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An Improved Protocol for Coupling Synthetic Peptides to Carrier Proteins for Antibody Production Using DMF to Solubilize Peptides

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We present an improved protocol for coupling synthetic peptides to carrier proteins. In this protocol, dimethylformamide is used as the solvent to solubilize peptides instead of phosphate-buffered saline (PBS) or 6 M guanidine-HCl/0.01 M phosphate buffer (pH 7). Additionally, the last desalting or dialyzing step to remove uncoupled peptides as in the traditional method is eliminated. Finally, 3 mL of 0.1 M ammonium bicarbonate is added to the carrier protein conjugated peptide solution to help the lyophilization process. Coupling of Cys-containing synthetic peptides to keyhole limpet hemocyanin or bovine serum albumin using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester are used as the test cases. This method produces high-quality antipeptide antibodies. Also, compared to the traditional method, this procedure is simpler and useful for peptides with solubility problems in PBS or 6 M guanidine-HCl.

Key Words: peptide, antibody, KLH, BSA, MBS, DMF, MALDI-TOF MS.

The use of synthetic peptides to generate antibodies has increased dramatically in recent years. The advantages of using peptides instead of proteins as antigens include ready availability[1–4] and the ease of producing antipeptide antibody specifically against protein isoforms or site-specific phosphorylated proteins. Although multiple antigen peptide systems,[5–7] which do not require a carrier protein, are being used to generate antipeptide antibodies, for routine mainstream production of antisera, synthetic peptides are coupled to carrier proteins (these conjugations normally elicit a strong immune response), which are used to immunize animals. Keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) conjugated peptides are two major kinds of antigens used to generate anti-peptide antibodies.[8–9]

In the traditional method, peptides are dissolved in phosphate-buffered saline (PBS) and then mixed with *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) conjugated KLH or BSA to make carrier protein conjugated peptides. If the peptide is not soluble in PBS, 6 M guanidine-HCl/0.01 M phosphate buffer (pH 7) can be used to dissolve the peptides. However, there are peptides that are insoluble in both buffers. We present here a procedure for making the carrier protein conjugated peptides using dimethylformamide (DMF) as the solvent to solubilize peptides instead of PBS or 6 M guanidine-HCl. Additionally, since the uncoupled peptide does not affect antibody production, the last desalting or dialyzing step to remove the uncoupled peptide in the traditional protocol is eliminated. Finally, 3 mL of 0.1 M ammonium bicarbonate is added to the carrier protein conjugated peptide solution before lyophilization, which helps the lyophilization process. We have successfully raised thousands of antipeptide antibodies using this procedure. Two test peptides, EMVAQLRNSSEPAKKC and RNTKGKRKGQGRPSPLAPC, are used in this report to demonstrate the effectiveness of this procedure.

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MATERIALS AND METHODS

Fmoc-amino acids and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were from AnaSpec (San Jose, CA). BSA, *m*-maleimidobenzoic

acid *N*-hydroxysuccinimide ester (MBS), 4-methyl morpholine, and other standard chemicals were from Sigma (St. Louis, MO). KLH was from Pierce (Rockford, IL). PD-10 column was from Pharmacia Bioscience (Piscataway, NJ).

Peptide Synthesis

Peptides were synthesized using Fmoc solid-phase synthesis chemistry on a Symphony Peptide Synthesizer (Protein Technologies, Tucson, AZ). The Fmoc group was removed by 20% piperidine, and Fmoc-amino acids were coupled using 0.1 M HBTU in DMF containing 0.4 M 4-methyl morpholine for 60 min. The resin-bound peptide was deprotected and cleaved from the resin using trifluoroacetic acid (TFA). Ethyl ether was added to precipitate the peptide from the TFA solution. The precipitated peptide was then lyophilized. The crude peptide was purified on a reversed-phase Vydac 218TP1010 C18 column (Hesperia, CA) using a BioCad Sprint (Applied Biosystems, Foster City, CA). A flow rate of 10 mL/min with solvent A (0.1% TFA in deionized water) and solvent B (0.1% TFA in acetonitrile) was used. The column was equilibrated with 5% solvent B. After sample loading, the column was eluted with a linear gradient from 5% solvent B to 100% solvent B in 60 min. The pure peptide fraction was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

Matrix-Assisted Laser Desorption/Donization Time-of-Flight (MALDI-TOF) Mass Spectra

Cyano-4-hydroxycinnamic acid (CHCA) or sinapinic acid (SA) was used as the matrix for MS analysis of peptide or protein solutions, respectively. Aliquots (1.3 μL) of the matrix solution (3–10 mg CHCA or SA in 1 mL aqueous solution of 50% [v/v] acetonitrile containing 0.1% [v/v] TFA) were mixed with the peptide or protein solution and spotted onto a MALDI-TOF target. A Voyager DE PRO Mass Spectrometer (Applied Biosystems, Foster City, CA) equipped with a 337-nm pulsed (~ 5 nsec) nitrogen laser was used to analyze the samples. Protein/peptide mass was measured using the positive-ion linear mode. Delayed extraction was carried out with an accelerating voltage of 25 kV, a grid voltage of 92%, a guide wire of 0.15%, and a delay time of 100–900 nsec. Mass spectra were obtained by averaging 100 laser shots (~ 1 μsec each shot). External mass calibration was performed using the peaks of a mixture of bradykinin fragment 1–7 at m/z 757, angiotensin II (human) at m/z 1046, P14R (synthetic peptide) at m/z 1533, adrenocorticotropic hormone fragment 18–39 (human) at m/z 2465, insulin oxidized B (bovine) at m/z 3494, insulin (bovine) at m/z 5735, cytochrome c (equine) at m/z 12362, apomyoglobin (equine) at m/z 16952, adolase (rabbit muscle) at m/z 39212, and albumin (bovine serum) at m/z 66430.

Coupling Peptides to KLH or BSA

The procedure for coupling peptides to KLH or BSA was as follows:

1. Dissolve 5 mg of KLH or BSA in 0.5 mL of 0.01 M phosphate buffer (pH 7).
2. Dissolve 3 mg of MBS in 200 μL DMF.
3. Add 70 μL of MBS solution to 0.5 mL of KLH solution.
4. After stirring or rotating for 30 min at room temperature, the KLH/MBS or BSA/MBS solution is passed through a PD-10 column using 0.05 M phosphate buffer (pH 6). Collect the 3.5 mL of purified KLH/MBS or BSA/MBS. Add 0.5 mL of water.
5. Dissolve 5 mg of peptide in 100 μL of DMF. Rapidly add 1 mL of purified KLH/MBS or BSA/MBS. Shake rapidly and immediately add 11 μL of 2 N NaOH.
6. Check the pH with pH paper. It should be 7.0–7.2. Too high pH or too low pH will stop the reaction between KLH/MBS or BSA/MBS and peptide. If needed, add immediately an appropriate amount of 0.5 N HCl or 2 N NaOH to change the pH.
7. Stir or rotate the solution 3 h or overnight at 4°C. Finally, add 3 mL of ammonium bicarbonate (0.1 M) before lyophilizing the reaction solution.

Antipeptide Antibody Production[8–9]

The rabbit was immunized subcutaneously with 1 mg peptide/carrier conjugate in 1 mL PBS mixed 1:1 with complete Freunds adjuvant. The rabbit was boosted with 0.5 mg peptide/carrier conjugate in 1 mL PBS mixed 1:1 with incomplete Freunds adjuvant at 4 wks and again at 8 wks. The rabbit antiserum was collected 10 d after the last immunization, and the serum was tested using enzyme-linked immunosorbent assay (ELISA).

Antipeptide Antibody Confirmation Using ELISA[8–9]

Synthetic peptide (2.5 μM) was prepared in carbonate buffer (pH 9.6) and incubated on a microtiter plate overnight at 4°C. Wells were washed three times with PBS containing 0.05% Tween-20. The remaining sites on the wells were blocked by BSA (10 mg/mL) in PBS/Tween-20 at 37°C for 1 h. After subsequent washing, 300 μL of 1:1000 dilutions of antipeptide antiserum was added to wells and incubated for 2 h at 37°C. The wells were washed three times with PBS/Tween-20 to remove the unbound antibody and then incubated with 1:500 dilution of alkaline phosphatase conjugate of goat anti-rabbit IgG in PBS/Tween-20 for 2 h at 37°C. Wells were washed three times with PBS/Tween-20, and 50 μL of enzyme substrate (p-

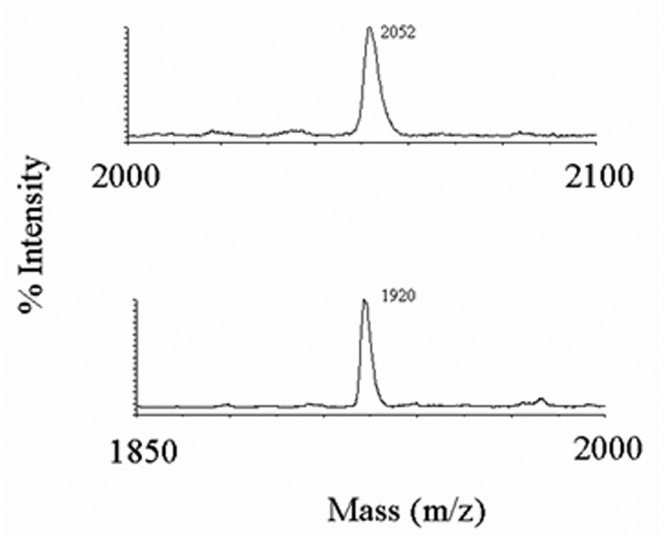

FIGURE 1

MALDI-TOF mass spectra of peptides EMVAQLRNSSEPAKKC (*m/z* 1920) and RNTKGKRKGQGRPSPLAPC (*m/z* 2052).

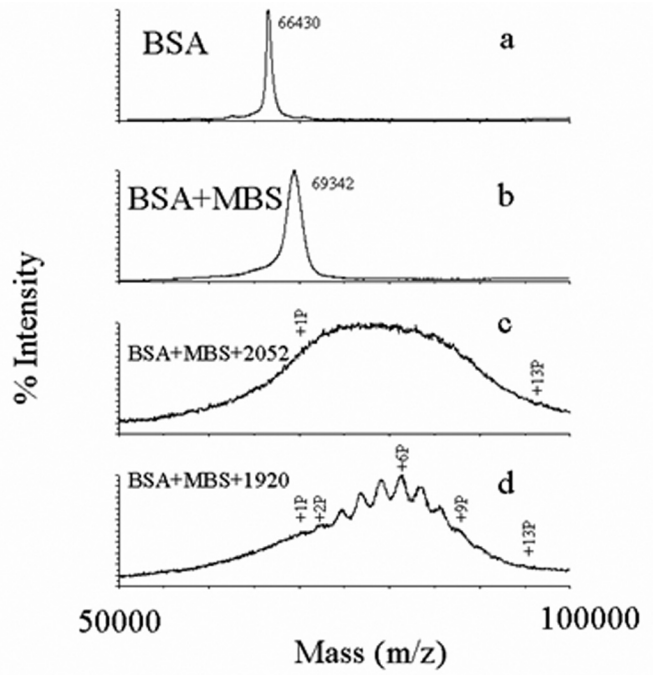

FIGURE 2

MALDI-TOF mass spectra of: **(a)** BSA; **(b)** BSA + *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS); **(c)** BSA + MBS + peptide RNTKGKRKGQGRPSPLAPC; **(d)** BSA + MBS + peptide EMVAQLRNSSEPAKKC. In this figure, +1P to +13P stands for +1 peptide to +13 peptides, respectively.

nitrophenyl phosphate) prepared in water was added for 10 min at 37°C. The plate was read at λ = 405 nm with a micotiter plate reader (StatFax-2100, Awareness Technology, Palm City, FL).

RESULTS AND DISCUSSION

The improved procedure for producing peptide/carrier protein conjugates used in this study consisted of three major modifications of the traditional protocol.

1. DMF was used as the solvent to solubilize peptides instead of PBS or 6 M guanidine-HCl/0.01 M phosphate buffer (pH 7).

2. The last desalting or dialyzing step to remove uncoupled peptides in the traditional method was eliminated.

3. Three milliliters of 0.1 M ammonium bicarbonate was added to the carrier protein conjugated peptide solution before lyophilization, which helped the lyophilization process.

Figure 1 shows the MALDI-TOF mass spectra of two test Cys-containing synthetic peptides, EMVAQLRNSSEPAKKC (*m/z* 1920) and RNTKGKRKGQGRPSPLAPC (*m/z* 2052). Since these two peptides contain internal amino acids Lys, Arg, and Glu, but no Tyr, other coupling reagents such as glutaraldehyde (which cross-links peptides through an amino group), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (which cross-links peptides through an amino or carboxylic acid group), or bis-diazotized benzidine (which cross-links peptides through a Tyr side chain) can not be used. These two peptides readily dissolve in DMF. However, if the DMF volume added exceeds 100 μL, the peptide solution may turn cloudy. Some peptides will give a cloudy appearance even if 100 μL of DMF is used. The reaction can be performed longer for such peptides. Some peptides in DMF will form a gel. The gel will dissolve upon mixing the peptide with KLH/MBS or BSA/MBS followed by sonication. It is best to add DMF/peptide to KLH or BSA solution slowly, rather than KLH or BSA to DMF/peptide. We have seldom encountered peptide solubility problems among thousands of peptides using DMF. In contrast, we encounter insoluble peptides from time to time using either PBS or 6 M guanidine-HCl/0.01 M phosphate buffer (pH 7).

Figure 2 shows MALDI-TOF mass spectra of BSA, BSA/MBS, and BSA/MBS + peptides. Results demonstrate that the use of DMF does not hinder the cross-linking between peptides and carrier proteins. BSA was used instead of KLH because KLH is difficult to detect by MALDI-TOF mass spectrometry. After BSA reacts with MBS in the first step of the method, ~13 MBSs have cross-linked to BSA, which is manifested by an increase of *m/z* 2912 from BSA (*m/z* 66430) to BSA/MBS (*m/z* 69432 = 66430 + [13 × 224]).

After mixing BSA/MBS with peptide EMVAQLRNSSEPAKKC (Figure 2d), a series of peaks were detected at *m/z* 71352, 73272, 75192, 77112, 79032, 80952, 82872, 84792, 86712, 88632, 88632, 90552, 92472, and

94392, corresponding to BSA/MBS +1 to +13 peptides, respectively. In similar fashion, after mixing BSA/MBS with peptide RNTKGKRKGQGRPSPLAPC (Figure 2c), a series of peaks were detected corresponding to BSA/MBS +1 to +13 peptides, respectively.

Studies also showed that the addition of 3 mL of the ammonium bicarbonate to the coupling solution in the improved procedure helped to dry the peptide/carrier protein into a powder. The powder was used to immunize rabbits, and produced polyclonal antibodies successfully. The antibodies all produced strong ELISA signals.

CONCLUSIONS

An improved protocol for cross-linking Cys-containing peptides to carrier proteins KLH or BSA for antipeptide antibody production was described. DMF, which increases the solubility of peptides, was used to solubilize peptides instead of PBS or 6 M guanidine-HCl/0.01 M phosphate buffer (pH 7). Since the uncoupled peptides do not interfere with the production of the antibody, the last desalting or dialyzing step to remove uncoupled peptides in the traditional method was eliminated. Finally, 3 mL of 0.1 M ammonium bicarbonate was added to the carrier protein conjugated peptide solution to aid the lyophilization process. Compared to the traditional method, this method is simpler and more useful for peptides with solubility problems in PBS or 6 M guanidine-HCl. We have been using this procedure to successfully create thousands of antipeptide antibodies.

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