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Fluorescence spectroscopy and principal component analysis of soy protein hydrolysate fractions and the potential to assess their antioxidant capacity characteristics

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ABSTRACT

The potential of intrinsic fluorescence and principal component analysis (PCA) to characterize the antioxidant capacity of soy protein hydrolysates (SPH) during sequential ultrafiltration (UF) and nanofiltration (NF) was evaluated. SPH was obtained by enzymatic hydrolysis of soy protein isolate. Antioxidant capacity was measured by Oxygen Radical Absorbance Capacity (ORAC) and Folin Ciocalteau Reagent (FCR) assays together with fluorescence excitation-emission matrices (EEM). PCA of the fluorescence EEMs revealed two principal components (PC_1-tryptophan, PC_2-tyrosine) that captured significant variance in the fluorescence spectra. Regression models between antioxidant capacity and PC_1 and PC_2 displayed strong linear correlations for NF fractions and a weak linear correlation for UF fractions. Clustering of UF and NF fractions according to $ORAC_{FPCA}$ and FCR_{FPCA} was observed. The ability of this method to extract information on contributions by tryptophan and tyrosine amino acid residues to the antioxidant capacity of SPH fractions was demonstrated.

1. Introduction

Antioxidant can be defined as substances that can significantly decrease the unfavorable effects of reactive species, such as oxidative free radicals, on typical human physiological functions (Halliwell & Gutteridge, 2015). Numerous well-established assays for measuring the antioxidant capacity of species are available (Apak et al., 2013). These assays can be classified as hydrogen atom transfer (HAT)- and electron transfer (ET)-assays. HAT-based assays, such as the Oxygen Radical Absorbance Capacity (ORAC), involve a complex scheme of reactions whereby an antioxidant and a substrate compete for peroxy radicals that are thermally generated by the breakdown of azo-compounds (Apak, Özyürek, Güçlü, & Çapanoğlu, 2016). In the ORAC assay, the antioxidant capacity of a specie is deduced from the fluorescence decay curve and the associated area under the curve (AUC) reflecting its oxidative degradation by peroxy radicals (Apak et al., 2007). ET assays, such as the Folin Ciocalteau's reducing capacity (FCR) and ferric reducing antioxidant power (FRAP) assays, involve a redox-potential probe (i.e., fluorescent or colored probe). Antioxidant capacity is thus measured by the reduction of an oxidant with a single electron transfer upon which a color change in solution can be observed and spectrophotometrically quantified (Apak et al., 2007). ET mechanisms employ non-physiological conditions (i.e., room temperature, pH conditions that are not representative of human physiology) to measure the reducing capacity of a molecule in the absence of reactive free radicals, whereas ORAC combines relative inhibition and time for inhibition of free radicals into one quantity to provide a superior indication of antioxidant capacity of a specie than ET mechanisms (Preedy, 2011; Cao & Prior, 1998).

Many proteins, such as soy proteins, can contain correct amino acid and peptide sequences for bioactive functions. However, these peptides are restricted from performing these functions within the sequence of their native protein by peptide bonds, which occupy the terminal ends of an amino acid's backbone structure (amino- and carboxyl-termini), and by side chain interactions between peptide chains. Upon liberation from their native protein sequence, certain peptides can fulfill antioxidant functions among other bioactive properties (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). A number of amino acid residues, including histidine, tyrosine, tryptophan, phenylalanine, proline, and leucine, have been identified as contributors to antioxidant

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Nomenclature

AAPH	2,2′-Azobis-2-methyl-propanimidamide dihydrochloride
AUC	Area under the curve
EEM	Excitation-emission matrix
ET	Electron transfer
Ex/Em	Excitation and emission wavelengths
FCR	Folin Ciocalteau Reagent assay
FCR_{FPCA}	Fluorescence- and PCA-estimated FCR
FRAP	Ferric reducing antioxidant power
HAT	Hydrogen atom transfer
MLRM	Multi-linear regression model
MWCO	Molecular weight cut off (kDa)
NF	Nanofiltration
OPA	O'phthaldialdehyde
ORAC	Oxygen Radical Absorbance Capacity assay
$ORAC_{FPCA}$	Fluorescence- and PCA-estimated ORAC
PC	Principal component
PCA	Principal component analysis
PMMA	Polymethylmethacrylate
PMT	Photomultiplier tube
RS	Rayleigh light scattering
SPH	Soy protein hydrolysate
SPI	Soy protein isolate
SSE	Sum of squared errors
TE	Trolox equivalents
TMP	Transmembrane pressure (Pa, N m^{-2})
TS	Total solid content (g L^{-1})
UF	Ultrafiltration

capacity in peptides (Hartmann & Meisel, 2007; Nimalaratne, Lopes-Lutz, Schieber, & Wu, 2011). One of the highly antioxidant peptides identified in soy protein hydrolysates is leucine-leucine-proline-histidine-histidine peptide (leu-leu-pro-his-his) (Chen et al., 1998). The his-his portion of this leu-leu-pro-his-his peptide was the primary contributor to its antioxidative property. It was found that pro-his-his, as an individual peptide, displayed the highest antioxidant capacity. Furthermore, the presence of a leucine or proline residue at the amino-terminus of a his-his-containing peptide enhanced the antioxidant capacity and hydrophobicity of the peptides (Hartmann & Meisel, 2007). Histidine and other aromatic amino acids contribute to antioxidant capacity, due to their ring structures (Nimalaratne et al., 2011). However, the antioxidant capacity of a histidine residue is greater within a peptide, compared to when it stands alone, due to synergistic effects with other amino acid residues, like those from proline and leucine.

ORAC antioxidant capacity assay is a time consuming method with multiple steps and prolonged analysis times. FCR assay is relatively less time consuming compared to the ORAC assay, but requires multiple steps during preparation and to obtain measurements for analysis (Margraf, Karnopp, Rosso, & Granato, 2015). Given the importance of these assays and the challenges faced with their use, novel and rapid methods to capture relative antioxidant capacities of samples are of interest in food, nutrition and medicine.

Intrinsic fluorescence refers to fluorescence caused by a chemical compound, known as a fluorophore, which can re-emit light at a higher wavelength upon excitation at a lower wavelength (Zhang, Müller, Wu, & Feld, 2000). Intrinsic fluorescence spectroscopy, a non-destructive analytical tool, presents many advantages and applications in biological processes; it is rapid, has high sensitivity, specificity, and reproducibility. Many naturally occurring fluorophores are present in the cellular environment including tyrosine, tryptophan, phenylalanine, retinol, and riboflavin (Christensen, Nørgaard, Bro, & Engelsen, 2006; Teixeira, Duarte, Carrondo, & Alves, 2011). Fluorescence analysis has been used to investigate protein structures (based on the intrinsic fluorescence of aromatic amino acids) (Bron, Ribeiro, Azzolini, Jacomino, & Machado, 2004; Christensen et al., 2006). At a given excitation wavelength (Ex), fluorescence intensities of a sample can be collected at a range of emission wavelengths (Em) to construct a fluorescence landscape, known as an excitation-emission matrix (EEM). The analysis of these EEMs is challenging due to the high volume of data points and the high degree of co-linearity present between intensity data captured at different excitation and emission wavelength combinations.

Multivariate statistical methods, such as principal component analysis (PCA), can be used for extracting specific and sensitive information from the fluorescence EEM intensity data. PCA is often used to capture variances and extract significant systematic trends in sample data sets that contain large amounts of variables (Christensen et al., 2006; Oliveira, Calado, Ares, & Granato, 2015; Peiris, Budman, Moresoli, & Legge, 2009; Zielinski et al., 2014). PCA can be used to observe the correlations between fluorescence signals and important bioprocess variables (Teixeira et al., 2011). A detailed description of PCA can be found in Eriksson, Johansson, Kettaneh-Wold, and Wold (2001).

The objectives of this work were to investigate the tryptophan and tyrosine content of soy protein hydrolysate fractions produced during sequential UF and NF membrane operations by intrinsic fluorescence spectroscopy and PCA, and evaluate potential correlations with the ORAC and FCR antioxidant capacity of these fractions.

2. Materials and methods

2.1. Materials

Soy protein isolate (SPI) PRO-FAM 974 powder was obtained from Archer Daniels Midland Company, Decatur, IL, USA. Pepsin from porcine stomach mucosa, pancreatin mixture from porcine pancreas, 99.9% sodium borate decahydrate (S9640-500G), sodium dodecyl sulfate (L4509-250G), phthaldialdehyde (P0657-5G), FCR reagent (F9252), 2,2′-azobis-2-methyl-propanimidamide dihydrochloride (AAPH, 440914-25G), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; 238813-1G) were obtained from Sigma-Aldrich, St. Louis, MO, USA. *L*-(+)-α-phenyl-glycine (2935-35-5) from MP Biomedical, Solon, OH, USA; Fluorescein (065-00252) from Wako Pure Chemical Industries, Osaka, Japan; and sodium carbonate (SX0400-1 500G) from EMD Chemicals, Gibbstown, NJ, USA. Hollow fibre polysulfone UF membrane module (UFP-10-E-4MA; 10 kDa MWCO, active area of 4.2×10^{-2} m^2) was purchased from Amersham Biosciences, Westborough, MA, USA. A G10 thin film composite NF membrane (2.5 kDa MWCO, active area of 1.4×10^{-2} m^2) was purchased from Sterlitech Corporation, Kent, WA, USA.

2.2. Preparation of soy protein hydrolysates

SPI was dissolved in MilliQ water to obtain a 3.12% (w/v) solution. The SPI solution was either subjected to heat treatment (95 °C during 5 min) or subjected to enzymatic hydrolysis as described