Structure characterization of the central repetitive domain of high molecular weight gluten proteins.

II. Characterization in solution and in the dry state

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Abstract

The structure of the central repetitive domain of high molecular weight (HMW) wheat gluten proteins was characterized in solution and in the dry state using HMW proteins Bx6 and Bx7 and a subcloned, bacterially expressed part of the repetitive domain of HMW Dx5. Model studies of the HMW consensus peptides PGQGQQ and GYYPTSPQQ formed the basis for the data analysis (van Dijk AA et al., 1997, *Protein Sci* 6:637–648). In solution, the repetitive domain contained a continuous nonoverlapping series of both type I and type II β -turns at positions predicted from the model studies; type II β -turns occurred at QPGQ and QQGY sequences and type I β -turns at YPTS and SPQQ. The subcloned part of the HMW Dx5 repetitive domain sometimes migrated as two bands on SDS-PAGE; we present evidence that this may be caused by a single amino acid insertion that disturbs the regular structure of β -turns. The type I β -turns are lost when the protein is dried on a solid surface, probably by conversion to type II β -turns. The homogeneous type II β -turn distribution is compatible with the formation of a β -spiral structure, which provides the protein with elastic properties. The β -turns and thus the β -spiral are stabilized by hydrogen bonds within and between turns. Reformation of this hydrogen bonding network after, e.g., mechanical disruption may be important for the elastic properties of gluten proteins.

Keywords: elastic properties; repetitive proteins; structural characterization; wheat gluten proteins

The central repetitive domain of high molecular weight proteins forms 60–80% of their amino acid sequence and is built from the consensus peptides PGQGQQ, GYYPTS(P/L)QQ, and GQQ (Anderson & Greene, 1989; Shewry et al., 1994). Its structural organization may consist of a series of β -turns that organize in a β -spiral structure (Tatham et al., 1984; Shewry et al., 1994); this idea is supported by scanning tunneling microscopy images of HMW Dx5 (Miles et al., 1991). The proposed β -spiral structure for HMW proteins is similar to that of elastin, where it forms the basis for the molecule's elastic properties (Urry, 1993). The structural analogy between HMW proteins and elastin suggests that the HMW proteins might have elastic properties as well; however, no

detailed structural characterization of the HMW repetitive domain has been performed. In the companion paper (van Dijk et al., 1997), we described the structure of the consensus sequences of the repetitive domain using cyclic and linear peptides. We showed that they are highly structured and contain a mixture of type I and II β -turns that are stabilized by multiple hydrogen bonds. In the present paper, we apply our findings to the characterization of the repetitive domain of HMW Bx6, HMW Bx7, and a subcloned part of the repetitive domain of HMW Dx5.

Results

Subcloning of dB

The HMW Dx5 repetitive domain consists, for more than 90%, of the consensus sequences GYYPTSPQQ and PGQGQQ; the GYYPTSPQQ/PGQGQQ-ratio is 0.26. A representative part of the repetitive domain of HMW Dx5 with a consensus ratio of 0.19 was subcloned from pUC9D5 in pSk⁺ using PCR as described in Materials and methods. The forward primer contained 19 bases corresponding to codons for residues P126–Q132 in HMW Dx5

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Abbreviations: FTIR, fourier transform infrared; HMW, high molecular weight; IPTG, isopropyl- β -D-thiogalactopyranoside.

Fig. 1. Amino acid sequence of dB. The β -turn regions were predicted using the data from Van Dijk et al. (1996). The QQGQ-sequence was not described in that study, because it is very similar to the β -turn sequence QQGY, which was described and has a comparable β -turn probability as calculated according to Wilmot and Thornton (1988). It is indicated as a β -turn.

(Anderson et al., 1989) and introduced a *Bgl* II restriction site at the 5' end. The reverse primer contained 22 bases, corresponding to codons for residues Q265–Q272 in HMW Dx5, introduced a *Bam*H I restriction site at the 3' end, and converted two rare GGA glycine codons to GGT (Sharp & Li, 1987). Special care was taken to design primers that hybridize at unique positions in the repetitive HMW sequence. Additional residues were introduced by incorporation of the restriction sites and of the C-terminal histidine-tag, resulting in the presence of two nonrepetitive stretches: residues M1–Q10 and Q150–H161 (Fig. 1). The protein was cloned in the *Bam*H I site of the expression vector pQE60, giving pQE60dB. The amino acid sequence of the final construct, named dB, is given in Figure 1.

Purification of dB

The dB protein was purified from bacterial cultures using Ni^{2+} -agarose chromatography as described in Materials and methods. The protein migrated as two bands at apparent molecular weights of 25 and 28 kDa (Fig. 2A); the relative intensity of the bands differed between preparations and, in some cases, only the band at 25 kDa was found. The dB preparation, containing two bands, was rechromatographed on Ni^{2+} -agarose, but the two bands eluted together again. Reversed-phase HPLC of the protein preparation showed a single peak that migrated as two bands on SDS-PAGE. The co-elution of the two bands on very different chromatography

systems indicates that they represent very similar proteins. The first 11 N-terminal residues were determined for several dB preparations containing both bands. All gave the sequence GGSPGQAS PQR, which confirms the protein's correct identity (Fig. 1); the N-terminal methionine is missing, indicating posttranslational modification. No significant sequencing heterogeneity was found, indicating that the protein preparations were homogeneous. The protein was subjected to mass spectrometry; preparations examined gave a single peak at a MW of 16,915 Da (± 1 Da) which is equal to the calculated MW for dB lacking the N-terminal methionine and confirms the results of the N-terminal sequence analysis. The dB protein was also subjected to transverse urea gradient SDS-PAGE (Fig. 2D); a continuous change in electrophoretic mobility was observed, very similar to that described for HMW proteins (Tatham et al. 1993; Hickman et al., 1994). The conformation of dB is destabilized gradually by urea, but the two bands remain separate at all urea concentrations.

Purification of HMW proteins

The HMW proteins were purified as described using traditional methods, resulting in better than 90% purity (Fig. 2B,C). HMW Bx6 migrated at an apparent MW of 96 kDa and HMW Bx7 at 94 kDa. The contaminations are most probably ω -gliadins which, are often found in HMW preparations and are very difficult to remove. No attempts were made to further purify these proteins.

Fig. 2. SDS-polyacrylamide gels of proteins. Apart from markers the gels represent dB (A: 15% gel); HMW Bx7 (B: 10% gel); HMW Bx6 (C: 10% gel). Transverse urea gradient (0–8 M urea) of dB (D: 10% gel).

Solubility of proteins

The dB protein was water soluble in 20 mM phosphate and acetate buffers in the pH range 3–9 and in the presence of NaCl (0–500 mM). The solubility behavior differed strongly from that of HMW Bx6 and HMW Bx7, which were only soluble in 10 mM acetate buffers at pH < 5.0. Higher pH values or the addition of NaCl (10 mM) resulted in precipitation, as is usually observed for HMW proteins.

FTIR of dB

FTIR spectra of dB in solution are presented in Figure 3A. At 25 °C, the amide I band is at $1,641\text{ cm}^{-1}$, compatible with a backbone carbonyl in a $1 \leftarrow 4$ hydrogen bond in a type I or II β -turn (Hollósi et al., 1994). The band shifts to $1,645\text{ cm}^{-1}$ when the temperature is raised to 80 °C (Fig. 3B), indicating loss of hydrogen bonding and thus of β -turn character. After cooling to 25 °C, the original spectrum was obtained again, indicating the reversibility of the changes (not shown). The band around $1,516\text{ cm}^{-1}$ originates from tyrosine residues.

CD spectroscopy of dB, HMW Bx6, and Bx7

The CD spectra of dB in solution are shown in Figure 4A as a function of temperature. When the temperature is increased, the 196 nm minimum (25 °C) is red shifted and its amplitude decreased; the 210–240-nm region shows an ellipticity increase with temperature, indicating structural changes. The spectra are independent of pH in the range pH 3–10 and in the presence of NaCl (0–500 mM) or glycerol (0–50%). The temperature dependence of the HMW Bx7 CD spectrum (Fig. 4B) is similar to that of dB, showing an ellipticity increase with temperature; the ellipticity in the 210–240-nm region is higher for the HMW protein than for dB, and the minimum at 25 °C is observed around 205 nm instead of 196 nm. The CD spectrum for HMW Bx6 is shown in Figure 4D; it is similar to that of HMW Bx7 (Fig. 4B) with a similar temperature dependence (not shown). Temperature-induced changes were reversible.

Conformation of dB and HMW Bx6 in the dry state

HMW proteins are deposited in protein bodies in wheat, where they occur in a state of low hydration. In order to mimic this state, we studied the proteins on a CaF_2 or quartz surface. The FTIR spectrum of dB (Fig. 3C) shows an amide I band at $1,638\text{ cm}^{-1}$, compatible with a $1 \leftarrow 4$ hydrogen bonding in a β -turn (Hollósi et al., 1994); it is 2 cm^{-1} lower than in solution (Fig. 3A), indicating increased hydrogen bonding character. The second derivative spectrum (Fig. 5) showed shoulders at $1,630$, $1,650$, and $1,658\text{ cm}^{-1}$, compatible with the presence of, e.g., β -sheet or unordered structures. The band at $1,620\text{ cm}^{-1}$ was also observed for several of the model peptides (van Dijk et al., 1997) and

probably indicates glutamine side chain amides; the weak $1,658\text{ cm}^{-1}$ band was also observed for the model peptide cyclo-[PGQGQQ]₂ (van Dijk et al., 1997) and possibly indicates less-ordered structures. The band at $1,516\text{ cm}^{-1}$ indicates the aromatic tyrosine side chain. The CD spectrum of dB on quartz (Fig. 4D) shows a strong band at 228 nm, which is indicative of a type B spectrum (Woody, 1974) and is associated with a type II β -turn.

Fig. 3. FTIR spectra of dB in solution at 25 °C (A) and 80 °C (B), and as a film on quartz at 25 °C (C). The protein was extensively dialyzed against water, freeze-dried, and redissolved in D₂O (~2 mg/mL); the pD was 4.0–4.5 and was not corrected for isotope effects. In the case of the dry protein, 100 μL of the solution was spotted on a CaF_2 disk (~4 cm²) and dried in air.

Fig. 4. CD spectra of dB (32 μ M) as a function of temperature in 20 mM NaPi pH 6.0 (A); HMW Bx7 (1.7 μ M) as a function of temperature in 10 mM HAc pH 3.5 (B); calculated spectrum of dB (C); HMW Bx6 in solution and on quartz and of dB on quartz (D). Dry protein samples were prepared as described in Materials and methods. The noise level at 250 nm was 0.1 mdegr for all samples; this increased gradually at lower wavelengths. Measurements were stopped at noise levels higher than 2 mdegrs.

The spectrum could only be recorded to 210 nm; at lower values, the rough sample surface caused too much scattering. The CD spectrum for HMW Bx6 on quartz (Fig. 4D) is similar to that of dB, but the minimum is at 222 nm instead of 228 nm for dB. The CD spectra of the dry proteins are very different from those in solution (Fig. 4B,D) indicating structural changes.

Discussion

Structure in solution

The structure of the HMW repetitive domain was studied using HMW Bx6 and HMW Bx7 and a subcloned part of the repetitive domain of HMW Dx5, called dB. The dB protein was readily soluble in water over a range of conditions, in contrast to HMW Bx6 and Bx7, which showed the usual poor solubility behavior of HMW proteins (Field et al., 1987; Shewry et al., 1994). The solubility of dB suggests that the repetitive domain of HMW proteins is readily soluble in water and that the poor water solubility of the HMW proteins resides in their N- and/or C-terminal domains.

The CD spectrum of dB showed little or no structure in the 210–240-nm region and could easily be interpreted as random coil; however, there are several reasons indicating that it represents

β -turn structure. First, the corresponding FTIR spectrum of dB showed an amide I band compatible with type I or II β -turns. Second, the CD spectrum of dB shows a typical temperature dependence; the ellipticity increases with temperature in a way that is very similar to that of the model peptides described in the companion paper (van Dijk et al., 1997). Third, the CD spectrum of dB could be simulated accurately using the spectra for the consensus sequences PGQGQQ and GYYPTSPQQ, also described in van Dijk et al. (1997); dB contains 16 PGQGQQ sequences and 3 GYYPTSPQQ sequences (Fig. 1). The calculated spectrum (Fig. 4C) is similar to the experimental one (Fig. 4A), with little ellipticity in the 210–240-nm region and a strong band at 196 nm. Differences between the calculated and experimental spectra are small and could be caused by variations in the consensus sequences in dB (Fig. 1) and the presence of nonrepetitive regions and additional GQQ sequences. It seems that the consensus sequences in dB adopt type I and type II β -turn conformations, like the model peptides. The temperature behavior of the CD spectrum of dB reflects the reversible destabilization of β -turn structure (van Dijk et al., 1997). HMW Bx6 and HMW Bx7 have a very similar temperature behavior, indicating that the repetitive domains of these proteins and dB behave similarly and suggesting that their structures are similar.

Fig. 5. Second derivative FTIR spectrum of dB in the dry state. The absorption spectrum is given in Figure 3C.

The CD spectra of HMW Bx6 and Bx7 have a higher amplitude than that for dB; in addition, a red-shift of the low wavelength minimum from 196 to 205 nm is observed. These differences are most probably caused by the presence of the N- and C-terminal domains of the HMW proteins, which are predicted to have a mixed α -helix/ β -sheet conformation (Shewry et al., 1994). Because α -helix and β -sheet structures have negative ellipticities in the 205–220-nm region, with a much higher absorption coefficient than β -turn structures (Perczel & Hollosi, 1996; Woody, 1996), even a relatively small amount of helix or sheet would disturb the β -turn spectrum of the repetitive domain. This interpretation is supported by the findings of Field et al. (1987), who recorded CD spectra of HMW Bx7 that are very similar to ours. When guanidine hydrochloride was added to destroy the structure of the N- and C-terminal domains, CD spectra were obtained that are very similar to that of dB (Field et al., 1987). Viscosimetric measurements also indicated that HMW Bx7 was still structured in the presence of guanidine hydrochloride, compatible with the presence of β -turn structure.

Structural changes upon drying

The low hydration state of HMW proteins was mimicked by drying the proteins on different surfaces (CaF₂ or quartz). FTIR and CD data indicate that this results in an increased β -turn stability and a type II β -turn character. In solution, both type I and type II β -turns are present; apparently, the type I β -turns are no longer present or are strongly reduced in number in the dry protein. They might have been converted to random coil structures, but second derivative analysis shows no prominent corresponding amide I band at 1,650 cm⁻¹. Alternatively, the type I could have been converted to type II β -turns. This is supported by the position of the amide I band at 1,638 cm⁻¹, exactly where it was observed for cyclo-[PGQGQPGQGQ], which contains only type II β -turns (van Dijk et al., 1997). The dry repetitive domain probably consists of a continuous series of these turns, which could organize into a so-called β -spiral, similar to that of elastin (Urry, 1993). The presence of this unusual supersecondary structure has been suggested previously from different studies (e.g., Tatham et al., 1984; Miles et al., 1991). Our hypothesis of the conversion of type I to type II β -turns is compatible with these observations. Pézolet et al. (1992)

recorded FTIR spectra of whole gluten in solution and in the dry state. They observed a band at 1,632 cm⁻¹, compatible with β -sheet structure, which was more prominent in the dry state than in solution, suggesting that the drying process results in β -sheet formation. The amide I band of dB in the dry state contained a weak band at 1,630 cm⁻¹ that is compatible with some formation of β -sheet structures, but not nearly as prominent as observed by Pézolet et al. (1992). Because HMW proteins are only one of the components of gluten, the β -sheet structures observed by Pézolet et al. (1992) are not necessarily related to the HMW proteins.

Two forms of the repetitive domain in dB

Figure 1 shows that dB is predicted to consist of a nearly continuous sequence of β -turns. There are only three regions where competing turn structures are predicted: residues 25–39, 46–60, and 84–97. In the first two regions, the turn at SPQQ overlaps turns at both its N- and C-terminal side, thus the probability of SPQQ occurring is very low. In the third region, a leucine insertion occurs at position 90, removing the overlap at the C-terminal side with the following turn. The SPQQ-turn consensus changes to SSQQL, which, however, has comparable probability as calculated according to Wilmot and Thornton (1988). Because the YPTS- and SSQQL-turn have comparable stabilities (van Dijk et al., 1997), both will occur. Formation of the SSQQL-turn is coupled to isomerization from *trans* to *cis* of the YPTS-proline (van Dijk et al., 1997); it disturbs the regular β -turn structure in dB and splits the protein into two parts. This might explain the two bands of dB that often occur on SDS-PAGE. One band would represent dB with the SSQQL-turn, the other one dB without the SSQQL-turn. Proline *trans/cis* isomerization in the YPTS sequence is, in this way, correlated to the appearance of the two bands. Because *trans/cis* isomerization is relatively insensitive to denaturants, it explains the presence of the two bands even in the presence of SDS and urea. Experiments to remove the leucine 90 are currently in progress to test the hypothesis. There is another well-documented example where a small disturbance of the repetitive structure results in a change in SDS-PAGE migration. Goldsbrough et al. (1989) exchanged parts of the repetitive domain between HMW By10 and HMW By12; they showed that the exchange of a sequence containing only six amino acid differences between the proteins causes a change in

SDS-PAGE mobility. Two mutations occur in a region similar to the regions with overlapping β -turns in dB (at residues 25–39, 46–60, and 84–97; Fig. 1). In By12, the mutations cause a strong reduction of the probability of occurrence of the overlapping β -turns at the N- and C-terminus of the SPQQ β -turn; in By10, this is not the case. In By12, the SPQQ β -turn will probably be formed, disturbing the regular β -turn structure that occurs in By10 and leading to a changed migration on SDS-PAGE; this is similar to the situation in dB.

Two bands have been seen on gels for a variety of HMW proteins, but usually no reference is made to the phenomenon (Shewry et al. 1984, 1992; Khelifi & Branlard, 1990; Doyen et al. 1994; Hou et al. 1996). Examples include the HMW Dx5, as expected, because dB was derived from this protein. We examined the repetitive domain of the sequenced HMW proteins (Ax1, Ax2, Bx7, Dx2, Dx5, By9, Dy10, Dy12) and found sequences that are likely to show the same type of disturbance of the regular β -turn structure as described above; each protein contains 1–3 of these regions, which always show an increased SPQQ turn probability relative to that of the preceding YPTS turn. Details of the analysis will be presented elsewhere. The two-band phenomenon seems to be common for HMW proteins and may reflect lack of homogeneity in the β -turn structure of the repetitive domain. The relative intensities of the bands differ between preparations for reasons that are not yet understood.

Elastic properties of the HMW repetitive domain

HMW proteins are essential to provide dough with visco-elastic properties; they are incorporated in a large, crosslinked protein (glutenin) network. Protein hydration in dough is relatively low and the β -spiral structure is probably present. Part of the observed elastic properties may reside in the β -spiral of HMW protein molecules in the network; this spiral structure can be described as a strongly hydrogen bonded network of β -turns. Hydrogen bonds not only stabilize turns, but probably also occur between side chains of different turns (van Dijk et al., 1997). Reformation of this hydrogen bonding network after, e.g., mechanical disruption could play a role in the visco-elastic properties that HMW proteins provide to dough. The mechanism differs strongly from that of elastin, in which hydration/dehydration of hydrophobic residues are the driving force for elasticity (Urry, 1993). The HMW repetitive domain and elastin therefore use the same structure for elasticity, but the driving force is completely different. This conclusion is compatible with the results of Belton et al. (1994), who concluded from NMR ^1H and ^2H relaxation times of elastin and HMW proteins that their mechanisms for elasticity must be different.

Materials and methods

Materials

Proteins were expressed in *Escherichia coli* strain SG13009 (pREP4). The pREP4 plasmid provides the cell with kanamycin resistance and high levels of the lac-repressor. The plasmid pUC9D5, described by Anderson et al. (1989) was a kind gift of P.R. Shewry, Long Ashton Research Station, Long Ashton, England. Ni^{2+} -agarose, the pQE60 and pREP4 vectors, and glass beads were obtained from QIAGEN; restriction enzymes, alkaline phosphatase, T4 DNA ligase, Klenow fragment of DNA polymerase, and IPTG were obtained from Boehringer. Tryptone and yeast extract

were obtained from DIFCO. Oligonucleotide primers were synthesized by Eurosequence BV, Groningen, Netherlands. Other chemicals were from Merck and were analytical grade.

Subcloning of a part of the repetitive domain of HMW Dx5 in pSk⁻ bluescript vector

The plasmid pUC9D5, containing the gene coding for HMW Dx5, was used to subclone a part of the repetitive domain using PCR with 5'-CTCAGATCTCCAGGCCAAGCTTCTCCGC-3' as the forward primer and 5'-CAGGGATCCTTGTGACCTTGACCGAGCTGC-3' as the reverse primer; priming regions are specified in Results. The nucleotides given in italics do not hybridize to the DNA and serve to introduce restriction sites (see Results); the nucleotides in bold indicate mismatches and introduce silent mutations. PCR was performed using 1 U *Taq* polymerase in 10 mM Tris.HCl, pH 8.3, 1.5 mM MgCl_2 , 50 mM KCl, 125 μM of each dATP, dCTP, dGTP, and dTTP, 0.01 $\mu\text{g}/\mu\text{L}$ of each primer, and 0.001 $\mu\text{g}/\mu\text{L}$ vector DNA. A 6-min cycle was used, consisting of 2 min at 92°C, 2 min at 60°C, and 2 min at 72°C, which was repeated 32 times. The PCR fragments were ligated in the T-tailed *EcoR* V site of pSk⁻ as described by Holton and Graham (1991); the ligation products were transformed to *E. coli* JM101 using the calcium chloride method (Sambrook et al., 1989). Positive colonies were identified using the β -galactosidase screening (Sambrook et al., 1989). The correct sequence of the cloned PCR fragments was verified by DNA sequencing of both strands using standard methods. The resulting plasmid was named pSkdB.

Construction of expression vectors

The dB was cloned in the expression vector pQE60; pSkdB was digested with *Bam*H I and *Bgl* II in the presence of alkaline phosphatase and pQE60 was digested with *Bam*H I. The fragments were purified from agarose gels and ligated; because *Bgl* II and *Bam*H I generate compatible ends, the dB fragment can have two orientations after ligation. The vectors with the correct orientation were identified by restriction analysis using the *Hind* III which has a site close to the 5' end of dB and one in the plasmid directly after the construct; the resulting vector was named pQE60dB.

Expression and purification of dB

E. coli SG13009 (pREP4) was transformed with pQE60dB and grown overnight in tubes at 37°C on TY medium (5 g/L yeast extract, 10 g/L tryptone, 5 g/L NaCl) containing 50 mg/mL ampicillin and 10 mg/mL kanamycin. Cultures were transferred to 1 L fresh TY-medium in flasks, induced with IPTG (1 mM) at $\text{OD}_{600} = 0.6$ and grown overnight at 37°C. Cells were harvested by centrifugation ($5,000 \times g$, 10 min, 4°C), resuspended in 20 mM NaPi , pH 8.0, to 10 mL/g cells wet weight, and disrupted using sonication or a French pressure cell ($>10,000$ psi). The supernatant after centrifugation ($10,000 \times g$; 1 h, 4°C) was collected and incubated for 1 h on a shaker with 1 mL Ni-agarose, equilibrated in the same buffer, per 50 mL supernatant. The suspension was poured into a column that was eluted with 80 mL buffer, followed by elution with 10 mL buffer portions containing 25, 50, and 100 mM imidazole; the protein eluted in the last step. It was dialyzed against 3×3 L 20 mM NaPi , pH 8.0, to remove imidazole. The isolation yielded 40 mg dB per liter of culture.

Purification of HMW Bx7

The HMW Bx6 and HMW Bx7 protein were purified at room temperature from Galahad 6 or Galahad 7 flour, defatted with petroleum ether; 5 g of flour were extracted for 10 min with 50 mL buffer containing 60 mM NaPi, pH 7.6, and 400 mM NaCl. The supernatant obtained after centrifugation ($400 \times g$, 10 min) was recentrifuged ($4,000 \times g$, 10 min); the HMW Bx7-containing pellets from both steps were collected, washed repeatedly with water, and then extracted with 50 mL 70% ethanol. The pellets were collected after centrifugation, freeze-dried, and suspended in 40 mL 50% isopropanol/1% DTT; after incubation (55 °C, 30 min) under stirring, the suspension was centrifuged as described before. Isopropanol was added to the supernatant to a final concentration of 60% and kept at 4 °C (60 min), after which it was centrifuged ($4,000 \times g$, 15 min). The isopropanol extraction was repeated, but with different incubation times in the first step (150 min at room temperature followed by 150 min at 55 °C). The HMW-containing pellet was washed twice with water and resuspended in 40 mL 50% degassed isopropanol/1% DTT and stirred for 2.5 h at room temperature and 2.5 h at 55 °C. The supernatant after centrifugation ($4,000 \times g$, 15 min) was brought to 60% isopropanol and stored at 4 °C for 1 h; the pellet, obtained after centrifugation ($4,000 \times g$, 20 min), contained the purified HMW proteins.

CD and FTIR

CD and FTIR data were recorded and processed as described in van Dijk et al. (1996). In addition to solution conditions, proteins were studied in the dry state. Proteins were dialyzed against 10 mM HAc, pH 3.6; 100 μ L of protein solution (0.2–2.0 mg/mL) was spread on a surface (quartz for CD, CaF₂ for FTIR) and dried overnight in air; the protein formed a transparent but rough film. CD and FTIR data were then recorded as for the liquid samples. The CD spectra of the precipitated samples were recorded at several orientations of the sample to exclude any orientation effects that might have occurred during drying. No angle-dependence was observed. Spectra of dry proteins were not normalized because the proteins are inhomogeneously distributed on the quartz surface. HMW Bx6-spectra were not normalized because its amino acid sequence is unknown; this prevents the usual protein quantitation via quantitative amino acid analysis. The amounts of HMW Bx6 were too low for accurate weight determination.

Mass spectrometry

Electrospray mass spectra of dB were obtained as described (van Dijk et al., 1997). Protein samples were dialyzed against water to remove salts, freeze dried, and redissolved in a 50/50 mixture water/acetonitrile containing 0.1% trifluoroacetic acid.

Other procedures

PAGE was performed according to Laemmli (1970); gels were stained in a solution containing 0.1% Coomassie brilliant blue and 12.5% trichloroacetic acid. Transverse urea 10% SDS-polyacrylamide gels were prepared using the Bio-Rad gradient delivery system and contained a linear gradient from 0 to 8 M urea. Stock solution were as described (Laemmli, 1970), containing 0 or 8 M urea. Protein concentrations were determined via quantitative

amino acid analysis by Eurosequence B.V., Groningen, Netherlands and are accurate within 10%.

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