Chemistry of Natural Compounds, Vol. 31, No. 6, 1995

DEVELOPMENT OF A METHOD FOR THE COMPLEX ISOLATION OF PHYSIOLOGICALLY ACTIVE COMPONENTS FROM BEE VENOM

Zh. F. Ziyavitdinov, U. K. Inogamov, N. Zh. Sagdiev, and Sh. I. Salikhov

UDC 577.112+547.993

A method is proposed for isolating the main components (melittin, apamin, and phospholipase A_2 from the venom of the bee Apis mellifera *using HPLC, and the synthesis of two sorbents for fractionating these peptides is described.*

Bee venom is widely used as a medicament in various diseases. [1]. However, to create highly specific and effective agents it is desirable to use the components of the venom in a highly purified state. In view of this, researchers are faced with the task of developing the simplest, fastest, and cheapest methods of fractionating the venom [2].

It is known that the venom of the bee *Apis mellifera* is a complex mixture of proteins and peptides exhibiting the most diverse biological activities. A number of proteins and peptides have been studied in detail, and the primary structures of the phospholipase A_2, melittin, apamin, and the MCD peptide have been determined [2-11]. More recently, new methods have been developed for isolating the components of bee venom, since these components are of interest for pharmacology [1]. Purification of the majority of the components is complicated by high surface activity, low molecular mass, and the presence of a large amount of complex substances [3, 11-14]. All this is leading to the use of different and incompatible methods of isolation by different groups of researchers.

Our aim was to improve the methods of fractionation, with a substantial increase in the yields of the components of the venom, and a decrease in the number of stages of the isolation process.

According to the results of HPLC (Fig. 1), whole bee venom contains substances differing greatly in hydrophobic properties. In the first stage, therefore, we used reversed-phase adsorbents based on silica gel. To check these presuppositions, we carried out the separation of bee venom on the sorbent LiChroprep RP-8 in a stepwise gradient of isopropanol (Fig. 2).

Five fractions were obtained which were characterized by the use of HPLC on a Nucleosil 100-5 C_{18} column. A disadvantage of this sorbent is that the low-molecular-mass colored substances of bee venom are irreversibly sorbed on the gel, which decreases the capacity and impairs the hydrodynamic properties of the column. This sorbent enables products to be obtained in a highly purified state, but a large amount of sorbent with a definite capacity stable in various buffer systems is necessary for semipreparative separation. For this purpose we used Polikhrom-1, but the capacity and hydrodynamic parameters of this gel proved to be inadequate for these purposes. We then synthesized a reversed-phase sorbent based on Silokhrom-80 by the direct modification of the silanol groups with octadecyltrichlorosilane in acetone at 50°C for 18 h.

The fractionation of the venom of the bee *Apis mellifera* with the aid of this sorbent gave fractions containing large amounts of impurities. In all probability, this was due to the fact that only 50% of the silanol groups had been modified by the octadecylsilane. The unmodified silanol groups also take part in separation, and it is this that was responsible for the nonspecific sorption of substances with a hydrophilic nature.

After the performance of the reaction of silica gel with the octadecylsilane, the residual silanol groups were modified with methyltrichlorosilane. The resulting sorbent was used for semipreparative separation, and this enabled us to obtain highly purified components of the bee venom.

A. S. Sadykov Institute of Biooganic Chemistry, Academy of Sciences of the Republic of Uzbekistan, Tashkent, fax (3712) 62 70 71. Translated from Khimiya Prirodnykh Soedinenii, No. 6, pp. 862-866, November-December, 1995. Original article submitted March 13, 1995.

 0009-3130/95/3106-0726$12.50

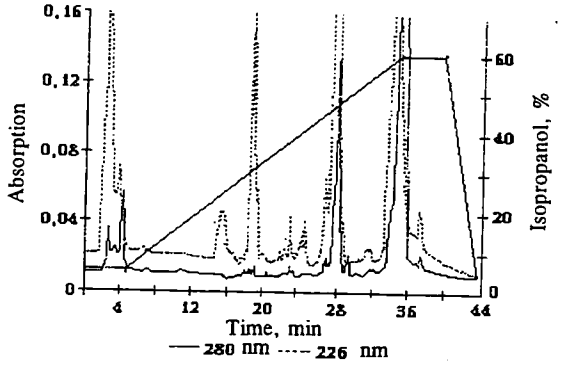


Fig. 1. High-performance liquid chromatography of the whole venom of the bee *Apis mellifera* on a column of 250/8/4 Nucleosil 100-5 C_{18}. Buffer A: 0.1% CF_3COOH; B: CH_3CN. Rate of flow 60 ml/h; absorption at 226 nm (280 nm); sensitivity 0.16 OU. Gradient, %B/min: 5/0-5; 60/35-40; 5/45.

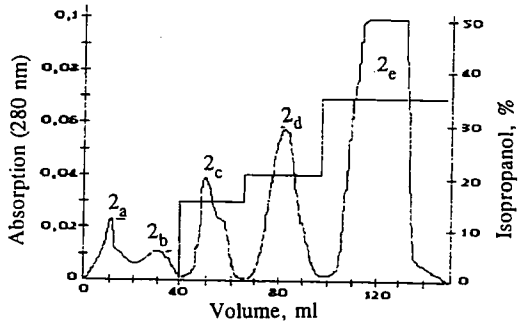


Fig. 2. Hydrophobic chromatography of the venom of the bee *Apis mellifera* on a column (1.0 × 10 cm) of the sorbent LiChroprep RP-8. Buffers: A) 0.1% CF_3COOH; B) isopropanol. Gradient — stepwise, %B: 10, 20, 35%. Rate of flow 60 ml/h, absorption at 280 nm; sensitivity 0.16 OU.

The first stage of separation of the whole venom was conducted under the following conditions. A solution of 2.7 g of the bee venom in 0.1% TFAA was added to a column (2.5 × 30 cm) of the gel Silokhrom-80 C_{18} equilibrated with the same buffer. After centrifugation at 6000*g* for 15 min, the sample was deposited on the column. The unsorbed fraction was washed out with the same buffer. Elution was conducted in a stepwise gradient of isopropanol (Fig. 3). Five fractions were obtained. Hydrophilic components and an excess of melittin in a complex with some other components of the venom (determined by HPLC) were eluted in fractions 3_a and 3_b. Phospholipase A_2 and melittin, respectively, were eluted in fractions 3_c and 3_d. It must be mentioned that the concentrations of phospholipase A_2 and melittin in fractions 3_c and 3_d exceeded 95% while Sigma commercial preparations have a purity of 85%. Low-molecular-mass pigments were eluted in fraction 3_e. The yield of melittin was 1 g, making 38.0% of the initial bee venom.

The second stage of separation made us of ion-exchange chromatography on the sorbent SP-Sephadex C-50. It was established in the course of preliminary investigations that this sorbent sorbs physiologically active components from bee venom irreversibly, which sharply lowers the final yield of product, and it has unsatisfactory hydrodynamic properties. We therefore then synthesized an ion-exchange sorbent from the hydrophilic support TSK HW 65f, which was modified by monochloroacetic acid in a strongly alkaline medium at 70°C for 0.5 h. The characteristics of the CM-TSK HW 65f gel synthesized are given in Table 1.

TABLE 1. Quantitative Characteristics of the Ion-Exchange Sorbent CM-TSK HW 65f

Sorbent concentration, g/ml	Sorbent taken, ml	Monochloroacetic acid added, g/ml	Capacity, mg/ml	
			ion-exchange	adsorption (for hemoglobin)
0.6	1.7	0.05	0.025	18
0.6	1.7	0.1	0.051	29
0.6	1.7	0.2	0.11	35
0.6	1.7	0.4	0.19	41
0.6	1.7	0.6	0.29	30
0.6	1.7	0.8	0.45	24

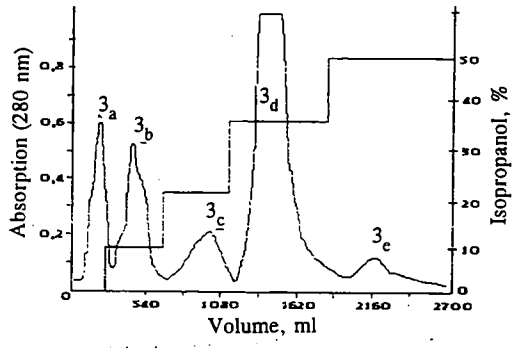


Fig. 3. Hydrophobic chromatography of *Apis mellifera* venom on a column (2.5 × 50 cm) with the sorbent Silokhrom-80 C_{18}. Buffers: A) 0.1% CF_3COOH; B) isopropanol. Gradient — stepwise, %B: 10, 20, 35, 50%. Rate of flow 180 ml/h; absorption at 280 nm; sensitivity 1 OU.

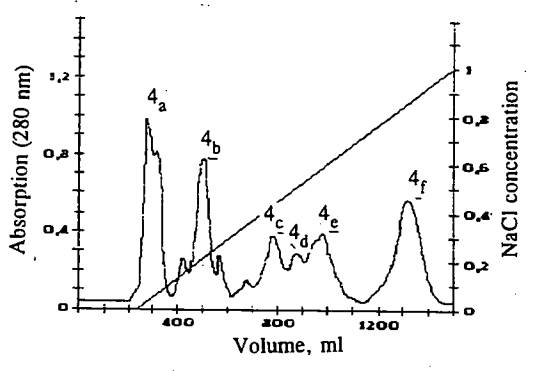
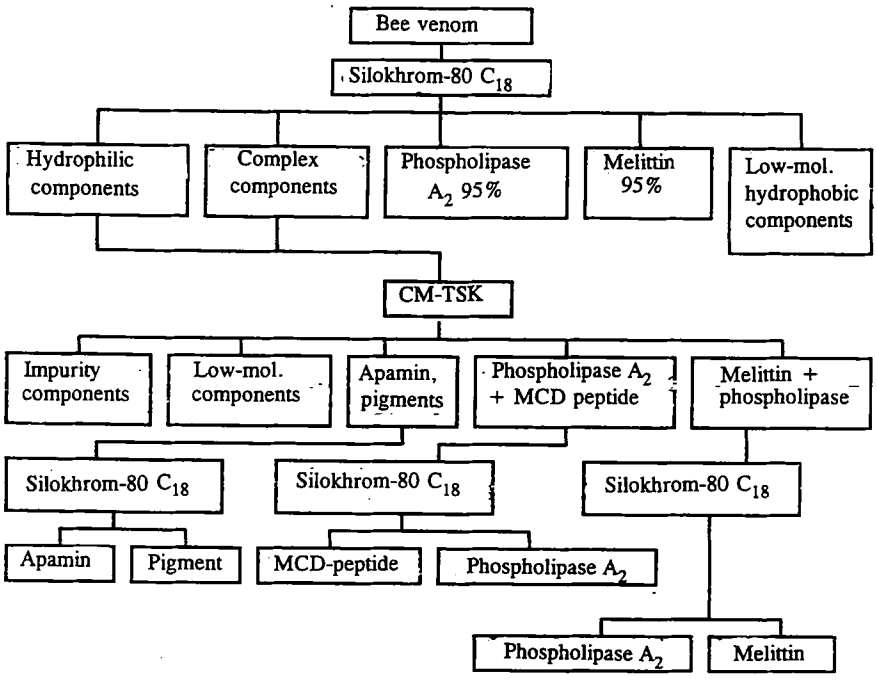


Fig. 4. Ion-exchange chromatography of fraction $3_a + 3_b$ on a column (2.5 × 50 cm) with the sorbent CM-TSK HW-65. Buffer 0.1 M ammonium acetate, pH 4.2. Gradient of 1 M NaCl in the same buffer. Rate of flow 60 ml/h, absorption at 280 nm, sensitivity 1.0 OU.

To isolate physiologically active substances from bee venom using ion-exchange chromatography on the sorbent CM-TSK HW-65f, combined fractions 3_a and 3_b were concentrated to minimum volume and deposited on a column (2.5 × 50 cm) of the sorbent equilibrated with 0.1 M ammonium acetate buffer, pH 4.2. Elution was conducted in a linear gradient of 1.0 M NaCl in the buffer (Fig. 4). Six fractions were obtained and were characterized by HPLC. It was established that apamin was eluted in fraction 4_d, the MCD peptide and phospholipase A_2 in 4_e, and melittin with trace amounts of phospholipase A_2

in fraction 4_f. The fractions were desalted on a column (1 × 10 cm) of the sorbent Silokhrom-80 C_{18} in an isopropanol gradient. After lyophilization, the fractions were analyzed by HPLC.

Thus, we have proposed a method for the complex isolation of the components of bee venom, which is shown in Scheme 1, and have synthesized two sorbents (Silokhrom C_{18} and CM-TSK HW 65f) for fractionating these peptides.



Scheme 1. Scheme of the complex isolation of the components of bee venom.

EXPERIMENTAL

Synthesis of the Hydrophobic Sorbent Silokhrom-80 C_{18}. Silokhrom-80 (200 g) was moistened with absolute acetone (200 ml), and 40 g of octadecyltrichlorosilane was added. The reaction mixture was incubated with constant stirring in a thermostat at 50°C for 18 h. It was then transferred to a Schott glass filter and was washed successively with acetone, 50% isopropanol, water, and acetone. To block the residual silanol groups, 80 g of methyltrichlorosilane was added. The reaction mixture was incubated at 50°C in a thermostat with constant stirring for 18 h. After being washed free from unbound methyltrichlorosilane, the sorbent was packed into a column and used for subsequent investigations.

Synthesis of CM-TSK HW 65f [15]. A mixture of 100 ml of TSK HW 65f and a solution of 90 g of caustic potash in 200 ml of water was cooled, and a solution of 30 g of monochloroacetic acid in 40 ml of water was added in four portions to the resulting suspension. The reaction mixture was carefully stirred and was heated in an oil bath at 70°C for 20 min. After cooling, the sorbent was washed with 10% acetic acid and with water, and then with ethyl alcohol (500-ml portions). The product obtained had a capacity of 0.6 meq/ml of sorbent.

Hydrophobic chromatography was conducted on a 2.5 × 30 cm column of the sorbent Silokhrom-80 C_{18} equilibrated with 0.1% TFAA. A solution of 2.7 g of the venom of the bee *Apis mellifera* in 50 ml of buffer was centrifuged at 6000*g* for 15 min, filtered through Millipore (0.4 μm), and deposited on the column at a rate of flow of 180 ml/h. Fractions were eluted in a stepwise gradient of isopropanol at concentrations of 10, 20, 35, and 50%. Fractions with a volume of 18 ml were collected in an Ultrorac II collector (LKB, Sweden).

Ion-exchange chromatography was conducted on a 2.5 × 50 cm column of the sorbent CM-TSK HW-675f equilibrated with 0.1 M ammonium acetate buffer, pH 4.2. The first and second fractions obtained after hydrophobic chromatography were combined, concentrated to minimum volume, and deposited on the column at a rate of flow of 60 ml/h. Fractions were eluted in a linear gradient of 1.0 N NaCl in the same buffer. Fractions with a volume of 18 ml were collected in an Ultrorac II collector (LKB, Sweden).

Desalting was achieved on a column (1 × 10 cm) column of the sorbent Silokhrom-80 C_{18}. The fractions obtained after ion-exchange chromatography were deposited on the column equilibrated with 0.1% TFAA at a rate of flow of 60 ml/h. After the column had been washed with the starting buffer, fractions were eluted with a stepwise gradient (10, 20, 35%) of isopropanol.

HPLC was conducted on a Du Pont 8800 chromatograph using a 250/8/4 Nucleosil 100-5 C_{18} column. Buffer A: 0.1% CF_3COOH; B: CH_3CN. Rate of flow 60 ml/h; absorption at 226 nm; sensitivity 0.16 OU; gradient, %B/min: 5/0-5; 60/35-40; 5/45.

REFERENCES

1. M. D. Mashkovskii, Drugs [in Russian], Meditsina, Moscow (1977) Vol. 2, p. 105.
2. J. Gauldie, J. M. Hanson, F. D. Rumjanek, R. A. Shipolini, and C. A. Vernon, Eur. J. Biochem., **61**, 376 (1976).
3. E. Habermann, Naunyn-Schmiederbergs Arch. Exp. Pathol. Pharmacol., **222**, 173 (1954).
4. E. Habermann and J. Jenisch, Hoppe-Seylers Z. Physiol. Chem., **348**, 37 (1976).
5. E. Habermann and J. Jenisch, Naunyn-Schmiederbergs Arch. Exp. Pathol. Pharmacol., **253**, 40 (1966).
6. E. Habermann and K. G. Reiz, Biochem. Z., **343**, 192 (1965).
7. R. A. Shipolini, A. F. Bradbury, G. L. Callewaert, and C. A. Vernon, J. Chem. Soc., Chem. Commun., 679 (1967).
8. G. L. Callewaert, R. A. Shipolini, and C. A. Vernon, FEBS Lett., **1**, 111 (1968).
9. H. Briethaupt and E. Habermann, Naunyn-Schmiederbergs Arch. Exp. Pathol. Pharmacol., **261**, 252 (1968).
10. C. A. Vernon, J. M. Hanson, and R. W. Brimblecombe, British Patent No. 1,314,823 (1969).
11. J. Gauldie, J. M. Hanson, R. A. Shipolini, and C. A. Vernon, Eur. J. Biochem., **83**, 405 (1978).
12. E. Habermann and K. Fischer, Eur. J. Biochem., **94**, 355 (1979).
13. I. A. Vladimirova and M. F. Shuba, Neurofiziologiya, **10**, 295 (1978).
14. B. S. Banks, C. E. Dempsey, F. L. Pearce, C. A. Vernon, and T. E. Wholley, Anal. Biochem., **116**, 48 (1981).
15. E. A. Peterson and H. A. Sober, J. Am. Chem. Soc., **78**, 751 (1956).