A Simple Method to Prepare Affinity Resins on Cellulose Basis

Karl Eisele, Fernando Dias Costa, Carlos Pascual, and Beatus Ofenloch-Hähnle

Physiologisch-chemisches Institut der Universität, D-7400 Tübingen, Hoppe-Seylerstr. 1, Bundesrepublik Deutschland

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Affinity resins with different spacer arms were synthesized analogues to the solid phase peptide synthesis using aminoethyl cellulose or carboxymethyl cellulose as matrix. The spacer arms could be varied in length and rigidity. Especially spacer arms consisting of poly amino acids can be synthesized with a defined amount of amino acid residues specifically in the low molecular weight ranges. The method is also applicable to other matrices which are not susceptible to 1 N HCl in glacial acetic acid, trethylamine, methylene chloride and dimethylformamide.

The synthesis of affinity resins with different spacer arms for the purification of androgen receptors is described as an example of the method.

Introduction

There are a number of methods described how to prepare affinity resins. The activation of the matrix is usually done by cyanogen bromide or by epoxydation [1–3]. The cyanogen bromide activated matrix has the disadvantage that the spacer arm or the ligand will be attached to the matrix *via* an isourea linkage, which is not very stable. Cyanogen bromide activation is accompanied by a crosslinking process of the matrix (*e.g.* using Sepharose), which is not always desired. In addition to this crosslinking imidocarbonates are still active bonds which behave like a time bomb and may react during chromatography. As to epoxy activated matrices, they have stable linkages to the spacer arm or to the ligand, but the rigidity of the spacer arm is not adjustable, because the usually used 1,4-bis-(2,3-epoxypropoxy)-butane is already a chain consisting of 7 carbon atoms and 3 oxygen atoms. This chain is not as rigid as sometimes necessary. Furtheron, spacer arms consisting of poly amino acids (*e.g.* poly lysine etc.) cannot be prepared in a defined length because the poly amino acids have to be synthesized separately by N-carboxyanhydride reaction (which yields a mixture of poly amino acid chains of different chain length). With the method described here, the chain length is well defined, especially in the low molecular weight range. What is said here about epoxy activated matrices is also true for AH- and CH-sepharose.

To find an affinity resin for a special isolation problem, the ligand, length and kind of spacer and the nature of the matrix are the variable parameters to gain the optimum resin.

The variations of the spacer arm can be done best in a simple and effective way with a modified technique of the solid phase peptide synthesis [4, 5] by the use of aminoethyl cellulose of carboxymethyl cellulose or other gels as matrix. The only prerequisites for application of the method described here is that the matrix which has been used is not susceptible to 1 N HCl in glacial acetic acid, triethylamine or dimethylformamide and that it bears amino- or carboxylic groups. The bridges linking the amino- or carboxylic groups to the molecule which forms the matrix-backbone should consist of less than three atoms (Fig. 1). To give an example of the method we present the synthesis of three affinity resins with different spacers for the purification of androgen receptors.

Materials and Methods

Aminoethyl cellulose (Servacel AE p.a.) with a capacity of 0.31 meq/g (Serva, Heidelberg, FRG), carboxymethyl cellulose with a capacity of 0.6 meq/g (Sigma, München, FRG). [1-^{14}C]acetic anhydride and [1,2,6,7,16,17-^{3}H]testosterone (New England Nuclear, Dreieich, FRG). All other reagents used were of analytical grade and purchased from E. Merck, Darmstadt, FRG, and from Serva, Heidelberg, FRG.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; BOC, *tert*-butyloxycarbonyl-; AUA, ω-aminoundecanoic acid.

Reprint requests to Prof. Dr. Eisele.

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PEM-buffer: 10 mM potassium phosphate, pH 7.4 containing 2 mM EDTA, 2 mM 2-mercaptoethanol.

DCC: dextran coated charcoal; 4% acid washed charcoal, 0.1% dextran 60 and 0.1% gelatine in PEM-buffer.

Protected amino acids

BOC-glycine was prepared following [6]. BOC-ω-aminoundecanoic acid was prepared at pH 10.2 following [6]. Crystallization was achieved from ethylacetat/petroleumether. The melting point was 70–71 °C.

Preparation of affinity resins

10 g (3.1 mmol amino groups) aminoethyl cellulose were preswollen in 100 ml methylene chloride (CH_2Cl_2) for 5 h with slightly shaking the reaction vessel. Then the CH_2Cl_2 was filtered off and the cellulose was treated with CH_2Cl_2 for 5 min three times. Thereafter 4.2 ml (30 mmol) triethylamine in 50 ml CH_2Cl_2 were added and shaken for 15 min. After this, the suspension was filtered and the cellulose washed with CH_2Cl_2 for 5 min three times. Then the BOC-amino acid (or dicarboxylic acid mono-*tert.*-butylester) 15 mmol and CH_2Cl_2 were added to the cellulose in order to form a shakeable slurry. After 15 min of agitation 15 mmol of dicyclohexylcarbodiimide were added and shaken over night. Then the reaction mixture was filtered and the cellulose washed successively with CH_2Cl_2 for 5 min three times, with ethanol (96%) for 5 min three times and CH_2Cl_2 for 5 min three times. The cellulose is dried and then 90 ml of 1 N HCl in glacial acetic acid is added and shaken for 40 min in order to deprotect the amino group (or in case of a dicarboxylic acid mono-*tert.*-butylester, the carboxylic group). After removing the HCl by suction the cellulose is washed with CH_2Cl_2. The deprotected carboxylic group (in case of the removal of *tert*-butylester) is now ready for coupling to amino acid esters (*e.g.* *tert*-butylester), to monoprotected diamines or to a ligand which has *e.g.* a free amino group. If the amino group (in case of the BOC-amino acid) was deprotected the cellulose is then treated for 15 min with 4.2 ml (30 mmol) triethylamine in 50 ml CH_2Cl_2. Thereafter the reagent was filtered off and the cellulose washed with CH_2Cl_2 for 5 min five times. The amino group is now ready for coupling either to another BOC-amino acid or to the ligand which needs at least one free carboxylic group (*e.g.* testosterone-17β-hemisuccinate). After each coupling reaction, before deprotection, treatment of the resin with glacial acetic acid and dicyclohexylcarbodiimide or with 4-nitrophenylacetate is useful to block unreacted amino groups (unreacted carboxylic groups may be blocked with *e.g.* ethanolamine and dicyclohexylcarbodiimide).

The deprotection- and coupling cycle is done for each residue of which the spacer arm is elongated.

Analytical methods

The amount of coupled spacer was determined (in case of a spacer arm consisting of amino acids) after hydrolysation of an aliquot of spacer bearing cellulose with 6 N HCl in a sealed tube at 110 °C for 36 h, with a "Kontron" amino acid analyzer.

The amount of testosterone was determined by hydrolysing an aliquot of the affinity resin in 1 N NaOH plus ethanol for 1 h at room temperature. After neutralization with 1 N HCl the suspension was centrifuged at $11\,000 \times g$ and the supernatant was stored. The pellet was washed six times with ethanol. The stored supernatant and the ethanol washings were combined and evaporated under reduced pressure. The residual solid was extracted several times with anhydrous ether. The ether was then evaporated and the testosterone dissolved in anhydrous ethanol. The testosterone concentration was measured photospectrometrically at 240 nm. The free amino groups which were not blocked by terminating reagents were measured by using [^{14}C]acetic acid.

[^{14}C]acetic anhydride was stored for 48 h in distilled water to split this molecule into [^{14}C]acetic acid. This radioactive acetic acid was diluted with a certain amount of radioinert acetic acid. Then an aliquot of the affinity resin was treated with this solution. Thereafter the acetic acid which was not bound to the resin was washed away with distilled water. Then the resin was treated with diluted NaOH. The [^{14}C]acetic acid which was eluted as sodium acetate by this procedure was measured in a toluol based scintillation cocktail containing 30% Triton X 100. The amount of the free amino groups can be calculated from the counts/minute measured.

"ON-kinetic"

The "ON-kinetic" was done by incubating a certain amount of affinity resin (200 mg of 1xAUD-, 229 mg of 10xAUD- and 291.9 mg of 4xGly-resin respectively) with androgen-receptor containing cytosol (its specific testosterone binding was already determined) at 4 °C. After certain time intervals an aliquot of cytosol-resin suspension was taken and centrifuged at 11 000 × *g*. The resulting pellet was washed with PEM-buffer and centrifuged again. The supernatants were combined and divided into two equal volumes. Specific binding/ml was evaluated analogues to Haase *et al.* [7]. The difference between the value of specific binding/ml of cytosol untreated with affinity resin and the value of specific binding/ml of affinity resin treated cytosol was expressed in percent. This procedure was done for ten time intervals (s. Fig. 2).

"OFF-kinetic"

The "OFF-kinetic" was done by incubating affinity resin with cytosol at 4 °C for 120 min. Thereafter the suspension was centrifuged at 11 000 × *g* and washed three times with PEM-buffer. The resin was then incubated with PEM-buffer saturated at 4 °C with radioinert testosteron. After certain time intervals an aliquot of the suspension was taken and centrifuged at 11 000 × *g* and washed with PEM-buffer. The combined supernatants were treated with dextran coated charcoal (DCC) and centrifuged at 11 000 × *g*. The supernatant was divided into two equal volumes. Specific binding/ml was evaluated following the method of Haase *et al.* [7]. This procedure was done for ten time intervals (s. Fig. 3).

Results and Discussion

The following affinity resins were prepared and tested as described above:

1) Testosterone-17β-hemisuccinyl-deca-ω-amino-undecanyol-aminoethyl-cellulose (10xAUA-resin). This resin has a very long spacer arm of defined length (s. Fig. 1). The amount of coupled ligand (testosterone) was 3.3 mg testosterone/g affinity resin, which is 11.5 µmol testosterone/g affinity resin.
2) Testosterone-17β-hemisuccinyl-tetra-glycyl-aminoethyl-cellulose (4xGly-resin). This resin has a shorter spacer arm, but this arm is very rigid because of the peptide bonds which are plain, stabilized by mesomerism and very close to each other (s. Fig. 1). The amount of coupled ligand (testosterone) was 2.1 mg testosterone/g affinity resin, which is 7.3 µmol testosterone/g affinity resin.

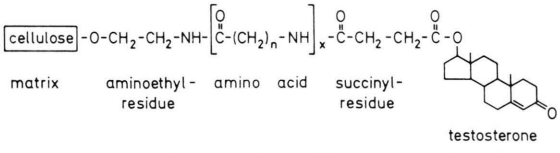

Fig. 1. General structure of the affinity resins described.

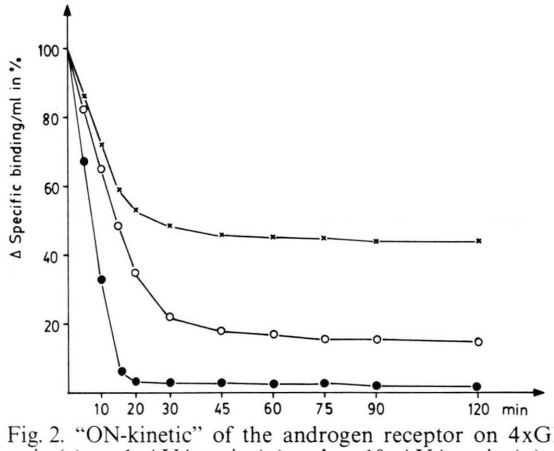

Fig. 2. "ON-kinetic" of the androgen receptor on 4xGly-resin (×), on 1xAUA-resin (○) and on 10xAUA-resin (●).

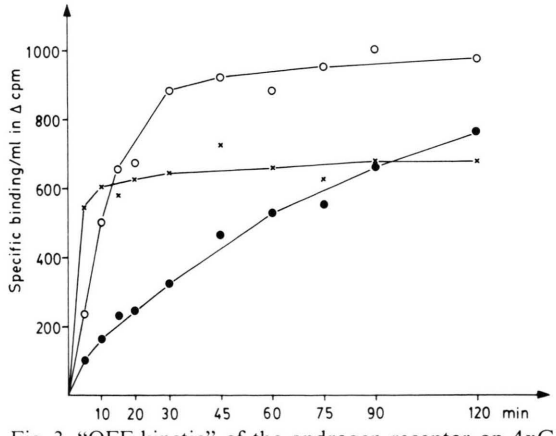

Fig. 3. "OFF-kinetic" of the androgen receptor on 4xGly-resin (×), on 1xAUA-resin (○) and on 10xAUA-resin (●).

3) Testosterone-17β-hemisuccinyl-ω-aminoundecanoyl-aminoethyl-cellulose (1xAUA-resin). This spacer arm is in the length comparable to the tetra-glycyl-arm but it is much more flexible (s. Fig. 1). The amount of coupled ligand (testosterone) was 3.87 mg testosterone/g affinity resin, which is 13.5 µmol testosterone/g affinity resin.

Amino groups were blocked by acetylation as described. Titration of the unblocked amino groups with [^{14}C]acetic acid showed < 0.03 µmol amino groups to be free. This is negligble compared with the amount of ligand coupled.

The "ON-kinetic" and the "OFF-kinetic" of the three affinity resins show how the length and the rigidity of the spacer arm influence the binding-kinetics of the androgen receptor to the affinity resin (s. Fig. 2 and Fig. 3).

Further experiences with the three affinity resins described here will be published elsewhere.

The advantage of the method described is, that spacer arms with all kinds of properties can be synthesized in a simple way directly at the matrix of the resin. The affinity resins described are very good for the batch technique but they are also usuable for affinity chromatography on columns.

Acknowledgement

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