Acid-responsive properties of fibrils from heat-induced whey protein concentrate

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ABSTRACT

The heat-induced fibrils of whey protein concentrate (WPC) have demonstrated an acid-responsive property; that is, the fibrils went through formation-depolymerization-reformation as pH was adjusted to 1.8, 6.5, and back to 1.8. We investigated the microstructure, driving force, and thermal stability of 3.0% (wt) WPC nanofibrils adjusted between pH 6.5 and 1.8 twice. The results showed that the nanofibrils had acid-responsive properties and good thermal stability after reheating for 10 h at 90°C and adjusting pH from 1.8 to 6.5 to 1.8. The content of WPC fibril aggregates was not much different with the prolongation of heating times during pH variation. Although the nanofibrils’ structure could be destroyed only by changing the pH, the essence of this destruction might only form fiber fragments, polymers that would restore a fibrous structure upon returning to pH 1.8. A described model for the acid-responsive assembly of fibrils of WPC was proposed. The fibrils went through formation-depolymerization-reformation by weaker noncovalent interactions (surface hydrophobicity) as pH changed from 1.8 to 6.5 back to 1.8. However, the fibrils lost the acid-responsive properties because much more S-S (disulfide) formation occurred when the solution was adjusted to pH 6.5 and reheated. Meanwhile, fibrils still possessed acid-responsive properties when reheated at pH 1.8, and the content of fibrils slightly increased with a further reduction of α-helix structure.

Key words: whey protein concentrate, fibrils, microstructure, acid responsive

INTRODUCTION

The study of protein aggregation has been important in relation to food production and biotechnology (Bauer et al., 2000). Many globular proteins have shown the ability to form long, thin fibrillar aggregates at pH 2.0 and low ionic strength, such as β-LG (Kavanagh et al., 2000; Ikeda and Morris, 2002; Veerman et al., 2002, 2003), whey protein concentrates (WPC; Wang et al., 2013), and whey protein isolates (WPI; Durand et al., 2002; Gosal et al., 2002; Arnaudov et al., 2003), after heating at high temperature for several hours. Arnaudov et al. (2003) described fibrils of β-LG at acidic pH in 3 main stages: an initial unfolding step, a step of linear fibrillar aggregation via nucleation and growth, and finally a step of random association of the fibrils. Ionic strength and pH also affect both the kinetics of fibril formation and the morphology of fibrils (Aymard et al., 1999; Bolder et al., 2006; Arnaudov and de Vries, 2007). With heat treatment at 80°C, added NaCl substantially accelerated β-LG denaturation at pH 2.5 (Schokker et al., 2000). Flexible fibrillar networks are formed at pH 3.35, and more branching is observed with an increase of ionic strength (Mudgal et al., 2009). Fibrils become shorter and more flexible and have a lower critical percolation concentration of β-LG fibril gel with increasing ionic strength (Mudgal et al., 2011). Surface hydrophobicity plays a dominant role in the formation of fibrils aggregates. Except the hydrophobic interactions, other noncovalent interactions such as ionic bonds, van der Waal’s force, and hydrogen bonds also stabilize the formation of fibrils, whereas disulfide interchange reactions have been shown to be inhibited at low pH (McKenzie et al., 1972). Disulfide bonding between β-LG molecules does not occur to any significant extent because cysteine residues are predominantly protonated (Otte et al., 2000; Alting et al., 2002). The formation of heat-induced nanofibrils is due to noncovalent interactions; therefore, its aggregation properties might be different from normal protein aggregates. The objective of the present study was to investigate the variation of properties between nanofibrils and normal protein aggregates of WPC through adjusting pH (pH 1.8, 6.5) and reheating (90°C, 10 h). Finally, a described model for the assembly and depolymerization of WPC fibrils was proposed at pH between 1.8 and 6.5.
MATERIALS AND METHODS

Materials

The WPC-80 (76.93% protein, 1.4% fat, 5.6% lactose, 4.62% ash) was purchased from Hilmar Cheese Co. (Hilmar, CA). Thioflavin T and 1-anilino-8-naphthalene sulfonate were purchased from Sigma-Aldrich (St. Louis, MO). 2,2'-Dinitro-5,5'-dithiodibenzoate (DTNB) was purchased from Merck (Darmstadt, Germany). All other reagents and chemicals were of analytical grade. All the reported results were averages of 3 separate experiments.

Solution Preparation

The WPC powder was dissolved in double-distilled water, and, after dissolving, the solution was adjusted to pH 2.0 by adding 6 M HCl. To remove any undissolved protein, the solution was centrifuged at 19,000 \( \times g \) (GL-21M centrifuge, Shanghai Precision Instruments Co. Ltd., Shanghai, China) for 30 min at 4°C. The nitrogen content of supernatants were determined by Kjeldahl analysis (N × 6.38; KDN-102C, Shanghai Qian Jian Instruments Co. Ltd.) and the supernatant was diluted into 3.0% (wt) solution; to adjust pH to 1.8 (6 M HCl, 0.1 M HCl), solutions were heated at 90°C for different periods of time (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h) in a water bath. After heat treatment, the samples were immediately cooled to room temperature by immersing in ice bath to form WPC nanofibrils (Ko and Gunasekaran, 2006; Bolder et al., 2007; Akkermans et al., 2008). Normal protein aggregates (pH 6.5) were formed by the same methods, except for adjusting pH (before centrifuging and heating treatment).

Transmission Electron Microscopy

The microstructure of heated protein dispersions was investigated by transmission electron microscopy (H-7650, Hitachi High-Technologies Corporation, Krefeld, Germany) according to the procedure of Krebs et al. (2009), with some modifications. Protein dispersion was diluted with 0.01 M phosphate buffer (pH 6.7), containing samples of varying total WPC (0.02, 0.01, 0.005, and 0.0025%, wt/vol) added to aliquots (20 μL) of 1-anilino-8-naphthalene sulfonate (8.0 mmol/L in the same buffer), vortexed, and kept in the dark for 20 min. The fluorescence emission intensities at 470 nm (whereas excitement was 390 nm) for each protein concentration were measured with fluorescence spectrophotometer (F-4500, Hitachi High-Technologies Corporation). The initial slope of the plot of fluorescence intensity versus protein concentration, which was calculated by linear regression (in all cases, \( R^2 >0.95 \)), was used as an index of the surface hydrophobicity of the protein sample evaluated. All measurements were performed in triplicate.

Turbidity

The turbidity of dispersions was measured using a UV spectrophotometer (TU-1800, Beijing Precision Instruments Co. Ltd., Beijing, China) according to the procedure of Kurganov (2002), with some modifications; the path length of sample cell used was 10 mm. The protein samples were diluted to the concentration of 1.5% (wt) in double-distilled water, vortexed, and had absorbance measured at 400 nm and room temperature; the double-distilled water was used as the blank and turbidity values were represented through absorbance values. All measurements were performed in triplicate.

Protein Surface Hydrophobicity

The surface hydrophobicity of the protein samples was determined by the method of Tubio et al. (2004). Protein dispersions were diluted with 0.01 M phosphate buffer (pH 6.7), containing samples of varying total WPC (0.02, 0.01, 0.005, and 0.0025%, wt/vol) added to aliquots (20 μL) of 1-anilino-8-naphthalene sulfonate (8.0 mmol/L in the same buffer), vortexed, and kept in the dark for 20 min. The fluorescence emission intensities at 470 nm (whereas excitement was 390 nm) for each protein concentration were measured with fluorescence spectrophotometer (F-4500, Hitachi High-Technologies Corporation). The initial slope of the plot of fluorescence intensity versus protein concentration, which was calculated by linear regression (in all cases, \( R^2 >0.95 \)), was used as an index of the surface hydrophobicity of the protein sample evaluated. All measurements were performed in triplicate.

Determination of Free Sulfhydryl Group

Free sulfhydryl group (SH) contents of protein samples were determined by the method of Shimada and Cheftel (1989), with some modifications. For free sulfhydryl group content determination, 5 mL of the Tris-Gly buffer (0.086 M Tris, 0.09 M glycine, 0.004 M EDTA, pH 8.0) containing 8 M urea were added to 0.3-mL protein samples (30 mg/mL). Then, 20 μL of DTNB (Merck) was added and absorbance was measured at 412 nm with UV spectrophotometer (UV-2401 PC, Shimadzu Corporation, Kyoto, Japan) after 15 min. The supernatants in buffer without DTNB were used as blanks. The calculation was as follows:

\[
\text{SH (μmol/g)} = \frac{(73.53 \times A_{412} \times D)}{C},
\]

where \( A_{412} \) is the absorbance at 412 nm, \( C \) is sample concentration (mg/mL), \( D \) is the dilution factor, and 73.53 is derived from 10^6/(1.36 \times 10^4); 1.36 \times 10^4 is the molar absorptivity (Ellman, 1959) and 10^6 is for...
the conversion from a molar basis to a millimolar per milliliter basis and from milligrams to grams of solids.

**Aggregate Rate**

The protein dispersions (3%, wt, protein basis) were heated at 90°C for 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h, normal aggregates were heated at 90°C at pH 6.5, fibril aggregates were heated at 90°C at pH 1.8. Twenty milliliters of each protein solution was transferred to 45-mL centrifuge tubes and then centrifuged at 19,000 $\times g$ for 30 min at 4°C; the protein concentration of the precipitation was determined by Kjeldahl analysis: $N \times 6.38$ (KDN-102C, Shanghai Qian Jian Instruments Co. Ltd.). The calculation was

$$\text{Aggregate rate} = \frac{C_t}{C_0}, \quad [2]$$

where $C_t$ and $C_0$ represent the protein concentration (mg/mL) after $t$ and 0 h, respectively. All measurements were performed in triplicate.

**Thioflavin T Fluorescence**

A thioflavin T (ThT) solution (800 mg/L) was prepared by dissolving ThT in 10 mM sodium phosphate buffer (pH 7.0) containing 200 mM NaCl and the solution was filtered (0.2 μm, Minisart, Sartorius, Gottingen, Germany) to remove undissolved ThT. The stock solution was stored at 4°C in a brown glass bottle covered with aluminum foil. Working solution was prepared by diluting the stock solution 50 fold in phosphate-NaCl buffer. Samples of 400 μL were added to 10 mL of ThT solution. The fluorescence of the samples was measured on a Hitachi F4500 fluorescence spectrometer (Tokyo, Japan) with excitation at 460 nm and emission at 490 nm (Akkermans et al., 2008); slit widths were 5 and 10 nm, respectively. All measurements were performed in triplicate.

**Circular Dichroism Spectroscopy**

Far-UV circular dichroism spectra were obtained using J-815 spectropolarimeter (Jasco Inc., Easton, MD) in 0.02-cm light path quartz cells by the method of Kurouski et al. (2012) with some modifications. All samples of 100 μL were diluted with 2.9 mL of ice-cold 0.005 M Tris-HCl buffer, pH 7.5, and stored on ice until the circular dichroism measurements the next day; the samples were scanned from 190 to 280 nm with a scan rate of 100 nm/min, and CD measurements were expressed as mean residue ellipticity ($\theta$) in degrees cm$^{-2}$dmol$^{-1}$. Protein α-helix was evaluated using the formula of Yang et al. (1986):

$$\alpha\text{-helix} (%) = \frac{-([\theta]_{222} + 3,000)}{33,000}. \quad [3]$$

All measurements were performed in triplicate.

**Acid Response**

We used the protein concentration of 3.0% (wt) WPC nanofibrils with different heating times (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h) as control samples. The 3.0% (wt) WPC nanofibrils were first adjusted to pH 6.5 (1 M NaOH, 0.1 M NaOH), first adjusted back to pH 1.8 (1 M HCl, 0.1 M HCl), then reheating the samples adjusted them back to pH 1.8 by the corresponding time (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 h); after reheating, nanofibrils were second adjusted to pH 6.5 (1 M NaOH, 0.1 M NaOH), second adjusted back to pH 1.8 (1 M HCl, 0.1 M HCl), and various indicators (surface hydrophobicity, free sulphydryl group, aggregate rate, thioflavin T fluorescence, turbidity) were determined at different stages.

**Statistical Analysis**

An ANOVA was conducted using Statistix 8.1 (Systat Software, Chicago, IL) and Microsoft Excel (version 2003, Microsoft Corp., Redmond, WA). Replicate means were considered significantly different at $P < 0.05$ unless stated. When significant differences were indicated by ANOVA, Tukey pair-wise comparisons were performed to indicate where the differences between properties existed.

**RESULTS**

**TEM**

The WPC nanofibrils had pH adjusted between 1.8 and 6.5 twice and the morphology of fibrils aggregation was examined using TEM (Figure 1). Nanofibril solutions were first adjusted to pH 6.5 and the nanofibril structure was destroyed, with fibril fragments aggregating into cluster-structure aggregates (Figure 1b) different from the structure of normal aggregates, which formed a particulate polymer by heating at a pH of 6.5 (Figure 2). The cluster-structure aggregates could reform fibril-structure aggregates after adjusting pH from 6.5 to 1.8 again (Figure 1c); the solutions adjusted back to pH 1.8 were reheated for 10 h and the fibrils also maintained fine-stranded morphology.
After the second adjustment of pH, the results were similar to the first treatment except more branches were noted (Figure 1e and f). We found that the nanofibrils had acid-responsive properties and good thermal stability after reheating.

**Turbidity**

We noted a gradual development of turbidity at 400 nm, along with acid-responsive processing and reheating (Figure 3). We observed that the optical density values of all samples were increased. A sharp increase of normal WPC aggregates (pH 6.5) was observed in the range of 58.53% at heating for 1 h, then gradually increased at 2 to 10 h, which were increased slowly and only increased by 29.11% at 10 h. The optical density values of fibril solutions adjusted to pH 6.5 twice were obviously higher than that of nanofibrils, which were similar to normal WPC aggregates. Conversely, the trend for fibril solutions first adjusted to pH 1.8 was the same as the nanofibrils. When the sample first adjusted to pH 1.8 was reheated for 10 h, the optical density values were similar to the initial values of fibrils and the TEM image showed that nanofibrils became thicker, branched, and formed more amyloid structure after reheating (Figure 1d). The optical density values of second change in fibrils back to pH 1.8 were similar to reheating treatment at first adjustment to pH 1.8 and the initial values of fibrils.

**Content of Protein Aggregation**

The amount of WPC aggregation was much different with the prolongation of heating times at 2 pH values (Figure 4). The normal WPC (pH 6.5) aggregated much faster than that of forming fibrils (pH 1.8), which was approximately 6 times faster compared with the amount of nanofibrils after heating for 10 h. It was interesting the content of protein aggregation increased slightly when the fibril solutions were adjusted to pH 6.5, and much lower than that of normal aggregates from nature WPC heated at a pH of 6.5. The results showed that the aggregate structure of fibril solutions when changing from pH 1.8 to 6.5 was different from reorganization of fibrils because more aggregation occurred. When the solutions changed back to pH 1.8 twice, the values were not changed. From the acid-responsive processing, we suggest that the depolymerization and reformation of fibrils did not lead to the destruction of fibril structure completely or form other aggregation structures. This inference could also be confirmed from the TEM results, as fibril fragments aggregated into cluster-structure aggregation with increasing pH (Figure 1) and fibrils could be reformed by decreasing pH.

**ThT**

Recently, it was found that ThT fluorescent molecules could be combined with the β-sheet structure of fibrils (i.e., measured fluorescence intensity was proportional to the content of fibrils; Groenning et al., 2007). The fluorescence intensity of pH 1.8 nanofibrils was significantly higher than that of normal WPC aggregates (pH 6.5; Figure 5). When the nanofibril solutions were first adjusted to pH 6.5, the ThT fluorescence intensity showed a ~36% decrease compared with nanofibrils (1.8), then the values rose again with a change to pH 1.8. After second adjustment to pH 6.5, the results were similar to the first adjustment without further reduction. We found intermediate values for the nanofibrils solutions that were first or second adjusted to pH 6.5 between that of normal WPC aggregates at pH 6.5 and nanofibrils at pH 1.8. The results showed that the structure of aggregates first or second adjusted to pH 6.5 were quite different from the normal aggregates at the same pH, although the nanofibril structure could be destroyed only by changing pH and the essence of this destruction might only form fiber fragment polymers that would restore a fibrous structure by readjusting to pH 1.8. Interestingly, the amount of fibril aggregates could be increased by 3% when the solutions were reheated for 10 h after adjusting back to pH 1.8 (Figure 5). The content of nanofibrils slightly increased with a further reduction of α-helix structure in WPC after reheating at a pH of 1.8 (Table 1). The ThT results showed that reheating treatment was more beneficial to increase the fibril content.

**DISCUSSION**

The fibrils of WPC went through formation-depolymerization-reformation by adjusting pH from 1.8 to 6.5 and back to 1.8, and the nanofibril structure was not destroyed thoroughly. This may be due in part to the major forces of protein molecules which were changed in the process of formation-depolymerization-reformation of nanofibrils. Previous studies deemed that surface hydrophobicity was the major force in formation of nanofibrils (Otte et al., 2000; Alting et al., 2002; Wang et al., 2013). No significant variation in surface hydrophobicity was noted in acid-responsive processing (Table 2), whereas the content of thiol groups did not change significantly in acid-responsive processing as pH changed from 1.8 to 6.5 to 1.8. But, compared with the preheating, the reheating treatment could lead to
Figure 1. Transmission electron micrographs (TEM) of the 3.0% (wt) whey protein concentrate nanofibrils at different pH and reheating treatment: (a) pH 1.8, (b) first adjusted to pH 6.5, (c) first changed back to pH 1.8, (d) reheating pH 1.8, (e) second adjusted to pH 6.5, and (f) second changed back to pH 1.8.
the reduction of surface hydrophobicity (~19.7%) and thiol groups (~23.31%) as the solutions of first changed back to pH 1.8 after reheating for 10 h. The reduction of thiol groups came from reheating to form disulfide bonds, which were still reformed fibrils at the second pH adjustment, without affecting acid-responsive properties of nanofibrils. Nevertheless, when pH changed from 1.8 to 6.5 after heating for 30 min, the reduction of thiol groups showed a ~56.39% decrease compared

**Figure 2.** Transmission electron micrographs (TEM) of the 3.0% (wt) whey protein concentrate (WPC) particulate aggregates and 3.0% (wt) WPC nanofibrils adjusted to pH 6.5. The samples (pH 6.5) were particulate aggregates (a) and nanofibrils adjusted to pH 6.5 (b; same micrograph as in panel b in Figure 1).

**Figure 3.** Variation of optical density at 400 nm for 3.0% (wt) whey protein concentrate (WPC) nanofibrils at different pH and reheating treatment. The samples (3%, wt, protein basis) were particulate aggregates (pH 6.5), nanofibrils (pH 1.8), first adjusted to pH 6.5, first changed back to pH 1.8, reheating changed back to pH 1.8 for 10 h at 90°C, second adjusted to pH 6.5, and second changed back to pH 1.8. Data are expressed as the mean ± SD of 3 replicates.

**Figure 4.** Fractional concentration of nonaggregated whey protein concentrate (WPC) by heating 3% (wt, protein basis) WPC solution versus heating time at 90°C with different pH and reheating treatment; $C_t/C_0$ is the ratio of whey protein concentration at time $t$ and time zero. Reaction conditions: The samples (3%, wt, protein basis) were particulate aggregates (pH 6.5), nanofibrils (pH 1.8), first adjusted to pH 6.5, first changed back to pH 1.8, reheating changed back to pH 1.8 for 10 h at 90°C, second adjusted to pH 6.5, and second changed back to pH 1.8. Data are expressed as the mean ± SD of 3 replicates.
with preheating, which led to complete destruction of the fibrils structure and fibrils never reforming.

Based on the results, we developed a new model of acid-responsive aggregation, described in Figure 6. Fibril solution was adjusted to pH 6.5, fibril fragments aggregated into large polymers through weaker noncovalent interactions (surface hydrophobicity), intermolecular repulsion strengthened because of increased surface charges when the pH was changed back to 1.8, and large polymers of noncovalent dissociated and led to the reformation of fibrils; however, this dissociation was not complete, which led to fibrils with a more branched and amyloid structure. Conversely, when the solutions were adjusted to pH 6.5 and reheated, the acid-responsive properties were lost because of much more covalent bond (disulfide bond) formation. The content of disulfide bonds in solutions adjusted to pH 6.5 and reheated for 30 min increased to 54.35% accompanied by the aggregation of fibril fragments, whereas pH 1.8 solutions increased only approximately 10.58% after reheating (Table 3). The aggregations from reheating at a pH of 6.5 were not depolymerized when the conditions changed back to pH 1.8; thus, we could not reform fibril structure. However, reheating the pH 1.8 fibril solution led to the formation of few disulfide bonds, which was not affected by the acid-responsive properties and could reform fibrils in the processing of pH adjusted between 6.5 and 1.8.

CONCLUSIONS

The WPC nanofibrils showed good acid-responsive properties when adjusting pH between 6.5 and 1.8 twice. The acid-responsive properties were due to protein molecules aggregating with each other by noncovalent bonds (surface hydrophobicity) into fibrils, which led to the variation of pH causing fibril formation-depolymerization-reformation. A model acid-responsive aggregation pathway was proposed as fibril fragments aggregated into large polymers through weaker noncovalent interactions (surface hydrophobicity) when pH was adjusted to 6.5; large polymers dissociated because of the strengthening of intermolecular repulsion led to reformation of fibrils when pH was adjusted back to 1.8. In contrast, solutions adjusted to pH 6.5 after reheating could not reform fibril structure because of many covalent bonds (disulfide bonds) formed between protein molecules. The content of nanofibrils slightly increased with the further reduction of α-helix structure in WPC after reheating at a pH of 1.8.

ACKNOWLEDGMENTS

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Table 1. Variation of α-helix of 3.0% (wt) whey protein concentrate (WPC) dispersions at 0 h, 3.0% (wt) WPC fibril dispersions (10 h), and 3.0% (wt) WPC nanofibril dispersions after reheating for 10 h

<table>
<thead>
<tr>
<th>Conditions</th>
<th>WPC material</th>
<th>Nanofibrils</th>
<th>Reheating nanofibrils</th>
</tr>
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<tbody>
<tr>
<td>α-Helix (%)</td>
<td>69.1 ± 0.34</td>
<td>34.7 ± 0.56</td>
<td>28.9 ± 0.72</td>
</tr>
</tbody>
</table>

Table 2. Changes in properties of whey protein concentrate nanofibrils (3.0%, wt, heating 10 h at 90°C) versus acid-responsive properties at pH 1.8

<table>
<thead>
<tr>
<th>Sample</th>
<th>Free sulfhydryl group (μmol/g)</th>
<th>Surface hydrophobicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 1.8 (nanofibrils)</td>
<td>16,596.66 ± 9.875</td>
<td>90.41 ± 2.233</td>
</tr>
<tr>
<td>Nanofibrils first changed back to pH 1.8</td>
<td>15,707.17 ± 30.158</td>
<td>86.17 ± 0.424</td>
</tr>
<tr>
<td>Reheating to pH 1.8 (10 h, 90°C)</td>
<td>12,945.32 ± 13.610</td>
<td>68.97 ± 1.753</td>
</tr>
<tr>
<td>Nanofibrils second changed back to pH 1.8</td>
<td>11,145.12 ± 10.520</td>
<td>64.86 ± 1.658</td>
</tr>
</tbody>
</table>

Different superscripts indicate significant difference at \( P < 0.05 \).
Figure 6. Proposed model of nanofibrils acid responsive pathway.

Table 3. Changes in properties of whey protein concentrate nanofibrils (3.0%, wt, heating 10 h at 90°C) versus acid-responsive properties at pH 6.5

<table>
<thead>
<tr>
<th>Sample</th>
<th>Turbidity</th>
<th>Free sulfhydryl group (μmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 1.8 (nanofibril)</td>
<td>0.956 ± 0.027&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16,596.66 ± 9.875&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Nanofibrils adjusted to pH 6.5</td>
<td>2.155 ± 0.126&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15,856.77 ± 10.543&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Reheating adjusted to pH 6.5 (30 min, 90°C)</td>
<td>2.788 ± 0.214&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7,238.26 ± 15.365&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Changed back to pH 1.8</td>
<td>2.534 ± 0.137&lt;sup&gt;c&lt;/sup&gt;</td>
<td>7,135.43 ± 11.987&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a–d</sup>Different superscripts indicate significant difference at <i>P</i> < 0.05.
REFERENCES


