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Characterization and Identification of a Chymotryptic Hydrolysate of Alpha-Lactalbumin Stimulating Cholecystokinin Release in STC-1 Cells

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Abstract Alpha-lactalbumin hydrolysate is of significant interest, due to its potential application as a source of bioactive peptides in nutraceutical and pharmaceutical domains. This study was focused on the cholecystokinin (CCK) family compounds which are small peptides involved in the satiety control. The action of chymotryptic hydrolysate of alpha-lactalbumin on cholecystokinin release from intestinal endocrine STC-1 cells was investigated. We demonstrated for the first time that a chymotryptic hydrolysate of alpha-lactalbumin was able to highly stimulate CCK-releasing activity from STC-1 cells. The peptidic hydrolysate was characterized by LC/MS and MS/MS, thus highlighting the presence of 11 fractions containing 21 peptides, each potentially having the desired activity.

Keywords Alpha-lactalbumin hydrolysate · Cholecystokinin · Enteroendocrine STC-1 cells · Peptides

Introduction

For many years, food proteins derived from various sources have been used for animal as well as human nutrition, but proteins can also be used in the food industry [1] to improve the functional properties such as taste, solubility, emulsification, and gelation. More recently, the pharmaceutical [2] and food industries [3] have shown increasing interest in peptides derived from proteins as components of functional foods. A wide range of biologically active peptides have already been isolated from milk proteins [4], soy proteins [5], gluten, or seafood hydrolysates [6, 7]. The alpha-lactalbumin is the second most abundant protein in bovine milk whey, its rate concentration being of 1.2 g/L of milk. Whey is a co-product of cheese production. Giving an added value to whey proteins has thus a potential economic interest. Alpha-lactalbumin consists of 123 amino acid residues forming a compact globular structure stabilized by four disulfide bonds [8]. As other proteins, the

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hydrolysis of the alpha-lactalbumin generates peptides presenting biological activities such as opioid [9], angiotensin-I-converting enzyme inhibitory [10], or antimicrobial [11].

Excess of food intake is a prominent contributor to several habit-related diseases like diabetes and obesity [12]. Thus, the control of food intake can possibly prevent increasing threats from these medical complications.

The satiety mechanism could follow different ways, including the plasmatic secretion of cholecystokinin (CCK) in presence of satietogen proteins or peptides. The plasmatic CCK transports the satiety signal from the gastrointestinal system to the brain [13].

Food intake suppression by CCK in the central nervous system is well established [13]. CCK is produced in the proximal small intestinal endocrine I cells and in the cerebral neurons, and secreted into the circulation. Gastrin peptides are mainly synthesized in antroduodenal G-cells from where they are released to blood to regulation. A characteristic feature of the two peptide systems is their common carboxyamidated C-terminal tetrapeptide sequence, Trp-Met-Asp-Phe-NH_2. This sequence also constitutes the minimal structure necessary for receptor binding and biological activity, although the potencies of both types of peptides depend upon their N-terminal extensions [14].

Several in vitro studies reported that peptones were able to stimulate CCK secretion [15] and gene transcription in STC-1 cell line [16, 17]. Because of their similarity with human CCK-secreting intestinal I cells, STC-1 cell line was described as one of the best in vitro model for the CCK secretion study [15]. However, only few studies reported the effect of food proteins (intact or hydrolyzed) on CCK release by STC-1 cells and the action of chymotryptic hydrolysate of alpha-lactalbumin on CCK secretion has not been studied yet.

The aim of this study was to investigate the biological activity of chymotryptic hydrolysate of alpha-lactalbumin, to demonstrate the release of cholecystokinin by STC-1 cells in vitro, then tests have been carried out to control the product cytotoxicity for future potential use in human food, and finally, the peptidic hydrolysate was characterized by liquid chromatography (LC)/mass spectrometry (MS) and MS/MS to identify the peptides present.

Materials and Methods

Materials

All common chemicals and solvents were of analytical grade from commercial sources. Dubelcco's modified Eagle's medium (DMEM) for STC-1 cell line, fetal calf serum, and other culture reagents were purchased from Dutscher (Brumath, France). Albumin acid hydrolysate from chicken egg (AEH), bovine serum albumin (BSA), alpha-lactalbumin, and chymotrypsin were purchased from Sigma Aldrich (Evry, France). The gastrin/CCK kit was from CisBio International (Cèze, France).

Hydrolysate Preparations

Alpha-lactalbumin solution (apoform) at 1 mg ml^{-1} was digested by chymotrypsin (E/S=1/100 mole/mole) at 37 °C in 0.1 M sodium phosphate buffer, pH 7.4 during 30 min, for obtaining a majority of intermediate peptides, which could have a potential in biological activities, such as CCK release. The enzymatic hydrolysis of alpha-lactalbumin was performed according to the *N*-Succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (Suc-AAPF-pNA) method, Suc-AAPF-pNA being a specific substrate for the determination of enzymatic activity obtained by chymotryptic hydrolysis [18]. Hydrolysis was stopped by adding HCl 2 M, at pH 3.0, to

inactivate the enzyme. Thus, hydrolysate was freeze dried and stored at −20 °C as powder before tests.

Determination of the Degree of Hydrolysis of Alpha-Lactalbumin

The degree of hydrolysis is defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds of alpha-lactalbumin. The cleavage of peptide bonds in the course of hydrolysis was quantified by the trinitrobenzene sulphonate method [19]. The corrected degree of hydrolysis is defined as the ratio of the number of cleaved peptide bonds (determined by the trinitrobenzene sulphonate method) to the total number of peptide bonds of corresponding to alpha-lactalbumin molecules effectively hydrolyzed. The respective proportions of hydrolyzed and non-hydrolyzed alpha-lactalbumin and then alpha-lactalbumin were deduced from the peak areas determined by RP-HPLC analysis.

Cell Line, Culture Conditions, and CCK Secretion Studies

The STC-1 cell line was a gratefully received gift from Dr Benoit Cudennec (Concarneau, France). STC-1 pluri-endocrine cell line was derived from an endocrine tumor developed in the small intestine of double transgenic mice expressing rat insulin promoter for the simian virus 40 large T antigen and the polyoma virus small T antigen [20]. STC-1 cell line is well known for the secretion of CCK phenomenon in intestine. Cells were grown in Dulbecco's modified Eagle's liquid medium supplement with 10% of fetal calf serum, 2 mM glutamine, 100 $U ml^{-1}$ penicillin, and 100 $\mu g\ ml^{-1}$ of streptomycin at 37 °C, in a 5% CO_2 atmosphere. Two days before the experiment, cells were seeded into 24-well culture plates. When sub-confluence was reached, after approximately 2 days of culture, the medium was replaced by the incubation buffer (4.5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM glucose, 140 mM NaCl, and 20 mM Hepes–Tris, pH 7.4), containing the hydrolysate solution. Cells were then incubated at 37 °C, in a 5% CO_2 atmosphere for 2 h; the incubation medium was collected, centrifuged (2,000×*g*, 7 min) and the supernatants kept at −20 °C before CCK determination.

CCK/Gastrin Assay

CCK assay was carried out using the Gask-Pr kit (CisBio International, France).

Gask-Pr is a radioimmunoassay kit for the direct quantitative determination of gastrin in human serum or plasma. The principle of the assay is based on competition between gastrin radiolabeled with iodine 125 and gastrin contained in the standards or samples to be assayed for a given limited number of anti-gastrin antibody binding sites. At the end of the incubation period, the amount of radiolabeled gastrin bound to the antibody is inversely proportional to the amount of non-radiolabeled gastrin originally present in the assay.

Cytotoxicity Studies

The effect of chymotryptic hydrolysate of alpha-lactalbumin on cell proliferation was measured using a colorimetric assay, the tetrazolium salt (XTT) II kit assay (Sigma Aldrich, France). This assay is based on the reduction of a tetrazolium salt into yellow formazan salt by active mitochondria. For proliferation tests, cells were transferred into a 96-well microtitre plate at a density of 6,000 cells by well into 150 μl of medium (DMEM) and in absence or in presence of proteins at different concentrations. After 48 h at 37 °C, in a 5 °C

CO_2 atmosphere, cells were incubated in the presence of XTT for 4 h. (Absorbance 490 nm, against 630 nm reference) in each well was measured in a microplate reader spectrophotometer (MRX Dynex, ThermoLabsystems, Issy Les Moulineaux, France). Results are expressed as percentages of basal growth activity.

HPLC Analyses

The liquid chromatographic system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC image 466 computer. Millennium® software was used to plot, acquire, and analyze chromatographic data.

All the chromatographic processes were performed with a Vydac C4 column (250 mm–10 mm), used at room temperature. The mobile phase was water/trifluoroacetic acid (1,000:1, *v*/*v*) as eluent A and acetonitrile/water/trifluoroacetic acid (600:400:1, *v*/*v*/*v*) as eluent B. The flow rate was 5 mL/min. Samples were filtered through 0.22 mm filters and then injected. The gradient applied was 0–67% (*v*/*v*) B over 30 min, then 67–87% (*v*/*v*) B over 35 min. On-line UV absorbance scans were performed between 200 and 300 nm at a rate of one spectrum per second with a resolution of 1.2 nm[21, 22].

Mass Spectrometry Analysis

MS and MS/MS measurements were performed in positive ion mode using electrospray ionization (ESI) and ESI/MS/MS, respectively. ESI mass spectrometry was performed using a triple quadrupole instrument from Applied Biosystems API 3000 (PE Sciex, Toronto, Canada). The system is controlled by the Analyst Software 1.4, allowing the control of the spectrometer, the analysis, and the processing of the data. Interpretations of MS–MS spectra were made with the Bioanalyst software. The freeze-dried samples were dissolved in acetonitrile/water (20/80 *v*/*v*) solvent containing formic acid 0.1% for the positive mode. The solution was injected (nebulized) uninterrupted, thanks to a pump (Model 22, Harward Apparatus, South Natick, USA) with a flow rate of 5 μL/min. The potential of ionization was of 5,000 V (volt) in positive mode. At the time of the recording of the spectrum, 30 scans on average were added (MCA mode) for each spectrum.

The gases used (nitrogen and air) were pure (up to 99%) and produced by a compressor Jun-Air 4000-40M and a Whatman model 75-72 nitrogen generator (Whatman Inc, Haverhill, MA, the USA). The polypropylene glycol was used for the calibration and the optimization of the machine system. The peptide sequence was determined from the CID spectrum of the protonated analyze $[M+H]^+$ by MS/MS experiments.

Results

Effect of Alpha-Lactalbumin Hydrolysate on CCK Release by STC-1 Cells

According to in vitro studies which reported that food proteins were able to stimulate the CCK secretion [15] and also that STC-1 cells were one of the best in vitro model for the CCK secretion study [15], it was decided to investigate the release of CCK by STC-1 cells in presence of various products. The cholecystokinin concentrations after 2 h exposure of STC-1 cells with either 2% *w*/*v* chymotryptic hydrolysate of alpha-lactalbumin, hen egg

albumin hydrolysate, bovine serum albumin, intact alpha-lactalbumin, or buffer (negative control to determine the basal CCK concentration) were compared. AEH and BSA have been reported by other authors to enhance CCK release by STC-1 cells [17] and were thus considered as positive controls. Interestingly, the CCK concentration after exposure to chymotryptic hydrolysate of alpha-lactalbumin (ALH) was significantly higher than after exposure to AEH, BSA, or intact alpha-lactalbumin (IL).

Effectively, as shown in Fig. 1, STC-1 basal CCK mean secretion (Ctrl) was 5.0 pM after 2 h of incubation. Thus, chymotryptic hydrolysate of alpha-lactalbumin induces a higher CCK release compared to the intact alpha-lactalbumin (more than two times at the concentration of 2% *w/v*). Also, as seen on Fig. 1, chymotryptic hydrolysate of alpha-lactalbumin induces a higher CCK release at each concentration in a dose-dependent fashion, compared to both positive controls and nonhydrolyzed lactalbumin. It is also shown that the chymotryptic hydrolysate of alpha-lactalbumin at similar concentration, allows a better release of CCK by STC-1 cells than positive controls. For example, at the highest concentration tested (2% *w/v*), STC-1 cells released two times more CCK in presence of ALH (83 pM) compared to the presence of AEH (39 pM), BSA (41 pM), or intact alpha-lactalbumin (36 pM).

This represented for bovine serum albumin and albumin hydrolysate from chicken egg, an 8-fold increase over the basal value, and for chymotryptic hydrolysate of alpha-lactalbumin a 10-fold increase at the concentration of 2% *w/v*.

Results have shown that chymotryptic hydrolysate of alpha-lactalbumin was able to highly stimulate CCK secretion by STC-1 cells.

Assessment of Alpha-Lactalbumin Hydrolysate Cytotoxicity on STC-1 Cells

The potential cytotoxicity of alpha-lactalbumin hydrolysate was assessed by monitoring proliferation of STC-1 cells for 48 h. Alpha-lactalbumin hydrolysate and positive controls (hydrolysate of albumin from chicken egg and bovine serum albumin) at 1% and 2% (*w/v*) (i.e., the same concentration than that used during the CCK concentration test) were added

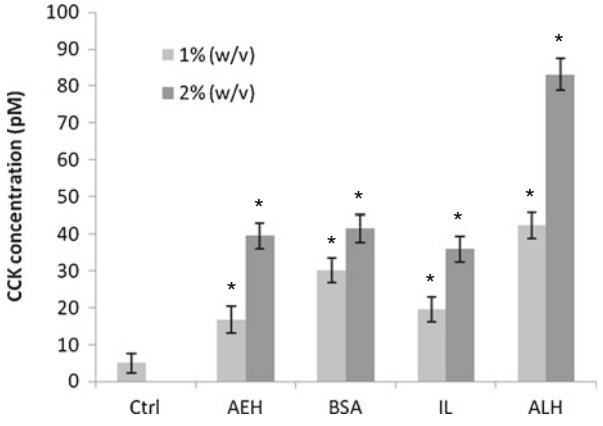

Fig. 1 Effect of alpha-lactalbumin hydrolysate (*ALH*) on CCK-releasing activity on STC-1 cells. Hydrolysate was tested at 1% *w/v* or 2% *w/v* on CCK release and compared to those obtained with albumin egg hydrolysate (*AEH*, a positive control), bovine serum albumin (*BSA*), and intact lactalbumin (unhydrolyzed)—*IL*. The negative control (*Ctrl*) is the buffer tested alone on the cells. Values are means ± SD of three repeated measurement. (*asterisk*): $p<0.05$ versus control

to the culture medium. Cell proliferation was estimated by performing a colorimetric test based on the reduction of a tetrazolium salt into yellow formazan by active mitochondria after 48 h incubation of cells in the presence or absence of proteins. The comparison of STC-1 cells proliferation in the presence of proteins and in their absence is presented in Fig. 2. On one hand, results have shown that the hydrolysate of albumin from chicken egg either at 1% (*w*/*v*) or 2% (*w*/*v*) did not inhibit STC-1 cells proliferation. On the other hand, bovine serum albumin and the chymotryptic hydrolysate of alpha-lactalbumin at 2% (*w*/*v*) significantly inhibited STC-1 cells proliferation, with a significant inhibitory but nontoxic effect (viable cells after 48 h). Bovine serum albumin at 1% (*w*/*v*) had no significant effect on STC-1 cells growth while chymotryptic hydrolysate of alpha-lactalbumin exerted a positive effect (71% increase). Further work will focus on the mechanism which could explain the inhibition of STC-1 cells proliferation of the proteins at 2% (*w*/*v*) and the absence of inhibition at 1% (*w*/*v*).

Alpha-Lactalbumin Hydrolysate Characterization by LC/MS and MS/MS

Most of the peptides of ALH were identified by LC-ESI-MS/MS and their sequence was compared to that of known bioactive peptides.

As shown on Fig. 3a, only one fraction is detected before hydrolysis by chymotrypsin. The analysis of the mass spectrum (Fig. 4) showed the presence of multi-charged species corresponding to a single mass of 14,183 Da, being the mass of the alpha-lactalbumin. This clearly indicates that in absence of the enzyme, only intact alpha-lactalbumin is present in the buffer.

After 30 min of chymotryptic hydrolysis, the hydrolysate of alpha-lactalbumin fractions contained 11 to 21 matching peptides identified (Fig. 3b and Table 1). Among these fractions, three are pure and contained only one peptide (the fractions 1, 4, and 11 which contained the fragment α32–36 (molecular weight 564 Da), α31–50 (molecular weight 2,142 Da), and α61–104 peptide (molecular weight 5,074 Da) of alpha-lactalbumin, respectively). As presented in Table 1, six fractions contained each two peptides, among which two peptides were previously described in the literature as being biologically active: the α105–110 fragment of alpha-lactalbumin (in the fraction 2) was previously described by Philanto-Lepalla in 1998 [10] as an inhibitor of angiotensin-I-converting enzyme, and α54–60 fragment previously described as immuno-modulator by Polverino de Laureto et al. in

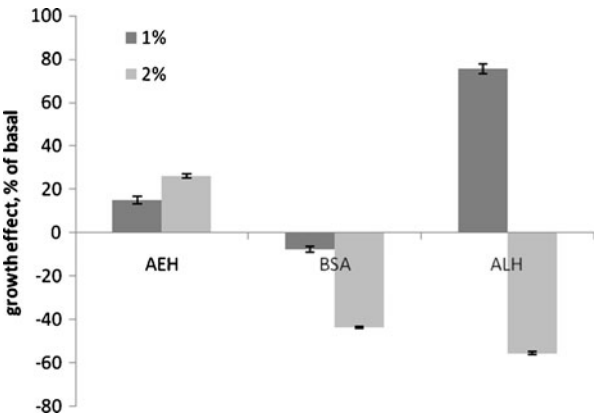

Fig. 2 Effect of AEH, BSA, and ALH tested at different concentrations (1% and 2% *w*/*v*) on STC-1 cells proliferation using a colorimetric assay (XTT based) after 48 h of incubation

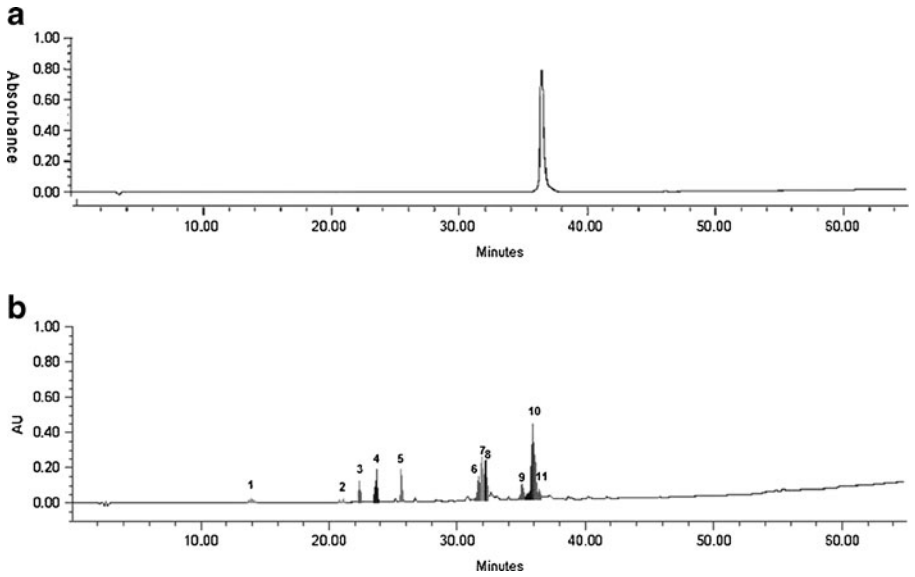

Fig. 3 **a** Chromatographic profile of alpha-lactalbumin obtained at time = 0 min, at 37 °C in phosphate buffer, by reverse-phase HPLC (absorbance at 215 nm). **b** Chromatographic profile of alpha-lactalbumin obtained at time = 30 min, at 37 °C in phosphate buffer, by reverse-phase HPLC (absorbance at 215 nm)

1999 [23]. The fractions 7 and 10 each contained three peptides. From these results, a mapping of the peptidic population was established (Fig. 5), allowing locating the cleavage sites of chymotrypsin. As shown on the mapping and as described in literature,

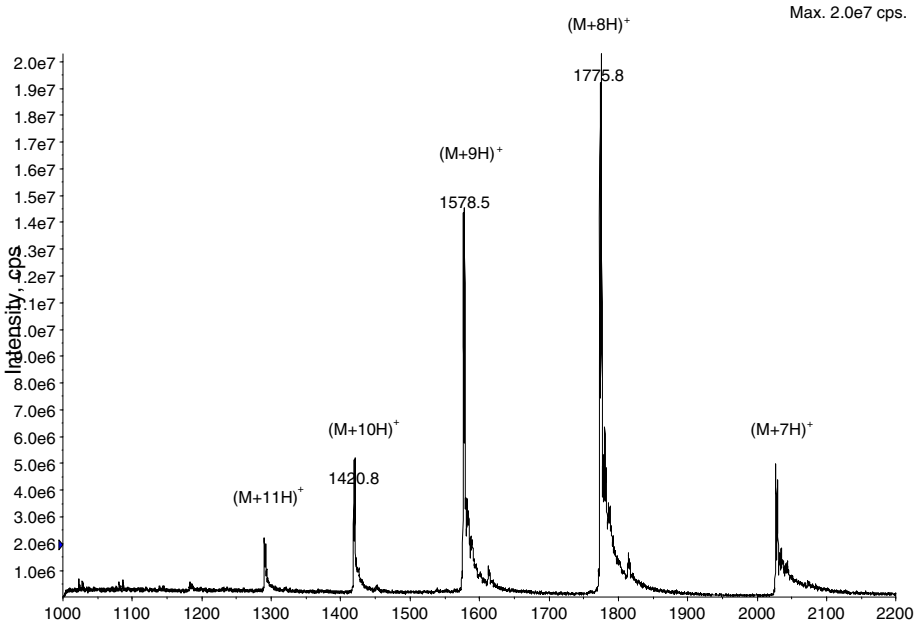

Fig. 4 ESI/MS spectrum of non hydrolyzed alpha-lactalbumin

Table 1 Masses and sequences of peptides obtained after 30 min of alpha-lactalbumin hydrolysis

Peptide fractions	Molecular weight (Da)	Peptide
1	564	32–36
2	652	105–110
	723	13–18
3	1,597	37–50
	1,121	10–18
4	2,142	32–50
5	914	54–60
	336	51–53
6	5,820	1–9/10–31/105–123
	5,186	1–9/10–31/111–123
7	4,717	1–9/19–31/105–123
	4,395	1–9/19–31/108–123
	4,083	1–9/19–31/111–123
8	5,800	1–31/105–123
	4,904	1–9/19–31/104–123
9	5,984	1–31/104–123
	4,883	61–103
10	14,183	1–123
	5,972	54–104
	5,778	54–103
11	5,074	61–104

chymotrypsin primary cleaves at the level of a tyrosine, a phenylalanine, or a tryptophan, and secondary at an histidine or a leucine [24], chymotrypsin cleaves the peptidic bonds after the amino acids mentioned. Polverino de Laureto et al. explained in 2002 that, because the conformation of a portion of apo-alpha-lactalbumin was in a state of molten globule (between residues 34 and 57), it was less structured, more flexible, and therefore more accessible to the enzyme [25]. They then showed that this region of alpha-lactalbumin was preferentially hydrolyzed by proteinase K or chymotrypsin.

The characterization of the active sample showed that the hydrolysis of alpha-lactalbumin by chymotrypsin under the conditions described, allowed getting 21 identified peptides which could have biological activity.

Discussion

In order to find appetite-suppressive molecules derived from alpha-lactalbumin proteins, their ability to stimulate CCK-releasing activity in the enteroendocrine STC-1 cell line was investigated. In this work, we demonstrated for the first time that chymotryptic hydrolysate of alpha-lactalbumin highly stimulated CCK secretion by STC-1 cells. The effect obtained with this enzymatic hydrolysate was compared to a commercial albumin egg hydrolysate, a commercial bovine albumin serum as positive controls [17], and the nonhydrolyzed corresponding protein. The chymotryptic hydrolysate from alpha-lactalbumin induced a 2.1-fold increase over the effect obtained with albumin hydrolysate from chicken egg or

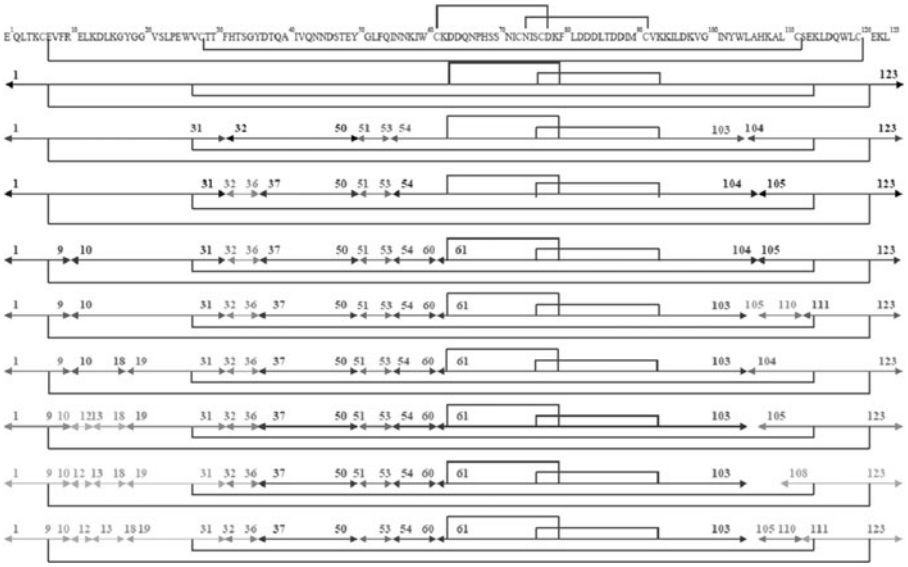

Fig. 5 Peptidic population representation after 30 min of chymotryptic hydrolysis of alpha-lactalbumin

with nonhydrolyzed alpha-lactalbumin. Toxicity of this hydrolysate was assayed and demonstrated that alpha-lactalbumin chymotryptic hydrolysate could highly stimulate cell proliferation at 1% *w*/*v*, or exert a significant inhibitory but nontoxic effect at 2%. The recent studies of Kim et al. [26] and Cudennec et al. [7] corroborated these results reporting that black soybean protein hydrolysate or fish protein hydrolysate, respectively, exerted a nontoxic inhibitory effect on cell growth in the same range as that obtained with alpha-lactalbumin hydrolysate.

Several studies showed that peptones from animal or vegetable origin were able to stimulate CCK release in STC-1 cells [15]. Thus, CCK-stimulating effects are mainly due to peptide molecules [15, 17]. The HPLC chromatogram obtained after 30 min of chymotryptic hydrolysis showed that alpha-lactalbumin hydrolysate contained different fractions probably being peptides according to the wavelength used for the detection. For further work it will be of interest to isolate from the mapping done, the biological molecules increasing CCK production by STC-1 cells and then, to assess the satietogenic activity in an in vivo test.

Conclusion

In summary, we demonstrated for the first time that chymotryptic hydrolysate of alpha-lactalbumin was able to highly stimulate CCK secretion in intestinal endocrine STC-1 cells and that this hydrolysate contained 11 fractions containing 21 isolated peptides. Future work will focus on isolation and identification of the biological molecules responsible for the satietogen activity. Thus, the satietogenic activity should now be assessed in vivo. If ALH is proposed as a potential functional food, it cannot be excluded (for example, other biases exist) that the fragments of alpha-lactalbumin stimulating CCK release are hydrolyzed into inactive fragments by pepsin in stomach or other proteolytic enzymes

than chymotrypsin in intestine. From an upgrading point of view, an industrial scale fraction exhibiting an activity favoring CCK production in vivo after ingestion would be of great interest in domains such as animal feeding or nutraceuticals.

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