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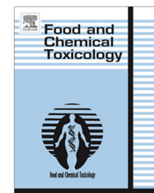

Effects of storage, processing and proteolytic digestion on microcystin-LR concentration in edible clams

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ABSTRACT

Accumulation of microcystin-LR (MC-LR) in edible aquatic organisms, particularly in bivalves, is widely documented. In this study, the effects of food storage and processing conditions on the free MC-LR concentration in clams (*Corbicula fluminea*) fed MC-LR-producing *Microcystis aeruginosa* (1×10^5 cell/mL) for four days, and the bioaccessibility of MC-LR after *in vitro* proteolytic digestion were investigated. The concentration of free MC-LR in clams decreased sequentially over the time with unrefrigerated and refrigerated storage and increased with freezing storage. Overall, cooking for short periods of time resulted in a significantly higher concentration ($P < 0.05$) of free MC-LR in clams, specifically microwave (MW) radiation treatment for 0.5 (57.5%) and 1 min (59%) and boiling treatment for 5 (163.4%) and 15 min (213.4%). The bioaccessibility of MC-LR after proteolytic digestion was reduced to 83%, potentially because of MC-LR degradation by pancreatic enzymes. Our results suggest that risk assessment based on direct comparison between MC-LR concentrations determined in raw food products and the tolerable daily intake (TDI) value set for the MC-LR might not be representative of true human exposure.

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1. Introduction

The occurrence of cyanobacterial blooms in freshwater is well recognized and documented. The main concerns for the environment and human health due to cyanobacterial blooms are the potential presence of high amounts of cyanotoxins in the water. Globally, the most studied cyanotoxins are microcystins (MCs), which are mainly produced by *Microcystis* but also by *Anabaena, Oscillatoria, Planktothrix, Nostoc*, and *Anabaenopsis* (Sivonen and Jones, 1999). Several structural variants of MCs have been identified. MC-LR (Fig. 1) is highlighted due to its toxicity and dominance in cyanobacterial blooms.

The main mechanism of MC-LR toxicity is the irreversible inhibition of serine/threonine protein phosphatases (PP) (PP1 and PP2A) (MacKintosh et al., 1990). The following two-step mechanism is involved in PP inhibition by MCs: (1) a rapid and reversible binding, followed by (2) a slower covalent binding (occurs over several hours) between the N-methyl-dehydroalanine (Mdha) residue of toxin and cysteine-273 of the catalytic subunit of PP1 (cysteine-266 of PP2A) (Craig et al., 1996; MacKintosh et al., 1995). MC-LR is preferentially taken up by hepatocytes due to active transport by the bile acid carrier transport system, a member of the family of Organic Anion Transporting Polypeptides (OATPs: human) (Fischer et al., 2005), with the liver as the main target. The formation of stable complexes between PP1/PP2A and MC-LR has been suggested to be critical for liver tumor promotion. Epidemiological studies in China may support this suggestion through the association of chronic exposure to MCs from contaminated drinking water with primary liver and colorectal cancer (Ueno et al., 1996; Zhou et al., 2002). Furthermore, the International Agency for Research on Cancer (IARC) classified MC-LR as "possibly carcinogenic to humans" (group 2B) (Grosse et al., 2006). Human health problems due to MC-LR are most likely associated with chronic exposure. Although the major source of long-term human exposure to MCs seems to be drinking water, exposure through

Abbreviations: ESI, electrospray; GST, glutathione-S-transferase; HACCP, Hazard Analysis Critical Control Points; HCl, hydrochloric acid; IARC, International Agency for Research on Cancer; LC–MS, Liquid Chromatography–Mass Spectrometry; MCs, microcystins; MC-LR, microcystin-LR; Mdha, N-methyl-dehydroalanine; MeOH, methanol; MRM, multiple reaction monitoring mode; MW, microwave; $NaHCO_3$, sodium bicarbonate; OATPs, Organic Anion Transporting Polypeptides; PDA, photoelectric diode array; PP, protein phosphatases; SD, standard deviation; SPE, solid-phase extraction; TDI, tolerable daily intake; WHO, World Health Organization; WW, wet weight.

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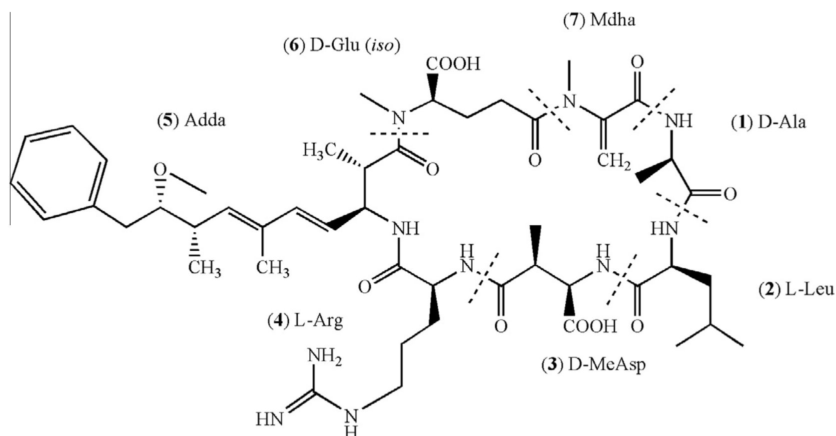

Fig. 1. The chemical structure of the heptapeptide MC-LR, where *D*-Ala is *D*-alanine (**1**), *L*-Leu is *L*-leucine (**2**), *D*-Me-Asp is *D*-erythro-*β*-methyl aspartic acid (**3**), *L*-Arg is *L*-arginine (**4**), Adda is the unusual amino acid (2s,3s,8s,9s)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoicacid (**5**), *D*-Glu is *D*-glutamic acid (**6**), and Mdha is N-methyl-dehydroalanine (**7**).

contaminated food must be further studied. Based on the potential for human health risks, the World Health Organization (WHO) established a provisional TDI of 40 ng/kg body weight for MC-LR. There are several reports of MCs accumulation in edible aquatic organisms (Amorim and Vasconcelos, 1999; Vasconcelos, 1995; Chen and Xie, 2005; Ibelings and Chorus, 2007). Bivalves (mussels and clams), which are filter-feeding organisms, may collect large amounts of toxic cyanobacterial cells. Bivalves seem to be insensitive to cyanotoxins, and, although most of them can detoxify MCs via the GST (glutathione-S-transferase) metabolic pathway (Pflugmacher et al., 1998; Vasconcelos et al., 2007), several studies have shown that MCs are stored in their organs (Vasconcelos, 1995; Amorim and Vasconcelos, 1999; Chen and Xie, 2005; Ibelings and Chorus, 2007). Furthermore, these organisms are usually eaten whole, which may enhance human exposure to MCs. Estimated daily intake of MCs was studied in four edible aquatic organisms, including clams, which seem to be unsafe for human consumption, once TDI proposed by the WHO was exceeded several times (Chen and Xie, 2005). Currently, most of the analytical methods used for MC-LR extraction from food matrices use organic solvents such as methanol (MeOH), which do not enable complete MC-LR (dissolved, non- and covalently bound) extraction (Williams et al., 1997). MeOH only retrieves dissolved MCs or those in non-covalent bonds (free), while covalently bound MCs are supposedly not available when food is consumed (Ibelings and Chorus, 2007; Smith et al., 2010). Therefore, it is accepted that only free MCs are relevant to human exposure estimation and risk assessment by contaminated food consumption. Nevertheless, the estimation of exposure to MCs as food contaminants has been based on the direct comparison of the concentration determined from studied organisms (raw food) with the TDI value (Chen and Xie, 2005; Ibelings and Chorus, 2007), assuming that the available concentration of MCs in raw and in ready-to-eat food products is similar. The risk assessment of human exposure to MCs through food must include detailed knowledge of the variation of the free MC content in the different steps of food storage (e.g., refrigeration, freezing) and processing (e.g., boiling, frying, microwaving), because food is generally consumed after such processing. Data on the effects of storage and processing practices on MC availability in food have been reported (Morais et al., 2008; Zhang et al., 2010; Guzmán-Guillén et al., 2011). For instance, Zhang et al. (2010) found that the mean concentration of MCs in bighead carp muscle was significantly increased after boiling. These findings suggest that the MC concentration in some contaminated foodstuffs may have been underestimated. Furthermore, once ingested, the food is subjected to the physical and chemical conditions of the stomach and small intestine, which may change the MC bioavailability. Bioaccessibility is one of the main factors limiting bioavailability. Bioaccessibility is defined as the fraction of the contaminant that is released from the food matrix by the action of digestive enzymes and is then available for absorption by the intestinal mucosa (Cabañero et al., 2004; Versantvoort et al., 2005). Bioaccessibility has been studied for several food chemical contaminants, such as mycotoxins (Versantvoort et al., 2005), mercury (Cabañero et al., 2004) and polychlorinated biphenyls (Xing et al., 2008). To our knowledge, there are no studies that estimate MC-LR bioaccessibility after *in vitro* proteolytic digestion. This study aims (1) to assess changes in the MC-LR concentration after common practices of food storage and processing as well as (2) the determination of MC-LR bioaccessibility after proteolytic digestion to create a more suitable estimation of human exposure to MC-LR through consumption of contaminated food.

2. Materials and methods

2.1. Reagents and chemicals

The mammalian enzymes pepsin (P7000), trypsin (T0303) and chymotrypsin (C4129) were purchased from Sigma–Aldrich (Spain). Aqueous solutions of hydrochloric acid (HCl) (37%) (Sigma, USA), phosphate buffer and sodium bicarbonate ($NaHCO_3$) (Sigma, USA) were prepared with ultrapure water supplied by a Millipore water purification system (0.0054 μS/cm) (MilliQ water). Acetic acid was purchased from Sigma (USA). The MeOH used for MC-LR extraction was analytical grade (Fisher Scientific, UK). All solvents used in LC–MS analysis were high-purity chromatography grade (LiChrosolv, Merck). Reagents used in the Z8 medium were analytical grade, and formic acid was LC–MS grade (Fisher Scientific, USA). MC-LR was used as the reference standard (lot no. SZBB069X, 95% purity, Sigma–Aldrich).

2.2. Biological material – cyanobacterial culture and clams

The exposure experiment was carried out with MC-LR-producing cells of *Microcystis aeruginosa* (LEGE 91094). Cyanobacteria were cultured to the exponential phase in Z8 medium (Kotai, 1972) (6 L flasks) under fluorescent light (light/dark cycle of 14/10 h) and a temperature of 25 ± 1 °C. *M. aeruginosa* LEGE 91094, produces MC-LR (95%) and low amounts of MC-LA and [D-Asp^3]-MC-LR (Vasconcelos, 1995). Specimens of Asian clams (*Corbicula fluminea*) (Müller, 1774), ranging from 25 to 30 mm in size, were collected in the estuary of River Minho (Valença, North Portugal). No MCs were detected in water where *C. fluminea* was collected (data not shown). The organisms were acclimated for one month prior to the experiment in 40 L aquaria with dechlorinated tap water. During this period, the organisms were fed twice a week with *Chlorella vulgaris* (1×10^5 cell/mL). The water was renewed weekly.

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