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EFFECT OF ENCAPSULATION ON DIGESTIVE ENZYMES TREATMENT OF CHICKEN EGG YOLK IGY ANTIBODIES

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ABSTRACT

The study involved analyzing the effects of various digestive enzymes including pepsin, trypsin, and chymotrypsin on the activity of Chicken egg yolk IgY antibodies generated against vaccine strain of rotavirus. When the IgY antibodies are proposed for oral passive therapy for gastrointestinal disorders including diarrhea, the stability of these antibodies against various digestive enzymes becomes very much essential. The experiments showed that IgY antibodies are highly affected by pepsin, whereas minimal effect was seen on trypsin and chymotrypsin treatments. Hence to protect the antibodies, an alternate method of encapsulating the antibodies were performed using chitosan-alginate beads and the encapsulated, non-encapsulated antibodies were exposed to all the three enzymes and the activity was checked by ELISA method. The results showed that the encapsulated IgY antibodies were well protected by pepsin treatment increasing its activity to 83% at pH 3.0 and 76% at pH 4.0 when compared to non-encapsulated. Similarly, when treated with trypsin and chymotrypsin, the encapsulated antibodies showed an activity from 63% to 81% and from 71% to 86% respectively at pH 8.0. Therefore, these anti rotavirus IgY antibodies can

be protected from digestive enzymes by alternate methods like encapsulation using chitosan-alginate for sustained release in the Gastro-Intestinal Tract and to neutralize the infection too which can be further extended as a feed supplement for infants and children.

KEYWORDS: IgY Antibodies, Encapsulation, Digestive Enzymes, Chitosan alginate beads.

INTRODUCTION

Chicken Egg yolk antibodies can be potentially used as an alternative to conventional mammalian antibodies by its ease in production without giving stress to the animals. They can also be used well in oral passive therapy in the form of nutraceuticals or as feed supplements to eliminate gastrointestinal tract disorders. Rotavirus, a virus responsible for a devastating number of children hospitalizations particularly in developing countries can be fought against using these antibodies to save a large number of children in countries where vaccination is unavailable or becomes ineffective due to many other reasons.

Studies in animal models have shown that Anti human rotavirus IgY antibodies were able to neutralize major strains of rotavirus and reduce the infection rate in experimentally infection induced mice models.[1] Also, the antibodies protected the neonatal piglet model from the virulent Wa G1P (8) strain of the virus. This animal model's digestive system mimics one of infants, proving its possible use in infants to control the infection.[2] The use of these antibodies for prophylactic and therapeutic purpose against human rotavirus infection in combination with probiotic bacteria like *Lactobacillus* has been proved that it can potentially replace the current rehydration therapy for rotavirus infection.[3]

IgY antibodies when used to treat gastrointestinal disorders like viral diarrhea, it becomes essential to check its stability in digestive enzymes present in the gastric condition of humans so that it can be effectively used for oral passive therapy approaches. Hence in this present research, the stability of Anti rotavirus IgY antibodies was checked against different digestive enzymes including pepsin, trypsin and chymotrypsin by checking the antibody activity through ELISA method.[4] Followed by, experimenting an alternate protective mechanism of encapsulating the antibodies using chitosan and sodium alginate to reduce the loss of antibody activity in the enzyme environment and to increase the sustained release in gastrointestinal Tract conditions was studied.[5,6] Therefore, the stability of IgY antibodies against digestive enzymes and the protective mechanism of encapsulation on the enzyme activity was well studied and the results were presented below.

MATERIALS AND METHODS

Generation of Antivac IgY antibodies: Antibodies against vaccine strain of rotavirus was generated by immunizing 21 weeks old white leghorn chickens with the commercially available rotavirus vaccine Rotavac (Manufactured by Bharat Biotech Intl. Ltd.,). Eggs were collected from immunized chickens, yolks separated and the egg yolk IgY antibodies were purified by PEG precipitation method as described by Polson *et al.*,1985.[7]

Effect of pepsin on Antivac IgY antibodies[4]: The Effect of pepsin on Antivac IgY antibodies was checked by dissolving digestive enzyme Pepsin (5µg/ml) in 0.07 M sodium acetate buffer and pH was adjusted to different pH range from pH1 to pH8 using 1N HCI & 1N NaOH. The dissolved enzyme solution was mixed with Antivac IgY solution (10mg/ml) in the ratio of pepsin to IgY as 1:20 and was incubated at 37°C for different time intervals from 0 to 4 hours. After appropriate incubation periods for 0-4 h, 0.45ml sample of the mixture was mixed with 0.05 ml of 5.0% sodium carbonate for inactivation of the enzyme. The remaining antibody activity was measured by the ELISA method at different pH range incubated at different time periods. Antibodies incubated at pH7 without the pepsin enzyme served as control which was considered as 100% activity.

Effect of Trypsin and Chymotrypsin on IgY Antibodies[8]: To examine the effects of enzymes trypsin and chymotrypsin, the Antivac IgY antibody was dissolved in 50mM Tris buffer containing 10mM $CaCI_2$, pH 8.0, at a concentration of 2 mg/ml. Trypsin and chymotrypsin were dissolved at a concentration of 2 mg/ml in the Tris buffer and immediately mixed with the IgY solution in the ratio of 1:20.The mixtures were incubated at 37°C for appropriate incubation time periods from 0-8 hours.0.45ml sample of the incubation mixture was mixed with 0.05 ml of phenylmethylsulphonyl fluoride solution (40mM in isopropanol) to inactivate the enzymes and the remaining antibody activity of the mixture was measured by ELISA method for each aliquot of sample at different incubation time. Antibody incubated at pH 8 without the enzymes served as control which was considered as 100% activity.[9]

Encapsulation of Antivac IgY antibodies: Encapsulation of antibodies were adopted to protect them from the detrimental effects of digestive enzymes and acidic pH by the method as described by Li *et al*., 2009 by use of Chitosan and sodium alginate. Briefly, 2% Sodium alginate was dissolved in distilled water and freeze-dried IgY powder was added at a loading rate of 25%. Encapsulation medium was prepared by Diluting the stock Chitosan solution

(1%) to a final concentration of 0.2% with 0.5% Calcium chloride solution. pH was adjusted to 3.5 with 4M NaOH. Microcapsules of IgY was prepared by adding 10ml of Sodium alginate IgY solution to 200ml of Encapsulation medium with stirring at 200rpm, a flow rate of 4ml/min through a 0.7mm needle with a distance of 8cm between the needle tip and the encapsulation medium. The microcapsules obtained were filtered, rinsed and stored in the refrigerator for further analysis (Fig.1). The encapsulated antibodies were treated with the digestive enzymes present in the GI tract along with the non-encapsulated ones to find out the protective effect of encapsulation on these antibodies against enzymes and acidic pH as described in the following experiments.

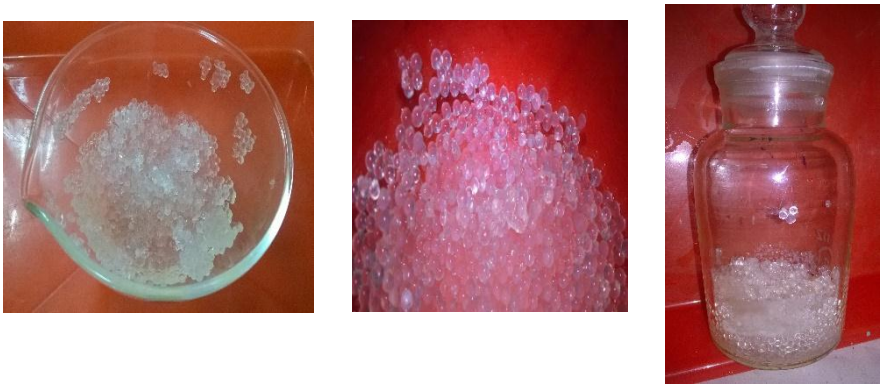


Fig.1 Encapsulation of IgY antibodies using Chitosan & Sodium alginate method.

Pepsin treatment of Encapsulated Antivac IgY antibody: The encapsulated and non-encapsulated antibodies were treated with pepsin as described previously at different pH range from pH1 to pH4 and being incubated for 0-4 hours' time period. Aliquots of samples were collected at each time interval and the enzyme pepsin was inactivated by the addition of 0.05 ml of 5.0% sodium carbonate and IgY antibody was released from the encapsulated beads by adding 10mg of the beads to 5 mL of IgY release solution (0.2 M NaHCO3, 0.06 M Na3C6H5O7.2H2O pH8.0) for 20-30 minutes. The % activity retained after pepsin treatment was analysed by performing ELISA and OD at 450nm was noted down. Non-encapsulated IgY antibodies treated with pepsin at different pH range at 0^{th} hour was considered as control with 100% activity.

Trypsin treatment of Encapsulated Antivac IgY antibody: Encapsulated and non-encapsulated antibodies were treated with trypsin at a concentration of 2mg/ml as described previously at pH 8 and were incubated for 0-8 hours' time period. Aliquots of samples were collected at each time interval and the enzyme trypsin was inactivated by the addition of 0.05

ml of Phenyl methyl sulfonyl fluoride solution (40mM in Isopropanol). Non-encapsulated IgY antibodies treated with trypsin at pH 8.0 served as a control with 100% activity.

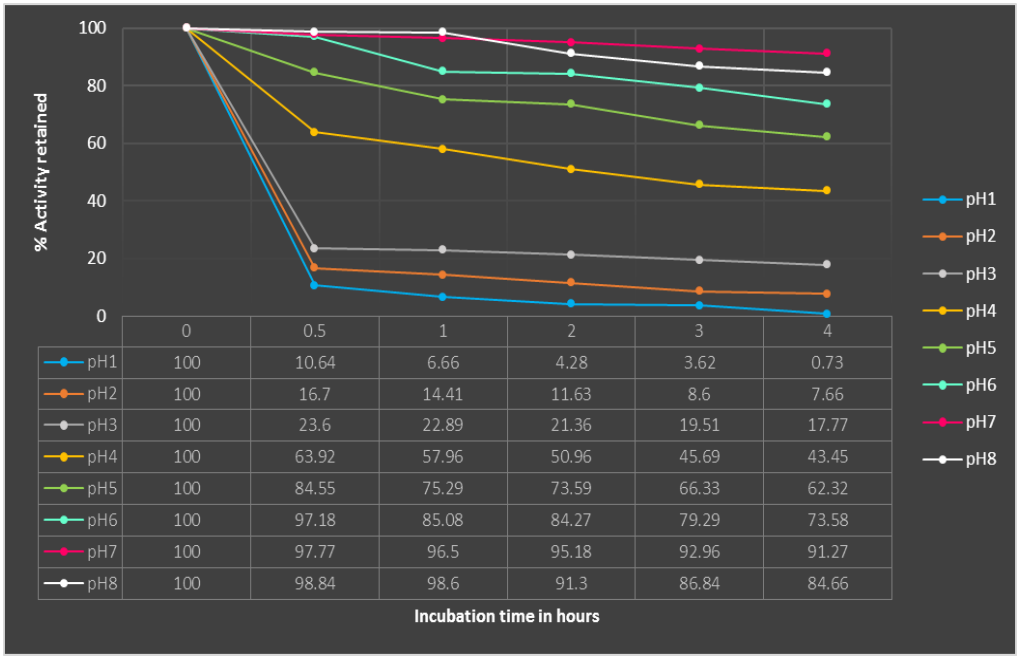
Chymotrypsin treatment on Encapsulated Antivac IgY antibody: To check the effect of encapsulation on chymotrypsin treatment on the antibodies, the encapsulated and non-encapsulated antibodies were treated with chymotrypsin at a concentration of 2mg/ml as described previously at pH 8 and were incubated for 0-8 hours' time period. Aliquots of samples were collected at each time interval and the enzyme chymotrypsin was inactivated by the addition of 0.05 ml of Phenyl methyl sulfonyl fluoride solution (40mM in Isopropanol). Non-encapsulated IgY antibodies treated with chymotrypsin at pH 8.0 served as a control with 100% activity.

The release of IgY antibody from Encapsulated Beads: After the trypsin and chymotrypsin treatment the IgY antibody was released from the encapsulated beads by adding 10mg of the beads to 5 mL of IgY release solution (0.2 M NaHCO3, 0.06 M Na3C6H5O7.2H2O pH8.0) for 20-30 minutes. The % activity retained after trypsin and chymotrypsin treatment was analysed by performing ELISA and OD at 450nm was noted down. Non-encapsulated IgY antibodies treated with chymotrypsin at pH8 was considered as control with 100% activity.

RESULTS AND DISCUSSION

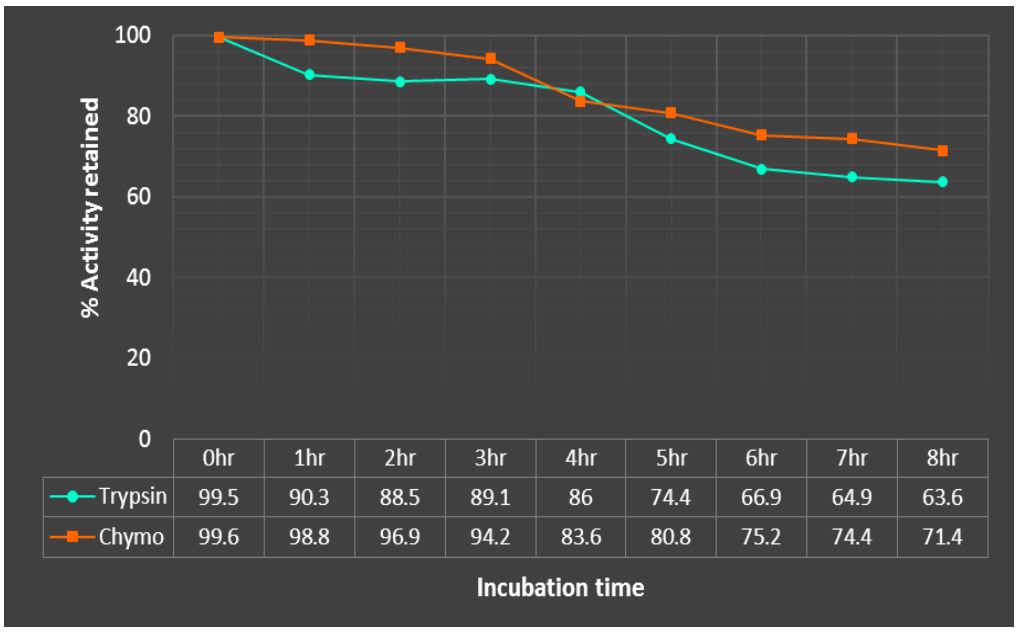
Effect of pepsin treatment on Antivac IgY antibodies: It was observed from the experiment that the effect of pepsin on Antivac IgY antibodies was greatly dependant on the pH of the solution and the activity was almost lost under pH3 whereas at pH 4.0 about 43% activity was retained even after 4 hours of incubation (Fig.2). At pH 6.0 to pH 8.0 nearly 73% to 84% activity was retained. Hence pepsin enzyme treatment at lower acidic pH greatly diminishes the antibody activity which makes it unsuitable for its use in oral therapy hence encapsulation method was adopted to protect it from acidic pH with pepsin exposure which mimics the gastric condition of humans as such.

Effect of Trypsin and Chymotrypsin treatment on IgY Antibodies: Treatment of Antivac IgY antibodies with enzymes trypsin and chymotrypsin incubated for nearly 8 hours showed that almost 63% and 71% of antibody activity was retained after the experiment respectively. The effects of trypsin and chymotrypsin at a pH of 8.0 on Antivac IgY antibodies were less significant when compared to pepsin treatment (Fig. 3).



	0	0.5	1	2	3	4
pH1	100	10.64	6.66	4.28	3.62	0.73
pH2	100	16.7	14.41	11.63	8.6	7.66
pH3	100	23.6	22.89	21.36	19.51	17.77
pH4	100	63.92	57.96	50.96	45.69	43.45
pH5	100	84.55	75.29	73.59	66.33	62.32
pH6	100	97.18	85.08	84.27	79.29	73.58
pH7	100	97.77	96.5	95.18	92.96	91.27
pH8	100	98.84	98.6	91.3	86.84	84.66

Fig 2: Effect of pepsin treatment on Antivac IgY antibodies with respect to pH and incubation time.



	0hr	1hr	2hr	3hr	4hr	5hr	6hr	7hr	8hr
Trypsin	99.5	90.3	88.5	89.1	86	74.4	66.9	64.9	63.6
Chymo	99.6	98.8	96.9	94.2	83.6	80.8	75.2	74.4	71.4

Fig 3: Effect of Trypsin & Chymotrypsin treatment on Antivac IgY Antibodies.

Hence to protect the antibody from the detrimental effect of pepsin with lower acidic pH of gastric conditions (when adopting for oral passive therapy approach) followed by exposure to trypsin and chymotrypsin in intestinal fluid, antibodies were encapsulated using Chitosan sodium alginate and the encapsulated vs non-encapsulated antibodies were exposed to similar enzymatic conditions and the results obtained were as follows.

Protective Effect of Encapsulation on pepsin treatment of Antivac IgY antibody: When comparing the antibody activity retained after 4 hours of incubating the encapsulated (E) and non-encapsulated (NE) IgY in pepsin, it was found that at pH1 about 73% of E IgY was retained in comparison to just 4% of NE IgY. Similarly, about 77%, 83% and 76% activity of E IgY was retained at pH2, pH3 and pH4 respectively, which clearly demonstrates the positive effect of encapsulation in protecting the antibody from pepsin and lower pH to a significant level (Fig.4).

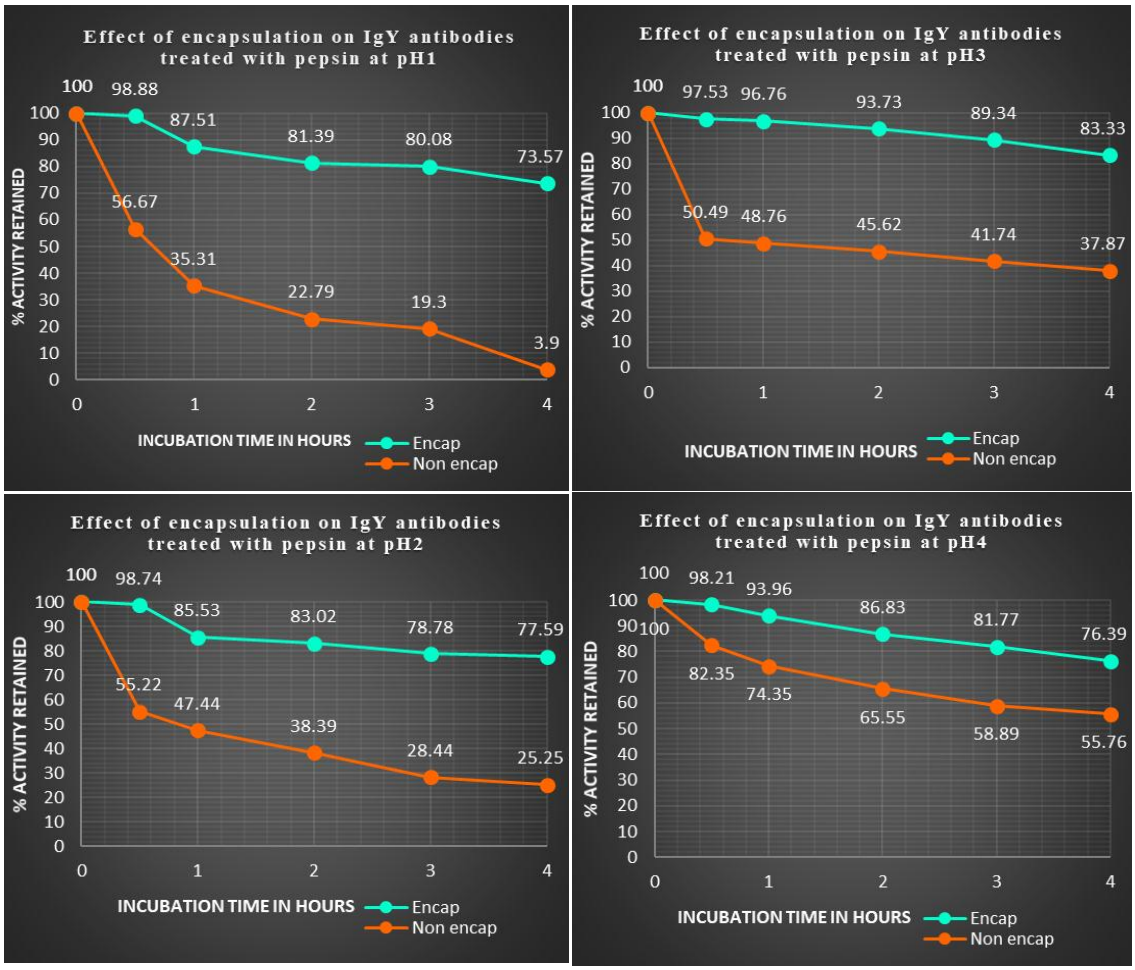
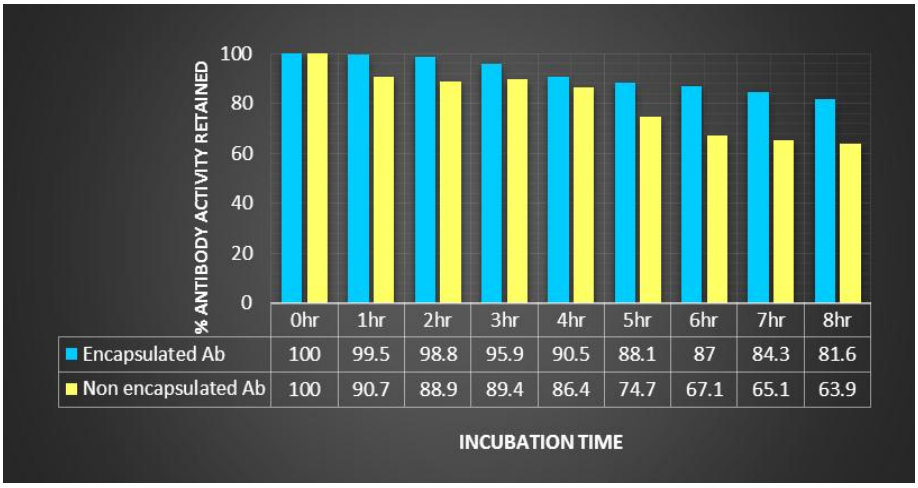


Fig 4: Efficacy of Encapsulation on IgY antibodies upon Pepsin Treatment at different pH (1-4).

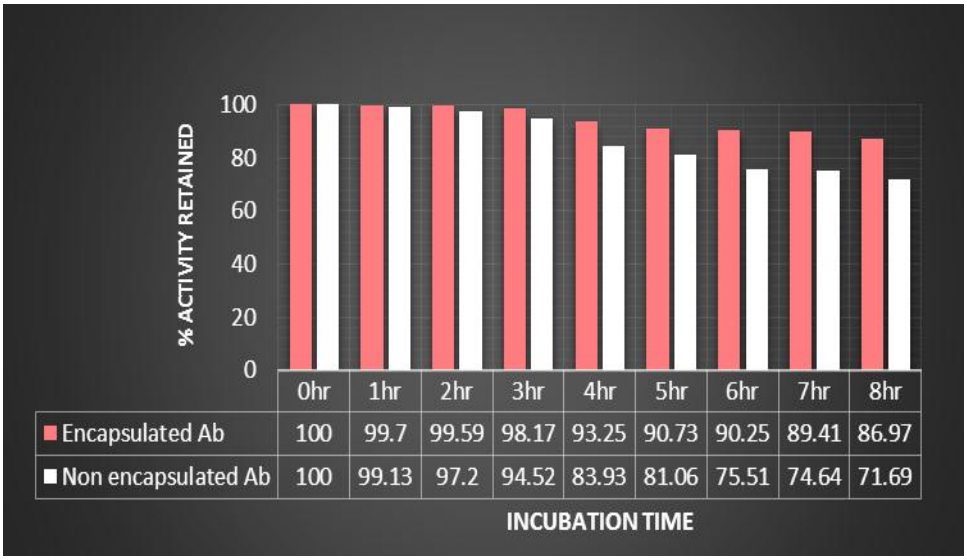
Protective Effect of Encapsulation on Trypsin treatment of Antivac IgY antibody: When comparing the antibody activity retained after 8 hours of incubating the encapsulated and non-encapsulated IgY in trypsin, it was found that about 81% of activity was retained by the encapsulated antibody whereas 63% was retained in the non-encapsulated control batch in the experiment which shows that even though trypsin had not much effect on the antibody, encapsulation has still increased the activity than previous studies(Fig.5).



	0hr	1hr	2hr	3hr	4hr	5hr	6hr	7hr	8hr
Encapsulated Ab	100	99.5	98.8	95.9	90.5	88.1	87	84.3	81.6
Non encapsulated Ab	100	90.7	88.9	89.4	86.4	74.7	67.1	65.1	63.9

Fig 5: Effect of Encapsulation on trypsin treatment of Antivac IgY antibody.

Protective Effect of Encapsulation on chymotrypsin treatment of Antivac IgY antibody. After 8 hours of incubation time, it was found that encapsulated and non-encapsulated samples retained its antibody activity around 86% and 71% respectively when treated with the enzyme chymotrypsin. Therefore, the Antivac IgY antibodies can be protected from digestive enzymes degradation and lower acidic pH conditions by encapsulating them by Chitosan alginate bead (Fig.6).



	0hr	1hr	2hr	3hr	4hr	5hr	6hr	7hr	8hr
Encapsulated Ab	100	99.7	99.59	98.17	93.25	90.73	90.25	89.41	86.97
Non encapsulated Ab	100	99.13	97.2	94.52	83.93	81.06	75.51	74.64	71.69

Fig 6: Effect of Encapsulation on Chymotrypsin treatment of Antivac IgY antibody.

CONCLUSION

Therefore, from these experiments, it was found that encapsulating the Antivac IgY antibodies to chitosan sodium alginate beads protects them from rapid digestion of pepsin together with lower acidic pH retaining its activity and also allowing for its sustained release

in gastric conditions. The activity of trypsin and chymotrypsin, even though they didn't have a significant destructive role with these antibodies, still the encapsulation procedure retained its activity to a certain increased level when compared to free antibodies when being exposed to these digestive enzymes. All these results prove that these Antirotavirus IgY antibodies in the form of encapsulated beads can be very well used for oral passive therapy approaches to control the rotavirus infection in children. Also, these antibodies can be used as nutraceuticals or as an efficient feed supplement to be delivered along with normal routine food to infants and children. This would prove to be an efficient alternative to the current rehydration therapy being offered for rotavirus infection. But still, the formulation of the feed supplement and the stability of these antibodies on the developed product needs to be studied in detail to take it to next future step.

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