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Effect of pH and Heat-Induced on the Yak B-Lactoglobulin Denaturation and Aggregation

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Abstract

The effect of pH and heating temperature on yak (β-Lactoglobulin) β-Lg denaturation/aggregation was investigated. Temperature and pH significantly influenced the rate and content of yak β-Lg denaturation/aggregation and the protein formation in solution. The content of native β-Lg monomer decreased with increase in temperature at all pH range. β-Lg was heated above 80°C the content of native β-Lg monomer at pH 4.5-5.5 was reduced markedly than higher pH range from 6.5 to 8.5. Meanwhile the formations and content of yak β-Lg solution were complication at pH from 6.5 to 8.5 after heated 80°C and 90°C. The trend of hydrodynamic diameter and particle size was similar. The particle size in yak β-Lg solution increased markedly form (52 and 75 nm) pH 8.5 to (345 and 489 nm) pH 4.5 when β-Lg was heated at 80°C and 90°C, respectively. pH and heating temperature are important parameters that can influence the heat-induced denaturation/aggregation and the characteristic of yak β-Lg and yak whey proteins. And these researches provide some basis date and results to better understanding the mechanism of yak β-Lg denaturation, disulphide-linked aggregation and precipitation.

Keywords: Yak milk; β-Lactoglobulin; Heat-induced; Size-exclusion chromatography; Particle size; Aggregates

Introduction

β-Lg is the major whey protein in bovine milk representing about 50% of the total whey protein. As a globular protein β-Lg monomer has single polypeptide chain of 162 amino acids with molecular weight of about 18.3 kDa [1]. The monomer has two disulphide-bonds and one free sulfhydryl group which buried in the native protein structure at pH<7.5 [2]. Under native conditions, β-Lg existed as a non-covalent dimer. Currently, it is thought that native dimer is in rapid equilibrium with native monomer. β-Lg is very important for it dominates the overall aggregation and gelatin behavior of whey proteins. As a result, most research on heat-induced aggregation and denaturation of whey proteins has focused mainly on the mechanism of aggregation of β-Lg. The native form of β-Lg assumes a monomeric configuration and may show different oligomerization states depending on pH [3].

Whey proteins are important food ingredients due to their nutritional and functional properties such as gelation and emulsification properties [4]. These functional properties are conferred on whey protein following denaturation and aggregation. The extent of denaturation may depend on pH, salt concentration, protein concentration, and heating temperature [3,5]. During denaturation, β-Lg unfolds cooperatively to expose side chain groups originally buried inside the molecular structure [6]. The denaturation of β-Lg has been attributed to covalent and non-covalent interactions which occur between exposed free thiol groups and disulphide bonds resulting in aggregation of denatured proteins [7]. Bovine whey proteins are popular because they have received much attention in terms of research. However, many other animals such as buffalo and yak can produce milk with desirable and superior nutritional properties compared to bovine milk.

Yak milk ranks third in economic importance after bovine and buffalo milk in China [8]. The production of yak milk has increased up to 40 million tons, but only about 25% of these productions is industrially processed [9]. According to previously research the protein (5.5-10%), whey protein (52.5 g/L) and β-Lg (6.3 g/L) contents were all higher than bovine milk [10-13]. Further, these authors reported that the physicochemical properties of casein micelles in yak milk differed significantly from that of bovine milk. Thus, the functionality of milk proteins may depend on animal breed since the protein composition of these animals may vary. Differences in whey protein composition and structure may thus affect their denaturation and aggregation during heating. Thus more information on yak milk is needed for the successful development of the yak dairy industry. Due to yak milk specific composition, yak milk could be a high-quality raw material for manufacturing food for infants, elderly and sectors of the population with particular needs. This research could be one of several ways of increasing the use of yak whey proteins in creating food gels. Therefore this paper reported the effect of heat-induced aggregation of yak β-Lg at different pH and temperatures.

Materials and Methods

Collection of yak milk samples

Yak milk samples were obtained from Hong Yuan country of Sichuan province. Milk samples were immediately stored in sterilized plastic containers and kept at 4°C until they reached the laboratory for subsequent experiments.

Isolation of yak β-Lactoglobulin

Yak whey β-Lg was isolated from fresh yak milk following the method described by Alomirah et al. [14] Yak milk β-Lg solution was dialysed extensively against water for 24 h at 4°C. The solution was

freeze dried and stored at -20°C until used. The purity of β-Lg assessed using electrophoretic and HPLC method was approximately 95%.

Heat treatment of β-Lactoglobulin

Dispersion of yak β-Lg (10 mg/mL) was prepared by dissolving freeze dried β-Lg in phosphate buffer (0.02 mol/L) at pH from 4.5 to 8.5. Dispersed samples at the different pH were heat-induced in a thermostatically controlled water bath. Four heating temperatures of 60°C, 70°C, 80°C and 90°C were used. Heating was done for 30 min. After heating, the samples were cooled with running water to room temperature (20°C). Thereafter, the samples were centrifuged for 30 min at 20,000 × g to remove the denatured β-Lg after heating. Samples were kept at 20°C prior to subsequent experiments.

Size-exclusion chromatography

The Size-exclusion chromatography (SEC) profiles of heated β-Lg were done according to the methods of Croguennec et al. [15] except that the column used was TSK G3000 SWXL (300 × 7.8 mm i.d.) (Lab quip, Lucan, Ireland) The heated samples were diluted 1 times in 20 mM sodium phosphate buffer and filtered through a 0.45 mm filter. The column was eluted with a phosphate buffer (0.10 mol/L, pH6.7) and sulphuric Sodium (0.10 mol/L) containing 0.05% of Sodiumazide at a flow rate of 1.0 ml/min. UV absorption was measured at 280nm.

SDS–PAGE

SDS–PAGE of heated samples was performed under non-reducing conditions (without DTT) using a Mini Protean II system (Bio Rad Laboratories, A Technologies, Dublin, Ireland) [16]. Gels were stained with Coomassie Brilliant Blue G250.

Dynamic light scattering

The hydrodynamic diameter of the aggregates was determined using a Zetasizer Nano system (Malvern Instruments Inc., Worcester, UK). The measurements were carried out at 25°C and detector position measuring at 173°C providing backscattering configuration which reduces scattering signals in protein solution samples. The cumulative method was used to find the mean size of a particle that corresponded to the mean of the volume distribution, 100 µL protein solution was diluted in 1 mL water and sample measured at 20°C.

Microscopic observations

Observations yak β-Lg aggregation with transmission electron microscope (TEM) operating at 80 kV (H-7650, HITACHI Company, Japan). A drop of sample was deposited onto a formvar-carbon-coated copper grid and excess of product was removed after 15 min using filter paper. The grid was then dried at room temperature for 20 min. Representative samples of the different systems studied were chosen for observations. Samples adjusted at pH 4.5, 5.5, 6.6, 7.5 and 8.5 heated at 90°C for 30 min.

Statistical analysis

All experiments were conducted in duplicate. Data were analysed using analysis of variance (ANOVA) and means were compared using Fischer's Least Significant Difference Test ($p<0.05$).

Results and Discussion

Effect of pH and temperature on native β-Lg monomer content

The native β-Lg monomer content of yak milk β-Lg heated at 20°C, 60°C, 70°C, 80°C and 90°C at pH from 4.5 to 8.5 (Figure 1) varied significantly. Yak milk β-Lg heated at 60 and 70°C at pH from 4.5 to 6.5 had similar native β-Lg monomer content (approx. 100%) compared to unheated (20°C) β-Lg of yak milk (100%). At 80 and 90°C, within the same pH range from 4.5 to 5.5, the native β-Lg monomer content progressively decreased reaching 0% at 90°C. The decrease in native β-Lg monomer content which began at 80°C suggests possible denaturation and aggregation of β-Lg. This seems plausible since of all the heated samples, only the sample heated at 80°C showed a progressive decrease in the native β-Lg monomer content from 92.46% to 41.02% at the pH range of 4.5 to 5.5. β-Lg has been reported to have a denaturation temperature of around 80°C, which may vary with pH [17].

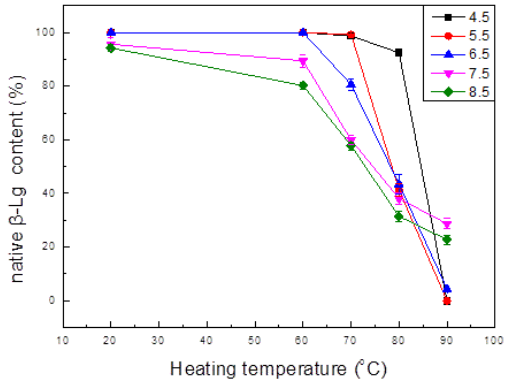
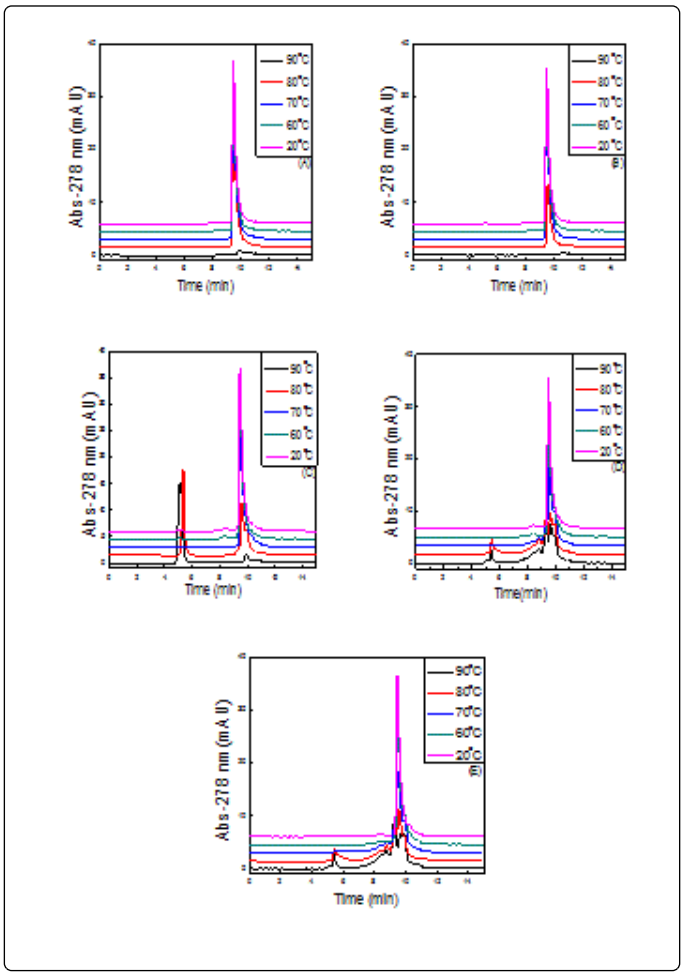


Figure 1: Effect of temperature and pH on the native β-Lg monomer content of yak milk.

In the pH range of 6.5 to 8.5 the native β-Lg monomer content generally decreased with increase in temperature. The highest decrease (approx. 96%) in native β-Lg monomer content was observed in the sample heated at pH 6.5. This is similar to those observed at pH 4.5-5.5 (Figure 1). The decrease in native β-Lg monomer content at low pH (≤ 6.5) has been previously associated with change in the tertiary and quaternary structures of β-Lg [18]. However, with increasing pH from 6.5 to 8.5, the native β-Lg monomer content showed a different trend when compared to those at lower pH 4.5 to 5.5. The content of native β-Lg monomer was decreased from pH 4.5 to 8.5 at 60°C, 70°C and 80°C. These results presumably could be attributed to the pH existence could increase stability and denaturation temperature of β-Lg [19,20]. Further, it was observed that the native β-Lg monomer content (94.21%) at pH 8.5 was similar to the content at pH 6.5 (100%). This may be due to the presence of fewer amounts of dimer compared to the monomer content of β-Lg. In comparison with samples heated at 90°C between pH 4.5 and 5.5, which showed nearly 0% native β-Lg monomer content, samples heated from pH 6.5 to 8.5 had some native β-Lg (4.45-22.72%) in solution.

SEC profile of yak β-Lg as affected by pH and temperature

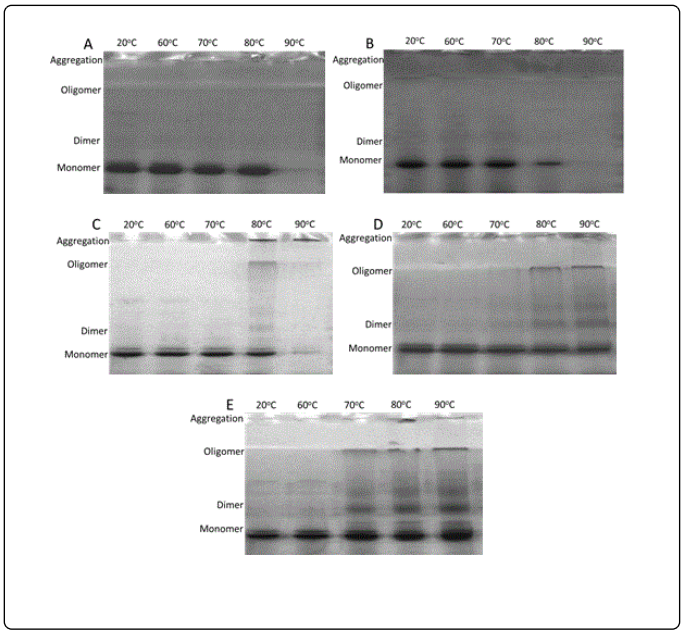
The elution profiles of yak β-Lg affected by pH and temperature is presented in Figure 2. Yak β-Lg heated between pH 4.5 and 5.5 showed similar elution pattern with a single peak at approximately 8.5 min corresponding to the native monomer form of Yak β-Lg. The native monomer contents of Yak β-Lg at 20°C, 60°C and 70°C within the lower pH range shown by the elution time were very similar. It is important to note that native β-Lg monomer content at 80°C decreased with increase in pH from 4.5 to 5.5 (Figure 1). The decrease in native β-Lg monomer content at 80°C confirms the denaturation of native β-Lg monomer content. At 90°C, the native β-Lg monomer content was nearly 0%. This trend agrees with the native β-Lg monomer content as shown in Figure 1. The near 0% at 90°C as shown by the chromatograms (Figure 2) confirms previous suggestion that all the native β-Lg content has been denatured.



Generally, heated β-Lg solution existed as a mixture of monomer and dimer in equilibrium at higher pH range of 6.5 to 8.5 (Figure 2). In addition to the peaks observed at lower pH, other peaks were observed when the pH of yak β-Lg was increased from 6.5 to 8.5. The elution time and number of peaks were depending on pH and temperature. Unheated β-Lg at 20°C and heated to 60°C and 70°C showed two additional peaks at about 8.5 and 9.5 min. These peaks correspond to the formation of dimer and native monomer respectively. The equilibrium between the monomeric and dimeric forms of β-Lg is reportedly dependent on temperature, protein concentration, pH and ionic strength [21,22]. At higher pH protein structure is weakened by the electrostatic repulsion of ionized groups within the protein molecule [3]. On heating the samples to 80°C and 90°C, additional two peaks appeared at approximately 5.3 and 9.6 min which are associated with aggregation and non-native monomer formation of β-Lg respectively. Thomas et al. reported bovine β-Lg heated at 85°C for different time regimes similarly observed oligomer and aggregate formation in the pH range used in this present study [23]. The native monomer content of β-Lg decreased with increase in temperature suggesting the formation of the non-native monomer content of β-Lg. This may account for the increase in non-native monomer content of β-Lg at 90°C as pH increased from 6.5 to 8.5. This explanation seems reasonable since aggregates were not formed when β-Lg was heated at lower temperatures of 20°C, 60°C and 70°C. Further, it was observed that the intensity of the peaks corresponding to the formation of aggregates, which appeared when β-Lg was heated at 80°C and 90°C reduced with increase in pH from 6.5 and remained almost the same between pH 7.5 to 8.5. A possible explanation for this occurrence is that pH has a stronger influence on aggregate formation than temperature. Many other authors reported that β-Lg is most sensitive to aggregation at low pH [24].

SDS-PAGE of yak β-Lg as affected by pH and temperature

SDS-PAGE under non-reducing conditions was used to further confirm the formation of non-native monomer, dimer and aggregate forms of β-Lg during heating (Figure 3). A major band was observed for unheated β-Lg and β-Lg heated at 60°C to 80°C between pH 4.5 and 5.5. This band which corresponds to β-Lg monomer decreased with increase in pH. The monomer band was not found in β-Lg heated at 90°C between pH 4.5 and 5.5. However, this band faintly appeared at pH 6.5 and increased thereafter up to pH 8.5 (Figure 3).

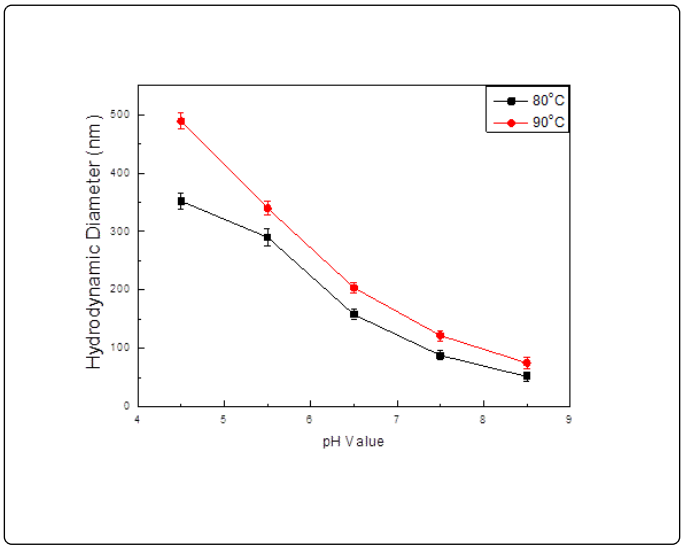


The increase in the monomer contents of β-Lg at 90°C agrees with the SEC profile of the monomer content formation of β-Lg which also increased at 90°C (Figure 2) [25]. A noteworthy band at pH 6.5 was observed when β-Lg was heated at 80°C and 90°C. This band presumably corresponds to the formation of aggregates since they appeared at molecular weights higher than 200 KDa. These aggregates fail to migrate into the stacking and separating gel suggesting that they

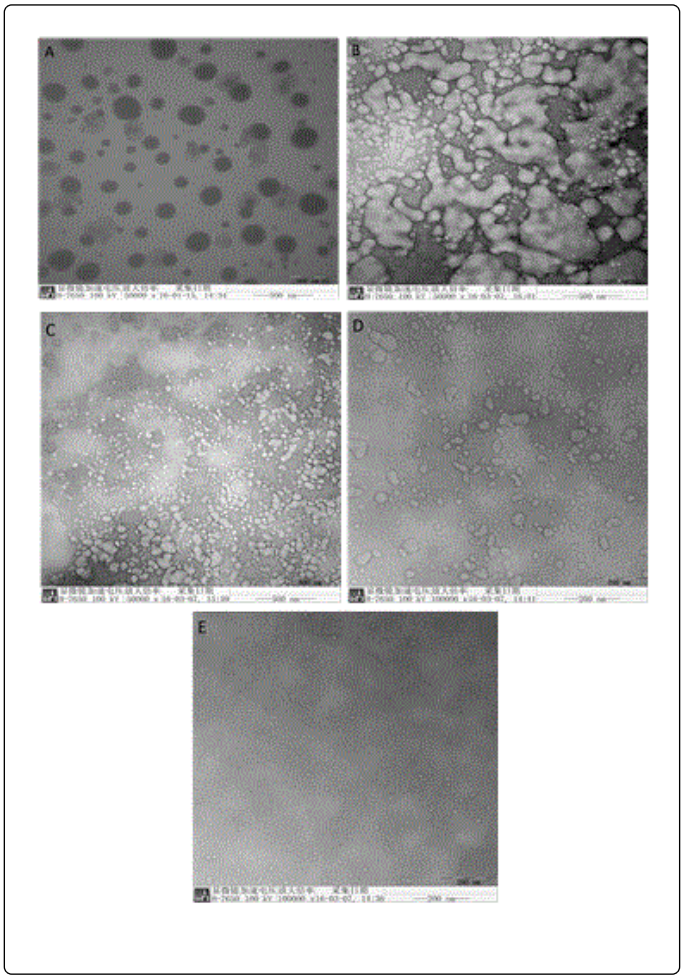
are of higher molecular weight. This further confirms the higher aggregate formation as observed in the SEC profiles for β-Lg heated at pH 6.5 (Figure 2). Heating temperature and pH are the major factors influencing the aggregation of β-Lg. During heating thiol/disulfide exchange reactions occur and are strongly dependent on pH [26], also observed polymeric aggregates with a molecular weight of about 300 KDa which failed to migrate into their stacking gel [27]. Another band which corresponds to the formation of β-Lg dimer was observed at between pH 6.5 and 8.5 (Figure 3). Although, the band faintly appeared at pH 6.5, 7.0 and 7.5, this band was very clear at pH 8.5 (Figure 3). The formation of monomeric and dimeric forms of β-Lg is largely dependent on pH as previously stated.

Aggregation morphology and hydrodynamic diameter

The hydrodynamic diameter (Dh) at 80°C and 90°C from pH 4.5 to 8.5 is presented in Figure 4. At pH 4.5 Dh was 381 nm and 548 nm at 80°C and 90°C respectively. Dh was decreased markedly with increasing pH, at pH 8.5 Dh was 52 nm and 75 nm and the value were the smallest. The value of Dh at pH 7.5 (88-126 nm) was similar with the result described by who found values close to 40 nm after heating at 65°C and heating β-Lg at 67.5°C in low ionic conditions [18,28,29]. The aggregation was influenced remarkably the β-Lg structural properties at different pH and temperature. The β-Lg aggregation and gelation at pH from 6.5 to 8.5 heated at 90°C was absence of spontaneous precipitation, were defined as stable heat-induced aggregation. The protein aggregation structure was led by protein charge and distribution, these all over affect the stability of aggregation meanwhile the content of protein aggregation was affected by pH and heating temperature. The β-Lg aggregation at 90°C observed by TEM is presented in Figure 5. At pH 4.5 the average length of aggregation was more than 500 nm. The aggregation was composed by some smaller aggregation which particle size ranged 200-300 nm. While the pH increased to 6.5 and 8.5 aggregation average length was 200 nm and 90 nm respectively, this protein aggregation structure was contributed by self-similar with fractal dimension formed by clusters of primary aggregates [30].



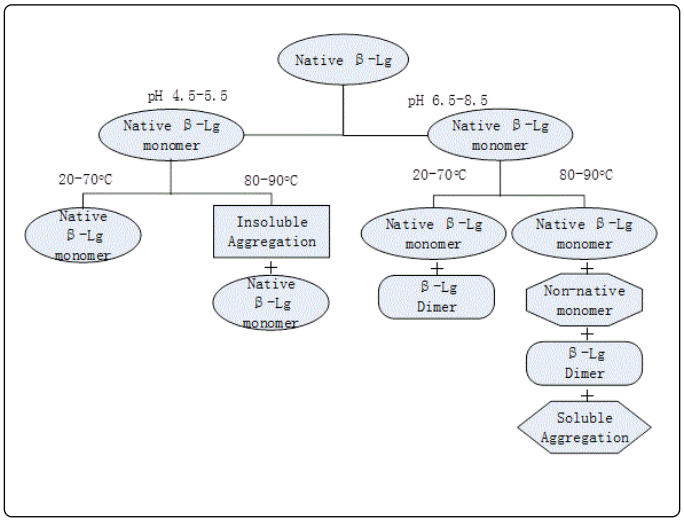
When yak β-Lg heated at 90°C, the aggregation size ranges from 50 to 1000 nm with major aggregation was about 300 nm. The results were similar with the results of bovine [31]. At lower pH micrographs showed the aggregates size was more than 1mm which consisted with diameter of 300 to 700 nm. The particulate structure was close to previous researches obtained the β-Lg heated at pH close to pI [32,33]. The aggregation and gelation of yak β-Lg was obviously effect by pHat lower pH the size of aggregation was increased remarkably, means that yak β-Lg has less stability at lower pH. The result of TEM was agreement with the DSL that aggregation particle size was increasing with pH decreased. The pH effect the surface charge, protein surface structural characteristic and polydispersity, protein aggregation surface charge distribution has a markedly effect with the structuring of aggregation and the stability of particle size. The results in was good agreement with (TEM) in this research report the soluble aggregation was formed at pH 7.0 [34].



Possible mechanism for the formation of β-Lg types and aggregation

According to previous research, β-Lg aggregation involved combination of two consecutive processes. Firstly, the β-Lg monomer aggregates into intermediate oligomers (di-,tri-and tetramer) and secondly the oligomers act as building blocks for aggregate formation [26,28]. In the experiments described above, pH had a large effect on aggregation of yak β-Lg, resulting in product of different composition. A possible explanation for the mechanism of β-Lg aggregation and the formation of native monomer, non-native monomer and dimer is summarized in Figure 6. The mechanism of aggregation under lower pH is considerably different at higher pH. At pH from 4.5 to 5.5 with heating temperature up to 80°C the composition of β-Lg was varied. In

solution these aggregates increased with increase in pH (Figure 2). At 90°C, β-Lg has substantially high content of insoluble aggregates and in solution β-Lg existing as native monomer. At higher pH range (6.5-8.5), β-Lg existed as native monomer and dimer at 20°C, 60°C and 70°C. An increase in temperature (80°C and 90°C) resulted in the formation of four forms of β-Lg: native monomer, non-native monomer, dimer and soluble aggregates (Figure 2).



Many events underlying the mechanism of the formation and aggregation of β-Lg have been described by many researchers [34-36]. The unfolding of β-Lg molecule is the initial step to aggregation, with a decrease in the rate of unfolding resulting in decreased aggregation rate. However, in this study, it appears that physical aggregation influenced protein aggregation than protein unfolding at lower pH (≤ 6). Further, electrostatic interactions also played significant role in the aggregation of β-Lg than hydrophobic and covalent interactions. This may have accounted for the formation of insoluble aggregates and little amount of non-native monomer at low pH. Many other authors reported little or only a small amount of non-native monomers that precipitated at low pH for β-Lg [15,26]. However, at pH above 6.0 the formation of larger soluble aggregates was favored, possibly due to linking of proteins by disulphide bonds as well as by intermolecular cross-links [35]. Low tendency for β-Lg aggregation at low pH suggest that intermolecular repulsion forces is dominant between the protein molecules previously articles identified that protein unfolding reactions would be the rate-limiting at low heating temperature and pH values close to the isoelectric point of the protein [17,37,38]. It is expected that protein aggregation would be low at higher temperature and pH values far from the isoelectric point [17]. With increase in pH, the rate of denaturation/aggregation of β-Lg aggregates decreased, because proteins had a higher propensity for polymerization through sulfhydryl/disulphide interchange reactions.

Conclusions

Temperature and pH significantly influenced the rate of yak β-Lg denaturation/aggregation and the protein formation in solution. However, pH had a major effect than temperature on yak β-Lg denaturation/aggregation. The formation of native, non-native, dimer and aggregates were confirmed by SEC and SDS PAGE. Aggregates formed at low pH (<6.5) were very large and insoluble. However, with increase in pH to 6.5, aggregates formed became soluble. No aggregation was formed below 80°C, but the aggregates formed at 80°C increased reaching the highest value at 90°C. There was less dimer formation between pH 4.5 and 5.5, while more dimer appeared at higher pH. Meanwhile pH affected the particle size at 80°C and 90°C was significantly. And these results provide some explanation of yak milk protein stability at different range of heating temperature and pH. Based on the results above, the application of yak β-Lg in foods will depend on the functionality desired with regards to the need to form soluble or insoluble aggregates.

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