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Non-enzymatic posttranslational modifications of bovine serum albumin by oxo-compounds investigated by high-performance liquid chromatography–mass spectrometry and capillary zone electrophoresis–mass spectrometry

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ABSTRACT

Non-enzymatic posttranslational modifications of bovine serum albumin (BSA) by various oxo-compounds (glucose, ribose, glyoxal and glutardialdehyde) have been investigated using high-performance liquid chromatography (HPLC) and capillary zone electrophoresis (CZE). Both of these methods used mass spectrometric (MS) detection. Three enzymes (trypsin, pepsin, proteinase K) were used to digest glycated BSA. The extent of modification depended on the selected oxo-compound. Reactivity increased progressively from glucose to glutardialdehyde (glucose < ribose < glyoxal < glutardialdehyde). Carboxymethylation of lysine (CML) was the main type of modification detected. The HPLC/MS method achieved higher coverage and a larger amount of CML was identified compared to CZE/MS.

1. Introduction

Peptide mapping is a common approach in proteomic research [1,2]. In this method various proteolytic enzymes are used, however trypsin is the most frequently applied. One of the common techniques used is based on the separation of peptides arising after enzymatic cleavage by high performance separation methods (HPLC, capillary electrophoresis).

Mass spectrometry (MS) is a highly efficient detection method for proteomic research, because it is universal, sensitive, selective and provides both structural and sequence information [3,4]. The introduction of electrospray ionization and matrix-assisted laser desorption ionization contributed to the great progress in the characterization of peptides and proteins [5,6].

In recent years, capillary electrophoresis (CE) has begun to come to the forefront, due to the fact that this method is characterized by a higher separation efficiency than HPLC [7–12]. An important factor is connecting CE with MS, especially with the above-mentioned soft ionization technique (ESI) [13]. This connection not only enables highly accurate determination of the relative molecular mass of separated peptides and proteins, but also provides important structural data on amino acid sequence, the locations of posttranslational modifications and non-covalent interactions [14,15]. ESI is the most widely used mode for connecting with CE, in particular for the following reasons: (i) multiple charged ions of high relative molecular mass can be detected, (ii) molecules can be transferred directly from the separation capillary into the mass spectrometer via the interface [16]. ESI is therefore suitable for the analysis of large biomolecules, which are difficult to evaporate and ionize. The CE–MS connection can be carried out in the following two ways: (i) sheath-flow, (ii) sheathless. The most common approach of this method is to generate a sheath liquid, its advantages being a simple approach and reproducible ordering. The end of the capillary is washed with a sheath liquid, allowing a more stable flow of liquid into the spectrometer and the capillary is grounded with a high voltage applied. The disadvantages are that this method leads to a dilution of the sample being analysed and to an increase in the signal background.

Posttranslational modifications of proteins are important reactions which significantly affect the function of proteins in the body. In principle, they can be divided into enzymatic and non-enzymatic modifications. Non-enzymatic reactions include glycation, which plays an important role in the chronic complications of diabetes mellitus, uremia, in the process of aging and degeneration of the brain.

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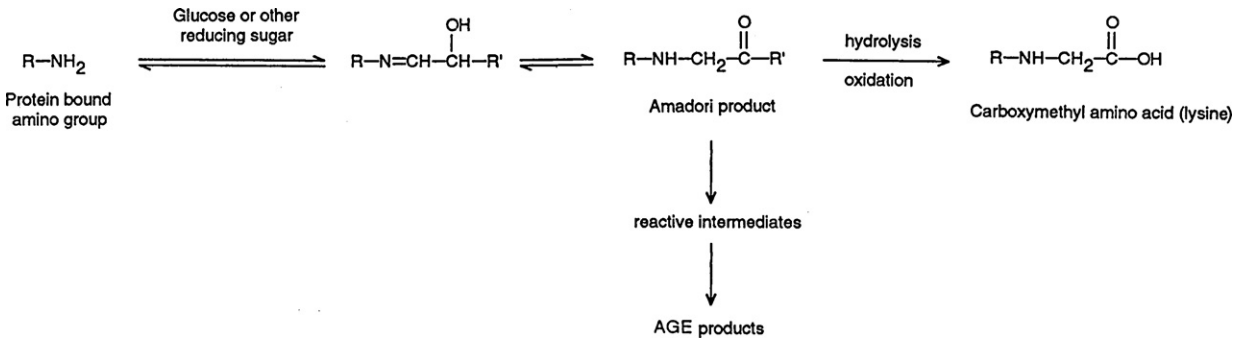

Fig. 1. Scheme of the non-enzymatic glycation of proteins: free amino groups of peptides/proteins react with the carbonyl groups of reducing sugars without the catalytic action of enzymes.

In the non-enzymatic glycation of proteins (earlier called non-enzymatic glycosylation), free amino groups of peptides/proteins react with the carbonyl groups of reducing sugars without the catalytic action of enzymes (Fig. 1). This reaction was first described by Louis Maillard [17], who observed a browning of proteins in their heating with sugars, and in his honor it is called the Maillard reaction. Initially it appeared that this reaction is only relevant in food chemistry, but in 1971 it was found that glycation takes place in every living organism [18], especially if the concentration of sugar in the blood is increased. Non-enzymatic glycation takes place in three steps, which may be called initiation, propagation and termination, analogous to free radical chain reactions.

In the first step, so-called early glycation products are formed. This involves the condensation of reducing sugars (mainly glucose, but also ribose, fructose, pentose, galactose, mannose, ascorbate and xylulose) with the ε-amino group of a lysine residue (or in some cases with the amino group of a terminal amino acid or arginine) and the formation of an unstable Schiff base, which rapidly rearranges to a highly stable ketoamine called the Amadori product [19]. This reaction does not require the participation of enzymes. The variables that regulate it *in vivo*, are the concentration of reducing sugar and protein, the half-life of the protein, the free amino group reactivity and the permeability of the cell walls to sugars. Under *in vivo* conditions, the Amadori product reaches equilibrium after approximately 15–20 days and it accumulates in both the long- and short-lived protein [20]. During propagation, Amadori products are resolved to carbonyl compounds (glyoxal, methylglyoxal, deoxyglukoson), which are highly reactive and can again react with the free amino groups of proteins, thus promoting a non-enzymatic glycation. In the last step, the above-mentioned propagators react with free amino groups and by oxidation, dehydration and cyclization reactions form advanced glycation end products (called AGEs). These products are thermodynamically stable and terminate glycation non-enzymatic reactions [21].

The amount of AGEs in the body reflects the equilibrium between their formation and catabolism (degradation in the tissues, renal excretion). A high level of AGEs forms in diabetic patients and their excretion is insufficient in patients with renal failure. In both groups, there is thus an accumulation of AGEs in plasma, which leads to the production of toxic substances, interaction with the basal membrane and bonds to lipoproteins and collagen. The pathological formation of cross linkages caused by AGEs leads to increased stiffness matrix proteins, limiting their functionality and increasing their resistance to proteolytic enzymes [22].

The aim of this work was a study of posttranslational modifications (namely carboxymethylation of lysine) in bovine serum albumin glycated by various oxo-compounds (glucose, ribose, glyoxal, glutardialdehyde). Special attention was paid to determining the exact location of these modifications.

2. Materials and methods

2.1. Instrumental

The HPLC apparatus used was a 1100 LC/MSD system (Agilent, Palo Alto, CA, USA) consisting of a degasser, a binary pump, an autosampler, a thermostated column and a diode array detector. The instrument was coupled to an ion-trap mass detector (Agilent, LC-MSD Trap XCT-Ultra) with atmospheric pressure electrospray ionization (API-ESI-MS).

The capillary electrophoresis Beckman P/ACE 5000 instrument (Beckman, Fullerton, CA, USA) was used with UV detection at 214 nm. Separation was done in a silica capillary with a total length of 100 cm × 50 μm i.d. The connection to the ion-trap mass spectrometer was done using a grounded needle with a sheath liquid.

2.2. Chemicals

Glucose, sodium dihydrogenphosphate, tris(hydroxymethyl) aminomethane hydrochloride (Tris–HCl) and toluene were purchased from Lachema (Brno, Czech Republic). Crystallized and lyophilized bovine serum albumin (BSA), trypsin, pepsin, proteinase K, ribose, dithiothreitol, iodoacetic acid, ammonium bicarbonate and formic acid were obtained from Sigma (St. Louis, MO, USA). Glyoxal, glutardialdehyde, disodium salt ethylendiammoniumtetraacetic acid and acetonitrile were from Merck (Darmstadt, Germany). Guanidine HCl was obtained from Appli Chem, Biochemica (Darmstadt, Germany). Sodium chloride was from Penta (Chrudim, Czech Republic), and acetic acid from Lach-Ner (Neratovice, Czech Republic).

All solutions were prepared in Milli-Q ultra pure water (Millipore, Bedford, MA, USA).

2.3. Sample preparation

2.3.1. Preparation of glycated and control BSA

Bovine serum albumin (BSA) was dissolved in phosphate buffer (0.2 mol/l NaH_2PO_4; pH 7.4) to a final concentration of 1 mg/ml. This solution was incubated (at 37 °C, 7 days) with one of the various selected substances containing an oxo-group (glucose, ribose, glyoxal and glutardialdehyde), whose concentration was 0.1 mol/l in the solution of BSA. A BSA control sample was prepared in a similar way, only without the added sugar. Due to the contamination which could occur during incubation, sodium azide and a thin layer of toluene were added to each sample. Dialysis followed after incubation and lasted 24 h.

2.3.2. Reduction and alkylation (carboxymethylation) of BSA

0.5 ml of pH 8.4 buffer (6 mol/l guanidine HCl; 1.2 mol/l Tris–HCl; 2.5 mmol/l Na_2EDTA) was added to a 5 mg lyophilized sample of BSA. Reduction was performed by adding 25 μl of 1 mol/l dithiothreitol. Samples were incubated for 30 min at 65 °C. The subsequent carboxymethylation took place using 60 μl of 1 mol/l iodoacetic acid (incubation at room temperature, 40 min in the dark). This reaction was stopped by adding 15 μl of 1 mol/l dithiothreitol.

2.3.3. Enzymatic digestion of BSA

BSA samples were desalted in Econo-Pac 10 DG columns (Bio-Rad Laboratories, Hercules, CA, USA) and lyophilized. Desalted BSA samples were digested by one of these enzymes: trypsin, pepsin or proteinase K. Blank samples were prepared by incubating the enzyme solution alone under identical conditions.

2.3.3.1. Digestion of BSA by trypsin. BSA samples were dissolved to a concentration of 5 mg/ml with 20 mmol/l ammonium bicarbonate buffer (pH 7.8) and treated with trypsin (1:50 enzyme:substrate ratio). Incubation was done at 37 °C for 2 h.

2.3.3.2. Digestion of BSA by pepsin. BSA samples were dissolved in acetic acid (0.5 mol/l; pH 2.5) to a concentration of 2 mg/ml and digested with pepsin (1:50 enzyme:substrate ratio). Incubation was done at 37 °C for 2 h.

2.3.3.3. Digestion of BSA by proteinase K. BSA samples were dissolved to a concentration of 5 mg/ml with 10 mmol/l Tris–HCl buffer (pH 7.5) and treated with proteinase K (1:50 enzyme:substrate ratio). Incubation was done at 37 °C for 2 h.

2.4. Conditions for HPLC/MS analysis

Chromatographic analysis of BSA digest was done in a Jupiter 4 μm Proteo 90 A column (250 × 2 mm i.d., Phenomenex, Torrence, CA, USA). A 20 μl sample was injected. Elution was obtained by applying a linear gradient between mobile phases A (water with 0.1% (v/v) formic acid) and B (acetonitrile with 0.085% formic acid). Separation was started with an isocratic elution for 2 min with 2% mobile phase B, followed by a gradient elution to 35% of B by 40 min. Finally the column was washed for was 10 min with 100% B. Before further analysis, the column was equilibrated with 2% B for 10 min. The flow rate was 0.25 ml/min. The column temperature was held at 25 °C and detection was done at 214 nm.

Atmospheric pressure ionization-electrospray ionization (API-ESI) positive mode ion-trap mass spectrometry was used. Operating conditions were: drying gas N_2 10 l/min, drying gas temperature 350 °C, nebulizator pressure 172.4 kPa.

2.5. Conditions for CE/MS analysis

Separations were performed at a voltage of 25 kV, samples of BSA were injected hydrodynamically (3.45 kPa; 300 s). Formic acid (0.25 mol/l) was used as the background electrolyte.

A Beckman P/ACE 5000 instrument was coupled with a grounded needle and a sheath liquid (5 mmol/l ammonium acetate/isopropanol (1:1, v/v), flow rate 3 μl/min) to the ion-trap mass spectrometer (Agilent, LC-MSD Trap XCT-Ultra). The following conditions were applied: drying gas N_2: 8 l/min; drying gas temperature: 150 °C; nebulizator pressure: 3.45 kPa. Before the first analysis, the capillary was washed with: 1 mol/l NaOH; water; 1 mol/l HCl; water; buffer. Each washing step lasted 20 min. Between runs, the capillary was only rinsed with the running buffer (5 min).

Table 1
Results of HPLC/MS analysis of control and glycated samples of BSA, digested by trypsin, pepsin, proteinase K: coverage and location of carboxymethyled lysine (CML).

	Trypsin		Pepsin		Proteinase K	
	Coverage	CML	Coverage	CML	Coverage	CML
Control sample	44%	–	65%	–	44%	–
Glucose	54%	140 401 412 455 489 548 561	69%	–	53%	140 – – – – – –
Ribose	53%	28 36 88 140 204 248 346 401 412 455 495 498 548 561	54%	138 412 489 568 597 – – – – – – – – –	56%	88 138 140 183 204 374 437 455 489 495 523 544 597 –
Glyoxal	53%	100 204 245 248 256 285 297 401 412 437 455 489 495 523 548	41%	130 309 318 420 489 523 568 597 – – – – – – –	44%	88 130 138 140 204 228 285 374 401 412 437 489 495 523 597
Glutardialdehyde	18%	–	20%	–	22%	–

Results from the MS (MS/MS) were interpreted using the software Spectrum Mill (Agilent v. 3.02). Searches were performed in the specific database for BSA extracted from the SwissProt database.

3. Results

3.1. HPLC/MS analysis of BSA digested by enzymes

Peptide maps of enzymatic digested BSA were obtained using HPLC techniques with MS and MS/MS detection. Trypsin, pepsin and proteinase K were used for cleavage. The extent of the post-translational modification depended on the nature of the modifiers used: glucose, being a mild modifier, offers a rich peptide profile, while ribose treatment led to a moderately modified protein more resistant to enzymatic cleavage than native BSA (Fig. 2). Reactions with dioxo-compounds (glyoxal, glutardialdehyde) produced modified proteins highly resistant to enzymatic cleavage. This resistance is caused by a cross-linking reaction of the dioxo-compounds with the free amino groups of two protein molecules.

Carboxymethylation of lysine (CML) was the main type of modification detected. The chromatographic positions of CML proteins caused by various modifiers are indicated with arrows in Fig. 2. Chromatographic separations of CML-peptides arising after modification by various oxo-compounds are given in Table 1. Both the

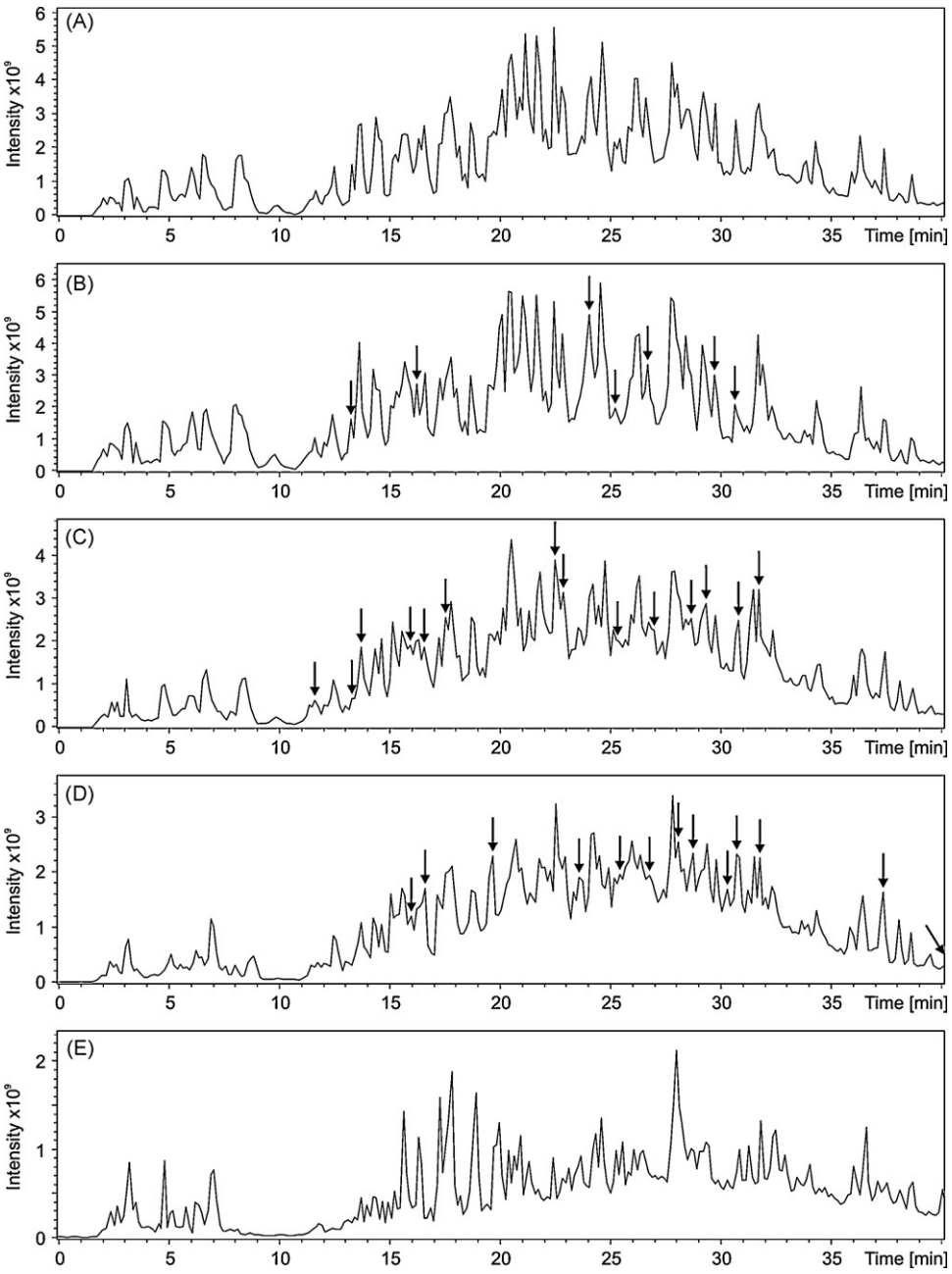

Fig. 2. HPLC/ESI-MS tryptic peptide maps of BSA: (A) control sample, BSA modified by (B) glucose, (C) ribose, (D) glyoxal, (E) glutardialdehyde (arrows indicate CML-modified peptides). For details see Section 2.

emergence of CML and coverage are strongly influenced by the modifier used. Samples modified by glutardialdehyde provided the minimum coverage. CMLs were not found in these samples, but oxidized methionines were (at position 111, 208 and 469).

Three enzymes (trypsin, pepsin, proteinase K) with different cleavage specificities were used to digest modified BSA. Trypsin preferentially digests the peptide bond at arginine and lysine. Pepsin digests mainly at phenylalanine, leucine, tryptophan, tyrosine, alanine, glutamic acid and glutamine. Proteinase K is not so strictly specific, it mainly digests at alanine, cysteine, glycine, methionine, phenylalanine, serine, tyrosine and tryptophan. Combining these three enzymes enabled a high protein coverage to be achieved and location of posttranslational modifications to be detected. The total coverage of BSA for the individual modifiers was: glucose 85%, ribose 82%, glyoxal 75% and glutardialdehyde 42%. The coverage of the unmodified control sample was 81%. The amino acid sequence of BSA modified by glucose and digested by three various enzymes is shown in Fig. 3. Identified peptides are in bold. Lysines and arginines, which may undergo glycation modifications, are underlined.

A highly reactive position in BSA mentioned in the literature is the peptide sequence 548–557 (KQTELVELLK) [23]. Lysine 548 is prone to carboxymethylation. Other reactive sites reported in the literature [24] are lysines at positions 88, 248, 256, 263, 309, 495, 528 and 597. Thanks to the digest with three different enzymes, not only was a higher coverage obtained, but also different carboxymethyled lysines. For example, CML occurs at position 140 in tryptic and proteinase-digested BSA. This shows the overlap of the peptides in the peptide mapping and founded reactivity proteins. Positions 401, 412, 455 are among the most reactive in addition to the above mentioned lysine at position 548. However, it should be

Fig. 3. Amino acid sequence of BSA modified by glucose (identified segments are in bold, lysines and arginines are underlined, modified lysines are highlighted in larger bold italic type letter). For details see Section 2.

noted, that the extent of modification is not 100%, this means that unmodified lysine can be observed as well as CML.

3.2. CE/MS analysis of BSA digested by enzyme

MS is widely used to determine protein sequence, posttranslational modifications, to determine the specific position of a modification when coupled with high performance separation techniques. CE is a separation technique with a high resolution capability. However, CE/MS is not a simple technique and always requires the optimization of various parameters (e.g. voltage in the capillary and flow of sheath liquid) [25].

A problem in CE/MS with the sheath-liquid configuration is the dilution of the samples by the sheath liquid and the subsequent reduction in sensitivity. A suitable solution can be sample preconcentration. In our approach, we used the dynamic pH junction technique. It is based on a longer sample injection time and the sample and the background buffer having different pH levels [26]. Samples are dissolved in the solution with a higher pH than the background buffer. A longer sample injection time creates a longer zone and the difference between the pH of the sample matrix and the basic buffer causes changes in their electrophoretic mobilities. The result is a narrowing of the bands at the buffer interface. This technique can increase sensitivity by a factor of 550–1000.

We used this method for the analysis of BSA glycation after trypsin digestion (Fig. 4). CML-modified peptides were identified (the positions of the CML arising after modification by various oxo-compounds are indicated with arrows). Table 2 shows the specific location of the CML-peptides in the BSA sequence and its coverage. This method achieved a lower coverage and a smaller number of CML were identified compared to the above-mentioned HPLC/MS. This lower sensitivity was caused by dilution of the analyte by the sheath liquid.

4. Discussion

This work deals with studying the separation of enzymatic digests of non-enzymatically modified (glycated) BSA by various oxo-compounds (glucose, ribose, glyoxal, glutardialdehyde). We have to mention that model *in vitro* system has been used in this work and therefore the results obtained have only a limited value for extracting conclusion for living systems (at least concentration of reactive oxo-compounds used is not at physiological level).

A mass spectrometer with electrospray ionization and an ion trap was used to determine the protein sequence, posttranslational modifications and to determine the exact location of these modifications. It was used together with HPLC and CE. The extent of modification depended on the selected oxo-compound. The reaction progressed in the order glucose, ribose, glyoxal and glutardialdehyde, with the mildest modifying factor being glucose. On the other hand, the most intensive modifications were found after BSA reaction with glutardialdehyde, when a large number of cross-links were formed and these complexes were highly resistant to enzymatic cleavage.

The HPLC–MS technique detected modifications occurring in the peptide sequences of enzymatic digests of glycated BSA. The main type of modification was the CML. It was confirmed that the most reactive glycated location is lysine 548. Other highly reactive sites were found on lysines 401, 412, and 455 (these positions were identical for all the modifiers except for glutardialdehyde). Another type of detected modification was oxidation of methionine. This modification was found only in samples modified by glutardialdehyde (at position 111, 208 and 469).

Table 2
Results of CE/MS analysis of control and glycated samples of BSA, digested by trypsin: coverage and location of carboxymethyled lysine (CML).

	Trypsin	
	Coverage	CML
Control sample	24%	–
Glucose	32%	–
Ribose	31%	36 401 412 455 495 498 548 561
Glyoxal	46%	36 100 245 248 256 401 455 548
Glutardialdehyde	6%	–

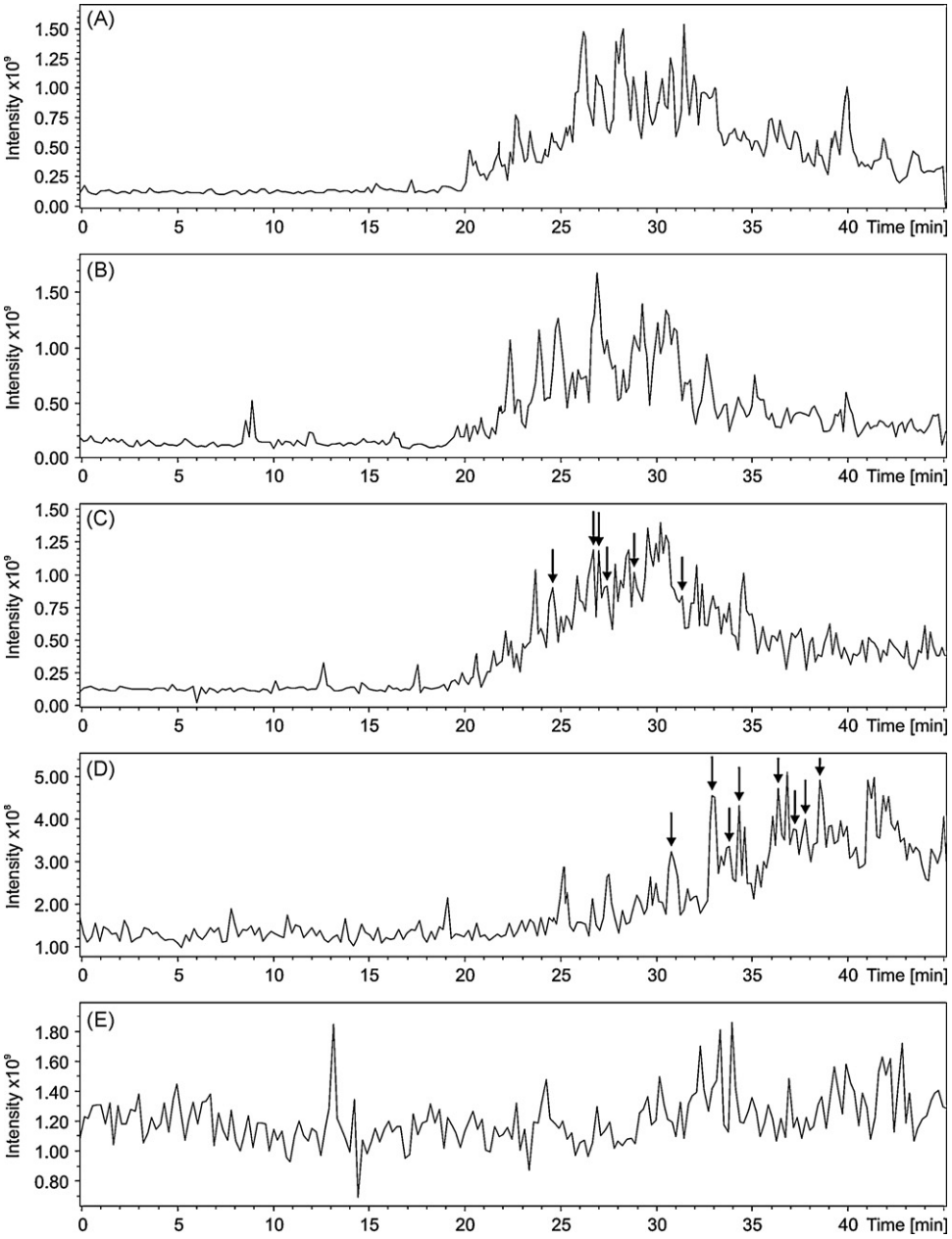

Fig. 4. CE/ESI-MS tryptic peptide maps of BSA: (A) control sample, BSA modified by (B) glucose, (C) ribose, (D) glyoxal, (E) glutardialdehyde (arrows indicate CML-modified peptides). For details see Section 2.

The lowest level of CML was detected with the CE–MS method (compared with HPLC–MS). Also, the coverage of the amino acid sequence was lower. Dilution of the analyte by the sheath liquid was a cause of significant reduction in sensitivity. The dynamic pH junction did not completely prevent this decrease in sensitivity.

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