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Structural analysis of the small chain of the 2S albumin, napin nIII, from rapeseed. Chemical and spectroscopic evidence of an intramolecular bond formation

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Napin nIII is a 2S albumin from rapeseed (*Brassica napus* L.) homologous to the major mustard allergen. It is composed of two different polypeptide chains linked by two disulphide bonds. The small chain has been isolated by reverse-phase HPLC after reduction of the native protein and its primary structure elucidated. This 37 residue polypeptide contains only two cysteines, at positions 10 and 23, which show a great tendency to form a non-native intramolecular disulphide bridge. The kinetic analysis of this process was performed by measuring the fluorescence emission of the single tryptophan residue of the molecule since its fluorescence intensity is about 30% decreased during disulphide formation. Small changes on the secondary structure of the polypeptide were measured by circular dichroism. The process is delayed in the presence of the reduced large chain of nIII. However, no dimer formation was detected under the conditions used, either between small chains or between the small and the large chains. Thus, the interchain disulphide formation in napin nIII should be considered as an early step during maturation of this multi-subunit seed protein.

Introduction

The 2S albumins are one of the main protein components from seeds. These storage molecules provide nitrogen for the germinating seedling. The interest in this class of proteins has increased through the last decade, mainly due to their remarkable nutritional value. Moreover, many of them cause allergenic responses in hypersensitive individuals [1–4]. This fact represents an additional interest, since these proteins would exhibit resistance against the activity of digestive proteinases, and would also be able to pass across

intestinal membranes and finally produce IgE secretion.

A significant part of the research about 2S albumins has been performed on rapeseed (*Brassica napus*), the main oilseed annual crop in the world. Four genes encoding 2S albumins from *B. napus* have been sequenced [5–7]. They show a quite similar sequence and are included in a multigene family containing more than 15 members. The earliest biosynthetic products for the expression of these genes are polypeptide precursors of 21 kDa which are processed by maturation proteinases, yielding the native proteins (napins). At least five 2S albumins can be isolated from *B. napus* seeds. All of them cross-react with polyclonal antisera obtained against *Sin a* I, the major allergen from yellow mustard [8]. Napins are composed of two different chains of about 4 kDa (small) and 9.5 kDa (large), linked by two disulphide bridges and arising from a single polypeptide precursor. This fact is of great significance in attempting to understand the post-translational processing of 2S albumins due to the folding-disulphide formation relationships potentially involved in such a process. Considering the proteolytic maturation

Abbreviations used: TFA, trifluoroacetic acid; 2-ME, 2-mercaptoethanol; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; PTH-, phenylthiohydantoin derivative; SC, small chain of napin nIII; LC, large chain of napin nIII; DTT, dithiothreitol; RNase, ribonuclease.

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of this class of proteins, we have recently proposed one involvement of β -turns at the cleavage site of napins [9]. Now, we study the structure of the small chain from napin nIII and the reactivity of its cysteine residues, in order to obtain new insights about conformation and maturation of this type of proteins.

Materials and Methods

Preparation of the nIII-small chain and chemical labelling

The napin nIII from *B. napus* seeds was obtained as previously described [8]. The two chains of napin nIII were separated as reported [9]. The native protein was reduced in 0.1% (v/v) TFA (pH 2.1) containing 5% (v/v) 2-ME, at 85°C, for 20 min. The sample was immediately centrifuged on a Beckman microfuge and the two chains separated on a μ Bondapak C-18 reverse-phase column (7.8 $\times$ 300 mm), by using a Beckman HPLC system, with a 20–50% (v/v) acetonitrile linear gradient in 0.1% (v/v) TFA, at a flow rate of 1.5 ml/min. When the nIII-small chain (SC) and its derivatives were chromatographically analyzed, a 0–35% (v/v) acetonitrile linear gradient was used. The effluent was continuously monitored at both 214 and 280 nm wavelengths, and 0.75 ml fractions were collected.

Radioactive labeling of SC was carried out by radioalkylation with [^{14} C]iodoacetamide as described [4], with minor modifications. Aliquots of 200 μ l (0.5–0.7 mg/ml protein concentration) were treated with 0.6 μ Ci of [^{14} C]iodoacetamide (53 μ Ci/ μ mol) in 0.2 M Tris-HCl buffer (pH 8.5) and the mixture incubated for 15 min at 25°C, in the absence of light. Unlabeled iodoacetamide was then added up to 150 mM final concentration, and the reaction was continued for another 60 min before its injection in the HPLC column.

Spectroscopic analysis

Ultraviolet-absorbance measurements were carried out on a Cary 118 spectrophotometer at 0.2 nm/s scanning speed and routinely in 1-cm optical-path cells.

Circular dichroism (CD) spectra were obtained on a Jobin Ivon Mark III dichrograph, fitted with a 250 W xenon lamp. The spectra in the far-ultraviolet (peptide bond region) were recorded at 0.2 nm/s scanning speed. Samples were analyzed in 0.05 cm optical-path cells. The polypeptide concentration was in the 0.1–0.5 mg/ml range. Mean residue weight ellipticities were calculated based on 111 as the average molecular weight per residue, obtained from the amino acid composition of SC and they were expressed in units of degree $\times$ cm ^{2} $\times$ dmol $^{-1}$. The reported CD values were the average of at least five independent determina-

tions. The secondary structure estimations were performed by computer fit according to the matrix formulation described [4] and by using the ellipticity reference values of [10].

Fluorescence measurements were performed on a Perkin-Elmer MPF-44E spectrofluorimeter fitted with a 150 W xenon arc and operated in the ratio mode. Spectra of the samples were recorded in 0.4 cm optical-path cells at 1 nm/s scanning speed, and by using 7 and 5 nm slit-widths for the excitation and emission beams, respectively. Analyses were performed in thermostated cells by using a Lauda circulating water bath, at 25°C for standard measurements and at different temperatures when required for specific studies. The samples were centrifuged on a Beckman microfuge prior to the spectroscopic determinations. Samples were prepared with absorbance at the corresponding excitation wavelength lower than 0.05, in order to avoid inner filter effects.

Quenching studies were performed by adding known vols. of 6 M recrystallized acrylamide on phosphate buffer (pH 7.2) to a solution of the sample at 40 μ g/ml. Emission spectra, for an excitation wavelength of 295 nm, were recorded for each quencher concentration. Fluorescence intensity values at 345 nm (maximum of emission) were used in a Stern-Volmer plot in order to obtain the quenching constants.

The samples were prepared in water Milli-Q and filtered through a Millipore filter (0.22 μ m pore diameter).

Sequence determination and predictive analyses

NH $_2$ -terminal Edman degradations of SC and isolated peptides were performed on an Applied Biosystems model 477A sequencer. The resulting PTH-amino acid derivatives were identified by using a model 120 A on-line PTH-analyzer and the standard Applied Biosystems program.

Tryptic digestion of SC was performed as previously described [4]; 30–50 nmol of reduced and lyophilized SC was dissolved in 0.2 M ammonium bicarbonate (pH 8.0). An enzyme/substrate weight ratio of 1:100 was used and the mixture was incubated at 37°C for 60 min. The digested material was lyophilized, dissolved in 0.1% TFA and centrifuged on a Beckman microfuge prior to injection in the HPLC column. The separation was performed on the system described above with a 0–15% (v/v) acetonitrile linear gradient in 0.1% TFA.

Secondary structure prediction, based on the determined amino acid sequence, has been performed according to Ref. 11. Several restrictions referring to the selection of β -bends have been considered [12]. Hydrophilicity and flexibility prediction profiles of SC were done according to Refs. 13 and 14, by using the EPI-PLOT microcomputer program [15].

Other analytical procedures

The protein concentration of the samples was determined by both amino acid analysis and spectrophotometric measurements. The hydrolyses were carried out at 105°C in evacuated and sealed tubes for 24 h, with 5.7 N HCl containing 0.1% (w/v) phenol. The analyses were performed on a Durrum D-500 amino acid analyzer. When the carboxyamidomethylated chain was hydrolysed, the colour value for *S*-carboxymethyl-cysteine was considered to be 92% that of aspartic acid. Total half-cystine was determined as cysteic acid after oxidation of the polypeptide with performic acid at -10°C [16]. Tryptophan was determined spectrophotometrically in 0.1 M NaOH [17,18].

The extinction coefficients (0.1% w/v, 1 cm, 280 nm) of 0.51, 1.23 and 0.20 were used for native nIII and its small and large chains, respectively.

Polyacrylamide gel electrophoresis in acidic conditions [19] was used for SC and its derivatives; 20% (w/v) polyacrylamide gels in 0.9 M acetic acid containing 2.5 M urea were employed. Samples were applied in 4–10 μ l of 0.9 M acetic acid containing 15% (w/v) sucrose; eventually, 5% (v/v) 2-ME was added and the samples were heated at 80°C for 20 min. SDS-PAGE was performed according to Ref. 20. The separation gel contained 19.3% (w/v) acrylamide, 0.074% (w/v) N,N'-methylene-bis-acrylamide, in 0.33 M Tris-HCl buffer (pH 8.8) containing 6 M urea, 0.1% (w/v) SDS and 75 mM NaCl. The presence of urea, SDS and NaCl, as well as a high acrylamide percentage were required for optimal separation of the analyzed low molecular weight polypeptides. The stacking gel contained 5% (w/v) acrylamide, 0.13% (w/v) N,N'-methylene-bis-acrylamide, in 0.0625 M Tris-HCl buffer (pH 6.8), containing 6 M urea and 0.1% (w/v) SDS. Samples were applied after heating at 80°C for 20 min, in 10–20 μ l of 80 mM Tris-HCl buffer (pH 6.8) containing 2.5% (w/v) SDS, 5% (v/v) 2-ME and 15% (w/v) sucrose.

Results

Intramolecular disulphide formation in the nIII-small chain

Purified nIII napin contains two polypeptide chains, which can be separated by reverse-phase chromatography after reduction of the two intermolecular disulphide bridges [9]. The reduced SC elutes at 25% (v/v) acetonitrile (Fig. 1a). But, the chromatographic behaviour of this chain changes upon incubation in 20 mM phosphate buffer (pH 7.2) at room temperature (Fig. 1b). A second peak eluting at 22% (v/v) acetonitrile appears. In these conditions, the change is completed after 4 h of incubation (Fig. 1c). Treatment of this modified form of SC with 5% (v/v) 2-ME, in 0.1% TFA, at 85°C for 20 min, restored the initial elution

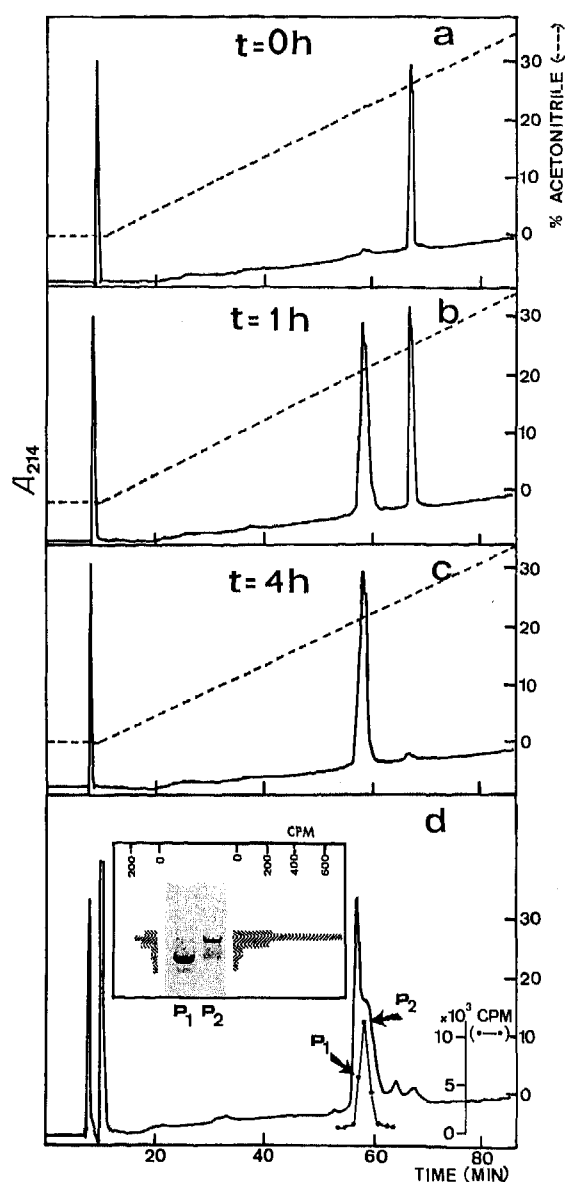


Fig. 1. Chromatographic behaviour of SC. Elution profiles of reverse-phase chromatography of isolated SC. A μ Bondapak column was used, with an acetonitrile gradient (0–35%) in 0.1% TFA (v/v), at 1.5 ml/min flow rate. (a) Zero time of incubation in 20 mM phosphate buffer, at 25°C; (b) and (c) after 1 h and 4 h incubation, respectively; (d) after 1 h incubation and further [^{14} C]iodoacetamide treatment; radioactivity counting (●—●) of fractions collected is shown. Inset: radioactivity counting of the 1 mm-sliced PAGE-gels from the P_1 and P_2 fractions eluted at 55 to 62 min on profile (d) is given by bars.

pattern. This result would be related to the formation of an intramolecular disulphide bridge affecting the only two free -SH groups in the isolated SC. Thus, the two cysteines of the small chain from nIII would exhibit a high tendency to form one intradisulphide linkage, in spite of they are involved in intermolecular bonds in the native napin.

Polyacrylamide gels of both forms of SC provide electrophoretic patterns (Fig. 2) which are in agree-

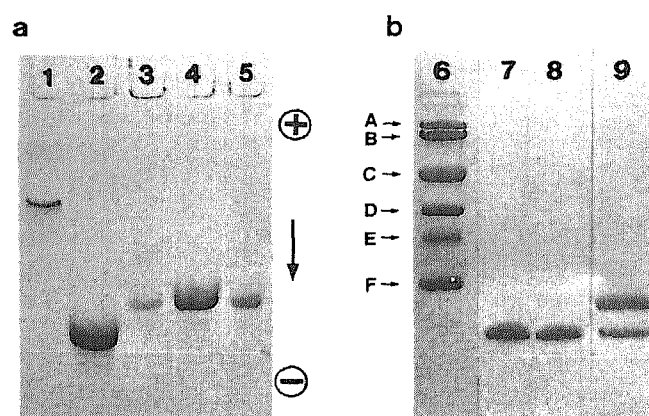


Fig. 2. Polyacrylamide gel electrophoresis patterns of the different forms of SC. (a) Acidic-PAGE in 0.5 M acetic acid, 2.5 M urea. (1) Cytochrome *c*, used as reference; (2) and (4) SC, modified form; (3) and (5) SC, original form. Samples in lanes (4) and (5) have been reduced with 2-ME. (b) SDS-urea-PAGE in which all of the samples were reduced with 2-ME: (6) proteins used as molecular mass standards: A, phosphorylase *b*, 94 kDa; B, albumin, 67 kDa; C, ovalbumin, 43 kDa; D, carbonic anhydrase, 30 kDa; E, trypsin inhibitor, 20.1 kDa; F, α -lactalbumin, 14.4 kDa; (7) modified SC; (8) original SC; (9) napin nIII.

ment with the chromatographic results. Isolated and modified forms of SC show different mobilities under acidic conditions (Fig. 2a, lanes 2 and 3). But the differences disappear after the treatment with 2-ME (lanes 4 and 5). These differences cannot be related to changes in molecular weight as deduced from the SDS-gels (Fig. 2b) and should be explained from changes in the compactness of the polypeptide upon intradisulphide formation.

The modified SC would exhibit a lower 'apparent hydrophobicity' than the original form, according to the corresponding reverse-phase elution patterns. Such a decrease in the hydrophobicity of the molecule should be a consequence of an oxidation-induced folding, resulting in less exposed hydrophobic residue side chains. Similar behaviour on reverse-phase columns has been described for the native and reduced structures of both lysozyme and pancreatic ribonuclease A [21], as well as for reduced and oxidized forms of Cys-containing synthetic peptides [22]. Moreover, a change on the hydrodynamic volume of a polypeptide chain could also modify its chromatographic behaviour [23]. Therefore, the decreased retention on the reverse-phase column of the modified SC could also be explained by a decreased molecular volume due to the new arrangement induced by the disulphide formation.

In order to obtain chemical evidence of the disulphide formation, modification of SC with [^{14} C]iodoacetamide was performed. SC, incubated for different lengths of time in phosphate buffer, was radioalkylated to label the residual free -SH groups. The obtained products were then chromatographed on the reverse-

phase column. The results obtained for the sample at 1 h of incubation in the phosphate buffer are given in Fig. 1d. The peak corresponding to the reduced SC, the last one in Fig. 1b, disappeared after alkylation appearing a relevant shoulder at 22% of acetonitrile (Fig. 1d). Radioactivity measurement of the eluted fractions, as well as the radioactivity scanning of the electrophoretic patterns of these fractions, show the presence of two peptide forms. One of them is not carboxyamidomethylated, since it lacks of radioactivity, eluting at the early fractions, and the other is the radioactive derivative which accounts for the above mentioned shoulder. This result demonstrates that intramolecular disulphide formation is involved in the chromatographic and electrophoretic changes observed upon incubation of SC at neutral pH and room temperature.

Amino acid sequence of the small chain of nIII

As indicated above, there are many napins resulting from the expression of several genes. Thus, we have determined the primary structure of SC (Fig. 3). This was accomplished by Edman degradation of the whole molecule and sequence analysis of the peptides resulting from the tryptic digestion. Two amino acid residues, Lys and Arg, were obtained in a similar yield at position 21 of SC. This heterogeneity could explain the asymmetric chromatographic peak obtained during the purification of the whole molecule. This possibility has been further corroborated, since the amino acid composition of the polypeptide eluting at the first fractions of such a peak was enriched in Lys whereas the tail of the peak was in Arg.

Finally, this primary structure is quite similar, but not identical to the ones deduced from the sequenced napin genes (Fig. 3).

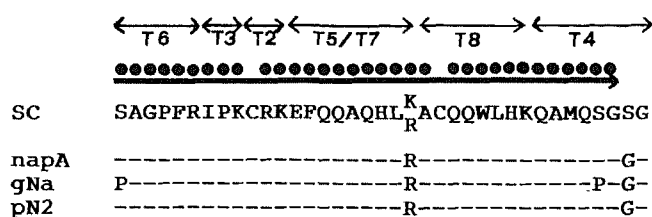


Fig. 3. Amino acid sequence of SC from *B. napus* and comparison with those deduced from napin genes. The horizontal gross arrow indicates the automated Edman degradation of the overall chain: (●) stands for the identified residues. The double arrows represent the peptides obtained by tryptic digestion, which were automatically sequenced. The numbering is referred to the elution position of the tryptic peptides in the chromatographic profile performed for their separation. The column used was a reverse-phase nucleosil C-18 and the elution was achieved by using an acetonitrile gradient in 0.1% TFA (other details are described in the Materials and Methods section). Peptide T-1 does not appear since it is a partial region of T-2. napA, gNa and pN2 are the sequences reported in Refs. 6, 7 and 5, respectively. (–) Stands for amino acid identical to that of the first aligned sequence.

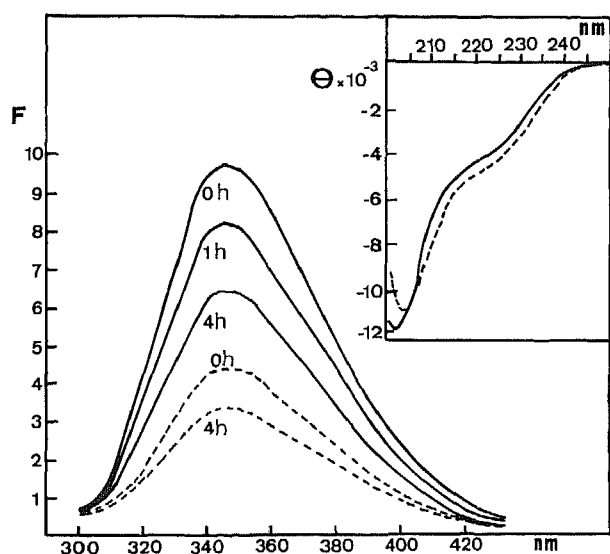


Fig. 4. Measurement of the intradisulphide formation of SC by spectroscopic analysis. Fluorescence emission spectra of SC for excitation at 275 nm (—) and 295 nm (---), at the indicated incubation times in 20 mM phosphate buffer (pH 7.2) and 25°C, at 40 $\mu\text{g}/\text{ml}$ of protein. The spectrum recorded at 4 h corresponds to the modified form of the chain. Fluorescence is expressed in arbitrary units. Inset: Circular dichroism spectra (θ , mean residue weight ellipticities in units of $\text{degree} \times \text{cm}^2 \times \text{dmol}^{-1}$) of the reduced (—), and totally modified form (---) of the chain in the conditions above described.

Circular dichroism and fluorescence spectroscopy studies

In order to analyze the conformational changes inherent to the disulphide formation, CD and fluorescence emission measurements of SC have been performed. The obtained spectra are given in Fig. 4. Small changes in the secondary structure of the polypeptide chain upon oxidation are deduced from the far-ultraviolet spectra of the two forms (inset Fig. 4). Analysis

TABLE I

Secondary structure analysis of the two forms of the small chain of nIII

Values are expressed as percentages of the overall residues in the polypeptide chain. The secondary structure analyses from the CD measurements were performed by using the reference parameters of [10].

	CD measurements	
	original SC	modified SC ^a
α -Helix	17.5	20.4
β -Structure	0.5	3.4
β -Turns	16.0	17.6
Aperiodic conformation	66.0	58.6
R.M.S. ^b	10.9	10.4

^a Totally modified form of SC, after 4 h of incubation at 25°C in 20 mM phosphate buffer, pH 7.2.

^b Root Mean Square error.

of these CD data according to Ref. 10 shows a slight increase in α -helix, β -structure and β -turn contents as well as decreased percentage of the random conformation (Table I), upon the spontaneous disulphide formation. Thus, the secondary structure of the polypeptide would not be greatly modified in spite of the observed spectral changes.

The fluorescence emission spectrum of the reduced SC (Fig. 4, zero time) shows a maximum at 345 nm upon excitation at both 275 and 295 nm. Both emission spectra have the same shape, thus revealing the absence of tyrosine emission which is in agreement with the presence of Trp as single fluorophore in the molecule. The position of the emission maximum is not conclusive in terms of solvent accessibility of fluorophores. Nevertheless, the single Trp of SC would not be extremely buried, since its maximum appears at

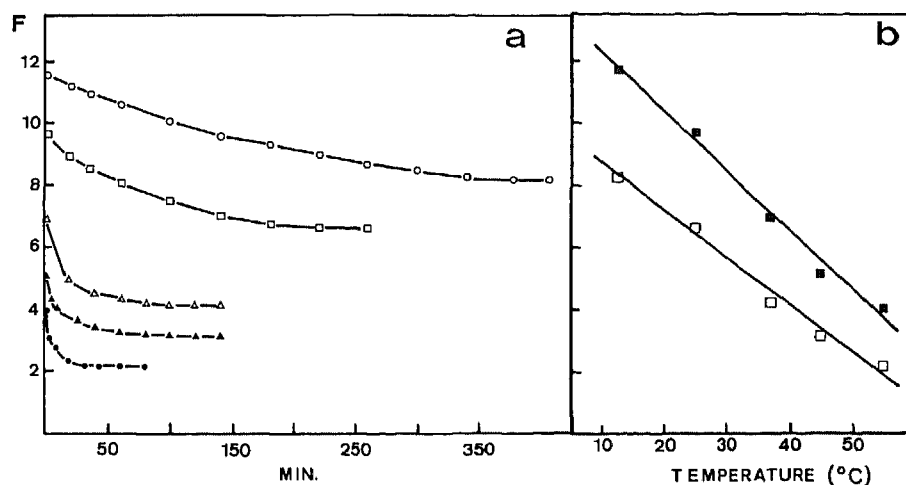


Fig. 5. Effect of the temperature on the modification of SC. *F* stands for fluorescence emission at 345 nm for excitation at 275 nm and is given in arbitrary units. (a) Fluorescence time variation for each assayed temperature: 12.5°C (○); 25°C (□); 37°C (Δ); 45°C (▲); and 55°C (●). All these experiments were carried out in 20 mM phosphate buffer (pH 7.2) at 40 $\mu\text{g}/\text{ml}$ protein concentration. (b) Fluorescence variation as a function of temperature for the original SC (■) corresponding to zero time in the modification and modified SC (□) to final time of the process.

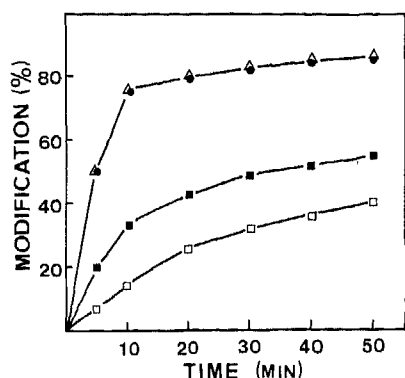


Fig. 6. Effect of different agents on the kinetic of the intradisulphide formation of SC: (Δ) SC alone, ($\bullet$) in the presence of $5 \mu\text{M}$ LC, ($\circ$) $5 \mu\text{M}$ pancreatic RNase A and ($\square$) $15 \mu\text{M}$ DTT. Modification is expressed as percentage of the fluorescence variation. 0% modification corresponds to the initial fluorescence emission at 345 nm (zero time) for excitation at 295 nm. The maximum fluorescence variation was considered as 100% modification (final time). All the experiments were carried out at 37°C and at $40 \mu\text{g/ml}$ ($10 \mu\text{M}$) SC concentration.

wavelengths near to that of exposed Trp-model systems. This Trp-fluorescence emission is quenched in a 30% upon the disulphide formation, as deduced from the spectra recorded at different times of incubation (Fig. 4).

The fluorescence emission decrease allows a kinetic analysis of the disulphide formation. The results obtained by measuring the time-course of the reaction are given in Fig. 5a, at different temperatures. The rate of the disulphide formation increases with temperature, being the process accomplished in 120 min at 37°C (Fig. 5a). Half-times ($t_{1/2}$) of the reaction at each temperature were calculated from double-reciprocal plots. The $t_{1/2}$ values range from 3.8 to 158 min, at 55 and 12°C , respectively. On the other hand, the disulphide formation of SC was also studied in dependence of the pH, but no differences from the data showed at pH 7.2 were obtained when the kinetic analysis were done in the 4.0 to 9.0 pH range.

When analysing the kinetic of the intradisulphide formation in SC in the presence of the reduced LC a clear modification of the time-course of the process is observed (Fig. 6). The $t_{1/2}$ of the reaction sensibly increases in the presence of LC. This occurs even at 0.5:1 LC/SC molar ratio. However, this observed delay can not be related to any interdisulphide formation, since no dimers, LC-SC or SC-SC or LC-LC, are detected. This has been demonstrated from the analyses of LC/SC mixtures in the range 0.5:1 to 2:1 molar ratios, at different reaction times, which have been performed by HPLC and both SDS-PAGE and PAGE at acidic pH. The presence of dithiothreitol (DTT) mimics the effect of LC (Fig. 6). In fact, the $t_{1/2}$ of the intradisulphide formation in SC is also increased in the presence of $15 \mu\text{M}$ DTT. This concentration of reagent

is similar to that of free -SH groups for the LC concentration studied. These results suggest that the effect of LC would be due to the reducing potential of its six half-cystines. This is corroborated by considering the effect of RNase A on the intradisulphide formation in SC. The results obtained in the presence of this protein, lacking of free -SH groups, would give information about the possible influence of protein-protein interactions in the studied reaction. RNase A has no effect on the time-course of the reaction (Fig. 6) at similar RNase A/SC molar ratio than the used when LC is considered.

Considering the initial and final fluorescence intensities as representative values for the two forms of the polypeptide, reduced and oxidized, the effect of the temperature on the quantum yield of the single tryptophan residue can be studied for both conformations. The result is given in Fig. 5b. The obtained linear plots reveal the absence of any thermally induced conformational transition for both polypeptide forms in the temperature range considered. The slopes obtained reveal slight differences on the micro-environment of the single tryptophan residue, upon the intradisulphide formation.

In order to analyze the solvent accesibility of this single Trp residue, quenching experiments have been performed. Acrylamide was used as collisional quencher, and the results obtained were analyzed by Stern-Volmer plots (Fig. 7). The calculated Stern-Volmer quenching constants (8.17 M^{-1} for reduced and oxydized polypeptides, respectively) are

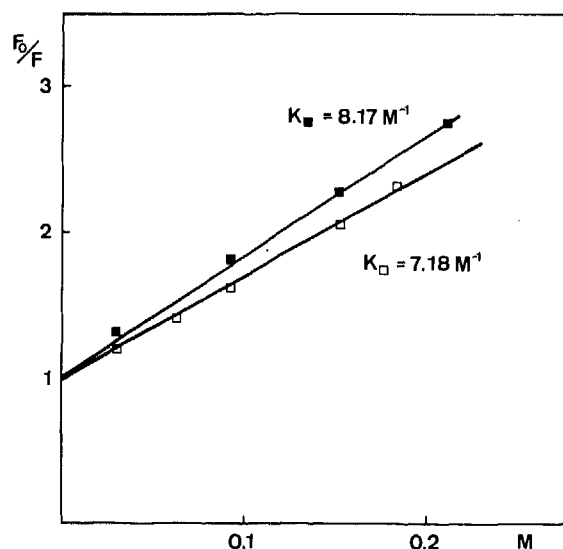


Fig. 7. Stern-Volmer plot, (F_0/F vs. acrylamide concentration) of reduced SC ($\blacksquare$) and modified SC ($\square$). The values of F , fluorescence at 345 nm, were obtained from the fluorescence spectra of both SC forms, at $40 \mu\text{g/ml}$ in 20 mM phosphate buffer, pH 7.2 and 25°C , for excitation at 295 nm. The F_0 value is considered as the fluorescence at 0 min of the reduced SC (See the Materials and Methods section).

Acrylamide concentration is expressed in molar units (M).

also slightly different for both SC forms in agreement with the above results. In both forms, the accessibility of the quencher to the single fluorophore is relatively high in comparison with other Trp-containing proteins [24].

Discussion

Napins are seed storage proteins from rapeseed, which are composed of two polypeptide chains resulting from proteolytic maturation of a single polypeptide precursor and linked through two disulphide bridges. These proteins are included in the group of 2S albumins from seeds. Actually, two different subunits -S-S-linked can be considered as the structural pattern for a significant part of the 2S albumins from seeds [4,25,26].

We have purified the napin nIII and the primary structure of its small chain has been determined. This polypeptide contains 37 amino acid residues and a high glutamine content (about 20%), as would correspond to its role in the seedling. The determined amino acid sequence is not included among those deduced from the known napin genes. This fact increases the relevance of the studies about the expression of the napin genes, as nIII is the most abundant 2S albumin from *B. napus* [8].

The nIII-small chain is a highly hydrophilic molecule, thus very stable in polar solvents. However, as herein demonstrated, the two cysteines of the molecule are very reactive and form an intramolecular disulphide bond in a rapid process. The resulting conformation is more compact than that of the reduced molecule, according to its hydrodynamic behaviour. But, they are not very different at level of the secondary structure, as deduced from CD measurements. Secondary structure prediction, based on the determined amino acid sequence, is given in Fig. 8. Only two β -turns and one α -helix segment appear as periodic structural motifs in this prediction. There are no regions of the molecule whose secondary structure propensities allow them to be apparently involved in conformational changes upon the disulphide formation, in agreement with the results obtained from the CD spectra.

It is remarkable the relatively high rate of formation of this intramolecular Cys-Cys bond (its $t_{1/2}$ at 55°C is 3.9 min). Half-times for disulphide bond formation were never found below 10–15 min for single-domain and short peptides under optimal conditions [27]. The conformational predictions done on the basis of the sequence obtained (Fig. 8), suggest great prominence and flexibility for the position occupied by Cys-10, which is predicted in β -turn conformation. This could facilitate the strict stereochemical requirements for the disulphide arrangement.

The single tryptophan residue of the molecule has been revealed as a very good marker to follow the

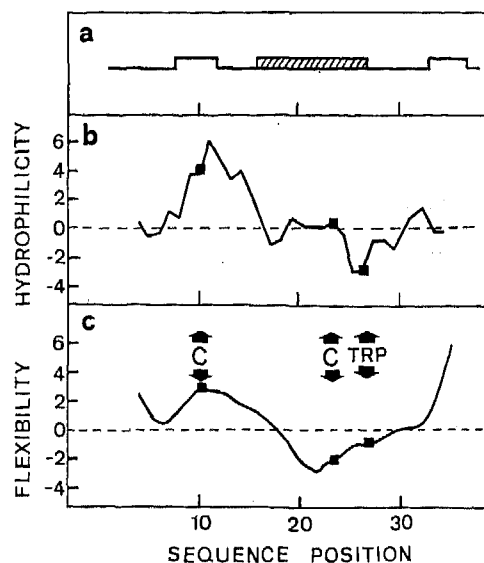


Fig. 8. Predictive analysis for the structure of SC. (a) Secondary structure prediction according to [11]. Lowered line: aperiodic conformation; uppered line: β -turn; and shaded zones: α -helix. No β -sheet was predicted for this sequence. (b) and (c) hydrophilicity and flexibility profiles, respectively, obtained according to Refs. 13 and 14, and the microcomputer program EPIPLOT [15]. A segment span of 7 was used for these calculations. The position of the two Cys and Trp are shown by arrows. Above and below of the reference line (— — —) correspond, respectively, to external and buried regions in profile (b) and mobile or conformed residues in (c).

conformational transition in the polypeptide. This fluorophore exhibits an increased quenching upon intramolecular disulphide formation. This may result from a change in the microenvironment of that residue, when polar amino acid residues near Cys-10 (Lys, Arg and Glu) become close to Trp-23 in the conformation of the oxidized SC. But, the disulphide bond formed in oxidized SC can also be responsible for the observed 30% decrease in the tryptophan quantum yield; in fact, disulphide bridges have been revealed as effective quenchers for indole fluorescence [28].

Numerous studies have been reported about disulphide bonds and protein stability [29,30]. The renaturation of proteins, in which the disulphide bridges have been maintained, frequently accounts for the reconstitution of their precise folding and biological activity. It seems that natural disulphide bonds stabilize the native conformation. However, no correct refolding is usually obtained in proteins which have been enzymatically processed. On the other hand, the importance of the folding stability increases when considering the secretory polypeptides whose organization as subunits linked by disulphide bonds is a common structural motive (polypeptide hormones, immunoglobulins, complement proteins). In spite of the non secretory nature of the seed storage proteins, they have been considered as secreted polypeptides and precursors with signal peptides have been reported for many 2S albumins

[5,31,32]. As in secretory proteins, disulphide bridges of napins could play an important role on their conformation stability, since they must be stored for long periods of time until the germination starts. Regarding the events which must occur during processing of prepro-napins to native structures, it could be easily compared with those for insulin. The elimination of the peptide segment linking the small and the large subunits on the precursor must be catalyzed by maturation proteinases. However, the strong tendency to form the intradisulphide bond in SC suggests that the proteolytic activity should occur after the correct arrangement of the interdisulphide bridges. Otherwise, the napin might be substrate of protein disulphide isomerase, which has been found in both smooth and rough endoplasmic reticulum of developing wheat endosperm [33]. The results obtained from the kinetic measurements of intradisulphide formation of SC in the presence of LC would be in agreement with these possibilities.

Considering that a significant part of the 2S albumins exhibits the same structural pattern than napin nIII, the maturation of this type of proteins should proceed similarly. The obtained results suggest that disulphide formation should be considered as an early step during the maturation of this type of multi-subunit seed proteins. This would be in agreement with that reported for secretory proteins from animal sources [34–36], thus suggesting the existence of a general processing pattern independent on the biological origin and function of the protein.

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