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Original article

Enzyme-linked immunosorbent assay of a linear, recombinant peptide designed for immunotherapy of Japanese cedar pollinosis

Daisuke Kozutsumi ^{a,*}, Kimiko Shimizu ^b, Keiko Morikubo ^b, Yukio Ohshiba ^a,
Taketo Yamaji ^a, Kohsuke Kino ^a

^a Research and Development Section, Pharmaceuticals Development Department, Meiji Dairies Corporation, 540 Naruda, Odawara, Kanagawa 250-0862, Japan

^b Food Science Institute, Division of Research and Development, Meiji Dairies Corporation, 540 Naruda, Odawara, Kanagawa 250-0862, Japan

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Abstract

Introduction: Cry-consensus peptide, a recombinant T-cell epitope peptide for immunotherapy of Japanese cedar pollinosis, is a linear peptide that does not have disulfide bonds because no cysteine residue exists in the molecule. We examined whether a sandwich enzyme-linked immunosorbent assay (ELISA) could be performed for linear peptides such as Cry-consensus peptide. **Methods:** The 3-dimensional conformation of Cry-consensus peptide was examined by ¹H NMR analysis. Nineteen monoclonal antibodies (mAbs) that recognized various domains of Cry-consensus peptide were established to use in a sandwich ELISA. The relationship between the recognition sites of mAbs and the sensitivity of the ELISA was investigated to optimize the selection of the combination of the capture and the detection antibodies. ELISA inhibitors in serum and plasma were also studied to improve the stability and the sensitivity of determination. **Results:** ¹H NMR analysis of Cry-consensus peptide suggested that Cry-consensus peptide molecule had no portions with rigid conformation. The sensitivity of the ELISA showed a good correlation with the distance between the respective binding sites of the capture and the detection antibodies. Human serum albumin and α 1-acid glycoprotein strongly inhibited the binding of the capture mAb to Cry-consensus peptide in a dose-dependent manner, and heparin also inhibited the binding in the concentration at which it is used as anticoagulant. Taken together, the findings indicated that an optimized method showed good linearity and minimal variation from 0 to 1000 ng/ml of Cry-consensus peptide. **Discussion:** These data indicate that this method is useful for monitoring Cry-consensus peptide concentrations in plasma or serum.

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1. Introduction

Recently, several immunogenic peptides have been reported as candidates for immunotherapy of allergic diseases such as asthma, atopic dermatitis and pollinosis (Carneiro et al., 2004; Immonen et al., 2005; Jahn-Schmid et al., 2002; Janssen et al., 2000; Smith, Alexander, Kay, Larche, & Robinson, 2004; Sone et al., 1998; Takai, Mori, Yuuki, Okudaira, & Okumura, 1999; Vrtala et al., 2000). These peptides have relatively short and linear sequences, and their quantitative determination is accordingly often very difficult in biological materials such as blood and tissues. ELISA is one of the most sensitive measurement methods, and has high

specificity since it is based on antigen–antibody binding. However, applying this technique to clinical and preclinical samples also poses several difficulties due to the features of these peptides.

Antigen recognition by antibodies is a very specific reaction that targets antigen-binding domains having rigid conformation, and thus ELISA is preferentially used as a method to determine the levels of various native proteins. In contrast, some recombinant proteins with unique sequences, such as T cell epitopes, exhibit low reactivity, and therefore improvement of the assay is often required (Shin, Lee, Kim, & Hahm, 1996). Cry-consensus peptide (Fig. 1) is a recombinant peptide consisting of linearly-joined T cell epitopes which were selected from the major allergens of Japanese cedar pollen (Sone et al., 1999), and it exhibits weak binding to the monoclonal antibodies established thus far (our unpublished data).

* Corresponding author. Tel.: +81 465 37 5211; fax: +81 465 37 0981.

E-mail address: daisuke_kozutsumi@meiji-milk.com (D. Kozutsumi).

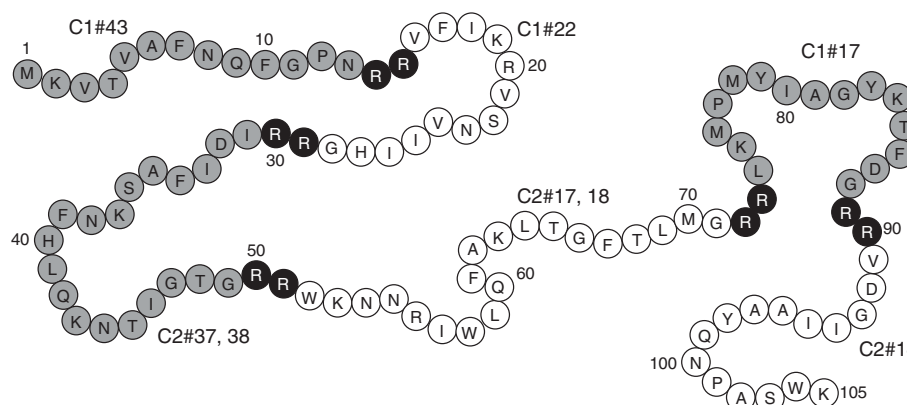


Fig. 1. Amino acid sequence of Cry-consensus peptide. The T cell epitopes (C1#43: 1–13, C1#22: 16–28 and C1#17: 74–88 from Cry j 1, and C2#37, 38: 31–49, C2#17, 18: 52–71 and C2#14: 91–105 from Cry j 2) are joined linearly with an arginine dimer motif between each pair of epitopes. The MHC class II restriction molecule for each epitope is as follows: peptides C1#43 and C2#17, 18: DPB1*0501, peptide C1#22: DRB5*0101, peptide #C2#37, 38: DPB1*0201/DRB4*0101, and peptide C2#14: DRB5*0101 (unknown for peptide 74–88).

Here, we report an ELISA method for Cry-consensus peptide determination with mAbs specific for Cry-consensus peptide. The 3-dimensional conformation of Cry-consensus peptide was confirmed by ^1H NMR analysis. Combinations of mAbs displaying different epitope specificities were identified and used to quantify Cry-consensus peptide in human serum using capture ELISA. Validation of the use of this method for quantitative determination is also discussed in this paper.

2. Methods

2.1. Human plasma

All subjects who donated blood for plasma preparation gave informed consent to participate in this study, which was approved by the Ethics Committee of Meiji Dairies Corporation.

2.2. NMR spectra of Cry-consensus peptide

Complete denaturation of Cry-consensus peptide and lysozyme (control protein) was performed in D_2O solution by gradual heating, and NMR spectra were recorded at 25–95 °C on an Inova 500 NMR (500 MHz, Varian, Palo Alto, CA, USA). The chemical shifts of signals were measured relative to the D_2O signal chosen as the internal standard (4.8 ppm).

2.3. TAK-201-specific monoclonal antibodies

The anti-Cry-consensus peptide mAbs listed in Table 1 were established in our laboratory and purified from the ascites of BALB/c-*nu/nu* mice (CLEA JAPAN Inc., Tokyo, Japan) that had been injected intraperitoneally with 1×10^7 cells/head of hybridoma clones producing the indicated mAb following Pristane (2,6,10,14-tetramethylpentadecane, Sigma-Aldrich, St. Louis, MO, USA) injection at a dose of 0.5 ml/head. Starting approximately 1 week after hybridoma transplantation, the ascites were harvested using a syringe with a 19G needle and then were pooled for purification of

the mAbs. Following filtration to remove debris, the mAbs were purified from the pooled ascites using a HiTrap Protein G Sepharose HP column (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The epitopes recognized by mAbs were identified by conventional ELISAs using plates coated with each T cell determinant.

2.4. Combinations of mAbs for the sandwich ELISA

The assay was a conventional sandwich detection with a capture and a detection mAb. Horseradish peroxidase (HRP)-conjugated E1.163 mAb (E1.163-HRP) was firstly chosen as the detection antibody. Briefly, the ELISA plate (PolySorp, Nunc, Roskilde, Denmark) was coated overnight at 4 °C with mAbs in 0.1 M carbonate buffer, pH 9.5 (K8.280, K6.208, K8.238, K8.212, G1.635, G1.432, E1.163, G1.67, and K6.121) at 1 $\mu\text{g}/100 \mu\text{L}/\text{well}$, respectively. The plate was washed and then blocked with 200 $\mu\text{L}/\text{well}$ of 10 $\times$ diluted BlockAce (Dainippon Pharmaceuticals, Osaka, Japan) for 1 h at room temperature (RT). After washing the plate again, the calibration samples spiked with 0, 5, 50, and 500 ng/mL of Cry-consensus peptide were added to the plate for 1 h at RT and the captured Cry-consensus peptide was detected by biotin-conjugated mAbs (K8.280, K6.208, G1.635, E1.163, and H1.429), respectively, and followed by streptavidin-peroxidase/*N*, *N*, *N*', *N*'-tetramethylbenzidine (TMB) detection. Among these combinations, biotin-conjugated E1.163 mAb was relatively sensitive, therefore E1.163 was selected to conjugate with HRP for preparing the detection antibody.

In the studies to investigate inhibitors of Cry-consensus peptide determination, K8.238 mAb was used as the capture antibody. The capture antibody was adsorbed overnight at 4 °C to the ELISA plates at 10 $\mu\text{g}/\text{ml}$ in 0.1 M carbonate buffer, pH 9.5 (100 $\mu\text{L}/\text{well}$). The plates were washed and then blocked with 200 $\mu\text{L}/\text{well}$ of 10 $\times$ diluted BlockAce for 1 h at RT. After washing the plates again, an aliquot of the test sample (100 μL) was added to each well, and the plates were incubated for 1 h at RT. The plates were washed once more, and 100 μL of E1.163-HRP was added to each well at an appropriate concentration.

Table 1
Anti-Cry-consensus peptide monoclonal antibodies

Clone	Recognition site in Cry-consensus peptide sequence (see Fig. 1)
K8.280	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
K8.32	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
K6.208	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
K8.238	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
K8.212	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
E1.237	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
E1.262	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
E1.431	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
E1.156	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
G1.635	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
G1.432	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
E1.584	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
E1.72	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
E1.163	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
G1.67	<u>C1#43</u> -(RR)-C1#22-(RR)-C2#37,38-(RR)-C2#17,18-(RR)-C1#17-(RR)-C2#14
K6.121	Unknown
H1.162	Unknown
H1.205	Unknown
H1.429	Unknown

The underlines represent the recognition sites of mAbs.

Isotype of the listed antibodies except for H1.205 is IgG1 and that of H1.205 is IgG2a.

RR: an arginine dimer motif.

The assay plates were further incubated for 1 h at RT and then washed, and bound E1.163 was developed with a TMB Substrate Kit (Pierce, Rockford, IL, USA) and detected at a wavelength of 450 nm. The results were the mean of duplicate determinations.

2.5. Optimization of the ELISA

For investigation of the optimal conditions for Cry-consensus peptide determination in blood, the ELISA was performed in the presence of several inhibitors present in plasma or serum samples as follows. Cry-consensus peptide solutions (100 ng/ml) were prepared in the following matrices: human plasma, human serum, 0.1 M citrate buffer (pH 1.5), 0.1 M phthalate buffer (pH 4.0), 0.1 M carbonate buffer (pH 9.5), and phosphate buffered saline (PBS, pH 7.2) with and without human serum albumin (HSA, 5–500 µM), α1-acid glycoprotein (α1-AGP, 3–30 µM), 0.3% sodium citrate, 10 U/ml heparin, 50 mM ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) and 50 mM ethylenediaminetetraacetic acid dipotassium salt (EDTA-2K). The results of the sandwich ELISA obtained with K8.238 and E1.163-HRP mAbs were the mean of triplicate determinations.

2.6. Method validation

Cry-consensus peptide concentration in plasma was measured using 10-fold diluted plasma with phosphate buffered saline (pH 7.2). The reproducibility of the calibration curve was determined for Cry-consensus peptide over the range of 0 to 1000 ng/ml. The reproducibility and freeze-thaw stability were measured in groups of five identical samples respectively spiked with Cry-consensus peptide at different concentrations within the calibration range. The variations in background

substances in plasma were examined with plasma from 6 healthy volunteers.

3. Results

3.1. Cry-consensus peptide has no rigid 3-dimensional folding

The 3-dimensional folding of Cry-consensus peptide was analyzed by ¹H NMR. The ¹H NMR spectra of Cry-consensus peptide showed no signal in the ranges indicating conventional conformations of proteins, and the patterns of the chromatograms were almost identical at all the treatment temperatures examined (Fig. 2a). In contrast, the signals indicating the 3-dimensional folding of lysozyme were diminished at 95 °C, a condition causing the denaturation of typical proteins, and then were recovered by refolding at 25 °C (Fig. 2b). Similar results were also obtained from our preliminary experiment using CD analysis (data not shown). These data suggest that Cry-consensus peptide has no 3-dimensional conformation within the molecule, and this result is also supported by the fact that the amino acid sequence does not include cysteine residues.

3.2. Recognition sites of Cry-consensus peptide-specific mAbs

The epitopes recognized by the mAbs we obtained are listed in Table 1. A total of 19 mAbs were obtained; but the binding sites of 4 mAbs (K6.121, H1.162, H1.205 and H1.429) were not identified. Half of the mAbs binding to the C-terminal region of Cry-consensus peptide recognized both the peptides C1#17 and C2#14, and therefore, it was considered that these mAbs bound to the domain joining these peptides, which includes an arginine dimer motif. We chose the E1.163 mAb as the detection antibody based on the results of testing various combinations of the mAbs (data not shown).

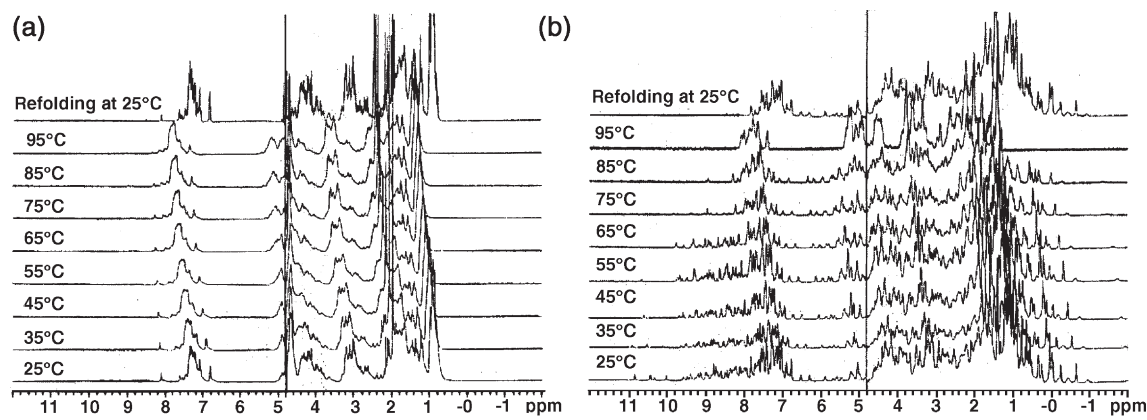


Fig. 2. Analysis of the 3-dimensional structure of proteins by ^1H NMR. Both Cry-consensus peptide (a) and lysozyme (b) were heated from 25 to 95 °C in D_2O and were then cooled to the initial temperature for refolding. Bold lines indicate the peak areas showing signals of proteins deriving from their 3-dimensional conformation.

3.3. Relationship between the spacing between the recognition sites and the sensitivity of the sandwich ELISA

The sensitivity of the sandwich ELISA was investigated using combinations of the E1.163-HRP mAb and the other mAbs. As shown in Fig 3a, mAbs of the K-series binding near the N-terminal or the middle regions of Cry-consensus peptide showed relatively high sensitivity in comparison with mAbs binding near the C-terminal or to unknown regions. The sensitivity was related to the spacing between the recognition

sites of the 2 mAbs with a high correlation coefficient (0.93). These results indicate that the sensitivity of the sandwich ELISA was improved by the appropriate spacing between the recognition sites of the capture and the detection mAbs in the primary sequence of Cry-consensus peptide, perhaps through a

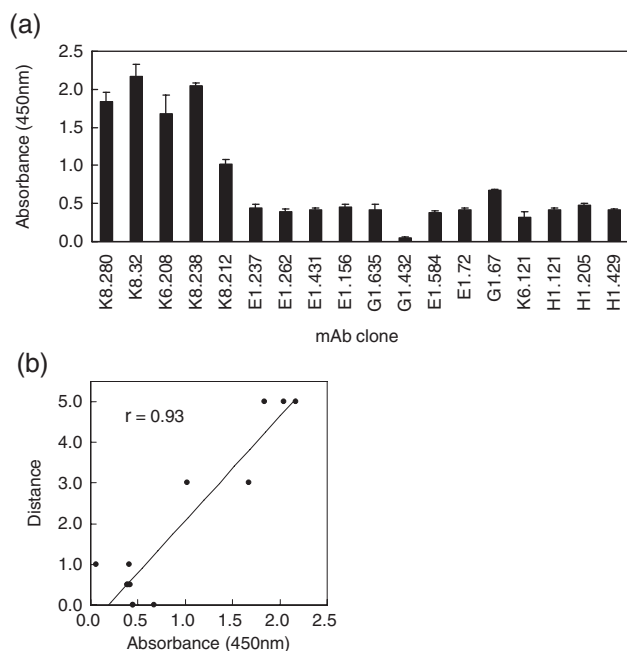


Fig. 3. Relationship between the spacing between two antibody recognition sites in the Cry-consensus peptide sequence and the sensitivity of the assay. (a) The mAbs listed in Table 1 were added at 10 $\mu\text{g}/\text{well}$ to 96-well microplates as the capture antibody. Captured Cry-consensus peptide (100 ng/ml) was detected by the E1.163-HRP mAb binding to the C-terminal epitope of Cry-consensus peptide (C2#14) as described in Methods. (b) Relationship between the assay sensitivity and the spacing of the two antibody recognition sites of the capture and the detection antibodies. Distance is the number of T cell determinants between the recognition sites of the two mAbs used in the ELISA.

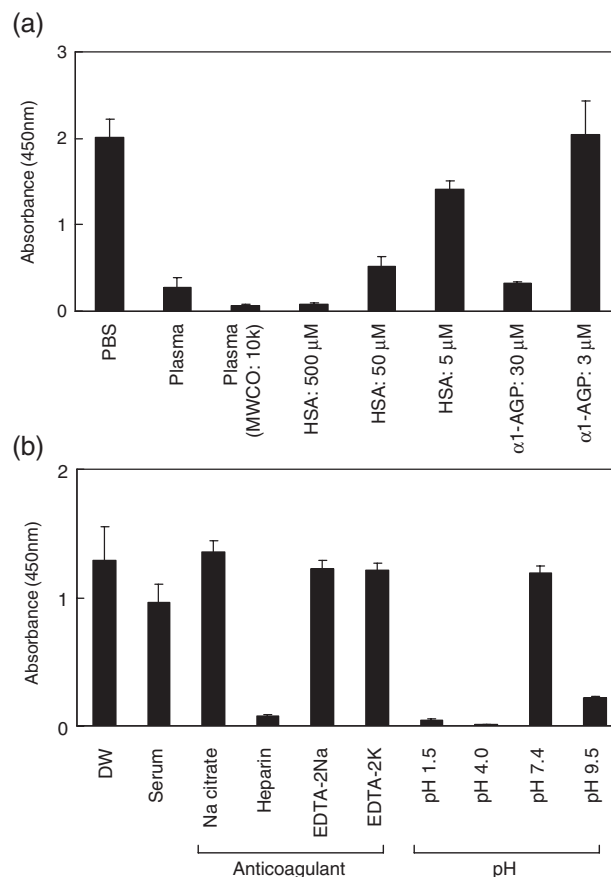


Fig. 4. Inhibitory factors affecting Cry-consensus peptide determination. The ELISA for Cry-consensus peptide was performed with K8.238 mAb as capture antibody and E1.163 mAb as detection antibody, under various conditions. Serum proteins and anticoagulants were added to PBS as a matrix, and pH was adjusted using citrate, phthalate, phosphate and carbonate buffers. Plasma and serum consisted of pooled materials from healthy subjects. DW; distilled water, MWCO; molecular weight cut-off.

mechanism similar to the avoidance of steric hindrance in folded proteins.

3.4. Sandwich ELISA inhibitory factors in human serum samples

The binding between the capture mAb (K8.238) used to coat the plates and Cry-consensus peptide was strongly inhibited when Cry-consensus peptide was mixed with human plasma (Fig. 4a), and therefore we investigated whether common molecules present in blood samples acted as inhibitors of this assay. As shown in Fig. 4a, the sensitivity of Cry-consensus peptide determination in plasma was further decreased by the addition of a plasma fraction with molecular weight of greater than 10 kDa, indicating that proteins in plasma might inhibit the detection of Cry-consensus peptide. Therefore, the contribution of HSA and α 1-AGP, major plasma proteins, to the reduction of the sensitivity was examined. HSA and α 1-AGP reduced the sensitivity strongly at their respective physiological concentrations 500 and 30 μ M, and the inhibitory effects were concentration dependent.

Next, the effects of anticoagulants included in plasma samples and the optimum pH of samples were also investigated. Among the anticoagulants examined, only heparin markedly inhibited Cry-consensus peptide detection, while the others did not interfere with the detection in the concentrations at which they are used as anticoagulants (Fig. 4b). The maximal sensitivity was obtained at pH 7.4, and under acidic conditions, K8.238 mAb exhibited almost no binding to Cry-consensus peptide. Taken together, these results imply that plasma samples for this assay must be prepared without heparin and must be diluted at least 10-fold with neutral buffer to obtain precise results, because α 1-AGP, which interferes with the assay, is a protein involved in inflammation whose concentration varies widely under physiological conditions.

3.5. Validation of the method of quantitative ELISA

The calibration curve of Cry-consensus peptide mixed with plasma was reproducible in the range 0–1000 ng/ml of Cry-consensus peptide, with a high correlation coefficient of >0.99 .

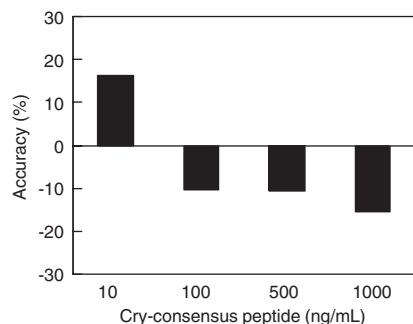


Fig. 5. Reproducibility of Cry-consensus peptide determination. Reproducibility of the assay was determined at concentrations of 10, 100, 500 and 1000 ng/mL Cry-consensus peptide. Spiked Cry-consensus peptide in five identical samples was measured for successive 5 days, and the mean accuracy of resulting values of the assay was calculated.

As shown in Fig. 5, the reproducibility was examined in groups of 5 identical samples spiked with Cry-consensus peptide at respective concentrations of 10, 100, 500 and 1000 ng/ml, and showed good results: 20% accuracy at all concentrations examined. Repeated freeze-thawing in plasma did not influence the stability of Cry-consensus peptide. Background factors that might vary among individual plasma samples obtained from 6 volunteers did not cause variation of the assay results (data not shown).

4. Discussion

There are many reports demonstrating the usefulness of sandwich ELISAs for the determination of proteins (Arilla et al., 2005; Gearhart, Middlemore, & Terry, 2006; Na et al., 2005; Njemini, Demanet, & Mets, 2005). However, all of those proteins contain cysteine residues in their amino acid sequences, and thus generally fold into a rigid conformation through intramolecular disulfide bonds. These rigid structures are essential for efficient recognition by antibodies, because the specificity of antigen-antibody binding is dependent on the existence of the 3-dimensional domains on the surface of molecules. However, the application of ELISA for linear peptides without rigid conformations has not been thoroughly investigated.

Cry-consensus peptide is a 105-amino-acid polypeptide that lacks cysteine residues and has a linear, non-rigid conformation (Fig. 1). Because of this feature, it was anticipated that the sensitivity of ELISA for Cry-consensus peptide measurement would be relatively low. The specificity of the assay is indispensable, especially for the determination of Cry-consensus peptide levels in blood samples. In practice, the lower limit of Cry-consensus peptide detection by an HPLC method is approximately 2 μ g/ml (data not shown), and thus while this ELISA is not as sensitive as conventional sandwich ELISA methods in general, it is superior to methods of measurement such as chromatography.

The findings of this study suggest that even for linear peptides such as Cry-consensus peptide, the sensitivity of ELISA is dependent on not only the amino acid sequences of sites recognized by antibodies but also the spacing between the recognition sites (Fig. 3). Generally, the sensitivity of sandwich ELISA is influenced by steric hindrance of antigen-antibody binding (Butler, Peterman, Suter, & Dierks, 1987; Cepica, Yason, & Ralling, 1990). It appears that in particular the avoidance of steric hindrance is essential for the establishment of sandwich ELISA for linear peptides with flexible conformation.

In the clinical or preclinical studies during drug development, the existence of inhibitors of antigen-antibody binding in plasma or serum is often a severe problem that interferes with determining the precise concentration of target compounds. The present study revealed that the ELISA for Cry-consensus peptide was also influenced by plasma proteins and by heparin used as an anticoagulant (Fig. 4). Especially in samples from patients with allergic diseases, it is important to consider the effect of α 1-AGP on the assay, because α 1-AGP levels in blood vary widely depending on the state of inflammation in vivo. However, our findings indicated that the inhibitory effect of α 1-

AGP was avoidable by 10-fold dilution of plasma or serum samples. In contrast, the inhibitory effect of HSA was not preventable by such dilution, but the plasma concentration of HSA is relatively constant among samples from various individuals, and therefore accurate values can be calculated using a calibration curve produced from tests with control plasma or serum as matrices. Taken together, our findings indicate that Cry-consensus peptide in plasma or serum can be measured accurately if 10-fold diluted samples are used and even if anticoagulants are present, except for heparin. Under these conditions, our examination of the accuracy of the determination showed good results regarding the repeatability, the reproducibility, the freeze-thaw stability and the lack of effect of plasma variability. The improvement of the sensitivity of this assay may be worthy of further study. Further, we think that the findings provided herein will also be useful for the evaluation of other clinically relevant linear peptides being developed as potential drugs, and also for linear peptides with non-clinical applications.

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