

Milk protein hydrolysates activate 5-HT_{2C} serotonin receptors: influence of the starting substrate and isolation of bioactive fractions

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Alice B. Nongonierma,^{†a} Harriët Schellekens,^{†bc} Timothy G. Dinan,^{bde}
John F. Cryan^{bdf} and Richard J. FitzGerald^{*a}

Milk protein hydrolysates generated with different starting substrates, including sodium caseinate (NaCN), acid casein (Acid CN), skim milk powder (SMP) and glycomacropeptide (GMP) were demonstrated to behave as serotonin 2C (5-HT_{2C}) receptor agonists. The 5-HT_{2C} receptor activating potential of NaCN hydrolysates correlated with an increased protein hydrolysis, most likely due to enhanced release of bioactive peptides over the time course of hydrolysis. In its unhydrolysed form, GMP was the only starting substrate showing 5-HT_{2C} serotonin receptor agonist activity. The 5-HT_{2C} serotonin receptor agonist activity of its corresponding hydrolysate (GMPH-240 min) was significantly higher ($P < 0.05$). Fractionation of the 240 min NaCNH using ultrafiltration (UF), solid-phase extraction (SPE), semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC) and isoelectric focusing (IEF) was carried out. Characterisation of the fractions obtained shows that the bioactive peptides had a relatively low molecular mass (<1 kDa), were hydrophobic in nature and had a pI between 8.6 and 13.2. These different physicochemical characteristics together with the stability of NaCNH-240 min to simulated intestinal digestion, allow prediction of a favourable outcome regarding the bioavailability of the bioactive peptides therein. These results reinforce the potential of milk-derived bioactive peptides to be developed into functional foods targeted at 5-HT_{2C} receptor modulation.

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1 Introduction

Alarming data for obesity incidence have been reported worldwide.¹ In 2008, it was reported that 1.5 billion adults were overweight, of which 500 million were obese.² In 2010, it was estimated that 43 million children under the age of five were overweight. In the European Union, it is estimated that 35.9% of adults are overweight and 17.2% are obese, with a higher proportion of males being overweight compared to females.³ Excess weight has been associated with negative health consequences including impaired glucose metabolism, together with dyslipidemia and hypertension, conditions which are generally categorised under the metabolic syndrome.^{1,4,5} Reduction of body mass can be achieved by decreasing food intake and increasing calorie expenditure through physical activity.

Nevertheless, this can constitute challenging alterations in the lifestyle of people who need to lose weight. Different strategies to counteract obesity have been proposed, these include modification of the diet combined with exercise and bariatric surgery.^{1,6–8} In addition, several anti-obesity pharmaceutical drugs have been developed over the past decade. However, most candidates have so far failed to reach the market, due to safety concerns and side effects.^{9–20} Currently, the only FDA-approved drug in the treatment of obesity is the over-the-counter available oral lipase inhibitor, orlistat (Xenical®; FDA approved 2007), albeit it is not as effective as other drugs in reducing body weight. Changes in diet, exercise and the pharmaceutical approach only allow short term reduction of body weight which generally does not last beyond a year often due to non-compliance of overweight people.¹ Weight loss strategies aimed at dietary alterations are still preferred over pharmacotherapies and are a lower risk alternative to bariatric procedures.¹ The inclusion of natural substances with appetite lowering properties in the diet have been proposed.^{21–25} Weight loss can be achieved by a modulation of appetite and satiety through the central nervous system (CNS) involving increased serotonin (5-hydroxytryptamine) neurotransmission.^{26–30} In addition, it has been established that central regulation of appetite can be achieved through 5-HT_{2C} serotonin receptor agonism.^{6,31} Expression of the 5-HT_{2C} serotonin receptors has been linked

^aDepartment of Life Sciences and Food for Health Ireland (FHI), University of Limerick, Castletroy, Limerick, Ireland. E-mail: dick.fitzgerald@ul.ie; Fax: + 353 (0) 61 331490; Tel: +353 (0) 61 202598

^bFood for Health Ireland, University College Cork, Cork, Ireland

^cSchool of Pharmacy, University College Cork, Cork, Ireland

^dLaboratory of Neurogastroenterology, Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland

^eDepartment of Psychiatry, University College Cork, Cork, Ireland

^fDepartment of Anatomy and Neuroscience, University College Cork, Cork, Ireland

[†] Both authors contributed equally to this work.

with suppression of appetite, reinforcing its role in body weight management.³⁰ Different serotonergic drugs targeting the CNS, including sibutramine, fluoxetine, *m*-chlorophenylpiperazine (mCPP) and d-fenfluramine, all act as appetite suppressants.^{32,33} In addition, specific drugs targeting serotonin receptors have also been developed to regulate appetite.⁶ However, these drugs often only give modest effects and the risks do not always outweigh the health benefits. Heart and pulmonary vasculature side-effects have been associated with non-specific effects, including off-target activation of other serotonin receptors (5-HT_{2A} and 5-HT_{2B}).^{6,30,31,34,35} Therefore, agonists which can selectively bind 5-HT_{2C} receptors while minimising side-effects would be beneficial for weight management. Currently, the first 5-HT_{2C} receptor specific drug, Lorcaserin, has been developed and is awaiting FDA approval.^{7,8,36,37} Development of non-pharmacological compounds from natural sources with serotonergic properties may help to avoid the side effects associated with the utilisation of synthetic drugs.

Milk has been associated to satiating properties and more particularly, milk proteins have been described as being more satiating than milk fats or carbohydrates.^{24,25,38} Satiety signals following protein intake occur in the gastrointestinal tract inducing slower gastric emptying, stimulation of gut hormone receptors and possibly opioid receptors.³⁸ Numerous biologically active peptides are encrypted within the primary structures of milk proteins.^{39–42} Bioactive milk peptides have been described as potential health promoting and disease risk reducing agents. Targeted utilisation of these peptides can beneficially modulate physiological systems within the human body. A diverse range of potential physiological targets have been identified, mostly using *in vitro* assays to date, for milk bioactive peptides. These include immunomodulatory, opioid, mineral binding/bone formation, hypotensive, antithrombic, anticancer, *etc.*, properties.^{39,43,44} The influence of whey protein intake on different satiating hormones has been reviewed by Luhovyy *et al.*²⁴ Milk bioactive peptides developed as natural ingredients with serotonin agonist properties have potential as weight loss management ingredients which could be incorporated in the diet. This may help to alleviate issues encountered with weight reducing drugs where compliance to the treatment can become an issue.³⁵

The aim of this study was to investigate the potential of milk derived peptides to activate the 5-HT_{2C} receptor in an *in vitro* cell-based assay system. The 5-HT_{2C} receptor belongs to the family of G protein coupled receptors operating *via* the G_{αq} signalling pathway. Activation of G_{αq} induces the stimulation of phospholipase C β , which leads to an increase in inositol triphosphate and elevation in intracellular Ca²⁺.^{6,45,46} Identification of 5-HT_{2C} receptor agonists can be achieved using fluorescent based assays to quantify an increase in intracellular calcium. Milk-derived hydrolysates were manufactured using a food-grade protocol with the view of developing ingredients for use in food-formulations. Hydrolysates arising from different casein-derived substrates were evaluated *in vitro* for their agonist properties towards 5-HT_{2C} serotonin receptors. Different fractionation protocols were applied to a bioactive sodium caseinate hydrolysate (NaCNH-240 min) in order to

selectively separate bioactive peptides from inactive peptides. Fractionation was carried out based on molecular mass, hydrophobicity or isoelectric point differences with the view to better understanding the physicochemical properties of the bioactive peptides therein.

2 Materials and methods

2.1 Reagents

Trifluoroacetic acid (TFA), phosphoric acid (H₃PO₄) and 5-hydroxytryptamine were obtained from Sigma Aldrich (Dublin, Ireland). Hydrochloric acid (HCl), sodium hydroxide (NaOH), HPLC grade water and acetonitrile (ACN) were from VWR (Dublin, Ireland). Solid-phase extraction (SPE) cartridges (Giga Tube StrataX, 33 μ m, 85 Å Polymeric RP 1 g per 12 mL) were obtained from Phenomenex (Cheshire, UK). The calcium sensitive fluorescent Ca4 dye was obtained from Molecular Devices Corporation (Sunnyvale, CA, USA). Human embryonic kidney 293 (HEK 293A) cells were from Invitrogen (Carlsbad, CA, USA). Hanks balanced salt solution (HBSS) and HEPES buffer were from Gibco (Grand Island, NY, USA). The skim milk powder (SMP, 25.8% (w/w) protein), sodium caseinate (NaCN, 90.4% (w/w) protein), acid casein (Acid CN, 89.0% (w/w) protein) and glycomacropeptide (GMP) were obtained from commercial suppliers. Corolase PP was obtained from AB enzymes (Darmstadt, Germany).

2.2 Hydrolysis of milk protein substrates and physicochemical characterisation of the hydrolysates

Hydrolysis of milk protein substrates. A schematic representation of the experimental procedure is given in Fig. 1. The starting substrates NaCN, Acid CN, and SMP were resuspended at 10% (w/w) on a protein basis in water. GMP was resuspended at 10% (w/w) powder in water. These solutions were dispersed under agitation at 50 °C for 1 h using an overhead stirrer (Heidolph RZR 1, Germany). The protein solutions were

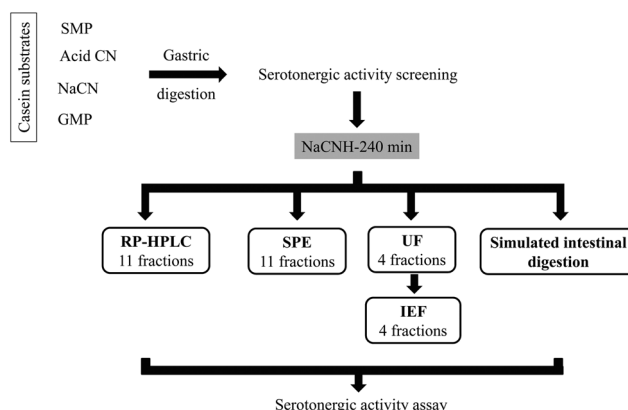


Fig. 1 Schematic representation of hydrolysis of the different milk protein substrates and simulated intestinal digestion and fractionation of the sodium caseinate hydrolysate (NaCNH-240 min). NaCN: sodium caseinate; Acid CN: acid casein; SMP: skim milk powder; GMP: glycomacropeptide; RP-HPLC: reverse-phase-high performance liquid chromatography; UF: ultrafiltration; SPE: solid-phase extraction; IEF: isoelectric focusing.

adjusted to pH 2.0 prior to hydrolysis using an aqueous solution of 1.0 N HCl. A control sample was removed from the protein dispersion and maintained at 50 °C for the duration of the hydrolysis reaction. NaCN, Acid CN, SMP and GMP were hydrolysed with pepsin at an enzyme to substrate ratio (E : S) of 1% (w/w), pH 2.0, 50 °C. Hydrolysis was conducted at a constant pH of 2.0 using a pH Stat (Titrand 843, Tiamo 1.4 Metrohm, Dublin, Ireland). For the NaCN hydrolysate, samples were withdrawn at different time intervals (10, 30, 60, 120 and 240 min) whereas hydrolysate samples for Acid CNH-240 min, GMPH-240 min and SMPH-240 min were withdrawn at 240 min. The enzyme was inactivated by heating at 90 °C for 20 min and the samples were subsequently freeze-dried (FreeZone 18 L, Labconco, Kansas City, U.S.A.) and stored at −20 °C until utilisation.

Simulated intestinal digestion. In order to study the stability of the bioactive peptides within NaCNH-240 min, simulated intestinal digestion (SID) was carried out according to Walsh *et al.*⁴⁷ Freeze-dried NaCNH-240 min was resuspended in water at 2% (w/v) on a protein basis at 37 °C for 30 min. Hydrolysis of NaCNH-240 min was carried out at 37 °C for 150 min at pH 7.5 with Corolase PP (E : S, 1.0%). The enzyme was inactivated by heating at 90 °C for 20 min and the samples were freeze-dried and stored at −20 °C until utilisation.

Reverse-phase high performance liquid chromatography (RP-HPLC), RP-ultra-performance liquid chromatography (RP-UPLC) and molecular mass distribution of peptides and proteins. Unhydrolysed milk protein (NaCN), hydrolysate sample (NaCNH) and fractions obtained following ultrafiltration (UF) of NaCNH-240 min were analysed by RP-HPLC with a liquid chromatograph (Waters, Dublin, Ireland) mounted with a 250 × 4.6 mm I.D., 5.0 µm particle size Jupiter C18 column attached to a C18 guard column (4 × 3 mm I.D., Phenomenex, Cheshire, UK). Detection of peptides and proteins was carried out at 214 nm. Separation of the peptides was carried out as described in Spellman *et al.*⁴⁸

Milk proteins (NaCN, Acid CN, SMP and GMP), hydrolysates (NaCNH, Acid CNH-240 min, SMPH-240 min and GMPH-240 min), SPE fractions and isoelectric focusing (IEF) fractions of NaCNH-240 min were analysed by liquid chromatography using a UPLC (Acquity, Waters, Dublin, Ireland) as described by Nongonierma and FitzGerald.⁴⁹ Separation of peptides and individual milk proteins was carried out at 30 °C, using a 2.1 × 100 mm, 1.7 µm Acquity UPLC C18 BEH column mounted with a 0.2 µm inline filter (Waters, Dublin, Ireland). Detection of peptides and proteins was carried out at 214 nm.

The molecular mass distribution profiles of the proteins and peptides present in the NaCN, NaCNH and associated UF fractions of NaCNH-240 min sample were determined by gel permeation chromatography-high performance liquid chromatography (GPC-HPLC) essentially as described by Spellman *et al.*⁴⁸ The freeze-dried samples were resuspended in the mobile phase (0.1% (v/v) TFA in 30% HPLC grade ACN) in HPLC grade water at a concentration of 0.25% (w/v) and filtered through 0.2 µm PTFE filters (VWR, Dublin, Ireland). A 600 × 7.5 mm I.D. TSK G2000 SW column attached to a 75 × 7.5 mm I.D. TSKgel SW guard column (Tosoh Bioscience, Stuttgart,

Germany) was used for the separation. Analysis of the samples was carried out in isocratic mode at a flow rate of 0.5 mL min^{−1} and the absorbance was monitored at 214 nm.

2.3 Fractionation of NaCNH-240 min

Hydrolysate sample NaCNH-240 min was fractionated by UF, SPE, semi-preparative RP-HPLC and IEF in order to isolate bioactive fractions present in this hydrolysate.

UF fractionation. NaCNH-240 min was fractionated using a UF unit (Sartoflow Alpha filtration system, Sartorius, Germany). Fractionation was carried out using two different membranes having 5 and 1 kDa nominal molecular weight cut-off (MWCO) values. The four fractions (permeates and retentates) collected were freeze-dried and stored at −20 °C until utilisation.

SPE fractionation. NaCNH-240 min was resuspended in HPLC grade water at a concentration of 0.5% (w/v) and applied on a Giga tube Strata X cartridge as described by Nongonierma and FitzGerald.⁴⁹ The SPE cartridge was connected to a manifold (Phenomenex, Cheshire, UK) to extract the different fractions under vacuum (15 mmHg). The procedure followed with the SPE cartridge was a modification of the fractionation technique described by Herraiz and Casal.⁵⁰ A volume of 30 mL NaCNH-240 min filtrate was slowly applied at the top of the SPE cartridge to allow adsorption of the peptides. The SPE cartridge was washed with water to remove any unbound material. Step-wise elution with different solutions of 5 to 50% (v/v) ACN in water were used to desorb the peptides from the SPE cartridge. This extraction procedure was repeated 3 times and the fractions eluted at the same ACN gradient were pooled. The eleven fractions generated were evaporated in a solvent evaporator (Genevac, EZ-2 Plus, Genevac Ltd., Ipswich, UK).

Semi-preparative RP-HPLC fractionation. Fractionation of NaCNH-240 min was carried out by semi-preparative RP-HPLC (Waters, Dublin, Ireland). NaCNH-240 min was resuspended at a concentration of 10% (w/v) in HPLC grade water and 500 µL were injected on a C18 semi-preparative column (250 × 15 mm I.D., 10 µm particle size, Phenomenex, Cheshire, UK) attached to a C18 guard column (Phenomenex, Cheshire, UK). Mobile phase A was HPLC grade water and phase B was 100% (v/v) ACN. The flow rate was set at 6 mL min^{−1} and separation of the peptides was carried out using the following linear gradient: 0–8 min: 100% A; 8–80 min: 100–30% A; 80–85 min: 30–0% A; 85–90 min: 0% A; 90–95 min: 0–100% A; 95–100 min: 100% A. The absorbance of the eluent was monitored at 214 nm. Eleven fractions were collected with a fraction collector (Waters, Dublin, Ireland) during each run. The semi-preparative procedure was carried out six times and fractions from the runs were pooled. The different fractions were evaporated in a solvent evaporator.

IEF fractionation. IEF was carried out using a Rotofor cell (Bio-Rad, Hercules, CA, USA) with the 5 kDa permeate of NaCNH-240 min (1.7% w/v) resuspended in distilled water containing 2.75 mL ampholyte Biolyte 3/10 (Bio-Rad, Alphatech, Wicklow, Ireland). The cathode and anode compartments of the Rotofor cell were filled with 0.1 M NaOH and H₃PO₄ solutions, respectively. Separation of peptides was carried out at 15 W

constant power for 4 h at 4 °C. These settings allowed migration of acidic peptides to the anode and basic peptides to the cathode until they reach their zwitterionic state and stabilize at a pH corresponding to their isoelectric point.^{51,52} Twenty different peptide fractions were collected and pooled depending on their pH value to make up four final fractions. The four fractions were freeze dried and stored at −18 °C.

2.4 Determination of 5-HT_{2C} receptor activation with the different milk protein hydrolysates and NaCNH-240 min fractions

The medium-throughput cellular based screening assay used in this study measured receptor mediated calcium uptake with a commercially available calcium sensitive fluorescent Ca4 dye. HEK 293A cells were stably transfected with two isoforms of the 5-HT_{2C} receptor, the unedited 5-HT_{2C}-INI and partially edited 5-HT_{2C}-VSV isoform. The partly edited 5-HT_{2C}-VSV, results from the distinctive ability of the 5-HT_{2C} receptor to be modified by post-transcriptional RNA editing⁵³ and is the most abundantly expressed 5-HT_{2C} receptor isoform in human brain regions, particularly in the hypothalamus.^{54,55} In addition, increased editing of the 5-HT_{2C} receptor has been associated with an altered feeding behaviour and fat mass, which supports the role of the 5-HT_{2C} in obesity.^{30,56,57} Stably transfected HEK 293A cells were seeded in 96 well plates at a density of 2.5×10^5 cells per mL at 100 μ L per well. Growth medium was removed after 24 h at 37 °C, 5% CO₂ and cells were incubated with 25 μ L of the assay buffer, containing HBSS supplemented with 20 mM HEPES buffer, and 25 μ L of Ca4 dye according to the manufacturer's protocol. Agonists (10 nM) and milk protein hydrolysates (0.005 to 1 mg mL^{−1}) were resuspended in assay buffer. Addition of assay buffer as blank control, endogenous ligand 5-hydroxytryptamine (5HT) as positive control and hydrolysate test solutions (25 μ L per well) was performed using the Flexstation II. Fluorescence readings were taken for 160 s in flex mode with excitation and emission wavelength of 485 and 525 nm, respectively. The relative increase in intracellular calcium (Ca²⁺) was calculated as the difference between maximum and baseline fluorescence ($V_{\max} - V_{\min}$). Unstimulated baseline emission obtained with the assay buffer was subtracted from ligand-induced emission and depicted as percentage relative fluorescent units (RFU) compared to the maximal calcium influx obtained by the 5HT positive control. Each agonist dose-response curve was constructed using ExcelTM software.

2.5 Statistical analysis

Mean multi comparison was carried out using a one way ANOVA followed by a Student Newman–Keuls test with SPSS (version 9, SPSS Inc., Chicago, IL, USA) at a significance level $P < 0.05$.

3 Results

3.1 Influence of hydrolysis time on serotonergic properties of NaCNH

The molecular mass distribution of NaCN and the different NaCNH was determined (Fig. 2a).

The GPC results showed that degradation of the different caseins present in NaCN (proteinaceous material >10 kDa) occurred during the time course of hydrolysis. The proportion of proteinaceous material decreased from 68.1 to 23.6% between 10 and 240 min hydrolysis while the proportion of peptides less than 1 kDa increased from 3.8 to 20.2% during the same time (Fig. 2a). NaCNH still contained some unhydrolysed caseins after 240 min hydrolysis. The four major caseins (*i.e.*, β -, α _{s1}-, α _{s2}- and κ -casein) were seen on the RP-HPLC profile of the NaCN substrate (Fig. 3a). After 240 min hydrolysis, the caseins were degraded following the hydrolytic activity of the enzyme, which resulted in the appearance of various peptide peaks on the RP-HPLC profile of NaCN-240 min (Fig. 3b). Unhydrolysed NaCN induced a small increase in intracellular calcium with 5-HT_{2C}-INI serotonin receptor whereas no change in intracellular calcium was seen with 5-HT_{2C}-VSV serotonin receptor (Fig. 2b). However, compared to NaCN, a greater increase in the intracellular calcium was seen with the different NaCNH (Fig. 2b). No calcium increase was seen in untransfected Hek293A cells, not expressing the 5-HT_{2C} receptor (data not shown). Over the time course of hydrolysis, serotonin 5HT_{2C} receptor mediated intracellular calcium increased from 49.1 ± 8.9 to $70.6 \pm 14.6\%$ and from 38.3 ± 5.9 to $61.9 \pm 8.9\%$ between 10 and 240 min with 5-HT_{2C}-INI and 5-HT_{2C}-VSV, respectively (Fig. 2b).

3.2 Influence of the starting substrate on serotonergic properties of milk protein hydrolysates

Three other starting substrates (Acid CN, GMP and SMP) were hydrolysed for 240 min. Three of the substrates allowed generation of hydrolysates (NaCNH-240 min, Acid CNH-240 min and

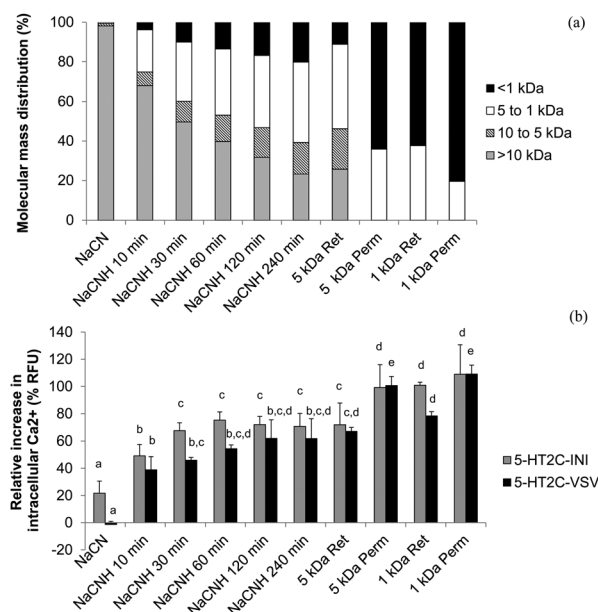


Fig. 2 (a) Molecular mass distribution profiles and (b) relative increase in 5-HT_{2C} receptor mediated intracellular Ca²⁺ in human embryonic kidney 293 (HEK 293A) cells. NaCN: unhydrolysed sodium caseinate, NaCNH x min: sodium caseinate hydrolysate withdrawn at x min, Ret: retentate obtained following ultrafiltration (UF) of NaCNH-240 min, Perm: UF permeate of NaCNH-240 min. In (b), values with different letters are significantly different ($P < 0.05$).

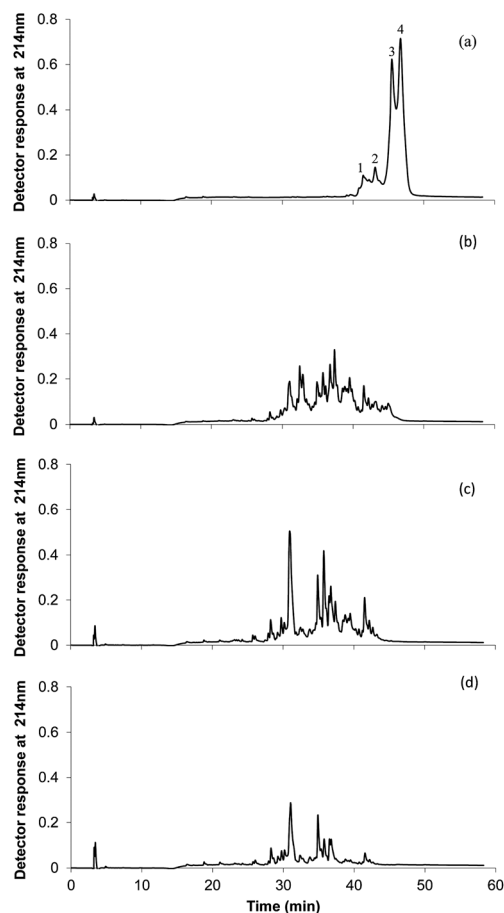


Fig. 3 Reverse-phase high performance liquid chromatography (RP-HPLC) profiles of unhydrolysed sodium caseinate (NaCN), sodium caseinate hydrolysates withdrawn at 240 min (NaCNH-240 min) and ultra-filtration (UF) fractions. (a) NaCN (b) NaCNH-240 min (c) 5 kDa permeate and (d) 1 kDa permeate. 1 – κ -casein, 2 – α ₂-casein, 3 – α ₁-casein, 4 – β -casein.

GMPH-240 min) with agonistic properties on the 5-HT_{2C} serotonin receptor. Intracellular calcium increase was higher with GMPH-240 min than with Acid CNH-240 min (Table 1). Interestingly, unhydrolysed GMP also behaved as an agonist for the 5-HT_{2C} serotonin receptor. However, the calcium increase measured with GMPH-240 min was significantly higher than that with GMP ($P < 0.05$). With SMPH-240 min, agonist properties were only observed with 5-HT_{2C-INI}, whereas intracellular calcium increase with 5-HT_{2C-VSV} was relatively low (Table 1). The low bioactivity observed with SMPH-240 min was probably due to the fact that this hydrolysate contains a lower concentration of bioactive peptides on a weight basis compared to the other hydrolysates. In fact, SMP contains 3 times less protein compared to NaCN or Acid CN.

Different fractionation procedures were applied to NaCNH-240 min hydrolysate in order to study the physicochemical properties of the bioactive peptides within this hydrolysate.

3.3 Fractionation of NaCNH-240 min by membrane fractionation, SPE, IEF and semi-preparative RP-HPLC

Membrane fractionation of NaCNH-240 min was carried out using a UF procedure where different MWCO membranes were employed (5 and 1 kDa). Four fractions with different molecular

Table 1 Relative increase in intracellular Ca²⁺ in human embryonic kidney 293 (HEK 293A) cells expressing the 5-HT_{2C} receptor treated with unhydrolysed and hydrolysed sodium caseinate (NaCN), acid casein (Acid CN), skim milk powder (SMP) and glycomacropeptide (GMP), and simulated intestinal digestion (SID) of the sodium caseinate hydrolysate NaCNH-240 min^a

Sample ^b	Relative increase in intracellular Ca ²⁺ (% RFU) ^c	
	Hek-5-HT _{2C-INI}	Hek-5-HT _{2C-VSV}
NaCN	2.0 ± 1.0 ^a	−3.1 ± 1.0 ^a
NaCNH-240 min	94.9 ± 3.1 ^c	82.7 ± 3.1 ^c
Acid CN	−0.3 ± 3.0 ^a	2.2 ± 1.1 ^a
Acid CNH-240 min	66.1 ± 5.5 ^b	38.4 ± 25.1 ^b
SMP	4.5 ± 2.8 ^a	5.0 ± 3.8 ^a
SMPH-240 min	42.6 ± 21.4 ^b	9.8 ± 6.4 ^a
GMP	43.4 ± 7.8 ^b	11.3 ± 2.9 ^a
GMPH-240 min	93.0 ± 18.3 ^c	74.7 ± 22.1 ^c
NaCNH-SID	57.4 ± 5.3 ^b	nd

^a nd: not determined. ^b NaCN: sodium caseinate; NaCNH-240 min: 240 min sodium casein hydrolysate; Acid CN: acid casein; Acid CNH-240 min: 240 min acid casein hydrolysate; SMP: skim milk powder; SMPH-240 min: 240 min skim milk powder hydrolysate; GMP: glycomacropeptide; GMPH-240 min: 240 min glycomacropeptide hydrolysate; NaCNH-SID: sample obtained following simulated intestinal digestion of NaCNH-240 min. All samples were tested at the concentration 0.5 mg mL^{−1}, except NaCN, NaCNH-240 min and NaCNH-SID which were tested at 1 mg mL^{−1}. ^c Relative increase in fluorescence units compared to the positive control 5-hydroxytryptamine tested at 10 nM. Average values ($n = 3$) ± SD. Within the same column, values with different superscript letters are significantly different ($P < 0.05$).

mass distribution profiles were obtained (Fig. 2a). As expected the 5 kDa retentate contained the proteinaceous material (>5 kDa) from NaCNH-240 min, whereas the 5 kDa permeate, 1 kDa retentate and 1 kDa permeate were free from proteins. The proportion of peptides less than 1 kDa was most abundant in the 1 kDa permeate (80.4%). The RP-HPLC profile of the 5 and 1 kDa permeates (Fig. 3c and d, respectively) showed a less complex peptide profile compared to that of NaCNH-240 min (Fig. 3b). This was due to the removal of large molecular mass peptides and proteins during the UF procedure. In addition, some peptide peaks were more abundant in the 5 and 1 kDa permeate compared to the NaCNH-240 min hydrolysate, showing enrichment in low molecular mass peptides upon UF. The volume concentration ratio (VCR) obtained following UF treatment with the 5 and 1 kDa membranes was 5.8 and 2.1, respectively. The RP profiles obtained using UPLC showed a better resolution of peptide peaks in the NaCNH-240 min (Fig. 4a) compared to RP-HPLC (Fig. 3b).

Bioactivity of the UF fractions was determined (Fig. 2b). With the 5-HT_{2C-INI} serotonin receptor, the retentates (5 and 1 kDa retentates) showed lower bioactivity than the permeates (5 and 1 kDa permeate) and bioactivity of the 5 and 1 kDa permeates were similar. Slight differences were seen with the 5-HT_{2C-VSV} serotonin receptor where bioactivity was lowest with the 5 kDa retentate, whereas the 5 kDa permeate, 1 kDa retentate and 1 kDa permeate displayed similar bioactivities. Dose–response curves depicting 5-HT_{2C} receptor mediated intracellular calcium influx following exposure to NaCNH-240 min, and its

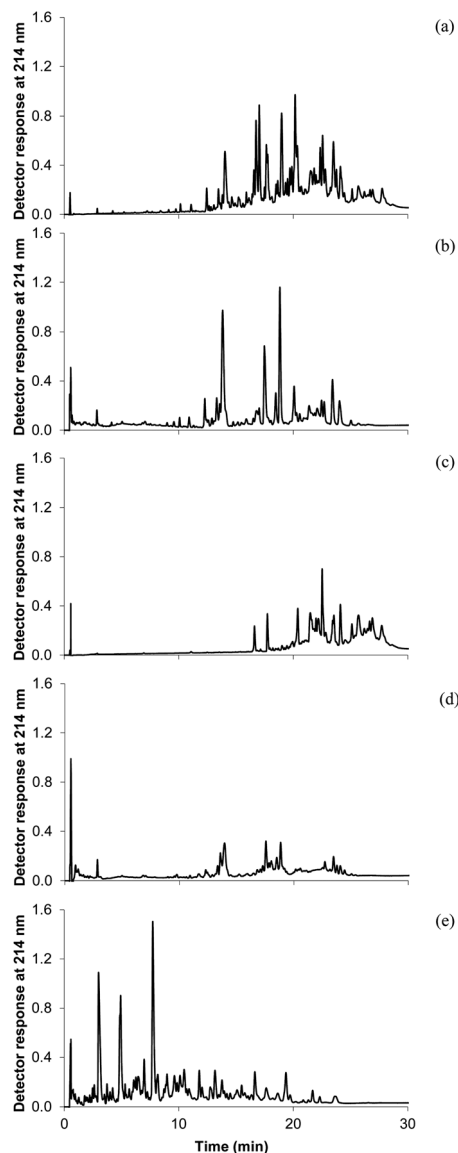


Fig. 4 Reverse-phase ultra-performance liquid chromatography (RP-UPLC) profiles of sodium caseinate hydrolysate (NaCNH-240 min), ultrafiltration (UF), solid phase extraction (SPE) and isoelectric focusing (IEF) fractions, and simulated intestinal digestion (SID) of the sodium caseinate hydrolysate NaCNH-240 min. (a) NaCNH-240 min, (b) UF 5 kDa permeate, (c) SPE X-40% ACN, (d) IEF 4 and (e) SID of NaCNH-240 min.

associated 5 and 1 kDa permeates are illustrated on Fig. 5. A dose-response relationship between the increase in intracellular calcium and the concentration of test substance was seen for these three samples, with the 5 and 1 kDa permeate both showing higher bioactivity than NaCNH-240 min. This result is in agreement with the enrichment in peptides observed upon UF fractionation. The 5 and 1 kDa permeates essentially showed superimposable dose-response curves, suggesting that the bioactive peptides had the same potency in both UF fractions of NaCNH-240 min.

The NaCNH-240 min hydrolysate was fractionated with SPE as described by Nongonierma and FitzGerald.⁴⁹ Recovery of peptide material using this procedure was 77.5%.

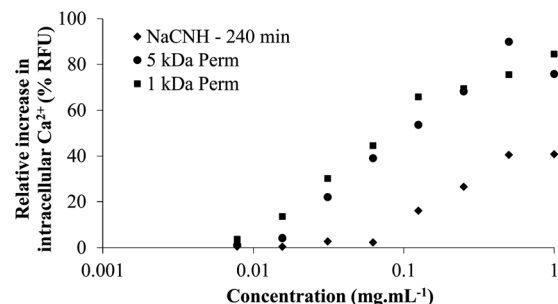


Fig. 5 Dose-response curves of sodium casein hydrolysate (NaCNH-240 min) and associated ultrafiltration fractions (UF 5 kDa permeate and UF 1 kDa permeate) on their 5-HT_{2C} induced calcium influx. Each point is the average of two measurements (confidence interval at 5%: 8.1, 6.5 and 3.4% for NaCNH-240 min, UF 5 and 1 kDa permeate, respectively) of relative increase in intracellular Ca²⁺ in human embryonic kidney 293 (HEK 293A) cells.

Although some peptide/protein material was lost during the SPE procedure, bioactivity was still seen within some of the fractions collected. Of the eleven fractions generated, the four fractions eluted between 35 and 50% ACN activated the 5-HT_{2C-VSV} serotonin receptor (Table 2). Activation observed with the fractions eluted with 35 and 40% ACN was not significantly different from that of NaCNH-240 min ($P \geq 0.05$). A significantly higher activation was observed with the fractions eluted at 45 and 50% ACN compared to NaCNH-240 min ($P < 0.05$). Activation induced by the fractions eluted between 35 and 50% ACN was not significantly different ($P \geq 0.05$). The RP profile of the SPE X-40% ACN fraction is shown in Fig. 4c. A lower number of peptide peaks is seen in this fraction compared to that of NaCNH-240 min (Fig. 4a) and its 5 kDa permeate (Fig. 4b). In addition, no peptide peaks with an elution time less than 15 min could be seen on the RP profile of SPE X-40% ACN which is in agreement with its relatively hydrophobic nature. Fractionation by IEF showed that two fractions out of four were bioactive. These were the fractions with the highest pH value, *i.e.* pH 8.6 and 13.2 (Table 2).

The electrolyte (Biolyte 3/10) employed to carry out the IEF fractionation did not seem to interfere with the assay as shown by the similar intracellular calcium values obtained with NaCNH-240 min with and without electrolyte with the 5-HT_{2C-VSV} serotonin receptor ($P \geq 0.05$, Table 2). The RP-UPLC profile of IEF fraction 4 (Fig. 4d) shows a wide range of peptide peaks eluting at different retention times. The RP profile of IEF 4 is also less complex than that of NaCNH-240 min. Bioactivity of this fraction was significantly higher than that of NaCNH-240 min with the 5-HT_{2C-VSV} serotonin receptor ($P < 0.05$, Table 2) whereas bioactivity of IEF 3 was not significantly different from NaCNH-240 min ($P \geq 0.05$, Table 2).

The fourth fractionation method used was semi-preparative RP-HPLC, which allowed isolation of 11 fractions with different peptide composition (Fig. 6a). Out of the 11 fractions isolated, the five fractions eluting between 31.5 (22.8% ACN-F5) and 84 min (69.5% ACN-F10) were bioactive (Fig. 6b).

3.4 Simulated intestinal digestion of NaCNH-240 min

A SID protocol was applied to NaCNH-240 min to study its stability to intestinal proteinase/peptidase activities. The

Table 2 Relative increase in intracellular Ca^{2+} in 5-HT_{2C} expressing human embryonic kidney 293 (HEK 293A) cells treated with sodium caseinate hydrolysate (NaCNH-240 min), fractions obtained on elution from solid-phase extraction (SPE) Strata X with different acetonitrile (ACN) concentrations and isoelectric focusing (IEF) fractions^a

Sample ^b	Relative increase in intracellular Ca^{2+} (% RFU) ^c	
	Hek-5-HT _{2C} -INI	Hek-5-HT _{2C} -VSV
NaCNH-240 min	94.9 ± 3.1 ^c	78.1 ± 14.4 ^b
SPE X-unbound	nd	3.7 ± 1.4 ^a
SPE X-5% ACN	nd	0.9 ± 0.5 ^a
SPE X-10% ACN	nd	2.7 ± 1.1 ^a
SPE X-15% ACN	nd	2.6 ± 0.7 ^a
SPE X-20% ACN	nd	3.7 ± 1.0 ^a
SPE X-25% ACN	nd	3.9 ± 0.6 ^a
SPE X-30% ACN	nd	5.4 ± 2.9 ^a
SPE X-35% ACN	nd	90.5 ± 8.8 ^{b,c}
SPE X-40% ACN	nd	86.7 ± 7.2 ^{b,c}
SPE X-45% ACN	nd	99.4 ± 11.0 ^c
SPE X-50% ACN	nd	98.7 ± 19.2 ^c
NaCNH-240 min + electrolyte	71.1 ± 5.8 ^b	75.9 ± 7.4 ^b
IEF 1	−8.5 ± 1.3 ^a	−4.3 ± 1.9 ^a
IEF 2	−3.8 ± 1.6 ^a	−5.1 ± 4.3 ^a
IEF 3	75.4 ± 7.5 ^b	82.6 ± 10.0 ^{b,c}
IEF 4	95.7 ± 13.6 ^c	100.9 ± 8.5 ^c

^a nd: not determined. ^b SPE X: fractions obtained on elution from solid-phase extraction with Strata X with different acetonitrile (ACN) concentrations; IEF: isoelectric focusing fractions. All fractions were tested at 1 mg mL^{−1}. ^c Relative increase in fluorescence units compared to the positive control 5-hydroxytryptamine tested at 10 nM. Average values ($n = 3$) ± SD. Within the same column, values with different superscript letters are significantly different ($P < 0.05$).

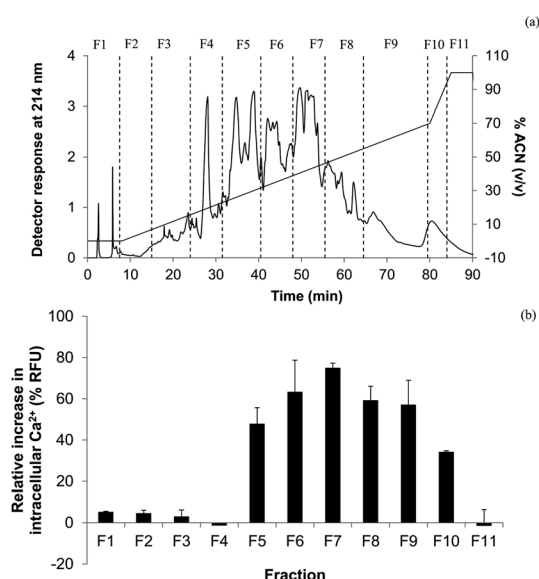


Fig. 6 Semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC) profile of the sodium caseinate hydrolysate NaCNH-240 min (a) and relative increase in intracellular Ca^{2+} in human embryonic kidney 293 (HEK 293A) cells expressing the 5-HT_{2C} receptor observed with the semi-preparative RP-HPLC fractions F1 to F11 (b). Values with different superscript letters are significantly different ($P < 0.05$).

RP-UPLC profile of NaCNH-SID (Fig. 4e) shows a significantly different peptide profile than NaCNH-240 min (Fig. 4a). The NaCNH-SID was still able to activate 5-HT_{2C}-INI serotonin receptor (Table 1) indicating that bioactive peptides within NaCNH-240 min may survive intestinal digestion *in vivo*. In addition, activation of the serotonin 5-HT_{2C}-INI receptor was not significantly different for NaCNH-SID and NaCNH-240 min ($P \geq 0.05$), which may suggest that the bioactive peptides within NaCNH-240 min are not further hydrolysed by the pancreatic enzyme activity or that their breakdown products are still able to stimulate serotonin 5-HT_{2C} receptors in the same fashion as the parent peptides.

4 Discussion

Different effects of milk proteins on serotonin release have been described in the literature. Human and bovine β -casomorphin-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) was shown to act as a 5-HT₂-serotonin receptor antagonist.⁵⁸ In addition, milk proteins such as α -lactalbumin are a good source of tryptophan (Trp), the precursor of serotonin. When diets rich in α -lactalbumin were administered to rats, an increase in the production of serotonin was seen. However, this did not translate into increased levels of extracellular serotonin.⁵⁹ Ingestion of α -lactalbumin has been correlated with an increase in hypothalamic serotonin release in rats⁵⁹ and in humans,⁶⁰ which has been linked to the high Trp content of α -lactalbumin compared to other milk proteins. Ingestion of α -lactalbumin has been correlated with a higher bioavailability of Trp which is the precursor of serotonin.⁶¹ To our knowledge, milk-derived bioactive peptides demonstrating direct 5-HT_{2C} serotonin receptor activation have not been reported in the literature to date. This study describes the generation of different milk protein hydrolysates and analysed their potential to increase intracellular calcium in HEK 293A cells engineered to express the 5-HT_{2C} receptor. An increase in activation of the 5-HT_{2C} serotonin receptor was seen over the time course of NaCN hydrolysis (Fig. 2b). Protein breakdown increased between 10 and 240 min (Fig. 2a), suggesting that release of bioactive peptides in time may have been responsible for the higher 5-HT_{2C} serotonin receptor activation observed. Hydrolysis has the potential to release specific peptide sequences with bioactive potential, previously encrypted within the protein structure.⁴⁸ As expected, enrichment in bioactive peptides correlated with the enrichment of smaller molecular mass peptides following the two UF steps. Bioactivity was found in the 5 and 1 kDa retentates possibly due to the fact that they still contained bioactive peptides as the UF procedure employed did not include a diafiltration step. The dose-response curves confirmed that the 5 and 1 kDa permeates were more potent than NaCNH-240 min (Fig. 5). No difference in potency was seen between the 5 and 1 kDa permeates, suggesting that bioactive peptides could be found within both fractions at similar concentrations. Similar potency in both fractions may also be related to the fact that their peptide composition does not dramatically differ, this was confirmed with the RP-HPLC (Fig. 3c and d) and molecular mass distribution profiles (Fig. 2a).

Milk protein hydrolysates generated with other starting materials were investigated for their potential to activate serotonin receptors. Unhydrolysed proteins such as Acid CN or SMP could not substantially activate the 5-HT_{2C} serotonin receptor (Table 1). GMP was shown to have 5-HT_{2C} receptor activating potential both in unhydrolysed and hydrolysed format (Table 1). GMP is a peptide which is released upon hydrolytic activity of chymosin notably during the renneting stage employed in the cheese making process. This peptide has been identified for its satiating properties when administered *in vivo*. Inhibition of gastric secretion and increased release of cholecystokinin (CCK) have been linked to the positive role of GMP on satiety.^{4,62,63} Nevertheless, this satiating effect of GMP does not always translate to a reduction of food intake.⁶⁴ Preload drinks containing whey proteins and GMP have been shown to enhance fullness ratings in human subjects compared to carbohydrates.⁶⁴ However, no differences in food intake at the lunch meal could be determined between the protein and carbohydrate preload.⁶⁴ Other conflicting results are reported in the literature notably when GMP was administered as a preload, showing no impact on satiety.⁶⁵ GMP was shown to increase the release of CCK, thereby mediating satiety signals *in vivo*.²⁴ A different mechanism is described here where GMP acts as an agonist for serotonin receptors. To our knowledge this is the first time that GMP has been shown to bind and activate the 5-HT_{2C} serotonin receptor. Furthermore, significantly higher 5-HT_{2C} serotonin receptor activation could be found with GMPH-240 min compared to GMP ($P < 0.05$). Enzymatic hydrolysis of GMP resulted in an increase in bioactivity. It is unlikely that intact GMP will reach the target serotonin receptors which are located in the enteric nervous system. Whereas hydrolysates, which contain lower molecular mass fragments, may cross the gut barrier and reach target organs outside of those present within the gut, provided that they are not degraded within the gut. This may be of significance for the management of satiety using GMP. By hydrolysing GMP, we demonstrated that the generation of bioactive peptides with serotonergic properties could be achieved. Therefore, GMP and GMP hydrolysates may have potential as multi-functional ingredients in weight management targeting both the gut as well as the enteric nervous system. However, it was shown that GMP alone is not critical in pre-meal whey-induced satiety. Nevertheless, it may have a unique role in compensatory intake regulation managing daily energy intake.⁶⁶

The stability of bioactive peptides to gastro-intestinal proteinases is a prerequisite for bioactivity *in vivo*.⁶⁷ SID of NaCNH-240 min was carried out in order to study the stability of the bioactive peptides to intestinal enzymes. There was no significant difference ($P \geq 0.05$) between the bioactive properties of NaCNH-SID and NaCNH-240 min (Table 1). This result suggests that the bioactive peptides present within NaCNH-240 min are likely to display their effect *in vivo*. It has also been suggested that different formulations, including the utilisation of surfactant agents, may be utilised to increase permeation of bioactive peptides in the gastro-intestinal tract.⁶⁷

Four fractionation techniques including UF, SPE, semi-preparative RP-HPLC and IEF were applied to NaCNH-240 min

in order to better characterise the bioactive peptides within this hydrolysate. UF fractionation yielded 4 fractions which were all able to stimulate 5-HT_{2C} serotonin receptor. However, the most potent samples were the 5 and 1 kDa permeates (Fig. 2b). Low molecular mass peptides can passively diffuse through the UF membranes. Recovery for the SPE procedure was of the same order as other studies.⁵⁰ For the SPE fractionation, fractions eluted with ACN concentrations between 35 and 50% (v/v) were bioactive (Table 2), which was in agreement with the results obtained with semi-preparative RP-HPLC with bioactive fractions eluting between 22.8 and 69.5% ACN (Fig. 6). SPE and semi-preparative RP-HPLC both allowed isolation of a restricted number of bioactive fractions. SPE allowed fractionation of larger quantities (>6 times more) than semi-preparative RP-HPLC. Isoelectric focusing also showed bioactivity in different fractions (pH 8.6 and 13.2 for IEF3 and IEF4, respectively), whereas no bioactivity was found within the lower pH fractions (pH 0.9 and 2.9 for IEF1 and IEF2, respectively). Significant peptide losses (up to 40%) were reported following precipitation during an IEF procedure.⁶⁸ Similarly, during the fractionation procedure used in this study, overall losses of 37.8% were obtained. The pH range for NaCNH-240 min fractions (between 0.5 and 13.6) was broader than that reported by Yata *et al.*⁶⁸ (between pH 3.0 and 10.0) with the same ampholyte Bio-lyte 3/10. Differences in pH range compared to Yata *et al.*⁶⁸ are likely due to composition differences between both hydrolysates, with low pH values possibly due to the HCl employed during pH stat control of NaCNH-240 min. Fractions displaying bioactive properties had a higher pH, *i.e.*, 8.6 and 13.2. Previous studies have described the utilisation of autofocusing (IEF conducted without ampholyte) in the isolation of bioactive peptides, notably for their immunostimulatory properties.^{51,52} Fractions isolated by Mercier *et al.*⁵² induced a higher lymphocyte secretion compared to the parent hydrolysate. This was explained by the fact that peptide-peptide interactions (hydrophobic and electrostatic interactions) can occur in milk protein hydrolysates,⁶⁹ resulting in lower bioactive potential of hydrolysates compared to fractions,⁵² which may have occurred within NaCNH-240 min herein. In addition, enrichment of bioactive peptides upon fractionation could be seen following analysis of the RP-UPLC profiles of these fractions (data not shown). No significant difference ($P \geq 0.05$) could be seen between the bioactivity of NaCNH-240 min and IEF 3 whereas IEF 4 was more potent ($P < 0.05$) than NaCNH-240 min (Table 2). Saturation of the intracellular calcium measured may have been reached in the assay with certain fractions. However, it is expected that IEF 3 and IEF 4 would yield more potent fractions than NaCNH-240 min as was seen with the UF permeates (Fig. 5).

The different fractionation techniques studied allowed us to establish that low molecular mass peptides with hydrophobic characteristics and a basic isoelectric point (pI) were able to stimulate the 5-HT_{2C} serotonin receptor in a HEK 293A cell culture model. Bioavailability of the peptides is crucial for their bioactivity as the target 5-HT_{2C} serotonin receptors are located in the enteric nervous system. It has been demonstrated that intestinal transport of intact peptides and even proteins occur

in vivo.⁷⁰ Intact peptides can be absorbed in the gastro-intestinal tract when they are stable to gastro-intestinal enzymes and are able to permeate through the intestinal mucosa.⁷⁰ Relatively hydrophobic peptides from milk such as Leu-Trp, Phe-Tyr, Ile-Tyr and Ile-Pro-Pro could reach the circulation without being degraded in the gastrointestinal tract.⁷¹ The small size of the bioactive peptides found within NaCNH-240 min may allow them to cross the gut barrier.⁷² SID of NaCNH-240 min revealed that bioactivity was maintained, which may suggest that *in vivo*, the bioactive components in the NaCNH-240 min hydrolysate would have the potential to survive intestinal digestion. In addition, the hydrophobic nature of the bioactive peptides of NaCNH-240 min make them good candidates for intestinal permeation.⁷³ Fractionation with IEF has demonstrated that the bioactive peptides within NaCNH-240 min have an isoelectric pH between 8.6 and 13.2, which suggests that these peptides will remain in solution in the small intestinal lumen which has been found to be between pH 5.5 and 7.6 in healthy subjects.⁷⁴ More research is needed on the role of these bioactive peptides on regulation of food intake.

5 Conclusions

The potential of a range of milk protein hydrolysates to activate serotonin receptors has been demonstrated *in vitro*. In most cases, enzymatic hydrolysis of the milk protein substrates was a prerequisite for 5-HT_{2C} serotonin receptor activation. However, intact GMP behaved as an agonist and was able to stimulate 5-HT_{2C} serotonin receptors. The 5-HT_{2C} serotonin receptor activation obtained with the GMP hydrolysate (GMPH-240 min) was however higher than that with GMP. Isolation of bioactive fractions of NaCNH-240 min allowed a better characterisation of the physicochemical properties of the bioactive peptides present within this hydrolysate. The small size, hydrophobic nature and basic pI of the bioactive peptides within NaCNH-240 min, together with the maintenance of bioactivity following SID suggest that these compounds are good candidates for intestinal absorption *in vivo*. Further studies on the *in vivo* effect on food intake are now warranted. Optimal dosage of NaCNH-240 min needs to be determined in humans in order to further formulate functional foods and dietetic products. The outcomes of this research indicate that milk-derived peptides may have potential as natural ingredients in the formulation of food-products designed to help in weight management.

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