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Recombinant pronapin precursor produced in *Pichia pastoris* displays structural and immunologic equivalent properties to its mature product isolated from rapeseed

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2S albumin storage proteins from rapeseed (*Brassica napus*), called napins, consist of two different polypeptide chains linked by disulphide bridges, which are derived by proteolytic cleavage from a single precursor. The precursor form of the napin BnIb (proBnIb) has been cloned using a PCR strategy and sequenced. The amino-acid sequence deduced from the clone includes 31 residues of the small chain and 75 of the large chain, which are connected by the peptide Ser-Glu-Asn. Expression of the cDNA encoding proBnIb has been carried out in the methylotrophic yeast *Pichia pastoris*. The induced protein was secreted to the extracellular medium at a yield of 80 $mg \cdot L^{-1}$ of culture and was purified by means of size-exclusion chromatography and reverse phase-HPLC. Recombinant proBnIb appeared properly folded as its molecular and spectroscopic properties were equivalent to those of the mature heterodimeric protein. As 2S albumin storage proteins from *Brassicaceae* have been shown to be type I allergy inducers, the immunological activity of the recombinant proBnIb was analysed as a measure of its structural integrity. The immunological properties of the recombinant precursor and the natural napin were indistinguishable by immunoblotting and ELISA inhibition using polyclonal antisera and sera of patients allergic to mustard and rapeseed. In conclusion, the recombinant expression of napin precursors in *P. pastoris* has been shown to be a successful method for high yield production of homogeneous and properly folded proteins whose polymorphism and complex maturation process limited hitherto their availability.

Keywords: 2S albumin; rapeseed; allergen; *Pichia pastoris*; BnIb precursor.

The economic interest on *Brassicaceae* seeds has increased in the last decade since *Brassica* represents one of the most important oil seed annual crops in the world, as well as one of the main sources for animal nutrition. Their 2S albumins represent a good model for studying expression and maturation processes in plant tissues [1]. The 2S albumin class is an abundant group of seed storage proteins widely distributed in numerous species, which have been isolated and characterized from several *Brassicaceae* as *Sinapis alba* (yellow mustard), *Brassica juncea* (oriental mustard), *Raphanus sativus* (radish), *Ricinus communis* (castor bean), *Arabidopsis thaliana* (thale cress) and *Brassica napus* (rapeseed) [2–7]. The 2S albumins from *B. napus*, called napins, are encoded by multigene families, whose products exhibit a high degree of sequence similarity. Members of this family constitute small (12–15 kDa) and basic (around pI 11.0) proteins composed of two different chains (small and large) linked by disulphide bridges, which are expressed as a single polypeptide precursor (preproprotein) [8]. The internal processed fragment (IPF), which connects both chains in the precursor, is eliminated by proteolytical cleavage together with N- and C-terminal extensions during the post-translational maturation of the preproprotein [1,8–10]. The most common and abundant napins have a molecular mass of 14–15 kDa (HMW-napins) and exhibit a high degree of polymorphism and sequence similarity. A smaller variant of 12 kDa (LMW-napin) is synthesized in low concentration in rapeseed, and two isoforms (BnIa and BnIb) were isolated from the seed [5]. The amino-acid sequences of BnIa and BnIb were determined by Edman degradation of peptides obtained by proteolytic treatment and showed a limited similarity to those of the HMW-napins [11] (Fig. 1).

Besides their biological role as nitrogen and sulfur donors, many 2S albumins display a broad spectrum of antifungal activities [12], calmodulin antagonist capability [13] and are also able to induce allergic reactions in hypersensitive subjects [3,7,14–16]. Type I allergies are common immunological disorders that affect > 20% of the population in industrialized countries. Seeds, as biological sources of allergens, have been involved in food and occupational allergies. The allergenic components of these sources are 2S albumins, mainly napin-type 2S albumins (NT2SA), whose high stability and solubility are important factors for being a food allergenic inductor [17,18]. Major allergens from yellow and oriental mustard seeds, castor bean and rapeseed, have been isolated and characterized [3,15,16,19,20].

Recombinant production of proteins is a useful strategy to obtain well defined and homogeneous materials for research or industrial purposes. Previous attempts at

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Abbreviations: IPF, internal processed fragment; LMW-napin, low molecular mass-napin; HMW-napin, high molecular mass-napin; NT2SA, napin-type 2S albumin; proBnIb, precursor form of the napin BnIb; rproBnIb, recombinant BnIb pronapin.

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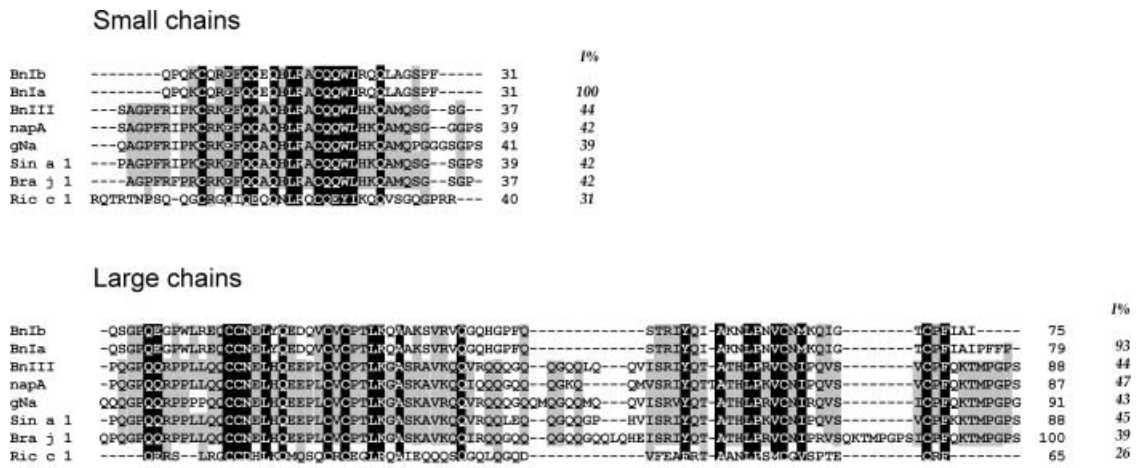

Fig. 1. Alignment of the sequences of the small and large chains of NT2SAs. Sequences of BnIb, BnIa, BnIII, napA, gNa, and the allergenic NT2SAs Sin a 1, Bra j 1 and Ric c 1 are shown. Sequences of the mature proteins (small and large chains) were used, except for sequences of napA and gNa, for which only DNA data are known (the deduced amino acid sequences were cut following their comparison with other HMW-napins from *Brassica napus*). Numbers on the right of the alignment correspond to the sequence position of each molecule. Dashes indicate gaps opened in the sequences for the best alignment. Shadowing of columns represents conservation among all the sequences, darker backgrounds stand for the highest values of conservation. The identity percentage (I%) is also presented.

expressing a properly folded napin in bacteria rendered poor results in terms of yield and solubility [21,22]. This work demonstrates that the heterodimeric 2S albumins can be expressed as their precursors in the eukaryotic expression system of the yeast *P. pastoris* and produced as a correctly folded secretion protein. The recombinant product obtained from the cDNA encoding the LMW-napin BnIb precursor (rproBnIb) was analyzed in terms of its structural and immunological equivalence to the mature protein. In addition, rproBnIb has been found to bind IgE from the sera from allergic individuals.

MATERIALS AND METHODS

Strains and plasmids

P. pastoris GS115 *his*4 strain (Invitrogen Corp.) was used as the host for transformations using the plasmid pPIC9. Bacterial *Escherichia coli* strains INVαF′ and TG1 were used, respectively, as hosts for cloning the PCR fragments in pCR2.1 (Invitrogen Corp.) and pPIC9 (Invitrogen Corp.).

Sera and antibodies

Sera from hypersensitive untreated individuals who exhibited positive skin-prick test and RAST (classes 3–6) to mustard seed extract, and a serum from a patient allergic to rapeseed flour were used to analyze the allergenic character of both natural and recombinant proteins. Polyclonal sera against recombinant pronapin BnIb and Sin a 1, the major allergen from yellow mustard seeds, were prepared by immunizing New Zealand white rabbits over a 6-week period by weekly injection of the protein (100 μg) in complete Freund's adjuvant. Mouse monoclonal anti-(human IgE) Ig was kindly donated by M. Lombardero (ALK-Abelló, Hørsholm, Denmark).

Isolation of total RNA and cDNA synthesis

Total RNA was isolated from rape seeds (Herborem) as described previously [23] with minor modifications. Rape seeds (0.5 g) were grinded and homogenized with a Polytron (Brinkman) in 5 mL of 2 M sodium citrate pH 7.0, containing 4 M guanidine thiocyanate, 0.5% sodium *N*-lauroylsarcosine. Single stranded-cDNA was synthesized from 10 μg total RNA using the first strand cDNA synthesis kit (Boehringer Mannhein), following the manufacturer's instructions.

Synthesis of oligodeoxynucleotides

Degenerate oligonucleotide primers used for cloning, NIB-1 and NIB-2, were designed based on the amino-acid sequence of BnIb napin obtained by Edman degradation. Sense primer NIB-1 (5′-cgtctcgagaaaagaCARCCNCARA ARTGYCAR-3′) corresponds to the first six amino-acid residues of the N-terminal of the small chain (QPQKCQ) and the antisense primer NIB-2 (5′-cggaattctta DATNGCDATRAANGGRCA-3′) corresponds to the six last residues of the C-terminal of the large chain (CPFIAI). Primers NIB-1 and NIB-2 contained a *Xho*I and *Eco*RI restriction sites (underlined), respectively. The sense primer contains a sequence that allows fusion of the proBnIb-encoding region in-frame with the sequence coding for the preprosequence of the α-mating factor present in the pPIC9 plasmid.

PCR-based cloning and sequence analysis

The cloning strategy was based on the PCR method using the synthesized cDNA as template and both sense and antisense primers (NIB-1 and NIB-2) and the *Taq*Gold DNA polymerase (Applied Biosystems PE) dissolved in

the PCR mixture. Amplification conditions were established previously [24] using 25 cycles of reaction and a hybridization temperature of 50 °C as minor modifications. The agarose-containing fragment was reamplified by using five cycles of denaturation at 94 °C (60 s), annealing at 47 °C (60 s), chain extension at 72 °C (90 s) and 25 cycles at 94 °C (60 s), 55 °C (90 s) and 72 °C (90 s). The fragment was purified by using the Magic PCR Prep kit (Promega) and ligated into a linearized pCR 2.1 vector. The construct was used to transform INVαF′ *E. coli* cells. Three clones were sequenced. DNA fragments were digested with *Xho*I/*Eco*RI restriction enzymes and subcloned into the same sites of the plasmid pPIC9 used to transform TG1 *E. coli* cells to obtain the construction pPIC9/proBnIb.

Transformation of *P. pastoris* GS115 and production of rproBnIb

pPIC9/proBnIb plasmid (5–10 μg) was linearized with *Bgl*II restriction enzyme, and the purified larger fragment was integrated by gene replacement in GS115 cells using lithium acetate treatment [25]. Transformed cells were incubated on minimal dextrose plates at 30 °C for 4–6 days until colonies appeared. Screening for His^+Mut^s phenotype, originated by homologous recombination at *AOX*1 locus, was performed by patching the His^+ colonies in replica-plating on minimal dextrose vs. minimal methanol plates. For the production of rproBnIb as secretion protein, selected (His^+Mut^s) transformed strains were processed as described previously [26]. The culture medium of GS115-induced cells was cleared by centrifugation at 3000 ***g*** at 4 °C. The presence of rproBnIb in the supernatant was analyzed by SDS/PAGE of aliquots taken at different times of culture (0, 24, 48, 72 h). Large-scale production of rproBnIb was performed in buffered methanol minimal medium using the colony that rendered the best yield in the small-scale experiments.

Purification of rproBnIb

The extracellular medium obtained after centrifugation of the yeast cells was subjected to dialysis against 20 mM ammonium bicarbonate pH 8.0 using dialysis membranes (6-8000 Spectra/Por) and lyophilized. Size-exclusion chromatography on a Sephadex G-50 column was used to fractionate the sample. Fractions containing rproBnIb, judged by SDS/PAGE, were lyophilized and chromatographed on a C-18 reverse-phase HPLC column with an acetonitrile gradient (30–50%) in 0.1% trifluoroacetic acid. Natural BnIb (nBnIb) was purified from rapeseed as described previously [5].

Analytical procedures

Composition and protein concentration of purified samples (1–2 nmol) were determined after hydrolysis with 5.7 M HCl at 105 °C for 24 h, in sealed tubes under vacuum and quantified on a Beckman 6300 amino-acid analyzer. Protein concentration of extracts was determined as described previously [27]. The N-terminal amino-acid sequence was determined using an Applied Biosystems model 477A sequencer. Mass spectrometry analyses were carried out on a Bruker Reflex II matrix-assisted laser-desorption ionization time-of-flight mass spectrometer, as described previously [28].

Spectroscopic analyses

Circular dichroism spectra were obtained in the far (195–250 nm) and near (250–350 nm) UV range on a Jasco J-715 spectropolarimeter, as described previously [28] with minor modifications. The protein concentration was in the 0.20–0.25 $mg{\cdot}mL^{-1}$ range for the far-UV and 1 $mg{\cdot}mL^{-1}$ for the near-UV spectra. Mean residue mass ellipticities were calculated based on 115.27 for nBnIb and 114.95 for rproBnIb as the average molecular mass/residue, obtained from the amino-acid composition, and expressed in terms of θ ($degree{\cdot}cm^2{\cdot}dmol^{-1}$).

Immunological characterization

SDS/PAGE was performed as described previously [29] using 15% polyacrylamide gels. Proteins were stained with Coomassie blue or electrophoretically transferred to nitrocellulose membranes. Immunodetection was achieved as described previously [28] using two rabbit polyclonal antisera raised against Sin a 1 and purified rproBnIb (diluted 1 : 1000 and 1 : 80 000, respectively), a pool of sera from patients allergic to mustard (diluted 1 : 10) and a serum from a patient allergic to rapeseed flour (diluted 1 : 10). The signal was developed by the ECL-Western-blotting reagent (Amersham corp.).

ELISA inhibition assays were performed as described previously [30]. After coating with 100 μL antigen (2 $\mu g{\cdot}mL^{-1}$), the plates were incubated with the pool of allergic sera (diluted 1 : 10) previously incubated with different concentrations of nBnIb or rproBnIb (0.001–100 μg) as inhibitors. This incubation was followed by a treatment with mouse monoclonal anti-(human IgE) Ig and horseradish peroxidase-labelled goat anti-(mouse IgG) Ig.

RESULTS

Cloning and sequencing of a cDNA codifying a precursor form of BnIb napin

The cDNA encoding a precursor of BnIb napin (proBnIb) was synthesized from rapeseed total RNA (0.5 μg) and amplified by PCR using two degenerate oligonucleotides corresponding to the N- and C-terminal ends of the small and large chains, respectively [11]. This fragment was ligated into the pCR2.1 plasmid and the construction was used to transform INVαF′ *E. coli* cells. The nucleotide sequences of three selected clones were determined confirming the absence of microheterogeneities (Fig. 2A). The deduced amino-acid sequence is composed of 109 residues, of which 31 and 75 correspond to the small and large chains, respectively, according to the data of the natural protein [11]. A high Cys/Gln residue content supports the nitrogen and sulfur storage role assigned to these proteins. These two sequences are linked by a short sequence (Ser-Glu-Asn). The alignment of this IPF with those of other 2S albumins is shown in Fig. 2B. Two differences were observed in the amino-acid sequence of the large chain in comparison to that of the natural napin; a Trp instead of Ser36 and a Ser substituting Trp43. These

A

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cagccacaaaagtgtcag agggagtttcagcaagagcaacacctaagagcttgccag   57
 Q  P  Q  K  C  Q  R  E  F  Q  Q  E  Q  H  L  R  A  C  Q
                          10
caatggatccgccagcaacttgcgggatcacccttcagcgaaaaccagtggggtcct   114
 Q  W  I  R  Q  Q  L  A  G  S  P  F  S  E  N  Q  W  G  P
 20                          30
cagcaggggccgtcgctacgcgagcaatgctgcaacgagctttaccaggaagaccaa   171
 Q  Q  G  P  S  L  R  E  Q  C  C  N  E  L  Y  Q  E  D  Q
    40                          50
gtgtgcgtttgccctactttgaaacaagcagccaagtcagttagggtccagggacag   228
 V  C  V  C  P  T  L  K  Q  A  A  K  S  V  R  V  Q  G  Q
       60                          70
cacggaccattccagtccacccgcatctaccagatcgctaagaacttgccaaacgtt   285
 H  G  P  F  Q  S  T  R  I  Y  Q  I  A  K  N  L  P  N  V
          80                          90
tgcaacatgaaacaaattggaaca tgtcctttcattgccatt                 327
 C  N  M  K  Q  I  G  T  C  P  F  I  A  I
             100
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B

2S albumin	*Internal processed fragment (IPF)*	*Ref.*
B.napus (*napA*)	W T L D G E F D F E D D M E N	[10]
S.alba (Sin a 1)	W T L D D E F D F E D D M E N	[19]
A.thaliana (*at2S2*)	* * L D D E F D L E D D I E N	[33]
R.sativus (*pBA3*)	W T L D D E F D F E D D M E N	[6]
***B.napus* (BnIb)**	* * * * * * * * * * * * **S E N**	
R.communis (Ric c 1)	* * * * * * * * * * * S D N	[34]

Fig. 2. Primary structure of proBnIb and comparison of its IPF with those of other NT2SA. (A) Nucleotide sequence of a cDNA clone of the proBnIb-encoding region. The deduced amino-acid sequence is also shown. The sequence contained in a box corresponds to the IPF connecting both chains. The sequences used as primers in the PCR cloning are underlined. GenBank accession number: AF448054. (B) IPF sequences corresponding to different NT2SAs.

changes must not be considered as artifacts of the PCR reaction because different clones have been sequenced from several PCR amplifications. The amino-acid composition of small and large chains of BnIb derived from the amino-acid sequence obtained by cloning fit well with that obtained by acidic hydrolysis of the natural protein.

Overproduction in *P. pastoris* and isolation of recombinant proBnIb

The construction pCR2.1/proBnIb was digested with the *Xho*I and *Eco*RI restriction enzymes and the released fragment was subcloned into the pPIC9 vector. The cDNA encoding proBnIb was inserted downstream of the AOX1 promoter and expressed in GS115 yeast cells. Soluble rproBnIb was efficiently secreted to the extracellular medium. The yield was around 80 mg of recombinant protein per L of culture. A time course of the production of this protein was followed analyzing the secreted medium by SDS/PAGE. A major band of 13.3 kDa apparent molecular mass appeared after 24 h of induction, reaching the highest level at 72 h (Fig. 3A). The protein band was able to bind to the Sin a 1-specific rabbit antiserum (Fig. 3B). After selection of the optimal conditions for proBnIb expression, the 'best producer' clone was used for the large-scale preparation. After exhaustive dialysis of the extracellular medium with a membrane cut-off of 8 kDa and lyophilization, a two-step procedure, using a size-exclusion fractionation in Sephadex G-50 and a reverse-phase HPLC C-18 column, was used to isolate the recombinant protein. The analysis by SDS/PAGE of the purified rproBnIb is shown in Fig. 4A. The final yield of the purified protein was 40 mg·L^{-1} of culture, calculated using the extinction coefficient at 280 nm of the natural protein ($\varepsilon_{280}^{0.1\%} = 1.15$).

Structural relationships between the natural and recombinant forms of the napin BnIb have been analyzed by

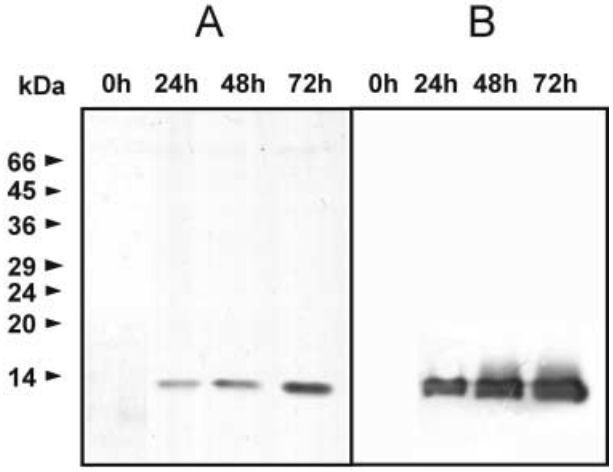

Fig. 3. Time course for the expression of rproBnIb in Pichia pastoris. Supernatants from cultures were harvested at different times and analyzed by: (A) Coomassie Blue staining after SDS/PAGE (B) Immunodetection with Sin a 1-specific polyclonal antiserum. Molecular-mass markers are indicated in kDa.

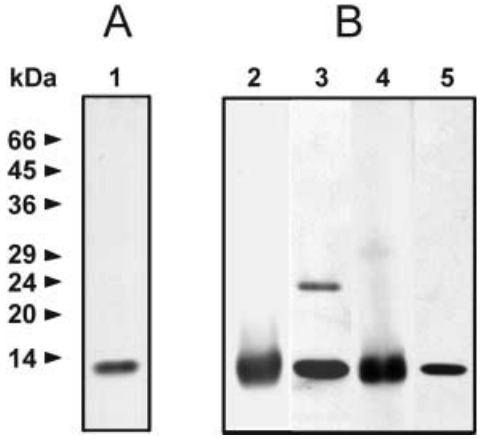

Fig. 4. SDS/PAGE analysis of purified rproBnIb. (A) Coomassie Blue staining of the purified protein after the HPLC step (lane 1). (B) Immunodetection with rabbit polyclonal antisera specific to Sin a 1 (lane 2) and to rproBnIb (lane 3); IgE-binding of a serum of a patient allergic to rapeseed flour (lane 4) and of a pool of sera allergic to yellow mustard (lane 5).

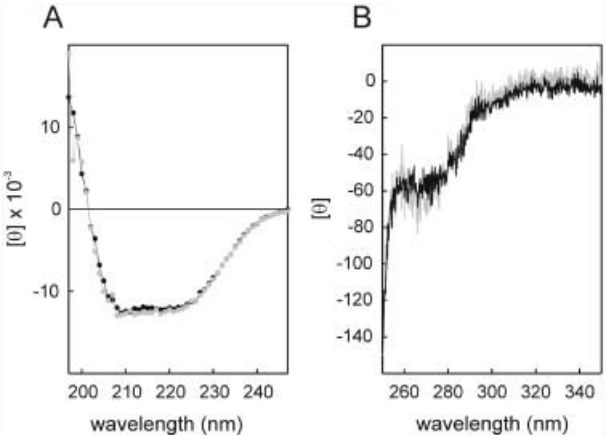

Fig. 5. Spectroscopic characterization of rproBnIb. (A) Far-UV (200–250 nm) and (B) near-UV (250–350 nm) CD spectra of rproBnIb (grey line) and nBnIb (black line). Ellipticity values (θ) are shown in degrees·cm^2·dmol^{-1}.

comparison of their antigenic properties. The recombinant form rproBnIb was recognized by the Sin a 1-specific polyclonal antiserum, as well as by a polyclonal antiserum raised in rabbit against the purified rproBnIb (Fig. 4B, lanes 2 and 3). The high affinity of the latter allowed to detect traces of a dimeric form of rproBnIb at 24 kDa. In addition, the purified recombinant napin was able to bind to the IgE antibodies present in a pool of sera of patients allergic to yellow mustard, which were sensitive to Sin a 1, and to those present in the serum of a patient hypersensitive to rapeseed flour (Fig. 4B, lanes 4 and 5).

Molecular characterization of rproBnIb

Purified rproBnIb exhibited an apparent molecular mass of 13.3 kDa in SDS/PAGE (Fig. 4A) and 12 518 Da as determined by mass spectrometry, which agrees with that deduced from the clone (12 512 Da). The amino-acid composition obtained by acidic hydrolysis and automatic analysis of the recombinant product also agrees with that calculated from the sequence deduced from the selected clone (data not shown). Edman degradation of the N-terminal end of rproBnIb resulted in a low yield (< 10%) of Gln. This behavior was identical to that of the protein obtained from the seeds and indicates the cyclation of the Gln-1 as pyroglutamate. This also indicated that the preprosequence of the α-factor was correctly processed in the yeast system.

Far- and near-UV CD spectra of both proteins provide information about the three-dimensional structure of proteins and therefore allow the comparison between rproBnIb and nBnIb conformations. No significant differences were detected either in the shape of the spectra or in the ellipticity values for both molecules. These spectra correspond to an all-helix protein (69% α helix content) with regions involving loops or turn like conformations, as it was reported by Rico *et al.* [31] for nBnIb (Fig. 5). These data confirm the correct folding of the recombinant protein at the levels of secondary and tertiary structures.

Immunological equivalence of rproBnIb and nBnIb

In order to quantify the IgG- and IgE-binding equivalences between nBnIb and rproBnIb, ELISA inhibition experiments were performed using the rproBnIb-specific polyclonal antiserum and a pool of sera from patients allergic to Sin a 1. As seen in Fig. 6, complete inhibition of the binding of the rproBnIb-specific IgG antibodies to rproBnIb-coated wells was reached when nBnIb was used as inhibitor, in a manner similar to rproBnIb. This result informs about the presence of common antigenic determinants in both proteins.

For the IgE-reactivity analysis, each protein (nBnIb and rpronBnIb) was immobilized to wells and their binding to the antibodies assayed after incubation of the allergic human sera with both proteins as inhibitors (Fig. 7). Complete inhibition was obtained with each form of the napin, indicating that they share the IgE epitopes. These results corroborated the immunological equivalence between both proteins.

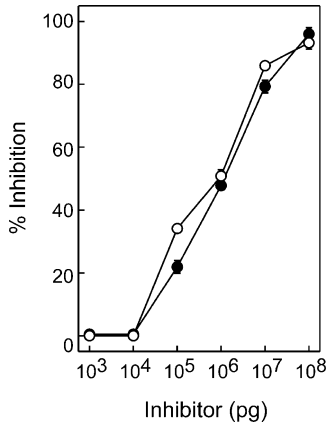

Fig. 6. IgG-binding equivalence between rproBnIb and nBnIb. ELISA inhibition assays of the binding of a rabbit polyclonal antiserum raised against rproBnIb to rproBnIb-coated wells. rproBnIb (●) and nBnIb (○) were used as inhibitors at different concentrations.

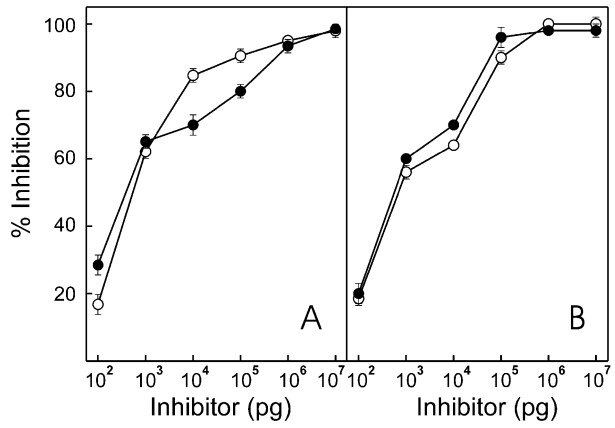

Fig. 7. IgE-binding analyses of rproBnIb and nBnIb. ELISA inhibition assays of the binding of IgE from sera of mustard-allergic patients to rproBnIb-coated (A) and nBnIb-coated (B) wells using rBnIb (●) and nBnIb (○) as inhibitors.

DISCUSSION

2S albumins constitute the major component of the total protein isolated from several dicotyledoneous seeds. Several functions or activities have been assigned to this family of proteins; nitrogen and sulfur storage, antifungal capacity, calmodulin antagonist activity and allergenicity [9,12–14]. The best known 2S albumins are napins, which belong to *B. napus*, one of the *Brassicaceae* members. Molecular organization and biological synthesis mechanisms of napins and related proteins (NT2SAs) have been two of the aims in the research on 2S albumins. As with many plant storage proteins, napins are synthesized as precursors that should be proteolytically processed before appearing in the mature form.

BnIb is an unusually small napin, but its sequence homology with the HMW-napins [8,11,15,19] and the identical circular dichroism spectra [3,8,32] suggest that all the NT2SAs have a similar three-dimensional structure. The amino-acid sequence of BnIb had been previously determined by Edman degradation [11], but no data were available on the nucleotide sequence of its specific DNA. Cloning and sequencing of the precursor proBnIb has allowed confirmation of the mild polymorphic character of BnI, contrary to the situation with most napins.

Interestingly, the amino-acid sequences of IPFs, which are removed from the precursor in the maturation process, have been shown to be highly conserved among different NT2SAs [33–35] than their own mature chains. The complete nucleotide sequence of proBnIb revealed that its IPF (Ser-Glu-Asn) is remarkably shorter than those of most NT2SAs. In HMW-napins, Sin a 1 and castor bean NT2SA, this is a 15-residue segment and 13 amino acids in arabidin (Fig. 2B). Only Ric c 1 contains a short IPF with an amino-acid sequence (Ser-Asp-Asn) similar to that of BnIb [36], despite the low sequence similarity between both mature proteins. A vacuolar cysteinyl-protease, which cleaves highly conserved Asn-X bonds of 2S proteins, has been proposed (37). This fact would be in agreement with the presence of an Asn conserved in the IPF of proBnIb. On the other hand, the importance of the propeptide sequence for the correct folding and processing of the pronapin has also been assessed [10]. D'Hondt and colleagues have demonstrated that arabidin is less efficiently folded when the IPF is missing or when it is mutated [38]. Previous to these reports, we showed that all our attempts for reconstituting the heterodimeric napins by combining the isolated chains failed [39], which supports the important role of the IPF region not only in the appropriate processing but also in the correct folding. In this context, the expression of the precursor forms of NT2SAs is the most reasonable strategy to produce recombinantly these functional proteins.

The HMW-napins, and 2S albumins in general, are the most abundant proteins in extracts of *Brassicaceae* seeds, but they are highly polymorphic. They can be purified in the order of milligrams but as a heterogeneous mixture of different isoforms [5,19,31]. In contrast, LMW-napins are mildly polymorphic, but they are barely produced in the seeds, purified with a very low yield and mostly contaminated with the HMW-napins. Recombinant production represents an efficient route to obtain this protein in amounts sufficient to carry out its structural and functional characterizations. Few such efforts have been carried out for the recombinant expression of heterodimeric 2S albumins or their precursors. The NT2SA Sin a 1, the major allergen from yellow mustard, was produced in *E. coli* as different fusion proteins [21,22]. A low amount of purified and soluble protein was always obtained because of the high tendency of the molecule to aggregate. Recently, another 2S gene napin (*napA*), which encodes a HMW-pronapin from *Brassica napus,* has been expressed in transgenic tobacco [10] and baculovirus [40] systems. Structural and immunological characterization of this pronapin and the mature form suggested that there are conformational differences between the molecules [41], perhaps attributable to the contribution of the IPF. Herein, the production of proBnIb in a recombinant soluble form using other eukaryotic system has been proven as a useful and reproductive alternative to obtain high amounts of 2S albumins in a functional form.

The strategy applied in this work is based on the use of the *P. pastoris* expression system, in which other proteins, several of them allergens such as Ole e 1, Cyn d 1 or Bla g 4 [28,42,43], have been produced in a correctly folded form. This eukaryotic system allows the formation of the correct disulphide bridges of proteins that exhibit high content of cysteine residues. By means of the fusion of the pronapin with the secretion signal of the α-factor from *Saccharomyces cerevisiae* and using a *P. pastoris* inducible expression system, we succeeded in overproducing proBnIb.

A priori this approach should involve remarkable difficulties, because the structural and functional properties of the recombinant pronapin could differ those expected for the heterodimeric natural protein. However, as it is shown by the spectroscopic studies, both molecules display equivalent features confirming that the IPF processing has little effect on the protein structure but should be essential in its folding. This is reasonable considering the short length of the IPF and would agree with the relevant role attributed to this peptide in the correct folding of the protein. The strategy of synthesizing precursors of proteins by recombinant technology instead their mature forms has been used with different goals. Interestingly, the house dust mite allergen Der p 1 has been successfully produced as an

enzymatically inactive precursor in insect cells, in order to avoid its protease capability [44].

IgG- and IgE-binding activities of rproBnIb were compared with those of nBnIb using ELISA. Results indicated that the antigenic properties of the napin are preserved in the recombinant form and its maturation is not necessary for the recognition by specific IgE or polyclonal antisera. Moreover, these data reveal that the region around the IPF is not involved in allergenic epitopes. From this work, it can be also deduced that BnIb shares antigenic determinants with Sin a 1, as the napin is able to bind to the Sin a 1-specific polyclonal antiserum. In addition, an allergenic character can be attributed to BnIb as it is recognized by IgE from sera of patients allergic to yellow mustard and by those from the serum of a patient allergic to rapeseed. The relevance of this protein as a type I allergy inducer in hypersensitive individuals, with implications in a potential cross-reactivity between mustard and rapeseed flours, will be the aim of future studies.

In conclusion, these results support the suitability of the precursor forms of 2S albumins produced in the eukaryotic system of the yeast *P. pastoris* for clinical purposes and scientific research as they exhibit properties equivalent to the natural proteins.

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