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# Separation of the Sticky Peptides from Membrane Proteins by High-Performance Liquid Chromatography in a Normal-Phase System[1]

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**The amphiphilic peptides obtained upon cleavage of membrane proteins, including numerous receptors, are recalcitrant to most separation techniques as a consequence of their limited solubility and tendency to aggregate and adsorb to surfaces. This paper describes HPLC systems that can separate these "sticky" peptides on silica and aminopropyl-modified silica columns with a mobile phase consisting of a mixture of chloroform/methanol/isopropylamine. The protocols developed have been applied to synthetic M1 and M2 peptides, which constitute part of the transmembrane domain of glutamate-gated ion-channel proteins. Four of these M1 and M2 peptides were separated from minor synthetic impurities, and a 23-mer was baseline separated from a 28-mer. The HPLC procedures have also led to purification of the 10 peptides resulting from cyanogen bromide cleavage of bacteriorhodopsin, peptides which have so far eluded HPLC separation despite numerous attempts. These HPLC protocols have been used to purify peptides ranging from 4 to 50 amino acids in high yield while the columns continued to resolve sharp peaks after more than 100 separation runs over a 6-month period. These new HPLC systems offer an efficient method for the isolation and analysis of this important albeit troublesome class of peptides.** 

The difficulties encountered in separating the sticky peptides obtained upon cleavage of membrane proteins have presented a constant obstacle in their structural studies. These peptides possess a mixed hydrophobic–hydrophilic (i.e., amphiphilic) character that makes them difficult to solubilize in common polar or nonpolar solvents, or leads to self-aggregation and adsorption to surfaces such as glassware and chromatographic columns. These problems have occasionally made it necessary to adopt extraordinary measures to generate the soluble monomers, including the complexation of peptides with inorganic salts (1) and the use of unusual solvent combinations (2).

Several soluble proteins, including cytochrome c′ and myohemerythrin, have a structural feature called the four $\alpha$-helix bundle, which is formed by the juxtaposition of four amphiphilic $\alpha$-helices (3). In this motif the hydrophobic side of each helix faces inward to facilitate interhelix binding, whereas the hydrophilic side faces outward to solvate with water. In contrast, most integral membrane proteins have a cluster of amphiphilic $\alpha$-helices that constitutes a transmembrane domain (4). In these motifs the hydrophilic face of the $\alpha$-helix is disposed inward, whereas the hydrophobic face is directed outward to interact with the lipid bilayer. Of the two types, the membrane proteins are generally more difficult to handle because of the greater overall hydrophobicity of their derived peptides (4).

The development of efficient methods for the purification of amphiphilic peptides has become absolutely essential to continue our structural studies of several integral membrane proteins employing photoaffinity labeling as the main technique (5,6). The sticky peptides derived from such proteins are generally refractory to traditional purification methods. Some success has been reported with reversed-phase HPLC (7–9), but

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these procedures have not been widely adopted in the research community. In one study, Khorana and coworkers were able to purify bacteriorhodopsin (BR)[5] peptides by using reversed-phase HPLC in conjunction with other methods (10). An ethanol gradient containing 5% formic acid was employed with a C-18 column for the HPLC; however, the sticky peptides eluted as broad, unresolved peaks. Photoaffinity studies of BR performed in our laboratory have also been constantly hampered by the extremely poor reversed-phase HPLC separation of the cleaved peptides (5,6).

Although silica-gel TLC is routinely used for peptide isolation (11), normal-phase HPLC has seldom been applied for the separation of membrane peptides. One article described silica HPLC of hydrophobic peptides in an isocratic mixture of cyclohexane/isopropanol/methanol, but the peptides eluted as broad peaks and short column lifetimes were observed (12). Alternatively, in an effort to avoid the difficult purification steps, tandem mass spectrometry (MS) has been applied to the unseparated mixture of 10 peptides resulting from cyanogen bromide (CNBr) cleavage of BR (Orlando *et al.*, manuscript in preparation). In this promising application, nine distinct peptide peaks were present in the tandem-MS, namely, peaks representing peptides 5, 2, 8, 1, 3, 7, 10, 9, and 6 (listed in sequence of increasing $M_r$, see Fig. 3 below). The one missing peak was that of the shortest peptide (4, a tetrapeptide), which was buried among the FAB matrix peaks in the low mass region. Importantly, tandem-MS was capable of directly sequencing all BR peptides with $M_r$ less than 3000, namely, all peaks except those of peptides 10, 9, and 6 (Orlando *et al.*, manuscript in preparation). This encouraging result has prompted ongoing studies on the application of tandem-MS to photoaffinity-labeled peptides. However, it remains imperative that preparative methods be developed for the purification of relatively large amounts of amphiphilic peptides, including synthetic peptides, for numerous purposes.

We report herein HPLC protocols to purify amphiphilic peptides on silica and aminopropyl-modified silica columns with a mobile phase consisting of a mixture of chloroform/methanol/isopropylamine, which is utilized in a linear gradient fashion. These procedures have led to high-yield, high-resolution separations of fairly complex mixtures of peptides while minimizing the degradation of columns. As exemplified in this report, the new HPLC systems will be useful for the isolation and characterization of certain troublesome members of this functionally important class of peptides.

[5] Abbreviations used: BR, bacteriorhodopsin; Cbz, carbobenzoxy; CNBr, cyanogen bromide; DCC, 1,3-dicyclohexylcarbodiimide; FAB, fast atom bombardment; Fmoc, 9-fluorenylmethoxycarbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; NMDA, *N*-methyl-D-aspartate; NMDA-1, NMDA peptide 1; NMDA-2, NMDA peptide 2; Quis, quisqualate; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; *t*-Boc, tertiary butyloxycarbonyl.

## MATERIALS AND METHODS

*Chemicals.* Synthetic peptides excepting the M1 and M2 peptides were purchased from Sigma, as was chymotrypsin. BR was purified from *Halobacterium halobium* and cleaved with CNBr according to standard procedures (13,10). HPLC-grade chloroform and methanol were from Fisher. Aldrich reagent-grade isopropylamine was distilled over calcium hydride before use.

*Peptide synthesis.* M1 and M2 peptides were synthesized on an Applied Biosystems Model 431A peptide synthesizer by solid-phase synthesis on a *p*-hydroxymethyl-phenoxymethyl polystyrene resin. The quisqualate M1 and M2 peptides were synthesized using HOBt-DCC activation, the M1 by Fmoc chemistry, and the M2 by *t*-Boc chemistry. The Asp and Arg side chains of the quisqualate M1 were protected with *t*-butyl and 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) groups, respectively, and the Arg of quisqualate M2 was protected by the mesitylene-2-sulfonyl (Mts) group. The NMDA peptides 1 and 2 were made using HBTU activation by Fmoc chemistry, the Glu and Asp side chains being protected with the *t*-butyl group. All peptides were cleaved from the resins with TFA and deprotected with TFA (87%), water (5%), thioanisole (5%), and ethanedithiol (2.5%) for 1.5 to 3 h.

*HPLC.* All separations were performed at room temperature on a Beckman 331 HPLC system. Solvent mixtures were degassed under an aspirator before use. The columns, μPorasil (silica) and μBondapak $NH_2$ (aminopropyl-modified silica), were obtained from the Waters Division of Millipore Corp. Peptides were dissolved in trifluoroethanol or 7/1/1 chloroform/methanol/isopropylamine (solvent A), sonicated briefly, and centrifuged at 14,000*g* in a microcentrifuge. Alternatively, samples were dissolved in 1/7/1 chloroform/methanol/isopropylamine (solvent B) and passed through a Waters Sep-Pak light $NH_2$ cartridge, then dried on a rotary evaporator and suspended in solvent A for injection onto the HPLC columns. The analytical scale columns had been preequilibrated to solvent A for at least 10 min at 1 ml/min (semiprep columns: 4 ml/min), and peptides were injected and the columns washed for 10 min with the same solvent. For separations employing the μBondapak $NH_2$ column, a linear gradient was developed over 45 min from 100% solvent A to 100% solvent B, whereas for the μPorasil silica column a 30-min gradient was developed from 100% solvent A to 50% solvent B. After the gradients the columns were washed for 10 min with the final solvent. Peptides were detected at 272 nm at a sensitivity of 0.5 absorbance unit full scale. Peaks were collected into glass round-bottom flasks or poly-

TABLE 1

Amphipathic M1 and M2 Peptides

| | | |
|---|---|---|
| | M1 peptide | |
| Quis | LALDIWCLVVGAFLLACLSLFALARFS | (27-mer) |
| | M2 peptides | |
| Quis | LVNQFSLSNSLWFVTGTLLRQGSGVNP | (27-mer) |
| NMDA-1 | EEEEEDALTLSSAMWFSWGVLLNSGIGE | (28-mer) |
| NMDA-2 | DALTLSSAMWFSWGVLLNSGIGE | (23-mer) |

*Note.* Sequences of synthetic peptides ($M_r$s determined by electrospray MS: Quis M1, 2932; Quis M2, 2936; NMDA-1 M2, 3100; NMDA-2 M2, 2454) corresponding to the M1 and M2 membrane-spanning segments of two glutamate-receptor subtypes. The M1 peptide represents a putative quisqualate (Quis) subtype from locust (18), whereas the M2 peptides represent the quisqualate and an NMDA subtype from rat (17); NMDA-1 is the five residue amino-terminal extension of NMDA-2. Hydrophobic residues are underscored to illustrate the amphipathic nature of these molecules. Hydrophobic amino acids require greater than 1.1 kcal/mol to be transferred from an $\alpha$-helix in the membrane interior to water (16).

propylene tubes and dried on a rotory evaporator or a Speed-Vac concentrator (Savant). The baseline change was caused by the changing refractive index of the solvent, and varied with the batch of solvent and the optics of the uv detector.

*Mass spectrometry.* Mass spectra were acquired on the first two sectors of a JEOL (Tokyo, Japan) HX/HX110A tandem four sector mass spectrometer, which was operated at 6 kV accelerating potential. Spectra are averaged profile data of 5–10 scans, which were acquired from 200 to 2500 $m/z$ at a rate that would scan from 1 to 6000 $m/z$ in 2 min as recorded by a JEOL Complement data system. A filtering rate of 100 Hz and an approximate resolution of 1000 were used in acquiring these spectra. Ions were produced in a JEOL MS-ESI electrospray ionization source. Samples were dissolved to an approximate concentration of 50 pmol/$\mu$l in a 1:1 mixture of water and methanol that contained 2% acetic acid and were introduced into the mass spectrometer at a flow rate of 2 ml/min. All measurements were performed on positively charged ions.

*Amino acid composition analysis.* Amino-acid compositions were determined on a Beckman Model 6300A analyzer. Peptides were hydrolyzed in 6 N HCl at 110°C for 24 h.

## RESULTS

A number of amphiphilic and hydrophobic peptides, including several purchased commercially, were employed to evaluate the HPLC conditions.

*Synthetic peptides.* The transmembrane domain of many ion-channel proteins consists of four peptides, M1/M2/M3/M4, which span the lipid bilayer in the $\alpha$-helical conformation (14,15). Table 1 shows the four synthetic M1 and M2 peptides of the quisqualate (Quis) and *N*-methyl-D-aspartate (NMDA) subtypes of glutamate receptors (17,18) which were employed in the present studies. The M2 peptides could not be purified by conventional reversed-phase HPLC procedures owing to their low solubility, aggregation, etc. These peptides have their hydrophobic amino acids underscored in Table 1 to illustrate the amphiphilic nature of the M2 helices, which surround the ion-channel pores with their hydrophilic faces directed inward (19). In contrast to the M2 peptides, the M1 is more hydrophobic because it is positioned away from the pore opening, further back into the lipid bilayer (19).

Figure 1A shows the HPLC separation of crude synthetic quisqualate M2 peptide on the $\mu$Bondapak aminopropyl column. Figure 1B is the HPLC trace after two semiprep-scale HPLC separations of the crude M2 peptide; it shows that the impurities appearing as shoulders on the main peak in Fig. 1A were removed by the two successive HPLC steps. All four synthetic M1 and M2 peptides were purified on the semiprep-scale aminopropyl column, the purity and identity being confirmed by electrospray MS and amino acid composition analysis. Approximately 5 mg of pure peptides was obtained upon combining the eluates of three or four HPLC runs, the recovery yield being over 95% as judged by uv spectrophotometry. The quisqualate M1 peak was much broader than the M2 peaks (data not shown), but the peptide was nonetheless baseline separated from the synthetic impurities. This M1 peptide eluted as a sharper peak using an alternative procedure, i.e., reverse-phased HPLC on a C-4 column with a mobile phase of 80% acetonitrile, 20% water (0.1% TFA), a system that failed to separate the M2 peptides.

Figure 2A shows the HPLC separation of the two NMDA peptides on the aminopropyl column, whereas Fig. 2B shows the separation on the $\mu$Porasil silica column. The NMDA-1 peptide is the five-residue amino-terminal extension of NMDA-2 (Table 1); the two peptides were baseline separated on both columns, although the aminopropyl column (Fig. 2A) resulted in better removal of the minor synthetic impurities appearing as shoulders on the main peptide peaks. The aminopropyl column generally provided sharper peaks and better resolution; however, the silica column is useful for certain separations in which a different profile is obtained.

*Peptides derived from BR, a membrane protein.* Figure 3 is a diagram of BR secondary structure showing the seven transmembrane $\alpha$-helices (A–G) and the Met residues where CNBr cleavage produces fragments 1–10. Separation of the CNBr fragments on the $\mu$Bondapak aminopropyl column gave the profile shown in Fig. 4. Peaks are labeled according to the fragment number, which was determined by electrospray MS (Table

2). The profile varied slightly in different trials, some overlapping peaks (i.e., fragments 7, 9, and 10) being somewhat better resolved in other experiments (Fig. 4, insert). Peak 2′ ($Thr^{24}$–$Met^{32}$) is a previously characterized truncation of peptide 2, which loses its three N-terminal amino acids as a result of hydrolysis by the 70% formic acid used in the CNBr cleavage (Table 2) (Orlando *et al.*, manuscript in preparation); the formic acid also led to formylation of some of the BR peptides, which were obtained as mono- to hexaformate esters.

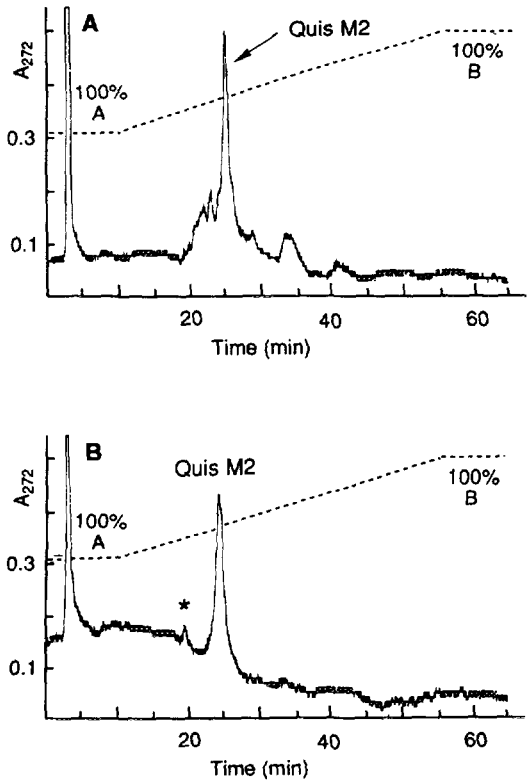


**FIG. 1.** HPLC purification of a crude synthetic M2 peptide. (A) HPLC trace of crude synthetic quisqualate M2 peptide (Table 1); Waters μBondapak aminopropyl column, analytical scale (300 × 3.9 mm, 10 μm, 125 Å; flow rate 1 ml/min),[a] retention time (in min) plotted against 272-nm absorbance. The synthetic contaminants, e.g., shorter peptides or peptides still retaining protective groups, appear as shoulders on the main M2 peptide peak. (B) HPLC trace of purified synthetic quisqualate M2 peptide; Waters μBondapak aminopropyl column, analytical scale.[a] Two HPLC cycles with the semiprep scale column (300 × 7.8 mm, 10 μm, 125 Å; flow rate 4 ml/min) yielded the essentially homogeneous peptide peak shown in (B). The main M2 peak was always accompanied by an additional small M2 peak (*) eluting at 19 min that had bound more weakly to the column. The homogeneity of the quisqualate M2 peak was confirmed by amino acid composition analysis. [[a]The peptide (~100 μg) dissolved in solvent A (7/1/1, chloroform/methanol/isopropylamine) was injected onto the column, which was washed for 10 min with the same solvent. After developing a linear gradient over 45 min from 100% solvent A to 100% solvent B (1/7/1, chloroform/methanol/isopropylamine), the column was washed for 10 min with solvent B. Peptides were detected at 272 nm at a sensitivity of 0.5 absorbance unit full scale.]

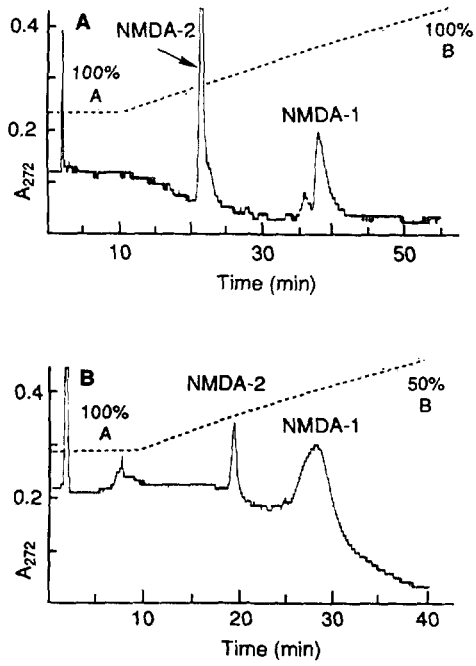


**FIG. 2.** HPLC separation of two synthetic M2 peptides. (A) Aminopropyl HPLC trace of NMDA-1 and NMDA-2 peptides (Table 1); Waters μBondapak aminopropyl column, analytical scale,[a] retention time (in min) plotted against 272 nm absorbance. (B) Silica HPLC trace of NMDA-1 and NMDA-2 peptides; Waters μPorasil silica column, analytical scale (150 × 3.9 mm, 10 μm, 125 Å; flow rate 1 ml/min).[b] [[a]Peptides were separated as described in the legend to Fig. 1A. [b]The peptides (~100 μg each) dissolved in solvent A (7/1/1, chloroform/methanol/isopropylamine) were injected onto the column, which was washed for 10 min with the same solvent. A linear gradient was developed over 30 min from 100% solvent A to 50% solvent B (solvent B: 7/1/1, chloroform/methanol/isopropylamine); the peptides were detected at 272 nm at a sensitivity of 0.5 absorbance units full scale.]

Figure 5 shows MS identification of two bisformates of peptide 5 (peaks 2F and 2F*), which were co-isolated with their unformylated counterparts (peaks 0F and 0F*) in the HPLC separation shown in Fig. 4. The formates were readily removed by cleavage of the ester linkages with 70% TFA. The mass spectrum shown in Fig. 5 also demonstrates that the BR peptides were obtained as carboxyl-terminal homoserine lactones (peaks 0F, 2F; Fig. 5, insert) and the corresponding hydrolyzed lactones, namely, carboxyl-terminal homoserines (peaks 0F*, 2F*). These residues are derived from Met as the end result of a process in which CNBr reacts with Met to bring about an intramolecular displacement of its methylthio group, which accompanies scission of the neighboring peptide bond to form the lactone shown in Fig. 5 (insert), which can then undergo hydrolysis (20).

The MS shown in Fig. 5 also demonstrates that the single HPLC run (Fig. 4) resulted in high purity BR peptide 5 (i.e., no peaks above 1200 Da). However, the large overlapping peaks in Fig. 4 eluting at around 23

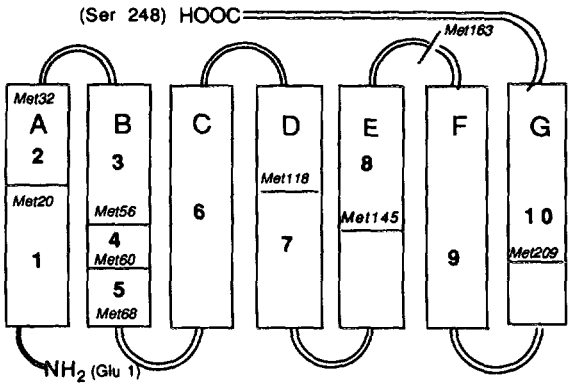


FIG. 3. Secondary structure of BR and cleavage sites. Schematic representation of BR secondary structure showing the seven membrane-spanning α-helices A–G and the CNBr cleavage sites that give rise to fragments 1–10.

and 32 min, respectively, were shown by MS and electrophoresis to be mixtures of fragments 1/3 and 7/9/10. It was found that a second HPLC step, this time on μPorasil silica, was capable of separating the two overlapping peaks into their respective component peptides, as exemplified for fragment 10 (Fig. 6). Thus, usage of the two consecutive HPLC procedures described has led to a clear separation of the peptides resulting from CNBr cleavage of BR for the first time.

Figure 7 shows that the BR peptides were eluted from the aminopropyl column (Fig. 4) according to chain length, the larger peptides being eluted later in the gradient. This relationship was linear (correlation coefficient = 0.984) for peptides of up to 27 amino acids.

## DISCUSSION

Most of the amphiphilic peptides investigated were insoluble in water and the following organic solvents

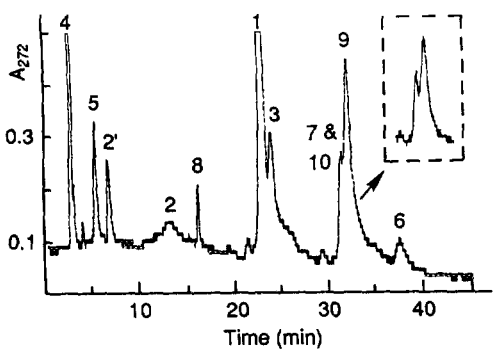


FIG. 4. HPLC separation of BR fragments. Aminopropyl HPLC trace of the 10 BR fragments (Fig. 3) obtained upon cleavage of 200 μg of BR.[a] Peaks are labeled according to the fragment number, which was determined by electrospray MS (Table 2). The insert is from a different trial, which gave somewhat better separation of fragments 7, 10, and 9. [[a]The peptide mixture was separated under conditions described in the legend to Fig. 1A.]

TABLE 2

Molecular Weights of HPLC-Purified BR Peptides Determined by MS

| Fragment no. (amino acids) | $M_r$ (Da) Calcd[a] | $M_r$ (Da) Experimental[b] |
|---|---|---|
| 1 (1–20) | 2226.7[c] | 2225.2 |
| 2 (21–32) | 1251.6 | 1252.3 |
| 3 (33–56) | 2510.0 | 2510.9 |
| 4 (57–60) | 465.6 | 466.1 |
| 5 (61–68) | 820.1 | 821.9 |
| 6 (69–118) | 5429.3 | 5429.0 |
| 7 (119–145) | 2915.5 | 2916.0 |
| 8 (146–163) | 2082.5 | 2083.1 |
| 9 (164–209) | 5068.0 | 5068.6 |
| 10 (210–248) | 3844.3 | 3844.5 |
| Fragment produced by acid hydrolysis | | |
| 2' (24–32) | 1024.3 | 1021.9 |

*Note.* Calculated and experimental molecular weights (in Da) of the HPLC isolated BR peptides (Fig. 4).

[a] Calculated average $M_r$ for peptides with carboxyl-terminal homoserine–lactone residue.

[b] Peptides were HPLC-purified and analyzed by MS as described in the legends to Figs. 4 and 5. The reported $M_r$s are averages of values determined for the singly and multiply charged ions of the major unformylated species.

[c] Hydrolyzed C-terminal homoserine-lactone residue, i.e., homoserine.

and solvent mixtures: acetonitrile, chloroform, methylene chloride, isopropanol, methanol, *N,N*-dimethylformamide, dimethylsulfoxide, *N,N'*-dimethylpropyleneurea, 1/1 methanol/chloroform, 5/2/2 methanol/chloroform/water, 1/1/0.002 acetonitrile/water/TFA, and 1/1/0.002 isopropanol/water/TFA. However, they were soluble in trifluoroethanol, 5/3/2 *n*-butanol/water/acetic acid, or chloroform/methanol/isopropylamine mixtures; the isopropylamine in the third system helped to increase the solubility of amphipathic peptides in these organic solvents. This ternary organic solvent mixture dissolved every peptide we tested except the extremely hydrophobic *N-t*-Boc-Phe–Ala–Ala–*p-nitro*–Phe–Phe–Val–Leu–4-hydroxymethylpyridine ester. The solubility of some peptides was affected by pretreatment, apparently through the formation of specific salts. For some peptides the generation of the isopropylammonium salt by pretreatment with aqueous isopropylammonium acetate improved the solubility.

Since an increase in solvent polarity effected elution of peptides from both column types in a similar fashion, the primary retention mode is normal phase. The longer peptides were eluted later in the gradients as a result of their greater number of polar groups, i.e., peptide bonds and side-chain groups. The carboxylates of Asp and Glu bind to the columns particularly tightly due to their an-

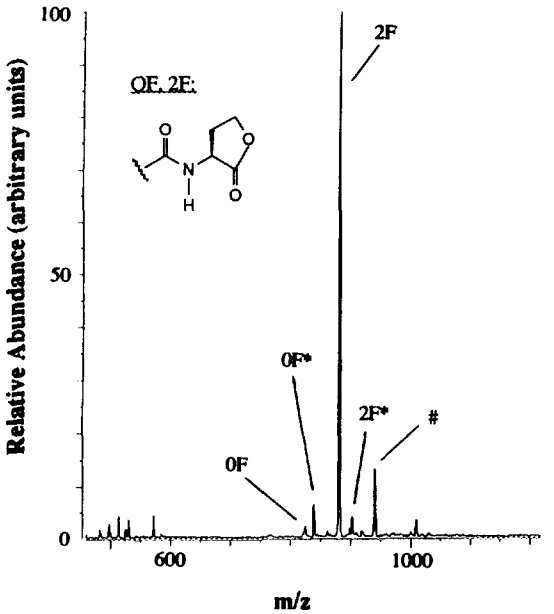


FIG. 5. Electrospray MS of BR fragment 5 after HPLC on the aminopropyl column (see Fig. 4).[a] The major spike 2F is the $(M + H)^+$ species of the bisformylated carboxyl-terminal homoserine lactone of peptide 5, whereas peak 0F is the unformylated homoserine lactone (insert) (20). Peaks 0F* and 2F* are the hydrolyzed lactones of peaks 0F and 2F, respectively, i.e., the corresponding carboxyl-terminal homoserines. The peak at $m/z$ 942 (i.e., #) could be the N-terminal Schiff's base (with formaldehyde) of the homoserine trisformate, or some other modified or complexed form of peptide 5. The series of peaks at $m/z$ = 450–550 are singly charged ions of peptide 4, a minor contaminant; no peaks higher than $m/z$ 1200 were present. [[a]Peptides were purified as described in the legend to Fig. 4, dried, dissolved in 49/49/2, methanol/water/acetic acid, and ionized by electrospray for $M_r$ determinations in the first two sectors of a four sector instrument.]

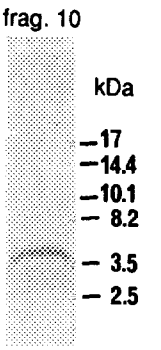


FIG. 6. Purity of BR fragment 10 after two-step HPLC purification. SDS–polyacrylamide gel showing fragment 10 (3.8 kDa) after HPLC with aminopropyl (Fig. 4) and then with silica columns.[a] Migration positions of molecular weight markers (Sigma MW-SDS-17) are also shown. [[a]Peptides that were purified as described in the legend to Fig. 4 then as in the legend to Fig. 2B were separated on a 16.5% polyacrylamide gel (with 10% spacer gel) in the presence of SDS (21) and silver stained using a Bio-Rad kit.]

ionic character, as exemplified by the strong retention of the quisqualate M1 peptide, which was eluted from the analytical-scale aminopropyl column after 57.4 min despite having only a small number of polar residues (Table 1). An earlier report (12) described a similar size-based HPLC separation of peptides on silica. The size-retention correlation shown in Fig. 7 might be breaking down for the longer peptides as a result of folding, which can cause deviation in the hydrophobicity–retention relationship for reversed-phase separations of peptides longer than ca. 15 amino acids by rendering some residues inaccessible to the column surface groups (22,23).

The HPLC procedures described above are best suited for the separation of intermediate-sized polypeptides comprising 5–50 amino acids, although smaller peptides can also be separated provided they are sufficiently polar to be retained on the columns. The small hydrophobic peptides *N*-Cbz-Val–Phe–methyl ester and *N*-Cbz-Val–Gly–Gly–benzyl ester were eluted with the solvent front from both the silica and aminopropyl columns. Likewise the BR fragment 4, a tetrapeptide, was eluted with the solvent front (Fig. 4); however, Phe and the dipeptides Gly–Tyr and Tyr–Arg were retained on both column types, being eluted as sharp peaks during the respective solvent gradients. The large polypeptide chymotrypsin (244 amino acids) was eluted from the aminopropyl column at the end of the solvent gradient, but the peak was too broad to be resolved from impurities if a crude mixture were to be separated.

The two column types maintained their resolving capabilities after more than 100 separation runs spanning a 6-month period. During this time they were stored in solvent A. It appeared that the column packing materials were not dissolving under these conditions because no peak broadening was observed over the duration of

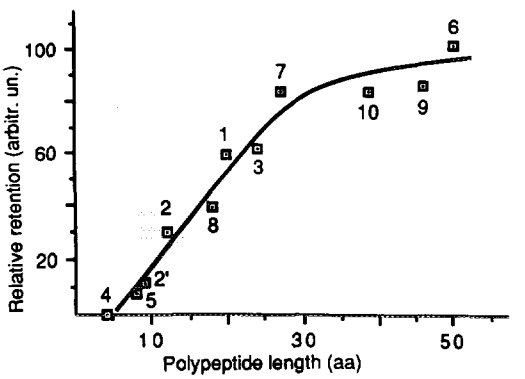


FIG. 7. Dependence of retention time on polypeptide chain length. The BR peptide length expressed in numbers of amino acid residues plotted against relative retention times (arbitrary units) on the μBondapak aminopropyl column (Fig. 4). The numbers indicate the BR fragments represented by each data point. A linear relationship (correlation coefficient = 0.984) for peptides of up to 27 amino acids is observed.

the study. The columns did eventually become contaminated as a result of the excessive through-put, as seen by the appearance of several "ghost peaks" and a trend to elute peaks earlier in the respective solvent gradients; however, repeated washing with the gradients regenerated the performance of both column types, which again eluted sharp and well-resolved peaks. These columns can thus handle a greater through-put than do many reversed-phase columns, which often deteriorate faster as a result of the irreversible adsorption of polypeptide material and the highly acidic conditions generally employed.

Certain membrane peptides, e.g., the quisqualate M1 mentioned above and an acetylcholine receptor M2 peptide, can be readily separated by HPLC on a C-4 column with conventional acetonitrile/water/0.1% TFA mobile phases (A. Kalivretenos, unpublished). However, with most membrane peptides such as the M2 (quisqualate and NMDA) and BR-derived peptides described above, the currently available reversed-phase HPLC procedures lead to poor resolution and low recovery yields as a result of limited solubility, irreversible adsorption, etc.; in such cases the systems described above should be investigated. In a direct comparison using the CNBr-derived BR fragments, the described aminopropyl HPLC procedure (Fig. 4) led to better-resolved and sharper peaks, higher recoveries, and more reproducible profiles than those of the published reversed-phase HPLC procedures (5,6,10).

In summary, we have developed improved HPLC protocols that can be employed for the purification of certain sticky peptides. These procedures should greatly facilitate structural studies of membrane proteins, particularly when used in conjunction with sequence analysis, photoaffinity labeling, or preparation of protein models from aggregates of synthetic peptides.

## ACKNOWLEDGMENTS

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