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DETERMINATION OF β-GALACTOSIDASE ACTIVITY IN THE INTESTINAL TRACT OF MICE BY ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ε-N-1-(1-DEOXYLACTULOSYL)-L-LYSINE AS SUBSTRATE

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SUMMARY

ε-N-1-(1-Deoxylactulosyl)-L-lysine was synthesized and used as a substrate to assay β-galactosidase activity. ε-N-1-(1-Deoxylactulosyl)-L-lysine and its degradation product ε-N-1-(1-deoxyfructosyl)-L-lysine were detected by high-voltage paper electrophoresis and ion-exchange high-performance liquid chromatography. The β-galactosidase activity in different parts of the intestinal tract of germ-free and control mice was determined and compared with a β-galactosidase activity which degrades lactose at pH 8.5 and 5.0 and which corresponded with bacterial and host enzymatic activities, respectively.

INTRODUCTION

The bacterial flora which is normally present in the gastrointestinal tract protects the host against potentially pathogenic microorganisms from the environment [1]. This mechanism is called colonization-resistance (CR) [2, 3]. This CR-associated protecting microflora might be strongly reduced by treatment with certain antibiotics. In order to monitor the bacterial infection risk during treatment of immunocompromised patients, the presence of the dipeptide β-aspartylglycine in faeces was used as an indicator of the degree of reduction of CR-associated intestinal microflora [4—6]. Analysis of β-aspartylglycine is routinely performed by high-voltage paper electrophoresis. During these analyses it was noticed that two other substances accumulated in complete absence of intestinal microflora (unpublished results). These two substances appeared to be identical to the spots observed by Ersser et al. [7] in the faecal contents of a germ-free infant and several newborn infants. These two

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substances were identified as ε-N-1-(1-deoxylactulosyl)-L-lysine (lactose-lysine) and ε-N-1-(1-deoxyfructosyl)-L-lysine (fructose-lysine), which are products of the Maillard reaction [8, 9]. This reaction takes place between a reducing sugar and the amino group of an amino acid. Whenever protein is heated in the presence of reducing sugars, for instance during processing of food, the free ε-amino group of lysine in proteins may react which results in a glycosylated protein. This carbohydrate-lysine product is liberated when the protein is digested. Particularly dried milk contains relatively large amounts of lactose-lysine and fructose-lysine [10]. The latter is the adduct of glucose and lysine after Amadori rearrangement [8, 9]. Erbersdobler and co-workers [11, 12] found that fructose-lysine was only degraded by microorganisms and not by the host. This fact makes fructose-lysine a possible marker of the absence of intestinal microflora. However, since fructose-lysine originates from processed food it was assumed that there was no direct relationship between the amount of fructose-lysine in the faeces and the disturbance of the intestinal flora. The bacterial enzymatic activities which degrade fructose-lysine and lactose-lysine might be more suitable indicators of a reduced bacterial flora.

Possible targets for such carbohydrate-lysine degrading enzymes are (1) cleavage of the carbohydrate-lysine bond, (2) degradation of the lysine moiety, (3) degradation of the carbohydrate part. Preliminary studies showed that the carbohydrate-lysine bond was hardly cleaved. Therefore this activity was not suitable for our purpose. Erbersdobler et al. [11] studied the microbial breakdown of fructose-lysine and found 20—40% deamination in 10 h, which gives an estimated 2—4% breakdown in 1 h. Since it is not possible to detect the deamination products fluorometrically with *o*-phthalaldehyde (OPA) and as will be shown in this paper, the β-galactosidase activity which cleaves lactose-lysine into β-galactose and fructose-lysine (further referred to as β-galactosidase) is much higher, the latter was chosen as a possible marker for the presence of intestinal microflora. In this β-galactosidase assay the cleavage of lactose coupled to lysine is measured. In order to couple lactose and lysine, these two substances are heated together. Since lysine has two amino groups, two adducts can be formed: α-N-1-(1-deoxylactulosyl)-L-lysine (α-lactose-lysine) and ε-lactose-lysine. If the heating is prolonged, adducts with more than one lactose molecule attached to lysine can be formed and brown polymeric products occur [8, 9]. These undesired side-reactions do not occur if a relatively short reaction time is chosen. The separation of α- and ε-lactose-lysine by semipreparative ion-exchange high-performance liquid chromatography (ion-exchange HPLC) will be described. In this study the cleavage of lactose-lysine is compared to the enzymatic cleavage of free lactose (further referred to as lactase activity). The relationship between these two types of β-galactosidase activity and the intestinal microflora will be discussed.

EXPERIMENTAL

Animals

Adult germ-free C3H mice were kept under germ-free isolation conditions in sterilized plastic isolators, fed with autoclaved SRM food (Hope Farms, Woerden, The Netherlands) and supplied with autoclaved drinking water. Adult

conventional C57B1/10 mice served as controls. These mice were fed RMH food (Hope Farms) supplied with normal tap water and housed under clean, conventional conditions.

Preparation of enzyme solutions

The animals were killed with ether and the entire intestine was removed. The small intestine was divided into two equally long segments. The segments were cut into pieces and a 0.5 volume of 10 m*M* sodium phosphate, pH 7.4, was added. The mixture was homogenized with a glass rod in a test tube and sonicated for 4 min at 80 W with a Branson B12 sonicator. The tube was chilled with crushed ice during sonication. The contents of cecum and colon were treated in the same way. After sonication, the samples were centrifuged for 40 min at 6000 *g*. The supernatants were dialyzed against 10 m*M* sodium phosphate, pH 7.4, and stored at −18°C.

Chemicals

L-Lysine—HCl was obtained from Calbiochem (San Diego, CA, U.S.A.); lactose was obtained from Lamers and Indemans ('s Hertogenbosch, The Netherlands); β-alanine was obtained from Aldrich Europe (Beerse, Belgium); Whatman 3MM chromatography paper was obtained from Whatman (Maidstone, U.K.); Durrum DC6A cation-exchange resin was obtained from Durrum (Sunnyvale, CA, U.S.A.). Absolute methanol was prepared as described by Gottschalk [13]. Other chemicals were purchased from Merck (Darmstadt, F.R.G.).

Chromatographic system

The chromatographic system consisted of a solvent delivery pump (Model LC20, Pye Unicam, Cambridge, U.K.) connected to a sample loop injector (Model 7125 Rheodyne, Berkeley, CA, U.S.A.). Fluorescence was monitored with a fluorescence detector (Model 420C Waters Assoc., Milford, MA, U.S.A.). The stainless-steel column was thermostated by means of a water bath.

Synthesis of ε-lactose-lysine

One gram of L-lysine—HCl, 9.5 g of lactose and 150 ml of absolute methanol were refluxed for 4 h. Unreacted lactose was removed by filtration. The methanol was evaporated in vacuo and the residue which contained lysine, α- and ε-lactose-lysine was dissolved in 50 ml of water. Batches of 1 ml were lyophilized and further purified by semipreparative HPLC. A batch was redissolved in 100 μl of 0.3 *M* pyridine acetate buffer, pH 3.4, of which 10 μl were injected onto a 100 × 4.6 mm column packed with Durrum DC6A cation-exchange resin (particle size, 11 ± 1 μm). Elution was performed with 0.3 *M* pyridine acetate, pH 3.4. The retention times of α- and ε-lactose-lysine were established by post-column derivatization with OPA and subsequent fluorometric detection (see Fig. 1) as described by Lee et al. [14]. Next, the remaining 90 μl of the batch were injected and the fractions containing α- and ε-lactose-lysine were collected directly from the outlet of the column at the appropriate times [15]. The elution positions of α- and ε-lactose-lysine were assigned by comparison with samples isolated from faeces and with

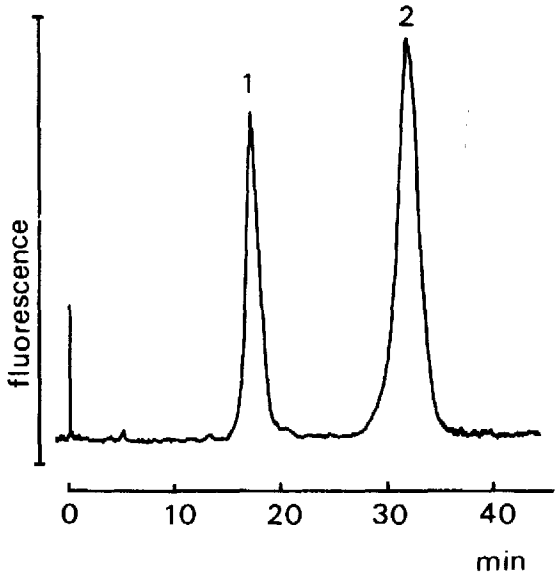

Fig. 1. Separation of α-lactose-lysine and ε-lactose-lysine on a cation-exchange column 100 × 4.6 mm with 0.3 *M* pyridine acetate, pH 3.4. Flow-rate, 0.5 ml/min; temperature, 50°C. Lysine eluted much later after the subsequent semipreparative run. 1 = α-Lactose-lysine; 2 = ε-lactose-lysine.

synthetic ε-lactose-lysine prepared from α-formyl-lysine after hydrolysis (1 *M* hydrochloric acid, 100°C, 15 min) of the protecting formyl group [16, 17]. The fractions were lyophilized and stored at −18°C in absolute methanol.

Amino acid analysis

Amino acid analyses were performed with an amino acid analyzer Liquimat III (Kontron, Zürich, Switzerland) using Pico buffers obtained from Pierce (Rockford, IL, U.S.A.).

β-Galactosidase assay

About 20 nmol of ε-lactose-lysine in methanol were dried under a stream of nitrogen; 10 μl of buffer and 10 μl of enzyme solution were added and the mixture was incubated for 1 h at 37°C. The following buffers were used: 0.1 *M* sodium acetate (pH 5.0); 0.1 *M* sodium maleate (pH 6.0); 0.1 *M* sodium phosphate (pH 7—7.5); 0.05 *M* sodium barbiturate (pH 8—9). The incubation was stopped by adding 100 μl of methanol containing 20 nmol of β-alanine as internal standard. Precipitated protein was removed by centrifugation at 6000 *g* for 15 min. A 50—100-μl aliquot of the supernatant was injected onto a 45 × 3.6 mm stainless-steel column packed with Durrum DC6A resin and eluted with pyridine—acetic acid—water (6:60:176, v/v).

Detection was performed with OPA as described by Lee et al. [14] (see Fig. 2). The amount of lactose-lysine and fructose-lysine was calculated from the peak height × peak width at half height assuming an equal specific fluorescence for both compounds. The percentage conversion of lactose-lysine to fructose-lysine in 1 h was taken as a measure for the β-galactosidase activity.

Alternatively, the incubation mixture was applied to Whatman 3MM chroma-

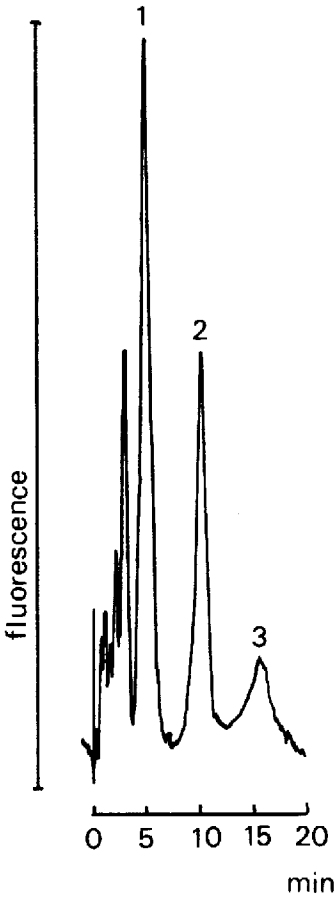

Fig. 2. Chromatogram obtained after injection of a β-galactosidase incubation mixture onto a 45 × 3.6 mm cation-exchange column. Mobile phase: pyridine—acetic acid—water (6:60:176, v/v). Flow-rate, 0.4 ml/min; temperature, 50°C. In front of lactose-lysine some free amino acids elute which are the result of proteolytic activity in the intestinal enzyme preparations. 1 = Lactose-lysine; 2 = β-alanine (internal standard); 3 = fructose-lysine.

tography paper and subjected to high-voltage paper electrophoresis at pH 3.5 for 1 h at 3000 V as described by Welling [6]. The spots were stained with ninhydrin and the β-galactosidase activities were estimated from the intensities of the lactose-lysine and fructose-lysine spots (see Fig. 3).

Lactase assay

Lactase activities were determined as described by Dahlquist [18].

RESULTS

Synthesis of lactose-lysine

After refluxing lactose and lysine, amino acid analysis showed that the reaction mixture contained 2.3% α-lactose-lysine, 7.3% ε-lactose-lysine and 90.4% lysine. Since no colour factor of lactose-lysine is known, the colour

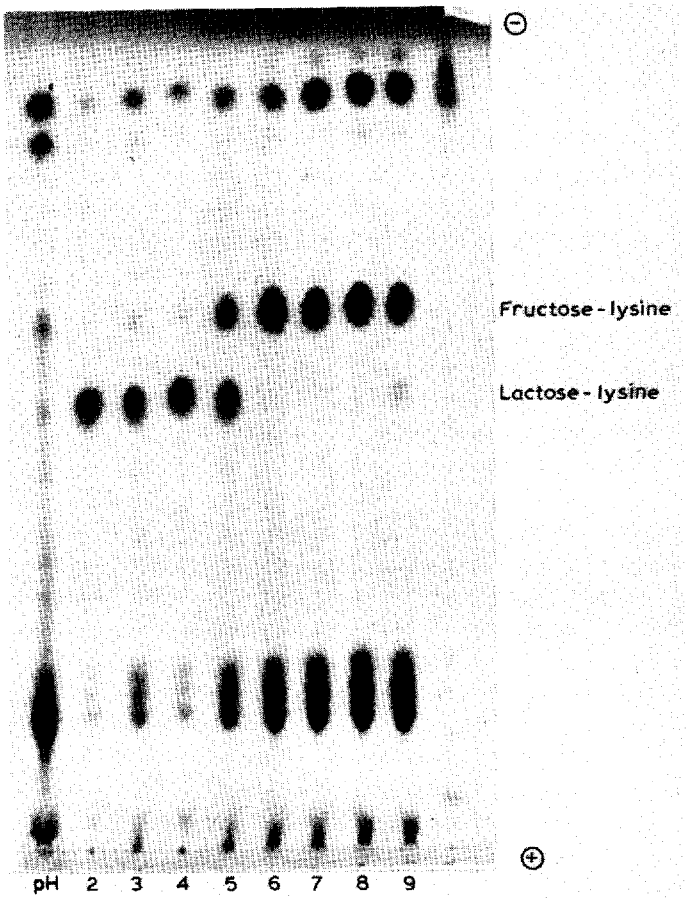

Fig. 3. High-voltage paper electrophoresis at pH 3.5 for 1 h at 3000 V. β-Galactosidase incubation mixtures at different pH values after overnight incubation. Because of complete degradation of lactose-lysine at pH 6—8, a shorter incubation time (1 h) was chosen.

factor of lysine was also used for lactose-lysine in this calculation. Amino acid analysis of lactose-lysine samples purified by HPLC showed no contamination.

Lactase and β-galactosidase activities

Influence of pH. pH—activity curves were made from a small intestinal sample and from a cecal sample obtained from conventional mice. Lactase in the cecum had an optimum pH of around 8, whereas lactase in the small intestine showed an optimum pH of 6.0. In the small intestine microorganisms do not contribute to any major extent to the production of disaccharidases such as lactase [19]. Therefore it was concluded that the lactase activity in the small intestine which showed a pH optimum of 6 was host-derived. The lactase in the cecum with an optimum pH of around 8 is more likely to be of bacterial origin since it was absent in the germ-free animals and the conventional cecum is known to contain large amounts of bacteria. The β-galactosidase (lactose-lysine cleaving) activity showed a broad pH optimum from pH 6 to 8. In various parts of the intestinal tract of germ-free and conventional mice this activity was determined at pH 7.5.

TABLE I

β-GALACTOSIDASE AND LACTASE ACTIVITIES IN DIFFERENT PARTS OF THE INTESTINAL TRACT OF ADULT GERM-FREE AND CONTROL MICE

Animals	Activity*	Small intestine		Cecum	Colon
		Upper	Lower		
Germ-free	β-Galactosidase	—	—	—	—
	Lactase pH 5.0	+	+	—	—
	Lactase pH 8.5	—	—	—	—
Control	β-Galactosidase	+	—	++	+
	Lactase pH 5.0	++	+	+	—
	Lactase pH 8.5	—	—	++	+

*Lactase: —, less than 0.01 U/ml; ++, more than 0.1 U/ml. β-Galactosidase: —, less than 2.5% degradation per hour; ++, more than 25% degradation per hour; +, intermediate values.

Distribution of activities in different parts of the gastrointestinal tract of germ-free and conventional mice. The β-galactosidase activity, given as percentage conversion per hour of lactose-lysine to fructose-lysine, in the upper small intestine, the lower small intestine, the cecum and colon of control mice was 9%, 0%, 28% and 13%, respectively. In the small intestine and colon of germ-free mice no β-galactosidase activity was found, while with an enzyme preparation from the cecum less than 2.5% of lactose-lysine was converted to fructose-lysine.

The results are summarized and compared with lactase activities in Table I. β-Galactosidase and lactase at pH 8.5 show a similar distribution pattern and are mainly present in cecum and colon, whereas lactase at pH 5.0 is mainly present in the small intestine. The latter activity was the only lactase or β-galactosidase activity found in the germ-free animals.

DISCUSSION

The semipreparative HPLC method used to purify lactose-lysine is essentially the same as that used by Weinstein et al. [15], except that we used as commercially available column packing. With slight modifications this method can be used for rapid purification of many amino acids or peptide-like substances. Since a volatile buffer is used, desalting is not necessary.

The use of lactose-lysine as substrate in the β-galactosidase assay has the advantage that it occurs naturally in diets which contain processed food, which is always the case with patients, and therefore reflects an in vivo situation. The amino group of lysine allows sensitive detection with amino acid reagents like OPA. Maximum sensitivity, however, was not achieved because of quenching of the fluorescence, probably due to the pyridine in the mobile phase. Attempts to separate lactose-lysine and its degradation product fructose-lysine in a reversed-phase HPLC system were not successful, probably because of the very similar hydrophobic part of the two molecules.

The results indicate that the β-galactosidase activity, determined in this way,

is not present in germ-free animals and therefore might be used as a marker for the presence of bacteria. The distribution of this β-galactosidase resembles lactase at pH 8.5, which is most likely also of bacterial origin. Examination of more samples is needed to establish these facts more firmly.

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