ENZYMIC DEPHOSPHORYLATION OF PEPSIN AND PEPSINOGEN

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ABSTRACT

It has been shown by the work presented in this paper that it is possible to dephosphorylate enzymically pepsin and pepsinogen with a variety of phosphatases. With the aid of a phosphodiesterase and the prostate phosphatase it has been established that the phosphorus in the two proteins is present as a diester and connects two sites of the peptide chain in a cyclic configuration. Removal of the phosphorus does not affect the proteolytic activity against hemoglobin or the synthetic substrate acetyl-L-phenylalanyl diiodotryosine, nor the pepsinogen pepsin transformation. However, an increase of the autodigestion of pepsin is observed.

It has long been known that pepsin with a molecular weight of 35,000 contains one atom of phosphorus per mole (1, 2). Inasmuch as this protein is representative of a group of enzymes which do not contain a prosthetic (non-amino acid) group, the question naturally suggested itself to test whether the presence of the phosphorus is essential for the proteolytic activity of this enzyme. In view of the fact that the author has developed methods for the characterization of phosphorus bonds in phosphoproteins (3) it was decided to attempt the removal of the pepsin-phosphorus enzymically thus leaving the protein molecule relatively intact. Details will be given here of these experiments which were carried out from 1952 to 1954 but heretofore have been mentioned only briefly (3–5). At the outset of this report it can, however, be stated that although some of the physicochemical properties are altered during the dephosphorylation process, there is no evidence that the phosphorus is essential for proteolysis nor does its presence influence the activation of the protein from its inactive presursor, pepsinogen.

Materials and Methods

Materials.—The pepsin used in this research was the Worthington crystalline product and a highly purified, non-crystalline pepsinogen preparation kindly supplied by Dr. Roger M. Herriott of the School of Hygiene and Public Health of Johns Hopkins University.

The enzymes, *i.e.* a prostate phosphatase and the alkaline phosphatase from calf intestine whose purification has been adequately described elsewhere (6, 7), were furnished by Dr. Gerhard Schmidt of the Boston Dispensary. The potato phosphatase

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and the phosphodiesterase from rattlesnake venom, *Crotalus adamanteus*, were prepared in this laboratory according to the procedures of Kornberg (8) and Sinsheimer and Koerner (9), respectively.

Methods.—The concentration of all protein solutions has been determined from nitrogen analyses by the Pregl micro-Kjeldahl method using the factor of 6.78 for conversion to dry weight. The phosphorus was estimated according to Lohmann and Jendrassik (10).

Electrophoresis.—For the electrophoretic measurements a cell of 11 ml. capacity and the apparatus described by Longsworth were used (11). Prior to electrophoresis at 0.5°C. the 0.7 per cent protein solutions were dialyzed at 5°C. for 1 to 2 days against large volumes of the appropriate buffers.

Enzyme Experiments.—In the dephosphorylation experiments pepsin was dissolved in a buffer of the desired pH. After addition of phosphatase, in concentrations of 0.2 per cent of the total protein, the mixture was incubated at 37°C. for various lengths of time. In order to determine the proteolytic activity of the pepsin substrate as well as the amount of inorganic phosphorus released during the dephosphorylation one aliquot of the reaction mixture was assayed for activity with the aid of the hemoglobin method (12). In some experiments the hydrolysis of the synthetic substrate, *N*-acetyl-L-phenylalanyl diiodotyrosine, was tested (13). A second sample was mixed with an equal volume of 20 per cent trichloroacetic acid and immersed in a boiling water bath for 5 minutes. The protein precipitate was then separated by filtration and the filtrate analyzed for phosphorus, and in certain cases, for non-protein nitrogen.

RESULTS

Dephosphorylation of Pepsin and Pepsinogen by Various Phosphatases.—In the first series of experiments the various enzymes which effected the removal of the ovalbumin-phosphorus were tested for their action on pepsin and pepsinogen. As shown in Table I, only the potato enzyme, at pH 5.6, and the intestinal phosphatase at pH 8.9, dephosphorylate these proteins. The action of the intestinal enzymes, at pH 5.6 and 6.0, respectively, and that of the two prostate phosphatases are negligible. The prostate enzyme with the activity optimum of pH 3.0 has recently been discovered in this laboratory and, if tested with low molecular weight substrates, found to be specific for the hydrolysis of phosphomonoesters of the O-P type.

In view of the peculiar pH stability range of pepsin, *i.e.* below pH 6.0, these studies, therefore, were limited to the use of the potato phosphatase.

Activity of Pepsin and the Phosphorus-Free Pepsin.—Having thus demonstrated that the pepsin-phosphorus can be removed enzymically, the two proteins were assayed for activity. The results of these tests are recorded in Fig. 1. Here, the proteolytic activity, expressed as optical density at 280 mμ, is plotted against the amount of pepsin present in the reaction mixture. A straight line relationship is obtained. Fig. 1 further indicates that the activity of three different phosphorus-free pepsin preparations and that of the starting materials are indistinguishable.

As shown in Fig. 2, a similar result is obtained on comparison of the rate of hydrolysis of the dipeptide, *N*-acetyl-L-phenylalanyl diiodotyrosine.

Pepsinogen-Pepsin Transformation.—In contrast to pepsin, its inactive precursor pepsinogen is stable in the alkaline pH range. The removal of the pepsinogen-phosphorus was therefore carried out with the aid of the intestinal phosphatase. On subsequent activation of the phosphorus-free pepsinogen, by acidification of the solution to pH 2.0, it could be demonstrated that the activity of the enzyme was identical with that of the control sample.

TABLE I

Dephosphorylation of Pepsin and Pepsinogen by Phosphatases of Various Origin

Enzyme	pH of optimum activity	Maximal amount of phosphorus liberated	
		Pepsin	Pepsinogen
(1)	(2)	(3)	(4)
		Per cent of total	
Prostate phosphatase I	5.6	1.5	0
Prostate phosphatase II	3.0	9.0	Not tested
Intestinal phosphatase I	5.6	3.0	Not tested
	9.0	100.0	100.0
Intestinal phosphatase II	6.0	6.0	0
Potato phosphatase	5.6	99.0	96.0

Thus, from the foregoing it follows that the phosphorus in these two proteins is unessential for the proteolytic activity of pepsin and for the pepsinogen → pepsin transformation.

Electrophoretic Behavior of Pepsin and of Phosphorus-Free Pepsin.—In the dephosphorylation studies of ovalbumin and α-casein (14, 15) it has been demonstrated that the enzymic removal of as little as one phosphate group influences considerably the electrophoretic characteristics of the protein. Moreover, as will be discussed below, electrophoretic analysis over a wide pH range presents a qualitative picture of the type of groups involved. It, therefore, seemed of interest to compare the mobilities of pepsin and of the phosphorus-free protein at different pH values. The results of these measurements in monovalent buffers of pH 1.0 to 6.0 are presented in Table II. Here, columns 1 and 2 list the composition and the pH of the solvents at 25°C., whereas in columns 3 and 4 are given the mobilities of pepsin and the phosphorus-free protein. The six pepsin preparations studied in this laboratory migrated as a single peak in the pH range of 1.0 to 6.0. Moreover, as shown already by Tiselius, Henschen, and Svensson (16) and confirmed with our preparations, pepsin in 0.1 N hydrochloric acid at pH 1.08 still moves anodically. This unusual behavior indicates the presence of a strongly acidic group that is still dissociated at this pH. That this can be attributed to the phosphate group is evident

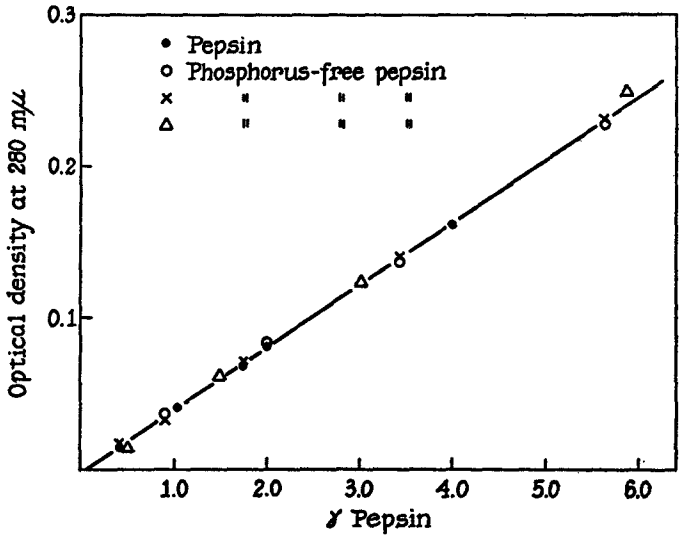


FIG. 1. Proteolytic activity of pepsin and phosphorus-free pepsin against hemoglobin as function of pepsin concentration.

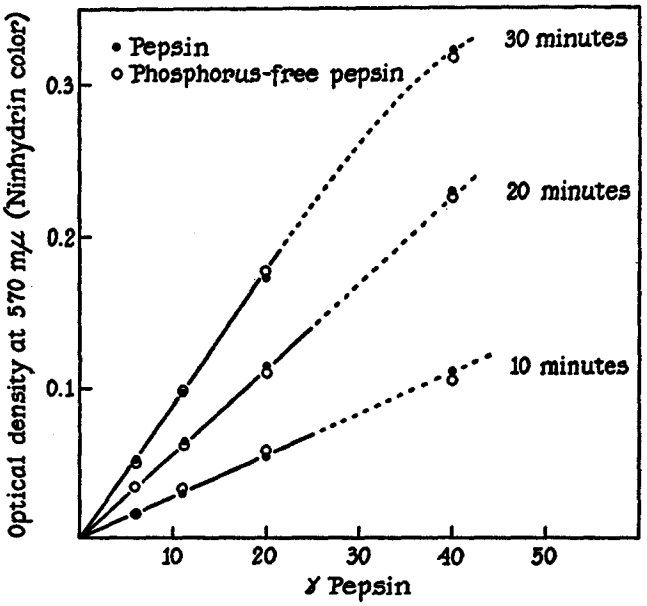


FIG. 2. Hydrolysis of *N*-acetyl-L-phenylalanyl diiodotyrosine by pepsin and phosphorus-free pepsin as function of pepsin concentration.

from the fact that the phosphorus-free pepsin, at pH 1.08 in 0.1 N hydrochloric acid is positively charged. Its isoelectric pH of 1.7 at 0.1 ionic strength was obtained by interpolation of several measurements in which the pH was varied from 1.1 to 2.5. If the mobilities of pepsin, (column 3), are compared with those of the phosphorus-free protein, (column 4), a constant difference, Δu, of 0.3 to 0.4 $\times$ 10^{-5} is obtained at all pH values below pH 5.0 (column 5).

TABLE II

Mobilities of Pepsin and Dephosphorylated Pepsin in Buffer Solutions of 0.1 Ionic Strength

Buffer	pH	Mobilities $\times 10^5$		Δu
		Pepsin	Phosphorus-free pepsin	
(1)	(2)	(3)	(4)	(5)
0.1 N HCl	1.0_8	−0.1	0.2	0.3
0.1 N HCl–0.1 M glycine	1.7_0	-0.3_5	0	0.3_5
0.1 N HCl–0.2 M glycine	2.5_0	−0.7	-0.3_2	0.3_8
0.1 N HCl–0.5 M glycine	3.0_5	−1.7	−1.3	0.4
0.02 N NaAc–0.1 N HAc–0.08 N NaCl	3.9_1	−4.1	-3.7_7	0.3_3
0.1 N NaAc–0.1 N HAc	4.6_4	-6.3_7	−6.1	0.2_7
0.1 N NaAc–0.01 N HAc	5.6_5	-9.1_8	−9.0	0.1_8
0.02 N NaCac–0.005 N HCac–0.08 N NaCl	5.7_2	−8.9	−8.6	0.3
0.02 N NaV–0.02 N HV–0.08 N NaCl	7.8_2	−7.8	-7.4_8	0.3_2

Ac = acetate; Cac = cacodylate; V = diethylbarbiturate.

Another point of interest emerges from the results given in Table II. On substitution of acetate by a cacodylate sodium chloride mixture of the same ionic strength and a similar pH, lower mobility values are observed in the cacodylate buffer. Likewise, in the pH range of 7.0 to 9.0 at which spontaneous inactivation (*i.e.*, denaturation) of pepsin occurs, a similar effect is noticed.[1] The mobility difference, Δu, of the pepsin and of the phosphorus-free protein, however, is identical with that given in Table II for the acidic buffers; *e.g.*, 0.3 to 0.4 $\times$ 10^{-5}. These results, therefore, can be taken as an indication that the interaction with buffer ions of these two closely related proteins is not affected by the presence of the phosphate group in the molecule but it is strongly influenced by the ionic composition of the solvent.

The Nature of the Phosphorus Bond of Pepsin.—As reported in a previous communication (14) the pH mobility curves of the three ovalbumins, A_1,

[1] Although pepsin in the pH range of 1.0 to 6.0 and 8.0 to 9.0, respectively, moves as a homogeneous protein, in the intermediate pH range it has three electrophoretic components. Similar results have been also obtained by Edelhoch (17).

A_2, and A_3, diverge until a constant mobility difference, $\Delta u = 0.6 \times 10^{-5}$, is found in the pH range of 7.0 to 9.0. If correlated with titration data, this mobility decrement at pH 7.0 indicates that on removal of each phosphorus atom of ovalbumin the net charge is altered by a value of -2, indicating that the phosphate groups of this protein are present in form of monoesters with two dissociable hydroxyls.

In contrast, as illustrated with the data given in Table II, the pH mobility curves of the two pepsins, although different from each other, do not diverge

TABLE III

Action of Prostate Phosphatase on Pepsin, Pretreated with Phosphodiesterase

Each reaction mixture contained 1 per cent pepsin, whereas the diesterase and prostate phosphatase content was 0.02 and 0.01 per cent, respectively.

Reaction mixture (1)	pH of reaction mixture (2)	Time of incubation at 37°C. (3)	Phosphorus released by enzyme (4)
		hrs.	*per cent*
Pepsin in 0.05 M $NaHCO_3$ + phosphodiesterase	8.2 → 7.8	3	0
Pepsin, phosphodiesterase-treated in acetate	5.8	12	0
Pepsin, phosphodiesterase-treated + prostate phosphatase	5.8	12	100
Pepsin in 0.05 M $NaHCO_3$, control	8.2 → 8.2	3	0
Pepsin (control) in acetate	5.8	12	0
Pepsin (control) + prostate phosphatase	5.8	12	0

at neutrality. These findings, taken together with the failure of the prostate and intestinal phosphatases to dephosphorylate pepsin at pH 5.6 and 6.0,[2] respectively, exclude the presence of a monoester of the O-P and N-P type (3) and suggest that the phosphorus of this protein occurs in the form of a diester with only one hydroxyl that can lose its proton. The diester nature of the phosphate group was confirmed as follows:—

If, as shown with the aid of Table III, pepsin in 0.1 N sodium bicarbonate is treated with a phosphodiesterase of snake venom, no inorganic phosphorus is set free. Since here the diester reaction is carried out in a weakly buffered solution, the shift of pH from 8.2 to 7.8 indicates the appearance of an acidic group. Subsequent exposure of the diesterase-treated pepsin to prostate phosphatase at pH 5.6 releases the pepsin-phosphorus. In view of the known specificity of these two phosphatases for the hydrolysis of O-P bonds when tested with low molecular weight substrates, one has to conclude that this diester is of the O-P-O type (3).

[2] The specificity of these enzymes for the hydrolysis of -O-P- and -N-P- monoesters has been discussed elsewhere (3).

As shown by the work of Williamson and Passmann (18), Van Vunakis and Herriott (19), and Heirwegh and Edman (20), pepsin is a protein with a single peptide chain. Therefore, the phosphorus must cross-link a portion of the chain into a cyclic loop. Hence, as illustrated in Fig. 3, if during the action of the

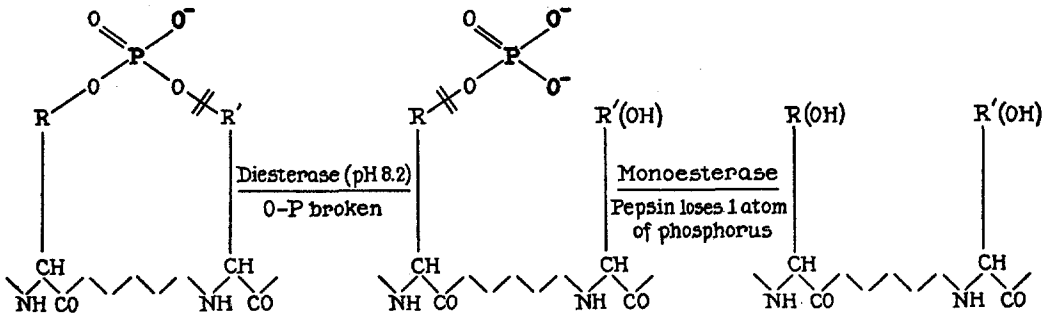
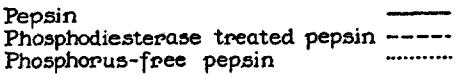


FIG. 3. Schematic presentation of stepwise dephosphorylation of pepsin.



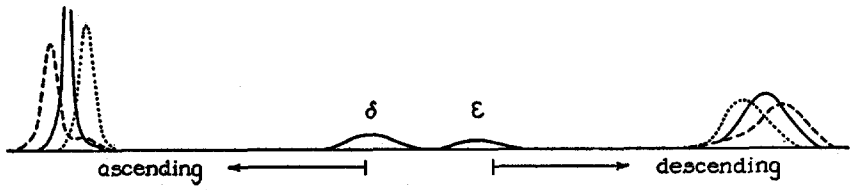


FIG. 4. Superimposed tracings of the electrophoretic patterns of pepsin, phosphodiesterase-treated pepsin, and phosphorus-free pepsin.

Electrophoresis was carried out at 1 per cent protein concentration in 0.1 N sodium bicarbonate at pH 8.2 for 9000 seconds at 6.5 volts per cm.

phosphodiesterase this cyclic bond is broken, the diester is converted into a monoester with the exposure of two acidic groups. Thus, it should be expected that in the pH range of 7.0 to 9.0 the diesterase-treated protein should move faster anodically than pepsin and the phosphorus-free protein more slowly. That this is the case is demonstrated with the aid of the superimposed tracings of the electrophoretic patterns obtained in 0.1 N sodium bicarbonate of pH 8.2 (Fig. 4). Here, the full line represents pepsin, the dashed line being the

pattern after treatment with the diesterase and the dotted one that of the phosphorus-free protein. From the relative position of these three peaks and the mobility values given in Table IV, it is clear that the mobility decrement is the same in each step; *i.e.*, $\Delta u = 0.3$ to 0.4×10^{-5} (columns 6 and 7).

In Table IV it is further shown that in an acetate buffer of pH 3.9 and 4.6, respectively, the mobilities of the diesterase-treated pepsin are identical with that of pepsin.[8] This result was to be expected in view of the fact that here the hydroxyl with a pK of 6.8 is undissociated and does not contribute to the net charge of the protein.

TABLE IV

Electrophoretic Mobilities of Diesterase-Treated Pepsin, Pepsin, and Phosphorus-Free Pepsin as Function of pH

Buffer	pH	Mobilities × 10^5			Δu	
		Diesterase-treated pepsin	Pepsin	Phosphorus-free pepsin	(3)-(4)	(5)-(4)
(1)	(2)	(3)	(4)	(5)	(6)	(7)
0.02 N NaAc–0.1 N HAc–0.08 N NaCl	3.9_1	-4.1_5	−4.1	-3.7_7	0	0.3_8
0.1 N NaAc–0.1 N HAc	4.6_4	-6.2_8	-6.3_7	−6.1	0	0.2_7
0.02 N NaV–0.02 N HV–0.08 N NaCl	7.8_2	−8.1	−7.8	-7.4_8	0.3	0.3_2
0.1 N $NaHCO_3$	8.2_2	−8.2	−7.9	−7.6	0.3	0.3

Ac = acetate; V = diethylbarbiturate.

Some Properties of the Phosphorus-Free Pepsin.—The most striking difference between the phosphorus-free pepsin and its parent substance is in the electrophoretic behavior. Nevertheless, it has not been possible to resolve a mixture of these two proteins into its components. Only one boundary is observed whose displacement at all pH values studied was intermediate to that of the two proteins. Resolution occurs, however, on mixing equal quantities of the phosphorus-free and diesterase-treated proteins. However, a comparison of the mobilities of the components of the mixture with the original materials reveals that also in this case protein-protein interaction has taken place.

Similarly, attempts to separate pepsin mixtures by chromatography have been unsuccessful. However, the following observations are of considerable interest. If pepsin is allowed to undergo autodigestion at pH values removed from those of optimal proteolysis, *i.e.* pH 1.5 to 2.5, and is subsequently passed through a column of 4 per cent linked Dowex 50, a newly formed component is

[8] In these experiments a phosphodiesterase prepared from saline extracts of prostate glands was used. This enzyme, in the presence of 10^{-4} M magnesium ions, is active at pH 5.8. Thus denaturation of pepsin was prevented.

isolated from the eluate. The amount of this second active component varies somewhat with the autodigestion. The eluate pattern of partially autolyzed, phosphorus-free pepsin is more complex in that it contains four to five components. Here too, a second active fraction is present with a slightly higher activity than that obtained from the partially autolyzed pepsin. From these observations it follows that the rate of autodigestion is higher in the case of the phosphorus-free protein than in the native protein.

DISCUSSION

In the present investigation it has been shown that the single phosphate group of pepsin and pepsinogen can be removed enzymically. Moreover, with the proper selection of two phosphatases, *e.g.* a diesterase from snake venom and the prostate phosphatase, it was demonstrated that the phosphorus in these two proteins is present in the form of a diester of the O-P-O type.[4] The occurrence of phosphodiesters in α- and β-casein has already been described. In these proteins the phosphorus serves to link peptide chains and its removal is accompanied by a disintegration of the protein into smaller units. In the case of pepsin, however, such a phenomenon is not observed. Here, the phosphorus serves to cross-link two sites of the peptide chain in a cyclic configuration and the experiments described here thus add a new type of intrachain bond to that of the disulfide linkages.

Although the removal of the phosphorus does not impair the peptic activtity nor affect the pepsinogen → pepsin transformation, some of the physicochemical properties have been altered and the rate of autodigestion of the phosphorus-free protein has increased considerably. It appears that the dephosphorylation process causes an opening of a cyclic loop in the peptide chain followed by a slight unfolding. A number of peptide bonds, hitherto not accessible to enzymic hydrolysis, are now exposed and are rapidly hydrolyzed. This could be demonstrated by an increased formation of trichloroacetic acid soluble nitrogen of the phosphorus-free pepsin and by a higher yield of dialyzable active fragments than has been found in the case of the parent protein (21). Therefore, it is not unlikely that the function of the phosphate group in pepsin is to stabilize the molecule and thus minimize its autodigestion.

The author is indebted to Dr. Gerhard Schmidt of the Boston Dispensary for generous samples of the prostate and intestinal phosphatases; to Dr. L. E. Baker for the synthetic substrate. My sincere thanks go to Mrs. Joan J. Berdick and Miss Barbara R. Hollingsworth for their assistance.

[4] As described by the author elsewhere (3) all potato phosphatase preparations contain small amounts of a diesterase active at pH 5.6 and specific for O-P bonds. Thus, the removal of the pepsin-phosphorus is due to a combined action of a mono- and diesterase.

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