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ORIGINAL CONTRIBUTION

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Rye bran bread intake elevates urinary excretion of ferulic acid in humans, but does not affect the susceptibility of LDL to oxidation *ex vivo*

Summary *Background* Rye bread contributes an important part of the whole grain intake in the Scandinavian diet. Ferulic acid is the major phenolic compound in rye bran and is an antioxidant *in vitro* and may, therefore, contribute to cardioprotective effects of whole grain consumption. *Aim of study* Firstly, to evaluate the bioavailability and potential antioxidative effects in humans of ferulic acid from rye. Secondly, to evaluate urine levels of ferulic acid as a possible biomarker of the ordinary dietary intake of ferulic acid. *Methods* We determined the urinary excretion of ferulic acid in 18 postmenopausal women after a dietary intake of rye bran or an inert wheat bran (control) in a crossover study (2 x 6 weeks with 4 weeks washout). The potential antioxidative effect of the rye bran intervention was investigated by measuring low-density lipoprotein (LDL) susceptibility to copper oxidation *ex vivo*. The subjects ingested rye bran enriched breads equivalent to ~10.2 mg ferulic acid per day. *Results* The urinary excretion of ferulic acid averaged ~ 4.8 mg per day during intervention with rye bran breads and ~1.9 mg per day on the control breads ($P = 0.002$). Rye bran intervention had no influence on lag time or propagation rate of the LDL oxidation *ex vivo*. *Conclusions* The present study demonstrated that ferulic acid from rye bran is bioavailable and that the urinary concentration of ferulic acid reflects the dietary intake of this hydroxycinnamic acid. Within the period of intervention, the elevated ferulic acid did not produce a measurable antioxidative effect on the subjects' LDL. It is suggested that the determination of ferulic acid in urine is a useful biomarker to assess the intake of ferulic acid from a regular diet.

Key words ferulic acid – rye – bioavailability – hydroxycinnamates – LDL oxidation – biomarker

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Introduction

Epidemiological studies have shown that dietary intake of whole grain foods is associated with reduced coronary heart disease [1–3]. In addition to dietary fiber, whole grain products may contain a variety of biologically active constituents, such as vitamin E and vitamin B6, folic acid, selenium, zinc, and phenolic compounds, which may contribute to the apparent cardioprotective effects of whole grains [4]. Rye bread contributes an important part of the whole grain and dietary fiber intake in the Scandinavian diet. In Denmark, the average consumption of rye bread is approximately 63 g/adult/day [5]. Phenolic extracts from rye, notably from rye bran, have been reported to inhibit LDL oxidation *in vitro* [6]. Ferulic acid is the major hydroxycinnamate in rye and 86 % of the total amount (~1000 μg/g) is found in the bran fraction [7], and this phenolic acid has been shown to possess antioxidant properties in various *in vitro* assays [8–13]. Ferulic acid is present in the cereal cell walls, where it is ester-linked to arabinoxylan. Ferulic acid may

EJN 463

be released from arabinoxylan by esterases located in the mucosa cells in the small intestine or by esterase activity of the colon bacteria [14, 15].

The physiological importance of ferulic acid depends on several factors, including its release from the cell wall arabinoxylan, its absorption, distribution, metabolism, and excretion. Very few studies have evaluated the bioavailability of ferulic acid from real foods in human subjects. Some data have been obtained from single dose consumptions of prunes, tomatoes, low-alcohol beer, and a wheat bran breakfast cereal using only a very limited number of subjects [16–19]. Bourne and Rice-Evans found that ferulic acid urinary excretion ranged from 2.7 to 5.5 mg after consumption of a single dose of tomatoes [17] and from 1.8 to 9.3 mg after consumption of 4 liters of low-alcohol beer over a period of 4 h [18]. The total urinary excretion of ferulic acid 24 h after consumption of 100 g wheat bran breakfast cereal ranged from ~ 5 to 14.5 mg in the six volunteers that were used in the investigation [19]. In the present study, we have examined the urinary excretion of ferulic acid after intake of rye bran enriched products in a human intervention study using a 6-week crossover design. Since the half-life of hydroxycinnamates in plasma is known to be short (2 h) [20], the urinary excretion of ferulic acid within 24 h was chosen as a biomarker for the bioavailable fraction of this compound from rye bran. The potential antioxidative effect of the rye bran intervention was investigated by measuring plasma low-density lipoprotein (LDL) susceptibility to copper oxidation *ex vivo*.

Subjects and methods

Subjects

Eighteen healthy, normo-lipidemic, non-smoking postmenopausal women between 55 and 71 years were recruited from the Danish civil registration system, in which all inhabitants in Denmark are registered. Postmenopausal women were selected for the study for two reasons: (i) women were targetted because the present study was part of a larger investigation on the phytoestrogenic effects of rye lignans that may be related to estrogenic effects; and (ii) postmenopausal women were selected to avoid any possible confounding effects from the subjects' cyclic, endogenous estrogen production. No vitamin or mineral supplements were allowed during or 2 months prior to the study. No medicine including antibacterial treatment or hormone replacement therapy was allowed 6 months prior to entering the study. None of the subjects consumed more than 14 alcoholic drinks per week. Body weight was measured in the two intervention periods at weeks 0, 3 and 6. Basic characteristics of the subjects are given in Table 1. The Ethical Committee of Frederiksberg and Copenhagen, Denmark approved the protocol (file no. KF 01–070/01) and written consent was obtained from all participants prior to the study.

Table 1 Basic characteristics of the subjects and their dietary fiber intake before and during intervention (average ± SEM)

	n = 18
Age (years)	63.3 ± 1.2
Weight (kg)	69.3 ± 3.2
BMI (kg/m^2)	25.1 ± 0.9
Dietary fiber intake:	
at baseline (g/day)	21.0 ± 1.5
during rye bran period (g/day)	24.5 ± 1.4
during Vitacell period (g/day)	26.0 ± 1.7

Study design

The study was a randomized crossover study with two 6-week intervention periods separated by a 4-week wash-out period. The subjects conducted a 4-day weighed food record 1 month prior to the study to assess their baseline habitual daily energy intake. The 4-day weighed food records were used to estimate the average intakes of energy and macronutrients only. The subjects were randomly assigned into two groups, and in the crossover design all subjects thus acted as their own control. One group was instructed to consume 250 g rye bran enriched products per day and the other group to consume 250 g control wheat products (Vitacell) per day for 6 weeks. Vitacell is a commercially available, purified wheat cellulose fiber containing no detectable amounts of ferulic acid (Vitacell® Wheat Fibre WF600, J. Rettenmaier & Söhne GMBH + Co., Rosenberg, Germany). The subjects could choose between three different types of rye bran or Vitacell enriched products: bread, muffin and crisp bread. The subjects replaced their normal bread consumption with the test breads and thereby maintained their usual eating patterns. After a 4-week wash-out period, during which the subjects consumed their habitual diet, the groups continued with the alternative bread for another 6-week period. To monitor the intakes of energy, dietary fiber, and macronutrients, the dietary intake of selected nutrients was assessed by a 4-day weighed food record during each period. Nutrient intakes were calculated by using a national food-composition database (Dankost 2000, Herlev, Denmark). The rye bran bread contributed with approximately 58.3 g rye bran per day, and the rye bran contained ~175 µg ferulic acid per gram dry matter as analyzed by HPLC (data not shown). This level corresponds to a daily intake of 10.2 mg ferulic acid during the rye bran period. No ferulic acid was detected in the control wheat bran (Vitacell) (data not shown).

European Journal of Nutrition (2004) Vol. 43, Number 4

Collection of urine samples and blood samples

At weeks 0 and 5 in both intervention periods, 48-h urine samples were collected in 2.5 L plastic containers containing 10 mL 10 % ascorbic acid (final concentration ~ 0.05 %) and 50 mL 1 mol/L HCl (final concentration ~30 mmol/L). After the first morning void, all urine was collected for the following 2 days, including the third morning's first void. Specific gravity and pH were measured in the pooled 48-h urine samples, before aliquots were weighed out for further analysis. The urine samples were overlaid with nitrogen and stored at –20°C. Blood samples were obtained before and at the end of each intervention period. The day before blood sampling, intake of alcohol and participation in sports were not allowed. Venous blood samples were collected into 10 mL tubes containing 0.1 % EDTA after an overnight fast (> 10 h). Samples were kept in the dark and on ice until centrifugation. The samples were centrifuged for 15 min at 3000 rpm and at 4°C. Plasma samples were then immediately aliquotted, overlaid with nitrogen, and frozen at –80°C for a maximum of 2 weeks prior to oxidation analysis.

Compliance

Compliance was assessed by a 4-day weighed food record during each intervention period.

Chemicals

Acetonitrile and methanol were of HPLC grade and obtained from Riedel-de Haën (Seelze, Germany). Ferulic acid, anisic acid, β-glucoronidase with known levels of arylsulfatase side activity (from Helix pomatia, type H-2, G-0876), and chemicals for Lowry protein determination were purchased from Sigma Aldrich Chemicals (St. Louis, USA).

Analysis of ferulic acid

Rye bran and Vitacell sample preparation

Brans were milled in a Retch Mill (model ZM1) and sieved through a 0.5 mm screen, suspended in buffer (0.08 mol/L phosphate, pH 6.0), combined with internal standard (anisic acid), and subjected to α-amylase treatment, alkaline hydrolysis, acidification, ethyl acetate extraction, and vacuum rotary evaporation (30°C) by the method of Andreasen et al. [21]. After the evaporation, the dry residue was dissolved in 10 mL methanol + 10 mL 0.02 mol/L phosphate, pH 2.15, and filtered into an HPLC vial through a syringe tip 0.45 µm nylon filter (Micro Filtration Systems, SRi Ltd., Gloucester, UK).

Urine samples

Urine samples were prepared according to the procedure described by Cremin et al. [16]. Urine samples were thawed and 2 mL were mixed with 0.05 mol/L sodium acetate buffer (pH 5.5) (0.45 mL) and 0.6 mol/L $CaCl_2$ solution (1 mL). Anisic acid was added as internal standard. Enzymatic hydrolysis was performed by addition of 32 µL β-glucoronidase/arylsulfatase to a final concentration of ~1050 U/mL and ~25 U/mL, respectively. To avoid undesirable microbial growth, azide (NaN_3) was added to a final weight of 0.02 % and the samples were incubated for 4 h at 37°C (nitrogen-flushed tubes, with shaking). After incubation, the samples were acidified with 6 mol/L HCl (20 µL) and extracted twice with ethyl acetate (4 + 3 mL). Phases were separated by centrifugation for 10 min at 4500g, 4°C. The combined ethyl acetate fractions were evaporated to dryness under nitrogen, redissolved in 50 % v/v aqueous methanol, and filtered through 0.45 µm nylon syringe tip filters (Micro Filtration Systems, SRi Ltd., Gloucester, UK) prior to HPLC analysis.

Quantification of ferulic acid by HPLC

Phenolic acids were analyzed by HPLC on a Hewlett-Packard 1100 instrument equipped with an RP C_{18} Novapak column and a diode array detector using a procedure described by Lamuela-Raventòs and Waterhouse [22]. Ferulic acid was identified by spectral and retention time analysis by comparison with an authentic ferulic acid standard, and then quantified from peak area at 316 nm using an external ferulic acid standard curve and anisic acid as internal standard.

Preparation and oxidation of LDL

LDL (1.019–1.063 g/mL) was isolated from plasma by density ultracentrifugation (18 h at 40 000 rpm, 4°C in a Beckman, L8–70M Ultracentrifuge) (Beckmann, Palo Alto, USA) by the procedure described by Princen et al. [23]. After determination of LDL protein concentration with the Lowry method [24], the individual LDL samples were diluted in 0.01 mol/L phosphate buffered saline with EDTA (PBS-EDTA), 0.15 mol/L NaCl, pH 7.4, 10 µmol/L EDTA to a standard protein concentration for the oxidation assay (see below), deoxygenated by flushing with N_2, and kept on ice. To minimize the loss of lipophilic antioxidants, the LDL was not dialysed prior to oxidation [25].

Susceptibility of LDL to oxidation in vitro

LDL, at a concentration of 0.08 mg protein/mL in PBS, pH 7.4 and EDTA diluted to 2.5 µmol/L, was oxidized in

the presence of 5 µmol/L $CuSO_4$ at 37°C in quartz cuvettes placed in a Pelletier-thermostat-controlled automatic sample changer in a UV/VIS spectrophotometer (Model λ20, Perkin Elmer, Norwalk, USA). The oxidation was evaluated by monitoring the conjugated diene lipid hydroperoxide formation by recording the absorbance at 234 nm every 30 s during 3 h [26]. The results were calculated after triplicate analysis and expressed as lag time and propagation rate computed from linear regression analyses of the data using the regression functions of the Microsoft Excel spreadsheet program (MS Office 2000).

Determination of LDL cholesterol

The concentration of LDL cholesterol was determined in plasma by enzymatic colorimetric methods, using a commercially available kit (Boehringer Mannheim, Mannheim, Germany), on a COBAS MIRA auto-analyzer (Roche, Basle, CH).

Statistics

Content of the ferulic acid in urine and the LDL oxidation parameters, lag time and propagation rate, in the Vitacell or rye bran diet groups were compared with the student's *t* test. Period effects were evaluated as described by Altman [27] by a paired *t* test comparing the two groups average of the differences ($\bar{e}_i$), i. e. $\bar{e}_1$ and – $\bar{e}_2$ (see Table 2). Treatment x period interaction was determined using the average ($\bar{a}_i$) of the mean value of all observations in each period, i. e., comparing $\bar{a}_1$ and $\bar{a}_2$ with a *t* test. If there was no difference in these tests between the two groups (level of rejection $P > 0.05$), it was assumed that influence of period and order of treatment were minimal, and the effect of the intervention could, thus, be determined by comparing d_1 with d_2 with a paired *t* test [27].

Results and discussion

The 4-day weighed food records indicated good compliance with the prescribed bread consumption. Compliance was similar within both rye bran and Vitacell intervention, as the subjects had an average intake (± SD) of test bread of 218 (± 32) g/day and 215 (± 47) g/day, respectively. The intakes of dietary fiber from rye bran and Vitacell during intervention were also similar (Table 1).

Excretion of ferulic acid

There were no period effects or any treatment-period interactions on the levels of ferulic acid detected in urine (Table 2); therefore, a two-sample *t* test was used to examine the differences between the two bread interventions. Ferulic acid was found in the urine at baseline and during the supplementation with both rye bran and Vitacell products. There were no significant differences between the urinary ferulic acid level in the period where the subjects were on their habitual diet and in the Vitacell intervention period ($P = 0.983$) (Table 3). However, as seen in Tables 2 and 3, the urinary excretion of ferulic acid was significantly higher during the intervention with rye bran compared with Vitacell intervention ($P = 0.002$) and vs. the baseline level ($P = 0.001$). The relative increase in ferulic acid excretion in urine after the rye bran consumption corresponds to approximately a doubling compared to both baseline and Vitacell intervention (Table 3). The significant increase in the amount of ferulic acid excreted in the urine after the

Table 3 Urinary ferulic acid excretion, LDL plasma cholesterol and LDL oxidation parameters (lag time and propagation rate) in postmenopausal women (n = 18) before (habitual diet) and after a 6-week intervention period with Vitacell (control) and rye bran (as average ± SD)

Biomarker	Habitual diet	Vitacell	Rye bran
Urinary ferulic acid (mg/day)	1.92 ± 2.41	1.94 ± 1.58	4.82 ± 3.46
LDL cholesterol (mmol/L)	3.78 ± 0.9	3.65 ± 0.9	3.78 ± 1.0
Lag time (min)	52.7 ± 5.6	51.3 ± 4.6	51.2 ± 5.2
Propagation rate x 10^{-4} (ΔOD/min)	268 ± 3.2	270 ± 3.1	267 ± 3.3

Table 2 Statistical evaluation of the effects of period, treatment x period and treatment (rye vs. Vitacell) in urinary ferulic acid, LDL cholesterol and LDL oxidation (lag time and propagation time) in postmenopausal women (n = 18) (average values and P-values)

	Period effect			Treatment-period effect			Treatment effect		
	$\bar{e}_1$	$-\bar{e}_2$	P-value	$\bar{a}_1$	$\bar{a}_2$	P-value	$\bar{e}_1$	$\bar{e}_2$	P-value
Urinary ferulic acid (mg/day)	1.36	3.22	0.098	1.99	0.72	0.079	1.36	–3.22	0.002
LDL cholesterol (mmol/L)	0.06	0.10	0.557	–0.08	–0.06	0.537	0.06	–0.10	0.315
Lag time (min)	–4.06	4.96	0.012	–2.26	–0.73	0.910	–4.06	–4.96	0.403
Propagation rate (ΔOD/min)	$4 \cdot 10^{-4}$	$2.6 \cdot 10^{-3}$	0.646	$3 \cdot 10^{-5}$	$1.1 \cdot 10^{-2}$	0.395	$4 \cdot 10^{-4}$	$-2.6 \cdot 10^{-3}$	0.312

European Journal of Nutrition (2004) Vol. 43, Number 4

rye bran intervention provides evidence for the absorption and bioavailability of ferulic acid from rye bran. Furthermore, the doubling of the level confirms our supposition that whole meal rye may provide an important dietary source of ferulic acid. The strong relationship between ferulic acid intake and urinary excretion has also been demonstrated in rat studies [28, 29]. Our data thus support the use of urinary ferulic acid excretion as a valid biomarker for its bioavailability. The estimated recovery of ferulic acid from the rye bran enriched products, i. e., the additional amount excreted in urine relative to the additional dietary intake of 10.2 mg ferulic acid/day during rye bran intervention, was approximately ~28 %. This estimated recovery is severalfold higher than the ~ 3 % reported recently after a single serving of wheat bran breakfast cereal [19], but in agreement with data obtained from single dose intakes of tomatoes, where a recovery of 11–25 % has been reported [17]. Although the levels of bound vs. free ferulic acid in the tomatoes [17] were not explicitly reported, we assume that the concordance between the ferulic acid recovery data of tomatoes and rye may be related to the fact that ferulic acid occurs as bound to polysaccharides in both foods. In tomatoes, ferulic acid is presumably bound to arabinose side chains of rhamnogalacturonan [30]. Adam et al. [28] studied the influence of complex cereals on ferulic acid absorption in rats. In accordance with the recent data on the wheat bran breakfast cereal [19], they found a lower recovery of ferulic acid in urine from cereals, where ferulic acid occurs bound to arabinoxylan, than when ferulic acid was present in the diet as the free acid. In beer, the ferulic acid presumably occurs in the free form, which could explain the relatively higher urinary ferulic acid recovery of 19–98 % obtained after consumption of low-alcohol beer [18]. Thus, from the available data, it appears that ferulic acid bound to polysaccharides has lower bioavailability than free ferulic acid.

The urinary ferulic acid concentration does, however, not necessarily reflect the absolute absorptive efficiency, as absorbed ferulic acid may be metabolized, stored, or excreted with bile [30]. In a perfusion study of rat intestine *in situ*, it was shown that 5–7 % of the absorbed ferulic acid was excreted with bile [28]. Besides hepatic metabolization and loss with feces, ferulic acid may be degraded by the intestinal microflora [31]. However, the correlation between intake of ferulic acid and urinary excretion found in our present study, in the available single dose human studies, and in rat studies supports the use of urinary ferulic acid excretion as a biomarker for its bioavailability [28, 29].

Effect of rye bran on LDL oxidation and LDL cholesterol

There were no period effects or treatment-period interaction on LDL cholesterol levels (Table 2); therefore, a two-sample *t* test was used to examine the differences between the rye bran and the control (Vitacell) bread intervention. No changes in LDL cholesterol levels were observed after rye bran bread consumption (Tables 2 and 3). A periodic effect was observed for lag time (Table 2) ($P = 0.012$), but not for propagation rate. There were no differences in compliance, but perhaps the periodic changes for lag time can be ascribed to a decrease in physical activity and/or seasonal changes in the diet from summer to winter, as the study was conducted from August to December. Rye bran intake had no effect on either lag time ($P = 0.403$) or propagation rate ($P = 0.312$) compared to Vitacell (Table 2). Furthermore, no significant changes in LDL cholesterol concentration or LDL oxidation parameters were found between the habitual diet period and the Vitacell intervention (Table 3).

Oxidation of LDL is important in the pathogenesis of atherosclerosis and LDL susceptibility to oxidative modification is modulated by its fatty acid composition and antioxidants incorporated into the particle plus antioxidants circulating in plasma [32]. *In vitro* studies have shown that ferulic acid and phenolic extracts of rye bran inhibits LDL oxidation [6, 10]. However, we were unable to detect any *in vivo* protective effect of rye bran intake on LDL susceptibility to oxidation *ex vivo* even though urinary excretion of ferulic acid increased significantly with rye bran intervention. One explanation for the missing effect could be that hydrophilic antioxidants, including ferulic acid, are separated from the LDL fraction during the ultra centrifugation. However, an effect on LDL's susceptibility to oxidation is still possible through a sparing effect on the lipophilic antioxidants incorporated in the LDL particles. *In vitro* studies with, for example, walnut polyphenolics [33] and green tea catechins [34] have shown a sparing effect on the lipophilic antioxidants incorporated in the LDL particles. On the other hand, it has been shown *in vitro* that the antioxidative effect of ferulic acid could not be ascribed to a sparing effect on α-tocopherol, as demonstrated for caffeic acid [11]. Furthermore, ferulic acid has been shown to be a less potent antioxidant *in vitro* than other hydroxycinnamates, such as caffeic acid and sinapic acid [9, 11]. However, no information is available on the relative antioxidant activity towards LDL of possible metabolites of ferulic acid. Another explanation could be that the plasma level of ferulic acid may be low after a 12-h fasting, as compared to the kinetics of other hydroxycinnamates, e. g., caffeic acid [20]. Azuma et al. [20] showed that the half-life of caffeic acid was only about 2 h and that it rapidly returned to undetectable

levels within 6 h after administration. Thus, the missing effect on LDL's resistance towards oxidation in our study could be that ferulic acid is present in too small amounts in plasma to exert a protective/sparing effect on other more lipophilic antioxidants. Alternatively, its eventual contribution may have been too insignificant to be measurable in our subjects that retained their regular eating habits during the intervention periods. The intake of ~10 mg ferulic acid per day via consumption of ~ 58 g rye bran per day for 6 weeks must be considered as a high intake. Rye kernels and, hence, products prepared from whole rye meal or rye bran constitute a rich source of ferulic acid: the kernels contain ~ 900–1170 μg ferulic acid per gram dry matter depending on the rye variety, with the highest concentrations in the bran [7]. Whole rye also harbors relatively high amounts of ferulic acid dehydrodimers, where the levels range from ~250 to 400 μg per gram dry matter with the 8-O-4'-DiFA [(*Z*)-β-{4-[-2-carboxyvinyl]-2-methoxy}-4-hydroxy-3-methoxycinnamic acid] being the most abundant dimer at concentrations ranging from ~125 to 200 μg per gram whole rye dry matter [7]. We have observed that the 8-O-4'-DiFA is a slightly better antioxidant than ferulic acid when tested at equimolar levels on human LDL oxidation *in vitro*. Nevertheless, the lower abundance of dehydrodimers coupled with their presumed low bioavailability make us believe that their physiological significance as antioxidants may be limited. Although the intake of ferulic acid (and its dehydrodimers) via consumption of high bran cereals may be substantial [19], a continued intake of significantly more than 10–15 mg of ferulic acid per day may be difficult to achieve via a normal diet using regular food products. Whether any circulating levels of ferulic acid in plasma can become high enough to exert *in vivo* effects against LDL oxidation in the long term thus remains to be demonstrated. Still, ferulic acid, and perhaps diferulic acids, may have beneficial effects on other mechanisms involved in atherosclerosis, such as inflammation, thrombosis, and lipid metabolism, for instance through an effect on cyclooxygenase and lipoxygenase, as showed for flavonoids [35–37]. More studies are needed to clarify any eventual, beneficial non-antioxidant effects of ferulic acid and ferulic acid dehydrodimers in humans.

Conclusion

In the present study, we have demonstrated that the urinary concentration of ferulic acid reflects the dietary intake of this hydroxycinnamic acid. The determination of ferulic acid in urine is, thus, a useful biomarker to assess the intake of ferulic acid from a regular diet. Although ferulic acid is an antioxidant [8, 12], it is not a very potent one compared to other phenolics and other hydroxy-cinnamates [6, 9], and we were unable to show any protective effects of the intake of ferulic acid or of any other components contained in rye bran on the LDL particles' susceptibility to oxidation *in vitro*. However, it cannot be excluded that ferulic acid or rye bran can have other antioxidative effects *in vivo*.

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