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Phytate Hydrolysis in Chicken's BBMVs: Influence of Age, Intestinal Site and pH

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ABSTRACT

Age effect, intestinal site and pH influence on phytate hydrolysis were assayed in brush border membrane vesicles isolated from chicks and layers proximal and distal intestinal segments using $MgCl_2$ precipitation and differential centrifugation. The ascertained BBMVs purity and functional integrity depicted alkaline phosphatase enzyme marker to range between 20-fold to 37-fold greater for chicks' and layers' proximal and distal BBMVs than in their mucosal homogenates, their integrity suggestive to presence of Na^+-dependent transport while electron micrograph showed a vesicular form. Both chicks' and layers' BBMVs, had significant decrease in phytate-P hydrolysed/mg.

Introduction

The significance of fowls' gut microflora in hydrolyzing phytate is less known, though bacteria populating caecum may provide a resource of phytase activity. However, microbial phytase was reported to have a broader pH activity range than plant phytases and therefore is more effective within the gastrointestinal environment [1]. The same authors have shown that wheat phytase is inactive at pH 3 and whether or not this inactivation is reversible when pH increases to 6 or 7 in the small intestine is not known. If it is irreversible, then any breakdown in phytate by plant phytases must occur prior to or within the proventriculus before the low pH inactivates the enzyme [2]. Several separate studies have revealed a substantial hydrolysis of phytate in brush border membrane vesicles (BBMVs). It occurred when pH range from 5 to 6.5 with a maximum hydrolysis at pH of 6 and that free inositol, but phytate was not reported to be actively transported across the intestinal mucosa by a mechanism dependent on sodium and energy [3] and is taken up in most tissues against a concentration gradient [4].

The available data on presence of phytase activity in intestinal secretions of poultry is scanty and inconclusive or conflicting. It prompts study to quantify hydrolysis of phytate down the digestive tract such that the physiology of lumenal phytate hydrolysis is understood. Subsequently, this study was undertaken to attest the role of intestinal phytases in phytate-P hydrolysis, as well as, to investigate the hypothesis that chicks' ability to hydrolyse phytate progresses with age of the bird, and that variations in pH along the digestive tract negatively influences dietary phytate hydrolysis.

Materials and Methods

Experimental Birds and Collections of Intestinal Mucosa

Fifty neonatal chicks of commercial laying strain were brooded up to 4 weeks of age and had ad libitum access to starter diet and water, with a 24 hours photoperiod. At day 28th 50 chicks plus 20 35-week-old-layers were randomly selected from the farm stock and fasted for 18hours, then sacrificed by cervical dislocation, dissected and the digestive tracts were excised into proximal and distal segments (Plate 1**).**

Plate 1. Intestinal segments cleared of the digesta.

The intestinal segments were inverted and cleared of their contents using cold normal saline. The entire mucosa was scraped off and samples were then pooled for respective intestinal sites that is to say proximal and the distal intestinal segments, loaded into individual vials and stored frozen at –70°C.

Preparation and Isolation of the BBMVs

After the preparations of the medium solutions, BBMVs were isolated from the respective mucosa scrapings according to the following procedures [5 and 6]:

1. The crude suspensions were then homogrnised on ice using a polytron homogenizer for 60 s.
2. To precipitate the connective tissue and poorly homogenized material, crude homogenates were centrifuged for 15 m at approximately 5400 rpm at 4ºC.
3. To the generated supernatant, MgCl2 was added to give the final concentration of 10 mM $MgCl_2$ ($MgCl_2$ precipitates basolateral and nuclear membranes) and the mixture was then stirred on ice for 15 min.
4. Then the mixture was centrifuged at approximately 5700 rpm, for 15 min at 4ºC

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5. To generate the crude brush border vesicles pellets, the supernatant was centrifuged at 15900 rpm, for 45 min at 4ºC, and the supernatant was discarded.
6. The crude brush border vesicles pellets were re-suspended in 4 ml suspension medium (300mM mannitol, 50 mM HEPES-Tris, pH 7.5) then homo genised with a glass-teflon homo geniser (6 strokes).
7. To generate the final BBMVs pellets, the homogenate was centrifuged as in 6, then the pellets were re-suspended and homo genised by passing through 23- and 25- gauge needles.
8. Suitable volumes of re-suspension medium containing the purified BBMVs were pooled and assayed for protein, then divided into aliquots and frozen in liquid nitrogen pending the assays.

BBMVs Protein Assay

Protein contents of the purified BBMVs were determined according to the Bio-Rad protein microassay procedure which is a dye-binding assay, based on differential colour change of a dye in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Reagents: Bovine Serum Albumen (No. A-9047, N = 15%, Lot 104F.0077), Dye Reagent Concentrate (Bio-Rad). Protein Standard Preparation and Measurement: 10 mg Bovine Serum Albumen (BSA) was weighed and mixed with 1ml of distilled water in a 1 ml Eppendorf tube, then 10 µl was added to 990µl of distilled water to form stock solution. 10, 40, 80, and 120 µl of the stock solution were pipetted into a set of 4 tubes and made up to 800µl by addition of distilled water. 0.8 ml of each of the standard BSA solutions was placed in a clean dry Eppendorf tube and then 0.2 ml of Dye Reagent Concentrate was added to each of the standards, this gave 1, 4, 8 and 12 µg of protein/ml respectively. They were gently vortex mixed and after 5 minutes read in a Spectrophotometer versus reagent blank at 595nm. The samples of BBMVs were measured as unknown following the same procedure as the standard.

Purity Assessment of the Isolated BBMVs

Determination of Alkaline Phosphatase Activity

The alkaline phosphatase as a marker enzyme was routinely determined for the respective segmental crude homogenates and the purified BBMVs for each bird group, following Sigma Diagnostics ALP kit(Sigma, ALP optimised, Alkaline Phosphatase EC 3.1.3.1 colorimetric test) immediately after vesicle collection, as well as, after vesicle purification.

Assay of D-Glucose Transport

All the four BBMVs samples were subjected to D-glucose uptake measurements at 25ºC by a rapid filtration technique [7].Chemicals and Medium Preparations: Mannitol, HEPES, Tris, MgSO4, NaSCN, KSCN, ^{14}C-D-glucose, filter paper, Phlorizin, Scintillation medium and Scintillation counter. Suspension Medium: Contained 300 mmol/l mannitol,0.1mmol/lMgSO4, and 20mmol/l HEPES/Tris, pH7.4. Incubation Medium: Contained 100 mmol/l mannitol,0.1mmol/l MgSO4, 20mmol/l HEPES/Tris, pH7.5 0.1mmol/l ^{14}C-D-glucose and 100mmol/l of either NaSCN or KSCN. Stop Solution: 150mmol/l KSCN,0.25 mmol/l phlorizin,20mmol/l HEPES/Tris, pH7.5. The Procedure: Duplicated BBMVs samples, proximal or distal BBMVs were loaded into suspension medium. Then 5 to 10 ml of the vesicle suspension (equivalent to 50 to 110 mg of protein) was added to 100 ml of the incubation medium. At a selected series of times (5 min interval), the uptake (reaction) was terminated by addition of ice-cold stop solution.

To determine uptake at time zero, the addition of stop solution preceded that of the incubation medium to the vesicles, then measurement was subtracted from the total radioactivity of each sample. To separate the BBMVs, 0.9 ml of reaction mixture was pipetted on pre-soaked cellulose nitrate filters (Millipore Filter, 0.22 µm pore size) and rinsed with a 5 ml ice-cold stop solution. The filter paper was then placed in a scintillation vial. The Na^+-dependent D-glucose transport was determined as the difference in the presence of Na^+ and the absence of Na^+ (presence of K^+). A time of 5 s was used to measure initial rates of transport of Na^+-dependent D-glucose.

Electron Microscopy

Samples for electron micrographs of the purified BBMVs were obtained by suspension of BBMVs in 0.1 M phosphate buffer (pH 7.4) fixed in added 2-glutaraldehyde for 2h at 4ºC, then centrifuged at 30000 g for 30 min. They were then post-fixed in 1% OsO_4 for 1hr, the pellets were dehydrated and embedded in Spurr resin, thin sectioned (70-90nm), stained with uranyl acetate followed by lead citrate, and examined in a transmission electron microscope. Procedure: 0.1 mM Phosphate buffers pH 7.4, Fixatives: Glutaraldehyde and Osmium tetroxide 50%, 70, 80, 90, and 100% Dry acetone, Epoxy resin. I. Fixation: Six duplicated samples of BBMVs, representatives for both chick and layer intestinal segments, were thawed and suspended in suspension buffer, then centrifuged at 15900 rpm for 30 minutes at 4ºC. The generated pellets were suspended in phosphate buffer, then fixed for 3–12 hrs at 4ºC by addition of glutaraldehyde to a final concentration of 3%. After centrifugation at 15900 rpm for 30 minutes at 4ºC, the fixed pellets were suspended in 0.1 mM phosphate buffer at 4ºC for 10 minutes, centrifuged at 15900 rpm for 30 minutes at 4ºC, then resuspended in 0.1 mM phosphate buffer and kept overnight, at 4ºC. Fixed pellets were obtained by centrifugation at 15900 rpm for 30 min, at 4ºC. They were then postfixed in 1% osmium tetroxide (OsO_4) in phosphate buffer for 2 h. at room temperature, after which the blackened pellets were repeatedly immersed for 10 minutes in 0.1 M phosphate buffer. II. Dehydration: The samples were dehydrated (water exclusion) through soaking in a series of de-hydration gradients of 50%, 70%, 80% and 90% acetone for 10 minutes each, then replaced by 100% dry acetone in a covered bottle, which was repeated twice for up to 1hr each, then a third time for 1 hr. III. Embedding: To exclude acetone and for thorough infiltration of the samples with epoxy resin, the samples pellets were embedded in a series of 3 mixtures of acetone: resin (2:1, 1:1 and 1:2 per 1 h each, respectively). Then immersed in fresh resin in uncovered vials and left overnight. The same procedure was repeated for 12 hrs overnight then for a final 12 hrs. IV. Polymerisation: The samples with pure epoxy resin mixture were then placed in a mould with identifications and dried at 60ºC in an oven for 48 hrs.

Sectioning and Staining for Electron Microscopy: The sample blocks after having been studied under a light microscope, were retrimmed (0.5–1.0 mm block face). The blocks were mounted in the ultramicrotome, precisely oriented to a diamond knife, and ultrathin sections were cut (70–90 nm), floated onto the surface of water and collected on “grids” of copper mesh. Then stained with uranyl acetate as the first stain, followed by lead acetate as the second staining, the stained sections were then examined in a transmission electron microscope and photographed [8].

Assays of Phytase, Phytate Hydrolysis and pH Dependency Assays Reagents: Sodium phytate, $MgCl_2$, 50mM Trichloro -acetic acid, water bath and a centrifuge. **Cocktail Buffer:** It contained; 50mM HEPES, 50mM Tris, 50mM 2-{N-morpho-lino} ethanesulphonic acid (MES), adjusted to pH6 with either NaOH or HCl. General Procedure: Appropriate aliquots of the respective vesicles were thawed, constituting 4 treatments: 2
ages X 2 BBMVs intestinal sites.

The aliquots of the respective types of the BBMVs (4 treatments) were appropriately diluted with normal saline to a concentration of 6 mg BBMVs protein/ ml. Then 10 µl aliquots of each of the diluted BBMVs aliquots were used in the assays.

Determination of Phytase and Rate of Phytate Hydrolysis

In determining the time course of phytate hydrolysis by chicks and layers purified BBMVs (2 ages x 2 intestinal vesicles sites x 6 time courses x 2 replication), 48 eppendorf microcentrifuge tubes were prepared in 4 sets of 12 tubes. Then 10 µl of each of the diluted BBMVs aliquots was pipetted into 2 eppendorf tubes, such that each treatment had 2 replications.

To an appropriate volume of the cocktail buffer a given weight of sodium phytate was added such that the incubation medium had 0.1mM sodium phytate solution. Then 110 µl of the incubation medium was added to each eppendorf tube containing 10 µl of the treatment BBMVs, to give a final volume of 120µl and a concentration of 0.5mg of BBMVs protein / ml. Each set of tubes was then incubated at 41ºC. The reaction was stopped immediately by addition of 80µl of ice-cold 50 mM trichloroacetic acid, after the 12 tubes per set had been incubated for 2.5, 5, 10, 15, 20, and 25 minutes in succession. The tubes were centrifuged at 730 g for 20 minutes and the supernatant was assayed for inorganic phosphorus [9].

The kinetics of brush-border phytase activities in the compared segments was also evaluated as in time course except that the media contained increasing levels of phytate (0.1, 0.2, 0.3, 0.4, 0.5, 0.6m*M* phytic acid).

Determination of pH dependency of phytate hydrolysis

The pH dependency of phytate hydrolysis by different BBMV was determined using the same conditions as in the previous determination, except that the buffer solution had its pH adjusted by addition of either NaOH or HCl such that the incubation media contained pH 2, 4, 6, 8, 10 or 12, then the released IP was measured [9].

Data analysis

The data generated from the three assays were subjected to analysis of variance for factorial experiment (2 ages x 2 intestinal vesicles sites x time courses, phytate or pH concentrations), using Genstat 5th edition [10] and the means of the significant variables were compared using the least significant difference [11].

Results

The finding of the study on the influence of age, intestinal site and pH-dependency on phytate hydrolysis in chickens' brush border membrane vesicles and prior to assays, the isolated BBMVs were first subjected to their enrichment and integrity, as well as, micro graphical examinations, as follows:

Alkaline Phosphatase Enzyme Marker

The purity of the brush border membrane vesicles, as evaluated from the enrichment of the brush-border membrane marker alkaline phosphatase are illustrated in Table 1, which shows that enrichment of the BBMVs compared with homogenate was found to be 27.5-fold and 29.5-fold greater for chick proximal brush border membrane vesicles (CPBBMVs) and chick distal brush border membrane vesicles (CDBBMVs), respectively, and for the layers' proximal brush border membrane vesicles (LPBBMVs) and distal brush border membrane vesicles (LDBBMVs) were 34.8-fold and 20.9-fold, respectively, greater than in their corresponding mucosal homogenates. However, recovery of alkaline phosphatase suggests that the enzyme is not activated by any isolation procedure and its observed enrichment reflects the degree of purity of the prepared BBMVs.

Table 1. Alkaline phosphatase activities in the preparations of the BBMVs.

Chicks Intestinal Segment			Layers Intestinal Segment	
	Proximal	Distal	Proximal	Distal
AlkalinePhosphatase	**(U/g protein)**			
Homogenate	53.6	30.0	23.2	37.7
BBMVs	1477.1	878.1	807.1	787.6
Enrichment factor	27.5	29.5	34.8	20.9

D-Glucose Uptake by BBMVs

Upon assessment of functional integrity of the isolated BBMVs, the start of glucose uptake occurred sooner in both layers proximal and distal BBMVs than in chick (Figure, 1). However, after 10 min the uptake became greater in chicks' preparations, which showed maximum uptakes at 20 min (distal) and 30 min (proximal), whilst, uptake declined after the first 10 minutes for both the layers proximal and distal BBMVs.

Maximum glucose uptake occurred after 10m and 30m of incubating the respective vesicles from the layers and chicks intestinal segments (Figure, 1) in which D-glucose levels were >40 and >50 pmol/mg protein, respectively. Thus, sodium enhanced glucose uptake, an indication of occurrence of Na^+-dependent active transport.

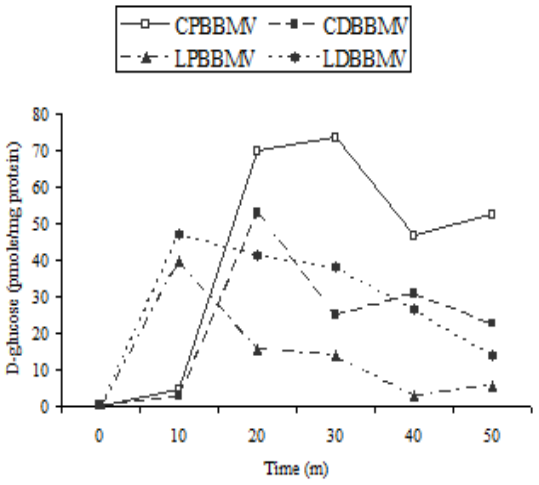

Figure 1. D-glucose uptake by BBMVs from chicks and layers intestinal segments. Data are means of duplicated samples.

Electron Microscopy of the Brush Border Membrane Vesicles

Electron micrograph of the purified proximal and distal BBMVs pellets obtained by the final centrifugation steps are presented in Plates 2 (A & B) and 3 (C & D) for the chicks and layers proximal and distal BBMVs, respectively. They reveal that the BBMVs preparations from the chicks and layers intestinal proximal and distal segments contained membrane vesicles with a prominent rod-like structure of microvilli, that are free from core materials,

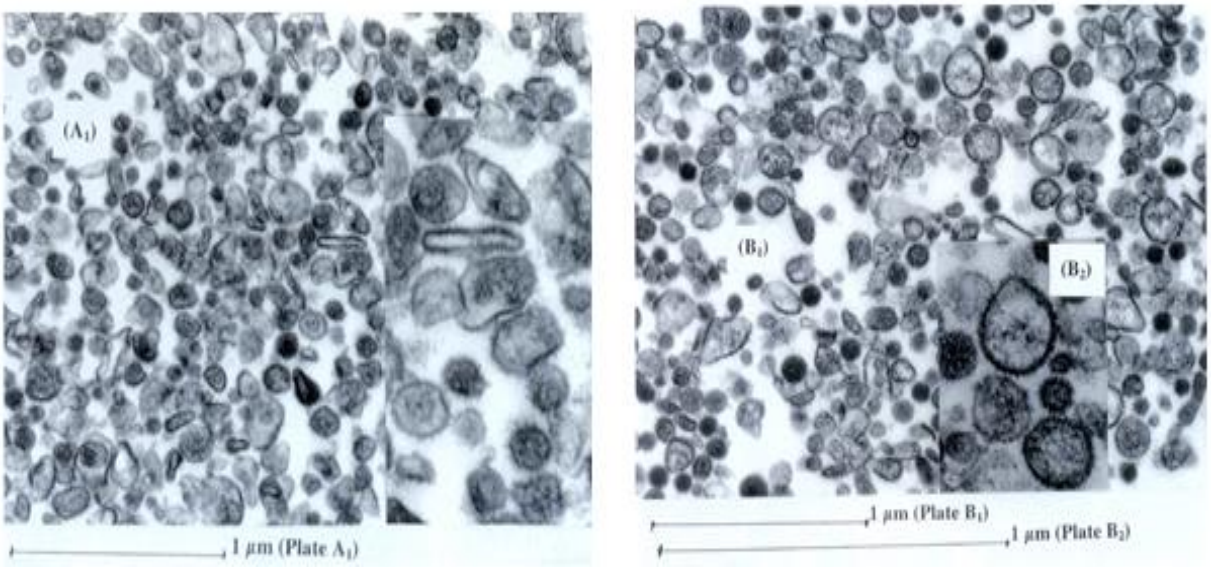

Plate 2. Electron micrograph of microvillus membrane for chicks' proximal intestine BBMVs (A) and chicks' distal intestine BBMVs (B). Bar A for plate A_1 for the magnification A_1. While in B, both bars B_1 and B_2 = 1 µm for the plate B_1 and inserted B_2.

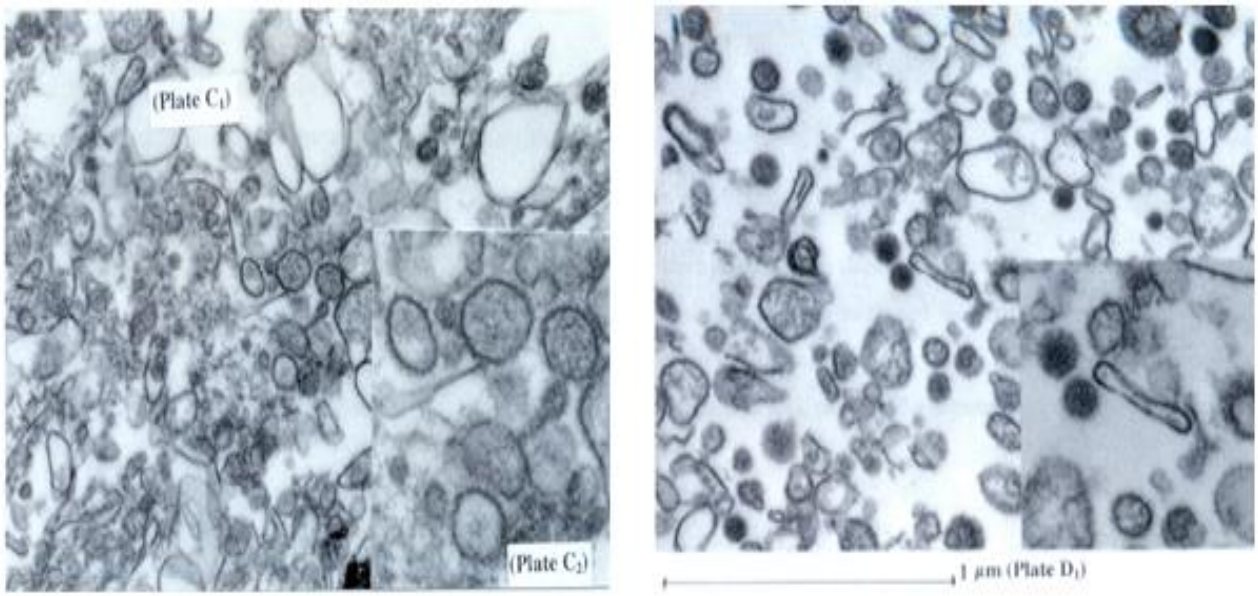

Plate 3. Electron micrograph of microvillus membrane for laying hens' proximal intestine BBMVs (C) and distal intestine BBMV (D). Insert in plate in (D) is for the lower magnifications. The bar = 1 µm.

consistent in both size and appearance and with no apparent contamination from other organelles and they range in diameters between 0.08 µm and 0.22 µm.

Phytate Hydrolysis in Growers' vs Layers' Proximal and Distal BBMVs

Results of phytate hydrolysis in both chicks and layers proximal and distal BBMVs are shown in Figure 2. The result depicts a decrease in phytate hydrolysis, or the release of inorganic phosphorus as a result of increase in phytate concentration. Both chicks' proximal and distal BBMVs showed significantly higher activities compared to layers, both proximal and distal BBMVs.

Chicks and layers showed a highly significantly difference on phytate hydrolysis between the lower phytate concentrations (0.1and 0.2mM phytate) and higher concentrations. Differences between proximal and distal segments tended to decrease at the higher concentrations for both chicks and layers, but chick proximal then distal samples maintained their higher activities compared to layers proximal and distal BBMVs.

Rate of Phytate Hydrolysis in Growers vs Layers BBMVs

The time courses of inorganic phosphorus being released from 0.1mM phytate are presented in Figure 3.

Under the assay conditions, the rate of inorganic phosphorus released increases for the first twenty minutes, and it is noted that all the assayed vesicles showed similar patterns.

In response to time course, chicks and layers BBMVs showed a significantly different increase in phytate-P hydrolysis ($P<0.01$) yields for the first 10 minutes, with chicks and layers distal BBMV showing the highest values, 48.14 and 42.0nmol Pi/mg protein at 20 and 15 minutes respectively.

Both ages depicted a significant phytate hydrolysis with more phosphate yielded from chicks BBMVs except at 15 min. It has been noted that both chicks and layers distal BBMVs depict higher activities in hydrolysing phytate, though chick distal BBMVs showed the highest phytate hydrolysis

pH Dependency of Growers and Layers Proximal and Distal BBMVs in Phytate Hydrolysis

The result of pH influence on phytate hydrolysis in both chicks and layers proximal and distal brush border membrane vesicles are shown in Figure 4.

At 0.1 nM phytic acid concentration and pH 6, the vesicles were significantly different (P<0.01) in their total phytase activity with distal vesicles showing lower values compared to the proximal. It seems that pH in the range of 6 to 8 gave slightly higher chicks BBMVs activity and hydrolysed phytate-P

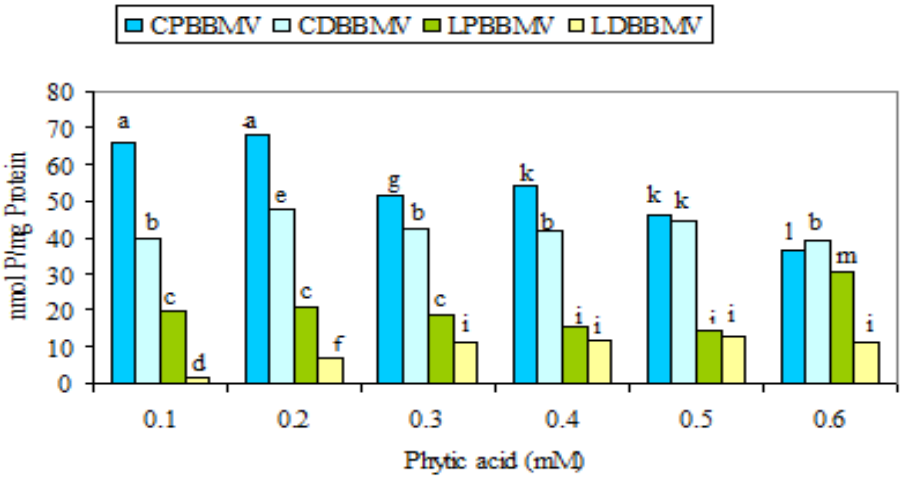

Figure 2.Phytate hydrolysis in chicks' vs. layers' proximal and distal BBMV.

The bars represent the mean, and the shared letters indicate no significant difference between samples Lsd at 5% is 0.75, 1.29, 1.83, and 2.85 for intestinal segment, phytic acid, phytic acid + segment, and age + phytic acid + segment, respectively**.**

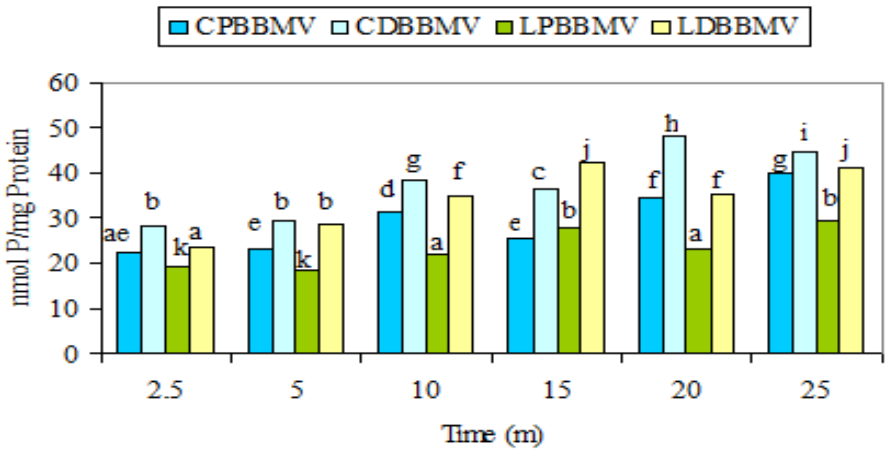

Figure 3. Initial rate of phytase activity for proximal and distal BBMVs for chicks . (CPBBMVs, CDBBMVs), and layers (LPBBMVs, LDBBMVs).

The bars represent the mean, and shared letters indicate no significant difference treatments. Lsd at 5% is 0.68, 0.96, 0.95, and 1.35 for intestinal segment, time, time + segment, and age + time segment, respectively.

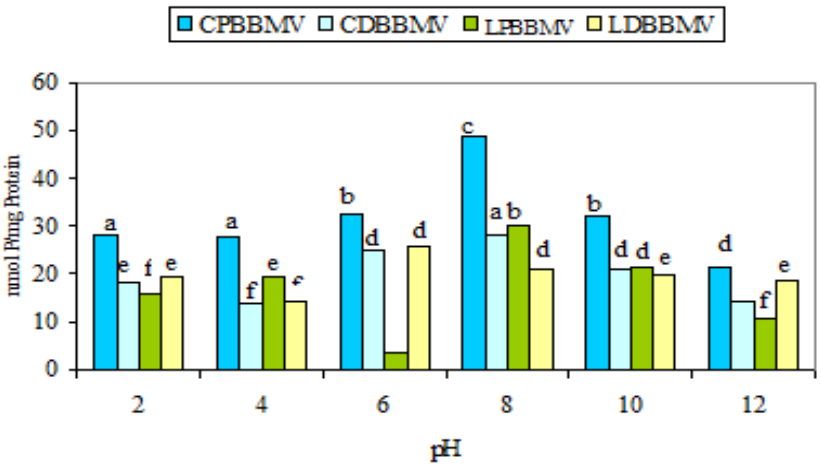

Figure 4 .Phytate pH dependency, and its hydrolysis in Brush Border Membrane Vesicles (BBMVs).

Data peak represents the treatment mean for the initial rate of 0.1mM phytate hydrolysis in both chicks and layers proximal and distal BBMVs. The bars represent the mean, and shared letters indicate no significant difference between phytase activities of treatments. Lsd at 5% is 1.04, 0.79, 0.96, 2.54, and 3.5 for intestinal segment, pH, pH + segment, and age +pH + segment, respectively.

Discussion

The purity of the isolated brush border membrane vesicles used in assaying the influences of age, intestinal site and pH on phytate hydrolysis using both $MgCl_2$ precipitation and differential centrifugation methods demonstrated that in first part of the fractionation, divalent cations have induced clumping which facilitate the removal of non-microvillar materials by low speed centrifugation. Later, Tris was used to disrupt the crude preparation of microvilli, it remove the microfilament cores and produces microvillus membrane vesicles. The added Mg was as effective as 10mM Ca in precipitating contaminating organelles from the homogenate [12].

Recovery of alkaline phosphatase activity in various fractions showed approximately 100%, indicating that the enzyme is not activated by any isolation step and that its observed enrichment actually reflects the degree of purity of the BBMVs preparation. The high enrichment of alkaline phosphatase in the respective BBMVs, which on average was 25-fold was consistent with the current isolates and suggest that the vesicles are primarily of brush-border origin and are not derived to any substantial extent from the basolateral domain of the enterocyte plasma membrane [12].

The ability shown to accumulate glucose against a concentration gradient indicates that the vesicles are tightly sealed. The subsequent drop in accumulated glucose with time is typical of Na^+ dependent transport into microvillus vesicles and reflects the dissipation of the initial sodium gradient [12]. However, the electron micrographs of purified BBMV reveal that the respective preparations from the distal and proximal intestinal segments for both the growers and laying hens contain membrane vesicles in the 0.2 to 0.5 µm size range, which are largely free of core materials. In both size and appearance the vesicles are similar in [7 and 12], this procedure proved to be adequate for fractionation of epithelial cells from the 4 weeks old growing chicks and 35 weeks old laying hen intestines.

Presence of phytase activity in mucosa of the small intestine of poultry [13,14] as well as its recovery from homogenates of the chick intestine [14] were well documented. But, whether or not the current obtained phytate hydrolysis by BBMVs was attributable to phytase or alkaline phosphatase is yet to be determined. Phytate may also act as a substrate for alkaline phosphatases (EC 3.1.3.1) and acid phosphatases (EC 3.1.3.2). However, previous authors have reported that intestinal phytase activity in rats was somewhat similar to but distinct from alkaline phosphatase [15] and it was suggested earlier that intestinal phytase activity could be a manifestation of such non-specific phosphatases [16].

The current result shows that phytate hydrolysis was greater at concentrations less than 0.3mM, with more phytase activity expressed especially in chicks proximal BBMVs, which contradicts the bulk of literature in favour of older birds. However, there is some evidence which indicates bacterial phytases may be active in digestive tract of poultry [17 and 18], since addition of lysed *E.coli* cellular material obtained from intestinal digesta to a phosphorus deficient corn-soybean meal diet was found to improve both growth and calcification in chicks; this response was attributed to phytase or similar enzymes of bacterial origin. If phytase from microbes resident in the digestive tract do hydrolyse phytate in poultry then the question arises whether it occurs at a site where phosphorus can be absorbed or not [2].

The activity of chick intestinal phytase activity was reported to be near maximum when the substrate concentration was about 0.25 mM sodium phytate (1.5mM phytate phosphorus) in the presence of 1.0mM $MgCl_2$ and pH about 7.2 [14], but substantial phytate hydrolysis was later observed to occur over the pH range from 5 to 6.5, with maximum hydrolysis of phytate at pH of 6. Phytine was previously found to be soluble at acidic pH, but has limited solubility in the more neutral pH region 5–8 [19]. The solubility of cation-phytate complexes in this region (1.33mM sodium phytate) by chicken intestinal mucosa was enhanced by the presence of Mg^{++} ions with slight change of optimum pH to more acidic [20], as inclusion of 25mM $MgCl_2$ in the medium was reported to double the rate of phytate hydrolysis [3]. The insolubility and stability of the mineral complexes in the gastrointestinal tract of monogastrics and birds is regarded as the major reason for the reduced bioavailability of some trace elements [21].

It could be deduced from the results of this homogenates that pH in the range between 6 and 8 is appropriate for the phytate-P hydrolysis, that is to say pH <6 might inhibit chicks BBMV activity in hydrolysing phytate bound phosphorus. *In vitro* studies with hamster small intestine have indicated that *myo*-inositol is actively transported against a concentration gradient, in a Na^+- and energy-dependent process, that may be distinct from glucose transport [22 and 23]. The effect of pH observed in this study showed high phytate hydrolysis activity at pH > 6 and < 8. Similar findings [3] revealed that substantial phytate hydrolysis occurred over the pH range from 5 to 6.5 with a maximum hydrolysis at pH 6. Inclusion of 25mM $MgCl_2$ in the medium doubled the rate of phytate hydrolysis, however, the specific and total activities of the intestinal brush border phytase were high in duodenum and decreased progressively down the length of the gut. A number of variables including source, pH and presence of metal ions have been suggested to control the activity of phytase in the gastrointestinal tract [2].

Both chicks and layers BBMV tended to show similar response patterns to increase in pH which seemed to agree with the reports of Maenz and Classen (1998) that the intestinal brush border vesicles prepared from broiler chicks and mature laying hens had comparable specific phytase activity. Furthermore, they disagree on the total activity of the brush border phytase activity and they reported that it was 35% higher in the small intestine of laying hens. Present findings show that both chicks proximal and distal BBMVs were rated higher than for layers; these differences probably stemmed from the fact that Maenz and Classen (1998) determined the total phytase activity in the whole chicken gastro-intestinal tract. Older birds might have adapted phytase, since rats were reported to adapt to phytate [24]. However, the adaptation in rats may result from either enhanced phytase or alkaline phosphatase synthesis by the gastrointestinal microflora stimulated by a lower level of phosphorus in the diet [24].

Intestinal brush border phytase could contribute to phytate-P digestibility and may be subjected to pH regulation and to the dietary phosphorus and vitamin D status of the chicken [3]. However, pH was noted as an extremely important factor determining the final solubility of the phytate. At pH 2.0 added calcium and zinc were completely soluble and not affected by protein sources or phytate [25]. Also, *in vitro* studies have shown that 0.5M calcium was ineffective as an inhibitor of microbial phytase at pH 5, while 0.05M calcium caused complete inhibition when the pH value of the medium was increased to 7.5 [26]. Moreover, mucosa phytase activity has been found in the intestine of many

species, probably it depend on calcium concentration [27], since in the presence of calcium in a molar ratio greater than 6:1 with phytate, small intestinal contents were found to contain phytase [27]. Thus, in the event of phytate escaping hydrolysis by mucosal phytase, bacteria in the large intestine are the most likely means of hydrolysis. In the present studies use was made of the same laying strain for both growing and mature chickens. Genotype could be a factor, but most reports on growing birds have involved the use of broiler strains, which could have been selected for different production characteristics to layers. Consequently, any differences in response could be due to genotype as well as to dietary manipulations. These have reduced a possible source of variation although there still been a difference in sexes. Perhaps, comparisons of BBMVs are more straightforward if the same strain were used, if so it might explain the differences from other reports with regard to age effect. It is scientifically observe that there are differences in food intake regulation between growing birds of layer and broiler strains which might reflect the differences in capacity to absorb nutrients. This could account for some of the differences reported here, but such queries can only be answered by direct comparisons.

In conclusion and given the condition of this study, the observed activity of brush border phytase seems to be higher in the proximal intestine and that growing chicks have shown higher capacity to digest phytate than the layer, however, comparisons of BBMVs could be more straightforward if the same strain is used. Furthermore, the hydrolysis of phytate-P by BBMVs may be influenced by dietary and gut regulation, but pH in the range between 6 and 8 seems appropriate for the phytate hydrolysis, i.e. pH <6 may inhibit chicks BBMVs phytase activity. But contribution of the total gut phyase, as well as, its differences from acid or alkaline is yet to be distinguished. Likewise, the process involve in regulation of phytate digestion and absorption that might require identification and characterisation of the enzyme or factors that may contribute to brush border phytase activity are yet to be determined.

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