Monitoring Coupling of Peptides to Carrier Proteins Using Biotinylated Peptide

D. Joseph Jerry
Baylor College of Medicine

INTRODUCTION

Protein conjugates have become important tools in molecular biology and medicine (16,17,21). Synthetic peptides coupled to carrier proteins provide versatile immunogens with which to obtain antibodies against restricted epitopes of proteins or protein sequences predicted from DNA sequences (11). The molar ratio of peptide/carrier is a critical parameter affecting the immunogenic activity of a peptide (2,9). Since efficiency of the coupling reaction can vary considerably among peptides (8%-60%; Reference 3), it is important to evaluate the peptide conjugate before investing the effort in immunizing animals with a poorly coupled product.

Several coupling approaches have been used for production of antipeptide antibodies. Heterobifunctional agents provide the advantage of specificity (1) and have been used most frequently to covalently couple peptides to carrier proteins (18). Typically, the carrier protein is activated by e- amines followed by specific reaction with the sulphydryl group of a cysteine residue that is included in the peptide of interest (11). The reaction can be monitored with radio-iodinated peptide, but iodinations generally employ oxidizing conditions that can destroy reactive groups (1,15) necessary for subsequent coupling reactions or biological activity of the product (1,19). Furthermore, the hazards associated with radio-iodinated conjugates render them unsuitable for immunization of animals or in vivo studies. Therefore, a nonradioactive method was developed.

In this method, peptides must contain free amine groups for biotin-labeling and a cysteine residue for crosslinking. Trace amounts of biotinylated peptide are then used to monitor coupling to a carrier protein that has been activated with a heterobifunctional coupling agent. After the coupled product is separated from the free peptide, incorporation of peptide can be evaluated by slot blot, immunoblot or competitive enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Materials

A fifteen amino acid residue peptide (ALKYDATKQKRKFS), deduced from the open reading frame in the cDNA sequence of D15K1 (8), was synthesized by standard Boc methods on a Model 430A synthesizer (Applied Biosystems [ABI], Foster City, CA) in the microchemistry laboratory at The Jackson Laboratory (Bar Harbor, ME).
A cysteine was introduced at the N-terminus to allow coupling. Ovalbumin (Sigma Grade V) and bovine serum albumin (recrystallized) were obtained from Sigma Chemical (St. Louis, MO). Keyhole limpet hemocyanin (KLH), sulfosuccinimidyl 4-(n-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), IODO-GEN®, and reagents for biotinylation and ELISA were purchased from Pierce (Rockford, IL). [125I]-NaI was purchased from ICN Biochemicals (Irvine, CA).

**Labeling Reactions**

Five micrograms of ovalbumin or peptide were reacted with [125I]-NaI in tubes coated with IODO-GEN at 23°C for 3 min essentially as described by Salacinski et al. (20). Iodinated ovalbumin was purified on a Sephadex G-25 column (Pharmacia Biotech, Piscataway, NJ) (10-ml bed volume) to remove free 125I, then repurified on a 20-ml G-25 column. Iodinated peptide was purified on a 6-ml Sephadex G-10 column, then repurified on a 16-ml Sephadex G-10 column. One milligram of peptide was reacted with 2 mg of NHS-LC-biotin in 200 μl of 50 mM sodium borate, pH 7.6, for 1 h at 23°C. Dithiothreitol was added to a final concentration of 5 mM and incubated for 10 min. Biotinylated peptide was purified twice on a Sephadex G-10 column (16 ml). The specific activity of the biotinylated peptide was approximately 1 nmol/g peptide as determined by the competitive ELISA described below.

**Coupling Reactions**

KLH was activated by incubating 5 mg (500 μl) of KLH with 1 mg of SMCC in 50 mM sodium phosphate, pH 7.2, for 40 min at 23°C with stirring. Bovine serum albumin (BSA) was activated similarly except 2 mg of SMCC and 50 mM sodium borate, pH 7.6, were used. Unreacted SMCC was removed by gel filtration chromatography on Sephadex G-25 (20-ml bed volume; 50 mM sodium phosphate, pH 6.5; 0.9% NaCl; 2.5 mM EDTA). The lower pH for chromatography was chosen to stabilize the maleimide group (14). Approximately 1 mg of SMCC-activated KLH or BSA was reacted with unlabeled peptide for 3 h at 23°C with stirring. Trace amounts of 125I-peptide and/or biotinylated peptide were included in the reaction (Figures 1 and 2). The reaction products were separated by gel filtration on a Sephadex G-50 column (20-ml bed volume). The absorbance at 280 nm and cpm were determined for 600-μl fractions.

**Slot Blot Analysis**

An aliquot of each fraction (50 to 100 μl) was slot blotted onto nitrocellulose (Millipore, Bedford, MA). Biotinylated peptide was detected using the ABC method (7) as follows. The filter was first incubated in PBS with 0.1% Tween 20 (Bio-Rad, Richmond, CA) for 20 min to block nonspecific sites, then washed in PBS three times for 5 min each. The avidin-biotin complex was prepared using a Vectastain kit (Vector Laboratories, Burlingame, CA) as described by supplier. The filter was incubated with the avidin-biotin complex for 15 min, then washed in
PBS three times. The membrane was incubated in substrate buffer containing 10 ml PBS, 2 ml of 4-chloro-naphthol solution (3 mg/ml in methanol) and 5 μl of 30% H₂O₂ until color was fully developed. To evaluate the relative binding characteristics of peptide and protein to the membrane, trace amounts of ¹²⁵I-labeled ovalbumin or peptide were added to stock solutions and serially diluted. One hundred microliters of dilutions were added to 500 μl PBS and slot blotted onto membranes. Each slot was cut out and counted in a Gamma 5000 counter (Beckman, Palo Alto, CA). Next, the pieces of membrane were washed two times in 2 ml PBS, 0.1% Tween 20 for 30 min to simulate the washing of the membrane during detection with biotin-avidin complex, then counted again.

**Biotin ELISA**

Biotinylated calf intestinal alkaline phosphatase (CIAP) (1280 U/mg; 5.3 mol biotin/mol CIAP) was dissolved in PBS containing 0.25% BSA to a final concentration of 100 μg/ml, then frozen in 100 μl aliquots. Each well of ELISA plates (Flow Laboratory, McLean, VA) was coated with 100 μl of avidin solution (5 μg/ml) for 18-24 h at 4°C. The solution was shaken out and 250 μl of 1% BSA in PBS was added. The plates were incubated 1 h at 37°C. Wells were washed 3 times with 300 μl PBS-Tween (PBS; 0.05% Tween 20). Next, 100 μl of standards or samples were added and incubated 1 h at 37°C. The standards consisted of 50 μl of biotinylated-CIAP (diluted to 1 μg/ml in PBS-Tween) and 50 μl of serial dilutions of biotin (0, 0.1, 0.15, 0.2, 0.3, 0.4, 0.5, 0.75, 1, 2, 5 or 10 pmol biotin). The samples contained 50 μl PBS-Tween, 50 μl of biotinylated-CIAP and 1 to 20 μl of sample. Following the incubation, the wells were washed 3 times followed by addition of 100 μl color solution. The color solution was prepared by dissolving 10 mg p-nitrophenyl phosphate in 10 ml substrate buffer (10 mM diethanolamine, 0.5 mM MgCl₂, pH 9.8). The reaction was incubated at 37°C until color had developed (30 min to 1 h), then the A₄₀₅ was determined. The data were plotted as absorbance vs. the log(concentration). The mass of biotin or biotinylated peptide in a sample was calculated using data from the linear portion of the curve.

**RESULTS**

The first objective was to compare coupling of ¹²⁵I-peptide and biotinylated peptide to KLH. In preliminary reactions, coupling of ¹²⁵I-peptide was barely detectable, while biotinylated peptide appeared to be efficiently coupled. To determine whether the ¹²⁵I-peptide itself was damaged during iodination or the preparation was contaminated with inhibitors of the coupling reaction, unlabeled peptide, biotinylated peptide, ¹²⁵I-peptide and SMCC-activated KLH were included.

**Table 1. Binding and Retention of ¹²⁵I-Labeled Ovalbumin and Peptide on Slot Blot**

<table>
<thead>
<tr>
<th>Mass Applied (μg)</th>
<th>Ovalbumin Bound (%)</th>
<th>Peptide Bound (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial⁹</td>
<td>After washes⁹</td>
</tr>
<tr>
<td>25</td>
<td>20.00</td>
<td>16.90</td>
</tr>
<tr>
<td>50</td>
<td>12.48</td>
<td>9.89</td>
</tr>
<tr>
<td>100</td>
<td>8.85</td>
<td>7.41</td>
</tr>
<tr>
<td>250</td>
<td>5.40</td>
<td>4.19</td>
</tr>
</tbody>
</table>

One hundred microliters of each dilution were slot blotted onto nitrocellulose. The slots were cut out, counted, washed two times in PBS with 0.1% Tween 20, then counted again. The amount of ovalbumin or peptide bound is expressed as a percent of the mass applied. The specific activities of ovalbumin and peptide were 1260 cpm/μg and 1389 cpm/μg, respectively.

⁹The percent of ovalbumin or peptide bound prior to washing.

Table 1: Binding and Retention of ¹²⁵I-Labeled Ovalbumin and Peptide on Slot Blot.

**Figure 2. Quantification of peptide coupled to BSA using biotinylated peptide.** The reaction contained unlabeled peptide (350 μg), biotinylated peptide (90 μg) and SMCC-activated BSA (1.2 mg). The products were fractionated on a Sephadex G-50 column, then an aliquot from each fraction was analyzed for biotin by competitive ELISA. Inset: Standard curve for the competitive ELISA.
Table 2. Effect of Carrier Protein Concentration on ELISA

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Absorbance ± SE (405 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KLH</td>
</tr>
<tr>
<td>0</td>
<td>2.181 ± 0.084</td>
</tr>
<tr>
<td>10</td>
<td>2.062 ± 0.105</td>
</tr>
<tr>
<td>20</td>
<td>2.354 ± 0.032</td>
</tr>
<tr>
<td>30</td>
<td>1.947 ± 0.020</td>
</tr>
<tr>
<td>66</td>
<td>1.510 ± 0.017</td>
</tr>
<tr>
<td>166</td>
<td>1.182 ± 0.002</td>
</tr>
</tbody>
</table>

Reactions contained 0.1 µg biotinylated-CIAP plus KLH or BSA added at the indicated concentration. The data are means of duplicate observations.

in a single reaction. The coupling reaction products were analyzed by gel filtration chromatography to separate coupled and free peptide, then an aliquot of each fraction was slot blotted onto nitrocellulose for detection of biotinylated peptide (Figure 1, A and B). Greater than 95% of the KLH was recovered in fractions 14–18 as determined by absorbance at 280 nm. Monomeric 125I-peptide eluted in fractions 24–35. Coupling of 125I-peptide to KLH was negligible, presumably due to oxidation of the thiols groups during iodination (15). In contrast to 125I-peptide, slot blot analysis revealed that the majority of the biotinylated peptide eluted with KLH in fractions 14–17 (Figure 1B; Coupling Reaction) indicative of successful coupling. To ensure that the peptide was not adsorbing to KLH in a noncovalent manner, a parallel reaction was performed in which no SMCC was added (Figure 1B; Sham Reaction). In the sham reaction, the majority of the biotinylated peptide was recovered in fractions eluting near the bed volume. The biotinylated peptide in the sham reaction eluted earlier than monomeric 125I-peptide in this and other experiments. This observation is consistent with dimerization of biotinylated peptide via disulfide bonds, whereas destruction of the thiols in 125I-peptide during iodination prevents dimerization.

Slot blot analysis assumes that both proteins and peptides bind to the membrane with similar efficiencies. Therefore, binding of peptide and a protein (ovalbumin) to nitrocellulose was determined by slot blotting 25 to 250 µg of peptide or ovalbumin containing tracer quantities of 125I-peptide or 125I-ovalbumin onto nitrocellulose. Following washes similar to those used for detection on slot blots, 16.90% to 4.19% of the ovalbumin remained bound to the filter (Table 1). The mass of peptide bound initially was much less than for ovalbumin; only 0.59 to 0.13% of the peptide was retained on the nitrocellulose after washing (Table 1). Therefore, slot blot analysis overestimates the coupling efficiency because of preferential binding and retention of proteins to membrane supports compared to peptides.

To estimate the mass of peptide coupled to carrier protein, a competitive ELISA for biotin was developed. Wells of ELISA plates were first coated with avidin followed by addition of biotinylated CIAP and increasing masses of biotin or sample. Binding of biotinylated CIAP was determined colorimetrically. Preliminary experiments demonstrated that coating the wells with 0.5 µg avidin in combination with 0.05 µg biotinylated CIAP resulted in the desired sensitivity. The limit of detection was <0.1 pmol biotin and was linear to 2 pmol biotin (Figure 2 inset). Biotinylated peptide produced a parallel curve (data not shown) indicating that the avidin-biotin interaction was unaffected by the covalent biotinylation of the peptide. The effects of carrier proteins on this assay are presented in Table 2. The presence of KLH at up to 10 µg/ml was tolerated in the assay; however, greater concentrations resulted in an incremental decrease in absorbance. In contrast, up to 166 µg/ml of BSA had negligible effects on the assay (Table 2).

To test the sensitivity of the competitive ELISA to monitor coupling, a reaction was set up that contained SMCC-activated BSA, unlabeled peptide and biotinylated peptide. The reaction was fractionated and each fraction was assayed for biotin by competitive ELISA. The A280 elution profile revealed both a BSA peak and a free-peptide peak, indicating that a small proportion of peptide was coupled (Figure 2). Likewise, the majority of the biotinylated peptide eluted at the bed volume, with a small amount eluting with BSA at the void volume. The coupling of peptide to BSA was 11% as determined by integrating the areas under the biotin elution profile. From these results the calculated incorporation was 1.4 mol peptide/mol BSA.

**DISCUSSION**

Immunization with peptide conjugates is an effective means to obtain antibodies against specific amino acid sequences. Since peptides by themselves are generally incapable of initiating a complete immune response, coupling to an immunogenic protein is desirable (4). Several factors influence the likelihood of successful production of antipeptide antibodies (4,6,22). Both KLH and BSA are suitable carrier proteins (4,18), and virtually any peptide of greater than 10 amino acid residues will provide a satisfactory antigen (11,18). N-terminal and C-terminal peptides are more likely to produce useful antisera (18), which is presumably related to the mobility of the termini of peptides and proteins (22). For this reason, heterobifunctional coupling reagents are preferred. Succi- midyl-maleimide heterobifunctional agents (e.g., SMCC) allow a cysteine residue to be added to either terminus of the peptide to achieve specific coupling between ε-amines of the carrier protein and the terminal sulhydryl group of cysteine without coupling to
internal sites on the peptide (11,18), and thereby exposing a mobile N- or C-terminal peptide that is very antigenic. The amount of peptide coupled to the carrier can also significantly affect the likelihood of a successful immune response. Low ratios of peptide/carrier may be insufficient, whereas high densities may induce tolerance effects (2,9). Optimal densities range from 5 and 30 mol of peptide/100 kDa of carrier (12,13).

Various methods have been used to estimate coupling efficiencies. Peptides can be labeled with $^{125}$I, but oxidizing conditions used can destroy the sulphydryl groups required for coupling with heterobifunctional agents and the hazards of radioactivity warrant a separate parallel reaction from that to be used for immunization. An alternative approach uses N-α-bromoacetylated peptides allowing quantification of coupling by release of S-carboxymethylcysteine upon acid hydrolysis (10). This procedure requires equipment and reagents that may not be readily available. Therefore, a method that utilizes biotinylated-peptide was developed.

Biotinylation did not destroy the sulphydryl moieties necessary for coupling, as shown in Figure 1. Slot blot analysis provides a rapid means to analyze the reaction product, but is not quantitative due to differential retention of peptides and proteins on nitrocellulose. UV-fixing of peptide to nylon membranes improved retention on membranes, but was not suitable for the purpose of quantification (data not shown). Likewise, immobilization of the peptide on ELISA plates was also inefficient.

To more accurately determine the mass of peptide coupled to the carrier protein, a competitive ELISA was used. In this assay, ELISA plates were coated with avidin and used to capture biotinylated-CIAP. When biotinylated-peptide is added to the reaction, it competes with biotinylated-CIAP for avidin binding sites. The concentration of biotinylated-peptide was inversely proportional to the log[biotin] or log[biotinylated-peptide] as shown in Figure 2 (inset). The elution profile of the coupling reaction can be rapidly analyzed by ELISA. The assay was sufficiently sensitive to detect coupling of approximately 1.4 molecules of peptide per molecule of BSA (Figure 2). Greater than 10 µg/ml of KLH in the ELISA reaction results in nonspecific effects (Table 2). This effect is not unexpected since KLH exists as large aggregates (4.5 × 10$^3$ to 1.3 × 10$^4$ kDa; Reference 5) that may cause “trapping.” Nonetheless, where 1 mol peptide/100 kDa KLH is desired, detection is possible by increasing the ratio of biotinylated peptide to unlabeled peptide in the reaction. The presence of trace amounts of biotinylated peptide in the immunogen was not detrimental to antibody production and did not result in any apparent artifacts when the antipeptide antiserum was used to detect the cellular protein encoded by DI5Kt1 by immunoblot (D.J. Jerry, unpublished observations).

Biotin-labeling of peptides offers a versatile means to monitor coupling reactions. Biotinylation is achieved using mild reaction conditions that maintain the integrity of essential functional groups and avoids the hazards associated with radiolabeling. The product is stable, and therefore allows analysis of the coupled product after storage. The product is easily analyzed by slot blot or immunoblot. For qualitative estimates, the coupled product can be analyzed by competitive ELISA.

ACKNOWLEDGMENTS

The author wishes to thank Leslie Kozak for his support and encouragement for this work. Fred Taylor provided the synthetic peptide and many helpful discussions. Charles Sidman and Valerie Scott assisted in the development of the ELISA. This project was supported in whole or in part by ACS IN-155 awarded by the American Cancer Society and Public Health Service Training Grant HD07065.

REFERENCES


Address correspondence to:
D. Joseph Jerry
Division of Molecular Virology
Baylor College of Medicine
One Baylor Plaza
Houston, TX 77030

For comments or questions, contact the author at the EMail address(s) below.
BioTechnet: DJERRY
INTERNET: DJERRY@BIOTECHNET.COM

HPLC '93
17th International Symposium on Column Liquid Chromatography
Hamburg, Germany
May 9-14, 1993
Advance Registration
Deadline: March 12
For Information Contact:
Gesellschaft Deutscher Chemiker
Abteilung TAGUNGEN
Warrentirappstr. 40-42
P.O. Box 900440
D-6000 Frankfurt am Main
Germany
Telephone: (49)-69-79 17-360
Fax: (49)-69-79 17-475

SPRINGER LABORATORY
Introducing a New Program From Springer-Verlag

J.H. PETERS and H. BAUMGARTEN (eds.)
MONOCLONAL ANTIBODIES
A Practical Guide
Monoclonal antibodies, in contrast to conventional antisera (polyclonal antibodies), are highly selective and can be produced in unlimited amounts of equal quality. They have evolved into one of the most powerful tools in biomedical research, diagnosis, and even therapy. This laboratory handbook focuses on procedures for hybridoma generation and gives detailed test protocols for the use of monoclonal antibodies. Contents include immunization, cell preparation, hybridization, cell culture and cloning of hybridomas, prevention of back-mutation, as well as purification, labeling, and characterization of the antibodies obtained. Practical hints and detailed product information together with detailed protocols for troubleshooting make this an exceptionally useful book.

1992/456 pp./$74 illus./Hardcover $69.00
ISBN 0-387-59842-0
All the Bioanalytical Labeling and Detection Techniques - Now in One Source!

C. KESSLER (ed.)
NONRADIOACTIVE LABELING AND DETECTION OF BIOMOLECULES
This book gathers together all the important nonradioactive labeling techniques for nucleic acids, proteins, glycoproteins and glycolipids, including DIG, Biotin, BrdU, Sulfone, Immunogold, Silver Enhancement, and SNAP as well as standard procedures for optical, chemical, biological, and electrochemical nanoscale and fluorescent detection. Additionally, applications for the use of non-isotopically labeled biomolecules are described. Specific protocols are given for blot, colony/plaque and in-situ hybridization formats, quantitative formats, as well as non-radioactive techniques for nucleic acid sequencing and amplification. Each chapter contains a short introduction, a detailed description of the method with lab protocols, troubleshooting tips and references.

1992/436 pp./$78 illus./Hardcover $89.00

PCR: CLINICAL DIAGNOSTICS AND RESEARCH
Contains complete information about PCR procedures, together with a discussion of contemporary opinions. Even experienced laboratories will welcome the clear presentation of demanding procedures such as the exact quantification of PCR products or the in-situ PCR. Protocols that have been tried and tested provide swift support for troubleshooting all sorts of procedural problems — many examples of problem areas found in diagnostically-oriented PCR are fully described. A discussion of the most important PCR equipment as well as the pros and cons of single individual reaction components which are necessary for performing PCR reactions is included. This lab book is clearly organized to give an introduction to each technique, brief background information, step-by-step instructions for carrying out the procedure and additional comments.

A. ROLFS, I. SCHULLER, U. FINCKH, and I. WEBER-ROLFS (eds.)

A. RADERBRUCH (ed.)
FLOW CYTOMETRY AND CELL SORTING
A Laboratory Manual
Practical aspects of flow cytometry and sorting are emphasized in this lab manual. The clear structure makes it easy to address specific problems fast. Both beginners and advanced users will find the "tips, tricks, and troubleshooting" feature especially useful. Following a chapter on operation of a flow cytometer, the book covers immunofluorescence (antibody-fluorochrome conjugation, multicolor analysis, staining principles, and Scatchard analysis), DNA and cell proliferation methods, cellular biochemistry, cell sorting (including chromosomes and large particles), and safety considerations.

A. ROLFS, I. SCHULLER, U. FINCKH, and I. WEBER-ROLFS (eds.)

CALL Toll-Free 1-800-SPRINGER (NJ call 201-348-4033) or FAX 201-348-4505.
Please mention S138 when ordering by phone, or
VISIT your local scientific bookstore.

REFERENCE #: S138

Vol. 14, No. 3 (1993)
Circle Reader Service No. 201