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ARTICLE

Polypeptide modification: An improved proglycinin design to stabilize oil-in-water emulsions

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This paper is dedicated to the memory of Professor Utsumi, who passed away on December 1, 2008.

Abstract

β-conglycinin and glycinin are soybean major seed storage proteins. Previous studies have shown that adding the extension region of β-conglycinin α subunit improves the emulsifying properties of proglycinin and confers more favourable characteristics than fusing the extension region of β-conglycinin α' subunit or the hypervariable regions (A4IV) of glycinin A1aB1b subunit. To evaluate the polypeptide properties, we designed mutants of A1aB1b subunits fused with truncated versions of A4IV (A4IVcut), α (αcut) or α' (α'cut) extension regions lacking the C-terminus 25 or 31 residues (A4IVC25, αC25 or α'C31), and also A4IVcut and α'cut with αC25 residues added (A4IVcut-αC25 and α'cut-αC25). All the modified proteins displayed conformations similar to the wild type. With good solubilities, the emulsion properties of the modified proteins were much better at ionic strength μ = 0.08 than at μ = 0.5. The modified A1aB1bαcut and A1aB1bα'cut showed poorer emulsion properties than those of A1aB1bα and A1aB1bα'. Replacing the hydrophobic A4IVC25 region of A1aB1bA4IV with hydrophilic αC25 created A1aB1bA4IVcut-αC25, which had the best emulsion stability among these proglycinin mutants. We found that addition of αC25 improves the emulsifying properties of two C-terminally truncated proglycinin variants, thereby illustrating its potential general utility. Our investigation showed that in order to improve the emulsifying ability and emulsion stability of a globular protein, the introduced polypeptide should (i) be highly hydrophilic, (ii) consist of multiple hydrophobic–strong hydrophilic regions comprising at least two alpha helixes, (iii) harbour a terminal α-helix at the end of the C-terminus, and (iv) have properties similar to those of αC25.

Keywords: emulsion; polypeptide properties; protein interface; protein engineering; glycinin.

Introduction

The use of soy protein products as functional ingredients is gaining increasing acceptance in food manufacturing from the standpoint of human nutrition and health (Anderson, Johnstone, & Cook-Newell, 1995; Anderson, Johnstone, & Cook-Newell, 1999; FDA, 1999; Kinsella, Damodaran, & German, 1985). Soybean protein isolates have been used in the production of yogurts, coffee creamers, whipped toppings, and infant formulas, which (totally or partially) substitute for milk proteins (Kolar, Cho, & Watrous, 1979). Soy proteins play different roles in food and non-food products owing to their beneficial physicochemical properties such as hydrophobicity, solubility, thermal stability, and emulsifying properties (Utsumi, 1992; Utsumi, Matsumura, & Mori, 1997). The emulsifying property of a protein is one of its most important functional properties in relation to its application in food systems (Dickinson, 1992). However, most plant storage proteins such as glycinin (Maruyama et al., 2004; Prak et al., 2005), beta-conglycinin (Maruyama et al., 2002), adzuki-derived 7S globulin (Fukuda et al., 2007), mungbean-derived 8Sα globulin (Torio et al., 2011), and coconut-derived 11S globulin (Angelia et al., 2010), have limited emulsifying properties. To improve emulsifying properties of proteins, many studies have been extensively investigated (Damodaran, 1997; Dickinson, Murray, & Stainsby, 1988; Graham & Phillips, 1976; Liu, Lee, & Damodaran, 1999; Palazolo, Mitidieri, & Wagner, 2003; Phillips, 1981). Attempts have also been made to improve the emulsifying properties of plant storage proteins by addition of emulsifiers (Burgess & Sahin,1998), heat and pressure treatments (Puppo et al., 2011; Tang, Chen, & Foegeding, 2011), addition of peptic enzyme-treated pectin (Huang et al., 2011), or by changing the pH, protein concentration, and ionic strength of the emulsion samples, individually or simultaneously (Burgess & Sahin, 1998; Karaca, Nickerson, & Low, 2011; Romero et al., 2011). During these investigations, many theories were proposed for understanding the emulsifying properties of proteins. However, studies attempting to improve the emulsifying properties of proteins have seen limited success. We have previously engineered a soybean protein that can be used for the production of physiologically active peptides (Prak et al., 2006; Prak & Utsumi, 2009) and have also extensively improved the emulsion properties of soybean proteins (Prak et al., 2007; Tandang et al., 2005). In contrast to other previous methods (Burgess & Sahin, 1998; Damodaran, 1997; Dickinson, Murray, & Stainsby, 1988; Graham & Phillips, 1976; Huang et al., 2011; Karaca, Nickerson, & Low, 2011; Liu, Lee & Damodaran, 1999; Palazolo, Mitidieri, & Wagner, 2003; Phillips, 1981; Puppo et al., 2011; Tang, Chen, & Foegeding, 2011; Romero et al., 2011), we have now improved the quality of soybean protein emulsions by introducing peptides or polypeptides. We have not resorted to the use of other emulsifiers or additional heat and pressure treatments to achieve this goal.

Soybean (*Glycine max* L.) protein is composed of two major components, glycinin (11S globulin) and β-conglycinin (7S globulin), accounting for 40% and 30% of the total seed proteins, respectively (Utsumi, 1992; Utsumi, Matsumura, & Mori, 1997). β-Conglycinin is a trimeric protein composed of three subunits: α (~67 kDa), α’ (~71 kDa), and β (~50 kDa). According to the amino acid sequences deduced from the nucleotide sequences, the α and α’ subunits harbour extension regions (125 and 141 amino acid residues for

α and α', respectively), in addition to the core regions (414–418 residues), which are common to all three subunits (Maruyama et al., 1998). The homology between the core regions of the subunits is ~71–87%, and between the α and α' extension regions is ~57%. The extension regions are rich in acidic amino acid residues. On the other hand, glycinin is a hexameric protein composed of five major subunits (A1aB1b, A1bB2, A2B1a, A3B4, and A5A4B3), each of which consists of an acidic (~30 kDa) and a basic (~20 kDa) polypeptide linked by a single disulphide bond, except for the acidic polypeptide A4 of A5A4B3 (Dickinson, Hussein, & Nielsen, 1989). The five subunits have been classified into two groups based on sequence homology. Group I comprises A1aB1b (53.6 kDa), A1bB2 (52.2 kDa), and A2B1a (52.4 kDa), and group II comprises A3B4 (55.4 kDa), and A5A4B3 (61.2 kDa). The homology of each subunit is more than 84% within a group and 45–49% among groups (Nielsen et al., 1989; Utsumi, Matsumura, & Mori, 1997). According to the amino acid sequences deduced from the nucleotide sequences of the five subunits, the main difference in the subunits is attributable to the presence of hypervariable regions at the C-termini of their acidic polypeptides and comprising 43, 29, 35, 70, and 103 amino acid residues for A1aB1b, A1bB2, A2B1a, A2B1a, A3B4, and A5A4B3, respectively (Adachi et al., 2001; Lawrence et al., 1994; Nielsen et al., 1989).

We found that the addition of various oligopeptides or polypeptides to A1aB1b resulted in improvements of emulsifying ability and emulsion stability (Prak et al., 2007; Tandang et al., 2005). Among the introduced polypeptides, only the β-conglycinin α extension region extensively improved the emulsion stabilities of the modified versions of A1aB1b. The α (125 aa) and α' (141 aa) extension regions had similar amino acid sequences and similar lengths (the α extension region is 16 amino acid residues shorter than the α' extension region), but the α extension region contained a more hydrophilic region consisting of the C-terminal 25 amino acid residues. The emulsion stability of A1aB1bα was better than that of A1aB1bα' (Prak et al., 2007). From this point of view, the 25 amino acid residues at the C-terminal end of the α extension region seemed important for the emulsion stability of proglycinins. To verify this, we removed 25 and 31 aa (αC25 and α'C31) from the C-terminal regions of A1aB1bα or A1aB1bα', respectively; and this created the less hydrophilic A1aB1bαcut and A1aB1bα'cut, respectively (Figs 1 and 2). The A4IV hypervariable region was more hydrophilic than the α and α' extension regions, but A1aB1bA4IV had a poorer emulsion stability than that of A1aB1bα and A1aB1bα' (Prak et al., 2007). For further investigation, we removed 25 aa (A4IVC25) from the A1aB1bA4IV C-terminus and created a new A1aB1bA4IVcut that had higher hydrophilicity than A1aB1bA4IV, A1aB1bαcut, and A1aB1bα'cut. We added αC25 to the C-terminus end of A1aB1bA4IVcut and A1aB1bα'cut, thus creating A1aB1bA4IVcut-αC25 and A1aB1bα'cut-αC25, respectively. To determine the polypeptide properties that are necessary for improving the emulsifying ability and emulsion stability of the proteins in oil-in-water emulsions, we expressed all the newly modified proteins in *Escherichia coli*. We then characterized their structural properties, and studied the corresponding physicochemical properties such as surface hydrophobicity, solubility, and emulsion property.

Materials and methods

Construction of expression plasmids for proglycinin mutants

The schematic representations of proglycinin A1aB1b wild type (WT) and their mutants are shown in Fig. 2. To construct the expression plasmids for the mutants, the expression plasmids pEA1aB1bA4IV, pEA1aB1bα, and pEA1aB1bα' (Prak et al., 2007), were used as templates for PCR. The different primers used for amplifying the desired mutant cDNAs by PCR using Pyrobest (Takara) are as follows: pEA1aB1bA4IVcut; pEA1aB1bA4IV as a template, 5'-*TAG*AATTCCGGATCCGAATTCGAGCTC-3' and 5'-TTCGCGGCTCTTGCGAGGTTG-3'. pEA1aB1bαcut; pEA1aB1bα as a template, 5'-AACGAGTGCCAGATCCAAAAACTC-3' and 5'-CTTCTGATGAGGTGGGCGTGG-3'. These pairs of primers were used for obtaining a DNA fragment encoding partial A1aB1b, starting from 270 nucleotides upstream of the *Avr*II restriction site (Prak & Utsumi, 2009) to its C-terminus–encoding region, in addition to the partial α extension region lacking the 25 amino acid residues from the C-terminus. pEA1aB1b was used as a template; whereas 5'-TAGAATTCCGGATCCGAATTCGAGCTC-3' and 5'-TGGTTTTATCACGCTCAGACCTCCTTTC-3' were used as primers. This pair of primers was used for the synthesis of DNA fragment containing pET-21d and a partial A1aB1b starting from the start codon to a position 440 nucleotides downstream of the *Avr*II restriction site. For pEA1aB1bA4IVcut-αC25, pEA1aB1bα was used as a template, whereas 5'- GAAGAGCGAAAGCAAGAGGAA-3' and 5'-TTGCTGATATTTTAGAAACTCTTGCTC-3' were used as primers. This pair of primers was used to get a DNA1 fragment encoding αC25 in pET-21d. Additionally, pEA1aB1bA4IV was used as a template, along with the primers 5'-TAGAATTCCGGATCCGAATTCGAGCTC-3' and 5'-TTCGCGGCTCTTGCGAGGTTG-3', respectively. This primer pair was used to get a DNA2 fragment encoding A1aB1bA4IVcut. For pEA1aB1bα'cut-αC25, pEA1aB1bα was used as a template; whereas 5'- GAAGAGCGAAAGCAAGAGGAA-3' and 5'-TTGCTGATATTTTAGAAACTCTTGCTC-3' were used as the primers. This primer pair was used to synthesize a DNA3 fragment encoding αC25 in pET-21d. pEA1aB1bα' was also used as a template, with the primers 5'-*TAG*AATTCCGGATCCGAATTCGAGCTC-3' and 5'-CTTTCCTTGGTGCTTTTCCTGC-3', respectively. This primer pair was used to get a DNA4 fragment encoding A1aB1bα'cut (stop codons are in italics).

The regions encoding pEA1aB1bA4IVcut and pEA1aB1bα'cut, and the DNA fragments were amplified by PCR. The DNAs encoding pEA1aB1bA4IVcut and pEA1aB1bα'cut were phosphorylated and self-ligated. The resulting short fragment for the construction of pEA1aB1bαcut was phosphorylated and the final construct was made by ligating the two corresponding fragments after digestion with *Avr*II. For the construction of pEA1aB1bA4IVcut-αC25 and pEA1aB1bα'cut-αC25, DNA fragment2 was phosphorylated and the constructs were made by ligating the two corresponding fragments after digestion with *Xba*I.

Protein expression

The expression plasmids were transformed into *E. coli* expression host–Origami(DE3). Culture and the expression conditions for A1aB1bαcut, A1aB1bA4IVcut, A1aB1bA4IVcut-αC25, A1aB1bα'cut, and A1aB1bα'cut-αC25 were identical to those for A1aB1bα, A1aB1bA4IV, and A1aB1bα', respectively, as described previously (Prak et al., 2007). The cells harbouring the individual expression plasmids were grown in LB

medium at 37°C. When A_{600} reached 0.4 to 0.6, 0.33 M NaCl was added to the culture and the protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) at 20°C. After cultivation, the cells were harvested by centrifugation at 9000*g* for 15 min at 4°C, and stored at -20°C. Proteins in the cell aliquots were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), using 11% acrylamide gel (Laemmli, 1970). The expressed recombinant proteins were identified based on their expected sizes, and this confirmed by western blotting (Prak et al., 2005), using the anti-glycinin antibody, followed by the goat-rabbit IgG-alkaline phosphatase conjugate (Promega).

Purification of mutant proteins

All purification steps were carried out at 4°C and centrifugation was carried out at 9000*g* for 20 min unless otherwise stated. The basic buffer used for all purification steps was buffer A (35 mM potassium phosphate, pH 7.6, 1 mM EDTA, 10 mM 2-mercaptoethanol (2ME), 0.1 mM (p-amidinophenyl)-methylsulfonyl fluoride, 1 μg/mL pepstatin A, 1 μg/mL leupeptin). Ammonium sulphate fractionation was performed according to the procedure of Green and Hughes (Green & Hughes, 1955). A1aB1bα and A1aB1bα'WT were purified as described previously (Prak et al., 2007). The frozen cells containing A1aB1bA4IVcut, A1aB1bαcut, A1aB1bα'cut, A1aB1bA4IVcut-αC25, and A1aB1bα'cut-αC25 were resuspended in buffer B (buffer A containing 1.0 M NaCl) at a density of 40 g/L of the buffer and lysed by sonication in an ice bath. The insoluble materials were removed by centrifugation. The expressed (modified) proteins were fractionated using ammonium sulphate as follows: 30% ammonium sulphate for A1aB1bαcut, A1aB1bα'cut, and A1aB1bα'cut-αC25, respectively; 35% ammonium sulphate for A1aB1bA4Ivcut, and A1aB1bA4IVcut-αC25, respectively. The precipitate was removed by centrifugation, and the soluble fraction containing the recombinant proteins was applied to a Toyopearl (Butyl-650M) (TOSOH, Japan) column (2.6 cm × 20 cm) equilibrated with buffer B containing 30% ammonium sulphate. Elution was carried out with a linear gradient (800 mL) of 30% to 0% of ammonium sulphate in buffer B. The fractions containing the modified proteins were pooled, and concentrated by VIVASPIN 20 MWCO 30.0 kDa (VIVASCIEN, Japan), and subsequently applied on a gel filtration column (HiPrep 26/60 Sephacryl S-300 HR) using buffer B as the mobile phase. The fractions containing the modified proteins were pooled and diluted 6.67 times with buffer C (buffer A without NaCl) to reduce the NaCl in the buffer to 0.15 M. The protein samples were then applied to a Mono Q HR 10/10 column (Pharmacia Biotech) equilibrated with buffer D (buffer A containing 0.15 M NaCl). Elution was performed with a linear gradient of 0.15 M to 0.5 M of NaCl in buffer A, over a period of 120 min at 2 mL/min.

The level of protein expression and the purity of the protein samples were analysed with a densitometric scan and estimated by analysing the gel image with ImageMaster 1D Elite, version 3.0 (Amersham-Pharmachia Biotech, Uppsala, Sweden).

Measurement of protein concentrations and self-assembly into trimers

The amount of protein in the samples was determined using a Protein Assay Rapid Kit (Wako), with bovine serum albumin (BSA) as a standard. The assay was performed according to the manufacturer's instructions. In brief, 3 mL of Color-producing Solution

was added to 50 μL of protein sample and protein sample buffer and mixed. The mixtures were left for 20 min at room temperature before measuring the absorbance at 600 nm. The amount of protein in the sample is calculated by using the net absorbance (net absorbance = absorbance of the protein sample – the absorbance of the protein sample buffer) and the corresponding BSA standard curve.

The self-assembly of each protein mutant was analysed using a HiPrep 16/60 Sephacryl S-300 HR column (Pharmacia Biotech) as described previously (Prak et al., 2005). For the above analysis, we used 500 μL of each sample at a concentration of 0.25 mg/mL in buffer E (35 mM sodium phosphate, pH 7.6, 0.4 M NaCl, 1 mM EDTA, 0.1 mM (p-amidinophenyl)-methylsulfonyl fluoride, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 0.02% NaN_3, and 10 mM 2ME), Buffer E was the mobile phase and the flow rate was 0.5 mL/min.

Differential scanning calorimetry

Differential Scanning Calorimetry (DSC) measurements of the samples were carried out as described previously (Prak et al., 2005), using 1 mg/mL of the sample in buffer E. Scanning was recorded using a Microcal MC-2 Ultra-Sensitive Microcalorimeter (Micro Cal Inc., Northampton, MA) at the rate of 1°C/min.

Surface hydrophobicity

Surface hydrophobicities of the samples were analysed as described previously (Prak et al., 2005), using butyl and phenyl sepharose columns (Amersham Bioscience, Sweden), and 500 μL of the samples (0.25 mg/mL) in buffer G (buffer E containing a 35% saturated of ammonium sulphate solution). The proteins were first eluted with a linear gradient of 35% to 0% of ammonium sulphate over a period of 55 min, and then with buffer E for 45 min at a flow rate of 0.25 mL/min.

Solubility analysis as a function of pH

All the samples were dialysed against buffer H (10 mM sodium phosphate, pH 7.6, 0.5 M NaCl, 1 mM EDTA, 0.1 mM (p-amidinophenyl)-methylsulfonyl fluoride, 1 μg/mL pepstatin A, 1 μg/mL leupeptin, 0.02% NaN_3, 10 mM 2ME). The experimental conditions were similar to those described previously (Prak et al., 2005). The protein samples (0.8 mg/mL) were adjusted to various pH values from 2.0 to11.0. The samples were then centrifuged at 20,000*g*, for 15 min at 4°C, after maintaining them at 4°C for 18 h, for the separation of soluble and insoluble fractions. The percentage solubility (soluble fraction) was determined by comparing the protein content of the resulting solution with the initial protein content of the sample (100% soluble).

Emulsifying property

The emulsifying properties of the samples were analysed as described previously (Prak et al., 2007) using 1.5 mL of the protein sample (0.5 mg/mL) at pH 7.6 in buffer E and buffer H for μ = 0.5 and 0.08, respectively. We added 0.25 ml of soybean oil to the sample just before homogenization. The mixed sample was homogenized for 30 s using a high speed homogenizer (Nichion Irikakikai Ltd.) set at 22,000 rpm and sonicated using sonication tip size 3, output control 3 on a constant duty cycle of an ultrasonic homogenizer (Nihonseiki Kaisha Ltd.) for 1 min. The emulsifying properties of the protein samples were analysed by measuring the particle size distribution and the mean particle diameter with a laser light scattering instrument (Model LA 500,

Horiba Sisakusho Ltd.). The stability of the emulsions was analysed by sealing the test tubes containing the emulsions and maintaining them at room temperature without agitation, and by visually observing them after 1 h, 20 h, 2 d, 5 d, 7 d, 14 d, and 20 d.

Results and discussions

Self-assembly of proglycinin mutants into trimers

To use these modified proteins for further analysis, it was necessary to confirm whether the modified proteins were able to fold with a conformation similar to the WT. To investigate this, the individual purified WTs and their modified versions were subjected to gel filtration chromatography using HiPrep 16/60 Sephacryl S-300 HR column at pH 7.6 and μ = 0.5. In a previous study, we found that modified A1aB1bα and A1aB1bα' can self-assemble into trimers (Prak et al., 2007). Table I shows that A1aB1bA4IVcut, A1aB1bαcut, and A1aB1bA4IVcut-αC25 eluted slower than A1aB1bα, and the elution times consistent with their molecular masses. The elutions of A1aB1bα'cut and A1aB1bα'cut-αC25 were slower than the elution of A1aB1bα'. A1aB1bα'cut and A1aB1bα'cut-αC25 were 10 aa bigger than A1aB1bαcut and A1aB1bα respectively. The elution time of A1aB1bα'cut 1.9 min more than that of A1aB1bαcut, and the elution time of A1aB1bα'cut-αC25 was 0.5 min more than that of A1aB1bα; these results were consistent with those for the mobility properties of A1aB1bα' (617aa; 97.0 min) and A1aB1bα (601aa; 96.6 min). The mobilities of A1aB1bα', A1aB1bα'cut, and A1aB1bα'cut-αC25 in the gel filtration column followed their molecular size, and these mobilities were similar to those of A2B1a, A2B1aα, and A2B1aα' (Prak et al., 2007), respectively. This indicates a slight difference in the molecular surfaces of A1aB1bα', A1aB1bα'cut, A1aB1bα'cut-αC25, and A1aB1b WT (Prak et al., 2005; Prak et al., 2007).

DSC analysis of the thermal stability (Table II) of the modified and original proteins showed that the T_m values of all the newly modified versions were slightly (0.1–1.6°C) lower or higher than those of the original, version, except for A1aB1bA4IVcut-αC25 (80.6 $\pm$ 0.20 °C), whose T_m was 5.9°C higher than that of A1aB1bA4IV (74.7 $\pm$ 0.34 °C). The T_m value of A1aB1bA4IVcut-αC25 was close to that of A1aB1bα'cut (79.9 $\pm$ 0.15 °C) (Prak et al., 2007). The thermal stability analysis data were consistent with the data from our previous study on the analogous modified versions of A1aB1bs (Adachi et al., 2003; Prak et al., 2007; Tandang et al., 2005), which indicated that all newly modified proteins probably assumed conformations similar to those of the parent proteins as well as of A1aB1b WT.

Surface hydrophobicity measurements

We employed two columns of phenyl and butyl sepharose for the measurement of surface hydrophobicity. Longer elution times of the sample corresponded to higher surface hydrophobicities. A protein with highest number of aliphatic residues on the surface will retain the longest in butyl sepharose column whereas a protein with highest number of aromatic residues on the surface will retain the longest in phenyl sepharose column. The surface hydrophobicities (Table III) of all the modified versions of the proteins were A1aB1bα'cut-αC25 $\geq$ A1aB1bA4IVcut-αC25 $\geq$ A1aB1bα'cut $\geq$ A1aB1bαcut = A1aB1bA4IVcut $>$ A1aB1bα, as analysed by using the butyl sepharose columns, and A1aB1bα'cut $>$ A1aB1bαcut $\geq$ A1aB1bα = A1aB1bα'cut-αC25 =

A1aB1bA4IVcut > A1aB1bA4IVcut-αC25, as analysed by using the phenyl sepharose columns. The percentages of aliphatic (A, V, L, I) and aromatic (Y, W, F) residues in each introduced polypeptide (% aliphatic; % aromatic residues) were α (3.2; 3.2), αcut (3.0; 4.0), α'cut (1.8; 3.6), α'cut-αC25 (2.1; 3.0), A4IVcut (1.3; 2.6), and A4IVcut-αC25 (2.0; 1.9). According to the percentage of the aliphatic and aromatic residues of the polypeptides added to the A1aB1b C-terminus, A1aB1bα and A1aB1bαcut was expected to elute the slowest from the butyl sepharose column, whereas A1aB1bαcut was expected to elute the slowest from the phenyl sepharose column, followed by A1aB1bα'cut. However, among the modified proteins, A1aB1bα'cut-αC25 (48.3 min) and A1aB1bA4IVcut-αC25 (48.1 min) eluted the slowest from the butyl sepharose column, and A1aB1bα'cut (68.9 min), followed by A1aB1bαcut (67.9 min), eluted the slowest from the phenyl sepharose column. The elution of the modified proteins from the sepharose column was likely dependent on the hydrophobicity of the polypeptides added to the A1aB1b C-terminus (Fig. 1). Previously, we found that the addition of 20 positively charged amino acid residues to the A1aB1b C-terminus resulted in an increase in the surface hydrophobicity of the protein (Prak et al., 2007). This was due to the interaction between the positive and negative amino acid residues at the disordered region II and IV, at the IE face (containing the interchain disulphide bond connecting the acidic and basic chains) of the protein (Adachi et al., 2001). Therefore, the difference in the surface hydrophobicity of the modified proteins (resulting into a slightly higher or lower elution time of 0.0–1.8 min) was not caused solely by an increase or decrease of the amount of hydrophobic amino acids introduced to the C-terminus of A1aB1b; other factors such as the specific interactions between the polypeptides and A1aB1b, and the nature of the introduced polypeptides in the solution, might have contributed to these changes.

Protein solubility as a function of pH

Solubility is a fundamental physicochemical property of food proteins (Bilgi & Çelik, 2004; Kinsella, 1979; Peng et al., 1984). We measured the solubility of A1aB1bα and that of the newly modified versions at high (μ = 0.5) and low (μ = 0.08) ionic strengths (Fig. 3). At μ = 0.5, the solubilities of A1aB1bα and A1aB1bA4IVcut-αC25 were quite similar. They both showed lower solubilities at pH 3.8 (20% and 0% for A1aB1bα and A1aB1bA4IVcut-αC25, respectively). At a pH < 3.0, while the other modified proteins were 80–90% soluble, the solubilities of A1aB1bα and A1aB1bA4IVcut-αC25 were only about 50%. A1aB1bA4IV had low (50%) solubility at pH 4.0, and high solubility at other (lower or higher) pH values. The solubilities of A1aB1bαcut, A1aB1bα'cut, and A1aB1bα'cut-αC25 were similar to each other and to the solubility of A1aB1bα' (Prak et al., 2007). The lowest solubility (~70%) was observed at pH 3.0–4.0. At μ = 0.08, the solubilities of A1aB1bα, A1aB1bA4IVcut, and A1aB1bA4IVcut-αC25 were similar, and they were more similar to the solubilities of A1aB1bA4IV and A5A4B3 (Prak et al., 2005; Prak et al., 2007) than to that of A1aB1b WT. Their solubility was nearly 0% at pH 3.8–5.6, but the solubility increased dramatically to ~80–100% at pH < 3.5 or pH > 6.0, except in the case of A1aB1bα, which had a solubility of ~40% at low pH. These results showed that the deletion of 25 aa from the C-termini of A1aB1bα and A1aB1bA4IV (A4IVC25 and αC25) and 31 aa from the A1aB1bα' C-terminus (α'C31), or the replacement of A4IVC25 or α'C31 with αC25, resulted in improved intrinsic solubilities of the newly modified proteins. The percentage of positively charged

residues in the polypeptides α'cut, α'cut-αC25, αcut, α, A4IVcut, and A4IVcut-αC25 were 48.5%, 41.9%, 36.2%, 31.6%, 29.4%, and 26.1%, respectively. At pH < 4.5, when histidine is positively charged, the solubilities of the mutants at μ = 0.08 were similar among A1aB1bα'cut, A1aB1bα'cut-αC25, and A1aB1bαcut, and also among A1aB1bA4IVcut, A1aB1bα, and A1aB1bA4IVcut-αC25. Along with our previous published results (Prak et al., 2007), these data suggest that the occupancy of the positively charged residues in the disordered/variable regions of a protein, directly affect the solubilities of the proteins at pH < 4.5, μ = 0.08.

Emulsifying property

The emulsifying properties of the A1aB1bα and the new modified versions were studied at pH 7.6 and at high (μ = 0.5) and low (μ = 0.08) ionic strengths. The investigation was based on two criteria: the emulsifying ability (Fig. 4) and the emulsion stability (Fig. 5). The emulsifying ability of the modified proteins at ionic strength μ = 0.5 was 2.7, 4.9, 5.5, 2.7, 4.4, and 1.5 μm and at μ = 0.08 was 1.5, 3.3, 3.8, 1.8, 2.2, and 1.3 μm for A1aB1bα, A1aB1bαcut, A1aB1bα'cut, A1aB1bα'cut-αC25, A1aB1bA4IVcut, and A1aB1bA4IVcut-αC25, respectively. Removal of the αC25 or α'C31 of A1aB1bα or A1aB1bα' resulted in poorer emulsions for A1aB1bαcut and A1aB1bα'cut. Replacement of α'C31 or A4IVC25 with αC25 improved the emulsifying abilities of A1aB1bα'cut-αC25 (2.7 and 1.8 μm) and A1aB1bA4IVcut-αC25 (1.5 and 1.3 μm) at high and low ionic strengths, as compared to A1aB1bα' and A1aB1bA4IV (Prak et al., 2007), respectively. The emulsifying property of A1aB1bA4IVcut-αC25 (1.5 μm) was better than that of A1aB1bα (2.7 μm). Figure 5 shows that the emulsion stabilities of A1aB1bα'cut and A1aB1bαcut at μ = 0.5 were less than 1 h. At μ = 0.08, A1aB1bαcut emulsion formed protein precipitates and attached to the wall of the emulsion tubes, whereas the A1aB1bα'cut formed a fluffy white protein-oil emulsion similar to the A1aB1bA4IVcut emulsions at both ionic strengths. The emulsion of the A1aB1bα'cut-αC25 was stable at μ = 0.5 for less than 1 d, but at μ = 0.08, it was still stable at 2 d. The emulsion of A1aB1bα and A1aB1bA4IVcut-αC25 at μ = 0.5 was stable for less than 2 days. The emulsion stability of A1aB1bA4IVcut-αC25 was much better than that of A1aB1bα at μ = 0.08. After 7 d, the A1aB1bA4IVcut-αC25 emulsion maintained its stability at the same level as in the first hour; the phase separation of the emulsion started to appear only after 20 d resulting a new record for the best emulsion stability of proglycinin mutants.

For investigating the polypeptide properties, at first the hydrophobicity profiles of the introduced polypeptides were analysed with the DNAsis program (Hitachi Software Engineering Co., Ltd, Japan) (Fig. 1), and the percentage of hydrophilic residues (D, E, K, R, H, S, Q, and N) in the C-terminus regions were calculated. The percentage of hydrophilic residues in the C-terminal regions was 80.5%, 83.4%, 86.5%, 96.0%, 75.2%, 70.7%, 79.5%, 75.4%, and 79.2%, for A4IV, A4IVcut, A4IVcut-αC25, αC25, α, αcut, α', α'cut, and α'cut-αC25, respectively. Although there was likely a correlation between the percentage of hydrophilicity and the emulsion stability, the range of hydrophilicity from the highest to the lowest for the introduced polypeptide were A4IVcut-αC25 > A4IVcut > A4IV > α' > α'cut-αC25 > α'cut > α > αcut, which does not totally correlate with the emulsion stability between groups of modified A4IV, α, and α' polypeptides. A4IVcut-αC25 has αC25 at the C-terminus end, like α (125 aa), but is

more hydrophilic (Fig. 1); its emulsion was also much more stable (Fig. 5). The extension regions of α and α' are very similar in their amino acid sequences and hydropathy profiles, except that α is slightly more hydrophilic than the α' extension region. Removal of their hydrophilic αC25 and α'C31 to create A1aB1bαcut and A1aB1bα'cut resulted in a reduced emulsion stability of these modified proteins from 7 d and 2 d to < 1 d. These data indicate that the replacement of hydrophobic oligopeptides with a hydrophilic αC25 oligopeptide at the C-terminus of a modified protein improves the emulsifying ability and emulsion stability. Investigators found that the FLEHAFSVDK oligopeptide from A1a-glycinin hydrolysate (Tsumura, Kugimiya, & Inouye, 2005) and synthetic peptides TFLQDLKEKVQQLTEALK and TVSQLQEYWTTLLSQIKTLLQQIKTS (Carey et al., 1994), showed good emulsifying activities. These suggested that the hydrophilicity at the C-terminus of the introduced polypeptide was important for the emulsifying properties, and that the composition of multiple hydrophobic-strong hydrophilic regions of the polypeptide contribute to the emulsion stability. In addition, it was found that an alpha helix in the peptide secondary structure (Brock & Enserm, 1994), the intermediate charged states of the peptides (Dexter, 2010) contributed to emulsion stability. We then looked at the secondary structure of the polypeptides. We assumed that there was no alpha helix structure in the first 45 aa of the polypeptides (Fig. 6) owing to the nature of the polypeptides that were derived from flexible regions that could not be observed by X-ray crystallography (Adachi et al., 2001; Maruyama et al., 2001). We observed that the length of alpha helix in the polypeptides was somewhat correlated to their in-group emulsifying property. As shown in Figs 1 and 6, A4IVcut was strongly hydrophilic but had no alpha helix in the structure, whilst αcut and α'cut had the lowest hydrophilicity amongst the introduced polypeptides and had 3 (composed of < 10 aa) and 2 (composed of > 10 aa) alpha helices respectively. These polypeptides had no αC25 in their structure. αC25 is very hydrophilic (96%) and formed alpha helix structures that were > 10 aa long (Fig. 6). A4IVcut-αC25 had the highest percentage of hydrophilic residues (86.5%) among the newly introduced peptides and had two alpha helices, each composed of more than 10 aa. Finally, we investigated the influence of the polypeptides on the surface of proglycinin. A1aB1b was highly soluble in the emulsion buffer at pH 7.6 (Prak et al., 2005). The surface of the proglycin trimeric protein is composed of IE face (containing the interchain disulfide bond connecting the acidic and basic chains), IA face (containing intrachain disulfide bond in the acidic chain) (Adachi et al., 2001; Jung et al., 1997) and disordered regions that are strongly hydrophilic. However, its core structure was strongly hydrophobic. In the process of emulsion preparation (homogenization and sonication), the secondary and tertiary structures of the protein might undergo some changes (Lee et al., 2007; Zhai et al., 2012) which may result in new interactions and absorption, and determination of emulsion stability (Damodaran, 1997; Dickinson, 1992). It has been suggested that the emulsion stability depends on the emulsion environment such as ionic strength and the nature of the protein (Steitz, Jaeger, & Klitzing, 2001; Wang et al., 1999; Utsumi, 1992; Utsumi, Matsumura, & Mori, 1997). Therefore, when a long polypeptide was added to A1aB1b (Fig. 7), one or more parts of the polypeptide interacted with parts of other polypeptides on the other modified A1aB1b molecules or with other parts of A1aB1b, as well as with oil and water. These interactions led to protein precipitation or the formation of a fluffy white material (Fig. 5). However, when an alpha helix (αC25) was added to the C-terminally truncated proglycinin variants, the interaction of the polypeptide with oil/water became stronger

and more stable (Brock & Enserm, 1994; Dexter, 2010) as illustrated in figure 7. The alpha helix held the oil and water apart, reduced non-specific interactions, and aided the proper formation of a globular protein. It acted as an emulsion stabilizer and held the modified protein molecules in an oil-water emulsion. A4IVcut-αC25 may play a role similar to that of the carbohydrate moieties of French bean 7s globulin (phaseolin) in emulsion (Kimura et al., 2010).

Conclusions

Addition of the A4IV hydrohypervariable, α', and α extension regions (with hydrophilicities ranging from high to low (A4IV > α > α')), to the A1aB1b C-terminus improved the emulsifying properties of the protein as shown in a previous study. The introduction of an α extension region significantly improved the protein emulsion stability, followed by a similar polypeptide, an α' extension region, and a totally different polypeptide A4IV. An interesting question was to address if the hydrophilicity and the 25 aa at the α C-terminus (αC25) play a crucial role in the protein interfacial behaviour necessary for stabilizing oil-in-water emulsions. New polypeptides were designed by removing and replacing 25 aa from the C-termini of α and A4IV, and 31 aa from the α' C-terminus with αC25 to create a more hydrophobic αcut and α'cut, or very highly hydrophilic A4IVcut polypeptides, for studying the role of αC25. As expected, in the absence of a special C-terminus containing the αC25 helix structure, the introduced polypeptides could not improve the emulsifying properties of the protein to stabilize oil-in-water emulsions. At low ionic strength, the protein either precipitated or formed a fluffy white substance. The creation of a highly hydrophilic polypeptide A4IVcut-αC25, composed of multiple hydrophobic-strong hydrophilic regions with αC25 at the C-terminus, resulted in a significant improvement of emulsifying property of A1aB1bA4IVcut-αC25 that helped in stabilizing the oil-in-water emulsion. The emulsion stability was still maintained after 20 d at room temperature and was much better than that of A1aB1bα. The evidence from this study suggests that to improve the emulsifying ability and the emulsion stability of proglycinin as well as of seed storage proteins, the introduced polypeptide should (i) be highly hydrophilic, (ii) be composed of multiple hydrophobic–strong hydrophilic regions, (iii) have at least two alpha helixes, each more than 10aa in length, at the end of its C-terminus, and (iv) the last alpha helix at the C-terminus should have properties similar to that of αC25.

These studies provide useful guidelines for designing an improved protein interface in order to stabilise oil-in-water emulsions. Future research should therefore be aimed at investigating obtaining emulsions with higher stability, resulting from the replacement of some of the A4IV-αC25 oligopeptides with one or more copies of αC25.

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Figure legends

Fig. 1. Hydrophobicity profiles of the polypeptides introduced to the A1aB1b C-terminus. The dashed boxes indicate what the deletions of the oligopeptides consisted of 25, 25, and 31 amino acid residues (aa) from the A5A4B3 A4IV hypervariable (103 aa), α (125 aa), and α' (141 aa) extension regions respectively, creating A4IVcut (78 aa), αcut (100 aa), and α'cut (110 aa). The closed boxes indicate the addition of an oligopeptide consisting of 25 amino acid residues from the C-terminus of the α extension region (αC25) to A4IVcut and α'cut, thus creating A4IVcut-αC25 (103 aa) and α'cut–αC25 (135 aa), respectively.

Fig. 2. Schematic diagram of proglycinin A1aB1b and the modified peptides. Roman numerals name the disordered regions of A1aB1b, shown in grey. Arabic numerals in the boxes indicate the number of amino acid residues introduced into the A1aB1b C-terminus (A4IVcut, αcut, α'cut, A4IVcut-αC25, and α'cut-αC25 consisted of 78, 100, 110, 103 (78 aa + 25 aa), and 135 (110 aa + 25 aa) amino acid residues respectively). The hydrophobicity profiles of the introduced polypeptides were analysed with the DNAsis program (Hitachi Software Engineering Co., Ltd, Japan).

Fig. 3. The pH dependence of the solubility of A1aB1b mutants at the ionic strengths of 0.5 and 0.08. Error bars represent the standard deviation from two to four separate experiments.

Fig. 4. Particle size distributions of emulsion of proglycinins and modified versions at ionic strengths of 0.5 and 0.08. The emulsifying ability of the proteins was analysed by measuring the particle size distribution and by calculating the mean droplet diameter of the emulsion samples, using a light scattering instrument. The smaller the particle size of the emulsion droplet, the better the emulsion. The values were means ± SD of three to ten independent experiments.

Fig. 5. Emulsion stability of modified proteins at ionic strengths of 0.5 and 0.08. The stability was analysed by sealing and maintaining the test tubes containing the emulsions at room temperature, between 1h and 20 days, without agitation. The emulsion stabilities of all the modified proteins were determined in two to six independent experiments from one to two independent purifications. The average emulsion stability of each modified protein sample is depicted.

Fig. 6. Prediction of the secondary structure of the introduced polypeptides. The prediction was analysed by the PSIPRED v3.0 protein structure prediction server (http://bioinf.cs.ucl.ac.uk/psipred/).

Fig. 7. Illustration of a stabilised A1aB1b mutant molecule in oil/water emulsion. The ribbon diagrams of the proglycinin A1aB1b homotrimer structure (PDB: 1FXZ) showing each monomer in black, dark grey, and grey, respectively. The Arabic numerals in each position indicate the residue numbers before the start and after the end of disordered regions I (residues 1–9), II (residues 92–109), III (residues 179–197), III' (residues 228–232), IV (residues 249–296), and V (residues 471-476), respectively. The disordered region V is shown in black dots. The introduced polypeptide is shown with a black dashed line. Black dots and black dashed lines are structural representations for

the estimation positions of the disordered region V and the introduced polypeptides which cannot be observed by X-ray crystallography. Empty ovals represent water molecules. Black ovals represent oil molecules.

Table I. Elution time of the modified proteins on a gel filtration column[a]							
	A1aB1bα	A1aB1bαcut	A1aB1bα'	A1aB1bα'cut	A1aB1bα'cut-αC25	A1aB1bA4IVcut	A1aB1bA4IVcut-αC25
Number of amino acid residues	601	576	617	586	611	554	579
Molecular mass (kDa)	67.0	65.9	70.9	67.3	70.4	63.1	66.2
Elution time (min)	96.6 ± 0.30	101.0 ± 0.05	97.0 ± 0.90	102.9 ± 0.15	97.5 ± 0.20	105.6 ± 0.25	99.9 ± 0.20

[a] The values are mean ± S.E of at least two independent experiments

Table II. DSC scans of modified proteins[a]

	A1aB1bα	A1aB1bα cut	A1aB1bα'cut	A1aB1bα'cut-αC25	A1aB1bA4IV cut	A1aB1bA4Ivcut-αC25
Denaturation temperature (°C)	79.2 ± 0.03	79.7 ± 0.05	79.9 ± 0.15	76.9 ± 0.20	74.8 ± 0.35	80.6 ± 0.20

[a] The values are mean ± S.E of at least two independent experiments

Table III. Elution time of modified proteins on hydrophobic column[a]

Hydrophobic column	A1aB1bα	A1aB1bα cut	A1aB1bα' cut	A1aB1bα'cut-αC25	A1aB1bA4IV cut	A1aB1bA4Ivcut-αC25
Butyl sepharose	46.5 ± 0.11***	47.5 ± 0.15	47.6 ± 0.13	48.3 ± 0.28***	47.5 ± 0.40	48.1 ± 0.20***
Phenyl sepharose	67.4 ± 0.15	67.9 ± 0.35	68.9 ± 0.15***	67.4 ± 0.20	67.4 ± 0.17	66.3 ± 0.43***

[a] The values are mean ± S.E of at least two independent experiments. There are significantly different on surface hydrophobicity between A1aB1bα and A1aB1bA4Ivcut-αC25, and between A1aB1bα and A1aB1bα'cut-αC25 using butyl sepharose column, and between A1aB1bα'cut and A1aB1bA4Ivcut-αC25 using phenyl sepharose column (***p < 0.001, one way ANOVA).

Figure 1.

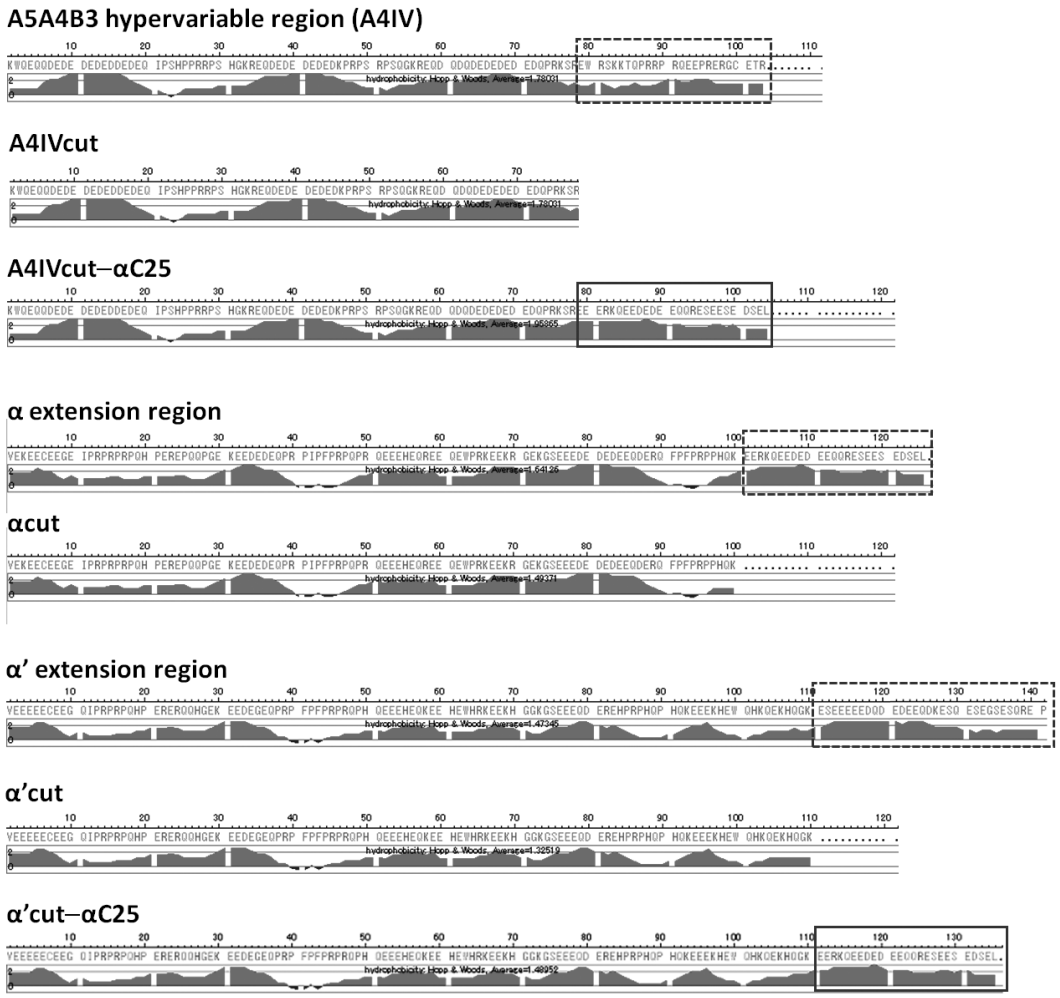
A5A4B3 hypervariable region (A4IV)
A4IVcut
A4IVcut–αC25
α extension region
αcut
α' extension region
α'cut
α'cut–αC25

Figure 2.

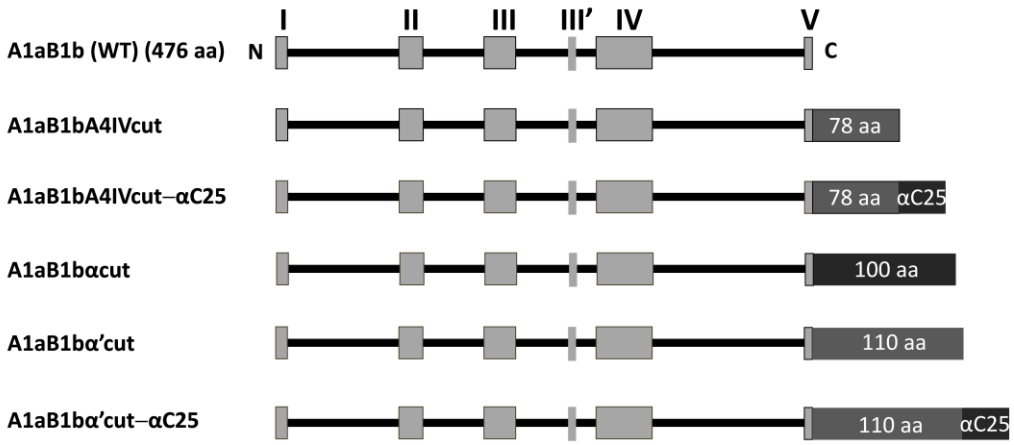
I
II
III
III'
IV
V
A1aB1b (WT) (476 aa)
N
C
A1aB1bA4IVcut
78 aa
A1aB1bA4IVcut–αC25
78 aa
αC25
A1aB1bαcut
100 aa
A1aB1bα'cut
110 aa
A1aB1bα'cut–αC25
110 aa
αC25

Figure 3.

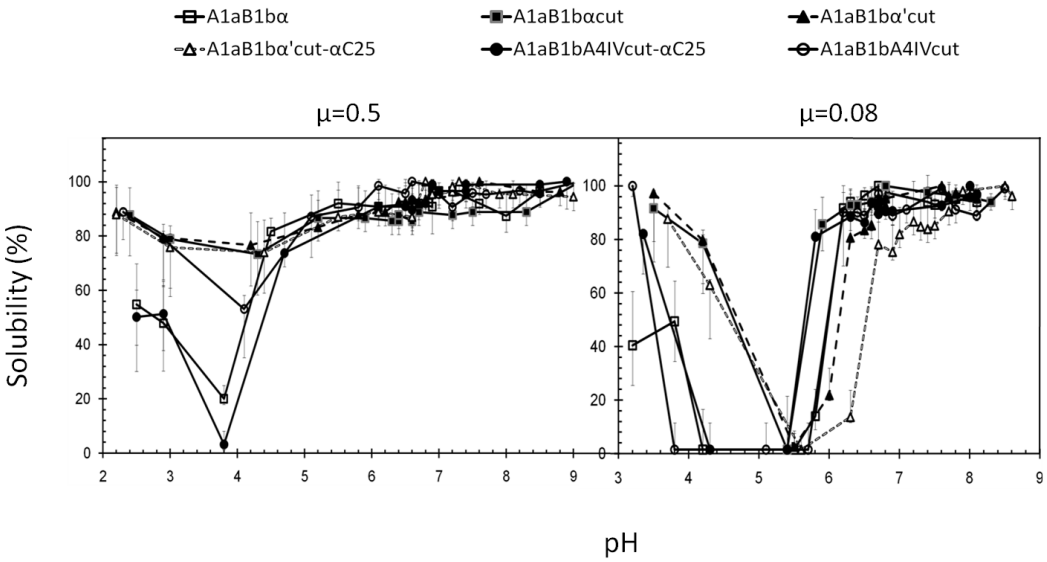
A1aB1bα
A1aB1bαcut
A1aB1bα'cut
A1aB1bα'cut-αC25
A1aB1bA4IVcut-αC25
A1aB1bA4IVcut
μ=0.5
μ=0.08
Solubility (%)
pH

Figure 4.

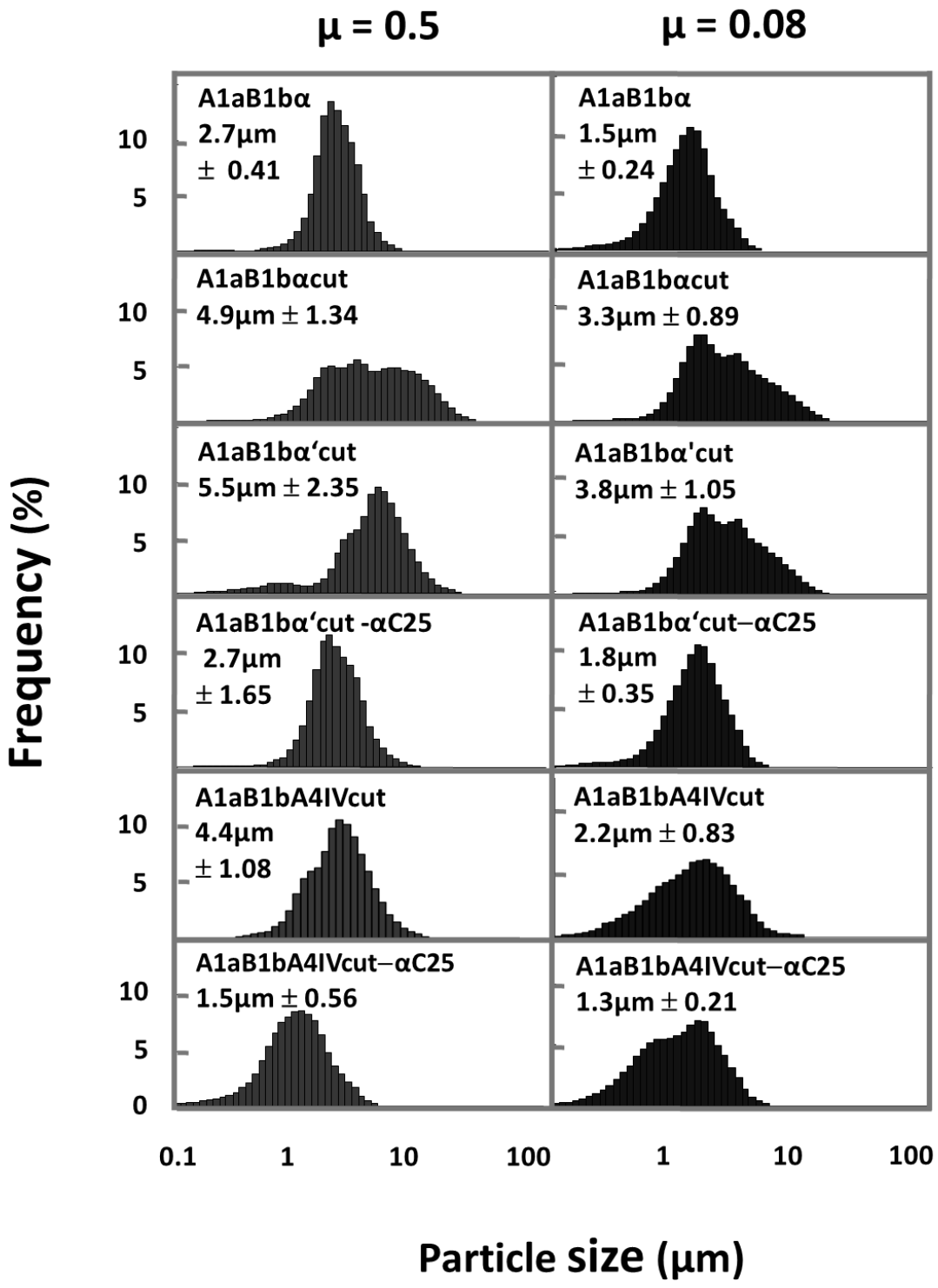
μ = 0.5
μ = 0.08
A1aB1bα
2.7μm
± 0.41
A1aB1bα
1.5μm
± 0.24
A1aB1bαcut
4.9μm ± 1.34
A1aB1bαcut
3.3μm ± 0.89
A1aB1bα'cut
5.5μm ± 2.35
A1aB1bα'cut
3.8μm ± 1.05
A1aB1bα'cut -αC25
2.7μm
± 1.65
A1aB1bα'cut–αC25
1.8μm
± 0.35
A1aB1bA4IVcut
4.4μm
± 1.08
A1aB1bA4IVcut
2.2μm ± 0.83
A1aB1bA4IVcut–αC25
1.5μm ± 0.56
A1aB1bA4IVcut–αC25
1.3μm ± 0.21
Frequency (%)
Particle size (μm)
0.1
1
10
100
0
5
10

Figure 5.

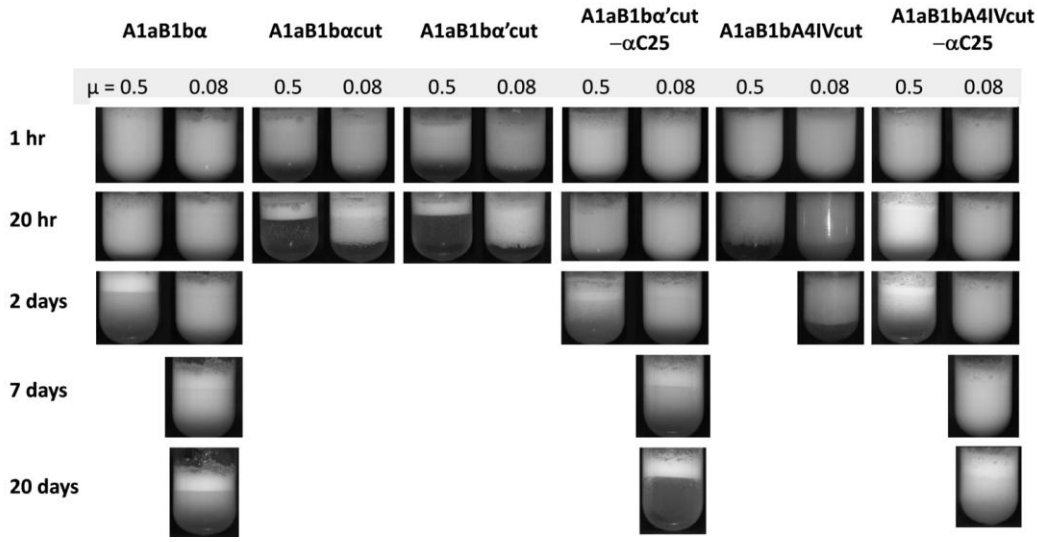
A1aB1bα
A1aB1bαcut
A1aB1bα'cut
A1aB1bα'cut −αC25
A1aB1bA4IVcut
A1aB1bA4IVcut −αC25
μ = 0.5
0.08
0.5
0.08
0.5
0.08
0.5
0.08
0.5
0.08
0.5
0.08
1 hr
20 hr
2 days
7 days
20 days

Figure 6.

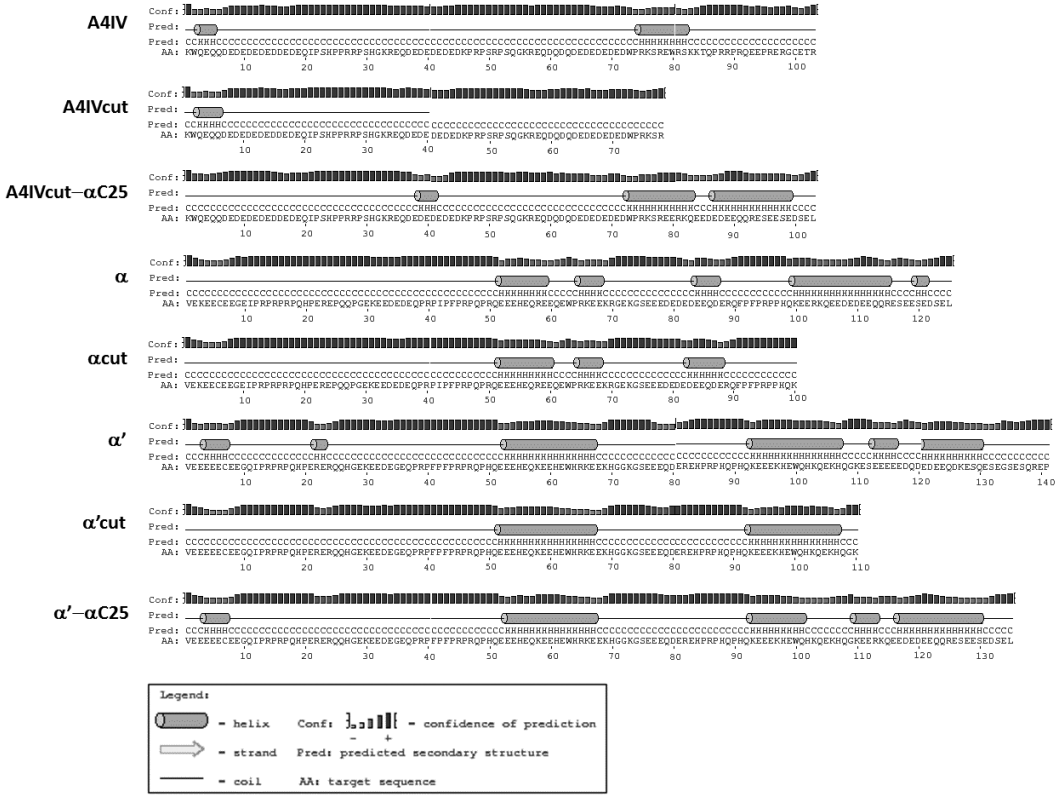
A4IV
A4IVcut
A4IVcut–αC25
α
αcut
α'
α'cut
α'–αC25
Conf:
Pred:
AA:
Legend:
= helix
= strand
= coil
Conf: = confidence of prediction
Pred: predicted secondary structure
AA: target sequence

Figure 7.