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Enzymatic preparation of mushroom dietary fibre: A comparison between analytical and industrial enzymes

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ABSTRACT

A comparative study on preparing dietary fibres (DFs) from three mushroom sclerotia, namely, *Pleurotus tuber-regium* (PTR), *Polyporus rhinocerus* (PR) and *Wolfiporia cocos* (WC), using analytical or industrial enzymes (including α-amylase, protease and amyloglucosidase), was conducted. Apart from enzyme activity and purity, their effects on the yield of sclerotial DF as well as its major components, such as β-glucans, chitin and resistant glycogen (RG), were investigated and compared. The activities of all industrial enzymes were significantly lower than those of their corresponding analytical ones, except for the Fungamyl® Super MA, which had the highest α-amylase activity (6395 U/g). However, this fungal α-amylase was less able to digest the three sclerotial glycogens when compared with the bacterial alternatives. Amongst all tested enzymes, only analytical and industrial amyloglucosidases were found to have significant amount of contaminating cellulase (7.05–7.07 U/ml) and lichenase (4.62–4.67 U/ml) activities, which would cause endo-depolymerization of the β-glucan-type cell wall components (3.39% reduction in glucose residue after RG correction) of the PTR, leading to a marked α-amylase hydrolysis of its otherwise physically-inaccessible cytoplasmic glycogen (20.3% reduction in RG content). Commercial production of the three novel sclerotial DFs, using the industrial enzymes, would be feasible since, in addition to their economic advantage, both the yield (PTR: 81.2%; PR: 86.5%; WC: 96.2% of sample DM) and total non-starch polysaccharide contents (PTR: 88.0%; PR: 92.5%; WC: 91.1% DF-rich materials of DM) of their resulting sclerotial DFs were comparable to the levels of those prepared using analytical enzymes.
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1. Introduction

The definition of dietary fibre (DF) proposed by the American Association of Cereal Chemists (AACC) defines DF as being made up of edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine, as well as having beneficial physiological effects, such as laxation, blood glucose attenuation and/or blood cholesterol attenuation (AACC Report, 2000). The increasing public awareness of DF's potential health benefits has undoubtedly encouraged food manufacturers to develop fibre-enriched or fibre-fortified food products such as snack foods, beverages, cookies and canned meat. (McKee & Latner, 2000; Sloan, 2001). Nowadays, most DF ingredients (such as cereals-based, fruits-based and legumes-based DF) originate from the by-products of their corresponding food processing (e.g. milling, juice extraction and de-hulling), followed by different refining steps (such as grinding, sieving, bleaching and defatting) in order to meet a wide range of customer requirements (Nelson, 2001). Because of the highly competitive market of fibre-enriched food products, exploration of alternative sources of DF, as well as DF preparation methods, is urgent.

Mushroom sclerotium, from a functional point of view, can be described as a nutrient-rich, multihyphal aggregated structure that can remain dormant during unfavourable conditions until the environment is suitable for its fruiting bodies to germinate (Willetts & Bullock, 1992; Wong & Cheung, 2008). The major fibrillar and amorphous components in the cell wall of its interwoven hyphae are chitin and β-glucans (with 1,3 and 1,6 linkages to different extents) (Backhouse & Willetts, 1984; Bullock, Willetts, & Ashford, 1980; Kohn & Grenville, 1989). The chemical composition of its extracellular matrix, which consists of highly hydrated materials expanding and filling the interhyphal spaces within the sclerotium, is also mainly comprised of a β-1,3 glucan backbone with β-1,6-linked side branches (Backhouse & Willetts, 1984; Bullock et al., 1980; Dubourdieu, Ribéreau-Gayon, & Fournet, 1981). Since chitin- and β-glucose-based polysaccharides cannot be digested and absorbed in the human intestine, the mushroom sclerotium obviously contains abundant cell wall and extra-cellular matrix materials that can be classified as DF (Cheung, 1997; Wasser & Weis, 1999). Our previous studies have found that the total DF content of three mushroom sclerotia, namely, *Pleurotus tuber-regium*

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(Fr.) Sing., *Polyporus rhinocerus* Cooke and *Wolfiporia cocos* (Schw.) Ryv. et Gilbn was ⩾80%, on a dry matter basis (DM), of which >95% was insoluble DF (Cheung, 1997; Cheung & Lee, 1998; Wong, Cheung, & Wu, 2003). These findings clearly indicated that these three mushroom sclerotia might act as a novel source of DF in the DF industry.

The most widely accepted method for total DF determination is the AOAC enzymatic-gravimetric method 985.29 (AOAC International, 2000) which involves the use of three analytical enzymes: heat stable α-amylase (EC 3.2.1.1), protease (EC 3.4.21.14) and amyloglucosidase (EC 3.2.1.3). Although, there are distinct advantages (e.g. energy saving, environmentally friendly and non-toxic) when using these enzymes for scale-up DF preparation in the food industry, their remarkably high cost, especially the protease, is a major concern. Recently, an enzymatic procedure, modified from the AOAC methods 985.29 for preparing the three novel sclerotial DFs, using industrial food grade enzymes, was developed in our laboratories (Wong & Cheung, 2005a). In this current experiment, a comparative study on preparing sclerotial DFs from *P. tuber-regium*, *P. rhinocerus* and *W. cocos*, using our previously developed enzymatic procedure with analytical or industrial enzymes, was conducted. Apart from determining the activity and purity of the tested enzymes, their effects on the yield of DF, as well as of its major components, e.g. β-glucan, resistant glycogen (RG) and chitin, were also investigated and compared. Since fungi usually secrete glycolytic enzymes to digest and utilise their storage glycogen for growth at different developmental stages (including the sclerotium), it would be worth whilst to find an alternative. An industrial fungal α-amylase namely, Fungamyl® Super MA, which is commonly used in the food industry, was applied in our previous study (Wong & Cheung, 2005a). The potential application of industrial enzymes for preparing the three sclerotial DFs in the food industry is therefore now considered.

2. Materials and methods

2.1. Sample preparation

Sclerotia of *P. tuber-regium*, *P. rhinocerus* and *W. cocos* were obtained from the Sanming Mycological Institute in the Fujian Province of China. All sclerotia were cleaned, dried and milled to a particle size of 0.5 mm, as previously described (Wong et al., 2003).

2.2. Analytical and industrial enzymes

Except for one highly purified amyloglucosidase (EC 3.2.1.3) that was bought from Megazyme (E-AMGDF, Megazyme International Ireland Ltd., Wicklow, Ireland), all analytical enzymes, including heat-stable α-amylase (EC 3.2.1.1), amyloglucosidase (EC 3.2.1.3) and protease (EC 3.4.21.14), were purchased from Sigma (catalogue nos. A3306, A9913 and P3910, respectively; Sigma Chemical Co., St. Louis, MO, USA). Industrial glycolytic enzymes, including Termamyl® Supra, Fungamyl® Super MA and Dextrozyme™ E, as well as industrial proteolytic enzymes, including Alcalase® 2,4 L FG and Protamex™, were kindly donated by Novozymes A/S (Bagsvaerd, Denmark). The origin of the analytical and industrial enzymes, as well as their individual working temperature and pH applied, in this study, are shown in Table 1.

In order to estimate and compare the activity, purity and efficacy of the analytical and industrial enzymes (Wong & Cheung, 2005a), in this study, all assays were performed under the same conditions (including the buffering system) as those described in the procedures originating from the AOAC Methods 985.29 (Table 1).

2.3. Enzyme activity

The activities of all tested α-amylases and amyloglucosidases were evaluated using the methods reported previously (McCleary, Bouhet, & Driguez, 1991; McCleary & Sturgeon, 2002; Sheenan & McClearly, 1988). In brief, after hydrolysing the highly purified end-blocked *p*-nitrophenyl malto-heptaoside (O-BNPG4, Megazyme) and *p*-nitrophenyl β-maltoside (N1884, Sigma) by the tested α-amylases and amyloglucosidases, respectively, their resulting *p*-nitrophenyl saccharides were then stoichiometrically broken down by excessive amounts of α-glucosidase (for α-amylase) or β-glucosidase (for amyloglucosidase) in order to release the free *p*-nitrophenol. The enzymatic reaction was terminated by addition of 1% tri-sodium phosphate (pH 11, for α-amylase) or 1% Trizma Base (pH 12, for amyloglucosidase) and the colour of phenolate developed (which was directly related to the levels of α-amylases and amyloglucosidases tested) was then measured colorimetrically at 400 nm (for α-amylase) or 410 nm (for amyloglucosidase). One unit of α-amylase or amyloglucosidase activity (U) is defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol per minute under defined assay conditions. Proteases activity was determined by measuring the absorbance of those soluble peptide

Table 1
Origin, manufacturer, working temperature, and pH of the tested analytical and industrial enzymes used for sclerotial DF preparation.

Enzyme	Grade	Origin(s)[a]	Manufacturer	Working temperature used in this study (°C)	Working pH used in this study[b]
α-Amylase					
Sigma A3306	Analytical	Bacterial (*Bacillus licheniformis*)	Sigma	98.0 ± 0.10	6.00 ± 0.20
Termamyl® Supra	Industrial	Bacterial (*Bacillus licheniformis*)	Novozymes A/S	98.0 ± 0.10	5.40 ± 0.20
Fungamyl® Super MA	Industrial	Fungal (*Aspergillus oryzae*)	Novozymes A/S	50.0 ± 0.10	5.00 ± 0.20
Amyloglucosidase					
Sigma A9913	Analytical	Fungal (*Aspergillus niger*)	Sigma	60.0 ± 0.10	4.30 ± 0.20
Megazyme E-AMGDF	Analytical	Fungal (*Aspergillus niger*)	Megazyme	60.0 ± 0.10	4.30 ± 0.20
Dextrozyme™ E	Industrial	Bacterial (*Bacillus deramificans*) & Fungal (*Aspergillus niger*)	Novozymes A/S	60.0 ± 0.10	4.30 ± 0.20
Protease					
Sigma P3910	Analytical	Bacterial (*Bacillus licheniformis*)	Sigma	60.0 ± 0.10	7.50 ± 0.20
Alcalase® 2,4 L FG	Industrial	Bacterial (*Bacillus licheniformis*)	Novozymes A/S	60.0 ± 0.10	7.50 ± 0.20
Protamex™	Industrial	Bacterial (*Bacillus* spp.)	Novozymes A/S	50.0 ± 0.10	6.50 ± 0.20

[a] According to the corresponding manufacturer's information.
[b] In order to estimate and compare the activity, purity and efficacy of the analytical and industrial enzymes during their preparation of the three novel sclerotial DFs using our previously developed enzymatic procedures (Wong & Cheung, 2005a), in this study, the buffering system used for all assays was the same as that described in the procedures, i.e. 0.08 M sodium phosphate buffer (pH 6.00 ± 0.20), followed by pH adjustment to the working pH of individual investigated enzymes.

Table 2
Purified substrates for detecting possible contaminating enzymes during sclerotial DF preparation.

Substrate	Manufacturer	Major contaminating enzyme(s) that can be detected[a]
Pachyman	Megazyme, P-PACHY	Exo-1,3-β-glucanase; endo-1,3-β-glucanase
Barley β-glucan	Megazyme, medium viscosity, P-BGBM	Lichenase (mixed linked endo-1,3;1,4-β-glucanase)
Carboxymethyl cellulose 4 M	Megazyme, P-CMC4M	Cellulase (endo-1,4-β-glucanase)
Chitin	Sigma, C9752	Chitinase

[a] According to the corresponding manufacturer's information.

materials in the filtered digesta after precipitation with 30% trichloroacetic acid (TCA) at 280 nm (McCleary, 1999; McCleary, 2000). A soluble soy protein isolate was used as the substrate (ICN Biochemicals, Inc., OH, USA) and the assay was standardised using a tyrosine standard curve (0–1000 μg of tyrosine). One protease unit (tyrosine equivalent U) is defined as the amount of enzyme required to liberate (and solubilise in TCA) 1 μmol of tyrosine equivalents per minute from the soluble soy protein isolate under defined conditions.

2.4. Enzyme purity

For assessing the purity, each analytical or industrial enzyme was evaluated for the presence of five potential contaminating enzymes (β-glucanases and chitinase) by separately incubating them with their corresponding highly purified substrates, as indicated in Table 2. In short, 50 mg of each purified substrate was dissolved in 5 ml of 0.08 M sodium phosphate buffer with the working pH of the individual analytical or industrial enzyme (Table 1). The mixture was then separately incubated with corresponding analytical or industrial enzymes (possessing the same standardised enzyme activity as shown in Table 4) for exactly 30 min at each individual working temperature (Table 1). All enzymatic reactions were terminated by adding 5 ml of 60 mM tri-sodium phosphate (pH 12), vortex-mixed and further adjusted to pH 4.0 (digesta from β-glucanases) or pH 5.0 (digesta from chitinase) with 1 M HCl. Subsequently, each diluted enzyme digest was centrifuged (1000*g*, 25 °C, 10 min) and 100 μl of supernatant were completely transferred into a new tube. For assessing the β-glucanases, the resulting β-linked gluco-oligosaccharides in the supernatant were further hydrolysed to glucose by incubation with 100 μl of β-glucosidase (0.8 U, EC 3.2.1.21, originating from *Aspergillus niger*; E-BGLUC, Megazyme) at 50 °C for 10 min. Consequently, the glucose liberated directly and indirectly from both exo- and endo-β-glucanases was quantified with a glucose assay kit (K-GLUC, Megazyme, Ireland). Similarly, in the case of chitinase, the resulting *N*-acetyl chitino-oligosaccharides in the supernatant were further hydrolysed to *N*-acetylglucosamine by incubation with 400 μl of β-*N*-acetylglucosaminidase [5 U, EC 3.2.1.52, originating from *Canavalia ensiformis* (Jack bean); A2264, Sigma] at 25 °C for 10 min and the amount of *N*-acetylglucosamine released in the digesta was determined by the widely used Schales procedure (Schales & Schales, 1945). One unit of side activity (U) is defined as the amount of contaminating enzyme required to release 1 μmol of glucose or *N*-acetylglucosamine per minute from the corresponding substrate under defined conditions.

2.5. Effects of different enzyme combinations on the yield of sclerotial DF and their major DF components

2.5.1. Preparation of sclerotial DF by different combinations of analytical and industrial enzymes

Except for the choice of α-amylase, protease and amyloglucosidase used, the three novel sclerotial DFs were prepared as described in our previously developed enzymatic procedures, starting with 54 g of powder from each mushroom sclerotium (Wong & Cheung, 2005a). As shown in Table 3, preparing the sclerotial DFs from *P. tuber-regium*, *P. rhinocerus* and *W. cocos*, using enzyme combination I (denoted as PTR1, PR1 and WC1, respectively), involved all analytical enzymes from Sigma that are commonly applied in the AOAC enzymatic-gravimetric methods. For enzyme combination II, it involved the use of the highly purified amyloglucosidase from Megazyme to substitute the Sigma one, since the amyloglucosidase from Sigma was found to possess substantial amount of contaminating cellulase and lichenase (Table 5), which might induce endo-depolymerization of the β-glucan-type DF component of the three sclerotia. The industrial enzyme combina-

Table 4
Enzyme activity of the tested analytical and industrial enzymes.[a]

Enzyme	Physical form of enzyme	Activity (U/ml or g of enzyme)	Enzyme activity (U) used in this study for each gramme of sample[b]
α-Amylase			
Sigma A3306	Liquid	3273 ± 63.26a	164 (50.0)[c]
Termamyl® Supra	Liquid	3934 ± 61.73a	164 (41.6)[d]
Fungamyl® Super MA	Powder	6395 ± 36.38b	164 (256.0; 100 mg/ml)[d]
Amyloglucosidase			
Sigma A9913	Liquid	619 ± 10.9a	61.9 (100)[c]
Megazyme E-AMGDF	Liquid	321 ± 13.8b	61.9 (193)[d]
Dextrozyme™ E	Liquid	454 ± 14.6c	61.9 (136)[d]
Protease			
Sigma P3910	Powder	8113 ± 29.2a	40.6 (100; 50 mg/ml)[c]
Alcalase® 2,4 L FG	Liquid	5101 ± 24.3b	40.6 (7.95)[d]
Protamex™	Powder	1801 ± 29.6c	40.6 (225; 100 mg/ml)[d]

[a] All activities of individual analytical and industrial enzymes were measured under the conditions described in our previously developed enzymatic procedures. Data are mean values of three determinations ± SD. For each group of enzymes, means in the column with different letters (a–c) are significantly different (one-way ANOVA using the Kruskal–Wallis Test, $p < 0.05$; Multiple comparisons, using Mann–Whitney U-Test with Bonferroni correction, $p < 0.025$).

[b] Enzyme activity (U) used in this study for each gramme of sample with reference to that of the corresponding one from Sigma.

[c] Figures in parentheses are volumes (ul) of Sigma's enzymes recommended for 1 g of sample in the AOAC method (AOAC International, 2000).

[d] Figures in parentheses are enzyme volumes (ul) used in this study for 1 g of sample, which providing the same enzyme activity as that of the corresponding one from Sigma.

Table 3
Combinations of the tested industrial and analytical enzymes for preparing the three sclerotial DFs.

Combination	Grade	α-Amylase	Protease	Amyloglucosidase	Sclerotial DFs
I	Analytical	Sigma A3306	Sigma P3910	Sigma A9913	PTR1 or PR1 or WC1[a]
II	Analytical	Sigma A3306	Sigma P3910	Megazyme E-AMGDF	PTR2 or PR2 or WC2
III	Industrial	Fungamyl® Super MA	Alcalase® 2,4 L FG	Dextrozyme™ E	PTR3
IV	Industrial	Fungamyl® Super MA	Protamex™	Dextrozyme™ E	PR4 or WC4

[a] PTRn or PRn or WCn with $n = 1, 2, 3$ or 4 representing the DFs obtained by corresponding enzyme combinations I, II, III or IV from the sclerotia of *P. tuber-regium* (PTR), *P. rhinocerus* (PR) or *W. cocos* (WC).

Table 5
Enzyme purity of the tested analytical and industrial enzymes.[a]

Enzyme	Exo- and endo-1,3-β-glucanase	Lichenase (mixed linked endo-1,3(4)-β-glucanase)	Cellulase (endo-1,4-β-glucanase)	Chitinase
α-Amylase				
Sigma A3306	ND[b]	ND	ND	ND
Termamyl® Supra	<0.001	<0.001	<0.001	<0.001
Fungamyl® Super MA	<0.001	<0.001	<0.001	<0.001
Amyloglucosidase				
Sigma A9913	ND	4.621	7.051	ND
Megazyme E-AMGDF	ND	0.021	0.047	ND
Dextrozyme™ E	<0.001	4.671	7.067	<0.001
Protease				
Sigma P3910	ND	ND	ND	ND
Alcalase® 2,4 L FG	<0.001	<0.001	<0.001	<0.001
Protamex™	<0.001	<0.001	<0.001	<0.001

[a] One unit of side activity is defined as the amount of contaminating enzyme required to release 1 μmol of glucose or *N*-acetylglucosamine per minute from corresponding substrate under defined conditions. Data are mean values of three determinations.
[b] ND = not detected.

tions used in our previously developed enzymatic procedures (Wong & Cheung, 2005a), for the *P. tuber-regium* as well as *P. rhinocerus* and *W. cocos* sclerotia, were Termamyl®, Supra-Alcalase® 2,4 L, FG-Dextrozyme™ E and Termamyl® Supra-Protamex™–Dextrozyme™ E. In enzyme combinations, III and IV, the bacterial α-amylase, Termamyl® Supra was substituted by another industrial one having a fungal origin, namely, Fungamyl® Super MA. The yield of the three novel sclerotial DFs resulting from the above-mentioned enzyme combinations (I, II, III or IV) were determined as reported previously (Wong & Cheung, 2005a).

2.5.2. Biochemical characterisation of the enzymatically prepared sclerotial DF

The glucose and *N*-acetyl-glucosamine residue, as well as the total non-starch polysaccharide (NSP) contents (the sum of all neutral and amino sugars, as well as uronic acid contents) of the three sclerotial DFs obtained were analysed as previously described (Wong et al., 2003). The content of resistant glycogen (RG) in each sclerotial DF was evaluated according to the AOAC Method 996.11. (AOAC International, 2000).

2.6. Statistical analysis

All data were presented as mean values of three determinations ± SD and analysed by non-parametric one-way ANOVA using the Kruskal–Wallis Test ($p < 0.05$). When the ANOVA indicated a significant effect, multiple comparisons amongst the samples were also performed by the Mann–Whitney U-Test with Bonferroni correction ($p < 0.025$) in order to detect significant differences amongst groups (SSPS, 1999).

3. Results and discussion

3.1. Enzyme activity

The activities of all industrial enzymes were significantly ($p < 0.025$) lower than those of their corresponding analytical ones from Sigma, except for the Fungamyl® Super MA, which had the highest α-amylase activity (6395 U/g). However, its activity was found to be far below the level (240,000 U/g) of the commercially available analytical α-amylase with the same fungal origin (*A. oryzae*, Megazyme). Since Sigma's α-amylase and Termamyl® Supra have the same bacterial origin (Table 1) and no significant difference was obtained between their activities (Table 4), this result might imply that the enzyme concentrations in these two preparations were similar. For both proteases (Sigma, Alcalase® 2,4 L FG and Protamex™) and amyloglucosidases (Sigma, Megazyme and Dextrozyme™ E) tested, the activities amongst each group of enzymes were significantly ($p < 0.025$) different from each other with descending order of activity: Sigma > Alcalase® 2,4 L FG > Protamex™ (ranging from 1801 to 8113 U/g or ml; Table 4) and Sigma > Dextrozyme™ E > Megazyme (ranging from 321.4–619.0 U/ml; Table 4), respectively. Although, Sigma's protease and Alcalase® 2,4 L FG share the same bacterial origin (Table 1), their activities were significantly different ($p < 0.025$), suggesting a difference in their enzyme concentration. McCleary (1999, 2000) reported that insufficient α-amylase concentration would overestimate the yield of TDF from samples particularly rich in resistant starch (RS) but the effect of different levels of amyloglucosidase and protease activities on the yield of TDF content was insignificant. In order to avoid the incomplete removal of non-DF materials (glycogen and proteins) of the three sclerotia during their DF preparation in 2.5.1, the activity of all industrial enzymes used was standardised with that of their corresponding Sigma ones (Table 4). For example, in the case of Dextrozyme™ E, 136.2 μl for each gramme of sample was used since this volume would provide the same amyloglucosidase activity (61.9 U) as did the 100 μl of Sigma A9913. As shown in Table 4, for providing the same enzyme activity as that of the Sigma, the volumes of the industrial glycolytic and proteolytic enzymes used were remarkably small (only ranging from 7.95 to 256 μl for each gramme of sample); thus, the corresponding cost of industrial enzymes compared to that of the analytical ones would be very economical, especially in the case of protease. This finding suggested that scale-up preparation of the three novel sclerotial DFs using the industrial enzymes would be a promising alternative.

3.2. Enzyme purity

Our previous study reported that the two major components of the three novel sclerotial DFs were chitin (glucosamine residue: 1.73–5.44% of total DF DM) and β-glucan (glucose residue: 77.7–88.6% of total DF DM) (Wong & Cheung, 2005a). Besides, the structures of their β-glucan-type DF components were species-dependent and susceptible to hydrolysis by exo-1,3-β-glucanase (EC 3.2.1.39), endo-1,3-β-glucanase (laminarinase, EC 3.2.1.58), mixed linked endo-1,3(4)-β-glucanase (lichenase, EC 3.2.1.73) and endo-1,4-β-glucanase (cellulase, EC 3.2.1.4) (Wong, Wong, Kwan, & Cheung, 2005). Hence, during enzymatic preparation of the three sclerotial DFs, contamination of the above-mentioned β-glucanases and chitinase (endo-*N*-acetyl-glucosaminase, EC 3.2.2.14) in the tested enzyme(s) might cause endo-/exo-depolymerization of the two major sclerotial DF components, which in turn underestimated the yield of the three sclerotial DFs. As shown in Table 5, amongst all tested enzymes, only Sigma's amyloglucosidases and Dextrozyme™ E were found to have significant amounts of contaminating cellulase (7.05–7.07 U/ml) and lichenase (4.62–4.67 U/ml) activities. Since their levels detected in the Megazyme's amyloglucosidase were extremely low (cellulase: 0.047 U/ml; lichenase: 0.021 U/ml, Table 5), the amyloglucosidase from Megazyme would likely be a better choice for preparing DF from sample particularly rich in cellulose or mixed linked β-glucans, e.g. cereals. According to Megazyme's product information, cellulase is not only able to hydrolyse the carboxymethyl cellulose 4 M, but also the mixed linked barley β-glucan at a slower rate. Since the working pH values (pH 4.30 ± 0.20, Table 1) of both analytical and industrial amyloglucosidases used in this study were similar to that of the cellulase (pH 4.5–5.0) but very different from

that of the lichenase (pH 6.5–7.0), the lichenase level detected in all tested amyloglucosidases would likely be partially due to the cellulase.

3.3. Effect of different enzyme combinations on the yield of sclerotial DF, as well as their major DF components

In contrast to *P. rhinocerus* and *W. cocos*, preparing *P. tuber-regium* DF by using the enzyme combination I (PTR1) not only markedly underestimated the yield (5.71%) ($p < 0.025$), but also significantly ($p < 0.025$) lowered its β-glucan-type DF components (about 3.39% reduction of β-glucose residue after RG correction, Table 6), when compared with the case of PTR2 (using the highly purified amyloglucosidase from Megazyme to substitute the Sigma one). Interestingly, a comparable reduction of yield (3.33%), as well as of β-glucan-type DF components (about 4.73% after RG correction, Table 6) was also observed in PTR3 which was prepared by the enzyme combination III, involving the use of the Dextrozyme™ E. As shown in Table 5, a high level of contaminating cellulase and lichenase activities, similar to that of the Sigma's amyloglucosidase, also was found in this enzyme. These findings might indicate that the two contaminating β-glucanases would only depolymerize the sclerotial β-glucan-type DF component of the *P. tuber-regium* but not those of the other two sclerotia, suggesting the involvement of a β-1,4 linkage in its β-glucan-type DF component, although, this is not commonly found in the hyphal cell wall and extracellular matrix of mushroom sclerotia (Willetts & Bullock, 1992). Our previous linkage analysis on the *P. tuber-regium* polysaccharides (an alkaline soluble fraction) also gave a similar result, suggesting the presence of a β-1,4 linkage apart from the typical β-1,3 and β-1,6 linkages (Cheung & Lee, 2000).

Unlike in many higher plants, granular glycogen, instead of starch, is present throughout the cytoplasm of the cortical and medullary hyphae at all stages of differentiation in sclerotia, and the granular glycogen fills the spaces between other storage bodies and organelles (Willetts & Bullock, 1992). Compared with PTR1, the significantly ($p < 0.025$) higher level of RG content found in the PTR2 (20.3%, Table 6) might be explained by the fact that the highly purified amyloglucosidase from Megazyme helped to maintain the integrity of the β-glucan-type cell wall component in the *P. tuber-regium* sclerotium, which acted as a physical barrier to protect the cytoplasmic glycogen from being digested by the external α-amylase, as previously suggested (Wong et al., 2003). Similarly, in the case of PR1 and WC1, since the high level of contaminating cellulase and lichenase in the amyloglucosidase from Sigma only exerted an insignificant effect on the β-glucan-type cell wall component of the *P. rhinocerus* and *W. cocos* sclerotia, the integrity of their cell walls would be retained, partially protecting their cytoplasmic glycogen. Furthermore, it is worth noting that, within the same mushroom species, sclerotial DFs prepared by both analytical and industrial enzyme combinations shared similar chitin (*N*-acetyl-glucosamine residue) and total NSP contents (Table 6), which were consistent with our previous findings using the industrial α-amylase, Termamyl® Supra (Wong & Cheung, 2005a).

In this study, the efficacy of an industrial fungal amylase, Fungamyl® Super MA was investigated, based on its glycogen-hydrolysing ability by determining the amount of RG content present in the three sclerotial DFs. As shown in Table 6, by using the Fungamyl® Super MA in enzyme combination III and IV, the RG content (ranging from 4.39 to 4.81 g/100 g) in the three sclerotial DFs (PTR3, PR4 and WC4) was notably ($p < 0.025$) higher than those (ranging from 3.71 to 3.91 g/100 g) resulting from the Sigma's α-amylase in combination I (PTR1, PR1 and WC1), as well as the bacterial α-amylase, Termamyl® Supra reported previously (ranging from 3.77 to 3.94 g/100 g; Wong & Cheung, 2005a). This finding implied that Fungamyl® Super MA might possess a weaker hydrolysing ability on the three sclerotial glycogens (under the same enzyme activity) than those of the two bacterial α-amylases (Sigma A3306 and Termamyl® Supra). The weaker hydrolysing ability of Fungamyl® Super MA might be attributed to its relatively lower working temperature suggested by the manufacturer (50 °C, Table 1). After gelatinization, a certain degree of retrogradation would likely occur when the gelatinized sclerotial glycogen was cooled to the relatively lower working temperature of the thermolabile Fungamyl® Super MA. The resulting gel formed is believed to hinder the enzymatic digestion of α-amylase. According to the manufacturer's recommended application, because of its thermolabile characteristics, Fungamyl® Super MA is commonly used in saccharification instead of liquefaction for manufacturing syrup with a high level (40–60%) of maltose.

Despite its fungal origin, the Fungamyl® Super MA was less able to digest the glycogen from the three mushroom sclerotia when compared with the bacterial ones (Sigma A3306 and Termamyl® Supra). However, this might have some nutritional implications. First, it is unknown whether the RG in the sclerotial DFs is resistant to the enzymatic digestion of human α-amylases or not. Second, numerous studies have reported that resistant starch/glycogen has several physiological benefits for humans (Niba, 2002; Topping

Table 6
The yield as well as glucose residue (Glc), resistant glycogen (RG), *N*-acetyl-glucosamine residue (Glc-NAc) and total non-starch polysaccharide (NSP) contents of the three sclerotial DFs prepared by different enzyme combinations.[a]

Enzyme combinations	Sclerotial DFs[b]	Yield[c] (g/100 g sample DM)	Glc (g/100 g DF rich materials DM)	RG (g/100 g DF rich materials DM)	Glc-NAc (g/100 g DF rich materials DM)	NSP (g/100 g DF rich materials DM)
I: Sigma A3306 ⇒ Sigma P3910 ⇒ Sigma A9913	PTR1	79.2 ± 0.88a	78.7 ± 1.12a	3.80 ± 0.16a	6.12 ± 0.12a	88.3 ± 0.02a
II: Sigma A3306 ⇒ Sigma P3910 ⇒ Megazyme E-AMGDF	PTR2	84.0 ± 0.79b	82.3 ± 1.13b	4.77 ± 0.18b	6.18 ± 0.16a	91.8 ± 0.15a
III: Fungamyl® Super MA ⇒ Alcalase® 2,4 L FG ⇒ Dextrozyme™ E	PTR3	81.2 ± 0.97a	78.5 ± 1.23a	4.64 ± 0.14b	6.09 ± 0.14a	88.0 ± 0.03a
I: Sigma A3306 ⇒ Sigma P3910 ⇒ Sigma A9913	PR1	85.8 ± 2.22a	87.3 ± 1.27a	3.91 ± 0.15a	1.92 ± 0.13a	91.0 ± 0.17a
II: Sigma A3306 ⇒ Sigma P3910 ⇒ Megazyme E-AMGDF	PR2	85.5 ± 1.64a	87.1 ± 1.18a	3.81 ± 0.17a	1.90 ± 0.15a	90.8 ± 0.10a
IV: Fungamyl® Super MA ⇒ Protamex™ ⇒ Dextrozyme™ E	PR4	86.5 ± 1.06a	88.8 ± 1.24a	4.81 ± 0.18b	1.89 ± 0.14a	92.5 ± 0.04a
I: Sigma A3306 ⇒ Sigma P3910 ⇒ Sigma A9913	WC1	95.7 ± 1.89a	85.1 ± 1.24a	3.71 ± 0.18a	1.51 ± 0.18a	90.2 ± 0.04a
II: Sigma A3306 ⇒ Sigma P3910 ⇒ Megazyme E-AMGDF	WC2	95.8 ± 1.07a	85.3 ± 1.21a	3.66 ± 0.14a	1.52 ± 0.19a	90.4 ± 0.11a
IV: Fungamyl® Super MA ⇒ Protamex™ ⇒ Dextrozyme™ E	WC4	96.2 ± 1.25a	86.3 ± 1.22a	4.39 ± 0.18b	1.43 ± 0.19a	91.1 ± 0.12a

[a] Data are mean values of three determinations ± SD. For each group of sclerotial DF prepared by different enzyme combinations, means in columns with different letters (a, b) are significantly different (one-way ANOVA using the Kruskal–Wallis Test, $p < 0.05$; Multiple comparisons, using Mann–Whitney U-Test with Bonferroni correction, $p < 0.025$).

[b] PTR*n* or PR*n* or WC*n* with $n = 1, 2, 3$ or 4 representing the DFs obtained by corresponding enzyme combinations I, II, III or IV from the sclerotia of *P. tuber-regium* (PTR), *P. rhinocerus* (PR) or *W. cocos* (WC).

[c] Yield = dietary fibre content after correction for residual protein and ash. Individual sclerotial DF was prepared from 54 g of its corresponding sclerotium powder, as descried in our previously developed enzymatic procedure (Wong & Cheung, 2005a).

& Clifton, 2001). Therefore, the amount of RG present in the sclerotial DFs might not be an appropriate criterion for choosing the most suitable industrial α-amylase. Nevertheless, in industrial practise, α-amylase is usually involved in both gelatinization (105–110 °C, 5 min) and liquefaction (95 °C, 90–120 min) because of its economic advantage. The heat-stable characteristics and relatively lower cost of the Termamyl® Supra would indicate its suitability for scale-up preparation of the three novel sclerotial DFs in the food industry.

4. Conclusions

Commercial production of the three novel sclerotial DFs, using the industrial enzymes, would be feasible since, in addition to their economic advantage, both the yield and total non-starch polysaccharide contents of their resulting sclerotial DFs were comparable to the levels of those prepared by using the analytical enzymes. Besides, the three novel sclerotial DFs not only had physicochemical and functional properties which were comparable to or even better than those of some commercial fibre rich supplements (Wong & Cheung, 2005a), but they were also found to have notably low *in vitro* mineral binding capacity (Wong & Cheung, 2005b). Amongst the three sclerotial DFs, *W. cocos* DF also exhibited remarkably high *in vitro* fermentability (Wong et al., 2005) and overall, improved Ca and Mg absorptions in ovariectomized rats fed a low Ca diet (Wong et al., 2006). As a result, the three β-glucan-rich sclerotial DFs offer a vast potential as functional food ingredients for enhancing Ca absorption or even lowering the risk of osteoporosis.

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