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Whey Powder: A Potential Anti-diarrheal Agent Through its Biofilm Formation

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Abstract: Whey, the natural product resulting from coagulation of milk is reported to have diverse pharmaceutical credentials. In the present investigation the anti-diarrhoeal activity of the whey powder was investigated. The Whey powder which was prepared using rennet powder and lactic acid, was studied against Magnesium sulphate-induced Diarrhea in Swiss Albino mice. Castor oil-induced enteropooling studies and *in vitro* biofilm-forming potentials of the whey powder were also carried out, as this is believed to contribute to the anti-diarrhoeal activities of the preparation. Anti-diarrhoeal activity was more pronounced in mice which received 250 mg kg b.wt. of whey powder when compared to those which received 500 mg kg^{-1} b.wt. The percentage inhibition of total number of feces in the 250 mg kg^{-1} b.wt. drug-treated group was 56.14%,whereas the animals which received 500 mg kg^{-1} b.wt. of whey powder showed 37.18% inhibition. The loperamide treated animal group showed 63.81% inhibition. In castor oil induced enteropooling, the percentage inhibition of intestinal content in the 250 mg kg^{-1} b.wt. drug-treated group was 61.42% against atropine-treated animal group that showed 26.24% inhibition. The whey powder also exhibited strong biofilm forming capacity with increase in concentration. The anti-diarrhoeal activity of whey preparation established herein is believed to be owing to certain active principles present in it or due to biofilm-forming capacity, which inhibits the attachment of mediators of diarrhoea to mucosal walls of the GI tract or due to interaction of diarrhoea inducing chemicals with whey peptides, which needs further investigation.

Key words: Biofilm, castor oil-induced, mucosal layer, whey powder

INTRODUCTION

Diarrhea has long been recognized as one of the most important causes of morbidity and mortality in developing countries (Walzem *et al*., 2002). It is characterized by increased bowel movements and wet stool and caused by imbalance between the absorptive and secretary mechanisms in the intestinal tract resulting in an excess loss of fluid in feces. It is a leading cause of malnutrition and death among pediatric subjects in developing countries of the modern world. Hence, research intended at reducing deaths due to diarrhea through oral rehydration therapy and potential therapeutic strategies are increasingly being attempted (Keusch *et al*., 2006). Many synthetic chemicals like, diphenoxylate, loperamide and antibiotics are available to treat diarrhea, although these agents are reported to impart minor subclinical side effects. A wide variety of antibiotics are commonly used for the treatment of serious infectious diarrhea caused by bacteria (Tumah, 2005). In recent years, multiple drug resistance, a threat to mankind, has developed due to indiscriminate use of existing antimicrobial drugs in the treatment of infectious diseases (Saeed *et al*., 2007). Due to the side effects of conventional medicine, the use of natural products has emerged as an alternative in healing and treatment of various diseases has been on the rise in the last few decades. Anti diarrheal drugs from natural origin are used in medicinal practices for treating food-borne diseases. Over 50% of all modern clinical drugs are of natural product origin (Suffness and Douros, 1982) and natural products play an important role in drug development programs in the pharmaceutical industry (Baker *et al*., 1995).

Since ancient times, gastrointestinal problems have been treated orally with whey liquid, based on traditional Siddha medicine. Whey is a popular natural dietary protein supplement, resulting from coagulation of milk, purported to provide antimicrobial activity, immune modulation, improved muscle strength and body composition and prevention of cardiovascular disease and osteoporosis (Kawakami, 1997).Intact whey contains several components with broad antimicrobial activity (Chatterton *et al*., 2006).Whey proteins contain bioactive components including α-lactoglobulin, β-lactalbumin,

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immunoglobulins, lysosymes and bovine serum albumin. Furthermore, antimicrobial peptides like glycomacropeptides, lactoferrin and lactoperoxidase may be generated from whey protein by proteolysis during gastrointestinal transit (Clare *et al*., 2003; Samie *et al*., 2005). The advantages of whey protein-derived antimicrobial peptides are that they are derived from a safe substance and they may be produced by naturally occurring enzyme activation. Research has shown that whey protein consumption could have significant salubrious health effects against certain immune deficiencies, neoplasm, cholesterol-associated manifestations and breast cancer (Marshall, 2004). With the above scenario an attempt was made in evaluating the anti diarrheal potentials of whey powder produced by a novel method in normal and magnesium sulphate-induced diarrheal rats.

MATERIALS AND METHODS

Materials and chemicals: The milk samples were obtained from the Tamilnadu Co-operative Milk Producers Federation Ltd, Aavin, Sholinganallur Chennai, India. All routine chemicals were obtained from SD Fine Chemicals Mumbai. Rennet powders, food grade lactic acid, BSA, were procured from Ranbaxy laboratories Ltd., Haryana, India.

Extraction of whey powder: To 1000 mL of cow's milk, stored at 4°C for 12 h, 1 5 mL of 20% food grade lactic acid and 0.001% of rennet powder was added and mixed well. Following a 30 min incubation of mixture in a 40°C water bath, casein and whey liquid were separated using a cheese cloth. The whey liquid collected was stored at 4°C until lyophilization. The yield of the lyophilized sample was found to be 6.25% w/v and it was stored at -20°C for further use.

Biochemical estimation of whey powder: Lactose was estimated using a titration method described by Folin and Denis (1918). Accordingly, the whey sample was titrated against the Benedict's quantitative reagent using standard lactose solution and the amount of lactose present in the whey powder was calculated. Estimation of total protein was performed by Lowry *et al.* (1951) method. The principle was based on the presence of phenolic group of tyrosine and tryptophan residues (amino acid) in the protein reacting with folin-ciocalteu reagent producing a blue purple color complex, with maximum absorption in the region of 660 nm wavelength.

Animals: Adult Albino rats (150-200 g) and Swiss Albino mice (20-35 g) of either sex were used in the pharmacological and toxicological studies. The inbred animals were obtained from animal facility extension at the C.L. Baid Mehta College of Pharmacy, Chennai, India. The animals were maintained in propylene cages in well-ventilated room with natural 12±1 h day-night cycle. They were fed balanced rodent pellet diet (Poultry Research Station, Nandam, Chennai-35) and tap water *ad libitum*, throughout the experimental period. The animals were housed for one week, prior to the experiments to acclimatize to laboratory conditions. The protocol was approved by Animal Ethics Committee constituted for the purpose, as per CPCSEA Guidelines.

Acute toxicity: Acute toxicity studies were conducted with the whey powder in Swiss albino mice by the Staircase method of Akhila and Alwar (2007). First group served as normal control. Whey powder was administered orally to different groups at the dose level of 250, 500, 1000 and 5000 mg kg^{-1} b.wt. All animals were observed for toxic symptoms.

Magnesium sulphate-induced diarrhoea: The Swiss albino mice were divided into 6 groups of six animals each and fasted for 18 h. Group I received single administration of 0.5 mL vehicle (1% v/v aqueous Tween 80) p.o., once and served as control. The groups II, III, IV received magnesium sulphate at the dose of 2 g kg^{-1}, p.o., for the induction of diarrhoea according to Doherty, (1981). Group III and IV received whey sample (250 and 500 mg kg^{-1} p.o., respectively) after 30 min of receiving magnesium sulphate. Group V received the standard drug loperamide (3 mg kg^{-1} p.o.) after 30 min of receiving magnesium sulphate. Group VI received only whey sample (500 mg kg^{-1} p.o.). Animals of all groups were placed separately in individual cages lined with filter paper. The filter papers were changed every hour and the severity of diarrhoea was assessed hourly for 4 h. The total number of faeces excreted and total weight of the faeces were recorded within the period of 4 h. The mean of the stools passed by the whey powder treated groups were compared with the group II animals that were given magnesium sulphate (2 g kg^{-1} b.wt.) p.o. The anti-diarrheal activity was determined in terms of percentage protection.

Castor oil-induced enteropooling: Intra luminal fluid accumulation was determined by the method described by Boominathan *et al.* (2005).The rats were fasted overnight and were divided into six groups of six animals each. Group I received single administration of 0.5 mL vehicle (1% v/v aqueous Tween 80) orally once and served as

control. The Groups II, III, IV received castor oil at dose of 1 mL per animal p.o. for the induction of diarrhea group III and IV received whey sample of 250 and 500 mg kg^{-1} p.o., respectively after 30 min of receiving castor oil. Group V received the standard drug atropine (3 mg kg^{-1} i.p.) one hour before the oral administration of castor oil. Group VI received only whey sample a dose of 500 mg kg^{-1} p.o. Two hours later, the rats were sacrificed under mild ether anesthesia. The edges of the intestine from pylorus to ceacum were tied with thread and the intestine was removed and weighed. The intestinal content was collected by milking into a graduated cylinder and reweighed to calculate the difference between the full and empty intestine.

***In vitro* biofilm formation:** *In vitro* biofilm formation was studied by a tissue culture plate assay method described by Christensen *et al.* (1985) with slight modifications. This is the most widely considered as a standard test for detection of biofilm formation by biomolecules. A stock solution of 500 mg mL^{-1} of whey powder in distilled water was prepared. Individual wells of sterile, polystyrene, 96 well-flat bottom tissue culture plate wells were filled with 0.2 mL aliquots of the whey powder and distilled water served as control. The tissue culture plates were incubated for 18 h at 37°C. After incubation, the contents of each well were gently removed by tapping the plates. The wells were washed four times with 0.2 mL of PBS (pH 7.2). Biofilm formed by whey powder in plate were fixed with sodium acetate (2%) and stained with crystal violet (0.1% w/v). Excess stain was rinsed off by thorough washing with de-ionized water and plates were kept for drying. Optical Densities (OD) of stained plates were determined with a micro plate ELISA auto reader at wavelength of 570 nm. These OD values were considered as an index of whey powder adhering to surface and forming biofilm. The experiment was performed in repetition for ten times, the data was then averaged standard error mean was calculated and compared with the standard table (Table 1) as described by Mathur *et al.* (2006).

Statistical analysis: Values reported are Mean±SE. The statistical analysis was carried out using Analysis of Variance (ANOVA) followed by Dunnett's test. p-values <0.05 were considered as significant (Woolson, 1987).

Table 1: Classification of biomolecules adherence by tissue culture plate method

Mean OD values	Adherence	Biofilm formation
<0.120	Non	Non/weak
0.120-0.240	Moderate	Moderate
>0.240	strong	High

RESULTS

Biochemical estimation of whey powder: Considering the possibility of protein turning carcinogenic on prolonged heating, in the present study, we standardized a non-thermal method of whey preparation using rennet powder and lactic acid, there by retaining the proteins in native form without denaturation. We were able to optimize our non thermal whey powder preparation using 15 mL of 20% food grade lactic acid and 0.001% of rennet powder. The lactose content in whey powder prepared herein was estimated as 6.5% whereas the protein content of the whey was found to be 0.9%.

Acute toxicity studies: Acute toxicity could not be determined since the whey powder up to the dose 5000 mg kg^{-1} b.wt. caused no considerable signs of toxicity in the animals tested. One-tenth of the upper dose was selected for the evaluation of the anti-diarrheal activity and it was fixed as 250 and 500 mg kg^{-1} b.wt.

Magnesium sulphate-induced diarrhea: The group II animals that did not receive whey powder but induced by magnesium sulphate showed typical signs of diarrhea such as watery stools and frequent defecation. A single oral dose of whey powder at the concentration of 250 and 500 mg kg^{-1} b.wt. in group III and group IV animals produced a significant decrease ($p<0.001$) in the severity of diarrhea in terms of reduction in the rate of defecation and consistency of feces in Swiss albino mice (Table 2). The percentage inhibition for the number of wet feces as well as weight of dry mass indicates the presence of anti-diarrheal activity in the whey powder as compared with that of magnesium sulphate induced diarrheal group. Experimental results reflect that the activity is more pronounced at the dose of 250 than 500 mg kg^{-1} b.wt. The anti-diarrheal effect of whey powder was similar to that of the standard drug, loperamide (3 mg kg^{-1} b.wt.) used in this study. The animals that received the whey powder alone did not show any signs of toxicity indicating that the administration of whey powder would be devoid of any visible side effect.

Castor oil-induced enteropooling: Whilst castor oil caused accumulation of water and electrolytes in intestinal loop, the whey powder at concentrations of 250 and 500 mg/mL/kg b.wt. significantly ($p<0.001$) inhibited castor oil-induced enteropooling. The volume and the weight of the intestinal contents were significantly decreased in group III and group IV animals when compared to the group II castor oil induced animals which showed the increased volume and weight of the intestinal contents (Table 3). The decrease in the volume and weight of intestinal content by whey powder was

Table 2: Effect of whey powder on magnesium sulphate induced diarrhea in swiss albino mice

Parameters	Group I tween 80 control	Group II $MgSO_4$ treated	Group III whey powder+ $MgSO_4$ treated (250 mg kg^{-1} b.w.)	Group IV whey powder+ $MgSO_4$ treated (500 mg kg^{-1} b.w.)	Group V loperamide+ $MgSO_4$ treated	Group VI whey powder treated (500mg kg^{-1} b.w)
Total number of feces	6±1.32	19.32±1.29	9±1.11a***	10.50±1.32b***	7.75±0.95	5.8±0.89c^{NS}
Total number of diarrheal feces	0	12.80±1.34	6.05±1.46a***	7.5±0.653b***	4±0.41	0
Inhibition (%) of total number of feces	0	0	56.14	37.18	63.81	0
Total weight of feces	2.30±0.2	9.39±0.18	4.19±0.16a***	5.90±1.69b***	2.95±0.93	2.43±0.2c^{NS}
Inhibition(%) of total weight of feces	80.6±0.05	0	62.07±1.17	50.96±0.56	70.61±1.13	80.1±0.58c^{NS}

Values are Mean±SEM from 6 animals in each group, Statistical significant test for comparison was done by ANOVA, followed by *post hoc* Dunnett's test. Comparison between: a: Group II vs group III,b: Group II vs group IV and c: Group I vs Group VI, p-values: *<0.05, **<0.01, ***<0.001, NS: Non significant

Table 3: Effect of whey powder on castor oil induced enteropooling in rats

Parameters	Group I tween 80 control	Group II castor oil treated	Group III whey powder+ castor oil treated (250 mg kg^{-1} b.wt.)	Group IV whey powder+ castor oil treated (500 mg kg^{-1} b.wt.)	Group V atropine+ Castor oil treated	Group VI whey powder treated (500mg kg^{-1} b.wt.)
Intestinal content in (mL)	1.584±1.02	4.42±0.04	1.954±0.05a**	2.654±0.08b**	1.289±0.27	1.667±0.06c^{NS}
Weight of the intestinal content in (g)	1.840±1.09	4.85±0.89	2.154±0.35a**	3.454±0.25b**	1.789±0.16	1.867±0.06c^{NS}
Inhibition of intestinal content (%)	30.12±1.27	0	61.42±1.04 a**	41.46±1.12b**	26.24±1.56	32.12±1.34 c^{NS}

Values are Mean±SEM from 6 animals in each group, Statistical significant test for comparison was done by ANOVA, followed by *post hoc* Dunnet's `t' test, Comparison between: a: Group II vs group III, b: Group II vs group IV and c: Group I vs Group VI, p-values: *<0.05, **<0.01, ***<0.001, NS: Non significant

Table 4: Effect of whey powder on Biofilm formation on tissue culture plate

Concentration of the whey powder (mg mL^{-1})	Mean OD values (595 um)	Adherence	Biofilm formation
25	0.048±1.53	Non	Non/weak
50	0.106±1.45	Non	Non/weak
100	0.156±2.93	Moderate	Moderate
200	0.248±2.56	Good	Good
400	0.371±2.46	Good	Good
500	0.416±1.83	Good	Good
600	0.457±2.67	Good	Good
700	0.505±3.12	Good	Good
800	0.628±1.59	Good	Good
900	0.698±2.33	Good	Good
1000	0.721±4.55	Good	Good

Each value represents the Mean±SE (n = 10), Mean OD values, >0.240 indicates good biofilm formation and strong adherence, 0.120-0.240 indicates moderate biofilm formation and adherence, <0.120 indicates non biofilm formation and adherence. At concentrations above 100 mg mL^{-1} the whey power showed OD values >0.240 in the tissue culture plate assay indicating its strong biofilm forming capacity and adherence

comparable to that of intra peritoneal injection of standard drug atropine sulphate at doses of 3 mg kg^{-1} b.wt. The group VI animals that received the whey powder alone did not show any signs of toxicity indicating that the administration of whey powder would be devoid of any visible side effect.

***In vitro* biofilm formation:** It has been observed that the whey powder has the tendency to form biofilm. The biofilm forming potential of any biomaterial was estimated in flat-bottomed tissue culture plates because they were the most convenient substrate, for their formation and adherence. Therefore, in the present investigation the biofilm forming capacity of the whey preparation was studied using polypropylene tissue culture plates which resemble the mucosal layers of gastro intestinal tract. Table 4 shows the biofilm forming potential of whey powder at different concentration.

DISCUSSION

Whey is a by-product in cheese manufacturing process and is usually prepared by a thermal process, wherein alteration in protein structure might occur by the heating as observed by aggregation and gelling of whey protein (Raikos, 2010). In general, protein profiles for heat-treated skimmed milk indicate that the denaturation of the total proteins begins at 40°C, accelerates with increasing temperature and becomes 95% complete at 85°C (Hong and Creamer, 2002). Considering the possibility of protein turning carcinogenic on prolonged heating, in the present study, we formulated a novel non-thermal method using rennet powder and lactic acid, thereby retaining the proteins in native form without denaturation. The presence of considerable quantities lactose and total protein of whey powder and its anti-diarrheal potential shows the presence of biomolecules intact because the biological components of whey and whey products depend on the methods of production, purification and concentration of lactic acid and rennet. Hence this method can be adapted for commercial preparations of whey powder.

Diarrhea results from an imbalance between the absorptive and secretary mechanisms in the intestinal tract accompanied by increased bowel movements and wet stool resulting in an excess loss of fluid in feces. The hallmark of certain diarrheal conditions is characterized by the presence of secretary components whereas others are characterized by hyper motility. A broad iota of underlying factors, viz., infective, immunological and nutritional have been known to involve in the perpetuation of diarrheal syndrome (Galvez *et al.,* 1995). In developing country like India, majority of people living in rural areas almost depend on the use natural biomolecules to treat their various health problems because of their cost effective nature. However, many biologically-derived materials are conveniently available in India (used in traditional folklore medicine) for the treatment of diarrhea. In establishing the pharmacological evaluation of a potential anti-diarrheal agent, the inhibition of experimentally induced diarrhea, reduction in the fecal output and gastrointestinal motility tests have remained the most commonly investigated parameters (Majumdar *et al*., 2000; Tangpu and Yadav, 2004; Venkatesan *et al*., 2005).

Magnesium sulphate has been reported to induce diarrhea by increasing the volume of intestinal content through prevention of reabsorption of water. It has been demonstrated that it promotes the release of cholecystokinin from the duodenal mucosa which increases the secretion and motility of small intestine and there by prevents the reabsorption of sodium chloride and water (Galvez *et al.,*1993). In this present study, it is observed that whey powder exhibited significant anti-diarrheal activity against magnesium sulphate induced diarrhea in rats. The individual or the cumulative effect of components of whey powder such as α-lacto globulin, β-lactalbumin, bovine serum albumin, lactoferrin, immunoglobulin, lactoperoxidase enzymes, glycomacropeptides, lactose and minerals may have the ability of inhibit the secretion and hypermotiliy caused by cholecystokinin in magnesium sulphate induced diarrheal rats. Rosaneli *et al.* (2002) has demonstrated that whey protein has a protective effect on gastric mucosa. According to him this effect is thought to be related to the sulfhydryl component particularly cystine and its link with glutamic acid in the production of glutathione which is one of the important antioxidant responsible for prevention of oxidative injury in the cells.

Whey protein was found to possess an anti-enteropooling effect in castor oil induced experimental diarrheal animals by reducing both the weight and volume of intestinal content. These effects are the direct consequences of reduced water and electrolyte secretion in small intestine suggesting that whey powder may enhance water and electrolyte absorption from intestinal lumen. More over hyper motility characterizes certain forms of diarrhea where the secretary component is not a causative factor (Chitme *et al*., 2003). Castor oil induced gastro intestinal hyper motility has been suggested due to the indirect activation of the cholinergic system (Brown and Taylor, 1996) since it is inhibited by atropine sulphate a known anti-cholinergic drug. In our present investigation, the research findings such as decrease in the volume, weight of the intestinal contents and reduction in the number of stools suggests that the components present in the whey powder has got the tendency of both inhibiting the hyper secretion and hyper motility.

The polypropylene plate (96-well tissue culture plate) was used to simulate the mucosal membrane in the gastrointestinal tract and it has been observed that the whey powder has got the tendency of biofilm formation in the tissue culture plates under proper physiological conditions. According to Walzem *et al.* (2002) most of the bio molecules especially proteins due to the interaction between the polypeptide chains undergo polymerization at certain conditions to form native film. So the possible mechanism responsible for the anti-diarrheal activity of the whey powder may be that it protects the intestinal mucosa by forming a thin biofilm layer and prevents the attachment of diarrhea causing chemicals to the receptors of intestinal mucosa. Kinsella (1984) also has already reported that most abundant and important component for film formation is Beta-lacto globulin fraction present in whey powder. Either this fraction alone or by the interaction with other fractions, the whey powder might exert its anti diarrheal activity by forming a thin bio film on the mucosal layers of the intestinal tract. The alternative possible mechanism could be that whey powder typically uses this biofilm formed due to the interaction of its various components as an Ultra filter where the diarrhea causing mediators are trapped and washed away from the intestine without causing any damage to the mucosal membrane.

CONCLUSION

Thus, the results of this investigation revealed the scientific basis for the potential use of whey powder in gastro intestinal disorders such as diarrhea. The whey protein, one of the by-products of milk industry is not properly utilized in spite of its various important bio molecules. Further research is to be carried out to fractionate and purify the whey derived peptides and the evaluation of each fraction for its *in vivo* biofilm forming potencies.

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