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Evaluation of recombinant human interferon α-2b structure and stability by in-gel tryptic digestion, H/D exchange and mass spectrometry

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Abstract

Stability and structure of recombinant interferon α-2b (*rHu*INF α-2b) was studied by mass spectrometry (MALDI-TOF and Q-TOF MS), chromatography (LC-UV-FLD-DAD, LC-MS) and CD spectroscopy. Besides analysis of the substance according to Ph. Eur. methods, two additional mass spectrometric methods were developed. The aim of both methods was to estimate structure–stability relationship connected to methionine oxidation or protein degradation. Preservation or degradation of protein structure was confirmed by H/D exchange in four separate experiments. Kinetics of deuterium incorporation into macromolecule was monitored over 2670 min. Isoforms of *rHu*INF α-2b were separated by 2D gel electrophoresis. In-gel digestion with trypsin and mass spectrometric analysis, performed on four separated isoforms at the mass corresponding to the mass of *rHu*INF α-2b with oxidized methionines, confirmed oxidation of all methionines to a different extent. Another four isoforms observed in 2D gel are most likely dimers of the same macromolecules with scrambled disulphide bridges. Oxidation and dimerisation are consequences of protein interaction with oxidizing reagents in polyacrilamide gel.

Keywords: Recombinant human interferon α-2b; Stability; MALDI-TOF MS; H/D exchange; 2D gel electrophoresis; Methionine oxidation

1. Introduction

Interferons are cytokines with a wide spectrum of biological activities including antiviral effect, cell growth inhibition and immunomodulatory properties [1,2]. There are three main types of human interferons according to the initial producer cell and immunospecificities: α (leucocytes), β (fibroblasts) and γ (immune). Alpha interferon has been approved for therapeutic use against hairy-cell leukemia, AIDS-related Kaposi's sarcoma and hepatitis C. It has also been found effective against chronic hepatitis B, a major cause of liver cancer and cirrhosis [3–5]. Although interferon was discovered in late 1950s, it became available in greater quantities after more than two decades through recombinant DNA technology [2]. Since then, genes for several subtypes of the alpha interferon have been cloned, but only interferon α-2a and α-2b were commercialized.

Purity of the recombinant human interferon (*rHu*INF) was investigated by different electrophoretic and chromatographic methods [6–9]. The purified and biologically active interferon was subject of numerous structural characterizations by mass spectrometry as it provides accurate molecular mass and sequence information. Different ionization techniques were used in previous *rHu*INF α-2b studies, e.g. fast atom bombardment [10], ^{252}Cf plasma desorption, Cs^+ liquid secondary ion mass spectrometry [11] and thermospray [12]. However, mild ionization gained by matrix-assisted laser desorption/ionization [13,14] and ESI (electrospray ionization) and TOF analyzers (time-of-flight analyzers) enabled protein analysis with high mass accuracy at the femtomole level [15].

Special care should be taken in characterization of proteins like *rHu*INF α-2b that can be easily degraded if not preserved on proper storage conditions (−80 °C). In this work set of analytical methods proposed by European Pharmacopoeia was upgraded by using mass spectrometry as an identification and characterization tool. Preservation of interferon α-2b three-dimensional structure in deuterated environment was

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investigated by MALDI-TOF operating in linear mode. MALDI produces predominately singly charged ions and is less sensitive to involatile salts than ESI [16]. Reflectron mode was used for detailed characterization of *rHu*INF α-2b isoforms separated by 2D gel electrophoresis.

2. Experimental

2.1. Chemicals

Acetonitrile, gradient grade for chromatography, Merck (Darmstadt, Germany); water MILLI Q quality (resistivity less than 18.2 MΩ cm at 25 °C and total organic carbon less than 5 ppb); trifluoroacetic acid, TFA, p.a., Merck (Darmstadt, Germany); potassium dihydrogenphosphate, crystalline, extra pure (Ph. Eur.), Merck (Darmstadt, Germany); matrices: sinapinic acid, SINA, p.a. and α-cyano-4-hydroxycinnamic acid, CHCA, p.a., Sigma–Aldrich (St. Louis, MO, USA); urea, p.a., Merck (Darmstadt, Germany); (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate, CHAPS, Amersham (Piscataway, NJ, USA); dithiothreitol, ultra, more than 99% pure, Sigma–Aldrich (St. Louis, MO, USA); ampholyte Pharmalyte, Amersham (Piscataway, NJ, USA); bromphenol blue, Bio-Rad (Hercules, CA, USA); sodium dodecyl sulfate, 99% pure (Bio-Rad, Hercules CA, USA); 2-Amino-2-hydroxymethyl-1,3-propanediol hydrochloride, Tris–HCl, p.a., Roche (Mannheim, Germany); interferon α-2b standard, certificated reference standard CRS, European Directorate for the Quality of Medicines (Strasbourg, France); trypsin from porcine pancreas, sequencing grade, Merck (Darmstadt, Germany); deuterium oxide, 99.9 at.% D, Sigma–Aldrich (St. Louis, MO, USA).

2.2. Spectroscopic analysis of secondary structure

CD spectrum of *rHu*INF α-2b water solution ($\gamma = 0.01$ mg ml^{-1}) was recorded with a CD spectrometer J-810 (Jasco, Tokyo, Japan) at 25 °C in the range 190–270 nm. Content of α-helix was calculated using Spectral Manager program.

2.3. Chromatographic analysis

Gel-filtration chromatography was performed on TSK 3000 SW column. Column temperature was 25 °C and sample temperature 7 °C. Injection volume of 0.02 mg ml^{-1} *rHu*INF α-2b solution (phosphate buffer, pH 6.7) was 50 μl. Mobile phase was the same as in Ph. Eur. [17]. Flow rate was 0.6 ml min^{-1} for 60 min. UV diode array detection at 214 and 280 nm was used. Excitation and emission spectra ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 348$ nm) were recorded by fluorescence detector.

Reversed-phase liquid chromatography was performed on ThermoHypersil PEP300 column, octadecyl – silica (C_{18}), 100 mm × 4.6 mm (5 μm), pore size 30 nm. Column temperature was 25 °C and sample temperature 5 °C. Mobile phases, gradient conditions and sample preparation were the same like in Ph. Eur. [17].

2.4. Mass spectrometric analysis

ESI MS spectra were obtained on Micromas Q-TOF Micro instrument (Manchester, UK), under the following conditions: positive ion mode, capillary voltage 3000 V, cone voltage 30 V, desolvation temperature 150 °C, source temperature 120 °C, cone gas 50 l h^{-1} and desolvation gas 450 l h^{-1}.

MALDI-TOF MS spectra in reflectron and linear mode were obtained on Applied Biosystems Voyager DE STR instrument (Foster City, CA). The laser wavelength was 337 nm (N_2 laser) and laser frequency amounted 20 Hz. Ions were accelerated to 2.0 kV. Delayed extraction was 150 ns. All peptide spectra were obtained in positive ion mode with reflectron. Calibration type was external and calibration was performed with bradykinin fragment 1–7, angiotensin II (human), ACTH fragment 18–39 (human) and insulin oxidized B chain (bovine), Sigma–Aldrich, St. Louis, MO, USA. Sample aliquot of 1 μl was mixed with 9 μl of CHCA matrix (10 mg ml^{-1} CHCA in 50% acetonitrile and 0.3% TFA in water, v/v). Volume of 1 μl was deposited onto the plate.

MALDI spectra of intact protein with linear detector were obtained in positive ion mode, acceleration voltage was 2.5 kV and delayed extraction was 600 ns. Calibration type was external and calibration was performed with aldolase (rabbit muscle) and albumin (bovine serum), Sigma–Aldrich, St. Louis, MO, USA. Volume of 13.3 μl *rHu*INF α-2b, $\gamma = 7.5$ mg ml^{-1} was diluted to 100 μl with D_2O (99.9% purity). Sample aliquot of 1 μl was mixed with 9 μl of sinapinic acid matrix (10 mg ml^{-1} SINA in 50% acetonitrile and D_2O, v/v). Volume of 1 μl was deposited onto the plate.

2.5. 2D gel electrophoresis

For IPG strips rehydratation following solutions were used: 8 mol l^{-1} urea, 2% CHAPS, 0.2% dithiothreitol, 0.5% Pharmalyte (pH 3–10) and 0.002% bromphenol blue. The solution of *rHu*INF α-2b (10 μl, $\gamma = 7.5$ mg ml^{-1}) was loaded during rehydratation process which lasted 10 h at 4 °C. Isoelectric focusing was carried out for 30 min at 500 V, 30 min at 1000 V and 100 min at 5000 V. The gel was equilibrated by soaking in 2% sodium dodecyl sulfate/30% glycerol/0.002% bromphenol blue/6 mol l^{-1} urea and 2-Amino-2-hydroxymethyl-1,3-propanediol hydrochloride solution for 15 min. Dithiothreitol solution (10 ml, $\gamma = 10$ mg ml^{-1}) was added during that time. Polyacrilamide gel (12%, thicknes of 1.5 mm) was used for SDS PAGE which lasted for 40 min at potential of 200 V and constant power of 400 W. Proteins were stained with solution of Coomasie Blue.

Excised gel spots were treated with trypsin according to the method described by Shevchenko et al. [18].

3. Results and discussion

3.1. Analysis of intact rHuINF α-2b

To examine the purity of recombinant human interferon α-2b, intact macromolecule was analyzed by circular dichroism

and liquid chromatography. Content of α-helix was calculated to be 56%, which is in agreement with data found in literature for native *rHu*INF α-2b [1,2]. Purity of interferon α-2b was examined by gel-filtration and reversed-phase chromatography with UV absorption and fluorescence detection. Contaminants, oxidized forms, dimers or polymers of the protein were not observed in amounts above 0.5%.

Accurate molecular mass of the protein was determined by mass spectrometry. Full scan MS spectra obtained by ESI showed signals from multiply charged ions of *rHu*INF α-2b and their adducts with one or more ions of trifluoroacetic acid from mobile phase (deconvoluted spectrum at *m*/*z* 19 265.73, 19 380.66, 19 493.52, etc.). Observed mass difference of 114 and 113 in deconvoluted spectra can be used as indication of good instrument calibration and accurate molecular mass determination [15]. Almost the same mass accuracy (*m*/*z* 19 265.63) was obtained by separate analysis performed on MALDI-TOF instrument with internal calibration.

3.2. Kinetics of H/D exchange

An aliquot of standard substance (*rHu*INF α-2b CRS) was tested for activity, structure characteristics and purity to confirm compliance with European Pharmacopoeia requirements [17]. Additionally, the standard substance was evaluated by chromatographic, spectroscopic and spectrometric methods (data not shown). Experimental results of numerous methods confirmed preservation of primary and secondary structure, activity and purity. Based on these results, quality of *rHu*INF α-2b CRS was confirmed to be within acceptable limits or theoretical values expected for the standard substance. Mass spectra of *rHu*INF dissolved in D_2O, obtained by MALDI-TOF MS in linear mode, can be used to estimate the extent of degradation during the storage period. Unstable proteins, like *rHu*INF α-2b, that require special storage conditions (−80 °C) could quite easily lose activity or active three-dimensional structure if the protein was frozen and unfrozen more than once. To define standard H/D exchange curve for properly stored *rHu*INF α-2b of preserved structure, the extent of H/D exchange at a given time was measured by mass spectrometry (Fig. 1).

Standard substance of *rHu*INF α-2b was incubated for 2670 min at 25 °C. Rate of H/D exchange at a given time revealed linear exponential curve expected for this type of experiment shown in Fig. 2 [19,20].

The period up to 500 min was the most important for defining the slope of the kinetic curve representing standard substance. In that time most of the amide protons were exchanged with deuterons forming the linear portion of the curve. After 500 min at 25 °C protein started to precipitate and finally settled down at the bottom of the reaction tube after 1000 min. Process of protein denaturation caused by exchange of deuterons and secondary and tertiary structure disruption lowered the amount of dissolved protein in solution over time and therefore its signal intensity in mass spectra. Nevertheless, trend of H/D exchange was continued up to 1300 min when the protein was almost completely saturated with deuterons (Fig. 2). Almost the equal curve profile was obtained for proteins stored at −20 °C (1 and 2 years

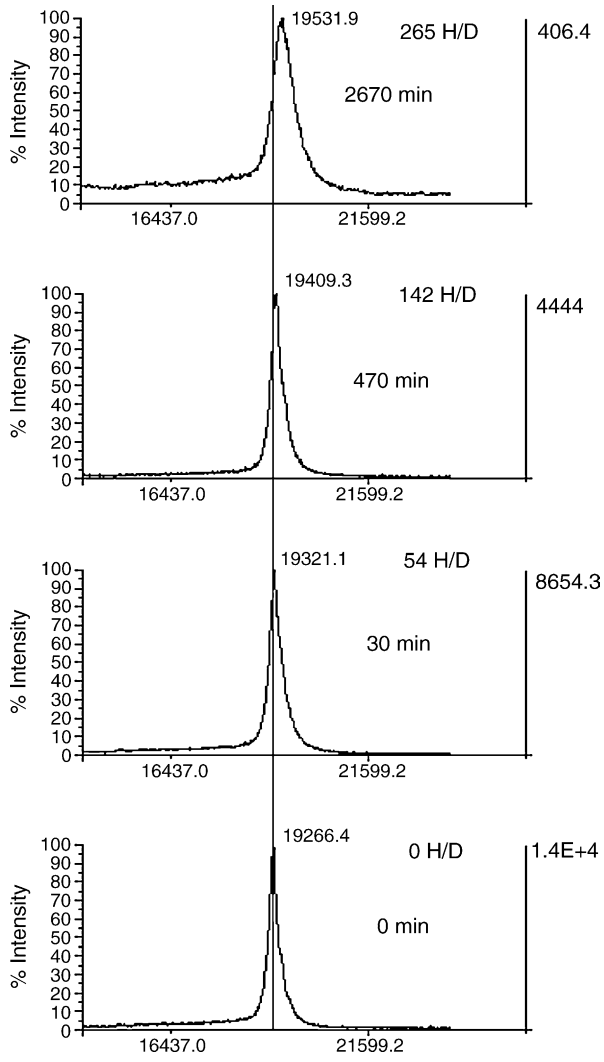

Fig. 1. Linear mode MALDI-TOF MS spectra of properly stored *rHu*INF α-2b obtained after 0, 30, 470 and 2670 min of H/D exchange. Samples were diluted and incubated in D_2O at 25 °C, except the sample denoted as 0 min, dissolved in water.

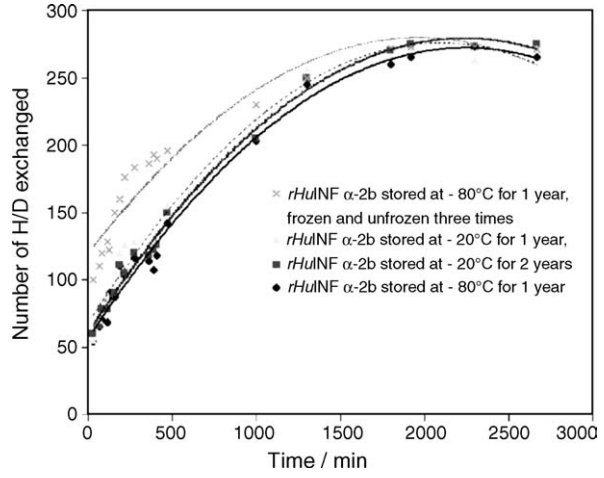

Fig. 2. The kinetics of deuterium incorporation into *rHu*INF α-2b at 25 °C. Samples were stored at −80 °C for 1 year (unfrozen once and frozen and unfrozen three times) and −20 °C for 1 and 2 years.

of storage), while *rHu*INF α-2b frozen and unfrozen three times exchanged twice more protons with deuterons in only 30 min relative to the standard curve.

Maximum measured number of incorporated deuterons was 275. Process of H/D exchange is time consuming and therefore, by raising the temperature for 10–20 °C in controlled environment, it can be accelerated. Differences between proteins stored at different conditions were clearly visible at 25 °C after 250 min of experiment (linear portion of the curve). The experiment time period of 2670 min was not necessary to establish structural differences, however it additionally confirmed validity of the method. Experimental error of ±5 Da did not significantly influence on the shape of the plotted curve. By this method, differences in primary structures, as well as denaturation or secondary and tertiary protein structural changes can be easily detected with minimal sample consumption. The experiment was repeated three times under the same conditions with different batches of standard substance properly stored at −80 °C. Compared results confirmed reproducibility of the method with maximum deviation of ± 8%.

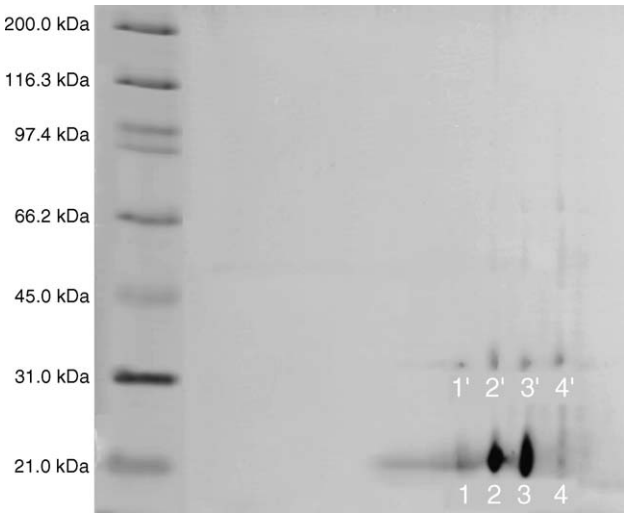

Fig. 3. 2D Coomassie-stained gel electrophoresis of *rHu*INF α-2b. Isoforms 1, 2, 3 and 4 were analyzed by MALDI-TOF mass spectrometry after in-gel trypsin digestion followed by peptide extraction.

3.3. In-gel tryptic digestion

The isoforms of macromolecule *rHu*INF α-2b were separated by 2D gel electrophoresis (Fig. 3). Four isoforms were observed at the mass corresponding to the mass of *rHu*INF α-2b and another four at the mass corresponding to the dimer of the same molecule (bands 1′, 2′, 3′ and 4′, Fig. 3). Series of isoforms were visible up to tetramers in trace amounts. The most probable cause of polymeric structures appearance was formation of intermolecular disulfide bridge between two or more macromolecules. Process of probable disulfide bonds reformation was observed in both 2D and SDS PAGE electrophoresis only when starting concentration of the analyzed sample was high (more than 0.5 mg ml^{-1}). Deamidation of glutamine (Gln) and asparagines (Asn), isomerization or racemization of aspartic (Asp) and glutamic acids (Glu) and oxidation of methionine (Met) are commonly observed chemical modification that could reduce in vitro or in vivo activity of recombinant proteins or cause immune response [21,22]. Deamidation is a common chemical modification resulting in the conversion of an asparagine residue to a mixture of isoaspartate and aspartate. Deamidation of glutamine residues can occur as well, but does so at a much lower rate. Oxidation of methionine residues leads to formation of methionine sulfoxide under mild oxidative

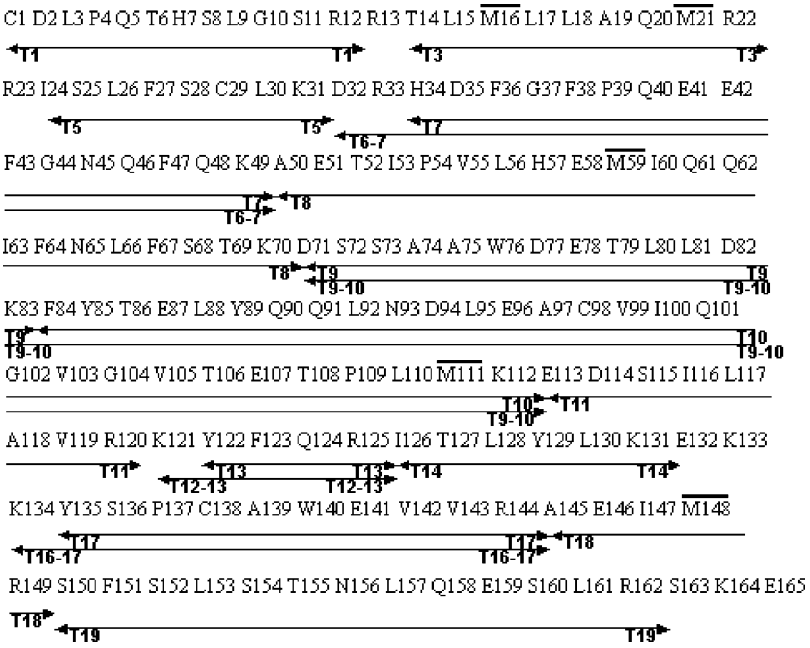

Fig. 4. Primary amino acid sequence of *rHu*INF α-2b and tryptic peptide fragments obtained after in-gel digestion. Five methionines that can be oxidized after 2D gel electrophoresis are additionally marked with lines.

conditions or methionne sulfone under severe and prolonged oxidative stress. Since *rHu*INF α-2b contains 12 Gln, 4 Asn and 5 Met residues, deamidation and oxidation take a part in protein degradation by influencing on structural and activity changes. Both processes were suggested to explain presence of different isoforms [1,23]. In this work, detailed structural characterization was performed. Bands isolated at mass corresponding to *rHu*INF α-2b were treated with trypsin. Tryptic digests of four separated isoforms (bands 1, 2, 3 and 4) were analyzed by mass spectrometry. Except external calibration, the spectra were additionally internally calibrated with matrix clusters and trypsin autolysis fragments [24]. This additional recalibration improved mass accuracy, especially at low signal intensity.

The process of deamidation is sequence-dependent [25], so in the case of complete peptide deamidation for each amino acid taking part in that process, there would be a mass difference of 1 Da between signals in the mass spectra. Sometimes, detection of partial deamidation by mass spectrometry can be extremely difficult, because signal difference of 0.0193 Da (^{12}CO and ^{13}CNH) could be obtained only by fourier transform ion cycloctron resonance mass spectrometry of high mass resolution (resolving power more than 250 000) [26]. Oxidation of methionine leads to mass difference of 16 Da in relation to not oxidized protein. Signal shifts in mass spectra of tryptic fragments (T13, T18, T3, T19, T7, T8 and T1-10) of four isolated bands were monitored. Each of these fragments has at least one amino acid that can be subjected to deamidation or oxidation. Tryptic fragments of *rHu*INF α-2b are shown in Fig. 4 and mass spectra of fragments T13, T18, T3, T19, T7 and T8 (band 2, tryptic digest) are shown in Fig. 5.

All Met-, Gln- and Asn-containing tryptic fragments for each isoform were correlated according to the signal intensity (Table 1).

The same isoforms obtained by isoelectric focusing were separated and analyzed by 2D gel electrophoresis and mass spectrometry. Unlike isoelectric focusing, 2D gel electrophoresis provided information on *rHu*INF α-2b aggregation, while structural analysis revealed differences between principal bands. Intact *rHu*INF α-2b previously tested for oxidized forms, which were below the limit of quantification, was analyzed by 2D gel

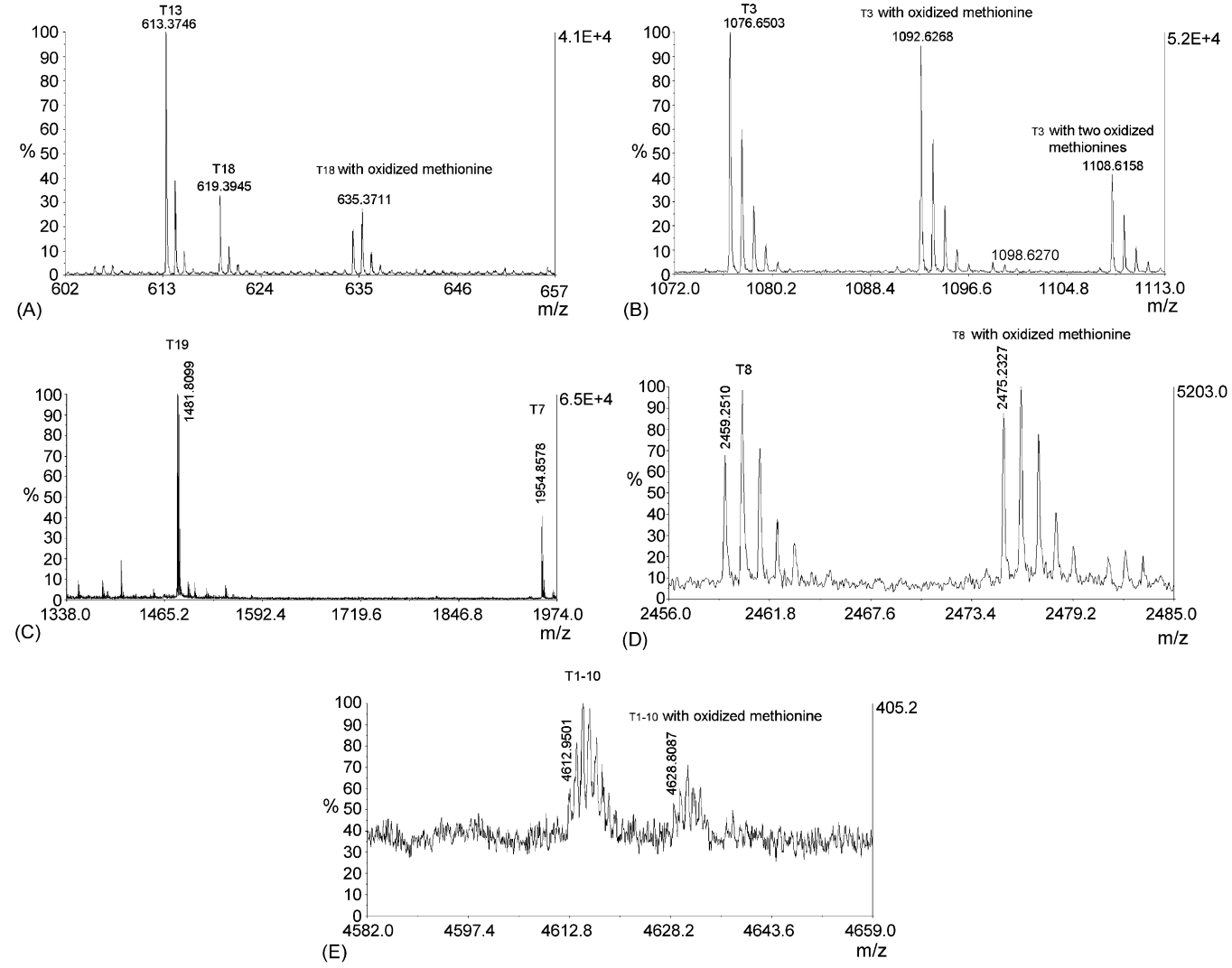

Fig. 5. Mass spectra (MALDI-TOF with reflectron) of tryptic peptides extracted from 2D gel after in-gel digestion of isoform 2. The *m*/*z* values of fragments T13 and T18 (A), T3 (B), T19 and T7 (C), T8 (D) and T1-10 (E) revealed different extent of methionine oxidation.

Table 1
Calculated and observed *m/z* of tryptic fragments extracted from the gel: T13, T18, T3, T19, T7, T8 and T1-10

Tryptic fragment *m/z* in silico	Amino acids subjected to deamidation or oxidation	Isoform 1 $[M+H]^+$ *m/z* observed changes	Isoform 2 $[M+H]^+$ *m/z* observed changes	Isoform 3 $[M+H]^+$ *m/z* observed changes	Isoform 4 $[M+H]^+$ *m/z* observed changes
T13 (612.30)	Q 124	613.26	613.38	613.46	613.49
T18 (618.32)	M 148	*619.23* (**635.25**)	619.39 (635.37)	619.43 *635.45*	*619.50 (635.48)*
T3 (1075.59)	M 16, Q 20 and M 21	1076.48 (**1092.43**, 1108.43)	**1076.65** (**1092.62,** *1108.43*)	**1076.78** (**1092.77,** *1108.77*)	*1076.83* (**1092.81**, *1108.80*)
T19 (1480.75)	N 156 and Q 158	1481.70	1481.81	1481.99	1482.04
T7 (1953.86)	Q 40, N 45, Q 46 and Q 48	1954.60	1954.86	1955.09	1955.17
T8 (2458.29)	Q 61, Q 62, N 65 and M 59	*2458.97(2474.94)*	**2459.25** (**2475.23**)	2459.09 (2475.06)	**2459.33** (**2475.35**)
T1-10 (4612.23)	Q 5, Q 90, Q 91, N 93, Q 101 and M111	*4612.98(4628.73)*	*4612.95(4628.81)*	*4612.82 (4628.85)*	Very low intensity signals

Each fragment contains at least one amino acid that can be subjected to deamidation or oxidation (Gln, Asn or Met). Methionine and oxidized methionine peptide fragments extracted from bands 1, 2, 3 and 4 relative to each other are marked with bold or italic numbers (high and low intensity signals, respectively).

electrophoresis and in-gel tryptic digestion. The results revealed high content of oxidized methionine-containing peptide fragments. Digestion with trypsin did not produce such an extensive oxidation. According to the previous studies, oxidation of methionine-containing peptide fragments as a result of proteolysis was not reported [10,11,15]. Under mild oxidizing conditions (0.05% hydrogen peroxide, v/v) in less then 1 h, *rHu*INF α-2b forms monomethionine sulfoxide variant at position Met111 [1,27]. Interaction of protein in polyacrilamide gel material with oxidizing reagents, especially sulfates (sodium dodecyl sulfate, 2-Amino-2-hydroxymethyl-1,3-propanediol hydrochloride solution, CHAPS, etc.), during prolonged period of 2D gel electrophoresis, was the most probable cause of so extensive *rHu*INF α-2b oxidation.

4. Conclusions

Monitoring H/D exchange of *rHu*INF α-2b in deuterated environment by MALDI-TOF MS can reveal the differences between properly and improperly stored proteins. Prior to H/D exchange experiment, the macromolecule was examined by standard set of methods proposed by European Pharmacopoeia and additionally by spectroscopy (fluorescence and circular dichroism) and mass spectrometry (analysis of native macromolecule, peptide mapping, impurity profiling, etc.). All methods confirmed high purity and primary and secondary macromolecule structure preservation of the certificated reference standard protein. Only after confirmation of structural integrity by numerous analyses, it was possible to conduct the experiment in order to define the standard H/D exchange curve related to expected conformational changes. Obtained linear exponential curve represented the starting point in comparative analysis between preserved and improperly stored or modified *rHu*INF α-2b.

Isoelectric focusing, very often used as a method for purity confirmation and identity of *rHu*INF α-2b provided a specified number of bands all of them in defined p*I* range (5.8–6.3). Bands of *rHu*INF α-2b were separated by 2D gel electrophoresis. Detailed characterization of protein fragments produced by tryptic digestion after isolation of peptides from the gel was performed by mass spectrometry. Interestingly, it was found that all isoforms contained methionine sulfoxides with different ratio of oxidized and unoxidized residues at different position in the macromolecule (Met16, Met21, Met59, Met111 and Met148). Deamidation of glutamines and asparagines, previously stated as possible cause of isoform formation was not observed [23].

Both developed methods are indicative for stability testing of recombinant human interferons providing valuable data on macromolecule structure.

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