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Hyperalkaline and Thermostable Phosphatase in *Thermus thermophilus*

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ABSTRACT

The phosphatases existing in the extreme thermophilic bacterium *Thermus thermophilus* have been studied. Utilizing ion exchange, hydrophobic, pseudoaffinity, and affinity chromatography, a number of distinct phosphatase activities were identified. At least four phosphatases, with optimum pH ranging between 5.0 and 11.5, were assayed with *p*-nitrophenylphosphate, and two with optimum pH between 7.0 and 11.0, with ^{32}P-casein as substrate. The authors have focused on the hyperalkaline phosphatase and have tried to purify and characterize it. This hyperalkaline phosphatase reaches a maximal level at the stationary phase of the growth, and is co-purified with alkaline phosphatase with optimum pH of 10.2. The enzymes present a relative mol wt of 65 and 58 kDa, respectively, as judged by SDS-PAGE and Sephadex G-150 column, and possess similar properties, indicating that they are isoforms. These enzymes barely function in the presence of tartrate, and are inhibited by EDTA, pyrophosphate, and molybdate. Among the metals tested, Hg^{2+} appeared as the strongest inhibitor of the hyperalkaline phosphatase. The two enzymes are thermostable and, upon treatment at 90°C for 10 min, 75% of their activity remains. The physiological role and function of these phosphatases need further investigation.

Index Entries: Phosphatases; hyperalkaline; thermostable; *Thermus thermophilus*.

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INTRODUCTION

Protein phosphorylation-dephosphorylation is a universal signal-transducing mechanism of all living systems. This reversible posttranslational modification of proteins proceeds via two categories of enzymes: protein kinases and protein phosphatases, respectively. The phosphorylation state of any such protein, and therefore the physiological processes it controls, may be the result of a balance between competing protein kinase and protein phosphatase activities *(1,2)*. The physiological significance of tyrosine kinase in the regulation of cell proliferation, and in transformation, has been well documented *(3,4)*.

Several acid phosphatases have been characterized in mammalian tissues *(5–7)*, some of which show phosphotyrosyl phosphatase activity *(7–11)*. The alkaline phosphatases (EC 3.1.3.1) are a family of enzymes found in all organisms from bacteria to man *(12,13)*. The *Escherichia coli* alkaline phosphatase is one of the better-characterized enzymes *(14,15)*, and many details of its tertiary structure, enzymology, biosynthesis, and regulation are understood quite well *(16–19)*.

The specificity of some dephosphorylating enzymes is now of biotechnological interest, because these enzymes work either at extreme pH values or at high temperatures. Therefore, purification of an enzyme with these properties presents genuine commercial opportunities and a valuable contribution to the field of biotechnology.

This article reports, for the first time, the existence of a hyperalkaline and thermostable phosphatase in the thermophilic microorganism *Thermus thermophilus*. The purification scheme and some of its properties are described as well.

MATERIALS AND METHODS

Materials

Tryptone and yeast extract were purchased from Oxoid (Unipath LTD, Hampshire, UK). Chromatographic materials were from Pharmacia and lysozyme from Boehringer (Mannheim, Germany). All other chemicals were purchased from Sigma (Steinheim, Germany).

Cultivation of *Thermus thermophilus*

T. thermophilus, strain HB_8, was grown at 65°C in a culture medium containing 0.3% (w/v) yeast extract, 0.5% (w/v) tryptone, 0.2% (w/v) NaCl, 2 μ*M* $FeCl_3$, 0.2 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, and 0.1% (w/v) glucose. The pH was adjusted to 7.0 with concentrated NaOH.

Preparation of Cell Extract

T. thermophilus cells (25 g) were suspended in 75 mL buffer A (50 m*M* Tris-HCl, pH 8.3). The cells were lysed by adding lysozyme (1 mg/mL final concentration) and incubated at room temperature for 30 min. DNase I was added at a final concentration of 0.1 mg/mL, and the mixture was stirred for 30 min at 37°C, then centrifuged at 10,000*g* for 10 min, and the supernatant was further used for the purification of phosphatases.

Enzyme Assay and Buffers

The enzyme activity was determined spectrophotometrically following the production of *p*-nitrophenol after incubation of the enzyme preparation with *p*-nitrophenylphosphate at 65°C.

The pH values for all buffers were measured at 25°C. The effect of temperature on pH for each buffer was taken into account, and all pH values were corrected to alkaline phosphatase assay temperature of 65°C by the −dpH/dt(unit/degree) coefficient, specific for each buffer.

Cell Growth

Cell growth was monitored by measuring the absorbance at 600 nm at a Perkin-Elmer spectrophotometer. The bacteria were harvested at the end of the logarithmic phase by centrifugation at 6000*g* for 10 min. Cells were washed twice with 0.9% (w/v) NaCl. The final yield was about 5 g wet cells/L of culture medium.

Definition of Unit and Specific Activity

One unit of phosphatase activity is defined as the amount of enzyme that catalyzes the release of 1 μmol *p*-nitrophenol/min of incubation at the above specified conditions. Specific activity was defined as the units per mg protein.

Purification of Hyperalkaline Phosphatase

Step 1: Crude 10,000 g Supernatant

The crude 10,000 g supernatant was prepared from 25 g of cells as described above.

Step 2: Ammonium Sulfate Fractionation

In the 10,000 g supernatant, solid ammonium sulfate was added to 25% of saturation. After 30 min, the mixture was centrifuged at 10,000*g* for 10 min, and to the supernatant solid ammonium sulfate was added until 60% of saturation. The mixture was stirred for 30 min and the 25–60%

ammonium sulfate pellet was obtained by centrifugation at 10,000*g* for 10 min. The 25–60% ammonium sulfate precipitate was suspended in buffer A, and further dialyzed overnight against the same buffer.

Step 3: DEAE-52 Cellulose Chromatography

The dialyzed sample was applied on a DEAE-cellulose (22 × 2.5 cm) column, which had been previously equilibrated with buffer A. The column was washed with 300 mL buffer A, and the enzyme was eluted with a 0–0.25 *M* linear gradient of NaCl in buffer A. Fractions with phosphatase activity were pooled and applied on the next column.

Step 4: Phenyl-Sepharose Chromatography

Fifty mL of enzyme solution from step 3 were applied on a phenyl-Sepharose (10 × 1.6 cm) column previously equilibrated with buffer A. Phosphatase activity was eluted with a 0–85% ethylene glycol in buffer A linear gradient. The active fractions were combined and applied on the next column.

Step 5: Cibacron Blue Chromatography

The enzyme solution (35 mL) from step 4 was applied on a Cibacron blue column (7 × 1.6 cm) equilibrated with buffer A. The enzyme was eluted with 0–2.0 *M* NaCl in buffer A linear gradient. The active fractions were pooled and concentrated to 5 mL by Amicon (Holland) ultrafiltration membrane (PM 10).

Step 6. Heparin-Sepharose Chromatography

The enzyme preparation from the previous step was applied on a heparin-Sepharose column (13 × 1.8 cm) equilibrated with buffer A. Fractions with phosphatase activity were eluted by a 0–0.25 *M* NaCl in buffer A linear gradient. The active fractions were pooled and concentrated to 2 mL by Amicon ultrafiltration (PM 10), and applied to a Sephadex G-150 column.

Step 7. Sephadex G-150 Column

The active concentrated fraction of the previous column was applied on a Sephadex G-150 column (60 × 2.4 cm) equilibrated with buffer A. Active fractions were concentrated to 2 mL, glycerol 10% v/v was added, and the solution was stored at −20°C.

Protein Phosphatase Assay

The protein phosphatase activity was assayed by the release of [^{32}P]phosphate from ^{32}P-casein, as described *(12)*. ^{32}P-Casein was prepared as previously reported *(20)*.

Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% acrylamide was performed according to Laemmli *(21)*. The gels were stained for protein with $AgNO_3$ *(22)*.

Localization of alkaline phosphatase after nondenaturing PAGE was performed as described *(23)*.

Con A-Sepharose Chromatography

The Con-Sepharose column was prepared as described *(24)*, and the column was equilibrated with buffer A with 0.5 *M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, and 1 m*M* $MnCl_2$.

Protein Determination

Protein was determined by Bradford's method *(25)* as modified by Baerden *(26)*, using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

This article describes an attempt to characterize and purify the phosphatases that exist in the extreme thermophilic bacterium, *T. thermophilus*. Four enzymes, with optimum pH 5.0, 7.5, 10.0, and 11.5, were assayed with *p*-nitrophenyl phosphate as substrate (Fig. 1). In addition, the same extract shows protein phosphatase activity at optimum pH of 7.0 and 11.0 with ^{32}P-casein as substrate.

The enzymes assayed with *p*-nitrophenyl phosphate show their maximal activities when the cells are at the stationary phase, as indicated at Fig. 2, and each one acts optimally at 65°C (data not shown). The authors have focused on the alkaline enzymes, which present biotechnological interest, and therefore have conducted studies on the hyperalkaline enzyme with the optimum pH 11.5.

The purification scheme developed for the hyperalkaline phosphatase is summarized at Table 1. The method includes ammonium sulfate precipitation (25–60% saturation), followed by a DEAE-52 column with 22-fold purification and a phenyl-Sepharose column with 72-fold purification. The hyperalkaline phosphatase was further purified with Cibacron-blue and a heparin-Sepharose columns.

A calculation of the yield and purification factor of the hyperalkaline phosphatase at the final step of purification with Sephadex G-150 column shows 5% recovery, with around 208-fold purification.

As shown at Fig. 1, four distinct peaks of phosphatase activity were revealed on the crude enzyme preparation. The picture of phosphatase

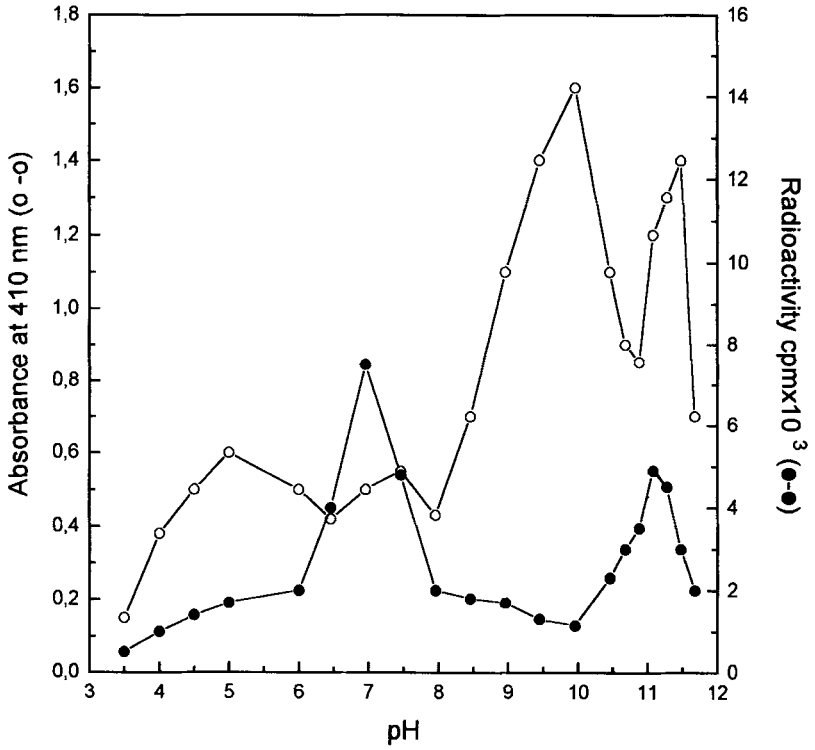

Fig. 1. Effect of pH on the phosphatase activity of the crude extract. For the different pHs, 50 m*M* of the following buffer solutions were used: CH_3COONH_4-CH_3COOH for pH 4.0–6.5; Tris-HCl for pH 6.5–9.0; glycine-NaOH for pH 9.0–10.5; and KCl-NaOH for pH 10.5–11.68. The pH values for the above buffers were measured at 25°C, and each value was adjusted to 65°C, as described in Materials and Methods.

activity is different in the partially purified preparation (Sephadex G-150 column), and shows clearly that two alkaline phosphatases are co-purified (Fig. 3).

The hyperalkaline phosphatase has a native M_r of approx 65 kDa, and reacts as a monomer, as determined by the gel filtration Sephadex G-150 column (data not shown). When the active fractions were combined, concentrated, and analyzed at different pHs, two phosphatases were obtained at pH 10.2 and 11.3, similar to that of Fig. 3, indicating that even after the step of gel filtration, the two enzymes are co-purified, possessing very close values of molecular mass.

The partially purified enzyme was further analyzed by PAGE (7.5% in acrylamide), under native conditions. Five gels were run as described in Materials and Methods. One gel was stained with $AgNO_3$, and the other four were cut in small pieces and analyzed with *p*-nitrophenyl phosphate at pH 9.8, 10.2, 11.3, and 11.5 (Fig. 4). This experiment shows that the two

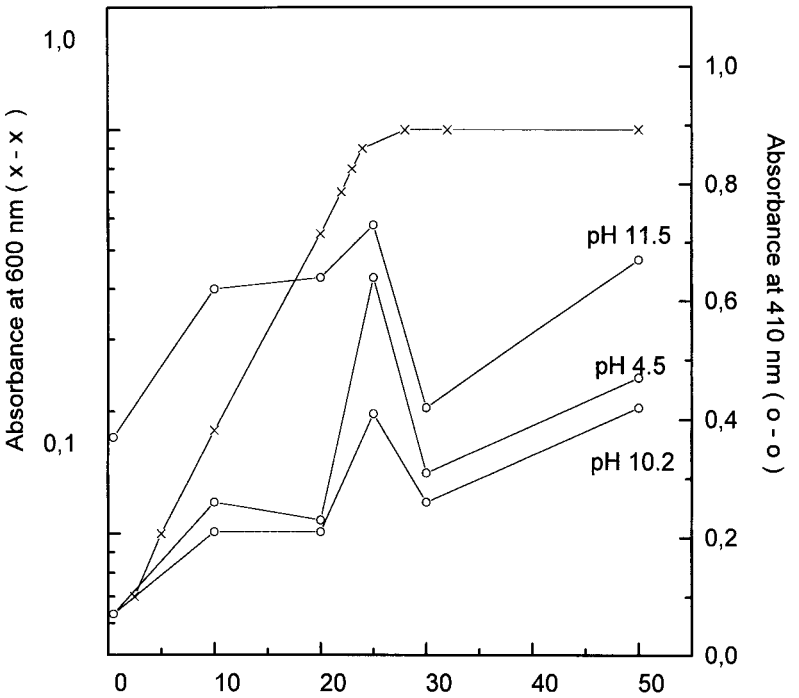

Fig. 2. Phosphatase activity during growth of *T. thermophilus*. Phosphatase activity (o-o) was measured at various times of growth at the pHs indicated at the figure. Growth was monitored by measuring the absorbance at 600 nm (x-x).

Table 1
Purification of Hyperalkaline Phosphatase from *T. thermophilus*

Purification step	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude	208.0	59,800	287.5	1	100
10,000 g sup					
$(NH_4)_2SO_4$	50.0	20,000	400	1.39	33
DEAE-52	2.7	17,500	6482	22.54	29
Phenyl-Sepharose	0.6	12,400	20,666	71.88	20
Cibacron-blue	0.3	9500	31,667	110.13	16
Heparin-Sepharose	0.2	8500	42,500	147.86	14
Sephadex G-150	0.05	3000	60,000	208.69	5

enzymes were not separated under these conditions. In addition, the hyperalkaline enzyme presents its optimal pH at 11.3, as indicated at Figs. 2 and 3. When the gels were run under native conditions and the enzyme was stained *in situ* at either pH 10.2 or 11.3, as described under Materials

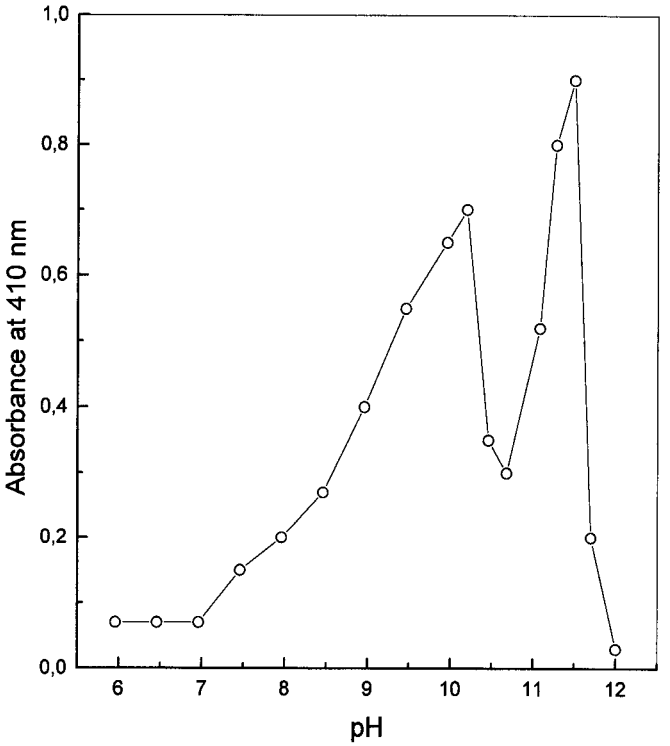

Fig. 3. Effect of pH on the partially purified hyperalkaline phosphatase.

and Methods, one band appeared, indicating that the two alkaline phosphatases run together in the same area of the gel.

The purity of the hyperalkaline phosphatase preparation was further analyzed by SDS-gel electrophoresis. At least four bands appeared in the SDS gel stained by $AgNO_3$ (Fig. 5, lane a). When this partially purified preparation (208-fold) was electrophoresed under native conditions, and the active band was electroeluted and subjected to SDS gel, two bands appeared, with molecular masses of 65 and 58 kDa (Fig. 5, lane b), probably corresponding to the alkaline phosphatases with pH optimum 11.3 and 10.2.

The two alkaline phosphatases did not bind to Con A-Sepharose, indicating that these enzymes do not contain carbohydrate moieties. Furthermore, these barely function in the presence of 0.1 *M* tartrate and are inhibited significantly in the presence of 1 m*M* EDTA, 5 m*M* pyrophosphate, or 10 m*M* molybdate (Table 2). In contrast, the hyperalkaline phosphatase is activated to different extent by metals, such as Mn^{2+}, Ni^{2+}, Pb^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, Co^{2+}, Cd^{2+}, Sr^{2+}, Hg^{2+} appeared as a strong inhibitor for the hyperalkaline enzyme. Fluoride, a widely recognized inhibitor of phosphoseryl protein phosphatases, inhibits about 47.6% the phosphatase with optimum pH 10.2; but it activates the hyperalkaline one by 82.8%.

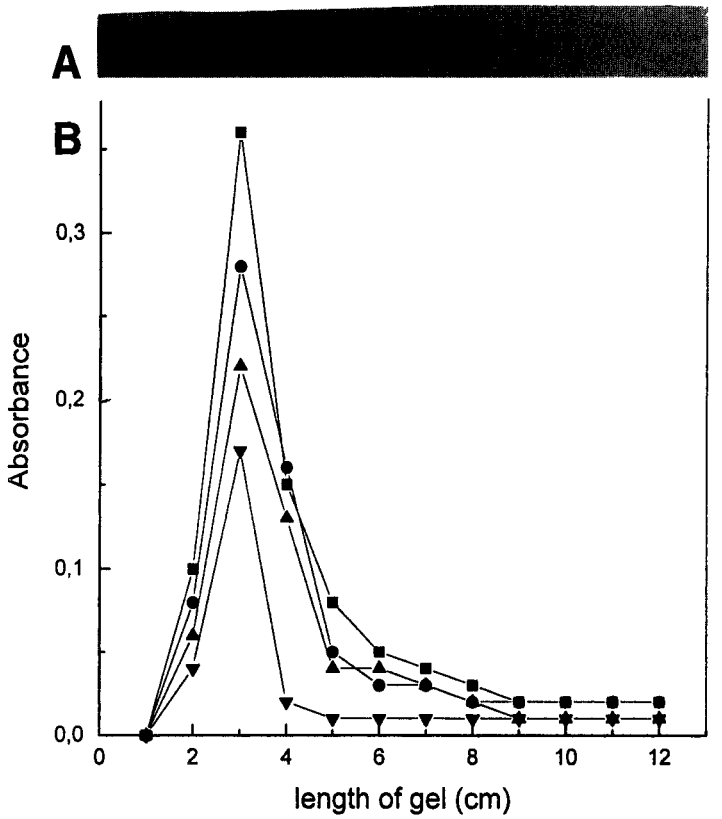

Fig. 4. PAGE of the partially purified hyperalkaline phosphatase. Samples prepared as in Table 1 (Sephadex G-150 step) were subjected to PAGE (7.5% in acrylamide). Gels were either **(A)** stained with $AgNO_3$ or **(B)** cut in small pieces, and enzymes activity was assayed at pH 9.8 (▲), 10.2 (●), 11.3 (■), and 11.5 (▼), as described under Materials and Methods.

The crude enzyme preparation is quite stable at high temperatures. By heating the enzyme preparation at 90°C for 10 min, about 75% of the initial activity remained at all the pHs tested. Similar results were obtained when the partially purified preparation was assayed at pH 10.2 or 11.3. The physiological role of these hyperalkaline enzymes, as well as their specificity toward many substrates and its applications to biotechnological processes, must be established. Therefore, further work is required to dissociate the two activities.

Recently, a rapid growth in the application of microbial enzymes in the areas of food or detergent chemistry has been observed. The stability that can be achieved with enzymes from thermophilic microorganisms will stifle one of the major criticisms of enzymes as reagents or commercial detergents.

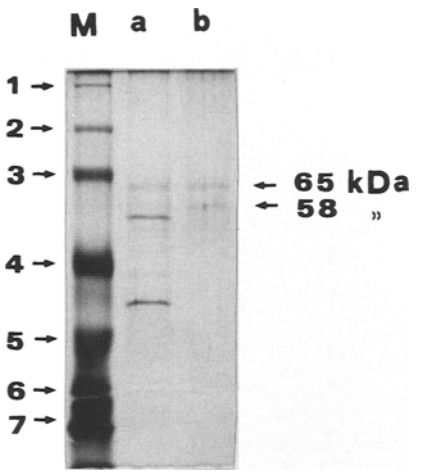

Fig. 5. SDS-PAGE of the partially purified hyperalkaline phosphatase. M: Protein markers for mol wt determination (Gibco-BRL): (1), myosin, H-chain, 200 kDa; (2) phosphorylase B, 97.4 kDa; (3) bovine serum albumin, 68 kDa; (4) ovalbumin, 43 kDa; (5) carbonic anhydrase, 29 kDa; (6) β-lactoglobulin 18.4; (7) lysozyme 14.3 kDa. Partially purified hyperalkaline phosphatase (step Sephadex G-150 of Table 1) was either electrophoresed (lane a) or run on a native PAGE, and the active band electro-eluted and electrophoresed (lane b).

Table 2
Effect of Various Substances on Phosphatases with Optimal pH 10.2 and 11.3

Substance	Concentration	Phosphatase pH 10.2	Activity % pH 11.3
Enzyme (control)		100	100
EDTA	1 m*M*	47.44	65
Tartrate	100 m*M*	8.66	18.15
Molybdate	10 m*M*	78.91	65.17
Pyrophosphate	5 m*M*	46.91	52.74
NaF	50 m*M*	52.37	182.76
$MgCl_2$	1 m*M*	52.55	187.69
$CaCl_2$	1 m*M*	63.65	123.07
$ZnCl_2$	1 m*M*	52.25	148.51
$PbCl_2$	1 m*M*	49.76	115
$NiCl_2$	1 m*M*	51.84	172.51
$MnCl_2$	1 m*M*	53.26	170.8
$HgCl_2$	1 m*M*	75.89	7.5
$CoCl_2$	1 m*M*	110.68	152.82
$CdCl_2$	1 m*M*	106.76	173.12
$SrCl_2$	1 m*M*	112.23	135.17

The past few years, much attention has focused on enzymes derived from thermophilic bacteria *(27)*. Therefore, the study of the function and stability of these enzymes must inevitably lead to valuable contributions to the field of biotechnology.

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