

## Age Dependent Changes in Activity of Intestinal Disaccharidases and Alkaline Phosphatase Activity in CD26 Deficient Mice

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The profile of intestinal brush-border membrane enzyme activity is described in several mice and rat species. Dipeptidyl peptidase IV (CD26/DPP IV) is as well an intestinal brush-border membrane hydrolase. In order to examine the consequences of CD26/DPP IV deficiency on the activity of other enzymes, CD26 deficient mice were investigated. The aim of this study was to determine the influence of age on intestinal brush-border disaccharidases (lactase, sucrase, and maltase), and alkaline phosphatase activity in CD26 deficient mice. The relationship between ageing and brush-border membrane enzymes activities was characterized in different small intestinal segments (duodenum, jejunum, ileum) in mice aged 2 weeks, 1, 2, 3, 6 and 12 months. The results of this study revealed that intestinal enzyme activities change statistically significantly with ageing in CD26 deficient mice. Interestingly, the horizontal patterns (duodenum to ileum) of their activities remain not affected by age.

*Keywords*

ageing  
CD26 deficient mice (CD26<sup>-/-</sup>)  
disaccharidases  
alkaline phosphatase

### INTRODUCTION

Prerequisites for normal metabolism and vital functions of the organism are digestion, hydrolysis and subsequent absorption of dietary nutrients. Adequate digestion and absorption depend on a wide spectrum of factors, including mechanical food mixing, enzyme production and activity, appropriate mucosal function, adequate blood supply, intestinal motility and ordinary microbial flora natural balance.<sup>1</sup>

The process of digestion is mediated by different hydrolases secreted in the gastrointestinal tract or produced by the enterocytes in the intestinal brush border membrane.<sup>2</sup> Intestinal disaccharidases are essential for the appropriate digestion of carbohydrates. Food carbo-

hydrates are hydrolyzed to monosaccharides before transport across the microvillus membrane. The digestion of disaccharides and some oligosaccharides is undertaken by a number of small intestinal brush border enzymes.<sup>3</sup> Lactase (lactase-phlorizinhydrolase, EC 3.2.1.23-62) is the absorptive enterocyte membrane glycoprotein essential for digestive hydrolysis of lactose in milk with a crucial role in the nutrition of suckling mice. Sucrase (sucrase-isomaltase, EC 3.2.1.48-10) is an  $\alpha$ -glucosidase that hydrolyzes sucrose, maltotriose and about 80 % of dietary maltose, while maltase (maltase-glucoamylase, EC 3.2.1.20) digests the remaining maltose to glucose.<sup>4</sup>

The intestinal alkaline phosphatase (ALP, EC 3.1.3.1) is a metalloenzyme generally accepted as a marker of epithelial cell differentiation. It hydrolyzes monophosphate

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esters at an alkaline pH.<sup>5</sup> Among the brush border enzymes of the small intestine, ALP is functionally involved in nutrient absorption and transport of long-chain fatty acids in the intestinal mucosa.<sup>6</sup>

Peptidases present in the brush-border membrane and in the cytosol of enterocytes of the small intestine hydrolyse peptides into amino acids. The dipeptidyl peptidase IV (DPP IV/CD26, EC 3.4.14.5) is a multifunctional glycoprotein expressed both in soluble forms in many biological fluids and on the surface of epithelial, endothelial and immune cells. It is a serine protease belonging to a subgroup of prolyl oligopeptidases. The DPP IV/CD26 as a brush border-associated hydrolase is unique among the peptidase family since its cleavage site includes a proline or, less frequently, an alanine in position 2 of the peptide N-terminus.<sup>7</sup> It has been shown that this enzyme is involved in the final steps of peptide metabolism. DPP IV/CD26 is important for digestion, because in general proline-containing peptides are resistant to hydrolysis by many of the known brush border proteases.<sup>8</sup> The role of DPP IV/CD26 has furthermore been proposed in the regulation of immune, inflammatory, nervous, and endocrine functions.<sup>9</sup>

The profile of intestinal brush-border membrane enzyme activity is documented in several mice and rat species.<sup>10–12</sup> But their activities, and especially the influence of age on their activities, are still not established in mice with targeted inactivation of the CD26 gene (CD26<sup>-/-</sup>). Therefore, the aim of this study was to determine the profile of three intestinal brush-border disaccharidases (lactase, sucrase, and maltase), and ALP activities in CD26<sup>-/-</sup> mice at different ages, from the suckling period, through maturation to senescence. Likewise, we wanted to determine if CD26 gene deficiency could influence the activity of investigated enzymes.

## EXPERIMENTAL

### *Ethical Considerations*

The experimental protocol was approved by the Ethical Committee of the School of Medicine, University of Rijeka, Croatia. The principles for care and use of laboratory animals were followed in the conduct of this study.

### *Animals*

Male CD26<sup>-/-</sup> mice generated on a C57BL/6 genetic background were used in this study. The single gene encoding both the transmembrane and the soluble form of CD26 was inactivated by homologous recombination in embryonic stem cells.<sup>13</sup> Animals were kindly provided by courtesy of Dr Didier Marguet (Centre d'Immunologie de Marseille Luminy, Parc Scientifique de Luminy, Marseille Cedex, France). Experimental animals were housed in plastic cages at the animal's facility, School of Medicine, University of Rijeka. Water and standard pellet food (MK, Complete Diet

for Laboratory Rats and Mice, Slovenia) were supplied *ad libitum*. Mice were maintained under a 12/12 h dark/light cycle at constant temperature (20 ± 1) °C and humidity (50 ± 5) %.

### *Experimental Procedure*

Animals were divided into six groups: 2 weeks, 1, 2, 3, 6 and 12 months old mice, totalling 6 to 8 animals per group. Mice mass was recorded weekly. Animals were sacrificed by cervical dislocation. In order to avoid diurnal variability, the experimental part was always performed between 8:00 and 9:00 am. The gastrointestinal tract was isolated and rinsed with isotonic cold saline thoroughly. The entire small intestine was removed, freed from adhering tissue and divided into three segments (duodenum, jejunum and ileum). Intestinal segments were weighed, their lengths were measured, and put on ice-cold glass plates. Each segment was slit open longitudinally. The mucosa was collected from the duodenum, jejunum and ileum by gently scraping with a glass slide. Brush border membrane fractions were prepared from mucosal scrapings according to Ahnen *et al.*<sup>14</sup> Aliquots were stored at -80 °C for further assays of enzyme activities.

### *Enzyme Activity Assays*

Intestinal disaccharidases (lactase, sucrase, maltase) and ALP activities were measured in the duodenal, jejunal and ileal intestinal segment in each group of differently aged mice. The relationship between ageing and brush-border membrane enzymes activities was characterized using a multiple-sampling technique that quantifies intestinal enzyme activity. Lactase, sucrase and maltase activities were determined according to the method of Dahlqvist,<sup>15</sup> by incubating the mucosal homogenates with the substrate (lactose, sucrose or maltose). The GOD-Perid method was used to measure the glucose released by disaccharidase hydrolysis. Intestinal ALP activity was determined using *p*-nitrophenylphosphate as a substrate with a commercial Sigma Diagnostic Assay Kit.<sup>16</sup> All measurements were performed at least in duplicate, using a Varian Cary 100 UV/Vis spectrophotometer (Cary, NC, USA).

Enzyme activities in homogenates were expressed as amount of substrate hydrolyzed per minute per 1 mg of protein (μmol min<sup>-1</sup> mg<sup>-1</sup>). Protein concentrations were determined according to the method of Bradford.<sup>17</sup> Bovine serum albumin (Sigma) was used as standard.

### *Statistical Analysis*

All data presented in the table and figures are expressed as mean ± standard deviation. Data were subjected to analysis of variance (ANOVA test), followed by the Scheffe Test. The value of *P* < 0.05 was considered as statistically significant. Statistical operations were performed using STATISTICA 6.1 (StatSoft, Inc., Tulsa, OK, USA).

TABLE I. Physiological data for 2 weeks, 1, 2, 3, 6 and 12 months aged CD26<sup>-/-</sup> mice<sup>(a)</sup>

	Age					
	2 weeks	1 month	2 months	3 months	6 months	12 months
Body mass / g	6.25 ± 0.85	10.45 ± 3.87	22.78 ± 2.34	28.48 ± 3.45	28.62 ± 1.23	39.92 ± 1.53
Intestine length / cm	13.90 ± 1.71	16.10 ± 2.84	21.30 ± 3.84	24.50 ± 4.35	25.38 ± 0.85	24.71 ± 1.67
Intestine mass / g	0.20 ± 0.05	0.49 ± 0.17	0.82 ± 0.11	0.98 ± 0.20	1.11 ± 0.10	1.18 ± 0.19
Wet mucosa mass / g						
Duodenum	0.06 ± 0.02	0.07 ± 0.02	0.08 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
Jejunum	0.06 ± 0.02	0.07 ± 0.02	0.06 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
Ileum	0.06 ± 0.02	0.07 ± 0.03	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
$\left( \frac{\text{Wet mucosa mass}}{\text{intestine length}} \right) / \text{mg cm}^{-1}$	12.76 ± 2.04	13.29 ± 2.81	9.27 ± 1.75	6.95 ± 0.60	6.27 ± 1.27	5.36 ± 1.71

<sup>(a)</sup> Data are expressed as mean ± standard deviation, *n* = 6–8 mice per group.

RESULTS

Data on mice physiological characteristics are summarized in Table I. Mice masses, intestine lengths and masses increased proportionally with mice age, as expected. The mucosa mass was the highest for mice aged 2 months in the duodenum, while in the jejunum and ileum, the highest mucosal masses were recorded for mice aged 1 month. Afterwards, a sequential decrease in mucosal masses with ageing was noticed in all intestinal segments. Likewise, after the most elevated mucosa mass/intestinal length ratio, which was achieved in mice aged one month in all intestinal segments, a coherent decrease was observed.

Disaccharidases (lactase, sucrase and maltase) and intestinal ALP activities were determined in three small intestinal segments in CD26<sup>-/-</sup> mice old 2 weeks, 1, 2, 3, 6 and 12 months. The time course of their duodenal, jejunal and ileal activities are shown in Figures 1 to 4. The ANOVA test revealed a significant effect of age on

analyzed enzyme activities in different segments of the small intestine (*P* < 0.05, Figures 1–4). A marked decrease in their activity with maturation was noticed.

Lactase activity in the duodenum, jejunum and ileum of differently aged mice is shown in Figure 1. The jejunum was the intestinal segment with highest lactase activity. With respect to age, the highest lactase activity values were observed in animals old 2 weeks, in each analyzed intestinal segment. A statistically significant decrease after age of 1 month and later was found (*P* < 0.05), showing a strong decrease in lactase activity all over the intestine after the suckling period. At age of 6 and 12 months, the lactase activity decreased about 98 % from activity values at 2 weeks and was almost undetectable in all intestinal segments.

Sucrase also showed the highest activity in jejunal segments of all differently aged mice groups (Figure 2). Contrary to lactase activity, sucrase activity increased 2–3 times to the age of 2 months, when its highest activities

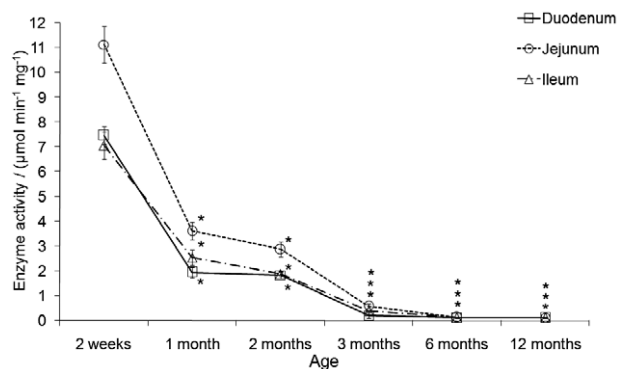


Figure 1. Lactase activity in duodenal, jejunal and ileal intestinal brush border membrane in CD26<sup>-/-</sup> mice old 2 weeks, 1, 2, 3, 6 and 12 months. Results are expressed as mean ± standard deviation; *n* = 6–8 mice per group. *P* < 0.05: statistically significant difference compared to 2 weeks old mice (\*).

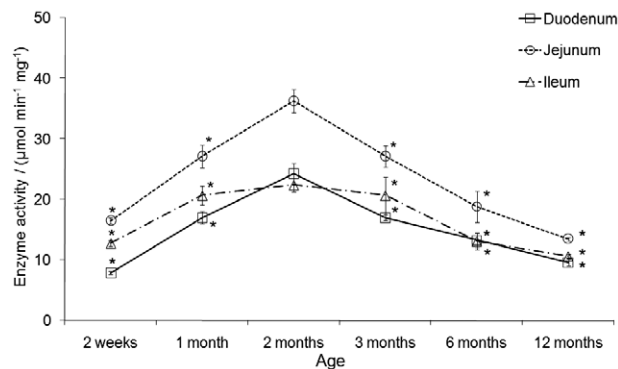


Figure 2. Sucrase activity in duodenal, jejunal and ileal intestinal brush border membrane in CD26<sup>-/-</sup> mice old 2 weeks, 1, 2, 3, 6 and 12 months. Results are expressed as mean ± standard deviation; *n* = 6–8 mice per group. *P* < 0.05: statistically significant difference compared to 2 months old mice (\*).

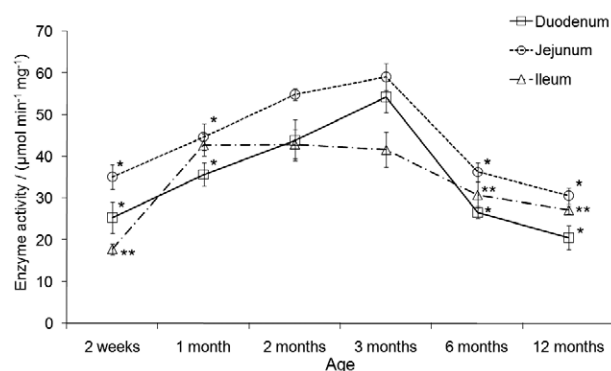


Figure 3. Maltase activity in duodenal, jejunal and ileal intestinal brush border membrane in  $CD26^{-/-}$  mice old 2 weeks, 1, 2, 3, 6 and 12 months. Results are expressed as mean  $\pm$  standard deviation;  $n = 6-8$  mice per group.  $P < 0.05$ : statistically significant difference compared to 3 months old mice (\*) and 1 month old mice (\*\*).

were detected, in all intestinal segments. These values were statistically significantly higher ( $P < 0.05$ ) in comparison with activities in all other age groups of mice in the whole small intestine. After this peak in sucrase activity was reached, a statistically significant decrease was found to occur with aging ( $P < 0.05$ ). In 12 months old animal, the sucrase activities decreased about 60 % in the duodenum and jejunum and 52 % in the ileum compared to its highest activities achieved at the age of 2 months.

Likewise, the influence of age on maltase activity was also detected in the duodenum, jejunum and ileum (Figure 3). Similarly to lactase and sucrase, the highest maltase activity was observed in the jejunum. On the other hand, with respect to age, its highest activity was reached in 3 months old animals in the jejunum and duodenum. But in the ileum, the highest maltase activity was detected in 1 month old animals. Maltase activities in 12 months old animals decreased about 62 % in the duodenum and 48 % in the jejunum, compared to the highest activity in the respective intestinal segments, noticed at the age of 3 months. In the ileum, the values decreased about 37 %, compared to the highest activity achieved at 1 months. In summary, statistically significant ( $P < 0.05$ ) variations were observed in maltase activity in different intestinal segments depending on the age of mice.

The time course of intestinal ALP activity is shown in Figure 4. The intestinal ALP activity decreased progressively along the intestine from duodenum to ileum. On the contrary to disaccharidases, the intestinal ALP activity was the highest in the duodenum. It reached the maximum of activity at the age of 1 month, in all analyzed intestinal segments. After that age, its activity started to decrease coherently ( $P < 0.05$ ). The lowest intestinal ALP activities were detected in 12 months old mice, showing a decrease of 74 % in the duodenum, 69 % in

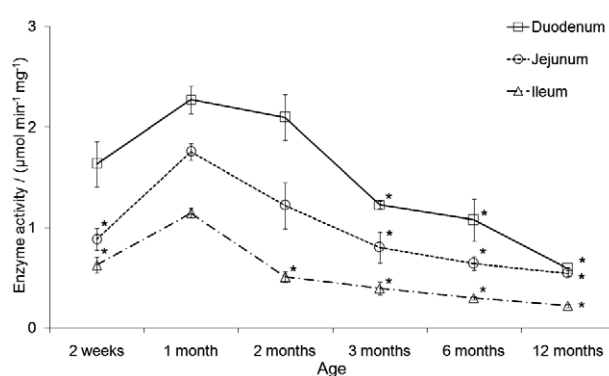


Figure 4. Alkaline phosphatase activity in duodenal, jejunal and ileal intestinal brush border membrane in  $CD26^{-/-}$  mice old 2 weeks, 1, 2, 3, 6 and 12 months. Results are expressed as mean  $\pm$  standard deviation;  $n = 6-8$  mice per group.  $P < 0.05$ : statistically significant difference compared to 1 month old mice (\*).

the jejunum and 81 % in the ileum in comparison to the activity present at the age of 1 month. An evident influence of age was noticeable in the whole small intestine, with most accentuated age-related changes in the ileum, where intestinal ALP activities were statistically significantly different ( $P < 0.05$ ) in all age groups in comparison with 1 month old mice. The same ALP activity pattern was found in all age groups, but showing a coherent decrease with ageing.

## DISCUSSION

$CD26^{-/-}$  mice showed normal behaviour and eating habits as wild type mice under standard laboratory conditions. The body mass of  $CD26^{-/-}$  mice was slightly lower than wild-type controls (C57BL/6 mice), as known in literature.<sup>13,18,19</sup> However, these mice are fertile and appear healthy despite a low DPP IV plasma level.<sup>20</sup> They display reduced N-terminal degradation of glucagon-like peptide 1, increased levels of insulin and improved glucose tolerance.<sup>13</sup>

The intestine mass and length in  $CD26^{-/-}$  mice were found to be similar to those in wild-type controls, but we noticed that  $CD26^{-/-}$  mice had higher mucosal mass in all three analysed intestinal segments in comparison with C57BL/6 mice.<sup>18</sup> This observation was the most pronounced in mice old 2 weeks and 1 month. After the age of 2 months, these values become similar for both groups of mice, showing a coherent decrease with ageing. It is interesting to notice that the mucosa mass/intestinal length ratios shown an even more emphasized difference in the same age groups.  $CD26^{-/-}$  mice old 2 weeks and 1 month had approximately two-fold higher mucosa mass/intestinal segment length ratios in comparison with C57BL/6 mice.



We focused on enzymes of the isolated brush-border membrane vesicles because of their importance in the processes of digestion and hydrolysis of nutrients. In all age groups the highest activities of disaccharidases were found in the jejunum while that of ALP in the duodenum. This observations agree with published results for BALB/c mice<sup>12</sup> and rats.<sup>21,22</sup>

The results presented herein revealed distinct age-related changes in the activity of analyzed enzymes. We have shown that intestinal disaccharidases and ALP activities change significantly with ageing. Meanwhile, the horizontal patterns (duodenum to ileum) of their activities remain not affected by age, which corresponds with published data.<sup>10</sup> Furthermore, Holt *et al.* showed that jejunal lactase activity falls gradually through the life span while jejunal sucrase, maltase and ALP specific activities do not fall gradually, but are reduced during senescence.<sup>23</sup> Intestinal lactase is expressed at highest levels in the jejunal segment of the small intestine shortly after birth and then declines considerably upon maturation in most mammal species including humans.<sup>24,25</sup> The results of our study agree with these data. The decline in brush border lactase activity in mammals is similar to the adulthood hypolactasia in humans. However, the mechanism underlying this process is not understood.<sup>26</sup> Nevertheless, the translation of mRNA to lactase is impaired in weaned animals, which may be responsible for the maturational decline in lactase activity in adult rat intestine.<sup>27</sup> Furthermore, it has been supposed that lactase gene is regulated at the translational and/or post-translational levels, but not at the transcriptional level.<sup>28</sup>

We noticed that CD26<sup>-/-</sup> mice have statistically significantly higher duodenal, jejunal and ileal lactase activity at the age of 2 weeks, 1 and 2 months, in comparison with their wild-type controls. At that age, the mucosa mass and especially the mucosa mass/intestinal segment length were also highest for CD26<sup>-/-</sup> mice in comparison with wild-type mice. The enhancement in mucosa mass could therefore be one possible explanation of the highest lactase activity in CD26<sup>-/-</sup> mice in the suckling and weaning period.

Early appearance of intestinal hydrolases is an important factor determining the capability of newborn mammals to assimilate ingested nutrients. While lactase is high and decreases rapidly at weaning, sucrase is undetectable and maltase is low in neonates.<sup>29</sup> Just before weaning, sucrase starts to appear and increases together with maltase to reach high adult levels.<sup>30,31</sup> The data on digestive enzymes activity obtained in our study matches with mice dietary habits. The maturational decline in lactase activity contrasts with a maturational increase in enzymatic activity of other intestinal hydrolases essential for digestion of solid foods.<sup>32</sup> These ontogenetic changes in enzyme expression seem to be a genetic

adaptation to dietary changes.<sup>29</sup> Our results confirm the known reciprocal shift in the intestinal activities of lactase and sucrase.

The observed lower intestinal activity of investigated enzymes in aged mice could be also caused by the cumulative effect of a reduced number of villus enterocytes synthesizing these enzymes. Previous studies which demonstrated that specific activities of several proximal small intestinal mucosal enzymes fall in the aging rat shown that this reduction was due to a delay in the full expression of activity of these enzymes during epithelial cell transit from the crypt onto the intestinal villus.<sup>23</sup> Additionally, the microbial environment is very important, since it is known that anaerobic bacteria can produce proteases that interfere with disaccharidases in the small intestine.<sup>33</sup>

Recently, there is increasing scientific interest for glucagon-like peptide 2 (GLP-2), a gut peptide that stimulates duodenal hydrolase activity and intestinal mucosal growth by inhibiting apoptosis and proteolysis.<sup>34</sup> GLP-2 stimulates jejunal sucrase and maltase mRNA abundance and activity levels in parenterally fed piglets.<sup>35</sup> Similarly, it was also found that activities of duodenal disaccharidases are increased by GLP-2 in mice.<sup>36</sup> It is known that GLP-2 is a substrate of DPP IV<sup>7</sup> and that mice and rats after administration of DPP IV inhibitors have higher GLP-2 concentrations.<sup>37</sup> However, we did not find any difference in the sucrase activity in the whole small intestine of CD26<sup>-/-</sup> mice in comparison with wild-type controls. Furthermore, maltase activity was significantly lower in CD26<sup>-/-</sup> mice in all the analyzed small intestinal segments compared to their controls. To our knowledge, such an effect has not previously been reported. Therefore, additional investigations should be done to give an explanation for these phenomena.

The intestinal ALP activity in our study showed a very coherent site- and age-dependence, which is in agreement with published data for mice<sup>12</sup> and rats.<sup>25</sup> It has been shown that dietary lactose increases the level of ALP activity, especially in the jejunum.<sup>38</sup> In our study, the highest ALP activity was found in 1-month-old mice, at the end of the suckling period, which supports previously reported findings. Variations in ALP activity during normal development may be due to the diet, age, or structural and enzymatic modifications during growth.<sup>39</sup> Some of these variations may be induced by hormones and conditions such as diabetes or starvation.<sup>40</sup>

Considering the importance of DPP IV/CD26 in the digestion and assimilation of proline-rich peptides, in the metabolism of several biologically important peptides (*e.g.* glucagon like peptides 1 and 2), and in modulation of the immune response, this research provides new data regarding the physiological consequences of the DPP IV/CD26 inactivity. This knowledge is important

and useful since DPP IV/CD26 activity emerges as a possible new diagnostic or prognostic marker.<sup>41</sup> Furthermore, pharmacological inhibition of this enzyme could be a new therapeutic strategy.<sup>7</sup>

## CONCLUSION

The study of variations of disaccharidases and ALP activities in the intestinal brush border membrane homogenates in CD26<sup>-/-</sup> mice revealed distinct age-related changes in the activities of investigated enzymes. Development of disaccharidase expression appears to be well matched with maturational changes in consumption of dietary substrates. All analysed enzymes showed a marked decrease in their activity upon maturation in the whole small intestine. Meanwhile, the horizontal pattern of enzyme activity (duodenum to ileum) was not affected by age. Concerning the outline of enzyme activities, our results concord with previous investigations which shown a drop in intestinal brush border membrane enzymes activity in aged rats and mice. The determination of enzyme activity in CD26 deficient mice at different ages provides new data regarding the physiological consequences of this peptidase inactivity. Finally, the results of the present study, together with our previous data, suggest that the deficiency in the CD26 gene could influence the lactase and maltase activity. However, future investigations should explain their causal connection.

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## SAŽETAK

### Utjecaj dobi na aktivnost crijevnih disaharidaza i alkalne fosfataze u CD26 negativnih miševa

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Aktivnosti enzima četkaste prevlake tankoga crijeva istraživane su u različitim sojevima miševa i štakora. Jedna od hidrolaza četkaste prevlake tankoga crijeva jest i dipeptidil-peptidaza IV (CD26/DPP IV). Kako bismo ispitali posljedice nedostatka CD26/DPP IV na aktivnost ostalih enzima, analizirali smo miševe dobivene inaktivacijom gena CD26. Cilj ovog istraživanja bio je utvrditi utjecaj dobi na aktivnost disaharidaza (laktaze, saharaze i maltaze) i alkalne fosfataze u tankom crijevu (duodenumu, jejunumu i ileumu) CD26 negativnih miševa starih 2 tjedna, 1, 2, 3, 6 i 12 mjeseci. Rezultati ovog istraživanja pokazuju da se starenjem statistički značajno mijenjaju aktivnosti svih ispitivanih enzima u tankom crijevu CD26 negativnih miševa. Zanimljivo je da starenje ne utječe na horizontalnu (od duodenuma do ileuma) distribuciju aktivnosti enzima.