Analytical techniques and formulation strategies for the therapeutic protein alkaline phosphatase

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Chapter 7

Investigations into the stabilization of drugs by sugar glasses: Delivery of an inulin-stabilized alkaline phosphatase in the intestinal lumen via the oral route

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Summary

In this study the possibility to deliver the acid sensitive enzyme alkaline phosphatase (AP) from calf intestine (CIAP) to the intestinal system by oral administration was investigated. Tablets were prepared and *in vitro* evaluated. Final proof of concept studies were performed in rats. This acid labile enzyme is potentially useful in the treatment of sepsis, a serious condition during which endotoxins can migrate into the blood stream. The CIAP was freeze-dried with inulin and subsequently compacted into round biconvex tablets with a diameter of 4 mm and a weight of 25-30 mg per tablet. The tablets were coated with an enteric coating in order to ensure their survival in the stomach.

In vitro evaluation of tablets containing alkaline phosphatase from bovine intestine (BIAP) was the first step in the development. It was found that tablets without enteric coating dissolved rapidly in 0.10 M HCl with total loss of enzymatic activity of the alkaline phosphatase. Tablets that were coated were stable for at least two hours in 0.10 M HCl, but dissolved rapidly when the pH was increased to 6.8. Furthermore, it was shown that the enzymatic activity of the released BIAP was fully preserved.

The *in vivo* test clearly showed that the oral administration of enteric coated tablets resulted in the release of enzymatically active CIAP in the intestinal lumen of rats. The location of the enhanced enzymatic activity of AP in the intestines varied with the time that had passed between the administration of the tablets and the sacrificing of the rats. Also, the level of enzymatic activity increased with an increasing number of tablets that were administered.

7.1 Introduction

Pharmaceutically active proteins have been applied for decades. However, not many therapeutic proteins were available until the 1980s. Since that time rapid developments in molecular biology resulted in a fast increase in the number of such proteins. Currently, the FDA has approved over thirty different recombinant DNA-derived proteins, e.g., erythropoietin, interferon alpha-2a/b, somatropin, and follitropin beta and many more are already in a far stage of development. This fast growth calls for the development of formulations that provide stability of the drug during manufacturing and subsequent storage and that also provide patient friendly administration.

Proteins are usually administered by subcutaneous injection, e.g., insulin and recombinant human growth hormone. The reason for this is the poor bioavailability of such drugs after oral administration [1]. However, if the therapeutic protein has its target in the intestinal tract it would be advantageous if it could be administered orally. One way to achieve this is to freeze-dry a solution of the protein with a sugar and then compact the product into tablets. Inclusion of the protein in the sugar glass protects it from degradation during further processing (e.g., compaction) and storage. Furthermore, the tablets need to be covered with an enteric coating in order to protect the active substance from the acidic environment in the stomach with its high enzymatic activity.

In previous studies it has been found that the disaccharide trehalose is a good stabilizer for proteins during freeze-drying and subsequent storage [2-9]. Recently, we showed that inulin, an oligosaccharide, is a good alternative for trehalose [10].

It was shown that inulin-stabilized protein could be processed into tablets of sufficient mechanical strength and low friability without any loss of enzymatic activity of the incorporated protein [11]. Furthermore, it was found that both the stabilizing capacity and the compaction behaviour of inulin were superior to trehalose.

In previous studies it was found that alkaline phosphatase (AP) is a highly promising therapeutic agent for the treatment of sepsis, which is caused by endotoxins produced by Gram-negative bacteria [12, 13]. Endotoxins can be detoxified by AP by the removal of a phosphate group from the lipid A moiety of the lipopolysaccharides. In case of sepsis the intestinal wall becomes more permeable, which leads to the translocation of

endotoxins from the intestinal lumen into the blood stream. This may further deteriorate the condition of the patient. Increasing the local AP activity may therefore be highly relevant to detoxify luminal endotoxin and prevent their translocation into the bloodstream. Also when a patient is already suffering from sepsis, the oral delivery of AP may still be quite relevant. Even when no endotoxin is present in the blood stream the presence of cytokines in the blood leads to increased vascular permeability in the intestinal wall, which ultimately might lead to a second wave of endotoxins entering the bloodstream. Moreover, reperfusion ischemia induced by the septic shock may damage the intestinal wall too, again leading to leakage of macromolecules from the intestinal lumen into the blood. This might be prevented by the administration of AP to the intestines.

In this study the possibility to deliver AP to the intestinal lumen via the oral route was investigated by the combination of a number of well-established techniques, such as freeze-drying, compaction and application of enteric coating.

7.2 Experimental

Materials

Inulin with a number/weight average degree of polymerization (DP_n/DP_w) of 23/26 was a gift from Sensus (Rosendaal, The Netherlands), alkaline phosphatase from calf intestine (CIAP) with a specific activity of 5937 U/mg was purchased from PharmAAware (Bunnik, The Netherlands). Alkaline phosphatase from bovine intestine (BIAP), para-nitrophenylphosphate (pNPP) and 2-amino-2-methyl-1,3-propanediol (ammediol) were purchased from Sigma-Aldrich (Steinheim, Germany). Triethyl citrate (citroflex) was from Fluka (Buchs, Switzerland). Sodium potassium tartrate and Folin reagent were purchased from Merck (Darmstadt, Germany). Copper sulfate was from Genfarma BV (Maarssen, The Netherlands) and bovine serum albumin (BSA) was from ICN Biochemicals (Aurora, Ohio, USA). Talc was from Genfarma (Zaandam, The Netherlands). Silicon antifoam suspension was from Boom (Meppel, The Netherlands). Eudragit L100-55 was from Röhm (Darmstadt, Germany). A Slide-A-Lyzer with a molecular weight cut off at Mw=3500 was from Pierce (Rockford, IL, USA). Sodium starch glycolate USP-NF

(Primojel) was from AVEBE (Veendam, The Netherlands). All other chemicals were purchased from commercial suppliers.

Dialysis

The CIAP was retrieved as a solution that contained glycerol. Since this substance strongly depresses the glass transition temperature of inulin it was removed by dialysis. A Slide-A-Lyzer (molecular weight cut-off: MW 3500) was filled with the sample solution (1 ml). Subsequently, dialysis was performed against 1 L of 5 mM Tris, 5 mM $MgSO_4$, pH 8.6 at 4 °C for two days, during which the dialysis medium was refreshed twice. The dialyzed sample was then transferred to an Eppendorf tube and stored in a refrigerator at 4 °C until use.

Determination of protein content

After dialysis the total protein concentration of the sample was determined according to the method of Lowry [14]. A calibration curve was produced in the concentration range 0-50 µg/ml using BSA. Solution D was prepared by mixing 9.6 ml of solution A (8 mg/ml sodium hydroxide and 40 mg/ml sodium carbonate in water) with 0.2 ml of solution B (10 mg/ml copper sulfate in water) and 0.2 ml of solution C (20 mg/ml sodium potassium tartrate in water). To 500 µl of sample, 500 µl of solution D was added. After 10 minutes 100 µl of Folin reagent (diluted 1:1 with water) was added to the mixture. The samples were then stored for 1 h in darkness. Subsequently, the absorbance of the samples at 700 nm was measured using a Philips UV 2100 spectrophotometer (Eindhoven, The Netherlands).

Activity assay of alkaline phosphatase

The activity of BIAP was determined by following the enzymatic conversion of the substrate pNPP to para-nitrophenol according to a previously published method [10]. A standard curve was prepared by measuring the activity of BIAP in the range 0 to 50 µg/ml in 0.05 M ammediol buffer pH 9.8. For the samples of the *in vitro* dissolution test the standard curve was prepared using the dissolution testing medium as solvent. The activity of the CIAP was determined using the same assay, but the activity was expressed in units (U): 1 U corresponds to the conversion of 1 µmol of substrate per min at 37 °C. In these calculations the molar absorption coefficient of the product (4-nitrophenol) at 405 nm was taken 18 450 L/mol·cm [15].

Freeze-drying

Solutions of 10% w/v of inulin, 10% w/v inulin/BIAP 19/1 w/w and 10% w/v inulin/CIAP (approximately 110 units/mg inulin), all in 0.05 M ammediol pH 9.8, were freeze-dried as described previously [11].

Tablet production

Freeze-dried material (inulin or AP/inulin) was ground to a fine powder and stored at 20 °C/0% relative humidity (RH) for at least 3 days. Subsequently, the material was stored at 20 °C/45% RH for at least 2 days. If primojel (5% w/w) was incorporated in the tablets, this was mixed with pestle and mortar followed by mixing with a Turbula mixer (Willy A. Bachofen AG Maschinenfabrik, Basel, Switzerland) for 30 min.

Round biconvex tablets with a diameter of 4 mm and a weight of about 25-30 mg per tablet were compacted. An automated hydraulic press from ESH Hydro Mooi (Appingedam, The Netherlands) was used to employ a compaction pressure of about 110 MPa. After compaction the tablets were stored in the vacuum desiccator at room temperature. The compaction behaviour of freeze-dried inulin has been described in an earlier publication [11].

Coating procedure

Tablets were coated with poly(methacrylic acid-co-methyl-methacrylate) (Eudragit® L100-55). The acidic groups are protonated at acidic pH, which leads to insolubility of the material. When the pH is increased the solubility increases due to deprotonation of the polymer. A 30% w/w suspension of Eudragit L100-55 was prepared in 0.14 M NaOH. 2.5 g of this suspension was mixed with isopropanol (2 g), talc (0.375 g), citroflex (0.075 g) and silicon antifoam (0.05 g). This suspension was used for the application of the first coating layer, which was performed by adding small drops (ca. 10 µl) of the liquid to each tablet. Each individual tablet was then rolled under a stream of warm air until the isopropanol had evaporated. Following the first layer, nine additional layers of coating were applied to a total coating weight of about 7 mg. The suspension for these layers was made in a similar way, except that water instead of isopropanol was used as solvent. After the coating procedure was complete the tablets were stored in a vacuum desiccator at room temperature until used for other experiments.

In vitro dissolution of tablets

The *in vitro* tests were performed in dissolution testing baths from Prolabo (Rowa Techniek B.V., Leiderdorp, The Netherlands). The dissolution behaviour of the tablets was evaluated first in 750 ml 0.10 M HCl at 37 °C and a rotation speed of 100 rpm for 120 min to test the acid resistance of the tablets. Subsequently, 250 ml 0.20 M Na_3PO_4 (preheated to a temperature of 37 °C) was added to the dissolution vessel to increase the pH to 6.8 (tablets without coating were only exposed to the acid stage). Throughout the test, 1.0 ml samples were taken at different time intervals. Each experiment was performed in triplicate. Immediately after being taken samples for the determination of enzymatic activity were diluted to 10 ml in 0.05 M ammediol pH 9.8 in order to stop the acid induced degradation.

The amount of dissolved inulin was determined by using the anthron reaction [16]. To 1.0 ml of standard or sample 2.0 ml of a solution of anthron in concentrated sulfuric acid (0.10% w/w) was added. Immediately after addition of the anthron solution the samples were mixed using a vortex. After 10 minutes the samples were placed in a water bath of 20 °C to cool the samples to room temperature. The absorbance at 625 nm of the samples was then measured using a Philips UV 2100 spectrophotometer (Eindhoven, The Netherlands).

In vivo test

Oral administration of CIAP tablets to rats

Animal experiments were conducted according to the guidelines provided by the Dutch Animal Protection Act, and were approved by the Committee for Animal Experimentation (DEC) of the University of Groningen. For all experiments male Male Wistar rats, 190 – 200 g on arrival were allowed to adapt for 1 week. During the experiment, i.e. from the time point of administration of the tablet(s) until sacrifice, rats were refrained from food. In the case of the 7.5 and 12.5 h time-interval studies, rats were also fasting during the night period prior to the experiment. Just prior to the administration of the tablets, rats were anaesthetized with isofluran/O_2/N_2O and the tablet(s) were placed at the back of the throat by a pair of tweezers and gently forced into the upper part of the esophagus by a bent, blunt probe. During recovery from anaesthesia, rats were stimulated to swallow by gentle throat massage.

Determination of the AP activity in the intestines

After a fixed time rats were again anaesthetized with isofluran/ O_2/N_2O and sacrificed. The stomach and intestine without the cecum were removed and faeces, if produced during the observation period, was collected. The small intestine (duodenum plus ileum) was cut in six equal fragments of about 150 mm whereas the large intestine (colon plus rectum) was analyzed as one fragment of about 80 mm to obtain a profile of AP activity from the whole intestine. Fragments (longitudinally cut open) and faeces were incubated for 1 h at 4 °C in a volume of 2 ml of 50 mM Tris/HCl pH 7.8 on a Denley 5 spiramix. After incubation, samples were vortexed briefly and centrifuged for 30 s to spin down large fragments and debris. The supernatant was then diluted 10-fold and assayed for alkaline phosphate activity as described above.

7.3 Results and discussion

Effect of dialysis and freeze-drying on the activity of calf intestinal alkaline phosphatase

In Table I the influence of various processes on the activity of BIAP incorporated in inulin sugar glass is given. As can be seen, the activity is not significantly affected by any of the processes. In Table II the activity of the CIAP before and after dialysis is given. The dialysis had no detrimental effect on the CIAP. It even seems that the specific activity increased after dialysis, but this is probably explained by the precision of the analytical methods used to determine the protein content and enzymatic activity. Also in Table II, the activity of the dialyzed CIAP incorporated in inulin sugar glass before and after compaction is given.

Table I. *Remaining relative enzymatic activity of BIAP incorporated in inulin sugar glass after various processes.*

Freeze-drying (%)	Grinding (%)	Pre-conditioning (%)	Compaction (%)	Coating (%)
108.9±0.4	110.3±0.3	103.8±1.1	99.5±2.8	106.6±2.3

Table II. *Remaining enzymatic activity of CIAP (*incorporated in inulin sugar glass) after various processes.*

Process step	Protein content	Activity	Specific activity
Before dialysis	5.59 mg/ml	33186 U/ml	5937 U/mg
After dialysis	5.34 mg/ml	32949 U/ml	6170 U/mg
*After freeze-drying	1.74 %w/w	47.3 U/mg product	2720 U/mg
*In tablets	1.74 %w/w	1276 U/tablet	2720 U/mg

As can be seen, only about 45% of the activity remains after the freeze-drying process. In previous studies this considerable loss of activity because of freeze-drying of AP with inulin has not been observed [10, 11]. However, in those studies rather impure BIAP was used whereas in the present study highly purified CIAP was employed. It is quite possible that the impurities present in that BIAP-sample also contributed to the stabilization of the protein during freeze-drying. It was for instance found by Ford and Allahiary that AP that was freeze-dried with serum albumin had 70% activity left after the process, while AP that was freeze-dried without albumin only had 5% activity remaining [17]. In a study by Millqvist-Fureby *et al*., it was shown that the activity of pepsin after freeze-drying was better preserved when the concentration of the enzyme was increased [18]. A similar trend was also found earlier by Izutsu *et al*., who found that for β-galactosidase the activity loss was smaller when the enzyme was freeze-dried from more concentrated enzyme solutions [19].

Dissolution of tablets

Since the highly purified CIAP was only available in limited quantities the initial tests were performed using tablets containing the readily available BIAP. The dissolution behaviour of non-coated tablets made of freeze-dried inulin/BIAP (19/1 w/w) was investigated as described above in 0.10 M HCl to simulate the stomach. The tablets dissolved completely within 1 h as shown in Fig. 1. However, no enzymatic activity of BIAP was found in any of the samples, which was expected since alkaline phosphatase rapidly loses its activity below pH 3.5 [20-22]. This result also demonstrates the need for a protective coating of the tablets that ensures their resistance against the acidic environment of the stomach.

Tablets prepared from freeze-dried inulin that were provided with an enteric coating did not dissolve during the 2-h exposure to 0.10 M HCl, but when the pH was increased to 6.8, which is the pH of the intestines, the

tablets immediately started dissolving. Dissolution, as measured from the inulin concentrations, was complete within 1 h (Fig. 1).

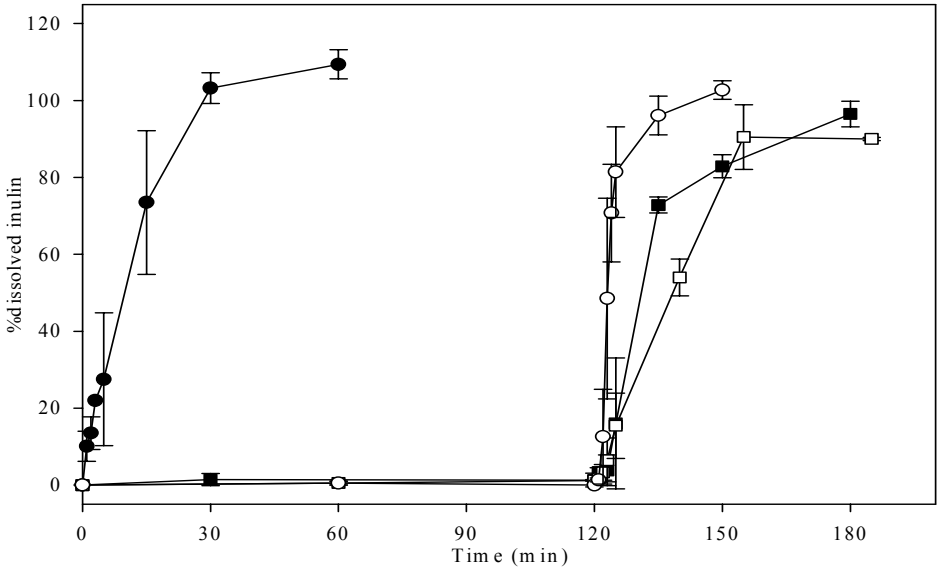

Fig. 1 *In-vitro dissolution testing of tablets measured as dissolved inulin. (•) inulin/BIAP (19/1 w/w), non-coated; (■) inulin, coated; (□) inulin/BIAP (19/1 w/w), coated; (○) inulin/primojel (95/5 w/w), coated. The pH was increased to 6.8 after2 h.*

The dissolution profile of tablets containing BIAP was similar to the dissolution profile of tablets that did not contain BIAP (Fig. 1). The protective function of the coating was also demonstrated by the fact that the activity of the BIAP was fully maintained, which shows that this approach enables the oral delivery of AP to the intestines. The dissolution curve of enzymatically active BIAP is shown in Fig. 2. It is clear that the release of BIAP matches the release of sugar from the tablets. The dissolution profile of the tablets containing the disintegrant primojel is given in Fig. 1. As expected, the addition of this substance increased the dissolution rate of the inulin tablets. The tablet dissolved within 15 min after the pH of the dissolution medium was increased to 6.8. Since the tablets that did not contain any primojel already had a satisfactory dissolution profile a decision was made not to use primojel for the *in vivo* studies.

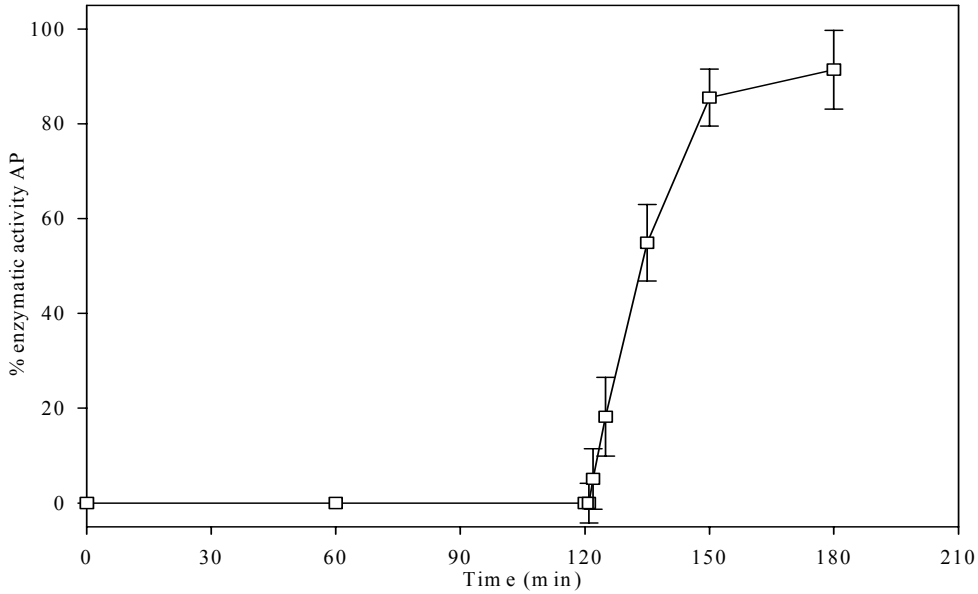

Fig. 2 *Amount of enzymatic activity that was found in the dissolution test of inulin/BIAP (19/1 w/w), coated tablets. The pH was increased to 6.8 after 2 h. 100% activity is defined as the level of activity that is expected based on the amount of BIAP present in the tablet.*

From these tests it was concluded that the application of the enteric coating provided the necessary protection of the alkaline phosphatase from the acidic environment in the stomach. During the 2-h exposure to acidic pH no inulin could be detected in the 0.10 M HCl. Moreover, the activity of the BIAP was fully maintained, confirming that no leakage of the medium into the tablet occurred. With these results in mind, tablets that contained CIAP were prepared for use in the *in vivo* experiments.

Administration of inulin/CIAP tablets to rats

Since the concentration of AP is already quite high in the intestines a very high dosis of AP has to be administered. Therefore, tablets containing highly purified and therefore highly active AP had to be prepared. AP with the purity and activity necessary was only available in limited amounts, which means that only a limited number of experiments could be performed. With the experiments that were performed at least a proof of concept study could be performed. The results of the *in vivo* tests are given in Fig. 3. After administration of a placebo tablet to the rat only the endogenous activity of alkaline phosphatase is found in samples from intestinal segments (Fig. 3a). The differences in AP activity in the different

samples reflect the natural profile in the intestine. First, the effect of time between the administration of the tablets and the sacrifying of the rats was investigated. After 7.5 h a considerable increase in enzymatic activity was found in the first fragment of the small intestine (Fig. 3b), and after 12.5 h (Fig. 3c) this increase was evident in the last fragment of the small intestine but, most strikingly, the enzymatic activity was very large in the faeces that had been produced by this rat. This was the only rat that produced faeces.

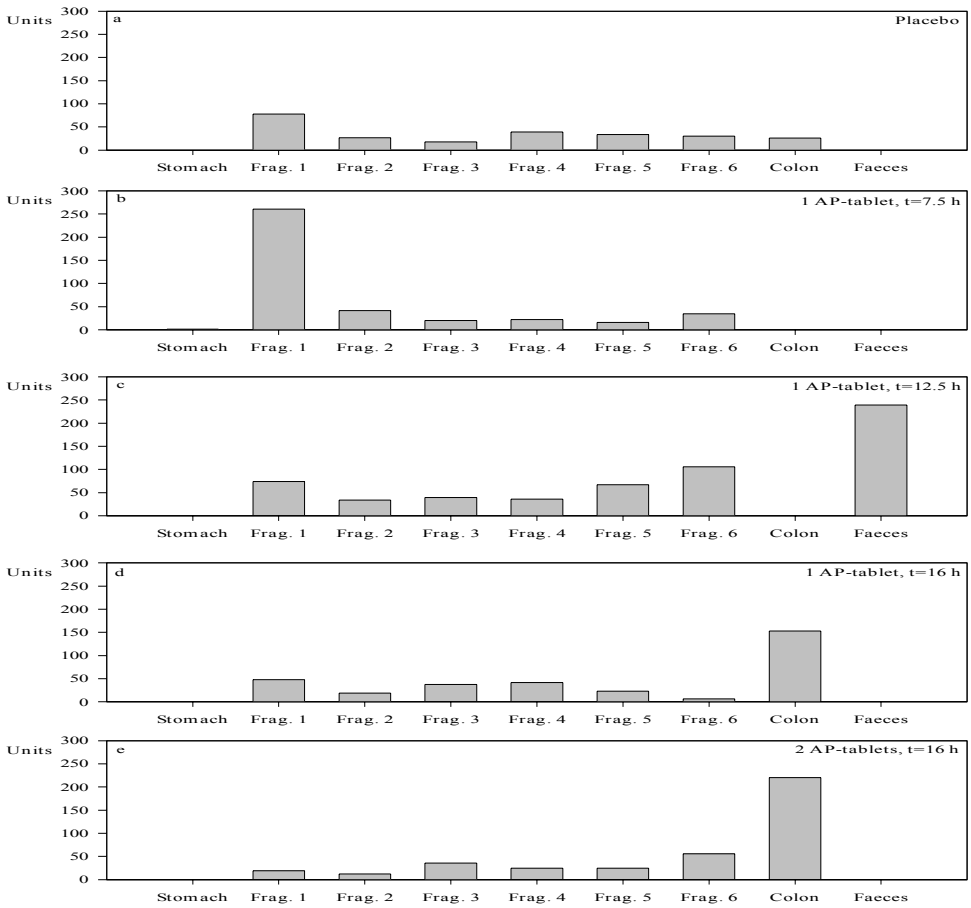

Fig. 3 *Results of the in-vivo tests. The enzymatic activity found after administration of: (a) the placebo tablet, rat sacrificed after 16 h; (b) one tablet with CIAP, rat sacrificed after 7.5 h; (c) one tablet with CIAP, rat sacrificed after 12.5 h; (d) one tablet with CIAP, rat sacrificed after 16 h; (e) two tablets with CIAP, rat sacrificed after 16 h.*

With a time-interval of 16 h between administration of tablets and sacrifice (Fig. 3d), all the increase in enzymatic activity was found in the colon.

Finally, the effect of two tablets on local AP activity in the intestine was examined, 16 h after oral application of the tablets. In this case the increase in enzymatic activity was also found in the last fragment of the small intestine and in the colon (Fig. 3e). It was also found that the increase was considerably higher compared to the one tablet administration (*cf.* Fig. 3d).

Even though each tablet contained approximately 1200-1300 units of CIAP only about one third of this was found in the enzymatic activity assays. This can be explained by the presence of trypsin and other digestive enzymes in the intestine, which most likely digested a substantial part of the administered AP. It is also possible that part of the active site of the AP was occupied by free inorganic phosphate and/or endotoxins that inevitably are present in the samples. These substances are known to attenuate the AP activity. In addition, phosphorylcholine, which also has been reported to act as a susbstrate for intestinal alkaline phosphatase [23], might have influenced the enzymatic activity of AP. These results indicate that if substantially less than 1200 units of CIAP is administered it is likely that no activity at all will be found back in the enzymatic activity assay.

7.4 Conclusions

Even though each technique used in this study is not new the combination of them is. The present study describes a combination of *in vitro* and *in vivo* studies, although the latter only provides a proof of concept. For the *in vivo* studies very pure enzyme preparations with very high specific activity was needed. These pure enzyme preparations can only be obtained in low yield, thus limiting the number of *in vivo* experiments.

It was shown that the inclusion in a sugar glass by freeze-drying, compaction to tablets and enteric coating, enable the intestinal delivery of inulin-stabilized AP to rats via the oral route. The successful delivery of this highly acid-sensitive enzyme opens the possibility to deliver other therapeutically interesting proteins to the intestines, where a local therapeutical effect can be achieved.

7.5 Acknowledgements

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7.6 References

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