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The purification and characterization of intracellular invertase obtained from pathogenic *Escherichia Coli*

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Summary

All the non-pathogenic strains of *Escherichia coli* tested failed to synthesize invertase. However, among the pathogenic *E. coli*, only 11% of them synthesized the enzyme. Invertase synthesis was best at pH 8.0, when the sole nitrogen source was peptone. The enzyme was induced by sucrose but repressed by glucose and fructose. The enzyme was partially purified by ammonium sulphate precipitation, followed by dialysis and gel permeation chromatography. The partially purified invertase possessed a molecular weight of 125,000 KD and an apparent km of approximately 2.94mM for sucrose. The enzyme was stimulated by Ca^{++} and Mg^{++}, inhibited by Cu^{++}, U^{++}, IAA and exhibited optimum activity at pH 6.5 at 40°C.

Résumé

Tous les efforts non-pathogène de l'Escherichia coli testés n'ont pas pu synthétiser l'invertase. Neanmoins, parmi le E. coli pathogènes, seul 11% d'eux ont synthétisé l'enzyme. La synthése invertase était la meilleure à pH 8.0, quand la source principale du nitrogène était peptone. L'enzyme a été incité par le sucrose mais réprimé par le glucose. L'enzyme a été partiellement purifié par la précipitation du sulphate d'ammonium, suivi du dialyse et de la pénétration du gel chromatographie. L'invertase partiellement purifié possédait une molécule du poids de 125,000 KD et un kilométrage apparent de, approximativement 2.94mM pour le sucrose. L'enzyme a été stimulé par Ca^{++} et Mg^{++}, inhibité par Cu^{++}, Hg^{++}, U^{++}, IAA et l'activité d'optimum exhibité à pH 6.5 a 40°C.

Introduction

Enzymes have not been reported to enhance the evasive and pathogenic properties of bacteria[1]. All faecal *Escherichia coli* have been found to produce maltase and lactase[2]. The enteropathogenic *E. coli* (EPEC) have similarly been reported to produce significantly higher amount of such enzymes than those produced by non-pathogenic strains of *E. coli*[3,4]. Similarly, Hashia *et al.* have shown that enzymes could play significant role in the pathogenesis of EPEC in childhood diarrhoea[5]. Invertase had been reported to be induced by sucrose[6] and sucrose is present in the human intestine[7]. Similarly both EPEC and Non-EPEC are present in the same environment[7]. Therefore, the possibilities of EPEC producing inducible invertase was therefore investigated. This paper thus described the production, purification and characterization of intracellular invertase produced by EPEC.

Materials and methods

Sources and collection of samples

Faecal samples from hundred with diarrhoea children under 2 years of age (60 females and 40 males) in Ile-Ife were screened for both pathogenic and non pathogenic *E. coli*. *E. coli* was identified by the technique of Cowan and Steel[8]. Pathogenic strains of *E. coli* were identified by serological techniques using 3 polyvalent commercial antisera prepared by Wellcome Laboratories, Beckenham, England[9,10].

Preparation of inoculum

Cell culture suspension of *E. coli* was prepared from 24 h. old nutrient agar slant cultures by the technique of Olusanya[11]. The cell suspension was adjusted to optical absorption of approximately 0.55 at 430nm on a cecil spectrophotometer and employed as the inoculum in all experiments, unless otherwise stated.

Culture conditions and growth of organism

The basal medium consisted of peptone (5g), K_2HPO_4 (2g), KH_2PO_4 (0.4g), Biotin (0.5mg), Thionine (0.5mg), Nicotinic aeid (1mg), $MgSO_4.H_2O$ (5mg), NaCl (10mg), $FeSO_4.7H_2O$ (2mg) and CaCl (5mg) per litre of distilled water.

The carbon source (sucrose) or any other carbon source employed was autoclaved separately and added to the basal medium to give a final concentration of 0.3% (W/V) unless otherwise stated. The pH was aseptically adjusted to pH 7.2 after autoclaving. Each 250ml flask contained 100ml of the medium. All cultures were incubated for the desired length of time at 35°C in an orbital incubator (gallenkamp) at 180 rpm. All experiments were performed in triplicate.

Preparation of bacterial culture for invertase

After incubation, the cells were separated from the growth medium by centrifugation at 10,000g for 15 minutes at 4°C. The cells were rinsed with three changes of 0.2M citrate phosphate buffer pH 7.0. The cells were shaken with toluene-alcohol (1:9v/v) for 5 minutes and the supernatant used as crude enzyme[2].

Invertase assay

Invertase was assayed by the glucose oxidase-toluidine technique of Olutiola and Cole[6] and one unit activity (U) is defined as the amount of enzyme in 1ml of reaction mixture that liberate 1µg glucose in 1 minute. The stoichiometry was determined as described by Olutiola and Cole[6].

Growth condition for catabolite repression and induction of invertase activity

E. coli was grown in the basal medium as described earlier, containing sucrose (0.3% w/v), sucrose plus glucose (0.15% + 0.15% w/v) or sucrose plus fructose (0.15% + 0.15% w/v). Experiments were also set up in which glucose (0.15%) or fructose (0.15%) was added to 0.15% sucrose medium after incubation for 4 hours. Induction experiments were similarly set up as follows: The organism was grown in medium containing glucose (0.15%) or fructose (0.15%) at the onset of experiment. After incubation for 4 hours, sucrose (0.15%) was added to some experimental flasks. Those without added sucrose served as control. All cultures were allowed to incubate for a maximum of 16 h. and all cultures were treated as described earlier for enzyme assays.

Ammonium sulphate precipitation

The crude enzyme preparation was precipitated with $(NH_4)_2SO_4$ between the limits of 60-90% saturation[12]. The precipitates were redissolved in 0.2M citrate phosphate buffer (pH 7.0) for 24 hours at 4°C. The dialysed filtrate was sterilized by membrane filtration before fractionation and assay procedure.

Gel permeation chromatography

A jacketed column was packed with Sephadex G-25 or G-100 to a height of 645mm (25mm diam) and cooled to 4°C. Preparation of the column was as described by Andrews[13]. The column was equilibrated and eluted on each occasion with 0.2M citrate phosphate buffer (pH 7.0) containing 5mM sodium azide. Application of samples to the column, collection and protein measurement of column affluent (5ml fraction) from both G-25 and G-100 were as previously described by Olutiola[14]. Each fraction was assayed as previously described.

Characterization of the invertase

The gas-liquid and paper chromatography technique of Olutiola and Cole[6] were used to determine the individual sugars present in the mixture.

The effects of pH, temperature, substrate concentrations, cations and chemicals on the activity of the partially purified invertase were determined by standard methods[11].

Results

Each of the 100 faecal samples from the children yielded *E. coli* but only 18 of them were pathogenic strains. Out of the 18 pathogenic strains, only two (11%) synthesized invertase whereas none of the non-pathogenic strains synthesized the enzyme.

Growth and invertase activity

The pathogenic *E. coli* grew in liquid synthetic medium containing sucrose as the sole carbon source, releasing into the medium a complex of proteins which exhibited invertase activity (Fig. 1). The growth of the organism was maximum in 12 h. while the production of the invertase was optimum at the 16th h. The organism utilized a number of carbohydrates for growth but invertase was produced only when the sole carbon source was sucrose (Table 1). The best nitrogen source for growth and enzyme production was peptone (Table 2). Optimum production of the enzyme occurred at pH 8.0 (Fig. 2).

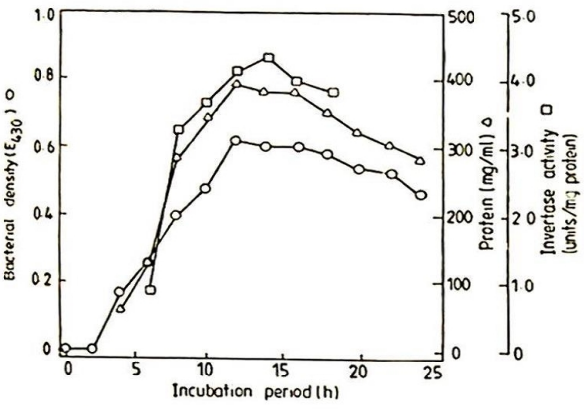

Fig. 1: Growth of *E. coli* in sucrose-peptone liquid medium, showing protein content and invertase activity of cell homogenate O, absorbance; Δ, protein; □, invertase activity

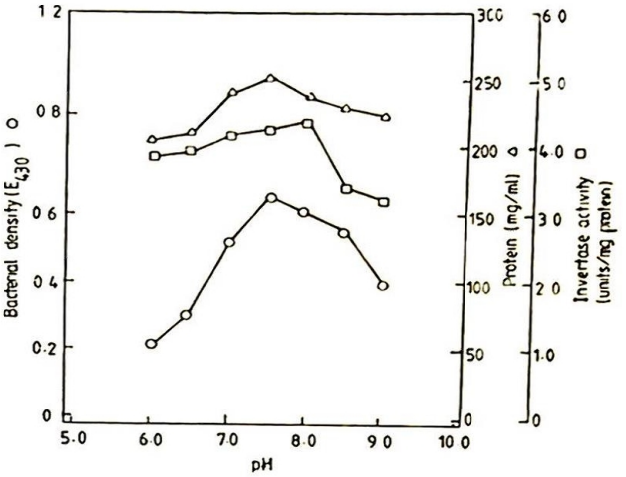

Fig. 2: Effect of pH on growth and production of invertase by E. coli in a sucrose-peptone liquid medium. O, absorbance; Δ, protein; □, invertase activity

Table 1: Growth of *E. coli* on different carbon sources and invertase activity of the cell homogenate

Carbon source	Growth (optical) density	Protein content (unit/ml)	Invertase activity (units/mg protein)
Fructose	0.61 ± 0.01*	195.00 ± 2.7*	0.0
Galactose	0.67 ± 0.01	299.00 ± 6.4	0.0
Glucose	0.62 ± 0.01	200.00 ± 5.77	0.0
Lactose	0.50 ± 0.01	197.00 ± 8.81	0.0
Maltose	0.54 ± 0.01	173.00 ± 4.40	0.0
Mannitol	0.59 ± 0.01	223.30 ± 2.33	0.0
Starch	0.64 ± 0.07	223.30± 8.81	0.0
Sucrose	0.80 ± 0.06	253.30 ± 6.67	4.40 ± 0.0*

*Each value is the mean of three replicates with standard error.

F (Growth) = 64.47**

F (Protein) = 14.91**

F (0.01) = 3.71

**Highly significant values

Table 2: Effect of source of nitrogen on growth and invertase activity of *E. coli*

Nitrogen source	Growth (optical) density	Protein content (mg/ml)	Invertase activity (units/mg protein)
Ammonium nitrate	0.56 ± 0.03*	230.00 ± 5.77*	1.92 ± 0.04*
Ammonium oxalate	0.57 ± 0.04	350.00 ± 5.77	1.91 ± 0.22
Ammonium sulphate	0.63 ± 0.02	312.00 ± 6.00	2.62 ± 0.16
Ammonium tartrate	0.64 ± 0.03	403.30 ± 8.82	3.58 ± 0.03
Peptone	0.82 ± 0.01	460.00 ± 5.77	4.42 ± 0.01
Potassium nitrate	0.48 ± 0.02	280.00 ± 9.99	3.57 ± 0.02
Sodium nitrate	0.39 ± 0.01	181.70 ± 4.38	1.41 ± 0.0
Tryptone	0.88 ± 0.30	483.00 ± 4.41	4.06 ± 0.08
Yeast extract	0.94 ± 0.02	537.00 ± 8.82	3.49 ± 0.11

*Each value is the mean of three replicates with standard error.

F (Growth) = 50.71** F (Protein) = 273.50**

F (Invertase) = 107.39**

F (0.01) = 3.71 **Highly significant values

Repression and induction of invertase activity

Experiments were carried out in which sucrose medium was supplemented with fructose or glucose after 0 h and 4 h respectively (Fig. 3). A decrease in invertase activity was observed in the presence of each of the two carbon sources, the repression being greatest when fructose or glucose was added to the sucrose medium at the onset of the experiment. The result has thus suggested that the inducible invertase of pathogenic *E. coli* may be under catabolite repressional control of glucose and fructose.

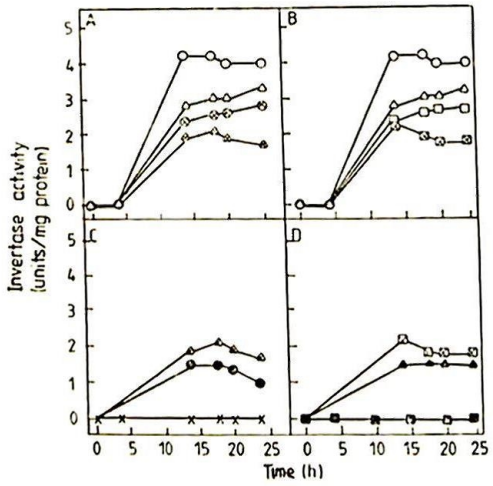

Fig. 3: Repression and induction of invertase activity of *E. coli* grown on single and mixed sources of carbon
A. ⧋, 0.15% sucrose plus 0.15% glucose; ⊗, 0.15% sucrose plus (after 4h) 0.15% glucose; Δ, 0.15% sucrose; O, 0.3% sucrose.
B. ⊠, 0.15% sucrose plus 0.15% fructose; □, 0.15% sucrose plus (after 4h) 0.15% fructose; Δ, 0.15% sucrose; O, 0.3% sucrose.
C. △, 0.15% glucose plus 0.15% sucrose; ■, 0.15% glucose plus (after 4h) 0.15% sucrose; X, 0.15% or 0.30% of glucose.
D. ⊠, 0.15% fructose plus 0.15% sucrose; ▲, 0.15% fructose plus (after 4h) 0.15% sucrose; ■, 0.15% or 0.30% fructose.

Type of invertase

Only glucose and fructose was detected by both gas-liquid chromatography and paper chromatography. The quantity of glucose produced equalled that of fructose when the glucose values obtained by the O-toluidine reagent were substracted from the total reducing sugar by the method described by Miller[15]. Thus the stoichiometry of the reaction was found to give a ratio of 1:1 of glucose to fructose, suggesting that the enzyme was an invertase[16].

Effect of pH and temperature

Invertase activity was optimum at 6.5. Activity was good both in alkaline and acid pH (Fig. 4). Invertase activity increased with increase in temperature achieving optimum at 30°C (Fig. 4). The enzyme was sensitive to heat, loosing all activities within 1 min. of heating at 60°C (Fig. 4). However Ca^{++}, K^{+} and albumen gave slight protection against heat inactivation.

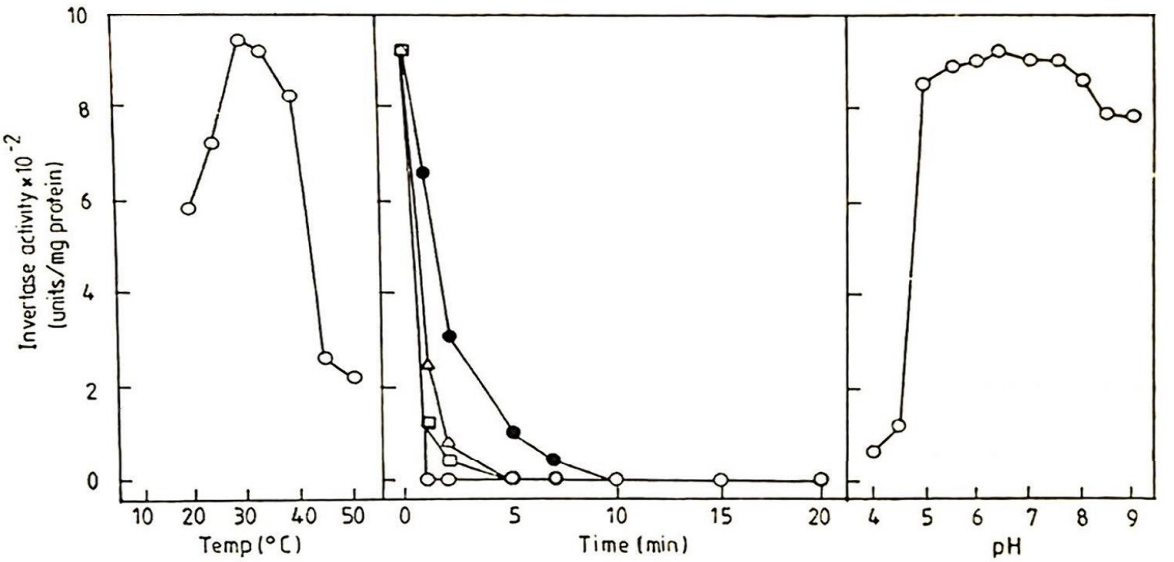

Fig. 4: Effect of temperature, heat (60°C) and pH on the activity of partially purified invertase (2nd ammonium sulphate fraction) obtained from cell homogenate of *E. coli*

Effect of substrate concentration

Invertase activity increased with an increase in concentration of sucrose, and saturating at 18mM sucrose. From the Lineweaver-Burk plot, the km for the hydrolysis of sucrose was approximately 2.94km (Fig. 5).

Hydrosis of carbohydrates

Specificity of *E. coli* invertase was tested against a number of carbohydrates (Table 3). Invertase activity was detected only when the substrate was sucrose, mannose or maltose, the highest activity being on sucrose.

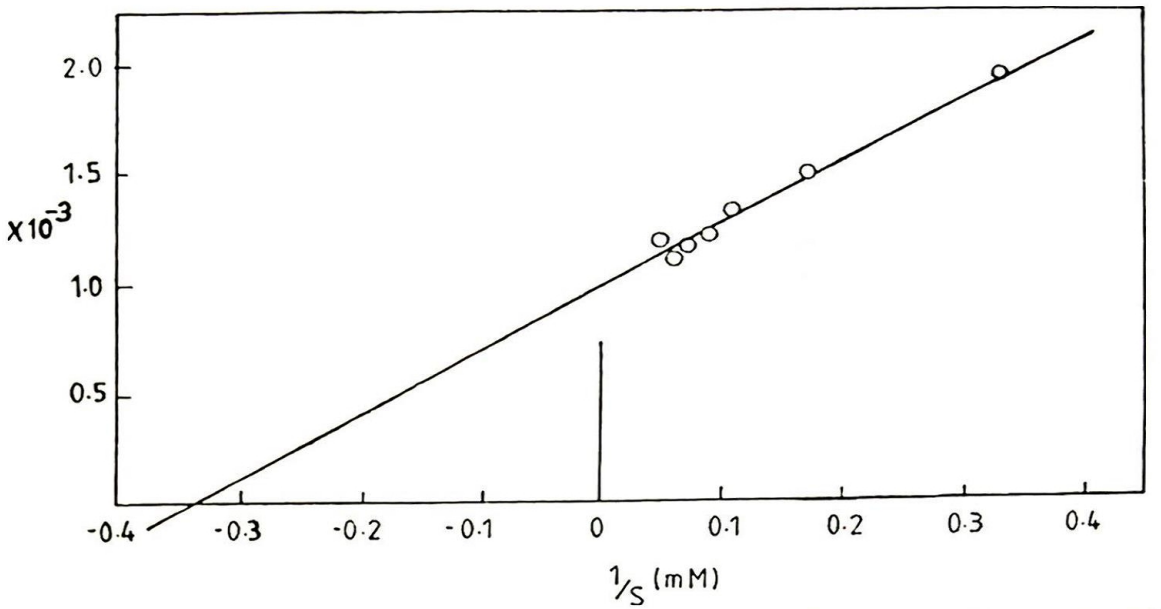

Fig. 5: Lineweaver-Burk plot of the hydrolysis of sucrose by the partially purified invertase (2nd ammonium sulphate fraction) obtained from cell homogenate of *E. coli* grown in sucrose-peptone liquid medium

Table 3: Activity of partially purified invertase (2nd ammonium sulphate fraction) of *E. coli* on different carbohydrates

Substrates	Invertase activity (units/mg protein)
Dulcitol	0.0
Inositol	0.0
Lactose	0.0
Maltose	96.16 ± 3.26*
Mannitol	0.0
Mannose	101.86 ± 6.53
Starch	0.0
Sucrose	910.5 ± 0.18

*Each value is the mean of three replicates with standard error.

Effect of cations and chemicals

Ca^{++} and Mg^{++} were stimulatory to the activity of invertase, whereas Cu^{++}, Hg^{++}, U^{++} and IAA were inhibitory (Fig. 6). Low concentration of Ba^{++} was stimulatory while higher concentrations were highly inhibitory.

Fractionation of Sephadex G-100

Fractionation of the concentrated culture filtrate on Sephadex G-100 led to three absorption peaks designated Aa, Ab and Ac with only Aa possessing invertase activity (Fig. 7). The molecular weight of this component (Aa) estimated from its elutian volume was approximately 125,000 KD using myoglobulin (MW7800) albumen (MW 45,000), haemoglobin (MW 68,000), glucose oxidase (MW 150,000) and kalatase (MW 240,000) as standards.

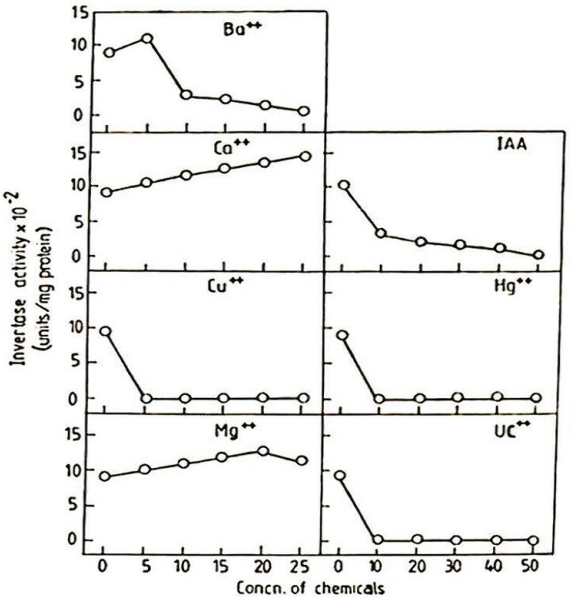

Fig. 6: Effect of chemicals on the activity of partially purified invertase (2nd ammonium sulphate fraction) obtained from cell homogenates of *E. coli*

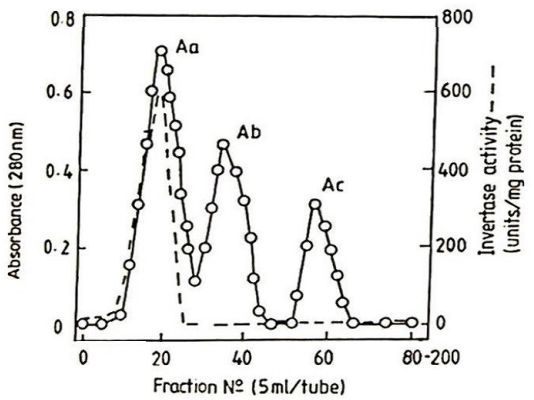

Fig. 7: Separation by gel filtration (sephadex G-100) of proteins fractions previously separated by gel filtration of Sephadex G-25 and enzymic activity of the fractions towards sucrose. 0------0, protein (E_{280}); ----, invertase activity

Partial purification

The purification as outlined in Table 4 showed that a final purification fold of 221 and activity of 911 units/mg protein were obtained.

Discussion

Previous report showed that the intestine is quite rich in sucrose[7]. Similarly the enzyme invertase has generally been reported to be inducible in the microbial sytem[6,16]. At the initial stage of the investigation therefore, we had anticipated that both EPEC and non-EPEC strains isolated from the intestine would be capable of synthesizing invertase. Our result showing that only 11% of the EPEC produced the enzyme and all the non EPEC did not synthesize it was quite unexpected. The inability of the non-EPEC and 89% of the EPEC to synthesize the enzyme is difficult to explain. One may however speculate that the inability to produce the enzyme could be linked to their invasive properties since enzyme production has been reported to enhance the invasiveness of bacterial agents[1].

Table 4: Partial purification of invertase from *E. coli*

Fractions	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Purification (Fold)
Crude extract	1753	425	4.12	100	1.0
Concentration (under vacuum at 30°C)	1734	321	5.4	98.9	1.3
Sephadex G-25	1535	51.9	29.6	87.6	7.2
1st $(NH_4)_2SO_4$ (60-90% displayed)	1421	12.8	111	81.1	26.9
Sephadex G-100 (Peak Aa)	1380	2.3	600	78.7	145.6
2nd $(NH_4)_2SO_4$ (60-90% dialysed)	1366	1.5	910.6	77.9	221.0

Glucose or fructose had repressing effect on the synthesis of invertase by pathogenic *E. coli* when added to sucrose growing culture or when sucrose was added to glucose or fructose growing culture. Thus the results suggest that under appropriate physiological conditions, glucose and fructose which could serve as souce of carbon and energy may repress the production of *E. coli* invertase. The results tend to support the theory of catabolite repression, a theory which postulates that formation of catabolite enzymes is sensitive to the concentration of one or more of the intermediate catabolites derived from the substrate of these enzymes[7].

The partially purified *E. coli* invertase exhibited a molecular weight of approximately 125,000 KD. Invertase from a number of other micro-organisms have also been reported to possess molecular weight range of approximately 40,000 to 160,000 KD [16,18]. The result indicates that Mg^{++} and Ca^{++} were stimulatory to the activity of the enzyme. A number of suggestions have been put forward to explain the stimulation of enzymic activity by cations. Baker and Borne[19] proposed that metal activation may involve the formation of binding organic molecules to proteins, thus stimulating the activity of the enzyme by forming essential part of the active centres of the enzyme protein. On the other hand, Viller and Spencer[20] proposed that metal ion may conbine with both substrate and the enzyme to form a substrate-metal-enzyme complex, with the metal acting as a link between the substrate and the enzyme. However, Hg^{++} and U^{++} were found to inhibit the activity of the *E. coli* invertase. Salts of heavy metals have been reported to inhibit enzyme activity by precipitating the enzyme and thereby making such precipitated enzymes inactive[17].

The activity of EPEC invertase was optimum at the pH 6.5 which is within the pH of 5.5-6.5 reported for other microbial invertase[16,21]. The optimum pH of the intestine has been reported to be at neutral pH[7]. Thus the presence of sucrose in the intestine[7] coupled with suitable pH will thus enhance the invasive properties of the EPEC strains capable of producing the enzyme.

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