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High-performance capillary electrophoresis for the determination of trypsin and chymotrypsin inhibitors and their association with trypsin, chymotrypsin and monoclonal antibodies

Anne Marie Arentoft

Chemistry Department, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C (Denmark)

Hanne Frøkiær

Department of Biochemistry and Nutrition, Technical University of Denmark, Building 224, DK-2800 Lyngby (Denmark)

Søren Michaelsen

Department of Research in Fur Animals, National Institute of Animal Science, Research Centre Foulum, P.O. Box 39, DK-8830 Tjele (Denmark)

Hilmer Sørensen*

Chemistry Department, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C (Denmark)

Susanne Sørensen

Department of Biochemistry and Nutrition, Technical University of Denmark, Building 224, DK-2800 Lyngby (Denmark)

ABSTRACT

High-performance capillary electrophoresis (HPCE) was adapted for the determination of Kunitz soybean trypsin inhibitor, Bowman Birk inhibitor from soybean and protein-type proteinase inhibitors from pea (*Pisum sativum* L.). The method was developed for the determination and characterization of the inhibitors, the enzymes trypsin and chymotrypsin and the monoclonal antibodies (mAbs) raised against the inhibitors, and also the inhibitor–enzyme and inhibitor–mAb association complexes. The results from studies involving the use of various types of buffers revealed the advantages of having zwitterions such as trimethylammoniumpropyl sulphonate (AccuPure) or taurine included in the buffer. The use of capillaries dynamically coated with zwitterions efficiently reduced the interactions of the proteins with the silica capillary surface, which was important for the analyses for trypsin, chymotrypsin and mAbs and their association complexes with the inhibitors. The influence of temperature, voltage, pH and buffer type on migration times, resolution, peak areas and number of theoretical plates was investigated for the proteins studied. The proposed HPCE method is very suitable for studies of proteinase inhibitors compared with traditional inhibitor studies, and it gives efficient protein separations with the possibility of 245 000 plates/m.

* Corresponding author.

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INTRODUCTION

Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) are produced in the small intestine from the proenzymes synthesized in the pancreatic glands of animals. These digestive enzymes vary in structure and properties depending on the animal species producing them and on the age of the animals [1,2]. Protein-type inhibitors for both trypsin and chymotrypsin are widespread in plants, foods and feeds, and these inhibitors have attracted much interest as they are considered to be the cause of various nutritional problems, as reviewed in several papers [3–6]. Efficient analytical methods are needed for the determination of the inhibitors, and for this purpose monoclonal antibodies (mAbs) have been produced against the inhibitors Kunitz soybean trypsin inhibitor (KSTI), Bowman Birk inhibitor (BBI) and pea (*Pisum sativum* L.) protein-type proteinase inhibitor (PPI) [7]. Information on the structures and properties of the individual BBI, KSTI and PPI and their interactions with mAbs, trypsin and chymotrypsin requires special attention, which raises the need for analytical methods with the potential for high resolution of the individual proteins.

High-performance capillary electrophoresis (HPCE) has proved useful for the determination of various proteins [8,9], and this technique in various modifications has been used for studies of mAbs [10,11]. However, efficient separations of proteins in fused-silica capillaries can be hampered by adsorption of proteins on the capillary walls if the buffer pH is higher than about 3, resulting in band broadening, low resolution and low recovery of the proteins [12]. These complications are especially expected to create problems in unmodified capillaries with proteins having p*I* values higher than the buffer pH. Various methods have therefore been considered to counteract protein adsorption on the capillary wall, including capillary surface treatments, electrophoresis in buffers with pH > p*I* for the proteins and the use of buffers with pH < 3 [12,13]. The use of zwitterionic compounds in the HPCE buffers is another promising approach to solve the problems [13], as is the use of micellar electrokinetic capillary chromatography (MECC), introduced by Terabe *et al.* [14] in 1984. Studies of interactions between native proteins using MECC require, however, micelles formed from surfactants other than sodium dodecyl sulphate to avoid the possibilities of changed or limited binding between the proteins.

This study was aimed at developing HPCE methods for studies of the individual KSTI, BBI and PPI and their interaction or binding to trypsin or chymotrypsin and also the mAbs raised against these inhibitors. An efficient HPCE method based on MECC with cholate and buffers containing zwitterionic compounds was developed. The parameters studied include systematic evaluations of the effects of buffer pH, electrolyte and modifier concentrations, temperature and voltage on migration time, peak area, number of theoretical plates and resolution.

EXPERIMENTAL

Apparatus

The apparatus used was an ABI Model 270 A-HT capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA) with a 760 mm × 0.05 mm I.D. fused-silica capillary. Detection was performed by on-column measurements of UV absorption at a position 530 mm from the injection end of the capillary. For data processing, a Shimadzu (Kyoto, Japan) Chromatopac C-R3A was used.

Samples and reagents

Bovine γ-globulin, Bowman Birk inhibitor (BBI) from soybean, Kunitz soybean trypsin inhibitor (Type I-S) (KSTI), porcine pancreas trypsin and bovine pancreas chymotrypsin were obtained from Sigma (St. Louis, MO, USA). Horseradish peroxidase (HRP) was purchased from Serva (Heidelberg, Germany) and β-lactoglobulin and α-lactalbumin (both from bovine milk) from BDH (Poole, UK). Proteinase inhibitors from pea and monoclonal antibodies raised against the inhibitors from pea and soybean were from the collection in this laboratory [7]. Trigonellinamide, disodium hydrogenphosphate, cholic acid and taurine (2-amino-

ethanesulphonic acid) were obtained from Sigma. Trimethylammoniumpropyl sulphonate (AccuPure Z1 Methyl reagent) was obtained from Waters (Milford, MA, USA). All chemicals were of analytical-reagent grade.

Procedure

All analyses with enzymes were made with solutions of enzymes in running buffer freshly prepared from standard solutions of enzymes in 1 m*M* HCl. The concentrations of the individual proteins ranged form 1 to 10 mg/ml.

Buffers with AccuPure as zwitterion contained 1 *M* AccuPure and 100 m*M* phosphate, with buffer of pH 7.0. Samples analysed with AccuPure as separation buffer were run at 10 kV and 28°C.

Other buffer solutions tested were based on phosphate, taurine and cholic acid with 1-propanol as modifier. The pH range tested was defined by considering the pK_a values of about 8.7 and 6.4 for taurine and cholic acid, respectively. The choice of buffers with cholate micelles was based on an interest in having solutions with positive effects on solubility and stability of the proteins, thus enhancing the possibility of binding reactions between proteins.

All buffers with taurine (50–300 m*M*) contained 35 m*M* cholate, which is well above the critical micelle concentration of cholate (*ca.* 10 m*M*). In addition, the buffers contained various amounts of sodium phosphate (25 and 100 m*M*) and 1-propanol (0–10%) as specified in the individual experiments. The samples were introduced from the positive end of the capillary by vacuum injection (1–3 s), resulting in injection volumes of a few nanolitres. Separations were performed at 30–40°C and 12–20 kV. On-column detection at 280 nm was applied. Standard conditions, while varying individual running parameters, were a temperature of 30°C, a voltage of 20 kV and electrolyte concentrations of 35 m*M* cholate and 100 m*M* sodium phosphate, a taurine concentration of 50 m*M* and a modifier concentration of 2% of 1-propanol. Buffer solutions were filtered through a 0.45-μm membrane filter before use.

The optimization of the MECC method for separation of proteins was performed using a mixture of proteins (HRP, γ-globulin, BBI, KSTI, β-lactoglobulin and α-lactalbumin). For the proteins run separately and for the binding studies, trigonellinamide was used as an internal standard. The protein mixture was dissolved in a buffer consisting of 50 m*M* phosphate, 35 m*M* cholate, 2% 1-propanol and 50 m*M* taurine (pH 8.7).

Calculations of relative migration time (*RMT*) and normalized peak area (*NA*) were performed according to the equations

$$RMT = MT_1/MT_2 \quad (1)$$

where MT_1 is the migration time of the actual protein and MT_2 that of α-lactalbumin, and

$$NA = A/MT \quad (2)$$

where A is the measured peak area. The number of theoretical plates (N) per metre of capillary and the resolution (R_s) was calculated as described elsewhere [15] with

$$N = 5.54(MT/\omega_{1/2})^2 \quad (3)$$

where $\omega_{1/2}$ is the peak width at half-height. To obtain the number of theoretical plates per metre of capillary, N is divided by the capillary length in metres from the injection end to the detector window. Washing with 0.1 *M* NaOH for 3 min and buffer for 5 min was performed between each analysis.

RESULTS AND DISCUSSION

Two separation systems with different zwitterions were investigated. The results obtained with AccuPure were comparable to those obtained with taurine as zwitterion. However, as taurine is much cheaper than AccuPure, the taurine system was used for optimization of the separation of proteins using zwitterions. Preliminary investigations with taurine buffers with and without cholate were performed, and it was decided to optimize on the system with cholate and taurine. As the critical micelle concentration of cholate (10 m*M*) was exceeded, the method presented is an MECC method.

The applied MECC method is based on the electrophoretic mobility of the analytes, elec-

troosmotic flow of the solvent and electrophoretic mobility of cholate micelles. The pH value of the applied buffer creates negatively charged silanol groups on the capillary wall. The positive ends of zwitterions compete with positively charged buffer ions and positively charged regions of macromolecules, thus preventing adsorption of proteins on the capillary wall. At the pH values applied, taurine is present as a zwitterion and therefore will not contribute to the conductivity of the operating buffer [13], whereas the cholate carboxylic acid group is present in its ionized form. Therefore, above the CMC there are possibilities of both ionic and hydrophobic interactions between cholate and proteins. Cholate in solution may be associated with hydrophobic protein domains adding negative charges to the molecules or by ion pairing with hydrophilic areas of the proteins adding hydrophobic groups to the proteins. The addition of negative charges on the proteins will both stabilize the molecules in the solution and reduce the possibilities of adsorption on the capillary wall.

Negatively charged cholate micelles move towards the anode in the opposite direction to the electroosmotic flow. With injection at the anode of negatively charged proteins the electroosmotic flow will be responsible for the migration of the proteins towards the cathode. Proteins with positive charges will be retarded by interaction with negatively charged cholate micelles or with the non-micellar associated free cholate in solution.

Modifications of the MECC method conditions implied changes of temperature, pH, voltage and composition of the buffer, including concentrations of phosphate and zwitterions.

The optimization studies consisted in the electrophoresis of several commercially available proteins with p*I* values from 4.5 to 9.5 and molecular masses from 8000 to 160 000.

Temperature

The influence of temperature was tested with a buffer of pH 8.0. Shorter migration times were seen with increasing temperature (Fig. 1), in agreement with the simultaneous decreases in the viscosity of the solvent in the capillary. The decrease in viscosity was also reflected by an

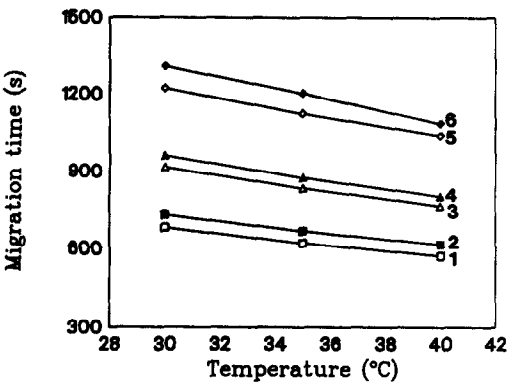

Fig. 1. Influence of temperature on migration times of proteins. Conditions: buffer, 35 m*M* cholate–100 m*M* phosphate–50 m*M* taurine (pH 8.0); voltage 20 kV; total length of capillary, 760 nm; length from injection end to detection, 530 mm; detection wavelength, 280 nm; vacuum injection for 1 s. 1 = HRP; 2 = γ-globulin; 3 = BBI; 4 = KSTI; 5 = β-lactoglobulin; 6 = α-lactalbumin.

increased current intensity. *RMT* values calculated relative to α-lactalbumin were almost unchanged from 30 to 40°C.

The *NA* values were not markedly affected by changes in temperature form 30 to 35°C, whereas a change from 35 to 40°C had different effects on the proteins present in the standard analyte solution. Corrections of peak areas (*NA*) were performed to eliminate the influence of alterations in migration time. However, under the present conditions, the corrections for changes in migration times were insufficient to keep the *NA* values constant for all of the proteins tested.

Increasing the temperature mainly affected the resolution of the proteins with the longest migration times, thus revealing several peaks originating from different proteins within the commercially available β-lactoglobulin and α-lactalbumin. The additional peaks are not believed to be a result of thermal denaturation at the applied electrolyte concentration, voltage and temperature. However, the improvement in peak separation at increased temperature was accompanied by reduced migration times, resulting in less space for baseline separations of proteins with closely related structures.

These conflicting effects are probably able to account for the different effects on the *N* values observed for the individual proteins when

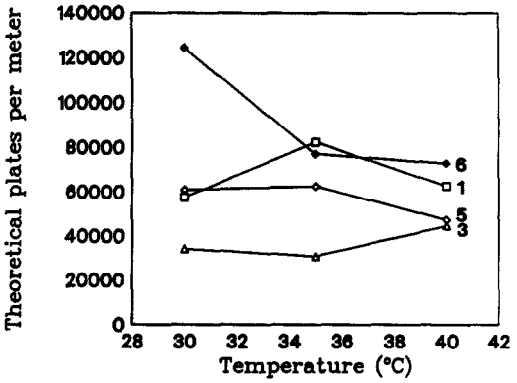

Fig. 2. Influence of temperature on theoretical plate number per metre of capillary. Numbers and other separation conditions as in Fig. 1.

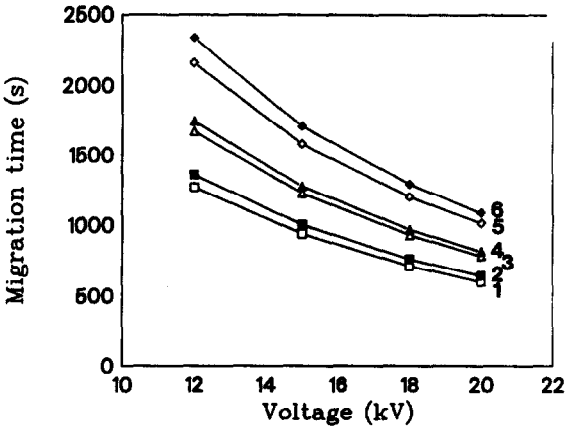

Fig. 3. Influence of voltage on migration times of proteins. Buffer pH, 8.7; temperature, 30°C. Numbers and other separation conditions as in Fig. 1.

changes in temperature were investigated, as illustrated in Fig. 2. The electropherograms forming the basis for the results presented in Fig. 2 were obtained using an injection time of 1 s in order to obtain a large number of theoretical plates. To evaluate the relative standard deviation, the analysis was also performed in triplicate with an injection time of 3 s. This resulted in relative standard deviations of 1–10% of the number of theoretical plates. With respect to migration time, the relative standard deviations were 0.2–0.8% with an injection time of 3 s.

Voltage

An increase in applied voltage resulted in a decrease in migration time, as illustrated in Fig. 3. The decreases were largest for compounds having long migration times, resulting in a considerable decrease in the total time of analysis at high voltage.

RMT values calculated relative to α-lactalbumin remained constant, whereas an increase in applied voltage had different effects on the *NA* values, with unchanged or decreasing *NA* values depending on the proteins in question. These differences could be due to the presence of several closely related proteins with overlapped peaks or to changing response factors of the proteins.

The *N* values were not greatly affected by an increase in applied voltage, although an increased field strength should lead to improved separation efficiency according to the theory of HPCE. This may be explained by limited removal of the developed heat, thus resulting in band broadening and less sharp peak profiles due to temperature increases in the capillary [8,16]. For α-lactalbumin an increase in *N* values was observed with increase in applied voltage, indicating less influence of increased temperature on band broadening for this protein.

pH

Changes in pH values were examined for a buffer containing 50 m*M* taurine. Changing the pH from 7.5 to 8.7 resulted in slightly lower migration times. This could be due to an increase in the electroosmotic flow because more of the surface silanols are ionized at pH 8.7 [13]. The *RMT* values (calculated relative to α-lactalbumin) remained constant. The *NA* values were not affected, except for α-lactalbumin, which showed a considerable decrease in *NA* values at pH 8.7, as demonstrated in Fig. 4.

Increased pH values also resulted in a large increase in *N* for α-lactalbumin. HRP and BBI showed minor decreases in *N* values, whereas the *N* values for the other proteins were less affected by changes in pH.

Electrolyte concentration

The effects of changes in the concentrations of cholate and phosphate ions in the running buffer

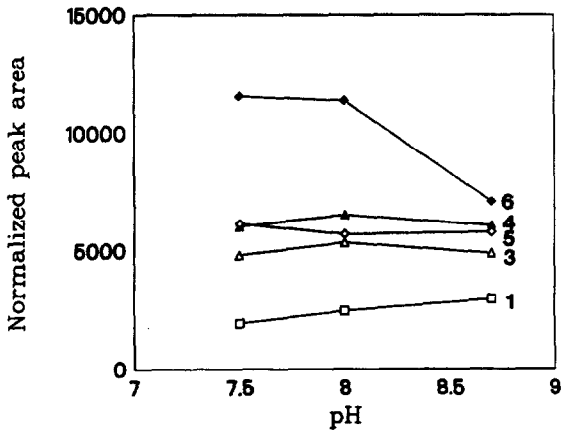

Fig. 4. Influence of buffer pH on normalized peak area. Temperature, 30°C. Numbers and other separation conditions as in Fig. 1.

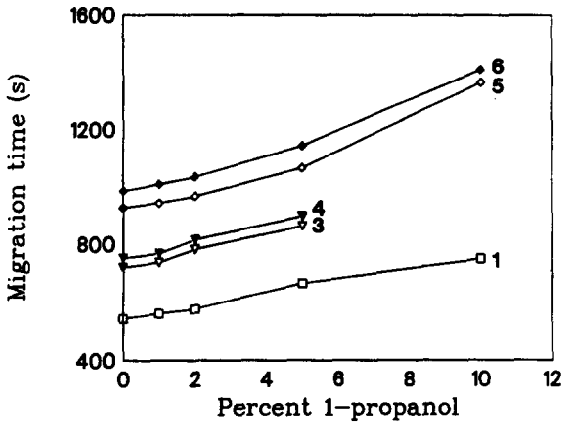

Fig. 5. Influence of 1-propanol concentration on migration time. Buffer pH, 8.7; temperature, 30°C. Numbers and other separation conditions as in Fig. 1.

were examined for a buffer containing 25 m*M* phosphate and 35 m*M* cholate and a buffer consisting of 100 m*M* phosphate and 35 m*M* cholate. With the electrolyte concentration in the latter buffer, the migration times and peak resolutions were appreciably improved and, therefore, this buffer was used in further analyses.

The zeta potential decreases with increasing electrolyte concentration, affecting the electroosmotic flow. The improved results with high electrolyte concentration could be explained by an increase in current followed by heat deposition, thus resulting in lower viscosity and shorter migration times for the proteins at high electrolyte concentrations. Moreover, a higher electrolyte concentration may result in a high degree of competition of electrolytes with proteins in the binding to the capillary wall dynamically coated with taurine.

Modifier concentration

The migration times increased with addition of 1-propanol to the running buffer (Fig. 5). The current decreased markedly at high concentrations of 1-propanol, which indicated a decreased electroosmotic flow. This explains the increased migration times seen on going from 0 to 10% of 1-propanol.

The *NA* values were essentially similar for 1 and 2% of 1-propanol added, whereas 5% of 1-propanol resulted in slight decreases in *NA* values.

No effects on *N* values were observed for KSTI and BBI, whereas the number of theoretical plates per metre of capillary for HRP, β-lactoglobulin and α-lactalbumin generally increased with the addition of up to 5% of 1-propanol and then decreased at 10% of 1-propanol (Fig. 6). As in Fig. 2, injection times of 1 s were used, resulting in high relative standard deviations.

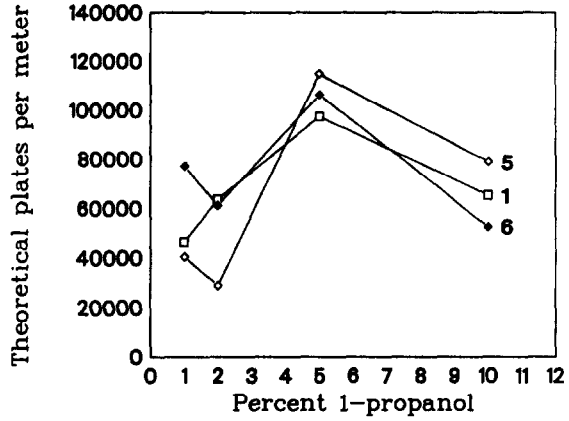

Fig. 6. Influence of 1-propanol concentration on theoretical plate number per metre of capillary. Buffer pH, 8.7; temperature, 30°C. Numbers and other separation conditions as in Fig. 1.

Taurine concentration

The influence of taurine concentration on the HPCE separation was examined for 50, 100 and 300 m*M* taurine. Slightly shorter migration times were seen with increasing taurine concentration. With respect to *NA* values the influence of taurine concentration showed individual effects on the examined proteins, as illustrated in Fig. 7.

The different behaviour of the proteins was also reflected by the influence on the theoretical plate number as some proteins were unaffected of changes in taurine concentration (γ-globulin, KSTI and BBI), whereas the others showed slightly increased *N* values with increasing taurine concentration.

Resolution (R_s) and N

The mixture of proteins used for optimization of the method did not consist solely of the proteins mentioned, as various other proteins were present in the commercially available proteins used in the standard mixtures. The results for *NA* and *N* values were therefore not as simple to interpret as when the individual proteins were analysed separately. As a consequence, the baseline separation was poorer and the normalized areas of the peaks were greater than those found for samples containing only a few proteins.

From the optimization investigations of the developed MECC method, some of the most relevant parameters were investigated for the individual proteins. Changes in voltage from 12 to 20 kV did not have a negative effect on *N* values, and 20 kV was chosen because of the reduced migration times obtained with this voltage. A buffer pH of 8.7 was chosen as increasing pH values resulted in a decrease in *MT* and a higher *N* value for α-lactalbumin. In Fig. 8 selected electropherograms of proteins from the standard analyte solution are shown. The presence of two peaks for β-lactoglobulin is probably due to the presence of both β-lactoglobulin A and B. Table I lists the *N* and R_s values obtained

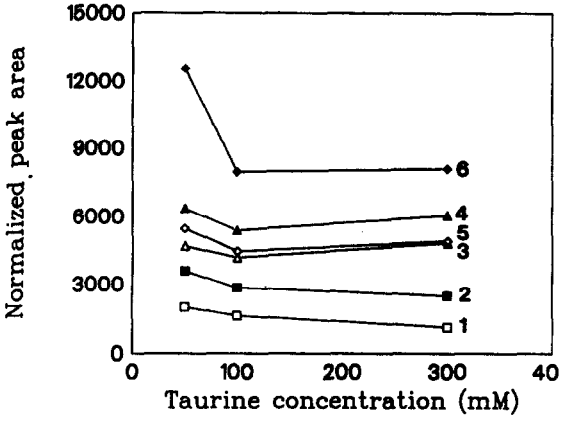

Fig. 7. Influence of taurine concentration on normalized peak area. Temperature, 30°C. Numbers and other separation conditions as in Fig. 1.

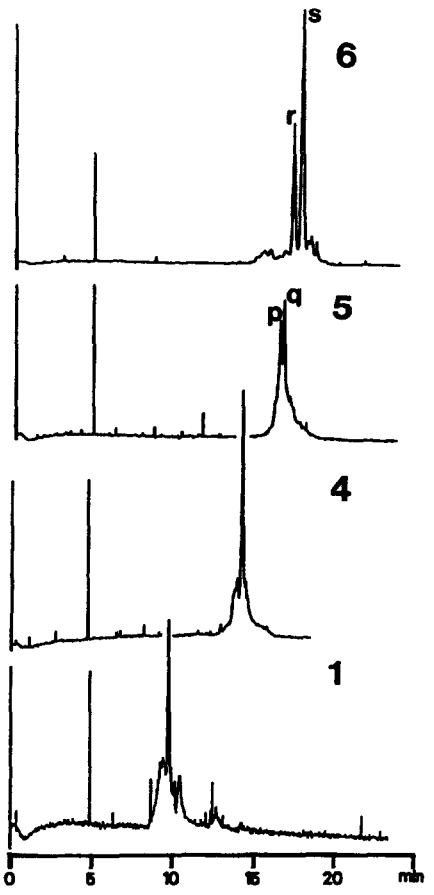

Fig. 8. Electropherograms of (1) horseradish peroxidase, (4) Kunitz soybean trypsin inhibitor, (5) β-lactoglobulin and (6) α-lactalbumin. Separation conditions as in Fig. 1 with buffer pH 8.7 and temperature 30°C; detection at 280 nm. For identification of peaks p, q, r and s, see Table I.

TABLE I

NUMBER OF THEORETICAL PLATES (N) PER METRE OF CAPILLARY AND RESOLUTION (R_s) FOR STANDARD ANALYTE PROTEINS UNDER DIFFERENT SEPARATION CONDITIONS

Conditions: all buffers contained 35 mM cholate and 100 mM phosphate in addition to the amounts of zwitterion and modifier indicated. Voltage, 20 kV; 1 s injection.

Protein		Zwitterion modifier and temperature					
		50 mM taurine, 2% 1-propanol, 30°C		300 mM taurine, 5% 1-propanol, 30°C		300 mM taurine, 5% 1-propanol, 35°C	
		N (m^{-1})	R_s	N (m^{-1})	R_s	N (m^{-1})	R_s
Horseradish peroxidase		46 830	–	116 521	–	136 478	–
Bowman birk inhibitor		75 974	–	114 107	–	129 254	–
Kunitz soybean trypin inhibitor[a]		96 396	–	136 544	–	36 285	–
β-Lactoglobulin[b]	p	59 219	0.64	113 275	1.15	36 285	0.76
	q	82 591		245 581		101 802	
α-Lactalbumin[b]	r	99 457	1.03	144 787	2.24	151 800	2.19
	s	104 936		151 713		149 751	
PPI[c]	t	208 781	0.66	392 779	1.03	376 519	0.68
	u	163 211		161 583		182 569	

[a] Electrophoresis of KSTI in the buffer with 300 mM taurine resulted in broad peaks around the main peak from KSTI which especially at 35°C strongly affected the N values.

[b] Peak letters as in Fig. 8.

[c] Two adjacent peaks (t and u) of several present in the electropherogram were chosen for the calculations.

with proteins run under different conditions in the developed MECC method.

It can be seen from Table I that the combination of 300 mM taurine with 5% of 1-propanol at 30°C resulted in the largest number of theoretical plates per metre of capillary and also the best resolutions between proteins. However, for KSTI the buffers with 300 mM taurine and 5% of 1-propanol resulted in considerable broadening at the peak base, although this phenomenon was not reflected in the N values, as there was no peak broadening at the peak half-height. Because of the undesirable peak broadening, the buffer containing 50 mM taurine was chosen for the binding studies.

Applications

The method described was applied to investigations of binding between inhibitors and enzymes and binding between inhibitors and antibodies, under conditions where all the investigated proteins were able to preserve their native structures and properties.

Fig. 9 shows electropherograms of trypsin, the proteinase inhibitors KSTI and BBI and monoclonal antibodies produced against these inhibitors, and also mixtures of inhibitors and antibodies or trypsin. Complexes between antibody and antigen in solution are believed to form rapidly (less than 30 s) and to be tightly bound [9]. From the sequence of electropherograms in Fig. 9A–C it appears that the migration time of the monoclonal antibody complex (C) was increased compared with the monoclonal antibody alone (A) as a result of binding between KSTI (B) and the monoclonal antibody produced against KSTI. In Fig. 9C the molar ratio of KSTI to antibody was 25:1 and therefore, as expected, all of the antibody seems to have formed a complex with KSTI, and a peak of unbound KSTI is present. Similarly, the binding of BBI (H) to a monoclonal antibody produced against BBI (G) resulted in a splitting

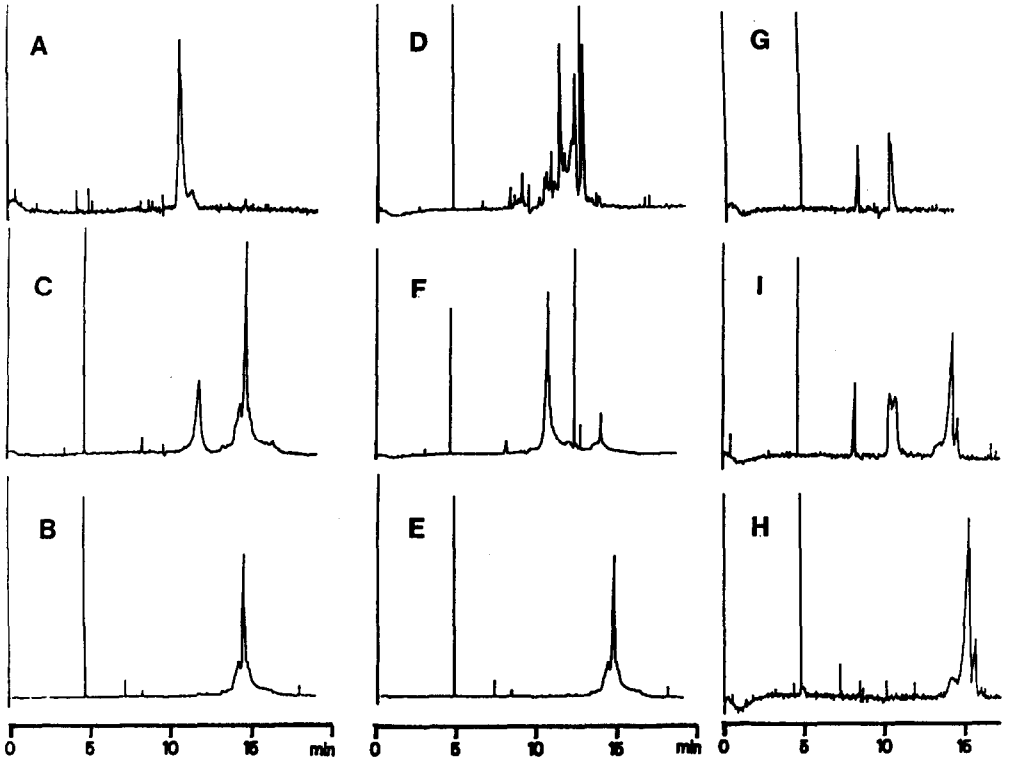

Fig. 9. Electropherograms obtained using buffer containing 50 m*M* taurine, 35 m*M* cholate, 100 m*M* phosphate and 2% 1-propanol at 20 kV, 30°C. Other conditions as in Fig. 8. (A) Monoclonal antibody against KSTI; (B) KSTI; (C) mixture of KSTI and monoclonal antibody against KSTI; (D) trypsin; (E) KSTI; (F) mixture of trypsin and KSTI; (G) monoclonal antibody against BBI; (H) BBI; (I) mixture of BBI and monoclonal antibody against BBI. The electropherograms were aligned according to the migration time of the internal standard, trigonellinamide (very sharp peak at *ca.* 5 min).

of the antibody peak (I), probably caused by the complex formation with BBI. Again, the molar ratio of antibody to inhibitor was 1:25 in the mixture, thus accounting for the presence of unbound BBI. The splitting of the antibody peak could be due to the presence of antibody with low or lost affinity for BBI, *e.g.*, caused by partial denaturation of the antibody during the purification steps. In the electropherograms in Fig. 9G and I the peaks at *ca.* 8 min. represent water from the samples. Other samples were dissolved in running buffer containing 50 m*M* phosphate.

An equimolar mixture of trypsin (D) with KSTI (E) also resulted in visible changes in the electropherogram of the mixture (F), indicating that binding between inhibitor and enzyme had occcurred. The presence of multiple peaks in the electropherogram of trypsin is probably due to several trypsin isoenzymes. In Fig. 9F only a complex peak and a small peak of residual KSTI appear, indicating that all isoenzymes present in D were able to form complexes with KSTI. Trypsin and KSTI have approximately equal molecular masses and the electrophoretic characteristics of KSTI therefore have a more pronounced influence on the electrophoretic mobility of the complex compared with the influence of KSTI on the complex of antibody and KSTI as the molecular mass of KSTI comprises only 13% of the antibody molecular mass. Similar results (not shown) were obtained with mixtures of BBI with trypsin and chymotrypsin and with mixtures of PPI and trypsin. With the chosen MECC system, the chymotrypsin and trypin electropherograms showed peaks with up to 250 000

and 91 000 theoretical plates per metre of capillary, respectively.

CONCLUSIONS

The optimum conditions for the separation of specific proteins vary with respect to especially taurine and 1-propanol concentrations.

With the applied MECC method it is possible to analyse mixtures of proteins with a wide range of p*I* values. The system allows the use of pH values below the p*I* values of proteins such as chymotrypsin and trypsin. Thereby, it is possible to study the complexes with enzyme inhibitors at the pH optimum of the enzymes. With respect to the antigen–antibody complexes the applied MECC method makes it possible physically to separate antibody–antigen complexes from unreacted reagents in non-denaturing environments. This gives the opportunity to prevent the problems associated with solid-phase immunoassays. The use of an internal standard (*e.g.*, trigonellinamide) gives the possibility of a relative determination of the amount of inhibitor before and after complex formation, thus giving information on, *e.g.*, which of several isoinhibitors have taken part in the complex formation.

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