Immunological Studies of Modified and Enzymically Degraded β -Lactoglobulin and α -Lactalbumin

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α -Lactalbumin and β -lactoglobulin were modified by dinitrophenylation, acetylation or esterification. Immune electrophoretic studies of the modified proteins indicated that a modification of amino groups did not necessarily inhibit the immunoprecipitation reaction with antibodies against the native proteins. Esterification of carboxyl groups in the proteins diminished their ability to form immunoprecipitates.

Studies of tryptic hydrolysates of native or acetylated β -lactoglobulin did not reveal any fragment with ability to precipitate with antibodies against the native protein. Similar studies on α -lactalbumin demonstrated several precipitation lines, but only one of them was obviously formed by native α -lactalbumin, whereas the others were due to trace impurities of serum albumin in the preparation.

Several studies have shown that chemical modification and enzymic degradation can be used in the investigations of groupings determining the antigenic properties of proteins, *e.g.* ribonuclease¹, human serum albumin²⁻⁴, bovine serum albumin⁵⁻⁹, ovalbumin¹⁰, rabbit γ -globulin¹¹, and human γ -globulin¹²⁻¹⁵.

The present report comprises a study of chemically modified and enzymically degraded α -lactalbumin and β -lactoglobulin from bovine milk whey. These proteins were chosen as they can be easily prepared, have low molecular weights and are fairly well-characterized.

MATERIALS AND METHODS

Preparation of proteins. α -Lactalbumin and β -lactoglobulin were prepared according to Aschaffenburg and Drewry¹⁶ and were recrystallized at least five times before use. The two proteins were both found to be pure by immune electrophoresis. Using very high concentrations of the α -lactalbumin preparation, however, a faint serum albumin precipitate was also formed. This impurity of serum albumin could be removed by gel filtration of the α -lactalbumin preparation on Sephadex G-75. The gel filtration procedure was essentially the same as the technique described below for gel filtration of tryptic hydrolysates except using a "Uvicord ultraviolet absorptiometer" (LKB-produkter AB,

Stockholm) for continuous registration in the filtrate of material absorbing ultraviolet light of wave length 253.7 $m\mu$ (spectral line obtained from a mercury vapour lamp).

2,4-Dinitrophenylated proteins (DNP-proteins). The proteins were dinitrophenylated according to Sanger¹⁷ by which procedure amino groups are known to react.

Acetylated proteins. Acetylation of amino groups was performed with acetic anhydride as described by Radichevich *et al.*¹⁸. Acetyl determination on the products were performed according to Fraenkel-Conrat *et al.*¹⁹.

Methoxylated proteins. Esterification of the carboxyl groups was performed by treatment of the proteins with dry methanol containing hydrochloric acid at 0° for 24 h¹⁹.

Tryptic degradation of the proteins. The protein (0.5 g) was dissolved in 10 ml 0.01 M tris(hydroxymethyl)-aminomethane-hydrochloric acid (THAM-HCl) buffer, pH 8.0, containing 0.05 M sodium chloride and incubated in a water bath at 37°C. The pH was adjusted to 8.25 and 25 mg of trypsin (2 × crystallized, 50 % w/w MgSO₄, obtained from Mann Research Lab., USA) was added. The degradation was followed by recording the amount of 0.05 M NaOH required to keep the pH at 8.25 (TT 1 titrator, Radiometer, Copenhagen). After about 4 h another 25 mg of trypsin was added. After 6 h the reaction was interrupted by freezing the incubation mixture at -20°C, where it was kept until use.

Gel filtration of tryptic hydrolysate of α -lactalbumin. Gel filtration according to Porath and Flodin²⁰ was performed on Sephadex G-50 (Pharmacia, Uppsala, Sweden) having a water regain of 4.9 per g dry gel. A column of the dimensions 5 × 46 cm was packed as described by Flodin²¹ and was equilibrated with the buffer used in the degradation experiments. 200–500 mg of hydrolysate was applied to the column and was filtrated with a flow rate of 40–60 ml/h. The filtrate was collected in 10 ml fractions, and the optical density of each fraction was determined at 280 $m\mu$ in a Zeiss spectrophotometer, model PMQ II using 1 cm cells. The filtration was carried out at a temperature of +4°C. The fractions obtained were pooled, desalted by filtration through a column of Sephadex G-25 which was equilibrated with a 0.03 M triethylammonium carbonate buffer pH 8.5 and finally they were lyophilized.

Immunological material and techniques. Rabbit immune sera against bovine milk and colostrum and the isolated proteins were produced by weekly subcutaneous injections on rabbits.

Agar gel electrophoresis and immune electrophoresis were performed according to Grabar and Williams²² in the modifications of Wadsworth and Hanson²³. Comparative immune electrophoresis was carried out according to Wadsworth and Hanson²³.

RESULTS

Studies of chemically modified proteins

Modification of amino groups was carried out in two ways, by treatment with Sanger's reagent giving DNP-proteins and by N-acetylation.

DNP- α -lactalbumin was soluble at pH 8.6. The preparation gave no detectable reaction with ninhydrin indicating a practically complete modification of amino groups. By gel electrophoresis it moved as a yellow spot with a much higher mobility than the untreated protein due to the absence of positively charged amino groups, which results by dinitrophenylation (Figs. 1 a and b). No immunoprecipitate was obtained with this derivative. By *acetylation of the α -lactalbumin* a similar change in mobility was observed but this derivative formed a precipitation line with anti-milk sera (Fig. 1 c). The acetyl determination on the acetylated α -lactalbumin indicated the presence of 14.5 acetyl groups per molecule of protein, which is in accordance with 12 lysin residues²⁴ and one N-terminal residue²⁵ in the protein. In addition no reaction was obtained with ninhydrin.

DNP- β -lactoglobulin, obtained as a poorly soluble product, moved as a broad zone extending from the starting basin, probably due to adsorption to

Fig. 1. Agar gel electrophoresis and immune electrophoresis of native (a) and modified (b–d) α -lactalbumin. Antiserum against bovine colostrum was employed.

Fig. 2. Agar gel electrophoresis and immune electrophoresis of native (a) and modified (b–d) β -lactoglobulin. Antiserum against bovine colostrum was employed.

the agar gel. In immune electrophoresis a complex pattern of lines was obtained (Fig. 2 b). As occurred with α -lactalbumin acetylation of β -lactoglobulin resulted in a fast moving component with ability to precipitate anti- β -lactoglobulin antibodies (Fig. 2c).

Carboxyl groups of the two proteins were modified by esterification. The methoxylated proteins did not move from the starting basin in gel electrophoresis. By immune electrophoresis both proteins gave a faint precipitation line at the starting basin. The precipitate obtained with methoxylated β -lactoglobulin had an additional maximum with the same electrophoretic localization as native β -lactoglobulin (Figs. 1 d and 2 d).

Fig. 3. Agar gel electrophoresis and immune electrophoresis of tryptic hydrolysate of α -lactalbumin.

Studies on proteolytically degraded proteins

Immune electrophoretic analysis of β -lactoglobulin hydrolysate after tryptic degradation showed in all experiments only one line situated in the same electrophoretic region as native β -lactoglobulin. A reaction of identity was also obtained with the native protein by comparative immune electrophoresis.

Tryptic degradation of α -lactalbumin prepared according to Aschaffenburg and Drewry gave rise to at least three precipitates in immune electrophoresis (Fig. 3). Precipitate A had the same localization as native α -lactalbumin. Precipitate B was found in the same electrophoretic region but localized somewhat farther from the immune serum basin. The latter precipitate showed a reaction of partial identity (or possibly of inhibition) with a precipitate, C,

Fig. 4. Gel filtration diagram of tryptic hydrolysate of α -lactalbumin, filtrated through Sephadex G-50. Column dimensions 5 $\times$ 46 cm.

Fig. 5. Immune electrophoresis of fractions I and II of the tryptic hydrolysate of α -lactalbumin obtained by gel filtration. Immune serum employed was anti-bovine colostrum serum.

situated farther towards the anode. Gel filtration of the enzymatic hydrolysate and subsequent lyophilization was performed to obtain concentrated peptide fractions (Fig. 4). Five fractions were obtained and were examined by immune electrophoresis. Fraction I was found to form only one precipitate in the same region as precipitate C, while fraction II formed a faint precipitate in the area of native α -lactalbumin (Fig. 5). With fractions III–V no precipitates were observed.

Comparative immune electrophoretic analyses of the α -lactalbumin hydrolysate and of bovine milk was performed and it was then found that the precipitate A identified with the α -lactalbumin line but the precipitate C showed a reaction of identity with the blood serum albumin precipitate. The

Fig. 6. Gel filtration of α -lactalbumin (5 times crystallized) filtrated through Sephadex G-75. Light transmission at 254 m μ continuously recorded by a "Uvicord" ultra-violet absorptiometer.

line B showed a reaction of partial identity with the serum albumin precipitate. The precipitate formed by fraction I (Fig. 5) identified with serum albumin. The precipitate formed by fraction II (Fig. 5) gave a reaction of identity with precipitate A, which identified with native α -lactalbumin.

Gel filtration of the crystalline α -lactalbumin on Sephadex G-75 (Fig. 6) revealed a small but definite impurity (fraction I) which had an electrophoretic mobility of serum albumin. The remaining large fraction (II in Fig. 6) was homogeneous α -lactalbumin in immune electrophoresis. Tryptic hydrolysis of the purified α -lactalbumin formed only a faint precipitate identifying with α -lactalbumin.

Tryptic degradation of acetylated α -lactalbumin and β -lactoglobulin gave similar precipitates as the unhydrolyzed protein derivatives.

DISCUSSION

In agreement with other reports^{3,4} acetylation of the amino groups in the proteins does not seem to affect the ability to precipitate with antibodies against the native proteins as judged from the immune electrophoretic results. The proteins used seem to have been completely substituted as seen from determination of their ninhydrin colour values and from acetyl determinations of the α -lactalbumin derivative.

The disappearance of the precipitation ability of α -lactalbumin induced by blocking of the amino groups with Sanger's reagent might be due to the introduction of a large number of aromatic residues causing a steric hindrance for the antigen-antibody reaction. DNP- β -lactoglobulin, however, gave a complex precipitation pattern. It has been reported that 1-fluoro-2,4-dinitrobenzene reacts incompletely with the ϵ -amino groups of lysine in β -lactoglobulin²⁶ and this might explain these precipitation patterns as the splitting of the precipitate may be caused by various degrees of substitution of the amino groups. Two precipitation lines have been obtained with an antigen extensively or partially diazotized or halogenated^{27,28}. The complex precipitation pattern might also be due to protein denaturation accompanying the modification procedure.

The localization of the methoxylated proteins at the starting basin after electrophoresis could be due to either a change in the mobility or to denaturation of the protein molecule by the treatment, causing aggregation of the protein molecules or adsorption to the agar gel. The esterified proteins showed an ability to form immuno-precipitates. No quantitative evaluation of precipitating ability or degree of esterification of the antigen was performed but it was noted that very faint precipitates were obtained with the esterified proteins. Sri Ram and Maurer⁷ and Steinbuch *et al.*⁴ have reported a diminished precipitating ability with increased degree of esterification of serum albumin.

The immunological studies of the enzymic hydrolysates did not give evidence that any fragments of β -lactoglobulin or α -lactalbumin with immuno-precipitating ability were present. These findings do not exclude, however, that an immunologically inert part of the antigen molecule might have been split off without change of the determinant groups or of the electrophoretic mobility of the antigen. The multiplicity of lines in the α -lactalbumin hydro-

lysates were due to the presence of trace amounts of blood serum albumin in the preparations, not demonstrable by zone electrophoresis or moving boundary electrophoresis. The serum albumin impurity in the α -lactalbumin preparation could be removed by gel filtration. Hydrolysates of this further purified α -lactalbumin only formed a faint α -lactalbumin precipitate.

Tryptic hydrolysates of the acetylated proteins each gave only one immunoprecipitate probably formed by unhydrolyzed proteins. It is known that blocking of the ϵ -amino groups of lysine inhibits the tryptic hydrolysis of the peptide bonds involving the lysin carboxyl groups¹⁹, thus resulting in hydrolysis only of peptide bonds formed by arginine carboxyl groups. The α -lactalbumin molecule has twelve lysine residues²⁴ and only one arginine residue²⁴ and it has been reported that only one peptide bond is split in acetylated α -lactalbumin by trypsin³⁰. Thus at least one relatively large peptide might be expected by the hydrolysis. The immunological experiments, however, did not demonstrate any fragment with retained immunoprecipitating ability.

Recently Richard *et al.*⁹ have claimed that serum albumin peptides with molecular weights of 3 200 to 6 800 can form immunoprecipitates. The concentration of peptides necessary for a precipitation reaction was about 1 000 times that of the albumin. In judging the results of Richard *et al.* the great sensitivity of the immunological techniques should be considered. Even extremely small impurities not revealed by physico-chemical homogeneity tests can give false results, especially when using very high concentrations. This is illustrated by our findings of albumin impurities in the five times recrystallized α -lactalbumin.

REFERENCES

1. Singer, S. J. and Richards, F. M. *J. Biol. Chem.* **234** (1959) 2911.
2. Lapresle, C. *Ann. Inst. Pasteur* **97** (1959) 626.
3. Houghton, P. B. and Martin, N. H. *Nature* **182** (1958) 329.
4. Steinbuch, M., Fine, J. M. and Battistini, A. *Clin. Chim. Acta* **5** (1960) 345.
5. Porter, R. R. *Biochem. J.* **66** (1957) 677.
6. Maurer, P. H. *Arch. Biochem. Biophys.* **79** (1959) 13.
7. Sri Ram, J. and Maurer, P. H. *Arch. Biochem. Biophys.* **80** (1959) 462.
8. Sri Ram, J. and Maurer, P. H. *Arch. Biochem. Biophys.* **83** (1959) 223.
9. Richard, A. J., Beck, S. and Hoch, H. *Arch. Biochem. Biophys.* **90** (1960) 309.
10. Kaminski, M. *Ann. Inst. Pasteur* **98** (1960) 51.
11. Porter, R. R. *Nature* **182** (1958) 670.
12. Augustin, R. and Hayward, B. J. *Nature* **187** (1960) 129.
13. Edelman, G. M., Heremans, J. F., Heremans, M.-Th. and Kunkel, H. G. *J. Exptl. Med.* **112** (1960) 203.
14. Hanson, L. Å. and Johansson, B. G. *Nature* **187** (1960) 599.
15. Burtin, P. *Proc. 8th Colloquium Bruges 1960, Protides of the Biological Fluids*. Elsevier, Amsterdam 1960, p. 119.
16. Aschaffenburg, R. and Drewry, J. *Biochem. J.* **65** (1957) 273.
17. Sanger, F. *Biochem. J.* **39** (1945) 507.
18. Radichevich, I., Becker, M. M., Eitingon, M., Gettler, V. H., Toralballa, G. C. and Galdwell, M. L. *J. Am. Chem. Soc.* **81** (1959) 2845.
19. Fraenkel-Conrat, H., Bean, R. S. and Lineweaver, H. *J. Biol. Chem.* **177** (1949) 385.
20. Porath, J. and Flodin, P. *Nature* **183** (1959) 1657.
21. Flodin, P. *J. Chromatography* **5** (1961) 103.
22. Grabar, P. and Williams, C. A. *Biochim. et Biophys. Acta* **10** (1953) 193.

23. Wadsworth, C. and Hanson, L. Å. *Intern. Arch. Allergy Appl. Immunol.* **17** (1960) 165.
24. Gordon, W. G. and Ziegler, J. *Arch. Biochem. Biophys.* **71** (1957) 473.
25. Yasomoku, K. T. and Wilcox, P. E. *J. Biol. Chem.* **231** (1958) 309.
26. Porter, R. R. *Biochim. et Biophys. Acta* **2** (1948) 105.
27. Augustin, R. *Immunology* **2** (1959) 148.
28. Wilson, M. W. and Pringle, B. H. *J. Immunol.* **77** (1956) 324.
29. Neurath, H. and Schwert, G. *Chem. Revs.* **46** (1950) 69.
30. Weil, L. and Telka, M. *Arch. Biochem. Biophys.* **71** (1957) 473.

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