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Isolation and characterization of modified species of a mutated (Cys^{125}–Ala) recombinant human interleukin-2☆

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Abstract

During purification of recombinant and mutated interleukin-2 (rhIL-2A^{125}) by reversed-phase–high-performance liquid chromatography, more and less hydrophobic fractions named MHF and LHF, respectively are discarded due to the presence of some unidentified forms of rhIL-2Ala^{125}. Using slow and linear gradients of acetonitrile, these fractions were further purified by RP-HPLC, analyzed by automatic Edman degradation, digested with trypsin and analyzed by electrospray ionization mass spectrometry. In all fractions, partial processing of the N-terminal Met residue was observed. In the LHF the Met^{104} was partially oxidized as sulfoxide. Combining the selective and reversible blocking of tryptic peptides and cation-exchange chromatography, two unexpected C-terminal peptides were selectively isolated. Automatic N-terminal sequencing showed that one of these corresponded to the C-terminal peptide of rhIL-2Ala^{125} linked to another 11 amino acids (AANDENYALAA) and the other corresponded to the C-terminal peptide of a truncated rhIL-2Ala^{125} without the C-terminal threonine residue and the extension of the 11 amino acids previously mentioned. MHF contained a mixture of four species of rhIL-2A^{125} monoacetylated at the N-terminus and at the ε-amino groups of internal Lys residues: 8, 32 and 48. Cys^{58} was found as free cysteine and also covalently linked to M_r 69 and 77 molecules. Covalent dimers of rhIL-2A^{125}, linked through disulfide bridges between Cys^{58} and Cys^{105} of different monomers were also found.

Keywords: Interleukins; Peptides

1. Introduction

In 1976, Morgan et al. [1] discovered human interleukin-2 (hIL-2). This cytokine is synthesized and secreted primarily by T-cells. It has direct effects on a number of immunological cells. hIL-2 can stimulate the growth, differentiation and activation of T, B and NK cells [2–4]. During the last decade IL-2 gained considerable attention as a therapeutic agent for certain forms of cancer [5,6]. In recent years, its safe use for immunotherapy has been recognized and clinical trials have denoted that the IL-2-based therapy is promising for chronic infectious diseases, especially for human immunodeficiency virus (HIV) infection and hepatitis C [7–9]. hIL-2 is a 133 amino acid polypeptide containing three Cys residues at positions 58, 105 and 125. A disulfide bond links

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Cys^{58} and Cys^{105}, while the Cys^{125} has a free thiol group.

Free Cys^{125} is not involved in the recognition of the IL-2 receptor [10] and due to the formation of aggregates or polymers its presence is troublesome for renaturing and purification. In fact, different procedures have been developed for the purification of human recombinant interleukin-2 (rhIL-2) from insoluble protein aggregates [11–14].

In order to avoid the undesirable formation of protein aggregates, mutants of rhIL-2 where the Cys^{125} was substituted for serine [15] or alanine (rhIL-$2Ala^{125}$) [16], have been reported. Reversed-phase–high-performance liquid chromatography (RP-HPLC) is the principal step for the purification of rhIL-2 [13,17,18].

Using RP-HPLC as the main step a procedure for purification of rhIL-$2A^{125}$ has been developed to remove certain unidentified species that are heterogeneous in size as determined by the sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis [16].

Here we describe the isolation and characterization of those modified species of rhIL-$2A^{125}$ by the successful combination of automatic Edman sequencing, peptide mapping, mass spectrometry and a previously developed method for the selective isolation of the C-terminal peptides [19].

2. Experimental

2.1. Materials

Acrylamide, *N*,*N*-dimethylacrylamide, tetrahydrofuran (THF), acetic acid, thioglycerol, glycerol, acetonitrile, Coomassie brilliant blue G-250 and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Merck (Darmstardt, Germany). Other reagents were purchased from different suppliers: sodium dodecyl sulfate and formic acid (BDH, Poole, UK), trifluoroacetic acid (TFA) (Pierce, Rockford, IL, USA), octylglucoside (Sigma, St. Louis, MO, USA) and maleic anhydride (Aldrich, Milwaukee, WI, USA). Xenon and argon gas for fast atom bombardment (FAB) MS were from Teisan (Osaka, Japan). PVDF filters were from Millipore (Bedford, MA, USA).

2.2. Production of recombinant rhIL-$2A^{125}$

A mutant hIL-2 protein, where Cys^{125} was replaced with alanine (rhIL-$2A^{125}$), was expressed at high levels in *Escherichia coli* JM101 harboring the pIL-2mA12 plasmid. This plasmid results from cloning the gene coding for the mature mutant protein in the pFP-15 plasmid [20]. rhIL-$2A^{125}$ was expressed as insoluble aggregates in inclusion bodies, and it was mostly isolated from contaminant proteins by a cell pellet washing procedure. The insoluble protein pellet was solubilized with 6 *M* guanidine hydrochloride (GuHCl) and renaturation was carried out using gel filtration chromatography (GFC) on Sephadex G-25. rhIL-$2A^{125}$ from GFC effluents was further purified by RP-HPLC, using a C_4 preparative column (5×25 cm) Vydac (Hesperia, USA). rhIL-$2A^{125}$ was eluted with a segmented linear gradient from 15 to 80% of acetonitrile containing 0.05% TFA at a flow-rate of 45 ml/min. The main peak (MP) was previously characterized by peptide mapping, MS analysis [21], and it corresponds to 95% pure rhIL-$2A^{125}$ estimated by electrophoresis with a specific activity ranging from $1.1 \cdot 10^7$ to $2.0 \cdot 10^7$ IU/mg [16].

2.3. Tryptic digestion of rhIL-$2A^{125}$

rhIL-$2A^{125}$ dissolved in 1% ammonium hydrogencarbonate (pH 8) at a concentration of 100 μg/ml was digested with trypsin (Serva, Heidelberg, Germany) at on enzyme/substrate ratio of 1:50 (w/w) for 5 h at 37 °C. The digestion was stopped by adding 500 μl of 0.1% aqueous solution of TFA and stored in a freezer at −20 °C until the chromatographic separation by RP-HPLC.

2.4. Endoproteinase Glu-C digestion

The tryptic peptides were dissolved in 20 μl of 1% ammonium hydrogencarbonate (pH 8) and digested for 4 h with endoproteinase Glu-C (Boehringer-Mannheim, Germany) at 37 °C using an enzyme–substrate ratio of 1:100 (w/w). The proteolytic digestion was stopped in the same way as the tryptic digestion.

2.5. RP-HPLC

The samples injected by using a Rheodyne 7725i injector (Cotati, USA) were separated by the gradient generated by a Merck–Hitachi L-7110 pump (Merck, Darmstadt, Germany) and detected at 226 nm by using an variable wavelength UV monitor from Knauer (Berlin, Germany). Buffer A was 0.1% (v/v) TFA in water and buffer B was 0.05% (v/v) TFA in acetonitrile. The solvents were degassed on-line by using a degasser (model L7612) from Knauer.

A reversed-phase C_{18} Vydac column (1×25 cm) was used for the isolation of rhIL-$2A^{125}$ species. The temperature was kept constant at 34 °C by using a column oven from Knauer. The less hydrophobic (LHF) and more hydrophobic fractions (MHF) containing modified species of rhIL-$2A^{125}$, were further separated by slow and linear gradients from 50 to 75% of buffer B in 32 min and 55–85% of buffer B in 55 min, respectively at 1 ml/min flow-rate.

Proteolytic peptides were separated by RP-HPLC using a C_8 wide pore Baker column (0.46×10 cm) (Phillipsburg, USA) and a linear gradient of 0–90% of buffer B in 90 min at a flow-rate of 0.8 ml/min. Data acquisition and processing was carried out with the Biocrom (CIGB, Havana, Cuba) or LaChrom from (Merck–Hitachi) software.

2.6. SDS–PAGE analysis

Electrophoresis was performed as described by Laemmli [22] by SDS–PAGE (15% SDS) at 30 mA for 3.5 h at room temperature under reducing or non-reducing conditions. The gels were stained with Coomasie blue dye.

2.7. N-terminal sequencing

Proteins and peptides were dissolved in 50% TFA and sequenced on a poly(vinylidene difluoride) (PVDF) membrane using a Knauer 810/816 sequencer (Berlin, Germany). Phenilthiohydantoin (PTH) derivatives were detected at 269 nm by online RP-HPLC.

2.8. FAB-MS

FAB-MS analysis was carried out with a JEOL JMS-HX110HF instrument equipped with a standard FAB ion source using a xenon-ionizing beam (1 kV). A mixture of glycerol–thioglycerol (1:1, w/w) was used as the matrix. The ion source was fixed at an accelerating potential of 10 kV. Mass spectra were acquired and processed using a JEOL JMA-DA5000 mass data analysis system. The collision induced dissociation (CID)-linked scan was acquired by scanning the electric (E) and magnetic field (B) keeping the B/E ratio constant. The collision gas was argon (Teisan, Osaka, Japan) and it was introduced into the collision cell to decrease the intensity of the precursor to one half the value.

2.9. Electrospray ionization mass spectrometry (ESI-MS)

The ESI-MS spectra of LHF species were obtained in a triple quadrupole mass spectrometer Sciex API III (Perkin-Elmer Sciex, Ontario, Canada). The samples were dissolved in 100 μl of a mixture of 0.5% formic acid, 50% acetonitrile in water and injected directly into the electrospray chamber at a rate of 5 μl/min by using a syringe pump from Harvard Apparatus (MA, USA).

The low-energy ESI-MS–MS spectra were acquired using a hybrid quadrupole orthogonal acceleration tandem mass spectrometer QTOF-2 from Micromass (Manchester, UK) fitted with a Z-spray nanoflow electrospray ion source. The mass spectrometer was operated with a source at 80 °C and a drying gas flow of 50 l/h was used. Peptides were dissolved in a solution of water–acetonitrile (50:50, v/v) containing 1% acetic acid to reach an approximate concentration of 5 pmol/μl. The peptide solution was infused onto the mass spectrometer at a flow-rate of 5 μl/min by using a syringe pump from Harvard Apparatus. To acquire the MS–MS spectra the first quadrupole was used to select the precursor ion within a window of 4–5 Thompson (Th). A pressure of $\sim 3 \cdot 10^{-4}$ Pa collision gas (argon) was used in the hexapole collision cell to yield the fragment ions. Appropriate collision energy was used to reduce the intensity of the precursor ion to more than half of its original intensity. Data acquisition and processing were performed using a MassLynx system from Micromass.

2.10. Selective isolation of C-terminal peptide

2.10.1. Selective blocking of N-terminal groups

The mixture of tryptic peptides was acidified, vacuum concentrated and dissolved in MES buffer (300 m*M*, pH 6) containing 60 m*M* of Gly to reach a final concentration of 130–150 m*M*. A 50-fold molar excess of maleic anhydride dissolved in THF, was added and the reaction was kept for 15 min at 0 °C under efficient stirring.

2.10.2. Cation-exchange chromatography

The cation-exchange chromatography was performed in a mini-column manually packed with 100 μl of the anionic resin EMD-650 (S) SO_3^- from Merck. The N-terminal blocked peptides were dissolved in 0.05% (v/v) TFA in water containing 0.5% of octylglucoside and applied onto the resin. After a short spin (5 s, approx. 5000 rev./min) using a microtubes centrifuge the non-retained fraction was collected and analyzed by RP-HPLC. The remaining tryptic peptides retained onto the column were eluted increasing the ionic strength of the buffer (1 *M* NaCl).

2.10.3. Peptide deblocking

Peptides contained in the non-retained fraction were vacuum dried, dissolved in a mixture of glacial acetic acid–formic acid (1:5.6, v/v), kept at 37 °C during 4 h, vacuum concentrated and spotted on the PVDF sequencer disk.

3. Results and discussion

3.1. RP-HPLC and SDS–PAGE analysis of LHF and MHF of rhIL-2Ala^{125}

During the purification of rhIL-2Ala^{125} by RP-HPLC, the LHF and the MHF (Fig. 1A) were collected and re-analyzed by using slow gradients RP-HPLC (Fig. 1B and C). LHF and MHF were resolved in four fractions each. Their analysis by SDS–PAGE (inserts of Fig. 1B and C) showed mass heterogeneity: f1, f2, f7 and f8 showed a component with higher apparent mass while f3, f4, f5 and f6 showed a major band that migrates at the expected size for rhIL-2Ala^{125}.

3.2. N-terminal sequencing of fractions f1–f8

Automatic N-terminal sequencing analysis of all fractions (f1–f8) showed two sequences, one corresponding to that expected for the intact rhIL-2Ala^{125} and the other to an additional Met at the first cycle, indicating the partial processing of this residue by *Escherichia coli*.

The high expression level reached during the fermentation process (18% total protein) does not allow an efficient action of the formyl-Met aminopeptidase [23] and also the presence of a proline residue located at the third position of the polypeptide chain [24], may hamper the efficient and complete removal of the initial Met.

3.3. Characterization of the individual fractions by ESI-MS

Molecular mass values of all fractions (f1–f8) were determined by ESI-MS and the results are summarized in Table 1. The molecular species of rhIL-2A^{125} found in each fraction (f1–f8) were denoted as a, b, c, etc.

3.4. Characterization of f1 and f2

The ESI-MS spectrum of f1 revealed two molecular species (f1a and f1b in Table 1). The mass difference between them was 131 u, corresponding to a Met residue (131.04 u). These results confirm the partial processing of the initial Met previously detected by Edman sequencing [21]. In the same way, the ESI-MS spectrum of f2 revealed two molecular species (f2a and f2b separated by 131 u) indicating the presence of an N-terminal methionine in the f2a species.

The molecular masses of the f1b and f2b determined experimentally were 1003 and 1104 u higher than the theoretical mass of rhIL-2Ala^{125}, respectively. These mass differences could be located at the C-terminal end of the protein because Edman sequencing demonstrated that the N-terminus of these species is identical to that of rhIL-2Ala^{125}.

Therefore, we applied a previously described method (Fig. 2) for the selective isolation of C-terminal peptide from proteins [19]. This methodology is based in the selective and reversible

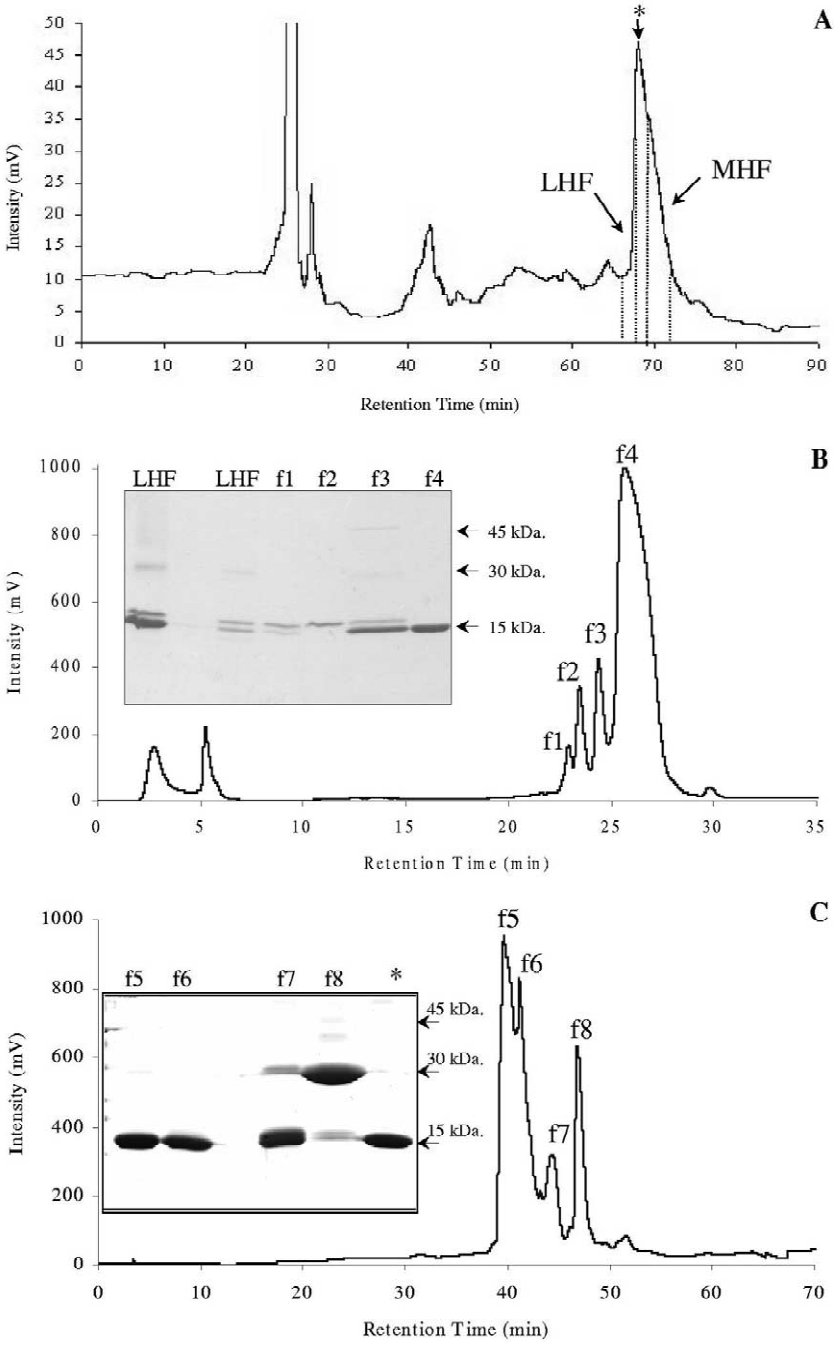

Fig. 1. (A) RP-HPLC purification of the rhIL-2A^{125} contained in washed-pellet. LHF and MHF indicate the less and more hydrophobic fractions analysed in the present work. The asterisk denotes the fraction of the purified rhIL-2A^{125}. (B) RP-HPLC of LHF obtained in the purification of rhIL-2Ala^{125}. The insert shows the SDS–PAGE analysis of LHF and individual fractions (f1–f4) previously isolated. (C) RP-HPLC of MHF obtained in the purification of rhIL-2Ala^{125}. The insert shows the SDS–PAGE analysis in non-reducing conditions of fractions f5–f8. Pure rhIL-2Ala^{125} isolated in (A) was applied on the lane labelled with an asterisk.

Table 1
ESI-MS analysis of LHF (f1–f4) and MHF (f5–f8)

Fraction		Species	Experimental molecular mass[a]	Molecular mass difference[b]	Assignment
LHF	f1	a	16518.00±2.9	1134.03	Met-(1-132)+AANDENYALAA[c]
		b	16387.00±1.8	1003.03	(1-132)+AANDENYALAA[c]
	f2	a	16619.90±1.0	1235.93	Met-(1-133)+AANDENYALAA[c]
		b	16488.90±1.0	1104.93	(1-133)+AANDENYALAA[c]
	f3	a	15531.10±1.8	147.13	Met-(1-133) [Met^{104} sulfoxide]
		b	15400.20±1.1	16.23	(1-133) [Met^{104} sulfoxide]
	f4	a	15515.90±1.1	131.93	Met-(1-133)
		b	15385.30±1.7	1.33	(1-133)
MHF	f5	a	15556.47±0.09	172.96	Met-(1-133)+Ac(Lys 8, 32 or 48)[d]
		b	15425.50±0.06	41.53	(1-133)+Ac(Lys 8, 32 or 48)[d]
	f6	a	15556.93±0.18	172.96	Ac-Met-(1-133)[e]
		b	15425.79±0.09	41.82	Ac-(1-133)
	f7	a	15584.92±0.03	200.95	Met-(1-133)+Cys^{58}–S-(69)[f]+Cys^{105}–SH[g]
		b	15453.74±0.12	69.77	(1-133)+Cys^{58}–S-(69)+Cys^{105}–SH[g]
		c	15559.25±0.20	175.28	Met-(1-133)+Ac(Lys^{32})[d]+2Cys(58,105)–SH[g]
		d	15428.05±0.13	44.08	(1-133)+Ac(Lys^{32})[d]+2Cys(58,105)–SH[g]
	f8	a	30767.44±0.32	15383.47	2×(1-133)[h]
		b	30898.57±0.11	15514.60	Met-(1-133)+(1-133)[h]
		c	31029.53±0.25	15645.56	2×Met-(1-133)[h]

[a] The experimental masses of fractions (f1–f4) and (f5–f8) were determined by using a triple quadrupole mass spectrometer from Sciex and a QTOF-2 from Micromass, respectively.

[b] Mass difference was calculated by subtracting the theoretical mass value of rhIL-$2A^{125}$ (15 383.97) from the experimental masses of the different species of the analyzed protein.

[c] The mechanism for adding these 11 amino acids to the C-terminal end of truncated proteins was previously described [26].

[d] Ac(Lys ≠) indicates a lysine residue acetylated at the ϵ-amino group. The numbers in parenthesis indicate the position of the modified lysine within the sequence of rhIL-$2A^{125}$.

[e] The protein contains an additional acetyl methionine residue at the N-terminus.

[f] An unknown modification increased the molecular mass of the cysteine residue by 69.

[g] The Cys residue with a free thiol group.

[h] Dimers of rhIL-$2A^{125}$ linked by intermolecular S–S bridges between Cys^{58} and Cys^{105}.

blocking of tryptic peptides with maleic anhydride and cation-exchange chromatography.

Using this procedure, the N-terminal blocked peptides at pH 2 are transformed into two pools: the single-charged peptides (internal peptides contain Lys and Arg at their C-terminus) and a neutral peptide (C-terminal peptide). These pools of peptides are separated by cation-exchange chromatography, the single-charged peptides are retained in the column while the C-terminal peptide is transformed as the maleyl-*N*-blocked peptide elutes in the non-retained fraction. Since f1 and f2 are mutually contaminated, to avoid loss of samples due to further purifications steps, we decided to apply the method to a mixture of f1 and f2.

The non-retained fraction was analyzed by RP-HPLC and two major peaks were obtained (Fig. 3). The intensity ratio of these peaks (1:2) was very similar to that obtained for f1:f2 during the RP-HPLC purification of LHF (Fig. 1B). This result suggested that peaks 1 and 2 contain the C-terminal peptides of f1 and f2. These peptides were deblocked by an acid treatment and analyzed by automatic Edman degradation. Two similar N-terminal sequences were obtained. Peak 2 contains a partial sequence of the C-terminal peptide of the rhIL-$2A^{125}$ (Ala^{125}–Thr^{133}) with an extension of 11 amino acids (AANDENYALAA, Fig. 3). The N-terminal sequence of peak 1 (Fig. 3) was similar to the sequence obtained for peak 2 but it did not contain the C-terminal Thr^{133}.

The calculated molecular mass of the eleven-residue peptide (1103.5 u) accounts for the experimental mass difference found between the rhIL-$2A^{125}$

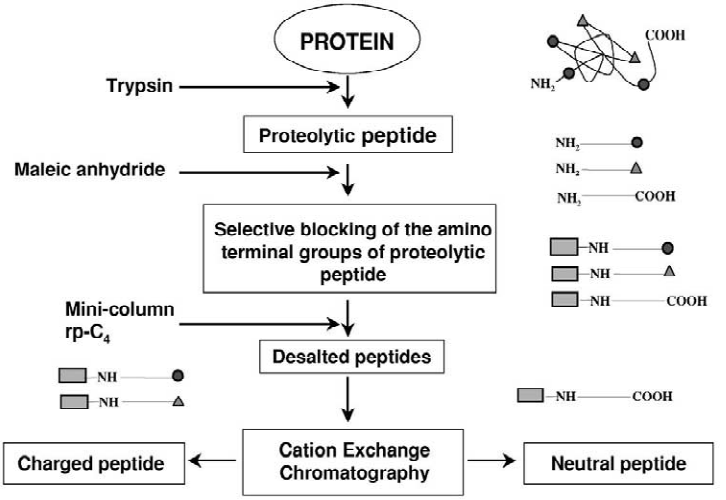

Fig. 2. The general strategy for the selective isolation of the C-terminal peptides of proteins based on the selective and reversible blocking of proteolytic peptides and cation-exchange chromatography [19]. The circles and triangles represent the basic amino acids Lys and Arg, respectively. The rectangles indicate a maleyl moiety attached to the N-terminal end of tryptic peptides.

and f2b (1104.93) considering the error of 0.01% for the ESI-MS measurement.

The experimental mass difference between the species f1b and f2b (101.9 u) is very close to the mass of the Thr residue (101.05 u) that was not detected during the N-terminal sequencing of the peak 1 (Fig. 3).

It should be noted that both C-terminal peptides of f1 and f2 were generated by a chymotryptic-like cleavage at the C-terminus of Phe[124] ([121]WITF-AQSIISTLT[133]). Hence, we conclude that f1 is the 132 amino acid rhIL-2A[125] (lacking the C-terminal Thr[133]) and fused to the polypeptide AAN-DENYALAA appended to its C-terminus (143 aa, total sequence length) [25]. Fraction f2 contains the rhIL-2A[125] plus 11 amino acids added to the C-terminus [25]. Besides, a partial processing of the initial methionine by *E. coli* was also observed in both cases.

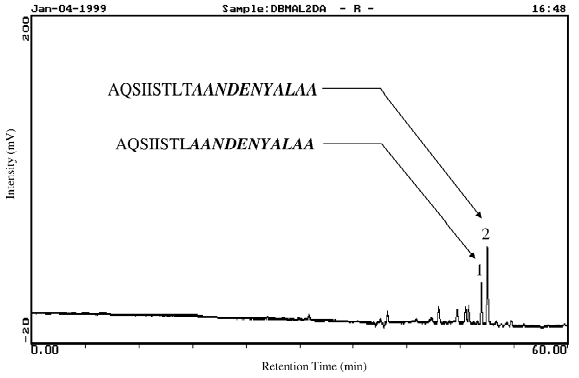

Fig. 3. RP-HPLC of the non-retained fraction obtained as the C-terminal peptides of f1 and f2 by using the strategy depicted in Fig. 2. The N-terminal sequences obtained for peaks 1 and 2 are indicated. The 11 amino acids (AANDENYALAA) appended to the C-terminal end of rhIL-2Ala[125] are typed in Italic-bold letters.

In 1994, Zafeer et al. [26] found this C-terminal extension (AANDENYALAA) on recombinant IL-2 expressed in *E. coli* but the species found in f1 had not been previously reported. This modification was also reported for truncated forms of interleukin-6 [27].

The addition of those 11 amino acids at the C-terminal end has been reported as a co-translational peptide tagging mechanism for eliminating proteins synthesized from damaged mRNAs and could be related with the high expression level of a recombinant protein [28].

3.5. Characterization of f3

The ESI-MS spectrum of f3 showed the presence of two species (f3a and f3b) of rhIL-2Ala[125] with the

initial Met partially processed (Table 1). The molecular specie f3b has a molecular mass that is 16 u higher than that expected for the rhIL-2Ala^{125} (Table 1). This mass difference suggested the presence of a Met sulfoxide form within the sequence. The rhIL-2A^{125} has four methionine residues located at positions 23, 39, 46 and 104.

In order to determine where this modification was located, the tryptic map of rhIL-2A^{125} and f3 were compared (Fig. 4). The peptides (Gly^{98}–Arg^{120}) and (His^{55}–Lys^{76}) which are linked by a disulfide bridge and contain the Met^{104} are missing in the tryptic map of f3. It was also observed that the fraction where the peptide Trp^{121}–Thr^{133} eluted has increased its intensity, suggesting that these two peptides co-eluted in the same fraction (see Fig. 4).

These peptides were further digested with endoproteinase Glu-C and separated by RP-HPLC. The FAB-MS spectrum of one fraction yields three main signals at m/z 1744.3, 1000.2 and 747.0. The signal at m/z 1000.2 matched with the expected molecular mass of His^{55}–Glu^{62} (1000.43). However, signals at m/z 1744.3 and 747.0 were 16 u higher than that expected for the peptides His^{55}–Glu^{62} and Thr^{101}–Glu^{106} linked by the S–S bond (1728.68) and the reduced peptide Thr^{101}–Glu^{106} (731.27), respectively (data not shown).

The CID mass spectrum of the peptide ^{102}Thr–Glu^{107} showed the loss of methyl sulphenic acid (–CH_3SOH, 64 u) from the precursor, which is a typical fragmentation for peptides containing Met sulfoxide residues (data not shown) [29,30]. Therefore, we concluded that Met^{104} in the f3 fraction is oxidized to sulfoxide [25]. Previous studies also suggested that this residue is particularly prone to oxidation in IL-2 when it is treated with chloramine-T [31].

3.6. Characterization of f4

The ESI-MS spectrum of f4 shows species (f4a and b, see Table 1) with molecular masses that are in good agreement with the expected for the rhIL-2A^{125} and Met-rhIL-2A^{125}.

The tryptic map of f4 was identical to that obtained for the intact rhIL-2A^{125} (data not shown). Taking into account the higher abundance of f4 with respect to f1–f3 (Fig. 1B), we can conclude that LHF is mainly composed of rhIL-2Ala^{125}.

3.7. Characterization of f5 and f6

The molecular masses of f5a and f5b also differ by 131 u, suggesting the presence of an additional N-terminal Met in f5a. The molecular masses of f5a

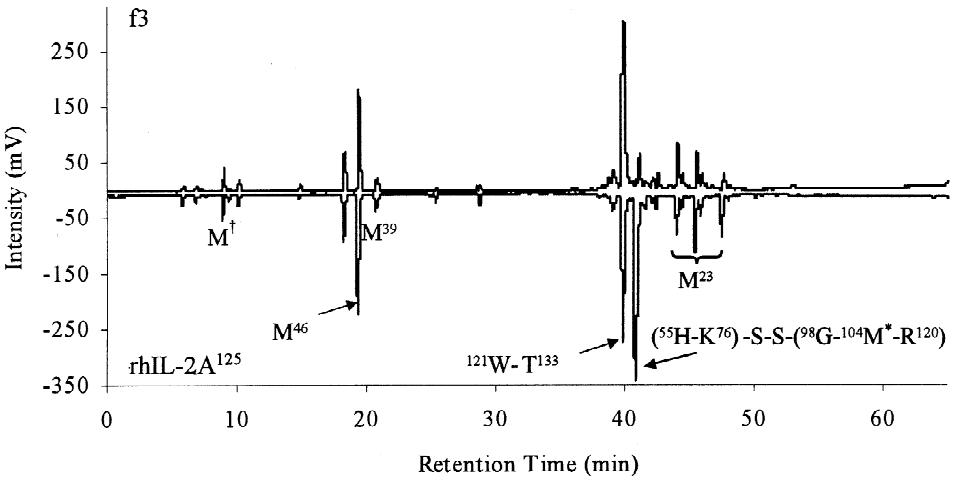

Fig. 4. The upper and lower chromatograms correspond to the tryptic maps of f3 and rhIL-2Ala^{125}, respectively. In the lower chromatogram, the fractions containing the four Met residues (M^{23}, M^{39}, M^{46} and M^{104}) of rhIL-2Ala^{125} are indicated. M† and *M^{104} indicate the initial Met partially processed by *E. coli* and Met sulfoxide residue located at position 104, respectively.

and f5b were 42 u higher than expected, therefore the presence of an acetyl group was suspected.

The tryptic map of f5 was very similar to the non-modified rhIL-$2A^{125}$ (data not shown). All peaks from the tryptic map were analyzed by ESI-MS. Two doubly-charged signals at m/z 540.26 and 428.22 and a quaternary-charged signal at m/z 798.68 that did not match with the mass values expected for the tryptic peptides of rhIL-$2A^{125}$ were found.

The signals at m/z 428.22, 540.26 and 798.68 were further subjected to ESI-MS–MS (Fig. 5A, B and C) and they corresponded to peptides, Phe^{44}–Lys^{49}, $Met–Ala^{1}$–Lys^{9}, and Lys^{8}–Lys^{35} containing the Lys^{48}, Lys^{8} and Lys^{32} acetylated at their ε-amino group, respectively. The position of this acetyl-Lys residue (*K**) in the ESI-MS–MS was determined by the loss of 170 u corresponding to the molecular mass of this modified residue and confirmed by the presence of several internal fragment ions containing *K**.

Though we found acetylation (Ac) at ε-amino groups of three lysine residues, it seems that there is only one acetylation per molecule because in the ESI-MS spectrum of f5, the molecular mass of rhIL-$2A^{125}$ increased by 84 (2×Ac) or 126 (3×Ac) (Table 1) were not found.

The formation of ε-*N*-acetyl-lysine in a protein was initially discovered in calf thymus histones H3 and H4 [32–35]. This altered amino acid has also been detected in other histones [36,37] and other classes of DNA-binding proteins [38,39]. ε-*N*-Acetyllysine had been detected in other natural proteins such as ferrodoxin from *Halobacterium halobium* [40], α-tubulin from *Chlamydomonas reinhardtii* [41], α-tubulin from 3T3 and HeLa cells [42] and mouse neuronal α-tubulin [43]. The ε-*N*-acetyllysine has been also identified in recombinant bovine and porcine somatotropin [44].

In eukaryotic systems the acetyl group is transferred to the ε-amine group of lysine [39,41] by an acetyltransferase enzyme, which uses acetyl-CoA as a substrate. However, the mechanism for acetylating Lys residues in *E. coli* has not been elucidated. The metabolic state of the cell could be related to the formation of ε-*N*-acetyl-Lys. The acetylation of Lys may be involved in signaling mechanism in *E. coli* either to direct the degradation of foreign proteins or to place proteins into inclusion bodies [44].

The ESI-MS spectrum of f6 was very similar to f5: an increment of 42 in comparison with the theoretical molecular mass of rhIL-$2A^{125}$ was observed (Table 1). In the tryptic map of f6 a signal at m/z 476.25 (2+) was detected; and its ESI-MS–MS spectrum showed the N-terminal peptide of rhIL-$2A^{125}$ containing an additional acetylated methionine residue at its N-terminus (f6a in Table 1 and Fig. 5D). We also found a doubly-charged signal at m/z 474.74 that was sequenced by ESI-MS–MS; it corresponds to the intact N-terminal peptide ($Ac-Ala^{1}$–Lys^{9}) of the acetylated protein (data not shown). N-terminal acetylation is one of the most frequently found postranslational modifications of proteins [45].

3.8. Characterization of f7

The ESI-MS spectrum of f7 was very complex, revealing the presence of several molecular species (see Table 1). The mass difference between the molecular masses of f7a and f7b (131.15 u) suggested the partial processing of the initial methionine but the molecular mass of f7a and f7b were 69 u higher than that expected.

The f7 fraction was digested with trypsin and the ESI-MS spectra of two different peaks in the chromatogram showed triply- and quaternarily-charged signals at m/z 877.78 and 826.18, respectively, that were not assigned to tryptic fragments. These peptides were fragmented, and their ESI-MS–MS spectra were deconvoluted in order to make easier the manual sequencing (Fig. 6A and B). The analysis of the ESI-MS–MS spectra revealed that signals at m/z 877.78 (3+) and 826.18 (4+) correspond to peptides $^{55}His–Lys^{76}$ and $^{48}Lys–Lys^{76}$, respectively, containing a modified cysteine residue with a molecular mass of 171 u.

We were not able to characterize the chemical structure of this modification covalently attached to Cys^{58} responsible for increasing its molecular mass by 68.

Since the molecular mass of f7b is 69 higher than that of rhIL-$2A^{125}$ we assumed that Cys^{105} should has a free thiol group to account for the experimental mass difference determined by ESI-MS. Therefore the f7a and b species were assigned to molecules of

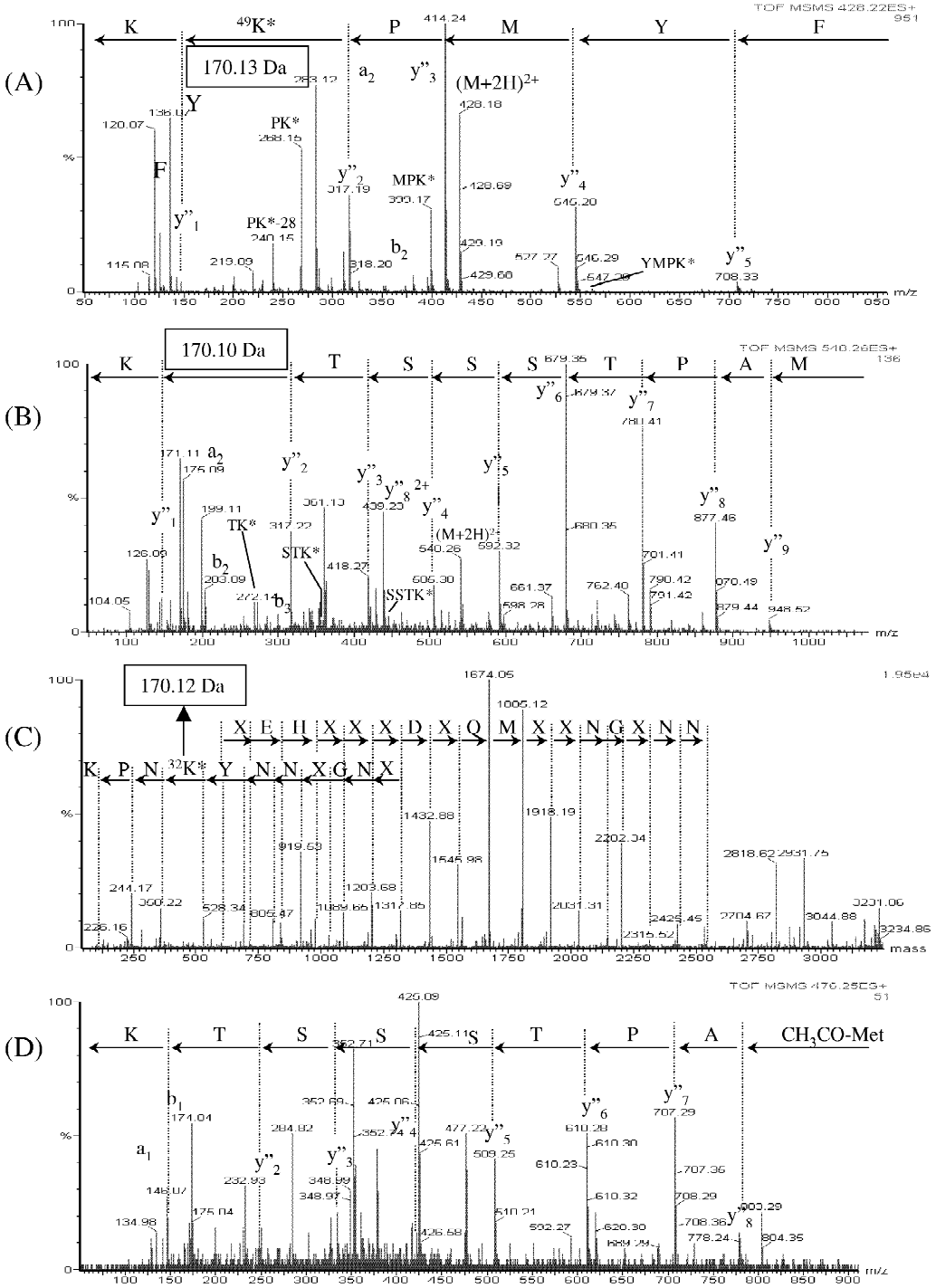

Fig. 5. ESI-MS–MS spectra of acetyl-lysine containing peptides isolated in fraction f5 ^{44}FYMP*K*^{49}K(A), M-^{1}APTSSST*K**^{9}K(B), ^{9}KTQLQLEHLLLDLQMILNGINNY*K**NP K^{35}(C). In all ESI-MS–MS spectra, *K** indicates an acetyl lysine residue within the peptide sequences. The ESI-MS–MS of the peptide 9-35 was deconvoluted (C) to make easier the manual sequencing. The ESI-MS spectrum shown in (D) corresponds to the N-terminal peptide of the rhIL2Ala^{125} containing an additional acetylated methionine residue obtained from the tryptic digest of f6.

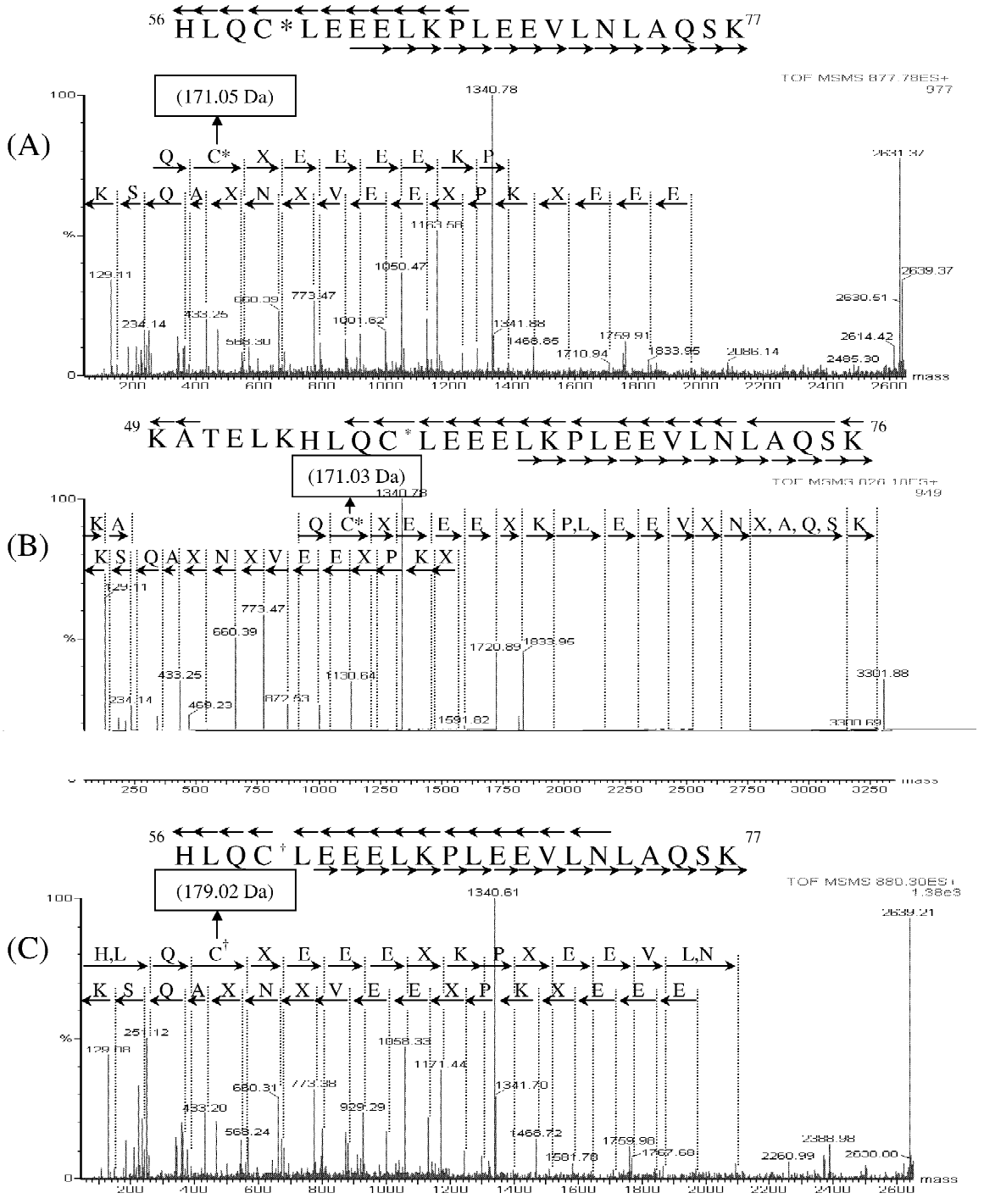

Fig. 6. Deconvoluted ESI-MS–MS spectra of peptides containing modified cysteine residues. C* and C† indicate cysteine residues with residual masses increased by 69 and 77 u, respectively. The mass values in boxes indicate the experimental mass determined for these modified Cys by subtracting the masses of the consecutive y''_n ions.

rhIL-2A^{125} carrying this unknown modification to Cys^{58} (69 u), a free Cys^{105} and a partial processing of the N-terminal Met.

In the tryptic digest of f7 another triply-charged peptide at m/z 880.29, was not assigned to specific cleavage of rhIL-2Ala^{125} and its sequence obtained by ESI-MS–MS (Fig. 6C) indicated that Cys^{58} is also covalently linked to a molecule that increases its molecular mass by 77 u.

It is important to point out, that although we found a tryptic peptide with a cysteine residue that had increased in mass by 77 u, in the ESI-MS spectrum of f7 we did not find any specie of rhIL-2A^{125} with this molecular mass increase.

In this fraction we found other modifications. Similarly to fraction f5 we found the Lys^{32} acetylated and also the quaternary-charged peptides ^{9}Lys–Lys^{35} (m/z 809.18) and ^{10}Thr–Lys^{35} (m/z 798.35) with Asn^{26} partially deamidated.

Since the ESI-MS spectrum of f7 showed two other molecular species: f7c and f7d having a 131 u mass difference and also considering that the molecular mass of f7d is 44 higher than that of rhIL-2A^{125}, they could be tentatively assigned to species of rhIL-2A^{125} containing an acetylated Lys^{32}, free Cys^{58} and Cys^{105} residues, and a partial processing of the initial Met.

In f7, species of rhIL-2A^{125} with an increasing molecular mass of 69 (Table 1) are the major components as judged by the intensity of the multiple-charged signals observed in the ESI-MS spectrum. Perhaps, this is the reason why minor species of rhIL-2hrA^{125} with very similar molecular masses (increased by 77) were not detected in the deconvoluted ESI-MS spectrum, and a higher resolving power mass spectrometer such as FT-MS [47,48] would be required to visualize these modified species of rhIL-2A^{125}.

3.9. Characterization of f8

Three species were clearly observed in this fraction with molecular masses of 30 767.44±0.32 (f8a), 30 898.57±0.11 (f8b) and 31 029.53±0.25 (f8c). Those mass values are approximately twice the molecular mass of rhIL-2A^{125} suggesting the formation of dimers (Table 1) and are in good agreement with the theoretical molecular masses expected for dimers of rhIL-2A^{125} linked by intermolecular disulfide bridges (30 767.95), a dimer of rhIL-2A^{125} containing a monomer with one additional methionine (30 899.15) and two methionine residues in each monomer (31 030.64).

This result is in good agreement with the SDS–PAGE analysis, where the f8 fraction shows a dimer in non-reducing conditions (Figs. 2C and 7A, lane 1) and when it was treated with reducing agents only the rhIL-2 Ala^{125} monomer was detected (Fig. 7A, lane 3). The dimer present in f8 was dissociated neither by treatment with 8 *M* urea nor the RP-HPLC conditions (data not shown). These results also support the previous conclusion that covalent bond (disulphide bridge) is formed between two monomers of rhIL-2 Ala^{125}.

The tryptic map of f8 was very similar to the non-modified rhIL-2 Ala^{125} (Fig. 7B) and the fraction that contains the peptides (His^{55}–Lys^{76}) and (Gly^{98}–Arg^{120}) linked by a disulphide bridge elutes in both chromatograms. The ESI-MS spectrum of the fraction labeled with an asterisk in the tryptic map of f8 (Fig. 7B) showed a quaternarily-charged signal at m/z 1297.62 that was assigned to the peptides previously mentioned linked by a disulphide bridge. This result suggests that an intermolecular disulfide bond links between Cys^{58} of a monomer and Cys^{105} of the second monomer and vice versa.

LC–MS analysis of the tryptic digest did not revealed the peptides from different monomers containing the same cysteine residues [(Cys^{58}–S–S–Cys^{58}) or (Cys^{105}–S–S–Cys^{105})].

It seems that the dimer was more resistant to the tryptic digestion than rhIL-2A^{125}; in fact several fractions of the tryptic digests were obtained with lower intensity, when both tryptic maps were compared (Fig. 7A and B). It is probable that dimer was formed during the refolding steps. The scrambled species via disulfide bridges were often obtained as inclusion bodies for recombinant proteins [46].

4. Conclusions

We have isolated, by RP-HPLC, seven fractions from LHF and MHF of rhIL2A^{125}, which contain

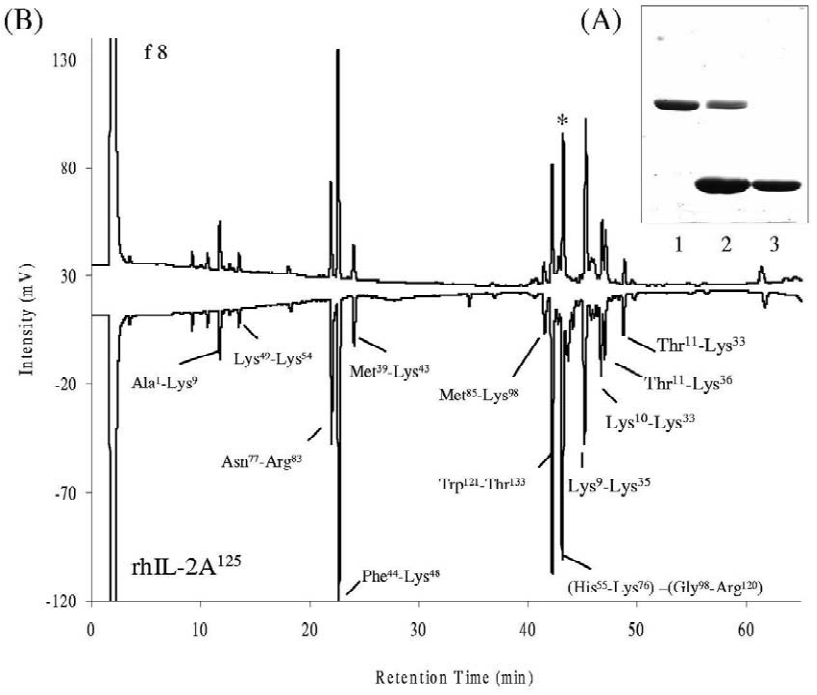

Fig. 7. (A) SDS–PAGE of non-reduced f8 (lane 1), MHF of preparative RP-HPLC (lane2), reduced f8 (lane 3). (B) Tryptic maps of f8 and rhIL-2Ala^{125}.

several modifications that were characterized by Edman degradation and mass spectrometry.

We successfully applied the methodology [19] for the selective isolation of modified C-terminal peptides of a rhIL2A^{125}. One of these modified species, a truncated molecule without the C-terminal Thr, but with the eleven amino acids (AANDENYALAA) appended, was not previously reported for rhIL2 expressed in *E. coli*.

The presence of three acetyl Lys within the sequence of rhIL-2A^{125} were clearly demonstrated by ESI-MS–MS, however, there are few studies on this modification in prokaryotes derived proteins, and the mechanism and the biological meaning for this modification remains as an open question.

We found a covalent dimer of rhIL2A^{125} covalently linked by S–S bridges of the different Cys of the independent monomers.

In all fractions we found partial processing of the initial Met. Perhaps the high expression level reached during fermentation and the presence of a Pro residue, at third position, does not allow *E. coli* to efficiently remove the initial Met during the protein synthesis [24].

We demonstrated, by ESI-MS–MS, two different modifications covalently linked to Cys that increased the molecular mass of the modified rhIL2A^{125} by 69 and 77 (based on –SS– form), however, since the low-energy MS–MS did not provide the side chain fragmentation, it was difficult to establish the chemical nature of such modifications.

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