Journal of Microbiological Methods 80 (2010) 44–48

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

A novel biosensor for the detection of zearalenone family mycotoxins in milk

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ARTICLE INFO

Article history:
Received 21 August 2009
Received in revised form 21 October 2009
Accepted 24 October 2009
Available online 1 November 2009

Keywords:
Zearalenone
Photinus pyralis
Luciferase reporter gene
Endocrine disrupter

ABSTRACT

In this study, a method for detecting estrogenic mycotoxin residues in milk was developed utilizing bioluminescent whole-cell biosensors. Milk products of various compositions were spiked with the estrogenic mycotoxins zearalenone and its metabolites zearalanone, α-zearalanol, β-zearalanol, α-zearalenol and β-zearalenol. The estrogenic response was detected by a whole-cell biosensor based on a genetically modified *Saccharomyces cerevisiae* strain that in the presence of an estrogenic compound produces firefly luciferase-enzyme and further light emission within a system provided with D-luciferin substrate. The results show that the yeast sensor reacts to mycotoxins with typical sigmoidal response at nanomolar concentrations. The response differs in different milk products with regard to the fat content of the milk. Due to short assay time of less than 3 h and automation the approach can be used as a bioavailability and activity screening method prior to more detailed chemical analysis.

1. Introduction

Mycotoxins are compounds produced by mould fungi in moist conditions. Approximately 25% of the world's crops are contaminated with mould or fungal growth and mycotoxins may be produced both before and after harvest (Bryden, 2007). In both humans and animals ingestion of food or feed contaminated by mycotoxins can lead to mycotoxicoses, the possible symptoms of which are acute intoxication, losses in productivity, reduced weight gain, immunosuppression and increased risk of cancer (Bryden, 2007; Fink-Gremmels, 1999).

The mycotoxin zearalenone produced by *Fusarium* species as well as its metabolites zearalanone, α-zearalanol, ß-zearalanol, α-zearalenol and ß-zearalenol are harmful for health mainly due to their estrogenic activity. These compounds are capable of binding the estrogen receptor and thus are competitive inhibitors for estrogen hormone causing e.g. problems in mammalian reproductive system (Katzenellenbogen et al., 1979; Le Guevel and Pakdel, 2001). Accordingly, these compounds can be regarded as endocrine disrupter compounds (EDCs) that can alter receptor-mediated process of steroids by mimicking or counteracting natural hormones (Degen et al., 2002).

Milk is a staple in many countries, and nutritionally important due to its high calcium content. So far, no maximum levels have been set for zearalenone concentration in milk or milk products. However, in 2005, the European Union set the maximum permissible levels of *Fusarium* toxins in certain other products: maximum zearalenone levels in unprocessed cereals other than maize and unprocessed maize are 100 μg kg^{-1} and 200 μg kg^{-1}, respectively (Commission regulation (EC) no 856/2005 of 6 June, 2005). In Russia, the maximum level of zearalenone in cereals for human food is 1000 μg kg^{-1} (Creppy, 2002). Cattle are exposed to estrogenic mycotoxins via naturally-contaminated feed or administration of zeranol (α-zearalanol) as a growth promoter. The latter is banned in the European Union (EU) (European Community Council Directive 96/22/EC of 29, April, 1996), but is permitted in the USA and Canada (Le Guevel and Pakdel, 2001). Ruminants have been regarded as tolerant to zearalenone exposure, because predominantly rumen protozoa are capable to converting mycotoxins into less toxic compounds (Fink-Gremmels, 2008; Kiesling et al., 1984). However, also zearalenone compounds have been detected in bovine milk (Mirocha et al., 1981; Prelusky et al., 1990; Coffey et al., 2009) and milk products (El-Hoshy, 1999). Moreover, recently it was reported (Seeling et al., 2005) that rumen metabolic capacity can be saturated depending on varying feeding regimes. The typical bovine diet mainly consists of roughage such as hay, but in the modern farming cows are fed high protein feed designed to bypass the rumen to increase milk yield. This might have an influence on the rumen microbial fermentation capability, therefore also the rumen capacity to metabolize zearalenone. Accordingly, monitoring the presence of mycotoxin in food products is vital in order to minimize the risk of exposure.

Conventionally mycotoxin concentrations have been monitored by chromatography and mass spectrometry (Berthiller et al., 2007; Choi et al., 2002; Nielsen and Thrane, 2001; Sørensen and Elbæk, 2005), as

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doi:10.1016/j.mimet.2009.10.017

well as enzyme immunoassays (De Saeger et al., 2002; Liu et al., 1985) and time-resolved fluorescence based immunoassays (Tuomola et al., 2002). These methods require a complex and time-consuming sample preparation often including extraction, filtration and dilution steps. In addition, the presence of mycotoxigenic fungi, or their DNA, has been detected using PCR (Marek et al., 2003; Niessen, 2007) and micro array -methods (Schmidt-Heydt and Geisen, 2007). These approaches do not measure mycotoxin content directly, and therefore may provide only limited information on the risk of or presence of mycotoxins in a given sample. Since these methods do not monitor the bioavailability of mycotoxins, they report only part of the biological effects.

Genetically modified (GM) yeast cells have been successfully used to evaluate toxicity (Hollis et al., 1999; Välimaa et al., 2008; Walmsley et al., 1997), estrogenicity (Coleman et al., 2004; De Boever et al., 2001; Ike et al., 2002; Layton et al., 2002; Leskinen et al., 2005; Routledge and Sumpter, 1996) and androgenicity of compounds (Michelini et al., 2005, 2008) including other endocrine disrupting compounds mimicking hormone-like molecules.

The sensor system for detecting hormone-like compounds used in this study is based on the well-known eukaryotic model organism *S. cerevisiae*, or baker's yeast. These engineered yeast cells (Leskinen et al., 2003, 2005) produce bioluminescence by the action of a modified firefly (*Photinus pyralis*) luciferase reporter gene (*luc*) inserted under the control of a hormone-responsive element (HRE). Constitutively expressed hormone receptors bind estrogenic ligands present in the cell, and this complex in turn binds the HRE to induce gene expression. Firefly luciferase catalyses the reaction:

$$\text{Luc} + \text{D} - \text{luciferin} + \text{ATP} \rightarrow \text{oxyluciferin} + \text{AMP} + \text{CO}_2 + \text{PPi} + \text{light}.$$

The resulting luminescence (yellowish light) can be measured very sensitively by adding D-luciferin substrate at pH of 5.0 into cells after an induction period of a few hours. The reporter protein luciferase has been modified to prevent its transportation into peroxisomes by removing the C-terminal peroxisomal targeting sequence (ser-lys-leu) (Gould et al., 1988) (Leskinen et al., 2003). This modification allows the luminescence measurement from intact, living cells.

In this study, our purpose was to apply our yeast assays in screening zearalenone mycotoxins in different milk products with different fat percentages.

2. Materials and methods

2.1. Chemicals and samples

Mycotoxins (zearalanone, α-zearalanol, β-zearalanol, zearalenone, α-zearalenol, and β–zearalenol), 17β-estradiol, adenine hemisulfate salt, L-leucine, L-histidine and citric acid and trisodium citrate were purchased from Sigma-Aldrich Co (St Louis, USA). From mycotoxins stock solutions were made in 100% ethanol. D-luciferin was purchased from Biothema (Handen, Sweden). Yeast nitrogen base w/o amino acids (Difco) and agar–agar (Difco) were from Voigt Global Distribution (Lawrence, KS, USA). Skim milk (0% fat) and coffee cream (19% fat) produced by Arla-Ingman Oy (Sipoo, Finland), as well as semi-skimmed milk (1.5% fat) and whole milk (3.5% fat) produced by Valio (Helsinki, Finland) were purchased from a local grocery store. Non-processed raw milk (on average 4% fat) was obtained from Valio Tampere dairy unit.

2.2. Yeast strain and cultivation

The yeast strain used in this study was *S. cerevisiae* BMAEREluc/ERα (Leskinen et al., 2003). In short, this strain was cultivated overnight at 30 °C with 300 rpm shaking in liquid synthetic dextrose medium (SD) that contains yeast nitrogen base w/o amino acids (6.7 $g l^{-1}$) supplemented with D-glucose 2% (w/v), adenine (5 $g l^{-1}$), and the essential amino acids L-histidine (2 $g l^{-1}$) and L-leucine (10 $g l^{-1}$). The yeast cells were grown overnight to the optical density (OD_{600}) of approximately 3.0. Before the start of the assay procedure the culture was diluted with the same medium to OD_{600} of 0.8. The optimal mycotoxin concentrations, used in the estrogen assay (the BMAEREluc/ERα strain), were determined in an assay measuring the effect of the samples concentration on the luminescence of the constitutively expressing luminescent strain BMA64luc (Leskinen et al., 2005; Välimaa et al., 2008) used for assay normalization. This procedure allowed us to exclude those concentrations that may cause non-specific toxicity of the mycotoxins and the stimulating effects of milk caused by nutrients such as sugars. The strain was cultivated as the BMAEREluc/ERα strain with the exception that the SD medium was supplemented with the additional amino acid, tryptophan (2 $g l^{-1}$).

2.3. Sample preparation

The stock solutions (10^{-3} M) of mycotoxins zearalanone, α-zearalanol, β-zearalanol, zearalenone, α-zearalenol, and β-zearalenol were prepared by dissolving the solid chemicals into 100% ethanol. Standard sets were prepared by diluting stock solutions in a ratio of 1:10 with the same solvent. Thus, the final mycotoxin concentration in vials varied from 10^{-7} M to 10^{-3} M. The standard sets were protected from light and stored at −20 °C until further use.

The sample sets were prepared by spiking milk products – skim milk, semi-skimmed milk, whole milk, raw milk or coffee cream – containing different fat percentages (0%, 1.5%, 3.5%, 4%, and 19%, respectively) with the mycotoxin standard sets in a proportion to 1:10. Thus, the final mycotoxin concentration of samples varied from 10^{-8} M to 10^{-4} M, and ethanol concentration was 10%. A similar ethanol concentration was also added to blank samples of each milk product. The absence of possible precipitation of protein by 10% ethanol was verified by mixing 100 µl of 100% ethanol and 900 µl of each milk product. No precipitation was observed after incubation in room temperature (25 °C). Sample sets from each of the mycotoxins and each milk product were prepared one day before assaying and were stored protected from light at 4 °C until the experiment was performed.

2.4. Assay procedure

Ten microliter aliquots of each sample set and reference samples were pipetted in triplicate into a white 96-well plate (Thermo Electron Corporation, Vantaa, Finland) with 10^{-8} M 17β-estradiol in 10% ethanol as a positive control. Ninety microliter aliquots of diluted yeast culture were then added into the wells containing samples. The final concentration of ethanol solvent in all assays was 1%. After 2.5 h incubation at 30 °C, 300 rpm, 100 µl aliquots of 1 mM D-luciferin (dissolved in 0.1 M Na-citrate buffer, pH 5) were pipetted into the wells. The resulting luminescence was measured immediately with a Chameleon Multilabel Detection Platform (Hidex Oy, Turku, Finland). Based on bioluminescence counts, the induction factors (IF) for each sample were calculated using Eq. (1)

$$\text{IF} = \frac{L_{\text{Sample}}}{L_{\text{Background}}} \quad (1)$$

where L_{Sample} is the luminescence emission (in relative light units, RLU) measured in yeast sensor system with sample and $L_{\text{Background}}$ is the luminescence emission (in RLU) measured in yeast sensor system with blank sample (solvent). $L_{\text{Background}}$ describes the non-specific background light production in samples. EC_{50} values (the concentrations yielding half of the maximum response) were calculated from

the dose response curves. The minimum response considered as positive (FI_{LOD}) was determined as suggested by Long and Winefordner (Long and Winefordner, 1983):

$$FI_{LOD} = \frac{2(X_B + 3SD)}{X_B} \quad (2)$$

where X_B is the mean background luminescence value of the sensor and SD is the standard deviation. The lowest detectable concentration of each ligand (limit of detection or LOD) was determined from the standard curve using FI_{LOD}.

3. Results

In this work, we have optimized the previously described estrogen-responsive yeast biosensor strain (Leskinen et al., 2003, 2005) for the measurement of estrogenic mycotoxin residues in milk. Milk products containing different fat percentages [skim milk (0% of fat), semi-skimmed milk (1.5% of fat), whole milk (3.5% of fat), raw, non-processed milk (on average 4% of fat) and coffee cream (19% of fat)] were spiked with the estrogenic mycotoxins zearalenone and its metabolites zearalanone, α-zearalanol, β-zearalanol, α-zearalenol and β-zearalenol. Optimized induction conditions were found to consist of 2.5 h incubation at 30 °C in the presence of 10 µl milk product at the volume of 100 µl in a microtiter plate well with continuous shaking under a plastic foil (data not shown). The extent of the estrogenic response of the estrogenicity was detected based on the concentration-dependant intensity of estrogenicity-induced bioluminescence emitted by the biosensor cells. The relative estrogenic potencies of these fungal compounds in the genetically modified yeast *S. cerevisiae* strain were also determined.

3.1. The relative estrogenic potencies and LOD

The relative estrogenic potencies (REP) for each compound were calculated as EC_{50} of 17β-estradiol equivalents dividing EC_{50} of 17β-estradiol by EC_{50} of the compound. The ranking order of mycotoxins based on REPs (Table 1) was as follows: α-zearalenol > α-zearalanol > zearalanone > zearalenone > β-zearalanol > > β-zearalenol. The lowest detectable concentration of a ligand, LOD was determined for each mycotoxin and the ranking order (Table 1) was as follows: α-zearalanol = α-zearalenol = zearalanone > zearalenone > β-zearalanol > β-zearalenol.

3.2. The measurement of estrogenic mycotoxin residues in different milk products

The response of yeast to mycotoxin concentrations in different artificially contaminated milk products was measured by bioluminescence emission of the reporter system described. The bioluminescence response of yeast sensor strain to mycotoxin concentrations in different milk products is described in EC_{50} values (nM) in Fig. 1. The results for ß-zearalenol, the least potent inducer, are not shown here. The results show clear difference between responses in different milk products. For most compounds the main trend was that the light emission decreased as the fat percentage of the sample increased.

The possible non-specific toxicity of the mycotoxins and stimulating effects of milk caused by nutrients such as sugar contents, were detected by measuring the effect of the samples on the constitutively luminescent control strain BMA64luc. Statistically non-significant differences in light production (results not shown) were found indicating that neither the fat content itself without any mycotoxin nor differences in the opacity of milk products had effects on the light production of the control strain.

As a typical response curve Fig. 2 shows the yeast response to zearalenone illustrated as the induction factors (RLU of sample/RLU of blank solvent). Fig. 3 illustrates the response of zearalanone at a concentration of 10^{-7} M. The highest light emission was detected from sample with the lowest amount of fat, and the response

Table 1
The limit of detection (LOD), EC_{50} [nM] and the relative estrogenic potency (REP) of the mycotoxins in our bioluminescent assay[a] compared to a fluorescent yeast assay[b] expressing human ERα.

Mycotoxin	EC_{50} [nM][a]	REP[a]	LOD [nM][a]	EC_{50} [nM][b]	REP[b]
α-zearalenol	41	3.7×10^{-2}	1	11	5.5×10^{-2}
α-zearalanol	62	2.4×10^{-2}	1	18	3.3×10^{-2}
Zearalanone	86	1.7×10^{-2}	2	40	1.5×10^{-2}
Zearalenone	264	5.7×10^{-3}	9	130	4.6×10^{-3}
β-zearalanol	515	2.9×10^{-3}	15	230	2.6×10^{-3}
β-zearalenol	1890	7.9×10^{-4}	71	230	2.6×10^{-3}

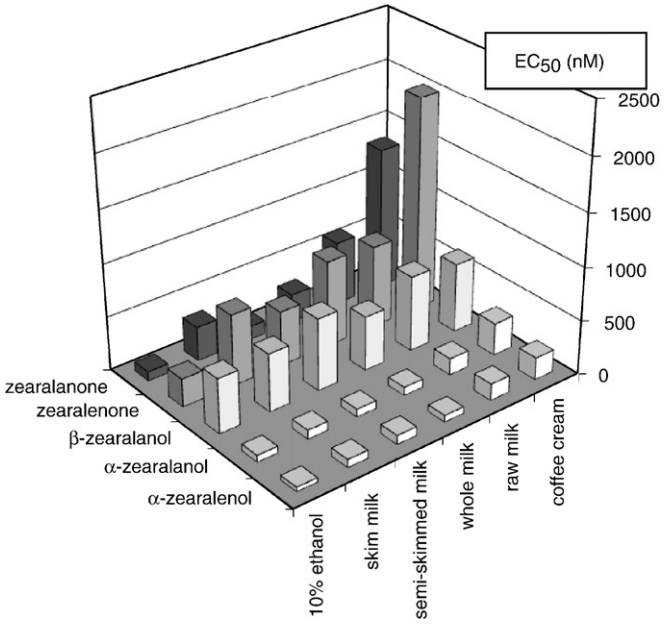

Fig. 1. The yeast response to mycotoxins. Zearalenone, zearalanone, α-zearalanol, ß-zearalanol and α-zearalenol were spiked into 10% ethanol and milk products (skim milk, semi-skimmed milk, whole milk, raw milk and coffee cream) with different fat percentages. For clarity reasons ß-zearalenol is excluded. Yeast sensor strain system was exposed to the samples and the bioluminescence response is described in EC_{50} values (nM).

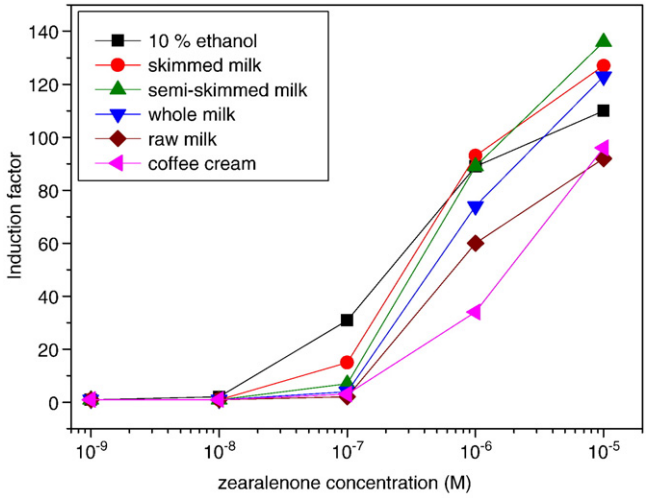

Fig. 2. The bioluminescence response curves of spiked zearalenone in 10% ethanol and milk products with different fat concentrations. For clarity, the error bars on the average of the order of 10% are not shown in the figure.

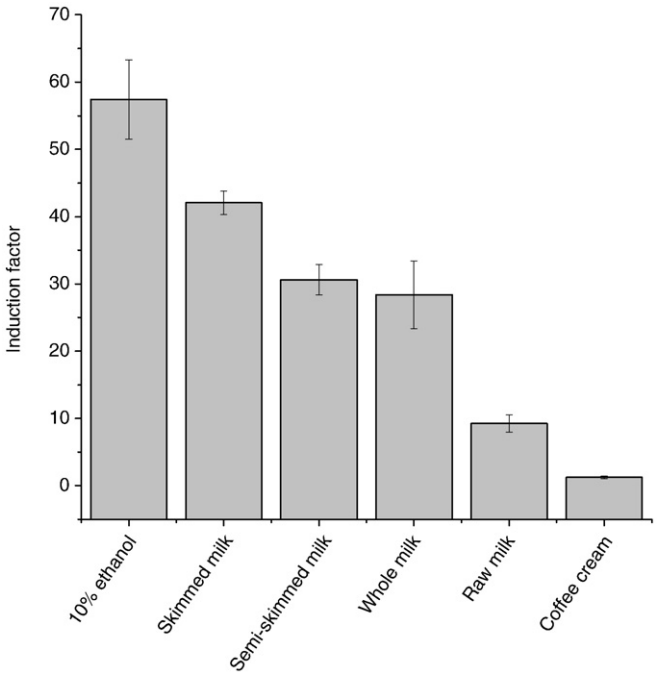

Fig. 3. Induction factors for zearalanone concentration of 10^{-7} M in milk product. The error bars represent the standard deviation of three independent measurements each with three replicates.

decreased as the fat percentage increased. Accordingly, the yeast system exposed to zearalanone in coffee cream produced lowest bioluminescence levels. Similar results were found for all the other zearalenone family molecules tested (data not shown). This underlines the need for proper controls. The standard deviation of response between three replicates of the same concentration was on average 10% in each assay. The results show that the yeast response to mycotoxins differs in different milk products and seem to have effect on luminescence response.

4. Discussion

In this work, we detected the estrogenic response of mycotoxins and residues of them in milk products by the yeast estrogen assay (Leskinen et al., 2003, 2005). A genetically modified, *S. cerevisiae* sensor strain in the presence of estrogen or an estrogenic compound produces firefly luciferase-enzyme and the luminescence produced is measured in the presence of D-luciferin substrate.

The relative estrogenic potencies (REP) for each compound were calculated as EC_{50} of 17β-estradiol equivalents divided by EC_{50} of the compound. The ranking order of mycotoxins based on REPs (Table 1) was as follows: α-zearalenol > α-zearalanol > zearalanone > zearalenone > β-zearalanol > > β-zearalenol. Similar REPs for the mycotoxins with exception of β-zearalenol have been reported by Bovee et al. (2004) and Le Guevel and Pakdel (2001). In our studies β-zearalenol was ten times less potent than β-zearalanol, while in the studies by Bovee et al. (2004) the compounds showed similar potency. The difference could be attributed to dissimilarities in assay methods or experimental conditions. The most prominent difference to Bovee et al. (2004) is found for β-zearalenol. This might be an indication of usage of different sensor proteins, *luc* vs. green fluorescent protein (GFP), which reports either in statically or cumulative manner, respectively. Also Bovee et al. (2004) as well as Le Guevel and Pakdel (2001) have inserted the receptor into the genome as single copy and their construct contains a relatively longer stretch of receptor gene. Compared to our construct the main difference seems to be higher copy number due to plasmid expression of the receptor gene. The method utilizing GFP (Bovee et al., 2004) needs an incubation period of 24 h compared to our rapid method of 2.5 h. Therefore, our method provides results during the working day.

The results highlight the sensitivity of our yeast biosensor system to low concentrations of zearalenone mycotoxins and their metabolites and biosensors sensitivity the inhibitory effects of increasing fat content on luminescence output. The results show that the yeast response to mycotoxins differs in milk products particularly milk with varying fat contents and seem to have effects only on absolute luminescence response. The reasons for the varying luminescence response to the same concentrations of mycotoxins could be attributed to the lipophilic character of the mycotoxins. Like estradiol (Pape-Zambito et al., 2007), lipophilic mycotoxins accumulate in the lipid fraction of milk leading to reduced entry into sensor cells. Therefore, a high fat content in the sample will interfere with the binding interactions between the mycotoxins and the receptor thus influencing the intensity of luminescence response. Our milk samples of 0–3.5% fat have similar responses to mycotoxins, but with raw milk and coffee cream the sensitivity decreases. These results show that 4% fat content might be the upper limit within which a reliable performance of the assay can be achieved. In a study of detection of tetracycline residues in raw bovine milk by luminescent bacteria, Kurittu et al. (2000) found that fat (3.5–6.1%) and also protein content of the milk had a modest inhibitory effect on the luminescence response.

We suggest that simple fat extraction methods during pre-analytical sample preparation steps should be done with samples of high fat content. A possible fat extraction method could be a non-solvent two-step centrifugation (Luna et al., 2005). In this rapid and simple method, milk fat was tempered at 20 °C for 20 min, and fat was separated by centrifugation at 17,800 ×*g* for 30 min and 19,300 ×*g* for 30 min. Because no solvent was used, milk samples could be used directly in whole-cell bioassays.

The LOD of our assay for different zearalenone compounds varied between 1 nM (α-zearalanol and α-zearalenol) and 258 nM (ß-zearalenol). Theoretically, if the carry over rate of zearalenone would be 1.924% or more (Coffey et al., 2009) we could detect zearalenone compounds from milk produced by a cow fed with highly contaminated feed and feeding has been continuous like in the studies of Mirocha et al. (1981) and Prelusky et al. (1990). They reported that zearalenone and its metabolites are excreted into milk when high doses of zearalenone were administered (Mirocha et al., 1981) and their concentrations rapidly decrease when exposure stops (Prelusky et al., 1990). However, it needs to take into account that these findings are based on results of animal feeding studies, while our results are based on studies of artificially contaminated milk products.

In this communication, our results demonstrate that we were able to detect zearalenone mycotoxins from artificially contaminated milk products at levels that might be expected in milk from cows that have ingested naturally-contaminated feed. However, the results can be obtained in real-time without any complicated fat extraction protocols directly from whole milk products. Furthermore, the assays can be done in a multi-well plate and the light emission produced by luciferase can be measured by automated counting devices. This allows our system to be used for convenient pre-screening purposes and providing very cheap and less-labour intensive means for detecting zearalenone compounds.

In conclusion, we have shown that our bioluminescence assay measuring bioavailability of compounds is capable to monitor the zearalenone mycotoxins in different artificially contaminated bovine milk products. We could easily detect the presence of estrogenic compounds from milk produced by a cow fed with highly contaminated feed and feeding has been continuous. The results show that the yeast response to the zearalenone compounds differs in different milk products especially with varying fat contents and seem to have effects only on absolute luminescence response but not on induction

factors. The signal over background noise regardless of the fat content of milk product suggests that this approach is suitable for an early-warning method for zearalenone contamination preceding higher priced conventional analytical chemistry methods.

We suggest that the assay could also be used for testing the presence of estrogenic mycotoxins in the milk of other ruminant species, such as dairy sheep, goats, and buffaloes, especially if the market for milk products from these animal species increases.

Acknowledgments

We are thankful to Mr. Sakari Halttunen, Tampere University of Technology for assisting in calculations and Mrs Nina Virolainen, Tampere University of Technology, for helpful comments and suggestions on the manuscript. The Raisio Tutkimussäätiö is acknowledged for their financial support (Välimaa).

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