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# Evaluation of *in silico* approach for prediction of presence of opioid peptides in wheat

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ABSTRACT

Opioid like morphine and codeine are used for the management of pain, but are associated with serious side-effects limiting their use. Wheat gluten proteins were assessed for the presence of opioid peptides on the basis of tyrosine and proline within their sequence. Eleven peptides were identified and occurrence of predicted sequences or their structural motifs were analysed using BIOPEP database and ranked using PeptideRanker. Based on higher peptide ranking, three sequences YPG, YYPG and YIPP were selected for determination of opioid activity by cAMP assay against μ and κ opioid receptors. Three peptides inhibited the production of cAMP to varied degree with $EC_{50}$ values of YPG, YYPG and YIPP were 5.3 mM, 1.5 mM and 2.9 mM for μ-opioid receptor, and 1.9 mM, 1.2 mM and 3.2 mM for κ-opioid receptor, respectively. The study showed that *in silico* approach can be used for the prediction of opioid peptides from gluten.

## 1. Introduction

Opioids, such as morphine and codeine, are the most common clinically used drugs for pain management (Janecka, Fichna, & Janecki, 2004; Teschemacher, 2003; Trescot, Datta, Lee, & Hansen, 2008). These opioids bind to opioid receptors present in the central and peripheral nervous system. However, they are often associated with side-effects like sedation, dizziness, nausea, vomiting, constipation, addiction, tolerance and respiratory depression (Benyamin et al., 2008). Opioids were considered to be alkaloid (derived from opium) only until discovery of endogenous opioid peptides in 1975 (Goldstein, Goldstein, & Cox, 1975; Hughes et al., 1975). These endogenous peptides and their modified forms have shown activity similar to alkaloids (Giordano et al., 2010; Mollica et al., 2014; Mollica et al., 2005; Mollica et al., 2013b; Mollica et al., 2011). However, exogenous opioid peptides or exorphins are naturally derived from food proteins (Stefanucci et al., 2016; Yoshikawa, 2013). These exogenous peptides are of particular interest as they are naturally derived from food, have possibly less side-effects (compared to synthetic drugs) and are inexpensive to produce (Garg, Nurgali, & Mishra, 2016; Udenigwe, Gong, & Wu, 2013). Most known bioactive peptides are small and non-immunogenic, as compared to larger peptides (6–25 amino acids) (Wang, Mejia, & Gonzalez, 2005). Hence, small peptides are researched more for their bioactivity and considered safe (Shahidi & Zhong, 2008).

Generally, bioactive peptides, including opioids, are produced by hydrolysis of food proteins during food processing (ripening, fermentation), storage (Choi, Sabikhi, Hassan, & Anand, 2012) and during gastrointestinal (GI) digestion (Garg et al., 2016; Stefanucci et al., 2016). The protein hydrolysate is then tested for bioactivity using *in vitro* and *in vivo* methods. Since these hydrolysates are mixtures of several peptides, their bioactivity results from the additive and synergistic effect of various components present. Bioactive hydrolysates containing mixture of peptides needs to be fractionated, purified and then tested for bioactivity (Udenigwe & Aluko, 2012). The whole process of preparing bioactive peptides from native proteins is tedious, time consuming and the peptide yields are low (Udenigwe, 2014), limiting and/or delaying their use in clinical applications.

Alternatively, bioinformatics tools can be used for predicting the presence of bioactive peptides in proteins (*in silico* approach) (Carrasco-Castilla, Hernández-Álvarez, Jiménez-Martínez, Gutiérrez-López, & Dávila-Ortiz, 2012; Holton, Pollastri, Shields, & Mooney, 2013; Lacroix & Li-Chan, 2012). Using this approach, one can search for potential precursors of bioactive peptides and select efficient proteolytic enzymes for their release from native protein sequences (Carrasco-Castilla et al., 2012; Udenigwe et al., 2013). In this approach, protein databases, such as, UniProtKB, SwissProt and TrEMBL can be used to access sequences of a food protein, and presence of bioactive peptides can be predicted using peptide databases BIOPEP and Pepbank (Udenigwe, 2014). The BIOPEP application contains a database of biologically active peptide sequences and a program enabling construction of profiles of the

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potential biological activity of protein fragments, calculation of quantitative descriptors as measures of the value of proteins as potential precursors of bioactive peptides, and prediction of bonds susceptible to hydrolysis by endopeptidases in a protein (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008). In fact, it has been successfully used for prediction of bioactive peptides from different food proteins having angiotensin converting enzyme inhibitory (ACE-I) activity (Cheung, Nakayama, Hsu, Samaranayaka, & Li-Chan, 2009; Dellafiora et al., 2015) and dipeptidyl peptidase-IV inhibitors (DPP-IV) (Lacroix & Li-Chan, 2012; Nongonierma, Mooney, Shields, & FitzGerald, 2014). PeptideRanker is a web based application and can predict the probability of a peptide being bioactive according to their score between 0 and 1 and can assist in the discovery of new bioactive peptides across many functional classes. Generally, any peptide over 0.5 threshold is labelled to be bioactive (Mooney, Haslam, Holton, Pollastri, & Shields, 2013; Mooney, Haslam, Pollastri, & Shields, 2012). Increasing the threshold from 0.5 to 0.8 reduces the number of false positive prediction from 16% to 6%, however, true positive rates also decrease (Mooney et al., 2012). If predicted probability is close to 1, the probability of peptide to be bioactive is significantly high (Mooney et al., 2012).

Bioinformatics approach is used for identification of structural patterns of peptides of known bioactivities. Presence of tryptophan in a peptide is associated with antioxidant activity (Chuan-Hsiao, Yin-Shiou, Shyr-Yi, & Wen-Chi, 2014) and carboxyl terminal alanine or proline containing peptides are DPP-IV inhibitors (Lacroix & Li-Chan, 2012). However, there is general lack of information for screening opioid peptides using bioinformatics approach. Wheat gluten contains exorphins; A5, A4, B5, B4 and C, having sequences GYYPT, GYYP, YGGW, YGGWL and YPISL, respectively (Fukudome & Yoshikawa, 1992; Zioudrou, Streaty, & Klee, 1979). Most of food derived opioid peptides have tyrosine and proline residues within them (Yoshikawa, 2013). Tyrosine (Y) is present either at the amino terminal or at the second position (as in gluten exorphins GYYPT and GYYP) and acts as part of the message domain to anchor the opioid peptide within the receptor (Heyl et al., 2003; Li et al., 2005). At position 1, Y acts as a dual hydrogen bond donor/acceptor with less acidic hydroxyl groups exhibiting stronger binding to opioid receptors. Moreover, steric bulk in the Y strengthens receptor binding by either a ligand conformational effect or enhanced van der Waals interactions with a loose receptor site (Heyl et al., 2003). Proline (P) acts as a spacer that fixes the peptide shape and induces other residues to assume proper spatial orientation for interacting with the opioid receptor (Cardillo, Gentilucci, Qasem, Sgarzi, & Spampinato, 2002). Peptides containing P also exhibit enhanced resistance to hydrolysis by enzymes of GI tract (Cardillo et al., 2002; Trivedi et al., 2014) and are therefore more likely to be active upon oral administration (Yang et al., 2001).

For peptides to exert opioid activity, they must bind to opioid receptors present within the central and enteric nervous systems. Opioid receptors belong to the superfamily of G protein coupled receptors (GPCRs) and on activation by opioid ligands, they inhibit adenylate cyclase enzyme (Garg et al., 2016; Gupta, Décaillot, & Devi, 2006) thus decreasing the production of cyclic adenosine monophosphate (cAMP) in the cells (Gupta et al., 2006). This decrease in concentration of cAMP in cells is used for screening opioid ligands (Huang, Kehner, Cowan, & Liu-Chen, 2001). This forms the basis of using cell lines transfected with opioid receptors for assaying the activity of peptides and using it for confirmation of peptides selected using bioinformatics approach.

The objective of this study was to search for opioid peptides in wheat gluten proteins based on the presence of tyrosine and proline, and use bioinformatics tools, BIOPEP and PeptideRanker to identity and rank these peptides for likelihood of having opioid activity. The identified peptides were then assayed for opioid activity by cAMP assay for confirmation of their bioactivity.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Cell culture media, Dulbecco's Modified Eagle's Medium (DMEM) containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), fetal bovine serum (FBS), antibiotic–antimycotic (100X) and hygromycin-B and phosphate buffer saline (PBS) – pH 7.2, were acquired from Life Technologies (Carlsbad, California, US). Lance cAMP detection reagents, and bovine serum albumin (BSA) stabiliser and optiplate were from Perkin Elmer Life Sciences (Cambridge, MA). 3-Isobutyl-1-methylxanthine (IBMX), trypsin-EDTA, forskolin, [D-Ala$^2$, N-MePhe$^4$, Gly-ol$^5$]-enkephalin (DAMGO) and dynorphin A and all other chemicals were purchased from Sigma Aldrich (Australia) unless otherwise stated. Custom peptides were synthesized from Mimotopes (Melbourne, Australia) at > 95% purity.

### 2.2. Cell lines

FlpIn CHO (Chinese hamster ovary) cells stably transfected with pOG44 vector encoding Flp recombinase and pDEST vector encoding either human μ or κ receptors were a kind gift from Dr. Meritxell Canals, Monash Institute of Pharmaceutical Sciences, Melbourne, Australia. The cells were transfected using polyethylenimine as transfection reagent and hygromycin-B (200 μg/mL) was used as a selection agent (Burford et al., 2015).

### 2.3. In silico analysis

#### 2.3.1. Sequences of wheat gluten proteins

The sequences of wheat storage proteins high molecular weight (HMW) and low molecular weight (LMW) glutenins and gliadins (alpha, gamma and omega) were accessed from UniProt database at http://www.uniprot.org/uniprot/ (Boutet, Lieberherr, Tognolli, Schneider, & Bairoch, 2007). These sequences were then searched for the presence of tri and oligo-peptides containing Y and P amino acids either consecutively or separated by a single amino acid.

#### 2.3.2. Peptide ranking and bioactivity prediction

Occurrence of predicted sequences or their structural motif thereof were analysed in known opioids using BIOPEP database (Minkiewicz et al., 2008). The PeptideRanker (Bioware.ucd.ie) was used to rank the predicted sequences according to bioactivity. A peptide having ranking closer to 1, increases its chances to be bioactive so they were selected to be tested for opioid activity.

### 2.4. Opioid activity assay

Opioid activity of the peptides was determined on the basis of cAMP assay. Cells were grown and maintained at 37 °C in a humidified incubator containing 5% $CO_2$ in DMEM, 10% FBS, 1% antibiotic–antimycotic and 200 μg/mL hygromycin-B (Burford et al., 2015). Cells were grown to 90% confluency, harvested and resuspended at $2 \times 10^6$ cells/mL in the media (DMEM + FBS + hygromycin B) and 100 μL of cells were seeded into sterile 96 well plates and incubated at 37 °C and 5% $CO_2$ overnight. The culture media in all the wells were replaced with stimulation buffer consisting of PBS, 50 mM IBMX and BSA stabiliser and incubated for 30 min before stimulation. Cells were stimulated at different concentrations of peptides in the presence of 10 μM forskolin for 30 min. The stimulation buffer containing peptides was then removed and 50 μL of ice cold 100% ethanol was added to each well. Ethanol was then evaporated and 75 μL of lysis buffer (0.3% tween-20, 5 mM HEPES and 0.1% BSA) was added to each well and the change in concentration of cAMP in the lysate was determined using Lance cAMP detection kit. 5 μL of lysate containing cAMP was mixed with 5 μL of Alexa flour-647 anti-cAMP antibody (stock antibody