AFFINITY CHROMATOGRAPHY OF PEPSIN USING PEPSTATIN FRAGMENTS AS LIGANDS OF SPECIFIC SORBENTS

E. N. Lysogorskaya, E. S. Oksenoit,
E. A. Morozova, I. N. Filonuk,
and V. M. Stepanov

UDC 577.156:543.544

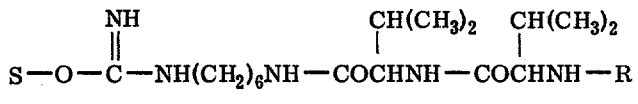
From a culture of actinomyces a N-acylated pentapeptide specifically inhibiting pepsin which has been called pepstatin has been isolated [1]. The results of a study of the mechanism of the action of pepstatin has shown that a large role in its interaction with pepsin is due to the hydroxy groups of the two 4-amino-3-hydroxy-6-methylheptanoic acid residues present in pepstatin and to hydrophobic interactions [2]. The N-terminal fragment of pepstatin is isovaleryl-L-valyl-L-valine. Apparently, this peptide, possessing high hydrophobicity, is capable of interacting with the substrate-binding zone in the pepsin molecule, participating in the formation of a pepstatin-pepsin bond.

In the present paper we consider the possibility of using valine peptides as ligands for the affinity chromatography of pepsin on hexamethylenediamine-Sepharose.

Four dipeptides differing in their acyl groups and in the configuration of the valine residues have been synthesized: isovaleryl- and benzyloxycarbonyl-L-valyl-D-valine and isovaleryl- and benzyloxycarbonyl-L-valyl-L-valine.

By using the cyanogen bromide activation method [3], hexamethylenediamine (HMDA) was attached to S Sepharose 4-B so as to space the ligands from the matrix in order to eliminate possible steric hindrance in the sorption of the enzyme. The dipeptides were covalently attached to HMDA-Sepharose after the conversion of the peptide into the p-nitrophenyl ester or by means of the water-soluble carbodiimide N-cyclohexyl-N'-[β-(4-methylmorpholinio)ethyl]carbodiimide p-toluenesulfonate (CMECDI). The results of an amino-acid analysis of the sorbents obtained showed that they contained 6-7 μmole of peptide per ml of gel.

In this way we obtained the following four sorbents:



R = $COCH_2CH(CH_3)_2$ (Iva) or $CO-OCH_2C_6H_5$ (Z)

I. S—HMDA—L—L—Iva; III. S—HMDA—L—L—Z

II. S—HMDA—D—L—Iva; IV. S—HMDA—D—L—Z

The sorption of the enzyme was carried out in 0.1 M acetate buffer, pH 4.5. For elution we used: I) a 1 M solution of sodium chloride in 0.1 M acetate buffer, pH 4.5, and II) a 20% solution of isopropanol in 1 M sodium chloride in the same buffer. The adsorbents obtained were first carefully washed with all the buffers that were used in the process of sorbing and eluting the enzyme. The course of chromatography was followed by determining the amount of protein from the absorption at 280 nm and the proteolytic activity by Anson's method [4]. The results of the chromatography of pepsin are given in Table 1.

Figure 1 shows the chromatography of pepsin with an initial specific activity of 17-19 units/mg of protein on isovaleryl-L-valyl-D-valyl-HMDA-Sepharose, with 43 mg of protein deposited on 1 ml of resin. The inactive protein was not retained on the column and was eluted by 0.1 M acetate buffer (2.3 ml; pH 4.5) and by 1 M sodium chloride solution. Desorption of the pepsin with 20% isopropanol gave an activity yield of the enzyme of

M. V. Lomonosov Moscow State University. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 387-392, May-June, 1977. Original article submitted January 4, 1977.

TABLE 1. Affinity Chromatography of Pepsin on HMDA-Sepharose with Covalently-Attached Valine Dipeptides

Expt.	Ligand	Initial specific act. of pepsin, units/mg	Deposited mg of protein/ml of sorbent	Buffer for elution of enzyme*	Activity yield of enzyme, %	Specific act. of pepsin aft. chromatography, units/mg
1	Isovaleryl-L-valyl-D-valine	17	43	I	14	73
				II	75	
2	Isovaleryl-L-valyl-D-valine	19	43	I	9,8	74,5
				II	85	
3	Benzyloxycarbonyl-L-valyl-D-valine	19	30	I	0,5	70
				II	96	
4	Benzyloxycarbonyl-L-valyl-D-valine	20	30	I	0,6	78
				II	91	
5	Isovaleryl-L-valyl-L-valine	13	127	I	62	45,5
				II	12	
6	Benzyloxycarbonyl-L-valyl-L-valine	12	86	I	8	45,5
				II	67	

*I) 1 M NaCl in 0.1 M acetate buffer (pH 4.5); II) 20% isopropanol in 1 M NaCl in acetate buffer (pH 4.5).

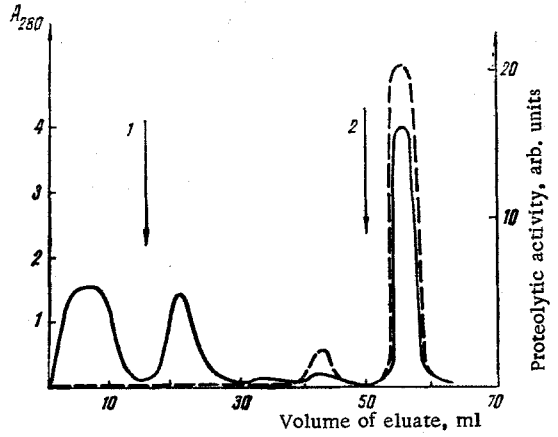


Fig. 1. Chromatography of pepsin on isovaleryl-L-valyl-D-valyl-HMDA-Sepharose (column 0.5 × 10 cm, beginning of elution shown by an arrow): 1) 1 M NaCl, pH 4.5; 2) 20% isopropanol in 1 M NaCl, pH 4.5. Full line) optical density of the elutae; dashed line) proteolytic activity. For the experimental conditions, see text.

75-85%. The specific activity rose to 74 units/mg of protein. Thus, the elimination of ballast protein raises the specific activity of pepsin fourfold (see Table 1, experiments 1 and 2).

Similar results were obtained in the chromatography of pepsin on Sepharose containing the dipeptide benzyloxycarbonyl-L-valyl-D-valine (Table 1, experiments 3 and 4). The enzyme was eluted by 20% isopropanol with a yield of 91-96% and with an increase in the specific activity to 70-78 units/mg of protein.

The interaction of pepsin and HMDA-Sepharose containing covalently attached isovaleryl-L-valyl-L-valine is less strong, elution taking place even with 1 M sodium chloride and giving a 62% yield with respect to enzymatic activity. The specific activity rose in this experiment from 13 to 45 units/mg through the elimination of ballast protein (Table 1, experiment 5). Elution with 20% isopropanol gave an additional 12% of active enzyme with the same specific activity.

The purification of pepsin on HMDA-Sepharose containing benzyloxycarbonyl-L-valyl-L-valine as ligand led to an increase in specific activity from 12 to 45.5 units/mg of protein. The enzyme was desorbed mainly by elution with isopropanol with a 67% activity yield. The ballast protein was easily washed out by the buffer and by sodium chloride solution, and the latter contained about 8% of the active enzyme (Table 1, experiment 6).

It follows from what has been said that pepsin is sorbed on all the sorbents obtained and its sorption has a specific nature, since the ballast protein is not bound under the same conditions.

The efficiency of the sorbents may be determined by the combined action of a number of factors:

a) hydrophobic interaction of the valine peptides with the hydrophobic sections forming the substrate-binding zone in the pepsin molecule. This interaction, of specific nature, apparently makes its contribution to the inhibiting action of pepstatin;

b) electrostatic interaction. Because of the possible incomplete acylation of the amino group in the hexamethylenediamine-Sepharose on condensation with the peptides under the conditions of chromatography, the sorbents may contain protonated amino groups, which leads to a stronger binding of the sorbents with the pepsin, bearing a negative charge. Protonated isourea groupings at the positions of attachment of the HMDA to the matrix may also take part in this interaction; and

c) with a considerable overloading of the column with ballast protein, the strength of binding of the enzyme to the sorbent falls (Table 1, experiment 5) and elution takes place under milder conditions (1 M sodium chloride in buffer), an enzyme being obtained with a specific activity not exceeding 45 units/mg of protein. Here the competing influence of the proteins, which must undoubtedly be taken into account, makes itself felt.

The sorbents investigated are effective in the purification of pepsin with a low initial specific activity when 30-40 mg of protein is deposited on 1 ml of sorbent (Table 1, experiments 1-4); in this case, a fourfold purification of the enzyme is achieved.

EXPERIMENTAL

Synthesis of the Peptides. Ethyl Ester of Isovaleryl-L-valyl-L-valine (I). To a solution of 16.5 mmole of the hydrochloride of the ethyl ester of L-valine in 200 ml of absolute nitromethane were added 16.5 mmole of triethylamine (TEA) and, after 30 min, 16.5 mmole of isovaleryl-L-valine and 19.8 mmole of dicyclohexylcarbodiimide (CDI). The mixture was left overnight at room temperature. After the complete elimination of the dicyclohexylurea, the filtrate was evaporated in vacuum to dryness, the residue was dissolved in ethyl acetate, and the solution was washed successively with 0.5 N HCl, water, 3% $NaHCO_3$, and water and was dried over Na_2SO_4 and evaporated in vacuum to dryness. After repeated treatment of the residue with absolute ether, 3.8 g (68%) of the peptide ester (I) was obtained with mp 121-123°C, $[\alpha]_D^4 -17.7°$ (c 1; ethanol). The dipeptide (I) had the composition $C_{17}H_{32}N_2O_4$. The substance was chromatographically and electrophoretically homogeneous.

Isovaleryl-L-valyl-L-valine (II). A mixture of 2 mmole of the dipeptide ether (I) and 8 ml of 0.5 N NaOH prepared by diluting 9 N aqueous alkali with ethanol was left at room temperature for 22 h. Then the solution was diluted with 50 ml of water, left for one hour in the refrigerator, and acidified with 1 N HCl. After an hour, the precipitate was separated off, washed with water, and dried in a vacuum desiccator over P_2O_5. It was recrystallized from hot methanol. The yield of dipeptide (II) was 78%, mp 190-191°C, $[\alpha]_D^4 -47.1°$ (c 1; methanol), composition $C_{15}H_{28}N_2O_4$. The substance was chromatographically and electrophoretically homogeneous.

According to the literature [5], mp 142-143.5°C, $[\alpha]_D^{20} -38.4°$ (c 1; methanol).

The same peptide that we obtained by means of the Schotten-Baumann reaction from L-valyl-L-valine and isovaleryl chloride was identical with (II) in all its properties.

The Ethyl Ester of Isovaleryl-L-valyl-D-valine (III) was obtained in a similar manner to (I) from the hydrochloride of the ethyl ester of D-valine and isovaleryl-L-valine. Yield of (III) 65%, mp 136-138°C, $[\alpha]_D^{24} -19.3°$ (c 1; ethanol), composition $C_{17}H_{32}N_2O_4$. The substance was chromatographically and electrophoretically homogeneous.

Isovaleryl-L-valyl-D-valine (IV) was obtained from (III) in a similar manner to (II). The yield of the dipeptide (IV) was 60%, mp 180°C, $[\alpha]_D^{24} -32.7°$ (c 1; ethanol).

The dipeptide (IV) had the composition $C_{15}H_{28}N_2O_4$ and was chromatographically and electrophoretically homogeneous.

The Ethyl Ester of Benzyloxycarbonyl-L-valyl-L-valine (V) was obtained from the hydrochloride of the ethyl ester of L-valine and benzyloxycarbonyl-L-valine in a similar manner to (I). The yield of the dipeptide ester (V) was 68%, mp 81-83°C. The dipeptide (V) had the composition $C_{20}H_{30}N_2O_5$ and was chromatographically and electrophoretically homogeneous.

Benzyloxycarbonyl-L-valyl-L-valine (VI) was obtained similarly to (II). The yield of the dipeptide (VI) was 70%, mp 129°C, $[\alpha]_D^{24} +8.5°$ (c 1; acetone). The substance was chromatographically and electrophoretically homogeneous. Literature data [6]: mp 132-135°C, $[\alpha]_D^{20} +7.4°$ (c 1; acetone).

The Ethyl Ester of Benzyloxycarbonyl-L-valyl-D-valine (VII) was obtained similarly to (I) from the hydrochloride of the ethyl ester of D-valine and benzyloxycarbonyl-L-valine. The yield of the dipeptide ester (VII) was 68%, mp 98°C. The dipeptide (VII) had the composition $C_{20}H_{30}N_2O_5$ and was chromatographically and electrophoretically homogeneous.

Benzyloxycarbonyl-L-valyl-D-valine (VIII) was obtained from (VII) similarly to (II). The yield of the dipeptide (VIII) was 73%, mp 179-180°C, $[\alpha]_D^{24}$ −10.2° (c 1; ethanol), and the substance was chromatographically and electrophoretically homogeneous. Literature data [6]: mp 184°C $[\alpha]_D^{21}$ −11.5° (c 1; ethanol).

p-Nitrophenyl Ester of Benzyloxycarbonyl-L-valyl-D-valine (IX). With stirring and cooling (0°C), 1.2 mmole of p-nitrophenol and 1.2 mmole of CDI were added to 1 mmole of (VIII) in 10 ml of absolute ethyl acetate. The mixture was left at room temperature for 1 h. Then the dicyclohexylurea was separated off and the solution was evaporated to dryness in vacuum. The oily residue was crystallized by treatment with absolute ether. After recrystallization from methanol, the yield of (IX) was 55%, mp 154-155°C, $[\alpha]_D^{24}$ +24.3° (c 0.8; DMFA). Substance (IX) had the composition $C_{24}H_{29}N_3O_7$ and was chromatographically and electrophoretically homogeneous.

Preparation of the Sorbents. Preparation of HMDA-Sepharose. Sepharose was activated with cyanogen bromide by Cuatrecasas's method [3] with a proportion of 250 mg of cyanogen bromide per 1 ml of Sepharose. Taking care that the temperature of the reaction mixture did not exceed 20°C, the pH of a suspension of 50 ml of Sepharose in 20 ml of water (initial pH 5) was brought to 11 by the careful addition of 8 M NaOH. Then 12 g of cyanogen bromide obtained immediately before the experiment was rapidly added to the Sepharose suspension and the pH of the medium was brought back to 11 by the addition of 8 M NaOH. The reaction mixture was poured rapidly on to a glass filter one quarter-filled with ice. The Sepharose was filtered off and washed with cooled 0.5 N $NaHCO_3$ solution (pH 10), and then to the Sepharose was rapidly added 50 ml of bicarbonate buffer (pH 10) and a solution of HMDA in 50 ml of cold water (previously titrated with 6 N HCl to pH 10). The mixture was stirred at 0°C for 2 h and at 4°C for 20 h. The HMDA-Sepharose was separated off, washed on the glass filter with 0.1 M $NaHCO_3$ (pH 10) and with water until the wash-waters were neutral and contained no free amine. The HMDA-Sepharose was left in the form of a suspension in water at 4°C.

Addition of the p-Nitrophenyl Ester of Benzyloxycarbonyl-L-valyl-L-valine (IX) to HMDA-Sepharose. A suspension of 5 ml of HMDA-Sepharose in 5 ml of 50% aqueous DMFA was prepared. After 15 minutes, the pH was brought to 9 with TEA and, with stirring, a solution of 120 mg of (IX) in 15 ml of aqueous DMFA was added, a pH of 9 being maintained with the addition of TEA. The suspension was stirred at 20°C for 2.5 h and was left overnight at 4°C. The sorbent was washed with 50% and 25% DMFA until the wash-waters showed no absorption peaks in the UV spectrum at 315 and 270 nm, and then with water to neutrality. The amount of added peptide, determined by means of an amino-acid analysis of a hydrolyzate, was 7 μmole per 1 ml of sorbent.

Addition of Isovaleryl-L-valyl-D-valine (IV) to HMDA-Sepharose. A suspension of 5 ml of HMDA-Sepharose in 5 ml of 50% DMFA was prepared. After 15 min, with stirring, a solution of 75 mg of the peptide (IV) in 4 ml of 50% DMFA, TEA to pH 4.8, and a solution of 265 mg of CME-CDI* in 2.5 ml of 50% DMFA were added. The mixture was stirred at 20°C for 22 h. The sorbent was washed with 50% and 25% DMFA until the peptide had completely disappeared from the wash-waters, and then with water to neutrality. An amino-acid analysis of a hydrolyzate showed that 1 ml of sorbent contained 6 μmole of peptide.

The Addition of Isovaleryl-L-valyl-L-valine (II) and Benzyloxycarbonyl-L-valyl-L-valine (VI) to DMFA-Sepharose was performed with the aid of the water-soluble carbodiimide CME-CDI as described in the preceding experiment. The sorbents obtained included 6-7 μmole of peptide per ml of gel.

The chromatography of pepsin on HMDA-Sepharose containing covalently attached isovaleryl-L-valyl-D-valine is shown in Fig. 1 (see Table 1, experiment 1). The results of the chromatography of pepsin on the other sorbents obtained are given in Table 1.

SUMMARY

1. Specific sorbents for the affinity chromatography of pepsin on hexamethylenediamine-Sepharose using valine peptides differing by the acyl groups and configurations of the valine residue have been synthesized.

2. The sorbents investigated are effective in the purification of pepsin with a low specific activity when 30-40 mg of protein is deposited on 1 ml of resin, a fourfold purification of the enzyme being achieved.

*N-Cyclohexyl-N'-[β-(4-methylmorpholio)ethyl]carbodiimide p-toluenesulfonate.

LITERATURE CITED

1. H. Umezawa, T. Aoyagi, H. Marishima, M. Matsuzaki, M. Hamado, and T. Takeuchi, J. Antibiotics, 23, No. 5, 259 (1970).
2. T. Aoyagi, H. Morishima, T. Takeuchi, and H. Umezawa, J. Antibiotics, 25, No. 4, 251 (1972).
3. P. Cuatrecasas, J. Biol. Chem., 245, 3059 (1970).
4. M. L. Anson, J. Gen. Physiol., 22, 79 (1938).
5. T. Aoyagi, H. Morishima, R. Nishizawa, S. Kinimoto, T. Takeuchi, H. Umezawa, and H. Ikezawa, J. Antibiotics, 25, No. 12, 689 (1972).
6. J. W. Hinman, E. L. Caron, and H. Christensen, J. Amer. Chem. Soc., 72, 1620 (1950); M. A. Niman and R. M. Herbst, J. Org. Chem., 15, 108 (1950).

ANALOGS OF OXYTOCIN WITH L- AND D-PENTAFLUOROPHENYLALANINES IN THE SECOND POSITION

O. A. Kaurov and M. P. Smirnova

UDC 547.466.1+547.583.1+541.69

On the basis of the results of a study of the biological activity of oxytocin analogs, we have previously put forward the hypothesis that the side chain of tyrosine, which is present in the second position of the hormone molecule, takes a direct part in hormone-receptor interaction, abandoning its original position in the receptor on stimulation of the chain of events leading to the biological effect [1], and therefore noncovalent interactions between the tyrosine and the receptor have an optimum nature. If the modification leads to a decrease in the intensity of noncovalent interactions, the affinity of the hormones for the receptor decreases correspondingly and, consequently, so does its biological activity. An increase in the intensity of interaction prevents the subsequent migration of the side chain, thus decreasing the capacity of the hormone-receptor complex formed for stimulating the biological effect. Consequently, such a modification may lead either to partial agonists or to antagonists of the natural hormone.

As can be seen from Table 1, a change in configuration of the modifying amino acid from L to D frequently leads to oxytocin antagonists. However, where the loss of biological activity is caused by an intensification of noncovalent interactions, the analogs with D-(amino acid)s either have a greater activity than the compound with the corresponding L-(amino acid)s (see compound (V)), or show more effective antagonism than [2-D-Phe]-oxytocin (compare compounds (IV) and VI)). If, however, the loss of biological activity is caused by a decrease in the affinity of the hormone for the receptor, the value of pA_2 for compounds with D-(amino acid)s is less than for [2-D-Phe]oxytocin (see compound (III)). The analog with phenylalanine in the second position of the oxytocin molecule occupies a boundary position between the groups of compounds under consideration.

It can also be seen from Table 1 that the interpretation of the results of a study of the dependence of the biological activity of the series of oxytocin analogs under consideration as a function of their structure is complicated by the fact that the structural modifications performed change the nature of the forces responsible for hormone-receptor interaction in an ambiguous manner. Any structural modification causes a change in some forms of noncovalent interactions and always to some degree or other changes the intensity of donor-acceptor interaction. And it is just this factor which apparently plays an important role in the stimulation of the biological effect.

In order to elucidate the influence of donor-acceptor interaction in the more or less pure form, it appeared to us to be of interest to synthesize analogs of oxytocin with a L- or D-pentafluorophenylalanine in position 2 and to compare their biological activities with those of [2-L- and -D-Phe]oxytocins.

A. A. Zhdanov Leningrad State University. Translated from Khimiya Prirodnykh Soedinenii, No. 3, pp. 392-398, May-June, 1977. Original article submitted February 16, 1977.