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Study of β-glucuronidase from Human Seminal Plasma: Purification and Properties[1)]

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—국문초록—

사람의 정액에 베타-글룩유로니다아제의 정제 및 그 성질에 관한 연구

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암모니움 썰페이트 분획분리법. 초미량여과. DEAE-셀루로오즈 및 젤 여과 크로마토그라피법 등을 사용하여 사람의 정액에서 베타 글룩유로니다아제를 분리하였다. 이 효소는 pH4.4와 온도 50 ~ 54°C에서 최고의 활동도를 나타내었다. 파라-나이트로페닐-디-글룩유로나이드를 기질로 사용하였을때 Km치는 0.59 mM이었다. 또한 이 효소는 수은이온, 구리이온, 은이온에 의해 활동도가 방해되고 반면에 망간이온 및 코발트 이온에 의해서 효소의 활동도가 증가되었다. 세파댁스 G-200 젤여과법에 의해 측정된 사람의 정액 베타글룩유로니다아제의 분자량은 400,000이었다. 이렇게 정제된 효소는 수정과정 중에서 카페시태시션의 연구에 도움을 주리라고 본다.

INTRODUCTION

In many tissues (de Duve et al, 1955) β-Glucuronidase is localized in microsomes, lysosomes and mitochondria (Dean R.T, 1974). This enzyme catalyzes in vitro the hydrolysis of the β-D-glycosidic bond of certain glucosiduronic acids (Musa, 1965) and a glucuronly transfer reaction (Fishman, 1967). It is known as a glycoprotein with multiple forms in bovine liver (Plapp & Cole, 1966, 1967), in cat liver lysosomes (Stahl & Touster, 1971), in rabbit liver (Dean, 1971) and in Mouse kidney (Swank & Ganschow, 1977).

Cook (1973) suggested that the precursor proteins of many lysosomal enzymes are glycosylated through Golgi Apparatus. Paigan (1961) had observed that a single structural gene for β-Glucuronidase was in the extract lysosomes and microsomes.

To elucidate the complex biochemical reactions in the process of mamalian fertilization, it is obviously essential to have purified enzyme. This enzyme identified in the seminal plasma preparation.

1) 이 연구는 1982년도 문교부 학술연구비 보조에 의해 수행되었음.

The work reported in the project is aimed at purification and characterization of β-Glucuronidase from human semen.

MATERIALS AND METHODS

(1) Materials

Human Semen obtained from Seoul National University Hospital, Korea University Medical Center and Catholic Medical School Hospital were used. It was stored at -70°C until used.

α-Nitrophenyl- β-D- glucuronides were purchased from Sigma Chemical Co.; Sephadex G-200 in bead form (40 to 120um) were from Pharmacia Fine Chemicals; DEAE-Cellulose were obtained from Sigma Chemical Co.

All other chemicals were of the highest purify available methods.

Protein concentration was determined by the method of Lowry et al (1951), with bovine semen Albumin as standard. The determination of β-glucuronidase activity was a modification of the method of Harris et al (1977) utilizing hydrolysis of α-nitrophenyl-β-D-Glucuronides as substrate in 0.2 M Acetate buffer at pH 4.4.

The assay was carried out in a total volume of 1 ml containing 0.5ml of substrate (2 10 M, pH 4.4.), 0.3ml of 0.2 M sodium acetate buffer pH 4.4. with 0.2m NaCl and 0.2ml of enzyme solution.

After Incubation in a water bath for 2hr at 37°C, the reaction was terminated by the addition of 0.5ml of 2M Glycine buffer pH 10.4 with 0.2 M NaCl, and the color developed was measured at 400 nm. One unit of the activity equals to the amount of enzyme which releases 1 μmol of α-nitrophenyl per min at 37°C under above conditions.

*** Other Enzyme Assay**

N-Acetyl glucosaminidase activity was monitored using α-nitrophenyl-N-Acetyl-D-Glucosaminide by the method of Tacentino and Maley, 1971. Arylsulfatase assay was carried out by using dipotassium 2-hydroxy-5-nitrophenyl sulfate by the method of Yang and Srivastava, 1976.

*** Step I. DEAE-Cellulose Chromatography**

The freeze-dried Seminal Plasma was dissolved on 0.05 M Tris-HCl buffer pH 7.2 and applied to the preequilibrated DEAE-Cellulose Column (3x16cm). The Column was washed with 360ml of 0.05 M Tris-HCl (pH 7.2) buffer and then with stepwise gradient of 0.1 M to 0.5 M NaCl were a applied at the rate of 45ml/hr.

The large protein peak showing β-Glucuronidase activity at about 0.1 M Nacl was pooled, dialyzed against 0.02 M sodium phosphate buffer pH 7 and concentrated to about 6ml with an Amicon Ultrafiltration Cell using a PM-10 filter. The specific activity was increased a 1.43 fold with high yield.

*** Step II. Gel filtration on Sephadex G-200**

The concentration fractions from DEAE-Cellulose Chromatography were applied to a Sephadex G-200 Column (1.7 x 30cm) equilibrated with 0.02 M Sodium phosphate buffer pH 7.2 at 4°C, and eluted with the same buffer with 140ml at the rate of 7.6ml/hr.

This enzyme was eluted as a single peak before the bulk of the protein comes. β-Glucuronidase acitive fractions was collected and concentrated to about 5.5ml. This step provides 2.65 fold increase in specific activity with an 55% yield. The enzyme was stored -20°C in the refrigerator.

*** Step III. DEAE-Cellulose Rechromatography**

The concentrated product from sephadex G-200 Chromatography was applied to a Column (3 x 18.5cm) which was equilibrated with 0.02 M Sodium phosphate buffer pH 7.2. The Column was developed with continuous gradient of

0-0.2 M Nacl to elute the enzyme.

β-Glucuronidase activity was present int the first protein peak, pooled, concentrated and stored at -20°C.

RESULTS

Step I. Preparation of Seminal Plasma and Ammonium Sulfate fractionation.

The freezed Human semen was centrifuged at 5,000 x g for 15min. The pooled supernant fraction was dialyzed against 0.04 M Sodium phosphate buffer pH 7.2. To the dialyzed supernant solution ("Seminal Plasma") was added 3% (12,000 x g) to 65% ammonium sulfate. For 30 min, the solution was centrifuged to yield a precipitate which contained the enzyme activity. The pellet was dissolved in a minimum

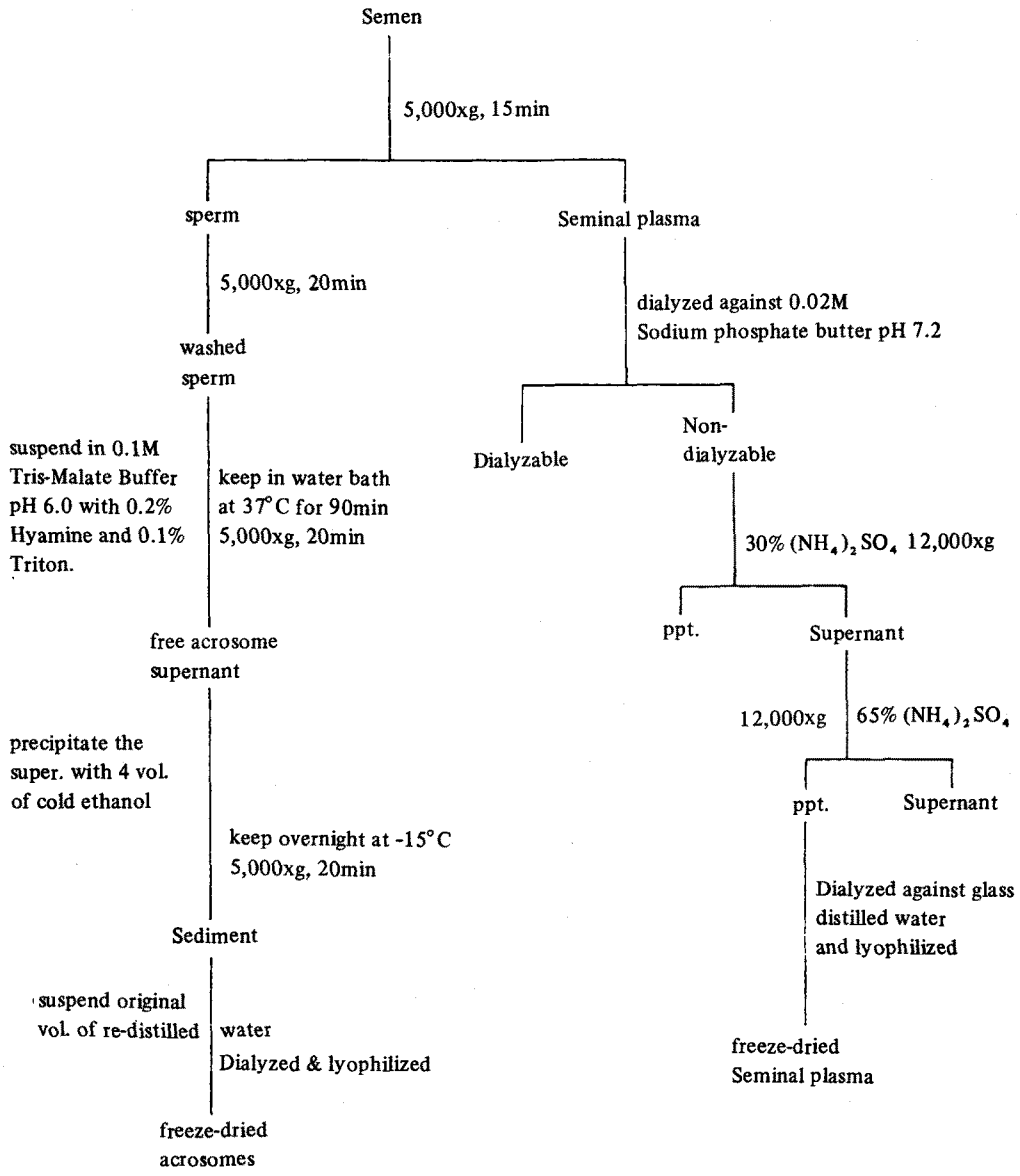

Fig. 1. Preparation of Extract for Chromatography.

amount of glass distilled water, and dialyzed extensively against the glass distilled water overnight at 4°C. The non-dialyzable material was freeze-dried and kept at -20°C. The specific activity of this crude enzyme was 0.6 mUnits/mg of protein. (Figure 1)

Step II. DEAE-Cellulose Chromatography

As shown in Figure 2. Several well separated protein peaks were obtained using a DEAE-Cellulose Column which is developed with 0.05 M Sodium Phosphate buffer pH 7.2 and with a stepwise Gradient of 0-0.5 M NaCl. β-Glucuronidase active fraction was collected, and appeared 0.56 mUnits/mg protein. (Figure 2)

Step III. Sephadex G-200 Chromatography

The concentrated enzyme was applied to the pre-equilibrated Column. β-Glucuronidase fraction was collected, and showed 1.57 mUnits/mg protein. (Figure 3)

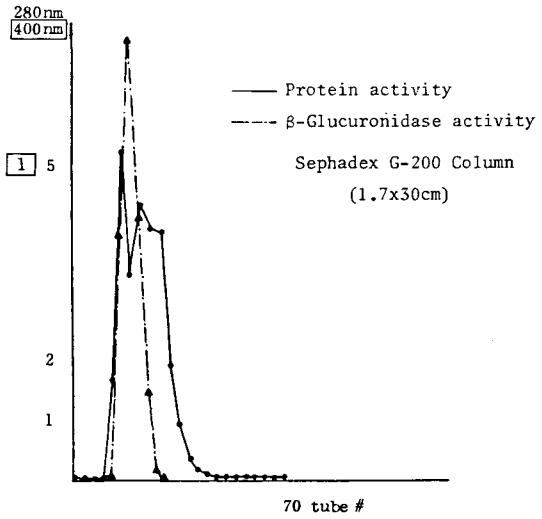

Fig. 3. Purification of β-Glucuronidase by Sephadex G-200 gel filtration. 6ml of sample solution in 0.02 M Na-phosphate buffer pH 7.2 was applied to the Sephadex G-200 Column (1.7 x 30cm) and eluted with the same buffer at the flow of 0.5ml/min. Fractions (2.0ml) were collected.

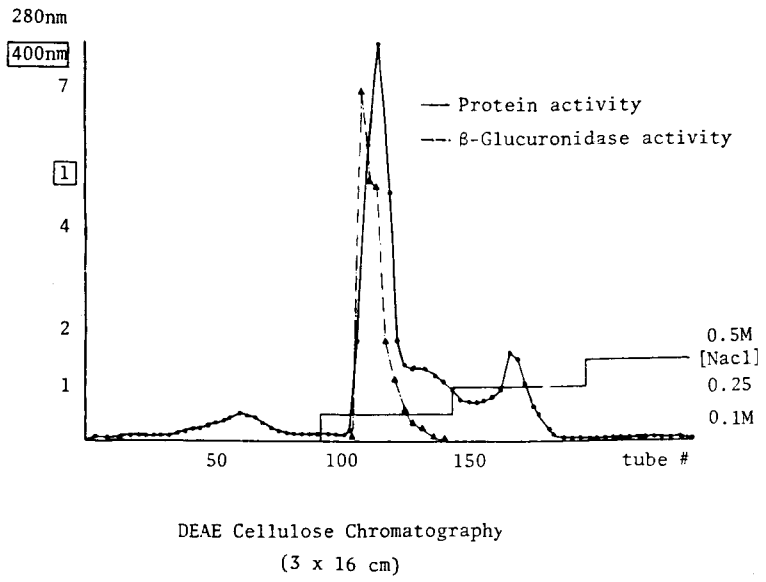

Fig. 2. Elution pattern of β-Glucuroidase from a DEAE-Cellulose Column (3 x 16cm). After washing using 360ml of the same buffer with a stepwise gradient of 0.1M to 0.5 M NaCl fractions of 4ml were collected.

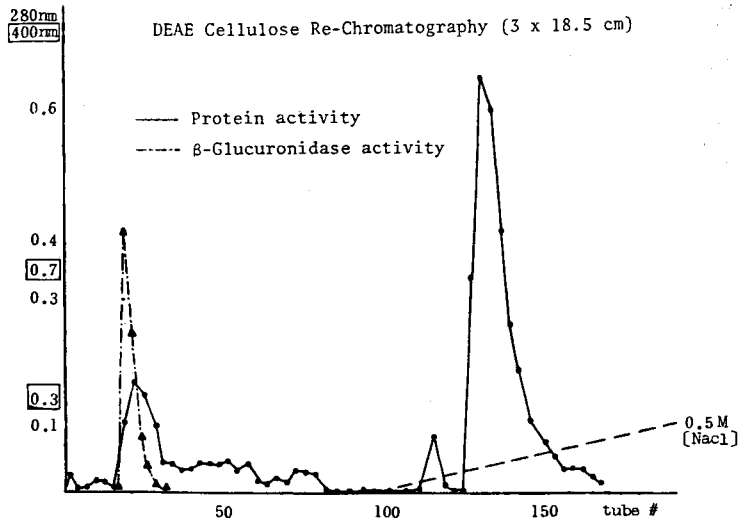

Fig. 4. Rechromatography on a DEAE-Cellulose Column (3 x 18.5 cm) Fractions of 3ml were collected.
The enzyme Unit is defined as micrograms of α-Nitrophenol released per hour at 37°C.

Step IV. DEAE-Cellulose Rechromatography

6ml of ultrafiltrated β-Glucuronidase fraction was applied to the pre-equilibrated Column. β-Glucuronidase activity was estimated to be 104.9 mUnits/mg protein. The Yield of this step was 41.3%. (Figure 4)

Step. V. Disc gel electrophoresis

The Degree of purity was tested by Disc gel electrophoreisis by the method of Brewer et al (1969). The finally obtained enzyme still showed several major components. The enzyme was partially purified. (Table I).

Table I. Summary of Purification of Human Seminal Plasma β-Glucuronidase

Step	Total mUnits	Protein (mg/ml)	Specific activity	Purification (fold)	Yield (%)
Lyophilized seminal plasma	300	20	0.6	1	100
Step I DEAE-Cellulose	293	4.5	0.86	1.43	97.7
Step II Sephadex G-200	89	2.57	1.57	2.62	55.6
Step III DEAE-Cellulose Re-chromatography	66	0.03	104.7	175	41.3

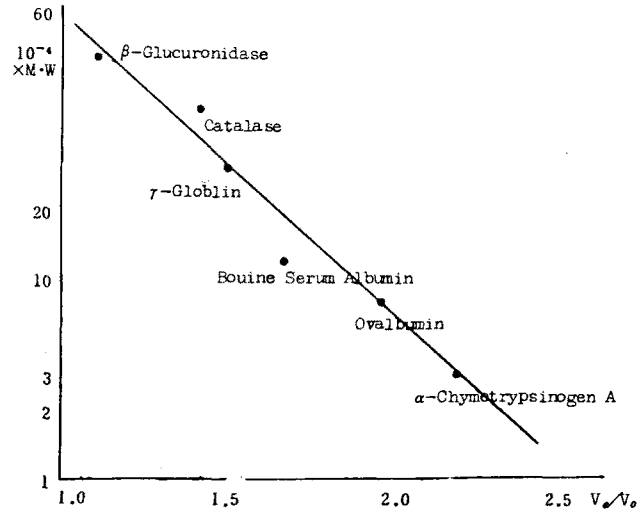

Fig. 5. Void volume were determined with Blue dextran 2000 Fractions of 2.2ml were collected. Molecular weight of β-Glucuronidase by Sephadex G-200 Chromatography

Step VI. Molecular weight estimation by gel filtration on Sephadex G-200

The Molecular weight of β-Glucuronidase was estimated by Sephadex G-200 Column (2.1 x 86.5cm). The eluting buffer was 0.05 M Tris-HCl buffer pH 7.2 with 0.5 NaCl, and the flow rate was 6.5ml/hr using the same buffer. The void volume of the Column using Blue dextran 2,000 was 75.2ml. The protein standards were Catalase (250,000). γ-globulin (150,000), Bovine Serum Albumin (67,000). Ovalbumin (47,000) and Chymotrypsinogen A (25,000).

Protein concentration in the elute was measured by reading the absorbance at 280nm. The elution volumes of the standard proteins were plotted against the log molecular weight (Figure 5). The estimated molecular weight of human remained plasma β-glucuronidase was approx. 400,000.

Estimation of the Molecular weight of Human Seminal-plasma β-Glucuronidase on a Sephadex G-200 Column.

Properties of partially purified Human Seminal Plasma β-glucuronidase

*** Effect of substrate Concentration**

The effect of α-nitrophenyl β-D-Glucuronide as substrate on enzyme activity at pH 4.4 is shown in Figure 7.

The reaction appears to obey normal Michaelis-Menten kinetics and has a Km value of 0.59 mM. (Figure 6)

*** Effect of pH**

With higher substrate concentration (2 x 10^{-3} M), the single pH optimum was at pH 4.4. When assays are run with lower substrate concentration (2 x 10^{-4} M), partially purified β-Glucuronidase has shown to have double pH optima. (Figure 7)

*** Effect of temperature**

As shown in Figure 8, the enzyme is stable at

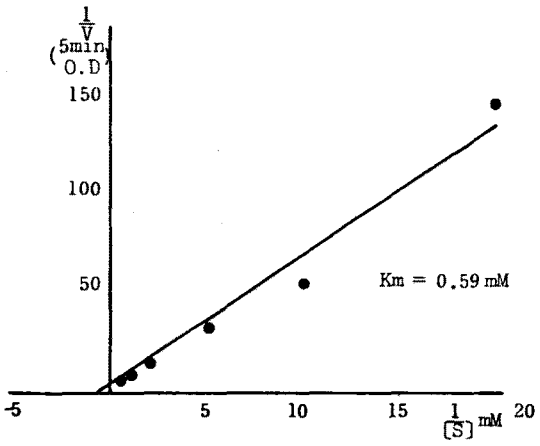

Fig. 6. Effect of substrate concentration on β-Glucuronidase activity. Km was computed from the Line weaver-Burk plot of 1/v versus 1/s. with substrate concentrations ranging from 5×10^{-5}M to 2×10^{-3}M.

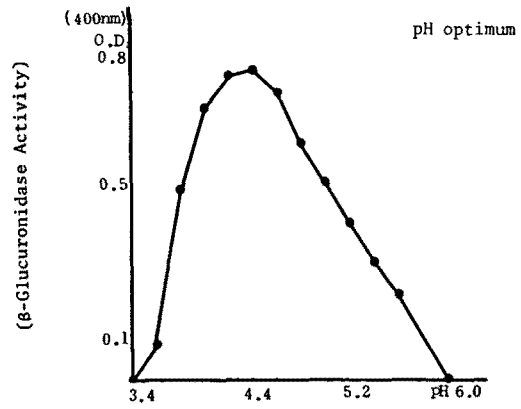

Fig. 7. Optimal pH for β-Glucuronidase activity. The enzyme activity was measured from around pH3 to 6 in 0.2 M sodium acetate buffer pH 4.4 containing 0.2 M NaCl The substrate concentrations were 2×10^{-3}M & 2×10^{-4}M.

60°C for 80 min. Under the standard assay conditions, α-nitrophenyl release was linear until 2hr at 37°C, β-Glucuronidase from Human Seminal Plasma showed temperature optimum at 50-54°C through all the pH and incubation time.

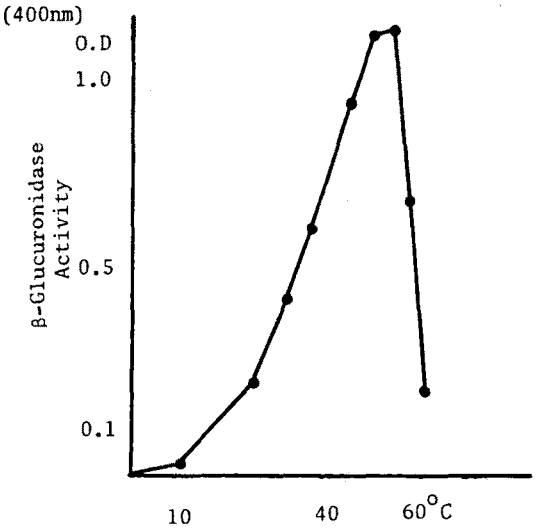

Fig. 8. β-Glucuronidase from Human Seminal plasma appears the maximum activity at 50-54°C.

DISCUSSION

The purification of β-Glucuronidase from bovine liver (Plapp & Cole, 1966), rat liver lysosomes (Stahl and Touster, 1971) and some other sources (Ohtsuka and Wakabayashi, 1970; preiss & Hilz, 1977) has been reported but the purification of Human Seminal Plasma Glucuronidase has not been previously reported. In our experiment the properties of the partially purified enzyme resembled those of that rat liver enzyme by Stahl & Touster (1971), and of the rabbit liver enzyme reported by Dean (1974). Our results showed a single pH optimum at pH 4.4 with 2×10^{-3}M, which is identical with the purified bovine liver enzyme (Plapp & Cole, 1966). The Km of enzyme was 0.59mM and this enzyme is highly stable at elavated temperature and extremes of pH. It can be concluded that β-Glucuronidase of Human Seminal Plasma is a tetramer as is the rat enzyme (Stahl & Touster 1971). Aggregates of β-Glucuronidase molecules were demonstrated in the electron microscope. The subunits were demons-

trated in the electron microscope. The subunits were demonstrated by variety of techniques involving dissociation or denaturation (Dean, 1974).

β-Glucuronidase from various sources is inhibited by low concentrations of mercuric, silver and cupric ions, Whilst being unaffected by anions such as fluoride, cyanide or iodoacetate (Fernley, 1962). Tappel et al (1967) also reported that Cholesterol and retinol give relatively high specific inhibition.

Multiple forms of β-Glucuronidase provide a continuing interest in enzyme kinetics, substate specifity, Glucuronyl transfer reactions and in various diseases.

In normal fertilization process, its role has not been clearly established although it may play an adjust role in penetration of the cumulus cell layer (Robert, 1974) and suggested that the conjugation of estrogenic hormone was involved. It is reported by Gwatkin (1971) that Hamster sperms are capacitated in vitro by β-Glucuronidase, whereas it is not required during the penetration period.

β-Glucuronidase from Human Seminal Plasma has pH optimum at pH 4.4. The maximum activity of human seminal plasma β-Glucuronidase showed that at 54°C The optimum activity at different Nacl concentrations was tested. Sodium chloride at the concentration of 0.3 M showed the highest activity of β-Glucuronidase.

Several different purification procedures were developed for obtaining highly active enzymes. To achieve purification of this enzyme, three techniques (DEAE-Cellulose, Sephadex G-200 on Gel filtration & DEAE-Rechromatography) are introduced. β-Glucuronidase exists in single form as determined by DEAE Column Chromatography and this is different from β-Glucuronidase of other source.

In this report β-Glucuronidase, abundant in human seminal plasma, was partially purified for the first time this source. Although its function still remains to be investigated, this enzyme have been postulated to be multifunctional.

SUMMARY

β-Glucuronidase from Human Seminal Plasma was partially purified by $(NH_4)_2SO_4$ fractionation, Chromatography on DEAE-Cellulose, Gel filtration on Sephadex G-200 and Rechromatography on DEAE-Cellulose. The partially purified enzyme showed one major band with some minor contaminant on Diso gel electrophoresis. This enzyme showed the maximum activity at pH 4.4 and optimum for its activity at 50-54°C. The Km value for α-Nitrophenyl-β-D-Glucuronide as substrate was 0.59mM. Hg^{2+}, Cu^{2+}, and Ag^{+} had inhibitory action for human Seminal Plasma β-Glucuronidase and Mn^{2+}, Co^{2+}, SO_2^{2-}, and PO_4^{2-} activated the enzyme activity.

According to the Gel filtr1tion on Sephadex G-200, the Molecular weight of Human Seminal Plasma β-Glucuronidase was approximately 400,000. The Purification and Characterization of this enzyme from Human Seminal Plasma will be useful for the stuides of the fertilization process.

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