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Research Article

IMMOBILIZATION OF ACID PHOSPHATASE (TYPE I) FROM WHEAT GERM ON GLUTARALDEHYDE ACTIVATED CHITOSAN BEADS: OPTIMIZATION AND CHARACTERIZATION#

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Abstract: Acid phosphatase from wheat germ (specific activity 1.327 U/mg protein) was used for immobilization on glutaraldehyde activated chitosan beads. Upon activation of chitosan beads, elongated fibers with pores were observed. The optimum percent immobilization obtained was 81.25%. The pH optimum of immobilized acid phosphatase was 5.5 with a shift of 0.5 units from the pH optimum of soluble enzyme (5.0). The values of K_m for *p*-nitrophenylphosphate with soluble and immobilized acid phosphatase were 0.107 and 0.32 mM, respectively. The V_{max} values for soluble and immobilized acid phosphatase were 1.644 and 1.85 µmol/min/mg protein, respectively. The optimum temperatures for soluble and immobilized acid phosphatase were 55 and 60°C, respectively. The immobilized acid phosphatase beads when stored at 4°C, retained 46.2% of enzyme activity after 49 days of storage. The same enzyme bead could be reused up to 10 cycles with 62.6% retention of activity. This is the first report of immobilization of acid phosphatase on crab shell chitosan beads. The method of immobilization reported here is simpler compared to previous reports of immobilization and the success obtained might ensure application in agriculture and medical diagnostics.

Key words: Acid phosphatase; wheat germ; immobilization; chitosan; kinetics; reusability

Introduction

Enzyme immobilization refers to the limitation of movement of enzyme by physical or chemical means on a suitable matrix. Enzymes are purified to homogeneity and the cost of production is high. Enzymes in the soluble form cannot be recovered from reaction mixture. However, the advantage with immobilized enzyme is that not only it can be separated from the reaction mixture but can also be reused for several cycles thereby reducing the cost of production. Different matrices are used in the process of immobilization.

Acid phosphatase (orthophosphoric monoesterphosphohydrolase, acid optimum; EC 3.1.3.2) (APase) catalyzes hydrolysis of a wide variety of phosphate esters and exhibit pH optimum below 6.0 (Vincent *et al.* 1992). There are few reports of APase immobilization on different matrices (Vaidya *et al.* 1987; Alteriis *et al.* 1988; Cantarella *et al.* 1988; Urso and Forter, 1996; Bautista *et al.* 1998; Marzadori *et al.* 1998; Wei *et al.* 2001; Ben-Knaz and Anvir, 2009; Kulkarni *et al.* 2010).

Chitosan is one of the commonly used matrices for immobilization of enzymes (Krajewska, 2004). It is a linear polysaccharide composed of β 1→4 linked D glucosamine and *N*-acetyl-D-glucosamine and is formed from deacetylation of chitin. The latter is the principal component of exoskeletons of crustaceans and insects and is the second most abundant biopolymer after cellulose (Bailey *et al.* 1999). Its features like hydrophilicity, biocompatibility and

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biodegradability make it an ideal support for enzyme immobilization (Payne and Sun, 1994). Glutaraldehyde has been used as a cross-linking reagent and for activation of chitosan beads. The glutaraldehyde activated chitosan beads are used for covalent attachment of enzyme. The other cross-linking agents include glyoxal (Martinez *et al.* 2007) and glutaric dialdehyde (El-Ghaffar and Hashem, 2009). There are reports of immobilization of APase on chitosan beads from shrimp shell wastes (Juang *et al.* 2001) and from cuttle fish wastes (Juang *et al.* 2002) cross-linked with glutaraldehyde or glyoxal. There are reports of use of composite beads of chitosan and activated clay for immobilization of APase (Chang and Juang, 2004) and composite beads of chitosan and ZrO_2 powders (Chang and Juang, 2007).

Wheat germ APase exists in multiple chromatographic and electrophoretic forms and are called isozymes (Joyce and Grisolia, 1960). A homogeneous isozyme from wheat germ has been isolated and characterized (Waymack and Van Etten, 1991). In the present paper we describe the optimization of immobilization of Type I APase from wheat germ on crab shell chitosan beads and its characterization. This is the first attempt of immobilization of APase on chitosan beads from crab shell.

Materials and Methods

Chitosan from crab shells with minimum 85% deacetylation, wheat germ APase (Type I) (1.327 U/mg), Coomassie Brilliant Blue G-250 and *p*-nitrophenylphosphate (p NPP) (Sigma Aldrich, USA); ethanol (Jebsen & Jessen Gmbh & Co. Germany) glacial acetic acid, orthophoshoric acid and glutaraldehyde (Sd. Fine Chemicals, India); sodium acetate, sodium hydroxide and *p*-nitrophenol (Hi Media, India); potassium hydroxide and Tris (hydroxymethyl) aminomethane (Sisco Research Laboratory, India) and hydrochloric acid (Qualigens Fine Chemicals, India) were procured from the sources mentioned within parenthesis. All solutions were prepared in de-ionized water from Milli-Q system (Millipore, USA).

Soluble APase activity assay and protein quantification- The APase activity was assayed by discontinuous spectrophotometric method using Varian Cary 50 spectrophotometer, Australia. The substrate used was pNPP and its conversion to *p*-nitrophenol was monitored at 405 nm. A stock solution of enzyme (4 mg/ml) was prepared by suspending 0.04 g in 10 ml of assay buffer (50 mM sodium acetate buffer, pH 5.0). A 1.0 mM solution of pNPP was prepared in assay buffer. The 2.0 ml assay solution contained 0.95 ml assay buffer and 1.0 ml of pNPP maintained at 37°C. The reaction was initiated by addition of an aliquot (0.05ml) of enzyme and incubated for 10 min. The reaction was terminated by addition of 1.0 ml of 0.1 N NaOH. Enzyme units were calculated using standard plot (A_{405} versus [*p*-nitrophenol]). One unit of APase was defined as the amount of enzyme releasing one µmol *p*-nitrophenol per min under the assay conditions. Protein content of soluble and in the washings of immobilized APase on chitosan beads was done according to method by Bradford (1976) with little modifications.

A chitosan (1.5%, w/v) solution was prepared by dissolving 1.5 g chitosan in 1.5 % glacial acetic acid solution at 60°C with continuous stirring. This solution was taken in a syringe and was allowed to fall drop wise into a 100 ml solution of pre-cool 1M KOH. Beads of uniform size and shape were formed. The beads were then washed with distilled water and stored in storage buffer (50 mM Tris HCl buffer, pH 7.5) at 4°C.

Optimization of immobilization of APase on chitosan beads- and activity assay - The optimization process included variation of three parameters namely glutaraldehyde concentration (1.0-4.0%), activation time for reaction of glutaraldehyde with chitosan bead (3-24 h) and coupling time of activated chitosan bead with enzyme (3-24 h). Glutaraldehyde activated chitosan beads were washed twice with storage buffer to remove the excess glutaraldehyde. The glutaraldehyde activated beads were suspended in 2.5 ml enzyme solution (4 mg/ml). The beads were then washed with buffer, till washings were free from unbound enzyme. The activity and protein content of chitosan beads were determined. The immobilization of enzyme is expressed in terms of % immobilization that is determined from the following formula:

$$\% \, immobilization = \frac{Specific\ activity\ of\ immobilized\ enzyme}{Specific\ activity\ of\ soluble\ enzyme} \times 100 \quad (1)$$

Where specific activity of immobilized enzyme = specific activity of soluble enzyme – specific activity of unbound enzyme.

The 2.0 ml test solution consisted of 1.0 ml pNPP and 1.0 ml assay buffer kept in a water bath maintained at 37°C. Two chitosan-enzyme beads were added to the test solution and the reaction was allowed to take place for 10 min. After 10 min, the beads were taken out and 1.0 ml of 0.1 N NaOH was added. The absorbance was recorded at 405nm.

Photoimages - The photograph of chitosan beads were recorded by Nikon Cool Pix L810 Camera with 5x magnification. Scanning electron micrographs (SEM) of chitosan beads surface were recorded on Scanning Electron Microscope SEM-JSM 6360, JEOL (Japan) in SAIF, NEHU, Shillong. The scanning was done at constant 20 kV, and micrographs at 9500 and 16000 magnifications were recorded.

Kinetic characterization of immobilized APase - The variation of enzyme activity with pH has been studied using 50 mM sodium acetate buffers, (pH 4.0- 7.0) at enzyme saturating concentrations of soluble pNPP. From the data, a plot of % relative activity versus pH was made along with that of soluble enzyme to determine pH optima. The rate of reaction was monitored at different concentrations of pNPP using 50 mM sodium acetate buffer, pH 5.5. From this data, Lineweaver-Burk plot (1/v versus 1/ [pNPP]) was made to determine values of K_m and V_{max}. Effect of temperature on immobilized APase activity has been studied in the range of 40 to 70°C using 50 mM sodium acetate buffer, pH 5.5. The reaction mixture was maintained at a particular temperature. From the data, a plot of relative % activity versus temperature was made to determine optimum temperature and was compared with that of soluble enzyme.

Storage stability of soluble and immobilized APase - The soluble APase was stored in assay buffer at 4°C and aliquots were withdrawn on different days and tested for residual activity. APase immobilized on glutaraldehyde activated chitosan beads were routinely stored in storage buffer at 4°C. Enzyme activity was routinely tested on different days using different beads. A plot of % residual activity versus time (Days) was made.

Reusability of immobilized APase - Activity assay of a particular bead was performed for several cycles and a plot of % residual activity versus cycles of reuse was made.

Experiments were conducted in triplicate and results represent the average of the three values and standard deviations have also been reported.

Results and Discussion

The plot of A_{405} versus [*p*-nitrophenol] revealed an absorbance of 0.1 corresponding to 20 nmole of *p*-nitrophenol (data not shown). The photographs of non-activated and 1% glutaraldehyde activated chitosan beads are shown in Figure 1. Upon activation, the color of beads became yellowish brown. The color was however more intense as 2% glutaraldehyde activated chitosan bead was used (Kumari and Kayastha, 2011). The scanning electron micrograph (SEM) of non-activated chitosan bead, glutaraldehyde activated chitosan bead and APase immobilized glutaraldehyde activated chitosan beads surface at 9,500 and 16,000 times magnification are shown in Figure 2a, b and c, respectively. The SEM of non-activated chitosan bead surface gave a condensed and granular appearance while on glutaraldehyde activated chitosan, elongated fibres were noticed on the surface along with pores. It has been suggested

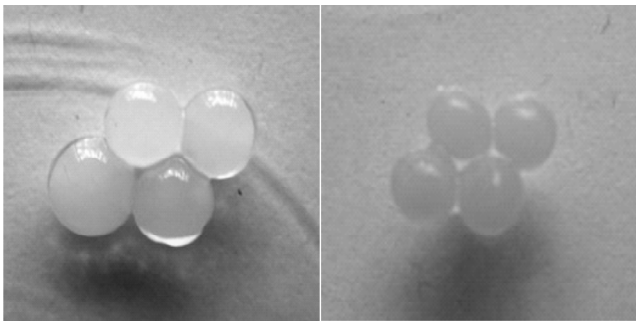

Figure 1: **Magnified photograph (5 x) of APase immobilized on chitosan beads using Nikon Cool Pix L810 Camera with 5x magnification. (a) Non-activated chitosan beads (b) Glutaraldehyde activated chitosan beads.**

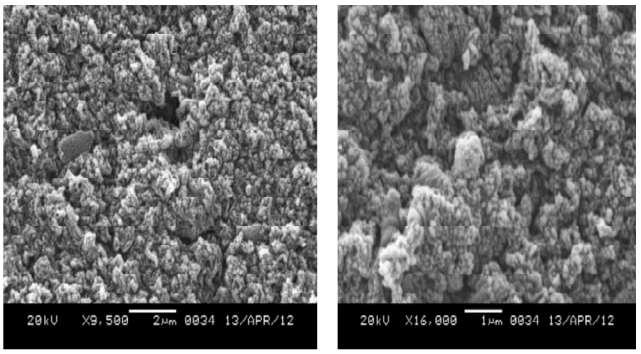

Figure 2a

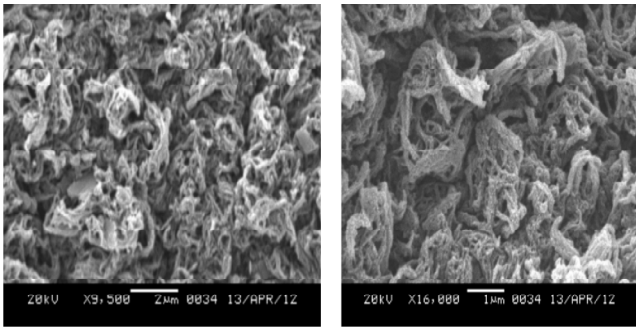

Figure 2b

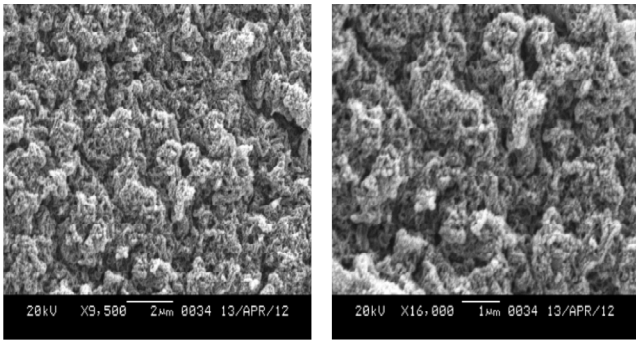

Figure 2c

Figure 2: **SEM of chitosan beads surface were recorded on Scanning Electron Microscope *SEM-JSM 6360, JEOL*. The scanning was done at constant 20 kV, and micrographs at 9500 and 16000 magnifications were recorded. (a) Non-activated chitosan beads (b) Glutaraldehyde activated chitosan beads (c) Glutaraldehyde activated chitosan beads after immobilization.**

that chemical modification brings about increase in porosity and accessibility to internal sites (Rorrer *et al.* 1993; Guibal *et al.*1995). The degree of elongation decreases upon immobilization.

In the present study, we have used 1.5% chitosan solution. Below 1.5% chitosan, beads were mechanically weak, whereas, above 1.5% chitosan, the beads were harder and difficult to allow passage of substrate. The results of optimization of immobilization of APase are given in Table 1. A decrease in % immobilization was observed when the concentration of glutaraldehyde was increased from 1 to 4%, keeping the activation and coupling time fixed. Therefore the chitosan bead activation was done at 1% glutaraldehyde concentration. The variation in activation time, practically had no effect on % immobilization, however maximum immobilization was observed when activation time was 6 h. The results of variation of coupling time suggests that maximum immobilization was observed when glutaraldehyde activated chitosan bead was allowed a 12 h coupling time. The % immobilization under optimal condition was 81.25%. There are some other reports on immobilization of APase on chitosan and other matrices with % immobilization around 80% (Juang *et al.* 2001; Zhu *et al.* 2010; Srivastava and Anand, 2014). The specific activity of immobilized enzyme was found to be 1.076 U/mg.

Table 1

[Glutaraldehyde] %	*Activation time h*	*Coupling time h*	*Immobilization %*
1	6	12	81.25 ± 0.9
2	6	12	54.6 ± 1.2
3	6	12	29.2 ± 1.1
4	6	12	14.6 ± 1.2
1	3	12	80.8 ± 1.1
1	6	12	81.25 ± 0.9
1	12	12	79.58 ± 0.9
1	24	12	80.00 ± 1.1
1	6	3	41.7 ± 0.9
1	6	6	45.0 ± 1.2
1	6	12	81.25 ± 0.9
1	6	24	77.9 ± 0.9

The result of effect of pH on relative activities of soluble and immobilized APase is shown in Figure 3. The immobilized enzyme showed a higher relative activity in comparison to soluble enzyme except at pH 5.0. The soluble APase showed pH optimum at pH 5.0. A shift has been observed however with immobilized APase (pH 5.5). The immobilized APase was therefore characterized further at pH 5.5. An increase in pH optimum by 0.5 units only has also been observed in some other immobilized APases (Kurita *et al.* 1997; Huang *et al.* 2005). In case of *Vigna aconitifolia*

APase immobilized on chitosan beads, an increase in pH optimum by 2.0 pH units has been reported (Srivastava and Anand, 2014).

The results of effect of temperature on relative activities of soluble and immobilized APase are shown in Figure 4. The soluble APase showed temperature optimum at 50°C and exhibited a drop in activity thereafter. The immobilized APase showed temperature optimum at 60°C. It is evident from the Figure 4 that in between 30 to 55°C, soluble APase showed better relative activity to immobilized APase, but beyond 55°C, immobilized APase showed better relative activity. The soluble and immobilized APase from *Vigna aconitifolia* showed temperature optimum at 60°C (Srivastava and Anand, 2014).

The Lineweaver-Burk plot of immobilized APase has been shown in Figure 5. The K_m values for soluble and immobilized APases were 0.107 and 0.32 mM, respectively. The increase in K_m values of immobilized APases has also been observed in literature (Marzadori *et al.* 1998; Yamato *et al.* 2000; Zhu *et al.* 2010; Srivastava and Anand 2014). Different reasons have been proposed for increase in K_m value like change in the conformation of enzyme after immobilization (Goldstein, 1976) or diffusional limitation of substrate (Reddy and Kayastha, 2006). The values of V_{max} for soluble and immobilized APases were 1.644 and 1.85 µmol/min/mg protein, respectively. From the plot it is evident that at low concentrations of pNPP, the rate of reaction catalyzed by immobilized APase was lower with respect to soluble APase. At higher concentrations of pNPP, the rate of reaction catalyzed by immobilized APase was higher in comparison to soluble APase.

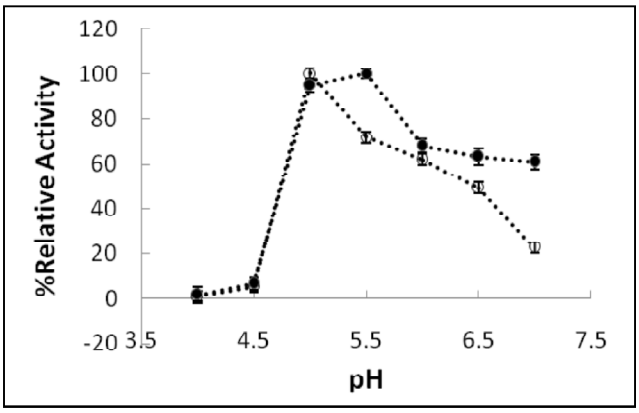

Figure 3: **Effect of variation of pH on relative activities of soluble (◦) and glutaraldehyde activated chitosan immobilized APase (•) bead catalyzed reaction.**

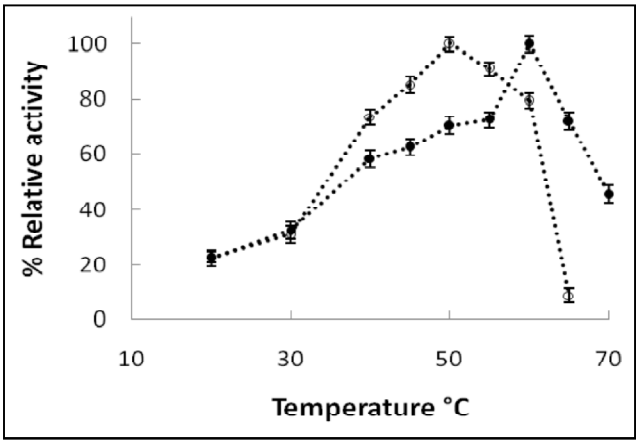

Figure 4: **Effect of variation of temperature on relative activities of soluble (◦) and glutaraldehyde activated chitosan immobilized APase (•) bead catalyzed reactions.**

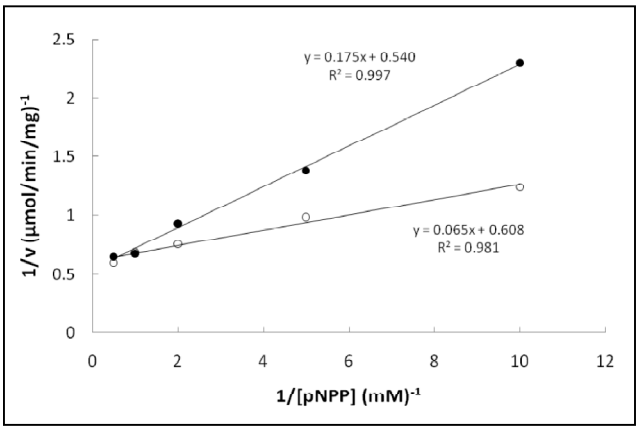

Figure 5: **Lineweaver-Burk plots of soluble (◦) and glutaraldehyde activated chitosan immobilized APase (•) bead catalyzed reaction rate at different pNPP concentrations.**

The results of storage stability of soluble and immobilized APase at 4°C are shown in Figure 6. It is evident that the immobilized APase showed better activity retention in comparison to soluble APase up to 7 weeks. The soluble APase retained only 11% activity on 49th day while immobilized APase retained 46% activity on 49th day. There are reports on low retention of activity in some immobilized APase (Marzadori *et al.* 1998; Juang *et al.* 2001). In some other immobilized APases, very high activity retention has been reported (Yamato *et al.* 2000; Juang *et al.* 2002).

The results of effect of reusability of a particular immobilized APase bead for different cycles of reuse are shown in Figure 7. More than

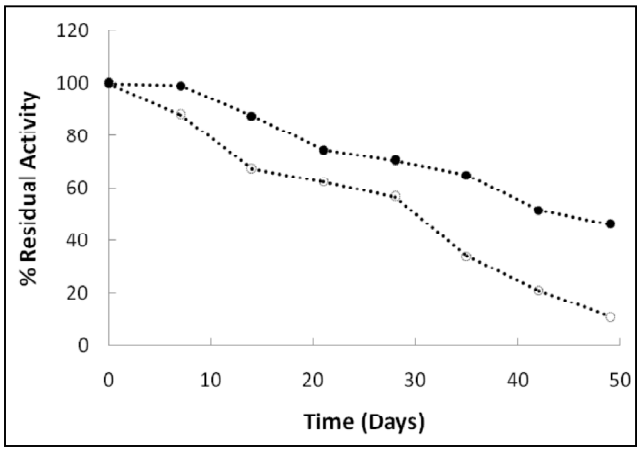

Figure 6: **Storage stability of soluble (○) glutaraldehyde activated chitosan immobilized APase (•) beads at 4°C.**

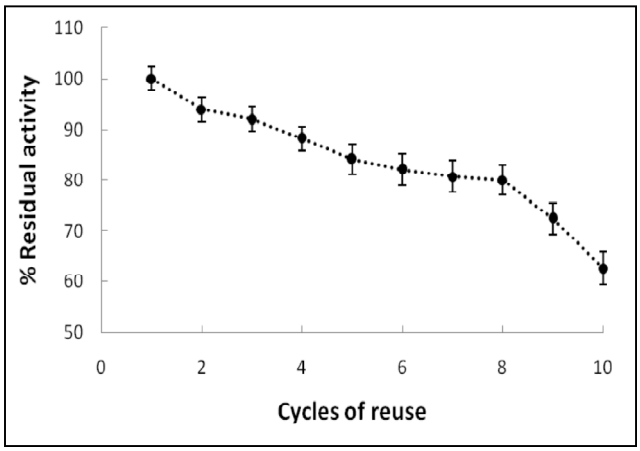

Figure 7: **Reusability of the same glutaraldehyde activated chitosan immobilized APase bead at 4°C.**

60% activity was retained after 10 cycles of reuse. There are reports of losses in activity in some other immobilized APases after 10 cycles of reuse (Kurita *et al.* 1997; Zhu *et al.* 2010). There are some other reports where high activity is retained after several cycles of reuse (Chang and Juang 2004; Srivastava and Anand, 2014). The loss in activity may be due to weakening in the binding of enzyme to the matrix.

Conclusion

The present work describes the optimization of immobilization of APase from wheat germ on glutaraldehyde activated chitosan beads from crab shell for the first time for better utility in future in applied research. The scanning electron micrograph clearly revealed the changes brought about during activation in the presence of glutaraldehyde. The immobilized APase showed better thermal stability and reusability for several cycles. The simple method of immobilization presented here is expected to further motivate use of immobilized acid phosphatase for such applications as metabolic marker enzyme of diseases as well as to improve phosphorus content of soil (Lai and Shin, 1993).

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Abbreviations

APase, acid phosphatase; APases, acid phosphatases; pNPP, *p*-nitrophenylphosphate; SEM, scanning electron micrograph

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