The Extracellular Thiaminase I of *Bacillus thiaminolyticus*. II. Preparation of the Antisera and Serological Properties*

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ABSTRACT: An antiserum to thiaminase I, prepared in rabbits, was demonstrated by a variety of precipitin reactions. The antiserum contained approximately 0.93 mg of antienzyme antibody/ml of serum. The antibody inhibited thiaminase I activity in a noncompetitive manner with respect to thiamine and was neither competitive nor noncompetitive with respect to aniline. A serological assay for thiaminase I using radial immunodiffusion was developed and the merits of this assay are discussed.

Thiaminase I (EC 2.5.1.2.), which catalyzes a base-exchange reaction using thiamine as a substrate (Murata, 1965), has been shown to be an extracellular enzyme in *Bacillus thiaminolyticus* (Douthit and Airth, 1966). The latter workers also have shown that this bacterium has a sole vitamin requirement for thiamine. The suggestion has been made that vitamin B_1 may regulate the synthesis of thiaminase I by repression (Wang and Airth, 1967).

Although the bacteria produce thiaminase I, no information is available regarding the role of this enzyme in intracellular processes. Indeed, preliminary studies have indicated that cell-free extracts of washed, sonicated cells contain little, if any enzymatic activity. However, one may postulate that these organisms possess inactive precursors of the enzyme which are activated after or upon release into the culture medium. On this premise, intracellular enzyme precursor(s) would be structurally and antigenically similar to extracellular thiaminase I. An antiserum specific for the purified enzyme would be useful in testing this possibility. This study describes the preparation and characterization of rabbit antithiaminase I. The effect of antibodies on the enzyme reaction are presented also.

Materials and Methods

Preparation of Thiaminase I. Crude thiaminase I was prepared as a 0–75% saturated ammonium sulfate precipitate from a filtrate of a 30-hr-old culture of *B. thiaminolyticus*. A combination of ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography results in a 200-fold purification of thiaminase I (Wittliff and Airth, 1968). The enzyme was judged homogeneous on the basis of ultracentrifugation and polyacrylamide gel electrophoresis. These preparations were used as antigen in the studies to be reported.

Enzyme Assay. Thiaminase I activity was measured by using a modification (Wittliff and Airth, 1968) of the method of Douthit and Airth (1966). An enzyme unit (U) is defined as that amount of enzyme which catalyzes the formation of 1 μmole of product in 1 min at 25°. Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine plasma albumin as a standard.

Preparation of Rabbit Antithiaminase I. Rabbit antisera to crude thiaminase I and purified thiaminase I were prepared in white New Zealand rabbits obtained from a local supplier. Two injections of 4 mg each of the crude enzyme incorporated in complete Freund's adjuvant were given intramuscularly at 3-week intervals. Five weeks later, the rabbits were boostered by a third injection containing 1 mg of protein with Freund's adjuvant. The rabbits were then bled by marginal ear vein punctures at weekly intervals for 4 weeks.

Rabbits receiving the purified enzyme were given a single intramuscular injection of 2.5 mg of the antigen incorporated in complete Freund's adjuvant. Two weeks later the rabbits were bled at weekly intervals by marginal ear vein puncture. Sera from weekly bleedings were tested for antibody content by the Ouchterlony gel diffusion method and were pooled as anticrude thiaminase or as antithiaminase I. The antisera were stored at −20°.

Precipitin Tests. Varying amounts (0–250 mU) of the purified thiaminase I in 0.05 M sodium phosphate buffer (pH 7.0) were added to a constant volume (100 μl) of rabbit antithiaminase I. The precipitating mixtures were adjusted to a final volume of 0.5 ml with 0.15 M sodium chloride and incubated at room temperature for 30 min and overnight at 4°. After centrifugation the supernatants were carefully removed and assayed for thiaminase I activity. The precipitates were washed three times with sufficient volumes of 0.15 M sodium chloride and

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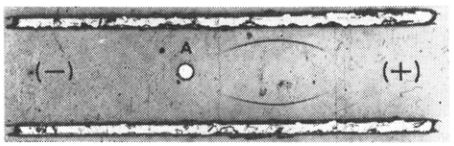

FIGURE 1: Immunoelectrophoresis of thiaminase I at pH 8.6. The well at A contained approximately 7 μg of purified enzyme. After electrophoresis, slots were cut in the gel and filled with antithiaminase I. The plate was incubated overnight in a humid chamber at 25°.

dissolved in 1.0 ml of 0.5 N sodium hydroxide. The amount of protein recovered as enzyme–antienzyme precipitate was determined from the absorption at 280 and 260 mμ according to the method of Warburg and Christian (1941).

Immunoelectrophoresis was performed according to the micromethod of Scheidegger (1955), using barbital buffer, pH 8.6, $\mu = 0.075$.

Radial Immunodiffusion Assay. Radial immunodiffusion (Mancini *et al.*, 1964) was employed to develop a quantitative assay for thiaminase I. Ionagar (15 ml) (1% in 0.15 M saline borate, pH 8.0) was cooled to 45–50° and 1 ml of undiluted antithiaminase I was added. The mixture was poured onto glass plates (8.2 × 10 cm) and allowed to solidify. Wells (approximately 3-mm diameter) were cut into the gels (approximately 20 wells/plate). Samples containing 0.6–6.0 μg of thiaminase I were placed in the wells with a microsyringe (Hamilton Syringe Co.) and the plates were incubated overnight at room temperature in a humid atmosphere.

As the antigen diffuses into the agar containing the antibody, a visible ring of precipitate develops about the wells. The reaction is usually complete in 14–20 hr. A Nikon microcomparator with a 10X objective allows for an easy and reproducible determination of the diameter of the diffusion rings.

Inhibition Studies. The capacity of rabbit antithiaminase I to inhibit the base-exchange reaction of thiaminase I was determined by preincubation of the enzyme (approximately 17 mU/reaction) for 10–20 min at 25° with varying amounts of antiserum (0–150 μl). As a control, normal rabbit serum replaced the antithiaminase I antiserum. When the thiamine concentration was varied, aniline concentration was 11.5×10^{-4} M. When varying aniline concentrations were used, the thiamine concentration (39.6×10^{-6} M) was kept constant.

Results

The antigenicity of purified thiaminase I was demonstrated by the successful production of antiserum in rabbits. Antiserum prepared against crude enzyme was multivalent in that several precipitin lines could be discerned after reaction with ammonium sulfate precipitated enzyme. Antiserum to the purified thiaminase I revealed a single band when tested against the crude or the purified enzyme preparations. Figure 1 is an immunoelectrophoresis pattern of reactivity of the antithiaminase I after electrophoresis of the purified enzyme. A single band at the anode was developed by the antiserum.

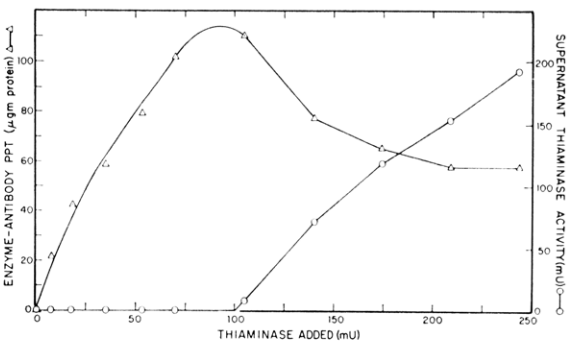

FIGURE 2: Titration of antithiaminase I with purified enzyme. Each tube contained 100 μl of antiserum, enzyme in varying amounts, and 0.15 M NaCl brought to a final volume of 0.5 ml. These mixtures were incubated at room temperature for 30 min and at 4° overnight. Protein estimates were performed on the washed precipitates; enzymatic activities were determined on the supernatants.

Figure 2 is a characteristic precipitin curve obtained when varying amounts of thiaminase I (as milliunits of enzyme activity) are added to a constant amount of antiserum. The equivalence point represents the maximum precipitation of antibody and antigen at an optimum ratio of antibody and antigen concentrations. Thus 110 μg of protein was precipitated by the addition of approximately 100 mU of thiaminase I to 100 μl of antiserum. Since the supernatants at equivalence contained no significant amount of thiaminase I activity, one can assume that the added enzyme (approximately 17 μg) was incorporated into the precipitate. Therefore, one can estimate the antibody content as 0.93 mg of antithiaminase I/ml of antiserum.

As expected from precipitin curve analysis and as evidence by the lack of activity in the supernatants, all of the enzyme added on the antigen deficiency side of the curve was precipitated. On the antigen excess side of the curve however, enzymatic activity was detected in the supernatants (Figure 2). The difference between the expected activity (thiaminase I added) and that recovered (supernatant thiaminase I) was attributed to loss of enzyme in the precipitate. From these results, an antibody unit was defined as the volume of antithiaminase I which removes one unit of thiaminase I from solution. Antiserum (1 ml) contained one antibody unit.

Figure 3 illustrates a time-course study wherein a constant amount of specific antiserum or normal rabbit serum was incubated with enzyme for varying periods of time prior to the addition of substrate. The results show that a maximum of 30% enzyme inhibition was attained after 10 min. Normal rabbit serum was not an effective inhibitor of thiaminase I.

Inhibition of thiaminase I activity with varying amounts of antithiaminase was linear within the range of 0–125 μl of antithiaminase antiserum (Figure 4). Anticrude thiaminase I was less effective as an inhibitor, presumably due to the decreased amount of antibody specific for the enzyme. The apparent enhanced inhibition by 150 μl of normal rabbit serum, anticrude thiaminase, and antithiaminase I was attributed to the limited sensitivity of the spectrophotometer at the high op-

TABLE I: The Effect of Thiamine Concentration on the Inhibition of Thiaminase I Activity by Antibody.[a]

Addn	Thiamine-HCl Concn (10^{-6} M)	Total Thiaminase I Act. (mμmoles of product/min)	% Inhibn	Inhibn (mμmoles of product/min)
AT	6.9	6.7	25.6	2.3
NRS	6.9	9.0		
AT	7.9	7.6	26.9	2.8
NRS	7.9	10.4		
AT	8.9	8.5	26.7	3.1
NRS	8.9	11.6		
AT	9.9	9.5	25.8	3.3
NRS	9.9	12.8		

[a] A constant amount (100 μl) of antithiaminase I (AT) or normal rabbit serum (NRS) was mixed with approximately 17 mU of thiaminase I and incubated 10–12 min. The activities of the mixtures were measured at 25° in the presence of different concentrations of thiamine; the initial aniline concentration was 11.5×10^{-4} M.

tical densities provided by nonspecific serum components.

To determine whether the inhibition provided by the antiserum represented competition with substrate for the enzyme catalytic site(s), enzyme inhibition experiments at various substrate concentrations were performed. As shown in Table I, an increase in thiamine concentration resulted in a proportional increase in the initial velocity of the reaction in the presence of antibody or normal rabbit serum. The per cent inhibition by the antibody is constant (approximately 26%) at the different thiamine concentrations, however, inhibition (mmicromoles of product per minute) increased with increasing substrate concentration. Lineweaver–Burk plots of these data indicated that the K_m values for thiamine are identical in the presence of antibody or normal rabbit serum, but that antibody lowers the maximum velocity (V_m) of the reaction. This would suggest that antithiaminase I acts as a noncompetitive inhibitor (Dixon and Webb, 1964) with respect to thiamine.

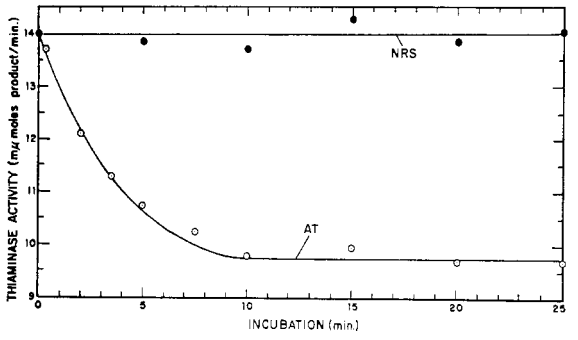

FIGURE 3: Time-course inhibition by antithiaminase I. Enzyme (14 mU) was preincubated at 25° with antithiaminase I (AT) or normal rabbit serum (NRS) for varying periods of time in 0.6 ml of 0.5 M sodium phosphate (pH 5.8). After incubation, aniline (3.45 μmoles) and thiamine (0.119 μmole) were added to initiate the enzymatic reaction.

Table II presents the results of an experiment in which the effect of aniline concentration on enzyme inhibition by antibody was measured. The enzyme activity in the presence of antibody at low aniline concentrations (3.8–5.7 $\times 10^{-4}$ M) was higher than the control. At an aniline concentration of 7.6×10^{-4} M, neither inhibition nor activation of thiaminase I activity was observed. However, when the aniline concentration was increased to 9.5×10^{-4} M or higher, inhibition of the enzyme activity by antithiaminase I was observed. A double-reciprocal plot of these data resulted in different K_m and V_m values for the control and the experimental, indicating that

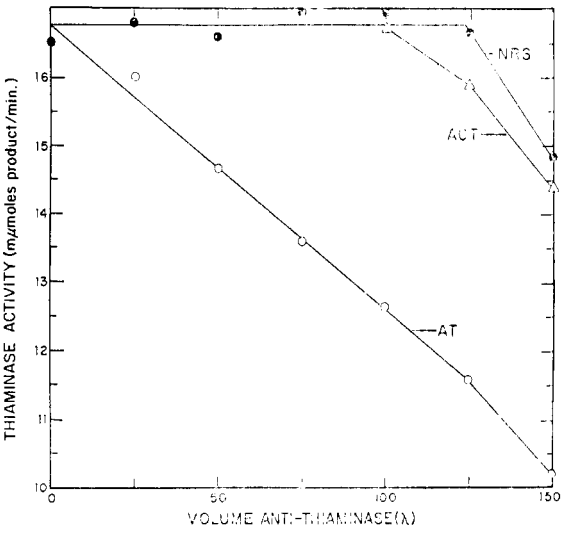

FIGURE 4: Inhibition of thiaminase I activity as a function of concentration of antithiaminase I (AT) and anticrude thiaminase (ACT). Normal rabbit serum (NRS) served as the control. Mixtures of the antibody and enzyme (16.5 mU/reaction) were incubated in sodium phosphate buffer (pH 5.8), for 10–12 min at 25°. The aniline (3.45 μmoles/reaction) was added and the reaction was initiated by thiamine (0.119 μmole/reaction).

TABLE II: The Effect of Aniline Concentration on the Inhibition of Thiaminase I Activity by Antibody.[a]

Addn	Aniline Concn (10^{-4} M)	Total Thiaminase I Act. (mμmoles of product/min)	% Inhibn	Inhibn (mμmoles of product/min)
AT	3.8	8.1	(−) 20.9[b]	(−) 1.4[b]
NRS	3.8	6.7		
AT	5.7	8.9	(−) 7.2[b]	(−) 0.6[b]
NRS	5.7	8.3		
AT	7.6	10.8	0.0	0.0
NRS	7.6	10.8		
AT	9.5	11.8	14.5	2.0
NRS	9.5	13.8		

[a] A constant amount (100 μl) of antithiaminase I (AT) or normal rabbit serum (NRS) was mixed with approximately 17 mU of thiaminase I and incubated 10–12 min. The activities of the mixtures were measured at 25° in the presence of different concentrations of aniline; the initial thiamine concentration was 39.6×10^{-6} M. [b] (−) per cent inhibition or (−) inhibition indicates an increase in enzyme activity.

antithiaminase I was neither a competitive nor a noncompetitive inhibitor (Dixon and Webb, 1964).

Previous results indicate that the inhibitory capacity of antithiaminase I is not sufficiently sensitive for quantitative studies of enzyme synthesis by repressed and derepressed cells. Precipitin analysis in agar however may prove to be a useful quantitative tool. Figure 5 illustrates the radial precipitin patterns obtained when varying amounts of enzyme were allowed to diffuse into the agar containing antithiaminase I. For these studies, antithiaminase was diluted 1:16 with melted agar. The sensitivity of this technique may be adjusted by varying the amount of antiserum incorporated into the agar.

Since the areas of the precipitin rings are proportional to the amount of enzyme placed in the wells, a plot of the radius squared *vs.* micrograms of the enzyme per well should result in a straight line. Figure 6 illustrates linearity over a protein concentration range of 0.6–6.0 μg.

Discussion

Extracellular thiaminase I from *B. thiaminolyticus* was found to be antigenic in rabbits. Precipitin bands which formed in gel diffusion reactions indicated that the enzyme preparations were homogeneous and that the rabbit antibody was specific for thiaminase I (Figure 1). Additional evidence for antibody specificity was provided by the precipitin curve (Figure 2). Titration of antithiaminase I with a purified enzyme preparation resulted in the typical decrease in the amount of precipitable antibody at antigen excess. Evidence for the participation of enzyme in antibody precipitation was provided by the loss of enzymatic activity in the supernatants. Specificity of antibody directed toward an enzyme has been reported by other workers (Kenney, 1962; McIvor and Moon, 1962; Cinader, 1963; Lehrer and Van Vunakis, 1965; Steers *et al.*, 1965; Beaty, 1966).

As pointed out by Cinader (1957, 1963) various mechanisms have been postulated for the inhibition of enzyme activity by an antibody: (1) binding of the antibody at the catalytic sites; (2) binding at a site other than the catalytic center, but proximal to it, which consequently block access to the active site; (3) aggregation

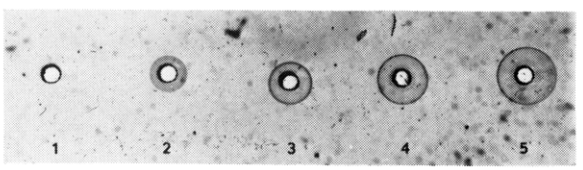

FIGURE 5: Radial immunodiffusion of thiaminase I. Varying amounts of enzyme ranging from 0.6 to 2.7 μg of protein were placed in wells 2–5; well 1 served as a control. The plate was incubated overnight in a humid chamber at room temperature. The radii of the precipitin rings were measured using a Nikon microcomparator with a 10X objective.

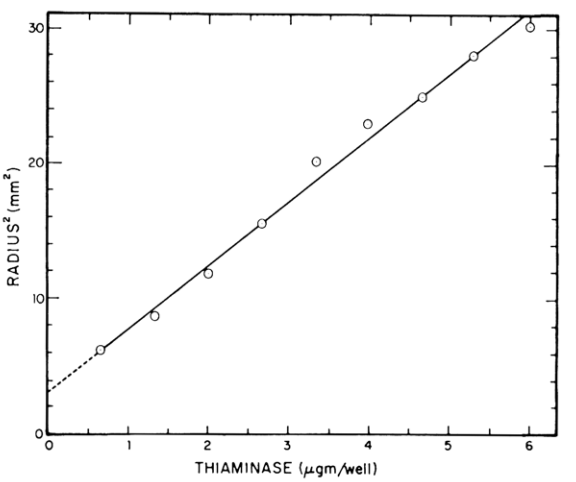

FIGURE 6: A plot of squares of the radii of the precipitin rings *vs.* the amount of enzyme in each well.

of the antigen–antibody complex which also blocks access to the active site; or (4) binding of the antibody induces a conformation change at the active site.

It was of interest to determine whether the nature of the inhibition of thiaminase I activity by antithiaminase I fell into any one of the above mechanisms. Although the inhibitory capacity of the antibody was linear with respect to concentration (Figure 4) inhibition was not a function of binding of antithiaminase I at the enzyme catalytic site(s). The velocities of the base-exchange reaction in the presence of antibody at different substrate concentrations indicate that antibody inhibition of thiaminase I is noncompetitive with respect to thiamine. A similar analysis for the other substrate, aniline, suggests that antibody was neither a classical competitive nor noncompetitive inhibitor.

Radial immunodiffusion was initially developed (Mancini *et al.*, 1964) to assay a single serum protein without employing tedious isolation procedures which frequently lead to undetermined losses. Quantitation of enzymes in culture medium or cell extracts frequently consists of assay methods which determine substrate utilization or product formation. Such techniques seldom provide data in terms of enzyme mass. The radial diffusion method, as applied to the assay of thiaminase I, has proven to be sensitive in detecting small amounts of enzyme ranging from 0.6 to 6.0 μg of protein (Figures 5 and 6). The success of radial diffusion, however, requires an antiserum which is specific for the antigen being assayed.

Preliminary studies with antithiaminase I indicated the presence of a cross-reacting material in the cell extracts of *B. thiaminolyticus*, the nature of which is currently under detailed investigation (L. Wang, J. L. Wittliff, and R. L. Airth, unpublished data).

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