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Antioxidant Activity of Alkylresorcinols from Rye Bran and Their Protective Effects on Cell Viability of PC-12 AC Cells

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ABSTRACT: Alkylresorcinols (ARs) are phenolic lipids that are present in high amounts in the bran layer of different cereals. Rye samples, cultivar Hazlet, and a white rye genotype, RT202, were analyzed for their antioxidant properties and AR content and composition, based on six fractions of the bran, where 1 was the outermost fraction and 6 was the bran fraction closest to the endosperm. Gas chromatography−mass spectrometry (GC-MS) analysis demonstrated that the most commonly found AR homologue in Hazlet rye is C19:0 and that the total amount of ARs decreases from the outermost to innermost fractions. The antioxidant activity using oxygen radical absorbance capacity (ORAC) for both white rye genotype RT202 and Hazlet brans was determined to decrease from the outermost fraction (136.05 μmol TE/g for Hazlet fraction 1 and 186.57 μmol TE/g for white rye genotype RT202 fraction 1) to the innermost fraction (9.84 μmol TE/g for Hazlet fraction 6 and 78.75 μmol TE/g for white rye genotype RT202 fraction 2). A positive relationship was seen with GC-MS results. Treatment of PC-12 AC cells with Hazlet fraction 1 increased mitochondrial biogenesis as determined using mitochondrial fluorescent dyes. In the presence of a prooxidant (AAPH), PC-12 AC cells were better protected from free radical attack when treated with Hazlet fraction 1 than with all other bran fractions. The results suggest that higher AR content in bran fractions confers antioxidant protection against free radical damage.

KEYWORDS: alkylresorcinols, reactive oxygen species, rat pheochromocytoma cells, oxidative stress, rye bran

INTRODUCTION

Free radicals and reactive oxygen species (ROS) are mainly produced through aerobic metabolism by mitochondria[1] but can also be produced by enzymes such as pyruvate, α-ketogluterate, acyl-coenzyme A, and isocitrate.[2] ROS damage proteins, nucleic acids, and membrane lipids in the cells through oxidation, and when ROS accumulate, cells undergo oxidative stress. The damage to cellular components causes diseases such as cancer,[3−5] coronary heart disease,[6,7] obesity,[8] and aging.[1] Carcinogenesis and heart diseases, which tend to be age related, are the outcome of elevated free radical levels. It is therefore crucial that free radical formation and removal are balanced.[9−11] ROS can be superoxide, hydrogen peroxide, alkyl peroxyl, lipid peroxyl, and hydroxyl radicals.[2]

Alkylresorcinols (ARs) are well-known antioxidants (Figure 1). They are amphiphilic phenolic lipids found in fungi, higher plants, bacteria, algae, and mosses.[12] They are derivatives of resorcinols and contain a long odd-numbered saturated hydrocarbon chain at position 5 of the phenolic ring, which can be 13−27 carbon atoms in length,[13] as opposed to fatty acids, which have even-numbered chains.

Many health benefits of a whole grain diet have been described. It is known that ARs possess antimicrobial, antibacterial, antifungal, and antitumor activities.[14] In addition, ARs can be used as biomarkers for whole grain rye and wheat intake as described by Linko and Adlercreutz.[15] The incorporation of ARs into human erythrocyte membranes was examined by monitoring AR levels after exposure to or avoidance of whole grain products.

In ARs, the long aliphatic side chain plays an important role in incorporation into cell membranes. ARs that contain long alkyl side chains have been shown to prevent peroxidation of fatty acids and phospholipids in liposomal membranes induced by ferrous ions.[13] The alkyl chain of ARs enables uptake into cellular membranes, whereas the phenolic 1,3-dihydroxybenzene ring[16] provides the antioxidant capacity.

ARs are found in the bran fraction of cereal grains such as wheat, rye, triticale, and barley[16] and in low amounts also in the endosperm.[17] The bran layer is the outer layer of cereal grains and comprises 20% of the kernel. The bran and germ layer are removed in refined grain products and thus lack ARs,[18] which makes ARs effective biomarkers for whole grain wheat and rye intake and markers for the presence of bran fractions in food products.[19] Rye bran is known to have a high amount of ARs with a range of 360−3200 μg/g, followed by triticale (580−1630 μg/g), wheat (317−1010 μg/g), and barley (44−500 μg/g).[16]

Mitochondrial biogenesis is a highly regulated process that is currently recognized as a response to cellular stress. Of particular importance is oxidative stress, as it has been shown that an increase in intracellular ROS results in decreased mitochondrial biogenesis.[20] Because the primary source of ROS within the cell is the mitochondria itself, any increase in intracellular ROS will result in the alteration of signaling pathways involved in down-regulating mitochondrial biogenesis. Mitochondrial uncoupling and caloric restriction have been shown to decrease mitochondrial-generated ROS and promote mitochondrial biogenesis.[21]

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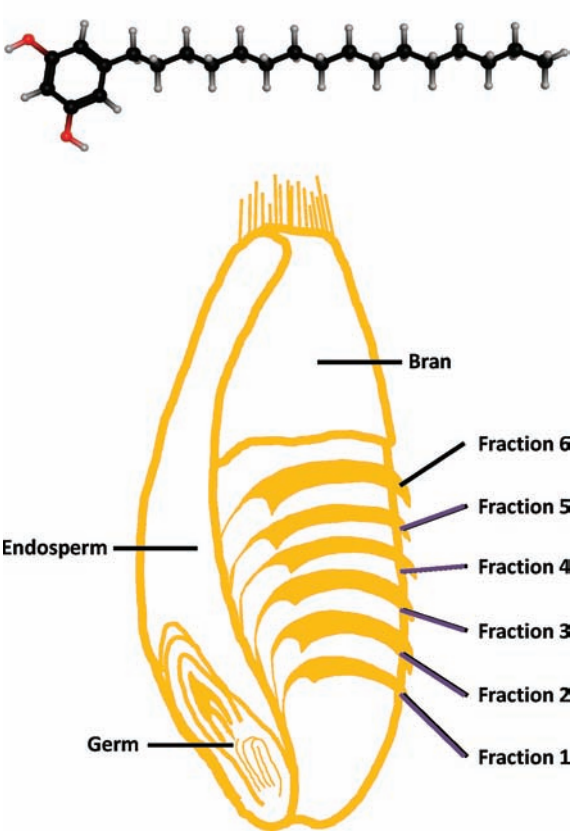

Figure 1. Chemical structure of ARs and the different bran fractions of a rye kernel.

The cytoprotective effects of ROS removal have been previously shown to activate the generation of mitochondria within cells. Antioxidants, taken in from dietary sources, may stimulate mitochondrial biogenesis by (a) removing the signaling molecules (ROS) involved in suppressing factors promoting biogenesis or (b) removing the sources of mitochondrial damage in general. Polyphenolic antioxidants, such as hydroxytyrosols found in extra virgin olive oil (responsible for the health benefits of the Mediterranean diet and the lower incidence of cardiovascular disease during diabetes), activate peroxisome proliferator-activated receptor coactivator 1, the factor responsible for mitochondrial biogenesis, leading to an enhancement of mitochondrial function and cellular defense from ROS.[22] As well, the same factors involved in mitochondrial biogenesis in cells are also responsible for up-regulating antioxidant defenses in mitochondria.

The cultivar Hazlet and the white rye genotype RT202 were used in studying ARs. Hazlet is a cultivar of winter rye (*Secale cereale* L.), which showed good winter survival and is well adapted to the Canadian prairies. Hazlet was selected for large seed size, floret fertility, and reduced plant height.[23] The white rye genotype RT202 was selected for white kernel color and winter habit.

The present study focuses on the different fractions of Hazlet and RT202 as opposed to the entire bran layer. The antioxidant activity, total AR content, and composition of AR homologues are determined for the different bran fractions.

Furthermore, the cytoprotective activity of Hazlet was determined in vitro, as well as its effect on mitochondrial biogenesis.

MATERIALS AND METHODS

Materials. The rye samples were developed and grown by Dr. Grant McLeod at Agriculture and Agri-Food Canada (AAFC) in Swift Current, SK. Rye bran fractionation was conducted by Dr. Nancy Ames at AAFC in Winnipeg, MB. Triticale (spring triticale, cultivar Ultima) grown in the Edmonton region of Alberta was received from Alberta Agriculture, Food and Rural Development (Edmonton, AB). The AR standards (C15:0, C17:0, C19:0, C21:0, C23:0, and C25:0) were obtained from ReseaChem GmbH (Burgdorf, Switzerland). Acetone was obtained from Caledon Laboratories Ltd. (Georgetown, ON). Mono- and dibasic potassium phosphate, fluorescein, Trolox, *trans*-cinnamic acid, rutin, 2,2′-azobis(2-methylpropionamidine)dihydrochloride (AAPH), phosphate-buffered saline (pH 7.4), trypan blue (0.4%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Folin−Ciocalteu reagent were obtained from Sigma-Aldrich (Oakville, ON). RPMI 1640 medium, F12 media, newborn calf serum, horse serum, fetal bovine serum, antibiotic/antimycotic (penicillin G sodium salt, streptomycin sulfate, and amphotericin B (P/S/A)), trypsin, DNase/RNase-free water, and Mito Tracker Deep Red FM were obtained from Invitrogen (Gibco) (Burlington, ON). Dimethyl sulfoxide (DMSO) was obtained from BioShop (Burlington, ON). Formaldehyde was obtained from BDH (Mississauga, ON). Trimethylsilyl, bis(trimethylsilyl)trifluoroacetamide, and 1% trimethylchlorosilane were purchased from VWR International (Ottawa, ON). Ethyl acetate was purchased from Carleton University Chemistry Stores (Ottawa, ON). Ferulic acid was obtained from Fluka Analytical (Sigma) (Oakville, ON). Sodium carbonate was obtained from Church & Dwight Canada Corp. (Mississauga, ON).

Pearling Preparation. Sequential bran fractionation for RT202 (spring × winter cross RT202) and winter rye (cultivar Hazlet) was achieved by abrading the outer layers of grain in a Satake TM 05 laboratory-scale pearler (Satake Co, Japan).[23] Initially, several 180 g batches of whole grain were abraded to remove 5% (by weight) of bran so that each batch yielded 9.0 g of 0−5% bran. The pearled rye kernels were pooled together, mixed thoroughly, and redivided into batches of 180 g. Again, each batch was processed in the Satake TM 05 to abrade a further 9.0 g (5%) of bran from each batch. The abraded bran was pooled together and labeled 5−10% bran, representing the layer between 5 and 10%. Hazlet was further pearled to obtain bran layers at 2.5% intervals, representing the layers between 10 and 12.5%, between 12.5 and 15%, between 15 and 17.5%, and between 17.5 and 20%. This process was repeated for every 2.5% bran layer up to the 20% bran layer. The different fractions were referred to in percent values and describe the distance from the outside of the bran layer to the center of the kernel. We named these fractions as follows: fraction 1 is 0−5%, fraction 2 is 5−10%, fraction 3 is 10−12.5%, fraction 4 is 12.5−15%, fraction 5 is 15−17.5%, and fraction 6 is 17.5−20%.

Extraction. ARs in rye samples were extracted by using acetone in a 1:40 w/v ratio according to the methods of Mullin et al.[24] and Ross et al.[16] After 24 h of stirring at room temperature, the samples were filtered using Whatman double filter paper. The precipitate was discarded, and the acetone was completely evaporated from the supernatant using a Brinkmann Rotavapor R110; the dried product was weighed and stored at −20 °C for further use. Each fraction was extracted and analyzed in triplicate.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC of ARs was measured according to the procedures described by Sim et al.[25] and Hosseinian et al.[26] Peroxyl radicals generated by AAPH and a fluorometric microplate reader (FLx800 Multi-Detection Microplate Reader with Gen5 software, BioTek Instruments) were used at an excitation wavelength of 485 nm and an emission wavelength of 525 nm. Trolox, *trans*-cinnamic acid, and the AR homologue C19:0 were used as standards, whereas rutin was used as a control. Bran extracts were prepared in a 1:4 w/v ratio with ORAC buffer (potassium phosphate buffer, pH 7.4). A microplate was prepared containing 20 μL of

Trolox/cinnamic acid/C19:0 standards, rutin control, and extract, as well as 120 μL of fluorescein solution. After 20 min of incubation, 60 μL of AAPH was added to a final concentration of 0.16 M in the assay, and data were obtained with the fluorescence reader. The area under the curve (AUC) was used to determine the ORAC value using a regression equation obtained from the Trolox/cinnamic acid/C19:0 standard curve. ORAC values were expressed as μmol Trolox equiv/g bran,[26] μmol cinnamic acid equiv/g, and μmol C19:0 equiv/g.

Gas Chromatography−Mass Spectrometry (GC-MS). GC-MS analysis was performed according to the method of Athukorala et al.[12] Ten microliter extracts were derivatized with trimethylsilyl using 200 μL of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane (TMCS) and 100 μL of ethyl acetate. The reaction mixture was heated at 75 °C for 30 min, allowed to cool, evaporated under continuous nitrogen flush, and redissolved in ethyl acetate for GC-MS analysis. The GC-MS analysis was conducted with a Hewlett-Packard HP 6890 Plus (GC) and a Hewlett-Packard HP 5973 (MS) equipped with a splitless injector and a network mass selective detector. A DB-17HT high-temperature capillary column was used (30 m × 0.25 mm i.d., 0.1 μm film thickness, J&W Scientific, Folsom, CA). Helium was used as the carrier gas with a flow rate of 1.3 mL/min, and the temperature of the injector was 300 °C. The initial oven temperature of 50 °C was held for 1 min, and the temperature was increased by 10 °C/min to 350 °C. A Hewlett-Packard HP 5973 (MS) was operated in the electron ionization (EI) mode at 70 eV, a source temperature of 280 °C, and a temperature of 150 °C, in the scan range of m/z 35−350. The MS was used to confirm the presence of AR homologues by their molecular ion peaks at m/z 320 (C15:0), 348 (C17:0), 376 (C19:0), 404 (C21:0), 432 (C23:0), and 460 (C25:0). Compounds were identified by comparing their spectra with those of relevant standards. AR C22:0 was used as the internal standard. Data were compared against the NIST (v.02) and Wiley (v.138) libraries (Palisade Corp., Newfield, NY).

Total Phenolics Content. A modified procedure of the Folin−Ciocalteu method[27] was used. Two hundred microliters of Hazlet extract was mixed with 1.9 mL of 10-fold-diluted Folin−Ciocalteu reagent, to which 1.9 mL of 60 g/L sodium carbonate solution was added. Absorbance was measured after 120 min at room temperature at 725 nm against distilled water (blank). Results are based on ferulic acid as the standard and expressed as ferulic acid equivalents.

Cell Culture. PC-12 AC cells were a gift from Dr. Steffany Bennett (Biochemistry, Microbiology and Immunology, University of Ottawa) and the American Type Culture Collection (ATCC). Cells were grown in RPMI 1640 media containing 10% horse serum, 5% newborn calf serum, and 3% P/S/A. Cells were maintained under normoxic conditions at 37 °C and 5% CO_2. The cell density was determined using trypan blue.

MTT Assay. One hundred microliters of PC-12 AC cells, at a density of 4.0 × 10^5 cells/mL, was plated into the inner wells of a 96-well cell culture microplate, whereas 200 μL of DNase- and RNase-free distilled water was added to the outer wells. The cells were then incubated for 24 h at 37 °C and 5% CO_2, after which the media were discarded and 100 μL of fresh medium was added. Serial dilutions of rye extract were prepared from a stock of 0.4 mg/100 μL made in DMSO. One microliter of rye extract was added to each well, with each concentration being plated six times. Cells were incubated for 1 h before 10 μL of 3 mM AAPH was added to each well. Cells were incubated for 24 h. The media were discarded and replaced with fresh media. Ten microliters of MTT (5 mg/mL) was added to each well, cells were incubated for 1 h before media were discarded, and 50 μL of DMSO was added to lyse the cells. Absorption values were obtained with the SpectraMax 340PC microplate reader at 570 nm with 630 nm background subtraction.

MitoTracker. F12 media containing 5% fetal bovine serum, 10% horse serum, and 1% P/S/A were used for cell culture studies. Two milliliters of PC-12 AC cells, at a density of 5.0 × 10^5 cells/mL, was plated into a six-well cell culture microplate onto coverslips. Cells were incubated for 24 h at 37 °C and 5% CO_2. The media were replaced with fresh media, and 20 μL of Hazlet fraction 1 was added to the wells at a concentration of 0, 0.025, or 0.4 mg/100 μL DMSO in duplicate. After 24 h of incubation, 10 μL of 500 nM MitoTracker Deep Red was added to each well. After 45

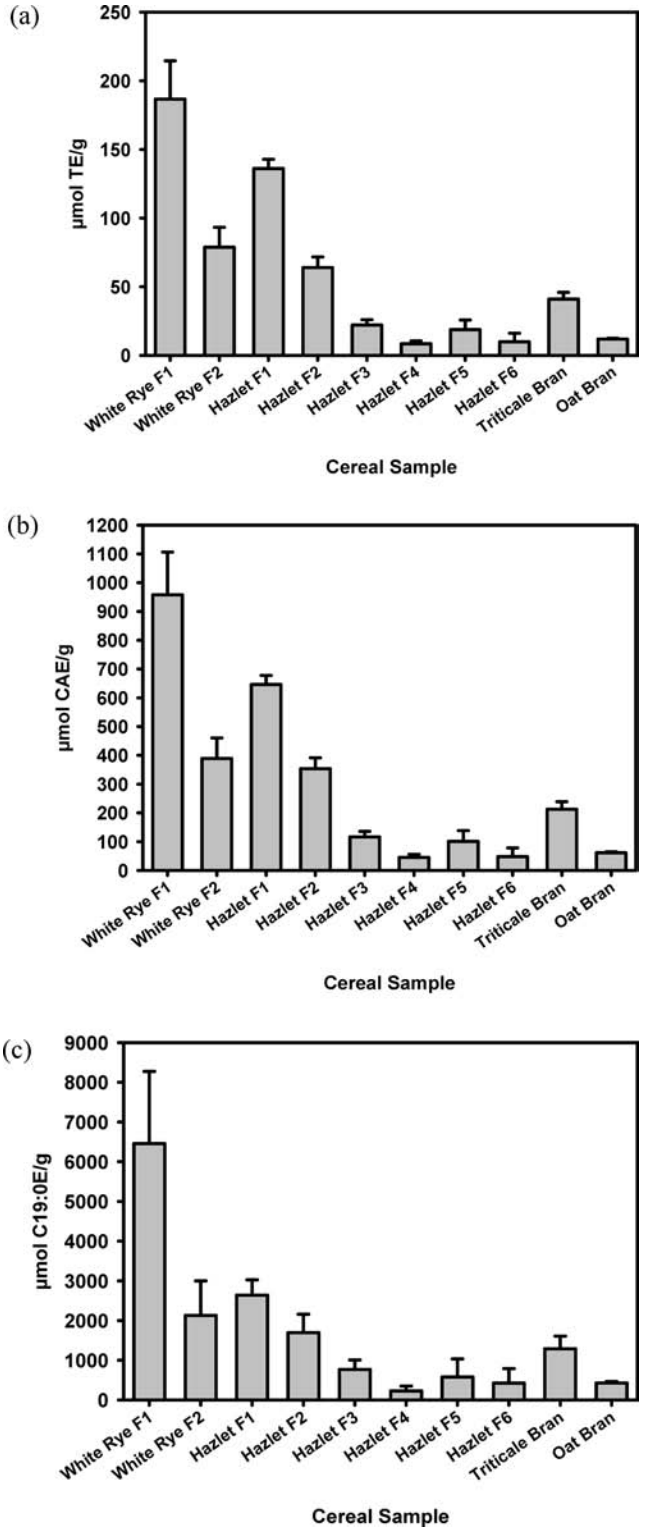

Figure 2. Antioxidant activity of different fractions (F1−6) of Hazlet, white rye, triticale, and oat bran using (a) Trolox (μmol TE/g), (b) cinnamic acid (μmol CAE/g), and (c) the AR standard C19:0 (μmol C19:0E/g) as standard.

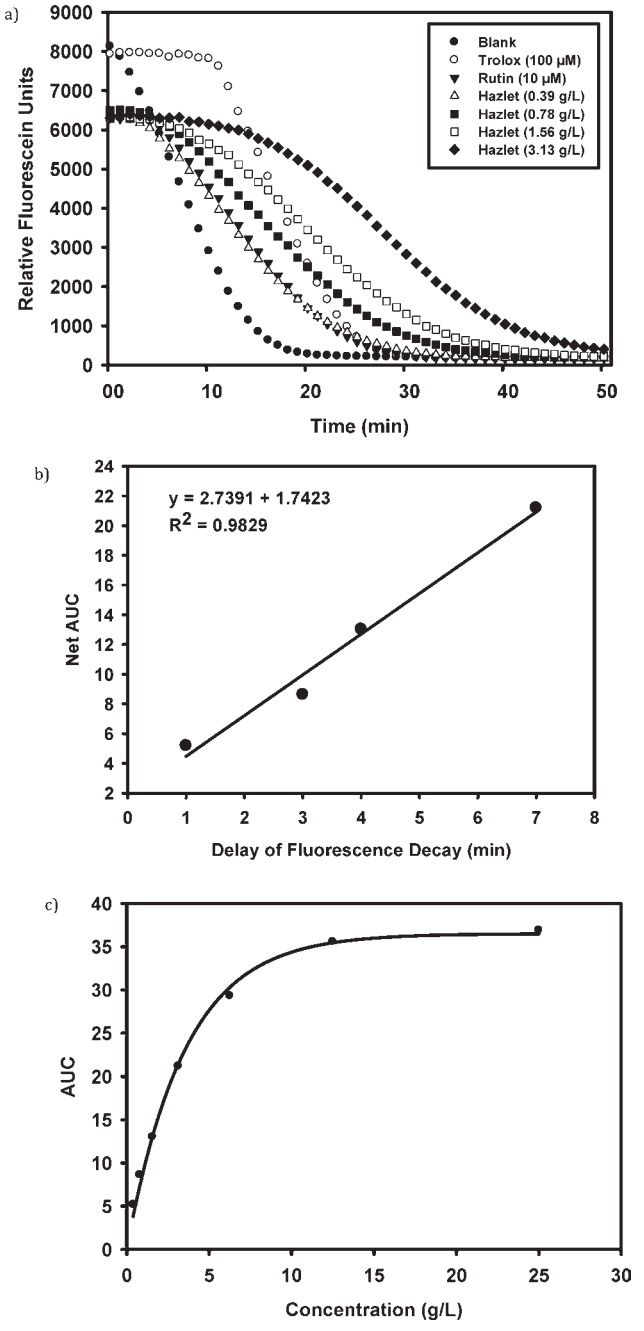

Figure 3. (a) Fluorescence decay curves of fluorescein in the presence of AAPH and Hazlet bran (fraction 1). (b) Correlation between the net area under the curve and delay of fluorescence in minutes of increasing concentration of Hazlet bran (fraction 1). (c) Relationship between the net area under the curve and different concentrations of Hazlet (fraction 1).

min of incubation, cells were fixed using 3.7% formaldehyde in 2 mL of F12 media and incubated for 15 min. The coverslips were then mounted on microscope slides, and edges were sealed with clear nail polish. Microscope slides were then stored at 3 °C until further use. MitoTracker images were obtained with the Nikon D-eclipse BOi.

RESULTS

Antioxidant Activity. The antioxidant activity using ORAC method was determined for ARs in each fraction of the bran layer of Hazlet, RT202, and the cereals triticale and oat bran. The outermost fraction of RT202, fraction 1, has the highest antioxidant activity, followed by the outermost fraction of Hazlet. The antioxidant activities of both RT202 and Hazlet (Figure 2) decrease with proximity to the inner endosperm. ORAC results have a similar pattern for standards Trolox, cinnamic acid, and the AR homologue C19:0, but with Trolox giving much lower antioxidant results than cinnamic acid and C19:0.

The antioxidant capacity increased with increasing concentration of Hazlet extract (Figure 3a). Even at the lowest concentration tested of Hazlet fraction 1 extract (0.39 g/L), there was still a small delay in fluorescence decay, similar to that of the antioxidant rutin.

As the delay of fluorescence decay increases for increasing concentrations of Hazlet fraction 1, the net AUC increases as well, as seen in Figure 3b. As the concentration of Hazlet fraction 1 increases, the AUC increases as well (Figure 3c).

GC-MS. Using GC-MS, the composition of AR homologues and the total amount of ARs in the three Hazlet fractions (1−3) were determined. ARs are separated on the basis of their alkyl chain length and degree of saturation because the phenolic head is always the same. In addition to the AR peaks (saturated side chains), other smaller peaks were present as well (belonging to unsaturated ARs), as seen in Figure 4b.

The AR composition of the three outermost rye fractions of Hazlet was determined using GC-MS, and the presence of AR homologues C15:0, C17:0, C19:0, C21:0, C23:0, and C25:0 was confirmed. As seen in Table 1, the AR homologue C19:0 was the most common for all three fractions, whereas C15:0 was the least common. More saturated ARs were present in the samples than unsaturated ARs. The C17:0/C21:0 ratio for all three fractions of Hazlet was 0.9.

The total amount of ARs in mg/100 g was determined for the three outermost bran fractions of Hazlet. As seen in Figure 5, the outermost fraction (1) has the largest amount of ARs at 159.75 mg/100 g, and it appears that the AR amount decreases as the bran fractions are closer to the endosperm of the kernel. Hazlet fraction 3 has less than half the amount of ARs and antioxidant activity compared to the 0−5% fraction (Figure 6).

Total Phenolic Content (TPC). Hazlet fraction 1 was determined to have a TPC of 129.61 mg of ferulic acid equiv/100 g.

MTT Assay. Cytoprotection of PC-12 AC cells by Hazlet fraction 1 was determined by means of the MTT assay. The MTT assay is a measure of mitochondrial function and metabolic activity as it relies on the activity of mitochondrial reductases. AAPH was used as a free radical generator to determine cytoprotection by Hazlet fractions. In the absence of AAPH, metabolic activity is higher, indicated by higher absorbance, than in the presence of AAPH as seen in Figure 7. This indicates that AAPH has indeed a negative effect on the cells by oxidizing cellular components. Increasing concentrations of Hazlet fraction 1 resulted in increased protection of metabolic activity in the presence of AAPH (Figure 7a) as inclusions of higher concentrations of extract brought MTT activity back to control (no AAPH) levels.

In the presence of AAPH, Hazlet fraction 2 did not show the same protective activity as Hazlet fraction 1 (Figure 7b) as viability/metabolic activity did not return to control levels. The MTT assay results also revealed that mitochondrial function was higher in the absence of extract and decreases with the inclusion of extract.

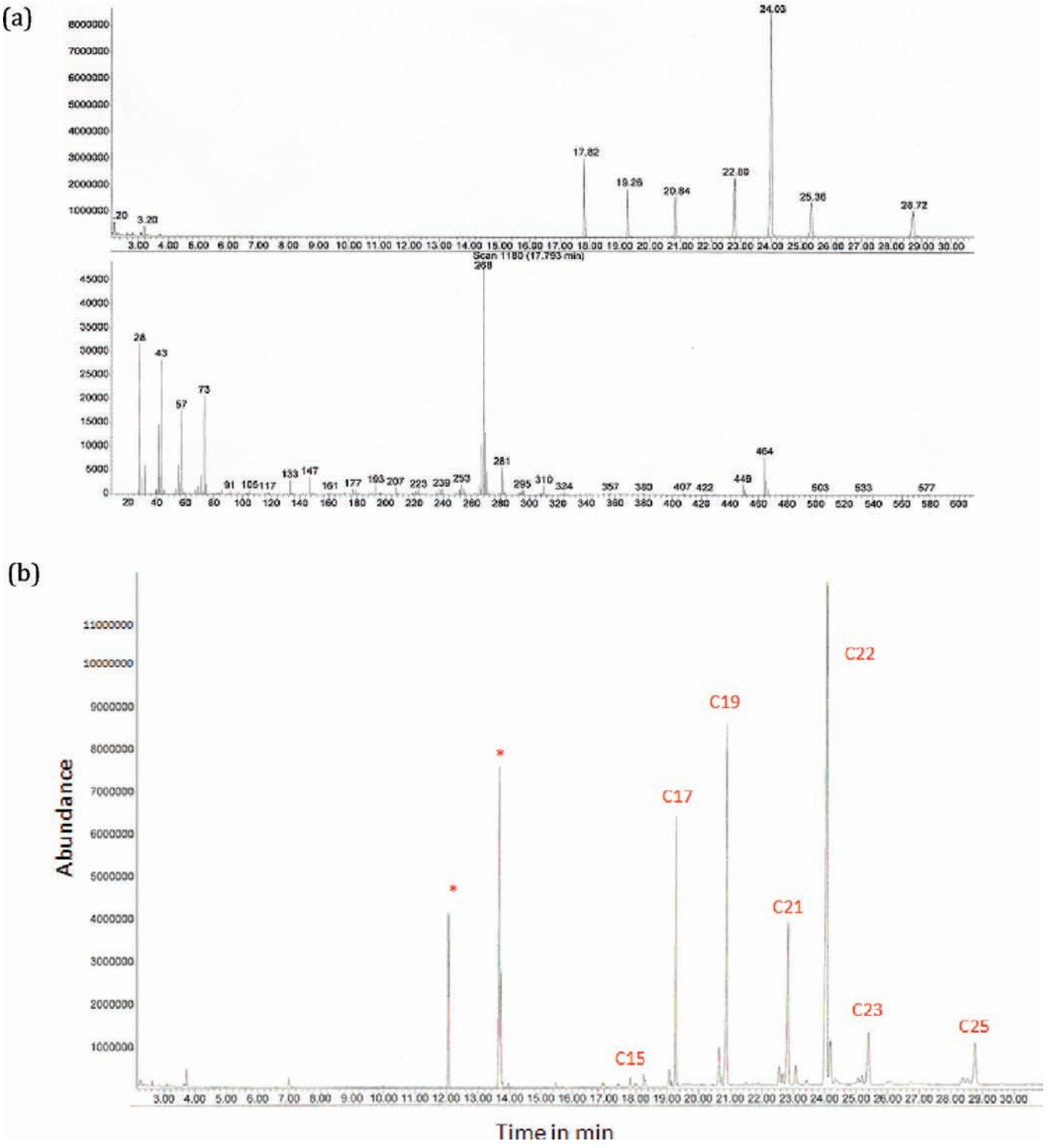

Figure 4. (a) GC-MS chromatogram for AR standards including ARs C15:0, C17:0, C19:0, C21:0, C23:0, and C25:0. (b) GC-MS chromatogram of ARs in Hazlet fraction 1. AR C22:0 is the internal standard. ∗, fatty acids including myristic (C14:0) and palmitic (C16:0) acids.

Effect of PC-12 AC Cells on Mitochondria. The effect of rye extract on mitochondria of PC-12 AC cells was determined using MitoTracker Deep Red. Panels a and b of Figure 8 show the MitoTracker stained untreated and extract (Hazlet fraction 1) treated PC-12 AC cells, respectively. Higher densities of mitochondria were seen in extract-treated cells than in nontreated cells. This suggests that mitochondrial biogenesis occurred in cells with extract treatment but not in those without.

DISCUSSION

ARs confer antioxidant activity and are present in highest amounts in rye bran, followed by triticale and wheat.[20] In this

Table 1. Relative Composition of AR Homologues (%) in Bran Fractions of Hazlet Rye Analyzed by GC-MS

sample	Hazlet fraction 1	Hazlet fraction 2	Hazlet fraction 3
alkylresorcinol homologues (%)			
5-*n*-heptadecylresorcinol C 15:0	0.85	0.94	1.60
5-*n*-heptadecylresorcinol C 17:0	17.77	16.19	17.91
5-*n*-nonadecanylresorcinol C 19:0	30.01	25.29	28.38
5-*n*-heneicosylresorcinol C 21:0	19.10	18.91	19.54
5-*n*-tricosylresorcinol C 23:0	8.91	9.19	8.37
5-*n*-pentacosylresorcinol C 25:0	8.22	10.82	8.32
unsaturated ARs	15.14	18.66	15.88
saturated ARs	84.86	81.34	84.12
C17/C21	0.93	0.86	0.92

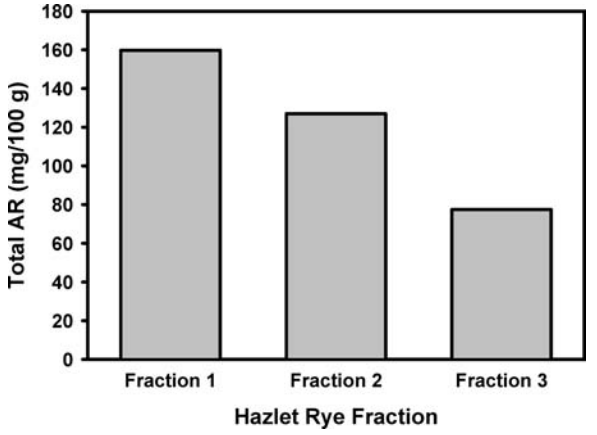

Figure 5. Total amount of ARs in the three outermost fractions of Hazlet rye determined through GC-MS analysis.

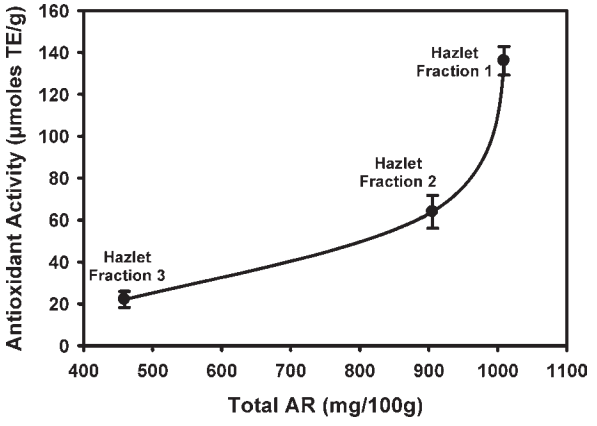

Figure 6. Relationship between the total amount of ARs and antioxidant activity of Hazlet fraction 1, 2 and 3.

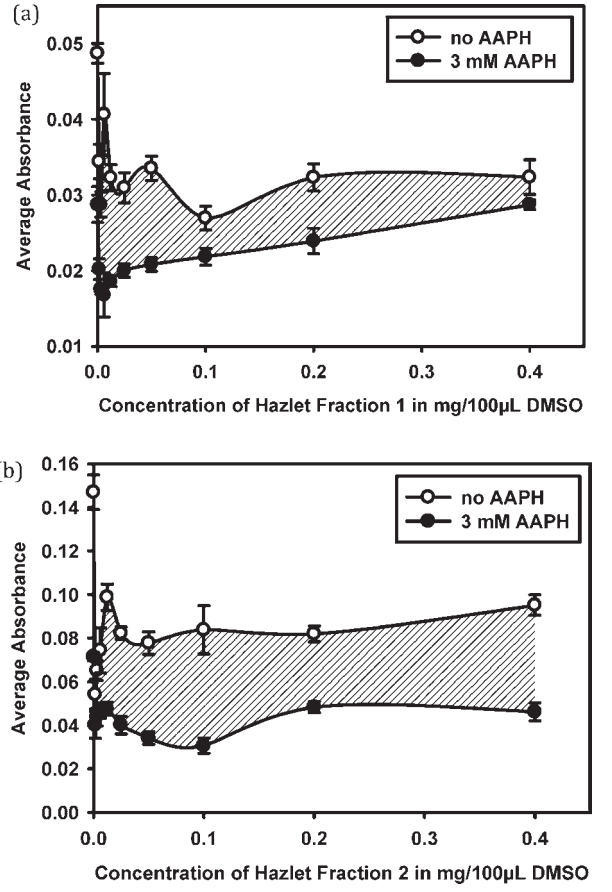

Figure 7. Cytoprotection of PC-12 AC cells by (a) Hazlet rye fraction 1 and (b) Hazlet fraction 2 in the absence and presence of 3 mM AAPH. Cells were plated out at a density of 4.0×10^5 cells/mL.

study, RT202 bran fractions had higher antioxidant activity than Hazlet bran fractions, with 186.57 and 136.05 μmol TE/g, respectively, for fraction 1 (Figure 2). Literature values for wheat bran show ORAC values of 124.29 μmol TE/g and lower.[28] Rye bran thus has higher antioxidant properties. Although Hazlet and RT202 have higher antioxidant activity than most other foods, such as fruits, vegetables, and nuts, they have much lower antioxidant properties than most spices, such as ground cinnamon and ground clove, with 2675.36 and 3144.46 μmol TE/g respectively.[29] Even though Hazlet and RT202 both have lower ORAC values than most spices, they can still be considered to have potent antioxidant capacity, because their ORAC values are larger than those of most foods that are known to be antioxidants, such as citrus fruits containing vitamin C.[30]

Triticale bran was used as a positive control in this study because it has a high amount of ARs and thus high antioxidant activity.[31] Because rye is known to have the highest amount of ARs, it could be assumed that antioxidant activity would be much

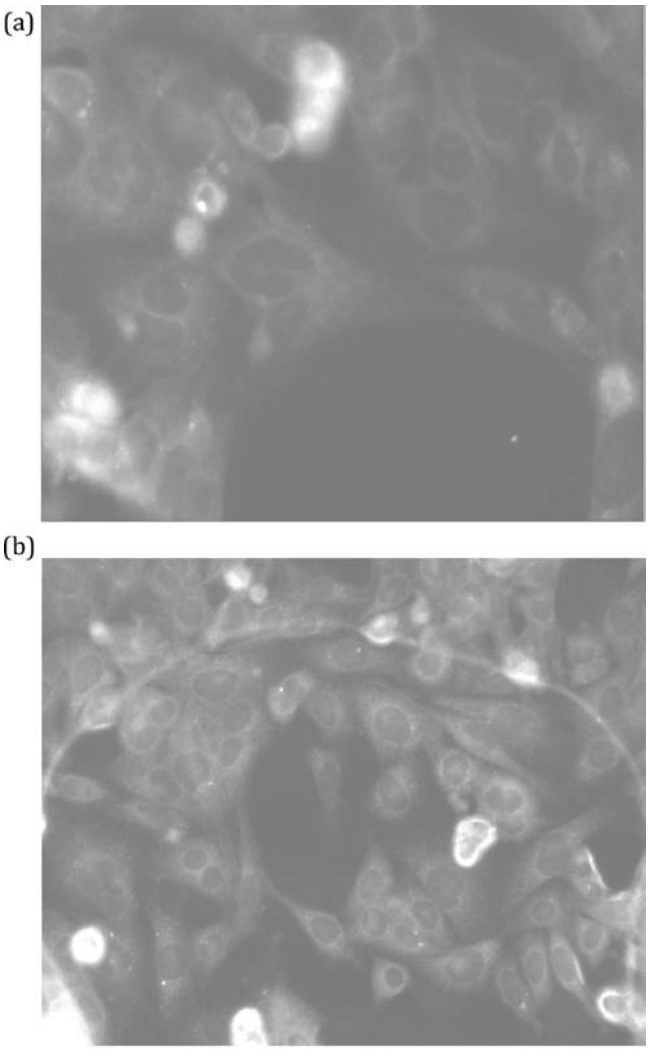

Figure 8. Mitochondria of (a) untreated PC-12 AC cells and (b) PC-12 AC cells treated with 0.4 mg/100 μL DMSO Hazlet rye fraction 1, stained with MitoTracker Deep Red FM (M22426).

higher for rye than it is for triticale. Oat was used as a negative control because it does not contain any ARs[16] and thus should have low antioxidant activity (Figure 2a). The low antioxidant activity of oat (11.81 μmol TE/g) (Figure 2a) may also be due to other compounds present in oat that give it its slight antioxidant properties, such as avenanthramides.[32] Hazlet fractions 4−6 also showed lower antioxidant activity than the outer portions of Hazlet (Figure 2a).

Trolox is a synthetic antioxidant and commonly used as a standard for antioxidant activity calculations. It was of interest to determine antioxidant activities of rye fractions when compared to a natural compound that is present in cereal bran and shows high antioxidant properties as well. Therefore, cinnamic acid was used as a standard, and whereas ORAC values follow the same trend as with Trolox, the antioxidant activity is higher overall (Figure 2b); that is, the antioxidant activity for Hazlet fraction 1 is 136.05 μmol TE/g when compared to Trolox and 656.01 μmol CAE/g when compared to cinnamic acid. The AR homologue C19:0 was used as well to assess the effect of a control compound that is structurally similar to the main compound. The antioxidant activity of all samples was higher using AR C19:0 (Figure 2c) compared to Trolox and cinnamic acid (Figure 2a,b), with values as high as 6456.47 μmol C19:0E/g for RT202 fraction 1. The structural similarity of ARs to AR C19:0 is responsible for the much higher antioxidant activity. Overall, in most studies, the antioxidant activity of phenolics is reported using only one standard, particularly Trolox. This study suggests that choosing a particular standard is crucial in the reporting of antioxidant activity, and this explains why antioxidant activity differs between standards.

The ORAC results obtained for Hazlet fraction 1 showed a delay in fluorescence decay as seen in Figure 3a. The greater the fluorescence delay, the higher the antioxidant activity of the compound. At the lowest concentration tested of Hazlet fraction 1, fluorescence decay was similar to that of rutin, which is a known antioxidant.[33] As fractions are further away from the outside of the kernel, higher concentrations of Hazlet are required to elicit the same delay in fluorescence decay (data not shown), indicating that antioxidant properties of ARs in the outermost fractions are highest.

The delay times of fluorescence decay of the Hazlet fraction 1 concentrations listed in Figure 3a were compared to the net AUC of the corresponding concentration of the fraction (Figure 3b). The net AUC, which is the area between the sample and blank, is larger when the delay time is larger. This is expected as the delay of fluorescence causes a larger gap between sample and blank and thus a larger AUC. As the concentration of Hazlet increases, the net AUC increases as well (Figure 3c). As seen in Figure 3a, the delay time of fluorescence is highest at the highest concentration of Hazlet causing a high AUC, as expected.

ARs have odd-numbered, saturated alkyl side chains ranging from 15 to 25 carbons in cereals.[34] Large GC-MS peaks with retention times of ≥17 min belong to saturated ARs of C17:0, C19:0, C21:0, C23:0, and C25:0. The main fragmentation ions of ARs were those at m/z 268 due to McLafferty rearrangement (Figure 4a), at m/z 267 due to β cleavage, and at m/z 281 due to γ cleavage.[35] Depending on injection of AR standards (C15:0−C25:0) and characteristic MS fragments, the saturated ARs are determined. Each saturated AR was confirmed by molecular ion as follows: at m/z 464 (C15:0), 492 (C17:0), 520 (C19:0), 548 (C21:0), 576 (C23:0), 604 (C25:0), and the molecular ion peak at m/z 268 for all ARs. The peaks with this characteristic m/z value were considered to be resorcinolic derivatives and were in agreement with literature results.[35] C15:0 was not found in the rye extract in great amounts and, thus, its peak is much smaller than those of the other AR homologues as seen in Figure 4b. The peak for the C15:0 homologue is also smaller than those of unsaturated ARs, such as alkenresorcinols. As well, the alkenresorcinol homologues C17:1, C19:1, C21:1, C23:1, and C25:1 were found in the rye extract. Smaller peaks that were present are possibly other AR analogues with mono- and diunsaturated aliphatic chains, undefined ARs that are less abundant, or other phenolics isolated from rye samples. Overall, the chromatogram is very clean, and the few large peaks are correctly identified as ARs. Furthermore, ARs were extracted with acetone, which is very selective and, thus, ARs should be the main component in the fractions. The data obtained for rye using GC-MS agree with literature values.[35] There are two large peaks at retention times of 12 and 13.7 min. These likely correspond to fatty acids such as myristic and palmitic acids. GC-MS results showed peaks that were mainly related to ARs. Other unknown peaks in Figure 4b could be

related to phenolics such as hydroxycinnamates because these are abundant in cereals as well and have shown antioxidant activity.[36]

The percent AR homologue values obtained and listed in Table 1 agree with the literature values, where the AR homologue C19:0 is known to be the main homologue in rye.[16,34] Wheat, triticale, and barley all have higher amounts of the C21:0 homologue. The ratio of C17:0/C21:0 indicates the type of cereal present, with ~1 for rye and ~0.1 for wheat.[37] Because the three Hazlet samples analyzed all had a ratio of ~0.9, it is safe to assume that the samples were pure.

TPC, for the outermost fraction of Hazlet rye, was determined to be 129.61 mg of ferulic acid equiv/100 g of sample. TPC can range from 65 to 300 mg/100 g in rye depending on the variety.[38] Generally, the higher the amount of total phenolics, the greater the antioxidant capacity, because phenolic compounds have high antioxidant activity. Of the different grain products, rye bran has the second highest TPC after wheat bran. Low antioxidant capacity grains such as oat bran or rice have low TPCs, with 33 and 12 mg of ferulic acid equiv/100 g of sample, respectively.[39]

The extraction method used was specific for ARs; it is, however, possible that other phenolics, such as cinnamic acid, were extracted in small quantities as well as other components as seen by the extra peaks in Figure 4b. ARs could then synergistically work with the other phenolics to increase the antioxidant activity. Because the TPC determined for Hazlet fraction 1 was high and falls within the expected range, it can be assumed that ARs contribute largely to the overall antioxidant activity, which is also supported by the ORAC results in Figure 2a.

ARs are known to provide high protection against free radicals in membranes by incorporating themselves directly in the membrane.[19] The most common phospholipids in membranes have an alkyl chain with ~18 carbons.[40] This would suggest that ARs in cereal bran are good candidates for incorporation into membranes and protect them from oxidation. This study showed that AR C19:0 was the highest amount (30.01% in Hazlet fraction 1, 25.29% in fraction 2, and 28.38% in fraction 3) of AR found in rye bran, indicating AR C19:0 may incorporate efficiently into membranes. Future studies need to determine which ARs have better functionality in human cell membranes.

The highest amount of ARs was found in Hazlet fraction 1 (1597.5 μg/g), whereas the lowest amount was found in fraction 3 (774.2 μg/g) as seen in Figure 5. This indicates that the outermost layer of the bran has a higher amount of ARs. Additionally, the value falls within the expected range.[34]

From a comparison of the results for antioxidant properties obtained using the ORAC assay and the total amount of ARs using GC-MS, it becomes clear that ARs are in fact the components that give rye its antioxidant activity, because antioxidant activity increases exponentially as the amount of ARs increases (Figure 6). A linear dose−response relationship was expected, but minor contamination of endosperm with bran during milling and fractionation of Hazlet rye bran fraction 2 could have caused the nonlinear relationship.

It seems reasonable that the outer fractions of the bran layer in rye kernels show higher antioxidant activity elicited by a higher amount of ARs, because kernels have known antifungal and antiparasitic activity.[41] The kernel uses ARs as a defense mechanism against fungi and parasites to protect the germ, which is the embryo and responsible for reproduction. Hence, having ARs in the outermost layer is crucial for plant survival.[42]

Because ARs are used as biomarkers for whole grain products, it can be assumed that baking and processing do not eliminate the antioxidant activity as they are still present in whole grain breads.[43] Michniewicz and Jankiewicz[43] studied the effect of hydrothermic treatment on many components, such as starch, sugars, proteins, and ARs, of the rye grains of two Polish rye grain varieties. Even autoclaving did not affect the amount of ARs present, which would indicate that baking should not alter ARs. However, Li et al.[44] determined that TPC, ORAC values, and total anthocyanins were reduced during the processing of purple wheat bran muffins, whereas these components were not affected in heat-treated purple wheat bran or muffin extracts. Because rye and wheat are similar, it is expected that rye would exhibit similar results. Studies done to assess the stability of ARs during processing in bread[16] and pasta[45] have found that ARs are stable, but are hard to extract as they form complexes with starch.

MTT is a cell viability assay that measures mitochondrial enzyme activity. Because it is a measure of mitochondrial activity, it is also used as a measure of mitochondrial biogenesis. In the cytoprotection studies, for both Hazlet fractions 1 and 2, it appears that MTT absorbances were highest when no rye extract was present and then decreased when extract was added. The addition of rye extract had no effect on cell viability, even at much higher concentrations (data not shown); it would appear that Hazlet decreases mitochondrial activity, rather than causing cell death. At much higher concentrations, Hazlet treatment resulted in a remarkable increase in cell proliferation, which also indicated that rye influences metabolic activity. As seen in Figure 7a, as the concentration of Hazlet increases, rye seems to increase the metabolic activity of the cells in the presence of AAPH to return it to a normal state, which represents antioxidant protection by Hazlet. To an extent, lower cellular metabolic activity results in a lower production of ROS, which would extend cellular lifespan, because ROS are known to negatively affect aging.[1] However, low mitochondrial metabolic activity also means a lower production of energy in the form of ATP, which is required to fuel most of the body's reactions. It is possible that higher concentrations of rye extract produce metabolic rates of cells that have a good balance between the production of ATP and ROS to cause a decrease in damage of cellular components by a decrease in ROS but to also keep an adequate level of energy. This would suggest that cells were more efficient in their utilization of oxygen such that fewer ROS were produced from normal aerobic metabolism.

Figure 7b shows that Hazlet fraction 2 does not have the same protective effect as Hazlet fraction 1. The outermost fraction of the Hazlet bran thus seems to have higher cytoprotective activity than the second outermost fraction. This finding correlates well with the results obtained using the ORAC assay and GC-MS.

Mitochondrial biogenesis occurred when PC-12 AC cells were treated with Hazlet fraction 1 (Figure 8). The total fluorescence was much lower in untreated cells than in cells treated with rye extract. Increased mitochondrial biogenesis may be the result of cells compensating for the decrease in metabolic activity by increasing cellular mitochondrial content. To maintain cellular ATP levels, PC-12 AC cells may increase their number of mitochondria, but the mitochondria are more efficient at oxygen utilization (i.e., fewer ROS are produced). Having more efficient mitochondria is therefore cytoprotective in that less damage is incurred. It was also shown that the number of mitochondria was reduced in Alzheimer's patients,[46] which suggests that having fewer, less efficient, mitochondria (and thus increased levels of ROS) may contribute to the development of neurodegenerative disorders.

The present study is important because it focuses on each individual fraction of the bran layer instead of the bran layer as a whole. It suggests that rye is a good dietary source of ARs, especially in the outer layer of the bran. Hazlet and RT202 contain a high amount of ARs in the outermost layer of the bran and lower levels in bran fractions closer to the endosperm. The amount of ARs gives rye its high antioxidant activity, which is greater than that of other cereals, such as triticale and wheat. The most common AR homologue in rye is C19:0, which allows for better incorporation into membranes and thus better protection from free radicals. Hazlet fraction 1 had a greater cytoprotective effect than Hazlet fraction 2 on PC-12 AC cells against AAPH. Hazlet causes a decrease in metabolic activity, which is balanced with an increase of mitochondrial biogenesis. The mechanism by which rye ARs effect this cellular change remains to be investigated.

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