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Studies of complexes between proteases, substrates and the protease inhibitor α_2-macroglobulin using capillary electrophoresis with laser-induced fluorescence detection

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Abstract

Capillary zone electrophoresis (CZE) with laser-induced fluorescence (LIF) detection is shown to constitute a unique technique for the investigation of the interaction between proteases, protease inhibitors and substrates. Under optimized analysis conditions, the formation of a complex between FITC-labelled proteases such as trypsin, plasmin, α-chymotrypsin and the (unlabelled) protease inhibitor α_2-macroglobulin was studied. This is not possible with UV detection, since under such conditions the complex cannot be distinguished from the unreacted protease inhibitor. Low ratios of FITC bonded to the proteases further complex formation, while high ratios often prevent the reaction. Complex formation shows a strong dependence on the incubation conditions (pH, salt concentration, temperature, incubation time). Once formed, however, the complexes are stable under CZE conditions (e.g., a pH of the electrophoresis buffer of 10.5) for at least 30 min. Treatment with sodium dodecyl sulfate (5 min at 90°C or 30 min at 75°C) does not destroy the complexes, whereas treatment with mercaptoethanol (reduction of disulfide bonds) eliminates the peak from the electropherogram. Both findings argue for the formation of a covalent bond between the protease and the inhibitor during complex formation. Since the reaction of the proteases with α_2-macroglobulin does not involve the binding site of the former, a residual proteolytic activity is still observed in the ensuing complex. The extent of the inhibition of the remaining trypsin activity in a trypsin – α_2-macroglobulin complex was established to depend on the molecular mass of the second trypsin inhibitor.

1. Introduction

Capillary electrophoresis (CE) offers a wide range of methods for the determination of peptides and proteins. High-resolution separations can be performed based on subtle differences in the physical properties of these substances, e.g., the mass-to-charge ratio, the size and the hydrophobicity. Concomitantly, CE is emerging as a tool for the investigation of biological and biochemical interactions between peptides and proteins or other specific components [1–7]. Among the advantages of using capillary electrophoresis in biological binding studies are the small sample volumes, the short separation times, the high selectivity and the possibility of on-line (column) detection. Especially when laser-induced fluorescence (LIF) detection is employed, highly specific investigations of minute amounts of sample become possible even in complex matrices [8,9]. Based on previous analysis of the interaction of FITC-labelled protein G and immunoglobulins [7], in this work the complex formation between protease and protease in-

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hibitors was studied and compared with the results achieved with traditional techniques.

2. Experimental

2.1. Capillary electrophoresis

All separations were performed on a Beckman P/ACE 2100 capillary electrophoresis system with either LIF detection (argon ion laser, excitation wavelength 488 nm, emission wavelength 560 nm) or with UV detection. Post-run data were analysed using System Gold software (Beckman) and exported to Excel 5.0 (Microsoft) for publication purposes. Details of the electrophoretic conditions are given later in the text and in the figure captions. Capillaries were supplied by CS-Chromatographie Service (Langerwehe, Germany).

2.2. Chemicals

α-2-Macroglobulin and non-labelled proteases and inhibitors were used as supplied by the manufacturer. The labelled proteins were purified by liquid chromatography (size-exclusion and affinity chromatography) prior to the binding studies.

Protein labelling with FITC was done according to the supplier's instructions. For analysis, samples were dissolved in 50 or 100 m*M* phosphate buffer (pH 7.4) containing 50 m*M* NaCl. Typically, samples were incubated at 30°C for the indicated given period of time.

All chemicals were obtained from Sigma (Deisenhofen, Germany).

3. Results and discussion

α-2-Macroglobulin is one of the major protease inhibitors found in the blood. It accounts for ca. 10% of the total trypsin-inhibiting capacity and constitutes between 3% and 4% of the total protein content of plasma. With a relative molecular mass of 725 000, α_2-macroglobulin is by far the largest of all known protease inhibitors. The molecule consists of four identical subunits. Among the unique properties of the molecule is an unusual thioester bond in its structure. Rather than having one specific physiological function, α_2-macroglobulin appears to be able to inhibit most known proteases. The inhibitor is consequently involved in modulating protease activity in a great variety of proteins and peptides in a manner unrelated to the protease-binding mechanism.

3.1. Complex formation between α_2-macroglobulin and FITC-labelled trypsin

When a mixture of α_2-macroglobulin and FITC-labelled trypsin is subjected to CE analysis without prior incubation, two signals are detected by the UV detector (Fig. 1A), while the LIF detector shows only one signal, the FITC-trypsin peak (Fig. 1B). After 5 min of incubation of the α_2-macroglobulin–FITC-trypsin mixture, a second peak appears in the electropherograms when LIF detection is used (Fig. 1C). Its height and area increase with increasing incubation time (Fig. 1D and E). This peak is the result of the formation of an affinity complex between the FITC-labelled protease and the protease inhibitor α_2-macroglobulin. The control, i.e., an electrophoresis of FITC-labelled trypsin without α_2-macroglobulin, shows no peak at this point (1.7 min) in the electropherogram, even after a 60-min incubation, thus confirming the signal to be the result of the formation of the affinity complex (Fig. 1F).

Irrespective of the incubation time, only two signals are found in the electropherograms recorded by the UV detector. As α_2-macroglobulin is considerably larger than FITC-labelled trypsin, its mass-to-charge ratio tends to dominate the electrophoretic properties of the complexes. As a consequence, the migration time of the complexes will be similar to that of the unreacted α_2-macroglobulin itself (see, e.g., Fig. 1A and E). Hence no differentiation between the complex and the unreacted α_2-macroglobulin is possible by UV detection, whereas such a differentiation is easily achieved by LIF detection.

On the basis of concentration vs. peak height/

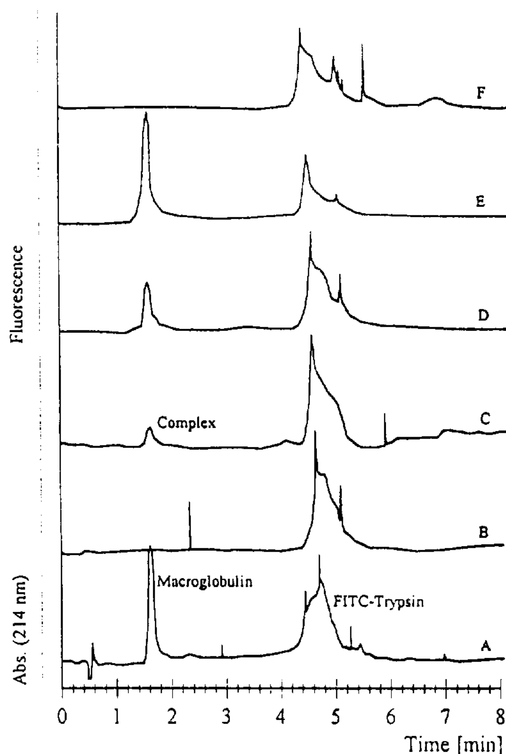

Fig. 1. Complex formation between α_2-macroglobulin and FITC-labelled trypsin. Capillary, 27 cm × 50 μm I.D.; buffer, 50 m*M* borate (pH 10.5); applied voltage, 12 kV; injection, 2 s; pressure, 50 mbar. (A) α_2-Macroglobulin and FITC-labelled trypsin with UV detection (214 nm); (B) FITC-labelled trypsin and the α_2-macroglobulin with LIF detection; (C) complex formation between FITC-labelled trypsin and α_2-macroglobulin after an incubation time of 5 min with LIF detection; (D) complex formation between FITC-labelled trypsin and α_2-macroglobulin after an incubation time of 30 min with LIF detection; (E) complex formation between FITC-labelled trypsin and α_2-macroglobulin after an incubation time of 60 min with LIF detection; (F) FITC-labelled trypsin as a control after an incubation time of 60 min with LIF detection.

area calibration runs for the two fluorescence signals, it can be deduced that the ratio of α_2-macroglobulin to trypsin in the affinity complex amounts to approximately two protease molecules per molecule of inhibitor. This corresponds to results achieved with conventional methods [10], while the finding that α_2-macroglobulin is capable of binding up to six trypsin molecules [11] cannot be confirmed.

The investigation of the influence of the incubation time showed that the reaction has reached equilibrium after ca. 60 min, since no further increase in the height and the area of the complex signal is observed for longer incubation times. An investigation of the influence of the pH of the incubation buffer and temperature on the complex formation showed that an increase in pH from 7 to 9 increases the rate of complex formation, and the optimum incubation temperature is ca. 30°C. As the mechanism of the protease inhibition by α_2-macroglobulin is not yet fully understood and the experimental conditions suggested here have still to be further optimized, these results were not quantified and the binding constants were not calculated.

Once formed, the complexes appear to be unusually stable. Other than, e.g., the immunocomplexes investigated previously [7], the protease–inhibitor complex stability seems to be independent of the CE separation conditions. Even under the harsh conditions employed, i.e., using an electrophoresis buffer of pH 10.5 and prolonging the separation times by utilizing increasingly longer capillaries, did not influence the separation performance or reduce the total amount of the complexes detected in this case (Fig. 2A–C).

The high stability of the affinity complex is mainly the result of the unique protease-binding mechanism of α_2-macroglobulin. A protease being inhibited by the α_2-macroglobulin is "trapped" by one of the two "bait regions" which are found approximately in the middle of the subunit chain of the inhibitor. On proteolytic cleavage of a single bond in each subunit, α_2-macroglobulin undergoes a conformational change to a more compact structure, leading to virtually irreversible binding of the respective protease. The active site of the protease, however, need not be involved in this reaction [12]. Although the exact nature of this mechanism is still under investigation, our results, i.e., the high stability of the complex over a prolonged period of time even at a high buffer pH, are in favour of the theory of entrapment of the protease by a conformational change of the inhibitor.

In addition to this reaction, another stabilizing

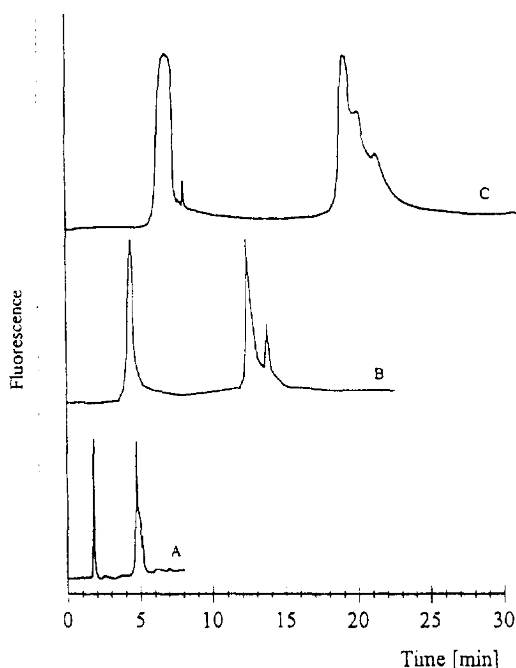

Fig. 2. Stability of the α_2-macroglobulin–FITC-trypsin complex with regard to the separation time. (A) Capillary, 27 cm × 50 μm I.D.; buffer, 50 m*M* borate (pH 10.5); applied voltage, 12 kV; injection, 2 s; pressure, 50 mbar; LIF detection. (B) Capillary, 47 cm × 50 μm I.D.; buffer, 50 m*M* borate (pH 10.5); applied voltage, 12 kV; injection, 2 s; pressure, 50 mbar; LIF detection. (C) Capillary, 67 cm × 50 μm I.D.; buffer, 50 m*M* borate (pH 10.5); applied voltage, 15 kV; injection, 2 s; pressure, 50 mbar; LIF detection.

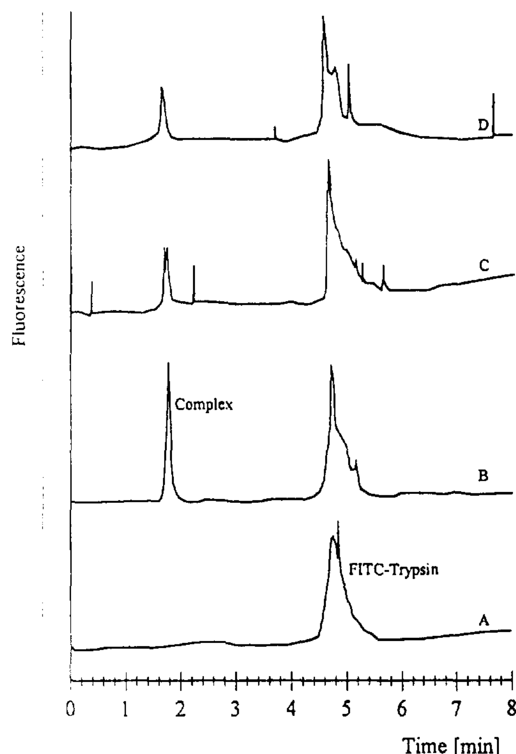

Fig. 3. Stability of the α_2-macroglobulin–FITC-trypsin complex with regard to covalent linkage between the proteins. Capillary, 27 cm × 50 μm I.D.; buffer, 50 m*M* borate (pH 10.5); applied voltage, 12 kV; injection, 2 s; pressure, 50 mbar; LIF detection. (A) FITC-labelled trypsin as a control after an incubation time of 60 min; (B) complex formation between FITC-labelled trypsin and α_2-macroglobulin with LIF detection after an incubation time of 60 min; (C) complex stabilaity between FITC-labelled trypsin and α_2-macroglobulin with LIF detection after an incubation time of 60 min and heating for 5 min at 90°C in the presence of 50 m*M* SDS; (D) complex stability between FITC-labelled trypsin and α_2-macroglobulin with LIF detection after an incubation time of 60 min and heating for 30 min at 75°C in the presence of 50 m*M* SDS.

mechanism has been recognized for protease–α_2-macroglobulin complexes [13]. On protease binding, the thioester bond, located roughly about two thirds of the distance from the NH_2-terminal end of the subunit chain, may break and the acyl group thus made available can form a covalent bond with any accessible primary amino group on the surface of the protease in question.

CE may once more serve to investigate the probability of this reaction. If an already formed complex (60-min incubation) is treated with sodium dodecyl sulfate (SDS) and heated for 5 min at 90°C (Fig. 3C) or for 30 min at 75°C (Fig. 3D), the affinity complex peak is still present in the electropherogram recorded afterwards, albeit at a lower concentration than without this treatment (Fig. 3B). This may be regarded an obvious indication for the stability of the complex. A further proof of this mechanism is given by the fact that the reduction of the disulfide bonds by mercaptoethanol in the presence of SDS eliminates the α_2-macroglobulin–complex peak,

whereas the FITC-trypsin signal concomitantly increases.

3.2. Complex formation between α_2-macroglobulin and other FITC-labelled proteases

A drawback in the application of CE using LIF detection is the necessity to use a labelling agent such as FITC. A high ratio of molecules of the labelling substance per molecule of protease results in the lowest possible detection limit. Concomitantly, labelling may strongly influence the biochemical and biophysical behavior of a protein, including its affinity for the protease inhibitor.

The impact of FITC labelling was demonstrated by CE analysis using several proteases with different ratios of bound FITC per protease molecule under the above-defined reaction conditions. α-Chymotrypsin with 4–7 molecules of FITC does not form complexes with α_2-macroglobulin (Fig. 4A), whereas the same protease labelled with only 1–3 molecules of FITC per molecule shows a considerable affinity to the inhibitor (Fig. 4B). The same effect of FITC-labelling can be observed for the complex formation between plasmin and α_2-macroglobulin (Fig. 4C and D). In this case labelling the plasmin with one or two molecules of FITC still allows complex formation, while three or more molecules of FITC prevent the reaction.

The observation that the effect of low FITC ratios on the affinity interaction is negligible, whereas a higher degree of FITC labelling causes considerable interference, may be interpreted in terms of steric hindrance in the "bait region" caused by the FITC. An effect of a putative blocking of the active site of the protease appears to be less likely, since the complex formation between protease and α_2-macroglobulin would be influenced independently of the particular FITC-to-protease ratio in this case. Nevertheless, all results of binding studies performed by CE with LIF detection should be considered with respect to the possible influence of the FITC and thus be interpreted carefully.

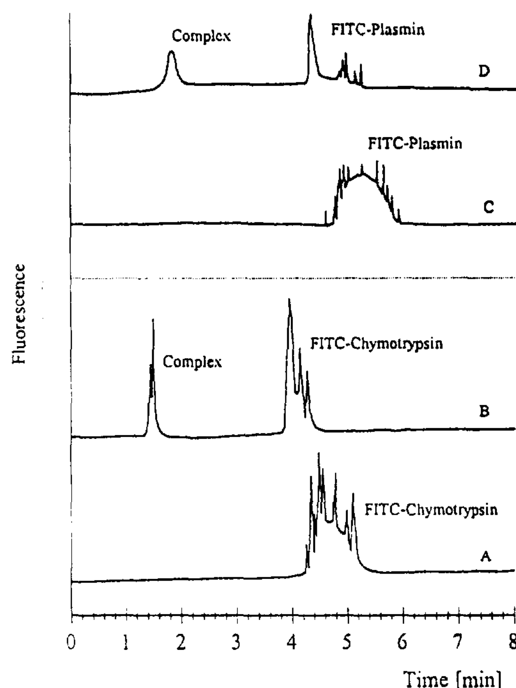

Fig. 4. Analysis of the α_2-macroglobulin–FITC-plasmin and α_2-macroglobulin–FITC-α-chymotrypsin complex formation with regard to the extent of FITC labelling. Capillary, 27 cm × 50 μm I.D., buffer, 50 mM borate (pH 10.5); applied voltage, 12 kV; injection, 2 s; pressure, 50 mbar; LIF detection. (A) Complex formation between FITC-labelled α-chymotrypsin (4–7 mol of FITC per mole of protein) and α_2-macroglobulin with LIF detection after an incubation time of 30 min; (B) complex formation between FITC-labelled α-chymotrypsin (1–3 mol of FITC per mole of protein) and α_2-macroglobulin with LIF detection after an incubation time of 30 min; (C) complex formation between FITC-labelled plasmin (3–8 mol of FITC per mole of protein) and α_2-macroglobulin with LIF detection after an incubation time of 30 min; (D) complex formation between FITC-labelled plasmin (1–2 mol of FITC per mole of protein) and α_2-macroglobulin with LIF detection after an incubation time of 30 min.

3.3. Complex formation between α_2-macroglobulin-entrapped trypsin and a second trypsin inhibitor

As the entrapment of a protease by α_2-macroglobulin does not necessarily involve the active site of the protease, an investigation of the residual protease activity (substrate or second

inhibitor) by CE is still possible. The protease aprotinin (M_r 6500) is, e.g., capable of reacting with an already bound and inhibited trypsin in the α_2-macroglobulin–protease complex. In Fig. 5A the separation of α_2-macroglobulin and trypsin is shown; detection was effected by measuring the UV activity of the substance to ensure the registration of all proteins. Fig. 5B–D show analyses for FITC-labelled aprotinin (B) alone and in the presence of (C) unlabelled trypsin and (D) α_2-macroglobulin. If the FITC-labelled aprotinin is incubated with the trypsin–α_2-macroglobulin complex, a "new" signal appears in the LIF electropherograms at approximately the migration time of the α_2-macroglobulin in the UV electropherogram (E). The size of this signal increases with increasing incubation time (F). Apparently a three-component complex between trypsin, α_2-macroglobulin and aprotinin has been formed, whose electromigration is once more strongly dominated by the properties of the α_2-macroglobulin. This complex can be easily studied by capillary electrophoresis.

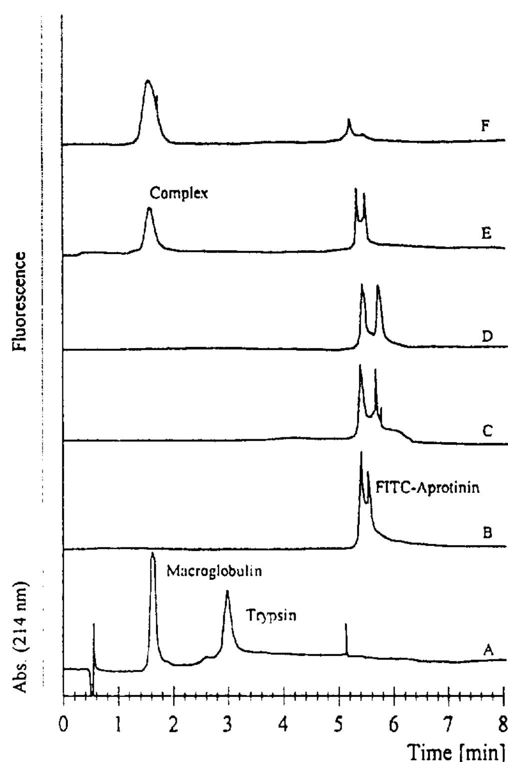

Fig. 5. Complex formation between a small FITC-labelled trypsin inhibitor (aprotinin) and the α_2-macroglobulin–trypsin complex. Capillary, 27 cm × 50 μm I.D.; buffer, 50 mM borate (pH 10.5); applied voltage, 12 kV; injection, 2 s; pressure, 50 mbar. (A) α_2-Macroglobulin and trypsin with UV detection (214 nm); (B) FITC-labelled aprotinin with LIF detection; (C) FITC-labelled aprotinin and trypsin after an incubation time of 30 min with LIF detection; (D) FITC-labelled aprotinin and α_2-macroglobulin after an incubation time of 30 min with LIF detection; (E) complex formation between FITC-labelled aprotinin and the α_2-macroglobulin–trypsin complex after an incubation time of 30 min with LIF detection; (F) complex formation between FITC-labelled aprotinin and the α_2-macroglobulin–trypsin complex after an incubation time of 60 min with LIF detection.

This effect is based on the unique inhibition reaction of α_2-macroglobulin, i.e., the entrapment of the protease in the "bait region" without inhibition of its reactive site. The still active protease trapped in the "α_2-macroglobulin bait" is capable of reacting with substrates (resulting in a reduced proteolytic activity of the protease [14]) or, as in the present case, with another protease inhibitor.

In view of the results concerning the influence of FITC on the complex formation, it is not surprising, that the size of the substrate and the second protease should be critical for the formation of the three-protein complex. Aprotinin with a relative molecular mass of 6500 is still capable of entering the "narrow bait region", while the much larger soybean trypsin inhibitor (M_r 21 700) is sterically hindered and therefore shows no reaction with the α_2-macroglobulin–trypsin complex (Fig. 6A–D). The results of the effect of the size of the substrate or the second protease inhibitor on the reaction of the substances with the α_2-macroglobulin–protease complex are again comparable to those achieved by conventional methods [15].

4. Conclusion

Capillary electrophoresis has proved to be a versatile tool for binding studies of proteases and the protease inhibitors. While it need not replace the traditional techniques, it can provide additional information with a unique speed and easy handling. Nevertheless, further optimization has

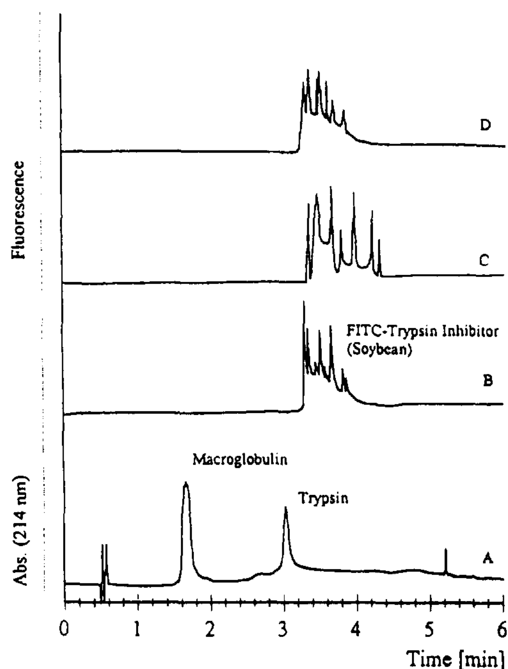

Fig. 6. Complex formation between a large FITC-labelled trypsin inhibitor (soybean) and the α_2-macroglobulin–trypsin complex. Capillary, 27 cm × 50 μm I.D.; buffer, 50 m*M* borate (pH 10.5), applied voltage, 12 kV; injection, 2 s; pressure, 50 mbar. (A) Separation of α_2-macroglobulin and trypsin with UV detection (214 nm); (B) FITC-labelled trypsin inhibitor with LIF detection; (C) FITC-labelled trypsin inhibitor and trypsin after an incubation time of 30 min with LIF detection; (D) FITC-labelled trypsin inhibitor the α_2-macroglobulin–trypsin complex after an incubation time of 30 min with LIF detection.

to be carried out with respect to the quantification problems of the binding assay in capillary electrophoresis.

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