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Short Communication

Silica sorbents with one- and two-site attached bacitracin in affinity chromatography

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ABSTRACT

It was found that the bifunctional oligopeptide bacitracin A can be attached to a silica surface by either one or two amino groups. The latter, in contrast to the former, is active in specific binding of proteinases.

INTRODUCTION

Silicas chemically modified by organic groups are extensively used in sorption, catalysis and chromatography [1]. However, many problems with the chemically bonded compound structure and composition are unresolved because they are very complicated. Particular difficulties arise when polyfunctional molecules are immobilized. Only a few studies have been devoted to detailed investigation of the bonded layer structure of silicas chemically modified by polyfunctional molecules [2–4]. This study deals with the preparation and characterization of affinity sorbents for proteinases on silica containing covalently bound residues of bacitracin A.

The cyclic oligopeptide bacitracin A is well known as an effective ligand in affinity chromatography of proteinases [5–7]. Literature data show that the selectivity of oligopeptide-bonded sorbents depends on both the type of support and the ligand–surface binding character. Often, immobilization of ligands leads to a loss of their affinity [7,8]. Such irreproducibility, in our opinion, may be related to the complicated ligand–surface binding character. Indeed, bacitracin contains two primary amino groups, and can react with a modified silica surface by either one or both of them to yield either a one- or two-site attachment of the ligand (Fig. 1).

EXPERIMENTAL

The silica support Silokhrom S-120 has an average pore diameter at 35–40 nm, a specific surface area of 116 m^2/g and an average particle size of 0.35–0.50 mm.

Epoxy-activated silica was prepared as described elsewhere [9] by treating Silokhrom S-120 with

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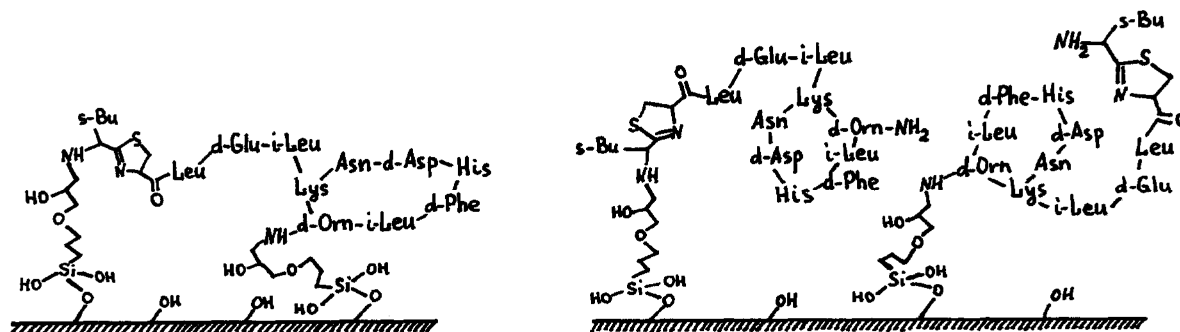
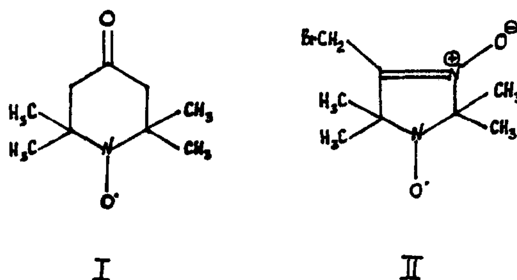


Fig. 1. Schematic representation of one-site and two-site attachment of bacitracin on the silica surface. s-Bu = *sec.*-C_4H_9; i = iso; d = optical isomer of amino acid.

γ-glycidoxypropyltriethoxysilane. Tosyl-activated silica was prepared from epoxy-activated silica by treatment with the tosyl chloride–pyridine following acidic hydrolysis of the epoxy groups [9].

Bacitracin-bonded silicas were synthesized by treating activated silicas with bacitracin (Serva) in aqueous solution (30 mg of bacitracin per gram of activated silica) for 3 days at room temperature. To remove unreacted active groups, the sorbents obtained were treated with an excess of aqueous Tris solution (pH 7.5). The characteristics of the synthesized activated silicas and affinity sorbents are given in Table I.



In order to introduce the radical **I** to bonded bacitracin molecules, the sorbent was treated with an excess of a boiling pentane solution of radical **I**. To label immobilized bacitracin with radical **II**, the sorbents were treated with a solution of **II** in acetonitrile in the presence of N,N-diisopropylethylamine. The molar amount of bonded **II** was less than the molar amount of bonded bacitracin.

Electronic reflectance spectra were obtained with a Specord M-40 UV–VIS spectrophotometer using a cuvette for solid species. ESR spectra were obtained with a model RE-1307 spectrometer (NPO EZNP, Chernogolovka, USSR). The sorbents were placed in the glass tube and evacuated to 0.01 Torr.

The bacitracin-bonded silica sorbents obtained were used in biospecific binding of human throm-

TABLE I

CHARACTERISTICS OF THE ACTIVATED SUPPORTS AND AFFINITY SORBENTS

Functional groups of the activated silica	Functional groups concentration × 10^6		$L'_{av.}$ [a] (nm)	Bonded bacitracin concentration × 10^6		Share of the one-site-bonded bacitracin (%)	Designation
	mol/g	mol/m^2		mol/g	mol/m^2		
Epoxy	104	0.9	1.45	6.0	0.05	100	Bc-E
Tosylate	244	2.1	0.96	6.0	0.05	22	Bc-T

[a] Average distance between functional groups of the surface, calculated from $L_{av.} = 1.075/p^{0.5}$, where p is the surface concentration of functional groups [14].

bin, pig pepsin, serine proteinase from *Thermoactinomyces vulgaris* and bovine trypsin. Sorption of proteinases was carried out under dynamic conditions. The column with sorbent was covered with the proteinase solution, which contained 0.5–3.0 mg of enzyme per millilitre of buffer solution [0.075 *M* HCl (pH 2.0) for pepsin and 0.05 *M* Tris–HCl–0.15 *M* NaCl (pH 8.0) for other enzymes].

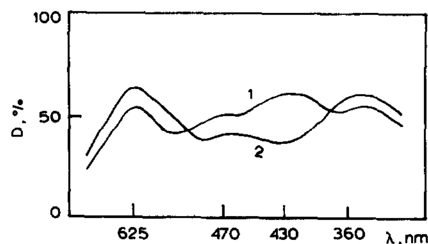


Fig. 3. Electronic reflectance spectra of the TCNQ-treated (1) Bc-E and (2) Bc-T sorbents.

RESULTS AND DISCUSSION

Typical chromatograms of the proteinases studied obtained on the synthesized sorbents are presented in Fig. 2. The data show that there is no binding of the studied proteinases with the Bc-E sorbent (for designations, see Table I). At the same time, there is quantitative binding of all the studied proteinases on the Bc-T sorbent (Table I). For the proteinases to be eluted one needs to change the eluent to 1 *M* NaCl solution containing 25% of isopropanol.

To investigate whether bacitracin is bound to the surface at one or two sites for the Bc-E and Bc-T sorbents, a method utilizing tetracyanoquinodimethane (TCNQ) was used. It has been shown [10,11] that reaction of TCNQ with bonded primary amino groups gives an adduct with maximum absorbance at 420–430 nm in their electronic reflectance spectra, whereas reaction with secondary or tertiary amino groups gives adducts having maximum absorbance at 340–380, 625–640 and 830–850 nm.

The electronic reflectance spectra of the Bc-E and Bc-T sorbents (before treatment with Tris treated with TCNQ are shown in Fig. 3. The presence of absorption in the 420–430 nm region for the Bc-E sorbent indicates that one-site binding of bacitracin is predominant, whereas for the Bc-T sorbent the absence of absorption in this region indicates that binding of bacitracin occurs at two sites.

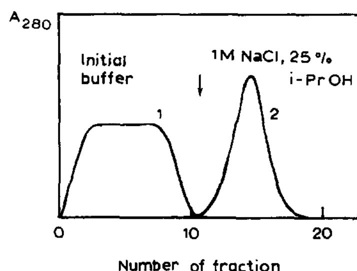


Fig. 2. Typical chromatograms of bovine trypsin on (1) Bc-E and (2) Bc-T sorbents. i-PrOH = Isopropanol.

It should be noted that the method mentioned above gives only qualitative information about the ligand–surface binding character. For quantitative data relevant to one- and two-site bacitracin binding, a spin-labelling method was used. Radical **I** is known to react readily with primary amino groups to yield Schiff bases [12], whereas secondary amino groups do not react with ketones under the conditions employed. Hence the molar amount of **I** that will be immobilized on the bacitracin-bonded silica surface will be equal to the molar amount of primary amino groups of attached bacitracin. The amount of **I** that is immobilized could be exactly determined by integrating the ESR spectra.

The results obtained show that for the Bc-E sorbent, all bacitracin molecules are one-site bonded, whereas for the Bc-T sorbent about 80% of the bacitracin molecules are two-site bonded (Table I). In our opinion, this result may be related to a greater average distance between bonded epoxy groups in the epoxy-activated silica compared with the average distance between bonded tosyl groups in the tosyl-activated silica (Table I).

It was of interest to compare the one- and two-site-bonded ligand conformational mobility. To investigate the dynamic behaviour of the bonded bacitracin, the spin-labelling method was applied with radicals **I** and **II** as spin labels. Radical **II** will react with any type of amino group present in the bonded bacitracin. As a measure of the conformational mobility, the correlation time τ_c was chosen. This value

TABLE II

CHARACTERISTICS OF THE ESR SPECTRA OF THE SPIN-LABELLED Bc-E AND Bc-T SORBENTS (MEDIUM: ISOPROPANOL)

Sorbent	Spin label	τ_c ("rapid" form) (s)	Share of "rapid" radicals (%)	τ_c ("slow" form) (s)
Bc-E	I	10^{-11}	100	–
Bc-T	II	10^{-11}	15	$2 \cdot 10^{-9}$

is the reciprocal of the radical rotation frequency [13] and can be easily obtained from ESR spectra.

The τ_c values of the spin-labeled samples are given in Table II. It is clear that for the sample labelled by **I** there are components conforming to only one type of rotational motion in the ESR spectrum. For radical **II**, there is superposition of the components, conforming to two types of motion, "rapid" and "slow". The τ_c of the radical **II** "rapid" form is equal to τ_c of the radical **I** (Table II). Accordingly, we suggest that "rapid" radicals (in the case of radical **II**) are attached to primary amino groups and "slow" radicals are attached to secondary amines. In addition, the share of "rapid" radicals is approximately equal to that expected for the given amount of one-site bonded bacitracin (Tables I and II). Therefore, it is possible to say that one-site-bonded bacitracin is more mobile than two-site bonded bacitracin.

CONCLUSIONS

The cyclic oligopeptide bacitracin A, containing two primary amino groups per molecule, can be chemically attached to a silica surface by either one or both amino groups. The sorbent containing one-site-attached bacitracin does not demonstrate specific binding with proteinases. However, the sorbent containing two-site-attached bacitracin specifically binds the studied proteinases.

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