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Capillary electrophoretic separation of proteins and peptides by ion-pairing with heptanesulfonic acid

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Abstract

Heptanesulfonic acid as ion-pairing agent was used for the separation of mixtures of low and high molecular mass peptides/proteins by capillary electrophoresis. The separation conditions used were: capillary 37 cm (30 cm to the detector) × 75 μm i.d., voltage 10 kV, phosphate buffer 50 mmol/l, ion-pairing agent heptanesulfonic acid at three different concentrations, namely, 0, 20 or 100 mmol/l, pH 2.5. The separation reflected the ion-pairing equilibria between the ion-pairing agent and the peptide/protein analytes. The influence of ion-pairing on sample mobility (running time) was more pronounced in case of the higher-molecular peptides as compared to the low molecular ones. This difference offers the possibility to separate low and high molecular peptides/proteins that under the absence of the ion-pairing agent would co-migrate. The principle of this approach was demonstrated on a randomly selected set of peptides/proteins; the practical applicability was demonstrated on a set of CNBr peptides arising from a naturally occurring mixture of collagen types I and III.

Keywords: Ion-pairing reagents; Peptides; Proteins; Heptanesulfonic acid

1. Introduction

The decisive mechanisms involved in separating complex protein/peptide mixtures is the effective charge and molecular mass of individual analytes involved. Changing the pH of the background electrolyte turned out quite advantageous but not sufficient to separate individual components of complex samples. The current practice prefers widely separations at very acid pH values (∼2.5). In order to increase the electrophoretic selectivity a number of authors attempted rather successfully to use ion-pairing interactions ([1,2], for review see [3]). The chemical nature of the ion-pairing reagents varies in a wide range comprising, besides others, tetramethylammonium chloride, tetraethylammonium chloride, tetrabutylammonium perchlorate, tetrahexylammonium chloride, butanesulfonic acid and decanesulfonic acid. For the separation of proteins/peptides hexanesulfonic acid appeared most promising [4]. As reported by Weldon et al. [5], using heptanesulfonic acid (or any alkylsulfonic acid) resulted in an almost two-fold resolution of tryptic peptides arising during enzymatic cleavage of cytochrome *c*. Rather surprisingly the resolution improvement resulting from the application of different ion-pairing agent was rather similar over a wide pH range (2.5–9.0). It was concluded that the interaction with anionic ion-pairing agents remains favorable owing to the positive charge of the zwitterionic peptide fragments. However, it was clearly pointed out that between two adjacent peptides the separation (selectivity) could be either better or worse depending on the ion-pairing equilibria between the ion-pairing agent and a particular peptide. On the other hand experiments done at neutral pH revealed that the changes in peptide profiles reflect a combination of selectivity resulting not only from the ion-pairing effect but from altered (decreased) interactions of the peptides followed with the inner surface of the capillary wall. The selectivity improvement with anionic ion-pairing reagents even at alkaline pHs strongly suggested that also hydrophobic interactions are involved in the final result, though they appeared to play a secondary role only. When a mixture of standard proteins (cytochrome *c*, albumin and ribonuclease) was used for testing the ion-pairing effect it seemed feasible to conclude that multiple ion-pairing interactions occur on the protein molecule provided that heptane or decanesulfonic acids were used as the ion-pairing agents. A partial loss of electrophoretic

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efficiency was ascribed to conformational changes and EOF reduction.

The results obtained previously by Rush et al. [6] fit perfectly the conclusions formulated later by Weldon et al. [5] upon separating tryptic peptides of erythropoietin: sodium phosphate buffer pH 2.5 (40 mmol/l) made 100 mmol/l with respect to heptanesulfonic acid revealed a clear-cut segregation of the resulting peptide profile into the faster moving section of non-glycosylated peptides followed by the glycosylated portion of the peptide mixture.

It was the aim of this investigation to reveal to what extent the presence of an ion-pairing reagent (heptanesulfonic acid) in the background electrolyte could help better separation of low and high molecular mass peptides/proteins in situations where the high and low molecular mass species co-migrate or overlap under standard separation conditions (i.e. in the absence of the ion-pairing reagent).

2. Experimental

2.1. Chemicals used

Sodium dihydrogen phosphate and hydrochloric acid were products of Lachema (Brno, Czech Republic) and were of p.a. quality. *n*-Heptanesulfonic acid was from Serva (Heidelberg, Germany) and ammonium bicarbonate was from Sigma (St. Louis, MO, USA), bromcyan (cyanogen bromide) and 2-mercaptoethanol were purchased from Merck (Darmstadt, Germany), formic acid was a product of Fluka (Buchs, Switzerland). All solutions were prepared in Milli-Q water (Millipore, Bedford, MA, USA).

2.2. Capillary electrophoresis

All runs were done with Beckman P/ACE instrument system 5500 (Beckman, Fullerton, CA, USA). Capillaries 37 cm (30 cm to the detector) × 75 μm i.d. were used (Polymicro Technologies, AZ, USA). Detection was performed by UV absorbance at 214 nm. Before analysis the capillary was washed 5 min with the background electrolyte. At the start and at the end of a set of analyses the capillary was stepwise washed by water (1 min), 1 mol/l NaOH (5 min), water (1 min), 3 mol/l HCl (5 min) and water (1 min). The sample was injected hydrodynamically (1 s, 3.45 kPa overpressure). The separation was run at 20 °C at applied voltage 10 kV.

Three types of background electrolytes were used.

(1) 50 mmol/l phosphate buffer, pH 2.5.
(2) 50 mmol/l phosphate buffer, 20 mmol/l heptanesulfonic acid, pH 2.5.
(3) 50 mmol/l phosphate buffer, 100 mmol/l heptanesulfonic acid, pH 2.5.

Before analysis background electrolytes were filtered using Millex-HV filter (Millipore), 0.45 μm.

2.3. Protein and peptide samples

The set of test analytes comprised the following proteins and peptides: cytochrome *c* (M_r 12 500), albumin from bovine serum (M_r 68 000) were products of Boehringer (Mannheim, Germany). Two poly-L-lysine samples were obtained from Sigma, the first had an average relative molecular mass 22 700 (by viscosity; degree of polymerisation, DP: 138) or 28 200 (by size-exclusion chromatography–low-angle laser light scattering (SEC–LALLS); DP: 172; molecular distribution: <10% smaller than 10 800 and <10% greater than 46 000) and the second polymer had an average relative molecular mass 4000 (by viscosity; DP: 19) or 3300 (by SEC-LALLS; DP: 16). Peptides Val-Ala-Ala-Phe (M_r 406.5), Phe-Gly-Phe-Gly (M_r 426.5), Gly-Gly-Tyr-Arg (M_r 451.5), Arg-Pro-Pro-Gly-Phe (Bradykinin fragment 1–5; M_r 572.7) and Tyr-Gly-Gly-Phe-Leu (Leucine enkephalin; M_r 555.6) were the products of Sigma. All samples were dissolved in water to the concentration of 1 mg/ml.

A set of peptides obtained by CNBr cleavage of rat tail tendon collagen was also used as model mixture. This sample contained fragments of both type I and III collagen and was prepared by the procedure described in our previous communication [7]. Briefly, samples (rat tail tendons) were incubated in 0.2 mol/l ammonium bicarbonate, pH 7.0, containing 25% (v/v) β-mercaptoethanol to reduce oxidised methionyl residues and after lyophilisation the samples were cleaved by CNBr in 70% (v/v) formic acid under nitrogen. Samples were lyophilised and then reconstituted in water to the concentration of 2 mg/ml.

The low molecular fraction (below 10 000 relative molecular mass) of CNBr collagen fragments was prepared by ultrafiltration through Microcon centrifugal filter devices (Microcon YM-10, regenerated cellulose with nominal molecular mass limit 10 000 relative molecular mass) by Amicon (Millipore) at 2000 × *g* for 10 min.

3. Results and discussion

The test mixture (as specified in Section 2) was composed of arbitrarily selected peptides and proteins that could be divided into three broad categories, namely (i) macromolecular species, cytochrome *c* (M_r 12 500), bovine serum albumin (M_r 68 000) and highly polymerized polylysine (M_r 28 200), (ii) relatively low molecular components (peptides) possessing an additional positively charged nitrogen containing functionality (amino or guanido) in addition to the N-terminal amine (i.e. Gly-Gly-Tyr-Arg and Arg-Pro-Pro-Gly-Phe), including short polylysine (M_r 3300), and (iii) peptides (low molecular species) possessing aromatic residues in their molecule but devoid of an additional, positively charged amino or guanido group (i.e. Val-Ala-Ala-Phe, Phe-Gly-Phe-Gly and Tyr-Gly-Gly-Phe-Leu). Separations effected in 50 mmol/l phosphate buffer pH 2.5 containing 0–100 mmol/l heptanesulfonic acid yielded

the profiles shown in Fig. 1. Introducing heptanesulfonic acid into the background electrolyte resulted in increasing the migration time of the macromolecular species (high molecular mass polylysine, cytochrome *c* and bovine serum albumin) which finally did not reach the detector's window at all, if the concentration of the heptanesulfonic acid was 100 mmol/l. The fact that this effect was observed even with the high molecular mass possessing polylysine indicates that at the 100 mmol/l concentration of the ion-pairing agent the equilibrium is shifted very much in direction of the ion-pair formation leaving very few (if any) positively charged amino groups in the analysed polymeric species that would be able to create cathodic mobility of this analyte. On the other hand the low molecular mass possessing polylysine

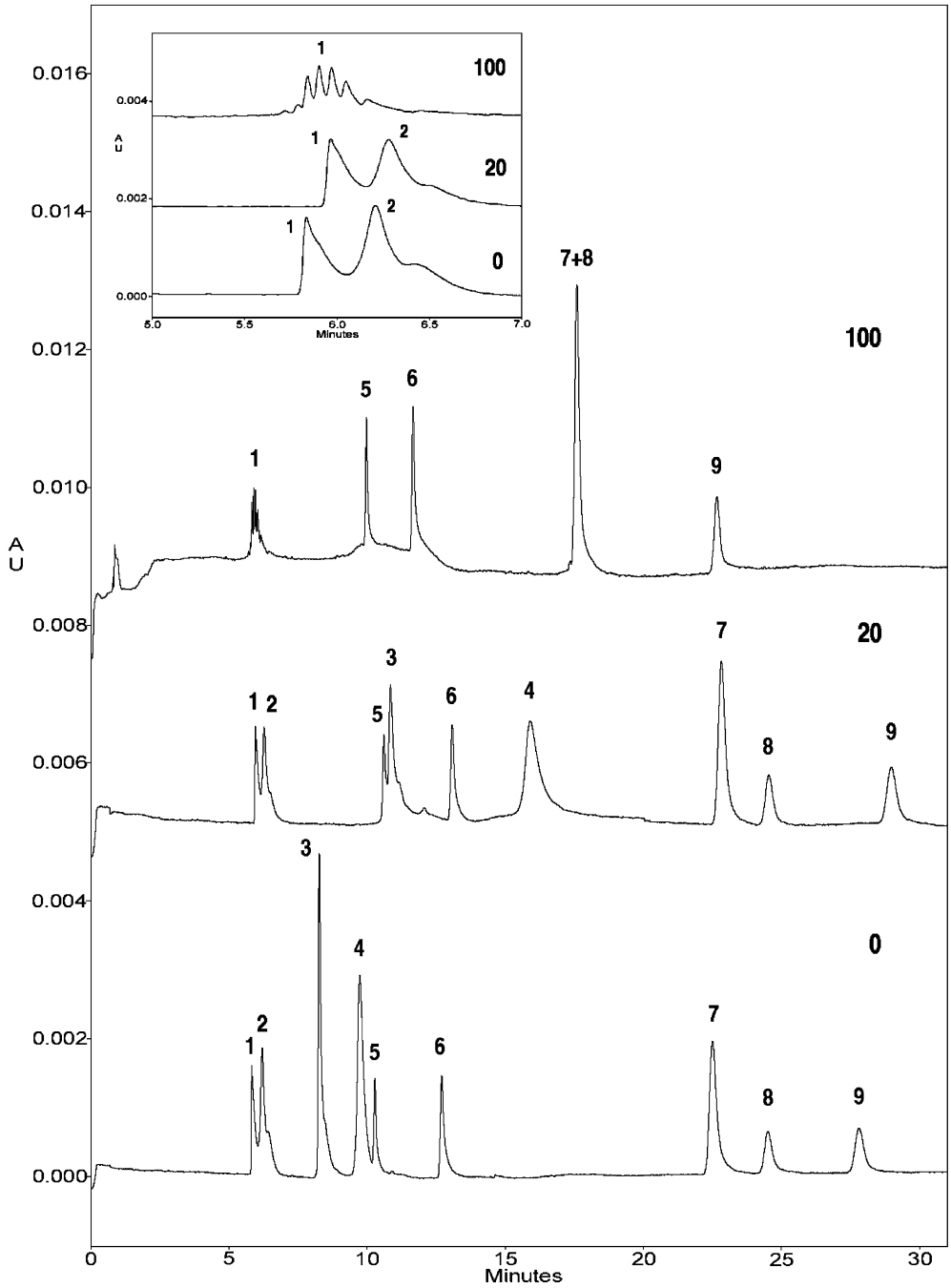

Fig. 1. Electropherograms of protein/peptide standards. Separation was done in 50 mmol/l phosphate buffer, pH 2.5 only (0), 50 mmol/l phosphate buffer, 20 mmol/l heptanesulfonic acid, pH 2.5 (20) and 50 mmol/l phosphate buffer, 100 mmol/l heptanesulfonic acid, pH 2.5 (100). Identification: (1) poly-L-lysine (average M_r 4000); (2) poly-L-lysine (average M_r 22 700); (3) cytochrome *c* (M_r 12 500); (4) albumin from bovine serum (M_r 68 000); (5) Gly-Gly-Tyr-Arg (M_r 451.5); (6) Arg-Pro-Pro-Gly-Phe (bradykinin fragment 1–5; M_r 572.7); (7) Phe-Gly-Phe-Gly (M_r 426.5); (8) Val-Ala-Ala-Phe (M_r 406.5); (9) Tyr-Gly-Gly-Phe-Leu (Leucine enkephalin; M_r 555.6). Inset: enlarged region 5–7 min.

retained its strong cathodic mobility even at the highest heptanesulfonic acid concentration in the background electrolyte. While at lower concentrations of the ion-pairing agent (or in its absence) only the separation of low and high molecular mass possessing lysine polymers was possible, at the highest concentration of heptanesulfonic acid in the background electrolyte the lysine sample exhibited further separation yielding several discrete (incompletely separated) peaks (see Fig. 1, inset).

Only minor changes in migration times were observed with peptides 5 and 6 possessing a single lysine or arginine in their structure.

While the slight increase in the migration time of peptide 6 in 20 mmol/l heptanesulfonic acid containing background electrolyte can be ascribed to ion-pair formation, the opposite effect observed with the highest concentration of the ion-pairing agent reflects probably the fact that under standard conditions (in the absence of the ion-pairing reagent)

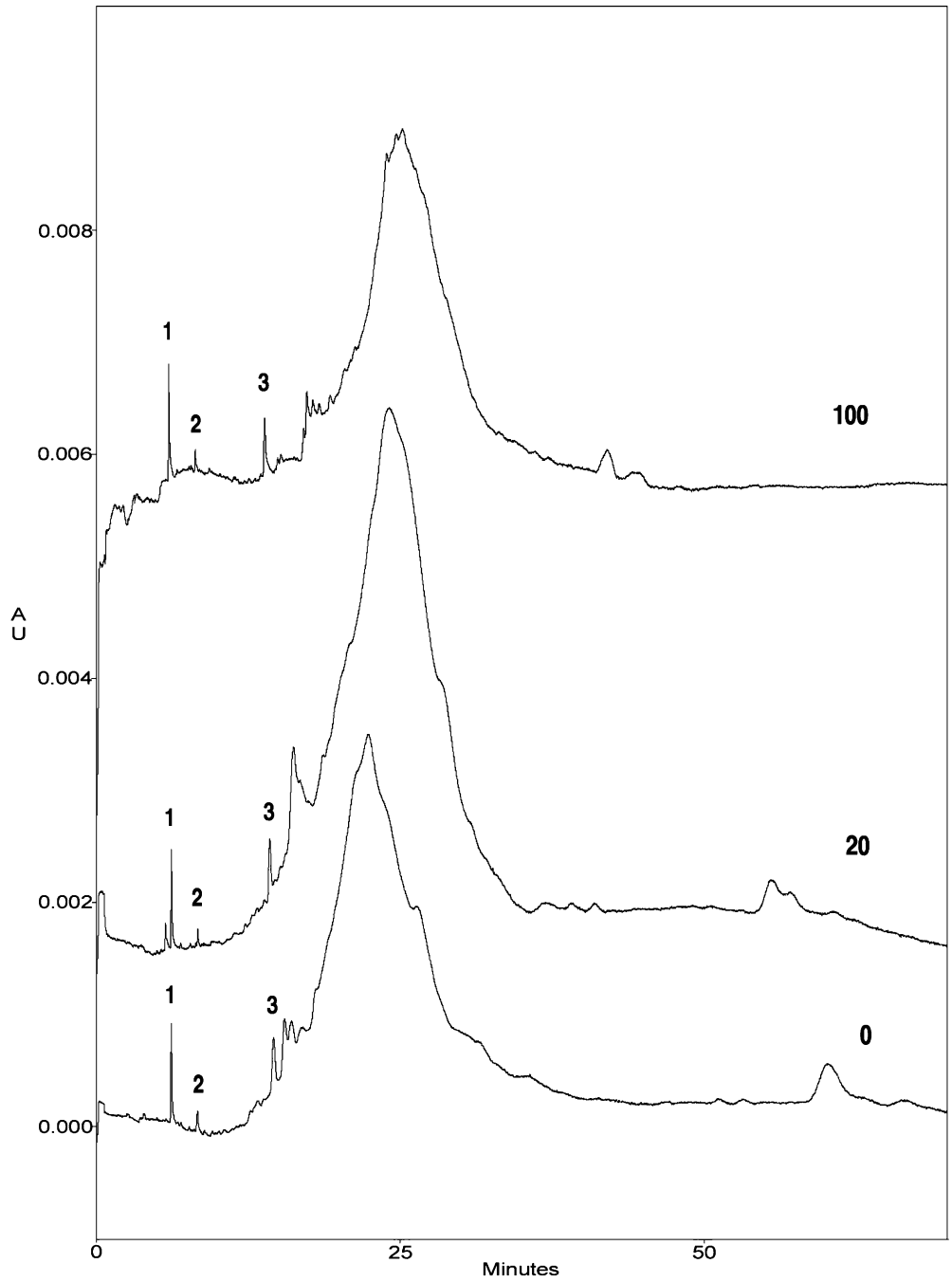

Fig. 2. Electropherograms of CNBr collagen fragments (rat tail tendon collagen). Separations were done in 50 mmol/l phosphate buffer, pH 2.5 only (0), 50 mmol/l phosphate buffer, 20 mmol/l heptanesulfonic acid, pH 2.5 (20) and 50 mmol/l phosphate buffer, 100 mmol/l heptanesulfonic acid, pH 2.5 (100). Numbers 1–3 represent the low molecular mass components of the mixture.

solute/wall interactions play a role in the separation of this particular peptide. If this is true then one would expect such effect to be even more pronounced with peptides devoid of lysine or arginine residues, however possessing an aromatic ring that is generally expected to be involved in hydrophobic solute/capillary wall interactions. As demonstrated by the behaviour of peptides 7–9, this is indeed what occurred.

Considering results mentioned above on the artificial test mixture it was reasonable to expect that this method of ion-pairing could be useful for the separation of a natural complex mixture of peptides/proteins with low and high molecular mass. CNBr peptides of collagen could serve as an example. The span of molecular mass of this set of peptides is really broad: from a few thousands of relative molecular mass to a 30×10^3 relative molecular mass or 60×10^3 relative molecular mass if the splitting of the collagen molecule is incomplete. In order to identify individual collagen types (details see, e.g. [8]) the low molecular mass peptides are particularly important as some of them are specific for individual collagen types. Owing to the large internal homogeneity of collagen it is expected to be difficult to separate large molecular mass fragments on the basis of charge differences and the ion-pairing principle only. Separation of this complex mixture has to be made on a long capillary (ca. 60/70 cm long, see [7,9]). To demonstrate the applicability of the ion-pairing principle in the case of complicated peptide system we used in the current experiments two-times shorter capillary (30/37 cm). Separation of collagen CNBr fragments in the phosphate buffer only is demonstrated in Fig. 2 (the zero indicated profile at the bottom of the figure). It is possible to see a dominant big and broad peak consisting of high molecular peptides (mainly peptides α_1(I)CB$_6$, α_1(I)CB$_7$, α_1(I)CB$_8$, α_2(I)CB$_4$ with relative molecular mass 20×10^3 to 29×10^3). The low molecular portion of the peptide mixture (around 3×10^3 relative molecular mass, typically α_1(I)CB$_2$, α_1(I)CB$_5$ and α_2(I)CB$_2$) are seen in front of the electropherogram partly overlapping with the big peak of the high molecular mass peptides. The main

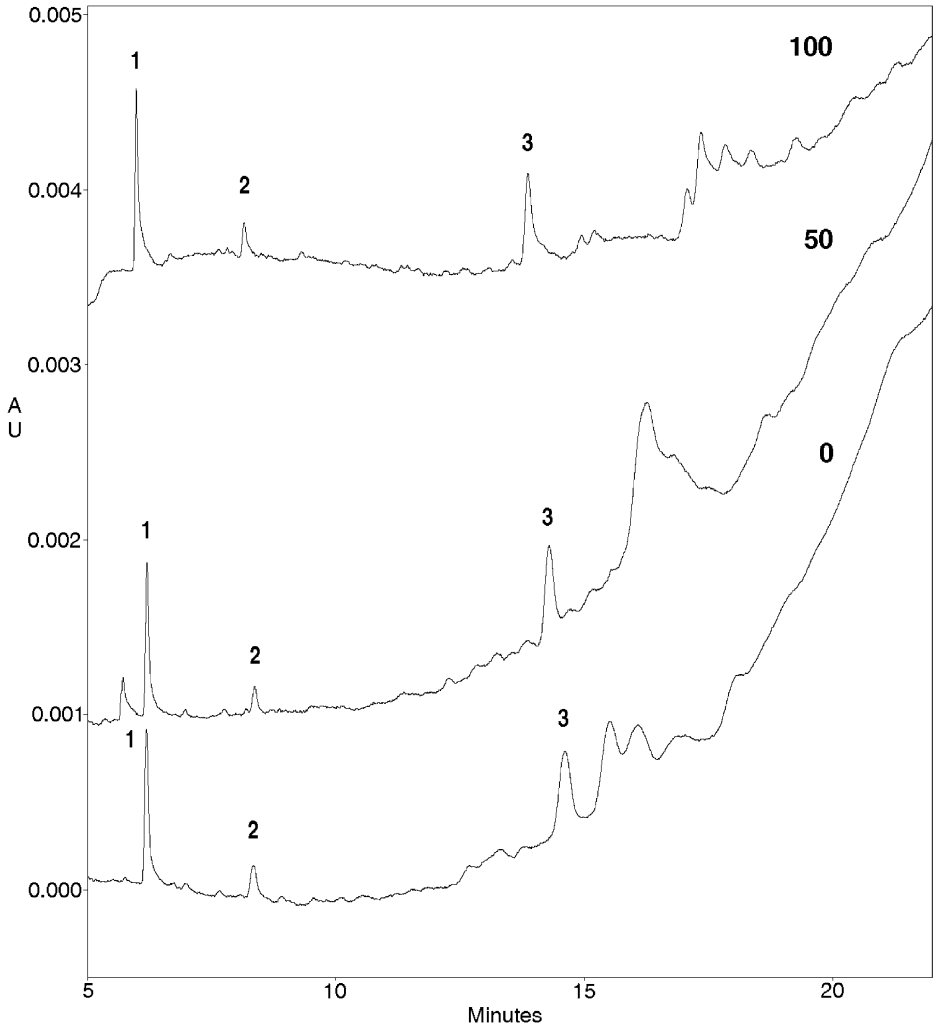

Fig. 3. Enlarged region of 12–22 min running times which involves the overlapping mixture of low and high molecular CNBr peptides (for details, see Fig. 2).

peaks in the front of the slope are α_1(I)CB$_2$, α_1(I)CB$_4$, α_1(III)CB$_2$, α_1(I)CB$_5$, α_2(I)CB$_2$ and α_1(I)CB$_3$. Detailed way of identification was published in our previous papers [7,9].

It is necessary to stress that in this electropherogram region low molecular mass containing peptides frequently occur in natural samples, depending on the source (tissue) from which the collagen preparation was obtained. These contaminants can be easily interchanged with collagen low molecular mass CNBr fragments. Precise identification of all peaks occurring in this region is recommended.

When the ion-pairing agent was added the separation changed. The change reflects the concentration of the

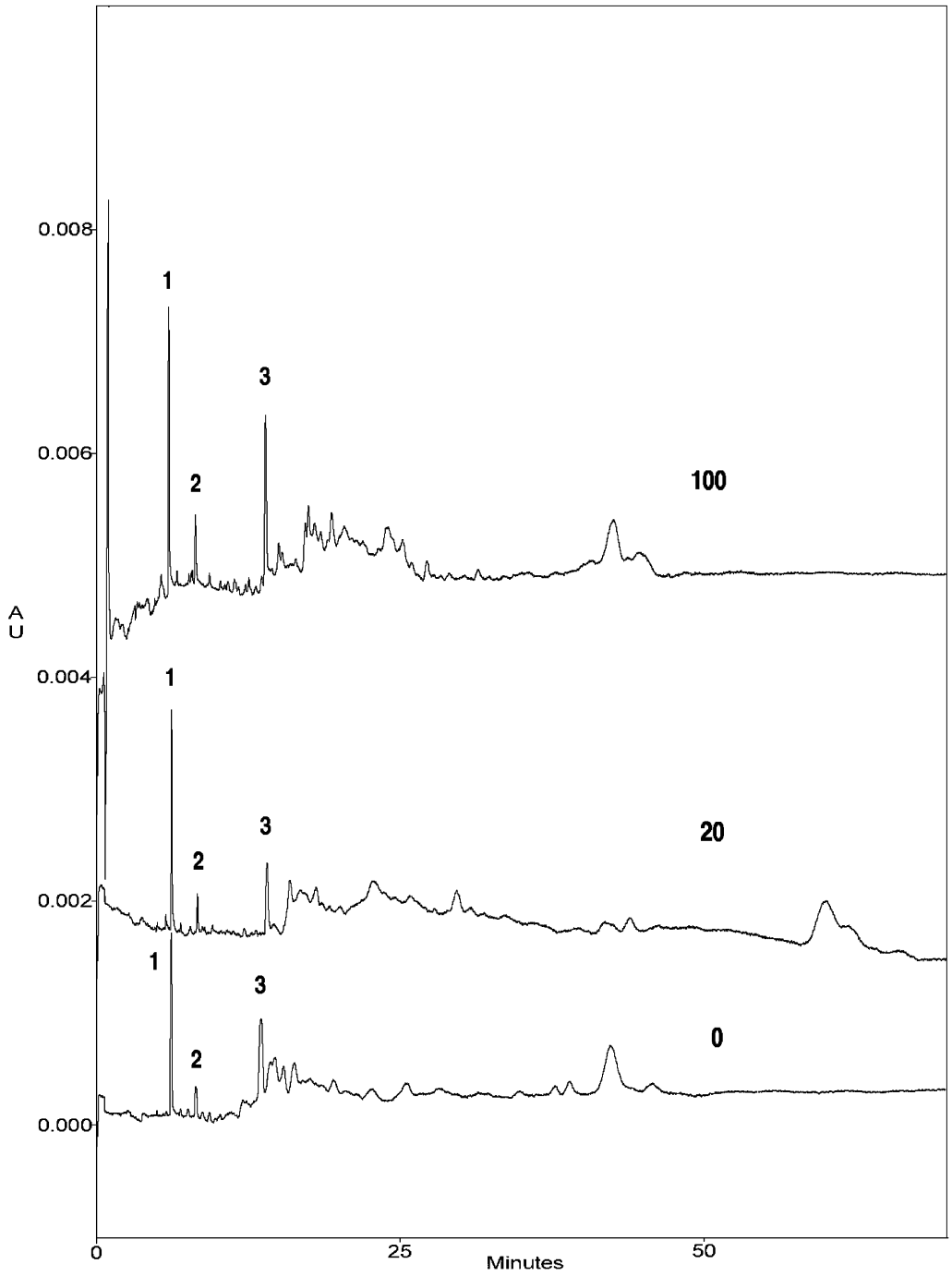

Fig. 4. Electropherograms of the low molecular part (below 10×10^3) of CNBr collagen fragments (rat tail tendon collagen). Separations were made in 50 mmol/l phosphate buffer, pH 2.5 only (0), 50 mmol/l phosphate buffer, 20 mmol/l heptanesulfonic acid, pH 2.5 (20) and 50 mmol/l phosphate buffer, 100 mmol/l heptanesulfonic acid, pH 2.5 (100).

ion-pairing agent used. In principle the situation is similar to that observed with the artificial test mixture. The separation depends obviously on the interaction of the heptanesulfonic acid with the peptides, i.e. on the ion-pairing equilibria between the ion-pairing agent and a particular peptide. This is visualized in Fig. 2 (curves 20 and 100). In this figure it is possible to see the same rearrangement of the peptide profile. This rearrangement starts at the lower concentration of heptanesulfonic acid (20 mmol/l) and is fully developed as the concentration of the heptanesulfonic acid is increased to its highest level (100 mmol/l) (at which the full saturation of peptides with the ion-pairing agent is expected). High molecular mass peptides exhibit slightly longer migration times (are more retained); simultaneously the order of shorter peptides (with lower-molecular mass) is altered. The main change of the profile can be seen at the slope of the big peak (for detailed view of this part, see Fig. 3). The reason of this rearrangement probably reflects the interactions with ion-pairing agent, as discussed above. Based on the results obtained with the artificial peptide mixture it appears feasible to propose that the higher-molecular species are more retained while at least some of the low molecular ones are not. Typically the position of the first peptide peak (α_1(I)CB_2) is only slightly influenced by the presence of the ion-pairing agent (if at all). Concomitantly with the practically complete removal of all the contaminating peptides after having added the ion-pairing agent to the background electrolyte allows a clear separation of low molecular mass peptides that comes from the different collagen species (I and III), for example α_1(I)CB_2 and α_1(III)CB_2. Application of this separation principle enables a better resolution of the shorter peptides than in the case of the use of phosphate buffer only. In comparison with previously published results [7] we obtained better and faster separation of the shorter peptides than in the case when two-times longer capillary with phosphate buffer only was used. A better view of the CNBr peptide profile can be seen in Fig. 4, where the separation of the low molecular part (below 10×10^3) is shown (after the peptide species above this limit were removed by ultrafiltration).

4. Conclusions

It can be concluded that the use of the ion-pairing principle exploiting heptanesulfonic acid appears useful for the separation of a complex mixture of low and high molecular peptides/proteins. This separation mode (ion-pairing) offers the possibility to detect clearly peptides which are normally (under non-pairing conditions) overshadowed by more dominant high molecular species. This was demonstrated by the separation of CNBr fragments of collagen, in which it was possible to separate low molecular peptides in the presence of dominant high molecular entities. The extent of the changes that could be expected was demonstrated on a set of randomly selected peptides/proteins.

Acknowledgements

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