Separation and some properties of temperature sensitive fraction in casein

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(Received for publication on October 11, 1967)

Chromatographic procedures using DEAE- or TEAE-cellulose columns have often been used for the fractionation of milk proteins. Several investigators reported the separation of α_s -, β - and κ -casein by column chromatography with buffer systems sometimes containing dissociation agents such as urea^{7,10}, and 2-mercaptoethanol¹⁰. However, most ignored the fraction which was not adsorbed on the columns and represented the first peak of the chromatograms. GROVES *et al.*² reported that the fraction was temperature sensitive. Otherwise information about this fraction is lacking.

The present paper describes a chromatographic procedure for the preparation of the temperature sensitive fraction (TSF) from acid casein, and some properties of the isolated fraction.

Materials and Methods

Preparation of acid casein:

Skim milk prepared from fresh, raw, pooled milk as described elsewhere³ was warmed to 37°C and acidified to pH 4.6 by the dropwise addition of 5% acetic acid under gentle agitation. The precipitate which formed in the acidified skim milk was separated on a Buchner funnel, redispersed in distilled water (about the same volume as original skim milk), dissolved at pH 7.7 by the dropwise addition of 1N NaOH, reprecipitated, and dissolved again. Two washes with acidified water (pH 4.5) followed each precipitation procedure.

The final casein solution was adjusted to pH 8.0 and dialyzed at 5°C against several changes of the buffer solution (0.027M 2-amino-2-methyl-1-propanol-0.0089M citric acid, pH 8.0) for 24 hours. The dialyzed casein solution was centrifuged (10,000G, 30 min at 1°C) to remove a trace of fatty substance and used as the casein solution for chromatography.

Column chromatography:

The chromatographic procedure described in previous paper³ was used with slight modifications as follows: The casein solution (20ml containing 385mg of protein) applied on the TEAE cellulose column (2.0×15cm) was eluted at 1°C or 30°C with a concave gradient of 2-amino-2-methyl-1-propanol (AMP)-citric acid buffer. Six hundred ml of starting buffer (pH 8.0), 300 ml of limiting buffer (pH 6.6) and 200ml of 0.2N NaOH was used. Additional elution with limiting buffer prior to the elution with NaOH was omitted. The composition of the buffer was slightly changed to ensure a proper pH. It was 0.027M AMP-0.0089M citric acid for starting buffer and 0.744 AMP-0.255M citric acid for limiting buffer.

Starch gel electrophoresis:

Gel composition and the bridge solution were the same as described by WAKE and BAWLDWIN¹¹. Vertical electrophoresis was carried out in a cold room (1°C) applying 1 mA/1×0.5 cm².

Preparation of TSF:

The casein solution (40ml containing 1.5g of protein) was applied to the column (2.8×15cm) and eluted with the starting buffer at 1°C. The portion eluted with the starting buffer was rechromatographed under the same conditions. Thus TSF, which was clear in the cold but turbid at room temperature (above 20°C) was obtained. Protein recoveries based on the absorbancy at 280m μ and 10% TCA-insoluble nitrogen were 5.1% and 4.7% of applied casein, respectively.

Treatment of TSF and measurement of turbidity:

The turbidity of TSF was examined at various temperatures and pH with a Hitachi EPU-2A spectrophotometer at 610m μ . The temperature of TSF contained in the glass cell of the spectrophotometer was adjusted by passing water from Yamato Coolnics through the wall of cell chamber. The pH of TSF was changed by the dropwise addition of 0.1-1M citric acid or 0.2-2M AMP. A Hitachi Horiba pH meter (type M-4) equipped with needle type electrode was used to measure the pH. To investigate the heat stability of TSF, it was heated in a test tube at 75°C for 15 min or 90°C for 10 min with subsequent cooling to 5°C. The turbidity of the treated TSF was measured as described above.

Electron micrographs and absorption spectrum:

The shape of the particles in the turbid TSF was examined with a Hitachi HU-11B electron microscopy using chromium shadowing⁹. The absorption spectrum of TSF (in 0.1N NaOH) was obtained with a Hitachi recording UV spectrophotometer. The tyrosine-tryptophane ratio was calculated from the spectrum by the method of BENCZE and SCHMID¹¹.

Results

The hatched area was subjected to rechromatography. The gradient curve was extrapolated from specific gravity measurement of the effluents. Absorbancy at 280 m μ was measured at 5 to 10°C. Turbidity of TSF at 610 m μ and 30°C is indicated by the broken line.

(A) and (B): Chromatograms of acid casein eluted at 1°C and 30°C, respectively
(C), (D) and (E): Rechromatograms of A-a, B-c, d and B-e, respectively.

Fig. 1. Chromatography of acid casein and subsequent rechromatography of some chromatographed fractions on TEAE cellulose column by gradient elution with AMP-citric acid buffer

Fig. 2. Starch gel electrophoresis (pH 8.6, 7M urea) of whole casein (W) and various effluent fractions of Fig. 1

Chromatograms of acid casein eluted at 1°C and 30°C and subsequent rechromatograms of some of the chromatographed fractions are shown in Fig. 1. Peaks in the figures were identified by starch gel electrophoresis (Fig. 2). The first peak eluted at 1°C (Fig. 1-A) contained TSF, but did not demonstrate the characteristic property, i. e. temperature sensitivity of turbidity. This property, however, did appear after rechromatography (Fig. 1-C). On the other hand, the first peak eluted at 30°C did not contain TSF (Fig. 1-B). Rechromatography of peak B-c, d and B-e, demonstrated that TSF had existed in peak B-c,d together with β - and κ -casein (Fig. 2), and only separated from the other components on rechromatograph at 1°C (Fig. 1-D). Peak B-e containing α -casein was not influenced by the temperature of elution, and seemed to have no direct relation to TSF.

The relation between turbidity and temperature of TSF at 3 levels of concentration is shown in Fig. 3. At the lowest concentration, the turbidity of TSF only appeared at a slightly

The solid lines and the dashed lines represent the turbidity obtained on warming and cooling, respectively.

Concentration: (1) 0.06%, (2) 0.04%, and (3) 0.02% protein (TCA-insoluble Kjeldahl N $\times$ 6.38)

Fig. 3. Turbidity of TSF in buffer solution at pH 8.0 as a response to temperature at three levels of concentration

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higher temperature. The changes in turbidity were reversible and could be treated as phase transitions. The turbidity of TSF depended largely on pH as well as temperature (Fig. 4). A visible precipitate was always observed near pH 4.5 regardless of temperature. The pH range, in which the protein of TSF precipitated, tended to expand at higher temperature. At the pH

Arrows show the formation of visible precipitate.
Fig. 4. Relation between the turbidity and pH for TSF
(0.06 % protein) measured at 8, 15 and 25°C

ranges lower than 4 and higher than 10, TSF was clear at all reasonable temperatures. Heated TSF was compared with non-heated TSF in respect to reversible changes of turbidity (Fig. 5). Heat treatments caused decreases in the turbidity to a certain extent, but the reversibility of turbidity was not altered.

The pH of the samples were adjusted to 7.0 after the heat treatment.
The solid lines represent the values obtained on warming and the dashed lines on cooling. (1) Control, (2) 75°C 15min, (3) 90°C 10min
Fig. 5. Effect of heat treatment on the turbidity of TSF (0.06% protein)

The electron micrograph of TSF, shows clearly defined particles that are almost spherical (Fig. 6). Since turbid TSF was used to prepare the electron microscopic specimen at room temperature, the particles represent TSF in the turbid state. The absorption spectrum of TSF is shown in Fig. 7. The tyrosin-tryptophane molar ratio (calculated from the spectrum was 2.55) was lower than the published data¹²⁾ for caseins.

Fig. 6. Electron micrograph on TSF (in turbid state) in distilled water

Fig. 7. Absorption spectrum of TSF in 0.1N NaOH

Discussion

Separation of TSF from other proteins in acid casein was achieved by column chromatography at low temperature (1°C). The property of formation of colloidal globules at room temperature, resulting in turbidity, is rather unusual in comparison with other milk proteins. However, a similar property was reported for tobacco-mosaic virus protein by LAUFFER⁵⁾. The tyrosin:tryptophane ratio and electrophoretic property as well as the temperature sensitivity demonstrated that TSF differed from casein components already reported. The temperature sensitivity is probably caused by the association-dissociation of relatively small proteins through weak bonds such as hydrophobic bonds.

The temperature dependent association of β -casein is well known. ROSE and COLVIN⁶⁾ observed that casein micelles from warm milk differ in electron microscopic appearance from those of cold milk. They explained it as follows: β -casein, which existed finely dispersed in the cold, deposited on the surface of the micelles when the milk was warmed to 35°C , and tended to link small micelles into clumps and strings, because β -casein is the only major component of milk known to vary in solubility or dispersing ability over this temperature range. The present paper, however, demonstrates that there is another component, i.e. TSF, which associates or dissociates on change of temperature. PAYENS⁶⁾ ascribed the thermal association of casein to hydrophobic interaction and this type of interaction would also account for the characteristic property of TSF.

The association-dissociation property of TSF and the effect of temperature on elution position of TSF on chromatography suggested the possibility that TSF also associates with other proteins, namely β - and κ -casein, and could therefore play an important role in the association between casein components. The sensitivity of TSF was demonstrable after heat treatment to 90°C , and might remain after an ordinary pasteurization process. The turbidity of TSF was also dependent on pH, showing a maximum at about the pH (at 25°C) of normal milk. These facts suggest that further investigations on TSF are necessary. Unpublished results⁴⁾ on gel filtration on TSF-preparations used in the present study revealed that the preparation still had some relatively large molecular weight material but further work will be required to understand its nature.

Summary

Acid casein was chromatographed on TEAE cellulose at 1°C and 30°C. A temperature sensitive fraction (TSF) was obtained by rechromatography of the first eluted peak at 1°C. There was no temperature sensitivity in the first eluted peak at 30°C. The TSF was also recovered from β - and κ -casein rich fractions at 30°C by rechromatography at 1°C. Protein recovery of TSF, based on the absorbancy at 280m μ , was 5.1% of the applied casein. The TSF was clear in cold but turbid at a room temperature. This clear-turbid relation was reversible.

The turbidity of TSF, which was measured at 610m μ , depended on the concentration and pH of the sample as well as temperature. Heat treatment (75°C 15min, 90°C 10min) caused no alteration of this specific reversible clear-turbid relation. The shape of turbid material in TSF at room temperature was spherical as observed by electron microscopy. The tyrosin-tryptophane molar ratio of TSF calculated from the absorption spectrum was 2.55.

Acknowledgment

The authors express their appreciation to Mr. K. MIKAWA, of the Faculty of Agriculture, Hokkaido University, for his technical help in the preparation of the electron micrograph. The authors are also grateful to Dr. T. YASUI, of the Faculty of Agriculture, Hokkaido University, for his kind advice and interest in this study.

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カゼイン中の Temperature sensitive fraction の調製と、 その二・三の性質について

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酸カゼイン中に、 16°C で急激に白濁し、冷却すると直ちに透明となる成分、すなわち、Temperature sensitive fraction (TSF) が認められたので、その分離を試み、白濁 $\rightleftharpoons$ 透明の関係、その他、若干の性質について実験を行なった。

新鮮脱脂乳の pH 4.6 沈澱物を分別し、水洗、溶解、再沈澱後 pH 7.7 で溶解し、カゼイン試料とした。これを 2・アミノ・2・メチル・1・プロパノール (AMP) - グエン酸緩衝液 (0.027 M - 0.0089 M, pH 8.0 ~ 0.744 M - 0.255 M, pH 6.6) を用いた凹型連続式溶出により、 1°C ならびに 30°C で TEAE セルロース・カラムクロマトグラフィーを行なった。 1°C の場合は、カラムに吸着されずに通過した最初の区分を、 30°C の場合は、 β -, κ -カゼインを主成分とする区分を、それぞれ 1°C で再

クロマトグラフィーを行ない、TSF を分離する事が出来た。TSF の調製を目的とし、カゼイン試料 40 ml (1.5g カゼイン) を、 1°C で TEAE セルロースカラム ($2.8 \times 15.0 \text{ cm}$) から、0.027 M AMP - 0.0089 M グエン酸緩衝液のみで溶出し、同緩衝液でふたたびクロマトグラフィーを行なった場合、収量は吸光度 ($280\text{m}\mu$) によればカゼインの 5.1%, 10% トリクロル酢酸不溶性窒素によれば 4.7% であった。

白濁 $\rightarrow$ 透明の可逆性は pH 6~10 の範囲で観察され、加熱 (75°C 15 分, 90°C 10 分) により失われなかった。電子顕微鏡像によれば、白濁時は球状粒子がみられた。紫外線吸収スペクトルから算出したチロシン/トリプトファン分子比は 2.55 であった。