International Standard Serial Number (ISSN): 2249-6807

International Journal of Institutional Pharmacy and Life Sciences 2(3): May-June 2012

INTERNATIONAL JOURNAL OF INSTITUTIONAL PHARMACY AND LIFE SCIENCES

Pharmaceutical Sciences **Research Article……!!!**

Received: 01-05-2012; Revised; Accepted: 11-05-2012

DECOMPOSITION OF PECTIN AND CROSS-LINKED PECTIN BY ENRICHED HUMAN FECAL CULTURES

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Keywords:

Colon specific oral dosage forms; in-vitro evaluation; pectinates; pectin de-polymerization; human fecal isolates; cross-linked pectin

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ABSTRACT

De-polymerization of gels of pectin by enriched human fecal cultures has been described. Various concentrations of calcium and ferrous pectinates were effectively degraded by enriched cultures under aerobic shaking incubation. Pectin de-polymerase activity was appreciable in culture supernatants and combination of cell extracts with culture supernatants did not improve de-polymerization of pectin. Enriched cultures of two different sources did not show significant difference in de-polymerizing action. Inclusion of enriched human fecal cultures in colonic simulating fluids has been suggested for in-vitro evaluation of colon specific oral dosage forms containing pectin as a component to achieve colon specificity.

INTRODUCTION

The necessity and advantages of colon-specific drug delivery systems have been well recognized and documented. Precise colon drug delivery requires that the triggering mechanism in the delivery system only respond to the physiological conditions particular to the colon. For a formulation to act as an effective colon specific drug delivery system, the primary condition is that a minimum amount of drug should be released in the environment of the upper gastrointestinal tract, *i.e.*, in stomach and small intestine. The normal transit time in the stomach is 2 h (though this may vary); while in the small intestine it is relatively constant and is around 3 h [1]. The usual colonic transit time varies from 20–30 h [2]. Thus, for a dosage form to be effective as a colon drug delivery system, the drug release is required to be retarded in the upper gastro intestinal tract conditions. Thereafter, the drug release should be complete within the next 20–30 h. The predominant species of anaerobic bacteria in the colon are the *Bacteroides, Bifidobacteria, Eubacteria, Clostridia*, and gram-positive cocci [3]. The organisms are known to degrade the polysaccharide excipients and cause release of drug in the colon. However, appropriate methods for in-vitro evaluation of polysaccharide containing dosage forms have not been developed [4]. With this investigation, we aimed to study the decomposition of pectin and cross-linked pectin by enriched human fecal cultures. The objective was to suggest suitable additives for in-vitro evaluation system for colon specific dosage forms with the help of human colonic bacterial isolates.

MATERIALS AND METHODS

Enrichment of bacterial culture: Feces sample from voluntary donors were collected in sterile vials and colon specific bacteria were enriched by inoculating one loopful of sample in sterile Fluid thioglycolate medium supplemented with 1% pectin and incubating for 24-48 h at 37 ºC. Anaerobic bacteria were isolated from yellow portion of the medium and used as a culture bacterium for polysaccharide degradation.

Preparation of Enzyme for pectin degradation: The test culture of colonic bacteria was grown for 48 hrs as described above for induction of appropriate enzymes. Culture was centrifuged at 5000 rpm for 10 min to pellet the cells. Separated culture supernatant and pellets suspended in phosphate buffer (0.02 M, pH 8.0) were used as source of enzyme. Alternatively, pellets were suspended in small volume of buffer, crushed with sterile glass pieces. Cell extracts in combination with culture supernatant were centrifuged to pellet cell debris and supernatant used as source of enzyme after filtration through 0.45 micron filter. Enzyme preparations (1ml) from three methods were separately inoculated to pectin solutions during degradation studies.

De-polymerization of pectin by colonic bacteria: Solution of 2% pectin was prepared in phosphate buffer (0.02 M, pH 8) and 50 ml solution distributed in different flask and sterilized by autoclaving. Appropriately induced fecal culture (5 ml) was inoculated and all the flasks incubate at 37 ºC under shaking condition. At different time intervals, reduction in viscosity was calculated. All the experiments were carried out in triplicates. Appropriate control tube for initial pectin was prepared. Percent reduction in viscosity of pectin was calculated using the formula (C – T/C) x 100 = % Reduction in pectin viscosity; Where, C = Time required for flow of initial pectin solution; T = Time required for flow of de-polymerized pectin.

Liquefaction of pectin by colon specific bacteria: Liquefaction of pectin was studied under both aerobic and anaerobic condition for which, 10% solution of pectin was distributed (10 ml) in different test tubes and sterilized by autoclaving. Culture Sample (3 ml) was inoculated and tubes were incubated under both aerobic and anaerobic condition at 37 ºC for 48 hr and liquefaction observed.

Degradation of beads of Pectin: For preparation of pectinates, solutions of different concentrations (5%, 7% & 10%) of pectin were prepared in tris buffer (0.2 M, pH 8). Beads were prepared by drop wise adding pectin solution in a chilled calcium chloride or ferric chloride solutions of respective concentrations. The beads prepared from 10 ml pectin solution were sterilized by autoclaving, suspended in 5 ml tris buffer (pH 8, 0.02M) and 3 ml of culture inoculated. The tubes were incubated in static and shaking mode under aerobic and anaerobic conditions at 37 ºC for 72 h and degradation of beads observed. The effect of concentration of pectin and enzyme on degradation was studied.

RESULTS AND DISCUSSION

The use of GI microflora as a mechanism of drug release in the colonic region has been of great interest to researchers in recent times.Majority of bacteria are present in distal gut although they are distributed throughout GI tract.Endogenous and exogenous substrates, such as carbohydrates and proteins, escape digestion in the upper GI tract but are metabolized by the enzymes secreted by colonic bacteria [5]. Use of polysaccharides offers an alternative substrate for the bacterial enzymes present in the colon. Most of the polymers used in pharmaceutical compositions and are considered generally regarded as safe (GRAS) excipients. Pectin alone and in combination with other polymers has been studied for colon-specific drug delivery. Pectin, when used alone, was needed in large quantities to control the release of the drug through the core [6]. To initiate the study, slurry of pectin was inoculated with the enzyme preparation from enriched human fecal culture and reduction in viscosity of pectin followed after specified intervals of time.

Table 1:Effect of incubation time on pectin de-polymerization by enriched human fecal samples

Incubation Time	% Reduction in viscosity ± Standard deviation	
	Sample 1	Sample 2
5hr	21.78 ± 1.24	22.32 ± 2.08
24hr	33.50 ± 2.32	32.47 ± 2.01
48hr	37.23 ± 3.70	33.65 ± 2.12
72hr	37.66 ± 0.20	30.70 ± 0.07

Data are mean of three replicates ± Standard deviation

The degradation of polysaccharides was measured by viscometer and percentage reduction in viscosity calculated. The results have been depicted in Table 1. A 22% reduction in viscosity was observed after 5 hr incubation which increased to 34% after 24hr incubation and 37% reduction was noted after 48 hr for sample 1. With sample 2, 22% reduction was observed after 5 hr incubation followed by 32% reduction after 24hr incubation and 34% reduction after 48 hr under aerobic shaking condition. It is clear from the table that peak hydrolysis was attained within 24 hrs and further incubation did not improve the liquefaction of pectin.

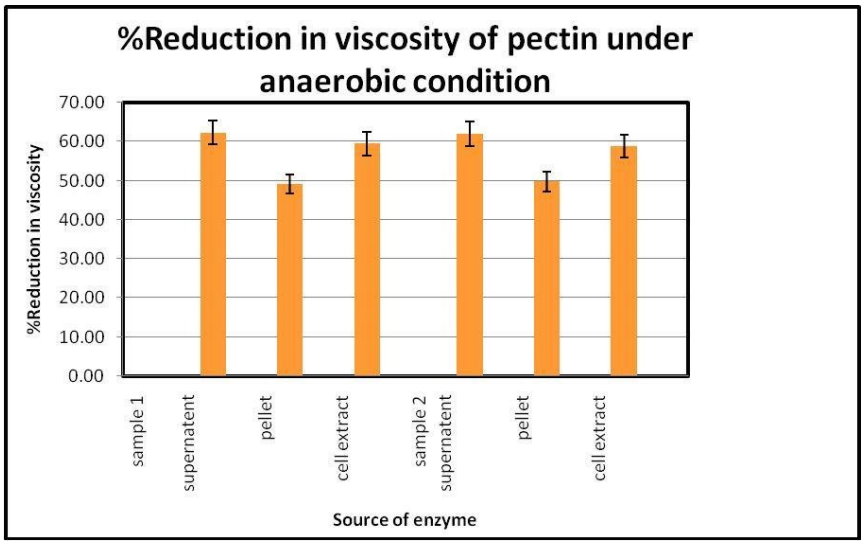

Figure1: Localization of pectin depolymerase activity of cultures.

De-polymerization of pectin was also studied by using different enzyme preparations from the culture in order to localize enzymatic acitivity (extracellular / cell associated) of the culture for pectin degradation. As is obvious from figure 1, a 62 % reduction in viscosity of pectin solution was obtained when culture supernatant was used as a source of enzyme. There was 50 % reduction in flask containing cell pellet as a source of enzyme while 60 % reduction was studied

in a flask containing combined supernatant and cell extract as a source of enzyme after 48hr incubation. The enzymatic activity was equally distributed in cells and culture supernatant and combination did not improve pectin hydrolysis to appreciable extent. Hence it was decided to directly use enriched liquid culture of organism for pectin hydrolysis in further experiments without any preparations.

Table 2: Liquefaction of pectin under aerobic and anaerobic incubation condition

	Aerobic incubation			**Anaerobic incubation**		
	Supernatant	**Pellet**	**Cell extract**	**Supernatant**	**Pellet**	**Cell extract**
Sample-1	++	+	++	+++	+	++
Sample-2	++	+	++	+++	+	++

No. of + designate score of liquefaction

Liquefaction study was also performed by using different enzyme preparation and incubation in aerobic and anaerobic conditions (table 2). Highest degradation was studied in the tube where supernatant were used as a source of enzyme whereas tube containing cell extract as a source of enzyme showed intermediate liquefaction. Lesser liquefaction was observed in the tube containing cell pellet as a source of enzyme. It is clear from the table that culture supernatants could be safely used for pectin hydrolysis under aerobic condition. Hence the enzyme does not require strict anaerobic media for activity, though it is secreted by an anaerobically amplified culture.For in vitro evaluation, any standardized evaluation technique is not available for evaluation of CDDS (Colonic Drug Delivery System) because an ideal in vitro model should posses the in-vivo conditions of GIT such as pH, volume, stirring, bacteria, enzymes, enzyme activity, and other components of food. Generally, these conditions are influenced by the diet, physical stress, and these factors make it difficult to design a standard in-vitro model. Most of the evaluation methods involve addition of rat caecal / fecal contents [7] which might not imitate the conditions of human colon. Use of purified enzymes has been recommended [8], but optimization of amount of enzyme and cost of enzymes affect the economy of the process. Some other methods describe use of human fecal contents with anaerobic incubation [9] which adds to complications of the evaluation method.Above tables and figures clearly suggest that enzymes secreted by the colonic organisms are capable of degradation of pectin under aerobic conditions. Hence this proves that the anaerobically grown culture of human fecal samples is suitable and can be utilized as supplements in the in-vitro evaluation methods under aerobic incubation for studying the release of drugs from colon specific oral dosage forms containing pectin.

The polysaccharide under study, i.e. pectin was gelled in presence of various gelling agents. The concentration of polysaccharide and gelling agents were varied in order to generate beads of varying hardness. Gels of pectin as calcium and ferric pectinates (Ca- and Fe- pectinates) of varying concentration were formed and their degradation by anaerobically enriched human fecal cultures under aerobic and anaerobic incubation in shaking and static conditions was studied.

Figure 2 shows the results of degradation of beads of calcium and ferrous pectinate pectin under aerobic condition. Under aerobic condition beads of calcium pectinate showed appreciable degradation as compared to beads of ferrous pectinate under both static and shaking condition. Similar degradation was observed for beads of calcium pectinate and ferrous pectinate under anaerobic condition with static incubation (table 3).

Table 3: Degradation of pectinate gels (5 %) by enriched cultures of human fecal samples

	Under Aerobic condition				**Under Anaerobic condition**	
	Static		**Shaking**		**Static**	
	Ca-pectinate	**Fe-pectinate**	**Ca-pectinate**	**Fe-pectinate**	**Ca-pectinate**	**Fe-pectinate**
Sample 1	+++	++	+++	+	++	++
Sample 2	+++	+	+++	++	++	++

+ (score of degradation)

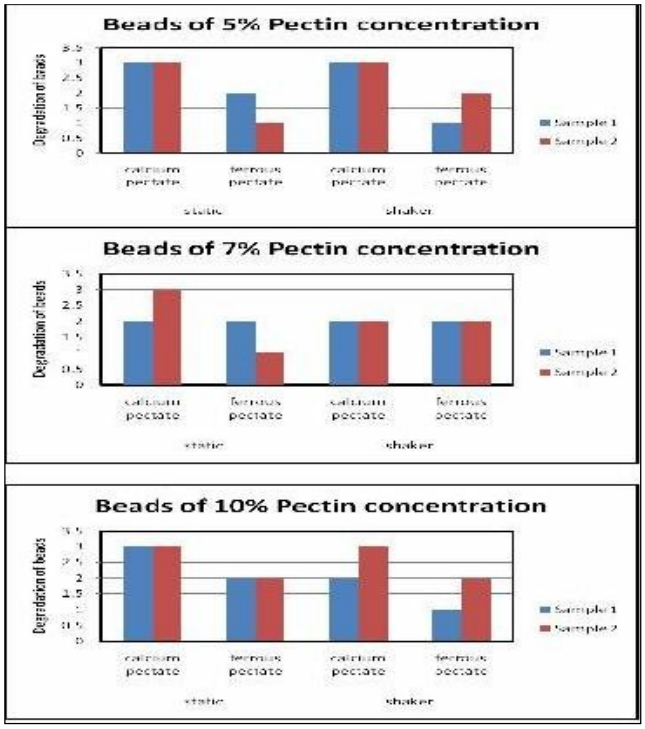

Fig.2: Score of degradation of beads of different concentration of calcium and ferrous pectinate under aerobic incubation.

The result indicated the effect of substrate concentration where the enzyme showed lesser degradation of higher concentration of pectin. Gel density and hardness increases with increasing concentration of gel. This might result into reduced degradation at higher concentration of gel. Degradation of 5 %, 7 % & 10 % beads of calcium and ferrous pectinate were studied under anaerobic and static incubation. It is obvious from table 4 that after 72 h incubation, almost similar degradation was observed in all the tubes.

Table 4: Effect of concentration on degradation of pectin beads under anaerobic and static incubation by enriched human fecal samples

Concentration	**5 %**		**7 %**		**10 %**	
	Ca-pectinate	**Fe-pectinate**	**Ca-pectinate**	**Fe-pectinate**	**Ca-pectinate**	**Fe-pectinate**
Sample 1	++	++	++	++	++	++
Sample 2	++	++	++	++	++	++

Degradation was also studied by varying enzyme concentration where 5 % and 7 % Ca- and Fe-pectinate beads were incubated with different amount of enzyme (5 ml / 7 ml). The results are shown in figure 3 and 4. It is evident from the figures that beads of calcium pectinate showed complete degradation with 7 ml enzyme. Ferrous pectinate beads showed comparatively less degradation when compared with calcium pectinate beads.

It should be noted that the beads of pectin were effectively degraded by enriched samples of human feces during present study. Most of the polysaccharide based colon specific drug delivery systems employ polymer matrices or coatings to achieve colon specificity. The swollen polymers have been effectively degraded by the human fecal cultures under aerobic shaking conditions and pose strong candidates for inclusion in the colonic simulating fluid for in-vitro evaluation of such dosage forms. These cultures closely mimic the colonic environment and are easy to develop and maintain. It should be noted that the two human fecal samples used throughout the study did not show much difference in response indicating the organisms show similar activity regardless of the source of isolation. Hence inclusion of anaerobically amplified enriched cultures of human feces in colonic simulating fluid shall yield observations that would be close to in-vivo evaluation methods.

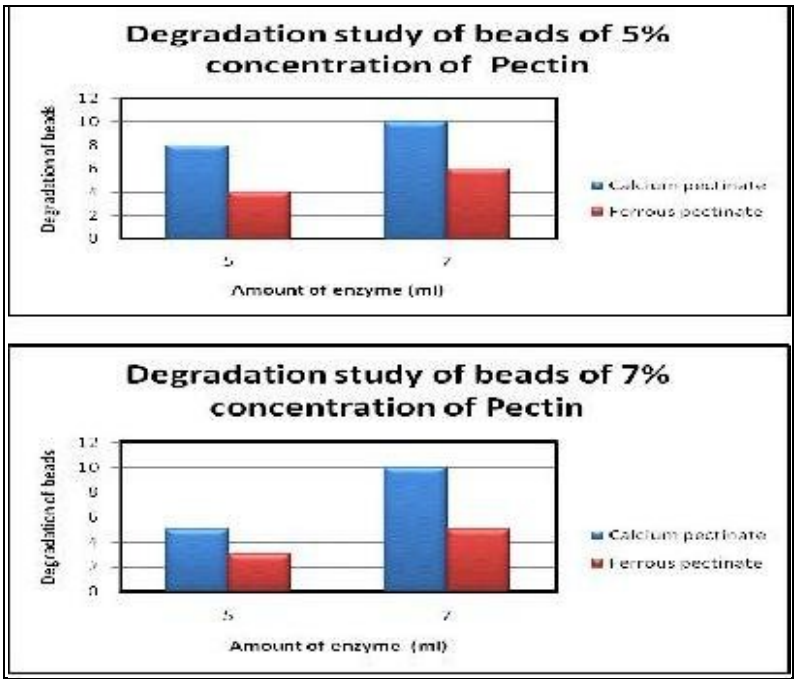

Figure 3: Effect of amount of enzyme on degradation of pectin beads.

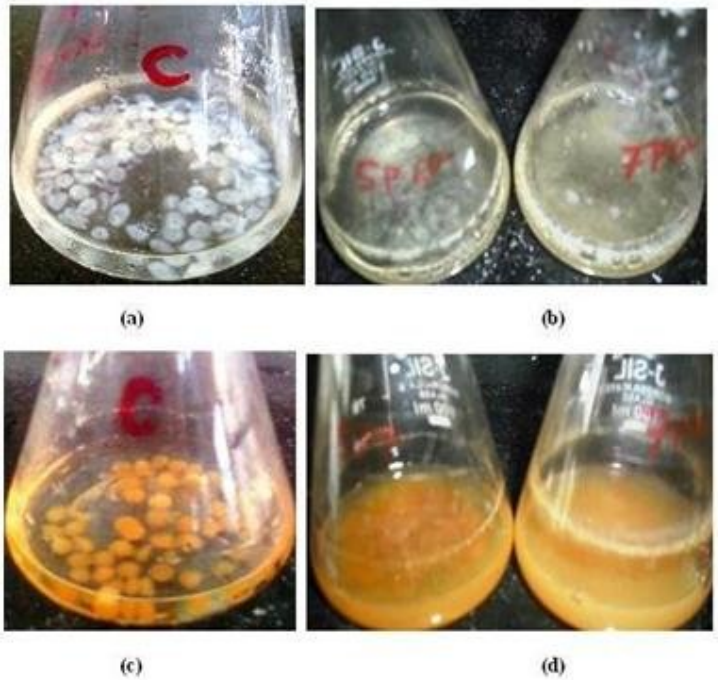

Figure 4: Degradation of beads of pectin under aerobic shaking condition

(a) Calcium pectinate beads before incubation with fecal samples

(b) Calcium pectinate beads after incubation with fecal samples

(c) Ferrous pectinate beads before incubation with fecal samples

(d) Ferrous pectinate beads after incubation with fecal samples

CONCLUSIONS

Liquid culture of enriched human fecal samples can be directly utilized for pectin hydrolysis. The human fecal samples after appropriate enrichment and amplification under anaerobic incubation could be efficiently used for de-polymerization of the above mentioned polysaccharide under aerobic condition. Hence, they can be used for the in-vitro evaluation systems that preferably and conveniently operate under aerobic conditions. The beads of gels of pectin could be efficiently degraded by the enriched cultures of human fecal samples. The nature of gelling agent influenced the degradation of the gel. Degradation of polysaccharide gels by human fecal cultures decreased on increasing concentration of the polysaccharide which suggests that proper optimization of enzyme (culture) concentration is required during setting up of the in-vitro evaluation system.

ACKNOWLEDGEMENT

Authors acknowledge the infrastructure, chemical, computational facility provided by Charutar Vidya Mandal, Vallabh Vidyanagar, Gujarat (India) for successful completion of this work.

REFERENCES

1. Rubinstein A. Natural polysaccharides as targeting tools of drugs to the human colon. Drug Development Research 2000; 50: 435–439. DOI: 10.1002/1098-2299.
2. Kinget R, Kalale W, Vervoort L, Colonic drug targeting. J. Drug Target 1998; 6: 129–149.
3. Englyst H N, Hay S and Macfarlane G T. Polysaccharide breakdown by mixed populations of human fecal bacteria. FEMS Microbiology Letters 1987; 45(3):163-171.
4. Pawar K S, Pawar S P,Bacterial polysaccharidases in colon specific drug delivery. International Journal of Pharmacuetical Sciences Review and Research 2011;.6(2): 188-196
5. Macfarlane S, Quigley M E, Newton D F and Macfarlane G T. Polysaccharide degradation by human intestinal bacteria during growth under multi-substrate limiting conditions in a three-stage continuous culture system. FEMS Microbiology Ecology 1998; 26: 231-243.
6. Jain A, Jain S K. Perspectives of Biodegradable Natural Polysaccharides for Site-Specific Drug Delivery to the Colon. J Pharm Pharmaceut Sci 2007;10 (1): 86-128.
7. Rama Prasad Y V, Krishnaiah Y S R, In vitro evaluation of guar gum as a carrier for colon-specific drug delivery. Journal of controlled release 1998; 51(2-3): 281-287.
8. Rubinstein A and Gliko-Kabir I. Synthesis and swelling dependent enzymatic degradation of borax modified guar gum for colonic delivery purpose. STP Pharm. Sci. 1995b; 5: 41–46.
9. Sawarkar S P and Deshpande S G, Simulation of Colonic Fluid for the In vitro Evaluation of Colon Specific Tablets, 2008 AAPS Annual Meeting and Exposition Online Itinerary.