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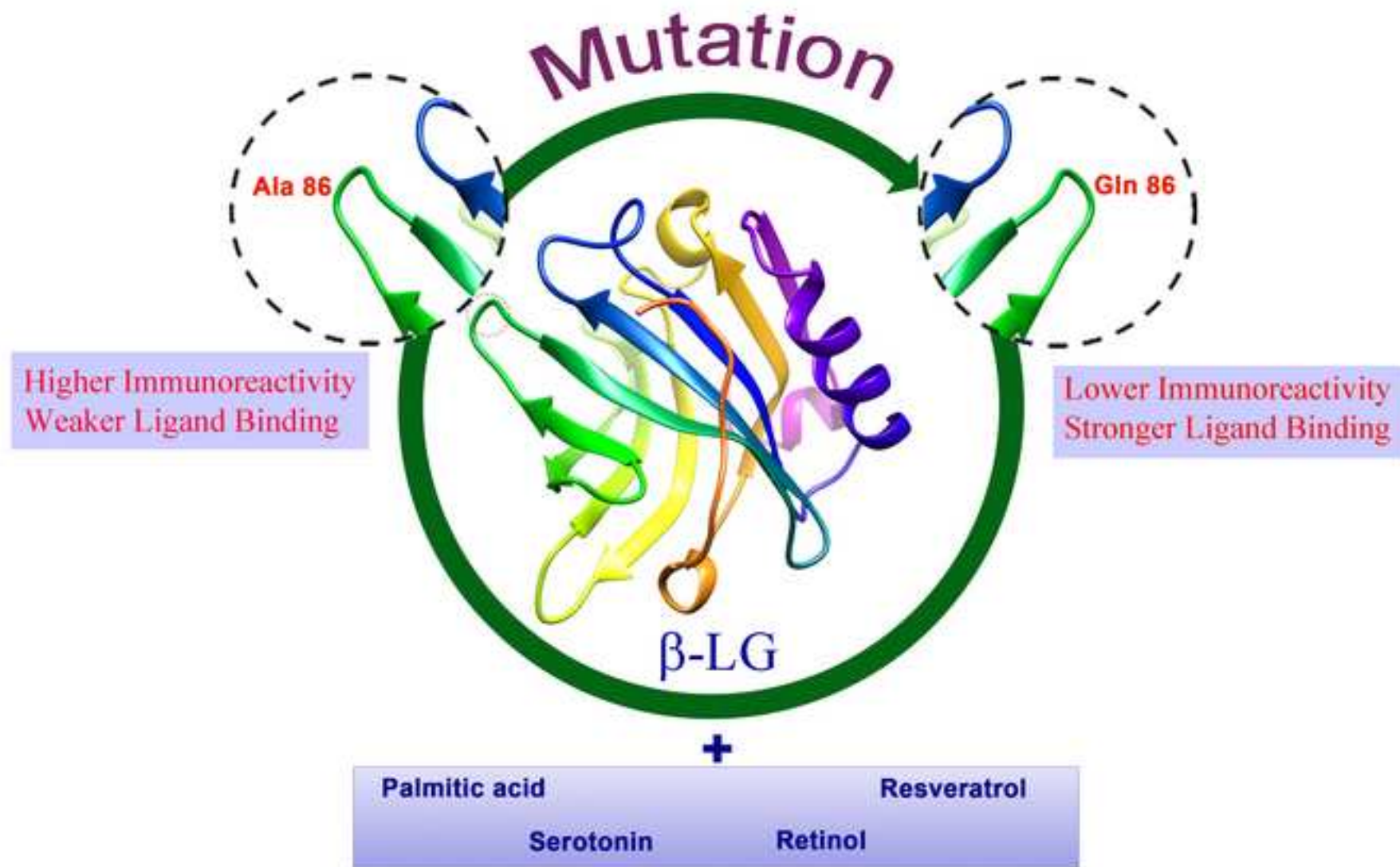
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Highlights

- 1- Wild-type and Ala86Gln mutant β -LG expressed in *P. pastoris* and purified in good yield.
- 2- The purity and native like folded structure of Ala86Gln confirmed by different techniques.
- 3- Ala86Gln β -LG had stronger binding affinity to ligands than native protein.
- 4- Ala86Gln mutant had attenuated allergenicity than native protein.
- 5- Alanine86 have significant role on the binding and immuoreactivity properties of β -LG.

The Ala86Gln mutant β -Lactoglobulin has increased ligand binding and decreased immunoreactivity properties

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ABSTRACT: β -Lactoglobulin (β -LG) is a member of lipocalin superfamily of transporters for small hydrophobic molecules. β -LG is also one of the major allergens in milk. Despite a lot of researches on decreasing of cow's milk allergenicity, the effects of the mutation of β -LG on its recognition by IgE from cow's milk allergy (CMA) patients have not been investigated. We described here the expression in the yeast *Pichia pastoris* of a mutant β -LG, in which Alanine86 was changed into Glutamine (Ala86Gln; a mutation on one of the major epitopes of the protein). The purity and native like folded structure of the recombinant Ala86Gln have been demonstrated using circular dichroism, HPLC, SDS-PAGE and mass spectrometry. The effect of the mutation on the binding of IgE from CMA patients to mutant protein was evaluated by ELISA methods and the results showed that the mutation of Ala86 was associated with weaker binding of IgE from CMA patients to Ala86Gln mutant protein. Subsequently, the binding of various ligands such as retinol, palmitic acid, resveratrol and serotonin, with native, recombinant wild type and Ala86Gln mutant β -LGs were investigated by fluorescence spectroscopy and an improvement on the binding affinity of the mutated protein to various ligands was observed.

Key word: β -Lactoglobulin; Allergy; Mutation; Ligand binding; Competitive ELISA; Circular dichroism.

1. Introduction

Food allergies are considered as the sixth problem of human health by World Health Organization (WHO). Due to a growing spread of food allergy in developed countries, it has become one of the most serious concerns in health issues [1,2]. Some of the recent investigations have demonstrated that allergenicity to certain foods or food ingredients affects both children and adults and within 2% to 10% of population is faced with food allergy [3]. For instance, in the USA, it is estimated that between 4.5 and 8% of the children aged below 2 years old are suffering from food allergies [4]. Food allergy happens when a person exhibits a high level of IgE directed against any ingested food antigen [5,6].

Among Food-induced allergies, milk allergy is one of the most prevalent hypersensitivities in young infants, with an estimated prevalence approximately 2-6 % [7,8]. Cow's milk allergy (CMA), defined as an immunologically mediated reaction against cow's milk antigens, is an important problem in pediatrics. Even though children outgrow CMA after 3 years of age, they might predispose the progress of other allergies in adults such as asthma, atopic eczema, rhinoconjunctivitis or egg allergy [9]. Allergic responses in CMA patients that could be run by entering of cow's milk proteins, are mediated by Th2 responses and release of allergen-specific IgE [10]. These IgE mediated reactions are led to clinical symptoms, resembling skin, respiratory tract, gastrointestinal tract, and systemic anaphylactic reactions [11,12].

To date, several proteins have been identified as milk official allergens. The most abundant cow's milk proteins are α_{S1} -, α_{S2} -, β -, and κ -caseins, β -lactoglobulin (β -LG) and α -lactalbumin. Caseins, β -LG and α -lactalbumin are the main allergens in cow's milk [13]. β -LG, the most abundant whey protein, is the most potent milk allergen and is recognized by the IgE of >90% of milk allergic patients. β -LG is a small globular protein of 162 amino acid residues with a

molecular mass of 18.36 kDa [14-16]. 3D-Crystallographic studies have shown that β -LG three-dimensional structure consists of nine anti-parallel β -sheet structures forming a so-called β -barrel (or calyx) stabilized by two disulfide bonds (one cysteine residue remaining free) [17-19].

Despite the function of β -LG in physiological condition is unknown, unique structure, its high water solubility and binding affinity to different molecules propounded β -LG as a carrier for various ligands such as drugs, fatty acids and vitamins [20-22]. Four potential binding sites have been reported for various ligands on β -LG: Calyx or the central cavity shaped by β -barrel, the hydrophobic pocket that is placed in a groove within the α -helix and β -barrel, a surface site near the entrance of the β -barrel close to Trp19-Arg124, and an area in the dimerization interface [23-25]. The interaction of ligands and proteins contributes to upgrade the biological properties and the solidity to both the protein and ligand. By far, interaction of β -LG with various compounds have been surveyed, including fatty acids [26], curcumin [27], surfactants [28], chemotherapeutic antibacterial drugs [29], vitamins [30], etc. It has therefore been proffered that β -LG could be utilized as a pertinent carrier in applications of controlled ligand delivery [31].

It has been found that food proteins with extreme resistance to structural changes caused by heat, low pH and hydrolysis by gastric fluid enzymes, have an increased probability of arriving to the intestinal mucosa where they can be absorbed and trigger a possible immune response [32,33]. Accordingly, factors such as low stomach pepsin activity at birth, an immature stomach acid generating mechanism and malfunction of pancreatic and intestinal enzymes contribute to the stability of β -LG by limiting its gastric proteolysis and therefore expose neonates to “allergic” responses [34,35]. Studies on epitope mapping of β -LG proposed several immunoreactive epitopes; residues 41-60, 102-124, 149-162 and 84-91 have been recognized as the common immunoreactive epitopes of the protein [36].

As long as β -LG is concerned, numerous studies on reduction of its allergenicity have been performed including heat treatment, enzymatic hydrolysis, pressure, and glycosylation, which focused on the modification of β -LG structure or destruction of its epitopes [37-40]. These procedures often destroy the 3D structure of β -LG and consequently affect its physiological function as a carrier protein. Recombinant mutant proteins, with mutation of key residues in binding sites of the allergen without interrupting the global structure, would be a possible solution for lucrative allergen-specific immunotherapy [41-46].

Despite a lot of researches on decreasing of cow's milk allergenicity, the effects of the mutation of β -LG, on its recognition by IgE from CMA patients have not been studied to date. Hence, in the present study, an expression system was developed for the production of recombinant Ala86Gln mutant β -LG (a mutation on the one of the major epitopes of the protein) and therewith the effect of the mutation on the binding of IgE from CMA patients was evaluated. Subsequently, the interactions of various ligands, such as retinol, palmitic acid, resveratrol and serotonin, with native, recombinant wild type and mutant β -LGs have been investigated using spectroscopic methods.

2. Materials and methods

2.1. Materials

The chemicals were of analytical grade with the highest degree of purity (Sigma-Aldrich and Merck chemical companies) and were used without further purification. All prepared solutions with double distilled water were kept at 4 °C before usage. β -LG variant A (as a pattern in all experiments) was acquired from homozygote cow's milk following the method of Maillart and Ribadeau Dumas [47] and it was >97% pure as judged from high performance liquid

chromatograms and polyacrylamide gel electrophoresis. β -LG was dissolved in aqueous solution containing 10 mM phosphate buffer pH 7.4. The protein concentration was determined spectrophotometrically by using molar extinction coefficient of $17600 \text{ M}^{-1}\text{cm}^{-1}$ (MW = 18.36 kD) at 280 nm [48]. The ligands solutions was prepared in ethanol/water 50 / 50% and diluted in 10 mM phosphate buffer pH 7.4, to various concentrations with the final ethanol concentration of 4%. The presence of 4% ethanol induces no major protein structural changes [49].

2.2. Recombinant protein expression and purification

The wild type (variant A) recombinant β -LG expression system, constructed before by Wilson et al. [50], was used as a template. Alanine86 was substituted by Glutamine (Ala86Gln) due to site-directed mutagenesis according to the QuickChange protocol from Stratagene (La Jolla, CA) with the following primers: 5' primer, 5'-GC-CTG-GTC-TGC-CAG-AGT-CTG-GTC-AGG-ACC-CC-3'; 3' primer, 5'-GG-GGT-CCT-GAC-CAG-ACT-CTG-GCA-GAC-CAG-GC-3'. Amplified DNA was transformed into Epicurian *Escherichia coli* XL1-blue cells (Stratagene) and plated for selection on Luria broth (LB)-agar plates (supplemented with 25 $\mu\text{g}/\text{mL}$ Zeocin). The miniprep kit from Qiagen (Venlo, The Netherlands) was utilized for purification of plasmids from positive clones and was sequenced to confirm the correct introduction of the mutation (MWG Biotech, Ebersberg, Germany). Each plasmid confirmed to involve the desired sequence was linearized with BspH1 and electroporation of *P. pastoris* was performed with a pulse voltage of 1.7 kV using an *E. coli* pulser (Bio-Rad). The best producing clones were selected by culture for 72 h in YPD medium (1% yeast extract, 2% peptone, 2% glucose).

The quantity of expressed protein was investigated with SDS-PAGE, and one of the strains with the highest level of expression was picked out for each recombinant β -LG. So, a fresh

colony of *P. pastoris* was grown overnight (37 °C, 200 rpm) in 40 mL of buffered glycerol complex medium (BMGY) supplemented by glucose (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 0.04% biotin, 2% glucose, 100 mM potassium phosphate buffer, pH 7.0, Zeocin™ 100 µg/mL). This culture was utilized to inoculate 2 L culture flasks containing 250 mL of BMGY each. Constitutive expression of the protein was performed at 37 °C under shaking (200 rpm) during 72 h with occasional pH readjustment with 1 M phosphate buffer, pH 7.0 and glucose addition. The protein expression during the culture was followed by SDS-PAGE. After 72 h, the broth of mass culture was centrifuged and filtration of the supernatant was performed with 5.0 µm pore size Millex-SV (Millipore, Bedford, MA). The filtered supernatant was dialyzed against distilled water on a modular tangential flow system (Vivaflow 50, Vivascience, Hanover, Germany).

Each type of recombinant β-LG in the supernatant was loaded onto an anion exchange column Q-Sepharose XL Streamline, (200 x 26 mm; Pharmacia, Peapack, NJ) equilibrated with 20 mM Tris-HCl buffer (pH 6.7) and eluted with a gradient of 0–1.0 M NaCl. The protein content of each fraction was confirmed by SDS-PAGE. The recombinant wild type (WT) and Ala86Gln mutant β-LGs were principally eluted at 0.3 M NaCl. These fractions containing β-LGs were concentrated by ultrafiltration (Vivaspin 20, 10 kDa MWCO, Vivascience) and passed through a Superdex 75 gel filtration column type XK26 (700 x 26 mm, Pharmacia) equilibrated with 50 mM sodium phosphate buffer, 0.1 M NaCl, pH 7.0, at a flow-rate of 4 mL/min. The solutions of purified β-LGs were dialyzed against distilled water and lyophilized. The purities of the recombinant WT and Ala86Gln mutant β-LGs were very high assessing from the patterns of SDS-PAGE, reverse-phase high-performance liquid chromatography (RP-HPLC) and electro-spray ionisation mass spectrometry (ESI-MS).

2.3. *Electro-spray ionisation mass spectrometry experiments*

ESI-MS experiments were performed on a Thermo Finnigan LCQ Advantage (San Jose, CA) by infusion at a flow-rate of 2.5 $\mu\text{L}/\text{min}$. Native, recombinant WT and Ala86Gln mutant β -LG samples were at 5 pmol/ μL in H_2O -acetonitrile (1/1, v/v) + 0.5 % formic acid.

2.4. *Reverse-phase high-performance liquid chromatography (RP-HPLC) experiments*

RP-HPLC experiments were carried out using a 1200 series Agilent HPLC and an Agilent automatic injector (Agilent Technologies, Palo Alto, CA), with a diode array detector set at 220 nm. Separation used an Alltech Prevail C_{18} column with particle size of 5 μm and dimensions of 4.6×150 mm (Grace, Illinois, USA), which was connected to a guard cartridge system and was operated at a flow rate of 0.3 mL/min at 20 $^{\circ}\text{C}$. All samples were filtered through a 0.45 μm syringe filter before injection. The injection volume was 50 μL , and samples were eluted using linear gradients of solvent A (10% v/v acetonitrile, 90% v/v H_2O with 0.1% v/v trifluoroacetic acid) and solvent B (90% v/v acetonitrile, 10% v/v water with 0.1% v/v trifluoroacetic acid). The mobile phase for the first 3 min was 100% A, then a linear gradient of 100% A to 50% A was applied using solvent B over 45 min, then a sharper gradient to 30% A over the next 5 min. This composition was retained for 5 min, and then returned to 100% A over 5 min. EZ Chrom Elite software (version 3.3.1) was used for data acquisition.

2.5. *SDS-PAGE electrophoresis experiments*

Proteins were analyzed by 12% SDS-PAGE. Briefly, proteins were mixed in loading buffer (60 mM Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.025% (w/v) bromophenol blue) and packed on 12% polyacrylamide gel containing 0.1% (w/v) SDS. Migration was executed for

about 1 h in a buffer containing 0.3% (w/v) Tris and 1.44% (w/v) glycine. The gels were stained with an aqueous solution comprising 50% (v/v) ethanol, 10% (v/v) glacial acetic acid and 0.25% (w/v) Coomassie brilliant blue and destained with a solution containing 10% (v/v) ethanol and 7% (v/v) glacial acetic acid. Electrophoresis experiments were carried out under reducing conditions in which 5% reducing agent β -mercaptoethanol (β -ME) was added to the sample buffer.

2.6. Circular dichroism experiments

Circular dichroism (CD) spectra were recorded on a CD6 dichrograph (Jobin Yvon, Longjumeau, France), using cells of the appropriate lengths and a scan time of 1 nm/sec. All measurements were carried out at 25 °C with thermostatically controlled cell holders. The instruments were calibrated with ammonium d-10-camphorsulfonic acid. The data were expressed as molar residue ellipticity $[\theta]$, which is defined as:

$$[\theta] = 100 \theta_{\text{obs}}/cl \quad (1)$$

where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue.mol.cm⁻³, and l is the length of the light path in cm. For measuring CD spectra in the far-UV region, the samples concentration was 1 mg/mL in 50 mM phosphate buffer pH 7.0, and the spectra were recorded between 190 and 260 nm using a quartz cuvette with 0.1 mm path length. Each spectrum was the accumulation of five successive measurements and buffer spectrum was subtracted for baseline correction.

A protein concentration of 0.5 mg/mL was used for near-UV CD spectra and the spectra were recorded between 260 and 350 nm using quartz cuvette with 1 cm path length. Four scans were

averaged per spectrum. The baseline was corrected by subtracting the corresponding solvent spectrum from sample spectrum.

2.7. β -LG titration with different ligands

Tryptophan fluorescence spectra were recorded on RF-5000 Shimadzu spectrofluorimeter with a cell compartment thermostated at 293 K. The excitation and emission slits were assigned at 5 nm. The interaction of different ligands involving retinol, resveratrol, palmitic acid, and serotonin, to the proteins (native, recombinant WT and Ala86Gln mutant β -LGs) were assessed by following the fluorescence quenching of protein tryptophan (at 335 nm). Intrinsic fluorescence experiments of three β -LGs were performed at constant protein concentration (5 μ M) titrated with different ligand concentrations to have at least a molar *ratio* of ligand/protein equal to 30. Emission spectra were recorded between 300 and 450 nm with an excitation wavelength of 287 nm and at a scan rate of 60 nm/min. All measurements were performed in 50 mM phosphate buffer pH 7.0. Fluorimetric measurements of the blank containing N-acetyl-L-tryptophanamide, titrated by the ligands were done according to Cogan et al. [51] The decrease in fluorescence intensity of N-acetyl-L-tryptophanamide solution during the titration is not due to the interaction between ligand and tryptophanamide, but it results from the inner filter effect as a consequence of ligand absorbance at 287 nm. After correction for the blank according to Cogan et al., differences in fluorescence intensity at 335 nm between the complex and free protein were monitored in order to measure the apparent dissociation constants of three β -LGs with different ligands. It was assumed that the changes in the fluorescence intensities depend on the amount of protein-ligand complexes. On the assumption that there are (n) substantive binding sites for quencher (L) on protein (P), the quenching reaction can be shown as following:



The binding constant (K_a) can be calculated as:

$$K_a = \frac{[L_nP]}{[P][L]^n} \quad (3)$$

where $[L]$ and $[P]$ refer to free protein and ligand concentrations, respectively, $[L_nP]$ is the concentration of non fluorescent fluorophore-quencher complex, and $[P_0]$ gives total protein concentration:

$$[L_nP] = [P_0] - [P] \quad (4)$$

$$K_a = \frac{[P_0] - [P]}{[P][L]^n} \quad (5)$$

The fluorescence intensity is proportional to the protein concentration as described:

$$\frac{[P_0]}{[P]} \propto \frac{[F_0]}{[F]} \quad (6)$$

Results from fluorescence measurements can be used to estimate the binding constant of ligand-protein complex and the binding number [52]. From equation 5:

$$\log \frac{F_0 - F}{F} = \log K_a + n \log [L] \quad (7)$$

where F_0 and F are the fluorescence emission intensities of protein without and with ligand, respectively (after subtracting the fluorescence intensity of ligand alone in buffer solution), $[L]$ is ligand concentration, K_a is the binding constant, and n is the binding number. The plot of $\log[(F_0 - F)/F]$ as a function of $\log [L]$ will be a linear plot with an intercept of $\log K_a$ and a slope of n .

2.8. Colorimetric ELISA (C-ELISA) experiments

Wells of 96 microtitration plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight with 100 μ L per well native, recombinant WT and Ala86Gln mutant β -LGs diluted to 5 μ g/mL in PBS, or with PBS in wells utilized as negative controls. Next, they were washed three times with PBS containing 0.1% (v/v) Tween-20 (PBS/T) and saturated for 2 h with 250 μ L of a solution of PBS/T containing 1% (w/v) polyvinyl alcohol (PBS/T/PVA). After three times washing with PBS/T, Plates were incubated for 90 min at room temperature with 100 μ L of mouse monoclonal anti- β -LG antibody (IgG) diluted to 1:100 in PBS/T/PVA from hybridoma supernatant. Mouse monoclonal anti- β -LG antibodies mAb37, mAb96, mAb100, and mAb116, which bind β -LG on four distinct epitopes independently of secondary and tertiary structures of β -LG, were produced and characterized in Nantes, INRA laboratory [53]. Plates were washed three times with PBS/T and incubated for 1 h at room temperature with a peroxidase conjugated antimouse IgG (Bio-Rad Laboratories, Hercules, CA) diluted 1:3000 in PBS/T/PVA. After three times washing with PBS/T, the secondary antibody binding was revealed by addition of 100 μ L of OPD (Sigma, 0.4 mg/mL in 50 mM citrate buffer, pH 5.5). The staining reaction was stopped after 20 min of incubation at room temperature using 100 μ L of 2 M H_2SO_4 . The absorbance was measured at 492 nm with the ELX₈₀₀ plate reader (BioTek Instruments, Inc., Winooski, VT).

2.9. Competitive ELISA experiments

A series of 24 sera from CMA patients presenting various symptoms were used. Total milk proteins and β -LG-specific IgE concentrations were determined with the Phadia ImmunoCAP System®. All of the sera had total milk proteins-specific IgE concentrations from <0.37 kAU/L to 62.6 kAU/L. For competitive ELISA the 96 micro titration plates (Nunc™) were coated

overnight with native β -LG diluted to 5 $\mu\text{g/mL}$ in PBS buffer. After coating, they were washed 3 times with PBS/T and saturated for 2 h with 250 μL of a solution of PBS/T/PVA. Plates were washed 3 times with PBS/T. Wells coated with native β -LG and in those coated with PBS as negative control, 100 μL of a pool of sera from patients preincubated for 2 h at 37 $^{\circ}\text{C}$ in the presence of increasing concentrations of competitor (native, recombinant WT and Ala86Gln mutant β -LG) were added. The final dilution of the serum was 1:20, and the final concentrations of the inhibitors ranged from 0 to 100 $\mu\text{g/mL}$ (1:10 serum pool dilution and inhibitor solution were mixed v/v to a final volume of 100 μL). After an overnight incubation at 4 $^{\circ}\text{C}$, the plates were washed 3 times with PBS/T and incubated for 2 h at room temperature with an alkaline-phosphatase conjugated polyclonal anti-human IgE (Sigma) diluted 1:1000 in PBS/T/PVA. The secondary antibody binding was revealed, after 3 times washings with PBS/T, by addition of 4-methylumbelliferyl phosphate (4-MUP) substrate diluted 1:5 in 1 M Tris-HCl, pH 9.8. The fluorescence emission was measured after 90 min incubation at temperature 37 $^{\circ}\text{C}$ with the FLX₈₀₀ plate reader (BioTek Instruments) fitted with a 360 nm excitation filter and a 440 nm emission filter. The concentration of β -LG (inhibitor) required to inhibit 50% of the total binding of serum Ig E (IC_{50}) was calculated from the inhibition curves by relating fluorescence intensity to inhibitor concentrations. Fluorescence data were corrected by subtracting the fluorescence of the control with no antigen.

3. Results and discussion

3.1. Production of recombinant proteins

Recombinant WT and Ala86Gln mutant β -LGs were produced and purified in methylotrophic yeast *P. pastoris*. According to the above protocol, recombinant WT and Ala86Gln β -LGs were expressed in final yields of 160 mg and 145 mg per 1 liter of culture medium, respectively. The results indicated that the addition of the amid side chain at Ala-86 hadn't any perceptible effect on the production yield, which was in agreement with previously described yields for recombinant β -LG production in *P.pastoris* [50,54].

The purity of recombinant proteins was confirmed by RP-HPLC and SDS-PAGE techniques. As shown in Figure 1, the elution time and the position of the native, recombinant WT and Ala86Gln mutant β -LG were around 34 min and confirmed the production of recombinant proteins. The integration of chromatographic peaks in Figure 1 demonstrated the purity above 95% for all produced proteins. The electrophoresis profile of recombinant WT and Ala86Gln mutant proteins in reducing conditions showed the existence of a single band with an apparent molecular mass of about 18 kDa corresponding to the monomer proteins and confirmed the purity of the both recombinant β -LGs (Figure 2).

[Fig. 1 and 2 to be inserted here]

The identity of the proteins was confirmed by ESI-mass spectrometry. The molecular weight of 18346.9, 18346.0 and 18404.4 Da has been calculated from the mass spectra of native, recombinant WT and Ala86Gln mutant β -LGs (Figure 3a, b, and c), respectively. No glycosylation was detected for recombinant WT and Ala86Gln mutant β -Lgs as is the case of β -Lg A purified from bovine milk. As it shown in Figure 3, analysis of recombinant proteins by mass spectrometry confirmed their purity, matching the calculated molecular mass with their mass theoretical, and the lack of post-translational modifications.

[Fig. 3a, b and c to be inserted here]

3.2. Circular dichroism results

The evolution of the secondary structure of β -LG through Ala86Gln mutation was analyzed by far-UV CD (Figure 4a). The far-UV CD spectrum of native, recombinant WT and Ala86Gln mutant β -LGs at pH 7.0 showed a minimum at 218 nm, which they are chiefly β -sheet proteins. As shown in Figure 4a, the spectra of both recombinant WT and mutant proteins were identical to that of native β -LG, showing that the recombinant proteins had the same secondary structure as native β -LG. Also the near-UV CD results showed that the recombinant WT protein with two minima at 285 and 293 nm was also very similar to that of the native β -LG, indicating similar tertiary structure of native and recombinant wild type proteins (Figure 4b). As shown in this figure, the near-UV CD spectra of recombinant Ala86Gln mutant β -LG represented slight differences compared to the native β -LG spectrum in the 260-280 nm regions, demonstrating that the mutation of Ala-86 did not affect the β -LG tertiary structure to any large extent and accordingly did not change the global conformation of protein.

[Fig. 4a and b to be inserted here]

3.3. β -LG/ligands interactions results

The changes in the binding properties of β -LG due to mutation were studied using retinol, resveratrol, palmitic acid, and serotonin as ligands. The intrinsic fluorescence of protein has been widely used to investigate the structural transition and binding properties of proteins in solution. β -LG has two tryptophan residues Trp-19 and Trp-61. Trp-19 is in an apolar environment and contributes to 80% of total fluorescence, while Trp-61 is partly exposed to aqueous solvent and has minor contribution to tryptophan fluorescence [55]. When other molecules interact with β -

LG, tryptophan fluorescence may change depending on the impact of such interaction on the protein conformation [56]. Figure 5 shows the fluorescence emission spectra of three kinds of β -LG in the presence of different concentrations of retinol at pH 7.0. The fluorescence spectra of other ligands were similar to that of retinol (data not shown). The decrease of fluorescence intensity of all β -LGs is observed as the concentration of the ligands increased. As it shown in Figure 5, the extent of the observed quenching of β -LG tryptophan fluorescence depends on β -LG type studied (native, recombinant WT and Ala86Gln mutant β -LGs). It also varies according to the applied ligands (retinol, resveratrol, palmitic acid, and serotonin) that could be explained by the structural differences between the studied ligands.

[Fig. 5 to be inserted here]

The decrease of fluorescence intensity of β -LG is monitored at 340 nm for ligand/ β -LG systems (Figure 5). The apparent binding constants of ligand/ β -LG complexes and the apparent molar ratio of ligand/ β -LG at saturation were calculated from the intercept and slope of the traced line acquired from plotting $\log [(F_0-F)/F]$ versus $\log [L]$ (the inset of Figure 5). The values of the average binding constant, K_a , for the interaction of three β -LGs with retinol, resveratrol, palmitic acid, and serotonin are presented in Table 1. As it shown in this table, the binding constants calculated for the β -LG/ligand adducts, suggested strong recombinant Ala86Gln mutant β -LG/ligand interactions compared to the native β -LG/ligand and recombinant WT β -LG/ligand complexes. Moreover, slight differences were perceived between native and recombinant WT β -LGs interactions with ligands, as it was expected. The results correlated with the binding constant found in literature for interaction of native β -LG with these ligands [23,57,58]. In fact, the native and mutant β -LGs diverge only by one amino acid at position 86 which is located at the entrance of the hydrophobic site of protein. Therefore it may affect the

binding properties of hydrophobic ligands to protein. In this work, a non-polar Alanine at position 86 is swapped by polar Glutamine in mutant protein, which could permit better interactions. Even though, a single mutation is not expected to significantly modify the secondary and tertiary structures of β -LG, but considering Alanine 86 is in the entrance of β -LG binding site, it can be effective in the binding of β -LG to ligands.

3.4. Response of patient sera to native, recombinant WT and Ala86Gln mutant β -LGs

The potential binding of IgE from patients having CMA on native, recombinant WT and Ala86Gln mutant β -LG was perused by competitive ELISA. Because mutation of β -LG causes modifications in the structure of protein, it was essential to check that recombinant WT and Ala86Gln mutant β -LGs were able to coat the wells as well as native β -LG. Accordingly the adsorption of recombinant WT and mutant β -LGs on microtitration plates was checked by C-ELISA, using four β -LG-specific monoclonal antibodies mAb37, mAb96, mAb100 and mAb116, which bind β -LG on four different epitopes independently of secondary and tertiary structures of β -LG.⁵³ The results showed that coating of microtitration plate wells with recombinant WT and Ala86Gln mutant β -LGs, were identical to what was detected with native β -LG (Figure 6).

[Fig. 6 to be inserted here]

For IgE binding inhibition experiments, a pool of sera constituted by 24 sera having CMA was preincubated with specific quantities of the inhibitors (native or recombinant WT or recombinant Ala86Gln mutant β -LGs). The results of competition between the native and recombinant proteins were plotted as dose-response curves. Lower binding of β -LG-specific IgE to Ala86Gln mutant β -LG was confirmed by IgE binding inhibition experiments (Figure 7). The

standard deviation was not reported on the graph because it was always <10%. As shown in Figure 7, recombinant Ala86Gln mutant β -LG had a shift to higher concentrations of the inhibitors than native and recombinant WT β -LGs. The calculated IC_{50} values were 0.46 and 0.41 μ g/mL for native and WT β -LGs, respectively, and increased due to mutation to reach 4.29 μ g/mL for Ala86Gln mutant β -LG (Table 2), indicating that mutated β -LG is less recognized (about 9 times) than native and WT β -LGs by IgE from CMA patients. Because the mutation caused slight modification on β -LG structure, it seems that the mutation of non-polar Alanine to polar Glutamine is responsible for the disappearance of one of the major epitopes of protein (84-92) as well as some conformational epitopes and, consequently, of reduced binding.

[Fig. 7 to be inserted here]

4. Conclusions

In recent years, several methods have been studied to reduce milk allergenicity including heat treatment, enzymatic hydrolysis, pressure, and glycosylation. These processes often destroy the 3D structure of β -LG and consequently affect its physiological function as a carrier protein. The mutation would be superior solution for decreasing protein allergenicity by elimination of allergen epitopes on the protein structure. The current study was carried out in order to the express in the yeast *Pichia pastoris* a mutant bovine β -LG, in which Alanine 86 (the residue that located near the main binding site of the protein) was changed into Glutamine to remove one of the major epitopes of β -LG. RP-HPLC and SDS-PAGE techniques demonstrated the purity above 95% for all produced recombinant proteins. Analysis of recombinant proteins by mass spectrometry confirmed their purity, matching the calculated molecular mass with their mass theoretical, and the lack of post-translational modifications. The native like folded structure of

the recombinant Ala86Gln mutant β -LG have been demonstrated using circular dichroism spectroscopy. The effect of the mutation on the binding of IgE from CMA patients was evaluated by ELISA methods and the results showed that mutated β -LG was about 9 times less recognized than native and recombinant WT β -LGs by IgE from CMA patients. Moreover, the interactions of various ligands, such as retinol, palmitic acid, resveratrol and serotonin, with native, recombinant wild type and mutant β -LGs have been investigated using fluorescence spectroscopy and the results illustrated an improvement on the binding affinity of the mutated protein to the ligands. The results of this study proved the significant role of Alanine 86 residue on the binding and allergenicity properties of β -LG. finally it can be concluded that mutated β -LG might act as a carrier to transport ligands in vitro without immunologically mediated reactions.

Abbreviations used

β -LG, β -lactoglobulin; WT, wild type; Ala, Alanine; Gln, Glutamine; CMA, cow's milk allergy; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electro-spray ionisation mass spectrometry; CD, circular dichroism; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Table 1. Binding affinity constant (K_a) and binding number (n) for the interaction of various ligands with native, recombinant wild-type, and Ala86Gln mutant β -LGs in 20 mM phosphate buffer pH 7.0 at 298 K.

Protein	Ligand	K_a (M)	n
Native	Retinol	$2.7 (\pm 0.07) \times 10^8$	$0.91 (\pm 0.06)$
	Palmitic Acid	$3.6 (\pm 0.10) \times 10^8$	$1.01 (\pm 0.02)$
	Resveratrol	$8.3 (\pm 0.19) \times 10^5$	$0.97 (\pm 0.04)$
	Serotonin	$4.9 (\pm 0.22) \times 10^5$	$1.14 (\pm 0.08)$
WT	Retinol	$3.1 (\pm 0.14) \times 10^8$	$0.94 (\pm 0.07)$
	Palmitic Acid	$3.9 (\pm 0.12) \times 10^8$	$0.96 (\pm 0.03)$
	Resveratrol	$8.5 (\pm 0.24) \times 10^5$	$0.91 (\pm 0.03)$
	Serotonin	$4.4 (\pm 0.17) \times 10^5$	$1.18 (\pm 0.11)$
Mutant	Retinol	$1.5 (\pm 0.13) \times 10^9$	$1.08 (\pm 0.06)$
	Palmitic Acid	$1.9 (\pm 0.16) \times 10^9$	$1.04 (\pm 0.09)$
	Resveratrol	$4.8 (\pm 0.21) \times 10^6$	$0.94 (\pm 0.05)$
	Serotonin	$9.6 (\pm 0.13) \times 10^5$	$1.09 (\pm 0.07)$

Table 2. Inhibition of IgE binding to native β -LG by native, recombinant wild-type, and Ala86Gln mutant β -LGs.

β -LG treatment	Native	WT	Mutant
IC ₅₀ ($\mu\text{g.mL}^{-1}$)	0.46	0.41	4.29

Figure Legends

Figure 1. RP-HPLC analysis of native, recombinant wild type, and Ala86Gln mutant β -LGs.

Figure 2. Analysis of 12% SDS-PAGE of native (1), recombinant wild type (2), and Ala86Gln mutant (3) β -LGs in 20 mM phosphate buffer pH 7.0 in reducing condition; M, molecular mass marker (kDa).

Figure 3. Electro-spray ionisation mass spectra of (a) native, (b) recombinant wild type, and (c) Ala86Gln mutant β -LGs. The highest peak was used to calculate the molecular weight of proteins.

Figure 4. Far-UV (a) and near-UV (b) circular dichroism spectra of native, recombinant wild type, and Ala86Gln mutant β -LGs in 20 mM phosphate buffer pH 7.0.

Figure 5. Fluorescence emission spectra of 5 μ M (a) native, (b) recombinant wild type, and (c) Ala86Gln mutant β -LGs in the presence of various concentrations of retinol in 20 mM phosphate buffer pH 7.0 at 298 K. Similar spectra were observed for other ligands, resveratrol, palmitic acid and serotonin.

Insets: The Stern-Volmer equation; $\log[(F_0-F)/F]$ vs. $\log [L]$ as per equation 7.

Figure 6. Adsorption of native, recombinant wild type, and Ala86Gln mutant β -LGs on ELISA plate: signal from the binding (optical density) of four anti- β -LG monoclonal antibodies in wells coated with native, recombinant wild type, and Ala86Gln mutant β -LGs.

Figure 7. Inhibition of anti- β -LG IgE in serum pool. Comparison between native, recombinant wild type, and Ala86Gln mutant β -LGs. The signal (AU) resulting from the binding of IgE on native β -LG coated plates is shown.

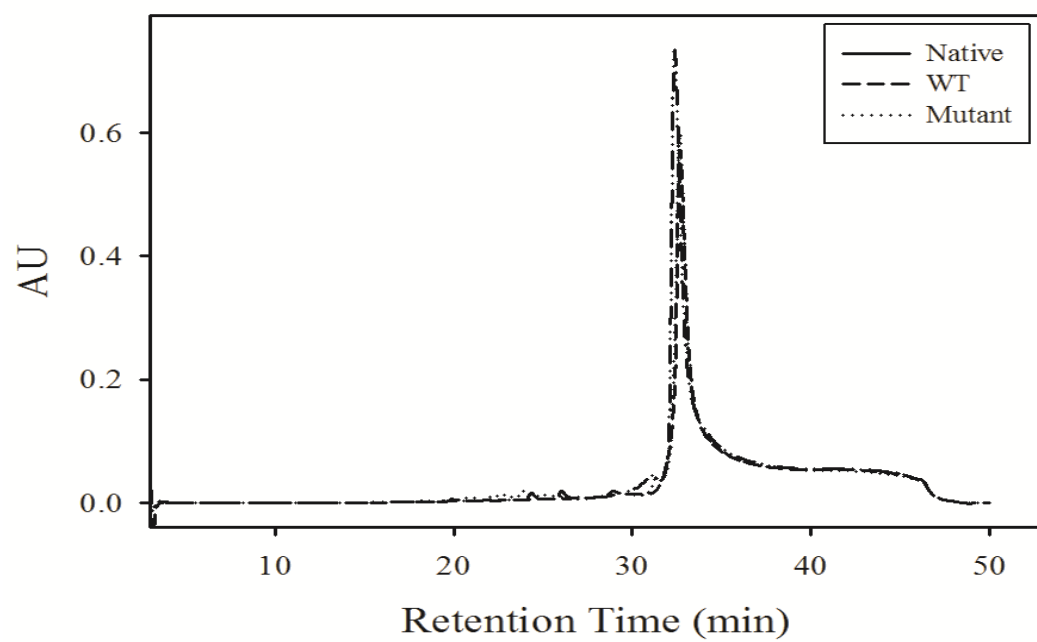
Figure 1

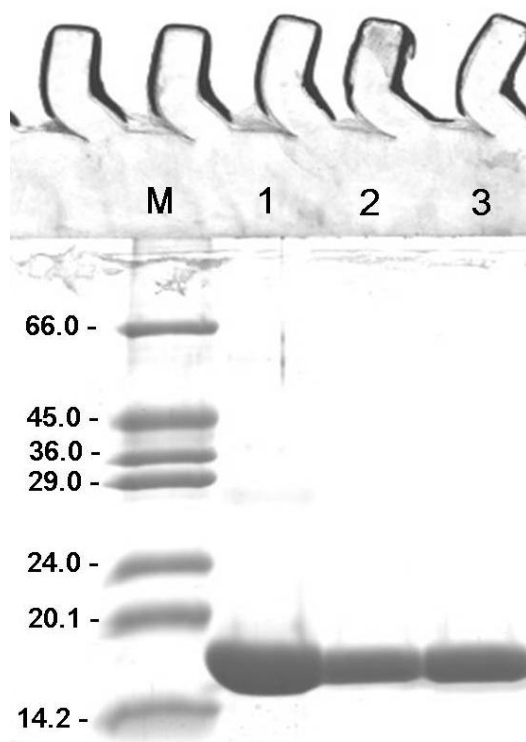
Figure 2

Figure 3a

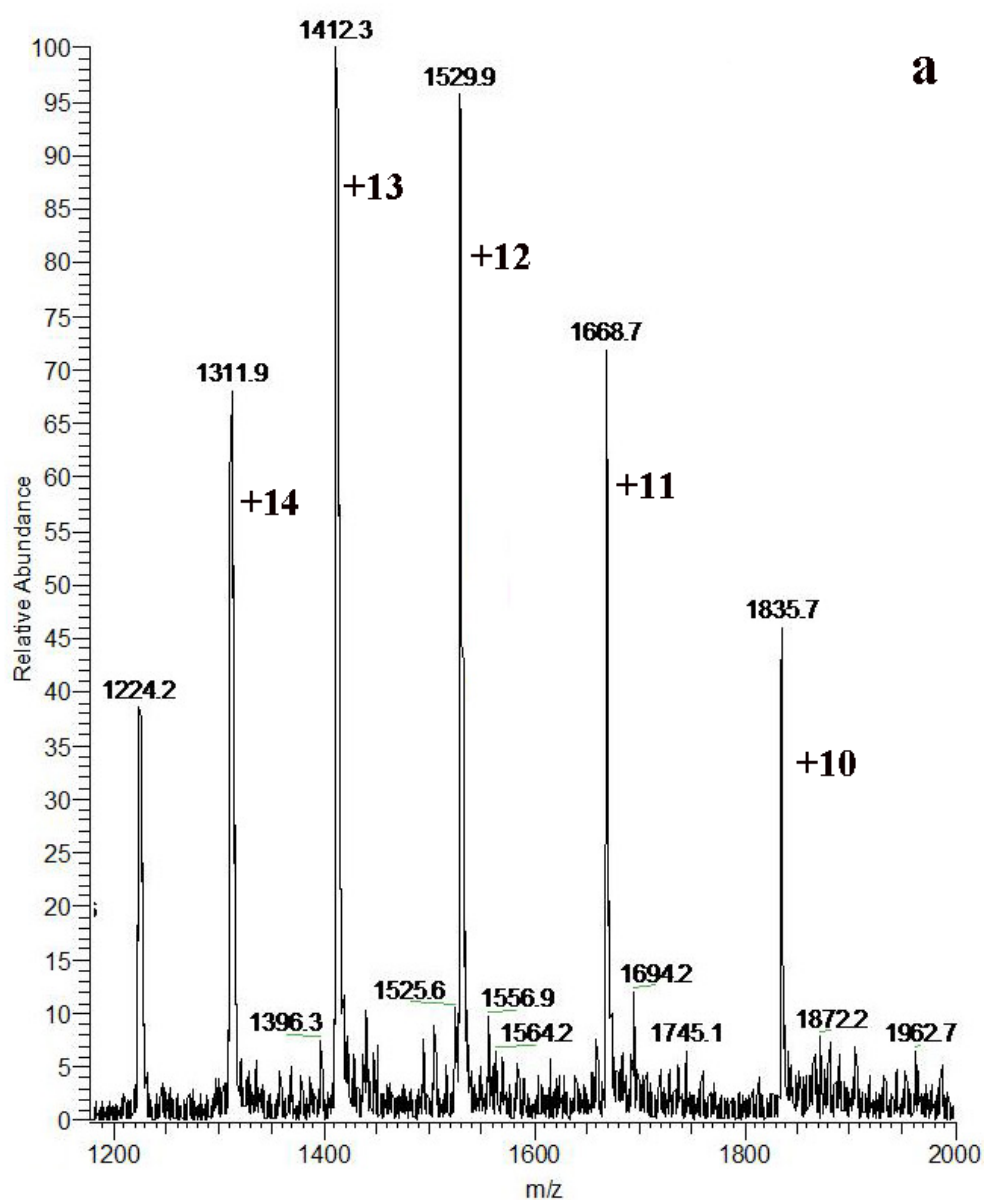


Figure 3b

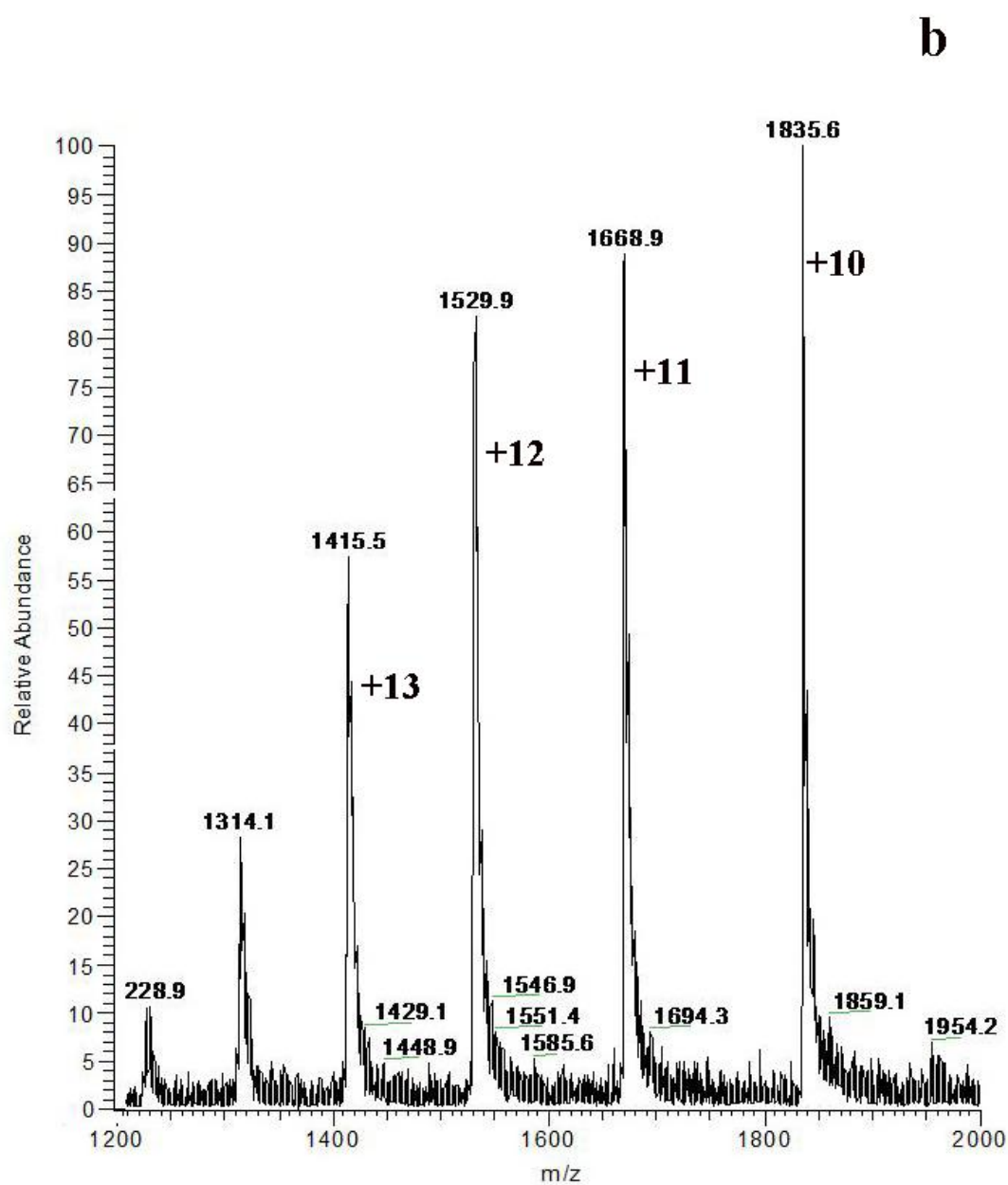


Figure 3c

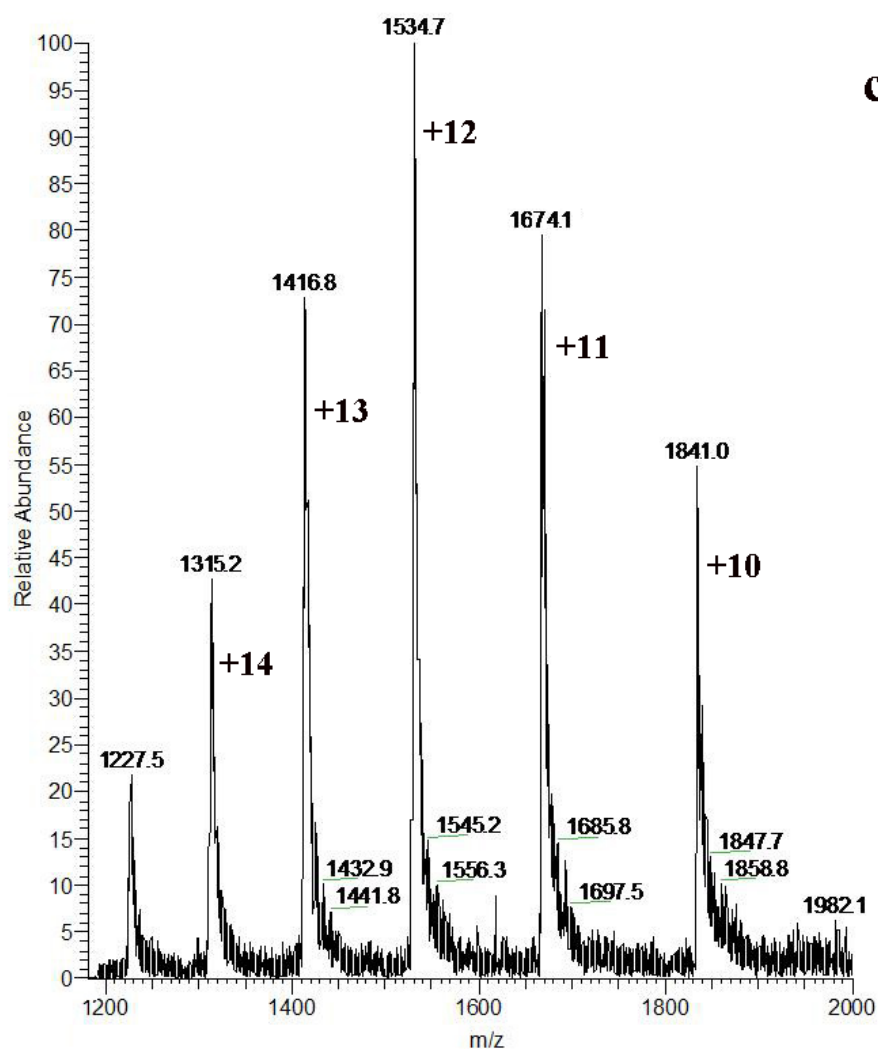


Figure 4a

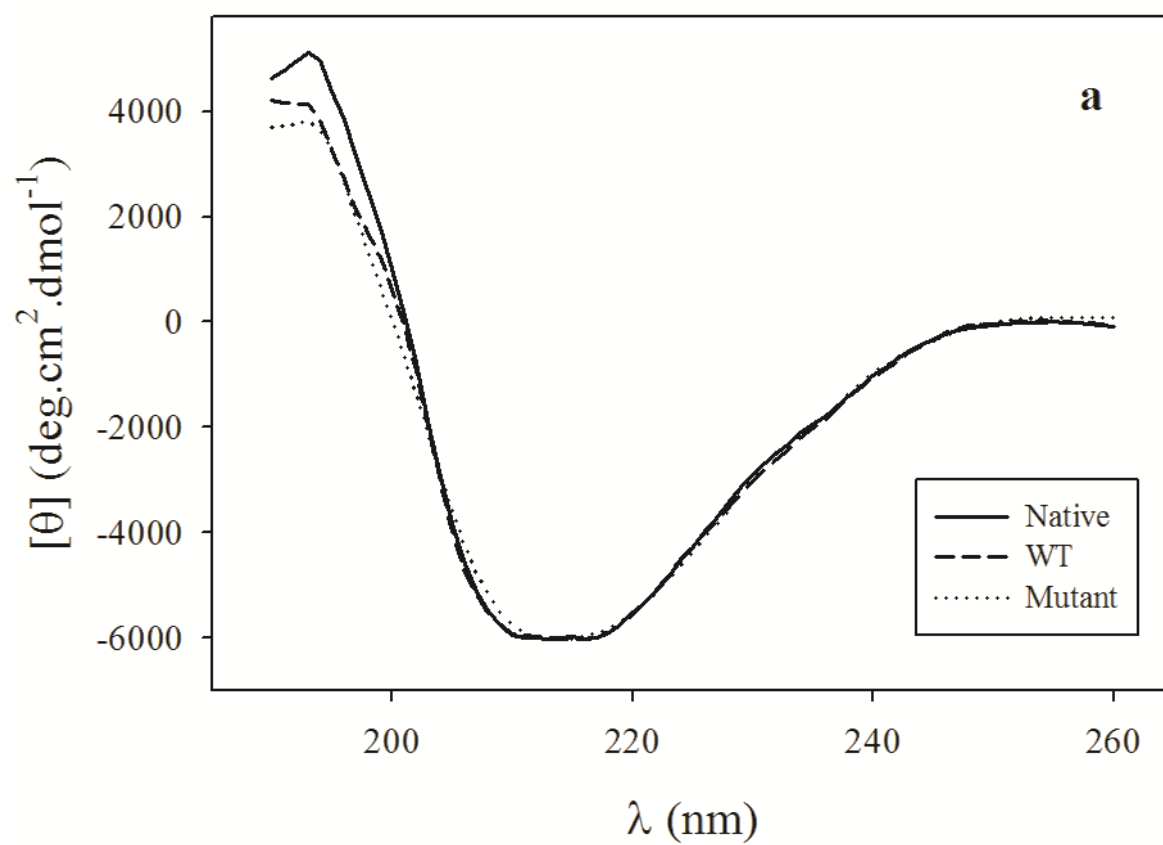


Figure 4b

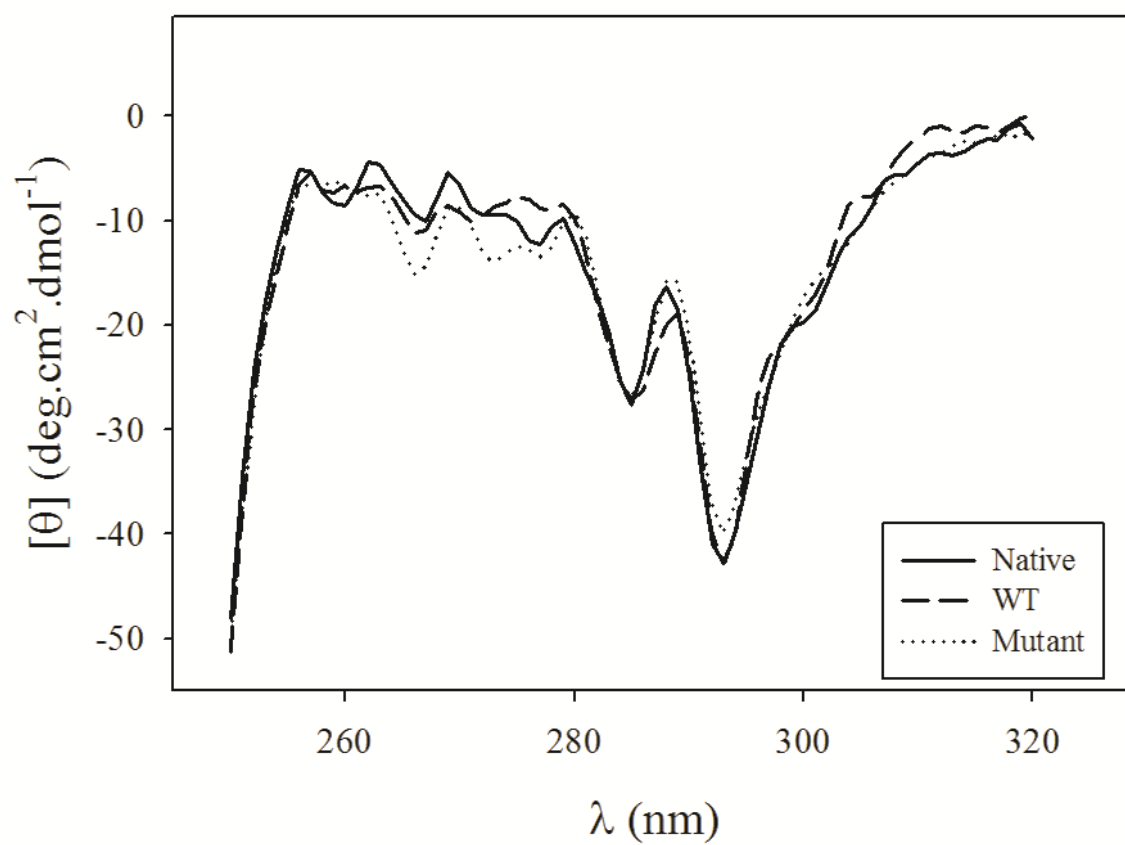


Figure 5

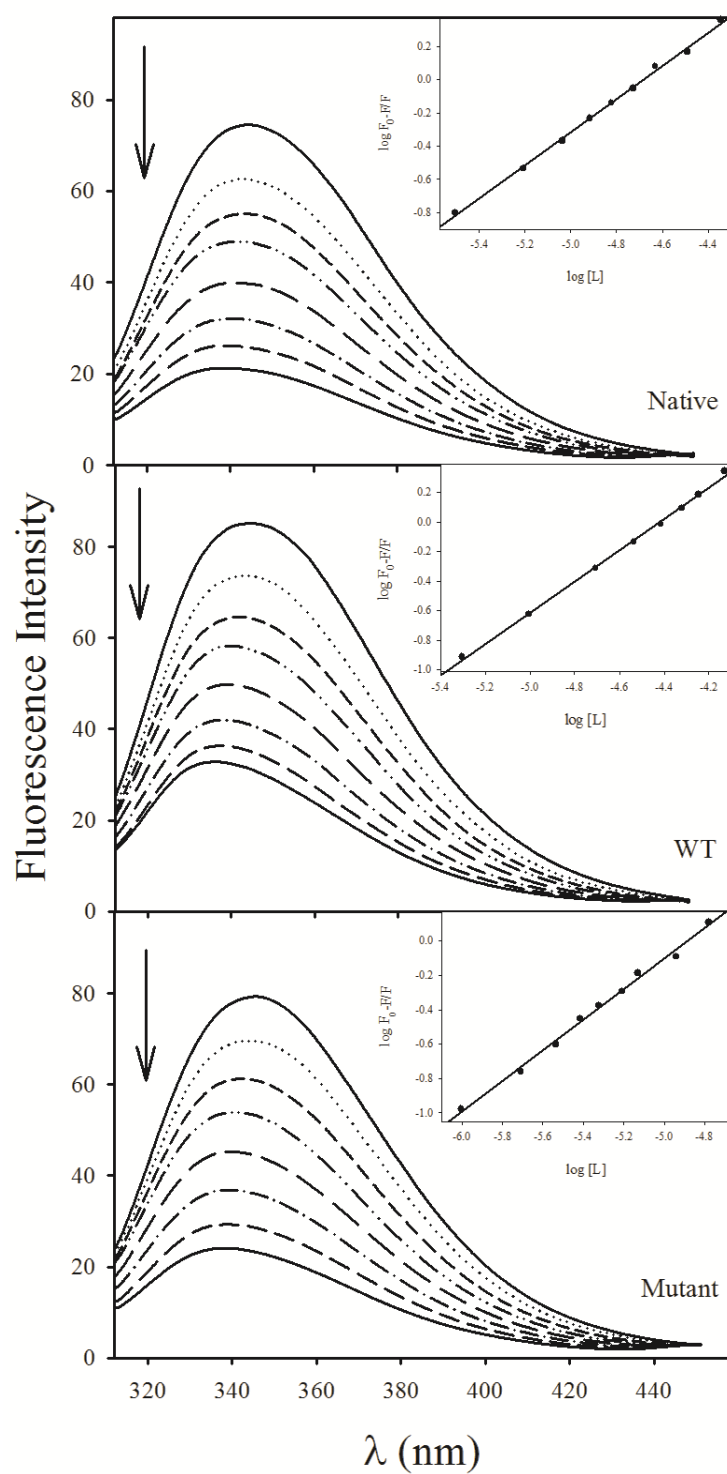


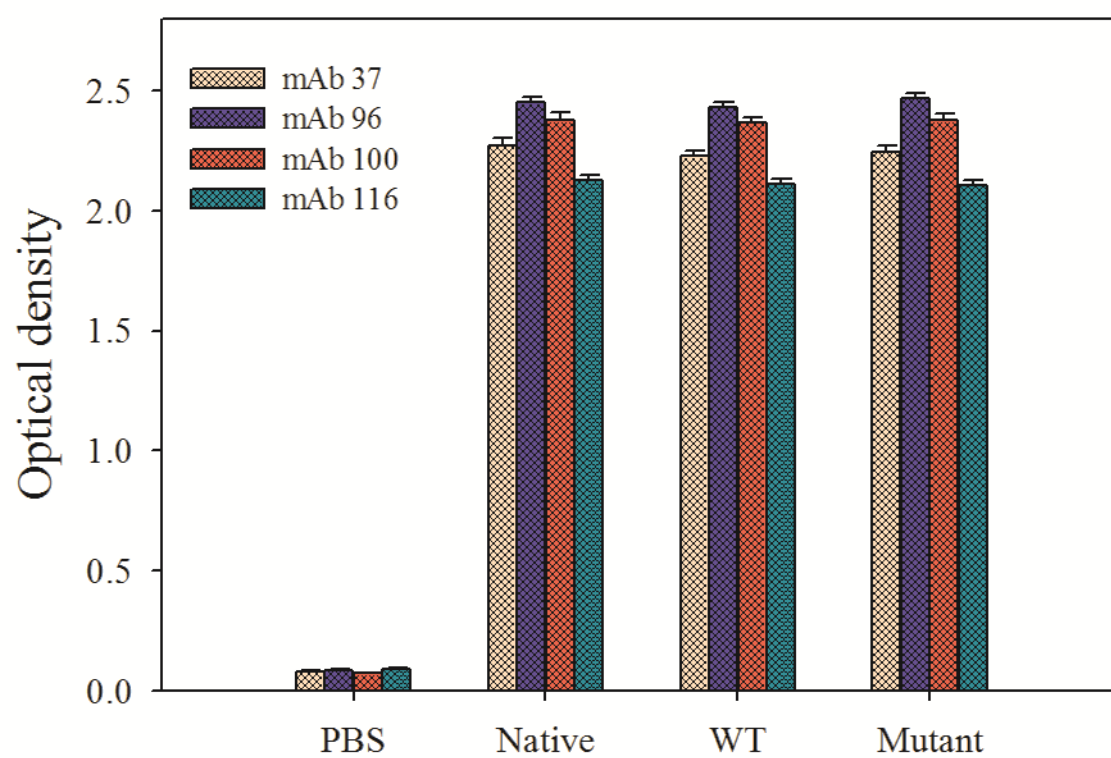
Figure 6

Figure 7

