purification of Urease from *Proteus mirabilis*

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(NJC)

(Received on 23/6/2008) (Accepted for publication 26/10/2008)

Abstract

Urease was extracted from uropathogenic *P.mirabilis* using Lauria agar and then purified by ion- exchange chromatography using DEAE-cellulose and gel filtration chromatography on Sephacryl S-200 column. The purification fold was 13.86 and the yield was 45.4%.

The molecular weight of urease was estimated by gel filtration and SDS-polyacrylamide gel electrophoresis. The native molecular weight of the enzyme using gel filtration was about 174000 Daltons whereas SDS-polyacrylamide gel electrophoresis revealed three bands and the molecular weight for these bands were (53.70, 31.68, 20.89) kDa respectively.

الخلاصة

تم استخلاص انزيم اليوريز من بكتريا *P.mirabilis* المسببة لالتهاب المجاري البولية في وسط اللوريا- اغار بعد ذلك تمت تنقيته بخطوتين تضمنت كروموتوغرافيا التبادل الايوني باستخدام المبادل DEAE-cellulose وكروموتوغرافيا الترشيح الهلامي باستخدام عمود Sephacryl S-200 . عدد مرات التنقية كانت 13.86 وبحصيلة انزيمية 45.4% .

تم تقدير الوزن الجزيئي لليوريز بواسطة الترشيح الهلامي والترحيل الكهربائي على هلام Polyacrylamide بوجود المادة الماسخة SDS . كان الوزن الجزيئي للانزيم باستعمال الترشيح الهلامي مقارب لـ 174000 دالتون بينما اظهر الترحيل الكهربائي على هلام polyacrylamide بوجود SDS ا ثلاث حزم وكان الوزن الجزيئي لكل حزمة (53.70 و31.68 و20.89) كيلودالتون.

Introduction

The enzyme urease (urea amidohydrolase; E.C. 3.5.1.5) occurs in a wide variety of tissues in man mainly the gastric mucosa, liver, kidney, erythrocytes, etc. as well as in bacteria, yeast , mold, plants and molluses [1]. Bacterial ureases, consisting of two or three subunits, share significant amino acid similarities with plant urease, consisting of a single subunit [2].

Urease is a nickel-containing enzyme; in plant, the enzyme contains two-nickel ions per subunit and the metal ions are apparently coordinated by oxygen and nitrogen ligands. Microbial ureases are distinguished from the jack *Brevibacter ammoniagenes* and *Bacillus pasteuri,* which are smaller in a subunit size and possess a single nickel ion per subunit. The *Selenomonas ruminantium* ureases are also small subunits but like the plant enzymes, they contain two nickel ions per subunit [3].

Urease was found in large amounts in jack beans, soybeans, and other members of the Leguminosae [3]. Jack bean urease is hexamer of

identical subunits known as an amino acid sequence [4]. Active site differences may also exist in the microbial and plant enzyme as shown by their differences in susceptibility to various inhibitors [4]. Nickel can be released from urease under acidic conditions, leading to irreversible loss of activity [5].

Urease synthesis is either constitutive or inducible [6,7]. Firstly, the enzyme is synthesized only in the presence of urea, showing that it is an inducible enzyme, during the study of the urease activity of *Proteus* it has been shown that this enzyme requires induction with urea that is inducible enzyme [8]. Other bacterial species appear to be produced constitutively and synthesis is not affected by addition or limitation of ammonia, urea or other nitrogenous compounds [5].

Secondly, the difference in rate of urease synthesis varies according to the carbon source provided. *Proteus retgerri* urease has been synthesized when glucose, succinate or lactate is used as a carbon source instead of glycerol [8].

These result shows that the enzyme formation is controlled by catabolite repression. Thirdly, the urease activity of cell grown in the presence of urea has been reduced to vary low level when ammonium is added to the medium. Thus, urease biosynthesis is controlled by end product repression or called feedback inhibition [8].

The bacterial urease enzyme generates ammonia and elevates the pH of the urine and the biofilm. Under these conditions, struvite (magnesium ammonium phosphate) and apatite (calcium phosphate) are formed and become trapped in the organic matrix which surrounds the cells. Therefore, the aim of this study extracting *P. mirabilis* urease and purified it by using ion exchange and gel filtration chromatography.

Materials and methods

Bacterial Isolates

Proteus mirabilis was obtained from Biology Department, College of Science, Baghdad University. These bacteria were isolated from urinary tract infection (UTI) patients.

Urease Extraction

The cells of *Proteus mirabilis* were grown at 37˚C with aeration in lauria agar medium supplemented with 0.1% urea pH 7.5.After 24 hours the cells were harvested from the surface of plates, the growths were flooded with 2 ml of (Phosphate buffer 20Mm, pH 7.5, Na_2-EDTA 1mM, β-marcaptoethanol 1mM, PEM), suspended cells were collected in a centrifuge tube and the process was repeated. The suspension was either used immediately or stored at -20˚C.The cells were collected by centrifugation; the pellet was drained and suspended in the same buffer (PEM) containing PMSF (1mM).The suspension was sonicated at iced-water for 1 minute. The sonicated solution was centrifuged at 10000 rpm for 30 minutes. The supernatant was removed with Pasteur pipette and used as crude enzyme. The urease activity and protein concentrations were determined.

Urease Assay [9]

Urease was assay according to [9]. Standard curve of ammonium chloride was done and different concentrations of ammonium chloride were prepared ranging from (0.01 to 1) mM, that was done by serial dilution the of the stock solution with buffer phosphate pH 7.5 (PEM). Indophenol assay was used for both standard curve and determination of urease in sample.

Protein Assay [10]

Different concentrations of bovine serum albumin were prepared ranging from (0 to 25) µg /ml . The linearity relationship between absorbance and concentration of protein was plotted and the standard curve was used to determine the concentration of protein. Protein concentration assay in the sample was determined FC:\WINDOWS\hinhem.scraccordin g to [10] and then the concentration of protein was calculated.

Purification of Urease

Ion exchange chromatography\ The crude enzyme 50 ml was loaded on DEAE-cellulose column(3×15)cm , then the column washed with 100 ml phosphate buffer pH 7.5 and the fractions were collected at a flow rate 30 ml /hrs. and 5ml for each tube. After that the proteins binding with gel were eluted using gradient (0-0.5M) KCl with (20 mM) phosphate buffer and pH 7.5. The absorbance of these fractions were measured at a wave length 280nm. The active fractions were combined and the volume was measured, then urease activity and protein contents were assayed.

Gel filtration chromatography \ The enzyme obtained from the ionexchange step was loaded on the Sephacryl S-200 column (1.5×65) at a flow rate 30 ml / hrs. and the eluted were collected in the form of 5 ml aliquots. The absorbance of these fractions were measured at the wave length of 280nm. The active fractions were combined and the volume was measured, then urease activity and protein contents were assayed.

Determination of the Molecular Weight of Urease

By gel filtration\ A 2 ml of blue dextran-2000 solutions (6 mg into 3 ml of PEM buffer pH 7.5) passed through the column then (20mM) PEM buffer pH 7.5 was added. Fractions of 5 ml were eluted and the absorbance at 600nm for each fraction was measured. The column void volume (Vo) was determined by estimating total volume of the fractions characterized with the starting point movement of the dextran to climax of absorbency of the blue dextran. A gel filtration chromatography was used for many standard proteins (bovine serum albumin, aldolase, catalase, ferrtin, thyroglobulin) to determine the molecular weight for each protein. The eluted fractions, which give a maximum absorbance at 280 nm, were determined and eluted volume (Ve) was calculated for each standard protein. The linearity between Ve /Vo versus log value of molecular weight of standard protein was plotted. The standard curve was used for determining the molecular weight of native urease. Sephacryl S-200 column (1.5×65)cm. which was used in the purification of urease was equilibrated for 24 hours with PEM buffer pH 7.5 at flow rate 30 ml/hrs.

By Polyacrylamide gel electrophoresis-SDS [11]

The gel was placed on the tank filled with reservoir buffer (3 gm of Tris-base HCl and 14.4 gm of glycine and 10% SDS in amount of distilled water then the volume was completed to 1L); a 50 µL of enzyme solution(250µL of enzyme (0.2 mg / ml) with 250µl of stock sample buffer in ependorff, then 25µl of β-marcaptoethanol was added, the mixture was heated in a boiling water bath for 5 minutes, then it was cooled at room temperature) was applied to the gel. Electrophoresis was conducted at 2 mA at 40 volt for 30 minutes in the stacking stage and 5 mA at 240 volt for 4-5 hours in the resolving stage with cooling at 4˚C. The gel was removed from the glass plate and put gently in a suitable tank then soaked with fixing

solution (10% trichloroacetic acid-40%methanol) for 3 hours. After that, it was stained with coomassie blue R-250 for 3 hour. The distaining was performed by soaking the gel into distaining solution(acetic acid with methanol and distilled water in ratio (1:4:5) for many times until enzyme band appeared. The movement of bromophenol blue from the starting point to the centre bond of dye was measured; the same movement of the standards proteins was measured from the starting point to the centre band of protein and the value of R_m was calculated as the following:

$$R_m = \frac{\text{Mobility of protien}}{\text{Mobility of bromophenol blue}}$$

Results and Discussion

Urease was extracted from *P.mirabilis* by using Lauria agar medium supplemented with 0.1% urea. This medium is the best for this purpose (urease production). Urease is synthesized in the presence of urea only, showing that it is an inducible enzyme [8]. Since urease is cytoplasmic enzyme [12], the cells were first disrupted by sonication for 1min. This technique was effective in cell disruption. Purification of urease includes two steps: ion-exchange and gel filtration chromatography.(Table1)**.** Ion –exchange chromatography was carried out using the anion exchanger DEAE-cellulose. Approximately 50 ml of crude extract was passed through a column. The first protein peak appeared in tube 20th that presented the maximum enzyme activity (Fig.1).The fractions were collected and proteins were detected at absorption 280nm and the enzyme activity was estimated, the peaks were observed.

The enzyme was further purified using gel filtration. The fractions which appeared enzyme activity from the above step was concentrated by using sucrose into 2 ml and applied to Sephacryle S-200 column. Two peaks were observed (Fig. 2). Fractions were collected and then proteins were detected at the absorption 280nm. The second peak showed the maximum enzyme activity (fractions 29).

The molecular weight of the native urease was estimated as 174 kDa as shown in (Fig.3) when using gel filtration and three subunit appeared and the molecular weight for these bands were (53.70, 31.68, and 20.89) kDa respectively as (Fig.4) when using SDS-electrophoresis.

Urease has been purified from a number of bacteria, Larson and Kallio [13] purified urease from *Bacillus pasteurii* by using ammonium sulfate and acetone. Magana-plaza *et al.,* [14] , studied *Proteus rettgeri* urease and purified it 42.8 fold with recovery 2.2% using Sephadex G-200, Hydroxyapatite and DEAE-Sephadex. Nakano *et al.,* [15] purified urease from *Brevibacterium ammoniagenes* 600-fold with a yield of about 10% using DEAE-cellulose and gel filtration chromatography. In another study, Mobley *et al.,* [16] purified urease from *Proteus penneri* using gel filtration chromatography; the crude urease was loaded on Sephacryl S-300 column. The fractions were collected at a flow rate 30ml/h. *Selenomonas ruminantium* urease was purified 592-fold with an overall recovery of 53% by using DEAE- Sepharose, Phenyl-Sepharose, Sephadex G-200 and fast protein liquid chromatography [17]. *K.aerogenes* urease was purified 24-fold with a yield 29% using ammonium sulfate and DEAE-cellulose [18] whereas Todd and Hausinger, [19] purified *K. aerogenes* urease 1070-fold with a yield 25% by a simple procedure

involving DEAE-Sepharose, phenyl-Sepharose, MonoQ, and Suprose 6 chromatographies. Ismail, [20] purified *P.mirabilis* urease 145.23 -fold with a yield 23.96% using DEAE-cellulose and Sephacryl S-200. In another study by Al-kanani [21], urease was purified from *Staphylococcus sapropyhticus* 46.25-fold with a yield 7.16 using Sephacryl S-300 as a second step of purification.

Table 1: The steps of purification of urease

Step of purification	Volume (ml)	Activity U/ml	Protein Mg/ml	Specific activity U/mg	Total Activity U	Yield	Purification Fold
Crude extract	50	2827.89	1.71	1653.74	141394.5	100	1
Ion exchange (DEAE-Cellulose)	20	6260.87	0.4	15652.18	125217.4	88.55	9.46
Gel filtration Sephacryl S-200	20	3210.60	0.14	22932.86	64212	45.41	13.86

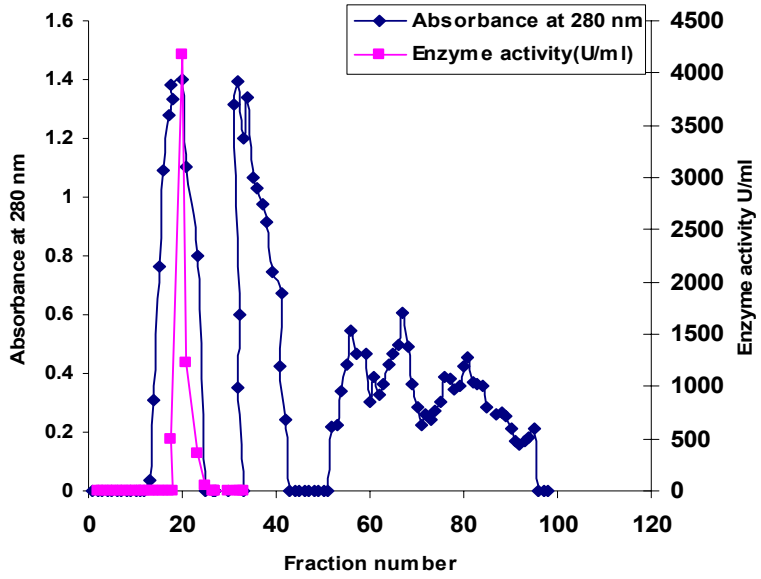

Fig. 1 ionexchange chromatography for purified *P.mirabilis* urease by using DEAE- cellulose column (3×15)cm.The column was calibrated with phosphate buffer pH 7.5 ,flow rate 30 ml / hrs.(5ml / fraction)

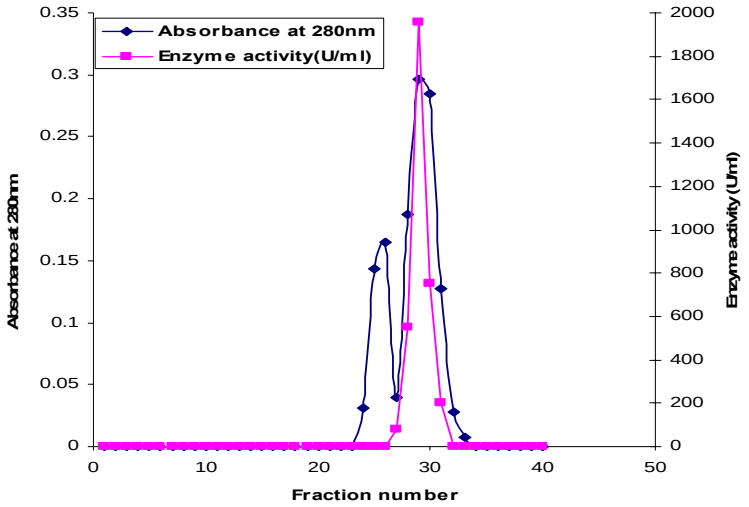

Fig. 2 gel filtration chromatography for purified *P.mirabilis* urease by using sephacryl S-200 column (1.5x65)cm . The column calibrated with phosphate buffer pH 7.5,flow rate 30 ml/hrs. (5ml/fraction)

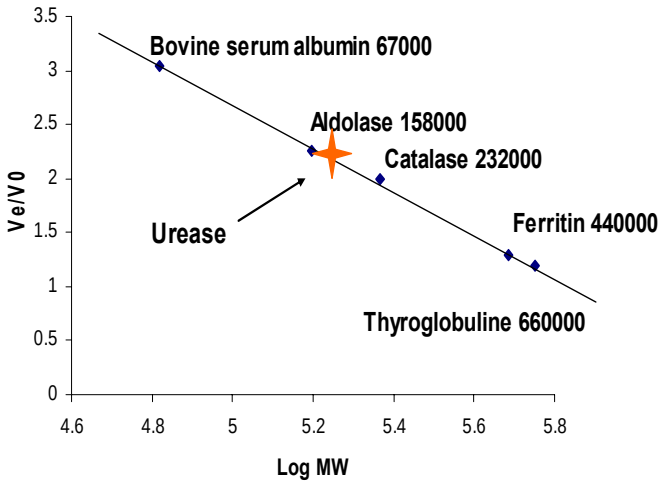

Fig.3 A plot of the logarithm molecular weights of known proteins versus elution volumes on a sephacryl S-200

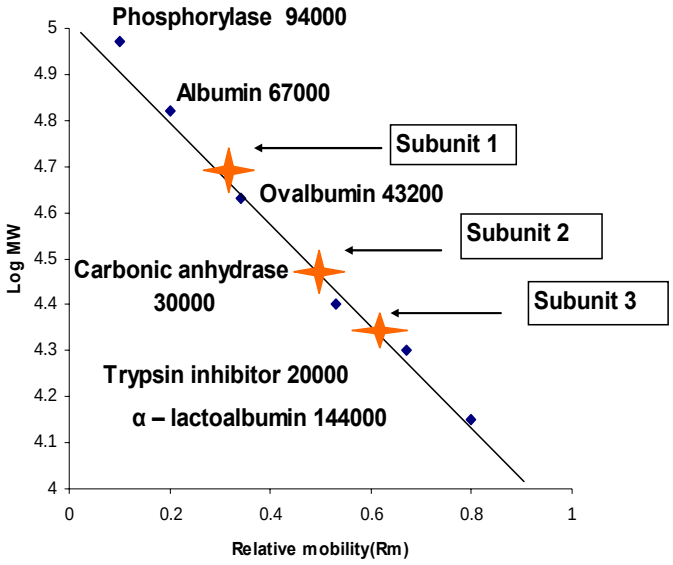

Fig4 Calibration curve for molecular weight. Estimation of urease enzyme by SDS-poly acrylamide gel electrophoresis using known molecular weight proteins.

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