Enzyme Immobilization on Glass Surface for the Development of Phosphate Detection Biosensors

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ABSTRACT

Alkaline Phosphatase was immobilized on aminated glass fiber disks by covalent bond in the vacuum process. In this procedure, amide bonds were formed between carboxyl groups on the enzyme and amino groups on the glass surface. 10% Glycidoxypropyle trimethoxysilane was the best coupling reagent which could help form bonds between carboxyl and amino group on the glass fiber disk. A 10% concentration of coupling reagent, pH 9.0 and 2 gram of silica were found to be the best conditions for coupling the enzyme over the glass surface showed the highest enzyme activity. The covalent attached immobilized enzyme not only retained its activity but also could be reused at least 4 times after washing without loss of enzyme activity. Immobilized enzyme showed nearly 16% loss of enzyme activity after the first trial. An average of 1.65 mg of reusable alkaline phosphatase was immobilized per gram of glass fiber. Phosphate elements were measured from water, raw milk and raw shrimp sample by the used of this alkaline phosphatase immobilized disk as a biosensor. Immobilized enzyme can converts substrate to product and then will converts it to a measurable signal. This study demonstrates the possibility of using such a glass disk for the development of biosensors application.

Keywords: Aminopropyl trimethoxysilane , Aminopropyle triethoxysilane, Chloroporpyle trimethoxysilane, Glycidoxypropyle trimethoxysilane, Alkaline phosphatase, *P*-nitrophenyl phoshphate, Immobilization, Biosensor.

1. INTRODUCTION

Biosensors based on immobilized enzymes have many applications in areas such as industry, biochemistry and immunology and enzymology , pharmaceutical (Marconi, 1978; Cheetham, 1985), where a wide range of fixation techniques have been developed and are continuously being improved. Of these techniques, covalent coupling on inorganic supports is one of the most commonly used due to its stability in achieving a good enzyme attachment (Weetall, 1993). Enzyme immobilization is carried out by activating a functional group, either on the protein surface or on the solid support, with chemical reagents (Lunblad, 1995). The general formula of covalent attachment with glass, silica, is R-Si-X. R is organofunctional group and X is hydrolysable group. The R group is separated from the silicon atom by a propyl chain and X is alkoxy group,eg : metoxy , ethoxy (Veliky.I.A et al 1994) .The support for enzyme immobilization may be a membrane, a water-insoluble solid, or a polymer matrix. Immobilization on glass surface is cheapest way for the application of biosensor. Alkaline phosphatase has been

immobilized on a variety of surfaces (Surinenaite et al., 1996; Wiley et al., 2001; Filmon et al., 2002) but, to our knowledge, from the previous report of immobilization on glass (Weetall, 1969), the porosity of the glass beads they used and organic solvents applied led to considerable enzyme inactivation. It was expected that with better flat surface of the glass fibre disk that problem could be overcome and more effective sensor produced. Glass has advantage that is dimensionally stable and easy to clean thoroughly to remove contaminants by sterilization, maximum enzyme loading, economics of its preparation and application and regeneration, easy to handling, low possibility to loss of enzyme activity, and nontoxic. The major objective of this study was to Optimization of the composition of selected coupling reagents for the immobilization of alkaline phosphatase on the glass surface and Application of the immobilized enzyme for analysis of phosphate element in fish, milk and water samples.

Kim et al (1996) considered biosensors to be an analytical device composed of biological element in intimate contact with physical transducer, which together relates the concentration of a target analyte to a measurable signal. Usually aim is to produce an electronic signal which is proportional in magnitude or frequency to the concentration of a specific analyte or group of analytes to which the biosensing element binds. Biosensors are composed of a detector and immobilized biocatalyst. Enzyme-based biosensors primarily rely on two operational mechanisms. The first mechanism involves the catalytic transformation of a pollutant (typically from a non-detectable form to a detectable form). The second mechanism involves the detection of pollutants that inhibit or mediate the enzyme's activity (Kim R. Rogers 2006). In this work the alkaline phosphates enzyme was used as a representative enzyme for immobilization on glass surface as its presence is easily selected due to its color reaction. For the same reason it is also tagged or conjugated to antibodies or antigen in Enzyme Linked Immunosorbant Assay (ELISA).

2. MATERIALS AND METHODS

Glass fiber filter disk (2.4 cm dia.) purchased from Whatman company (cat no is 1820024). Silicon powder, four coupling agents Aminopopyl trimethoxy silane (APTMS), Aminopropyl triethoxy silane (APTES), chloropropyl trimethoxy silane (CPTMS) and Glycidoxypropyl trimethoxy silane (GOPS), Enzyme : Alkaline phosphatase, Substrate : *P* nitrophenyl phosphate and fish sample (shrimp),milk and water where all used in the cause of the experiment.

2.1 Optimization of enzyme immobilization on glass surface by using selected coupling reagents on the glass surface

2.1.1 Activation of silica filter disks

The glass fiber disks were refluxed in 12N HCl for 3 hours. With the help of a Buchner funnel, Glass fiber filter disks washed with excess distilled water and dried in oven at 80°C for about 40 minutes.

2.1.2 Amination process

The activated glass filter disks were animated as described by Russel et al. (2005) the glass fibre disks were fitted over a pressure equalizer funnel. Then different amount of silicon powder (2gm, 4gm, and 6gm) and 0.05 ml triethylamine was added over the disks. Then 0.1 M NaCl, toluene and 2 ml of coupling reagents (APTMS, APTES, CPTMS, and

GOPS) were mixed by using magnetic stirrer. This solution was added on the disk as a washing solution of silica by syringe in the pressure equalizer flask.

Three different concentrations 10 %(v/v), 20 %(v/v) and 30 %(v/v) solutions of APTMS, APTES, CPTMS, GOPS in toluene were used for the second time refluxed of glass disk after amination for 18 hours. After that toluene, acetone and then distilled water was used respectively to rinsed the disks.

2.1.3 Immobilization of alkaline phosphatase

A 10 mg/ml aqueous stock of bovine intestinal mucosa alkaline phosphatase was prepared and adjusted to different pH 7.0, 8.0 and 9.0 by using 1N NaOH. In 200 μl of these stock solutions glass fibre disks was soaked for approximately 10 second. These moist activated filter disks were lyophilized. After that the dried ALPase-disks were sealed under vacuum ($\approx$ 50 m Torr) and incubated at 80°C for 96 hours as described by Russel et al. (2005). Immobilized disks were rinsed in a Buchner funnel with excess 0.1M NaCl and distilled water and then lyophilized and then stored at 4°C.

2.1.4 Immobilized alkaline phosphatase (ALPase) activity assay

Standard method with p-nitrophenylphosphate (pNPP) as a substrate was used to determine ALPase activity. A stock solution of 350 μM pNPP in 25 mM glycine was prepared and adjusted to pH 9.6 with 1 N NaOH. The immobilized glass fiber filter disks were put into the test tube. At time zero, 20 ml of pNPP solution was added to the test tube with constant stirring. Every two minutes, a 90 μl aliquot was removed from the mix and placed in a well of a microtiter plate containing 10 μl of a stop solution composed of 0.1M NaOH and 0.1M EDTA. The appearance of the color indicated enzyme reaction. The enzyme activity of the sample was determined from the absorbance at 405nm of the ensuing solution measured using a spectrophotometer. The activity of immobilized ALPase was calculated using the formula:

$$Enzyme\ Activity = \frac{A_{405}(V_{reaction} / V_{aliquot})}{\beta}$$

Where, A_{405} is the absorbance at 405 nm

$V_{aliquot}$ The volume of the aliquot read (0.1ml)

$V_{reaction}$ The volume of the reaction (20ml) and

β is the extinction coefficient for the *para*-nitrophenol (pNP (18.5 at 405nm))

EU is the enzyme activity in micromoles of pNPP.

2.1.5 Quantification of enzyme in soluble form

20mL of the 350 μM pNPP substrate and free soluble ALPase were used to generate a standard activity curve. Initially the substrate was stirred vigorously and 0.5, 1, 5, 20, 100 μg of soluble ALPase from stock solution were added and incubated for 30 minutes and then the change in absorbance at 405nm measured. 5 different concentrations of soluble ALPase were examined, each in duplicate, and the mean values plotted on a standard curve. This standard curve was used to estimate the amount of immobilized ALPase activity. Between each trial the filter disk containing the immobilized ALPase were rinsed

three times with excess (approximately 3× 10 mL) 1M NaCl and distilled water by placing the disk in a Buchner funnel attached to a water aspirator.

2.2 Sensitivity and stability of the immobilized enzyme activity

In this steps only one specific condition that was found to be best in experiment A (10% coupling reagents, silica amount 2 gram and pH 9.0) was used and made 4 trials for each immobilized glass disk. All the experimental steps were same as experiment A. Optical Density was taken in every minute of total 25 minutes. So there were 25 readings. The results were compared within all 4 coupling reagents. The loss of enzyme activity and the amount of enzyme bound after immobilization was also calculated. (Diethanolamine Assay) Alkaline Phosphatase for quantification to determine of loss of activity due to immobilization.

Calculation:

$$\text{Units/ml enzyme} = \frac{(\Delta A405nm\_Test - \Delta A405nm\_Blank)(3.1)(df)}{18.5 \times 0.1}$$

3.1 = Volume (in milliliters) of assay
0.1 = Dilution factor (df)
18.5 = Mill molar extinction coefficient of p-Nitro phenol at 405 nm
0.1 = Volume (in milliliters) of enzyme used

$$\text{Units/mg solid} = \frac{units/ml\_enzyme}{mg\_solid/ml\_enzyme}$$

$$\text{Units/mg enzyme} = \frac{units/ml\_enzyme}{mg\_enzyme/ml\_enzyme}$$

This method varied from previous method that a buffer was used to maintain pH. One unit enzyme will hydrolyze 1.0 μmole of p-nitrophenyl phosphate per minute at 37°C. Enzymatic activity was expressed as EU/ml for the soluble enzyme. The bound enzyme activity was measured directly in the derivative and it was expressed as EU/g of glass. The specific activity was defined (i) as the ratio between the activity expressed in EU/ml and the concentration in mg of enzyme/ml, for soluble enzyme;(ii) as the ratio between the activity expressed in EU/g of glass and the concentration of immobilized enzyme in mg/g of glass for immobilized enzyme. Binding efficiency is the percentage of initial free enzymatic activity that remains attached to glass after immobilization.

2.3 Application of immobilized enzyme for phosphate detection

Same amount of phosphate was inoculated into the same amount of samples for making a standard curve. The amount of phosphate and the intensity of yellow color were measured by the comparison with standard curve.

2.3.1 Fish sample tasted

Fish sample (shrimp) was taken for detection of Nirophenyl Phosphate. 5 gm fresh and rotted shrimp were homogenized in 15 ml distilled water. After homogenized it was filtrated and made the total volume made up to 25ml by distilled water. Aliquot was mixed with diethanolamine buffer pH 9.8.After 10 minutes this mixture was applied to each filter disks and observed the reaction color. Yellow color of the reaction was indicated the presence of phosphate elements.

2.3.2 Milk sample tested

2 ml pasteurized and spoiled milk sample were centrifuged for 10 min at 3000 rpm for the separation of fat layer. Penetrated fat layer with pipette and samples was take out (milk) from cream. Skimmed milk was filtered through a small, moist, blue band filter and 0.1 ml of the filtrate mixed with diethanolamine (Bergmeyer 1974). This mixture was applied over the immobilized glass surface and observed the yellow color formation observed.

2.3.3 Water sample tested

Water samples used was different Tap water at BPT, distilled water and mineral water were (Nestle, Namthip, ThipSiam, and Flowave) for examination of the presence of Phosphate elements. 3 ml of water sample was mixed with diethanolamine buffer. 200 µl of water buffer solution were dropped into the surface of immobilized disk. Observed the yellow color was formation for 10 minutes over the immobilized disk.

3. RESULTS AND DISCUSSION

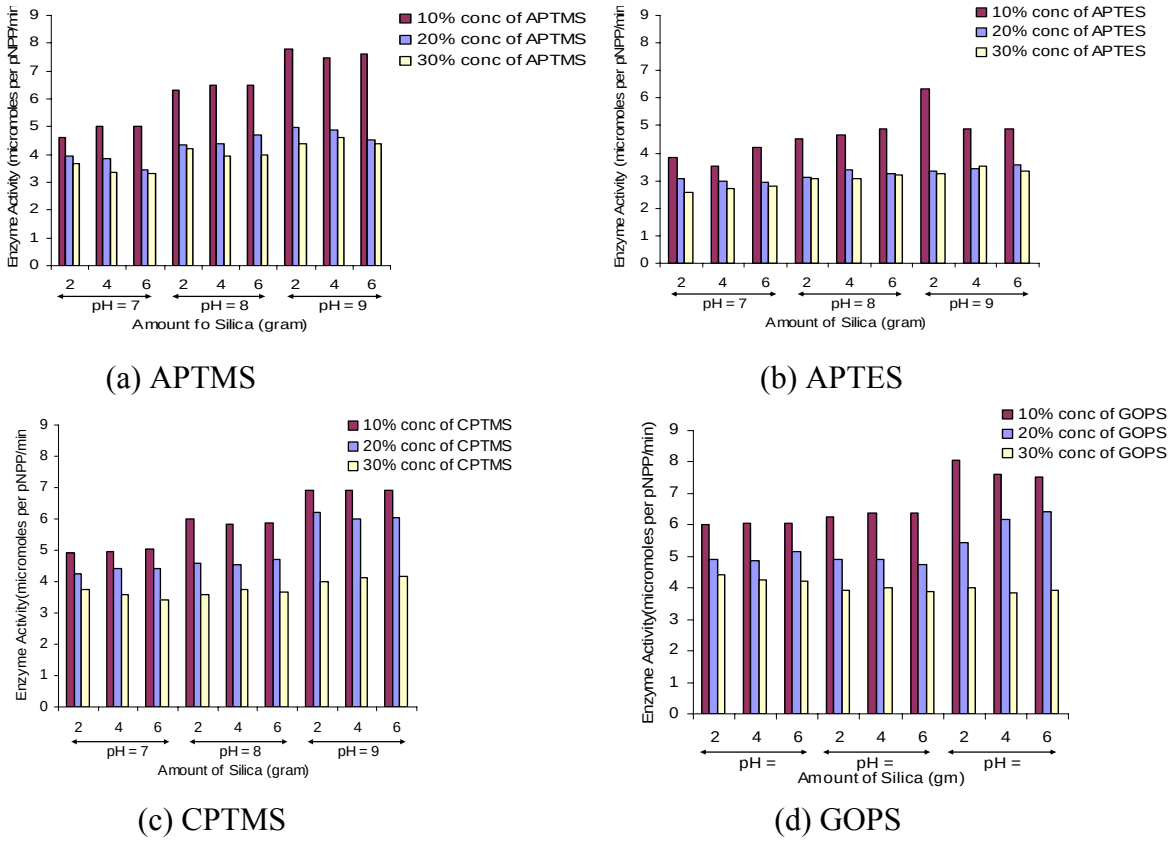
The enzyme alkaline phosphatase is a non-specific phosphatase unlike most enzymes it recognizes a wide variety of molecules as substrates that are found in bacteria, fungi, and higher animals. The assay of alkaline phosphatase activity takes advantage of the fact that the enzyme is non-specific, and utilizes the non-biological substrate *p*-nitrophenyl phosphate to give *p*-nitrophenol upon hydrolysis. Activity assays of alkaline phosphatase with *p*-nitrophenyl phosphate are terminated by the addition of NaOH. This serves two functions: first, it stops the enzyme-catalyzed reaction by changing the pH to one unsuitable for the enzyme (the enzyme is denatured at high pH and so unable to function). Secondly, it dephosphorylates the *p*-nitrophenol to give the yellow colored *p*-nitrophenylate.

Alkaline phosphatase immobilized on glass surface coupled by APTMS, APTES, CPTMS and GOPS by covalent bond in vacuum condition with the loss of hydrochloric acid. The optimum pH for coupling depends on the amino acid composition of the protein, as proteins with free thiols show better protein loading at a higher pH ('Tresyl-Activated Sepharise' Pharmacia Fine Chemicals, Uppsala, Sweden). Higher pH is recommended for maximum enzyme activity is retained (Nilsson & Mosbach, 1981)

3.1 Optimization of immobilized enzyme

In our experiment, immobilization of enzyme was done at pH 7.0 to 9.0. pH value of 9.0 is the higher for maximum amount of enzyme immobilized that retain on the surface of

glass. The results with 10% concentration APTMS coupling reagent in 7, 8, 9 and amount of silica 2, 4, 6 gram as shown in figure 1(a) . Best condition for immobilized alkaline phosphatase was found by using different coupling reagents. Figure 1 (a) shows that 10% concentration of APTMS gives highest enzyme activity of 7.8 micromoles of pNPP at pH 9.0 and the amount of silica was 2 gram for amination.



(a) APTMS (b) APTES

(c) CPTMS (d) GOPS

Figure 1: Activity of enzyme immobilization with 10%, 20% and 30% (a) APTMS , (b) APTES, (c) CPTMS, (d) GOPS in pH 7, 8, 9 and amount of silica 2, 4, 6 gram.

For 20% concentration of coupling the highest enzyme activity was 5.0 at pH 9.0 and amount of silica was 2 gm. Similarly highest enzyme activity shown in figure 1 (b), for 30% concentration at pH 9.0 but the amount of silica was 4 gram. 10% Concentration of APTES gives highest enzyme activity of 7.0 micromoles per pNPP at pH 9.0 and the amount of silica is 2 gram for amination shown in the same figure. For 20% concentration the highest enzyme activity is 3.6 micromoles per pNPP at pH 9.0 and amount of silica is 6 gm. Similarly, highest enzyme activity of 3.5 micromoles per pNPP is obtained at 30% concentration and pH value of 9.0 but the amount of silica is 6 gram. The experiments were done with different concentration of CPTMS and different levels of pH and amount of silica.

Best condition for ALPase immobilized by Chloropropyl trimethoxy silane at 10%, 20% and 30% respectively is shown in figure 1 (c). 10% concentration of CPTMS gives the

highest enzyme activity of 6.9 micromoles per pNPP at pH 9.0 for all the different amount of silica (2,4,6 gram) for amination. For 20% concentration the highest enzyme activity is 6.2 micromoles per pNPP at pH 9.0 and amount of silica is 4 gm. Similarly highest enzyme activity of 4.2 micromoles per pNPP is shown at 30% concentration at pH value of 9.0 but the amount of silica is 6 gram.

10% Concentration of GOPS gave the highest enzyme activity of 8.1 micromoles per pNPP at pH 9.0 and the amount of silica is 2 gram for amination. For 20% concentration the highest enzyme activity is 6.4 micromoles per pNPP at pH 9.0 and amount of silica is 6 gm. Highest enzyme activity of 4.4 micromoles per pNPP for 30% concentration at pH 7.0 is shown in figure 1 (d) and the amount of silica is 2 gram. Sometimes damage appeared with APTMS, APTES, CPTMS and GOPS over the glass fibre disk surface during the use of silica amount of 4 and 6 gram.

In this experiment, highest value of enzyme activity was found at the pH 9.0 in all steps. But with GOPS highest enzyme activity was obtained at pH 7.0. In order to find the optimum condition for getting the highest enzyme activity univariate analysis was done for each of the four chemical used in this thesis work. From statistical results, it was evident that best condition for getting the highest enzyme activity for CPTMS was 10% concentration with pH value 9 and the amount of silica is 2 grams. 2 grams of silica was taken for saving some chemicals as both will give the same result. The highest enzyme activity for GOPS is 10% concentration with pH value 9 and the amount of silica was 2 grams. This is exactly same condition like the previous three chemicals. It was found from this analysis of four chemicals that there is only one best condition exists and it is 10% concentration with pH value 9 and the amount of silica is 2 grams with high significant level except APTMS.

Weetall reported (1969) report, the soluble enzyme equivalent is indicative of the reaction conditions (amount of immobilized enzyme, time of reaction, etc.) necessary to achieve substrate hydrolysis. It is, therefore, a more useful value than, for example, the absolute amount of enzyme immobilized. In homogeneity of the reaction mixture necessarily results when one component of the enzyme–substrate is immobilized on a solid support and some reduction in the apparent specific activity (enzymatic activity under a given set of reaction conditions per absolute amount of protein immobilized) is both expected and routinely observed (Zingaro and Uziel, 1970). However, since enzyme immobilization has the potential to physically alter the ability of individual enzyme molecules to catalyze reactions when they do come into contact with substrate. The glass-enzyme complex developed in the present work can be used as a high-performance biocatalyst for various chemical processing applications, particularly in organic media. It needed very low amount of enzyme for Immobilization on glass surface. The best conditions for each coupling reagent in term of the highest enzyme activity of the immobilized samples were used in the next experiment for further optimization.

The result of the experiments was to use to further optimize the enzyme reaction and determine the sensitivity and stability of the immobilized enzyme.. The activity of immobilized enzyme was in 4 trials for 25 minutes. The disks were the washed and put

back new substrate solution for another 25 minutes (trial 2). This was done for four times. The same series of experiments were carried out with the four coupling reagents.

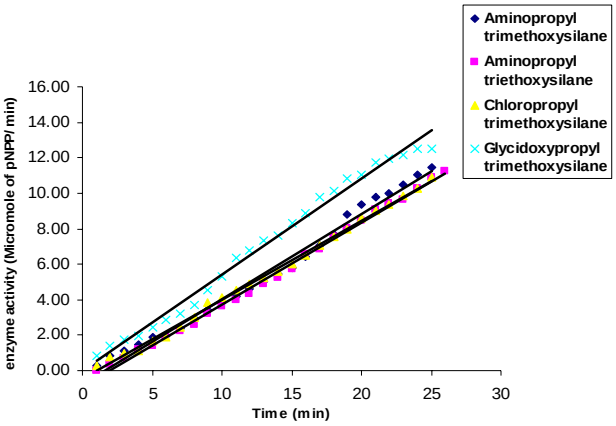


Figure 2: Summary of the activity of immobilized APLase for 4 coupling reagent with 4 trials for 25 minutes

Figure 2 is showing the best results with the different coupling reagents for enzyme immobilization on glass surfaces. GOPS is the best coupling reagent in this experiment because it shows highest enzyme activity than other three reagents. 25 samples were taken every minute in a trial. The ALPase dephosphorylated nearly 15 µmol pNPP after immobilized by GOPS in 25 min. But the rate of dephosphorylation was nearly 12 µmol for the others three coupling reagents. From the statistical analysis, comparable all data for 4 coupling reagents were not significant. So we can use any of four chemical for the analysis of sensitivity and stability experiment.

This figure 3 shows the final activity of immobilized enzyme comparing all coupling reagents after 25 minutes of test. The disks were washed between each trial. GOPS was shown highest enzyme activity after 4 trails. In summary, 10% GOPS is the best coupling reagent for immobilization of ALPase. And the best condition for immobilization of ALPase is pH 9.0 and the amount of silica used was 2 gram for amination in glass surface for getting highest enzyme activity.

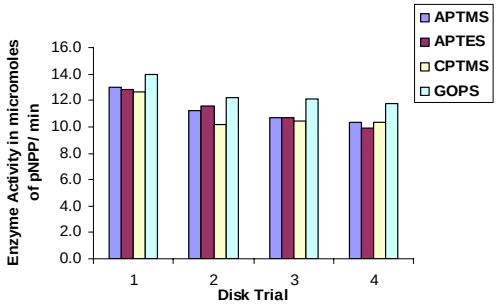


Figure 3: Final enzyme activity of Immobilized APLase on disks (n=4). Each disk was assayed four times and rinsed between successive trials.

Table 1 shows the percentage loss of enzyme after immobilized in each trial. For APTMS, it gave 9.23% loss after first trial. On the other hand APTES 9.38%, CPTMS 19% and GOPS 12.40% have shown loss after 1st trial. In the last trial APTMS, APTES, CPTMS have shown over 20%, 22% and 18% loss respectively, where GOPS gave 15% loss with in 25 minute after 4 trials. A similar experiment was also conducted by Weetall (1969). The result of the experiment shown the alkaline phosphatase activity loss was 19.3 percentages after 60 and 120 minute. Taylor (2005) also found in his experiment that the disk lost approximately 60% of alkaline phosphatase activity after immobilization

Table 1: Loss of activity of enzyme after immobilized as calculation for final value.

No. of Trail	APTMS	% Loss	APTES	% Loss	CPTMS	% Loss	GOPS	% Loss
1	13		12.8		12.6		13.95	
2	11.8	**9.23**	11.6	**9.38**	10.2	**19.05**	12.22	**12.40**
3	10.7	**17.69**	10.7	**16.41**	10.4	**17.46**	12.11	**13.19**
4	10.3	**20.77**	9.9	**22.66**	10.3	**18.25**	11.8	**15.56**
Average		15.90		16.15		18.25		13.72

An Average loss of 16%, 16%, 18% and 14% for APTMS, APTES, and CPTMS and GOPS respectively is shown in the table 1. Therefore it is obvious that there are small amounts of alkaline phosphates loss after immobilization by GOPS on glass fibre disk incubated under vacuum condition.

With GOPS as the coupling reagents the enzyme is more stable and retained activity even after repeated washing. The total amount of protein immobilized per gram of glass fibre was also determined. Three different amounts of silica powder and 2.4 cm glass fibre disk were used in this enzyme immobilization. An amount of glass deceased, the rate of dephosphorylation per micromole of pNPP was increased in case of every coupling reagents that shown in experiment 1. Highest enzyme activity is 1.23 µmol per pNPP when the amount of silica is 2 gram. 2.09 mg enzyme per gram of fibre glass is remaining after immobilization that indicates the binding efficiency approximately 82%.

Table 2: Amount of enzyme bound after immobilization

Amount of silica (Gm)	Initial enzyme (mg/ml)	Initial free enzyme activity U/ml	Bound enzyme (mg/g of fibre glass)	Immobilized Enzyme activity U/g	Binding Efficiency %
2	2.59	1.5	2.09	1.23	82
4	2.35	1.07	1.79	0.51	48
6	2.15	0.92	1.05	0.46	50

This value compares to the value obtained by Taylor 2005 who reported that startly 2.16 mg protein per gram of fibre the remained immobilized to the soluble equivalent of reusable immobilized ALPase of 1.4 mg per gram of fibre after reuse. This indicates that approximately 67% of the apparent specific activity of the soluble enzyme can be

retained after immobilization and several reuses. Alkaline phosphatase has been immobilized on a variety of surfaces (Surinenaite *et al*., 1996; Wiley *et al*., 2001; Filmon *et al*., 2002). But, in 1969, Weetall reported that 0.74 mg of ALPase per gram of glass could be immobilized by adding the enzyme to glass beads chemically activated with diazo groups. This value compares favourably with examples of ALPase immobilized on other surfaces (1% of specific activity retained on an ethylene-maleic anhydride co-polymer (Zingaro and Uziel, 1970) and 56–72% on various vanacryls (Brown and Joyeau, 1974) as well as to values obtained for acid phosphatases immobilized on plain and glycerol coated glass (4 and 14%, respectively (Van Hekken et al., 1990). But the results in this study showed a larger amount of active immobilized enzyme and the difference with respect to the amount of active immobilized enzyme with previous study may not be significant. The results of experiment do demonstrate that the extent of covalent immobilization achieved by the in vacuum process is at least comparable to that obtained by the immobilization processes using conventional chemical activation. Immobilized enzyme has a good effect in stability compare to free enzyme up to 6 days after all the enzyme preparations were stored at 0-4ºC it was happening in a decreasing amount. It should be considered an amount of loss of activity. The immobilized enzyme had much better storage stability than the free enzyme.

3.2 Application of immobilized enzyme disks for detection of Phosphatase

Fig 4 (a) is shown the texture of the immobilized disk is hard, nearly same as a glass texture. The appearance of the disk is transparent and yellow color indicates the presence of phosphate. These experiments were done with shrimp, milk and water sample for testing phosphate compounds. There is no color formation after the application of cooked shrimp sample on the immobilized disks. No color formation over the disk indicates there is no phosphate substrate that can react with immobilized alkaline phosphatase. Phosphate was not found in pasteurized milk and also cooked shrimp. During heat treatment of milk and shrimp the phosphate is inactivated. There was phosphate element in the raw shrimp and raw milk because yellow color formation after applied the sample over the immobilized disks. Tap water from toilet was taken and phosphate was found in that water. Four samples of tap water showed negative result (no color formation after applying in the immobilized disk). There are no phosphate elements in drinking water and also in distilled water because the glass surface remained colorless. All qualitative result is shown in the table 3. Quantitative results for milk and shrimp is shown in table 4. There was no phosphate element in the water sample.

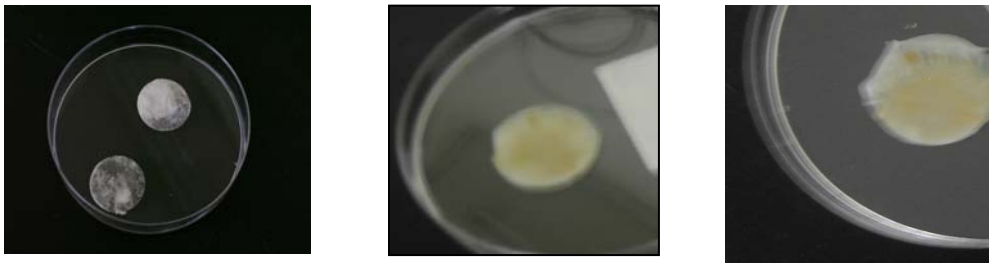


Figure 4: (a) Alkaline phosphates immobilized glass disk (b) Testing of shrimp sample in immobilized glass disk color indicated the presence of phosphate element (c) disk tested with spoiled milk and color formation.

Known amount of phosphate was inoculated in to all of three samples and make a standard curve figure 5(a) and 5(b) by measured color intensity by a colorimeter. Figure 6 shows the linearity of the color with time by using different concentration of phosphate in milk, shrimp, and water sample respectively.

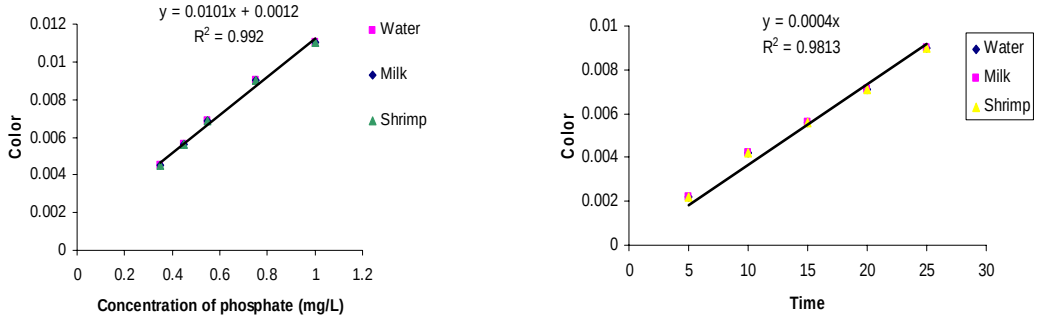
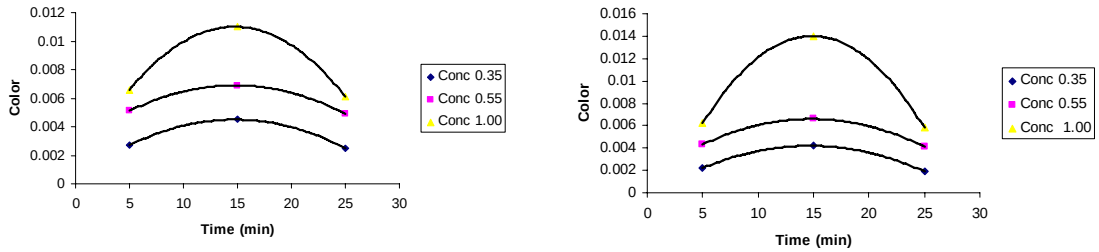
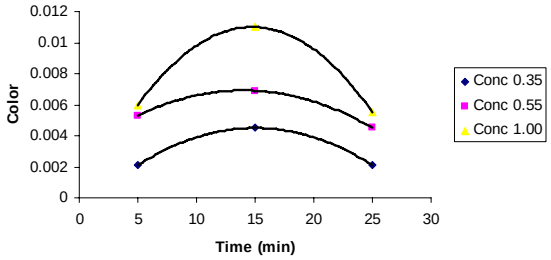


Figure 5: (a) Standard Curve for phosphate detection and (b) for color intensity



(a) Milk (b) Shrimp



(c) Water

Figure 6: Linear relationship between time and color in different sample (a) milk, (b) shrimp (c) water

Table 3: Amount of Phosphate after applied of immobilized enzyme with different samples

Sample no	Milk	Shrimp
	Amount of Phosphate mg/L	Amount of Phosphate mg/L
1	0.54	0.66
2	0.80	0.72
3	0.88	
4	0.92	0.88
5	0.73	0.75

4. CONCLUSION

Attachment of enzymes to the surface of glass fibre is a technique used for developing biosensors. This attachment occurs by the covalent bond or physical adsorption. It has been used widely as one of the traditional enzyme immobilization technologies. Although glass provides desirable mechanical strength and thermochemical stability, it is difficult to achieve high enzyme loading as required for efficient bioprocess application (Ping Wang et al; 2001). The development could potentially enhance the area of rapid detection using biosensor (N.S.Hobson, I.Tothill, A.P.F.Turner 1996). In our procedure, amide bonds were formed between carboxyl groups on the protein and amino group on the glass surface was attempted.

Results of the research prove that the Glycidoxypropyle trimethoxysilane was the best coupling among four reagents and could helped form bond between carboxyl and amino group on the glass fiber disk. A pH of 9.0, concentration of coupling reagent of 10% and an amount of silica 2 gram were the best condition for coupling the enzyme over the glass surface which showed the highest enzyme activity. Storage activity were examined between immobilized and free enzyme. Immobilized and free enzyme both showed nearly the same activity in the experiment of different pH. The storage life of immobilized enzyme is much better than free enzyme.

Results of the research also indicate that filter disks containing immobilized enzymes have the potential for repeated use without significant loss of activity. Approximately 16% loss of the ALPase activity was removed after the first trial but there was virtually no further loss of activity in subsequent trials. But this loss varies for the coupling reagents and amount of silica. An average of 1.64 mg of reusable alkaline phosphatase was immobilized per gram of glass fiber was probably with GOPS as coupling reagent. It was expected that the washing steps in the process before trial one would remove any non-covalently associated protein. While the source of the loss of activity is not clear, it may be that, despite the attempts to remove any non-covalently bound enzyme using 0.1M NaCl, some still remains associated with the glass matrix during the first trial. Regardless of the mechanism, the covalently immobilized enzyme remaining after the first trial and washing was stable. The losses of activity with four subsequent washes of the disk were minimal. Despite the gaps in interpretation of the specific activity of inhomogeneous mixtures, these data suggest that vacuum immobilization process is at

least as effective as more than traditional chemical methods of immobilization in preserving the activity of the enzyme molecules immobilized on the glass. Some other parameters (pH and storage activity) were examined between immobilized and free enzyme. Immobilized and free enzyme both showed nearly the same activity in the experiment of different pH. The storage life of immobilized enzyme is much better than free enzyme.

This immobilized disk was applied as a biosensor. Phosphate was detected from water sample, milk and shrimp sample by the used of alkaline phosphates immobilized disk. The result of this experiment proves that, raw milk and shrimp has some phosphate element which is toxic. So it is better to drink pasteurized milk or after heat treatment and cooked shrimp. Because phosphate element will be destroyed after heat treatment

The success with ALPase demonstrates that this immobilization process should be applicable between other soluble proteins containing carboxyl groups and other solid supports with free amino groups. The covalent bonded enzyme could be used for the development of biosensor applications. Immobilized Alkaline phosphatase disk could be apply on several medical samples or some other food sample for example barley, wheat, rice, oats, rye, fruit juices for the detection of phosphate elements. The recommendations could be made for the possible further study, could be developed a transducer and a sensor when display a quantitative result for the detection of toxic phosphate elements as biosensor application. Immobilization on glass surface for other enzymes which are responsible for detection of food borne pathogen should be done and should be developed a transducer and a sensor when display a quantitative result for the detection of toxic phosphate elements as biosensor application for recommendations could be made for the possible further study.

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