The pH Dependence of the Dephosphorylated Pepsin-Catalyzed Hydrolysis of N-Acetyl-L-phenylalanyl-L-tyrosine Methyl Ester[1a]

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Abstract: The pH dependencies of the kinetic constants for the dephosphorylated pepsin catalyzed hydrolysis of N-acetyl-L-phenylalanyl-L-tyrosine methyl ester were determined in the pH range 1.12–5.39 at 25°. The dephosphorylated pepsin was prepared by enzymatically dephosphorylating pepsinogen with potato phosphatase at pH 5.60 and then activating this pepsinogen using the procedure of Rajagopalan, Moore, and Stein. The pH *vs.* k_{cat} curve is bell-shaped and depends on a pair of catalytic groups with pK_a's of 2.1 and 4.6. The catalytic behavior of this dephosphorylated pepsin is compared to that of "regular" pepsin. A discussion of the role of and type of bonding of this phosphate group in pepsin concludes the paper.

A previous report by us presented the kinetics of the pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe[2] at various pH's between 1 and 5.[3] The enzyme preparations used in these studies were commercial twice-crystallized pepsin and pepsin obtained from the activation of commercial pepsinogen. Both protein preparations were isolated from swine. However it has been reported recently that commercial swine pepsin and pepsinogen are heterogeneous preparations containing a few per cent of three similar but different proteins.[4] One of the main differences between pepsin and the minor component proteins, pepsins B, C, and D, is that pepsin contains a phosphate group whereas the minor proteins do not. In some very interesting studies, Perlmann[5] reported that enzymatically dephosphorylated pepsin was still catalytically active toward hemoglobin and Ac-Phe-I_2Tyr at pH 5.6. The aim of the present work is twofold. The first is to prepare a more homogeneous pepsin preparation by completely removing the phosphate group. Secondly, we wanted to obtain a clearer understanding of the role of the phosphate group in pepsin catalysis by studying the pH dependencies of the kinetic constants of the dephosphorylated pepsin catalyzed hydrolysis of Ac-Phe-Tyr-OMe.

Experimental Section

Materials. Pepsinogen, Ac-Phe-Tyr-OMe, and all buffer solutions have been previously described.[3] The concentrations of a protein solution were determined spectrophotometrically at 278 mμ assuming molar absorptivities of 50,900 for pepsin[6] and 56,000 for pepsinogen.[7] In this study we assumed that the molar absorptivities for pepsin and pepsinogen did not change upon dephosphorylation. The nonspecific phosphomonoesterase from potatoes (EC 3.1.3.2), potato phosphatase, was pepared from California-Long White Shafter potatoes according to the method of Hsu.[8] The only significant modification in our preparation was that during the tannic acid fractionation and subsequent heat denaturation; the phosphate activity was assayed using *p*-nitrophenyl phosphate (Sigma Chemical Co.). By using this rate assay sufficient tannic acid could be added to cause precipitation of protein with 20% loss in activity. This precipitated protein was filtered and discarded. To the filtrate was added additional tannic acid to cause further precipitation of protein with 90% loss in activity. This precipitated protein fraction was filtered and retained for further purification. During the final heat denaturation step, the protein solution was heated at 60° for just the length of time to allow 40–50% loss of activity as monitored by the rate assay. In general, the rate assay was carried out by adding 50 μl of phosphatase solution to 3.0 ml of a 4.4×10^{-4} *M* *p*-nitrophenyl phosphate solution dissolved in a 0.2 *M* (pH 5.0) acetate buffer. The enzymatic production of *p*-nitrophenol was followed for *ca.* 5 min at 340 mμ on a Cary 14 recording spectrophotometer. A blank solution of *p*-nitrophenyl phosphate was required for the reference cell. The weight of lyophilized potato phosphatase obtained from 20 lb of potatoes was 150 mg. A 50-μl aliquot of a solution of potato phosphatase (1.0 mg protein/ml) gave a rate in the preceding assay of 1.25×10^{-3} absorbance units/sec. The absorbance of this protein solution (1 mg protein/ml) was 1.175 at 280 nm and the over-all purification factor was 140.

Determination of Inorganic Phosphate. The method of Chen and coworkers[9] for the determination of inorganic phosphate in urine samples was slightly modified for our use with pepsinogen and pepsin. Their reagent C was prepared by mixing 1 vol of 6 *N* sulfuric acid with 2 vol of distilled water, 1 vol of 2.5% (w/v) ammonium molybdate and 1 vol of 10% (w/v) ascorbic acid. This reagent was prepared fresh every 4 hr and each individual component solution was prepared fresh every week. The standard curve of moles of phosphate *vs.* absorbance at 820 mμ of the reduced phosphomolybdate complex is shown in Figure 1. This curve was determined as follows: to 1 ml of 10^{-4} *M* pepsin solution was added 1 ml of a standard phosphate solution and 1 ml of 20% (w/v) trichloroacetic acid. This mixture was heated at 60° for 15 min to effect denaturation of the pepsin, and cooled and centrifuged at 1500 rpm for 10 min. The liquid containing the inorganic phosphate was decanted and a 2-ml aliquot was added to 3 ml of reagent C and 1 ml of water. This final solution was thoroughly mixed, placed in a constant temperature bath at 36° for 2 hr to develop the blue color and read without further dilution at 820 mμ. The substitution of pepsinogen for pepsin had no effect on the production of the blue phosphomolybdate complex but did require a higher denaturation temperature of 85°.

Preparation of Dephosphorylated Pepsin. The dephosphorylated pepsin used for the kinetic studies was always obtained from pep-

(1) (a) This research was supported in part by Grant No. GM 12022 of the National Institutes of Health and the New York State Research Foundation; (b) author to whom all inquiries should be addressed at the Department of Chemistry, Kenyon College, Gambier, Ohio 43022; (c) National Science Foundation undergraduate summer research participants.

(2) Abbreviations used: Phe, L-phenylalanine; Tyr, L-tyrosine; I_2Tyr, L-diiodotyrosine; Ser, L-serine; Ala, L-alanine; Leu, L-leucine; Glu, L-glutamic acid; Thr, L-threonine; Ac, acetyl; OMe, methyl ester.

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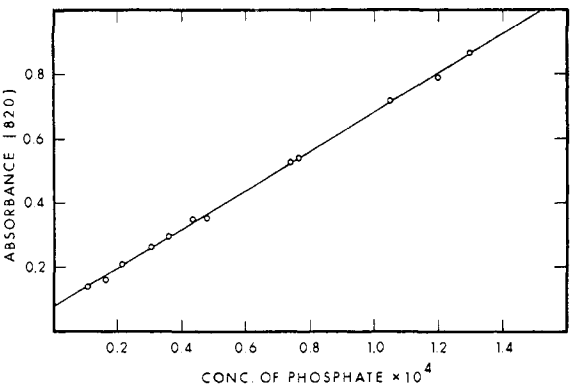

Figure 1. Standard curve for the determination of inorganic phosphate liberated during the enzymatic dephosphorylation of pepsinogen.

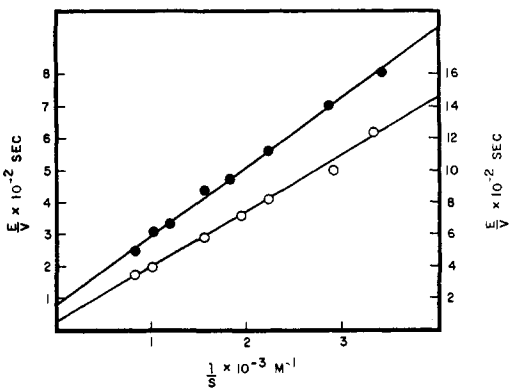

Figure 2. Typical Lineweaver-Burk plots for the deposphorylated pepsin catalyzed hydrolysis of Ac-Phe-Tyr-OMe: pH 3.41 (○), left-hand scale; pH 5.39, (●), right-hand scale.

sinogen. Our initial approach was to prepare pepsin from pepsinogen using the method of Rajogopalan[10] and then dephosphorylate this pepsin with potato phosphatase in a pH 5.0 acetate buffer at 36° for 19 hr. However, these dephosphorylated pepsin solutions gave erratic kinetic results indicating that significant denaturation of pepsin had occurred during the time required for dephosphorylation. This method was abandoned and all dephosphorylated pepsin used for the kinetic studies reported herein was produced by the following method.

Approximately 85 mg of pepsinogen and 4 mg of potato phosphatase were dissolved in 9 ml of a 0.03 *M* (pH 5.6) acetate buffer. The solution was placed in a water bath at 36° for exactly 19 hr. The concentration of inorganic phosphate was determined on a 1-ml aliquot, using the previously described procedure and Figure 1, by substituting 1 ml of water for the 1 ml of standard phosphate solution. The 19-hr dephosphorylation period was determined to be a convenient time to observe as much dephosphorylation of pepsinogen as was going to occur. The yield of inorganic phosphate was always 86–90% based on the known concentration of pepsinogen. This same 86–90% yield of phosphate was also observed when either commercial twice-crystallized pepsin or pepsin obtained from pepsinogen were enzymatically dephosphorylated. The less than 100% inorganic phosphate liberation is consistent with the work of Ryle[4] who showed that both commercial pepsinogen and pepsin preparations contain 5–10% of pepsins with no phosphate group. After the 19-hr dephosphorylation period, the dephosphorylated pepsinogen was activated to pepsin, purified according to the method of Rajogopalan,[10] and used directly for the kinetic experiments.

Kinetics of Dephosphorylation. The rate of dephosphorylation at 36° of 40 ml of a 6×10^{-5} *M* pepsin solution at pH 5.0 containing 4 mg of potato phosphatase was measured. The results, when plotted as a first-order reaction, were linear to 3 half-lives giving a rate constant for dephosphorylation of $7.1 \pm 0.7 \times 10^{-3}$ min^{-1}. A similar kinetic study of a 6×10^{-5} *M* pepsinogen solution at pH 5.6 containing 5 mg of potato phosphatase yielded a first-order plot linear over 4 half-lives with a rate constant of $7.9 \pm 0.6 \times 10^{-3}$ min^{-1}.

Lineweaver-Burk Plots and Kinetic Schemes. The initial velocities (rates) were determined by the previously described[3] automatic ninhydrin method of Lenard and coworkers.[11] The kinetic parameters listed in Table I for the dephosphorylated pepsin catalyzed hydrolysis of Ac-Phe-Tyr-OMe were obtained from this initial rate data by applying a least-squares computer program to the Lineweaver-Burk[12] form of the Michaelis-Menton equation. The error in k_{cat} reflects the error in the intercept and the error in K_m is a summation of the errors in the slope and intercept. A weighted least-squares analysis[13] of the Lineweaver-Burk plots produced excellent agreement with the simple least-squares analysis. Figure 2 shows typical Lineweaver-Burk plots for the dephosphorylated pepsin-catalyzed hydrolysis of Ac-Phe-Tyr-OMe at pH's 3.41 and 5.39. The pH-k_{cat} bell-shaped curve shown in Figure 3 was

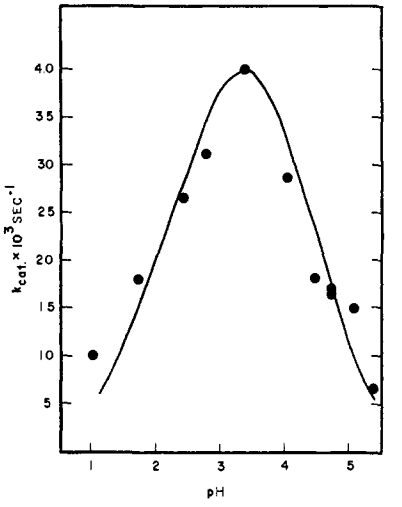

Figure 3. The pH-k_{cat} profile for the deposphorylated pepsin catalyzed hydrolysis of Ac-Phe-Tyr-OMe in 3.03% (v/v) dioxane-water.

determined according to procedures described in Dixon and Webb[14] and eq 1.

$$k_{cat} = \frac{k_{cat}(\text{limited})}{1 + H/K_1' + K_2'/H} \quad (1)$$

Amino Acid Analysis of the Dephosphorylated Pepsin. The dephosphorylated activated pepsin that was obtained directly from the SE-Sephadex column in a pH 4.4 acetate buffer was placed in ampoules which were diluted with the appropriate quantity of concentrated hydrochloric acid to produce a 6 *N* acid solution. The ampoules were sealed under vacuum in a nitrogen atmosphere and heated at 110° for 24, 48, and 72 hr. Duplicate amino acid analyses of the pepsin hydrolysates were obtained on a BIOCAL BC 200 automatic analyzer. Except for serine, the amino acid analyses of these dephosphorylated pepsin hydrolysates agreed with the amino acid composition of pepsin as reported by Rajogopalan.[10] The serine analysis was 5–6 residues low presumably due to extensive decomposition during the acid hydrolysis.

Activity of the Enzymes. The pepsin obtained by the activation of pepsinogen and the dephosphorylated activated pepsin were assayed with hemoglobin at pH 1.8 using a modified procedure.[3,10] Both pepsin preparations gave the same activity toward hemoglobin under these assay conditions. The phosphodiesterase activity of the potato phosphatase was assayed by a spectrophotometric method at pH 5.6 using adenylyl-(3¹–5¹)-adenosine as the substrate.[15] The potato phosphatase showed no diesterase activity toward this substrate.

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Table I. The Kinetics of the Dephosphorylated Pepsin Catalyzed Hydrolysis of Ac-Phe-Tyr-OMe in Aqueous Media[a]

pH	$k_{cat} \times 10^3$ sec^{-1}	$K_m \times 10^3$ M	Buffer
1.12	10.0 ± 1.4	1.5 ± 0.3	HCl
1.74	17.9 ± 2.0	2.0 ± 0.3	HCl
2.46	26.3 ± 4.0	3.1 ± 0.6	Phosphate
2.79	31.1 ± 9.4	3.7 ± 1.4	Phosphate
2.79	31.4 ± 10.1	3.7 ± 1.4	Citrate
3.41	40.0 ± 12.7	6.9 ± 2.5	Citrate
4.05	28.4 ± 5.8	5.3 ± 1.3	Citrate
4.47	18.0 ± 2.0	3.6 ± 0.5	Acetate
4.74	17.1 ± 5.6	4.5 ± 1.6	Acetate
4.74	16.3 ± 2.0	4.3 ± 0.7	Acetate
5.10	15.0 ± 5.7	5.1 ± 2.0	Acetate
5.39	6.2 ± 2.7	2.7 ± 0.3	Acetate

[a] 3.03 % (v/v) dioxane-water; 25.0°; $[E_0] = 2\text{–}6 \times 10^{-8}$ M, $[S_0] = 0.26\text{–}1.3 \times 10^{-3}$ M.

Discussion

In a series of papers, Ryle and coworkers[4] have isolated and characterized a number of minor pepsinogens from pig gastric mucosa. Pepsin B, obtained by activation of pepsinogen B, possesses activity with Ac-Phe-I_2Tyr but little activity with hemoglobin. The zymogen contained two components, by electrophoreses, one of which probably contains a phosphate group. Pepsinogen C and pepsin C contain no phosphate group and pepsin C exhibits activity with the substrate, hemoglobin. The amino acid analyses of both these proteins show some differences in amino acid composition from the major proteins isolated from gastric mucosa, pepsinogen A and pepsin A. Both pepsinogen A and pepsin A contain a phosphate group. More recently, these same workers[4] found that commercial pepsinogen contains *ca.* 5% of a minor zymogen, pepsinogen D, which is similar to if not identical with dephosphorylated pepsinogen A. It was also shown that commercial pepsin contains a pepsin D which accounts for *ca.* 10% of the enzymatic activity of the commercial preparation. This pepsin D shows activity with hemoglobin and Ac-Phe-I_2Tyr and probably arises from activation of pepsinogen D. The presence of pepsinogen D in the commercial preparation accounts for our observation that phosphate liberated from pepsinogen by the phosphatase is only 86–90% of the theoretical amount based on the known pepsinogen concentration (*cf.* Experimental Section).

In order to determine the effect of removing the phosphate group on pepsin activity and to gain additional information on the type of phosphate bonding in pepsin, monoester *vs.* diester, we have prepared dephosphorylated pepsin. This dephosphorylated pepsin should be a more "homogeneous" preparation for use in the kinetic studies. The bell-shaped pH–k_{cat} profile for the dephosphorylated pepsin catalyzed hydrolysis of Ac-Phe-Tyr-OMe is shown in Figure 3. A similar bell-shaped curve was obtained for the regular pepsin-catalyzed hydrolysis of this same substrate.[3] The limiting rate constants and pK_a's associated with the catalytic groups controlling the dephosphorylated pepsin and regular pepsin-catalyzed hydrolyses of Ac-Phe-Tyr-OMe are summarized in Table II. A comparison of the k_{cat}(lim) shows that the dephosphorylated activated pepsin is 89% as active as the regular activated pepsin. Perlmann[5] has also observed that the removal of the phosphate group does not reduce pepsin activity toward hemoglobin and Ac-Phe-I_2Tyr. A further examination of Table II shows that the pK_1' and pK_2' have increased 0.5 pK_a unit in going from activated pepsin to dephosphorylated activated pepsin. Denburg and coworkers[16] found pK_1' and pK_2', respectively, to be 1.35 and 4.15 for the twice-crystallized pepsin-catalyzed hydrolysis of a neutral substrate, Ac-Phe-Tyr-NH_2. If one considers that the errors in the pK_a's are of the order of ±0.2 pK_a units, then the pK_a's of the catalytically important groups in dephosphorylated pepsin have not been significantly altered. These small shifts in pK_a's are probably due to small changes in the conformation of pepsin upon dephosphorylation. We have interpreted the small decrease in k_{cat}(lim) 10% and the small shift in pK_1' and pK_2' with dephosphorylation of pepsin to mean that the phosphate group in pepsin is *not* involved in its catalytic process (mechanism of action). In addition, the removal of the phosphate group also has no apparent effect on the enzyme–substrate binding constants. The K_m's for the "regular" and dephosphorylated pepsin binding of Ac-Phe-Tyr-OMe do not vary by more than a factor of 2.[3] Finally, we can say that the abnormally low pK_a's (in the range 1–2) observed for the left-hand side of the pH–k_{cat} profiles cannot be due to the ionization of a phosphate group.

Table II. Limiting Rate Constants and pK_a's Associated with the Catalytic Groups Controlling the Pepsin-Catalyzed Hydrolysis of Ac-Phe-Tyr-OMe

Pepsin preparation	k_{cat}(lim) × 10^3 sec^{-1}	pK_1'	pK_2'
Twice crystallized pepsin[a,b]	24.6 ± 3.6	1.6	3.5
Activated pepsin[b,c]	51.9 ± 7.0	1.6	4.1
Dephosphorylated activated pepsin[d]	45.1 ± 8.1	2.1	4.6

[a] The pepsin was a commercial twice-crystallized preparation. [b] Reference 3. [c] This pepsin was obtained from the activation of pepsinogen. [d] This work.

It has been known for some time that the major component proteins of commercial pepsinogen and pepsin contain a phosphate group presumably bonded in a diester linkage.[5] This phosphate group is bonded to a serine contained in the following peptide sequence:[17] Glu-Ala-Thr-Ser-P-Glu-Glu-Leu. However, we feel that the following data provides evidence that the phosphate group in pepsin could be in the *monoester* form.[18]

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(18) The arguments found in ref 5 which support the conclusion that the phosphate group in pepsin is in a diester linkage are as follows. (a) Incubation of pepsin with a phosphodiesterase from rattlesnake venom, *Crotalus adamanteus*, does not liberate phosphate. However, when this mixture is subsequently treated with prostate phosphatase, exhibiting no diesterase activity, 1 mol of phosphate is liberated. Direct incubation of pepsin with prostate phosphatase results in the liberation of only a few per cent of the total phosphate. (b) A comparison of the electrophoretic mobility of pepsin with dephosphorylated pepsin indicates a net change of one unit of charge as would be expected for a diester phosphate linkage. (c) Finally, the 100% release of phosphate obtained by treatment of pepsin with potato phosphatase was attributed to small amounts of diester active enzyme(s). Alternatively, we have found that highly purified potato phosphatase exhibiting little or no diesterase activity cleanly removes the phosphate group from pepsin and pepsinogen. At this time we are not able to rationalize these two divergent conclusions. When the entire linear sequence of amino acids in pepsin is established and the bonding of the phosphate group is known, this point will be resolved.

(a) The removal of the phosphate group results in only small changes in the k_{cat}(lim) and pK_a's for the pepsin-catalyzed hydrolysis of one neutral substrate, Ac-Phe-Tyr-OMe. If the phosphate group were in the diester linkage, and therefore expected to function like a disulfide bridge to connect two different sections of the enzyme, one might expect that its removal would have a greater effect on pepsin catalysis due to a major change in the conformation of the enzyme. Also, the removal of the phosphate group did not diminish pepsin's activity toward hemoglobin at pH 1.8 (this work) and pH 5.6.[5]

(b) Only one phosphorus-containing amino acid sequence has been isolated from the hydrolysis of pepsin.[17,19] Because of the hydrolysis procedures employed for this sequence analysis, it could be argued that the diester linkage was destroyed during the hydrolysis and purification steps. Alternatively, one could argue that if a diester phosphate linkage were to be observed, it should be stable to the mild conditions employed for this sequence analysis. These conditions were enzymatic digestions of the reduced carboxymethylated pepsin, followed by ion exchange chromatography, paper electrophoresis, and paper chromatography.

(c) The phosphate groups in pepsin and pepsinogen were removed by a potato phosphomonoesterase. In this work it has been shown that the potato phosphatase will not catalyze the hydrolysis of adenylyl-(3^1–5^1) adenosine while Hsu[8] reported that it had no alkaline pyrophosphate activity nor phosphodiesterase activity as assayed at pH 5.0 and 8.7 with *p*-nitrophenylthymidine 5^1-phosphate.[20]

At this point, it is not clear whether the phosphate group in pepsin serves any function at all. The phosphate group is not involved in the activation of pepsinogen to pepsin as shown by the identical amino acid analysis of dephosphorylated activated pepsin and "regular" activated pepsin. It has been reported not to be in the 41 end amino-terminal amino acids of pepsinogen[21] nor in the 27 carboxyl-terminal end residues of pepsin which do not contain a serine.[22] It is probably on the surface of the pepsin molecule since it is a polar group and easily removed by the potato phosphatase. Finally, the phosphorylated pepsinogen and pepsin is a homogeneous preparation (at least with respect to the environment of the phosphate group) as evidenced by the almost identical first-order kinetics of dephosphorylation (*cf.* Experimental Section). In summary we have presented evidence that the phosphate group in pepsinogen and pepsin could be in the monoester linkage and is *not* involved in the mechanism of pepsin action.

Previously we reported[5] that the k_{cat}/K_m–pH profiles for the commercial twice-crystallized pepsin and pepsin obtained from the activation of pepsinogen-catalyzed hydrolysis of Ac-Phe-Tyr-OMe were slightly curved with a maximum around pH 2.5 and flat, respectively. Alternatively, Denburg[16] reported an excellent bell-shaped pK–k_{cat}/K_m profile for the twice-crystallized pepsin-catalyzed hydrolysis of Ac-Phe-Typ-NH_2. We hoped that the preparation of a more "homogeneous" pepsin, by removing the phosphate group, would produce some insight into this observed difference in k_{cat}/K_m with this type of pepsin. The k_{cat}/K_m data which can be obtained from Table I, generally do increase with increasing pH. However, it is still not clear whether the k_{cat}/K_m data decrease at low pH for the dephosphorylated pepsin. Since the k_{cat}/K_m–pH data for the dephosphorylated pepsin catalyzed hydrolysis of Ac-Phe-Tyr-OMe produced a curve which was neither flat nor bell shaped, we have concluded that the presence or absence of the phosphate group in the previously used pepsin preparations does not account for the different pH–k_{cat}/K_m curves.

Acknowledgment. The authors thank Professor F. J. Kézdy for many suggestions regarding the preparation of the potato phosphatase and Mr. Robert Heindrickson of the University of Chicago for the amino acid analyses.

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(20) A sample of potato phosphomonoesterase (kindly supplied by Dr. Kézdy) removed the phosphate group from pepsin and pepsinogen with the same efficiency as the potato phosphatase prepared by us for this work. In addition, Dr. Kézdy has furnished us with a sample of potato phosphomonoesterase with additional DEAE cellulose chromatogaphic purification (over 1000-fold purification factor) which also cleanly removes the phosphate group with 90% phosphate liberation.

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