Antiaflatoxigenic property of food grade antioxidants under different conditions of water activity in peanut grains

Maria A. Passone a, Silvia Resnik b, Miriam G. Etcheverry a,*

a (CONICET) Departamento de Microbiología e Inmunología, Facultad de Ciencias Exactas, Físico Químicas y Naturales, Universidad Nacional de Río Cuarto, Ruta Nacional 36, km 601, 5800, Córdoba, Argentina
b (CIC) Departamento de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Buenos Aires, Argentina

Received 3 November 2006; received in revised form 20 February 2007; accepted 7 May 2007

Abstract

Analytical grade (AG) and industrial grade (IG) of three-food grade antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl paraben (PP) were analyzed to prove their fungitoxic effect on Aspergillus section Flavi strains. The effect of interactions among 10 antioxidant treatments at water activity levels (0.982, 0.955, 0.937 aW) for 11 and 35 days of incubation and at 25 °C in peanut grains on mycelial growth (CFU g⁻¹) and aflatoxin B1 (AFB1) accumulation were evaluated. Both antioxidant grade treatments had a significant effect (Pb0.001) on fungal count. All antioxidant treatments showed the highest effectiveness on control of growth of peanut aflatoxigenic strains at 0.937 aW and at 11 days of incubation. Overall, AG and IG binary mixtures M3 (20+10 mM), M4 (20+20 mM) and ternary mixtures M5 (10+10+10 mM), M6 (10+20+10 mM), M7 (20+10+10 mM) and M8 (20+20+10 mM) were the treatments most effective at inhibiting growth of Aspergillus section Flavi strains. Industrial grade BHA 10 and 20 mM, binary mixtures M1 (10+10 mM), M2 (10+20 mM), M3 (20+10 mM), M4 (20+20 mM) and ternary mixtures M5 (10+10+10 mM), M6 (10+20+10 mM), M7 (20+10+10 mM) and M8 (20+20+10 mM) completely inhibited AFB1 production. The studied results suggest that IG antioxidant mixtures have potential for controlling growth of these mycotoxigenic species and prevent aflatoxin accumulation at the peanut storage system.

© 2007 Elsevier B.V. All rights reserved.

Keywords: Industrial grade antioxidants; Analytical grade antioxidants; Aflatoxins; Peanut

1. Introduction

The best-known mycotoxins are the aflatoxins, which are produced by certain strains of Aspergillus flavus Link, A. parasiticus Speare, A. nomius Kurtzman, A. pseudotamarii and A. bombycis (Horn et al., 1996; Orth, 1997; Ito et al., 1999; Pettersson, 2000). These fungi can infect almost any foodstuff and have been found on most agricultural products. There is thus a potential for the occurrence of aflatoxins in a wide variety of foods, mainly groundnuts and many other nuts (Bathnagar and García, 2001). This family of secondary metabolites has attracted considerable research because of their extreme toxicity, mutagenicity, carcinogenicity (IARC, 1993), significant reductions in crop yield and economic losses (Gourama and Bullerman, 1995; Gqaleni et al., 1996). Among all classes of aflatoxins, aflatoxin B1 (AFB1) is known to be most significant in terms of animal and human health risk (Coulombe, 1993; Salunkhe et al., 1987). In populations where regulations are not adequately enforced and have relatively high exposure, a role for aflatoxins as a risk factor for primary liver cancer in humans has repeatedly been suggested (Robens and Richard, 1992). Recent evidence indicates that aflatoxins are involved in the very high incidence of primary liver cancer in parts of Africa, Southeast Asia and China (Pitt, 2004). Optimum conditions for aflatoxin production by these species is at 33 °C and 0.99 aW, while that for growth is 35 °C and 0.95 aW (Hill et al., 1985). Peanut grains
are usually into storage at relatively low moisture content (less than 9.5%). If the $a_w$ (or equilibrium relative humidity; ERH) of the grains remains below 0.60, moulds are unable to grow and the stored grain will be stable. However, if temperature and moisture gradients develop in the store by the insects and rodents biological activity, localized pockets of higher moisture may develop, opening the way for mould germination, growth and consequently aflatoxin production (Hocking, 2003).

It is for this reason that much effort has been devoted to the search for new antifungal materials for use in food and grain (Filip et al., 2003; López García et al., 2003). However, disappointingly few of them have been developed for use in foods (Shelef, 1984). Synthetic antioxidants, namely food grade antioxidants (JECFA, 1996) are products widely used as preservatives especially in rich foods in oils or fats, because these antioxidants exhibit an exceptional stress oxidative protection. Furthermore, a report from studies conducted by Farnochei et al. (2005) on natural maize grains has shown that synthetic antioxidants, butylated hydroxyanisole (BHA) and propyl paraben (PP) exhibited antifungal activity. In previous studies it has been determined the effectiveness of the food grade antioxidants butylated hydroxytoluene (BHT), trihydroxybutyrophenone (THB), PP and BHA at concentrations of 1, 10 and 20 mmol g$^{-1}$ on germination, growth and aflatoxin $B_1$ accumulation by Aspergillus section Flavi. The antioxidants BHA, PP and BHT were effective fungal inhibitors to A. flavus and A. parasiticus strains in vitro on peanut extract meal agar, but PP and BHT sprayed on irradiated peanut only showed a solid fungal control when applied in combined form (Passone et al., 2005, 2006).

The present study was undertaken to compare the antifungal activity of analytical and industrial grade antioxidants mixtures of BHA, PP and BHT against Aspergillus section Flavi in natural peanut grains under different water activity conditions.

2. Materials and methods

2.1. Fungal isolates

Two isolates belonging to the genus Aspergillus, A. parasiticus CHG24 and A. flavus CHG46, were used in these experiments. The strains were isolated from peanut soil (Barros et al., 2005). It had been demonstrated that these strains were aflatoxin producers in peanut meal extract agar (PMEA). In this medium, aflatoxin $B_1$ production by A. parasiticus CHG24 was 13.07 $μg$ g$^{-1}$ and the production by A. flavus CHG46 was 0.12 $μg$ g$^{-1}$ (Passone et al., 2005). The isolates were maintained at 4 °C on slants of malt extract agar (MEA) and in 15% glycerol at −80 °C.

2.2. Substrate

Natural peanut grains with initial water content of 0.582 $a_w$ and AFB$_1$ free were used throughout this study and kept at 4 °C. The peanuts were rehydrated to achieve the required $a_w$ (0.982, 0.955, 0.937) by addition of sterile distilled water using a moisture absorption curve for the grain. The $a_w$ of the grains was determined with a Thermoconstantar Novasina TH 200 (Novasina, Zurich, Switzerland).

2.3. Antioxidants

The following antioxidants were used: benzoic acid, 2,3-(tert-butyl)-4 hydroxyanisole (BHA); $n$-propyl $p$-hydroxybenzoate (PP); 2,6-di (tert-butyl)-p-cresol (BHT). Analytical grade (AG) antioxidants (99.5 to 99.9% of purity) were obtained from Sigma Chemical (Dorset, U.K.) and industrial grade (IG) antioxidants were obtained from Eastman Chemical Company. Industrial grade antioxidants PP and BHT had a purity of 99%, containing as contaminants $ash<0.02%, arsenic<3 μg g^{-1}$ and heavy metals $<10 μg g^{-1}$. Butylated hydroxyanisole had a purity of 98.5% containing as trace elements sulfated ash $<0.01%, citric acid<2500 μg g^{-1}$, arsenic $<3 μg g^{-1}$ and heavy metals $<10 μg g^{-1}$. These contaminating compounds did not exceed the levels allowed by JECFA (1996). Stock solutions of BHA, PP and BHT (10 and 20 mmol l$^{-1}$) were prepared in 950 ml ethyl alcohol/distilled water. The antioxidants mixtures used in this assay were: BHA (10 and 20 mM); BHA–PP mixtures M1 (10+10 mM), M2 (10+20 mM), M3 (20+10 mM) and M4 (20+20 mM) and BHA–PP–BHT mixtures M5 (10+10+10 mM), M6 (10+20+10 mM), M7 (20+10+10 mM) and M8 (20+20+10 mM).

2.4. Inoculation and incubation conditions

Seventy grams of natural peanut grains were dispensed as a monolayer into sterile Petri dishes. Peanut grains plates of different $a_w$ values were amended with the appropriate antioxidant according to the treatment. The substrate was conditioned with the appropriate amount of water and kept at 4 °C for 48 h with periodic shaking to allow absorption and equilibrium. Fungi were grown on MEA for 5 days at 30 °C to obtain heavily sporulating cultures. Peanut grains were inoculated with 1 ml of spore suspensions ($1\times10^8$ spores ml$^{-1}$). Petri

<table>
<thead>
<tr>
<th>Factor</th>
<th>df$^a$</th>
<th>MS$^b$</th>
<th>$F$ value$^c$</th>
<th>$P&gt;F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T$</td>
<td>10</td>
<td>367.45</td>
<td>79461.73</td>
<td>0.0001</td>
</tr>
<tr>
<td>$G$</td>
<td>1</td>
<td>124.26</td>
<td>26870.75</td>
<td>0.0001</td>
</tr>
<tr>
<td>$I$</td>
<td>1</td>
<td>109.59</td>
<td>23698.26</td>
<td>0.0001</td>
</tr>
<tr>
<td>$a_w$</td>
<td>2</td>
<td>54.66</td>
<td>11820.14</td>
<td>0.0001</td>
</tr>
<tr>
<td>$I^*a_w$</td>
<td>2</td>
<td>4.46</td>
<td>964.20</td>
<td>0.0001</td>
</tr>
<tr>
<td>$I^*G$</td>
<td>1</td>
<td>3.46</td>
<td>748.92</td>
<td>0.0001</td>
</tr>
<tr>
<td>$I^*T$</td>
<td>10</td>
<td>15.00</td>
<td>3244.23</td>
<td>0.0001</td>
</tr>
<tr>
<td>$a_w^*G$</td>
<td>2</td>
<td>62.60</td>
<td>13536.74</td>
<td>0.0001</td>
</tr>
<tr>
<td>$a_w^*T$</td>
<td>20</td>
<td>18.63</td>
<td>4029.05</td>
<td>0.0001</td>
</tr>
<tr>
<td>$G^*T$</td>
<td>10</td>
<td>25.31</td>
<td>5473.37</td>
<td>0.0001</td>
</tr>
<tr>
<td>$I^*a_w^*G$</td>
<td>2</td>
<td>9.08</td>
<td>1963.12</td>
<td>0.0001</td>
</tr>
<tr>
<td>$I^*a_w^*T$</td>
<td>20</td>
<td>7.94</td>
<td>1718.02</td>
<td>0.0001</td>
</tr>
<tr>
<td>$I^*G^*T$</td>
<td>10</td>
<td>1.55</td>
<td>357.72</td>
<td>0.0001</td>
</tr>
<tr>
<td>$a_w^*G^*T$</td>
<td>20</td>
<td>18.56</td>
<td>4013.13</td>
<td>0.0001</td>
</tr>
<tr>
<td>$I^*a_w^*G^*T$</td>
<td>20</td>
<td>5.04</td>
<td>1089.83</td>
<td>0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>264</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ df = degrees of freedom.
$^b$ MS = mean squares.
$^c$ Significant at $P<0.001$. 

dishes containing peanut at the same $a_W$ were enclosed together in sealed plastic containers. Each container had beakers with a glycerol/water solution at the same $a_W$ as the peanut grains, to maintain constant the relative humidity. The experiments were all carried out with three separate replicates per treatment. The cultures were incubated at 28 °C. Peanut fungal colonization was analyzed at the end of the incubation period (11 and 35 days).

2.5. Aspergillus section Flavi populations

The colonization of the grains was assessed as colony forming unit per gram of peanut grains (CFU g$^{-1}$) after 11 and 35 days of incubation. A sample (10 g) was taken from each treatment and shaken for 5 min with 90 ml of 1 g l$^{-1}$ peptone/distilled water plus 0.06 g l$^{-1}$ of Tween 80. Serial decimal
dilutions until $10^{-9}$ for control samples and until $10^{-4}$ for treated samples were done. A 0.1 ml aliquot of the three last serial decimal dilutions of each treatment was spread on the surface of the solid media *A. flavus* and *parasiticus* agar (AFPA) by triplicate (Pitt and Hocking, 1997). Plates were incubated in darkness at 30 °C for 48 h. The colony that showed orange/yellow reverse colors were counted and the average was reported as colony forming units of *Aspergillus* section *Flavi* per gram (CFU g$^{-1}$) of peanut.

### 2.6. Aflatoxin B$_1$ analyses

After 11 and 35 days of incubation, 50 g of peanut samples were quantitatively determined by HPLC following the
methodology of detection of Truckssess et al. (1994). Samples were analyzed by extracting AFB1 adding acetoniitrile/water (90+10 by volume) and shaking milled peanut grains and solvent for 30 min on an orbital shaker (New Brunswick, Scientific Co., Inc.). A 3 ml aliquot of each extract was transferred to a multifunctional column (Mycosep 224 MFC, Torrance, CA, USA) connected to an HP1046A programmable fluorescence detector, and the quantification was done by a Hewlett Packard workstation. Chromatographic separations were performed on a stainless steel, C18 reverse-phase column and 200 μl aliquot of filtered extract and AFB1 standard was derivatized with 700 μl of trifluoroacetic acid/acetic acid/water (20+10 by volume). The derivatized aflatoxins (50 μl solution) were analyzed using a reversed-phase HPLC/fluorescence detection system. The HPLC system consisted of a Hewlett Packard 1100 pump (Hewlett Packard, Palo Alto, CA, USA) connected to an HP1046 A programmable fluorescence detector, and the quantification was done by a Hewlett Packard workstation. Chromatographic separations were performed on a stainless steel, C18 reverse-phase column (150 mm×4.6 mm i.d., 5 μm particle size, Luna-Phenomenex, Torrance, CA, USA). Water/methanol/acetoniitrile (4+1+1 by volume) was used as the mobile phase, at a flow rate of 1.5 ml min⁻¹. Aflatoxin derivative fluorescence was recorded at excitation and emission wavelengths of 360 and 440 nm, respectively. Standard curves were constructed with different levels of AFB1. The mean recovery percentage for AFB1 was 94.5%. The limit of detection of the analytical method was 1 ng g⁻¹.

2.7. Statistical analyses

Statistical analyses were made using SigmaStat program Version 3.10. Copyright© 2004 Systat Software, Inc. Means of Aspergillus section Flavi colony forming units grown on AFPA medium and AFB1 accumulation were determined by analyses of variance (ANOVA) (\(P<0.001\)). The significant differences between the control and treatments were established by using Duncan’s New Multiple Range Test at \(P<0.005\). Fisher's LSD Method (\(\alpha=0.05\)) was applied to compare significant differences between treatment and control means for AFB1 accumulation.

3. Results

3.1. Determination of Aspergillus species from section Flavi

Statistical analyses on growth of Aspergillus section Flavi; incubation time, water activity (\(\alpha_W\)), antioxidant grade, antioxidant treatments and two-; three- and four-way interactions indicated that were statistically significant (Table 1). The major effect was produced by antioxidant treatments and antioxidant grade. Colony forming units per gram of peanut (CFU g⁻¹) of Aspergillus section Flavi strains at different \(\alpha_W\) levels and incubation times in the presence of BHA, PP and BHT (10, 20 mmol g⁻¹) are shown in Figs. 1 and 2. Control samples counts on selective and differential medium (AFPA) at 0.982 \(\alpha_W\) and at 11 days of incubation were about 1×10¹⁰ CFU g⁻¹ in peanut inoculated with the strains CHG24 and CHG46, while counts were reduced in 1 and 2 log units at 0.955 and 0.937 \(\alpha_W\), respectively. Control samples showed similar counts at both incubation periods; nevertheless, this parameter caused substantial impact on antioxidant inhibitory activity. The major effect of antioxidant treatments was observed at 11 of incubation; furthermore, mycelial growth was always more inhibited at lower \(\alpha_W\) values. Some treatments with IG antioxidants at 0.982 \(\alpha_W\) as such: BHA (10 and 20 mM), binary mixtures M1 (10+10 mM) and M2 (10+20 mM) were more effective than treatments with AG antioxidants. Binary mixtures M3 (20+10 mM) and M4 (20+20 mM) caused complete inhibition of growth of Aspergillus section Flavi at all \(\alpha_W\) values, as did ternary mixtures M5 (10+10+10 mM), M6 (10+20+20+10 mM), M7 (20+10+10 mM) and M8 (20+20+10 mM) (data not shown). Binary mixtures M1 and M2 gave greater than 35.9 and 44.6% of Aspergillus section Flavi growth reductions at 11 days of incubation and all \(\alpha_W\) values tested, regardless of antioxidant grade. The application of both BHA grade (10 and 20 mM) at 35 days of incubation and 0.982 \(\alpha_W\) was less effective against aflatoxigenic strains, showing growth reduction percentages from 0.8 to 72.56% and from 5.9 to 100%, respectively.

3.2. Effect of antioxidants on aflatoxin B₁ production

The AFB₁ accumulation levels in peanut grains inoculated with \(10^4\) spores ml⁻¹ of A. parasiticus CHG24 and A. flavus CHG46 in the presence of the studied 20 treatments, are shown in Table 2. At \(\alpha_W\) 0.982 and 0.955 control samples accumulated AFB1. However, AFB1 accumulation was not detected at 0.937 \(\alpha_W\). The increase of this metabolite, 1.2 and 23.5 times, was observed at the end of the incubation period in peanut controls inoculated with CHG24 and CHG46, respectively. Industrial grade antioxidants were more effective than AG, showing a total inhibition of AFB1 accumulation at both incubation time and at 0.982, 0.955 and 0.937 \(\alpha_W\). In general, those treatments with AG antioxidants that reduce mycelial growth, stimulated

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Influence of treatments on aflatoxin B₁ (AFB₁) production by Aspergillus section Flavi strains at different water activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB₁ (μg g⁻¹)</td>
<td>0.982 (\alpha_W)</td>
</tr>
<tr>
<td></td>
<td>11 days</td>
</tr>
<tr>
<td>AG</td>
<td>IG</td>
</tr>
<tr>
<td>Control²</td>
<td>36.1ᵃ</td>
</tr>
<tr>
<td>BHA10 mM</td>
<td>16.7ᵇ</td>
</tr>
<tr>
<td>BHA20 mM</td>
<td>n.d.ᵉ</td>
</tr>
<tr>
<td>Control³</td>
<td>7.1ᵃ</td>
</tr>
<tr>
<td>BHA10 mM</td>
<td>3.6ᵇ</td>
</tr>
<tr>
<td>BHA20 mM</td>
<td>1.5ᵃ</td>
</tr>
</tbody>
</table>

n.d.: not detected.

² Mean in the same group (incubation time) are not significantly different (Fisher LSD Method at \( \alpha=0.05 \)).
³ AG: analytical grade; IG: industrial grade; BHA: butylated hydroxyanisole.
⁴ Peanut grains inoculated with A. parasiticus CHG24 (1×10⁴ spores ml⁻¹).
⁵ M1: BHA–PP (10+10 mM).
AFB1 production. This behavior was observed with the treatments BHA 10 mM (0.982 aw/35 days; 0.955 aw/11 and 35 days); BHA 20 mM (0.982 and 0.955 aw/35 days) and binary mixture M1 (10+10 mM) (0.955 aw/35 days), while binary mixtures M2 (10+20 mM), M3 (20+10 mM), M4 (20+20 mM) and ternary mixtures M5 (10+10+10 mM), M6 (10+20+10 mM), M7 (20+20+10 mM), M8 (20+20+10 mM) completely inhibited AFB1 accumulation (data not shown).

4. Discussion

In this study unsterilized peanuts were inoculated with Aspergillus section Flavi spores to ensure that aflatoxin-producing species were present. This study showed that mycelial growth of Aspergillus section Flavi from peanut grains was found to be significantly influenced by treatments with IG and AG antioxidants, incubation time, aw values and their interactions. At 11 days of incubation, BHA 10 and 20 mM significantly reduced the mycelial growth of Aspergillus section Flavi, but this effect was not extended by further incubation. With inshell groundnuts, 0.3 and 0.5% ammonium propionate was demonstrated to exert a selective effect on the natural mycoflora. This effect was evident with 0.3% propionic acid and controlled potentially aflatoxigenic fungi up to day 14 of incubation (Calori-Dominguez et al., 1996). When high moisture maize was heavily inoculated with A. flavus/parasiticus and treated with 10 mM cinnamic acid and above, growth and production of aflatoxin were completely inhibited (Nesici and Etcheverry, 2006). It has been reported that subinhibitory doses together with inadequate distribution of preservative, specially at low aw values, could enhance fungal growth on the treated material with an initial low level of contamination (Smith and Moss, 1985; Lacey, 1989). For example, population of Aspergillus species increased with concentration of propionate mixtures (Marin et al., 2000), trihydroxybutyrophenone (THB) (Passone et al., 2005), peppermint and boldus (Bluma et al., 2006). It is known that antioxidants are usually combined to take advantage of their differing proprieties (Mazzani et al., 1998; Rivera-Carriles et al., 2005). In the present work, it was evident that the antifungal effect of BHA when it is combined with PP and with PP and BHT increased to achieve total growth inhibition. At the highest studied aw value (0.982), IG antioxidants showed more antifungal activity compared to AG, while this behavior was not observed at the other two aw assayed. However, AFB1 accumulation was significantly different affected (P<0.001) by the treatments with IG and AG antioxidants. The amounts of AFB1 accumulated in inoculated peanut grains and treated with AG BHA 10 mM were higher than the control values. Nevertheless, all IG treatments totally inhibited AFB1 accumulation at 0.982, 0.955 and 0.937 aw values. The main contaminant substance of IG BHA is the antioxidative activity of chemicals used in this work. On the other hand, this contaminant (citric acid) could chelate metals such as Zn (Turgut et al., 2004) that is essential in the aflatoxin pathway biosynthesis (Ehrlrich et al., 1999). On an economic point of view, the application cost of IG antioxidants is 91% cheaper than AG antioxidants (Eastman Chemical Company, 2006b; Sigma-Aldrich, 2006).

The present study showed significant antifungal and antiaflatoxigenic activities of IG BHA +PP and BHA +PP+BHT mixtures. Therefore, it may be suggested that binary mixtures M2 (10+20 mM), M3 (20+10 mM), M4 (20+20 mM) and ternary mixtures M5 (10+10+10 mM), M6 (10+20+10 mM), M7 (20+20+10 mM), M8 (20+20+10 mM) could be used in peanut storage agroecosystems to control fungal growth, prevent aflatoxin contamination and increase the shelf life.

Acknowledgements

This research received financial support from Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto (Res. No. 077/03, granted during 2003–2004), Agencia Córdoba Ciencia (granted during 2003–2004) and FONCYT PICT (No. 08-14551).

References


