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TARGETED IMMOBILIZATION OF NEUROTRANSMITTERS AND NEUROPEPTIDES ON AGAROSE AND ON ACRYLEX POLYMERS

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SUMMARY

A new strategy was devised for the targeted immobilization of ligands on aminohexyl- and carboxyhexyl-agarose. Selectively protected neurotransmitter amino acids and neuropeptides were coupled to amino or carboxyl group-containing agarose derivatives using activated esters, mixed anhydrides or carbodiimides. After coupling, agarose beads were dehydrated and the protecting groups were cleaved in non-aqueous media with acids (trifluoroacetic acid, formic acid). Agarose beads were rehydrated and applied for affinity chromatography and cell surface recognition. The same compounds were coupled to derivatized polyacrylamide beads containing primary amino (Acrylex A), acyl hydrazide (Acrylex AH-100) or carboxyl (Acrylex C-100) groups. Protecting groups were removed by acidolytic cleavage. Oxytocin, vasopressin, tetra- and pentagastrin, cholecystokinin, leucine-enkephalin and carboxyl-bearing derivatives of the neurotransmitters noradrenaline, dopamine, histamine, serotonin, acetylcholine and γ-aminobutyric acid were immobilized on agarose and on derivatized polyacrylamide gels.

INTRODUCTION

Affinity chromatography is the method of choice for the isolation of specific hormone and neurotransmitter receptors. The highly specific interaction between a hormone (or neurotransmitter) and a receptor requires affinity adsorbents, in which ligands are coupled to the matrix in a targeted manner. Selective immobilization instead of random coupling prevents the attachment

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of ligands via functional groups essential for the receptor interactions. Previous preparative methods for affinity adsorbents containing polypeptide hormones have involved the direct reaction of natural or synthetic hormones with activated agarose (Sepharose) derivatives. Such procedures result in randomly coupled peptide-agarose adsorbents with heterogeneous structures [1—4]. More recently, selective immobilization methods were used to attach adrenocorticotropin (ACTH) 5-24, Val^5-angiotensin-II-amide [5] and Lys^8-vasopressin [6] to affinity matrices. Hofmann and co-workers [7, 8] reported a targeted attachment of ACTH and insulin by complexing biotinyl-ACTH 1-25 amide and N$^{\alpha}$-B1-biotinyl-insulin, respectively, to avidin-Sepharose. However, these methods are not suitable for the targeted attachment of small molecules such as trifunctional amino acids and neurotransmitters [catecholamines, serotonin, histamine and γ-aminobutyric acid (GABA)] to agarose. The methods recently available [9] make possible the attachment of small molecules via one of the functional groups important for biological activity. Such neurotransmitter-Sepharose adsorbents are known from the literature [10, 11], but their applicability in biospecific adsorption is questionable. Similarly, there are a number of methods for the immobilization of peptides on a polymer matrix, but no general method has been developed for the targeted attachment of polyfunctional amino acids and peptides resulting in a chemically homogeneous ligand–polymer system.

The aim of this work was to use new derivatized polyacrylamide gels as matrices and to develop a general method for the unambiguous coupling of polyfunctional neurotransmitters and peptides to agarose and derivatized polyacrylamides.

Hydrophilic polyacrylamide gels are suitable for forming uniform beads with excellent chromatographic properties. We have developed new ways of copolymerization for the synthesis of functional groups containing polyacrylamide beads. A primary amino group was built into the polymer by using N-aminoethylacrylamide in the polymerization mixture (Acrylex A). Similarly, the addition of acrylic acid or acryl hydrazide to the polymerization mixture (acrylamide-N,N-methylenebisacrylamide) results in the formation of copolymers containing carboxyl (Acrylex C) and acyl hydrazide (Acrylex AH)

TABLE I

STRUCTURE AND PROPERTIES OF ACRYLEX MATRICES

Type of Acrylex	Structure	Concentration of the functional group (mmol/g dry weight)	Diameter of beads (μm)	Swelling (ml/g)	Exclusion limit (Dalton)
Acrylex A	$-[CH_2-CH(CONH_2)]_n-CH_2-CH(CONH-C_2H_4NH_2)-[CH_2-CH(CONH_2)]_m$	2	50–100	20	100 000
Acrylex AH-100	$-[CH_2-CH(CONH_2)]_n-CH_2-CH(CONHNH_2)-[CH_2-CH(CONH_2)]_m$	4.9 ± 0.2	100–320	8	100 000
Acrylex C-100	$-[CH_2-CH(CONH_2)]_n-CH_2-CH(COOH)-[CH_2-CH(CONH_2)]_m$	6.2 ± 0.3	100–320	100	100 000

groups. The structures and properties of the Acrylex matrices are shown in Table I. These polyacrylamide matrices are particularly applicable for the immobilization of enzymes [12–16].

We have found that partially protected polyfunctional compounds having one free functional group are particularly applicable for targeted immobilization. All the functional groups of a neurotransmitter molecule were saved during coupling by temporary protective groups. A new carboxyl group was introduced into the neutrotransmitter molecule and coupling was performed via this free extra carboxyl function. The following carboxyl-bearing compounds were used by targeted immobilization of neurotransmitters: 3,4-dihydroxyphenylalanine (DOPA) instead of dopamine; 3,4-dihydroxyphenylserine (DOPS) instead of noradrenaline; 5-hydroxytryptophan instead of serotonin; histidine instead of histamine; glutamic acid instead of GABA; succinylcholine instead of acetylcholine.

After coupling via the free function, protecting groups could be cleaved by acidolysis without any damage of the beads, resulting in immobilized neurotransmitters with free functional groups. Peptides could be immobilized in a similar way.

EXPERIMENTAL

Derivatized polyacrylamides (Acrylex) were purchased from Reanal Fine Chemicals (Budapest, Hungary) and Sepharose 4B and Sepharose CL 4B from Pharmacia (Uppsala, Sweden). N-Protected carboxyl-bearing neurotransmitters were synthesized in our laboratory. Trifluoroacetic acid and formic acid were purchased from Merck (Darmstadt, F.R.G.), di-*tert.*-butyl pyrocarbonate and cyanogen bromide from Fluka (Buchs, Switzerland) and α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl (Ddz) hydrazide from Boehringer Ingelheim (F.R.G.). Oxytocin was a generous gift from the Gedeon Richter (Budapest, Hungary). Tetragastrin, pentagastrin, arginine^8-vasopressin (AVP) and cholecystokinin octapeptide were synthesized in our laboratory [17, 18]. Thin-layer chromatographic (TLC) control was performed on Merck Kieselgel plates (HF 254). Chromatographic systems were: A, ethyl acetate–(*n*-butanol–acetic acid–water, 75:10:24), 95:5 (v/v); B, ethyl acetate–(pyridine–acetic acid–water, 20:6:11), 95:5 (v/v).

Synthesis of N-protected carboxyl-bearing neurotransmitters

*N-tert.-Butyloxycarbonyl-*L*-3,4-dihydroxyphenylalanine, dicyclohexylamine salt (Boc-*L*-DOPA · DCHA).* L-DOPA (980 mg, 5 mmol) was dissolved in 15 ml of dioxane–water (1:1), and ascorbic acid (100 mg), and 1 *M* sodium hydroxide (5 ml) were added. The mixture was reacted with di-*tert.*-butyl pyrocarbonate (1.3 g, 6 mmol) at 20° C overnight in the dark. After the reaction was complete, the reaction mixture was diluted with water (50 ml), the excess reagent was extracted with ethyl acetate, and the aqueous phase was acidified to pH 3.0 with 0.5 *M* hydrochloric acid at 0° C. Boc-L-DOPA was extracted three times with ethyl acetate, the organic phase was dried with anhydrous sodium sulphate and evaporated to 15 ml, and dicyclohexylamine (0.9 ml, 5 mmol) was added. The DCHA salt was crystallized after addition of diethyl ether (30

ml). Filtration and drying yielded 950 mg (40%) of pure Boc-DOPA · DCHA salt; m.p. 145—146°C, $[\alpha]_D^{20} = -25.3°$ (c = 1, methanol).

Boc-D-DOPA · DCHA. Boc-D-DOPA · DCHA was similarly synthesized from 5 mmol of D-DOPA, yielding 1200 mg (52%) of pure product; m.p. 143—145°C, $[\alpha]_D^{20} = +26.3°$ (*c* = 1, methanol), R_F A = 0.42.

N-tert.-Butyloxycarbonyl-D-5-hydroxytryptophan, dicyclohexylamine salt (Boc-D-5OH-Trp · DCHA). D-5-Hydroxytryptophan (220 mg, 1 mmol) was reacted with di-*tert.*-butyl pyrocarbonate (250 mg, 1.1 mmol) as described above. Formation of the DCHA salt yielded 317 mg (68%) of pure material; m.p. 200– 206°C (decomposition), R_F A = 0.52.

α,α-Dimethyl-3,5-dimethoxybenzyloxycarbonyl-3,4-dihydroxyphenylserine (Ddz-DOPS). DL-DOPA (50 mg, 0.25 mmol) was dissolved in 0.5 ml dimethylformamide (DMF) together with Triton-B (0.11 ml, 40% methanol solution). Ddz azide (Ddz-N_3) (89 mg, 0.3 mmol) and triethylamine (0.11 ml) were added. The reaction mixture was stirred for 20 h at 40°C, and evaporated to dryness. The residue was dissolved in water (10 ml), and excess Ddz-N_3 was extracted with ethyl acetate. The aqueous phase was acidified with 0.5 *M* potassium hydrogen sulphate, and Ddz-DOPS was extracted three times with ethyl acetate. After drying (anhydrous sodium sulphate) and evaporation, the oily residue was purified on a Merck preparative TLC plate [solvent systems: a, chloroform—methanol (9:1); b, system B]. The pure product was dissolved from the silica with ethyl acetate, and evaporation of the solvent and crystallization from ethyl acetetate—diethyl ether yielded pure product (43 mg, 39%); m.p. 101—105°C (decomposition), R_F B = 0.25.

Succinylcholine. Succinylcholine was synthesized from succinyldicholine via partial hydrolysis according to the literature [19], and was purified by means of column chromatography: yield 38%, m.p. 159–163°C.

Coupling of N-protected amino acids and neurotransmitters to aminohexyl-agarose beads (general procedure)

Aminohexyl-agarose (AH-Sepharose, 12 g wet weight) was washed ten times with distilled water, and with DMF—water mixtures containing an increasing amount of DMF (5, 10, 20 and 40%). The beads were suspended in a DMF—water—acetone (2:1:2) mixture (25 ml). In the meantime, an N-protected amino acid or neutrotransmitter (0.5 mmol) was reacted in DMF (1 ml) with isobutyl chloroformate (0.065 ml, 0.5 mmol) and triethylamine (0.070 ml, 0.5 mmol) at −10°C for 5 min. The solution of the mixed anhydride formed in this reaction was added to the suspension of AH-Sepharose beads. After the reaction was complete (+4°C, within 4 h), the beads were thoroughly washed with a mixture of DMF—water—acetone (2:1:2) and dehydrated by washing with the following mixtures: 60% acetone in water; acetone; 20, 40, 60 and 80% dichloromethane in acetone; dichloromethane. The acid-labile protecting groups were cleaved with 50% trifluoroacetic acid in dichloromethane, washed with dichloromethane and rehydrated by washing with the following mixtures: 80, 60, 40 and 20% acetone in water; pure water. The concentrations of the amino acids and neutotransmitters were determined either through amino acid analysis after hydrolysis of a sample (20 mg) of Sepharose beads with mercaptoethanesulphonic acid [20], or with a UV

TABLE II

NEUROTRANSMITTERS AND PEPTIDES IMMOBILIZED ON AH-SEPHAROSE, ACRYLEX A AND AH-100

Structure of compound immobilized	Appropriate neurotransmitter	Reagent used	Ligand concentration (m*M*)
3,4-Dihydroxyphenylseryl-AH-Sepharose	Noradrenaline	Ddz-DOPS	2.0
3,4-Dihydroxyphenylalanyl-AH-Sepharose	Dopamine	Boc-DOPA (L and D)	6.7
D-5-Hydroxytryptophyl-AH-Sepharose	Serotonin	Boc-D-5OH-Trp	2.2
D-Histidyl-AH-Sepharose	Histamine	Boc-His(Boc)	3.4
D-Glutamyl-AH-Sepharose	GABA	Boc-D-Glu(OBu^t)	7.95
Succinylcholine-AH-Sepharose	Acetylcholine	$(CH_3)_3N(CH_2)_2OCO(CH_2)_2COOH$	2.5
3,4-Dihydroxyphenylseryl-Acrylex AH-100	Noradrenaline	Ddz-DOPS	2.0
D-3,4-Dihydroxyphenylalanyl-Acrylex A	Dopamine	Boc-D-DOPA	3.5
D-5-Hydroxytryptophyl-Acrylex A	Serotonin	Boc-D-5OH-Trp	1.8
L-Histidyl-Acrylex A	Histamine	Boc-His(Boc)	3.2
Succinylcholine-Acrylex A	Acetylcholine	$(CH_3)_3NCH_2CH_2OCOCH_2CH_2COOH$	2.5
D-Glutamyl-Acrylex A	GABA	Boc-D-Glu(OBu^t)	1.1

spectrophotometer using 0.1-cm cells. Results are given in Table II.

N-Protected, carboxyl-bearing neurotransmitters were coupled to primary amino group containing Acrylex A and AH-100 in a similar way using mixed anhydride activation in a DMF—dioxane mixture; protecting groups were cleaved simply with 50% trifluoroacetic acid in dichloromethane. Results are given in Table III.

TABLE III

PEPTIDES IMMOBILIZED ON CH-SEPHAROSE AND ON ACRYLEX C-100

Structure of compound immobilized	Active compound and solvent used*	Ligand concentration (m*M*)
CH-Sepharose-oxytocin	CH-Sepharose-OSu, water	2.3
CH-Sepharose-vasopressin	CH-Sepharose-OSu, water	2.2
CH-Sepharose-cholecystokinin-8	CH-Sepharose-OSu, water	2.0
CH-Sepharose-tetragastrin	CH-Sepharose-OPCP, dioxane	5.0
CH-Sepharose-pentagastrin	CH-Sepharose-OPCP, dioxane	3.73
CH-Sepharose-cholecystokinin (unsulphated)	CH-Sepharose-OPCP, dioxane	1.5
Acrylex C-tetragastrin	Acrylex C-100-OSu, dioxane	3.0
Acrylex C-pentagastrin	Acrylex C-100-OSu, dioxane	2.8
Acrylex C-Arg^8-vasopressin	Acrylex C-100-OSu, water	4.5
Acrylex C-oxytocin	Acrylex C-100-OSu, water	2.0
Acrylex C-cholecystokinin	Acrylex C-100-OSu, water	2.5
Acrylex C-Leu-enkephalin	Acrylex C-100-OSu, dioxane	6.3

*OSu = N-hydroxysuccinimide ester; OPCP = pentachlorophenyl ester.

Coupling of polypeptides to 6-carboxyhexyl-agarose beads (general procedure)

In these experiments, the N-hydroxysuccinimide ester of 6-carboxyhexyl-agarose [21] ("activated CH-Sepharose 4B") and the pentachlorophenyl ester of 6-carboxyhexyl-agarose were used. The latter was synthesized as follows: 25 g of 6-carboxyhexyl-agarose (CH-Sepharose 4B) were reacted with 25 ml of 0.1 *M* dicyclohexyl-carbodiimide and 25 ml of 0.1 *M* pentachlorophenol in anhydrous dioxane (90 min, 20°C). The dicyclohexylurea precipitated was removed by extensive washing of the beads with a 1:1 dioxane–methanol mixture and the active ester was stored at +4°C in a 1:1 suspension of anhydrous dioxane.

Peptides (oxytocin, vasopressin and cholecystokinin-8) were coupled to the

N-hydroxysuccinimide ester of 6-carboxyhexyl-agarose as follows. Activated agarose (10 g wet weight) was suspended in 10 ml of 0.1 *M* phosphate buffer (pH 7.6), and 0.1 mmol of peptide was added, dissolved in 10 ml of phosphate buffer (pH 7.6). After shaking for 24 h at +4°C, the residual active ester groups were eliminated by reacting the beads with 10 ml of 1 *M* glycine solution (pH 9.0) for 2 h. The adsorbent was finally washed exhaustively with phosphate-buffered saline (PBS) solution. If necessary, protecting groups were removed with 50% trifluoroacetic acid in dichloromethane. Concentrations of the ligands were determined by amino acid analysis and/or UV spectrophotometry (Table III). Water-insoluble peptides (tetra- and pentagastrin, non-sulphated cholecystokinin-8) were coupled to the pentachlorophenyl ester of 6-carboxy-hexyl-agarose. Activated agarose (5 g dry weight) suspended in 10 ml of DMF, and 0.014 ml of triethylamine and 0.1 mmol of peptide dissolved in 5 ml of DMF were added. After reaction overnight at 20°C, the residual active ester groups were eliminated by reacting the beads with 20 ml of 1 *M* glycine solution (pH 9.0) for 2 h, and the adsorbent was washed thoroughly with PBS solution. The ligand concentration was determined by means of amino acid analysis and/or UV spectrophotometry (Table III).

Peptides were coupled in a similar way on Acrylex 100 beads (after activation of the carboxyl group of the polymer chain as the N-hydroxysuccinimide ester) in DMF or in a dioxane—water mixture (1:1). Results are given in Table III.

Gastric parietal cell surface recognition by immobilized ligands

Parietal cells were isolated from canine gastric mucose according to Scholes et al. [22]. After the last centrifugation, the cell suspension was diluted with 4 vols. of buffer A [pH 7.4, Krebs—Ringer containing bovine serum albumin (1%), amino acid supplement, glucose (0.1%), Ca^{2+} and Mg^{2+}]. Neurotransmitter-Sepharose and peptide-Sepharose beads were washed thoroughly with PBS, centrifuged, and diluted with 9 vols. of buffer A. For the cell surface recognition technique, 100 μl of diluted Sepharose beads were mixed in a vial with 10 μl of diluted parietal cell suspension in buffer A, and after a 3-min

TABLE IV

BINDING OF CANINE PARIETAL CELLS ON PEPTIDE-SEPHAROSE AND NEUROTRANSMITTER-SEPHAROSE BEADS

Adsorbent	Neurotransmitter or hormone on the surface	Binding of cells on the beads (%)
1 Sepharose 4B	—	21
2 Butyl-AH-Sepharose 4B	—	25
3 Histidyl-AH-Sepharose 4B	Histamine	48
4 Succinylcholine-Sepharose 4B	Acetylcholine	49
5 5-OH-Tryptophyl-AH-Sepharose 4B	Serotonin	24
6 3,4-Dihydroxyphenylseryl-AH-Sepharose 4B	Noradrenaline	19
7 3,4-Dihydroxyphenylalanyl-AH-Sepharose 4B	Dopamine	22
8 Pentagastrin-CH-Sepharose 4B	Gastrin-4	45

incubation the number of cells adsorbed on the surface of the agarose beads was counted at magnification × 200 under a microscope. Counting was repeated nine times. Results are listed in Table IV.

Application of the adsorbents in affinity chromatography

Cholecystokinin-CH-agarose (3 ml) was washed in a little column with phosphate buffer (0.05 *M*, pH 7.4) for 6 h. Anti-cholecystokinin antiserum (1 ml) (raised in a rabbit) was loaded onto the column without prepurification. The column was left overnight at +4°C and elution was performed with 75 ml of phosphate buffer (0.05 *M*, pH 7.4). Two fractions were active; low-affinity antibodies were eluted in a volume of 5–8 ml, high-affinity antibodies in an elution volume of 40—44 ml. Fractions were dialysed and lyophylized. The purified high-affinity anti-cholecystokinin antibody fraction was used in the immunocytochemistry.

RESULTS AND DISCUSSION

Our new method, the coupling of partially protected polyfunctional compounds via the only free functional group on agarose or derivatized polyacrylamide beads, proved to be applicable for the targeted immobilization of neurotransmitters and polypeptides. All kinds of polyfunctional compounds can be immobilized in this way, if the necessary protected derivatives are available. The method is based on two facts: (i) activation of the only carboxyl group of a protected substance as a mixed anhydride leads to unequivocal coupling instead of random immobilization; (ii) acid-labile protecting groups [Boc, Ddz, *tert.*-butyl (Bu^t), etc.] can be cleaved from the immobilized polyfunctional compounds without any damage to the polysaccharide or polyacrylamide beads in organic solvents. The latter finding was surprising, for it had been expected that the agarose structure would be severely damaged in strong acids such as trifluoroacetic acid.

We found that acidolytic cleavage of glycosyl bonds between the monosaccharide units of agarose takes place only in aqueous solutions or strong acids; trifluoroacetic acid treatment in dichloromethane does not hydrolyse the polysaccharide chains. Our method results in chemically uniform, well characterized, homogeneous affinity adsorbents with excellent chromatographic properties, in which all the functional groups necessary for biological interactions remain free.

In some cases it is necessary to introduce an additional functional group into the compounds to be selectively immobilized. For example, carboxyl-bearing neurotransmitters (e.g. succinylcholine, histidine, glutamic acid, DOPA, DOPS and 5-hydroxytryptophan) were used instead of the original neurotransmitters (acetylcholine, histamine, GABA, dopamine, noradrenaline and serotonin) in order to preserve all the functional groups necessary for receptor—transmitter interactions. In some cases, the D-isomers of carboxylated neurotransmitters were used because of their better enzymic resistance.

Using mixed anhydride activation, we attained a ligand concentration of ca. 10^{-3} *M* in almost every case, which is high enough for work with affinity techniques. Our new product, the pentachlorophenyl ester of carboxyhexyl-

Sepharose 4B, is particularly applicable in cases of immobilization of water- and dioxane-insoluble peptides.

The applicability of our new adsorbents was proved using a known technique of cell surface recognition by the immobilized ligands in our laboratory. Affinity adsorbents have frequently been used recently in histochemistry, biochemistry and physiology, intact viable cells being reacted with Sepharose beads containing immobilized hormones and neurotransmitters [23]. Receptors on the cell surface interact with immobilized ligands [24]; in this way, cells can bind to affinity adsorbents specifically. We have found that isolated parietal cells of canine mucosa are specifically bound to immobilized penta- and tetragastrin, histamine and acetylcholine, but not to serotonin- or noradrenaline-Sepharose or untreated Sepharose beads. This finding is in accordance with the theory that parietal cells contain histamine, acetylcholine and gastrin receptors on their surface. Cell surface recognition, if well standardized, seems to be a sensitive method for receptor investigation. Hormone—receptor or neurotransmitter—receptor interaction could be investigated in this way using different media (effects of pH, ionic strength, composition of the media, etc.) and different affinity adsorbents. The further development and standardization of the cell surface recognition technique are in progress in our laboratory.

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