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CHROMATOGRAPHIC ANALYSIS OF HUMAN SEMINAL ANGIOTENSIN I CONVERTING ENZYME ISOFORMS

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ABSTRACT

Separation of isoforms of angiotensin I converting enzyme (EC 3.4.15.1, ACE) from human semen was attempted by chromatographic procedures. The crude ACE was fractionated on DEAE-Sephadex A-25 and Sephadex G-200 columns. Two ACE components were separated out in the first DEAE-SEphadex A-25 chromatography. The two ACE preparations (S-I and S-II) were separately applied to gel filtration through a Sephadex G-200 column. S-I was further separated into two peaks. Elution of the first peak showed MW about 150 KD, while the second peak corresponded to MW about 100KD. S-II was eluted as a single peak at an elution position corresponding to MW aabout 150 KD. At least three ACE components were separated out from the seminal extract.

INTRODUCTION

Angiotensin I converting enzyme (ACE) is a dipeptidyl carboxypeptidase which releases dipeptides from carboxyl termini of many peptides. ACE is a metallo-peptidase that has a saccharide moiety in its molecule[1]. The ACEs in lung and kidney are considered to work as a converting factor of angiotensin I into vasopressive angiotensin II and an inactivating factor of vasodilative kinins [1,2]. The pulmonary and renal ACEs, therefore, are considered to be an important key enzyme in blood pressure regulation via renin-angiotensin and kallikrein-kinin systems.

It is well known that ACEs are also distributed in a great amount in male reproductive organs including testis, epididymis and prostate of various mammalian species[3-5]. In human, the genital ACE appears in ejaculated semen[3,6]. This prompted scientists in a field related to andrology to study relationship between seminal ACE content and pathophysiological status. Human seminal ACE, however, is regarded as a mixture of ACEs from multiple sex organs. Because, testes, epididymes and prostate which supply seminal components have been reported to contain ACE in human[5].

In this context, measurement of seminal ACE content was done for each seminal ACE component from a single organ to give some information for the pathophysio- logical assessment of that particular organ. For this purpose. Search for a technique to fractionate seminal ACE components and identify origins of each component is very helpful. In the present study, we have established a chromatographic fractionation method that can be used to separate three different ACE components from human seminal extract.

EXPERIMENTAL

Chemicals :

N^{α}-hippuryl-L-Leu (HHL) and hippuric acid was obtained from Peptide Institute Inc. (Osaka, Japan).

DEAE-Sephadex A-25 and Sephadex G-200 were obtained from Pharmacia Japan (Tokyo, Japan). Blue-cellulofine was a product from Selkagaku-kogyo Co. Ltd. (Tokyo,Japan). Other chemicals used in the present study were of extra pure analytical grade.

ACE assay :

Enzyme activity of ACE was measured by spectrophotometric assay using HHL[7] Two hundred µl of substrate solution (6.25 mM HHL in 125 mM borate-bicarbonate buffer, pH 8.3, containing I M NaCl) was incubated with 50 µl of sample solution for 30 min at 37°C. After enzyme reaction was stopped by adding 250 µl of 0.1 N HCl, hippuric acid which had been enzymatically splitted from HHL was extracted from the reaction mixture with ethyl acetate. After mixing the reaction mixture and ethyl acetate vigorously for 15 sec, the emulsion was subjected to centrifugation at 1.000 xg. Aliquot of the upper ethyl acetate phase was taken and evaporated to dryness in a glass tube settled in a boiling water. Residual materials was restored with distilled water and the absorbance of the solution was measured at λ 228 nm for determination of hippuric acid. After background value that had been obtained by the same procedure without incubating the enzyme. Amount of hippuric acid was calculated by subtracting the test from background. A calibration line was prepared with authentic hippuric acid at the sametime, of the assay.

Seminal specimen :

Human ejaculated semen (100-200 ml) was collected from healthy donors of normospermia by masturbation. Seminal samples were stored at -20°C until they were used for ACE extraction. The frozen stocked semen was thawed at room temperature and the procedures men- tioned below were carried out at 4°C. Sodium deoxycholate was added to the thawed semen to adjust its final concentration 1.25% (W/V). The seminal sample was gently stirred in a glassware for I h. The insoluble material including spermatozoa were removed by centrifugation at 10.000 xg for 1 h. The supernatent was dialyzed against 10 mM Tris-HCl buffer (pH 8.0). The insoluble material generated during dialysis was removed by centrifugation. Solid ammonium sulfate was gradually added to the supernatent to obtain 75% ammonium sulfate saturation. After being stirred for 1 h, precipitated material was collected by centrifugation and the pellet was dissolved in 10 mM Tris-HCl buffer (pH 8.0). After ammonium sulfate remaining in the sample was removed by dialysis against 10 mM Tris-HCl buffer (pH 8.3), the dialysate was applied to a blue-cellulofine column which had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0). Non-retained fraction was pooled and used as a starting material for further chromatographic analyses.

RESULTS AND DISCUSSION

Among the different body fluids, semen had been demonstrated to contain greater amount of ACE activity than blood plasma and urine. Table 1 shows contents of ACE activity (HHL hydrolyzing activity) in three different human body fluids. The results of chromatography of human semen on a DEAE-Sephadex A-25 column was shown in Fig. 1.

ACE was separated into two peaks, the first was eluted at conductivity of 10 mmho/cm and the second was eluted at conductivity of 15 mmho/cm by an elution method with a linear NaCl gradient. First and second ACE components (fractions shown by brackets were pooled) were marked as S-I and S-II, respectively. S-I and S-II were separately concentrated by ammonium sulfate 80% saturation.

In order to evaluate specificity of the HHL hydrolyzing activity (observed in the S-I and S-II samples), we examined effects of captopril, a specific ACE inhibitor on the enzyme activity. As shown in Fig. 2, either the HHL

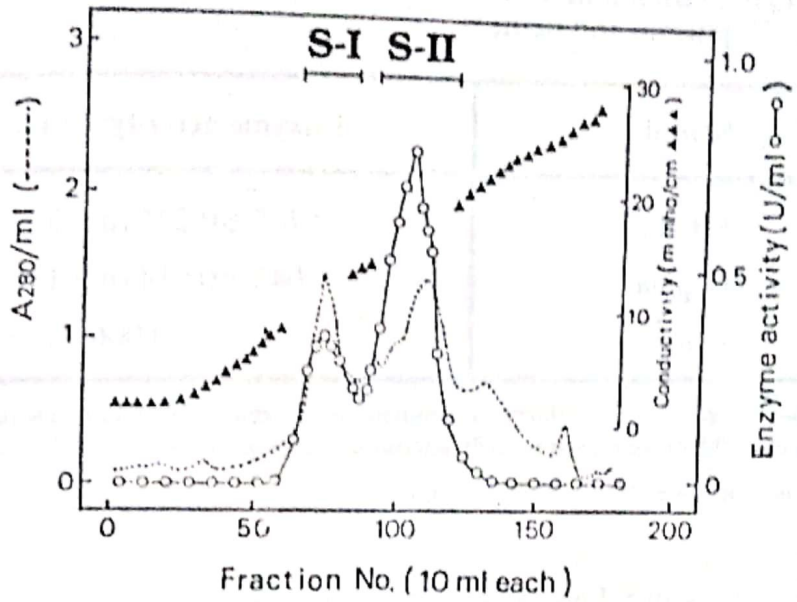

Fig. (1): DEAE-Sephadex A-25 chromato- graphy of human seminal ACE from Blue-cellulose chromatography.

A non-retained materials from a Blue-cellulose column was concentrated by salting out with ammonium sulfate. A half of the concentrated materials were applied to a DEAE-Sephadex A-25 column (2.6 x 36 cm) which had been equilibrated with 25 mM Tris/HCl buffer, pH 8.0. Afte the column was washed with 500 ml of the buffer, adsorbed materials were eluted by a linear gradient of NaCl (from 0M to 0.3 M). Fractions with each bracket was pooled as S-I or S-II.

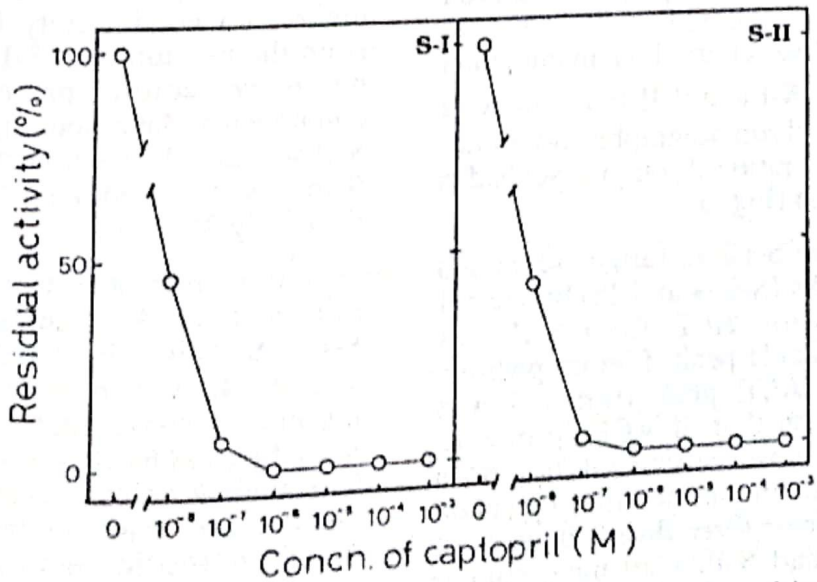

Fig. (2): Effect of captopril on the HHL hydrolyzing activity found in S-I and S-II fractions from human seminal ejaculate.

HHL hydrolyzing activity assay for S-I and S-II was carried out with or without captopril of various concentra- tions in order to evaluate the enzyme activity in these fractions as ACE. One mU of each enzyme was used in the assay.

Table (1): Contents of HHL hydrolyzing activity in human semen, plasma and urine.

Sample	Enzyme activity (μ/ml)
Semen	2.705 ± 0.275 (n = 3)*
Plasma	0.041 ± 0.014 (n = 13)**
Urine	< 0.006

* Value is mean ± S.D. of thtree determinations. Frozen stocked human semen of 100, 150 and 200 ml were used in each determination, respectively.

** Value is mean ± S.D. of 13 normal donors.

hydrolyzing activity in S-I and S-II were strongly inhibited by adding captopril in the assay medium. The activity was completely blocked at the captopril concentration of 10-6M. We observed a dose dependant relationship between residual HHL hydrolyzing activity and captopril in S-I and S-II fractions. This suggests that there was no possibility that the two fractions contain HHL hydrolyzing enzyme other than ACE. Furthermore, these results of the inhibitory effect of captopril observed here with the seminal ACEs were in paralel with that reported on mammalian (rat) ACE[8]. S-I and S-II fractions were subjected to chromatographic fractionation by gel filtration through a Sephadex G-200 column (Fig. 3).

ACE from S-I was further separated into two peaks (S-Ia and S-Ib) by the gel filtration, while ACE from S-II was eluted as a single peak. Elution position of the first ACE peak from S-I was coincided with that of ACE from S-II. From these observations, it has been revealed that human seminal ejaculate contains at least three different types of ACE. S-Ia and S-Ib may have similar electrostatic properties as they were eluted from the DEAE-Sephadex A-25 column at the same ion conductivity. However, they have different molecular sizes as they were separated by gel filtration through the Sephadex G-200 column. Elution positions of S-Ia and S-Ib were very similar to those that had been reported for rat pulmonary ACE (MW,150 kilodaltons) and testicular ACE (MW,100 kilodaltons), respectively[8] S-II, another ACE component from human seminal ejaculate was found to have similar molecule from S-Ia because it was separated from the S-I components by DEAE-Sephadex A-25 chromatography, suggesting that S-II has different electrostatic property. As S-II required higher ion conductivity for its elution from the column than S-I did, S-II may be more acidic protein than S-I components. An amount ratio of S-I and S-II was 26:74. A ratio between S-Ia and S-Ib was estimated to be 2.3:1 from peak areas (Fig.3).

From these observation, an amount ratio of three ACE components (S-Ia : S-Ib : S-II) was finally estimated to be 18:7.8:74. We repeated the similar chromatographic analyses three times with different batches of seminal sample. The amount ratios of each component in second and third analyses were very similar to that in the first experiment as described above (data are not shown). Human seminal ejaculate is a mixture of the secreta from different sex organs and accessory genital glands. It is well known that ACE is distributed in various male reproductive organs including prostate, epididymes and testis of

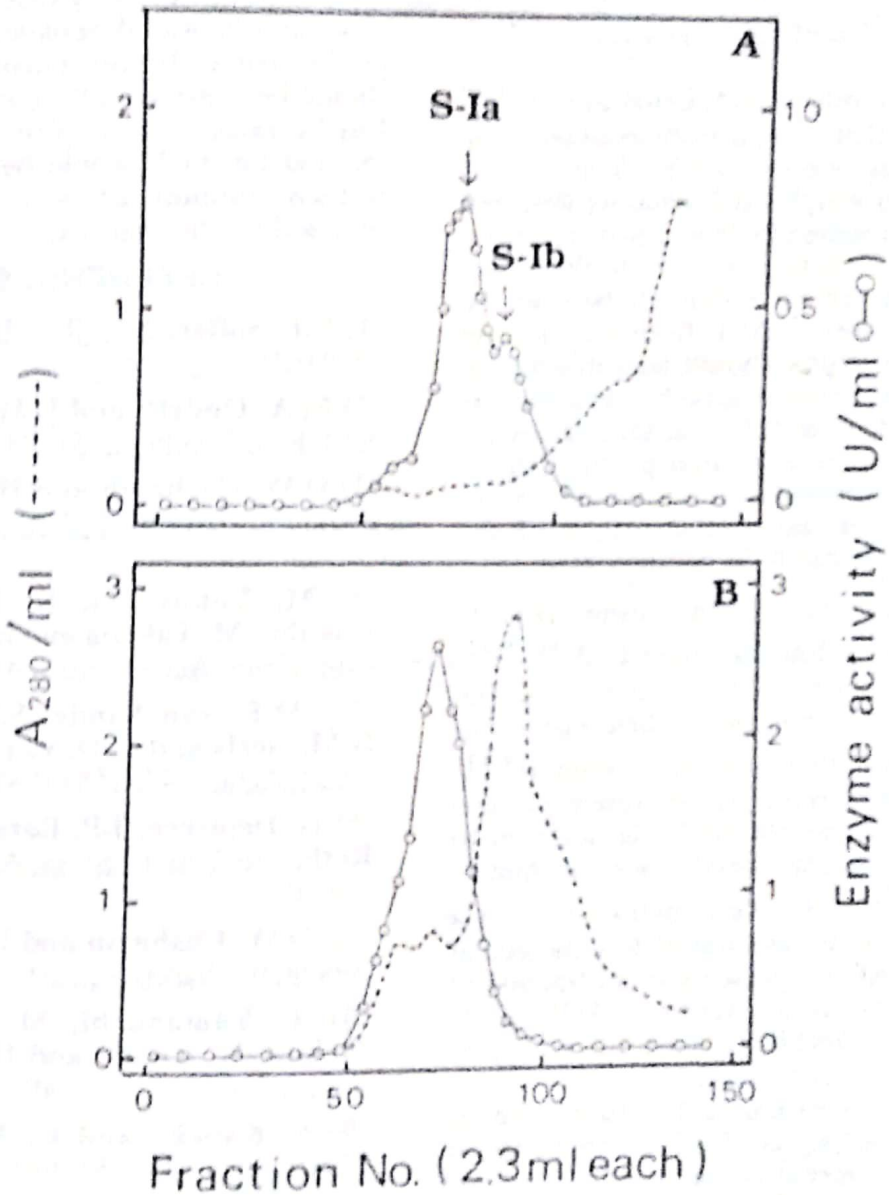

Fig. (3): Sephadex G-200 gel filtration of S-I and S-II.

S-I and S-II from DEAE-Sephadex A-25 chromatography was separately applied onto a Sephadex G-200 column (2.5 x 85 cm) which had been equilibrated with 25 mM Tris/HCl buffer, pH 8.3, containing 0.1 M NaCl. The sample was gel filtered through a column at a flow rate of 0.4 ml/min.

human[5]. Among them, prostate is reported to contain the largest amount of ACE, followed by epididymes and testis[5]. Human prostatic ACE was reported to have similar molecular weight as that of pulmonary enzyme[9]

On the other hand, Lanzillo et al.[10] reported that human testis contains low molecular weight ACE. The higher molecular weight ACEs that we detected in human semen (S-Ia and S-II) might be derived from prostate, while the lower molecular weight S-Ib might be from the testis. However, ACE from each human male sex organ should be subjected to the same chromatographic analyses as we had done with human seminal ACEs. Identification of elution position in the chromatographic analyses would give us information about the origin of the human seminal ACE components.

There has been some reports concerning human seminal ACE[9-12]. Among them, three groups did not describe the presence of heterogenity of ACE in human seminal ejaculate[9-11]. Miska et al. reported the presence of low molecular weight ACEs in addition to high molecular weight ones in human semen[12]. As demonstrated in the present study, amount of low molecular weight ACE represents small fraction of total human an seminal ACE (only 7.8%). Therefore, careful and fine separation of to component from other sources is essential for future better understanding of ACE components. It has been revealed that multiple seminal ACE components can be detected in human seminal samples. Each ACE component should be identified and quantified separately in order to elucidate pathophysiological relationship of the ACE component with certain andrological disease.

For clinical application of the present chromatographic analyses,we need some technical improvement in addition to determinaton of origin of each ACE component. We used conventional chromatographic methods that are time consuming, although separation achieved by that method was satisfactory. Rapid separation by high performance liquid chromatography should be considered for clinical routine. Furthermore, more sensitive assay method for ACE would be essential to reduce amount of seminal sample required for the analysis.

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التحليل الكروماتوجرافى لنظائر محول انزيم الانجيوتنسين - ١ فى السائل المنوى للإنسان

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تمت محاولة فصل نظائر محول انزيم الانجيوتنسين-١ من السائل المنوى للأنسان بأستخدام طرق الفصل الكروماتوجرافى . وقد تم استخلاص محول انزيم الانجيوتنسين من السائل المنوى الكامل المحتوى على الحيوانات المنوية فى وجود ٢٥ر٠ ٪ من دى أوكسى كولات الصوديوم وتكثيفه . وتم تجزئة محول انزيم الأنجيوتنسين الخام باستخدام طرق الفصل الكروماتوجرافى على جيل DEAE سيفادكس أ-٢٥ وأعمدة السيفادكس ج - ٢٠٠. وتم فصل أثنين من مكونات محول انزيم الأنجيوتنسين عن طريق كروماتوجرافيا الجيل على DEAE سيفادكس أ-٢٥.

واعتبرت هذه الطريقة مفيدة فى تقييم الأهمية الفسيولوجية المرضية لمحول أنزيم الأنجيوتنسين فى مجال علم الذكورة.