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Treatment of Cereal Products with a Tailored Preparation of *Trichoderma* Enzymes Increases the Amount of Soluble Dietary Fiber

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Nutritionists recommend increasing the intake of soluble dietary fiber (SDF), which is very low in most cereal-based products. Conversion of insoluble DF (IDF) into SDF can be achieved by chemical treatments, but this affects the sensorial properties of the products. In this study, the possibility of getting a substantial increase of SDF from cereal products using a tailored preparation of *Trichoderma* enzymes is reported. Enzymes were produced cultivating *Trichoderma* using durum wheat fiber (DWF) and barley spent grain (BSG) as unique carbon sources. Many *Trichoderma* strains were screened, and the hydrolysis conditions able to increase by enzymatic treatment the amount of SDF in DWF and BSG were determined. Results demonstrate in both products that it is possible to triple the amount of SDF without a marked decrease of total DF. The enzymatic treatment also causes the release of hydroxycinnamic acids, mainly ferulic acid, that are linked to the polysaccharides chains. This increases the free phenolic concentration, the water-soluble antioxidant activity, and, in turn, the phenol compounds bioavailability.

KEYWORDS: ***Trichoderma*** **enzyme; durum wheat; barley spent grain; dietary fiber; ferulic acid**

INTRODUCTION

Dietary fiber (DF) includes cellulose and lignin, hemicellulose, pectins, gums, and other plant polysaccharides and oligosaccharides. It is defined as "edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" (*1*). DF is conventionally classified in two categories according to its water solubility: IDF, insoluble dietary fiber (cellulose, part of hemicellulose, and lignin) and SDF, soluble dietary fiber (pentosans, pectins, gums, and mucilage).

DF of many cereals is mainly insoluble as the polysaccharide fraction of cereal primary cell walls is dominated by arabinoxylans and cellulose. In barley mixed-linked β-glucans are present, whereas the dicotyledonous primary cell walls are rich in pectins, xyloglucans, and cellulose (*2*). In wheat and barley β-glucans constitute the most abundant nonstarch polysaccharide component of the endosperm cell wall accounting for approximately 3.0−4.5% of the total grain weight. Structurally, these β-glucans are comprised of β-(1,3) and β-(1,4) linkages exhibiting distinct structural and physicochemical features.

Many studies have pointed out that cereal polysaccharide fractions are associated with a substantial amount of phenolic compounds (*3*, *4*). Therefore, the concept of a DF−antioxidant complex has been introduced to better describe this food component and to stress the possible nutritional effects of this dietary component (*5*, *6*).

Hydroxycinnamic acids bound to plant cell wall polymers, polysaccharides, and lignin may have physiological effects due to their antioxidant activity (*7*). In cereals these compounds are linked via ester bonds to the arabinoxylan residues of cell plant walls (*8*, *2*). The carboxylic group of ferulic acid is usually attached to the primary hydroxyl group at the C5 position of α-L-arabinofuranosyl residues (*9*). In addition, parts of the ferulic acid may be bound to lignin through ether bonds (*10*) giving a heterogeneous and insoluble molecular network.

The bioaccessibility and of the bioavailability of these compounds has still to be assessed, although some in vitro (*11*) and animal (*12*) studies gave interesting indications. The phenolic compounds bound to the polysaccharide backbone cannot be absorbed when part of these macromolecules. However, endogenous esterases of the upper and lower gastrointestinal tract are able to cleave the bonds and may release

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free phenolic compounds into the gut lumen, thus promoting their absorption (*13*).

In this framework it should be noted that ferulic acid can dimerize by oxidative coupling, even when part of a polysaccharide complex. This phenomenon which is known to affect the mechanical properties of cereal products could also affect the nutritional value of the polysaccharide−antioxidant complex (*14, 2*)

From a nutritional point of view it is recommended to increase the intake of DF, particularly of soluble DF which is considered to be too low in most cereal-based products. Conversion of IDF into SDF can be achieved by chemical treatments, which unfortunately resulted in the alteration of the sensorial properties of the final product. We have recently shown that a mechanical separation of durum wheat bran could produce fractions having quite a different ratio of DSF/IDF (*15*). In any case, by using the mechanical procedure it was not possible to obtain a fraction having more than 3.5% of SDF.

The present study was conducted to evaluate the possible application of enzymes produced by *Trichoderma* species, to obtain a significant increase of SDF by the hydrolysis of components of cereal pericarp cell walls. *Trichoderma* species are efficient producers of extra-cellular enzymes, generating a large battery of chitinases, glucanases, xylanases and cellulases able to hydrolyze a variety of plant materials. Cellulase of *Trichoderma reesei* is able to grow on crude corn fiber; in particular very high levels of xylanase activity were obtained when the fungal strains were cultured on corn fiber arabinoxylan medium (*16*). The production of specific enzymes can be induced by the substrate used to promote the growth. Silveira et al. (*17*) verified enzyme activity of crude extracts of *Trichoderma harzianum* grown in a solid medium containing wheat bran as a carbon source.

In this paper, the ability of *Trichoderma spp.* to produce specific cell wall hydrolytic enzymes upon cultivation on durum wheat fiber (DWF) and barley spent grain (BSG) was monitored. Various *Trichoderma* strains were screened to estimate their ability to produce enzymes having high specific activity toward cereal DF. The enzymes were used to treat DF-rich cereal products obtaining a significant conversion of IDF into SDF. The enzymatic treatment also caused the release of the hydroxycinnamic acids linked to the polysaccharide chains, thus increasing the water soluble antioxidant activity.

MATERIALS AND METHODS

Fungal Strains and Substrates. *Trichoderma* strains listed in **Table 1** have been used in this study. They belong to the collection of the Department of ARBOPAVE of the University Federico II of Naples. The strain P1 ATCC 74058 (American Type Cultures collection) derived from strain 107 of *T. atroviride* was isolated on a medium containing 500 ppm of iprodione. Strain 107 of *T. viride* was isolated from wood by C. Dennis in Norfolk (England) and has been selected by Tronsmo (*18*) for its ability to grow at low temperature. *T. atroviviride* strain P1 (ATCC 74058) was selected based on its biocontrol ability (*19*). The B05.10 strain is an aploide *Botrytis fuckeliana* obtained from P. Tudzinsky (University of Munster, Germany). Durum wheat fiber (DWF) was obtained from Barilla G. & R. F.lli S.p.A (Parma Italy); barley spent grain (BSG) was obtained by Ringnes AS (Oslo, Norway); and coffee silverskin was a gift from Illycaffè (Trieste, Italy).

Culture Conditions. Dual step cultures were used. In the first step, spore suspensions were inoculated into 250 mL flasks containing 50 mL of PDA (Potato Dextrose Agar, Merck). After 7 days, 14 days, and 21 days at 25 °C mycelia were collected. The spores have been collected by using 10 mL of sterile distilled water and have been used for the inoculum. The concentration of the conidia for the inoculum has been determined using a Bright-linens apparatus (Sigma). Flasks subsequently containing 1 L of PDB have been inoculated with a suspension of conidia and left in agitation 7 days at 25 °C. The produced biomass was collected by the aid of a sieve and transferred to 1 L of pH 6.6 salt medium (*20*) containing different concentrations of the following carbon source: glycerol; freeze-dried edible fungi (*Pleroutus*); DWF and BSG.

Table 1. Ability of *Trichoderma* Strains To Grow on Medium Where DF-Rich Cereal Product Were the Unique Carbon Source

Trichoderma	durum wheat fiber (DWF)	barley spent grain (BSG)
T. harzianum T22	+++	++++
T. harzianum T24	++	++
T. harzianum T39	++	+++
T. harzianum Th6	+	++
T. viride	+++	+++
T. virens G-4	−	+
T. virens G-6	+	+
T. virens G-41	+	+
T. atroviride 11	++	+++
T. atroviride P1	+++	+++
T. reesei 67	+++	++++
T. reesei ATCC 5875	++	+++
T. reesei 62	++	+++
T. asperellum DAOM230803	−	−
T. koningii TUB F-938	−	+
Botrytis cinerea	+++	+++

The cultures have been incubated at 25 °C with agitation at 150 rpm in a 1-L flask. Culture filtrates were collected after 7 days and dialyzed against distilled water for 48 h at 4 °C, using membranes with molecular exclusion of 6−8 kDa. Protein containing fractions were lyophilized and stored at −20 °C. The growth of *Trichoderma* strains was assayed on different carbohydrate substrates, concentrations, and times. DWF and BSG were tested as substrates at concentrations ranging from 1 to 5%, and growth periods from 4 to 20 days were tested.

Enzymatic Activities. Extracellular culture filtrates were filtered through a 0.22-μm membrane filter (Millipore, Bradford, MA), dialyzed against distilled water for 24 h at 4 °C, concentrated about 20-fold with polyethyleneglycol (8000 MW; Fluka Biochemika, Buchs, Switzerland), and stored at −20 °C with 20% glycerol until use. N-Acetylglucosaminidase activity (NAGase) and glucanolytic (glucanase) activity were performed as described by Harman et al. (*20*) and Lorito et al. (*21*), measuring the release of *p*-nitrophenol from *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide and from *p*-nitrophenyl β-D-glucopyranoside, respectively (all from Sigma, St. Louis, MO). 1,3-β-Glucanase activity was determined by measuring the release of reducing sugar from laminarin (Sigma, St. Louis, MO). The standard assay contained 250 μL of enzyme solution and 250 μL of a 0.1% (w/v) solution of laminarin in 50 mM pH 6.7 potassium phosphate buffer. After incubation at 30 °C for 1 h, the reducing sugars produced were determined by the method described by Ashwell (*22*). Cellulosolytic and xylanolytic activity were measured using cellazyme C tablets and xylazime AX tablets respectively in accordance to the manufacturer's protocol (Megazyme).

One unit of 1,3-β-glucanase activity was defined as the amount that catalyzed the release of 1 nmol of glucose equivalents per min under the above condition. One unit of NAGase or glucanolytic activity corresponds to 0.1 absorbance unit measured at 405 nm after 30 min of incubation at 50 °C and 37 °C, respectively. One unit of cellulosolytic activity corresponds to 0.1 absorbance unit measured at 600 nm after incubation at 40 °C for 10 min. Xylanolytic activity was determined by using the manufacturer's standard curve and converting absorbance (590 nm) to milli-Somogyi units of activity.

Condition of DF Solubilization. To test the efficacy of enzymes produced by different strains of *Trichoderma* in the hydrolysis of polysaccharides present in the DF-rich cereal materials, different conditions were tested, in particular the amount of water and the concentration of total protein. Twenty, 50, and 100 μg mL^{-1} of total protein obtained from *Trichoderma* grown on DWF or BSG were added

to 1 g of the various substrates and dissolved in 3 mL, 15 mL, and 30 mL of water. The system was placed in agitation for 24 or 28 h. All DF solubilization experiments were conducted in quadruplicate, two samples with enzymes and two without it as controls.

Determination of Soluble and Insoluble Fiber. The amount of SDF and IDF have been determined according to a gravimetric enzymatic method as previously described by Prosky et al. (*23*) (AOAC Method 991.43).

An enzymatic kit to determine fiber amount was from Megazyme; it was constituted by α-amylase (E-BLAAM) with an activity of 3000 Ceralpha units/mL, protease (E-BSPRT) at a concentration of 50 mg/mL (350 tyrosine units/mL), and amyloglucosidase (E-AMGDF) with an activity of 200 *p*-NP-*β*-maltoside units/mL.

The procedure consists of two main steps: enzymatic digestion and filtration. The first step is common for all fiber, instead the filtration changes depending on the type of fiber investigated (TDF, IDF, SDF). The analytical protocol is described in detail elsewhere (*15*).

Total Carbohydrates. Total carbohydrates were determined by a phenol-sulfuric acid reaction (*24*): 500 μL of the sample was placed in a glass tube, added with 500 μL of 3% phenol and 2.5 mL of concentrated H_2SO_4, and mixed. After 10 min, samples were mixed again and incubated at 30 °C for 20 min. Then the absorbance at 485 nm against pure water was measured. Results were expressed in μg mL^{-1} of glucose.

Determination of Total Phenolic Compounds by the Folin-Ciocalteus Method. The amount of total phenolic compounds was determined by using the Folin-Ciocalteus reagent (*25*). Samples (125 μL, three replicates) were introduced into test tubes; 125 μL of Folin's reagent and 500 μL of distilled deionized water were added. After 6 min, 1.25 mL of sodium carbonate (7.5%) was added. The tubes were mixed and allowed to stand for 90 min. Absorption at 765 nm was measured. Results was expressed as gallic acid equivalents (GAE) in mg g^{-1} of dry material.

Determination of Proteins. Dialyzed culture filtrates of the various *Trichoderma* strains were tested for the total protein concentrations by colorimetric assay (Bio-Rad assay) and analyzed by SDS-PAGE (*26*).

Antioxidant Activity (ABTS). The determination of the radical scavenging activity was performed by the ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid, Fluka) method as described by Pellegrini et al. (*27*). At the end of incubation with the enzymes cereal samples were centrifuged at 4000*g*, and the supernatants were diluted in ethanol and tested. The antioxidant activity was expressed as mmol Trolox per 100 g of fresh matter.

Determination of Ferulic Acid. Total ferulic acid in cereals before and after enzyme treatment was determined by the LC/MS/MS system (API 3000, MDS SCIEX). LC analysis was performed using a system consisting of a series of 200 binary pumps (Perkin Elmer, U.S.A.). The mass spectrometer is equipped with a Model 11 syringe pump (Harvard, Apparatus, Holliston, MA). A turbo Ionspray source, with the nebulizer temperature set at 450 °C, was used. The collision-induced dissociation was carried out using nitrogen as collision gas. The analysis was performed using a Prodigy column 5μ ODS3 100A, 250 × 4.60 mm (Phenomenex, U.S.A.). The solvent system was water 0.1% formic acid (solvent A) and methanol (solvent B). The linear solvent gradient was as follows: 0−10 min 95% A 5% B, 10−12 min 55% A 45% B, 12−15 min 45% A 55% B, 15−17 min 0% A 100% B, 17−22 min 0% A 100% B, 22−24 min 95% A 5% B returned to initial conditions. The acquisition has been carried out by the MRM (**m**ultiple **r**eaction **m**onitoring) system, in negative mode. The specific transition ions used for quantification of ferulic acid were as follows: *m*/*z* 193→*m*/*z* 134 and *m*/*z* 193→*m*/*z* 178. To promote ionization of the precursor ion, the voltage applied was 4500, while the collision energy (CE) and the collision cell exit potential (CXP) were optimized for each transition.

Data acquisition and processing were performed using Analyst software 1.4. The amount of ferulic acid was expressed in mg per kg of material (ppm).

Statistical Analysis. Statistical analysis of data was performed on the original data by analysis of variance and tested for significance by the Turkey test, which allowed a multiple comparison among the data to individualize the significant differences. Differences were considered significant if $p < 0.05$.

Table 2. Production of Total Protein and Glucanolytic Activity by Four *Trichoderma* Strains Cultivated Using Different Conditions.

strains	substrate concn (%)	time of growth (days)	total protein (μg/mL)	1,3-β glucanolytic (E.U. per μg of protein)
T. harzianum 22	0.5	7	50.8	1.15
		14	54.2	1.21
T. harzianum 22	1	7	76.6	1.94
		14	80.4	2.02
T. harzianum 22	2	7	75.8	1.85
		14	78.2	1.68
T. reesei 67	0.5	7	97.6	1.45
		14	99.5	1.56
T. reesei 67	1	7	120.5	2.56
		14	123.5	2.48
T. reesei 67	2	7	121.3	2.21
		14	125.7	2.04
T. atroviride P1	0.5	7	nd	nd
		14	nd	nd
T. atroviride P1	1	7	56.9	1.70
		14	65.3	1.58
T. atroviride P1	2	7	60.3	1.45
		14	62.1	1.76
T. viride	0.5	7	45.9	0.98
		14	47.9	1.23
T. viride	1	7	52.3	1.53
		14	55.9	1.71
T. viride	2	7	55.7	1.47
		14	60.8	1.32

RESULTS AND DISCUSSION

A screening of several strains belonging to different *Trichoderma* species was performed to select those able to efficiently use DWF or BSG as a unique carbon source. The working hypothesis was that the fungal strains showing a massive growth in these conditions and were also able to produce a relevant amount of polysaccharide hydrolytic enzymes. As shown in **Table 1** *Trichoderma* strains are generally able to grow on these substrates, but some species perform better than others. To find the suitable fungi for this work four *Trichoderma* strains were selected. The parameters used for the first screening were glucanolytic activity and total protein production; they were measured at different cultivation conditions: time of growth and concentration of substrate. In **Table 2** the results obtained from this set of experiments are summarized. From these data it can be concluded that *T. harzianum* 22 (T22) and *T. reesei* 67 (T67) are the most promising strains. In fact, they gave the highest production of total proteins, T22 up to 80 mg L^{-1} and T67 up to 125 mg L^{-1}, and the highest value of glucanolytic activity, which reached 2.02 and 2.56 enzymatic units per μg of total protein for T22 and T67, respectively.

Data of **Table 2** also reported the optimal conditions to obtain the production of the hydrolytic enzymes from *Trichoderma*: a substrate concentration of 1 g per 100 mL of growth medium and a cultivation time of 7 days were selected. Both lower or higher concentrations of substrate (0.5% and 2%) resulted in lower glucanolytic activity, while the total protein production and the glucanolytic activity did not increase after 14 days of growth. In particular, for T22 and T67 no differences in the production of total protein using a concentration of substrates of 1% and 2% were observed; while an increase between 0.5% and 1% was found.

In the following experiments the two most promising strains were cultivated using the optimized conditions of growth (1 g of fiber per 100 mL of growth medium and 7 days of cultivation), and the complete pattern of hydrolytic enzymes

Table 3. Activity of Cell Wall Hydrolytic Enzymes Produced by *Trichoderma harzianum* 22 and *Trichoderma reesei* 67 Grown on Different Substrates[a]

enzyme activity	*Trichoderma harzianum 22 (T22)*				*Trichoderma reesei 67 (T67)*			
	BSG 1%	DWF 1%	*Pleroutus* 0.5%	glycerol 1%	BSG 1%	DWF 1%	*Pleroutus* 0.5%	glycerol 1%
glucanase	68.2	73.17	60.9	19.5	95.0	100	87.8	29.3
NAGase	41.3	46.5	37.9	17.2	92.0	100	86.2	62.1
cellulase	69.8	100	97.7	44.2	65.1	81.4	76.7	41.9
1,3-β-glucanase	93.6	77.2	51.6	27.1	86.2	100	56.2	34.3
xylanase	100	90.1	10.2	2.1	71.1	72.9	10.1	5.2

[a] Numbers are the percentage of the maximum value for each row.

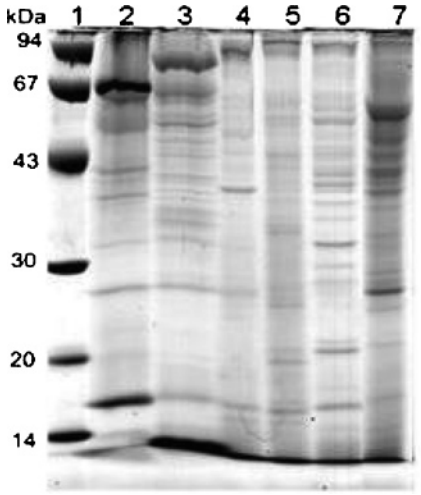

Figure 1. SDS-PAGE of total protein present in the culture filtrates of *Trichoderma* strains collected after 7 days of growth on glycerol, dry *Pleroutus*, and DWF. Lane 1, molecular weight markers; lane 2, T22 cultivated on glycerol; lane 3, T67 cultivated on glycerol, lane 4, T22 cultivated on dry *Pleroutus*; lane 5, T67 cultivated on dry *Pleroutus*; lane 6, T22 cultivated on DWF; lane 7, T67 cultivated on DWF.

was investigated. Moreover, the use of DWF or BSG as a unique carbon source was compared with two well-known methods to induce the production of fungal enzymes, namely the use of mushroom mycelium (lyophilized *Pleroutus*) and glycerol. Results are reported in **Table 3**, while the SDS-PAGE of total protein produced by the two selected strains cultivated on the various substrates is shown in **Figure 1**. The main finding is that both protein expression and enzymatic activities are strongly enhanced by the presence of DWF and BSG, while only a moderate increase with respect to the control is observed with dry *Pleroutus*. Among the different enzymatic activities assayed, the 1,3-β-glucanase and the xylanase had the highest rate of increase in the presence of DWF and BSG. There are no differences in the single enzyme activities between the two cereal materials; however, the data in **Table 3** demonstrate that these two *Trichoderma* strains are quite efficient in adapting their enzyme production to the type of carbon source present in the medium.

Previous attempts to regulate the *Trichoderma* enzyme production have been conducted by using different substrates as the sole carbon source. Xin-Liang and co-workers (*28*) used corn fiber byproducts derived from the fermentation for industrial ethanol production. Donzelli et al. (*29*) studied the relationships between the production of enzymes from *Trichoderma* strain P1 and different cultivation parameters. These works have reported that the enzyme activities were influenced by the substrates, the pH, and the available water. In particular, chitin and scleroglucan were very effective as inducers of enzyme production, a result that agrees with our observations, where DF-rich substrates were potent inducers for production of hydrolytic enzymes.

Table 4. Setup of the Experimental Conditions To Perform the DF Hydrolysis on the DWF[a]

time (h)	amt of enzyme (μg mL^{-1})	amt of substrate (%)	% TDF of DWF	
			T22	*T67*
control		33.3	63.2 ± 1.5	60.9 ± 1.2
		6.6	63.8 ± 1.7	61.3 ± 1.5
		3.3	63.5 ± 1.2	61.2 ± 1.9
24	20	33.3	62.0 ± 0.9	58.2 ± 2.0
		6.6	60.5 ± 1.5	57.5 ± 1.3
		3.3	62.3 ± 1.2	58.6 ± 1.6
24	50	33.3	60.9 ± 0.8	58.9 ± 1.3
		6.6	***57.6*** **± 0.9**	***55.6*** **± 1.9**
		3.3	57.7 ± 1.3	55.5 ± 1.5
24	100	33.3	59.3 ± 1.6	59.6 ± 1.8
		6.6	55.0 ± 2.1	55.8 ± 2.4
		3.3	55.8 ± 2.3	55.2 ± 2.1
48	20	33.3	60.8 ± 2.0	57.9 ± 1.8
		6.6	59.8 ± 1.8	56.7 ± 1.6
		3.3	59.5 ± 1.5	56.2 ± 1.4
48	50	33.3	59.5 ± 2.3	57.7 ± 1.5
		6.6	56.5 ± 1.2	55.4 ± 1.3
		3.3	56.9 ± 1.2	55.2 ± 1.8
48	100	33.3	60.3 ± 1.6	58.8 ± 2.0
		6.6	55.0 ± 1.8	55.2 ± 1.2
		3.3	55.3 ± 1.3	54.9 ± 1.3

[a] The optimal hydrolytic conditions are highlighted in the bold row.

The final objective of this work was to use the enzymes produced by T22 and T67 to modify the DF composition of cereal products. In particular, the aim was to increase the percentage of SDF in DWF and BSG without causing a relevant drop of the TDF concentration. This goal could be achieved using an enzymatic preparation where a high activity of endo-hydrolases and a low activity of exo-hydrolases are present.

Results of the trials carried out with the enzymatic preparation obtained as above-described are shown in **Table 4** and **Figure 2**. Data indicated that the experimental conditions of the hydrolysis played a fundamental role: the concentration of SDF after the treatment ranged between 3% and 9% depending on conditions. To optimize the DF hydrolysis, different enzyme amounts (20, 50, and 100 μg mL^{-1}) and incubation times (24 and 48 h) were tested. The experiments were carried out with different water quantities (final substrate concentration from 3.3 to 33.3%).

From the results shown in **Table 4** and **Figure 2** the following can be concluded. (1) The amount of water is a key factor in determining the final result: a concentration of substrate of 6.6% (1 g of material in 15 mL of water) gave the most satisfactory results. When the substrate concentration rose to 33% (a value near the water holding capacity of the cereal product) very low enzyme hydrolytic activity was detected. On the other hand, a

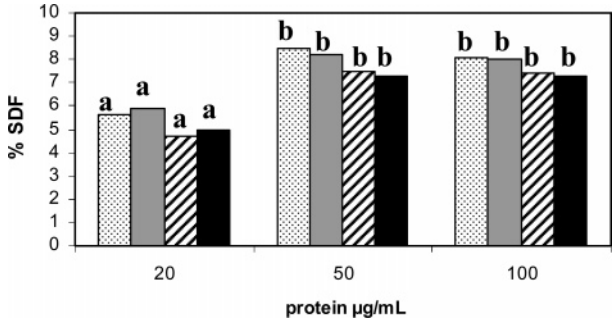

Figure 2. Amount of SDF of DWF after treatment for 24 and 48 h with different amounts of hydrolytic enzymes (20, 50, and 100 μg mL^{-1}) obtained from T22 and T67. Dotted bars: T22 enzymes for 24 h; gray bars: T22 enzymes for 48 h; diagonal pattern bars: T67 enzymes for 24 h; solid black bars: T67 enzymes for 48 h. Bars with the same letter are not statistically different (p<0.05).

Table 5. Proximal Chemical Composition of Dietary Fiber Rich Material

compositional parameters	coffee silverskin (CS)	durum wheat fiber (DWF)	barley spent grain (BSG)
proteins	18.6 ± 0.6	8 ± 0.3	10.4 ± 0.5
fats	2.2 ± 0.1	3.0 ± 1.0	1.4 ± 0.8
carbohydrates	62.1 ± 1.6	70 ± 0.4	70 ± 0.5
ashes	7 ± 0.2	4 ± 1.0	nr
TDF	62.4 ± 0.6	65 ± 0.5	74 ± 0.2
IDF	53.7 ± 0.2	60 ± 0.8	70 ± 1.0
SDF	8.8 ± 0.4	3 ± 1.0	3 ± 0.5

more diluted hydrolysis environment did not give a substantial advantage and required much more enzyme to obtain the same final protein concentration. (2) A significant increase of the amount of SDF in DWF was detected by augmenting the crude enzyme from 20 to 50 μg mL^{-1} up to 100 μg mL^{-1} (**Figure 2**). However, the increase from 50 μg mL^{-1} to 100 μg mL^{-1} of the enzyme concentration also causes a decrease of TDF (**Table 4**). (3) No significant differences were obtained by extending the incubation time from 24 to 48 h. Therefore, the shortest incubation time of 24 h could be used. (4) The strain T22 at a protein concentration of 50 μg mL^{-1} gave a better performance than T67, in terms of the highest concentration of SDF observed (**Figure 2**).

Therefore only the T22 strain was adopted in the final experiments, although T67, giving a higher production of total protein, could be very useful in a perspective scaling up of the system.

The optimized conditions of hydrolysis were adopted to verify the ability of the enzymes to act on different DF-rich materials, namely DWG, BSG, and coffee silverskin (CS). The chemical composition of the polysaccharide fraction of barley and durum wheat is quite similar (*30*), while the CS is a completely different DF-rich product (*31*), that is the way it was taken as a control for enzyme specificity. The proximal composition of the three materials is reported in **Table 5**: all substrates have TDF above 60%, and the native CS also has a considerable amount of SDF.

The amount of SDF before and after enzymatic hydrolysis in the three substrates with different enzyme concentrations are shown in **Figure 3A**. SDF concentration raised to 8.1 and 8.7% on BSG and DWF, respectively. This increase on approximately 250% could not be obtained without using chemical treatments. This result indicates that tailored enzyme preparations permit the use of a DF-rich cereal product as an important source of

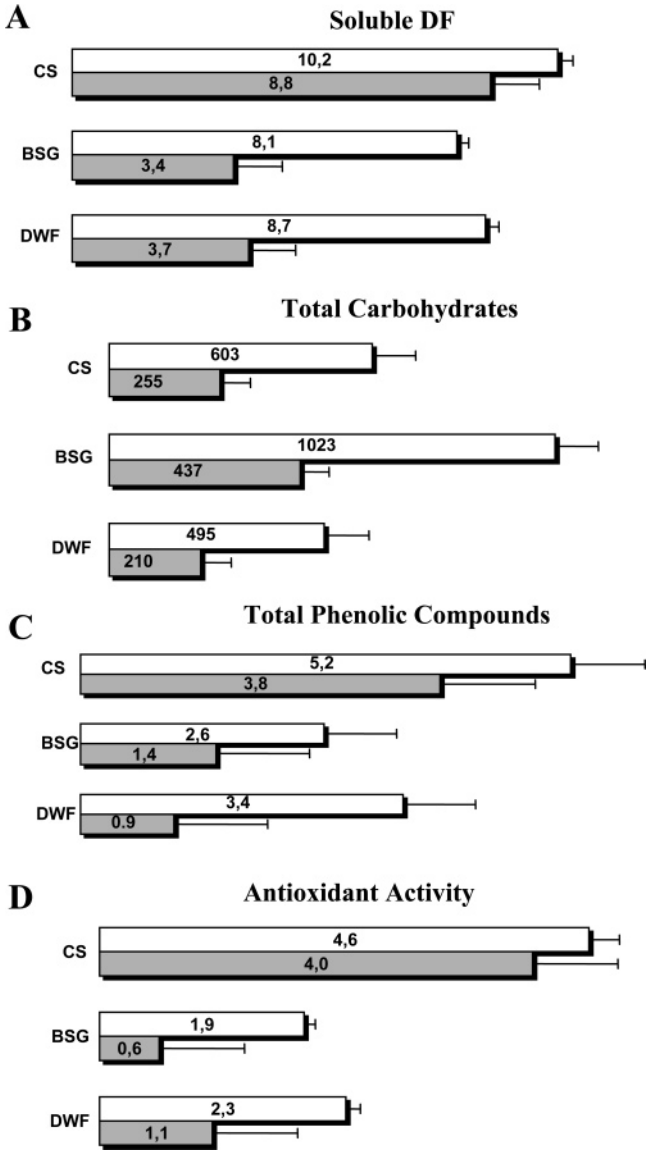

Figure 3. Effect of the treatment by T22 enzymes on the DWF, BSG, and CS. Gray bars: controls; white bars: samples after enzymatic treatment. **A**, amount of SDF (%); **B**, amount of total carbohydrates (μg mL^{-1} of glucose); **C**, amount of phenol compounds (equivalent of gallic acid mg g^{-1}); **D**, antioxidant activities measured by ABTS method (mmol Trolox per 100 g of fresh weight). All values are the means of three replicates with standard deviations (SD).

SDF. The increase of SDF upon enzymatic treatment was comparable in DWF and in BSG. On the contrary, the enzyme preparation had very little effect on coffee silverskin, which can be explained considering the different composition and chemical structure of this material.

Data of **Figure 3B**,**C** demonstrate that the amount of SDF produced corresponded not only to the released carbohydrates, measured as total carbohydrate, but to also the phenolic compounds measured by the Folin-Ciocalteau method. It is reasonable that both oligosaccharides and feruloyl oligosaccharides were detected with these colorimetric assays. Also the antioxidant activity measured by ABTS assay in the water fraction after enzymes treatment (**Figure 3D**) showed a marked increase. Comparing the different material it could be noted that while the observed increase of the total phenolic compound was higher in DWF, the increase of antioxidant activity was higher in BSG.

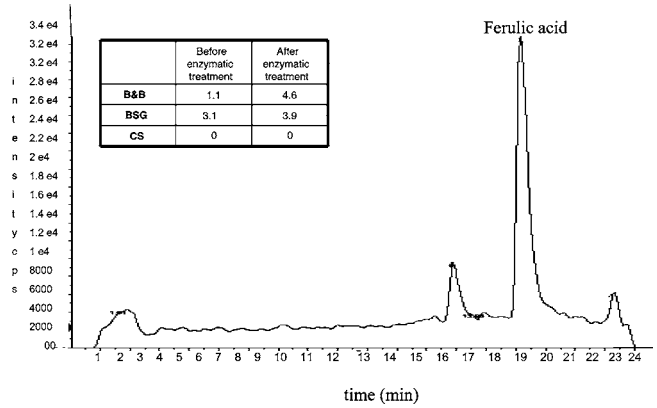

Figure 4. LC-MS-MS chromatogram of ferulic acid. In the insert table the calculated value for free ferulic acid before and after enzymatic treatment on DWF, BSG, and CS are reported. All values are in mg kg^{-1}. All values are the means of three replicates.

It is well-known that the most abundant phenolic compound in cereal-based DF is ferulic acid (FA), which was quantitifed before and after enzymatic treatment by LC-MS/MS using MRM detection. The results are reported in the inset table of **Figure 4**. They confirmed a dramatic increase of FA concentration as a consequence of the enzymatic treatment on DWF. The concentration of free FA of DWF jumped from 1.1 mg kg^{-1} before to 4.6 enzymatic treatment to 4.6 mg kg^{-1} after the treatment. A total amount of FA of 11.6 mg kg^{-1} in DWF was measured after complete hydrolysis obtained with NaOH. Therefore, it can be calculated that while water extractable FA is only 10% in the native DWF, this percentage increases four times after the treatment with the fungal enzymes. Interestingly, only a moderate increase was detectable in BSG (from 3.1 to 3.9 mg kg^{-1}): this finding combined with the results showed in **Figure 3D** suggests that the BSG material contains less potent antioxidant compounds and/or that the *Trichoderma* esterases are more specific toward DWF. As expected, no FA is present in CS where chlorogenic acid and its derivatives are mainly present (*31*).

In conclusion, *T. harzianum* 22 and *T. reesei* 67 efficiently grew on BSG or DWF as the sole carbon sources producing a large amount of cereal DF hydrolytic enzymes. It was possible to regulate their cultivation conditions in order to obtain a cocktail of enzymes having high endo-hydrolytic and low eso-hydrolytic activities. Using T22 enzymes on DWF in the appropriate conditions, we obtained up to 8.7% of SDF without decreasing the TDF below 59%. Similar results could be obtained also on BSG. The conversion of cereal IDF into SDF corresponds to the release of hydroxycinnamic acids linked to polysaccharides chains. In particular 40% of the total FA present in the DWF was found in the soluble fraction after enzymatic treatment in the optimized hydrolysis conditions developed in this work.

The possibility to obtain at industrial scale SDF from cereal products has many potential applications, and future studies on enzyme-treated plant material may demonstrate the technological and nutritional advantages of this process. Besides the effects related to the presence of SDF, the release in free form of phenolic acids previously bound to the fiber can promote their absorption. It should be noted that phenolic acids from cereals constitutes a relevant part of dietary antioxidants. Moreover a partially solubilized antioxidant DF, even if not directly absorbed in the upper gut, could be more accessible, thus better fermented than the native fiber, by the lower gut microflora.

ABBREVIATIONS USED

T22, *Trichoderma harzianum* strain 22; T67, *Trichoderma reesei* strain 67; DF, dietary fiber; SDF, soluble dietary fiber; IDF, insoluble dietary fiber; BSG, barley spent grain; DWF, durum wheat fiber.

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