Preparation and Amino Acid Composition of Enzymically Dephosphorylated Casein

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(*Received* 16 *July* 1956)

Plimmer & Bayliss (1906) were the first to show that casein could be completely dephosphorylated by incubation at 37° with 1% sodium hydroxide for a period of 24 hr. Acidification of the digest after the completion of dephosphorylation precipitated a substance resembling casein. Rimington (1927) analysed the dephosphorylated protein and noticed little difference in composition from that of casein except in amide and arginine nitrogen. The protein, termed dephosphorized caseinogen, gave all the colour reactions of casein. Plimmer & Lawton (1939) called attention to the fact that the action of alkali on casein was not as simple as appeared from the experiments of Rimington. These workers observed a splitting up of the casein molecule by alkali, resulting in the formation of the protein termed dephosphorized caseinogen by Rimington and a proteose precipitable by half saturation with ammonium sulphate. The dephosphocasein which accounted for about 52% of the casein nitrogen and the dephosphocaseose which accounted for nearly a third of casein nitrogen had more or less the same amino acid composition as casein (Macara & Plimmer, 1940).

Experiments carried out in this Laboratory on the action of alkali on proteins indicate that, besides casein, other proteins are also degraded by prolonged incubation with alkali, resulting in the liberation of a significant amount of acid-soluble nitrogen (Sundararajan, 1956). Further, the racemization of amino acids and the hydrolysis of amide linkages in the protein brought about by the action of alkali render this reagent unsuitable for the study of the dephosphorylation of proteins.

Enzymic methods have been used in recent years in studies of this type. Thus the use of citrus-fruit phosphatase by Mecham & Olcott (1949) for bringing about the dephosphorylation of phosvitin and the preparation of dephosphorylated ovalbumin by Perlmann (1952) through the use of an intestinal phosphatase preparation are typical of this new type of approach. The discovery of phosphoprotein phosphatase by Harris (1946) provided the protein chemist with a new and far more effective tool for bringing about modification of proteins.

The availability of purified preparation of phosphoprotein phosphatase from ox spleen (Sundararajan & Sarma, 1954*a*) has led in the present investigation to a study of its usefulness as a specific dephosphorylating agent for casein. In preliminary experiments it was observed that casein solutions, incubated with purified preparations of the enzyme at pH 6·0, developed a milkiness, followed by the gradual formation of a precipitate within a few hours. After about 24 hr. the precipitate had settled down at the bottom of the tube, leaving a clear supernatant. Later these investigations were repeated with larger portions of casein and the precipitated protein was separated and estimated for its phosphorus content. The precipitate was consistently found to be free from phosphorus (Sundararajan & Sarma, 1954*b*). Since the enzyme preparations used to bring about dephosphorylation should be free from proteolytic activity so as to avoid undesirable side reactions, attention was next devoted to the preparation of protein phosphatase devoid of proteolytic activity. An effective method was to heat the enzyme preparation to 80° for 5 min. This treatment brought about selective inactivation of the proteolytic activity, leaving the phosphatase activity unaffected. The present paper gives details of this procedure and of the preparation of dephosphorylated casein through the use of this enzyme. The amino acid make-up of the dephosphorylated protein and the changes accompanying the enzymic dephosphorylation of casein have also been investigated.

METHODS

Measurement of phosphatase activity. Methods for the determination of phosphatase activity and definition of unit of enzyme activity are as described in a previous paper (Sundararajan & Sarma, 1954*a*).

Measurement of proteolytic activity. Proteolytic activity was determined by estimation of nitrogen in the trichloroacetic acid filtrate from enzyme digests according to the method of Koch & McMeekin (1924).

Analyses of proteins. Analyses of casein and dephosphorylated casein for the various constituents were carried out simultaneously and under identical conditions so as to make strict comparison of values possible. All analyses were carried out on air-dried protein samples. Moisture determinations were made as suggested by Chibnall, Rees & Williams (1943). Total nitrogen was determined according to the micro-Kjeldahl method of Chibnall *et al.* (1943), the

ammonia being estimated by distillation into boric acid (Ma & Zuazaga, 1942). Phosphorus determinations were carried out by the method of Fiske & Subbarow (1925) after digestion with sulphuric acid.

Amino acid analyses. Hydrolysis of the protein was carried out in 20% (w/w) hydrochloric acid at 110° for 24 hr. The acid was removed by repeated distillations *in vacuo* and the hydrolysate made up to a suitable volume.

Tyrosine and tryptophan estimations were carried out on alkali hydrolysates of the protein according to the methods of Bernhart (1938) and Horn & Jones (1945) respectively.

Cystine was determined according to the procedure of Kassell & Brand (1938) and methionine by the method of Horn, Jones & Blum (1946).

Arginine and histidine were estimated after a preliminary separation of these amino acids from the protein hydrolysate by precipitation with phosphotungstic acid (Van Slyke, Hiller & MacFayden, 1941). The colorimetric procedure of Macpherson (1946) was then employed for the determination of these amino acids.

Hydroxyamino acids were estimated by periodate oxidation. Threonine was estimated titrimetrically as acetaldehyde after periodate oxidation (Rees, 1946). Serine was determined by oxidation with periodate, distillation of formaldehyde by the method of Boyd & Logan (1942), and estimation of formaldehyde photometrically with chromotropic acid according to the micromethod of Frisell, Meech & Mackenzie (1954).

Glycine was estimated according to the procedure of Alexander, Landwehr & Seligman (1945).

Glutamic acid was determined manometrically as carbon dioxide after enzymic decarboxylation with *Clostridium welchii* SR12 according to Meister, Sober & Tice (1951).

Microbiological assay methods were employed for the estimation of leucine, isoleucine, valine, proline, phenylalanine, lysine, alanine and aspartic acid. Alanine was determined according to Sauberlich & Baumann (1949) and aspartic acid by the procedure of Hac & Snell (1945). Analyses of the other amino acids were carried out essentially according to the procedure outlined by Barton-Wright (1952).

RESULTS

Preparation of protease-free phosphoprotein phosphatase

Phosphoprotein phosphatase was purified from ox spleen according to the method described previously (Sundararajan & Sarma, 1954*a*). A minor modification in the original method was introduced in that the extraction of the enzyme from the tissue was carried out at pH 5·0 instead of at pH 5·8. The bulk of the inactive protein material was rendered insoluble by this procedure and sharp separation of the enzyme in the ammonium sulphate fractionation step was obtained. An outline of the modified procedure along with the purification obtained is given in Table 1. The purified preparation exhibited some amount of proteolytic activity on testing with casein and egg albumin as substrates. Heat denaturation was found, however, to be quite effective for the elimination of this activity. For this purpose, the enzyme solution, buffered at pH 5·0, was heated in a water bath maintained at 80°. At the end of 5 min. the solution was quickly cooled to room temperature and filtered. This treatment completely destroyed the proteolytic activity, leaving the phosphatase activity relatively unaffected. The amounts of inorganic phosphorus and acid-soluble nitrogen released from casein by such preparation during a 24 hr. incubation period are indicated in Table 2. It will be seen that a small amount of acid-soluble nitrogen is formed even after the heat-treatment. No such liberation was found, however, with egg albumin as substrate. The nature of acid-soluble nitrogen released from casein has been characterized by paper chromatography and is discussed in a later section.

Table 1. *Purification of phosphoprotein phosphatase from ox spleen*

Stage	Volume (ml.)	Total activity (units)	Overall yield (%)	Specific activity*
1	2550	9775	—	0·5
2	1635	6810	70·0	1·4
3	65	5230	53·5	139·0
4	40	3307	33·8	773·0

* Activity units/mg. of protein nitrogen.

Summary of procedures

(1) Ox spleen was homogenized with 2·5 volumes of saline solution containing 0·2 M acetate buffer at pH 5·0. (2) The supernatant was obtained on centrifuging of homogenate. (3) The supernatant was fractionated with ammonium sulphate. The precipitate obtained at 40–80% saturation was separated and dialysed. The precipitate separating on dialysis was extracted with saline solution at pH 5·0 and filtered. (4) The filtrate from (3) was fractionated with acetone. The precipitate obtained at 50–66% (v/v) acetone at 0° was extracted with saline at pH 5·0 and filtered.

Table 2. *Selective heat denaturation of proteolytic activity*

Activities determined with 0·1 ml. of purified phosphoprotein phosphatase preparation with casein as substrate (1 ml. of 10% solution) and thioglycollic acid (0·001 M) as activator. Incubation time, 24 hr.

Enzyme	Inorganic phosphorus released (μg.)	Acid-soluble nitrogen formed (μg.)	Nitrogen solubilized (%)
Untreated preparation	470	1350·0	9·6
Preparation held for 5 min. in a water bath at 80°	454	307·5	2·2

Preparation of dephosphorylated casein

Casein (25 g. of Light White Soluble, British Drug Houses, Ltd.) was added in small portions to 800 ml. of water contained in a beaker, with continuous stirring. After the protein had gone into solution, 10 g. of Celite Analytical Filter-Aid (diatomaceous silica filter aid L665 obtained from Fisher Scientific Co., U.S.A.) were added, the suspension stirred well and filtered through a layer of Celite on a Büchner funnel. The clear filtrate was transferred to a 2 l. bottle. A molar solution of acetate buffer (pH 5·8, 50 ml.) was added, followed by 10 ml. of 0·1 M thioglycollic acid. Enough water was added to bring the solution to a final volume (including volume of enzyme solution) of 1 l. A purified phosphatase preparation from ox spleen (15 ml., specific activity about 700 units/mg. of protein nitrogen) was added and, after the addition of 25 ml. of toluene, the bottle was stoppered well and placed in an incubator at 37°. After 24 hr. a second portion (15 ml.) of enzyme solution along with thioglycollic acid (0·001 M final concentration) was added. A precipitate had begun to separate by this time. At the end of 48 hr., the precipitated protein was collected on the centrifuge and washed well with water.

For purification, the protein was suspended in water and brought into solution by the dropwise addition of dilute alkali (N) with continuous stirring. The solution was filtered through a layer of paper pulp on a Büchner funnel. The dephosphorylated casein was precipitated from this solution by the dropwise addition of N hydrochloric acid to pH 6·0. The precipitated protein was collected by centrifuging, washed repeatedly with water and dried with acetone. The air-dry product weighed about 15 g.

The protein is a white amorphous powder lighter than casein. It is relatively insoluble in water. It is, however, soluble in dilute alkali and its solubility in dilute acids is greater than that of casein. It is precipitated from its solution in alkali by acidifying to pH 6·0. There appears to be thus a shift in the isoelectric point of casein to the alkaline side during its dephosphorylation, presumably owing to the removal of the phosphoric acid groups.

The supernatant solution from dephosphorylated casein gave a small amount of precipitate (0·38 g.) when adjusted to pH 4·8. The filtrate from this precipitate gave, on half saturation with ammonium sulphate, additional amounts of a precipitate (0·4 g.). The nature of this product has not been investigated in detail.

During the dephosphorylation of casein, small amounts of acid-soluble nitrogen were also liberated, which corresponded to about 2% of the casein nitrogen. The nature of this product has been investigated by the paper-chromatographic technique.

Characterization of acid-soluble nitrogen formed during dephosphorylation of casein

A solution (50 ml.) containing 1 g. of casein, acetate buffer of pH 5·8 (0·1 M) and thioglycollic acid (0·001 M) was incubated with 1 ml. of a purified preparation of phosphoprotein phosphatase. After incubation at 37° for 24 hr., an additional amount (1 ml.) of enzyme was added. After 48 hr., the precipitated protein was collected by centrifuging. The precipitate was washed twice with water. The supernatant and the washings were combined and deproteinized by the addition of trichloroacetic acid. After being kept in a boiling-water bath for 15 min., the solution was filtered from the small amount of precipitate formed. The filtrate was shaken with several portions of ether in a separating funnel to remove trichloroacetic acid. The aqueous layer, after the final extraction, was drawn off and evaporated to dryness *in vacuo*. The small amount of residue obtained was dissolved in 2·5 ml. of water and filtered and the filtrate preserved for chromatographic analysis. Substrate blanks were carried out simultaneously. The experimental conditions were the same, with the difference that the substrate was incubated in the absence of the enzyme.

For paper-chromatographic examination, a 20–30 μl. portion of the test solution was applied as a spot on to a Whatman no. 1 paper (23 cm. × 40 cm.). The development of the chromatogram was carried out according to the ascending technique of Williams & Kirby (1948), with *n*-butanol–acetic acid–water (4:1:5) as solvent system. After the chromatogram had been run for 24 hr., the sheet was dried at room temperature. For the detection of the spots, the sheet was sprayed with a 0·1% (w/v) solution of ninhydrin in *n*-butanol. Spots were revealed by leaving the

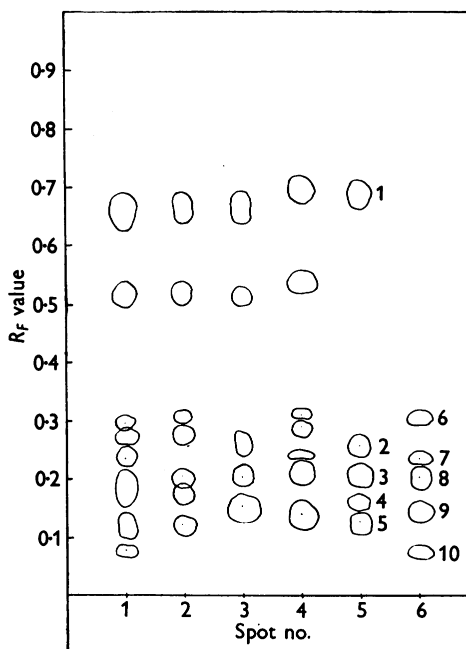

Fig. 1. Paper chromatogram of hydrolysates of peptides released during enzymic dephosphorylation of casein. Spots 1–4 represent hydrolysate of peptides having the R_F values indicated in the text. Spots 5 and 6 represent mixtures of authentic samples of the following amino acids: (1) isoleucine, (2) threonine, (3) serine, (4) arginine, (5) lysine, (6) alanine, (7) glycine, (8) glutamic acid, (9) histidine and (10) cystine.

paper in an oven at 100° for a few minutes. Four ninhydrin-positive spots were obtained in this way. With the solvent system used, they had the following R_F values: (1) 0·12, (2) 0·21, (3) 0·27 and (4) 0·89. Spot no. 4, which had the highest R_F value, was also found to be the most intense. In the blank experiment, a faint spot corresponding to an R_F of 0·89 was the only one obtained.

For further characterization, portions (about 20–30 μl.) of the test solution were applied as successive spots (five in number) to a Whatman no. 1 filter sheet (23 cm. × 40 cm.) and the chromatogram was run for a period of 24 hr., as described above. The sheet was taken out and the ninhydrin-positive spots, located with the aid of guide strips, were cut out from the paper. Spots having the same R_F values were combined and eluted from the paper with water. The eluates were hydrolysed in a sealed tube with 2N hydrochloric acid at 110° for a period of 24 hr. The product in each case was taken up in a small amount of water and analysed by paper chromatography, with the butanol–acetic acid–water solvent system. A diagrammatic representation of the chromatogram obtained with the acid hydrolysates of the different peptides is presented in Fig. 1. In the figure are also indicated the spots obtained with several authentic samples of amino acids.

Amino acid composition of dephosphorylated casein

Results of amino acid determinations on casein and dephosphorylated casein are given in Table 3. The values for dephosphorylated casein represent the average of results obtained with two different samples of the protein. The samples had more or less the same amino acid composition, the divergence in values being well within experimental error (below 2 % for chemical methods and below 5 % for the microbiological-assay methods). The analytical results presented in the table have all been corrected for moisture. The figures for serine and threonine include corrections made for the decomposition of these amino acids during acid hydrolysis of the protein, the correction factors worked out by Rees (1946) (100/89·5 for serine and 100/94·7 for threonine) having been applied for this purpose.

Table 3. *Amino acid composition of dephosphorylated casein*

Values are expressed in g./100 g. of the dry protein.

Constituent	Dephos-phorylated casein	Casein: Present investigation	Casein: Literature value*
Total nitrogen	15·55	15·56	15·63
Total phosphorus	0·04	0·80	0·86
Alanine	3·26	3·38	3·00
Glycine	1·68	1·90	2·70
Valine	6·89	6·94	7·20
Leucine	9·43	9·54	9·20
Isoleucine	5·76	6·13	6·10
Proline	12·22	11·72	11·30
Phenylalanine	5·10	4·98	5·00
Tyrosine	7·21	6·32	6·30
Tryptophan	1·46	1·31	1·20
Serine	7·02	6·27	6·30
Threonine	5·27	5·28	4·90
Cystine	0·24	0·35	0·34
Methionine	2·99	2·93	2·80
Arginine	4·12	3·92	4·10
Histidine	3·06	3·03	3·10
Lysine	7·86	8·18	8·20
Aspartic acid	7·34	7·66	7·10
Glutamic acid	20·73	21·62	22·40

* Values taken from Gordon, Semmett, Cable & Morris (1949).

DISCUSSION

The enzymic dephosphorylation of casein is accompanied by the liberation of a small but significant amount of acid-soluble nitrogen. Paper-chromatographic examination of the nitrogenous product reveals that it is composed of a mixture of peptides (Fig. 1). Since the phosphatase preparation used in these studies was free from proteolytic activity and did not liberate acid-soluble nitrogen from egg albumin, it appears probable that the formation of acid-soluble nitrogen from casein is linked up with its dephosphorylation. Liberation of peptide material during enzymic dephosphorylation of casein has also been observed by Perlmann (1954*a*, *b*), and she has made the suggestion that, as with the disulphide bridge, phosphodiester and pyrophosphate linkages can cross-link peptides in the intact protein. Disruption of the linkage by means of a suitable phosphatase preparation would thus result in the liberation of peptides. The results obtained in the present investigation are in conformity with this suggestion. Additional evidence for this view is provided by the fact that the peptides formed during the dephosphorylation of casein contain, as common constituents, glutamic acid, isoleucine and serine—amino acids which are also present in the phosphopeptones obtained from casein by various workers (Rimington, 1941; Damodaran & Ramachandran, 1941).

The results presented in Table 3 show that the dephosphorylated casein has approximately the same amino acid composition as casein. Significant deviations are observed only with respect to a few amino acids. Thus the dephosphorylated protein contains somewhat higher amounts of serine, tyrosine and tryptophan and less cystine and glycine than casein. Dephosphorylated casein contains about 7 % of serine, as against the value of 6·3 % obtained for casein. These values include corrections made for the decomposition of serine during acid hydrolysis of the protein. The corrected values for casein may be low, in view of the greater lability to acid hydrolysis of serine combined as phosphoserine in phosphoproteins, as compared with that of free serine (Damodaran & Ramachandran, 1941; Nicolet, Shinn & Saidel, 1942; Mecham & Olcott, 1949). Thus Nicolet *et al.* (1942) give 7·38 % as the

probable serine content of casein, compared with 5·5% found on direct acid hydrolysis. The serine content of dephosphorylated casein is close to the value suggested by these workers for casein. These results taken with the findings of Mecham & Olcott (1949) indicate that the true serine content of phosphoproteins is best obtained when the estimation is carried out on the dephosphorylated protein.

Dephosphorylated casein prepared by the enzymic and chemical methods respectively differ in their content of most of the amino acids. Most striking perhaps are the observed differences in the contents of arginine, tryptophan, aspartic acid, cystine and the hydroxyamino acids and the presence of less than 1% of valine in alkali-treated casein (Macara & Plimmer, 1940). The alkali-dephosphorylated protein has also a lower nitrogen content. This has been attributed by various workers to the hydrolysis of the acid amide linkage (Rimington, 1927; Macara & Plimmer, 1940). The use of alkali as a dephosphorylating agent suffers from the additional disadvantage in that the casein molecule is split. Failure to obtain appreciable amounts of products, other than dephosphorylated casein, in enzymic digests of casein indicates that no such drastic changes take place during the enzymic dephosphorylation of casein. This view is also supported by results obtained on the amino acid composition of the enzymically dephosphorylated casein. The protein has also been found to be readily attacked by proteolytic enzymes (Sundararajan, 1956). Alkali-dephosphorylated casein is attacked by these enzymes with difficulty, possibly owing to some racemization having taken place during the treatment with alkali (Rimington, 1927). The results obtained in the present study thus indicate the advantages of enzymic over alkali dephosphorylation methods and emphasize the importance of phosphoprotein phosphatase as a specific tool for the study of dephosphorylated proteins.

SUMMARY

1. The preparation from ox spleen of a phosphoprotein phosphatase free from proteolytic activity and suitable as a dephosphorylating agent for casein is described.

2. The preparation and some properties of dephosphorylated casein are described. In the preparation of the protein, advantage has been taken of the insolubility of the dephosphorylated casein at pH 6·0, which is also the optimum pH for the enzymic dephosphorylation.

3. Acid-soluble nitrogen formed during the enzymic dephosphorylation of casein has been determined and the nature of the nitrogenous product investigated by paper chromatography.

4. Dephosphorylated casein has been analysed for amino acids. Comparison of the values with those obtained for casein shows that the amino acid composition of casein and of its dephosphorylated product are about the same.

5. It is concluded from these studies that during the enzymic dephosphorylation of casein the protein remains relatively intact and that the changes, if any, brought about by this treatment are not of a drastic nature.

6. The relative merits of alkali and enzymic dephosphorylation methods are discussed.

One of us (T.A.S.) is indebted to the Government of India for the award of a Senior Research Scholarship during the tenure of which these investigations were carried out.

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