a-Lactalbumin (a-LA) & β-Lactoglobulin (β-LG) are major protein moieties of bovine whey proteins. As much of the process involves heat treatment during the preparation of milk on an industrial scale, the unpredictable measures of the process are an essential issue in determining the quality of the milk. The purpose of the present study was to investigate the major change(s) of whey proteins in the processed milk using a simple approach. By a native-polyacrylamide gel electrophoresis (PAGE), β-LG in processed milk was found to be extensively denatured, but not α-LA. Such denaturation was presumably associated with the heating procedure used in the process. The kinetics of thermal denaturation of purified α-LG or α-LA at various temperatures was further studied using a circular dichroic (CD) analysis. The maximal changes of ellipticity at 205 nm were correlated to the heating temperature and time. There were no significant changes in CD spectrum of β-LG at the temperature below 60 ℃ within 500 s, mild changes occurred at 70-80 ℃, but the most pronounced changes were at 90-95 ℃ in a time-dependent fashion. Westernblot analysis revealed that the large multiple forms of β-LG were markedly present in processed milk, but not in the raw milk. Therefore, using a simple native PAGE and Westernblot, β-LG may be served as a marker for evaluating the thermal process of manufactured milk.

Keywords: α-Lactalbumin, β-Lactoglobulin, Native-PAGE, Westernblot

Molten globules are thought to be general intermediates in protein folding and unfolding (1). α-LA or β-LG is one of the major protein moieties of bovine whey proteins, both of them are the most investigated models for understanding the mechanism involved in protein stability, folding and unfolding upon the heating. Recent studies have shown that milk α-LA and β-LG may induce apoptosis in tumor cells (2,3), and possess
immunomodulatory (4-6) and hypcholesterolemic effect (7). As much of the process involves heat treatment during the preparation of milk on an industrial scale, the unpredictable nature of the process has therefore been an essential issue in affecting the physiologic role of the $\alpha$-LA and $\beta$-LG. For this reason, we attempted to investigate some major changes of the whey proteins in the processed milk. In fact, there were no such reports that have been documented concerning the processed milk thus far. In the present study, we demonstrated that there was a substantial loss of $\beta$-LG in the whey proteins using a native-PAGE. Since the manufacturers do not disclose the processing procedures of the milk, we established the thermal denaturation curves of the loss of $\alpha$-LA and $\beta$-LG with respective to their heating time and temperatures. Some of the thermal denaturation of each respective $\alpha$-LA or $\beta$-LG has previously been investigated (8,9) ; but the detailed information regarding to their correlation with the kinetic changes in CD spectra at various temperatures has not been simultaneously reported. The present study provided a reference value, in general, for the study of the correlation of conformational changes and electrophoretic properties in both $\alpha$-LA and $\beta$-LG. In addition, the Westernblot analysis on the changes in some molecular form of $\beta$-LG in the raw and processed milk, which has not been reported previously, is also investigated.

結果與討論
Result

To identify the possible difference in protein profiles between raw and dairy processed milk, freshly prepared whey samples were initially analyzed on a native-PAGE. As shown in Fig. 1, using the same batch of the milk before and after manufactured process, there was a marked decrease in two acidic proteins in processed milk as compared to that of raw milk. N-terminal sequence analysis of these proteins revealed that they were the isoforms of $\beta$-LG (Table 1) consisting to the chemical characteristics of $\beta$-LG previously reported (14). In the next experiment, we examined the whey samples on a denatured SDS-PAGE using a PAGE procedure without heat. Similarly, the $\beta$-LG (at a molecular weight about 18 kDa) was substantially reduced (Fig. 2). It is worthy to mention that one additional band corresponding to a molecular weight about 25 kDa was observed in the processed milk sample (Fig. 2), but it was not related to $\beta$-LG as assessed by a Westernblot (described later). It might be derived from the other protein moiety during the manufacturing process. Thus, our data suggested that the overall negative charges as well as the molecular form of $\beta$-LG were altered during the process of dairy milk.

Using a native-PAGE in the next experiment, we determined the feature of whey proteins in other 4 major brands of processed milk purchased from the market in Taiwan (total 5). Fig. 3 shows that the $\beta$-LG component, but not $\alpha$-LA, was extensively denatured in all 5 samples. However, the $\beta$-LG remained almost intact in 4 randomly chosen brands from the US market (Fig. 3). In addition, 3 brands of powdered milk imported from Australia, New Zealand, and Denmark were also analyzed (Fig. 3). The Denmark brand exhibited some extent of denaturation in $\beta$-LG, but not from that of Australia and New Zealand. Thus, a simple native-PAGE may be used to impart technological difference involved in the process of dairy milk.

To test the hypothesis that the overall change in negative charges of $\beta$-LG was caused by a heat treatment in the processed milk, we heated the isolated $\beta$-LG and $\alpha$-LA at 90 $^\circ$C overtime. On native-PAGE analysis, the acidic property of $\beta$-LG was altered in a time dependent manner (Fig. 4). A marked change in heat treatment was observed in $\beta$-LG over time up from 30 s. The major extra-band (Fig. 4) from the denaturation was $\beta$-LG as confirmed by a Westernblot (described later). Whereas, the $\alpha$-LA was more resistant (240 s) as compared to $\beta$-LG. We further examined the thermal changes in each purified $\beta$-LG or $\alpha$-LA at various temperatures over time using
the same native-PAGE. Following the integration using a digital image system from the gel, there was no significant change in β-LG below 60 °C over a period of 500 s (Fig. 5A). Some mild changes occurred at 70-80 °C, but the most pronounced changes occurred between 90 and 95 °C and was in a time-dependent fashion. The native form of β-LG was almost abolished when heating was proceeded for longer than 240 s. Similarly, the thermal denaturation curves of α-LA were constructed, but the severity was much less than that of β-LG in both time and temperature responses (Fig. 5B).

Therefore, our data suggested that β-LG suffered more changes in overall structure than did α-LA upon the heating. To further support this hypothesis, we monitored the structural changes in β-LG and α-LA using a CD spectral measurement. Fig. 6 shows that β-LG exhibited more disordered structure at the temperature up to 80 and 95 °C than that of α-LA. The maximal ellipticity changes within our experimental ranges (time vs. temperature) are depicted in Fig. 7. Thus, the conformational changes of β-LG may be responsible for the overall changes of electromobility as that found in native-PAGE shown in Fig. 1.

Finally, we employed a polyclonal antibody prepared against native β-LG to further trace the heated β-LG on a Western blot. This polyclonal antibody reacted equally to the native and heated β-LG on a competitive ELISA indicating that the antibody was capable of recognizing heat denatured antigen (data not shown). Using purified β-LG, the Westernblot demonstrated that both charged property (native-PAGE) (Fig. 8A) and molecular forms (SDS-PAGE) (Fig. 8B) of β-LG were significantly altered upon the heating. Large molecular forms (such as aggregates) of immunoreactive β-LG were demonstrated in Fig. 8B. While, there was no immunoreactive β-LG at 25 kDa (Fig. 8B) further indicating that heat treatment on β-LG did not generate a 25kDa as that displayed in the processed milk (SDS-PAGE in Fig. 2). Furthermore, Western blot on commercially processed milk (whey proteins), both domestic and imported powdered milk revealed changes of molecular properties in β-LG as characterized by a native-PAGE (Fig. 9A). One single and no detectable alternation of β-LG was present in raw milk (Fig. 9A). Formation of multiple forms of large molecular weight of β-LG was observed in processed milk as characterized by a SDS-PAGE (Fig. 9B). However, such polymerization was less associated in powdered milk. Again, only raw milk and 4 brands from US revealed undetectable changes.

Discussion

Recent studies (4,5) have indicated that whey protein α-LA appeared to possess immunomodulatory properties, conferring increased resistance to the growth of tumors. On the other hand, β-LG is associated with the hypocholesterolemic effect (5,7). Because almost all dairy processes require heat treatments, information on the heat stability of milk with a modified protein component becomes an essential subject. It is conceivable that the dairy industry accounts for a large number of the knowledge and nature of molten globules, since the industry is concerned with improving the process of whole milk and concentrates as well as extending the products used for nutritional source (15). Our original purpose of the present study was to characterize the major changes of α-LA and β-LG, if any, of whey proteins in processed milk. Our study, however, revealed that the heating procedures used in domestic milk was significantly different from those in other countries. Whether or not it may commercially influence the textured dairy products (e.g. yogurt and cheese yield) or nutritional role is presently beyond our objective. β-LG was found severely denatured. If it was due to the excessive heating, it is certainly of worthy to avoid undesirable effects, such as the formation of deposits on walls of heating equipment (16) and the impaired renneting properties (17). Detailed knowledge of the denaturation behavior of β-LG is required to promote the
positive effects and to minimize the deleterious ones (18). In practice, we have found that the deposit of milk remnants did produce the damage on the radiators in some facility of our local dairy manufacturers. Since ⍺-LG can form clot during the heating process (19-26), it may be responsible for such destructible event. The thermal denaturation curves (Fig. 4) and CD spectra (Fig. 5) show that the denaturation of ⍺-LG was more rapid and extensive than that of ⍺-LA in both time and temperature response. The result, however, is consistent with the previous reports where the extent of polymerization was limited in ⍺-LA (26). The relatively weak hydrophobic interactions occur between unfolded ⍺-LA that makes it high resistant to thermal changes as compared to ⍺-LG (4).

Although the kinetics of denaturation of whey proteins in milk have been studied by native-PAGE (27), no information is available on the detailed kinetics of heat-induced conformational changes in ⍺-LA and ⍺-LG as judged by both CD and native-PAGE simultaneously (50-95 °C for 15s to 15 min). The present study may provide a valuable reference, in general, for the study of the correlation of conformational changes and electrophoretic properties in both ⍺-LA and ⍺-LG.

It is of worth to mention that only the whey proteins, rather than whole milk, were chosen for native-PAGE in the present study. This was due to the lipids (micelles) and casein in whole milk that can considerably affect the performance of gel electrophoresis as described by the others (10,27-29). Furthermore, since some ⍺-LG could associate or polymerize with micelle and casein fractions in overheated whole milk (9,19,30-33), this association is responsible for the partial loss of ⍺-LG in whey protein fraction. Evidently, it may explain the substantial loss of ⍺-LG found in our domestically processed milk (Figs. 1 and 2), in which some loss of ⍺-LG was not completely recovered in gel electrophoresis. Likewise, the immnoreactive ⍺-LG blotted as in multiple large forms (Fig. 9A and 9B) may sorely represent the partial loss of total ⍺-LG in the processed milk. Nevertheless, the result clearly demonstrated that the Westernblot technique was relatively sensitive in detecting the thermal changes in ⍺-LG, which has not been reported previously. It also suggests that the electrophoretic changes of ⍺-LG found in native-PAGE were structurally irreversible. Thus, using a simple native PAGE as well as a Westernblot, ⍺-LG may be used as a marker for the quality control in thermal process of manufactured milk.

參考文獻


Table 1: Amino sequence of acidic proteins isolated from the native-PAGE shown in Fig. 1

<table>
<thead>
<tr>
<th></th>
<th>Amino sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac1</td>
<td>LIVTQTMKGLDIQKVAGTWY</td>
</tr>
<tr>
<td>Ac2</td>
<td>LIVTQTMKGLDIQKVAGTWY</td>
</tr>
</tbody>
</table>

*Both ac1 and ac2 proteins are the isoforms of β-LG as reported (15).*
Fig. 1. Native-PAGE analysis on whey proteins obtained from raw (A) and processed (B) milk. Ten ug of the protein sample were loaded onto each lane. There was a marked decrease in two acidic proteins in the processed milk as compared to that in the raw milk. These two acidic-proteins were subsequently eluted from a transfer blot followed by an amino acid sequencing and was identified as two isoforms of β-LG (Table 1).
Fig. 2. SDS-PAGE analysis on whey proteins obtained from raw (A) and processed (B) milk. Ten μg of the protein sample were loaded onto each lane without a conventional pre-heating procedure. M: Markers with each molecular weight standard expressed in kDa, while Lane C was a purified standard of β-LG (18.5 kDa) and α-LA (14 kDa), respectively.
Fig. 3. Native-PAGE analysis on whey proteins obtained from raw, processed, and powdered milk. Lane 1: Freshly prepared raw milk (from Taiwan); Lanes 2 to 6: Processed milk (5 major brands from Taiwan); Lanes 7 to 9: Powdered milk (from Denmark, Australia, and New Zealand, respectively); Lanes 10 to 13: Processed milk (4 brands from US). Whey proteins loaded on lanes 1 and 2 were freshly prepared from the same batch, and were obtained from a university dairy farm before and after the process.
Fig. 4. Native-PAGE analysis on isolated β-LG and α-LA (1mg/mL) heated at 95 °C over time. Lane 1: Native β-LG and α-LA without heat; Lanes 2 to 7: Heated β-LG and α-LA at various time from 30, 60, 120, 240, 480, and 960 seconds, respectively. Ten ug of each protein were loaded on the native gel.
Fig. 5. Effect of heating temperature and time on the loss of α-LA (A) and β-LG (B). The loss of each protein upon the heating was extrapolated from the native-PAGE (similar to that conducted in Fig. 4), while using the sample without heat as 0 % loss. The experiment was conducted independently at various temperatures as indicated: 50 (●); 60 (○); 70 (■); 80 (□); 90 (▲); and 95 (△).
Fig. 6. Circular dichroic spectra of heated α-LA and β-LG at various temperatures. A: α-LA heated at 50 (-----), 60 (-----), 70 (------), 80 (-----), 90 (-----) and 95°C (-----) for 15 s to 15 min; B: β-LG heated at 50 (-----), 60 (-----), 70 (------), 80 (-----), 90 (-----) and 95°C (-----) for 15 s to 15 min. Notably, the spectrum of unheated was identical to that heated at 50°C for 15 sec (data not shown). α-LA appears to be more resistant to heat than that of LG.
Fig. 7. Thermal denaturation curves based on the maximal changes of the ellipticity of \( a \)-LA (A) and \( \beta \)-LG (B) heated at 50 (\( \square \)), 60 (\( \square \)), 70 (\( \square \)), 80 (\( \square \)), 90 (\( \square \)) and 95 (\( \square \)) \( ^\circ \)C. The data were extrapolated from Fig. 6.
Fig. 8. Characterization of heated β-LG using a Western blot analysis on native-PAGE (A) and SDS-PAGE (B). The experiment was carried out by heating purified β-LG (1mg/ml) at 95°C prior to the reaction with a polyclonal β-LG antibody. Time course was expressed in seconds as indicated. In panel B, there were no breakdown or aggregates of β-LG on the initial sample (time 0) and no aggregate was observed at 25 kDa as that shown in Fig. 2.
Fig. 9. Characterization of the β-LG component following a heat on whey proteins of raw, processed, and powdered milk using a Western blot analysis on native-PAGE (A) and SDS-PAGE (B). The specificity of the antibody was confirmed by the formation of a single band against the whole whey proteins. Lane 1: Freshly prepared raw milk (from Taiwan); Lanes 2 to 6: Five major brands of processed milk (from Taiwan); Lanes 7 to 9: powdered milk (from Australia, New Zealand, and Denmark, respectively); Lanes 10 to 13: Four brands of processed milk (from US). Again, there was no immunoreactive β-LG at 25kDa. Notably, the decrease in β-LG was not as sharp as that characterized on Coomassie blue staining due to the extremely high sensitivity of the immunoblot.
行政院國家科學委員會補助專題研究計畫成果報告

利用噬菌體崇組抗體檢測品質

計畫類別：
個人型計畫   整合型計畫
計畫編號：NSC-90-2313-B-009-001
執行期間：90年8月1日至91年7月31日

計畫主持人：毛仁淡
共同計畫主持人：
計畫參與人員：廖純沂、陳文亮

本成果報告包括以下應繳支附件
? 赴國外出差或研習心得報告一份
? 赴大陸地區出差或研習心得報告一份
? 出席國際學術會議心得報告及發表之論文各一份
? 國際合作計畫研究計畫國外研究報告書一份

執行單位：交通大學生物科技所

中華民國九十年一月十七日