Polymerization of Unprotected Synthetic Peptides: A View toward Synthetic Peptide Vaccines

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Abstract: A generic method is reported for the assembly of multi-peptide polymers in which peptides are synthesized in the solid phase, the N-terminal residue acryloylated, and the derivitized peptides cleaved, purified and finally polymerized by free radical induced polymerization. The high molecular weight polymers generated in this way have individual peptides pendant from a backbone support. Incorporation of 6-aminohexanoyl or other residue(s) at the N-terminus of the peptide prior to acryloylation allows the peptide to be distanced from the polymer backbone and incorporation of acryloylated reagents into the polymerization mixture also permits distancing of pendant peptides along the length of the backbone support. The polymerization process results in highly antigenic artificial proteins as measured by ELISA. Because this approach allows the incorporation of the same or combinations of different purified peptides into polymers, it lends itself to the assembly of potential vaccine candidates containing epitopes from single or multiple pathogens into a single covalent structure.

Introduction

Synthetic peptides are widely used to generate site-specific antibodies, a fact which has stimulated considerable interest in evaluating their use as vaccine candidates. The advantages of this approach include safety, as there is no requirement for infectious material, and the ability to chemically define the product. Although these and other factors make synthetic peptide vaccines an attractive alternative to present vaccine preparations, currently only two peptide-based vaccines have been used in the field.[1,2] The general finding that large molecules are better immunogens than small peptides may in part explain the lack of progress with synthetic peptide vaccines. Furthermore, the requirement for T cell epitopes necessary to provide help for production of antibodies directed against B cell epitopes present in the immunogen demands that multiple and different epitopes are presented to the immune system in a single covalent structure. Traditionally this has been achieved by coupling a peptide epitope to a protein carrier such as keyhole limpet hemocyanin or tetanus toxoid using chemical coupling agents. Although such peptide−protein constructs can produce high titres of anti-peptide antibodies, irrelevant anti-protein carrier antibodies are also produced. The conjugation chemistry used can also lead to changes in the peptide epitope affecting antigenic and immunogenic properties.[3] Furthermore, prior exposure of the individual to the protein carrier can reduce the antibody response to the peptide of interest by carrier-induced epitope specific suppression.[4−7]

To circumvent these problems, alternative strategies have been developed such as the tandem assembly of helper T cell and B cell peptide epitopes.[8−12] Dicyclohexylcarbodiimide and 1-hydroxybenzotriazole chemistry has also been used to produce multiple peptide repeats of protected peptides.[13] The tandem synthesis of B and T cell epitopes may not produce the desired immune response because the relative positions of the epitopes can drastically affect the construct's immunogenicity[14,15] and the linkage of one peptide epitope to another can potentially create novel determinants at the junction thereby inducing inappropriate immune responses.[16,17]

An alternative strategy is the multiple antigenic peptide (MAP) approach[18] which has the advantage that several copies of peptide epitopes can be simultaneously synthesized onto an oligolysine support. Such MAP's are, however, often heterogeneous and to overcome this problem ligation chemistries have been introduced,[19,20] in which peptides are functionalized with

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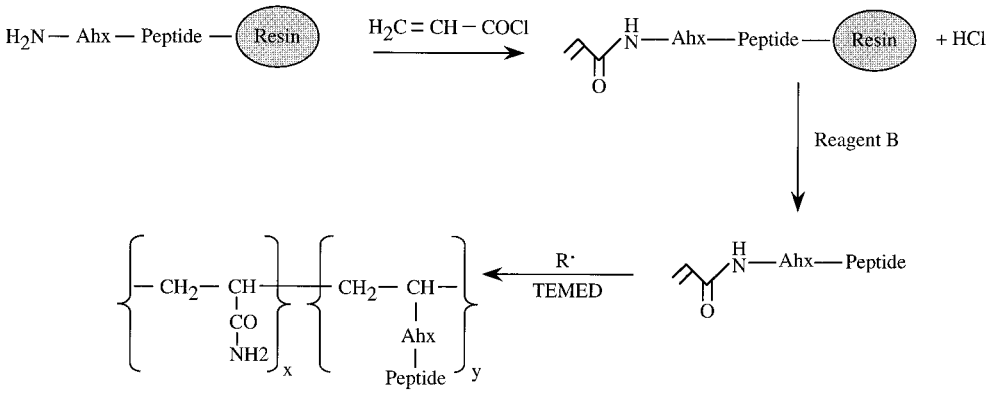
Table 1. Amino Acid Sequences of Peptides Used

amino acid sequence[a]	designation	origin
TLKLATG[b]	P1	influenza virus
GMRNVPEKQT	P2	influenza virus
ALNNRFQIKGVELKS	P3	influenza virus
PKYVKQNTLKLATG	P4	influenza virus
PKYVKQNTLKLATGMRNVPEKQT	P5	influenza virus
DCTLIDALLGDPH	P6	influenza virus
TYQRTRALV	P7	influenza virus
SQHWSYGLRPG	P8	LHRH[c]
DRAAGQPAGDRAAGQPAGDR	P9	malaria parasite
$(NANP)_5$	P10	malaria parasite
FNNTVSFWLRVPKVSASHLE	P11	tetanus toxin
QYIKANSFIGITEL	P12	tetanus toxin
LRRDLDASREAKKQVEKALE	P13	group A streptococcus
YIYADGKMVNEALVRQGLAK	P14	staphylococcal nuclease
DLIAYLKQATAK	P15	pigeon cytochrome C
GFGA	P16	model peptide

[a] Single letter code. [b] All peptides were assembled as the carboxyamide except P2, P5, and P7 which were synthesized with a free −COOH at the C-terminus. [c] Luteinising hormone releasing hormone.

Scheme 1



a weak nucleophilic base and subsequently reacted with an oligolysine support derivatized with aldehyde groups (forming oxime, hydrazone, and thiazolidine linkages). The valency and number of different peptides that can be incorporated into MAP constructs using these approaches is, nevertheless, limited and we therefore sought a simple and generic method for the synthesis of multivalent peptide-based macromolecules in which very large numbers of the same or different peptide epitopes could be incorporated.

Here, we describe a general protocol for the incorporation of the same or different pure synthetic peptides into a high molecular weight artificial protein. This incorporation of multiple B and T cell epitopes into polymeric molecules offers a new approach for the generation of synthetic peptide-based vaccines.

Results and Discussion

Synthesis of Acryloyl Peptides. Peptide sequences varying in length from 4 to 23 residues (Table 1) were assembled by solid-phase peptide synthesis employing Fmoc chemistry. In general, 6-aminohexanoic acid (Ahx) was introduced as the last "residue" and the N-terminus then acylated by acryloylation. In this way Ahx functions as a spacer to distance the acryloyl group from the peptide.

Three techniques for acryloylating resin-bound peptides were evaluated. Two methods involving the coupling of acrylic acid to the N-terminus either by a symmetrical anhydride route[21] or by using *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate and *N*-hydroxybenzotriazole activation gave poor yields of pure *N*-acryloyl peptides. Consistent and high yields of pure *N*-acryloyl peptides are, however, obtained by acylation of the peptide N-terminal amino group with acryloyl chloride (Scheme 1).

As can be seen from Figure 1, the acryloylation of peptide yields material with an increased retention time compared to the non-acryloylated peptide when measured by reverse-phase chromatography. Reagent B[24] was used for the cleavage of *N*-acryloyl peptides from the resin support and side chain deprotection because the use of reagent R[25] resulted in the addition of thioanisole to the double bond. The crude acryloylated peptides were purified by reverse-phase chromatography and gave the expected amino acid analysis. Typical ^{1}H NMR resonances for the acryloyl group were obtained with a variety of *N*-acryloylated peptides. A typical spectrum is shown (Figure 2) for the model peptide *N*-acryloyl GFGA (P16).

Although the majority of peptides are N-terminally acryloylated, peptides P4 and P6 have been synthesized with the acryloyl group at their C-terminus. This was achieved by the acryloylation of the ϵ-amino group of lysine which was introduced at the C-terminal position as Fmoc-Lys-(Mtt)-COOH.

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(25) Peptide NH_2-Gly-Phe-Gly-Ala, when acryloylated with acryloyl chloride and then cleaved with reagent R (TFA:thioanisole:anisole:ethanedithiol 90:5:3:2), gave an *m/z* value 124 daltons greater than expected by FAB mass spectrometry, a result consistent with thioanisole addition to the double bond.

Table 2. Amino Acid Composition of Peptide Polymers

	polymers of							
	P2		P8		P6 + P7		P1 + P3 + P14 + P15	
amino acid[a]	residues found	residues expected	residues found	residues expected	residues found	residues expected[b]	residues found	residues expected[b]
D or N	0.9	1	0	0	2.8	3	5.2	5
E or Q	2.0	2	1.1	1	1.0	1	5.6	5
S	0	0	1.9	2	2.0	2	1.3	1
H	0	0	0.9	1	2.2	2	0	0
G	0.9	1	1.8	2	3.2	3	4.2	4
T	1.1	1	0	0	0.9	1	3.3	3
A	0	0	0	0	0.9	1	8.3	8
P	1.0	1	1.0	1	1.9	2	0	0
Y	0	0	0.9	1	1.3	1	3.3	3
R	1.1	1	1.0	1	1.0	1	2.3	2
V	1.0	1	0	0	0	0	0	0
M	0.9	1	0	0	0	0	0.8	1
I	0	0	0	0	0.9	1	3.2	3
L	0	0	1.0	1	3.9	4	8.4	8
F	0	0	0	0	0	0	1.5	1
C	0	0	0	0	0.9	1	0	0
K	1.0	1	0	0	0	0	7.4	7

[a] Single letter code. [b] Assuming stoichiometric incorporation of individual peptides into polymer.

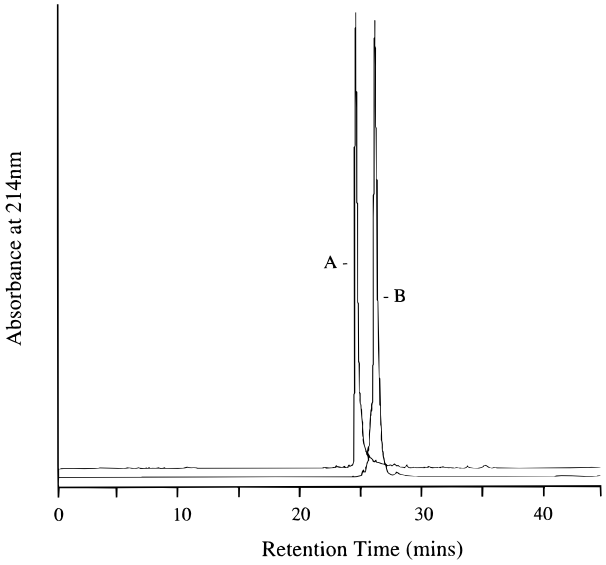

Figure 1. Analytical reverse-phase HPLC chromatograms of crude H_2N-P8 (A) and crude *N*-acryloyl-P8 (B) using a Vydac C18 column (4.6 × 300 mm) installed in a Waters HPLC system. The chromatogram was developed at a flow rate of 1 mL min^{-1} using a gradient, 0−100% solvent B developed over 30 min.

The ability to synthesize N- or C-terminally acryloylated peptides allows for opposite orientations of a peptide within a polymer.

Polymerization of Synthetic Peptides. A simple protocol (Scheme 1), similar to that used in routine preparation of polyacrylamide gels, was adapted to assemble linear polyacrylamides bearing pendant peptides. This procedure yields high molecular weight, water soluble peptide polymers. The polymerization of short (tetra or penta) peptides has been reported[21,22] using similar methodology but in those studies high temperatures, which may be detrimental to biological activity, were used and the side chain protecting groups, which can introduce potential solubility problems, were left in place during the polymerization process. The assembly of polyacrylamides bearing sialic acid groups has also been reported.[23]

The polymerization reaction was routinely carried out in degassed 6 M guanidine−HCl (GuHCl) and 2 mM EDTA in 0.5 M Tris (pH 8.3) although solvents such as 1.5 M Tris−HCl (pH 8.8) and 8 M urea were also successfully employed. A 50-fold molar excess of acrylamide over the amount of acryloylated peptide was introduced to allow stretches of polyacrylamide to be interspersed between the various acryloyl peptide units with the aim of minimizing steric interactions between the peptide chains and to maximize water solubility of the overall polymer. This was not always necessary because peptides P2, P9, P10, and P13 incorporating Ahx were polymerized successfully in the absence of added acrylamide. Polymerization was initiated by addition of ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) and the reaction mixture allowed to stand for 18 h at room temperature under N_2. Time course analysis of the polymerization process indicated that incorporation of peptide into polymer is rapid with evidence that some peptide is incorporated into polymer within 15 min. Figure 3 shows high molecular weight peptide containing material formed when $CH_2{=}CHCO-P8$ but not when NH_2-P8 was subjected to the polymerization conditions. Each of the peptide polymers examined eluted in the void volume of a column (1 × 30 cm) of Superose 6 which has an exclusion molecular weight of approximately 4 × 10^7 Da for globular structures. Amino acid analysis of the polymers indicated that 80 to 97% of acryloylated monomeric peptides was incorporated into the polymer. The data also indicate that different peptides may be incorporated into the same polymeric structure and the ratios of these, when co-polymerized in equimolar amounts, is approximately equivalent (Table 2). This technique was successfully applied to the copolymerization of the following combinations of peptides: P1 + P3; P2 + P3; P1 + P2 + P3; P1 + P4; P2 + P4; P1 + P2 + P4; P8 + P11; P9 + P11; P9 + P10; P9 + P10 + P11; P1 + P3 + P14 + P15; P1 + P14; and P1 + P15.

Antigenicity of Synthetic Peptide Polymers. A potential concern of free radical-induced polymerization of unprotected peptide monomers is that their side-chain functions may be altered affecting their antigenicity and immunogenicity. To examine this possibility, polymerized P8 and polymerized P2, both of which contain side-chain functional groups likely to be affected by free radicals, were coated on plastic microtitre trays and probed with antiserum directed against P8, or a monoclonal antibody (MAb 1/1) which is specific for P2.[26,27] The results (Figure 4) demonstrate that each antibody was capable of binding efficiently to determinants within the polymers indicat-

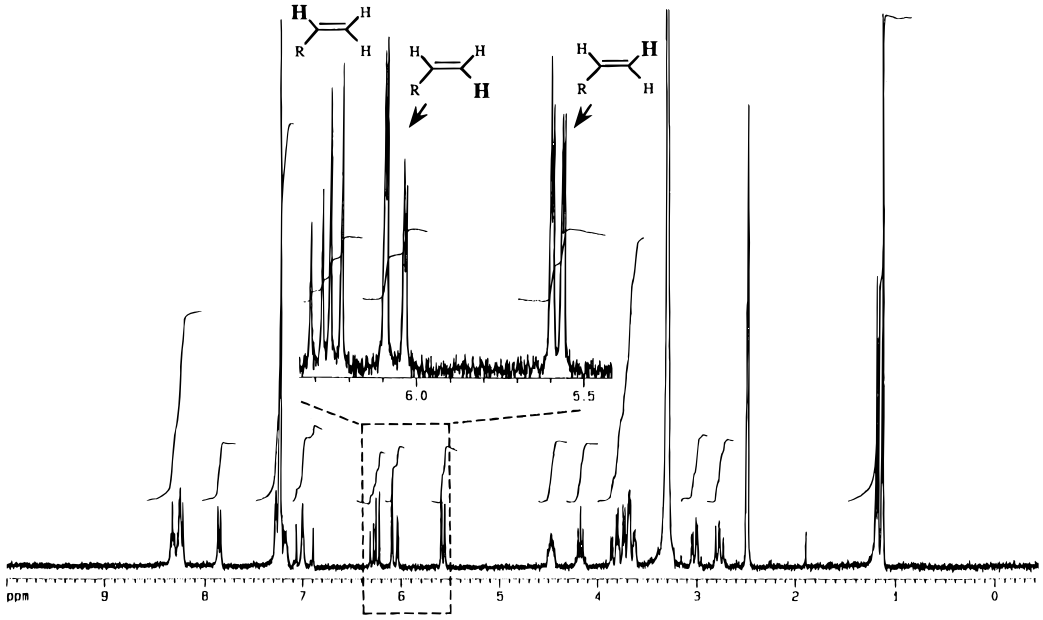

Figure 2. ^{1}H NMR of *N*-acryloyl-GFGA. The *N*-acryloylated peptide was cleaved from the resin using reagent B, purified by RP-HPLC, and examined by ^{1}H NMR. The characteristic chemical shifts for the acryloyl group (^{1}H NMR (d_6-DMSO) 6.27 (dd, 1H, J_{trans} = 17.3 Hz, J_{cis} = 10.34 Hz, **CH**=CH_2), 6.08 (dd, 1H, J = 17.13 Hz, J_{gem} = 1.8 Hz, H-trans), 5.57 (dd, 1H, J = 11.16 Hz, J_{gem} = 1.4 Hz, H-cis)) are indicated.

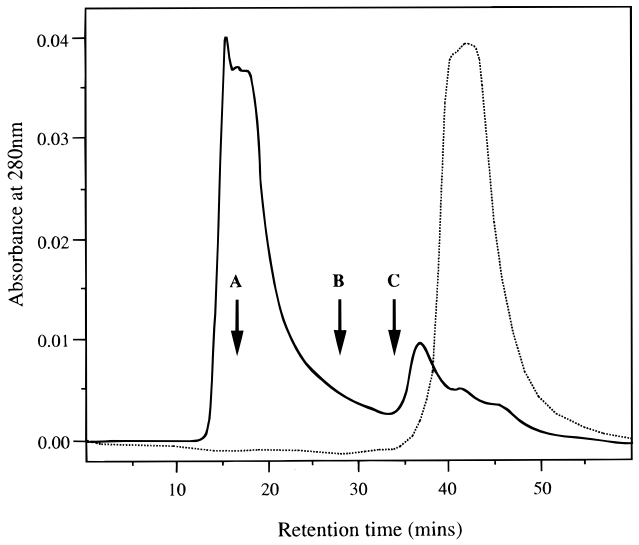

Figure 3. Gel permeation chromatography of monomeric and polymeric peptides. CH_2=CHCO−P6 (—) and NH_2−P6 (···) were subjected to the polymerization protocol and introduced to a column (1 × 30 cm) of Superdex 200 and the chromatogram developed at a flow rate of 0.5 mL min^{-1} in 50 mM NH_4HCO_3. The column effluent was monitored at 280 nm to detect tryptophan and tyrosine residues. The arrows indicate the retention time of the molecular weight standards: thyroglobulin 669 kDa (A), bovine serum albumin 67 kDa (B), and ribonuclease 13.7 kDa (C).

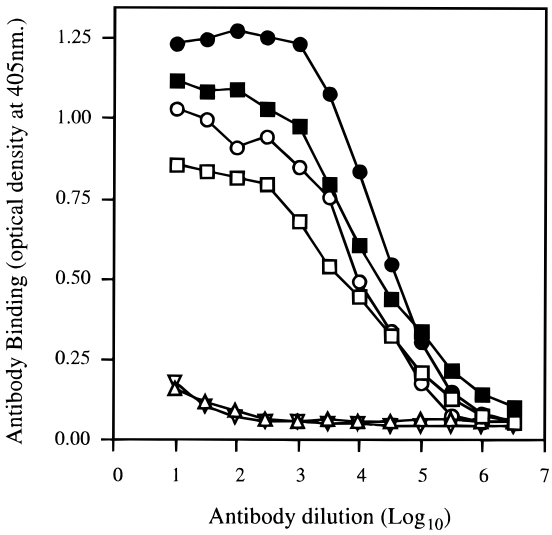

Figure 4. Antigenic integrity of peptides and peptide polymers. The binding of anti-P8 antisera to polymerized P8 (■), P8 monomer (□), or polyacrylamide (△) and the binding of MAb 1/1 to polymerized P5 (●), P5 monomer (○), or polyacrylamide (▽) was examined by ELISA.

ing that the epitopes remain antigenically intact despite exposure to free radical.

An advantage of polymerizing synthetic peptides is that the multiple copies of a peptide that are present in a single molecule could confer enhanced antigenicity over peptide monomers.[28] To evaluate this, an inhibition ELISA, which is independent of any difference in the ability of the antigens to bind to the microtitre plate, was used to compare the antigenic properties of monomeric and polymeric peptides. P2 and polymerized P2 were used to inhibit the binding of MAb 1/1 to P5-coated microtitre plates and similarly, P8 monomer and polymerized P8 were used to inhibit the binding of the anti-P8 antiserum to P8-coated plates. As can be seen in Figure 5, polymeric peptides are more antigenic than peptide monomers. A polymer of P4 (which does not contain epitopes recognized by MAb 1/1 or anti-P8 antiserum) did not inhibit the binding of these antibodies to their relevant peptides indicating that increased inhibition caused by the peptide polymers is a consequence of the presentation of multiple copies of each peptide determinant. The results in Figures 4 and 5 demonstrate that polymerized peptides not only retain antigenic integrity but are more antigenic than the corresponding peptide monomer, presumably because multiple copies of the same antigenic determinant allows high avidity interaction with antibody.

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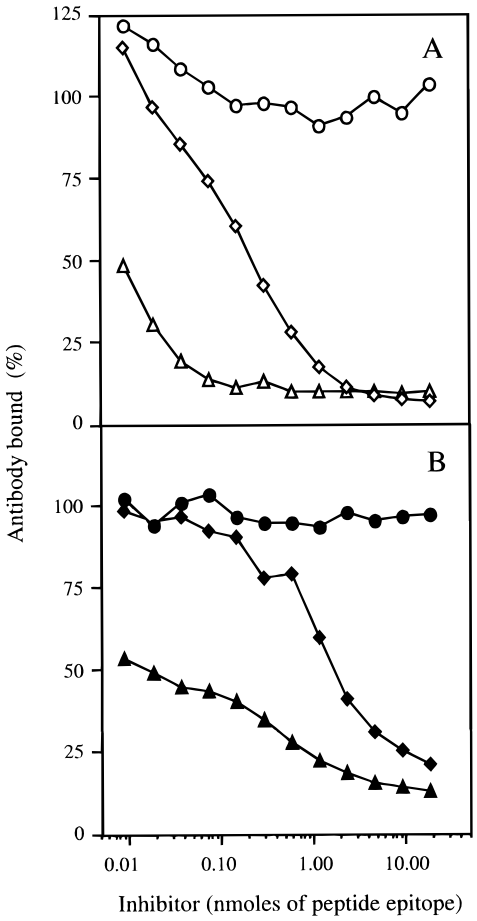

Figure 5. Ability of soluble monomeric and polymeric peptides to inhibit antigen−antibody binding. Dilutions of peptide or peptide polymer were mixed with a constant amount of antibody and added to wells of a microtiter tray coated with peptide antigen. Binding of the antibody was measured by ELISA and the result expressed as the percentage of the antibody bound in the absence of inhibitor. (A) P2 monomer (◇), polymerized P2 (△), and polymerized P4 (○) were incubated with MAb 1/1 and binding assessed on P5 coated wells. (B) P8 monomer (◆), polymerized P8 (▲), and polymerized P4 (●) were incubated with anti-P8 antisera and binding assessed on P8-coated wells.

Conclusion

The synthesis and polymerization of unprotected acryloyl peptides provides a practical generic method for the synthesis of peptide macromolecules. High yields of acryloyl peptides are obtained by using acryloyl chloride to derivatize the N-terminus of protected peptides still attached to the resin support followed by cleavage and deprotection using reagent B. The inclusion of 6-aminohexanoic acid as a spacer between the peptide and the acryloyl group allows the polymerization of certain peptides in the absence of acrylamide. Free radical polymerization does not affect the antigenic integrity of peptides, and peptide polymers are more antigenic than monomeric peptides. An advantage of this approach is that any number of the same or different acryloyl peptides should be able to be assembled into a polymer with the expectation that the overall antigenic activity of the construct will be largely determined by the nature of the peptide units which are pendant from a hydrocarbon chain. By polymerizing a mixture of B cell and T cell peptide epitopes it may be possible to assemble a construct in which all or most of the important epitopes of a pathogen or of several pathogens are represented. This is particularly important for those organisms such as the malaria parasite, HIV, and influenza virus and group A streptococci which occur as serologically diverse strains.

Experimental Section

Materials and Methods. Unless otherwise stated chemicals were of peptide synthesis grade or its equivalent. *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIPEA), *N*,*N*-dimethylformamide (DMF), piperidine, trifluoroacetic acid (TFA), and 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids were obtained from Auspep Pty Ltd (Melbourne, Australia). Thioanisole, anisole, ethanedithiol (EDT), triisopropylsilane (TIPS), and acryloyl chloride were obtained from Aldrich (New South Wales, Australia). 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) was obtained from Sigma Chemical Company (New South Wales, Australia) and acrylamide, ammonium persulfate, TEMED, and tris(hydroxymethyl)aminomethane (Tris) were obtained from BioRad (Richmond, CA). Phenol, dichloromethane (DCM), and diethyl ether were obtained from BDH (Poole, UK).

Solid-Phase Peptide Synthesis. Peptides were synthesized manually or using an automatic peptide synthesizer (either a Novasyn Crystal, Novabiochem, U.K., or a 9050 Plus PepSynthesiser, Milligen, Millford, MA). Standard solid-phase peptide synthesis protocols for Fmoc chemistry were used throughout. Peptides were assembled as the C-terminal carboxyl or carboxyamide form using Novasyn KA 100 or KR 100 resins (Calbiochem-Novabiochem, New South Wales, Australia), respectively. Coupling was accomplished with HBTU/HOBt activation using 4 equiv of amino acids and 6 equiv of DIPEA. The Fmoc group was removed by 20% piperidine in DMF or 2.5% DBU in DMF. Cleavage of peptides from the resin support was performed using reagent B (88% TFA, 5% phenol, 5% water, 2% TIPS) for 2 or 4 h depending on the arginine content of the peptide. After cleavage the resin was removed by filtration and the filtrate concentrated to approximately 1 mL under a stream of nitrogen. After the peptide products were precipitated in cold ether, they were centrifuged and washed three times. The peptide precipitate was then dissolved in 5 to 10 mL of water containing 0.1% v/v TFA and insoluble residue removed by centrifugation.

***N*-Acryloylation of Peptides.** Resins bearing peptides were swollen in a minimum amount of anhydrous, de-aerated DMF and acryloylated under nitrogen. After cooling on ice, a 20-fold molar excess of DIPEA in 0.5 mL of DMF and a 10-fold molar excess of acryloyl chloride in 0.5 mL of DMF were added to the resin. The mixture was stirred for 1 h on ice and then for a further 1 h at room temperature. The progress of acryloylation was monitored by the trinitrobenzenesulfonic acid (TNBSA) test. When a negative TNBSA test was returned the resin was washed (5 × in DMF, 3 × in DCM, and 3 × in diethyl ether). The resin was then dried under vacuum.

Peptides P4 and P6 were assembled with Fmoc-Lys(Mtt)-OH (Calbiochem-Novabiochem, New South Wales, Australia) at their C-terminus and Boc-Pro-OH and Boc-Asp(OtBu)-OH at the N-terminus of P4 and P6, respectively. After assembly of the peptide, the Mtt group was removed with 1% TFA containing 5% TIPS in DCM and Fmoc-Ahx then coupled to the free ϵ-amino group using HBTU activation. The Fmoc group was removed from the Ahx with 2.5% DBU in DMF and the exposed amino group acryloylated as above.

Purification of Peptide Monomers. Purification of synthesized peptides was performed using a Pharmacia C18 Pep RPC column (1.6 × 10 cm) installed in a Fast Protein Liquid Chromatography (FPLC) system (Amrad-Pharmacia Pty. Ltd., Victoria, Australia). Chromatograms were developed at a flow of 4 mL min^{-1} using 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) as the limit buffer. All peptides were eluted with a linear gradient of 10−30% solvent B formed over 40 min. Analytical HPLC was carried out using a Vydac C18 column (4.6 × 300 mm) installed in a Waters HPLC system. Chromatograms were developed using solvent A and solvent B at a flow rate of 1 mL min^{-1} and a 0−100% linear gradient of solvent B formed over 30 min. Material eluted from columns was detected by determining the absorbance at 214 or 280 nm.

^{1}H NMR spectra of the *N*-acryloyl peptides were recorded at 300 MHz on a Bruker-AM300 spectrometer (in DMSO-d_6). FAB mass spectra were recorded using a JOEL LMS-DX 300 spectrometer using an acceleration voltage of 3 keV with an FAB primary ion energy of 6 keV and emission current of 20 A. Xenon was employed as the bombardment gas. The scan range was 100−1500 *m*/*z*.

Copolymerization of Acryloyl Peptides with Acrylamide. General conditions for all polymerizations were as follows: a 1:50 molar ratio of acryloyl peptide(s) and the co-monomer acrylamide (final concentration of 5% w/v) were mixed in degassed 6 M guanidine−HCl (GuHCl) in 2 mM EDTA and 0.5 M Tris (pH 8.3). Polymerization was initiated by addition of ammonium persulfate at a concentration of 2% of the molar concentration of acrylamide present and 5 μL of a 20% v/v solution of TEMED. Polymerization was allowed to proceed for 18 h under nitrogen at room temperature.

Purification and Partial Characterization of Peptide Polymers. Peptide polymers were isolated by gel permeation chromatography (GPC) using a column (1.6 × 60 cm) of Superdex 200 installed in a FPLC system. Chromatography was carried out at a flow rate of 3 mL min^{-1} in 50 mM NH_4HCO_3. All polymers eluted in the void volume. Peptide polymers isolated in this way were then lyophilized.

Amino acid composition of the peptide monomers and polymers was confirmed by amino acid analysis of purified material. Peptide material was hydrolyzed (0.001% w/v phenol in 6 N HCl for 24 h at 110 °C) and the hydrolysate derivatized with Fmoc-Cl. Amino acid analysis was carried out using a GBC Aminomate system using fluorometric detection.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISAs were performed as described[29] using a solution (5 μg mL^{-1}) of peptide or peptide polymer to coat wells of flat-bottomed polyvinyl microtitre plates (Microtiter, Dynatech Laboratories, VA). Bound antibody was then detected by incubation with horseradish peroxidase-conjugated (HRPO) rabbit immunoglobulin (Ig) directed against mouse Ig (DAKO, Denmark) or HRPO donkey Ig directed to sheep Ig (DAKO, Denmark) for 1.5 h. After washing, substrate (0.2 mM 2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid) in 50 mM citric acid at pH 4.0 containing 0.004% v/v hydrogen peroxide) was added and after color had developed the optical density (OD) at 405 nm was measured using a Titertek Multiskan MC (Flow Laboratories, Melbourne, Australia).

Inhibition ELISAs were carried out using a $^1/_{1500}$ dilution of MAb 1/1 or anti-P8 antiserum incubated with known concentrations of inhibitor (soluble peptide or polymer) for 2 h and then transferred to flat-bottomed polyvinyl microtiter plates coated with P5 or P8. Following overnight incubation, the plates were washed and the ELISA developed as above.

Antibodies. The preparation and properties of monoclonal antibody MAb 1/1 has been described elsewhere.[26] MAb 1/1 was raised against the synthetic peptide representing the C-terminal 24 residues (^{305}CPKYVKQNTLKLATGMRNVPEKQT^{328}) of the heavy chain (HA_1) of the hemagglutinin of influenza virus A/Memphis/1/71 (H3) and is specific for the B cell determinant RNVPEKQT.[27] Hyperimmune serum (HIS) was raised in sheep to luteinising hormone releasing hormone (LH-RH; P8) and was a generous gift from Dr. M. McNarmara (CSL Ltd., Melbourne, Australia).

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