

Development and Characterization of Chicken Egg Yolk Antibodies *Igy* Against Porcine Pancreatic Lipase as a Preliminary Step to Control Obesity

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Abstract:

Obesity, once seen as a major clinical problem in high-income countries has been a significant health issue even in developing countries and urban populations in recent decades. Issues related to obesity including hypertension, cardiovascular issues are highly critical that it leads to the high mortality rate in most of the countries. Obesity observed in children is of greater concern to be treated with surgeries and drugs in younger ages leading to side effects in later years needs to be addressed very carefully. Hence the present study attempted towards antibody generation towards lipase enzyme which is significant in the clinical condition of obesity. Anti-lipase IgY chicken antibodies were developed generated in experimental white leghorn chickens and purified by water dilution method followed by the desalting procedure. Antibodies were characterized for total protein estimation by the Lowry method as 56mg/mL and by the Bradford method as 90mg/mL. Purity and the molecular weight of the porcine pancreatic lipase antigen and IgY antibodies were found to be 65kDa and 180kDa respectively. The antibody titer of anti-lipase IgY antibodies as determined by Indirect ELISA revealed the highest titer on the 63rd day of immunization with an increasing peak up to the 35th day of immunization. Hence the present study has opened up the idea of antibody therapy for treating obesity against working upon the key enzyme lipase with initial characterization of the generated anti-lipase IgY antibodies. Further experimental studies on animal models followed by human clinical trials need to be critically studied on the mechanism of action and effectiveness of these antibodies in controlling the enzyme activity and in turn the clinical condition of obesity.

Keywords: Obesity, Anti-Lipase, IgY antibodies, Chicken egg yolk antibodies, Indirect ELISA

Introduction: Obesity is seen as a growing problem in high-income countries, but today it is reported as a major issue in even developing countries and urban environments. The world population review stated by WHO shows that more than 3 million people face death each year on being obese. Obesity increases the risk of diabetes, cancer, and coronary artery diseases. Apart from this people facing obesity also are prone to diseases such as high blood pressure, sleep apnea, osteoarthritis, and many kidney diseases (Freedman et al., 1999). Obesity may also be linked to improper sexual development in adolescents (Pulgarón 2013), various factors including genes, cells, metabolism, physiology behavior, and social habits of the individual (Bose et al., 2007), health and lifestyle of people in a particular geographical location (James 1995). Obesity seems to be one of the most serious risk factors of some high mortality rate diseases (Parikh et al., 2007).

Surgical technique such as sleeve gastrectomy proves to be a promising modern, safe and effective method to treat obesity with a low mortality rate (Kheirvari et al., 2020). Other surgical methods include laparoscopic adjustable gastric banding (LAGB), Roux-en-Y gastric bypass (Jacques Himpens et al., 2011) standard biliopancreatic diversion, Duodenal switch diversion, gastric plication, vertical banded gastroplasty, mini-gastric bypass, and many more (Angrisani et al., 2015). As for the mode of action of synthetic drugs, there exist targets such as appetite suppression through Phentermine (Kang et al., 2010), sympathomimetics such as Diethylpropion (Glazer 2001), Endocannabinoid blockers including Rimonabant and Taranabant (Marzo et al., 2004), Noradrenaline or serotonin reuptake inhibitors such as Sibutramine (Rucker et al., 2007), and synthetic lipase inhibitors such as Orlistat and Cetilistat (Roßner et al., 2000, Kopelman & Groot, 2009).

Modern technology of controlling enzymes using antibodies (Łupicka-Słowik et al., 2017) developed by immunologists etches the way to develop antibodies for obesity. Pancreatic lipase is one of the targets for the FDA approved synthetic drug Orlistat. Targeting this enzyme by egg yolk antibodies (IgY) to prevent the breakage of triglycerides in the food into free fatty acids which get accumulated in the host body (Tarigan & Kusumorini 2016) could promise a new therapy for obesity. Also suggested that the porcine pancreatic lipase be a better antigen since it has 10 times higher activity than human

pancreatic lipase (Xiao et al., 2013). The main aim of this study is to develop antibodies against purified Porcine Pancreatic Lipase as executed by (Hirose et al., 2013) and characterize it as a preliminary step to control obesity.

Materials and methods

Experimental birds: Twenty-one week's old white leghorn chickens purchased from Chandran's Poultry Farm, Coimbatore were used for the studies. Birds were maintained in separate cages in the animal house and were regularly fed with sterile corn-meal feed and water. Birds were allowed to be acclimatized to the new environment for few days before starting the experiments.

Antigen Preparation & Immunization: Porcine pancreatic lipase Type II L3126-100G (Sigma Life sciences) at a concentration of 5mg/ml in 1X Phosphate Buffer Saline (PBS) was used as an antigen to immunize a total of five chickens by intramuscular injection at multiple sites of the breast muscles. 2 unimmunized birds served as the control group. 0.5mL of the prepared porcine pancreatic lipase (5mg/ml) was mixed homogeneously with 0.5 ml of Freund's complete adjuvant for first immunization (Meenatchisundaram, et al., 2009). Antigen and the adjuvant were taken in a 2mL sterile syringe connected by a sterile tube, mixed gently till the appearance of a milky white emulsion. Followed by first immunization, booster doses were given as per the given schedule (Table 1) with Freund's incomplete adjuvant.

Table 1: Immunization schedule

Antigen dosage	Days	Antigen-adjuvant volume	Lipase antigen concentration	Adjuvant used	Adjuvant volume
Dose I	0	0.5ml	5mg/ml	FCA*	0.5ml
Dose II	14			FIA*	
Dose III	28			FIA	
Booster Doses	Immunized after every 14 days interval in the same procedure.				

FCA: Freund's complete adjuvant; **FIA:** Freund's incomplete adjuvant.

Egg collection, Purification and desalting of IgY Antibodies:

Eggs from the immunized and unimmunized chickens were collected, disinfected with 70% ethanol, labeled and stored at 4 °C until processed. Under the aseptic condition, eggs

were broken carefully with sterile forceps and yolks were separated from the albumin by placing it on a sterile filter paper and removing the excess of albumin from the yolk. The yolk sac was punctured and the yolk contents were collected in a measuring cylinder and the volume was noted down.

To the collected volume of yolk, added 1:7 ratio of sterile distilled water and the pH of the emulsion was adjusted to 5 using 1N NaOH and 1N HCl and incubated at -20°C overnight. The emulsion was thawed at 4°C in a refrigerator overnight the next day, separating the water-soluble fraction from the rest of the contents by sedimentation overnight. The emulsion was equally distributed into 50mL falcon tubes and centrifuged at 10000 rpm for 20 minutes at 4°C. Collected the supernatant and measured to which 8.8% of NaCl was added and dissolved completely. Adjusted the pH to pH 4.0 using 1N HCl and 1N NaOH and incubated at 4°C for 2 hours to precipitate the proteins. Centrifuged the samples at 6000 rpm for 15 minutes at 4°C, discarded the supernatant and the pellets were air-dried. Pellets containing the anti-lipase IgY antibodies were dissolved using 1X PBS and stored at -20°C (Hodek et al., 2013). Purified anti-lipase IgY Antibodies were desalted by dialysis against 1X PBS buffer. Dialysis was carried out for about 16 hours with two rounds of buffer changes. Dialyzed IgY antibodies were stored at 20°C until further characterization.

Characterization of anti-lipase IgY antibodies:

Protein estimation:Total protein content of the purified anti-lipase IgY antibodies were estimated as per the protocol described by Lowry et al., 1951 and Bradford et al., 1976 respectively. Bovine serum albumin protein standards were prepared from 20 µg to 100 µg and the standard curve was plotted against which the absorbance of IgY antibody determined spectrophotometrically at 660 nm against the blank was plotted to determine the total protein concentration. The cupric ions of alkaline copper sulfate reagent react with proteins to produce a blue coloration which is detected in the presence of Folin Ciocalteu reagent in the method of Lowry et al.,

Bradford et al., reported to be a more sensitive method of protein estimation were carried out with BSA Standards (10 µg to 1000 µg) against the IgY sample. 3 mL of Bradford's reagent was added to all the tubes and incubated at room temperature in dark condition and the absorbance was determined spectrophotometrically at 595 nm against 4 mL of Bradford Reagent as blank.

SDS PAGE: Molecular weight and the purity of the Porcine pancreatic lipase antigen and purified anti-lipase IgY antibodies were determined by SDS PAGE analysis described by Laemmli et al., 1970. Sodium dodecyl sulfate denatures proteins into its primary straight chain polypeptides and the Poly-acryl amide gels- 4% stacking gel and 10% resolving gel allow the separation of the polypeptides under the influence of an electric potential across the electrophoresis chamber.

Standard broad range protein marker ranging from 3.5 kDa to 205 kDa (GeNei PMWB 0110475001730), 10 μ L of Porcine Pancreatic Lipase (5mg/ml) and 0.5 μ L of purified IgY were made upto 15 μ L using 5X sample buffer, incubated at 100° C in a water bath for 5 minutes and short spun to collect the contents within microcentrifuge tubes and loaded on to the SDS poly-acrylamide gels. Samples were electrophoresed at 75V for approx. 150minutes. The gel was stained with Coomassie Brilliant Blue G staining and de-stained visualize the separated protein bands of various expected band sizes.

Indirect ELISA:

Specificity of the anti-lipase IgY antibodies against commercial Porcine Pancreatic Lipase antigen was assayed by Indirect ELISA procedure (Wang et al., 2000). Coated the micro titer wells with 100 μ L of Porcine Pancreatic Lipase antigen (5mg/mL in 1X PBS) and incubated at 4°C overnight. After overnight incubation, wells were washed thrice with 300 μ L of 1X PBST and blocked with 3% skimmed milk in 1X PBST, incubated at room temperature for 2hours. The washing procedure was repeated once. Anti-lipase IgY antibodies were diluted as 1:200, 1:400, 1:800 up to 1,02,400 using 3% skimmed milk and 100 μ L was added to the blocked wells. 100 μ L of 3% skimmed milk in PBST served as blank. Incubate the plate at room temperature for 1hour and washed again. 100 μ L of Rabbit anti-chicken IgG conjugated with horseradish peroxidase (1:1000 using 1X PBS) was added to each well and incubated for 1 hour at room temperature.

The wells were washed five times to remove any excess unbound secondary antibody and 100 μ L of TMB (3,3',5,5'-Tetramethylbenzidine)chromogenic substrate was added and incubated for 20minutes in dark condition and observed for blue color development. The reaction was stopped by the addition of 100 μ L of 4N sulfuric acid and OD was recorded at 450nm in a microplate reader. The protocol was repeated for IgG antibodies present in the serum of the immunized chicken as a quantitative analysis of the antibody titer developed during the 14th week of immunization.

Results

Eggs collected from the immunized and unimmunized control group of chickens were used to purify the antibodies by water dilution method followed by salt precipitation method allowing the antibodies and other smaller proteins to precipitate from the water-soluble fractions of the yolk. Approximate 2.8mL of purified antibodies were obtained post-dialysis procedure which was used for further characterization studies.

Protein estimation by Lowry et al., 1951:

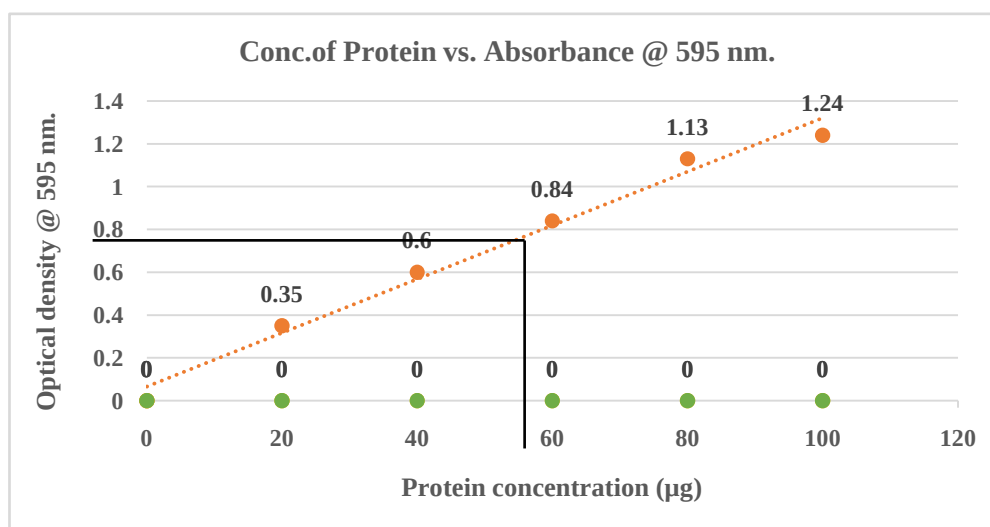
The concentration of anti-lipase IgY antibodies present in the purified samples was calculated by protein estimation. In the method of Lowry, the addition of Folin Ciocalteu reagent changed the color of the solution in all the tubes to fluorescent green which upon incubation turned Prussian blue indicating the presence of proteins. The visible increase in the contrast of the color in the standard tubes is indicative of the increase in the concentration of protein standard. Optical density values read at 595 nm spectrophotometrically against the blank are as mentioned in table 2.

Table 2: Protein estimation by Lowry et al., 1951 method:

S. No.	Vol. of Working Std. (μL)	Conc. of Protein (μg)	Vol. of distilled water added (μL)	Vol. of alkaline Copper sulfate + Folin Ciocalteu Reagent added (μL)	Incubation time (minutes)	Optical density at 595 nm.
B.	0	0	1000	4000 + 500	45	0.00
1.	200	200	800			0.35
2.	400	400	600			0.60
3.	600	600	400			0.84
4.	800	800	200			1.13
5.	1000	1000	0			1.24
S-10.	10	x	990			0.80

The standard graph plotted with protein concentration against absorbance at 595nm produced as a straight line and the values of IgY standard plotted on the standard graph indicated the protein concentration of purified anti-lipase IgY antibodies with 56 μg in 10 μL of the sample (Figure 1). Thus, the concentration of the purified anti-lipase IgY is estimated to be 56 mg/mL according to Lowry et al., 1951.

Figure 1: Protein concentration of anti-lipase IgY antibodies estimated by Lowry et al., 1951:

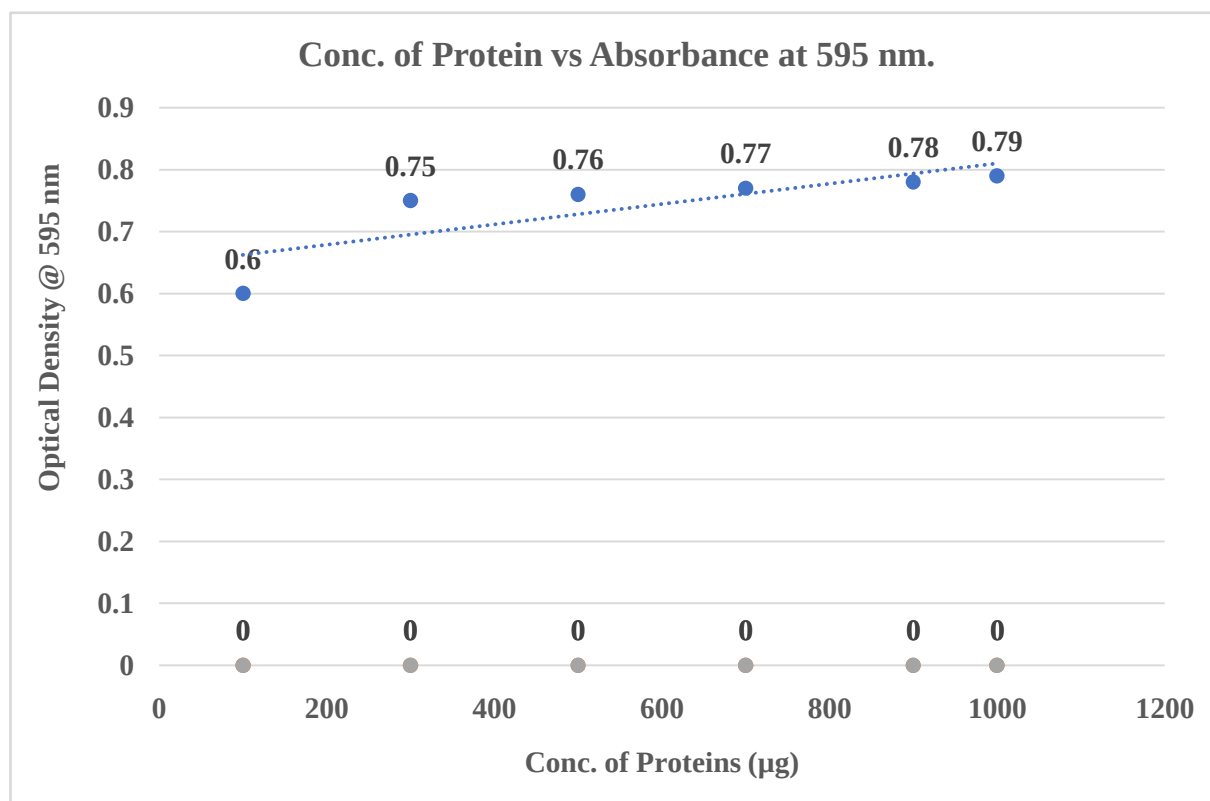


Protein estimation by Bradford method: Spectrophotometric analysis of the protein standards showed an orderly increase in absorbance from 30 µg standard onwards with no deviation of values at all. The absorbance of the unknown sample was read exactly like one of the standard values which indicative of the accuracy of the assay (Table 3). However, a standard graph of concentration versus absorbance was plotted to furnish a straight trend line. Thus, the concentration of the IgY present in the purified IgY sample is estimated to be 90 mg/ml according to Bradford et al., 1976.

Table 3: Protein estimation by Bradford method:

S. No.	Vol. of Working Std. (µL)	Conc. of Protein (µg)	Vol. of distilled water added (µL)	Vol. of Bradford reagent added (µL)	Incubation time (minutes)	Optical density at 595 nm.
B.	0	0	1000	5000	45	0.00
1.	100	100	900			0.60
2.	300	300	700			0.75
3.	500	500	500			0.76
4.	700	700	300			0.77
5.	900	900	100			0.78
6.	1000	1000	0			0.79
S-10.	10	x	990			0.78

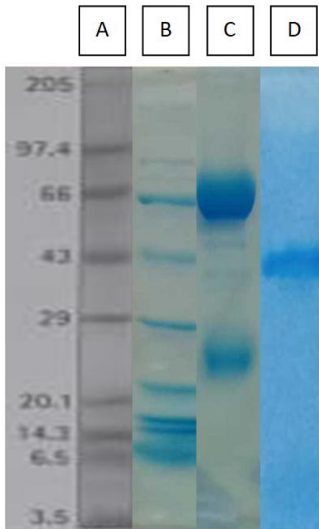
Figure 2: Protein concentration of anti-lipase IgY antibodies estimated by Bradford method:



SDS PAGE Analysis: The molecular weight and purity of the commercially obtained porcine pancreatic lipase antigen and the developed anti-lipase IgY from egg yolks of the immunized eggs were checked by SDS PAGE analysis and the gel was electrophoresed at 75V for 175minutes. The protein bands were stained with Coomassie Brilliant Blue G250 for 1 hour and de-stained overnight with little modification in the protocol as stated by (Laemmli, 1970).

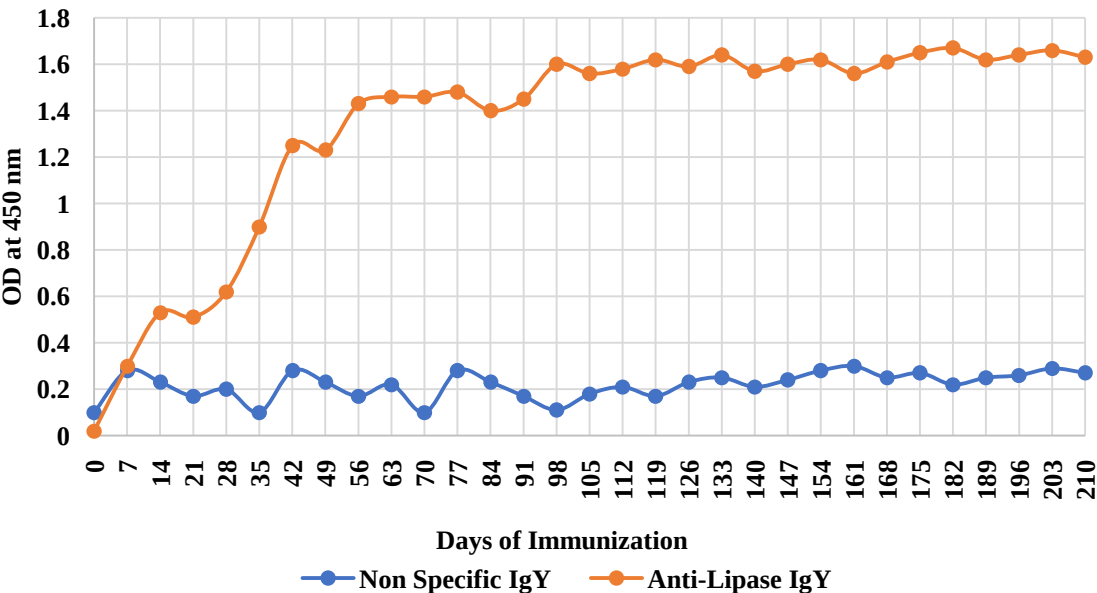
The first lane of the gel contains the broad range protein marker (GeNei Broad Range PMWB 0110475001730) (Lane B), Lane C contains the IgY antibody, Lane D contains the antigen Porcine Pancreatic Lipase. On comparing with the molecular weight of the protein marker, it was observed that the antigen was approximately 48 kDa in size. The light chain of the anti-lipase IgY weighs around 27 kDa and the heavy chain weighs about 60 kDa (Figure 3).

Figure 3: SDS PAGE Analysis of antigen and the antibody samples against standard protein ladder:



Indirect ELISA:Antibodytiter levels of anti-lipase IgY antibody was estimated by indirect ELISA as described by (H., 2002) with slight modifications. The antibody titer was observed to increase at the time of booster immunizations. The comparative results showed that the antibody titer increased during immunization (Figure 4).

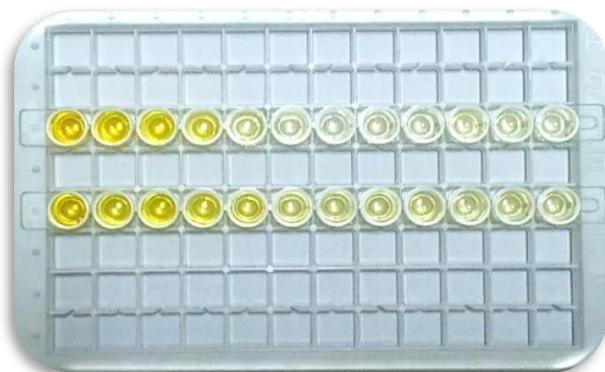
Figure 4: Quantification of IgY antibody titer determined by Indirect ELISA



The IgY levels were observed to increase 2 weeks post-immunization and the upsurge continued till the 35th day after which the titer level remained unchanged till the 49th day after

immunization. A peak anti-lipase IgY titer of 1.02 at OD 450 nm was observed on the 63rd day after immunization of the chicken (Figure 5).

Figure 5: Antibody titer observed in serum vs egg yolk



First row- IgG antibody concentration from the serum samples.

Second row- IgY antibody concentration from the egg yolk samples.

OD Values observed from the microplate reader at 450nm showed that IgY samples on the 63rd day had a peak value of 1.02 as highlighted in the Table 4 below.

Table 4: OD Values observed at 450nm for the purified antibodies:

S. No	Dilutions	IgG in Serum (OD at 450 nm)	IgY in Yolk (OD at 450 nm)
1.	1: 200	2.576	1.704
2.	1: 400	1.818	0.827
3.	1: 800	1.059	0.960
4.	1: 1600	0.527	0.602
5.	1: 3200	0.264	0.464
6.	1: 6400	0.162	0.316
7.	1: 12800	0.115	0.263
8.	1: 25600	0.131	0.249
9.	1: 51200	0.143	0.267
10.	1: 102400	0.192	0.248
11.	1: 204800	0.154	0.227
12.	Blank	0.142	0.214

Discussion& Conclusion:

The study involved the development of anti-lipase IgY antibodies against Porcine pancreatic lipase similar to Hirose, et al., 2013. Antibodies were successfully developed in the animal model from the 35th day up to a higher titer level observed on the 63rd day of the

experiment. Chickens immunized with the commercially purchased Porcine Pancreatic lipase antigen did not show any visible response of loss of appetite or fatigue upon immunization and regular egg-laying performance was monitored and collected for the antibody purification process.

IgY antibodies were purified by water dilution method rather than a routine process of PEG precipitation method as described by Polson et al., 1980, due to the time-consuming process involved in it and also the yield of IgY finally was seen to be less when compared to the water dilution technique (Meulenaer & Huyghebaert, 2001). The concentration of proteins when estimated through Lowry et al., 1951 and Bradford et al., 1976 methods, showed a significantly high value for proteins indicative of its highly soluble nature. Lowry et al., 1951 showed a high concentration of proteins of about 56 µg per 10 µL of the sample. However, the optical density readings when compared to Bradford was noticed to be less accurate. Bradford et al., 1976 showed an accurate result and the concentration was calculated to be higher, of about 90 µg per 10 µL of the IgY sample.

SDS PAGE analysis revealed the purity of the antigen with a single band corresponding to the 65kDa molecular weight and anti-lipase IgY antibodies being split up into heavy chain and light chain primary structures with an added molecular weight of ~ 88 kDa in the gel. IgY antibodies possess 2 heavy chains and 2 light chains connected with the help of disulfide bonds with a total molecular weight of ~180 kDa. The antibody titer and the specificity of the purified antibodies by Indirect ELISA concluded that the IgY raised from the immunized eggs were highly specific to the porcine pancreatic lipase enzyme and the highest titer value was observed on the 63rd after immunization.

Various diseases have been treated by oral administration of IgY antibodies in both human and veterinary medicine (Chalghoumi et al., 2009). The development of polyclonal antibodies has not been more economic and animal ethics encouraging than IgY technology. (R., et al., 1996). Passive immunity against many viral and microbial infections have been targeted in previous literature. Immunization against eukaryotic enzymes and the development of antibodies for the prevention of a disease like obesity is a novel concept not explored by many (Tarigan et al., 2016). The activity of porcine pancreatic lipase is reported to be 10 folds higher than human pancreatic lipase. (Xiao et al., 2013) Thus, its administration as prophylaxis for obesity may be suggested to humans also after sufficient clinical trials on animal models. IgY has been trusted as an alternative to mammalian

antibodies (Michael et al., 2010) and due to its phylogenetic relations to humans, little has been known about the negative immune responses (Torche et al., 2006).

A report on avian antibodies has concluded that IgY develops the general protective immune response along with an anaphylactic IgE response (Harley 2016). IGY Life Sciences, Canada, in their recent advertisement features have proclaimed the standardizations for the development of IgY against COVID 19 as a nasal spray formulation and an enteric capsule. Thus, in the long run, anti-lipase IgY is anticipated to be put to use as a prophylaxis for obesity in variable formulations such as chewable tablets, along with dietary products or even as a feed-additive to produce lean meat. Further animal model studies on the effectiveness of IgY and its negative impact is needed to convert the investigated antibody into a product that is safe to consume.

Conflict of interest: The authors declare no conflict of interest.

Acknowledgements: The author would like to thank the management of PSGCAS, Coimbatore for providing all the necessary facilities to carry out the experiments.

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