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New indole oxygenase from the leaves of *Tecoma stans* L. Part II: Immunological characterization

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Abstract

Antibodies raised against indole oxygenase from the leaves of *Tecoma stans* did not crossreact with indole oxygenase from *Jasminum grandiflorum*. *Tecoma* indole oxygenase–IgG immunoprecipitate has retained 90% of the original activity. The K_m and V_{max} of indole oxygenase in the immuno complex were comparable to those of the free enzyme. The enzyme was protected against heat inactivation by antibodies in the immunoprecipitate.

Key words: Indole oxygenase, *Tecoma stans*.

1. Introduction

In recent years, immunological investigation of macromolecules, particularly in the study of immune response toward protein antigens at the molecular level, has made significant progress. For solving the paramount problem of elucidating the antigenic specificity determinants and their structural parameters, enzymes have been used extensively as important vehicles[1]. The use of enzymes, rather than proteins, as antigens, offers an advantage, because the antigens possess biological activity that resides in limited area of the molecule, and antibodies specific toward this or related regions may have an effect on the catalytic activity.

Though a few indole-metabolising enzymes are known[2-5], no immunological characterization was reported. Indole oxygenases from *Tecoma stans*[6] and *Jasminum grandiflorum*[4] catalyze the same reaction, *viz.*, the conversion of indole to anthranilic acid, but differ considerably in their properties. Indole oxygenase from *J. grandiflorum*[4] is a cuproflavoprotein and is completely inhibited by both thiol compounds and reagents, while the oxygenase from *T. stans*[6] is not. In this paper, we report immunological characterization of indole oxygenase from *T. stans* and investigate immunological similarity with indole oxygenase from *J. grandiflorum*[4].

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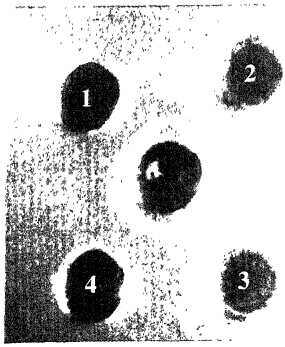

FIG 1. Ouchterlony double immunodiffusion. The central well contained antibodies and the surrounding wells (1 and 3) contained purified enzyme and (2 and 4) 20–50% ammonium sulfate fraction. The plates were developed at 4°C.

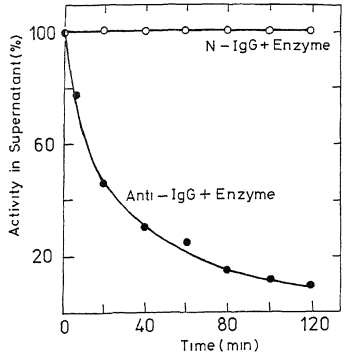

FIG 2. Time course of antigen–antibody complex formation.

2. Results

2.1. *Homogeneity of the antigen*

The enzyme used throughout the investigation was prepared by affinity chromatography[7]. The homogeneity of the antigen, the indole oxygenase from the leaves of *Tecoma stans*, was established by polyacrylamide gel electrophoresis and immunoelectrophoresis. Subsequent to the preparation of the anti-IgG fraction, the specificity of the antigen–antibody system was tested by Ouchterlony double immunodiffusion (Fig. 1). The formation of a single precipitin line in the tests with both partially purified and purified enzyme indicated that the immunizing antigen was pure within the limits of detectability.

2.2. *Time course of complex formation*

To investigate the time required for complete precipitation of the antigen by the specific antibodies a time course of complex formation was followed. The enzyme (1 mg) was incubated at 37°C in 50 mM sodium phosphate buffer containing 0·15 M saline, pH 7·4, with an excess of IgG isolated from the antiserum. At different time intervals the samples were centrifuged and the activity in the supernatant was determined under standard conditions. Normal IgG was added to the control samples which contained the same amount of enzyme. More than 50% of the enzyme was precipitated within 20 min (Fig. 2). The disappearance of the enzyme activity from the supernatant was a consequence of the complete binding of the enzyme to the antibody which is finally being precipitated.

Note: All the methods are described in detail in previous papers[6,7,9].

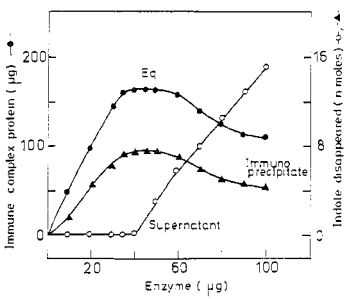

FIG. 3. Quantitative precipitation of the *Tecoma* leaf indole oxygenase by IgG-antibody.

Different concentrations of the enzyme were taken in 50 mM sodium phosphate buffer containing 0·15 M saline, pH 7·4 and 125 μg of the IgG-antibodies were added. The tubes were incubated at 37°C for 30 min and subsequently incubated at 4°C for 12 h. The precipitate was removed by centrifugation at 3,000 × *g* for 10 min at 4°C. The immunoprecipitates were then dissolved in 0·2 N NaOH and the protein content was estimated. The enzyme activity in the immunoprecipitate (parallel experiment) was measured by suspending the precipitate in 0·1 M citric acid–0·2 M sodium phosphate buffer, pH 5·2, and assayed under standard conditions.

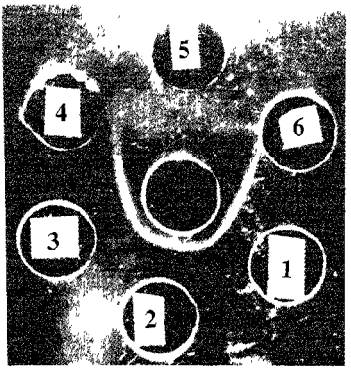

FIG. 4. Ouchterlony double immunodiffusion test.

2.3. *Quantitative precipitin test*

A fairly characteristic bell-shaped precipitin curve was obtained (Fig. 3), when increasing amounts of the purified *Tecoma* leaf indole oxygenase (TIO) was added to a constant amount of the anti-enzyme globulin (125 μg) in a final volume of 500 μl. The immunoprecipitate was separated by centrifugation at 3,000 × *g* for 10 min at 4°C and the protein was estimated by the method of Lowry *et al*[8]. The enzyme activity in the supernatant of each fraction was measured under standard conditions except that the whole supernatant (500 μl) was used for assaying the activity. It is clear from Fig. 3 that at the equivalance point (Eq), no enzyme activity could be detected in the supernatant, indicating that all of the enzyme activity had been precipitated. However, the enzyme activity was found in the region of antigen excess, as expected. It was interesting that the insoluble *Tecoma* leaf enzyme–IgG antibody complex, TIO-Ab, when dispersed in 0·1 M citric acid–0·2 M sodium phosphate buffer, pH 5·2, had 90% of the original activity. As evidenced from Fig. 3, the enzyme activity increases in the precipitate and attains a maximum value at the

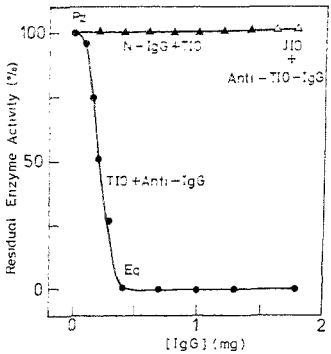

FIG. 5 Interactions of anti-tecoma leaf indole oxygenase–IgG with the purified indole oxygenases from *Tecoma* and *Jasminum*[4] leaves.

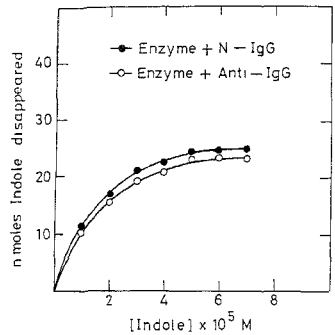

FIG 6. Indole saturation pattern of *Tecoma* leaf indole oxygenase–antibody complex.

equivalence point. However, the enzyme activity decreases in the precipitate in the antigen-excess region because of the formation of the soluble antigen–antibody complex and hence the decrease in the amount of the protein itself.

2.4. *Crossreactivity of jasmine leaf indole oxygenase with Tecoma leaf indole oxygenase antibodies*

Indole oxygenase from the leaves of *Jasminum grandiflorum* was purified according to the procedure of Divakar *et al*[4]. When Ouchterlony double immunodiffusion test was performed, using *Tecoma* leaf indole oxygenase–IgG antibodies in the central well and indole oxygenases from the leaves of *Tecoma stans* and *jasminum grandiflorum* in the surrounding wells (60μg in each), a single precipitin line was obtained with *Tecoma* leaf indole oxygenase while the jasmine enzyme did not crossreact with the antibodies raised against *Tecoma* enzyme (Fig. 4). There may not be any apparent gross homology between these two enzymes catalyzing the same reaction.

2.5. *Neutralization of indole oxygenase from Tecoma stans by its antibodies*

Different amounts of the IgG antibodies (0–2 mg) were incubated for 30 min at 37°C and for 12 h at 4°C with 100 μg of *Tecoma* leaf indole oxygenase, TIO, and 100 μg of jasmine indole oxygenase, JIO. In a control experiment, 100 μg of *Tecoma* leaf enzyme was incubated with increasing amounts of (0–2 mg) normal IgG (pre-immune) under identical conditions. The precipitated immune complex was separated by centrifugation at 3,000 × *g* for 10 min and the enzyme activity in the supernatant solution was estimated. The activity in the absence of added IgG antibodies was normalized to 100. A typical neutralization curve was obtained when increasing quantities of the IgG antibodies were added to a fixed amount of the *Tecoma* leaf indole oxygenase

(Fig. 5). After a small plateau region (Pz), there is a linear decrease in the enzyme activity in the supernatant on adding increasing amounts of the antibodies. This linear phase reflects the interaction of the antibody molecules with the enzyme and estimates the neutralizing titre (Eq) of the antiserum. The jasmine enzyme was not crossreacting and hence no decrease of the enzyme in the supernatant. Comparable amounts of normal rabbit IgG had no effect on the activity of the enzymes from *Tecoma* and *Jasminum* leaves. Pre-incubation of the enzymes with indole prior to the addition of antibodies did not have any effect on the immunocomplex formation.

2.6. *Kinetic constants of the enzyme–antibody complex*

The enzyme (1 mg) was incubated with 5 mg of anti-(Tecoma leaf enzyme) antibodies in a final volume of 8 ml, for 30 min at 37°C and for 12 h at 4°C. The immunoprecipitate obtained on centrifugation at 3,000 × *g* for 10 min was dispersed in 10 ml of 0·1 M citric acid–0·2 M sodium phosphate buffer, pH 5·2. This dispersed precipitate (corresponding to 100 μg/ml reaction mixture of the free enzyme) was used for the indole saturation experiment. A mixture of 1 mg of *Tecoma* enzyme and 5 mg of rabbit pre-immune IgG, treated similarly, served as control for this experiment. The enzyme–IgG antibody complex showed a hyperbolic saturation pattern with indole, as does the free enzyme + pre-immune IgG with indole (Fig. 6). K_m and V_{max} values for indole thus determined were 18·5 μM and 32 nmoles/15 min from Lineweaver-Burk plot. These results are in good agreement with the values obtained for the free enzyme[6] and the enzyme treated with pre-immune IgG.

2.7. *Thermal inactivation of the enzyme–antibody complex*

Tecoma leaf indole oxygenase antibody complex obtained as described for the above experiment was dispersed uniformly in 0·1 M citric acid–0·2 M sodium phosphate buffer, pH 5·2. This dispersion was heated in a water bath at 70°C and aliquots (0·5 ml) withdrawn at regular time intervals were rapidly cooled on ice and assayed for the enzyme activity at 30°C. A mixture of the enzyme and pre-immune IgG or the enzyme alone in buffer, treated identically, served as controls. The activity of the enzyme which was not heated was taken as 100. The free enzyme and the enzyme treated with pre-immune IgG are rapidly inactivated at identical rates at 70°C, with complete loss of the activity occurring in 5 min (Fig. 7). In contrast, the enzyme–IgG antibody complex was heat stable and retained nearly 50% of the original activity when heated to 70°C for 25 minutes.

3. Discussion

The recovery of the catalytic activity (approximately 90%) in the insoluble *Tecoma* leaf indole oxygenase–IgG antibody complex (Fig. 3) suggested that (i) substrates could diffuse through the immuno-complex to reach the active site; (ii) the antibody induced conformational change did not alter the stereoscopic orientation of the amino acids at the active site; or (iii) the antigenic determinant site might be located distal to the substrate-binding site. The final argument seems to be substantiated by

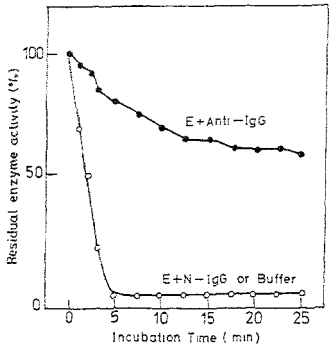

FIG. 7. Heat inactivation pattern of the *Tecoma* leaf indole oxygenase antibody complex.

the observation that indole failed to protect the enzyme against precipitation by antibodies, although it caused a conformational change as observed by fluorescence experiments[9]. It is worth noting that in several cases substrates and substrate analogs protect the enzyme against antibody inhibition, *e.g.*, the *Staphylococcal* nuclease was protected against antibody inactivation by substrate analogs[10] and mung bean glutamine synthetase was partially protected against antibody inhibition by glutamate and ATP[11]. In contrast, the addition of malate to *Staphylococcal* malic enzyme resulted in enhanced antibody-mediated inhibition of catalytic activity[12]. Further, the K_m and V_{max} values seen with both free enzyme and the enzyme in the complex with antibody are the same. These results suggested that the catalytic site did not take part in eliciting antibody formation in rabbits either directly or indirectly or, in other words, the catalytic site did not confer immunogenic specificity to the indole oxygenase from the leaves of *Tecoma stans*.

The antibodies raised against *Tecoma* indole oxygenase did not complex with *Jasminum* indole oxygenase as evidenced by the absence of a precipitin line on Ouchterlony double immunodiffusion (Fig. 4) and immunoelectrophoresis (figure not given). Fujioka[13] showed that guinea pig antiglobulin prepared against one of the isoenzymes (cytosolic or mitochondrial) of rabbit liver serine hydroxymethyl transferase, does not inhibit the activity of the other, although it inhibits the corresponding enzyme, suggesting that the two isoenzymes are immunologically distinct. Prager and Wilson[14,15] demonstrated that the immunological crossreactivity of homologous proteins is closely related to their sequence homology. It is sometimes believed that immunological crossreaction may be taken as corroborating evidence for conformational homology[16].

The protection of *Tecoma* leaf indole oxygenase by its specific antibodies against heat inactivation suggested that (i) the antibody might 'freeze' the enzyme in the most active conformation or (ii) the binding of antibody prevented the initiation of denaturation, by blocking the initiation domain on the enzyme. Similar observations were reported in the case of the monkey liver serine hydroxymethyl transferase[17]; the human erythrocyte acetylcholine esterase[18,19]; mouse catalase[20]; *E. coli* β-galactosidase[21]; yeast alcohol dehydrogenase[22] and *B. cereus* β-lactamase[23].

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