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Immobilization of α-human atrial natriuretic peptide on insoluble supports and affinity purification of specific antibodies from a polyclonal goat anti-α-human atrial natriuretic peptide serum

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ABSTRACT

α-Human atrial natriuretic peptide (α-hANP) was covalently coupled via single attachment onto two different insoluble matrices. Controlled-pore glass–α-hANP matrices were well suited for the purification of monospecific antibodies, whereas Enzacryl AA–α-hANP did not withstand the inevitable chemical and physical stresses during affinity purification.

INTRODUCTION

Human atrial natriuretic peptide (hANP) is one of the recently discovered heat-stable vasoactive peptides. It is produced and processed in the cardiac atrium. The main target organs of the peptide hormone are the kidneys, the adrenal cortex, smooth vascular musculature and several regions of the central nervous system. The fragment of interest is α-hANP (28 amino acids), which is derived from the prohormone γ-hANP (126 amino acids) by carboxyterminal proteolytic cleavage. Several other circulating forms have been described[1–3]. The half-life of α-hANP in circulating blood is about 3–5 min[4]; most of the peptide is removed from the bloodstream by the kidneys and the liver[5]. The standard procedure for the determination of α-hANP in plasma or urine is a radioimmunoassay of an extract of urine or EDTA–plasma obtained by use of Sep-Pak C_{18} cartridges[6–9]. Various cross-reactions with its metabolites or degradation products cannot be excluded[9–14].

The use of affinity chromatography or an adapted version thereof should yield

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antibodies of known specificity, to be tested for their applicability in radioimmunoassays[15].

EXPERIMENTAL

Materials

The following were used: α-hANP (CB-PP051041) and [^{125}I]-α-hANP tracer (AW-A3103) (Biomedica, Vienna, Austria); Enzacryl AA 70871 (00-12506/8) (Koch Light, Hatfield, U.K.); controlled-pore glass (CPG) (44741) (Serva, Heidelberg, F.R.G.); RIAzid radioimmunoassay (Henning, Berlin, F.R.G.); EDTA disodium salt, 99% pure (Sigma, Deisenhofen, F.R.G.); BSA (Sigma, St. Louis, MO, U.S.A.); and powdered charcoal, highest purity (2184), Triton X-100, for scintillation measurement, citric acid monohydrate and disodium hydrogenphosphate (Merck, Darmstadt, F.R.G.).

Coupling procedure

Enzacryl AA. Enzacryl AA was activated with 2 *M* hydrochloric acid for 15 min at 4°C, followed by treatment with a 2% sodium nitrite solution for 20 min at 4°C. After washing with 10 m*M* phosphate buffer (pH 7.0) the activated Enzacryl AA was incubated with α-hANP for 48 h. The immobilizate was washed with 10 m*M* sodium acetate buffer (pH 4) to remove unreacted peptide, treated with 3 *M* sodium acetate buffer (pH 4) in order to destroy any unreacted spacers, washed repeatedly with 10 m*M* sodium acetate buffer (pH 4) and stored at 4°C for use (Fig. 1). This matrix is referred to as Enzacryl AA–α-hANP.

Controlled-pore glass (CPG) beads. The CPG surface[16] was treated as follows: 10 g of CPG were incubated with 3% nitric acid for 12 days at room temperature (after degassing) in order to produce free silanol groups. After two washing steps with distilled water, 250 ml of 10% aqueous 3-aminopropyltriethoxysilane were added to

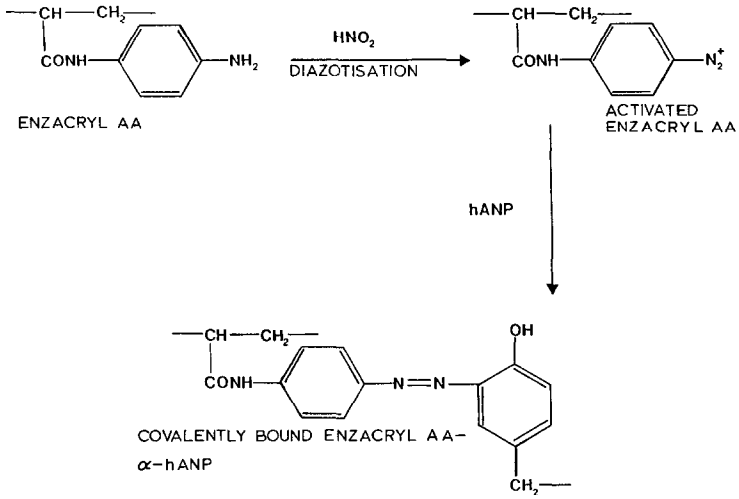

Fig. 1. Diazo coupling of the C-terminal tyrosine of α-hANP to Enzacryl AA.

the support while shaking vigorously. The pH was adjusted to 3.5 using 6 *M* hydrochloric acid and the suspension was evacuated in a water-bath at 75°C for 2 h. The glass beads were filtered off, thoroughly washed with distilled water and dried overnight at 115°C.

The free amino groups introduced by this reaction were used to link glutardialdehyde spacers to the glass beads (Fig. 2). After degassing, the CPG suspension was treated with a 10% glutardialdehyde solution under a fume-hood, rotated at room temperature for 4 h, filtered and washed extensively with ice-cold water. The structure was reduced using aqueous sodium tetrahydroborate (the support is now stable under water at 4°C) prior to diazotisation (Fig. 3) and the peptide hormone covalently bound to the activated support employing reaction conditions analogous to those used with Enzacryl AA. Unreacted sites were saturated by addition of 0.01 *M* hydrochloric acid or a 1:1 mixture of normal goat serum and phosphate-buffered saline (PBS) [without bovine serum albumin (BSA)]. Subsequent treatment was analogous to that for Enzacryl AA.

In addition, a control matrix without α-hANP was prepared. The reaction conditions and post-coupling treatment were identical with those described above with the exception that the buffer solution used for the coupling procedure contained no α-hANP. The control matrix is referred to as CPG and the affinity purification matrix as CPG–α-hANP.

Affinity purification of anti-α-hANP antibodies

Either polyclonal goat anti-α-hANP serum or the antibody fraction thereof

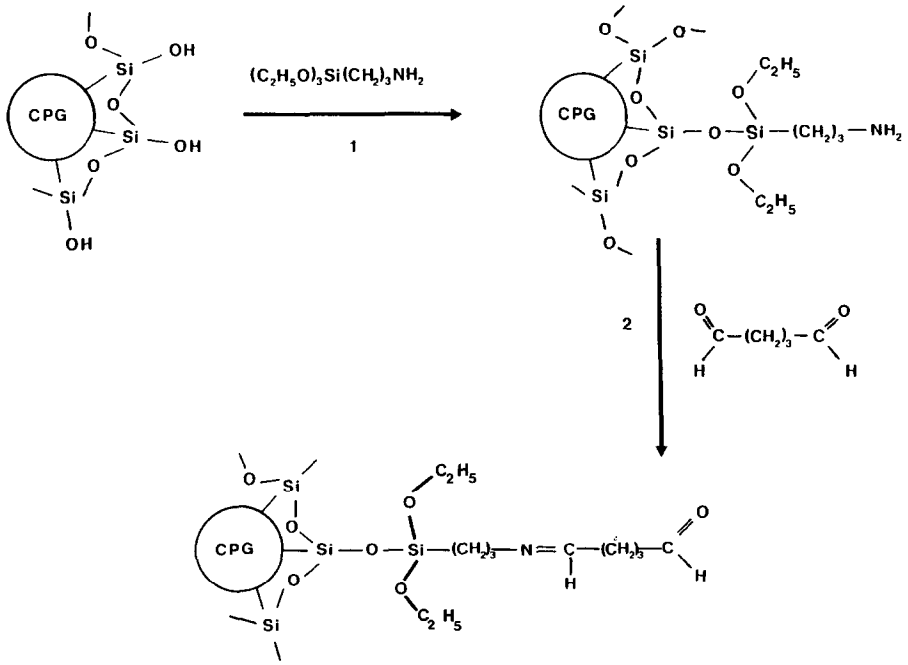

Fig. 2. Derivatization of the active surface silanol groups of CPG using 3-aminopropyltriethoxysilane (1) and the addition of glutardialdehyde to provide a spacer group (2).

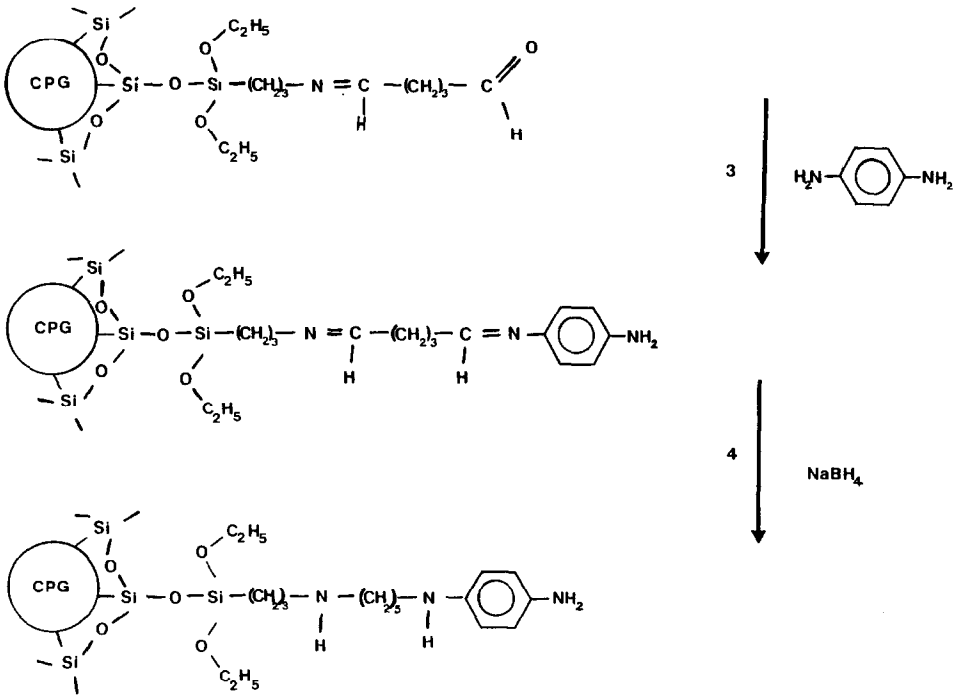

Fig. 3. Processing of the spacer's terminal aldehyde by reaction with 1,4-diaminobenzene (3) and reduction of the carbon–nitrogen double bonds (4), in order to provide a suitably reactive site for the subsequent coupling procedure (Fig. 2).

(obtained by ammonium sulphate precipitation) was used. An adapted version of affinity chromatography was used in this study.

Enzacryl AA–α-hANP. A batch technique was used at 4°C throughout. The antibodies (or antiserum) were applied at pH 7 in Tris–HCl or McIlvaine buffer solutions (McIlvaine-buffer, pH 2.6, 7.0, 7.6: 0.1 *M* citric acid monohydrate–0.2 *M* disodium hydrogenphosphate) and allowed to bind to the matrix. The excess antibodies and other serum components were removed by thorough washing with the loading buffer. Following this, the antibody–immobilized antigen complex was dissociated by use of McIlvaine buffer (pH 2.6). The supernatant was collected in this buffer at pH 7.6; the same buffer was used to neutralize the matrix (Fig. 4).

CPG–α-hANP. Three types of matrices were used: CPG–α-hANP pretreated with dilute hydrochloric acid and the control matrix CPG (see *Coupling procedure*).

The batch technique was carried out at room temperature. Antibodies in PBS (pH 7.4) and also the antiserum were allowed to thaw at 4°C before being applied to the matrix at pH 7. McIlvaine buffer (pH 7) was used to wash out excess antibodies and serum components after the matrix had been allowed to interact with the sample for 1 h. The affinity chromatographic matrix was then washed with PBS (without BSA), in order to remove any unspecifically adsorbed material. McIlvaine buffer (pH 2.6) was used to elute the matrix-bound antibody, which was collected in the same buffer at pH 7.6 (Figs. 5 and 6)[15].

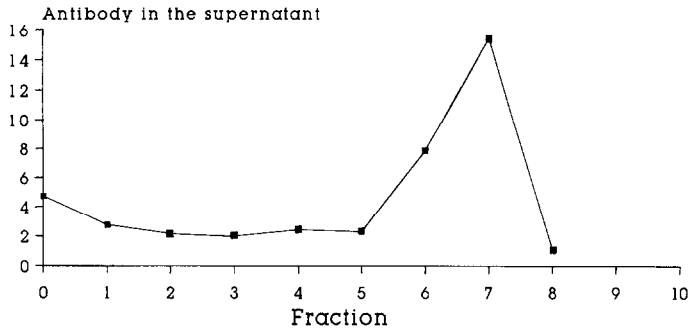

Fig. 4. Batch affinity purification steps employed when using the Enzacryl AA–α-hANP matrices: 0, application of the antibody fraction or antiserum; 1–5, washing with buffer to remove excess of antibodies or other serum components; 6,7, elution of the bound specific antibodies; 8, neutralization of the matrix used.

Treatment of the supernatants obtained by use of the batch technique. The batch technique results in a number of supernatants being collected. An aliquot of each was treated with a constant amount of tracer and subsequently processed as below. A plot of cpm in the supernatant against the fraction number provides a discontinuous elution profile for the affinity purification procedure (see Figs. 4–6).

Determination of antibody titre. A constant amount of [^{125}I]-α-hANP tracer (antigen) was added to a dilution series of the antibody in PBS, mixed well and kept at 4°C overnight (the AB + AG $\rightleftharpoons$ ABAG reaction will have reached equilibrium by then). A 2% (w/v) suspension of charcoal in radioimmunoassay (RIA) buffer [RIA buffer: sodium phosphate buffer (pH 7.4) containing 1 m*M* EDTA (disodium salt), 0.3% BSA and 0.1% Triton X-100] was agitated at 4°C overnight in order to block the macromolecule complex adsorption sites of the charcoal particles. The suspension was centrifuged at 5000 rpm for 15 min, the supernatant discarded and the pellet resuspended in RIA buffer to make a 2% (w/v) suspension. A constant volume of this suspension was used to adsorb the excess of unbound tracer and was centrifuged off as

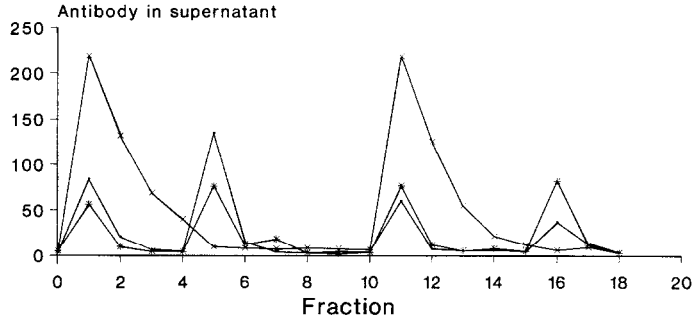

Fig. 5. Batch affinity purification steps employed when using the CPG–α-hANP matrices pretreated with normal goat serum: 1,11, application of the antibody fraction or antiserum; 2–4, 12–15, washing with two buffers to remove excess antibodies or other serum components and any adsorbed material; 5,6, 16,17, elution of the specific anti-α-hANP antibodies bound to the matrix; 7–10,18, neutralization and additional washing of the matrix used. ● = Antibody/CPG–α-hANP; × = antiserum/CPG; ∗ = antiserum/CPG–α-hANP.

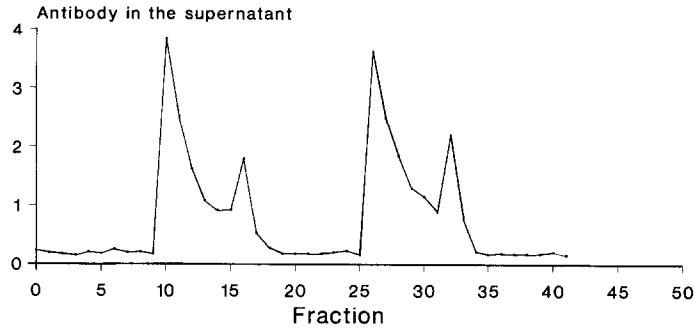

Fig. 6. Batch affinity purification steps employed when using the CPG–α-hANP matrices pretreated with 0.01 *M* HCl: 0–9, 18–25, 34–41, conditioning and neutralization of the matrix before sample application; 10,26, application of the antibody fraction or the antiserum; 11–15, 27–31, washing with buffer in order to remove excess antibodies or other serum components; 16,17, 32,33, elution of the specific anti-α-hANP antibodies bound to the affinity matrix.

above after incubation at 4°C for 30 min. The charcoal-bound excess of tracer in the pellet and the antibody-bound tracer in the supernatant were counted separately in a gamma-counter (Canberra Packard, Model A 5410). The data were plotted as cpm in the supernatant (or pellet) against the dilution (not shown).

RESULTS AND DISCUSSION

An adapted version of affinity chromatography was used throughout this study. Owing to the nature of Enzacryl AA, its use in column form would not have suited our purposes. Chemically modified CPGs exhibit a large available surface area onto which synthetic α-hANP may be immobilized by a stable covalent bond, thus specifically oriented to allow optimum antibody recognition. By use of the batch technique, the small amounts of the prepared affinity chromatographic matrix needed to isolate large amounts of antibodies are more easily handled, with optimum recovery.

The spacer group (aromatic amine) which is already present as an integral part of the Enzacryl AA polymer may be diazotized and coupled to the peptide hormone directly, whereas the CPG beads require treatment in order to be of use. Dilute nitric acid provides the free silanol groups required for the subsequent even coating of the CPG surface by use of 3-aminopropyltetraethoxysilane. This reaction in turn provides the aliphatic amine necessary for the introduction of the spacer, glutardialdehyde. The free aldehyde group obtained reacts with 1,4-diaminobenzene, followed by reduction with sodium tetrahydroborate, thus completing the spacer, while making available the aromatic amine for the peptide coupling procedure (as for Enzacryl AA). The adapted CPGs may be stored indefinitely under water at 4°C before coupling to α-hANP. After removing excess of unreacted peptide, the CPG beads were treated with dilute hydrochloric acid or a 1:1 mixture or normal goat serum and PBS (without BSA) in order to block the residual adsorption sites (mainly unreacted spacers).

To show that the antibodies eluted from the matrix are not simply due to reversible adsorption, a control matrix was prepared by simply neglecting to add any α-hANP to the coupling buffer; all other steps were identical with those for the

production of the CPG–α-hANP matrices. The control matrix was subjected to the same batch procedure as the affinity chromatographic matrices.

As can be seen in Figs. 5 and 6, the serum or antibody fraction applied to the control matrix was washed out quantitatively, with no reversibly adsorbed antibodies being eluted. This implies that the eluate peaks obtained during the batch procedure are due only to specific anti-α-hANP antibodies.

The influence of pH on the loading of the matrix was found to be minimal; the affinity of the antibodies to Enzacryl AA–α-hANP suffices to resist several (up to seven) washes with buffers of different ionic strength at pH 7. Elution of the antibodies was performed at pH 2.6. It is necessary to neutralize the eluted antibodies immediately in order to avoid their denaturation (Fig. 4).

Enzacryl AA–α-hANP was found to be suitable for the storage of the specific anti-α-hANP antibodies at 4°C over a long period of time. This was achieved by simply interrupting the batch procedure before the elution step. These highly purified antibodies would normally be very unstable at such temperatures without a stabilizer, but their biological activity proved to be intact on elution after a 4-month storage period; the antibodies may be eluted as required.

Unfortunately, the Enzacryl AA matrix tended to produce large amounts of fines during the washing and elution procedure, rendering its re-use impossible. Therefore, CPG beads were tested. This immobilizate showed no problems during repeated adsorption and desorption. No decrease in capacity was observed after re-use (Figs. 5 and 6). The antibody titres[15] found were dilutions of 1:4096, 1:8192 and 1:600 for goat anti-α-hANP, antibody fraction of goat anti-α-hANP and antibody eluate sources, respectively.

The prepared CPG–α-hANP matrix was found to be ideal for the isolation of biologically active anti-α-hANP-antibodies. The batch purification was easy to handle, clean and efficient. The matrix should prove suitable for use in a column, *i.e.*, for classical affinity chromatography.

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