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PRODUCTION, PURIFICATION, CHARACTERIZATION AND IMMUNOLOGICAL STUDIES OF *E.COLI* E-U_{10} PENICILLIN AMIDASE

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ABSTRACT: **Penicillin amidase enzyme (PA) was isolated from the culture filtrate of one isolated strain of *E.coli* E-U_{10} grown on optimized medium after 24 hours incubation. The enzyme was purified to homogeneity by sequential ammonium sulfate fractionation and ion exchange and gel filtration chromatography. On using Disc-PAGE, the final purification fraction showed only one distinctive band indicating high purity. On using SDS-PAGE the final fraction showed two distinctive units with molecular weight of 22000 and 59000 Dalton. The optimal pH, temperature and time course for activity were 7.5, 37°C and 10 minutes respectively. The enzyme with penicillin G as substrate had a K_m of 13 mM and V_{max} 3.92 U/ml. The purified protein was rich in alanine, lysine, glutamic, aspartic, serine, histidine, threonine and glycine. Antisera specific to the tested pure enzyme was prepared through immunization technique. The addition of anti-*E.coli* E-U_{10} penicillin amidase to the antibiotic increases the sensitivity of the tested bacterium towards the resistant penicillin G antibiotic.**

Key words: Production, purification, immunological studies, penicillin amidase, *E.coli* E-U_{10}

INTRODUCTION

Penicillin amidase (penicillin amidohydrolase or penicillin acylase) is of considerable industrial importance, primarily in the production of 6-aminopenicillanic acid (6-APA) as an intermediate in the manufacture of semisynthetic penicillins (virden, 1990). Ampicillin and cephalexin are medically and commercially important semisynthetic ß-lactam antibiotics that are synthesized through the condensation of D- (–) - α-aminophenylacetic acid with 6-aminopenicillanic acid (6-APA) or 7-aminodeacetoxycephalosporanic acid (7-ADCA) respectively. Several enzymes catalyze the above condensations (Konecyn.1981 and Nam *et al*, 1985). One of these enzymes , the penicillin amidase (EC 3.5.1.11) of *Escherichia coli*, has

been well characterized (Svedas *et al*,1980). This enzyme catalyzes the formation of amide bonds through an acyl-enzyme intermediate (Kasche, 1985). In the synthesis of ampicillin, the enzyme reacts with D - (–) - α-aminophenylacetic acid to form an acyl-enzyme intermediate. The D - (–) - α-aminophenylacetyl moeity is subsequently transferred to 6-APA to yield ampicillin. The formation of the acyl-enzyme intermediate is the rate-limiting step for both the synthesis and hydrolysis of ampicillin (Forney and Wong, 1989). On the other hand, the bacterial resistance to penicillin results from a modification of either the antibiotic itself or a specific target structure in the bacterial cell. Modification of the antibiotic structure is effectively achieved by β-lactamases or by penicillin amidases. β-lactamases open the β-lactam ring hydrolytically, thus converting the antibiotic to the inactive penicilloic acid. Penicillin amidase removes the acyl side chain but leaves the penicillin nucleus intact.

Because of insufficient information on technical and economic feasibility of penicillin amidase production. Our objective was biphasic, firstly: elaborate production, purification and characterization of the *E.coli* E-U_{10}, penicillin amidase to be used in the manufacture of semisynthetic penicillins. Secondly: studying the bacterial resistance to penicillin by serological studies on the pure penicillin amidase enzyme that increases the sensitivity of the bacterium towards the same antibiotic.

MATERIALS AND METHODS

Organism and cultivation conditions

E.coli E-U_{10} was kindly supplied by Bacteriology Department, Faculty of medicine, Alexandria University. Stock cultures were maintained at 4°C on columbia agar base slants of the following composition gl^{-1}: Peptone, 23.0; starch, 1.0; sodium chloride, 5.0; agar, 10.0. The optimized medium used in cell propagation and enzyme production was the modified basal salt medium (Gang and Shaikh 1976) with the following composition gl^{-1} : K_2HPO_4, 14; $MgSO_4$, 0.2; mannitol, 6; ascorbic acid, 0.001; yeast extract, 10; phenylacetic acid, 0.25 and uric acid 1mmol. Transfers from single slant cultures of 48 hours old were taken into 50ml aliquots of the basal medium dispersed in 250ml Erlenmeyer flasks to initiate growth. Standard inocula of 2% (v/v) were taken when substantial turbidity (~ 0.5 OD_{540} units) was observed after overnight incubation at 37°C under shaken conditions. The cultivation of the organism was performed in 250ml Erlenmeyer Flasks containing 50ml of the basal medium, pH 7.5 the antibiotic substrate as an inducer was added after autoclaving and cooling of the medium at a level of 0.5mg/l (v/v). flasks were incubated on rotary shaker (280 shakes/min., amplitude 1cm)at 37°C for 24 hours.

Biomass yield

Cell dry weight was calculated turbidometrically at 540 nm from a standard curve which plotted dry weight against turbidity (Daumy *et al*, 1985a).

Extraction of the crude enzyme

The culture was centrifuged (12000 rpm for 20 minutes) using a cooling centrifuge. The supernatant was collected, representing the extracellular (EC) enzyme. The cells were washed with distilled water and suspended in 10ml, 0.05 M phosphate buffer pH 7.5. The bacterial cells were disrupted by sonication (Brown Sonifier) in an ice bath until all the cells were disintegrated. The homogenate was centrifuged at 12000 rpm for 30 min. at 4°C. The supernatant thus obtained was taken as a crude intracellular enzyme (IC) (Daumy *et al*, 1985a)

Enzyme assay

The penicillin amidase activity was determined by measuring the amount of 6 aminopenicillanic acid (6-APA) produced in a reaction mixture containing 20 mg/ml benzyl penicillin (penicillin G) in 0.1 M phosphate buffer, pH 7.5, when incubated at 37°C. The assay depends on the reaction of the free amino group of 6-APA with p-dimethylaminobenzaldehyde to form Coloned Schiffs base (Balasingham *et al*, 1972).

Enzyme purification

Ammonium sulfate precipitation

Proteins precipitated at 60% saturation of ammonium sulfate were then collected and centrifuged (12000 rpm for 20 min.). The crude enzyme obtained after dialyzing against 0.01 M phosphate buffer pH 7.

Ion- exchange and gel filtration chromatography.

The dialyzed ammonium sulfate fraction was concentrated using Amicon 8400 ultra filtration and then applied to an anion exchange (DEAE-Sephadex A_{50}) column (2.7 x 50 cm) which had been equilibrated with 0.01 M phosphate buffer pH 7. The adsorbed proteins were eluted by a linear gradient of NaCl from (0.01-0.05 M) in the same buffer. The practically purified penicillin amidase enzyme sample was then subjected to gel filtration on Sephadex Bio-Gel P-6 column (2.5 x 50 cm) for further purification.

Gel electrophoresis

Protein purity and molecular weight estimation of the purified enzyme was performed. Disc and SDS-PAGE according to the method of stegemann *et al*, (1987) using 15% and 8.5% acrylamide respectively. Protein bands were stained with commassie blue R-250

Characterization of purified penicillin amidase enzyme

pH and temperature optima

Substrate was used in the penicillin amidase assay (20 mg/ml penicillin G) at 37°C for 10 min. Enzyme activity at pH and temperature optima was tested at time intervals.

pH and temperature stability

The purified enzyme in 20mM citric-citrate pH 5 and phosphate (Na_2HPO_4-NaH_2PO_4, pH's 6 & 7.5) buffers were incubated at 40°C for 10 hours. Temperature stability was determined in the same buffer system at 37 and 45°C for 6 hours respectively. Samples withdrawn at intervals for both cases were assayed at 37°C and pH 7.5 for residual activities.

Determination of kinetic parameters

Enzyme (56U each) was incubated with various concentrations of penicillin G (0-56 m mol) in 0.1M phosphate buffer pH 7.5 at 37°C. kinetic parameters K_m and V_{max} were calculated by linear regression from Lineweaver-Burk plots.Aliquots of 0.25-200ml of the pure enzyme preparation each was added to 1.0ml substrate and incubated at 37°C for 10 min.

Determination of amino-acids content

Amino acid composition of the pure penicillin amidase enzyme was detected by Bechman Amino Acid Analyzer (119 GL) according to the method of Speckman *et al*, (1958).

Immunological studies of the pure enzyme

Antiserum specific to the tested enzyme was prepared by subcutaneous injections of rabbits (2-3 kg in weight) each received two injections with 0.36 mg protein /ml purified enzyme emulsified in complete Freund adjuvant. The antisera were collected after 4 weeks and stored at 15°C until required.Immunodiffusion studies were occurred according to Ouchterlony double diffusion technique (1953). The inhibitory effect of anti *E.coli* E-U_{10} penicillin amidase towards bacterial growth was tested using single disc diffusion method (Knox and Smith, 1961).

RESULTS AND DISCUSSION

Enzyme production

Nutritional and environmental factors affecting penicillin amidase production by *E.coli* E-U_{10} have been studied previously (Mabrouk, A. 1998). The optimum conditions for enzyme production determined were thus followed and described in materials and methods. A typical time course of penicillin amidase production by the strain shown in fig. 1. The enzyme increased rapidly with incubation time during the first 24 hours. It was found that, the logarithmic phase of *E.coli* E-U_{10} seems to extent to the end of the 24th hour of incubation under shaking. Activities of both IC and EC appeared first and reached peak values of 27.86 and 21.25 U/ml respectively. The enzyme activities decreased with time soon after the maximum activities of the enzyme had been attained. The loss of enzyme activity may be due to poisoning, denaturation and/or decomposition of penicillin amidase as a result of interaction with other components in the medium. This

results are in accordance with that obtained by Roa *et al*, (1994).

Purification of penicillin amidase

Culture supernatant of a 24 hour old grown culture was subjected to a three-step purification scheme involving ammonium sulfate precipitation, anion exchange and gel filtration chromatography. About 86% of total enzyme activity was found in 60% saturation ammonium sulfate precipitate. Anion exchange chromatography of ammonium sulfate-fractionated proteins (fig.2a) resulted in a protein content reduction by 98.2% and the specific activity was increased by 110.3 times. The purification profile on DEAE-Sephadex A_{50} column indicated that *E.coli* E-U_{10} penicillin amidase not firmly bound to the gel since it was eluted at low NaCl concentration. The yeild percentage (63.3%) indicated that the purification step preserved most of the enzyme activity and the specific activity was found to be 70.17 U/mg protein. The enzyme was purified 110.3 fold indicating high purification factor and good efficiency of DEAE- Sephadex A_{50} column in purifying *E.coli* E-U_{10} penicillin amidase. The fractions with penicillin amidase activity were collected concentrated dialyzed and further purified by gel filtration on Bio Gel P-6 (fig.2b), the protein content was reduced by 99.1% and the specific activity was increased by 278.14 times. The purity of the proteins obtained at chromatographic steps was examined by Disc-PAGE (fig. 3).

The electrophoretic pattern of the Bio-Gel P-6 fraction gave one band indicating high purity of the isolated enzyme. On applying SDS-PAGE, the final fraction showed two distinctive bands with molecular weight of 22000 and 59000 Dalton (fig. 4). These results are in agreement with those obtained by Lindsay and Pain (1990), Martin *et al*, (1991) and Prieto *et al*, (1992).

A summary of the purification results is shown in table 1. The purification procedure gave about 278.14 fold overall purification and approximately 40.8% recovery of activity. The specific activity of purified enzyme at 37°C was 176.9 U/mg^{-1} protein. Penicillin amidase preparations of *A. viscosus* 8895 GU subjected to all these steps of purification techniques, were purified 2.31 fold with 6.7% recovery (Ohashi *et al.*, 1988).

Kinetic parameters

The enzyme kinetics study, indicated that the enzyme activity dependent on the enzyme and substrate concentration. As the substrate concentrations were increased, the initial reaction rate of activities were fast. When the relation between the enzyme activity and substrate concentration was analyzed by Lineweaver-Burk analysis, it was found that K_m is 13.51 mM and V_{max} is 3.92 U/ml (fig. 5a) indicating much higher hydrolyzing activity of *E.coli* E-U_{10} penicillin amidase against penicillin G. These results are in agreement with that obtained by Roa *et al*, (1994), and in contrary with those obtained by Forney *et al*,

(1989).The effect of pH on penicillin amidase activity was determined in five different buffers with pH values ranging from 2.0-12.0 (fig. 5b). The enzyme was most active at pHs around 6-8 at 37°C but lost the activity at pH greater than 8. The enzyme retained 75% activity after 8-10 hours of incubation at optimum pH 7.5.The optimum temperature for enzyme activity was around 37°C as reported by Ohashi *et al*, (1988). The enzyme was less stable at pH 8 than at pH 7.5. The thermostability of this enzyme at optimum temperature showed it lost 50% activity after 4.5 hours at pH 7.5 where as the stability decreased at pH 8 as the residual activity was reduced to 28%. The enzyme possessed a temperature optimum of 37°C after 10min incubation (fig. 5c,d) which was higher than 25°C of *K. citrophila* mutant (Martin *et al*., 1990). It showed a moderate thermal stability at 37°C. Further more, the enzyme had maximum activities within (6-8), but it lost activity at pH value higher than 9 which is probably near the iso-electric point of the enzyme. The quality of *E.coli* E-U_{10} penicillin amidase has been assessed for its amino acids constituents. Thus all amino acids were detected in adequate levels. Relatively higher amounts of alanine, lysine, glutamic, aspartic, serine, histidine and threonine. The tested protein proved to be rich in glycine and leucine (table 2).

In a trial for the formation of anti *E.coli* E-U_{10} penicillin amidase through immunization and Ouchterlony double diffusion, it was found that the antigen and antibody molecules diffuse out of there respective wells into the agar at a rate inversely proportional to their molecular weights, if the molecules have no affinity for the agar itself. The two reactants diffused and reacted together to form a line of precipitation (precipitin line) (fig.6.).*E.coli* E-U_{10} was resistant towards penicillin G antibiotic, but when anti *E.coli* E-U_{10} penicillin amidase was added to this antibiotic, it renders the bacterium sensitive to the antibiotic(fig. 7), due to the high spectrum of activity of the isolated antibody. The inhibitory effect of the isolated antibody towards bacterial growth may be due to the block of the protein synthesis and hence the penicillin amidase synthesis which catalyse the hydrolysis of penicillin G into 6-aminopenicillanic acid (6-APA) and phenylacetic acid (PAP) (Daumy *et al*, 1985b). So it can be used in the therapeutic measures through combination with penicillin G during industrial preparations to improve its antimicrobial spectrum (El-Sayed *et al*., 1995).

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Table 1: Purification of penicillin amidase of *E.coli* E-U_{10}.

	Collected volume (ml)	Activity (U / ml)	Protein (mg/ml)	Total activity (U)	Total protein (mg)	Specific activity (U / mg protein)	Purification factor	Yield %
Crude extract	300	26.0	40.88	7800	12264.0	0.636	0.00	100
Precipitation by $(NH_4)_2SO_4$	200	33.55	2.027	6710	405.4	16.55	26.02	86
Anion exchange chromatography on DEAE- Sephadex A50	100	49.40	0.704	4940	70.4	70.17	110.3	63.3
Bio Gel P-6 column	50	63.7	0.36	3185	18.0	176.9	278.14	40.8

Table 2: Amino acid contents (mole %) of the purified enzyme as compared with FAO reference.

Amino acids	*E. coli* E-U_{10} penicillin amidase (mole %)	FAO
Aspartic	7.31	-
Threonine	4.97	2.8
Serine	5.46	-
Glutamic	7.62	-
Proline	1.78	-
Glycine	11.98	-
Alanine	8.34	-
Valine	4.90	4.2
Methionine	1.26	2.2
Isoleucine	4.34	4.2
Leucine	10.39	4.8
Tyrosine	0.96	-
Phenylalanine	3.10	2.8
Histidine	5.13	-
Lysine	7.80	4.2
Arginine	3.24	-

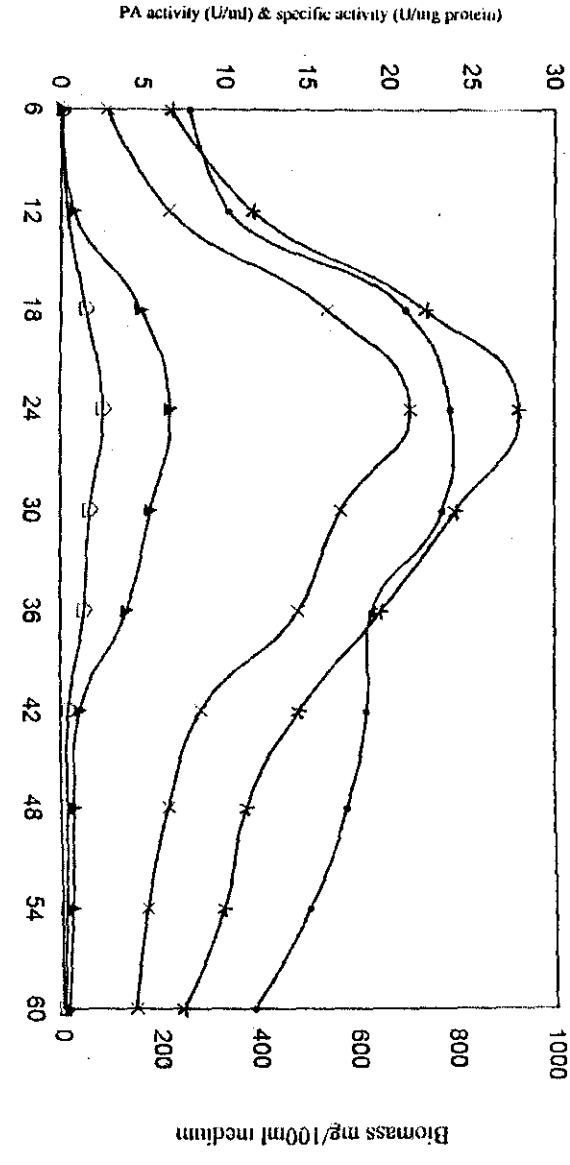

Fig 1: Biomass and enzyme activities of fermentation broth during a batch cultivation of *E.coli* ES-110 under optimal medium composition and cultivation conditions.
IC and EC (inter and extra cellular enzyme activities respectively)
PA (penicillin amidase)

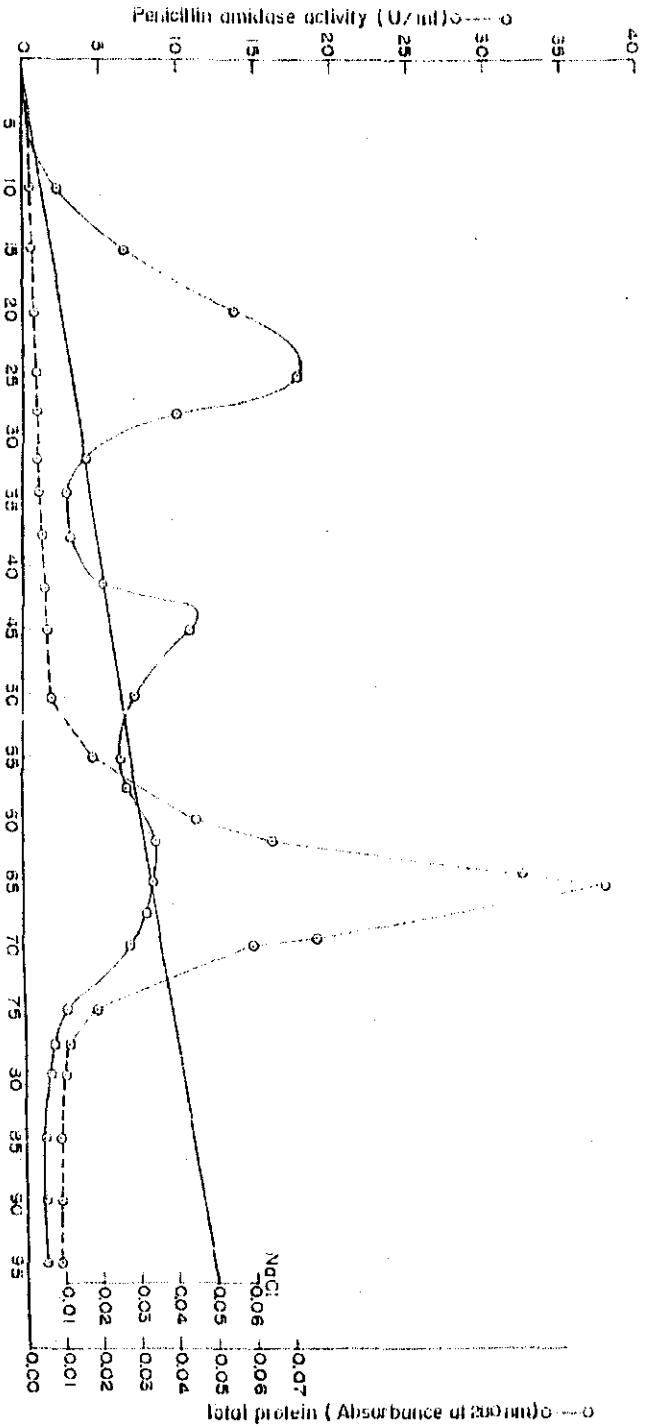

Fig.2a: First DEAE-Sephadex A_{50} chromatography of penicillin amidase. The dialyzed ammonium sulphate precipitate was chromatographed on DEAE-Sephadex A_{50} column by elution with a linear gradient of NaCl. Total protein was monitored at 280 nm. The fractions were assayed for penicillin amidase activity.

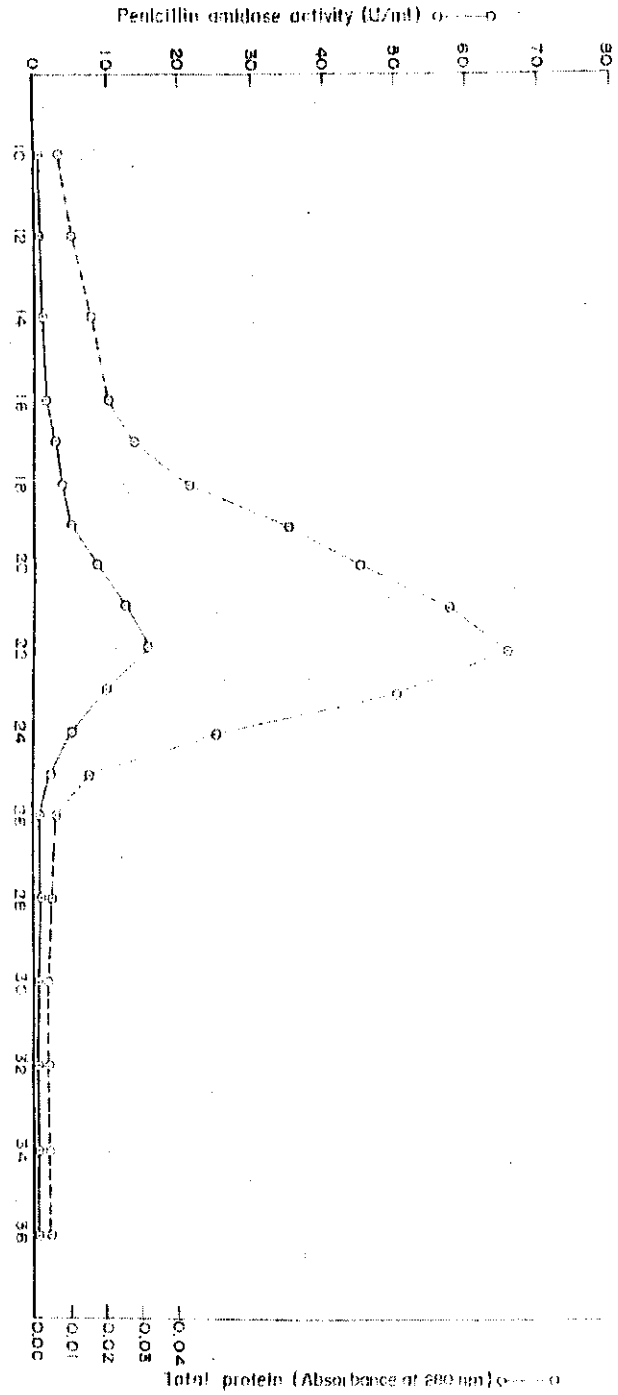

Fig. 2b: Second Bio Gel column of penicillin amidase. The first DEAE-Sephadex A_{50} collected fractions were applied to Bio Gel P-6. Total protein was monitored at 280 nm . The fractions were assayed for enzyme activity.

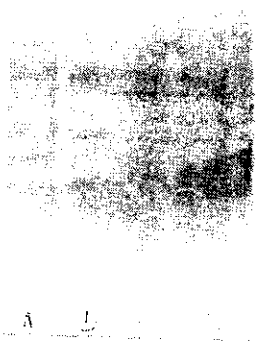

Fig. 3: Disc PAGE of A: Crude extract; B: Ammonium sulphate extract; C: DEAE-Sephadex A_{50} fraction and D: Gel filtration Bio Gel P-6 fraction

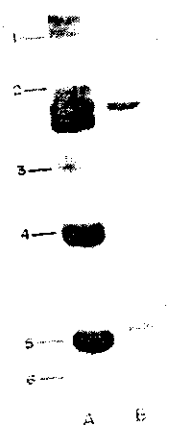

Fig.4: Molecular weight determination by using SDS/PAGE
A: Standard protein:
1:Phosphorylase (97,400 D);
2: BSA (67000D);
3: Ovalbumin (42000 D);
4: Carbon anhydrase (31000 D);
5: Soya bean trypsin inhibator (21000 D).
B: Gel filtration Bio Gel P-6 fraction.

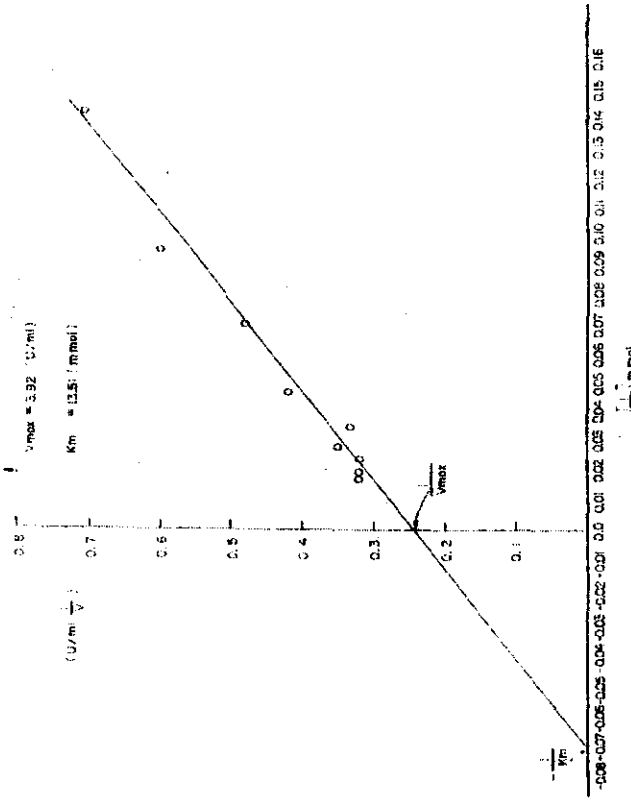

Fig. 5a: Line weaver-Burk plot of *E.coli* E-U$_{in}$ penicillin amidase activity Versus penicillin G.

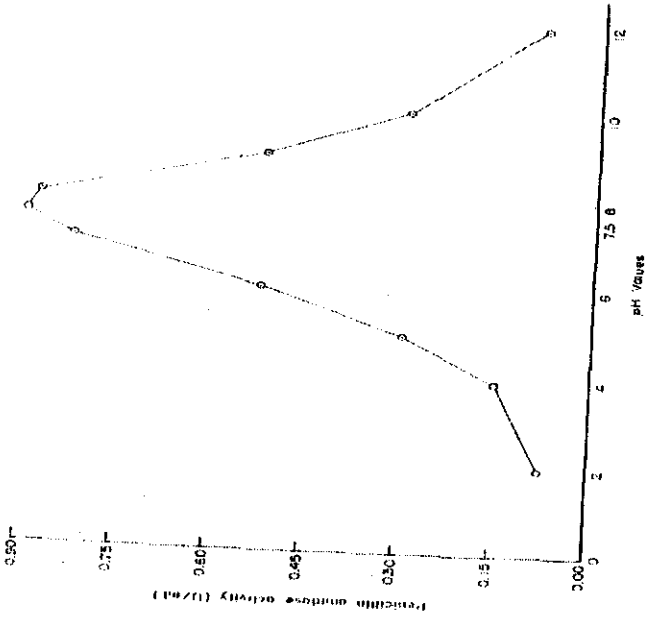

Fig. 5b: Effect of pH on *E coli* E-U$_{in}$ penicillin amidase.

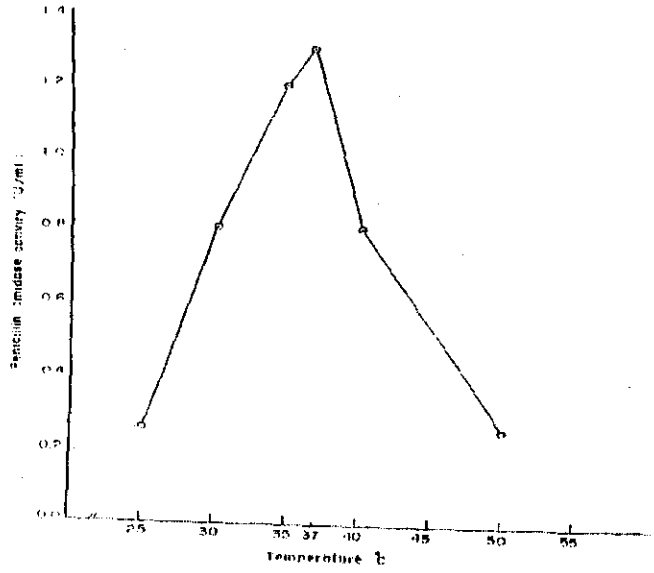

Fig.5e: Effect of incubation temperature on the activity of *E.coli* E-U_{10} Penicillin amidase.

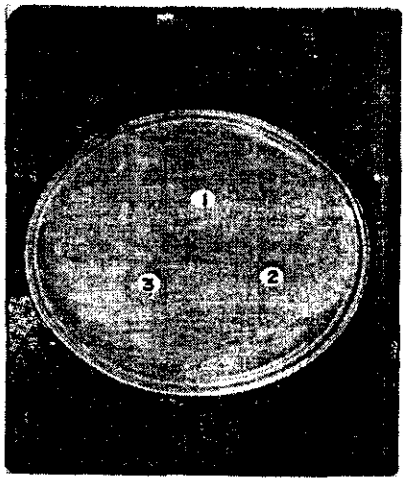

Fig.7: The inhibitory effect of anti- *E.coli* E-U_{10} penicillin amidase towards bacterial growth)
1. Penicillin G
2. Penicillin G + 10 ul anti- *E.coli* E-U_{10}
3. Penicillin G + 20 ul anti- *E.coli* E-U_{10}

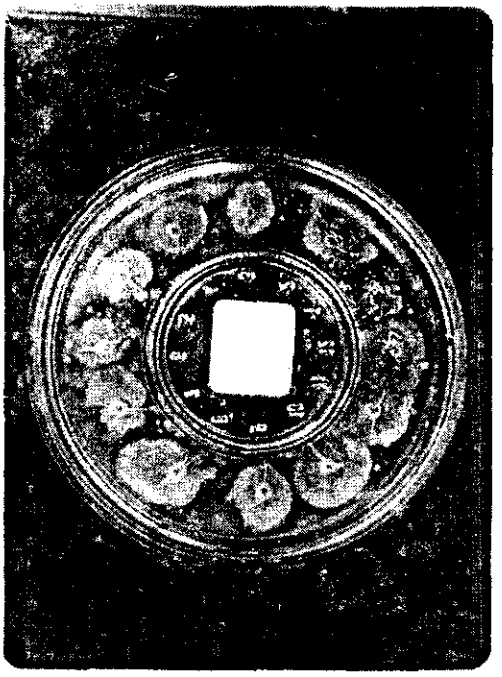

Fig.6: Antigen-antibody reaction can be measured by the formation of insoluble complex: Ouchterlony double diffusion (the precipitin reaction)