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# AFFINITY CHROMATOGRAPHY OF CARBOXYLIC PROTEINASES ON A POLYMERIC SORBENT CONTAINING GRAMICIDIN-C AS LIGAND

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A new polymeric sorbent for proteinases has been synthesized by the radical copolymerization of N-vinylpyrrolidone, bis-$N^{\delta}$-acryloylgramicidin C, and N,N'-methylenebisacrylamide. Biospecific chromatography on the new sorbent has enabled an industrial preparation of procine pepsin to be purified by a factor of 2.5. With the aid of the new sorbent, a carboxylic proteinase has been isolated from the industrial preparation Tsellolignorin with a 15-fold purification factor.

Affinity chromatography on sorbents consisting of insoluble supports (Sepharose, Aminosililochrom) covalently linked with the polypeptide antibiotic gramicidin-C is being widely used for the isolation and purification of carboxylic proteinases [1, 2]. However, the low solubility of the antibiotic in water leads to great losses of it during the synthesis of the sorbents, a consequence of which is the low degree of inclusion of the ligand even at high concentrations of gramicidin-C. It is impossible to overcome this difficulty by using organic solvents in the synthesis, since, as a rule, the polysaccharide matrix withstands only relatively low concentrations of them. The maximum inclusion of gramicidin-C in the synthesis of the Sepharose sorbents is 5-8 μmole/ml of sorbent.

The sorbents based on Aminosilochrom [2] are resistant to the action of organic solvents but their synthesis requires a large excess of antibiotic, and the inclusion of the ligand is also low.

To synthesize sorbents with a regulable inclusion and distribution of the ligand it is desirable to use the method of the ternary copolymerization of the monomers forming the matrix and of the ligand modified by the introduction into its molecule of a residue containing a double bond [3]. As the monomers we used N-vinylpyrrolidone, N,N'-methylenebisacrylamide, and bis-$N^{\delta}$-acryloylgramicidin-C. As can be seen from Table 1, a change in the ratio of the monomers led to different inclusions of gramicidin-C in the polymeric matrix.

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TABLE 1. Some Characteristics of the Sorbents Obtained

| Sorbent | AG/VP* molar ratio in the mixture of monomers | Specific volume of swelling, ml/g of dry sorbent | Theoretical amount of AG in the copolymer, μmole/g of sorbent† | Amount of AG in the copolymer from results of amino acid analysis | |
|---|---|---|---|---|---|
| | | | | μmole/g of sorbent | μmole/ml of gel |
| 1 | 1:100 | 16 | 70 | 74 | 4.6 |
| 2 | 1:80 | 16 | 88 | 87.7 | 5,4 |
| 3 | 1:60 | 9,5 | 124 | 44 | 5 |
| 4 | 1:50 | 8,5 | 139 | 71 | 8 |
| 5 | 1:40 | 8 | 165 | 93 | 12 |
| 6 | 1:30 | 7,8 | 205 | 119 | 15 |
| 7 | 1:20 | 7,7 | 283 | 157 | 20 |

* AG) Bis-$N^{\delta}$-acryloylgramicidin-C; VP) N-vinylpyrrolidone.
† Calculated on the assumption of the quantitative inclusion of gramicidin-C in the copolymer.

All the sorbents obtained possessed approximately the same sorption capacity but the most economic syntheses permitting 100% inclusion of the antibiotic to be achieved were those in which 80- and 100-fold excesses of E-vinylpyrrolidone in relation to the bis-$N^{\delta}$-acryloylgramicidin-C, were taken.

The sorbents that we obtained swelled well in water, were stable over a wide pH range, were not broken down by organic solvents, and were not affected by enzymes. The absence of a charge from the matrix brought the possibility of nonspecific sorption to a minimum. In contrast to chromatography on the sorbents described previously, the sorption of porcine pepsin on the new synthetic polymeric sorbent took place at pH 2.1, while the elution of the active enzyme was carried out under mild conditions by changing the pH of the buffer solution from 2.1 to 5.2 and did not require the use of salt solutions or alcoholic solutions. Apparently, only that conformation of the pepsin that is realized at a pH close to the pH optimum of the action of the enzyme, ensures correspondence of the matrix-bound ligand to the zone of binding of the pepsin.

A single chromatographic separation of an industrial preparation of porcine pepsin permitted a highly active enzyme (55 activity units/optical unit) to be isolated in quantitative yield with a purification factor of 2.5 (Fig. 1). The chromatography on the new sorbent of the industrial preparation Tsellolignorin, which contains carboxylic and serine proteinases together with a group of cellulolytic enzymes, permitted the carboxylic proteinases to be isolated with a purification factor of 15 (Fig. 2).

## EXPERIMENTAL

The enzymes used were porcine pepsin (BC 3.4.2.3.1) from the Olaine chemical reagents factory, and the cellulolytic industrial preparation Tsellolignorin P10Kh from a culture of the microscopic fungus Trichoderma lignorum. The proteolytic activity of the pepsin was determined from the hydrolysis of hemoglobin [4]. The proteolytic activity of the carboxylic proteinase from Trichoderma lignorum was determined from the curdling of milk [5]. The inclusion of gramicidin-C was determined from the results of amino acid analysis of a hydrolysate of the sorbent (5.7 N HCl, 105°C, 72 h) on a Biocal BC-200 analyzer (FRG).

Synthesis of Bis-$N^{\delta}$-acryloylgramicidin-C. A solution of 1.2 g (4 mmole) of gramicidin-C dihydrochloride in 120 ml of chloroform was washed with a 3% solution of sodium bicarbonate (3 × 30 ml) and then with water to neutrality. The solution was evaporated to dryness, the residue was dissolved in 100 ml of chloroform, and the solution was treated with 0.38 ml (4 mmole) of absolute triethylamine. Then 0.31 ml (4 mmole) of acryloyl chloride was added, slowly, with stirring to the solution and the reaction mixture was left at room temperature for 2 h and was then washed with a 3% solution of sodium bicarbonate (3 × 30 ml) and with water to neutrality and was evaporated to dryness, after which the dry residue was dissolved in methanol and was reprecipitated with ether. The precipitate was filtered off and dried in a vacuum desiccator over $P_2O_5$. Yield 0.99 g (81%), mp 275°C (with decomposition). $R_f$ 0.42 (TLC on Silufol; chloroform–ethanol (9:1); on electrophoresis (pH 2.5, 1000 V, 1 h) the substance remained at the start.

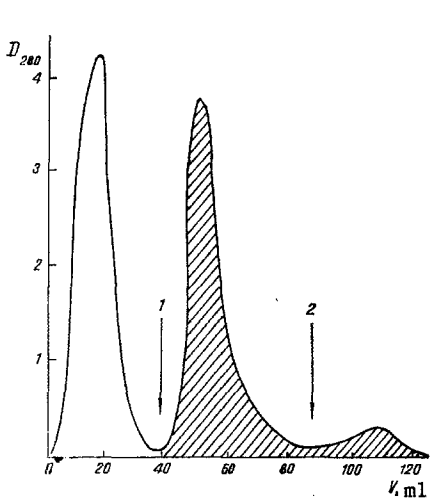


Fig. 1

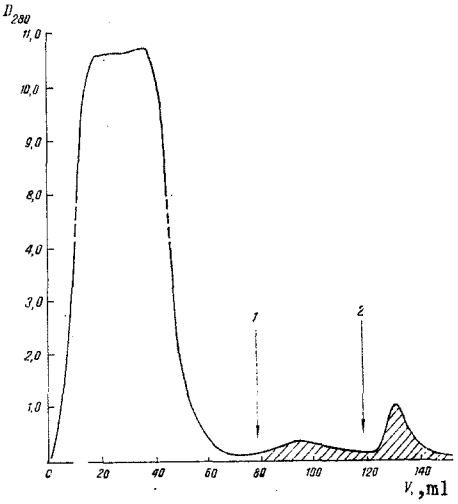


Fig. 2

Fig. 1. Chromatography of an industrial preparation of porcine pepsin on a polymeric sorbent containing 4.6 μmole/ml of gramicidin-C: column (1 × 3.5 cm) equilibrated with 0.05 M glycine-HCl buffer, pH 2.1. The amount of protein deposited was 25 mg in 12.5 ml of 0.05 M glycine-HCl buffer, pH 2.1. The arrows show the beginning of elution: 1) 0.3 M acetate buffer, pH 5.1; 2) 25% isopropanol in 0.3 M acetate buffer, pH 5.2. The peaks containing the activity are hatched.

Fig. 2. Chromatography of an industrial preparation of Trichoderma ligorum on a polymeric sorbent containing 5 μmole/ml of gramicidin-C: column (1 × 1.5 cm) equilibrated with 0.05 M glycine-HCl buffer, pH 2.1. The amount of preparation deposited was 1 g in 50 ml of 0.05 M glycine-HCl buffer, pH 2.1. The arrows show the beginnings of elution with: 1) 0.3 M acetate buffer, pH 5.2; and 2) 20% of isopropanol in 0.3 M acetate buffer, pH 5.2.

Found %: C 63.94; H 7.58; N 12.52. $C_{66}H_{98}N_{12}O_{12}$. Calculated %: C 63.34; H 7.89, N 13.43.

Chromatography of Porcine Pepsin on a Sorbent Containing 4.6 μmole/ml of Ligand. A solution of 30 mg of pepsin with a specific activity of 24 activity units/optical unit in 15 ml of 0.05 M glycine-HCl buffer, pH 2.1, was filtered, and 12.5 ml of the filtrate was deposited on a column containing sorbent 1 (Table 1) (1 × 3.5 cm) equilibrated with the same buffer. The initial buffer eluted inactive impurities (55% of the amount of protein deposited) and a small amount (3.5%) of active protein. Enzyme with a specific activity of 55 activity units/optical unit was eluted by 0.3 M acetate buffer, pH 5.1, with a yield of 36% in terms of protein and 91% in terms of activity. The residual protein (5%) with a specific activity of 28 activity units/optical unit was eluted with 25% isopropanol in 0.3 M acetate buffer, pH 5.1. The total yield in terms of protein was 96% and in terms of activity 100%.

Isolation of a Carboxylic Proteinase from the Industrial Preparation Tsellolignorin. A solution of 1 g of the industrial preparation from Trichoderma lignorum in 50 ml of 0.05 ml of 0.05 M glycine-HCl buffer, pH 2.1 was centrifuged (18,000 rpm, 15 min), and 45 ml of the enzyme solution with a specific activity of 3.8 activity units/optical unit was deposited on a column containing sorbent 3 (Table 1) (1 × 1.5 cm) equilibrated with the same buffer. The initial buffer eluted a pigment and other inactive impurities amounting to 88% of the protein deposited. On elution with 0.3 M acetate buffer, pH 5.2, enzyme with a specific activity of 60 activity units/optical unit was desorbed with a yield of 1% in terms of protein and 14% in terms of activity. The residual protein, with a specific activity of 31 activity units/optical unit was eluted with 20% isopropanol in 0.3 M acetate buffer, pH 5.2, with a yield of 2% in terms of protein and 70% in terms of activity. The total yield was 91% in terms of protein and 84% in terms of activity,

SUMMARY

1. A new polymeric sorbent for proteinases has been synthesized by the radical copolymerization of N-vinylpyrrolidone, bis-$N^{\delta}$-acryloylgramicidin-C, and N,N'-methylenebisacrylamide.

2. Biospecific chromatography on the new sorbent has permitted the 2.5-fold purification of an industrial preparation of porcine pepsin.

3. With the aid of the new sorbent, a carboxylic proteinase has been isolated from the industrial preparation Tsellolignorin at a purification factor of 15.

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# SPIN-LABEL STUDY OF THE CONFORMATIONAL STATES OF POLYPEPTIDE MODELS OF HISTONES

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The conformational states of the regular polypeptides $(Gly\text{-}Lys\text{-}Gly)_n$, $(Ala\text{-}Orn\text{-}Gly)_n$, and $(Ala\text{-}Orn\text{-}Ala)_n$ have been studied by the spin-label method. Their behavior in solutions of guanidine hydrochloride and urea and in solutions of salts of bivalent metals does not contradict the presence of an extended levohelical conformation in their polypeptide chains. In $CaCl_2$ (5 N) solutions the polypeptides exhibit aggregation properties. A study of the behavior of the poly peptide at these temperatures has shown that with a rise in temperature there is a monotonic change in the structures of the polypeptide chains that is characteristic for a conformation of the polyproline-II type. Differences have been observed in the behavior of glycine- and alanine-containing polypeptides in the presence of sodium dodecyl sulfate with a change in the temperature.

To understand the molecular mechanisms of the interaction of histones with DNA in the formation of DNP complexes it is important to know the potential structural possibilities of the polypeptide chains of the histones participating in this interaction. According to modern ideas on the structure of chromatin [1, 2], such sections in histones are the N- and C-terminal segments which are enriched with basic amino acids. One of the approaches to the study of the conformation of features of the terminal sections of histones is the use of model polypeptides of regular structure with given sequences of amino acid residues.

With this aim, we have synthesized the polypeptides $(Gly\text{-}Lys\text{-}Gly)_n$, $(Gly\text{-}Orn\text{-}Gly)_n$, $(Ala\text{-}Orn\text{-}Gly)_n$, and $(Ala\text{-}Orn\text{-}Ala)_n$ [3, 4] modeling the N-terminal sections of bovine histones enriched with glycine and basic amino acid residues, and the C-terminal sections of histones H1 enriched with alanine and basic amino acid residues [5-7].

The conformational states of the regular polypeptides synthesized were investigated by the spin-label method. This method has found wide use in the solution of such problems as

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