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Development of Enzyme Immuno Assay for Analysis of Free Prostate Specific Antigen in Serum

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Abstract: Recent reports indicate that the clinical usefulness of prostate specific antigen (PSA), particulary in the differentiation of benign prostate hyperplasia from prostate cancer, can be improved by measuring the amount of free PSA in serum. Measuring free PSA is especially useful in attempts to improve diagnositc performance of PSA in the diagnostic gray zone of total PSA. The objective of this study was to develop free PSA assay kit using sandwich microplate enzyme immunoassay format. We chose a test format with polyclonal anti-PSA antibodies coated on the wells and monoclonal anti-free PSA antibodies for quantification to gain higher test sensitivity. We adpoted 50 uL of specimen and 2 hours of first incubation time with detecting antibody for free PSA EIA format using microplate. The within-day and between-day precision (%CV) in the high and low concentration ranges were below 4%. The correlation coefficient between in-house free PSA assay and commercial assay kit was r=0.9965 (slope=0.0984, y intercept=0.0173, N=27). No hook effect was found by 40 ng/mL and correlation coefficient (r) value of the fitted linear regression was over 0.995. The recovery tests were in the range of 98.9~104.1% for free PSA. In conclusion, in-house free PSA enzyme immuno assay is cost effective, simple and rapid and could be useful for the prognosis after theraphy as well as for the differential diagnosis between prostate cancer and benign prostate hyperplasia.

Key Words: Free PSA, EIA

INTRODUCTION

Prostate-specific antigen (PSA) is a 32 kDa single chain glycoprotein serine protease with a chymotrypsin like specificity exclusively produced by the secretory epithelium of the prostate gland(8). PSA is normally secreted into the seminal fluid, and plays a functional role in the cleavage of the seminal vesicle proteins[1]. Only low level of PSA is normally present in the blood stream, and increasing serum concentrations are indicative of prostatic pathology, including benign prostatic hyperplasia and cancer of the prostate[3,4]. Determination of total PSA has widely been used for detection and management of patients with prostatic cancer, and considered as the superior serological marker for prostate cancer[15,17]. Even though the total PSA assay is relatively tissue specific, it is unfortunately not specific for prostate cancer. Patients with benign prostate diseases, such as benign prostate hyperplasia and prostatitis, also may produce total PSA with values that may overlap with those found in

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prostate cancer[13]. The ability to identify patients with elevated total PSA values, though only benign cases of a disease, would help to avoid the performance of many unnecessary biopsies[11]. It was recognised only recently that serum total PSA exists at least in two major forms namely free PSA and PSA-α_1-antichymotrypsin (PSA-ACT) complex[1,6,9]. Free PSA occurs in lower concentration and is probably an inactive form of PSA. It has been known that in most cases sera from patients with prostate cancer contains over 95% PSA-ACT complex, only a very small portion of the serum total PSA is in the free form[12]. It turned out that sera from benign prostate diseases were usually associated with higher percentage of free PSA, whereas a smaller percentage of free PSA was usually found in sera from patients with prostate cancer[2]. Therefore, measuring free PSA could be useful in attempts to differentiate between benign and malignant prostate diseases[5]. Recently several commercialized kits using various principles have been introduced to determine serum free PSA. In this study, an enzyme immuno assay for free PSA on the microplate following sandwich format was established.

MATERIALS AND METHODS

Bovine serum albumin (BSA), tween 20, casein, tetramethylbenzidine (TMB) substrate and thimerosal were all purchased from Sigma (St Louis, Mo, USA). Microplates used for this assays were obtained from Nunc Co. (Roskilde, Denmark). The polyclonal anti-PSA and monoclonal anti-free PSA were purchased from DAKO Co. (Glostrup, Denmark). Sheep anti-rabbit IgG horseradish peroxide conjugate solution was obtained from Biodesign Co. (Kennebunk, Maine).

Coating the microplate

For coating to the 96 well microplate, 100 μL of polyclonal anti-PSA (5 μg/mL in 0.1 M carbonate buffer, Dako Co, Glostrup, Denmark) was added to each well. After overnight incubation at 4℃, the coating solution was removed and dried. Then 250 μL of blocking solution (0.05% casein in PBS) was added to each well.

Preparation of calibrator

For free PSA determination, sera at various free PSA levels obtained from Seoul Clinical Laboratory were pooled and used for control. Calibrator grade free PSA antigen isolated and purified from LNCaP cells was from Dr. Wu in University of Utah (USA). The free PSA value of the calibrator was assayed by commercial kit (CanAg Co., Gothenburg, Sweden).

Assay procedure for the determination of free PSA

Fifty microliter of anti-free PSA solution were dispensed to each well and 50 μL of assay standard, control or samples were added to assigned well of microplate. After incubation for 2 hours at room temperature with constant shaking on rotator, each well was washed three times with 250 μL of wash solution (PBS containing 0.05% tween-20). Then 100 μL of anti-mouse HRP conjugate (1:5,000 dilution) was added. After incubation for 1 hour at room temperature, each well was washed three times with 250 μL of wash solution. One hundred microliter of TMB substrate solution was added to each well and incubated for 10 min at room temperature. Finally 100 μL of stop solution (1 N H_2SO_4) was added and the absorbance of free PSA was measured at 450 nm within 15 minutes.

Specimens

Forteen serum specimens obtained from Seoul Clinical Laboratory were used for the comparison study.

Calculation

Within-run assay and between-run assay vari-

ations were monitored with three control preparations (representing high, low and middle range values).

RESULTS

Adjustment of free PSA EIA parameters

As shown in Fig. 1, increase in the sample volume from 10 μl to 50 μl represented apparently higher absorbance. By increasing the first incubation time with detection antibody from 1 hour to 2 hour, the absorbance was increased, whereas the second incubation time did not show a significant effect on absorbance (Fig. 2). In order to complete the assay rapidly, we decided to adopt 2 hour/ 1 hour arrangement (first incubation time with detecting antibody/ second incubation time with IgG-HRP conjugate).

Table 1. Precision test of free PSA assay

Between-Run assay

Controls	Mean	CV (%)	Replicate No.
High	4.63±0.14	3.10	4
Medium	1.34±0.04	2.64	4
Low	0.46±0.02	3.69	4

Within-Run assay

Controls	Mean	CV (%)	Replicate No.
High	4.40±0.14	3.21	9
Medium	1.36±0.05	3.38	9
Low	0.43±0.01	3.29	9

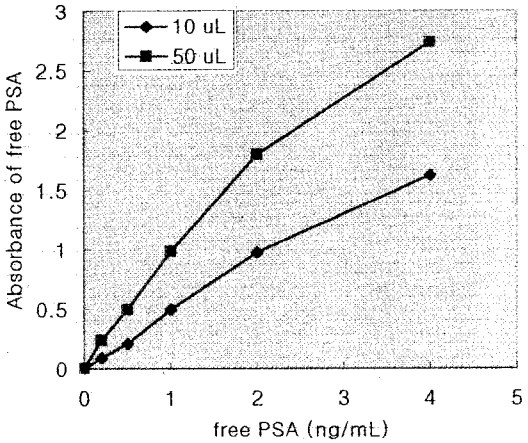

Fig. 1. Effects of the sample volume in free PSA assay.

Precision and detection limit of free PSA assay

Intra-assay (within run) and inter-assay (between run) precision tests were shown in Table 1. The coefficient variation (CV) of precision tests of control samples were lower than 4.0%. The detection limit of the free PSA EIA assay using 50 μL of sample is less than 0.1 ng/mL defined as the concentration corresponding to the mean of the absorbance values for the zero standard±2 standard deviations (mean value± 2 SD).

Recovery and linear regression tests

Spiked serum samples were prepared by adding aliquots of a sample with highly elevated free PSA to normal serum samples. The re-

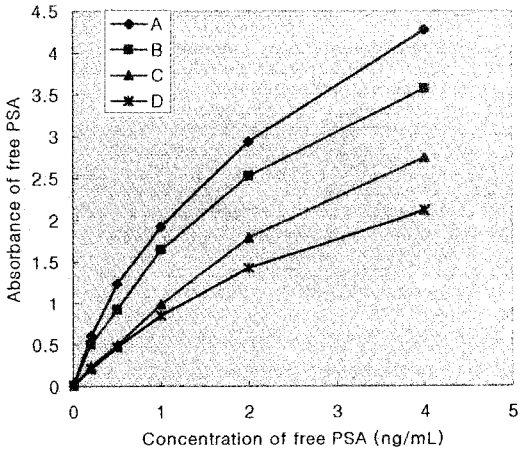

Fig. 2. Differences of absorbance in accordance with incubation time in free PSA assay.

TYPE	First incubation time with detection antibody	Incubation time with HRP conjugate
A	3 hour	1 hour
B	2 hour	2 hour
C	2 hour	1 hour
D	1 hour	30 min

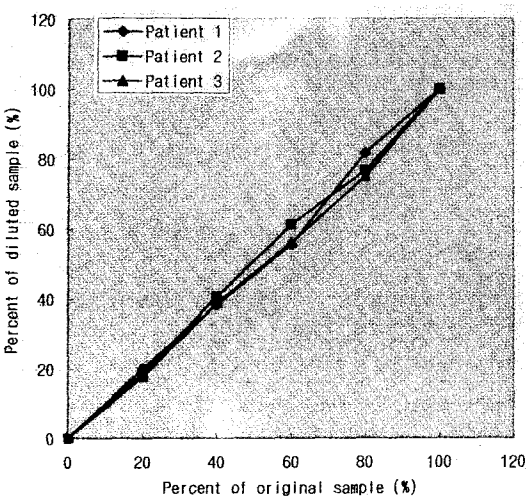

Fig. 3. Linear regression test of free PSA assay in three patient samples.

Patient 1: y=0.998x+1.015 r=0.996
Patient 2: y=0.997x−1.028 r=0.997
Patient 3: y=0.965x+0.724 r=0.995

Table 2. Recovery test of free PSA assay

Added (ng/mL)	Expected (ng/mL)	Measured (ng/mL)	Recovery (%)
S_1		0.75	
S_1 + 3.2	3.95	3.91	98.9
S_1 + 2.5	3.25	3.31	101.8
S_1 + 1.2	1.95	1.96	100.5
S_1 + 0.7	1.45	1.51	104.1

covery of the free PSA antigen were in the range 98.9~104.1% for free PSA (Table 2). For linearity test, patient samples were serially diluted to 100%, 80%, 60%, 40%, 20% with sample diluent. Mean correlation coefficient value (r) of three patient samples were over 0.995 (Fig. 3). No hook effect has been noticed with samples up to 40 ng/mL.

Correlation with commercialized EIA kit

The results using in-house free PSA EIA were correlated well with those from the commercialized assay at both low and elevated free PSA concentrations. Fig. 4 shows the overall correlation at a PSA concentration range from zero to 4 ng/mL (r=0.9965, slope=0.0984,

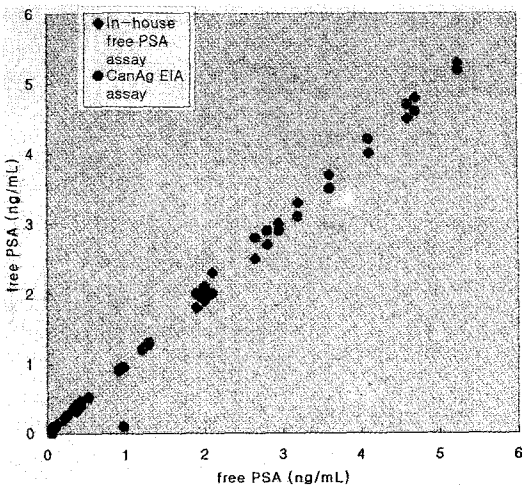

Fig. 4. Correlation study between in-house free PSA assay and CanAg EIA kit.

y=0.0984x0.0+1734, r=0.9965

y intercept=0.0173, N=27).

DISCUSSION

PSA has been shown to form stable complexes with different anti-proteases, and the dominating proportion of PSA in patient serum occur in complex with antichymotrypsin (PSA-ACT)[12]. However there were large variations in the relation between free PSA and PSA-ACT complex between different individuals. Studies have also indicated that the proportion of free PSA was higher in benign prostatic disease as compared with prostatic cancer[2]. The discovery that PSA exists in the serum in different molecular forms and the development of assays for free PSA represent a major advancement in the diagnosis of early, curable prostate cancer[13,16]. Catalona and coworkers[5] reported that high concentration of free PSA was closely related with prostatic cancer when the total PSA value was in the normal ranges. However, the measurement of free PSA alone can be misleading, because very high free PSA could be found in cancer patients. When the total PSA value is in the range of 4.0 to 10.0 ng/mL, analysis of free PSA improves the

specificity of the PSA test[7,14].

Several recent reports of the clinical significance of free PSA led to develop free PSA commercialized kits by immuno assay with sandwich or competitive principle with radioisotope or enzyme labeling. Recently environmental and biohazard problem are making laboratories to convert radioisotopic immunoassay (RIA) to enzyme immuno assay (EIA)[10]. Other chemiluminescent immunoassay (CLIA) reagent was developed by some commercial company. However its application could be limited due to need for an expensive equipment.

In the present study, microplate EIA of free PSA was developed using sandwich format. Microplate EIA format in our opinion is the most cost effective and it can be applicable by manual method or using any auto microplate system. Moreover, the higher affinity between the coated antibody and antigen in the specimen showed the higher sensitivity of the assay. Therefore the use of polyclonal antibody-conjugated polystyrene plates and sandwich design we adopted for free PSA assay allowed the coverage of a wide linear range of free PSA concentration without sacrificing accuracy at both low and high levels[19]. Several other assay parameters can be optimized to improve the sensitivity of microplate assay, such as the total reaction volume, the surface area of the solid phase, the concentration of the sample, the incubation time between the coated antibody and free PSA, between the antibody-bound free PSA and the detecting antibody, and the final volume of the color development system for the enzymatic reaction[18]. To develop the sensitive and simple free PSA EIA assay, we tried to adjust several parameters. Two different sample volumes from each specimen, 10 μL and 50 μL, were tried for the assay in order not only to further increase test sensitivity and improve precision at both low and highly elevated free PSA conentrations, but also to widen the assay concentration range. The 10 μL of specimen showed a distinct decrease of absorbance, whereas proper optical density was observed using 50 μL of specimen (Fig. 1). We also compared the first and second incubation times with detecting antibody and enzyme conjugate, respectively (Fig. 2). In this experiment, the detecting antibody was added for the first incubation time because concentration of free PSA is very low in serum. The reaction of one hour of first incubation was too weak, whereas two hour incubation represented a better standared curve (Fig. 2). Therefore, 50 μL of specimen and 2 hour/1 hour incubation time (detecting antibody/ enzyme conjugate) were adopted in our microplate EIA format. The possibility of prolonging the incubation time any further became impractical as far as turn-around time was concerned. The in-house free PSA EIA kit represented below 4% of coefficient variations (%CV) in precision tests including between-run assay and within-run assay (Table 1). Recovery test was in the range of 98.9%～104.1% (Table 2) and the coefficient correlations of linear regression in three patient samples were over 0.995 (Fig. 3). The results of random samples (N=27) of in-house free PSA assay were consistant to those of commercialized assay with low and high range of concentration (r=0.9965) (Fig. 4).

Even though EIA method is useful for routine laboratory work, it can produce the "hook effect" when the specimen contains highly elevated concentrations of free PSA. When an assay with the sandwich design involves only one incubation, the excessive amounts of free PSA will bind and saturate both the solid phase-coated and the enzyme-conjugated antibodies simultaneously, preventing from the binding of the enzyme-conjugated antibody to the solid phase[19]. Consequently a false low value will be produced. The fact that the hook effect is still observable at extremely elevated free PSA concentrations indicates that excess free PSA cannot be removed entirely by washing. The "hook effect" was not found in our

free PSA assay by 40 ng/mL. From this study, a free PSA enzyme immuno assay developed by using microplate format is so simple and rapid it could be useful for routine clinical laboratory work.

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=국문초록=

혈청 유리형 전립선항원 (free PSA) 측정을 위한 효소면역측정법의 개발

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전립선특이항원 (PSA: Prostate Specific Antigen)은 전립선 세포에서 분비되는 단백질로서 장기 특이성이 높아 전립선암의 조기진단 및 치료의 예후를 판단하기 위해 임상에서 널리 사용되고 있는 혈청 종양표지검사의 대상항원이다. 그러나 혈청 PSA치는 전립선암 뿐 아니라 양성 전립선질환인 전립선비대증 (BPH), 전립선 경색, 전립선 염증 등에서도 상승될 수 있다. 혈청 내의 PSA는 여러 가지 분자형태로 이루어져 있으며 대표적으로 α_1-antichymotrypsin과 결합된 결합형 PSA (PSA-ACT)와 유리형 PSA (free PSA)로 나눌 수 있다. 전립선암 환자의 혈청에는 결합형 PSA가 95% 이상으로 매우 높고 유리형 PSA의 분포는 매우 작은 반면 전립성비대증에서는 유리형 PSA의 농도가 높아지므로, 결합형 PSA와 동시에 유리형 PSA를 측정하면 종양표지검사로서의 특이성과 예민도를 높일 수 있다는 보고가 있어, 최근 혈청내의 유리형 PSA의 측정의 중요성이 임상적으로 대두되고 있다. 이에 본 연구에서는 sandwich 원리로 유리형 PSA 효소면역측정법 (EIA) kit를 개발하고 그 유용성을 검토한 결과, 고, 저농도에서의 일내, 일간 변이계수 (CV)는 4% 이하였으며 상품화된 free PSA kit와 비교하였을 때 두 방법간의 상관계수는 0.9965으로 매우 양호하였다. 또한 농도가 높은 세 환자의 검체를 희석하여 직선성 검사를 하였을 때 상관계수가 모두 0.995이상으로 나타났다. 또한 microplate 법에서 문제될 수 있는 hook effect는 유리형 PSA 농도가 40 ng/mL까지 나타나지 않았으며, 98.9%~104.1%의 회수율을 나타내었다. 따라서 본 연구에서 개발한 면역측정법 kit는 혈청 중의 유리형 PSA를 정확하고 간편하게 분석할 수 있으므로 임상 실험실에서 전립선암이나 전립선질환의 진단 및 치료효과의 판정에 유용하게 사용될 수 있을 것으로 기대된다.

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