

ORIGINAL COMMUNICATION

Phloem fortification in rye bread elevates serum enterolactone level

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Objective: To analyse the lignan content of phloem powder enriched rye bread and to study the dose–response relationship of the effect of dietary plant lignans derived from phloem on intestinal production of enterolactone by measuring enterolactone concentration in serum.

Design: A randomized double-blind supplementation trial.

Subjects: Seventy-five non-smoking men recruited by newspaper advertisements.

Intervention: Subjects were randomized to three study groups receiving either rye bread high in phloem (HP, 14% of rye flour substituted with phloem powder), rye bread low in phloem (LP, 7% of rye flour substituted with phloem powder) or placebo rye bread. Participants consumed 70 g of study bread daily for 4 weeks and provided serum samples for enterolactone analysis at baseline and at the end of the intervention.

Results: There was a significant increase in serum enterolactone concentration in the LP and HP groups compared with the placebo group ($P=0.009$ and $P=0.003$, respectively). Considerable interindividual differences were observed in the response to dietary lignans within the study groups.

Conclusions: Our results indicate that plant lignans attached to insoluble fibre layer in phloem can be further metabolized and converted to enterolactone presumably by the bacteria present in the colon. Phloem powder is useful source of lignans for functional foods aimed to elevate serum enterolactone levels.

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Contributors: MV participated in designing and implemented the study protocol, undertook most of the data analysis and drafted the paper. JM participated in designing and carrying out the study. TN carried out the lignan analysis of the food stuffs. SV participated in designing and contributed to the analysis of the food record data. THR participated in designing and brought up to date the dietary data base. RS as physician examined the participants. HA developed and supervised the enterolactone assays. JTS designed and initiated the study and edited the manuscript. All investigators contributed to the writing of the paper.

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Introduction

The arrival of polyphenol enriched nutraceuticals to the market has created a need to analyse the content of different polyphenols in the products and their bioavailability in the medium in which they are present. In Finland the use of phloem bread and other newly available phloem-enriched food products has become an interesting curiosity from the past for some consumers. The high content of fibre and polyphenols in phloem powder is assumed to bring potential health benefits.

Polyphenols like lignans and flavonoids are often postulated to explain some of the association of diets high in whole grain, fruit and vegetables with reduced risk of cardiovascular disease (Anderson & Hanna, 1999; Ness & Powles,

1997). Dietary plant lignans are converted by the intestinal microflora to enterolactone which can thereby be measured in serum and urine (Adlercreutz & Mazur, 1997). In a prospective study we observed high serum enterolactone to be protective against acute coronary events (Vanharanta *et al*, 1999). Prospective data, although inconsistent, also suggest that a high intake of flavonoids might reduce the risk of death from coronary heart disease (CHD) (Hertog *et al*, 1993; Knekt *et al*, 1996). However, the measurement of flavonoid metabolites in biological fluids has been more challenging than that of lignans, mostly due to the diversity of flavonoid metabolites. Thus until now no consensus has been reached concerning a standardized analytical method. Altogether the knowledge on the bioavailability and metabolism of polyphenols is unfortunately still very limited.

Phloem powder produced from the inner bark layer of the pine tree was used to compensate for the shortage of flour in bread-making during times of famine in Finland in the beginning of the twentieth century and earlier. Phloem powder consists mainly of insoluble fibre (80%) and contains high concentrations of different phenolic compounds such as lignans and flavonoids. In gymnosperms like pine tree, lignans are constituents of the fibre component (Ayles & Loike, 1990). Consequently it is not clear whether plant lignans in phloem become digested and are thus available to the gut flora to produce enterolactone. To address this uncertainty and to study the impact of dietary lignans on serum enterolactone level, we conducted a randomized double-blind trial on the effect of phloem enriched rye bread on serum enterolactone level.

Material and methods

Subjects

Seventy-five non-smoking voluntary men aged 30–69 y were recruited from the Kuopio area in eastern Finland through newspaper advertisements. Potential participants were screened in an initial telephone interview for the following inclusion criteria: (1) no severe obesity (body mass index (BMI) < 32 kg/m²); (2) increased serum cholesterol concentration (total cholesterol 6–9 mmol/l); (3) no regular use of any drug with antioxidative or lipid lowering properties; (4) no chronic diseases such as diabetes, CHD or other major illness; and (5) willingness to consume 70 g of dried rye bread/day for 4 weeks. All criteria were ascertained prior to entering the study and participants were also examined by a physician. A written informed consent approved by the local Ethics Committee was obtained from all subjects. The study protocol was approved by the Research Ethics Committee of the University of Kuopio.

Study design

The study was a 4-week randomized double-blind supplementation study. Subjects were randomly assigned to one of the three study groups, each consuming different study

bread: a rye bread high in phloem powder (HP group), a rye bread low in phloem powder (LP group) or placebo rye bread (placebo group). In the bread consumed by the LP and HP groups, 7 and 14% of the rye flour, respectively, was substituted with phloem powder. Due to logistics, the study was conducted in two parts. First, 15 volunteers were randomized into the placebo and LP groups. The second part was started immediately after the first part. In the second phase of the study we recruited and randomized 15 more volunteers to the placebo and LP groups and added the HC group to the study with 15 volunteers, the total number of participants then being 75. The daily amount of study bread was 70 g, which was instructed to be consumed evenly throughout the day.

Four weeks prior to the study subjects were asked to discontinue tea and red wine drinking and the consumption of chocolate and antioxidant supplements (selenium, vitamins C and E and β -carotene). Other than these, subjects were advised to maintain their dietary and other lifestyle habits such as exercise unchanged. It was emphasized to the study subjects not to cut down on their normal bread consumption. A 4-day food record (with one weekend day included) was required during the week before the intervention and during the last week of intervention period to control for possible confounding changes in the diet and to check the compliance to given instructions. Food records were first checked by a nutritionist together with the subject and then analysed by using the Micro-Nutrica[®] nutrient calculation software (Version 2.5) which mainly uses Finnish database values of foods. Collecting of fasting blood samples was carried out at the baseline and after the 4-week intervention period together with blood pressure and weight measurements. Determination of serum enterolactone concentration was based on time-resolved fluoroimmunoassay (TR-FIA), described previously (Adlercreutz *et al*, 1998; Stumpf *et al*, 2000) and the measurements were carried out after the conclusion of both of the study phases. Blood pressure was measured manually in sitting position after a rest of 10 min, three measurements at 3 min intervals and weight was measured using electronic scales. Serum cholesterol was determined with an enzymatic colorimetric test (Konelab, Espoo, Finland).

Lignan analysis of the phloem powder and of the study breads

Lignan content of the phloem powder and of the study breads was determined applying the GC-MS method previously described (Mazur *et al*, 1996). The method used was developed to analyse secoisolariciresinol (Seco) and matairesinol (Mat) from food samples and it was not separately optimized for the analysis of the newly discovered rye lignans: lariciresinol (Lari), pinoresinol (Pin), syringaresinol (Syr) and isolariciresinol (IsoL). Acid hydrolysis included in the method apparently partly destroyed other new rye lignans but did not affect the amount of isolariciresinol (IsoL).

Therefore the presented results concerning the newly discovered lignans are approximations, the true values being slightly higher.

Statistical analysis

Data were analysed using the SPSS for Windows statistical package (version 10.0). Values are expressed as mean \pm s.d. The statistical significance of the heterogeneity of means across the three treatment groups and differences between the groups were tested with one-way analysis of covariance, entering the baseline serum enterolactone as a covariate.

Results

All 75 men recruited for the study completed the 4 week supplementation period and provided the necessary blood samples. Two participants were excluded, one due to pathological concentrations of serum triglycerides (8.8 mmol/l) and the other due to exceptionally high level of serum enterolactone (337 nmol/l), leaving 73 men for the final analysis. The final analysis included 29 men in the placebo group, 29 men in the LP group and 15 men in the HP group.

The baseline characteristics of the subjects were as follows: age 50.7 ± 10.9 y, BMI 25.8 ± 2.7 kg/m², serum total cholesterol 6.8 ± 1.0 mmol/l, systolic blood pressure 130 ± 10 mmHg, diastolic blood pressure 83 ± 9 mmHg and dietary intake of fibre 26 ± 9 g/day. No significant changes were observed in any of these measured parameters (data not shown) and nobody reported of any adverse effects during the study period. According to the two 4 day food records the subjects' compliance to given dietary and lifestyle instructions during the experiment was excellent. As all subjects consumed fairly large amounts of rye bread on a regular basis, most thought that the additional amount of rye bread made no difference to their normal diet.

The mean serum enterolactone concentration at baseline was 31.5 ± 27.5 nmol/l. Enterolactone concentrations at baseline between the three study groups tended to differ from each other but not significantly ($P=0.052$). These differences were not explained by fibre intake, which was rather high for all but did not differ between the groups ($P=0.79$). No association was seen between serum entero-

lactone level and fibre intake (Pearson correlation coefficient 0.036).

The lignan content of the phloem powder and of the study breads is shown in Table 1. The total amount of lignans and total amount of mammalian lignan precursors are presented. Syr was the most abundant lignan in the rye bread used as the placebo. The phloem powder-fortified rye bread contained very high concentrations of Seco but also significant amounts of other lignans. Consumption of study breads increased the serum enterolactone concentration in all groups (by 1.9 nmol/l in placebo, 25.3 nmol/l in LP and 27.1 nmol/l in HP; Figure 1.). The heterogeneity of the increase between the groups was statistically significant ($P=0.001$). Also in an analysis of covariance taking the baseline differences into account, the heterogeneity of the enterolactone concentration change was highly significant across the three groups ($P=0.005$). The difference of change between the placebo and the LP group ($P=0.009$) as well as that between the placebo and the HP group ($P=0.003$) was significant. The increase of serum enterolactone concentration did not differ between the LP and HP group ($P=0.98$).

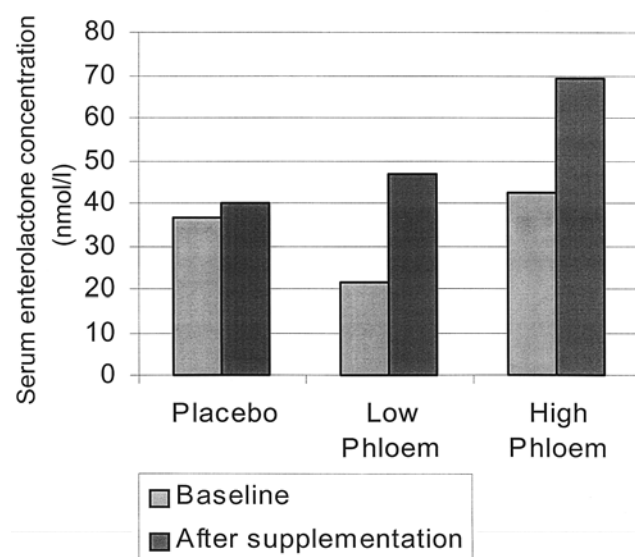


Figure 1 Serum enterolactone concentrations at baseline and after the supplementation separately for the three study groups.

Table 1 Lignan contents of the phloem powder used as the fortification, placebo bread and phloem-fortified rye breads

	Seco	Mat	Lar	Pin	Syr	IsoL	Total lignans	Total precursors ^c
Phloem powder (nmol/g) ^a	2089	7	12	19	9	60	2196	2136
Placebo (nmol/70 g) ^b	79	60	93	93	433	240	998	758
Low phloem (nmol/70 g) ^b	11 493	61	148	120	509	610	12 941	12 331
High phloem (nmol/70 g) ^b	19 729	164	180	140	560	865	21 638	20 773

Seco, secoisolariciresinol, Mat, matairesinol, Lar, lariciresinol, Pin, pinoresinol, Syr, syringaresinol, IsoL, isolariciresinol.

^aValues are given in nmol/g of dry phloem powder.

^bValues are given in nmol/70 g, which was the amount of daily bread supplementation.

^cIsolariciresinol is not included.

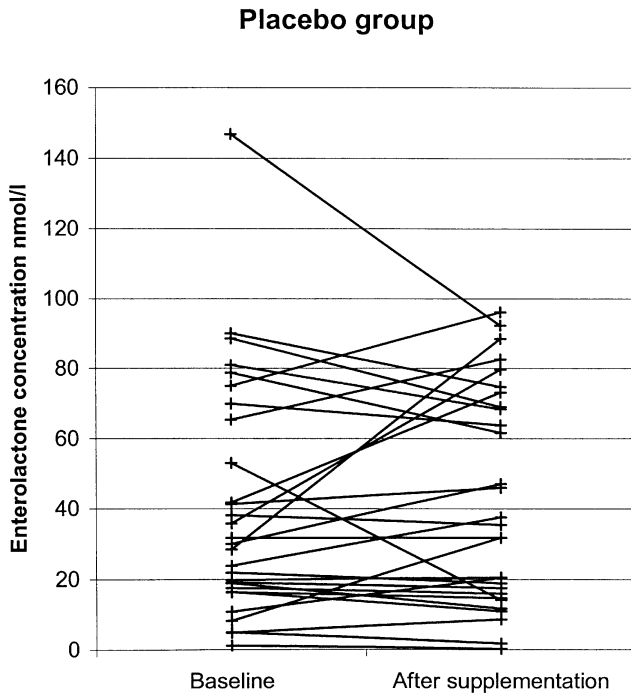


Figure 2 The individual changes of serum enterolactone concentration during the study in the three study groups.

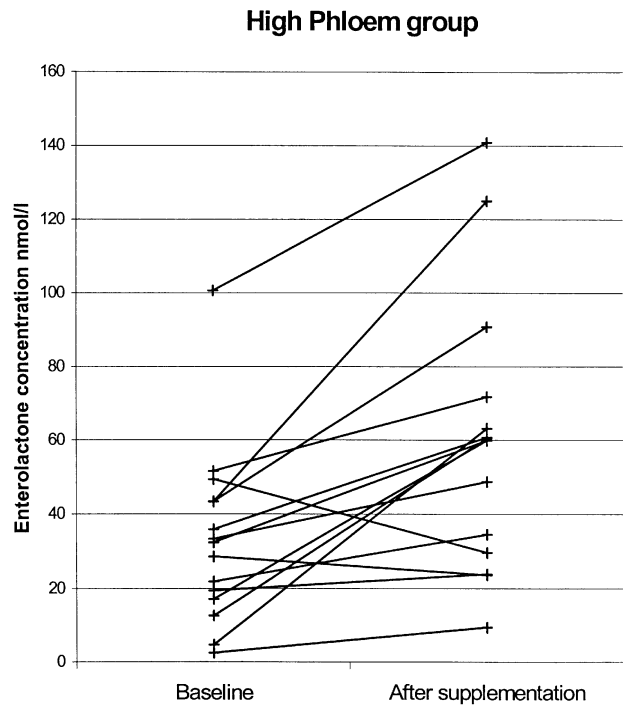


Figure 4 The individual changes of serum enterolactone concentration during the study in the three study groups.

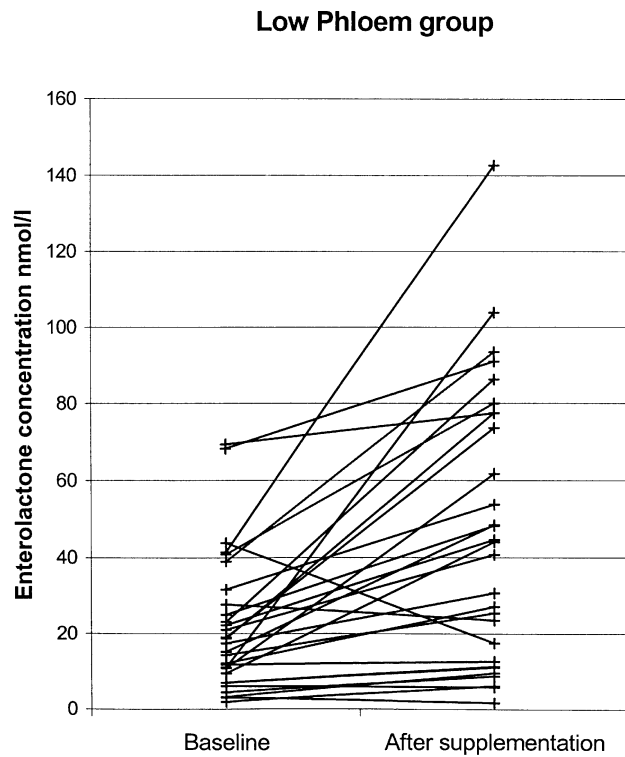


Figure 3 The individual changes of serum enterolactone concentration during the study in the three study groups.

Substantial individual differences were observed in the response to study breads (Figure 2). The ranges of enterolactone concentration changes in the groups were as follows: $-54.5-60.0$ nmol/l (placebo), $-26.2-101.3$ nmol/l (LP), $-19.6-81.8$ nmol/l (HP).

Discussion

The recognition of phloem powder as a concentrate of polyphenols such as lignans and flavonoids and as a source of fibre has inspired food manufacturers to develop and produce phloem powder containing functional foods with claimed health benefit. Our aim was to determine the content of lignans (Seco, Mat, Pin, Syr, Lar, IsoL) in rye bread fortified with phloem and to assess its effect on the level of serum enterolactone, a marker of their bioavailability from the insoluble medium.

Rye bread is known to contain Seco and Mat, which are converted to mammalian lignans enterodiol and enterolactone by intestinal microflora. Recently, it was found out that rye contains other plant lignans as well. The newly found rye lignans Lar, Pin and Syr were tested *in vitro* with faecal bacteria, which demonstrated that they were converted to mammalian lignans enterodiol and enterolactone like Seco and Mat (Heinonen *et al*, 2001). Exceptionally, however, one

of the new rye lignans, IsoL, was not converted to mammalian lignans. To our knowledge in previous studies the content of Pin, Lar and Syr in the supplemented food stuffs has not been known, which has complicated the estimation of the dose-response relationship of the effect of dietary lignans on serum enterolactone concentration. According to our analysis, the phloem fortification of the rye bread dough significantly increases the level of all lignans in the bread but the increment on the level of Seco is by far the greatest (Table 1). The amount of lignans in phloem-fortified bread was proportional to the added amount of phloem powder. Only the amount of Pin was lower than what would have been expected. The heat or the mechanical stress during the baking process may have affected the amount of Pin, which has a labile structure. On the other hand, the differences in the amount of Pin may occur also due to the GC-MS method, which was optimized only for Seco and Mat.

Phloem powder could also be used for lignan supplementation as such. The slightly bitter taste that the unprocessed phloem typically has can be modified by ethanol extraction. Ethanol removes the components causing the bitter taste, but it does not affect notably the amount of lignans.

In a recently published cross-over study (Juntunen *et al*, 2000) subjects were given approximately 200 g/day of rye or wheat bread for a 4 week period. The amount of rye bread consumed on average at baseline was doubled during the study period and the rye bread supplementation added 22 g of fibre to the diet. However, in that study the serum enterolactone concentration remained unchanged. It can be speculated that the large supplemental amount of rye bread might have reduced constipation symptoms for some and thus shortened the transit time through the colon. Consequently, the absorption of lignans may have become incomplete, as suspected by the authors of a cross-sectional study showing an association between elevated serum enterolactone concentration and constipation (Kilkkinen *et al*, 2001). In the current study the serum enterolactone level dropped only in few subjects in all groups during the supplementation period. This might be an indication of the speculated effect of fibre on intestinal motility.

We have previously reported that men with high serum enterolactone levels (> 30.1 nmol/l) have a lower risk of acute coronary events than men with concentrations lower than 7.2 nmol/l (Vanharanta *et al*, 1999). The implication of this is that elevated serum enterolactone levels might provide protection against CHD. In the current study all men were non-smokers and regular consumers of rye bread. Both of these factors are likely to explain the high mean baseline serum enterolactone concentration of 31.5 nmol/l, which is somewhat higher compared with those measured in the mentioned cross-over study (28.1 nmol/l) and with what has been reported for Finnish men in a cross-sectional national survey (13.8 nmol/l); (Juntunen *et al*, 2000; Kilkkinen *et al*, 2001). In the baseline food recordings the participants' intake of fibre (26 g/day) is slightly greater than average intake of fibre of Finnish men (23 g/day), which

can additionally explain the high baseline concentrations of enterolactone in our study participants (National Public Health Institute, 1998).

Flaxseed, the most abundant source of plant lignans known to exist, has been studied most in the previous clinical trials aimed to test the effect of dietary lignans on serum enterolactone level. The only previous study in men to show an increase of the serum enterolactone level as a response to dietary supplementation was carried out in four individuals, the supplement being 15 g of flaxseed (Morton *et al*, 1997). Flaxseed, which contains up to 34% of fat, makes this oil seed a distinct source of lignans as compared with phloem powder, which consists mainly of insoluble fibre and contains almost no fat. Interestingly, the results of the present study indicate that the lignans attached to the insoluble fibre complex in phloem are effectively converted to enterolactone. This might indicate that other phenolic substances that are constituents of insoluble phloem fibre are likely to become bioavailable as well. The use of other concentrated sources of plant lignans, apart from flaxseed, might be of interest in studies in which an increase in energy intake or exposure to cyanogenic glucosides present in flaxseed must be avoided (Rosling, 1993).

The considerable interindividual differences in enterolactone production also observed in the current study have been reported previously. In a dietary supplementation study in which 23 women were given 25 g of flaxseed daily for 2 weeks, the overall range of plasma concentrations of enterolactone after the experiment was 140–819 nmol/l (Morton *et al*, 1994). Although the subjects continued consuming their regular diets during the experimental period, which most likely varied in lignan content, the differences in serum enterolactone persisted even after significant addition of dietary precursors. The same was the case in another study in which the range of urinary enterolactone concentration widened further following the vegetable and fruit supplementation (Hutchins *et al*, 1995). In agreement with these observations our study clearly demonstrates the interindividual differences in serum enterolactone level which do not seem to disappear with supplementation of plant lignans. Additionally, the differences in the magnitude of the response to dietary lignans suggest the presence of other determinants in addition to the dietary ones. The microflora in the colon is likely to be the most important of these.

In summary, this study demonstrated that phloem powder is a concentrated source of a variety of plant lignans and that the insoluble fibre complex surrounding the lignans in phloem powder does not obstruct the production of enterolactone by the intestinal bacteria. Thus, the phloem powder is a useful source of lignans for functional foods aimed to elevate serum enterolactone level.

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