

Inhibitory effects of extractives from leaves of *Morus alba* on human and rat small intestinal disaccharidase activity

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The inhibitory effect on human and rat intestinal disaccharidase by the extractive from the leaves of *Morus alba* (ELM) containing 0.24% 1-deoxynojirimycin equivalent and its inhibitory activities were investigated by the modified Dahlqvist method. In the presence of 1000-fold diluted ELM solution, the sucrase activity of four human samples was inhibited by 96% and that of maltase and isomaltase by 95 and 99%, respectively. The activities of trehalase and lactase were inhibited by 44 and 38%, respectively. The human disaccharidase activities varied from sample to sample because the samples were obtained from different resected regions after surgery. However, the ratio of the inhibitory effect for sucrase, maltase, isomaltase, trehalase and lactase was very similar among the four samples, and also that of resembled rat intestinal disaccharides. The inhibitory constant of the 1-deoxynojirimycin equivalent for sucrase, maltase and isomaltase was 2.1×10^{-4} , 2.5×10^{-4} and 4.5×10^{-4} mM, respectively, and these inhibitory activities were shown, using rat brush border membrane vesicles, to be competitive. These results demonstrate that digestion is inhibited when an appropriate amount of ELM is orally ingested with sucrose or polysaccharide in man. When ELM was orally administered in a sucrose solution to fasted rats, the elevation in blood glucose was significantly suppressed, depending on the concentration of ELM given. These results suggest that ELM could be used as an ingredient in health foods and in foods that help to prevent diabetes.

Inhibitory effects: Human disaccharidase: *Morus alba* extractive

Morus alba has been used for centuries in Japan as an infusion tea and as a diet for silkworms. It has already been shown in an animal study that 1-deoxynojirimycin (DNJ) and its derivatives, which are D-glucose analogues, inhibit intestinal α -glucosidases and pancreatic α -glucosidases, as well as inhibiting the processing of oligosaccharides (Fuhrmann *et al.* 1985; Asano *et al.* 1994a,b; Dong *et al.* 1996). Of these inhibitors, DNJ, N-methyl-1-deoxynojirimycin, fagomine (1,2-dideoxynojirimycin), 1,4-dideoxy-1,4-imino-D-arabinitol, Calystegin B2 (1 α ,2 β ,3 α ,4 β -tetrahydroxy-*nor*-tropane), 1,4-dideoxy-1,4-imino-(2-*o*- β -D-glucopyranosyl)-D-arabinitol, and 2-*o*- α -D-galactopyranosyl-1-deoxynojirimycin are distributed in the leaves and roots of *Morus* (Asano *et al.* 1994a,b).

D-Glucose analogues such as boglibose, miglitol and acarbose, with nitrogen-in-rings, have been already used as medicines for the treatment of diabetes (Katsilambros *et al.* 1986; Taylor *et al.* 1986; Raimbaud *et al.* 1992; Bjarnason *et al.* 1996; Drent *et al.* 2002; Mazzaferro *et al.* 2003; Yasuda *et al.* 2003). They inhibit intestinal α -glucosidase and therefore suppress the response of both blood glucose and insulin secretion.

Few studies have been done on the health effects in man of extractive from the leaves of *Morus alba* (ELM; Kimura *et al.* 1995; Miyahara *et al.* 1996, 2004). The aims of this study

were to clarify whether ELM inhibits small intestinal disaccharidase in man, to determine the inhibitory activities and to compare these activities in man and rats. Furthermore, the suppressive effect on the increment of blood glucose in rats was clarified. We believe that the results of this study could be applied to the prevention of diabetes and to clinical support for those with the condition.

Materials and methods

Extractives from the leaves of *Morus alba*

The ELM was kindly provided by Toyotama Healthy Food Co., Ltd (Tokyo, Japan). To prepare the solution, the leaves were extracted with 50% ethanol, the ethanol was removed, and the resulting extractive was stored at -80°C . The original extract solution contained 0.24% DNJ and a small amount of several kinds of DNJ derivative measured using LC-MS. It has been clarified that this extraction is not associated with any toxicity or with any haematological, blood biochemical or pathological abnormalities in rats (Miyazawa *et al.* 2003). Before being used for the experiments, the original solution was centrifuged at 20 000 g for 30 min at 4°C to remove the impurities and was then filtered (Whatman paper No.1; Whatman Japan KK, Tokyo, Japan).

Abbreviations: AUC, area under the curve; BBMV, brush border membrane vesicles; DNJ, 1-deoxynojirimycin; ELM, extractive from the leaves of *Morus alba*; K_i , inhibitory constant; V_{max} , maximum velocity.

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Preparation of human homogenate of intestinal mucosa

Fragments of the human small intestine were obtained from four patients who had undergone dissection of a part of their gastrointestinal tract in hospital, the region dissected varying among patients. The patients were operated on for bladder cancer. In all of the small intestinal fragments used in this experiment, the malignant tissue was not determined.

After the fragments had been washed with ice-cold 0.9% (w/v) NaCl, they were stored at -80°C . Before use, the fragments were thawed and washed with ice-cold saline. The mucosa around the pathological tissue was scraped off with a glass slide, weighed and homogenized in ice-cold 0.9% NaCl (5% wet w/v), using a homogenizer (Polytron; Kinematica Inc., Littan-Lucerne Switzerland). All of these homogenates were separated into small plastic tubes and stored at -80°C until use. Prior to the assay, the homogenates were rehomogenized.

Animals

In order to prepare the small intestinal homogenate, ten male Wistar rats (SeacYoshitomi Inc., Oita, Japan), each weighing 200 g, were housed and fed a standard solid diet (MF diets; Oriental Yeast Co., Osaka, Japan) and tap water *ad libitum* for 2 weeks. The animal quarters were air-conditioned ($22-24^{\circ}\text{C}$, 50% humidity) and maintained on a 12 h light-dark cycle.

To isolate the brush border membrane vesicles (BBMV) from the small intestinal mucosa, twenty male Wistar rats (SeacYoshitomi Inc.), each weighing 200 g, were used. The rats were housed individually and maintained under the same conditions as mentioned earlier. In order to administer the sucrose solution containing ELM, male Wistar rats (200 g each; SeacYoshitomi Inc.) were housed individually for 5–7 d in the same conditions as mentioned earlier.

Preparation of homogenates of rat intestinal mucosa

After 12 h fasting, the rats were killed by decapitation. The small intestines were immediately removed, slit opened and washed with ice-cold saline. The mucosa was scraped off with slide glasses on ice-chilled glass plates, weighed and homogenized in ice-cold 0.9% NaCl (10% wet w/v) using a homogenizer (Polytron; Kinematica Inc.). All of the homogenates were stored at -80°C until assay. Prior to assay, the homogenates were rehomogenized and diluted to adequate concentrations.

Animals and preparation of brush border membrane vesicles

After 12 h fasting, the rats were killed by decapitation. The small intestinal BBMV were prepared by the modified method of Kessler *et al.* (1978). The BBMV were suspended in 0.9% (w/v) NaCl and stored at -80°C until assay. Prior to assay, the BBMV were gently homogenized using a Potter Teflon homogenizer.

Determination of inhibition by ELM on disaccharidase activity

The disaccharidase activity was assayed by Oku's method (Oku *et al.* 1982), which is the modified method of Dahlqvist

using glucose oxidase (Dahlqvist, 1964). Glucose was used as a standard. Substrates such as sucrose, maltose, palatinose, trehalose and lactose were dissolved to prepare 56 mM solution in 0.1 M-sodium maleate buffer (pH 6.0). The enzymatic solution was diluted to an adequate concentration to determine the activity. The ELM was diluted to an adequate concentration and added to the assay system. Palatinose, which has a α -1,6 glycosidic linkage and is hydrolysed by isomaltase, was used instead of isomaltose.

For evaluation in the enzymatic kinetic study, an adequate concentration of substrate solution was dissolved in 0.1 M-sodium maleate buffer (pH 6.0). Protein was measured by the method of Bradford (1976) with bovine serum albumin used as a standard.

Inhibitory effect of ELM on elevation of blood glucose by sucrose ingestion in rats

After overnight fasting for 12–14 h, 2.5 ml sucrose solution (0.4 g/ml) dissolved in the original or five-fold diluted ELM solution was administered to the rats (body weight 230–250 g) using magensonde. Blood was collected from the tail vein with a haematocrit tube before administration and at 30 min intervals for 2.5 h after administration. Sera were obtained by centrifugation at 11 000 g for 30 min at room temperature. Blood glucose was measured by Trinder's method using glucose oxidase (Trinder, 1969).

Reagents and chemicals

Maltose (over 99.8% purity) and trehalose (over 99.9% purity) were kindly provided by Hayashibara Biochemical Laboratories, Inc. (Okayama, Japan), and the palatinose (purity, 99%) was provided by Mitsui Sugar Co. Ltd. (Tokyo, Japan). Sucrose, lactose and glucose were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All of these chemicals were of analytical grade.

Statistical analysis and calculation of results

The units of enzyme activity are reported with specific activity (μmoles), which is the number of micromoles of substrate hydrolysed per milligram of protein for 1 h (μmol substrate hydrolysed/mg protein per hour) at 37°C (pH 6.0). The disaccharidase activity in five rats was calculated as the mean and standard deviation. Differences in the inhibitory effect on disaccharidase activity and blood glucose level were compared with and without ELM, and the dose-dependency of ELM was compared using cumulative increasing areas under the curve (AUC) for 150 min. A P value < 0.05 was considered to be significant with paired Student's t testing and ANOVA, using SPSS for Japan version 10.0 (SPSS Inc., Tokyo, Japan).

Ethics

The study protocol was approved by the respective committees of Siebold University of Nagasaki and Juzenkai Hospital in Nagasaki, Japan. Each patient who donated intestinal tissue gave his or her written informed consent to participate in this study. The rat experiments were performed under the guidelines on the care and use of laboratory animals of Siebold Uni-

versity of Nagasaki. All the experiments were carried out in the Laboratory of Public Health Nutrition, Siebold University of Nagasaki.

Results

Inhibitory effect of ELM on disaccharidase activity of human small intestinal homogenates

The inhibitory effects on human small intestinal disaccharidase are shown in Figs. 1 and 2. The human disaccharidase activity varied from sample to sample because different resected regions of the intestine were used.

The range of sucrase activity in the four human samples was 0.3–1.0 μ moles in the presence of ELM and 9.0–25.4 μ moles in the absence of ELM. The range of maltase activity was 1.5–5.7 μ moles and 47.5–99.4 μ moles in the presence and absence of ELM, respectively. The activity of isomaltase was 0–0.1 μ moles with ELM and 2.5–6.5 μ moles without ELM. Trehalase and lactase activities were also decreased from 0.9–8.2 to 0.3–5.1 μ moles and from 0.2–1.1 to 0.1–0.7 μ moles, respectively, by the addition of ELM.

The ratios of the inhibitory effect on sucrase, maltase, isomaltase, trehalase and lactase were very similar among the four samples, despite the fact that the human disaccharidase activities varied from sample to sample. The inhibition ratio with the 1000-fold diluted ELM solution was more than 90% of the full activity of each type of sucrase, maltase

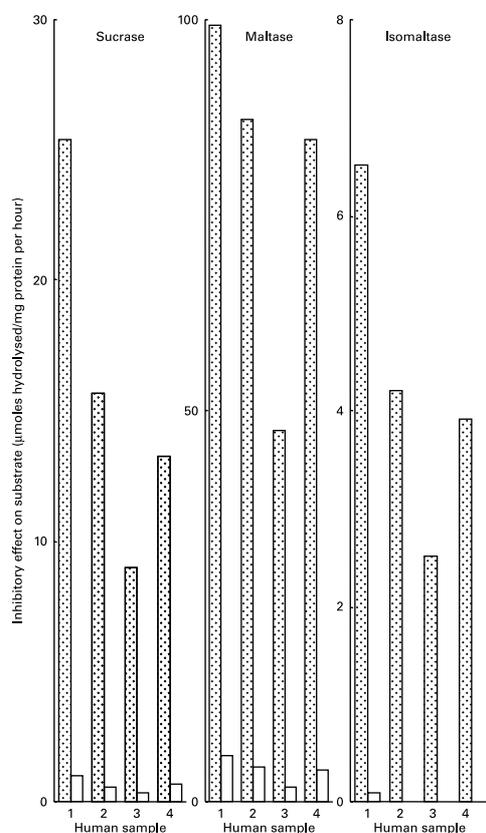


Fig. 1. Inhibitory effect of the extractive from *Morus alba* on human intestinal sucrase, maltase and isomaltase. Sucrase, maltase and isomaltase activities without *Morus alba* extractive (▨) were inhibited by the addition of 1000-fold diluted *Morus alba* extractive (□) in man.

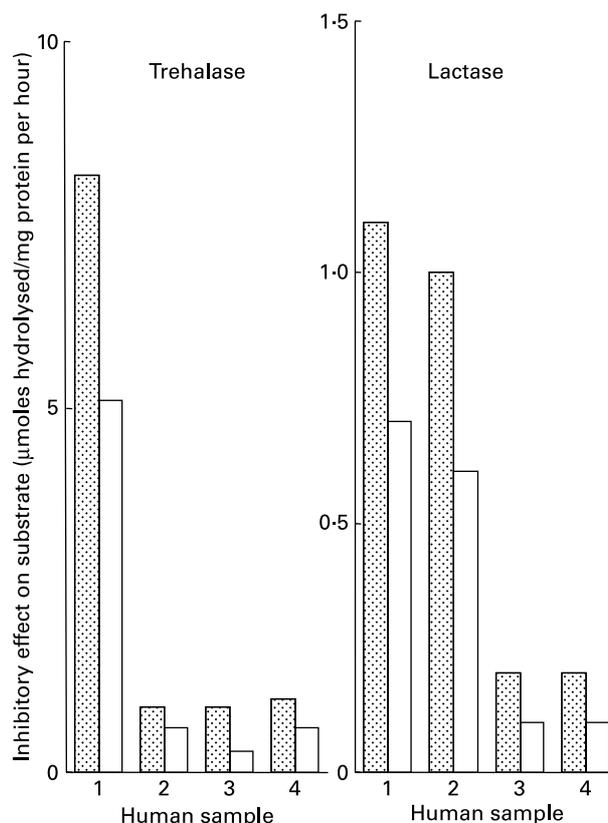


Fig. 2. Inhibitory effect of the extractive from *Morus alba* on human intestinal trehalase and lactase. Trehalase and lactase activities without *Morus alba* extractive (▨) were inhibited by the addition of 1000-fold diluted *Morus alba* extractive (□) in man.

and palatinase, respectively (Fig. 1). However, the inhibition ratio for trehalase and lactase was less than 50% (Fig. 2). These results demonstrate that inhibition by ELM is more conspicuous for sucrase, maltase and isomaltase than for trehalase and lactase.

Comparison of the inhibitory effect of ELM on intestinal disaccharidase activity between human subjects and rats

A comparison of the inhibitory effect of ELM on intestinal disaccharidase activity between human subjects and rats is shown in Fig. 3. When 1000-fold diluted ELM solution was added to the assay system, the ratio of inhibition by ELM for sucrase activity was 95.9 (SD 0.6) % for human subjects and 92.5 (SD 2.1) % for rats. The ratio of inhibition for maltase and isomaltase activity was 95.1 (SD 0.6) % and 99.4 (SD 0.6) % for human subjects and 95.4 (SD 1.9) % and 92.6 (SD 0.6) % for rats, respectively. The ratio of inhibition for the trehalase and lactase activities was 44.3 (SD 15.7) % and 37.8 (SD 7.1) % for human subjects and 36.7 (SD 6.4) % and 36.2 (SD 8.2) % for rats, respectively. When the same concentration of ELM was used, the inhibitory effect on rat sucrase and maltase was similar to that for human sucrase and maltase. The inhibitory effect on isomaltase, trehalase and lactase was a little bit stronger for human subjects than for rats, but there was no significant difference in the inhibition ratio between human subjects and rats.

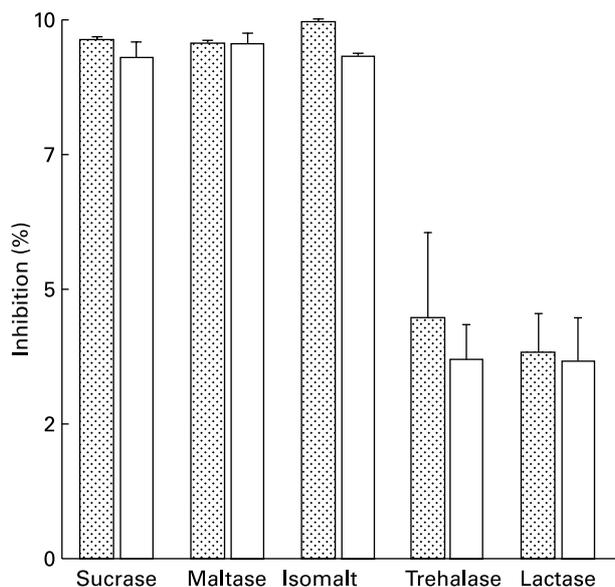


Fig. 3. Comparison of the inhibitory ratio of the extractive from *Morus alba* against intestinal disaccharidase in man and rat. Data were expressed as means with standard deviation shown by vertical bars. The ratio of inhibition by *Morus alba* extractive on disaccharidase activity was not significantly different between human subjects (▨) and rat (□), respectively.

Kinetic analysis of inhibition by ELM on sucrase, maltase and isomaltase using rat brush border membrane vesicles

The inhibitory activities of ELM on the sucrase, maltase and isomaltase activity of the rat small intestine were observed using BBMV (Fig. 4). The K_m and maximum velocity (V_{max}) for each disaccharidase and the inhibitory constant (K_i) by ELM were calculated using Lineweaver–Burk plots. Inhibition by ELM was calculated as DNJ equivalents in the ELM.

The K_i of DNJ equivalent was 2.1×10^{-4} mM for sucrase, 2.5×10^{-4} mM for maltase and 4.5×10^{-4} mM for isomaltase.

Among the three disaccharidases, the inhibitory effect was the strongest for sucrase activity. Linear regressions with or without the inhibitor of these three disaccharidases converged on the y-axis. The inhibitory activities of ELM were competitive on sucrase, maltase and isomaltase. With the inhibitory activities of ELM against trehalase and lactase, the linear regressions with or without the inhibitor did not converge on the y-axis but crossed at different points on the y-axis in terms of the different concentrations of ELM (data not shown).

The K_m (mM) and V_{max} (μ moles substrate hydrolysed/mg protein per hour) for these three disaccharidases are summarised in Table 1. The K_m and V_{max} were 37.9 and 153.9 for sucrase, 4.3 and 416.7 for maltase, and 4.5 and 27.2 for isomaltase, respectively.

Suppressive effect of ELM on the increment of serum glucose in rats

The response of blood glucose after the administration of the sucrose solution containing the original or five-fold diluted ELM solution is shown in Fig. 5. At 30 min after the administration of 2.5 ml (sucrose 1 g) sucrose solution (0.4 mg/ml), the blood glucose level was significantly increased compared with the situation before ingestion ($P < 0.05$); this was maintained until 60 min thereafter, before spontaneously decreasing to the basal level. In the case of sucrose (1 g) containing five-fold diluted ELM solution, the blood glucose levels at both 30 and 60 min after administration were significantly suppressed compared with that of the control ($P < 0.05$). The administration of sucrose containing the original ELM solution strongly suppressed the blood glucose. Cumulative increases in blood glucose were calculated as AUC for 150 min. The AUC for the sucrose-alone administration was 1872 mg/100 ml, and for the sucrose containing original or five-fold diluted ELM solutions was 1299 and 436 mg/100 ml, respectively. ELM

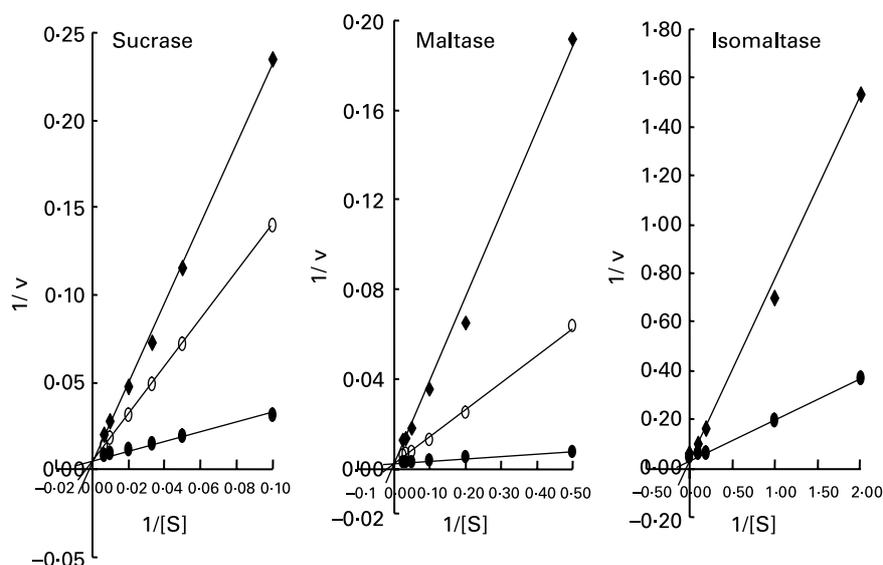


Fig. 4. Kinetic analysis of sucrase, maltase and isomaltase in rat intestinal brush border membrane vesicles by Lineweaver–Burk plot. Velocity (v) measured in μ moles substrate hydrolyzed/mg protein per hour; Substrate [S] measure in mM. The inhibitory activity on sucrase, maltase and isomaltase of *Morus alba* extractive was competitive.

Table 1. Maximum velocity (V_{\max}) and K_m of sucrase, maltase and isomaltase and inhibitory constant (K_i) by the extractive from the leaves of *Morus alba* in rat small intestinal brush border membrane vesicles

	Sucrase	Maltase	Isomaltase
V_{\max}	153.9	416.7	27.2
K_m	37.9	4.3	4.5
K_i	2.1×10^{-4}	2.5×10^{-4}	4.5×10^{-4}

V_{\max} measured in μ moles substrate hydrolysed/mg protein per hour; K_m and K_i measured in mM.

V_{\max} , K_m and K_i are calculated by Lineweaver–Burk plot.

suppressed blood glucose elevation in a dose-dependent manner.

Discussion

The inhibitory effect of ELM on human intestinal disaccharidase and the inhibitory activities of ELM were investigated. Sucrase, maltase and isomaltase of the human small intestine were conspicuously inhibited by ELM, whereas trehalase and lactase were not so strongly inhibited. The inhibitory effects became remarkable as the concentration of ELM increased. The samples varied from person to person because of the difference in the dissected region obtained. However, the ratios of inhibition for human intestinal disaccharidase by ELM were very similar to each other, and also similar to the inhibitory effect seen in rats in the presence of the same concentrations of ELM.

All of the inhibitory activities by ELM for sucrase, maltase and isomaltase of the rat small intestine were competitive inhibitory activities because the linear regressions with or without ELM had the same points of intersection on the y-axis using a Lineweaver–Burk plot. The results suggest

that ELM competitively inhibits the active site of human intestinal disaccharidase. The K_i of DNJ equivalent was 2.1×10^{-4} mM for sucrase, 2.5×10^{-4} mM for maltase and 4.5×10^{-4} mM for isomaltase. The inhibitory effect of ELM on human and rat intestinal sucrase is extremely strong. ELM contains 0.24% DNJ and its derivatives with pyranoses and furanoses, which have nitrogen in the ring. It has already been shown that DNJ strongly inhibits intestinal α -glucosidases (Fuhrmann *et al.* 1985; Asano *et al.* 1994a,b; Dong *et al.* 1996). Therefore, the inhibitory effect seen in this study was considered to be due to the presence of DNJ and its derivatives.

It was clarified in this study that sucrase, maltase and isomaltase from the human small intestine are conspicuously and competitively inhibited by ELM, and that sucrase activity is more inhibited than that of the other disaccharidases. These results suggest that ELM might be suppressing the elevation in blood glucose that results from the ingestion of carbohydrates such as sucrose. In fact, the increase in blood glucose following sucrose administration was significantly lower with the addition of ELM in rats, and the suppressive effect was dependent on the quantity of the ELM used. When 6 mg ELM equivalent were administered with 1 g sucrose to a rat, the suppressive effect was very strong, and the elevation of blood glucose was completely suppressed. However, the administration of 1.2 mg ELM equivalent induced approximately half of the suppressive effect in the presence of the same amount of sucrose. Therefore, we considered the ratio of ELM to carbohydrate to be very important for suppressing increments in blood glucose caused by the ingestion of carbohydrates. ELM shows a similar inhibitory effect for human sucrase, maltase and isomaltase as it does for rats. Therefore, when ELM is ingested with carbohydrates, such as sucrose in human subjects, the suppressive effect on the elevation of blood glucose might be induced, just as it is in rats.

The saccharide, which escapes the digestion in the small intestine, may reach the large intestine, where it is fermented by intestinal microbes. It is therefore possible for ELM to function in a prebiotic manner, as non-digestible oligosaccharides act (Holt *et al.* 1996, Dehghan-Kooshkghazi & Mathers, 2004).

The number of patients with diabetes or a prediabetic condition is currently increasing in Japan and other countries. Therefore, it is crucial to develop functional foods that have a suppressive effect on both the blood glucose response and on the secretion of insulin. ELM could be used as an ingredient in health food and in a functional food that prevents diabetes mellitus.

Conclusion

The inhibitory effect on human intestinal disaccharidase of ELM and its inhibitory activities was investigated. The activity of sucrase, maltase and isomaltase in four human samples was conspicuously and completely inhibited. The activities of trehalase and lactase were also remarkably decreased, but not as strongly as for sucrase, maltase and isomaltase. The inhibitory effect on sucrase, maltase and isomaltase was found to be competitive. Increments in blood glucose level caused by the administration of sucrose were significantly suppressed by the addition of ELM in rats. These results demonstrate that if human subjects orally ingest the appropriate amount of ELM with di- or

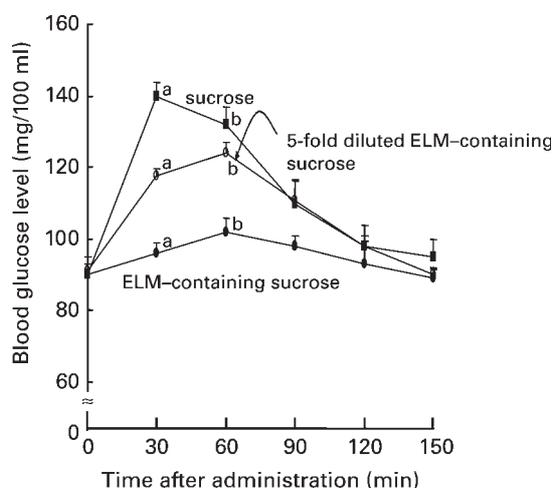


Fig. 5. Suppressive effect of *Morus alba* extractive on increments in serum glucose level in rats. Data were expressed as mean values with standard deviations shown by vertical bars. ^{a,b}Blood glucose levels after the administration of ELM (●) or five-fold diluted ELM (○) solution including 0.4 g/ml of sucrose were significantly less than those after sucrose administration (■; $P < 0.05$, by paired Student's *t* test).

polysaccharides, digestion in the small intestine should be inhibited. ELM can thus be used as an ingredient in health food that is marketed as stimulating both the response of blood glucose and the secretion of insulin.

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