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Journal of Chromatography A, 816 (1998) 11–20

JOURNAL OF CHROMATOGRAPHY A

Synthesis and purification of hydrophobic peptides for use in biomimetic ion channels

Sean Y. Kassim, Iliana M. Restrepo, Aristotle G. Kalivretenos*

Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore, MD 21250, USA

Abstract

The synthesis and subsequent purification of several hydrophobic peptides is described. These peptides include the 24-residue M3 transmembrane domain of the rat connexin 32 protein, a peptide sequence that contains only seven amino acids with hydrophilic side-chains (71% hydrophobic). Moreover, for comparison, a much smaller hydrophobic octapeptide, designed to exist with α-helical secondary structure, was also studied. Optimum conditions for the RP-HPLC purification of these peptides was dependent on peptide length and solubility properties.

Keywords: Peptides

1. Introduction

Ion transport across cell membranes is a fundamental biological process. The mechanism of signal transmission in the mammalian central nervous system (CNS) is through ion transport by way of highly selective ion channel proteins in excitable (neuronal) membranes [1]. Ion flow into and out of the cell is controlled by either ligand-gated ion channels [e.g. the nicotinic acetylcholine (nACh-R), the γ-aminobutyric acid (GABA-R) and glutamate (Glu-R) receptor proteins] [2,3], or highly selective voltage-dependent ion channel proteins (Na^+, K^+ and Ca^{2+} channels) [3,4]. Cell-to-cell communication in other mammalian tissues, cardiac tissue in particular, is mediated by transport through ion channel proteins (connexons) at gap junctions [6]. Abnormalities in the function of ion channels have been linked to neurological [7–11] and cardiac dysfunction [12]. As such, they have become targets in the search for therapeutic agents for these diseases.

In general, ion channel proteins are multi-subunit, membrane-bound proteins that contain a number of transmembrane domains of amphiphilic α-helical secondary structure [4,5]. The subunits aggregate in an ordered fashion about a central axis in the membrane, in which the hydrophilic faces of these membrane-spanning α-helices form the ion channel. Due to their complexity and lack of availability in pure form, molecular level structural studies of these proteins have focused on peptide bundle models [13,14].

Synthetic ion channels have been formed using purported naturally occurring ion channel peptide sequences from ion channel proteins, including the nACh-R [15], the Na^+ channel [16] and a Ca^{2+} channel [17]. Synthetic ion channels, consisting of preformed peptide bundles prepared using template assembled synthetic protein (TASP) methodology [18] have been prepared for the nACh-R [19], an L-type Ca^{2+} channel [17] and the pore-forming toxin, mellitin [20]. Moreover, the focus of our research

*Corresponding author.

0021-9673/98/$19.00
PII: S0021-9673(98)00250-7

has been the study of channel-forming peptides and the use of segment condensation reactions for the synthesis of peptide bundles [21]. In general, the pore-forming peptides have a high percentage of hydrophobic amino acids and can be difficult to synthesize and purify. In this report, we describe in detail our efforts for the synthesis and purification of an extremely hydrophobic 24-residue channel peptide corresponding to the rat connexin 32 M3 transmembrane domain, as well as a hydrophobic α-helical octapeptide.

2. Experimental

2.1. General

Dichloromethane was distilled from calcium hydride. *N*,*N*-Dimethylformamide (DMF), *N*-methyl-2-pyrrolidinone (NMP) and trifluoroacetic acid (TFA) were all of peptide synthesis grade and used as obtained. Acetonitrile (MeCN), isopropanol (iPrOH) and methanol (MeOH) were of HPLC grade. Deionized and distilled water was filtered through a 0.45-μm membrane filter before use in HPLC. N_{α}-Fmoc (fluorenylmethoxycarbonyl) amino acids and peptide synthesis resins were purchased from Bachem or Novabiochem. Other starting materials and reagents were obtained either from Aldrich or from other commercial suppliers and were used as obtained, unless otherwise noted. Matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass spectra were obtained using a Kratos Kompact MALDI III spectrometer in the reflectron mode. Fast atom bombardment (FAB) mass spectra were obtained by the Mass Spectrometry Laboratory at the University of Maryland, College Park, MD, USA.

3. RP-HPLC analysis and purification

3.1. Instrumentation

RP-HPLC analysis and purification were performed using a Waters binary gradient chromatography system (two Waters 510 pumps, a Waters UK6 injector, a Waters 486 UV–Vis detector, a Waters column oven; Waters, Milford, MA, USA) controlled by the Millenium chromatography work-station. Analytical RP-HPLC was carried out using a flow-rate of 1 ml/min, detection at 220 nm and a column temperature of 30°C, unless otherwise noted.

3.2. Columns

Two columns were utilized for analytical RP-HPLC: (1) A Phenomenex Jupiter C_{18} reversed-phase column (250×4.6 mm, 300 Å pore size, 5 μm particle size; Phenomenex, Torrance, CA, USA); (2) A Vydac C_4 reversed-phase protein and peptide column (250×4.6 mm, 300 Å pore size, 5 μm particle size; Separations Group, Hesperia, CA, USA). Peptide purification was carried out using either a Vydac C_{18} reversed-phase protein and peptide semi-preparative column (250×10 mm, 300 Å pore size, 5 μm particle size, 30°C, Separations Group) or a Phenomenex Jupiter C_{18} semi-preparative reversed-phase column (250×10 mm, 300 Å pore size, 5 μm particle size).

4. Solid phase peptide synthesis.

4.1. Manual peptide synthesis

Manual solid-phase peptide synthesis (SPPS) was completed using a solid-phase reaction flask and a shaker for agitation. Peptides were prepared on a 0.1-mmol scale utilizing Fmoc chemistry on a Rink Amide MBHA resin [22], which allows cleavage of the peptide from the resin as the C-terminal amide, unless otherwise noted. Two coupling methods were employed: (1) The use of preformed symmetric anhydrides and (2) 1-hydroxybenzotriazole (HOBT)-mediated coupling of pentafluorophenyl esters, unless otherwise noted [23]. The efficiency of the coupling steps was monitored using the Kaiser test [24]. Incomplete couplings were repeated to ensure quantitative yield.

4.2. Automated peptide synthesis

Automated SPPS was completed using an ABI 432 Synergy peptide synthesizer [Applied Biosystems (ABI), Foster City, CA, USA]. The peptides

were prepared on a 0.025-mmol scale utilizing Fmoc chemistry and ABI's standard coupling protocol [2-(1-*H*-benzotriazol-1-yl) - 1,1,3,3-tetramethyluronium-hexafluorophosphate (HBTU) activation] [25]. To ensure high coupling efficiencies during automated peptide synthesis, the ABI 432 synthesizer employs a conductivity feedback monitoring system that adjusts reaction times to optimize coupling efficiency.

4.3. Cleavage and deprotection of resin-bound peptides

Completed peptides were Fmoc deprotected at the N-terminus, followed by cleavage from the resin and complete side-chain deprotection using a TFA-based cleavage cocktail containing cation scavenging agents [26].

4.4. Peptide characterization

The composition of synthetic peptides was determined by FAB (cation mode) or MALDI–TOF mass spectrometry (MS).

4.5. Rat connexin M3 peptide

The 24-residue peptide was synthesized on a pre-substituted alkoxybenzyl resin [27] using the ABI 432 peptide synthesizer. Upon completion of the synthesis (the ABI 432 carries out the N-terminus Fmoc deprotection step), a portion of the resin (0.0277 g) was cooled to 0°C, followed by the addition of the cleavage cocktail (5 ml TFA, 0.375 g phenol, 0.25 ml deionized water, 0.25 ml thioanisole and 0.125 ml ethanedithiol) [26], also at 0°C, to the resin. The cold bath was removed and the suspension was stirred for 4 h at 25°C. The suspension was then filtered, and the filtrate was concentrated to ~2 ml. The concentrated filtrate was then added dropwise to two 50 ml conical centrifuge tubes, each containing 40 ml of diethyl ether at 0°C, to precipitate the crude peptide. The precipitated peptide was centrifuged for 5 min (IEC clinical centrifuge, highest speed, 5125 *g*) and the supernatant was discarded. The pellet was resuspended in diethyl ether, centrifuged as before and again the supernatant was discarded. The crude peptide was suspended in water, frozen and lyophilized to yield 0.0086 g of crude peptide. For analysis and purification, the peptide was dissolved in 2,2,2-trifluoroethanol (TFE), and diluted with water to provide a TFE–water (1:1, v/v) mixture. Purification by RP-HPLC [Vydac C_{18} protein and peptide semi-preparative column, 30°C, 2 ml/min, 220 nm, 0–75% iPrOH (0.1% TFA) over 25 min, 75% iPrOH (0.1% TFA) for 25 min; retention time, 36 min] yielded a white fluffy powder after lyophilization. The composition of the peptide was confirmed via FAB–MS (calculated for $C_{148}H_{211}N_{29}O_{32}S$ M^+ 2938, found $[M+1]^+$ 2941).

4.6. AcNH–CLSSLLSL–CONH$_2$

The octapeptide was synthesized on a Rink amide MBHA resin via manual SPPS. It was necessary to repeat the couplings of **S**6, **L**7 and **C**8 numerous times to achieve complete coupling based on the Kaiser test [24]. Due to incomplete coupling of **C**8, the more reactive benzotriazole-1-yl-oxy-tris(dimethylamino)phosphoniumhexafluorophosphate (BOP) activation chemistry [28] and the use of *N*-methyl-2-pyrrolidinone (NMP) as solvent were substituted for the standard coupling conditions. Once completed, the N-terminus of the resin-bound peptide was Fmoc deprotected, followed by acetylation with acetic anhydride in dichloromethane. The peptide was cleaved from the resin and side-chain deprotected in a manner analogous to that for the rat connexin 32 M3 peptide. The crude peptide was suspended in water, frozen and lyophilized to yield a crude powder. For analysis and purification, the peptide was dissolved in TFE. Purification by RP-HPLC [Phenomenex C_{18} semi-preparative reversed-phase column, 30°C, 3 ml/min, 220 nm, 20–80% MeCN (0.1% TFA) over 30 min, 80% MeCN (0.1% TFA) for 20 min; retention time, 31.5 min] yielded a white fluffy powder after lyophilization. The composition of the peptide was confirmed via MALDI–TOF–MS (calculated for $C_{38}H_{69}N_9O_{12}S$, M^+ 875, found $[M+Na]^+$ 899).

5. Results and discussion

As part of ongoing research directed at studies of ion transport, we have synthesized and studied the channel-forming properties of several hydrophobic

nACh-R M2:	E K M S T A I S V L L A Q A V F L L L T S Q R
NMDA M2:	E D A L T L S S A M W F S W G V L L N S G I G
Ca^{2+} S3:	C D P W N V F D F L I V I G S I I D V I L S E
KAIN M3:	C P K A L S G R V I T S I W W L F S L V L L A
	C Y F A N L S L

Fig. 1. Transmembrane domains from ion channel proteins.

peptides based on native ion channel proteins. Our first efforts focused on the nACh-R M2 domain as per Oiko et al. [15]. We synthesized the 23-residue M2 domain of *Torpedo californica* using Fmoc SPPS and an alkoxybenzyl resin. Resin cleavage and subsequent RP-HPLC (C_4 column, MeCN–water–0.1% TFA gradient) yielded pure peptide that displayed channel activity that was analogous to literature reports [29]. Based on this result, we have prepared transmembrane peptides from other ion channel proteins, including the M2 domain of rat N-methyl-D-aspartate (NMDA) receptor protein [30], the M3 domain of a goldfish kainate receptor protein [31], and the S3 domain of a voltage-dependent calcium channel [17]. The sequences of these peptides are given in Fig. 1.

Along with this work, we have pursued studies directed at confirming the ion channel domain of the rat liver connexin 32 protein found at gap junctions. Gap junctions are areas where the cell membranes of two neighboring cells are in close apposition to each other (~1.5 nm) and, when viewed using an electron microscope, appear to have a large number of proteins embedded in the membrane [6]. Each cell of the pair expresses one connexon, or hemichannel, and the extracellular loops of these hemichannels interact, in a mechanism that has yet to be determined, to form a channel between the two that is permeable to small molecules [M_r up to ~1000, including second messengers cAMP and inositol triphosphate (IP3)] and water-soluble metabolites, as well as ions such as Na^+, K^+, Ca^{2+} and Cl^- [6]. It has been shown that each connexon is a hexamer of identical subunits (connexins), and that the gap junction channel is a dodecamer, formed by one hexameric connexon from each cell [32]. The primary amino acid sequence of many connexins is known, although due to its abundance, the rat liver connexin 32 has been studied in great detail. Both the C-terminus and the N-terminus have been shown to be cytocellular and, from hydrophobicity plots, it has been predicted that the protein spans the membrane four times, and that the region identified as M3 may line the channel pore (Fig. 2) [33–35]. Unfortunately, very little is understood about the three-dimensional structure of the connexon on a molecular level. To provide evidence for the involvement of the M3 domain of the rat connexin 32 protein in ion channel formation, we synthesized this peptide, as well as the M1 domain as a control, for subsequent conductance studies.

Standard Fmoc solid-phase peptide chemistry was utilized for the synthesis of all peptides, although the reaction conditions and reagents were modified to provide the most efficient preparation of these peptides. Once the crude peptides were obtained, they were dissolved in TFE and diluted with water. The peptides were then purified by RP-HPLC, using either MeCN or iPrOH in water containing 0.1% (v/v) TFA. For a typical peptide, aqueous MeCN was initially used as the mobile phase for purification. The nACh-R M2 peptide and the NMDA-R1 M2 peptide were efficiently purified in this manner. The other peptides, including the Ca^{2+} S3 peptide,

M1:	G R V W L S V I F I F R I M V L V V A A E S V
M2	V H M A V L L A P T S V L I L Q L S W L R V H
M3	G ***T*** L W W ***T*** Y V I ***S*** V V F ***R*** L L F ***E*** A V F M Y V
M4:	V E A V N L I I C I G S A A L M F V T F V T K E

Fig. 2. Predicted transmembrane domains for the rat connexin 32 protein.

the KAIN M3 peptide and the M1 and M3 peptides of the rat connexin 32 protein were not amenable to purification under these conditions. Interestingly, these peptides were all less soluble than the nACh-R M2 and NMDA-R1 M2 peptides in a variety of solvents, including 0–50% (v/v) acetic acid in water, TFE and dimethyl sulfoxide (DMSO), based on qualitative observations. These peptides were much more efficiently purified using aqueous iPrOH as the mobile phase in RP-HPLC. Below, we describe in detail the successful synthesis and purification of the rat connexin M3 peptide, which has been adopted as the typical protocol employed in our laboratory for hydrophobic peptides. For comparison purposes, the synthesis and purification of a shorter hydrophobic octapeptide is described as well.

The peptide corresponding to the M3 domain of the rat connexin 32 ion channel protein presented a significant synthetic challenge due its hydrophobic nature. The 24-residue sequence contains only seven amino acids with hydrophilic side-chains (Y was included as a hydrophilic residue in this calculation). When depicted in a helical wheel diagram, the five amino acids highlighted in Fig. 2 (**T**, **T**, **S**, **R** and **E**) appear on one face of the helix, as expected for an amphipathic structure. The extremely hydrophobic nature of this peptide (71% hydrophobic residues) was of concern due to difficulties that can arise in the solid-phase synthesis of hydrophobic peptides due to folding of the growing peptide chain on the resin, or interchain interactions resulting in inefficient couplings. We first attempted to synthesize the M3 peptide utilizing manual SPPS via the batch method. The synthesis was carried out on a 0.1-mmol scale utilizing Fmoc chemistry on a pre-substituted alkoxybenzyl resin. The synthesis failed after the eighth coupling, and subsequent cleavage of the peptide from the resin, RP-HPLC purification and MALDI MS analysis confirmed the composition of the product as a nonapeptide corresponding to the first nine amino acids of the M3 peptide starting from the C-terminus.

As an alternative synthetic method, we utilized an ABI 432 Synergy automated peptide synthesizer. The major advantage of this instrument (outside of the automation) is the use of the continuous flow synthetic method. Instead of reactions occurring by agitation of a reaction vessel, the reagents are continually pumped through the resin for reaction. In general, the resins used in continuous flow applications have lower active site loadings (less chance of interchain interactions) and good diffusion properties (access to growing peptide chains). The M3 peptide was synthesized using Fmoc chemistry on a pre-substituted continuous flow alkoxybenzyl resin with the ABI instrument. The completed resin-bound peptide was subjected to TFA cleavage from the resin and, after precipitation, was collected by lyophilization. As expected, the crude peptide was insoluble in water, but could be dissolved in neat TFE with sonication, and was diluted in a TFE–water (1:1, v/v) solution for RP-HPLC analysis and purification.

Our efforts to purify hydrophobic peptides have centered around the use of RP-HPLC methods, although recent reports describe the use of ion-exchange chromatography [36] and hydrophilic interaction chromatography [37] for this type of application. RP-HPLC using aqueous iPrOH with high percentages of formic acid has also been successfully used for the purification of hydrophobic transmembrane peptides, although care must be taken to avoid peptide modification under these conditions [38,39]. To identify the optimum separation conditions, both C_{18} and C_4 stationary phases were used, mobile phases containing MeCN or iPrOH with water (0.1% TFA) were applied, as well as high-temperature conditions. The results of analytical RP-HPLC separations of the crude rat connexin M3 peptide (utilizing a 1-mg/ml peptide solution in TFE–water, 1:1, v/v) are given in Figs. 3 and 4. Based on these studies, it was obvious that the MeCN–water (0.1% TFA) mobile phase was inappropriate, regardless of whether the C_{18} or the C_4 HPLC column was used. This was surprising due to the earlier success of this method in the purification of the nACh-R M2 peptide. It was expected that the decreased adsorption of the hydrophobic peptide on the C_4 surface would enhance the resolution. Increasing the temperature under these conditions (Fig. 4) increased the resolution of the desired product, although it still appeared as a very broad absorbance. Ultimately, the optimum conditions are as given in Fig. 3c, utilizing the C_{18} HPLC column and the iPrOH–water (0.1% TFA) mobile phase (peptide product retention time, 27.7 min). Using these conditions, mg quantities of

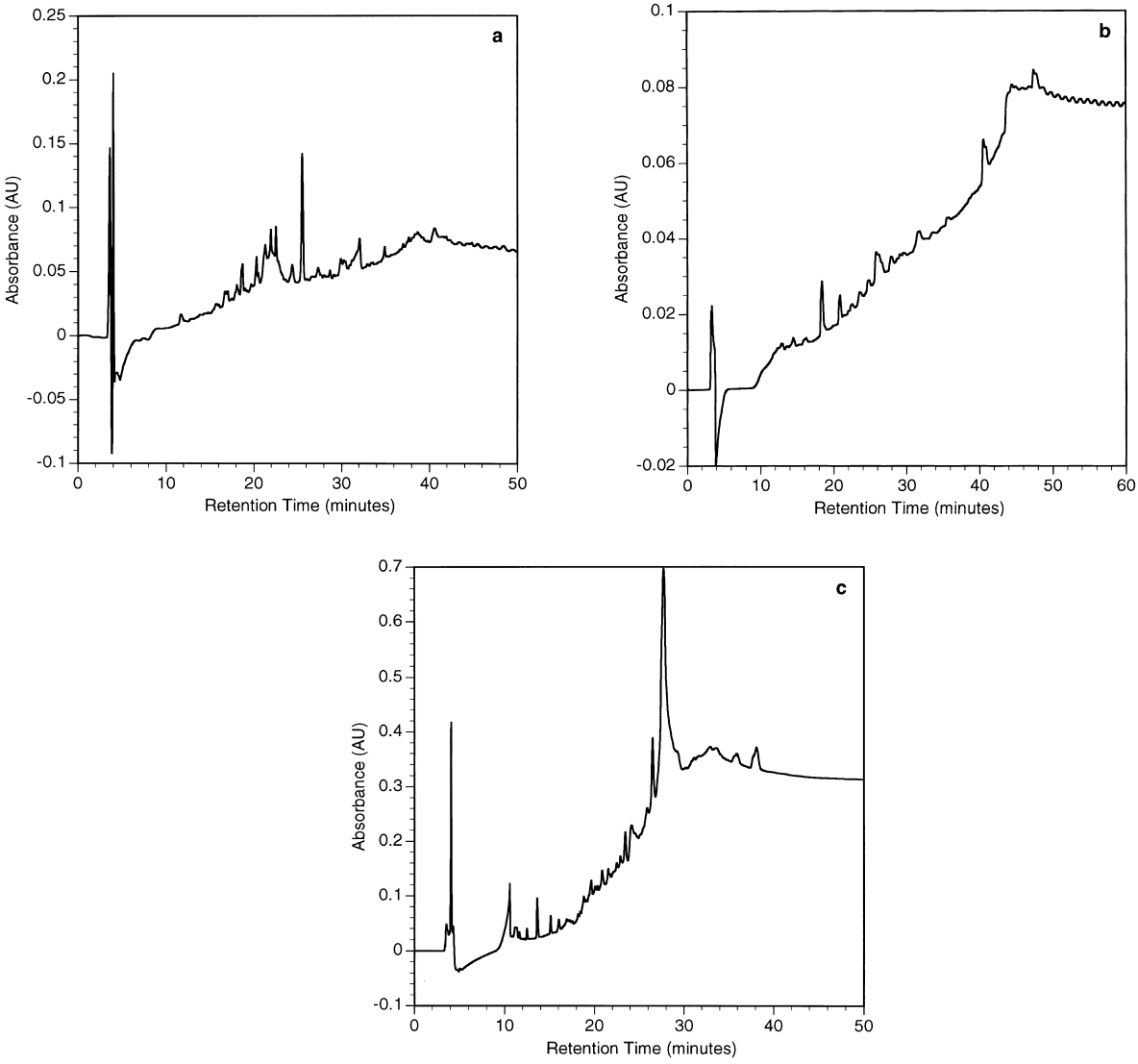

Fig. 3. RP-HPLC analysis of crude rat connexin M3 peptide. (a) Phenomenex Jupiter C_{18} column; 20–80% MeCN (0.1% TFA) over 30 min, 80% MeCN (0.1% TFA) for 20 min; injection volume, 250 μl. (b) Vydac C_4 column; 0–80% MeCN (0.1% TFA) over 40 min, 80% MeCN (0.1% TFA) for 20 min; injection volume, 50 μl. (c) Phenomenex Jupiter C_{18} column; 0–75% iPrOH (0.1% TFA) over 25 min, 75% iPrOH (0.1% TFA) for 25 min; injection volume, 250 μl.

the M3 peptide were purified and the composition was confirmed by FAB–MS. The purified M3 peptide has recently been shown to form channels in artificial bilayers [40].

A second goal of our ongoing work is the synthesis of hydrophobic peptide bundles utilizing the TASP methodology that we have developed [21]. Kienker and Lear [41] recently reported single channel studies of a peptide [AcNH–$(LSSLLSL)_3$–$CONH_2$] designed with just two amino acids (Leu and Ser) in a repeating unit to yield an amphiphilic α-helical peptide. With an interest in preparing tetrameric bundles of this peptide using our segment condensation chemistry, we initially envisioned the synthesis of a simpler bundle comprised of an octapeptide (CLSSLLSL), to optimize the synthetic

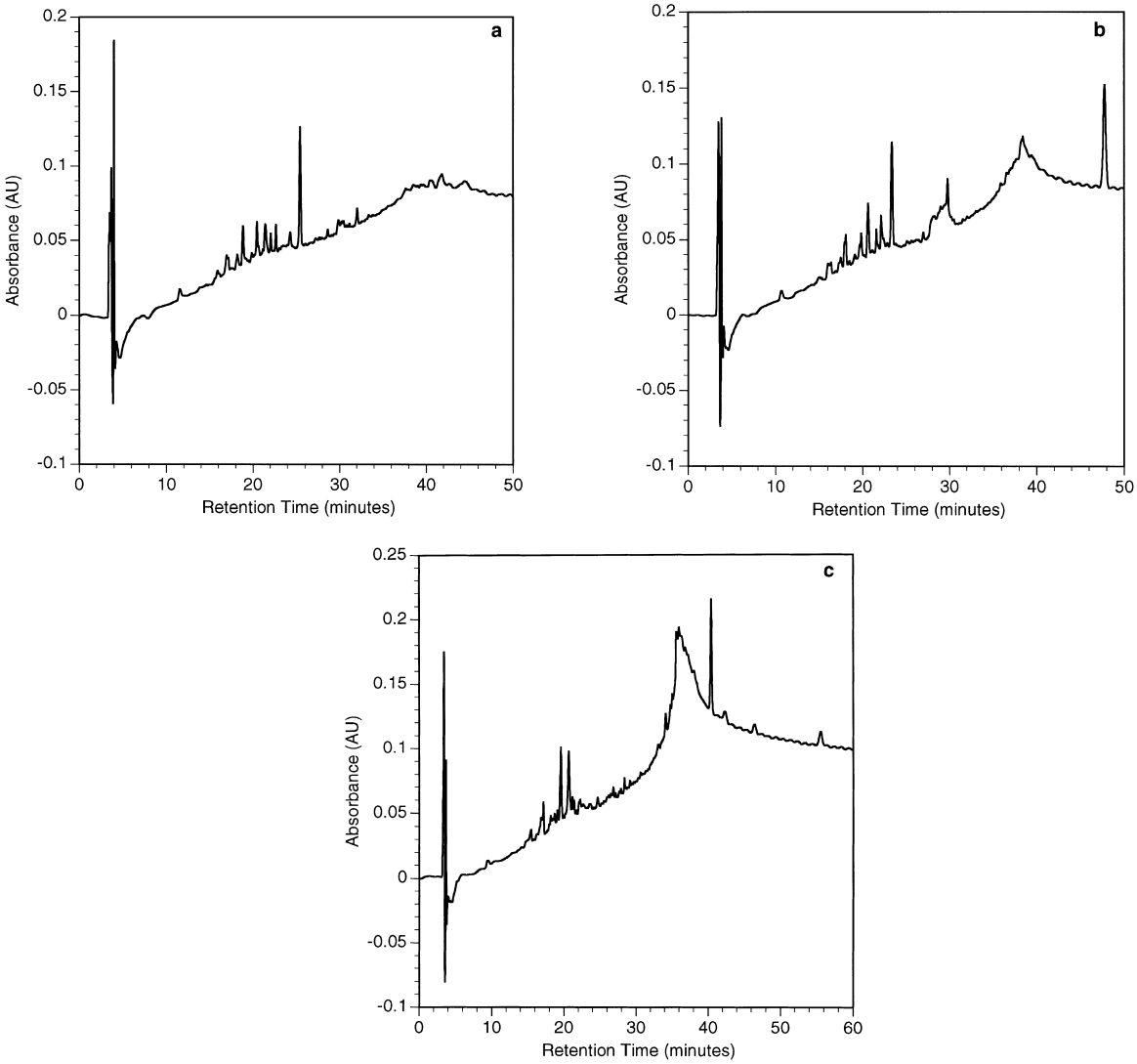

Fig. 4. Effect of temperature on RP-HPLC analysis of crude rat connexin M3 peptide. (a) Phenomenex Jupiter C_{18} column; 20–80% MeCN (0.1% TFA) over 30 min, 80% MeCN (0.1% TFA) for 20 min; 30°C; injection volume, 250 μl. (b) Phenomenex Jupiter C_{18} column; 20–80% MeCN (0.1% TFA) over 30 min, 80% MeCN (0.1% TFA) for 20 min; 50°C; injection volume, 250 μl. (c) Phenomenex Jupiter C_{18} column; 20–80% MeCN (0.1% TFA) over 30 min, 80% MeCN (0.1% TFA) for 20 min; 75°C; injection volume, 250 μl.

method. To this end, we attempted the synthesis and purification of the CLSSLLSL peptide for bundle formation.

Based on the successful synthesis of the M3 peptide, the CLSSLLSL peptide was synthesized in an analogous manner to that used for the M3 peptide, using the ABI instrument. The completed resin-bound peptide was cleaved from the resin, precipitated, and obtained by lyophilization. Unfortunately, we recovered very minimal amounts of crude peptide product from the cleavage reaction, and subsequent RP-HPLC analysis indicated a mixture of products. The synthesis may have been inhibited due to the propensity of the peptide to fold into its α-helical secondary structure. Although the ABI instrument compensates for difficult couplings by increasing

reaction times, it could not adjust enough under standard protocols to efficiently synthesize the peptide. Rather than modify the coupling protocol, we carried out the synthesis utilizing manual SPPS via the batch method. Once completed, the peptide was cleaved from the resin and the crude peptide was isolated as before. The crude peptide was dissolved in neat TFE for RP-HPLC analysis and purification.

As compared to the M3 peptide, it was expected that the hydrophobic octapeptide would be efficiently separated using the MeCN–water (0.1% TFA) mobile phase and the C_{18} HPLC column, due to its low relatively low molecular mass (<1000). Results of RP-HPLC analysis of the crude octapeptide, given in Fig. 5, confirm this hypothesis. The octapeptide was subsequently purified using a C_{18} HPLC column and

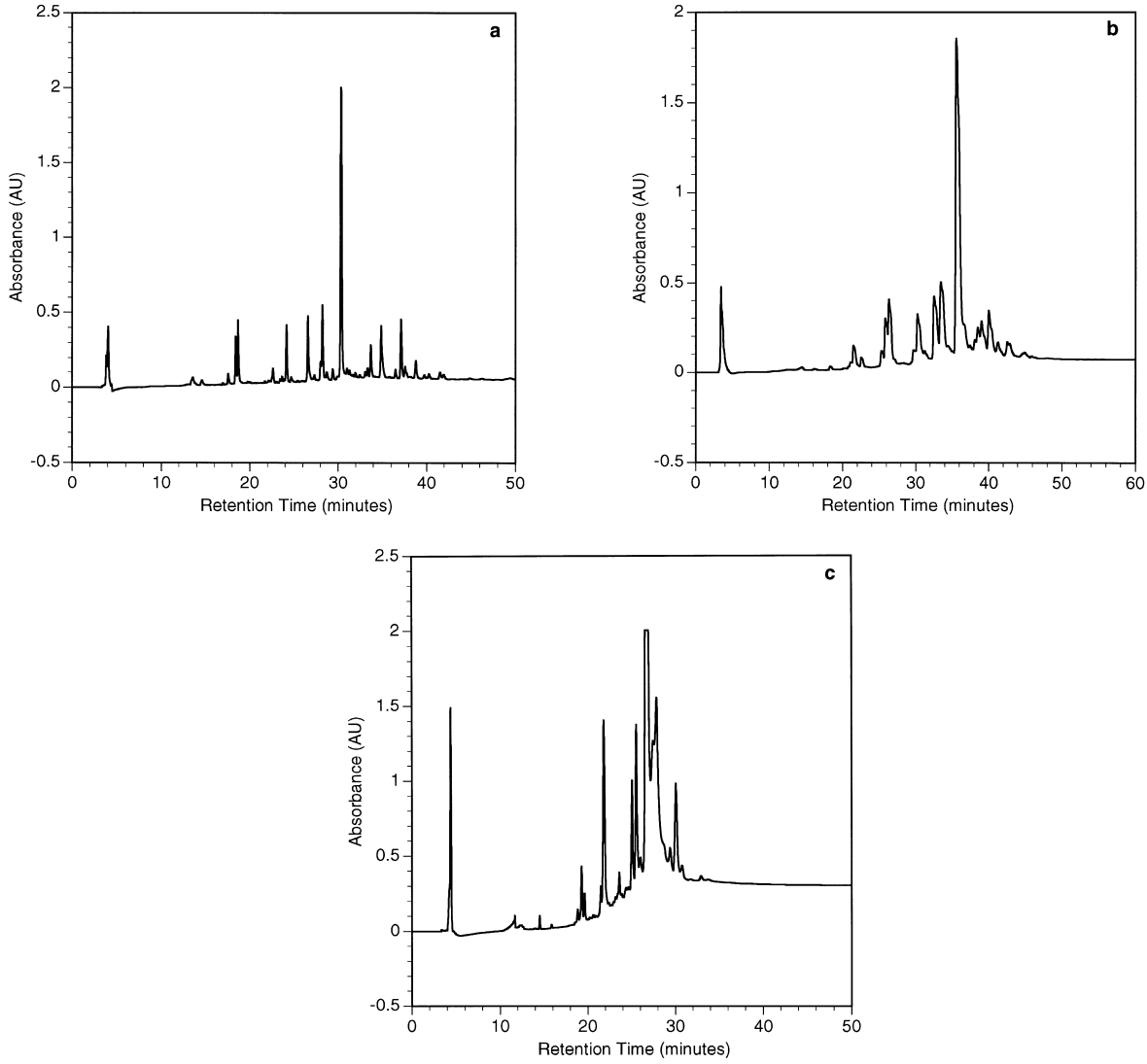

Fig. 5. RP-HPLC analysis of crude CLSSLLSL peptide. (a) Phenomenex Jupiter C_{18} column; 20–80% MeCN (0.1% TFA) over 30 min, 80% MeCN (0.1% TFA) for 20 min; injection volume, 50 μl. (b) Vydac C_4 column; 0–80% MeCN (0.1% TFA) over 40 min, 80% MeCN (0.1% TFA) for 20 min; injection volume, 50 μl. (c) Phenomenex Jupiter C_{18} column; 0–75% iPrOH (0.1% TFA) over 25 min, 75% iPrOH (0.1% TFA) for 25 min; injection volume, 50 μl.

the MeCN–water (0.1% TFA) mobile phase (Fig. 5a; peptide product retention time, 30.3 min) to yield a pure peptide, the composition of which was confirmed by MALDI–TOF–MS.

6. Conclusion

In summary, we have prepared and purified hydrophobic transmembrane peptides, ranging in size from 23–31 amino acids, from ion channel proteins. In general, as the solubility of these peptides decreased (increased hydrophobicity), purification was much more efficient using RP-HPLC with iPrOH–water–0.1% TFA as the mobile phase. In particular, an extremely hydrophobic 24-residue peptide (71% hydrophobic residues), corresponding to the rat connexin M3 domain, was synthesized using the continuous flow Fmoc method and was purified using C_{18} RP-HPLC with gradients of iPrOH–water–0.1% TFA. The M3 peptide was subsequently shown to display ion channel activity in planar lipid bilayers. In contrast, a much shorter hydrophobic octapeptide (CLSSLLSL) proved more difficult to synthesize, although C_{18} RP-HPLC with gradients of MeCN–water–0.1% TFA provided good resolution of peptide products for purification, due to its smaller size and molecular mass. Overall, we find the use of aqueous iPrOH–TFA to be highly efficient for the purification of hydrophobic transmembrane peptides, and that the choice of purification conditions for a given hydrophobic peptide sequence can be predicted based on its length, as well as its solubility properties.

Acknowledgements

The authors thank the University of Maryland, Baltimore County for financial support.

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