



Formulation and *In Vitro* Evaluation of Multiple Emulsion for An Oral Delivery of Cysteine Protease

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Abstract

Multiple emulsion is novel approach of drug delivery system for enhancement of bioavailability and pharmacological activity. It is important to prevent the problem of oral drug delivery system and they are stabilized by using of combination of hydrophilic and lipophilic surfactant. In this project the most critical challenge in oral delivery of ficin is to preserve the formulation during its passage through the stomach without denaturation. The key to success of digestive proteins as pharmaceuticals is to have in place an efficient site-specific pH dependent drug delivery system that allows the protein to gain access to the target site at the right time and for an appropriate duration.

Keywords

Multiple emulsion, Proteolytic enzyme, Ficin, Eudragit S100 polymer

INTRODUCTION:

Dietary proteins are the essential for growth, repair, and regulation of homeostasis. However, many people are intolerant of such foods, which includes baked beans soup, soyabean, and meat tenderizer. This intolerance can lead to uncomfortable and embarrassing symptoms, such as flatulence, belching, diarrhoea, constipation, malnutrition, food allergies anaemia, undigested food in stool, chronic intestinal parasites, and abnormal flora. These symptoms usually occur during achlorhydria and pancreatin insufficiency. Therefore, the need for a

protein – digesting supplement arises. Now a days, the demand for digestive aids has increased, but the supply of pepsin has decreased. Thus plant – derived proteases like papain and ficin is a food grade, highly active endolytic cysteine protease derived from carica papaya and *ficus recemosa* respectively. Its broad substrate specificity and ability to hydrolyse small peptides as well as large peptide protein make ficin and papain an ideal enzymatic supplement. enzymes that play the central role in protein degradation by hydrolysing peptide bonds were known as “proteases” or “peptide hydrolase”.



Fig.No.1: Ficus Racemosa plant

MATERIAL AND METHODS:

Materials used for research work were as shown in Table no .1.

Instruments used for research work were as shown in Table no 2.

Table No: -1: List of materials and chemicals

Name	Supplier
Ficin powder as a proteolytic agent obtained from Raw latex of ficus raecmosa	
Ethyl Acetate	Rankem Pvt. Ltd.
Methanol	Rankem Pvt.Ltd. Mumbai
Eudragit S 100	Alkem Pvt. Ltd. Mumbai
Trifluoroacetic acid	S.D. Fine chemicals Mumbai
Potassium Dihydrogen Phosphate	S.D. Fine chemicals Mumbai
Sodium hydroxide	S. D. Fine chemicals Mumbai
HCL	S.D. fine chemicals Mumbai
Polysorbate20	S. D. Fine chemicals Mumbai
Distilled water	Lab
Disodium EDTA	S. D. Fine chemicals Mumbai
Cysteine hydrochloride	S.D. Fine chemicals Mumbai
Citric acid	S.D. Fine chemical Mumbai
Polyvinyl alcohol	S.D. Fine chemical Mumbai
Lactose	S. D. Fine chemicals Mumbai
Ethanol	Rankem Pvt.Ltd.
Dichloromethane	S.D. Fine chemicals, Mumbai
Isopropanol	S.D. Fine chemicals, Mumbai

Table No: -2: List of Equipment

INSTRUMENT	Model	Manufacturer
UV-Visible Spectrophotometer	UV-1800	Shimadzu
Hot –air oven	BTI29	Bio technique, India
Electronic Balance	BL-220H	Shimadzu Co. Japan
FT-IR	IR-Affinity	Shimadzu Co-Japan
Brookfield viscometer	DV3T02346	Brookfield, USA
Magnetic stirrer	IML	Remi India Ltd.
Stability chamber	GMP	LABLINE
Probe sonicator	F-250	Frontile Sonicator
Digital Weighing balance	AA-2200	ANAMADE Instrument
Centrifugation	-	Biotra centrifuge
pH meter	MK VI	Systronics
Freezer	GL-A82SPZL	LG Refrigerator

Methods: Solvent evaporation method.

Table No: -3: Formula of Multiple Emulsion

Material	Functional category	Formulation Batches (%w/v)								Phases volume
		ME1	ME2	ME3	ME4	ME5	ME6	ME7	ME8	
		0.2		0.4		0.2		0.4		
Cysteine protease ficin	Proteolytic Agent	0.0199	0.199	0.398	0.398	0.199	0.199	0.398	0.398	W1 0.4 ml Internal aqueous phase
Polysorbate 20 3% w/v	Primary emulsifier	0.006	0.006	0.012	0.012	0.006	0.006	0.012	0.006	
Lactose 0.16% w/v	Cryoprotectant	0.00032	0.00032	0.00064	0.00064	0.00032	0.00032	0.00064	0.00064	
D: E: I 5:6:4	Solvents	5	5	5	5	5	5	5	5	O Organic Phase 5ml
Eudragit S 100	Acrylic polymer	0.1	0.1	0.2	0.2	0.1	0.1	0.2	0.2	
PVA1% w/v solution	Stabilizer	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	W2 External aqueous phase 25ml
Lactose 1.6% w/v	Cryoprotectant	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5	30 ml
Total										

D: E: I – Dichloromethane: Ethanol: Isopropanol

EXPERIMENTATION

Method of preparation: Multiple emulsion was prepared by two step emulsification process: a) Preparation of primary emulsification b) Secondary emulsification.

Procedure: 10 ml of distilled water containing an aqueous ficin at a concentration of 2851 mg/ml was prepared. The internal aqueous phase (W1, 0.4ml, 0.2 ml) containing ficin, 3% v/v polysorbate 20 (dispersing agent), and 0.16% w/v lactose as a cryoprotectant was emulsified with 5 ml of organic phase for one minute using an ultrasonic disrupter. Temperature was maintained at 4°C, using an ice bath during emulsification. The organic phase consisted of 200 mg of polymer Eudragit S 100 in 5 ml of a mixed solvent system of dichloromethane, ethanol, and isopropanol in ratio of 5:6:4. The resulting primary emulsion (w₁/organic phase) was added drop by drop to the external aqueous (W2 25 ml) of 1% W/V polyvinyl alcohol and 1.6 % w/v lactose solution. Emulsification was continued using

an ultrasonic disrupter for two minutes to form a multiple emulsion (w₁/organic phase/w₂) at 4°C in an ice bath.¹

Determination of absorption maxima (λ max) for Ficin:

The solution of Ficin was found to exhibit maximum absorption at 223nm (shown in Fig 7.5) in phosphate buffer pH6.8 after scanning on the spectrophotometer which was reported as absorption maximum.

Calibration Curve of Ficin

A standard solution of 100µg/ml is prepared by dissolving 10 mg of Ficin in 100 ml pH 6.8 phosphate buffer. From the above stock solution, aliquots of 1-5 ml were withdrawn and were diluted with pH 6.8 phosphate buffer up to 10 ml to get solutions of 10, 20, 30, 40, 50 µg/ml concentrations respectively. The resultant dilutions were analysed by UV at 223 nm and absorbance values were noted for each dilution. Finally, a graph of absorbance V/s concentration was plotted.

Table No: -4: Data for calibration curve of Ficin in phosphate buffer pH 6.8.

Sr. No.	Concentration $\mu\text{g/ml}$	Absorbance at 223 nm
1)	10	0.159
2)	20	0.257
3)	30	0.376
4)	40	0.458
5)	50	0.458

Table No: -5: FT-IR Interpretation

Components	Observed peak	Standard peaks	Functional group
Ficin	1285.30	1350-1000	C-N
	3454.51	3500-3100	N-H
	1265.30	1550-1350	N=O
	1145.75	1300-1000	C-O
	3390.86	3400-2400	O-H
	2357.01	2550	S-H
	1614.42	1725 -1700	COOH
	3441.01	3000-2850	CH ₃
	1232.51	1465	CH ₂
	3371.27	3500-3100	NH ₃

Table No: -6: PH of multiple emulsion formulation

Sr. No	Formulation code	pH
1	ME1	6.2
2	ME2	6.7
3	ME3	6.2
4	ME4	6.8
5	ME5	6.7
6	ME6	6.8
7	ME7	6.7
8	ME8	7.2

Table No: -7: Melting point of formulation

Drug	Melting point
Emulsion	118°C

Table No: -8: Optimization of stirring time for primary and secondary emulsion.

Batches	Stirring time for primary emulsion	Stirring time for secondary emulsion 5min	% phase separation
ME1	35	5	3.44
ME2	30	5	3.72
ME3	25	5	3.44
ME4	25	3	No phase separation
ME5	25	6	3.44
ME6	25	3	No phase separation
ME7	25	2	4
ME8	25	3	No phase separation

Table No: -9: Optimization of stirring speed for primary and secondary emulsion.

Batches	Stirring speed for primary emulsion 30 min	Stirring speed for secondary emulsion 5min	% phase separation
ME1	1500	600	4
ME2	2000	600	3.46
ME3	2500	600	3.87
ME4	3000	600	3.46
ME5	3500	600	3.74
ME6	4000	600	0
ME7	4500	600	371
ME8	4000	600	0

Table No: -10: % Entrapment efficiency of Multiple emulsion

Sr. No.	Formulation	% Entrapment Efficiency
1	ME2	87.54
2	ME4	83.85
3	ME 6	88.15
4	ME8	87.10

Table No: -11: Zeta potential

Formulation	Zeta potential (mV)
Multiple emulsion ME8	-21.71

Table No: -12: % ficin content

Formulation Batches	Ficin content
ME2	0.012
ME4	0.015
ME6	0.017
ME8	0.019

Table No: -13: Partition coefficient

Sr. no	Solvent system	Partition coefficient
1	n-toluene/ distilled water	0.356(Hydrophilic)

Table No: -14: In-vitro ficin diffusion study

Time (hour)	Formulations % ficin diffusion				
	ME2	ME4	ME6	ME8	Marketed
1	3.60	1.38	2.55	4.82	3.12
2	8.37	3.31	6.706	9.70	8.20
3	16.74	6.54	12.01	14.99	17.19
4	28.48	11.58	18.54	22.44	27.41
5	43.68	17.64	25.86	30.57	39.45
6	60.63	24.41	34.79	40.60	53.81
7	78.20	32.37	43.86	51.55	69.86
8	99.28	41.09	54.86	63.64	86.30

Table No: -15: % Entrapment efficiency of Multiple emulsion

Sr. No.	Formulation Batches	% Entrapment Efficiency
1	ME2	91.04
2	ME4	94.27
3	ME 6	91.48
4	ME8	95.38

Table No: -16: Melting point of formulation

Formulation Batch	Melting point
Multiple Emulsion 6	118±10c

Table No: -17: Partition coefficient

Sr.No	Solvent system	Partition coefficient
1	n-toluene/ distilled water	0.356

Table No: -18: After Stability Evaluation of Multiple Emulsion formulation

Sr. No	Parameter	Temperature conditions		
		4 ⁰ -5 ⁰ C	25 ⁰ ±2C	40 ⁰ ±2C
1	Physical appearance	No change	No change	No change
2	pH	6.8±0.04	6.8±0.01	6.8±0.03
3	Viscosity	18000±107	18000±100	18000±100
4	Ficin Content	0.017±0.1	0.017±0.1	0.017±0.3
5	Entrapment efficiency	91.48±0.2	91.48±0.1	91.48±0.4

(*Mean ± SD n=3)

RESULT AND DISCUSSION

Characterization of Ficin by FT-IR:

Ficin is the proteolytic enzyme which come under the class of endolytic cysteine protease, cysteine containing functional group N-H- having peak range 3500-3100, CH₂-1465, CH₃-3000-28500, COO-1725-1700, SH – 2550, NH-33500-3100.

Viscosity: Viscosity of multiple emulsion follows the non-Newtonian flow.

pH: pH of all prepared multiple emulsion formulation was determined by using digital pH meter. pH values of multiple w/o/w emulsions

containing ficin at to range from 7.2± 0.03 to 6.8±0.04 in samples kept at room temperature. For samples kept at 40⁰c, the pH ranged from 7.2±0.04 to 6.8±0.01 for ME8 and ME6 respectively.

Melting point analysis

Melting point of pure drug was found to be 118⁰c with the help Thieles tube containing liquid paraffin and capillary containing formulation.

% Phase separation: Formulations (ME1 to ME8) were stored for one week and observed the % phase separation, which was determined by following equation

$$\% \text{ Phase Separation} = \frac{100 (V_{\text{sep}})}{\text{Vol. of batch}} \left[\frac{(V_1 + V_2)}{(V_1 + V_2 + V_0)} \right]$$

V₁, V₂, V₀ represents the volume of internal phase, dispersed phase, and middle oil phase respectively.

Entrapment efficiency: Percentage entrapment efficiency is indicative of the percentage of crude drug that has been encapsulated within the polymer powder with respect to the feed drug concentrations. The result of percentage entrapment efficiencies as shown in table no. 7.11 it was found that Ficin could be entrapped with high efficiencies in the formulation due to its lipophilic nature. The average entrapment of Ficin in the formulated multiple emulsion was found to be 87.10 calculated in triplicates.

Zeta potential determination:

Zeta potential of the multiple emulsion formulation found to be (shown in fig 7.9) which indicates that formulation is stable.

Zeta potential of the multiple emulsion was analysed by using Malvern zeta sizer. Multiple emulsion was taken in cuvette diluted with distilled water and analysed. Zeta potential observed-21.71 mV, which

makes multiple emulsion stable due to the negative charge present.

Particle size analysis:

Size distribution analysis was done to determine average particle size of multiple emulsion. The average particle size of optimized multiple emulsion was found to be 55.65 d.nm. From the results of particles size analysis (Figure No.7.10) it can concluded that the particle size of multiple emulsion is homogenous having polydispersity index is 0.391.

Ficin content determination:

A 0.5 gm of the prepared multiple emulsion was taken in centrifuge tube containing 5 ml of buffer. Shake the tube for 2 min. 1ml of that stock was withdrawn diluted to 100 ml with buffer and filtered. Total ficin content was determined by UV spectrophotometer at 223nm.

Partition Coefficient: Partition Coefficient studies are carried out to find out extent of drug transfer in the aqueous and the other non-aqueous layer. This phenomenon usually is done to obtain the drug concentration in either layer. Partition coefficient value of ficin also revealed its hydrophilic nature which is given in following.

In- vitro diffusion study: In vitro release profiles of ficin from its various multiple emulsion formulations are represented in following table 7.15. The higher drug release was observed with formulations ME8 and ME6 at pH7 after 8 hours this may be due to presence of maximum conc. of polymer as compared to other formulation.

Entrapment efficiency:

Percentage entrapment efficiency is indicative of the percentage of crude ficin that has been encapsulated within the polymer powder with respect to the feed ficin concentrations. The result of percentage entrapment efficiencies as shown in table no. 7.10 it was found that less internal aqueous phase having high efficiencies in the formulation ME6, the average entrapment of Ficin in the formulated multiple emulsion was found to be 91.48% this batch shows good result as compared to other batches.

Final optimization of Batch:

Formulation batch was optimized on the basis better results of Ficin content, Entrapment efficiency and In vitro diffusion study which showing better results as compared to other batches so batch ME 6 was optimized.

Melting point analysis: Melting point of cysteine protease in batch ME6 was found to be $118 \pm 1^\circ\text{C}$.

Zeta potential:

Zeta potential of the batch ME6 multiple emulsion formulation is found to be (shown in Fig 7.11) which indicates that formulation is stable. Zeta potential of the multiple emulsion was analysed by using Malvern zeta sizer. Zeta potential observed -21.71 mV, which makes multiple emulsion stable due to the negative charge present. The general dividing line between stable and unstable suspensions is generally taken at either + 30 or -30 mV. Particles with zeta potentials more positive than +30 mV or more negative than -30 mV are normally considered stable.

Particle size analysis:

Size distribution analysis was done to determine average particle size of batch ME6 multiple emulsion. The average particle size of optimized multiple emulsion was found to be 55.65 d. nm and having polydispersity index 0.391 indicate its almost homogeneity. The standard range of polydispersity index is 0.15-0.3 indicates reasonable size homogeneity.

Partition coefficient: Partition coefficient studies are carried out to find out batch ME 6 extent of cysteine protease ficin transfer in the aqueous and the other non-aqueous layer. Partition coefficient value of ficin also revealed its hydrophilic nature which is given in following table 7.14.

Stability study:

Stability study of optimized batch ME6 formulation containing *ficus racemosa* latex powder as proteolytic agent was evaluated, and this study was performed in three different conditions i. e refrigerator, room temperature and oven on 30 days. On these days' time interval were re-evaluation test physical appearance i e colour, odour, and liquefaction, pH, and viscosity, ficin content and entrapment efficiency test was given. The results are showing in following table no 18.

Batch ME6 was selected based on result which showing good viscosity, optimum pH, Ficin content, and after stability study there was no change in above mentioned parameter According to this result optimized batch was successfully evaluated the stability study.

SUMMARY:

From the first day of collection and purification of raw material i.e. natural latex, the physical appearance suggested that the latex has milky fluid containing proteolytic enzyme which are used in digestion of protein or breakdown of protein. *Ficus racemosa* grows all over India in many forests and hilly areas. It is frequently available around water streams and is also cultivated.

Proteolytic enzymes have great medical and pharmaceutical importance due to their key role in biological process and in the life- cycle of many pathogens. Proteases are extensively applied enzymes in several sectors of industry and Biotechnology, peptide synthesis, digestion of unwanted proteins during nucleic acid purification, cell culturing and tissue dissociation, diagnostic and therapy, Proteolytic digestion of proteins in proteomics.

As ethyl acetate and methanol are used for purification to form free flowing powder characterized by physicochemical property.

In the second phase of research, the formulation development and evaluation of oral drug delivery of Proteolytic enzyme multiple emulsion formulation latex powder i.e. ficin used as active moiety ME6 batch was optimized as compared to other batches having polymer emulsifier ratio is optimum and showing the drug release maximum, stable at different temperature and homogeneous dispersion, showing better result of viscosity, pH.

CONCLUSION:

On comparison formulation Batch ME6 can be considered as the best formulation for the preparation of multiple emulsion using Eudragit S 100 polymer, prepared emulsion having small particle size, good stability and maximum entrapment efficiency. The drug release was also sustained upto 8hrs. So batch ME6 pH is compatible with intestine pH and formulation release in the colon because of maximum absorption occur due to large surface area.

Ficin shows extensive proteolytic activity towards proteins, short chain peptides, amino acids, in the field of food and medicine make an ideal enzyme supplement.

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