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**Formation of nano-fibrils from the A, B and C variants of bovine β-lactoglobulin**

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**Abstract**

This study investigated the self-assembly of purified β-Lg genetic variants A, B and C into amyloid-like fibrils. β-Lg solutions (1% w/v) were heated at 80°C and pH 2 and were analyzed for the presence of fibrils using the thioflavin T assay. Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to follow heat-induced acid hydrolysis of β-Lg monomers. Fibrils separated from heated solutions were characterized by SDS-PAGE and transmission electron microscopy. The substitution of amino acid residues in β-Lg variants A, B and C did not significantly affect the kinetics of acid hydrolysis, self-assembly kinetics, or the morphology of the fibrils. The fibrils from β-Lg A, B and C were, however, slightly different in peptide compositions. These differences may be explained on the basis of amino acid substitutions, in particular the Asp64 of β-Lg A that is Gly in variants B and C.
1. Introduction

The bovine whey protein $\beta$-lactoglobulin ($\beta$-Lg) self-assembles into long, un-branched amyloid-like fibrils when heated above its denaturation temperature at pH 2 and at low ionic strength (Akkermans et al., 2008). Fibril formation under these conditions follows nucleation-dependent polymerization kinetics, with a 3-phase sigmoidal growth curve (Bromley, Krebs, & Donald, 2005) and fibrils formed at protein concentrations below 3 % w/v consist of peptides released from the acid hydrolysis of $\beta$-Lg (Akkermans et al., 2008).

$\beta$-Lg exists in two common genetic variants A and B, and also as a less common C form. These differ from each other due to substitutions in their amino-acid sequences (Sawyer, 2003). The differences in the sequences occur at residue 59 (Gln in variants A and B vs. His in variant C), residue 64 (Asp in variant A vs. Gly in B and C), and residue 118 (Val in variant A vs. Ala in B and C) (Sawyer, 2003). Although the native structure of the three $\beta$-Lg variants is largely similar (Bewley et al., 1997), the amino-acid substitutions influence their heat stability, aggregation propensity and the properties of aggregates formed upon heating at neutral pH (Manderson, Creamer, & Hardman, 1999; Nielsen, Singh, & Latham, 1996; Qin et al., 1999), although differences were not apparent at pH 2 (Le Bon, Durand, & Nicolai, 2002). Previous studies of $\beta$-Lg fibril formation have used either whey protein isolate (Bolder et al., 2007) or a mixture of $\beta$-Lg A and B (Akkermans et al., 2008; Hettiarachchi et al., 2012). It is not known, therefore, whether the genetic variation in $\beta$-Lg A, B and C will have any effect on their self-assembly.

The sites of substitutions in the sequences of $\beta$-Lg A, B and C have been found in fibril-forming peptides (Akkermans et al., 2008; Dave et al., 2013; Hettiarachchi et al., 2012). Under the highly acidic conditions of self-assembly, the Asp64 substitution in $\beta$-Lg A would provide an additional site for acid hydrolysis that is not present in variants B and C. The substitution of Asp 49 and Asp101 in the sequence of chicken lysozyme enhanced its self-assembly at pH 2, in comparison to equine lysozyme (Gln49) and turkey lysozyme (Gly101), by facilitating acid hydrolysis (Krebs et al., 2004; Mishra et al., 2007). In addition, valine (Val) residues in the sequence facilitate the adoption of $\beta$-sheet conformations (Street & Mayo, 1999). Thus, the net effect of Asp64 and Val119 substitutions in $\beta$-Lg A may enhance the self-assembly kinetics. Similarly, the His at position 59 in $\beta$-Lg C would increase the net positive charge on the monomer at pH 2, which may affect the electrostatic interactions between peptides, and thereby alter the kinetics of self-assembly or the morphology of fibrils. To test these possibilities, we have investigated the self-assembly of the purified A, B and C variants of $\beta$-Lg.

2. Materials and Methods

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and unless otherwise stated all reagents were prepared using Milli Q® water. The genetic variants of $\beta$-Lg A, B and C were prepared by the method of Manderson, Hardman, and Creamer (1998) and were characterized by electrospray ionization mass spectrometry (ESI-MS) (Dave et al., 2014). For self-assembly studies, the protocols for sample and fibril preparations were as per the methods described by Dave et al. (2013). The heated samples were analyzed by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the Thioflavin T (ThT) assay to detect acid hydrolysis and the presence of fibrils respectively. The protocols for these analyses were as per published methods (Dave et al., 2013; Loveday et al., 2010). The residual intensities of the unhydrolyzed $\beta$-Lg monomers in the SDS-PAGE gels were fitted with the first-order exponential function in Eq. 1 to calculate the rates of hydrolysis ($k_h$). The molecular weights of peptide bands were estimated using the methodology described in Dave et al. (2013).
\[ I = I_0 e^{-kt} \]  

The ThT fluorescence intensities in the heated samples were fitted with the sigmoidal model represented in Eq. 2 (Morris, et al., 2008) using SigmaPlot 12.0 software (Systat Software Inc., Chicago, IL, USA). The kinetic parameters of self-assembly (\( t_{\text{lag}} \), the lag time; \( \left( \frac{df}{dt} \right)_{\text{max}} \), the maximum rate of fluorescence increase; \( t_{1/2\text{ max}} \), time required to attain half the maximum fluorescence) were computed from Eq. 2 to 4 (Dave, et al., 2014; Loveday, et al., 2010). The value of the empirical constant \( \alpha \) in Eq. 2 represented \( f_{\text{max}} \), the fitted maximum fluorescence.

\[
f_t = \alpha - \frac{\beta + \alpha}{1 + \frac{p}{\gamma} \exp \left[ t(\beta + \gamma) \right]}
\]  

where \( f_t \) denotes fluorescence at time \( t \) (h), and \( \alpha, \beta \) and \( \gamma \) are constants.

\[
t_{\text{lag}} = \frac{1}{\beta + \gamma} \left( \ln \left( \frac{\alpha}{\beta} \right) - 4 \frac{\alpha}{\beta + \gamma} + 2 \right)
\]  

\[
\left( \frac{df}{dt} \right)_{\text{max}} = \frac{\left( \frac{\beta}{\gamma} + \alpha \right)(\beta + \alpha\gamma)}{4}
\]  

\[
t_{1/2\text{ max}} = \frac{\ln(2 + \alpha\gamma)}{(\beta + \alpha\gamma)}
\]  

The morphology of the fibrils was examined by transmission electron microscopy (TEM) using the protocol described by Loveday, et al. (2010). The composition of the fibrils was investigated by first separating them from heated solutions using ultracentrifugation according to the methodology described by Dave et al. (Dave, et al., 2014), then analyzing them with SDS-PAGE.

3. Results and Discussion

3.1 Heat-induced acid hydrolysis

The heat-induced acid hydrolysis in \( \beta \)-Lg samples was investigated by analyzing the heated samples with reducing SDS-PAGE. All heated samples showed progressive acid hydrolysis of \( \beta \)-Lg monomers into several peptide bands with increasing heating times (results not shown). The kinetics of acid hydrolysis followed first-order exponential kinetics and the rate constants \( k_t \), calculated from Eq. 1, are given in Table 1. The acid hydrolysis of \( \beta \)-Lg during self-assembly occurs by the cleavage of Asp-X or X-Asp peptide bonds in the sequence (Akkermans, et al., 2008; Dave, et al., 2013; Hettiarachchi, et al., 2012) and promotes self-assembly by generating assembly-capable peptides that form the building blocks of fibrils (Dave, et al., 2013). The differences in \( k_t \) for \( \beta \)-Lg A, B and C were not significant (\( P=0.47 \), likelihood ratio test) which indicates that the additional site of acid hydrolysis (Asp64, \( \beta \)-Lg A) did not affect the overall rate of hydrolysis of \( \beta \)-Lg A. Thus, at any given heating time, the samples of \( \beta \)-Lg A, B and C had approximately similar concentrations of assembly-capable peptides.

3.3 Self-assembly kinetics

The self-assembly of \( \beta \)-Lg A, B and C into fibrils was studied using the ThT assay (Figure 1). All variants showed a distinct lag phase of around 4 h duration, followed by a rapid increase in the fluorescence intensity and then a plateau after long heating times (18 h). The differences in the self-assembly of \( \beta \)-Lg A, B and C into fibrils were not significant (\( \rho = 0.52 \), likelihood ratio test). The kinetic parameters describing their self-assembly are listed in Table 1.
Figure 1. Thioflavin T fluorescence intensities at 486 nm in the heated solutions of $\beta$-Lg A (solid lines), B (dashed lines) and C (dotted lines). The concentration of $\beta$-Lg in all samples was 1% w/v and the samples were heated at 80°C and pH 2 for different heating times. Error bars represent the standard error of measurements from two experiments with two replicates in each. Curves indicate fit to Eq. 2 of Loveday et al. (Loveday, et al., 2010).

Table 1. Kinetic parameters representing the rates of acid hydrolysis and self-assembly calculated from Eq. 1 to 5: $k_h$, rate of hydrolysis; $t_{lag}$, lag time; $(df/dt)_{max}$, maximum rate of fluorescence increase; $t_{1/2\ max}$, time for fluorescence to reach half of its maximum value; $f_{max}$, maximum fluorescence. Numbers in parentheses indicate standard deviations.

<table>
<thead>
<tr>
<th>$\beta$-Lg variant</th>
<th>Molecular mass (Da)$^a$</th>
<th>$k_h \times 10^3$ (min$^{-1}$)</th>
<th>$t_{lag}$ (h)</th>
<th>$(df/dt)_{max} \times 10^2$ (FU.h$^{-1}$)</th>
<th>$t_{1/2\ max}$ (h)</th>
<th>$f_{max} \times 10^3$ (FU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>18364</td>
<td>2.18 (0.05)</td>
<td>4</td>
<td>2.2 (0.3)</td>
<td>8.5 (0.4)</td>
<td>2.0 (0.08)</td>
</tr>
<tr>
<td>B</td>
<td>18278</td>
<td>2.30 (0.09)</td>
<td>4</td>
<td>2.8 (0.3)</td>
<td>7.0 (0.3)</td>
<td>1.9 (0.07)</td>
</tr>
<tr>
<td>C</td>
<td>18278</td>
<td>2.21 (0.08)</td>
<td>4</td>
<td>2.4 (0.4)</td>
<td>7.1 (0.4)</td>
<td>2.1 (0.10)</td>
</tr>
</tbody>
</table>

$a$: mass corresponding to the single and the largest peak in the de-convoluted electrospray ionization mass spectrometry (ESI MS) spectra of $\beta$-Lg A, B and C.

The similarity in the $t_{lag}$ (experimental and calculated) for $\beta$-Lg A, B and C self-assembly, may be attributed to the similar overall rates of monomer hydrolysis and sequence homology in the N-terminal region (residues 1-58) of the variants. This region forms the core of fibrils and plays an important role in nucleation (Dave, et al., 2013; Dave, et al., 2014). The $(df/dt)_{max}$ and $f_{max}$ for heated $\beta$-Lg A, B and C were also similar, suggesting that the peptide-peptide interactions leading to the growth of the nuclei into fully-formed fibrils were unaffected by the differences in the primary structure. This indicates that the effects of the amino acid
substitutions in $\beta$-Lg A and C were too minor to significantly impact the peptide-peptide interactions during self-assembly.

3.4 Characterization of the fibrils

Fibrils from $\beta$-Lg A, B and C were long, unbranched and semi-flexible. Furthermore, the fibrils from all three variants were uniform in diameter and up to 1 µm in length (Figure 2). These features of the fibrils were very similar to those reported previously (Akkermans, et al., 2008; Bolder, et al., 2007).

Figure 2. Transmission electron microscopy images of fibrils from $\beta$-Lg A, B and C after 24 h at 80°C.
The composition of the fibrils was investigated by analyzing purified fibrils by SDS-PAGE after ultracentrifugal separation. The SDS-PAGE analysis showed that the fibrils from all three variants consisted of a few preferentially-accumulated peptides (Figure 3). There were minor differences among the variants in the number and intensity of bands corresponding to peptides in the fibrils. Fibrils from β-Lg A had an additional band, labeled ‘R’ in Figure 3, which was not present in fibrils assembled from β-Lg B or C. Similarly, the intensity of the peptide band marked ‘C’ in Figure 3 was higher in fibrils from β-Lg A than those from β-Lg B and C. Fibrils from β-Lg B produced two additional weak bands (region ‘i’ in Figure 3) compared to those from β-Lg A and β-Lg C.

![Figure 3](image_url)

**Figure 3.** Reducing tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis of fibrils formed after heating β-Lg (1% w/v) solutions at 80°C, pH 2 for 12 h and separated by ultracentrifugation at 2.3 x 10⁶ g for 60 minutes at 20°C. Pellets were washed thrice by repeated cycles of re-suspending the pellet in water (pH 2) and ultracentrifugation. M₀: molecular weight marker; S: supernatant; P: pellet containing fibrils. Molecular weights in kDa. See text for descriptions of ‘i’ and bands A to E and R.

These differences in the peptide make-up of fibrils may be explained on the basis of the amino acid substitutions in these variants. Based on the in-gel digestion ESI-MS/MS data for the SDS-PAGE peptide bands of fibrils reported previously (Dave, et al., 2013), the possible sequences present in Bands A to E and R were constructed (Table 2). In β-Lg A, the preferential cleavage of the peptide bond involving Asp64 would be expected to result in two distinct additional peptides (band R: seq. 65-136, band B: seq. 1-64 and band C: seq. 12-64) in comparison to those in β-Lg B and C.
Table 2. Possible sequences present in the peptide bands of fibrils from β-Lg A, B and C, as shown in Figure 3.

<table>
<thead>
<tr>
<th>β-Lg Variant</th>
<th>Band</th>
<th>Location</th>
<th>Possible Sequence[^a,^b]</th>
<th>Predicted Mass[^c]</th>
<th>SDS-PAGE Mass[^d]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, B, C</td>
<td>A</td>
<td>12-96</td>
<td>(D)IQKVAGTWYSLAMAASDILLDAQSAVLPRYYVEELKPTPEGDL EILL#KWEN$ECAQKKIIAEKTIPAVFKIDALNENKVLVD(T)</td>
<td>9.3</td>
<td>8.9</td>
</tr>
<tr>
<td>A, B, C</td>
<td>A</td>
<td>54-130</td>
<td>(D)EILLQKWEN$ECAQKKIIAEKTIPAVFKIDALNENKVLVD TDY KYLLFCMENSAPEQSL@CQCLVRTPEVD(V)</td>
<td>8.9</td>
<td>8.9</td>
</tr>
<tr>
<td>A, B, C</td>
<td>A</td>
<td>85-162</td>
<td>(I)DALNENKVLVD TDY KYYLLFCMENSAPEQSL@CQCLVRTPEVDDEALEKFDKALPMHRLSFNP'TQLEEECHI(−)</td>
<td>8.9</td>
<td>8.9</td>
</tr>
<tr>
<td>A, B, C</td>
<td>A</td>
<td>65-136</td>
<td>(D)ECAQKKIIAEKTIPAVFKIDALNENKVLVD TDY KYYLLFCMEN SAPEQSLVCQCLVRTPEVDDEALEKF(D)</td>
<td>8.2</td>
<td>8.0</td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>1-64</td>
<td>(−)LIVTQMKGLDIIQKVAGTWYSLAMAASDILLDAQSAVLPRYYVE ELKPTPEGDLEILQDKWEND(E)</td>
<td>7.1</td>
<td>7.2</td>
</tr>
<tr>
<td>A, B, C</td>
<td>B</td>
<td>34-98</td>
<td>(D)AQSAVLPRYYVEELKPTPEGDLEILL#KWEN$ECAQKKIIAEKTIPAVFKIDALNENKVLVD(Y)</td>
<td>7.1</td>
<td>7.2</td>
</tr>
<tr>
<td>A, B, C</td>
<td>C</td>
<td>1-53</td>
<td>(−)LIVTQMKGLDIIQKVAGTWYSLAMAASDILLDAQSAVLPRYYVE ELKPTPEGD(L)</td>
<td>5.7</td>
<td>5.7</td>
</tr>
<tr>
<td>A</td>
<td>C</td>
<td>12-64</td>
<td>(D)IQKVAGTWYSLAMAASDILLDAQSAVLPRYYVEELKPTPEGDLEILLQDKWEND(E)</td>
<td>5.9</td>
<td>5.7</td>
</tr>
<tr>
<td>A, B, C</td>
<td>C</td>
<td>34-84</td>
<td>(D)AQSAVLPRYYVEELKPTPEGDLEILLQKWEN$ECAQKKIIAEKTIPAVFKIDALNENKVLVD(E)</td>
<td>5.7</td>
<td>5.7</td>
</tr>
<tr>
<td>A, B, C</td>
<td>D</td>
<td>1-52</td>
<td>(−)LIVTQMKGLDIIQKVAGTWYSLAMAASDILLDAQSAVLPRYYVE ELKPTPEG(D)</td>
<td>5.6</td>
<td>5.2</td>
</tr>
<tr>
<td>A, B, C</td>
<td>D</td>
<td>53-96</td>
<td>(G)DLEILL#KWEN$ECAQKKIIAEKTIPAVFKIDALNENKVLVD(T)</td>
<td>5.1</td>
<td>5.2</td>
</tr>
<tr>
<td>A, B, C</td>
<td>E</td>
<td>12-52</td>
<td>(D)IQKVAGTWYSLAMAASDILLDAQSAVLPRYYVEELKPTPEG(D)</td>
<td>4.4</td>
<td>4.4</td>
</tr>
<tr>
<td>A, B, C</td>
<td>E</td>
<td>1-33</td>
<td>(−)LIVTQMKGLDIIQKVAGTWYSLAMAASDILLDAQSAVLPRYYVEELKPTPEG(D)</td>
<td>3.6</td>
<td>4.4</td>
</tr>
</tbody>
</table>

[^a]: locations and possible sequences in peptide bands predicted from the sequences found experimentally by in-gel digestion MS/MS of SDS-PAGE peptide bands of fibrils (Dave, et al., 2013); [^b]: only peptide bonds involving aspartic acid residues (D) were considered as sites of acid hydrolysis; [^c]: predicted molecular weights calculated from ExPAsy compute pl/MW resource tool (Lupton, 1996); [^d]: calculated from the equation, Log (molecular weight) = -1.69 (Rf) + 5.16, derived from the standard curve of log (molecular weight) vs relative mobility (Rf) plotted from molecular weight markers in Figure 3.

Letters in the parentheses indicate the amino acid residue adjacent to the peptide while (−) indicates either, 'N' terminal or 'C' terminal end of the β-Lg. a: locations and possible sequences in peptide bands predicted from the sequences found experimentally by in-gel digestion MS/MS of SDS-PAGE peptide bands of fibrils (Dave, et al., 2013); b: only peptide bonds involving aspartic acid residues (D) were considered as sites of acid hydrolysis; c: predicted molecular weights calculated from ExPAsy compute pl/MW resource tool (Lupton, 1996); d: calculated from the equation, Log (molecular weight) = -1.69 (Rf) + 5.16, derived from the standard curve of log (molecular weight) vs relative mobility (Rf) plotted from molecular weight markers in Figure 3.
4. Conclusions

In this study we have shown that the genetic substitution of amino acids in β-Lg had no measureable effect the kinetics of acid hydrolysis or fibril assembly. Fibrils of β-Lg A, B and C were similar in morphology; however, we observed minor differences in the peptide composition of the fibrils, which may be attributed to the amino-acid substitutions in these variants.

Acknowledgements

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References.


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