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STUDIES ON EFFECT OF DRUGS ON HUMAN LIVER GLUCOAMYLASE

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ABSTRACT

Human liver glucoamylase was purified using four convenyional steps viz Fractional precipitation with ammonium sulfate, ion exchange chromatography on DEAE cellulose, molecular sieving on bio gel P-100 and poly acrylamide gel electrophpresis technique, even though the molecular size of the isoenzymes was very close to each other. Human liver glucoamylase was immobilized on CNBr activated agaropection. Human liver glucoamylase was characterized and showed pH optima of 4.4 and 5.6; temperature optima 50^{o}C and 45^{o}C; Km characteristics towards starch 26.6 mg/mL and 15.4 mg/mL for free and immobilized. The drug penicillin competitively inhibited the human liver glucoamylase activity while streptomycin accelerated the activity of reaction.

Key words: Starch, Glucoamylase, Peniciline, Streptomycin.

INTRODUCTION

The liver in the intact animal assists to maintain the normal level of blood glucose in three ways[1], firstly by regulation of reversible reaction Glucose ↔ Glycogen (liver), secondly by regulating new glucose formation mainly from non-nitrogenous residues of amino acids produced from protein and the glycerol from fat, thirdly by regulating the removal of glucose from blood and deposition as fat in liver. Liver can be described as blood glucostat reacting to maintain the blood glucose within normal range. E.C.3.2.1.1 α amylase hydrolyses α-1,4 glucan link in polysaccharides in random manner, the α-1,4 links in outer and inner chains beyond the branch point. Thus it causes rapid depolymerisation of glycogen to oligosac-charides[2]. E.C.3.2.1.3 is designated as glucoamylase or amyloglucosidase or γ-amylase. Glucoamylase hydrolyses α-1,4 linkage in polysaccharides so as degrading glycogen. The effect of substances, which can remove successive glucose units from non-reducing end of chain[3]. The α-1,6 glucan linkage is also hydrolysed by glucoamylase. Liver glycogen plays an important role in maintaining the normal level of blood glucose. The

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amylases are mainly involved in either activate or inhibit the amylases is of prime interest, as they may directly affect the level of blood glucose. An attempt has been made to study the effect of certain drugs on activity of liver glucoamylase.

EXPERIMENTAL

Protein estimation was carried out using Lowry et al. procedure[4]. Glucose estimation was carried out using 3,5 dinitro salicylic method[5]. All operations were carried out at 4°C, except where stated. The human liver washed until the exterior surface was free of blood, by using portions of 0.25 M sucrose, 0.2 mM EDTA buffer pH 7.2. The small pieces of liver were homoginised with sucrose-EDTA buffer, the homogenate was centrifused and the supernatant containing glucoamylase activity was processed to achieve further purification. The fractional precipitation of protein at varying concentration of ammonium sulfate was in separating the glucoamylase fraction from protein solution.

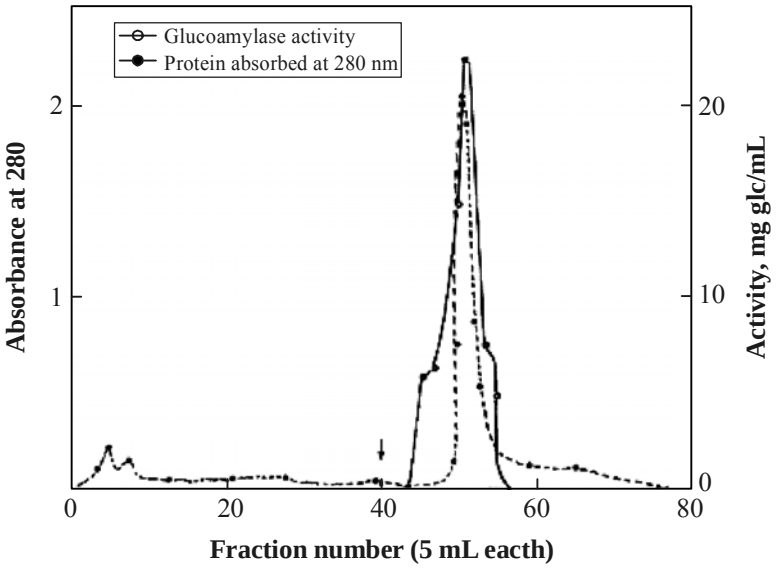

Fig. 1: Enzyme solution 5 mL column size 24 x 2 cm, equilibration and Elution with tris HCl butter pH 7.0, flow rate 40 mL/hr.

The precipitate was dissolved in minimum quantity of 5 mM -Tris-2 mM EDTA – 5 mMB-mercaptoethanol buffer pH 7.2 and dialysed. The separation of protein from a mixture of proteins is possible in making the use of its ionic characteristics. The DEAE cellulose was reconditioned and is equillibriated with the Tris-HCl buffer pH 7.0. The chromatographic column 24 x 2 cm was filled by transferring the activated DEAE cellulose slurry. After washing the column, the pH of effluent and influent was checked, whether they were the

same. Dialysed glucoamylase protein fraction was applied onto the surface of DEAE cellulose. The fractions of 5 mL each were collected at a flow rate 40 mL/hr so as to get good resolution. The Tris - HCl 0.25 M KCl buffer was used to elute enzyme. The protein estimation of each fraction was carried out with U.V. spectrophotometer at 280 nm. The glucoamylase activity of fractions was determined using 3,5 dinitro salicylic acid reagent method. The profile of DEAE cellulose column was obtained by plotting Fig. 1.

The fractions from DEAE cellulose column chromatography, containing highest glucoamylase activity were pooled and dialysed against distilled water. The resulting enzyme solution was lyophilized and stored in freeze. The poly acrylamide gel (Bio gel P-100) employed for gel chromatography. The swollen gel beads were suspended in 0.1 M potassium acetate 0.2 M KCl pH 4.2 and equilibriated, deareated and filled in a LKB column of size 70 x 1.6 cm. The flow rate was adjusted to 12 mL/hr. The protein solution of lyophilized powder in acetate buffer was loaded. After loading 1 mL fractions were collected at flow rate 12 mL/hr. Protein content in each fraction was estimated by Lowry technique, glucoamylase activity of fractions was determined by 3,5 dinitro salicylic acid reagent method. The elution profile was obtained Fig. 2.

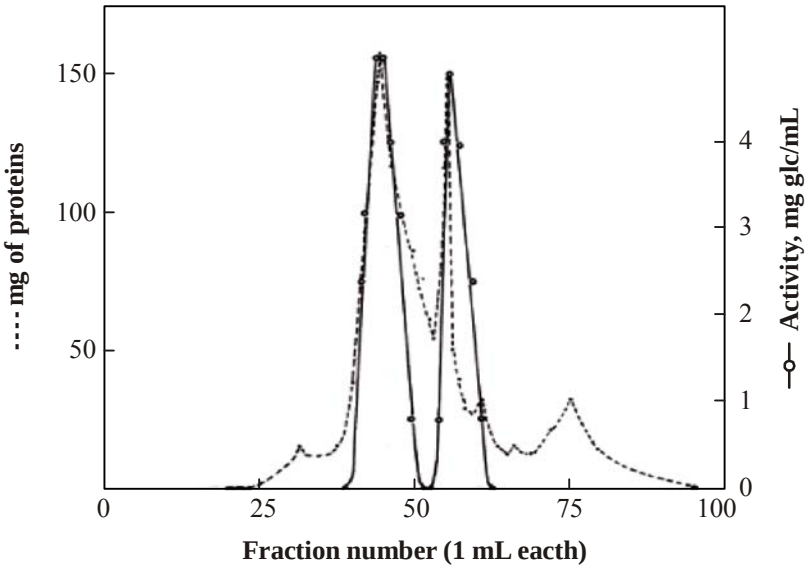

Fig. 2: Enzyme solution 0.8 mL column size 70 x 16 cm, equilibration and elution with 0.1 M pot acetate butter pH 4.2, flow rate 12 mL/hr.

The purification is calculated as ratio of specific activity at each step relative to that of the homogenate fraction, Table 1.

Table 1: Purification of human liver glucoamylase

Fraction	Protein conc. mg/mL	Total protein mg	Specific activity	Purification
Homogenate	15.00	15,000	0.029	-
Extract	14.00	11,200	0.10	3
Ammonium sulphate fractionation dialysed	8.7	609	0.252	8.689
DEAE cellulose pool	0.733	47.64	10.91	376.2
Lyophilized & redissolved	0.52	1.04	51.53	1777.0
Gel filtration bio-gel P-100				
Peak I	0.146	0.146	35.6	1227.5
Peak II	0.118	0.118	40.6	1400.0

The 0.25 M Glycine NaOH buffer pH 8.5 and TEMED were mixed. 28% acrylamide solution in water and N-N′ methylene bis acrylamide water solution were prepared and mixed. The polymerization is initiated by addition of pinch of ammo. persulfate. The resulting solution was poured in gel tubes of 9.5 X O.5 cm, all operations are carried out at room temperature. The sample was prepared in 50% glycerol, Bromophenol blue was added to one of tubes as tracking dye. The electrophoresis was carried at constant current 3 mA/tube till tracking dye reaches the bottom of tubes. The gels are removed and stained with 0.25% amido black. Then destaining is done in 7% glacial acetic acid prepared in 7% ethanol, washings are carried out till protein bands are clearly visible. The position of bands with respective peak fractions as shown in Fig. 3.

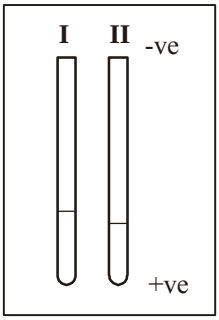

Fig. 3: Electrophoresis in poly acrylamide gel of human liver glucoamylase 100 μL, Run carried out at pH 8.5, 3 m.A. per tube, 2.5 hr at room temp.

Immobilization of enzyme: Free human liver glucoamylase is immobilized by simple method, on cynogen bromide activated agaropectin. Agar is fractionated to agarose and agaropectin. 2-4% agar in 10 M urea treated with alcohol at 55^{o}C, using mechanical stirring for few hours. The over-night solution centrifuged volume made upto 100% with alcohol. Agaropectin is washed with water to neutral pH. Immobilisation is carried out at 4^{o}C. The pH of agaropectin suspension is maintained at 11.2 during the addition of CNBr and later by alkali solution. After few hours refrigeration agropectin is washed to remove excess of CNBr till pH comes to neutral. The activated agaropectin was mixed with enzyme solution, the mixture stirred for few hours and supernatant is checked for the absence of protein and enzyme activity.

Characterization

The various parameters were studied and dependence enzyme with respect to pH, temperature, substrate concentration were determined in free and immobilized enzyme form. pH: 0.1 M potassium acetate, 0.25 M KCl buffer pH 3 to 6, phosphate buffer pH 5 to 8 were used for solutions with different pH values. Assays were carried by 3,5 dinitro salicylic reagent method; plots of pH against enzyme activity were shown in Fig. 4. Dependance of glucoamylase activity on temperature: The reaction mixture contains the buffers, enzymes, substrate similar pH dependence assay. The difference was only in the temperatures of each tube incubation period. The temperature was in range of 10 to 70^{o}C. Dependance of glucoamylase activity on substrate concentration : 2% stock substrate solution is used. The concentration of starch in increasing order was present in reaction mixtures, volumes were adjusted with buffer. Liberated glucose assay done with 3,5 dinitro salicylic method. Dependance of glucoamylase activity on drugs: The activity was altered by the presence of toxic compounds. In the present investigation the drugs used are Penicillin and Streptomycin. The enzyme was pre-incubated with 60 micro gram drug, then it was mixed with increasing concentration of substrate and reaction was carried out under standard conditions assay. The product formed was estimated using 3,5 dinitro salicylic acid reagent. The same experiment was carried out with drug concentration of 120 micro gram and results were displayed in Fig. 5.

RESULTS AND DISCUSSION

Human liver glucoamylase was purified to more than thousand folds by using simple techniques like ammo. sulfate fractionation. Ion-exchange chromatography with DEAE cellulose, further purification was achieved with Gel filtration onto Bio gel P-100. Molecular sieving on Bio gel P-100 resolved the enzyme into two activity peaks. The PAGE supported the presence of iso-enzymes. It is evident from Table 1 that two iso-enzymes show lower

specific activity when separated from each other on gel filtration, while combined specific activity of both the isoenzymes in the previous purification step is much higher. It refers that combination of the isoenzymes of glucoamylase was more efficient than the separated isoenzymes. The two glucoamylases from P. oxalicum were observed by Yamasaki et al.[6] Similar results were observed with pig liver glucoamylase by Matsui and Chiba[7]. Glucoamylase from rat liver was isolated and characterized by Stetton[9]. Rosenfield and Ushakova[10] isolated α-amylase free glucoamylase from rat liver, the enzyme showed maximum activity at pH 4.5 and had a molecular weight 90,000 daltons. The enzyme of smilar activity was isolated and purified from rat liver by Jeffrey et al.[11] displaying the maximum activity at pH 3.7 for maltose, pH 4.2 for isomaltose, pH 4.4 for glycogen. The glucoamylase from cat liver displaying maximum activity at pH 4.5 and molecular weight of 107,000[12].

In present study, it has been observed that pH optima of free and immobilized enzyme were 4.4 and 5.6 as in Fig. 4.

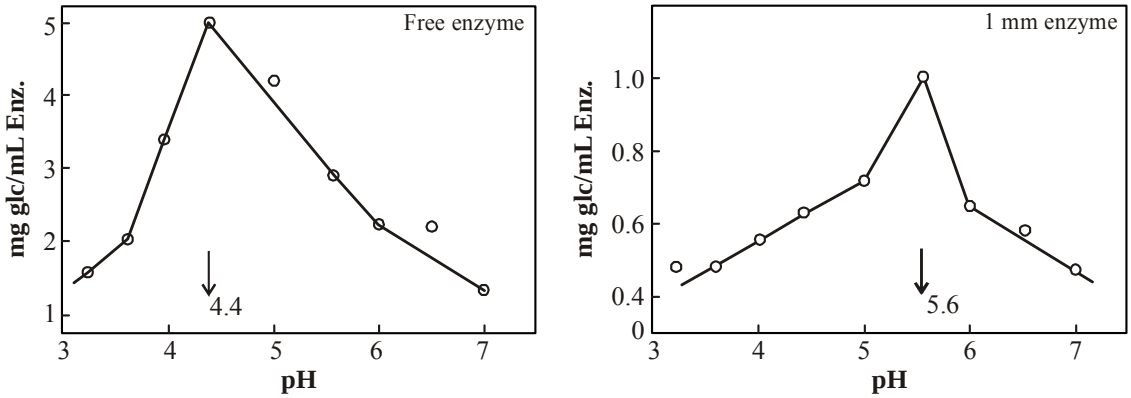

Fig. 4: Glucoamylase activity —o— mg glc./mL Enz. at 37°C/20', pot. Acetate butter pH 4.2

After immobilization pH dependence shifted to a higher value. The shift is belived to be due to the aggregation of the anions at the centre of the immobilized molecule. The nature of curve indicates that the enzyme was sensitive to pH. Temperature effects several factors that alter the velocity of enzymic reaction, like stability, actual velocity of breakdown complex, substrate enzyme affinity, pH function of any or all the components. The optimum activity of human liver glucoamylase was observed at 50°C and of immobilized enzyme at 45°C. The values of activation energy of free and immobilized enzyme observed were 1176.4, 515.34 and 1517.2, 761, respectively. The results are summarized in Table 2.

Table 2: Characterization of human liver glucoamylase

S. No.	Parameters	Free enzyme	Immobilized enzyme
1	Temperature (^{o}C)	50	45
2	Activation energy in calories/mole	1176.4 & 615.34	1517.2 & 761.0
3	pH	4.4	5.6
4	Km (mg/mL)		
	(i) Starch	26.4	15.4
	(ii) Maltose	11.4	16.6

Rao et al.[8] calculated the enery of activation from Arrhenius plot as 61.5 KJ/mole. The enzyme was found to be themostable. The Km characteristics to free and immobilized human liver glucoamylase enyzme toward starch 26.6 g/mL 15.4 mg/mL; towards maltose 11.4 mg/mL & 16.6 mg/mL, respectively. The lowering of Km value on immobilized enzyme indicates the increase in affinity to substrate. The effect of penicillin on human liver glucoamylase is shown in Fig. 5.

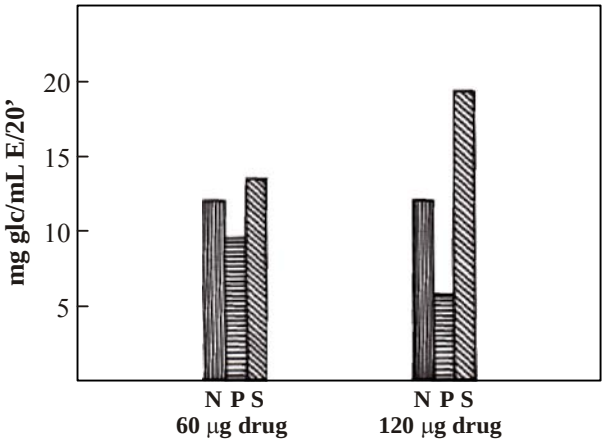

Fig. 5: Effect of drugs on human liver glycoamylase

N – Normal activity column; P – Penicillin affected activity column;
S – Streptomycin affected activity column

The drug showed the increasing appearant Km of the reaction therefore, it would be a competitive inhibition. It is of interest to note that though penicillin does not have a proper structural similarity to substrate, the kinetics indicate a competitive inhibition, which may be due to the non-covalent ineeteracton of penicillin with one of the amino acids involved in the

active center of the enzyme. The effect of streptomycin on human liver glucoamylase is shown in Fig. 5. It was observed that the velocity of glucoamylase reaction increased with decrease of Km. The streptomycin would be a activator enzyme activity. The action of these two drugs on human liver glucoamylase is of great importance since both drugs are widely used in the treatment of various diseases. The limitation of this investigation, does not pursue studies *in vivo*, the inhibiting or activating effect of these antibiotics on human liver glcoamylase especially in patients suffering from any of the diseases of glycogen metabolism could lead to serious clinical manifestations.

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REFERENCES

1. C. Keele and E. Neil, Samson Wright′ s Applied Physiology, EL 135, 11th Edn. (1965) p. 389.
2. D. J. Candy, Biological Functions of Carbohydrates, Blackie-Glasgow (1980) pp. 113-115.
3. L. L. Philips and M. L. Codwell, J. Am. Chem. Soc., **73**, 3559-3563 (1951).
4. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randal, Protein Measurement with Folin Phenol Reagent, J. Biol. Chem., **195**, 265 (1951).
5. G. Noel Ting and P. Bernfield, Assay conditions for α- and β-Amylase Methods in Enzymology, **Vol. I** (1988) p. 149.
6. Y. Yamasaki, Y. Suzuki and J. Ozawa, Purification and Properties of Two Forms of Glucoamylase from Penicillium Oxalicum, Agric. Biol. Chem., **41(5)**, 755-762 (1977).

7. H. Matsui and S. Chiba, Purification and Some Properties of Pig Liver Acid α-Glucosidlase, Agric. Biol. Chem., **47(4)**, 707-713 (1983).

8. V. Baseweswara Rao, N. V. S. Sastri and N. V. Subba Rao, Purificayio and Characterisation of a Thermolabile Glucoamylase from the Thermophilic Fungus Theromtces Lanuginous, Biochem. J., **193**, 379-387 (1981).

9. M. Stetton, Liver Glucoamylase Extraction, S. Am. Chem. Soc., **81**, 1437-1441 (1959).

10. E. L. Rosenfield and N. A. Ushakova, Purification of Rat Liver Neutral γ-γamylase, Biokhimia, **31(5)**, 1001-1006 (1966).

11. P. Jeffrey, D. H. Brown and B. I. Brown, Studies of Lysosomal α-Glucosidase I Purification and Properties of Rat Liver Enzyme, Biochemistry, **9(6)**, 1403-1415 (1970).

12. T. Tomako, N. Inokuchi and M. Irie, Purification and Characterisation of Glucoamylase from A. Saitoi, Bio. Ceh. Tokyo, **89(1)**, 125-134 (1981).

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