



CHARACTERIZATION AND PURIFICATION OF UREASE ENZYME FROM NEW *PROTEUS MIRABILIS* STRAIN

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ABSTRACT

Proteus mirabilis bacterium is a common etiologic agent of urinary tract infections and produces large amounts of urease enzyme (EC 3.5.1.5). The urease enzyme producing new *Proteus mirabilis* isolate was isolated from positive sample collected from Khartoum hospital. In this study's the enzyme was extracted from the bacterial isolate by distilled water and purified by gel-permeation. Urease enzyme activity was measured in SDS page based on cresol red and assayed spectrophotometrically. The recovery percentage of pure urease enzyme was in range 55-98%. The *Bacterium* protein extraction retained urease enzymatic activity and showed the typical subunits of M_r 66000, 45000, 29000, and 15000 when subjected to sodium dodecyl sulfatepolyacrylamide gel electrophoresis.

Keywords: Purification; *Proteus mirabilis*; Urease enzyme, Characterization

1. INTRODUCTION

The Bacterium *Proteus mirabilis* is a common etiologic agent of urinary tract infections, especially in the elderly catheterized population, and is second only to *Escherichia coli* as a cause of urinary tract infections [1]. The ammonia generated by urease is responsible for the increase in urine pH and subsequent formation of struvite and apatite stones which are a hallmark of *P. mirabilis* infection. Urease has been shown to be a critical virulence factor in vivo in the CBA mouse model of ascending urinary tract infection [2, 3]. Among the virulence factors characterized until today is the urease M_r 380.000, which helps the pathogen to survive in the acidic environment of the stomach [4, 5]. In addition, urease is involved in chemotactic reaction of the bacterium and reduces opsonization by human complement [6, 7]. The enzyme consists of six subunits UreA (M_r 30.000) and subunits UreB [8]. Unlike other bacterial ureases the enzyme is found not only in the cytoplasm of the cell but also on the bacterial surface [9, 10]. The main aim of the study was the isolation and characterization of urease enzyme from new *Proteus mirabilis* strain

2. MATERIAL AND METHOD

2.1. Bacterial Isolation

Proteus mirabilis strain was isolated from positive sample provided by urinary tract infection (UTI) department, Khartoum hospital.

2.2. Molecular identification.

The *Bacterium* DNA was extracted according to method [11]. The extracted DNA was subjected to PCR amplification using 16S rRNA gene, specific primer of *P. mirabilis* (forward primer), AAC TGG AGG AAG GTG GGG AT and (reverse primer). AGG AGG TGA TCC AAC CGC Amplifications were performed in 25 μ l reaction mixtures containing the template DNA 1 μ l (50 ng), 1 μ l for each of the primers (20 pm), dNTPs 2.5 μ l (2.5 mM), Taq DNA polymerase 0.2 μ l (1.25 unit/ μ l) and 10x Taq buffer 2.5 μ l (fermentas).

The mixture was subjected to the following amplification conditions: 4 min at 94 °C, followed by 30 cycles of 94 °C for 25 Sec, 62 °C for 30 S and 72 °C for 1 min, and ended by a final extension step at 72 °C for 5 min. The PCR products were electrophoresed on 2% agarose gels and the target band was excised and purified with DNA gel Extraction Kit (MBI Fermentas).

2.3. Nucleotide sequence and accession number

The PCR product of the 16S rRNA gene was sequenced using automated DNA sequence by Macrogen (macrogen.com, Korea). The obtained DNA nucleotide sequence was subjected to DNA analysis using blast DNA (www.ncbi.com). The DNA sequence was deposited in Gene Bank under the accession number KF233427 (www.ncbi.com).

2.4. Urease Extraction

The cells of *Proteus mirabilis* were grown at 37°C with aeration in Lauria agar medium (L.B) supplemented with 1% urea (pH 7.5).

After 72 hours incubation, the cells were harvested from the surface of plates, the cells were flooded with 2 ml of Phosphate Buffer (pH 7.5) (20 mM, Na₂-EDTA 1mM, 1mM, β-Mercaptoethanol), suspended cells were collected in a centrifuge tube and the process was repeated several times.

The suspension was sonicated on ice for 1 minute. The sonicated solution was centrifuged at 10,000 rpm for 30 minutes. The supernatant was removed with Pasteur pipette and used as crude enzyme. The urease activity and protein concentrations were determined according to method [12].

2.5. Determination of protein content

To determine the protein concentration, standard colorimetric method according to Bradford Method was used [12]. A 10 μ/L of sample were mixed with 990 μ/L of Bradford solution which contained of 20 mg Coomassie Brilliant Blue G-250 (Serva, Heidelberg, Germany) dissolved in 10 ml of 95% ethanol and 20 ml of 85% phosphoric acid was added and the volume was adjusted to 200 ml with distilled water and the absorbance was measured at O.D. 595 nm. A 10 μ/L of 0.9% NaCl served as blank. Moreover, Bovine serum albumin (BSA) (Sigma Aldrich) in the range of 0.125 to 20.0 mg/ml was utilized to generate a standard curve.

2.6. Enzyme Purification

2.6.1. Ammonium Sulphate Precipitation

The organisms were grown for 72 hours as described before and the cells were separated by centrifugation (10,000 rpm/15 minutes), the supernatant was fractionated by precipitation with ammonium sulfate (70% saturation). All subsequent steps were carried out at 4°C. The protein was resuspended in gel-per-meation buffer) 50 mM NaH₂PO₄, 150 mM NaCl, 1 mM EDTA) pH 7, and dialyzed against the same Buffer.

2.6.2. Mono Q and Sephadex G-100 Gel Filtration Chromatography

The protein pellet obtained after saturation with ammonium sulphate (70%) was dissolved in gel-permeation buffer and filtered by 0.45 Millipore filter before loading into Mono Q (GE Healthcare Life science) and Sephadex G-100 column (2.4 × 75 cm) equilibrated with gel-permeation buffer (50 mM NaH₂PO₄, 150 mM NaCl, 1 mM EDTA). The flow-rate was 1 ml/min and 4 ml fractions were collected continuously after V₀ was reached. After screening the fractions for urease enzyme, the contents of positive tubes corresponding to a single peak were pooled.

2.7. Urease-screening

After each column chromatography 20 μl of each fraction were mixed with 300 μl of testing Buffer (3 mM NaH₂PO₄,

110 mM urea, 7 mg phenol red, adjusted to pH 6.8) [8] in a 96-well flat bottom microtiter plate using a micropipette. Blank wells received 20 μ/L chromatography buffer. The plate was immediately covered with a sticky seal and incubated at 37°C. In wells containing urease the color slowly changed from yellow to pink. The absorbance at 560 nm was quantitatively determined in a micro plate reader (Dynatech MR7000, Guernsey, UK) by serial measurement at different time points up to 20 min.

2.8. Determination of urease specific activity

To quantify urease activity more precisely 600 μ/L of testing buffer were placed in a 1ml semi-micro cuvette (Brand, Wertheim, Germany) and the absorbance was set to zero in a spectrophotometer at 560 nm. Without removing the cuvette 5–50 μ/L, sample was added and mixed immediately. With a maximum delay of 15 sec. the absorbance was determined sequentially every 6 sec. for period of 3 min. If the change in absorbance (ΔA) was 0.2, the assay was repeated with an increased volume of sample. Absorbance was plotted against time and ΔA/t was calculated from the linear part of the enzyme reaction curve. The corresponding amount of ammonia was assessed by a serial dilution of a purchased 25% ammonia solution. After determination of the protein content the specific activity (mM ammonia / mg protein/min) could be calculated.

2.9. Electrophoresis SDS page

The urease enzyme was precipitated by ammonium sulphate and purified by column and analyzed by SDS-PAGE according to Maniatis Method [13]. Denaturation of proteins was done by diluting the samples with sample Buffer (1:2) and heating the mixtures at 75°C for 20 min. After electrophoresis, gels were stained in staining solution (10% acetic acid, 25% Methanol & 0.25 g/ml Coomassie Brilliant Blue G-250) over night and destained in 30- 10% Methanol and acetic acid over night. Standard proteins served as markers for molecular mass (fermenas).

2.10. Detection of enzymatic activity within gels

2.10.1. Preparation of samples for SDS-PAGE

After resuspending the pellet in a small volume of solubilisation buffer, the cells were sonicated (8 kHz, 50% cycle for 1 min). The solubilisation buffer contained 0.625 M Tris- HC1 (pH 6.8), sodium dodecylsulphate (SDS) 3% w/v, 2-mercaptoethanol 5% w/v, glycerol 10% v/v and bromophenol blue 0.01% w/v. Cell debris were removed from the sonicate by centrifugation at 13,000 g and the cell free extract was dissolved in solubilisation buffer and stored in apendorfe tub at -20°C.

Extracts containing urease enzyme were analyzed by SDS PAGE in a modification of the method of Senior [14]. The cell-free extracts were applied to a slab gel comprising a stacking gel of acrylamide 4.5% w/v and a resolving gel of

acrylamide either 5 or 6% w/v. The samples were stacked at 10 mA and separated at 20 mA constant current. The gels were then washed several time with a filtered solution of disodium ethylene diamine tetraacetic acid (EDTA) 0.1 % w/v and cresol red indicator (0.02 % w/v) until the gel turned yellow, indicating neutral or slightly acidic conditions. Gels were developed by discarding the washing solution and immersing them in a small volume of urea 1.5 % w/v. Areas of urease activity appeared as cherry-red bands against the yellow background due to the local production of alkali as a result of enzymic degradation of urea.

3. RESULTS AND DISCUSSION

Highest cytoplasmic and extracellular urease activities during the growth of *P.mirabilis* were carried out. The highest cytoplasmic urease activity was observed at 72 hours when used Lauria agar (LB) medium supplemented with 0.1% urea. The previous studies concluded that that L.B is best medium for urease production [15, 16].

Optimising enzyme release by sonication is very important. According to the Larson [17] Manual, a power of 8 kHz, 50% cycle for 1 min is recommended for sonication of bacterial cells. In this study, sonication was done at 4°C and the cells were suspended in phosphate buffer.

The protein concentrations in the extracellular fraction and intracellular fraction were evaluated by the Bradford assay. A typical profile for biosynthesis of proteins during bacterial growth was observed. The Maximal activity of protein unit was 200mg/ml (Fig 1).

Each concentrated extract was filtered through a 0.45 µm pore size filter. Each filtrate was then subjected to Mono Q (GE Healthcare Life science) and Sephadex G-100 column (2.4 × 75 cm) equilibrated with gel-permeation buffer. The fractions were screened for urease activity. The fractions that were urease-positive were pooled and assayed for activity. The enzyme purified fold was 4.5 with recovery between 55-98 %, These results were similar to 2.2 and 42.8 % which is also reported by some studies [18, 19] and different to that obtained by others [8, 10, 20].

For evaluation of protein content and assay activity, specific activity, fold purification and enzyme recovery were calculated and the results were summarized in (Table 1)

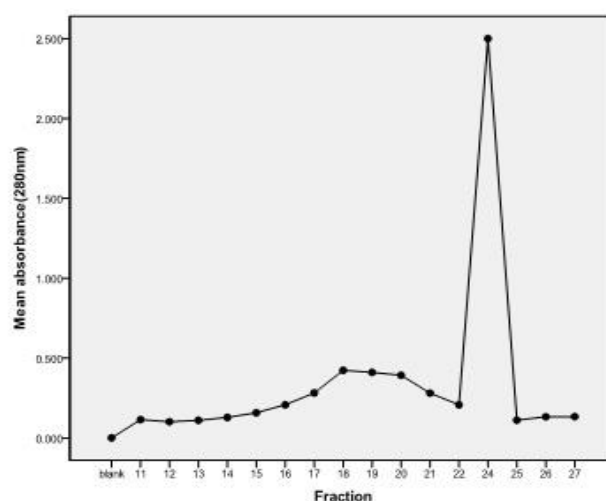
Table 1. Purification of urease enzyme from *P.mirabilis*

Fraction	Volume (ml)	Total protein (mg)	Specific activity (units/mg)	Fold purification	% yield
Crud	50	64.06	0.550	1.0	100
Ammonium sulfate	50	69.60	0.624	1.3	40
Sephadex G-100	20	49.80	2.500	4.5	98

Protein was extracted from *P.mirabilis* by a brief suspension in distilled water. Protein concentration was determined by the Bradford method with BSA as a standard. The amount of protein in parentheses is the amount which was actually subjected to the next column. Total recovery means the amount of protein when protease hydrolysis urea. Urease activity per min was determined by a spectrophotometric assay based on ammonia production from the linear part of the curve .fold purification, divided the specific activity of each fraction by the specific activity found in the crud.

The molecular weight markers, crude extract, and Mono Q and a Sephadex G-100 extract were loaded in lanes 1, 2 and 3 respectively, while lane 4 was loaded with pooled fractions obtained from the Sephadex G-100 chromatography. A faint band at molecular weight 66, 45, 29, and 15 kDa was seen (Fig. 2) with the pooled fractions from the size-exclusion chromatography, while very distinct bands were observed in the crude extract (obtained after sonication) and the ammonium sulphate fraction. The molecular weight values of these subunits are similar to those as reported [21].

Finally the urease enzyme activity was detected in SDS-PAGE (Fig 3). These results were similar to those as reported earlier [14].



Size-exclusion chromatography of *P.mirabilis* proteins on Mono Q and Sephadex G-100 26/60 column (2.4 × 75 cm) equilibrated with running buffer (50 mM NaHPO₄, 150 mM NaCl, 1 mM EDTA, pH 7) at 1 ml/min. 4-ml volume of water extracted proteins was applied to the column after adjusting the salt concentration to running Buffer. Proteins were detected by absorbance at 280 nm, urease activity was determined in an aliquot of the elution fractions (4 ml) semiquantitatively by phenol red based on spectrophotometric assay.

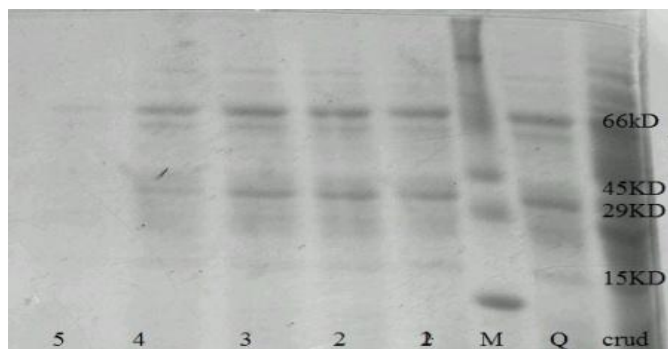


Fig. 2: Positive fractions after column chromatography SDS–PAGE using a 12% separation gel. Denaturation of proteins was done by diluting the samples (adjusted to 2 mg/ml) with sample buffer (1:2) and heating the mixtures at 95°C for 5 min. 15µ/ml volume was loaded on the gel and electrophoresis was done at 100 V for 3h. The gel was stained in staining solution (10% acetic acid, 25% Methanol, 0.25 g/l Coomassie Brilliant Blue G-250) for overnight and destained in 30-10% methanol and acetic acid overnight(M), Low-molecular-mass standard and (1-5) fraction collection.



Fig. 3: Detection of urease activity by SDS–PAGE using a 6% separation gel. Denaturation of proteins was done by diluting the samples (adjusted to 2 mg/ml) with sample buffer (1:2). 15µ/ml volume was loaded on the gel and electrophoresis was done at 25 V for 6h. The gels were then washed in several changes of a filtered solution of disodium ethylenediaminetetraacetic acid (EDTA) 0.1% w/v and cresol red indicator 0.02% w/v until the gel turned yellow, indicating neutral or slightly acidic conditions. Gels were developed by discarding the washing solution and immersing them in a small volume of urea 1.5% w/v. Areas of urease activity appeared as cherry-red bands against the yellow background due to the local production of alkali as a result of enzymic degradation of urea.

4. CONCLUSION

Maximum extracellular and intracellular urease activity was attained on culturing for 72 hours. The highly significant urease activity observed after sonication of the cells indicates that the high activity of urease enzyme is located in the cytoplasm. The Maximal activity of protein unit production by *P.mirabilis* strain was 200mg/ml.

5. ACKNOWLEDGEMENTS

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