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Short communication

Immunogenicity differences between intact insulin and a peptide representing the C-terminal region

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Abstract

Immunization of rabbits using a peptide corresponding to the 12 amino acids of the C-terminal region of porcine insulin B-chain produced an anti-peptide antibody reactive with the native insulin, as well as with the peptide. It was also found that the adsorption characteristics of the anti-peptide antibody against insulin and peptides, including its reactivity against the insulin A-chain, were different from those of anti-native insulin antibodies. The reactivity against the insulin A-chain was raised by maturation of the anti-peptide antibody. These results show that peptide immunization may cause different immuno-responses, and have some advantages for obtaining antibodies possessing desirable characteristics. © 1997 Elsevier Science S.A.

Keywords: Peptide immunization; Anti-peptide antibody; Insulin; Immunogenicity

1. Introduction

In recent papers [1,2], we have reported on the immuno-recognition of anti-peptide antibodies and their application to the separation and detection of biomolecules. By using synthesized peptides, anti-peptide antibodies which react with target proteins may be obtained without purified antigens, because antibodies against short peptides consisting of the surface portions of proteins can, in some cases, bind to the native proteins [3–5]. Such anti-peptide antibodies are useful for immunoaffinity purification and immunodetection in cases where a sufficient amount of antigen (target protein) of suitable purity for immunization is not available. We applied anti-peptide antibodies to the purification of rice α-amylases secreted from recombinant yeasts, and postulated that peptide immunization would be a useful strategy when target proteins have biological activity and/or toxicity, or possess a very high degree of homology with the corresponding proteins of immunized animals [2]. We found that a synthetic peptide, PC-InB12P, which corresponds to residues 19–30 of the B-chain of porcine insulin, caused different immuno-responses from those of intact porcine insulin. High homology between the insulin used as an antigen (porcine) and the host insulin (rabbit) resulted in raising an anti-insulin antibody of low affinity, because they differ in only one amino acid (the C-terminal amino acid of the insulin B-chain). However, the peptide having the same amino acid sequence as the C-terminal region of porcine insulin could raise an anti-peptide antibody which bound with porcine insulin with 10-fold higher affinity than that of anti-porcine insulin antibody.

Such different immuno-responses of peptide antigens could be of interest in terms of auto-immunity. Much clinical and epidemiological evidence indicates that infections are important in inducing auto-immunity. Particular viral infections frequently precede auto-immune myocarditis and type I diabetes (IDDM) [6,7]. Wuchenpfennig and Strominger and co-workers found that some viral and bacterial mimicry peptides could efficiently stimulate myelin basic protein-specific T cell clones from multiple sclerosis (MS) patients, indicating that molecular mimicry of this immunodominant self-peptide by viruses presents a possible mechanism for the induction of auto-immunity in MS [8].

Here, we compare the characteristics of an anti-peptide antibody against the C-terminal region of porcine insulin B-chain with those of anti-native insulin antibodies by using substituted peptides, native insulin and an insulin A-chain, and clarify the differences in the immunorecognition of these antibodies.

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Table 1
Primary structures of insulins used in this work

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
A-chain																														
Rabbit	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile	Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn									
Porcine																														
Sheep					Ala		Gly	Val																						
B-chain																														
Rabbit	Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala	Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ser
Porcine																														Ala
Sheep																														Ala

Only residues differing from rabbit insulin are shown for the insulins of the other species.

2. Materials and methods

2.1. Materials

The insulins and peptides used for immunization, measurement of adsorption characteristics and ELISA were porcine and sheep insulin, and peptides representing the C-terminal region of porcine insulin B-chain and synthesized porcine insulin A-chain. Their primary structures are summarized in Tables 1 and 2. PC-InB12P is a peptide representing residues 19–30 of porcine insulin B-chain and was used for immunization to obtain an anti-peptide antibody. The peptides PC-InB11P, PC-InB11P (A–T), PC-InB11P (P–G) and PC-InB11P (Y–T), were used to measure the adsorption characteristics of the obtained anti-peptide and anti-insulin antibodies. PC-InB11P corresponds to residues 20–30 of the B-chain (porcine); the other three peptides were obtained by single amino acid replacements. PC-InB8P corresponds to residues 23–30. A peptide representing residues 1–21 of porcine insulin A-chain (P-InA21P) was also synthesized. The peptides were all synthesized by the solid-phase method (431A, Applied Biosystems, Foster, USA) and purified by an HPLC system (LC-10A, Shimadzu, Kyoto, Japan) and a reverse-phase column. The sequences of the synthesized peptides were confirmed by a peptide sequencer (Procise 492, Applied Biosystems). Crystalline porcine insulin and sheep insulin were obtained from Sigma (St. Louis, USA). The purities of the peptides and insulin exceeded 90%. Crystalline *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) was also obtained from Sigma. The chemicals used were of reagent grade.

2.2. Preparation of antibodies

The peptide PC-InB12P was coupled to a keyhole limpet hemocyanin (KLH) with glutaraldehyde according to the procedure of Mariani et al. [9] or goat serum albumin (GSA) with MBS according to the procedure of Liu et al. [10]. Rabbits were immunized with a mixture of an antigen solution (1 mg peptide cm^{-3}) and Freund's complete adjuvant (1 cm^3 each). Booster injections were given 3 times in a similar manner at 8-day intervals. To obtain anti-insulin antibodies, rabbits were immuninized with a mixture of an insulin solution (2 mg insulin cm^{-3}) and Freund's complete adjuvant (1 cm^3 each). Booster injections with incomplete Freund's adjuvant were given 5 times for porcine insulin and 3 times for sheep insulin at 7-day intervals. In the case of insulin immunization, a 20% glucose solution (20 cm^3) was administered at every immunization by subcutaneous injection, and a 5% sucrose solution was given per os to rabbits to avoid severe hypoglycemia.

Specific antibodies were purified from pooled sera by affinity chromatography using antigen-coupled Sepharose 4B. To obtain immunoadsorbents, antibodies were coupled to CNBr-activated Sepharose 4B (Pharmacia LKB Biotech, Uppsala, Sweden). The amounts of antibodies coupled were 0.42 and 0.74 mg cm^{-3} bed for anti-PC-InB12P and anti-sheep insulin antibodies, respectively.

2.3. Conventional ELISA

The following indirect ELISA protocol was used [11]. Microtiter plates with 96 flat-bottomed wells (Coster 3590,

Table 2
Primary structures of peptides used in this work

	1	2	3	4	5	6	7	8	9	10	11	12
PC-InB12P	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro	Lys	Ala
PC-InB11P	–											
PC-InB8P	–	–	–	–								
PC-InB11P(Y–T)	–							Thr				
PC-InB11P(P–G)	–									Gly		
PC-InB11P(A–T)	–											Thr

PC-InB12P corresponds to residues 19–30 of porcine insulin. Only a residues differing from PC-InB12P are shown for the substituted peptides.

Data Packing, Cambridge, MA) were used for ELISA. Two types of buffer solutions, phosphate-buffered saline (PBS) and PBS containing 0.05 vol.% Tween 20 (PBST), were used for washing the plates. Each well was coated with 100 mm^3 of various concentrations of antigen solution in PBS for 20 h at 4 °C. After washing with PBS, all wells were coated for 1 h with 200 mm^3 of 4-fold diluted Block Ace (Snow Brand Milk, Sapporo, Japan) to decrease non-specific adsorption. After washing with PBST, various concentrations of antibody were added to the wells (50 mm^3/well) and incubated for 1 h at room temperature. After washing with PBST, each well was incubated with 100 mm^3 of ×2000 diluted anti-rabbit IgG-horseradish peroxidase solutions in 10-fold diluted Block Ace for 1 h. After washing with PBST, colour was developed using 100 mm^3/well of ABTS solution (0.3 mg cm^{-3} in 0.1 M citrate buffer containing 0.01% H_2O_2, pH = 4.1). The absorbance of each well was recorded by a microplate reader (MTP-32, Corona, Ibaraki, Japan) at 415 or 405 nm.

2.4. Measurement of adsorption capacity

The immunoadsorbent (about 5 cm^3) was packed in an adsorption column 1.26 cm in diameter which was equilibrated with an equilibration buffer (PBS, pH 7.6) at a flow rate of 1–2 cm^3 min^{-1} and 23 ± 2 °C. A solution containing the peptide or insulin (0.2–3.5 nmol cm^{-3} in PBS) was applied to the column. After washing with the equilibration buffer, the adsorbed peptide or insulin was eluted by 0.1 M HCl. The absorbance of the effluent solution at 215 nm (peptide) or 280 nm (insulin) was continuously measured by a spectrophotometer (SPD-6AV, Shimadzu). The total amount adsorbed was obtained by numerical integration of breakthrough curves, assuming the total void fraction of the packed bed to be 0.96.

3. Results and discussion

3.1. Binding characteristics of anti-peptide and anti-insulin antibodies

Fig. 1 shows adsorption isotherms of the peptides and insulin to anti-PC-InB12P antibody (Fig. 1(a)) and anti-sheep insulin antibody (Fig. 1(b)). These antibodies were purified using the respective antigens, i.e. PC-InB12P for anti-PC-InB12P antibody and sheep insulin for anti-sheep insulin. Fig. 1(a) shows that the anti-peptide antibody adsorbed insulin as well as the peptides. The adsorption capacity of PC-InB8P was the same that of PC-InB11P. In the case of PC-InB11P (A–T), however, in which Ala at the C-terminal was replaced by Thr, the adsorption capacity was about 30% lower than that of PC-InB11P, while the capacities of PC-InB11P (P–G) and PC-InB11P (Y–T), in which a single amino acid was also replaced, were only one-fourth.

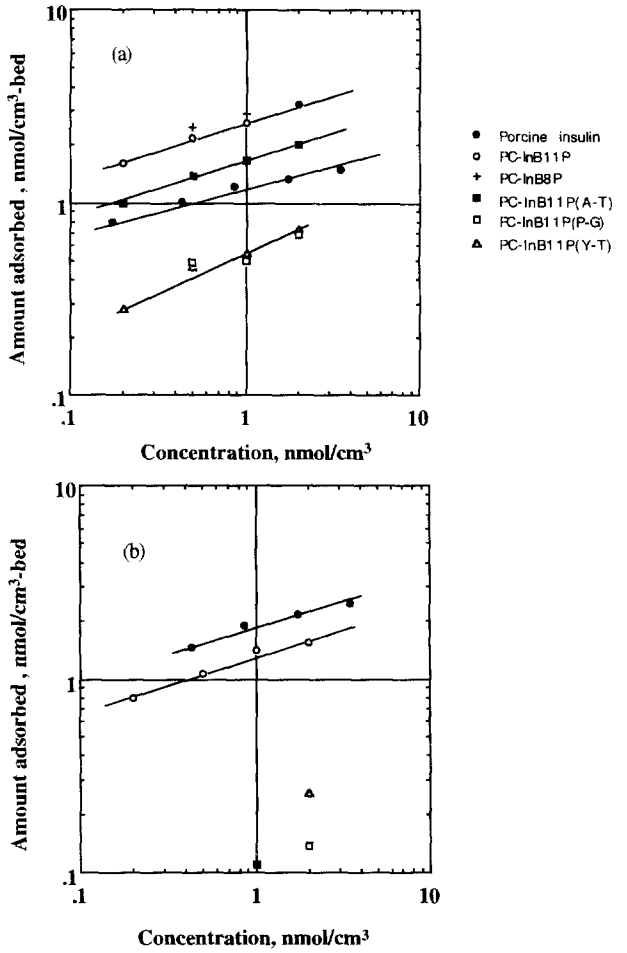

Fig. 1. Adsorption equilibria of peptides and insulin to anti-PC-InB12P antibody (a) and anti-sheep insulin antibody (b).

Fig. 1(b) shows that PC-InB11P, which corresponds to a part of the B-chain of porcine insulin, was adsorbed by anti-sheep insulin antibody, indicating that some parts of anti-sheep insulin antibody can bind to the C-terminal region of the insulin B-chain. The adsorption capacities of PC-InB11P (P–G) and PC-InB11P (A–T) were less than one-tenth that of PC-InB11P, and that of PC-InB11P (Y–T) was only slightly better. The figure shows that the adsorption capacities of these peptides increased in order of the position of the substituted amino acid, starting from Ala, at the C-terminal of PC-InB11P. This is quite different from the case of anti-PC-InB12P antibody, indicating that the C-terminal amino acid (at position 30 of the insulin B-chain) is strongly recognized by the fraction of anti-sheep insulin antibody recognising the B-chain. This may be due to the fact that the B-chains of sheep and rabbit (immunized animal) insulin differ in only one amino acid, at position 30.

Anti-PC-InB12P antibody recognized fewer than 8 amino acids of the C-terminal region and showed the same degree of affinity to Tyr at position 26 and Pro at 28. The effect of replacing Ala at position 30 was minor in comparison with replacements of Tyr and Pro. The structural variety of the peptide resulting from conjugation to KLH or GSA might

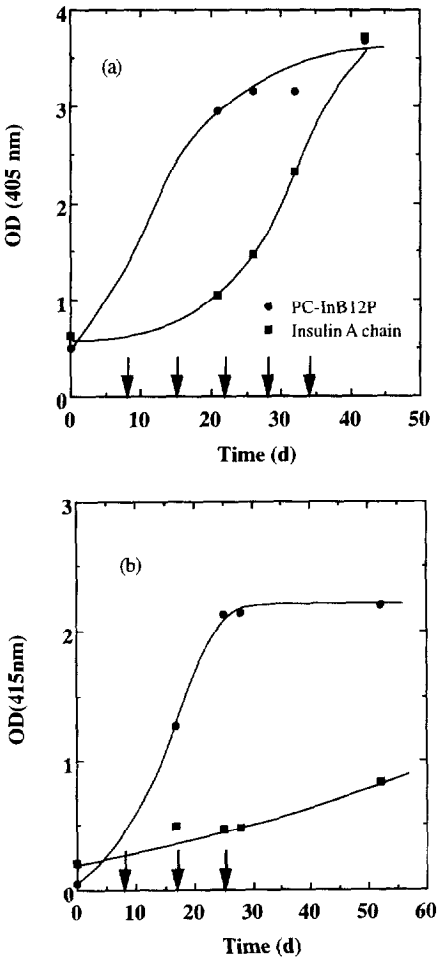

Fig. 2. Time courses of titer increases for anti-porcine insulin antibody (a) and anti-PC-InB12P antibody (b). Antigen concentration: 10 $\mu g\ cm^{-3}$. Antigen: ●, PC-InB12P; ■, P-InA21P. Arrows indicate the times of booster injections.

change the immunogenicity against immunized rabbits and raise an antibody of different characteristics.

3.2. Time course of antiserum titer

Fig. 2 shows the titers of antisera raised against porcine insulin (Fig. 2(a)) and PC-InB12P (Fig. 2(b)) on ELISA using PC-InB12P and P-InA21P as antigens. The titer of anti-porcine insulin serum against PC-InB12P increased rapidly after the third immunization. Although the porcine and rabbit (immunized animal) insulin B-chains, differ in only one amino acid, at position 30, the reactivity against P-InA21P also increased gradually after the fifth immunization. This means that porcine insulin A-chain, which has the same sequence as that of the immunized animal, showed some degree of immunogenicity, and gradually raised antibody populations binding to the A-chain on repeated immunization. Similarly, in the case of anti-PC-InB12P serum, the titer against P-InA21P increased slightly after the fourth immunization, although the synthesized antigen PC-InB12P was perfectly free of contamination with insulin A-chain. The titer increase against P-InA21P may be caused by rabbit insulin A-chain which exists in the rabbit itself.

3.3. Characteristics of antibodies purified from anti-PC-InB12P serum

Two types of antibodies were purified from the antisera against PC-InB12P by use of affinity columns packed with PC-InB12P-coupled Sepharose 4B and P-InA21P-coupled Sepharose 4B. The amounts of specific antibodies purified by P-InA21P and PC-InB12P were 0.17 and 0.31 mg cm^{-3} serum, respectively. The amount of antibody adsorbed by P-InA21P in the antisera was significantly higher than in the cases of antibodies in antisera against other peptides and proteins with sequences completely different to that of insulin (typically in the range 0.02–0.03 mg cm^{-3} serum). These results clearly indicate that immunization with PC-InB12P, i.e. a part of the insulin B-chain, enabled antibody to be raised against the insulin A-chain.

The specific bindings of these purified antibodies were tested using indirect ELISA. Fig. 3, in which the absorbance at 415 nm after 30 min incubation is plotted against the antibody concentration, shows the reactivity of these antibodies against PC-InB12P and P-InA21P. The antibody purified by using PC-InB12P reacted strongly with PC-InB12P but did not react with P-InA21P. On the other hand, the antibody purified by P-InA21P reacted with both P-InA21P and PC-InB12P. The antibody purified using P-InA21P showed stronger affinity with PC-InB12P, indicating that the reactivity of this antibody against the A-chain was acquired with the maturation of antibody populations reactive with PC-InB12P.

It is interesting that immunizing rabbits with PC-InB12P raised the anti-insulin A-chain antibody, because PC-InB12P represents residues 19–30 of porcine insulin, which only dif-

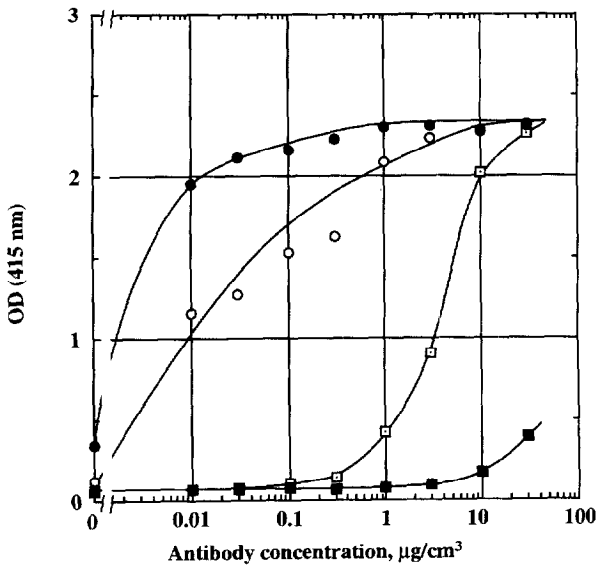

Fig. 3. ELISA of antibodies purified by PC-InB12P (■, ●) and P-InA21P (□, ○) from anti-PC-InB21P serum. Antigen concentration: 10 $\mu g\ cm^{-3}$. Antigens: (○, ●), PC-InB12P; (□, ■), P-InA21P.

fers from rabbit insulin in having Ala at the C-terminal. This anti-insulin A-chain antibody was probably raised by the rabbit insulin A-chain. Immunizing rabbits with human insulin is known to raise at an early stage an antibody reacting only with human insulin, which with maturation can react with both human and rabbit insulin. A similar immune-response might occur when rabbits are immunized with porcine insulin. Recognition of the C-terminal region of the B-chain shifted to the A-chain in the vicinity of this antigenic site on the insulin molecule. Thus, anti-PC-InB12P antibody might cause recognition of the A-chain through the insulin molecule of the immunized host. Although the mechanism is still unclear, the structural variety of the peptide caused by conjugation to KLH or GSA might change its immunogenicity against immunized rabbits and could trigger the immuno-response despite the high similarity with the host insulin.

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References

[1] S. Katoh, Y. Sohma, M. Terashima, E. Sada, Immunorecognition of anti-peptide antibodies and application to separation of biomolecules, in: S. Furusaki (ed.), Biochemical Engineering for 2001, Springer-Verlag, Tokyo, 1992, pp. 509-512.
[2] S. Katoh, M. Terashima, K. Tomioka, N. Hikida, J. Immunol. Methods 191 (1996) 33–38.
[3] Lerner, R.A. (1984), Adv. Immunol 36 (1984) 1–44.
[4] T. Tanaka, D.J. Slamon, M.J. Cline, Proc. Natl. Acad. Sci. USA 82 (1985) 3400–3404.
[5] D.M. Wojchowski, J.M. Sue, A.J. Sytkowski, Biochim. Biophys. 913 (1987) 170–178.
[6] N.R. Rose, L.J. Wolfgram, A. Herskowitz, K.W. Beisel, Ann. NY Acad. Sci. 475 (1986) 146–156.
[7] C.G. Ray, J.P. Palmer, J.R. Crossley, R.H. Williams, Clin. Endocrinol. 12 (1980) 375–378.
[8] K.W. Wucherpfennig, J.L. Strominger, Cell 80 (1990) 695–705.
[9] M. Mariani, L. Bracci, R. Presentini, D. Nucci, P. Neri, G. Antoni, Mol. Immunol. 24 (1987) 297–303.
[10] F.-T. Liu, M. Zinnecker, T. Hamaoka, D.H. Katz, Biochemistry 18 (1979) 690–697.
[11] K. Tomioka, H. Fukuda, S. Katoh, J. Ferment. Bioeng. 79 (1995) 284–286.