

## Synthetic Peptides as Antigens for Antibody Production

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### Summary

The use of synthetic peptide immunogens as a means to generate specific immunological reagents for a variety of purposes has increased markedly in recent years. In this chapter, we outline some of the salient factors to be considered when designing peptide immunogens and describe basic methodologies for the conjugation of short synthetic peptides to immunogenic carrier proteins.

**Key Words:** Synthetic; peptide; antibody; antigen; polyclonal; immunogen; carrier; conjugation.

### 1. Introduction

There is an ongoing requirement in cell and molecular biology for the preparation of antibodies to use as probes for specific proteins. Two main strategies exist to raise appropriate antibodies. A complementary deoxyribonucleic acid (cDNA), or gene sequence encoding the protein of interest can be expressed in a heterologous species,

From: *Methods in Molecular Biology*, vol. 295: *Immunochemical Protocols*, Third Edition.  
Edited by: R. Burns © Humana Press Inc., Totowa, NJ

usually bacteria, and the resultant purified protein used as an immunogen. Glutathione-S-transferase fusion proteins, for example, have been extensively used as immunogens. Alternatively, small synthetic peptides can be synthesized that contain amino acid sequences derived from the cDNA acid or gene. Such anti-peptide antibodies crossreact with the corresponding intact native protein with surprisingly high frequency and have the additional advantage that the epitope recognized by the antibody is already well defined (**I**). In this way, antibodies can be raised against novel gene products that are specifically directed against sites of interest, for example, unique regions, highly conserved regions, active sites, extracellular domains, intracellular domains or regions of posttranslational modification, such as phosphorylation sites. Moreover, the ready availability of the peptide immunogen against which the antibody was raised means that sera can be rapidly and easily screened, for example, using an enzyme-linked immunosorbent assay for anti-peptide activity. Free peptide can also be used to block antibody binding and so demonstrate immunological specificity, and it may be coupled to a solid support (e.g., agarose) to generate an affinity matrix for antibody purification. In this chapter, we describe the basic principles behind the design, synthesis, and use of synthetic peptides as immunogens and in this and the following chapter outline some of the basic methods used in our laboratories. These methods have been used for several years, with a considerable degree of success, by groups in our institute and elsewhere.

### ***1.1. Choosing Peptide Sequences***

Many peptide sequences can be immunogenic, but not all are equally effective at eliciting antibodies that crossreact with the intact cognate protein (we term these crossreactive peptides). There is no guarantee that antibodies raised against a particular synthetic peptide will crossreact with the intact protein from which the sequence is derived. In our experience the probability of generating a successful anti-protein antibody by the methods outlined is approx 50%. Many factors can influence the success of using peptide

immunogens to raise antiprotein antibodies. These include elements such as the number of peptides from one protein sequence to be used and the number of animals available for immunization (both of which may be determined by existing resources); the availability and accuracy of sequence data, the predicted secondary structure of the intact protein and finally, the ease of synthesis of specific sequences. Continual improvements to synthesis methodologies means that the latter aspect is less significant than in the past, although certain sequences can still be problematic (*see Subheading 1.2.*). Despite these potential reservations, there are a number of ways of improving one's chances of success (*see Subheading 1.1.1.–1.1.3.*).

#### *1.1.1. Predicted Structure of the Whole Protein*

There is a wide range of predictive algorithms available that can provide data on antigenicity, hydrophilicity, flexibility, surface probability, and charge distribution over a given amino acid sequence. The algorithms of Chou and Fasman and of Robson and Garnier (2,3) have provided a basis for many secondary structure predictive algorithms that can give a good idea of where regions of particular secondary structure, such as  $\alpha$ -helix,  $\beta$ -sheet, turns, and coils are likely to form. For example, the proteomics server of the Swiss Institute of Bioinformatics (<http://www.expasy.ch/>) provides access to primary and secondary structure analysis tools via the Expert Protein Analysis System (ExPASy). Other prediction scales include the Turn scales of Pellequer and Westhof (4). These are based on the occurrence of amino acids within turns. The level of correctly predicted antigenicity using this program is high (70%), but the number of predicted antigenic sites per protein is smaller than for other programs. In general, however, there is rather poor correlation between amino acid type and secondary structure with similar folds able to be made by sequences with only 20% identity. The relative merits of different predictive scales is discussed in depth elsewhere (5).

Primary amino acid sequences can also indicate consensus sequences that may be sites of posttranslational modification (e.g.,

*O*- and *N*-linked glycosylation sites and sites of phosphorylation) and that may therefore be immunologically unavailable in the fully mature protein. Clearly, accessibility on the external surface of the intact protein is, overall, the most important requirement for a cross-reactive peptide. Very frequently, the C-terminus of a protein, although often not a region of strongly predicted secondary structure, is exposed, and this sequence makes a good first choice. However, the C-terminus occasionally forms the membrane anchoring region of some membrane-bound proteins and in these cases would generally be too hydrophobic to consider. The N-terminus of a protein can also prove to be a good candidate sequence, but in our experience is a less reliable choice than the C-terminus and may be modified or truncated. Regions with too high a charge or hydrophilicity are sometimes not as effective as might be expected, probably because almost all known antibody combining sites make contact with their epitope via polar and Van der Waal's bonds and not ionic interactions. Hydrophilic  $\alpha$ -helical regions can be good peptide epitopes because, provided the synthetic peptide is itself long enough to form a helix, it often assumes an identical conformation to that in the intact protein.

### *1.1.2. Specific Requirements*

By their nature, antipeptide antibodies are site-directed probes for proteins. Both the sequence and position of the antibody epitope is predefined. Indeed, the technique of "epitope tagging" exploits the existence of an antibody with specificity for a given linear peptide epitope that can be expressed in the context of a fusion protein (6). It is, therefore, possible to target antipeptide antibodies to specific regions of interest in the intact protein, such as areas of high conservation to identify additional members of a protein family; or areas of hypervariability in order to unambiguously identify a particular family member. The increasing reliability of synthesis of, for example, phosphopeptides means that sites of posttranslational modification can also be analyzed. Antibodies that recognize both degenerate and specific consensus phosphorylation motifs are avail-

able commercially and antibodies raised against a specific phosphopeptide have been used as tools to recognize novel phosphorylation targets (7,8). When selecting a peptide to produce a phosphospecific antibody, it is preferable to localize the phosphorylated residue close to the middle of the peptide to reduce the likelihood of producing an immunodominant epitope containing nonphospho amino acid sequence. Other functional or regulatory regions of a protein, such as binding sites, transmembrane domains or signal sequences may also be targeted. However, factors, such as hydrophilicity and secondary structure, may affect the success of any given peptide immunogen.

### *1.1.3. Immunological Requirements*

Peptides of 10–20 amino acids are optimal as antigens and our standard is approx 15 residues. Short peptides (less than approx 7 residues) are probably of insufficient size to function as epitopes. Larger peptides may adopt their own specific conformation (that is often immunodominant over any primary structural determinants), which may not be reflected in the conformation of the sequence within the intact protein. Given the previous criteria, it is possible to say that almost all peptide sequences are immunogenic if presented to the immune system in the right way (*see Subheading 1.3.*), but that not all will generate cross-reactive antibodies. Probably the most important factors in optimizing one's chances of making useful antibodies to a protein of interest are to use several peptides from different regions of the protein sequence and to immunize more than one animal with each peptide. Different animals within the same group frequently respond differently to the same immunogen. In addition, a given antipeptide antibody may sometimes work well in one assay, for example, Western blotting, but not in another, for example, immunoprecipitation.

### *1.1.4. Synthesis Requirements*

The chemical difficulties of synthesizing certain amino acid sequences can be complex. In general, hydrophilic sequences are

more soluble and easier to synthesize (and are more likely to be exposed on the surface of the intact molecule). There appears to be little requirement for a high degree of purity for peptide immunogens. Our experience is that peptides of 75% purity, or sometimes even less, generate effective polyclonal antisera, although criteria may need to be more stringent when making monoclonal antibodies.

## **1.2. Peptide Synthesis**

Peptides can be purchased from several companies specializing in contract synthesis and if only a few are required, this is the most straightforward way to obtain the desired reagents. Custom synthesis of peptides can be expensive, with specific modifications costing even more. However, in-house synthesis is labor-intensive, requires significant knowledge of peptide chemistry and, if performed using an automated machine, involves large capital expenditure. In general, acquisition of an automated peptide synthesizer is probably best suited to laboratories or institutes with substantial and ongoing requirements for synthetic peptides, and preferably with their own dedicated personnel. An in-house peptide synthesis facility is a particularly attractive alternative to custom synthesis because it allows much greater flexibility in the design and production of peptides. This can be important if specially derivatized peptides are needed, or if, for example, chemically defined immunogens such as multiple antigen peptides are to be synthesized (**9,10**).

### *1.2.1. Principles of Peptide Synthesis*

Solid-phase peptide synthesis is based on the sequential addition of protected amino acids onto an insoluble support. Addition proceeds from carboxy terminus to amino terminus. The first amino acid is attached to a solid support by a linker and, if necessary, side-chain amino acid function is protected throughout chain assembly. The carboxy group of the in-coming, acylating amino acid is activated for coupling while its amino group is protected temporarily for each coupling step and then deprotected for the next cycle. The

cycles of deprotection and coupling are continued until the amino acid chain is complete. The peptide is then cleaved from the solid support and the amino acid side-chains are deprotected to give the final peptide product. In general, once a peptide is made the sequence cannot be altered. Modifications such as acetylation, phosphorylation or the introduction of additional residues (e.g., for use in conjugation) should be planned before synthesis. The modifications can then be incorporated into the synthesis procedure. For example, phosphopeptides are synthesized using amino acid residues with specially derivatized side-chains. The final product is usually evaluated by reverse-phase high-performance liquid chromatography (C8 or C18 columns with water/acetonitrile gradients) and mass spectrometry. For antibody production, a suitable amount of peptide is about 50 mg. This allows for affinity purification of antibodies and antibody blocking experiments, to demonstrate antibody specificity, as well as immunization. The synthesis of most peptide sequences in the region of 20 residues in length is currently considered to be quite routine. There are, however, always exceptions. Certain sequences can be extremely difficult to synthesize and may require alternative synthetic strategies (*11,12*).

### ***1.3. Conjugation of Peptides to Carrier Proteins***

In general, short peptides are poor immunogens, so it is necessary to conjugate them covalently to immunogenic carrier proteins to raise effective anti-peptide antibodies. These carrier proteins provide necessary major histocompatibility complex class II/T-cell receptor epitopes while the peptides can then serve as B-cell determinants. Keyhole limpet hemocyanin (KLH) and thyroglobulin are examples of carriers that are commonly used to generate polyclonal anti-peptide antibodies. We generally avoid using bovine serum albumin because the high levels of anti-bovine serum albumin antibody generated can interfere with subsequent studies on tissue culture cells grown in media containing bovine sera.

The peptides are covalently conjugated to the carrier molecule using an appropriate bifunctional reagent—the most straightforward

coupling methodologies involve the amine or sulfhydryl groups of the peptide. Substantial antibody titres are also usually generated against determinants present on the carrier molecules. In general, such anticarrier antibodies present few problems in polyclonal anti-peptide antibodies and may anyway, be adsorbed out on a matrix of carrier bound to agarose. When making monoclonal antibodies, however, the substantial anticarrier response may mask the frequently weaker anti-peptide response, resulting in few peptide-specific hybridomas being isolated. A variety of alternative approaches to the use of conventional peptide-carrier conjugates have been developed including, for example, the multiple antigenic peptide (MAP) system (9). The MAP system makes use of the epsilon-amino group of lysine residues to generate a branched core matrix that can be used as a scaffold for subsequent peptide synthesis. This system can be employed to deliver high densities of single, defined peptide antigens or to generate B-cell and T-cell epitopes attached to the same MAP scaffold (10). Nevertheless, MAP synthesis products can be difficult to analyze by high-performance liquid chromatography and mass spectrometry because of their large mass.

The most straightforward carrier-peptide conjugation procedure uses glutaraldehyde as the bifunctional reagent, which crosslinks amino groups on both carrier and peptide. In our experience, glutaraldehyde conjugation is reliable, easy and effective, and generates good anti-peptide antibodies even with short peptides. *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) can be used to crosslink the thiol group of cysteine on the peptide to an amino group on the carrier. The MBS method generates a somewhat better defined conjugate, but it involves a slightly more involved procedure and requires the presence of a reduced cysteine residue at one end of the peptide (this is frequently added to the sequence during synthesis specifically for conjugation purposes). If the chosen peptide sequence contains an internal cysteine residue, coupling via MBS should be avoided.



## 2. Materials

### 2.1. Conjugation of Peptides

1. KLH: purchased as a solution in 50% glycerol (Calbiochem) and stored at 4°C. If obtained as an ammonium sulfate suspension the KLH will require extensive dialysis against borate buffered saline (20 mM Na Borate/144 mM NaCl) containing 50% glycerol.
2. Glutaraldehyde (Sigma, Grade 1) stock: is a 25% solution divided into 1-mL aliquots. It is stored at -20°C and never re-frozen.
3. Sodium bicarbonate stock (10X): a 1-M solution adjusted to pH 9.6 with HCl.
4. Glycine ethyl ester hydrochloride (Sigma): make up as a 1-M stock and adjust to pH 8.0 with NaOH.
5. MBS "Sulfo-MBS" version of this reagent is water-soluble and, therefore, preferable (Pierce).
6. 0.1 M Sodium phosphate buffer, pH 6.0: mix 12 mL of 1 M disodium hydrogen phosphate with 88 mL of 1 M sodium dihydrogen phosphate and make up to 1 L with water.
7. Sephadex G25 (APBiotech).
8. Sodium borohydride.
9. Borate buffer: 0.1-M boric acid solution adjusted to pH 8.0 with NaOH.
10. 1 M HCl.
11. 1 M NaOH.
12. Acetone.
13. Saline: 0.9% NaCl.
14. Ammonium hydrogen carbonate, pH 7.5.

## 3. Methods

### 3.1. Glutaraldehyde Conjugation Method (see Notes 1 and 2)

1. Weigh out the peptide and an equal weight of KLH (or thyroglobulin) carrier. This gives an approximate ratio of 40–150 molecules of peptide to each molecule of carrier (2 mg of peptide per animal is ample).

2. Dissolve the peptide and carrier protein in 0.1 *M* (1X) sodium bicarbonate using 1 mL for every 2 mg of carrier protein.
3. Thaw out a fresh vial of glutaraldehyde and add to the peptide-carrier solution to a final concentration of 0.05%. Mix in a glass tube, stirring with a magnetic stirring bar; keep at room temperature overnight in the dark (wrap the tube in foil). The solution will usually turn a pale yellow color. Occasionally the solution will turn pale brown or orange—this reflects the fact that peptide preparations sometimes contain traces of chemical scavenger reagents used in the final cleavage of the peptide from the resin and is not a cause for concern.
4. Either: dialyze against double distilled water for 12 h and lyophilize the coupled carrier. Assess yield by weighing the lyophilized material to determine the percentage of peptide coupled.

Or, because coupling efficiency is usually reasonable, and not too critical, it is easier to do the following: add 1 *M* glycine ethyl ester to a final concentration of 0.1 *M* and leave for 30 min at room temperature. Then, precipitate the coupled carrier with 4–5 vol of ice-cold acetone at  $-70^{\circ}\text{C}$  for 30 min. Briefly warm at room temperature and pellet the protein at 10,000g for 10 min at room temperature, pour off the acetone, air dry the pellet, and redisperse it in saline at 1 mg carrier/mL. As the pelleted protein is rather sticky, this is best done using a Dounce<sup>TM</sup> homogenizer. Conjugates can be stored at  $-20^{\circ}\text{C}$  and rehomogenized before use.

### **3.2. MBS Coupling Method (see Notes 2–4)**

1. Dissolve 15–20 mg of carrier protein in a small amount of phosphate-buffered saline (about 1 mL).
2. Dissolve 5 mg of MBS in a small amount of dimethylformamide (about 0.75 mL) or for Sulfo-MBS, dissolve in a small amount of sterile water.
3. When crosslinker and carrier are completely dissolved, mix well and leave at room temperature for 1 h.
4. Desalt on a 20-mL Sephadex G25 column using 0.1 *M* sodium phosphate buffer, pH 6.0. Collect 2-mL fractions. Read the optical density (OD) of the fractions at 280 nm. Keep the two fractions with the highest OD<sub>280</sub>.
5. Meanwhile, reduce the peptide.

- a. Make up fresh 5 mg/mL solution of sodium borohydride and store on ice.
  - b. Dissolve 15–20 mg of peptide in minimum amount of 0.1 M borate buffer, pH 8.0.
  - c. Add 100 mL of sodium borohydride to the dissolved peptide, mix well, and stand on ice for 5 min.
  - d. Lower pH by adding 1 M HCl (approx five drops), mix, and leave on ice for a further 5 min.
  - e. Add equal number of drops of 1 M NaOH and check that the pH is between 6 and 7. If not, then adjust with 1 M NaOH or 1 M HCl. (10 mL is approx 0.5 of a pH unit).
6. Add desalted crosslinker/carrier to reduced peptide and leave overnight at room temperature.
  7. If the conjugate becomes insoluble, precipitate completely with 4–5 vol of ice-cold acetone at  $-70^{\circ}\text{C}$  for 30 min. Briefly warm at room temperature. Pour off the supernatant and air dry. Resuspend in saline as in **Subheading 3.1.4**. Alternatively, if the conjugate remains soluble, then desalt the solution on a 20-mL Sephadex G25 column using ammonium hydrogen carbonate buffer, pH 7.5. Collect 2-mL fractions and pool those of  $\text{OD}_{280} > 0.4$ . Conjugates can be stored at  $-20^{\circ}\text{C}$  and rehomogenized before use.

#### 4. Notes

1. It is often worth adding a cysteine residue at the C-terminus of the peptide to give the option of coupling via MBS as well as via glutaraldehyde.
2. During glutaraldehyde conjugation it is vital to exclude any buffers containing amino, imino (e.g., Tris-HCl), ammonium or azide moieties as these will inhibit the cross-linking reaction. If the peptide or carrier is insoluble in coupling buffer, sodium dodecyl sulphate may be added to 0.1% without affecting the conjugation. Occasionally, a peptide-carrier conjugate becomes less soluble as the conjugation reaction proceeds. This does not appear to affect the efficacy of the final product and is usually, therefore, no cause for concern.
3. If, during MBS coupling, the DMF concentration exceeds 30% the KLH will come out of solution. KLH concentrations in excess of 20 mg/mL will also lead to insolubility. Use the Sulfo-MBS derivative if possible.

4. If many conjugation reactions are required, the MBS activation of KLH can be scaled up and performed batchwise. Alternatively, if only a few conjugates are needed, Maleimide-activated KLH is commercially available (Calbiochem or Pierce). This leaves only the final addition of peptide to be performed.

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<http://www.springer.com/978-1-58829-274-2>

Immunochemical Protocols

Burns, R. (Ed.)

2005, X, 318 p. 22 illus., Hardcover

ISBN: 978-1-58829-274-2

A product of Humana Press