Studies on Casein

1. PURIFICATION AND PROPERTIES OF PHOSPHOPROTEIN PHOSPHATASE

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Harris (1946) discovered in frog's eggs an enzyme catalysing the dephosphorylation of phosphoproteins. Since then this enzyme, phosphoprotein phosphatase, has been studied in crude tissue extracts by a number of workers (e.g. Feinstein & Volk, 1949; Norberg, 1950). Foote & Kind (1953) described the purification of a similar enzyme from chick embryo, and Sundararajan & Sarma (1954*a, b*) and Roche, Thoai & Pin (1953) used ox and rat spleens as starting materials for their preparation. To study the phosphorus linkages in casein an enzyme was required which could be easily prepared in good yield and be free of both protease and phosphomonoesterase activity. It was also desirable to have an enzyme which would be stable at low temperatures for long periods. None of the published methods fulfilled these requirements. The method of Sundararajan & Sarma (1954*a*) for preparing the enzyme was very time-consuming, whereas that of Roche *et al.* (1953) led to very unstable preparations.

A new method which gave a stable, partially purified enzyme free of protease and β -glycerol phosphatase activity was therefore developed. This simplified method, together with some of the properties of the enzyme preparation, is described in the present paper. The following paper (Hofman, 1958) presents the results of a detailed study on the specificity of the enzyme and its action on various casein preparations. A preliminary account of some of these results has been given (Hofman, 1955).

MATERIALS AND METHODS

Ox spleen. Spleens were obtained from newly slaughtered animals. They were immediately chilled with ice and stored at -15° .

Substrates. In this and the following paper (Hofman, 1958) the term 'unfractionated casein' will be taken to mean the mixture of α -, β - and γ -caseins which is obtained from milk by precipitation at pH 4.6 essentially according to the procedure of Dunn (1949). It was prepared from fresh skim milk of Ayrshire cows. Disodium phenyl phosphate and disodium β -glycerol phosphate were commercial products.

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Phosphoprotein phosphatase activity. The enzyme activity was measured by incubating 4 ml. of a solution of casein containing 10 μ g. atoms of casein P/ml. in 0.1N-acetic acid-sodium acetate buffer, pH 5.9, with 1 ml. of an appropriately diluted enzyme solution at 37° for 30 min. The inorganic P liberated was determined by the method of Martin & Doty (1949), who precipitated protein with trichloroacetic acid and silicotungstic acid. This method was preferred to the conventional methods of Fiske & Subbarow (1925) or Berenblum & Chain (1938) because, on incubating casein with crude tissue extracts, breakdown products were formed which interfered with the development of the molybdenum blue. Silicotungstate completely removed these interfering substances. Similar observations have been made by Mattenheimer, Nitschmann & Zahler (1952). Controls were carried out by incubating (a) casein and water, (b) enzyme and water.

Phosphomonoesterase activity. This was determined by substituting a solution of disodium β -glycerol phosphate, containing 10 μ g. atoms of bound P/ml. in acetate buffer, for the casein solution.

Unit of phosphatase activity. One unit of phosphoprotein phosphatase and phosphomonoesterase activity is defined as the amount of enzyme which liberates 1 μ g. of P/min. under the conditions just described.

Activity of proteolytic enzymes. The non-protein N soluble in 4% (w/v) trichloroacetic acid, after incubation of 4 ml. of casein solution with 1 ml. of suitably diluted enzyme solution for periods of from 30 min. to 24 hr., was determined by a micro-Kjeldahl method (Hiller, Plazin & Van Slyke, 1948).

Units of proteolytic activity. One unit is defined as the amount of enzyme which liberates 1 μ g. of N/min.

Specific activity. This is expressed in units/mg. of protein N of the enzyme solution.

Nitrogen determinations. Protein N was calculated from the difference between the total N in the sample before and after deproteinization, as determined by a micro-Kjeldahl method (Hiller *et al.* 1948).

Purification of phosphoprotein phosphatase

In preliminary experiments in which the method of purification developed by Sundararajan & Sarma (1954*a*) was used it was difficult to separate the enzyme from insoluble matter of the spleen. These authors used 0.5M-NaCl in acetate buffer to keep the enzyme in suspension, and cell debris and nuclei were removed by centrifuging. The enzyme was then precipitated as a gel with ammonium sulphate. This gel could not be centrifuged and had to be filtered for long periods. Further experiments showed that the enzyme could be brought into solution by a treatment with butanol according to the method of Morton (1953).

The following procedure for the purification was finally adopted.

Extraction of enzyme. Frozen spleen was skinned and minced. The mince (100 g.) was homogenized in a top-drive Blender with 900 ml. of cold 0.5M-sodium chloride in 0.05M-sodium acetate-acetic acid buffer, pH 5.9. The homogenate was centrifuged at 900 g for 30 min. and the precipitate discarded. The supernatant extract (ES) (900 ml.) was cooled to 0° and shaken with 450 ml. of cold butanol for 5 min. The emulsion was centrifuged at 1500 g for 15 min., when it separated into three layers. The bottom layer containing the enzyme (450 ml.) was collected by suction through a capillary and the intermediate layer (600 ml.) extracted with 500 ml. of the buffered NaCl solution.

The combined extracts after the butanol treatment (1270 ml.) were cooled to 2° and acetone (330 ml.) was added to give an acetone concentration of about 20% (v/v). After standing at -15° overnight the solution contained an inactive gelatinous precipitate, which was centrifuged off and discarded. Acetone (940 ml.) was added to the clear supernatant liquid (1600 ml.) to give an acetone concentration of about 50% (v/v) and the mixture was left again at -15° overnight. A red-brown precipitate which settled contained almost all the enzyme activity and was removed by centrifuging. After most of the acetone had been removed by draining, the precipitate was dissolved in 115 ml. of the buffered NaCl solution. Some insoluble, inactive material was removed by centrifuging and a clear red-brown solution (A_0) was obtained. The solution A_0 was dialysed at 2° for 48 hr. against frequent changes of distilled water. A flocculent precipitate settled and was centrifuged off. The clear red-brown supernatant (A_1) contained some of the phosphoprotein phosphatase activity and all the β -glycerol phosphatase and protease activity of A_0 . No attempt was made to separate these enzyme activities further, and the solution was discarded. The precipitate was dissolved in 80 ml. of 0.5M-sodium chloride-0.05M-sodium acetate buffer, pH 5.9, and centrifuged to remove some inactive, insoluble material. The yellowish supernatant (B_1) was slightly opalescent. It contained the larger part of the phosphoprotein phosphatase activity, and was free from β -glycerol phosphatase activity (determined as described, with a 24 hr. incubation period). Attempts were made to purify the enzyme further by adsorption on calcium phosphate gel, Hyflo Supercel, kaolin and alumina gel C_v , but in every case the activity was lost. The solution B_1 was used in the studies of the properties of the enzyme described in the following sections, except where otherwise stated.

The results obtained in a typical purification experiment are summarized in Table 1. As the table shows, the enzyme was purified about 60-fold (1.37-82 units/mg. of N) and was obtained in 21% yield.

RESULTS

Properties of phosphoprotein phosphatase

Specificity. A detailed study on the specificity of the enzyme is given in the following paper (Hofman, 1958). It may be mentioned, however, that the enzyme readily liberates inorganic phosphate, not only from casein but also from phenyl phosphate and a number of other organic phosphates as well as from inorganic pyrophosphate, but not from β -glycerol phosphate.

Optimum pH. The influence of the pH on the activity of the enzyme was determined with casein, phenyl phosphate and inorganic pyrophosphate as substrates. As Fig. 1 shows, the optimum for the three substrates is around pH 5.0-5.5. Owing to the insolubility of the casein below pH 5 the activity with this substrate could not be measured at lower values. Even between pH 5 and 6 it is difficult to obtain solutions of casein. For this reason almost all the incubation experiments were carried out at pH 5.9 rather than at the optimum, pH 5.0-5.5; pH 5.9 was accordingly also used when substrates other than casein were incubated.

Effect of pH on stability of phosphoprotein phosphatase. As Fig. 2 shows, the enzyme is stable for 24 hr. at room temperature only in the narrow range pH 5.5-6.0, but loses little activity in the range pH 4-8. At lower and higher pH values, however, inactivation is very marked. Similar experiments were carried out with a crude spleen extract (preparation ES). They showed that the crude enzyme is very labile, particularly outside the range pH 5.5-6.5. This finding is in close agreement with the result of Norberg (1950), who studied the inactivation of phosphoprotein phosphatase with liver homogenates and extracts. The purified enzyme obtained by Sundararajan & Sarma (1954a) seemed to be less stable than the present preparation. The results, however, are not

Table 1. *Summary of a typical enzyme purification*

Fraction	Vol. (ml.)	Protein N (mg./ml.)	Phospho- protein phosphatase activity (units/ml.)	Specific activity (units/mg. of N)	Overall yield (%)	β -Glycerol phosphatase activity (units/ml.)	Protease activity (units/ml.)
Spleen homogenate	1000	2.7	3.7	1.37	100	3.1	38
Extract ES	870	1.7	3.5	2.03	82	2.8	33
Acetone fraction A_0	119	0.9	13.7	15.3	44	0.7	16
Dialysis: precipitate B_1	84	0.11	9.4	82	21	0.08	0.1
supernatant A_1	164	0.66	5.5	8.3	—	0.4	—

strictly comparable, as the authors studied the stability of their enzyme at 37° and in the presence of thioglycolic acid as activator.

Effect of temperature on stability of phosphoprotein phosphatase. Roche *et al.* (1953) prepared a phosphoprotein phosphatase from calf spleen and found that it was extremely thermolabile, losing its activity after standing for a few days at temperatures near 0°. It is interesting therefore to find that the present preparation B_1 is stable at temperatures up to 70° (Table 2). No loss in activity was found when the enzyme was incubated at this temperature for 4 hr. At 75°, however, the enzyme is rapidly inactivated. After incubation for 5 min. only 27 % of the original activity is left.

The enzyme was very stable when frozen, retaining full activity at -15° for 2 years. In

agreement with Norberg (1950) the unpurified enzyme was found to be rapidly inactivated by short exposure to temperatures above 40°.

Electrophoresis on filter paper. In order to find whether the actions of the enzyme preparation B_1 on phenyl phosphate and casein were due to the same enzyme, an attempt was made to separate the two activities from each other by electrophoresis on filter paper. Experiments were carried out at various pH values in an apparatus described by Latner (1952). No separation of the two activities was observed. Fig. 3 shows the result of a typical experiment made at pH 8.6. It will be seen that the two activities travelled together. When the filter-paper strips were stained with Naphthalene Black 12B 200 (Flynn & De Mayo, 1951) a band of protein was observed which was well separated from the band carrying the enzymic activities, indicating that the enzyme was contaminated with a considerable amount of inactive material.

Activation. No detailed study on the activation and inhibition of the enzymic activities was undertaken, but in agreement with Sundararajan & Sarma (1954*a*) it was found that mm-thioglycolic acid activated the enzyme. Table 3 shows that

Fig. 1. pH-activity curve. Substrate solution (4 ml.) containing 10 μ g. atoms of P/ml. in acetate or veronal-acetate buffer was incubated with 0.75 ml. of water and 0.25 ml. of enzyme (B_1). O, Casein; Δ , phenyl phosphate; $\square$, inorganic pyrophosphate.

Fig. 2. Stability of enzyme at various pH values. A volume (1 ml.) of enzyme solution (B_1) was adjusted to the desired pH with NaOH or HCl and left standing at room temp. for 24 hr. After readjusting to pH 5.9 the solution was assayed as described under Materials and Methods.

Table 2. Stability of phosphoprotein phosphatase at different temperatures

Enzyme solution (B_1 , 1 ml.) was incubated at a given temperature. After a selected time the activity was determined as described under Materials and Methods.

Temp. of incubation	Time (min.)	Activity (% of original)
20°	1440	101
37°	1440	95
65°	1080	96
70°	240	104
75°	5	27
75°	30	13.5
100°	2	0

Fig. 3. Electrophoresis of purified enzyme. A volume (0.1 ml.) of enzyme was examined on Whatman no. 31 filter-paper strip, 2 cm. wide. Conditions: veronal buffer, pH 8.6, $I=0.1$; run for 10 hr. at +2°, 3.7 v/cm. Activity was determined on 0.5 cm. segments. Substrates: Δ , casein; O, phenyl phosphate. Arrows indicate the position of two main protein bands, as revealed by staining with 'Naphthalene Black 12B 200'. No quantitative estimation of the protein was made.

thioglycollic acid increased not only the activity of the enzyme towards casein, but also towards inorganic pyrophosphate and phenyl phosphate, thus providing additional evidence for the possibility that the action on the various substrates was due to the same enzyme. Table 3 also shows that magnesium had no effect on the activity with any of the three substrates, either in the presence or the absence of thioglycollic acid.

Effect of substrate concentration. The dependence of the enzymic activity on the substrate concentration was studied with both casein and phenyl phosphate as substrates. When the experimental results were treated according to Lineweaver & Burk (1934), Michaelis constants of 3 mM for casein phosphorus were obtained for casein and 2 mM for phenyl phosphate. The figure for casein is slightly higher than the value of 2 mM found by Sundararajan & Sarma (1954*a*) but agrees with the value found by Norberg (1950).

DISCUSSION

A comparison of the present enzyme with that of Sundararajan & Sarma (1954*a*) strongly suggests that the two are identical. Both are activated by thioglycollic acid and have about the same pH optimum. The Michaelis constants show a small discrepancy, but this could be due to slightly different experimental conditions. Sundararajan & Sarma (1954*b*) also isolated an enzyme from rat spleen, for which a Michaelis constant of 0.55 mM was found. These authors concluded from their studies on the specificity of the enzyme that it was an enzyme quite distinct from the phosphomonoesterases. The results presented in this paper do not support this claim. As is shown in the following paper (Hofman, 1958), the enzyme is more active on phenyl phosphate than on casein. The evidence given here suggests that the two activities are due to the same enzyme, because (a) they could not be separated by electrophoresis, (b) they are both activated by thioglycollic acid, and Mg^{2+} ions have no effect, and (c) their pH optimum lies in the same range. It would have been more satisfactory, of course, to show that the ratio of the two activities

during purification had remained the same. However, owing to the presence of a strong non-specific phosphomonoesterase up to the second last stage of the purification, as shown by the splitting of β -glycerol phosphate (Table 1), the ratio of phenyl phosphatase to casein phosphatase activity could not be determined.

The pyrophosphatase activity of the present preparation also shows optimum activity in the pH range 5.0–5.5 and is activated by thioglycollic acid, indicating that it might be due to the same enzyme.

The phosphoprotein phosphatase which was isolated by Harris (1946) from frog's eggs seems to be different from the present enzyme since it showed no activity towards phenyl phosphate.

The phosphoprotein phosphatase which Roche and his colleagues (Roche *et al.* 1953; Thoai, Roche & Pin, 1954) described differs from the present enzyme in at least four properties: (a) it is extremely labile towards organic solvents as well as to temperature; (b) it liberates phenol from diphenyl phosphate whereas the present enzyme is inactive on this substrate (Hofman, 1958); (c) it is inactive towards phenyl phosphate; (d) it liberates only part of the phosphate from casein whereas the present enzyme effects complete hydrolysis.

Roche, Thoai & Thim (1952) described the preparation of phosphomonoesterases from rat muscle and pig erythrocytes. Starting from material which was highly active towards α - and β -glycerol phosphates and phenyl phosphate, they obtained at first preparations which had lost their activity towards β -glycerol phosphate and then some which were active only towards phenyl phosphate. The authors suggested that this separation of activities might be due to the selective destruction in one enzyme only of the configurations responsible for the formation of substrate–enzyme complex with any one substrate, rather than to the separation of three enzymes with narrow substrate specificity. The present experiments demonstrate that, at least with the spleen enzymes, two enzymes can be separated, one which acts on phenyl phosphate, other aromatic phosphates and casein, but not on β -glycerol phosphate, and the other which acts on

Table 3. *Effect of thioglycollic acid and Mg^{2+} ions on enzyme activity*

A volume (1 ml.) of substrate containing 3×10^{-3} g. atoms of P/l. was incubated with 0.025 ml. of enzyme and mM-thioglycollic acid or mM- $MgCl_2$ or both (final concentrations), in a final vol. of 1.25 ml. After 10 min. at 37° the P liberated was determined.

Substrate	P liberated (μ g./ml.)			
	No addition	$MgCl_2$	Thioglycollic acid	$MgCl_2$ + thioglycollic acid
Casein	3.3	3.6	11	10
Phenyl phosphate	9	8	20	21
Pyrophosphate	6	5	11	10

β -glycerol phosphate. This separation is shown in Table 1, where most of the phosphoprotein phosphatase and some of the phenyl phosphatase activity of A_0 appears in B_1 , whereas all the β -glycerol phosphatase activity is found in A_1 . The last-named enzyme has not been further studied, but it would clearly be desirable to investigate its properties.

SUMMARY

1. An improved method for the preparation of phosphoprotein phosphatase from ox spleen is described. The enzyme was purified 60-fold with a yield of 21 %.

2. The enzyme preparation was free from proteolytic activity and had no action on β -glycerol phosphate.

3. The enzyme was active on caseins, phenyl phosphate and inorganic pyrophosphate. Hence it cannot be described as a specific phosphoprotein phosphatase.

4. The pH optimum with caseins and phenyl phosphate was about pH 5.5.

5. The enzyme was stable over a wide range of pH and at temperatures below 70°.

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REFERENCES

- Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
 Dunn, M. S. (1949). *Biochem. Prep.* **1**, 22.
 Feinstein, R. N. & Volk, M. E. (1949). *J. biol. Chem.* **177**, 339.
 Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
 Flynn, F. V. & De Mayo, P. (1951). *Lancet*, **2**, 235.
 Foote, M. W. & Kind, C. A. (1953). *Arch. Biochem. Biophys.* **46**, 254.
 Harris, D. L. (1946). *J. biol. Chem.* **165**, 541.
 Hiller, A., Plazin, J. & Van Slyke, D. D. (1948). *J. biol. Chem.* **176**, 1401.
 Hofman, T. (1955). *Abstr. 3rd Int. Congr. Biochem., Brussels*, p. 26, nos. 4-9.
 Hofman, T. (1958). *Biochem. J.* **69**, 139.
 Latner, A. L. (1952). *Biochem. J.* **51**, xii.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Martin, J. B. & Doty, D. M. (1949). *Analyt. Chem.* **21**, 965.
 Mattenheimer, H., Nitschmann, H. & Zahler, P. (1952). *Helv. chim. acta*, **35**, 1970.
 Morton, R. K. (1953). *Biochem. J.* **55**, 795.
 Norberg, B. (1950). *Acta chem. scand.* **4**, 1206.
 Roche, J., Thoai, N.-V. & Pin, P. (1953). *C.R. Soc. Biol., Paris*, **147**, 409.
 Roche, J., Thoai, N.-V. & Thiêm, N. V. (1952). *C.R. Soc. Biol., Paris*, **146**, 227.
 Sundararajan, T. A. & Sarma, P. S. (1954a). *Biochem. J.* **56**, 125.
 Sundararajan, T. A. & Sarma, P. S. (1954b). *Biochim. biophys. Acta*, **13**, 588.
 Thoai, N.-V., Roche, J. & Pin, P. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 483.

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2. THE ACTION OF PHOSPHATASES ON CASEINS AND LOW-MOLECULAR-WEIGHT PHOSPHATES*

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Although a number of studies on the nature of the phosphorus linkages in phosphoproteins and in caseins in particular have been made during the past 30 years, no definite conclusions about the site and nature of the phosphorus linkages can as yet be drawn. That the phosphorus may be linked to the hydroxyamino acids serine and threonine was suggested by the isolation of *O*-serine phosphate by Lipmann (1933) and *O*-threonine phosphate by de Verdier (1953). However, the isolation procedure of these compounds did not exclude the possibility that the phosphate group had migrated

to the hydroxyl group of serine and threonine during hydrolysis. This was suggested by Plapinger & Wagner-Jauregg (1953), who showed that serine phosphorylated with a diisopropyl phosphate residue on the amino group was converted on hydrolysis into *O*-serine phosphate. A number of authors (see review by Perlmann, 1955) prepared, from enzymically degraded casein, phosphopeptides which always contained serine and phosphate groups with a varying number of other acids. New interest was aroused in the nature of the phosphorus linkages in caseins by the findings of Perlmann (1952b). This author employed phosphatases to remove, selectively, the phosphate groups from ovalbumin, caseins and pepsin.

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