Journal of Chromatography, 559 (1991) 489–497
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 420

Capillary isotachophoresis with concentration-gradient detection

An application to the separation of synthetic peptides

THERESA McDONNELL and JANUSZ PAWLISZYN*
The Guelph–Waterloo Centre for Graduate Work in Chemistry, University of Waterloo, Waterloo, Ontario N2L 3G1 (Canada)

ABSTRACT

The purity of several synthetic peptides was determined by capillary isotachophoresis with the concentration-gradient detector. The results of the separations are complimentary to those obtained by reversed-phase high-performance liquid chromatography. Detection of low concentration of underivatized peptides (nmol) is facilitated by the universal and low volume optical detector. A simple slider injector was designed for this inexpensive system.

INTRODUCTION

Interest in high-resolution capillary isotachophoresis (cITP) has been rapidly growing over the past few decades [1–7]. This method performs the separation of small quantities of ionic components of a sample in a complex buffer system. It has significant applications in biochemical and medical separations. Instrumentation for cITP traditionally includes a PTFE separation capillary, an injector and one or more detectors [1]. Common injectors include valves and taps which both yield reproducible injection volumes with little or no mixing, but which are complex and expensive. Widely used detectors include UV absorption, and those based on conductivity and fluorescence [1,2]. These measure respective zone properties and have poor resolution; therefore, the performance of the technique is often limited by the detection system.

In this paper we describe simple cITP instrumentation which was constructed in-lab with a few starting materials and access to a machine shop. In our design we use the universal concentration-gradient detector which detects the refractive index gradients associated with high concentration gradients formed at the narrow boundary between two zones of analytes [8–11]. Applications of this inexpensive instrumentation to the separation and determination of the purity of synthetic peptides are described.

0021-9673/91/$03.50

EXPERIMENTAL

The ITP system consisted of an injector and separation capillary, and the concentration-gradient detection system consisting of a laser, a focusing lens and a position sensor [12]. The laser beam is focused directly into the separation capillary and is then intercepted by the position sensor.

Capillary isotachophoresis instrumentation

Fig. 1 shows the assembly of the homemade sliding injector. It was built in a student science shop. Two polytetrafluoroethylene resin rods, 10 cm × 1.9 cm diameter are require to construct the plunger and the house. The size can be varied based on the dimensions required.

The plunger was made first, by machining one rod on a lathe to the required diameter, in this case 6.4 mm. The plunger must be machined to a smooth surface so that it can easily slide into the housing. A "head" is left on one end of the plunger (2 in Fig. 1), while the other end remains bare so that it can slide into the injector housing. The length of the plunger must be longer than the housing by a distance slightly greater than the predetermined sliding distance. The exact length of the plunger is adjusted later. The house for the plunger is made next using the second PTFE rod. A hole is drilled through the rod with a diameter about 0.25 mm smaller than that of the plunger. Then the hole is smoothed with a reamer on the lathe so that the plunger fits in tightly. A tight fit allows vertical motion of the plunger upon applying a force, but prevents free movement of the plunger as well as leakage between the plunger and the

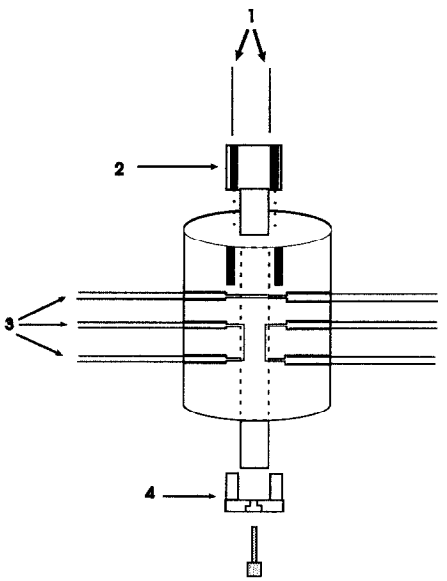

Fig. 1. Diagram of the injector showing the plunger in its housing. 1 = Two rods extending from the head of plunger down into the house which guides the movement of the plunger and prevents horizontal rotation; 2 = plunger head; 3 = capillaries joined tightly into holes drilled in housing, by a butt-type connection; 4 = screw cap on plunger.

house. Two aluminium rods were inserted through the plunger head, down into the housing to prevent horizontal rotation during the sliding motion. These are shown in Fig. 2 and labelled 1.

Three parallel holes are drilled through one side of the housing, and then through the opposite face, with a diameter equal to that of the separation capillary, in this case 0.30 mm. The plunger, aligned in the housing, is also marked with drill bits to map out the placement of the grooves and canal, and is then removed in order to drill the canal and to cut the grooves. The holes in the housing sides are enlarged partway into the housing with a diameter equal to the outer diameter of the capillaries used, 0.76 mm. The plunger and housing are depicted, partially assembled, in Fig. 1. The capillaries fit into the housing by a butt-type connection (Fig. 1). Next, a screw cap is made for the plunger end, which determines the exact distance that the plunger slides.

The diameter of the hole drilled through the plunger, 0.30 mm, as well as the diameter of the plunger, 6.4 mm, determine the volume of the liquid injected. In this injector, about 0.5 μl is injected between the leading and tailing electrolyte. This volume can be varied by replacing the entire injector. Alternatively, a different plunger with a different-diameter hole drilled through it can replace the existing one.

During construction of the injector, care must be taken so that dead volumes are not created between the plunger and housing which may attract either micro gas bubbles from the electrolyte, or a thin layer of liquid. Either situation would decrease reproducibility and affect zone length. The dead volumes can be eliminated by a properly constructed tight fit or by placing narrow O-rings on the plunger brackering the sample hole.

To prepare for an ITP separation, the injector was first rinsed with leading electrolyte (LE), tailing electrolyte (TE) and sample. This was done while the injector was in the up position (Fig. 2A). The LE was filled by syringe action into the capillaries on one face of the injector, and the TE on the other. For example, the tailing electrolyte was filled in through capillary 3 on the bottom (Fig. 2A) up through the small groove

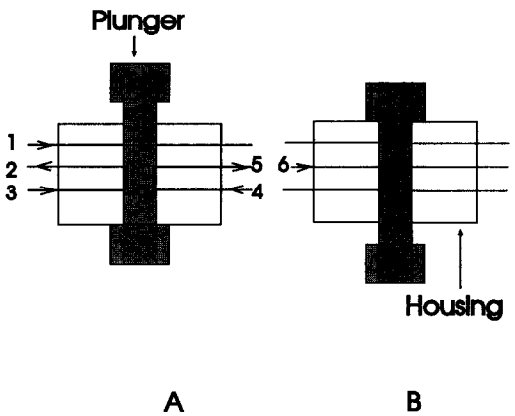

Fig. 2. Two stages of operation of the injector. (A) Stage 1. The plunger is in the up position in the housing, the rinse/fill position of the LE, TE and sample. (B) Stage 2. The plunger slides down in housing. The sample trapped in the plunger is now sandwiched between the LE and TE. The ITP separation follows. 1 = Fill with sample; 2,3 = fill with TE in through 3 and out through 2; 4,5 = fill with LE in through 4 and out through 5; 6 = separation capillary with sample in plunger.

cut in the plunger and out of the centre drainage capillary 2. Similarly, on the opposite side of the injector, the leading electrolyte is filled in through 4 on the bottom and out through 5. Excess LE exists through the separation capillary. The sample is rinsed into capillary 1, through the canal in plunger and exits by a drain capillary. When the rinsing is complete the two ends of the separation capillary are attached to two buffer reservoirs containing membranes.

The sample in the canal of the plunger is then injected (Fig. 2B) as the plunger slides down. The canal in the plunger is now aligned with the middle holes in the housing, 2 and 5. The canal, capillaries 2 and 5 now form the separation capillary 6 in Fig. 2B. The sample is now trapped between the LE contained in 5, and the TE in 2. The voltage is immediately applied.

The separation capillary is made of tetrafluoroethylene (TFE, Cole Parmer, Chicago, IL, USA) tubing, 0.30 mm I.D. × 0.76 mm O.D. The total length of the capillary is 33.0 cm and the length from the injector to the detector is 12.0 cm. This separation capillary was isolated on an XYZ translation stage (Model MR3 linear XYZ translation stage, Klinger Scientific, Montreal, Canada). Two buffer reservoirs were located at either end of the separation capillary. In both reservoirs, membranes (type EPA; Osmonics, Minnetonka, MN, USA) are used to prevent hydrodynamic flow within the capillary. If present, this flow will interfere with the separation. The injector, XYZ translation stage and detector were fastened securely to a stable table, to eliminate any vibrations or movements which would interfere with the optical detector [12].

The current is monitored at the grounded end of the separation capillary, by measuring a drop of voltage across a 1-kΩ resistor, using a digital multimeter (HM 8011-2, Hameg, Germany). A voltage-stabilized high-voltage power supply (Spellman, Plainview, NY, USA) is used to drive the separation. A plexiglass box houses the high-voltage end to shield the user and instruments. The potential is applied to platinum wires embedded into the buffer reservoirs. The negative end of the capillary is grounded.

Gradient detection instrumentation

Concentration-gradient detection was used and is described in further detail elsewhere [8–12]. The optical arrangement consists of a light source which is a helium–neon laser (Model GLG5261, NEC, Japan) and photodiode position sensor. The laser beam is focused to a small spot size of 40 μm directly into the separation capillary, by a focusing lens of focal length 38.10 mm. The probe beam is arranged so that the far field intensity profile points to the centre between two photodiodes placed close together [11]. When irradiated uniformly, the photodiodes generate equal amounts of photocurrent. Upon encountering a concentration gradient, for example at the boundary between two zones, the laser probe beam is deflected and the amount of light reaching the diodes is not equal. The difference in photocurrent associated with the two diodes corresponds to the magnitude of deflection of the laser beam. The difference in photocurrent generated by the diodes is converted to a voltage by a single operational amplifier. The data is collected by an IBM DACA board, in a PC-AT compatible personal computer, using the software ASYST (Asyst Softwares Technology, Rochester, NY, USA).

Reagents

For anionic separations, HCl (ACS reagent grade, Caledon Lab., Georgetown, Canada) was used as leading electrolyte. Counter ion was ammediol, 2-amino-2-(hydroxymethyl)-1,3-propanediol (J. T. Baker, Phillipsburg, NJ, USA). The ammediol was recrystallized twice from ethanol. An additive was added as stabilizer to the leading electrolyte, in this case 0.05% (w/v) polyvinyl alcohol (PVA, molecular mass 10 000; Sigma, St. Louis, MO, USA) was added.

All solutions were prepared from deionized water. The leading electrolyte was prepared by first dissolving the stabilizer in deionized water, and then adding HCl to the desired concentration. Counter ions were added last, and the pH of the solution was monitored by a Corning pH meter (Model 220, Suffolk, UK). Enough counter ion was added to reach a pH of 8.9.

The tailing electrolyte was β-alanine (Sigma), adjusted to a pH of 10.3 with $Ba(OH)_2$ (BDH, Toronto, Canada).

Solutions were filtered twice using 0.2-μm pore size cellulose acetate filters (Sartorius, Göttingen, Germany), and degassed before use by purging with helium. If solutions are not degassed, microbubbles from during the separation which block the passage of current and hinder the reproducibility of the separations. In addition, the presence of CO_2 in the solution will degrade the performance of the separation by the formation of carbonate ion [1]. Carbonate can be separated as a band immediately behind chloride ion and before the first sample, or it may interfere with the separation and distort zones. The effects of carbonate ion are decreased by the use of high-pH tailing electrolyte. The tailing electrolyte must therefore be bubble-free which is effectively done by purging with helium between separations. Also, between injections the separation capillary was washed twice with leading and tailing electrolyte. If the capillary is not rinsed twice, the separations are not reproducible.

DISCUSSION

Fig. 3 illustrates the trace generated when the concentration gradient detector is applied to detect the three boundaries formed in a cITP separation of a mixture of two amino acids between a leading and tailing ion. In this separation, the refractive index of each successive zone was higher than the previous one, from leading to tailing ion. This creates three positive deflections of the probe beam as the zone boundaries migrate by the detector. The broad base in the last valine/tailing electrolyte peak, corresponding to the boundary, is present because of a large temperature gradient between these two zones. In ITP separations, each of the zones has a characteristic temperature associated with it. If the difference in these temperatures is large (dT/dx), then the corresponding large refractive index gradient (dn/dx) is expected to distort the concentration-gradient information. However, it is easy to distinguish between the peaks associated with the concentration and temperature gradients since the latter will be broader, because of higher thermal diffusivities compared to mass diffusivities.

The concentration of analytes in separated zones is governed by the Kahlrausch regulating function which states that the concentration of a pure zone of one ionic species becomes adapted to the concentration of the LE. At a steady state, the concentration of components in zones is similar in magnitude and independent of the initial concentration of components in the injected sample mixture. At low concentra-

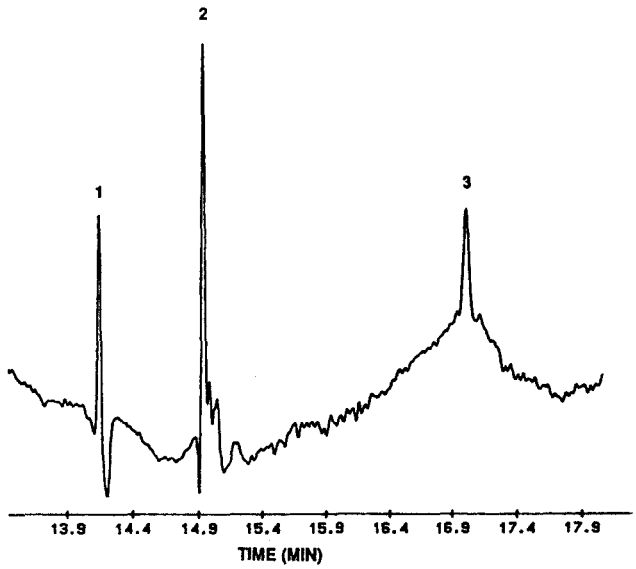

Fig. 3. cITP separation of two amino acids with concentration gradient detection. The leading ion is chloride ion (3 m*M*), counter ion is ammediol pH 8.9, tailing ion is β-alanine (3.0 m*M*) adjusted to pH 10.3 with $Ba(OH)_2$. Sample components are glutamic acid 0.3 m*M*, and valine 0.3 m*M*. Applied voltage is 5 kV.

tion of LE (2 m*M* chloride ion) and low concentration of sample, the concentrating effect of the LE on the sample will not be large. The final volume of each component and therefore the final zone length will be decreased only slightly since mass balance must be applied. For example, four components of a crude preparation of a synthetic peptide (not purified after solid-phase synthesis) were separated by cITP and detected with the high spatial resolution gradient detector (Fig. 4A) at a low LE concentration. This figure shows three major components forming the longest zones, composed of the peptide of interest and possible peptide fragments. The peptides are marked by boundaries 2–3, 3–4 and 4–5. The less than 0.5 mm zone marked by boundaries 1–2 is an impurity comprising about 7% of the total crude preparation. Similar results have been obtained by reversed-phase high-performance liquid chromatography (HPLC); however, the separation required a much longer separation time and more expensive instrumentation. Notice that the gradient detector is capable of marking the 0.5-mm zone indicating its high resolution. Fig. 4 illustrates the excellent compatibility of combining the universal non-destructive concentration gradient detector with cITP to determine the degree of peptide purity. Pure fractions can be collected for micro-preparative work, for example to determine biological activity, since the diameter of the capillaries used are large. cITP with the gradient detector offers an alternative method to reversed-phase HPLC for the separation and collection of pure components of a sample. HPLC, the most widely used chromatographic method for pure sample collection, primarily separates analytes on the basis of polarity. In contrast, cITP separates analytes on the basis of differences in ionic mobility in an electric field and therefore offers a method of separation complimentary to that available by HPLC [7].

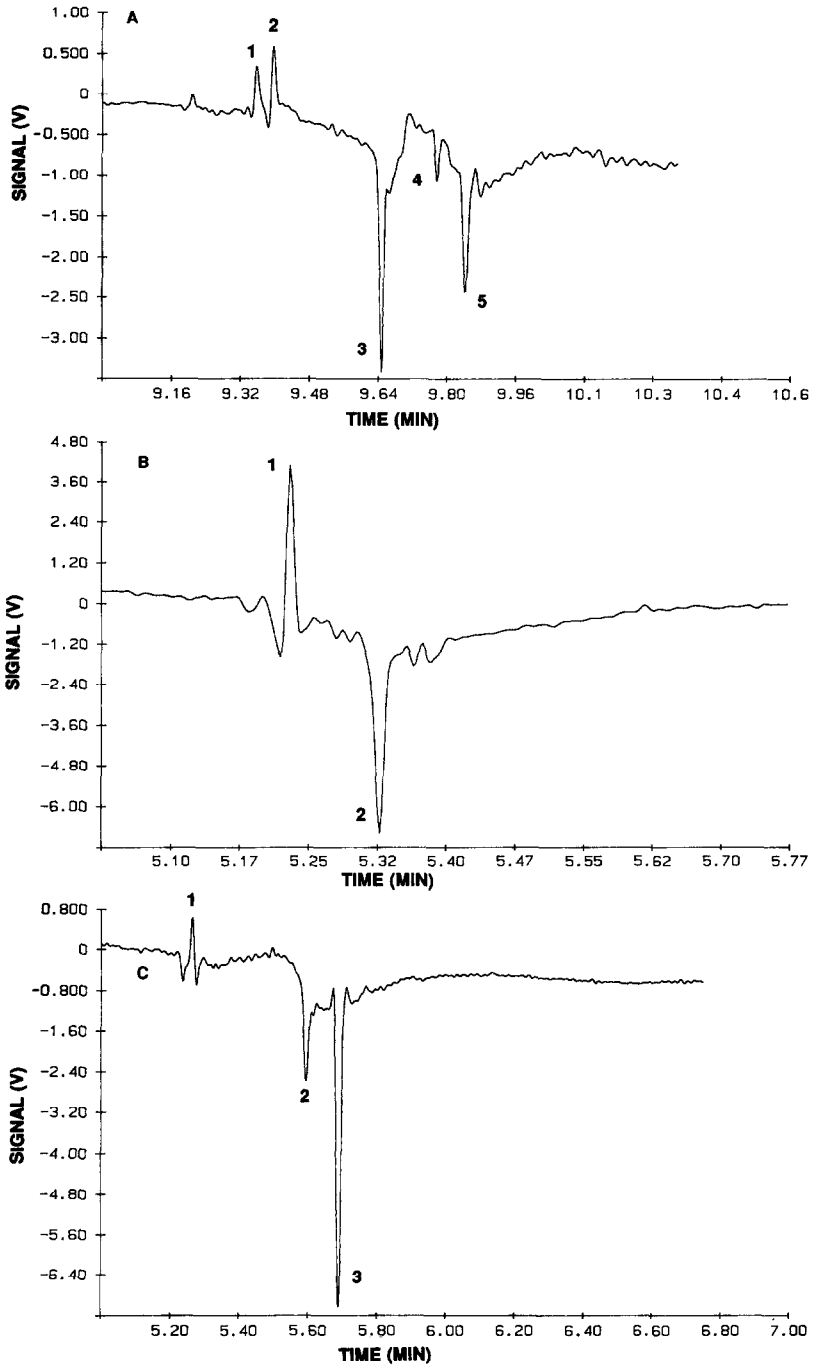

Fig. 4. cITP of crude synthetic peptide. Leading ion is chloride ion 2 mM, counter ion is ammediol pH 8.9, tailing ion is β-alanine 1.6 mM adjusted to pH 10.3 with $Ba(OH)_2$. (A) Injection of 0.5 μl of 0.6 mM concentration of a crude preparation of the linear peptide, 12 kV. (B) Injection of 0.5 μl of 0.3 mM concentration of the purified linear peptide, 12.5 kV. (C) Injection of 0.5 μl of 0.6 mM concentration of two other components of the crude preparation from (A), 13.5 kV.

In addition, the gradient detector can be used to mark the zones of pure sample components to facilitate effective identification with other spectroscopic methods [6].

Stages of a peptide purification scheme were examined with ITP and the concentration-gradient detector. The peptide of interest, Glu–Pro–Val–Thr–Leu–Asp–Leu–Lys–Tyr, formed in the crude separation shown in Fig. 4A has biological activity making it is possible therapeutic agent towards conditions such as emphysema and connective-tissue disorders. This peptide was isolated by reversed-phase HPLC, and the degree of purity of 0.2 nmol of the peptide was determined (Fig. 4B) by ITP with the concentration-gradient detector. The presence of two major boundaries, labelled 1–2 indicate one component, the peptide of interest. On either side of the boundaries marking the peptide zone there are a few small peaks. These are likely impurities which have formed into their own distinct zones. The signal-to-noise ratio of these peaks is very small versus the boundaries labelled as 1–2. The concentration-gradient detector gives a signal related to the molecular-mass difference between species, and therefore generates a large signal with a large molecular-weight difference and a corresponding small signal, such as the ones seen on either side of the two major peaks, with a small molecular-weight difference [8]. The small refractive index gradient of the unlabelled peaks indicates that these impurities have only small differences in refractive index between them and the LE or TE. These must be much smaller in size and molecular mass than the peptide and could be small ions, amino acids or fragments of peptides.

Other major fractions from the peptide synthesis, which were not the peptides of interest forming the main product, were also collected after separation by reversed-phase HPLC. They were isolated in order to check for possible biological activity. These were separated by ITP and detected by the concentration gradient detector (Fig. 4C). The three boundaries labelled as 1–2–3 map out the two major fractions, as zone 1–2 and zone 2–3. At least one of these two pure fractions is composed of a peptide which may also have some biological activity. The first boundary, 1, between the leading ion and highest-mobility component, shows a dip in the baseline around the boundary. This occurs because of a large temperature difference between the two zones, as discussed previously.

A portion of the peptide of interest was cyclized yielding a crude mixture of approximately 80% cyclized and 20% linear peptide. This crude mixture was separated using ITP (Fig. 5) under conditions similar to those previously described of high voltage and low concentrations. Three major boundaries are present and some smaller signals from impurities such as ions or peptide fragments. The first zone marked by boundaries 1–2 comprises 20% of the total distance between peaks 1–3, having a zone length of 1.0 mm. This is the linear unreacted peptide. The second zone marked by boundaries 2–3 has a zone length of 4.0 mm, comprising about 80% of the total distance and is the desired cyclized peptide. The results from ITP with the concentration gradient detector confirm the chromatogram obtained for the same mixture by reversed-phase HPLC.

The simple instrumentation described in this paper is a valuable and practical tool for the determination of peptide purity and collection of samples for micro-preparative work. The instrumentation can be easily adapted to capillary isoelectric focusing, a similar electrophoretic technique which has both self-sharpening and concentrating properties. In this method sharp zones of analytes along a pH gradient

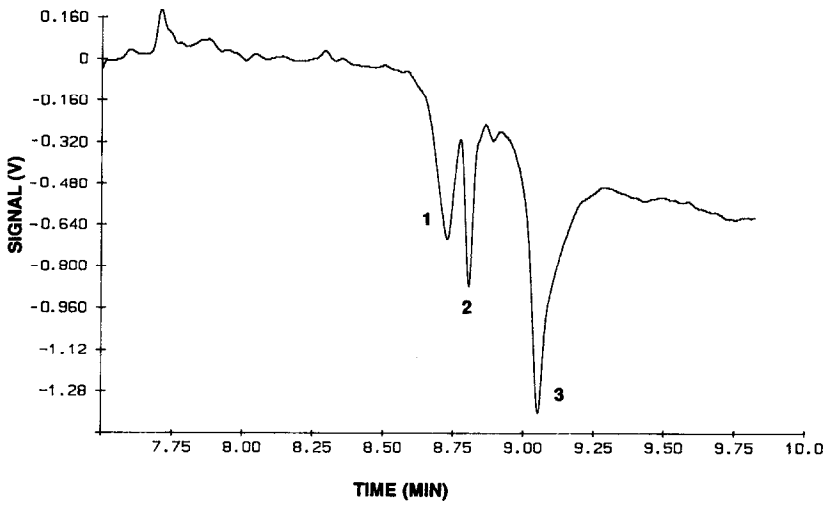

Fig. 5. cITP separation of a cyclized and linear peptide, 13 kV. Leading ion is chloride ion 2 m*M*, counter ion is ammediol pH 8.9, tailing ion is β-alanine 1.6 m*M*, adjusted to pH 10.3 with $Ba(OH)_2$. Injection was 0.5 μl of 0.45 m*M* of the mixture.

are formed, which can be effectively detected with the concentration-gradient detector. The cost of the system can be decreased to about US$ 150 by replacing the HeNe laser with a diode laser.

ACKNOWLEDGEMENTS

The Natural Sciences and Engineering Research Council of Canada supported this work. Beckman Instruments Inc. provided the high-voltage power supply. Dr. Lajoie, Peter Belshaw and Anna Crivici provided the synthesized peptides.

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