

SOME PROPERTIES OF PHOTOOXIDIZED κ -CASEIN

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CHARLES A. ZITTLE

Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, U. S. D. A., Philadelphia, Pennsylvania

ABSTRACT

Photooxidation of κ -casein to the extent of 15 moles of O_2 per 30,000 g oxidizes 100% of the histidine and 70% of the tryptophan. κ -Casein oxidized to this degree has lost completely its ability to stabilize α_s -casein and it is not acted on by rennin. κ -Casein to which photooxidized κ -casein is added (1:1) is not precipitated by calcium ions when acted on by rennin. Photooxidation of κ -casein decreases the electrophoretic mobility in starch gel at pH 8.6, but on cellulose acetate the electrophoretic mobility is greater than that of the native κ -casein.

Previous reports (10, 12) have described some of the marked changes that occur in the properties of α_s - and β -caseins when they are photooxidized. κ -Casein has now been photooxidized and some of the changes in properties are described herein. The most interesting of these changes are the loss of ability to stabilize α_s -casein against precipitation by calcium ions, the resistance of the photooxidized κ -casein to the action of rennin, and the stabilization of para- κ -casein by the photooxidized κ -casein.

MATERIALS AND METHODS

κ -Casein. The κ -casein was isolated from casein from pooled milk by the sulfuric acid method and freed of minor contaminants by precipitation with ethanol (11). It was free of other components on starch gel electrophoresis and was typical κ -casein in its ability to stabilize α_s -casein (see later).

Photooxidation. Photooxidation of the κ -casein was done with methylene blue in the solution in illuminated Warburg flasks with an atmosphere of air. The reaction was followed manometrically. Details of the method have been described (12). In recent experiments an automated calculating and recording attachment for the Warburg apparatus (5) has been used.

Starch gel electrophoresis, ultraviolet absorption, and amino acid analytical methods have been referred to previously (12). Electrophoresis was also done on cellulose acetate (3).

Stabilization of α_s -casein by κ -casein against precipitation with calcium ions. The method used was a slight modification of that previously described (9). A 0.5% solution of α_s -casein at pH 7.8 is used; 1.5-ml portions of this solution are placed in 15-ml centrifuge tubes followed by water to make the final volume, after the other additions, 5.0 ml. The desired amount

of κ -casein is added next (for the unmodified κ -casein this is about 0.4 ml of a 0.25% solution to stabilize completely the α_s -casein). Finally, 0.5 ml of 0.1 M calcium chloride is added, which gives a final pH of about 6.8. The mixture is stirred, kept at 30 C for 15 min, then centrifuged at about $3,000 \times g$ for 5 min. Samples of the supernatant solutions are withdrawn, appropriate dilutions made (usually 1:5), and one drop of 10N NaOH added to clarify the solutions and bring the pH to 13. The casein in solution is determined from the light absorption at 280 $m\mu$. The amount of κ -casein used in the test is deducted from the casein in solution and the results expressed as per cent of α_s -casein in solution.

Sialic acid determination. The sialic acid content of the κ -caseins was determined by the method of Warren (6). Results were quantitated by use of a molar absorption coefficient of 57,000 for the colored sialic acid derivative (6).

Action of rennin. The action of rennin (a 1:30,000 commercial preparation was used) on κ -casein and photooxidized κ -casein has been followed in various ways. Qualitatively the action could be followed visually by the appearance of a precipitate at pH 6.0, or at the higher pH of 6.7 by the appearance of a precipitate with 0.1 M NaCl or 0.01 M $CaCl_2$ present. The ratio of rennin to casein was about 1:150. The visual observations were quantitated by removing the precipitate by centrifugation and estimating the protein remaining in solution from the ultraviolet absorption at 280 $m\mu$. Since the product of rennin action on photooxidized κ -casein might not be insoluble under these conditions, experiments were also tried using precipitation with acetate buffer (1); both κ -casein and para- κ -casein are precipitated, but the split products of rennin action are soluble. The native κ -casein pre-

cipitates at pH 4.7 (1), but the photooxidized κ -casein did not precipitate at this pH but did precipitate when the pH was adjusted to 4.3. Use of acetate buffer of pH 4.3 was effective in precipitating the photooxidized κ -casein. Soluble material resulting from rennin action was estimated from the ultraviolet absorption at 210 $m\mu$. Trichloroacetic acid (TCA) (5 or 12%) precipitation was also used to follow the action of rennin. TCA interfered with the use of ultraviolet absorption; therefore, the TCA-soluble material resulting from the action of rennin on κ -caseins was estimated with Nessler's reagent after digestion of the sample with H_2SO_4 and a drop of 30% H_2O_2 , if necessary. In some experiments the NH_3 released by digestion of the sample was estimated by titration with standard HCl.

RESULTS

The course of oxygen uptake by 20 mg of κ -casein at pH 8.5, with 1.0 mg of methylene blue present, when illuminated, is shown in Figure 1. A change in the slope of the curve

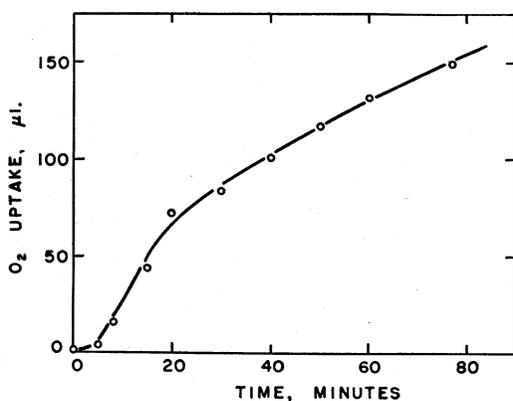


FIG. 1. Photooxidation of κ -casein: oxygen uptake in microliters of O_2 per minute with 20 mg of κ -casein.

(Figure 1) is apparent at an O_2 uptake of 80 μ l. The total O_2 taken up in this experiment (Figure 1) at 75 min is 10.0 moles per 30,000 g of κ -casein. Other similar experiments were stopped at the desired degree of oxidation and a number of runs were pooled to give material for subsequent investigations of the properties of the photooxidized κ -caseins. κ -Caseins of the following degrees of oxidation (moles of O_2 per 30,000 g κ -caseins) were prepared for the studies to be described: 3.0, 7.5, 13.8, and 15.0. The methylene blue in these solutions was adsorbed on to IRC-50 resin (NH_4^+ form), salts were removed by dialysis, and the photooxidized κ -casein recovered by freeze-drying.

The ultraviolet absorption of the photooxidized caseins is shown in Figure 2 for a concentration of 1 mg per milliliter and pH 7.0 in 1-cm cells. The most marked change from the photooxidation is the increased absorption at the shorter wave lengths, particularly 250 $m\mu$.

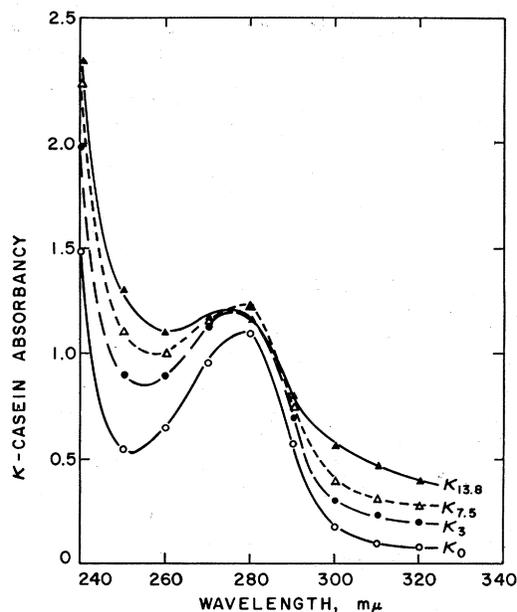


FIG. 2. Ultraviolet absorbance of κ -casein photooxidized to various degrees. One milligram per milliliter, at pH 7.0, in 1-cm cell. \circ κ -Casein, \bullet κ -casein photooxidized 3.0 moles O_2 per 30,000 g, Δ 7.5 moles O_2 , \blacktriangle 13.8 moles O_2 .

The quantitative changes in certain amino acids in the course of the photooxidation of κ -casein are shown in Figure 3. The tryptophan was determined by a chemical method (4b); the other amino acids were determined by standard column chromatography of 24-hr acid hydrolysates (4a). The change in the sialic acid content is shown also.

Starch gel electrophoresis of photooxidized κ -caseins (3 to 15 moles O_2 per 30,000 g) showed that all were considerably reduced in mobility, with a large part remaining at the starting line. Electrophoresis of photooxidized κ -casein (15 moles O_2 per 30,000 g) on cellulose acetate (Figure 4), however, showed that the mobility of the photooxidized casein was about 25% greater than that of the unoxidized.

The stabilization of α_s -casein against precipitation by calcium ions is shown in Figure 5, together with the effect of photooxidation on this property of κ -casein. Photooxidation ex-

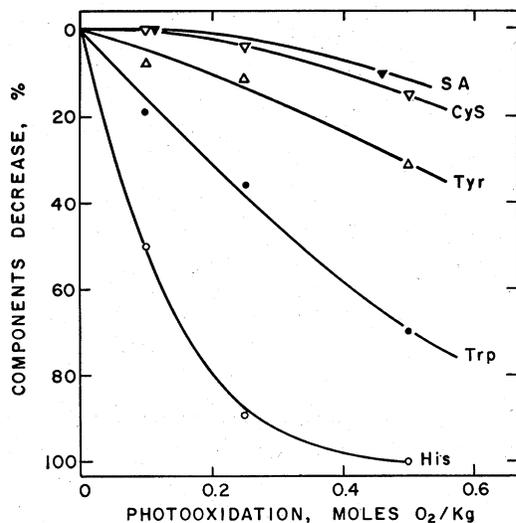


FIG. 3. Compositional change in κ -casein on photooxidation. (Per cent decrease in particular components).

○ Histidine ○ Tryptophan
 △ Tyrosine ▽ 1/2 Cystine
 ▼ Sialic Acid

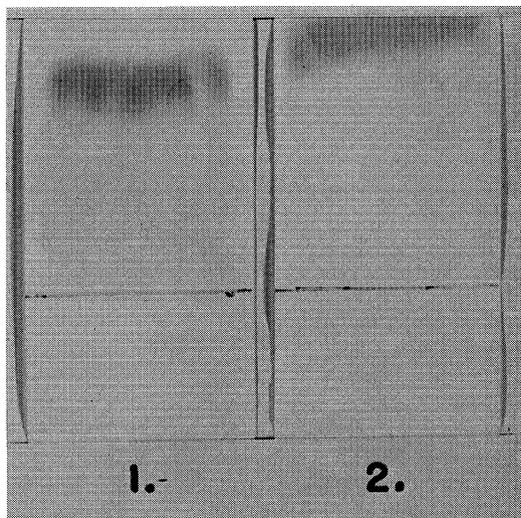


FIG. 4. Electrophoresis of κ -casein (1) and photooxidized κ -casein (15 moles O₂ per 30,000 g) (2) on cellulose acetate.

ceeding 3 moles of O₂ per 30,000 g of κ -casein very markedly reduces the ability of κ -casein to stabilize α_s -casein. Photooxidation to the extent of 15 moles gives a κ -casein product completely unable to stabilize α_s -casein.

Rennin acting on the photooxidized κ -casein did not give the precipitate characteristic of κ -casein (11), either at pH 6.0 or 6.7, with NaCl or with CaCl₂ present. Since the speed

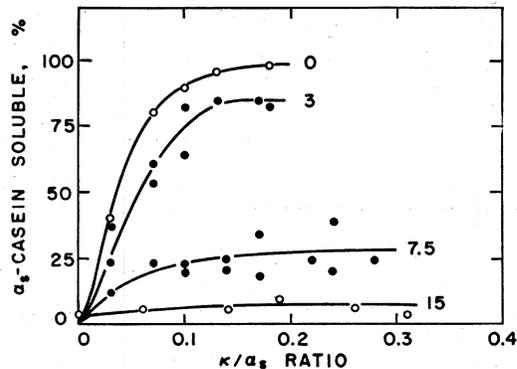


FIG. 5. Stabilization of α_s -casein by κ -casein and photooxidized κ -caseins. The numbers of each curve represent the degree of oxidation as moles of O₂ per 30,000 g κ -casein.

and extent of precipitation is most pronounced with calcium ions, this was considered another example of the poor precipitation of photooxidized caseins with calcium ions (10, 12). An investigation of the soluble products resulting from the action of rennin on the κ -casein showed, however, that rennin was not acting on photooxidized κ -casein (7.5 moles O₂ per 30,000 g) (Figure 6). This was evident when trichloroacetic acid was used as a precipitant and the soluble products were estimated from nitrogen determinations [absorption measurements at 280 $\mu\mu$ cannot be used to estimate this proteinaceous material, since it does not contain aromatic amino acids (4)]. This was

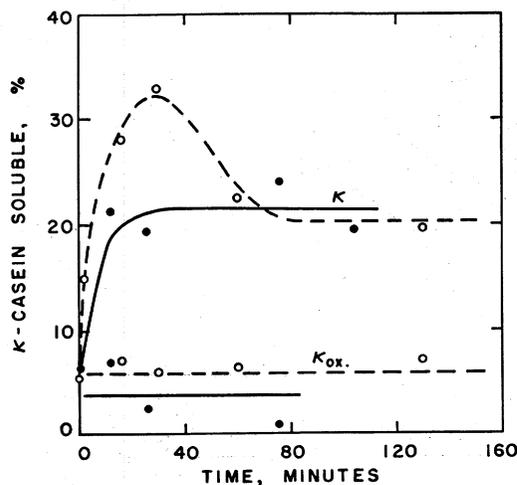


FIG. 6. Action of rennin on κ -casein (κ , top curves) and on photooxidized κ -casein (7.5 moles O₂ per 30,000 g) (κ_{ox} , bottom curves) measured by increase in solubility in 5% trichloroacetic acid (●—●) or at pH of minimum solubility (see text) (○—○).

also evident when the κ -casein was precipitated with acetate buffer (1). The amount of the κ -caseins becoming soluble after rennin action was estimated from the light absorption at 210 $m\mu$ (absorption at this wave length is due to the peptide bonds and is about the same for all proteins). As shown in Figure 6, about 22% of the κ -casein became soluble in the course of rennin action, but no such solubility increase occurred with the photooxidized κ -casein. κ -Casein oxidized by hydrogen peroxide does not precipitate when acted on by rennin, but this oxidized κ -casein still retains its ability to stabilize α_s -casein against precipitation with calcium ions (2).

In the course of the rennin studies it was observed that, when photooxidized κ -casein (7.5 moles O_2 per 30,000 g) was present with the native κ -casein, the action of rennin did not lead to precipitation with calcium ions. When the photooxidized κ -casein to κ -casein ratio was 1:1 the solution did not even become turbid, and a decrease in turbidity could be observed when the ratio was as little as 0.1:1. This could be due to inhibition of the rennin by the photooxidized κ -casein or to nonprecipitation of the para- κ -casein, perhaps because of interaction with the oxidized κ -casein. The data in Figure 7 show that the latter is the probable explanation, since soluble products are released to the same extent from κ -casein and from κ -casein to which photooxidized κ -casein is added.

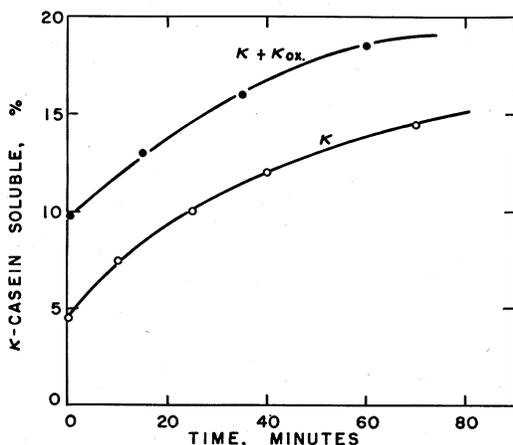


Fig. 7. Action of rennin on κ -casein without (\circ — \circ) and with (\bullet — \bullet) photooxidized κ -casein (7.5 moles O_2 per 30,000 g) present (1:1), measured by increase in solubility in 12% TCA.

DISCUSSION

The oxygen uptake in the course of the photooxidation of κ -casein (Figure 1) is characteris-

tic for this type of reaction (12). The break in the slope of the curve apparent at an O_2 uptake of 80 μ corresponds to an O_2 uptake of 5.4 moles per 30,000 g κ -casein. At this point most of the histidine has been oxidized and oxidation of tryptophan and other amino acids proceeds at a slower rate.

Photooxidation of κ -casein brings about a marked increase in the ultraviolet absorption (Figure 2) in the 250 $m\mu$ region. This can be attributed to oxidation of the aromatic amino acids histidine (8), tryptophan, and tyrosine (7). Actual analysis of the κ -casein before and after photooxidation shows this to be the case (Figure 3). The changes in the sialic acid and cystine are relatively negligible. Histidine and tryptophan are most rapidly and extensively oxidized and, presumably, the oxidations of these amino acids contribute most to the changes in properties observed. The change in properties may not, however, be a direct effect, but reflect a change in state of the oxidized κ -casein, such as aggregation. Starch gel urea electrophoresis at pH 8.6 shows that these oxidized κ -caseins have very little mobility. Actually, an increase in mobility was anticipated, since this was observed with photooxidized α_s -casein [(10), unpublished studies] and since the pH of minimum solubility of the oxidized κ -casein was shifted to pH 4.3. Electrophoretic studies at pH 8.6 on cellulose acetate paper confirmed this expectation. Here, where electrophoresis is on the surface and mobility is not hindered by pore size, the mobility of the oxidized κ -casein is about 25% greater than the mobility of the unoxidized κ -casein (Figure 4). The electrophoretic results suggest that the oxidized κ -casein is probably more highly aggregated than the native κ -casein.

Photooxidation of κ -casein markedly decreases its ability to stabilize α_s -casein against precipitation with calcium ions (Figure 5). Aside from the complication of aggregation, the results suggest that histidine and perhaps tryptophan have an important role in the association of κ -casein with α_s -casein. Results to be discussed below suggest that the oxidized κ -casein, even though aggregated, is capable of interacting with other casein components.

The inability of rennin to act on photooxidized κ -casein was first evident when κ -casein was treated with rennin in the presence of neutral salts or calcium ions. Native κ -casein forms insoluble para- κ -casein when treated in this manner. Nonprecipitation could be due to nonprecipitation with calcium ions, even though rennin acted on the oxidized κ -casein. Photooxidized α_s - and β -caseins precipitate very

poorly with calcium ions (10, 12). The estimation of solubility changes in the rennin experiments by adjustment of the pH with acetic acid to the pH of minimum solubility and also by the use of trichloroacetic acid as a precipitant showed that photooxidized κ -casein was indeed not acted on by rennin (Figure 6). An explanation is not apparent for the hump in the isoelectric solubility curve of the native κ -casein. It may be due to a transient derivative of rennin action. These experiments suggest that the site on κ -casein sensitive to rennin is not available to rennin action in the oxidized derivative, either because of inaccessibility or perhaps because of interference due to chemical alteration at or near the rennin-sensitive bond.

κ -Casein to which photooxidized casein was added did not precipitate with calcium ions when it was treated with rennin. This could be due to inhibition of the rennin by the photooxidized κ -casein or result from nonprecipitation of the para- κ -casein, perhaps because of interaction with the oxidized κ -casein. A study of the action of rennin in this system showed that the rennin apparently acted normally on the κ -casein, as was evident from the increase in the TCA-soluble fraction (Figure 7). This suggests that the second explanation above is probably valid: para- κ -casein is formed by rennin action but the photooxidized κ -casein prevents its precipitation. In a sense, the para- κ -casein, oxidized κ -casein system is probably similar to the α_s -casein, κ -casein system; that is, nonprecipitation is due to interaction of the components, one of which cannot be precipitated by calcium ions. For oxidized κ -casein to be effective in this system, either aggregated, oxidized κ -casein or dissociated units must interact with the native κ -casein.

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