—Regular Article—

Enteric Microsphere Formulations of Papain for Oral Delivery

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Enteric microspheres formulations of papain were prepared by w/o/w emulsion solvent evaporation using hydroxypropyl methylcellulose phthalate (HPMCP), Eudragit L 100 and Eudragit S 100, to avoid gastric inactivation of papain. Smaller internal and external aqueous phase volume provided maximum encapsulation efficiency (74.49–79.76 %), least particle size (52.4–60.2 μm) and 21–26% loss of enzyme activity. Release studies in 0.1 N HCl confirmed the gastro-resistance of formulations. The anionic microspheres, zeta potential between -18.21 and -20.06 mV, aggregated in 0.1 N HCl (*i.e.*, gastric pH 1.2), due to protonation of carboxylic groups of enteric polymer and loss of surface charge with subsequent change in zeta potential. The aggregates being <500 μm size would not impede gastric emptying. However, at pH$>$5.0 (duodenal pH) the microspheres showed de-aggregation due to restoration of surface charge. HPMCP and Eudragit L 100 microspheres facilitated almost complete release of papain within an hour at pH 6.0 and 6.8, respectively while Eudragit S 100 microspheres released 84.56% papain at pH 7.4, following Higuchi kinetics. FTIR spectroscopy revealed entrapment of enzyme; PXRD & DSC indicated amorphous character and SEM showed spherical shape of microspheres. In simulated gastro-intestinal pH condition, HPMCP, Eudragit L 100 and Eudragit S 100 microspheres showed good digestion of paneer and milk protein. Thus, enteric microspheres formulations could serve as potential carrier for oral enzyme delivery. Stability studies indicated the formulations with around 5% overage would ensure 2 years shelf life at room temperature.

Key words——enzyme; hydroxypropyl methylcellulose phthalate (HPMCP); Eudragit L 100; Eudragit S 100; pH sensitive polymer; zeta potential

INTRODUCTION

Proteins in diet are essential for growth, repair and for regulating the homeostasis of the body functions. But many people are intolerant to such foods including milk, cheese, yogurt, baked beans, bean soup, eggs, chicken, fish, meat, *etc*. This intolerance can lead to uncomfortable and embarrassing symptoms such as flatulence, bloating, belching, diarrhoea/constipation, malnutrition, food allergies, anaemia, undigested food in stool, chronic intestinal parasites and abnormal flora. These symptoms usually occur during achlorhydria and/or pancreatin insufficiency.[1,2] So, the need of protein digesting supplement arises to overcome the deficiency manifestations.[3] Now a day's demand of protein digesting aids has increased on the other hand supply of pepsin (prepared from hog mucosa) has decreased. Thus, the plant source derived proteases like papain can be used as supplement as there will be no scarcity relative to its supply.[4] Papain is a food grade, highly active endolytic cysteine protease (EC 3.4.22.2) derived from *Carica papaya*. Its broad substrate specificity and the ability to hydrolyze small peptides as well as large proteins make papain an ideal enzymatic supplement.[5] The optimum pH value for the activity of papain is in the range of 5.0–9.0, varying with different substrates.[6] Papain is almost inactive at gastric pH of 1.2.[7] Therefore, the ideal place for papain delivery is the intestine, where pH is in the range of 5.0–8.0. However, specific characteristics of papain (being a protein) like hydrophilic nature; complex structure and insufficient stability in gastrointestinal tract (GIT) are the major obstacles in oral delivery of papain.[8]

The key to the success of digestive proteins as pharmaceuticals is to have in place an efficient site specific pH dependent drug delivery system that allows the protein drug to gain access to the target site at right time and for the proper duration. pH sensitive poly-

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mers (water insoluble at low pH, water–soluble at high pH) are of particular interest as the release rate of drug can be triggered by the pH of the environment.[9] Enteric formulation of papain is more rational than immediate release commercial papain products since the former would protect the acid labile enzyme from gastric pH and deliver it to its site of action *i.e.*, intestine. Enteric coated microspheres of pancreatin of 1.0 to 1.2 mm in diameter showed 25% higher therapeutic effectiveness compared with 1.8–2.0 mm microspheres.[10] Stead *et al.*[11] also stated that faecal fat excretion was reduced by 44% with increased coefficient of fat absorption with enteric coated microspheres of pancreatin compared to enteric coated tablet. Therefore, the design of microparticulate systems has received increasing attention for oral delivery of these biomolecules.[12] Thus, the objective of the present study was to prepare microparticulate formulations of papain for pH dependent site specific release using pH sensitive polymers (hydroxylpropyl methylcellulose phthalate, Eudragit L 100 and Eudragit S 100).

MATERIAL AND METHODS

Hydroxypropyl methylcellulose phthalate (HPMCP) (Mol. wt 45 kDa) and methacrylic acid copolymers *i.e.*, Eudragit L 100 and Eudragit S 100 (average Mol. wt 135 kDa) was received as gift from Jubilant Organosys, India. Potassium dihydrogen phosphate, sodium hydroxide, hydrochloric acid (Qualigens Fine Chemicals, Mumbai, India) and papain (source *Carica papaya*), casien, tyrosine and trichloroacetic acid (98.0%) (Himedia Laboratories Pvt. Ltd., Mumbai, India) were used as received. Disodium ethylenediaminetetraacetate (99.5%), cysteine hydrochloride (99.0%), and citric acid (98.0%), polyvinyl alcohol, polysorbate 20 and lactose were purchased from S. D. Fine-Chem Ltd., Mumbai, India. Micro BCA™ protein assay kit (Pierce, Rockford, IL, USA) was procured from Thermo Scientific, India. Ethanol, dichloromethane (DCM) and iso-propanol (IPA) were received from Merck, Germany. Saras paneer (ingredient: milk solids) and Saras tonned milk was procured from Jaipur Zila Dugdh Utpadak Sahakari Sangh Ltd., Rajasthan, India. All other chemicals and solvents were of analytical grade and were used without further purification. Double-distilled water was used throughout the study.

Preparation of Microspheres Papain loaded microspheres were prepared by double-emulsion solvent evaporation technique. An aqueous solution of papain of 141.06 mg/ml concentration was prepared. The internal aqueous phase (IAP) (W_1) (0.4 or 0.2 or 0.1 or 0.05 ml) (Table 1) containing papain, 3% v/v polysorbate 20 (dispersing agent) and 0.16% w/v lactose as cryoprotectant was emulsified with 5 ml of organic phase for 1 min using an ultrasonic disruptor (30 W output power, 40% duty cycle) (Branson Sonifier® 450, Danbury, USA). Temperature was maintained at 4℃, using an ice-bath during emulsification. The organic phase (O) consisted of 200 mg of polymer (HPMCP or Eudragit L 100 or Eudragit S 100) in 5 ml of a mixed solvent system of DCM: Ethanol: IPA in a ratio of 5 : 6 : 4.[13] The resulting primary emulsion (W_1/O) was added drop by drop to external aqueous phase (EAP) (W_2) (100 or 50 or 25 ml) (Table 1) of 1% w/v polyvinyl alcohol (PVA) and 1.6% w/v lactose solution. The aqueous PVA solution acts as an emulsion stabilizer. Emulsification was continued using a homogenizer (750 W) (Virtis, SENTRY™ Microprocessor) at 10000 rpm for 7 min to form a multiple emulsion ($W_1/O/W_2$) at 4℃ in an ice bath. The resulting $W_1/O/W_2$ emulsion was stirred at room temperature for 16–18 h with a magnetic stirrer to allow the solvent to evaporate. The microspheres were collected and washed 3 times with distilled water by centrifugation at 10000 *g* for 20 min at 4℃. The microspheres were re-suspended in distilled water and lyophilized for 24 h. The final product was stored in desiccator at 25℃. Different formulations prepared by varying the IAP: O: EAP ratios as well as polymer were coded as shown in Table 1. The optimised formulation prepared with 50 μl IAP: 5 ml O: 25 ml of EAP (*i.e*, HM_4, LM_4, SM_4) was subjected to further studies.

Characterization of Microspheres

Enzyme Assay The proteolytic activity of papain was estimated by modified casein digestion method of USP XXVII in the presence of cysteine hydrochloride. Appropriately diluted standard papain solution in phosphate-cysteine disodium ethylenediaminetetraacetate buffer was added to 5 ml of buffered 1% w/v casein substrate (pH 6.0±0.1). After incubation at 37℃ for 20 min in a shaking water bath, the reaction was stopped by the addition of 3 ml of 30% w/v trichloroacetic acid solution. The tubes were allowed to stand for 30–40 min at 40℃ in water bath, to allow

Table 1. Different Batches of Microspheres Prepared with HPMCP, Eudragit L 100 and Eudragit S 100 Polymer

Polymer	Formulation code	Volume of IAP (W_1) ml	W_1 : O	Volume of EAP (W_2) ml	O : W_2
HPMCP	HM_1	0.4	1 : 12.5	25	1 : 5
	HM_2	0.2	1 : 25	25	1 : 5
	HM_3	0.1	1 : 50	25	1 : 5
	HM_4	0.05	1 : 100	25	1 : 5
	HM_5	0.05	1 : 100	50	1 : 10
	HM_6	0.05	1 : 100	100	1 : 20
Eudragit L 100	LM_1	0.4	1 : 12.5	25	1 : 5
	LM_2	0.2	1 : 25	25	1 : 5
	LM_3	0.1	1 : 50	25	1 : 5
	LM_4	0.05	1 : 100	25	1 : 5
	LM_5	0.05	1 : 100	50	1 : 10
	LM_6	0.05	1 : 100	100	1 : 20
Eudragit S 100	SM_1	0.4	1 : 12.5	25	1 : 5
	SM_2	0.2	1 : 25	25	1 : 5
	SM_3	0.1	1 : 50	25	1 : 5
	SM_4	0.05	1 : 100	25	1 : 5
	SM_5	0.05	1 : 100	50	1 : 10
	SM_6	0.05	1 : 100	100	1 : 20

complete coagulation of the precipitated protein. Thereafter, the supernatant containing digested amino acids was filtered through Whatman filter paper no. 42 by discarding first 3 ml of filtrate. The absorbance of the filtrate was measured at 280 nm using UV–VIS Spectrophotometer (Lab-India® UV 3000$^+$, India) against the tyrosine standard plot of absorbance versus tyrosine concentration (μg/ml). The papain activity was expressed in terms of Casein Digestion Unit (CDU). A Casein Digestion Unit (CDU) is the microgram of tyrosine liberated in 1 min by 1 mg enzyme under assay conditions.

Protein Content Estimation Protein content of samples was determined using Micro BCA™ protein assay kit (Pierce, Rockford, IL, USA).

Estimation of Encapsulation Efficiency Ten milligram of microspheres were accurately weighed and dissolved in 1 ml of ethanol-phosphate buffer (pH 7.4) mixture (1 : 1). The resulting solution was analyzed for papain content by measuring absorbance in an ELISA at 540 nm using Micro BCA™ protein assay kit (Pierce, Rockford, IL, USA) for protein estimation. Results were expressed as mean±S.D. of three experiments. Encapsulation efficiency was calculated as

$$\text{Actual loading}(\%) = \text{mg of encapsulated papain /100 mg microspheres} \quad (1)$$

$$\text{Encapsulation efficiency}(\%) = (\text{actual enzyme loaded /theoretical enzyme loading}) \times 100 \quad (2)$$

Enteric Nature of Microspheres This test was performed to determine the extent of drug release in the acidic environment of the stomach (*i.e.*, pH 1–3). An accurately weighed sample ($n=3$) of papain loaded HPMCP, Eudragit L 100 or Eudragit S 100 microspheres equivalent to 10 mg of papain was introduced into 20 ml of 0.1 N HCl equilibrated at 37±0.5℃ in a shaking water bath at 100 shakes per minute. Samples were withdrawn after 120 min and protein content was determined using Micro BCA™ protein assay kit.

In Vitro Drug Release *In vitro* release of papain from enteric coated microspheres was conducted to study the effect of pH on drug release. An accurately

weighed sample ($n=3$) equivalent to about 10 mg of papain was transferred to 20 ml prepared dissolution media (pH 6.0, 6.8 or 7.4 phosphate buffer), maintained at 37±0.5°C in a shaking water bath at 100 shakes per minute. At pre-determined time intervals, 1 ml sample was withdrawn followed by replacement of withdrawn volume by fresh phosphate buffer. Enzyme content was estimated using Micro BCA™ protein assay kit of protein estimation to confirm the integrity of protein molecule. Results were expressed as mean±S.D. of three experiments.

Release Kinetics The mechanism of drug release was investigated by fitting the drug release data into Higuchi's dissolution model. An approximation of the Higuchi's equation[14] can be obtained by plotting the amount of drug released *vs.* square root of time expressed by

$$W = K\sqrt{t} \quad (3)$$

where W is the % of drug released at time t (min) and K is the Higuichi release rate constant. The goodness of fit of drug release was evaluated by linear regression.

Particle Size and Zeta Potential Measurement Freeze-dried microspheres were dispersed in water after treatment in an ultrasonic disperser (Seishin) for 1 min. The z-average mean diameter of papain loaded microparticles in aqueous dispersion was determined by laser diffractometry using a Malvern Mastersizer S (Malvern Instruments, France). Each value quoted was the average of determinations on three independent samples.

The surface charge of optimised enteric microspheres was determined by zeta potential measurement using Zetasizer Nano ZS (Malvern Instruments, U.K.). Samples were appropriately diluted and dispersed in distilled water for zeta potential measurement. Each value quoted was the average of determinations on three independent samples. Change in the behaviour of microspheres with change in the pH *i.e.*, pH 1.2 to 5.3 of the release media was analyzed using an optical imaging system (Nikon Eclipse TS 100; Nikon, Tokyo, Japan), equipped with a Sony camera (Hyper HAD model SSC-DC38DP; Elvetec, Templemars, France) and the Optimas 6.0 software (Media Cybernetics, Silver Spring, USA).

Digestion of Paneer and Milk Protein Paneer and milk samples containing an amount equivalent to 450 mg casein was taken for determining the proteolytic activity of papain. Paneer (2.25 g) passed through sieve no. 22 or toned milk (17.14 ml) was mixed with 20 ml of 0.1 N HCl and optimised formulation of enteric microspheres (HM_4, LM_4, SM_4) equivalent to 1.0 mg of papain was added and the mixture was maintained at 37±0.5°C in a shaking water bath at 100 shakes per minute for 2 h followed by increase of the pH to 6.0 by addition of disodium hydrogen phosphate and kept for 2 h. Subsequently the pH was raised from pH 6.0 to 6.8 and kept for 2 h followed by further increase in pH from pH 6.8 to 7.4 and maintained for 2 h. At predetermined time interval; 1 ml sample was withdrawn followed by replacement of withdrawn volume by fresh phosphate buffer. Samples were centrifuged at 17000 rpm for 30 min and the supernatant was estimated for tyrosine content, as described previously.

Fourier-transform-infrared Spectroscopy (FTIR) FTIR spectra of papain powder, HPMCP, Eudragit L 100, Eudragit S 100 and papain loaded HPMCP (HM_4), Eudragit L 100 (LM_4) and Eudragit S 100 (SM_4) microspheres were obtained using a FTIR spectrometer (FTIR-8400S, Shimadzu, Japan) as KBr pellets in the range of 4000–400 cm^{-1}.

Powder X-ray Diffraction (PXRD) The X-ray diffraction patterns for papain, HPMCP, Eudragit L 100, Eudragit S 100 and papain loaded HPMCP (HM_4), Eudragit L 100 (LM_4) and Eudragit S 100 (SM_4) microspheres were recorded in a X-ray diffractometer (Siemens, Model D5000, Germany) using $CuK\alpha_1$ radiation of wavelength 1.5406 Å, generated at 45 kV, 40 mA, by measuring the angle of diffraction over the range of 3.0 to 45.0° 2θ.

Differential Scanning Calorimetry (DSC) The thermal characteristics of papain powder, HPMCP, Eudragit L 100, Eudragit S 100 and papain loaded HPMCP (HM_4), Eudragit L 100 (LM_4) and Eudragit S 100 (SM_4) microspheres was determined using a differential scanning calorimeter (DSC–60, Shimadzu, Japan). Samples were crimped in a standard aluminium pan and heated from 40 to 250°C at a heating rate 10°C/min under constant purging of nitrogen at 30 ml/min.

Scanning Electron Microscopy (SEM) Particle morphology was analyzed by scanning electron microscopy (LEO 435 VP) using an acceleration voltage of 2 kV. The surface microscopic structure of microspheres was also investigated. Particles were mounted on brass stubs using carbon paste. SEM photographs were taken with variable pressure scan-

ning electron microscope at the required magnification at room temperature.

Stability Testing Accurately weighed papain parent bulk or selected microspheres (HM_4, LM_4 and SM_4) equivalent to 10 mg of papain were filled into hard gelatin capsules (size 3). The capsules were packed in amber coloured glass bottles and subjected to stability testing according to the International Conference on Harmonization guidelines for zone III and IV. The packed containers of prepared capsules of papain parent bulk or microspheres were kept for accelerated (40±2℃/75±5% relative humidity) and long term (30±2℃/65±5% relative humidity) stability for 6 months and 12 months, respectively. Samples kept under accelerated storage conditions were withdrawn at 0 day, 6 weeks, 3 and 6 months and papain activity were estimated. Similarly, samples stored at 30±2℃/65±5% were withdrawn at 0 day, 3, 6, 9 and 12 months, and analysed for papain activity. Visual inspection of samples for discoloration of capsule content was also done after completion of stability study.

RESULTS AND DISCUSSION

Formulation Variables Effect of formulation variables on yield, encapsulation efficiency and particle size of HPMCP, Eudragit L 100 and Eudragit S 100 microspheres prepared by double emulsion solvent evaporation technique is shown in Table 2. With respect to the total amount of papain used for microsphere preparation, microspheres made with Eudragit L 100 and Eudragit S 100 encapsulated larger amount of papain compared to HPMCP (Table 2). It could be due to difference in the molecular weight of polymers. Low molecular weight polymers incorporate smaller amount of protein than those with higher molecular weight.[15,16] This can be explained by the different viscosities of the polymer solution since those with low viscosity are easier to disperse and form a thinner layer when deposited on microspheres of the inner protein solution.[17,18] This enables the protein to migrate towards the external

Table 2. Effect of Formulation Variables on Yield, Encapsulation Efficiency and Particle Size of HPMCP, Eudragit L 100 and Eudragit S 100 Microspheres Prepared by Double Emulsion Solvent Evaporation Technique

Formulation code	Yield (%) (mean±S.D.)	Theoretical loading (%)	Actual loading (%)	Encapsulation efficiency (mean±S.D.) (%)	Particle size (mean±S.D.) μm
HM_1	54.81±3.26	21.95	2.71±0.39	12.35±1.77	530.4±45
HM_2	58.10±3.75	12.34	2.70±0.36	21.88±2.93	212.6±24
HM_3	60.05±4.09	6.58	2.67±0.34	40.58±5.31	108.7±23
HM_4	61.86±3.19	3.41	2.54±0.11	74.49±3.09	52.4±20
HM_5	63.58±3.52	3.41	2.40±0.10	70.38±2.94	82.2±19
HM_6	60.99±3.62	3.41	2.30±0.10	67.45±3.38	108.3±32
LM_1	65.09±3.19	21.95	2.98±0.24	13.58±1.11	600.2±27
LM_2	58.92±3.51	12.34	3.03±0.15	24.55±1.23	235.1±34
LM_3	61.53±3.56	6.58	2.90±0.27	44.07±4.05	111.1±35
LM_4	63.88±4.10	3.41	2.67±0.11	78.30±3.23	60.2±23
LM_5	61.72±3.50	3.41	2.55±0.13	74.78±3.92	84.4±24
LM_6	60.78±2.86	3.41	2.42±0.18	71.08±5.40	119.8±11
SM_1	60.41±2.81	21.95	3.09±0.22	14.08±1.01	608.7±27
SM_2	64.97±2.80	12.34	3.11±0.27	25.20±2.14	231.3±29
SM_3	63.11±2.61	6.58	2.87±0.23	43.62±3.48	118.4±18
SM_4	60.54±3.64	3.41	2.72±0.12	79.76±3.66	58.2±18
SM_5	63.36±2.56	3.41	2.57±0.15	75.37±4.51	86.2±21
SM_6	61.72±2.75	3.41	2.51±0.11	73.61±3.23	127.8±32

aqueous medium resulting in low entrapment of the protein.

The type of polymer and volume of IAP affected the encapsulation efficiency of papain loaded microspheres. It was observed that as the volume of IAP was decreased from 0.4 to 0.05 ml, the encapsulation efficiency increased and particle size decreased in case of all the polymeric particles but the extent varied slightly. The mean diameter of microspheres varied from 52.4±20 μm to 608.7±27 μm with varying IAP volume from 50 μl to 400 μl in case of all the three polymers used in study. Employment of 50 μl IAP provided highest papain loading efficiency and least particle size for HPMCP (74.49±3.09%, 52.4±20 μm), Eudragit L 100 (78.30±3.23%, 60.2±23 μm) and Eudragit S 100 microspheres (79.76±3.66%, 58.2±18 μm) respectively (Table 2). Thus, smaller volume of IAP is desirable for higher encapsulation of papain and particles of smaller size. This improved encapsulation efficiency may be simply due to greater proportion of polymer with respect to the amount of the enzyme. On the other hand, the increase in polymer load leads to a shorter time for the composition of the polymer solution to reach the viscous (gelation) boundary, resulting in rapid film like membrane formation on the droplets periphery. If the film like polymeric membrane is quickly solidified, the microparticle structure is more fixed and thus solvent and non-solvent counter diffusion is delayed. As a consequence, less water may be allowed to diffuse into the dispersed phase and fewer drugs (enzyme) will be carried by solvent into the aqueous phase.[19] The optimized ratio of IAP to O was 1 : 100 *i.e.*, 50 μl of IAP and 5 ml of O.

Effect of change in volume of EAP on encapsulation efficiency of microspheres was also studied. As the volume of EAP was increased from 25 ml to 100 ml, the encapsulation efficiency decreased and particle size increased (Table 2). Thus, for maximum encapsulation of microspheres optimized ratio of IAP (W_1): O: EAP (W_2) was 1: 100: 500 (*i.e.*, 50 μl IAP: 5 ml O: 25 ml of EAP).

Jain *et al.*[20,21] encapsulated porcine insulin in Eudragit L 100 and Eudragit S 100 microspheres by w/o /w emulsion solvent evaporation and observed actual drug loading of 0.45 and 0.43% while the encapsulation efficiency was 84.5 and 81.8% respectively. In the present experiment the actual drug loading of papain in Eudragit L 100 and Eudragit S 100 microspheres was 2.67–2.72% and the corresponding encapsulation efficiency was 78.30 and 79.76%. The higher drug loading observed in the present study appears to be due to employment of higher concentration of papain in the internal aqueous phase (IAP) compared with insulin (141.06 mg/ml of papain against 32 mg/ml of insulin). Since encapsulation efficiency is the ratio of actual drug loading and theoretical drug loading, increase in theoretical drug loading would reduce encapsulation efficiency and the same could account for the slightly lower encapsulation efficiency of papain in the microspheres. The encapsulation studies of insulin (mol. wt. 5.8 kDa) and papain (mol. wt 23 kDa) in Eudragit L 100 and Eudragit S 100 microspheres suggest that the molecular weight of the protein does not have any significant affect on encapsulation.

The yield of microspheres obtained from different batches of different polymers varied from 54.81% to 65.09% irrespective of the enzyme loading. The low yield of microspheres could be attributed to losses occurring during various steps of processing, such as sticking of the polymeric solution to the glass container, loss of microspheres during the washing step *etc*. Loss due to sticking could be minimised by using apparatus made of plastic or polyethylene.

During microspheres preparation, the papain activity decreased from 887.92 CDU to 700.42 CDU, 682.92 CDU and 527.04 CDU for HPMCP, Eudragit L 100 and Eudragit S 100 microspheres, respectively. About 21–26% loss in enzyme activity was observed under the conditions of the experiment for optimised formulations of three different polymers. This might be due to the denaturant effect of the mixed organic solvent system, mechanical stress exerted by the probe during sonication and exposure to vacuum during lyophilization.

Enteric Nature of Microspheres Studies with the optimized formulations revealed the release of papain from papain loaded microspheres was mainly influenced by the nature of polymer as well as the pH of the surrounding media. Absolute enteric coating could not be achieved as 7.34, 6.05 and 4.78% of papain was released from HPMCP (HM_4), Eudragit L 100 (LM_4) and Eudragit S 100 (SM_4) microspheres, respectively, in 0.1 N HCl (pH 1.2) in 2 h. This might have resulted from the leaching of drug from microspheres, where insufficient coating might have occurred. Since the polymers are insoluble in the release media of pH 1.2, the microspheres were only

slightly swollen and remained intact in this case. Available studies where papain was immobilized in ionotropically crosslinked sodium alginate and kappa-carrageenan gel beads reported that 90% of papain was released from formulation within 50 minutes in simulated gastric fluid.[22,23] As a result papain would remain almost inactive as well as lose its structural integrity at low pH of stomach (pH 1.2). Hence sufficient amount of papain would not reach to the target site for digestion of peptides/proteins. Thus, use of enteric polymer matrix system for oral papain would be satisfactory (as the enzyme is not released from the optimised formulation in significant amount in the stomach) in maintaining the structural integrity of enzyme during transit through stomach.

The microspheres had negative charge contributed by the carboxylic groups of phthalic acid (pKa≈4.47) and methacrylic acid (pKa≈4.23) residues in the enteric polymer backbone. The zeta potential of aqueous dispersion of microspheres was found between −18.21 and −20.06 mV. It was observed that the zeta potential of microspheres was changed in 0.1 N HCl (0.1473–0.2210 mV) (Table 3). Optical microscopy showed aggregation of microspheres in 0.1 N HCl (pH 1.2) (Fig. 1). We know that the carboxylic groups of anionic particles get protonated if the pH is below the pK_a of the carboxylic acid leading to decrease in surface charge of particles. Reduction in surface charge diminishes electrostatic repulsion and increases the Van der Waals force of attraction between the particles facilitating aggregation.[24] Thus it appears quite natural that the microspheres would aggregate in 0.1 N HCl having pH 1.2 which is below the pK_a of phthalic acid (pKa≈4.47) and methacrylic acid (pKa≈4.23).[25] Addition of 0.5% soybean oil did not have any appreciable effect on zeta potential in 0.1 N HCl or aggregation. It has been reported that stomach retains food particles until these are fragmented into particles smaller than 0.5 mm in diameter.[26] Since the size of aggregated microspheres was less than 500 μm, there should not be any significant delay in gastric emptying of the particles. Subsequent to gastric emptying the microspheres would enter duodenum where the pH is>5.0. Accordingly, as the pH of the dispersion of microspheres in 0.1 N HCl was raised to 5.3, de-aggregation of particles was observed due to pH-induced deprotonation of carboxylic groups and restoration of negative charge. The result suggests that the microspheres on being emptied by the stomach into duodenum having pH>5.0 would undergo de-aggregation resulting in increase in effective surface area of the

Table 3. Zeta Potential of Optimised Formulations of Enteric Microspheres

Formulation	Zeta potential (mV) (mean±S.D.)	
	Distilled water	0.1 N HCl
HM_4	−20.06±2.68	0.2210±2.35
LM_4	−18.34±3.06	0.1473±2.56
SM_4	−18.21±2.28	0.1682±3.38

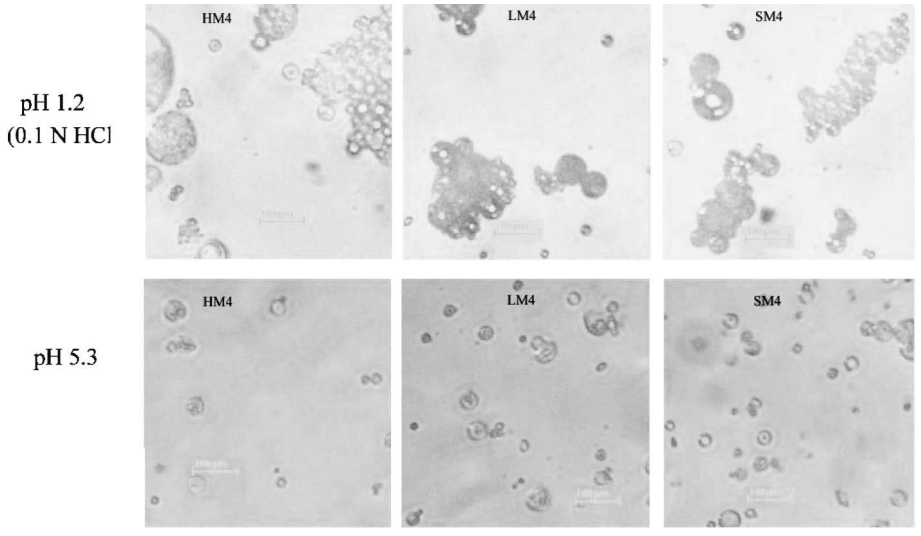

Fig. 1. Effect of Change in pH from pH 1.2 to 5.3 on the Aggregation and Deaggregation of Optimised Papain Loaded Enteric Microspheres of HPMCP (HM_4), Eudragit L 100 (LM_4) and Eudragit S 100 (SM_4)

particles.

***In Vitro* Release Profile** *In vitro* release study from all the optimized batches of microspheres showed 4.78–7.34% of drug release in 0.1 N HCl (pH 1.2) in 2 h. Next, the drug release was studied at pH 6.0, 6.8 or 7.4 for HPMCP, Eudragit L 100 and Eudragit S 100 microspheres, respectively. HPMCP microspheres (HM_4) showed almost complete drug release in an hour at all the pH studied whereas Eudragit L 100 (LM_4) microspheres showed 17.89% drug release at pH 6.0 and complete release at pH 6.8 and 7.4, respectively (Fig. 2). Eudragit S 100 (SM_4) microspheres showed only 12.45–15.46% release at pH 6.0 and 6.8 against 84.56% at pH 7.4 in an hour. However, complete release of drug from SM_4 microsphers at pH 7.4 was obtained in 1 h 20 min. The drug release from microspheres is consistent with the pH-dependent solubility of the polymers as HPMCP dissolves at pH around 5.5 while Eudragit L 100 and Eudragit S 100 dissolve at pH$>$6.0 and pH$>$7.0 respectively.[27]

Release Kinetics Microspheres are diffusion systems in which the drug is dispersed uniformly throughout the polymer matrix. The release mechanism of drug from microspheres was evaluated. The (%) cumulative release of papain increased with increase of time until the complete disintegration of microspheres. Accordingly, a plot of amount of drug released *vs.* square root of time for the formulations showed linear rise indicating a diffusion controlled release, following Higuchi kinetics with regression coefficient R^2=0.988 for HPMCP microspheres, R^2 =0.979 for Eudragit L 100 microspheres and R^2= 0.965 for Eudragit S 100 microspheres.

Digestion of Paneer and Milk Protein Milk and paneer are the most common source of protein consumed as food in Indian subcontinent. Thus, assuming a maximum gastro-intestinal transit time of 12 h through the absorptive areas of GIT,[28] paneer and milk protein digesting ability of HM_4, LM_4 and SM_4 formulations was evaluated in pH progressive media (simulating gastro-intestinal pH condition). Substrate (milk or paneer) was successively exposed to microspheres at pH 1.2 for 2 h, pH 6.0 for 2 h, pH 6.8 for 2 h and pH 7.4 for 2 h. It was observed that 8.54±3.45 and 0.27±2.31 mg of tyrosine was formed from paneer with HM_4 and LM_4 at pH 6.0 after 2 h whereas 8.67±3.42 and 0.33±2.24 mg of tyrosine was formed from milk. Subsequently, as the pH was raised to 6.8, total amount of tyrosine formed from paneer was 8.59±3.68, 8.48±4.92 and 0.07± 2.73 mg with HM_4, LM_4, SM_4 in 2 h while 8.69± 3.27, 8.57±3.96 and 0.06±3.13 mg of tyrosine was formed from milk. With further increase in pH from 6.8 to 7.4, total amount of tyrosine formed from paneer was 8.601±3.22, 8.638±3.84 and 8.433± 3.65 mg with HM_4, LM_4 and SM_4, respectively after 2 h whereas the corresponding tyrosine formed from milk were 8.709±3.48, 8.728±3.64 and 8.472±2.38 mg. Thus, HPMCP and Eudragit L 100 microspheres

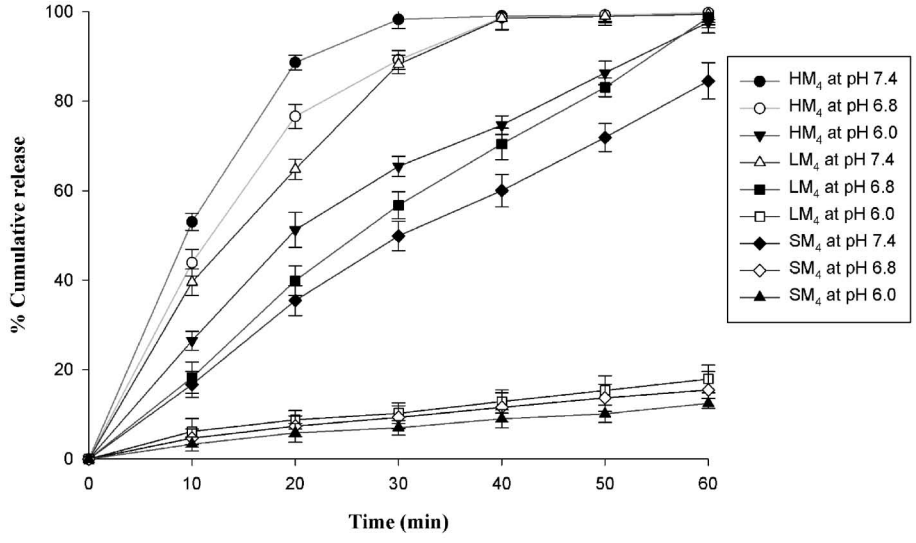

Fig. 2. Effect of pH on the Release of Enzyme from the Optimized Formulation of Papain Loaded Microspheres of HPMCP (HM_4), Eudragit L 100 (LM_4) and Eudragit S 100 (SM_4) at pH 6.0, 6.8 and 7.4 Respectively

would have good digestion power of proteins/peptides at pH 6.0 and 6.8 respectively, during the short gastro-intestinal transit time of 4–6 h whereas Eudragit S 100 microspheres require longer time for digestion. This indicates that in an *in vivo* situation, as the microspheres will pass through stomach to intestine, uniform mixing of microspheres with chyme would facilitate the digestion of proteins.

Fourier-transform-infrared Spectroscopy (FTIR) FTIR spectra of Eudragit L 100, Eudragit S 100, HPMCP, papain and optimised papain loaded Eudragit L 100, Eudragit S 100 and HPMCP microspheres are shown in Fig. 3. Eudragit L 100 polymer contains both carboxylic acid and ester groups. Therefore the spectrum showed overlapping carbonyl vibrations of ester group at 1728.2 cm^{-1}. The peak at ~1728.2 cm^{-1} could be attributed to stretching vibrations of the ester group carbonyl. Since Eudragit S 100 is a methylmethacrylate polymer similar to Eudragit L 100, the spectrum of Eudragit S 100 resembled the spectrum of Eudragit L 100 with slight variations in peak positions. HPMCP polymer showed various distinct peaks: very broad band in the region of 3500–3250 cm^{-1} having peak at 3438 cm^{-1} due to polyhydroxy $(-OH)_n$ group; 1735.3 cm^{-1} due to C=O stretching; 1064.4 cm^{-1} due to C–O stretch of cyclic ethers. Papain also showed various distinct peaks: one predominant band around 3450–3225 cm^{-1} having peak at 3300 cm^{-1}due to N–H stretch of secondary N –substituted amide; weak peak at 2981 cm^{-1} due to C–H stretching, medium bands at 1600–1500 cm^{-1} due to C–C, 868 cm^{-1} and 850 cm^{-1} due to p-substituted aromatic out-of-plane C–H deformation of aromatic residues of tryptophan or tyrosine; 1429 cm^{-1} and 1321 cm^{-1} due to C–H deformation of alkyl chains of amino acids; 1654.2 cm^{-1} due to C=O stretch of carboxylate anion and amide group; and strong peaks between 1150–1050 cm^{-1} and 705–570 cm^{-1} due to C–S stretch of sulphides and disulfides.

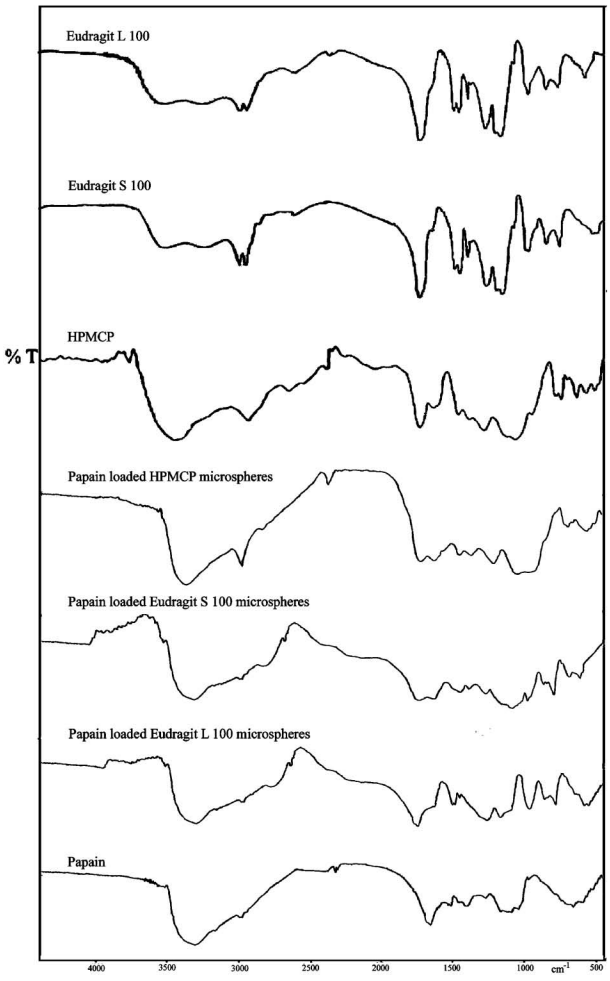

Fig. 3. The FTIR Spectra of Eudragit L 100, Eudragit S 100, HPMCP, Papain Powder and Optimised Formulation of Papain Loaded HPMCP (HM_4), Eudragit L 100 (LM_4) and Eudragit S 100 (SM_4) Microspheres

With the incorporation of papain, the spectra of papain loaded enteric polymer microspheres showed peaks at 3386.2 cm^{-1} for substituted secondary amide, 1145 cm^{-1} and 600 cm^{-1} due to C–S stretch of sulphides and disulfides. In the spectrum of HPMCP microspheres, the C=O stretching appeared at 1642.6 cm^{-1} while in the spectra of Eudragit L 100 and Eudragit S 100 microspheres a broad peak appeared at 1652 cm^{-1} similar to that of papain powder which appears to be a dilution effect of polymer as papain content of microspheres was around 2.54–2.72%.

Powder X-ray diffraction (PXRD) Figure 4 shows the powder diffraction patterns for papain, HPMCP, Eudragit L 100, Eudragit S 100 and optimised papain loaded microspheres of HPMCP, Eudragit L 100 and Eudragit S 100. The diffractograms of papain and polymers indicated amorphous structure. The diffractograms of microspheres also indicated amorphous structure and the diffractograms of microspheres appeared identical to HPMCP, Eudragit L 100 and Eudragit S 100 polymers.

Differential Scanning Calorimetry (DSC) The DSC thermograms of papain, HPMCP, Eudragit L 100, Eudragit S 100 and optimised papain loaded microspheres of HPMCP, Eudragit L 100 and Eudragit S 100 are shown in Fig. 5. Papain exhibited two broad endotherms with peaks at 74.49℃ and

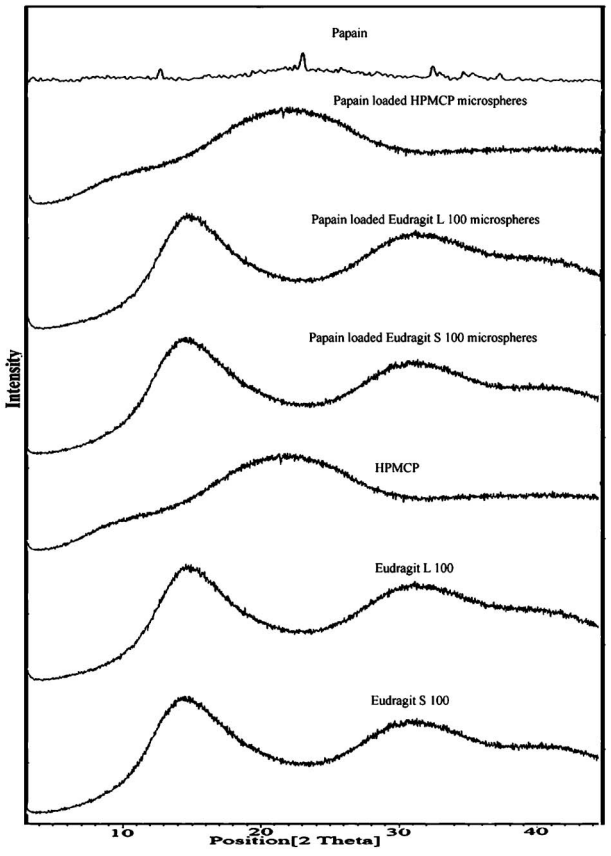

Fig. 4. XRD Pattern of Eudragit S 100, Eudragit L 100, HPMCP, Papain Powder and Optimised Formulation of Papain Loaded HPMCP (HM_4), Eudragit L 100 (LM_4) and Eudragit S 100 (SM_4) Microspheres

174.47℃. Eudragit L 100 and Eudragit S 100 showed broad endotherms with peaks at 216.2℃ and 188.51 ℃ respectively while HPMCP exhibited two broad endotherms having peaks at 198.75℃ and 211.57℃, which are typical of amorphous material. DSC thermograms of papain loaded polymeric microspheres were similar to the thermograms of respective polymers but the polymeric peaks were shifted to lower temperatures as papain was present as an impurity (2.54–2.72%). However, thermograms did not show any melting peak of papain, and the effect appears to be a dilution effect contributed by the amorphous polymer. The powder XRD of papain-loaded microspheres also showed amorphous structure similar to polymer.

Morphology of Particles Particles were analysed by scanning electron microscope to observe the morphology of microspheres. The microspheres made of polymer Eudragit L 100, Eudragit S 100 and HPMCP containing papain were spherical and had smooth surface (Fig. 6).

Stability Study Tables 4 and 5 present the results of accelerated and long-term stability studies of microspheres and unentrapped papain formulations. The microspheres formulations HM_4 and SM_4 showed around 92% papain content on storage under accelerated conditions (*i.e.*, 40℃/75% RH) for 6 months while LM_4 and unentrapped papain formulations showed 93 and 73% drug content respectively (Table 4). HM_4, LM_4 and SM_4, however, showed around 92% drug content when stored at 30℃/65% RH for 12 months against 72.55% drug content for unentrapped papain formulation. The results suggest improved stability of the enzyme on entrapment in polymer. On the basis of first order degradation rate constants, the calculated t_{90} of LM_4, SM_4 and HM_4 at 30℃/65% RH would be 514, 482 and 479 days respectively (Table 5). The K_{calc}/t_{90} values suggest that optimised formulations will not provide 2 years shelf life (t_{90}) of the product and might need some overages resulting in higher initial drug concentration. Thus, to ensure 2 years shelf life, formulations HM_4, LM_4 and SM_4 would need 4.30–5.44% overage (Table 5) while capsules containing unentrapped papain require around 71% overage. The colour of bulk papain powder *i.e.*, unentrapped papain changed from pale buff to light brown. On the contrary papain loaded microspheres formulations did not show any colour change. Thus, the stability of papain entrapped in microspheres was significantly improved than the bulk papain powder.

CONCLUSIONS

Papain was successfully encapsulated in the enteric microspheres by double emulsion solvent evaporation, optimizing various formulation parameters in order to attain maximum encapsulation efficiency, enteric nature, spherical shape, almost monodispersed particle size distribution and optimum *in vitro* release profile. *In vitro* release profiles confirmed their gastroresistance, thus allowing pH dependent release of papain in the GIT. Among the microspheres of HPMCP, Eudragit L 100 and Eudragit S 100, HPMCP and Eudragit L 100 microspheres disintegrated and showed complete release of papain in the small intestinal pH within an hour whereas Eudragit S 100 microspheres needed higher pH and longer time for drug release. HPMCP, Eudragit L 100 and Eudragit S 100 microspheres showed good digestion of

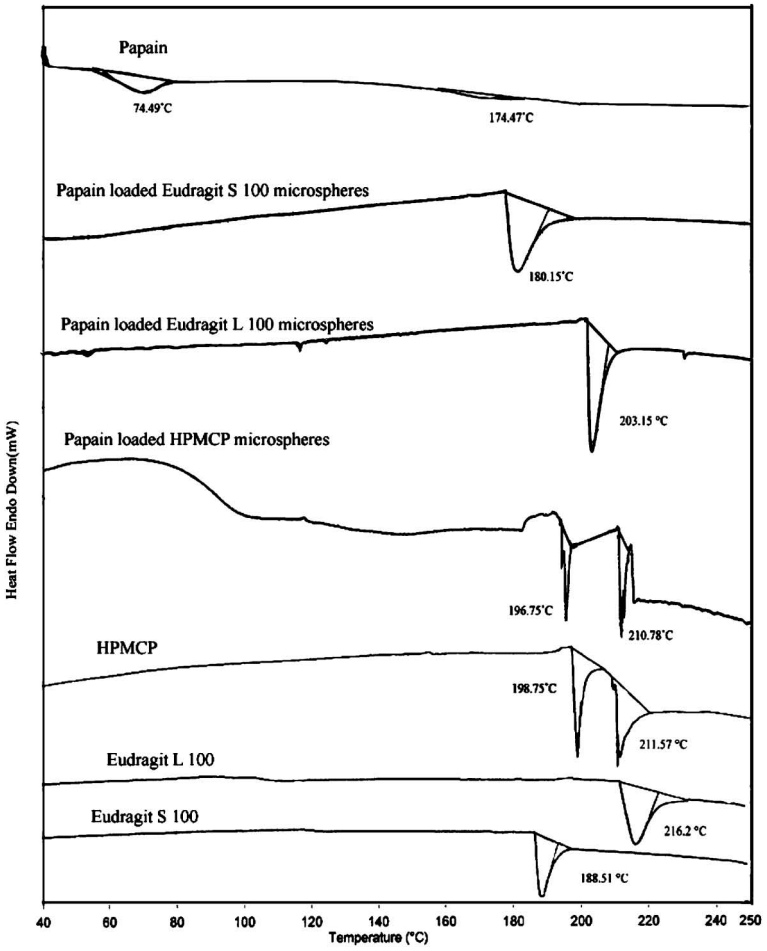

Fig. 5. The DSC Thermograms of Eudragit S 100, Eudragit L 100, HPMCP, Papain Powder and Optimised Formulations of Papain Loaded Eudragit S 100 (SM_4), Eudragit L 100 (LM_4) and HPMCP (HM_4) Microspheres

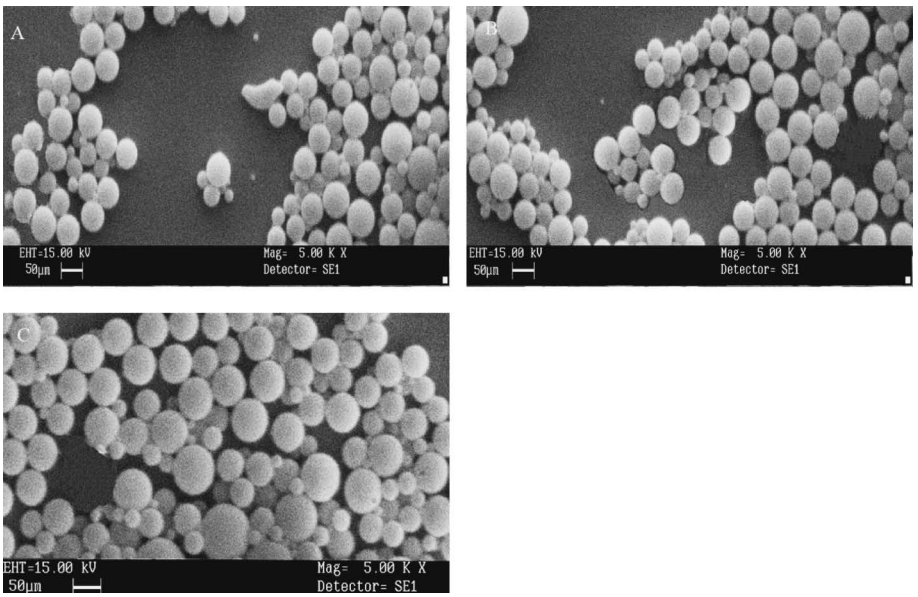

Fig. 6. SEM Photographs of Optimised Formulations of Papain Loaded HPMCP (A), Eudragit L 100 (B) and Eudragit S 100 Microspheres (C)

Table 4. Stability of Free Papain and Papain Loaded Microspheres of HPMCP (HM_4), Eudragit L 100 (LM_4) and Eudragit S 100 (SM_4) under Accelerated Storage Conditions (40±2℃/75±5% RH)

Formulation code	% Papain activity remaining				K_{calc}
	0 D	6 W	3 M	6 M	$(day)^{-1}$
Papain	100.00±2.26	91.93±3.20	82.78±3.19	73.58±2.46	1.71×10^{-3}
HM_4	100.00±4.19	98.10±3.19	95.42±5.45	92.09±3.13	4.58×10^{-4}
LM_4	100.00±3.77	98.05±3.63	95.97±3.26	93.65±2.42	4.15×10^{-4}
SM_4	100.00±3.11	97.69±3.15	95.13±1.99	92.38±1.80	4.40×10^{-4}

Values are mean±S.D. ($n=3$), W: weeks, M: months, K_{calc}: calculated first-order degradation rate constant.

Table 5. Stability of Free Papain and Papain Loaded Microspheres of HPMCP (HM_4), Eudragit L 100 (LM_4) and Eudragit S 100 (SM_4) under Room Temperature Storage (30±2℃/65±5% RH)

Formulation code	% Papain activity remaining					K_{calc}	t_{90} (Days)	Int_{calc}
	0 D	3 M	6 M	9 M	12 M	$(day)^{-1}$		for 2 years
Papain	100.00±2.26	94.56±2.32	87.42±3.05	79.21±2.87	72.55±3.38	8.79×10^{-4}	118.27	170.95
HM^4	100.00±4.19	98.69±3.00	96.49±3.79	94.82±3.04	92.39±2.43	2.17×10^{-4}	479.50	105.44
LM_4	100.00±3.77	98.66±3.61	96.95±3.46	94.87±3.95	92.62±3.21	2.02×10^{-4}	514.85	104.30
SM_4	100.00±2.96	98.25±3.47	96.69±3.49	94.44±3.33	92.44±3.42	2.15×10^{-4}	482.80	105.29

Values are mean±S.D. ($n=3$), D: days, M: months, K_{calc}: calculated first-order degradation rate constant, t_{90}: time to reach 90% of initial drug concentration, Int_{calc}: calculated initial drug concentration for shelf life (t_{90}) of 2 years.

paneer and milk protein in simulated gastro-intestinal pH condition. Thus, enteric microspheres formulations could serve as potential carrier for oral delivery of papain to facilitate the digestion of peptides/proteins. Stability studies indicated the formulations with around 5% overage would ensure 2 years shelf life at room temperature.

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