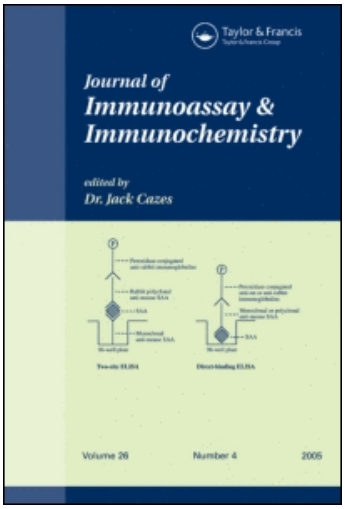

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Direct Enzyme-Linked Immunosorbent Assay of Anti-Peptide Antibodies Using Capture of Biotinylated Peptides by Immobilized Avidin

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DIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY OF ANTI-PEPTIDE ANTIBODIES USING CAPTURE OF BIOTINYLATED PEPTIDES BY IMMOBILIZED AVIDIN

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ABSTRACT

Synthetic peptides were prepared by a solid-phase method and biotinylated selectively and in high yield at the amino terminus prior to peptide deprotection and detachment from synthesis resin. It was shown that peptides biotinylated in this manner could be bound by avidin immobilized on a plastic surface and used to detect anti-peptide antibodies in an enzyme-linked immunosorbent assay. The advantages of this method compared to conventional immunoassay techniques for anti-peptide antibodies are discussed.

INTRODUCTION

The exceptionally strong binding of the water-soluble vitamin biotin by the egg-white protein avidin (1) has been utilized widely for protein labelling, detection and purification. The success of such methods derives partly from the fact that most polypeptides can be readily biotinylated and that the covalently linked

biotin moieties retain their capacity to be bound by avidin.

Biotinylation of polypeptides is usually carried out with active ester derivatives of biotin. The reaction is thus non-specific in that any nucleophilic group in the polypeptide may react. In the case of synthetic peptides, biotin can be introduced specifically at the amino terminus after solid-phase synthesis and before side-chain deprotection and cleavage (2). Such on-resin biotinylations are particularly efficient and convenient. An important application of on-resin biotinylation, *viz.* the one-step purification of chemically synthesized proteins present in low yield, has recently been demonstrated (3).

The detection of antigen-antibody interactions in immunochemical assays can be amplified by the use of the avidin-biotin system. In these procedures biotinylated antigens and/or antibodies are used, together with avidin as a bridge; amplification is provided due to several biotin binding sites being present on each avidin molecule.

Antibodies against synthetic peptides have recently become valuable tools in such applications as immunoaffinity isolation of proteins, study of protein-protein interactions, diagnostics, *etc.* The screening of such antibody preparations requires simple and

reliable techniques. In our experience, synthetic peptide antigens, particularly if these are hydrophilic and containing less then ca. 20 amino acid residues, are difficult to immobilize on plastic surfaces for the purposes of sufficiently sensitive direct immunosorbent assays. Such assays are often required to evaluate and compare titres of anti-peptide sera or for the study of protein antigenic sites. 'Sandwich' assay procedures are not usually advisable, as small peptides are unlikely to be multivalent antigens. Alternatively, but from a practical viewpoint, less satisfactorily, peptide antigens can be conjugated to carrier proteins and immobilized in this form, or they can be bound covalently to chemically activated surfaces (4). A different approach is the use of certain peptide synthesis resins, together with acid-stable linkage agents between resin and peptide, yielding water-soluble peptidyl resin complexes which can be immobilized directly on microtitre plates (5).

We wish to report a direct enzyme-linked immunosorbent assay (ELISA) procedure which avoids the problems of peptide antigen immobilization. This technique takes advantage of the convenient on-resin peptide biotinylation. Instead of immobilizing the peptides directly on the solid phase, corresponding biotinylated peptides are captured by immobilized avidin.

MATERIALS AND METHODS

Chemicals

9-Fluorenylmethyloxycarbonyl-amino acid (Fmoc-amino acid) derivatives and synthesis resin (Pepsyn KA, 0.1 meq/g) were obtained from MilliGen, Lane Cove, N.S.W. Peptide synthesis grade N,N-dimethylformamide (DMF) and trifluoroacetic acid (TFA) were from Auspep Pty. Ltd., South Melbourne, Vic. Other synthesis reagents and solvents were of the highest grade available. Egg white avidin, biotin-ε-aminocaproic acid N-hydroxysuccinimide ester (biotin-X-NHS) and affinity-purified (H- and L-chain specific) anti-mouse IgG-horseradish peroxidase (HRP) conjugate were purchased from Calbiochem Corp., La Jolla, Ca., USA. Streptavidin-biotinylated HRP complex was bought from Amersham Australia Pty. Ltd., North Ryde, N.S.W. Biotin-NHS, bovine serum albumin (BSA), keyhole limpet haemocyanin (KLH), HRP, o-phenylenediamine, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) and Tween-20 were supplied by Sigma Chemical Co., St. Louis, Mo., USA; 2,4,6-trinitrobenzensulphonic acid (TNBSA) and N,N-diisopropylethylamine (DIPEA) by Fluka AG, Buchs, Switzerland.

Peptide Synthesis

Continuous-flow Fmoc-polyamide solid-phase synthesis was carried out semimanually, essentially as previously described (6). Oxo-benzotriazine (Ser and Thr) and pentafluorophenyl esters (all other amino acids) of the Fmoc-L-amino acids were used in the coupling reactions. Amino acid side chains were protected as t-butyl esters (Asp and Glu), ethers (Ser, Thr and Tyr) and urethanes (Lys and His). The guanidino and thiol groups of arginine and cysteine were protected by the 4-methoxy-2,3,6-trimethylbenzenesulphonyl and trityl groups, respectively. Resin samples were removed after each coupling step for TNBSA colour testing (7). Cleavage and side-chain deprotection was accomplished for appropriate lengths of time with TFA, containing suitable mixtures of scavenger chemicals. The peptides were purified successively by gel filtration in 10 % (v/v) aqueous acetic acid on Sephadex G-10 and reverse phase high-performance liquid chromatography (HPLC) with water-acetonitrile gradients (containing 0.1 % (v/v) TFA). The integrity of purified materials was ascertained by amino acid analysis.

Immunizations

Peptides (25 mg) were coupled to KLH (10 mg) with MBS via cysteine thiol groups (8). Groups of one-week

old female Swiss albino mice were immunized intraperitoneally, each with 100 μg peptide-KLH conjugate in 200 μL phosphate-buffered saline (PBS; 10 mM phosphate, 150 mM NaCl, pH 7.4). Booster injections were given after 4, 9 and 16 days. Blood was collected 5 days after the last booster. Free peptides (50 μg/injection) were administered with Freund's adjuvant (complete adjuvant for initial injection, incomplete for boosters).

Biotinylation Reactions

Aliquots (100 mg) of the dry side-chain protected peptidyl resins were swollen in 1 mL of DMF. The solvent was then decanted and 22 mg biotin-X-NHS (approximately corresponding to a 5-fold molar excess over free amino groups on the peptidyl resin) in 1 mL DMF was added. The reaction vessels were then flushed with nitrogen and incubated at 45 ^{o}C for 18 h. At the end of this period the peptidyl resin samples were filtered, washed successively with 20 mL each of DMF, dichloromethane and diethyl ether, and then dried *in vacuo*. The biotinylated peptides were cleaved from the resins and purified in the same manner as described above. BSA and HRP were biotinylated as follows: 100 mg of the proteins were dissolved in 3 mL PBS. To the stirred

solutions were added slowly 20 mg biotin-NHS in 1 mL DMF. The mixtures were stirred for a further 4 h at room temperature and were then dialysed against several changes of PBS.

Testing of Biotinylation Reactions

After biotinylation and DMF-washing, samples (ca. 2 mg) were removed from the peptidyl resins for testing of the extent of reaction of the N-terminal amino groups. The samples were added to 0.5 mL each of freshly prepared solutions containing 10 % (v/v) DIPEA and 1 % (w/v) TNBSA in DMF. The samples were observed for colour development. Separate peptidyl resin aliquots were tested for the presence of biotinyl groups. The resin samples were transferred to 5 mL PBS, containing 0.1 % (w/v) Tween-20. After 20 min they were filtered and incubated for 3 h at room temperature in 0.2 mL 1/500 diluted (with 4 % (w/v) BSA, 0.05 % (w/v) Tween-20 in PBS) streptavidin-biotinylated HRP complex. The samples were filtered again and washed on the sinter with 5 x 2 mL (2 min each) 0.05 % (w/v) Tween-20 in PBS. The colour was developed as described in the ELISA section below. For both tests corresponding non-biotinylated peptidyl resin samples served as controls.

ELISA

The wells of microtitre plates (PVC, high binding; from Flow Laboratories, Melbourne, Vic.) were coated with the appropriate antigen (i.e. 30 nmol free peptide or 50 μg peptide-BSA) or 12 μg avidin, dissolved in 150 μL PBS, overnight at 4 °C. After aspirating, the wells were washed with 0.05 % (w/v) Tween-20 in PBS (3 x 5 min). In order to minimize non-specific binding in the subsequent steps, blocking was performed for 3 h at room temperature with 200 μL/well of 4 % (w/v) BSA, 0.05 % (w/v) Tween-20 in PBS. Dilutions of biotinylated peptides (15 nmol/well), antisera and anti-mouse IgG-HRP conjugate (1/2,000) were also made into this blocking solution. Incubations (150 μL/well) were always 2 h long, followed by washing cycles as above. Colour development was with 150 μL/well of 0.5 mg/mL o-phenylenediamine, 0.01 % (v/v) H_2O_2 in 0.1 M phosphate/citrate buffer, pH 5, in the dark. The reactions were stopped by the addition of 50 μL/well of 4-M H_2SO_4. The colour was measured immediately in a Titertek Multiscan plate reader at 492 nm. Absorbance readings were corrected for background (determined with non-immune sera).

RESULTS

We chose four model peptides ranging in length from 9 to 22 amino acid residues and of varying hydro-

TABLE 1

Synthetic Peptides Studied

Peptide	Sequence[a]	Average hydrophilicity[b]
A	CISSMKKHA	4.64
B	CWVDNEEDIDVIIKA	2.96
C	CLKGNNLIWTLKDSAGEA	2.71
D	CEKIIKTIDNFLEKRYEKWIEA	2.01

[a] N-terminal Cys residues were incorporated for carrier protein conjugation, and C-terminal Ala residues for facile resin attachment.

[b] As calculated by the hydrophilicity scale of Parker et al. (12) for the amino acids.

philicities (**Table 1**). The sequences of the peptides were taken from that of tetanus toxin (9) and were used in a study of immunochemical aspects of tetanus toxin.

On-resin biotinylations were carried out with the extended bridge biotin derivative biotinyl-ε-amino-caproic acid-N-hydroxysuccinimide ester (10). At 45 °C, the reactions were nearly complete after two hours and biotinylation was quantitative, as judged by the TNBSA test, after overnight incubation. The presence on the resin of biotin groups available for binding by avidin was further confirmed by testing with streptavidin-biotinylated HRP complex. The resistance of the biotin moiety to liquid hydrogen fluoride (containing thiol

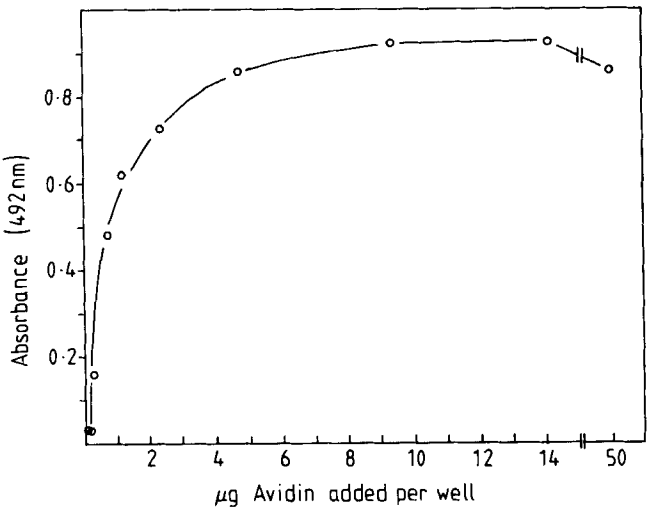

FIGURE 1 Various amounts of avidin in 150 μL PBS were added to the wells of microtitre plates. The plates were kept in the refrigerator overnight. After blocking and washing, bound avidin was detected by incubation with biotinylated HRP and colour developed by exposure to enzyme substrate.

scavengers to prevent modifications of the biotin thioether function) cleavage has been demonstrated (2,3). We employed the milder TFA cleavage method which is used in the Fmoc/t-butyl protection scheme for solid-phase peptide synthesis.

The coating of microtitre plate wells (**Fig. 1**) was performed with 12 μg avidin in PBS. Using 50 mM sodium bicarbonate, pH 9.6, or 50 mM sodium acetate, pH 5.5 as coating buffers gave somewhat lower binding. Immobilization of BSA-biotin, followed by saturation

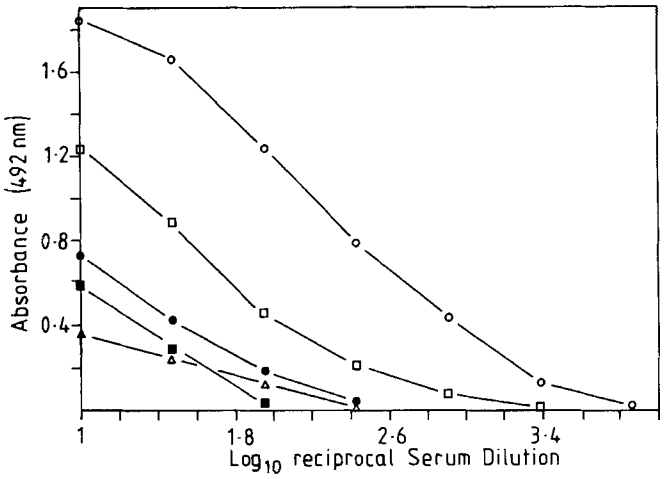

FIGURE 2 The binding of anti-peptide **A** IgG in pooled mouse sera to peptide **A** immobilized in various ways on microtitre plates was measured by ELISA:

Serum raised against	Peptide immobilized as	Symbol
Peptide-KLH	Free peptide	△
Free peptide	Peptide-BSA	●
Peptide-KLH	Peptide-BSA	○
Free peptide	Avidin/biotin-peptide	■
Peptide-KLH	Avidin/biotin-peptide	□

with avidin, also apparently did not increase the number of available biotin binding sites in the wells.

As can be seen from **Fig. 2**, in the case of the small and strongly hydrophilic peptide **A** (**Table 1**), the apparent titre of anti-peptide-KLH serum is very low when the free peptide is coated. Furthermore, when serum raised against the free peptide was assayed in the same manner, no measurable titre was detected. Antibodies raised against the free peptide could be

TABLE 2

Titres of Anti-Peptide IgG Antibodies in Antisera Raised Against Peptide-KLH Conjugates as Measured Using Assay Procedures with Immobilized Peptides, their BSA-Conjugates and Avidin-Captured Biotinylated Peptides

Peptide	Titres[a]		
	Immobilized:		
	Free peptide	BSA-peptide	Avidin/biotin-peptide
A	240	7680	2430
B	480	4860	2430
C	960	15360	7680
D	1920	7680	3840

[a] Reciprocal of highest serum dilution giving statistically significant above-background ELISA absorbance reading

detected, however, when the peptide-BSA conjugate or the avidin/biotin-peptide was used in the assay.

The differences between apparent titres of anti-peptide antibodies, using plates coated with either free peptides or avidin/biotin-peptides, were less pronounced for the longer and more hydrophobic peptides (**Table 2**), as expected. Here again using capture of biotinylated peptides with immobilized avidin resulted in titres approaching those from assays with immobilized BSA-peptides.

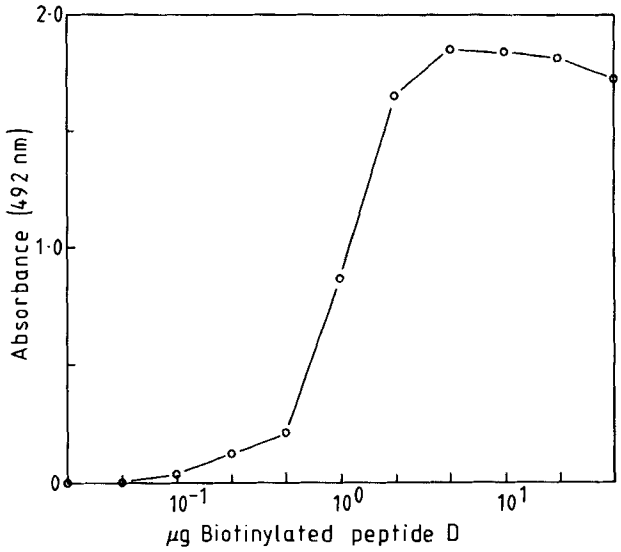

FIGURE 3 The effect of adding increasing quantities of biotinylated peptide D to immobilized avidin (12 μg/well), keeping the concentration of anti-peptide D serum constant (serum dilution of 1/200), was determined by ELISA.

The optimal concentration of the biotin-peptides for the avidin capture assays was determined by titration of the biotin-peptides at a single anti-peptide serum dilution. The results from such an experiment are shown in **Fig. 3** for peptide **D**. In each case the lowest amount of biotin-peptide giving maximal binding corresponded to 10 - 15 nmol.

Intra-assay coefficients of variation for the assays using the novel avidin/biotin-peptide technique, determined by assaying the same dilutions of each serum

in six wells of a plate, were found to lie below 7 % throughout. Interassay coefficients, using a uniform colour development time of 10 min, for the same sera, were found to range from 20 to 25 %.

DISCUSSION

The assay procedures described constitute a simple and efficient way of measuring specific anti-peptide antibodies in sera raised against free peptides or their conjugates. Furthermore, since the amount of avidin coated per well can be standardized and the quantitative nature of biotinylation reactions of peptides verified, the method reported here permits comparisons between the immunogenicities of various peptide materials. This is not the case with peptide-BSA (or other carrier protein) conjugates, because the molar ratios between carrier and peptide are difficult to standardize and determine. Furthermore, different conjugates may not coat with the same efficiency. When cross-reactivity of anti-protein antibodies with different peptides is to be determined quantitatively, the presence of equimolar amounts of peptides is important. This can be ensured by the use of avidin capture of biotin-peptides.

It is clear that in order to avoid loss of detection of some anti-peptide antibodies in an immunoassay through structural or conformational modification of the antigen, attachment of the immunogenic carrier usually necessary for antibody production, as well as attachment of the carrier used for immobilization, should be at the same point. The desired specificity of attachment for the purposes of the proposed avidin/biotin-peptide assays can be obtained by placement of a cysteine residue at the amino terminus of the synthetic peptide. The immunogen can then be prepared by coupling the peptide to a carrier *via* the cysteine side-chain thiol group with the aid of a suitable heterobifunctional reagent. Immobilization of the peptide is *via* the same cysteine residue, since the biotin moiety is introduced at the amino terminus during peptide synthesis.

The use of BSA-peptide conjugates in ELISA's is well established and results in slighly more sensitive assays. However, this advantage is outweighed by those of reliable on-resin peptide biotinylation, which is preferable to the laborious and sometimes difficult carrier protein conjugation. We recently reported that preparation of peptide-protein conjugates for the purposes of raising anti-peptide antibodies in laboratory animals can also be avoided by the use of peptidyl

resin immunogens (11). Using also the ELISA procedure described here it is thus possible to produce and screen anti-peptide antibodies simply and rapidly.

A further possible application of biotin-peptides is the determination of the subclass and isotype of anti-peptide antibodies. Such antibodies could be captured by immobilized anti-sublass/isotype antibodies and detected via the biotin label of the peptides.

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