

Expression and folding of an interleukin-2-proinsulin fusion protein and its conversion into insulin by a single step enzymatic removal of the C-peptide and the N-terminal fused sequence

Lila R. Castellanos-Serra*, Eugenio Hardy, Raimundo Ubieta, Nelson S. Vispo, Cesar Fernandez, Vladimir Besada, Viviana Falcon, Marta Gonzalez, Alicia Santos, Gudelia Perez, Alejandro Silva, Luis Herrera

Center for Genetic Engineering and Biotechnology, P.O. Box 6162, La Habana, Cuba

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Abstract We report the expression in *E. coli* of a proinsulin fusion protein carrying a modified interleukin-2 N-terminal peptide linked to the N-terminus of proinsulin by a lysine residue. The key aspects investigated were: (a) the expression of the fused IL2-PI gene, (b) the folding efficiency of the insulin precursor when still carrying the N-fused peptide and (c) the selectivity of the enzymatic cleavage reaction with trypsin in order to remove simultaneously the C-peptide and the N-terminal extension. It was found that this construction expresses the chimeric proinsulin at high level (20%) as inclusion bodies; the fused protein was refolded at 100–200 $\mu\text{g/ml}$ to yield about 80% of correctly folded proinsulin and then it was converted into insulin by prolonged reaction (5 h) with trypsin and carboxypeptidase B at a low enzyme/substrate rate (1:600). This approach is based on a single enzymatic reaction for the removal of both the N-terminal fused peptide and the C-peptide and avoids the use of toxic cyanogen bromide.

Key words: Recombinant insulin; Proinsulin folding; Enzymatic processing

1. Introduction

Human insulin was the first recombinant protein expressed in bacteria to be produced at industrial scale, more than 10 years ago [1]. As early attempts to obtain recombinant insulin by the expression of the proinsulin gene (coding for the sequence B-chain-C-peptide-A chain) in bacteria failed due to the rapid intracellular degradation of the protein, insulin was attained as a fusion or chimeric protein, carrying at its N-terminus a polypeptide resistant to intracellular degradation [1–3]. This sequence is linked to the B-chain N-terminus by a methionine residue. For these constructions, removal of the N-terminal fused peptide is accomplished at an early stage in the process by chemical cleavage with cyanogen bromide at the methionine residue [1]. This approach requires two independent cleavage reactions: firstly, prior to folding, for removing the fused peptide, and then, after folding, for removing the C-peptide.

Due to the increasing demand of human insulin [4] and the complexity of handling large amounts of cyanogen bromide at the industry, the search for a process that could avoid the use

of this highly toxic product has been under research. Recently, two alternative approaches are described. One consists of the biosynthesis of an inverted proinsulin molecule, A-C-B, and its conversion into human insulin by enzymatic cleavage of the C-peptide [5,6]. In contrast to the 'normal' proinsulin, the inverted proinsulin is stable. A second approach is based on the selective removal of a short N-terminal extension from a previously folded insulin precursor by the action of dipeptidyl amino peptidase-1 [7].

We explore here a different approach. A fused proinsulin precursor (IL2-PI) is constructed that bears a tryptic site (lysine) at the end of the N-fused peptide, instead of the traditional methionine residue. Folding is accomplished at the stage of the chimeric proinsulin molecule and then both the N-fused sequence and the C-peptide are removed simultaneously, in a single step, by reaction with trypsin. As fused peptide, a modified N-terminal region of human interleukin-2 (residues 1 to 22), followed by an hexapeptide, was designed (Fig. 1). A related sequence yielded high expression levels when fused to the antigenic determinants of HIV-1 proteins in *E. coli* [8]. It was found that the IL2-PI construction expresses the chimeric proinsulin at high level (20%) as inclusion bodies; this fused protein was folded at low protein concentration (100–200 $\mu\text{g/ml}$) to yield about 80% of correctly folded proinsulin and then it was converted into insulin by prolonged reaction (5 h) with trypsin and carboxypeptidase B at a low enzyme/substrate rate (1:600).

2. Materials and methods

Synthetic oligonucleotides and restriction enzymes were supplied by HeberBiotec (Cuba). The proinsulin gene was synthesized by Jimenez et al. [9]. Sequence grade proteinases were from Boehringer Mannheim (Germany); all other reagents were analytical grade from commercial suppliers.

2.1. Plasmid construction and cell culture

The prototrophic *E. coli* K12 strain W3110 (F[−]) was transformed according to the method of Hanahan [10] with plasmid pISL-31. This is a vector derived from plasmid pBR322, coding for the N-terminal sequence of interleukin-2 (residues 1 to 22) in which lysine residues 8 and 9 in the IL-2 sequence have been replaced by alanine and glutamine, respectively, followed by a pentapeptide, a lysine residue and the proinsulin (PI) sequence (Fig. 1). The expression was regulated by the *trp* promoter. The recombinant strain was grown overnight in Luria Bertani medium in the presence of tryptophan (100 $\mu\text{g/ml}$). Then, 500 ml of culture were inoculated into 5 l fermentation medium, containing 1% M9 salts, 2% casein hydrolysate, 0.4% glucose, 1% tryptone and 50 $\mu\text{g/ml}$ ampicillin and grown under aeration at 37°C. After 12 h, the cells were harvested and kept at −70°C until use.

*Corresponding author. Fax: (53) (7) 336008.
E-mail: protchem@serverdos.cigb.edu.cu

2.2. Isolation and purification of IL2-PI

All procedures were done at 0–4°C. Centrifugations were done at 3,000 rpm for 20 min. Twenty grams of harvested cells were homogenized in 100 ml of the standard buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride), supplemented with 1 M urea and 1% Triton X-100 and disrupted by sonic oscillation (3 cycles, 3 min each). After centrifugation, the sediment was resuspended, agitated and centrifuged successively in 25 ml of the standard buffer supplemented with 4 M urea and 2% Triton X-100, and then twice in 25 ml of the standard buffer. The sediment was solubilized with 12 ml of the standard buffer supplemented with 8 M urea (pH 8.9), stirred for 30 min and centrifuged. The supernatant was diluted with the same buffer to a final concentration of about 3 mg/ml. Cysteine groups were converted into their S-sulfonates by oxidative sulfitolysis, accomplished by the addition of sodium sulfite and sodium tetrathionate (20 eq. sulfite/10eq. tetrathionate/1 eq. cysteine). After 6 h, the reaction was stopped by dilution with water to a final urea concentration of 2 M, and by acidification at pH 4.0 with 2 N HCl. The IL2-PI S-hexasulfonate was collected as an abundant white precipitate, which was washed twice with 0.01% HCl and kept at –20°C or alternatively, it was immediately purified by anion exchange chromatography on a Q-Sepharose Fast Flow column (Pharmacia, Sweden). The column was equilibrated in the loading buffer (8 M urea, 20 mM Tris, 0.2 M NaCl, pH 8.9), the fusion protein hexasulfonate was dissolved and loaded at a protein concentration of about 3 mg/ml and the column was eluted with an increasing NaCl gradient. IL2-PI S-hexasulfonate eluted at about

0.6 M NaCl and then precipitated by dilution of urea to a final concentration of 2 M after acidification at pH 4.0.

2.3. Folding of the fusion protein and its enzymatic conversion into insulin

IL2-PI S-hexasulfonate was dissolved in the folding buffer (50 mM glycine, 1 mM EDTA, pH 10.5) and the solution was degassed. In order to exchange the sulfonate groups and to induce disulfide bond formation, β -mercaptoethanol was added (1.5 eq. per eq. of S-sulfonate) [1]. Folding was performed at protein concentrations varying from 25 μ g/ml to 1 mg/ml, at 4°C for varied times from 2 h to 24 h. To stop the reaction, the pH was adjusted to 4.0. Folding products were isolated by reversed phase HPLC, lyophilized, dissolved in 200 mM Tris, pH 9.0, to a protein concentration of 1 mg/ml and digested with trypsin (E/S: 1:600, w/w) or trypsin plus carboxypeptidase B (E/S: 1:600) at 37°C for 5 h. In order to confirm the structure of the main product, the peak corresponding to human insulin as well as a human insulin standard were cleaved with Glu-C endoproteinase at 25°C for 5 h (E/S: 1:20, w/w; in 0.2 M Tris, pH 8.0) and the digestion products were isolated by reversed phase HPLC and analyzed by fast atom bombardment mass spectrometry (FAB-MS).

2.4. Ultrastructural studies

The sample consisting of pelleted cells (2×10^7 cell) from transformed W3110 *E. coli* was fixed with 3.2% glutaraldehyde, and post-fixed for 1 h in 1% OsO₄. Then, it was rinsed with 0.1 M PBS, pH 7.2, and dehydrated in increasing ethanol concentrations. The embedding was in Spurr. The blocks were sectioned with an ultramicrotome (LKB 2188) and the ultrathin sections were placed on 400 mesh grids without membrane. All sections were examined in a Jeol-JEM 2000 EX electron microscope.

2.5. Fast atom bombardment mass spectroscopy

Mass spectra were recorded on a JEOL JMS-HX110 mass spectrometer equipped with a standard FAB ion source using a xenon ionizing beam (4 kV). Mixtures of glycerol and thioglycerol (1:1) and dithiothreitol and dithioerythritol (5:1) were used as matrix. The ion source was fixed at 10 kV accelerating potential and data acquired with a JEOL JMA-DA5000 mass data analysis system.

2.6. Other analytical procedures

Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) was performed on a 15% acrylamide gel according to Laemmli [11]. Sulfitolysis reactions were analyzed by PAGE in 8 M Urea on 7.5% polyacrylamide gels without SDS. Proteins were visualized by Coomassie blue staining. Protein recoveries were calculated on a weight basis, as determined by the Bradford method. Reversed phase HPLC was accomplished with dual wavelength at 214 nm and 280 nm with automatic data processing by using a Vydac C-4 column for protein analysis and a Vydac C-18 column (250×4.6 mm) for peptide mapping. Solvents and gradients were: for peptide mapping: A: 0.1% aqueous TFA, B: 0.1% TFA in acetonitrile (0% B to 60% B in 120 min); for sulfonated IL2-PI, A: 0.1% sodium phosphate, pH 7.0, B: 40% (A) + 60% acetonitrile (25% B to 65% B in 40 min); for folding products, A: 72% 0.15 M ammonium sulfate pH 4.0 + 28% acetonitrile, B: 40% 0.15 M ammonium sulfate pH 4.0 + 60% acetonitrile (10% B to 45% B in 10 min and then to 100% B in 55 min).

3. Results and discussion

A major problem in the production of human proinsulin in *E. coli* is its intracellular degradation. In order to achieve protein stability, PI has been expressed as a fusion protein which is resistant to proteolysis, the N-terminal extension being artificially engineered or selected from bacterial proteins [1–3].

A system for the overexpression of heterologous proteins in *E. coli* was recently developed, based on the fusion of the N-terminus (residues 1 to 62) of human interleukin-2 to several target proteins. This system allowed high expression (20% to 30%) of the immunodominant regions of HIV-1 and HIV-2 proteins [8]. In a previous design, we fused the PI gene to the IL-2 (1–62) sequence. This construction yielded about 30% of

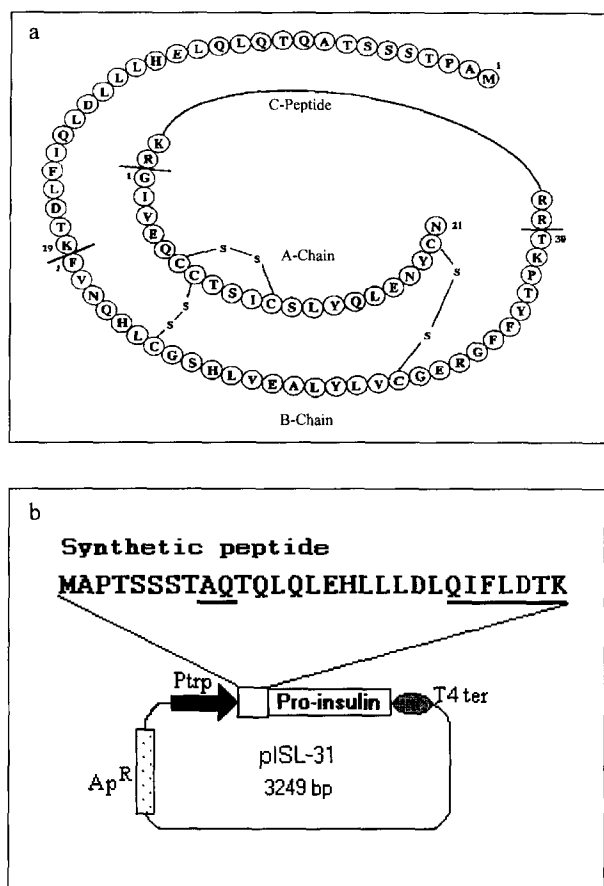


Fig. 1. (a) The chimeric IL2-PI sequence: a N-terminal fused peptide containing residues 1 to 22 of human interleukin-2, in which Lys⁸ and Lys⁹ have been replaced by Ala and Gln respectively, followed by an hexapeptide (QIFLDTK), the insulin B-chain (Phe¹ to Thr³⁰), the proinsulin C-peptide and the insulin A-chain (Gly¹ to Asn²¹). The additional methionine incorporated at the N-terminus of the IL-2 sequence is partially removed during biosynthesis. (Molecular mass: 12732.2 Da, insulin represents 46% of the molecular mass for the fusion protein.) (b) Genetic construction on plasmid pISL-31.

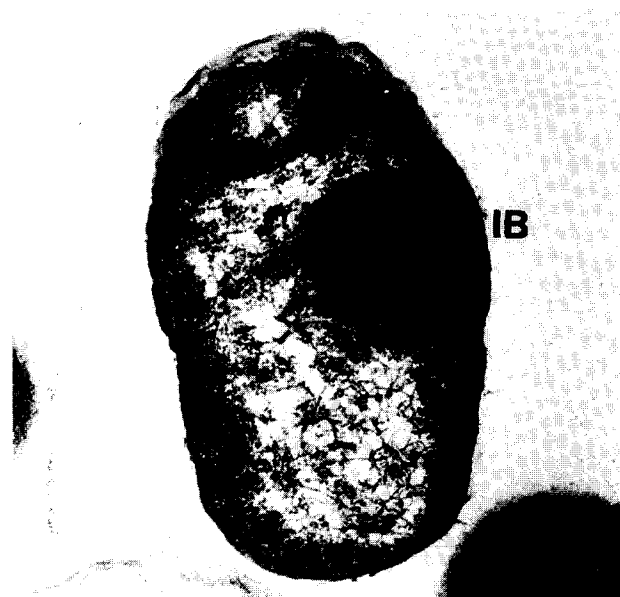


Fig. 2. Microphotograph showing at ultrastructural level the presence of an inclusion body (IB) inside the *E. coli* transformed cell, corresponding to the IL-2PI protein.

the chimeric protein but resulted in a very low solubility that diffculted further purification and folding (unpublished results). Therefore, a shorter fusion sequence was then designed, corresponding to a modified IL2 N-terminal region (1 to 22) in which two lysine residues were mutated to suppress these additional tryptic sites. In order to introduce a tryptic cleavage site at the end of the fused sequence, a hexapeptide containing lysine at its C-terminus was linked to the N-terminus of B-chain in the PI sequence (Fig. 1).

3.1. Expression and isolation of IL-2PI as inclusion bodies

The transformed strain *E. coli* W3110 containing plasmid pISL-31 was able to express the fusion protein IL2-PI as was evidenced by SDS-PAGE, densitometry and Western blot of disrupted cell extracts. The expressed protein accumulated at the cytosol in morphological distinct inclusion bodies identified by electron microscopy, and represented about 20% of the total cellular protein (Fig. 2).

Different concentrations of urea (from 2 M to 8 M) in Tris-EDTA buffer were evaluated for the selective extraction of *E. coli* contaminants from the insoluble protein fraction. It was concluded that one extraction with 4 M urea during 3 h sub-

Table 1
Molecular ions of peptide fragments corresponding to the enzymatic digestion of folding products 1 and 2 (Fig. 4)

Peak	Mass + H ⁺ (AMU)	Assignment
1	859.0	B-chain (23–29)
2	3148.5	C-peptide
3	4865.2	des-octa-insulin
4	5808.7	insulin
5	3112.1	N-extension
6	3243.3	N-extension + Met
7	2380.2	A-chain oxidized
8	2485.9	B-chain (1–22) oxidized

stantially eliminated most of the contaminants, the pellet was enriched in IL2-PI up to about 80% (Fig. 3), while no losses of IL2-PI in the supernatant were observed by electrophoresis. According to this, 200 mg of IL2-PI were obtained from 20 g wet weight cells. After purification by reversed phase chromatography, the primary structure of the fusion protein was verified by peptide mapping, FAB MS and automatic sequencing.

3.2. Oxidative sulfitolysis and purification of IL2-PI hexasulfonate

An efficient procedure for renaturing recombinant proinsulin was first developed by Frank and co-workers [1]; this procedure consists of (a) removal of the fused N-terminal sequence from the chimeric protein by cleavage with cyanogen bromide, (b) conversion of PI into its *S*-hexasulfonate, which is purified at this stage by anion exchange chromatography and (c) single step non-oxidative transformation of PI-*S*-sulfonate at basic pH into renatured proinsulin presenting its three disulfide bridges correctly arranged. In the present approach, a key point is the simultaneous removal of the fused sequence and the C-peptide in a single step after protein renaturation, therefore sulfitolysis was accomplished at the stage of the fused precursor. In order to establish optimal reaction conditions, oxidative sulfitolysis of IL2-PI was carried out at variable molar ratios for sodium sulfite/Cys-SH groups (from 1/1 to 50/1), with a constant proportion between sulfite and tetrathionate equal to 1/0.5. It was observed that conversion of IL2-PI into its hexasulfonate was complete in 1 h at room temperature, at a sulfite to cysteine molar ratio of 20/1. The fusion protein *S*-hexasulfonate was purified by anion exchange chromatography. The loading capacity of the matrix was about 50 mg of total protein per ml of gel. Equilibration of the matrix in 8 M urea was essential for the efficient retention of the protein during loading. The non-retained fraction contained only contaminant proteins while IL2-PI hexasulfonate eluted by increasing NaCl concentration as a highly pure fraction (over 98% as evaluated by reversed phase HPLC at neutral pH and 8 M urea PAGE).

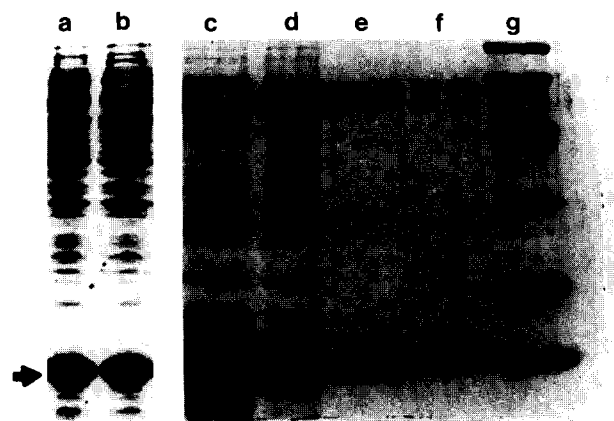


Fig. 3. SDS-15% polyacrylamide gel electrophoresis of IL2-PI fusion protein. (a,b) The initial crude extract of *E. coli* proteins containing the overexpressed (about 20%) fusion protein IL2-PI; (c,d) the inclusion bodies were extracted with (c) 2 M or (d) 4 M urea in Tris buffer. After selective extraction at 4 M urea, the protein was recovered with relatively high purity (approximately 80%); (e,f) the folded IL2-PI, isolated by reversed phase HPLC (corresponds to fraction 1, Fig. 4a); (g) molecular weight markers, the including cytochrome *c* (lower band).

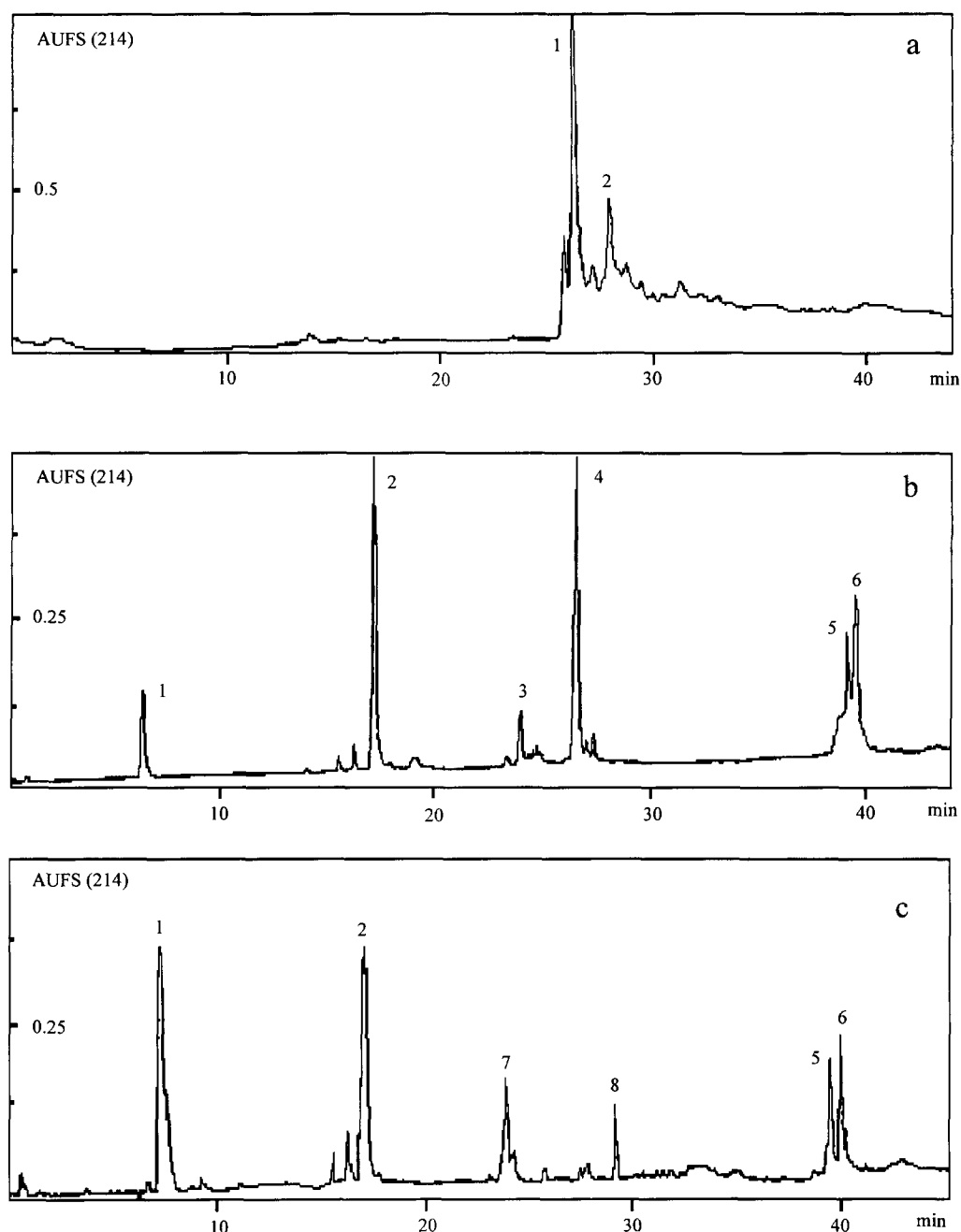


Fig. 4. Folding of the fusion protein IL2-PI. (a) HPLC of the folding reaction after 6 h. (Protein concentration: 100 $\mu\text{g}/\text{ml}$.) (b) Peptide map of fraction 1 in Fig. 4a, digested with trypsin and carboxypeptidase B. (c) Tryptic map of fraction 2 in Fig. 4a. The molecular ions of peptides are shown in Table 1.

3.3. Folding of the IL2-PI fusion protein

The conversion of the *S*-hexasulfonate into the renatured fusion protein was done according to the procedure previously reported for PI [1], which is based on the sulfonate exchange reaction with a thiol reagent. Analysis of the folding process by reversed phase HPLC with water/acetonitrile/TFA as solvents, resulted in poor resolution but was highly improved by using a gradient of acetonitrile in aqueous ammonium sulfate at pH 4.0.

Two main fractions were resolved (Fig. 4a) in a relative proportion of 80:20, as determined by protein quantitation

after HPLC separation. Fraction 1 was digested with trypsin and carboxypeptidase B (Fig. 4b). Mass values of the peptides are presented in Table 1 and corresponded to insulin, C-peptide and two peaks (5 and 6, Fig. 4b) at higher retention time, from the fused IL-2 peptide, differing by a methionine residue at their NH₂-terminus (generated by partial intracellular processing of the N-terminal residue in bacteria). Two minor components (1 and 3, Fig. 4b) were generated by internal cleavage at Arg²² (B-chain). Thus, the major compound, with lower retention time on HPLC corresponded to the fusion protein with the correctly folded PI domain.

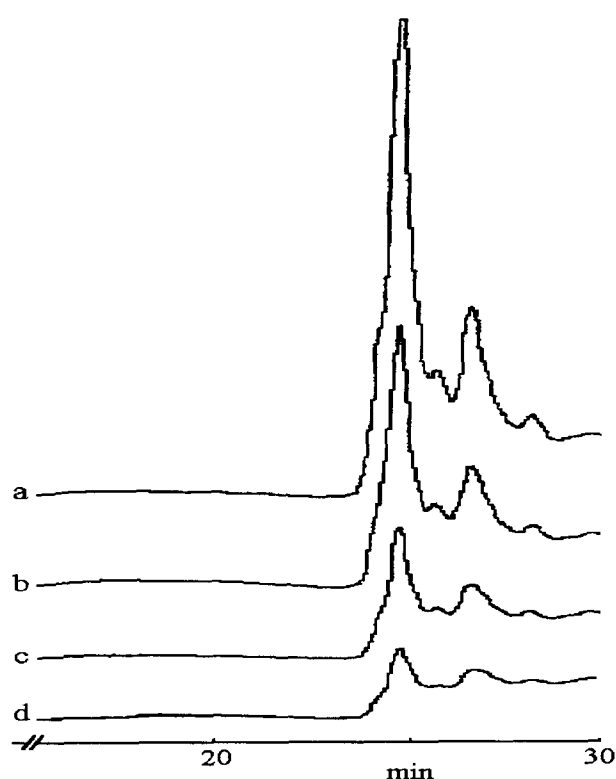


Fig. 5. Effect of protein concentration on the folding reaction. Folding concentrations are: (a) 200 $\mu\text{g/ml}$; (b) 400 $\mu\text{g/ml}$; (c) 600 $\mu\text{g/ml}$; (d) 800 $\mu\text{g/ml}$. In all cases the injected amount corresponded to the soluble fraction after centrifugation of 100 μg protein.

The analysis of tryptic peptides from the minor product (fraction 2, Fig. 4a) indicated the absence of interchain disulfide bridges (Fig. 4c and Table 1); peak 7 corresponded by its mass values to A-chain presenting two intramolecular disulfide bridges, while peak 8 corresponded to the Phe¹–Arg²² (B-chain) fragment with one disulfide bridge. The minor folding product corresponds then to a misfolded PI domain, in which interchain disulfide bridges are absent and replaced by intra-chain bridges. The amino acid composition of all tryptic peptides, derived from fraction 1 and fraction 2 was determined and confirmed the assignments made by FAB-MS.

Reaction time was not critical on the yield of folded IL2-PI. After 6 h at 4°C the reaction showed steady yields as observed by the HPLC profile when it was monitored during 24 h; further experiments were evaluated after 6 h of reaction. A very critical factor in the efficiency of the folding reaction was IL-PI S-sulfonate concentration. When this parameter was varied from 25 $\mu\text{g/ml}$ to 1 mg/ml, it was found that, as protein concentration increased, folding yields decreased dramatically (Fig. 5). Thus, at a concentration of 800 $\mu\text{g/ml}$, the yield of renatured IL2-PI was only 15% referred to the yield at 200 $\mu\text{g/ml}$. At the same time, the relative proportion of fractions 1 to 2 decreased significantly and a precipitate was observed that corresponded to higher molecular weight aggregates as evidenced by SDS-PAGE under non-reductive conditions. Therefore, protein concentration under folding for this fusion PI construction was fixed at 100 $\mu\text{g/ml}$. In order to look for an increase in folding efficiency at higher protein concentration, the effect of several

additives in the buffer was evaluated. Chaotropic agents at low molar concentration (urea 0.5 M to 2 M, and guanidinium hydrochloride 0.5 M to 2 M), a modification of solvent polarity by addition of ethanol (2% to 20%) and the influence of zinc cations that could coordinate to the folding domain of proinsulin (0.1 to 6 equivalents per mol of IL2-PI S-hexasulfonate), resulted in no substantial improvement or in a decrease in folding yield.

3.4. Conditions for the enzymatic cleavage of IL2-PI

An essential aim of the present approach was the simultaneous removal of both the N-terminal fused peptide and the C-peptide by trypsin digestion in presence of carboxypeptidase B. Due to the existence of two potential tryptic cleavage sites inside the insulin molecule (Arg²² and Lys²⁹ in B-chain), it was necessary to search for highly selective reaction conditions. The explored experimental parameters included buffer pH (from 7.0 to 10.5), trypsin/substrate ratio (from 1:50 to 1:1000), digestion time (from 10 min to 6 h) and temperature (4°C, 28°C and 37°C). It was found that, while cleavage of the C-peptide proceeded quantitatively in all the pH range investigated, the pH was critical on the removal of the N-terminal peptide and best results (over 90% as estimated by reversed phase HPLC) were attained at pH 9.0 and 9.5 (Fig. 4b).

Modification of trypsin/substrate ratio resulted in severe changes in HPLC profiles. Best results, corresponding to minimal internal cleavage inside B-chain and maximal removal of the N-terminal peptide, were obtained for a trypsin/substrate ratio in the range of 1/400 to 1/600. On the other hand, while the C-peptide was totally removed after 10 min (pH 9.0, E/S = 1/600, 37°C), removal of the N-terminal extension was completed only after prolonged reaction during 5 h (Fig. 4a). Under these conditions (pH 9.0, E/S = 1:600, 5 h, 37°C) des-octa-insulin (due to cleavage at Arg²²) represented about 3%–8% as evaluated from several independent experiments. Variation in this proportion could be related to differences in the specific activity of different batches of trypsin. Variations in temperature between 4°C and 37°C affected mainly the rate of the enzymatic digestion, and only slightly, the proportion between human insulin and des-octa-insulin.

Confirmation of the primary structure and disulfide bonds of the obtained recombinant insulin was made by a comparative HPLC mapping followed by FAB-MS analysis of the Glu-C endoproteinase digestion of our product and a human insulin standard. Identical HPLC maps were observed, and closely matched to those reported by Frank and coworkers [1].

The molecular ion $m/z = 1378.0$ (corresponding to the fragment A^{18–21} + B^{14–21}) and the molecular ion $m/z = 2967.9$ (corresponding to fragment A^{5–17} + B^{1–13}) confirmed the correct arrangement of the interchain disulfide bonds. The internal disulfide bond in A-chain (Cys⁶–Cys¹¹) although not directly verified, was deduced to be correct based on the identity of HPLC Glu-C endoproteinase maps for the recombinant human insulin and the human insulin standard.

4. Final comments

As an attractive approach for processing insulin precursors in *E. coli*, it was conceived to fold the fusion protein, carrying a tryptic site at the end of the N-terminal extension, and then, to accomplish in a single step the removal of the C-peptide and

the N-terminal extension. It was found that the chimeric LI2-PI protein could be correctly folded with yields about 80%. Nevertheless, while for PI S-hexasulfonate an effective process is reported at 2 mg/ml [1], here the protein concentration on folding had to be kept at 100–200 µg/ml. The way how this short peptide affected so drastically the folding behavior of the PI domain is not clear. The second objective of the present approach, the search for selective trypsination conditions to remove the stabilizer and the C-peptide, required a careful evaluation over an extensive set of experimental conditions. While a complete removal of the C-peptide took place always efficiently, conditions for an efficient cleavage of the N-terminal fused sequence that at the same time preserved B-chain integrity were difficult to achieve and, finally, were found by prolonging the reaction time up to 5 h while reducing appreciably the enzyme/substrate ratio.

The presented approach explored a route to the production of recombinant insulin in bacteria that avoids the use of cyanogen bromide. The search for other N-terminal fused sequences that may provide higher yields on folding at increased protein concentration is under study.

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