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Ecotoxicological survey of MNEI and Y65R-MNEI proteins as new potential high-intensity sweeteners

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Abstract Low-calorie sweeteners are widespread. They are routinely introduced into commonly consumed food such as diet sodas, cereals, and sugar-free desserts. Recent data revealed the presence in considerable quantities of some of these artificial sweeteners in water samples qualifying them as a class of potential new emerging contaminants. This study aimed at evaluating the ecotoxicity profile of MNEI and Y65R-MNEI, two engineered products derived from the natural protein monellin, employing representative test organism such as *Daphnia magna*, *Ceriodaphnia dubia*, and *Raphidocelis subcapitata*. Potential genotoxicity and mutagenicity effects on *Salmonella typhimurium* (strain TA97a, TA98, TA100, and TA1535) and *Escherichia coli* (strain WP2 pKM101) were evaluated. No genotoxicity effects were detected, whereas slight mutagenicity was highlighted by TA98 *S. typhimurium*. Ecotoxicity results evidenced effects

approximately up to 14 and 20% with microalgae at 500 mg/L of MNEI and Y65R-MNEI, in that order. Macrophytes and crustaceans showed no significant effects. No median effective concentrations were determined. Overall, MNEI and Y65R-MNEI can be classified as not acutely toxic for the environment.

Keywords Sweet proteins · MNEI · Y65R-MNEI · High-intensity sweeteners · Toxicity · Mutagenicity · Genotoxicity

Introduction

Dietary sweeteners (DSWs) to replace sucrose are a current goal of food and feed industry keeping flavor and reducing calories and the risk for dental caries (Kroger et al. 2006; Whitehouse et al. 2008; Subedi and Kannan 2014). However, their safety for human health and the environment has been controversial (Schiffman 2012; Suez et al. 2014; Di Luccia et al. 2015). Synthetic sweeteners could induce toxic, genotoxic, and carcinogenic effects as demonstrated for many food additives (Hobbs et al. 2012). The US Food and Drug Administration (FDA) banned cyclamate in 1970 from all dietary foods due to its potential to induce carcinogenesis in experimental animals. Chronic exposure to saccharin resulted in induction of bladder cancer in rats (Schoenig and Anderson 1985; Weihrauch and Diehl 2004). Plant proteins that interact with the taste receptors may represent a promising alternative to the artificial sweeteners (ASWs). Originally found into fruits of tropical plants (for a review, see Picone and Temussi 2012), sweet proteins including monellin, brazzein, and thaumatin have been not much exploited as commercial products. Among all, monellin is one of the best-characterized sweet proteins isolated so far. However, to the best of our

Highlights • MNEI and Y65R-MNEI are promising safe low-calories sweeteners.

- Sweeteners are potentially emerging contaminants in aquatic environments.
- Genotoxicity, mutagenicity, and ecotoxicity of MNEI and Y65R-MNEI have been assessed.
- MNEI and Y65R-MNEI have slight ecotoxicity effect with microalgae.
- Overall, MNEI and Y65R-MNEI can be classified as not acutely toxic.

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knowledge, no environmental fate and ecotoxicological profile has been reported for this sweetener so far. Natural monellin consists of two separate polypeptide chains, A and B, made of 44 of 50 amino acids, respectively, forming a single domain through non-covalent interactions (Morris and Cagan 1972). Being many thousands of times sweeter than sucrose on a molar basis (Morris and Cagan 1972), monellin has high potential as high intensity sweetener, because it can combine a marked and persistent sweet taste sensation with very low caloric power. Due to the low thermal resistance of natural monellin, which dissociates and loses its sweetness at about 50 °C, single-chain constructs have been engineered to increase the stability of the protein, also in view of its potential applications in food and beverages. In particular, the protein-dubbed MNEI, representing the target of our study, has been obtained by linking the two monellin chain by a Gly-Phe dipeptide linker (Kim et al. 1989). Moreover, in silico studies of the interaction between MNEI and the sweet taste receptor lead to the design of the charge mutant Y65R-MNEI (Esposito et al. 2006). In Y65R-MNEI, the tyrosine residue at position 65 has been replaced by an arginine, which was found sweeter than the parent protein in a wide range of pH (Rega et al. 2015), thus representing an even better candidate for the development of new low-calorie sweeteners and excipients for food and pharmaceutical preparations.

Preliminary structural studies, by using CD and NMR spectroscopies in solution at pH 7.0, revealed a high degree of similarity between MNEI and the newly discovered sweeter mutant Y65R-MNEI (Esposito et al. 2006). In order to find the best conditions for potential alimentary applications, we have subsequently investigated the structural and functional behaviors of the two proteins in chemical and physical conditions resembling those of common sweet drinks, i.e., acidic pH and temperatures typical of coffee and fruit juices (Rega, et al. 2015). Our results confirmed that the mutant Y65R-MNEI, designed to interact more efficiently with the sweet receptor T1R2/T1R3, is definitely sweeter than the parent protein, at both neutral and acidic pH. Recently, a more efficient, cheap, and fast protocol has been optimized for the biotechnological production of MNEI (Leone et al. 2015), opening the way to the scale-up of the process towards industrial applications as low-calorie protein-based sweeteners. However, before to proceed with the industrial production, the environmental effects of these sweeteners when released into the environment need to be carefully evaluated.

The fate of commercial dietary sweeteners (DSWs) in wastewater treatment plants (WWTPs) is almost unknown with just few studies dealing with ASWs and potential removal rates <2% like for sucralose, aspartame, saccharin, and acesulfame (Subedi and Kannan 2014). Recent environmental impact studies have stated the widespread distribution of saccharin, acesulfame, cyclamate, and sucralose in water cycle levels that classifies them as “new emerging pollutants”

(Kokotou et al. 2012). Little information is available for long-term consequences of sweeteners distribution in aquatic ecosystems as well as their by-products. Sucralose concentrations $\leq 0.5 \mu\text{g/L}$ altered the physiology and motility of *Daphnia magna* (Wiklund et al. 2012), interfered with plant photosynthesis (Lubick 2008), and resulted in an increase in the intake of food by marine copepod, *Calanus glacialis* (Hjorth et al. 2010).

The aim of this study was to investigate the effects of MNEI and its sweeter mutant Y65R-MNEI considering a battery of toxicity tests. Aquatic organisms (*D. magna* and *Ceriodaphnia dubia*) were used to assess potential residuals at the discharge (liquid phase), while terrestrial ones (*Lepidium sativum*) were employed to evaluate the effects of MNEI and Y65R-MNEI in sludge after agricultural land disposal. Further investigations were carried out to assess potential genotoxicity on *Salmonella typhimurium* (umu-test and Ames-test) (Ames and Nikaido 1985). Since little is known about possible mechanism of biota alteration induced by these sweeteners, the antibacterial ability of sweet proteins in order to define them as selective agents was evaluated as well.

Material and methods

Proteins expression and purification

Recombinant MNEI and Y65R-MNEI were expressed in *Escherichia coli* strain BL21(DE3) cells harboring the pET-22b+ plasmid, as previously described (Rega et al. 2015). Protein yield in LB medium was on average 25 mg/L of culture. Protein samples were extensively dialyzed against double distilled water, and their purity and folding were assessed by SDS-PAGE, gel-filtration, circular dichroism analysis, and mass spectrometry. Molecular weights of MNEI and Y65R-MNEI are 11,271.8 and 11,264.8, respectively. The protein concentration was estimated on the basis of ultraviolet absorbance at 280 nm, using a calculated molar extinction coefficient of 1.41 and 1.29 cm/mg/mL for MNEI and Y65R-MNEI, respectively.

Treatment of protein samples for toxicity assessment and physico-chemical analysis

Proteins for the test concentrations and the negative control were initially dissolved at a concentration of 1 mg/mL in hydrochloric acid (HCl) 1×10^{-3} M to maximize protein solubility and then diluted in appropriate buffer to obtain the final stock solutions (HCl concentration $<1 \times 10^{-5}$ M). pH was checked before and after addition of test samples to avoid pH alterations. Potassium dichromate (0.01 to 50 mg/L) in the toxicity assays, sodium azide (5 ng/mL) in fluctuation Ames medium, and nitroquinoline-1-oxide (50 $\mu\text{g/L}$) in the

umu-test were used as positive controls. The concentration of the two sweet proteins was monitored up to 7 days, in each medium, and only a low deviation between nominal and effective concentrations was found (Table 1).

Antibacterial activity

The antibacterial activity of the sweet protein was tested against seven bacteria (*Staphylococcus aureus* ATCC 6538, *Streptococcus faecalis* ATCC 33186, *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *E. coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *S. typhimurium* ATCC 14028) from the American Type Culture Collection (ATCC; Rockville, MD, USA) reconstituted according to standard methods (ISO 2014).

Mutagenicity and genotoxicity assay with *S. typhimurium*

The Muta-Chromoplate kit was used to evaluate the mutagenicity (Ames et al. 1975).

The fluctuation test was performed using *S. typhimurium* strains TA100 observing the potential reverse mutation from amino acid (histidine) auxotrophy to prototrophy after exposure to mutagens (EBPI 2005). Overnight bacteria cultures in exponential phase were exposed to different samples concentration in the presence of bromocresol purple and then dispensed into 96-well microtiter plates. The plates were incubated at 37 °C for 3 to 5 days. After this period, the number of wells that turned into yellow was counted. All yellow wells

Table 1 MNEI and Y65R-MNEI proteins effects on bacterial development (*Salmonella typhimurium* and *Escherichia coli*) at various exposure concentrations

Dose (mg/plate)	Number of colonies/plate (mean ± SD)				
	<i>Salmonella typhimurium</i>				<i>Escherichia coli</i>
	TA97a	TA98	TA100	TA1535	WP2 pkM101
MNEI					
5	14 ^a	28	n.a.	22 ^a	33 ^a
10	22 ^a	26	42 ^a	26 ^a	30 ^a
100	20 ^a	33	60 ^a	25 ^a	35 ^a
500	33	36	n.a.	37	45 ^a
Y65R-MNEI					
5	15 ^a	18	n.a.	10 ^a	55 ^a
10	24 ^a	22	32	10 ^a	60
100	25 ^a	34	60 ^a	20 ^a	72
500	30 ^a	37	n.a.	23 ^a	67

Three independent assays were performed and SD represents standard deviation; $p < 0.05$ versus control

n.a. not available

^a H_0 verified = not significantly different from control

were considered positive, and all purple wells were scored as negative.

For the Ames test assay, positive responses required a dose-related increase in the number of revertant colonies/plate for one or more strains. Negative response was defined as no dose-related increase in the revertant colonies number. Also, a positive score required that the number of revertant colonies/plate for at least one of the treatment concentrations was twice the number of colonies/plate in the vehicle control. Revertant colonies (his+ revertants) were counted after incubation at 37 °C for 72 h. The number of his+ revertant colonies in each sample was determined as a mean value of the three plates. The results were expressed as a mutagenicity ratio (MR), i.e., the ratio of the number of *S. typhimurium* revertants grown in the presence of the tested sample to the number of spontaneously appeared revertants. The sample was considered mutagenic when $MR \geq 2$ (Piekarska and Karpinska-Smulikowska 2007). Chi-square analysis was used for statistical evaluation of the treated plates versus the control plates.

Genotoxicity assay with *S. typhimurium*

The umu test was performed according to standard procedure (ISO 2000) and was developed for the detection of genotoxic materials that cause damage to a cell's DNA. In this assay, a modified strain of *S. typhimurium* TA1535/pSK 1002 bacteria was used, whereby a β -galactosidase gene was linked to SOS-DNA response. Bacterial cultures were grown overnight at 37 °C and then diluted in tryptone-glucose-ampicillin (TGA) medium until cells entered logarithmic phase. After an incubation period of 2 h with the test substance, induction of genotoxicity, expressed as β -galactosidase activity by colorimetric endpoint was determined by mean of application of the *O*-nitrophenyl galactopyranoside substrate and was measured as the absorbance at 420 nm. Growth was measured from the absorbance at 600 nm. The result was calculated as an induction ratio (IR): $(1/G) \times US$, where *G* was the growth and *US* the relative enzyme activity. The sample was considered genotoxic when IR was greater than 1.5 (ISO 2000).

Antimicrobial activity assays

The Clinical and Laboratory Standards Institute documents were used as methodology guidelines to determine antimicrobial activity of MNEI and Y65R-MNEI via disk diffusion assay (DDA). Bacterial strains were grown on Mueller-Hinton agar (MHA) plates (Oxoid, UK), and inoculum suspension was prepared in Mueller-Hinton broth (MHB) (Oxoid, UK): isolated colonies selected from an 18- to 24-h were incubated in agar plate, and then bacterial concentration of each strain was adjusted to 1×10^8 and 1×10^5 cells/mL. McFarland standards were used (Fuchs et al. 1995). Within 15 min after adjusting the turbidity of the inoculum

suspension, a sterile cotton swab was dipped into the adjusted suspension and the dried surface of an MHA plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum before applying the compost-impregnated disks (“Blank Disks,” Whatman GE Healthcare, USA, 13 mm in diameter). Each disk was pressed down to ensure complete contact with the agar surface and impregnated with 35 µL of each compounds (5 mg/mL). The plates were incubated at 37 °C for 16 to 18 h. Thereafter, the size of the halo from the inhibition growth was measured. The positive controls was performed according to the (CLSI, M100-S17) documents. Each experiment was repeated three times.

Toxicity tests

The acute bioassay with *D. magna* was conducted according to the previously reported procedure (Guida et al. 2008; ISO 2012a). The test was considered valid if the immobilization in the control did not exceed 10%.

Moreover, *D. magna* heart rate (second molt organisms) was carried out according to Baylor (1942). Cotton fibers were placed into a 1.5-mL glass well allowing some

movement, but preventing swimming. Water fleas were monitored singly in five replicates. After a 120-s acclimation period, heart rate was counted using a camera mounted on a stereomicroscope (Leica EZ4 HD) and connected to a computer. Multiple heart rate readings were taken per each flea, starting 30 s after immobilization and continuing every 30 s per 60 min. Finally, organisms were discarded.

The chronic toxic bioassay with *C. dubia* was carried out according to the standard procedure (ISO 2008). At the end of 7 days, the results of the bioassay were considered acceptable if the concentration of dissolved oxygen in different treatments was maintained at a saturation value ≥40% and the survival of the control animals was ≥80%.

The growth inhibition test was assessed following the standard procedure (ISO 2012b). Exponentially, growing algae (10⁴ cell/mL) were exposed to various concentrations of the test item over a period of 72 h under defined conditions as described in (Guida et al. 2008) and conducted in six replicates. Results were expressed as the mean (± standard deviation) of the percentage inhibition of the cell growth (% I) at sample compared with negative control (*p* ≤ 0.05).

The test with *L. sativum* was performed following the standard protocol (OECD 2006). Germination and growth experiments were conducted in aqueous solutions at controlled pH,

Table 2 MNEI and Y65R-MNEI proteins mutagenicity (*Salmonella typhimurium* and *Escherichia coli*) and induction ratio (*S. typhimurium*) at various exposure concentrations; failed thresholds are evidenced in red

Dose (mg/plate)	Mutagenicity Ratio (MR)					Induction ratio (IR)
	<i>S. typhimurium</i>				<i>E. coli</i>	
MNEI	TA97a	TA98	TA100	TA1535	WP2 pkM101	<i>S. typhimurium</i> UMU-CHROMOTEST™
5	-	1.6	-	-	-	0.2
10	-	1.4	-	-	-	0.2
100	-	1.8	-	-	-	0.9
500	1.9	2.0	-	1.9	-	1.1

Dose (mg/plate)	Mutagenicity Ratio (MR)					Induction ratio (IR)
	<i>S. typhimurium</i>				<i>E. coli</i>	
Y65R-MNEI	TA97a	TA98	TA100	TA1535	WP2 pkM101	<i>S. typhimurium</i> UMU-CHROMOTEST™
5	-	1.0	-	-	-	0.3
10	-	1.2	0.6	-	1.4	0.4
100	-	1.9	-	-	1.7	0.9
500	-	2.1	-	-	1.6	1.1

The sample was considered mutagenic when MR ≥ 2. The sample was considered mutagenic when IR ≥ 1

– MR was not calculated when H₀ was verified

in three replicate experiments. Parallel controls were performed. The index of growth (IG %) was calculated by multiplying the germinated seed number exposed to sample (G1) and length of roots exposed to sample (L1) divided by the factor obtained from the multiplication of germinated seed number exposed to negative control medium (Gc) and length of root exposed to negative control medium (Lc).

Data analysis

The data for the study were calculated by linear regression analysis of transformed protein concentration as natural logarithm data and percentage of response. Chi-square analysis (Gilbert 1980) was used to evaluate statistical significant differences between exposure groups and control groups for antibacterial activity. Quantitative data were tested using Student's test and chi-square. The $p < 0.05$ was considered statistically significant. The significance of differences between average effect values of other different experimental treatments and controls was assessed by the analysis of variance (ANOVA) considering a significance threshold level always set at 5%. When ANOVA revealed significant differences among treatments, *post-hoc* tests were carried out with Dunnett's method and Tukey's test. Statistical analyses were

performed using Microsoft® Excel 2013/XLSTAT®-Pro (version 7.2, 2003, Addinsoft, Inc., Brooklyn, NY, USA).

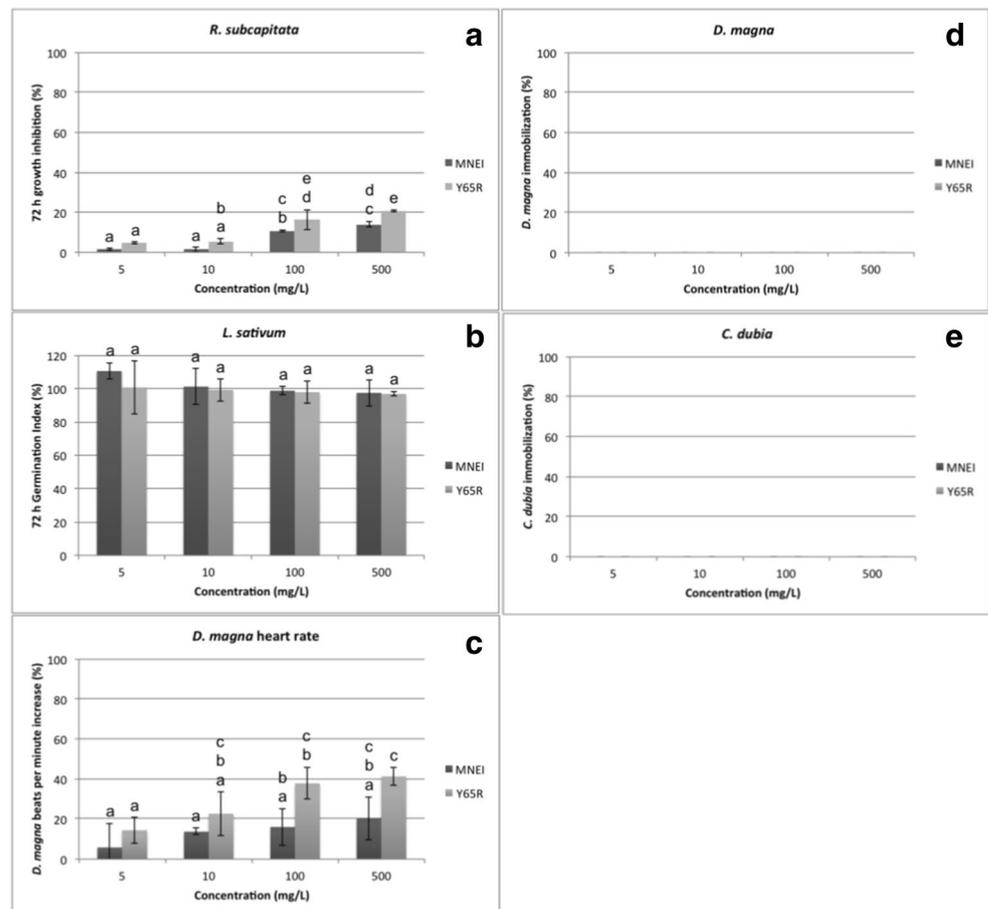
Results and discussion

All negative and positive controls' results were in accordance with the respective reference protocols. DDA did not provide any significant result compared to negative controls. Thus, no relevant antimicrobial activity was obtained by MNEI and Y65R-MNEI.

Antibacterial activity was reported in Table 1 for both MNEI and Y65R-MNEI considering all *S. typhimurium* strains and *E. coli*. Results evidenced no significant differences ($p < 0.05$) between treatment concentrations (5, 10, 50, and 100 mg/plate) and negative controls. Frequently, at 500 mg/plate, both MNEI and Y65R-MNEI stimulated bacterial growth. Overall, both sweeteners generated no relevant antibacterial effects.

Mutagenicity and genotoxicity results are summarized in Table 2 as MR for all *S. typhimurium* strains and *E. coli* and as IR for *S. typhimurium* Umu-Chromotest™. No relevant mutagenicity was detected except for TA98 *S. typhimurium* at 500 mg/plate for both MNEI (MR = 2) and Y65R-MNEI

Fig. 1 Ecotoxicological effects of MNEI and Y65R-MNEI to *Raphidocelis subcapitata* (a), *Lepidium sativum* (b), *Daphnia magna* heart rate (c) and mortality (d), and *Ceriodaphnia dubia* (e) mortality; data with different letters (a, b, c, and d) are significantly different (Tukey's test, $p < 0.05$)



(MR = 2.1). The IR reported no significant genotoxic consequences for both sweeteners. These results are of special interest when compared with studies carried out on other natural compounds used as sweeteners in the food and drug industry. For instance, toxicological studies have shown that stevioside, one of the steviol glycosides, does not have mutagenic, teratogenic, or carcinogenic effects (Pól et al. 2007). However, concern has been expressed in recent publications that steviol glycosides may be mutagenic based on selected studies representing a small fraction of the overall database suggesting that further in vivo genotoxicity studies are required to complete their safety profiles (Urban et al. 2013).

Recently, by site-directed mutagenesis, modification of the N-terminus of monellin, without the need of methanol induction in *Pichia pastoris* expression system, indicates the possibility for large-scale production of this sweet-tasting protein (Cai et al. 2016).

Ecotoxicological data are displayed in Fig. 1 considering the effects on *R. subcapitata*, *L. sativum*, *D. magna*, and *C. dubia*.

About *R. subcapitata* (Fig. 1a), growth inhibition effects increased with increasing exposure concentration up to 21% for Y65R-MNEI. Effects were not significantly different after 5 and 50 mg/L of MNEI and Y65R-MNEI treatments. Significant differences ($p < 0.05$) were observed at 100 and 500 mg/L of MNEI and Y65R-MNEI, being Y65R-MNEI slightly more toxic. About *L. sativum* (Fig. 1b), the germination index showed no significant differences between the different protein amounts considered as exposure treatments (5–500 mg/L) and between MNEI and Y65R-MNEI exposures.

Immobilization effects on *D. magna* (Fig. 1d) and *C. dubia* (Fig. 1e) did not produce any significant effect, but *D. magna* heart rate monitoring (Fig. 1c) evidenced a stress related condition only for Y65R-MNEI ≥ 100 mg/L. Similar results were found about other natural sweeteners such as for instance lactose, leading to heart arrhythmia in *Daphnia* (Campbell et al. 2004).

According to Zucker (1985) and Libralato et al. (2010), MNEI and Y65R-MNEI can be ranked as practically not acutely toxic ($EC_{50} > 100$ mg/L) presenting no immediate ecotoxicological side effects.

More investigations are required to better understand the slight levels of mutagenicity with TA98 *S. typhimurium* and to explore potential long-term chronic effects.

Conclusions

Based on our toxicity data, the environmental effects of MNEI and Y65R-MNEI, sweet proteins derived from monellin, are perfectly comparable and seem to be negligible into freshwater ecosystems up to 500 mg/L. Neither antibacterial nor genotoxicity were highlighted. Only slight mutagenicity effects were observed for both compounds with TA98 *S. typhimurium*. Nevertheless, they can be considered as

practically not acute toxic, but long-term toxicity tests are required to look for potential chronic effects.

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