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Journal of Chromatography A, 846 (1999) 143–156

JOURNAL OF CHROMATOGRAPHY A

Characterization of the isoforms of PIXY321, a granulocyte-macrophage-colony stimulating factor–interleukin-3 fusion protein, separated by preparative isoelectric focusing on immobilized pH gradients

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Abstract

We present here the purification and the characterization of the isoforms of PIXY321, a genetically engineered fusion of granulocyte-macrophage-colony stimulating factor and interleukin-3 expressed in yeast. The isoforms of PIXY321 were isolated using preparative isoelectric focusing (IEF) on immobilized pH gradients. Analysis of the collected fractions on analytical IEF gels showed that PIXY321 was resolved into four discrete isoforms of isoelectric point (p*I*) 5.0, 5.1, 5.2 and 5.3 with excellent yields. Subsequent analysis of purified isoforms of PIXY321 by peptide mapping and mass spectrometry linked the microheterogeneity of the original molecule to three parameters, the presence of deamidated residues, charged glycans and the pattern of O-linked glycosylation along the peptide sequence. This last parameter emphasizes the role of conformational aspects as key factors influencing the apparent isoelectric point of protein isoforms.

Keywords: Preparative electrophoresis; Glycosylation; Proteins; Mannose; PIXY321

1. Introduction

Elucidation of the molecular basis of protein microheterogeneity, a difficult challenge of protein chemistry, requires separating sizable amounts of the isoforms observed on analytical isoelectric focusing (IEF) gels. We present here the purification and the characterization of the isoforms of a recombinant human molecule expressed in *Sacharomyces cerevisiae*. PIXY321, a fusion of granulocyte-macrophage-colony stimulating factor (GM-CSF) and interleukin-3 (IL-3), has been developed as a potential drug for the treatment of chemotherapy-induced myelosuppression [1]. PIXY321 is a glycoprotein expressed in yeast, under the control of the α-factor system, as an M_r 35 000 monomer [2]. The consensus sites of N-linked glycosylation originally present in the human molecules have been removed by site directed mutagenesis. The characterization of the yeast-expressed PIXY321 showed that the glycoprotein carries four sites of O-glycosylation, one in the GM-CSF portion and three in the IL-3 portion [3]. PIXY321 separates on analytical IEF gels with immobilized pH gradients (IPGs) into four discrete isoforms of isoelectric point (p*I*) 5.0, 5.1, 5.2 and 5.3.

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00127-2

Preparative isolation of isoforms visualized on analytical IEF gels presents a challenging separation problem. A narrow set of chromatographic techniques can be used, such as ion-exchange [4,5] or chromatofocusing [6,7], but the success in resolving subtle charge differences in otherwise identical proteins is often limited. Preparative isoelectric focusing in a liquid vein using immobilized pH gradients, an electrophoretic technique developed by Righetti and co-workers [8,9,10], offers an interesting alternative to chromatographic methods and overcomes the limitations of preparative isolation from gel matrices. Isoelectric membranes, of defined pH values, are prepared by mixing appropriate amounts of acrylamido buffers of known p*K* values [11,12]. A set of isoelectric membranes generates a pH gradient across consecutive chambers. The goal of the experiment is to force the molecules to migrate through the membranes, under application of an electric field, and focus them at their p*I* values inside the chamber delimited by membranes with bracketing pH values. We present the results we obtained for the PIXY321 molecule and the analytical characterization of the purified isoforms.

2. Experimental

2.1. Preparative IEF

Preparative focusing was performed in a multicompartment electrolyzer (Isoprime, Hoefer Pharmacia Biotech) that uses isoelectric membranes with predetermined pH values. A specific pH for each membrane was generated by the use of a combination of acrylamido buffers (Fluka or Pharmacia) of defined p*K* values as calculated using DR.PH software (Pharmacia). Acrylamide solutions were prepared at each pH value by mixing the appropriate combination of acrylamido buffer reagents with the acrylamide monomer (5% final) and the cross linking reagent (acrylamide–bisacrylamide, 92:8). Then the polyacrylamide gels were allowed to polymerize on glass microfiber filters (Whatman).

PIXY321 membranes were prepared at the following pH values: 4.50, 4.84, 5.03, 5.18, 5.36, 5.50, 5.65. The buffering capacity was calculated at 6 mequiv./l per pH unit. The membranes delimited eight chambers. The chambers were filled with 30 ml of deionized water and the instrument was run for 2 h prior to loading. PIXY321 was solubilized at 0.5 mg/ml and loaded in a single chamber. In order to limit the insolubility of isoforms at their isoelectric points, the solubilizing agents glycerol and CHAPS (cholamidopropyldimethylammoniopropanesulfonate), were added. Separations were conducted in 20% glycerol–2% CHAPS. Total protein load was 15 mg. Electrophoresis limits were set at 3500 V, 20 mA, 4 W with separation times ranging from 16 to 96 h at 4°C.

2.2. Analytical IEF

Isoform separations were evaluated by concentrating aliquots of each fraction from the instrument and analyzing on Novex (pH 3–7) pre-cast IEF gels. Electrophoresis was carried out for 2 h at 2 W (500 V limit). Gels were fixed using 12% trichloroacetic acid and stained with a colloidal Coomassie blue stain.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

PIXY321 was analyzed by SDS–PAGE using 4–20% acrylamide gels (Novex). The electrophoresis was performed at 30 mA constant current until the bromophenol blue dye reached the bottom of the gel. The gel was stained with 0.2% Coomassie blue R250 in water–methanol–acetic acid (50:43:7, v/v) solution.

2.4. Peptide mapping

Reduced-alkylated PIXY321 (150 μg) was solubilized at 1 μg/μl in 150 μl 25 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.5 and digested by 2% (w/w) Lys-C endoproteinase for 1 h at 37°C. The reaction was stopped by diluting to 500 μl with boiling water and holding the sample at 100°C for 2 min. The mixture was then frozen and stored at −20°C until analysis. The digest was injected on a C_{18} column (250×4.6 mm, 5 μm, 300 Å, Vydac) equilibrated in water–0.1% trifluoroacetic acid (TFA) (solvent A) and the peptides separated by the following gradient

of acetonitrile–0.1% TFA (solvent B): 0–15% B in 10 min, 15–25% B in 10 min, 25% B isocratic for 5 min, 25–32% B in 7 min, 32% B isocratic for 10 min, 32–40% B in 15 min, 40–70% B in 30 min. The peptide elution was monitored at 220 nm.

2.5. Monosaccharide composition

A 200-μg amount of PIXY321 was hydrolyzed with 2 *M* TFA for 1 h at 100°C, dried under vacuum and taken back in solution with 100 μl of water. Monosaccharide standards were isocratically eluted with 17 m*M* sodium hydroxide on a Carbopac PA1 column (250×4 mm, Dionex) and monitored using a pulsed amperometric detector. A 20-μl sample of the hydrolysate was injected on the column under the same conditions and the detected sugars, by comparison to standards, were identified as only mannose residues. Quantification by peak integration revealed a monosaccharide composition for PIXY321 of 7–8 mol mannose residues/mol protein.

2.6. Deglycosylation

A 100-μg amount of PIXY321 solubilized at 10 μ*M* in 100 m*M* sodium acetate, 2 m*M* zinc chloride, pH 5.0 was deglycosylated using 2 U of α-mannosidase 1-2,3,6 (Oxford Glycosciences) for 24 h at 37°C. The deglycosylated molecule was separated from reactants and stabilizers by reversed-phase high-performance liquid chromatography (RP-HPLC).

2.7. Electrospray mass spectrometry

Electrospray mass spectrometry (ES-MS) measurements were obtained on a Finnigan TSQ700 triple quadrupole instrument with positive-ion detection mode. Liquid chromatography (LC)–MS experiments were performed using a splitter device to adjust the elution solvent flow entering the ion source to 10 μl/min. High voltage (5 kV) was applied to the electrospray needle and nitrogen gas was used as an auxillary and sheath gas for aerosol formation. Evaporation of the solvent occurred in the heated capillary maintained at 250°C. Scanning was performed from m/z 400 to m/z 2400 in 4 s.

2.8. N-Terminal sequencing

Microsequencing was performed on an Applied Biosystems 470A gas-phase protein sequencer and the phenylthiohydantoin derivatives were analyzed by on-line chromatography with an Applied Biosystems 120A HPLC system. The ratio of the two PIXY321 N-terminal forms, full length and the molecule starting at Ala3, was calculated using the repetitive yields of proline residues.

2.9. Bioassay

The biological activity of PIXY321 was assessed by its capacity to stimulate the proliferation of TF-1 cells, an erythroleukemia cell line dependent on EPO, GM-CSF and IL-3 [13]. PIXY321 stimulates a dose-dependent proliferation of the cells, measured by incorporation of tritiated thymidine into their DNA, allowing for quantitation of the cytokine activity.

3. Results

3.1. Expression and purification of PIXY321

We previously described the production, purification and characterization of PIXY321 in yeast [3]. Briefly, the plasmid coding for PIXY321 was expressed in yeast under the control of the leader and signal sequence of the yeast α-factor [2]. The prepro-α sequence that directs the glycosylation and secretion of the precursor molecule in yeast is cleaved by the Kex2 protease to release the native PIXY321 in the culture medium. PIXY321 production was scaled up in fermentors from 10 to 1200 l and the protein was purified by four successive steps of ion-exchange chromatography [3]. The final purified product runs at an apparent molecular mass (M_r) of 35 000 on SDS–PAGE with 98% purity as estimated by scanning densitometry (Fig. 1, second lane). The final purified PIXY321 elutes as a single peak, 99% pure by integration, on analytical RP-HPLC and shows no aggregates or low-molecular-mass contaminants when analyzed by size-exclusion HPLC [3]. The final purified PIXY321 molecule was used in the present work as starting material for the

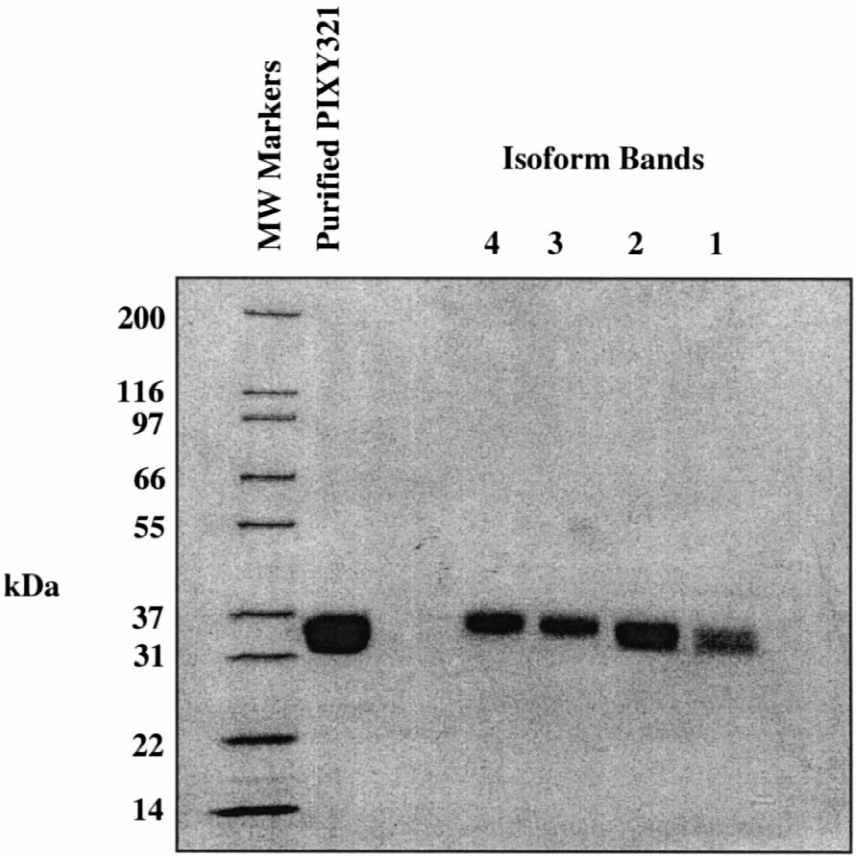

Fig. 1. SDS–PAGE of purified PIXY321 starting material and separated PIXY321 isoforms. The fractions separated by preparative IEF are labeled as bands 1, 2, 3 and 4. Samples were loaded on a Novex SDS–PAGE 4–20% pre-cast gel and run at 30 mA constant current. The gel was stained with 0.2% Coomassie Blue R250 in a water–methanol–acetic acid solution. Molecular masses are compared against Mark12 molecular mass (Mw) standards (Novex) (kDa=kilodalton).

preparative separation of four discrete isoforms visualized on analytical IEF gels (Fig. 2, second lane).

3.2. Purification and characterization of PIXY321 isoforms

3.2.1. Purification

Use of the viscosity enhancing reagents, glycerol and CHAPS, was necessary to improve the separation of the molecules. These additives reduce the precipitation of isoforms on the membranes, control the fluid transport and limit the movement of water by electroendosmosis toward the anode [10]. The purified PIXY321 starting material was loaded on the acidic side, at the appropriate position for the most acidic form (chamber 3, p*I* membranes=4.84–5.03) and allowed to run for 16–48 h. The experiment was designed to minimize the movement of molecules. Analysis of the collected fractions by analytical IEF showed that after the first run, the acid isoform (band 4) was still in chamber 3 whereas the basic isoform (band 1) was found isolated in chamber 5 (p*I* membranes=5.18–5.36). Chamber 4 (p*I* membranes=5.03–5.18) contained a mixture of bands 2, 3 and 4. Fractions containing pure isoform were removed for storage and the impure fractions were recycled in the instrument (chamber 3) for an extended run up to 96 h. Analytical IEF showed that band 3 was the major component of chamber 4

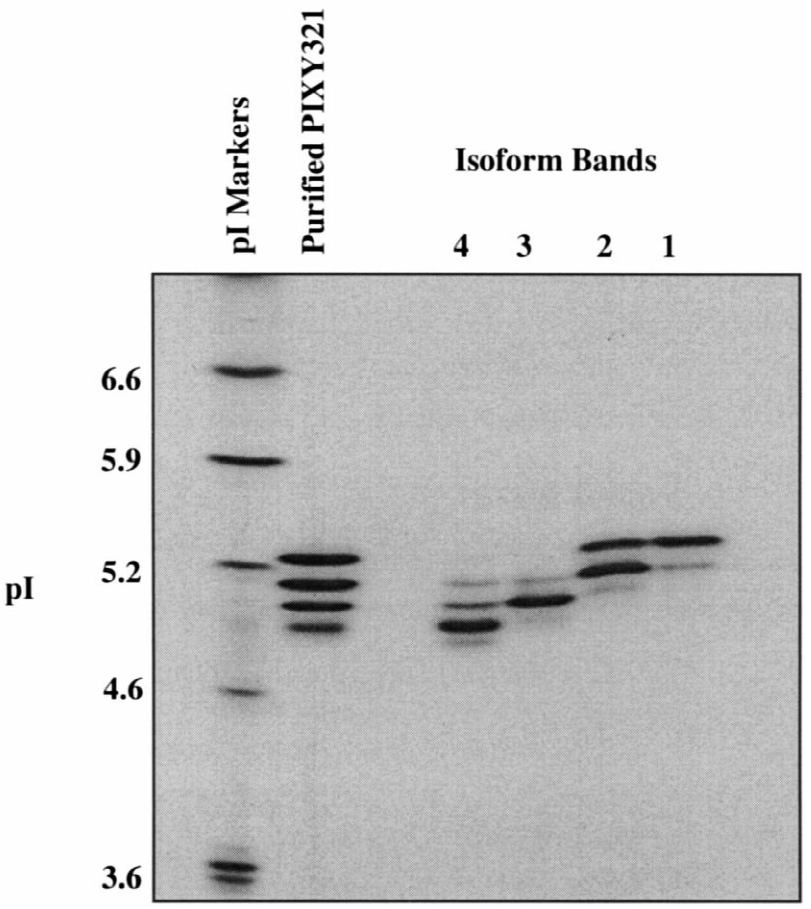

Fig. 2. IEF gel of purified PIXY321 starting material and separated PIXY321 isoforms. The fractions separated by preparative IEF are labeled as bands 1, 2, 3 and 4. Samples were loaded on a Novex (pH 3–7) pre-cast IEF gel and electrophoresis was performed for 2 h at 500 V, 2 W. Gels were fixed using 12% trichloroacetic acid and stained with a colloidal Coomassie Blue stain. Isoform p*I* values are compared with IEF calibration (pH 3–10) markers (Pharmacia).

whereas band 2 was mainly found in chamber 5. Each fraction was separately re-run until complete separation was obtained. PIXY321 was resolved into four major bands (Fig. 2) with yields estimated at 90% by absorbance measurement at 280 nm. SDS–PAGE analysis of the isoforms sorted by increasing p*I* correlated with a decrease in apparent mass (Fig. 1).

The purified PIXY321 molecule is isolated as a mixture of two N-terminal forms due to the action of the yeast protease ysc IV (Ste 13 gene product) involved in the maturation of natural α-factor in yeast [3]. Although the biological role of Ysc IV in α-factor expression is to remove (Glu/Asp)–Ala extensions present in α-factor precursor [14], the Ala–Pro dipeptide sequence at the N-terminus of PIXY321 is also an excellent substrate [15]. Consequently, Ysc IV activity generates a mixture of two N-terminal species of PIXY321, full-length and a molecule starting at Ala3. N-Terminal sequencing was performed to evaluate the distribution of the two N-terminal species in each isoform. The result shows that the relative percentage of the full-length molecule increases with decreasing p*I* (Table 1). The biological activity of each isoform was measured and the TF-1 bioassay indicated that the different isoforms had similar potency (Table 2).

Purified isoforms 1, 2, 3 and 4 were further

Table 1
N-Terminal sequencing of PIXY321 isoforms, showing an increase in the relative percentage of the full-length molecule to the truncated molecule with decreasing p*I*[a]

PIXY321 sample	Purified PIXY321	Band 1 (p*I* 5.3)	Band 2 (p*I* 5.2)	Band 3 (p*I* 5.1)	Band 4 (p*I* 5.0)
Full length (%)	60.0	55.5	59.8	63.2	69.4
Ala3 (%)	40.0	44.5	40.2	36.8	30.6

[a] The ratio between the two forms was calculated using the repetitive yields of proline residues.

analyzed by Lys-C peptide mapping and LC–MS (Figs. 3 and 4), a methodology previously used to characterize the purified PIXY321 molecule [3].

3.2.2. Analysis of modified amino acids

Comparative peptide maps of the four purified isoforms showed that peak 11 (eluting at 45 min, Fig. 4), absent in the most basic isoform, was raised in the most acidic form at the expense of peak 10 (Fig. 4). These two peptides, of mass 4419.77 u and 4420.88 u respectively, represented the same fragment 167–204 (L9, theoretical mass=4420.00 u) with a 1 mass unit difference. This mass difference was explained by the presence of a deamidated residue in peak 11. The deamidation position was localized by complete N-terminal sequencing of both peaks. N-Terminal sequencing of peak 10 showed a normal response for all seven Asn residues. The sequencing of peak 11 showed that the residue Asn176 was modified. Other Asn residues along the fragment were present at normal levels.

3.2.3. Analysis of O-linked glycosylation

3.2.3.1. Neutral glycans

Monosaccharide compositional analysis showed that, as expected for an O-glycosylated protein expressed in yeast, mannose was the only sugar detected in PIXY321. Characterization of the purified PIXY321 molecule showed that O-glycosylated structures were located on four Lys-C peptides, L1 (N-terminal) at Ser9, L7, L11 at site Thr227 and L14 (C-terminal) at Ser267. On the Lys-C peptide map (Fig. 4) these 4 glycopeptides are positioned as follows. Peptide L1 elutes in two peaks, 17 and 18 (75 min). Peptide L7 is found associated with peaks 13, 14 and 15 (50 min). Peptide L11 elutes in two peaks 7 and 9 (35–40 min). Finally peptide L14 is scattered in numerous small peaks eluting early in the chromatogram (12–25 min). Each glycosylated peptide represented under these peaks was analyzed in each individual LC–MS of the purified isoforms (Fig. 4).

The scans of peak 18 exhibited two series of ions that were deconvoluted to two masses of 7256.41 u and 7088.21 u, corresponding to the masses of two non-glycosylated fragments L1, 1–63 and 3–63 (theoretical masses=7199.09 u and 7030.89 u, respectively), with two carboxymethyl-cysteine residues (CM-Cys). Peak integration showed that peak 18 decreased from 30% of the total L1 area (sum of peaks 17 and 18) in the most basic isoform (band 1, p*I* 5.3) to 10% in the most acidic isoform (band 4, p*I* 5.0). The spectrum obtained for peak 17 exhibited a complexity typical of glycosylation heterogeneity (Fig. 5). Analysis of the scans across the peak demonstrated that peak 17 corresponded to the progressive elution of glycopeptides with masses ranging from 7418.83 u to 8876.87 u. These masses correspond to peptide L1 carrying a distribution from

Table 2
Bioactivity of PIXY321 isoforms, assessed by the ability of the protein to stimulate the proliferation of TF-1 cells. The TF-1 assay results indicate that the isoforms have similar biological activity[a]

PIXY321 sample	Purified PIXY321	Band 1 (p*I* 5.3)	Band 2 (p*I* 5.2)	Band 3 (p*I* 5.1)	Band 4 (p*I* 5.0)
Specific activity (U/μg)	$1.41 \cdot 10^5$	$1.44 \cdot 10^5$	$1.49 \cdot 10^5$	$1.25 \cdot 10^5$	$1.23 \cdot 10^5$

[a] TF-1 is an erythroleukemia cell line dependent on erythropoiethin (EPO), GM-CSF and IL-3 [13].

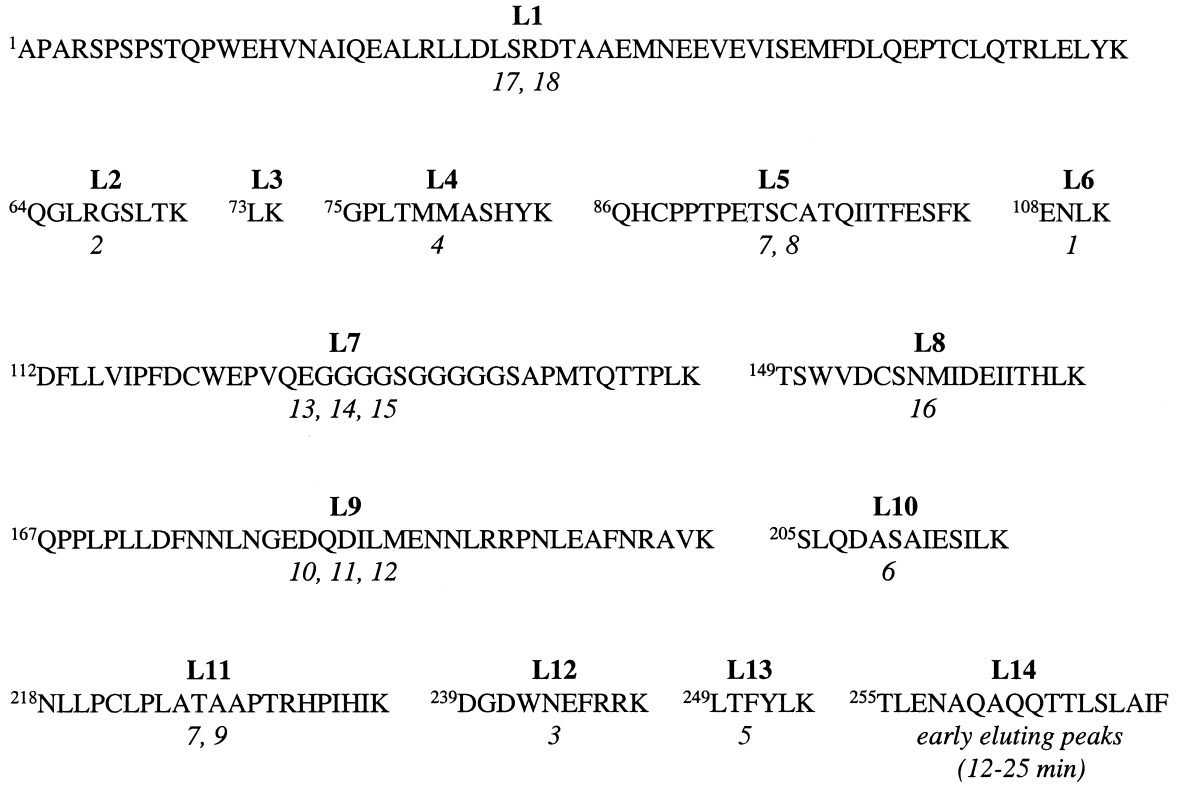

Fig. 3. Amino acid sequence of PIXY321 with Lys-C fragments numbered in bold above the sequence. The positions of the first amino acid residue of each fragment are noted in superscript. The major peaks of the peptide map (labeled in Fig. 4, bottom chromatogram) are given below the sequence in italics.

one to 10 mannose residues (Fig. 5). The major form, with four mannose residues, was the same on all the isoforms. The acid isoforms carried the highest glycosylated peptides, up to 10 mannose residues, whereas the basic isoforms had smaller glycan structures. The overall variation of glycosylation on the N-terminal peptide was quantified by peak integration (Figs. 4 and 6). The extent of glycosylation on glycopeptide L1 (peak 17) was increased up to 90% with decreasing p*I*, a variation concomitant with the progressive diminution of non-glycosylated peptide L1 (peak 18) to 10%. Conversely, the glycosylation of glycopeptide L1 (peak 17) represents only 70% of all N-terminal peptides, associated to 30% of non-glycosylated counterparts (peak 18), in the most basic isoform (Figs. 4 and 6).

The deconvolution of the spectrum of peak 15, eluting at 52 min (Fig. 4), gave the mass 3764.94 u, corresponding to the fragment 112–148 (L7, theoretical mass=3707.17 u) with one CM-Cys. The more hydrophilic glycosylated equivalents of this fragment eluted under peaks 13 and 14 with the form L7+2 mannoses as the main species. The relative ratio of glycosylated versus non-glycosylated L7 varied dramatically (Fig. 4). In band 1 (p*I* 5.3), peak integration showed that the non-glycosylated peptide L7 (peak 15) represented 75% of all fragments L7 while in the most acidic form (band 4, p*I* 5.0) it only accounted for 35%.

Sensitive MS measurements showed that peptide L14 was separated in several species due to C-terminal amino acid sequence variation. The C-terminal fragments, relatively difficult to visualize on the UV trace (Fig. 4, 12–25 min), were followed on the mass detector by their doubly charged ions. Two populations were represented. The non-glycosylated fragments (−4) and (−6), missing four and six residues at the C-terminus respectively, were de-

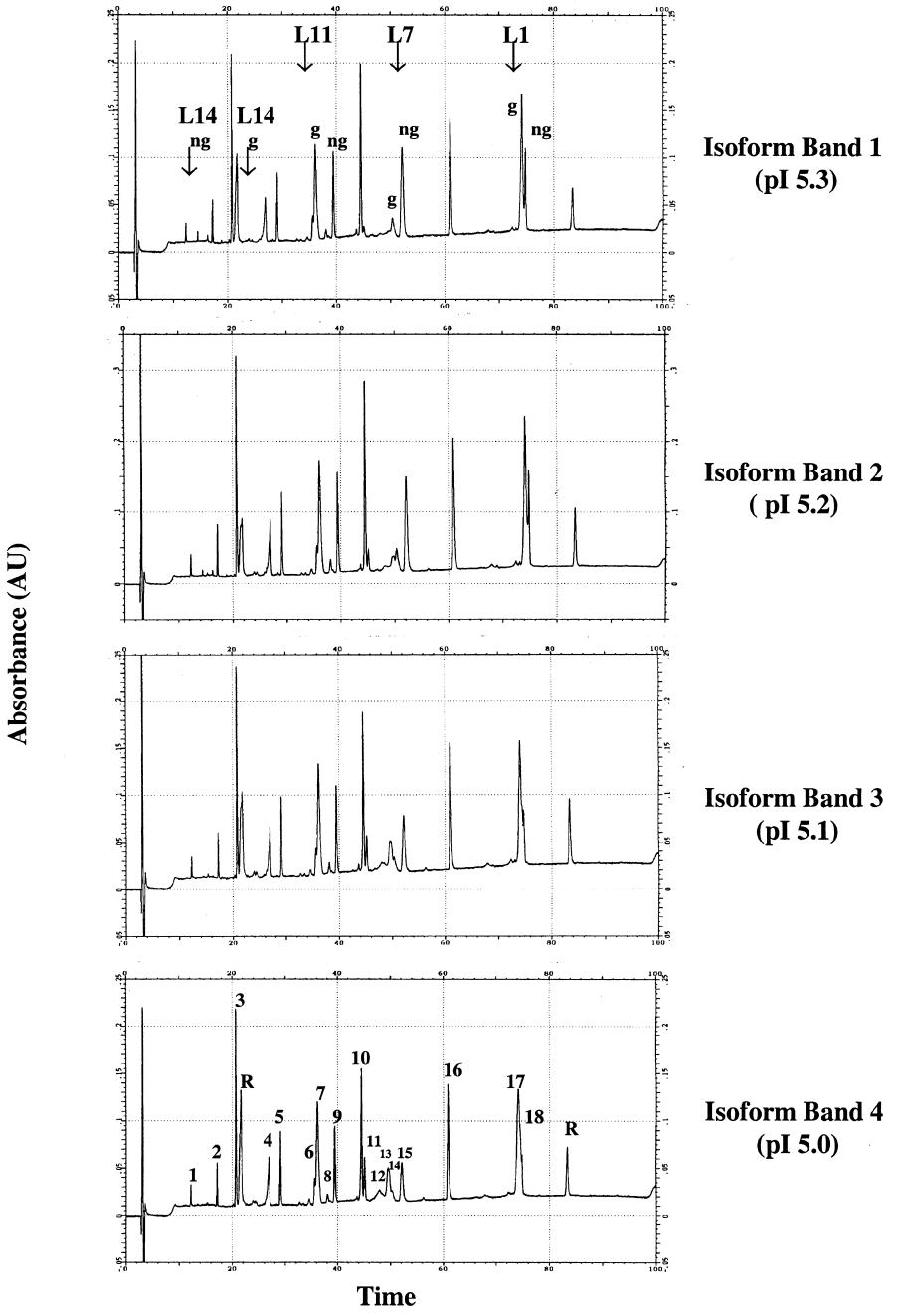

Fig. 4. Peptide maps of PIXY321 isoforms (g=glycosylated, ng=non-glycosylated, R=reagents). The reduced-alkylated isoform fractions were digested with 2% (w/w) Lys-C endoproteinase in 25 m*M* Tris–HCl, 1 m*M* EDTA, pH 8.5 for 1 h at 37°C. The digestion was stopped by adding boiling water and holding the digest at 100°C for 2 min. RP-HPLC analysis was conducted with a C_{18} column (Vydac, 250×4.6 mm, 5 μm, 300 Å) using the following gradient of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) and monitoring the absorbance at 220 nm: 0–15% B in 10 min, 15–25% B in 10 min, 25% B isocratic for 5 min, 25–32% B in 7 min, 32% B isocratic for 10 min, 32–40% B in 15 min, 40–70% B in 30 min. The Lys-C peptides that carry on O-glycosylation site, L1, L7, L11 and L14 are indicated with an arrow (upper chromatogram).

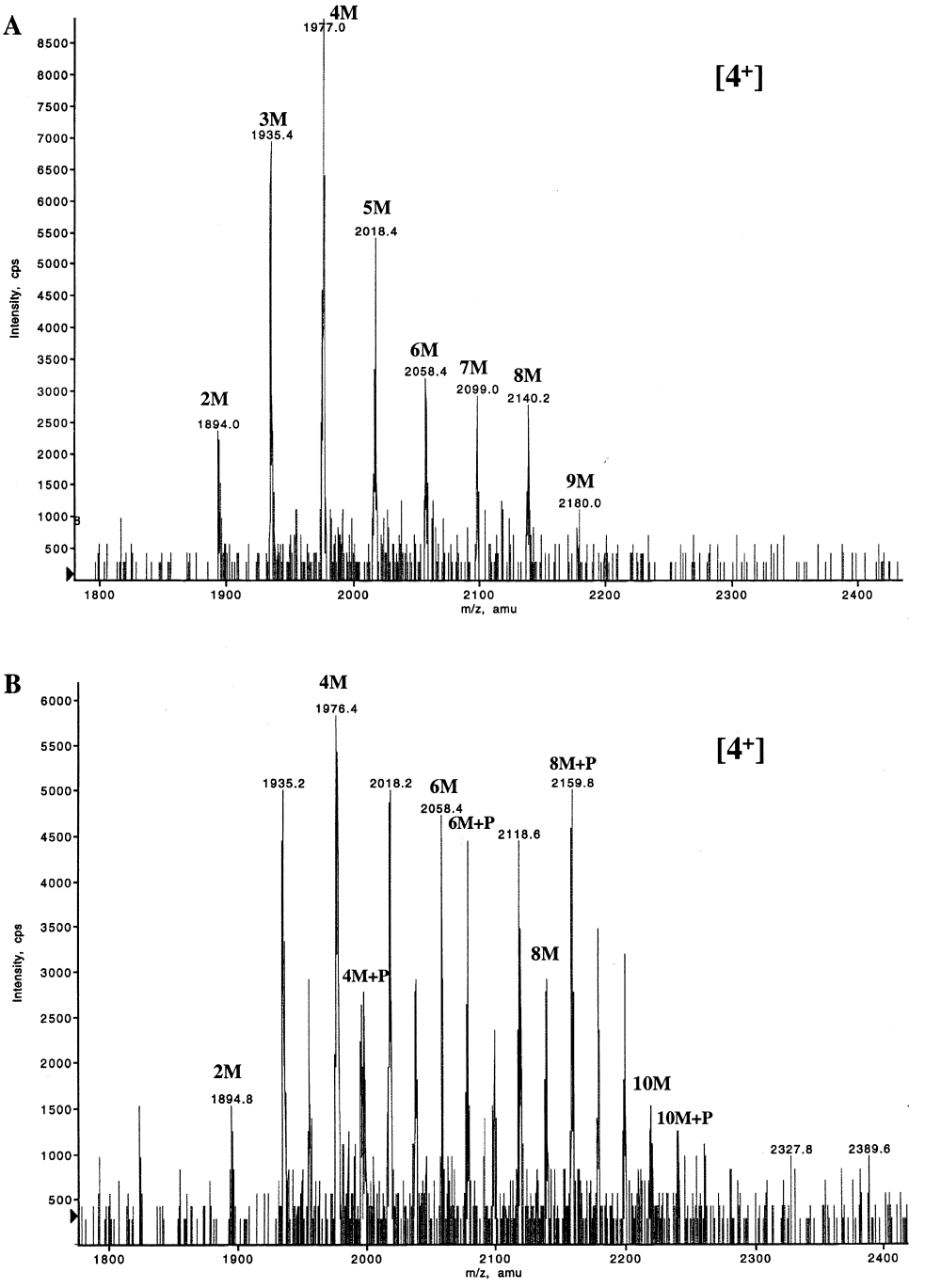

Fig. 5. Mass spectra of glycopeptide L1 (peak 17) in (A) basic band 1 (p*I* 5.3) and (B) acidic band 4 (p*I* 5.0). Only the m/z signals of the quadruply charged ions are represented in the figure. The spacing between two signals in spectrum A corresponds to the mass of one mannose residue. The masses of basic band 1 (spectrum A) correspond to L1 carrying from two to nine mannose residues. The signals of acidic band 4 (spectrum B) correspond to two series of ions. One series is equivalent to the ions displayed in spectrum A. The second series of ions, 80 mass units apart, is consistent with the presence of one phosphate residue. For simplicity, only the peaks corresponding to even numbers of mannose residues are labeled in spectrum B.

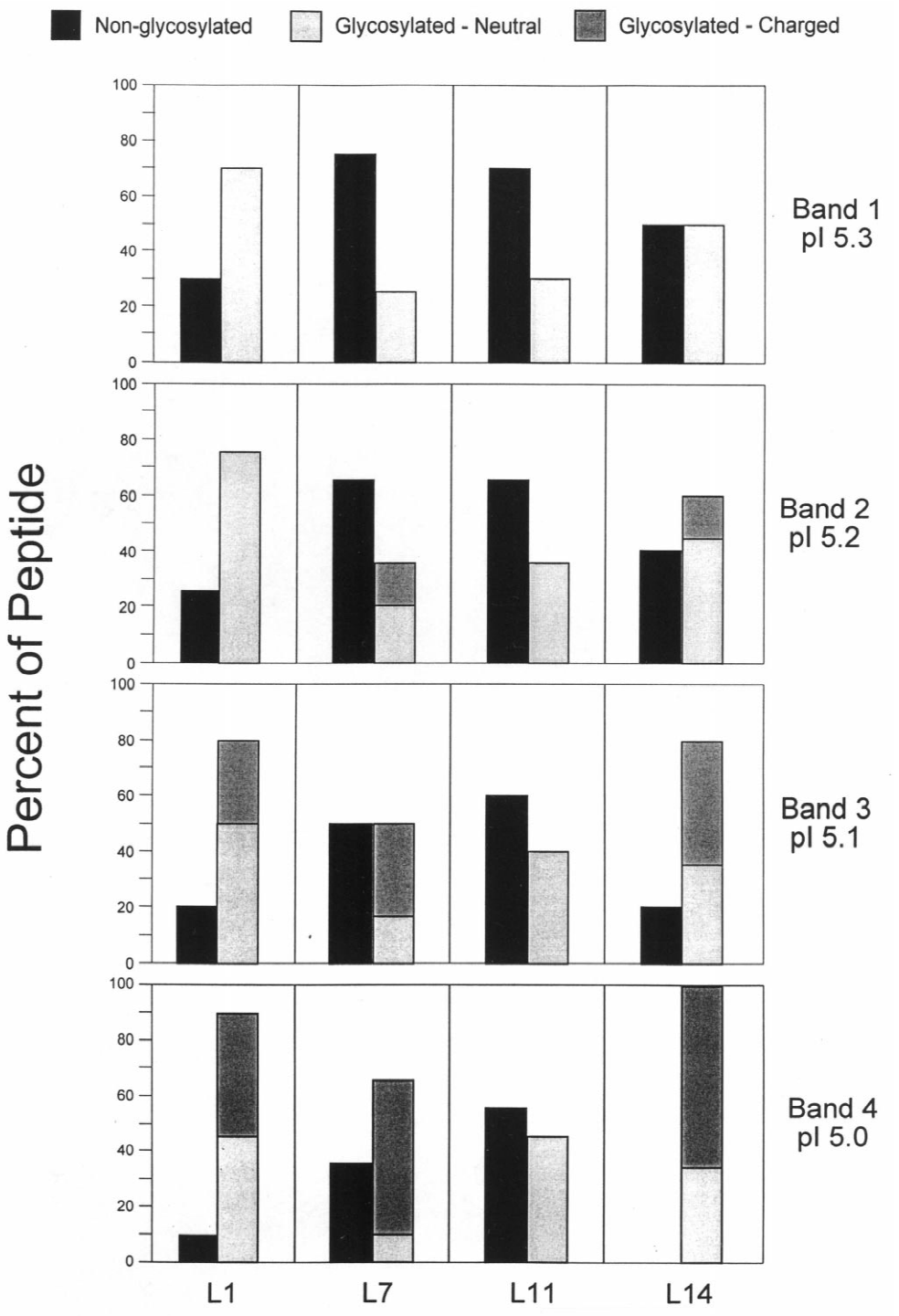

Fig. 6. Schematic illustration summarizing the glycosylation content for L1, L7, L11 and L14. The *x*-axis represents the glycosylated and non-glycosylated peptides L1, L7, L11 and L14. The sum of glycosylated and non-glycosylated forms is equal to 100%. The percentage of each form is represented for each isoform.

tected between peaks 1 and 2 (12–15 min), while non-glycosylated fragment (−5) missing five residues, was between peaks 2 and 3 (15–21 min). These C-terminal peptides were visible in the map of the basic isoform but absent in the acidic form (Fig. 4). The glycosylated C-terminal fragments were detected between peaks 3 and 4 (21–25 min). The glycosylated peak (−3) was a very minor form. The structures of glycosylated fragment (−2), carrying three to five mannoses, and fragment (−1), carrying larger glycans up to nine mannose residues, were constant throughout the four isoforms.

The mass spectrum of peak 9, eluting at 40 min (Fig. 4), could be deconvoluted to a mass of 2334.36 u, expected for the fragment L11, 218–238 (theoretical mass=2276.79 u), carrying a CM-Cys. The glycosylated counterparts of this fragment elute earlier, under peaks 6 and 7. The deconvolution of the data gave the masses corresponding to fragment L11 carrying one to four mannose residues. The glycan structures on the O-glycosylated peptide L11 exhibited a little variation throughout the four isoforms.

3.2.3.2. Charged glycans

The microheterogeneity was also related to the appearance of additional glycopeptide masses, 80 mass units apart from the original masses. Glycopeptide L1 in basic band 1 (p*I* 5.3) gave a clean distribution of evenly distributed quadruply charged ions that were deconvoluted to masses corresponding to peptide L1 carrying from one to nine mannose residues (Fig. 5). In acidic bands 3 and 4 (p*I* 5.1 and 5.0), the signal was still present but doubled with another spectrum exhibiting a similar type of ion distribution, 80 mass units apart (Fig. 5). Similar types of additional masses were observed in the peptide maps of acidic isoforms for the fragments L7 and L11. In the case of L7, the spectrum was deconvoluted into masses varying from 1 (Man+80 u) to 8 (Man+80 u) with the major ions representing (2 Man+80 u). In the case of peptide L14, the signal was deconvoluted into masses of C-terminal fragment (−1) carrying four to nine (Man+80 u). These glycopeptides were only found on the two most acidic isoforms. No signal at +80 u was found associated with the C-terminal fragment (−2). This mass increase on the glycopeptides could correspond to the presence of either a phosphate or a sulfate residue. A single modification (phosphate or sulfate) was observed per glycan structure on peptides L1, L7 and L14.

A literature search revealed that the modification of O-linked mannose by addition of phosphate in *Saccharomyces cerevisiae* has been recently described in two recombinant proteins, the leech-derived tryptase inhibitor [16] and the Bar secretion domain, a molecule engineered for the export of foreign proteins in yeast [17]. By opposition, to our knowledge, evidence for the presence of sulfated mannose on O-linked yeast glycans has never been documented. It is therefore likely that the modification on the glycan structure on peptides L1, L7 and L14 is a phosphate group.

LC–MS of deglycosylated PIXY321, prepared by treatment with α-mannosidase 1-2,3,6, an exoglycosidase with broad specificity toward various types of α-linked mannose residues, showed that neutral glycopeptides are shifted to non-glycosylated peptides. However, as the enzyme does not remove phosphorylated mannose residues [17], charged glycopeptides, although reduced in size, remained visible. For peptide L1, in addition to the signals of the two non-glycosylated peptides 1–63 and 3–63, the ion at m/z 1916.2 was present. This ion corresponded to peptide L1+2 mannoses+phosphate. The α-mannosidase treated glycopeptide L7 was found under the form of a peptide associated with one mannose and one phosphate. Treatment of PIXY321 with α-mannosidase showed that the phosphate group is found on the innermost mannose residue or the second position. Similar types of mannosidase treatment aimed at locating phosphate addition on *Saccharomyces cerevisiae* glycoproteins have been previously described [16,17].

Overall, comparison of the peptide maps showed that more acidic isoforms are more heavily glycosylated. This increased addition of glycan structures on acidic isoforms explains the relative mass differences observed by SDS–PAGE (Fig. 2). A summary of the glycosylation content at each site for each glycoform is presented in a schematic form in Fig. 6.

4. Discussion

Even in a highly purified form, most proteins demonstrate some degree of heterogeneity. A better

understanding of the microheterogeneities present and their impact on the final product requires the preparative isolation of pure isoforms and the characterization of the isolated molecules.

We have applied a preparative IEF method based on immobilized pH gradients to isolate sizable amounts of PIXY321 isoforms. We have found that this method offered many distinct advantages. Versatility in the design of the isoelectric membranes allows an easy adjustment of the pH intervals between chambers. During the progress of the separation process, intermediate results give an indication of the appropriate pH of the membranes to prepare. Flexibility in use of separation time and electric parameters allows the modulation of experimental conditions. Intermediate aliquots are easily taken in order to monitor the progress of the separation. Re-loading of partially purified fractions in the same experimental setting can lead to a successful separation as shown in the case of PIXY321. Recovery of the final purified isoforms is obtained in simple solutions compatible with downstream analytical techniques, such as matrix-assisted laser desorption ionization (MALDI), proteolytic digests and LC–MS characterization. Finally, an unique resolving power, not achievable by any other method, can be obtained by appropriate selection of immobiline mixtures. In theory, membranes differing by as little as 0.001 pH units can be crafted [10]. In summary, a high degree of flexibility exists to achieve a successful separation of isoforms.

Some limitations may also be experienced with this method. Although the experimental protocols were designed to minimize protein movement, by loading the starting material in the chamber that corresponded to the isoelectric point of the acid species, the very principle of the method calls for a passage of the molecules through the membranes. Precipitation of the molecules close to their isoelectric point during their interaction with the barrier between chambers can adversely affect the purification yield. Addition of solubilizing reagents and attention to membrane preparation, notably adjusting the acrylamide concentration and acrylamide–bisacrylamide ratio, are factors to consider to overcome this problem. The final yield was excellent in the case of PIXY321 but can be less satisfying in the case of larger molecules. We must note that the intrinsic features of the molecule play a crucial role. PIXY321 appears relatively soluble at its isoelectric point and physical interactions with the membranes are expected to be of little concern for an M_r 35 000 monomer.

Overall, preparative IEF on immobilized pH gradients is a much needed and powerful tool to separate molecules with subtle differences. Successful application of the method in the separation described here for PIXY321, along with other examples [9,18], provides a strong case for the use of this technique in the characterization of biopharmaceuticals.

Separation of protein isoforms is especially useful for biological activity studies. It was important to prove that PIXY321 isoforms had similar bioactivity and the distribution of microheterogeneities had no detrimental effect on the final potency of the product.

Analysis of the purified isoforms showed that the microheterogeneity of PIXY321 can be explained by three factors. First, part of the microheterogeneity was related to deamidation. The presence of a deamidated residue, located on residue Asn176, was observed in the two acidic forms. Based on computer calculations (Mass ProMac 1.5.3 program), a single charge difference, the replacement of an Asn residue by an Asp residue at position 176, correlates with a variation of 0.1 pH unit between the isolectric points of the isoforms of 271-residue long PIXY321. Second, the microheterogeneity involved the presence of charged glycans. Four glycopeptides, L1, L7, L11 and L14, were characterized on each isoform. The basic form, band 1 (p*I* 5.3), was found to be glycosylated on four positions by neutral mannose only. Three extra charges, in the form of phosphorylated glycopeptides L1, L7 and L14, appear progressively on band 2 (two sites) and band 3 and 4 (three sites). A quantification of only one phosphate per site was obtained. The fourth glycopeptide, L11, carried neutral glycans on each isoform.

The phosphorylation of O-linked mannose in *Saccharomyces cerevisiae* has been recently documented in two recombinant proteins, the leech-derived tryptase inhibitor [16] and the Bar secretion domain [17]. In the first study, it was shown that the modification was predominantly present at the inner most mannose residue but also occured in the second, third, fourth and fifth positions in oligo-

saccharide comprising one to ten Man residues. Likewise, one phosphate group in PIXY321 was found associated with mannosidic oligomers varying from one to 10 residues. Treatment of the molecule with α-mannosidase located the modification mainly at the innermost and second mannose positions.

Interestingly, the third factor involved in the isoform distribution was found to be glycosylation occupancy. Detailed analysis of the glycosylated fragments on the peptide map correlated position of the isoform bands with extent of glycosylation. Fig. 6 shows that globally on the entire protein the difference in glycosylation site occupancy between the basic and the acidic isoforms varies from 30% to 60%. In the acidic band 4, the N-terminal (L1) and C-terminal (L14) peptides are essentially completely glycosylated, whereas in the basic band 1, 50% of these peptides are free of any glycan structures. Obviously, these differences in site occupancy have an effect on the conformation of the protein. Our experimental results showed that, while the most basic form carried zero charge, there was no qualitative difference in the other isoforms and that the same type of charged glycans were present on all the other molecules. We could have more easily imagined another scenario where the presence of isoforms would have been based on a simple charge increase from 0 charge for the basic isoform to 1, 2 and finally 3 charges on the increasingly acidic isoforms. Actually, we showed that the extent of post-translational modification accounts for the difference between the isoforms. The progressive increase in glycan content does not correlate with an increased diffusion of the molecule on analytical IEF gels but rather results in a well defined difference in the apparent isoelectric point. The conformational aspect therefore appears very important to define the separation of PIXY321 by isoelectric focusing. We can speculate that the glycan structures have to reach a critical size for the molecule to move from one band to the next. This threshold may be related to a shielding effect that affects certain charged amino acid residues located at the surface of the protein and important for the apparent p*I* of the molecule.

Additionally, our results showed that the purified isoforms were still a mixture of molecules with heterogeneities in the peptide sequence at the N- and C-termini. The characteristics of the protein sequence, the absence of charged residues at both ends, are such that these sequence heterogeneities have no influence on the final charge of the molecule. However, the purified isoforms exhibited a very different distribution of N- and C-termini as described in the text. Therefore an additional factor, likely to be related to an increased protection of more heavily glycosylated species against proteolytic attack, appeared to be associated with the distribution of PIXY321 isoforms.

In conclusion, the ability to preparatively isolate purified isoforms of PIXY321 was critical in our quest for the complete characterization of the microheterogeneities of this biopharmaceutical molecule.

Acknowledgements

We thank Mary Gerhart for N-terminal sequencing and Gary Carlton for help with the art work. PIXY321 is prepared by the Immunex Corporation.

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