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ARTICLE · JUNE 2015

DOI: 10.5604/18982395.1157568

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Journal of Pre-Clinical and Clinical Research, 2014, Vol 8, No 2, 61-66
www.jpccr.eu

Novel potential of pancreatic-like enzymes of microbial origin in exocrine pancreatic insufficiency – study on a pig model

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Szwiec K, Goncharova K, Valverde-Piedra JL, Ushakova G, Kovalenko T, Osadchenko I, Kardas M, Świeboda P, Podgurniak P, Winiarczyk M, Grochowska-Niedworok E, Lozinska L, Filip R, Pierzynowski SG. Novel potential of pancreatic-like enzymes of microbial origin in exocrine pancreatic insufficiency – study on a pig model. J Pre-Clin Clin Res. 2015; 9(1): 61–66.

Abstract

Introduction. The standard porcine-derived pancreatic enzyme replacement therapy (PERT) is a lifesaving treatment for patients with diseases causing exocrine pancreatic insufficiency (EPI). An attempt to replace PERT with microbial enzymes were undertaken. The aim was to highlight whether the mode of application, mixed with food or applied directly to the stomach, of pancreatic-like enzymes of microbial origin (PLEM) can affect their activity along the gastrointestinal tract.

Materials and method. The activity of amylase, lipase and proteinase in the stomach, duodenum and ileum were tested in EPI pigs (n=6) after supplementation of PLEM, either orally – before and during feed consumption – or via the stomach – before and during feed consumption. Healthy pigs not treated with PLEM (n=3) served as controls. Activity of the enzymes measured in the chyme were obtained together with the digesta pH. Activity of the enzymatic residues in the stool samples was also checked.

Results. The highest pancreatic enzyme activities were found in the duodenum of the healthy pigs (amylase 162,68 kU/mL, lipase 507,34 kU/mL and protolitic (trypsin) activity 357,60 kU/mL). Nevertheless, the microbial enzymes remained also active along the entire length of the GIT – including stomach in EPI pigs, regardless of their route of administration. However, activity level was significantly lower.

Discussion. Results indicate that the activity pattern of PLEM in the small intestine mimics the activity of the natural endogenous pancreatic enzymes in healthy pigs. The most physiological features of PLEM were observed when enzymes were offered orally. The magnitude of PLEM activity in the stomach of EPI pigs was essential and significantly higher than that measured in healthy pigs, thus being somewhat not physiological, and for health reasons of the patients should be further explored. Interestingly, specific trypsin-like activity was measured in all parts of the GIT after PLEM application. However, proteolytic activity of the experimental proteaze in *in vitro* studies did not exhibit trypsin-like activity.

Key words

microbial enzymes, exocrine pancreatic insufficiency, porcine model, enzyme activity

INTRODUCTION

Exocrine pancreatic insufficiency (EPI) is a major consequence of diseases that lead to loss of pancreatic parenchyma (pancreatitis, cystic fibrosis, or obstruction of the main pancreatic duct, decreased pancreatic stimulation celiac disease), and/or acid-mediated inactivation of pancreatic enzymes (Zollinger-Ellison syndrome). In addition, gastrointestinal and pancreatic surgical resections (e.g. gastrectomy or duodenopancreatectomy) are frequent causes of pancreatic exocrine insufficiency [1]. Low secretion of pancreatic enzymes is also observed in pre-term and/or full term human babies [2,3] and the elderly [4, 5]. The treatment for EPI is currently symptom-oriented, based on pain reduction, and in chronic cases is often combined with pancreatic enzyme substitution. Pancreatic enzyme replacement therapy (PERT) of porcine origin was approved by the FDA in 2010 [6]; however, pancreatic enzymes of animal origin (Creon, Pancreatin, etc.) have been used as drugs in Europe more than 100 years. Many studies have been performed to evaluate the correct dosing of pancreatic enzymes of porcine or bovine origin; however, even while receiving high doses of PERT, **some patients were ineffectual** to receive sufficient amount of nutrients [7]. Factors that may account for the sub-optimal outcomes following the use of porcine enzyme preparations include: diminished delivery of enzymes to the duodenum due to the enteric coating formulation, fibrosing colonopathy, and impurities in different batches of PERT with potential

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Received: 24 April 2015; accepted: 28 May 2015

biological contamination. In addition, the high pill burden also plays a role in patient compliance [8, 9]. Interestingly, pancreatic-like enzymes of microbial origin (PLEM) seem to exceed porcine-derived enzymes with respect to certain properties, including the high activity and wide pH range, purity and compacted activity per gram of product, as well as the cheap and relatively fast production of the enzymes. Both PERT and PLEM have been investigated in human therapy and animals studies; the clinical responses observed in treated patients were positive and yielded good therapeutic prognoses [8, 10, 11].

The activity of exogenous enzymes within the gastrointestinal tract (GIT) is poorly described. To the best of the authors' knowledge, the activity and dynamic of the PLEM used in the presented study have not previously been reported. The porcine model of EPI is highly relevant for human studies due to the many physiological and anatomical similarities between humans and pigs, including the transit time of feed through the GIT [12, 13, 14].

OBJECTIVE

The aim of the presented study was to measure the ***in vivo*** activity of PLEM (alpha amylase, lipase and protease) along the GIT of pigs with EPI provided directly to the stomach and with meal, compare to the activity of the endogenous pancreatic enzymes of healthy control pigs.

MATERIALS AND METHODS

All phases of the research were approved by the local Ethical Committee and Court in Lund, Sweden. The animals were weaned to a diet formulated for young growing pigs (Vaxtill Solo 330, Sweden), which was enriched with extra fat (18%), according to a recipe previously described by Donaldson et al. [15]. Throughout the study, the pigs were fed an amount equivalent to 2% of body weight per meal, equals to a feed amount these animals would receive in production [16]. Water was provided *ad libitum*. Following surgery, the pigs were housed individually in pens (22±2 °C, 70±5% humidity, 12:12 light: dark cycle).

Castrated male pigs (40 days old) were randomly divided into three groups: shoats with intact pancreas – Healthy Group (n=3), shoats with surgically induced EPI – Group EPI (n=6) and shoats, which underwent EPI and received PLEM supplementation – Group EPI+E (n=6). Surgical procedure was conducted two weeks after weaning, including pancreatic duct ligation to induce exocrine pancreatic insufficiency (EPI and EPI + E groups), and cannulation of GIT fistulas of the stomach, duodenum, and ileum for all groups of animals [17, 18]. The EPI pigs developed symptoms of EPI (arrested growth, steatorrhea) about one month post-surgery [12].

Enzyme administration and sampling. Each PLEM dose per meal contained 520 active lipase kilo units (*Burgholderia cepacia*), 400 active protease kilo units (*Aspergillus melleus*) and 60 active amylase kilo units (*Aspergillus oryzae*). The enzymes were obtained from Amano (Amano Enzyme, USA). The experimental period commenced when the pigs from the EPI group developed pancreatic insufficiency, which was confirmed upon the presence of the growth arrest and steatorrhea.

The enzyme mixture was incorporated into 20 g of fat-free yoghurt (0.5% fat, 4% protein, natural yogurt (Skanem, Sweden) administered to the pigs around morning feeding time. The enzyme-yoghurt mixture was provided to the EPI pigs from both group EPI and EPI+E 4 times during the study, via different routes and time of administration (Tab. 1). The pigs from the Healthy Group were also fed the same feed and amount of yogurt, but without enzymes. The level of enzyme supplementation was based on a previously published study, in which a significant digestibility improvement was observed in the same EPI pig model, following enzyme supplementation [17].

Table 1. Experimental design, including specific sequence, route and timing of enzymes administration to exocrine pancreatic insufficient pigs

DAY	ROUTE	TIME	EPI pigs	HEALTHY pigs
0	O	DF	Y	Y
1	IG	DF	PLEM + Y	Y
4	O	BF	PLEM + Y	Y
7	IG	BF	PLEM + Y	Y
10	O	DF	PLEM + Y	Y

Each PLEM dose per meal contained 52,000 active lipase units (*Burgholderia cepacia*), 40,000 active protease units (*Aspergillus melleus*) and 60,000 active amylase units (*Aspergillus oryzae*). Digesta samplings were scheduled with intervals, allowing for evacuation of enzyme residues from the gastrointestinal tract.
EPI – exocrine pancreatic insufficient pigs; PLEM – pancreatic like enzymes of microbial origin; Y = yougurt; O – orally; IG – intragastricaly; DF – during feeding; BF – before feeding

Digesta samples were collected from all experimental animals, before and after yogurt administration (with or without PLEM). A total of 9 collections were made from each cannulised part of the GIT on each of the 5 experimental days. During the first collection, the digesta samples from the stomach, duodenum and ileum of EPI, EPI+E and Healthy Group pigs were collected before administration of the yogurt. The subsequent 8 collections of digesta samples from each GIT cannula were collected at 5, 30, 60, 120, 180, 240, 300, and 360 min after administration of the yogurt. Following digesta, collections were scheduled similarly within the time of sample collection, although differed in ways and timing of enzymes administration (Tab. 1). Thus, a total of 27 samples per day (approximately 2 mL each) were collected from each pig. Immediately following collection of the digesta samples, the pH of each sample was recorded (Jenway 370, Bibby Scientific Limited, UK) and the samples were then stored at -20 °C until further analysis.

Analysis of enzyme activity. Amylase activity was measured in the supernatant of homogenised digesta samples using commercial colorimetric test kits (Amylase Liquid Stable Reagent, Infinity™, Thermo Scientific¸ USA). Lipase activity in the digesta samples was measured using an assay (Randox Laboratories Ltd., UK). Homogenised samples were well mixed prior to performing the assay (Vortex MS 3), the lipase activity was then measured in triplicate. Protease activity was measured calorimetrically using Nα-Benzoyl-DL-Arginine + p-Nitroaniline (BAPNA), following the method described by Fritz et al. [19]. This method was chosen as the basal standard laboratory test to measure specific trypsin and trypsin-like activity (proteinase). The maximal activity of each enzyme measured in the digesta samples was expressed as the mean maximal value (P_{max}) obtained at a particular time point. Area under the curve (AUC) was used to report

overall enzyme activity in the EPI pigs, compared to that of the Healthy Group pigs. Total enzyme activity was calculated by adding all the results obtained from the digesta samples collected within each group of pigs (EPI, EPI+E and Healthy Group).

Statistical analysis. The differences between experimental groups were assessed using Statgraphics plus v. 4.1. (Statpoint Technologies, Inc., USA). The results of the enzyme activities are presented as the means and standard errors (mean±SE), and were subjected to a two-way analysis of variance followed by a Tukey *post-hoc* test. AUC and total enzyme activity were expressed as mean±SD, and subjected to two-way analysis of variance. In all statistical analyses $p<0.05$ was considered significant.

RESULTS

Activity of PLEM measured as the area under the curve (Tab. 2) in the GIT digesta samples from EPI pigs after enzyme administration (EPI+E), was significantly lower ($p < 0.05$) than in the control pigs, but significantly higher ($p < 0.05$) than in EPI pigs not receiving the enzymes.

Table 2. AUC for amylase, lipase and protease activity measured in digesta samples collected from the stomach, duodenum and ileum of EPI (Exocrine pancreatic insufficient pigs), EPI+E (Exocrine pancreatic insufficient pigs supplemented with enzymes) and contol pigs (Healthy Group)

Enzyme	GIT segment	EPI	Healthy	EPI+E			
				Before feeding		During feeding	
				O	IG	O	IG
Amylase	Stomach	11.10[a]	6.67[a]	41.48[b]	27.04[b]	27.26[b]	42.16[b]
	Duodenum	9.07[a]	119.60[b]	40.29[c]	28.56[c]	44.66[c]	36.27[c]
	Ileum	8.92[a]	31.01[b]	13.74[c]	18.14[c]	15.43[c]	11.32[c]
	Total	29.10[a]	158.29[b]	95.50[c]	73.75[c]	87.35[c]	89.75[c]
Lipase	Stomach	113.10[a]	651.84[b]	NA	544.21[b]	NA	501.37[b]
	Duodenum	77.41[a]	903.21[b]	NA	430.23[b]	NA	879.27[b]
	Ileum	227.46[a]	235.45[a]	NA	177.15[a]	NA	86.26[a]
	Total	417.98[a]	1790.51[b]	NA	1151.59[b]	NA	1466.90[b]
Protease	Stomach	13.06[a]	26.50[a]	41.40[a]	37.21[a]	77.50[a]	24.87[a]
	Duodenum	7.69[a]	650.17[b]	46.41[c]	58.59[c]	47.08[c]	50.58[c]
	Ileum	26.75[a]	57.84[a]	56.55[a]	46.39[a]	65.29[a]	30.09[a]
	Total	47.52[a]	735.53[b]	144.36[a]	142.19[a]	189.88[a]	105.55[a]

Different lowercase letters indicate statistically significant differences for $p<0.05$
NA = not analysed; O – orally; IG – intragastrically

Activity of endogenous enzymes in Healthy Group pigs. As expected, the amylase and protease activity in the stomach samples of control pigs (Healthy Group) were low: amylase P_{max} 0.68–2.92 kU/mL (Fig. 1) and protease P_{max} from 2.13–16.18 kU/mL (Fig. 3). Stomach lipase activity was high (P_{max} 235.03±12.06 kU/mL) and remained high until 5 hours after feeding (P_{max} 204.33±30.65 kU/mL) (Fig. 2). The highest pancreatic enzyme activity was measured in the duodenum of healthy pigs and differed significantly from values obtained from EPI and EPI+E groups. The AUC for amylase was more than twice as high in pigs from the Healthy Group, compared to EPI and EPI+E pigs groups ($p<0.05$). The AUC, for protease

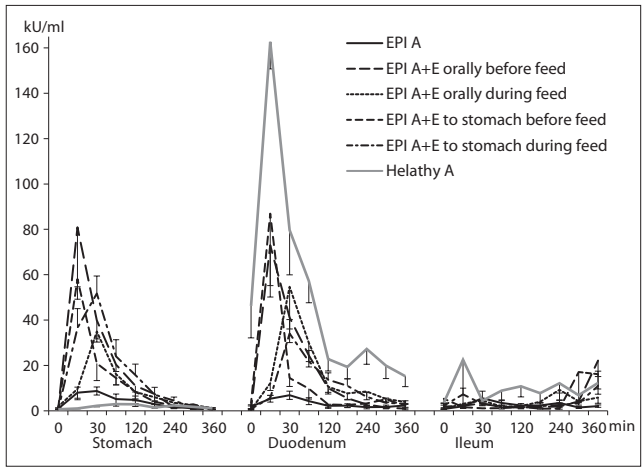

Figure 1. Endogenous and exogenous amylase (A) activity in the stomach, duodenum and ileum of EPI A (Exocrine pancreatic insufficient pigs), EPIA+E (Exocrine pancreatic insufficient pigs supplemented with enzymes) and contol pigs (Healthy A). Values presented as P_{max} (mean ± SEM)

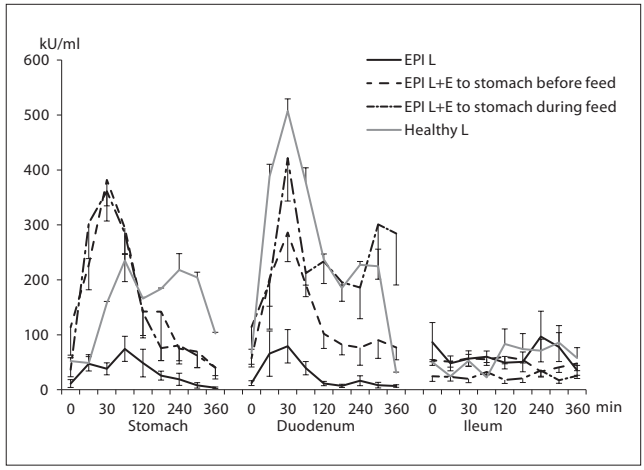

Figure 2. Endogenous and exogenous Lipase (L) activity in the stomach, duodenum and ileum of EPI L (Exocrine pancreatic insufficient pigs); EPI L+E (Exocrine pancreatic insufficient pigs supplemented with enzymes) and contol pigs (Healthy L). Values presented as P_{max} (mean ± SEM)

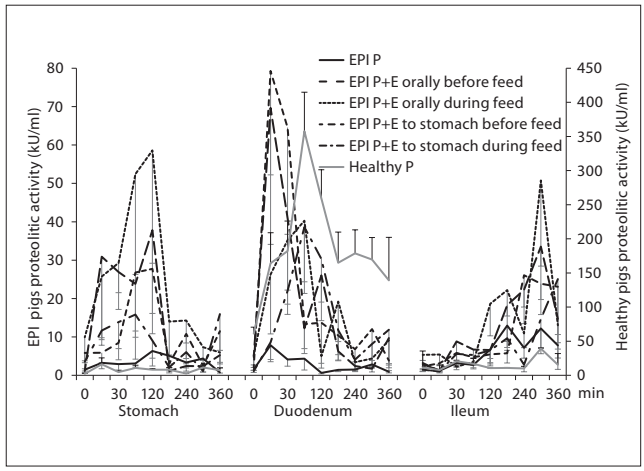

Figure 3. Endogenous and exogenous proteinase (P) activity in the stomach, duodenum and ileum of EPI P (Exocrine pancreatic insufficient pigs), EPI P+E (Exocrine pancreatic insufficient pigs supplemented with enzymes) and contol pigs (Healthy P). Values presented as P_{max} (mean ± SEM)

was more than 10 times higher in healthy pigs, compared to that observed in the EPI and EPI+E pig groups ($p<0.05$). Lipase activity was significantly higher in animals from

the Healthy Group than in EPI pigs (Tab. 2). The activity of enzymes measured in the ileum was significantly smaller. However, a general increase in ileal enzyme activity was observed after 120 minutes following feeding (Fig. 1–3).

Enzyme activity in EPI pigs. Endogenous amylase in the stomach (P_{max} 8.74 kU/mL), duodenum (P_{max} 6.87 kU/mL) and ileum (P_{max} 5.56 kU/mL) of EPI pigs was low, as expected (Fig. 1). Similar results were obtained for protease activity in the stomach (P_{max} 6.37 kU/mL), duodenum (P_{max} 7.84 kU/mL) and ileum (P_{max} 12.95 kU/mL) (Fig. 3). Changes in the dynamic of amylase and protease activity, within the time elapsed between digesta sample collection from the various sections of the GIT, were not significant. Stomach (P_{max} 74.38 kU.mL) and duodenal (P_{max} 79.11 kU.mL) lipase activity measured after feeding was much higher than before feeding (Fig. 2).

Activity of PLEM in EPI+E pigs. PLEM were recovered in the collected digesta samples. Also, the amplitude of the PLEM activity varied between different scenarios of enzyme administration.

Amylase activity. The highest activity of amylase in the stomach samples of EPI+E pigs was detected when PLEM was orally administrated before feed, P_{max} 80.95 kU/mL (Fig. 1), which was significantly higher than that observed in EPI pigs, and more than twice as high as that observed following the other routes of enzyme administration. In the duodenum, the highest amylase activity was measured when enzymes were administered via the gastric tube, before the feed, P_{max} 86.96 kU/mL ($p<0.05$) (Fig. 1).

Lipase activity. Exogenous lipase activity in the stomach was similar in EPI+E group of pigs when enzymes were administered before and during feeding, which was significantly higher ($p<0.05$) in comparison to EPI group and higher when PLEM was administered before feeding (P_{max}381.85 kU/mL). In contrary, the highest lipase activity in the duodenum was obtained when enzymes were administered during feeding (P_{max}421,6 kU/mL, $p<0.05$) (Fig. 2).

No significant differences in lipase activity in the digesta samples collected from the ileum were observed between the different routes of PLEM administration, or between the EPI and control pigs (Healthy Group).

Protease activity. Microbial protease activity measured in the stomach was significantly higher in EPI+E pigs than in EPI and Healthy Group of pigs after the PLEM was administered orally, during feeding ($p<0.05$) (Fig. 3). The highest exogenous protease activity in the duodenal samples was observed following the administration of PLEM to the stomach, before feeding (P_{max} 79.3 kU/mL, $p<0.05$). No significant differences of ileal protease activity in the digesta samples were observed between the various pig groups. However, the protease activity measured 5 hours following PLEM administration was more than twice as high than that observed in the first collected samples.

pH of digested samples. The mean pH values of the stomach samples collected from the Healthy Group and EPI pigs were similar (3.34±1.47 and 3.23±1.35) and no significant differences were observed. However, the pH measured in samples collected from Healthy Group was generally higher than that observed in EPI and EPI+E groups. The mean pH of duodenal samples from healthy pigs was higher (6.5±1.0) that that observed in EPI (6.1±1.3) and EPI+E (5.7±1.1) pigs. Following an overnight fast, the mean pH of the samples collected from the stomach of EPI pigs was lower (1.5±0.5) than that observed in the other pig groups (Healthy 2.1±0.9 and EPI+E pigs 2.2±0.8). The mean pH values of samples collected from the ileum did not differ significantly between the experimental groups.

DISCUSSION

Adequate enzyme substitution allows for the appropriate digestion and absorption of feed, including essential nutrients and fat-soluble vitamins. The improved utilization of nutrients and the right choice of enzyme supplementation are important for people with EPI and other digestive enzyme impairment-related syndromes [20]. Microbial enzymes can be used in human therapy in place of the porcine/bovine derived enzymes, specifically in young patients, as this allows for the pill burden to be substantially lowered.

Pancreatic enzymes are naturally produced in inactive forms which, among others, are activated in the presence of bile salts, enterokinase and narrow optimal pH, e.g. pancreatic enzymes are irreversibly deactivated in low stomach pH. Enzymes of microbial origin used in the presented study have a wide pH range in which they are active and require no activation within the recipient. It has been shown that PLEM are already active in the stomach that consecutively prolongs the time available for effective digestion. However, it raised the question whether such activity is harmful for the stomach mucosa. On the other hand, the gastric mucosa secretes its own active enzymes, e.g. lipase and pepsins. Moreover, PLEM remain active in the ileum for up to 5–6 h after administration, and active residues were found in faecal samples up to 24 h following administration (data not shown). The pattern of enzyme activity is closely associated with type of feed and intestine transit time. Intestinal muscular contractions shift chyme, water and enzymes along the GIT, so the enzyme activity is found deeper within the intestine as time passes from consumption [21]. In a study on rats conducted by Owyang et al. (1986), it was shown that after PDL surgery the intestine transit time can be altered [22]. This has not been previously confirmed in the EPI pig model, for which similar enzymatic activity time patterns were observed in both EPI and healthy pigs.

In the presented study, different scenarios of time and route of PLEM administration were investigated. The presence of digestive enzymes within the gut lumen prior to the arrival of the ingested feed bolus is essential in order to ensure the highest level of enzyme activity. This mirrors the natural condition in which enzymes are released after the cephalic phase of pancreatic enzyme stimulation [23]. The highest activity level for all microbial enzymes was measured immediately after their administration in the duodenum. This was consistent even when enzymes were given directly into the stomach and exposed to its low pH (range 1.45–2.86).

The adaptive ability of the GIT was observed when pH was closely investigated. Gradual increases in the pH of the duodenum content of EPI pigs indicates that single doses of PLEM altered some GIT parameters, or caused a possible

adaptation to impaired digestion. It has been speculated that in EPI pigs after pancreatic duct ligation, duodenal pH decreases to that observed in the stomach contents, which has not been confirmed to-date [24]. The pH of the GIT chyme in the EPI pigs increased in the time frame between the first and the last enzyme dosing. Further long-term PLEM substitution studies are required to confirm whether enzyme substitution may restore the natural pH in the GIT.

Konturek et al. showed that only 1/10 of the total enzymes produced by the pancreas is required to digest all the essential nutrients from diet [25]. In the current study, the P_{max} values of enzymes in the EPI+E groups were lower than that observed in control animals (Healthy Group). Even though the dose of PLEM administered to the EPI pigs was significantly lower than the amount of endogenously secreted pancreatic enzymes in control pigs, it was shown that PLEM activity pattern from the stomach through to the lower ileum complied with activity of pancreatic enzymes in healthy pigs. Also, Carriere et al. [26] suggested that gastric lipase cannot replace the pancreatic lipase under conditions of pancreatic insufficiency. However, the presented study shows that the gastric lipase activity in EPI pigs before enzyme administration was higher than that observed in healthy pigs. Moreover, gastric lipase remained active in both the duodenum and the ileum on a similar level in the upper and lower GIT in EPI pigs.

In vitro tested proteases derived from *Nocardiopsis prasina* and *Bacillus subtilis* display higher effectiveness compared to the Pancreatin (porcine pancreatic enzyme) in the digestion of soya and casein proteins (data not shown). According to Sikkens *et al*., the microbial proteases might serve as appropriate substitutions in the enzymatic treatment of pancreatic insufficiency-related cases [27]. The effectiveness of microbial pancreatic-like enzymes has also been proved in *in vivo* studies. Authors of the present study previously have shown that after 3 consecutive weeks of enzyme replacement therapy, EPI pigs became more active, and the EPI-related symptoms including arrested growth and steatorrhea were reversed and EPI pigs resembled controls [18, 28].

To summarize, the administered enzyme dosage, consisting of 60 kU amylase, 520 kU of lipase and 400 kU protease, remained active in the GIT with maximal recovery in the duodenum, and closely mimicked activity of endogenously-secreted pancreatic enzymes. Amylase recovery was very close to 100%. Lipase and protease recovery reached over 80% and 20%, respectively. Interestingly, trypsin activity was measured using a trypsin-specific method in the chime along the entire length of the gut, including stomachs of EPI + E pigs following PLEM treatment [19]. However, during the *in vitro* tests of PLEM used in the trial, the protease activity lacked any trypsin-like activity (data not shown). Also, the chyme from EPI pigs not treated with PLEM did not show trypsin-like activity in different trypsin-like activity assay (data not shown). These observations require further investigation; however, they indicate the potential existence of a mechanism responsible for switching-on the trypsin-like activity in the GIT environment of protease originally not attributed with such activity. However, it is more likely that PLEM treatment stimulates the production of proteinase in the GIT mucosa exhibit trypsin-like activity.

Previous studies have shown that the gastro-intestinal tract is highly responsive to various conditions and different feed compositions [29]. Slupecka et al. [30] showed a significant increase in the length and thickness of the intestine in young, suckling piglets receiving additional exogenous digestive enzymes. Since the intestine is one of the largest organs in the body, it is obvious that it has significant importance, and apparent changes in this organ have a large influence on other systems and organs.

PLEM are a sufficient source of digestive enzymes and can be used in therapy as an effective alternative for treatment of pancreatic insufficiency in adults and children with EPI. Moreover, PLEM are stable in the GIT compartment; however, given with a meal they exhibit the best activity and imitate the activity pattern of pancreatic enzymes secreted in healthy pigs, although with the somewhat controversial advantage that their potential is shown already in the stomach.

Acknowledgments

This work was supported by grants from the Director Albert Påhlsson Foundation in Malmö and Anara AB, Sweden.

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