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Protein digestomic analysis reveals the bioactivity of deer antler velvet in simulated gastrointestinal digestion

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Gly-Pro-*p*-nitroanilide hydrochloride (PubChem CID: 16219380)
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o-Phthalaldehyde (PubChem CID: 4807)
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ABSTRACT

Proteins are the most prominent bioactive component in deer antler velvet. The aim of the present study was to track the fate of protein of antler velvet by protein digestomics. The peptide profile identified by LC-MS/MS and the *in vitro* bioactivity of antler velvet aqueous extract (AAE) were investigated in simulated gastrointestinal digestion. A total of 23, 387 and 417 peptides in AAE, gastric and pancreatic digests were identified using LC-MS/MS, respectively. Collagens, the predominant proteins, released 34 peptides in gastric digests and 146 peptides in pancreatic digests. The gastric and pancreatic digests presented dipeptidyl peptidase IV (DPP-IV) and prolyl endopeptidase (PEP) inhibition activities. Four peptides from digests were proved to be DPP-IV and PEP inhibitory peptides. The results showed that the peptides released from antler velvet protein contributed to the bioactivity of antler velvet during digestion.

1. Introduction

Deer antler velvet is the cartilaginous tissue of male antlers prior to ossification. Deer antler grows rapidly and completes growth, calcification, velvet shedding and antler casting in three months (Kierdorf, Li, & Price, 2009). Antler growth is a stem-cell mediated process and stem cells proteins were different than those of somatic cells (Li, Harper, Puddick, Wang, & McMahon, 2012). According to proteomics analyses of red deer antlers by Park et al. (2004), ~130 proteomes identified in antler velvet were dissimilar to those from other type tissues. The crude protein content of antler velvet in sika deer ranges from 53.9 to 76.4% (Jeon et al., 2009). Gao et al. (2010) identified 416 proteins from antler velvet and Sui et al. (2013) increased the number of identified proteins from antler velvet to 1423 using LC-MS/MS. The *in vitro* and animal studies proved that polypeptides and proteins are the important bioactive component in antler velvet (Lee et al., 2015; Xin, Zhang, Zhang, Lin, & Zhou, 2013; Zha et al., 2013; Zhao, Luo, Wang, & Ji, 2011). The protein hydrolysates generated by mixture enzymes of pancreatin-pepsin and alcalase and neutrase from aqueous antler velvet extracts presented *in vitro* anti-oxidant activity (Zhao et al., 2011). The velvet antler hydrolysates produced using alcalase exhibited anti-inflammatory activity effects in zebrafish as well as *in vitro* using cell lines (Lee et al., 2015). A natural polypeptide isolated from antler velvet with a molecular weight of 3215.8 promoted the proliferation of epidermal cells and NIH3T3 cell line (Xin et al., 2013). A native 3.2 kDa polypeptide from antler velvet also presented potential immunomodulatory effects on the immune system of mice (Zha et al., 2013). The nature polypeptides of antler velvet demonstrated anti-osteoporosis activities on osteoarthritic chondrocytes in rabbit (Zhang et al., 2011).

Antler velvet possesses immunomodulatory, anti-aging and several other health benefits and has been used as Chinese medicine for a thousand years (Wu et al., 2013). Antler velvet is typically decocted with hot water, and subsequently orally administered. Proteins are the main components of aqueous extract. Aqueous antler extract showed anti-bone resorption in adjuvant-induced arthritic rats (Kim et al.,

Abbreviations: AAE, antler velvet aqueous extract; ACN, acetonitrile; DPP-IV, dipeptidyl peptidase IV; FA, formic acid; GDAAE, gastric digest of AAE; OPA, *o*-Phthalaldehyde; PDAAE, pancreatic digest of AAE; PEP, prolyl endopeptidase; SPE, solid-phase extraction; TFA, trifluoroacetic acid

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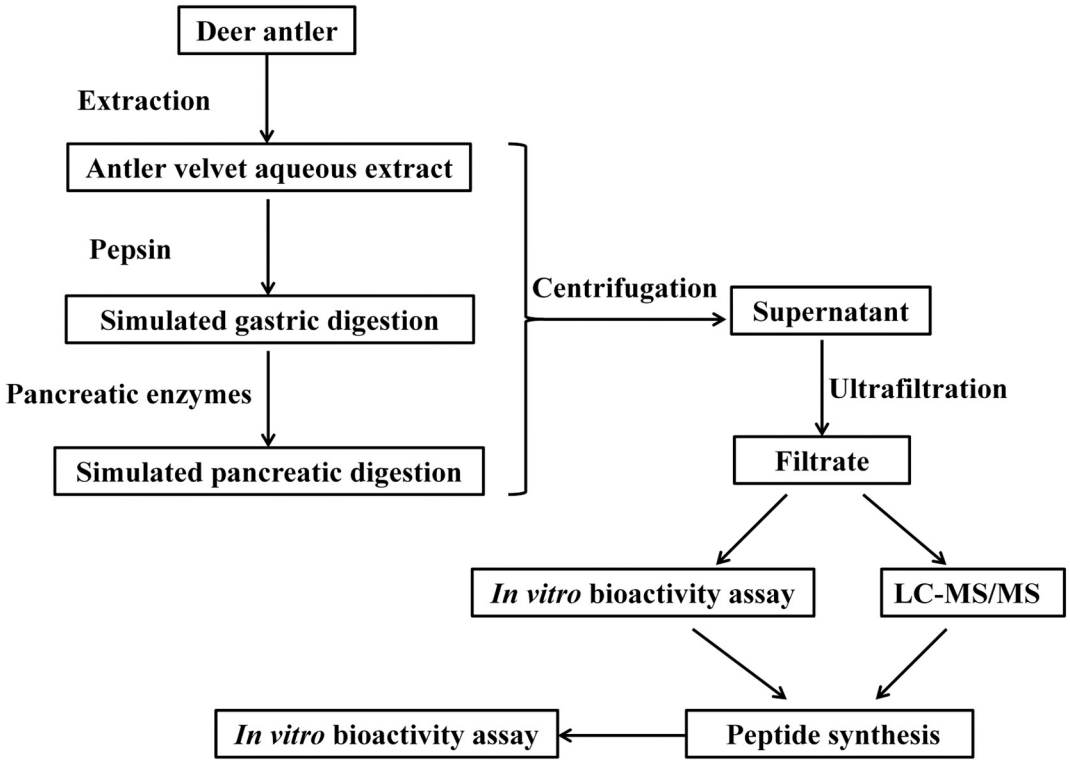

Fig. 1. Schematic overview of the analytical strategy to identify peptides from AAE in the simulated gastrointestinal digestion.

2005) and *in vitro* antioxidant activity (Zhao et al., 2010; Zhou & Li, 2009). The aqueous antler extract significantly restored scopolamine-induced memory impairments in mice and elevated oxidative damage in the brain (Lee et al., 2010). Aforementioned bioactivities of aqueous antler velvet extract are based on *in vitro* or animal experiments. The polypeptides and proteins in aqueous antler velvet extracts would be digested by gastrointestinal enzymes to peptides and amino acids in the human gastrointestinal tract. The fates of polypeptides and proteins of aqueous extract in the human gastrointestinal tract play key roles on their bioactivities in the human body.

Protein digestomics is the integrated platform to study food-digestion and derived bioactive peptides (Picariello, Mamone, Nitride, Addeo, & Ferranti, 2013). MS-based proteomics and peptidomics has been applied to track and identify peptides that survive in the gastrointestinal tract (Jin et al., 2016; Saavedra, Hebert, Minahk, & Ferranti, 2013; Sánchez-Rivera, Martínez-Maqueda, Cruz-Huerta, Miralles, & Recio, 2014; Sayd, Chambon, & Santé-Lhoutellier, 2016). To our knowledge, no peptide profiling of bioactivity analysis of peptides released from antler velvet proteins in the human gastrointestinal tract has been reported. In the present study, we conducted a comprehensive analysis of the peptide profile and bioactivity of antler velvet aqueous extract (AAE) in simulated gastrointestinal digestion. The peptide profile of antler velvet in simulated gastrointestinal digestion was analyzed using LC-MS/MS. The bioactivities of AAE in gastrointestinal digests were evaluated through prolyl peptidases inhibitory activities. Prolyl peptidases are a class of serine protease that cleaves proteins and peptides after proline residues, and these enzymes are considered potential targets for drug discovery (Rosenblum & Kozarich, 2003). Dipeptidyl peptidase IV (DPP-IV) and prolyl endopeptidase (PEP) are two prolyl peptidases that play important roles in human health (Rosenblum & Kozarich, 2003). DPP-IV inhibitors have emerged as an effective treatment option for type-2 diabetes (Drucker & Nauck, 2006). PEP, also known as prolyl oligopeptidase, is a highly conserved serine protease. PEP plays a role in the metabolism of proline-containing neuropeptides and is associated with the induction of amnesia (Fulop, Bocskei, & Polgar, 1998). PEP activity is altered in neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease (Garcia-Horsman, Mannisto, & Venalainen, 2007). PEP inhibitors are potential drugs for the reversion of the memory of Alzheimer's disease patients. The potential DPP-IV and PEP inhibitory peptides in AAE, gastric digest of AAE (GDAAE) and pancreatic digest of AAE (PDAAE) were synthesized and their activities were tested.

2. Materials and methods

2.1. Materials and reagents

Deer antler velvet was collected in Xifeng, Liaoning Province, China. Pepsin (from porcine gastric mucosa, 400–800 U/mg), trypsin (from bovine pancreas, ≥ 10,000 U/mg), α-chymotrypsin (from bovine pancreas, ≥ 40 U/mg), elastase (from porcine pancreas, ≥ 4 U/mg), trifluoroacetic acid (TFA), dipeptidyl peptidase IV (DPP-IV, from porcine kidney), diprotin A (Ile-Pro-Ile), Gly-Pro-*p*-nitroanilide hydrochloride, PEP (from *Flavobacterium* sp.), sodium valproate, and Z-Gly-Pro-*p*-nitroanilide were purchased from Sigma (St. Louis, MO, USA). *o*-Phthalaldehyde (OPA) and 2-mercaptoethanol were purchased from J & K Scientific Ltd. (Beijing, China). Methanol (HPLC grade) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Acetonitrile (ACN, HPLC grade) was purchased from Merck (Darmstadt, Germany). The water used in these experiments was purified using a Milli-Q system from Millipore Company (Bedford, MA, USA). Other chemicals were purchased from Kermel (Tianjin, China).

2.2. Sample preparation and gastric-pancreatic digestion

The workflow of antler velvet extraction and analysis was shown in Fig. 1. Fresh deer antler velvet was collected from male sika deer (*Cervus Nippon* Temminck) and blood was immediately removed using a vacuum pump. Blood-free antler velvet was lyophilized and subsequently powdered, and 1 g of antler velvet powder was extracted with