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Discovery and quantification of bioactive peptides in fermented cucumber by direct analysis IR-MALDESI mass spectrometry and LC-QQQ-MS☆

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ABSTRACT

Bioactive peptides have been identified in lactic acid bacteria fermented foods including cultured milk, sourdough, and cured meats; however, their presence has not been investigated in fermented vegetables. In this study, infrared, matrix-assisted laser desorption electrospray ionization (IR-MALDESI) mass spectrometry (MS) was employed to identify bioactive peptides in fermented cucumber. Natural and starter culture fermented cucumbers were prepared in triplicate in sodium chloride brines and compared to acidified cucumbers. Putative matches of known food-derived bioactive peptides were identified by direct analysis using IR-MALDESI-MS. Peptides were confirmed by IR-MALDESI MS/MS and quantified by LC-MS/MS. Three angiotensin converting enzyme (ACE) inhibitory peptides, IPP (0.42–0.49 mg/kg), LPP (0.30–0.33 mg/kg), and VPP (0.32–0.35 mg/kg) were formed in fermented cucumbers. A fourth ACE inhibitory peptide, KP (0.93–1.5 mg/kg), was enhanced 3–5 fold in fermented cucumbers compared with acidified cucumbers. This work demonstrates that lactic acid bacteria fermentation can enhance bioactive peptide content in vegetables.

1. Introduction

Worldwide, consumption of fermented foods is commonly perceived as healthful. Research on the potential health benefits of fermented foods has primarily focused on the ingestion of live microorganisms and subsequent probiotic effect. More recently, emphasis has been placed on the discovery of health-promoting compounds derived from microbial activity, known as bioactives. Lactic acid bacteria (LAB) are the most prominent microbial group responsible for fermentation of meat, dairy, grains, and vegetables. Apart from their primary metabolic role of converting sugars to acid, LAB are fastidious microorganisms that possess complex proteolytic systems. These systems include cell envelope proteases to hydrolyze food proteins, transport systems to uptake peptides, and intracellular peptidases to metabolize peptides into amino acids and nitrogen essential for survival (Savijoki, Ingmer & Varmanen, 2006). During LAB fermentation, hydrolysis of food proteins leads to the formation of both free amino acids and peptides.

Bioactive peptides are sequences of amino acids encrypted in a latent form within food proteins that are liberated via enzymatic hydrolysis by one of three means: application of exogenous proteases during food processing; digestive enzymes post-consumption; or microbial fermentation (Meisel & Bockelmann, 1999). The resulting short peptides (2–20 amino acids) that contain specific sequences of amino acids exert biological activity locally within the gastrointestinal tract or systemically in the blood and organs (Kussmann & Van Bladeren, 2011). Reported benefits include antioxidative, antithrombotic, antihypertensive, hypocholesterolemic, or immunomodulatory effects (Gibbs, Zougman, Masse, & Mulligan, 2004; Karnjanapratum et al., 2017; Kayser & Meisel, 1996; Koyama et al., 2013; Nagaoka et al., 2001; Seppo, Jauhiainen, Poussa, & Korpela, 2003), and the growing interest in natural alternatives to chemical pharmaceuticals has led researchers to investigate bioactive peptide formation for either therapeutic consumption of foods or commericialization as active pharmaceutical ingredients. While much research has been done on

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bioactive peptides in meats, fermented dairy and select grains, there is limited information regarding their presence in raw or fermented vegetables.

Cucumber pickles are the most commonly consumed fermented vegetable in the United States. Commercially, fresh cucumbers are submerged in high-salt brine (0.6–1.7 M NaCl) in large capacity vats and undergo fermentation by the LAB naturally present on the cucumbers until less than 0.05% sugar remains. Microorganisms responsible for cucumber fermentation typically include *Lactobacillus plantarum or Lb. pentosus, Lb. brevis*, *Enterococcus faecalis*, *Leuconostoc mesenteroides*, and *Pediococcus cerevisiae* (likely *Pediococcus pentosaceus* and/or *Pediococcus acidilactici* after recent reclassification) (Pérez-Díaz et al., 2013, chap. 51). Inoculation of cucumber fermentations with a known starter culture is not common practice but has been performed in both laboratory and industrial settings. During fermentation, between 110 and 140 mM lactic acid is produced and the pH equilibrates near 3.2–3.6 (Pérez-Díaz et al., 2013, chap. 51). Many cucumber pickle products are stored for extended periods of time and/or pasteurized prior to consumption; therefore, health-promoting properties of these foods depend on the chemical composition rather than the presence of live LAB. In addition to fermented cucumber pickles, non-fermented, acidified cucumber pickles are commonly consumed. The latter are produced by packing fresh whole or sliced cucumbers into jars and covering them with an acidified brine, typically containing acetic acid from vinegar, salt, and sodium benzoate and/or potassium sorbate to prevent fermentation and spoilage. Cucumbers contain 0.65% protein (USDA Food Composition Database, https://ndb.nal.usda.gov/ndb/search/list, Accessed 10.10.16) that may serve as a substrate for microbial or endogenous enzymes in the production of bioactive peptides, and we hypothesize that fermented cucumbers possess greater concentrations of bioactive peptides than raw or acidified cucumbers due to the fermentation process.

Discovery of bioactive peptides in plant-based foods typically employs either bioactivity-guided or targeted approaches. In a bioactivity-guided approach, samples undergo several stages of separation and fractionation using combinations of size-exclusion (SEC), ion-exchange (IEX), or high performance liquid chromatography (HPLC) for fractionation of samples prior to bioactivity testing (Panchaud, Affolter, & Kussmann, 2012; White, Sanders, & Davis, 2014). Collected fractions are tested in vitro for specific bioactivities and those with the highest activity are further fractionated and analyzed. Peptide sequences in the final fractions are identified by comparison to synthetic standards using LC tandem mass spectrometry (LC-MS/MS) and in some cases MS^3. Conversely, targeted workflows exclude bioactivity testing and consist of analyzing peptide standards by $LC\text{-}MS^n$ to obtain separation and spectral data for comparison to food samples. Bütikofer, Meyer, Sieber, & Wechsler (2007) and Solieri, Rutella, & Tagliazucchi (2015) utilized this targeted route to confirm and quantify IPP and VPP in fermented dairy products, and Yamamoto et al. (2014) screened soy sauce for 337 hypothesized dipeptides, confirming the presence of 237. While these two approaches are commonly used for bioactive peptide discovery, they often require lengthy method development and extensive sample preparation, including lyophilization, cryopulverization, desalting, precipitation, filtration and solvent extraction (Lee, Bae, Lee, & Yang, 2006; Rizzello, Cassone, Di Cagno, & Gobbetti, 2008).

Infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI) mass spectrometry (MS) is a salt-tolerant, atmospheric pressure, soft-ionization technology capable of ionizing analytes directly desorped from intact tissue samples using endogenous water as the energy-absorbing matrix (Bokhart & Muddiman, 2016; Sampson, Murray, & Muddiman, 2009). Direct analysis IR-MALDESI circumvents sample preparation and separation steps for biological samples and can be used for mass spectrometry imaging (MSI) in which a molecule's spatial location within the tissue is displayed as a heat map. This novel method has been demonstrated for identification of small molecules in fermented cucumbers, which are not directly amenable to traditional ESI due to their high salt (1 M NaCl) content (Ekelöf, McMurtrie, Nazari, Johanningsmeier, & Muddiman, 2017). The objectives of this study were to: 1) apply direct analysis IR-MALDESI MS for identifying small bioactive peptides in raw, acidified, and fermented cucumbers; and 2) determine whether bioactive di- and tri-peptides are formed as a consequence of lactic acid fermentation.

2. Materials and methods

2.1. Chemicals and materials

Pickling cucumbers, pickling salt (sodium chloride, NaCl, ≥ 99%), and vinegar (acetic acid, 20%) were obtained from Mount Olive Pickle Company (Mount Olive, NC, USA). Calcium chloride ($CaCl_2$, ≥ 93%), hydrochloric acid (HCl, ≥ 37%), sulfuric acid (H_2SO_4 3 N) and lactic acid (≥ 85%) were purchased from Sigma-Aldrich (St. Louis, MO, USA); calcium hydroxide ($Ca(OH)_2$, ≥ 95%) was purchased from Fisher Scientific (Hampton, NH, USA); and sodium benzoate (≥ 99%) was purchased from Acros Organics (Waltham, MA, USA).

For IR-MALDESI analyses, LC-MS-grade methanol and water were purchased from Burdick and Jackson (Muskegon, MI, USA); and MS-grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Nitrogen gas (≥ 99.999%) for the higher energy collision (HCD) cell was purchased from Arc3 Gases (Raleigh, NC, USA). Glass slides were purchased from VWR (Radnor, PA, USA).

For LC-MS/MS analyses, LC-MS grade methanol and water were purchased from Fisher Scientific (Hampton, NH, USA). Peptide standards were purchased from Bachem (Bubendorf, Switzerland): isoleucine-proline-proline (IPP, ≥ 98%), valine-proline-proline (VPP, ≥ 99.5%), leucine-proline-proline (LPP, ≥ 99%), arginine-tyrosine (RY, ≥ 99%), and lysine-proline (KP, ≥ 99%). Amicon Ultra-0.5 filters with a 3 kDa cutoff were purchased from Fisher Scientific (Hampton, NH, USA).

2.2. Experimental design

Four treatments were prepared: raw cucumber, acidified cucumber, naturally fermented cucumber, and starter culture fermented cucumber. Treatments were independently replicated in triplicate from one lot of pickling cucumbers. The acidified cucumbers served as a control by mimicking the salt and acid content of a fermented cucumber while preventing fermentation with the addition of sodium benzoate.

2.3. Brining and fermentation of cucumbers

Size 2B pickling cucumbers (3.5–3.8 cm diameter) were rinsed, packed into 1.36 L glass jars, and covered with brine (55:45 cucumber:brine ratio). Jars were sealed with a septum fitted lid to allow for brine sampling with a syringe. Cucumbers fermented with a starter culture were brined and inoculated with *Lactobacillus pentosus* strain LA0445 (Food Science Research Unit Culture Collection, USDA-ARS, Raleigh, NC, USA) to a final concentration of 6.4×10^5 CFU/mL prior to sealing the jars. Acidification and fermentation brines were prepared so that the equilibrated concentrations in the brined cucumbers were 0.684 M NaCl, 12 mM $CaCl_2$, 18 mM $Ca(OH)_2$, and 53 mM acetic acid. Acidified cucumber brines also contained lactic acid to mimic fermented cucumber acid content (110 mM, equilibrated), sodium benzoate to prevent fermentation (8 mM, equilibrated), and were adjusted with HCl to pH 2.75 so the cucumbers would reach a final pH of 3.25 after equilibration. Brined cucumbers were incubated at 28 °C for 6 weeks. On day 43, three cucumbers were sampled from each replicate treatment, cut into 2 cm cross sections, then into three lobes, and stored at −80 °C. Approximately 200 g of cucumber from each treatment and replicate was blended into a slurry. Raw cucumbers were prepared similarly the same day that brined treatments were packed. All samples were stored immediately at −80 °C until the time of analysis.