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Identification of the Wheat Seed Protein CM3 as a Highly Active Emulsifier Using a Novel Functional Screen

SIMON M. GILBERT,*,† GARY R. BURNETT,‡ E. N. CLARE MILLS,‡ PETER S. BELTON,§ PETER R. SHEWRY,† AND ARTHUR S. TATHAM†

Long Ashton Research Station, Department of Agricultural Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF, U.K., Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, U.K., and School of Chemical Sciences, University of East Anglia, Norwich, NR4 7TJ, U.K.

Lyophilized albumin protein fractions were prepared from flour of four varieties of wheat: *Triticum aestivum* cvs. Mercia and Riband, *Triticum aestivum* var. *spelta*, and *Triticum turgidum* var. *durum* (Kamut). The dry powders were redissolved in sodium phosphate buffers at pH 3.0, 6.5, or 8.0 and at ionic strengths of 0.1 or 1.0 M to a concentration of 0.1% (w/v). Emulsions formed by sonication of protein solutions with *n*-hexadecane were aged at room temperature and separated into aqueous, interstitial, and interfacial phases. The distinct emulsion components were lyophilized and analyzed by RP-HPLC. A protein was observed to be preferentially located in the interfacial component and subsequently purified from a total albumin fraction and identified by N-terminal sequencing as CM3, an α-amylase inhibitor subunit. Measurement of the equilibrium surface tension of CM3 as a function of protein concentration demonstrated that it was at least as active as bovine β-lactoglobulin, an established protein emulsifier. Furthermore, measurement of the surface dilational elastic modulus at an air/water interface demonstrated the formation of a viscoelastic film, while fluorescence and FT-IR spectroscopic measurements on adsorbed and nonadsorbed CM3 suggest that the secondary structure is essentially unchanged upon adsorption to an oil/water interface. It is concluded that functional screening is a valid approach to identify novel protein emulsifiers in complex mixtures.

KEYWORDS: α-Amylase inhibitor; emulsifier; CM3; wheat

INTRODUCTION

The extent to which an emulsion may be stabilized in a food system is influenced by the composition and conformation of the constituent proteins and their interactions with other components. In addition, processing may impact the functional properties of constituent proteins (*1*). Protein solubility is widely regarded as an important characteristic of a functional protein, enabling it to interact with other ingredients including water (*2*). Moreover, for a protein to be a good emulsifier, it must have a combination of hydrophobic regions to absorb onto an oil/water interface, hydrophilic regions that may protrude into the aqueous phase and prevent coalescence by steric repulsion, a high charge density to allow electrostatic repulsion, and the ability to give good coverage and therefore provide mechanical strength (*3*).

There is a perceived need to identify and characterize functionally active plant proteins to provide alternatives to animal products. Crop plants are an obvious focus for this search because they are readily available with well-established systems for production, harvest, storage, and fractionation and enjoy widespread consumer acceptance. Wheat has been exploited for thousands of years (*4*) largely because of the gluten proteins, which confer viscoelastic properties that enable dough to be used for breadmaking and the production of a range of other foods. Consequently, the structures and properties of the gluten proteins have been studied in detail in order to determine the biochemical and molecular bases for their functional properties (*5*−*7*). In contrast, little work has been carried out on wheat proteins that are soluble in water or in dilute saline (0.5−1.0 M NaCl), although together they constitute up to 30% of the total grain nitrogen (*8*). Consequently, these proteins are essentially wasted when wheat flour is fractionated to give gluten and starch.

The albumin (water soluble) and globulin (salt soluble) protein fractions of wheat are complex mixtures (*5*), and the identification of novel functional proteins by purifying and testing individual components would be a formidable task. We have, therefore, utilized a novel strategy to identify wheat albumin proteins with functional properties by exploiting their inherent ability to stabilize emulsions as the basis for a functional screen.

* To whom correspondence should be addressed (telephone (44) 1275 549274; fax (44) 1275 394281; e-mail simon.gilbert@ bbsrc.ac.uk).
† University of Bristol.
‡ Institute of Food Research.
§ University of East Anglia.

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MATERIALS AND METHODS

Milling and Defatting. *T. aestivum* cvs. Mercia and Riband were obtained as seed, and the moisture content was adjusted to 15% w/w. After conditioning for 1 h, the grain was passed through a roller mill (Quadromat Junior, Brabender) and the white flour was separated from the bran. Kamut (*T. turgidum* var. *durum*) was obtained as seed, and wholemeal semolina was produced by passage through a cyclone mill equipped with a 0.12 mm sieve. Spelt (*T. aestivum* var. *spelta*) flour was obtained ready-milled from a commercial supplier. Each sample was treated with chloroform to yield 200 g of defatted flour.

Protein Extraction and Fractionation. Each defatted flour sample (200 g) was extracted once for 1 h at 21 °C with 1 L of 50 mM Tris-HCl buffer, pH 8.0, containing 1 M NaCl and 1 mM phenylmethylsulfonyl fluoride (PMSF) using gentle mechanical stirring. The resulting slurry was clarified by centrifugation at 10000*g* and 4 °C for 10 min, and the supernatant was transferred to dialysis tubing with a nominal molecular mass cutoff of 3500 Da. Following exhaustive dialysis against water, the supernatant (containing albumins) was separated from the pellet (containing globulins) and lyophilized.

Preparation of Emulsion Components. Stock solutions of each albumin fraction were prepared by dissolving the lyophilized extract in 10 mM sodium phosphate buffer to a final concentration of 1 mg mL^{-1}. Buffers were prepared at pH 3.0, 6.5, and 8.0 and at ionic strengths of 0.1 and 1.0 M (NaCl), giving six stock solutions for each albumin extract. Emulsions were prepared by adding 4.5 mL of *n*-hexadecane ($C_{16}H_{34}$) to 7.5 mL of stock solution in a 15 mL centrifuge tube and sonicating with an Ultrasonic XL 2020 sonicator fitted with a Microtip 419 probe (Heat Systems) set to deliver 85 W and operated for 10 s with a pulse time of 1 s and a rest time of 9 s. Emulsions were left to stand for 4 h at 25 °C before the aqueous phases (designated 4 h aq) were removed with a pipet and transferred to fresh tubes for freeze-drying. The remaining emulsions were left for a further 44 h at the same temperature, after which the aqueous phases (designated 48 h aq) were removed and lyophilized. The emulsions were then gently washed with 5 mL of the appropriate buffer and left for a further hour before the aqueous phases ("interstitial components") were removed and lyophilized. The remaining emulsions were broken by storing at −20 °C for at least 24 h and then incubating for at least 1 h at 25 °C, after which the aqueous phases ("emulsion components") were removed and lyophilized.

HPLC Analysis of Emulsion Components. Solvent A was a 0.07% (v/v) aqueous solution of trifluoroacetic acid (TFA), and solvent B was a 0.05% (v/v) solution of TFA in CH_3CN. The lyophilized emulsion components were dissolved in 0.7 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 3 M urea, and then the mixture was passed through a 0.2 μm filter. After loading 250 μL of sample onto a Prosphere C18 5 μm, 300 Å analytical HPLC column (250 mm × 4.6 mm) (Alltech) maintained at 45 °C, the constituent proteins were eluted with the following gradient system: 0−5 min, 20% solvent B in solvent A; then 5−50 min, 20−50% solvent B in solvent A. The column was washed by adjusting the concentration of solvent B in solvent A to 100% and then to 0% and finally to 20% (v/v) to reequilibrate prior to the next analysis.

Purification of CM3. An amount of 1.3 g of lyophilized albumin fraction from cv. Mercia was applied to a 1 m column of Sephacryl S-200HR (Amersham-Pharmacia) preequilibrated in 50 mM sodium phosphate buffer, pH 8.0 (packed bed volume of 1500 mL). Elution occurred overnight at 4 °C with the same buffer using a flow rate of 1 mL min^{-1}. Pooled fractions were dialyzed against water, lyophilized, and analyzed by SDS−PAGE under reducing and nonreducing conditions and by HPLC. Fractions enriched for CM3 were further purified by preparative RP-HPLC using a Prosphere C18 5 μm, 300 Å column (250 mm × 10 mm) (Alltech). The HPLC-purified protein was applied to an SDS−PAGE gel using a Tris-tricine (*9*) and then electrophoretically transferred to ProBlott membrane for N-terminal sequence analysis. The molecular mass was determined by ES-MS.

Functionality Testing. The surface tension of solutions of CM3 dissolved in 10 mM sodium phosphate buffer, pH 7.0, was measured using the pendent drop technique (*10*) as a function of protein concentration. Photographic images of a drop of protein solution hanging from a hydrophobic needle were acquired using a digital camera (Pulnix TM500) as a function of time. The surface tensions of the solutions were calculated from analysis of the drop shape by the selected-plane method. Interfacial tensions between the protein solution and *n*-hexadecane (Sigma, >99% purity) were also measured by creating a drop in the oil as opposed to in air. Equilibrium surface tensions (γ) were taken 15 min after drop formation. No further reduction in surface tension was observed after this period. All measurements were made at room temperature (~22 °C).

Surface dilation measurements of proteins adsorbed at the air/water interface were made according to a published procedure (*11*). This technique utilized a roughened glass cylinder (10 cm in diameter) that was periodically raised and lowered in a vessel containing buffer and a surface film of protein while the surface tension was measured by the Wilhelmy plate method. The cylinder oscillation was sinusoidal, with a periodicity of 0.8 rad s^{-1} and an amplitude that caused a 5% oscillation of the surface area of the protein film. The protein films were created by spreading protein solution (1 mg mL^{-1}) onto a buffer surface (10 mM sodium phosphate buffer, pH 7.0). The dilational elastic moduli recorded are the stable values obtained typically 20 min after spreading.

Fluorescence emission spectra were accumulated between 290 and 500 nm using a Perkin-Elmer LS50B fluorimeter equipped with a front-face sampling accessory. Use of the front-face geometry allowed for the collection of fluorescent light from opaque samples (*12*). An excitation wavelength of 280 nm was used with a 290 nm cutoff emission filter to reduce the effect of scattered light (Rayleigh and Mie scattering). The scan speed was 250 nm min^{-1}, with a 2.5 nm slit width for emission and a 5 nm slit width for excitation. Spectra were the average of 10 scans, subjected to Savitsky−Golay filtering.

Fourier transform infrared (FT-IR) spectra were recorded using a Nicolet Magna 860 spectrometer equipped with an MCT detector and an ATR cell (ZnSe crystal). Spectra were averages of 256 scans recorded at a resolution of 2 cm^{-1}. Since the band due to the HOH deformation of water overlaps with the protein absorbance band of interest (the amide I band), the solutions and emulsions to be analyzed by FT-IR were prepared in D_2O (Aldrich, 99.9% D atom, with a surface tension greater than 72.6 mN m^{-1}). The contributions of nonprotein material (D_2O and *n*-hexadecane) were manually subtracted. The labile protons of buffer salts were replaced by deuterons, by contacting the protonated form with D_2O and then removing the water by rotary evaporation. Since the broad amide I band is difficult to interpret, band-narrowing by Fourier self-deconvolution (FSD) was carried out using Omnic 5.2 software with a bandwidth of 16 cm^{-1} and an enhancement factor of 2.3. IR band assignments were then made in accordance with published work (*13*, *14*). Protein solutions and emulsions were prepared 24 h before measurement to ensure that all exchangeable protons in the protein had been replaced by deuterons.

Emulsions for spectroscopic study were prepared by sonicating a 5 mg mL^{-1} solution of protein in buffer solution (10 mM sodium phosphate (deuterated), pD 7.0) with 40% (v/v) *n*-hexadecane (Sigma, >99% purity). Prior to spectroscopic measurements, the emulsions were washed to remove interstitial (nonadsorbed) protein by centrifugation at 7500*g* for 5 min and removal of the noncreamed subphase. More buffer was then added, and the emulsion was shaken to redisperse the droplets. This process was repeated for a total of three washes. This process has been shown to remove interstitial protein while leaving the emulsion drop size unchanged (*15*, *16*).

RESULTS

Development of a Novel Functional Screen. To identify proteins with emulsifying activity, albumin fractions prepared from wheat grain were used with *n*-hexadecane to prepare a variety of emulsions as described above. The protein fractions were derived from two commercial varieties of bread wheat (Mercia and Riband), Kamut (*T. turgidum* var. *durum*), and spelt (*T. turgidum* var. *spelta*).

The emulsions formed with these different fractions and conditions provided components corresponding to the aqueous,

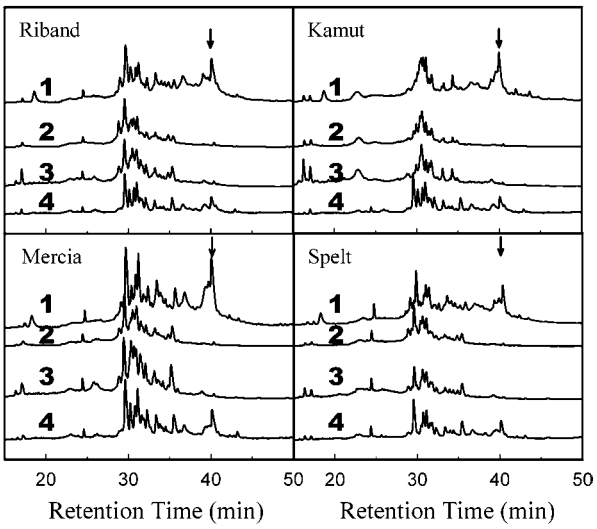

Figure 1. Reversed-phase HPLC analysis of emulsion components. Emulsions were prepared from the albumin fractions isolated from the wheat varieties indicated and analyzed as described in Materials and Methods: (trace 1) emulsion constituents remaining after washing; (trace 2) interstitial constituents; (trace 3) aqueous constituents; (trace 4) stock albumin solution.

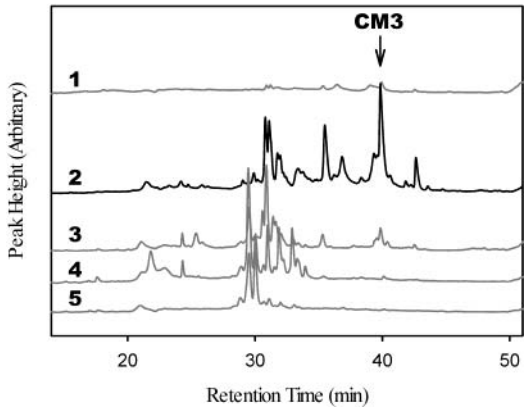

Figure 2. RP-HPLC analysis of pooled, lyophilized Mercia albumin fractions separated by gel-permeation chromatography on Sephacryl S200HR. Traces 1–5 show the pooled fractions in order of elution from the column.

interstitial, and emulsion phases as described in Materials and Methods. The individual emulsion components were then separated by RP-HPLC using the total albumin fractions as controls. Typical results obtained using albumin fractions from the four lines at pH 8.0 and 0.1 M ionic strength are shown in **Figure 1**. Although most of the major protein peaks are present in all the emulsion components, some differences in proportions are apparent. In particular, a peak eluting at about 40 min (arrowed in **Figure 1**) was clearly enriched in the interfacial phases of the emulsions shown in **Figure 1** and of those made under other conditions of pH (3.0, 6.5) and ionic strength (1.0 M) (results not shown).

Purification of the "40 min" Protein and Identification as CM3. The "40 min" protein was partially purified by HPLC separation of emulsion components obtained from cv. Mercia albumins, separated by SDS−PAGE, and transferred to a ProBlott membrane. N-terminal amino acid sequencing showed that it was identical to the wheat albumin CM3 for the first 10 residues (Ser-Gly-Ser-Xaa-Val-Pro-Gly-Val-Ala-Phe-), allowing for failure to recover Cys at position 4 and some uncertainty at position 1. A greater amount of the protein was then purified from a total albumin fraction from cv. Mercia. Separation on a

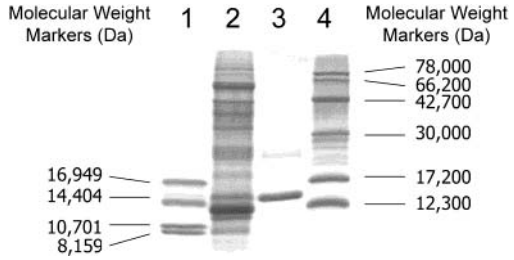

Figure 3. SDS–PAGE analysis of CM3 purified from Mercia. Lanes 1 and 4 are molecular weight markers. Lane 2 is a total albumin fraction from Mercia, and lane 3 is purified CM3.

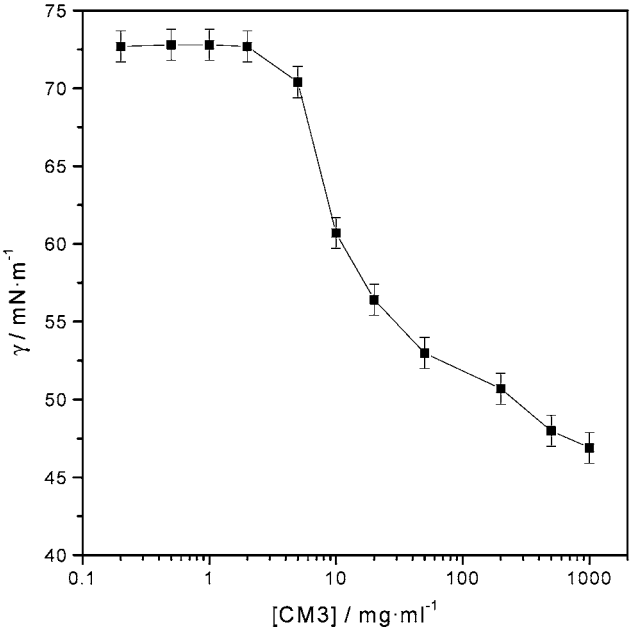

Figure 4. Plot of the surface tension of aqueous CM3 solutions (in 10 mM sodium phosphate buffer, pH 7.0) as a function of concentration. The line is a guide for the eye only. Errors are ±1 mN m^{-1}.

column of Sephacryl S200HR gave five pooled fractions, which were dialyzed against water, lyophilized, and analyzed by RP-HPLC (**Figure 2**). This showed that fraction 2 was enriched in CM3, which was subsequently purified by preparative RP-HPLC. N-terminal amino acid sequencing confirmed that this protein was identical to the wheat albumin CM3, the residue at position 1 being unambiguously assigned on this occasion. The purified protein gave a predominant band of M_r about 15 000 when separated by SDS−PAGE (**Figure 3**) together with a more slowly migrating band that may represent a minor contaminant or an aggregated form (i.e., a dimer).

The mass determined by ES-MS was 15 539 compared with a calculated mass of 15 822 for CM3, based on the sequence of a cloned cDNA (*17*) and assuming all the Cys residues are present as intrachain disulfide bonds. The difference in mass (283) may suggest some C-terminal cleavage or some inaccuracies in the published sequence.

Surface Activity of CM3. The equilibrium surface tensions of pure CM3 solutions as a function of protein concentration are shown in **Figure 4**. At low concentrations (<2 μg mL^{-1}), the surface tension was equivalent to that of pure buffer solution. However, at greater concentrations, the protein is surface-active, decreasing the surface tension by 26 mN m^{-1} at a concentration of 1 mg mL^{-1}. This behavior is mirrored in the interfacial tensions between water and *n*-hexadecane (**Figure 5**). This relationship between bulk protein concentration and the onset

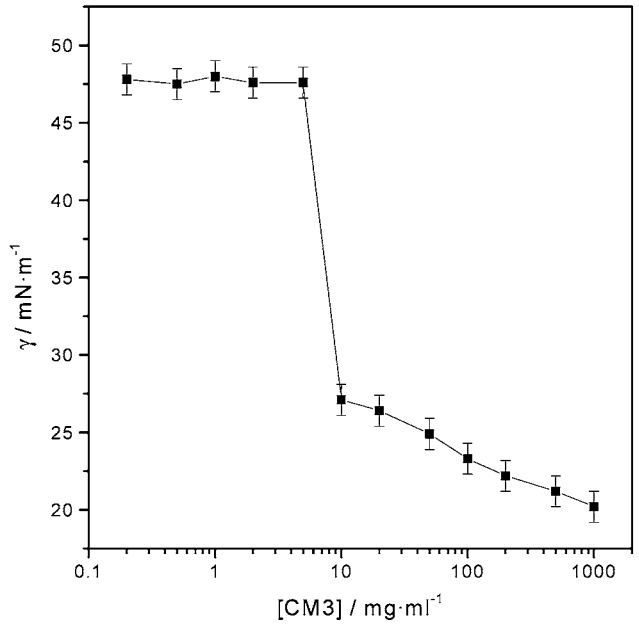

Figure 5. Plot of the interfacial tension between aqueous CM3 solutions (in 10 mM sodium phosphate buffer, pH 7.0) and *n*-hexadecane as a function of concentration. The line is a guide for the eye only. Errors are ±1 mN m^{-1}.

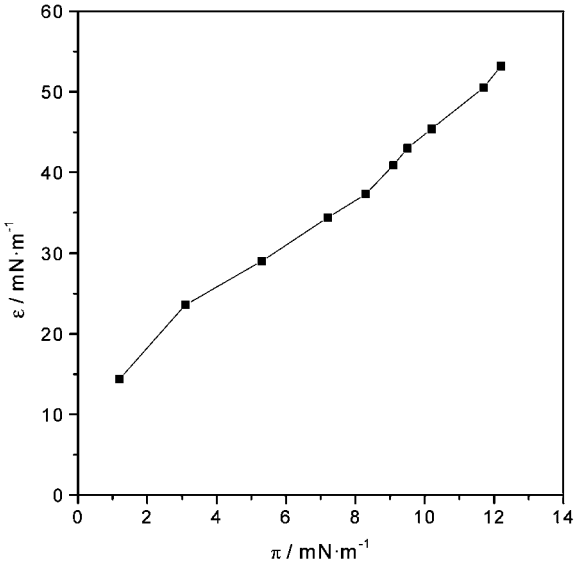

Figure 6. Plot of surface dilational elastic modulus (ϵ) versus surface pressure (π) for CM3 spread onto aqueous 10 mM sodium phosphate buffer, pH 7.0, from a 1 mg mL^{-1} solution in the same buffer. The line is a guide for the eye only. Error bars fall within the size of the data symbols.

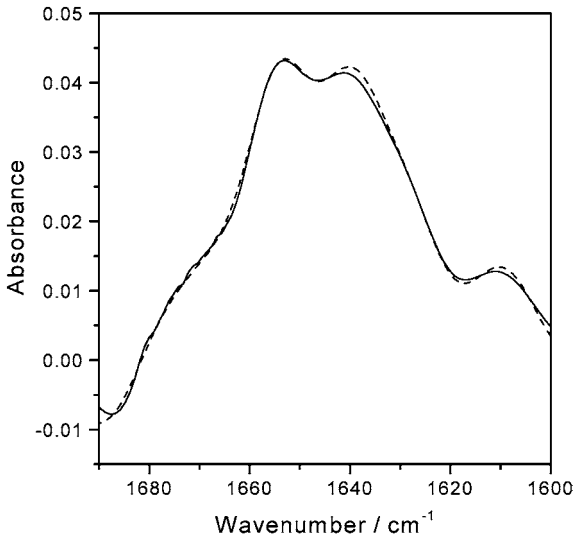

Figure 7. FSD-enhanced FT-IR spectra of amide I region of 5 mg mL^{-1} CM3 solution in D_2O and 10 mM sodium phosphate at pD 7.0 (solid line) and CM3-stabilized emulsion prepared from 5 mg cm^{-3} (in D_2O with 10 mM sodium phosphate at pD 7) CM3 and 40% (v/v) *n*-hexadecane (dashed line).

of surface tension reduction is not unusual. It is believed that a critical concentration of protein has to adsorb at the air/water interface before surface tension reduction occurs, an observation that has been rationalized in terms of requiring sufficient protein at the interface for intermolecular interactions to occur (*13*). The large reduction of the surface tension by CM3 at higher concentrations suggests that this protein is at least as surface-active as other 2S albumins (e.g., from sunflower seeds (*14*) and bovine β-lactoglobulin (β-lg) (*15*)). However, the ability of a protein to reduce the surface tension between two immiscible phases does not, in itself, confer an ability to stabilize an emulsion. It is believed that a degree of intermolecular interaction must occur between the protein molecules on the surface in order to form a viscoelastic monolayer. It is this monolayer that provides the steric barrier to the coalescence of emulsion drops. The surface dilational elastic modulus (ϵ) is a measure of the interactions between protein molecules at the surface. Proteins that are good emulsifiers and foaming agents are expected to have high elastic modulus because the proteins are better able to reduce film rupture and droplet coalescence (*20*). The surface dilational elastic modulus of CM3 that has been spread at the water/air interface is shown in **Figure 6** as a function of surface pressure (defined as the surface tension of the buffer minus the surface tension of the sample). This plot shows a roughly linear increase in ϵ as the surface pressure increases, suggesting that CM3 is able to form a viscoelastic film at the air/water interface. It should be noted that the surface dilational viscous modulus was simultaneously calculated for this system and was found to be essentially zero at all surface pressures. This is the expected behavior for protein monolayers.

Spectroscopic Studies of Adsorbed and Nonadsorbed CM3. The adsorption of protein to a fluid interface is generally considered to be an irreversible process involving irreversible denaturation of the protein (*21*). The ability of CM3 to adsorb to liquid interfaces forming a viscoelastic film has been demonstrated. It is generally thought that emulsion stability, in part, arises from intermolecular bonding between adjacent protein molecules at the interface. These interactions can include β-sheet formation (*15*, *16*, *22*, *23*), intermolecular disulfide bond formation (*24*−*26*), and electrostatic interactions (*27*), which interlock adjacent protein molecules at the interface to form a gel-like film. It has been argued that these interactions usually result in a degree of change in secondary structure of the protein (*14*, *28*−*32*), which can be most easily evaluated by comparison of the FT-IR spectra of adsorbed and nonadsorbed protein. It is therefore pertinent to consider what, if any, protein denaturation occurs when CM3 adsorbs to an emulsion interface. Analysis of the FT-IR spectra of CM3 solution and CM3 at the oil/water interface (**Figure 7**) shows that the CM3 secondary structure is essentially unchanged on emulsification. These spectra suggest that CM3 predominantly contains α-helix (1635 cm^{-1}) and random structure (1640 cm^{-1}), with a small amount of β-sheet (1610 cm^{-1}); however, there are no bands that can be assigned

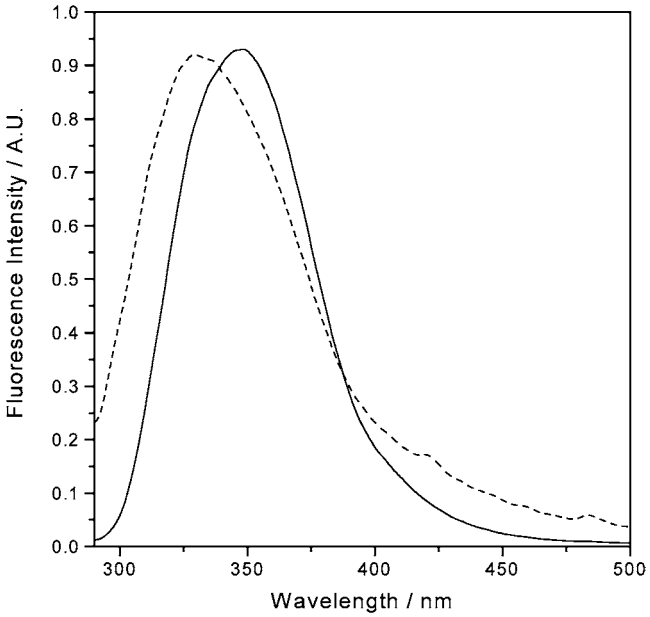

Figure 8. Front-faced fluorescence emission spectra of a 5 mg mL^{-1} solution in D_2O and 10 mM sodium phosphate at pD 7.O (solid line) and a CM3-stabilized emulsion prepared from 5 mg L^{-1} CM3 (in D_2O with 10 mM sodium phosphate pD 7.0) and 40% (v/v) *n*-hexadecane (dashed line). Excitation was at 280 nm.

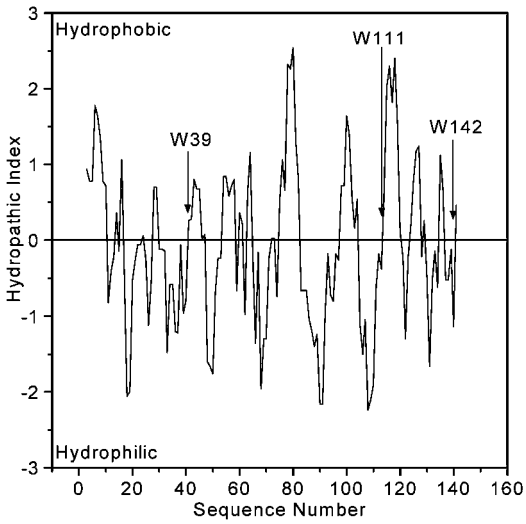

Figure 9. Hydrophobicity plot for CM3, calculated by the method of Kyle and Doolittle (*44*), with a window size of five residues. The arrows show the locations of the three tryptophan residues (W39, W111, and W142), all which lie within the hydrophobic regions of the protein.

to intermolecular β-sheet structure (1620−1630 cm^{-1}). This finding is, to our knowledge, unique in that we could find no other example in the literature of a protein emulsifier that does not undergo some denaturation upon adsorption to a liquid/liquid interface. The secondary structure measured by FT-IR is in excellent agreement with a modeled structure for CM3 (unpublished results), which predicts four α-helices connected by random structure and only one small region of antiparallel β-sheet. Moreover, the predicted structure contains five disulfide bonds that would significantly restrict the amount of possible denaturation. There are also no free sulfhydryl groups available to facilitate intermolecular disulfide bond formation. Any structural change would therefore be expected to be restricted to a general reorientation of the loops of random structure. It would, therefore, seem that no measurable secondary structure changes occur during the adsorption of CM3 to the oil/water interface even though dilational rheology measurements indicate the formation of a viscoelastic monolayer. This could indicate that either electrostatic forces or nonspecific entanglement of unordered structure is causing intermolecular interactions. Further studies (especially surface shear rheological measurements) on CM3 systems could be useful in the elucidation of the nature of the intermolecular interactions involved.

Fluorescence spectroscopy was also employed to investigate the solvation of CM3 at an emulsion interface. The intrinsic fluorescence of proteins predominates from tryptophan residues, the fluorescent properties of which are strongly dependent on the effective polarity of the medium surrounding the indole ring of the tryptophan residue. It has been reasoned (*33*) that three spectral classes of tryptophan can be distinguished. Tryptophan in a nonpolar environment (i.e., buried in the core of a globular protein) fluoresces between 330 and 332 nm; tryptophan completely exposed to water (i.e., at the protein surface) fluoresces between 350 and 353 nm, while fluorescence in the 340−342 nm range is attributed to a partially hydrated tryptophan. The fluorescence spectrum of a 5 mg mL^{-1} solution of CM3 (**Figure 8**) shows a maximum emission at 348.5 nm, suggesting that the three tryptophan residues present in CM3 (*17*) reside in a predominantly solvated environment. Although it is generally unusual for hydrophobic residues (such as tryptophan) to reside on the surface of a protein, the relatively hydrophobic nature of CM3 would suggest that some hydrophobic residues must reside at the surface.

The fluorescence emission spectrum of CM3 located at the oil/water interface shows a distinct blue shift relative to that of the solution, with a peak maximum of 329.0 nm, suggesting that the tryptophan residues have relocated to a nonpolar environment. This could be due to a significant change in the global structure of the protein to accommodate the tryptophan residues in the center of the protein or due to the residues being located either in the oil phase of the emulsion or at the oil/water interface. Since it is known from the FT-IR spectra that no significant secondary structure change occurs, it is probable that the tryptophan residues are located either at the interface or in the oil phase. Previous studies with a 2S globular protein from sunflower seed have shown that the tryptophan residue in this protein locates in the oil phase when stabilizing an emulsion. The fact that the three tryptophan residues in CM3 are located in the hydrophobic regions of the protein, as envisaged by the hydropathic plot shown in **Figure 9**, suggests that these entire hydrophobic regions could be located either in the oil-phase or at the interface.

It should be noted that although not enough CM3 was purified for a full emulsion stability study, the emulsions prepared for fluorescence and FT-IR measurements were stable to both creaming and phase separation for a period of at least 24 h. This further demonstrates the effectiveness of CM3 as an emulsifier, especially considering the large amount of oil (40% v/v) stabilized in these emulsions.

DISCUSSION

We have used a functional screening procedure to demonstrate that the albumin CM3 is highly active at interfaces with excellent emulsifying properties. CM3 is a subunit of a tetrameric inhibitor of exogenous α-amylase enzymes, forming part of a larger family of trypsin/α-amylase inhibitors from cereal seeds (*34*). CM3 was initially identified and designated as a CM protein (based on its solubility in chloroform/methanol mixtures) as

early as 1969 (*35*) and has since been studied in some detail. Nevertheless, the present study provides the first report of its unusually high surface activity. The functional screen employed here, however, selects only proteins that are able to stabilize emulsions and not those that are merely surface-active. In this case, CM3 is considered to be a good emulsifier in that it is able both to reduce surface tension and to form a strong viscoelastic monolayer at the interface, although the exact mechanism by which CM3 forms such a monolayer remains unclear. This confirms the value of the functional screening approach for the identification of novel functional proteins and the demonstration of new properties of a previously identified protein.

Despite the need for new protein emulsifiers, it is highly unlikely that CM3 will be used commercially in the future. This is because CM3 has been demonstrated to bind IgE in serum from some patients exhibiting atopic dermatitis resulting from ingestion of wheat (*36*) while related α-amylase/trypsin inhibitors may also be allergenic when ingested (*37*) or inhaled, the latter leading to bakers' asthma (*38*−*43*). The procedure remains a viable approach for identifying surface-active proteins in other plant species and tissues.

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