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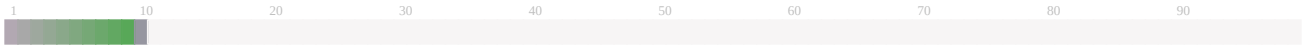

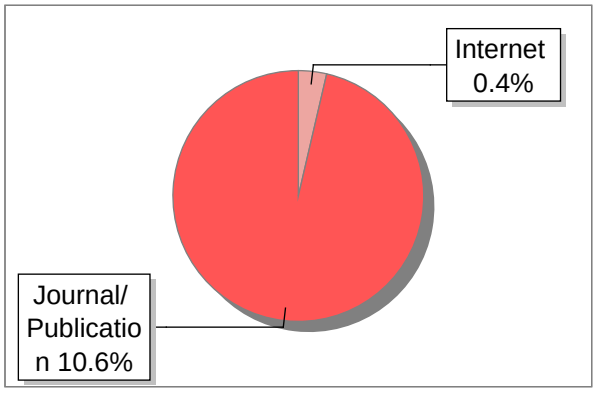

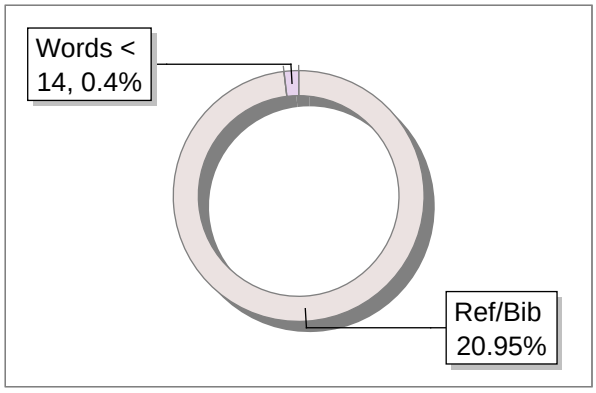

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Full length article

Mucus permeating self-emulsifying drug delivery systems (SEDDS): About the impact of mucolytic enzymes

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ABSTRACT

This study aimed to improve the mucus permeating properties of self-emulsifying drug delivery systems (SEDDS) by anchoring lipidized bromelain, papain and trypsin using palmitoyl chloride. SEDDS containing enzyme-palmitate conjugates were characterized regarding droplet size and zeta potential. Their mucus permeating properties were evaluated by Transwell diffusion and rotating tube method using fluorescein diacetate (FDA) as marker. Degree of substitution of modified enzymes was 35.3%, 47.8% and 38.5% for bromelain-palmitate, papain-palmitate and trypsin-palmitate, respectively. SEDDS as control and SEDDS containing enzyme-palmitate conjugates displayed a droplet size less than 50 nm and 180–312 nm as well as a zeta potential of −3 to −4 and −4 to −5 mV, respectively. The highest percentage of permeation was achieved by introducing 5% papain-palmitate into SEDDS. It could enhance the mucus permeation of SEDDS in porcine intestinal mucus 4.6-fold and 2-fold as evaluated by Transwell diffusion and rotating tube method, respectively. It is concluded that mucus permeation of SEDDS can be strongly improved by incorporation of enzyme-palmitate conjugates.

1. Introduction

Self-emulsifying drug delivery systems (SEDDS) are promising formulations for improving oral bioavailability of drugs. These formulations consist of oils, surfactants and co-solvents spontaneously dispersing in gastrointestinal medium forming oil-in-water emulsions. SEDDS show high potential as carriers for lipophilic, poorly soluble drugs [1] and even hydrophilic drugs that need to be protected towards the gastrointestinal environment [2]. SEDDS can also improve the permeation behavior of drugs across the mucus due to their ability to avoid interactions between drug molecules and mucin glycoproteins [3].

Mucus mainly consists of water and mucus glycoprotein, so-called mucins. This material has sticky and viscoelastic properties providing protection for the underlying epithelium [4]. The molecular weight and protein structure of mucus glycoproteins are mainly responsible for the viscous properties of mucus. A decrease in viscosity of mucus can be achieved by breakage the disulfide bonds converting glycoproteins from tertiary structure to secondary one or by reducing their length via proteolytic cleavage [5,6]. Decreasing mucus viscosity by reducing glycoprotein chain length can be achieved by utilizing proteolytic enzymes such as papain, bromelain and trypsin as shown in previous research [7]. Anchoring proteolytic enzymes on the surface of SEDDS droplets might therefore increase their mucus permeability.

It was therefore the aim of this study to develop a novel drug delivery system which can surmount the intestinal mucus barrier by combining SEDDS with proteolytic enzymes. At present study, three kinds of enzymes were incorporated into SEDDS including bromelain, papain and trypsin. To be incorporated into SEDDS, enzymes were modified by acylation using palmitoyl chloride as acylating agent. The impact of resulting enzyme-palmitate conju-

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gates on the mucus permeation behavior of SEDDS was evaluated by Transwell diffusion and rotating tube method.

2. Materials and methods

2.1. Materials

Bromelain (from pineapple stem), papain (from Carica papaya), trypsin (from bovine pancreas), propylenglycol, Kollipor EL and fluoroscein diacetate (FDA) were obtained from Sigma Aldrich (Vienna, Austria). Captex 355 was obtained from ABITEC Corporation (Columbus, OH 43215 USA). Palmitoyl chloride from Acros Organics (New Jersey, USA) was used as acylating agent. Mucus was collected from porcine intestine which was obtained freshly from slaughter house, Innsbruck, Austria.

2.2. Methods

2.2.1. Synthesis of enzyme-palmitate conjugates

Enzyme-palmitate conjugates were synthesized according to a method described by Xue and co-workers [8]. Three kinds of enzymes including bromelain, papain and trypsin were used for this synthesis. Each enzyme was dissolved in 0.1 M phosphate buffer pH 8.0 at a concentration of 3 mg/ml using thermomixer. Palmitoyl chloride solution in acetone at a concentration of 100 mg/ml was added dropwise into enzyme solution at a volume ratio of 1:40. The pH was maintained at 8 by addition of 1 M NaOH. The reaction was conducted for 90 min at room temperature and produced suspension. Afterwards, the modified enzymes suspension was dialyzed against water for 24 h followed by lyophilization. Enzyme-palmitate conjugates were stored at 4 ◦C prior to use.

2.2.2. Characterization of enzyme-palmitate conjugates using FTIR and ninhydrin assay

Functional groups of acyl enzymes were determined using FTIR (PerkinElmer Spectrum 100 FTIR Spectrometers, Waltham, USA). Unscreambler X (CAMO Software AS, Oslo, Norway) was used as software to record IR spectrum of products.

Characterization of enzyme-palmitate conjugates using ninhydrin assay was conducted as follows. Each enzyme-palmitate conjugate solution in DMSO at a concentration of 10 mg/ml was diluted using deionized water to achieve standard solution at a concentration of 40, 400, 800 and 1500 μg/ml. Equal volume of 4 M acetic buffer pH 5.5 was augmented to the standard solution followed by addition of ninhydrin solution at twice volume prior to 15 min incubation at 90 ◦C for 15 min. Subsequently, absorbance at wavelength of 570 nm was recorded using microreader Tecan Infinite M 200. A linear regression of absorbance as a function of concentration was calculated. As control, ninhydrin assay was also performed for each native enzyme. Free amine fraction of enzyme-palmitate conjugates was calculated by dividing the linear regression slope of each enzyme-palmitate conjugate with slope of native enzymes [9], [10]. Degree of substitution (DS) of acyl enzymes was determined using Eq. (1).

$$DS = (1 - free amino group) x 100\% \quad (1)$$

2.2.3. Preparation and characterization of SEDDS

SEDDS consisted of Captex 355 as oil, Kollipor EL as surfactant and propylenglycol as co-solvent. This composition and preparation method were adapted from Efiana and co-workers with modification [11]. SEDDS were prepared at room temperature by mixing oil, co-solvent and surfactant at a weight ratio of 2:3:5 for 30 min using magnetic stirrer (Hotplate Stirrer Stuart) at the rate of 500 rpm. This mixture showed clear solution. SEDDS was dispersed in 0.1 M phosphate buffered saline (PBS) pH 6.8 with a volume ratio of 1:100 by stirring at 50 rpm prior to be evaluated regarding nanoemulsion droplet size and zeta potential (Nicomp™ 380 ZLS Santa Barbara California USA).

2.2.4. Preparation and characterization of SEDDS enzyme-palmitate conjugates

Each enzyme-palmitate conjugate was dispersed in oleic acid at a concentration of 10% (m/v). Subsequently, equal volume of enzyme-palmitate conjugates dispersion and SEDDS were mixed using vortex for 10 min followed by sonication for 6 h at room temperature using Bandelin Sonorex at a frequency of 35 kHz. Droplet size of SEDDS enzyme-palmitate conjugates was determined after dispersing in 0.1 M PBS pH 6.8 at a volume ratio of 1:100 immediately and at 4 h. Enzyme-palmitate conjugates SEDDS were stored in refrigerator at 4 ◦C.

2.2.5. Determination of enzyme-palmitate payload in SEDDS

Enzyme-SEDDS preconcentrate containing 5% of enzyme-palmitate conjugate was centrifuged at 12110 rcf. Afterwards, the supernatant was discarded and the precipitate containing un-dissolved enzyme-palmitate was washed using acetone. Subsequently, the precipitate was dissolved in DMSO prior to quantification using Bradford method. Bradford reagent consisted of coomassie brilliant blue G-250 at a concentration of 0.1 mg/ml in 5% ethanol and 8.5% phosphoric acid [12]. The amount of undissolved enzyme-palmitate was determined using a series of calibration curves. The payload of enzyme-palmitate in SEDDS was calculated by subtracting the amount of un-dissolved enzyme-palmitate in the preconcentrate from the total amount added.

2.2.6. Measurement of enzymes activity

Activity of enzymes and of enzyme-palmitate conjugates was determined by casein hydrolytic method [8]. Native enzymes as well as enzyme-palmitate conjugates were dissolved or dispersed in 50 mM phosphate buffer pH 8 containing 0.4 mM EDTA dehydrate and 1 mM L-cysteine at a concentration of 400 μg/ml. Equal volume of 20 mg/ml casein solution in 50 mM phosphate buffer pH 8 and twice volume of 10 mM PBS pH 8 were added prior to mixing in thermomixer at 37 ◦C for 30 min in rate of 750 rpm. Afterwards, trichloroacetic acid (TCA) was added at the final concentration of 2.9% followed by centrifugation at 12110 rcf for 10 min. SEDDS enzyme-palmitate conjugates dispersion in 50 mM phosphate buffer containing equal mass of enzyme-palmitate conjugates were processed as well. The concentration of tyrosine in the supernatant was detected using spectrophotometer at 280 nm wavelength. Standard curve of tyrosine was constructed to measure the mass of tyrosine produced from casein hydrolysis and the activity of enzymes was calculated using Eq. (2).

$$Enzyme\ activity\ (unit\ per\ mg\ enzyme) = \frac{mmol\ tyrosine\ released}{30\ minute.\ mg\ enzyme} \quad (2)$$

2.2.7. Preparation of intestinal porcine mucus

Intestinal porcine mucus was prepared as described previously [13]. Intestine was cut into short segments around 10 cm and cleaned from debris. Subsequently, the intestine was cut longitudinally and mucus was collected. Thereafter, 5 mL of sodium chloride 0.1 M was added into 1 g of mucus prior to stirring with mild agitation for 1 h. Centrifugation was conducted at 14556 rcf for 2 h at a temperature of 4 ◦C to separate the debris from mucus. Supernatant was discarded and mucus was retained. The refining process was repeated and the purified mucus was collected and stored in refrigerator at −20 ◦C prior to use.

The viscosity of purified mucus was determined and compared to the native one. It was confirmed that the viscosity of mucus with and without refining using NaCl are 15.1 4.5 Pa*s and 18.6 2.9

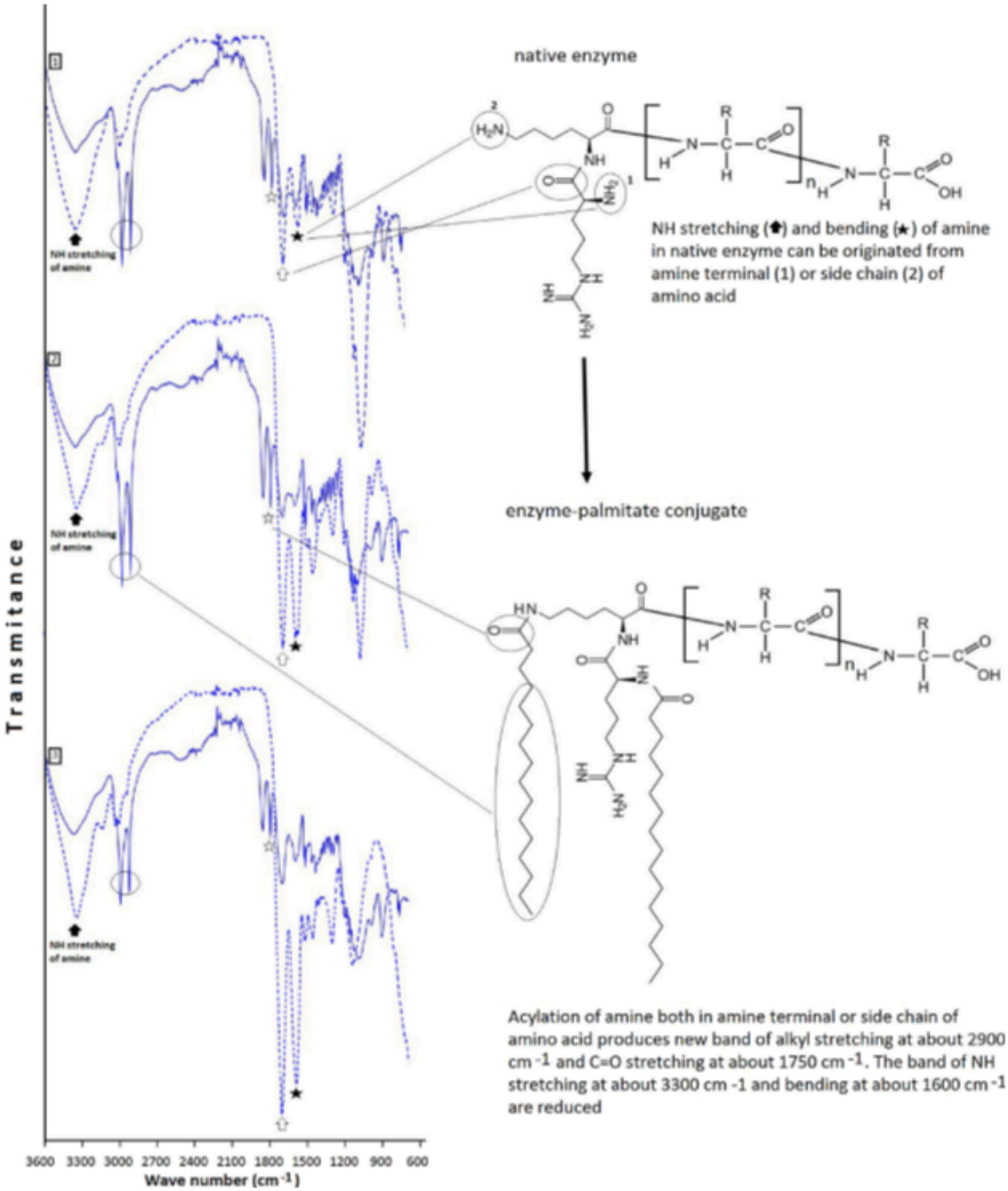

Fig. 1. FTIR spectrum of native enzymes (strip line) and enzyme-palmitate conjugates (bold line) of bromelain (1), papain (2) and trypsin (3). The functional groups presented by the peak are displayed bellow arrows or directed by curves connecting the peak to the functional group of the native enzyme as well as enzyme-palmitate conjugates.

Pa*s, respectively. The independent sample *t*-test showed that there is not any significant difference. It means that the refinement of mucus did not influence mucus viscosity.

2.2.8. Permeation study of SEDDS enzyme-palmitate conjugates

2.2.8.1. Labelling of SEDDS and SEDDS enzyme-palmitate conjugates using fluorescein diacetate (FDA). Prior to permeation study, SEDDS and SEDDS enzyme-palmitate conjugates were labelled with FDA at a final concentration of 1%. The process was performed using vortex for 5 min followed by sonication for 10 min and incubation for 20 min at the temperature of 25 ◦C.

2.2.8.2. Permeation study of SEDDS enzyme-palmitate conjugates using transwell diffusion method. Permeation study was performed using 24-well plates (Greiner-BioOne, Kremsm̈ unster, Upper Austria, Austria) [13,14]. SEDDS enzyme-palmitate conjugates as sample and SEDDS without enzymes as control were dispersed in 0.1 M PBS pH 6.8 at a ratio of 1:100. Prior to permeation process,

50 μL of mucus was filled in the Transwell and acceptor chamber was filled with 500 μL of 0.1 M PBS pH 6.8. The permeation was started by filling donor chamber with 250 μL of sample. The experiment was conducted on a shaking board (Vibramax 100; Heidolph Instruments, Schwabach, Bavaria, Germany) at 300 rpm in the incubator at 37 °C. About 100 μL of acceptor medium was withdrawn at 0, 1, 2, 3 and 4 h and replaced with 0.1 M PBS pH 6.8 at the same volume and temperature. To determine the amount of FDA in the sample, 20 μL of 5 M NaOH was added followed by incubation at 37 °C for 30 min. Afterwards, fluorescence was determined using microplate reader Tecan at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. As 100% control, the permeation process of each FDA labelled composition was also conducted without mucus. Percentage of FDA permeated was defined as percent ratio of FDA amount in acceptor compartment of sample treatment to 100% control at each sampling time.

2.2.8.3. Permeation study of enzyme-palmitate conjugates SEDDS using rotating tube method. Rotating tube is another way to describe the ability of SEDDS droplets to diffuse in the mucus [15,16]. Tube with a diameter of 4 mm and a length of 50 mm was filled with 300 μL of mucus and the end was covered with stopper. Subsequently, on the opposite end, 50 μL of sample were placed and also covered with stopper. SEDDS enzyme-palmitate conjugates as sample and SEDDS without enzymes as control were dispersed in 0.1 M PBS pH 6.8 at a ratio of 1:100. The experiment was conducted in the incubator at 37 °C by holding the tubes on the rotor with continuous horizontal rotation (50 rpm). After 4 h of experiment, tubes were taken from the rotor and frozen at −80 °C for 1 h. Subsequently, tubes were cut into 10 slices of 2 mm length, starting from the side which was filled with sample. FDA in the mucus of each tube slice was extracted by addition of 400 μL of 5 M sodium hydroxide and incubating at 37 °C for 30 min followed by centrifugation at 12110 rcf. The FDA concentration was determined using the same method applied in Transwell permeation above. As 100% control, initial amount of FDA in donor compartment was determined as well. Percentage of FDA permeated was calculated by percent ratio of FDA amount in each slice of mucus to 100% control.

2.3. Statistical data analysis

Independent sample *t*-test was used to analyze the differences of percentage of FDA permeated using Transwell diffusion method as well as rotating tube method between compositions including SEDDS and SEDDS enzyme-palmitate conjugates. All statistical analyses were performed using SPSS 17 at 95% confidence level ($p < 0.05$).

3. Results

3.1. Characterization of enzyme-palmitate conjugates using FTIR and ninhydrin assay

Acylation by following Schotten-Boumann reaction was successfully performed. The hydrogen of primary amino groups available at the N terminus as well as on lysine and arginine side chains of the protein can be substituted with the acyl group of acyl chloride [17]. Fig. 1 shows that the acyl group is covalently bound in the reaction product by the appearance of a CH stretching duplet band at a high intensity. The acylation is processed in protein primary amino groups as shown by reducing of its NH bending and stretching. Concur with FTIR spectrum the ability of enzyme-palmitate conjugates to interact with ninhydrin reagent was lower than in case of the native enzyme.

Based on the ninhydrin assay data, the degree of primary amino proton substitution with palmitoyl group could be calculated as shown in Table 1. In the present research, 35%-50% degree of acylation could be achieved.

Table 1
Degree of substitution of enzymes calculated from ninhydrin assay.

Enzymes	Degree of substitusion (%)
Bromelain	35.3
Papain	47.8
Trypsin	38.5

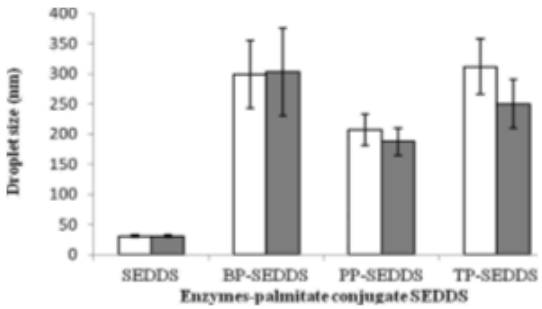

Fig. 2. Comparison of the droplet size of SEDDS and 5% enzyme-palmitate conjugates in SEDDS composition after dispersing in PBS pH 6.8 at 0 (white bars) and 4 h (grey bars). Indicated values are means SD of three experiments. BP-SEDDS (bromelain-palmitate SEDDS), PP-SEDDS (papain-palmitate SEDDS) and TP-SEDDS (trypsin-palmitate SEDDS).

3.2. Preparation and characterization of SEDDS enzyme-palmitate conjugates

In general, the miscibility of enzyme with SEDDS composition was low due to the hydrophilic nature of enzyme. The disability of enzyme to configure SEDDS droplets as a corona due to poor surfactant properties was an additional challenge in the surface modification of SEDDS droplets. In the present research, enzyme-palmitate conjugates could be successfully mixed with SEDDS. SEDDS containing enzyme-palmitate conjugates could be dispersed and evaluated according their properties such as droplet size and zeta potential. The increase in droplet size as shown in Fig. 2 was a consequence of the incorporation of enzyme-palmitate in SEDDS droplets. Trypsin-palmitate SEDDS showed a droplet size of 312 nm which was the highest droplet size among others.

On the other hand, SEDDS droplets should preferably exhibit mucoadhesive properties to trigger attaching process on the surface of mucus. Conversely, SEDDS droplets have to be mobile in the mucus in order to reach the underlying membrane [3]. Zeta potential plays a crucial role on adhesion and mobility properties of droplets. If the nanocarrier is highly negatively charged, the adhesion step will be difficult. On the contrary, a positively charged nanocarrier can easier adhere on mucus surface, but it is likely tightly bound to sialic- and sulfonic acid substructures of mucus unable to reach the underlying membrane. The value of zeta potential should be slightly negative or around zero to penetrate the mucus straightforwardly. In present research, enzyme-palmitate conjugates which were anchored on the surface of droplets decreased the zeta potential around 1.6-fold as shown in Fig. 3.

3.3. Payload determination of enzyme-palmitate in SEDDS

Payload of enzyme-palmitate describes the maximum amount of enzyme-palmitate conjugate which can be dissolved in SEDDS. The payload of bromelain-palmitate, papain-palmitate and trypsin-palmitate can be seen in Table 2. The concentration of

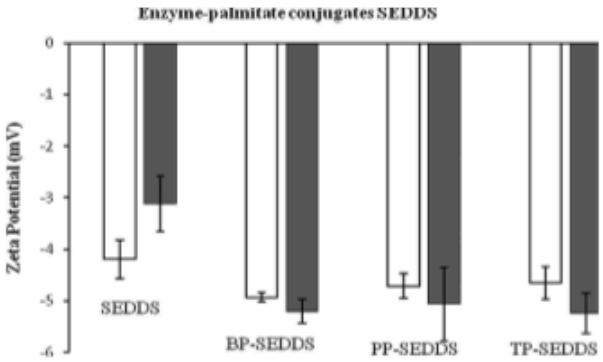

Fig. 3. Zeta potential of SEDDS and 5% enzyme-palmitate conjugates in SEDDS composition after dispersing in PBS pH 6.8 at 0 (white bars) and 4 h (grey bars). Indicated values are means SD of three experiments. BP-SEDDS (bromelain-palmitate SEDDS), PP-SEDDS (papain-palmitate SEDDS) and TP-SEDDS (trypsin-palmitate SEDDS).

Table 2
Payload of enzyme-palmitate conjugate in SEDDS.

Enzyme	Payload (% weight)
Bromelain-palmitate	4.6 0.04
Papain-palmitate	4.5 0.03
Trypsin-palmitate	4.4 0.05

enzyme-palmitate in SEDDS composition was 5% and the payload was about 4.5%. Therefore, around 90% of enzyme-palmitate conjugate introduced into SEDDS was dissolved.

3.4. Measurement of enzymes activity

The activity of enzymes and modified enzymes is shown in Fig. 4. Bromelain showed the lowest decline in activity (2% of the native) followed by trypsin (20% of the native). Papain showed the highest decline in activity (64% of the native). The activity of papain-palmitate immobilized on SEDDS droplets decreased intensively in comparison to enzyme-palmitate conjugates before anchoring, whereas trypsin-palmitate showed the least diminution. Merging with the previous data about the reduction of activity due to acylation, it can be generalized that attaching acyl group around the

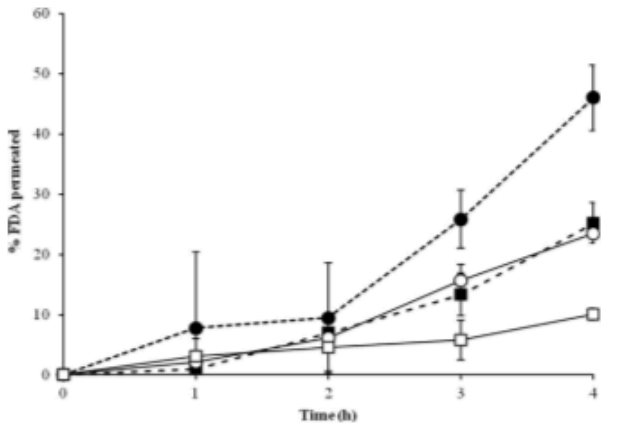

Fig. 5. Mucus permeation of SEDDS droplets (□), BP-SEDDS (bromelain-palmitate SEDDS: ○), TP-SEDDS (trypsin-palmitate SEDDS: ■) and PP-SEDDS (papain-palmitate SEDDS: ●) droplets containing FDA label in transport studies using Transwell method for 4 h of experiment. Indicated values are means SD of three experiments.

active side decreased the activity of enzyme. It was owing to the alteration of active side conformation.

The effect of sonication on enzymatic activity is depending on the type of enzyme, temperature and frequency, rather than on duration of ultrasound [18]. For homogenization of enzyme-palmitate and SEDDS ultrasound at a frequency of 35 kHz being regarded as low frequency [19] was therefore applied at room temperature for six hours. According to literature data this low frequency and moderate temperature should not harm enzymatic activity [20,21].

3.5. Permeation study of SEDDS enzyme-palmitate conjugates using transwell diffusion method

To compare the effect of mucolytic activity of enzyme after anchoring into SEDDS droplets on transport efficiency, mucus permeation using Transwell diffusion chamber was performed and the result is shown in permeation profile in Fig. 5.

Featuring SEDDS droplets using papain-palmitate significantly ($p < 0.05$) increased the droplet permeation 4.6-fold, whereas bromelain-palmitate as well as trypsin-palmitate enhanced per-

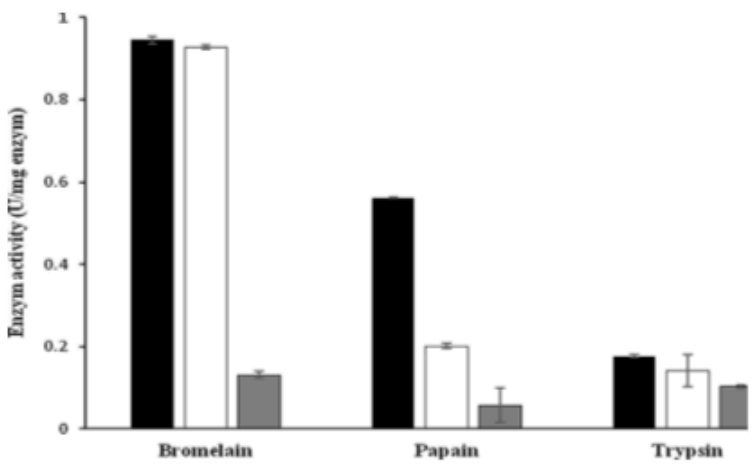

Fig. 4. Casein hydrolysis activity of native enzymes (black bars), enzyme-palmitate conjugates (white bars) and acylated followed by immobilized on SEDDS droplets (grey bars). Indicated values are means SD of three experiments.

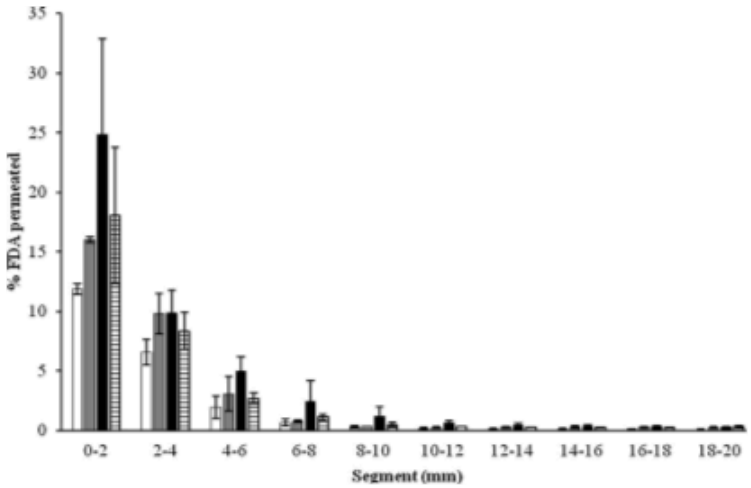

Fig. 6. Mucus permeation of SEDDS (white bars), BP-SEDDS (bromelain-palmitate SEDDS: grey bars), PP-SEDDS (papain-palmitate SEDDS: black bars) and TP-SEDDS (trypsin-palmitate SEDDS: strip bars) droplets containing FDA label in transport studies using rotating tube method after 4 h of incubation. Indicated values are means SD of three experiments.

meation 2.5-fold compared to SEDDS as control at the fourth hours of experiment.

3.6. Permeation study of SEDDS enzyme-palmitate conjugates using rotating tube method

Rotating tube method showed that introducing enzyme-palmitate conjugates into SEDDS formulation increased the permeation of FDA in all mucus segments as shown in mucus permeation experiments in Fig. 6.

Area under curve of segment length-percentage of FDA permeation (AUC) was calculated from Fig. 6 and used as a parameter of permeation efficiency. AUC of SEDDS, bromelain-palmitate SEDDS, papain-palmitate SEDDS and trypsin-palmitate SEDDS are 43.9, 62.7, 90.7 and 64.1 mm%, respectively. Based on AUC calculation, the incorporation of papain-palmitate into SEDDS increased droplet permeability by 2.2-fold, followed by bromelain-palmitate at 1.5-fold and trypsin-palmitate at 1.4-fold compared to SEDDS without any enzyme. Statistical analysis showed that introducing enzyme-palmitate conjugates into SEDDS composition increased significantly ($p < 0.05$) the total FDA permeated into mucus compared to SEDDS as control.

4. Discussion

In the present studies, the ability of proteolytic enzymes on SEDDS to overcome the high viscosity of mucus on intestinal surface was proven. Prior to the incorporation in SEDDS, certain enzymes including bromelain, papain and trypsin were modified to increase their lipophilicity. The modification was performed via acylation using palmitoyl chloride as acylating agent. The modified enzyme, so-called enzyme-palmitate conjugates, showed appropriate lipophilicity to be introduced into SEDDS. A length of 16 carbon atoms of acyl chain turned out to be sufficient to immobilize these enzymes on the surface of nanodroplets generated by SEDDS dispersion. Two processes namely acylation and immobilization on SEDDS reduce the activity of enzymes as a rational consequence.

To maintain a sufficient high mucolytic activity of enzymes, degree of acyl substitution had to be controlled. Increasing the degree of acyl substitution, on the one hand, enhances the feasibility of enzyme-palmitate conjugates of being anchor on the droplets after SEDDS dispersion. However, high degree of substitution decreases proteolytic activity. The ideal degree of substitution is not known yet. However, a 60–70% degree of substitution resulted in a dramatic decrease in enzymatic activity (data not shown). When the concentration of fatty acid chloride in the synthesis process was decreased, a lower degree of substitution was obtained. As a result the activity of enzyme-conjugates was comparatively higher.

Diminishing in enzyme activity as a consequence of palmitate substitution is triggered by at least two alterations, (1) enzyme conformation changes particularly at the active side, (2) and bulkiness close to the active side hindering the substrate to access it [22,23].

The decrease of enzyme-palmitate conjugates activity in comparison to the native enzyme is determined by the amino acid sequence and the active side of enzyme. Enzyme acylation reduced the activity of bromelain and trypsin to a lower level compared to papain. It can be explained by the amino acid sequence of active side or around the active side. The changes in conformation as well as in bulkiness of the active side are the determining factors for activity reduction. The chance of altering both factors will likely increase when acylation is taking place more closely to the active side – i.e. one or two amino acids before or after it.

Two active sides of bromelain are cysteine-26 and histidine-158 [24] which are not the target of acylation. The four amino acids around cysteine-26 are glycine, twice of alanine and tryptophan, whereas around histidine-158 are leucine, asparagine, alanine and valine [24]. All of them are not the target side of acylation. The same explanation can be given for trypsin containing three active sides as follows (the active sides are numbered with its position); AAH(48)CY, DND(92)IM and GDS(190)GG [25]. None of them are the target of acylation.

Different situation is presented for papain bearing three active sides. Amino acids position around the first and second side of action, i.e. GSC(158)WA and VDH(292)AV [26], are not the target of acylation, but the amino acid around the third active side, IKN(308)SW [26], contains an amino acid being target for acylation. Lysine (K) has primary amino groups in the side chain. As a result, the activity of papain decreased intensively after acylation.

Mucus permeation studies using Transwell membrane can be used to analyze only the effect of enzyme activity by arranging

an appropriate control as described in the methods in Section 2. Fig. 5 shows that immobilized papain-palmitate results in the highest mucus permeating properties of nanodroplets. The mucolytic activity of papain increased the permeation of SEDDS droplets 4.6-fold compared to the control. This increase in permeation is even higher than that achieved in case of poly acrylic acid (PAA) and PLGA nanoparticles. Immobilization of papain on these polymers led just to a 2.5-fold and 3-fold improvement over the corresponding unmodified control particles, respectively [14,27].

In another study, the effect of papain and bromelain immobilized on PAA nanoparticles was evaluated regarding mucus permeating properties using the rotating tube method. The content of enzyme decorated nanoparticles was thereby in all segments higher than in case of particles without an enzyme. In comparison to the mucus permeating properties achieved by enzyme-palmitate conjugates described herein, however, this effect was also lower [16].

In addition to this mucolytic effect of enzymes being anchored on nanodroplets, droplet size and zeta potential influence the mucus permeation behavior as well. As highlighted in Figs. 2 and 3 the incorporation of enzyme-fatty acid conjugates increased the droplet size and altered zeta potential. Results of mucus permeation studies via the rotating tube method, however, demonstrated higher mucus permeating properties of all enzyme decorated nanodroplets in comparison to unmodified SEDDS although they were 6-to 10-fold smaller in size. In contrast to the strong impact of enzyme-fatty acid conjugates on droplet size, their impact on zeta potential was quite minor. Generally, a negative zeta potential seems to be advantageous, as the mucus has a negative net charge and consequently ionic interactions with the nanodroplets can be avoided [28,29].

Mucin representing the main component of mucus consists of oligosaccharides being bound to a protein backbone on serine- and threonine-rich subdomains. These regions are heavily glycosylated and the carbohydrate side chains play a crucial role in order to protect these regions towards cleavage by proteases. Other regions are cysteine-rich and due to disulfide substructures also hardly accessible for proteases [3,26]. Papain and bromelain are cysteine proteases [27,28] attacking un-glycosylated regions, while trypsin, a serine protease digestive enzyme [29] assaults the glycosylated regions. As papain showed both in the Transwell and rotating tube studies higher mucus permeation than trypsin, it seems plausible that the protective effect of oligosaccharide side chains on mucins is higher than that of disulfide substructures.

All of SEDDS enzyme-palmitate conjugates exhibited significant increasing of mucus permeation compared to SEDDS as control. Papain-palmitate SEDDS displayed two advantages of penetration, i.e. high enzyme activity observed by Transwell method and small droplet size. As a result, papain-palmitate SEDDS produced the greatest mucus permeation in rotating tube method. This result supplements the previous study to utilize papain enzyme as surface modifier of drug nanocarrier systems.

5. Conclusion

Incorporation of enzyme-palmitate conjugates in SEDDS could improve the capability of SEDDS to permeate intestinal mucus due to the capability of enzymes to cleavage glycoprotein in the mucus. Furthermore, SEDDS as promising drug carrier systems have capability to bring lipophilic drugs closer to the absorption membrane. Integration of bromelain-palmitate, papain-palmitate as well as trypsin-palmitate into SEDDS increased significantly ($p < 0.05$) mucus permeation compared to SEDDS as control. These findings suggest that SEDDS enzyme-palmitate conjugates can be used as mucus penetrating carrier systems for further development of drug delivery.

Conflict of interest

There is no conflict of interest associated with this research.

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