RESEARCH PAPER

Received 17th April 1996
Indian J. Pharm. Sci., 1997,59(1)pp. 13-17

Amylase Immobilised in Water-in-oil-in-water based liquid Surfactant Membrane

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An effort was made to immobilise amylase, a digestive enzyme in liquid surfactant membrane. The system was based on typical w/o/w emulsions. Organic membrane was composed of cotton seed oil containing additives such as Calcium, Stearate, lecithin and Cetyl Alcohol. The incorporated additives facilitated the selective transport of substrate i.e. starch to the internal aqueous phase containing amylase. However, the enzyme remained immobilised in the internal aqueous phase. The activity and the stability of the system(s) were found to be related to water : oil ratio in primary emulsion and incorporated additives. The system(s) were subjected to stability studies at different temperature conditions. The efforts of ageing on in vitro activity and viscosity of different system(s) was observed. The system(s) were found to be effectively stable at room temperature.

ENZYMES were used in various biotechnological processes, in replacement therapy after surgical removal of digestive organs as well as in digestive system disorders[1,2]. Enzyme immobilisation has become an important area in the field of biocatalysis and medicine as a way to reduce the production cost and enhancing enzyme stability. Several methods have been introduced and improved, including adsorption on or covalent binding to polymer or porous supports, inter or intramolecular cross-linking with bi- or multifunctional reagents, entrapment in microcapsules, immobilisation in solid membrane reactors and reverse micelle systems[3-5].

Immobilisation technique utilising liquid surfactant membrane technology was first described by Li[6] in 1986. Several characteristics of liquid membranes and enzyme emulsions; including transport effect, membrane stability and process modelling have been extensively reviewed and discussed[7]. Enzyme emulsions combine specific enzymatic reactions with selective transport through the organic phase which is achieved by additives. The transport generally depends on the chemical behaviour of permeants and is independent of their molecular weight.

The objective of present study was to develop a system containing immobilised enzyme in internal aqueous phase of multiple emulsion which could act as a circulating carrier and provide the enzyme whenever needed by the substrate.

EXPERIMENTAL

Materials

Amylase (A & B mixture) was obtained from Rallies (India) Ltd. Soya lecithin was procured from Sigma Chemical Co., St. Louis, USA. Starch was obtained from Loba Chemie, Bombay. All other ingredients used were of A. R. grade (B.D.H.), unless otherwise stated.

Test for starch permeability

The permeability of starch through oil phase (cotton seed oil) was determined by inverted U tube method[5]. One arm of the tube was filled with starch solubilized in water and other arm with phosphate

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buffer saline (PBS) pH 7.4. The Curved space joining two arms was filled with oil to be tested. The effect of various additives viz., stearic acid, cetyl alcohol,span 80, lecithin, calcium stearate and glyceryl tristearate on permeability behaviour was elucidated (Table 1). The amount of starch permeated was determined by colorimetry[8].

Preparation of multiple emulsion

The emulsions were prepared by the method suggested by Adeyeye and Price[9]. The primary w/o emulsion was prepared using different water to oil ratios viz., 1:1 (1 DME), 1:2 (2 DME) and 1:2 with higher amount of additives (2 DMEH). The oil and aqueous phase (PBS) with emulsifier were stirred at 4000 rpm for 5 minutes. This w/o emulsion was then dispersed in PBS containing 1% w/w Tween 80 at low speed stirring (25 rpm). The prepared emulsions were then characterised for various parameters as discussed below.

Percent drug entrapment

The emulsion was broken by centrifugation at 1000 rpm for 8 min. the aqueous phase was separated and incubated with starch solution at 37° for 1 h. The percent starch digested was determent colorimetrically. Similar activity of equivalent amount of amylase as in aqueous phase of the emulation was determined by incubating with starch solution at 37° for 1 h. The reaction was arrested by adding 1 ml of conc. hydrochloric acid and amount of undigested starch was determined. Then percent amylase entrapped was determined using formula.

$$\% \text{ amylase entrapped} = \frac{\% \text{ starch digested after breaking the emulsion}}{\% \text{ starch digested before emulsification}}$$

The size distribution

The size distribution of emulsion gloubles was determined microscopically using calibrated ocular piece (Leitz, Biomed Microscope, Germany).

Table 1
Effect of additives of Starch Permeability in Oil Phase (Cotton seed oil)

Additives	Permeation
3% w/w span 80+0.1% w/w calcium stearate	No permeation
3% w/w span 80+0.1% w/w cetyl alcohol + 0.1% w/w stearic acid	No permeation
3% w/w span 80+0.1% w/w stearic acid + 0.1% w/w calcium stearate + 0.1% w/w glyceryltristearate	Trace amount
3% w/w span 80 + 0.1% w/w cetyl alcohol + 0.1% w/w calcium stearate + 0.1% w/w glyceryltristearate + 2% w/w soya lecithin	Large amount

Enzyme leakage through oil barrier

The emulsion was taken in a blunt end glass tube provided with treated cellophane membrance at one end. The tube was dipped in a beaker containing phosphate buffered saline pH 7.4. The beaker was kept on a magnetic stirrer at 37%. The samples were withdrawn periodically at intervals of 0.5,2,4,6,12 and 16 h and were incubated with starch solution. The amount of starch undigested was then determined.

In vitro enzyme activity

Twenty ml of the multiple emulsion was incubated with starch solution. The mixture was stirred moderately with help of magnetic stirrer. The samples were withdrawn periodically and amount of starch undigested was determined.

Stability studies

The effect of ageing and temperature was studied on the viscosity of emulsions. The emulsions were kept at 5±2° and 38±2°. The viscosity was measured at different time intervals (Brookfield viscometer).

Table 2
Characterization of Different Emulsions

Emulsion	Percent Amylase entrapped ± S.D.*	Average globule size (μm) ± S.D.*
1 DME	65.0 ± 2.0	7.10 ± 0.5
2 DME	61.0 ± 0.95	7.88 ± 0.7
2 DMEH	56.8 ± 1.3	7.36 ± 0.4

* n = 3

Table 3
Effect of Ageing on Viscosity

Temperature °C	Emulsion	Viscosity in cps after time (days)					
		0	7	14	21	28	35
	1 DME	51.2	50.2	49.4	48.8	48.6	48.3
5 + 2	2 DME	50.4	49.8	49.2	48.6	48.2	47.9
	2 DMEH	50.3	49.4	48.9	48.4	48.0	47.2
	Time (days) ⟶		1	2	3	4	5
	1 DME		51.2	48.8	48.0	46.4	46.0
38 + 2	2 DME		50.0	48.0	47.0	45.6	43.2
	2 DMEH		49.6	47.2	46.4	44.7	42.6

The effect of ageing on sedimentation volume ratio was studied. The height of emulsion sediment was noted at different time intervals and sedimentation volume ratio was calculated using formula Vu/Vo, where Vu=ultimate volume and Vo=original volume. The effect of ageing and temperature on enzyme activity was also elucidated. The emulsions were kept at 5±2° and 38±2°, samples were withdrawn and activity was recorded after incubating the withdrawn sample with starch solution and determining the amount of starch digested.

RESULTS AND DISCUSSION

Multiple emulsion of amylase was successfully formulated. Cotton seed oil was selected as oil phase because of its biocompatibility. The effect of various additives on permeation of starch through oilphase was studied. Span 80 (3% w/w) was used as emulsifier, calcium stearate and soya lecithin were found to enhance permeation of starch through oil phase. Cetyl alcohol acted as viscosity enhancer and helped in immobilisation of amylase in internal aqueous phase. Lecthin and calcium stearate probably enhanced starch permeability due to reverse micellar orientation. Percent entrapment of amylase in emulsion was determined (Table 2). 1 DME emulsion was found to have maximum entrapment (65%) followed by 2 DME (61%) and 2DMEH (57%). Average globule size was recorded to be 71.9 μm in 1 DME emulsion, 7.88 μm in 2 DME and 7.36 μm in 2 DMEH emulsion respectively (Table 2).

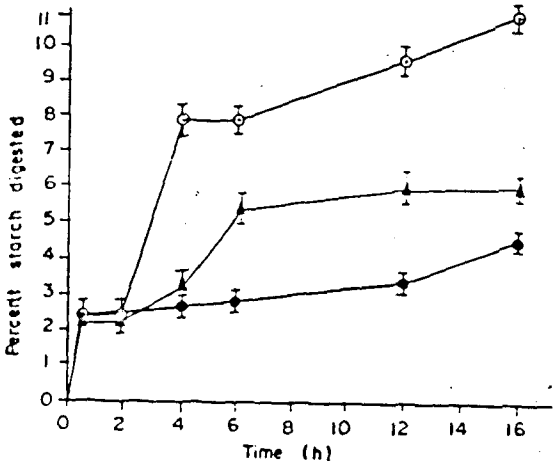

Fig. 1: Enzyme Leakage Into External Outer Phase of Emulsions
O–O 1 DME Δ—Δ 2 DME O—O 2 DMEH

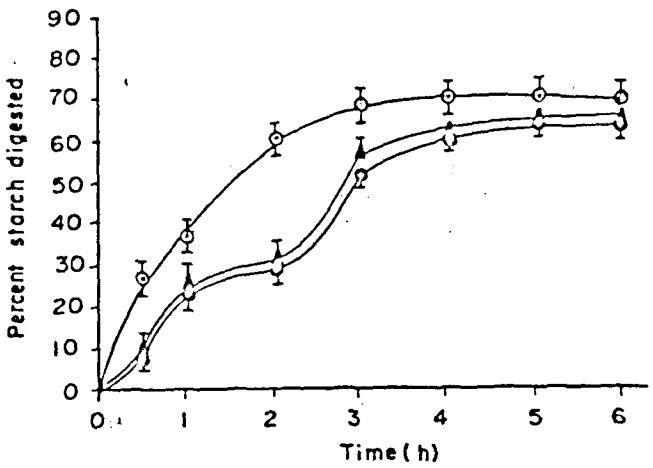

Fig. 2 : In Vitro Enzyme Activity of Different Emulsions
O–O 1 DME Δ--Δ 2DME O--O 2 DMEH

The thickness of oil layer was less in 1 DME emulsion and so the globule size. Increased amount of surfactant resulted in decreased globule size probably due to dispersion of oil and water to form smaller primary emulsion globules.

Enzyme leakage profile of different emulsions was studied (Fig.1). 1 DME emulsion showed maximum leakage possibly due to thin oil barrier in comparison to 2 DME or 2 DMEH emulsions. However, incorporation of sodium alginate, gelatine (gelling agents) and cetyl alcohol (viscosity enhancer) resulted in decreased leakage. This can be attributed to retardation of partitioning of enzyme into the outer external phase.

In vitro enzyme activity of different emulsions was determined (Fig.2). 1 DME emulsion was found to be more active than 2 DME or 2 DMEH emulsions. 1 DME emulsion digested 72% starch in 6 h in comparison to 2 DME emulsion, which digested 68% and 66% of starch in 6h respectively. This is possibly due to the fact that substrate (starch) has to travel a shorted path (thin oil barrier) in case of 1 DME emulsion. 2 DMEH emulsion exhibited marginally less activity than 2 DME, because larger amount of surfactant probably retarded the transport of starch across oil barrier to reach the enzyme.

Effect of ageing on viscosity (in centipoise) of the emulsion was observed at 8±2° and 38±2° temperature (Table 3).

The viscosity of emulsions invariably changed at both temperature but the change was more pronounced at higher temperature. This is probably due to high kinematic energy. Among different emulsion, 1 DME exhibited less change in viscocity at 5±2° possibly due to swelling of globules. The emulsions were more stable at low temperature, probably due to solubility of surfactant and viscosity characteristics of oil phase. At 38±2°, 2 DME emulsion showed more pronounced viscosity change due to large globule size and hence relatively less change in size. Sedimentation volume ratio at 5±2° after 35 days was found to be 0.82 for 1 DME and 0.75 for 2 DME emulsion. The study indicated that 1 DME emulsion is more stable. This can be attributed to smaller globule size in 1 DME emulsion and hence low sedimentation rate. Moreover, change in viscosity of 1 DME emulsion was less which contributed to stability of emulsion.

Effect of ageing on amylase activity was elucidated at 5±2° and 38±2° temperature (Table 4). After storage for 35 days at 5±2°, the activity (amount of starch digested) of 2 DME emulsion was found more then 1 DME emulsion 2 DME emulsion digested

Table 4
Effect of Ageing on Amylase activity

Temperature °C	Emulsion	% amount of starch digested after time (days)					
		1	7	14	21	28	35
	1 DME	70.0	68.0	66.0	65.0	65.0	64.0
5 ± 2	2 DME	68.0	67.8	67.0	66.9	66.5	66.5
	(days)⟶	1	2	4	6	8	10	14
	1 DME	68.0	56.0	42.0	36.0	31.0	28.0	24.0
38 ± 2	2 DME	65.0	63.0	44.0	38.0	33.0	30.0	26.0

67% of starch, while 1 DME emulsion digested 64% of starch. This may be attributed to efficient immobilisation of amylase in case of 2 DME emulsion. At 38±2° remarkable decrease in activity of emulsions was noted. 2 DME emulsion could digest only 26% starch, while 1 DME emulsion 24% of starch after a period of 14 days. The emulsions were more stable at low temperature.

In conclusion the study indicated a relation between amylase activity and emulsion globule size. Where, the letter was noted to be dependent on water-oil ration used in the preparation of primary emulsion. It is inferred that water-oil ration and its optimization is critical in multiple emulsion system stability which could be monitored. Thus, can be utilized in drug release control while globule size viz-a-viz ultimate stabilisation of multiple emulsion system. It was further noted that on comparision, formulation 2 DME was established to be better as far as enzyme activity, and stability are concerned. The system was found substrate responsive, therefore, if long circulatory attributes could be incorporated it can possibly be used as an autoprocessor unit. The system demonstrated interesting results. Thus, holds for promises for their possible use in modern therapeutics.

ACKNOWLEDGEMENT

The authors are thankful to CSIR, New Delhi, for awarding Senior Research Fellowship to C. P. Jain.

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