Purification and Properties of Phosphoprotein Phosphatase from Ox Spleen

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Harris (1946) detected for the first time, in frog's eggs, an enzyme capable of effecting a specific dephosphorylation of phosphoproteins and accordingly termed it phosphoprotein phosphatase. A similar enzyme was found to be present in rat tissues by Feinstein & Volk (1949) who also studied some of its properties. The enzyme was not activated by metal ions, nor did it lose its activity when dialysed. A characteristic property of the enzyme was its activation by reducing agents. This was, however, contradicted by Norberg (1950) who, in his experiments, could find no activation. On account of the divergent views on this question. and since the previous investigators used crude tissue extracts as the enzymic material in their studies, the problem has been re-investigated with a purer enzyme preparation. The object of the present study was also to establish the existence of phosphoprotein phosphatase in animal tissues as an independent enzyme quite distinct from the phosphomonoesterases. Accordingly a method has been evolved for the purification of the enzyme from ox spleen. The final product obtained represents a 200-fold purification and can be considered to be the purest specimen of the enzyme so far obtained.

EXPERIMENTAL

Materials

Substrates. Casein prepared according to the method of Cohn & Hendry (1930) was used routinely as substrate. Phosvitin and vitellin were prepared from egg yolk according to the methods of Mecham & Olcott (1949) and of Calvery & White (1931), respectively. Phosphopeptone was prepared in the form of its barium salt from a peptic-tryptic digest of casein according to the method of Damodaran & Ramachandran (1941). The glycerophosphate employed was the β-isomer obtained from British Drug Houses Ltd. (B.D.H.).

Activator. Thioglycollic acid (B.D.H.) was used as an activator, unless otherwise stated. The enzyme was found to exert its maximum activity in the presence of 0·001 M-thioglycollic acid.

Buffer. Michaelis veronal-acetate buffer.

Methods

Measurement of enzyme activity. The test mixture employed for the measurement of the enzyme activity was made up as follows: 1 ml. of the mixture contained 10 μmoles casein P, 1 μmole activator, 20 μmoles buffer at pH 6 and varying amounts of the enzyme solution. The mixtures were incubated at 37° for 30 min. After deproteinization with trichloroacetic acid (TCA), the activity was followed by the estimation of the liberated inorganic P by the method of Fiske & SubbaRow (1925). Controls with casein and water and blank values with water and sample were run at the same time.

Unit of enzymic activity. This was defined as the amount of enzyme catalysing the splitting of 1 μg. of inorganic P/min. at 37° and at pH 6 from a test mixture containing 10 μmoles of casein P/ml.

Specific activity. This was expressed in units/mg. protein N.

Determination of protein nitrogen. The protein was first precipitated from the test solution by TCA. When washed free from ammonia the N content was determined by the micro-Kjeldahl method (Pregl, 1945).

RESULTS

Purification of phosphoprotein phosphatase

Ox spleen was preferred as starting material for the preparation of the enzyme on account of its ready availability and relative abundance.

Extraction of the enzyme. Spleens from freshly slaughtered oxen were removed and conveyed from the slaughter house

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

Step	Fraction	Volume (ml.)	Total activity (units)	Overall yield (%)	Specific activity
1	Original saline extract	350	3410	—	1·35
2 and 3	Fraction *A*	130	1023	30	3·25
2 and 3	Fraction *B*	27	1087	32	5·19
4	Fraction A_1	10	35	1·0	—
	Fraction A_2	50	520	15	—
	Fraction *C*	25	1324	39	37·3
5	Fraction *D*	25	417	12·2	28·5
	Fraction *E*	12·5	675	19·8	254

mixed with ice. After a preliminary mincing of the tissue in a meat chopper, a weighed amount of the minced tissue was ground well in a Waring Blendor with 2·5 times its weight of 0·5 M-NaCl, buffered at pH 5·8. The use of this medium facilitates the almost quantitative extraction of the enzyme besides ensuring its stability. The tissue suspension was centrifuged for 15 min. at 2000 rev./min. The supernatant was opalescent and reddish brown in colour.

Ammonium sulphate fractionation. The saline extract was cooled to 10° and solid $(NH_4)_2SO_4$ was added gradually with mechanical stirring to 0·3 saturation (21·9 g./100 ml.). Most of the protein was separated at this concentration (fraction *A*). The precipitate was separated by filtration through fluted filter papers and allowed to drain in the ice chest (10°). The filtrate was next treated with $(NH_4)_2SO_4$ to 0·6 saturation and the precipitate separated as before (fraction *B*). A portion of the filtrate from 0·6 saturation was treated with $(NH_4)_2SO_4$ to full saturation. The precipitate contained very little of the activity and hence was discarded.

Precipitation of the enzyme by dialysis. Fractions *A* and *B* were taken up separately in water and dialysed at 10° in cellophan sacks against frequent changes of distilled water. A flocculent precipitate separated in each case at the end of 24 hr. and settled down in the sacks after the completion of dialysis in about 72 hr. The precipitates were separated by centrifugation, taken up separately in 0·5 M-NaCl at pH 5·8, and the activities of the different fractions were estimated as usual. The precipitates in each case were found to contain most of the activities of the respective fractions, whereas the supernatants had negligible activities.

Refractionation with ammonium sulphate of 0·3 saturation fraction. The precipitate which separated on dialysis of fraction *A* was dissolved in a suitable amount of 0·5 M-NaCl and filtered through fluted filter paper. The filtrate was subjected to a refractionation with $(NH_4)_2SO_4$. This time most of the activity was found to have gone over to the 0·6 fraction (fraction A_2). The 0·3 fraction (fraction A_1) contained little activity and consequently was rejected. The appreciable amount of activity found in the 0·3 fraction during the first fractionation (fraction *A*) is obviously due to the adsorption of the enzyme on the bulky precipitate formed, rather than due to any precipitation of the enzyme at this salt concentration.

Fraction A_2 was taken up in water and dialysed as before. The precipitate which separated during dialysis was added to the corresponding fraction obtained in the first $(NH_4)_2SO_4$ fractionation. The pooled precipitates were extracted once with 0·5 M-NaCl. The extract (fraction *C*) containing high phosphatase activity was finally subjected to an acetone fractionation.

Table 2. *Relative action of the different fractions towards casein and glycerophosphate*

(Activities of the crude spleen extract and of the purified enzyme were tested on 0·5 and 0·1 ml. samples, respectively. Final concentration of substrates corresponded to 10 μmoles of organic P/ml.)

Expt. no.	Enzyme source	Specific activity with Casein	Specific activity with Glycero-phosphate
1	Crude spleen extract	1·07	0·52
	Final purified product	190·5	10·04
2	Crude spleen extract	1·10	0·61
	Final purified product	205	23·33

Fractionation with acetone. To a measured amount of the saline extract (fraction *C*), cooled in a freezing mixture of ice and salt, ice-cold acetone (B.D.H., A.R. grade) was added dropwise with vigorous mechanical stirring until the acetone concentration was 50%. The precipitate formed was separated on a centrifuge. Further quantities of acetone were added to the supernatant as before till the acetone concentration reached 66%. The precipitate formed was removed by centrifugation. The two fractions were dissolved separately in suitable amounts of 0·5 M-NaCl, centrifuged and the activities of the water-clear supernatants (fractions *D* and *E*) were estimated as usual.

The results obtained in a typical fractionation experiment are presented in Table 1. The precipitate separating between 50 and 66% acetone concentration (fraction *E*) will be seen to have the highest activity, representing approximately a 200-fold purification. This fraction has been used throughout this work for the study of the properties of the enzyme and will be referred to as the purified enzyme.

The enzymic properties of phosphoprotein phosphatase

Specificity

Relative action of the crude and purified enzyme preparations towards casein and glycerophosphate. The object of the experiment was to see whether phosphoprotein phosphatase could be differentiated from acid phosphomonoesterases on the basis of its action on the different substrates. The results presented in Table 2 show that on purification of the enzyme there is a marked decrease in activity with

glycerophosphate as substrate and an increase with casein. The hydrolysis of the two substrates must hence be assumed to be brought about by two different enzymes.

Action of the purified enzyme on other phosphoproteins. Since the enzyme has a preferential action on casein, it would be of interest to see whether it can attack the other phosphoproteins with equal vigour. It is evident from the results given in Table 3 that both phosvitin and vitellin are dephosphorylated by the enzyme, though not to the same extent as casein. The figures for vitellin are perhaps too low, since only a part of the substrate was present in the dissolved state in this case. No appreciable dephosphorylation took place with phosphopeptone. It is of interest to note that neither phosphopeptone nor phosphoserine is attacked by this enzyme (Norberg, 1950; Feinstein & Volk, 1949). Evidently the enzyme cannot attack casein after considerable proteolytic degradation.

Kinetics of enzymic action

Fig. 1 presents the time/activity curves of our purified enzyme preparation. A linear relationship is found to hold good in the initial stages in both the preparations studied. The main reason for the slowing down of the reaction may be the influence of the split products on enzymic action. It is of interest to note in this connexion that the activity of the enzyme is diminished by added inorganic phosphorus (Norberg, 1950).

Effect of substrate concentration on enzymic activity. The dependence of enzyme activity upon substrate concentration is shown in Fig. 2. The activity of the enzyme is found to increase at first but remains constant at a substrate concentration corresponding to about 10 μmoles of casein phosphorus/ml. Treatment of the results according to the method of Lineweaver & Burk (1934) gives a Michaelis constant of 2 mM of casein phosphorus. This figure is somewhat lower than the value of 3 mM obtained by Norberg (1950) for rat-spleen phosphatase.

Optimum pH. The effect of pH on the rate of the enzymic hydrolysis of casein is shown in Fig. 3. The optimum pH lies in the region of pH 6·0. Hydrolysis is negligible below pH 4·5 and above pH 7.

Table 3. *Action of the purified enzyme on some phosphoproteins*

(0·1 ml. of the purified enzyme preparation was used for each experiment. All the substrates were present in a final concentration corresponding to 10 μmoles/ml. of organic P. Phosvitin and vitellin were weighed directly into the reaction tubes. Other substrates were added in the form of solution.)

Substrate	μg. of inorg. P liberated in 30 min. by Enzyme preparation *A*	Enzyme preparation *B*
Casein	121	130
Phosvitin	57	74
Vitellin	30	36
Phosphopeptone*	12	—

* Quantitatively freed from barium.

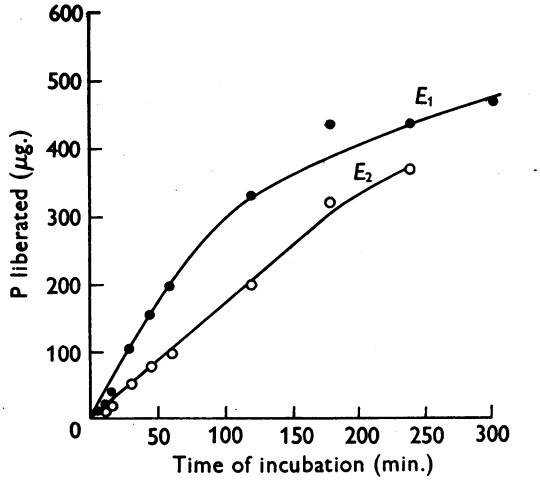

Fig. 1. Time/activity curves of phosphoprotein phosphatase. Conditions as in test mixture (see Experimental). E_1 and E_2 are curves for two different enzyme preparations having specific activities of 254 and 86 units, respectively. 0·1 ml. of the preparation was used for each experiment.

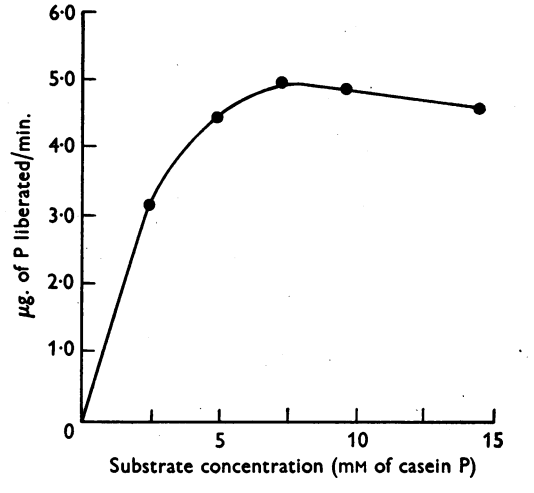

Fig. 2. Substrate concentration and enzyme activity. Conditions as in test mixture. 0·1 ml. of the purified enzyme was used for each experiment. Incubation time, 30 min. at 37°.

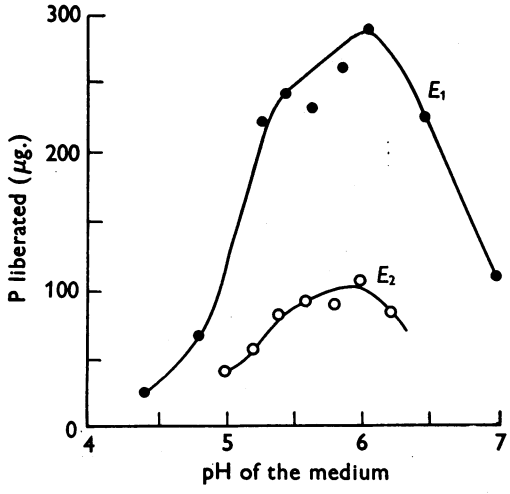

Fig. 3. Effect of pH on enzyme activity. Conditions as in 'test mixture'. 0·1 ml. of the purified enzyme was used for each experiment. Incubation time, 30 min. at 37°. E_1 and E_2 are curves for two different enzyme preparations.

Table 4. *Stability of the enzyme at various* pH *values*

(A sample (0·1 ml.) of the enzyme preparation mixed with buffer of the desired pH was incubated for 1 hr. at 37° with and without activator as indicated below. After readjusting the pH to 6·0, activities of the preparations were assayed as usual with casein as substrate.)

	Loss in activity (%)	
pH of incubation	Thioglycollic acid added before incubation	Thioglycollic acid added after incubation
5·0	1·0	0
6·0	18·6	0
7·0	64·3	24·3
8·0	82·9	32·9

Effect of pH *on stability of the enzyme*

The stability of the enzyme preparation at four different pH values has been studied. Two sets of experiments were carried out. In one series, the enzyme preparation, brought to the desired pH by means of buffer, was incubated in the presence of activator at 37° for 1 hr. In the second series the activator was added only after the completion of this preliminary incubation. The solutions were assayed for phosphatase activity as usual after bringing the pH back to 6·0. From the results presented in Table 4 it appears that the enzyme is more stable at pH 5 than at pH 6, and that it undergoes appreciable inactivation at pH 7 and above. Further, thioglycollic acid has no protective action when it is added before incubation. It can, however, bring about a marked reactivation when it is added after incubation.

Table 5. *Effect of metal ions on enzyme activity*

(0·1 ml. of the pure enzyme preparation was incubated with the indicated metal ion and buffer at pH 6 for 15 min. at 30°. Activity was then measured as usual with casein as substrate. No activator was added in the case of the heavy metal ions.)

Substance added	Molarity	Activation (+) or inhibition (−) (%)
NaCl	0·01	−3·0
K_2SO_4	0·01	−8·3
$MgCl_2$	0·01	−4·2
$MnSO_4$	0·01	+2·8
$CaCl_2$	0·01	−8·3
$BaCl_2$	0·01	+2·8
$CuSO_4$	0·01	−75·0
—	0·001	−65·5
—	0·00025	−69·0
Lead acetate	0·01	−65·5
$HgCl_2$	0·01	−28·0
$ZnSO_4$	0·01	−100·0

Activators and inhibitors of the enzyme

Effect of metal ions. The results summarized in Table 5 show the effect of various metal ions on the activity of the enzyme. Thioglycollic acid was used as an activator for the first six metal ions. No activator was added in the case of heavy metal ions, since if the latter act by combining with the —SH group of the enzyme protein, the presence of thiol substances will naturally counteract their action. From the results shown it will be seen that whereas none of the metal ions produce any appreciable activation of the enzyme, inhibition is observed with ions of all heavy metals. Copper and zinc are especially inhibitory, the former ion exerting its inhibitory effect even when present at a concentration of $2{\cdot}5 \times 10^{-4}$M. Inhibition at such low concentration suggests that the sulphydryl group of the enzyme protein may be the point of attack in these cases.

Effect of oxidizing and reducing agents. Feinstein & Volk (1949) have reported a marked activation of rat-spleen phosphoprotein phosphatase by reducing agents and an inhibition by oxidizing agents. Norberg (1950), however, could find no activation of the enzyme in the presence of 0·01M ascorbic acid. Table 6 gives a list of oxidizing and reducing agents employed in the present investigation. All these reagents were present in a final concentration of 0·001M and were not found to interfere in the colorimetric estimation of phosphorus. This was confirmed by carrying out the estimation in the absence of these reagents according to the method of Delory (1938).

From the results presented in Table 6, it will be seen that while cyanide produced an inhibition, all reducing agents produce considerable activation, the extent of activation depending upon the activity

of the enzyme preparation. Thus preparations having low activity are activated to a greater extent than the highly active ones. A reverse effect is observed with oxidizing agents which inhibit preparations of high activity to a greater extent. These results can be explained by the sulphydryl nature of the enzyme. Thus while the action of oxidizing agents consists in the oxidation of —SH to inactive —S—S— groups, a reverse effect is brought about by reducing agents. It may be further surmised that the degree of activation of the enzyme depends on the number of thiol groups present in the reduced state. The relative ease with which the enzyme is influenced by these various reagents shows that the active thiol groups of the enzyme protein are readily available for oxidation-reduction purposes.

Effect of some thiol- and amino-group inhibitors. The effect of some of these reagents on the activity of the enzyme is shown in Table 7. Inhibition is found to occur in all the cases. The inhibitory effects of alloxan, maleic acid and arsenite were completely abolished by the presence of 2 mg./ml. of cysteine in the reaction mixture. These substances thus seem to exert their effect by affecting the —SH group of the enzyme protein. Inhibition by formaldehyde and nitrous acid suggests further that amino groups may also be necessary for the activity of the enzyme.

Table 6. *Effect of oxidizing and reducing agents*

(0·1 ml. of the enzyme + buffer at pH 6 + the indicated substance in a final concentration of 0·001 M incubated at 30° for 15 min. After addition of substrate, incubation was continued at 37° for 30 min. with active enzyme preparations (E_1 and E_3), and for 60 min. in the case of weak enzyme preparation (E_2). Enzyme activity units are given in brackets. These units are expressed in terms of μg. of inorganic phosphorus liberated in 30 min. in the absence of activator.)

	Activation (+) or inhibition (−) (%)		
Substance	E_1 (56 units)	E_2 (17 units)	E_3 (133 units)
Oxidizing agents			
Ferricyanide	−87·5	−45·5	—
Hydrogen peroxide	−45	−29	—
Ferric chloride (0·01 M)	−100	−44	—
Reducing agents			
Ascorbic acid	+175	+508	+90
Cysteine	+97·5	+376	—
Thioglycollic acid	+107·5	+596	+92
Sodium cyanide	−47·5	−29	−32

Table 7. *Effect of some thiol- and amino-group inhibitors*

(0·1 ml. of the enzyme + buffer at pH 6 + indicated substance were incubated at 30° for 15 min. After addition of substrate, incubation was continued at 37° for 30 min.)

Substance	Molarity	Inhibition (%)
Alloxan	0·001	64
Maleic acid	0·001	42
Arsenite	0·001	38
Formaldehyde	0·01	38
Sodium nitrite	0·01	55

DISCUSSION

From the activation and inhibition studies it would appear that the enzyme is inhibited by heavy-metal ions, oxidizing agents and thiol inhibitors. Iodoacetic acid has been reported to be a strong inhibitor for the enzyme (Norberg, 1950). Further, the enzyme is activated to an appreciable extent by reducing agents. Put together, these facts point towards the indispensability of sulphydryl groups for the activity of the enzyme. No satisfactory metal-ion activator has so far been found for the enzyme. Furthermore the enzyme is unaffected by dialysis, and therefore no dialysable coenzyme seems to be necessary for enzyme activity.

It will be interesting at this stage to compare the properties of phosphoprotein phosphatase with that of the acid phosphomonoesterase occurring in ox spleen (Davies, 1934). Both exert their maximum activity at about pH 6 and have their maximum stability at pH 5–6. Magnesium has no action on either of them. However, although the former appears to be a sulphydryl enzyme, none of the phosphomonoesterases has so far been found to require sulphydryl groups for its activity. Moreover, it has been found possible in the present investigation to separate the two enzymes from each other to a certain extent (Table 2). Finally, the inability of our purified enzyme preparation to dephosphorylate phosphopeptone to any appreciable extent rules out the possibility of the enzyme being identical with any of the phosphomonoesterases which, as is known, hydrolyse phosphopeptone but not casein (Schmidt & Thannhauser, 1943; Perlmann, 1952). It is obvious, however, that the action of this enzyme on other phosphorus esters has to be studied before any conclusion can be drawn as regards its absolute specificity. Also the exact physiological role of the enzyme remains to be investigated. A preliminary step in this direction has been made by Norberg (1951).

SUMMARY

1. Phosphoprotein phosphatase has been shown, for the first time, to be present in cattle tissues.

2. A procedure has been worked out for the partial purification of the enzyme from ox spleen. Using a combination of ammonium sulphate- and acetone-fractionation procedures, a 200-fold purifi-

cation of the enzyme with an over-all yield of 20% was effected.

3. A purified preparation of the enzyme attacked all three phosphoproteins studied, but had no appreciable action on glycerophosphate or casein phosphopeptone. The enzyme is hence a true phosphoprotein phosphatase, distinct from the acid phosphomonoesterase.

4. Optimum enzyme activity was found at pH 6·0 and at a substrate concentration corresponding to about 10 μmoles/ml. of casein phosphorus with 0·001 M thioglycollic acid as activator.

5. From the activation and inhibition studies it is deduced that sulphydryl and amino groups are essential for the activity of the enzyme. However the enzyme requires no dialysable coenzyme for its activity.

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