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Oriented immobilization of peptide ligands on solid supports

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ABSTRACT

A synthetic procedure was developed for the direct immobilization on preactivated affinity supports of peptidic ligands requiring free α-amino groups to recognize their targets properly. The peptidic ligand is assembled by solid-phase peptide synthesis on an octa-branched heptalysine core through a polyglycine spacer, similarly to the method developed for the production of multiple antigenic peptides. After deblocking from the resin, peptide is dialysed, lyophylized and used directly for coupling to preactivated supports. Following immobilization, only a limited number of peptide chains are covalently linked to the solid phase, leaving the remainder facing the mobile phase and sufficiently spaced to interact properly. This procedure was applied successfully to the design, synthesis and oriented immobilization of a multimeric tripeptide ligand (Met–Tyr–Phe) for affinity purification of bovine neurophysin.

INTRODUCTION

Retention of the molecular recognition properties of immobilized ligands requires their orientation on the solid phase [1,2]. A vast number of peptidic ligands used for polypeptide purification need complete accessibility of the α-amino groups, often involved in stabilizing interactions in the forming complex [3]. In these instances, to achieve full retention of recognition properties, is necessary to immobilize the peptidic ligand through its C-terminal carboxyl group, following an often laborious route which requires reversible protection of all the ligand amino groups, activation of the ligand C-terminal carboxyl group with usually the formation of an active ester, coupling to an amino group-derivatized solid support and removal of the α-amino protecting groups. If the peptide ligand bears other carboxyl groups in the side-chains, the activation cannot be directed towards a specific group and the peptide will be randomly immobilized through all the activatable carboxyl groups. A procedure has been developed to overcome these problems, based on the direct synthesis of the peptide ligand in a multimeric form starting from a polydentate lysinic core, similarly to the syntesis of multiple antigenic peptides for antisera production [4]. This procedure has been applied to the design, synthesis and immobilization of a tripeptide Met–Tyr–Phe, able to associate bovine neurophysins non-covalently [3,5]. Neurophysins are a class of small (10 000 dalton) neuroendocrine proteins which form multimolecular non-covalent complexes with the neurohypophysial peptide hormones oxitocin and vasopressin, and also di- and tripeptide analogues of the α-amino terminal sequence of the hormones [6]. Peptides containing only the first three residues of the hormones contribute almost two thirds of the binding free energy of the natural hormones, and binding interactions at the side-chain in position 1 appear to be hydrophobic and an aromatic side-chain in position 2 is necessary for binding [7]. Removal of the free α-amino group in position 1 by substitution with H or by derivatization diminishes binding to neurophysins by a factor of at least 100. The requirement of availability of the peptidyl α-amino group precluded the non-directed coupling of peptides, hormones and analogues to solid supports for affinity column preparation [8].

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EXPERIMENTAL

Materials

9-Fluorenylmethoxycarbonyl (Fmoc)-derivatized amino acids and 4-hydroxymethylphenoxyacetic (HMP) resin for solid-phase peptide synthesis were purchased from Novabiochem (Laufelfingen, Switzerland). High-performance liquid chromatographic (HPLC)-grade, dichloromethane (DCM), N-methylpyrrolidone (NMP), methanol, water and acetonitrile were purchased from Merck (Darmstadt, Germany) and trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA). Reagents used as scavengers during deblocking of peptides from resins, such as phenol, thioanisole and ethanedithiol, were obtained from Aldrich (Milan, Italy). Eupergit C 30N, an acrylic support for affinity chromatography preactivated with epoxy groups, was obtained from Rohm (Weiterstadt, Germany). Glass affinity columns used for packing peptide derivatized support were purchased from Omni (Cambridge, UK). Freeze-dried bovine posterior pituitary glands were obtained from Peel-Freeze (Roger, AR, USA).

Solid-phase synthesis

Octameric MYFGGGGG ligand (8[MYF-G_5] (MYF = Met–Tyr–Phe) was synthesized by solid-phase peptide synthesis following the Fmoc methodology [9] on a fully automated Model 431 A Applied Biosystems synthesis, software version 1:1.

The solvent systems for dissolving derivatized amino acids were NMP–DCM mixtures and peptide chains were assembled from subsequent coupling of dicyclohexylcarbodiimide (DCC)–hydroxybenzotriazole-activated amino acids on HMP resin. The attachment of the first amino acid, glycine, to the resin was accomplished automatically using 4-dimethylaminopyridine as catalyst. On the Gly–HMP resin, three subsequent coupling cycles of Fmoc-Lys(Fmoc) were performed, and then the MYFGGGGG chain was assembled. After completion of the synthesis cycles, the peptide resin was dried overnight under vacuum. Peptide was cleaved from the resin (200 mg) using 10 ml of TFA–phenol–thioanisole–ethanedithiol (95:2:2:1) and incubating at room temperature for 1.5 h. The mixture was then filtered and vacuum-concentrated to 1 ml and peptidic material was precipitated by adding 10 ml of cold diethyl ether. The collected precipitate was dissolved in 20 ml of 0.1 *M* acetate acid, extensively dialysed against 0.1 *M* acetic acid and lyophilized. The peptide 8[G_5], structurally identical with 8[MYF-G_5] except for the first three MYF N-terminal residues, was synthesized and purified similarly as a control.

Affinity column preparation

Immobilization of 8[MYF-G_5] on Eupergit C 30N was carried out by incubating 10 mg of peptide dissolved 10 ml of 0.1 *M* $NaHCO_3$–0.5 *M* NaCl (pH 8.5) with 1.0 g of support with agitation for 24 h. The extent of peptide incorporation was followed by reversed-phase (RP) HPLC, indicating that 95% of the starting peptide was support bound. Subsequently, the resin was repeatedly washed with 0.1 *M* Tris (pH 8.5) and slurry packed on an 80 × 6.6 mm I.D. glass column. In a similar way were prepared control columns, incubating 1.0 g of Eupergit C 30N with 10 ml of 0.5 *M* Tris (pH 8.5) or 10 mg of MYF-NH_2 or 10 mg of 8[G_5].

Determination of binding constants

Dissociation constants for the interaction between immobilized 8[MYF-G_5] and soluble neurophysin (NP) were determined by competitive elution experiments [10,11]. The extent of retardation of the NP elution volume on the (8[MYF-G_5])Eupergit column was measured in the presence of increasing amounts of MYF-NH_2 dissolved in the elution buffer. Data were plotted according to the equation

$$\frac{1}{V - V_0} = \frac{K_{M/P}}{M_T} + \frac{[L]K_{P/L}}{M_T K_{M/P}} \quad (1)$$

where V is the elution volume of NP, V_0 the column void volume, $K_{M/P}$ the dissociation constant for the interaction between immobilized 8[MYF-G_5] and soluble NP, [L] the concentration of MYF-NH_2 in the buffer, $K_{P/L}$ the dissociation constant for the interaction between soluble NP and soluble MYF-NH_2 and M_T the total amount of immobilized 8[MYF-G_5].

Amino acid analysis

The amino acid composition of synthetic peptides, peptide fragments obtained by neurophysin proteolytic digestion and the peptide loading of de-

rivatized supports were evaluated by RP-HPLC analysis of Fmoc-derivatized acid hydrolysates [12].

Purification of NP II from bovine pituitary glands

Crude neurophysin mixtures were obtained from freeze-dried bovine pituitaries by acid extraction, sodium chloride precipitation and gel filtration on Sephadex G-25 in 1 *M* formic acid [13]. Isolation of bovine neurophysin II isoforms from mixtures of neurophysin I and other non-NP-related peptides was achieved by ion-exchange chromatography on a DEAE-Trisacryl column (70 × 2.4 cm I.D.) using a linear gradient from 0.1 *M* (1 l) to 0.5 *M* (1 l) ammonium acetate (pH 6.0). The identity of the purified neurophysin II was confirmed by amino acid analysis and tryptic mapping of performic acid-oxidized material, under the conditions described previously [14]. In detail, samples of neurophysin II (100 μg) were treated with 100 μl of performic acid, incubated for 3 h at 4°C and then diluted with water and lyophilized. Trypsin digestion was performed by incubating the performic acid-oxidized protein with 2% L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK) trypsin for 2 h at 37°C in 0.1 *M* NH_4HCO_3 (pH 8.6). At the end the mixture was lyophilized and subjected to RP-HPLC analysis. Fragments corresponding to neurophysin II residues 1–8, 9–18, 21–43, 44–66 and 67–93, purified by RP-HPLC, were identified by amino acid analysis.

High-performance electrophoresis

Determination of the molecular weight of 8[MYF-G_5] was carried out by high-performance electrophoresis on an ABI Model 230A instrument using a 50 × 2.5 mm I.D. 12% polyacrylamide gel tube. Gels, prepared according to the manufacturer's instructions in the native and denaturing form, were run at 1.5 mA, and the effluent was monitored by measuring the UV absorbance at 225 nm. Under these conditions, the multimeric peptide eluted after 135 min, bromophenol blue (700 dalton) after 50 min, neurophysin (10 000 dalton) after 120 min and α-lactalbumin (14 200 dalton) after 130 min.

Thermolysin treatment of 8[MYF-G_5] and (8[MYF-G_5])Eupergit

Samples of 8[MYF-G_5] (100 μg) and (8[MYF-G_5])Eupergit (50 mg) were treated with 0.1 μg of thermolysin (TLN) in 1 ml of 0.1 *M* Tris (pH 7.0). The mixtures were incubated for 3 h at room temperature under agitation. Subsequently, aliquots of 100 μl were analysed by RP-HPLC and compared with the digestion products of MYF-NH_2 (50 μg) obtained by similar treatment.

RESULTS

Design, synthesis and characterisation of 8[MYF-G_5]

The tripeptide vasopressin analogue Met–Tyr–Phe was synthesized starting from an octadentate branching heptalysine core, obtained after three subsequent couplings of Fmoc–Lys(Fmoc) on a Gly–HMP resin. A pentaglycine spacer was introduced before the MYF sequence to provide augmented accessibility of the tripeptide for NP interaction. The structure of the resulting product, denoted 8[MYF-G_5], is shown in Fig. 1. After cleavage from the resin, peptide was purified by dialysis against 0.1 *M* acetic acid and lyophilized. The purified material gave a satisfactory amino acid analysis (M 6.9, Y 6.5, F 7.4, G 41.3, K 7.2). RP-HPLC analysis of dialysed material provided a non-homogeneous profile (Fig. 2, top), probably indicative of some deletions occurred during synthesis, caused by the steric hindrance created by the chains growing in close proximity from the polylysinic core. The non-homogeneity of the elution profile could also be enhanced by non-homogeneous types of interac-

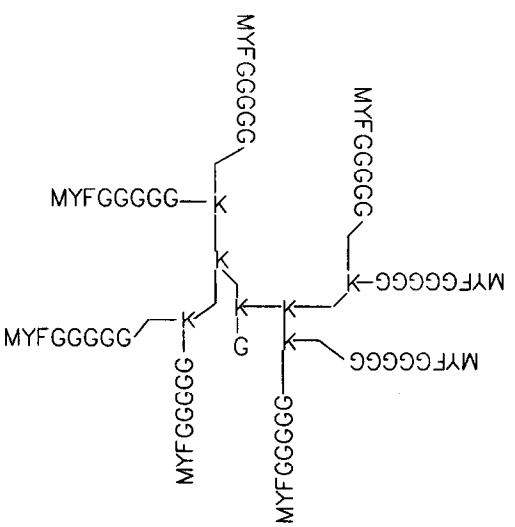

Fig. 1. Structure of 8[MYF-G_5].

tions of the branched peptide with the stationary phase of the reversed-phase column (C_4, 300 Å pore size). Determination of the molecular weight of 8[MYF-G_5] on a calibrated gel filtration column (Fig. 2, bottom) provided a value higher than the expected theoretical value (8000 dalton). The same behaviour was observed in gel electrophoresis under denaturing and non-denaturing conditions. This effect could be explained by a larger molecular volume filled by the 8[MYF-G_5] molecule compared with that occupied by a folded protein of the same molecular weight. Incorporation of the tripeptide at the head of the pentaglycine octamer was also confirmed comparing the digestion products of 8[MYF-G_5] and MYF-NH_2 treated with TLN (Fig. 3). Under the same proteolysis conditions, the dipeptide MY-COOH is obtained in both instances, indicating that the tripeptide MYF is correctly assembled in 8[MYF-G_5] after the polyglycine spacer.

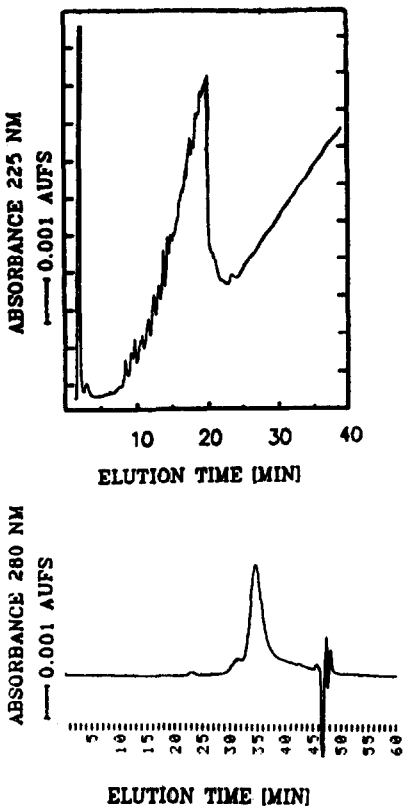

Fig. 2. Chromatographic characterization of 8[MYF-G_5]. Top: RP-HPLC profile of dialysed 8[MYF-G_5]. The sample (50 μg) was eluted on a Brownlee Labs. C_4 RP-HPLC column (50 × 3 mm I.D.) equilibrated at a flow-rate of 0.5 ml/min with acetonitrile–water–(5:95:0.1), with a linear gradient of acetonitrile from 5% to 60% in 30 min. The effluent was monitored at 225 nm (0.1 a.u.f.s.). Bottom: gel filtration profile of dialysed 8[MYF-G_5]. The sample (100 μg) was eluted on a TSK-SW 2000 gel filtration column (300 × 4.6 mm I.D.) equilibrated at a flow-rate of 0.5 ml/min with 0.1 *M* acetic acid. The effluent was monitored at 280 nm (0.01 a.u.f.s.).

Recognition properties of immobilized 8[MYF-G_5]

The binding characteristics of immobilized 8[MYF-G_5] were first evaluated by zonal and competitive elutions of ion-exchange chromatographically purified NP on the (8[MYF-G_5]) Eupergit high-performance liquid affinity chromatography (HPLAC) column. A small amount of NP (100 μg) was applied to the column equilibrated at a flow-rate of 1 ml/min with 0.1 *M* ammonium acetate (pH 5.7), monitoring the effluent at 280 nm, and after 25

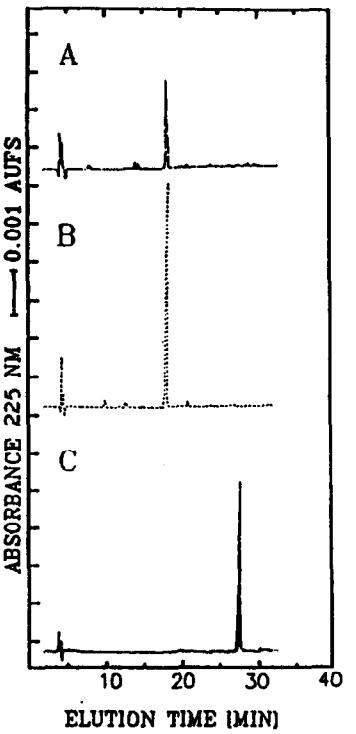

Fig. 3. Thermolysin proteolytic degradation of 8[MYF-G_5]. RP-HPLC profile of (C) MYF-NH_2, (B) TLN-treated MYF-NH_2 and (A) TLN-treated 8[MYF-G_5] obtained on a Zorbax C_{18} column (250 × 4.6 mm I.D.) using a linear gradient from 5 to 50% acetonitrile in 30 min. Eupergit-immobilized 8[MYF-G_5] after TLN treatment provided the same peptide fragment as in chromatogram A.

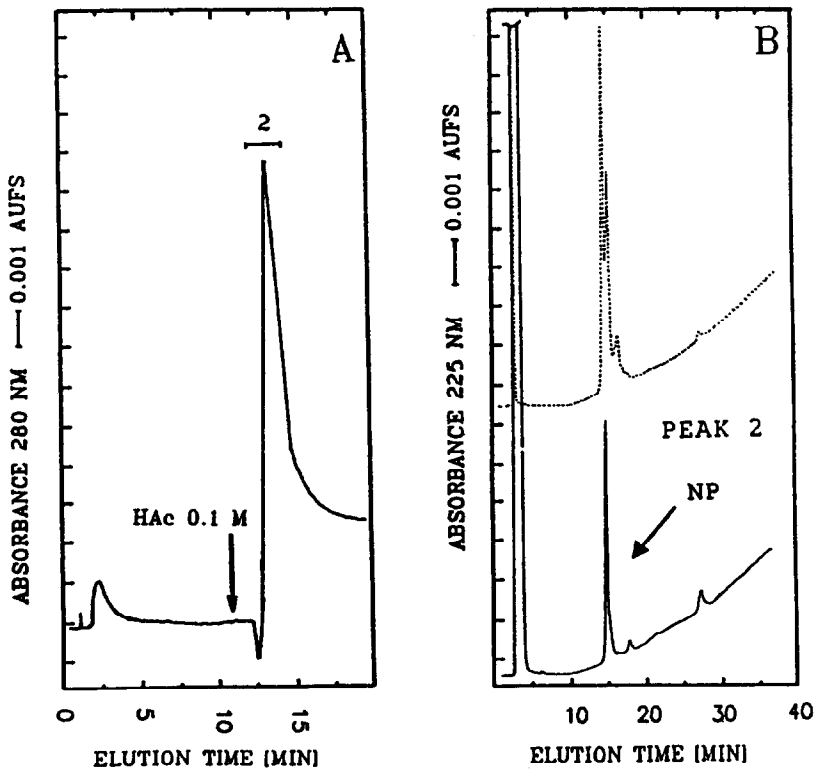

Fig. 4. Binding of partially purified NP to (8[MYF-G_5])Eupergit. (A) HPLAC profile of 100 μg of NP obtained after ion-exchange chromatography on the (8[MYF-G_5])Eupergit column. The column was equilibrated at a flow-rate of 1.0 ml/min with 0.2 *M* ammonium acetate (pH 5.7). At the position indicated by the arrow, the eluent was changed to 0.1 *M* acetic acid. Eluted material, denoted peak 2, was collected for RP-HPLC analysis. (B) RP-HPLC analysis of 50 μg of NP after the ion-exchange chromatographic step (top) and HPLAC-purified peak 2 (bottom).

min the eluent was changed to 0.1 *M* acetic acid (Fig. 4A). Bound material, denoted peak 2, was collected and analysed by RP-HPLC (Fig. 4B, bottom) and compared with the starting material (Fig. 4B, top). The affinity column retained NP almost quantitatively (90%) and allowed the removal of closely eluting RP-HPLC contaminants. Under the identical elution conditions and column geometry, a (Tris)Eupergit column failed to retain any NP sample applied (Fig. 5A). Similarly, columns of the same overall dimensions prepared by immobilizing MYF-NH_2, or 8[G_5], similar in structure to 8[MYF-G_5] but missing the N-terminal tripeptide MYF, did not retain NP under the conditions tested (Fig. 5B and C). Further, treatment of Eupergit-immobilized 8[MYF-G_5] with thermolysin released the MY-NH_2 dipeptide in solution, as reported in Fig. 3, providing clear evidence that not all the chains are covalently bound to the support. Overall, these results indicate (a) that the interaction between NP and 8[MYF-G_5] occurs through the N-terminal tripeptide moiety MYF, and not with the polyglycine core, (b) that the interaction with the tripeptide occurs only if the α-amino group is not covalently linked to the support, and consequently (c) that not all of the eight peptide chains in 8[MYF-G_5] are covalently linked to the solid support, and some of them face the mobile phase and can interact properly with NP.

Further evidence of interaction specificity was shown by the competitive effect of MYF-NH_2 dissolved in the elution buffer. According to eqn. 1, in this situation the elution volume of NP should be reduced proportionally to the amount of MYF-NH_2 in the buffer (Fig. 6). The same amount of NP (50 μg) was applied to the column equilibrated with

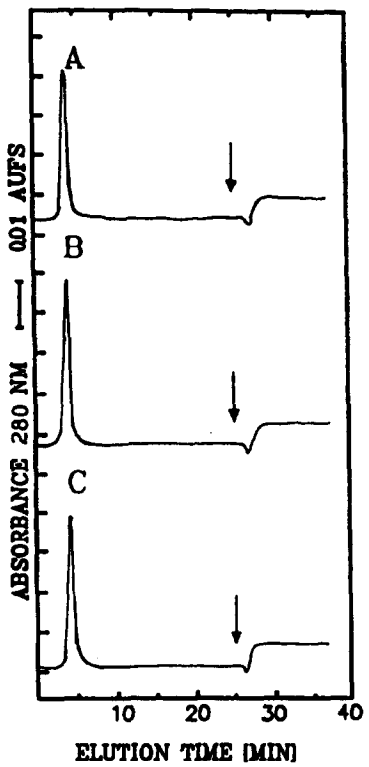

Fig. 5. Elution profile of ion-exchange purified NP on control columns. HPLAC profiles of 100 μg of NP on (A) [MYF-NH_2]Eupergit, (B) [Tris]Eupergit and (C) (8[MYF-G_5])Eupergit. Columns were equilibrated at a flow-rate of 1.0 ml/min with 0.2 *M* ammonium acetate (pH 5.7). At the positions indicated by the arrows, the eluent was changed to 0.1 *M* acetic acid.

0.1 *M* ammonium acetate (pH 5.7) and increasing amounts of MYF-NH_2, and data plotted according to eqn. 1 allowed the determination of dissociation constants for immobilized 8[MYF-G_5]–soluble NP ($K_{M/P}$ = 3.1 · 10^{-4} *M*) and soluble NP–soluble MYF-NH_2 ($K_{P/L}$ = 6.1 · 10^{-4} *M*) interactions. The amount of immobilized MYF-G_5 (M_T = 30 μmol) used for calculations of dissociation constants was determined by quantitative amino acid analysis of the derivatized support, assuming 8[MYF-G_5] to be octavalent, and does not take into account the unknown number of chains covalently linked to the solid support and thus not available for the interaction. The correct dissociation constant values can range from *ca.* 2.7 · 10^{-4} *M*, for only one chain linked to the solid phase (M_T = 26 μmol), to 0.4 · 10^{-4} *M*, for seven chains linked to the solid phase (M_T = 4 μmol). The close agreement of the dissociation constant values determined by competitive elution experiments with the value obtained fully in solution by other methods (K_{DISS} = 1.3 · 10^{-4} *M*) [2], and by analytical affinity chromatography on Met–Tyr–Phe–Affi-Gel 102 [13] (K_{DISS} = 1.2 · 10^{-4} *M*) suggest that immobilized 8[MYF-G_5] retains similar binding characteristics to the tripeptide MYF-NH_2 in solution.

Further, the multi-branched ligand recognizes NP probably as a monomer, because NP, able to self-associate in solution, in its dimeric form binds the hormone analogue MYF-NH_2 with a one order

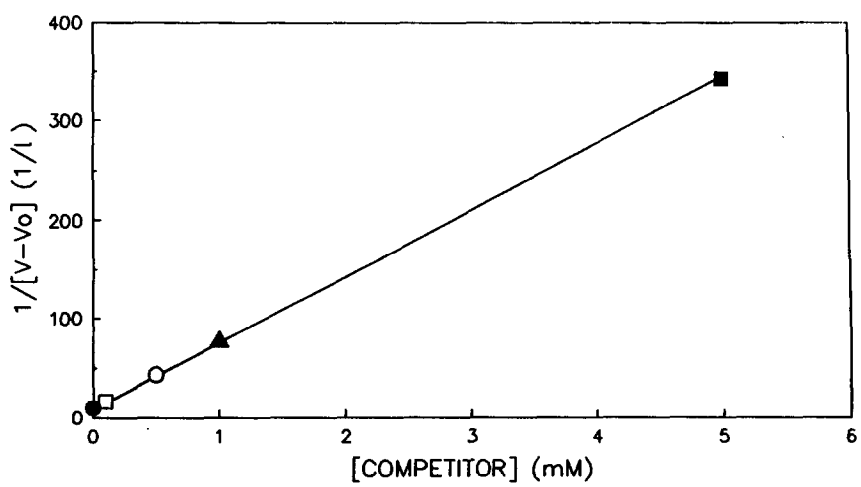

Fig. 6. Competitive elution of NP on the (8[MYF-G_5])Eupergit column. The column was equilibrated with 0.2 *M* ammonium acetate (pH 5.7) containing different amounts of MYF-NH_2 and 50 μg of NP were applied, monitoring the effluent at 280 nm. The dependence of NP retardation on MYF-NH_2 concentration in the elution buffer is plotted according to eqn. 1.

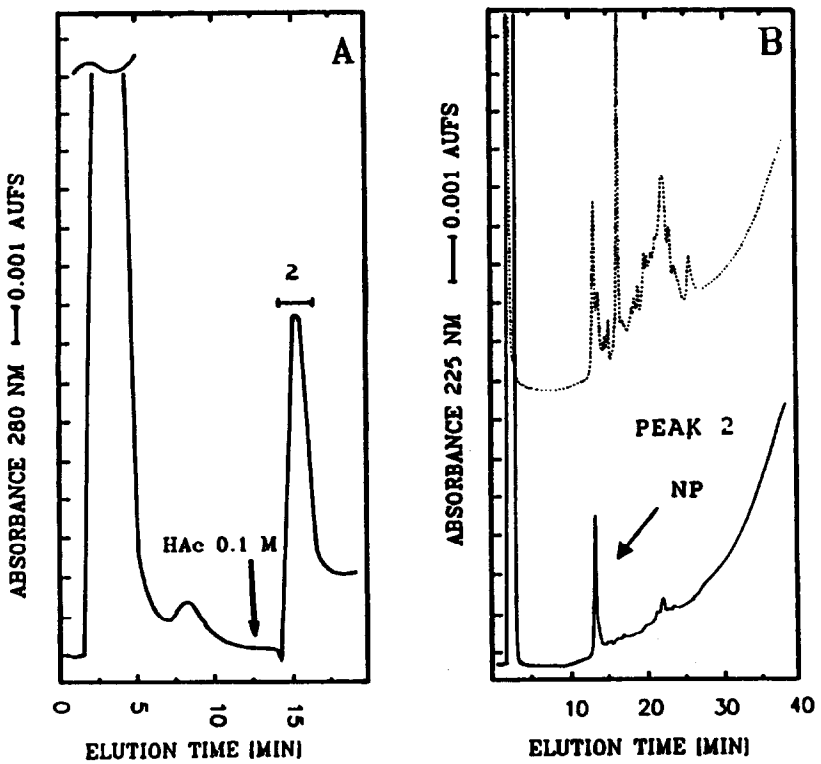

Fig. 7. HPLAC purification of NP from crude extracts. (A) HPLAC profile of crude extract applied to the (8[MYF-G_5])Eupergit column, equilibrated with 0.2 *M* ammonium acetate (pH 5.7). At the position indicated by the arrow, the eluent was changed to 0.1 *M* acetic acid. Eluted material, denoted peak 2, was collected for RP-HPLC analysis. (B) RP-HPLC analysis of crude extract (top) and HPLAC-purified peak 2 (bottom).

of magnitude higher affinity than monomeric NP [15].

Purification of NP from crude extract

To examine the selectivity of immobilized 8[MYF-G_5]–NP recognition, a crude protein mixture (1 mg/ml total proteins) was prepared combining together different *Escherichia coli* extracts, spiked with a small amount of NP (50 μg/ml), and applied to the 8[MYF-G_5]Eupergit column. After elution of unbound material, the eluent was changed to 0.1 *M* acetic acid (Fig. 7A), and bound material, denoted peak 2, was collected for RP-HPLC analysis (Fig. 7B, bottom) and compared with the crude starting material (Fig. 7B, top). The overall recovery was close to 90%, and most contaminants were efficiently removed. The same column has been used for more than 30 runs without any apparent decrease in capacity.

DISCUSSION

The non-directed coupling of neurohypophysial peptide hormones to solid supports has precluded their interaction with neurophysin, as this process depends on the availability of the peptidyl α-amino groups. This problem has been overcome by the coupling of a reversible acetone-blocked lysine vasopressin to cyanogen bromide-activated agarose and subsequent deprotection [16], and by DCC-mediated incorporation of α-nitrophenylsulphenyl–Met–Tyr–Phe–COOH to aminohexylagarose and subsequent deprotection [17]. Either procedure required complex synthetic steps. In the latter instance, moreover, the introduction of the aminohexyl spacer produced aspecific interactions with neurophysin at low ionic strength. Direct immobilization of branched peptidic ligands on preactivated solid supports may represent an actractive alterna-

tive to C-terminal covalent linkage for oriented coupling. In the immobilized form, multi-branched peptidic ligands are properly oriented for interactions with target molecules requiring ligand-free α-amino groups, and at the same time are sufficiently spaced from the solid support through the entire length of the chains involved in the covalent linkage with the preactivated solid phase. Further, in the case of poorly soluble ligands, the branched polylysinic core can be designed with the incorporation of charged residues, such as Arg or Glu, to improve the solubility of the final product.

Current automated instrumentation for solid-phase peptide synthesis allows an easy and convenient preparation of multimeric ligands in very short time. Preactivated solid supports for ligand immobilization and subsequent column preparation are also widely available.

In the case system analysed here, immobilized 8[MYF-G_5] retained binding properties for neurophysin similarly to carboxyl-terminal immobilized monomeric MYF-COOH. Recognition was selective, as evidenced by the ability of immobilized 8[MYF-G_5] to purifying NP from a crude mixture, and specific, as demonstrated by the competing effect of MYF-NH_2 in the buffer. The absolute values of the dissociation constants determined using competitive elutions on the 8[MYF-G_5] column cannot be very accurate because the exact number of MYF-GY_5 chains covalently linked cannot be precisely calculated, and hence the amount of M_T that is functionally active. In any event, the relative magnitude of $K_{M/P}$ and $K_{P/L}$ is correct, because the same M_T value is used in both calculations. The close similarity of the dissociation constants obtained for the interaction between immobilized 8[MYF-G_5] and soluble NP and between soluble NP and soluble MYF-NH_2 indicates that the presence of the pentaglycine spacer in 8[MYF-G_5] does not noticeably affect the recognition.

The method developed can be easily adapted to other ligands requiring C-terminal immobilization for proper functioning. Spacer length, incorporation of solubilizing residues and a reduced or augmented number of chains linked to the central core can be selected according to the characteristics of the ligand under consideration.

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