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Establishment and clinical application of a highly sensitive enzyme immunoassay for determination of *N*-acetyl-seryl-aspartyl-lysyl-proline

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N*-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP) is a natural inhibitor of pluripotent hematopoietic stem cell proliferation and is normally found in human plasma. Because AcSDKP is hydrolyzed by the *N*-terminal active site of angiotensin converting enzyme and partially eliminated in urine, its plasma level is a result of a complex balance between its production, hydrolysis by ACE, and renal elimination. In this study, we attempted to establish an enzyme immunoassay (EIA) for quantifying AcSDKP-like immunoreactive substance (IS), which is applicable for monitoring plasma AcSDKP levels in healthy subjects and patients with chronic renal failure. Using β-D-galactosidase-labeled Gly-γAbu-SDKP as a marker antigen, an anti-rabbit IgG-coated immunoplate as a bound/free separator and 4-methylumbelliferyl-β-D-galactopyranoside as a fluorogenic substrate, a highly sensitive and specific EIA was developed for the quantification of AcSDKP-IS in human plasma. The lower limit of quantification was 0.32 fmol/well, and the sharp inhibition competitive EIA calibration curve obtained was linear between 8.0 and 513 fmol/ml. This EIA was so sensitive that only 10 μl plasma sample was required for a single assay. The coefficients of variation (reproducibility) for human plasma concentrations of 0.2 and 2.1 pmol/ml were 7.2 and 7.7%, respectively, for inter-assay and 13.3 and 7.8% for intra-assay comparisons. Plasma AcSDKP-IS level was significantly higher in patients with chronic renal failure (0.92 ± 0.39 pmol/ml) compared with healthy subjects (0.29 ± 0.07 pmol/ml). These results suggest that our EIA may be useful to evaluate plasma AcSDKP level as a biomarker in various patients. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: *N*-acetyl-seryl-aspartyl-lysyl-proline; AcSDKP; Ac-SDKP; enzyme immunoassay; clinical application; chronic renal failure

Introduction

N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP, Figure 1) is an endogenous peptide released from its precursor (thymosin-β4) by proline oligopeptidase [1,2]. AcSDKP is a natural inhibitor of pluripotent hematopoietic stem cell proliferation that prevents cells from entering S-phase from G1 of the cell cycle [3,4] and is normally found in human plasma [5]. AcSDKP has been shown to suppress renal fibroblasts [6] and inhibit collagen deposition in cardiac fibroblasts in mice [7]. In the past, AcSDKP was shown to originate from the bone marrow [8], but recent studies in mice have demonstrated that both AcSDKP and its precursor are ubiquitously distributed in tissues including the lung, kidney, and heart [9]. Because AcSDKP is hydrolyzed by the *N*-terminal active site of ACE [10,11] and partially eliminated in urine [12], its plasma level is a result of a complex balance between its production, hydrolysis by ACE, and renal elimination. AcSDKP hydrolysis is blocked by ACE inhibitors (ACEIs) such as captopril and lisinopril *in vitro* [10,11] and *in vivo* [13,14]. After a single administration to normal subjects or during long-term treatment in hypertensive patients, an ACE inhibitor increases plasma AcSDKP levels by fivefold to sixfold compared with control subjects or patients [13,14]. In patients with chronic heart failure treated with ACEI, plasma AcSDKP levels are significantly higher in anemic than in non-anemic patients and clearly correlate with erythroid progenitor cell proliferation [15]. Furthermore, plasma AcSDKP levels have been shown to increase in patients with chronic renal failure, suggesting that accumulated AcSDKP may be partially involved in nephrogenic anemia because of reduced renal function in patients with chronic renal failure [16,17]. In addition, plasma AcSDKP levels in patients with chronic renal failure treated with ACEI are significantly higher compared with non-ACEI-treated patients, indicating that ACEI may increase

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Journal of PeptideScience

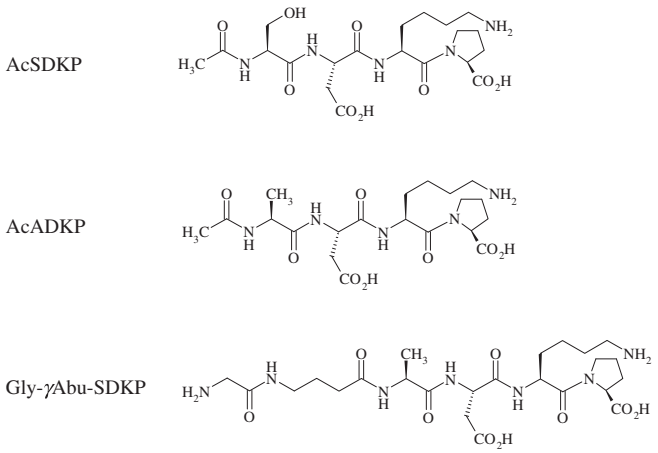

Figure 1. Chemical compositions of AcSDKP, AcADKP, and Gly-γ Abu-SDKP.

the risk of anemia in patients with chronic renal failure [17]. Whereas plasma AcSDKP levels in chronic renal failure have been reported by several studies, the characteristic of plasma AcSDKP level in acute renal failure remain largely unknown.

Several studies have reported different methods for the quantification of AcSDKP. Pradelles *et al*. [5,9] reported an enzyme immunoassay (EIA) for the investigation of the distribution of AcSDKP. This method is relatively specific but has limited sensitivity (lower limit of quantification: 0.3 pmol/ml), so that 1 ml of sample is needed for a single measurement. Junot *et al*. [18] reported an assay to screen endogenous AcSDKP in human plasma and urine using liquid chromatography with electrospray mass spectrometry. This method has the demerits of low recovery in plasma (64%) and low sensitivity (lower limit of quantification: 10.3 pmol/ml), requiring 2 ml of sample for a single measurement. Recently, Inoue *et al*. [19] reported a method for the quantification of AcSDKP in human plasma, which employs solid phase extraction, liquid chromatography (LC) separation, and stable isotope dilution electrospray tandem mass spectrometry. This method has good recovery and precision, but the preparation of sample is cumbersome and 500 μl of sample is needed for a single measurement because of limited sensitivity (lower limit of quantification: 0.21 pmol/ml). Given this situation, more sensitive and reliable methods for the quantification of AcSDKP are required.

In this study, we established a sensitive and specific EIA for quantifying AcSDKP-like immunoreactive substance (IS), which is applicable to monitoring plasma AcSDKP levels in healthy subjects and patients with chronic renal failure.

Materials and Methods

Materials

Synthetic AcSDKP was purchased from Bachem (Bubendorf, Switzerland). Synthetic peptide *N*-acetyl-alanyl-aspartyl-lysyl-proline (AcADKP) (Figure 1) was purchased from Sigma-Aldrich (MO, USA). Antiserum to AcSDKP (A03881) was purchased from Bertin Pharma (Paris, France). Goat affinity purified antibody to rabbit IgGs (whole molecule) (55641) was purchased from ICN Pharmaceuticals (Aurora, OH, USA). 4-Methylumbelliferyl-β-D-galactopyranoside (MUG) and *N*-(ε-maleimidocaproyloxy) (EMC)-succinimide were purchased from Sigma (St Louis, MO, USA). *β*eta-D-galactosidase (β-Gal) was purchased from Boerhinger Mannheim (Mannheim, Germany). All other reagents were analytical reagent grade from commercial sources.

Subjects

Plasma samples were collected from healthy volunteers ($n = 6$) and patients with chronic renal failure having proteinuria and creatinine clearance less than 30 ml/min ($n = 6$). All six healthy subjects were male, aged 25–32 years (median 27) and weighing 56–70 kg (median 62). No healthy subject received any medication during 1 month before the study. The patients comprised four males and two females, aged 34–68 years (median 50) with creatinine clearance of 6.6–11.5 ml/min (median 8.3) and weighing 34–75 kg (median 59). All patients were hemodialyzed three times a week and received medications for hypertension and hyperphosphatemia, but none were prescribed ACEI during more than a year before this study and throughout the whole study. The study was approved by the Ethics Committee of Oita University. Each subject received information about the scientific purpose of the study and gave informed consent.

Venous blood samples were collected at three time points (8:00, 14:00, 22:00) in the healthy subjects and at one-time point (8:00) in patients with chronic renal failure. All blood samples were centrifuged and plasma samples frozen at −40 °C within 30 min of peripheral venipuncture.

Preparation of Plasma Extracts

Plasma samples were extracted using a modification of the method of Pradelles *et al*. [5]. Five hundred μl of methanol was added to 10 μl of plasma sample and vortexed. After centrifugation at 1500 g for 15 min at 4 °C, the supernatant was decanted into a clean test tube and evaporated to dryness under a stream of nitrogen. The extracts were reconstituted with 100 μl of assay buffer (see succeeding texts) prior to analysis. A standard AcSDKP solution was added to AcSDKP-free human plasma before extraction to examine the recovery and reproducibility of the assay system [20].

Synthesis of Gly-γAbu-SDKP

In order to prepare an enzyme-labeled antigen, we synthesized the glycyl-γ-aminobutanoyl (γAbu)-seryl-aspartyl-lysyl-proline peptide (Gly-γAbu-SDKP), an AcSDKP derivative (Figure 1). Gly-γAbu-SDKP was prepared by standard 9-fluorenylmethoxycarbonyl (Fmoc)-based solid phase peptide synthesis. The respective amino acids were condensed manually in a stepwise manner using diisopropylcarbodiimide *N*-hydroxybenzotriazole on a Pro-preloaded 2-chlorotrityl resin (Novabiochem, San Diego, CA). The following side chain protecting groups were used: Asp, and Ser, *t*-butyl; Lys, *t*-butoxycarbonyl. Resulting protected peptide resins were deprotected and cleaved from the resin using a TFA/thioanisole/m-cresol/ethanedithiol/H_2O mixture (80:5:5:5:5, v/v) at room temperature for 3 h. Crude peptides were precipitated and washed with diethyl ether and then purified by HPLC using a Mightysil RP-18 column (Kanto Chemical Co., Inc., Tokyo, Japan) with a gradient of H_2O and acetonitrile containing 0.1% TFA. The purity and identity of the synthetic peptides were confirmed by HPLC and by electrospray ionization mass

spectroscopy. Mass spectroscopy was performed at the Central Analysis Center, Tokyo University of Pharmacy and Life Sciences.

Preparation of Enzyme-Labeled Antigen

Gly-γAbu-SDKP was conjugated with β-Gal by EMC-succinimide according to the method of Kitagawa *et al.* [21]. In brief, Gly-γ Abu-SDKP (1.0 mg) dissolved in 0.05 M phosphate buffer pH 7.0 (0.20 ml) was mixed with EMC-succinimide (1.0 mg) in tetrahydrofuran (0.20 ml) at room temperature (20 °C) for 60 min. The EMC-peptide obtained was purified by separation through a Sephadex G-25 column (1.5 × 50 cm) pre-equilibrated with 0.05 M phosphate buffer (pH 7.0), which was also used to elute the column. Individual fractions (1.8 ml each) that showed an absorbance at 220 nm were collected. The purified EMC-peptide fractions were combined with β-Gal (1.0 mg) by mixing at room temperature for 60 min. The β-Gal conjugates were applied to a Sephacryl S-300 column (1.5 × 52 cm) and eluted with 0.05 M phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$. Individual fractions (1.8 ml each) that showed an absorbance at 220 nm were collected. The fractions containing β-Gal activity were collected and stored at 4 °C after the addition of 0.2% BSA and 0.1% sodium azide.

EIA Procedure for AcSDKP-IS

The EIA was used to assay AcSDKP-IS in plasma. The assay was performed by a delayed addition method. Separation of bound and free antigens was performed on an anti-rabbit IgG-coated immunoplate (Nunc-Immuno Module Maxisorp F8, InterMed, Denmark).

The assay buffer was 0.05 M phosphate buffer (pH 7.0) containing 0.5% BSA, 1 mM $MgCl_2$, and 250 KIU/ml aprotinin. Diluted antiserum (100 μl) and the sample (100 μl of plasma extract or standard) were mixed and incubated at 4 °C for 24 h. Diluted enzyme-labeled antigen (50 μl) was then added, and the mixture was incubated at 4 °C for an additional 24 h. One hundred microliters of the antigen-antibody solution for each sample was transferred to a well of the secondary antibody-coated immunoplate. After incubating at 4 °C overnight, the wells were washed with 0.01 M phosphate buffer (pH 7.0) containing 0.15 M NaCl and 0.05% Tween 20. Then, 200 ml of 0.1 mM MUG in 0.05 M phosphate buffer (pH 7.0) containing 1 mM $MgCl_2$ was added to each well. The plate was incubated at 37 °C for 180 min, and fluorescence intensity (λ_{Ex} 360 nm, λ_{Em} 450 nm) of the fluorescent product, 4-methylumbelliferon, was measured with an SH-9000 microplate reader (Corona Electric, Ibaraki, Japan).

HPLC of Plasma Extracts

HPLC was performed using a Waters Alliance model e2695 system (Waters, Milford, MA, USA) and a reversed-phase C18 packed column (Cosmosil 5 C18, Nacalai Tesque, Kyoto, Japan). Ten microliters of plasma sample, extracted by methanol as described earlier, was reconstituted with 100 μl of 0.1% TFA and passed through the column. AcSDKP-IS was eluted with a linear gradient of acetonitrile (from 1 to 10% over 40 min) in 0.1% TFA. The flow rate was 1.0 ml/min and the fraction size was 1.0 ml. Eluted fractions were concentrated by spin-vacuum evaporation, lyophilized, and reconstituted to 100 μl with the assay buffer. The fractions were assayed using the AcSDKP EIA.

Influences of EDTA and Heparin

Calibration curves using normal assay buffer, assay buffer containing EDTA (5×10^{-3} M) and assay buffer containing heparin (20 units/ml) were compared. Split blood samples collected in EDTA and heparin tubes were obtained from six healthy volunteers at the same time (three time points for each subject, total 18 time points) and compared. Ten microliters of 10^{-3} M captopril was added immediately into a heparinized blood sample to prevent degradation of AcSDKP [10]. Stability of AcSDKP at room temperature was evaluated in samples of EDTA-plasma, heparin-plasma and heparin-plasma with added captopril, obtained by prompt centrifugation after blood collection from six healthy volunteers.

Comparison with Conventional EIA

Plasma AcSDKP-IS levels of heparin-plasma obtained from six healthy volunteers (18 samples) were assayed by a conventional EIA and our EIA and compared. Conventional EIA was performed using the methods previously reported by Pradelles *et al.* [5].

Data Analysis and Statistics

All values are expressed as mean ± standard derivation. AcSDKP-IS levels are presented in pmol/ml, as in previous reports [5,12-17]. The relationships between AcSDKP-IS levels in EDTA-plasma and heparin-plasma and AcSDKP-IS levels quantified by conventional EIA and our EIA were analyzed by Pearson's product–moment correlation coefficient. Comparison of circadian profiles of plasma AcSDKP-IS levels was conducted by Tukey–Kramer test. Difference in plasma AcSDKP levels between the healthy subjects and patients with chronic renal failure was analyzed by Welch's *t*-test. A *p*-value less than 0.05 was considered statistically significant. Statistical analyses were performed using the SPSS software package (version 17.0; SPSS Inc., IL, USA).

Results

Standard Curve

A typical calibration curve for AcSDKP-IS EIA is shown in Figure 2, which depicts the enzyme activity when given concentrations

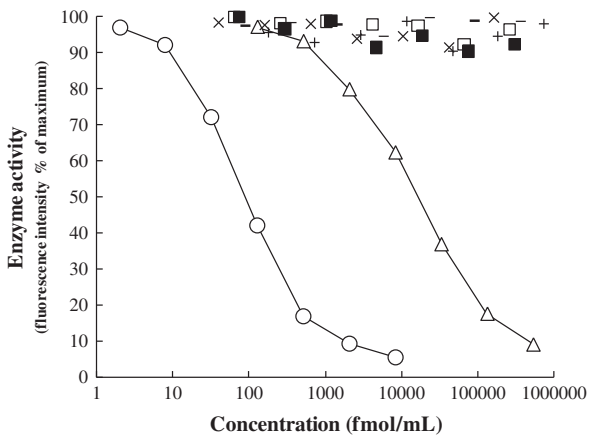

Figure 2. Displacement curves of AcSDKP (○), AcADKP (Δ), and other endogenous peptides (human calcitonin gene-related peptide (□), vasoactive intestinal peptide (■), substance P (+), motilin (−), and adrenomedullin (×)) in the EIA by competition between Gly-γAbu-SDKP conjugated with β-Gal and AcSDKP for antiserum A03881

Journal of PeptideScience

(*x*-axis) of AcSDKP in the standard prepared in assay buffer compete with enzyme-linked Gly-γAbu-SDKP for the antiserum to AcSDKP (A03881). When plotted as a semi-logarithmic function, the linear portion of the calibration curve was found to be between 8.0 and 513 fmol/ml AcSDKP in buffer. The minimum amount of AcSDKP detectable by this EIA system was 0.8 fmol (0.32 fmol/well), and the 50% inhibitory concentration (IC50) of the calibration curve was 73.3 fmol/ml.

Specificity of Antiserum to AcSDKP (A03881)

The immunological specificity of the antiserum to AcSDKP; A03881, was examined by EIA using Gly-γAbu-SDKP conjugated with β-Gal. The displacement curves of AcSDKP, AcADKP, and other endogenous peptides (human calcitonin gene-related peptide, vasoactive intestinal peptide, substance P, motilin, and adrenomedullin) are shown in Figure 2. AcADKP exhibited cross-reactivity with synthetic AcSDKP. Other endogenous peptides showed minimal inhibition of the binding of β-Gal-conjugated Gly-γAbu-SDKP with antiserum A03881.

Measurement of AcSDKP-IS in Human Plasma by EIA

The recovery rates of human EDTA-plasma AcSDKP for two concentrations (0.2 and 2.1 pmol/ml) lying within the proposed detectable range of this EIA were 95.4 ± 4.0 and 98.1 ± 3.5%, respectively ($n = 6$, mean ± standard derivation). The reproducibility expressed as coefficient of variation for human plasma concentrations of 0.2 and 2.1 pmol/ml using this AcSDKP EIA was 7.2 and 7.7%, respectively, in inter-assay ($n = 6$), and 13.3 and 7.8% for intra-assay ($n = 6$) comparisons.

Human plasma extracts were subjected to reversed-phase HPLC to examine the presence of AcSDKP-IS molecular variants in human plasma. The elution profile revealed the presence of a main immunoreactive peak (arrow) eluted at a position corresponding to AcSDKP standard (Figure 3).

Influences of EDTA and Heparin on AcSDKP-IS EIA

The influences of EDTA and heparin, the major anticoagulants for plasma, on the AcSDKP EIA were evaluated. Calibration curves for AcSDKP prepared in normal assay buffer, assay buffer containing EDTA (5×10^{-3} M) and assay buffer containing heparin (20 units/ml) are shown in Figure 4. There were little variations among the three curves. Figure 5 shows the relationship between AcSDKP-IS levels in EDTA-plasma and heparin-plasma collected from six healthy volunteers (18 points), showing a good correlation between the two ($y = 1.017x - 0.001$, $r = 0.85$). The stability of AcSDKP at room temperature in samples of EDTA-plasma, heparin-plasma, and heparin-plasma with captopril is shown in Figure 6. AcSDKP was stable for up to 2 h after blood sampling in samples of EDTA-plasma and heparin-plasma with added captopril. On the other hand, AcSDKP in heparin-plasma without captopril decreased gradually from 0.5 h after sampling.

Comparison with Conventional EIA

Figure 7 shows the relationship between AcSDKP-IS levels in heparin-plasma obtained from six healthy volunteers (18 samples) quantified by a conventional EIA and our EIA. A good correlation was obtained between the two assays ($y = 0.931x - 0.052$, $r = 0.92$).

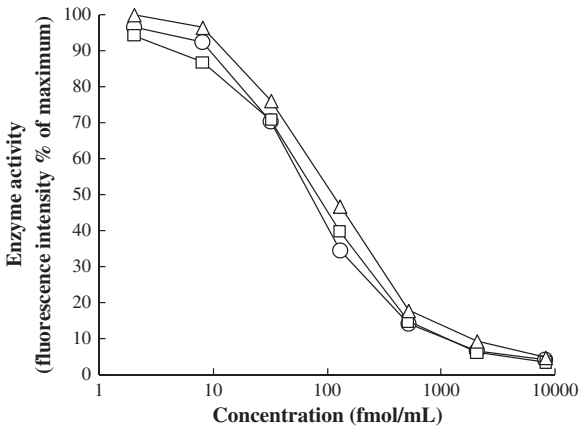

Figure 4. Inhibition curves of AcSDKP using normal assay buffer (○), assay buffer containing EDTA (Δ), and assay buffer containing heparin (□).

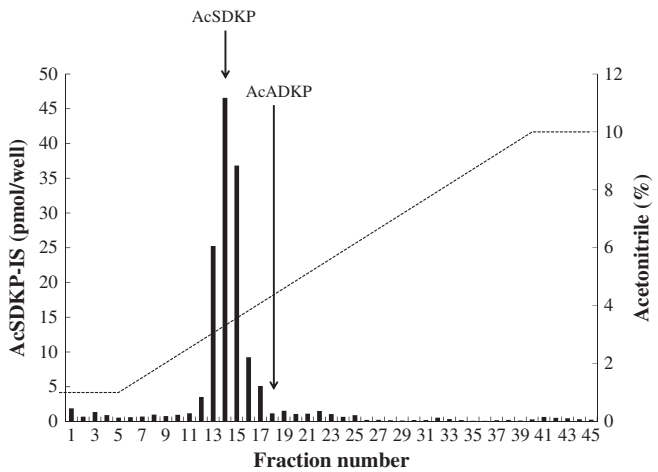

Figure 3. HPLC elution profiles of AcSDKP-IS in human plasma. Synthetic AcSDKP and AcADKP are run as separate chromatograms under the same conditions (indicated by the arrows).

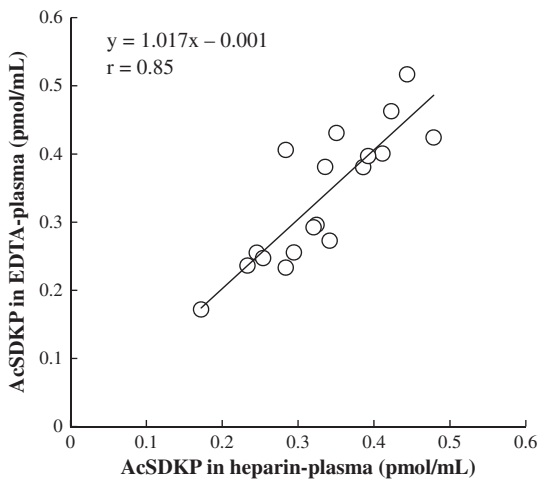

Figure 5. Relationship of AcSDKP levels in EDTA-plasma and heparin-plasma collected from six healthy volunteers.

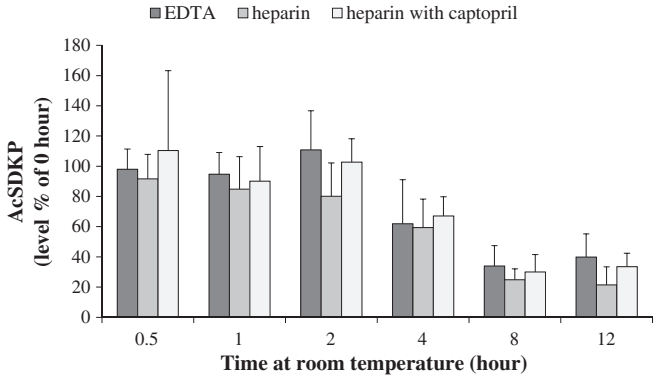

Figure 6. Stability of the AcSDKP at room temperature in samples prepared in EDTA-plasma, heparin-plasma, and heparin-plasma with captopril.

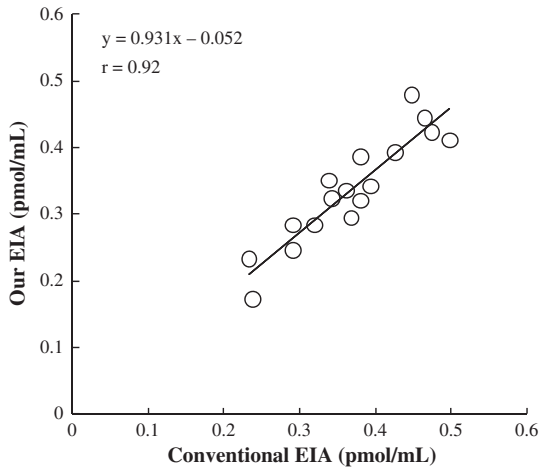

Figure 7. Relationship of plasma AcSDKP-IS levels quantified by conventional EIA and our EIA.

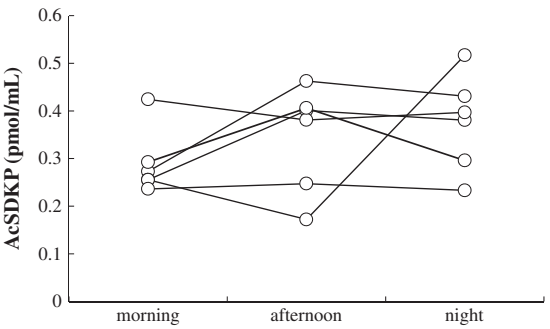

Figure 8. Circadian profiles of plasma AcSDKP-IS levels in six healthy subjects.

Plasma AcSDKP-IS Levels in Healthy Subjects and Patients with Chronic Renal Failure

The circadian profiles of plasma AcSDKP-IS levels in healthy subjects are shown in Figure 8. Plasma AcSDKP-IS levels were 0.29 ± 0.07 pmol/ml at 8:00, 0.34 ± 0.11 pmol/mL at 14:00, and 0.38 ± 0.11 pmol/ml at 22:00, with no significant differences. The AcSDKP-IS levels in EDTA-plasma at 8:00 in six healthy subjects and six patients with chronic renal failure are shown in Figure 9. Plasma AcSDKP-IS level was significantly higher in

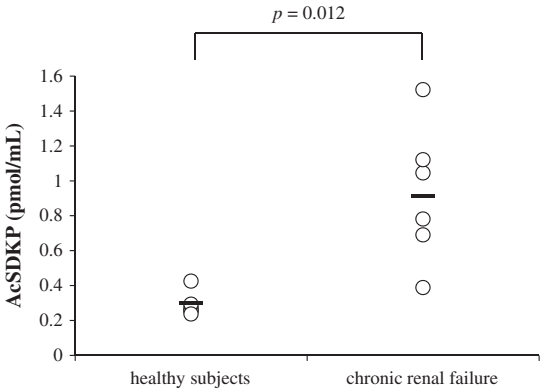

Figure 9. AcSDKP-IS levels at 8:00 in six healthy subjects and six patients with chronic renal failure.

patients with chronic renal failure (0.92 ± 0.39 pmol/ml) compared with healthy subjects (0.29 ± 0.07 pmol/ml).

Discussion

Using β-Gal-labeled Gly-γAbu-SDKP as a marker antigen, an anti-rabbit IgG-coated immunoplate as a bound/free separator, and MUG as a fluorogenic substrate, we developed a highly sensitive and specific EIA for the quantification of AcSDKP in human plasma. Because AcSDKP has no free amino group in the *N*-terminus, it is difficult to prepare an AcSDKP-linked enzyme while maintaining the structure of AcSDKP. To overcome this issue, we used Gly-γAbu-SDKP that has a free amino group in the *N*-terminus, thus permitting the linkage of this AcSDKP derivative to an enzyme using a maleimide type bifunctional cross-linking reagent.

Our EIA for AcSDKP-IS is highly sensitive; the lower limit of quantification is 0.32 fmol/well, and the sharp calibration curve is linear between 8.0 and 513 fmol/ml. Our EIA for AcSDKP-IS is so sensitive that only 10 µl plasma sample is needed for a single assay (detectable range in plasma: between 0.08 and 5.1 pmol/ml). The required sample volume is thus dramatically reduced compared with the traditional methods [5,9,18,19]. Therefore, our EIA that requires small blood sample for determining plasma level of AcSDKP may have wide clinical application. Furthermore, this AcSDKP EIA has no cross-reactivity with other endogenous peptides, although there is little cross-reactivity with AcADKP (Figure 2). AcADKP is a minor peptide released from the precursor thymosin-β10 and is present in very low levels in human plasma (lower than 0.21 pmol/ml) [19]. Therefore, it is unlikely that AcADKP will affect the measurement of plasma AcSDKP levels in our EIA. In fact, main immunoreactive peak was not observed at a position corresponding to standard AcADKP in the elution profiles of AcSDKP-IS by HPLC (Figure 3). With regard to practicability, our EIA allows simultaneous measurement of many samples (96 wells) by using an anti-rabbit IgG-coated immunoplate as the bound/free separator. We applied the novel EIA to detect AcSDKP in human plasma. The recovery and reproducibility (inter-assay and intra-assay coefficient of variation) of this EIA for assaying plasma samples were satisfactory.

It is important to use appropriate anticoagulants for obtaining plasma samples when venous blood was collected because they may cross-react with AcSDKP or affect the

Journal of PeptideScience

stability of AcSDKP in blood samples. As shown in Figure 4, EDTA and heparin did not affect the calibration curve for AcSDKP-IS. Furthermore, there was good correlation between EDTA-plasma and heparin-plasma samples in healthy subjects (Figure 5). These results suggest that both EDTA and heparin do not affect this EIA for AcSDKP. Our stability study of AcSDKP-IS in plasma sample revealed that AcSDKP-IS levels were stable for 2 h after blood sampling in EDTA-plasma and heparin-plasma with captopril (Figure 6), indicating that preparation of plasma extracts should be completed within 2 h at room temperature. The stability of AcSDKP was comparable in EDTA-plasma and heparin-plasma with captopril, showing that AcSDKP is stable in EDTA-plasma without the addition of captopril. Previous study demonstrated that EDTA, a metalloproteinase inhibitor, inhibits the hydrolysis of AcSDKP *in vitro* [10]. Taken together, these results suggest that EDTA-plasma and heparin-plasma with captopril are suitable for the measurement of plasma AcSDKP levels.

A good correlation between plasma AcSDKP levels quantified by conventional EIA and liquid chromatography with electrospray mass spectrometry has been reported [18]. In this study, we also demonstrated a good correlation between plasma AcSDKP-IS levels quantified by a conventional EIA and our EIA (Figure 7). This finding further validates that our EIA is as accurate as the conventional methods.

A previous study reported that plasma AcSDKP-IS levels at baseline did not vary during the day in healthy volunteers [22]. The present study similarly demonstrated minor circadian fluctuation of plasma AcSDKP levels (Figure 8). As shown in Figure 9, plasma AcSDKP-IS level was significantly higher in patients with chronic renal failure (0.92 ± 0.39 pmol/ml) compared with healthy subjects (0.29 ± 0.07 pmol/ml). These results were consistent with the previous studies that reported the elevation of plasma AcSDKP levels in patients with chronic renal failure [16,17]. Based on these findings, our EIA is suitable for measuring plasma AcSDKP levels ranging from healthy subjects with lower levels to patients with chronic renal failure who have higher levels. Therefore, our EIA may have wide clinical application.

In conclusion, we established a highly sensitive EIA for the measurement of plasma AcSDKP level and applied it for the measurement of plasma AcSDKP levels in healthy subjects and patients with chronic renal failure. This EIA requires only 10 μl of plasma sample for a single assay. The assay may be useful to evaluate plasma AcSDKP level as a biomarker in a variety of clinical settings.

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