Number 4 Issue 3 June 2017

International Biotechnology Color Journal

A Scientific Peer Reviewed Journal with Focus on BIOTECHNOLOGY
and Covering Its Many Hues, Tints, Tones & Shades

Special issue:
Selected papers from 3rd Biotechnology Summit, 2016

From october 24th to 28th, 2016 at Cd. Obregón, Sonora, Mexico

Produced and hosted by *Centro de Investigación Científica de Yucatán, A.C.*,
in collaboration with *Universidad Nacional Autónoma de México*,
and the *International Foundation for Biotechnology Research & Early Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, Technology & Society*
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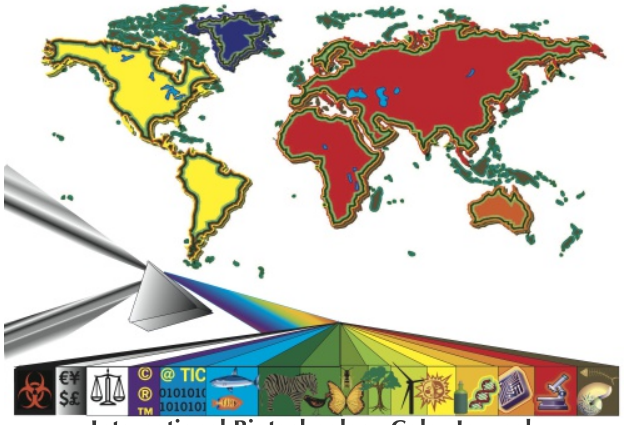
The IFBR&ESCHNSAST&S is a civil association and nonprofit organization.

Its main goal is to help bringing together efficient biotechnological solutions for many human problems, with the need for environmentally friendly and sustainable processes.

As means to such goal, the Foundation counts on education to create social awareness to biotechnology's benefits and risks, and to promote the formation of highly qualified professionals and research scientists.

The constitutive act of the foundation was signed in the Heroic City of *Huajuapan de León, Oaxaca*, on September 14th, 2009, at the *NOTARÍA PÚBLICA No. 61 de los ESTADOS UNIDOS MEXICANOS*. As .



International Biotechnology Color Journal

Susana Lozano Muñiz
President of the Foundation

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Editorial section

International Biotechnology Color Journal (IBCJ) is an electronic Open Access journal, devoted to rapidly publishing full peer-reviewed articles covering all the fields of biotechnology. The central focus of IBCJ is to publish scientific reports, though it also provides a forum for reviews, short notes on relevant findings, essays on novel technical advances or relevant updates, book reviews, scientific meeting reports, and letters to the editor. Instructions for every type of contribution are presented in the journal's Homepage and in PDF format in the last issue of each year.

The Editorial Board of IBCJ is fully committed to publish articles innovating in all areas of biotechnology. Contributions are reviewed from a rigorous optic of scientific criticism; thus, any original contribution that fits within the scope of the journal and promotes the advancement of biotechnology are particularly welcome.

Due to a lack of contributions worth publishing, this number is appearing with two years delay, but we hope the renewal of the staff, currently taking place will make this journal more active and future issues will be prublished in time from 2018.

Editorial comments to the contents in this issue

Chief editor.

In this special issue of the International Biotechnology Color Journal, we are particularly glad to some selected papers in extenso from the Biotechnology summit 2016, held in Cd. Obregon, Sononra, Mexico, from the 24th to the 28th of october, 2016.

Sonora state has a quiet atmosphere with notably diverse natural ecosystems, and is also a part of Mexico rich in industrial activity, a parfect frame for the international conference Biotechnology Summit 2016.

This meeting was organized with the support of the "International Foundation for Biotechnology Research & Early Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, Technology & Society", and "Instituto Tecnológico de Sonora."

To IBCJ now publiches selected papers from those oral presentations were the authors were willing to contribute with in extenso papers.

The papers presented here relate to three very different aspects of Biotechnology, such as food safety, legislation and brewing.

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SELECTED PAPERS[1] IN EXTENSO FROM THE BIOTECHNOLOGY SUMMIT 2016

The Meeting was organized by an International comitte and hosted by the "International Foundation for Biotechnology Research & Early Stimulation in the Culture of Health, Nutrition, Sport, Art, Science, Technology & Society," *and the "Instituto Tecnológico de Sonora".*

The Biotechnology Summit 2016 was held in Cd. Obregón, Sonora, Mexico, on October 24 to 28, 2016.

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The Threats to Food Fafety and Biocontrol of Aflatoxins

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ABSTRACT

Aflatoxins are a serious food safety concern for human and animal health. Much attention should be paid to the dietary exposure to this toxins in order to minimize the risk of aflatoxin contamination in the food chain. Although the research on aflatoxins was started more than 50 years ago, aflatoxins contamination has not been completely eliminated due to numerous factors including biological, genetic, biochemical, environmental, and economical reasons. Safe food of high quality for the consumers is the ultimate goal. Among the reported strategies to manage aflatoxin contamination into food and feed, the biological control seems to be the most promising approach, depending on their biological origin, and on the use of living organisms or their derivatives. The use of new materials like enzymes, peptides, essential oils or antagonistic microorganisms of aflatoxigenic fungi, appear as very effective tools against aflatoxin contaminations, and more importantly, they are safe for human and animal health, and friendly to the ecosystems and the environment.

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Abbreviations:

AF, aflatoxin; LAB, lactic acid bacteria; GRAS, generally recongnized as safe

Keywords

Aflatoxin; Bacillus megaterium; Aspergillus flavus; Biocontrol.

Green: Wildlife, Ecology & Environment Biotechnology

Introduction

Aflatoxins, the most potent naturally occurring hepatocarcinogens, are a group of polyketide-derived mycotoxins and mainly consist of B_1, B_2, G_1, G_2 (Figure 1), as identified by their chromatographic and fluorescent properties (Keller et al, 2005). The ultraviolet absorption spectra of the four compounds are very similar, showing maxima at 223, 265 and 363 mμ, respectively; while the maxima of the fluorescence emission spectra of these four compounds differ slightly: the reported value for aflatoxins B_1, B_2 was 425 mμ and for G_1, G_2 was 450 mμ. Aflatoxins have been recognized as a food safety problem since 1960, because in this year 100,000 turkeys died after feeding on groundnut infected with Aspergillus flavus. In this incident, aflatoxins were initially isolated and identified as the causative toxins (Asao et al, 1965; D'Mello and Macdonald, 1997). Aflatoxins are produced by species in the genus Aspergillus and are a family of secondary metabolites. The common producers of aflatoxins include A. flavus, A. parasiticus, A. nomius, A. tamarii, A. pseudotamarii, A. bombycis, and A. Ochraceoroseus (Cary et al, 2005). From these fungi A. flavus and A. parasiticus are the most important aflatoxingenic species. The A. flavus mainly produces aflatoxin B_1 and B_2, and by contrast, A. parasiticus produces all four types of the aflatoxins. The biosynthesis of aflatoxins is influenced by temperature, moisture, substrates, the host, etc. The optimum temperatures for the production of aflatoxins by A. flavus and A. parasiticus were found to be between 25 and 32 °C.

Aflatoxins are highly carcinogenic, leading to fetal mis-development and miscarriage, suppressing immune systems and can directly modify the structure of DNA (Razzaghi-abyaneh et al, 2013). Among aflatoxins, aflatoxin B_1 is the most widespread and the most toxic one. The toxic effects of aflatoxins in human and animals can be clasified into two types based on the dose, which determines their mode of action: acute aflatoxicosis and chronic aflatoxicosis. When high levels of aflatoxins are consumed at about 10 to 20 mg of aflatoxin per day in adults, acute aflatoxicosis is induced. The symptoms include quick and progressive jaundice, edema of the limbs, pain, vomiting, necrosis, cirrhosis and probably even causing acute liver failure and death. While the chronic aflatoxicosis is the results of long exposure to aflatoxins in the diet at a low level, is rarely noticed, and its common signs are cancer, reduced rates of growth and some other pathological conditions (Nierman et al, 2008).

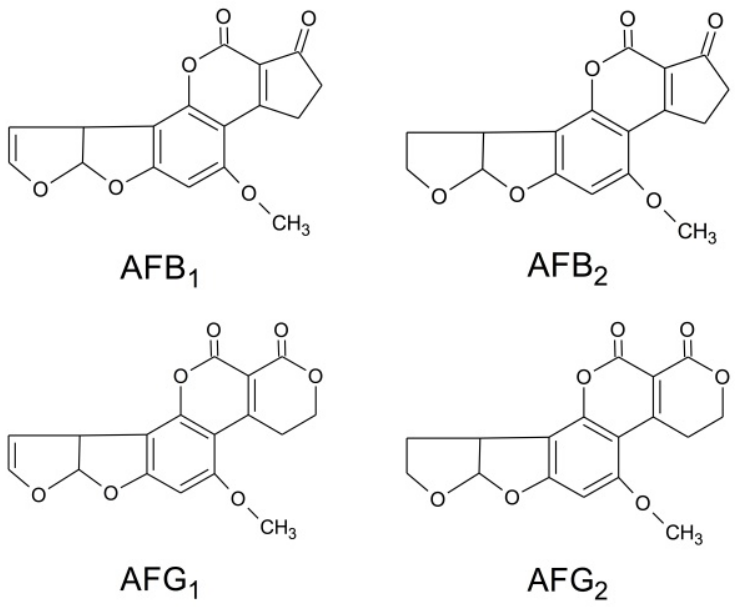


Figure 1. The chemical structures of the four types of aflatoxins.

Aflatoxins have been detected in various food commodities and within a wide geographical area. The zone between 35N and 35S latitudes is considered the area of higher occurence of Apsrgillus sp. contamination, and this region is where most developing countries are located. The geographical aflatoxin contamination boundaries may expand northward due to global warming. Those countries previously considered free of aflatoxin contamination, are now facing it as an emerging problem in need of attention. Aflatoxins mainly invade crops such as maize, peanuts, tree nuts, cottonseed and some other species. AFM_1 is a modified form of aflatoxin B_1 and has also been found in milk and milk products (Wild and Gong, 2010). Aflatoxins are structurally stable compounds and can remain stable for a long time in food commodities (Lizárraga-Paulín et al, 2011). Aflatoxin B_1 was classified as a group I carcinogen since 1987 by the International Agency for Research on cancer (Roebuck, 2004), and a group I carcinogenic agent since 1993 in

combination with exposure to hepatitis B virus (Castegnaro and McGregor, 1998). Williams et al. (2004) estimated an approximate of 4.5 billion population from developing countries chronically exposed to aflatoxins. Obviously, aflatoxin contamination of food and feed are both issues of economic losses and public health.

Aflatoxins : an emerging problem

New aflatoxigenic fungi were isolated

Except the certain strains of A. flavus and A. parasiticus, several strains of A. nomius, A. pseudotamarii, A. bombycis, and A. ochraceroseus as well as two Emericella species have also been reported to be capable of producing aflatoxins (Bennet and Klich, 2003; Yan et al, 2004). It is a well stablished fact that A. flavus when colonizing the aerial parts of plants does produce aflatoxins (Blankson and Mill-Robertson, 2016). Varga et al. (2011) isolated two new aflatoxin producing species A. pseudocaelatus sp. nov. and A. speudonomius sp. nov. Soare et al. (2012) described three new aflatoxin-producing species, A. mottae, A. sergii and A. transmontanensis, which were isolated from Portuguese almonds and maize.

Aflatoxins can cause severe diseases in human and animal

Except for causing liver cancer in human and animal, aflatoxins do present additional toxic effects, which are frequently neglected by the scientific research community. Human individuals chronically exposed to u undetermined amounts of aflatoxins have compromised immunity and altered assimilaton of protein and some critical micronutrients (Williams et al, 2004). Being aflatoxin B_1 the most prevalent, many different metabolic pathways for its detoxification have been described in liver (Figure 2). In the liver, AFB_1 is mainly metabolized to AFB_1-8,9-exo-epoxide and 8,9-endo-epoxide and the exo-epoxide can bind to DNA to induce mutations. Aflatoxins can also bind to RNA and can inhibit protein biosynthesis by interfering amino acid translation (Wild et al,2002). AFB_1 is also metabolized to AFP_1 and AFQ_1 which are excreted in urine and feces. Both, cows and goats, can convert AFB_1 to AFM1. The three metabolites of AFB_1, are several-fold less toxic than AFB_1 and can be detoxified by conjugation with taurocholic and glucuronic acids (Nabney et al, 1967; Buchi et al, 1973; Bassir and Osiyemi, 1967).

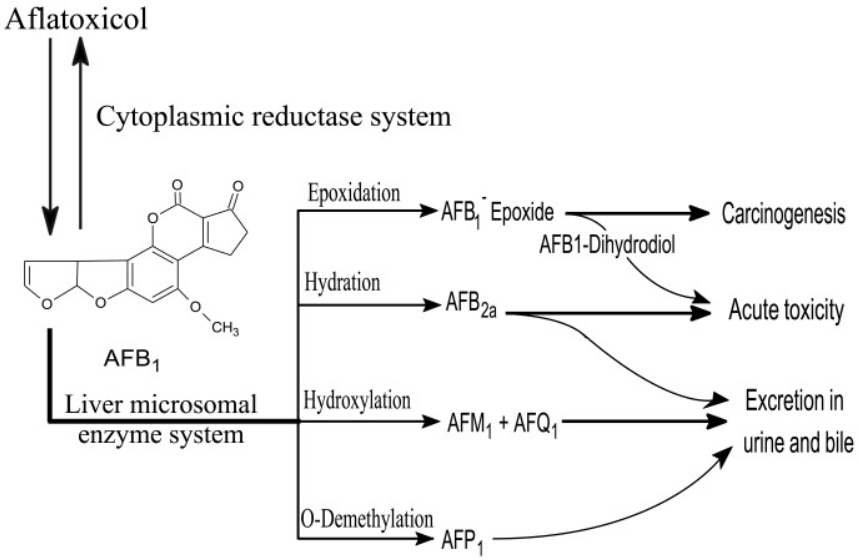


Figure 2. Metabolism of aflatoxin in liver. Adapted from Dhanasekaran et al. (2011).

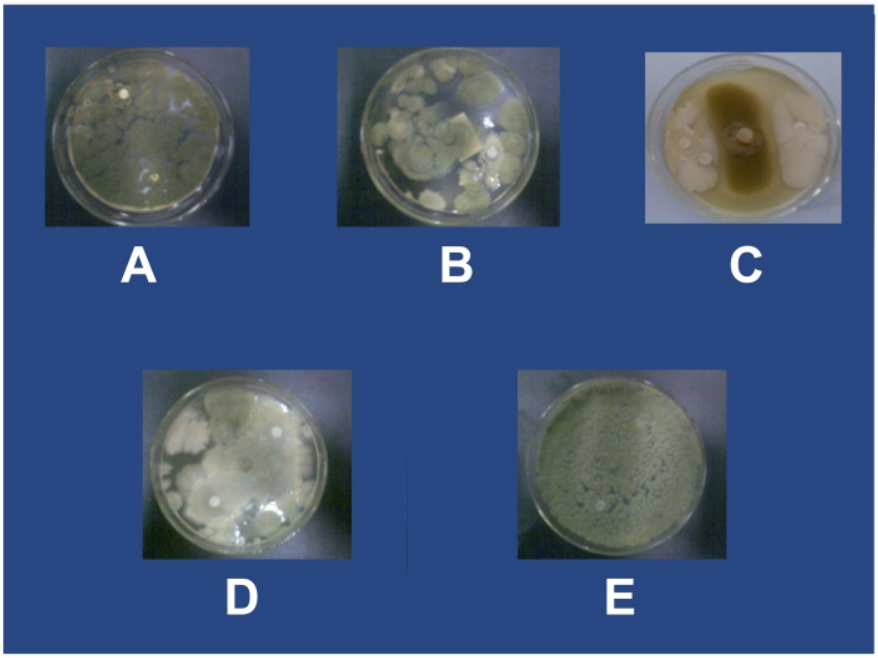
In addition, aflatoxins can also cause immunologic suppression and nutritional interference. In animal tests, AFB_1 has been shown to induce thymic aplasia, reduce T-lymphocyte function and number, suppress phagocytic activity, and reduce complement activity (Reddy et al, 1987; Richard et al, 1978). When exposed to aflatoxins, the effectiveness of vaccination is decreased for the effect of aflatoxins on the immune system. The growth rate is decreased as a

result of chronic aflatoxin exposure due to aflatoxin convalent binding to DNA and decrease protein synthesis (Edd, 1973). Gong et al (2003) reported that aflatoxins are directly related to underweight, the clinical cause of which was malnutrition and other diseases. Exposure to aflatoxins can decrease immunoglobulins concentration which provide vital protection against infections. And the decreasing in immunoglobulins is a reason for concern with respect to HIV transmission.

Obviously, the contamination of aflatoxins is an emerging problem. Aflatoxins has become a global health issue due to the consequences due to the consumption of this toxin by animals and human beings. It is clear that humans are commonly exposed to aflatoxins in areas where regulations are not enforced. Effective measures should be taken to prevent and control aflatoxicosis, and this requires the commitment of sufficient resources and the collaboration between the agriculture and public health communities, as well as proper regulatory policies by the local, regional, national, and international governmental agencies.

Biocontrol of aflatoxins

In recent years, increased attention has been devoted to prevent and control aflatoxins in crops. Given its long history, it will be a hard work for us to virtually eliminate the global distress caused by aflatoxin contamination of crops and agricultural commodities. Physical and chemical methods are not widely accepted because of their low efficiency or environmental concerns (Jermnak et al, 2012, kong et al, 2014). By contrast, the biological control is efficient, less hazardous, and thus believed to be the most promising method for diminishing the threat of aflatoxin contamination both preharvest and postharvest (Razzaghi-Abyaneh, 2013). Beneficial metabolites of miroorganisms, plants and insects and essential oil from medicinal plants and herbs have attracted a high degree of attention due to their relatively safe status and enrichment (Razaghi-Abyaneh et al, 2008). Several extracts from Teucrium ramosissimum possess antimutagenic activity against aflatoxin B_1 (Sghaier et al, 2011). Akacid$^{®plus}$, methyl syringate and Satureja hortensis L. essential oil have been reported for their inhibitory effects on aflatoxin production or on the growth of aflatoxigenic fungi (Jermnak et al, 2012; Razzaghi-Abyaneh et al, 2006).



The use of various antagonistic microorganisms such as fungi, bacteria, and actinomycetes as biological control agents, has been the most potential strategy for an effective reduction of aflatoxin contamination. Terrestrial bacteria identified from agricultural crops can have versatile effects against aflatoxigenic fungi. Doener et al, (2003) reported that three strains of A. flavus and A. parasiticus that cannot produce aflatoxins, were effective biocontrol agents to reduce preharvest aflatoxin contamination in peanuts. The maize endophyte Acremonium zeae is considered an antagonistic microorganism to aflatoxin producing fungi such as A. flavus and Fusarium verticillioides (Wicklow et al, 2005). A

Figure 3. Inhibitory effect on the growth of *A. flauvs by B. megaterium*. The treatments were as following: (**A**) autoclaved culture; (**B**) culture filtrate; (**C**) 1 $\times 10^8$ CFU/ml unwashed cell culture mixture; (**D**) 1×10^8 CFU/ml washed cell suspension; (**E**) sterile distilled water as a control.

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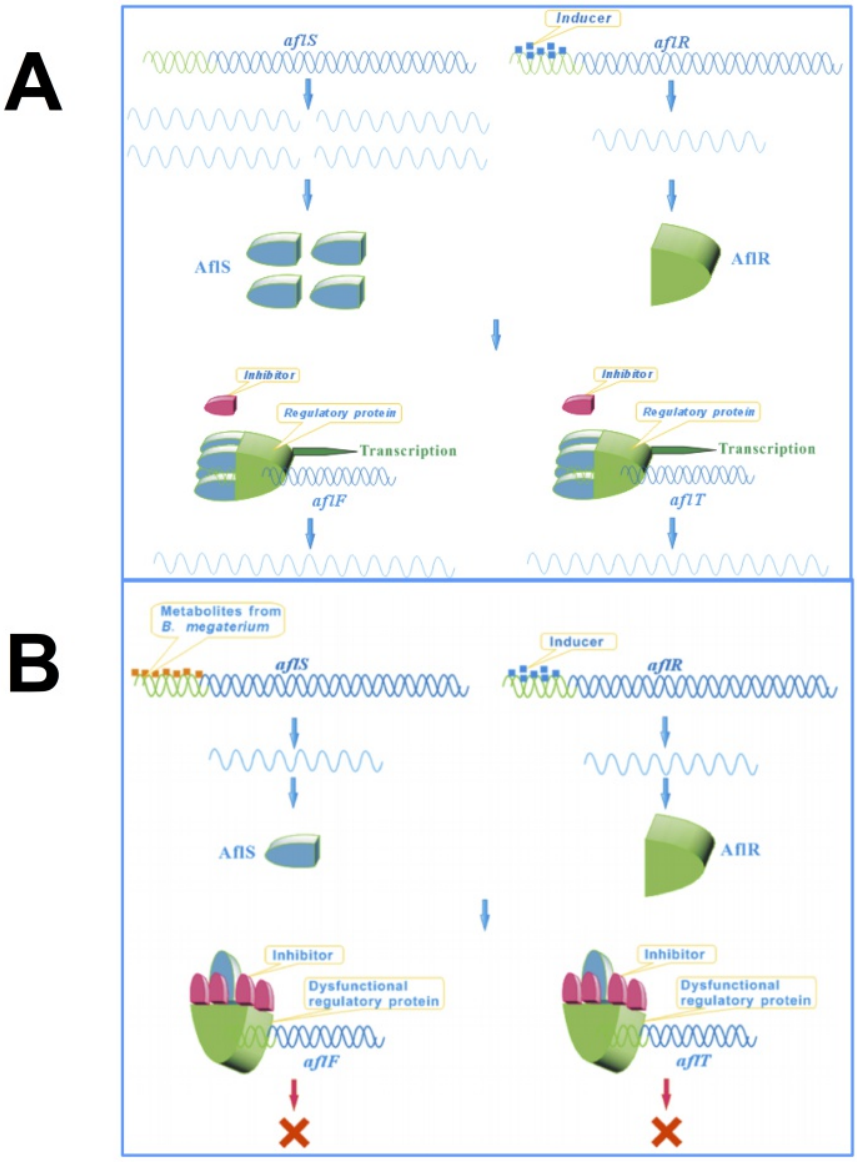
cyclodipeptide (L-leucyl-L-propyl) produced by Achromobaceter xylosoxidans has been purified and can siginificantly inhibit aflatoxin production by A. parasiticus (Yan et al, 2004). Muhialdin et al (2016) reported that the peptide FPSHTGMSVPPP can inhibit the growth of the A. flavus MD3. Sun et al (2015) found that the cinnamaldehyde (CIN) extracted from Cinnamomum spp. can inhibit AFB_1 production in a dose-dependent fashion and postpone spore germination of A. flavus.



Effect of B. megaterium on germination and growth of A. flavus in vitro

Kong et al. (2010) reported that a strain of marine Bacillus megaterium isolated from the Yellow Sea of East China can significantly reduce the biosynthesis of aflatoxins and expression of aflR and aflS genes. To evaluate the interactions between the antagonist and the pathogen in culture, five different treatments of B. megaterium were tested. It was demonstrated that the washed cell suspention of B. megaterium at 10^8 CFU/ml significantly inhibit the growth of A. flavus compared to the control (Figure 3).

Gene chip analysis indicated A. flavus genes that were down-regulated by co-cultivation with B. megaterium across the entire fungal genome and some specifically within the aflatoxin pathway gene cluster (aflF, aflT, aflS, aflJ, aflL, aflX). Importantly, the expression of the regulatory gene aflS was significantly down-regulated during co-cultivation. As a result, the AflR/AflS-dependent aflatoxin pathway gene transcription cannot be activated and no aflatoxin was produced (Figure 4).

Detoxification of aflatoxins

To cope with the negative impact of aflatoxins on health,the prevention of toxin formation, its elimination, inactivation or the reduction of toxin bioavailability in contaminated products are also important strategies, which can be achieved with

Figure 4 Schematic representing the theoretical inhibition mechanism by *B. megaterium*. *B. megaterium* inhibited aflatoxin biosynthesis through down-regulating the aflS gene expression in *A. flavus*. (**a**) Under normal aflatoxin-producing condition in PDB and MM (control). (**b**) Under *B. megaterium* inhibited condition in PDB and MM (treatment). Effect of B. megaterium on germination and growth of *A. flavus* in vitro. Taken from Kong et al. (2014), with authors' permission.

physical, chemical, and biological control methods. A variety of physical methods are widely adopted for the degradation of aflatoxins, such as heat, absorption,, extraction, gamma rays and ultraviolet light irradiation (Das and Mishra, 2000; Kabak et al, 2006). A number of chemical methods have been screened for their ability to alter the chemical structure of aflatoxins, for example, ozonization, ammoniation, treatment with formaldehyde and calcium hydroxide, or exposure to chlorine gas and hydrogen peroxide (Luo et al., 2014; Saalia and Phillips, 2011). However, both physical and chemical methods have limitations, such as certain nutrients may be destroyed in the process, expensive equipment may be required or concerns derived from the safe usage of the equipments. While, biological control methods using microorganism such as yeasts, filamentous fungi, bacteria, algae, etc., on aflatoxins offers an attractive alternative for the control of aflatoxins in foods and feeds due to their efficiency,

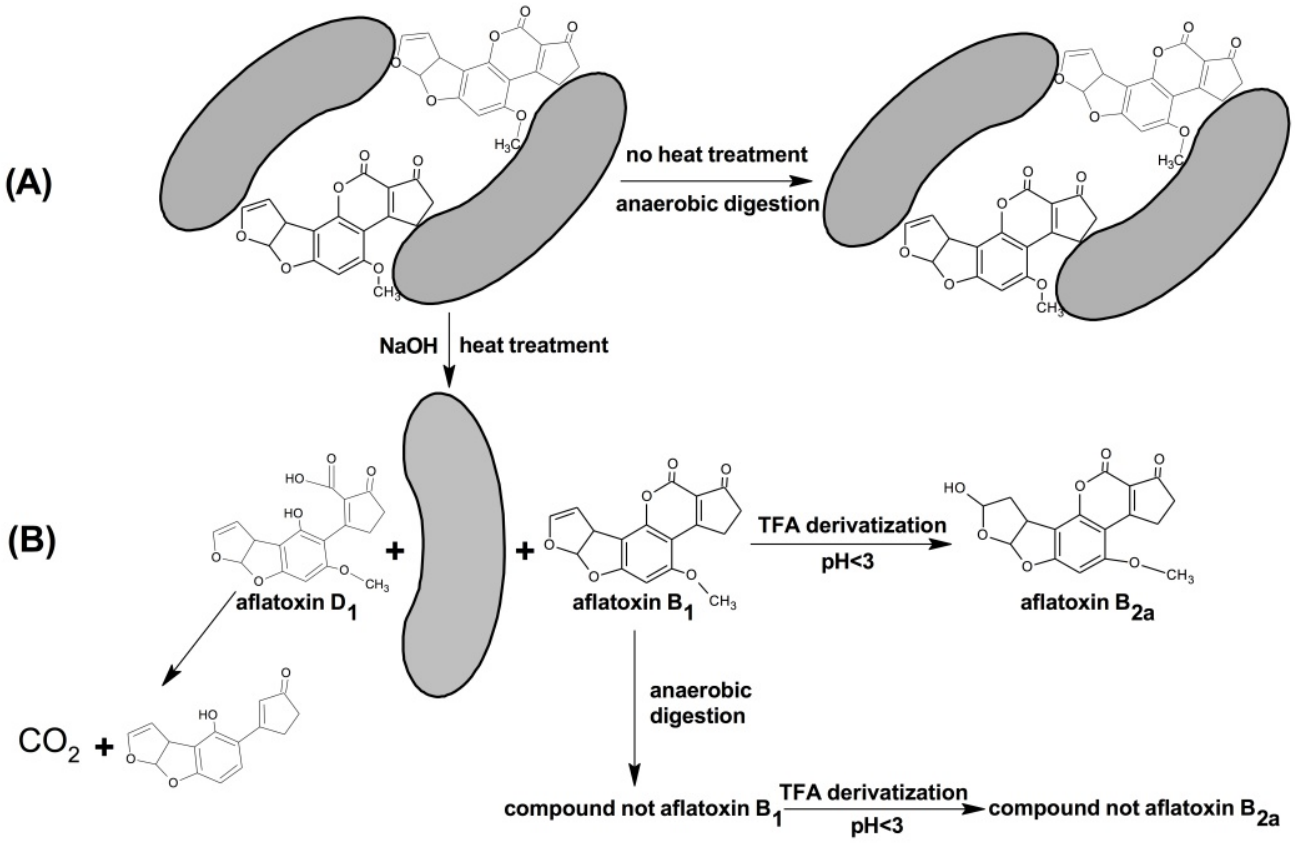


Figure 5. Schematic representing the theoretical aflatoxin B1 biotransformation process by anaerobic solid fermentation. (A) Peanut meal proteins firmly combine with AFB_1 and most AFB_1 couldn't be broken down by direct anaerobic solid fermentation. (B) Alkaline heat treatment could break the linkage of peanut meal proteins and AFB_1. And then partial of AFB_1 could be converted into AFD_1; most of AFB_1 could be biotransformed by anaerobic digestion. When the AFB_1 is derived by trifluroacetic acid (TFA), the AFB_1 is transformed into AFB_{2a} which could be easily detected by HPLC with a fluorescent detector. Taken from Chen et al. (2015), with authors' permission.

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specificity, cost-effectiveness, and environmental friendliness (Fazeli,2009). Among the types of studied microorganisms, lactic acid bacteria and yeasts have been the best.

Most of lactic acid bacteria (LAB), due in large part to their Generally Recongnized as Safe (GRAS) status and their uses as probiotics, are of particular interest for aflatoxins sequestration (Hernandez-Mendoza et al., 2009). In the 1960s, the first bacterium Flavobacterium aurantiacum B-184 was proved to have the ability to irreversibly remove aflatoxins from solution (Ciegle, et al. 1966). Shahin (2007) reported that 27 strains of Lactococcus spp. and 15 strains of Streptococcus spp. were tested for their aflatoxin removal ability using viable and non-viable preparations in buffered solution and different vegetable oils. And the results showed, the removal rates of L. lactis and S. thermophilus in buffered solution were 54.85% and 81.0%, respectively. While in vegetable oils viable L. lactis cells removed from 71% to 86.7%, the non-viable cells removed 100% of the toxin in all the oils tested. With S. Thermophilus, the viable cells removed from 66.5% to 91.5% of the toxin, and non-viable ones, from 81.7% to 96.8%. Both viable and non-viable cells are able to remove aflatoxins in aqueous solutions, demonstrating that cells may physically bind to aflatoxins. And the integrity of the bacterial cell wall is important in the process of aflatoxin binding to both viable and non-viable cells (Hernandez-Mendoza et al, 2009). Mainly polysaccharide and peptidoglycan components of the bacterial cell wall have been shown to covalently binds aflatoxins. In addition to the type of bacterial strain, other factors in inactivation treatment such as heat-pretreatment and acid-pretreatment, bacterial countsthe bacterial species, pH, incubation temperature, addition of nutrients, and the solvents used, can also influence the formation and stability of the LAB/aflatoxin complex (Oluwafemi and Silva, 2009; Peltonem et al, 2001).

Saccharomyces cerevisiae is the best known species of yeast, and has also been studied for their ability to remove aflatoxins from contaminated media. Joannis-Cassan et al (2011) reported that in pH 3, 37ºC, and 15 minutes of contact condition, the removal rates of aflatoxins from 2.5% to 49.3%, depended on aflatoxin concentration and on the yeasts-based products used,. The ability of S. cereviciae to adsorb AFB_1 in contaminated cron was analyzed. The author found that the adsorption process was inversely proportional to the aflatoxin concentration, that is, the higher the AFB_1 concentration in the medium, the lower the efficiency of AFB_1 removal by S. cereviciae (Madrigal-Santillán et al, 2006). Immobilized S. cereviciae cells were also investigated for their ability to remove AFB_1. It was observed that the cell counts in the medium had an important influence on the amount of aflatoxins removed (40% and 70% of removal for concentration of 10 ng/ml and 20 ng/ml, respectively). Other authors concluded that the treatment of S. cereviciae cell with heat at 60ºC and 120ºC, and in the presence of acid can increase their ability to bind AFB_1 from medium (Shetty et al, 2007; Rahaie et al, 2010). As for the possible binding mechanisms, it has been shown that the weak hydrogen bonds and van der Waals contacts between the aflatoxins and β-D-glucans components of the yeast cell wall are responsible for the absorption process (Jouany et al, 2005).

But the removal mechanisms are not limited to the formation of complexs of cell wall with aflatoxins. We have reported that the S. thermophilus and L. delbrueckii subsp. Bulgaricus can biotransform the aflatoxins in solid medium (Chen et al, 2015; Figure 5). It was shown that the capability of biotransforming aflatoxins by this method could reach 100%, and this results means that it is possible to biotransform the toxins by fermentation, and not only depending on its reversible absortion by the cells.

Conclusion

In conclusion, biodegradation of aflatoxins is an promising choice for preserving foods and feeds quality and safety. Lactic acid bacteria and yeast have a huge potential for practical application in preventing aflatoxin contamination. However, the mechanisms of how the cell-aflatoxin complex formed are not clear yet. Because the cell-aflatoxin bond may be broken with changes in the pH and temperature conditions, it is necessary to determine the behavior of strains in the different environmental conditions. And it is also a wise choice to biotransform aflatoxins to non-toxic components.

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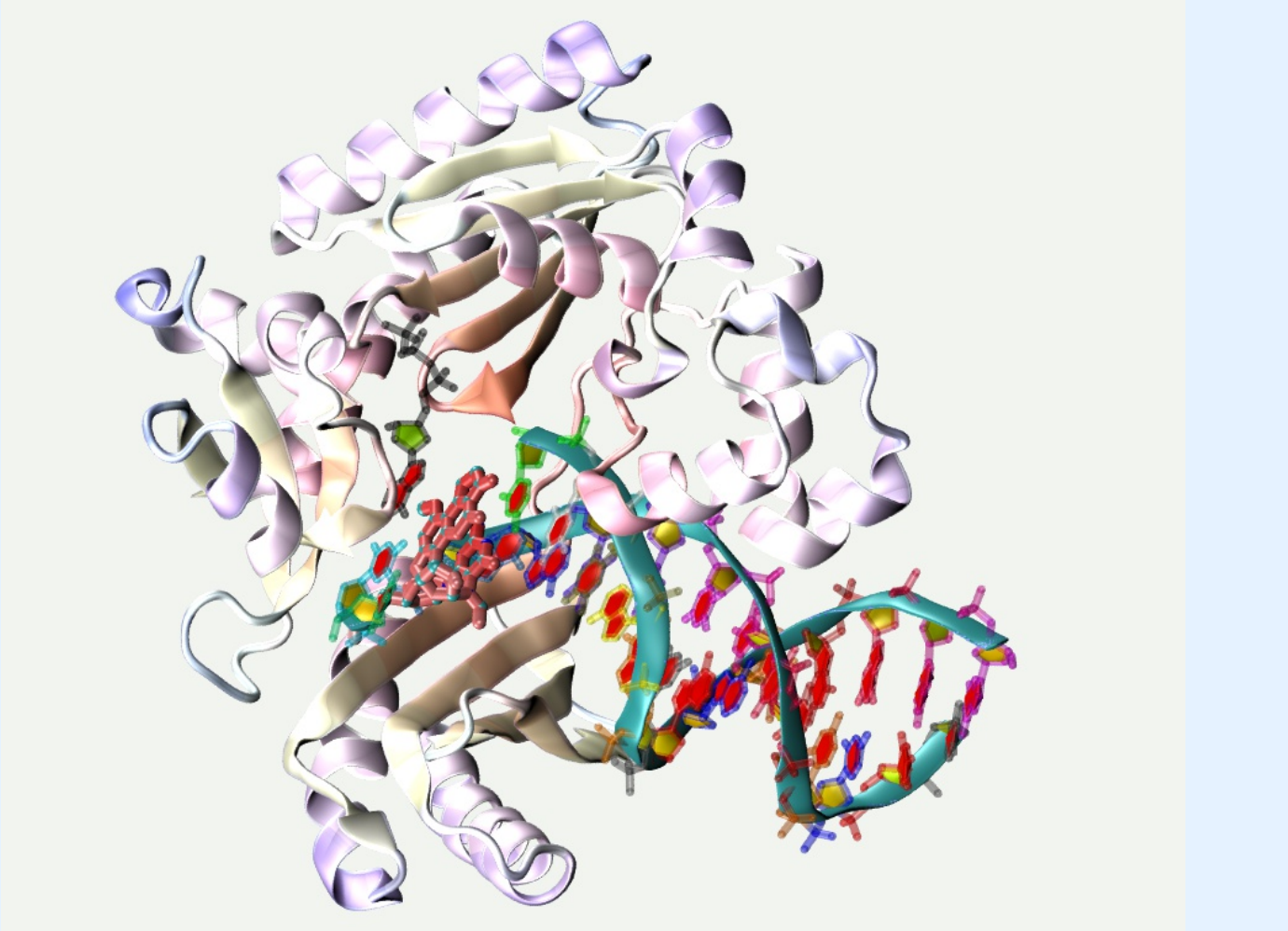

Related graphics. Schematic representing the theoretical three-dimensional structure of the Quaternary Aflatoxin B_1-DNA-DNA polymerase IV complex with incoming dATP. [Banerjee S, Brown KL, Egli M, Stone MP (2011) Bypass of aflatoxin B1 adducts by the Sulfolobus solfataricus DNA polymerase IV. J Am Chem Soc 133: 12556-68, DOI:/10.1021/ja2015668 , PMID: 21790157]. The coordinates were taken from PDBid 3PW0 [Banerjee S, Brown KL, Egli M, Stone MP (2010) Ternary complex of Aflatoxin B1 Adduct modified DNA (AFB1-FAPY) with DNA Polymerase IV and incoming dATP, DOI:/10.2210/pdb3pw0/pdb]. The image was generated by IBCJ staff with the aid of VMD software [Humphrey W , Dalke A , Schulten K (1996) VMD: visual molecular dynamics. J Mol Graph 14: 33-38, PMID: 8744570].

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Intellectual Property Right Issues in Biotechnology: Some Recent Court Cases in the United States

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ABSTRACT

There have been several court cases involving biotechnological patent eligibility issues in the United States during the last ten years. This review compiles the issues, the contentious court cases involving gene patents of plants, animals and humans and the outcome of the judicial decisions.

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Purple: Patents & Intelectual Property Rights

Keywords

Genes and Patents, Patenting Human Genes, Patent-related Court Cases.

Abbreviations

CAFC, Court of Appeals for the Federal Circuit; PCR, Polymerase Chain Reaction; AMP, The Association for Molecular Pathology; US PTO, United States Patent & Trademark Office; ACLU, American Civil Liberties Union; NCI, National Cancer Institute; PBTC, The Pediatric Brain Tumor Consortium; USFDA, US Department of Agriculture.

Introduction

There have been some contentious court cases involving biotechnological patent issues in the United States. One is the patent eligibility case taken up by the Supreme Court in 2010 known as Bilski v. Kappos to affirm the 9-3 majority ruling by the Court of Appeals for the Federal Circuit (CAFC) in 2008 that business methods such as how to hedge commodity-associated risks are not patent eligible because they do not meet the court's machine or transformation test as well as reflect more on the mental calculations rather than inventive procedures.Another interesting case that the Court ruled in a 7-2 opinion in June, 2011, is known as Stanford v. Roche on the patent right ownership by academic institutions (or small businesses) on inventions that are federally funded under the University and Small Business Patent Procedures Act of 1980, commonly known as the Bayh-Dole Act. In this decision, the Supreme Court sided on the part of Roche Molecular Systems, who was sued by the Stanford University for infringing its patents on the use of the PCR (Polymerase Chain Reaction) technique in the detection and quantitation of HIV viral DNA in infected patients.The Supreme Court affirmed the ruling by the CAFC that the patent rights under the 1790 US Patent Laws belong to the inventor and not to an entity where the inventor was employed simply because the entity was funded under the Bayh-Dole Act and was entitled to any intellectual property created under such federal funding.In this case, the Stanford researcher signed a separate agreement with a company Cetus where the Stanford employee went to learn how to use PCR techniques for screening of HIV viral DNA and gave up the right of ownership to Cetus which was later acquired by Roche.

Another patent-related case involving the patenting of a natural process is that of Mayo Collaborative Services v. Prometheus Laboratories which involves thiopurine drug dosing in order to determine the optimum level of the drug in the blood of a patient for the treatment of autoimmune diseases.When Mayo started to develop a similar diagnostic test, Prometheus sued Mayo alleging patent infringement.A District Court granted Mayo summary judgment, contending that the Prometheus patents claimed natural phenomena and were patent ineligible.In 2009, the CAFC, which used a criterion called machine or transformation test, reversed the District Court ruling and affirmed the patent eligibility of the Prometheus patents.On appeal, the Supreme Court remanded the case back to the CAFC in view of the Court's decision on Bilski v. Kappos. The CAFC, however, reaffirmed its earlier decision. On further appeal, a unanimous Supreme Court in March, 2012, reversed the CAFC decision, pointing out that such procedures of drug dosing follow basically the laws of nature reflecting a natural phenomenonand are patent ineligible.

Gene Related Patent Cases

Early in 2013, there were two cases involving patenting of genes, both plant and human, that were under consideration by the US Supreme Court.These cases are different from the previously-mentioned cases in that both the cases involve patenting of DNA and genes, both plant and human, raising a different set of questions.One case is known as Bowman v. Monsanto while the other is known as The Association for Molecular Pathology (AMP) v. Myriad Genetics, Inc.

The Monsanto case involves a genetically-engineered soybean plant called ROUNDUP Ready which resists a weed killing herbicide glyphosate, also called ROUNDUP, produced and marketed by Monsanto.The modified soybean plant can resist the weed killer because it has been engineered by introducing in the plant genome a bacterial (Agrobacterium) gene that encodes an enzyme capable of inactivating glyphosate. Thus the ROUNDUP resistant (Ready) soybean plant can grow in presence of the weed killer by inactivating it but other weeds cannot.However, once the soybean plants are grown, harvested and sent to grain elevators for sales as animal feed or other uses, the seeds harboring the bacterial gene can be re-used for growth of the ROUNDUP Ready soybean.Thus the seeds have the self-replicating glyphosate-inactivating gene for which Monsanto holds patent rights and the farmers are obligated under a contractual agreement with Monsanto not to use the engineered seeds after the first harvest unless they buy new seeds from Monsanto every year or pay royalty for re-use.

An Indiana farmer, Vernon Hugh Bowman, who signed such a contract with Monsanto, however, used a different approach when planting a second round of soybean plants.Around 1999 onwards, he bought a mixture of seeds, not just the ROUNDUP Ready seeds but hoping to find some ROUNDUP Ready seeds in the mixture, from a grain elevator and planted the

seeds with the ROUNDUP weed killer to allow any resistant variety to grow.When a few such seeds did allow the plant growth (because these seeds contained Monsanto patent-protected technology), Mr. Bowman used these seeds for growing soybean plants.Once Monsanto became aware of this use around 2007, it sued Mr. Bowman for patent infringement, as Monsanto has done earlier with other farmers. A District Court in Indiana sided with Monsanto regarding the validity of its patent and awarded the company significant monetary compensation.On appeal, the CAFC agreed with the District Court decision, rejecting the doctrine of the patent exhaustion defense put forward by Mr. Bowman's lawyers.This doctrine implies that a patent is basically exhausted when the patented product is sold for the first time.The Court rejected the exhaustion on the validity of self-replicating patented entities that can carry the patented gene for every application.Indeed, such a doctrine was affirmed by the Supreme Court in a 2008 case involving computer chips manufactured by Intel and licensed from LG Electronics.In this case, the Supreme Court ruled that once Intel sold the chips to various computer manufacturers, the patent doctrine of exhaustion curtailed LG's rights to the chips.

After affirmation of the District Court ruling by CAFC, Mr. Bowman appealed to the Supreme Court for reversal.However, the Supreme Court in May, 2013, decided on the side of Monsanto. The Court rejected Mr. Bowman's arguments that once sold, the rights to the soybean seeds with the patented gene are exhausted.The Court upheld the patent eligibility of the self-replicating ROUNDUP Ready soybean seeds for future applications as well and thus gave Monsanto, as well as the agricultural biotechnology industry, a major boost with regard to the ownership of their intellectual property.

This Supreme Court victory for Monsanto is in contrast to another patent-enforcement related controversy of the same ROUNDUP Ready soybean plant in Europe, and this decision points to the unique role of DNA and genes as patentable material. The European case involves patent eligibility claims of the ROUNDUP resistance gene present in cooked soybean.Unlike the Bowman case where Mr. Bowman was growing the ROUNDUP Ready soybean plants in presence of the glyphosate herbicide, where the gene was functionally active to help the plant become glyphosate-resistant, the Monsanto lawsuit in Europe involved the presence of the DNA of the gene in a non-functional form in soy meals exported by an Argentinian company to a Dutch group in the Netherlands.The ROUNDUP Ready soybean meal containing the patented bacterial glyphosate-resistance gene was, however, not functioning in its role in promoting growth of the soybean plants in presence of the herbicide. Thus while Monsanto demonstrated the presence of the glyphosate-resistant gene in the soymeal and sued for patent protection, the European Court of Justice cited article 9 of the Directive 98/44/EC of the EU Patent Law which mandates that the patented DNA must perform its function in the material where it is present. The European Court of Justice, therefore, ruled against Monsanto because of the absence of the intended function of the ROUNDUP Ready gene present in the soymeal, thus providing an interesting perspective of the patent eligibility of a gene, only when tied to its functionality.

Patent Eligibility of Human Genes: *The Association for Molecular Pathology v. US PTO/Myriad Genetics*

Many human diseases such as cystic fibrosis, sickle cell anemia and hundred others are due to mutations in our genes (1). Cancers are a clear example, where sequencing of more than hundred tumor genomes has demonstrated the presence of about 50 mutations for pancreatic cancer, 100 or more mutations in lung and melanoma, and a varying numbers for others. Thus cancer is basically a disease of the old age where accumulation of such mutations, particularly in some key genes where the mutations are known as driver mutations while the others are called passenger mutations, can lead to cancer.For example, for the skin cancer melanoma, noted for its metastatic (movement to other organs) propensity, mutations in a gene known as PREX2 are more frequently found, making PREX2 appearing like a driver mutation (2). Besides old age, certain chemicals or radiation, such as nicotine in tobacco smoke or UV radiations in sunlight can trigger cancer.Thus mutations in genes that encode enzymes responsible for the metabolism and excretion of environmental xenobiotic compounds and carcinogens, such as N-Acetyl-Transferase (NAT1 and NAT2), Glutathione-S-Transferase (GST M1, M3, P1, T1 etc) or cytochrome P50 (CYP1A1, CYP2D6, etc), have been implicated in the causation of bladder, colon or lung cancers (3).Besides old age and exposure to environmental toxicants, a third way to cancer susceptibility is genetic inheritance from parents of certain mutations that can in turn confer cancer susceptibility.Thus knowing the presence of such inherited genetic mutations in one's genome can allow a potentially susceptible person to take precautionary measures, as prenatal or postnatal genetic screenings have shown (1).Often, the developers of such diagnostic testing procedures patent their methods to prevent others from carrying out the screening of the mutations for commercial gain, and then are alleged to charge high fees or limit the testing by others, thereby fostering frustrations among clinicians, patients and advocacy groups.This was the case when the Canavan Foundation and parents of

children suffering from Canavan disease, a central nervous system disorder due to mutations in chromosome 17 in a gene encoding an enzyme aspartoacylase, brought a lawsuit in 2000 in the District Court of Chicago against Miami Children's Hospital alleging high costs of testing because of limitations put on other clinicians for carrying out the patented testing.Such cases have created confusions and apprehensions in the general public about the value and ethics of the patenting of human genes and mutations (4).

Another example of a similar lawsuit because of alleged high cost of genetic testing and the limits of such testing by other clinicians is the case known as AMP et al v. US PTO et al (5).Around 1995-2000, the University of Utah Research Foundation and a company Myriad Genetics in Salt Lake City filed several patent applications to cover the role of two genes known as BRCA1 and BRCA2 where certain frameshift and deletion/genetic rearrangement mutations led to a very high (about 50 to 80%) incidence of breast or ovarian cancers in women where the normal incidence is about 8% or less (5).The claims in these patents related to isolation, purification and sequence determination of a gene BRCA1 present on chromosome 17 and a second gene BRCA2 on chromosome 13, where certain mutations were determined to foster susceptibility to breast, or with a lower frequency to ovarian, cancers.Seven such patents were issued between 1997 and 2000 on BRCA1 and BRCA2 genes, whereupon Myriad Genetics was alleged to charge high fees for testing and prevent other clinicians and medical groups to conduct the genetic tests under an acceptable licensing agreement with Myriad Genetics.Myriad Genetics was also alleged not to allow women with a family history of breast or ovarian cancer, who were diagnosed by Myriad Genetics to harbor the BRCA mutations, to have the tests conducted by a second testing group to have an independent evaluation.

Frustrated with Myriad Genetics' strict enforcement of its patent rights on BRCA1 and BRCA2 genes, a group of patients, clinicians, the Association for Molecular Pathology (AMP), American Civil Liberties Union (ACLU) and the Public Patent Foundation brought a lawsuit in May, 2009, in the District Court of the Southern District of New York against the United States Patent & Trademark Office (US PTO), Myriad Genetics and the University of Utah Research Foundation.They argued that the US PTO should not have issued the BRCA gene patents to the patentees since these human genes are products of nature, common to mankind and should not be the products of commerce.In March 2010, Judge Robert Sweet of the District Court in Manhattan granted a summary judgment in favor of the plaintiffs, revoking the seven patents issued to Myriad Genetics.

In June, 2010, Myriad Genetics appealed the District Court ruling to the CAFC for reversal, arguing that the isolated and purified BRCA genes were not the same as they occurred in the human genome and required considerable human ingenuity and intervention.The isolated DNA had free 5' and 3'ends that were not free when the genes were part of the genome.The 3-judge CAFC panel in July 2011 then reversed the New York District Court ruling, upholding the patent eligibility of isolated and purified human genes under 35 USC section 101.The panel unanimously held the c-DNA forms to be patent eligible while isolated and purified DNA fragments led to a split 2-1 decision with one judge finding the scope of the structural changes insufficient for patent eligibility.The CAFC held a negative view of the patentability of the sequence comparison for defining mutations in the BRCA genes, however, asserting that such sequence comparisons are mental exercises that do not fall under the patent eligibility laws.

After the issuance of the CAFC decision, the parties approached the Supreme Court for a final resolution, submitting a petition for writ of certiorari in December 2011, which was granted.However, in March 2012, the Supreme Court vacated and remanded the AMP v. Myriad Genetics case, No. 11-725, back to the CAFC to reconsider its decision based on the Supreme Court's ruling on Mayo Collaborative Services v. Prometheus Laboratories where the Supreme Court held the Prometheus diagnostic tests to be patent-ineligible, as mentioned earlier.In August 2012, however, the 3-judge panel of the CAFC reaffirmed its earlier decision on the patent eligibility of the isolated and purified BRCA1 and BRCA2 genes as distinct chemical entities, as opposed to the genes on the chromosome, thus leading the plaintiffs to appeal to the Supreme Court for a final resolution of the case.On June 13, 2013, in a unanimous decision, the Supreme Court held that isolated and purified BRCA1 and BRCA2 genes are naturally occurring DNA segments without any modifications and are not patent eligible.The Court ruled that any cDNA, that is different from the naturally-occurring intron-containing DNA, will be patent eligible provided it satisfies the statutory requirements of patentability.This decision thus reversed the 2-1 majority decision by the CAFC that Myriad's claims on isolated and purified BRCA genes were patent eligible.The Supreme Court acknowledged that Myriad found an important and useful gene(s), but separating that gene from its surrounding genetic material was not an act of invention, contending that ground breaking, innovative or even brilliant discovery does not necessarily satisfy the section 101 inquiry.

A more recent court case involving the patent eligibility issue of a diagnostic procedure is known as Sequenom, Inc. v. Ariosa Diagnostic Inc., Natera, Inc. & DNA Diagnostic Center, Inc., involving judicial exception categories of ‘natural phenomena’ and ‘law of nature’. The case involved a patent on Sequenom invention of using maternal blood, serum or plasma to

amplify selectively the paternally-inherited sequences in fetal DNA, enabling the technology developer to diagnose the gender or any potential genetic defect in a baby without using invasive procedures to draw samples of amniotic fluid from the amniotic sac of the mother. Sequenom's '540 patent covered this test, known as MateriT21 test, but was rejected for patent eligibility by the Court of Appeals for the Federal Circuit (CAFC) on the ground of a previous Supreme Court decision on Mayo Collaborative Services v. Prometheus Laboratories, Inc. (132 S. Ct. 1289, 2012). Interestingly, the CAFC recognized the importance of the claims in the Sequenom patent but considered the claims as reflecting a natural phenomenon/process using commonly used isolation, detection and DNA sequence techniques and therefore patent ineligible under the Supreme Court's Mayo decision. Sequenom has since filed an appeal to the Supreme Court to consider the case.

Concluding Remarks

Nearly 8% of breast cancer positive women harbor BRCA mutations.Since about 8 to 10% of all women will likely get breast cancer in their lifetime, about 1% or slightly less of all women will develop breast cancer due to BRCA mutations.This is indeed a huge number that requires urgent remedy.The first remedy is, of course, to determine who are the most vulnerable persons, particularly with a family history of breast or ovarian cancers.This requires the genetic screening for the presence of the BRCA mutations, which has led to the patent-related monopoly problem.We have previously argued (5) that the US PTO, as dictated by their revised guidelines of 2001 requiring specific, substantial and credible utility of a gene to be patent eligible under 35 USC section 101 and 112, should not have granted patents on the non-mutated BRCA1 and BRCA2 genes used as reference genes to determine the nature and number of BRCA gene mutations/deletions/genetic rearrangements in the genomes of vulnerable women.However, we have also argued (5, 6) that determination of the mutations in the BRCA1 and BRCA2 genes, even though involving nucleotide sequence comparison-related mental exercises, deserves patent protection based on the Supreme Court precedent Diamond v. Diehr, 450 US 175 (1981).This Supreme Court decision allowed patentability of mental exercises when such exercises are tied to a useful invention (optimum time of curing of rubber in this instance).Given the enormous importance of the screening of BRCA1 and BRCA2 mutations in vulnerable women to allow them to take preventive measures, we argued that the screening for mutations, but not the reference non-mutated genes, should be patent eligible.A simple example will illustrate this.A company comes up with a more colorful and more fragrant genetically-modified rose.When demonstrating the improved nature of the genetically-engineered rose for patent protection, the company makes a comparative study of the brilliance of color and more fragrant nature of the genetically modified rose with a common garden-variety rose. The company then asks for patent protection of both the reference garden-variety rose and the genetically modified rose, thus having the patent-assured limited monopoly on all roses, natural and genetically modified.

A second and equally, if not more, important consideration is what to do if or when a woman, particularly when a teen-age girl, with a family history of breast or ovarian cancer is tested positive for the presence of the mutated BRCA genes.One choice is not to take any particular preventive measure but to remain vigilant with the hope that the cancer never makes an appearance, or if detected early, can be surgically removed without any aftereffect.One concern is the emergence of ovarian cancer which usually becomes detectable only after its metastatic migration to other body parts, leading to the appearance of symptoms.However, it should be noted that there are emerging protein-based diagnostic tests for ovarian cancer such as OVA1 approved by the FDA. This test uses a set of biomarkers and appropriate software to predict the probability of having ovarian cancer which could be useful for early detection.A less desirable and traumatic approach, used sometimes, is the surgical removal of the breasts and the ovary to prevent the onset of the cancers.This is particularly devastating for young women of child bearing age, as recently reported in many national newspapers.Ideally, the BRCA mutation-positive women should have access to drugs that are non-toxic for long term use with both cancer therapeutic and preventive activities.While no such drug currently exists, there appears to be on the horizon potential candidate drugs, such as p28, with some of these desirable properties (6, 7). p28, a peptide of 28 amino acids, has recently been shown in phase I clinical trials in 15 stage IV cancer patients with solid metastatic tumors to have no significant toxicity even at the highest dose but considerable beneficial effect including partial and sometimes complete regression of the tumors that were resistant to all conventional drugs (6, 7).These patients had an average life expectancy of less than six months, but after the termination of the trials, 3 patients were alive beyond two and three years with

their tumors regressed partially or completely (7). In a second phase I trial sponsored by the National Cancer Institute (NCI) and the Pediatric Brain Tumor Consortium (PBTC), p28 has shown acceptable toxicity in such pediatric brain tumor patients but with certain efficacy involving certain types of brain tumors (http://clinicaltrials.gov/ct2/show/NCT01975116) allowing the USFDA to approve the designation of p28 as an orphan drug for glioma. It's not known if the tumors that were uniquely susceptible to p28 harbored any particular type of driver or passenger mutations that conferred such susceptibility in the stage IV cancer patients.While this therapeutic activity of p28 with no toxicity but some efficacy against the drug-resistant metastatic tumors is encouraging, more important is the fact that its therapeutic activity aside, p28 has been shown to have cancer preventive activity as well (6).As we suggested earlier (6), this combined therapeutic and preventive activity without any demonstration of toxicity can be tested in mouse models.Introduction of activated oncogenes such as H-RAS, AKT and Tp53 through Cre-loxP-controlled lentiviral vectors can trigger brain tumor glioblastoma in all the mice (8) while the introduction of activated oncogenes K-RAS and Tp53can trigger adenocarcinomas of the lungs in mice.Oral and esophageal cancers can also be induced in male CBA mice when given a carcinogen 4-nitroquinoline-1 -oxide in drinking water for several weeks (9).Another carcinogen azoxymethane induces tumor formation when inflammation is induced in interleukin 10-deficient (IL 10 -/-) mice which in turn promotes colonization by genotoxic gut bacteria (10).p28 at various doses can be given to these mice through intravenous injections, or through nasal spray for lung cancers, to examine its potency in preventing the induction of cancers in such susceptible mice.If p28 can be shown to be functional in preventing the emergence of cancer in mice models, it can then be tested in BRCA1/BRCA2 mutation-harboring women in long term experiments to determine if p28 can be effective in significantly reducing the incidence of breast or ovarian cancers in women taking intravenous injections of p28 once a week, as opposed to women not taking p28.If the initial results look encouraging, further modifications of p28 can be made using polymers such as polyethylene glycol to produce an oral variety of p28 for long term consumption.Ultimately, if all these potential experiments show some degree of success, that will demonstrate the importance of the genetic screening of BRCA mutations in vulnerable women for them to take non-surgical preventive measures. We have recently written a book, a science fiction, entitled ‘Three Daughters, Three Journeys: Quest for Cancer Cure’ (Stanford Publishers, Singapore, in press) where we speculated how young girls, inheriting the BRCA mutations, will likely deal with the problem in the future.

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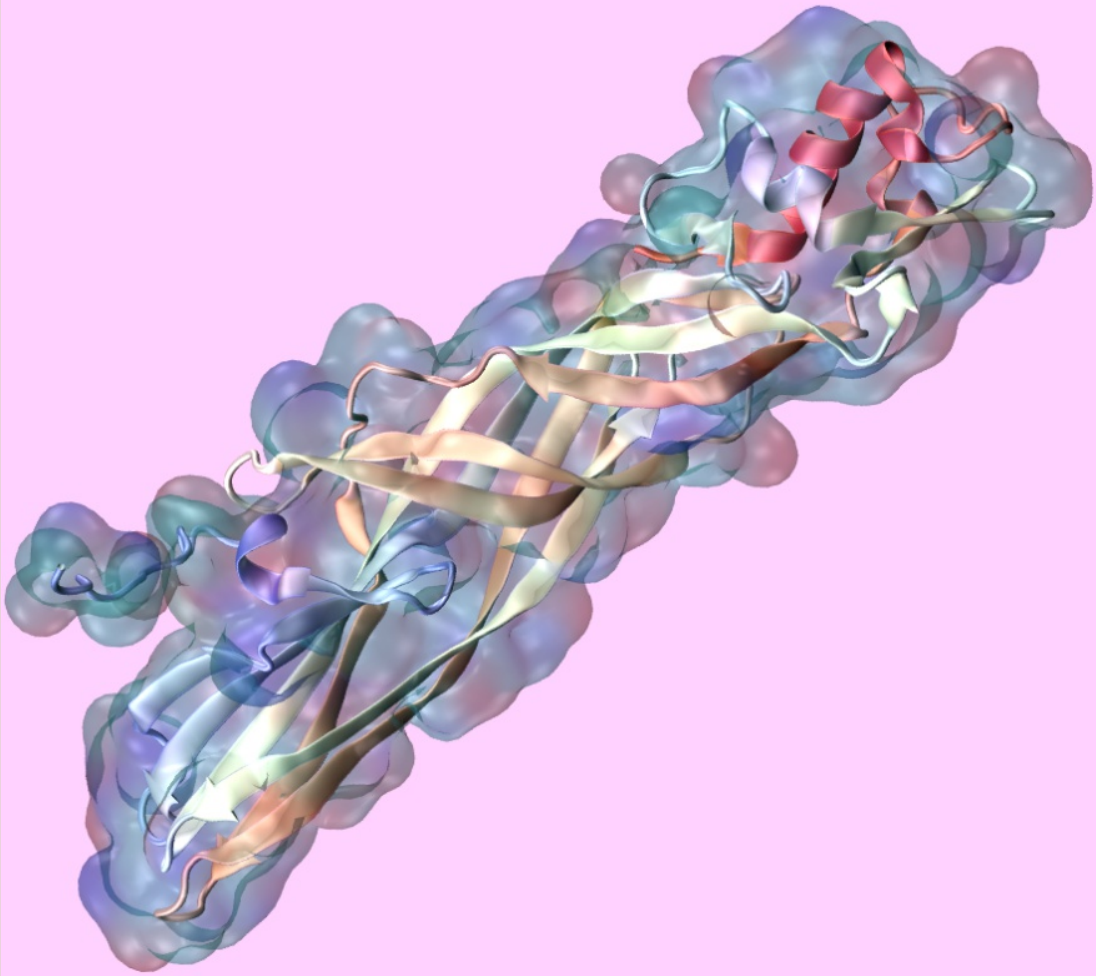

Related graphics. Schematic representing the three-dimensional structure of the Cry51Aa1 toxin from Bacillus thuringiensis. This toxin is one of the proteins expressed in insect-resistant transgenic crops [Xu C, Chinte U, Chen L, Yao Q, Meng Y, Zhou D, Bi L, Rose J, Adang MJ, Wang B, Yu Z, Sun M (2015) Crystal structure of Cry51Aa1: A potential novel insecticidal aerolysin-type β-pore-forming toxin from Bacillus thuringiensis. Biochem Biophys Res Commun 462:184-9, DOI:/10.1016/j.bbrc.2015.04.068, PMID: 25957471]. The coordinates were taken from PDBid 4PKM [Xu C, Chinte U, Chen L, Yao Q , Meng Y, Zhou D, Bi L, Rose J, Adang MJ, Wang B, Yu Z, Sun M (2015) Crystal structure of Bacillus thuringiensis Cry51Aa1 protoxin at 1.65 Å resolution. DOI:/10.2210/pdb4pkm/pdb]. The image was generated by IBCJ staff with the aid of VMD software (Humphrey W, Dalke A, Schulten K (1996) VMD: visual molecular dynamics. J Mol Graph 14: 33-38, PMID: 8744570).

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Evaluation of Protease Activity from Two Brewing Malts

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ABSTRACT

Alcoholic fermentation is a set of biochemical transformations in which, certain yeast strains are responsible for transforming the carbohydrates present in wort to a liquid containing alcohol and carbon dioxide. Therefore, the processing of wort is a high-impact process, in which proteases play a vital role, because their hydrolytic activity enriches worts in amino acids and peptides, essential to fermentation. The role of these biomolecules is well known, but there are still fundamental questions about the variation of the content and type of these proteins. In this study, we analyzed the protease activity of two malts using zymograms and we found differences in the profile and level of activity of proteases sensitives to E64 plus PMSF inhibition.

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Abbreviations:

M1, Mexican brewing malt sample 1; M2, Mexican brewing malt sample 2; PMSF, ;Phenylmethanesulfonyl fluoride E-64, trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane; 2D, bidimensional.

Key words

Brewing malt; zymogram; proteases; proteome

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INTRODUCTION

Biotechnology has been part of preparation of beer since the beginning. The study of the brewing process has contributed to improve the knowledge of important features of beer, mainly in areas like raw material quality, taste, aroma, appearance and microbiological quality. These features impact shelf-life, production and demand.

The need to combine agronomic aspects with industrial quality makes malting barley a complex crop, in relation to its genetic improvement. Therefore, the absence of a reliable marker of malt quality, could cost a beer company a substantial amount per year in expenses process adjustments, required to compensate for the low quality of the raw material. Malt proteases play an important role because these determine the type and concentration of amino acids in the wort, which are metabolized by yeast, generating ester type flavors and modifying the flavor profile of beer. Because we do not have a full knowledge and control about the synthesis of compounds influencing the characteristics of beer, a better characterization of malt is necessary regarding the levels of expression of yeast genes involved in the production of volatile compounds in the beer, and the relationship between proteases and amino acids present in the wort. Because of this, we decided to study the protease activity in malt to help to brewer to accept, reject or combine malt types using specific molecular markers as selection criteria to control the characteristics of final beer.

MATERIALS AND METHODS

Materials

Two samples of Mexican brewing malt named M1 and M2, were provided by Cervecería Cuauhtémoc-Moctezuma. Samples were stored at -20 °C until use.

Protein extraction

0.52 g of ground malt (50µm) were homogenized in 1 ml of sodium acetate buffer 50 mM pH 5.0 or 25 mM Tris-HCl pH 7.5 by vortex for 30 min, alternating in ice. Extractions were followed by 30 min centrifugation at 4°C and 10000 rpm. Supernatant was dialyzed against the same buffer for 24 h at 4°C (Wrobel and Jones, 1992) Total protein content was measured with Bradford method and stored at -20°C until used.

Electrophoretic separations one dimensional SDS-PAGE

Protein profiles were analyzed by 12% SDS-polyacrylamide gel electrophoresis. 10 µg of total soluble protein were mixed with 1 volume Laemmli sample buffer without β-mercaptoethanol (He, 2011). Gels were stained with Coomassie Brilliant Blue R250.

Proteolytic activity profile

Protease activity profile was studied by zymograms in two different ways:

Native zymograms sandwich-type: 100 µg of sample were mixed with loading buffer and separated in 12% polyacrylamide gel electrophoresis using non-reducing conditions (Vandooren et al., 2013). After separations, gels were transfer to 1% gelatin copolymerized polyacrilmide gel and incubated overnight in buffer 25mM Tris-HCl, 5mM $MgCl_2$ pH 5.0, 7.0 and 9.0 at 40°C. Clear bands of protease activity were visualized after Coomassie Brilliant Blue R250 staining.

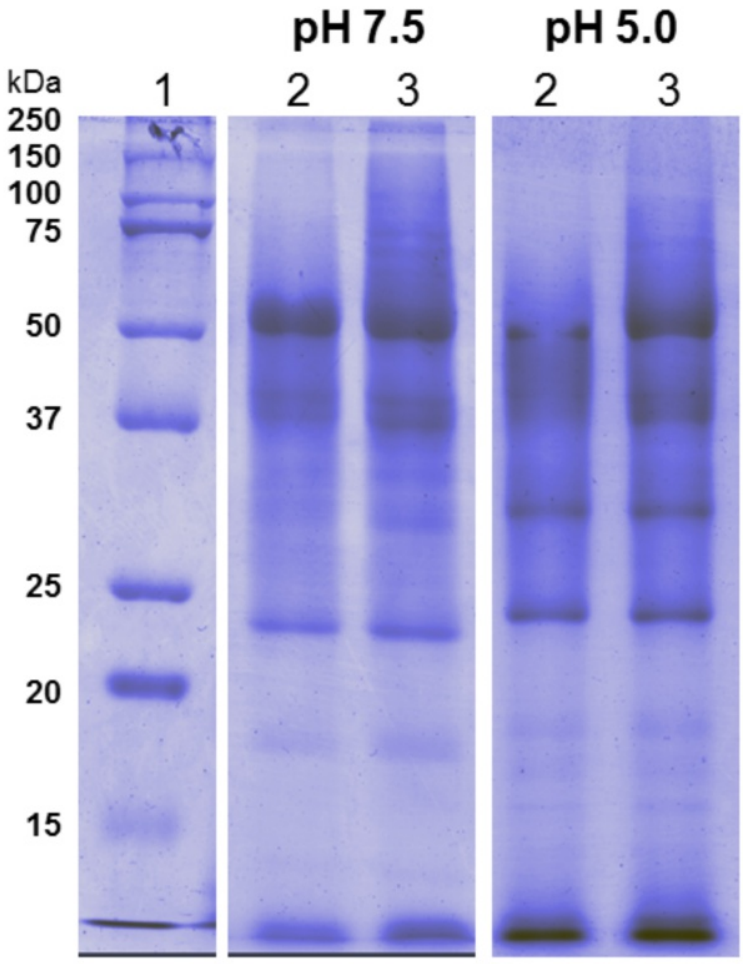

Figure 1. Proteins profile of malt extracted with 25 mM Tris-HCl pH 7.5 and acetates 0.05M pH 5.0. 1. Molecular weight marker Kaleidoscope (BioRad); 2. Malt M1. 3. Malt M2.

2D-zymograms: 2000 µg of sample were precipitated with 100% ice-cooled acetone, separated by centrifugation for 10 min at 12000 rpm and 4°C, washed twice in 80% acetone, air-dried and resuspended in rehydratation buffer (8M Urea, 2% Chaps, 0.2% IPG buffer (GE Healthcare)). The samples were passively absorbed during the rehydration step for 15 h into ReadyStrip™ IPG 7 cm IPG strips, pH 3-10 (Bio Rad) at 10°C. IEF was performed in Protean IEF Cell System (Bio Rad) for a total of 14 kVh. After IEF, strips were equilibrated for 30 min with equilibration buffer (375 mM Tris-HCl pH 8.8, 6M urea, 20% glycerol, 2% SDS), and subjected to 12% SDS-PAGE with 0.2% gelatin copolymerized as substrate. The gels were incubated for 16 h at 40°C in sodium acetate buffer 50 mM pH 5.0 and stained with Coomassie Brilliant Blue R250 and the protease "spots" were identified in the gel by the clear zones.

Activity inhibition studies

The effect of activity inhibitors 10 mM PMSF and 10 µM E-64 was studied based in the reported for Zhang and Jones (1995). 100 µg of sample were separated by 10% polyacrilamyde electrophoresis in reducing conditions with 0.1% gelatin copolymerized as substrate. After separations, the gels were incubated 16 h at 40°C in sodium acetate buffer 50 mM pH 5.0 in the presence or absence of inhibitors. The gels were stained with Coomassie Brilliant Blue R250.

RESULTS AND DISCUSSION

Protein profiling malt

Protein extractions of both malts were performed at pH 5.0 and 7.5 following the methodology described for Wrobel and Jones (1992) and Ostergaard et al. (2004) to compare the protein profile in both malts. Malts are very similar respect their protein profile with some differences respect to the buffer used (Figure 1). With sodium acetate buffer pH 5.0 we could extract more intensity bands and three different bands with low molecular weight (<20 kDa), we decided used this buffer for the proteolytic assays due to the similarity of pH during the mashing process of the beer production (Sopanen et al. (1980)).

Proteolytic activity profile

The differences in the proteinase profile of malts was studied by zymography using substrate gelatin. The Figure 2 shows the results using sandwich-type zymograms where differences in both malts is very clear, malt M1 shows three proteolytic activity bands around 150 and 242 kDa while malt M2 shows only one around 40 kDa, these results are like reported by Jones and Marinac (2002).

The 2D-zymograms were used for a better knowledge about the proteases presents in both malts. Malt M1 shows more intense activity than malt M2 (Figure 3). Both malts share one group of two activity-spots and four clustered activity spots with higher level of activity in malt M1 than malt M2. Two activity spots are present only in malt M1 and no different spots are present in malt M2. All activity spots have pI value of 4.0-6.5.

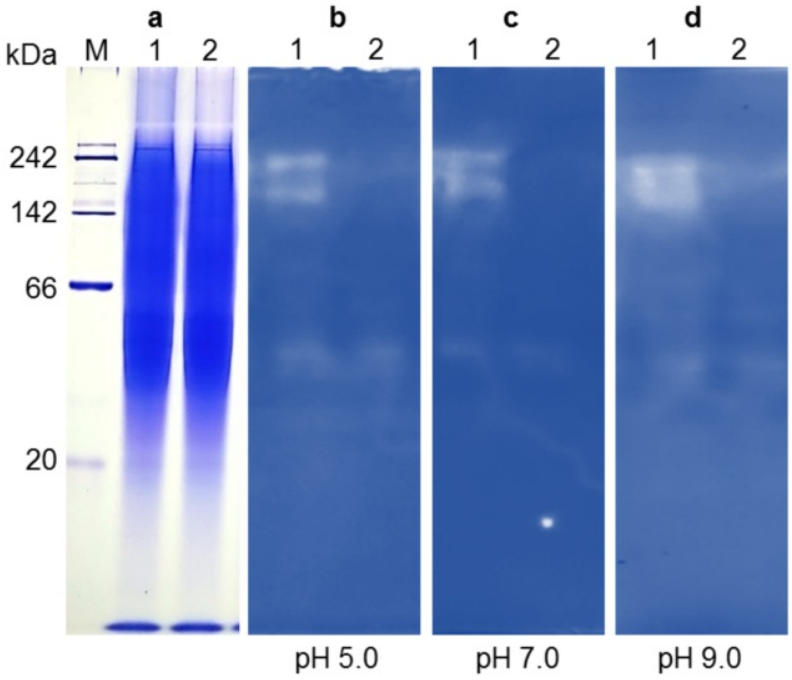

Figure 2. Effect of pH on the proteolytic activity in zymograms sandwich-type. (a) shows the protein profile of different malts after transfer to copolymerized substrate gel. (b, c) and (d) show the proteolytic activity in 1% gelatin zymogram at different pH values. M. Native molecular weight marker (Invitrogen). 1. Malt M1. 2. Malt M2.

Activity inhibition studies

To determine the type of protease activity presents in each malt, we used specific protease inhibitors: E-64 [10 µM] for cysteinyl-type protease activity and PMSF [10mM] serine-type protease activity (Zhang and Jones, 1995). With no inhibitors used, both malts show 2 zones of activity, high molecular weight zone (>100 kDa) and low molecular weight zone (<100 kDa) (Figure 4-A). When E-64 is used, the low molecular weight zone activity is inhibited completely (Figure 4-B). In the presence of PMSF, the high molecular weight activity is inhibited almost completely (Figure 4-C) and both zones were inhibited completely with the combined inhibitors (Figure 4-D). So, we assume that proteases present in both malts are a combination of two types: cysteinyl-type protease in the low molecular weight zone and serine-type in the high weight molecular zone.

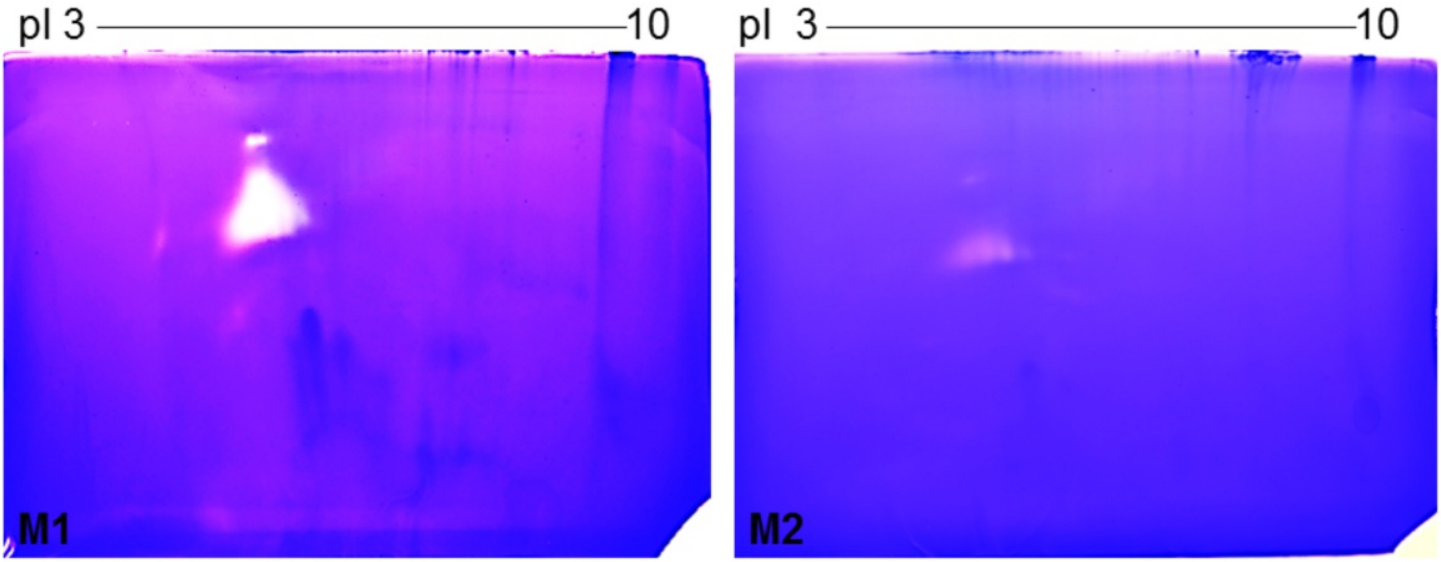

Figure 3. 2D-Zymograms separations of proteinases in malts. pI. Isoelectric point scale. M1. Malt M1. M2. Malt M2.

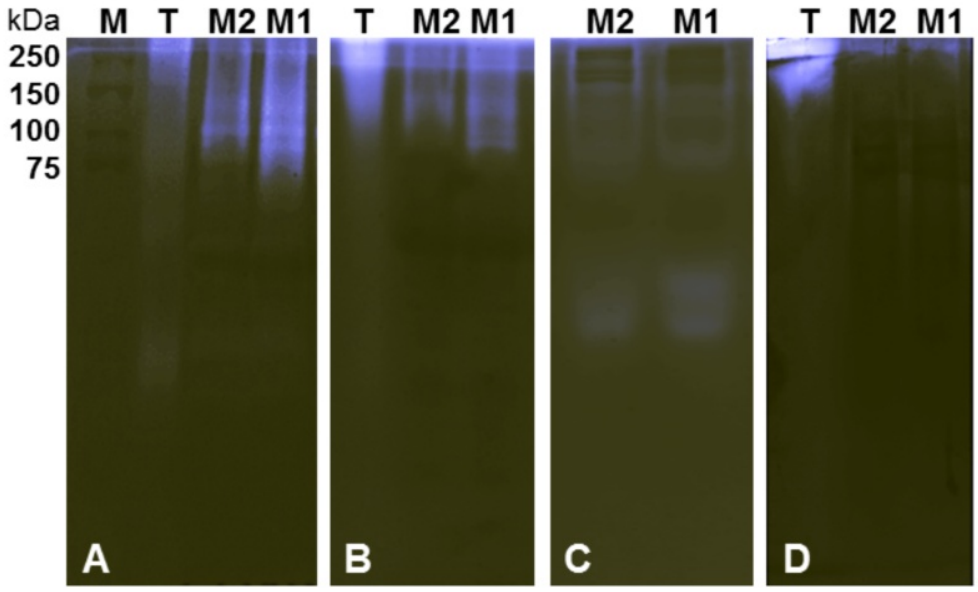

Figure 4. Effect of specific inhibitors PMSF and E64 on the proteolytic activity. (A) No inhibitors, (B) E-64 [10 µM], (C) PMSF [10mM] and (D) Combined E-64 [10 µM] and PMSF [10mM]. M. Molecular weight marker Kaleidoscope (Biorad). T. Trypsin 0.02 µg/ml. M1. Malt 1. M2. Malt 2.

CONCLUSIONS

In the present study, we could determine the protein profile of malt samples extracted at pH 5.0 and pH 7.5, using the sodium acetate buffer 50 mM pH 5.0 extraction, we saw more intensity bands and three different bands with low molecular weight (<20 kDa). By zymography techniques, we studied the proteases presents in malts and we found that malt M1 has more proteolytic activity bands at different pH conditions with molecular weight of 150 and 240 kDa and one of 40 kDa while malt M2 has only 1 band of approximately 40 kDa. To a better understood of the proteases in both malts, 2D-separations were carried on and we could saw activity spots with pI value of 4.0-6.5 and both malts share one group of two activity-spots and four clustered activity spots with different level of activity, higher in malt M1. These clustered spots could be isoforms of the same proteinases. Besides, malt M1 has two activity spots that are not present in malt M2. With inhibitor studies, we concluded that the proteases present in both malts are a combination of two types: cysteinyl activity-type protease and serine activity-type protease according to the specific inhibition in the presence of E-64 and PMSF. We are now in the process of studying other types of protease activity to characterize the "protease-omics" of both malts to identify which could serve as markers of quality

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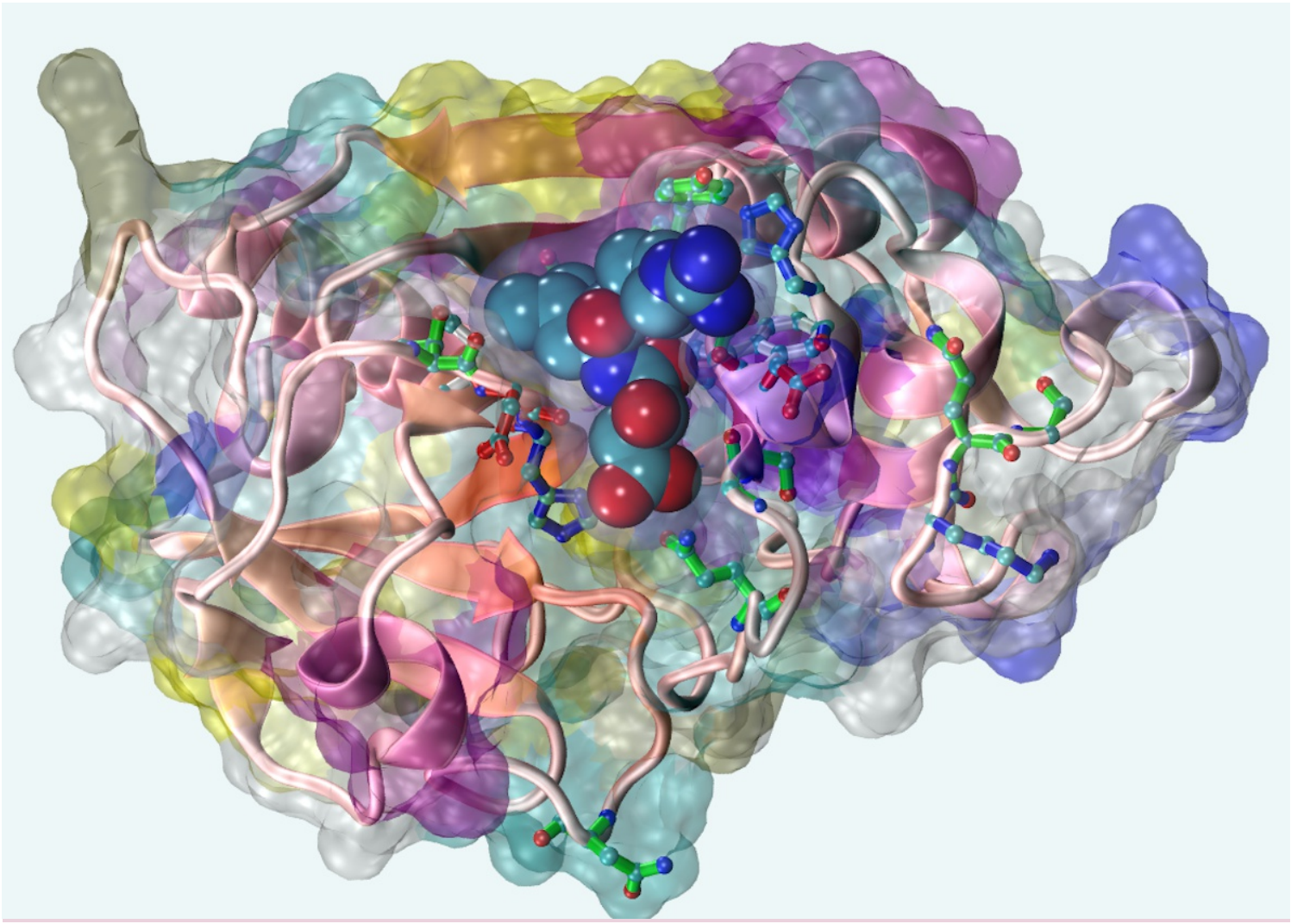

Relative graphics. Schematic representing the X-ray diffraction three-dimensional structure of the protease Mexicain from Jacaratia mexicana. The structure of specific cystein-protease inhibitor E64 (trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane; 2D, bidimensional) is shown as van der Walls spheres. The coordinates were taken from PDBid 2BDZ [Gavira Ja , Oliver-Salvador Mc , Gonzalez-Ramirez La , Soriano-Garcia M , Garcia-Ruiz Jm (2005) Mexicain from Jacaratia mexicana. , DOI:/10.2210/pdb2bdz/pdb]. The image was generated by IBCJ staff with the aid of VMD software (Humphrey W , Dalke A , Schulten K (1996) VMD: visual molecular dynamics. J Mol Graph 14: 33-38, PMID: 8744570).

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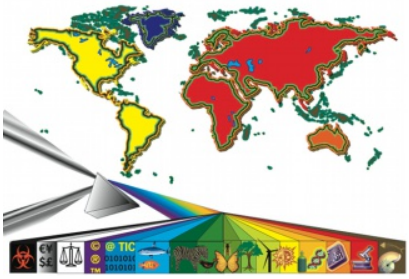

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