Ferulic Acid Esterase Activities of Typical Intestinal Bacteria

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Ferulic acid esterase activity arose in the ceca of rats that were fed on acid hydrolysate of refined corn bran. The main component of this hydrolysate was soluble ferulic acid arabinoxylan ester. In order to determine the relationship between the ferulic acid esterase activity and intestinal bacteria, the ferulic acid esterase activities from the four kinds of typical bacteria in the intestine were measured. Ferulic acid arabinose ester (LMW) and ferulic acid arabinoxylan ester (HMW) were used as substrates. The enzyme from *Lactobacillus acidophilus* exhibited the highest activity when LMW was used as a substrate. However, when HMW was used, all enzymes from these bacteria exhibited trace activities. At the same time, *Bifidobacterium bifidum* showed high xylanase and arabinofuranosidase activities. It was suggested that the xylanase and the arabinofuranosidase from bacteria such as *B. bifidum* attacked HMW and degraded it to lower molecules at first. The ferulic acid esterase from bacteria such as *L. acidophilus* might then act to release ferulic acid in the cecum.

Keywords: ferulic acid esterase, intestinal bacteria, *Lactobacillus*, *Bifidobacterium*

Many investigations on dietary fiber have been carried out, and evidence is emerging that dietary fiber has an influence on nutrition because it is fermented, absorbed and has various functions (Ebihara & Kiriya, 1990; Ayano, 1992). Cereals such as rice, wheat and corn, are the main suppliers of dietary fiber. Their structures have been studied in detail (Brillouet *et al.*, 1982; Shibuya, 1984; Kamisaka *et al.*, 1990; Shiba *et al.*, 1992). It has become apparent that the corn bran cell wall mainly consists of ferulic acid arabinoxylan ester, that is, the component of decomposed corn bran is *O*-(5-*O*-feruloyl- α -L-arabinofuranosyl)-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose (Kato, 1992; Kato & Nevins, 1985). On the other hand, soluble ferulic acid arabinoxylan ester was obtained by the hydrolysis of refined corn bran (RCB). This compound showed antioxidative activities against autooxidation of linoleic acid and lipid peroxidation of rat liver microsomal system induced by CCl₄ (Ohta *et al.*, 1994). This compound also showed antioxidative activities against LDL oxidation systems, and these antioxidant properties came from ferulic acid, which was present as ferulic acid arabinoxylan ester. Further, ferulic acid and ferulic acid arabinose ester were absorbed from the tract to the circulation system (Ohta *et al.*, 1997).

Recently, ferulic acid esterases have been isolated from ruminant microorganisms, soil bacteria and fungi (MacKenzie & Bilous, 1988; Borneman *et al.*, 1992; Faulds & Williamson, 1994). Ferulic acid esterases from human feces were also studied (Faulds & Williamson, 1995; Kroon *et al.*, 1996; 1997). These studies pointed out that ferulic acid esterase can solubilize ferulic acid from plant fiber and that the rate of solubilization of ferulic acid from insoluble plant cell walls depends on the plant sources.

In a previous paper, we reported that serum cholesterol levels tended to be suppressed when rats were fed on the hydrolysate of RCB with high cholesterol diets. At the same time, ferulic acid esterase activity arose in the ceca of the rats. Further, xylanase, arabinofuranosidase and ferulic acid esterase might act synergistically in the cecum (Nishizawa *et al.*, 1997).

The objective of this study was to measure ferulic acid esterase activities of typical bacteria which exist in the cecum (Aoe *et al.*, 1988) in order to determine the relationship between the ferulic acid esterase activity and intestinal bacteria in the cecum. These activities were compared with that of the cecal contents of the rats.

Materials and Methods

Materials RCB was kindly provided by Nihon Shokuhin Kako Co., Ltd. (Shizuoka). All chemicals used in this experiments were of reagent grade.

Strains *Lactobacillus acidophilus* (IFO 13951T, *L. acidophilus*), *Lactobacillus brevis* (IFO 13110, *L. brevis*), *Lactobacillus rhamnosus* (IFO 14710, *L. rhamnosus*) and *Bifidobacterium bifidum* (IFO 14252T, *B. bifidum*) were obtained from the Institute For Fermentation, Osaka (IFO, Osaka). These strains were chosen from the strains that were entrusted to IFO. The cecal contents were obtained from the rats that were fed on the acid hydrolysate of RCB for 10 days. The main component of the hydrolysate was ferulic acid arabinoxylan ester (Nishizawa *et al.*, 1997).

Cultivation A preculture of *L. acidophilus* was grown stationarily at 37°C for one night in medium (0.5% polypeptone, 0.5% yeast extract, 0.5% glucose, 0.2% lactose, 0.05% Tween 80, 0.1% MgSO₄ · 7H₂O, pH 6.6-7.0). The culture was inoculated into the same medium and cultivated at 37°C for 48 h without aeration or agitation. *L. brevis* and *L. rham-*

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nosus were grown following the procedure used for *L. acidophilus*, but the medium contained 0.5% polypeptone, 0.5% yeast extract, 0.5% glucose and 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (pH 6.6–7.0). *B. bifidum* and the cecal contents of the rats were also cultivated anaerobically as the same as *L. acidophilus*, except that the medium contained 5.9% GAM broth (pH 7.3) (Nissui, Tokyo). After the cultivation, the cells were collected by centrifugation (3000 rpm, 10 min), washed three times with 10 mM acetate buffer (pH 5.0) to remove the culture medium and were stored at -20°C .

Enzyme preparations The cells were suspended in 10 mM acetate buffer (pH 5.0) and were disrupted by sonification at 20 kHz for 3 min (1 min, 3 times). This solution was centrifuged (3000 rpm, 10 min) and the supernatant was used for the measurements of enzymatic activities. Protein concentrations were estimated by the method described by Lowry (1951), using bovine albumin as the standard.

Preparation of substrates RCB (5 g) was heated in 200 ml of 30 mM oxalic acid solution in a boiling water bath for 3 h. After cooling in water, the insoluble fraction was removed by filtration and the filtrate was evaporated under reduced pressure. The hydrolysate was fractionated on Sephadex LH-20 (column dimensions, 15 mm i.d. $\times$ 200 cm; solvent, 50 v/v% aqueous methanol). The absorbance at 320 nm of each fraction was measured (Shimadzu UV-1200 UV-VIS spectrophotometer). Figure 1 shows the results of gel filtration. Fraction A is ferulic acid arabinoxylan ester (high molecular weight fraction, HMW), and fraction B is ferulic acid arabinose ester (low molecular weight fraction, LMW), which were identified by TLC and NMR analyses (Ohta *et al.*, 1994). These fractions were collected, evaporated under reduced pressure and dissolved in 10 mM acetate buffer (pH 5.0). The concentration of the solution was also determined by measuring the absorbance at 320 nm.

Enzyme assays

Ferulic acid esterase activity The enzyme solution (0.2 ml) was mixed with a substrate in 10 mM acetate buffer (pH 5.0, 0.1 ml) and incubated at 37°C . After the incubation, the reaction mixtures were placed in a boiling water bath for 5 min. Following centrifugation ($6500 \times g$, 10 min), the ferulic

acid concentration of the supernatant fractions was determined by high-performance liquid chromatography (Hitachi Model L-6200) with a column (μ -Bondapak C18, 125 Å, 10 μm , 3.9×300 mm, Waters). The mobile phase was 50 v/v% aqueous methanol and ran at a flow rate of 1 ml/min. Ferulic acid was detected with a UV detector (Hitachi Model L-4200) set at 320 nm (Hatfield *et al.*, 1991).

Xylanase activity The enzyme solution (0.1 ml) was mixed with 1% oat speltis xylan (Sigma) in 10 mM acetate buffer (pH 5.0, 0.5 ml) and was incubated at 37°C for 19 h. The liberated reducing sugar was determined according to the Somogyi-Nelson method (Somogyi, 1952; Nelson, 1944). Xylose was used as the standard.

Arabinofuranosidase activity The enzyme solution (0.1 ml) was mixed with 0.1% *p*-nitrophenyl- α -L-arabinofuranoside (Sigma) in 10 mM acetate buffer (pH 5.0, 0.2 ml) and was incubated at 37°C for 19 h. The reaction was terminated by the addition of 1% Na_2CO_3 solution (1 ml). The liberated *p*-nitrophenol was measured by the absorption of 420 nm.

Results

Ferulic acid esterase activity Ferulic acid esterase activities of bacteria are shown in Table 1. When the substrate was LMW, the enzyme from *L. acidophilus* exhibited the highest activity. The enzymes from other bacteria showed lower activities or only trace activities. When the substrate was HMW, the enzymatic activities were traces.

Xylanase activity and arabinofuranosidase activity Table 2 shows xylanase and arabinofuranosidase activities of bacteria. Only the enzymes from *B. bifidum* and the cecum contents exhibited xylanase activity, and the enzyme from *B. bifidum* showed higher activity than that of the cecum contents. The enzymes from the cecum contents and *B. bifidum* exhibited arabinofuranosidase activity, and the

Fig. 1. Gel filtration chromatogram of RCB hydrolysate on Sephadex LH-20. Fraction A was a high molecular weight fraction (ferulic acid arabinoxylan ester, HMW), and fraction B was a low molecular weight fraction (ferulic acid arabinose ester, LMW). Measuring conditions were described in the text.

Table 1. Ferulic acid esterase activity (μmole ferulic acid/mg protein).

Bacteria	LMW	HMW
<i>L. acidophilus</i>	21.4	trace
<i>L. brevis</i>	0.50	trace
<i>L. rhamnosus</i>	trace	trace
<i>B. bifidum</i>	trace	trace
Cecum contents	0.96	trace

Ferulic acid esterase activity was measured after 19-h incubation at 37°C . Substrates were LMW and HMW. Data are mean values ($n=2$). "trace" means that the activity was less than $0.2 \mu\text{mole}$ ferulic acid/mg protein.

Table 2. Xylanase activity and arabinofuranosidase activity.

Bacteria	Xylanase activity (μmole xylose/mg protein)	Arabinofuranosidase activity (μmole <i>p</i> -nitrophenol/mg protein)
<i>L. acidophilus</i>	ND	trace
<i>L. brevis</i>	ND	trace
<i>L. rhamnosus</i>	ND	trace
<i>B. bifidum</i>	6.60	1.28
Cecum contents	0.19	3.18

The enzymatic activity was measured after 19-h incubation as described in the text. Data are the mean values ($n=3$). "ND" means not detected and "trace" means the activity was less than $0.5 \mu\text{mole}$ /mg protein.

Fig. 2. Effect of pH on ferulic acid esterase activity. The enzyme solution (0.2 ml) was mixed with 0.1 ml of LMW solution and 0.5 ml McIlvaine buffer and was incubated at various pHs at 37°C as described in the text. Incubation times were 3 h and 1 h for enzymes from the cecum contents and *L. acidophilus*, respectively. ●, cecum contents; ▲, *L. acidophilus*.

enzyme from the cecum contents showed higher activity than that of *B. bifidum*.

Effect of pH and temperature on ferulic acid esterase activity Ferulic acid esterase activity from *L. acidophilus* and the cecum contents were measured using McIlvaine buffer at various pHs. Figure 2 shows the effect of pH on ferulic acid esterase activity. The optimum pH was observed from 6.0 to 6.6 in the enzyme from *L. acidophilus*. The enzyme from the cecum contents showed the same tendency, but this range was slightly wider than that of *L. acidophilus*.

Figure 3 shows the effect of temperature when the enzymes from *L. acidophilus* and the cecum contents were incubated in McIlvaine buffer at pH 6.0. The optimum temperature was from 37°C to 45°C in the enzyme from *L. acidophilus* and from 30°C to 45°C in that of the cecum contents. The range observed in the cecum contents was slightly wider than that of *L. acidophilus*. The optimum temperature ranges of these enzymes almost overlapped, but the enzyme from the cecum had a wider ranges than that of *L. acidophilus*.

Discussion

L. acidophilus, *L. brevis*, *L. rhamnosus* and *B. bifidum* were typical bacteria in intestine (Mitsuoka, 1995). Aoe *et al.* (1988) reported that *Lactobacillus* and *Bifidobacterium* existed in the feces of rats after the prebreeding, and the number of *Bifidobacterium* increased significantly when the rats were fed on hemicellulose from rice bran. The rats used in this experiment were the same strain (Sprague-Dawley) male rats as in the experiment of Aoe *et al.*, and the main component of dietary fiber was similar, that is, the main component of hemicellulose from rice bran was arabinoxylan

Fig. 3. Effect of temperature on ferulic acid esterase activity. The enzyme solution (0.2 ml) was mixed with 0.1 ml of LMW solution and 0.5 ml McIlvaine buffer (pH 6.0) and was incubated at various temperature as described in the text. Incubation times were 3 h and 1 h for enzymes from the cecum contents and *L. acidophilus*, respectively. ●, cecum contents; ▲, *L. acidophilus*.

and that of the hydrolysate of RCB was ferulic acid arabinoxylan ester, in which 4 w/w% ferulic acid was esterified to arabinoxylan. Therefore, *Bifidobacterium* and *Lactobacillus* were supposed to exist from the beginning of the experiment, and bacteria in the cecum changed as in the experiment of Aoe *et al.*

The ferulic acid esterase activities of these bacteria were measured and compared with that of the cecum contents of the rats. When LMW was used as a substrate, the enzyme from *L. acidophilus* exhibited the highest activity, but enzymes from other bacteria exhibited lower or trace esterase activities. This indicated that the ferulic acid esterase activity in the cecum was caused by bacteria such as *L. acidophilus*. All enzymes showed trace activities when HMW was used as a substrate. The remarkable difference between LMW and HMW was the size of the molecules. That is, as mentioned before, the main components of LMW and HMW were ferulic acid arabinose ester and ferulic acid arabinoxylan ester, respectively (Ohta *et al.*, 1994). MacKenzie and Bilous (1988) studied the ferulic acid esterase activity from the fungus *Schizophyllum commune* and reported that the enhancement of ferulic acid esterase was due to the size or inaccessibility of the materials bearing the ferulic acid residue. In our experiment, the ferulic acid esterase could attack small molecules easier than large ones.

There are many species of bacteria in the cecum. One has a high xylanase activity and another has a high arabinofuranosidase activity. There are also bacteria that have a high ferulic acid esterase activity such as *L. acidophilus*. At first, the enzymes from bacteria, which have a high xylanase or arabinofuranosidase activity, might attack and degrade

HMW to smaller molecules. Next, ferulic acid esterase from other bacteria might attack these smaller molecules and ferulic acid would be released. The enzymes from various kinds of bacteria might act synergistically in the cecum.

In order to obtain more details on this degrading mechanism, xylanase activity and arabinofuranosidase activity of enzymes from these bacteria were measured (Table 2). *B. bifidum* and the cecum contents exhibited both enzymatic activities, and enzymes from other bacteria either showed trace activities or none. There are many kinds of bacteria and the concentration of *B. bifidum* was diluted in the cecum. As a result, an enzyme from the cecal contents might exhibit lower xylanase activity than that of *B. bifidum*. However, in the case of arabinofuranosidase activity, there might be many kinds of bacteria that exhibited high arabinofuranosidase activity. For this reason, the arabinofuranosidase activity from the cecum contents became higher than that of *B. bifidum*. However, in this experiment, the concentration of *B. bifidum* in the cecum was not measured. This should be elucidated.

Figures 2 and 3 show the effects of pH and temperature on ferulic acid esterase activities of enzymes from *L. acidophilus* and the cecum contents. The optimum pH and temperature almost overlapped, that is, pH 6.0–7.0 and 37–45°C, respectively. However, the optimum ranges of pH and temperature of the enzyme from the cecum contents were wider than those of *L. acidophilus*. Many kinds of bacteria in the cecum might relate to these phenomena, and *L. acidophilus* might be one kind of relating bacterium. The pH in the cecum is acidic because dietary fiber is degraded by intestinal bacteria and short-chain fatty acids are produced. In addition, the temperature of the body is about 37°C. It might be more effective for *L. acidophilus* to release ferulic acid in the cecum.

By the way, ferulic acid is well-known to have an antioxidant potential because it readily forms a resonance-stabilized phenoxy radical, and this radical has the ability to terminate free radical chain reactions (Graf, 1992). Furthermore, ferulic acid arabinoxylan ester has antioxidant activity against the autoxidation of linoleic acid and lipid peroxidation of rat liver microsomal system induced by CCl_4 *in vitro*, and it is absorbed from the digestive tract into the vascular system (Ohta *et al.*, 1994, 1997). The more the bacteria such as *L. acidophilus* and *B. bifidum* increase in the cecum, the more favorable this would be for the body.

The existence of ferulic acid esterase in typical intestinal bacteria was shown in this study, but whether ferulic acid esterase activity was induced in *L. acidophilus* or whether the number of *L. acidophilus* was increased in the cecum of the rat was not examined. This should be elucidated. Furthermore, this enzyme was not purified and characterized. Although the bacteria used in this experiment belong to the same species or related species, their enzymatic activities were different. It was not clear in this experiment that these differences came from the differences in species, cultivation methods or other cultivating conditions. They should be elucidated in the near future.

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