Analytical isoelectric focusing of rat intestinal brush-border enzymes: postnatal changes and effect of neuraminidase in vitro

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Increase in pI values was found between 11 and 30 days of postnatal life in papain-solubilized brush-border enzymes of rat small intestine by means of thin-layer analytical isoelectric focusing in agarose gel. Treatment with neuraminidase converted the acidic forms of enzymes from 11- and 20-day-old rats into the zymogram patterns identical with the adult, more basic forms. The zymograms of the respective enzymes are the same in 30-, 60- and 74-day-old animals and do not change on treatment with neuraminidase.

Brush-border enzyme *Isoelectric focusing* *pI changes* *Sialic acid* *Postnatal development* *Rat small intestine*

1. INTRODUCTION

Marked changes of intestinal brush-border structure and its enzymic activities are observed in the rat during the transition from suckling to weaning in the third and fourth week after birth [1]; e.g., a decrease in sialic acid content of rat intestinal brush-border membrane was found to occur between 5 and 60 postnatal days [2]. In young animals certain brush-border enzymes appear to be more sialylated than their adult forms. This regards fetal alkaline phosphatase (EC 3.1.3.1) [3] and fetal γ-glutamyltransferase (EC 2.3.2.2) [4], although contradictory data were reported for the latter enzyme of human origin [5]. Changes in sialylation of cell membranes may reflect mitotic and differentiation processes [6]. Treatment with neuraminidase resulted in a changed electrophoretic mobility of the higher M_r intracellular precursors of pig intestinal aminopeptidase M (EC 3.4.11.2) and sucrase-isomaltase (EC 3.2.1.48 – 10) [7]. Sialic acid-rich fetal form of γ-glutamyltransferase was found in undifferentiated cryptal cells, from which the enterocytes originate [4].

Bound sialic acids contribute to the negative charge of membrane glycoproteins. Our aim was to investigate the pI changes of solubilized brush-border enzymes during postnatal development of the rat.

Abbreviations: Nap, naphthyl; 2-NNap, 2-naphthylamide; 2-NNapOMe, 4-methoxy-2-naphthylamide; Ind, 4-Cl-5-Br-3-indolyl; CBZ-, carbobenzoxy-; FBB, fast blue B salt; HPR, hexazonium-*p*-rosaniline; HF, hexazotized fuchsin; IEF, isoelectric focusing

2. MATERIALS AND METHODS

2.1. *Solubilization of brush-border proteins*

Intestinal brush-border membranes were prepared from mucosal scrapings of rat jejunum, essentially as in [8]. Rats of both sexes were 11, 20, 30, 60 and 74 days old. The isolated brush-border preparations from individual age groups were treated with papain (from *Papaya latex*, 2 × cryst., Koch-Light Labs, Colnbrook) at 37°C for 60 min (33 μg/mg protein; activated with cysteine hydrochloride 3.3 μg/mg protein). The 105000 × *g* (4°C) supernatant was dialyzed against 10 mM KCl

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before application to the isoelectric focusing gel. Protein concentration was 1–3 g/l.

2.2. *Treatment of brush-border proteins with neuraminidase*

When testing for the presence of sialic acids bound to the enzymes of the solubilized brush-border fraction, the samples were incubated with neuraminidase from *Clostridium perfringens* (0.6 units/mg dry powder, Boehringer, Mannheim). The incubation of neuraminidase-treated (60 munits/ml with 20 mM $CaCl_2$, pH 5.6) and control samples proceeded at room temperature overnight, before isoelectric focusing was started. The neuraminidase preparation displayed none of the enzymic activities of the samples.

2.3. *Analytical isoelectric focusing*

Thin-layer analytical isoelectric focusing was performed in Agarose IEF (Pharmacia Fine Chemicals, Uppsala) with Pharmalyte™ (Pharmacia) of pH intervals 3–10, or 4–6.5 according to the instructions of the producer [9]. Isoelectric Focusing Calibration Kits (Pharmacia) were employed for pI estimation of separated enzymes.

2.4. *Histochemical demonstration of enzyme activities in the gels*

For zymograms the unfixed gels were incubated in optimized media [10–12]. The following enzyme activities were demonstrated employing azo-coupling reactions; i.e., incubation of gels at 20°C for 1 h in staining solutions containing the corresponding substrates (0.5–1 mM): α-glycosidases, using 6-Br-2-Nap–α-D-Glc*p* (Lachema, Brno) or 2-Nap–αD-Glc*p* (Serva); β-glycosidases, employing 1-Nap–β-D-Glc*p* (Fluka AG, Buchs); aminopeptidase M using L-Leu-2-NNapOMe or L-Ala–2-NNapOMe (Bachem, Feinchemikalien, Bubendorf); dipeptidyl peptidase IV (EC 3.4.14.5) using Gly–L-Pro–2-NNapOMe (Bachem); aminopeptidase A (EC 3.4.11.7) employing 1-L-Glu–2-NNapOMe (Bachem); and γ-glutamyltransferase, using 5-L-Glu–2-NNapOMe (Bachem) and Gly–Gly (Lachema). Enteropeptidase (EC 3.4.21.9) was demonstrated in agarose by a modified sandwich method [13] using *N*-CBZ–Gly–Gly–L-Arg–2-NNapOMe (Bachem) as trypsin substrate [14]. HPR or HF according to [10] or FBB (Lachema) were used as diazonium salts (1 mg/ml incubation medium). β-Galactosidase (EC 3.2.1.23) was demonstrated by the indigogenic method with Ind-β-D-Fuc*p* (Cyclo Chemical Division, Travenol Labs, Los Angeles) [10,15–17].

3. RESULTS AND DISCUSSION

Zymograms of solubilized intestinal brush-borders from rats of different ages exhibited an increase in pI of all investigated enzymes between 11 and 30 days of life (table 1; fig.1,2). The most pronounced changes were found in γ-glutamyltransferase, dipeptidyl peptidase IV and enteropeptidase, with pI shift representing 2–13, 1.5–2 and 1–1.5 pH units, respectively. A smaller change exists in aminopeptidase M and A and in α-glycosidases (ΔpI = 0.3–0.5) and the least difference was observed in β-glycosidases (ΔpI = 0.1–0.2). The zymogram patterns of the respective enzymes were identical in 30-, 6- and 74-day-old animals.

Treatment with neuraminidase in vitro converts the acidic forms of all investigated enzymes from 11-day- and 20-day-old rats into the more basic

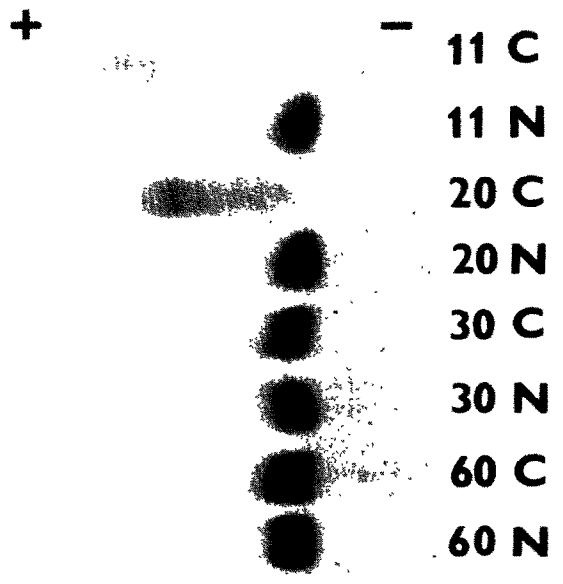

Fig.1. Isoelectric focusing of brush-border dipeptidyl peptidase IV, pharmalyte pH 3–10; stained with Gly–L-Pro–2-NNapOMe, FBB (pH 7.2). Numbers indicate the age of rats in days; C = controls; N = treated with neuraminidase.

Table 1

pI changes[a] and other characteristics of intestinal brush-border enzymes during postnatal development of the rat

Enzyme	Substrate	Postnatal days		
		11	20	30[b]
α-Glycosidases	6-Br-2-Nap–α-D-Glc*p*	–	4.2 –4.8	4.55[c]; 4.8[d]
	2-Nap–α-D-Glc*p*	–	4.2 –4.8	4.55[c]; 4.8[d]
β-Glycosidases	1-Nap–β-D-Glc*p*	4.5 –4.7	*4.7 –4.8* (5.0; 5.2)	4.8
	Ind–β-D-Fuc*p*		4.7 –4.8	4.8
Peptidases				
Aminopeptidase M	L-Leu–2-NNapOMe	3.8 –4.6	4.1 –4.75	4.65–*4.75*
	L-Ala–2-NNapOMe	4.1 –4.6	4.3; 4.4; *4.5; 4.6; 4.7*	4.65; 4.7; *4.75*
Aminopeptidase A	1-L-Glu–2-NNapOMe	4.1 –4.75	4.4 –4.75	4.7; 4.75; *4.8*
Dipeptidyl peptidase IV	Gly–L-Pro–2-NNapOMe	3.65–5.2[e]	4.15–5.75[e]	5.4 –5.8[e]
Enteropeptidase	Trypsinogen	3.0 –4.2	3.0 –4.3	4.3
Aminoacyltransferase				
γ-Glutamyltransferase	5-L-Glu–2-NNapOMe + Gly–Gly	4.3 –7.35[f]	4.55–7.35[f]	6.85; *7.15; 7.35;* 7.75

[a] pI values estimated with IEF-pI Calibration Kit (section 2). The values of the strongest bands are printed in italics. Values of very weak bands are in parentheses. Broader range of pI indicates that no distinct maxima were apparent on the zymogram

[b] Identical values found also in 60- and 74-day-old animals

[c] 50°C 1 h; heat-stable

[d] 50°C 1 h; heat-labile

[e] Large heterogeneity

[f] At least 10 maxima

(adult) ones. No such effect was observed in older age groups (fig.1). In young rats the neuraminidase action resulted in zymogram patterns of adult animals. This suggests that the increase in pI during postnatal development is most probably caused by the progressive loss of bound sialic acids. This conclusion is supported in [2], where the author found 4-fold higher values of bound sialic acid in the intestinal brush borders of 5-day-old rats than in 60-day-old animals. In older rats the level remained constant. In our experiments the observed pI shift did not continue in the weaning period close to the age of 30 postnatal days.

Some microheterogeneity could still be observed in zymograms of peptidases (fig.1) and of γ-glutamyltransferase, but not in those of α- and β-glycosidases (table 1; fig.2) of adult rats. In α-glycosidases two bands belonging to different enzymes were observed in zymograms of adult animals. The heat-labile one with pI 4.8 corresponds to sucrase-isomaltase [18]; the other with pI 4.55 represents the heat-resistant glucoamylase (EC 3.2.1.20) [18]. Sucrase-isomaltase is known to be absent in baby rats. There was almost no α-glycosidase activity demonstrable with the substrate used here in zymograms of 11-day-old animals. In β-glycosidases the single band observed in adult animals with β-fucoside as substrate [15] confirms the homogeneity of lactase-phlorizin hydrolase (EC 3.2.1.23 – 62). In addition the β-glucosidase activity displaying the same pI suggests the existence of an enzyme complex [16,17]. The heterogeneity in enzyme patterns was reduced by neuraminidase action on all brush-border enzymes from young rats but it was not influenced by this treatment in zymograms of adult animals (fig.1).

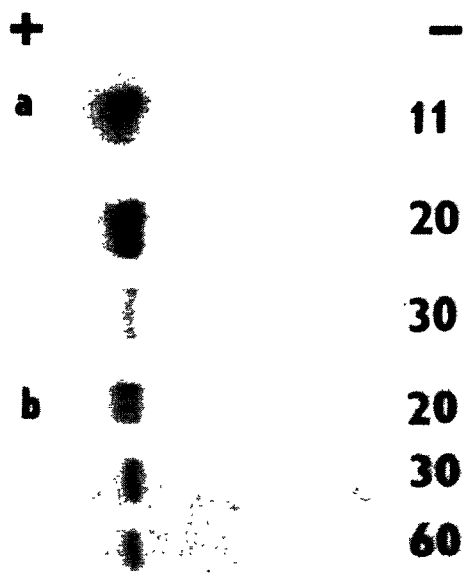

Fig.2a. Isoelectric focusing of brush-border β-glycosidases; pharmalyte pH 3–10; stained with 1-Nap–β-D-Glc*p*, HPR (pH 6.5). 2b. Stained with Ind–β-D-Fuc*p* (indigogenic method) (pH 5.5). Numbers indicate the age of rats in days.

The explanation of this residual heterogeneity of proteases and γ-glutamyltransferase requires further study, including comparison of various solubilization procedures.

When the solubilized brush-border fractions from 11-day- and 60-day-old rats were mixed together and incubated overnight at 20°C, no pI shift in the individual zymograms was observed, the two being clearly separated. This may be an argument against the formation of reversible enzyme–acid glycoprotein complexes or against other factors present in the investigated material, which would lead to differences in zymogram patterns from different age groups in vitro [19].

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