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Note

Purification of a synthetic peptide with the aid of covalent chromatography

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Efficient purification of synthetic peptides poses problems of varying complexity; the standard techniques used were recently reviewed[1]. In an attempt to overcome some special problems associated with the purification of peptides prepared by solid-phase peptide synthesis (SPPS)[2], Krieger *et al.*[3] recently devised a procedure based on an affinity strategy, which they utilized for the preliminary purification of two medium-sized fragments of ribonuclease and histone H4. Their procedure, the organomercurial Cys-Met tactic, involves attaching Cys-Met to the amino end of the peptide. The Cys serves as an affinity label, organomercurial-agarose as an affinity receptor, and Met as a temporary binding group which, after purification of the peptide has been effected, can be split off together with the affinity label.

In this communication, we propose the application of covalent chromatography based on thiol–disulphide interchange[4,5] as an alternative to the use of organomercurial agarose in the purification of a synthetic peptide. A detailed procedure applied to a peptide corresponding to residues 33–48 of allergen M from cod[6] is given below.

EXPERIMENTAL

Materials

Thiopropyl-Sepharose 6B with a capacity of approximately 20 μmoles/ml swollen gel was obtained from Pharmacia (Uppsala, Sweden). The peptide Cys-Met-Val-Gly-Leu-Asp-Ala-Phe-Ser-Ala-Asp-Glu-Leu-Lys-Lys-Leu-Phe-Lys was prepared by using SPPS and split off from the support with hydrogen fluoride; analytical data for the crude product are given in Table I. Special attention is drawn to the high value for Lys and the low value for Val.

Procedure

A portion (100 mg) of the crude Cys-Met-hexadecapeptide, which, according to amino acid analysis, contained 21 μmoles of Cys (Table I), was dissolved in 3 ml of 6 *M* guanidine hydrochloride containing 1 m*M* EDTA, and the pH was adjusted to 8.5 by the addition of 2 *M* Tris. Nitrogen was passed through the solution for several

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TABLE I

AMINO ACID ANALYSES*

Amino acid (theory)	*Crude peptide*	*After gel-filtration*	*After covalent chromatography*		
			*A***	*C***	*D***
Lys (3)	3.50	3.64	3.58	2.81	2.97
Phe (2)	2.13	1.94	1.90	2.09	1.94
Leu (3)	3.31	3.08	3.14	2.58	3.08
Glu (1)	1.07	1.09	1.12	1.32	0.99
Asp (2)	1.97	2.03	1.78	1.93	2.07
Ala (2)	1.88	1.97	1.77	2.01	1.97
Ser (1)	0.93	0.95	0.88	0.99	1.00
Gly (1)	0.94	1.04	0.82	1.56	1.04
Val (1)	0.79	0.99	0.59	1.16	0.99
Met (1)	0.78***	0.97***	0.44***	0.90***	0.97***
Cys (1)	0.70§	0.58§	0.25§	0.64§	0.99§
Peptide (μmoles)	31.1	28.0	9.9	4.2	10.3
Cys (μmoles)	21.1	16.2	2.8	2.7	10.2

* After oxidation with performic acid followed by hydrolysis in evacuated tubes for 24 h at 110° with 6 *M* HCl containing 1% (w/v) of phenol.

** Analyses were performed on the de-salted fractions indicated in Fig. 1.

*** Determined as methionine sulphone.

§ Determined as cysteic acid.

minutes, then dithioerythritol [DTE; 194 mg (1.3 mmole)] was added and allowed to react in a nitrogen atmosphere for 24 h. The peptide was desalted by gel filtration on Sephadex G-10 (1.6 × 90 cm) in 0.1 *M* ammonium acetate, pH 5.0, containing 1 m*M* EDTA and 0.5 m*M* DTE. The flow-rate was 65 ml/h and 3-ml fractions were collected and kept in closed tubes under nitrogen. Those fractions (19 to 25) corresponding to the void-volume peak were combined and immediately passed at 12 ml/h through a column (1.6 × 4.5 cm) of Thiopropyl-Sepharose 6B (prepared from 2.0 g of freeze-dried material and equilibrated with 0.1 *M* ammonium acetate–1 m*M* EDTA (pH 5.0) (Fig. 1). The column was washed with 50–150 ml each of the following buffers:

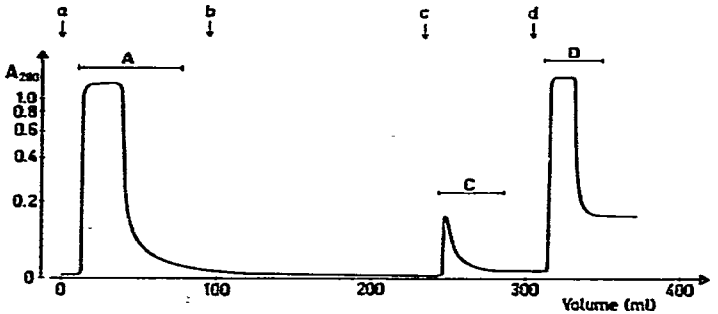

Fig. 1. Covalent chromatography of reduced and de-salted Cys-Met-hexadecapeptide on a Thiopropyl-Sepharose 6B column (1.6 × 4.5 cm). Elution was performed at 12 ml/h with (a) 0.1 *M* ammonium acetate–1 m*M* EDTA (pH 5.0), (b) 0.1 *M* sodium acetate–0.3 *M* sodium chloride–1 m*M* EDTA (pH 5.0), (c) 0.1 *M* ammonium acetate (pH 8.0), and (d) 0.1 *M* ammonium acetate–20 m*M* DTE (pH 8.0).

(a) 0.1 *M* ammonium acetate–1 m*M* EDTA (pH 5.0); (b) 0.1 *M* sodium acetate–0.3 *M* sodium chloride–1 m*M* EDTA (pH 5.0); and (c) 0.1 *M* ammonium acetate (pH 8.0). The unretarded material (*A*), and that released by washing at pH 8 (*C*), was recovered by freeze-drying the corresponding fractions and then de-salted on Sephadex G-10 (1.6 × 30 cm) in 0.1 *M* ammonium acetate (pH 5.0). The covalently bound peptide was desorbed from the Thiopropyl-Sepharose 6B column by elution with (d) 0.1 *M* ammonium acetate (pH 8.0) containing 20 m*M* DTE, 4-ml fractions being collected at a flow-rate of 12 ml/h. Those fractions containing peptide material (*D*) were combined and de-salted as above to yield 23 mg of purified product, which contained 10 μmoles of Cys.

RESULTS AND DISCUSSION

In SPPS, terminated peptides are sometimes formed in unacceptable quantities; the reason for this may be steric as well as chemical. In practice, however, it seems as if a number of peptide chains in the polymer permanently stop growing at different stages during a synthesis. The strategy mentioned above involves the attachment of an affinity label to the normal (still growing) chains, thus allowing them to be selectively distinguished from the terminated ones. As affinity label, Cys-Met has so far been used, the reason for this choice being the possibility of exploiting the special affinity of the thiol group for mercury and the susceptibility of methionyl bonds to specific cleavage by cyanogen bromide to furnish the desired peptide. In this work, Cys-Met was also chosen for similar reasons.

Inspection of Table I, which summarizes the results of amino acid analyses, reveals that the composition of the desorbed peptide is close to that expected. The material released from the column by washing with pH 8 buffer and the unretarded material both contained peptide. The latter fraction was reduced with DTE, gel-filtered, and passed through Thiopropyl-Sepharose 6B as described above. No material was adsorbed to the column in this case, suggesting the presence of only non-reducible thiol derivatives, probably as a consequence of incomplete deprotection in the hydrogen fluoride step. The striking drop in cysteine content that accompanies the first gel-filtration can be explained by the presence of non-covalently bound Cys in the crude peptide. Boc-Cys(Bz1OMe) was the amino acid residue introduced in the last coupling step, and it is plausible that excess of reagent (and/or its side products) were so strongly adsorbed to the matrix as to preclude their complete removal in the ensuing washing procedure. It should be emphasized that virtually no material is irreversibly lost during covalent chromatography as applied here. Of the 16.2 μmoles of Cys-containing peptide applied to the Thiopropyl-Sepharose 6B column, 15.7 μmoles are recovered in different fractions of the effluent.

Similar, but even more striking, results were obtained when a different (poorer) crude peptide preparation was treated under identical experimental conditions.

As an affinity receptor, Thiopropyl-Sepharose 6B compares favourably with organomercurial agarose; the higher binding capacity of Thiopropyl-Sepharose 6B permits the use of small columns and prevents undue dilution of bound peptide in the desorption step. Desorption can be effected under milder conditions (20 m*M* DTE *vs.* 0.4 *M* mercaptoethanol). The release of pyridin-2-thione can be utilized to monitor the coupling reaction spectrophotometrically. Thiol–disulphide interchange as applied

in the present approach is compatible with acetamidomethyl protection of cysteine side chains. On the other hand, this blocking group is removed by mercuric agents.

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