Short Communication

STUDY OF ALKALINE PHOSPHATASE FROM HUMAN HYDATIDIFORM MOLE

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ABSTRACT

Objective: Study was performed on purification of alkaline phosphatase from Hydatiform mole.

Methodology: The sample of mole Hydatidiform subjects from Taleghani Hospital, Tehran, Iran was studied. An Alkaline phosphatase from Human Hydatidiform mole was purified by a protocol involving solubilization using precipitation by butanol, acetone, Ammonium sulphate, Sephadex G_{200}, Ion exchange chromatography and preparative electrophoresis .

Results: The enzyme was purified 800-fold to apparent homogeneity. This enzyme has 5.2% carbohyrate content. The optimum Temperature and pH were 40 centigrade degree and 10.4, respectively.

Conclusion: Human Hydatidiform mole alkaline phosphatase is a novel alkaline phosphatase.

KEY WORDS: Alkaline Phosphatase, Hydatidiform mole, Ion exchange.

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INTRODUCTION

Alkaline Phosphatase is synthesized by Kidney, liver, bone, Intestine and Placenta.[1,2] Alkaline Phosphatase, at high levels of activity, has long been associated with calcifying tissues; however, its role in the calcification process remains unclear.[3] Liver, bone and Intestine Alkaline Phosphatase are the isoenzymes of Ap most commonly found in serum. Human Alkaline Phosphatase (hpALP EC: 3.1.3.1), an isoenzyme of the Alkaline Phosphatase (AP) group of enzymes, ordinary is synthesized in the placenta.[4] For alkaline phosphatase synthetic substrate p-nitrophenyl phosphate is used as substrate.[1]

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METHODOLOGY

Acrylamide, glycine (Hayashi Pure Chemical Ind. Ltd., Japan); N, N^{l}-Methylene bisacrylamide and N, N, N^{l}, N^{l}-tetramethylethylene diamine (Eastman Organic Chemicals, New York); Sodium dodecylsulphate, ß-mercaptoethanol, ß-Alanine, Polyethylene glycol 6000 (Sisco); CEoomassie Brilliant blue R-250, Sephadex G_{200}, Bio-gel P-100 and Blue dextran 2000, CM-cellulose, (Pharmacia Fine Chemicals, Sweden); Sodium azide (Reidel, Germany); Ammonium per-sulphate (May and Baker Ltd., England); Ethylenediamine tetra acetic acid (E. Merck, Germany); Bromophenol blue.

Purification procedures in all operations were carried out at 4°C. The buffer used in the purification procedures was tris buffer (pH 10.5, 0.025 M).

Step I: 100gr of Hydatidiform mole washed and was homogenized in a mixer with saline (0.85% NACl), the homogenate was then mixed with 1-butanol (10ml/50ml of homogenate); the mixture was stirred for one hour at 4°C and the butanol layer was removed by centrifugation at 12.080g. The treatment was repeated three / four times to ensure complete removal of lipids and pigments.[6]

Step II: The homogenate was mixed with equal amount of cold acetone 0°C under stirring. The resulting precipitate was collected by centrifugation at 12.080g. The precipitate was extracted with same saline (100ml), with stirring for 5-6 hours at 4°C. The suspension was centrifuged at (12,080g) at 4°C, the supernatant was collected and the residue was re-extracted with saline as before.[7]

Step III: The combined supernatants were pooled and subjected to fractional precipitation with ammonium sulfate, essentially according to the procedure of Agrawal and Goldstein.[8] The protein fraction precipitated between 30%-80% ammonium sulfate saturation was collected by centrifugation at 4°C, dissolved in minimum amount of distilled water, extensively dialyzed against distilled water and finally against saline I. The dialyzed protein solution was centrifuged 12,080g at 4°C and the clear solution containing glycosidase and phosphatase activities (Fraction A) was preserved at -20°C.

Step IV: Gel filtration of Fraction A on Sephadex G_{200}. Fraction A (»20mg protein) was dissolved in 5ml of tris buffer (pH 10.5, 0.1 M) and loaded on a column of Sephadex G_{200} (2.5×27cm) equilibrated in the same buffer and fractions of 3ml were collected. The fractions were analyzed for protein as well as for enzyme activities. The fractions rich in enzyme activities were pooled together for next step.[9]

Step-V: Ion exchange chromatography of Gel filtration fraction on DEAE cellulose. Alkaline Phosphatase from wash off fraction was further purified by ion exchange chromatography on DEAE cellulose column. Enzyme was eluted with discontinuous gradient of NaCl (0.01-1 M.).[6]

Step VI: Preparative Electrophoretic analysis. The enzymatically active pools from before step of purification analysed by the PAGE system of Laemmli.[10] The electrophoresis was performed using two parallel continuous 7.5% gels at 4°C. After an electrophoretic run, one gel was stained for protein with Coomassie Brilliant Blue.[5,11]

Protein Concentration and Carbohydrate Content: Protein was quantified by the method of Lowry et al with bovine serum albumin as standard.[12] Protein was also measured by absorbance at 280nm assuming that A_{280} = 1.0 corresponds to 1mg/ml.[13] Phenol sulphuric acid method was used for determination of carbohydrate using glucose as standard.[14]

Estimation of Alkaline phosphatase activity: 200ml of p-nitrophenyl phosphate disodium salt solution (2mM) was incubated with an enzyme solution in the presence of tris buffer (pH 10.5, 0.1 M) in a total volume of 500ml at 30°C+/- 0.1. The reaction was arrested after suitable time period by addition of 2.5ml borate buffer (pH 9.5, 0.1 M). The liberated p-nitrophenol was monitored spectrophotometrically at 405nm.[6]

Influence of pH on enzyme activities: 100µl of enzyme (≈ 10 units) was incubated with 200µl of tris buffer of pH ranging between pH 3.0 to pH 6.0 for 1 hour and 200µl p-nitrophenyl substrates were added. Reaction was arrested after suitable time period by addition of 2.5ml of borate buffer (pH 9.5, 0.1M). The released p-nitrophenol was estimated at 405nm.[6]

Influence of temperature on enzyme activities: 100ìl of enzyme was incubated with 200µl of tris buffer of optimum pH at different temperatures ranging between 0°C -100°C for 30 min. 200µl of substrate was added. The reaction was arrested after suitable time period by addition of borate buffer (pH 9.5, 0.1M) and released p-nitrophenol was monitored at 405nm.[6]

Table-I: Purification of Human Hydatidiform mole

Purification step	*Total Protein mg*	*Total units*	*Specific activity (u/mg)*	*Purification Fold*
Saline Extract	10000	3280	0.33	1
Butanol extract	702	3021	4.3	13
Sephadex G 200 Chromatography	494	2918	5.9	17.86
Ammonium sulphate	193	2345	12.1	36.6
Acetone precipitate	116	1859	15.9	48.18
DEAE Cellulose Chromatography	7.14	183	25.6	76.8
Preparative electrophorosis	0.52	105.8	201.9	611.8

RESULTS

The first purification step of the crude preparation by gel permeation chromatography gave three peaks. The enzymatic activity of the three peaks was assayed, as well as that of the fraction-A preparation. The fraction-A has prepared from saline extract when subjected to preparative gel filtration on sephadex G_{200}. However high fold increase in activity was observed (Table-I). Enzyme activity containing fractions were pooled and used for further purification. Chromatography profile of reach fractions of enzyme of sephadex G_{200} on DEAE cellulose column, Alkaline phosphatase could be eluted from the column by gradient elution with NaCl concentration gradient between 0.01 and 0.1M was able to separate Alkaline phosphatase. Preparative electrophoresis was chosen as the next step of purification. Alkaline phosphatase and other enzymes could be eluted from the column of gel electrophoresis was able to separate alkaline phosphatase and other enzymes (Figure-1). The purity and homogeneity of the purified enzyme was checked by electrophoresis in native PAGE (pH 8.3). Alkaline phosphatase was further subjected to gel filtration on sephadex G_{200} when a single peak was obtained for enzyme indicating homogeneity. Enzyme contains 5.2% carbohydrate. With ñ-nitrophenyl phosphate in tris buffer as substrate alkaline phosphatase shows maximum activity at pH 10.4 and optimum temperature 40°C.

Figure-1: 1 Page (pH = 8.3) profile of Native Electrophoresis purified Alkaline phosphatase from Human Hydatidiform mole.

DISCUSSION

Human Hydatidiform mole alkaline phosphatase has special characterization namely optimum temperature and pH, carbohydrate contain that these characterization are difference with another alkaline phosphatase.

CONCLUSION

Human Hydatidiform mole alkaline phosphatase is a novel alkaline phosphatase.

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