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Capillary electrophoresis analysis of concanavalin A and its succinyl derivative*

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Abstract: A high-performance capillary electrophoresis (CE) system has been developed for concanavalin A (Con A) and its succinyl derivative (SCA). Under the CE system, the tetramer, trimer, dimer, monomer and protein fragments of CA were separated in less than 50 min. SCA was resolved into more than 10 components which were believed to be isomeric succinyl derivatives of Con A. The CE system is a simple and sensitive analytical technique to profile the composition of Con A and SCA preparations. The minimum concentration of Con A in water detectable by the CE system is 1 $\mu g\ ml^{-1}$.

Keywords: *Capillary electrophoresis; concanavalin A; succinyl concanavalin A.*

Introduction

Concanavalin A (Con A) is a cell agglutinating protein (lectin) isolated from Jack bean (*Canavalia ensiformis*). Its first isolation dates back to 1919 [1] and since then the chemical and biochemical properties have been extensively studied. Its cell agglutinating and sugar binding abilities have made Con A an invaluable tool for life scientists [2]. The anti-viral [3–6] and anti-tumour [7–10] activities of Con A have made it a potential candidate for anti-viral and anti-tumour chemotherapy. However, Con A preparations contain protein fragments and multimers which are interchangeable under different solution conditions. This causes some confusion in understanding the biological activity of Con A. To understand the behaviour of Con A more fruitfully, its more stable succinyl derivative (SCA), a dimer, has been prepared [11].

Con A is a globular protein composed of identical subunits with a molecular weight of about 27,000 daltons [12, 13]. Each subunit contains 237 amino acid residues [13–15], one calcium, one magnesium ion and one binding site for a specific saccharide [16, 17]. All Con A preparations are mixtures of at least two molecular species (dimer and tetramer) with different sugar binding valency. Depending on the solution condition, the ratio of the two species varies [13, 18]. Between pH 2 and 6, Con A exists mainly as a dimer of about 55,000 daltons; at pH above 6 it is predominantly a tetramer of 110,000 daltons [13, 19, 20]. Because the binding activity of Con A multimers are different, it is useful to know the molecular composition of Con A preparations.

The molecular species of Con A has been investigated by affinity chromatography, high-performance affinity chromatography (HPAC), SDS polyacrylamide gel electrophoresis (SDSPAGE) and isoelectric focusing (IEF). Sephadex affinity chromatography [21] has been used to separate the tetramer from the dimer, but the process was time consuming. HPAC improved the speed substantially but the separation was only partial [22]. SDSPAGE separated the intact subunit of Con A from its protein fragments [17]. However, multimers of Con A could not be distinguished due to the denaturation of protein during the SDSPAGE process [11]. IEF, although it gave distinct bands for the dimer, tetramer and protein fragments of Con A [16, 23], was laborious. Because high-performance capillary electrophoresis (CE) is simple, efficient and sensitive, it should be an ideal technique for Con A analysis.

To the authors' knowledge, no analysis of Con A or SCA by CE has been reported. In this paper, an efficient CE method for the analysis of Con A and SCA is presented.

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Experimental

Reagents and materials

Highly purified lyophilized powder Con A (type IV, C2010) and SCA (L3885) samples were purchased from Sigma Chemical Co. They were used without further purification. Sample solutions were prepared by dissolving 2–5 mg in 1.00 ml of solvent.

Tris(hydroxymethyl)aminomethane (TRIS) and boric acid were purchased from Mallinkrodt. Urea was from Aldrich Chemical Co. and (ethylenedinitrilo)tetraacetic acid (EDTA) was from Matheson Coleman & Bell. The chemicals were reagent grade.

Sample and buffer solutions were prepared with distilled water.

Capillary electrophoresis (CE)

CE was performed on a Bio-Rad HPE 100 High Performance Electrophoresis System using 200 × 0.025 mm or 500 × 0.05 mm glass capillaries coated with a covalently bonded linear polymer. Loading was done electrophoretically at 6 or 12 kV for 6 s. The run voltage was kept at 6 or 9 kV. The higher voltage was used for the longer capillary. Run buffer was pH 8.4, 0.089 M TRIS-boric acid containing 0.002 M EDTA and 7 M urea (the final pH of this solution was 8.9). Direction of movement of ions was toward the positive electrode. Detection was by UV at 230 nm and the data was collected and processed by a Baseline 810 Integration System (Dynamic Solution).

Results and Discussion

Polymer coating

Separation of protein by CE using untreated glass capillary is complicated by the adsorption of protein onto the capillary wall and by electro-osmosis [24]. These complications lead to band broadening, distorted peak shapes and reduced separation efficiencies. Protein adsorption and electro-osmosis in CE, however, can be reduced or eliminated by using a capillary whose inner wall has been coated with a linear polymer of polyacrylamide [25] or by using a buffer whose pH is above the pI of the protein [26]. Since the pI of Con A is about 8 [23], it was decided to use a high pH (8.9) buffer and a linear polymer coated capillary to develop the Con A separation. The patented coated capillary cartridge (Bio-Rad, Richmond, CA) was designed specifically to eliminate protein adsorption and electro-osmosis. Indeed, using mesityl oxide and acetone as electro-osmotic flow markers, as suggested by Beckers *et al.* [27], the electro-osmotic mobility of our system was less than -5×10^{-6} cm^2 V^{-1} s^{-1} (marker peaks were not observed within 200 min when detection was at either electrode). The mean electrophorectic mobility of Con A sample under identical conditions was 50×10^{-6} cm^2 V^{-1} s^{-1} (based on the mean migration time of 20 min towards the positive electrode). The coated capillary cartridge appeared very stable under the operating conditions. After continuous use for at least 3 months, the capillary cartridge showed no noticeable deterioration in performance.

Con A

A typical electropherogram of a freshly prepared aqueous solution (A) of Con A (Fig. 1) shows two major components at 19 (V) and 40 min (VII), three minors at 14 (II), 17 (IV) and 22 min (VI), and two traces at 12 (I) and 16 min (III). The CE profile of freshly prepared solutions of Con A in 0.01 M boric acid (pH 5.5, B), 0.01 M TRIS–0.01 M boric acid (pH 8.8, C), 0.01 M TRIS-borate (pH 9.3, D), 0.01 M TRIS (pH 10.1, E), and the CE run buffer (pH 8.9, F) are all similar to that shown in Fig. 1, except for the relative intensities of V and VII. Table 1 presents the relative peak areas for samples A–F. Except in the run buffer (F), VII increased slightly with the pH of the sample solution at the expense of V. Compositions for samples A–F remained fairly constant for 4 h, indicating that Con A was reasonably stable in each of the solution media.

Con A is known to exist as mixtures of its tetramer and dimer plus three low molecular weight protein fragments [13, 14, 19, 20]. At pH below 6, the dimer predominates while the tetramer is favoured at higher pH. In the presence of 6 M protein denaturants, such as guanidine and urea, Con A is denatured to the monomer [11]. Therefore, VII, which is favoured at higher pH, is probably the tetramer and V is the dimer. Figure 2(a) is the CE profile of a freshly prepared solution of Con A in 7 M urea (pH 8.4, G). A new major peak (VIII) appeared at 9 min with a significant reduction of VII. After 4 h of room temperature storage, this urea solution, G, gave a CE profile [Fig. 2(b)] which showed VIII to be the

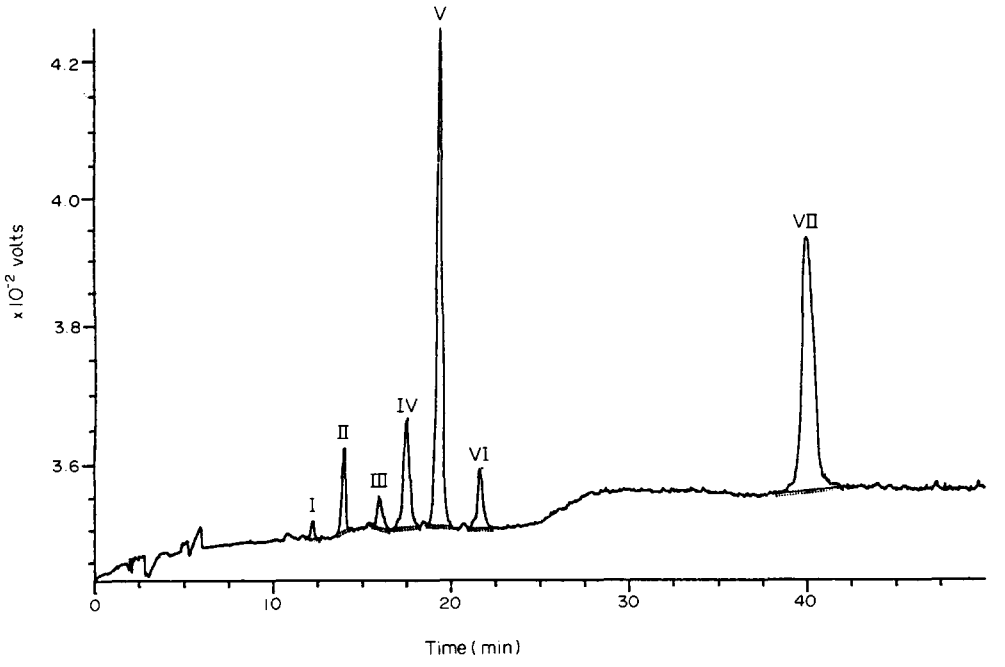

Figure 1
Electropherogram of a water solution of Con A (5 mg ml^{-1}). See text for CE conditions. Sample loading: electrophoretic, 6 s at 6 kV. Capillary: 200 × 0.025 mm. Run voltage: 6 kV.

Table 1
Relative intensity of Con A components by CE

Solution medium	Relative peak intensity I	II	III	IV	V	VI	VII
A Distilled water	0.7	3.9	2.3	9.6	34.3	4.8	44.4
B pH 5.5 buffer*	0.6	3.7	2.2	8.9	34.2	4.2	46.2
C pH 8.8 buffer†	0.7	4.0	1.9	8.1	32.1	4.7	48.5
D pH 9.3 buffer†	0.7	3.9	2.1	8.4	30.7	4.8	49.3
E pH 10.1 buffer‡	0.8	5.0	2.1	8.1	22.6	5.3	56.1
F CE run buffer§	1.1	5.8	2.3	11.7	48.1	4.3	26.6

See text for CE conditions.
*0.01 M boric acid, pH unadjusted.
†0.01 M TRIS, pH adjusted with boric acid.
‡0.01 M TRIS, pH unadjusted.
§0.089 M TRIS, 0.089 M boric acid, 0.002 M EDTA and 7 M urea, final pH of 8.9.

major component. VII and V were drastically reduced. This affirmed that VII and V were the tetramer and dimer, respectively, of Con A. In the 7 M urea solution, the tetramer (VII) and dimer (V) were denatured to the monomer (VIII). This denaturing process, however, was inhibited by the presence of EDTA. Table 2 lists the relative peak intensities of Con A in various solution media containing 7 M urea. The monomer (VIII) was detected only in solutions containing urea but not EDTA (G, H). The identification of the monomer (VIII), dimer (V) and tetramer (VII) is consistent with their relative mobility of 9, 19 and 40 min, respectively.

Cunningham *et al.* [17] reported the formation of a precipitate when Con A was incubated in 1% NH_4HCO_3 (pH 7.4) at 37°C for 15 h. The precipitate contained Con A intact subunits (mol wt 27,000) enriched with the protein fragments while the supernatant contained only the intact subunit. Because Cunningham *et al.* determined the molecular weight by sedimentation under a denaturing condition (7 M guanidine), the intact subunits in the supernatant and precipitate could have existed as mixtures of molecular aggregates. In this study the incubation was repeated in order to further identify the Con A components separated by our CE system. The resulting

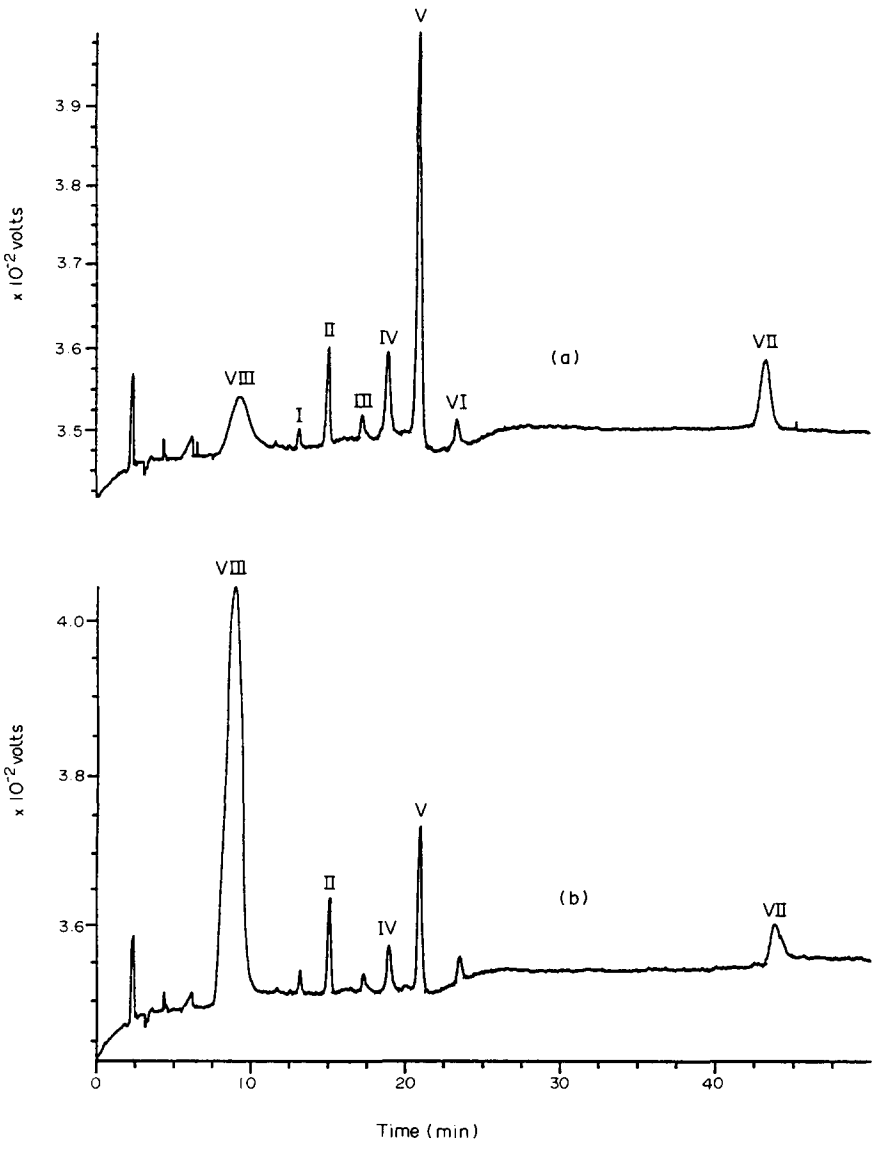

Figure 2
Electropherograms of a (a) fresh and (b) 4.5 h old 7 M urea solution of Con A (5 mg ml^{-1}). See text for CE conditions. Sample loading: electrophoretic, 6 s at 6 kV. Capillary: 200 × 0.025 mm. Run voltage: 6 kV.

Table 2
Effect of urea and EDTA on the relative intensity of Con A components

Solution medium	Relative peak intensity							
	I	II	III	IV	V	VI	VII	VIII
F 7 M urea + 0.002 M EDTA in TBA*	1.1	5.8	2.3	11.7	48.1	4.3	26.6	0.0
G 7 M urea in water pH 8.4	1.2	6.3	2.2	9.0	35.5	3.0	15.6	25.0
H 7 M urea in TBA,† pH 9.2	2.0	12.0	4.3	14.1	43.7	1.8	5.7	13.1
I 0.002 M EDTA in TBA,* pH 8.4	0.8	6.1	3.0	15.3	56.0	6.4	12.5	0.0
J 0.004 M EDTA in TBA,* pH 5.7	0.8	3.9	1.8	9.8	47.8	3.6	32.3	0.0
K 1% NH_4HCO_3, pH 7.4	0.6	6.4	1.7	11.8	48.0	5.3	26.2	0.0

See text for CE conditions.
*0.089 M TRIS, 0.089 M boric acid.
†0.01 M TRIS, 0.01 M boric acid.

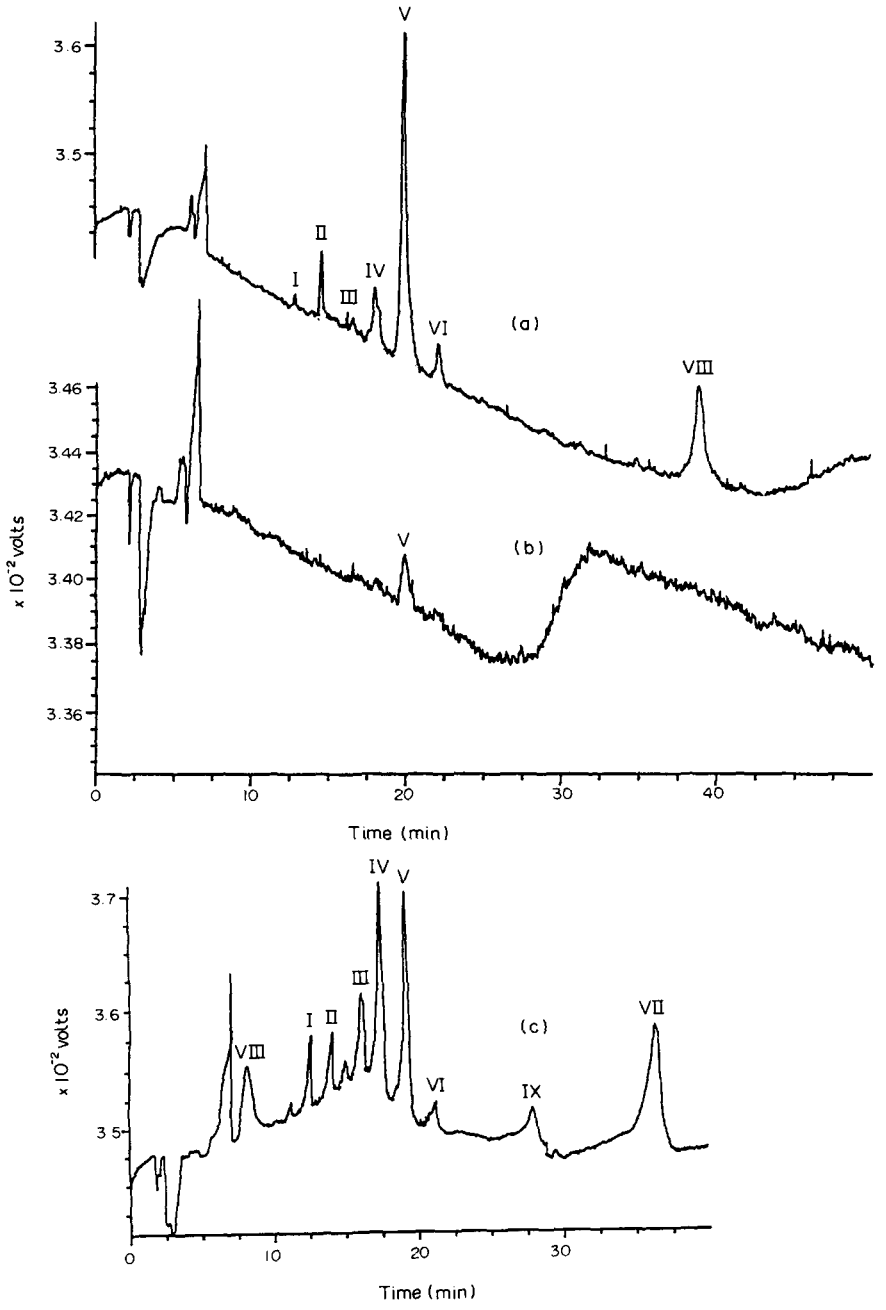

Figure 3
Electropherograms of (a) a fresh 1% NH_4HCO_3 solution of Con A (5 mg ml^{-1}), (b) the supernatant and (c) the precipitate (in water) resulting from 15 h of 35°C incubation of the NH_4HCO_3 solution. See text for CE conditions. Sample loading: electrophoretic, 6 s at 6 kV. Capillary: 200 × 0.025 mm. Run voltage: 6 kV.

precipitate was separated from the supernatant by centrifugation. The CE profile of the supernatant and the precipitate are shown in Fig. 3, along with that of the pre-incubated bicarbonate solution. Due to low analyte concentration after precipitation, only the dimer (V) was detected in the supernatant [Fig. 3(b)]. In the precipitate [Fig. 3(c)], I, III and IV were significantly enriched and, therefore, would be the low molecular weight protein fragments of Con A. In addition to the decrease of V (dimer), two new components VIII (9 min, 13%) and IX (28 min, 8%), appeared in the CE profile of the precipitate. IX was also detected in a day-old run buffer solution of Con A (F). Like VIII, it must be a denatured product of Con A multimers. Based on its mobility, IX is probably the trimer.

The detection sensitivity of the CE system depends on the concentration and the medium of sample solutions. Because sample loading was done electrophoretically, the charge status of the analyte and the presence of buffer salts in the sample solution influenced the amount of sample being loaded. Detection sensitivity was best when the sample solution contained no salt or salt ions identical to but at lower concentration than those used in the run buffer. The pH of the sample solution should be at or above the pI of the analyte. This has been reported as about 8 for Con A [23]. The minimum concentration of Con A in water detectable by the CE system presented in this paper was 1 μg ml^{-1}. This sensitivity limit was based on the dimer peak (V) at S/N ratio of 2.

SCA

Separation of SCA samples by the CE system is presented in Fig. 4. At least 10 significant components are observed. The profiles are identical, whether the SCA solution was prepared in water or in the CE run buffer. Ageing both solutions at 25°C for 72 h resulted in CE profiles identical to those of the fresh solutions. This observation is consistent with an earlier report [28] that SCA exists as a dimer and is resistant to denaturation. The CE results indicate that SCA is not a single component as reported by slab gel electrophoresis studies [28–30].

SCA is formed by the action of succinic anhydride on the ε-amino group of the lysine residues in Con A. The succinylation process resulted in covalent coupling of 10 succinyl groups per subunit of Con A [11]. Since each Con A subunit has 12 lysine residues [17], many succinyl derivatives are possible. It is possible that the multiple peaks of SCA separated by the CE system represent different site/degree of Con A succinylation.

Conclusion

This paper presents an efficient CE system for the analysis of Con A and SCA composition. The system separates the monomer, dimer, trimer and tetramer of Con A from its low molecular weight protein fragments. A freshly prepared Con A solution contains the dimer and tetramer as the major and the protein fragments as minor components. Denaturing the Con A samples with urea or incubation resulted in the formation of the trimer and the monomer. Minimum concentration of Con A in water detected by the CE system is 1 μg ml^{-1}.

Contrary to slab gel electrophoresis results, CE results obtained here demonstrate that SCA samples are not homogeneous but a mixture of at least 10 components. The multiple components are probably Con A of different site and degree of succinylation.

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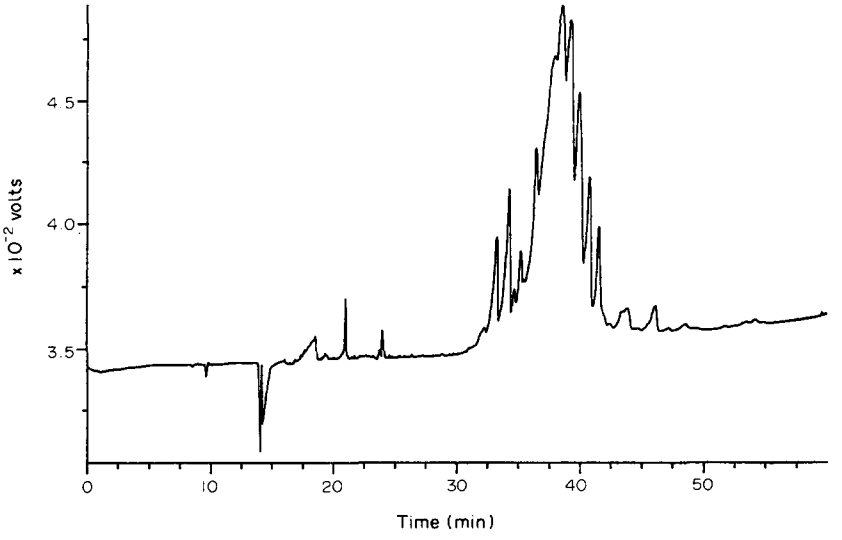

Figure 4
Electropherogram of a water solution of SCA (5 mg ml^{-1}). See text for CE conditions. Sample loading: electrophoretic, 8 s at 9 kV. Capillary: 500 × 0.05 mm. Run voltage: 12 kV.

References

[1] J.B. Sumner, *J. Biol. Chem.* **37**, 137–142 (1919).
[2] H. Bittiger and H.P. Schnebli (Eds), *Concanavalin A as a Tool.* Wiley Interscience, N.Y. (1976).
[3] J.M. Zarling and S.S. Tevethia, *Virology* **45**, 313–316 (1971).
[4] S.S. Tevethia, S. Lowry, W.E. Rawls, J.L. Melnick and V. McMillan, *J. Gen. Virol.* **15**, 93–97 (1972).
[5] G. Poste and P. Reeve, *Nature* (New Biol.) **237**, 113–114 (1972).
[6] E. Penhoet, C. Olsen, S. Carlson, M. Lacorbiere and G.L. Nicolson, *Biochemistry* **13**, 3561–3566 (1974).
[7] P.B. Dent, *J. Nat. Cancer Inst.* **46**, 763–773 (1971).
[8] P. Ralph and I. Nakoinz, *J. Nat. Cancer Inst.* **51**, 883–890 (1975).
[9] M. Inbar, H. Ben-Bassat and L. Sachs, *Int. J. Cancer* **9**, 143–149 (1972).
[10] J. Shoham, M. Inbar and L. Sachs, *Nature* **227**, 1244–1246 (1970).
[11] G.R. Gunther, J.L. Wang, I. Yahara, B.A. Cunningham and G.M. Edelman, *Proc. Nat. Acad. Sci. USA* **70**, 1012–1016 (1973).
[12] M.O.J. Olson and I.E. Leiner, *Biochemistry* **6**, 105–111 (1967).
[13] J.L. Wang, B.A. Cunningham and G.M. Edelman, *Proc. Nat. Acad. Sci. USA* **68**, 1130–1134 (1971).
[14] A.J. Kalb and A. Lustig, *Biochim. Biophys. Acta* **168**, 366–367 (1968).
[15] A.J. Kalb and A. Levitzki, *Biochem. J.* **109**, 669–672 (1968).
[16] J.B. Sumner and S.F. Howell, *J. Bacteriol.* **32**, 227–237 (1936).
[17] B.A. Cunningham, J.L. Wang, M.N. Pflumm and G.M. Edelman, *Biochemistry* **11**, 3233–3239 (1972).
[18] Y. Abe, M. Iwabuchi and I.I. Ishii, *Biochem. Biophys. Res. Commun.* **45**, 1271–1278 (1971).
[19] G.H. Mckanzie, W.H. Sawyer and L.W. Nichol, *Biochim. Biophys. Acta* **263**, 283–293 (1972).
[20] G.M. Edelman, B.A. Cunningham, G.N. Reeke Jr, J.W. Becker, M.J. Waxdal and J.L. Wang, *Proc. Nat. Acad. Sci. USA* **69**, 2580–2584 (1972).
[21] Y. Oda, K. Kasai and S. Ishii, *J. Biochem.* **89**, 285–289 (1981).
[22] Y. Abe and S.I. Ishii, *J. Chromatogr.* **510**, 95–100 (1990).
[23] L. Bhattacharyya and F. Brewer, *J. Chromatogr.* **502**, 131–142 (1990).
[24] J.W. Jorgenson and K.D. Lukacs, *Science* **222**, 266–272 (1983).
[25] S. Hjerten, *J. Chromatogr.* **347**, 191–198 (1985).
[26] H.H. Lauer and D. McManigill, *Analyt. Chem.* **58**, 166–170 (1986).
[27] J.L. Becker, F.M. Everaerts and M.T. Ackermans, *J. Chromatogr.* **537**, 407–428 (1991).
[28] M.N. Pflumm and S. Beychok, *Biochemistry* **13**, 4982–4987 (1974).
[29] K. Weber and M. Osborn, *J. Biol. Chem.* **244**, 4406–4412 (1969).
[30] B.J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404–427 (1964).

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