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Sandwich-type immunoassay with an affinity column coupled to anti-peptide antibodies

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Abstract

A new sandwich-type immunoassay method utilizing an anti-peptide antibody column and a fluorescence-labeled antibody (FLA) was proposed and applied to the measurement of the concentrations of insulin and insulin B-chain. In this method an immunoadsorbent coupled with an anti-peptide antibody was packed in a small column. A target protein was adsorbed on the anti-peptide antibody in the column, coupled with the FLA as a secondary antibody in sandwich manner and then eluted by an eluent containing an antigen peptide to the anti-peptide antibody. The concentration of the target protein was determined form the fluorescent intensity of the eluted FLA. It was possible to determine the concentrations within 30 min, and the higher sensitivity was obtained in comparison with the direct method using an immunoaffinity column. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The specific and strong immunointeraction between antigen and antibody has widely been used in assay methods of many biological materials. In bioprocesses, monitoring and control of the concentrations of nutrients, metabolites and bioproducts are important for effective production, and thus rapid and accurate assay methods of these materials with high sensitivity are desired. Although enzyme-linked immunosorbent assay (ELISA) is the most popular immunoassay method among many assay methods based on the immunointeraction, it requires many repeated incubation and washing steps for separation of bound and free antibodies and antigens. Therefore, this method is too time consuming to apply to process monitoring and control in bioindustries.

Immunoassay methods using liposomes coupled with antigen or antibody (liposome immune lysis assay, LILA) have been proposed as homogeneous and rapid methods [1,2]. Assay methods utilizing an immunoaffinity chromatography column may also offer a rapid and specific immunoassay method. An affinity column coupled with a specific antibody as a ligand can adsorb a corresponding antigen from a sample solution. From the area or height of the eluted peak from the affinity column, the concentration of the antigen can be determined (direct method) [3]. The sensitivity of the direct method, however, is relatively low because of the detection method utilizing a spectrophotometer, and other components adsorbed non-specifically to the column can affect the accuracy of the measurement. To

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avoid the latter drawback, Nilsson et al. proposed a flow-injection ELISA [4]. In this method an enzyme-labeled antigen was bound to an affinity column competitively with the antigen in a sample solution. Then a substrate solution was supplied to the column, and the amount of the substrate reacted by the labeled antigen was measured. This method still not has a high sensitivity.

A sandwich-type immunoassay method utilizing fluorescence-labeled antibodies or antigens can improve the sensitivity but causes two main drawbacks [5]. One is the subtle change in the fluorescent intensity of labeling agents under elution conditions of different pH values or ion compositions usually used in affinity operation. The other is large amounts of non-specific adsorption of fluorescence-labeled antibodies (FLAs) to immunoadsorbents because of the interaction of labeling agents with carriers of affinity ligands and also with affinity ligands themselves.

In the present work, we propose a new sandwich-type immunoassay method utilizing anti-peptide antibody columns and FLAs. In this method the effect of non-specific adsorption of FLAs was minimized by the specific elution of target proteins with eluent containing antigen peptides for the anti-peptide antibodies [6]. By such specific elution, FLAs nonspecifically adsorbed should not be eluted and do not affect the fluorescent intensity of eluted peaks. Two types of anti-peptide antibodies recognizing C- and N-terminal regions of bovine insulin B-chain were used for this sandwich-type immunoassay.

2. Materials and methods

2.1. Materials

Peptides and insulin used for immunization and measurement were insulin from bovine (Sigma), bovine insulin B chain (Nacalai Tesque) and peptides against C-terminal and N-terminal regions of insulin B chain, which are summarized in Fig. 1. PC-InB12P is a peptide which represents residues 19–30 of bovine insulin B chain and was used for immunization to obtain an anti-PC-InB12P antibody. PC-InB11P, which corresponds to residues 20–30 of insulin B-chain, was used for measurement of adsorption characteristics. PN-InB7K, which is a peptide representing residues 1–7 of insulin B chain and having an additional lysine residue, was used for immunization to obtain an anti-PN-InB7K antibody. PN-InB6KK was used for measurement of adsorption characteristics and a specific eluent. These peptides were synthesized by the solid-phase method (Applied Biosystems, 431A) and purified by a HPLC system (Shimadzu LC-10A) and a reversed-phase column. The sequences of the synthesized peptides were confirmed by a peptide sequencer (Applied Biosystems, Procise 492). The other chemicals used were of reagent grade.

2.2. Preparation of immunoadsorbent used for assay

The peptide was coupled to keyhole limpet

PC-InB12P (C-terminal region of bovine insulin B-chain)

Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala

PC-InB11P

PN-InB7K (N-terminal region of bovine insulin B-chain)

Phe-Val-Asn-Gln-His-Leu-Cys-Lys

PN-InB6KK

Phe-Val-Asn-Gln-His-Leu-Lys-Lys

Fig. 1. Peptides used for immunization and specific elution.

hemocyanin (KLH) with glutaraldehyde according to the procedure previously reported [7], and a mixture of an antigen solution (1 mg peptide cm^{-3}) and Freund's complete adjuvant (1 cm^3 each) was immunized to rabbits. Booster injections were repeated several times in a similar way at 10-day intervals.

Specific anti-peptide antibodies (anti-PC-InB12P and anti-PN-InB7K) were purified by use of Sepharose 4B coupled with antigens (PC-InB11P and PN-InB6KK). Specific antibodies were coupled to Formyl Cellulofine (Chisso) to obtain immunoadsorbents. The prepared immunoadsorbent was packed in a column of 4.6 mm I.D. and 30 mm long.

2.3. Coupling of fluorescein isothiocyanate and FLA

Anti-peptide antibodies were labeled by fluorescein isothiocyanate (FITC). After equilibration with a phosphate buffer (20 m*M*+0.15 *M* NaCl, pH 7.6), anti-peptide antiserum was applied to a column packed with Sepharose 4B coupled with the antigen peptide. After washing with the phosphate buffer, a carbonate buffer (50 m*M*, pH 9.0) was applied. Then a FITC solution (100 mg cm^{-3} in the carbonate buffer, pH 9.0, three times the bed volume) was applied to the column, and the column was kept at 4°C for 12 h. After washing with the phosphate buffer, FLA was eluted by 0.1 *M* HCl, collected in a bottle containing 5 cm^3 of a Tris–HCl buffer (1 *M*, pH 8.5) and dialyzed overnight against the phosphate buffer.

2.4. Assay methods using anti-peptide antibody and FLA

The concentrations of insulin and insulin B-chain in the phosphate buffer (pH 7.6) were measured by the following two methods using the affinity column coupled with the anti-peptide antibodies, as shown in Fig. 2 for the case of the anti-PN-InB7K column. In the procedure 1, 100 μl of a sample solution containing various concentrations of insulin (or insulin B chain) was injected into the anti-peptide antibody column (30 mm long) equilibrated with the phosphate buffer supplied by an HPLC pump (Shimadzu LC-10A) with a flow-rate of 1 cm^3 min^{-1}. After washing with the phosphate buffer for 5 min, 5 μl of a FLA solution (0.295 mg–IgG cm^{-3} or 0.17 mg–IgG cm^{-3} of fluorescence-labeled anti-PC-InB12P) was injected into the column and bound with insulin (insulin B-chain) adsorbed by the immunoadsorbent packed in the column. After washing for 10 min, the bound insulin (insulin B-chain) and FLA were eluted by 2 cm^3 of an eluent containing 0.2 mg cm^{-3} of the antigen peptide (PN-InB-6KK for anti-PN-InB7K column and PC-InB11P for anti-PC-InB12P column), and the fluorescent intensity of the eluted peak was monitored by a spectrofluorometer (RF-535, Shimadzu) at excitation wavelength 490 nm and emission wavelength 525 nm. The remaining FLA and adsorbed antigen peptide were eluted by 0.1 *M* HCl, and the column was reequilibrated by the phosphate buffer for the next measurement.

In the second procedure (procedure 2), 100 μl of a sample solution was mixed with 50 μl of a FLA solution and equilibrated for 5 min, and 100 μl of the mixture was injected into the column. After washing for 10 min, the bound insulin and FLA were eluted by the antigen peptide and 0.1 *M* HCl in the same way as the procedure 1. This procedure is a kind of the simultaneous sandwich method and simpler than the procedure 1.

In some experiments the effects of the concentration and amount of the eluent, as well as the effect of liquid flow-rate at the elution step, were studied.

2.5. Determination of adsorption equilibrium

The immunoadsorbent was packed in an adsorption column of 1.26 cm diameter, and the column was equilibrated with the phosphate buffer (pH 7.6) at a flow-rate of 1–2 cm^3 min^{-1} and 23±2°C. The antigen solutions of various concentrations were applied to the column. After washing with the equilibration buffer, the adsorbed antigen was eluted by 0.1 *M* HCl. The absorbance of the effluent solution at 215 nm was continuously measured by a spectrophotometer. The total amount of adsorbed antigen was obtained by numerical integration of breakthrough curves, assuming that the total void fraction of the packed bed is 0.95.

According to Pauling et al. [8], the effective association constant K_0 for polyclonal antibody can

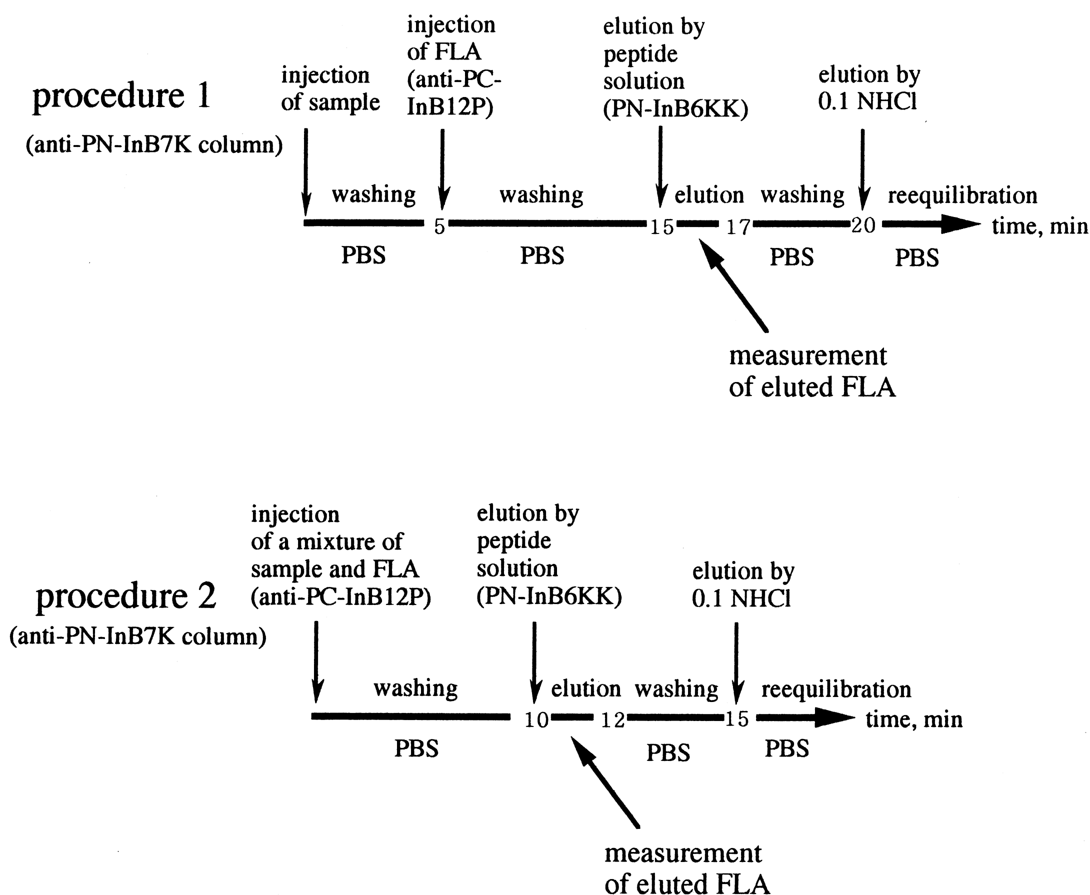

Fig. 2. Two assay procedures used in this work.

be estimated by assuming that the free energy of antigen–antibody combination can be described by the normal distribution function:

$$y = 1 - \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{e^{-\alpha^2}}{1 + K_0 C\, e^{\alpha\sigma}}\, d\alpha \qquad (1)$$

$$\alpha = \ln(K/K_0)/\sigma \qquad (2)$$

where C is the liquid phase concentration of free antigen; K is the association constant; σ is the heterogeneity of K; and y is the fractional saturation of antibody.

3. Results and discussion

Fig. 3 shows adsorption isotherms of bovine insulin B chain and insulin to anti-PC-InB12P and anti-PN-InB-7K antibodies, and the calculated values of K_0 and σ are summarized in Table 1. Two anti-PC-InB12P antibodies from different sera were used in this work, but the isotherms of one of them (anti-PC-InB12P-2) are shown in Fig. 3. Both anti-PC-InB-12P and anti-PN-InB7K antibodies showed higher adsorption capacities and association constants for insulin B-chain than bovine insulin.

The results obtained by the procedure 1 (anti-PN-InB-7K column and FITC-labeled anti-PC-InB12P-1, 0.295 mg IgG cm^{-3}) are shown in Fig. 4, in which the peak height of FLA eluted from the anti-PN-InB7K column with 2 cm^3 of the PN-InB6KK solution (0.2 mg cm^{-3}) was plotted against the concentration of insulin B-chain in the range from 0.1 $\mu g\, cm^{-3}$ to 5 $\mu g\, cm^{-3}$. The small peak height

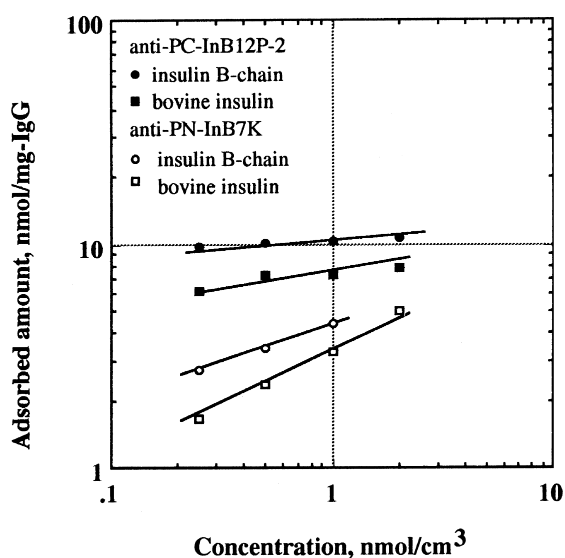

Fig. 3. Adsorption equilibria of bovine insulin and insulin B chain to anti-PC-InB12P-2 and anti-PN-InB7K antibodies.

obtained by the elution without injection of the sample solution was subtracted from the observed peak heights. The peak height first increased linearly with the insulin B chain concentration and reached a plateau. The reproducibility of the measurements was ±15%, and the lower limit of measurable concentration was 0.1 mg cm^{-3}, which is lower than one-fiftieth in the case of the direct method.

From the measurement of the absorbance of the effluent from the column, the pass fraction of injected insulin B chain from the column was negligibly small. This is because the immunoadsorbent packed in the column (0.66 mg–IgG/0.5 cm^3 adsorbent) had an enough adsorption capacity to adsorb insulin B-chain injected. Adsorbed FLA was specifically eluted by PN-InB-6KK in this case. It might seem possible to elute specifically FLA by PC-InB11P, since FLA adsorbed should be eluted by PC-InB11P. This elution method, however, gave extremely large intensities of eluted peaks, because FLA was eluted also from insulin B-chain molecules nonspecifically adsorbed on the support or ligands, which is schematically shown in Fig. 5. Thus, it is necessary to elute by the antigen peptide corresponding to the antibody coupled to the support in order to avoid the effect of nonspecific adsorption of the target protein.

With use of anti-PC-InB-12P column, FITC labeled anti-PN-InB-7K antibody and PC-InB-11P, similar results were obtained (data not shown), but the peak heights were lower than those in the former case. Since the association constant of anti-PN-InB-7K antibody was lower than those of anti-PC-InB-

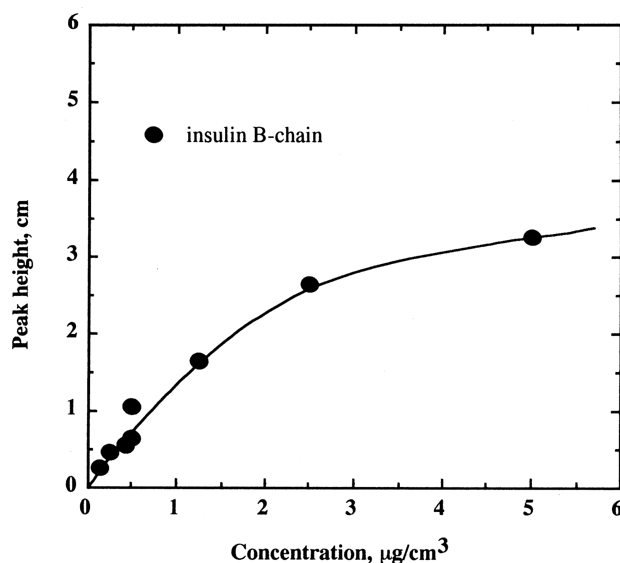

Fig. 4. Measurements of insulin B-chain by procedure 1 (anti-PN-InB-7K column; 0.66 mg–IgG/0.5 cm^3, FITC-labeled anti-PC-InB12P-1 antibody; 0.295 mg–IgG cm^{-3}, liquid flow-rate; 1 cm^3 min^{-1}, eluent; 2 cm^3 of 0.2 mg cm^{-3} PN-InB-6KK).

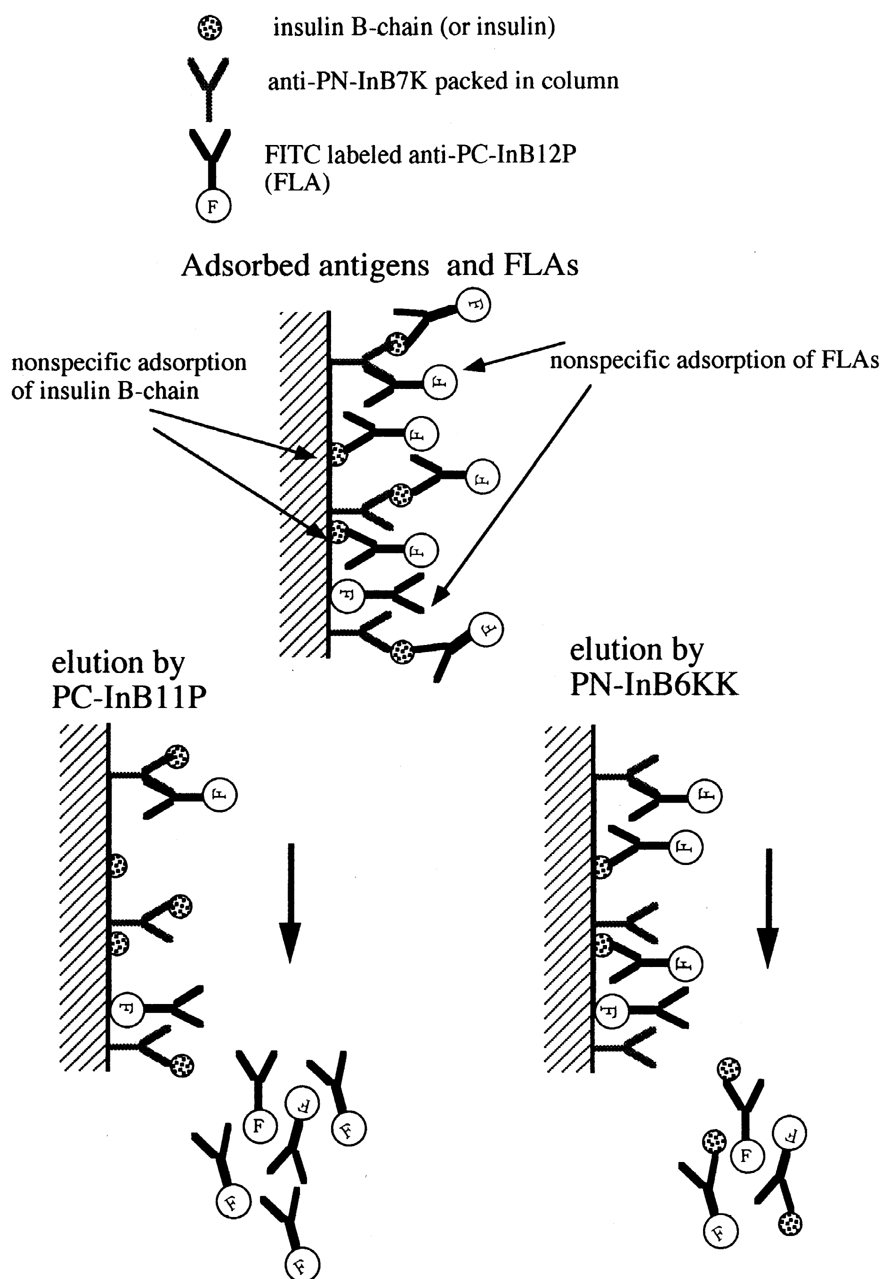

Fig. 5. Difference in elution schemes between PN-InB6KK and PC-InB11P.

12P antibodies, as shown in Table 1, less amount of FLA could bind to a definite amount of insulin B-chain adsorbed on anti-PC-InB-12P antibody column.

Fig. 6 shows the results obtained by the procedure 2 (anti-PN-InB-7K column and FITC-labeled anti-PC-InB12P-2, 0.17 mg–IgG cm^{-3}). In this case the peak heights increased linearly from 0.1 $\mu g\ cm^{-3}$ to 3 $\mu g\ cm^{-3}$ in the measurement of insulin B-chain and were higher than those in the procedure 1,

Table 1
Average association constant and heterogeneity index

Antibody	Antigen	K_0 (M^{-1})	σ
anti-PC-InB12P-1	Insulin B chain	$7.9\cdot10^{6}$	2
anti-PC-InB12P-2	Insulin B chain	$3.4\cdot10^{7}$	2
	Insulin	$1.8\cdot10^{7}$	2
anti-PN-InB7K	Insulin B chain	$1.2\cdot10^{6}$	2
	Insulin	$8.0\cdot10^{5}$	2

because a larger amount of FLA was mixed with the sample solutions. The sensitivity was almost the same in the procedure 1. The peak heights in the measurement of insulin were lower than one third of those in insulin B chain because of the lower adsorption capacity and association constant.

4. Discussion

The effects of the concentration and volume of the eluents used in specific elution of FLA are summarized in Table 2. With increase in the concentration and volume, the peak heights increased, however the effects of the volume were slight. The effects of the concentration in the range from 0.1 to 0.2 mg cm^{-3} and of the liquid flow-rate in the range from 1 to 2 cm^{3} min^{-1} (data not shown) were also slight. Therefore, the elution condition using 2 cm^{3} of the 0.2 mg cm^{-3} eluent at a flow-rate of 1 cm^{3} min^{-1} is reasonable for the requirement of obtaining relatively high outputs with saving the antigen peptide.

Table 2
Effects of concentration and volume of eluent on peak height (peak height: cm, anti-PN-InB7K column; FLA: anti-PC-InB12P-2 antibody, eluent: PN-InB6KK)

Peptide concentration (mg cm^{-3})	Volume of eluent (cm^{3})		
	0.5	1.0	2.0
0.05	–	–	3.5
0.1	–	–	4.7
0.2	–	4.3	4.5
0.4	4.5	5.4	5.9

In procedure 2 the antigen in a sample was equilibrated with FLA before injection to the anti-peptide antibody column. Thus, there should be some relationships between the peak height of eluted FLA and the concentration of FLA coupled with the antigen, since all the antigen supplied to the column was adsorbed, as stated above. The equilibrium concentrations of FLA coupled with insulin B-chain in samples at various concentrations of insulin B-

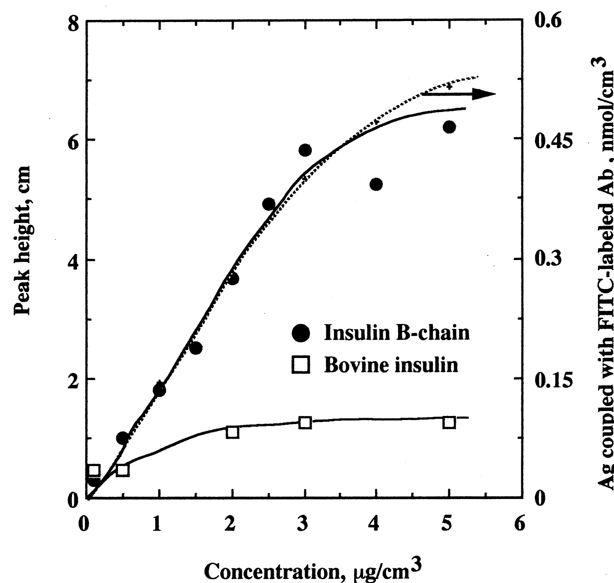

Fig. 6. Measurements of insulin B chain and bovine insulin by procedure 2 (anti-PN-InB7K column; 0.66 mg–IgG/0.5 cm^{3}, FITC-labeled anti-PC-InB12P-2 antibody; 0.17 mg–IgG cm^{-3}, liquid flow-rate; 1 cm^{3} min^{-1}, eluent; 2 cm^{3} of 0.2 mg cm^{-3} PN-InB6KK).

chain were calculated by use of Eqs. (1) and (2) and the values of K_0 and σ for anti-PC-InB12P-2 antibody. The dotted line in Fig. 6 shows the calculated concentrations plotted against the concentration of insulin B-chain and agrees well with the curve showing the dependence of the peak height on the concentration. Although only a part of FLA coupled to the column was eluted under the present elution condition, the peak height was correlated to the equilibrium concentration of the antigen coupled with FLA.

5. Conclusions

By the proposed method using anti-peptide antibodies and the specific elution with antigen peptides, the concentration of the target antigens were measured without the effect of nonspecific adsorption of fluorescence-labeled secondary antibodies. The sensitivity and accuracy were much improved in comparison with those of the direct method. This method enables rapid monitoring of the concentrations of biomaterials and real-time control of bioprocesses depending on the measured values, and can be applied to any targets, in case a specific eluent of the target from an antibody is found. The target should be adsorbed by a column coupled with this antibody and be eluted by the specific eluent, which can be a mimotope or a competing agent with the target.

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