General method for the rapid solid-phase synthesis of large numbers of peptides: Specificity of antigen–antibody interaction at the level of individual amino acids

(simultaneous multiple-peptide synthesis)

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ABSTRACT A novel yet simple method is described that facilitates the synthesis of large numbers of peptides to the extent that the synthesis process need no longer be the limiting factor in many studies involving peptides. By using the methods described, 10–20 mg of 248 different 13-residue peptides representing single amino acid variants of a segment of the hemagglutinin protein (HA1) have been prepared and characterized in less than 4 weeks. Through examination of the binding of these analogs to monoclonal antibodies raised against residues 75–110 of HA1, it was found that a single amino acid, aspartic acid at position 101, is of unique importance to the interaction. Two other residues, aspartic acid-104 and alanine-106, were found to play a lesser but significant role in the binding interaction. Other single positional residue variations appear to be of little or no importance.

Over the past 5 years there has been an escalating need for synthetic peptides in a wide variety of applications, including the development of synthetic peptide vaccines (1–5), the detailed study of antigen–antibody interactions (6–11), the preparation of optimal analogs of biologically active peptides (12–14), the optimization of peptide antigens of clinical diagnostic utility (15–17), the mapping of protein products of brain-specific genes (18, 19), and the study of protein conformational parameters (20). In the majority of these studies, the limiting factor has been the availability and cost of the desired peptides. Clearly, these studies would be greatly facilitated if synthetic methods were available that would permit the synthesis of larger numbers of peptides in a more cost efficient manner and in a shorter period of time per peptide. While it is currently possible to produce large numbers of peptides (i.e., hundreds) using available methods, the effort is so time-consuming and expensive that to actually carry out this number of syntheses is impractical, even in especially well-equipped laboratories. The first part of this paper describes a general method that can be utilized for the rapid (2–6 weeks) synthesis of large numbers of peptides (>100) in quantities that satisfy most needs (>10 mg).

It is known from recent studies that antibodies raised against synthetic peptides frequently react with sites in the parent protein (1, 2, 11). These findings allow more detailed understanding of immunological interactions. Thus, instead of defining the reactivity of a protein antigen at the whole protein or subunit level, antigen–antibody interactions can be localized within the antigen to a short sequence of immunological importance (7, 11). The next step is the refinement of our understanding of the antigenic sites of proteins or peptides at the level of individual amino acids. To illustrate the utility of the synthesis procedures presented here, the second part of this paper presents a detailed study of peptide

antigen–antibody interactions. In the example presented, 248 individual peptides were synthesized and used in a study of one particular antigen–antibody interaction. The peptide analogs were variations of a specific sequence within the influenza hemagglutinin (HA) molecule (HA1, residues 98–110). By using standard ELISA procedures, an examination was conducted of changes that occur in the binding of the various analogs with monoclonal antibodies raised against a longer parent sequence found in HA1 (residues 75–110). It was possible, by using the methods described, to distinguish exactly which of the amino acids in this linear sequence were of importance in the peptide's interaction with its corresponding antibodies.

MATERIALS AND METHODS

Synthesis of Peptides. General methods. Standard Boc (*t*-butoxycarbonyl) amino acid resin (50–100 mg; 0.2–0.8 meq/gm) was contained in polypropylene 74- μ m mesh packets having approximate dimensions of 15 $\times$ 20 mm. After a number was placed at the top of the unsealed bag with a black marking pen, the packet was closed and the number was permanently sealed into the polypropylene to give an easily readable label for each bag. These packets are currently available from Biosearch (San Rafael, CA). The resins used included, but were not limited to, standard benzyl-linked polystyrene resins (21), spacer-linked styrene resins (22), polyamide resins (23), or macroreticular resins (6). These resin packets can be used for simultaneous multiple-peptide syntheses (SMPS)—that is, syntheses in which many different peptides are produced concurrently—or for multiple-analog peptide syntheses (MAPS)—syntheses in which many analogs of a particular peptide are produced concurrently. The standard deprotecting, neutralization, coupling, and wash protocols used in this laboratory were variations of Merrifield's original solid-phase procedures (21) and are described in detail elsewhere (24, 25).

Synthesis of analogs having single amino acid variations. By using various peptide synthesizers or completely manual methods, 40–80 individual packets containing the desired starting resin were carried through their common Boc-removal, washing, and neutralization steps. After their methylene chloride washes to remove excess base, the packets containing the neutralized peptide resins were removed from the reaction vessel and added to solutions containing performed symmetrical anhydrides of the next protected amino acid (26). The individual coupling steps were carried out for 60 min with stirring at room temperature. After completion of the coupling steps, the resin packets were returned to the reaction vessel, and the synthesis process was continued through additional cycles of common wash, deprotection, neutralization, and coupling steps until the syntheses were

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Abbreviation: HA, hemagglutinin.

completed. Specific variations of analogous peptides, such as single residue-replacement or -omission analogs or chain-lengthened or -shortened analogs, were easily accomplished by removing the individual coded packets at the point of variation during the synthesis, carrying out the desired variation separately, and, if appropriate, returning the packet to the common reaction vessel for completion. After the preparation of a series of protected peptide resins, the resin-filled packets were washed thoroughly, dried, and weighed to give an initial indication of coupling completion. The protected peptide resins, still contained within their packets, were then cleaved by using conventional HF/anisole procedures (24) in a vessel modified to allow cleavage of 20 peptide resins at once. After extraction of the residual anisole with ether or ethyl acetate, the peptides were extracted from the resin packets by 5% acetic acid and either were lyophilized directly or were put through a Sephadex G-10 desalting column prior to lyophilization. The crude peptides were characterized by HPLC and found to have an average purity of 84% (70–94%). For the studies described here, the peptides were not purified further. The amino acid compositions of the individual peptides were determined by using an LKB model 4150 amino acid analyzer after the hydrolysis of the peptides in constant-boiling HCl for 24 hr at 110°C. All values were between $\pm 10\%$ of theory.

ELISA Methods and Materials. ELISAs for determination of the percentage of binding of the analogs relative to the control peptide were carried out with 10 nmol of each peptide adsorbed to separate wells of 96-well microtiter plates (Costar 1/2 area plates). Peptides were adsorbed to microtiter plates by incubation in 0.6 M sodium bicarbonate/0.03 M sodium carbonate buffer, pH 9.6, at room temperature for 8 hr. Then the plates were washed 10 times with deionized water to remove unbound peptide. Blocking to prevent nonspecific adsorption of the antisera was accomplished by incubating the plates with 100 μ l of 1% bovine serum albumin (Sigma) in phosphate-buffered saline (albumin/saline) per well for 1 hr at 37°C. Antibodies were bound to the analogs by incubating at 37°C for 1 hr with 25 μ l of a 1:10 dilution of the cell-free supernatant prepared from the hybridoma cell line 20C01 (7, 11). After 10 washes with deionized water to

FIG. 1. Illustration of a mesh packet containing resin.

remove unbound antibody, horseradish peroxidase-conjugated rabbit anti-mouse IgG (Zymed Laboratories, Burlingame, CA) was added to each well at a dilution of 1:3000 in albumin/saline and incubated 1 hr at 37°C. Excess conjugate antibody was removed by washing as before with deionized water. The amount of conjugate antibody bound in each well was quantitated by reaction for 20 min with 100 μ l of a freshly prepared developing solution of 24 mg of *o*-phenylenediamine (Pitman-Moore, Washington Crossing, NJ) and 1.0 ml of 3% hydrogen peroxide in 50 ml of deionized water. The resulting color was read at 495 nm by a Titertek multiscan spectrophotometer (Flow Laboratories).

RESULTS

Peptide Synthesis. A series of 247 replacement analogs and 13 identical controls of the 13-amino acid sequence corresponding to residues 98–110 (Y P Y D V P D Y A S L R S; standard single-letter amino acid code) of HA1 were prepared. Fig. 1 illustrates the type of packet used in their preparation. A wide variety of sizes, shapes, and materials can be used for these packets without significantly changing the efficacy of the synthesis. The amount of resin used for a given synthesis relative to the size of the packet was such that

Table 1. Amino acid analysis of Y P [] D V P D Y A S L R S series of peptides

Peptide no.	Amino acid in position []	Amino acid composition of peptide with substitution at [],* % of theoretical value								
		A	2D	L	2P	2S [†]	V	2Y [†]	R	[]
1	A	100	99.4	98	95.4	93.2	94.2	94.8	101.2	100
2	C [†]	100	95.1	101.0	98.7	91.3	101.1	92.8	98.2	90.0
3	D	100	96.7	92.6	97.9	90.0	100.2	99.0	98.8	96.7
4	E	100	100.3	99.5	100	94.0	91.4	91.1	96.1	93.1
5	F	100	91	109.4	99.5	93.1	100.6	90	92.9	98.7
6	G	100	99	107.9	106.7	96.4	103.5	101.3	100.6	93.7
7	H [†]	100	91.2	103.8	99.0	90.6	92.1	102.4	100.0	101.2
8	I	100	96.4	103.5	95.2	91.7	100.4	94.1	95.2	96.2
9	K	100	99.5	97.9	91.5	92.7	96.6	90.5	99.3	109.0
10	L	100	98.0	102.9	103.4	97.4	94.7	97.4	98.1	110.0
11	M	100	102.5	104.3	100.7	95.2	93.9	93.8	103.4	91.2
12	N	100	92.9	91.9	93.5	90.6	96.1	91.6	92.3	91.9
13	P	100	92.5	93.0	90.1	92.3	90.4	90.6	96.3	100.1
14	Q	100	109.4	94.0	101.2	97.3	92.6	94.4	102.9	103.7
15	R	100	94.7	101.0	102.1	94.8	99.1	90.7	95.1	92.7
16	S [†]	100	98.5	99.8	103.6	97.3	95.8	91.2	98.2	97.3
17	T [†]	100	103.2	107.5	107.8	98.7	104.0	96.9	100.2	97.0
18	V	100	108.5	99.7	96.6	101.6	96.0	96.2	99	96.1
19	W [†]	100	96.9	98.7	103.8	98.1	93.4	91.0	97.4	92.0
20	Y [†]	100	99.7	97.2	96.3	98.1	95.6	91.2	97.8	109.2

Amino acid analysis of a set of 20 single amino acid replacement peptides made by multiple-analog peptide synthesis.

*For peptides 1–20, no other contaminating amino acids were present at the lowest level of sensitivity (<0.1%) of the amino acid analysis.

[†]Uncorrected for destruction during hydrolysis.

FIG. 2. Typical HPLC analyses of analogs of HA1, residues 98–110. Position number 100 was varied to prepare arginine (A), threonine (B), tyrosine (C), isoleucine (D), and phenylalanine (E) analogs. The peptides were examined in crude form directly following HF cleavage and lyophilization. Buffer A was 20% acetonitrile/80% H_2O /0.1% CF_3COOH ; buffer B was 80% acetonitrile/20% H_2O /0.1% CF_3COOH .

the swelling of the resin was readily accommodated by the space in the packet. By using these packets, 260 individual peptides were synthesized with a total of >3380 coupling steps in a period of 4 weeks. Each peptide was obtained in 10- to 20-mg quantities. By comparison of the results of both HPLC and AAA, the purity of the peptides prepared by multianalog peptide synthesis or simultaneous multipolypeptide synthesis was found to be as good or better than the purity of those peptides prepared by individual free resin syntheses. Representative amino acid analyses of the replacement series

at position 100 are given in Table 1, and the HPLC chromatograms are shown in Fig. 2. The HPLC information was gathered on the crude peptides, and no attempt was made to optimize any of the syntheses. It is important to note that the amino acid analysis data (Table 1) and the HPLC data (Fig. 2) clearly show that no cross-contamination at a given position occurred for different peptides prepared concurrently.

ELISA Results. An indication of the extent of binding of the various analogs with antibodies produced by a single monoclonal cell line, relative to the binding of control peptides, is given in Table 2 and Fig. 3. The ability of a particular analog to bind to the monoclonal antibody is represented as a percentage of the optical density relative to the control peptide bound to the same plate. For amino acid variations at positions 98 (Y), 99 (P), 100 (Y), 102 (V), 103 (P), 105 (Y), 107 (S), 108 (L), 109 (R), or 110 (S), there was no significant difference in the binding relative to the control sequence as determined by ELISA. A dramatic loss of binding ability relative to the control was seen for peptides in which the aspartic acid at position 101 was replaced with any other amino acid. Significant binding remained only with replacement of aspartic acid with asparagine, glutamic acid, and glutamine. Although it is not immediately obvious from Table 2 or Fig. 3 *Upper*, a small but significant loss of binding ability occurred when aspartic acid at position 104 and alanine at position 106 were replaced with other amino acids. This is more readily apparent in Fig. 3 *Lower*, which shows the percentage of decreased binding relative to the control peptide. The data in Fig. 3 *Upper* and *Lower* are arranged in the order of decreasing hydrophobicity (27). Within the limits of resolution, little correlation was found between the binding ability and hydrophobicity of the individual amino acids. Since the peptides prepared ranged in purity from 70% to 95% (average, 84%), the effect of the variation in the amount of antigen on the plate was considered. By using the control peptides, representative binding curves showed that a decrease by as much as a factor of 50 in the amount of antigen on the plate caused no difference in the resulting amount of bound antibody (data not included).

Table 2. Percentage binding of monoclonal antibodies (20C01) to a replacement series of peptides

Natural amino acids	Position (amino acid) replaced in Y P Y D V P D Y A S L R S												
	1 (Y)	2 (P)	3 (Y)	4 (D)	5 (V)	6 (P)	7 (D)	8 (Y)	9 (A)	10 (S)	11 (L)	12 (R)	13 (S)
W	99.1	107.2	104.5	9.4	71.0	64.7	33.1	76.0	21.0	47.6	100.9	94.0	101.0
F	92.3	109.3	96.8	7.3	80.7	86.1	39.4	114.4	107.8	67.5	97.3	84.8	103.2
L	99.8	102.3	94.1	9.0	90.7	72.1	62.0	88.9	32.6	73.4	100.0	97.9	110.1
I	101.2	101.7	87.6	7.8	84.9	74.8	81.1	107.4	73.3	70.7	109.0	101.7	106.8
Y	100.0	109.3	100.0	7.7	84.0	79.5	63.7	100.0	30.0	77.3	101.2	107.2	98.3
V	120.2	119.9	96.5	8.2	100.0	89.2	67.9	93.2	48.4	83.1	111.9	104.1	103.5
C	97.2	102.9	96.2	8.8	81.0	60.6	42.9	94.4	28.1	87.6	106.9	107.8	121.3
M	100.0	112.4	104.7	9.9	87.1	68.5	49.5	103.1	31.2	79.8	114.6	110.9	107.3
P	105.5	100.0	103.1	6.3	77.1	100.0	77.2	109.3	54.1	48.5	102.7	116.1	106.1
A	101.7	108.6	76.3	9.9	93.6	66.5	73.4	80.4	100.0	107.4	97.9	113.8	99.4
E	114.0	115.6	106.3	26.9	88.2	101.6	67.3	105.8	36.1	94.1	111.9	98.1	108.9
G	132.1	114.9	79.2	7.2	102.7	99.4	62.1	102.6	76.4	93.2	107.9	112.6	100.4
R	113.2	117.8	108.2	9.7	86.8	76.3	54.8	106.2	32.7	81.4	105.5	100.0	98.5
H	111.4	90.2	73.2	22.0	95.4	87.0	64.8	83.5	24.9	89.2	105.5	108.9	112.2
D	100.6	106.3	107.9	100.0	96.4	98.3	100.0	131.6	57.4	107.0	100.1	114.4	109.1
T	118.8	109.2	87.6	10.9	97.7	101.7	58.4	78.8	58.6	90.5	103.2	103.5	100.7
K	103.8	111.3	103.4	6.3	88.5	86.7	58.0	118.0	49.6	74.2	87.9	90.7	114.0
Q	138.3	103.4	116.7	11.3	93.3	91.0	71.5	128.0	32.6	99.2	107.4	109.2	126.3
S	127.7	108.1	100.0	13.0	90.0	85.0	73.1	111.8	94.5	100.0	108.2	104.0	100.0
N	102.6	116.0	107.5	34.7	115.3	100.3	78.6	150.7	53.9	92.9	111.9	108.6	107.7

Binding of monoclonal antibodies to the individual complete set of replacement analogs, expressed as a percentage relative to binding to the control peptide (Y P Y D V P D Y A S L R S).

FIG. 3. (Upper) This figure illustrates the binding of the monoclonal antibodies (20C01) to the analogs formed by replacement of the positions in the HA1 sequence Y P Y D V P D Y A S L R S with each of the 20 naturally occurring amino acids. Each separate graph illustrates the binding of antibody to the replacement set of analogs for the position indicated below the graph. Each vertical line on the graph represents the percentage of OD of one replacement analog relative to that of the standard HA1 sequence above bound to the same plate. The replacement analogs are arranged on the ordinate of each histogram in the order of decreasing hydrophobicity from left to right as follows: W-F-L-I-Y-V-C-M-P-A-E-G-R-H-D-T-K-Q-S-N. (Lower) This figure is an inverted representation of the graphs shown in Upper. The individual lines illustrate the percentage of decreased binding relative to that of the control HA1 sequence. Binding of $\geq 100\%$ of analogs relative to that of the control are given a value of 0 (0% decreased binding).

DISCUSSION

The method of solid-phase peptide synthesis as presented by Merrifield in 1963 (21), has changed little from the original conception and almost universally is still used today. This method, which over the past 20 years has been of unparalleled utility, uses a single reservoir that contains a polymeric solid support having the initial COOH-terminal amino acid of the desired peptide covalently attached to it. The coupling of additional amino acids is a process that uses repetitive, easily automated washing, deprotection, and neutralization steps. Although all steps except the coupling reaction are identical, only a single peptide is generally prepared at a given time. To utilize the common steps in the synthesis procedure, a method has been described previously in which two different-sized resins were used and later separated, enabling two syntheses to be carried out simultaneously (28); also an apparatus has been devised that permits four concurrent manual syntheses (29). However, these procedures are somewhat limited in scope and practicality. An interesting method that has been presented recently involves the synthesis of peptides permanently bound to a solid support (9, 10) and interaction of these support-bound peptides with antisera in

an ELISA-type assay. While of obvious utility, this method of peptide synthesis as presented (9, 10) appears to have several limitations: only microgram amounts of peptide can be generated, these peptides are not removable from the solid support, the effect of the solid support on the antigen-antibody interaction is unclear, large unexplained variations in the antibody binding behavior of identical control peptides were found, and there is no provision for accurately varying the concentration of antigen on the solid support. Studies in this laboratory clearly indicate that the relative concentration of antigen is of critical importance in antigen-antibody interaction (data not shown).

The methods presented in this paper for the production of peptides use a procedure in which the individual resins for the solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in solid-phase methods. This approach can be used successfully with all resins commonly employed for solid-phase peptide synthesis (6, 21-23). The data presented establish that this means of synthesis is able to produce useful quantities of free peptides of quality equal to those produced by conventional methods, at a much faster rate, and with more cost-effective use of materials. For 50 representative samples from the 248 different sequences prepared, having variations along the entire chain, the purity of the individual crude peptides was comparable to that obtained by other methods of synthesis and ranged between 70% and 95% (average, 84%). It is important to note that the HPLC and amino acid analysis data clearly show that no cross-contamination occurred with different amino acids at a given position for peptides prepared concurrently. By using these methods in conjunction with automated synthesizers, it is possible to accomplish between four and eight couplings per day on at least 100 different peptides, or as many as 800 amino acid couplings in 1 day. In addition, a completely manual procedure can be used in which the various solvents are contained in separate vessels, and a basket carrying the resin packets is moved from one solvent reservoir to another. This procedure potentially allows a much larger number of syntheses (i.e., >500-1000) to be conducted simultaneously.

To illustrate the scope and potential of this new method, the peptides synthesized were used to determine the role of individual amino acids in the antigen-antibody interaction of an antigenic peptide sequence of influenza HA1, residues 98-110. Recent research has shown that monoclonal antibodies raised against HA1 residues 75-110 were able to bind strongly to the native HA molecule as well as to the parent peptide (7, 11). Studies of the binding of these monoclonal antibodies by using peptides of overlapping sequences within the parent region (residues 75-110) further localized the binding of eight of the monoclonal antibodies to the region of residues 98-110 (7). The importance of the individual amino acids in the sequence to the binding with the monoclonal antibodies remained to be determined. For this determination, 247 analogs of the 13-residue sequence corresponding to residues 98-110 of the HA molecule (Y P Y D V P D Y A S L R S), as well as 13 identical control peptides, were synthesized by the methods described above.

The use of these analogs in an ELISA binding study shows conclusively that positions 101 (B), 104 (B), and 106 (A) play a role in the binding of the monoclonal antibodies with this 13-residue peptide. The residue at position 101 was found to be of specific and unique importance in that substitution of this aspartic acid for any other residues produced a dramatic loss of ability to bind to the antibodies. Although residues 104 and 106 appear to be less sensitive to replacement by other amino acids, they are significant in that the average decrease in binding of antibodies to the peptides substituted in these

positions was 25% and 55%, respectively. Preliminary results from solution binding studies using an immunoprecipitation assay corroborate the ELISA data except that, in addition to residues 101, 104, and 106, the tyrosine at position 105 seems to have a role in the binding of the peptide to the antibody. The difference between the ELISA and immunoprecipitation data is being explored further.

The variation in the ELISA data for the 13 identical control peptides was 10%. This value is an indication of the random error inherent in the procedures used. Since several of the peptides had activities that were in the range of 120–135% of the controls, the question arose as to whether these peptides are actually interacting with the monoclonal antibodies to this extent or whether this is an artifact of the ELISA procedure. Preliminary results of studies in which these binding-enhancing amino acids are combined into single peptides indicate that this information can be used to prepare optimized peptide antigens. Other studies utilizing the synthetic procedures presented here have been carried out. These include the preparations of multiple analogs of a number of biologically active peptides for *in vivo* and *in vitro* studies, and the preparation of peptides for use in the study of discontinuous determinants in protein–antibody interaction. The fact that a single amino acid change in peptides can eliminate a peptide antigen–antibody interaction takes on greater significance when one considers the fact that effective immunity against a given influenza strain can be circumvented by “antigenic drift,” a process in which very few changes in linear chain sequence produce a “new” influenza strain that can elude the host’s protective immunity (30).

In summary, this work illustrates a new procedure for preparing peptides that greatly facilitates the production of large numbers of specific peptides. The study of antigen–antibody interactions with the peptides synthesized by this method demonstrates the utility and potential of the method for these studies as well as for other applications requiring synthetic peptides.

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