

Effect of mucin on β -lactoglobulin and lactose interaction

Hilal Yılmaz

Department of Biotechnology, Bartın University, Kutlubey Campus, 74100, Bartın, Turkey

Research Article

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Hilal Yılmaz,

Email: hicelebioglu@bartin.edu.tr**Abstract**

Functions of mucin, as the major macromolecular component in saliva or gastric fluids, are drawing increasing attention in the context of understanding the oral processing or digestion of dairy foods at the molecular level. This study was designed to investigate the interactions between β -lactoglobulin (BLG)-lactose, mucin-lactose and BLG-lactose-mucin at the molecular level under different temperature and pH conditions using fluorescence spectroscopy in combination with scanning electron microscopes (SEM). It is the first study of its kind. There was no lactose-dependent quenching on BLG fluorophore in the range of 0–10 mM lactose concentration. On the contrary, there was a continuous increase in the fluorescence intensity of the BLG protein when the lactose concentration increased, especially at 25°C. BLG-lactose complex became thermally unstable at 37 and 45°C. Moreover, BLG exhibited a pH dependent conformational change and had higher fluorescence intensity at pH 3 than pH 6.8. The fluorescence result was in correspondence with SEM images where we observed lactose crystals gathering around and on the BLG molecule, but lactose molecules could not be seen in the presence of mucin. It was anticipated that mucin molecules interacted with BLG-lactose complex *via* electrostatic attraction and formed an extra protective layer around the BLG molecules to avoid solvent exposure.

The milk from dairy cows contains 12–14% solid matter composed of proteins (2.5–4%), fat (3–5%), and lactose (~5%) (Osorio *et al.*, 2016). Milk is used in the food industry in many different ways. Not only is milk a very nutritious food but also the by-products released during the production of dairy products carry additional nutritional properties. The best example is the whey, which is released as a waste/by-product during cheese processing. 10 kilos of milk are used to produce 1 kilo of cheese, and approximately 9 kilos of whey are released as a result of the process. Whey contains high amounts of protein. Among them β -lactoglobulin (BLG) represents about 50% of the total whey proteins in bovine milk and possesses high levels of secondary, tertiary and, in most cases, quaternary structures (Kinsella and Whitehead, 1989; Edwards *et al.*, 2009). In addition to protein, whey powders include lactose sugar. Whey is often an ingredient of choice as an additive in the food industry due to its multi-purpose functional properties (Huffman, 1996). Before its industrial processing in different food products, the interactions of the ingredients in whey and their response to changing process conditions such as temperature, pH and humidity must be known. As a result of the studies carried out for this purpose, it has been observed that the milk sugar lactose in whey is affected by the temperature and the water content, and it has been stated that possible changes in lactose molecules affect the shelf life of this type of food product (Vuataz, 2002).

Similarly, BLG protein is known to be affected by temperature and pH changes of the environment (Aymard *et al.*, 1996; Fang and Dalgleish, 1997). Not only changes in ambient conditions, but also the interaction of BLG protein with other molecules, affect its functional properties and taste (Guichard and Langourieux, 2000). In recent studies, especially on taste and oral processing, it has been reported that BLG interacts with the mucin protein in the saliva (Çelebioğlu *et al.*, 2015) and, as a result of this interaction, the model food products containing BLG change the friction coefficient in the oral environment and create changes in taste perception (Vardhanabhuti *et al.*, 2011; Çelebioğlu *et al.*, 2016; Çelebioğlu *et al.*, 2020). It is stated that BLG protein also interacts with lactose sugar and this interaction continues during the drying process and storage conditions (Thomas *et al.*, 2004a). It has been shown that the BLG-lactose interaction stabilizes lactose crystallization (Costantino *et al.*, 1998) and thus prevents the formation of undesirable properties like particle caking, Maillard reaction, lipid oxidation and so on (Burin *et al.*, 2000; Özkan *et al.*, 2003; Thomas *et al.*, 2004b). Lactose crystallization in products such as milk powders increases the amount of free fat molecules (Kim *et al.*, 1981). In order to prevent lactose crystallization, the use of high molecular weight molecules such as proteins (Levine and Slade, 1986; Nasirpour *et al.*, 2006) or sugars other than lactose have been recommended in the literature (Nasirpour *et al.*, 2007).

At this point, the interaction of lactose with BLG and mucin protein is an interesting subject to investigate. Although there are some studies related to lactose and BLG as mentioned above,

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there is no literature on the interaction of lactose and mucin protein. Since mucin protein has both a high molecular weight and a highly glycosylated structure (Bansil and Turner, 2006), its interaction with lactose can be used functionally. Mucin, as the major salivary protein, is an important protein related to oral food processing and digestion. Through the various stages of oral processing and digestion, foods continuously mix with saliva and mucins interact with food components (Çelebioğlu *et al.*, 2020). Therefore, the mucin–lactose interaction or the change of BLG–lactose interaction in the presence of mucin may give important clues about the taste and bioavailability properties of a wide range of products such as milk/whey powder containing lactose and BLG.

In line with all these motivations, this study aimed to investigate the interactions of BLG–lactose, mucin–lactose and BLG–lactose–mucin at molecular level. Different temperatures and pH values were used to compare the conditions of different processing and digestion environments. The fluorescence spectroscopy technique was chosen as the optimum technique for this research. Fluorescence spectroscopy is based on the presence of intrinsic fluorophores such as tryptophan (Trp, the dominant fluorophore) and tyrosin (Tyr) residues, in which the emission spectra (both intensity and λ_{max}), the maximum peak positions) are sensitive to the local environment of the Trp residues, and can thus provide useful information on both conformational and structural changes of proteins (Lakowicz, 2006). Recently, the interaction of BLG and mucin was studied with fluorescence, resulting in useful and interesting clues about the way these two proteins interact (Yılmaz *et al.*, 2021). However, there is no integrating study examining the interaction of lactose, BLG and mucin molecules with fluorescence spectroscopy. It is envisaged that explaining the molecular level interactions between them will contribute to food process optimizations to produce functional products with improved sensory properties and bioavailability.

Material and methods

Sample preparation

BLG from bovine milk, lactose and porcine gastric mucin (PGM) (Type III) were purchased from Sigma Aldrich and were used as received. Protein solutions with the concentration of 1 mg/ml were prepared by dissolving proteins in 100 mM phosphate-buffered saline (PBS) solutions. By addition of HCl or NaOH as appropriate, the pH values of the buffer solutions were adjusted to pH 6.8, which may represent the milk, oral and intestine pH, and adjusted to pH 3 to represent gastric/stomach environment. The pH 6.8 and pH 3, where BLG is negatively and positively charged respectively, were also useful to observed the charged dependent interaction behavior of the protein. All buffer solutions were filtered (polyethersulfone 0.20 mm).

A dilution series of lactose solutions (0.7–23.4 μM) was prepared in PBS solutions at pH 6.8 and 3. For each data point, 1 ml of the appropriate lactose solution was added into 1 ml BLG or PGM (1 mg/ml) solution. The change in fluorescence emission intensity was measured after 2 min of adding mucin to the BLG. The addition of a constant volume of quencher to the protein solution avoided complications due to dilution effects within titration type experiments.

Fluorescence spectroscopy measurement

All intrinsic fluorescence measurements were carried out using an FS5 Spectrofluorometer (Edinburgh Instruments, Livingston, UK)

with a 150 W Xenon lamp and a single photon counting photomultiplier (PMT) detector (Hamamatsu, R928P). The excitation wavelength range (λ_{ex}) was at 280 nm and the emission wavelength range was from 290 to 420 nm (measured every 2 nm). Other settings of the instrument were; a slit width of 2 nm (for both excitation and emission) and a photomultiplier (PMT) detector voltage of 1245 V.

The change in fluorescence emission intensity of samples at three different temperature conditions (25, 37, and 45°C) were measured to determine quenching capacity, binding, and thermodynamic parameters. To account for the possibility that lactose might absorb energy at the established emission wavelength, a blank was made for each lactose concentration, where the lactose solution was replaced by PBS. The respective spectrum of each lactose solution was then subtracted from the emission spectrum of the corresponding mixture (Brandão *et al.*, 2017). Each measurement was repeated in triplicate and the mean and standard deviation were calculated. To correct the inner filter effect, the suggested method of Birdsall *et al.* (1983) was used but it should be noted that no significant changes on the measured data were observed because the studied concentrations were low enough to not have an impact on the linearity of emitted light.

Scanning electron microscopy, SEM

The samples were freeze-dried before the test. To do this, the final protein–lactose mixtures were placed into 15-mL round-bottomed flasks and were frozen at -40°C , then the samples were freeze-dried (Vertical Freeze Dryer Biobase BK-FD18P-T, High-tech Zone, Jinan, Shandong, China) for 36 h. Water content of all samples was measured after freeze-drying and samples had less than 2% water (dry basis). A scanning electron microscope (TESCAN-MAIA3) operating at 5 kV accelerating voltage was used to examine the surface of the mixture of protein and lactose samples. By using double sided adhesive tape, thin layers of powder samples were mounted on aluminum stubs and then sputter-coated with carbon and with gold/palladium.

Statistical analysis

Data obtained in this study was expressed as the mean \pm standard deviation of triplicate measurements. Data were statistically analyzed for multiple comparisons using SPSS software (version 28, IBM SPSS Inc., Armonk, NY, USA) for analysis of variance (ANOVA). Duncan's novel multiple-range test was applied to compare different samples, with significance established at $P < 0.05$.

Results and discussion

Fluorescence intensities of BLG and lactose mixtures

Changes in the intrinsic fluorescence intensities (FI) of milk/whey protein BLG in the presence of increasing concentration of milk sugar lactose are shown in Figure 1. There was a linear increase in BLG FI when the lactose concentration increased from zero to 10 mM according to the values measured at both pH 3 and pH 6.8 at room temperature. The increase has also been observed at 37 and 45°C, but at these temperatures the FI changes depending on the concentration are irregular.

The indole group of Trp alone has λ_{max} at around 340 nm but λ_{max} for BLG was observed at a shorter wavelength ($\lambda_{\text{em}} = 326$). This usually means that the main fluorescing residue of the

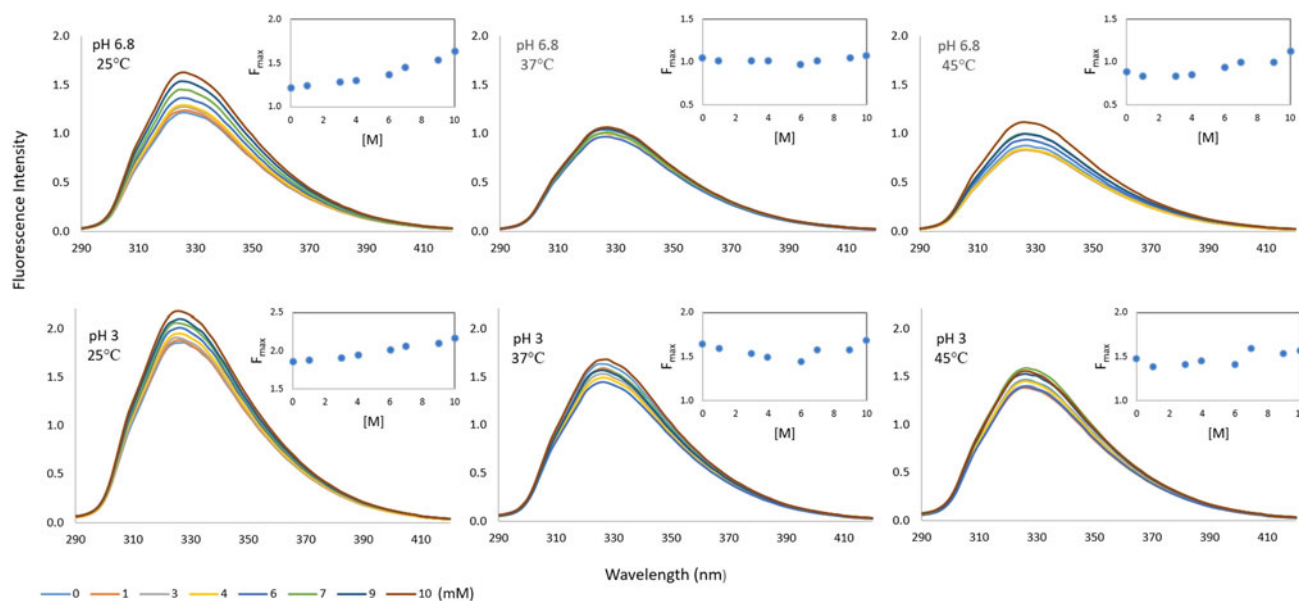


Fig. 1. Fluorescence intensity spectra of BLG in the presence of lactose (0–10 mM) at different pH (pH 3 and 6.8) and temperature conditions (25, 37, and 45 °C).

protein is shielded from aqueous solvent. The main fluorescing residue of BLG is Trp-19, which is located in the more hydrophobic environment of the protein molecule (Liang and Subirade, 2012). However, there was no change in λ_{\max} value depending on both pH and temperature, which may indicate that the pH and temperature changes do not affect the environment of the fluorescent Trp residue of BLG significantly, regardless of lactose existence. The emission maximum of Trp is highly dependent on the details of its surrounding environment but indole can also display a substantial spectral shift upon forming a hydrogen bond to the imino nitrogen (Lakowicz, 2006). According to Arakawa *et al.* (2001) a direct interaction between lactose and the polar groups of proteins is formed, especially with hydrogen bonds. Thomas *et al.* (2004a) suggested that this type of interaction may develop between lactose and milk proteins in whey powders and influence lactose crystallization by stabilizing the model whey powders against lactose crystallization.

There was no lactose-dependent quenching of the BLG fluorophore in the range of 0–10 mM lactose concentration. On the contrary, there was a continuous increase in the F_{\max} value of the BLG protein when the lactose concentration increased, especially at 25°C. This may be a sign that in the presence of lactose molecules, Trp residues of BLG are better protected from the solvent and giving higher FI. Similarly, a rise in FI of ligand-binding

protein like glucose/galactose-binding protein have been observed in the literature. Pickup *et al.* (2013), for instance, mentioned the glucose-induced three-dimensional change in glucose/galactose-binding protein into a fluorescence change that is related to glucose concentration.

Some processing and storage conditions may affect this molecular interaction. For instance, freeze drying could develop hydrogen bonds between lactose and BLG. In addition, Maillard reaction may occur due to covalent interaction between lactose and BLG during storage of BLG and lactose containing powders. However, FI measurements in this study have been performed without any freeze drying or storage but still a sign of interaction between lactose and BLG was observed.

Temperature and pH dependent change in fluorescence intensity

The effect of temperature on the changes in FI was compared in the absence of lactose and at the highest lactose concentration in the BLG protein solution (Table 1). In both cases, the highest FI was measured at 25°C, while the FI decreased as the temperature increased. These temperature dependent changes are similar at pH 3 and pH 6.8. However, when the temperature was increased from 25 to 37°C in the presence of lactose, the FI decreased more rapidly and an extra increase in the temperature to 45°C had no further effect on the FI.

The percentage changes in F_{\max} at pH 6.8 were calculated as 33.3, 2.8 and 27.1% at the temperature of 25, 37 and 45°C, respectively. The same calculation was also applied for pH 3 and the results were 17.1, 2.6 and 6.1% at the temperature of 25, 37 and 45°C, respectively. The results indicated that the changes at 25°C were the most drastic one at both pH conditions. In addition to the smallest change in FI at 37°C, there is no significant difference according to the pH value.

The adverse temperature dependence of FI as in our BLG-lactose complex indicated that the stability of the complex decreases with rising temperature. In other words, BLG-lactose interaction boosts the FI but the complex due to this interaction

Table 1. The maximum fluorescence intensity values (F_{\max}) of BLG alone and BLG–lactose mixture to compare the changes with temperature and pH

	25°C	37°C	45°C
BLG			
pH 3.0	1.86×10^6	1.63×10^6	1.47×10^6
pH 6.8	1.22×10^6	1.04×10^6	0.87×10^6
BLG-Lactose			
pH 3.0	2.18×10^6	1.68×10^6	1.56×10^6
pH 6.8	1.62×10^6	1.07×10^6	1.12×10^6

F_{\max}

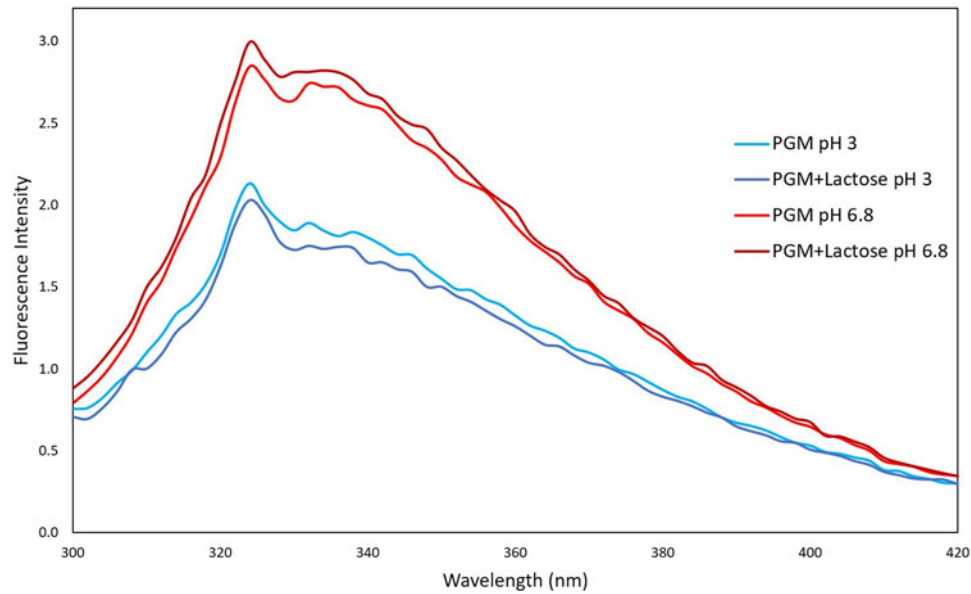


Fig. 2. Fluorescence intensity spectra of mucin with and without lactose (10 mM) at different pH (pH 3 and 6.8) conditions.

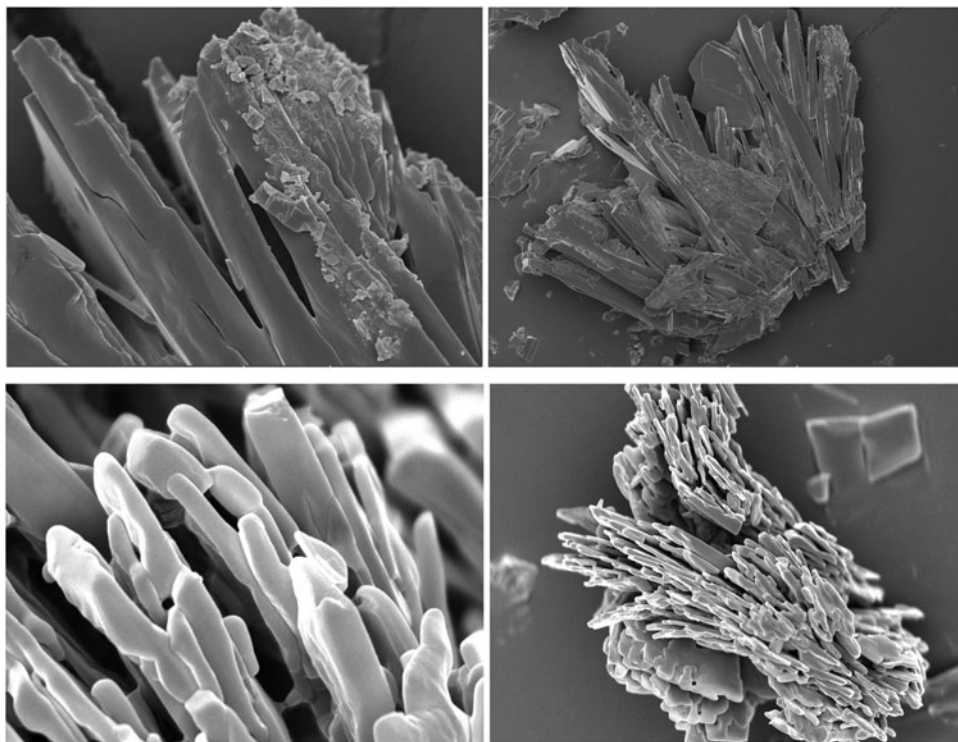


Fig. 3. Scanning electron micrographs of (a) BLG-lactose and (b) PGM-lactose.

is thermally unstable. It should be noted that the lactose effect on FI at 37°C is not as drastic as at 45°C (Table 1), which means that the decrease in FI at 37°C was mainly due to BLG's instability. Ghalandari *et al.* (2014) showed that total fluorescence of BLG was more accessible to quencher at 37°C compared to 25°C. Moreover, Thomas *et al.* (2004a) mentioned that the lactose in whey powders produced by spray drying or freeze-drying was very sensitive to changes in temperature and water content.

The pH dependent FI changes can be monitored more clearly in Table 1. Regardless of the presence of lactose, the highest FI was obtained at pH 3 at all temperatures. In our previous studies, it was observed that BLG had a higher FI at pH 3 due to the preserved Trp residues within 'hydrophobic pockets'. This is explained by the charged dependent conformational change of BLG at pH 3 where BLG is overall positively charged and more hydrophobic in the sense that nonpolar solutes are more

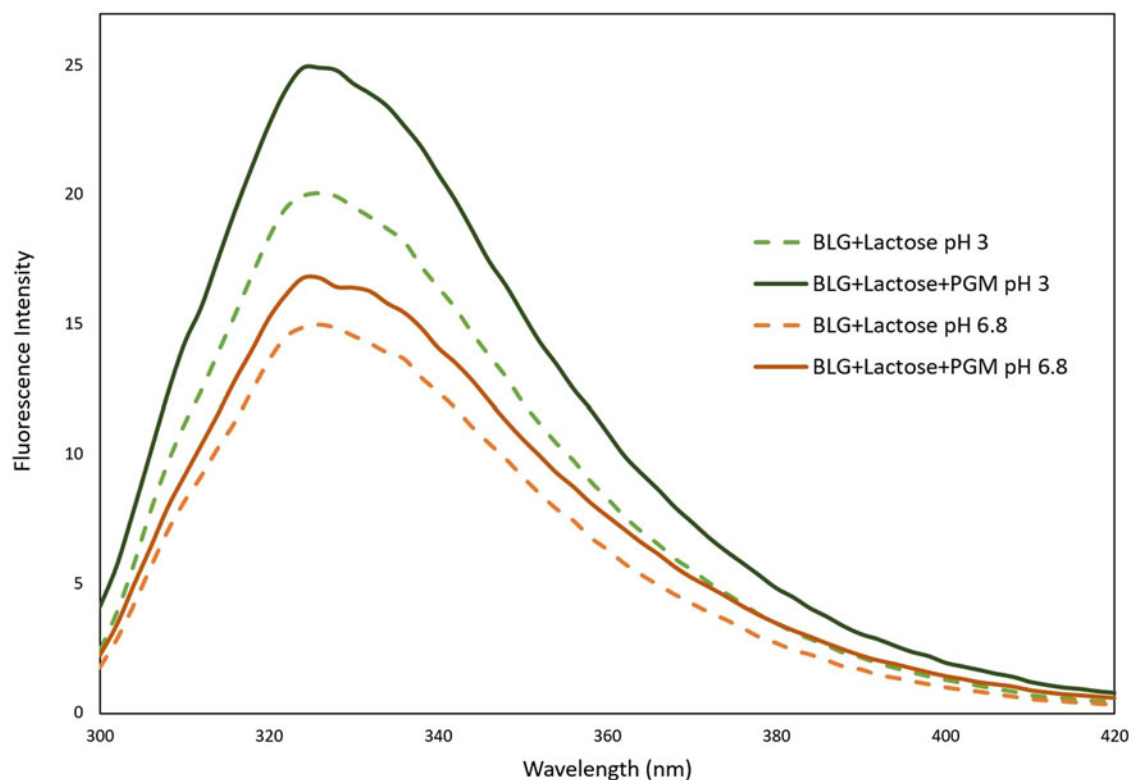


Fig. 4. Fluorescence intensity spectra of BLG, lactose and mucin mixture at pH 3 and pH 6.8.

effectively shielded from aqueous solvents and a more compact configuration is formulated (Cardamone and Puri, 1992). Meanwhile, at close to pH 3, BLGs are known to be mainly monomeric, yet they are mainly dimeric at a neutral pH (Busti *et al.*, 1998) and this pH dependent monomer–dimer transition may also change the FI of BLG. Mills and Creamer (1975) reported that Trp fluorescence of BLG was reduced in intensity by association of the monomeric proteins into dimers, which is consistent with the trend shown in Table 1.

Fluorescence intensities of mucin and lactose mixtures

When we examined the pH-dependent change of the interaction of lactose with mucin protein by fluorescence spectroscopy technique, unlike the lactose–BLG mixture, the lactose–mucin mixture gave a higher FI at pH 6.8 (Fig. 2). Since the isoelectric point of mucin is around 2, the mucin becomes more negative when the pH is 6.8. However, at pH 3, the mucin molecules become protonated and the negative charge value decreases. This was shown in the result of our previous zeta potential studies (Yılmaz *et al.*, 2021).

It is well known that gastric mucin undergoes a conformational change from a random coil conformation at $\text{pH} \geq 4$ to an anisotropic, extended conformation at $\text{pH} < 4$ where mucin molecules cluster together (Cao *et al.*, 1999; Celli *et al.*, 2020). Moreover, at low pH values, mucin molecules have increasing exposure of hydrophobic binding sites due to conformational transitions (Smith and LaMont, 1984).

According to Figure 2, it was observed that the presence of lactose did not have a significant effect on the mucin protein FI value. For this reason, no trace of interaction was found between mucin and lactose according to fluorescence spectroscopy results.

SEM images of BLG–lactose and mucin–lactose mixtures

SEM was used as a complementary technique to confirm the fluorescence spectroscopy results of protein–lactose systems. When BLG–lactose and mucin–lactose interactions were examined with SEM images, we observed lactose crystals gathered around and on the BLG molecule, but lactose molecules could not be seen in the presence of mucin (Fig. 3). The SEM results support the fluorescence results in the sense that lactose molecules surrounding the BLG surface probably protected the fluorophore against solvent quenching, at least to some extent. The same does not happen with mucin. Presumably, lactose molecules vanished inside the comparatively large mucin molecules and so had no significant effect on mucin fluorescence.

Effect of mucin on fluorescence intensity of BLG–lactose mixtures

The pH-dependent FI change of mucin is negligible when compared to the FI change of BLG protein. This was also shown in our previous study; the FI of mucin is very low when compared to BLG. Since mucin is a random coil conformation, the Tyr residues of mucin molecules are quenched due to solvent exposure. The effect of mucin on the lactose–BLG interaction was examined according to the fluorescence results given in Figure 4. An increase of BLG FI has already been shown in the presence of lactose (Fig. 1), but interestingly this increase became more dramatic when mucin was added.

According to our previous study, mucin causes a decrease in the FI of BLG (Yılmaz *et al.*, 2021). However, the increase of FI in the presence of lactose can be explained as follows: lactose binds to BLG, forming a protective barrier around it. When

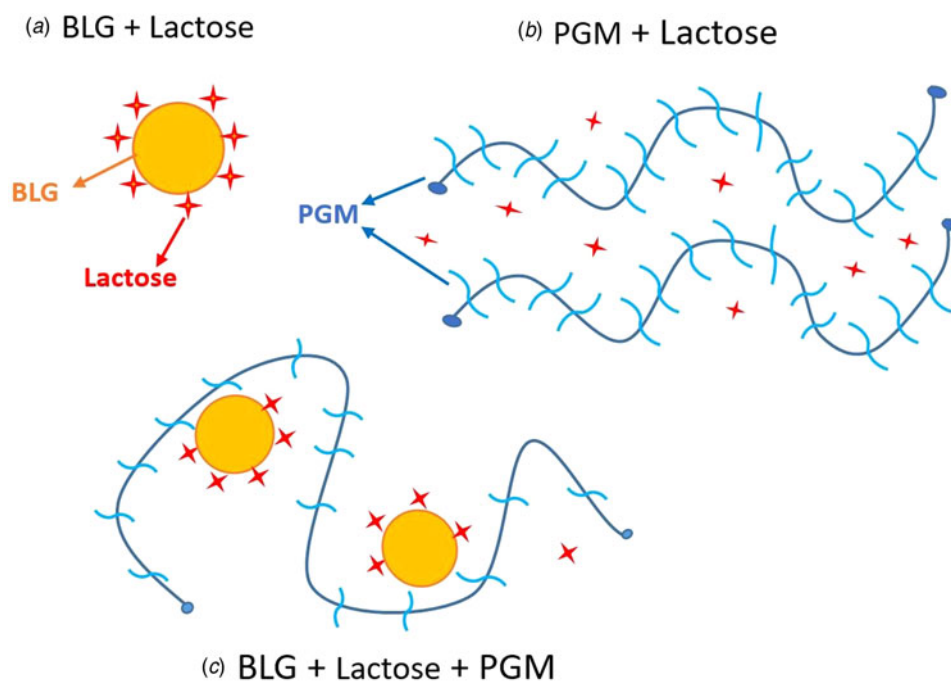


Fig. 5. Illustration of the interactions between BLG–lactose, PGM–lactose, and BLG–lactose–PGM.

mucin was added after BLG–lactose interaction, the mucin could not bind to BLG and could not change its conformation, because lactose molecules were already bound with BLG. Thus, lactose reduces the binding between BLG and mucin. The fact that mucin caused an extra increase in the FI of BLG may be due to the inability of mucin to form hydrophobic interactions with BLG in the presence of lactose, meaning that mucin would only have electrostatic and hydrogen bond interactions with BLG. Lack of hydrophobic interaction could mean that Trp residues in the hydrophobic part of the BLG molecules continued to be preserved from solvent related quenching. The high FI increase at pH 3 supports the presence of the electrostatic interaction.

When mucin is added to the BLG–lactose mixture, it interacts through electrostatic attraction and the mucin molecules form an extra protective layer around the BLG molecules, reducing exposure to solvent. Therefore, the protected Trp residues (probably not only in the hydrophobic part but also in the outer Trp residues of BLG) result in higher FI. Our prediction of BLG–lactose, mucin–lactose and BLG–lactose–mucin interactions based on both fluorescence and SEM results is illustrated in Figure 5.

The electrostatic interaction with the proteins can facilitate re-folding of the proteins at low pH (Ali *et al.*, 1999). Not only the BLG but also the unfolded terminal regions of PGM at pH 3 (Cao *et al.*, 1999; Madsen *et al.*, 2016) may undergo folding again after binding with BLG, or with BLG–lactose complex. Thus, the indole in BLG Trp can be buried in the hydrophobic protein matrix.

In conclusion, a lactose induced increase in BLG FI has been indicated by our fluorescence measurements, but the complex due to this interaction is thermally unstable. A higher FI at pH 3 is explained by the charged dependent conformational change of BLG at pH 3 where BLG is overall positively charged and more hydrophobic in the sense that nonpolar solutes are more effectively shielded from aqueous solvents. By contrast, lactose

does not have a significant effect on the mucin protein FI value. When BLG–lactose and mucin–lactose interactions are examined with SEM images, lactose crystals can be seen gathered around and on the BLG molecule, but lactose molecules cannot be seen in the presence of mucin. Mucin added to the BLG–lactose mixture, interacts through electrostatic attraction and the mucin molecules form an extra protective layer around the BLG molecules, thereby reducing solvent exposure. As the Trp residues are protected, FI is increased. As the molecular-level interactions of milk proteins, milk sugars and salivary proteins are critical for a full understanding of the textural, sensory and nutritional properties of food products during digestion, the findings of this study may provide a valuable background for dairy food processing and related applications of BLG–lactose systems.

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