

The structure and properties of Napin-seed storage protein from rape (*Brassica napus* L.)

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Purification to homogeneity of 2S albumin storage protein from rape seeds (napin) was achieved and its secondary structure and conformational stability were characterised by circular dichroism (CD), and high-sensitivity differential scanning calorimetry (HS-DSC). Deconvolution of the far-UV CD spectrum of napin revealed about 25% of α -helix and 38% of β -sheet structure at neutral and slightly acid pH. HS-DSC data show a single peak of protein denaturation with maximum at 101 °C and 88 °C at pH 6.0 and 3.0, respectively. This discloses very

high stability of napin tertiary structure. The thermal denaturation of napin is irreversible due to secondary processes such as hydrophobic aggregation of the unfolded protein. The deconvolution of the transition profile at pH 3.0 carried out with assumption that napin aggregation does not contribute to the transition parameters allows to distinguish two constituent transitions which may be attributed to two different domains in napin folding.

1 Introduction

Proteins in rape seed constitute up to 20–25% of dry seed weight [1]. Two most abundant are 12S and 2S proteins termed cruciferins and napins, respectively [2]. High molecular weight cruciferins are neutral proteins composed of several subunits. Napins are low molecular weight basic proteins built of two disulphide-linked polypeptide chains [1–3]. They may represent from 15 to 45% of total rape seed protein depending on variety [4]. They are issued from initial translation product which is a 20 kDa precursor protein truncated in two peptide chains of 10 and 4 kDa forming mature protein [1, 5]. Its precise processing consists of: first the removal of the N-terminal signal peptide from the precursor and second precursor cleavage which gives two disulphide-linked peptide chains [1, 6]. Removal of negatively charged prosequences is neutralising electrostatic charges and results in a mature protein with very basic character (pI of approx. 11) [10–12]. However, removal of prosequences has little effect on the structure of mature protein because reactivity of pronapin and napin with a polyclonal antiserum showed that the majority of napin antigenic epitopes can be detected in pronapin [13].

Napins are products of multigenic family of 10–16 genes [8]. Four napin gene sequences, napA [8], napB [7], BngNAP1 [10], and gNa [9], and three c-DNA sequences, pN1, pN2 [5] and pNAP1 [1] are known. Mass spectroscopy was used to identify which products of this multigenic family are expressed the most [11]. It was found that napin proteins BngNAP1, napA and short chain of gNa correspond to known gene sequences. Two other short chains named BngNAP1', gNa' and long chains BngNAP1B, BngNAP1C and gNaA differ by 1–4 amino acids from sequences BngNAP1 and gNa, respectively. Recently, additional napin-type protein was isolated and characterised on the amino acid level. The amino acid sequence determined by Edman degradation [12] shows only about 50% homology with major class of napin proteins. Three dimensional structure of this protein was determined by ^1H NMR. Its proposed structure

contains five helices and a C-terminal loop arranged in a right-handed helix [13]. The study presented here was undertaken to get information about the structure of napin in solution and its conformational stability.

2 Materials and methods

2.1 Isolation and purification of napin

500 g of rape seeds (*B. napus* var. Bor) were homogenised and extracted with buffer A (50 mM NaH_2PO_4 , pH 7.0, 1 mM EDTA) and centrifuged for 1 h (all centrifugation were at 4000 rpm ($2500 \times g$)). Solid $(\text{NH}_4)_2\text{SO}_4$ was added to 400 ml of supernatant, up to 30% of saturation and centrifuged. The supernatant was saturated again to 75% with $(\text{NH}_4)_2\text{SO}_4$ and centrifuged. The pellet was dissolved in 50 ml of buffer A, dialysed against the same buffer, and desalted by gel chromatography on Sephadex G-50 column. Fractions containing napin were collected, reprecipitated with $(\text{NH}_4)_2\text{SO}_4$ (75% of saturation) and centrifuged. Crude napin was dissolved in buffer B (same as buffer A, but pH 7.4), dialysed against it, loaded onto a CM-Sephadex C-50 column and eluted with a gradient of 0.15–0.35 M NaCl in buffer B. Fractions containing napin were pooled, precipitated with $(\text{NH}_4)_2\text{SO}_4$, redissolved in buffer A, and dialysed. SDS-PAGE of napin after dialysis shows one band which is resolved into two components in the presence of mercaptoethanol (Fig. 1). Gel-filtration HPLC showed, however, more complex elution profile of napin (Fig. 2). This surprising heterogeneity of the napin sample was also indicated by reverse-phase HPLC (data not shown). Spectral analysis of the elution patterns revealed the presence of high-molecular components showing absorbency maximum at 240–270 nm. This may indicate that some part of the napin is complexed with ligands absorbing in this spectral range. Consequently, napin was further purified by cation-exchange chromatography using gradient up to 1 M NaCl at pH 5.0 (Fig. 3). The fractions with $\lambda_{\text{max}} = 280$ nm corresponding to the central part of the peak were collected, concentrated and analysed.

2.2 Solutions for spectrophotometric and calorimetric measurements

The solutions were prepared by dialysis of the napin stock solution against desired buffer at 4 °C over night. Protein concentration in buffer after dialysis was measured spectrophotometrically using $E_{276}^{\text{nm}} = 13890 \text{ M}^{-1} \text{ cm}^{-1}$, which was obtained by the Edelhoch procedure [14].

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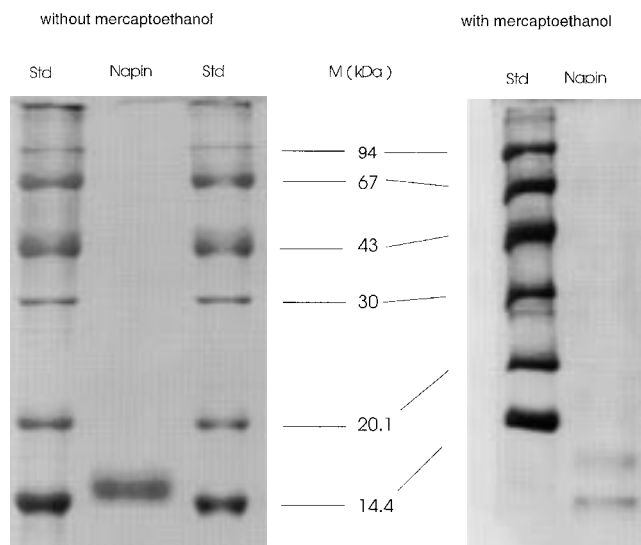


Figure 1. SDS-PAGE of napin before additional purification by ion-exchange HPLC.

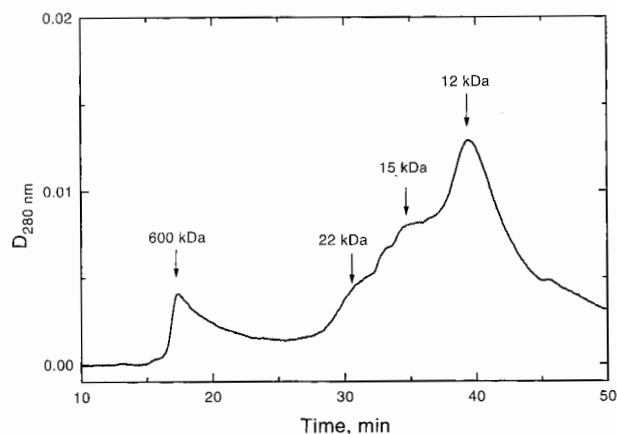


Figure 2. Gel-filtration elution profile of napin before additional purification by ion-exchange HPLC.

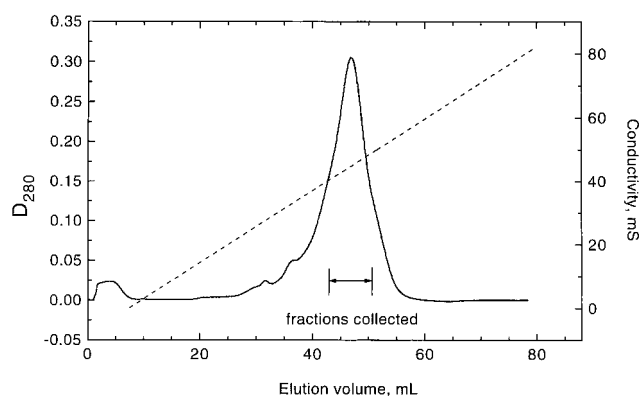


Figure 3. Ion-exchange chromatogram of napin at pH 5.0.

2.3 HPLC

TSK-G2000SWXL column (Interchim) was used for gel-filtration chromatography. Elution was carried out at the rate 0.3 ml/min using 50 mM potassium phosphate, 0.2 M NaCl as an eluent at pH 7.0. The column was calibrated with six protein standards covering the molecular weight range from 12.4 to 669 kDa. Reverse-phase HPLC was performed on C8 Nucleosil (300 Å, 5 mm, 70/3, Macherey

Nagel) column using 3% ACN, 97% H₂O, 0.1% TFA as solution A and 60% ACN, 40% H₂O, 0.09% TFA as solution B. Ion-exchange HPLC was carried out on the column Poros 20 HS (Perceptive Biosynthesis) applying 0–1 M NaCl gradient in 25 mM acetate buffer at pH 5.0.

2.3 CD spectra

CD spectra were measured at 20 °C using a Jobin Yvon CD6 dichrograph from 180 to 260 nm. Spectra were averages of 3 accumulated scans with subtraction of the baseline. Cylindrical cells used had a pathlength of 0.02 cm. Protein concentrations were of about 0.6 mg/mL in 10 mM phosphate buffer with pH 7.0 and 5.5. Molar residue ellipticity was calculated assuming a mean residue mass of napin $M_r = 131.8$. Deconvolution of the far-UV spectra was done using the *Provencher-Glökner* algorithm [15].

2.4 HS-DSC

Calorimetric studies were carried out with the high-sensitivity differential scanning microcalorimeter VP-DSC (MicroCal, USA) within the temperature range 5–125 °C at the heating rate 1K/min and excess pressure 0.25 Mpa. 10 mM phosphate containing 1 mM EDTA and 40 mM glycine were used as buffer solutions at pH 6.0 and 3.0, respectively. Primary data processing and deconvolution of calorimetric curves were carried out using Origin 2.0 software. Molar heat capacity was calculated assuming a molecular weight of napin $M = 14.5$ kDa.

3 Results and discussion

Napin proteins obtained from rape seeds are mixture of closely related proteins very difficult to separate [11]. Mass spectrometry analysis revealed that some of these proteins are the products of the genes identified earlier napA [8], BngNAP1 [10] and of a short chain of gNa [9]. Other identified fragments differ only slightly. These new variants are BngNAP1', BngNAP1A and gNaA (short chains) and BngNAP1B, BngNAP1C and gNaA (long chains) [11]. It was also found that the short chain is connected via two disulphide bonds to the long one. Two other disulphide bonds are present in the long chain [3, 11, 13].

In Fig. 4 the far-UV CD spectra of napin at pH 7.0 and 5.5 are shown. Molar ellipticity displays a maximum at 192 nm and two minima at 209 and 220 nm. The positions of the bands as well as their intensities do not change while changing pH from 7.0 to 5.5. Deconvolution of the spectra gives about 25% of α -helix and 38% of β -sheet structure (average values for two pH) (Fig. 4). These data are consistent with previous studies of napin and its precursor displaying very little structural changes at different pH [16]. They differ, however, significantly from the results of CD spectra analysis of napin reported earlier [17]. This is due to the use of different spectral interval during prediction [18].

Calorimetric measurements were carried out with napin samples before and after final purification by ion-exchange chromatography (Fig. 5). It can be seen that heat capacity curve of highly purified napin differs substantially from the less purified sample, first of all, by the position and the slope of the base line below transition area. The negative slope of calorimetric curve and abnormally low value of the apparent heat capacity of napin before final purification are indicative of a significant amounts of non-protein ligands bound to napin. Taking into account very basic nature of napin one may suggest that it can form electrostatic complexes with lipids, nucleic acids or dyes.

The slope of heat capacity curve for purified napin is slightly positive as it is usually observed for globular proteins. However,

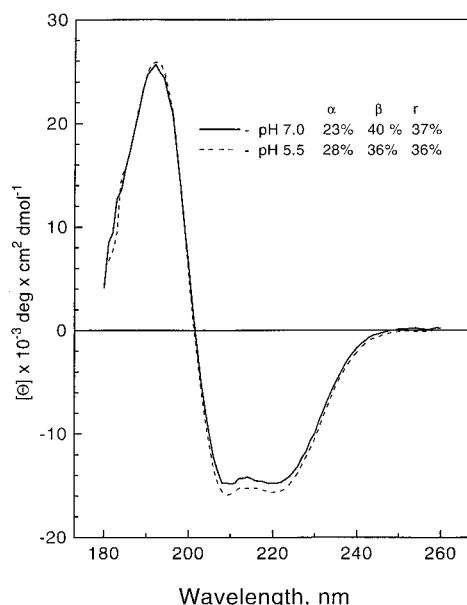


Figure 4. CD spectra and different secondary structure content of napin at pH 7.0 and pH 5.5.

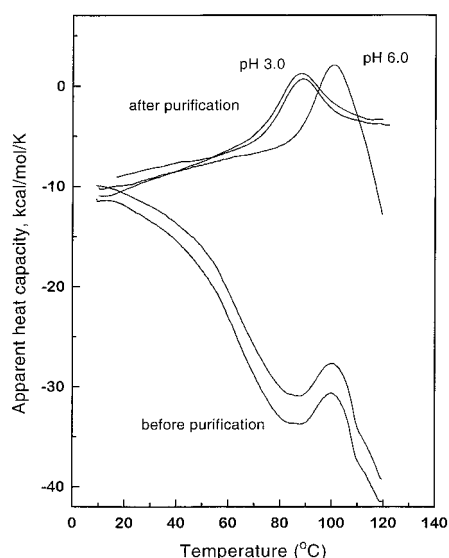


Figure 5. Partial heat capacity curves of napin at pH 6.0 before and after additional purification by ion-exchange HPLC.

it is difficult to calculate precisely the transition enthalpy at pH 6.0 because the denaturation of napin is accompanied by exothermic heat effect of aggregation (Fig. 5). The transition peak is not seen during reheating of the solution in calorimeter indicating thus irreversible character of napin denaturation. Gel-filtration of napin solution taken from calorimetric cell after heating shows appearance of polymeric forms of the protein. Presence of disulphide polymers in the heated solution was not detected by SDS-PAGE. This suggests that hydrophobic effect is a major cause of napin aggregation after unfolding.

In order to minimise aggregation phenomenon calorimetric measurements were carried out with purified napin at pH 3.0 (Fig. 5). Thermogram does not show any exothermic effects at pH 3.0, however, the observed transition is still irreversible. It

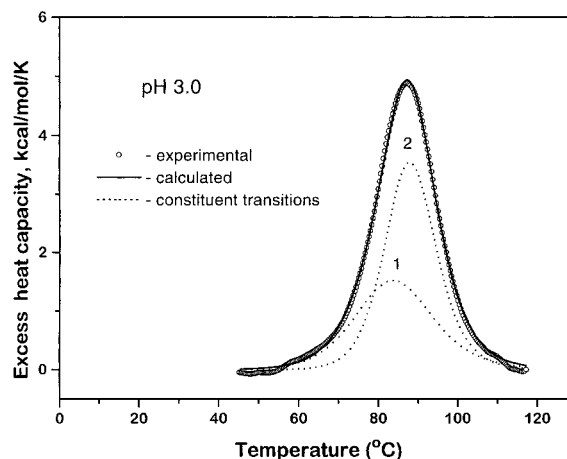


Figure 6. Excess heat capacity curve of napin at pH 3.0 and result of its deconvolution.

was shown for several proteins that slow post-denaturational processes like polymerisation of polypeptide chains do not affect markedly the denaturation parameters. This means that calorimetric parameters for irreversible transition $N \rightleftharpoons D \rightleftharpoons I$ are close to thermodynamic parameters for the reversible stage $N \rightleftharpoons D$. The excess heat capacity of purified napin at pH 3.0 is shown in Fig. 6. Deconvolution of this curve based on the two-state model resulted in two transitions (Fig. 6) with different parameters: transition 1 – with temperature $T_{d1} = 84.3^\circ\text{C}$ and enthalpy $\Delta H_1 = 39.4$ kcal/mol and transition 2 – with temperature $T_{d2} = 88.3^\circ\text{C}$ and enthalpy $\Delta H_2 = 60.6$ kcal/mol. These transitions may probably be attributed to the independent unfolding of two structural domains of napin molecule. This conclusion could be made if conformational stability of napin variants composing the sample under study does not differ substantially. In connection with this it would be interesting to have an insight into the three-dimensional structure of napin.

Three-dimensional structure of one member of the napin family was solved recently [13]. Sequence similarity between this protein and other known up to now napins amounts to only ~50%. The analysis of the disulphide bond alignment suggests, however, that structure of all these molecules may be similar. Also other proteins from different plant species belonging to so called napin-type 2S albumins (NT2SA) exhibit high structural similarity even in case of high polymorphism of their sequences [13]. This supports the hypothesis that complex profile of the denaturation transition of napin is rather due to its domain structure than to its heterogeneity.

In conclusion, it is necessary to turn the attention to the unusually high thermal stability of napin. Because of its extremely basic nature, napin has a large positive charge at pH 3.0. However, the denaturation temperature of napin at these conditions is high – 88°C what is indicative of its very stable folding.

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Received: 2 September 1997.

Accepted: 9 February 1998.