Properties of TS-casein in aqueous alcohol solutions

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In the last paper⁴⁾, TS-casein was separated from temperature sensitive fraction by gel filtration on Sephadex G-100, and such properties as amino acid composition, approximate molecular weight and turbidity were reported. For the convenience of further studies of this protein, the procedure of the separation was modified, and properties of TS-casein in alcohol solutions, especially in aqueous ethanol, were investigated in the present paper.

Materials and Methods

Preparation of TS-casein: Temperature sensitive fraction (TSF) was used as a starting material to separate TS-casein. The same procedure as described in the previous paper⁵⁾ was carried to separate TSF from acid casein. TSF in 0.027 M 2-amino-2-methyl-1-propanol-0.0089 M citric acid buffer (pH 8.0) was concentrated by means of ultrafiltration through a membranefilter (Sartorius). The concentrate was applied on a TEAE cellulose column of which temperature was held at 40°C. The concentration procedure could be omitted by mixing TSF directly with wet TEAE cellulose, which had been equilibrated in the buffer solution, then the mixture was warmed to 40°C, under continuous stirring, and poured into a glass column. Then, 100 ml of the above mentioned buffer solution was passed through at 40°C. After the elution, the column was held in a cold room (2-4°C) overnight, and then, TS-casein was eluted by the buffer solution at 2-4°C. Each ten milliliter amount of the effluent was collected and their absorbancies at 280 m μ (at 10°C) and 610 m μ (at 30°C) were measured by Hitachi EPU-2A spectrophotometer.

Starch gel electrophoresis: Method of SCHMIDT⁷⁾ was used for the preparation of starch gel. Horizontal electrophoresis was carried out in a cold room (2-4°C) with 1 mA/1 $\times$ 0.5 cm² applied.

Electrofocusing: Electrophoresis was carried out at 2°C in an electrofocusing column (LKB-Producter) according to the procedure of VESTERBERG and SVENSSON⁸⁾ with a modification which added urea at 5 M as final concentration in both of a dense and a less dense solution. A carrier ampholyte, pH 3-10 (LKB-Producter) was used for the formation of a pH gradient. An effluent monitor (Hitachi 034) and a pH meter (Hitachi F 5 equipped with micro flow-type electrode Horiba 6901-25 T) were also used for the measurement of absorbancy and pH, respectively.

Measurement of turbidity: The turbidity was examined as described previously⁴⁾.

Stability in aqueous ethanol: TS-casein and whole casein were dialyzed against 0.05 M phosphate buffer (pH 7.0) for 24 hours. The precipitate formed during dialysis was removed by filtration (Toyo filter paper No. 2). Two milliliter of the filtrate (protein concentration 0.96 mg/ml) was mixed with 2 ml of aqueous ethanol (10-99.5%) and left for 24 hours. The

mixtures were centrifuged (1,000 G, for 10 min.), and nitrogen in 3 ml of the supernatant was determined by the micro Kjeldahl method. All procedures were carried out in a cold room (2–4°C) except for the determination of nitrogen. TS-casein was also dialyzed against deionized water for 24 hours. Two milliliter of the TS-casein solution was mixed with 1 ml of a buffer solution (0.084 M–0.064 M citric acid–0.032 M–0.19 M Na_2HPO_4 , for pH 2.9–7.6, 0.028 M–0.043 M sodium borate–0.045 M–0.015 M HCl, for pH 7.9–9.1), and 1 ml of absolute ethanol was added. Fifty percent ethanol solutions were also made by mixing 1.5 ml of TS-casein, 0.5 ml of every buffer solution and 2 ml of absolute ethanol. The mixtures were analyzed as described above. After centrifugation the pH of the supernatant was measured at 2–4°C by a pH meter (Hitachi M4) which was equipped with a needle type electrode (Horiba 6201).

Results

TSF obtained from acid casein by TEAE cellulose column chromatography was applied to another temperature-controlled TEAE cellulose column. An elution diagram of TSF is seen in Fig. 1. Column temperature was controlled as shown in the figure. The eluates at peaks of the diagram were subjected to starch gel electrophoresis (Fig. 2). Elution at 40°C permitted the column to adsorb TS-casein and removed impurities (Peak a) from it. It had been shown by another experiment that the column temperature of 30°C was not enough for all of the TS-casein to be adsorbed. Temperature change from 40°C to 20°C permitted a considerable part of TS-casein to elute as Peak b (Fig. 1). All of the TS-casein which remained in the column was eluted from it by holding the column temperature at 2–4°C. The

Fig. 1. A chromatogram of TSF on TEAE cellulose

Column temperature was varied as indicated in the figure. A solid line and a dotted line indicate absorbance at 280 mμ (at 10°C) and 610 mμ (at 30°C) respectively. Ten milliliter fractions were collected at a flow rate of 50 ml/hr. Buffer: 0.027M 2-amino-2-methyl-1-propanol-0.0089M citric acid, pH 8.0.

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Fig. 2. Starch gel electrophoresis of fractions obtained from Fig. 1 (a-d) and whole casein (w)
Tris-citrate buffer, pH 8.6 with 7M urea and 0.022M 2-mercaptoethanol (SCHMIDT⁷³)

Fig. 3. Gel filtration through Sephadex G-100 of TS-casein (A) and impurities (B)

Buffer: 0.027M 2-amino-2-methyl-1-propanol-0.0089M citric acid, pH 8.0 column size: 2.8×4.8cm. Five milliliter fractions were collected at a flow rate of 24 ml/hr in a cold room (2-4°C)

elution buffer was not changed throughout the entire course. TS-casein and impurities separated by this procedure were applied on a Sephadex G-100 column (Fig. 3). Peak a and d were absent in Fig. 3 (above), although a trace of Peak b was observed.

Fig. 4 shows an elution pattern of TS-casein from an electrofocusing column, with 5M urea, at 2°C. TS-casein consisted of two components in respect to isoelectric point, pH 6.7 and pH 7.3. An experiment with no urea caused precipitation at the end of the electrophoresis which disturbed the formation of the peaks in the pattern.

Fig. 4. Elution pattern of TS-casein from an electrofocusing column

Fig. 5. Effect of various alcohols on the turbidity of TS-casein

Alcohol concentration: 5% (v/v)
Buffer: 0.05 M phosphate, pH 7.0

Fig. 6. Effect of ethanol concentration on the stability of TS-casein and whole casein

Buffer: 0.05 M phosphate, pH 7.0

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Mixtures of TS-casein (protein concentration 0.35 mg/ml) in 0.05 M phosphate buffer solution (pH 7.0) and alcohols (methanol, ethanol, n-propanol, n-butanol) were made at a final alcohol concentration of 5% in volume, and their turbidities at the temperature between 10 and 55°C were measured (Fig. 5). The temperature at which the turbidity increased rapidly was lowered by addition of alcohols. On the other hand, the maximum turbidities of the mixtures were lower than that of control to which deionized water was added instead of alcohol.

Aliquot portions of TS-casein (protein concentration 0.96 mg/ml) and whole casein (0.95 mg/ml) in 0.05 M phosphate buffer solution (pH 7.0) were mixed with an equal volume of 10-99.5% ethanol to observe stability of the protein in various concentrations of aqueous ethanol solutions. The results are presented in Fig. 6. Whole casein was not precipitated up to final ethanol concentration of 50%. While, in the case of TS-casein in the existence of ethanol at 15-45% concentrations, visible precipitates were formed after 24 hours at 2-4°C. In Fig. 6, minimum stability was observed at 25% aqueous ethanol. The bottom part of the curve, however, distributes in considerably wide range from 20 to 40% ethanol concentrations. TS-casein was completely solubilized when ethanol concentration reached to 50%. The stability of TS-casein in 25% and 50% ethanol solutions was further observed, in a pH from 3.6 to 10.5 (Fig. 7). Protein in supernatant decreased, when the ethanol concentration was 25%, in a wide pH range, i.e. from pH 4.1 to 8.8, amounting 35-55% of total protein. At the ethanol concentration of 50%, minimum recovery was observed at pH 5.9-6.9 and the amount of protein in supernatant was higher than the case of 25% ethanol in a wide pH range as seen in the figure.

Fig. 7. Effect of pH on the stability of TS-casein in 25% and 50% aqueous ethanol

Discussion

TS-casein was separated from temperature sensitive fraction by adsorption on TEAE cellulose at 40°C. When a TS-casein solution is warmed, self-association is caused to form stable micelles^{8,4)}. With the co-existence of TEAE cellulose at this condition, TS-casein interacted more closely with the adsorbent than the association of TS-casein itself. The

technique was convenient and almost all of the TS-casein was recovered by only lowering the temperature of the adsorbent from 40°C to 2-4°C. The technique also provided somewhat an unusual method in regard to the use of ion-exchange cellulose, when applied to temperature-sensitive protein such as TS-casein. The adsorption, however, was not clearly understood whether on account of the increase in ionic attraction between protein and adsorbent at higher temperature or the increase in hydrophobic force which was studied thermodynamically by NÉMETHY and SHERAGA⁶⁾.

Thus separated TS-casein was the same for the most part as that of previously described⁴⁾ in respect to starch gel electrophoresis and Sephadex gel filtration. Isoelectric focussing experiment revealed that the protein consisted mainly of two components which differed in their isoelectric points in 5 M urea. This difference seems to be due to the genetic polymorphism of TS-casein that was recently observed by GROVES³⁾, rather than due to the existence of another protein such as γ - or R-casein because the two peaks were almost the same in amount and such amount of two components were not observed by starch gel electrophoresis.

Addition of alcohols to 5% concentration in TS-casein solution affected a turbidity curve in each case. Effect of alcohols, in this case, should be considered from both sides i.e. alteration of water structure surrounding protein and action on protein itself.

In a low alcohol concentration and at below room-temperature, hydrogen-bonded water structure so called clusters⁶⁾ may be altered, and this may cause the changes of the stability of side chain of the protein molecule in contact with the solvent.

Aqueous ethanol is often employed for the detection of stability of milk on some manufacturing treatments. ARIMA *et al.*¹⁾ reported that the minimum concentration of calcium ion in order to obtain a clear detection of stability of casein in ethanol solutions was about 5 mM. It was confirmed by our experiment that whole casein is stable in aqueous ethanol in the absence of calcium. In the case of TS-casein, however, it was found out that calcium was not necessary for precipitation in ethanol solutions. This seems to be related with the previous observation that the turbidity was not influenced by the addition of calcium⁴⁾.

Minimum stability was obtained when the ethanol concentration was 25%. In this solvent, TS-casein precipitated in a wide pH range from 4.1-8.8. The pH independence of the low solubility in a 25% ethanol solution suggests participation of non-ionic binding, namely, hydrophobic binding, between protein molecules. Probably, conformational changes of the protein in this solution may make these binding easier.

The increase in ethanol concentration would be favoured for the solvation of the nonpolar side chains. A high ratio of the hydrophobic groups of amino acids⁴⁾ seems to make the protein stabilize in a high concentration of ethanol.

It would be noticeable that TS-casein precipitated at similar concentration of ethanol to the case of alcohol-test of milk.

Summary

TS-casein was separated from temperature sensitive fraction by adsorption on TEAE cellulose at 40°C and recovered by cooling the adsorbent to 2-4°C in the same buffer solution. TS-casein separated by this procedure agreed with that previously described in respect to gel filtration on Sephadex G-100, and consisted of a major and two minor components by starch gel electrophoresis in the presence of 7 M urea and 0.022 M 2-mercaptoethanol. An electro-

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focusing experiment showed two major components (isoelectric point: 6.7 and 7.3) in the existence of 5 M urea.

Each addition of 5% (at the final concentration) methanol, ethanol, n-propanol, and n-buthanol to TS-casein solution affected its turbidity curve. In a 25% ethanol solution, TS-casein precipitated in a wide pH range (4.1-8.8). The minimum stability in 50% ethanol was observed at pH 5.9-6.9 and the stability was higher than in 25% ethanol except in this pH range.

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TS-カゼインのアルコール溶液中における性質について

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前報において、Temperature sensitive fraction (TSF) よりセファデックス G-100 を用いたゲルろ過により TS-カゼインを分離して、そのアミノ酸組成、分子量、濁度におよぼす塩類の影響などについて報告したが、本蛋白質の性質をさらに研究するため、分離方法を改善し、アルコール溶液中における本蛋白質の性質について、主に安定性の面から調べるため本実験を行なった。

酸カゼインより TSF の調製までは同様であるが、本報では TSF を 40°C に保たれた TEAE セルロースカラムに加え、同温度で 2-アミノ-2-メチル-1-プロパノールクエン酸緩衝液 (pH 8.0) で溶出させた後 (区分 1)、カラム温度を 20°C (区分 2)、さらに 2~4°C (区分 3) に下げて同緩衝液で溶出した。濁度およびでんぷんゲル電気泳動によりしらべた結果、区分 1 には TS-カゼインは含まれず、区分 2 および 3 に存在することがわかった。これより、40°C で TEAE セルロースに吸着し、2~4°C で溶出される成分を TS-カゼインとして実験に供した。本成分はセファデックス G-100 を用いたゲルろ過では前報と溶出位置が一致し、7 M 尿素、0.022 M 2-メルカプトエタノールを含むでんぷんゲル電

気泳動では主要成分の外に少量の 2 成分が認められた。エレクトロフォーカシングによれば、5 M 尿素中で等電点が pH 6.7 と 7.3 の 2 成分が認められた。TS-カゼイン溶液にメタノール、エタノール、n-プロパノール、n-ブタノールを最終濃度で 5% になるように加えると、いずれも対照より低い温度で濁度が上昇したが、最高濁度は低かった。TS-カゼインおよび全カゼインを種々のエタノール濃度と種々の pH の溶液中において一夜放置 (2~4°C) した後、遠沈 (1,000 G, 10 分) して上澄の蛋白質含量をマイクロケルダール法により測定した結果、pH 7.0 の緩衝液に透析した時は、全カゼインはエタノール濃度 50% まで沈殿を生じなかったが、TS-カゼインは 15~45% で沈殿を生じ、25% で最も沈殿が多かったが、50% では再び可溶性となった。エタノール濃度 25% の場合、pH 4.1~8.8 で TS-カゼインの 45-65% が沈殿し、pH 3.6 以下と 9.5 以上で可溶性であった。また、エタノール濃度 50% の時では、pH 6 付近で最も沈殿が多かったが (60%) これ以外の pH 域では、25% の時より沈殿率が少なかった。以上の性質は前報で報告した疎水性アミノ酸が多いことと何らかの関連性があるものと思われた。