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Fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) as a model protein drug: opportunities and drawbacks

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Received November 17, 2005, accepted December 17, 2005

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Pharmazie 61: 770–774 (2006)

In the therapy of various diseases, parenterally administered protein drugs are of steadily rising importance. In order to reduce the application frequency, these proteins can be incorporated into drug delivery systems, e.g. biodegradable microparticles from poly(lactic-co-glycolic acid) (PLGA). To evaluate the characteristics of these vehicles, fluorescent labelled proteins like fluorescein isothiocyanate labelled bovine serum albumin (FITC-BSA) may be used as model drugs to allow the visualisation of the protein localisation within the microparticle and the detection of microparticles in cell cultures or tissues. However, the quantification of protein by fluorescence spectroscopy failed. In this study we focused on the mechanism of fluorescence dequenching in a multi-FITC-labelled protein and its impact on a reliable protein determination.

1. Introduction

The steadily rising importance of protein drugs can be seen in an increasing number of such products on the market, e.g. infliximab (Remicade®), etanercept (Enbrel®) and adalimumab (Humira®) for the therapy of rheumatoid arthritis or interferone beta-1a (Avonex®, Rebif®) and interferone beta-1b (Betaferon®) in the treatment of multiple sclerosis. However, the need of parenteral administration up to every second day is a main drawback of substances like these. During the last two decades, biodegradable microparticles from poly(lactic-co-glycolic acid) (PLGA) have been proposed as suitable delivery devices to reduce the application frequency, not only for conventional drugs, but in particular for proteins due to their rapid degradation. There are numerous reports in the literature on the microencapsulation of both therapeutic and model proteins. Typically model proteins are chosen to study protein stability and polymer characteristics of such microparticles (Conway and Alpar 1996; Mäder et al. 1998; Sah 1999; Castellanos et al. 2001; Jiang et al. 2002; Li and Schwendeman 2005; Bilati et al. 2005a). Labelling the proteins with a fluorescent dye like fluorescein may allow (i) visualisation of the protein localisation within the microparticle (Wischke and Borchert 2006), (ii) detection of microparticles in cell cultures or tissues (Wischke et al. 2006) and (iii) high sensitive quantification of the released model protein (Cohen et al. 1991; Frangione-Beebe et al. 2001; Bilati et al. 2005b).

Beside the field of microencapsulation, fluorescent labelled proteins are extensively employed in life science research, e.g. labelled antibodies for fluorescence microscopy and flow cytometry. A large number of fluorescent dyes is available (Haughland 2002), but fluorescein is one of the most frequently used fluorophores for labelling proteins. Fluorescein isothiocyanate (FITC) labelled species of antibodies and other proteins can be easily purchased from distributors and bear several fluorophores per protein molecule.

In this study we showed the potential of FITC-labelled bovine serum albumin (FITC-BSA) as a model protein in microencapsulation. But the main focus of this work was directed on the problem of fluorescence dequenching within a multi-FITC-labelled BSA and its impact on a reliable protein determination.

2. Investigations, results and discussion

2.1. Opportunities of FITC-BSA in microencapsulation

By the encapsulation of FITC-BSA and the examination of fluorescence distribution by confocal laser scanning microscopy it is possible to experience the protein localisation within the microparticles. Moreover one can conclude on the morphology of the microparticles (microspheres or microcapsules) and the efficiency of primary emulsification in a double-emulsion solvent-evaporation based microencapsulation procedure (Wischke and Borchert 2006). A homogeneous fluorescence of the particles indicates the appearance of microspheres, which contain more than one protein loaded cavity (Fig. 1a). Contrary, a microcapsule would show a large central hole and the protein localised near the surface of the particle (Fig. 1b). Furthermore the protein distribution may also give information on the release behaviour of the microparticles, because microcapsules were expected to show a faster protein delivery.

To visualise the particle's localisation in the cell culture and to allow the study of the phagocytosis of microparticles by human monocytes or dendritic cells, we had to adjust fluorescence intensity of the microparticles by encapsulating different amounts of FITC-BSA. While loading the micropar-

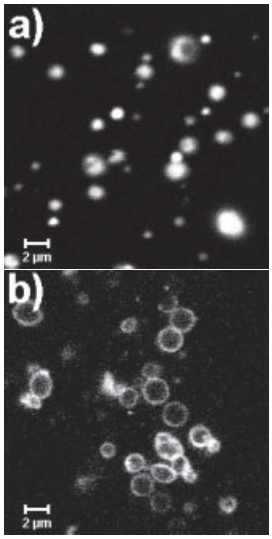

Fig. 1: Confocal laser scanning micrographs showing the protein distribution inside the microparticles. (a) microspheres (ultrasonic homogenizer for w_1/o), (b) microcapsules (Ultra-Turrax® for w_1/o)

ticles with 0.1% labelled protein did not allow particle detection by flow cytometry, entrapping 1% FITC-BSA gave an optimal fluorescence. On examination with a simple fluorescence microscope, the whole particles were shining. After incubation with human monocytes, the particles were visible inside the cells (Fig. 2). Using flow cytometry, the uptake of the microparticles could be quantified by measuring the fluorescence intensity of the cells (Fig. 3). In a future study, the impact of particle surface modification on the extent of particle phagocytosis will be examined.

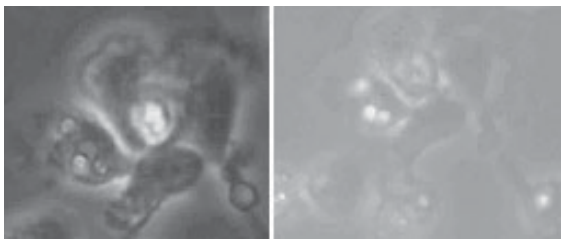

Fig. 2: Microscopic pictures of monocytes after microparticle phagocytosis. (left) light microscopy, (right) fluorescence microscopy

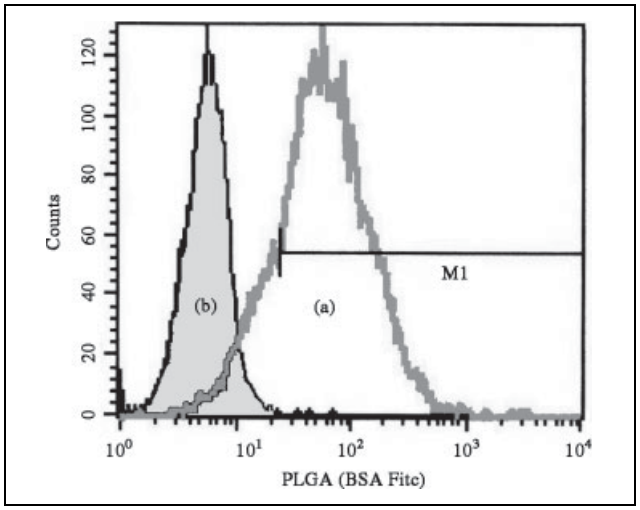

Fig. 3: Uptake of microparticles into human immature dendritic cells. Cell fluorescence measured by flow cytometry (a) following phagocytosis of FITC-BSA loaded microparticles compared to (b) nontreated cells

2.2. Protein release studies

In a few papers, FITC-BSA was described as a model protein in microencapsulation and the absorption of fluorescein was employed to quantify the amount of entrapped or released drug (Cohen et al. 1991; Frangione-Beebe et al. 2001; Bilati et al. 2005b). However, in our study on microparticles with a very high burst release, fluorescence spectroscopy detected a cumulative release of more than 100% within 7 days (Fig. 4). Similar results could be obtained with other microparticle batches, too. Since release levels much above 100% are not reliable, we performed protein quantification either by a silver-dithizone protein assay or by ELISA in further release studies. Both, the silver-dithizone assay and the ELISA showed about 90% cumulative release within 7 days. We assumed that this is the true release profile and concluded, that fluorescence spectroscopy was not appropriate to quantify the release of FITC-BSA .

When standard solutions of FITC-BSA were incubated at 37 °C, a steady increase in fluorescence intensity could be found (Fig. 5). We assumed, that structural changes within the FITC-BSA might be the reason for that.

2.3. Studies on FITC-BSA standards

To study this phenomena, standard solutions of 100 µg/ml FITC-BSA in Tris buffer pH 7 were prepared under aseptic conditions with sterilized materials and kept at 37 °C for up to 28 days. Subjecting this samples to SDS-PAGE,

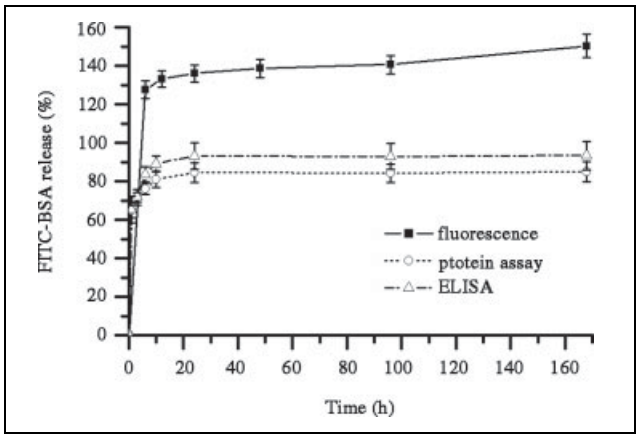

Fig. 4: Determination of the FITC-BSA release from microparticles produced by using Ultra-Turrax® for w_1/o emulsion ($n = 3$, median and range)

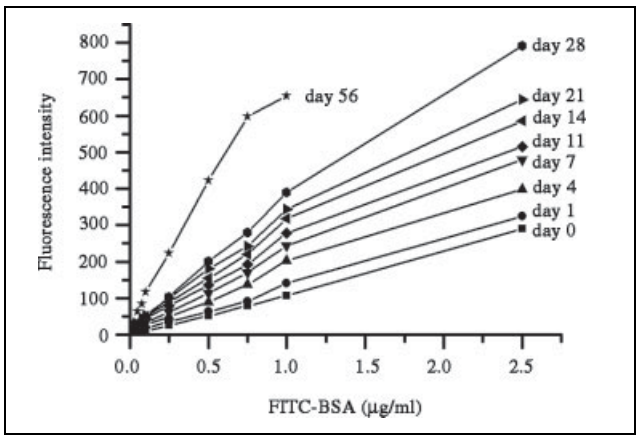

Fig. 5: Fluorescence intensity of FITC-BSA standard solutions in Tris buffer (pH 7) after incubation at 37 °C up to 56 days

the FITC-BSA band could be found at about 70 kDa (Fig. 6). Additional protein bands were visible, the most intensive ones appeared at higher molecular weight and corresponded to dimers and oligomers of FITC-BSA, which typically appear in commercial BSA preparations (Janatova et al. 1968; Folta-Stogniew and Williams 1999; Hunter and Carta 2001). Following incubation at 37 °C, the intensity of this bands decreased and a new band of 10 kDa or less could be found.

To additionally clarify the results of the SDS-PAGE, the FITC-BSA standard samples were analysed by size exclusion chromatography (SEC) using a coupling of an UV and a fluorescence detector to get information about the amount of protein (UV absorption at 280 nm) causing a certain fluorescence emission. As can be seen from the fluorimetric determination depicted in Fig. 7a, the left shoulder of the main peak, corresponding to the FITC-BSA dimer decreased when FITC-BSA was incubated at 37 °C. Contrary three peaks at higher retention times increased. By extrapolation, the molecular weights of these fractions were estimated to be about 4700, 3500, and 2800 Da respectively. The right shoulder of the main peak, corresponding to a molecular weight of about 37 kDa, increased initially and got smaller at further incubation. The curves obtained from UV detection (Fig. 7b) had a similar shape for the FITC-BSA dimer, monomer, and the ~37 kDa fragment. Contrary to the findings from fluorescence detection, no peaks appeared at higher retention times.

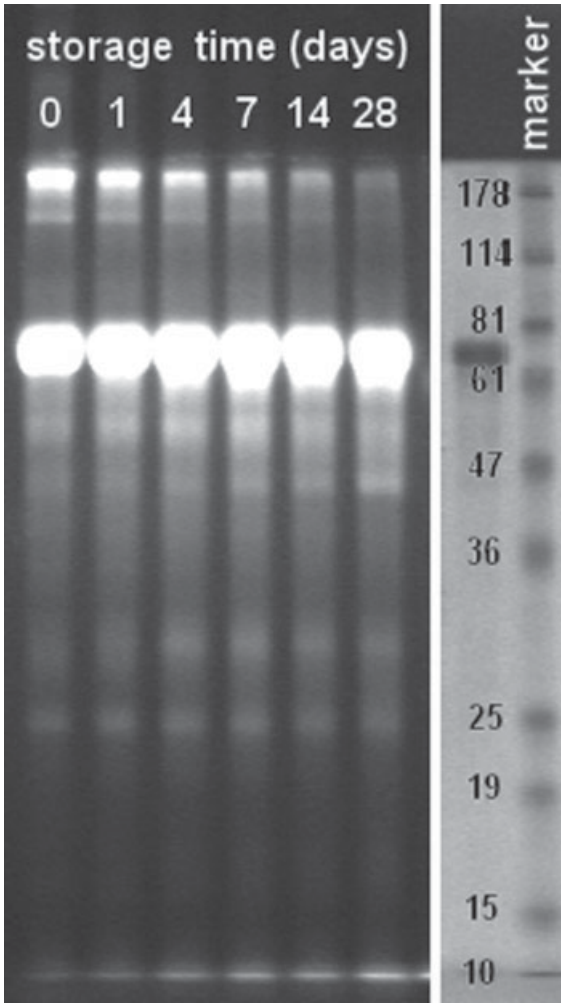

Fig. 6: Impact of storage time at 37 °C on FITC-BSA standards (SDS-PAGE)

To determine the molecular weights of the minor bands visible at the SDS-PAGE, we used the mass spectrometry. Since BSA with a molecular weight of 66430 Da (Hirayama et al. 1990) was labelled tenfold with FITC (MW 389 Da), a molecular weight of 70.31 kDa was expected for FITC-BSA. As can be seen from Fig. 8, the main peak could be found in good agreement at m/z = 70370. The double charged species was visible at m/z = 35352 and superimposed the ~37 kDa fragment visible in SEC. The

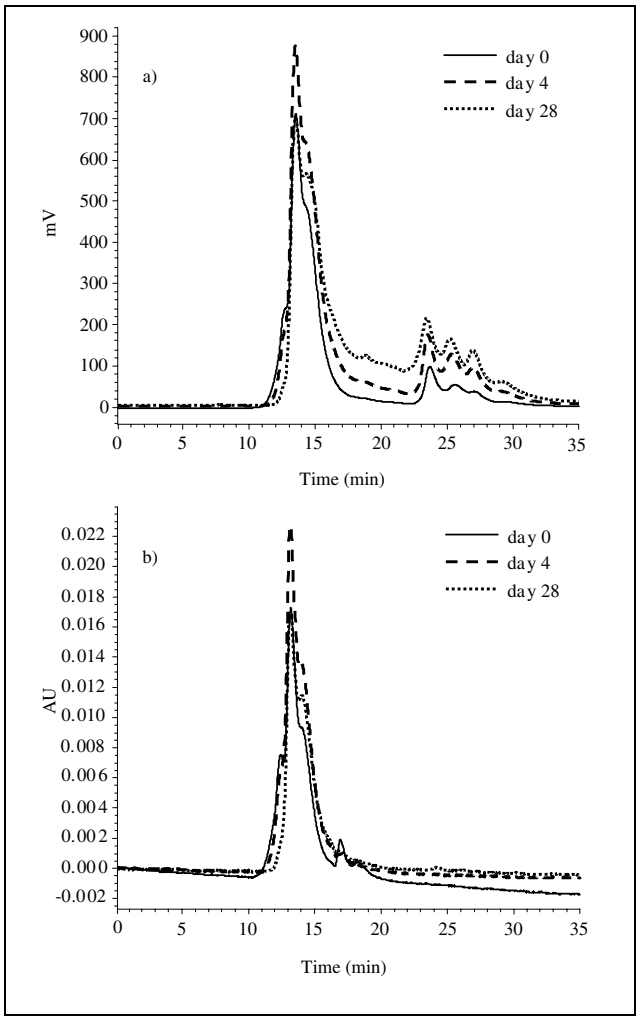

Fig. 7: Size exclusion chromatography of FITC-BSA standards that were stored at 37 °C for 0, 4, or 28 days. (a) Fluorescence emission at 525 nm, (b) UV absorption at 280 nm

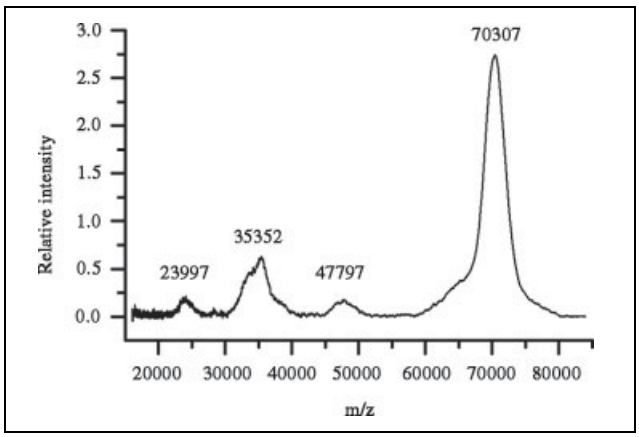

Fig. 8: Mass spectrum of FITC-BSA

triple charged protein (z = 3) was visible at m/z = 23997. One additional fragment could be found at m/z = 47797. It is assumed that this fragment appears from limited proteolysis of BSA as described at low pH by Wilson and Forster (1971), which resulted in a single 46 kDa fragment from the N terminus of BSA and two fragments of 21 and 24 kDa from the C terminus. In the case of FITC-BSA, the N terminal fragment shifted to a higher m/z due to the coupled FITC and the two C terminal fragments were most probably superimposed by the FITC-BSA fragment with z = 3. However, the intensity of the bands from these fragments was not changed during incubation at 37 °C as shown in Fig. 6. Therefore they should not be responsible for the increase in fluorescence intensity.

Overall our results implicate, that during the incubation of FITC-BSA at 37 °C, two main processes happened. On the one hand, FITC-BSA dimers dissociated and more monomers appeared. One the other hand, FITC-BSA was degraded to a minimum extent. However, the resulting fragments with a molecular weight below 5 kDa showed a very high fluorescence intensity. We assumed, that the protein hydrolysis caused a change in the environment of the fluorescein, allowing a more intense fluorescence emission.

2.4. Mechanism of dequenching

As already mentioned, the used FITC-BSA has a fluorophore/protein ratio (F/P) of 10. According to the information of the manufacturer the high F/P ratio should be advantageous, since this would increase the sensitivity of detection. However, data from the literature show the opposite. Labelling of the Fab fragments of antibodies with an increasing amount of FITC resulted in a decrease of the relative fluorescence of each fluorophore. Moreover the absolute fluorescence of the whole protein showed a maximum at F/P = 4 and decreased at higher F/P ratios (Der-Balian et al. 1988).

In a multiple-labelled protein, the FITC molecules are bound next to each other at the surface of the protein. FITC molecules can now associate and form non fluorescent dimers, which can be classified as a special case of concentration quenching (Förster and König 1957; Chen and Knutson 1988). Beside this, resonance energy transfer (RET) occurs from the excited state of a fluorescent FITC monomer to a non fluorescent dimer, resulting in an additional loss of fluorescence intensity of multiple-FITC-labelled proteins. The so-called Förster distance, describing the distance at which RET is 50% efficient (Lakowicz 1999), is known to be in the range of 20 to 42 Å for fluorescein (Kawski 1983). Since BSA has a heart-shaped structure of about 45 × 60 Å at neutral pH (Carter and Ho 1994), RET can easily happen. Moreover the energy homotransfer is very effective in FITC-labelled proteins, because fluorescein displays a small Stokes' shift, i.e. small wavelength difference between the maximum of absorption and emission (Lakowicz 1999). In consequence, a multiple-FITC-labelled protein shows high fluorescence quenching.

Concerning this, the increase in the fluorescence intensity of FITC-BSA after incubation at 37 °C can be explained by fluorescence dequenching. Since FITC-BSA is a highly quenched protein, hydrolysis of only a few molecules will result in a noticeable increase in fluorescence intensity (Fig. 9). This can explain our findings in SEC (Fig. 7), where a clear fluorescence peak could be detected for the completely dequenched fragments <5 kDa, while the protein concentration was below the detection limit. For proof of concept, we performed protein hydrolysis by adding trypsin or proteinase K to a solution of FITC-BSA at 37 °C. As presented in Fig. 10, this treatment resulted in an up to 70 fold increased fluorescence intensity.

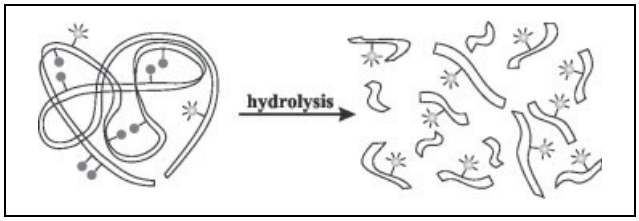

Fig. 9: Scheme of fluorescence dequenching following protein hydrolysis

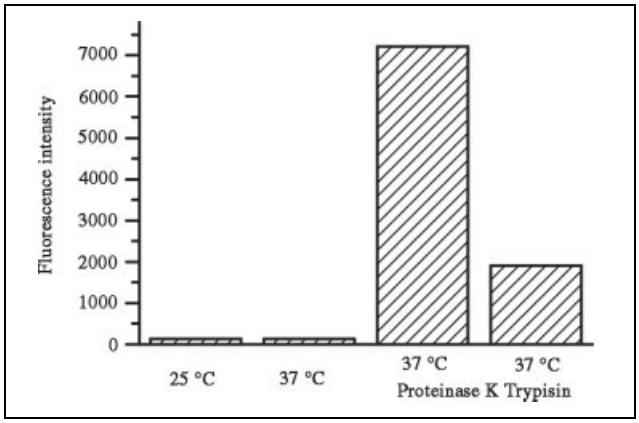

Fig. 10: Fluorescence intensity of FITC-BSA after 10 min incubation in 10 mM Tris buffer pH 7.0 without and with proteolytic enzymes

In conclusion, fluorimetric quantification of FITC-BSA and other multiple FITC-labelled proteins should only be performed, when dequenching effects can be excluded. The use of other fluorescent dyes with less self-quenching phenomena or a carefully labelling of proteins with only one or two FITC molecules might be a way to avoid the problems described herein.

3. Experimental

3.1. Materials

Poly(D,L-lactic-co-glycolic acid), Resomer® RG 502H, was a gift from Boehringer Ingelheim (Ingelheim, Germany). As a model protein we encapsulated bovine serum albumin, bearing fluorescein isothiocyanate groups (FITC-BSA, fluorophor/protein ratio = 10/1), which was purchased from Sigma (Taufkirchen, Germany). Solutions of 10 mg/ml FITC-BSA were prepared in 10 mM Tris buffer pH 7 and diluted, if necessary. All other chemicals were of analytical grade.

3.2. Microparticle preparation

Microparticles were prepared in a w/o/w double emulsion solvent evaporation procedure as described elsewhere (Wischke et al. 2006). Briefly, primary w_1/o emulsification of the FITC-BSA solution in a 5% solution of PLGA in dichloromethane was performed using either an Ultra-Turrax® or ultrasound. Emulsification of the w_1/o emulsion in a 0.25% solution of polyvinyl alcohol (w_2 phase) to form a w_1/o/w_2 emulsion was performed using a static micromixer (Institut für Mikrotechnik, Mainz, Germany). Following solvent evaporation, microparticles were lyophilized.

3.3. Protein distribution

The protein distribution inside the microparticles was detected by confocal laser scanning microscopy (LSM 510 meta, Axiovert 100, Zeiss, Germany).

3.4. Cell studies

Monocytes were isolated from human blood by isopycnic separation on Ficoll™ (Amersham Biosciences, Uppsala, Sweden). Dendritic cells were derived from monocytes as described elsewhere (Wischke et al. 2006).

Lyophilized microparticles were resuspended and 50 µg FITC-labelled particles were added per ml cell suspension (10^6 cells/ml). After 20 h, fluorescence spectroscopy and flow cytometry analysis were performed.

3.5. Release study and protein quantification

Lyophilized microparticles (10 mg) were suspended in 1.5 ml 10 mM Tris buffer pH 7.0, incubated at 37 °C and the supernatant was collected at certain times and replaced by fresh media (Wischke and Borchert 2006). Protein concentration was determined by (i) fluorescence spectroscopy (excitation 485 nm, emission 535 nm, LS 50 spectrometer, Perkin Elmer, Milano, Italy), (ii) a slightly modified silver-dithizone protein assay (Boratynski 1985), or (iii) an enzyme linked immunosorbent assay (ELISA) using an enzyme labelled albumin antibody (R1048HRP, Acris Antibodies, Hiddenhausen, Germany) and liquid gelatin as blocking solution (Serva Electrophoresis, Heidelberg, Germany) as described elsewhere (Wischke and Borchert 2006).

3.6. Analysis of FITC-BSA standards

Standard solutions of FITC-BSA were prepared in 10 mM Tris buffer pH 7.0 and incubated at 37 °C for up to 56 days. After that, samples were frozen. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% Tris-SDS gels using Tris-Glycin-SDS running buffer in a vertical Gibco V15-17 apparatus (Grand Island, New York, USA) and a 4X β-mercaptoethanol sample buffer. Size exclusion chromatography (SEC) was performed on a SynChropak 300 A column with a mobile phase of 0.05 M Tris/HCl buffer pH 7.5 containing 0.25 M NaCl and a flow rate of 0.2 ml/min on a Waters HPLC system. Mass spectrometry (MS) was performed on a PBS II SELDI-TOF MS (Ciphergen Biosystems Ltd., Guildford, UK) using a reverse phase chip and sinapinic acid for sample preparation.

3.7. Proteolysis

Proteolysis of 12.5 µg/ml FITC-BSA in 10 mM Tris buffer pH 7 was performed at 37 °C for 10 min using either 250 µg/ml proteinase K or 25 µg/ml trypsin. Following incubation, the fluorescence intensity of the samples was measured immediately.

Acknowledgements: We are grateful to Mrs. Fuhrman-Selter, Mrs. Hurtienne, Mrs. Juhls, Mr. Brosig, Mr. Lorenzen, Mr. Mothes, Mr. Oksche, and Mr. Hermann for support in recording spectral data.

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