APPLICATION OF CELLULOSE PAPER AS SUPPORT MATERIAL IN SIMULTANEOUS SOLID PHASE PEPTIDE SYNTHESIS

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Cellulose paper was esterified with N^{α}-Fmoc-protected amino acid chlorides providing a chemically stable segmental support, the utility of which for simultaneous solid phase peptide synthesis was demonstrated by the synthesis of several sets of model peptides following different synthetic strategies.

Synthetic peptides* are of growing interest as valuable tools for molecular biology and immunology. Especially this applies to the localization, elucidation and characterization of binding sites by which proteins interact with their ligands such as receptors and antibodies. The epitope mapping using short overlapping synthetic partial sequences of proteins as well as the preparation of replacement sets of antigenic determinants are efficient methods of localization and characterization of sequential determinants. However, this requires plenty of synthetic peptides in relatively small quantities (nmol to μmol-scale). This increased need of peptides also demands an appropriate enlargement of synthetic capacities.

Utilizing the advantages of solid phase peptide synthesis and using a mechanically stable and compact support material simultaneous peptide synthesis by support--segmentation has been developed[1-3]. The synthesis of different peptides on distinct pieces of support material permits the selective combination, separation and resorting of the support pieces, thus enabling identical processes as deprotection, wash and coupling of the same amino acid to be carried out together with following resorting of the support pieces for separate coupling reactions.

This approach has been introduced into peptide synthesis by H. M. Geysen[1], who synthesized 208 hexapeptides on polyethylene rods without final cleavage of the peptides from the support. This method was designed to provide large numbers of peptides in nmol-quantities for screening tests using a solid phase immuno assay. The so called "tea bag" method, demonstrated by R. A. Houghten[2] with the syn-

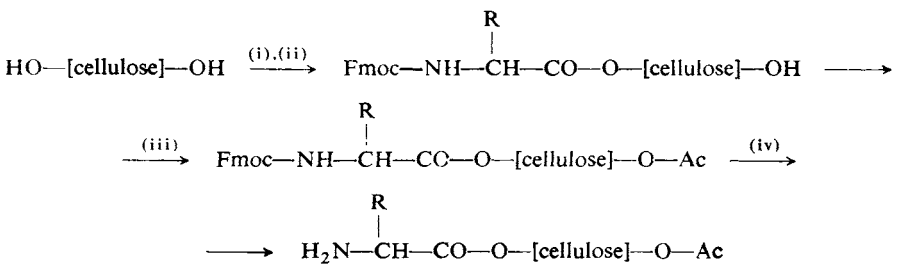
* Nomenclature and abbreviations follow the suggestions of IUPAC–IUB (Eur. J. Biochem. *138*, 9 (1984)). Further abbreviations: Ahx, ε-aminohexanoic acid; DCM, dichloromethane; DMF, N,N-dimethylformamide; DIPEA, diisopropylethylamine; TFA, trifluoroacetic acid.

thesis of 248 13-residue peptides, works on the usual polystyrene resin. The support segmentation is realized by sealing the resin amounts for each peptide into polypropylene bags.

Cellulose paper meets the basic requirements to a support material for simultaneous synthesis. Furthermore, it can be variably cutted and therefore the size of the support pieces can be chosen individually. Cellulose paper is a rather cheap and convenient to handle material. It has already been applied to simultaneous synthesis of oligonucleotides[4].

Recently, R. Frank[3] demonstrated its utility as support material for simultaneous peptide synthesis with the synthesis of 13 heptapeptides on paper disks under continuous flow conditions. Because of the acid labile peptide-paper linkage of this support its applicability is limited to synthetic strategies using base-labile protecting groups (Fmoc) for the temporary N^{α}-protection of amino acids.

We report here the easy to perform preparation of a paper support as well as the synthesis of model peptides on it following different synthetic strategies. The functionalization of the paper (Whatman 540) was realized by esterification of OH-groups of the cellulose with Fmoc-amino acid chlorides[5] after alkaline pretreatment of the paper (see Scheme 1).



SCHEME 1

Preparation of the cellulose paper support: (i) 1M-NaOH, (ii) Fmoc-amino acid chloride, (iii) acetic anhydride/pyridine, (iv) piperidine/DMF; R = H, CH_3

The substitution of the cellulose with amino groups can be determined by photometric measurement of the Fmoc-cleavage product (dibenzofulvene–piperidine adduct) and was found to amount to 1 to 2 μmol/cm^2. The chemical stability of the cellulose ester bond was checked by following treatments:

(i) 25% TFA in DCM 3 h at RT

(ii) 20% piperidine in DMF 3 h at RT, followed by coupling of an Fmoc-amino acid.

After both treatments, corresponding to (i) 12 and (ii) 18 coupling cycles, only small decrease of substitution ((i): 9% and (ii): 6%) was detectable, thus indicating that the given cellulose support is applicable to different synthetic strategies (N^{α}-Boc-

-protection with acidic deprotection as well as N^{α}-Fmoc-protection with alkaline deprotection).

However, the mechanical stability of the paper support towards acids is limited. It gets destroyed or even dissolved by stronger acids such as methanesulfonic or trifluoromethanesulfonic acid, which are also used as deprotection or cleavage reagents in peptide synthesis.

Before starting peptide synthesis the paper support can be furnished with an appropriate spacer or anchor group according to the purpose the peptides shall be prepared for. If they are designed to get support-fixed tested (cellulose paper is widespread used as suppport material in solid phase immuno-assays) a spacer such as an ω-amino acid can be coupled to provide a certain distance between the support and the peptide. If the synthesis is to be carried out in a preparative sense (including cleavage and isolation of the peptides) it is possible to insert a selectively cleavable anchor group such as *p*-alkoxybenzyl ester handles[6]. This is an advantageous feature of the paper support in comparison with polyethylene rods or tea bags which are designed either for support-fixed testing (rods) or for cleavage of the synthesized peptides (tea bags) only.

On the paper support, penta- to undecapeptides were synthesized with an average purity of the crude products of 70 to 80%. Two sets of model peptides will be demonstrated here.

set A: *I*, Tyr-Val-Pro-Lys-Ahx-Ala-OH
II, Tyr-Glu-Gly-Thr-Ahx-Ala-OH
III, Tyr-Lys-Gln-Ile-Ahx-Ala-OH

set B: *IV*, Tyr-Pro-Thr-Lys-Phe-Leu-Gly-Lys-Ala-Phe-Val-OH
V, Tyr-Pro-Ala-Gly-Val-Leu-Ala-Thr-Pro-Phe-Val-OH
VI, Tyr-Leu-Ala-Lys-Val-Pro-Gly-Thr-Ala-Phe-Leu-OH
VII, Tyr-Leu-Thr-Gly-Phe-Pro-Ala-Lys-Pro-Phe-Leu-OH
VIII, Lys-Pro-Lys-Pro-Gly-Gly-Phe-Phe-Gly-Leu-Leu-OH

Peptides *I*, *II* and *III* were synthesized simultaneously on 3 pieces of H_2N—CH-(CH_3)—CO—O-cellulose paper (Ala-paper; each piece 3 × 3 cm; 1·2 μmol/cm^2), using preactivated HOBt esters of Boc-amino acids (with the exception of Fmoc--Lys(Boc), Fmoc-Pro and Fmoc-Val).

Course and completion of couplings were checked by bromophenol blue – monitoring[7]. The benzyl side chain protecting groups of Glu and Thr (*II*) were cleaved by boron tris(trifluoroacetate)[8]. The peptides were removed from the paper by alkaline cleavage of the cellulose ester and analysed by HPLC (see Fig. 1). Some of the polar side peaks probably do not correspond to peptidic impurities since the same peaks also appear on the HPLC-profile of the "cleavage product" of blank paper. The identity of the main peaks was confirmed by amino acid analysis (see Table I).

TABLE I

Amino acid analyses of the main HPLC-peaks of *I*– *VIII*

Amino acid	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
Val	1·12	—	—	1·04	1·97	0·98	—	—
Leu	—	—	—	0·92	0·94	1·94	1·90	1·87
Phe	—	—	—	2·05	1·03	1·02	2·09	2·16
Ala	1·0	1·0	1·0	1·0	2·0	2·0	1·0	—
Pro	0·97	—	—	0·92	1·81	0·92	1·72	1·85
Lys	1·06	—	1·1	1·93	—	0·95	0·95	1·99
Thr	—	1·1	—	0·91	0·94	0·96	0·91	—
Gly	—	1·04	—	1·05	1·10	1·07	1·06	3·0
Tyr	0·83	0·86	0·9	1·01	0·99	0·98	0·97	—
Ahx	0·97	0·98	0·96	—	—	—	—	—
Glu	—	0·95	—	—	—	—	—	—
Ile	—	—	1·1	—	—	—	—	—
Gln	—	—	1·13	—	—	—	—	—

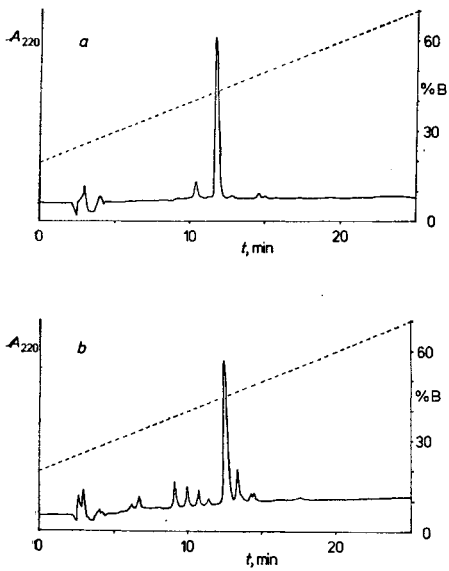


FIG. 1

HPLC-profiles of *I* (*a*) and *III* (*b*) (LiChrosphere C_8 column 25 × 0·4 cm, particle size 5 μm, gradient: A 0·05% TFA, B 0·05% TFA/CH_3CN, 3 : 7)

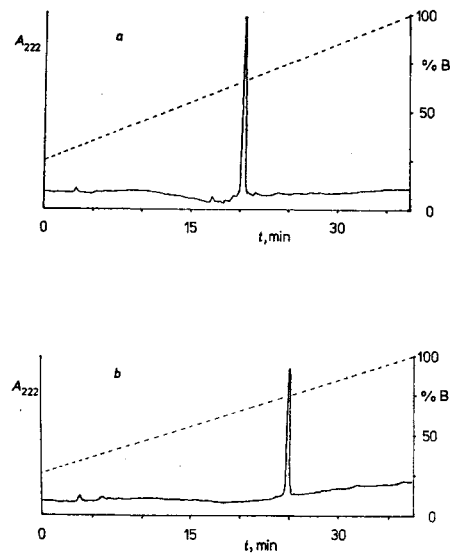


FIG. 2

HPLC-profiles of *IV* (*a*) and *V* (*b*) (Separon SIX C_{18} column 20 × 0·4 cm, particle size 7 μm, Gradient: A 0·05% TFA, B CH_3OH)

TABLE II

FAB-MS data of the main HPLC-peaks of *IV*– *VIII*

Mass value	*IV*	*V*	*VI*	*VII*	*VIII*
Theoretical (M)	1 270	1 134	1 179	1 253	1 160
Obtained ($M + H^+$)	1 271	1 135	1 180	1 254	1 161

TABLE III

Coupling cycles for Boc- and Fmoc-amino acids

Step	Reagent	Time, min
	Boc-amino acids	
1. deprotection	3 ml 25% TFA in DCM (2% anisole)	15
2. wash	2 × 5 ml DCM	2 × 3
3. neutralization	2 × 3 ml 5% DIPEA in DCM	2 × 3
4. wash	2 × 5 ml DCM	2 × 3
	resorting of the papers	
5. coupling	Boc-AA-OBt-ester[a] (0·25M in DMF)	60 to 120
6. wash	2 × 5 ml DMF	2 × 3
7. wash	2 × 5 ml DCM	2 × 3
	Fmoc-amino acids	
1. deprotection	3 ml 20% piperidine in DMF	10
2. wash	2 × 5 ml DMF	2 × 3
3. wash	2 × 5 ml DCM	2 × 3
	resorting of the papers	
4. coupling	Fmoc-AA-OBt-ester[a] (0·25M in DMF)	60 to 120
5. wash	2 × 5 ml DMF	2 × 3
6. wash	2 × 5 ml DCM	2 × 3

[a] The volume of acylation reagent depends on the size of the support pieces; set A: 1 ml, set B: 0·5 ml.

Peptides *IV* to *VIII* were synthesized simultaneously on 5 pieces of $H_2N—CH_2—CO—$O-cellulose paper (Gly-paper; each piece 1 cm^2; 0·8 μmol/cm^2) using pre-activated HOBt esters of Fmoc-amino acids (side chain protection: Lys(Boc), Thr(Bu^t). The first amino acids (Val and Leu) were coupled bound to an activated acid labile handle (Fmoc-amino acid ester of 4-(hydroxymethyl)phenoxypropionic acid trichlorophenyl ester[6]).

The synthesized peptides were removed from the paper by acidic cleavage of the anchor-bond and analysed by HPLC (see Fig. 2). The identity of the main peaks was confirmed by amino acid analysis and FAB mass spectrometry (see Tables I and II). The obtained analytical data of the synthesized peptides demonstrate the utility of the paper support for simultaneous peptide synthesis following both "classical" Boc/benzyl- and orthogonal Fmoc/Bu^t-strategies.

EXPERIMENTAL

Preparation of the Cellulose Paper Support

Fmoc-amino acid chlorides were prepared according to ref.[5]. Whatman 540 paper (5 × 5 cm) was shaken in stoppered glass vessels successively in (i) 3 ml 1M-NaOH (30 min), (ii) 3 ml 0·25M Fmoc-Ala-Cl or Fmoc-Gly-Cl in toluene/acetone 1 : 1 (v/v) (30 min), (iii) 30% acetic acid in acetone (5 min), (iv) acetone, water, ethanol and DCM (each 5 ml, 3 min) and dried between filter paper. Remaining susceptible OH-groups were acetylated in 3 ml acetic anhydride/pyridine 1 : 2 (v/v) (1 h), followed by washing with DMF and DCM (each 5 ml, 3 min).

For determination of the degree of substitution 0·2 cm^2 of the paper was shaken in 3 ml 20% piperidine in DMF (15 min) and absorption of this solution was read at 301 nm (ε = 8 100 in 20% piperidine/DMF).

Peptide Synthesis

Amino acid derivatives were prepared in our laboratory. Synthesis of the *p*-alkoxybenzylester handle as well as its coupling to the starting amino acids in set B were performed according to ref.[6]. Couplings were carried out batchwise in stoppered glass vessels according to Table III.

Analytical Methods

HPLC-analyses were carried out on a Knauer device (set A) and on a Spectra Physics SP 8700 device (set B), respectively. Amino acid analysis was performed after acidic hydrolysis of the peptides (6M-HCl, 110°C, 20 h) on an automatic analyzer (Durrum 500). FAB MS analysis was carried out on a ZAB EQ spectrometer (VG Analytical, Manchester) with xenon at 8 kV as the bombarding gas.

Cleavage of the Benzyl Side Chain Protecting Groups

The peptide-paper was shaken in 3 ml 1M boron tris(trifluoroacetate) in TFA (0·1% thioanisole) for 1 h, followed by washing with TFA, DCM, 5% DIPEA in DCM, DCM (each 5 ml, 3 min) and dried.

Cleavage of the Peptides from the Support

I—III: The peptide-papers were shaken in 1 ml 1M-NaOH for 1 h and washed with another ml of 1M-NaOH. The combined cleavage and wash solutions were neutralized with 1M-HCl, desalted by gel filtration on Sephadex G 10 and lyophilized yielding 5 to 7 mg of crude peptide.

IV—VIII: The peptide-papers were shaken in 1 ml 75% TFA in DCM (2% anisole) for 2 h and washed with another ml of TFA/DCM and 10 ml water. The combined cleavage and wash solutions were evaporated to remove DCM and TFA, extracted with 10 ml ether, again evaporated and lyophilized yielding 0·7 to 1 mg of crude peptide, which were purified by HPLC on a Vydac C_{18} column (25 × 1 cm, particle size 10 μm, gradient: 25—100% methanol/0·05% TFA in 75 min).

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