USE OF SEPHADEX LH-20 FOR SOLID-PHASE SYNTHESIS OF OXYTOCIN

I. É. Zel'tser, A. A. Antonov,
V. N. Karel'skii, E. A. Prikhodkina,
A. M. Ovsepyan, and E. E. Zhuravleva

UDC 547.964.4:543.544

The solid-phase synthesis of oxytocin on commercial Sephadex LH-20 is described. The biological activity of the product obtained was 132 AU/mg, and its oxytocin content, according to HPLC, was 28.2%. It was shown that the amount of oxytocin and the biological activity do not depend on the degree of addition of Gly^9 to the polymer matrix within the range of 0.6-1.6 mmoles of Gly^9/g of resin; after regeneration, the Sephadex LH-20 can be re-used for solid-phase synthesis of oxytocin.

Synthetic oxytocin is obtained both by the classical or liquid-phase method and by the solid-phase method, the main disadvantage of which is the formation of shortened and other erroneous sequences because of the incomplete occurrence of the condensation reaction and of side reactions. Recently, in connection with an improvement of methods for the purification of peptides and the developments of preparative HPLC permitting the separation of peptides of close molecular masses and structures, the solid-phase method of obtaining peptides has begun to acquire practical importance.

In the present paper we consider the stepwise solid-phase synthesis of oxytocin on commercial Sephadex LH-20, which has been used previously for the preparation by the solid-phase method of enkephalin and its analogs [1], and pentadecalysine and the nonapeptide analog of luliberin [2].

The first amino acid, BOCGlyOH or the dipeptide BOCLeuGlyOH was attached to the polymeric matrix by the carbodiimide method in the presence of an equivalent of pyridine [1] or with the use of BOC_2O in the presence of an equivalent of pyridine [3]. After the elimination of the N^{α}-BOC protective group from the C-terminal Gly^9 or Leu^8 by the action of 1 N p-toluenesulfonic acid in AcOH, the number of free NH_2 groups in the glycyl- or leucyl-glycyl-Sephadex was determined by Dorman's method [4]. The results obtained are shown in Table 1. As can be seen from the figures given, the N^{α}-BOC-dipeptide added to the polymeric matrix considerably less well than the N^{α}-BOC-(amino acid). The further growth of the polypeptide chain was carried out by the carbodiimide method, with the exception of the asparagine and glutamine residues, which were added by the activated-ester method. The capacity of the peptidylpolymer determined after condensation, acylation, and deblocking [4] remained constant after each cycle. The nonapeptide obtained was split off from the Sephadex by ammonolysis in MeOH–DMFA (5:1.5), and the N^{α}-BOC-S,S'-dibenzylnonapeptide amide with the sequence of oxytocin (I), after being purified by recrystallization, was identical in its physicochemical constants with the product obtained by the liquid-phase method (Table 2) [5]. The elimination of the S-benzyl protective groups by the action of sodium in liquid ammonia and of the N^{α}-BOC group by the action of HCl/AcOH followed by oxidation in water gave a mixture of peptides (II) containing oxytocin which was analyzed by the method of reversed-phase HPLC and biologically (see Table 2).

On deblocking followed by oxidation of the unpurified peptide (I), samples were obtained in which the amount of oxytocin was, according to HPLC and biological activity, practically the same as for samples obtained from recrystallized (I) (see lines 3-6 in Table 2), but the yield of oxytocin from 1 g of resin was approximately 1.5 times less.

The oxidation of the peptides was carried out in aqueous solution without stirring (c 1 mg/ml) at room temperature for 15 days (see Table 2). When air was bubbled into a solution of sample (I) (see Table 2) in 0.01 M ammonium acetate solution for 2 h and then,

All-Union Scientific-Research Institute of the Technology of Blood Substitutes and Hormone Preparations, Moscow. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 106-113, January-February, 1989. Original article submitted March 28, 1988.

 0009-3130/89/2501-0094 $12.50

TABLE 1. Conditions for the Addition of BOCGlyOH or BOCLeu-GlyOH to Sephadex LH-20

N^{α}-BOC-(amino acid) or -peptide	Condensing agent	Reaction time, h		Amount of free NH_2 groups, mmole/g of resin
		with stirring	without stirring	
BOCLeuGlyOH	BOC_2O	—	72	0,283
BOCLeuGlyOH	BOC_2O	7	65	0,445
BOCLeuGlyOH	BOC_2O	12	55	0,215
BOCLeuGLyOH	DCHC	5	17	0,126
BOCLeuGlyOH	DCHC	11	19	0,121
BOCGlyOH	DCHC	3 5	17	0,756
BOCGlyOH*	DCHC	7	17	0 705
BOCGlyOH	DCHC	3	20	0 605
BOCGlyOH**	DCHC	9	15	1,558
BOCGlyOH	DCHC	3	21	0,802

*Re-use of the Sephadex LH-20 after the separation of the N^{α}-BOC-S,S'-dibenzyloxytocin from the resin.
**The reaction was performed with a fourfold excess of BOCGlyOH and condensing agent (see the Experimental part).

with stirring, for 3.5 h, according to [6], a mixture of peptides was obtained with, according to HPLC, 11.5% of oxytocin and a biological activity of 60 U/mg.

Chromatographic analysis was carried out on an Ultrasphere ODS column using aqueous acetonitrile as eluent [7]. Figure 1 shows chromatograms of the mixture of peptides before (a) and after (b) oxidation. On the chromatogram of the mixture of peptides obtained after oxidation a signal appeared with a retention time of ~8 min, which was identified as oxytocin. The amount of oxytocin in the mixture of peptides obtained was determined from a calibration graph of the dependence of the concentration of a standard solution of oxytocin in water on the area of the peak on a chromatogram [7].

Biological activities of the samples of oxytocin were determined by a method based on the capacity of oxytocin for lowering the arterial pressure in birds [8].

We investigated the dependence of the concentration of peptide on the resin and also the influence of the acylation of the amino groups that had not reacted after condensation

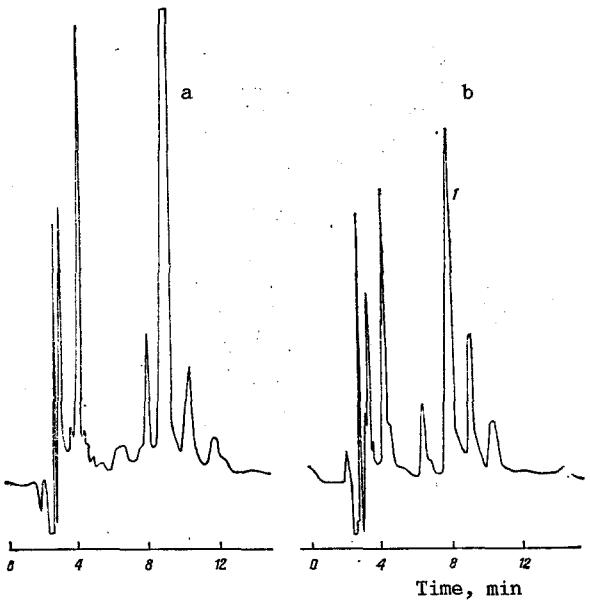

Fig. 1. Chromatogram of the mixture of peptides before (a) and after (b) oxidation. Conditions of separation: Ultrasphere ODS' column (4.6 × 240 mm); eluent – 0.1% solution of CF_3COOH–MeCN (76:24); temperature 20°C; rate of flow of eluent 1.5 ml/min; detection at 235 nm; 1) oxytocin.

TABLE 2. Conditions and Results of the Synthesis of Oxytocin on Sephadex LH-20

Experiment No.	Conditions of synthesis		Yield of (I)			Phys.-chem. constants of (I) after recrystallization		Amount of peptide (II) obtained from 1 g of resin, mg	Amount of oxytocin (before HPLC) in the mixture of peptides, %	Biol. activity, U/mg
	conc. of Gly^9, mmole/g of resin	acyl'd, NH_2 gr. after condens.	before crystallization, g/g resin	after recrystallization, g/g resin	after recrystallization, % on Gly^9	$[\alpha]_D^{20}$ (c 1; AcOH)	mp., °C			
1	0,756	+, apart from Asn, Gln	0,850	0,456	46,8	—60,5	233—236	195,3	20,0	90
2*	0,705	—	0,730	0,374	41,1	—58,0	233—235	99,8	25,5	131
3	0,605	—	0,635	0,437	56,0	—51,0	239—241	140,4	28,2	89
4	0,605	—	0,635	(I) was not recrystallized before deblocking				88,9	18,4	Not detected
5	1,558	+	1,705	0,723	36,2	—52,0	229—231	300,0	28,2	132
6	1,558	+	1,705	(I) was recrystallized before deblocking				232,2	26,4	131,8

*Re-use of Sephadex LH-20.

TABLE 3. Program for Addition of Leu[8], Pro[7], Cys[6], Ile[3], and Tyr[2]

Stage	Reagent	Volume, ml	Time for the operation, min	Number of repetitions
1	CH_2Cl_2	20	2	2
2	Condensation: twofold excess of BOC-(amino acid) in CH_2Cl_2, twofold excess of DCHC (1N solution in CH_2Cl_2)	25	2 H with stirring by a current of nitrogen and 18 h without stirring	1
3	CH_2Cl_2	20	2	2
4	MeOH	20	2	2
5	$CHCl_3$	20	2	2
6	Acylation: 1.4 ml of Ac_2O and 2 ml of Et_3N in $CHCl_3$	25	30	1
7	$CHCl_3$	20	2	2
8	MeOH	20	2	2
9	AcOH	20	2	2
10	Deblocking: 1 N solution of p-toluenesulfonic acid in AcOH	20	2 h	1
11	AcOH	20	2	2
12	MeOH	20	2	2
13	$CHCl_3$	20	2	2
14	10% solution of Et_3N in $CHCl_3$	20	5	1
15	$CHCl_3$	20	10	1
16	CH_2Cl_2	20	2	2
17	Determination of free NH_2 groups [4]	20	2	2

on the yield and biological activity of the end-product. As can be seen from the results obtained (see lines 1, 3, and 5 in Table 2) a doubling of the concentration of peptide on the resin did not lead to a change in the amount of oxytocin in the mixture of peptides and to a change in biological activity. Methylation of the NH_2 groups that had not reacted after the addition of the BOC-(amino acid)s likewise did not affect the yield and biological activity of the oxytocin in the mixture of peptides obtained (see lines 1 and 3 in Table 2).

The Sephadex LH-20 obtained after the ammonolysis of the peptidylpolymer was used again for the synthesis of oxytocin under the same conditions. The IR spectrum of the peptidyl-Sephadex contained bands at 1650 and 1520 cm^{-1} corresponding to the amide (I) and amide (II) vibrations of a peptide group [9] and also a band at 1740 cm^{-1} corresponding to the $\nu(C{=}O)$ vibrations of an ester bond [9]. The amide bands disappeared after the ammonolysis of the peptidylpolymer, while the band at 1740 cm^{-1} disappeared only after the hydrazinolysis of the resin.

After the addition of the first amino acid (Gly^9) to such a Sephadex and the removal of the N^{α}-BOC protective group, the concentration of NH_2 groups (mmole/g of resin) was the same as after the addition under the same conditions of Gly^9 to commercial Sephadex (see Tables 1 and 2). The yields and chemical constants of the N^{α}-BOC-S,S'-dibenzyloxytocin obtained both on the commercial Sephadex and on that isolated after the synthesis were identical (see Table 2). Furthermore, the mixture of peptides obtained after the deblocking and oxidation contained the same amounts of oxytocin according to HPLC results and possessed the same biological activity as that synthesized with the use of the fresh commercial Sephadex. Thus, Sephadex LH-20 can be used repeatedly for the solid-phase synthesis of peptides, which raises the amount of peptide that can be obtained from 1 g of resin (see Table 2).

EXPERIMENTAL

The IR spectra of the substances, molded into tablets with KBr, were recorded on a Perkin-Elmer model 682 instrument. Melting points of the samples were determined in open capillaries without correction, and angles of optical rotation on a polarimeter.

Sephadex LH-20 (Pharmacia) and derivatives of L-(amino acid)s were used.

HPLC analysis was carried out on a Beckman-Altex model 110A instrument equipped with a pump and an injector (model 210) with a 20-μl loop, and also with a Uvidec-100 spectrophotomer (Jasco) and a Chromatopac-PIA integrator (Shimadzu). An Ultrasphere ODS,

TABLE 4. Program for Adding Asn^5 and Gln^4

Stage	Reagent	Volume, ml	Time for the operation, min	Number of repetitions
1	DMFA	20	2	2
2	Condensation: fourfold excess of the p-nitrophenyl ester of a BOC-(amino acid) in DMFA	25	24 h	1
3	DMFA	20	2	2
4	MeOH	20	2	2
5	$CHCl_3$	20	2	2
6—17	For stages 6–17, see Table 3			

4.6 × 250 nm column (Altex) was used. The amount of sample introduced was 20-40 μg (20 μl of a 0.1-0.2% solution) for the materials under investigation. The samples for analysis were dissolved in the eluent, which included acetonitrile (Merck) of "for chromatography" grade and distilled water that had been passed through ion-exchange resins. Synthetic oxytocin purified by preparative HPLC, with a biological activity of 500 U/mg, was used as the standard. The amount of oxytocin in the mixture of peptides was determined according to [7].

Biological activities of the samples were determined by a method based on the capacity of oxytocin for lowering the arterial pressure in birds. For this purpose, with the aid of a pressure sensor (Nihon-Konden) the arterial pressure in the femoral arteries of chickens weighing 2.5 kg narcotized with urethane was measured. The preparation was injected into the femoral vein, and the change in the mean pressure on the introduction of doses differing by a factor of two of the preparations under investigations and the standard were measured. The activity was calculated from the formula

$$R = \text{antilog}\frac{0.3(T_1 + T_2 - S_2 - S_1)}{T_2 - T_1 + S_2 - S_1},$$

where R is the relative activity of the preparation, U/mg;

T_1 and T_2 are the mean values in the change in the arterial pressure on the injection of a smaller or larger dose of the preparation under test;

S_1 and S_2 are the mean changes in the arterial pressure on the injection of a smaller or larger dose of the standard preparation; and

0.3 is the logarithm of the ratio between the doses.

To obtain the absolute activity of a preparation, the relative activity was multiplied by the activity of the standard. The activity found had to be not less than 80% and not more than 125% of that of the standard. The determination of the activity of each sample was carried out not less than three times.

1. Preparation of the Glycylpolymer. A reactor for solid-phase synthesis was charged with 2 g of LH-20 resin, which was washed with CH_2Cl_2 (20 ml × 2 min), and then 0.7 g (4 mmole) of BOCGlyOH, 0.48 ml (6 mmole) of pyridine in 20 ml of CH_2Cl_2, and 6 ml of a 1 N solution of DCHC in CH_2Cl_2 was added. The reaction mixture was stirred with a current of nitrogen at room temperature for 5 h and was left for 12 h, and it was then filtered and the resin was washed successively with CH_2Cl_2, 2 × (20 ml × 2 min); MeOH, 2 × (20 ml × 2 min); and AcOH, 2 × (20 ml × 2 min). Then 20 ml of a 1 N solution of p-toluenesulfonic acid in AcOH was added to the resin, the mixture was stirred with a current of nitrogen for 2 h and was filtered off, and it was washed successively with AcOH, 2 × (20 ml × 2 min); MeOH, 2 × (20 ml × 2 min); $CHCl_3$, 2 × (20 ml × 2 min); a 10% solution of Et_3N in $CHCl_3$, 20 ml × 5 min and 20 ml × 10 min; and with $CHCl_3$, 2 × (20 ml × 2 min). According to Dorman's method [4], the amount of glycine was 0.756 mmole/g of resin. To obtain a glycylpolymer with a capacity of 1.558 mmole of NH_2 groups/g of resin, 1 g of LH-20 resin was treated with 4 mmole of BOCGlyOH, 6 mmole of pyridine, and 6 mmole of DCHC in 25 ml of CH_2Cl_2.

2. Preparation of N^{ε}-tert-Butoxycarbonyl-(S-benzyl)cysteinyltyrosylisoleucylglutaminyl-asparaginyl-(S-benzyl)cysteinylprolylleucylglycyl-Sephadex. To the glycylpolymer were added, successively, Leu^8, Pro^7, and Cys^6 by the program given in Table 3, Asn^5, and Gln^6 by the program given in Table 4, Ile^3 by the program of Table 3, Tyr^2 by the program of

Table 3 using a two- to five-fold excess of BOCTyrOH, and Cys[1] by the program of Table 3 without stages 6-17 of acylation and deblocking. The peptidylpolymer obtained was washed with CH_2Cl_2, 2 × (20 ml × 2 min) and with ether, 2 × (20 ml × 2 min), and was removed from the reactor, and dried at 0.1 mm Hg and 40°C to constant weight. The yield of peptidylpolymer was 3.75 g.

3. Separation of the Peptide from the Resin. Preparation of N^{α}-tert-Butoxycarbonyl-S,S'-dibenzyloxytocin. To 150 mg of MeOH–DMFA (5:1.5) saturated with ammonia (c 0.3-0.5 g/ml) was added 3.75 g of the peptidyl-Sephadex, and the mixture was left at room temperature without stirring for 24 h and was then stirred for 8 h and left again without stirring for 18 h. The solid matter was filtered off and was washed with MeOH (2 × 10 ml), hot (50°C) DMFA (3 × 10 ml), MeOH (2 × 10 ml), and ether, (2 × 10 ml). The filtrate was evaporated in vacuum and the residue and the resin were dried at 0.1 mm Hg and 40°C to constant weight. This gave a mixture of peptides containing 1.700 g of (I) and 2.050 g of Sephadex. Compound (I) was recrystallized from MeOH (see Table 2).

4. Deblocking of the SH Groups of Cysteine. Preparation of N^{α}-BOC-S,S'-Dihydrooxytocin. With boiling and stirring, 20 ml of a 0.4 mM solution of metallic sodium in liquid ammonia was added to a solution of 0.85 g ($6.59 \cdot 10^{-4}$ mole) of (I), obtained after recrystallization, in 75 ml of liquid ammonia until the blue coloration persisted for 45 sec, and then 1.6 g of ammonium acetate was added to achieve decolorization, and the ammonia was evaporated off in a current of nitrogen. The residue was treated with 70 ml of 5% AcOH solution and was filtered off and was washed with 5% AcOH (2 × 15 ml), H_2O (2 × 10 ml), acetone (2 × 15 ml), and ether (2 × 15 ml) and was dried at 0.1 mm Hg and 40°C to constant weight. This gave 0.45 g of N^{α}-BOC-S,S'-dihydrooxytocin (mp 227-229°C; $[\alpha]_D^{20}$ − 53° (c 1; AcOH)).

5. Preparation of S,S'-Dihydrooxytocin Hydrochloride. A solution of 0.45 g of N^{α}-BOC-S,S'-dihydrooxytocin in 7 ml of glacial AcOH was treated with 4 ml of 2.58 N solution of HCl in glacial AcOH and the mixture was kept for 4 min, after which 80 ml of ether was added and the precipitate was filtered off, washed with ether (2 × 15 ml) and dried at 0.1 mm Hg and 40°C to constant weight. This gave 0.408 g of S,S'-dihydroxytocin hydrochloride (mp 186-192°C, $[\alpha]_D^{20}$ −61 to −66° (c 1; H_2O).

6. Oxidation of S,S'-Dihydrooxytocin Hydrochloride. Preparation of Oxytocin. A solution of 0.05 g of S,S'-dihydrooxytocin in 50 ml of distilled water was filtered and left at room temperature in a flask closed with a polyethylene stopper for 15 days and was then lyophilized. This gave 0.048 g of a white powder, which was analyzed by the HPLC method and biologically for its oxytocin content (see Table 2).

7. Hydrazinolysis of the Sephadex. To 2.05 g of the Sephadex obtained after the ammonolysis of the peptidyl-Sephadex (see paragraph 3), was added 2 ml of hydrazine hydrate in 20 ml of MeOH; the mixture was stirred at room temperature for three days, and then the resin was filtered off, washed with MeOH (3 × 10 ml) and with ether (2 × 10 ml) and was dried at 0.1 mm Hg and 40°C to constant weight. This gave 1.95 g of Sephadex LH-20.

SUMMARY

1. A mixture of peptides with biological activity of 132 Eu/mg, the amount of oxytocin in which was, according to HPLC, 28.2%, has been obtained by the solid-phase method on Sephadex LH-20.

2. It has been shown that the amount of oxytocin in the product obtained and its biological activity do not depend on the concentration of Gly^9 in the glycyl-Sephadex in the range of 0.6-1.6 mmole/g of resin.

3. It has been shown that the Sephadex LH-20 can be re-used for the solid-phase synthesis of oxytocin.

LITERATURE CITED

1. G. P. Vlasov and A. Yu. Bilibin, USSR Inventor's Certificate No. 250153, cl. C 07 c 104/52; Byull Izobret., No. 26, p. 24 (1969); G. P. Vlasov, V. A. Gusel', N. Yu. Kozhevnikova, V. N. Pavlov, N. G. Illarionova, I. B. Ditkovskaya, E. N. Krasnova, M. Yu. Dorosh, Yu. B. Moskvicheva, I. G. Denisov, and O. S. Veselkina, Bioorg. Khim., 12, No. 5, 591 (1986).

2. G. I. Aukone, I. L. Gibiete, R. M. Stamere, and U. O. Kalei, in: Abstracts of Lectures at the Vth All-Union Conference on Methods of Obtaining and Analyzing Biochemical Preparations [in Russian], Riga (1987), p. 280.
3. V. F. Pozdnev, Bioorg. Khim., 11, No. 5, 583 (1985); V. F. Pozdnev, N. N. Podgornova, I. K. Yakaite, and U. O. Kalei, Bioorg. Khim., 12, No. 7, 898 (1986).
4. L. C. Dorman, Tetrahedron Lett., No. 28, 2319 (1969).
5. K. Sturm, R. Geiger, and W. Siedel, Swiss Patent No. 481883, cl. C 07 c 103/52 (1969).
6. K. Sturm, R. Geiger, and W. Siedel, Swiss Patent No. 462190, cl. C 07 c 103/52 (1968).
7. I. É. Zel'tser, A. A. Antonov, A. K. Ivanov, V. P. Pakhomov, and V. N. Karel'skii, Khim. Prir. Soedin., 892 (1987).
8. I. A. Éskin, Principles of the Physiology of the Endocrine Glands [in Russian], Vysshaya Shkola (1975), p. 237.
9. L. Bellamy, Infrared Spectra of Complex Molecules, 2nd edn., Wiley, New York (1958) [Russian translation, IL, Moscow (1963), pp. 258 and 319].

WATER-SOLUBLE PROTEINS OF THE SEEDS OF SOME VARIETIES OF CASTOR-OIL PLANT

D. A. Khashimov, Kh. G. Alimov,
P. Kh. Yuldashev, T. V. Chernenko,
M. Talipova, and G. V. Podkuichenko

UDC 665.117.4.093.5

The water-soluble proteins of twelve varieties of castor-oil plant have been studied by electrophoresis. The high hemagglutinating activities of proteins of the seeds is connected with the presence of an electrophoretic component having R_f 0.042.

The castor-oil plant is widely used in many sectors of industry and medicine [1]. An investigation of the fatty acid composition of castor oil has shown that the level of ricinoleic (12-hydroxyoleic) acid in various subspecies of the castor-oil plant ranges from 83.6 to 90%, while palmitic, stearic, oleic, and linoleic acids are present in small amounts [2].

The seeds of the castor-oil plant (castor beans) contain a considerable amount of protein, but the presence of certain toxic groups of proteins called phytohemagglutins greatly decreases their fodder value [3].

At the present time, work is being done on the creation of new varieties of castor-oil plant with a decreased amount of toxic substances [4]. The aim of our investigation was an electrophoretic study of the water-soluble proteins of different varieties of castor-oil plant and their hemagglutinating activity, which is of great interest for domestic selection.

The seeds were ground and their oil [5] and protein [6] contents and hemagglutination titers [7] were determined. The results of the analyses are given in Table 1. The varieties differed considerably with respect to their oil and protein contents and agglutination titers. The oil content of the seeds of the individual varieties ranged from 43.1 to 52.7%, the protein content from 20.1 to 32.2%, and the agglutination titer from 1:64 to 1:1024. The lowest agglutination titers were found in samples 1, 2, 3, and 9, the protein contents of which were high.

The results of the electrophoretic investigation of the protein spectrum of the water-soluble fractions of individual seeds are shown in Table 2 in the form of the relative

Institute of the Chemistry of Plant Substances, Uzbek SSR Academy of Sciences, Tashkent. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 113-116, January-February, 1989. Original article submitted April 12, 1988.

 0009-3130/89/2501-0100 $12.50