Effect of p-chloromercuribenzoate on protein interaction in heated skimmilk

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Heating milk results in the various changes on milk proteins and the interaction between protein fractions is also induced. The association of casein with β -lactoglobulin on heating was reported^{1,2,8,14}.

SAWYER *et al.*⁷⁾ and PURKAYASTHA *et al.*⁴⁾ investigated the role of the sulfhydryl (SH) groups in β -lactoglobulin in formation of protein complex and showed the preventive effect of N-ethylmaleimide on this reaction. TRAUTMAN and SWANSON⁹⁾ also reported the effect of p-chloromercuribenzoate during forewarming to manufacture evaporated milk on the formation of protein complex, which might influence the stability of evaporated milk.

In this report, p-chloromercuribenzoate (PCMB) was added to milk and its effect on the heat denaturation of proteins in heated milk was investigated.

Materials and Methods

Milk and casein samples: Pooled skimmilk was used throughout this work. Both κ - and α_s -casein fractions were prepared from skimmilk by the urea-sulfuric acid method of ZITTLE and CUSTER¹³⁾.

Heat treatment of milk: Skimmilk was heated in the tightly closed Erlenmeyer flask or test tube by using a water bath at temperatures ranging from 65°C to 95°C. Then it was cooled rapidly in an ice bath.

Treatment of milk with PCMB: More than 98% pure p-chloromercuribenzoic acid was obtained from Daiichi Pure Chemicals Co., which was dissolved with *N* NaOH before use. Skimmilk was made 0.2 mM with PCMB by adding 5 ml of 2.2 mM PCMB to 50 ml of skimmilk. This concentration of PCMB is approximately equivalent to that of SH group of milk reported previously¹²⁾. The colorimetric method of SASAGO *et al.*⁶⁾ to determine the SH content in milk by using PCMB and dithizone was modified and applied for the estimation of the residual PCMB which did not bind with SH group in skimmilk. The modification made was to make determination of PCMB in the absence of urea.

Sephadex gel filtration: Sephadex G-100 was hydrated in an excess of pH 6.78 phosphate buffer (0.10 ionic strength) for 24 hr and added to a 2.7 cm by 29 cm chromatographic column. Five ml of skimmilk was added to the column and eluted at the room temperature with phosphate buffer, the flow rate being adjusted to 75 ml/hr. Each 5 ml fraction was collected and absorbancy at 253.7 m μ was measured. The relative area for each peak was calculated by determining either nitrogen or UV absorption of each respective pattern peak.

Polyacrylamide gel electrophoresis (PAE): It was done in the horizontal electrophoresis cell (Fuji Riken Co.). The gel was composed of 7% Cyanogum in pH 9.2 Tris-borate-4.5 M

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urea buffer containing EDTA and mercaptoethanol¹⁰. Protein sample (30 μ l of a 2% solution) was applied to the gel and run at 200 V for 7 hours. The gel was cooled with ice during the run.

Results and Discussion

When skimmilk containing 0.2 mM PCMB was held at 4°C, the reaction of PCMB with SH group of skimmilk proceeded as shown in Fig. 1. The reaction reached maximum after about 8 hr at 4°C, when PCMB bound to SH was 0.15~0.17 mM. As the value of 0.18~0.20 mM was reported for SH content of milk¹², most of SH was considered to have bound with the added PCMB. SASAGO *et al.*⁶ have shown that the reaction between PCMB and milk or β -lactoglobulin reached a maximum value in about 24 hr in phosphate buffer at pH 7.

Fig. 1. Effect of holding at 4°C on the reaction between PCMB and skimmilk

Heating milk results in the denaturation of whey protein and decreases the non-casein nitrogen (NCN). Skimmilk made 0.2 mM with PCMB was heated and NCN was determined by the method of ROWLAND⁵ and expressed as % of NCN of the unheated skimmilk. The results were shown in Fig. 2. When heated at 80°C for 10 min, NCN for skimmilk treated with

Fig. 2. Effect of PCMB treatment on the content of non-casein nitrogen (NCN) in heated skimmilk. NCN of unheated skimmilk was expressed as 100 %
 —●— heated without the addition of PCMB
 —○— heated soon after the addition of PCMB
 —×— heated after 8 hr's holding at 4°C with PCMB
 —△— heated after 24 hr's holding at 4°C with PCMB

PCMB for 8 and 24 hr at 4°C before heating were 89.5% and 85.2% of that of unheated milk, respectively. These values were about 20% more than that of milk heated in the absence of PCMB. For the milk heated for 95°C for 5 min, the similar difference in NCN was also observed between PCMB-treated and untreated (control) milk. From these results, it is assumed that PCMB prevents the denaturation of whey protein and retains more NCN in heated milk. But, when skimmilk was heated at 80°C or 95°C soon after the addition of PCMB, such effect of PCMB to prevent whey protein denaturation on heating milk was not found. The difference between NCN of these skimmilk samples and that of control milk were only about 4%.

When skimmilk was heated at 65°C, 80°C or 95°C soon after the addition of 0.2 mm PCMB, the reaction of PCMB was, as shown in Table 1, about 0.15 mm. This value does not differ much from the results (Fig. 1) obtained with unheated milk held at 4°C for more than 8 hr in the presence of PCMB. In spite of that the amount of reacted PCMB was apparently same, however, the effect of PCMB to prevent the heat denaturation of whey protein was not found in NCN determination of skimmilk which was heated soon after the addition of PCMB. These results suggest the possibility that, in such a milk sample, PCMB binds rather to other activated sites of protein than to SH. There might be a competition between SH and other heat activated sites of protein to react with PCMB.

Table 1. Effect of heating on the reaction of PCMB in skimmilk

Temperature	Unheated	65°C			80°C			95°C
Time (min)		10	20	30	2	5	10	For an instant
Reacted PCMB (mm)	0.017	0.115	0.128	0.153	0.144	0.162	0.189	0.156

Fig. 3. Sephadex G-100 gel filtration patterns of skimmilk

Top: unheated

Middle: heated at 95°C for 5 min

Bottom: heated at 95°C for 5 min after 24 hr's holding
at 4°C with PCMB

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The gel filtration pattern of skimmilk by Sephadex G-100 is shown in Fig. 3. Unheated skimmilk was fractionated by Sephadex G-100 to four components. When milk was heated at 95°C for 5 min, Components II and III decreased and their boundary became obscure. For the milk treated with PCMB at 4°C for 24 hr and heated at 95°C for 5 min, Components II and III combined each other to make a single peak, the relative area of which was greater than with milk heated in the absence of PCMB. For the milk samples heated at 65°C or 80°C with and without the added PCMB, the similar Sephadex G-100 gel filtration patterns were obtained.

Table 2. Effect of heating and PCMB on the Sephadex G-100 gel filtration patterns of skimmilk

Sample preparations		Relative area of each component (% of total) calculated					
Heating condition	PCMB (mm)	by nitrogen determination			by UV absorption		
		I	II + III	IV	I	II + III	IV
Unheated	0	69.3	23.2	7.5	59.3	16.2	24.5
65°C, 30 min	0	76.6	14.8	8.6	53.3	15.9	30.8
	0.2	71.4	20.9	7.7	56.1	16.6	27.3
80°C, 10 min	0	74.9	17.0	8.1	59.4	13.8	26.8
	0.2	73.0	17.7	9.3	57.0	16.7	26.3
95°C, 5 min	0	82.7	9.4	7.9	64.5	10.5	25.0
	0.2	72.4	17.6	10.0	60.3	15.2	24.5

The relative area of each component was tabulated in Table 2. The differences were observed between the relative area of peaks of gel filtration patterns calculated by nitrogen determination and UV absorption. It is due to the difference in relative proportion of aromatic amino acids in each peak³⁾.

The sum of relative area of Components II and III, calculated either by nitrogen or UV absorption, decreased in heated milk. In the milk heated after treatment with PCMB, the decrease in the relative area of Components II and III was less than with milk heated in the absence of PCMB.

According to MORR *et al.*³⁾, Component I in fractionation of skimmilk with Sephadex G-100 contains caseinate and large sized serum protein such as globulins. Components II and III are mainly β -lactoglobulin and α -lactalbumin, respectively, and Component IV is non protein fraction. The fact that the relative area for Components II and III in Sephadex pattern of heated milk is smaller than with the unheated milk is due to the denaturation and aggregation of whey proteins. The decrease of Components II and III is consistent with the lower NCN content in heated milk. The added PCMB seems to possess a preventive effect on such a type of heat denaturation of whey protein.

In the PAE pattern of Component I of heated skimmilk, the band corresponding to κ -casein disappeared (Fig. 4). But, with milk heated after PCMB treatment, κ -casein was detected in PAE pattern. This result suggests that κ -casein is one of the fractions to interact with whey protein on heating milk.

In the previous paper¹¹⁾, it was shown that κ -casein fraction prepared from heated milk did not possess the α_s -casein stabilizing ability. The loss of stabilizing ability of κ -casein was thought not to be due to the heat denaturation of κ -casein itself, but to be resulted from the

Fig. 4. Polyacrylamide gel electrophoresis patterns of Component I of skim milk fractionated with Sephadex G-100 (see Fig. 3)
 Top: Component I of unheated skim milk
 Middle: Component I of skim milk heated at 95°C for 5 min
 Bottom: Component I of skim milk heated at 95°C for 5 min after 24 hr's holding at 4°C with PCMB

Fig. 5. α_s -Casein stabilizing ability of κ -casein fractions
 — $\circ$ — κ -casein fraction prepared from unheated skim milk
 — Δ — κ -casein fraction prepared from skim milk heated at 80°C for 10 min
 — $\bullet$ — κ -casein fraction prepared from skim milk heated at 80°C for 10 min after 24 hr's holding at 4°C with PCMB

heat-induced interaction of κ -casein and whey protein. The κ -casein fraction prepared from milk heated at 80°C for 10 min after treatment with PCMB was tested for α_s -casein stabilizing ability by using the method described previously¹¹. As shown in Fig. 5, it retained the full stabilizing ability.

The loss of the stabilizing ability of κ -casein fraction of heated milk, the decrease of NCN, disappearance of κ -casein band in PAE pattern and the decrease of whey protein fraction in Sephadex gel filtration pattern were observed in heated milk. These changes were considerably prevented by the treatment of milk with PCMB before heating. PCMB may combine with SH groups in milk and prevent the formation of protein complex. Though the mechanism of interaction of whey protein with casein on heating had not been clearly explained yet, SH had been thought to play an important role. SAWYER *et al.*⁷) suggested the formation of an intermolecular disulfide bond between two proteins, and found the preventive effect of N-ethylmaleimide on this reaction.

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The reaction of PCMB with SH group of milk or β -lactoglobulin is rather slow in the absence of protein denaturing agents⁹. When milk was heated soon after the addition of PCMB, the preventive effect of PCMB on the protein interaction was not observed. The formation of intermolecular disulfide bond might proceed faster than the reaction of PCMB with SH in heated milk.

Summary

The effect of PCMB on the interaction of milk proteins induced by heat-treatment was investigated.

Skimmilk was made 0.2 mm with PCMB and held at 4°C for 8 to 24 hr before heating. PCMB treated milk was then heated at temperatures ranging from 65°C to 95°C and the nitrogen distribution, Sephadex gel filtration, PAE patterns and the stabilizing ability of κ -casein prepared from it were studied.

The decrease of NCN by heating milk at 80°C for 10 min and 95°C for 5 min were 10.5 % and 14.8 %, respectively, in PCMB treated milk, while those of control (heated in the absence of PCMB) milk were 31.9 % and 39.5 %, respectively. This preventive effect of PCMB on the heat denaturation of whey protein was also found in Sephadex G-100 gel filtration patterns of heated milk, that is, the relative area of components corresponding to whey proteins was larger in PCMB treated milk than with control milk.

The results of PAE showed that κ -casein interacted with other protein fractions in heated milk, this interaction being inhibited in PCMB treated milk.

κ -Casein prepared from heated milk did not possess the α_s -casein stabilizing ability, but, from heated PCMB treated milk, κ -casein with the full stabilizing ability was obtained.

When skimmilk was heated soon after the addition of PCMB, the preventive effects of PCMB on whey protein denaturation were not found. In this case, PCMB might react non-specifically with the heat activated sites other than SH in heated milk.

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牛乳蛋白質の加熱変性におよぼすパラクロル水銀安息香酸 (PCMB) の影響

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牛乳加熱時の蛋白質の相互作用に対する PCMB の影響を調べた。

脱脂乳に PCMB を 0.2 mm 添加し、4°C で 8~24 時間放置したのち、65°~95°C で加熱したものについて、非カゼイン態窒素 (NCN) を測定し、さらに Sephadex G-100 によるゲル濾過、ポリアクリルアミドゲル電気泳動 (PAE) を行なうと共に、 κ -カゼインを分離してその α_s -カゼイン安定化力を調べた。

PCMB で処理した脱脂乳は 80°C、10 分または 95°C、5 分の加熱により、NCN は 10.5 % または 14.8 % の減少を示すのみで、PCMB を添加しないで加熱した脱脂乳では 31.9 % または 39.5 % の減少が認められたのにくらべ、明らかに差がある。このようなホエー蛋白質の熱変性を阻止する PCMB の効果は Sephadex G-100 によるゲル濾過でも認められた。すなわち、加熱脱脂乳の

Sephadex による分別において、ホエー蛋白質に相当する成分 II および III の相対量が減少するが、PCMB 処理後に加熱した場合はその減少度は小さくなる。成分 I はカゼインに相当するが、加熱脱脂乳の成分 I の PAE では κ -カゼインに相当する成分が消失し、PCMB 処理後に加熱すればその成分が認められた。また、PCMB 処理後に加熱した脱脂乳から調製した κ -カゼインは α_s -カゼイン安定化力を保有していた。

以上のようにホエー蛋白質の変性で説明できるような牛乳加熱時の諸変化に対し、PCMB が阻止作用を有することが確認された。ただし、PCMB を添加した直後に加熱した場合にはその阻止作用は認められず、この場合には PCMB は SH 基ではなく、加熱により活性化した他の反応基と結合している可能性も考えられる。