Inactivation of an Alkaline Bacillopeptidase by Pentasodium Triphosphate

W. SCHREIBER, Biochemische Laboratorien,
Henkel and Cie GmbH, Düsseldorf, Germany

ABSTRACT

The inactivation of an alkaline microbial protease by pentasodium triphosphate (TPP) follows first order kinetics, the apparent activation energy being the same from pH 7-10 with and without added TPP. Almost exclusively low molecular peptides were observed as reaction products.

INTRODUCTION

Pancreatic enzymes, formerly contained in enzymatic detergents, have been replaced totally by enzymes of microbial origin. The reasons for this are the better stability of microbial enzymes under washing conditions and their easier production on the technical scale by submersed culture methods. Most of these enzymes used in detergents are bacterial exoproteases (1) and are produced using organisms of the genus bacillus. They are similar in their characteristics to subtilopeptidase A (EC 3.4.4.16), first isolated by Güntelberg and Ottesen (2). Proteases of some fungi, e.g., genus *aspergillus* or *streptomyces*, also seem to be suitable for industrial use. These proteases fulfill the requirements of the detergent producers: they have an optimum in the alkaline pH range, are stable for a sufficiently long time up to 60 C, and are compatible with most surfactants. However under special conditions not identical to those experienced during the washing process, these proteases too may be inactivated to a substantial degree by some agents found in most surfactants. These are: (a) chelating agents of organic, e.g., EDTA, or inorganic nature (pentasodium triphosphate); (b) anionic surfactants with sulfate or sulfonate groups; and (c) oxidants such as perborate or chlorine-producing substances (1,3-6). The examination of the inactivation of the proteases by these agents has been performed up to now almost exclusively under standardized conditions with a fixed incubation time. We felt that a kinetic investigation of the inactivation reaction would bring about a better understanding of its mechanism and therefore undertook an examination of the inactivation of an alkaline bacillopeptidase of the serine type (7) from a strain of *Bac. subtilis* by pentasodium triphosphate (TPP), which constitutes as a builder a main ingredient of modern presoak and heavy duty detergents.

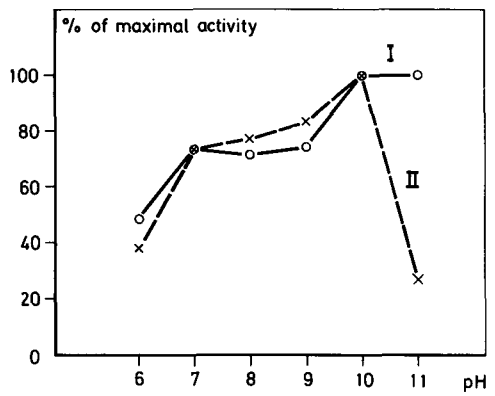

FIG. 1. pH profile of the alkaline bacillopeptidase, incubating for 15 min at 50 C without (I) and with previous incubation in 5 mM EDTA for 3 min at 35 C (II), 30 μg protein per milliliter.

MATERIALS AND METHODS

Substances

The alkaline protease was isolated by precipitation with Na_2SO_4 from filtered fermenting broths of a *Bac. subtilis* strain and was used without further purification. TPP was of technical grade (Thermphos NW, Knapsack Griesheim) and contained minor amounts of higher and lower condensed phosphates. All other substances were of analytical grade.

Gel Chromatography

Using sephadex gels and columns from Pharmacia, a Uvicord photometer and an Ultrorac fraction collector from LKB Produkters, we followed standard procedures for gel chromatography (8). Solutions were preserved by addition of 0.01% merthiolate. The buffer used was 0.2 M phosphate, its pH being 6.5.

Proteolytic Assay

A procedure analogous to that published by van Raay et al. (9) was used. It consisted of the incubation of solutions of protease (5 ml) with casein (15 ml) at pH 8.5 and 50 C; precipitation of undigested casein by trichloracetic acid-acetic acid-sodium acetate (15 ml); and spectrophotometry of the filtrate in the UV range. The concentrations of protease in the kinetic runs were such that the samples withdrawn could be directly used for the enzymatic assay. The substrate solution was prepared by dissolving 6 g casein with 50 ml 0.3 M tris buffer of pH 8.5 in a synthetic tap water of 15 deg german hardness at 50-70 C, adjusting to pH 8.5 and filling up to 500 ml. The synthetic tap water was 1.975 mM in $CaCl_2$ and 0.69 mM in $MgCl_2$, the precipitating reagent was 0.44 M in trichloracetic acid, 0.51 M in acetic acid, and 0.22 M in sodium acetate.

The amount of proteolytic activity in the sample is calculated by inserting the difference of the optical densities at 275 and 300 nm (ΔE) in the formula: ΔE x 20 = activity (PE/ml). Hence one proteolytic unit (PE) is one-fiftieth of the amount of protease contained in a sample of 5 ml, which will give a ΔE of 0.5 (9). With kinetic runs at pH 10 it was necessary to adjust the substrate solution to pH 8.2 in order to reach pH 8.5 in the enzymatic assay.

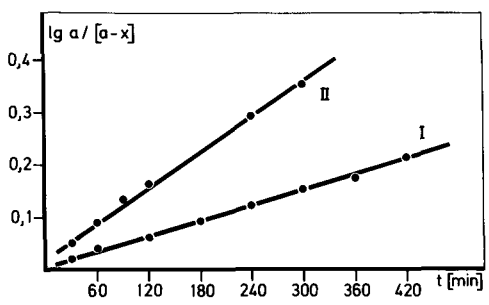

FIG. 2. Inactivation kinetics of an alkaline bacterial protease by 0.2% pentasodium triphosphate at pH 10 and 30 C (I) and 33 C (II). Ca^{2+}- and Mg^{2+}-concentrations 0.66 mM and 0.23 mM, respectively, 10-40 μg protein per milliliter.

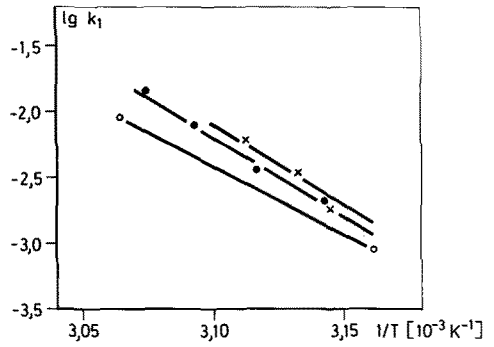

FIG. 3. *Arrhenius* plots for the inactivation of alkaline bacterial protease at various pH values in absence of pentasodium triphosphate (o—o = pH 7; •---• = pH 9; x - - - x = pH 10). Ca^{2+}- and Mg^{2+}-concentrations 0.66 mM and 0.23 mM, respectively, 10-40 μg protein per milliliter.

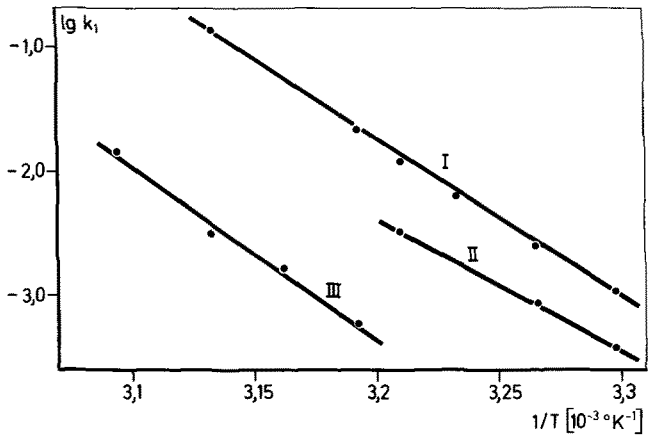

FIG. 4. *Arrhenius* plots for the inactivation of alkaline bacterial protease by 0.2% pentasodium triphosphate at various pH values (I = pH 10; II = pH 9; III = pH 7). Ca^{2+}- and Mg^{2+}-concentrations 0.66 mM and 0.23 mM, respectively, 10-40 μg protein per milliliter.

Optimum-pH

The optimum-pH of the protease was measured by incubating a protease solution with a set of casein solutions, which were prepared by dissolving 6 g casein in 500 ml of various buffers (10). The molarity of the buffers was 0.08-0.12 M; their ionic strength had been adjusted to 0.3 by addition of NaCl. The incubation time was 15 min and the incubation temperature 50 C, all pH values being measured at this temperature. The volumes of the samples, of the substrate solution and the precipitating reagent, their composition, and the mode of measurement and calculation were the same as in Proteolytic Assay.

Kinetic Experiments

Ten milliliters enzyme solution, 10 ml buffer and 10 ml of a 0.6% TPP solution were mixed and incubated in a constant temperature bath. The enzyme solution and the buffers were prepared using distilled water, whereas the TPP solution was prepared using the described synthetic tap water (9). Concentration of calcium and magnesiums ions in the inactivation mixtures were hence 0.66 mM and 0.23 mM, respectively; concentration of TPP, if present, was 5.44 mM. Buffers used at pH 9 and 10 were prepared by mixing 4 mM $NaHCO_3$ and 4 mM Na_2CO_3. At pH 7 equal amounts of a 4 mM solution of $NaHCO_3$ and of 0.6% TPP were adjusted with HCl. All pH values were measured at the incubation temperature. Samples of 5 ml withdrawn at fixed intervals were analyzed for enzymatic activity as described under Proteolytic Assay.

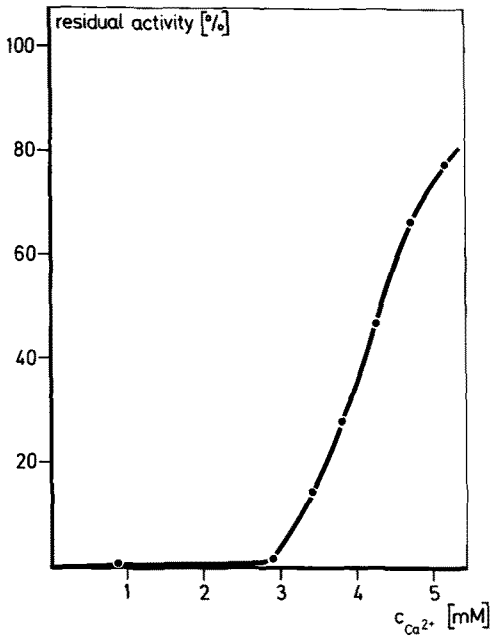

FIG. 5. Residual activity of alkaline bacterial protease after incubation with 0.2% pentasodium triphosphate for 30 min at pH 10 and 50 C, adding various amounts of $CaCl_2$ x 2 H_2O, 15 μg protein per milliliter.

Preparative Inactivation of the Protease

Six-tenths gram protease with 700 PE/mg were incubated at pH 10/50 C for half an hour in 60 ml of a 0.2% TPP solution in presence of 0.66 mM Ca^{2+} and 0.23 mM Mg^{2+}. A minor amount of residual activity (2% of the original value) disappeared after adding a solution of 50 mg phenylmethanesulfonylfluoride in 5 ml acetone.

RESULTS

Nature of the Protease

By gel chromatography the molecular weight of the protease has been determined as ca. 24,000; the protease is inhibited by phenylmethanesulfonylfluoride and shows no serious changes of its pH profile by previous incubation of its solutions with EDTA (Fig. 1). These properties, the shape of its pH profile, and its optimum-pH lead to the assumption that this protease belongs to the group B of alkaline bacillopeptidases in the nomenclature of Keay et al. (11).

Kinetics of Inactivation

The results of the inactivation experiments, performed at low protein concentrations (10-40 μg/ml) and in presence of 0.66 mM Ca^{2+} and 0.23 mM Mg^{2+}, were plotted as log $a/(a-x)$ against t, an example being given in Figure 2. The linearity of these graphs up to 50-75% reaction is compatible with pseudo first order kinetics (12). Using regression analysis we calculated from the slope of these lines the first order rate constants (13), their values being given in Table I. They increase with increasing pH and temperature. *Arrhenius* plots (14) of the different sets of rate constants indicate that in all our experiments the resulting lines have almost the same slope (Figs. 3,4).

Gel Chromatography of Inactivation Products

We separated the mixture of enzyme and its low molecular fission products produced on inactivation with TPP by chromatography on a column of Sephadex Gel 25, which was standardized by chromatography of marker proteins such as bovine serum albumine, ovalbumine,

TABLE I

First Order Rate Constants for the Inactivation of Alkaline Bacillopeptidase

In absence of TPP[a]			In presence of 0.2% TPP[a]		
pH	Temperature, C	$10^3 \times k_1$ (min^{-1})	pH	Temperature, C	$10^3 \times k_1$ (min^{-1})
7	43	0.88	7	40	0.63
	53	9.1		43	1.6
9	33	0.55		46	3.4
	45	2.1		50	14.6
	47.5	3.6	9	30	0.38
	50	8.0		33	0.85
	52	14		38	3.4
10	44.5	1.8	10	30	1.09
	46	3.4		33	2.58
	48	6.0		36	6.24
				38	12.2
				40	20.5
				43	55.4
				46	147

[a]TPP = pentasodium triphosphate.

chymotrypsinogen A, trypsin, cytochrom C and bacitracin. Independently of whether the enzyme precipitated by sodium sulfate (ca. 75% material of molecular weight around 25,000) or a purified enzyme obtained by gel chromatography was used for the inactivation experiments, we found almost exclusively lower molecular peptides with a molecular weight under 2000 as inactivation products.

Protection by Calcium Ions

An addition of a substantial amount of Ca^{2+} ions to the inactivation mixture of enzyme and 0.2% TPP solution overcomes the inactivating effect of the TPP (Fig. 5). This protecting effect is seen only at levels of Ca^{2+} seldom found in natural waters.

DISCUSSION

In spite of the fact that we used a preparation of the protease prepared by simple precipitation with sodium sulfate, which contained some low molecular peptides (ca. 20%) and a certain amount of sodium sulfate (ca. 40%), we will try to draw some conclusions from the results mentioned above. We believe the results of this study will not be influenced to a high degree by using this grade of enzyme, since we found no neutral protease nor amylase in this preparation. Furthermore over the concentration range used (10-40 µg/ml), the sodium sulfate and lower peptides in the enzyme concentrate should not interfere seriously with our measurements.

The observed first order characteristics of the inactivation reaction are compatible with a rate determining step in the inactivation process involving only one enzyme molecule. This rate determining step leads to an activated enzyme molecule ("enzyme*" in the scheme shown below) and this activated molecule either undergoes a direct but somewhat slower attack by a second enzyme molecule with concomitant fission into small peptides; or it is transformed in a slow reaction into a denatured protein molecule, which is then split by another still active enzyme molecule. A series of reactions of this kind is consistent with the observation of only small peptides as inactivation products. The relative resistance of native enzymes against proteolytic attack, and the easy cleavage of denatured enzymes inferred from these observations and from the first order rate is well known with a number of enzymes (15). The following scheme shows these reaction sequences:

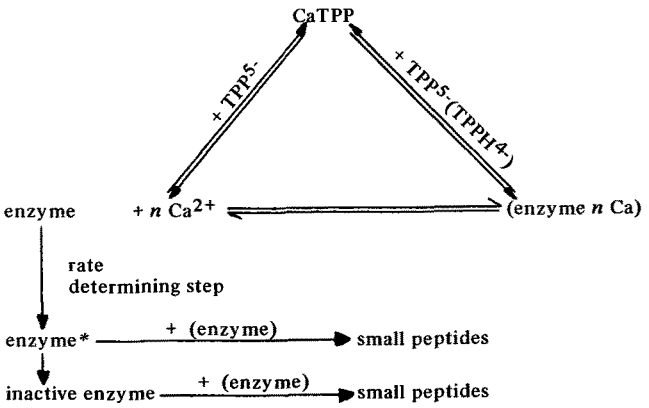

TABLE II

"Activation Parameters" for the Inactivation of Alkaline Bacillopeptidase

	$(\Delta \lg k_1):(\Delta\ 1/T)$	
pH	In absence of TPP[a]	In presence of 0.2% TPP[a]
7	10,500	12,600
9	12,500	11,300
10	12,700	12,800

[a]TPP = pentasodium triphosphate.

By calculating "activation parameters" from the slopes of the *Arrhenius* plots nearly identical values are obtained (Table II). (We refrain from calculating a value for the activation energy of the inactivation reaction since in any case there exist equilibria which precede the rate determining step and whose reaction enthalpies will enter this calculated activation energy to an unknown percentage. [This situation parallels the situation found with enzymatically catalyzed reactions, see Ref. 18.]) From these not very differing values it may be inferred that the rate determining step, and possibly preceding equilibria, are the same with and without added TPP. In other words if there is no direct inactivating attack of TPP on the enzyme molecule, then the observed enhancing effect of TPP on the overall rate may consist, e.g. of an influence on the equilibrium between free enzyme and a stabilized enzyme containing Ca^{2+}-ions.

Consistent with these ideas is the observed influence of changing the pH value in inactivations with and without added TPP (Figs. 3,4): without TPP there is almost no variation in overall inactivation rate, whereas in presence of TPP the rate increases with increasing pH. These observations can be explained as follows: the TPP penta-anion is monoprotonated with a pK of 8.6 (16) and the stability

constant of the Ca^{2+}-TPP complex drops by 2-3 powers of ten on adding one proton to the TPP ligand (17). The result of these reactions is an increase of the concentration of unbound Ca^{2+} and a decrease of the concentration of the TPP^{5-}anion in Ca^{2+}-TPP-solutions with rising pH. The same holds for higher or lower condensed phosphates.

Either now this increase of the pool of Ca^{2+} ions with falling pH stabilizes directly the enzyme by displacing the equilibrium between free enzyme and stabilized Ca^{2+}-enzyme in favor of the latter, or alternatively only the TPP-pentaanions are capable of attacking the Ca^{2+}-protease under removal of Ca^{2+}-ions.

The beneficial influence of Ca^{2+}-ions on the stabilization of the enzyme against inactivation by TPP demonstrated in Figure 5 is in agreement with the postulated inactivation scheme: it begins shortly before reaching a molar ratio of 1:1 for TPP/Ca^{2+}. The stabilizing effect of Ca^{2+}-ions on proteases of animal or microbial origin is well known (19,20) and has not been investigated in this work.

ACKNOWLEDGMENTS

W. Schneider performed the regression analysis and I. Hagenau helped with the kinetic experiments.

REFERENCES

1. Langguth, R.P., and L.W. Mecey, Chem. Spec. Mfr. Ass. Proc. Annu. Meet. 55:92 (1969).
2. Güntelberg, A.V., and M. Ottesen, Nature 170:802 (1952); C. R. Trav. Lab. Carlsberg, Sér. Chim. 29:36 (1954).
3. Hoogerheide, I.C., Fette, Seifen, Anstrichm. 70:743 (1968).
4. Wieg, A.J., Process Biochem. 4(2):30 (1969).
5. Duesing, K.P., Fette, Seifen, Anstrichm. 69:738 (1967).
6. Green, S.R., Soap Chem. Spec. 44(5):86 (1968).
7. Hartley, B.S., Ann. Rev. Biochem. 29:45 (1960).
8. Determann, H., "Gelchromatographie," Springer-Verlag, Berlin 1967; Specials by Deutsche Pharmacia GmbH, Frankfurt/Main.
9. Van Raay, H.G., H. Saran and H. Verbeek, Tenside 7:125 (1970).
10. Stauff, J., and R. Jaenicke, in "Biochemisches Taschenbuch," Vol. 2, Edited by H.M. Rauen, Springer-Verlag, Berlin, 1964.
11. Keay, L., P. Moser and B.S. Wildi, Biotechnol. Bioeng. 12:213 (1970).
12. Frost, A.A., and R.G. Pearson, "Kinetik und Mechanismen homogener chemischer Reaktionen," Verlag Chemie, Weinheim, 1964.
13. Sachs, L., "Statistische Auswertungsmethoden," Springer-Verlag, Berlin, 1969.
14. Laidler, K., "Chemical Kinetics," McGraw-Hill, London, 1965.
15. Okunuki, K., Advan. Enzymol. 23:29 (1961).
16. Perrin, D.D., "Dissociation Constants of Inorganic Acids and Bases in Aqueous Solution," Butterworth, London, 1969.
17. Sillén, L.G., and A.E. Martell, "Stability Constants of Metal-Ion Complexes," The Chemical Society, London, 1964.
18. Dawes, E.A., in "Comprehensive Biochemistry," Vol. 12, Edited by M. Florkin and E.M. Stotz, Elsevier Publishing Co., Amsterdam, 1964.
19. Desnuelle, P., in "The Enzymes," Vol. 4, Edited by P.D. Boyer, H. Lardy and K. Myrbäck, Academic Press, New York, 1960.
20. Schindler, F.J., and C.E. Neubeck, Chem. Spec. Mrf. Ass. Proc. Annu. Meet. 55:89 (1969).

[Received June 28, 1971]