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PURIFICATION BY DEAE-SEPHADEX CHROMATOGRAPHY OF THREE HEXAPEPTIDES SYNTHESIZED BY THE SOLID-PHASE TECHNIQUE

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SUMMARY

Solid-phase peptide synthesis according to Merrifield is a widely used technique that is often described as uncomplicated, at least for the syntheses of peptides composed of a small number of amino acid residues. Three hexapeptide analogues were synthesized using an automated synthesizer and analysed by preparative ion-exchange chromatography, monitoring the eluate absorbance at 206 and 280 nm. The results obtained indicated the need for careful purification of oligopeptide preparations synthesized by this technique.

INTRODUCTION

Many peptides synthesized on a preparative scale by the solid-phase technique of Merrifield[1] seem to be insufficiently characterized in terms of homogeneity. Monitoring systems, developed to determine yields of coupling and deblocking, have mainly been devoted to analytical purposes, although some are applicable to and have been used for preparative work[2]. Gel filtration, which as a criterion of purity and a means of fractionation for synthetic oligopeptides is generally not recommended, appears to be the method most widely used for the purification of crude preparations, but only seldom in combination with more efficient techniques (for a review, see Meienhofer[2]).

Peptides containing tyrosine and glutamic acid residues are known to be potentially troublesome in solid-phase synthesis, because of the risk of side-reactions during coupling, deblocking and cleavage procedures[3–8]. This paper reports the synthesis and purification by means of ion-exchange chromatography on DEAE-Sephadex of three hexapeptide analogues designed as models of the branched polypeptide antigen poly(L-Tyr, L-Glu)-poly(DL-Ala)--poly(L-Lys)[9–12]. Common synthetic strategies were applied using an automated synthesizer, not equipped with monitoring devices.

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EXPERIMENTAL

Attachment to the solid support

The resin (Bio-Beads S-X2, 200–400 mesh; Bio-Rad Labs., Richmond, Calif., U.S.A.) suspended in 1,2-dichloroethane was chloromethylated as described by Losse *et al.*[13] using freshly distilled monochlorodimethyl ether and tin(IV) chloride. The degree of substitution was determined by Volhard titrations according to Stewart and Young[14] (1.10 mmole of Cl per gram of chloromethylated resin). Boc-Gly-OH, prepared by the method of Schnabel[15], was coupled by refluxing the resin for 42 h in absolute ethanol, in the presence of Boc-amino acid and triethylamine[13]. Potentiometric titrations[16] indicated a degree of substitution of 0.32 mmole of Gly per gram of peptide resin, compared with 0.33 mmole/g determined by amino acid analysis performed directly on the hydrolysed peptide resin (see Table I for details).

Syntheses

Three hexapeptides (general formula H-Tyr-Glu-X-Ala-Ala-Gly-OH, where X = Ala, Glu or Tyr) were prepared using an automated peptide synthesizer (Schwartz/Mann, Orangeburg, N.Y., U.S.A.) developed by Brunfeldt *et al.*[17].

The program for the synthesis of one of the hexapeptides, containing 600 operations, is shown in Fig. 1. At step No. 317 codes H, J or N were used to activate

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A039A039A039E039E059A039A039A039F039F039F039B139G159
B139B139B139B139B139B139
H15P1779B139B139B139C139C139C139C139C139C139
A039A039A039E039E059A039A039A039F039F039F039B139G159
B139B139B139B139B139B139
P13H1779B139B139B139C139C139C139C139C139C139
A039A039A039E039E059A039A039A039F039F039F039B139G159
B139B139B139B139B139B139
N15P1779B139B139B139C139C139C139C139C139C139
A039A039A039E039E059A039A039A039F039F039F039B139G159
B139B139B139B139B139B139
J15P1779B139B139B139C139C139C139C139C139C139
A039A039A039E039E059A039A039A039F039F039F039B139G159
B139B139B139B139B139B139
N15P1779B139B139B139C139C139C139C139C139C139=
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Fig. 1. Program for the synthesis of H-Tyr-Glu-Tyr-Ala-Ala-Gly-OH, containing 600 operations coded on punched tape; a reader activates through a control unit the appropriate reagent or solvent reservoir, from where the material is forced to a metering device by nitrogen pressure. Controlled by photocells, the pressure is switched off at a pre-determined level in the metering device, and the reagent/solvent is drained into the reaction flask containing the peptide-resin. The reactor is shaken for an appropriate time, and drained using nitrogen pressure. Codes: A, HOAc; B, methylene chloride; C, ethanol; E, HCl–HOAc (1 *M*); F, methylene chloride; G, Et_3N–methylene chloride (10%); H, Boc-Ala-OH; N, Boc-Tyr(Bzl)-OH; P, dicyclohexylcarbodiimide; 0 and 1, metering drain; 3, 5 and 7, reactor shaken for 3, 5 and 120 min, respectively; 9, reactor drain; =, stop.

reservoirs containing Boc-Ala-OH, Boc-Glu(OBzl)-OH or Boc-Tyr(Bzl)-OH, respectively, synthesized[10] by the method of Schnabel[15]. Four equivalents of Boc derivatives and the coupling reagent, dicyclohexylcarbodiimide (DCC), were used throughout. All solvents were added in an amount of 10 ml per gram of resin; a pre-made 10% solution of triethylamine in dichloromethane was used in the neutralization steps; 8.7 g of resin were used in each synthesis, corresponding to 2.8 mmole of peptide.

Cleavage

The cleavage of the peptide from the resin and deprotection of functional groups were accomplished by treatment of the peptide resin, while suspended in anhydrous trifluoroacetic acid, with a stream of dry, bromine-free hydrogen bromide for 3 × 40 min at room temperature. Anisole (50-fold excess) was added as a nucleophile scavenger[14]. The collected eluates were evaporated and extracted with diethyl ether to remove anisole, the residue was re-dissolved in small amounts of aqueous acetic acid (10%), and the solution was passed through an ion-exchange column (Amberlite IR-45, Ac^-). Ninhydrin-positive fractions (spot test) were collected, evaporated and finally freeze-dried.

Purification

The peptides were further purified on Sephadex G-10 columns (120 × 1.5 cm) by elution with deionized water. The material from a fairly symmetrical peak was collected, evaporated and freeze-dried. The peptides were then subjected to ion-exchange chromatography on DEAE-Sephadex A-25 (Pharmacia, Uppsala, Sweden)[8]. After application of the peptide (100–200 mg) in 0.01 *M* phosphate buffer (pH 7.0), the columns (30 × 1.5 cm) were washed with 150–200 ml of the initial buffer, and the peptides were eluted using a linear salt gradient produced by a three-channel pump (Pharmacia P-3), flow-rate 35 ml/h. The eluate was monitored simultaneously at 206 and 280 nm using a Uvicord III UV Absorptiometer (LKB, Bromma, Sweden). An identical column was run in parallel in order to feed the reference cell. Fractions were pooled as indicated, evaporated and desalted in 0.1 *M* ammonia solution on Sephadex G-10 columns (120 × 1.5 cm). The peptides were identified by amino acid analysis.

RESULTS AND DISCUSSION

The completion of an entire sequence could be accomplished in 50–60 h without any discontinuations caused by technical shortcomings. The syntheses were carried out at room temperature using pre-made reagent solutions.

Despite extensive purification of the crude peptide preparation, including elution from an Amberlite IR-45 column and further fractionation on Sephadex G-10, DEAE ion-exchange chromatography revealed a number of peaks absorbing mainly at 206 nm.

In the light of recent attempts to optimize coupling and deblocking reactions using different monitoring devices[2], it is of interest to discuss the origin of the components observed, whether these sequences are due to truncations or other side-reactions during the stepwise addition of amino acid residues, or whether they should be ascribed to side-reactions caused by the cleavage procedure.

After completion of the entire sequence, the peptide resin was subjected to amino acid analysis (Table I). The observed lack of tyrosine, however, cannot serve as a clear indication of a possible truncation of the peptide chain before addition of the last tyrosine residue. A significant degradation of tyrosine during hydrolysis can be observed by comparing the differing results obtained when different hydrolysis conditions are applied (Table I).

TABLE I

AMINO ACID ANALYSES OF PEPTIDES BEFORE DEPROTECTION AND CLEAVAGE FROM THE RESIN COMPARED WITH CLEAVED, DEAE-FRACTIONATED PREPARATIONS

All hydrolyses were performed in evacuated, sealed vials using a Beckman Model 120 C amino acid analyzer.

Peptide	*Amino acid analyses (Tyr:Glu:Ala:Gly)*	
	Resin-bound peptide	*DEAE-fractionated peptide*
H-Tyr-Glu-Ala-Ala-Ala-Gly-OH	0.67:1.01:2.98:1.00* 0.81:0.97:2.99:1.00**	1.00:1.02:3.04:1.00***
H-Tyr-Glu-Glu-Ala-Ala-Gly-OH	0.56:2.02:2.23:1.00* 0.71:2.01:2.22:1.00**	0.98:2.05:2.04:1.00***
H-Tyr-Glu-Tyr-Ala-Ala-Gly-OH	1.68:0.97:1.99:1.00*	2.02:1.07:2.02:1.00***

* Hydrolysed 96 h, 6 *M* HCl–HOAc (1:1), 110°.
** Hydrolysed 96 h, 12 *M* HCl, 110°.
*** Hydrolysed 24 h, 6 *M* HCl–propionic acid (1:1), 110°.

DEAE ion-exchange chromatography of Sephadex-fractionated preparations of the hexapeptides H-Tyr-Glu-Ala-Ala-Ala-Gly-OH and H-Tyr-Glu-Glu-Ala-Ala-Gly-OH gave one major peak absorbing at 280 nm, eluted at a salt concentration of *ca.* 0.14 *M* NaCl (Figs. 2 and 3). Minor peaks eluted at higher salt concentrations might be ascribed to 3-benzylated tyrosine-containing peptides known to be formed during cleavage of peptide from the resin[4]. With H-Tyr-Glu-Ala-Ala-Ala-Gly-OH (Fig. 2), fractions absorbing only at 206 nm might be due to H-Glu-Ala-Ala-Ala-Gly-OH, truncated owing to incomplete removal of the Boc-protecting group before addition of the tyrosine derivative, or to pGlu-Ala-Ala-Ala-Gly-OH that has been terminated by cyclization of the α-amino group to 2-pyrrolidone-5-carboxylic acid (pyroglutamic acid, pGlu)[3,8]. While hydrogen fluoride together with anisole as the cleavage reagent at room temperature has been reported to cause the formation of considerable amounts of peptides containing an anisole acylation product of glutamic acid[5–8], the use of the less acidic hydrogen bromide is reported to cause the formation of only minor amounts of the pyroglutamic acid containing peptides during cleavage[6].

The DEAE chromatogram of H-Tyr-Glu-Tyr-Ala-Ala-Gly-OH (Fig. 4) appeared to be more complex, however, depicting a number of peaks probably due to side-reactions identical with those already described. The postulated truncation of the peptide chain due either to incomplete removal of the Boc-group or to pGlu formation in the synthesis of H-Tyr-Glu-Ala-Ala-Ala-Gly-OH is supported by the finding of an analogous peak in Fig. 4, but here displaying an additional absorbance

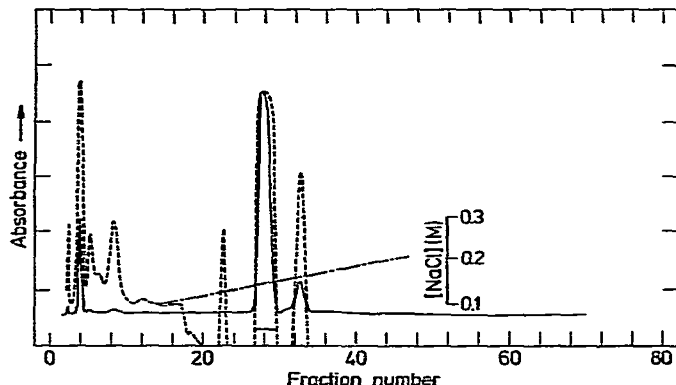

Fig. 2. DEAE-Sephadex chromatography of Sephadex G-10 fractionated H-Tyr-Glu-Ala-Ala-Ala-Gly-OH. Column dimensions, 30 × 1.5 cm, equilibrated with phosphate buffer (0.01 *M*, pH 7.0) and eluted with a linear gradient (2000 ml total) to 0.5 *M* NaCl. Fraction volume, 12.5 ml; flow-rate, 35 ml/h; temperature, 24° (fractions were collected at 4°). Absorbance was simultaneously read at 206 nm (dashed line) and 280 nm, using a Uvicord III UV Absorptiometer (LKB); an identical column was run in parallel, feeding the reference cell. Fractions were pooled as indicated.

at 280 nm due to a tyrosine residue at the 4-position of the peptide. The shoulder observed in the main peak in Fig. 4 has not been further analysed, but might be caused by incomplete resolution of the 3-benzylated tyrosine analogue.

In conclusion, DEAE ion-exchange chromatography seems to be suitable for both preparative fractionation and analytical resolution of the complex mixtures formed by the solid-phase synthesis even of peptides composed of a relatively small number of amino acids. The results indicate that due consideration must be given to side-reactions caused by cleavage of the peptide from the solid support. This appears to be especially important taking into account the considerable possibilities, through different monitoring systems now at hand, for control and optimization of coupling and deblocking reactions during synthesis[2]. A major improvement in solid-phase peptide synthesis has been the replacement of O-benzyltyrosine by O-(2,6-dichlorobenzyl)tyrosine, known to be far less susceptible to the rearrangement causing

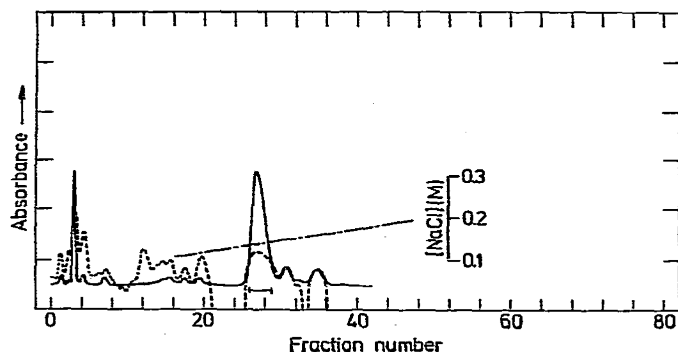

Fig. 3. DEAE-Sephadex chromatographic separation of Sephadex G-10 fractionated crude H-Tyr-Glu-Glu-Ala-Ala-Gly-OH. Details as in Fig. 2.

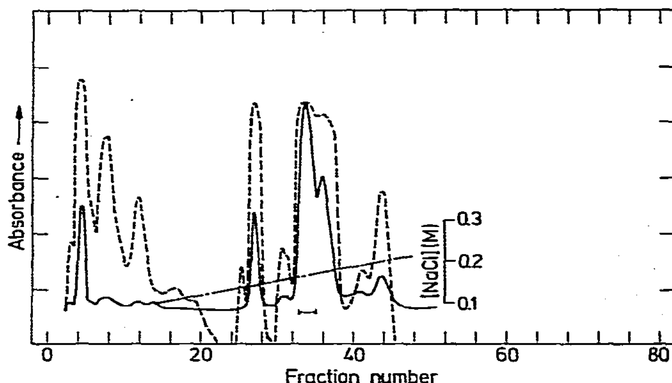

Fig. 4. DEAE-Sephadex chromatographic separation of Sephadex G-10 fractionated crude H-Tyr-Glu-Tyr-Ala-Ala-Gly-OH. Details as in Fig. 2.

ring benzylation[4]. The truncation of the peptide chain caused either by cyclization to pyroglutamic acid or the failure of total removal of the Boc-group from the N-terminal glutamic acid[3,8] will be further studied.

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