

The Action of Phosphatases on Casein Fractions

Edwin B. Kalan and Marie Telka

From the Eastern Regional Research Laboratory, Eastern Utilization Research and Development Division, Agricultural Research Service, United States Department of Agriculture, Philadelphia, Pennsylvania

INTRODUCTION

The occurrence of different types of phosphorus linkages in casein fractions has been postulated by Perlmann (1-3). Thoai *et al.* (4) have also reported the existence of at least two different phosphorus bonds in whole casein. These findings are in contrast to those of Sundararajan *et al.* (5, 6) who have concluded that the phosphate bonds of α -, β -, and unfractionated casein are identical, without specifying the exact nature of the bond. The similarity of the phosphate bonds of α - and β -casein from two different species has been reported by Hofman (7). That the phosphate bond in casein fractions is a monoester is suggested by the work of Peterson *et al.* (8) and Hipp *et al.* (9), as well as by the recent publication of Hofman (24).

The present paper reports the liberation of orthophosphate from α -, β -, and unfractionated casein by means of three different phosphatase preparations. Though the final amount of phosphorus released by each preparation under the conditions reported varies, the rate and final amount are not influenced by prior incubation with phosphodiesterase or pyrophosphatase. The results, without revealing the exact nature of the phosphorus linkage, indicate that phosphorus is bonded in α -, β -, and unfractionated casein in a similar manner.

METHODS

Determination of Proteolysis

The extent of proteolysis accompanying phosphate liberation was measured by the increase of ninhydrin-positive material of the entire reaction mixture (10). A 0.1-ml. aliquot, containing 0.5 or 1.0 mg. of the casein fraction under study, was added to 1.0 ml. of the modified ninhydrin reagent of Moore and Stein (10) plus 0.9 ml. water at 0°C. The capped tubes were heated for 15 min. in a boiling water bath, cooled before a fan, and finally diluted with 10 ml. of 1:1 alcohol-water. The tubes were read in the Beckman spectrophotometer, model B, at 570 m μ , and the results were quantitated by comparison with a standard tyrosine curve. In all instances,

the degree of proteolysis determined by this method was very low (a maximum of about nine peptide bonds per 30,000 mol. wt. was observed). However, since the ninhydrin value of the intact caseins was quite significant and since the color yield per mole of peptide may not necessarily be equivalent to the standard, the quantitative data are to be taken only as approximations of the degree of proteolysis.

Determination of Phosphate Liberation

The amount of phosphate liberated was determined by a modification of the method of Fiske and SubbaRow (11). McDonald and Hall (12) have noted that when casein is partially degraded, breakdown products are not always completely removed by protein precipitants. The subsequent addition of molybdate to such solutions leads to the formation of a cloudy precipitate which interferes with the phosphorus analysis. To avoid this hazard, the phosphorus determination was carried out as follows. A 1.0-ml. aliquot of the reaction mixture was added to 0.9 ml. of 20% trichloroacetic acid plus 0.8 ml. of 10 *N* H₂SO₄ kept in an ice bath. Then 0.1 ml. of 10% Na tungstate.2H₂O was added, and the precipitate was removed by centrifugation in the cold. An amount of 1.4 ml., equivalent to 2.5 mg. protein, was taken for color development according to Fiske-SubbaRow except that no further addition of 10 *N* H₂SO₄ was required. These filtrates gave clear solutions upon addition of molybdate, but the blue color was not too stable, and the tubes were read 10-15 min. after addition of the chromogen. The method described has given good linearity and reproducibility from 5 to 20 μ g. phosphorus in a final volume of 10 ml.

Proteolysis and phosphate liberated were always measured simultaneously at the time intervals indicated.

MATERIALS

Substrates

The casein fractions, though not homogenous proteins, were prepared in these laboratories (13, 14) and are comparable to those used in the studies of Perlmann (1-3) and Sundararajan *et al.* (5, 6). The unfractionated casein had a phosphorus content of 0.8%, α -casein 1.0%, and β -casein 0.6%.

Enzymes

Snake venom phosphodiesterase was prepared by the method of Sinsheimer and Koerner (15). The preparation was carried through the second acetone cycle and had 1.9 mg. protein/ml. One-tenth milliliter liberated 0.09 μ mole *p*-nitrophenol measured spectrophotometrically (15) in 30 min. from 3 ml. of solution containing 0.5 μ mole Ca[bis(*p*-nitrophenyl) phosphate]₂, 30 μ moles MgCl₂, and 315 μ moles of Veronal buffer pH 9.4. The enzyme had no activity toward phenyl phosphate, adenylic acid, and *o*-carboxyphenyl phosphate.

Yeast inorganic pyrophosphatase was prepared according to the method of Heppel and Hilmoe (16). A loss of activity was suffered during the alcohol fractionation because of inadequate temperature control. The final preparation had a protein concentration of 0.15 mg./ml. and a specific activity of 2010 (16). The enzyme was free of mono- and diesterase activity.

Wheat germ acid phosphatase was a commercial preparation which was prepared essentially according to the method of Singer (17). Of this preparation, 150 μ g. liberated 0.34 μ mole salicylic acid measured spectrophotometrically (18) in 20 min. from 3 ml. of solution containing 1.5 μ moles of *o*-carboxyphenyl phosphate, 10 μ moles

MgCl₂, and 380 μmoles of acetate buffer pH 5.0. The enzyme had no diesterase activity toward diphenyl phosphate and bis(p-nitrophenyl) phosphate under essentially similar conditions.

Calf intestinal mucosa phosphatase was a commercial alkaline phosphatase. Of this preparation, 50 μg. liberated 0.21 μmole salicylic acid measured spectrophotometrically (18) in 10 min. from 3 ml. of solution containing 1.5 μmoles *o*-carboxyphenyl phosphate, 10 μmoles MgCl₂, and 300 μmoles of Veronal buffer pH 9.4. The preparation had no detectable diesterase activity toward diphenyl and bis(p-nitrophenyl) phosphate under similar conditions.

Another calf intestinal mucosa phosphatase (19) was obtained through the courtesy of Dr. C. A. Zittle of this laboratory. This preparation is known to contain a phosphodiesterase activity, but this activity can be virtually eliminated if the reaction is carried out at pH 5.5-6.0. In all experiments where this enzyme was used the pH was 6.0. This enzyme preparation has no detectable activity toward synthetic substrates at acid pH. However, 2 μg. of this preparation liberated 0.18 μmole salicylic acid measured spectrophotometrically (18) in 20 min. from 3 ml. of solution containing 1.5 μmoles *o*-carboxyphenyl phosphate, 10 μmoles MgCl₂, and 300 μmoles of glycine buffer pH 9.1. It is of interest to note that this enzyme liberated phosphate from α-casein over the pH range 5.7-8.9 with a broad maximum occurring at pH 7.4.

To minimize proteolysis, the last three enzymes described were treated as follows. Four milligrams enzyme was dissolved in a solution containing 0.2 ml. of 0.1 *M* diisopropyl fluophosphate in *n*-propyl alcohol, 0.1 ml. of Veronal buffer 0.15 *M* pH 9.0 plus water, and 0.1 *N* NaOH to bring the final volume to 10 ml. and pH to 8.0-8.5. The addition of base was required because the diisopropyl fluophosphate lowered the pH of the solutions below the optimum pH range for reaction with the known proteolytic enzymes. The solutions were kept at 4°C. overnight and used the following day. Fresh solutions were prepared prior to each experiment.

EXPERIMENTAL

The following experiments were designed to detect differences in the release of phosphate from the casein fractions. Such differences would be expected on the basis of Perlmann's conclusions (1-3).

Phosphodiesterase Experiment

Of the casein fraction under study, 200 mg. was dissolved in water by means of careful addition of 1 *N* NaOH to pH 9-9.3. To this was added 1.0 ml. of 0.1 *M* MgCl₂, 1.0 ml. of snake venom phosphodiesterase (enzyme/substrate = 1/105) and additional water to a final volume of 20 ml. A control tube contained no enzyme. The solutions were then incubated under toluene for 18 hr. at 36° C. The pH, inorganic phosphate released, and proteolysis were measured at 0 and 18 hr. as described above. At the end of 18 hr., four 4.0-ml. aliquots were removed from each tube. To each aliquot was added 1.0 ml. of acetate buffer 0.2 *M* pH 5.0. The first tube of each set of four was the control to which was added water and 0.1 *N* NaOH (0.2 ml.) to bring the final volume to 8.0 ml. and pH to 6.0. The second tube of each set contained 1.0 ml. of wheat germ acid phosphatase which had been treated with diisopropyl fluophosphate as described above (enzyme/substrate = 1/105). The third tube contained 1.0 ml. of the commercial alkaline phosphatase preparation and the fourth tube 1.0 ml. of the phosphoesterase preparation (19). The eight tubes, all adjusted to pH 6 and a final volume of 8 ml., were incubated under toluene at 36°C., and aliquots were taken at indicated intervals to measure phosphate released and the extent of proteolysis.

Pyrophosphatase Experiment

This experiment was essentially the same as the preceding except as noted. The same quantity, 200 mg. of each fraction, was dissolved in 18 ml. of Veronal-acetate buffer pH 7.2 (20). Then 1.0 ml. of 0.1 M MgCl₂ was added and 1.0 ml. of yeast inorganic pyrophosphatase (enzyme/substrate = 1/1300). A control tube contained no enzyme. The solutions were incubated under toluene for 2 hr. at 36° C. Inorganic phosphate released and proteolysis were measured at 0 and 2 hr. At the end of 2 hr., aliquots were removed as described above, and the experiment was carried out in a fashion identical with the above except for differences in some of the time intervals.

RESULTS

The change in pH and orthophosphate liberated by preincubation of α -, β -, and unfractionated casein with the phosphodiesterase preparation are given in Table I. It is noted that the diesterase caused a shift in pH, as has been reported (1-3), without release of inorganic phosphate. However, a small degree of proteolysis is observed (about 3-4 bonds split), and, in the case of β - and unfractionated casein, turbidities which persist for the entire period of incubation are observed. This turbidity develops very early (after 1 hr.) with the β -fraction and somewhat later in the case of unfractionated casein, probably because of the lower concentration of β -fraction in whole casein. No satisfactory reason has been found for the pH shift noted. However, it is not necessary that the shift be associated with the hydrolysis of phosphate bonds. Koshland *et al.* (21) have demonstrated that the fall in pH, which solutions of tobacco mosaic virus undergo upon heating or treating with long-chain alkyl sulfates, is probably due to unmasking of acid groups. These workers were unable to find evidence for phosphate-bond hydrolysis accompanying the fall in pH in the virus system.

TABLE I
Phosphate Released and Change in pH Brought about by Preincubation
of Casein Fractions with Snake Venom

Casein fraction ^a	pH _i	pH _f	Δ pH	Net change	Per cent of total P released
Whole casein + diesterase	9.1	7.9	-1.2	-1.2	0
Whole casein - diesterase	9.3	9.3	0		0
α -Casein + diesterase	9.0	7.9	-1.10	-1.1	0
α -Casein - diesterase	9.2	9.2	0		0
β -Casein + diesterase	9.2	8.0	-1.2	-1.4	0
β -Casein - diesterase	9.2	9.4	+ .2		0

^a One per cent solution of each fraction incubated for 18 hr. at 36°C. at enzyme/substrate = 1/105 in a nonbuffered system.

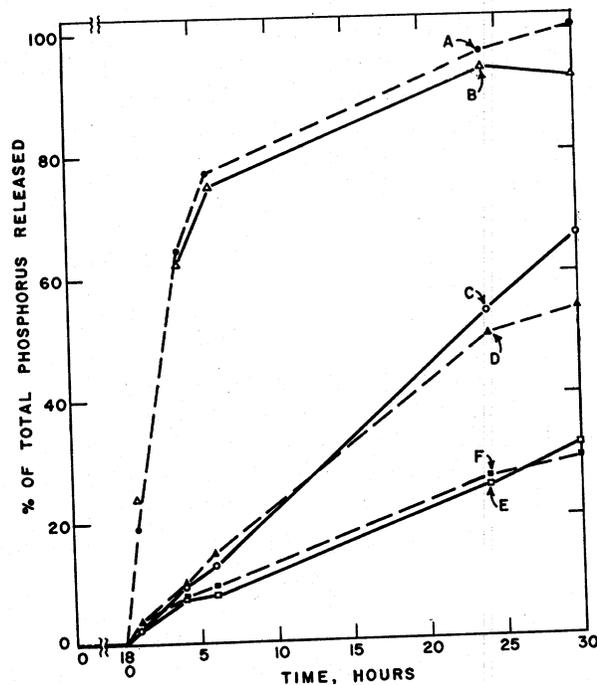


FIG. 1. The release of orthophosphate from unfractionated casein by phosphatase preparations following preincubation with and without snake venom diesterase (S.V.D.) (see text for experimental details). A: S.V.D. + phosphoesterase of Zittle (Z); B: -S.V.D. + Z; C: S.V.D. + commercial alkaline phosphatase (P); D: -S.V.D. + P; E: S.V.D. + wheat germ phosphatase (W); F: -S.V.D. + W.

Figure 1 is a record of the orthophosphate released by three different phosphomonoesterase preparations from unfractionated casein after preincubation with and without phosphodiesterase. This incubation was carried out at pH 6, and the phosphate released was measured at the indicated time intervals. It is clear from the graph that the final amount and rate of phosphate released from each fraction is independent of prior incubation with the diesterase under the conditions described. The amount and rate under the conditions described does, however, vary with the phosphomonoesterase preparation, but it appears that each monoesterase handles the three caseins in essentially the same manner with respect to phosphate release. It was found in an independent experiment that both the wheat germ preparation and the commercial alkaline phosphatase continued to catalyze the liberation of orthophosphate after 53 hr. of incubation. In the case of the wheat germ preparation, about 60% of the total phosphorus was liberated, whereas 85-95% was released with the

commercial alkaline phosphatase. This result indicates that if the incubation were allowed to continue for a sufficiently long period of time, all of the phosphorus would be released. This can also be inferred from the graph, which shows an increasing phosphate release with these two enzyme preparations after 30 hr. This can also be taken as evidence that there are no phosphate bonds resistant to the action of these enzymes. Similar curves were obtained with α - and β -casein. It can be noted that after 6 hr. of incubation, there was little or no proteolysis (0-2 bonds split), and, at the end of 30 hr., the proteolysis increased (about 5-9 bonds split) but was in no sense found to be correlated with the phosphatase activity in any of the experiments.

When the casein fractions were preincubated with yeast inorganic pyrophosphatase, no proteolysis or orthophosphate liberation was observed after 2 hr. The initial pH was 7.2, and no final pH measurement was made since the system was buffered; no turbidities or precipitates were observed. Figure 2 shows the release of orthophosphate by the monoesterases from the whole casein fraction after preincubation with and without pyrophos-

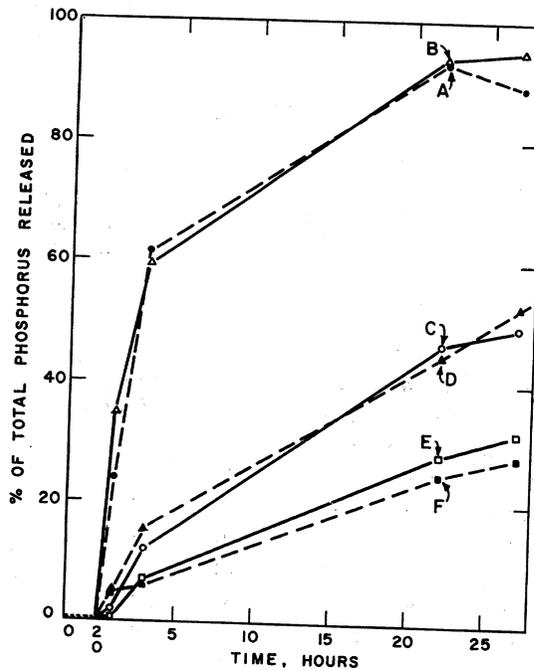


FIG. 2. The release of orthophosphate from whole casein by phosphatase preparations following preincubation with and without yeast inorganic pyrophosphatase (Y.I.P.) (see text for experimental details). A: Y.I.P. + Z; B: -Y.I.P. + P; C: Y.I.P. + P; D: -Y.I.P. + P; E: Y.I.P. + W; F: -Y.I.P. + W.

phatase. Again, it appears that the final amount and rate of the phosphate released is a function of the monoesterase and is independent of prior incubation with pyrophosphatase. The present results are in good agreement with those obtained in the diesterase experiment. Similar results were obtained with α - and β -casein. The degree of proteolysis, as in the previous experiment, was very low (about 3-5 bonds split).

It is of interest to note that the monoesterase preparations caused the formation of precipitates or turbidities during the incubation. The wheat germ preparation caused the α -casein fraction to precipitate most strongly, whereas Dr. Zittle's preparation caused the β fraction to precipitate most strongly. In each instance, turbidities were also observed with the whole casein fraction, reflecting its composition of 20-25% β - and 75-80% α -casein. In addition, the commercial alkaline phosphatase preparation also caused turbidities. Despite the occurrence of such phenomena, the phosphate released by each phosphatase was easily measurable and reproducible. On occasion, the precipitates interfered with proteolysis determinations because of the inability to obtain a uniform sample where the protein had settled out.

DISCUSSION

Perlmann (1, 2) has reported that 40% of the phosphorus of α -casein is present as phosphomonoester (R-O-P), 40% as phosphodiester (R-N-P-O-R), and 20% as pyrophosphate (R-O-P-O-P-R). At least 72% of the phosphorus of β -casein is present as phosphodiester (R-O-P-O-R) (3). These findings were based on enzymic studies with a variety of enzymes and, in particular, with a prostatic acid phosphatase preparation. Such a preparation was not used in the present study. With regard to enzyme specificity, Schmidt (22) has pointed out that while diphenylphosphate is resistant to prostatic phosphatase, the corresponding *p*-nitro derivative, bis(*p*-nitrophenyl) phosphate is hydrolyzed at a considerable rate. Schmidt has also concluded that the question as to whether the phosphatase-resistant groups of phosphoproteins are diesterified required further investigation. The results of the present study indicate that α -, β -, and unfractionated casein release phosphate in a similar manner when treated with each of three different phosphomonoesterase preparations. The amount and rate of orthophosphate liberated appears to be independent of prior incubation with snake venom diesterase and yeast inorganic pyrophosphatase and appears to be a function only of the nature of the monoesterase preparations under the conditions of experiments described. These findings lead to the conclusion that the phosphate bonds of α -, β -, and whole casein are identical, without specifying their exact nature. This conclusion is not in harmony with that of Perlmann (1), who has postulated three different

types of bonds in α -casein (2) and a fourth type in β -casein (3). Thoai *et al.* (4) have concluded that phosphomonoesters and phosphodiester are present in whole casein. This finding was based on the phosphate liberated by two different enzyme preparations from whole casein, α -casein, and three different casein fractions obtained by proteolytic degradation. The spleen preparation, having only diesterase activity (on the basis of very limited specificity studies), liberated from whole casein and α -casein the same amount of orthophosphate in the same time intervals as did the potato preparation, having only monoesterase activity (again on the basis of very limited specificity studies). The conclusions of these workers are difficult to reconcile with their experimental facts. On the other hand, Sampath Kumar *et al.* (5) have treated α -, β -, and unfractionated casein with a spleen phosphatase and found that the pH maximum and Michaelis' constant were the same for the three fractions with this enzyme. They obtained 80% of the phosphorus of the fractions as orthophosphate after 3 hr. incubation. They have concluded that the phosphorus bond is the same in all casein fractions studied. Using a similar preparation, Hofman (7) was able to liberate 100% of the phosphorus from α - and β -casein from cow's and goat's milk. In addition, Sundararajan and Sarma (6) found that two different vegetable acid phosphatases (ground nut and soya bean) also handle the caseins in identical fashion, though the rate and amount of phosphorus liberated depends on the enzyme employed. These findings (5-7), plus the present study, support the hypothesis of a single type of phosphorus linkage in α -, β -, and whole casein. Evidence that this linkage is a monoester comes from the results presented by Peterson *et al.* (8) and Hipp *et al.* (9), as well as from those of Hofman (24).

Peterson *et al.* (8) have isolated a large phosphopeptide from β -casein after limited tryptic action (20 min). These workers have found that the phosphorus and hydroxyamino acids are present in a 1:1 molar ratio. Since the phosphopeptide isolated accounts for 75% of the phosphorus originally present in the β fraction, this implies that essentially all of the phosphorus in this fraction exists as monoester.

Hipp *et al.* (9) have measured the titration curves of α - and β -casein. It was found that the number of ionizable groups determined at two different pH's by the titration method is in perfect agreement with the number of ionizable groups calculated from the amino acid composition and phosphorus content determined by Gordon *et al.* (23). This agreement between the titration data and analytical data is possible only if the phosphorus is present as the monoester; all other types of linkages would lead to discrepancies.

Hofman (24) has shown on the basis of enzymic and chemical evidence that the most probable phosphorus linkages in casein fractions are mono-

ester. However, because of the conflicting evidence from enzymic studies, Hofman (24) concluded that any hypothesis on the way in which phosphorus is bound in casein must be treated with reserve.

SUMMARY

Data have been presented to support the hypothesis that the phosphate linkages in α -, β -, and unfractionated casein are identical.

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