Food Chemistry 141 (2013) 644–653

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Inhibition of dipeptidyl peptidase IV and xanthine oxidase by amino acids and dipeptides

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ARTICLE INFO

Article history:
Received 4 December 2012
Received in revised form 11 January 2013
Accepted 26 February 2013
Available online 7 March 2013

Keywords:
Dipeptidyl peptidase IV inhibitors
Xanthine oxidase inhibitors
Amino acids
Dipeptides
Milk
Predictive modelling
AutoDock Vina
Intestinal stability

ABSTRACT

Xanthine oxidase (XO) and dipeptidyl peptidase IV (DPP-IV) inhibition by amino acids and dipeptides was studied. Trp and Trp-containing dipeptides (Arg-Trp, Trp-Val, Val-Trp, Lys-Trp and Ile-Trp) inhibited XO. Three amino acids (Met, Leu and Trp) and eight dipeptides (Phe-Leu, Trp-Val, His-Leu, Glu-Lys, Ala-Leu, Val-Ala, Ser-Leu and Gly-Leu) inhibited DPP-IV. Trp and Trp-Val were multifunctional inhibitors of XO and DPP-IV. Lineweaver and Burk analysis showed that Trp was a non-competitive inhibitor of XO and a competitive inhibitor of DPP-IV. Molecular docking with Autodock Vina was used to better understand the interaction of the peptides with the active site of the enzyme. Because of the non-competitive inhibition observed, docking of Trp-Val to the secondary binding sites of XO and DPP-IV is required. Trp-Val was predicted to be intestinally neutral (between 25% and 75% peptide remaining after 60 min simulated intestinal digestion). These results are of significance for the reduction of reactive oxygen species (ROS) and the increase of the half-life of incretins by food-derived peptides.

1. Introduction

Various potential health promoting and disease risk reducing activities for milk proteins and milk-derived peptides have been reported (FitzGerald & Meisel, 2003; Pihlanto, 2006). Insulin secretion from pancreatic beta cells in the presence of glucose is influenced by incretins such as glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1). Enzymatic degradation of these hormones by dipeptidyl peptidase IV (DDP-IV) activity can significantly reduce their level *in vivo* (Guasch et al., 2012). It has been reported that peptides with 2–8 amino acid containing hydrophobic amino acid residues, including Pro residues, can inhibit DPP-IV. It has also been shown that milk derived peptides can inhibit DPP-IV activity (Lacroix & Li-Chan, 2012a, 2012b; Nongonierma & FitzGerald, 2013). Food protein derived DDP-IV inhibitory peptides may play a role in type 2 diabetes management by increasing the half-life of GIP and GLP-1 (Lacroix & Li-Chan, 2012a).

Cardiovascular and renal disease are complications associated with type 2 diabetes and insulin resistance syndrome. A link between hyperuricemia, the development of atherosclerosis, hypertension and insulin resistance has been proposed (Hayden & Tyagi, 2001). In type 2 diabetes patients, the enzyme-based antioxidant systems involving superoxide dismutase, glutathione peroxidase and catalase may be depleted arising from increased oxidative stress (Hayden & Tyagi, 2001). An increase in the concentration of reactive oxygen species (ROS) in endothelial cells can result in endothelial injury. Supplementation of anti-oxidants through dietary intake has been proposed as a means to counteract oxidative stress. The *in vitro* anti-oxidative properties of milk proteins and peptides have been reviewed (Pihlanto, 2006; Power, Jakeman, & FitzGerald, 2012). An increase in the concentration of ROS can occur during the oxidation of xanthine to uric acid catalysed by xanthine oxidase (XO). It has recently been shown that Trp-containing dipeptides inhibit XO activity (Nongonierma & FitzGerald, 2012).

Quantitative structure activity relationship (QSAR) and molecular docking approaches have been used to predict peptide binding to proteins (Pripp, 2007). While molecular docking strategies have been widely used as virtual screening tools for the development of active substances in the pharmaceutical sector, there appears to be limited use of this approach in the study of dietary compounds

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http://dx.doi.org/10.1016/j.foodchem.2013.02.115

(Pripp, 2007). Some studies have used docking strategies to identify new peptide sequences with angiotensin converting enzyme (ACE) inhibitory properties or to better understand the interaction of peptides with ACE (Norris, Casey, FitzGerald, Shields, & Mooney, 2012; Pripp, 2007). The peptide docking strategy developed by Norris et al. (2012) led to the discovery of two new dipeptide sequences (Asp-Trp and Trp-Pro) with potent *in vitro* ACE inhibitory properties. A good agreement between Vina score and IC_{50} value was found for some dipeptides.

No previous docking studies of peptides with XO have been conducted to our knowledge. Molecular docking of various flavonoids with XO has been carried out (Umamaheswari et al., 2012). Various potent DPP-IV inhibitors originating from natural products have recently been identified using a virtual screening procedure. A further increase in the DPP-IV inhibitory properties was achieved using derivatives of the most potent inhibitor (Guasch et al., 2012). In a recent study, amaranth derived peptides with $\geqslant$13 amino acid residues have been docked with DPP-IV. The mechanism of inhibition of these relatively large peptides has been shown to involve blockage of the active DPP-IV dimer formation (Velarde-Salcedo et al., 2013).

Given the link between diabetes and elevated ROS, it was decided to study the role of amino acids and dipeptides in controlling the activity of DPP-IV and XO. The aim of this study was therefore to determine the potential of amino acids and dipeptides to act as natural XO and DPP-IV inhibitors. Protein–ligand docking (LIGPLOTs) and virtual screening with Autodock Vina were used to assess the XO and DPP-IV inhibitory properties of amino acids and dipeptides. Furthermore, the type of XO and DPP-IV inhibition mediated by the amino acids and dipeptides was determined using Lineweaver and Burk kinetic analysis.

2. Materials and methods

2.1. Reagents

Porcine DPP-IV ($\geqslant$10 units/mg protein), Gly-Pro-para-nitroaniline (pNA), sodium phosphate monobasic, sodium phosphate dibasic, tris(hydroxymethyl)aminomethane (TRIS), ethylenediamine tretracetic acid (EDTA), hydroxylamine phosphate, xanthine, Allopurinol and bovine XO (0.1–0.4 units/mg protein) were obtained from Sigma Aldrich (Dublin, Ireland). The amino acids and synthetic peptides Trp, Met, Ala, Val, Cys, Leu, His, Ile, Arg, Thr, Glu, Tyr, Asp, Asn, Phe-Leu, His-Leu, Gly-Gln and Ile-Pro-Ile (Diprotin A) were obtained from Sigma-Aldrich, while Arg-Trp, Lys-Trp, Trp-Val, Val-Trp, Ile-Trp, Ser-Leu, Ala-Leu, Asp-Lys, Val-Ala, Glu-Lys, Gly-Leu and Ser-Phe were from Bachem (Bubendorf, Switzerland). Pro was from Merck (Darmstadt, Germany), Phe, Glu and Ser were from Sdn BHD (Pandamaran, Klang, Malaysia), Gly was from Fisher Scientific (Dublin, Ireland) and Lys was from SAFC Biosciences (Kansas, USA).

2.2. XO inhibition assay

The test samples (amino acids and dipeptides) were dispersed in HPLC grade water at concentrations ranging from 0.001 to 2.0 mg ml^{-1}. The XO inhibition assay was carried out as essentially described by Nongonierma and FitzGerald (2012). Briefly, test samples (50 μl) were pipetted onto a 96 well microplate (Sarstedt, Dublin, Ireland) containing EDTA (final concentration 12.5 μM), hydroxylamine phosphate (final concentration 25 μM) and xanthine (final concentration 0.125 mM). The reaction was initiated by adding 50 μl of XO (0.1 U ml^{-1}). Each sample was analysed in quadruplicate. The microplate was incubated at 37 °C for 30 min in a microplate reader (Biotek Synergy HT, Winoosky, VT, USA), absorbance of the uric acid formed was monitored at 290 nm. The XO IC_{50} values (concentration of active compound required to observe 50% inhibition of XO) were determined by plotting the percentage inhibition as a function of the concentration of test compound. The mode of inhibition of the different compounds was investigated using Lineweaver and Burk kinetic analysis by measuring the initial rate of the reaction at different xanthine concentrations between 0.0125 and 0.1250 mM without inhibitors and in the presence of inhibitors at their IC_{50} concentrations. K_m and V_{max} values were deducted from the Lineweaver and Burk double reciprocal plots.

2.3. DPP-IV inhibition assay

The test samples were dispersed in HPLC grade water at concentrations ranging from 0.01 to 1.25 mg ml^{-1}. The DPP-IV inhibition assay was carried out as described by Lacroix and Li-Chan (2012a) and Nongonierma and FitzGerald (2013). Test samples (25 μl) were pipetted onto a 96 well microplate containing Gly-Pro-pNA (final concentration 0.200 mM). The negative control contained 100 mM Tris–HCl buffer pH 8.0 (25 μl) and Gly-Pro-pNA. The reaction was initiated by the addition of DPP-IV (final concentration 0.0025 U ml^{-1}). Diprotin A was used as a positive control. Each sample was analysed in triplicate. The microplate was incubated at 37 °C for 60 min in a microplate reader, and absorbance of the released pNA was monitored at 405 nm. The IC_{50} values for DPP-IV and the mode of inhibition of the different samples were determined as described in Nongonierma and FitzGerald (2013). The initial rate of reaction was measured at different Gly-Pro-pNA concentrations between 0.200 and 0.600 mM without inhibitors and in the presence of inhibitors at their IC_{50} concentrations.

2.4. Computational analysis

AutoDock Vina (Trott & Olsen, 2010) was used to dock all 20 amino acids and 400 dipeptides to Protein Data Bank (PDB) structures 3BDJ, the crystal structure of bovine milk XO with a covalently bound oxipurinol inhibitor (Okamoto, Eger, Nishino, Pai, & Nishino, 2008), 1WCY, the crystal structure of human DPP-IV in complex with Diprotin A (Hiramatsu et al., 2005) and 1ORW, the crystal structure of porcine dipeptidyl peptidase IV (CD26) in complex with a peptidomimetic inhibitor (Engel et al., 2003). Ten ligand inhibitors of XO were taken from their respective PDB files (Table 1) and docked to the PDB structure 3BDJ. Six ligand inhibitors of DPP-IV were taken from their respective PDB files (Table 1) and docked to the PDB structures 1WCY and 1ORW. The initial poses of the PDB-formatted structures of amino acids and dipeptides were generated using the Open Babel Package, version 2.1.1 (O'Boyle et al., 2011). AutoDockTools (Morris et al., 2009) was used to prepare the ligands and the two receptors, and to determine the 'search space'. Amino acids, dipeptides and ligand inhibitors were then docked with the PDB structures giving a Vina score, i.e. the predicted affinity of the molecule to bind to the PDB structure, calculated in kcal mol^{-1}. A more negative score indicates that a molecule (ligand) is more likely to dock with the structure (enzyme) and achieve more favorable interactions. LIGPLOTs were generated for the highest ranked Vina docking pose for each amino acid, dipeptide and inhibitor (using their PDB files as input and the best Vina docking poses) according to the protocol described by Wallace, Laskowski, and Thornton (1995). Dipeptides were also assessed for their intestinal stability using an amino acid clustering model adopted from Foltz, Van Buren, Klaffke, and Duchateau (2009). This model rates dipeptides as 'stable' (>75% peptide remaining after 60 min simulated intestinal digestion – SID), 'neutral' (between 25% and 75% peptide remaining after 60 min SID) or 'unstable' (<25% peptide remaining after 60 min SID) with regard