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In vitro inhibition of platelet aggregation by peptides derived from oat (*Avena sativa* L.), highland barley (*Hordeum vulgare* Linn. var. nudum Hook. f.), and buckwheat (*Fagopyrum esculentum* Moench) proteins

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ABSTRACT

Bioactive compounds present in foods could have beneficial effects on human health. In this study, we report the capacity of peptides released from oat, highland barley, and buckwheat proteins after enzymatic digestion to inhibit platelet aggregation *in vitro*. All hydrolysates showed high antiplatelet activity, with IC_{50} values of 0.282 mg/ml (oat flour gastrointestinal hydrolysate, 6 h) to 2.496 mg/ml (highland barley glutelin tryptic hydrolysate, 14 h) in a dose-dependent manner. Thirty-eight peptides with more than seven residues were identified in the tryptic hydrolysates of oat globulin. Results of computational modeling revealed that nine peptides, including ALPIDVLANAYR, EFLLAGNNKR, GEEFGAFTPK, QLAQIPR, LQAFEPLR, ALPVDVLANAYR, GEEFDAFTPK, QKEFLLAGNNK, and TNPNSMVSHIAGK bound the cyclooxygenase-1 active centers with low binding energy (−6.5 to −7.5 kcal/mol). This is the first report to identify antiplatelet peptides from grain hydrolysates and the binding modes at the molecular level, leading to their possible use as functional food ingredients to prevent thrombosis.

1. Introduction

Cardiovascular diseases are the leading cause of mortality and morbidity worldwide, accounting for nearly 30% of global deaths (Lindholm & Mendis, 2007). Intravascular thrombosis, such as coronary thrombosis, deep venous thrombosis, and acute arterial thrombosis, is a major cardiovascular disease. The general pathogenesis of thrombosis includes platelet activation, subsequent adhesion, release of granule content, response to multiple agonists, and platelet aggregation (Kong et al., 2014). Patients with coronary heart disease tend to have increased platelet reactivity. Therefore, antiplatelet therapy is a promising approach for preventing thrombosis.

Insect proteins are abundant sources of anti-platelet peptides, including hirudin from leeches (Salzet, 2002), the KRDS peptide from human lactotransferrin (Mazoyer et al., 1990), *Agkistrodon acutus* venom (Kong et al., 2009), and Ser-Gln-Leu from centipedes (Kong, Huang, Shao, Li, & Wei, 2013). Plant proteins also contain antiplatelet peptides, such as proteases from the medicinal

Abbreviations: AA, arachidonic acid; ASA, acetylsalicylic acid; COX1, cyclooxygenase-1; NS, normal saline; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TXA_2, thromboxane-A_2; TXB_2, thromboxane-B_2; WP, washed platelet; GO, gastrointestinal hydrolysate of oat flour; GH, gastrointestinal hydrolysate of highland barley flour; GB, gastrointestinal hydrolysate of buckwheat flour; TOGB, tryptic hydrolysate of oat globulin.

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mushroom *Ganoderma lucidum* (Kumaran, Palani, Nishanthi, & Kaviyarasan, 2011) and Trp-Gly-Cys from *Inonotus obliquus* (Hyun, Jeong, Lee, Park, & Lee, 2006). Grains are important protein sources for humans. However, the antiplatelet peptides SSGE and DEE have to date been detected only in soybean (Lee & Kim, 2005). More information on cereal proteins needs to be uncovered.

Oat (*Avena sativa* L.), highland barley (*Hordeum vulgare* Linn. var. nudum Hook. f.), and buckwheat (*Fagopyrum esculentum* Moench) are food staples for people living in northwest China. Oat and buckwheat, which are popular in the northwest region of China, can be made into various foods, such as noodles, pies, and pancakes, whereas highland barley is used in the Tibet region. Current studies indicate that a long-term consumption of these grains will produce such health benefits as lowering blood cholesterol and preventing coronary disease, tumor growth, and diabetes (Guo, Zhu, Zhang, & Yao, 2010; He et al., 1995; Murphy et al., 2004; Pins & Kaur, 2006; Tong et al., 2014). The main active components are attributed to the β-glucan fraction in oats and highland barley, and to the flavonoid fraction in buckwheat (Christa & Soral-Smietana, 2008; Delaney et al., 2003; Storsley, Izydorczyk, You, Biliaderis, & Rossnagel, 2003). As one of the main constituents, protein level varies from 12.4% to 24.5% in oat, 7.68% to 17.52% in highland barley and 9.19% to 17% in buckwheat, respectively (Chang, Alli, Konishi, & Ziomek, 2011; Hui, Hou, LI, Liu, & Zhu, 2005; Wang P. Z. & Zhang S. H., 1997). However, little information on the benefits of these proteins is available.

The aim of this study was to clarify the antiplatelet activity of peptides derived from oat, buckwheat, and barley proteins. The peptides encrypted in the three cereal storage proteins were released using *in vitro* gastrointestinal digestion, trypsin and alcalase digestions, and their *in vitro* platelet aggregation inhibitory activities were evaluated. High activity oat globulin tryptic hydrolysates were identified using nano-liquid chromatography–electro spray ionization tandem mass spectroscopy (nano-LC–ESI-MS/MS). Docking modeling was carried out to predict the molecular mechanisms of the interactions between the oat peptides and cyclooxygenase-1 (COX1).

2. Materials and methods

2.1. Materials

Oat (*A. sativa* L.), highland barley (*H. vulgare* Linn. var. nudum Hook. f.), and buckwheat (*F. esculentum* Moench) were purchased in Shanxi, China and were harvested in autumn of 2013. Oat, highland barley, and buckwheat flour were obtained by crushing and sieving the three cereals (60-mesh sieve). The flours were stored at −20 °C until use.

Blood was collected from healthy volunteer donors (age, 20–25 years) after approval from the Beijing Forestry University Ethical Review Committee (permission number: 2013XL001-3) and with the informed consent of all volunteer donors. The volunteer donors fasted overnight prior to blood collection from a vein in the morning, and the fresh blood was collected into plastic tubes containing 3.8% sodium citrate and used within 3 h.

Porcine pepsin, trypsin, pancreatin, arachidonic acid (AA), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Alcalase was obtained from Novozyme (Beijing, China). Acetylsalicylic acid (aspirin, ASA) was purchased from Alfa-Aesar Co. (Tianjin, China). Human COX1 and thromboxane-B_2 (TXB_2) enzyme immunoassay (EIA) kits were purchased from Feng-xiang Biological Co. (Shanghai, China). All other reagents were of analytical grade.

2.2. Protein extraction

The albumin, globulin, and glutelin fractions were extracted sequentially according to a method reported previously (Chang et al., 2011). A sample (50 g) of oat, highland barley, or buckwheat flour was mixed with distilled water (200 ml). The mixture was allowed to stand with intermittent stirring for 2 h and then centrifuged to separate the mixture into the S1 supernatant and the R1 residue. S1 was filtered through fine glass wool and precipitated by adding 1 M HCl until pH 4.1 was reached, followed by centrifugation (8,000*g*, 20 min); this albumin fraction (highland barley albumin and buckwheat albumin) was obtained after lyophilizing the precipitate and discarding the supernatant. R1 was mixed with 5% NaCl (200 ml), stirred for 2 h, and centrifuged (8,000*g*, 20 min) to obtain the S2 supernatant and the R2 residue. S2 was filtered through fine glass wool and precipitated by adding 1 M HCl until pH 4.3 was reached, followed by centrifugation (8,000*g*, 20 min); this globulin fraction (oat globulin) was recovered after lyophilizing the precipitate and discarding the supernatant. R2 was mixed with 0.1 M NaOH (200 ml), stirred for 2 h, and centrifuged (8,000*g*, 20 min). The supernatant was filtered through fine glass wool and precipitated by adding 1 M HCl until pH 4.8 was reached, followed by centrifugation (8,000*g*, 20 min); this glutelin fraction (oat glutelin, highland barley glutelin, or buckwheat glutelin) was recovered after lyophilizing the precipitate and discarding the supernatant. All freeze-dried protein isolates were stored at −20 °C until further use.

2.3. In vitro gastrointestinal digestion simulation

An *in vitro* gastrointestinal digestion model was established as suggested previously (Velarde-Salcedo et al., 2013). Briefly, 1 g of sample (defatted flour from raw oat, highland barley, and buckwheat) was resuspended in 20-ml 0.03 M NaCl (pH 2.0). The suspensions were heated in a water bath at 80 °C for 5 min to inactivate the bacteria and proteases and cooled to room temperature. Porcine pepsin, which was dissolved previously in 0.03 M NaCl (pH 2.0), was added at a 1:20 ratio (w/w enzyme:substrate ratio), and the samples were digested at a constant pH for 3 h at 37 °C. pH was adjusted to 7.5. A mixture of trypsin and pancreatin was prepared (1:1 w/w trypsin:pancreatin ratio in 0.1 N $NaHCO_3$), added to the digestive solution, and incubated at a constant pH for another 3 h (1:20 w/w enzyme:substrate ratio). Suspensions were obtained during the digestion process. Digestion was stopped by heating the suspensions at 75 °C for 20 min. The hydrolysates were centrifuged at 13,000*g* for 30 min, and the supernatant was stored at −20 °C until analysis the next day. Protein concentrations were determined using a Lowry-based DC Protein Assay (Bio-Rad, Hercules, CA, USA) using BSA as the standard.

2.4. Tryptic digestion of grain proteins

The oat globulin and glutelin fractions, the highland barley albumin and glutelin fractions, and the buckwheat albumin and glutelin fractions were subjected to tryptic digestion. Trypsin from porcine pancreas was used at a trypsin:protein (w/w) ratio 1:50. The digestions were carried out in 100 mM Tris buffer (pH 8.0) incubated in a Thermomixer (Eppendorf, Hamburg, Germany) for 14 h at 37 °C. The reaction was terminated by freezing. The hydrolysate was centrifuged at 13,000*g* for 30 min, the supernatant was collected, and protein concentration was determined using the Lowry-based DC Protein Assay.