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Studies on the synthesis of the toxins, pardaxin, δ-toxin and their analogues by solid-phase methods

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Abstract. Studies in our laboratory have been directed towards understanding the mechanism of action of two hydrophobic toxins, pardaxin comprising 33 residues and δ-toxin comprising 26 residues. Since isolation of these peptides in large amounts from natural sources is not convenient, we have explored synthetic approaches to get these peptides as well as their analogs. We have used chemistry specific to fluorenylmethoxycarbonyl (Fmoc) and *t*-butyloxycarbonyl (Boc) amino acids. Synthesis specific for Fmoc amino acids was carried out manually as well as on a semi-automated continuous flow peptide synthesizer. Synthesis specific for Boc amino acids was carried out manually. The protocols used by us have yielded 15–33 residue peptides which are of high purity. Even in peptides where heterogeneity was present, pure peptide could be obtained in good yields using simple gradients in fast performance liquid chromatography. The synthesis of pardaxin, δ-toxin and several analogs should help in identifying the molecular determinants of biological activity.

Keywords. Pardaxin, δ-toxin; toxins; peptide synthesis; solid-phase methods.

1. Introduction

Peptides are an important component of molecular armaments used by species across the evolutionary scale to defend themselves against invaders (Bevins and Zasloff 1990; Boman 1991; Lehrer *et al* 1991; Saberwal and Nagaraj 1994). Many such peptides are composed of 15–40 residues and exert their activity by altering the permeability properties of membranes (Saberwal and Nagaraj 1994). Their mechanism of action does not involve any chiral recognition. Peptides like melittin from bee venom and δ-toxin from *S. aureus* are potent hemolytic agents (Bernheimer and Rudy 1986), whereas peptides like cecropins, magainins and defensins which are a part of the host defence mechanism of insects, amphibians and mammals respectively possess antibacterial activity (Saberwal and Nagaraj 1994). Studies in our laboratory have been directed towards understanding the mechanism of action of two toxins, pardaxin from *P. pavoninus* (Thompson *et al* 1986) and δ-toxin from *S. aureus* (Fitton *et al* 1980). Since isolation of these peptides from natural sources is not convenient, we adopted a synthetic approach to get these peptides which are composed of 33- and 26-residues respectively. Our intention has also been to get shorter segments of these

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peptides to gain insight into minimal requirements for biological activity. In our synthetic approach, we have attempted to optimize protocols so that the final product did not require extensive purification. We describe in this paper, the results of our synthetic studies on pardaxin and δ-toxin. Peptides used in the study are summarized in table 1.

2. Experimental procedures

2.1 *Materials*

Merrifields resin (polystyrene codivinyl benzene) was from Peninsula Labs, USA, polyamide resins were from Nova Biochem, *p*-hydroxymethylphenoxymethyl polystyrene resin was from Applied Biosystems, USA. All Fmoc amino acids were either from Nova Biochem, UK or Applied Biosystems, USA. Boc amino acids without side chain protection were prepared by the Boc ON procedure (Itoh *et al* 1975). Boc amino acids with protected side chains were from Peninsula Labs, USA. The main solvents were · dichloromethane (CH_2Cl_2), N,N′-dimethylformamide (DMF), N-methylpyrrolidone (NMP). Other reagents were diisopropylethyl amine (DIEA) (Sigma) distilled over ninhydrin, N,N′-dicyclohexylcarbodimide (DCC) (Sigma), 1-hydroxybenzotriazole (HOBT), trifluoroacetic acid (TFA), distilled before use, *m*-cresol, thioanisole ethane dithiol and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU).

2.2 *Attachment of C-terminal amino acid to resin*

Boc amino acids were attached to chloromethyl resin by the cesium salt procedure of Gisin (1973). The extent of substitution was estimated by the picric acid method (Taylor and Kaiser 1987). A substitution of 0·3 mmol/gm was used for the synthesis of P14. The substitution for the synthesis of D15K and D15E was 0·25 mmol/gm for each. When Fmoc amino acids were used and the mode of synthesis was by continuous flow, the substitution used was $\sim$ 0·1 mmol/gm and the scale of synthesis was 0·05 mmol. The level of substitution was determined by monitoring absorption of the Fmoc group at 290 nm (Meienhofer *et al* 1979). When synthesis was done manually using Fmoc chemistry, the substitution was $\sim$ 0·82 mmol/gm.

2.3 *Synthesis*

Synthesis was performed manually in a reaction vessel that has been previously described (Stewart and Young 1984) and in a Pharmacia LKB Biolynx 4175 Peptide Synthesizer which involves continuous flow conditions. Synthesis involving Boc chemistry was done only manually whereas synthesis employing Fmoc chemistry was done on the synthesizer and by manual operations. When Boc chemistry was employed, the side chain protecting groups were benzyl for Ser and Thr, 2-Cl benzyloxy carbonyl for Lys. In the case of Fmoc chemistry the side chain protecting groups were *t*-butyl for Ser, Thr and Boc for Lys. After synthesis, cleavage from resin was achieved by the following methods depending on the resin used (i) transesterification, (ii) treatment with TFA:thioanisole:metacresol:ethanedithiol (10:1:1:5), (iii) treatment with methanol saturated with ammonia.

2.4 *Peptide purification and characterization*

Peptides were checked for purity by fast performance liquid chromatography (FPLC) on reverse phase C18 pepRPC 5/5 (Pharmacia) columns using the solvent systems: A = 0·1% TFA in water and B = 0·1% TFA in CH_3CN. Generally, gradients were chosen to give optimum separation. Detection was at 214 nm. Pure peptides were obtained by repeated runs on the pepRPC column. Amino acid composition of the peptides were confirmed by amino acid analysis on a LKB Alpha Plus 4151 Analyzer after hydrolysis with 6N HCl, 6N HCl:TFA:1:1 in evacuated sealed tubes at 110°C for 24 h and 15 h respectively. Sequence analysis of the peptides was done on an Applied Biosystems 473A Sequencer by stepwise Edman degradation.

3. Results and discussion

The synthesis of 14–15 residue peptides corresponding to N-terminal and central regions of pardaxin was attempted first using Merrifield's chloromethyl resin and CH_2Cl_2 as solvent for the deprotection, coupling and neutralization steps. The protocol for this approach was essentially as described earlier (Reddy *et al* 1985; Reddy and Nagaraj 1986). The crude peptide obtained after cleavage from the resin was subjected to FPLC analysis. The profile shown in figure 1 indicates considerable heterogeneity. In order to effect baseline separation of the peaks, a near isocratic gradient was employed as shown in figure 1b. The peak of interest was determined after collection from multiple FPLC runs and amino acid analysis. An optimized flattened gradient was used to collect the peak of interest as shown in figure 2a. The bunched peaks at the early and latter part of the run were not of interest and were therefore not fractionated. Peptides collected by baseline separation were reinjected to ascertain purity. Figure 2b shows the FPLC trace of peptide thus purified. It is evident that pure peptide can be obtained by choosing a proper gradient to effect

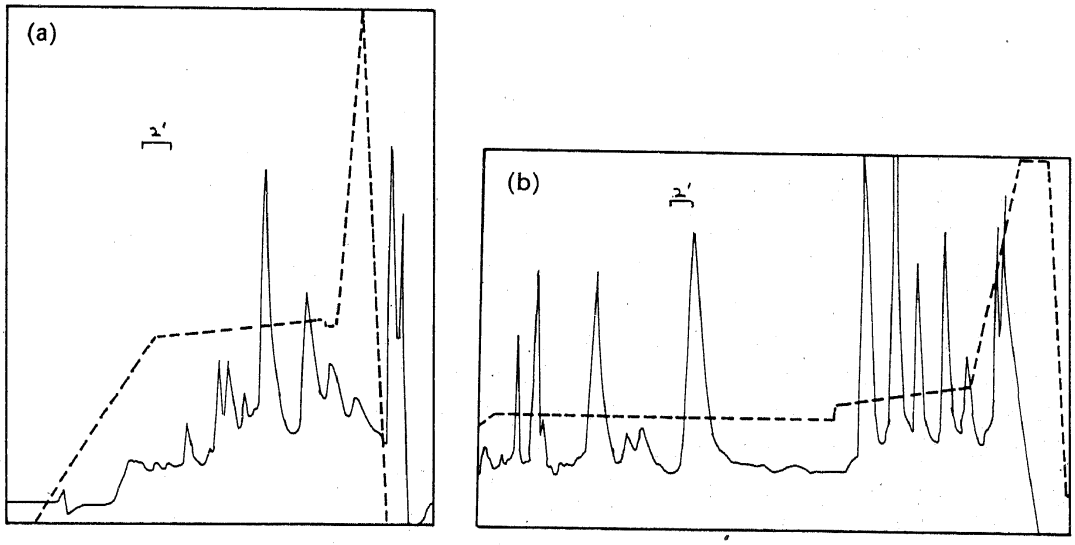

Figure 1. FPLC analysis of crude 14, 15 peptides corresponding to pardaxin synthesized by solid-phase methods, manually using CH_2Cl_2 as solvent for all operations. (**a**) 0–36% B in 9', 36–40% B in 14'. (**b**) 30% B in 34', 34–40% B in 14'.

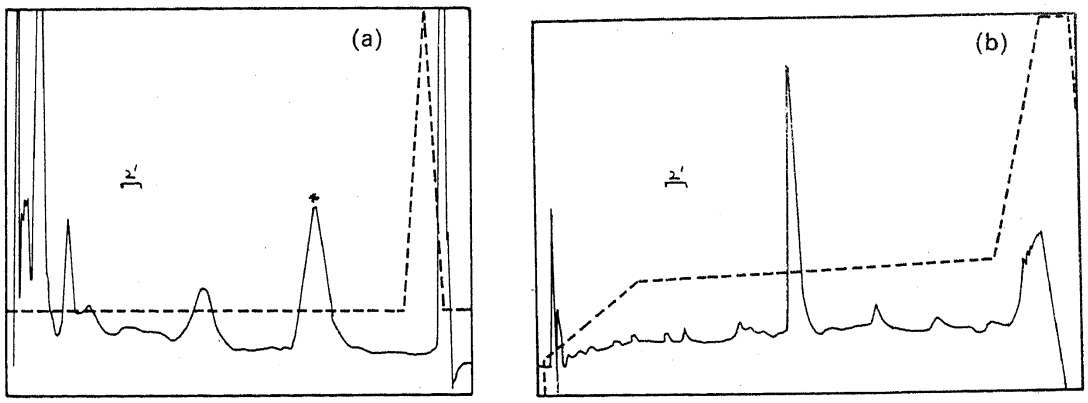

Figure 2. FPLC profiles of crude 14, 15 peptides corresponding to pardaxin synthesized as indicated in legend to figure 1, used for collection and rechecking (**a**) an optimized flattened gradient to collect peak of interest (indicated by an asterisk, B = 22%). (**b**) reinjection for purity check. B = 30–34% in 24'.

Table 1. Primary structures of pardaxin, δ-toxin and their analogues[a].

Pardaxin	:	GFFALIPKIISSPLFKTLLSAVGSALSSSGEQE
P33A	:	GFFALIAKIISSPLFKTLLSAVGSALSSSGEQE
P18	:	GFFALIPKIISSPLFKTL-$CONH_2$
P18A	:	GFFALIAKIISSPLFKTL-$CONH_2$
P15	:	GFFALIPKIISSPLF-CONH
P15A	:	GFFALIAKIISSPLF-$CONH_2$
P14	:	IISSPLFKTLLSAV
δ-toxin	:	FormylMAQDIISTIGDLVKWIIDTVNKFTKK
D16	:	DLVKWIIDTVNKFTKK
D16C	:	IISTIGDLVKWIIDTV
D16KD	:	IISTIGKLVKWIIDTV
D16DK	:	IISTIGDLVKWIIKTV
D16KK	:	IISTIGKLVKWIIKTV
D15K	:	GTAISKAQDIISTIG
D15E	:	GTAISEAQDIISTIG

[a]Underlined amino acids indicate changes from the parent sequence

baseline separation. The yield after FPLC purification was < 10% making recovery of pure peptide a tedious, time-consuming and expensive process. Hence, alternate strategies were tried for the synthesis of other peptides listed in table 1.

Synthesis of peptides employing Fmoc amino acids is being increasingly used (Atherton and Sheppard 1989). Here, couplings can be effected by active esters or symmetric anhydrides of Fmoc amino acids. Deprotection of Fmoc is achieved by the base, piperidine. The peptide after synthesis is cleaved off the resin by either dilute TFA or TFA:H_2O (95:5 v/v), or TFA and scavengers depending on the side chain protecting groups used and methanolic NH_3. Pardaxin and analogues have been synthesized using Fmoc chemistry in a continuous flow, LKB Biolynx Peptide Synthesizer. The protocols employed are described in table 2. Pardaxin and its 'A' analog P33A were synthesized using HBTU activated Fmoc amino acid esters. FPLC traces of the crude peptide are shown in figures 3 and 4. Only one major peak is

Table 2. Protocol for synthesis of peptides using Fmoc amino acids after attachment of C-terminal amino acid to the resin[a]

1. Wash, DMF (15 ml) (time = 5′ at flow rate of 3 ml/min)
2. Deprotection, 20% piperidine in DMF (30 ml) (time = 10′ at flow rate of 3 ml/min)
3. Wash, DMF (conditions as in 1)
4. Equilibrate resin with NMP (~ 5 ml)
5. Coupling by one of the following methods[a,b]
   (i) Preformed symmetric anhydride in DMF, 1 h, 3·5 eq.
   (ii) Preformed HOBt active ester, 1 h, 3·5 eq.
   (iii) Fmoc amino acid + HOBT + HBTU mixed in equivalent amounts followed by DIEA also in equivalent amount, in NMP. The constituents were mixed just before addition to the resin. After introduction of amino acid, recirculation for 30′ at 2 ml/min.
6. Steps 1–5 repeated

[a]The symmetric anhydrides and HOBT active esters were synthesized as described by Atherton and Sheppard (1989)
[b]When synthesis was performed manually, the volume in which the Fmoc amino acid was dissolved was 10 ml. When the synthesizer was used, the volume was 1·5 and 2·5 ml for 0·5 g and 1 g of resin respectively

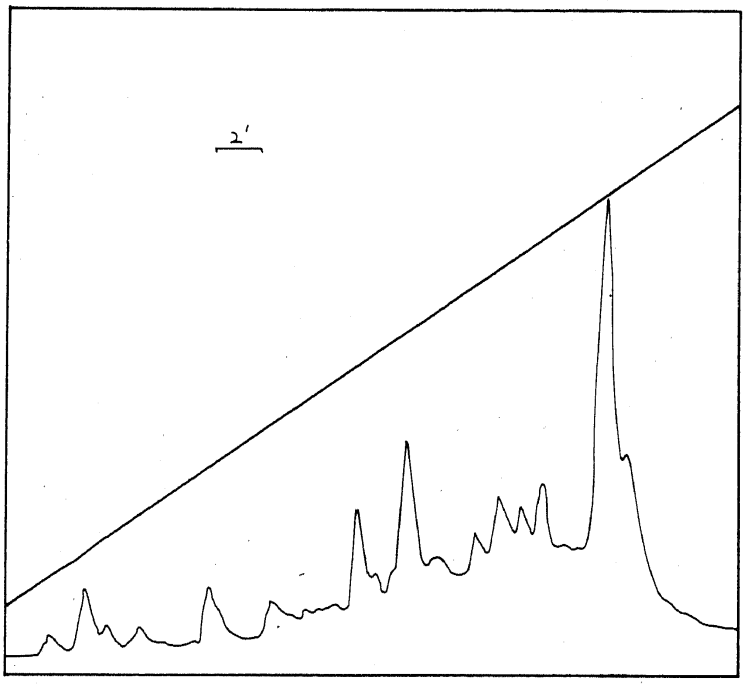

Figure 3. FPLC analysis of crude synthetic pardaxin B = 0–100% in 60′. No peaks were observed from B = 0·20% or beyond 80% B.

evident. Although there are impurities, it is evident from the FPLC profiles that it should be easy to separate the major component from the rest. The major peak was collected for both pardaxin and its analogue P33A and rechromatographed to confirm their homogeneity. FPLC analysis of the purified peptides indicated that the collected peaks are homogeneous as shown in figure 5. Purity of the peptides was also confirmed by amino acids analysis and sequencing. The synthesis has clearly gone off well as purification could be effected by an uncomplicated straightforward linear gradient.

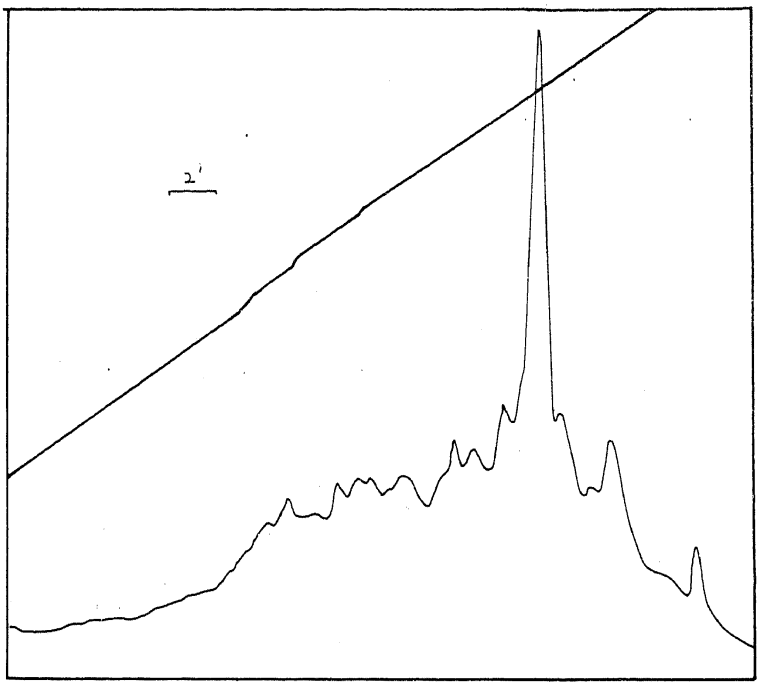

Figure 4. FPLC analysis of crude P33A. B = 0–100% in 60′. No peaks were observed in the region other than that shown in the figure.

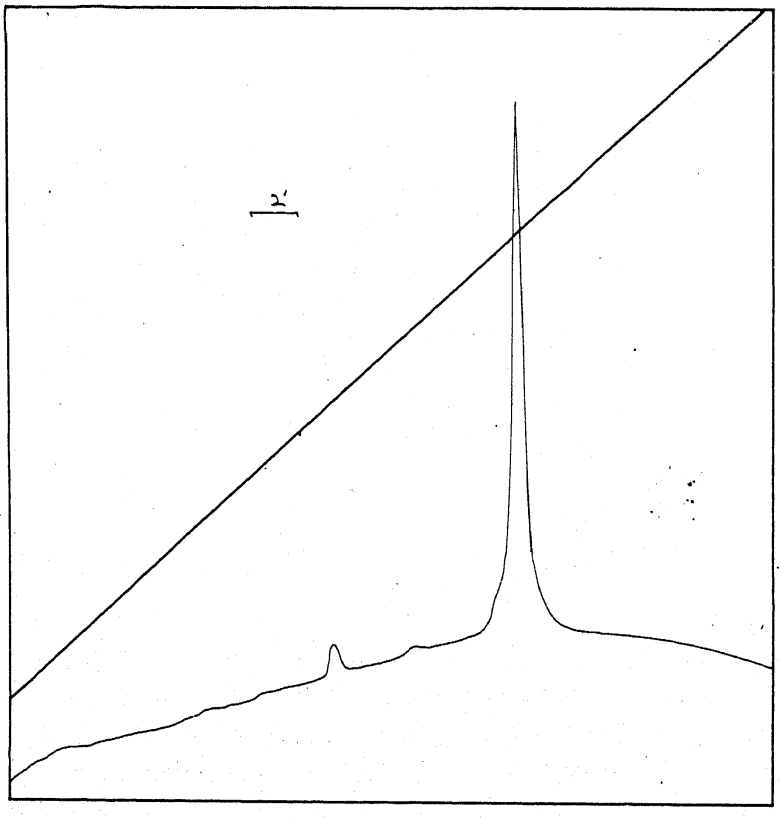

Figure 5. Rerun of purified pardaxin collected from the profile shown in figure 3. 0–100% B in 44′.

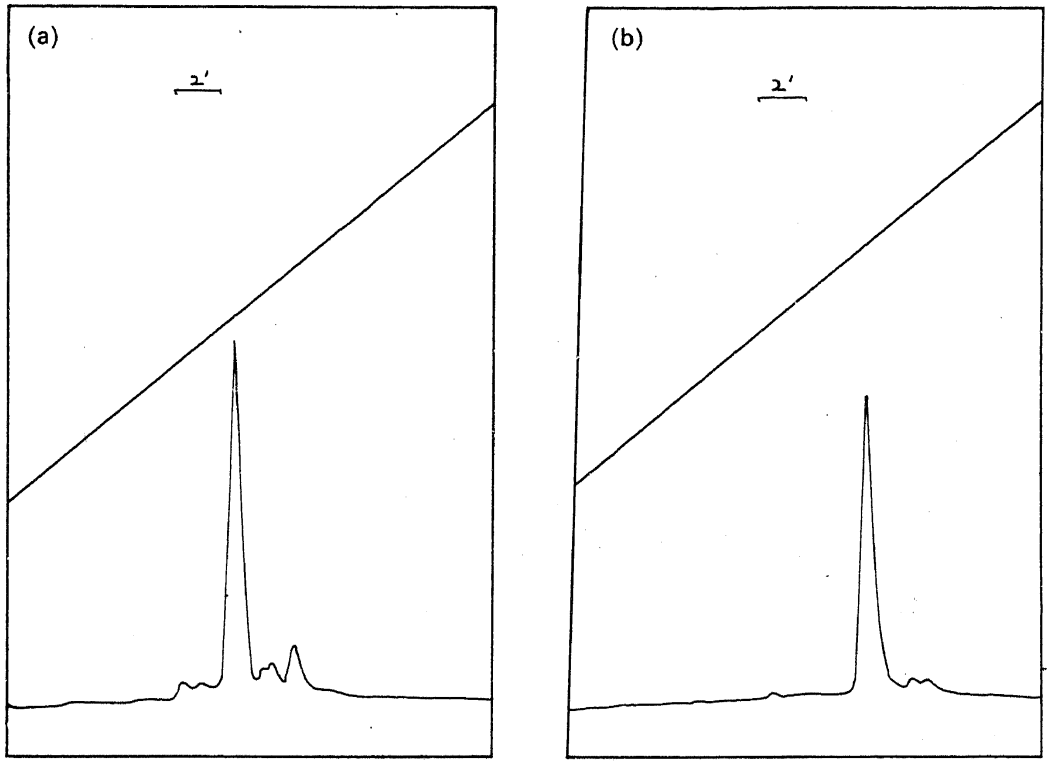

Figure 6. FPLC profiles of crude 18 residue peptides. (**a**) P18, (**b**) P18A. 0–100% B in 44′ for both analysis.

The shorter 15 and 18 residue peptides were synthesized using HOBT active esters. The esters were prepared *in situ* and added to resin. The FPLC traces of the crude 18-residue peptides P18 and P18A are shown in figure 6. It is evident that even the crude peptides exhibit homogeneity. FPLC traces indicate that crude P15 and P15A were also quite pure. The synthesis of P14 with C-terminal amide was also attempted on KB resin (Nova Biochem). While the protocols for synthesis were as described in table 2, deprotection was effected suspending the resin-bound peptide in methanolic NH_3 for 12 h. On filtering off the resin and evaporating methanol, the crude peptide was obtained. FPLC analysis of the peptide shown in figure 7 indicated two peaks. Amino acid analysis indicated that the peak with the greater area had the expected amino acid composition for P14. The peak with less area had one Phe residue less than expected for P14. It is evident that by Fmoc chemistry, fairly pure peptide has been obtained as compared to the synthesis using Boc chemistry protocols. Amino acid analysis of P14 indicates recovery of all the expected amino acids. However, with P15, P15A, P18 and P18A, the C-terminal amino acids were not recovered. On careful analysis of the NMR spectrum it was observed that P14 had a C-terminal ester rather than amide. Although it has been suggested that KB resin on ammonolysis yields C-terminal amide, we have observed that this procedure yields predominantly methyl esters.

All the peptides yielded satisfactory amino acid and sequence analysis as reflected by the amino acid analysis data presented in table 3. Thus, assembly of peptide chains in DMF and NMP with amino acid activation by DCC/HOBT and HBTU has yielded peptides of high purify or afforded easy purification as compared to synthesis

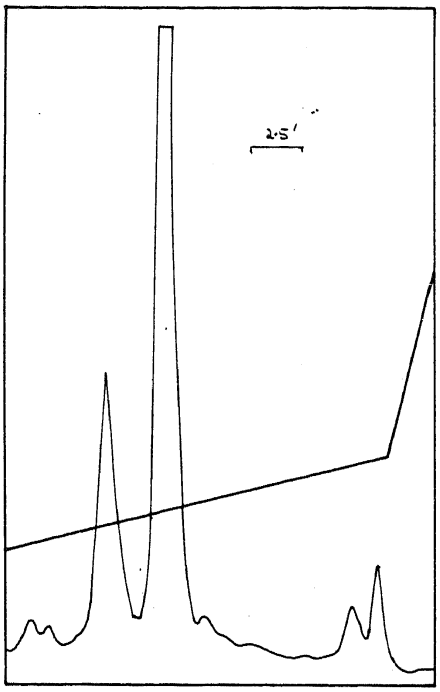

Figure 7. FPLC analysis of crude P14 amide synthesized using Fmoc chemistry under continuous flow conditions. 20–50% B in 50′.

Table 3. Amino acid analysis data for synthetic pardaxin and related peptides[a,b].

Peptide	G	F	A	L	I	P	K	S	T	V	E
Pardaxin	2·98	2·89	3·02	5·25	2·69	1·92	1·91	4·96	0·67	1·09	3·3
	(3)	(3)	(3)	(5)	(3)	(2)	(2)	(7)	(1)	(1)	(3)
P33A	3·30	2·89	3·66	5·1	2·72	0·91	1·86	6·33	0·89	1·04	3·18
	(3)	(3)	(4)	(5)	(3)	(1)	(2)	(7)	(1)	(1)	(3)
P18	1·09	2·00	1·1	1·97	1·89	2·00	1·81	1·94	0·96	—	—
	(1)	(3)	(1)	(3)	(3)	(2)	(2)	(2)	(1)	—	—
P18A	1·08	2·05	2·06	1·94	1·89	1·16	1·87	1·91	0·97	—	—
	(1)	(3)	(2)	(3)	(3)	(1)	(2)	(2)	(1)	—	—
P15	1·03	1·93	1·02	1·92	2·17	1·80	0·95	1·72	—	—	—
	(1)	(3)	(1)	(2)	(3)	(2)	(1)	(2)	—	—	—
P15A	1·23	2·03	2·01	2·0	2·15	0·92	0·94	1·98	—	—	—
	(1)	(3)	(2)	(2)	(3)	(1)	(1)	(2)	—	—	—
P14(Am)	—	1·02	1·07	3·11	0·50	0·90	1·00	2·83	1·01	0·99	—
		(1)	(1)	(3)	(2)	(1)	(1)	(3)	(1)	(1)	—

[a]Values in parentheses indicate theoretical values. S and T values not corrected for loss during hydrolysis

[b]The presence of 3 F residues were confirmed in P15 and P15A by 1H NMR at 300 MHz by relayed COSY experiments

in CH_2Cl_2 in the case of Boc chemistry. Very good yields of pardaxin and P33A were obtained after FPLC purification.

3.1 *δ-toxin and fragments*

δ-toxin is a 26-residue peptide secreted by *S. aureus* having potent hemolytic activity (Bernheimer and Rudy 1986). Almost all the work on this toxin has been done with the one isolated from the microorganism. The peptide tends to aggregate into oligomers and is surface active (Bernheimer and Rudy 1986). Synthesis of the peptide on Merrifield resin employing CH_2Cl_2 as solvent for all the operations and DCC as the coupling reagent did not yield satisfactory results. The crude peptide was too heterogeneous to attempt purification. Even the C-terminal 16-residue peptide could not be synthesized satisfactorily on Merrifield resin. Hence, synthesis using Fmoc chemistry was attempted under continuous flow conditions in the semi-automated peptide synthesizer. The C-terminal 16-residue segment and δ-toxin were synthesized using preformed HOBT active esters as described in table 2. FPLC analysis of the crude peptides is shown in figure 8. While the C-terminal segment is reasonably pure, the δ-toxin is heterogeneous but with a very prominent peak. The major peak was collected and on analysis corresponded to δ-toxin. This was rechromatographed to assess purity and FPLC analysis indicated that the peptide was homogeneous. Peptides corresponding to central segments of δ-toxin were

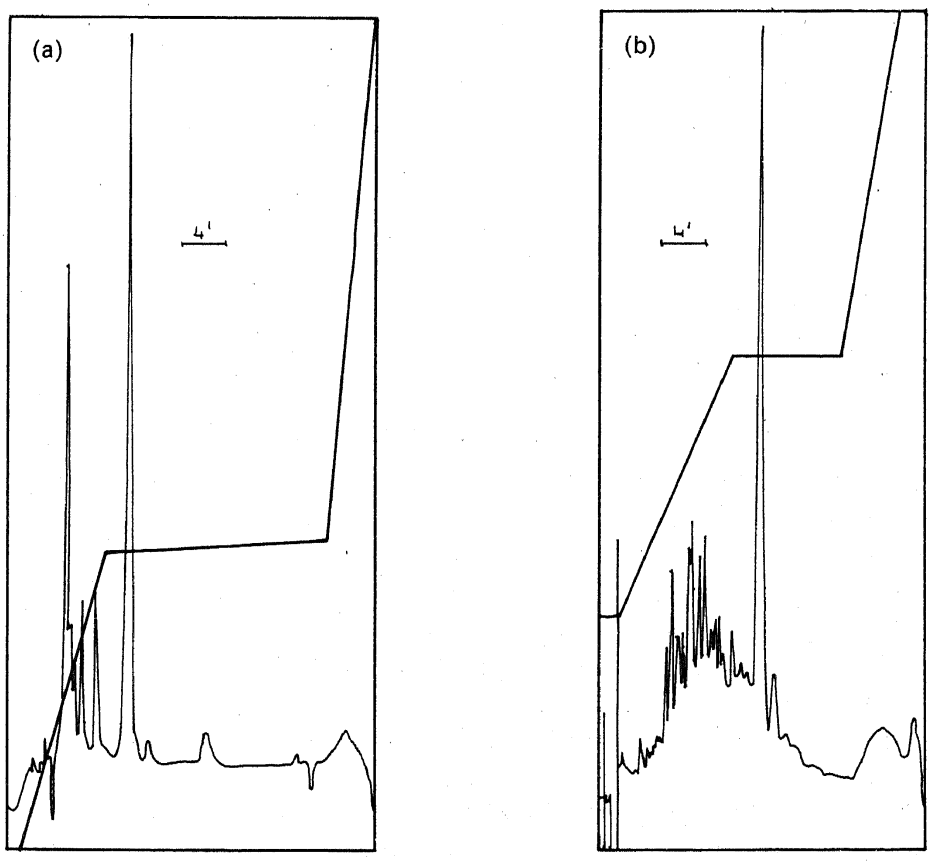

Figure 8. FPLC analysis of crude δ-toxin related peptides (**a**) D16 0–39% B in 8′, 39–40% B in 20′. (b) δ-toxin 30–60% B in 10′, 60% B for 10′.

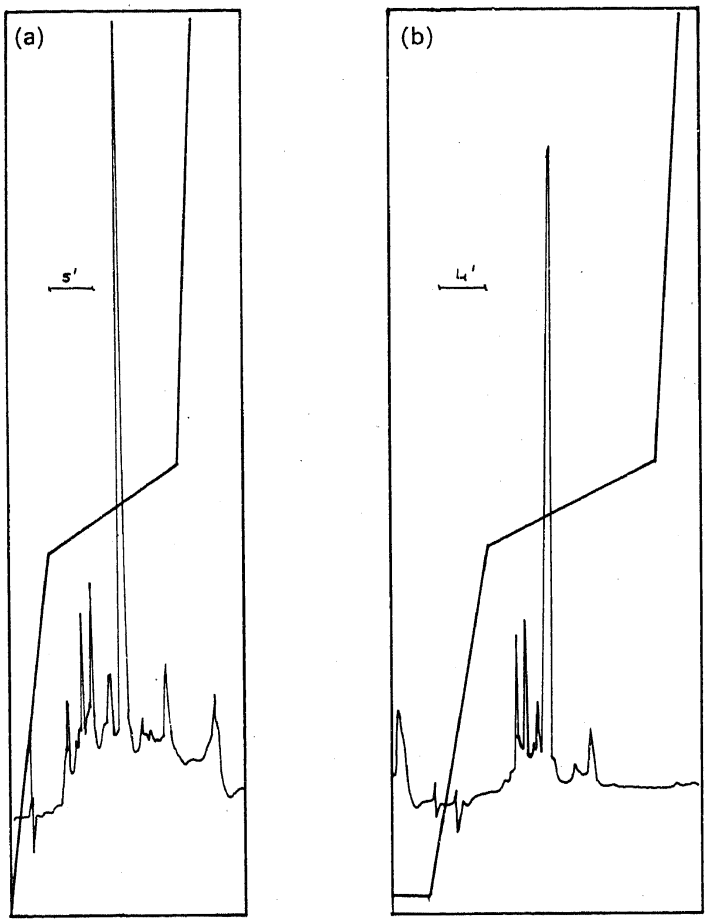

Figure 9. FPLC analysis of crude peptides corresponding to analogues of the central segment of δ-toxin prepared using Fmoc protocol under continuous flow conditions. (**a**) D16DK, 0–40% B in 5', 40–50% B in 15'. (b) D16KK, 0–40% B in 5', 40–50% B in 15'.

synthesized on the semi-automated machine. The FPLC traces shown in figure 9 indicate that crude peptides are not very impure. The major peak corresponds to the desired peptide and could be easily obtained by repeated injections and collection. The rerun showed a single peak without any contaminants.

The semi-automated LKB synthesizer operates on the principle that all operations including washing, deprotection and coupling are done under continuous flow conditions. In order to examine how the protocol shown in table 2 works when polystyrene-based resin is used and all operations are performed manually, peptides D16DK and D16KK were synthesized. The FPLC traces of the crude peptides showed very little impurity. The major peak in both cases corresponded to the desired peptide. It is evident that the synthesis has proceeded as well as in the case of synthesis with continuous flow conditions. We also wished to explore the synthesis of peptides on Merrifield resin employing alternate strategies, since synthesis of hydrophobic peptides with CH_2Cl_2 did not yield satisfactory results. We tried synthesis using NMP as solvent for coupling and DIEA in DMF for neutralization after deprotection with TFA. The protocol is outlined in table 4. FPLC analysis of the crude peptides indicated that the peptides are considerably pure as in the case of peptide shown in figure 9. Their composition was confirmed by amino acid and sequence analysis.

Table 4. Protocol for the synthesis of peptides using Boc amino acids.

1. De-protection, 30% TFA in CH_2Cl_2, 10 ml, 15′
2. Washes with CH_2Cl_2, 10 ml, 5 × 5′
3. Washes with DMF, 10 ml, 5 × 5′
4. Neutralization, 5% DIEA in DMF, 10 ml, 5′
5. Washes with NMP, 10 ml, 5 × 5′
6[a]. Coupling, preformed Boc amino acid HOBT ester in NMP, 10 ml 40′
   DMSO to make 15% in NMP 15′; DIEA, 3·8 equivalents 5′
7. Wash, 30% methanol in DMF, 3 × 5′
8. Washes with NMP, 10 ml 3 × 5′
9. Coupling step repeated
10. Step 7 repeated
11. Washes, CH_2Cl_2, 10 ml, 5 × 5′
12. Steps 1–10 repeated

[a]Boc amino acid HOBT active esters were prepared in the same way as Fmoc amino acids and used immediately.

Table 5. Amino acid analysis of δ-toxin and related peptides[a,b].

Peptide	A	E	D	I	S	T	G	L	V	K	F
δ-toxin	1·00 (1)	1·10 (1)	3·90 (4)	2·98 (5)	1·08 (1)	2·92 (3)	1·22 (1)	1·00 (1)	1·89 (2)	4·00 (4)	1·06 (1)
D16	—	—	3·03 (3)	1·63 (2)	—	1·81 (2)	—	0·97 (1)	2·15 (2)	4·00 (4)	0·85 (1)
D16C	—	—	2·07 (2)	1·58 (5)	1·11 (1)	1·66 (2)	1·06 (1)	1·02 (1)	1·43 (2)	0·97 (1)	—
D16KD	—	—	1·15 (1)	1·76 (5)	1·26 (1)	1·85 (2)	1·15 (1)	1·04 (1)	1·73 (2)	1·94 (2)	—
D16DK	—	—	1·33 (1)	1·84 (5)	1·29 (1)	2·04 (2)	1·20 (1)	1·16 (1)	1·71 (2)	1·84 (2)	—
D16KK	—	—	—	2·10 (5)	1·27 (1)	2·07 (2)	1·24 (1)	1·11 (1)	1·85 (2)	2·97 (3)	—
D15K	1·92 (2)	1·00 (1)	1·00 (1)	3·47 (4)	1·36 (2)	1·7 (2)	2·10 (2)	—	—	1·0 (1)	—
D15E	1·84 (2)	2·10 (2)	0·95 (1)	3·44 (4)	1·44 (2)	1·73 (2)	2·05 (2)	—	—	—	—

[a]Values in parentheses indicate theoretical values. S and T values not corrected for loss during hydrolysis
[b]Presence of W was confirmed by UV spectroscopy

Thus, even using Merrifield's resin and manual operations, it should be possible to get fairly pure peptides using NMP as solvent for coupling and DIEA in DMF for neutralization.

The results of amino acid analysis for the peptides related to δ-toxin are summarized in table 5. While values are somewhat low for Ile and Leu, the others are as expected. In all cases, deprotection from the resin yielded peptides in amounts as would be expected from the initial substitution. The yield of purified peptide obtained by FPLC purification in the case of δ-toxin was 85%.

Presently, medium-sized as well as large proteins can be obtained in large quantities by recombinant DNA methods (Wetzel and Goeddel 1983). However, this approach is limited to synthesis with L-amino acids. Although solution phase methods have been responsible for development of peptide chemistry (Finn and Hofmann 1976), it is of little practical use when large quantities are required in a short period of time. Solution phase methods are very time-consuming due to the large number of intermediate purification steps (Finn and Hofmann 1976). The considerable amount of time and effort spent on solution phase synthesis of RNase A (Yajima and Fujii 1981) and lysozyme (Galpin *et al* 1981) argues against this approach as a practical means of generating peptides for biological research. Synthesis by solid-phase methods, originally developed by Merrifield (1986) has revolutionized peptide synthesis by cutting down time as well as opening up the scope for automation. However, the problem of getting homogeneous peptide is still a formidable challenge. In recent years, with improved automation, peptide synthesis has become user-friendly. In fact, proteins like interleukin (Clark-Lewis *et al* 1986) and AIDS proteases (Wlodawer *et al* 1989; Milton *et al* 1992) have been synthesized. The synthetic AIDS proteases have also been crystallized and their structures determined by X-ray analysis (Wlodawer *et al* 1989) indicating a high degree of purity of the synthetic product. A careful examination of these reports indicate that although the actual synthesis takes a very short time, there is no detailed protocol outlined. In fact, most of these reports mention that protocols have been *optimized* for the sequence. In this paper, we have described in detail our efforts on the synthesis of 16–33 residue hydrophobic peptides corresponding to the toxins pardaxin and δ-toxin by semi-automated as well as by manual methods. We have used only single couplings with only $\sim 3{\cdot}5$ equivalents excess when Fmoc chemistry was employed and have been able to get even crude peptides with high purity. We have also optimized a protocol for synthesis using Boc chemistry manually.

The successful synthesis and purification of pardaxin, δ-toxin and several analogues would help in correlating the structure of these toxins with function and help in identifying the molecular determinants of biological activity. Efforts in this direction are currently underway in our laboratory.

Acknowledgement

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