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Antioxidant potential of edible mushroom (*Agaricus bisporus*) protein hydrolysates and their ultrafiltration fractions

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ABSTRACT

Mushroom protein isolate (MPI) from *Agaricus bisporus* was hydrolyzed using Alcalase, Pancreatin, Flavourzyme, Alcalase-Pancreatin and Alcalase-Flavourzyme. The obtained hydrolysates (MPHs) were ultrafiltered to generate peptide fractions (UFs) of molecular sizes (<1, 1–3, 3–5 and 5–10 kDa). The electrophoretic profile results indicated that the enzymatic systems were efficient in hydrolyzing the MPI into low molecular weight peptides. Hydrolysate yields of >57% and protein recoveries of >43% were obtained. Effective concentration that scavenged 50% (EC_{50}) of DPPH radicals was similar for the MPHs while inhibition against linoleic acid oxidation was strongest (66.49%) for Alcalase-Flavourzyme hydrolysate on day 5 of incubation. UFs exhibited a concentration-dependent FRAP, with the highest activity for fractions from Alcalase and Pancreatin recorded in 1–3 kDa. The antioxidant activities of MPHs and their UFs suggested that they could be potential bioactive ingredients for use in the formulation of functional foods as well as natural antioxidants in lipid food systems.

1. Introduction

Free radicals are constantly generated through the normal physiological reactions in humans for diverse functions like signaling roles and providing defense against infections (Dhaval, Yadav, & Purwar, 2016). However, when produced in excess, the normal cellular physiological functioning is altered due to protein malformation, DNA mutation, oxidation of membrane phospholipids and modification of the low density lipoproteins (Alaiz, Beppu, Ohishi, & Kikugawa, 1994; Lee, Koo, & Min, 2004). This leads to several neurodegenerative disorders, diabetes, arthritis, atherosclerosis, cancer, Alzheimer's disease, Parkinson's disease and ageing problems (Halliwell, 1994). Moreover, free radical-mediated lipid peroxidation in food products is a major global problem in the food industry (Sarmadi & Ismail, 2010). When the endogenous protection system under certain circumstances fails, oxidative stress sets in resulting into an unstable cellular state (Maritim, Sanders, & Watkins, 2003). In food systems, synthetic antioxidants including butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), tertiary butyl hydroquinone (TBHQ) and propyl gallate (PG) have been used to prevent lipid peroxidation (Girgih et al., 2015). However, the use of synthetic antioxidants has been put under strict regulation due to their potential health risks associated with long-term use (Ndhlala, Moyo, & Van Staden, 2010). Thus, natural antioxidants from food protein-derived bioactive peptides has been a focus of attention of numerous studies. These bioactive peptides can be released from the native protein molecule through gastrointestinal digestion, *in vitro* chemically, enzymatic hydrolysis and fermentation (Boschin, Scigliuolo, Resta, & Arnoldi, 2014; Onuh, Girgih, Aluko, & Aliani, 2014).

Numerous studies have reported antioxidant properties of enzymatic hydrolysates from various protein sources; chicken skin (Onuh et al., 2014), bambara groundnut (Arise et al., 2016), black bean (Evangelho et al., 2017) and corn (Jin, Liu, Zheng, Wang, & He, 2016). The antioxidant activities of these hydrolysates have been reported to depend on enzyme specificity, molecular weight, the degree of hydrolysis and amino acid composition (Alashi et al., 2014; He, Girgih, Malomo, Ju, & Aluko, 2013).

Agaricus bisporus (white button mushroom) is an edible fungus and the world's leading cultivated mushroom with yields accounting for 70% of the total edible fungi (Kalac, 2013). Its worldwide consumption is attributed to its delicious taste and flavour in addition to being a rich source of nutrients including proteins, essential amino acids, minerals and vitamin B (Liu, Jia, Kan, & Jin, 2013). Several bioactivities from *A. bisporus* have been reported including ACE inhibitory activity (Lau, Abdullah, Shuib, & Aminudin, 2014), hypoglycaemic (Mao, Mao, & Meng, 2013) antioxidant and

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antimicrobial (Smolskaitė, Venskutonis, & Talou, 2015). However, no reports have been made on the antioxidant potential of *A. bisporus* protein hydrolysate(s). In this study, *A. bisporus* protein isolate was hydrolyzed using single (Alcalase, Pancreatin and Flavourzyme) and sequential (Alcalase-Pancreatin and Alcalase-Flavourzyme) enzymatic processes. The aim was to generate mushroom protein hydrolysates (MPHs), fractionate the hydrolysates into peptides of various molecular weights and evaluate the potential antioxidant activity of these samples using different *in vitro* antioxidant evaluation systems.

2. Material and methods

2.1. Materials

Fresh fruiting bodies of *A. bisporus* were purchased from a local market (Nanjing, China). Proteases Alcalase 2.4U/g and Flavourzyme 500MG were from Solarbio Life Sciences (Beijing, China). Pancreatin 4.6x10^{6}U/g was bought from Ryon Biological Technology (Shanghai, China). Reduced L-glutathione (GSH), ultrafiltration cassettes with 1, 3, 5, and 10 kDa molecular weight cut-off (MWCO) sizes were procured from Millipore Corp. (Billerica, MA, USA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 30% and 40% Acrylamide/Bis were obtained from Sigma-Aldrich (St. Louis, MO, USA) Ferrozine iron reagent, linoleic acid, and Tricine were from Macklin Biochemical (Shanghai, China). Other reagents used were of analytical grade.

2.2. Sample Preparation

The fruiting bodies were washed, sliced and immediately freeze dried after which they were ground to pass through an 80-mesh standard screen. The powder was packed in air-tight plastic bags and stored at −20 °C.

2.2.1. Protein extraction

Protein extraction was done according to Jeurink, Noguera, Savelkoul, and Wichers (2008) with modifications. The mushroom powder was added to 5% ice-cold acetic acid solution (1:15, w/v) in the presence of 2-mercaptoethanol (0.1%) and stirred for 3 h. The slurry was centrifuged at 15,500*g* for 15 min at 4 °C, supernatant filtered with Whatman filter paper No. 5, and the solubilized proteins precipitated overnight (12 h) at 4 °C by the addition of ammonium sulphate to 75% saturation. The mixture was centrifuged at 25,000*g* for 20 min at 4 °C and pellets re-dissolved in a small volume of distilled water. Re-dissolved extract was desalted by dialyzing (10 kDa) against distilled water for 24 h at 4 °C. The dialysate was lyophilized to produce mushroom protein isolate (MPI) powder which was stored at −20 °C. Protein content of MPI was determined by the modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978) with bovine serum albumin used as a standard.

2.2.2. Preparation of mushroom protein isolate hydrolysates (MPH)

The MPI was hydrolyzed separately with Alcalase, Flavourzyme and Pancreatin at their optimum hydrolysis conditions (Table 1). MPI (5% w/v, protein content basis) was dispersed in deionized water in a 250 mL glass beaker that was placed on a magnetic stirring hot plate equipped with an external temperature probe and a pH electrode for temperature and pH control, respectively. The mixture was heated to the appropriate temperature and the pH adjusted using 1 M NaOH and allowed to equilibrate for 30 min. Enzymatic hydrolysis was initiated by addition of each enzyme at an enzyme/substrate ratio of 2.5% (w/w, protein basis). Hydrolysis was carried out at the stated conditions (Table 1) maintaining constant pH of the reaction mixture by the pH-stat technique with 0.5 M NaOH. For sequential hydrolysis, Alcalase was used first, reaction terminated after 2 h by immersing the digest in boiling water for 10 min, cooling to the appropriate temperature and adjusting the pH for the subsequent enzyme (Pancreatin or Flavourzyme) and allowing to equilibrate for 30 min. Hydrolysis with the second enzyme was done for 2 h, maintaining temperature and pH constant for each enzyme. Alkaline consumption over the 4 h hydrolysis period was recorded after every 15 min. To terminate the hydrolysis, pH was adjusted to 7.0 with 1 M NaOH or 1 M HCl and the digest immediately immersed in boiling water for 15 min. After cooling to room temperature, the undigested proteins were precipitated by centrifugation at 21,000*g* for 20 min at 4 °C. The supernatants were lyophilized as mushroom protein hydrolysates (MPHs) powders (AH, PH, FH, APH and AFH for Alcalase, Pancreatin, Flavourzyme, Alcalase-Pancreatin and Alcalase-Flavourzyme hydrolysates, respectively). MPHs were stored at −20 °C. The protein content of the lyophilized MPHs was determined as described in section 2.2.1. MPH percent yields and protein recovery were determined as follows:

$$\textit{MPH yield}\ (\%) = \left[\frac{\textit{weight of hydrolysate}}{\textit{weight of MPI used for hydrolysis}}\right] \times 100$$

$$\textit{MPH protein recovery}\ (\%) = \left[\frac{\textit{weight of protein in hydrolysate}}{\textit{weight of protein in MPI used for hydrolysis}}\right] \times 100$$

2.2.3. Ultrafiltration of the hydrolysates

A 0.3% solution (w/v) of MPHs was prepared in distilled water. The solution was ultrafiltered by sequentially passing it through Pellicon 2 Mini cassettes with MWCO of 1, 3, 5, and 10 kDa. The retentate from 1 kDa was passed through 3 kDa; retentate from 3 kDa was passed through 5 kDa whose retentate was finally passed through the 10 kDa cassette. Permeates from each cassette were collected as ultrafiltered peptide fractions (UFs) with molecular weight (MW) distributions of <1, 1–3, 3–5 and 5–10 kDa, respectively. The UFs were concentrated by rotary evaporation (45 °C), freeze dried and stored at −20 °C. The protein content of the UFs was determined as described in section 2.2.1 while the UF yields and protein recovery were calculated as follows:

$$\textit{UF yield}\ (\%) = \left[\frac{\textit{weight of UF}}{\textit{weight of respective hydrolysate}}\right] \times 100$$

$$\textit{UF protein recovery}\ (\%) = \left[\frac{\textit{weight of protein in UF}}{\textit{weight of protein in respective hydrolysate}}\right] \times 100$$

Table 1
Hydrolysis conditions for the various enzymatic hydrolyses.

	Alcalase	Pancreatin	Flavourzyme	Alcalase-Pancreatin	Alcalase-Flavourzyme
Temp (°C)	50	37	50	50 then 37	50
pH	9.0	7.5	7.0	9.0 then 7.5	9.0 then 7.0
Time (h)	4	4	4	2 then 2	2 then 2
E/S ratio (w/w)	2.5/100	2.5/100	2.5/100	2.5/100	2.5/100
Substrate concentration (w/v)	5/100	5/100	5/100	5/100	5/100