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APPLICATION OF FREE FLOW ELECTROPHORESIS TO THE PREPARATIVE PURIFICATION OF BASIC PROTEINS FROM AN *E. COLI* CELL EXTRACT

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SUMMARY

The application of free flow electrophoresis (FFE) to the purification of a basic protein from a complex protein mixture was investigated. For this purpose lysozyme (E.C. 3.2.1.17) from hen egg white, serving as a model for a basic protein, was added to a crude *E. coli* cell extract and reisolated. For three techniques of FFE (zone electrophoresis, isoelectric focusing and field step electrophoresis), suitable electrolyte systems were developed. The purity, purification factor, recovery and throughput were determined for the optimized experiments. A combination of field step electrophoresis and zone electrophoresis gave the best purification factor (9.5) and the highest recovery (95%). The purification factors achieved in zone electrophoresis and isoelectric focusing were comparable to each other and ranged from 3.5 to 4.75. In isoelectric focusing, 94% of the enzyme activity was recovered. Zone electrophoresis gave recoveries of 82% and 87%, respectively. Purities of more than 95% were achieved with all the techniques described. With the exception of zone electrophoresis, all the techniques effected a concentration of the enzyme during the separation. Zone electrophoresis and field step electrophoresis were very simple in application.

INTRODUCTION

With the developments in biotechnology in recent years, the requirements placed on the purity of proteins for pharmaceutical applications have increased considerably. New and efficient methods for the preparative purification of proteins had to be developed in order to reduce the cost of large-scale production. In addition to the commonly used purification techniques based on precipitation or chromatography, electrophoresis takes advantage of two individual characteristics of proteins, *viz.*, the mobility in an electric field depending primarily on the charge of the molecules and the isoelectric point.

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For the purification of small amounts, a flat-bed electrophoresis system was developed, running in the zone electrophoresis[1] or the isoelectric focusing[2] mode. Sepharose or agarose was recommended as a stabilizing gel to minimize gravitational and diffusional instabilities. After the electrophoretic run, the gel has to be sliced and the proteins must be eluted from each slice.

For the purification of small ions and also for living cells or particles, free flow electrophoresis (FFE), pioneered by Hannig[3–5] as zone electrophoresis, was developed in the last two decades. Despite the advantages of this method, such as continuous use and the absence of carrier materials, which improve the recovery of active proteins, FFE has not been popular for the purification of biopolymers in the milligrams to grams range. With the development of new techniques[6–9], including isoelectric focusing (IEF), field step electrophoresis (FSE) and isotachophoresis (ITP), allowing higher sample throughputs at higher resolutions, FFE has become more and more interesting for application in research and industry. Additionally, these new techniques concentrate diluted sample compounds after the separation. An extensive overview of the technique and applications of FFE is given in ref. 10.

This paper demonstrates the suitability of FFE techniques for the purification of a basic protein from a crude *E. coli* protein extract. Lysozyme, as a model for interesting pharmaceutically active proteins with low molecular weights and high p*I* values such as the interleukins, was added to an *E. coli* protein extract and reisolated in a single step.

EXPERIMENTAL

Chemicals

All chemicals were of analytical-reagent grade unless indicated otherwise.

Arginine, aspartic acid, glutamine, glycine, hydrochloric acid, potassium chloride, potassium sulphate, *Micrococcus luteus* lyophil. cells and urea were purchased from Serva (Heidelberg, F.R.G.). E. coli cells were cultivated by BASF (Ludwigshafen, F.R.G.). All other substances were obtained from Merck (Darmstadt, F.R.G.).

Analytical methods

The enzymatic activity of lysozyme was determined according to Shugar[11] by measuring the change in turbidity of a *Micrococcus luteus* suspension at 450 nm and 25°C, using a Hitachi Model 100-60 photometer. The total protein concentration was determined by measuring the absorbance at 280 nm with chicken egg albumin as a standard[12]. For the measurement of pH and conductivity a WTW 530 pH meter and a WTW LF 521 conductimeter, respectively, from WTW (Lauda, F.R.G.) were employed.

Preparation of the sample

About 20 g of *E. coli* cells were suspended in 100 ml of 0.1 mol/l Tris–HCl (pH 7.0). After cell breakage by sonification and removal of the cell debris by centrifugation, 0.4% polyethyleneimine was added to the supernatant to precipitate the polynucleic acids. The resulting suspension was stirred for 30 min at room temperature and the precipitate was centrifuged. The conductivity of the sample solution was

reduced by dialysis against 0.1 mol/l glycine. The addition of lysozyme to the protein mixture caused precipitation, which could be prevented by adding at least 5 mol/l urea or 0.02 mol/l potassium sulphate.

Electrophoresis apparatus

The FFE apparatus and the operating conditions have been described in detail elsewhere[13].

RESULTS AND DISCUSSION

Although basic proteins tend to adsorb to supporting media in chromatography, they easily aggregate with oppositely charged proteins in free solutions, as was observed for the lysozyme with *E. coli* proteins. To achieve successful separations of lysozyme from the contaminating proteins, these hydrophilic protein interactions had to be suppressed by using inorganic salts or, *e.g.*, urea[14,15].

If lysozyme was added to a crude *E. coli* cell extract, precipitation of the enzyme with *E. coli* proteins could be observed. The addition of urea at concentrations of nearly 5 mol/l or potassium sulphate at more than 0.02 mol/l to the *E. coli* protein mixture prevented the precipitation of lysozyme with bacterial proteins or led to the dissolution of already precipitated proteins. The dependence of the enzyme solubility on the concentration of urea and potassium sulphate is illustrated in Fig. 1.

Zone electrophoresis

The electrophoretic mobility of proteins depends strongly on the pH of the dissolving medium. Investigations on mobility in zone electrophoresis (ZE) at different pH values revealed the general shape of the mobility curve for a protein in a polyacrylamide gel. With a decrease in pH from 11 (p*I* of lysozyme) to 7.5, the mobility of the enzyme increased steadily and changed only slightly at pH values lower than 7.5. The separation of lysozyme from contaminating *E. coli* proteins by ZE

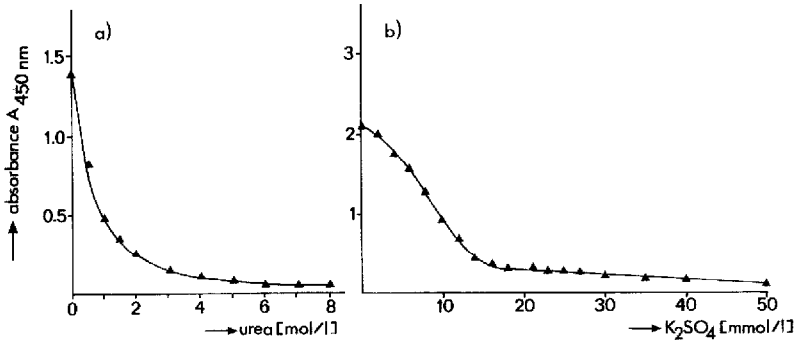

Fig. 1. Solubility of the lysozyme–*E. coli* protein complex depending on the concentration of (a) urea and (b) potassium sulphate. Solid lysozyme at a concentration of 2 mg/ml was dissolved in the dialysed *E. coli* cell extract with a protein concentration of (a) 14 and (b) 20 mg/ml and a conductivity of 0.4 mS/cm. After the addition of the various amounts of urea and potassium sulphate, the turbidity of the solution was determined by measuring the absorption at 450 nm.

succeeded very easily at pH 7.5 (Fig. 2). As most of the bacterial proteins had p*I* values below 7.5, they migrated towards the anode whereas lysozyme moved towards the cathode, with the consequence that almost all contaminating proteins could be separated. In many experiments two lysozyme peaks with equal biological activities were measured. Neither separation by polyacrylamide gel electrophoresis (PAGE) nor the A_{450}/A_{280} ratio gave any indications of different enzyme structures.

The peak shape of the lysozyme bands showed sharp fronting, whereas the bacterial proteins migrated as a surprisingly narrow band. The latter peak shape might have its origin in the high concentration of urea, which probably supports ionic interactions between the proteins. This assumption is also confirmed by the experiment but with potassium sulphate instead of urea as the solubilizing agent (Fig. 3). As the power supply used was limited to a maximum current of 0.2 A, the voltage and the flow-rate had to be changed compared with the experimental conditions given in Fig. 2. Potassium sulphate enhanced the polarity of the background electrolyte and probably induced the breakage of ionic protein interactions of the *E. coli* proteins, resulting in a broad peak shape ranging over more than 30 fractions.

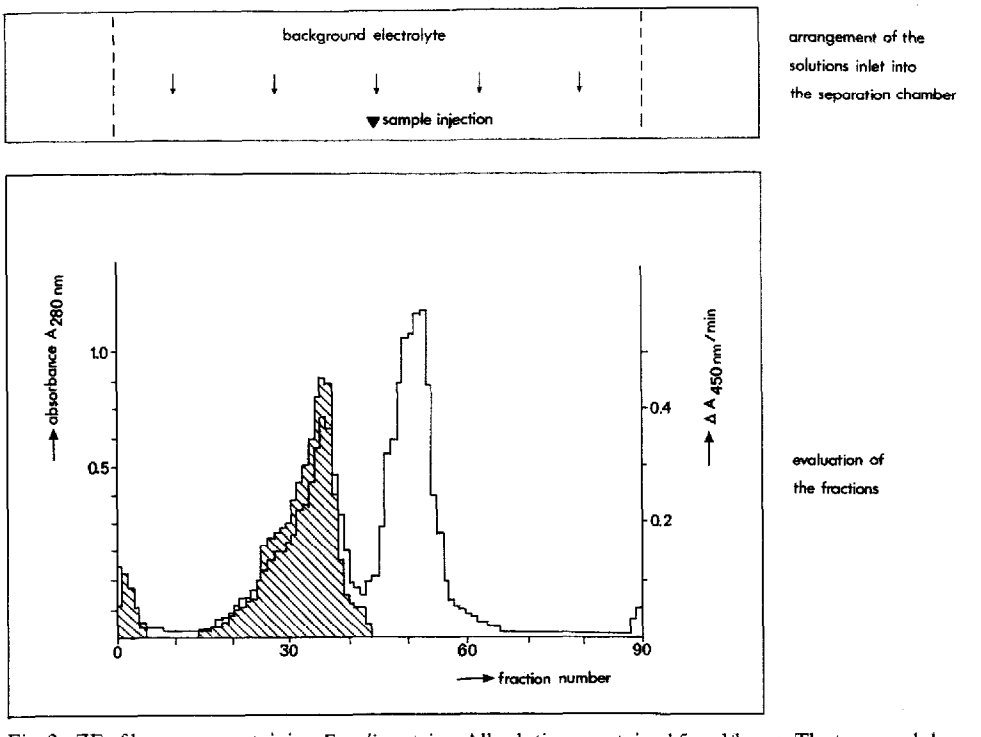

Fig. 2. ZE of lysozyme containing *E. coli* proteins. All solutions contained 5 mol/l urea. The top panel shows the inlet configuration and the lower panel shows the distribution of the enzyme activity (dashed area, as ΔA_{450}/min) and the total protein distribution (as A_{280}). The background electrolyte consisted of 0.01 mol/l Tris, adjusted to pH 7.5 with hydrochloric acid. The sample contained 10 mg/ml lysozyme and 28 mg/ml bacterial proteins. The field strength was 60 V/cm. The flow-rate was 190 ml/h and the sample was injected at 2 ml/h.

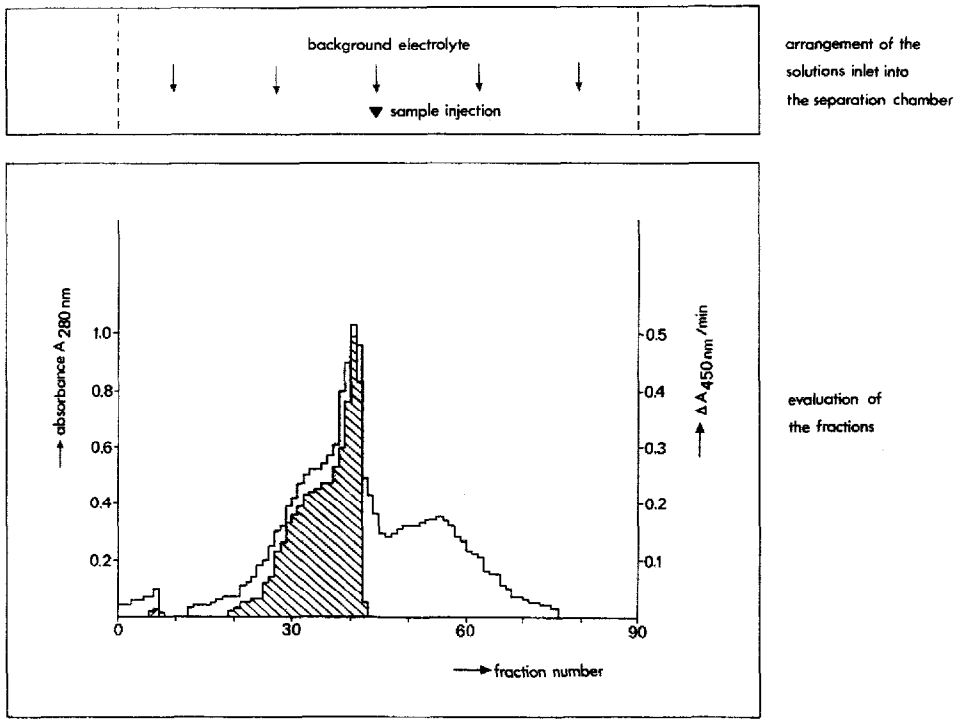

Fig. 3. ZE of lysozyme containing *E. coli* proteins. The top panel shows the inlet configuration and the lower panel shows the enzyme activity (dashed area, as ΔA_{450}/min) and the total protein distribution (as A_{280}). All solutions were adjusted to 0.02 mol/l potassium sulphate. The background electrolyte was prepared according to Fig. 2. The sample contained 10 mg/ml lysozyme and 18 mg/ml bacterial proteins. The field strength was 45 V/cm. The flow-rate was 100 ml/h and the sample was injected at 1 ml/h.

Isoelectric focusing

In addition to the recycling isoelectric focusing apparatus (RIEF) of Bier and Egen[16], which operates batchwise, Manzoni[17] developed IEF in a stepwise pH gradient running in the continuous mode. On pumping simple buffer solutions as parallel liquid films through the separation chamber there arises a stepwise pH profile according to the different pH values of the buffers. The migration of the enzyme within the pH step is based on the same principle as the migration in ZE. However, as in ZE almost the total transportation of charges is taken over by the ground electrolyte (carrier electrolyte), the migration velocity of the sample compounds, depending on the ionic strength of the buffer, is decreased compared with that in stepwise IEF. The focusing effect of this technique in only a few fractions allows one to add the sample as a broad zone into the top of the chamber. High ionic strengths of the sample solutions are unnecessary in this technique, because the sample compounds participate to a great extent in the transportation of current in the dosing zone of the sample. Shorter residence times or pH values with lower enzyme mobilities can be adjusted.

The separation of the enzyme from the bacterial protein mixture succeeded when using a cathodic buffer solution of pH 12.3 and a residence time of 7 min (Fig. 4). Most

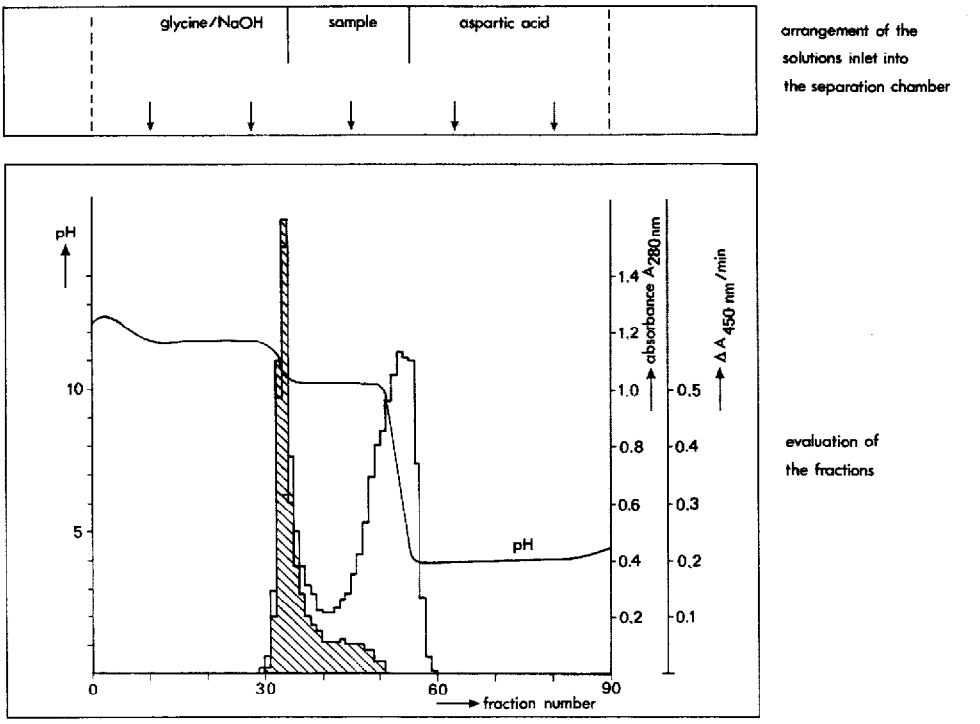

Fig. 4. IEF of 0.01 mg/ml lysozyme containing 0.3 mg/ml *E. coli* proteins. The arrangement of the inlet system is given at the top and the evaluation of the fractions is shown below; the dashed area indicates the enzyme activity as ΔA_{450}/min and the total protein concentration is given as A_{280}. All buffers contained 5 mol/l urea. Arginine–glutamine (0.03 mol/l each), pH 10.0, served as solvent for the sample. The concentration of glycine was 0.05 mol/l, adjusted to pH 12.3 with sodium hydroxide; the anolyte was 0.03 mol/l aspartic acid, pH 3.7. At a field strength of 30 V/cm the flow-rate was adjusted to 215 ml/h.

of the enzyme activity was well focused on few fractions and only weak tailing remained. Longer residence times in order to remove this tailing decreased the slope of the pH interfaces and diminished the concentration factor of the enzyme.

Field step electrophoresis

FSE is a simple and powerful technique for the concentration of diluted samples[18] and for these investigations it was often used to increase the protein concentration in fractions after the separation. The concentration factors depended on the zone width of the sample and could reach values of 10–20.

As the resolution of this technique was low for the purification of lysozyme, it was combined with ZE, as illustrated schematically in Fig. 5. In the separation buffer between the sample zone and the catholyte the sample compounds could be separated according to their different mobilities. The optimized experimental results are shown in Fig. 6. Nearly all the lysozyme was found in only three fractions. The enzyme was five-fold more concentrated than the injected sample and almost free from contaminants.

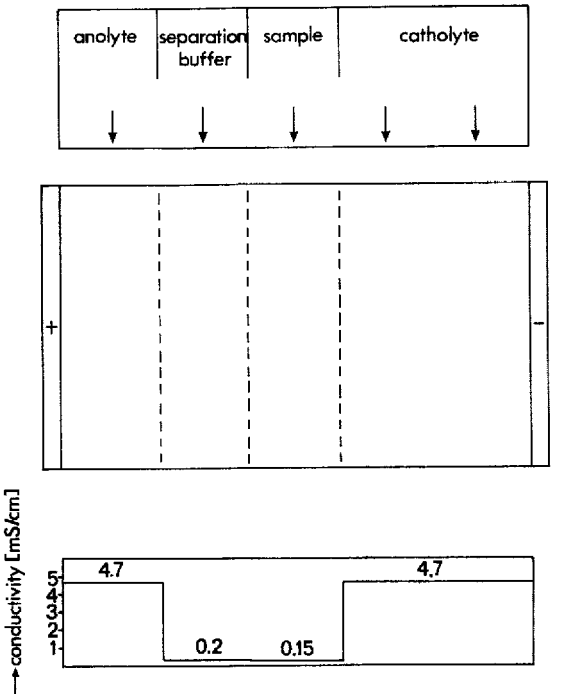

Fig. 5. Schematic representation of FSE combined with ZE. Above: the inlet system with anolyte and catholyte characterized by high conductivities, the separation zone with a conductivity adjusted according to the mobilities of the sample compounds and the sample solution with low conductivity. Below: conductivity profile across the separation chamber.

Comparison of results

All optimized experiments described abive were evaluated in terms of the purification factor, recovery, throughput and purity. The results are summarized in Table I.

All methods gave very high purities, irrespective of the purity of the sample before the separation. The recovery of enzyme activity was the highest for the focusing techniques IEF and FSE + ZE, but ZE also gave high recoveries of more than 80%. The combination of FSE and ZE achieved the best results. As no problems arise with protein precipitations, samples of much higher concentration than in IEF can be separated. ZE provided a low throughput of the sample volumes but, owing to the dilution of the sample compounds during the separation, high protein concentrations could be employed. Also, this technique was easy to apply. IEF was limited with regard to the concentration of the sample compounds because of the low solubility of the proteins at their isoelectric points. The resolving power of IEF is lower than that of the combined FSE and ZE. Separations of lysozyme from *E. coli* proteins can probably be performed with inorganic salt solutions instead of urea, as demonstrated with potassium sulphate in ZE.

Concerning chromatographic techniques for protein purification, FFE should

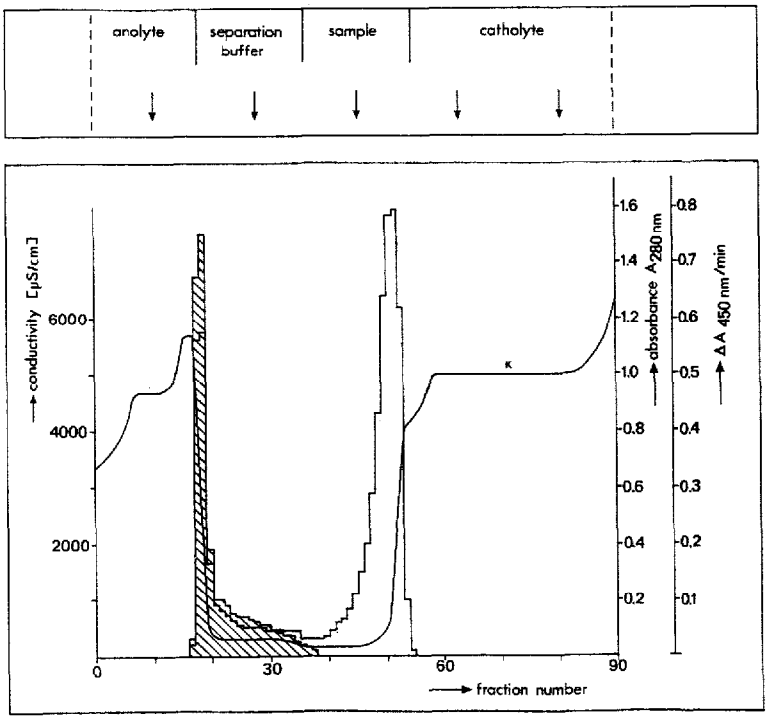

Fig. 6. FSE combined with ZE of lysozyme containing *E. coli* proteins. The top panel shows the inlet configuration and the lower panel shows the enzyme activity (dashed area, as ΔA_{450}/min) and the total protein distribution (as A_{280}). All solutions contained 5 mol/l urea. The anolyte and catholyte was Tris (0.1 mol/l) adjusted to pH 7.6 with hydrochloric acid, conductivity 4.7 mS/cm; the separation buffer was Tris (5 mmol/l)–HCl, pH 7.7, conductivity 0.17 mS/cm. The sample buffer was Tris (3 mmol/l)–HCl, pH 7.6, conductivity 0.15 mS/cm, with 0.17 mg/ml lysozyme and 1.5 mg/ml bacterial proteins. Field strength, 45 V/cm; flow-rate, 300 ml/h.

TABLE I

PURIFICATION OF LYSOZYME FROM CONTAMINATING *E. COLI* INTRACELLULAR PROTEINS BY ZE, IEF AND FSE COMBINED WITH ZE

Method	*Purification factor*[a]	*Recovery*[b] *(%)*	*Throughput (ml sample/h)*	*Purity*[c] *(%)*
ZE (urea)	3.7	87	2[d]	>95
ZE (K_2SO_4)	3.5	82	1[d]	>95
IEF	4.75	94	43	>95
FSE + ZE	9.5	95	60	>95

[a] Purification factor: purity after the separation/purity before the separation.
[b] Recovery: enzyme activity after FFE/enzyme activity before FFE × 100 (%).
[c] Purity: mg lysozyme/mg total protein × 100 (%).
[d] For ZE the protein concentration of the sample can be chosen as high as possible, as the sample will be diluted in the elctrophoretic run.

not been regarded as a competing but, owing to the different separation mechanisms, as a complementary technique within a purification scheme. For this purpose FFE is distinguished by high recoveries, high separation power and easy application.

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