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Study of correlations between fine structure of bonded layer and affinity properties of silicas with attached cyclic oligopeptides

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Abstract

The cyclic oligopeptides bacitracin A and gramicidin S were immobilized on epoxy-, tosyloxy- and halogenoalkyl-activated silicas. The sorbents obtained were tested for affinity binding of proteinases. The effects of spacer length, ligand loading and the character of ligand attachment (one- or poly-site) on the biospecific characteristics have been studied.

Keywords: Stationary phases, LC; Silica; Affinity adsorbents; Oligopeptides; Peptides; Trypsin; Thrombin; Subtilisin

1. Introduction

In recent years, affinity chromatography has become one of the most promising techniques for the separation and the purification of biopolymers [1–3]. Modified silicas have good potential for use as biospecific sorbents. In fact, silica supports have good hydrodynamic characteristics, they do not swell in organic solvents and are non-biodegradable. Besides, silicas are commercially available and rather cheap. However, it should be noted that until recently silica sorbents have not been widely used in affinity chromatography. Little is known about the correlations between the affinity properties and the structure of surface bonded layers of such sorbents.

Silica supports, in contrast with those commonly used in affinity chromatography, organic polymers (polycarbohydrates, polyacrylamide, polystyrene etc.) can have a strong influence on both the properties of grafted affinity ligands and the interactions between substrate and sorbent. This influence is sharply pronounced for the supports which have insufficiently shielded surfaces and, therefore, are capable of strong non specific interactions both with grafted ligands and with substrate sorbing.

In our opinion, the following problems in the study of affinity silica sorbents are of special interest. First, how does the manner of ligands grafting influence the affinity properties of the sorbent? It would appear reasonable that the more mobile the attached ligand, the more accessible it can be for the substrate. However, mobile ligands can strongly interact with the surface of silica, and the more mobile the ligand, the stronger the interactions can be. These interactions can result in the distortion of the native structure of the ligand.

Second, the surface concentration of the attached ligands can also have an influence on the affinity properties of the sorbent. Of course, an increase of the ligand loading can lead to an increase in affinity

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binding, however, possible ligand–ligand, substrate–substrate and other interactions can significantly complicate the picture. This problem is quite insufficiently studied not only for silicas, but even for commonly used organopolymeric supports.

This paper studies the correlations between the grafted layer structure and the biospecific properties of the silica sorbents by the example of the bacitracin- and gramicidin-bonded silicas.

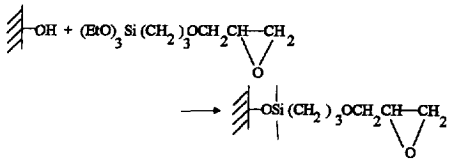
2. Experimental

2.1. Silica

The initial silica support Silokhrom S-120 (Luminophor, Stavropol, Russia) has an average pore diameter of 35–40 nm, a BET specific surface area of 116 m^2/g and the pore volume of 1.05 ml/g. The fraction of 0.35–0.50 mm was used.

2.2. Epoxy-activated silica

Modification was carried out with the use of 3-glycidooxypropyl-triethoxysilane [3–9]:



A 1 g amount of silica was stirred in 50 ml 0.1 *M* NaOAc buffer solution (pH 5.5) containing 0.2 ml 3-glycidooxypropyltriethoxysilane (GOPTES) (Reakhim, Russia) at 90°C for 1.5 h. Then the sorbent was consequently washed with distilled water, acetonitrile and diethyl ether.

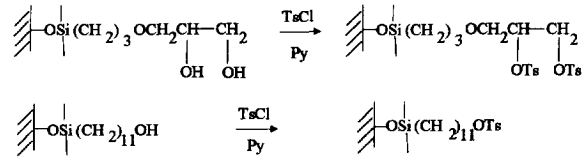
2.3. Tosyl-activated silicas

A 1 g amount of epoxy-activated silica was stirred with 100 ml of water containing sulfuric acid (pH 2.0) for 2 h. Then the sorbent was washed with distilled water up to neutral reaction of the supernatant. The sorbent obtained contains attached diol groups.

A 1 g amount of a diol-silica or a silica, containing attached $-(CH_2)_{11}OH$ groups (synthesis was described in our previous work [9]), was washed on a glass Schott filter with dry acetone.

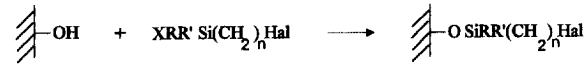
Then the sorbent was added to the mixture containing 5 ml of dry acetone, 0.5 g TsCl and 0.3 g of dry pyridine (all from Reakhim, Russia). The mixture was allowed to stand in an ice bath for 24 h. Then the sorbent was washed with acetone (6–8 times× 10–15 ml).

The tosylation schemes were the following [10]:



2.4. Halogenoalkyl-activated silicas

Modification was carried out according to the reaction [11–14]:



$n = 1$, R = CH_3, R′ = Cl, Hal = Br;

$n = 3, 5$, R = R′ = Cl, Hal = Br;

$n = 11$, R = R′ = Cl, Hal = Cl

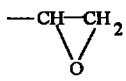
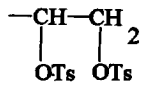
A 1 g amount of vacuum-dried silica was stirred in boiling anhydrous toluene containing 1 mmol of corresponding chlorosilane (for syntheses see Ref. [9]) for 10 h. Then the sorbents were extracted with toluene, acetone, acetone–water (1:1), acetone and washed in Soxhlet apparatus with diethyl ether for 6 days.

Concentrations of attached functional groups were estimated on the basis of titration of sorbents with diethylamine, or sodium thiosulfate [9]. Characteristics of the activated silicas obtained are given in Table 1.

2.5. Immobilization of bacitracin

Bacitracin A (Serva) was dissolved in distilled water (0.9 mg/ml). A 3 g amount of corresponding activated silica was treated with the desired amount of this solution (from 0.1 to 30 mg of bacitracin per gram of activated silica). The reaction volume was

Table 1
Characteristics of the activated silicas synthesized

Des.	Functional groups of the activated silica	Concentration of attached functional groups (groups per nm^2)	d_{av} (nm)
Epoxy		0.55	1.45
bis-OTs		1.25[a]	0.95
11-OTs	$-(CH_2)_{11}OTs$	1.33	0.97
1-Br	$-CH_2Br$	2.15	0.74
3-Br	$-(CH_2)_3Br$	2.20	0.72
5-Br	$-(CH_2)_5Br$	2.14	0.74

[a] Assuming tosylation of both hydroxy groups.

increased with distilled water up to 100 ml, if needed. The mixture was allowed to stand for 3 days at room temperature without stirring. The sorbents obtained were washed with distilled water (2×100 ml).

To remove the remaining active groups, the sorbents obtained were treated with an excess of saturated aqueous solution of Tris·HCl (pH 7.5) during one day. Finally, the sorbents were consequently washed with distilled water (2×100 ml), isopropanol (2×100 ml), pentane (2×100 ml) and dried at room temperature on glass filter.

2.6. Immobilization of gramicidin

Gramicidin S (Kh.F.Z., Krasnoyarsk, Russia) was dissolved in isopropanol (0.9 mg/ml). A 3 g amount of corresponding activated silica was treated with the desired amount of this solution (from 0.1 to 30 mg of gramicidin per gram of activated silica). The reaction volume was increased with isopropanol up to 100 ml, if needed. The mixture was allowed to stand for 3 days at room temperature without stirring. The sorbents obtained were washed with isopropanol (100 ml) and distilled water (2×100 ml).

Blocking of the unreacted groups with Tris·HCl and final washing were done as for bacitracin sorbents (see above).

Exception was for sorbents containing long chain bonded groups ($C_{11}OTs$). For this highly hydrophobic sorbent, isopropanol–aqueous Tris·HCl solution (1:3) was used for removal of unreacted tosylate groups.

2.7. Determination of concentration of immobilized peptides by combustion analysis

C, H analysis was done on Carlo Erba CHN analyser for all types of the sorbents. The concentration of attached ligands was determined as follows [9,13]:

$$c(\text{groups per nm}^2) = 6\times 10^5\,\Delta P_C/[(1200\,n_C - W\,\Delta P_C)S]$$

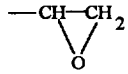
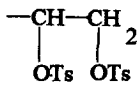
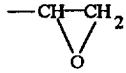
where c is the concentration of attached peptides, ΔP_C is the increase of carbon percentage in the sample due to ligand immobilization, n_C is the number of carbon atoms in the peptide, W is the formula weight of the peptide, and S is the specific surface area of the silica support (m^2/g).

Characteristics of the affinity sorbents synthesized are summarized in Table 2.

2.8. Affinity chromatography

The traditional column chromatography technique was used. (This technique implies that all the enzyme binds with the sorbent and does not move along the column during the elution with initial

Table 2
Characteristics of affinity sorbents synthesized

No.	Active groups on the initial silica	Ligand attached	Concentration of the ligand attached[a]	
			mg/g	groups per nm^2 $\times 10^2$
1		Bacitracin	0.1–10.0	0.03–3.0
2		-"-	0.1–10.3	0.03–3.1
3	$-(CH_2)_{11}OTs$		0.1–18.7	0.03–5.6
4	$-CH_2Br$	-"-	0.1–13.0	0.03–3.9
5	$-(CH_2)_3Br$	-"-	0.1–18.5	0.03–5.6
6	$-(CH_2)_5Br$	-"-	0.1–12.6	0.03–3.8
7		Gramicidin	0.1–12.0	0.04–4.8
8	$-CH_2Br$	-"-	0.1–5.1	0.04–2.0
9	$-(CH_2)_3Br$	-"-	0.1–9.2	0.04–3.7
10	-(CHz)SBr	-"-	0.1–7.1	0.04–2.8

[a] Four samples have been obtained within the range of concentration given for each type of activated silicas and each ligand. Totally forty sorbents have been studied for affinity binding of propteinases.

buffer. The elution of the enzyme occurs after the change of the eluent, and the enzyme moves together with the front of the eluent.) The column (40×4 mm) with sorbent was covered with the proteinase solution, which contained 0.5–3.0 mg of enzyme per milliliter of buffer solution (0.05 *M* Tris·HCl, 0.15 *M* NaCl, pH 8.0). The following enzymes were studied: bovine trypsin (Spofa, Prague), subtilisin (alkali proteinase of *Bacillus subtilis*, Ladyzhinsky ZFP, USSR) and human thrombin (Diagnostikum, Lvov, USSR). Sorption of proteinases was carried out under dynamic conditions. Elution was carried out using 1 *M* NaCl aqueous solution containing 25% of iso-propyl alcohol. The flow-rate was 0.05–0.15 ml/cm^2 min. The total amount of the protein eluted *M* (gram) and its activity *E* (units of activity) were also determined.

It should be noted that the sorbents studied are quite stable under the chromatographic conditions used. The decrease of affinity binding was revealed only after 2000–3000 elutions.

2.9. Determination of specific activity of the proteinases

2.9.1. Thrombin

Prothrombin complex was isolated from Kohn III fraction of human plasma using PEG-115 (ZTOS, Ivano-Frankivsk, Ukraine). Prothrombin activation was done using purified tissue thromboplastin. Starting coagulative activity was 125–225 NIH units per mg of the protein.

The coagulative activity was obtained from the coagulation time of 1 ml of 0.1% solution of fibrinogen in 0.01 *M* Tris·HCl (pH 7.3), containing 0.15 *M* NaCl, at 37°C. The amount of the enzyme yielding a fibrinogen clot for 15 s was taken as 1 NIH unit.

2.9.2. Trypsin

A 43.5 mg amount of $N^{(\alpha)}$-Bzl-dl-Arg-*p*NA·HCl were dissolved in 2 ml of DMFA under heating, then 100 ml of 0.05 *M* Tris·HCl (pH 8.0) were added.

The resulting concentration of the substrate is 0.001 *M*.

A 2 ml volume of the substrate and 0.1 ml trypsin solution were placed into a test tube, and then the mixture was allowed to stand at 37°C for 1 min. After this the reaction was stopped by the addition of 1 ml of 30% acetic acid. The activity of the enzyme was determined by an increase of extinction at 405 nm. The specific activity was determined using the Eq. (1):

$$S = (E_{405} - E'_{405})/V\ c\ t, \quad (1)$$

where S is the specific activity of trypsin, E_{405} is the extinction of the sample under the study at 405 nm, E'_{405} is the extinction of the control sample at 405 nm, V is the volume of the sample under study, c is the concentration of this sample, and t is the time of the reaction.

2.9.3. Subtilisin

A 500 mg amount of human haemoglobin was dissolved in 20 ml 0.05 *M* Tris·HCl (pH 8.0). Then the solution was filtered and placed into test tubes (1 ml of the solution per tube). The test tubes were allowed to stand at 37°C for 15 min. Then the solution of the enzyme was added to the test tubes and the mixture was allowed to stand at 37°C for 15 min. The reaction was stopped by an addition of 5 ml of 5% trichloroacetic acid. The residue was filtered, and the extinction at 280 nm was measured. Specific activity was determined using Eq. (1).

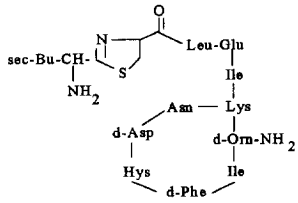
2.10. Spin labeling and ESR measurements

Spin labeling procedures and ESR measurements were described in our earlier paper [14].

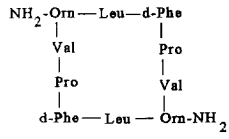
3. Results and discussion

3.1. One- and two-site attachment of the ligands

The structures of the ligands studied are as follows:



Bacitracin A



Gramicidin S

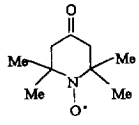
One can see that these ligands contain two primary amino groups per molecule. Thus, they can react with a modified silica by either one or both of them, to yield one- or two-site attachment (Fig. 1A resp. Fig. 1C). Indeed, it was demonstrated [14–18] that polyfunctional ligands (in particular bacitracin [14]) can be attached to a surface either by one- or by two- or more sites.

In the present work we used three types of activated silicas for the immobilization of the ligands (Table 1). The main distinction between these carriers lies in the different surface concentration (C_s) of active groups and, therefore, in average distances (d_{av}) between attached groups. Note that d_{av} and C_s are related with the following equation [19,20]:

$$d_{av}(\text{nm}) = 1.075/C_s(\text{groups per nm}^2)$$

Evidently, d_{av} can markedly affect the binding manner of polyfunctional ligands.

To investigate whether the ligands are bound to the surface by one or two sites, the spin labeling method was used. The essence of the method is that a sorbent is treated with an excess of radical I [14].



Radical I

Radical I is known to react readily with primary amino groups yielding Schiff bases. At the same time, it does not react with secondary and tertiary amines under mild conditions [21–23]. Hence, the amount of I that is immobilized is equal to the amount of one-site attached ligands. The amount of I and, therefore, the share of one-site attachment can be determined by ESR spectroscopy.

The results obtained (Table 3) show that for epoxy-activated silicas practically 100% of ligands

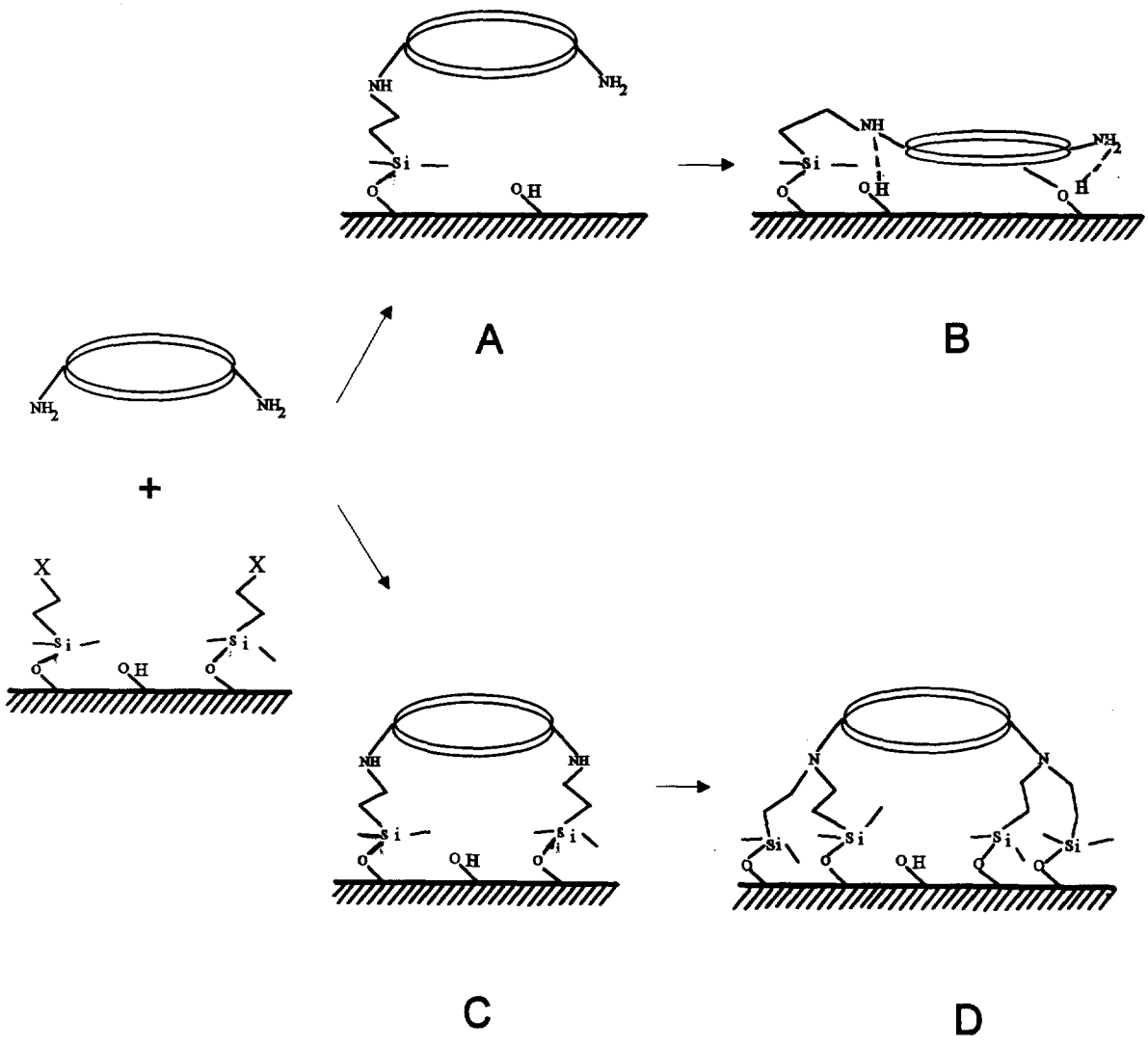


Fig. 1. Interactions of bacitracin and gramicidin with the surface of activated silicas. A – one site attachment; B – interaction between the ligand attached and the surface silanols; C – two-site attachement, once alkylated aminogroups; D – two-site attachment, twice alkylated aminogroups. Peptide rings of the ligands are presented schematically.

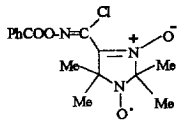
are one-site attached. At the same time, for bromoalkyl- and tosyl-activated silicas, the share of one-site ligand attachment is far smaller. It should be noted that the labeling with radical I was not done for all the sorbents. However, we believe that the data given in Table 3 are sufficient to demonstrate the difference between epoxy-sorbents (with no affinity properties) and other sorbents (with satisfactory affinity properties). We also believe this data to be sufficient to connect these properties with the number of bonds between ligand and support.

The next step of the study is the following. Does the alkylation of the ligands yield once alkylated aminogroups (Fig. 1C) or does this result in twice alkylated aminogroups of the ligands (Fig. 1D)? The question is whether attached aminogroups of the

Table 3
Share of one-site binding and affinity activity of the sorbents studied

Functional group of initial activated silica	Ligand attached	Average distance between bonded active groups (nm)	Share of one-site binding (%)	Activity in affinity binding of proteinases studied
—CH—CH_2 (O)	Bacitracin A	1.45	96±12	No
-$(CH_2)_{11}$OTs	-"-	0.97	22±3	Yes
—CH(OTs)—CH(OTs)—CH_2	-"-	0.95	23±3	Yes
-$(CH_2)_3$Br	-"-	0.72	0	Yes
—CH—CH_2 (O)	Gramicidin S	1.45	110±14	No
-CH_2Br	-"-	0.74	62±8	Yes
-$(CH_2)_3$Br	-"-	0.72	54±7	Yes

ligands are secondary or textiary? To study the problem we used radical II.



Radical II

It is common knowledge that oximoyl chlorides interact both with primary and secondary amines and do not react with tertiary ones. The results obtained showed that radical II did not immobilize on the sorbents synthesized from halogenoalkyl-activated silicas. Thus, practically all aminogroups of the attached ligands are at least twice alkylated (Fig. 1D).

3.2. Correlation between affinity activity and type of the ligand attachment

All the sorbents synthesized were studied in affinity binding – of human thrombin, bovine trypsin and subtilisin. The results obtained show that all the sorbents prepared from epoxy-activated silicas do not demonstrate specific binding of the proteinases studied over a wide range of ligand loading (Nos. 1 and 7, Table 2). However, the other sorbents (Nos. 2–6 and 8–10, Table 2) do exhibit biospecific binding of the enzymes studied.

We suggest that the loss in biospecific activity lies in the difference of the ligand binding manner. Namely, the greater average distance d_{av} for the epoxy-activated silica leads to the predominant formation of the one-site attached ligands. The latter has more mobility than two-site ones [14], and are likely to interact strongly with surface silanol groups yielding so-called arch structures (Fig. 1B). These structures are known to effect rather markedly the properties of the attached compounds [14,24–27]. We believe such interactions can distort the native spatial structure of the ligands. The latter can disturb the biospecific binding of enzymes. On the other hand, the ligands attached via two sites can hardly interact with surface silanols and, therefore, save its native conformation and affinity (Table 3).

It should be noted that we have no direct evidence that one-site attached ligands form arch structures more readily than two-site attached ones. However,

in our previous study [14] we have demonstrated that one-site attached ligand (bacitracin) is more flexible than the two-site attached one. Thus, we believe that one-site attached ligands have more chances to interact with surface silanol groups and to form arch structures. Besides, it should be noted that the remaining reactive groups of the sorbents were blocked with Tris–oxymethylaminomethane (Tris). The bulky molecules of Tris evidently decrease the probability of interaction between surface and non-flexible two-site attached ligand.

3.3. Effect of spacer length on affinity properties of the sorbents

The effect of spacer length on the biospecific properties was studied for the sorbents prepared from halogenoalkyl-activated silicas. The most available modifiers, which contain one, three, five and eleven methylene units, were used for the preparation of activated silicas.

The data on the biospecific binding of trypsin on these sorbents are presented at Fig. 2. As one can see, the maximal activity is observed for the sorbent which contains a spacer of three methylene groups.

We suggest the decrease in the biospecific binding for long-spacered sorbents is associated with the non specific sorption of proteins on the spacer. Besides, formation of arch structures is favoured by long flexible spacers [27]. On the other hand, an extremely short spacer (one methylene group) is not sufficient in the separation of a ligand from a surface. In our opinion, the latter also leads to the formation of unwanted arch structures. In addition, the closely spaced silica surface can prevent the biospecific sorption of an enzyme.

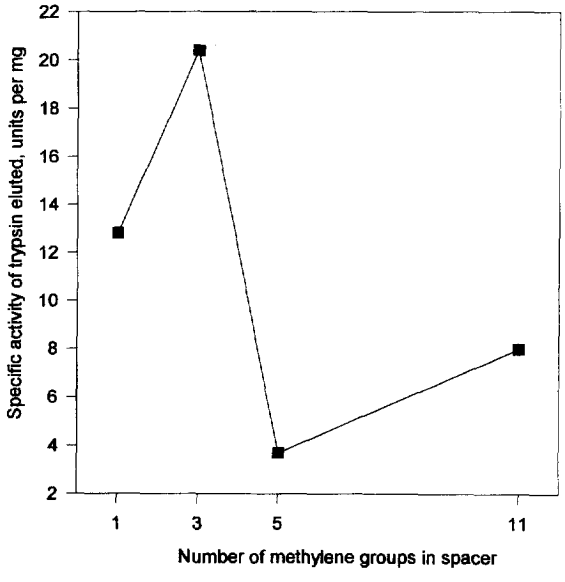


Fig. 2. Biospecific binding of trypsin with the bacitracin-bonded sorbents as a function of spacer length.

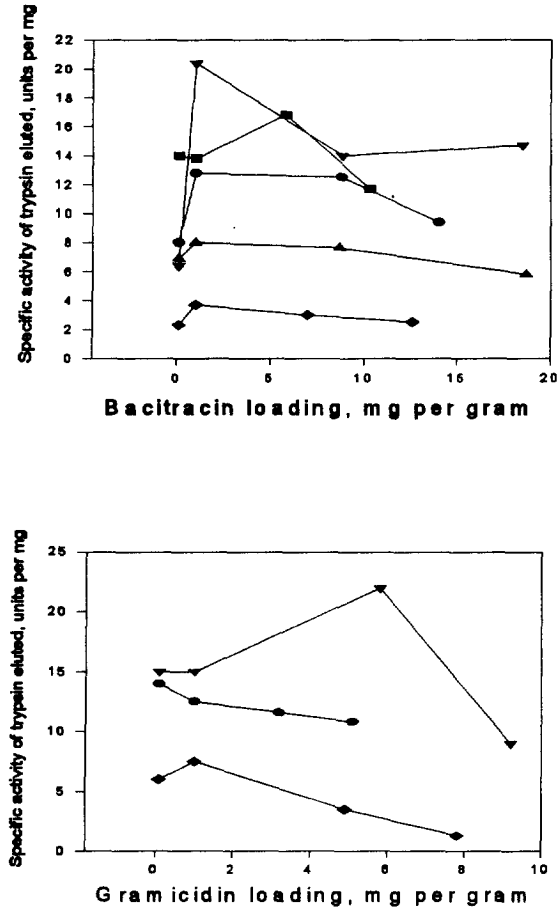


Fig. 3. Biospecific binding of trypsin on the bacitracin-bonded sorbents (top) and on the gramicidin-bonded sorbents (bottom). Functional groups of activated silica (designated as in Table 1): (▼) 3-Br; (●) 1-Br; (■) *bis*-OTs; (♦) 5-Br; (▲) 11-OTs.

We would like to note that the data obtained are not quite sufficient to draw the conclusion that the best spacer should contain exactly three methylene units. Maybe spacers of two or four methylenes would be better. Evidently, further investigation of this matter is desirable.

3.4. Effect of ligand loading on affinity properties of the sorbents

Fig. 3 describes the biospecific sorption of trypsin on the sorbents studied. The data demonstrates the progressive decrease of a specific activity of the enzyme eluted at a high ligand loading. We assume that such a reduction is associated with steric interactions between the enzyme and the attached neighboring ligands (steric hindrances). Analysis of possible reasons for this phenomenon can be found in Ref. [28]. It should be stressed that the dependencies presented in Fig. 3 have only four experimental points in each curve. Apparently, the maximum of the dependencies lies between the second and third point concentrations of a ligand.

For subtilisin, the results obtained are similar to those described in the literature [1–3]. Namely, the specific activity S (units of activity per mg) of the enzyme eluted (and its total amount) increases to a definite limit as the ligand loading is increased (Fig. 4). Such behavior can be associated with the formation of an enzyme monolayer on the surface [1–3].

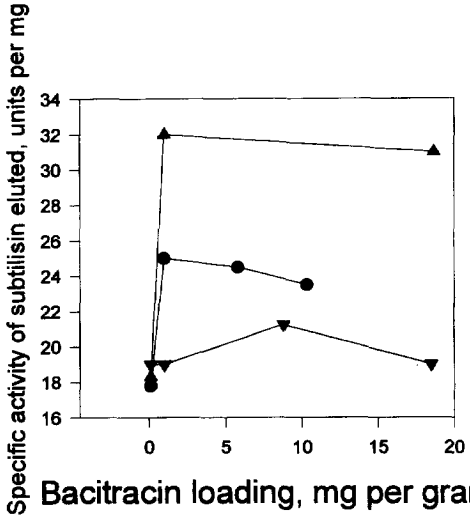


Fig. 4. Biospecific binding of subtilisin on the bacitracin bonded sorbents. Functional groups of activated silica (designated as in Table 1): (▼) 3-Br; (●) 1-Br; (■) *bis*-OTs; (▲) 11-OTs.

In the case of thrombin, the data obtained are very complex (Fig. 5). We believe the contributions of nonspecific interactions between the enzyme and bonded hydrophobic and ionogenic groups are considerable.

4. Conclusions

Silica sorbents containing immobilized cyclic oligopeptides bacitracin A and gramicidin S were synthesized. The sorbents obtained were studied for affinity binding of bovine trypsin, human thrombin and subtilisin.

It was demonstrated that:

(i) The ligands studied can be immobilized on the surface of activated silica by either one- or by two sites. The latter depends on the average distance between the active groups on the surface. It was shown that good biospecific activity was obtained for the sorbents containing the ligands attached to a surface by at least two sites. One-site attached ligands are shown to lose their affinity towards the enzyme studied.

(ii) The dependence of affinity properties on polymethylene spacer length are shown to have an extremum in the range of medium spacer length. Among the sorbents studied, the best one contains the ligands attached to a silica via a spacer of three methylene groups.

(iii) The dependence of affinity properties versus ligand loading for trypsin are also demonstrated to have an extremum.

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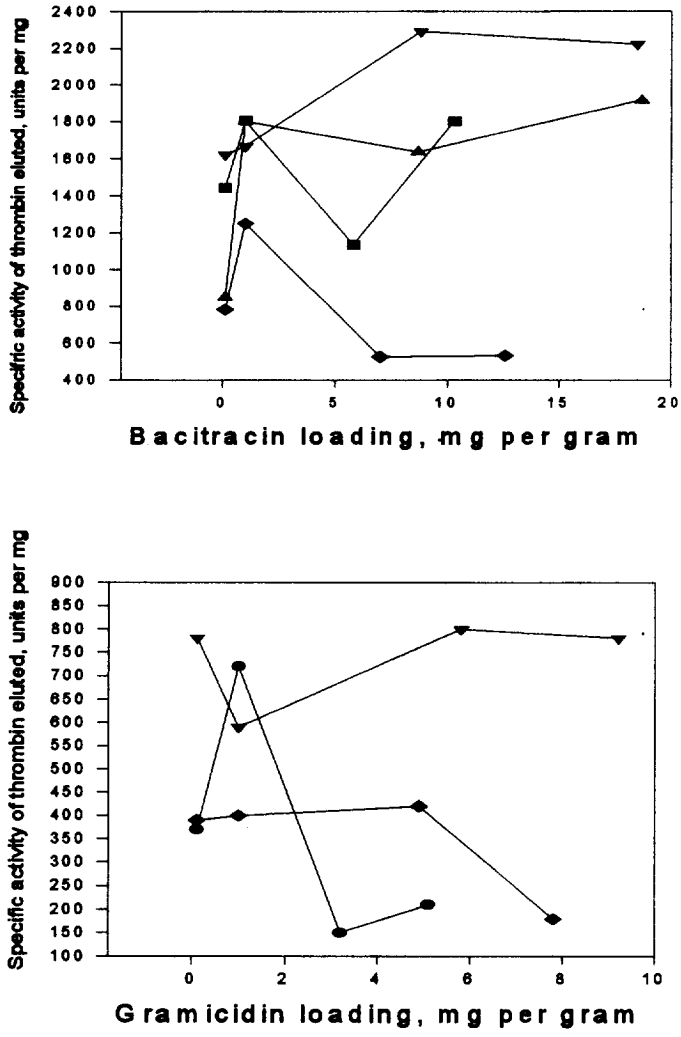

Fig. 5. Biospecific binding of thrombin on the bacitracin-bonded sorbents (top) and on the gramicidin-bonded sorbents (bottom). Functional groups of activated silica (designated as in Table 1): (▼) 3-Br; (●) 1-Br; (♦) 5-Br; (▲) 11-OTs.

References

[1] J. Turkova, Bioaffinity Chromatography, Journal of Chromatography Library, v. 55, Elsevier, Amsterdam, 1993.
[2] L.A. Osterman, Khromatografiya Belkov i Nukleinovykh Kislot (Chromatography of Proteins and Nucleic Acids), Nauka, Moscow, 1985, 536 p.
[3] P.D.G. Dean, W.S. Johnson, F.A. Midle (Eds.), Affinity Chromatography, IRL Press, Washington, DC, 1985.
[4] F. Regnier, R. Noel, J. Chromatogr. Sci. 14 (1976) 316.
[5] D. Wu, R.R. Walters, J. Chromatogr. 458 (1988) 169.
[6] M.E. Landgrebe, D. Wu, R.R. Walters, Anal. Chem. 58 (1988) 1607.
[7] A.G. Livingston, H.A. Chase, J. Chromatogr. 481 (1989) 159.
[8] D.J. Phillips, B. Bell-Alden, M. Cava, E.R. Grover, W.H. Mandeville, R. Mastico, W. Wawlivich, G. Vella, A. Weston, J. Chromatogr. 536 (1991) 95.
[9] P.G. Mingalyov, A.Yu. Fadeev, J. Chromatogr. 719 (1996) 291.
[10] K. Ernst-Cabrera, M. Wilchek, J. Chromatogr. 397 (1987) 187.
[11] G.B. Cox, C.R. Lossombe, M.L. Slutt, K. Sugden, J.A. Upfield, J. Chromatogr. 117 (1976) 269.
[12] P. Roumeliotis, K.K. Unger, A.A. Kurganov, V.A. Davankov, J. Chromatogr. 255 (1983) 51.

[13] G.V. Lisichkin (Ed.), Modifitsirovannye Kreznnezemy v Sorbtsii, Katalize i Khromatografii (Modified Silicas in Sorption, Catalysis and Chromatography), Khimia, Moscow, 1986.
[14] A.Yu. Fadeev, P.G. Mingalyov, S.M. Staroverov, E.V. Lunina, G.V. Lisichkin, A.V. Gaida, V.A. Monastyrsky, J. Chromatogr. 596 (1992) 114.
[15] G.L. Kamalov, O.V. Zakolodyazhnaya, A.V. Manolova, Yu.V. Gavsevich, R.N. Lozitskaya, Yu.O. Zakolodyazhnaya, Zh. Obshch. Khim. 62 (1992) 687.
[16] M.A. Ditzler, G. Doherty, S. Sieber, R. Allston, Anal. Chim. Acta. 142 (1982) 305.
[17] L.A. Belyakova, I.N. Polonskaya, A.Ye. Pestunovich, N.N. Vlasova, M.G. Voronkov, Dokl. Acad. Nauk SSSR 299 (1988) 643.
[18] K. Jinno, K. Yamamoto, H. Nagashima, T. Ueda, K. Itoh, J. Chromatogr. 517 (1990) 193.
[19] K.K. Unger, Packings and Stationary Phases in Chromatographic Techniques, Marcel Dekker, New York, 1990.
[20] A.Yu. Fadeev, S.M. Staroverov, J. Chromatogr. 447 (1988) 103.
[21] K. Murayama, J. Synth. Org. Chem. Jap. 29 (1971) 366.
[22] K. Murayama, J. Suzuki and S. Amoru, Jpn. Patent 7100103, 1971.
[23] L.D. Hall, J.C. Waterton, J. Amer. Chem. Soc. 101 (1979) 3697.
[24] D.E. Leyden, E.S. Kendal, L.C. Burggraff, F.L. Pern, M. de Bello, Anal. Chem. 54 (1982) 101.
[25] G.V. Kudryavtsev, G.V. Lisichkin, V.M. Ivanov, Zh. Anal. Khim 38 (1983) 22.
[26] S.G. Caravajal, D.E. Leyden, G.R. Quinting, G.E. Maciel, Anal. Chem. 60 (1988) 1776.
[27] P.G. Mingalyov, A.Yu. Fadeev, S.M. Staroverov, G.V. Lisichkin, E.V. Lunina, J. Chromatogr. 646 (1993) 267.
[28] P.G. Mingalyov, A.Yu. Fadeev, J. Chromatogr. A 764 (1997) 21.