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Bioactive hydrolysates from bovine blood globulins: Generation, characterisation, and *in silico* prediction of toxicity and allergenicity

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ABSTRACT

Two protein fractions rich in γ-globulins (FI) and α- and β-globulins (FII) were generated from bovine blood and hydrolysed with the enzyme papain. The generated hydrolysates showed *in vitro* angiotensin-I-converting enzyme (ACE-I), renin, and dipeptidyl peptidase-IV (DPP-IV) inhibitory activities. A total of 626 and 2246 peptides were identified by LC-MS/MS from the 1 kDa fractions of FI and FII, and the potential toxicity and allergenicity of these peptides were assessed *in silico* using three independent predictive approaches. All of the peptides identified from the bioactive blood protein fractions FI and FII were predicted to be non-toxic. However, 72 peptides from FI and 492 peptides from FII were identified as potential allergens with at least two predictive approaches. Results suggested that papain hydrolysates of FI and FII contain potential allergenic peptides, and those products containing such hydrolysates should be labelled correctly in line with Food and Consumer legislation – Regulation (EU) No.1169/2011.

1. Introduction

Bioactive peptides are short sequences of amino acids that are inactive within the sequence of the parent protein but have positive impacts on systems of the body once released by the action of microorganisms, enzymes, or acids (Korhonen & Pihlanto, 2006). Although blood is an excellent raw material for the generation of angiotensin-I-converting enzyme (ACE-I; EC3.4.15.1), renin (EC 3.4.23.15), and DPP-IV (DPP-IV; EC 3.4.14.5) inhibitory peptides (Bah, Bekhit, Carne, & McConnell, 2013; Lafarga & Hayes, 2014), few bioactive hydrolysates and peptides have been generated from bovine blood globulins to date. These included trypsin and alcalase hydrolysates of bovine globulins which previously showed ACE-I IC_{50} values of 8.14 and 7.11 mg/mL, respectively (Hyun & Shin, 2000).

Inhibition of ACE-I and renin plays a key role in the treatment of hypertension and inhibition of DPP-IV has potential

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Abbreviations: EFSA, European Food Safety Authority; ACE-I, angiotensin-I-converting enzyme; DPP-IV, dipeptidyl peptidase-IV; GIP, gastric-inhibitory peptide; GLP-1, glucagon-like peptide-1; MWCO, molecular weight cut-off; MS, mass spectrometry; LC, liquid chromatography; FA, formic acid; DMSO, dimethyl sulfoxide; ACN, acetonitrile; TCA, tri-chloroacetic acid; MW, molecular weight; SD, standard deviation; FAO, Food and Agriculture Organisation; WHO, World Health Organisation; SEM, standard error of the mean

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for use in the treatment of type-2 diabetes, hypercholesterolemia, and insulin resistance as demonstrated in a number of studies to date (Dicker, 2011; Juillerat-Jeanneret, 2014). Bioactive hydrolysates and peptides generated from blood proteins show potential for use as health-promoting ingredients in functional ingredients, and the identification of novel bioactive peptides with health-promoting properties would not only provide a commercial opportunity for many companies but may also have a role in improving public health. However, although the processes used for the generation of protein hydrolysates are common in the food industry and usually use food-grade materials, processing aids, and equipment, proteins and peptides can be toxic and produce allergic reactions after ingestion (Wang & De Mejia, 2005). Indeed, some of the most important toxic substances in inedible mushrooms are peptides (Yilmaz et al., 2014), and melittin, a peptide of 26 amino acids in length, is the principal active component of bee venom (Jamasbi et al., 2014). Peptides can also cause allergic reactions in humans as peptides can be used as allergens in specific allergen immunotherapy (Larché, 2007).

The term *food allergy* refers to an immune response directed towards food and can be defined as an "adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food" (Burks et al., 2012). The antibody responsible for most allergic reactions (type I hypersensitivities) belongs to the IgE isotype (Ribatti, 2016). Food allergy consists of two separate phases: sensitisation and elicitation. During the sensitisation phase, the immune system responds to the presence of an allergen with food-specific IgE antibody production by plasma cells that have differentiated from allergen-specific B lymphocytes (Burks et al., 2012). These IgE antibodies interact with low- and high-affinity IgE receptors (FcεRII/CD23 and FcεR1, respectively) on the surface of tissue mast cells or basophils, and once the concentration of IgE antibodies on the mast cell and basophile is high enough, the elicitation phase can occur (Stone, Prussin, & Metcalfe, 2010). The binding and cross-linking of the ingested (or inhaled) protein allergen with the specific IgE present on the cell membrane result in the release of histamine and other mediators of the inflammatory response triggering the various symptoms of allergy (Burks et al., 2012; Mittag et al., 2006; Panda, Tetteh, Pramod, & Goodman, 2015). Although the toxicity and the allergenicity of food products must be assessed *in vitro* and *in vivo*, the European Food Safety Authority (EFSA) favours the use of *in silico* tools for initial prediction of potential allergens from food proteins (Christer et al., 2010). In addition, *in silico* tools can be used to predict the toxicity of peptides (Gupta et al., 2013).

The aim of this study was to generate and characterise novel bioactive blood protein hydrolysates from bovine γ-, α-, and β-globulins which are currently underused as functional food ingredients. Two protein-rich fractions containing these proteins were generated from bovine blood by cold ethanol precipitation. These fractions were hydrolysed with the food grade enzyme papain (EC 3.4.22.2) and enriched using 1, 3, and 10 kDa molecular weight cut-off (MWCO) filters. Peptides contained in the generated 1 kDa fractions were characterised by LC-MS/MS. The identified peptides were assessed for potential toxicity and allergenicity *in silico*.

2. Materials and methods

2.1. Materials and reagents

Formic acid (FA), ethanol, dimethyl sulfoxide (DMSO), acetonitrile (ACN), tri-chloroacetic acid (TCA), sodium citrate, papain from *Carica papaya*, the specific renin inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe, and the ACE-I inhibitor Captopril® were supplied by Sigma Aldrich (Dublin, Ireland). The DPP-IV inhibitor screening assay kit, containing the DPP-IV inhibitor sitagliptin, and the renin inhibitor screening assay kit were supplied by Cambridge BioSciences (Cambridge, England, UK). The ACE-I inhibition assay kit was supplied by NBS Biologicals Ltd. (Cambridgeshire, England, UK). All other chemicals used were of analytical grade.

2.2. Blood collection and globulin fractionation procedure

Whole bovine blood was collected at time of slaughter under hygienic conditions from the abattoir at the Teagasc Food Research Centre, Ashtown, Dublin 15, Ireland. All animals slaughtered were Charolais cross heifer breed and were aged between 23 and 24 months at the time of slaughter. Sodium citrate solution was used as an anticoagulant and was added immediately to blood following collection at a final concentration of 1.5% (w/v). Blood was chilled to 4 °C and handled carefully to minimise haemolysis.

Whole blood cells were separated from plasma by centrifugation at 4 °C and 10000 × g for 10 minutes using a Sigma 6K10 centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). Plasma was kept at 4 °C, filtered through glass wool and freeze-dried using an industrial scale freeze-drier FD 80 model (Cuddon Engineering, Marlborough, New Zealand). The temperature was maintained at less than 35 °C during the freeze-drying process. Two fractions enriched for γ- and α- and β-globulins were obtained from the freeze dried extract by precipitation following a previously described method (Álvarez, Bances, Rendueles, & Díaz, 2009). Briefly, a protein fraction, rich in γ-globulins, labelled as FI, was precipitated by addition of ethanol to the serum at a final concentration of 19% (v/v). The pH was adjusted to 7.2 by addition of NaOH. Furthermore, a protein fraction, rich in α- and β-globulins, labelled as FII, was precipitated by adjustment of the pH to 5.5 and subsequent addition of ethanol to a final concentration of 40% (v/v). Ethanol was added drop by drop and the process was carried out in an ice bath to minimise protein denaturation. The generated protein-rich fractions FI and FII were separated by centrifugation at 4 °C and 10,000× g for 5 minutes, re-suspended in Milli Q water, frozen, and freeze-dried.

The total protein content was determined in duplicate using a LECO FP628 Protein analyser (LECO Corp., MI, USA) based on the Dumas method and according to AOAC method 992.15, 1990. The conversion factor of 6.25 was used to convert total nitrogen to protein. The total protein content and yields were calculated per litre of blood throughout the separation. Moisture and ash content were determined gravimetrically in accordance with previously described methods (Kolar, 1992).