ELSEVIER

Journal of Chromatography A, 672 (1994) 231–236

JOURNAL OF CHROMATOGRAPHY A

Separation of pig liver esterase isoenzymes and subunits by capillary zone electrophoresis in the presence of fluorinated surfactants

Å. Emmer, M. Jansson, J. Roeraade*

Department of Analytical Chemistry, Royal Institute of Technology, S-100 44 Stockholm, Sweden

(First received November 23rd, 1993; revised manuscript received February 23rd, 1994)

Abstract

An attempt was made to separate the isoenzymes and subunits of pig liver esterase by capillary zone electrophoresis. This enzyme is a complex mixture and is strongly adsorbed on a fused-silica capillary. However, by simply adding a cationic fluorosurfactant to the running buffer, adsorption was significantly reduced. The effects of adding a zwitterionic and a neutral fluorosurfactant were also investigated. Large changes in the elution pattern were observed when using different combinations of these additives. Mixtures of different fluorosurfactants added to the running buffer can therefore be utilized in strategies for optimization of the separation selectivity.

1. Introduction

Pig liver esterase (PLE) is an enzyme of considerable interest as a chiral catalyst in asymmetric organic synthesis. Even though studies of PLE have been reported by several workers [1,2], the exact molecular structure has remained unknown. This is mainly due to the extreme complexity of this enzyme, which consists of several isoenzymes and subunits [3].

It is highly desirable to be able to improve the catalytic performance of PLE in organic synthesis. This could be achieved by means of protein engineering. However, this has not yet been possible because of the uncertainties regarding the molecular structure and the difficulties in separating the different isoenzymes [3].

Capillary zone electrophoresis (CZE) is rapidly advancing as a powerful technique for the separation of proteins. However, wall adsorption is a basic problem associated with such separations and it frequently leads to broadened and distorted peaks. Several ways of improving this situation have been proposed [4–12]. However, the efficiency of these suggestions has mostly been demonstrated for molecules such as peptides and small model proteins and very little has been reported on free-flow CZE separations of large proteins.

Even a molecule such as lysozyme, which is often used as a model compound and is considered "difficult" in terms of wall adsorption owing to its high p*I*, is a relatively small and compact protein. Large proteins usually have a more complex tertiary (three-dimensional) and quarternary structure (combinations of different subunits) and therefore a more complex charge distribution. Consequently, the mechanisms of

* Corresponding author.

0021-9673/94/$07.00
SSDI 0021-9673(94)00186-D

wall adsorption become unpredictable. For example, regional charge heterogeneities within the molecule can cause adsorption of solutes under conditions where these have the same net charge as the wall [13]. Large proteins also have more charged sites that can cause electrostatic interactions and, once such proteins have been adsorbed, desorption will be very difficult.

Previously, we proposed a new concept in which a cationic fluorosurfactant was added to the running buffer. Strongly reduced protein adsorption, high efficiencies and excellent reproducibility were observed for smaller model proteins [14,15]. Other workers have also been able successfully to confirm our findings [16]. The unique properties of the fluorosurfactant prompted us to extend our studies to larger, more complex proteins. Apart from the cationic surfactant, this work also included studies with a zwitterionic and a neutral fluorosurfactant. Pig liver esterase was chosen as a model for these studies.

2. Experimental

A laboratory-made CZE apparatus was utilized. A UV detector (Linear Instruments, Reno, NV, USA) was employed at 230 nm for on-column detection. Injections were carried out by timer-controlled electromigration. New capillaries (fused silica, 100 cm × 50 μm I.D.) were flushed with 0.4 *M* NaOH for 30–60 min and then to neutrality with water and finally with running buffer for 20–30 min. The surfactants FC134 [$C_8F_{17}O_2NH(CH_2)_3N^+(CH_3)_3I^-$], FC430 (non-ionic fluoroaliphatic polymeric esters) (3M, St Paul, MN, USA) and a zwitterionic fluorosurfactant [$F(CF_2CF_2)_{3-8}CH_2CH(OCOCH_3)CH_2N^+(CH_3)_2CH_2CO_2^-$] (DuPont, Wilmington, DE, USA) were added to the running buffer, either separate or in various combinations at concentrations ranging between 50 and 400 μg/ml.

The experiments were carried out with a crude sample of PLE (Sigma, St. Louis, MO, USA) with a total protein concentration of 0.5 mg/ml. The enzymatic activity of PLE in presence of fluorosurfactants was assayed at pH 7 in 0.05 *M* phosphate buffer. After desalting on a PD-10 gel filtration column (Pharmacia, Uppsala, Sweden), spectrophotometric measurement at 405 nm was carried out. The conditions used were 2 m*M* *p*-nitrophenyl acetate, 5% dimethyl sulphoxide and PLE (1.4 μg/ml) dissolved in phosphate buffer.

3. Results and discussion

PLE is an acidic protein and is a complex mixture of at least six isoenzymes (M_r *ca.* 180 000, p*I* = 4.7–5.6), each consisting of trimers of different combinations of at least seven subunits (M_r *ca.* 60 000). PLE is strongly adsorbed on an untreated fused-silica capillary. Our initial attempts to operate at a pH of 7, according to the concept of Lauer and McManigill [4], failed. This method uses the fact that proteins are amphoteric and are repelled from the negatively charged silica surface if the pH of the buffer medium is above the p*I* of the protein. At pH 7, PLE has a net negative charge and should therefore not interact with the silica wall. However, the adsorption of PLE is severe. In fact, no relevant peaks are recognized in the electropherogram. This suggests that there are positively charged local domains in the molecule, which are attracted to the wall. A remedy for this problem could be to increase the pH. However, operation at pH extremes is not recommended, owing to the risk of protein denaturation. Also, the pH is a crucial parameter for selectivity optimization, and optimum selectivity in CZE is usually achieved near the p*I*. Under such conditions, however, PLE is strongly adsorbed on the silica wall. Also, other complications, such as precipitation of the protein, could occur when the pH of the buffer is close to the p*I* of the protein.

We have shown previously that the use of a cationic fluorosurfactant buffer additive decreases the wall adsorption considerably [14,15]. Using this concept, we also noted a drastic decrease in the adsorption of PLE at pH con-

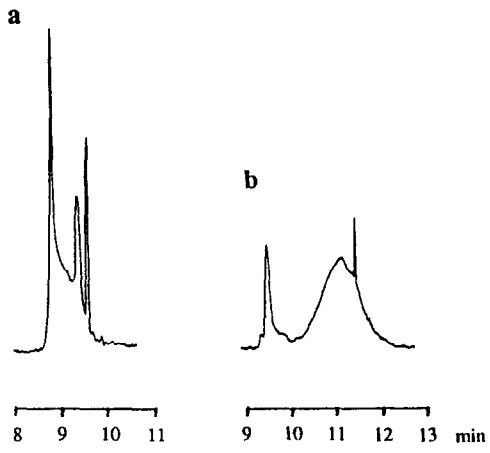

Fig. 1. Electropherograms of pig liver esterase in the presence of a cationic fluorosurfactant (FC134). Running buffer, 0.05 *M* acetate–phosphate with 100 μg/ml of FC134 added; buffer pH, (a) 4 and (b) 3.5; field strength, −30 kV; injection, electromigration at −20 kV for 10 s.

ditions just below the p*I* of the enzyme (Fig. 1). The reason for this improvement can in the first instance be explained by the formation of an admicellar bilayer or hemimicelles on the silica surface [15–19]. Thereby, the charge of the surface becomes positive. An indication of this is that the direction of the electroosmotic flow is reversed. PLE, which has a net positive charge under the actual pH conditions, should therefore be electrostatically repelled from the surface. However, negatively charged local regions of the protein molecules could still interact with the wall in different ways. We believe that these regions are shielded by bilayers and/or micelles of the fluorosurfactant and thus electrostatically repelled from the bilayer on the silica surface. Further, permanent adsorption of the protein on the bilayer is less probable, as the formation of the admicellar layer is a dynamic process, with a

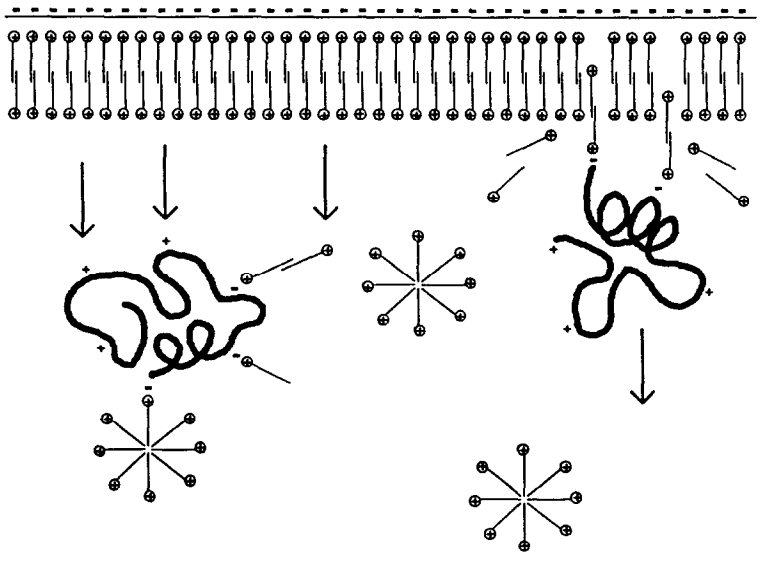

Fig. 2. Schematic diagram of some proposed interactions between a protein and the capillary wall in presence of a cationic fluorosurfactant. This must be seen as a dynamic process, with a continuous interchange of surfactant molecules. The negatively charged surface becomes positively charged owing to the formation of a bilayer of surfactant. The left part illustrates possible interactions between a protein molecule and the fluorosurfactant. The fluorosurfactant is present in the form of aggregates of different sizes up to complete micelles. The negatively charged centra of the protein will attract the positively charged surfactant monomers and aggregates. In its turn, these aggregates will attract other surfactant molecules by hydrophobic interaction. Thus the protein is shielded by positive charges, and consequently repelled from the positively charged wall. The right part shows the dynamic exchange of fluorosurfactant molecules between the surface bilayer and the protein molecule.

continuous interchange of the fluorosurfactant molecules. Fig. 2 shows a schematic diagram of these proposed mechanisms.

We also tested the effects of adding a zwitterionic fluorosurfactant. This amphoteric compound has a p*I* of about 8 [20]. Therefore, at a pH below this value, it will be positively charged and will act in a similar way to the cationic surfactant. However, significant changes in selectivity were observed on adding different combinations of the zwitterionic and the cationic fluorosurfactant to the running buffer for the PLE capillary electrophoretic separation (Fig. 3). We attribute these selectivity changes to the formation of mixed micelles in the buffer solution, on the silica surface and around the negatively charged local sites of the protein [21]. As the two fluorosurfactants have a different chemical composition and molecular structure, changes in the electrostatic interactions and corresponding electrophoretic mobility are to be expected.

As mentioned earlier, the buffer pH is a central parameter for optimization of the separation selectivity, as it affects the magnitude of the net charge and influences molecular conformations and charge distribution. For a complex protein such as PLE, this is further complicated by dissociation of the isoenzymes into subunits. It can therefore be expected that the separation patterns of PLE at different pH values of the running buffer will be very dissimilar. This can also be seen in Fig. 1.

Further, we attempted to improve the selectivity of the separation by adding a neutral fluorosurfactant. The results are shown in Fig. 4. Owing to their extremely non-polar character, one can expect a significant interaction between the fluorinated carbon chains of the charged and the neutral surfactants [22]. It is therefore likely that the neutral fluorosurfactant is also partially incorporated in the adsorbed admicellar double layer [21]. This would lead to a decrease in the

Fig. 3. (a) Electropherogram of PLE in the presence of a zwitterionic fluorosurfactant; (b) same as (a) but with addition of the cationic fluorosurfactant. Running buffer, 0.01 *M* acetate (pH 3.5) with (a) 400 μg/ml of zwitterionic fluorosurfactant added and (b) 200 μg/ml of zwitterionic and 50 μg/ml of cationic fluorosurfactant added; injection, electromigration at (a) −20 kV for 10 s and (b) −10 kV for 10 s. Other conditions as in Fig. 1.

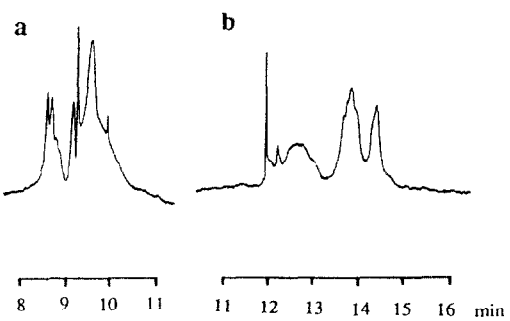

Fig. 4. Influence of the addition of a neutral fluorosurfactant on the separation of pig liver esterase. Running buffer: (a) 0.02 *M* acetate with 400 μg/ml of zwitterionic and 100 μg/ml of cationic fluorosurfactant added; (b) same as (a) but with an additional 200 μg/ml of neutral fluorosurfactant. Injection, electromigration at −10 kV for 10 s. Other conditions as in Fig. 1.

charge of the wall and result in a decrease in the electroosmotic flow. This is in accordance with our observations, as shown in Fig. 4. However, part of the decrease of the electroosmotic flow must also be attributed to an increased viscosity of the buffer solution.

Unfortunately, the PLE sample proved to be too complex to obtain a distinct separation of the enzyme units. The best results were obtained when a mixture of the three fluorosurfactants was added to the buffer (Fig. 4b), where about fifteen signals can be recognized. Several of the compounds overlap in the form of broad peaks. We believe that this could be partially due to the association and dissociation of subunits during the electrophoretic transport in the column. Another contributing factor is that the crude PLE sample is likely to contain a number of unspecified contaminants. The most feasible way to improve the separation would be to include a prefractionation, *e.g.*, by isoelectric focusing.

In previous studies [14], we assumed that hydrophobic interactions between fluorosurfactants and proteins are likely to be minimal, in view of the fact that the fluorocarbon chains have a lipophobic character. In order to investigate this assumption in practice, an enzymatic activity test with PLE in the presence of the fluorosurfactants was carried out. The results are presented in Table 1, and show that the enzymatic activity of PLE is completely preserved in the presence of the neutral fluorosurfactant. As expected, the charged fluorosurfactants interact with the proteins. However, at the low concentrations where wall deactivation for CZE is optimum, the enzymatic activity of PLE is to a large extent preserved.

4. Conclusions

CZE separation of native proteins such as PLE is not straightforward, owing to the complexity of such proteins and their unpredictable wall adsorption behaviour. Owing to the diversities in configuration, size, p*I* and charge distribution of the different isoenzymes and subunits, it is very difficult to design a model for optimum separation. Prefractionation schemes (*e.g.*, using isoelectric focusing) should be advantageous in this context.

Fluorosurfactants, used as buffer additives, can be successfully employed to suppress wall adsorption, while allowing operation at an optional buffer pH, preferably near the p*I* of the enzyme.

Significant differences in elution patterns are obtained with combinations of cationic, zwitterionic and neutral fluorosurfactants, which can be exploited for experimental optimization of the separation selectivity. Interactions between the fluorosurfactant additives and the enzyme were shown to be relatively moderate, as demonstrated by enzyme assays.

Several alternative methods to suppress adsorption, *e.g.*, the use of permanent wall coatings, could also prove to be effective. However, the advantage of the fluorosurfactant concept is

Table 1
Enzymatic activity of PLE in the presence of fluorosurfactants

Buffer additive	Activity (ΔA/min)	Relative activity
No additive	0.18	1
Cationic fluorosurfactant	0.11	0.61
Non-ionic fluorosurfactant	0.18	1
Zwitterionic fluorosurfactant	0.11	0.61

The enzymatic activity was monitored by the change in absorbance ($\lambda = 405$ nm) per minute (ΔA/min). Fluorosurfactant concentrations: 100 μg/ml for the cationic, 200 μg/ml for the non-ionic and 400 μg/ml for the zwitterionic surfactant. The results were corrected for non-enzymatic self-hydrolysis by running a parallel blank without enzyme.

its simplicity. Moreover, a very good reproducibility of migration times is obtained even with a sample such as crude PLE, because the surfactant layer on the wall is continuously renewed.

5. References

[1] E. Heymann and W. Junge, *Eur. J. Biochem.*, 95 (1979) 509.
[2] D. Farb and W.P. Jencks, *Arch. Biochem. Biophys.*, 203 (1980) 214.
[3] N. Öhrner, A. Mattson, T. Norin and K. Hult, *Biocatalysis*, 4 (1990) 81.
[4] H.H. Lauer and D. McManigill, *Anal. Chem.*, 58 (1986) 166.
[5] R.M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
[6] J.S. Green and J.W. Jorgenson, *J. Chromatogr.*, 478 (1989) 63.
[7] M. Bushey and J.W. Jorgenson, *J. Chromatogr.*, 480 (1989) 301.
[8] S.A. Swedberg, *Anal. Biochem.*, 185 (1990) 51.
[9] G.J.M. Bruin, R. Huisden, J.C. Kraak and H. Poppe, *J. Chromatogr.*, 480 (1989) 339.
[10] J.K. Towns and F.E. Regnier, *J. Chromatogr.*, 516 (1990) 69.
[11] Z. Zhao, A. Malik and M.L. Lee, *J. Microcol. Sep.*, 4 (1992) 411.
[12] J.T. Smith and Z. El Rassi, *J. High Resolut. Chromatogr.*, 15 (1992) 573.
[13] M. Wahlgren and T. Arnebrant, *TIBTECH*, 9 (1991) 201.
[14] Å. Emmer, M. Jansson and J. Roeraade, *J. Chromatogr.*, 547 (1991) 544.
[15] Å. Emmer, M. Jansson and J. Roeraade, *J. High Resolut. Chromatogr.*, 14 (1991) 738.
[16] W.G.H.M. Muijselaar, C.H.M.M. de Bruijn and F.M. Everaerts, *J. Chromatogr.*, 605 (1992) 115.
[17] M.A. Yeskie and J.H. Harwell, *J. Phys. Chem.*, 92 (1988) 2346.
[18] H. Rupprecht and T. Gu, *Colloid Polym. Sci.*, 269 (1991) 506.
[19] J.M. Cases and F. Villieras, *Langmuir*, 8 (1992) 1251.
[20] Å. Emmer and J. Roeraade, *J. Liq. Chromatogr.*, in press.
[21] J.H. Harwell and J.F. Scamehorn, in K. Ogino and M. Abe (Editors), *Mixed Surfactant Systems* (*Surfactant Science Series*, Vol. 46), Marcel Dekker, New York, 1993, pp. 263–281.
[22] F. MacRitchie, *Adv. Colloid Interface Sci.*, 25 (1986) 341.