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Short communication

Capillary zone electrophoresis method development for the analysis of *Hippeastrum* hybrid agglutinin samples

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ABSTRACT

A capillary zone electrophoresis (CZE) method was developed aiming the analysis of *Hippeastrum* hybrid agglutinin (HHA) samples. HHA is presently being tested as a vaginal microbicide to prevent HIV transmission. It acts by direct binding to mannose residues that are abundantly present on the HIV gp120 envelope and so interrupts the virus entry process. The final CZE method employs 50 mM sodium tetraborate (pH 9.9) as background electrolyte. In this condition, a cluster of about 30 isoform peaks is obtained, with very repeatable patterns. RSDs in the order of 0.2% for the migration time and detection sensitivity in the order of 70 $\mu g\,ml^{-1}$ were achieved.

1. Introduction

Topical microbicides can be formulated as gels, foams, films or vaginal rings and are presently being developed as a possible new approach to prevent HIV infection. More than 60 potential microbicides are being tested in preclinical and clinical trials [1–3], including the plant lectins derived from *Hippeastrum* hybrid (Amaryllis).

Lectins are proteins and glycoproteins widely distributed in nature and present in cyanobacteria, sea corals, algae, plants, invertebrates and vertebrates [4]. They represent a very heterogeneous group and are often responsible for pronounced physiological responses. By an extrapolation of plant lectin definition, lectins are proteins possessing at least one noncatalytic domain that binds reversibly to specific mono- or oligosaccharides [5]. Since carbohydrates at cellular receptors play a key role in the binding of ligands, the carbohydrate specificity can be extended to cell specificity. In fact, lectins are able to promote agglutination of specific cells, and for this reason they are frequently called carbohydrate binding proteins (CBPs) and/or agglutinins. *Hippeastrum* hybrid agglutinin (HHA) is a mannose binding protein (more specifically α-(1,3) and α-(1,6)-mannose oligomers) [6,7].

The viral entry is nowadays one of the most promising targets for anti-HIV chemotherapy. This process is highly dependent on the interaction of the viral glycoproteins gp120 and gp41 with the cellular receptor CD4 and, as a second step, a co-receptor, mainly CXCR4 and/or CCR5 [4,8–12].

HHA is able to bind to the glycans on the HIV-1 gp120 envelope protein, thereby hindering the interaction of the virus to the cellular receptors and so interrupting the viral entry process (EC_{50} varying from 3.2 $\mu g\,ml^{-1}$ to 0.16 $\mu g\,ml^{-1}$ for different HIV strains, including resistant ones) [13–16].

HHA is a promising candidate for use in vaginal formulations because it is stable at body temperature and at vaginal pH. Also, HHA is odourless, colourless, tasteless, does not present human red blood cell agglutinating activity, is not cytotoxic, not antimetabolically active and not mitogenic to human primary T-lymphocytes at 10^2 to 10^3 fold its antiviral active concentration [4,13].

HHA is a tetrameric protein composed of four sub-units of circa 12.5 kDa encoded by a gene family. The tertiary and quaternary structure of HHA has already been determined and at least eight isoforms have been cloned and are available in the Genbank/EMBL [13,17–20]. Genbank data of these eight isoforms can be found in Table 1: accession numbers, number of amino acids, MW and the estimated p*I* values (calculated with a Genbank/EMBL tool).

In order to develop a successful vaginal formulation of HHA for the prevention of HIV transmission, the availability of analytical methods for HHA is crucial, e.g. for release studies, quality control and stability studies. Despite the rich literature behind HHA and

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Table 1
Genbank/EMBL accession numbers, isoelectric point, molecular weight and length of the HHA isolectins.

Accession number	p*I*[a]	MW	Length
M88124	8.50	12606.30	115
M88125	8.91	11879.43	109
M88126	8.50	12607.30	115
M88129	8.50	12497.13	116
M88130	7.69	11593.98	109
M88131	7.69	11593.98	109
M88132	7.69	11593.98	109
M88133	5.14	11827.22	109

[a] Isoelectric point estimated according to the respective amino acid sequences.

other promising CBPs, there are still no analytical tools described in the literature for routine analysis of these substances. Recently a CE–MS method for the analysis of another anti-HIV CBP, namely *Urtica* dioica agglutinin (UDA), has been described in the first report on using CE for analysis of plant lectins [21]. The purpose of the present study was to develop a simple and reliable CE method, using relatively inexpensive and accessible techniques, suitable for the analysis of HHA samples.

2. Experimental

HHA was kindly provided by the Laboratory for Biochemistry and Glycobiology of the University of Ghent (Belgium). The material provided was isolated and highly purified (mainly based on affinity chromatography in a mannose-immobilized column) as described elsewhere [6,7].

Experiments were carried out with two different batches of HHA. The samples were dissolved in the background electrolyte (BGE) prior to the experiment or dissolved in 50 mM tetraborate solution and stored at 7 °C for at most 7 days. The dried bulk HHA was stored at 7 °C.

All the chemicals used were of analytical grade. Sodium hydroxide and sodium dihydrogen phosphate were purchased from Riedel-de Haën (Seelze, Germany). Sodium hippurate was obtained from Sigma–Aldrich (St. Louis, MO, USA). Sodium tetraborate decahydrate, phosphoric acid 85%, ammonium acetate, sodium acetate and ethanol were obtained from Acros Organics (Geel, Belgium). Boric acid, methanol, acetone and acetonitrile were purchased from Fisher Chemicals (Leicestershire, UK). All solutions used for the experiments were prepared with ultrapure Milli-Q water (Millipore, Milford, MA, USA).

Tetraborate buffers were prepared with sodium tetraborate or boric acid (with the molarity corrected for tetraborate) and the pH was adjusted with solutions of sodium hydroxide. The pH values of buffers were measured and adjusted with the aid of a pH-meter Metrohm 691 (Herisau, Switzerland). For buffers containing organic modifiers, the reported pH corresponds to the aqueous solution before the addition of the organic solvent.

Experiments were performed on a P/ACE™ MDQ equipment with diode array detector and the data acquisition was done by means of 32 Karat™ 4.0 software (both Beckman–Coulter, Fullerton, CA, USA). The capillaries used were purchased from Polymicro Technologies (Phoenix, AZ, USA). All of the capillaries used were uncoated fused silica, 50 μm id, 375 μm od, 60 cm long (50 cm effective). New capillaries were conditioned at 25 °C by rinsing with 1 M NaOH (5 min), 0.1 M NaOH (30 min) and water (5 min). Daily conditioning was performed at a temperature of 25 °C by rinsing with water (5 min), 0.1 M NaOH (10 min), water (2 min) and BGE (10 min). Between each run the capillary was rinsed at 25 °C with 0.01 M NaOH (1 min) and BGE (2 min). All the rinsing procedures were performed by applying a pressure of 138 kPa. The inlet/outlet vials were replaced every three runs.

Acetone (0.2%) was used as neutral marker to identify the electroosmotic flow (EOF) migration time in different BGEs.

3. Results and discussion

3.1. Method development

Gel filtration experiments have demonstrated that HHA is a protein of about 50 kDa, but, when analysed by SDS-PAGE, one single spot of about 12.5 kDa is revealed, which suggests that HHA is a tetramer composed of four identical units [18,19]. When analysed by ion exchange chromatography (IEC) and isoelectric focusing electrophoresis (IEF) [18] a mixture of isolectins is assumed by the occurrence of several spots in IEF, indicating isoelectric points (p*I*)s ranging from 3.90 to 5.45, and a complex and repeatable pattern in IEC.

The two main modes of CE applied to peptides and proteins analysis are capillary gel electrophoresis (CGE) and capillary isoelectric focusing (CIEF), typically used for MW or p*I* determination, respectively. However, the simplest and most popular CE mode is capillary zone electrophoresis (CZE), which, when applied to protein analysis, could provide a combination of the information obtained by CGE and CIEF, since the migration of the proteins in free solution is dependent on the p*I* and on the MW of the protein.

Several buffers with different pHs were screened as BGE for the CZE separation of HHA (data not shown). In acidic conditions (phosphate buffer pH 2.5), slightly under the experimentally determined p*I* [18], the molecules are positively charged and migrate in the same direction of the EOF; however, due to the low velocities of both EOF and sample, the result is an undefined and broad cluster. Upon increasing the buffer pH (acetate buffer pH 4.8), the HHA cluster was observed just after the EOF, but not more than an elevation of the baseline was obtained. According to the experimental p*I* data, most of the isoforms have a net charge very close to zero at pH 4.8, and the proteins should migrate together with the EOF. At this pH, the interaction of the proteins with the capillary wall can explain the undefined baseline elevation after the EOF: the neutral proteins are carried by the EOF, but due to their interaction with the wall it takes some time for the EOF to completely drag-out the HHA. At neutral pH (phosphate buffer pH 7.0), a broad and undefined elevation after the EOF was detected, though more pronounced as compared to pH 4.8. In alkaline conditions (tetraborate buffer pH 9.3 and phosphate buffer pH 12.0), the undefined signals observed at pHs 4.8 and 7.0 were replaced by a complex cluster of not completely resolved peaks; the separation was best at pH 9.3. The improvement in the separation and peak shape at alkaline conditions was expected due to the reduction of the protein–wall interaction (both are negatively charged).

Since the best separation of HHA was obtained using the tetraborate buffer (pH 9.3), the whole tetraborate buffering range was examined in more detail (Fig. 1). The observed gain in resolution and peak shape when increasing the pH from 8.2 to 10.2 was related to the observed increase of HHA's electrophoretic mobility and consequent decrease of its apparent mobility. Thereafter other sodium tetraborate buffers (with pH values not shown in Fig. 1) were tested and pH 9.9 was selected as optimum.

Augmenting the ionic strength from 10 mM to 150 mM slightly improved the peak shape and the resolution (results not shown). Higher ionic strengths led to higher currents, making Joule heating the main limitation in using concentrated BGE in CE. A BGE containing 50 mM sodium tetraborate, pH 9.9 showed a good compromise between analysis time and resolution, allowing the application of up to 26 kV without compromising Joule heating (with a typical current of 103 μA).

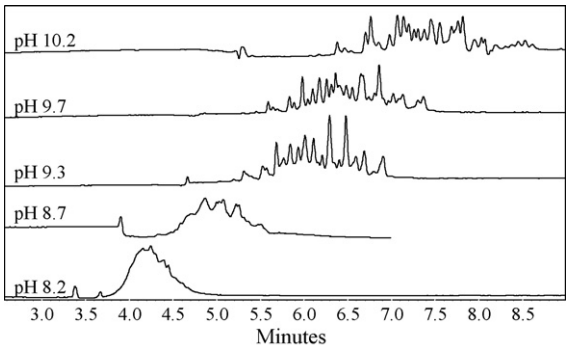


Fig. 1. pH influence on the separation of HHA. CE: capillary, uncoated fused silica capillary 50 μm id, 375 μm od, 60 cm long (50 cm effective); voltage, 25 kV; temperature, 25 °C; detection, UV 190 nm; injection, inlet pressure 1.38 kPa for 4 s; BGE, 50 mM sodium tetraborate; sample, HHA 2.04 mg ml^{-1} in BGE pH 9.3.

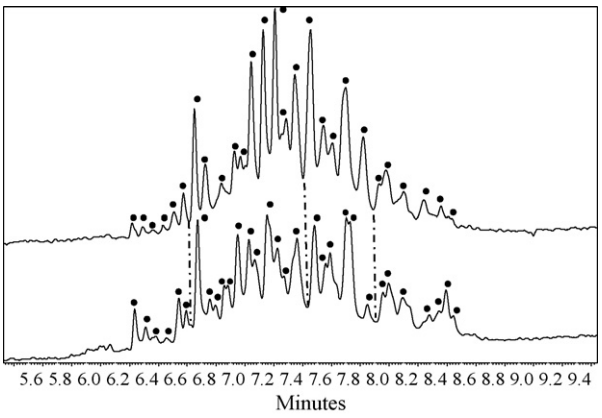


Fig. 2. Separation profile of two different batches of HHA. The black dots spotting the peaks and the three dashed lines are simply for eye guiding. 27 peaks can be visualized in one batch (superior line) and 31 peaks can be visualized in the second batch (inferior line). CE: injection, inlet pressure 3.45 kPa for 5 s; BGE, 50 mM sodium tetraborate pH 9.9; sample, HHA 2.0 mg ml^{-1} (for both batches) in BGE. Other conditions are the same as in Fig. 1.

Different temperatures from 15 °C to 50 °C were tried out in steps of 5 °C (data not shown). At low temperature, the migration time and the cluster time frame increased, with some peak broadening. At higher temperatures the migration time was decreased and the separation of the isolectins was prejudiced. A good compromise was obtained at 25 °C.

The presence of organic modifiers in the BGE can significantly alter a CE separation. For the HHA separation, BGEs containing 0, 5, 10 and 20% of methanol, ethanol or acetonitrile were compared (data not shown). All three organic solvents resulted in a reduced EOF mobility, and, consequently, increased analysis times. The separation window increased with the concentration of the organic modifiers, accompanied however by some peak broadening. Apparently none of the organic modifiers caused changes in the selectivity of the peaks.

Although some improvement in the resolution was observed with the addition of organic modifiers, it was not enough to promote baseline resolution and/or to separate considerably more HHA isoforms. As a conclusion, the use of methanol, acetonitrile or ethanol could be useful when some improvement in the resolution is necessary, but does not seem mandatory for the analysis of HHA samples.

To summarise, a system simply composed of 50 mM sodium tetraborate pH 9.9 was chosen for further evaluation of the HHA analysis by CZE. To correct for injection errors, an internal standard was adopted; among several tested substances, hippuric acid was suitable due to its good stability and good separation from the HHA cluster.

3.2. HHA cluster interpretation

To check whether different HHA batches would produce exactly the same profile, two batches were analysed under the same conditions (Fig. 2). The majority of the peaks could be observed for both batches, albeit in different proportions. Fluctuations in the relative expression of the isoforms can be expected in distinct HHA extracts. The migration time of the cluster was very similar for both batches.

What exactly does the observed cluster represent? As mentioned before, the samples represent a highly purified mixture of the isolectins. In order to associate the observed pattern to the different isolectins reported in Genbank (Table 1) a combinatorial analysis was performed.

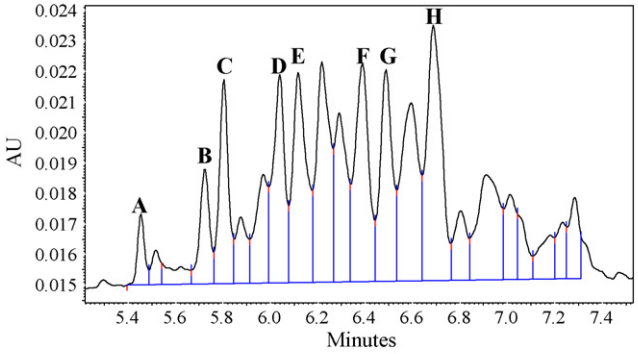
The HHA is a tetrameric protein formed by a combination of eight different isoforms. This means that, in principle, it is possible to have proteins with all four monomers expressed by one gene or proteins with all of the monomers expressed by different genes. In the light of this statement, one can conclude that it represents a combination with repetitions, which is expressed by the following equation:

$$C(n, k) = \frac{(n + k - 1)!}{k!(n - 1)!}$$

where $C(n, k)$ is the number of possible combinations of k objects that can be chosen from n objects. In the present case, n is the number of isoforms ($n = 8$) to form a tetramer ($k = 4$).

Solving $C(8, 4)$, 330 combinations are possible for HHA, which is far more than observed in the electropherograms. However, some considerations and approximations should be adopted: (1) although the estimated p*I* values for the isoforms (Table 1) are considerably different from the experimental p*I* values found for the tetramers [18], it can be assumed that the p*I* shift of each isoform when forming the quaternary structure will be proportional for each isolectin; (2) the CZE separation of HHA, as discussed before, is based on the charge, which is related to the p*I*, and radius (MW and solvatation) of the analytes; (3) as reported in Table 1, some of the isoforms possess practically the same values of p*I* and MW; as a result, the separation of some isoforms from each other would be very difficult, even with CE being a high efficiency technique; (4) taking into account considerations (1)–(3), it is possible to consider the isoforms M88124, M88126 and M88129 as one and the isoforms M88130, M88131 and M88132 as one as well.

Thus, following approximation (4), the number of isoforms to be considered in the combinatorial analysis is four instead of eight. Solving the simplified model $C(4, 4)$ reveals that the number of "separable" combinations is reduced to 35, which is close to the number of peaks that can be distinguished in the HHA cluster after CZE separation (Fig. 2). It could be considered that some of the possible combinations are less probable than others (assuming all isoforms are similarly expressed), especially the ones containing multiple units of the isolectins M88125 and M88133. In fact, the combinations 4× M88125 and 4× M88133, could be present at very low levels. Furthermore, the isoforms M88133 and M88125 are the isoforms with the lowest and highest p*I* values, respectively. This is in good agreement with the obtained cluster shape, more populated in the central region than in the extremities, where the less abundant isoforms combinations are situated. Concluding, the combinatorial analysis suggests that the peaks observed in the cluster are indeed tetramers with different isoforms composition and most of the peaks have multiple combinations.



	Migration time (min)		Corrected area	Sensitivity
	Average	RSD (%)	RSD (%)	LOD (μg.ml^{-1})
Peak A	5.45	0.18	3.99	67
Peak B	5.72	0.18	7.15	42
Peak C	5.79	0.17	4.48	24
Peak D	6.03	0.18	4.11	24
Peak E	6.11	0.19	3.11	24
Peak F	6.38	0.18	3.51	22
Peak G	6.48	0.20	4.49	23
Peak H	6.68	1.19	4.55	19

Fig. 3. (Electropherogram) Peaks chosen for determination of repeatability and sensitivity. CE: voltage, 26 kV; injection, inlet pressure 3.45 kPa for 5 s; sample, HHA 1.33 mg ml^{-1}, sodium hippurate (internal standard—not visible in the zoomed time frame) 29.2 μg ml^{-1} in BGE. Other conditions are the same as in Fig. 1. (Table) Migration time and corrected peak area (peak area divided by internal standard area) repeatability and sensitivity. LOD: S/N = 3, n = 9.

3.3. Method validation

Prior to the application of the CZE approach to the analysis of HHA, the analytical capability of the CZE separation was validated with respect to repeatability, sensitivity and linearity.

In the profile of HHA, eight peaks (A–H) were chosen to determine repeatability and sensitivity (Fig. 3). The limit of detection (LOD) can be calculated as the concentration giving S/N = 3. The LOD obtained for peak A, a small peak in the cluster, is in the order of 67 μg ml^{-1}, expressed in total concentration of HHA. Typical gel formulations under investigation employ HHA at concentrations from 0.5 mg g^{-1} to 5.0 mg g^{-1}, which makes the method sensitive enough for HHA determination in gel samples. The repeatability was verified by calculating the relative standard deviation (RSD) of the migration time and the HHA area corrected by the internal standard (hippuric acid 29.2 μg ml^{-1}) in nine replicates. Good repeatability was achieved for the migration time considering several replicates. The deviations in the areas of individual peaks are partly due to integration difficulties. The RSD obtained for the integration of the whole cluster was 1.8%. The table embedded in Fig. 3 shows the repeatability and sensitivity results obtained for the eight chosen peaks.

Finally, the linearity for the whole cluster area of HHA was evaluated, since differences in the isoforms proportion are possible from batch to batch. This approach showed good linearity, with a regression equation expressed as $y = 3.5289x - 0.0587$, $R^2 = 0.9993$ (obtained with 0.12 mg ml^{-1}, 0.24 mg ml^{-1}, 0.48 mg ml^{-1}, 0.96 mg ml^{-1}, 1.92 mg ml^{-1} and 3.83 mg ml^{-1} of HHA, injected in duplicate).

Finally, the feasibility of the CZE methodology to assess HHA in a typical gel formulation was checked. The tested gel contained HHA 5 mg g^{-1} and was composed of propylparaben (0.2 mg g^{-1}), methylparaben (1.8 mg g^{-1}), hydroxyethyl cellulose (HEC, 15 mg g^{-1}), glycerol (50 mg g^{-1}), lactic acid 85% solution (0.6 mg g^{-1}), NaOH (to adjust to pH 4.5) and water. The excipients were well separated from the HHA cluster (Fig. 4). Slight modifications, namely some peak broadening and some loss in the peak resolution, were observed in the HHA cluster when comparing with bulk HHA cluster. This might be caused by an interaction with some compounds of the gel (most probably the viscosity enhancer HEC) in the beginning of the separation. These "distortions" cannot be explained by differences in the proportion of isoforms, since exactly the same HHA batch used for gel preparation was used as standard. The fact that more diluted gel samples led to more similar profiles (data not shown) enforces the idea that the separation is slightly affected by the sample plug.

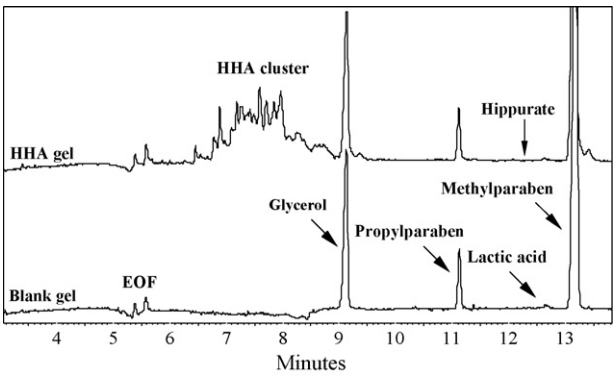


Fig. 4. Electropherograms obtained from a gel formulation containing 5 mg g^{-1} of HHA and from a blank formulation. CE: injection, inlet pressure 3.45 kPa for 5 s; sample, diluted gels (1:3). The expected position for hippurate (internal standard—not added to these samples) is indicated. Other conditions are the same as in Fig. 1.

4. Conclusion

In this work a straightforward CZE method was developed for the analysis of HHA samples. Although the method did not achieve full baseline separation, it did enable fast (10 min) separation of the isolectins, providing a kind of fingerprint cluster capable even to discern different HHA batches. The developed method showed a good repeatability, linearity and sensitivity. It was illustrated that the method can also serve for HHA analysis in the development of microbicide formulations. Although the above conditions were found as optimal, small adjustments in the BGE may be needed in the future depending upon changes in the gel formulation.

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