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Short Communication

Ion-exchange chromatography with a soft sorbent operating in a pressurized column

M.B. Baru and I.V. Kozlovsky-Vagenina*

Branch of Shemyakin Institute of Bioorganic Chemistry, Russian Academy of Sciences, Puschino, Moscow Region 142292 (Russian Federation)

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ABSTRACT

A standard Pharmacia column was modified to enable pressurization with inert gas. The column moveable adaptor can follow the gel as the volume changes under pressure; thus, "dead volume-free" operation is achieved. A trial separation of natural bee venom components was carried out on the modified column with CM-Sephadex C_{25} in a pH and ionic strength gradient. It was found that, at 54 kPa pressure, the elution times of the bee venom constituents phospholipase a_2, apamin and melittin are reduced and the resolution is improved.

INTRODUCTION

Pressurization, widely used with rigid sorbents to improve packing [1,2] and optimize separation, is considered inapplicable to soft gels, though dextran gels, for instance, are known to be able to withstand pressures up to 1700 kPa [3]. In order to provide dead volume-free operation and optimize soft gel packing we made a minor improvement to the standard Pharmacia column by applying inert gas pressure to the gel bed via the moveable adaptor. Pressure can be adjusted to make the adaptor follow the gel volume variation during gel shrinking or swelling; additional pressurizing can also be accomplished. Such dead volume elimination at the column head is important when concentrated protein mixtures are applied to soft gel columns during gel filtration and in gradient elution from soft ion exchangers. Usually the ion exchange process is thought to depend least of all chromatographic methods on the packing quality. It was of interest to see if the zero dead volume column operation with additional pressure influences the pH and ionic strength gradient elution efficiency.

MATERIALS AND METHODS

A continuous-flow reaction system for biopolymer solid-phase synthesis served as a prototype of our device [4]. The moving piston is pressed close to the sorbent with preset force. Fig. 1 shows the modified $C_{16/20}$ column (Cat. No. 19-5101-01, Pharmacia, Sweden) with moveable adaptor AC 16 (1) (Cat. No. 19-5109-01), thermostat jacket JC 16/20 (2) (Cat. No. 19-5105-

* Corresponding author.
E-mail: Biochem@sovamsu.sovusa.com.

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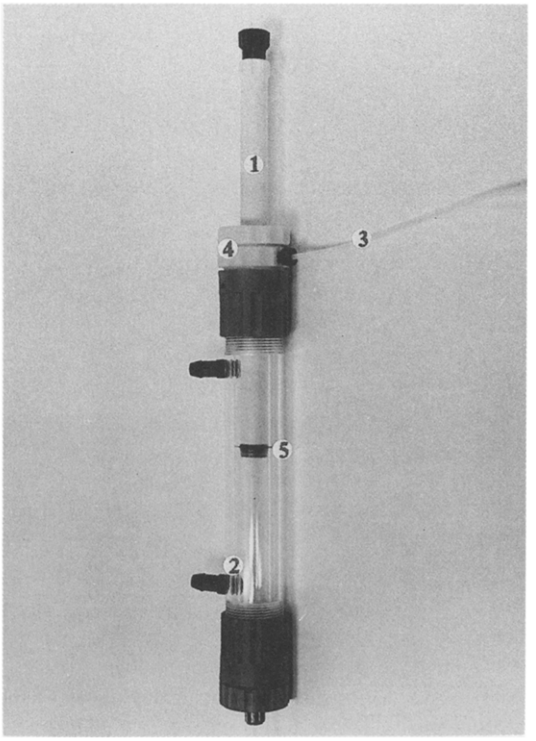

Fig. 1. General layout of the modified Pharmacia "dead volume-free" operated column: 1 = moveable adaptor; 2 = thermostat jacket; 3 = tubing for gas inlet; 4 = modified unit with fluoroplastic collar.

01) and a PTFE tube for inert gas feeding (3). The modified unit (4) was made from Caprolon (poly-ε-caproamid). The net gas pressure moving the adaptor was calculated by adding the pressure applied directly to the sorbent to the pressure component due to friction of the standard O-ring rubber seal (5) on the glass wall. The friction value determined experimentally depends on the wear of the O-ring seal and its retention against the column glass walls. In order to reduce friction the column glass walls were thoroughly silanized, as described by Stewart and Young [5]. The adaptor surface was polished in order to minimize the adaptor friction on the fluoroplastic collar edge. The fluoroplastic collar was inserted into the modified unit (4) in order to prevent the gas from leaking from the cavity. The moving adaptor gas pressure was chosen to overcome friction, thus minimizing the dead volume and also applying to the sorbent a pressure of 50–100 kPa.

The pressurized column was tested by separating crude natural bee venom into its basic components [6]: phospholipase a_2, melittin and apamin. The crude bee venom was a gift from Apis (Moscow, Russia). It was dissolved in 0.05 *M* triethylammonium acetate, pH 6.7 or 5.6 (25 mg in 3 ml), filtered through cotton and applied to the CM-Sephadex C_{25} column (Pharmacia, Sweden) equilibrated with the appropriate buffer. The starting gel volume was about 12 ml.

The non-adsorbed components of the sample were isocratically eluted. Then the chromatogram was developed at room temperature for 540 min with a pH and ionic strength linear gradient to 0.1 *M* triethylammonium acetate, pH 10, with 1.5 *M* NaCl. The flow-rate was 60 ml/h. The effluent was collected in 2-ml fractions. Detection at 226 nm was carried out with a Uvicord SII (LKB, Sweden). In experiments with pressure the column was packed and the chromatography was carried out under nitrogen pressure of 54 or 93 kPa.

The resulting bee venom constituents were identified by their HPLC retention time values and amino acid analysis data. Fractions corresponding to the eluting peak maxima were analysed by RP-HPLC with a Gilson analytical gradient system (Gilson Medical Electronics, Villiers-le-Bel, France) on a Synchropack C_4 column (250 × 4.1 mm I.D., 6.5 μm particle size; Alltech, USA). The linear gradient from 0.1% trifluoroacetic acid (TFA) to 70% acetonitrile (plus 0.1% TFA) in 35 min was used. The solvent flow-rate was 1.0 ml/min. The samples were analysed for amino acid composition with a Pico-Tag instrument (Waters, Milford, MA, USA).

RESULTS AND DISCUSSION

Figs. 2 and 3 depict the chromatographic separation of the bee venom components at initial pH values of 5.6 and 6.7, under pressure and without it. Phospholipase a_2, apamin and melittin are adsorbed at both pH values (p*I* 10.5,

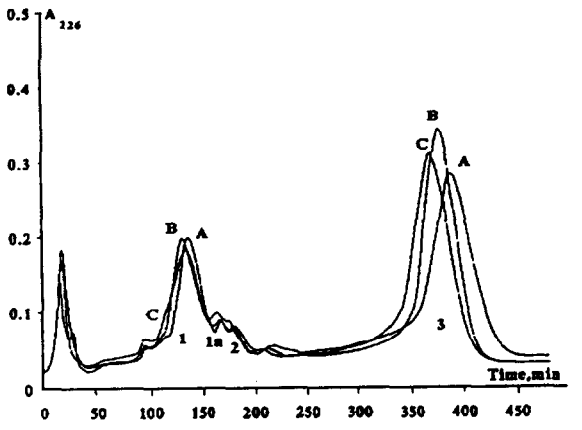

Fig. 2. Elution profile of bee venom at initial pH 5.6: (A) without external pressure; (B) with external pressure 54 kPa; (C) with external pressure 93 kPa. See text for chromatographic conditions.

7.9 and 12.1, respectively [6]); the front peak of non-adsorbed components was not analysed. According to amino acid analysis the first, second and the third peaks correspond to phospholipase a_2, apamin and melittin, respectively (see Table I).

The overall chromatogram character with pressure application remains practically the same, but the retention times and resolution values differ at both pH values (see Table II). It is seen that separation efficiency at the initial pH 5.6 rises slightly at 54 kPa, but at further pressure increase to 93 kPa the resolution becomes lower while the retention times remain practically the same as at 54 kPa. The initial pH 5.6 seems not to be favourable for separation of the bee venom components because basic peptides and protein are too strongly adsorbed, and elute slowly with the gradient; besides, phospholipase a_2 emerges as two peaks (peaks 1 and 1a, Fig. 2).

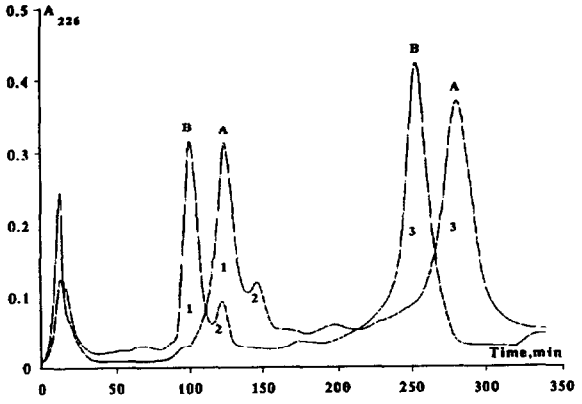

Fig. 3. Elution profile of bee venom at initial pH 6.7: (A) without external pressure; (B) with external pressure 54 kPa. See text for chromatographic conditions.

The separation appears to improve significantly when the initial pH is 6.7. The three substances emerge as three separate peaks and also elute more quickly than at the initial pH 5.6 (see Table II). The positive influence of 54 kPa pressure is also more pronounced. Resolution for peaks 1 and 2 rises under pressure approximately two-fold (see Table II), and the concentration of peak maximum fractions increases 1.7-fold for the first two peaks and 1.2-fold for the third one according to HPLC analysis.

The separation improvement under 54 kPa pressure is probably caused by facilitation of ion diffusion to the sites of reaction and increase in the available pore volume, resulting in more uniform eluent distribution in the bed volume. Diffusion facilitation is of critical importance, as the rate of ion exchange on weak acid gels is controlled by the rate of cation diffusion through the exchanger particles to the sites of reaction and diffusion of the displaced ions out of the interior of the exchanger [7]. Also, pressure application prevents channelling, which is most pronounced upon gel contraction [8].

The determination of pressure optimum for a chromatographic separation and the elucidation of the exact mechanism of the observed phenomena need further studies.

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TABLE I

AMINO ACID ANALYSIS DATA OF THE BEE VENOM CONSTITUENTS

a = Analytical and t = theoretical numbers of amino acids.

	Apamin		Phospholipase a_2		Melittin	
	a	t	a	t	a	t
Asp/Asn	1.04	1	12.5	16	0.14	–
Glu/Gln	3.48	3	4.7	6	2.27	2
Ser	0.03	–	7.8	10	1.15	1
Gly	0.09	–	9.4	12	3.56	3
His	0.95	1	4.7	6	–	–
Arg/Thr	3.21	2 + 1	11.7	15	3.92	4
Ala	3/ref[a]	3	3.1	4	2/ref[a]	2
Pro	1.14	1	3.9	5	1.10	1
Tyr	0.03	–	6.3	8	0.06	–
Val	0.06	–	3.9	5	1.70	2
Met	–	–	2.3	3	–	–
Cys	2.08	4	6.3	8	–	–
Ile	–	–	3.1	4	2.58	3
Leu	0.78	1	7.0	9	3.50	4
Phe	–	–	3.9	5	–	–
Lys	0.84	1	8.6	11	2.49	3

[a] Reference values used for calculation of amino acid quantity.

TABLE II

CHARACTERISTICS OF SEPARATION EFFICIENCY

Separation conditions	Peak No.	Retention time (min)	Resolution	Total resolution
Initial pH 5.6				
Without pressure	1	134		
t_0 = 13 min	2	183	1.14	
	3	405	3.01	4.15
54 kPa	1	128		
t_0 = 8 min	2	174	1.28	
	3	366	4.57	5.85
93 kPa	1	128		
t_0 = 9 min	2	171	1.02	
	3	362	4.15	5.17
Initial pH 6.7				
Without pressure	1	125		
t_0 = 12 min	2	145	0.83	
	3	286	3.71	4.54
54 kPa	1	98		
t_0 = 10 min	2	120	1.57	
	3	250	4.33	5.90

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