Journal of Chromatography B, 878 (2010) 1125–1134

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Capillary electrophoresis separation and matrix-assisted laser desorption/ionization mass spectrometry characterization of bovine serum albumin–fluorescein isothiocyanate conjugates

Johan Jacksén[a], Kenneth Dahl[a], Ann-Therese Karlberg[b], Theres Redeby[b], Åsa Emmer[a,*]

[a] Division of Analytical Chemistry, Department of Chemistry, School of Chemical Science and Engineering, Royal Institute of Technology (KTH), Teknikringen 36, SE-100 44 Stockholm, Sweden
[b] Dermatochemistry and Skin Allergy, Department of Chemistry, University of Gothenburg, SE-412 96 Gothenburg, Sweden

ARTICLE INFO

Article history:
Received 12 November 2009
Accepted 12 March 2010
Available online 19 March 2010

Keywords:
Capillary electrophoresis
MALDI-TOF-MS
Contact allergy
Peptide-fluorescein isothiocyanate adducts
Bovine serum albumin

ABSTRACT

A protocol using enzymatic digestion, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and capillary electrophoresis with laser induced fluorescence detection (CE-LIF) for the investigation of the binding of the fluorescent contact allergen fluorescein isothiocyanate (FITC) to the 66 kDa large protein bovine serum albumin (BSA), as a model system for protein–hapten binding in the skin, is presented. Mass spectra of BSA–FITC digestions, using trypsin and chymotrypsin, respectively, provided sequence coverage of 97%. To investigate the number of FITC-bound peptides using CE-LIF separation, three different buffer salts at four different pH levels were evaluated. The use of 20 mM sodium citrate pH 6.5 as well as 20 mM sodium phosphate pH 6.5 or pH 7.5 as background electrolyte revealed high numbers of peptides with at least one bound FITC. The effect of the electrolyte counter ion on MALDI-MS was investigated and was found to have effect on the MALDI spectra signal-to-noise (S/N) at 50 mM but not at 10 mM. Of the 60 theoretical FITC-binding sites in BSA this MALDI-MS protocol presents 30 defined, 28 possible and 2 non-binding sites for FITC.

1. Introduction

Studies of interactions between small organic molecules and proteins are interesting in several aspects. Labeling of proteins with fluorescent dyes are used in cell biology research [1] as well as for sensitive detection associated with separation techniques [2]. Further, knowledge about protein conjugation with small molecules is central in drug development [3,4] but also when investigating processes leading to contact allergy [5], which is the area of interest behind the present study. Contact allergy is caused by skin contact with low molecular weight chemicals (called haptens in this context), which penetrate the skin and either directly or after metabolic modification bind to macromolecules such as proteins in the skin [6]. The protein–hapten complexes are then further processed into antigens, resulting in antigen-specific T-cell formation. When entered into the circulation these T-cells constitute a permanent biochemical memory and a skin inflammation (eczema) will result at re-exposure to the same hapten. Due to the scarce knowledge about what protein(s) are involved in the hapten binding relevant for antigen formation, studies of protein–hapten interactions are currently limited to model proteins like serum albumin [7], cytokeratin or cofilin [8].

Fluorescein isothiocyanate (FITC) is a potent skin sensitizer [9]. Furthermore, FITC is extensively used for fluorescence labeling of antibodies and other proteins for optical tracking [10]. The binding sites for FITC are primary amino groups of the amino acid lysine and the protein N-terminal [10,11]. That is, the binding takes place *via* a nucleophilic attack by the amino groups at lysine or the N-terminal. Thus, for a protein with n lysine residues, the number of potential protein-FITC adducts will be $2^{n+1} - 1$. This results in an immense number of combinations when the number of binding sites is high, *e.g.* in bovine serum albumin (BSA) where $n = 59$, there are 1.15×10^{18} possible versions of BSA–FITC adducts. However, the true number of adducts formed between a specific protein–hapten pair may be reduced and even controlled in *in vitro* experiments through reaction parameters such as protein:hapten molar ratio, pH, reaction time and temperature [11–16]. Furthermore, at *in vivo* exposure of skin to FITC all theoretical binding sites are not available for binding due to protein location, three dimensional structure and binding site microenvironment. In addition to this, other biological factors such as metabolism can influence the results. Which of these still vastly numerous possible binding sites trigger sensitization is at present not known. In pursue for this information, characterization of defined model substances

* Corresponding author. Tel.: +46 8 7906407; fax: +46 8 108425.
E-mail address: aae@kth.se (Å. Emmer).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.03.024

and the development of appropriate analytical tools are necessary.

Serum albumin is commercially available at low cost, is water soluble and has several important biological functions. It is also well studied and used as a model protein for a diversity of purposes including protein–hapten conjugate studies [17,18]. Consequently, FITC-bound serum albumin is a commonly used fluorescent marker and can be bought already labeled. The average degree of substitution has been determined, *e.g.* by absorbance and fluorescence spectroscopy [12,19,20], gel electrophoresis [19,20], size exclusion and ion exchange chromatography [12,19,20] or capillary isotachophoresis [21]. However, despite the frequent use of BSA–FITC, the actual FITC-binding sites have only been investigated to a very limited extent [20] since this task is complicated by the many potential binding sites. As serum albumin is the most abundant protein in skin and FITC is a known skin sensitizer, the conjugation between FITC and serum albumin can be studied as a model system for protein–hapten binding in skin.

Mass spectrometry has earlier been used for investigations of protein–hapten adducts. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and electrospray ionization (ESI) mass spectrometry has facilitated the identification of binding targets for different hapten–protein combinations [5,8]. In ESI multiply charged ions are frequently occurring complicating the spectra, though. For complex samples, such as the ones expected subsequent to protein–hapten binding, hyphenation to, *e.g.* nano-LC is necessary.

In the present work, we have developed a protocol using enzymatic digestion, MALDI-TOF-MS and capillary electrophoresis with laser induced fluorescence detection (CE-LIF) for the investigation of BSA–FITC conjugates. An additional aim has been to optimize both analytical techniques for future CE-MALDI off-line coupling [22].

2. Experimental

2.1. Chemicals and materials

Bovine serum albumin, fluorescein isothiocyanate labeled BSA, trypsin, chymotrypsin, the MALDI calibration peptides: substance P, insulin chain B oxidized (from bovine pancreas), insulin from bovine pancreas, leucine enkephalin and Tyr-bradykinin (for investigation of buffer influence on MALDI signals), or bradykinin fragment 1–7, angiotensin II, ACTH (adrenocorticotropic hormone) fragment 18–39 and insulin (for all other MALDI-MS analyses) as well as dithiothreitol (DTT), iodoacetamide (IAA), trifluoro acetic acid (TFA), acetonitrile (ACN), hydrochloric acid, sodium hydroxide, ammonium hydrocarbonate (NH_4HCO_3), phosphoric acid, disodium phosphate, sodium dihydrogen phosphate, citric acid, sodium citrate, acetic acid, sodium acetate, ammonium phosphate and potassium phosphate were obtained from Sigma–Aldrich (Stockholm, Sweden). Mesityloxide was purchased from VWR (Stockholm, Sweden). The matrix 2,5-dihydroxy benzoic acid (DHB) was obtained from Bruker Daltonics (Bremen, Germany). The MALDI plate used for evaluating the buffer counter ion effect on the MALDI signals was a teflon concentration plate from Bruker Daltonics (MTP AnchorChip var/384). A custom made prestructured MALDI plate [22,23] was used for all other MALDI-MS analyses. ZipTip^{TM} C18 and the Synergy 185 system for purification of the water used were from Millipore (Bedford, USA).

2.2. Instrumentation

A Bruker Reflex III MALDI-TOF instrument with a SCOUT 384 ion source in reflector mode and control software from Bruker Daltonics was utilized. A total of 200 shots were collected with a laser intensity of about 60%. Internal calibration was performed with a method optimized for m/z 1637 resulting in a mass accuracy of 6 ppm (monoisotopic mass) for bradykinin 1–7 (m/z 757), 64 ppm (monoisotopic mass) for angiotensin II (m/z 1047), 26 ppm (average mass) for ACTH fragment 18–39 (m/z 2465) and 241 ppm (average mass) for insulin (m/z 5736), and a resolution (FWHM, full width at half maximum) of 4100 for bradykinin 1–7 and angiotensin II, 9500 for ACTH fragment 18–39 and 1200 for insulin. The software XTOF (Bruker) was utilized to generate the signal-to-noise (S/N) values. For peptide identification the proteomics software tool PeptideMass at ExPaSy Proteomics Server (http://www.expasy.ch/) was utilized for generation of peptide lists. Five missed cleavages were allowed. The peptide lists were manually compared to detected MALDI-MS peaks with a mass difference tolerance of ±1 Da. The capillary electrophoresis instrument (P/ACE System 5500, Beckman Instruments, Fullerton, CA, USA) was connected to a LIF detector (Beckman P/ACE System laser Module, argon, 3 mW). Wavelengths used were 488 nm (excitation) and 520 nm (fluorescence). The 50/375 μm (id/od) silica capillaries (Composite Metal Service Ltd., Ilkley, UK) had an effective length of 120.4 cm and a total length of 127 cm. Capillaries were rinsed (140 kPa) with 1 M sodium hydroxide, water and running buffer (30 min each) before use. Between runs, the buffer was exchanged and the capillary rinsed for 5 min. When changing the type of buffer and at the beginning of each day, the rinse was extended to 30 min. Electrophoresis was performed in ambient temperature in positive mode using a voltage of +25 kV and the samples were injected applying +10 kV during 5 s. Peak picking was performed using Beckman P/ACE station version 1.0 with integration parameters as follows: width 2 (defines the width of the narrowest peaks registered), threshold 15,000 (determines the peak start and stop, and is defined as a change in the signal voltage per unit time) and shoulder sensitivity 500 (enables detection of shoulders on larger peaks).

For determinations of electroosmotic mobility a custom-built capillary electrophoresis equipment was used, comprising a Plexiglas box, a 30 kV high voltage supply constructed from a Spellman CZE 100 (Plainview, NY, USA), a UVIS 2000 UV-detector from LINEAR (Reno, NV, USA) and a compressed air system (140 kPa) for rinsing of the capillary. The mobility of mesityloxide was monitored at 254 nm, using the software program EZChrome 6.8 (Scientific Software, San Ramon, CA, USA). The same type of fused silica capillaries (total length 60 cm, effective length 40 cm) and the rinsing procedures described above were utilized. Electrophoresis was performed in ambient temperature in positive mode using a voltage of +15 kV and the samples were injected applying +15 kV during 5 s.

2.3. Digestion and sample preparation

Protein samples of 100 μl each with a concentration of 1 mg/ml BSA or BSA–FITC in 50 mM NH_4HCO_3 were reduced by adding 5 μl 200 mM DTT in 100 mM NH_4HCO_3 and were left at room temperature for 1 h. Free thiol groups were alkylated by adding 4 μl 1 M IAA in 100 mM NH_4HCO_3 and left at room temperature for 1 h. The alkylation was stopped by adding 20 μl 200 mM DTT in 100 mM NH_4HCO_3 and left for one hour at room temperature. To dilute the DTT concentration to compatibility with the enzyme, 175 μl 100 mM NH_4HCO_3 were added to each vial. The digestions were performed based on the instructions from the manufacturer, with some modifications. Specifically, a stock solution of 12.5 mg/ml trypsin in 50 mM NH_4HCO_3 pH 8 was added to each sample to obtain an enzyme:protein ratio of 1:20 (w/w) whereupon the samples were kept at 37 °C in the dark for 24 h. For digestion with chymotrypsin, the enzyme stock solution was prepared at 1 mg/ml in cold 1 mM hydrochloric acid and added immediately to the samples to obtain an enzyme:protein ratio of 1:60 (w/w) whereupon

the samples were kept at room temperature in the dark for 24 h. Digested samples were stored at −20 °C and those containing FITC were kept in the dark by wrapping the sample vial in aluminum foil during handling and storage. Prior to MALDI-TOF-MS analysis, samples were desalted and concentrated by ZipTip™ C18. In short, rinsing was performed three times each with 10 μl acetonitrile (ACN), 10 μl 0.1% trifluoro acetic acid (TFA) and the sample solution containing 10 μl sample and 1 μl 5% TFA. The sample was eluted with MALDI matrix dissolved in 1:1 ACN:0.1% TFA and transferred to the MALDI plate. During the counter ion study the elution from the ZipTip™ were performed into 1:1 ACN:0.1% TFA solution. The matrix DHB was prepared separately as 20 mg/ml in 1:1 ACN:0.1% TFA. The sample and the matrix were applied to the MALDI plate in a 1:1 ratio using a dried droplet procedure.

For evaluating the buffer counter ion effect on the MALDI signals, a 0.5 μl trypsin digested BSA–FITC sample eluted from ZipTip™ C18 was mixed with 0.5 μl of the DHB matrix solution and 0.5 μl solutions of 10 or 50 mM, ammonium, potassium or sodium phosphate, all adjusted with phosphoric acid to a similar pH (pH 3.4). The number of sample spots was 20 for 10 mM solution and 16 for 50 mM solution for each salt. The same procedure was used to compare sodium phosphate and sodium acetate.

2.4. Background electrolyte preparation

The concentration of all CE background electrolytes (BGEs) was 20 mM with a pH of 4.5, 5.5, 6.5 or 7.5. Acetate, citrate and phosphate were used as buffer ions, all with sodium as counter ion. Stock solutions of the buffer salts and their corresponding acids were prepared at a concentration of 200 mM each. The BGEs were prepared from the stock solutions and water to acquire the final pH values and buffer ion concentrations. At the same concentration the ionic strength will be higher for citrate and phosphate than for the monovalent acetate.

3. Results and discussion

3.1. MALDI-TOF-MS analysis

Initially, intact (not hydrolyzed) BSA and BSA–FITC were analyzed by MALDI-MS (data not shown). A comparison between the molecular weight of the labeled and non-labeled BSA suggested an approximate binding ratio of 2–3 FITC molecules per BSA molecule. However, later in this work it is shown that individual peptides from enzymatic digestions of BSA–FITC were found binding more than three FITC molecules, that is, there are some BSA–FITC molecules with higher conjugation ratios. According to the manufacturer's specifications, the BSA–FITC conjugate has a degree of substitution of 7–12 moles of FITC per mole albumin measured using absorbance spectroscopy. This kind of discrepancies between methods has been observed earlier [17] and probably depends on that MALDI only detects the covalent conjugates in contrast to other methods that may additionally detect non-covalent complexes. On the other hand, the establishment of the centroid peak in the MALDI analysis is obstructed by the broad peaks obtained, which influence the precision of the ratio determination. When comparing the MALDI spectra from BSA and BSA–FITC we experienced that conjugation of FITC to BSA impairs the sensitivity. This may be due to that the ionization efficiency is decreased when the free and most easily protonated lysine residues are exchanged for FITC entities and thereby blocked [11,24].

Although this type of analysis reveals the presence of conjugation between a hapten and a protein, information of the specific binding sites is not accessible. Therefore, to gather more details about both which lysine residues in the BSA molecule that undergo labeling and the diversity of the formed conjugate molecules, enzymatic proteolysis was conducted in order to divide the large BSA molecule into smaller peptides where individual lysine residues could more easily be probed. Separate digestions of BSA–FITC were performed with trypsin or chymotrypsin as cleaving agents. To identify where in the BSA sequence the incorporation of FITC had taken place, the peptide samples resulting from these digestions were analyzed by MALDI-TOF-MS. A typical mass spectrum is shown in Fig. 1. The observed peaks were identified through matching with theoretical peptide m/z values for up to five missed cleavage positions. This provided a sequence coverage of 97% on an amino acid basis when combining data from both the tryptic digest (TD) and the chymotryptic digest (CTD) including peptides both with and without any attached FITC units. Based on number of detected amino acids the sequence coverage was 91% for TD solely and 66% for CTD, while based on detected peptides 92% for TD and 70% for CTD. This means that a major part of the protein is covered and that it is possible to get a broad representation of the binding of FITC to BSA.

Detected peptides corresponding to theoretical m/z values where one or more FITC are bound are illustrated in Fig. 2 for the tryptic digestion and in Fig. 3 for the chymotryptic digestion. Out of the 60 theoretical binding sites in BSA, 44 possibly occupied binding sites were identified through the tryptic peptides (Fig. 2). Twenty four of these 44 were unambiguously found to be bound to one or several FITC molecules. For example, in the sequence of peptide t2–3 (Fig. 2) there is only one lysine (K^{12}) and the m/z value of the detected peptide corresponds to the theoretical value of t2–3 plus one bound FITC. It can therefore be concluded that FITC does bind to K^{12}. Such defined FITC-binding positions are lysine number 12, 20, 51, 106, 159, 173, 180, 187, 204, 211, 221, 224, 239, 280, 285, 350, 362, 388, 431, 439, 465, 499, 523 and 563. Complementary FITC-binding data is acquired through the chymotrypsin peptides, where data show 32 possibly occupied binding sites for FITC (Fig. 3). Here, 6 additionally defined FITC-bound lysine residues were iden-

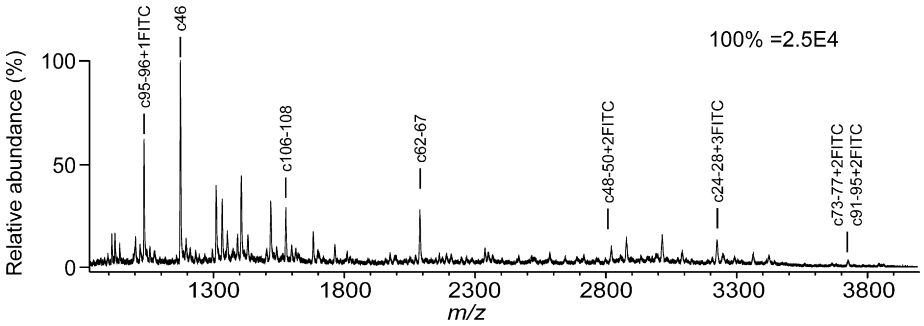

Fig. 1. MALDI mass spectrum of chymotrypsin digested BSA–FITC. Examples of detected peptides with 0 to 3 FITC entities are indicated (see Fig. 3 and Tables 2 and 4 for more details on the peptides). The peptides are designated with c for chymotrypsin and the sequential number. The peak at m/z 3732 could represent either of two peptides (Table 4).

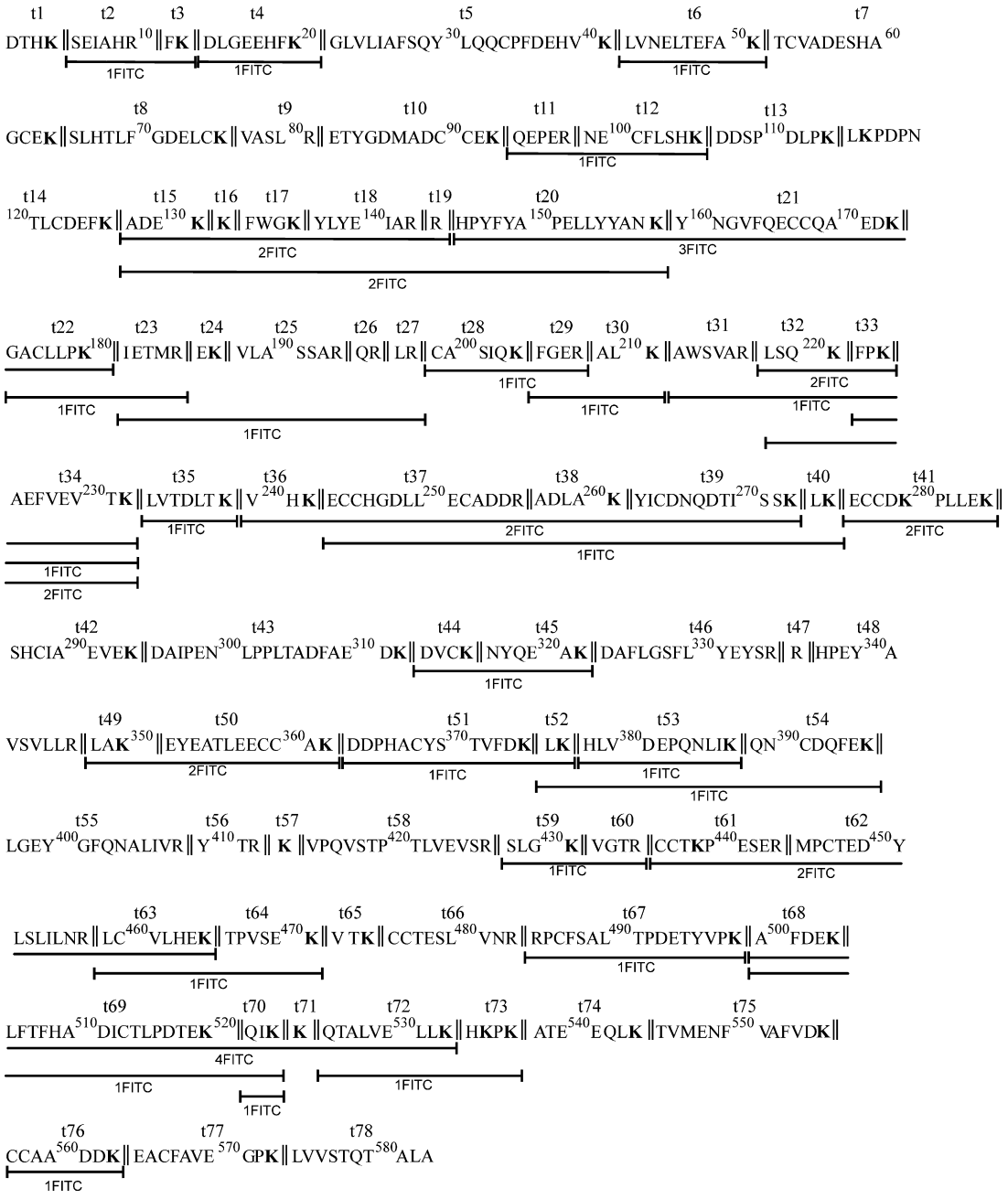

Fig. 2. A schematic representation of the BSA amino acid sequence with the peptides obtained from a tryptic BSA–FITC digest indicated. Possible cleavage sites (after arginine, R, and lysine, K) are marked with double vertical lines. Lysines are in bold. For clarity amino acid numbering is included for every 10th amino acid. Each peptide is labeled with *t* for tryptic digest and the sequential number. Peptides underlined are identified as containing FITC using MALDI-MS. The number of FITC incorporated is given below the corresponding peptide.

tified, at position 41, 93, 136, 242, 322 and 504 (Fig. 3), resulting in a total of 30 defined binding sites combining TD and CTD data. However, FITC is not necessarily conjugated to these sites in every single BSA–FITC molecule.

The identified tryptic and chymotryptic peptides without any bound FITC are listed in Table 1 and Table 2, respectively. These are peptides that include potential FITC-binding sites but which are vacant from FITC in some of the protein molecules in the BSA–FITC population.

An example, illustrating that peptides containing potential binding sites were detected both including conjugated FITC (Figs. 2 and 3) and lacking FITC (Tables 1 and 2), is peptide t1–3 (Fig. 2). It can be seen in Table 1 that the above mentioned defined binding site K^{12} within this peptide has also been detected without bound FITC in some part of the BSA–FITC population. The data presented in Tables 1 and 2 together with Figs. 2 and 3 shows that this is the case for all of the 30 defined FITC-binding sites. That is, all lysine residues identified as actually binding FITC (Figs. 2 and 3) have also been detected without FITC (Tables 1 and 2). This implies that the substitution is far from complete and that there is a high probability for a vast heterogeneity among the BSA–FITC molecules. The fact that many different binding sites have been defined (Figs. 2 and 3), while the average binding ratio was low, supports this conclusion.

Comparing results obtained using trypsin in contrast to using chymotrypsin as digestion enzyme, indicates that the trypsin method is more informative. A plausible explanation to this is the higher specificity [25] of trypsin facilitating identification of enzyme specific peptides.

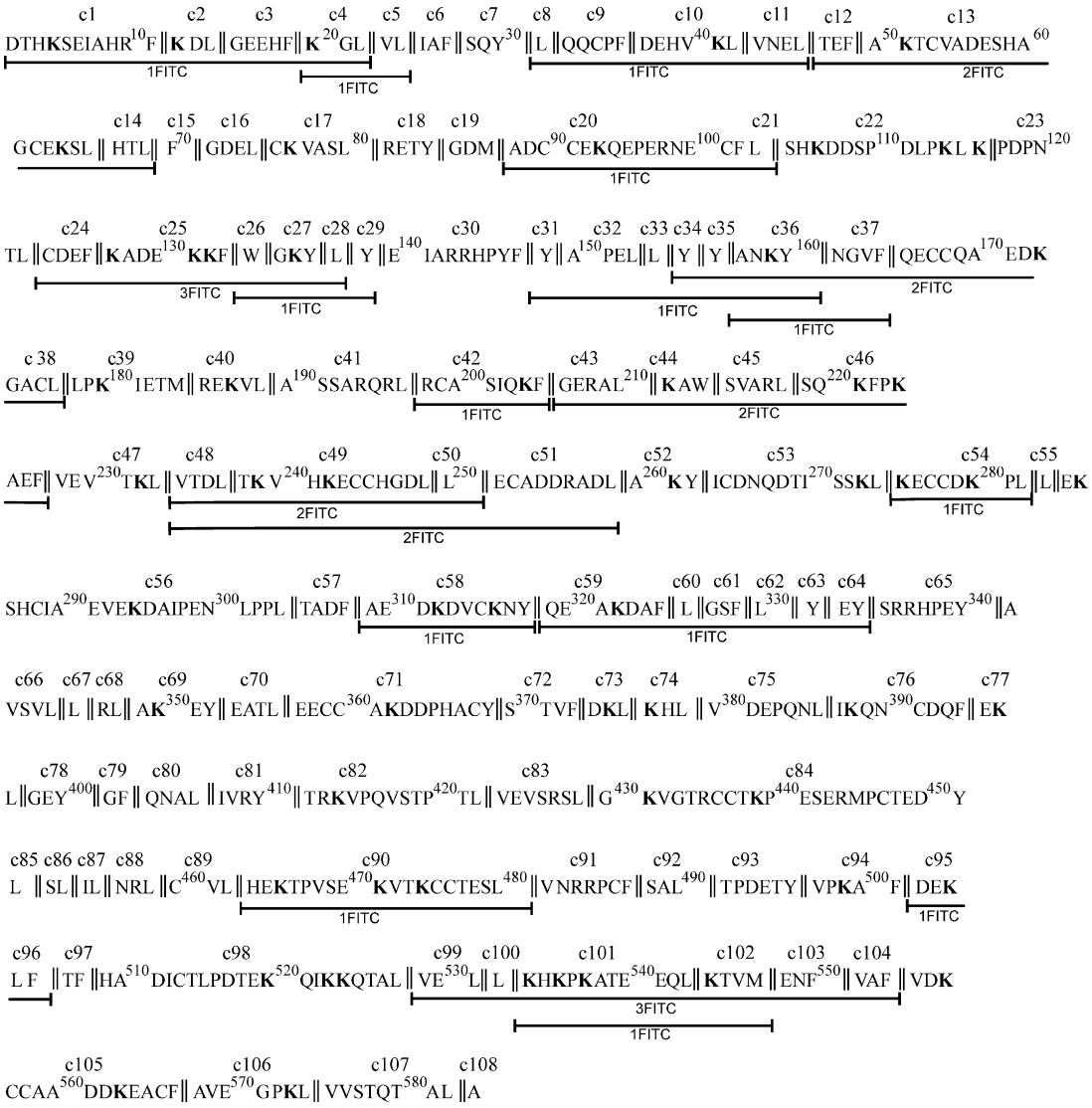

Fig. 3. A schematic representation of the BSA amino acid sequence with the peptides obtained from a chymotryptic BSA–FITC digest indicated. Possible cleavage sites (carboxylic side of leucine, L; methionine, M; phenylalanine, F; tryptophan, W; tyrosine, Y) are marked with double vertical lines. Lysines are in bold. For clarity amino acid numbering is included for every 10th amino acid. Each peptide is labeled with c for chymotryptic digest and the sequential number. Peptides underlined are identified as containing FITC using MALDI-MS. The number of FITC incorporated is given below the corresponding peptide.

In addition to the 30 identified binding sites from the combined data from the trypsin and chymotrypsin digests (Figs. 2 and 3), there are detected peptides which correspond to two or more theoretical *m*/*z* values (Tables 3 and 4). From the collected data it can be concluded that there are only two sites where no FITC binding was detected at all: 76 and 114. Despite the high sequence coverage there is also one site (116) which has not been detected either with or without bound FITC. Consequently, nothing is known about the binding to this specific site but it is still possible that this could be an additional binding site. To sum up, there are 28 lysines where binding is possible but remains uncertain.

Altogether, the data presented here show 2 non-binding, 28 possible and 30 defined binding sites. In relation to the estimated binding ratio of 2–3 FITC/BSA mentioned above or even the value of 7–12 FITC/BSA given by the manufacturer these data strengthen the assumption that the conjugation is rather deficient and the heterogeneity is very high. Similar phenomena have earlier been observed with different techniques by a number of research groups for BSA–FITC [19], FITC conjugated to other proteins [13,14] and other haptens conjugated to BSA [26].

Much work remains before correlations between specific protein–hapten configurations and allergy processes can be evaluated. Nonetheless, from the presented data it can be concluded that there is a low degree of discrimination in where FITC binds to a large protein like albumin when the reaction has been performed *in vitro*. Specific binding sites that are more prone to be occupied by a FITC entity than others can not be pinpointed. Even so, a more expressed predisposition for specific binding sites could be expected in albumin and other large proteins *in vivo*, where the number of exposed binding sites is limited by the native folding of the proteins. Although the true image of the protein–FITC adducts presented to the immune system obviously is highly complex, the presented results point to a significantly lower number of protein–FITC versions than theoretically possible.

3.2. CE-LIF separation

A CE-LIF method was developed and optimized for the separation of trypsin digested BSA–FITC to obtain further information of the extent of FITC conjugation to BSA. A future coupling of the CE method to MALDI-MS has also been regarded during this develop-

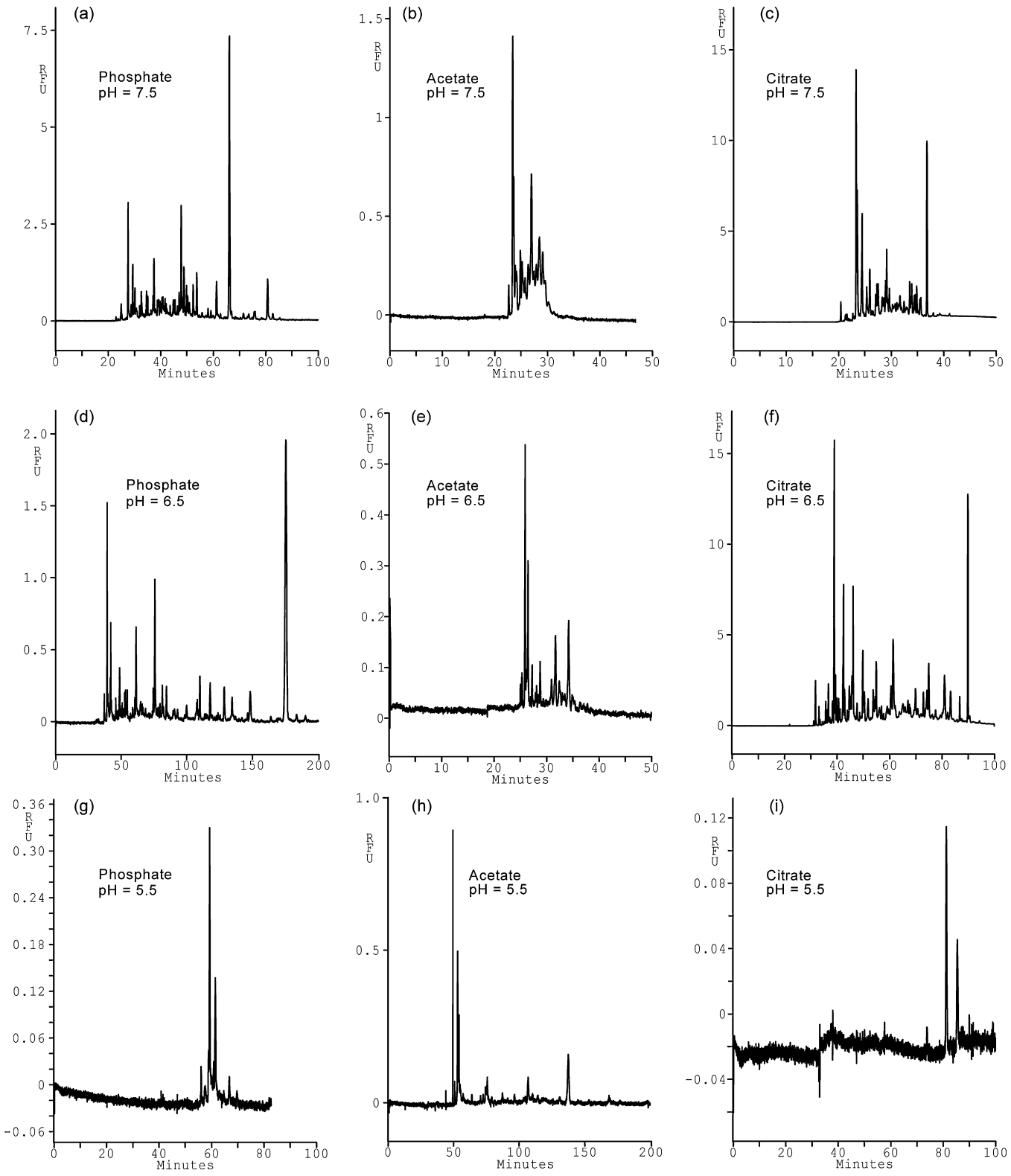

Fig. 4. CE-LIF separations of trypsin digested BSA–FITC using three sodium running buffers at 20 mM: (a, d and g) phosphate, (b, e and h) acetate, and (c, f and i) citrate, at three pH levels: (a–c) pH 7.5, (d–f) pH 6.5 and (g–i) 5.5. The voltage was +25 kV. The *y*-axis units are relative fluorescence units (RFU). The number of detected peaks were: (a) 103, (b) 17, (c)35, (d) 102, (e) 16, (f) 98, (g) 10, (h) 30 and (i) 6 determined using integration parameters as follows: width 2, threshold 15,000 and shoulder sensitivity 500.

ment. Thus, both the CE performance and the compatibility with MALDI-MS had to be taken into consideration during optimization. Laser induced fluorescence detection was chosen as this is both sensitive and selective for peptides with bound FITC. The composition of the BGE has a considerable impact on the CE performance both generally and for peptides specifically [27]. Factors such as buffer type, buffer concentration, ionic strength, buffer capacity and pH influence both selectivity and resolution. Considering peptides, buffer pH is particularly important for optimizing selectivity and avoiding analyte adsorption at the capillary inner wall although

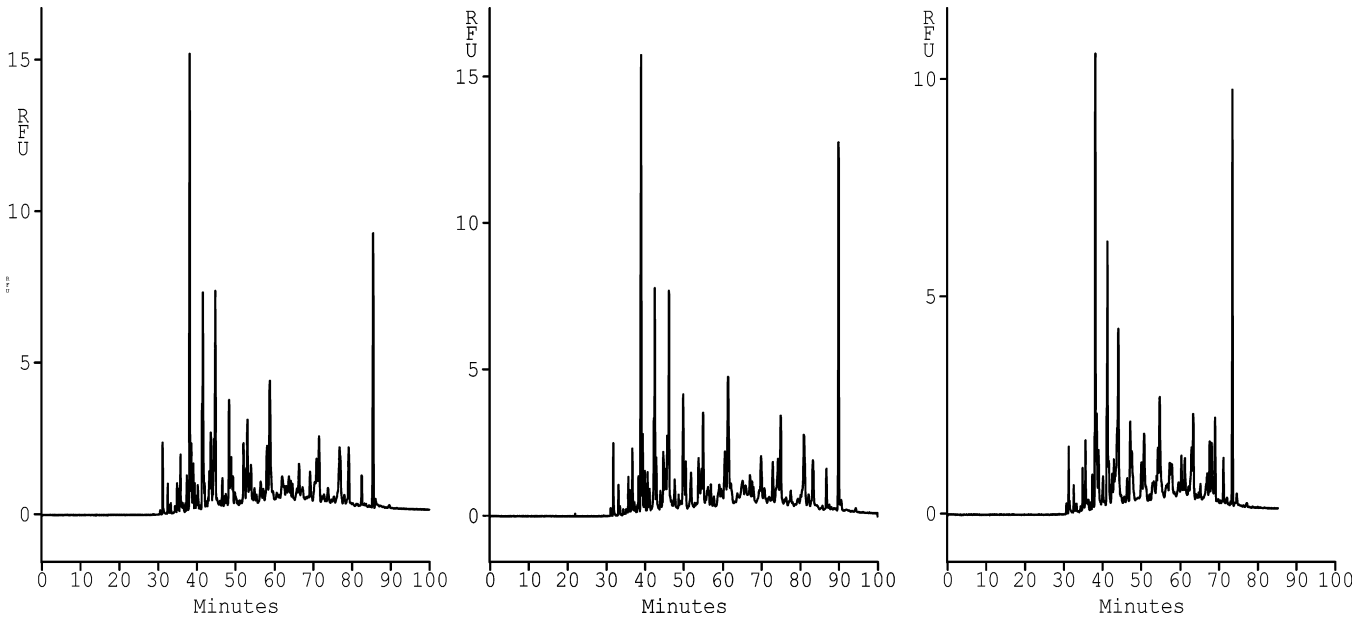

Fig. 5. Three consecutive CE separations of trypsin digested BSA–FITC using 20 mM citrate (pH 6.5) as BGE. The voltage was +25 kV, no temperature control was available. The *y*-axis units are relative fluorescence units (RFUs).

Table 1
Peptides from trypsin digestion of BSA–FITC detected using MALDI-TOF-MS.

Peptide	Theoretical *m*/*z*	Observed *m*/*z*
t1	500.2	500.1
t1–2	1193.6	1193.8
t1–3	1468.8	1468.4
t3–4	1249.6	1249.8
t5	2492.3	2491.4
t6	1163.6	1163.5
t8	1419.7	1419.8
t11–13	2541.2	2541.6
t17–18	1445.8	1445.1
t17–19	1601.9	1602.0
t18	927.5	927.9
t18–20	2953.5	2953.0
t22–26	2614.4	2613.6
t27–29	1464.8	1465.2
t27–30	1777.0	1777.1
t27–31	2447.3	2447.6
t29	508.3	508.0
t29–32	1947.1	1947.4
t30–31	1001.6	1001.4
t35–38	3382.6	3383.2
t36–38	2612.2	2612.3
t38–40	2183.1	2183.7
t42–46	5793.7	5793.3
t44–47	2959.4	2959.7
t46	1567.7	1568.2
t47–48	1439.8	1440.1
t48–49	1595.9	1596.7
t50–51	2867.4	2867.1
t56–57	567.3	567.3
t56–60	2858.6	2858.9
t57–58	1639.9	1640.3
t57–59	2025.2	2025.7
t57–60	2438.4	2438.6
t64–66	2108.0	2108.5
t65–66	1466.7	1466.2
t66	1138.5	1138.5
t73–74	1308.7	1309.5
t74–76	3019.4	3020.3
t76–77	1927.8	1927.9

Listed are *m*/*z* values corresponding within ±1 Da to peptides without any bound FITC.

other factors also influence the final separation [27,28]. Several BGE compositions were therefore evaluated in the present study. Sodium phosphate, acetate and citrate buffers at a concentration of 20 mM and four different pH levels were used (Table 5). As expected the electroosmotic mobility (μ_{EOF}) increased with increased pH for all three buffers. This will affect the total analysis time as will the choice of buffer. Resulting electropherograms at three pH values can be seen in Fig. 4. Some of these BGE have a rather low buffer capacity (below 5 mM/pH; acetate and citrate pH 6.5 and pH 7.5 and phosphate pH 4.5, and pH 5.5), which could impair CE performance and reproducibility [29]. This is due to the fact that pH changes can occur within the capillary affecting mobility and selectivity.

Table 2
Peptides from chymotrypsin digestion of BSA–FITC detected using MALDI-TOF-MS.

Peptide	Theoretical *m*/*z*	Observed *m*/*z*
c1	1339.9	1340.7
c2–4	1273.3	1272.7
c9–11	1855.4	1855.9
c10–12	1572.1	1572.8
c12–14	2592.2	2591.2
c13–14	2214.9	2214.0
c14	517.2	517.3
c19–20	2274.3	2274.9
c42	1008.7	1009.5
c46	1080.6	1081.6
c62–67	2095.7	2095.1
c64–65	1237.5	1236.6
c64–66	1705.7	1705.9
c65–66	1412.9	1413.8
c74–75	1192.0	1192.6
c75–79	2771.7	2771.3
c79–81	1180.1	1180.6
c91	948.6	948.5
c92–96	2170.2	2171.1
c94–98	3732.1	3733.0
c98–99	2651.5	2651.4
c101–103	2157.7	2158.1
c102	478.1	478.3
c105–108	3252.8	3253.6
c106–108	1583.3	1583.9
c107–108	890.5	889.5

Listed are *m*/*z* values corresponding within ±1 Da to peptides without any bound FITC.

Table 3
Peptides from trypsin digestion of BSA–FITC detected using MALDI-TOF-MS. Listed are observed *m*/*z* values corresponding within ±1 Da to several peptides with or without FITC.

Observed *m*/*z*	Peptide	Theoretical *m*/*z*	Number of bound FITC	Theoretical *m*/*z* with bound FITC
720.2	t49	331.2	1	720.6
	t30	331.2	1	720.6
817.8	t74	818.4	0	818.4
	t59–60	817.5	0	817.5
906.5	t23–24	906.5	0	906.5
	t38	517.3	1	906.7
	t70–71	516.4	1	905.8
1001.9	t30–31	1001.6	0	1001.6
	t78	1002.6	0	1002.6
1206.9	t74	818.4	1	1207.8
	t59–60	817.5	1	1206.9
1391.2	t30–31	1001.6	1	1391.0
	t78	1002.6	1	1392.0
1444.1	t16–17	665.4	2	1444.2
	t39	1443.6	0	1443.6
1479.5	t10	1478.5	0	1478.5
	t55	1479.8	0	1479.8
1512.0	t58	1511.8	0	1511.8
	t70–72	1512.0	0	1512.0
1555.1	t61	1166.5	1	1555.9
	t51	1554.7	0	1554.7
1868.9	t63–65	1868.0	0	1868.0
	t10	1478.5	1	1867.9
1897.4	t28–30	1507.8	1	1897.2
	t58–59	1897.1	0	1897.1
1922.3	t71–72	1142.7	2	1921.5
	t40–41	1532.8	1	1922.2
1946.9	t8–9	1946.0	0	1946.0
	t29–32	1947.1	0	1947.1
2060.7	t26–30	2061.1	0	2061.1
	t56–58	2060.1	0	2060.1
2073.5	t39–40	1684.8	1	2074.2
	t33–34	1294.7	2	2073.5
3589.7	t66–68	3590.7	0	3590.7
	t31–34	2421.3	3	3589.5
3816.7	t53–55	3815.9	0	3815.9
	t50–51	3038.2	2	3817.0
5734.7	t50–53	4566.1	3	5734.3
	t54–59	4955.6	2	5734.4
5928.5	t8–13	5927.7	0	5927.7
	t20–25	5928.9	0	5928.9
	t49–54	5927.8	0	5927.8

An increase in buffer capacity can be obtained by raising the concentration, albeit to the cost of high currents and long analysis times, which was the case when a concentration of 50 mM was used in the present investigation (data not shown). Nevertheless, reproducibility of the peak pattern was found to be satisfactory even for these BGE as can be seen, *e.g.* in Fig. 5, where citrate at pH 6.5 was used. The migration time reproducibility was 1% RSD (relative standard deviation) for an early peak and 10% RSD for a peak at the end of the electropherogram. The reason for this discrepancy is not known but the lack of temperature control in the system could be a contributing factor. Similar or slightly decreased reproducibility was obtained using acetate at pH 5.5 and phosphate at pH 6.5 both having higher buffer capacity.

Lower pH values (pH 4.5, data not shown, and pH 5.5, Fig. 4) were included in this study because the ionization in a future fraction collection for off-line coupling to MALDI-TOF-MS would benefit from an acidic background electrolyte. However, the fluorescence of FITC is highly pH dependent with a significant decrease in signal intensity at pH below 7 [30]. This can be seen in Fig. 4 comparing the sensitivity obtained using BGE of different pH. Therefore, it is more difficult to evaluate the separation performance at lower pH only using LIF detection.

The number of peaks obtained varied both with BGE anion and pH (Fig. 4). The highest number of peptides was detected using sodium phosphate at pH 7.5 (103 peaks) and pH 6.5 (102 peaks), and sodium citrate at pH 6.5 (98 peaks) as BGE. When choosing between these three options a lower pH may be advantageous considering coupling to MALDI-MS. Moreover, citrate is preferred referring to that this BGE offered a considerably shorter analysis time than phosphate at the same pH. It should be considered that the current is much higher using citrate (25 μA at pH 6.5 and +25 kV) than using phosphate (10 μA at pH 6.5 and +25 kV), though, which

Table 4
Peptides from chymotrypsin digestion of BSA–FITC detected using MALDI-TOF-MS. Listed are observed *m*/*z* values corresponding within ±1 Da to several peptides with or without FITC.

Observed *m*/*z*	Peptide	Theoretical *m*/*z* without FITC	Number of bounded FITC	Theoretical *m*/*z* with bound FITC
930.5	c43–44	930.3	0	930.3
	c44–45	930.3	0	930.3
1399.6	c63–65	1398.8	0	1398.8
	c25–27	1398.8	0	1398.8
1583.3	c68–70	1193.7	1	1583.1
	c94–96	1193.7	1	1583.1
1982.0	c62–66	1981.7	0	1981.7
	c63–67	1981.7	0	1981.7
2694.4	c47–50	2694.7	0	2694.7
	c11–13	2694.7	0	2694.7
2771.7	c24–26	1602.7	3	2770.9
	c44–46	1993.1	2	2771.9
	c105–106	2383.1	1	2772.5
3214.5	c18–20	2824.1	1	3213.5
	c58–61	2435.1	2	3213.9
3325.0	c55–57	2936.5	1	3325.9
	c101–102	1768.0	4	3325.6
3732.1	c73–77	2952.5	2	3731.3
	c91–95	2953.5	2	3732.3

could affect analysis time and reproducibility. The use of sodium acetate at these pH values resulted in electropherograms disclosing considerably fewer peaks than phosphate and citrate (between 16 and 30 peaks). Interestingly, this BGE has a lower ionic strength than the phosphate and citrate BGE. This property has a substantial influence on the selectivity [29]. At pH 5.5 the sensitivity has decreased drastically and it is difficult to decide which of the three BGEs that is most promising, even though the highest number of peaks is detected using acetate (30) compared to phosphate (10 peaks) and citrate (6 peaks). Indeed, at pH 4.5 the sensitivity was so low that no or merely single peaks could be detected using any of the buffers. Nevertheless, even though the use of acetate as BGE at pH 5.5 resulted in the highest number of detected peaks the choice of the BGE when proceeding with coupling of CE to MALDI-MS is not straightforward. This must therefore be further investigated in future studies when MS is available for detection.

Peptides without FITC pass undetected using the LIF detector as only fluorescent analytes are detected by this technique. This implies that the peaks detected in Figs. 4 and 5 represent peptides with at least one bound FITC unit. This means that utilizing, *e.g.* citrate at pH 6.5 (Figs. 4f and 5) approximately one hundred fluorescent tryptic peptides were revealed. Taking into account the 33 peptides represented in Fig. 2 as well as the number (20) of possible peptides containing one or more FITC in Table 3 a maximum of 53 fluorescent TD peptides was detected by MALDI-TOF-MS. Since more peptides were detected by CE-LIF than by MALDI-TOF-MS, this suggests that not all peptides with bound FITC are represented in Fig. 2 or Table 3. One possible explanation for this is signal suppression in the MALDI-MS analysis. Another explanation is that peptides containing the same number of FITC units but in different positions can be separated in CE due to slightly different conformations even though they have the same mass to charge ratio [11]. Consequently, it is possible that even more FITC-bound peptides would be detected by MS if the complex peptide mixture was first separated by CE and thereby reducing MALDI signal suppression and distinguishing between peptides with the same mass to charge ratio. Still, the CE results confirm the complexity and heterogeneity of the FITC conjugation to BSA also discovered using MALDI-MS.

Table 5
Electroosmotic mobility (μ_{EOF}) in $cm^2/V\,s \times 10^{-6}$ for the three buffers at four pH values.

Buffer	pH 4.5	pH 5.5	pH 6.5	pH 7.5
Acetate	386 ± 0.6%[a]	511 ± 8.3%	588 ± 4.0%	652 ± 5.7%
Citrate	137 ± 9.0%	189 ± 1.5%	319 ± 3.1%	496 ± 5.6%
Phosphate	189 ± 17%	296 ± 7.2%	454 ± 1.5%	578 ± 2.3%

[a] RSD, relative standard deviation, $n = 4$. No temperature control was available.

3.3. Buffer counter ion effect on MALDI-TOF spectra

In the future goal of an off-line connection between the CE separation and MALDI-TOF-MS detection, the same buffer salt will be present in both techniques. It is well known that the buffer counter ion plays a central role in MALDI signal suppression and cluster formation [31], but few extensive studies have been performed on this topic. Therefore, the effects of the common buffer counter ions, ammonium, sodium and potassium, on the MALDI signal were evaluated here. Five tryptic peptide peaks were selected to cover the sample mass range: *m*/*z* 928 (t18), *m*/*z* 1480 (t10 or t55), *m*/*z* 1568 (t46), *m*/*z* 1640 (t57–58) and 2070 (t41 plus 2 FITC). No significant difference could be seen between the three salts at 10 mM (data not shown), whereas the S/N of the selected peptides were affected to varying degrees by the presence of different salt solutions at the higher concentration of 50 mM (Fig. 6). The salt resulting in consistently high S/N despite this high concentration was ammonium phosphate with sodium phosphate as the second best alternative. This was mainly a consequence of lower noise levels. As discussed in Section 3.2 the choice of BGE anion influences the CE performance substantially. In order to evaluate the effect on the MALDI analysis sodium phosphate and sodium acetate were also compared. The influence of the buffer anion on the S/N was examined to complement the foundation for selection of BGE in a coupled system. No significant differences between phosphate and acetate could be discovered, though, which suggests that the choice of buffer anion is not as crucial as the choice of counter ion, as it comes to MALDI-MS analysis of BSA–FITC peptides.

As discussed in Section 3.2 several aspects have to be regarded optimizing the CE separation. Thus, it is clear that the choice of type and concentration of BGE should be thoroughly considered in order to get the best performance possible both in the CE separation

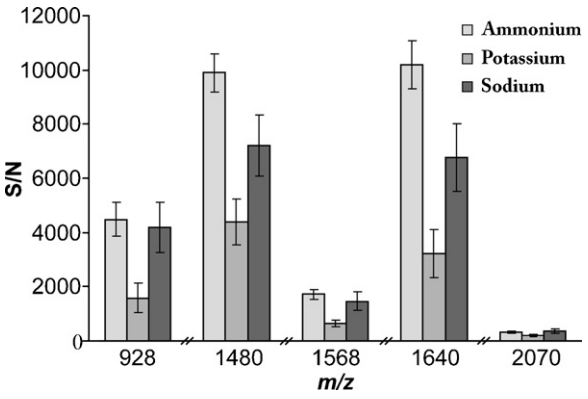

Fig. 6. MALDI-MS S/N values as a function of the three different counter ions: Na^+, K^+ and NH_4^+. The salt concentration for the ions was 50 mM, the anion was phosphate, the pH 3.4 and 16 spots were analyzed for each salt. Five *m*/*z* values were selected to cover the mass range. The standard deviation is shown for each *m*/*z* value and counter ion. The sample was trypsin digested BSA–FITC.

and the MALDI-MS analysis. Even though a salt concentration of 10 mM did not influence the MALDI-MS S/N ratio it should be kept in mind that an off-line coupling between CE and MALDI-MS can influence the accumulation of the salt. Additionally, the choice of MALDI matrix is central for a successful analysis, and thus a vital parameter for the coupling between CE and MALDI-MS. Research regarding this is presently ongoing in our laboratory.

4. Conclusions

Methods for MALDI-TOF-MS and CE-LIF analysis were optimized to examine the conjugation between the fluorescent contact allergen FITC and the model protein BSA. The heterogeneous population of conjugated BSA–FITC was digested by trypsin and chymotrypsin, respectively. MALDI-TOF-MS provided a sequence coverage of 97% and 30 lysine sites were established as actually binding FITC. Furthermore, potential FITC conjugates corresponding to 28 sites were found, while two sites without any bound FITC were identified.

A high number of peaks could be detected using sodium phosphate at pH 7.5 and pH 6.5, and sodium citrate at pH 6.5 as BGE in CE separation of trypsin digested BSA–FITC, suggesting that all FITC peptides are not detected with MALDI-MS. A future off-line coupling between CE and MALDI-MS may improve this, but demands a proper choice of the type of BGE, concentration and pH. In addition to influence of the BGE on CE performance, it was shown that the use of potassium as counter ion impaired the S/N ratio in the MALDI-MS. For optimal results an electrolyte adapted both to the CE separation and the MALDI-MS characterization is crucial.

Acknowledgements

The Swedish Research Council and the Nanochemistry program, funded by the Swedish Foundation for Strategic Research, and Göteborg Science Centre for Molecular Skin Research are gratefully acknowledged for financial support. Preliminary studies by Mikaela Michael and Christoffer Jarméus are appreciated. Theres Redeby gratefully acknowledges the Department of Analytical Chemistry at Stockholm University for providing work space and facilities.

References

[1] K.M. Marks, G.P. Nolan, Nat. Methods 3 (2006) 591.
[2] M. Lacroix, V. Poinsot, C. Fournier, F. Couderc, Electrophoresis 26 (2005) 2608.
[3] A. Sulkowska, J. Mol. Struct. 614 (2002) 227.
[4] T. Kosa, T. Maruyama, M. Otagiri, Pharm. Res. 14 (1997) 1607.
[5] M. Aleksic, C.K. Pease, D.A. Basketter, M. Panico, H.R. Morris, A. Dell, Toxicol. In Vitro 21 (2007) 723.
[6] A.T. Karlberg, M. Andresen Bergström, A. Börje, K. Luthman, J.L.G. Nilsson, Chem. Res. Toxicol. 21 (2008) 53.
[7] M. Divkovic, C.K. Pease, G.F. Gerberick, D.A. Basketter, Contact Dermatitis 53 (2005) 189.
[8] M. Aleksic, C.K. Pease, D.A. Basketter, M. Panico, H.R. Morris, A. Dell, Toxicol. In Vitro 22 (2008) 1169.
[9] D.W. Roberts, A.O. Aptula, G. Patlewicz, Chem. Res. Toxicol. 20 (2007) 44.
[10] M. Brinkley, Bioconjugate Chem. 3 (1992) 2.
[11] P.R. Banks, D.M. Paquette, J. Chromatogr. A 693 (1995) 145.
[12] A. Gajraj, R.Y. Ofoli, Langmuir 16 (2000) 8085.
[13] G. Grunwaldt, S. Haebel, C. Spitz, M. Steup, R. Menzel, J. Photochem. Photobiol. B 67 (2002) 177.
[14] T. Konecsni, F. Kilár, J. Chromatogr. A 1051 (2004) 135.
[15] J. Tuls, L. Geren, F. Millett, J. Biol. Chem. 264 (1989) 16421.
[16] A. Feltus, N.G. Hentz, S. Daunert, J. Chromatogr. A 918 (2001) 381.
[17] M. Adamczyk, A. Buko, Y.-Y. Chen, J.R. Fishpaugh, J.C. Gebler, D.D. Johnson, Bioconjugate Chem. 5 (1994) 631.
[18] K.V. Singh, J. Kaur, G.C. Varshney, M. Raje, C.R. Suri, Bioconjugate Chem. 15 (2004) 168.
[19] A.M. Landel, Anal. Biochem. 73 (1976) 280.
[20] L.-O. Andersson, A. Rehnstrom, D.L. Eaker, Eur. J. Biochem. 20 (1971) 371.
[21] R. Einarsson, R. Karlsson, E. Åkerblom, J. Chromatogr. 284 (1984) 143.
[22] J. Jacksen, T. Redeby, Å. Emmer, J. Sep. Sci. 29 (2006) 288.
[23] T. Redeby, J. Roeraade, Å. Emmer, Rapid Commun. Mass Spectrom. 18 (2004) 1161.
[24] J. Lu, R. Zenobi, Anal. Biochem. 269 (1999) 312.
[25] T. Inagami, H. Mitsuda, J. Biol. Chem. 239 (1963) 1388.
[26] S. Safi, Z. Asfari, A. Hagège, J. Chromatogr. A 1173 (2007) 159.
[27] G.M. Janini, H.J. Issaq, in: N.A. Guzman (Ed.), Capillary Electrophoresis Technology, Chromatographic Science Series, vol. 64, Marcel Dekker, Inc., New York, 1993, p. 119.
[28] G.M. Janini, H.J. Issaq, Chromatographia Suppl. 53 (2001) S18.
[29] J.C. Reijenga, T.P.E.M. Verheggen, J.H.P.A. Martens, F.M. Everaerts, J. Chromatogr. A 744 (1996) 147.
[30] R. Sjoback, J. Nygren, M. Kubista, Spectrochim. Acta A 51 (1995) L7.
[31] B.O. Keller, L. Li, J. Am. Soc. Mass Spectrom. 11 (2000) 88.