

Detoxification of Aflatoxin B1 by Extracellular Enzymes of *Aspergillus oryzae* KKB4

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ABSTRACT

Aflatoxin B1 (AFB1) is the common mycotoxin in food from tropical countries and the most harmful mycotoxin to human health. Detoxification is important step in food processing, in order to get foods free from AFB1, because of the resistance of this mycotoxin against the ordinary processing conditions. The ability of Aspergillus oryzae KKB4 on detoxification of AFB1 was evaluated. The strain used in this was an indigenous proteolytic Aspergillus, isolated from koji, the intermediate product of soy sauce fermentation. Preliminary test indicated that the crude extracellular enzymes produced by the mold able to inactivate or detoxify AFB1. Gel filtration of the enzyme extract resulted in five protein fractions and all of them able to decrease and detoxify AFB1. The highest specific activity was 3.79 µg AFB1/mg enzyme protein/20 hours. The detoxification products have no observed toxicity effects. It was supposed that the structure of AFB1 was changed and part of AFB1 bound with protein of enzymes.

Key words : Detoxification, aflatoxin, extracellular enzymes, Aspergillus.

INTRODUCTION

The most important aspect on food supply chains and food processing is how to keep foods safe from hazard such as toxins. Mycotoxins,

secondary metabolites produced by molds, are toxic to vertebrates and human (Frisvad and Thrane, 1995). Aflatoxin B1 (AFB1) is the most commonly found mycotoxin in food grain and legumes from tropical countries and the one of the most harmful mycotoxin to human health. The humid tropical conditions and improper post harvest handling resulted in favorable conditions for the growth of aflatoxigenic mold. This circumstances could lead to contributed high AFB1 contamination level of Indonesian grain commodities, especially on during rainy season (Sardjono, *et al.* 1992; Sardjono, *et al.* 1995, Sardjono, 2003; Yamashita *et al.* 1995; and Norhayati *et al.* 1998).

Detoxification is the important step on food processing in order to reduce or eliminate AFB1 from the products, because the molecule of AFB1 is relatively resistant under common processing conditions (Kamimura, 1999). Previous publications have reported biological degradation of AFB1 by using molds (Djien, 1974; Knol, 1998), *Flavobacterium aurantiacum* (Ciegler, 1996; Lillehoj, 1967; Smiley, 2000); *Lactobacillus* and *Propionibacteria* (El-Nezami *et al.* 2000).

It was known from previous research that indigenous proteolytic *Aspergillus* isolated from "koji", identified as *Aspergillus oryzae* KKB4, was able to degrade AFB1 on synthetic medium. In addition, the rate of decreasing on AFB1 was linear with the rate of mycelium growth (Sardjono, *et al.* 1992) and other compound were produced during degradation (unpublished data). The role

of the strain on AFB1 detoxification was not yet known, but it may be extra cellular enzymes takes role on detoxification. The aim of this research was to determine the ability of the extra cellular enzymes produced by the strain on detoxification of AFB1.

MATERIALS AND METHODS

Microorganism

Indigenous proteolytic *Aspergillus oryzae* KKB4 was used in this research. Strain was stored under freeze dried condition, and resuscitation was done before used.

Medium

Potato Dextrose Agar (Oxoid) was used for spore production, and Modified Glucose Ammonium Nitrate medium was used for enzyme production, consist of 50 g glucose, 2.4 g ammonium nitrate, 2.0 g MgSO₄·7H₂O, 1.3 mL of 5% CaCl₂ solution, 1.3 mL mixed of (2% ZnSO₄, 0.2% CuSO₄ and 0.1% CoNO₃·6H₂O), 0,5 g Yeast extract for 1 liter medium (Sardjono, et al., 1992).

Production of extra cellularenzymes

Freeze dried culture was resuscitated in potato infusion (Oxoid) for 24 hrs. The culture was transfered to PDA slant, incubated in 30C for 7 days. Spores were harvested using 0.05% tween 80, separated by centrifugation, and were re-suspended in peptone water. The spore concentration was 10⁷ spore/mL. The amount of 100 mL Glucose Ammonium Nitrate medium in 250 mL flask was inoculated by 1 mL of spore suspension, incubated in room temperature on Recipro shaker NR-10 at 115 stroke/min for 7 days. Filtrate was obtained by filtration of broth using Whatman 41 filter paper, kept at 4C for enzyme separation. Ammonium sulfate was used for saturation followed by ultracentrifugation at 32.000 G at 4C for 30 min. Protein was suspended in 0.05M phosphate buffer, pH=7.0. Dialysis was done at 4C for 18 hrs in dialysis bag with a cut off at 12.000. Toyopearl 35F was used for gel filtration in a column (20 mm X 600 mm), eluted with

0.05M phosphate buffer pH=7.0, at the rate of 3mL / tube / hr , 4C. Each fraction from gel filtration was used for detoxification of AFB1.

Detoxification method

The amount of 130 ng AFB1 in small tube ependorf was reacted with 500 µL enzyme solution from each fraction. Detoxification was done at 30C , pH=7 for 20 hrs in dark condition (Smiley and Draughon, 2000). The same method was used for preheated enzymes, in order to determine the activity of enzymes after heat treatment at 90C for 15 minutes. Aflatoxin was extracted with 500 µL chloroform and quantitatively analyzed on TLC plate by using Cammac TLC scanner-3.

Toxicity test of detoxification products

This test is important to confirm that the products of reaction between extracellular enzyme and AFB1 is safe or has no toxic effect. *Bacillus megaterium*, the most sensitive microorganism to AFB1, was used as indicator for the test (Burmeister and Hesseltine, 1966). The amount of 5 mL nutrient broth in 10 mL test tube was added with 500 µL reaction products and inoculated with 0.5 mL starter of *B. megaterium* and incubated at 30C on recipro shaker NR-10 at 115 stroke/min for 24 hrs. The failure of bacteria to reproduce indicate that the reaction products still have toxic effect. The failure to reproduce was shown by elongation of bacteria cells. For comparison, *B. megaterium* were grown on medium containing 0.25 µg/mL and 2.5 µg/mL AFB1 for 24 hrs.

RESULTS AND DISCUSSION

Enzyme separation.

From gel filtration, five protein fraction were obtained (Fig. 1). Similar pattern were obtained for 5 different batch of samples. They were assigned as Fraction A (1-7), Fraction B (8-11), Fraction C (12-22), Fraction D (23-29) and Fraction E (30-41). All of these fractions were tested for the activity on degrading and detoxifying AFB1

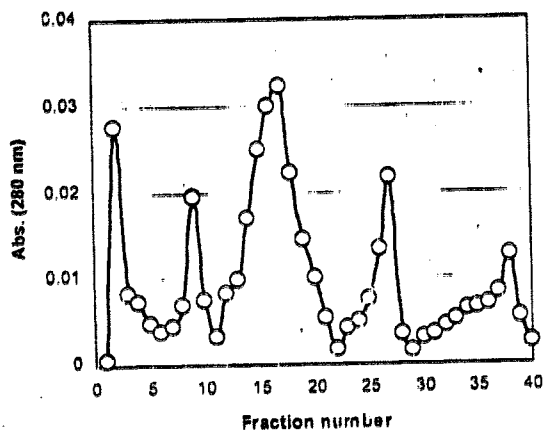


Table 1. The result of gel filtration with Toyopearl 35F

Activities of extracellular enzymes for degrading and detoxification of AFB1

The activity of each protein fraction on AFB1 degradation and detoxification is shown in Table 1. It was shown that all of enzymes fraction have activity on detoxification of AFB1 and the highest specific activity was observed in Fraction B (3.79 $\mu\text{g AFB1} / \text{mg enzyme protein} / 20 \text{ hrs}$). The enzyme was inactivated by heating as indicated by relatively small fraction of AFB1 was degraded. From AFB1 balance, it was found that certain amount of AFB1 was not remain in chloroform extract, it may be bound on the surface of enzymes protein, either heated or unheated enzymes. The amount of bound AFB1 was not proportional with the protein content of enzymes. The enzyme conformation may have significant role on different capability of enzyme to bind AFB1.

Table 1. Activities of enzymes fraction

AFB1 in medium (ppb)	Fraction	AFB1 after reaction (ppb)	Total activity ($\mu\text{g AFB1/mL/20 hrs}$)	Enzyme protein ($\mu\text{g/mL}$)	Specific activity ($\mu\text{g AFB1/mg protein/20 hrs}$)
260	A	132.08	75.84	13.7	5.5
260	B	136.32	52.24	13.78	3.79
260	C	143.60	44.96	29.8	1.17
260	D	137.6	65.12	41.16	1.56
260	E	138.72	55.92	41.16	1.39

Toxicity of AFB1 detoxification products

Toxicity of AFB1 on *B. megaterium* was observed by the failure of bacteria to reproduce and cell became longer and longer. It was shown from Fig 2B that 0.25 $\mu\text{g/mL}$ of AFB1 makes bacteria cell became longer (6 – 7 μm) compared the normal cell (3 – 4 μm), while the addition of 37.5 ppb AFB1 cells failed to reproduce, and in higher concentration, cell were lyses (Fig2). This suggests that fraction A and B of extracellular enzyme effectively detoxified AFB1, as indicated by the normal growth of *B. megaterium* (Fig. 3B and 4B). Similar results were obtained for fraction C, D and E (figures not shown). However, when enzymes were heated at 90C for 15 minutes before reaction with AFB1, intoxication of *B. megaterium* was observed as indicated by failure to reproduce, bacteria cells became longer than the normal cell (Fig. 3C and 4C). It means that reaction products obtained from heated enzyme and AFB1 still toxic to *B. megaterium*. Heating at 90C inactivated the enzymes and failed to detoxify AFB1.

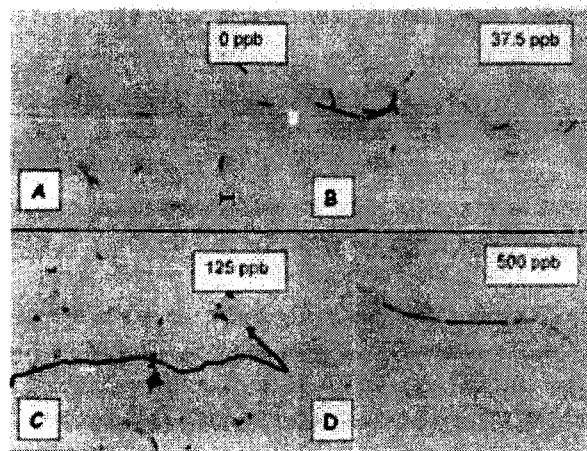


Figure 2. Cell of *Bacillus megaterium* grown in medium spiked with AFB1 (bar = 3 μm)

- A. Normal growth of cells (0 ppb)
- B. Cells failed to reproduce, become longer (37.5 ppb)
- C. Cell became very long (very hard intoxicated) (125 ppb)
- D. Cells are lysis (500 ppb)

CONCLUSION

Extracellular enzymes produced by *Aspergillus oryzae* KKB4 able to degrade and detoxify AFB1. The highest activities was observed in fraction D from gel filtration 15.65 ng AFB1 / μ g enzyme protein / 20 hrs. Heating at 90C for 15 min inactivated the enzymes. It may suggest that AFB1 was changed in its structure and part of it are bound with enzymes protein, either heated or unheated enzymes. Further research on characterization of enzymes and elucidation of functional groups of AFB1 molecule is still underway.

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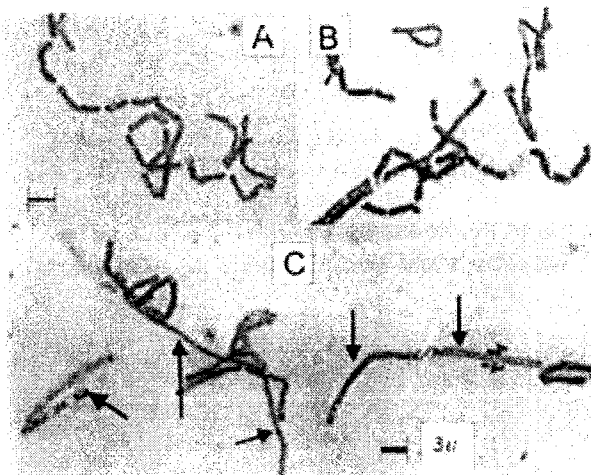


Figure 3. Toxicity test of detoxification products of AFB1 and Fraction A of extra cellular enzymes.

- Normal growth of *Bacillus megaterium*.
- Cell were grown in medium containing the detoxification (reaction) product of AFB1 and fraction A of extracellular enzyme.
- Cell were grown in medium containing the reaction product of AFB1 and heated fraction A of extracellular enzyme

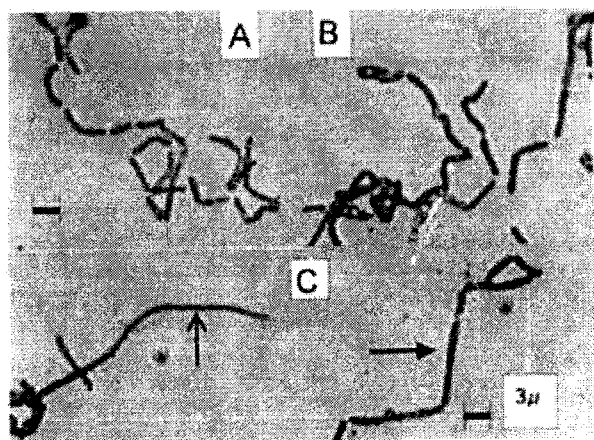


Figure 4. Toxicity test of detoxification products between AFB1 and Fraction B of extracellular enzymes.

- Normal growth of *Bacillus megaterium*.
- Cell were grown in medium containing the reaction product of AFB1 and fraction B of extracellular enzyme
- Cell were grown in medium containing the reaction product of AFB1 and heated fraction B of extracellular enzymes.

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