Disulphide structure of a sunflower seed albumin: conserved and variant disulphide bonds in the cereal prolamin superfamily

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Abstract Disulphide mapping of a methionine-rich 2S albumin from sunflower seeds showed four intra-chain disulphide bonds which are homologous with those in a related heterodimeric albumin from lupin seeds (conglutin δ). Similar conserved disulphide bonds are also present in α-gliadin and γ-gliadin storage proteins of wheat, but a lower level of conservation is present in a further related group of proteins, the cereal inhibitors of α-amylase and trypsin. These differences may relate to the different functions of the proteins.

Key words: Seed; Cereal; Sunflower; Wheat; Albumin; Inhibitor; Gliadin; Disulfide bond

1. Introduction

The cereal prolamin superfamily of seed proteins comprises three major groups of proteins: the storage prolamins of barley, wheat and rye, the cereal inhibitors of α-amylase and trypsin and 2S albumin storage proteins present in seeds of a range of dicotyledonous plants [1–3]. Although these three groups of proteins differ widely in their properties (including M_r and amino acid sequences) they are characterized by the presence of conserved cysteine residues. This conservation is assumed to reflect a crucial role of these cysteine residues in stabilizing the protein structure via disulphide bond formation, but this remains to be established as few components have been disulphide mapped, and no detailed comparisons have been reported.

2S albumin storage proteins have been characterized from a diverse range of plants, including legumes (lupins, peas), crucifers (radish, oilseed rape, *Arabidopsis*), cotton, sunflower, Brazil nut and castor bean [4]. These are typically heterodimeric proteins, with large and small subunits of M_r about 9000 and 4000, respectively, which arise from post-translational proteolysis of a precursor protein. One such component has been disulphide mapped, the conglutin δ of lupin. However, post-translational proteolysis does not occur in sunflower, and the albumin fraction consists of monomeric proteins of M_r about 10000–18000 [5,6]. We have therefore determined the disulphide structure of one such sunflower albumin, a methionine-rich component SFA8 [7]. The pattern of disulphide bonds is identical to that in the heterodimeric conglutin δ [8] and comparison with structures reported for members of the prolamin and inhibitor groups shows the presence of conserved and variant disulphide bonds in this superfamily of seed proteins.

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2. Materials and methods

2.1. Materials

5 M cyanogen bromide in acetonitrile was obtained from Aldrich. Guanidine hydrochloride and Tris were obtained from Sigma. Sequence grade trifluoroacetic acid (TFA) was from Applied Biosystems and HPLC grade acetonitrile from Merck. Water was purified using a Milli Q System (Millipore). All other reagents were analytical grade.

2.2. Protein purification

SFA8 was purified from sunflower Hybrid 246 as described by [7] with an additional final separation by RP-HPLC on a SynChropak RP-P C18 Semi-preparative column (10×250 mm) with a linear gradient (25% to 55%) of acetonitrile containing 0.07% (v/v) TFA in 0.05% (v/v) aqueous TFA.

2.3. Cyanogen bromide cleavage

0.5 mg (≅40 nmol) of purified SFA8 was dissolved in 100 μl of 70% (v/v) TFA and 25 μl of 5 M CNBr in acetonitrile (about 200 molar excess over methionine residues) added. The reaction mixture was flushed with argon and incubated at 22°C for 18 h in the dark. After drying the peptides were dissolved in 50 μl of 5 M guanidine hydrochloride in 0.1% (v/v) TFA and separated by RP-HPLC using an Aquapore RP-300 C8 column (4.6×220 mm) with a linear gradient (0 to 40%) of acetonitrile containing 0.08% (v/v) TFA in 0.1% (v/v) aqueous TFA.

2.4. Identification of disulphide-bonded peptides

About 10% of each dried fraction was redissolved in 50 μl of 0.1 M Tris-HCl buffer, pH 8, containing 5 mM EDTA and 5 M guanidine hydrochloride. Reduction of disulphide bonds and alkylation of sulphydryl groups was carried out by adding 1.4 μmol of dithiothreitol dissolved in 1 μl of distilled water, standing for 30 min at 22°C, and adding 1 μl (9.3 μmol) of 4-vinylpyridine. The reaction mixture was then separated by RP-HPLC as described for the cyanogen bromide digest, in order to identify fractions giving two peaks.

2.5. N-terminal amino acid sequencing

N-terminal amino acid sequence analysis was carried out with a Model 816 protein sequencer (Knauer, Berlin) equipped with a Model 120A PTH Analyser (Applied Biosystems) operated according to the manufacturer's instructions. Peptide samples were applied onto Immobilon membrane in 30% (v/v) acetonitrile containing 0.1% (v/v) TFA.

3. Results

N-terminal amino acid sequencing of the SFA8 fraction purified from sunflower Hybrid 246 showed that it was identical for 20 residues to the sequence reported by Kortt et al. [7]. The latter showed the presence of eight cysteine residues and 16 methionines in a protein of 103 residues (Fig. 1A). With the exception of two adjacent residues (Cys-51 and Cys-52), all the cysteines are separated by at least one methionine. The purified protein was therefore cleaved with cyanogen bromide and 11 fractions were purified by RP-HPLC (Fig. 2). Re-separation of each fraction after reduction and

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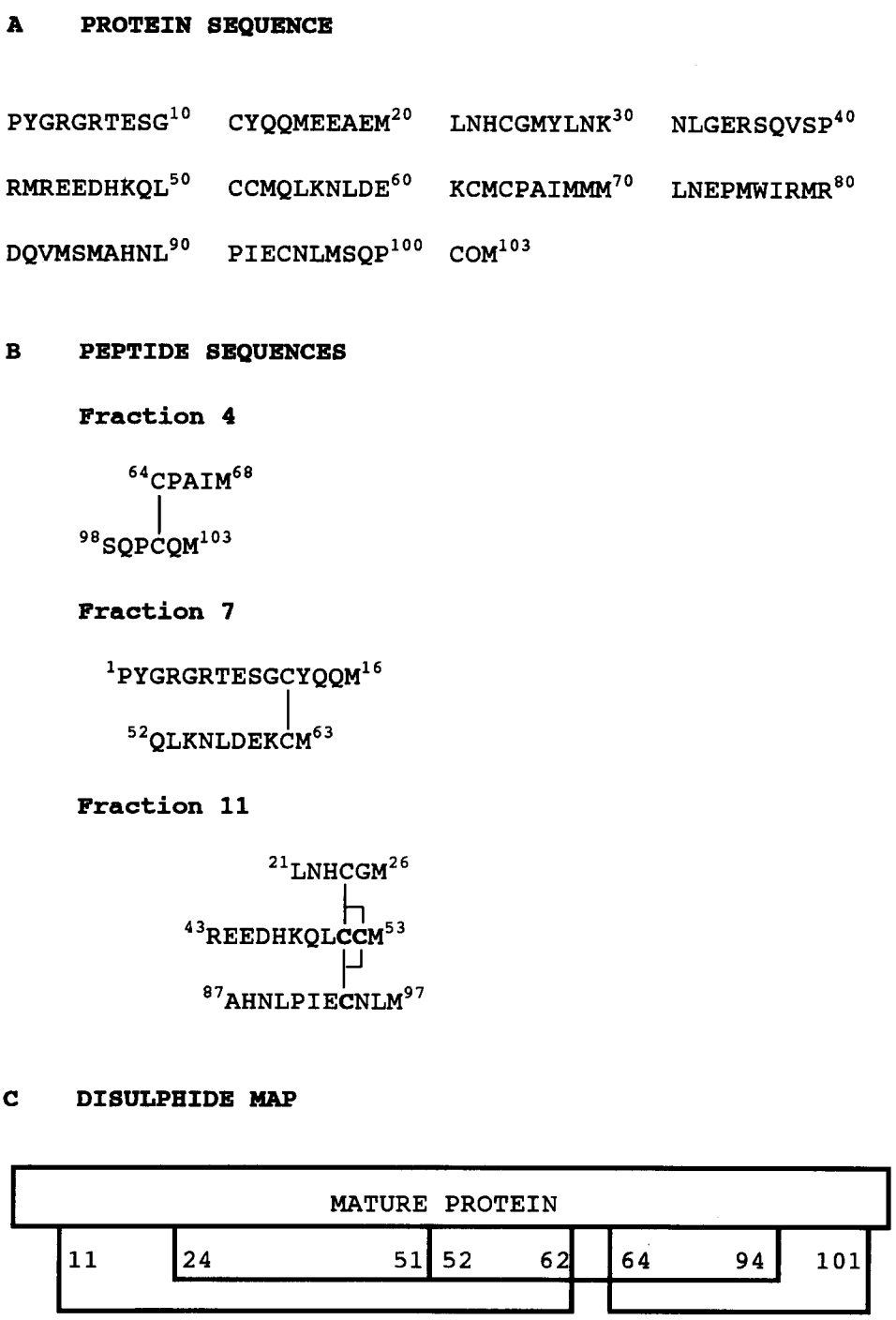

Fig. 1. Disulphide bond mapping of the sunflower albumin SFA8. (A) The protein sequence reported by Kortt et al. [7]. (B) The sequences of disulphide-bonded peptides isolated as fractions 4, 7 and 11. (C) Disulphide map of the protein.

alkylation showed that three of them (nos. 4, 7, 11) consisted of two or more peptides linked by disulphide bonds. These fractions were therefore subjected to N-terminal sequence analysis, both before and after reduction of disulphide bonds. This showed that fraction 4 consisted of two peptides linked by a disulphide bond between Cys-64 and Cys-101 and fraction 7 of two peptides with a disulphide bond between Cys-11 and Cys-62. Fraction 11 consisted of three peptides with Cys-24 and Cys-94 linked to the adjacent cysteine residues Cys-51 and Cys-52, but it was impossible to identify the precise bonds involving these two residues (Fig. 1B). The disulphide structure of SFA8 was therefore established as shown in Fig. 1C.

4. Discussion

The disulphide structure of one heterodimeric 2S albumin, namely conglutin δ from lupin which has nine cysteines, has

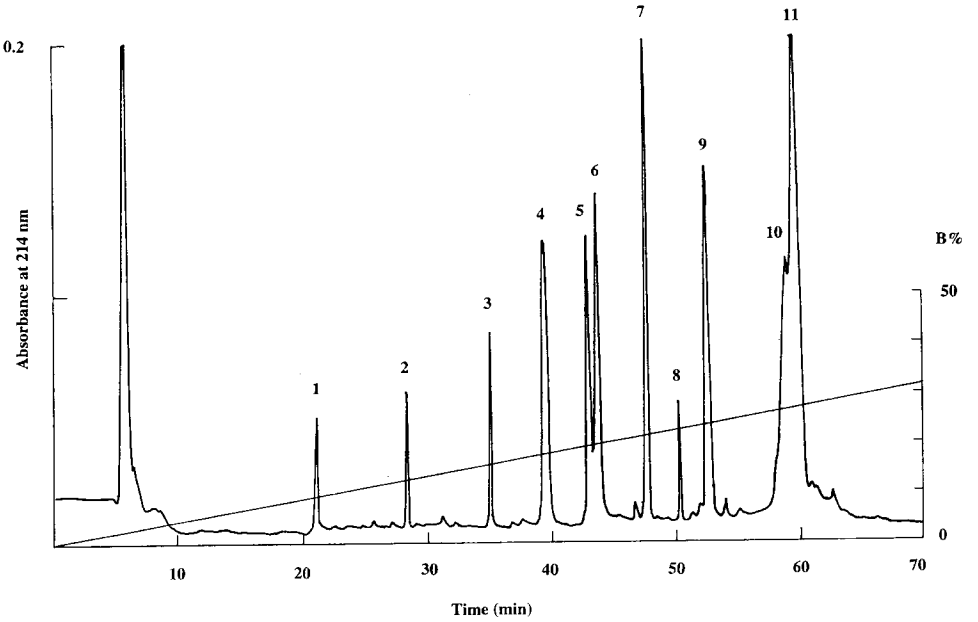

Fig. 2. Separation of products of cyanogen bromide cleavage of intact SFA8 (approx. 0.5 mg) by reversed-phase HPLC on an Aquapore RP-300, C8 column (4.6×220 mm). Peptides were eluted with a linear gradient of acetonitrile in the presence to TFA (from 0% B to 40% B for 90 min) at a flow rate of 0.5 ml/min. Solvent A: 0.1% (v/v) aqueous TFA; solvent B: acetonitrile containing, 0.08% (v/v) TFA. Fractions 4, 7 and 11 contained disulphide-bonded peptides.

been reported [8]. Despite a low level of overall sequence identity to SFA8 (17% homology), eight of the cysteine residues in conglutin δ are clearly homologous, based on position and sequence context, to those in SFA8 (Fig. 3). These cysteine residues, called A-H in Fig. 3, also form the same 4 disulphide bonds, two of which are inter-chain in conglutin δ. In neither case was it possible to discriminate between the bonds involving the adjacent cysteine residues (C and D). The ninth cysteine residue in conglutin δ, Cys-45, is not present in SFA8 and is unpaired. This is called Cys J in Fig. 3.

Six of the eight cysteine residues present in SFA8 and conglutin δ (cysteines B, C, D, F, G, H) are also conserved in the C-terminal domains of two types of monomeric wheat prolamin, called α- and β-gliadins (Fig. 3). These six conserved cysteine residues also form the same three disulphide bonds in the α- and β-gliadins as in the 2S albumins (B to C or D, C or D to G, F to H), while the γ-gliadin also contains an additional pair of cysteine residues (called K and L in Fig. 3) which form a fourth disulphide bond [9–11].

The disulphide structures of three cereal α-amylase/trypsin inhibitors have been reported: the monomeric 0.28 (WAI-0.28) [12] and tetrameric 0.53 (WAI-0.53) [13] α-amylase inhibitors from wheat (Fig. 3), and a bifunctional α-amylase/trypsin inhibitor (RBI) from ragi (Indian finger millet) [14] (not shown). Again it is possible to identify conserved cysteine residues homologous to those present in the 2S albumins and the gliadins, but the patterns of disulphide bond formation are not identical.

WAI-0.28 and RBI both have 10 cysteine residues, corresponding to cysteines A to H and K in the gliadins and the 2S albumins and a single unique cysteine residue called M. The disulphide maps of these proteins were determined using NMR spectroscopy (RBI) and mass spectroscopy (WAI-0.28), showing identical patterns. Also, in both cases, it was possible to distinguish between disulphide bonds involving the adjacent cysteine residues (C and D), in contrast to the other proteins. These studies show that 4 of the 5 disulphide bonds (A to E, B to C, D to G and F to H) are conserved, with the fifth bond between cysteine K (which is paired with L in the γ-gliadin) and M. The third inhibitor that has been disulphide-mapped, WAI-0.53, shares 9 cysteine residues with WAI-0.28 and RBI, lacking cysteine residue E. The absence of cysteine E is associated with the formation of a new disulphide bond between cysteines A and H, leaving cysteine F unpaired.

These comparisons show a high degree of conservation of disulphide bonds within and between the gliadins and 2S albumins, involving cysteine residues B to C/D, C/D to G and F to H. In addition, the γ-gliadin and 2S albumins each contain a single additional disulphide bond, between cysteines K to L and A to E, respectively. However, only two disulphide bonds involving the adjacent cysteine residues (C/D) are also conserved in the inhibitors, with differences between RBI/WAI-0.28 on the one hand and WAI-0.53 on the other. Thus, their different disulphide bond patterns may relate to the maintenance of active sites for their target enzymes.

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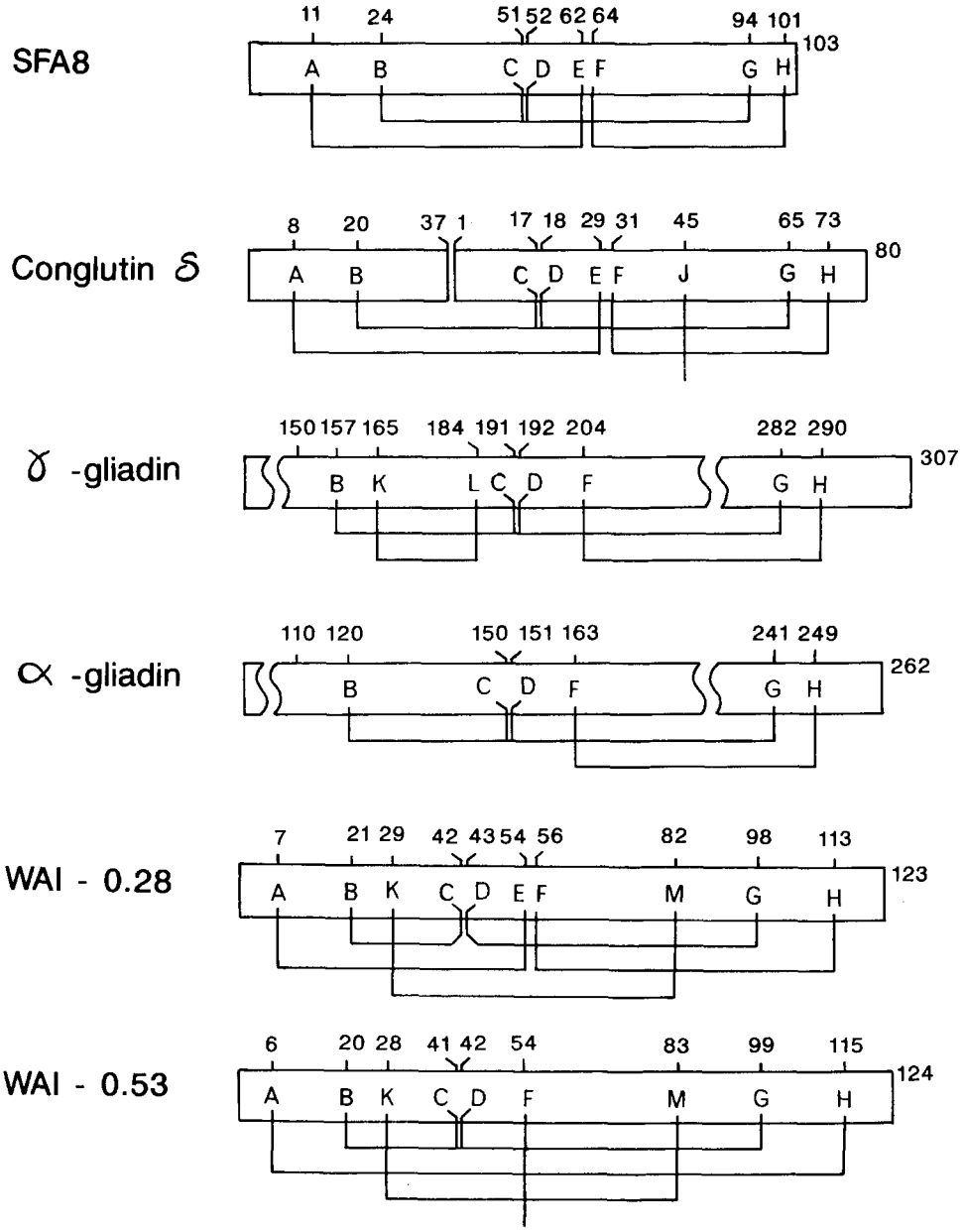

Fig. 3. Comparison of the patterns of disulphide bonds formed by 2S albumins, cereal inhibitors and gliadins. Cysteine residues are labelled A–M as discussed in the text. The disulphide maps for conglutin δ, WAI-0.28, WAI-0.53, α-gliadin and γ-gliadin are reported in [8–14].

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