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In vitro degradation of aflatoxin B_1 by horse radish peroxidase

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Abstract

Aflatoxin B_1 (AFB_1), extracted from *Aspergillus flavus* ATCC 15517, was detoxified up to 60% by commercial horse radish peroxidase (HRP) (200 U/mg) in vitro. Partially purified peroxidase enzymes from freshly harvested radish root having 20 and 30 U/mg of protein were made to act upon the toxin in vitro and showed approximately 30 and 38% conversion, respectively. The optimum enzymatic reaction occurred in 50 mM phosphate buffer at 20°C, pH6, incubation time 60 min and normal pressure. Hydrogen peroxide in 5 mM concentration was used as oxidising agent in all the reactions performed. The reaction product was tested for its toxicity on *Bacillus megaterium* and other micro-organisms. Toxicity tests resulted in 97 and 30% growth of bacilli when 200 and 30 U/mg enzyme was used for the reaction, respectively.

1. Introduction

Aflatoxins, a group of secondary metabolites produced by the fungi *Aspergillus flavus* and *A. parasiticus*, are mutagenic (McCann, Edmund, Yamasaki & Ames, 1975), hepatotoxic (Lee, Dunn, Delucca & Ciegler, 1981) and hepatocarcinogenic (Butler, 1965) in a wide variety of animal species. In liquid culture, these fungi normally have a period of active synthesis and accumulation of the aflatoxin in the medium (Hamid & Smith, 1987). They also produce toxin using several food grains as solid substrates (Sharma, Trivedi, Wadodkar, Murthy & Punjarath, 1994). Enzymes or enzyme systems are known to be involved in the degradative metabolism of several xenobiotic molecules (Sariaslani, 1991); for example, the fungal cytochrome P-450 mono-oxygenase enzyme system is involved in the catabolism of naphthalene (Cerniglia & Gibson, 1978), progesterone (Ghosh & Samanta, 1981) and benzo(a)pyrene (Ghosh, Dutta, Samanta & Mishra, 1983). Plant peroxidases such as lignin peroxidase from white rot fungi, *Phanerochaete chrysosporium*, has been reported to oxidise a wide range of toxins, including polycyclic aromatics and polychlorinated phenols (Georgiou, 1987). Apart from several works on physicochemical (Samarajeewa, Sen, Cohen & Wei, 1990) and biological detoxification of the aflatoxin molecule (Ciegler, Lillehoj, Petersen & Hall, 1966) much of the work has been performed on in vivo detoxification of the toxin by the AFB_1-metabolising aldehyde reductase and alpha class glutathione S transferase reactions in the presence of chemoprotectors as the inducer of the enzymes (Ellis, Judah, Neal & Hayes, 1993; McLellan, Judah, Neal & Hayes, 1994). In this work, an attempt has been made to identify the enzyme which can detoxify aflatoxin B_1 in vitro. To achieve this, the enzymatic reaction at various concentrations of horse radish peroxidase (HRP) and AFB_1, at different temperatures, pH values and incubation times was studied in the presence of hydrogen peroxide as an oxidising agent. The reaction conditions were optimised. The current study also aims to recommend a natural source of HRP enzyme which can be used to degrade AFB_1.

2. Materials and methods

2.1. Microorganisms and culture conditions

Aspergillus flavus ATCC 15517 was used throughout the study and was maintained on potato dextrose agar (PDA) slants at 4°C. For inoculum production, the cultures were grown on PDA at 30°C for 10 days. Spores were harvested with a sterile 0.001% Tween 80 solution, washed with sterile distilled water and made up to a

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final spore concentration of 2×10^6 spores/ml. Sucrose low salt (SL) medium (Reddy, Viswanathan & Venkitasubramanian, 1971) was used for all experiments to produce AFB_1. *Bacillus megaterium* MTCC 2444 was obtained from the Institute of Microbial Technology, Chandigarh, India, and maintained on nutrient agar slants at 4°C. For inoculum production, the spore suspension was prepared by washing the spores with 3 ml phosphate buffer and then inoculated with 2×10^7 cells/ml into sterile nutrient broth media. Aflatoxin standards and crystalline horse radish peroxidase enzyme were procured from Sigma Chemicals, USA. All chemicals were of analytical grade.

2.2. Aflatoxin production and assay

AFB_1, produced by *A. flavus* ATCC 15517 in sucrose low salt medium, was extracted by chloroform and purified by column chromatography following official CB methods of analysis [Association of Official Analytical Chemists (AOAC) 1995]. The amount of aflatoxin in the extracts was determined initially by thin-layer chromatography (TLC), coated with 0.25 mm silica gel G 254 (E. Merck). The unknown concentration of AFB_1 was quantified against a standard curve obtained by plotting the fluorescence intensity vs concentration at 363 nm excitation wavelength using a Perkin–Elmer luminescence spectrophotometer model LS 50 B.

2.3. Preparation of enzyme from horse radish and assay

Freshly harvested roots of radish were crushed and the juice was extracted through fine mesh, which was used directly for the reaction as crude juice. This was further purified by 68 and 31.2% ammonium sulfate precipitation, respectively, along with subsequent dialysis (Keilin & Hartee, 1951) after each step. Total protein content of the extract was measured by the Bradford method (Bradford, 1976) and units were determined by guaiacol assay (Devlin, 1953). One unit of enzyme forms 1 mg of tetraguaiacol from guaiacol in 60 s at pH 6 at 20°C. As the reaction of HRP with AFB_1 was slow, the specific activity of the enzyme has been calculated as the gram moles of substrate converted (AFB_1) per milligram of protein per hour. Henceforth, in all the following experiments, the commercially procured enzyme, having 200 U/mg protein, is referred to as enzyme A, the fraction obtained by 68% ammonium sulfate precipitation having 20 U/mg as enzyme B and fraction obtained by 31.2% ammonium sulfate precipitation, having 30 U/mg, as enzyme C.

2.4. Determination of concentration of hydrogen peroxide

Hydrogen peroxide was directly estimated by the method of Hildebrandt and Roots (1975). For this technique, 1 ml aliquots of reaction mixture were transferred to test tubes, to which were added 10 mM ferroammonium sulfate (0.2 ml) and 2.5 M potassium thiocyanate (0.1 ml). Thus, ferric thiocyanate was formed upon oxidation of Fe^{2+} to Fe^{3+} by hydrogen peroxide. The optical density of the reaction mixture was measured at 480 nm and compared to the control.

2.5. Toxicity test

Determination of the effect of AFB_1 on the growth of various microorganisms was carried out with special reference to *B. megaterium*. The bacteria having 2×10^7 cells/ml concentration were inoculated into 150 ml of nutrient broth and incubated in the presence of 5 ml of reaction mixture containing no toxin as control set and 1 mM AFB_1 as the highest level of toxin. In three more sets of experiments, enzymes A, B and C were added to 10 ml of 1 mM AFB_1 and incubated up to 60 min at room temperature. From each of the reaction mixtures, 5 ml was taken out and inoculated into three separate sets of sterile culture media and incubated up to 12 h on an orbital shaker at 200 rpm. The effect of in vitro enzyme detoxification of AFB_1 was confirmed by growth inhibition of bacilli.

3. Results and discussion

In search of a cheap and convenient source of plant peroxidase, freshly harvested roots of horse radish were chosen. The data obtained from the purification process show that the raw juice has only 0.6 U enzyme/mg of protein, which is very low. Two consecutive ammonium sulfate precipitation processes, along with subsequent dialyses against pure water, give better enzyme recovery (Table 1). Further experiments were done using the pure and partially purified enzymes. To optimise the reaction of AFB_1 with horse radish peroxidase, concentration of substrate and enzyme were varied over a wide range of pH and incubation times.

To optimise the substrate concentration, AFB_1 was varied from 0.2 to 1.8 mM to observe the velocity of reaction by the peroxidase enzyme. In a 10 ml volume of reaction mixture, 2 U of enzyme was added to the substrate in 50 mM phosphate buffer at pH 6, 20°C temperature and normal pressure. From Fig. 1 it can be seen that the initial, as well as the maximum, velocity of reaction, in the case of enzyme A, is greater than partially-purified enzymes B and C. Although the rate of reaction shows an increasing trend in higher substrate concentration, the per cent conversion becomes almost stable beyond 1 mM. Up to 0.3 mM substrate concentration, no significant reaction was observed.

Optimisation of enzyme concentration is a crucial step because the cost of detoxification will be less depending

Table 1
Summary of HRP enzyme purification process

Enzyme fraction	Total volume (ml)	Conc. of total protein (mg/ml)	Total protein (mg)	Recovery (%)	Units (mg^{-1})
Crude extract	100	0.21	21	–	0.6
68% Ammonium sulfate pptn.	10	1.8	18	100	20
31.2% ammonium sulfate pptn.	5	2.2	11	61	30
Commercial enzyme	–	–	–	–	200

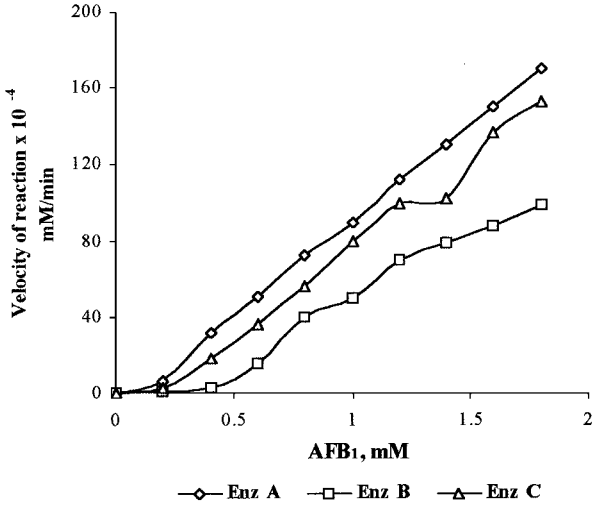

Fig. 1. Effect of substrate concentration on HRP–AFB_1 reaction.

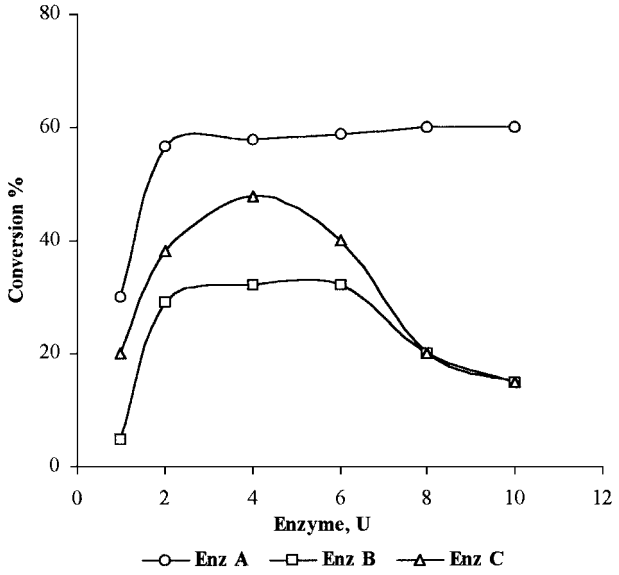

Fig. 2. Change in percentage conversion with increase in enzyme units.

on the use of optimum concentration of the enzyme. The reaction was set up using 1 mM AFB_1 as substrate in 50 mM phosphate buffer, pH 6, at room temperature. Enzymes A, B and C were added to 10 ml of reaction mixture in varying concentrations and incubated for 1 h. In the case of pure enzyme A, the velocity of reaction increases up to 8 U of enzyme, but after that no observable increase in rate occurs. The enzymes A and B have a steep increase in activity up to 4 U. With increase in the enzyme concentration, the activity as well as per cent conversion, is decreased (Fig. 2). This may be due to substrate saturation or some inhibitory factor. An enzyme concentration of 2 U/mM of substrate was considered as optimum for the following experiments.

To optimise the incubation time, optimum substrate and enzyme concentrations were used for the reaction in 50 mM phosphate buffer at pH 6 and 20°C. The peroxidase reacts very quickly with its substrate, so the activity of the enzyme (2 U/mM) was measured starting from 10 s to 65 min. Up to 15 min no significant reaction had started in the case of enzymes A or B. However, enzyme C showed 0.07% conversion. With increase in time, the velocity of reaction was also increased up to 30 min in the case of enzyme A, 50 min in the case of enzyme B and up to 60 min in the case of enzyme C. The rate decreased beyond 60 min of incubation in all cases and drops markedly down in the case of enzyme B as shown in Fig. 3. This may be due to the presence of various plant proteolytic enzymes and inhibitors in the raw horse radish juice which act upon the peroxidase. To get the highest percentage of substrate conversion, an incubation time of 60 min can be considered as optimum.

HRP is stable over a wide range of pH but, as it plays a key role in controlling the rate of reaction, pH of the reaction mixture was varied from extremely acidic to the basic range. From Fig. 4, it is clear that the optimum pH is 6 in all cases. The very high rate of degradation at pH 2 and similarity in the rates of reaction with partially pure, and commercially-obtained enzyme, suggests that the degradation of AFB_1 may not be due to enzymatic action only, but also due to instability of the AFB_1 molecule at the extreme pH. HRP enzyme may also become denatured at pH 2.

It is essential to compare the specific activity of the enzyme A to partially purified enzymes B and C. From Table 2, it can be concluded that it is worth using partially purified enzyme rather than highly expensive commercial enzyme and, for optimisation of reaction conditions on a laboratory scale, AFB_1 purified from cell free extracts can be used conveniently, as the results obtained by using more expensive AFB_1 from commercial sources are almost similar.

In all the above cases, reaction was set up at 20°C, taking 1 mM AFB_1 as substrate in the presence of 5

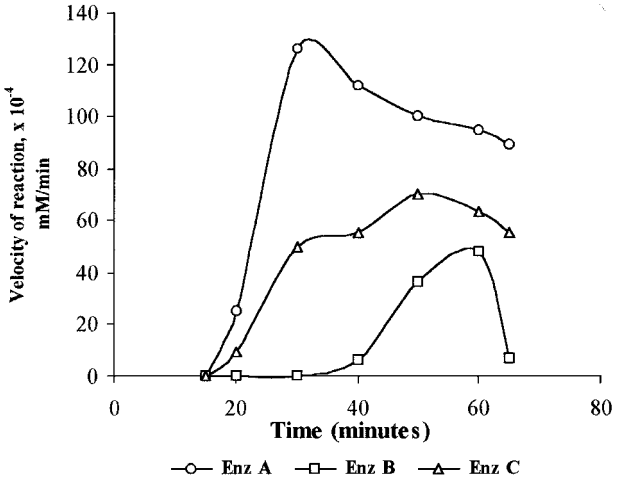

Fig. 3. Effect of incubation time concentration on HRP–AFB_1 reaction.

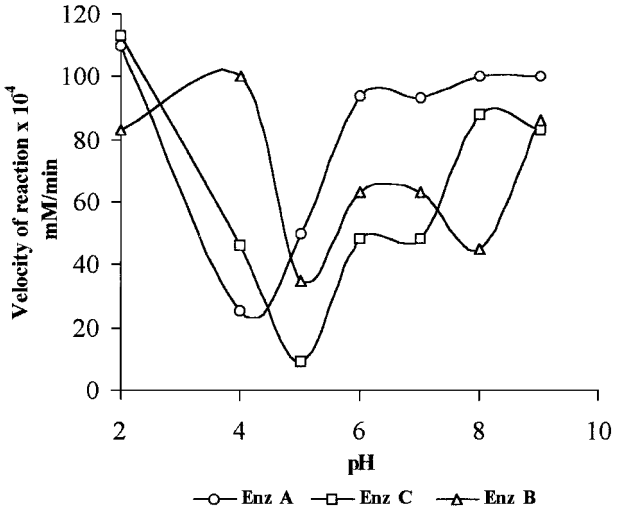

Fig. 4. Change in reaction velocity with pH.

mM hydrogen peroxide as oxidising agent, 2 U of enzyme and volume being made up to 10 ml with 50 mM phosphate buffer at pH 6. The rate of consumption of hydrogen peroxide observed in the peroxidation reaction gives a negative slope with increase in time as seen in Fig. 5. This supports the consumption of the oxidising agent in the peroxidation reaction.

During the toxicity test, initially 5 ml of 1 mM AFB_1 solution was added to 150 ml of liquid culture of *B. megaterium*, which gives a final concentration of about 10 μg AFB_1 ml^{-1}. To an optimum reaction set up as stated earlier, enzymes A, C and B were added. After the reaction, 5 ml of each reaction mixture was added to 150 ml culture, which gives approximately 4.5, 6.4 and 7.37 μg/ml of AFB_1 concentration, respectively. The inhibitory effect was highest in the case of the initial reaction mixture containing no enzyme and lowest in the case of mixtures containing no toxin. As the more purified enzymes were used for the reaction, toxicity

Table 2
Summary of reaction of HRP with AFB_1

Enzyme fraction	Initial conc. of AFB_1 (μg/ml)	Final conc. of AFB_1 (μg/ml)	Degradation (%)	Specific activity$\times 10^{-6}$ (g mol/mg/1/h)
Raw juice	312	29	5.8	0.017
Enzyme B	312	221	29.1	0.580
Enzyme C	312	192	38.3	6.3
Enzyme A	312	141	54.7	0.055
Enzyme C[a]	312[a]	180	42.2	7.05
Enzyme A[a]	312[a]	135	56.6	0.056

[a] Fractions were acted upon AFB_1 purified from *Aspergillus flavus* ATCC 15517 grown in sucrose low salt medium.

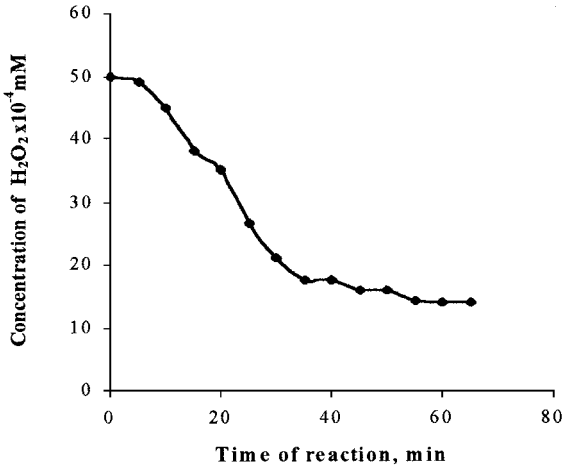

Fig. 5. Consumption of H_2O_2 with time of reaction.

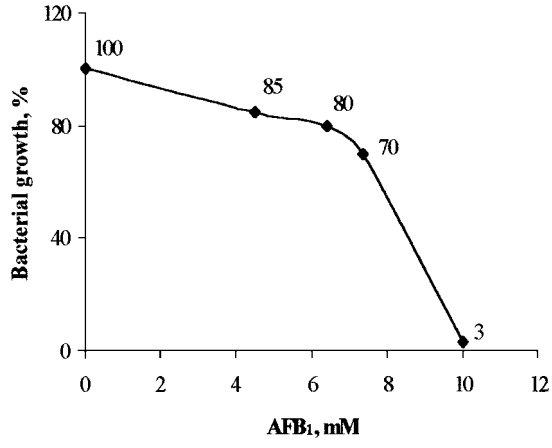

Fig. 6. Growth inhibition of *Bacillus megaterium*.

decreased, resulting in less inhibition of growth. The inhibitory effect starts at 4.5 μg/ml of AFB_1 but markedly slows growth beyond 7.37 μg/ml of AFB_1 and drops to 3% growth in the case of 10 μg/ml of toxin, leading to complete inhibition with further increase in concentration. A simple curve in Fig. 6 shows the increase in growth inhibition with increase in toxin concentration. The same results were obtained with

several other bacteria and fungi including *B. subtilis*, *Asperigillus niger* and *Trichoderma viride* (data not shown).

4. Conclusions

Raw juice of radish, having 0.6 U/mg of protein, has a very low specific activity and very short enzyme stability. This may be due to autodigestion of the enzyme in the presence of other plant proteases or inhibitors in the raw juice. Addition of crude juice to substrate did not show any significant reaction. The enzyme was further purified but, to minimise the cost of purification as well as the detoxification process, only partial purification was achieved. In vitro enzymatic detoxification is free from the possibility of further toxification which may occur after using microbiological methods. In vivo metabolism of ingested AFB_1 by some hepatocellular enzymes induced by antioxidants, such as ethoxyquin or phenobarbital, is promising but in vitro detoxification has more commercial importance. Moreover, cruciferous plants used here as enzyme sources also add 1,2 dithiole-3-thione which is a natural antioxidant and H_2O_2, used as oxidising agent, inhibits fungal growth. The pure enzyme shows much higher activity but cannot bring down the toxin concentration to the threshold level. Also, the purification process escalates the cost of the enzyme. So, work is in progress to find out a possible solution to the aflatoxin problem which may be a combination of simple physical or chemical detoxification process supported by HRP enzyme or some other enzymatic reaction. This may be done initially by treatment of the aflatoxin-infected sample by short term heat treatment, or brief exposure to UV radiation followed by enzymatic reactions which may result in complete removal of the toxin with no adverse effect on quality.

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