A novel staining method for detecting phytase activity

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Abstract

Differential agar media for the detection of microbial phytase activity use the disappearance of precipitated calcium or sodium phytate as an indication of enzyme activity. When this technique was applied to the study of ruminal bacteria, it became apparent that the method was unable to differentiate between phytase activity and acid production. Strong positive reactions (zones of clearing around microbial colonies) observed for acid producing, anaerobic bacteria, such as Streptococcus bovis, were not corroborated by subsequent quantitative assays. Experimentation revealed that acidic solutions generated false positive results on the selected differential medium. Empirical studies undertaken to find a solution to this limitation determined the false positive results could be eliminated through a two step counterstaining treatment (cobalt chloride and ammonium molybdate/ammonium vanadate) which reprecipitates acid solubilized phytate. This report discusses the application of the developed two step counterstaining treatment for the screening of phytase producing ruminal bacteria as well as its use in phytase zymogram assays. © 1999 Published by Elsevier Science B.V.

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1. Introduction

Phytic acid [myo-inositol hexakis(dihydrogen phosphate)], the predominant form of total phosphate found in cereal grains and oilseed meals (Reddy et al., 1982), passes largely intact through the monogastric digestive tract. Swine and poultry diets must be supplemented with inorganic phosphate, while phytate phosphorus is excreted in manure and contributes to eutrophication of surface waters in areas of the world with intensive monogastric livestock production (Common, 1989; Wodzinski and Ullah, 1996). Amendment of monogastric animal rations with enzymes is one solution currently employed to overcome the inefficiencies and pollution caused by dietary constituents (Campbell and Bedford, 1992; Wodzinski and Ullah, 1996) and phytase amendment has been adopted recently in areas with intensive monogastric livestock production.

The majority of phytase research has been directed at the characterization, production and application of Aspergillus niger phytases. Although phytate degrading activities have been described for a number of plants, animals, fungi and aerobic bacteria (Rapoport et al., 1941; Shieh and Ware, 1968; Howson and Davis, 1983; Shimizu, 1992; Wodzinski and Ullah,
(1996), many possible sources of novel phytases remain unexplored. Despite reports of phytase activity in the rumen (Raun et al., 1956; Morse et al., 1992), the microbial inhabitants of this ecosystem have been largely ignored in the search for novel phytases with unique biochemical properties.

Suitable enzyme assays are necessary prerequisites for screening large numbers of microbial isolates such as would be found in complex ecosystems like the rumen. Although effective for characterizing individual isolates, standard solution assays for measuring phytase activity (Shimizu, 1992; Van Hartingsveldt et al., 1993) are unsatisfactory for the rapid screening of a complex mixture of microbial populations. A more suitable approach is the use of a differential plating medium. One method may be to use non-specific chromogenic phosphatase substrates, such as those used for histochemical and molecular biology applications, including 5-bromo-4-chloro-3-indolyl phosphate (BCIP), naphthol AS phosphates (West et al., 1990) and phenolphthalein diphosphate/methyl green (Riccio et al., 1997). Unfortunately, this approach lacks the desired specificity. A more specific method for detecting phytase activity would rely on the disappearance of precipitated calcium or sodium phytate as an indication of enzyme activity. Microorganisms expressing phytases produce zones of clearing on agar media containing sodium or calcium phytate (Shieh and Ware, 1968; Howson and Davis, 1983). However, the solid medium assays described in the literature were found to be unsatisfactory for screening anaerobic bacteria for phytase activity because of the false positive reactions of acid producing bacteria such as Streptococcus bovis. To overcome this problem, a two step counterstaining procedure can be used in which solid agar medium is flooded first with an aqueous cobalt chloride solution and second by an aqueous ammonium molybdate/ammonium vanadate solution. This staining technique has also proven to be useful in the detection of phytase activity in situ in polyacrylamide gels.

2. Materials and methods

2.1. Sodium phytate stability

Stabilities of sodium phytate solutions were determined by measuring the release of free phosphate following sterilization through a syringe filter (0.2 μm, cellulose acetate) or in an autoclave (121°C at 15 psi for 20 min). Released orthophosphate (P<sub>i</sub>) in the reaction mixture was measured by a modification of the method of Fiske and Subbarow (1925). Colour reagent (750 μl), prepared daily by mixing four volumes of 1.5% (w/v) ammonium molybdate in a 5.5% (v/v) sulfuric acid solution and one volume of a 2.7% (w/v) ferrous sulfate solution, was added to the sample solution (750 μl) and the production of phosphomolybdate was measured spectrophotometrically at 700 nm.

2.2. Phytase assays

Sample solutions (culture filtrates, cell suspensions, lysates, washes or distilled water blanks) were assayed for phytase activity by incubating 150 μl of the solution with 600 μl of substrate solution [0.2% (w/v) sodium phytate (Sigma, St Louis, MO, USA) in 0.1 M sodium acetate buffer, pH 5.0] for 30 min at 39°C (Shimizu, 1992). The reaction was stopped by adding 750 μl of a 5% (w/v) trichloroacetic acid solution and the free phosphate determined as above. Results were compared to a standard curve prepared with inorganic phosphate (K<sub>2</sub>HPO<sub>4</sub>). An improved phytase plate assay was developed which eliminated false positive results caused by microbial acid production. Bacterial isolates were grown under anaerobic conditions in an anaerobic chamber (90% CO<sub>2</sub> and 10% H<sub>2</sub>) on modified Scott and Dehority (1965) agar medium containing 10% (v/v) rumen fluid, carbohydrates (0.25% glucose, 0.25% cellobiose, and 0.3% starch), 1.8% (w/v) agar and 1.0% (w/v) sodium phytate (phytate agar) for 5 d at 39°C. Following growth, the petri plates were removed from the anaerobic chamber and under aerobic conditions the colonies were washed from the agar surface and the petri plates were flooded with a 2% (w/v) aqueous cobalt chloride solution. After a 5 min incubation at room temperature the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium vanadate solution. Following a 5 min incubation, the ammonium molybdate/ammonium vanadate solution was removed and the plates examined for zones of clearing.
2.3. SDS-PAGE and phytase zymogram analysis

Samples were mixed with sample loading buffer [62.5 mM Tris buffer (pH 6.8) containing 0.05% bromphenol blue, 0.72 M 2-β-mercaptoethanol, 10% glycerol and 2% sodium dodecyl sulfate; Laemmli, 1970] in a microtube and the tubes placed in a boiling water bath for 5 min. The denatured samples were resolved by SDS-PAGE on a 10% separating gel topped with a 4% stacking gel (Laemmli, 1970). Following electrophoresis, the gels were either stained for proteins or phytase activity. Protein bands were detected by submerging the gels in 0.1% (w/v) Coomassie Brilliant Blue in an aqueous methanol (40%), acetic acid (10%) solution and destained in an aqueous methanol (40%), acetic acid (10%) solution. Zymograms were prepared by soaking the gels first in 1% Triton X-100 for 1 h at room temperature and then in 0.1 M sodium acetate buffer (pH 5.0) for 1 h at 4°C. Phytase activity was detected by incubating the gels for 16 h in a 0.1 M sodium acetate buffer (pH 5.0) containing 0.4% (w/v) sodium phytate. Activity bands were visualized by immersing the gel in a 2% (w/v) aqueous cobalt chloride solution. After a 5 min incubation at room temperature the cobalt chloride solution was replaced with a freshly prepared solution containing equal volumes of a 6.25% (w/v) aqueous ammonium molybdate solution and 0.42% (w/v) ammonium vanadate solution. Phytase activity was evident as zones of clearing in an opaque background.

3. Results and discussion

3.1. Stability of sodium phytate as a phytate substrate

Preliminary experiments with phytate revealed that orthophosphate was released from phytate salts during autoclaving. This phenomenon is problematic as it not only interferes with the phosphate assay by increasing the background but may also release enough phosphate to inhibit phytase activity or repress phytase expression. Shieh and Ware (1968) observed a decline of 50% in the phytase activity of A. ficium NRRL 3135 when the phosphorus content of a corn starch medium was increased from 40 to 60 μg/ml. Further increasing of the phosphorus content of the medium from 40 to 140 μg/ml decreased phytase activity from 11.5 to 0.5 U/ml. Subsequent studies by Van Hartingsveldt et al. (1993) have demonstrated that A. ficium NRRL 3135 phyA expression is regulated at the transcriptional level (i.e., low levels of phosphate derepress phyA expression).

Initial stability experiments examined the orthophosphate content in a 1.0% (w/v) sodium phytate solution before and after autoclaving (30 min at 121°C and 15 psi). Orthophosphate content of the 1.0% aqueous solution increased from 4.1 μg/ml before autoclaving to 72.7 μg/ml after autoclaving. When a complex medium such as PSM (0.5% sodium phytate) (Howson and Davis, 1983) was autoclaved the release of orthophosphate was even greater. Orthophosphate content of PSM broth before autoclaving was 2.0 μg/ml compared to 216.3 μg/ml after autoclaving.

In a second study the release of orthophosphate from 1% sodium phytate in HEPES buffer (0.1 M, pH 6.5) and sodium acetate buffer (0.1 M, pH 5.0) after filter sterilization and autoclaving was examined. As observed in the previous experiment, autoclaving released orthophosphate from phytate. Inorganic phosphate levels of the 1% sodium phytate in HEPES or sodium acetate buffers were 215 and 270 μg/ml following autoclaving compared to undetectable levels of orthophosphate released following filter sterilization. The stability of a phytate solution over time was examined with Scott and Dehority medium containing 1% sodium phytate. In this case filter sterilized sodium phytate was added after the medium was autoclaved. Orthophosphate release over the time course of the experiment is shown in Fig. 1. The results of this study show that the amount of orthophosphate present in the medium increases with time. However, the rate of increase is low and most likely will not interfere with phytase studies. Further studies are in progress to determine the effects of various compounds and pH on orthophosphate release from phytate during autoclaving, but it is clear even at this point that caution must be taken with media preparation if the release of orthophosphate is to be minimized.

3.2. Phytase differential stain

When searching complex microbial communities
for novel enzymatic phenotypes, it is useful to have the ability to rapidly screen large numbers of isolates. In our survey of the rumen ecosystem for phytase producing bacteria we used a modification of the differential phytase screening medium reported by Howson and Davis (1983). On this medium putative phytase producing isolates could be identified by a distinct zone of clearing around the colony. The presence of phytase activity could be confirmed for some of the isolates, however this phenotype could not be replicated in broth culture for about 80% of the putative phytase positive isolates (unpublished data). Upon further examination it became apparent these isolates were also acid producers and the reduction of pH in the region immediately surrounding the acid producing colonies resulted in false positive results on the differential phytase screening medium. Perhaps this is not surprising as hydrochloric acid has long been used for the solubilization of phytate (Rather, 1917). The effect of pH on insoluble sodium phytate complexes can be readily demonstrated by adjusting the pH of a sodium phytate solution. While the precise mechanism involved in the counterstaining is not clearly defined we can speculate about the chelation capacity of phytate with cobalt and studies on phytic acid–metal complexes have shown the pH dependency of these complexes and the relative binding of different cations with phytate (Maddaiah et al., 1964; Vohra et al., 1965). Examination of other salts for the ability to form complexes less sensitive to a pH reduction found none better than cobalt chloride (unpublished data). The addition of the molybdate/vanadate solution changes the colour from pink to yellow and enhances contrast.

The effect of acid production was tested by cultivating Selenomonas ruminantium JY35 (high phytase producer) and Streptococcus bovis (high acid production) on the differential phytate agar medium. After 5 days incubation, zones of clearing were evident around the colonies of both microorganisms (Fig. 2). This figure demonstrates that the clearing
zone caused by acid could be mistaken for phytase activity, unless the counterstaining technique was utilized. Plate tests performed with commercially obtained *Aspergillus ficuum* phytase and weak HCl solutions supported this conclusion. Both created clearing zones on the solid medium, however only the zone of clearing remained around the *A. ficuum* phytase treatment following the counterstaining treatment (unpublished data).

Based on these results, we have applied this screening method with isolated anaerobic bacteria from rumen contents (Yanke et al., 1998). The success of this study has demonstrated the utility of this technique as a method for isolating novel phytase producing microorganisms while avoiding the false positive reactions associated with acid production.

### 3.3. Phytase zymogram

Following the success of the cobalt chloride/ammonium molybdate/ammonium vanadate counterstaining technique for the elimination of false positives obtained with the phytate differential medium it was decided to adapt this technique to a phytase zymogram. Non-specific phosphatase chromogenic substrates may be used to identify phytase active protein bands (Van Hartingsveldt et al., 1993). However, the limitation of this method is a lack of specificity. Protein extracts from *S. ruminantium* JY35 were resolved on standard Laemmlli gels, renatured and incubated with sodium phytate in situ.

![Fig. 3. Zymogram developed for phytase activity. Washed cells from a 1 ml culture of *S. ruminantium* JY35 (lanes B, C) and low molecular weight markers (lane A, Bio-Rad Laboratories) were resolved by SDS-PAGE in a 10% polyacrylamide gel. Proteins in lane C were stained for phytase activity. Proteins in lanes A and B were stained with Coomassie Brilliant Blue. The sizes of the low molecular weight markers are indicated to the left of the gel.](image-url)
When the phytate impregnated gels were exposed to the counterstaining reagents, insoluble phytate complexes formed where phytate was present. Zones of clearing remained in the immediate vicinity of active phytase protein bands, demonstrating a requirement for intact phytate in precipitate formation (Fig. 3). Thus, this counterstaining method has proven applicable in screening for phytase activity on solid medium and in the detection of phytase activity on polyacrylamide gels. While the mechanism is not defined we feel its successful application with both the solid medium and agarose gels employed here suggests it is a specific result between the metal ions and the phytate, involving chelated complexes of a pH dependent nature.

References


