High-Level Expression of a Wheat LMW Glutenin Subunit Using a Baculovirus System

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A wheat gene encoding a low molecular weight (LMW) subunit of glutenin was expressed in cultured insect cells using a baculovirus vector. The LMW subunit accounted for 25–30% of the extracted protein; 30–50 mg was readily purified from 1 L of culture by solubility in 50% (v/v) aqueous propan-1-ol followed by salt precipitation. The plant signal sequence was apparently cleaved, and the protein accumulated as disulfide-bonded polymers in dense deposits within the lumen of the rough endoplasmic reticulum. The expressed protein was less soluble than LMW subunits prepared from wheat, and over 90% was irreversibly absorbed to a column of CM-cellulose. However, the protein eluted from the column did show more typical solubility properties and could be refolded to give a mixture of monomers and disulfide-stabilized polymers using slow dialysis or rapid dilution methods. Circular dichroism spectroscopy of the refolded protein showed secondary structure contents similar to LMW subunits purified from wheat.

INTRODUCTION

The low molecular weight (LMW) subunits of wheat glutenin account for about 40% of the total gluten fraction and for 60–70% of the glutenins (Seilmeier et al., 1991). They consist of a number of individual components which are classified into three groups (B–D) on the basis of their behavior on SDS–PAGE and 2-D IEF/SDS–PAGE (Payne and Corfield, 1979; Jackson et al., 1983). Although the precise relationships of these three groups are still not established, preliminary studies indicate that the C group consists mainly of proteins related to α-type and γ-type gliadins (Tao and Kasarda, 1989; Lew et al., 1992), while the D group may be most closely related to ω-gliadins (Payne et al., 1988). In contrast, the B group appears to be structurally distinct (although related more closely to the α-type and γ-type gliadins than to the HMW glutenin subunits and ω-gliadins) and, thus, can be considered to comprise true LMW subunits.

Detailed characterization of LMW subunits has been limited by the difficulty in preparing pure components in reasonable quantities, previously reported studies being carried out on mixed fractions (Shewry et al., 1983; Tatham et al., 1987) or on small amounts of protein transferred from 2-D gels (Tao and Kasarda, 1989) or prepared by RP-HPLC (Lew et al., 1992). However, the isolation of cDNA (Okita et al., 1985) and genomic (Colot et al., 1989; Cassidy and Dvorak, 1991) clones has provided the complete amino acid sequences of B-type LMW subunits and has also opened the way to studying their structure and functionality using expression in heterologous systems and protein engineering.

We have therefore established a high level expression system for a LMW subunit, using a baculovirus-derived vector in cultured insect cells. This system was selected because of its high expression levels (up to 50% of the total protein) (Emery and Bishop, 1987) and its suitability for expressing insoluble proteins (the polyhedrin protein produced by the wild-type virus also being insoluble). In addition, it was considered important to use a eukaryotic system to ensure that authentic post-translational processing (including disulfide bond formation) occurred. The results of these studies, including the characterization of the expressed protein, are discussed here.

MATERIALS AND METHODS

Colot et al. (1989) described the isolation of the clone LMWG-1D1 containing an LMW glutenin subunit gene from chromosome 1D of bread wheat. A 950-bp fragment containing the complete gene cloned into the *Sma*I site of pUC19 (named pLMW) was obtained from Dr. V. Colot (IPSR Cambridge Laboratory, Norwich, U.K.). This fragment was excised with *Eco*RI and *Bam*HI and subcloned into the baculovirus transfer vector pAcYM1, after cutting with *Bam*HI. Putative positive clones were identified by colony hybridization. The orientation of the insert was confirmed by restriction enzyme digestion, and the sequences of the 5′ and 3′ junctions were determined using the dideoxy method. The resulting recombinant transfer vector (pAcLMW1) was used to coinfect cultured cells of *Spodoptera frugiperda* with wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV). Potential recombinants were identified by visual screening for plaques lacking polyhedrin. Virus stocks from 12 purified polyhedrin-minus plaques were used to reinfect cultured cells, which were harvested after 4 days and extracted with RIPA buffer [50 mM Tris-HCl, pH 7.4, containing 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 150 mM NaCl, 10 mM EDTA, and 0.1% (w/v) SDS].

Samples for SDS–PAGE were dissolved in 10 mM Tris-HCl, pH 6.9, with 10% (w/v) SDS, 25% (v/v) glycerol, and 0.02% (w/v) Bromophenol Blue (sample buffer). SDS–PAGE was carried out using 10% Laemmli (1970) gels and proteins detected either by staining with Coomassie BBR250 or by western blotting using a semidry electroblotting apparatus and a wide specificity polyclonal antiserum raised against γ-gliadin (Festenstein et al., 1987) followed by goat anti-rabbit antibody conjugated to alkaline

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phosphatase. Ion-exchange chromatography on CM-cellulose in 4 M urea at pH 4.6 was carried out as described by Shewry et al. (1981).

For immunogold labeling, cells were fixed for 1 h in 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5. After being washed in NPV–PBS [0.82% (w/v) NaCl, 0.2% (w/v) KCl, 0.02% KH_2PO_4, 0.114% (w/v) Na_2HPO_4, pH 7.3] at pH 7.5, the cells were dehydrated in a series of ethanol solutions (50%, 70%, 90% v/v) for 5 min each and then transferred to LR-White resin for 3–4 h before being embedded in gelatin capsules with fresh LR-White resin. Polymerization was at 50 °C overnight, after which the blocks were sectioned and mounted on 400-mesh nickel grids. Sections were incubated for 5 min in blocking solution [0.1% (w/v) bovine serum albumin in NVS-PBS] and then floated on a 1:200 dilution of polyclonal antiserum in blocking solution for 1 h. After being washed three times in blocking solution they were floated on a 1:20 dilution of secondary antibody (goat anti-rabbit conjugated to 10-nm gold particles) and then washed three times in blocking solution, one time in NPV–PBS, and one time in sterile water. They were then stained in uranyl acetate and lead citrate for 20 min each.

In vitro refolding was carried out using two methods: dialysis and rapid dilution. For the dialysis method, the protein was dissolved at 1 mg/mL in 0.1 M Tris-HCl buffer, pH 8.0, containing 8 M urea, 0.1 mM dithiothreitol, 1 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride (PMSF) and incubated at room temperature for 2 h to allow reduction. The pH was then reduced to 3 by the addition of acetic acid and the solution diluted to 0.5 mg/mL before dialysis into 0.1 M acetic acid at 4 °C. The solution was then diluted to 0.1 mg/mL and dialyzed for 24 h against refolding buffer (0.1 M Tris-HCl, pH 8.0, with 1 mM EDTA, 0.5 M dimethylformamide, 2 M urea, 3 mM reduced glutathione, 0.3 mM oxidised glutathione, and 0.1 mM PMSF). The protein was then dialyzed into 0.1 M acetic acid and lyophilized. In the rapid dilution method the reduced protein in 0.1 M acetic acid was injected directly into the folding buffer to give a final concentration of 10 μg/mL. After 1 h of incubation at 20 °C, this solution was dialyzed against 0.1 M acetic acid and lyophilized. Samples for SDS–PAGE were removed before dialysis into 0.1 M acetic acid and iodoacetamide added to 100 mM to block free thiol groups and thus minimize any rearrangements. They were then dialyzed against 0.1 M acetic acid and lyophilized.

A total LMW subunit fraction was prepared from wheat cv. Chinese Spring. Defatted flour was extracted with 1 M NaCl to remove albumins and globulins, washed with water, extracted with 50% (v/v) aqueous propan-1-ol and 2% (v/v) acetic acid (10:1 solvent/flour) for 3 × 1 h at room temperature, and centrifuged and the supernatant dialyzed against distilled water and lyophilized. The prolamin mixture was dissolved in 8 M urea containing 1% (v/v) acetic acid and chromatographed on Sephacryl S300 in the same buffer. The excluded peak, corresponding to polymers consisting of LMW glutenin subunits, was dialyzed against distilled water and freeze-dried. The proteins were reduced and alkylated with 4-vinylpyridine (Friedman et al., 1970).

Circular dichroism (CD) spectroscopy was performed on a JASCO J600 spectropolarimeter at 20 °C. The spectra are solvent and baseline corrected. The concentration of the Chinese Spring LMW subunits was determined by weight, corrected for the protein content (% N × 5.75) determined by mass spectroscopy (Europa Scientific Tracermass). For the expressed protein 1 mg was dissolved in 1 mL of TFE, magnetically stirred for 1 h, and centrifuged and the supernatant used for spectroscopy. The solution concentration was estimated by comparison of the absorbance at 280 nm with that of the Chinese Spring LMW subunits. The results are expressed as $\Delta\epsilon$ (cm^{-1} mol^{-1}).

RESULTS AND DISCUSSION

Isolation of Recombinant Baculovirus. A LMW subunit gene from chromosome 1D of bread wheat (Colot et al., 1989) was selected for expression. This encodes a protein of 307 residues including a putative signal peptide sequence of 20 residues [see Lew et al. (1992)]. The gene was inserted into a "shuttle" transfer vector and used to coinfect cultured insect cells with wild-type baculovirus. Visual screening identified plaques not expressing polyhedrin (the protein produced by the wild-type baculovirus), and 12 of these were used to prepare virus stocks. The stocks were then used to reinfect cultured insect cells, which were harvested after 4 days and extracted with buffer containing SDS and Triton X-100. SDS–PAGE of these extracts under reducing conditions showed the presence of bands which corresponded in M_r to LMW glutenin and reacted on western blotting (results not shown) with a wide specificity prolamin antiserum raised against wheat γ-gliadin (Festenstein et al., 1987). One recombinant virus, which was named AcLMW1, was used to produce a high-titer stock for detailed studies.

Processing and Deposition of LMW Subunit. In wheat, the gluten proteins, including the LMW subunits of glutenin, are cotranslationally transported into the ER, with the cleavage of a signal peptide. From there, they may be transported via the Golgi apparatus to the vacuole to form protein bodies or may remain in the ER lumen, which becomes distended to form a second population of protein bodies of ER origin. It is not clear at present whether these two populations of protein bodies contain different groups or proportions of gluten proteins or whether their formation may relate to the age or stage of development of the tissue [see Shewry (1993)]. The only post-translational processing that occurs is disulfide bond formation, either intrachain disulfide bonds in the monomeric α-gliadins and γ-gliadins or interchain and intrachain disulfide bonds in the glutenin subunits which are present in high M_r polymers. These bonds are presumably formed in the lumen of the ER, where the enzyme protein disulfide isomerase is present (Freedman, 1989).

It was decided, therefore, to determine whether the same pathway of processing and deposition occurred when the LMW subunit was expressed in the insect cells. To determine whether the protein was present in polymers, a log-phase culture of insect cells was infected with a high-titer stock of AcLMW1 and then harvested and extracted with RIPA lysis buffer. The extracts were then boiled with SDS–PAGE sample buffer, with and without 10% (v/v) 2-mercaptoethanol. SDS–PAGE showed a major band corresponding to the LMW glutenin (identified by its M_r and by western blotting) in the reduced sample only (indicated by an arrow in Figure 1a), where it accounted for about 25–30% of the total extracted protein. This band was not observed with the unreduced sample (Figure 1b), which contained a large amount of insoluble material that was removed by centrifugation before electrophoresis.

Previous studies of LMW glutenin subunits from wheat cv. Chinese Spring showed that a number of individual subunits had blocked N termini (Tao and Kasarda, 1989). To determine whether the protein synthesized in cultured insect cells was also blocked, two separate samples of protein, extracted from SDS–PAGE separations or purified by ion-exchange chromatography (see below), were subjected to automated Edman degradation using an Applied Biosystems Model 471 pulsed liquid-phase sequencer. In both cases, only traces of amino acids were released from each cycle, indicating that the protein was indeed N-terminally blocked. Because of this, it was not possible to confirm that signal peptide cleavage had occurred. However, immunogold labeling of tissue sections showed that the protein was concentrated within dense cytoplasmic inclusions which appeared to be derived from the rough ER (Figure 2).

Since cotranslational cleavage of the signal peptide occurs as proteins are transported into the lumen of the

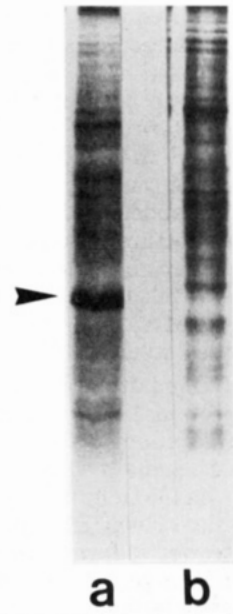

Figure 1. SDS–PAGE of total extracts of cultured insect cells infected with the recombinant baculovirus Ac LMW1 expressing the LMW subunit protein. Samples were extracted without reducing agent and separated after reduction with β-mercaptoethanol (track a) and without reduction (track b). The arrow in track a indicates the LMW subunit protein.

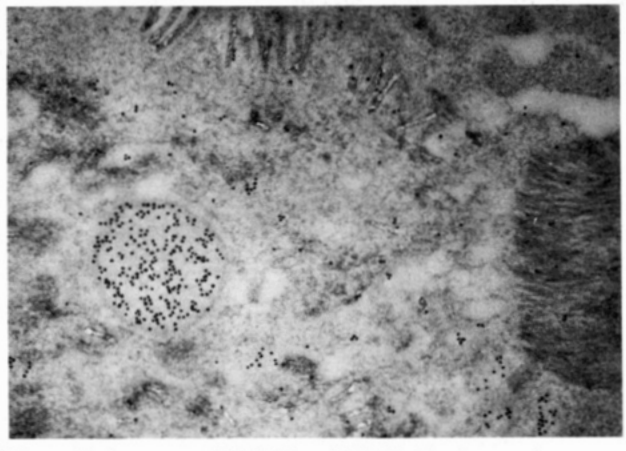

Figure 2. Immunogold labeling of LMW subunit protein present in cultured insect cells infected with the recombinant baculovirus AcLMW1, using an antibody to the related γ-gliadins (Festenstein et al., 1987) of wheat. Note that the LMW subunit protein is present in dense inclusions. Magnification 53 000×. (This figure is reproduced at 60% of the original.)

ER, it is probable that such cleavage also took place with the LMW subunit expressed in insect cells. More detailed studies, for example, by mass spectroscopy of peptide fragments, would be required to confirm this.

The results of these studies indicate, therefore, that the LMW subunit synthesized in the cultured cells is transported into the lumen of the ER, where it is assembled into high M_r polymers which accumulate to give dense deposits. This is similar to the formation of ER-derived protein bodies in the developing endosperm of wheat.

Purification and Characterization of LMW Subunit. Suspension cultures of infected cells were disrupted by freeze/thaw (2–3×) or sonication (6 × 30 s) and extracted with 50% (v/v) aqueous propan-1-ol with 2% (v/v) 2-mercaptoethanol at 60 °C. Preliminary experiments demonstrated that extraction was less efficient in the absence of 2-mercaptoethanol or at lower temperatures. A temperature of 60 °C would not be expected to affect the protein structure, as prolamins have been shown to

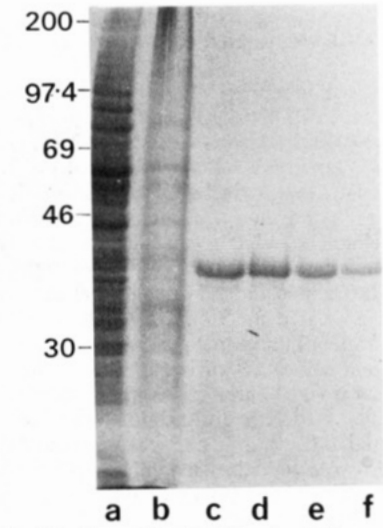

Figure 3. Purification of the LMW subunit from cultured insect cells infected with the recombinant baculovirus AcLMW1: (a) total cell proteins; (b) total cell proteins after extraction with 50% (v/v) aqueous propan-2-ol + 2% (v/v) 2-mercaptoethanol at 60 °C; (c–f) increasing dilutions of the LMW subunit protein extracted with the above solvent and then precipitated by the addition of two volumes of 1.5 M NaCl followed by standing overnight at 4 °C. The positions of M_r marker proteins are indicated; they are myosin (200 000), phosphorylase *b* (97 400), bovine serum albumin (69 000), ovalbumin (46 000), and carbonic anhydrase (30 000).

undergo fully reversible conformational transitions at temperatures up to 80 °C (Tatham and Shewry, 1985). The protein was readily precipitated from the extract by adding two volumes of aqueous 1.5 M NaCl and allowing it to stand for 24 h at 4 °C. SDS–PAGE showed a single major band (Figure 3) which accounted for over 95% of the preparation. The total yield varied from about 30 to 50 mg/L of culture.

Although the protein preparation was sufficiently pure for detailed conformational studies, it was found to differ considerably in its solubility properties from LMW subunit fractions prepared from grain. For example, it would not redissolve in 50% (v/v) aqueous propan-1-ol or 0.1 M acetic acid, even in the presence of 2-mercaptoethanol, while fractions purified from grain readily dissolve in these solvents. Because of this insolubility it was not possible to carry out analyses of protein conformation.

Anomalous solubility of expressed proteins may result from incorrect folding or from the formation of complexes with other compounds which may be removed by ion-exchange chromatography (Creighton and Darby, 1990). It was therefore decided to purify the LMW subunit to homogeneity using ion-exchange chromatography on CM-cellulose and then to refold it *in vitro*.

The expressed protein was initially dissolved in 8 M urea with 4% (v/v) 2-mercaptoethanol. It was then diluted to 4 M urea in 10 mM glycine/acetate buffer, pH 4.6, and applied to a column of CM-cellulose equilibrated in the same buffer. Elution was with a linear gradient of 0–0.4 M NaCl. The LMW glutenin subunit was eluted as a single peak between 0.08 and 0.1 M NaCl (as expected from the properties of LMW subunits extracted from wheat grain) and was pure as determined by SDS–PAGE. However, the yield was very poor for two reasons. Only about half of the extracted subunit could be dissolved and applied to the column, and only 10–20% of this was eluted. Thus, the total yield of purified subunit from 1 L of culture was only 1–3 mg. The remaining LMW subunit was not eluted even by 1 M NaCl, indicating that it was very tightly bound.

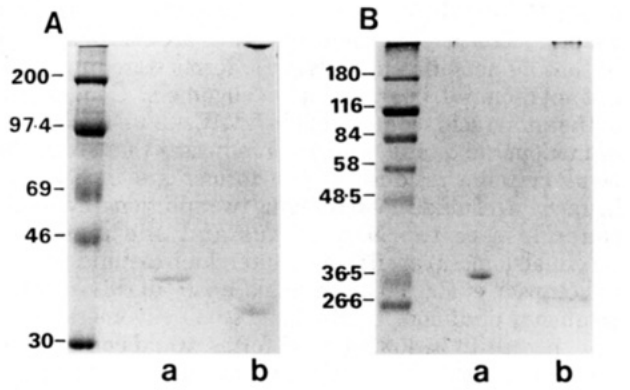

Figure 4. SDS–PAGE of LMW subunit under reducing conditions (tracks a) and after refolding (tracks b) using the dialysis (part A) or rapid dilution (part B) procedures. The positions of M_r marker proteins are indicated, they are myosin (200 000), phosphorylase *b* (97 400), bovine serum albumin (69 000), ovalbumin (46 000), and carbonic anhydrase (30 000) in part A and α_2-macroglobulin (180 000), β-galactosidase (116 000), fructose 6-phosphate kinase (84 000), pyruvate kinase (58 000), fumarase (48 5000), lactate dehydrogenase (36 500), and triose phosphate isomerase (26 600) in part B.

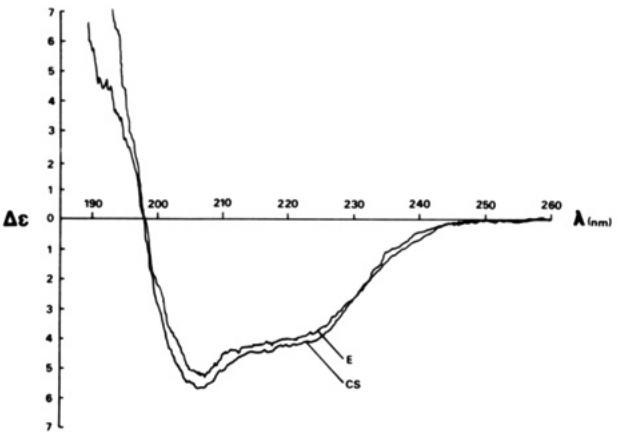

Figure 5. Far-UV circular dichroism spectroscopy of the expressed LMW subunit and a total LMW subunit fraction purified from cv. Chinese Spring. The Chinese Spring (CS) spectrum was determined using a solution of 1 mg/mL. When the expressed protein (E) was dissolved at a similar concentration the spectral intensity was very low, presumably because much of the protein was insoluble. The spectrum presented has therefore been adjusted to represent 1 mg/mL of dissolved protein, based on comparison of the absorption with that of the Chinese Spring fraction.

The recombinant protein eluted from the CM-cellulose column was more readily soluble, being comparable in its properties to fractions purified from grain. It was therefore refolded *in vitro*, using either dialysis or rapid dilution into refolding buffer containing 2 M urea and 0.5 M dimethylformamide to maintain solubility and reduce subunit interactions and a mixture of reduced and oxidized glutathione as an oxido-shuffling system. The two methods gave essentially identical results (Figure 4). Whereas the reduced protein migrated as a single sharp band, the refolded protein showed the presence of monomeric and polymeric forms. The monomeric refolded protein migrated faster than the reduced protein, which was presumably due to the presence of interchain disulfide bonds resulting in a more compact conformation. In contrast, the polymeric protein was of very high M_r and failed to enter the separating gel. Neither method gave appreciable amounts of oligomers (e.g., dimers, trimers, or tetramers). It was not possible to determine the precise proportions of monomeric and polymeric proteins in the refolded preparations as much of the polymeric protein failed to enter the separating gel. However, it is clear from Figure 4 that the proportion of monomeric protein was low.

An aliquot of LMW subunit protein refolded using the rapid dilution method was used for CD spectroscopy, being compared with a reduced and pyridylethylated total LMW subunit fraction from wheat cv. Chinese Spring (the cultivar that was used to isolate the LMWG-1D1 clone). Trifluoroethanol (TFE) was selected as a solvent because its low dielectric constant may mimic the environment of the protein within the protein bodies of the seed, where the water content is low and hydrogen bonding and hydrophobic interactions are favored.

The spectra of the two fractions (Figure 5) were essentially similar to each other and to those previously reported for LMW subunit fractions from other cultivars of wheat (Tatham et al., 1987), with negative maxima at about 207 and 221–223 nm. These spectra indicate high contents of α-helix, which can be calculated by deconvolution (Chen et al., 1972) as about 32% for the expressed protein and 35% for the total fraction. The calculated contents of β-sheet were lower, about 24% and 22%, respectively. Similar contents of α-helix (32–37%) and β-sheet (18–24%) were previously determined by CD spectroscopy of mixed LMW subunit fractions from four cultivars (Tatham et al., 1987). In addition, secondary structure prediction using the method of Chou and Fasman (1978) and the sequence of a closely related LMW subunit (Okita et al., 1985) gave about 37% α-helix and 15% β-sheet (Tatham et al., 1987). However, the spectral intensity of the refolded LMW subunit fraction was much less than that of the fraction purified from wheat (see legend to Figure 5), indicating that the refolded protein only accounted for about 15% of the total weight.

It is clear, therefore, that most, if not all, of the LMW subunit expressed in cultured insect cells was incorrectly folded. However, a small proportion of this was successfully refolded *in vitro* to give protein with similar contents of α-helix and β-sheet to that purified from wheat.

GENERAL DISCUSSION

We have obtained high level expression of a wheat LMW subunit in cultured insect cells using a *Baculovirus* vector. This expression system has not been used previously for cereal prolamins, but the levels obtained here (about 20–30% of the extracted proteins) compare well with those reported for other proteins (up to 50% of total cell protein). Similarly, although there have been no other reports of LMW subunit expression in heterologous systems, the yields (30–50 mg/L of culture) are appreciably higher than those reported for α-gliadin and γ-gliadin in yeast (up to 4 mg/L) (Neill et al., 1987; Blechl et al., 1992; Pratt et al., 1991; Scheets and Hedgcoth, 1989) and comparable with those obtained for C hordein (30 mg/L) and wheat HMW glutenin subunits in *E. coli* (Galili, 1989; Tamas et al., 1994).

In addition to providing high expression levels, the *Baculovirus*/insect cell culture system is particularly suited to the expression of proteins from eukaryotes because the eukaryotic nature of the insect cells should facilitate the production of proteins which are correctly folded and processed. In wheat, the LMW subunits undergo signal peptide cleavage on the rough ER and are then folded and assembled into disulfide-bonded polymers within the ER lumen. Subsequently, they may remain within the lumen or be transported via the Golgi apparatus to the vacuole. Signal peptide cleavage probably also occurs in the cultured insect cells, and the protein accumulates in dense deposits

which appear to be derived from the rough ER. The synthesis of interchain disulfide bonds also takes place to form high M_r polymers, and it is probable that some intrachain disulfide bonds are also synthesized. However, lack of knowledge of the precise disulfide bonding patterns of the proteins deposited in wheat means that it is not possible to conclude whether the disulfide bonds formed in the cultured insect cells are correct or incorrect.

There is, however, clear evidence from the solubility properties of the reduced LMW subunit, and from its tight binding to the CM-cellulose column, that a substantial proportion of the protein is incorrectly folded. This agrees with our own previous expression studies of a monomeric γ-gliadin in yeast (Pratt et al., 1991). In this case, the protein also had low solubility, was tightly absorbed to CM-cellulose, and was present as a range of disulfide-bonded oligomers and polymers as well as monomer. It is therefore probable that incorrect disulfide bonds were also formed with the LMW subunit.

Other reported studies of prolamin expression in heterologous systems have not, in general, included detailed characterization of the expressed protein, so it is difficult to conclude whether incorrect folding is a general phenomenon for all prolamin types. However, preliminary studies from our laboratory indicate that there is at least one exception. This is C hordein of barley, a representative of the S-poor group of prolamins which also include the ω-gliadins of wheat and ω-secalins of rye. C hordein expressed in *E. coli* using the pET 3d vector system was found to be correctly folded as demonstrated by its solubility, retention on RP-HPLC, and CD spectrum over a range of temperatures (Tamas et al., 1994). This may relate to the structure of C hordein which, together with other S-poor prolamins, differs from all other cereal prolamins in two respects. Firstly, it does not contain cysteine residues, and secondly, it consists almost entirely of repeated sequences, with only short nonrepetitive sequences at the N and C termini. We consider the latter feature to be most important, as cysteine-containing mutants of C hordein are also correctly folded and form interchain disulfide bonds (Tamas et al., 1994).

Repeated sequences are also present in other prolamins of wheat and barley, and in all cases these appear to adopt unusual secondary structures which are rich in β-turns and/or poly(L-proline) II-like structure. However, in the other prolamin groups (the S-rich prolamins including the α-gliadins, γ-gliadins, and LMW subunits of wheat, and the HMW prolamins including the HMW subunits of wheat) these sequences are accompanied by nonrepetitive domains which are largely globular (with α-helix and β-sheet structures) and also contain most of the cysteine residues involved in the formation of interchain and intrachain disulfide bonds. It is probable that misfolding of these globular domains is responsible for the unusual solubility properties of the expressed prolamins and may also lead to the formation of incorrect disulfide bonds. It is known that molecular chaperones play key roles in the folding of monomeric proteins and assembly of oligomeric proteins in other systems and that some chaperones (e.g., the immunoglobulin binding protein BiP) are located in the lumen of the ER (Gatenby, 1992; Gething and Sambrook, 1992). The failure to produce correctly folded prolamins by expression in heterologous eukaryotic systems may, therefore, relate to failure to interact with the heterologous chaperones or the presence of such chaperones in limiting amounts.

Some correctly folded subunit was produced by our *in vitro* refolding procedures, but this only accounted for about 15% of the protein eluted from the CM-cellulose column. The *in vitro* refolding results are also of interest in that monomeric and polymeric forms were produced, but not dimers, trimers, or higher oligomers. Comparison of the amino acid sequence of the LMW subunit with those of monomeric α- and γ-gliadins indicates that six of the eight cysteine residues in the former are involved in intrachain disulfide bonds, leaving two additional cysteines (one close to the N terminus and one in the C-terminal domain) available for interchain disulfide bonds (Thompson et al., 1993; Lew et al., 1992). If this is so, the frequency of dimers, trimers, and small oligomers would be expected to be low as these forms would contain free cysteines which are absent from prolamins synthesized *in planta*. Our failure to observe such oligomers when the protein was refolded *in vitro* is therefore consistent with this hypothesis, but the presence of appreciable amounts of monomers is not. It can only be proposed that the latter arose from incorrect disulfide bonding resulting in the formation of four rather than three intrachain disulfide bonds.

In conclusion, the studies reported here have succeeded to the extent that high levels of LMW protein have been produced, but with the major drawback that most, if not all, is incorrectly folded. Nevertheless, they do provide a sound basis for future studies, in which correctly folded protein could be produced by coexpression with chaperones or other proteins (e.g., peptidyl prolyl *cis,trans* isomerase) from wheat or *in vitro* refolding in the presence of such proteins.

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