料分析を行うプロトコールを作成することとした。

・一部の試料が 8 機関より少なくなっても室間共同試験が即失敗とは考えず、専門家委員会でプロトコルが妥当か判断している場合がある。

・LOD、LOQ 算出時の有効数字の取り扱いを統一すべきである。

・各機関の報告してきた LOQ を ng/g 単位に再計算すると機関 10 の 0.05ng/g、機関 3 の 0.025ng/g 以外は 0.01ng/g 以下となった。

・この分析法の LOQ は 0.01ppb、適用可能範囲は 0.01ppb～1.0ppb となる。

・今回のコラボの分析法は試験法として問題ないので、通知法として採用できるとの評価が得られた。

(2) 総アフラトキシン分析のクライテリア(案)について　資料 2-4(案), 2-5 (農薬)

・LC/MS/MS を使用できるようにとの目的で作成している。アフラトキシン B1 も同じ理由で審議することとなった。原案を審議し、作成した。

・添加量はそれぞれ 10ppb と 1ppb とする。

・アフラトキシンは発がん性があるのであまり高濃度ではリスクが高い。

・総アフラトキシン分析の対象食品は落花生、アーモンド、ピーナッツ、ヘーゼルナッツとする。B1 は全食品である。

(3)　総アフラトキシン分析通知法 (案) について

・総アフラトキシンのイムノアフィニティー法について：イムノアフィニティーカラムに負荷する前に水希釈が良い場合が多い。抽出前に NaCl を加えることとする。希釈については注解に記載する。

Ⅱ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
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Ⅲ. 研究成果の刊行物・別刷

An inter-laboratory study to validate quantitative and qualitative immunoassay kits for screening test of aflatoxin B_1 in corn

Yoshiko SUGITA-KONISHI[1], Shingo NIIMI[2] and Kei-ichi SUGIYAMA[1]

[1] Division of Microbiology, National Institute of Health Sciences
(1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan)
[2] Division of Biological Chemistry and Biologicals, National Institute of Health Sciences
(1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan)

Collaborators: Eiichi ISHIKURO[3], Toshitsugu TANAKA[4], Yoshitsugu SUGIURA[4], Yoshinori ITOH[5] and Masahiro NAKAJIMA[6]

[3] The Fertilizer and Feed Inspection Service
(2-1, Shinntoshin, Chuo-ku, Saitama, 330-9733, Japan)
[4] Kobe Institute of Health
(4-6 Minatojima-Nakamachi, Chuo-ku, Kobe, Hyogo 650-0046, Japan)
[5] Division of Biomedical Food Research, National Institute of Health Sciences
(1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan)
[6] Nagoya city Public Health Research Institute
(1-11, Hagiyama, Mizuho-ku, Nagaya 467-8615, Japan)

Summary

To validate commercial kit for screening of the presence of AFB_1 in corn, an inter-laboratory study was conducted to evaluate three quantitative and two qualitative immunoassay kits designed for the detection of aflatoxins. Four laboratories performed a screen for the presence of aflatoxin B_1 (AFB_1) in corn. As for the quantitative kits, repeatability relative standard deviation (RSD_r) and reproducibility relative standard deviation (RSD_R) were estimated. One laboratory evaluated the lot variation of each quantitative kit. All kits used in this study showed that the RSD_r and RSD_R were less than 23.3 % and 35.7 %, respectively, in spiked or naturally contaminated corn samples. As for the qualitative kits, neither false positive nor false negative results were found in corn samples (blank, spiked or naturally contaminated samples) in any laboratories. The RSDs in the lot variation of the same quantitative kit was less than 46.5 % in both two brands. The results appeared to indicate that all kits tested in this study could be validated for the screening of the presence of AFB_1 in corn, and were also available for the first step of the detection of AFB_1 at levels of more than 5 ng/g.

Key words : inter-laboratory study, immunoassay kit, aflatoxin, corn

(Received: January 15, 2007, Accepted: April 23, 2007)

Introduction

Aflatoxins (AFs: Aflatoxin B_1, B_2, G_1 and G_2) produced by *Aspergillus flavus* and *A. parasiticus* are well-known as carcinogenic mycotoxins. Especially, Aflatoxin B_1 (AFB_1) is the most frequently encountered among AFs and possesses a high toxic potential[1] responsible for human liver cancer. The contamination of AFs has been found in corn, beans, peanut, tree nuts as well as in animal feed[2].

Many analytical methods have been conducted to detect AFs in foods and feeds[3,4], however, most of them require sophisticated equipment and are time-consuming. Recently commercially available immunoassay kits were introduced for the detection of AFs. These assays are timesaving and do not use expensive instruments. The positive and negative results can be visualized directly or measured by a micro-well reader. These rapid analytical immunoassay kits for mycotoxins have been supplied from several chemical companies, and it is convenience to detect the target easily. The commercial kits are divided into two analytical types. One is a quantitative determination such as enzyme-linked immunosorbent assay (ELISA) and quantitative lateral flow test. The other is a qualitative rapid detection such as rapid membrane-based enzyme immunoassay.

To estimate the efficiency of commercial immunoassay kits in screening of corn containing AFB_1 exceed the regulatory limit, we carried out four inter-laboratory studies using blank, spiked, and naturally contaminated corn. Additionally, the variation of lots in the same kit was carried out by one of the participating laboratories. Using the same samples, the assays were performed in triplicate in each lot of the kit.

Materials and Methods

Immunoassay kits Table 1 summarizes the specification of 5 commercial kits for this study with target chemicals and detectable ranges. The kits A, C and E are for total aflatoxins, while kits B and D are only for AFB_1. As commercial kits, Agra Quant Total Aflatoxin Assay 4/40 (Romer Lab.), ROSA Aflatoxin quantitative (Charm Sciences inc.), RIDASCREEN FAST aflatoxin (R-biopharm Rhone Ltd.), AFLACARD B_1 (R-biopharm Rhone Ltd., 4 ng/g Cut-off.) and AgraStrip™ Total Aflatoxin Test (Romer Lab., 4 ng/g Cut-off) were used. Due to the Food Sanitation Law of Japan that defines the guideline to regulate only AFB_1 in all foods, AFB_1 was used as a spiked aflatoxin into analytical samples throughout this inter-laboratory study.

Table 1. The specification of the kit tested in the inter-laboratory study

Kit	Quantitative/qualitative	Range for detection (ng/g)	Target for detection
Kit A	quantitavtive (ELISA)	1 ~ 20	total aflatoxin
Kit B	quantitavtive (ELISA)	0 ~ 45	aflatoxin B_1
Kit C	quantitavtive (lateral-flow)	0 ~ 100	total aflatoxin
Kit D	qualitative	> 4	aflatoxin B_1
Kit E	qualitative	> 4	total aflatoxin

Sample preparation and test procedure Blank corn kernels, which contained less than 1 ng/g of AFs, were obtained from the Fertilizer and Feed Inspection Service, Japan. The corn kernels were ground in a grinder to a mesh size of 1 mm. Two spiked samples were prepared by adding an appropriate volume of AFB_1 (1 μg/ml) dissolved in acetonitrile to 10 g of blank material. Final concentrations of AFB_1 were adjusted to 5 ng/g and 10 ng/g. Natural contaminated corn containing 10.7 ± 2.2 ng/g of AFB_1 alone was purchased from R-biopharm Rhone Ltd. as a standard material.

Sample extraction and test procedures were carried out according to the manufacturer's instruction in each kit.

Inter-laboratory study This study has been performed by four laboratories, National Institute of Health Sciences (division of microbiology, division of biological chemistry and biologicals, and division of biomedical food research), Kobe Institute of Health, Nagoya city Public Health Research Institute and Japan Food Research Laboratories. National Institute of Health Sciences (division of microbiology and division of biological chemistry and biologicals) conducted the study containing preparation of all samples and protocols and others were participants. Kits and samples (blank or naturally contaminated corn) were stored at 2-8 ℃ after being received. Test samples were measured with their own micro-well reader at suitable wavelength in the quantitative assay, and the interpretation of the results was visually determined in the qualitative assay. In these experiments, duplicate analyses were performed for each kit.

Statistics Parameters of precision, which are the inter-laboratory means, RSDr, RSD_R, were deduced using an analysis of variances according to the AOAC guideline[5].

Results and Discussion

The aim of this study was to evaluate whether these kits are suitable for the screening of AFB_1-contaminated corn at levels of more than 10 ng/g. Taking account the unexpected errors in the steps of extraction and analytical practice, the cut-off value of the results was set at around 5 ng/g. Therefore in all kits validated in this study, the range for detection covered 5 ng/g.

In Table 2-1, the RSD_r of two quantitative kits (A and B) showed that the RSD_r was 12.7 and 16.6 % for 5 ng/g of AFB_1 spiked sample, 6.2 and 7.4 % for 10 ng/g spiked, and 4.8 and 16.4 % for naturally contaminated samples, respectively, while the RSD_R of kit A and kit B were 20.5 and 20.0 %, 14.6 and 14.5 %, and 13.3 and 30.6 %, respectively. The correlation coefficients of kits A and B in four laboratories were 0.982-1.0 and 0.995-0.996, respectively. Kit C, which is a quantitative lamina flow kit, showed that RSD_r and RSD_R were less than 23.3 % and 35.7 %, respectively. The data indicated that the RSD_r and RSD_R values of kit C were higher than those of kits A and B. In the qualitative kits, shown in Table 2-2, kits D and E showed neither false positive in blank corn nor false negative in spiked or naturally contaminated samples. The results obtained here demonstrated that the quantitative and qualitative kits tested in this study were successfully utilized for the detection of AFB_1 in aflatoxin-contaminated corn.

In Table 3, the results of the variation between different lots of the same kit are shown. The RSD between lots of the kit A was 11.4 % for the spiked corn with 5 ng/g AFB_1, 3.5 % for the spiked corn with 10 ng/g AFB_1, and 10.9 % for naturally contaminated corn. In kit C of the lateral flow method, the

Table 2-1. Interlaboratory study results for determination of AFB_1 in corn by immunoassay tests (Quantitative kit)

	Laboratory No.	Laboratory 1		Laboratory 2		Laboraty 3		Laboratory 4		Results		
	Number of test	test 1 (ng/g)	test 2 (ng/g)	test 1 (ng/g)	test 2 (ng/g)	test 1 (ng/g)	test 2 (ng/g)	test 1 (ng/g)	test 2 (ng/g)	Mean (ng/g)	RSDr (%)	RSD_R (%)
Kit A	Blank	ND	ND	ND	ND	ND	ND	ND	ND	–	–	–
	5 ng/g spike	2.2	3.4	4.4	4.5	4.3	3.8	3.8	3.5	3.7	12.7	20.5
	10 ng/g spike	7.5	7.5	8.4	9.0	7.9	7.3	6.7	5.7	7.5	6.2	14.6
	naturally contamination	8.9	8.2	7.1	7.4	9.2	8.7	7.1	6.6	7.9	4.8	13.3
Kit B	Blank	ND	ND	ND	ND	1.78	1.78	ND	ND	–	–	–
	5 ng/g spike	5.0	5.5	4.1	4.8	4.5	5.7	2.9	4.4	4.6	16.6	20.0
	10 ng/g spike	8.0	7.5	7.0	8.1	9.5	8.7	6.3	6.8	7.7	7.7	14.5
	naturally contaminated	8.3	7.6	3.7	7.5	11.3	11.6	9.2	8.5	8.5	16.4	30.6
Kit C	Blank	1	1	1	1	ND	ND	1	1	–	–	–
	5 ng/g spike	8	5	3	4	4	4	5	6	4.5	23.3	38
	10 ng/g spike	19	12	7	13	14	11	8	12	11.2	2.7	31.2
	naturally contaminated	12	15	5	15	10	9	7	8	9	12.2	35.7

ND: Not determined
RSDr: repeatability relative standard deviation
RSD_R: reproducibility relative standard deviation

Table 2-2. Interlaboratory study results for determination of AFB_1 in corn by immunoassay tests (Qualitative kit)

	Laboratory No.	Laboratory 1		Laboratory 2		Laboraty 3		Laboratory 4	
	Number of test	test 1 (ng/g)	test 2 (ng/g)	test 1 (ng/g)	test 2 (ng/g)	test 1 (ng/g)	test 2 (ng/g)	test 1 (ng/g)	test 2 (ng/g)
Kit D	Blank	–	–	–	–	–	–	–	–
	5 ng/g spike	+	+	+	+	+	+	+	+
	10 ng/g spike	+	+	+	+	+	+	+	+
	naturally contaminated	+	+	+	+	+	+	+	+
Kit E	Blank	–	–	–	–	–	–	–	–
	5 ng/g spike	+	+	+	+	+	+	+	+
	10 ng/g spike	+	+	+	+	+	+	+	+
	naturally contaminated	+	+	+	+	+	+	+	+

ND: Not determined
RSDr: repeatability relative standard deviation
RSD_R: reproducibility relative standard deviation

RSD of corns spiked with 5 ng/g, 10 ng/g, and naturally contaminated corn was 24 %, 46.5 %, and 4.4 %, respectively. In the kits D and E, no positive result appeared in blank sample and no negative result was recognized in corn spiked with 5 ng/g, 10 ng/g or naturally contaminated (data not shown). As for kit B, the manufacturing company supplies only one lot for this study.

In the quantitative assay, the results suggested that kits A and B were more robust than kit C, but they needed a more complicate procedure than kit C. Taking the performance of these kits into account, the users have to choose a kit which is suitable for their own purpose.

Taken together, 5 commercially available immunoassay kits were subjected to an inter-laboratory study involving 4 participants, and the results obtained in the present study validated the accuracy and reproducibility of the quantitative kits for AFB_1 in corn, showing that all kits are also able to screen the presence of AFB_1 in corn.

Table 3. The lot variation of quantitative kits

Kit	Sample	Mean (ng/g)	RSD (%)
Kit A	Blank	-	-
	5 ng/g spike	4.1	11.4
	10 ng/g spike	7.9	3.5
	naturally contaminated	9.5	10.9
Kit C	Blank	-	-
	5 ng/g spike	5.5	24.1
	10 ng/g spike	15.0	46.5
	naturally contaminated	8.8	4.4

RSD: relative standard deviation

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Toxicon 50 (2007) 339–348

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The effect of feeding piglets with the diet containing green tea extracts or coumarin on *in vitro* metabolism of aflatoxin B1 by their tissues ☆

P. Tulayakul, K.S. Dong, J.Y. Li, N. Manabe, S. Kumagai*

Graduate School of Agricultural and Life Sciences, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan

Received 2 October 2006; received in revised form 18 April 2007; accepted 18 April 2007
Available online 22 April 2007

Abstract

To clarify whether enzymes involved in aflatoxin B1 (AFB1) metabolism in pigs respond to antioxidant agents, the effect of feeding piglets with diets containing green tea extracts (Sunphenon) and coumarin on *in vitro* AFB1 metabolism by their liver and intestinal tissues was studied. The results showed that coumarin reduced AFB1–DNA adduct formation by both liver and intestinal microsomes, while Sunphenon did not have any effects. Both coumarin and Sunphenon enhanced the glutathione *S*-transferase (GST) activity to conjugate AFB1 to glutathione GSH in the intestine, although no effects were noted in the liver. Changes of the expression of mRNA of GSTA2 and GSTO1 were not in parallel with the observed changes of GST activity, suggesting that other GST subtypes are involved in the GST activity toward AFB1. As for lipophilic-free AFB1 metabolites, coumarin reduced the liver microsomal conversion of AFB1 to aflatoxin M1 (AFM1) and aflatoxin Q1 (AFQ1), but Sunphenon exerted no effects. Both coumarin and Sunphenon enhanced the conversion of AFB1 to aflatoxicol in the liver. All the results suggest that feeding with a diet containing coumarin affects AFB1 metabolism to enhance AFB1 detoxification through the suppression of P450 enzyme activity in the liver and the enhancement of GST activity in the intestine. Feeding with a diet containing Sunphenon enhances AFB1 detoxification, but the effects are noted mainly in the intestine.

Keywords: Green tea; Coumarin; Aflatoxin B1; Pig; Glutathione *S*-transferase; DNA adduct

1. Introduction

Up until the present, the eradication of aflatoxin B1 (AFB1) contamination in agricultural products has been difficult, especially in drought, hot and humid regions. Climate changes toward an increased atmosphere temperature of the earth make it even more difficult. Therefore, one of the effective methods to overcome the toxic and carcinogenic effects of AFB1 is to enhance AFB1 metabolism toward its detoxification in humans or animals.

☆ *Ethical statement*: All experimental procedures with animals for this study were in accordance with the Guidelines of the University of Tokyo for the care and use of animals for research purposes, and were approved by an ethic committee of the University.

*Corresponding author. Laboratory of Veterinary Public Health, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan. Fax: +81 3 5841 5469.
E-mail address: askuma@mail.ecc.u-tokyo.ac.jp (S. Kumagai).

0041-0101/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2007.04.005

There have been some trials of the intervention of chemo-protective agents, such as ethoxyquin, dithiolethiones, oltripraz and butylated hydroxytoluene, against AFB1-induced DNA damage and carcinogenesis by inducing class I and II enzymes (Manson et al., 1997). Among such agents, green tea polyphenols have recently been reported to be a potent antioxidant and beneficial in oxidative stress, and to inhibit the initiation of AFB1-induced hepatocarcinogenesis in rats and mice (Qin et al., 1997; Chen et al., 2004; Raza and John, 2005). However, the effect of green tea polyphenols on AFB1 metabolism in farm animals is unknown.

The other antioxidant, coumarin (1,2-benzopyrone), is known to be a natural product contained in a variety of plants (Lake et al., 1989; Born et al., 1997, 2003; Beamand et al., 1998). It has been reported that feeding with a diet containing coumarin can prevent the initiation of AFB1 hepatocarcinogenesis in association with the induction of AFB1-aldehyde reductase (AFAR) and μ-class glutathione transferase (GST)A5, π-class GSTP1 and NAD(P)H quinine oxido-reductase in rat liver (Kelly et al., 2000a, b). However, little is known about the effect of these chemicals on AFB1 metabolism or on its adverse effects in farm animals.

To clarify whether enzymes involved in AFB1 metabolism in pigs respond to antioxidant agents, the effect of feeding piglets with diets containing green tea extracts (Sunphenon) or coumarin on *in vitro* AFB1 metabolism by their liver and intestinal tissues was studied.

2. Materials and methods

2.1. Chemicals

[^{3}H]-AFB1 (sp. activity, 18.6 Ci/mmol; 99.6% purity) was purchased from Moravek Biochemicals (California, USA). Green tea extracts (Sunphenon BG: contains 91.3% polyphenol and 76.6% catechin, which contains 9.6% epigallocatechin (EGC), 45.9% epigallo catechin gallate (EGCG), 5.3% epicatechin (EC), 8.6% epicatechin gallate (ECG) and others, w/w) were kindly gifted by Taiyo Kagaku Co., Ltd. (Mie, Japan). Standard AFB1, aflatoxin P1 (AFP1), aflatoxin Q1 (AFQ1), aflatoxin M1 (AFM1), aflatoxicol (AFL), glucose-6-phosphate (G-6-P), G-6-P dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADPH), ethylenediamine-tetraacetate (EDTA), glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), *trans*-4-phenyl-3-buten-2-one (*t*-PBO), ethacrynic acid (EA) and coumarin were obtained from Sigma Aldrich Chemical Co., Ltd. (St. Louis, MO, USA). Cumene hydroperoxide was from Aldrich Chemical Co., Ltd. AFB1-dialdehyde and -dialcohol were synthesized from the AFB1 exo-epoxide according to the method of Guengerich et al. (2001), and confirmed with LC/MS (Tulayakul et al., 2005; Agilent 1100 Series, Germany; ESI-TOF, Tokyo, Japan). The mixture of AFB1-endo and exo-epoxides was chemically synthesized according to the method of Raney et al. (1992). Other chemicals were of analytical grade and obtained from regular commercial sources.

2.2. Animals and treatments

Nine 1-month-old female piglets (Landrace × Large Yorkshire) were purchased from Kadoi Co., Ltd. (Ibaraki, Japan) and were acclimatized for a week under 12 h light on/off conditions with water and feed available *ad libitum*. They were divided into three experimental groups (three piglets in each) as follows: group 1 (control group), received a nursery complete diet (Genki Series); group 2 (Sunphenon group), received a diet supplemented with 0.2% (w/w) green tea extracts (Sunphenon); group 3 (coumarin group), received a diet supplemented with 0.5% (w/w) feed of coumarin. The nursery complete diet with or without the supplement was replaced with phase II diet (Pure Life II-K) containing no supplement or the same supplement after the first 10-day phase I diet period according to the company's recommendation.

2.3. Experimental design

After 3 weeks of feeding trials, all piglets received an intravenous (i.v.) injection of ketamine (10 mg/kg b.wt) and medetomidine (80 μg/kg b.wt), and were euthanized by the i.v. injection of 3 M potassium chloride. Livers and small intestines (jejunum region) were removed, frozen in liquid nitrogen and stored at −80 °C until use. Microsomes and cytosolic fractions were prepared as described previously (Bammler et al., 2000; Esaki and Kumagai, 2002).

2.4. Microsomal activity to form AFB1–DNA adduct

The microsomal activity to form the AFB1–DNA adduct was determined according to the method of Kono and Kumagai (1995). Briefly, 1 mg protein of the microsomes fraction prepared from various organs of piglets was pre-incubated for 5 min at 37 °C with 0.1 mg of calf thymus DNA dissolved in 0.8 ml of 0.1 M potassium phosphate buffer solution (pH 7.4) containing 2 mM NADPH, 5.6 mM G-6-P and 0.5 units of G-6-P dehydrogenase. The pre-incubated mixture was mixed with 200,000 dpm of [^{3}H]-AFB1 dissolved in 10 μl of dimethyl sulfoxide and incubated for 10, 30 or 60 min. The DNA was precipitated with ethanol, and then the radioactivity of [^{3}H]-AFB1 in each sample was counted with a scintillation counter (LSC-5100; Aloka Co., Ltd., Tokyo, Japan) using ACSII (Amersham Pharmacia Biotech UK Co., Ltd., Chalfont St. Giles, Bucks, UK) as a scintillator to determine the amount of [^{3}H]-AFB1–DNA adduct.

2.5. Conversion of AFB1 to free lipophilic metabolites

The determination of the microsomal activity to convert AFB1 to the other free lipophilic metabolites in various organs of piglet was carried out according to the method of Esaki and Kumagai (2002). Briefly, the microsome fraction from various organs was pre-incubated at 37 °C for 5 min with the same buffer as above. The reaction was started by the addition of 5 μg of AFB1 dissolved in DMSO and was stopped by adding chloroform after incubation for 30 min. The chloroform fraction was dried and dissolved in ethanol, and analyzed for free metabolites with HPLC with a Pegasil ODS column (4.6 × 250 mm; Senshu Scientific Co., Ltd., Tokyo, Japan) with the same condition as described in Tulayakul et al. (2006).

2.6. Cytosolic GST activity toward AFB1

The cytosolic GST activity toward AFB1 was determined by the method of Esaki and Kumagai (2002). Briefly, the reaction mixture (0.45 ml) containing the hamster liver microsomes (1 mg protein), the cytosolic fractions from various organs (1 mg protein), 2 mM NADPH, G-6-P dehydrogenase (1 unit), 10 mM G-6-P, 5 mM $MgCl_2$, 1 mM GSH and 1 mM EDTA in 0.1 M potassium phosphate buffer (pH 7.4) were pre-incubated at 37 °C for 5 min. The reaction was started by the addition of 5 μg of AFB1 dissolved in 10 μl DMSO. The reaction mixture was terminated with chloroform at 2 min of incubation. The aqueous layer was loaded on a Sep-Pak C18 column (Waters Corp., Massachusetts, USA), and the AFB1–GSH fraction was eluted with distilled water and loaded on a Pirkle-Concept Chiral column packed with D-phenylglycine covalently bound to aminopropyl silica (4.6 mm × 250 mm; Regis Technologies Inc., Morton Grove, IL, USA) in the HPLC system for the determination of AFB1–glutathione conjugate, as described in Tulayakul et al. (2006).

2.7. AFB1-dialdehyde reductase activity

AFB1-dialdehyde reductase activity in various organs in the piglet was determined. Briefly, AFB1 dialdehyde as a substrate was incubated with cytosol for 30 s at 37 °C. AFB1-dialcohol was analyzed by HPLC using with the same conditions as those for AFB1–GSH conjugate determination.

2.8. Determination of GST activity toward the substrates other than AFB1

The GST activity toward different substrates was determined according to the method of Habig et al. (1974). Substrates used in this study were 1-chloro-2,4-dinitrobenzene (CDNB), ethacrynic acid (EA), *trans*-4-phenyl-3-buten-2-one (*t*-PBO) and cumene hydroperoxide. The specific activity of GST is expressed in nmol/min/mg protein. The protein concentrations of the cytosol or microsome preparations were determined with a spectrophotometer (U-1500; Hitachi Co. Ltd., Tokyo, Japan) using a reagent for protein assays (Bio-Rad Protein Assay, Bio-Rad Lab., Hercules, CA, USA).

2.9. RNA extraction and quantification

Total RNA was prepared from frozen liver and small intestinal tissues using the RNeasy mini RNA extraction kit (RNeasy Mini kit, Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The small residual amount of DNA remaining was removed using the RNase-Free DNase Set (Qiagen). The concentration was determined by measuring the absorbance at 260 nm in a spectrophometer and purified RNA was kept in water at −80 °C until use.

2.10. Real-time RT-PCR (q RT-PCR)

The housekeeping sequences of GADPH genes for use as internal standards in porcine were referenced from Duvigneau et al. (2003). For the internal standard, a series of diluted cDNA (starting concentration of 60 ng/μl) of the livers from Sunphenon-treated piglets were used.

For each target gene, primers and probes were selected and designed with TaqMan® probe. Primers and probe were selected using the following criteria: (1) forward and reverse primers were placed on two consecutive exons of the gene where possible, (2) no G in the 5′-terminus for the hybridization probe, (3) no more than three Gs or Cs within the last five nucleotides in the 3′-termini of primers and (4) the hybridization probe is within 10 bp of the closest primer. All primers and probe sequences are listed in Table 1.

Real-time RT-PCR (q RT-PCR) was performed in a 96-well PCR plate using TaqMan Assay two-step RT-PCR. cDNA (10–100 ng/μl) in distilled water was supplemented with 2 × TaqMan Universal PCR master mix, 10 μM synthesized primers, 5 μM probe and 1 μl of cDNA (about 10–100 ng/μl) to give a final reaction volume of 50 μl. The amplification conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C using a sequencing detection system (ABI PRISM 7000; Applied Biosystems Co., Ltd., Foster, CA, USA). The final analysis was performed by comparison of the mean quantity of the target gene in relation to the internal control.

2.11. Statistical analysis

The values were expressed as the mean ± S.E., except for gene expression by real-time RT-PCR, in which the mean ± S.D. of three piglets was used. Statistical significance was determined using Students-*t* test with the control using the statistic function in Microsoft Office Excel 2003 for Windows XP.

3. Results

3.1. AFB1–DNA adduct formation

The effect of Sunphenon and coumarin on the formation of the AFB1–DNA adduct by piglet tissues was examined using microsomal fractions of the liver and intestine from the three different groups of piglets. The levels of the AFB1–DNA adduct formed by the liver and intestinal microsomes were significantly lower in coumarin-treated group than the control group (Fig. 1A and B). However, no significant differences were noted between the Sunphenon-treated and control groups. Thus, the result of this study showed that coumarin reduced DNA adduct formation by both liver and intestinal microsomes, while Sunphenon did not have any effects on DNA adduct formation in both organs.

3.2. Free lipophilic metabolites

Fig. 2 shows the lipophilic metabolites of AFB1 formed by the liver and intestinal microsomes from three groups of piglets. Coumarin treatment reduced AFQ1 and AFM1 formation by liver microsomes, but Sunphenon treatment exerted no effects on AFB1 metabolism to AFM1 and AFQ1 by liver microsomes. As for intestinal microsomes, Sunphenon treatment increased AFQ1 formation, while coumarin treatment increased AFB2α formation.

Fig. 3A and B shows aflatoxicol formation by microsomes and cytosol, respectively. Coumarin treatment increased remarkably aflatoxicol

Table 1
Primers and probe used for the determination of gene expression by real-time RT-PCR

Target gene	Forward primer	Reverse primer	Probes
GSTA2	5′-GGC CAT CCT CAA CTA CAT CGC-3′	5′-GCT CTC TCC TTG GCG TCC TTC-3′	5′-ACC AAG TAC AAC CTC TAC G-MGB-3′
GSTO1	5′-GCC TGA TCC GTG TCT ACA GCA T-3′	5′-GGC ATT CAG GAC CAG GAG AGT-3′	5′-CTG TCC TTT CGC CCA GAG-MGB-3′
GADPH	5′-TTG CCC TCA ACG ACC ACT TC-3′	5′-CAC CCT GTT GCT GTA GCC AAA-3′	5′-CAA GCT CAT TTC CTG GTA CG-MGB-3′

MGB: minor groove binder.

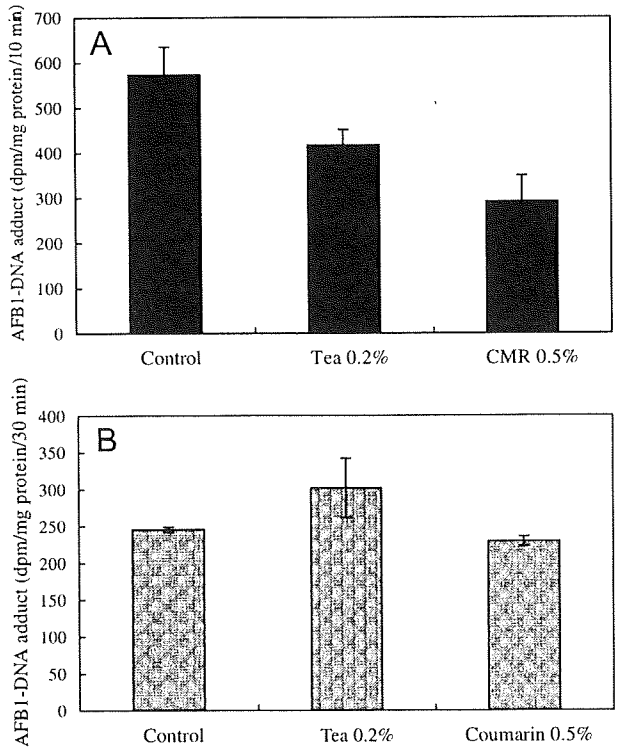

Fig. 1. AFB1–DNA adduct formation by the microsomes fraction in liver (A) and intestine (B). *, **: Significantly different from each control group ($p<0.05$ and 0.01), respectively.

formation by liver microsomes and cytosol. Sunphenon induced also the increase in aflatoxicol formation by liver microsomes, although the effect was smaller than that of coumarin. In the intestinal tissues, neither treatment affected the AFB1 metabolism to aflatoxicol.

3.3. AFB1–GSH conjugate

The GST activity in cytosol to conjugate AFB1 to GSH is shown as the amount of AFB1–GSH conjugate in Fig. 4. The GST activity of the liver cytosol was not different among the groups. The GST activity of the intestinal cytosol at 32 μM of AFB1 was higher in the Sunphenon- and coumarin-treated groups than in the control group, although there were no differences in GST activity at 128 μM AFB1 among the groups.

3.4. Reductase activity toward AFB1-dialdehyde

The cytosolic reductase activity to convert AFB1-dialdehyde to AFB1-dialcohol in the liver and intestine with various treatments is shown in Fig. 5. The intestinal reductase activity at 15 μM AFB1-dialdehyde was lower in the coumarin-treated than the control group. There was no significant difference among groups in the intestinal activity at 50 μM AFB1-dialdehyde and in the liver activity at both 15 and 50 μM AFB1-dialdehyde.

3.5. GST activity towards various substrates

The specific activities of liver and intestinal GST in Sunphenon or coumarin treated, or control piglets are given in Table 2. CDNB served as a universal substrate (Habig et al., 1974; Kunze, 1997). The specific activity toward CDNB was increased by Sunphenon- and coumarin-treatments in both liver and intestinal tissues. The specific activity toward EA was reduced in the intestinal tissue for both Sunphenon and coumarin treated groups, but not in the livers in both treatment groups. The activity toward *t*-PBO was elevated in the livers from the Sunpehnon-treated group. However, there was no significant change in the activity of both the liver and intestine toward cumene hydroperoxide by any treatment.

3.6. Real-time RT-PCR of GSTA2 and GSTO1 expression in piglets

The results of the quantitative RT-PCR studies of the expression of pig GSTA2 and GSTO1 in the livers and intestinal tissues are shown in Fig. 6. The GSTA2 expression in the liver was significantly lower in the Sunphenon-treated group than in the control group. The GSTO1 expression in the intestine was significantly higher in the Sunphenon-treated group than the control group. No significant difference was noted between the treatment and control groups in the GSTA2 expression in the intestine or in the GSTO1 expression in the liver. Also, no significant difference was noted in the GSTA2 expression in the liver or in the GSTO1 expression in the intestine between the coumarin-treated and control groups.

4. Discussion

Studies of supplementation of green tea extracts and coumarin in feed, and its effects on the toxicity and hepatocarcinogenesis of AFB1 in animal species have been limited to rodents. The liver cancer caused by AFB1 has posed serious problems in tropical and subtropical countries (Groopman et al., 1988). Natural substances that can prevent

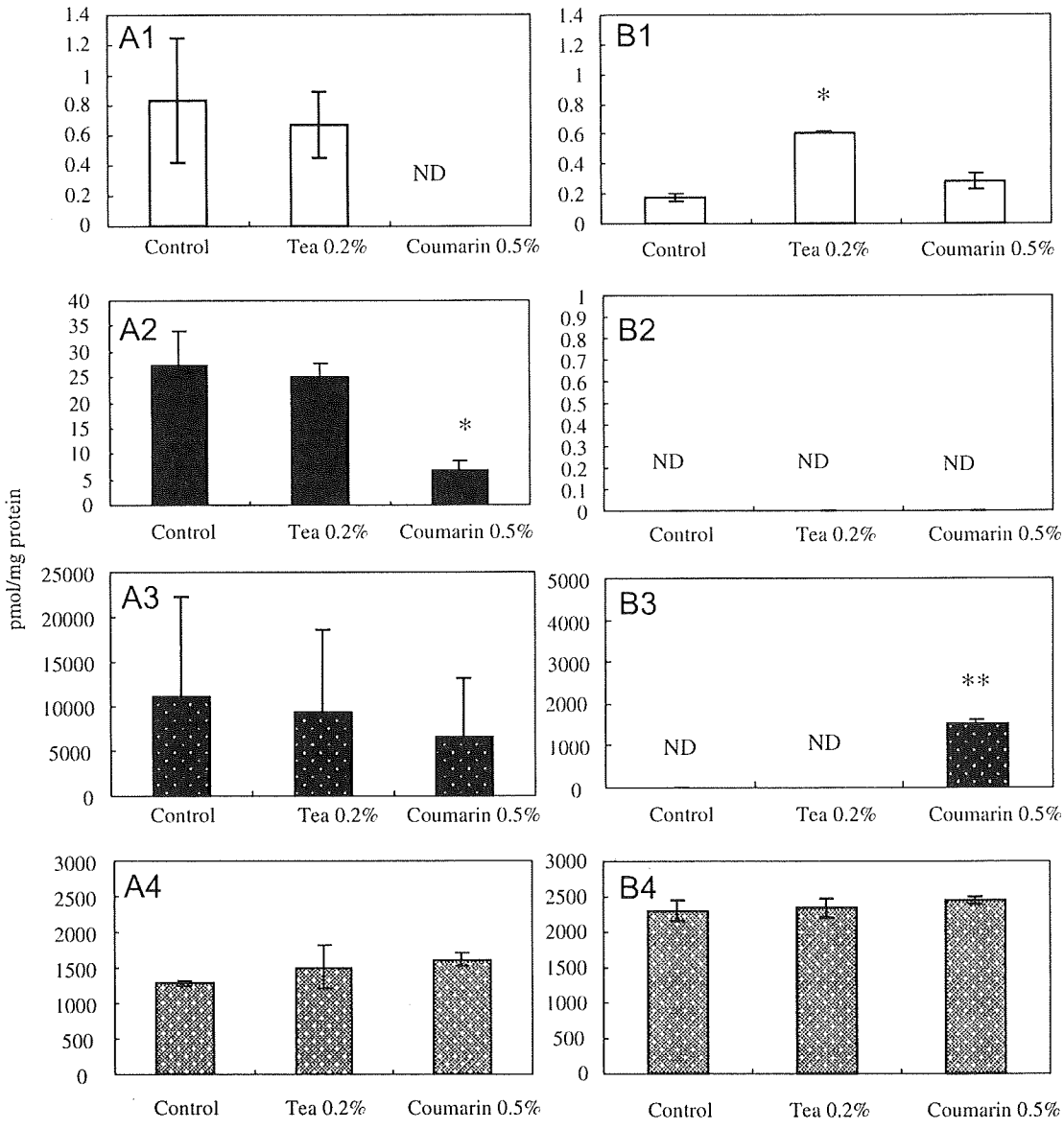

Fig. 2. Formation of lipophilic metabolites of AFB1 incubated for 30 min with the microsome fraction from liver (A) and intestine (B) of the control and Sunphenon- and coumarin-treated piglets: Panels 1, 2, 3 and 4 show AFQ1, AFM1, AFB2α and AFP1, respectively. ND is non-detectable (<0.2 pmol/mg protein). *, **: Significantly different from control ($p<0.05$ and 0.01), respectively.

AFB1 hepatocarcinogenesis and hepatotoxicity would be helpful to human and animal health with minimal cost in foods and feed.

The result of this study showed that coumarin treatment caused the reduction of the metabolic activity of microsomes to convert AFB1 to the AFB1–DNA adduct, AFQ1 and AFM1, but enhanced the activity to convert AFB1 to aflatoxicol in the liver organ. A recent study using human liver microsomes has shown that CYP3A4, 3A5, 3A7 and 1A2 enzymes have more important roles in the conversion of AFB1 to AFB1-epoxide in this order (Kamdem et al., 2006). Therefore, the suppression of these P450 enzymes might be involved in the observed reduction of the formation of AFB1–DNA adduct in coumarin treated piglets. The enhanced aflatoxicol formation may also contribute to the reduction of the AFB1–DNA adduct, because direct interaction of aflatoxicol-epoxide with DNA is minor compared with AFB1-epoxide (Loveland et al., 1987). The reduction of AFB1 conversion to AFQ1 and AFM1 may be caused by the suppression of CYP3A4 and CYP1A1, because these enzymes in pig hepatocytes have been found to