ELSEVIER

Talanta 44 (1997) 2051–2058

Talanta

Spectrophotometric determination of enzymatically generated hydrogen peroxide using Sol-Gel immobilized horseradish peroxidase

Faida A. El-Essi [a], Ali Z. Abu Zuhri [a], Suleiman I. Al-Khalil [a], Monzir S. Abdel-Latif [b,*]

[a] *Chemistry Department, Faculty of Science, An-Najah National University, P.O. Box 707, Nablus, Palestine*
[b] *Chemistry Department, The Islamic University, P.O. Box 108, Gaza, Palestinian Authority, Israel*

Received 27 August 1996; received in revised form 6 January 1997; accepted 14 January 1997

Abstract

Peroxidase entrapment in different Sol-Gel matrices was successful. The enzyme did not show a decrease in activity for at least 2 months as well as storage at room temperature and dry condition for periods exceeding 3 weeks. It was evident that the enzymatic activity was a function in the type of the alkoxysilane precursor. In addition, the optimum temperature which resulted in maximum enzymatic activity was also dependent on the type of Sol-Gel matrix. Excellent results were obtained for the determination of glucose in serum samples using soluble glucose oxidase in conjunction with the Sol-Gel entrapped peroxidase. The enzymatically produced hydrogen peroxide is oxidized by the entrapped peroxidase yielding oxygen which oxidizes the faint blue variamine blue into the intensely violet colored species (the molar absorptivity is about 1.8×10^4 l mol^{-1} cm^{-1}). The characteristics of this chromogenic system as well as optimized conditions for its use in the spectrophotometric determination of enzymatically generated hydrogen peroxide were investigated. Excellent agreement between the results obtained by the proposed method and the widely used standard method, utilizing a commercial reagents kit, was always observed. © 1997 Elsevier Science B.V.

Keywords: Sol-Gel; Enzyme immobilization; Peroxidase; Variamine blue

1. Introduction

The high specificity of enzymes in catalyzing some chemical reactions is an important feature that has been utilized for the selective and accurate determination of many substrates. Analytes are usually selectively converted into other species which can be easily monitored. Many enzymatic reactions result in the generation of hydrogen peroxide as a by-product of the conversion of appropriate substrates. Therefore, there has been a great deal of emphasis on the development of sensitive and highly reliable methods for the quantitative determination of hydrogen peroxide concentration which is an indirect indication of analyte concentration [1–3]. Several electrochemi-

* Corresponding author.

PII S0039-9140(97)00032-5

cal, spectrophotometric, and luminescence methods were suggested and procedures based on these methods were optimized for such systems. However, enzymes are usually expensive and their use as homogeneous catalysts is, most of the time, costly. In addition, the disposal of these active catalysts can impose environmental problems in many situations. Therefore, it is justified that efforts be directed towards designing approaches to immobilize these biomolecules so that they can be reused as well as be confined to a specific matrix [4]. Several methods for the immobilization of enzymes were suggested using different approaches. Physical adsorption of enzymes to an activated solid support is an important method of enzyme immobilization. However, these preparations are usually unstable and enzymes tend to come off in solution. Covalent binding of enzymes to solid surfaces like membranes or other solid supports is one of the most widely used approaches. However, several steps for the preparation of an adequate functional group on the solid support are usually involved prior to the final crosslinking step. These procedures are usually complicated and require good experience in this field. In addition, the enzymatic activity decreases appreciably and the immobilized enzymes do not retain their activity over long periods, beside some other disadvantages [4]. Recently, some biomaterials were trapped into a silicate glass matrix under mild conditions [5–8]. This was accomplished by the Sol-Gel method where room temperature polymerization of an alkoxysilane, after partial hydrolysis of the alkoxy groups results in a silicate polymer gel. The thus obtained gel solidifies with time as the polymerization continues and ultimately a glass-like silicate solid matrix is obtained. When an enzyme or any biomaterial is added to the silane precursor after partial hydrolysis, these materials can be entrapped into the polymeric matrix after complete solidification. Fortunately, enzymes show considerable retention of their activity in such immobilization matrices. Small substrate molecules can diffuse into the pores of the polymeric matrix and reach the active site of the enzyme and thus be converted to other simpler species which can diffuse back into solution where it can be detected. The relatively large size of the enzyme assures minimum leaching from the solid matrix. The Sol-Gel process for enzyme immobilization seems very promising especially due to the mild conditions that are used for the immobilization process as well as the simplicity of the procedure involved. In addition, the immobilization matrix can be optimized so that transparent monoliths, thin films or fine particles are formed depending on the ultimate application of the preparation. Detailed description of the Sol-Gel process can be found in the many books and reviews on this topic [9–16].

Braun et al. [6] suggested that the encapsulation of glucose oxidase in conjunction with peroxidase and a chromogen in a tetramethoxysilane Sol-Gel matrix was good for the determination of glucose. However, no analytical data was presented. Yamanaka et al. [7] investigated the activity of glucose oxidase entrapped in a Sol-Gel matrix using a conventional photometric scheme for detection of the generated peroxide. The turnover number and the dissociation constant were reported to be similar and two-fold greater than the corresponding values for the native enzyme, respectively. Also, storage conditions were reported to have significant influence on the activity of the enzyme.

In this study, we present results on a new chromogenic system for the determination of hydrogen peroxide as well as results of peroxidase entrapment in different silicate glass matrices prepared by the Sol-Gel method. The system is optimized for the determination of enzymatically generated hydrogen peroxide produced from the bioconversion of glucose by glucose oxidase. Details of the system characterization as well as results of the determination of glucose in serum blood samples will be presented.

2. Experimental section

2.1. Instrumentation

Absorbance measurements were recorded using a double beam Perkin-Elmer Lambda 15 UV-Vis spectrophotometer. The instrument is microprocessor based with excellent performance char-

acteristics. Also, a Spectronic 20D spectrophotometer was occasionally used for absorbance measurements. The pH of the different solutions was adjusted using a Hanna digital pH meter. Temperature controlled studies were conducted using a conventional water bath.

2.2. Chemicals and reagents

3-[Trimethoxysilyl]-1-propanthiol (TMSP), triethoxypropylsilane (TEPS), 3-aminopropyltriethoxysilane (APES), 3-chloropropyltriethoxysilane (CPES), triethoxyvinylsilane (TEVS), horseradish peroxidase (180 U mg^{-1}), and glucose oxidase from *Aspergillus niger* (100 U mg^{-1}) were purchased from Merck Chemical Company, Germany. Disodium hydrogen phosphate, citric acid, sodium citrate, and succinic acid were obtained from Reidel de Haen, Germany. Variamine blue was obtained from UCB Chemicals, Belgium. Glucose standards and controls as well as commercial kits for glucose determination were purchased from Sigma Chemical Company, USA. Thymol-indophenol, thionine, methylviologen, phenosafranine, and 7,8-benzoflavone were from BDH, England. All chemicals and reagents used in this study were of the analytical grade and were used as received without further purification. Distilled, deionized water was used throughout this work.

3. Procedures

3.1. Preparation of a variamine blue working solution

Variamine blue is slightly soluble in water yielding a light bluish solution which darkens with time. The procedure for the preparation of a working solution of variamine blue involved the transfer of about 90 mg of the material into a beaker followed by the addition of about 40 ml of distilled water. The solution was heated with stirring to about 80°C. The solution was then filtered and the final volume was then adjusted to 50 ml by addition of distilled water. The thus prepared solution contains about 80 mg of variamine blue. This solution was kept for 1 week before use since it was observed that the absorbance of this solution increased with time for the first 5 days before stabilization. Therefore, we recommend the storage of the freshly prepared variamine blue for a period exceeding 5 days before actual use so that reproducible readings can be achieved.

3.2. Preparation of the Sol-Gel matrix

3.2.1. Sol stock solution

First, 2.25 ml of the appropriate alkoxysilane derivative was transferred into a small glass bottle followed by addition of 0.7 ml of distilled water and 50 μl of 0.1 M HCl. The bottle was firmly capped and the mixture was shaken for about 3 h. At this stage, partial hydrolysis of the alkoxysilane derivative was accomplished. The thus prepared partially hydrolyzed silane derivatives were kept in capped bottles at room temperature.

3.2.2. Peroxidase entrapment

The entrapment procedure involved the mixing of a 1:1 ratio of the previously prepared Sol stock solution and a buffered peroxidase solution (0.1 M phosphate buffer at pH 6). The solution was then shaken so as to obtain a homogeneous mixture which was allowed to solidify at room temperature. The formation of the Sol-Gel starts few minutes after mixing and complete solidification (constant weight) after about 5–10 days. The Sol-Gel immobilized peroxidase was kept at room temperature for at least 2 weeks before washing with successive solutions of 1.0 M NaCl, 0.1 M phosphate buffer at pH 6, and plenty of distilled water, respectively. This washing procedure is necessary to wash out any residual or surface adsorbed peroxidase.

3.3. Analytical procedure for peroxide determination

The general procedure for the quantitation of hydrogen peroxide involved the transfer of 2–4 ml of the buffer solution into a test tube containing the Sol-Gel entrapped peroxidase. An appropriate known amount of peroxide (or glucose and glucose oxidase) was then added and the enzy-

matic reaction was allowed to proceed for 10 min. Just before the absorbance measurements, a 100-μl portion of the variamine blue working solution was added. The absorbance of the oxidized variamine blue (violet color) was measured immediately at 550 nm against a blank.

4. Results and discussion

Initially, experimental conditions were optimized in order to obtain a sensitive chromogenic system for the determination of hydrogen peroxide using soluble peroxidase. Several redox systems were tried in an attempt to find a good system for such determinations. Variamine blue resulted in a very sensitive system for the determination of hydrogen peroxide while thymol-indophenol, thionine, methylviologen, phenosafranine, and 7,8-benzoflavone did not result in any appreciable signal. Therefore, all the following experiments were done using variamine blue.

4.1. Effect of pH on absorbance

Variamine blue is oxidized by hydrogen peroxide in presence of peroxidase. The resulting violet color was found to be highly dependent on the pH of the buffer solution. Fig. 1 shows the relationship between the absorbance signal and the pH of the buffer solution. It can be concluded from the figure that very little oxidation takes place at pH values above pH 7 and essentially no observable oxidation takes place at pH values above pH 8. Lower pH values result in more improved absorbance signals than measurements performed at higher pH values while optimum pH of the buffer solution occurs at about pH 5. Therefore, all subsequent studies were performed with buffer solutions at pH 5 even though this pH value does not match the optimum pH for maximum peroxidase activity. This could possibly be justified since the activity of peroxidase used at pH 5 is still very high although not highest possible.

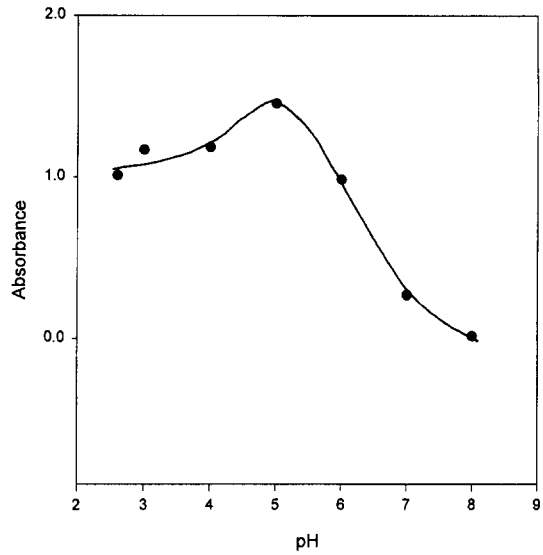

Fig. 1. Effect of the pH on the absorbance. Conditions: 0.1 M buffer solution, 5×10^{-5} M peroxide, 2.5 U peroxidase, and 3×10^{-4} M variamine blue. The absorbance signal was monitored at 550 nm.

4.2. Effect of buffer concentration

The concentration of the buffer solution used also affects the value of the absorbance signal as can be seen in Fig. 2. It can be shown from this figure that there was an increase in the ab-

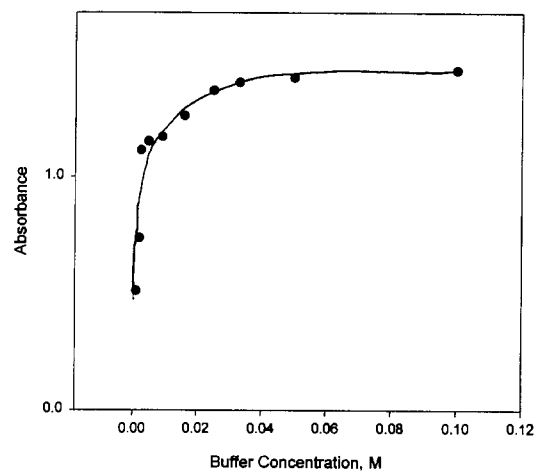

Fig. 2. Effect of buffer concentration on the absorbance signal. Conditions: buffer solutions at pH 5 but different concentrations. Other conditions are the same as in Fig. 1.

sorbance signal as the concentration of the buffer solution was increased at pH 5. However, the increase in the absorbance signal with concentration was not linear and absorbance increased only slightly at buffer concentrations greater than 3.3×10^{-2} M, and a plateau is essentially obtained at buffer concentrations exceeding 3.6×10^{-2} M. It is also clear from the figure that large increase in the absorbance signal was obtained as the buffer concentration was increased in the lower range of buffer concentration. Therefore, it seems advantageous to use higher buffer concentrations as low concentrations of buffer result in lower absorbance signals. A 0.1 M buffer concentration was judged suitable and was thus used in all further experiments.

It should also be indicated that buffer type was found to affect the absorbance signal where it was observed that buffers containing acetate or citrate resulted in improved absorbance signals while those containing succinate resulted in lower results.

4.3. Effect of variamine blue concentration

As expected, an increase in the variamine blue concentration resulted in an increase in the value of the absorbance signal. This is due to formation of more of the oxidized chromogen so that the equilibrium expression could be satisfied. Fig. 3 shows the dependence of the absorption signal on the variamine blue concentration. However, it is noteworthy to indicate that the background absorbance increased as the concentration of variamine blue was increased. Therefore, a variamine blue concentration of about 3×10^{-4} M was selected and was thus used in all subsequent experiments.

4.4. Effect of temperature

It is well established that temperature has pronounced effects on the activity of enzymes and thus the rate of enzymatic reactions. The temperature effects on peroxidase activity is depicted in Fig. 4. It can be shown from this figure that the optimum temperature for the determination of hydrogen peroxide, in presence of peroxidase and

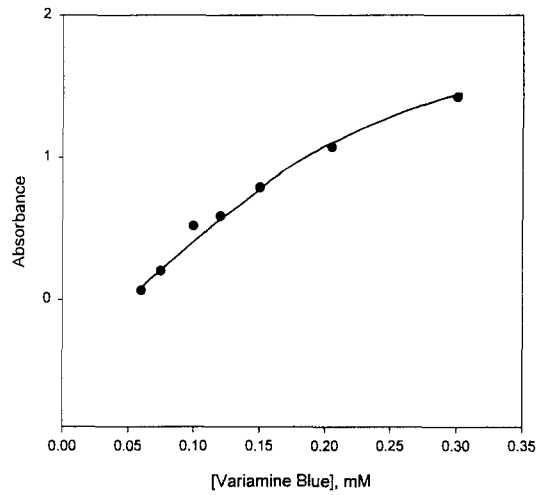

Fig. 3. Effect of the concentration of variamine blue on the absorbance signal. Conditions: 0.1 M buffer solution at pH 5. Other conditions are the same as in Fig. 1.

using variamine blue as the chromogenic material, is about 30°C. However, Fig. 4 also suggests a relatively small dependence of the absorbance signal on temperature at lower temperatures. Abrupt changes in the value of the absorbance signal was observed at temperatures exceeding about 33°C. Unless otherwise indicated, room temperature, 25°C was used throughout this work.

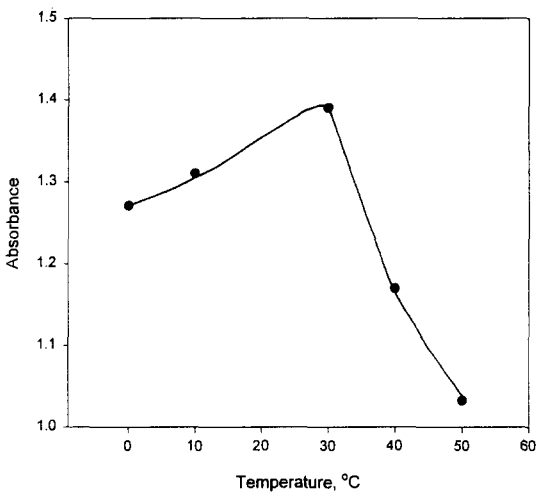

Fig. 4. Effect of temperature on the absorbance signal. Conditions: same as in Fig. 3.

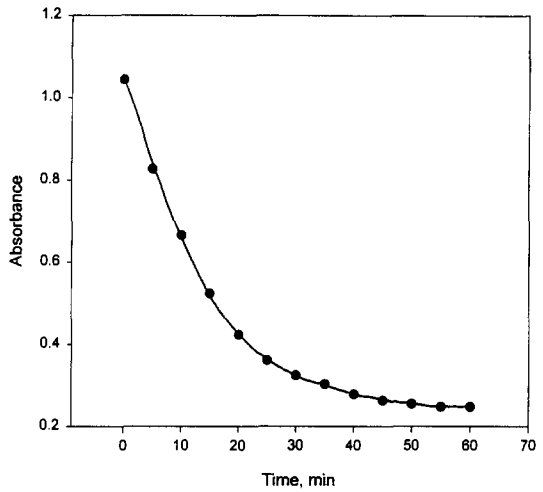

Fig. 5. Stability of the oxidized variamine blue with time. Conditions: same as in Fig. 4 except for the peroxide concentration (3.6×10^{-5} M).

4.5. Stability of the oxidized variamine blue

A very interesting behavior of oxidized variamine blue absorption with time can be seen in Fig. 5. It is clear from this figure that sensitive results can only be obtained when measurements are done immediately after the addition of the last reagent. This is because the absorbance signal starts to deteriorate with time. Initially, we suspected that the oxidized variamine blue was light sensitive. However, running a parallel experiment in a system completely isolated from light resulted in the same absorbance as that which was exposed to light. Therefore, this roles out the possibility of light instability of the oxidized chromogen. Unfortunately, the instability of the oxidized species can not be explained at this time. Throughout this study, absorbance measurements were done immediately after the addition of variamine blue, peroxidase, or hydrogen peroxide, depending on the experiment of interest.

4.6. Sensitivity of the variamine blue system

The variamine blue system showed a higher sensitivity for the determination of hydrogen peroxide, in presence of peroxidase, than the 4-aminophenazone system which is used, in a commercial kit, as the chromogen for hydrogen peroxide determination in presence of peroxidase. The molar absorptivity of the oxidized variamine blue (1.8×10^4 l mol^{-1} cm^{-1}) was about twice that of the 4-aminophenazone. Therefore, the optimized variamine blue system was subsequently used for the determination of enzymatically generated hydrogen peroxide using entrapped peroxidase.

4.7. Dependence of peroxidase activity on the entrapment matrix

Four different entrapment matrices were used to check whether peroxidase activity changes with the type of matrix in which it is immobilized. The composition of these matrices is detailed below:

Matrix I: 200 μl of the Sol containing 3-[trimethoxysilyl]-1-propanthiol was mixed with 300 μl of the Sol containing 3-chloropropyltriethoxysilane, 300 μl of peroxidase, and 200 μl of a papain solution (0.4 mg).

Matrix II: 500 μl of the Sol containing 3-chloropropyltriethoxysilane was mixed with 400 μl of peroxidase and 100 μl of papain (0.2 mg).

Matrix III: 300 μl of the Sol containing triethoxyvinylsilane was mixed with 200 μl of the Sol containing 3-chloropropyltriethoxysilane, 250 μl of peroxidase, and 250 μl of papain (0.25 mg).

Matrix IV: 400 μl of the Sol containing triethoxyvinylsilane was mixed with 100 μl of the Sol containing 3-chloropropyltriethoxysilane and 300 μl of peroxidase, but no papain.

All four matrices were left in a desiccator, at room temperature, for 20–23 days although full solidification occurred within 5–10 days. The weights of the samples after washing and drying were 0.168, 0.183, 0.096, and 0.201 g, respectively.

It is worth mentioning that the preparation of the Sol-Gel matrices was always reproducible under the specified conditions. Throughout this work, many different matrices were successfully and reproducibly prepared using the described procedures.

The procedure for the determination of hydrogen peroxide concentration involved soaking the appropriate solid matrix in 2.5 ml of buffer solution at pH 5 followed by addition of 30 μl of the

peroxide solution. The reaction was allowed to proceed for a specific period of time then a 100 μl portion of the variamine blue solution was added. The absorbance signal of oxidized variamine blue was immediately measured at 550 nm. Fig. 6 shows the relationship between the absorbance value and reaction time. It is clear from the figure that Matrix II shows highest absorbance value and lowest reaction time. This may suggest that hydrogen peroxide can easily diffuse through that matrix and reach the active site of the enzyme and thus be converted to oxygen which oxidizes the chromogenic material, resulting in the intense violet color. The three other matrices show approximate equilibration times but still the absorbance value varies for each one, where the absorbance measured using Matrix I was greater than that produced when Matrix III was used. The absorbance value was lowest when Matrix IV was used. It may be interesting to indicate that peroxide activity appears to be proportional to the amount of the 3-chloropropyltriethoxysilane precursor in the entrapment matrix. Therefore, it is clear that the entrapment matrix significantly affects the enzymatic activity and thus the value of the absorbance signal and the equilibration time as well.

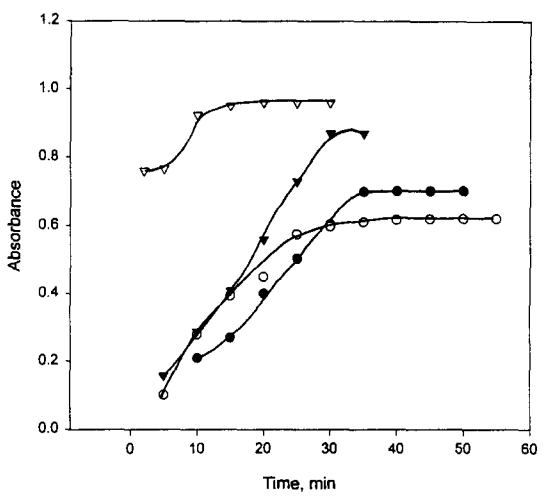

Fig. 6. Effect of the Sol-Gel matrix on the immobilized peroxidase activity. Matrix I (▼), Matrix II (△), Matrix III (●), and Matrix IV (○).

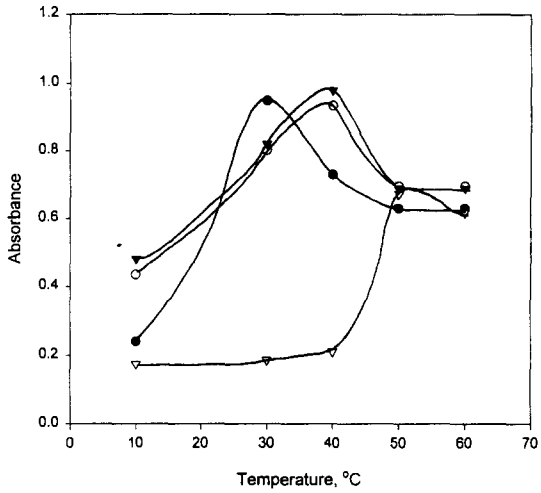

Fig. 7. Effect of temperature on the Sol-Gel entrapped peroxidase. Matrix I (●), Matrix II (▼), Matrix III (○), and Matrix IV (△).

4.8. *Effect of temperature on immobilized peroxidase*

Immobilized peroxidase in different matrices showed a rather interesting behavior at different temperatures as compared to the behavior of the enzyme in solution. The matrix composition seems to be very influential in the determination of the optimum temperature which results in maximum enzymatic activity. The optimum temperature for peroxidase was very similar to that of the soluble enzyme when the TMSP silane derivative was used. However, a significant shift in the optimum temperature of peroxidase (about 20°C) was observed when peroxidase was entrapped in a matrix which has TEVS as the predominant silane derivative but without papain as an additive (Fig. 7). Using comparable amounts of papain with the CPES or a mixture of TEVS and CPES silane derivatives resulted in a comparable shift in the optimum temperature of peroxidase that is about 10°C. Papain was used as an additive to the silane matrix hoping to improve the stability and enhance the activity of peroxidase in the Sol-Gel matrix. According to our experience and observations, proteins like papain or bovine serum albumin (BSA) improve the stability of some enzymes especially at solid surface matrices. We attribute

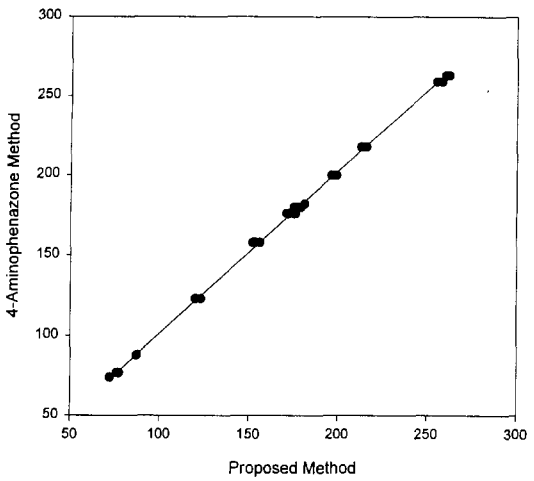

Fig. 8. Determination of serum glucose by the proposed method and the 4-aminophenazone method (34 experiments).

this to decreased interactions of the enzyme with the solid support due to competition between the enzyme and the additive for the very active surface of the solid support.

4.9. Determination of glucose in serum samples

The Sol-Gel immobilized peroxidase was used for the determination of glucose in serum. The blood samples were collected from a nearby clinic and were analyzed by both the standard method as well as our proposed method. The standard method uses a commercially supplied kit with 4-aminophenazone as the chromogenic material while the proposed method uses variamine blue in addition to Sol-Gel immobilized peroxidase. Excellent agreement between the two methods was always observed which is demonstrated in Fig. 8. The absolute difference between the results in the two methods was always less than 3% for any individual sample which can also be attributed to delay in doing the measurement by the proposed method due to time required to transfer the serum samples from the clinic to our laboratories.

4.10. Stability of the Sol-Gel immobilized peroxidase

After the formation of the solid matrix incorporating the enzyme, the Sol-Gel enzymatic matrix was kept in a desiccator, at room temperature, for 2 weeks. Then the different Sol-Gel preparations were used for the analysis of the peroxide/glucose samples for 35 days. No change in the performance characteristics of the Sol-Gel immobilized enzyme was observed. This may suggest excellent stability of the entrapped enzyme inside the Sol-Gel matrix. Although a long-term stability study was not performed, it is anticipated from initial results of about 2 month that these enzymatic preparations will be stable for longer periods. Even after the analysis of about 50 serum samples for glucose, consistent and reproducible results were always obtained. The relative standard deviation for repetitive determinations ($n = 10$) was less than 1% in all cases. It should also be indicated that the Sol-Gel enzymatic preparations were kept refrigerated at the end of each working day.

References

[1] S. Igarashi, W.L. Hinze, Anal. Chem. 60 (1988) 446.
[2] M.S. Abdel-Latif, G.G. Guilbault, Anal. Chem. 60 (1988) 2671.
[3] M.S. Abdel-Latif, A.A. Suleiman, G.G. Guilbault, Anal. Lett. 21 (1988) 943.
[4] R.F. Taylor, Protein Immobilization: Fundamentals and Applications, Marcel Dekker, New York, 1991.
[5] R. Zusman, C. Rottman, M. Ottolenghi, D.J. Avnir, J. Non-Cryst. Solids 122 (1990) 107.
[6] S. Braun, S. Shtelzer, S. Rappoport, D.J. Avnir, J. Non-Cryst. Solids 147 (1992) 739.
[7] S. Yamanaka, F. Nishida, L. Ellerby, C. Nishida, B. Dunn, J. Valentine, J. Zink, J. Chem. Mater. 4 (1992) 495.
[8] R. Wang, U. Narang, P. Prasad, F. Bright, Anal. Chem. 65 (1993) 2671.
[9] C.J. Brinker, G.W. Sherer, Sol-Gel Science, Academic Press, New York, 1989.
[10] M. Yamane, T.J. Kujima, J. Non-Cryst. Solids 44 (1981) 181.
[11] D.L. Wood, E.M.J. Rabinovich, J. Non-Cryst. Solids 107 (1989) 199.
[12] H.J. Shmidt, J. Non-Cryst. Solids 73 (1985) 681.
[13] R. Assink, B.J. Kay, J. Non-Cryst. Solids 99 (1988) 359.
[14] C.J. Brinker, J. Non-Cryst. Solids 100 (1988) 31.
[15] L. Hench, J. West, Chem. Rev. 90 (1990) 33.
[16] P. Wanadi, W. Davis, C.J. Cummins, J. Am. Chem. Soc. 117 (1995) 2110.