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Non-enzymatic posttranslational modifications of bovine serum albumin by oxo-compounds investigated by chromatographic and electrophoretic methods

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Abstract

Non-enzymatic posttranslational modifications of bovine serum albumin (BSA) by oxo-compounds, particularly glucose, ribose, glyoxal and glutardialdehyde, have been investigated using a set of modern chromatographic and electrophoretic separation methods. High-performance liquid chromatography (HPLC) alternatively with UV spectrophotometric (diode array) or mass spectrometric (MS) detection, polyacrylamide gel electrophoresis (PAGE) with Coomassie brilliant blue staining detection, and capillary zone electrophoresis (CZE) with UV spectrophotometric detection have been employed for the investigation of the chemical and structural changes of BSA caused by its reaction with the above oxo-compounds exhibiting different degree of reactivity. The extent of modifications was found to be dependent on the nature of the oxo-compound used and progressed in the glucose < ribose < glyoxal < glutaraldehyde order. With the aid of HPLC/UV/MS and CZE/UV tryptic peptide mapping and amino acid analysis of both unmodified and modified BSA it was revealed that the mildest modification resulted from the reaction of BSA with glucose, in this case presumably only monofunctional derivatives have arisen, whereas the most intensive modifications were found after BSA reaction with glutardialdehyde, which resulted in high degree of both inter- and intra-molecular cross-linking, due to which no unmodified peptides were detected after tryptic cleavage of BSA modified by this agent.

Keywords: Bovine serum albumin; Oxo-compounds; Non-enzymatic posttranslational modifications

1. Introduction

Posttranslational non-enzymatic modifications of proteins (as well as of lipoproteins and glycoproteins) represent important reactions of proteins, which are believed to play an important role in several physiological and pathophysiological processes, such as, e.g. ageing [1,2]. Non-enzymatic chemical reactions that occur between an oxo-group stemming typically from reducing sugars or arising from lipid oxidation/peroxidation) and free amino groups of proteins/peptides lead to the formation of a plethora of still poorly characterized reaction products. The so-called Maillard reaction [3] is traditionally of interest in food chemistry. Basically it is a reaction of simple sugars (their carbonyl groups) and amino acids (free amino groups), which involves several steps leading to the so-called Maillard reaction products. Its counterpart under in vivo conditions is better known as the "glycation" reaction. In the initial stage of Maillard reaction, typically glucose reacts with an amine (amino group of amino acid) to make a labile Schiff base that rearranges to the Amadori product [2]. The Schiff base is highly prone to oxidation and free radical generation, which leads to the formation of oxoaldehydes (glyoxal and methylglyoxal). Amadori product is a precursor of 3-deoxyglucosone, 1-deoxyglucosone and, most importantly the lysine-linked 1,4-dideoxy-5,6-dioxoglucosone from which glucosepane cross-links are synthesized. In the last step the so-called "advanced glycation

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end products" (AGEs) are formed [2]. As mentioned, only a few compounds involved in this set of reactions have been characterized so far. The reason is that numerous intermediates in this pathway are not stable under the hydrolyzing conditions and cannot be easily isolated (if at all). Pentosidine, the main cross-linking agent is best known so far [2]. Other compounds are N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL), pyrraline, and glyoxal lysine amide (GOLA) [4]. AGEs have been studied in connection with diabetes [5], aging, endothelial dysfunction and vascular diseases. Of the fluorescent products pentosidine (excitation/emission 335/385 nm) has been fully characterized [6]. CML can be formed by an oxidative cleavage of the Amadori product fructose-lysine [7,8], and by a reaction of proteins with the peroxidation products of polyunsaturated fatty acids [9] or dicarbonyl compounds like glyoxal [10,11]. Non-enzymatic protein glyco-oxidation [12] is an important chemical process, which occurs naturally in biological fluids and tissues with far reaching physiological consequences [13,14].

Serum albumins represent in this context a frequently studied model proteins [15]. Basically they are heterogeneous serum proteins; in human serum albumin (HSA) four major reasons of this heterogeneity are considered: (a) polymerization; (b) differences in the thiol content; (c) the presence of bound compounds; (d) occurrence of and/or postsynthetic modifications of the protein [16–18]. Small amounts of dimers and polymers of HSA are normally present in plasma, probably arising from disulphide linkages [19]. Many different compounds can also bind HSA and contribute to its heterogeneity [17,20]. Finally, different mutants of HSA have been reported to contribute to its heterogeneity [21]. Also several other post-translational modifications (including deamidation, acetylation, etc.) have been suggested to contribute to the heterogeneity of HSA [16].

Regarding its chemical features, HSA is a single chain protein (585 amino acid residues) containing a total of 17 disulphide bridges and a free Cys-34 residue [22]. It is also rich in histidine residues (∼18) and contains one tryptophan residue. In HSA the principal glycation site is Lys 525 accounting for about 33% of the overall glycation, in the sequentially highly homological bovine serum albumin (BSA) glycation was shown to occur in the peptide sequence covering the region 548–557. The most likely residue to be glycated is Lys 548, however it accounts for less than 20% of the modified lysine residues [23].

It should be emphasized that the literary information regards mainly HSA while the corresponding information on BSA is not commonly available.

In general, there are three strategies applicable to revealing non-enzymatic post-translational modification of a protein. The drawbacks of analyzing hydrolytically stable adducts have been briefly mentioned and the topic was reviewed in detail in [2]. Separation technologies for unfragmented modified proteins can be another route. This approach was also reviewed [1]. A number of standard separation procedures have been used for this purpose, particularly polyacrylamide gel electrophoresis (PAGE), gel permeation chromatography and borate affinity chromatography. The fact that some of the arising adducts exhibit typical fluorescence (as mentioned already) can be exploited for detection.

While the resistance of modified proteins to hydrolysis is a serious restriction for all methods involving hydrolysis, separation of intact proteins suffers from mostly very small differences in physico-chemical characteristics between modified and unmodified protein making thus any separation a difficult task.

In this communication we attempted to show the differences resulting from using different oxo-moiety possessing modifiers, particularly glucose, ribose, glyoxal and glutardialdehyde, applied to the same model protein, BSA, and to demonstrate (exploiting proteolytic cleavage), how the modification susceptible amino acid residues are distributed along the polypeptide chain.

2. Materials and methods

2.1. Instrumental

The HPLC apparatus used was a HP 1100 LC/MSD system (formerly Hewlett-Packard, now Agilent, Palo Alto, CA, USA) consisting of a degasser, a binary pump, an autosampler, a thermostatted column compartment, a spectrophotometric diode array detector and an atmospheric pressure ionization–electrospray ionization mass spectrometric detector (API–ESI–MS).

Capillary zone electrophoresis (CZE) was performed in the P/ACE 5000 instrument (Beckman Instruments, Fullerton, CA, USA), equipped with bare fused silica capillaries of 40/47 cm or 50/57 cm × 75 μm i.d., and UV-spectrophotometric detector set at 214 nm.

Mini-Protean 3 Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA) was used for PAGE.

2.2. Chemicals

Glycine was purchased from Reanal (Budapest, Hungary); BIS (*N*,*N*-methylenebis-acrylamide) and acrylamide was obtained from Sigma (St. Louis, MO, USA); ammonium persulfate (APS); bromophenol blue (3,3,5,5-tetrabromophenolsulfonephthalein); tetramethylethylenediamine (TEMED); 2-mercaptoethanol; glycerol, SDS and the low molecular mass protein markers (ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, lactalbumin) were purchased from Sigma as well. Crystallized and lyophilized bovine serum albumin, pepsin, trypsin, glucose, ribose, glyoxal (40% (v/v) solution in water), glutardialdehyde (50% (v/v) solution in water), diglycine and tetraglycine were obtained from the same source. Phenylisothiocyanate (PITC) was from Pierce (Rockford, IL, USA).

Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl); methanol and acetonitrile for HPLC (gradient grade) were from Merck (Darmstadt, Germany), all other chemicals were purchased from Lachema (Brno, Czech Republic) in an analytical grade (p.a.) quality; Milli-Q water (Millipore, Bedford, MA, USA) was used throughout this study.

2.3. Sample preparation

2.3.1. Procedure for BSA modification

BSA was dissolved in 0.2 M phosphate buffer pH 7.4 at the concentration of 1 mg/ml and incubated at 37 °C (96 h) with the studied oxo-compounds (glucose, ribose, glyoxal or glutardialdehyde). The concentration of all reactants was 100 mmol/l. BSA incubated under the same conditions in the buffer only served as a control.

2.3.2. Enzymatic digestion of BSA by trypsin

BSA samples were desalted on the Econo-Pac 10 DG columns (Bio-Rad Laboratories, Hercules, CA, USA) and lyophilized. Desalted BSA samples were diluted to the concentration 3 mg/ml with 20 mmol/l ammonium bicarbonate buffer (pH 7.8) and treated with trypsin (1:50 enzyme:substrate ratio). Blank samples were prepared by incubation of the enzyme solution only under identical conditions. Incubation was done at 37 °C for 36 h. Next the samples were centrifuged for 5 min at 2000 × *g* and the supernatants removed and stored at −18 °C.

2.3.3. Peptide standards for spiking of the CZE runs

Diglycine (0.48 mg) and 0.48 mg tetraglycine were dissolved in 100 μl of the running buffer; for spiking 2 μl diglycine and 1 μl tetraglycine were added to 6 μl of the sample as internal standards to compare migration times among individual samples.

Table 1
Total amino acid composition (amino acid residues per 100 amino acids in peptide chain) of modified BSA before the digestion by trypsin

Amino acid	A	B	C	D	E	T
Asx	9.8	7.4	10.2	11.0	11.8	8.9
Glx	13.9	12.8	15.6	17.6	17.9	13.1
Ser	4.5	5.8	5.5	3.2	3.6	5.3
Gly	3.8	8.1	5.3	5.3	4.9	2.8
His	2.5	1.9	1.9	2.6	2.3	2.8
Arg	3.3	2.6	1.7	1.3	2.5	4.3
Thr	5.3	7.4	5.6	3.2	3.2	5.8
Ala	8.2	8.0	9.2	9.7	10.1	7.8
Pro	4.6	8.4	5.0	4.9	5.0	4.6
Tyr	3.9	2.0	3.4	2.3	2.7	3.5
Val	6.4	7.6	7.5	7.8	7.4	6.3
Met	0.5	2.2	0.4	0.5	0.3	0.8
Cys	3.5	3.3	2.8	2.4	1.8	5.8
Ile	2.6	3.1	3.1	3.5	3.2	2.5
Leu	12.7	12.0	15.4	17.0	16.4	10.8
Phe	4.9	4.4	5.6	6.3	5.8	5.0
Lys	9.5	3.1	1.7	1.7	1.0	9.9

Averaged values from three measurements are presented: A, unmodified BSA; B, BSA modified with glucose; C, BSA modified with ribose; D, BSA modified with glyoxal; E, BSA modified with glutaraldehyde; T, theoretical composition of BSA from SwissProt database.

2.4. Conditions for HPLC/UV and HPLC/MS

2.4.1. HPLC of the whole BSA

Chromatographic analysis of the whole BSA was done on a Supelcosil LC-304 column (25 cm × 4.6 mm i.d., 30 nm pore size, 5 μm) (Supelco, Bellefonte, PA). A 10 μl sample

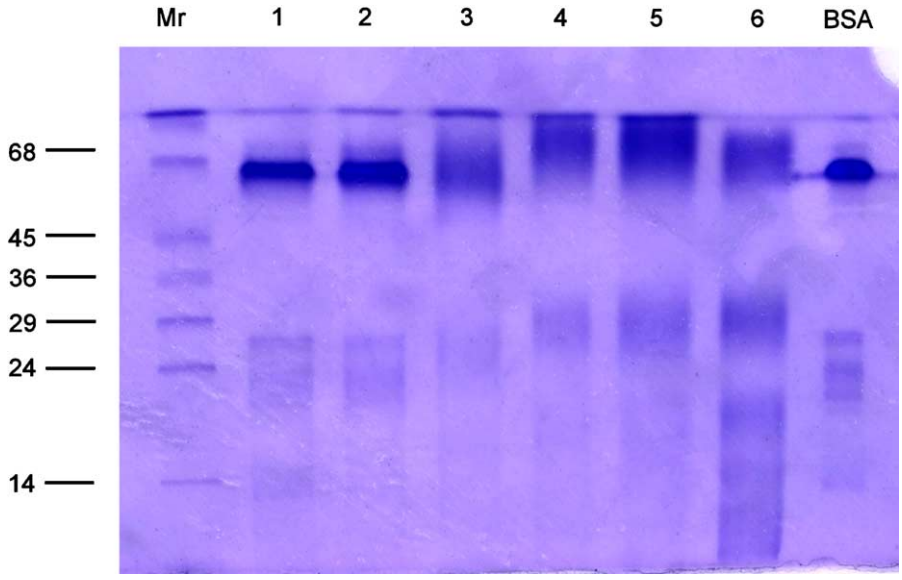

Fig. 1. Polyacrylamide gel electrophoresis (PAGE) analysis of unmodified and by oxo-compounds modified BSA. Left lane: low M_r markers: bovine serum albumin (M_r 68,000), ovalbumin (M_r 45,000), glyceraldehydes-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000), α-lactalbumin (M_r 14,000); (lane 1) 4 μl unmodified BSA; (lane 2) 4 μl BSA modified by glucose; (lane 3) 4 μl BSA modified by ribose; (lane 4) 4 μl BSA modified by glyoxal; (lane 5) 8 μl BSA modified by glyoxal; (lane 6) 4 μl BSA modified by glutaraldehyde; and right lane: 1 mg/ml BSA 0.5 μl.

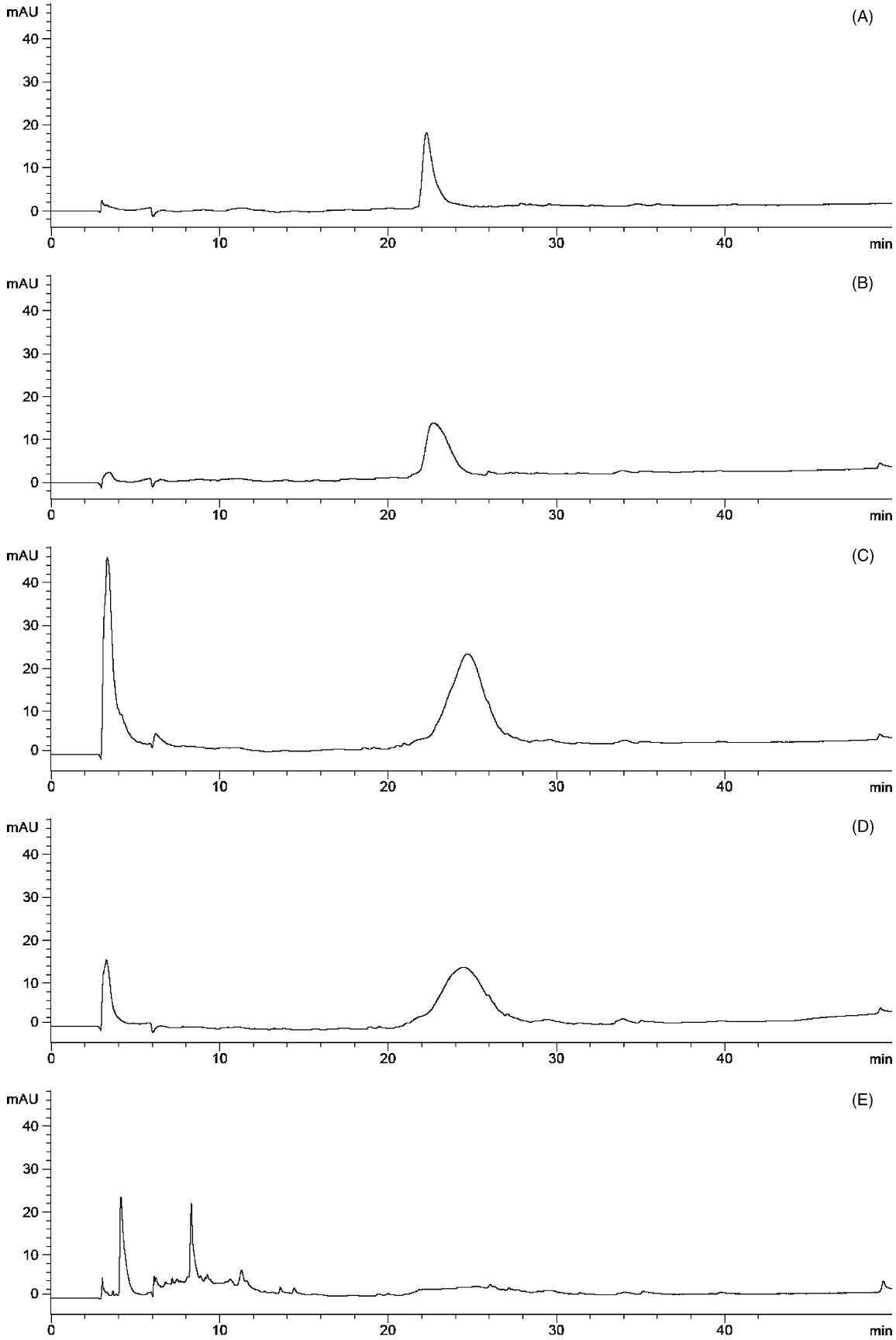

Fig. 2. HPLC/UV analysis of unmodified BSA (A) (albumin was eluted at 22 min), and BSA modified by glucose (B), ribose (C), glyoxal (D), glutaraldehyde (E), *X*-axis: retention time, *Y*-axis: absorbance at 280 nm.

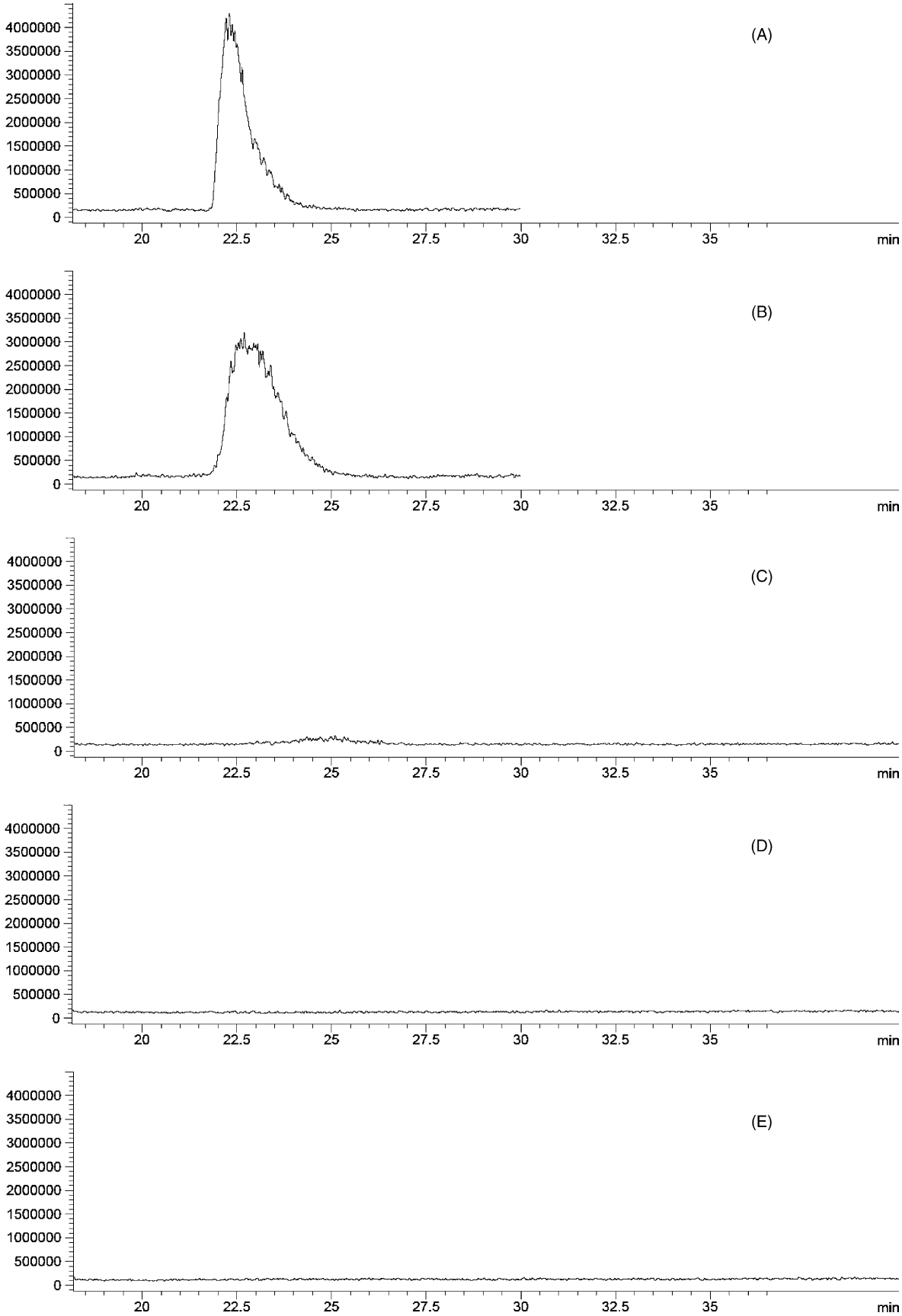

Fig. 3. HPLC/ESI-MS analysis of unmodified BSA (A) (albumin was eluted at 22 min), and BSA modified by glucose (B), ribose (C), glyoxal (D), glutaraldehyde (E), *X*-axis: retention time, *Y*-axis: total ion current.

was injected. Elution was obtained by applying a linear gradient between mobile phases A (water with 1% (v/v) formic acid) and B (methanol–water 75:25 with 0.75% (v/v) formic acid) (0–100% (v/v) B over 50 min at a flow-rate 1 ml/min, followed by elution with 100% B for 5 min). Before the next run the column was washed with buffer A for 20 min. Column temperature was held at 25 °C and detection was done at 280 nm.

2.4.2. HPLC of the tryptic digests of BSA

A Zorbax Eclipse XDB-C18 column (150 mm × 2.1 mm i.d., 10 nm pore size, 5 μm), Rockland Technologies (Hewlett-Packard) was used for the separation of tryptic peptides of BSA: a 10 μl samples were injected. Elution was achieved by a linear gradient between mobile phase A (water with 0.1% formic acid) and B (methanol–acetonitrile 1:1 with 0.075% formic acid). Gradient started from 0 to 100% B over 50 min with a flow-rate of 0.25 ml/min followed by elution with 100% B for 5 min. Equilibration before the next run was achieved by 15 min washing with buffer A (column temperature 25 °C, detection at 214 nm).

2.4.3. API–ESI mass spectrometry

Atmospheric pressure ionization–electrospray ionization (API–ESI) positive mode mass spectrometry was used. Operating conditions (optimized by FIA): drying gas (N_2), 10 l/min; drying gas temperature, 350 °C; nebulizator pressure, 1.7×10^5 Pa (25 psi); capillary voltage, 4500 V; ions were observed at mass range *m*/*z* 200–1500; fragmentor was set at 80 V.

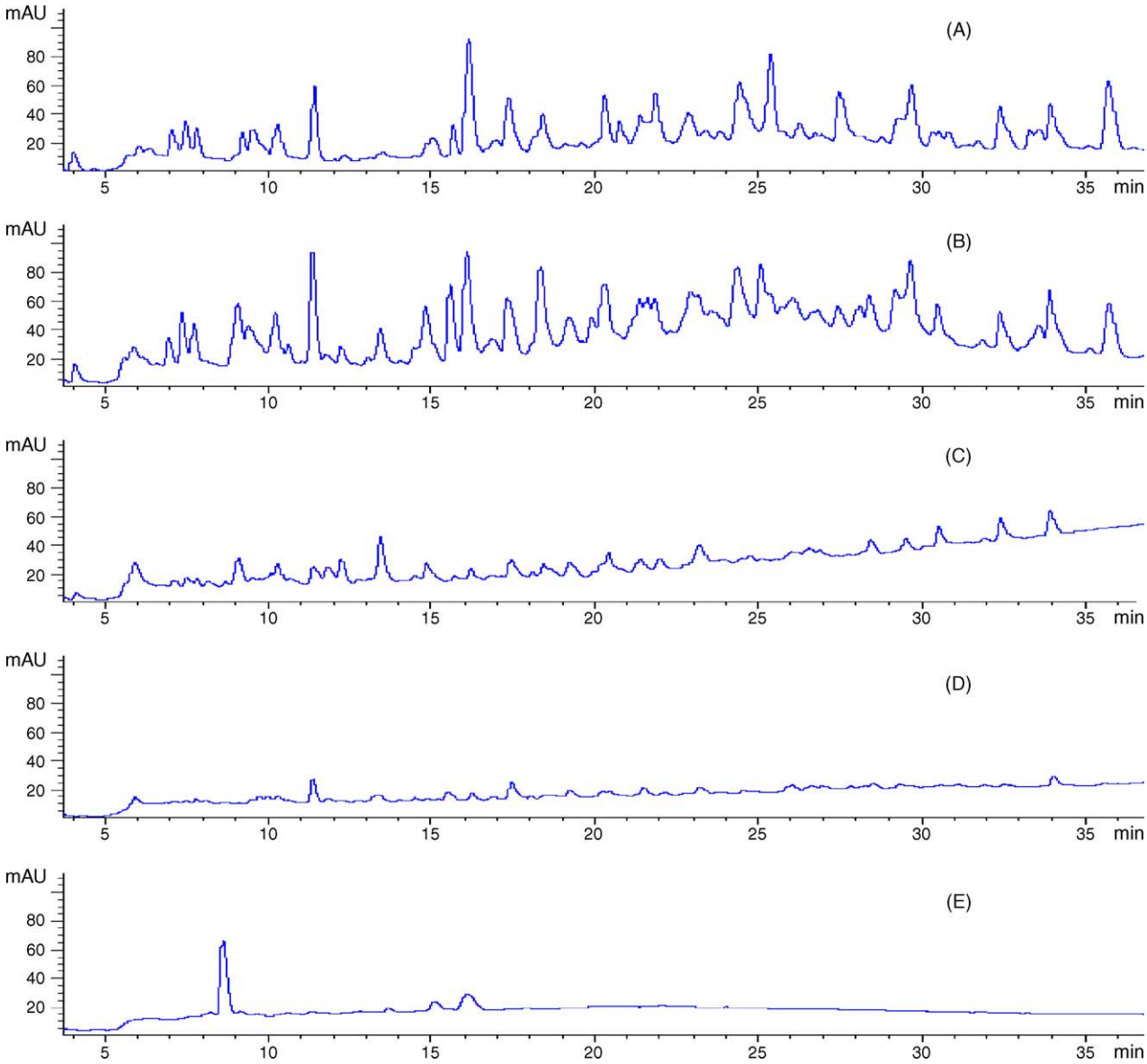

Fig. 4. HPLC/UV tryptic peptide maps of unmodified BSA (A), and of BSA modified by glucose (B), ribose (C), glyoxal (D), glutaraldehyde (E), *X*-axis: retention time, *Y*-axis: absorbance at 214 nm.

2.5. Conditions for CZE

2.5.1. CZE of the whole BSA

Bare fused silica capillary of 47 cm (40 cm to the detector) × 75 μm i.d. was used. Capillary was conditioned stepwise by 1 mol/l NaOH (30 min), water (1 min), 1 mol/l HCl (30 min) and water (1 min). Phosphate buffer (50 mmol/l, pH 2.5) was used as background electrolyte. The BSA samples were diluted with water (1:1) and injected hydrodynamically 1 s at 3.45 kPa. Before analysis, the capillary was washed for 4 min with the background electrolyte. All separations were run at 10 kV and 20 °C.

2.5.2. CZE of the tryptic digess of BSA

Bare fused silica capillary of 47/40 cm (for the separation at pH 2.5) or 57/50 cm (for runs at pH 7.0) × 75 μm i.d. were used. In both cases the capillary was conditioned stepwise with 1 mol/l NaOH (30 min), water (1 min), 1 mol/l HCl (30 min) and water (1 min). Two 50 mmol/l phosphate running buffer were used: pH 7.0 and 2.5. CE separations were run at 15 kV (pH 7.0) and 10 kV (pH 2.5), and at the temperature 20 °C. Between runs the capillary was conditioned with the running buffer (5 min) only. The samples of BSA digested by trypsin were diluted with water (1:1) and injected hydrodynamically 1 s at 3.45 kPa.

2.6. Polyacrylamide gel electrophoresis (PAGE)

The separating gel (10%) was prepared by mixing 2.45 ml water, 1.25 ml Tris–HCl (1.5 mol/l, pH 8.8), 50 μl SDS (10% (m/v)), 1.25 ml acrylamide (30% (m/v)) and bis-methylenacrylamide (0.8% (m/v)), 25 μl APS (10% (m/v)) and 2.5 μl TEMED. This gel was polymerized for 45–60 min at room temperature. Next the stacking gel (4%) was pre-

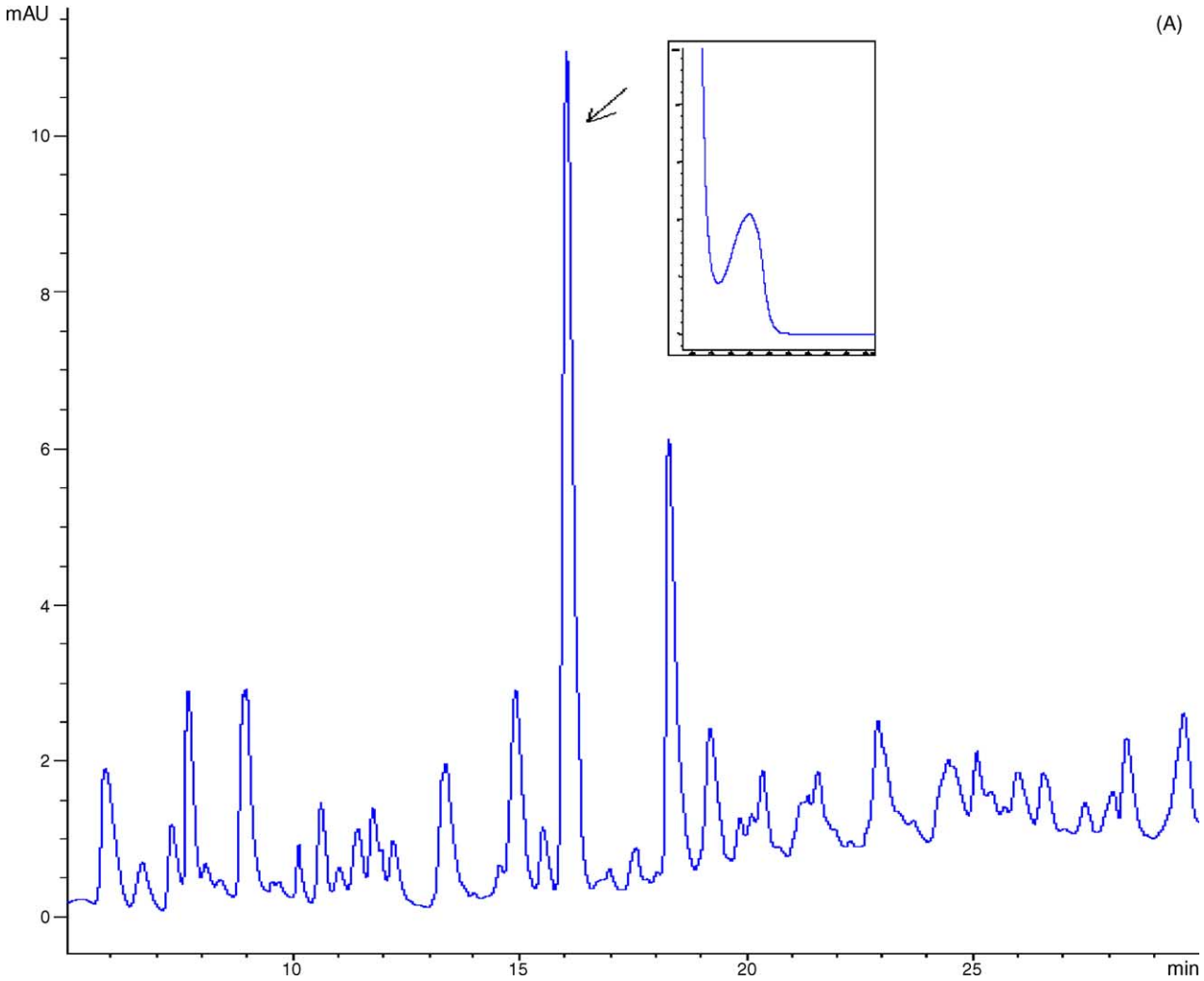

Fig. 5. HPLC/UV tryptic peptide maps of modified BSA and changes in UV-absorption spectra. (A) BSA modified by glucose and detected at 280 nm (spectrum of the peak with retention time 16 min). (B) BSA modified by ribose and detected at 280 nm (spectrum of the peak with retention time 19.3 min). (C) BSA modified by glutaraldehyde and detected at 278 nm (spectrum of the peak with retention time 9.5 min; peak at 8.6 min had two absorption maxima at 224 and 251 nm). *X*-axis: retention time, *Y*-axis: absorbance at the above specified detection wavelength. Inset: spectrum at 210–450 nm.

pared by mixing 3.05 ml water, 1.25 ml Tris–HCl (0.5 mol/l, pH 6.8), 50 μl SDS (10% (m/v)), 0.65 ml acrylamide (30%) and bis-methylenacrylamide (0.8%), 25 μl APS (10% (m/v)) and 5 μl TEMED. This gel polymerized within 35–45 min. After the samples were applied electrophoresis was run at 150 V over the gel. Finally the gel was stained by a solution of Coomassie brilliant blue (0.25% (m/v)) and destained in 1% (v/v) acetic acid for 120 min. The resulting separation was scanned by flat scanner (hp scanjet 7400c; Hewlett-Packard).

2.7. Amino acid analysis

Amino acid analyses were carried out routinely using a PICO-TAG Amino Acid Analysis System (Waters, Milford, MA, USA). The method exploits the precolumn derivatization with phenylisothiocyanate (done according to the manufacturer's instructions) followed by the separation of the arising products by HPLC on a reversed-phase column (C18; Pico-Tag column 300 × 3.9 i.d. mm; Waters) using acetate buffer (pH 6.4, 0.14 mol/l)–acetonitrile gradient (according to manufacturer's procedure). Protein hydrolysis was done by HCl vapors (6 mol/l HCl with 2% phenol) for 20 h at 110 °C in a vial under vacuum (after a nitrogen flush).

3. Results and discussion

3.1. Analysis of the whole BSA

Polyacrylamide gel electrophoresis of bovine serum albumin modified by the different oxo-moiety possessing compounds is shown in Fig. 1. While intact BSA and BSA subjected to the reaction with glucose shows a single rather sharp band at about 66×10^3 relative molecular mass, treatment with any of the other reactive components revealed a small shift of the BSA band to higher rel. mol. mass range with concomitant broadening of the zone. This appears indicative of several incompletely resolved molecular species present in the zones corresponding to modified BSA.

HPLC/UV analyses of both unmodified and modified BSA shown in Fig. 2 revealed a rather sharp peak corresponding to unmodified BSA; not even a slight change in retention time was observed after glucose treatment, however a slight increase in retention time was seen with ribose and glyoxal treated samples; no peak was eluted in the case of BSA treatment by glutaraldehyde. Similarly to the electrophoretic patterns also the chromatographic peaks obtained after glucose, ribose and glyoxal treatment were broadened in comparison to the peak of the untreated BSA.

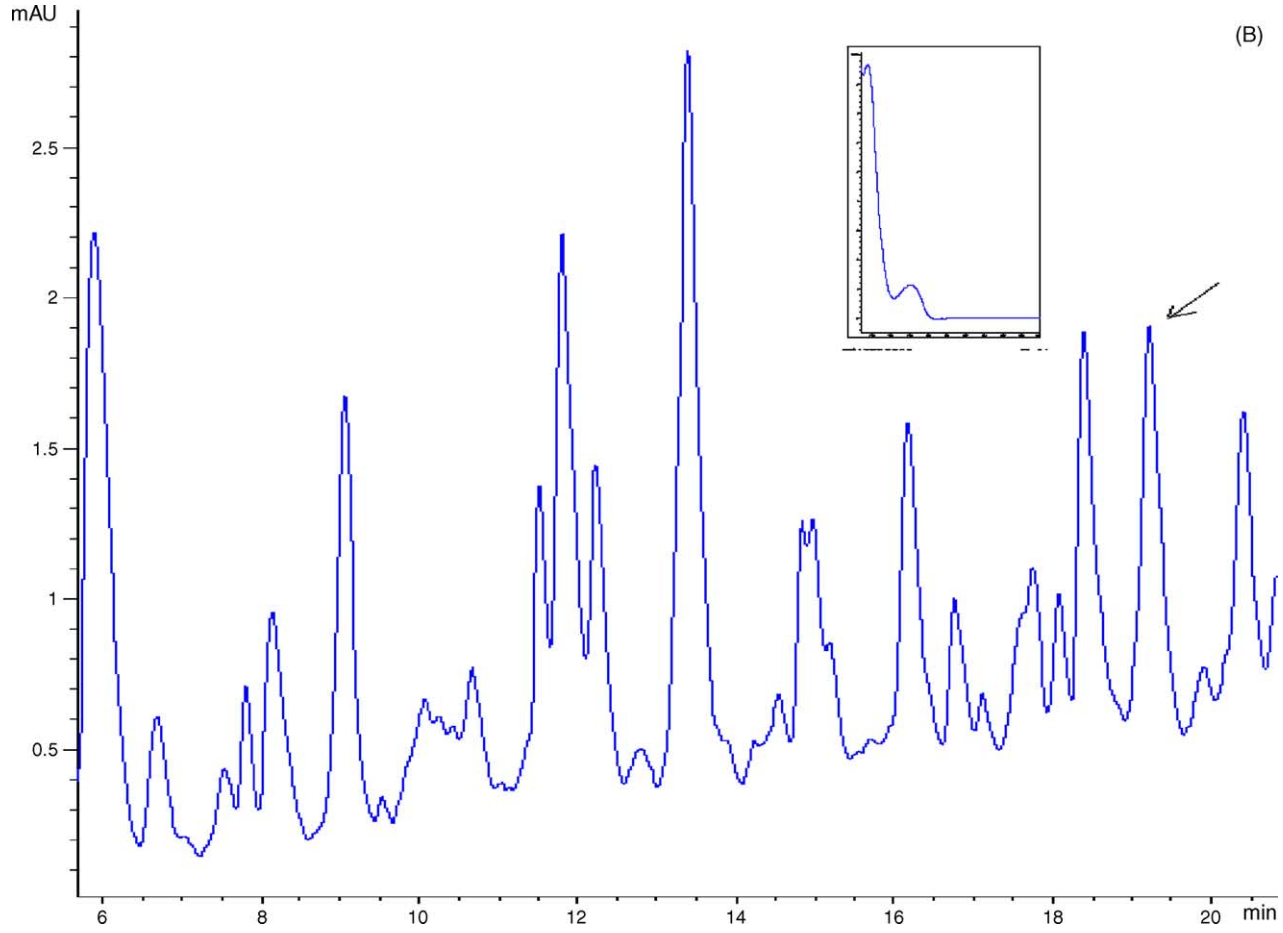

Fig. 5. (*Continued*)

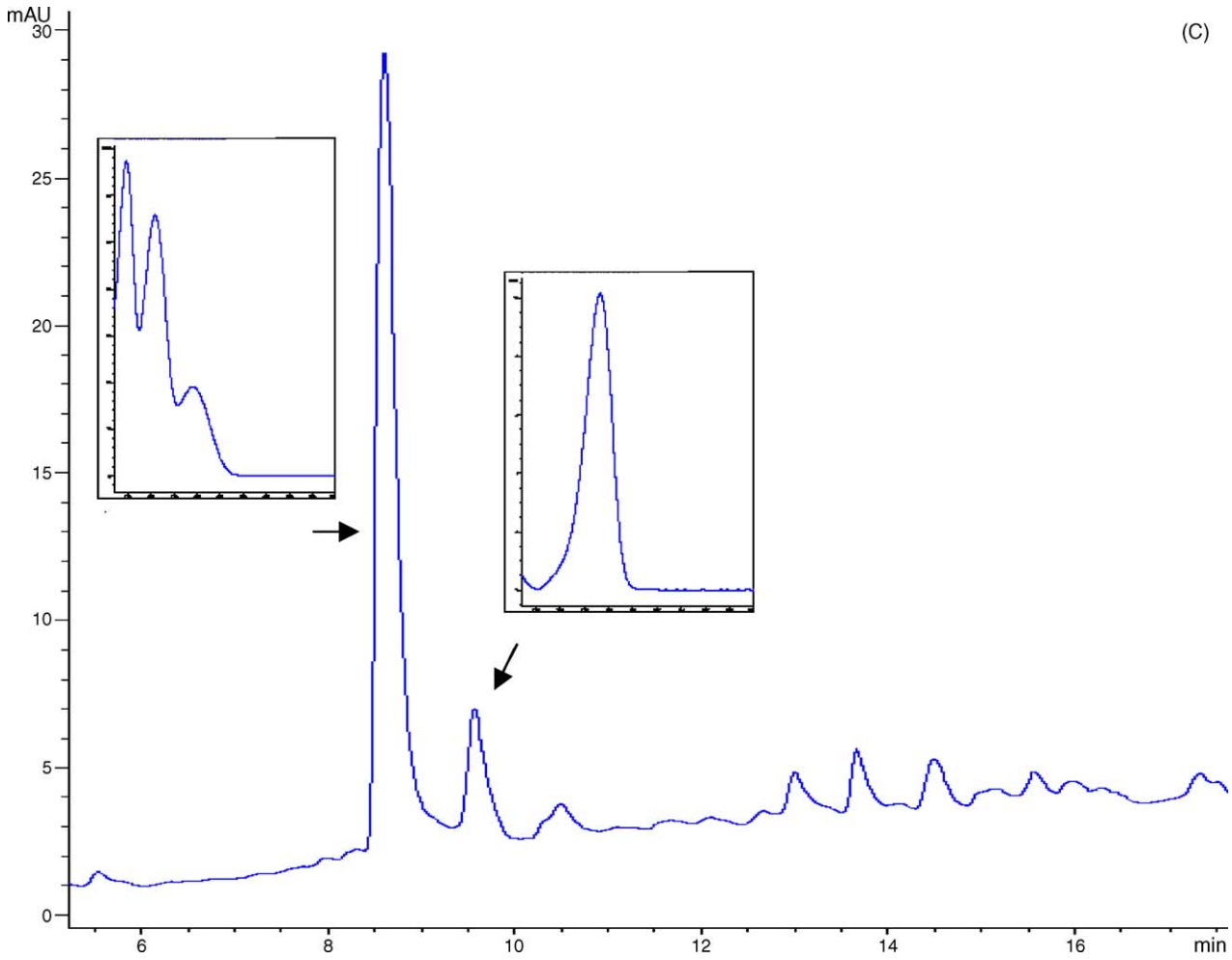

Fig. 5. (*Continued*).

Using ESI-MS as the detection mode in HPLC analyses of BSA samples revealed a peak with the intact protein sample as well as with the sample, in which glucose-treated BSA was present. No peaks were revealed in BSA samples treated with ribose, glyoxal or glutaraldehyde. As the ESI-MS detection is unable to detect proteins of rel. mol. mass higher than 100,000, it can be concluded that except glucose, all other oxo-moiety possessing modifiers caused distinct polymerization of the protein, the most efficient in this respect being apparently glutaraldehyde (Fig. 3).

The nature of the faint zones in the PAGE analyses with a higher mobility than the main protein zone is difficult to assess. It may be conceivable to propose that they represent impurities present in the original BSA sample, perhaps modified by the particular treatment. The nature of the sharp peak(s) appearing in the chromatographic profiles (with UV detection) at the beginning of the run was difficult to assess.

On the other hand, the control amino acid analyses of native and treated BSA revealed a dramatic decrease in the content of lysine and a less dramatic decrease in arginine in all BSA samples treated with the oxo-moiety possessing compounds (Table 1). The relative proportions of arginine and lysine decreases are different with different oxo-moiety possessing reactants; the largest preference for lysine being revealed with the glutaraldehyde treatment.

3.2. Analysis of tryptic digests of BSA

A further insight into the BSA modifications was obtained by tryptic peptide mapping. The applied separation methods were HPLC with either UV or MS detection and capillary electrophoresis. As shown in Fig. 4 the extent of the modification depends on the nature of the modifiers used: glucose, being a mild modifier, offers a rich profile of tryptic peptides, while ribose treatment led to a moderately modified protein more resistant to enzymatic cleavage than native BSA. Reaction with dioxo-compounds, i.e. glyoxal and glutaraldehyde, resulted in a modified protein highly resistant to enzymatic cleavage. It is conceivable to propose that this resistance is caused by a cross-linking reaction(s) of the dioxo-compounds with two free protein amino-groups; such cross-linking could be both inter-or intramolecular; no matter which type of the cross-link is involved a decrease in susceptibility to proteolytic cleavage will be the result in either case.

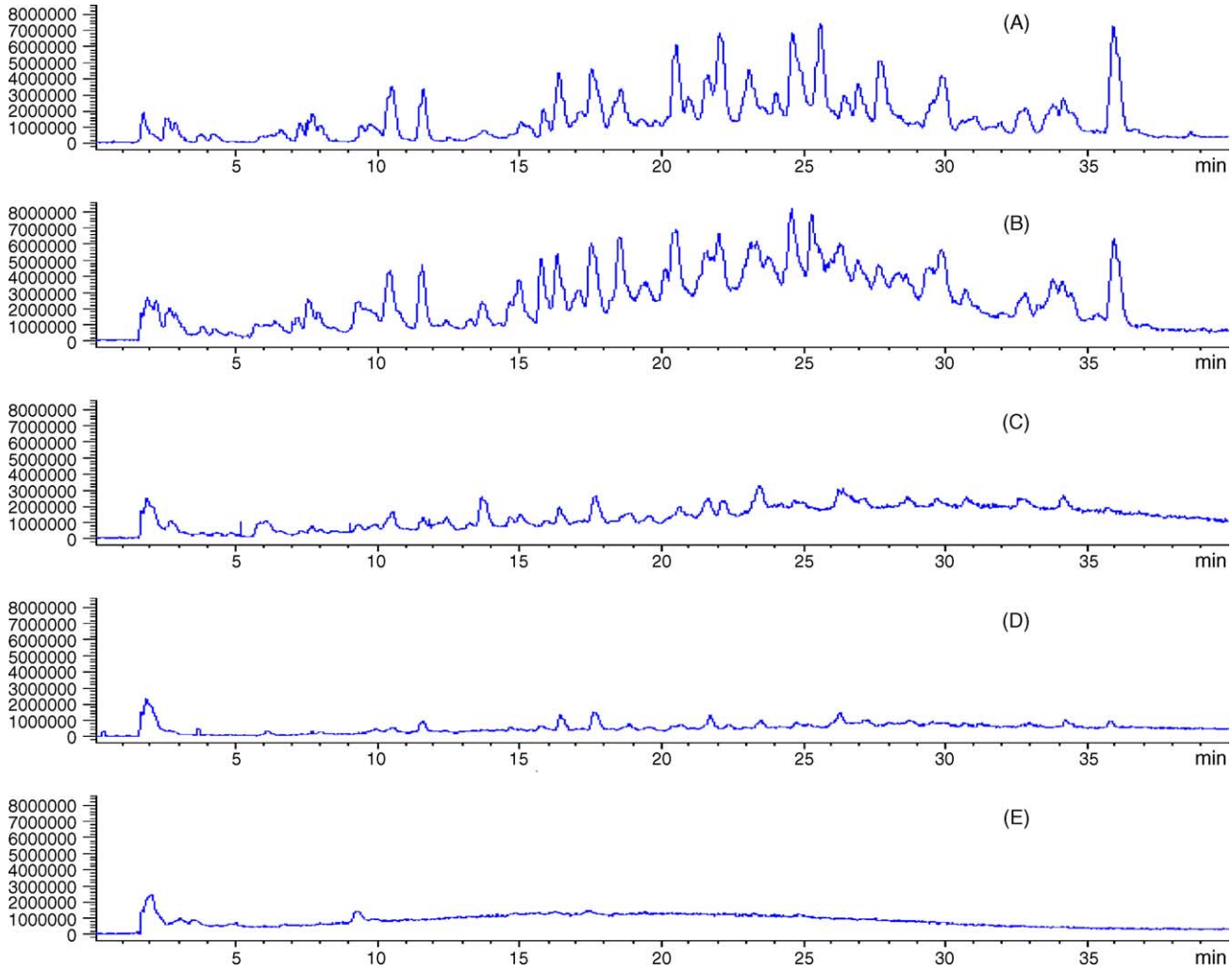

Fig. 6. HPLC/ESI-MS tryptic peptide maps of unmodified BSA (A), and BSA modified by glucose (B), ribose (C), glyoxal (D), glutaraldehyde (E). *X*-axis: retention time, *Y*-axis: total ion current.

Additional information about the released peptides and changes in their structure can be obtained from their UV–vis absorption spectra. It has been reported that the reaction of peptides with oxo-moiety possessing compounds leads to coloured products [1,2]; in some cases the change is even visible by naked eye. The result of a search for peptides with modified UV spectra is shown in Fig. 5A–C (only sections of the total profile where the spectral changes can be detected are shown). Glucose (Fig. 5A), ribose (Fig. 5B) and glutaraldehyde (Fig. 5C) as protein modifiers were investigated. In all these profiles peptides exhibiting absorption at 280 nm were found. No such peptides could be found in the tryptic peptides of unmodified serum albumin.

If ESI-MS was used for detection of the individual tryptic peptides in the chromatographic profile, the patterns shown in Fig. 6 were obtained. In general, the profiles are generally similar to those shown in Fig. 4; rich peptide profiles are seen (as expected) with unmodified or glucose modified BSA. A more detailed analysis of the set of trypsin released peptides is presented in Fig. 7A–E, in which the peptide maps obtained from untreated, glucose, ribose, glyoxal and glutaraldehyde are shown. The unmodified peptides are marked by numbers. The respective relative molecular masses are summarized in Table 2.

As published previously [23], the peptide sequence 548–557 (KQTALVELLK) containing the glycation site Lys 548 is very reactive. In our analysis this peptide occurred under peak number 30 (Fig. 7A) and (as expected) disappeared after the reaction with glucose. Other reactive lysine residues sensitive to glucose modification are Lys 309 (fragment 300–309, peak 21), Lys 597 (fragment 588–607, peak 18), in addition, arginine 220 (fragment 219–220, peak 1) also exhibited some sensitivity to modification by glucose.

On the other hand, there are some glucose-unreactive peptides that disappear only after the reaction with glutaraldehyde, which, as demonstrated, is the most reactive compound of the set of oxo-compounds investigated. These peptides possess lysines at positions 88 (fragment 76–88, peak 15), 248 (fragment 246–248, peak 13), 256 (fragment 249–256, peak 22), 263 (fragment 257–263, peak 20), 495 (fragment

Table 2
The comparison of relative molecular masses

Peak no.	Fragment. amino acid composition	A	B	C	D	E
1	30 (219–220), QR 69 (496–498), VTK	**302.1** **346.1** **552.2**	*763.6*; 4832.8; 2423 **346.1**	*763.6*; *1187.4*	*763.6*; *1187.4*	*763.6*; 677.6; 1559.2
2	34 (233–235), ALK or 53 (372–374), LAK 31 (221–222), LR 74 (545–547), QIK 36 (242–245, LSQK	**330.2** **287.1** **387.2** **474.2**	**330.1** **287.1** **387.2** **474.2**	**330.1** **287.1** **387.1**	**330.2** **287.2**	0
3	64 (456–459), VGTR 7 (35–36), FK	**431.2** **293.1** **332.1**	**431.2** **293.1**	**431.2** **293.1**	**431.1**	0
4	63 (452–455), SLGK	**403.2**	**403.2**	**403.2**	0	0
5	60 (434–436), YTR 28 (210–211), EK	**438.2** **275.1**	**438.2** **275.1**	**438.2** **275.1**	0	0
6		**487.2**	0	0	0	0
7		**424.1**	0	0	0	0
8	42 (281–285) ADLAK	**516.2**	**516.2**	**516.2**; *553.2*	*553.2*	0
9	6 (29–34), SEIAH R 40 (264–266), VHK	**711.4** **382.1**	**711.4** **382.2**	**711.3** **382.1**	0	0
10	68 (490–495), TPVSE K	**659.4**	**659.3**	**659.3**	**659.2**	0
11	49 (341–346), NYQEA K	**751.3**	**751.3**	**751.2**	0	0
12	33 (229–232), FGER	**507.2** **360.1**	**507.2**	0	8583; 817.3	263.1; 345.1

Table 2 (*Continued*)

Peak no.	Fragment. amino acid composition	A	B	C	D	E
13	72 (524–528), AFDEK	**608.2**	**608.2**; 7525; 537	**680.1**	**680.1**	0
	37 (246–248), FPK	**390.1**	**390.1**	**390.1**	**390.1**	
14	78 (562–568), ATEEQ LK	**817.5**	**817.4**; 576.3	**817.7**		0
	13 (101–105), VASLR	**544.3**	**544.3**	**544.3**	**544.3**	
		445.2	**445.2**	**445.1**		
15	11 (76–88), TCAVD ESHAG CEK	**1346.9**	**1346.9**	**1346.9**	**1346.7**	0
	27 (205–209), IETMR	**648.3**	**648.3**			
16	5 (25–28), DTHK	**499.3**	**499.3**	**499.4**		0
		1661.2				
17	17 (131–138), DDSPD LPK	**885.5**	**885.5**; *957.5*; *701.3*; 836.4; *645.3*	*957.5*; *701.3*; 597.2; *645.3*; 570.4	0	0
18	81 to 82 (588–607), EACFA VEGPK LVVST QTALA	**2032.4**	0	0	0	0
19	8 (37–44), DLGEE HFK	**973.7**	**973.5**	0	0	0
20	39 (257–263), LVTDL TK	**788.6**	**788.5**	**788.5**	**788.5**; 576.3	0
	64 (456–459), VGTR	**431.2**	**431.2**			
	35 (236–241), AWSVA R	**688.4**	**688.3**	**688.3**		
21	45 (300–309), ECCDK PLLEK	**1175**	1021.7	0	0	0
22	38 (249–256), AEFVE VTK	**921.7**	**921.6**	**921.6**; 532.2; 841.4	**921.6**; 721.3	*361.2*
23		**1940**	0	0	841.5	0
24		**3815.7**	**3815.5**; 914.3; 3574.4	0	0	0
25	57 (402–412), HLVDE PQNLI K	**1305**	**1305**; 18983.5; 19157.6; 2223	**1305**; 793.3	0	0

26	22 (161–167), YLYEI AR	**926.7**	**926.3**	**926.3**	0	0
27	82 (598–607), LVVST QTALA	**1001.8**	**1001.5**	**1001.5**; *1023.5*	*1023.4*; **1001.6**	0
28	61 to 62 (437–451), KVPQV STPTL VEVSR	**1639.4**	**1639.4**; 3345	**1639.5**	0	0
29	51 to 52 (360–371), RHPEY AVSVL LR	**1439.2**	**1439.2**; 4624.4; 3684.9	1511.3; 1697.3; *528.2*; 784	*528.2*	0
30	75 to 76 (548–557), KQTAL VELLK	**1142**	0	0	0	0
31	10 (66–75), LVNEL TEFAK	**1162.9**	**1162.9**; 4338.8; 1712.8; 1304; 30221.6	**1162.7**	0	0
32		**5188.7**	**5188.8**; 3699.4	1282.7	0	0
33	62 to 65 (438–468), VPQVS TPTLV EVSRS LGKVG TRCCT KPESE R	**3342**	3851; 19243.7; *584.3*	*584.3*	*2156.9*	0
34	76 (549–557), QTALV ELLK	**1013.8**	**1013.8**; *963.4*	*963.4*	*963.3*	0
35	59 (421–433), LGEYG FQNAL IVR	**1479.2**	**1478.9**; 3832.5; *526.2*	0	0	0
36	14 to 17 (106–138), ETYGD MADCC EKQEP ERNEC FLSHK DDSPD LPK	**3831**	**3832.5**	*755.3*; *561.3*; *777.3*; *526.2*	561.3; *755.3*	0
37	47 (319–336), DAIPE NLPPL TADFA EDK	**1956**	**1955.5**	0	**1955.8**	0
38		**5264.7**	**5264.9**	*851.3*; *873.3*; *720.3*	0	0
39		**2506.2**	*1014.4*; 833.4; 5634.6	*1014.4*; 1036.4	0	0
40	45 (300–309), ECCDK PLLEK	**1177.8**	*996.5*; *501.6*; **1177.9**	**1177.8**; *996.5*	*996.5*	0
41		**4476.3**	**4476.1**	0	0	0
42	29 (212–218), VLTSS AR	**731.4**	**731.4**; *1031.4*	*1031.4*; **731.4**	**731.4**; *1031.3*	0
43		**4984.8**	**4984.8**	*1750.5*	*1750.6*	0

M_r of peptide fragments obtained from HPLC–ESI/MS tryptic peptide mapping of unmodified (A) and modified BSA (B–E). Only M_r values of the most significant peptides are presented. Determination of the fragment composition and M_r values of individual peptide peaks: A, unmodified BSA; B, BSA modified with glucose; C, BSA modified with ribose; D, BSA modified with glyoxal; E, BSA modified with glutaraldehyde. Numbers in bold refer to peptides from unmodified BSA. For peak identification see Fig. 6.

490–495, peak 10), 528 (fragment 524–528, peak 13), and arginines 105 (fragment 101–105, peak 14), 218 (fragment 212–218, peak 42), 222 (fragment 221–222, peak 2), 459 (fragment 456–459, peak 3).

Capillary zone electrophoresis (CZE) offers another view on the nature of the adducts/cross-links of BSA arising from its reactions with the above oxo-compounds. CZE analysis of tryptic peptides of BSA in phosphate buffer, pH 7.0 (Fig. 8) offers an incomplete separation of peptides of the unmodified albumin. Modification of BSA by glucose rearranges this profile in such a way that the peaks in the front of the separation profile are decreased while a massive cluster of peaks appears at the end of the profile. Modifications with other (more reactive) oxo-compounds did not yield any peak in the CZE run at all. This behavior is probably caused by the well-known sticking/interaction of proteins with the capil-

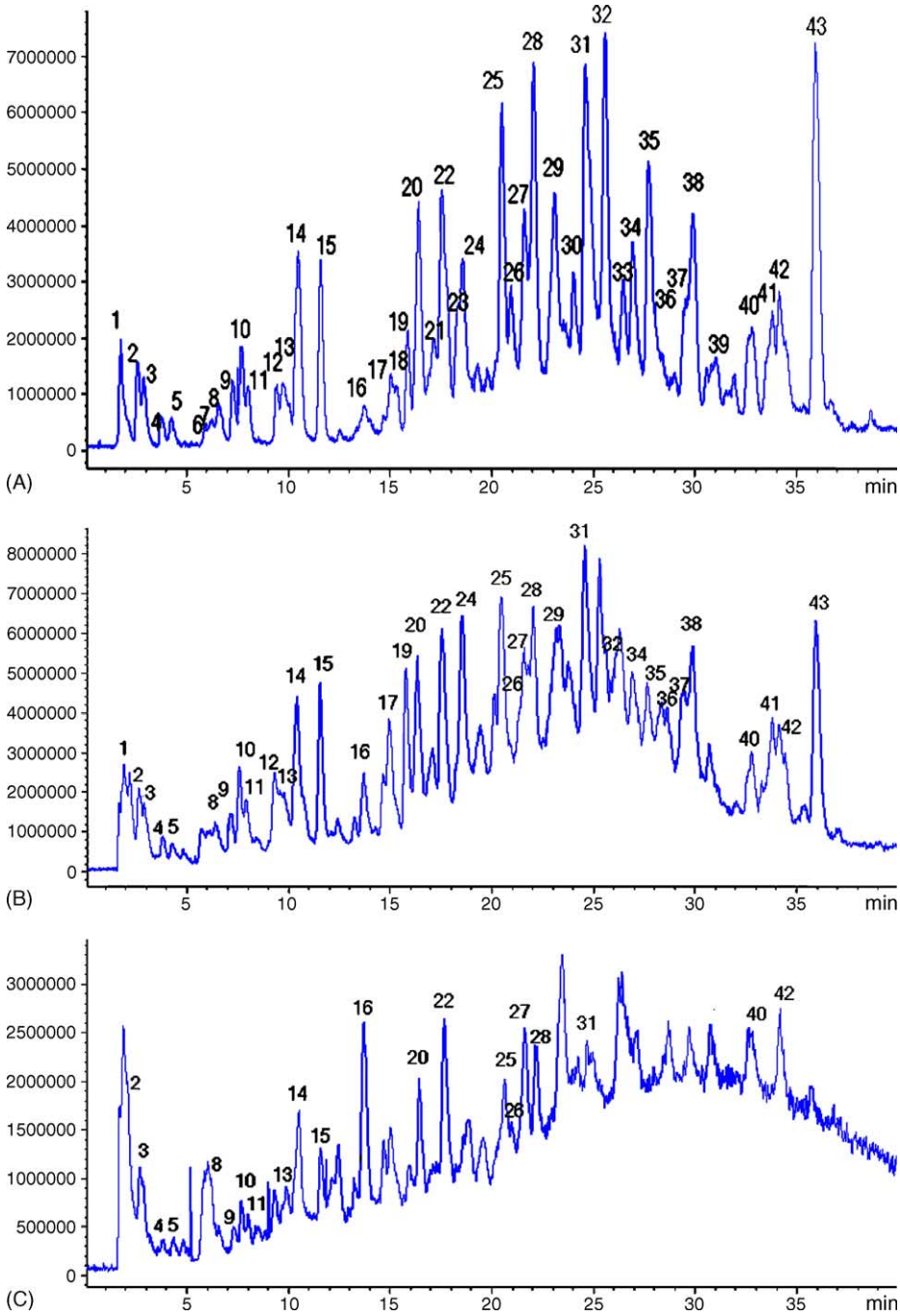

Fig. 7. HPLC/ESI-MS tryptic peptide maps of unmodified BSA (A), and BSA modified by glucose (B), ribose (C), glyoxal (D), glutaraldehyde (E). *X*-axis: retention time, *Y*-axis: total ion current. Peak numbers refer to the unmodified tryptic peptides, i.e. tryptic peptides of unmodified BSA. Individual peaks are specified in Table 2.

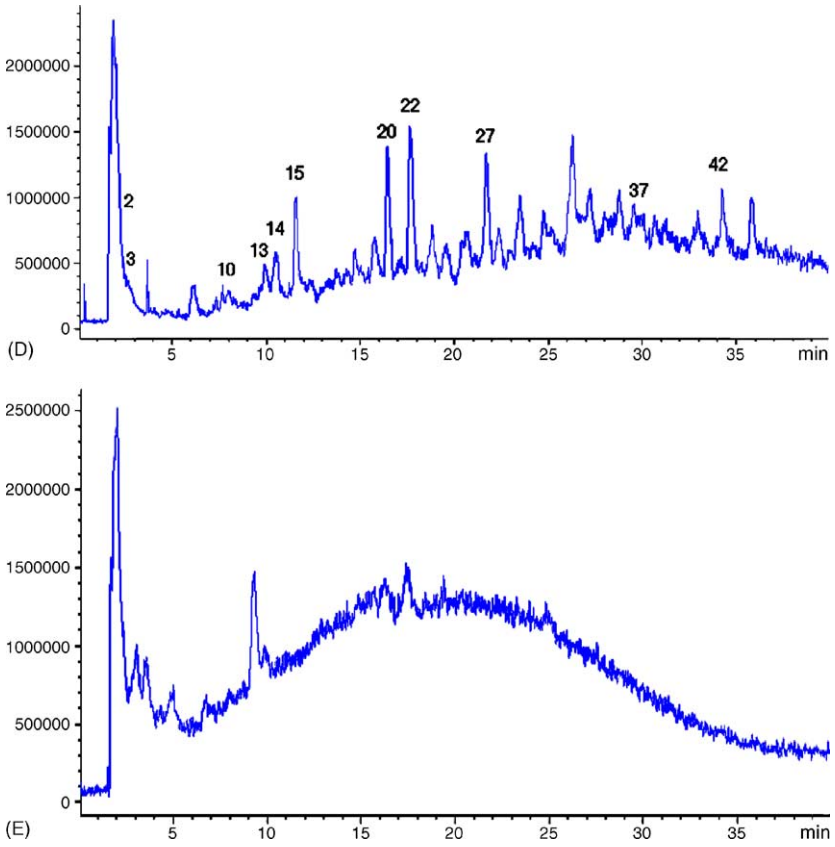

Fig. 7. (*Continued*).

lary wall at this pH. Protein modifications alter the properties of the proteins in such a way that sticking to the negatively charged wall of the capillary is more intense. In the case of the relatively mild effect of glucose modification this effect is probably less profound. It appears feasible to propose that the monotopical modifications possess a smaller deteriorative effect on the arising profile. The separation profile is changed but still the individual peaks can be resolved. Modifiers with higher reactivity lead to more profound changes of the released peptides, which cannot be separated at this pH.

CZE separations of BSA digests in phosphate buffer at pH 2.5 (Fig. 9) provides a well resolved complex peptide maps. At this pH sticking of peptides to the capillary wall is suppressed. BSA modified by glucose gives a similar map as unmodified albumin. There are no significant changes in the profile (number of peaks), however, changes in area/height of some peaks do occur. Considerably more distinct changes were observed after modification of BSA with other (more reactive) oxo-compounds as could have been expected. In principle this is the same result as in the case of HPLC separations. It is also possible to observe some bump on the electrophoretic profile probably owing to the presence of cross-linked peptides perhaps interacting with the capillary wall. It could be mentioned that due to sticking and interaction with the capillary wall (in principle, it is some kind of open-tubular capillary electrochromatography) not all peptide peaks could be observed.

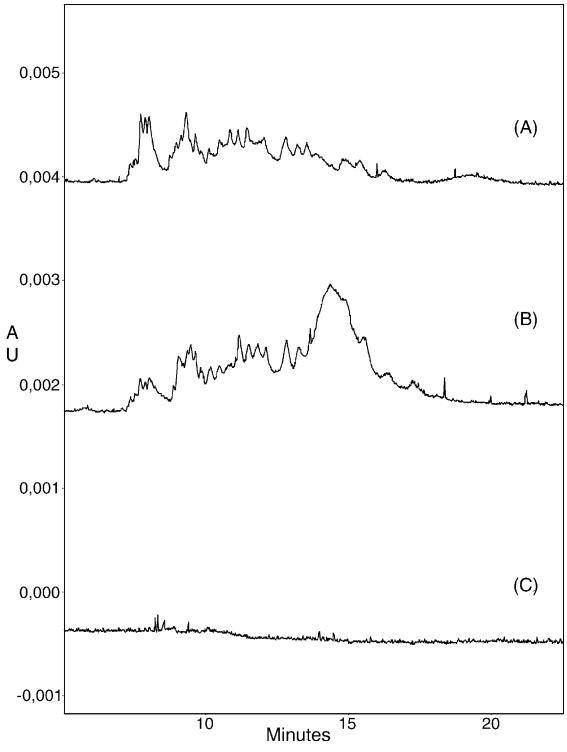

Fig. 8. CZE/UV tryptic peptide maps of unmodified and modified BSA at pH 7.0. Unmodified BSA (A), BSA modified by glucose (B) and ribose (C). CZE separations were performed in 50 mmol/l phosphate buffer, pH 7.0, using the 50/57 cm fused silica capillary.

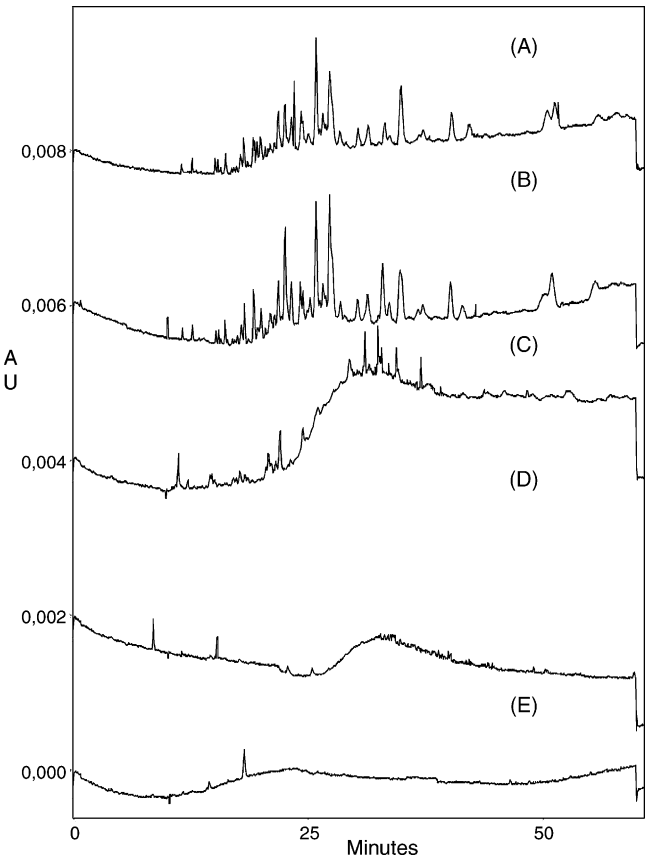

Fig. 9. CZE/UV tryptic peptide maps of unmodified and modified BSA at pH 2.5. Unmodified BSA (A), BSA modified by glucose (B), ribose(C), glyoxal (D), glutaraldehyde (E). CZE separations were performed in 50 mmol/l phosphate buffer, pH 2.5 using the 40/47 cm fused silica capillary.

From the capillary electrophoretic experiments it can be concluded that after the mild modification (relatively short time exposition to glucose) monofunctionally bounded compounds are preferentially formed which fully supports the chromatographic results described earlier in this communication.

4. Conclusions

Application of the set of chromatographic and electrophoretic methods, HPLC/UV, HPLC/MS, PAGE, CZE/UV, allowed effective investigation of the non-enzymatic post-translational modifications of BSA by oxo-compounds. These modifications represent important reactions of proteins, involved in several physiological processes, such as, e.g. ageing. The extent of these modifications was found to be dependent on the nature of oxo-compound used; the reaction progressed in the glucose < ribose < glyoxal < glutardialdehyde order. The mildest modification was observed after the reaction of BSA with glucose; in this case presumably only monofunctional derivatives were formed. On the other hand, the most intensive modifications were found after BSA reaction with glutardialdehyde, which resulted in high degree of both inter- and intra-molecular cross-linking. The changes in the HPLC/UV, HPLC/MS and CZE/UV tryptic peptide maps of both unmodified and modified BSA are indicative of a structural dependence of the reaction between a free amino group of protein and the oxo-compounds; apparently there are peptide sequences which are less or more sensitive to this reaction. Both monofunctional modifications and cross-link formation can be involved depending on the modified sequence and the chemical nature of the modifier.

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