First investigation of potential adverse effects and environmental impact of new sugar replacers derived from the sweet protein monellin

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Sweet proteins represent a class of natural molecules extremely interesting for their potential use as safe low-calories sweeteners for individuals who need to control sugar intake, such as obese or diabetic subjects.

Natural sweet proteins:

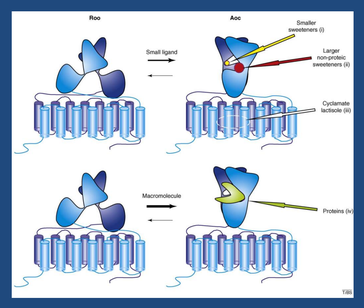
Sweet proteins are all found in unrelated tropical plants and, apart from the common tropical plant origin, sweet proteins show no functional similarity among them either in sequence or in three-dimensional structure.

Protein	Plant origin	Relative Sweetness
Monellin	Dioscoreophyllum cumminsii	100,000
Thaumatin	Thaumatococcus danielli	100,000
Brazzein	Pentadiplandra brazzeana B.	17,000
Mabinlin	Capparis masaikai Levl.	400

(molar, referred to sucrose)

Why are sweet proteins sweet?

According to the mechanism termed "wedge model" [1,2], sweet proteins activate the sweet-taste receptor (TR) by binding to an external site and thus stabilizing the active form of the sweet receptor (Aoc) even in the absence of small molecular mass sweeteners .



Monellin:

Natural monellin consists of two separate polypeptide chains, A (44aa) and B (50aa), linked together through non-covalent interactions. When heated above 50°C denatures loosing sweetening power.

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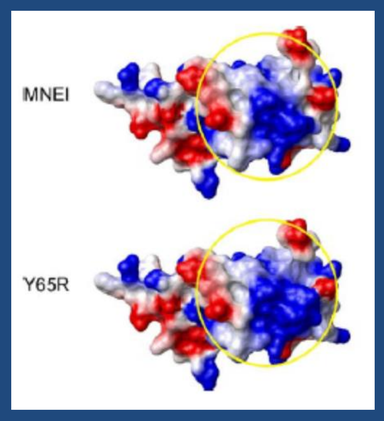
Recombinant single chain constructs, obtained by joining the two chains either by a direct amide bond (SCM) or by the insertion of a dipeptide linker (MNEI) were generated and found to be comparatively sweet as native monellin but with increased thermal stability which make the protein more suitable for the conditions employed in food processing.



A super-sweet MNEI mutant : Y65R

In silico studies of the interaction between MNEI and the sweet taste receptor lead to the design of charged mutants of MNEI. Among them, the mutant Y65R was proven particularly promising for its high sweetness [4].

⬇

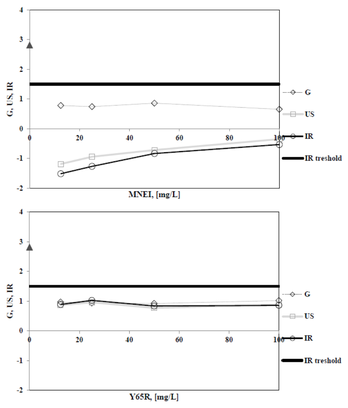
We have recently reported a thorough physico-chemical comparison of Y65R and MNEI [5], together with an extensive evaluation of their sweetness in different experimental conditions chosen to reproduce those of some common sweet drinks, which we believe could constitute the first applicative target of new sweeteners structurally derived from monellin.

Given the growing global consumption of artificial sweeteners, 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"

Preliminary safety evaluation

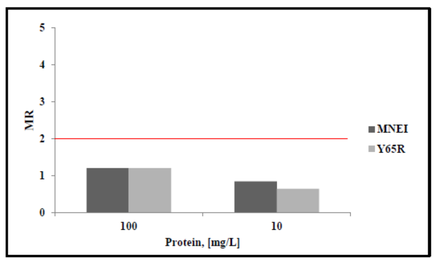
The present study aims at the assessment of the ecotoxicity profile of two protein-based sweeteners derived from the natural sweet protein monellin, MNEI and its Y65R mutant, on representative organisms belonging to different trophic levels. Potential effects on the nervous system were evaluated by measuring the heart rate of *Daphnia magna*. Genotoxicity, mutagenicity and antibacterial activity effects of these two protein were evaluated. Enzymatic proteolysis experiments indicate that both proteins are readily degraded in small fragments, thus facilitating their biological turnover.

Genotoxicity assay with *Salmonella typhimurium* strain TA1535/pSK 1002



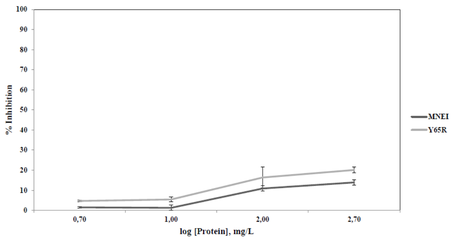
IR (Induction Ratio), G (Growth) and US (activity of β-galactosidase) values for MNEI and Y65R. IR values ≥1.5 indicate genotoxicity, ▲= positive control. Values are means of 3 replicates and are statistically different from the control ($P < 0.05$, Student's t-test).

Mutagenicity fluctuation test using *Salmonella typhimurium* strains TA100



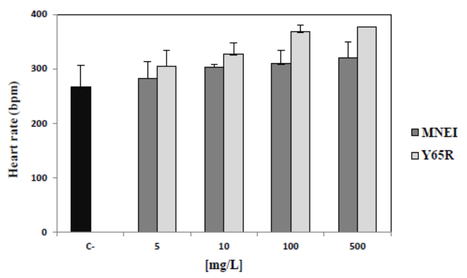
Mutagenic ratio (MR) of fluctuation values ≥2 indicate mutagenicity. *$P < 0.01$ mean difference levels comparing with the control group (Test chi).

Inhibiiton (I) of *Pseudokirchneriella subcapitata* cell growth after 72h



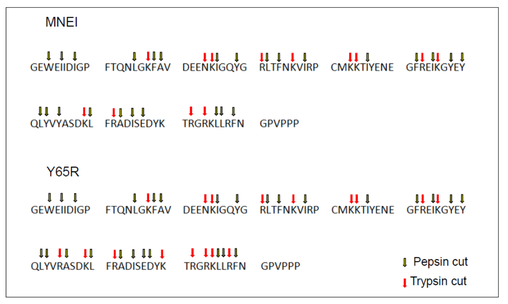
I values < 20% indicate no toxicity; I values <50% indicate slight toxicity and I values >50% indicate toxicity. *$P < 0.01$ mean difference levels of MNEI compared to Y65R at the same concentration (Student's t-test).

D. magna heart rate change in MNEI and Y65R at different concentrations



C- is the basal heart rate in the negative control and bars represents Dev.ST.

Proteases cleavage sites



Black and yellow arrows indicate pepsin cleavage site and red arrows indicate trypsin cleavage site.

These results demonstrate a low hazard and risk potential toward several aquatic organism and absence of genotoxic and mutagenic effects thus encouraging the marketability of MNEI and Y65R as new low calories and environmental safe sweeteners.

Future perspectives:

It has been previously reported that under certain condition MNEI forms irreversible protein aggregates [7]. We believe that the combined effect sweetness-high thermal stability of MNEI and its novel derivative Y65R may represent promising potential for large-scale production of edible films. Such films could be used for food wraps, layers between food components, or heat-sealed to form sacks, sachets, pouches, or bags to contain dry foods or pre weighed ingredients. Edible protein-based films obtained by thermoplastic processes provide the opportunity to fulfill the consumers' demands and expectations of new food packaging systems that are convenient and environmentally friendly.

References:

[1] Temussi, P.A. *FEBS, Letters* 526 (2002) 1^4; [2] Temussi, P.A. *Cell., Mol. Life Sci.* (2006), 63, 1876.; [3] Spadaccini, R. *et al., J. Mol. Biol.* (2001), 305, 505; [4] Esposito, V. *et al., J. Mol. Biol.* (2006), 360, 448; [5] Rega, M. F. *et al., Food Chem. (2015), 1179-1186;* [6] Lange, F. T., *et al. Analytical and Bioanalytical Chemistry,* (2012), *403*(9), 2503-2518; [7] Esposito V, *et al., Biochemistry* (2010), 49(13):2805-2810.