The Role of Extracellular Enzymes Produced by *Aspergillus oryzae* KKB4 in Biodegradation of Aflatoxin B1

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ABSTRACT

Previous research showed that Aspergillus oryzae KKB4 able to degrade aflatoxin B1 (AFB1) during its growth. According to detoxification test, it was known that extracellular enzymes produced by this mold was able to detoxify AFB1. In this study, the role of these enzymes in biodegradation of AFB1 were further studied. Biodegradation products of AFB1 were analyzed using HPLC and the destruction of functional groups were analyzed using IR spectrophotometer. Molecular weight and enzymes activity were also determined. The result showed that extra cellular enzymes of Aspergillus oryzae KKB4 consist of five fractions with molecular weight of 14.2, 20, 23, 29 and 36 kDalton. All enzyme fractions were able to degrade AFB1 with the highest specific activity 3.79 ìg AFB1/mg enzyme protein/20 hrs. The HPLC analysis indicated that biodegradation product of AFB1 was different from untreated AFB1. The infrared spectrum suggest that enzymes degraded lactone ring, cyclo-pentanone reduction and openined difuran ring.

Keywords: Aspergillus oryzae, extra cellular enzymes, aflatoxin B1, detoxification.

INTRODUCTION

The contamination of foods and feedstuffs with aflatoxins is a serious problem for human and livestock health. 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 make high AFB1 contamination level of Indonesian grain commodities, especially during rainy season (Sardjono, *et al.* 1992; Sardjono, *et al.* 1995, Sardjono, 2003; Yamashita *et al.* 1995; and Norhayati *et al.* 1998). Many experiments therefore have been performed to reduce the level of aflatoxin in contaminated crops.

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 food 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). *Rhizopus*

spp were able to transform 87% of AFB1 into non-fluorescent substances of yet unknown nature. *Rhizopus oryzae* and *R. oligosporus* have been reported capable of reducing the cyclopentanone moiety, resulting in formation of aflatoxicol (Janssen et al., 1997). Dairy strains of Lactic acid bacteria and Bifidobacteria able to bind AFB1, more than 50% AFB1 was bound throughout a 72 hours incubation period and reaction is reversible (Peltonen et al., 2001). Preliminary evidence was found that AFB1 degradation by *Flavobacterium aurantiacum* is enzymatic(Smiley et al., 2000). In lactic acid fermentation at pH d" 4.0, AFB1 is readily converted into AFB2a (Janssen et al., 1997) and into AFB2 (Shukla et al., 2002).

Our previous study indicated that *Aspergillus oryzae* isolated from koji, reduced the AFB1 content during its growth in AFB1 contaminated media. The rate of decreasing AFB1 was similar with its mycelium growth (Sardjono et al, 2004). The extra cellular enzyme of the strain have therefore been performed to detoxify AFB1(submitted to the Indonesian Food and Nutrition Progress). The objective of the study was to characterize the molecular weight of the extracelular enzymes and to observe the change of AFB1 molecule. The biodegradation of functional group of AFB1 were determined by Infra Red spectroscopy, Rf value on TLC and the retention time of HPLC analysis.

MATERIALS AND METHODS

Culture

Strain was originally obtained from koji (Sardjono et al., 2004), and currently kept at the Laboratory of Biotechnology Faculty of Agricultural Technology, stored under freeze dried condition. Strain was resuscitated in potato infusion (Oxoid) for 24 hours, and transferred on Potato Dextrose Agar (PDA) slant.

Preparation of extra cellular enzymes

Production of spore for an inoculum using PDA slant. Incubation was carried out at 30ÚC for 7 days Spores were collected using 10 ml of 0.05% Tween 80. After centrifugation, spores were resuspended in peptone water and adjusted the concentration to be 10^7spore/ml.

Production of extracellular enzyme using Glucose Ammonium Nitrate (GAN) liquid medium. One milliliter of spore suspension was inoculated into 100 ml liquid medium in 250 mL flask. Incubation was done at room temperature on Recipro shaker NR-10 at 115 strokes/minute for 7 days.

Extracellular enzymes was found by separation of spent medium using Whatman filter paper 41. Precipitation of protein was carried out using Ammonium sulfate, followed by ultracentrifugation at 32,000 *G* for 30 minutes. All works were carried out at 4ÚC. Pellet was suspended in 0,05M phosphate buffer pH = 7.0

Preparation of crude enzymes. Dialysis was done at 4ÚC for 18 hours in dialysis bag with a cut of at 12,000. Toyopearl 35F was used for gel filtration in a column (20X600 mm), eluted with 0,05M phosphate buffer pH = 7, at flow rate of 3 ml/tube/hr. Each fraction of enzyme from gel filtration was collected for evaluation their capability to degrade AFB1. Enzymes were stored in freeze dried condition.

Enzymes activity and their capability to degrade AFB1.

The amount of 130 µl standard solution of AFB1 (10 µg/ml) was transferred into eppendorf tube. After solvent was evaporated, 500 µl enzymes solution (0.5 g freeze dried in 1 ml 0.05 M Phosphate buffer pH=7.0) was added and mixed well. Duplicate samples used for each of enzymes fractions. The reaction between AFB1 and enzymes was done in dark condition at 30ÚC for 20 hrs (Smiley and Draughon, 2000). Reaction between AFB1 and phosphate buffer was used for control. Enzymatic reaction was stopped by heated the sample at 90ÚC (water bath) for 15 minutes. Aflatoxin was extracted with 500 µl chloroform and quantitatively analyzed on TLC plate by using Cammag TLC scanner-3. Enzyme activity was difined as µg AFB1 degraded/mg protein/20 hours. The same method was used for preheated enzymes

at 90ÚC for 15 minutes, in order to know the effect of heat on the enzymes activity.

Analysis of biodegradation products.

Biodegradation products of AFB1 by each of enzymes fraction were analyzed using HPLC and the functional groups were analyzed using Infra Red Spectrophotometer. Beckman HPLC with reverse phase ODS-80 column (250 x 4.6 mm), particle size 5 µm was used for analysis. Wave length of emission was 435 nm and excitation at 365 nm., with fluorescence detector. Methanol was used as mobile phase at 0.5 ml/ min.

Molecular weight of enzymes fraction

Sodium Dodecyl Sulphate Polyacrilamide Gel Electrophoresis (SDS-PAGE) used for separation of enzymes fraction based on molecular weight. Running was done in Bio-Rads apparatus with 60 mA 220 volt. Protein was stained with Coomassie Brilliant Blue and the molecular weights estimated using molecular weight protein kit as standard.

RESULTS AND DISCUSSION

Extracellular enzymes of *Aspergillus oryzae* KKB4

From 5 different batches of separation with gel filtration, it was found that extracellular enzymes of *A. oryzae* consist of 5 protein fractions (Figure 1). According to the results of SDS-PAGE as shown in Figure 2, the molecular weight of fraction A (36 kDalton), fraction B (29 kdalton), fraction C (23 kdalton), fraction D (20 kDalton) and fraction E (14.2 kDalton).

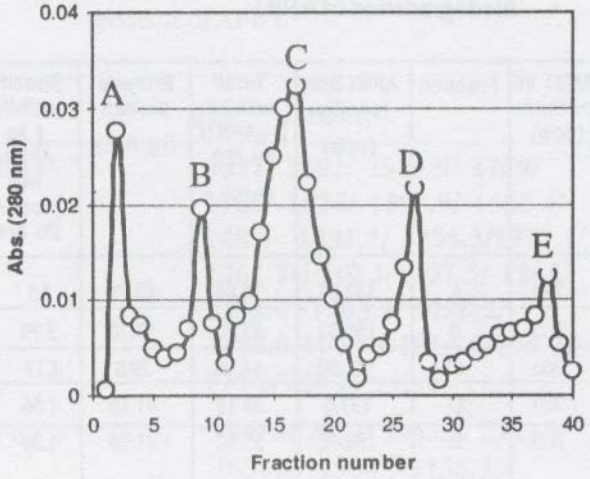

Figure 1. Gel filtration of extra cellular enzyme

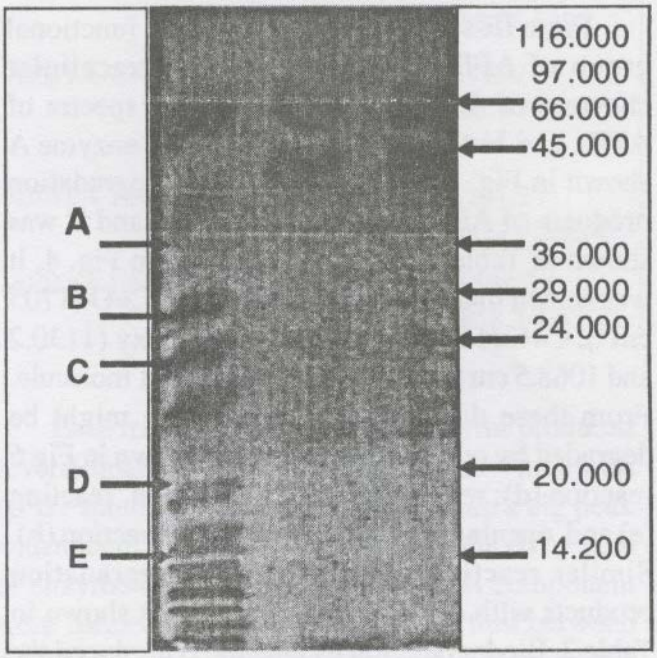

Figure 2. SDS-PAGE electrophoresis of extra cellular enzyme 1. sample batch1, 2. sample batch 2, 3. MW Marker

All of these fractions were tested for the activity on degrading of AFB1. The activities of each fraction were shown in Table 1. It was shown that AFB1 had been degraded by each fraction of enzymes. Their activity were different with the highest activity was found in fraction A with 3.79 ìg AFB1/mg protein/20 hrs. Heated enzymes at 90ÚC for 15 minutes did not show their activities on degrading of AFB1 (data not written).

Table1. The ability each fraction of extra cellular enzyme on biodegradation of AFB1

AFB1 in medium (ppb)	Fraction	AFB1 after reaction (ppb)	Total activity (1gAFB1/ mL/20 hrs)	Enzyme protein (1g /mL)	Specific activity (1g AFB1/ mg protein/ 20 hrs)
260	A	132.08	75.84	13.78	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

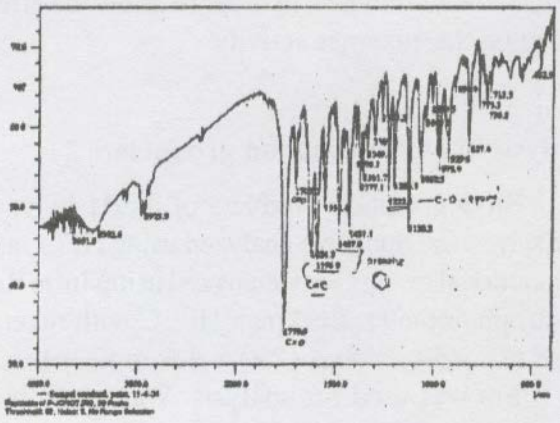

Figure 3. Spectrum of AFB1

Functional groups of biodegradation products

From IR spectra, it was shown that functional group of AFB1 was changed by extracellular enzymes of *A. oryzae* KKB4. The IR spectra of AFB1 and biodegradation of AFB1 by enzyme A shown in Fig. 3 and Fig. 4, while biodegradation products of AFB1 by enzyme B, C, D and E was shown in Table 1. If Fig. 3 compared to Fig. 4, it was shown that the functional group of C=O (1705 cm^{-1}), C=C (1624 cm^{-1})and C-O-C epoxy (1130.2 and 1068.5 cm^{-1}) disappeared from AFB1 molecule. From these data, the AFB1 molecule might be degraded by opening lacton ring as shown in Fig 6 reaction (d); reduction of cyclopentanon, reaction (e) and opening bisfuranoid structure, reaction (b). Similar reaction was found in biodegradation products with other enzyme fraction as shown in Table 2. Biodegradation by enzyme B produced the less functional groups, and from chromatogram of HPLC as shown in Fig. 5a, this enzyme produced the higher number of small fraction compared to the other. Because of the concentration was very small, it was very difficult to elucidate the structure of biodegradation products.

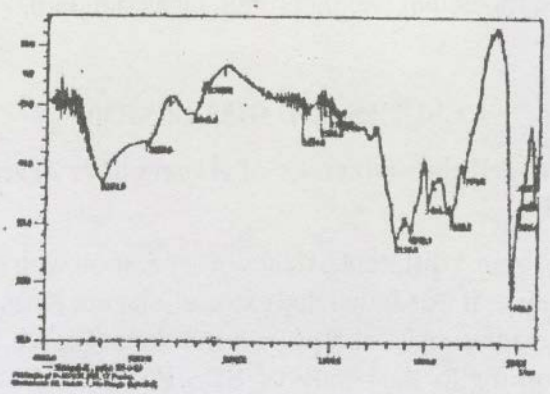

Figure 4. Spectrum of biodegradation of AFBI by enzyme A

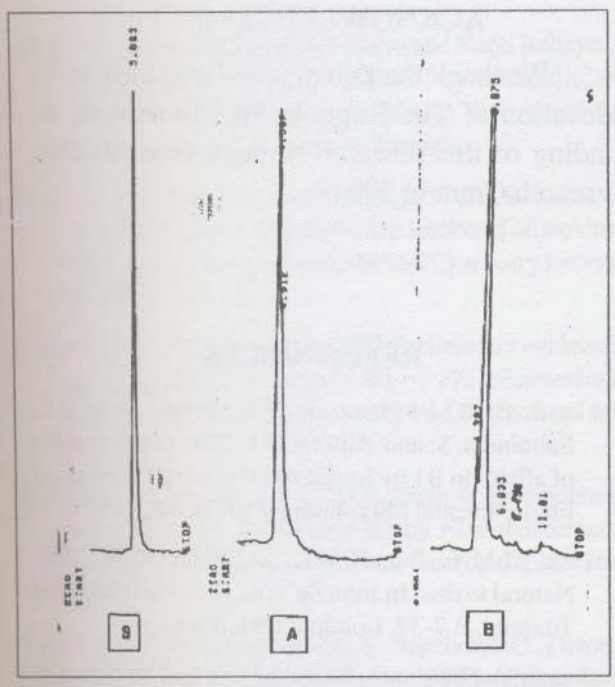

Figure 5a. Chromatogram of AFB1 (S), biodegradation product of AFB1 by fraction A (A) and biodegradation product of AFB1 by fraction B (B)

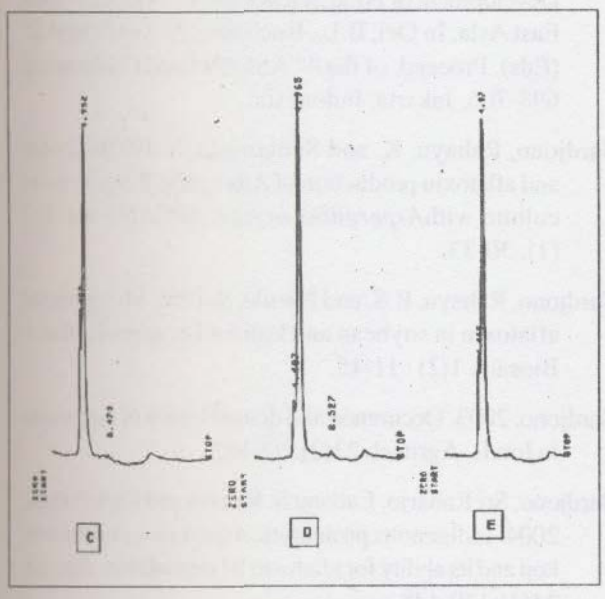

Figure 5b. Chromatogram of biodegradation product of AFB1 by fraction C(C); biodegradation product of AFB1 by fraction D (D) and biodegradation product of AFB1 by fraction E (E)

Table 2. IR spectrum data of AFB1 and biodegradation products of AFB1

	IRv^{KBr} max/cm
AFB1	3651/ 3392/ 2923.9/ **1759/ 1705/ 1624/ 1596.9/** 1558.4/ **1487/ 1431.1/ 1398.3/1377.1/ 1361.7**/1349.4/1307.5/ **1265/ 1232.4 /1203.5/ 1130.2/ 1068.5/1043.4/1014.5**/894.9/ 775.3/736.8/ 715.5
AFB1 + ENZ A	3409.9/ 2922/ 2443.6/ 2302.8/ 1654.8/ 1560.3/1155.3 / 1078.1/985.6/ 860.2/ 794.6/ 532.3
AFB1 + ENZ B	3413.8/ 1654/ 1508.2/ 1458/ 1170.7/ 1076.2/ 858.3/ 530
AFB1 + ENZ C	3392.6/ 2923.9/ 2449.4/ 1541.0/ 1298 / 1166.9 / 1076.2/ 860.2/530.4
AFB1 + ENZ D	3413.8 / 1654.8 / 1157.2 / 1076.2 / 947.0 / 860.2 / 530.4
AFB1 + ENZ E	2885.3 / 1654.8 / 1508.2 / 1166.9 / 1076.2 / 858.3 / 532.3

Each fraction of extracellular enzyme produced several small peak with different retention time with AFB1 standard, but in general there are a big peak with retention time closer to AFB1 produced by each of enzyme fraction (Table 2). Several component were formed in small part after reaction between AFB1 and extra cellular enzymes, but the major component were obtained closer to AFB1 standard (Table 3, bold). The retention time was less than AFB1. It was indicated that AFB1 had been changed into relatively more polar compound. Very small peaks were shown in reaction product between AFB1 with enzyme B, D and E and we did not prove it (Figure 5a and Figure 5b).

The most interested finding was that the toxic C-O-C epoxy group was disappeared from AFB1 molecule. It had been proved that the reaction products had no toxic effect according to the results of toxicity test of biodegradation products towards *Bacillus megaterium* (Sardjono et al, 2004).

Tabel 3. Retention time of peak of biodegradation products of AFB1 analyzed by HPLC

SAMPLE	TIME (minute)									
STANDART AFB1						5,088				
AFB1 + enzyme A			4,54	4,912						
AFB1 + enzyme B	4,387				5,075		6,833	7,74	8,458	11,01
AFB1 + enzyme C			4,457	4,952					8,473	
AFB1 + enzyme D		4,468		4,97						
AFB1 + enzyme E		4,407		4,965					8,527	

Detoxification of AFB1 with extra cellular enzymes of *Aspergillus oryzae* KKB4 is effective. HPLC and IR spectra confirmed that biodegradation products of AFB1 was a different substance from AFB1. It was also consistent with previous result on TLC plate it was different Rf value with AFB1 (data not shown). It is preliminary evidence that biodegradation by mold is enzymatic and according to the results, extracellular enzymes of *A. oryzae* capable on degrading of AFB1 probably through change in bisfuranoid structure, opening lactone ring and reduction of cyclopentanon. Change in bisfuranoid structure of AFB1 also supported by the result of toxicity test by using *B.megaterium* , that biological degradation of AFB1 by extracellular enzymes of *A. oryzae* KKB4 had no toxic effect.

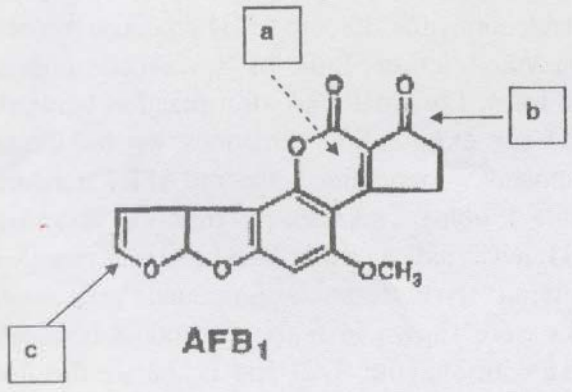

Figure 6. Proposed biodegradation of AFB1
a. degradation of lacton ring
b. reduction of cyclopentanone
c. opening difuran ring

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