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Screening of soy and milk protein hydrolysates for their ability to activate the CCK1 receptor

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Abstract

The cholecystokinin receptor-type 1 (CCK1R) is a G-protein coupled receptor localized in the animal gastrointestinal tract. Receptor activation by the natural peptide ligand CCK leads to a feeling of satiety. In this study, hydrolysates from soy and milk proteins were evaluated for their potential to activate CCK1R, assuming that bioactive peptides with a satiogenic effect can be used as an effective therapeutic strategy for obesity. Different protein hydrolysates were screened with a cell-based bioassay, which relies on the generation of a fluorescent signal upon receptor activation. Fluorescence was monitored using a fluorescence plate reader and confocal microscopy. Results from the fluorescence plate reader were biased by background autofluorescence of the protein hydrolysate matrices, which makes the fluorescence plate reader inappropriate for the evaluation of complex formulations. Measurements with the confocal microscope resulted in reliable and specific results. The latter approach showed that the gastrointestinal digested 7S fraction of soy protein demonstrates CCK1R activity.

Keywords: cholecystokinin receptor, lorglumide, soy, casein, protein hydrolysates, bioactive peptides

1. INTRODUCTION

The cholecystokinin receptor-type 1 (CCK1R) is a G-protein coupled receptor, which is expressed in different cell types of the animal gastrointestinal tract, including vagal afferent cells and muscle cells of the intestines and stomach [2,3,15]. Activation of this receptor by the natural peptide ligand cholecystokinin (CCK) can induce a feeling of satiety [12]. There are indications that bioactive peptides from food protein evoke a similar effect [4] and could therefore be used as an ingredient for functional foods with a satiogenic effect. This has relevance for the treatment and prevention of obesity [1,19].

Previously, we described a cell-based bioassay to assess the CCK1R-activating potential of pure compounds [20]. The technique is based on the fluorescence measurement of a CCK1R-induced intracellular calcium increase with a plate reader and results were validated using confocal microscopy. The natural ligand CCK8S and a partial agonist JMV-180 were used to activate the CCK1R signaling cascade. We now evaluated the performance of this assay to screen more complex formulations such as protein hydrolysates for their ability to activate the CCK1R. The potential of the fluorescence plate reader for the screening of such complex matrices was investigated and compared with the confocal microscope. We focused on soy and milk proteins, well-known and convenient food proteins of which beneficial health effects have been proven [6,9,10,13], but which differ significantly in source and structure.

2. MATERIALS AND METHODS

2.1 *Cell lines and chemicals*

CHO (Chinese Hamster Ovary) cells functionally expressing the rat CCK1R (CHO-CCK1R) were established by Prof. Peter Willems [18] and native CHO-K1 cells were obtained from Prof. Georges Leclercq (Ghent University Hospital, Department of Clinic Biology, Microbiology and Immunology, Ghent, Belgium). Advanced Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) (DMEM-F12), fetal bovine serum (FBS), geneticin (G-

418 antibiotic), Fluo-4AM, Pluronic F-127 and Hank's buffered salt solution (HBSS) were purchased from Invitrogen (Paisley, UK), probenecid, lorglumide ((±)-4-[(3,4-dichlorobenzoyl)amino]-5-(dipentylamino)-5-oxopentanoic acid sodium salt; CR-1409), bovine serum albumin (BSA), HEPES, hexane, sodium bisulfite, α-lactalbumin, κ-casein, pepsin, trypsin and chymotrypsin from Sigma-Aldrich (Bornem, Belgium/St.-Louis, MO), sulfated cholecystokinin octapeptide (CCK8S) from Research Inc. (Barnegat, NJ), and clear black bottom 96-well plates from Greiner (Frickenhausen, Germany). Soybeans were purchased in the local grocery store (Heuschen & Schrouff OFT B.V., Canada). Enzymatic soy hydrolysates E110, AM41, A2SC and A3SC were obtained from Organotechnie (La Courneuve, France).

2.2 *Preparation of 7S fraction from soy protein*

The 7S fraction from soybean protein (β-conglycinin) was extracted following a method based on pH precipitation described by Liu et al. [11]. In brief, soybean seeds were ground with a coffee mill and defatted with hexane to obtain defatted soybean flour. The soybean flour was extracted twice with 0.03 M Tris-HCl (pH 8.5) for 1 h at 45°C in a ratio of 15% (w/v). Subsequently, sodium bisulfite was added to the extraction product to a concentration of 0.01 M. The solution was kept overnight at 4°C and centrifuged. Next, NaCl was added to a concentration of 0.25 M to the supernatants and the solution was centrifuged again. The pH of the obtained supernatants was adjusted to 4.8, which caused the 7S protein fraction to precipitate. The precipitate was dialyzed over pure water and subsequently freeze-dried.

2.3 *Hydrolysis simulating gastrointestinal peptic digestion*

An *in vitro* gastrointestinal digestion was performed on α-lactalbumin, κ-casein and the 7S soy proteins as described before [21]. Briefly, the lyophilized proteins were dissolved in distilled water (4% w/v), pH was lowered to 2 and pepsin was added in a ratio of 0.4% (w/w) to the sample. The solution was kept at 37°C and shaken for 2 h. Next, the pH was set to 6.5

and trypsin and chymotrypsin were added also in a ratio of 0.4% (w/w), and the solution was shaken for 2.5 h at 37°C. The reaction was terminated by heating the samples for 15 min to 80°C.

2.4 *Cell-based bioassay to screen for CCK1R activity*

The determination of the intracellular free Ca^{2+} concentration was performed as described before [20]. In brief, CHO-CCK1R and CHO-K1 cells, seeded in a 96-well plate, were loaded with a cell-permeant fluorescent dye Fluo-4AM. As receptor activation leads to a rapid increase in intracellular calcium concentration (within seconds), the fluorescence intensity was measured kinetically using two different setups: a fluorescence plate reader (Infinite Pro200; Tecan, Männedorf, Switzerland) and a confocal laser scanning microscopy system (Nikon A1r; Nikon Instruments Inc., Paris, France).

Samples were added *in fluxo*: after 6 s of acquisition, the protein hydrolysate sample or CCK8S was added instantaneously while the acquisition continued for another 34 s. For each sample concentration, measurements were repeated in five wells for both cell types (technical replicates) and every experiment was repeated 2-4 times (biological replicates). For inhibition experiments, lorglumide was added at a final concentration of 50 µM, 30 min prior to acquisition.

2.5 *Image analysis*

Confocal recordings were analyzed as previously described [20]. For population average measurements, the average fluorescence intensity (per pixel) was measured for the entire image using ImageJ freeware (National Institute of Health, Bethesda, MD). For single-cell analysis, all cells in an image were automatically segmented and assigned as regions of interest (ROI) of which the average intensities were analyzed using Matlab 7.10.0 (R2010a, The Mathworks Inc., Natick, MA).

2.6 *Data analysis and statistics*

Data analysis was performed in the same way for both measuring devices. Per time point i, fluorescence measurements (F_i) were normalized by dividing with the average fluorescence before sample addition (F_0). The average normalized fluorescence values (F_i/F_0) of 5 technical replicates of CHO-K1 were substracted from the average normalized fluorescence values obtained for CHO-CCK1R cells. Consequently, the relative fluorescence (RF) was calculated using the following formula: $RF_i = \frac{F_i(CHO-CCK1R)}{F_0(CHO-CCK1R)} - \frac{F_i(CHO-K1)}{F_0(CHO-K1)}$. Next, the net response (NR) was calculated as the total relative fluorescence from the moment of sample addition to end of the acquisition: $NR = \sum_{i=6s}^{40s} RF_i$. This net response was expressed as a percentage (mean ± SEM) of the net response induced by 1 nM CCK8S. Statistical analyses were performed using one-sample Student's *t*-tests, two-sample Student's *t*-test or non-parametric Wilcoxon rank sum tests. Differences between net responses were considered significant for p-values smaller than 0.05. Sigmoid dose-response curves were generated for the net response versus sample concentration relationships and the EC_{50} value (the sample concentration at which 50% of the maximum response was reached) was calculated using Prism v4 software (GraphPad Prism, La Jolla, CA) [24].

Specifically for confocal microscopy, the robustness of the single cell analysis was determined using an *in silico* approach, which calculates the minimal numbers of cells to determine the net response accurately [20]. To this end, selected image data sets containing all individual cell responses were progressively eroded by omitting one single, randomly selected cell at a time, down to one single cell, and by calculating the average response with each step. This was repeated 100 times on permutated data sets to obtain a distribution that represents the variability within the data set. From these distributions, the coefficient of variation (COV= standard deviation/mean) was derived and plotted against the number of cells.

3. RESULTS

3.1 *The fluorescence plate reader fails to give accurate results for complex matrices of protein hydrolysates*

First, four commercial crude soy hydrolysates E110, AM41, A2SC and A3SC were tested for their potential to induce a CCK1R-mediated calcium flux. Measurements were performed with the fluorescence plate reader since this setup proved to be a fast and reliable tool for pure compounds [20]. A net response was measured for these hydrolysates between 90 and 300%. However, there was a high variability (25 to 40%) and the fluorescence profiles showed atypical kinetics. Upon addition of the protein hydrolysates an immediate (within 0.3 s) increase in fluorescence was observed. The increase was 5 to 7 times higher than that observed for CCK8S and this was the case both for CHO-CCK1R and CHO-K1 cells (Fig. 1a). Moreover the fluorescence level remained stable throughout the acquisition period, irrespective of the administered dose (data not shown). For comparison, addition of CCK8S resulted in an exponential increase of the fluorescence signal in CHO-CCK1R cells with a delay of minimum 3 s after sample addition and no significant signal increase in CHO-K1 cells. This suggested that the crude protein hydrolysates induced a non-specific rise in fluorescence, possibly due to the presence of an autofluorescent component.

Since it was difficult to pinpoint the exact origin of the strong (auto-)fluorescence in the complex hydrolysates, we decided to measure the fluorescence kinetics in hydrolysates of purified soy and milk proteins. The gastrointestinal digested 7S fraction from soy protein (1 g/l), gastrointestinal digested κ-casein from milk (3 g/l) and gastrointestinal digested α-lactalbumin (3 g/l) from milk were evaluated. The fluorescence profiles of these hydrolysates demonstrated more reliable kinetics than those of the crude hydrolysates: the fluorescence increase was lower than that induced by 1 nM CCK8S, reached a maximum response after several seconds and gradually faded (Fig. 1b). In addition, the fluorescence increase of CHO-

CCK1R was significantly higher than that of CHO-K1 cells. For the 7S soy hydrolysate, a net response of 13.4±3.0% (n=5, p=0.01) was measured, which was significantly different from zero. Net responses were obtained of 7.7±1.4% (n=2, p=0.11) for the κ-casein hydrolysate and 19.9±4.2% (n=2, p=0.13) for the α-lactalbumin hydrolysate, but these were not significantly different from zero.

3.2 *Confocal microscopy allows to measure CCK1R activation accurately, excluding false positives*

3.2.1 Only the 7S soy hydrolysate shows a significant net response

In our previous work with pure compounds [20], we noted that it was important to validate the data from the plate reader with a confocal microscope, especially in a context of strong autofluorescence background. In addition, especially for crude hydrolysates, highly variable results were obtained with the plate reader, as reported above. Therefore, the experiments with the soy and milk protein hydrolysates were repeated using confocal microscopy and the population-average response was calculated. To this end, the average pixel intensity of an image comprising 150 to 250 cells was measured per well. Interestingly, the high responses found with the fluorescence plate reader for the crude soy hydrolysates were not reproduced with the confocal microscope. Compared to the CCK8S-induced response, a delayed and limited increase in fluorescence was observed (Fig. 2a). CHO-CCK1R cells and CHO-K1 cells reacted alike, resulting in a low net response for all hydrolysates. The net responses for soy hydrolysate E110, AM41, A2SC and A3SC were not higher than 7.7% and none were significantly different from zero. Taken together, these results showed that the crude hydrolysates failed to induce a CCK1R-mediated Ca^{2+}-flux.

As for the hydrolysates from purified proteins, confocal analysis of the 7S soy hydrolysate (1 g/l) showed a significant net response of 14.3±1.8% (n=4). Moreover, the fluorescence profiles demonstrated reliable kinetics. An exponential increase was observed in

fluorescence signal, which was significantly higher for CHO-CCK1R than for CHO-K1 cells; it reached a maximum and subsequently decreased (Fig. 2b). For this hydrolysate, a dose-response curve was established (Fig. 3), from which an EC_{50} value was calculated of 0.069 mg/l (95% confidence interval: 0.028-0.170 mg/l; R^2=0.79).

For the milk κ-casein hydrolysate, a net response of 10.6±5.1% (n=2) was measured, but it was not significantly different from zero (p=0.28). The net response of the milk α-lactalbumin hydrolysate was negligible.

3.2.2 Single-cell variations for the 7S soy hydrolysate

To complement the aforementioned population-average approach and obtain a better insight in the actual single-cell response, we measured fluorescence kinetics in individual cells by means of automated image analysis. As documented before [20], CHO-CCK1R cells treated with 1 nM CCK8S showed a strong, highly synchronous fluorescence profile. This profile was characterized by a steep increase in fluorescence signal, reaching a maximum at 3 s after the sample addition and a subsequent gradual decrease. In contrast, CHO-K1 cells showed no significant response to the CCK8S stimulation, except for a sporadic non-specific fluctuation in calcium flux. When exposed to varying concentrations of the 7S soy hydrolysate, CHO-CCK1R cells demonstrated intensity fluctuations with a strong intercellular variability, both in magnitude and time point of the maximum fluorescence intensity. The time lag between the moment of sample addition and the moment of maximum fluorescence intensity became progressively shorter with increasing hydrolysate concentration. In parallel, the response of individual cells became more synchronous at higher concentrations. Notably, CHO-K1 cells also showed pronounced individual cell responses, which were significantly different from CCK8S-treated CHO-K1 cells for concentrations of 7S soy hydrolysate higher than 6.3E-02 g/l. These fluctuations were lower and less abundant than those observed in CHO-CCK1R cells, but much more pronounced than basal calcium fluctuations

(Supplementary Fig. 1). Despite the inherent variability and fluctuations in both cell types, all doses, except for 3.9E-03 g/l, induced a response in CHO-CCK1R cells, which was significantly different from the dose-matched CHO-K1 controls (Fig. 4a).

As a measure for the robustness of the single cell analysis, we determined the numbers of cells required to obtain an accurate estimate of the average net response on representative datasets of the 7S soy hydrolysate. When the coefficient of variation of the net response was plotted against the numbers of cells that were included (as described in the material and methods), a strong exponential decrease was observed for increasing numbers of cells (Fig. 4b). On average, across the entire dataset, 15±2 cells were required to determine the net response (±5%) with 95% confidence.

Finally, single cell analysis on image datasets of the soy hydrolysate E110 showed significant intensity fluctuations, which were equally abundant in CHO-CCK1R and CHO-K1 cells. Comparison with the fluorescence profiles of background regions (without cells) confirmed that the fluorescence kinetics as measured by the population-average method, were predominantly caused by non-specific calcium fluxes (Supplementary Fig. 2).

3.2.3 The effect of lorglumide on the 7S soy hydrolysate

To test the specificity of the CCK1R response elicited by the 7S soy hydrolysate (1 g/l), confocal microscopy experiments were repeated in the presence of 50 µM lorglumide, a known antagonist of CCK1R. The fluorescence profiles of lorglumide-treated CHO-CCK1R cells showed a significant decrease compared to the profiles of cells without lorglumide pretreatment. But surprisingly, this decrease was also observed with the CHO-CCK1R cells (Fig. 2b), and as a result, the net response showed no significant change between lorglumide-treated and non-treated cells.

4. DISCUSSION

In this study, we have screened raw soy hydrolysates and purified soy and milk proteins for

their potential to activate the CCK1R. First, crude protein hydrolysates were tested, which were commercially available. A primary screen was performed with a fluorescence plate reader. However, this device produced dubious results. Very high fluorescence responses were measured for the complex matrices, which mainly seemed to be attributed to background autofluorescence within the sample rather than to a CCK1R-induced Ca^{2+}-increase. Why CHO-CCK1R cells showed a higher non-specific fluorescence response than CHO-K1, when measured with the plate reader is still unclear. Theoretically, the subtraction of the relative fluorescence of the CHO-K1 cells from that of the CHO-CCK1R cells should correct for non-CCK1R-induced Ca^{2+} increases and sample autofluorescence. One possibility is the presence of technical inconsistencies such as non-linearity effects in the detection of strong fluorescence (saturation). Visual inspection of the complex hydrolysates with a widefield microscope confirmed that the formulations showed strong autofluorescence (data not shown). However, in confocal microscopy the excessive signal increases, caused by the autofluorescent components in the sample, were not detected. This is presumably a result of its optical sectioning capacity. By reducing the field depth to the bottom of the well, registration becomes mostly restricted to signals stemming from the cells. However, in the fluorescence plate reader, fluorescence is measured across the entire well, which may obscure more subtle fluorescence increases at the level of the cells due to autofluorescence of the sample. Indeed, single cell analysis of the crude E110 protein hydrolysate showed that there were cellular responses, even though they were non-specific and occurred both in CHO-CCK1R and CHO-K1 cells. Hence, we conclude that confocal microscopy allows for measuring a more specific response and testing higher sample concentrations when working with autofluorescent samples.

As for the hydrolysates from purified proteins, the 7S soy hydrolysate yielded a significant response with a fluorescence profile pointing to a real CCK1R activation. Single

cell fluctuations showed resemblance to those observed after administration of low concentrations of CCK8S or partial agonists such as JMV-180 [20], suggesting partial activation of the CCK1R. The EC_{50} value amounted to 66 mg/l which is in the same range as the EC_{50} value of a commercial soy protein hydrolysate (Quest International, Naarden, the Netherlands) described by Foltz et al. [4]. Unfortunately there were some confounding factors, which complicate the interpretation of the data. First, the 7S soy hydrolysate was not completely specific since it induced a less pronounced but significant response in CHO-K1 cells. Activation of CHO-K1 cells was also observed for other hydrolysates, albeit to a variable extent, e.g. for soy hydrolysate E110. This is in contrast to the results of Foltz et al. who reported no activation of CHO-K1 cells upon hydrolysate treatment [4]. Our results suggest that not only CCK1R but also other receptors might become activated, which induce an intracellular calcium flux. In fact, many receptors (other GPCRs, receptor tyrosine kinase-type receptors…) use a similar signaling machinery involving Ca^{2+} as a second messenger [7,8,16,17]. Activation of these receptors may complicate downstream effects and obscure the envisioned cellular outcome. A second complication was discovered after lorglumide treatment. While a decrease was observed in the fluorescence kinetics of CHO-CCK1R cells, pointing to a specific CCK1R activation, we also found an unexpected decrease in CHO-K1 cells. This indicates that lorglumide might also inhibit other receptors, as was previously suggested by Gaudreau et al. for opioid receptors [5].

Interestingly, it has been reported that hydrolysates and peptides of the 7S fraction of soy protein are also capable of releasing CCK from STC-1 cells and reducing appetite in rats [14,22,23]. Therefore it might be possible that the appetite-suppressing effect of the 7S soy hydrolysates described in the latter studies have a double mode of action, i.e. stimulation of the release of CCK on the one hand and direct activation of the CCK1R on the other hand. As such, hydrolysates from the 7S fraction from soy protein form a promising candidate

ingredient for functional foods as a helpful tool for weight control.

In conclusion, the fluorescence plate reader seems less suited to measure complex formulations and therefore can only be used to perform a rough primary screen. Confocal microscopy is crucial to exclude false positive and to distinguish specific from non-specific effects. Moreover, an equally high accuracy was obtained with pure compounds [20], indicating that the confocal microscope is as reliable for measuring complex formulations as it is for pure compounds. Using confocal microscopy, we discovered that the gastrointestinal digested 7S fraction from soy protein contains CCK1R activity. We also have indications that CCK1R-activating bioactive peptides might be released from κ-casein in milk, since substantial activity was seen, although not significant at the tested concentrations in the current experiments.

Further research is needed to increase the *in vitro* net responses of the protein hydrolysates, which includes optimization of hydrolysis and purification of the active fractions and peptides. Finally, validation of the effect of the active components in an *in vivo* model is imperative. To our knowledge, this study is the first in which the effect of non-pharmaceutical CCK1R agonists, i.e. food protein hydrolysates, is analyzed in detail at the cellular level. This knowledge may facilitate the screening and discovery of novel products with CCK1R activity, thereby contributing to the battle against obesity.

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FIGURE LEGENDS

Fig. 1. Normalized fluorescence kinetics (F_i/F_0) for CHO-CCK1R and CHO-K1 in cell populations monitored with a plate reader. Results for sample as well as for 1 nM are shown. The curves represent the mean of 5 technical replicates (wells). (a) Representative curve for soy hydrolysate E110 (3 g/l). (b) Representative curve for 7S soy hydrolysate (1 g/l).

Fig. 2. Normalized fluorescence kinetics (F_i/F_0) for CHO-CCK1R and CHO-K1 in cell populations monitored with a resonant scanning confocal microscope. Results for sample as well as for 1 nM are shown. The curves represent the mean of 5 technical replicates. (a) Representative curves for soy hydrolysate E110 (3 g/l). (b) Representative curves for 7S soy hydrolysate (1 g/l). Dotted lines represent the fluorescence kinetics of the experiments performed in the presence of lorglumide.

Fig. 3. Dose-dependent CCK1R-mediated calcium fluxes in cell populations monitored with a resonant scanning confocal microscope. (a) Representative relative fluorescence kinetics (RF) of increasing concentrations of 7S soy hydrolysate (0.0039-1 g/l). The curves represent the mean of 5 technical replicates. (b) Dose-response curve for 7S soy hydrolysate based on 2 experiments (biological replicates) in which the measurements for each concentration were repeated 5 times, expressed as a percentage of the maximum net response, i.e. the net response induced by 1 nM CCK8S.

Fig. 4. Dose-dependent calcium-mediated fluorescence fluxes in individual cells monitored with a resonant scanning confocal microscope. (a) Boxplot representing the single-cell response of CHO-CCK1R and CHO-K1 cells to increasing concentrations of the 7S soy hydrolysate (0.0039-1 g/l), measured as the average intensity of individual cells and expressed as a percentage of the maximum response, i.e., the net response induced by 1 nM CCK8S. The boxplots are based on the results of five technical replicates. Stars indicate significant differences from the dose-matched CHO-K1 control (3.9E-03 g/l: p= 9.1E-01, 1.6E-02 g/l:

p=1.8E-02, 6.3E-02 g/l: p=1.0E-02, 2.5E-01 g/l p=3.8E-02, 1.0 g/l p=4.8E-05). (b) *In silico* erosion. Per condition, the response was calculated for all cells within one representative image per concentration of 7S soy hydrolysate. Next, cells were progressively removed from permutated data sets, one by one, down to the single cell and per step the average net response was calculated. This was repeated 100 times per condition. Every line represents one complete erosion cycle. (c) From the eroded data sets the coefficient of variation (COV) was calculated and plotted as a function of the number of cells. The inset shows the same plot represented on a logarithmic scale to facilitate discrimination of the exponential part of the plot (cell numbers <20).

Supplementary material

Supplementary Fig. 1. Inter-individual and temporal variation of normalized fluorescence kinetics (F_i/F_0) in response to varying doses of the 7S soy hydrolysate and 1 nM CCK8S, monitored in CHO-CCK1R and CHO-K1 cells with a resonant scanning confocal microscope. Every plot displays the kinetic fluorescence profiles of all cells of one arbitrarily selected recording.

Supplementary Fig. 2. Normalized fluorescence kinetics (F_i/F_0) in response to 3 g/l of the soy hydrolysate E110, monitored in CHO-CCK1R (a) and CHO-K1 (b) cells with a resonant scanning confocal microscope. Kinetic curves are displayed for one representative recording of each cell type. The left plot shows the average signal of the whole image, the middle plot shows the kinetics of the average signal per cell for all cells and the right plot displays the average signal of an image region where no cells are present (background). The relative stability of the background signal and the pronounced intensity fluctuations of individual cells confirm that the measured response across the whole image can mostly be attributed to a cellular component (calcium fluxes).