Small intestinal beta-galactosidase activity in the horse

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SUMMARY Two enzymes having lactase activity are present in the equine small intestine. The first, the digestive enzyme, neutral beta-galactosidase, declines in activity from birth to three years, disappearing completely between 3 and 4 years of age. The other, the soluble lysosomal enzyme, acid beta-galactosidase, having affinity for lactose and a synthetic beta-galactoside, shows a decrease in activity in the first three months of life and thereafter varies little in activity and represents the lactase enzyme in the adult horse. This pattern may parallel the development of lactase activity in many other mammals and in the majority of the world's human population.

In foals as in many other newborn mammals, the milk sugar, lactose, is the principal energy source and is hydrolysed into the readily absorbed monosaccharides, glucose and galactose, by the enzyme, lactase (beta-galactosidase, E.C. 3.2.1. 23), located in the brush border of the enterocytes of the small intestinal mucosa (Kretchmer, 1972). Roberts, Hill, and Kidder (1973) described the distribution of lactase in the equine small intestine, and the development of the enzyme from early foetal to adult life. Differing optimum pH values for lactase activity from mucosal homogenates of young and adult horses indicated that more than one enzyme could be contributing to the hydrolysis of lactose.

Following the description of two enzymes with lactase activity in the small intestinal mucosa of the calf (Heilskov, 1956), the small intestine of many other animals has been shown to contain more than one beta-galactosidase (Asp, Dahlqvist, and Koldovský, 1969). In the rat, one of these enzymes, optimum pH 3-4, is mainly soluble and has high affinity for the synthetic hetero beta-galactosides, the other, localized in the brush border, hydrolyses lactose more rapidly than the hetero beta-galactosides and has optimum pH 5.5-6.0 (Asp and Dahlqvist, 1968). Three enzymes have been demonstrated in the human, the brush border digestive enzyme, neutral beta-galactosidase acting at a neutral pH with greater affinity for lactose, and two hetero beta-galactosidases present in the lysosomes or cytoplasm having greater

affinity for the sythetic substrates (Asp, Dahlqvist, and Koldovský, 1970).

The purpose of the present study was to characterize the equine beta-galactosidases with respect to pH optima, substrate specificity, enzyme inhibition, differential ultracentrifugation and solubilization in small intestinal mucosa from horses early in foetal development to maturity.

Materials and Methods

EQUINE MUCOSAL HOMOGENATES

These were prepared from freshly obtained or deep-frozen samples of foetal, young foal, or adult equine small intestine (Roberts and Cotchin, 1973). The mucosa was scraped off with a metal spatula and homogenized with 40 volumes of chilled deionized water by sonication (Soniprobe, type 1130A, Dawe Instruments Ltd, England) in a tube surrounded by crushed ice. The homogenate was used immediately or stored at -20°C until required and any further dilutions were made with cold deionized water.

PARA CHLOROMERCURIBENZOATE (P-CMB) INHIBITION STUDIES

The method of Koldovský, Asp, and Dahlqvist (1969) for the separate assay of acid and neutral beta-galactosidases by p-CMB inhibition of the acid enzyme was used. The following buffered substrates were prepared: 0.1 M lactose solution in 0.1 M citrate/phosphate buffer for pH 3.0-4.6, and in 0.1 M maleate buffer for pH 5.0-7.0, with and without 0.2 mM p-CMB (parachloromercuri-

benzoic acid, BDH Ltd, Poole), and 0.1 M cellobiose solutions at pH 4.2 and 6.0 in the respective buffers with and without the presence of 0.2 mM p-CMB. The enzyme activity was expressed as units per gram of wet mucosa per minute at 37°C.

ASSAY OF 6-BROMO-2-NAPHTHYL BETA-GALACTOSIDASE (BNG-ASE) ACTIVITY

The procedure of Dahlqvist, Bull, and Gustafsson (1965), using the substrate 6-bromo-2-naphthyl beta-galactoside (BNG), was employed. An homogenate concentration of 1 in 400 or 1 in 800 was required. Buffers covering a range pH 3.0-7.0 consisted of 0.1 M citrate/phosphate pH 3.0-3.4, 0.1 M acetate buffer pH 3.8-5.4, and 0.1 M maleate buffer pH 5.8-7.0 with and without the presence of 0.2 mM p-CMB. The enzyme activity was expressed in units of micromoles of substrate hydrolysed per minute by 1 ml of the enzyme solution.

ULTRACENTRIFUGATION AND SOLUBILIZATION

Separation of the beta-galactosidases by ultracentrifugation and subsequent solubilization of the originally particle bound enzyme fraction with crystalline papain solution (Sigma Chemical Co, St Louis, Mo, USA) in the presence of cysteine hydrochloride (BDH Chemicals Ltd, Poole) was performed by the method of Asp *et al* (1969).

DEFINITION OF ENZYME ACTIVITIES

Total beta-galactosidase or lactase activity was the calculated enzyme activity determined with the substrate lactose at the stated pH. The p-CMB resistant activity represented the neutral beta-galactosidase, being the lactase activity in the presence of p-CMB at the stated pH, and this activity subtracted from the total activity represented the p-CMB sensitive or acid beta-galactosidase activity at that pH.

Results

EFFECT OF p-CMB ON BETA-GALACTOSIDASE ACTIVITY IN THE EQUINE FOETUS

Mucosal homogenates from the jejunum and ileum of three foetuses were examined for beta-galactosidase activity. In the jejunal mucosa of a 4-month foetus, optimal beta-galactosidase activity was at pH 4.2-4.6, the total activity being reduced by 20% at pH 6.0 (fig 1a). In the presence of the inhibitor, optimal activity was at pH 6.0-6.4; this p-CMB resistant component represented almost 80% of the total activity at pH 6.0, whereas the p-CMB sensitive component had a pH optimum 3.8-4.2. However, in the ileal mucosa (fig 1b) both the total beta-

Fig 1 Effect of p-CMB on the activity curves of lactase in the equine foetus (37) 175 days.

galactosidase and p-CMB sensitive enzyme had pH optima 4.2-4.6, the p-CMB-resistant component having an optimum pH 6.0 and providing 60% of the total activity at that pH. These pH activity curves indicated that two distinct enzymes contributed to the total beta-galactosidase activity in the intestinal mucosa of the equine foetus, and that the proportion of acid enzyme (optimum pH 4.2) to neutral beta-galactosidase (optimum pH 6.0) was greater in the ileum than in the jejunum.

BETA-GALACTOSIDASES IN THE YOUNG HORSE

Mucosal homogenates from the jejunum, and occasionally the lower jejunum and ileum of nine

horses from birth to 3 years old were examined. With lactose as substrate, optimal activity of total lactase and the p-CMB-resistant component providing over 90% of the total activity was at pH 6.0 (fig 2a). The p-CMB-sensitive acid beta-galactosidase, pH optimum 4.2-4.6, was present at a very low level. The affinity of the beta-galactosidases for BNG using the same homogenates demonstrated optimal activity of BNG-ase at pH 4.2, whilst in the presence of p-CMB almost complete inhibition of BNG-ase was produced throughout the pH range, minimal residual activity being present at pH 6.0 (fig 2b). However, beta-galactosidase activity for the beta-glucoside, cellobiose, more nearly paralleled that for lactose. Cellobiase activity had an optimum pH 6.0, was barely discernible at pH 4.2, and was unaffected by the presence of 0.2 mM p-CMB in the substrate solution.

Fig 2 Beta-galactosidase activity in the jejunal mucosa of a young horse (30). Comparison of optimum pH with lactose and BNG as substrates.

BETA-GALACTOSIDASES OF ADULT HORSES

Beta-galactosidase activity was examined in the small intestinal mucosa of seven horses over 4 years of age. Irrespective of the site sampled, optimal beta-galactosidase activity in the adult small intestine occurred at pH 4.2 with lactose as substrate, the activity being 90.7-98.8% (mean 94.9%) sensitive to 0.2 mM p-CMB (fig 3a). Although slight residual activity was demonstrated (fig 3a), this may reflect inaccuracies with the method rather than a p-CMB-resistant enzyme. The p-CMB-sensitive acid beta-galactosidase represented virtually all the lactase determined in the

Fig 3 Beta-galactosidase activity in the jejunal mucosa of an aged horse (10). Comparison of optimum pH with lactose and BNG as substrates.

mucosa of the adult. Highest levels of acid beta-galactosidase were in the proximal small intestine, and this enzyme exerted 23-25% maximal activity at pH 6.0 throughout the small intestine. With BNG as substrates enzyme activity was optimal at pH 4.2 with 55-60% of this level present at pH 6.0. Furthermore BNG-ase was completely inhibited by 0.2 mM p-CMB at all pH values (fig 3b). Cellobiase was not detected in mucosal homogenates from the adult small intestine.

INFLUENCE OF AGE ON BETA-GALACTOSIDASE ACTIVITIES

Significant changes in the development of the equine beta-galactosidases occurred from early in foetal life (estimated gestational age 110 days) through to maturity (fig 4). Neutral beta-galactosidase detected in the earliest foetus reached maximum activity at birth thereafter steadily decreasing for three years, until at 4 years of age and subsequently, the enzyme could not be demonstrated in the small intestine. Acid beta-galactosidase present in the jejunal and ileal mucosa of the 110-day foetus had almost trebled in activity by 228 days and further increased until birth. The proportion of acid to neutral enzyme was consistently greater in the ileal than in the jejunal mucosa. The maximum recorded activity was at 2 days of age, 6.29 units of acid beta-galactosidase compared with 14.58 units of the neutral enzyme. By 15 weeks of age, acid beta-galactosidase activity in the jejunum and ileum was 1.18 and 1.01 units respectively, the range of activity from this age to adult being 0.57 to 2.23 units determined in the presence of p-CMB at pH 4.2.

LARGE INTESTINAL BETA-GALACTOSIDASE ACTIVITY

Mucosal homogenates prepared from all sections of the caecum and colon, and assayed for lactase, had far higher activity at pH 4.2 than at pH 6.0, and the almost total inhibition by 0.2 mM p-CMB indicated that the enzyme was acid beta-galactosidase.

ULTRACENTRIFUGATION AND SOLUBILIZATION OF EQUINE BETA-GALACTOSIDASES

When a mucosal homogenate from a young horse had been ultracentrifuged at 100 000g for 60 minutes only 4.8-7.1% (mean 6.2%) of the neutral beta-galactosidase activity, but 26.3-54.3% (mean 42.4%) of the acid and 63% of the BNG-ase activity were found in the supernatant. The sediment contained 81.3-95.2% (mean 89.1%) of the neutral, 13.7-67.8% (mean 42.0%) of the acid, and 17.4-29.2% (mean 23.3%) of the BNG-ase activities. By contrast, from centrifuged adult mucosal homogenates the supernatant contained 54.7-60.6% (mean 57.6%) of the acid enzyme, 50.6-58.6% (mean 54.6%) of the BNG-ase, some 30 to 34% of these activities being sedimented with the pellet, but the neutral enzyme was absent from supernatant and sediment.

Incubation with papain partly solubilized the originally particle-bound neutral beta-galactosidase in the young horse, the clear supernatant after further centrifugation at 100 000g for 60 minutes being used as the solubilized enzyme preparation. Considerable inactivation of the original activity must have occurred during incubation as only 21% of the original neutral beta-galactosidase (measured

Fig 4 Development of beta-galactosidase activities in the horse.

with lactose in the presence of p-CMB at pH 6.0) appeared in the supernatant. Gel filtration chromatography of these equine enzyme fractions was markedly unsuccessful for further separation and purification.

Discussion

There is substantial evidence provided in this investigation for the existence of two beta-galactosidases hydrolysing lactose in the small intestinal mucosa of horses. The procedure adopted for their characterization in crude homogenates was based on differences in the optimum pH, substrate specification, and sensitivity to parachloromercuribenzoate as defined by Asp and Dahlqvist (1972) in the human. Unfortunately, gel filtration chromatography did not assist the separation and partial purification of these equine enzymes (Roberts, 1972). In the foetus and in the young horse the two enzymes contributed to the pH activity curves whereas in the adult horse of 4 years and older only the acid beta-galactosidase was present. The neutral beta-galactosidase, pH optimum 6.0, was resistant to 0.2 mM p-CMB with lactose as substrate, did not show affinity for BNG, and was at least 90% particle bound on ultracentrifugation. This enzyme represented the true lactase of the small intestine and was very similar to the neutral enzyme of the rat (Asp and Dahlqvist, 1968) and man (Asp *et al.*, 1969). Under optimal conditions for the solubilization of human lactase only 30% of the originally particle-bound activity could be recovered in soluble form (Asp *et al.*, 1969), this comparing with 21% for the corresponding enzyme in the young horse.

The acid beta-galactosidase present in mucosal homogenates of horses of all ages had a pH optimum 4.2, was sensitive to 0.2 mM p-CMB in the substrate solutions, and had affinity for lactose and BNG, termed BNG-ase, with the latter substrate although exerted by the same enzyme. The enzyme represented the soluble fraction although 42% of the activity determined with lactose was sedimented after initial ultracentrifugation. However, 63% of the BNG-ase activity was in the supernatant and only 23% in the sediment following centrifugation comparable with the findings for human BNG-ase activity (Asp *et al.*, 1969). This equine acid beta-galactosidase showed similarities to the acid enzyme described in the rat (Koldovský *et al.*, 1969), the pig (Sato and Yamashina, 1971), and the human (Asp, 1971). The beta-glucosidase, cellobiase, probably exerted by the same brush border enzyme responsible for lactase activity (Dahlqvist and Lindberg, 1966) was demonstrated in the small

intestine of foetal and young horses but not adults. Cellobiase like the neutral beta-galactosidase had optimum pH 6.0, was not inhibited by 0.2 mM p-CMB, was barely discernible in mucosal homogenates from adults, and the beta-glucosidase/neutral beta-galactosidase activity ratio was consistently 1 to 5 throughout the small intestine (Roberts, 1972) as reported in the human (Asp *et al.*, 1969).

A third enzyme, hetero beta-galactosidase, presumably lysosomal, was described in the small intestine of the human (Asp *et al.*, 1970), monkey (Swaminathan and Radhakrishnan, 1969), and pig (Sato and Yamashina, 1971) having an optimum pH 5.5-6.0, except in the monkey, pH 7.0, and was inhibited by 0.2 mM p-CMB. This enzyme, absent in the rat (Asp, 1971), was not detected in the horse although this absence might be dependent on the substrates used in the investigation. No specific substrate exists for the hetero beta-galactosidase (Asp and Dahlqvist, 1972); however, BNG was used because in the human, of the two lysosomal enzymes hydrolysing this substrate, the acid, but not the hetero beta-galactosidase, also hydrolysed lactose (Alpers, 1969).

The inhibition of equine acid beta-galactosidase by p-CMB indicated that this enzyme, like the beta-galactosidase of *Escherichia coli* and the acid enzymes of the rat and human, was dependent on intact thiol groups for hydrolytic activity (Asp *et al.*, 1969). This soluble enzyme, located in the enterocyte lysosomes, the number of which could affect the levels of activity in different parts of the tract (Asp, 1971), is also found in many other organs and tissues throughout the animal body usually associated with lysosomal particles (Furth and Robinson, 1965). Physiologically, the enzyme is concerned with intracellular metabolism, possibly of glycosaminoglycans or glycolipids, whereas the hetero beta-galactosidase could influence glucocerebroside metabolism (Asp, Berg, Dahlqvist, Jussila, and Salmi, 1971).

The established properties of the equine and human beta-galactosidases are compared in the table. The pattern of beta-galactosidase activity in the equine small intestine closely parallels that present in other mammals, including humans with racial lactose intolerance in traditional non-milk-drinking areas, representing the major proportion of the world's population (Kretchmer, 1972). In this condition, which becomes evident as early as 4 years of age in Bantu children, the brush border enzyme is missing or barely detectable from this age despite being present at comparable levels to normal white persons at birth whilst the acid enzyme is unaffected (Cook, 1967).

The present findings are in agreement with lactose

Properties	Equine Beta-galactosidase		Human Beta-galactosidase		
	Acid	Neutral	Acid	Hetero	Neutral
Localization	Cytoplasmic or lysosomal Soluble Present in supernatant after centrifugation at 100 000 g for 60 min	Presumably in brush border Particulate Major fraction sediments after centrifugation at 100 000 g for 60 min	Lysosomal Soluble	Presumably cytoplasmic Soluble	Presumably in brush border Particulate
Substrates	Lactose, BNG	Lactose, cellobiose	Lactose, ONPG PNPG, BNG Inhibited	ONPG, PNPB BNG, PG Inhibited	Lactose, ONPG PNPG, cellobiose Not inhibited
Reaction with 0.2 mM p-CMB	Inhibited	Not inhibited			
pH Optimum	4.2	6.0	4.4-5	5.5-6.0	5.5-6.0
Activity at acid pH optimum	Maximal	26% of activity at pH 6.0 (in young horse)	Maximal	Probably none	Negligible
Activity at neutral pH optimum	23% of activity at pH 4.2	Maximal	25% of activity at pH 4.2	Maximal	Maximal

Table A comparison of some of the properties of equine and human small intestinal beta-galactosidases

tolerance tests performed on adult horses (Roberts, 1972), which revealed no increase in plasma glucose levels, although a marked elevation occurred on dosing with the constituent monosaccharides, further confirming the absence of a functional lactose-hydrolytic enzyme in the small intestine of the adult horse. Some of the horses exhibited signs of mild abdominal discomfort and transient diarrhoea after the lactose load, whereas younger horses were able to digest this disaccharide.

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