Chapter – 3

Pressure – A Novel Tool for Immobilization of Protein
3.1 INTRODUCTION

Attachment of biomolecules on solid matrices have become part and parcel of several biological applications including diagnostics, genomics, proteomics, glycomics and high-throughput drug screening (Ma and Horiuchi, 2006; Dyukova et al., 2006; Houseman et al., 2002). Several techniques have been developed for immobilization of proteins which are mainly based on physical, covalent and bioaffinity interaction of which covalent immobilization is popular due to its stability, reproducibility and rapidity (Rusmini et al., 2007). Covalent immobilization of biomolecules onto the polymer matrix is conventionally performed at a certain temperature, usually at 37°C in a thermal incubator. Nevertheless, some researchers choose untraditional method of immobilization bypassing thermal incubation. Recent advances in the chemistry of photoactivable hetero-functional reagents have made it possible to immobilize proteins by light energy. Usually, UV light of 300 nm or above is used for photoimmobilization of proteins, as this range of radiation is not harmful to biological molecules (Naqvi and Nahar, 2004; Ito, 2006; Kumar and Nahar, 2007). Nahar and Bora, (2004) used microwave energy for rapid immobilization of enzymes onto activated surface. Sharma et al., (2011) applied ultrasound energy instead of thermal incubation for immobilization of proteins on polymer surfaces. However, in all the above methods, heat is either applied or generated during immobilization. This is not desirable, particularly when thermo-labile enzyme is used, also, during small scale immobilization, buffer may evaporate leading to unsatisfactory immobilization.

Pressure has been used as a tool in biotechnology for food processing, pharmaceutical, medical sciences and inactivation of biological agents for sterilization of biopharmaceuticals, or medical compound (Masson et al., 2001). Very high pressure causes inactivation of vegetative microorganisms through membrane modifications, inactivation of key enzymes, and inhibition of protein biosynthesis (Abe et al., 1999). Pressure is also being used as a tool for the study of structural and functional aspects of biomolecules. Pressure can alter the structure and function of biomolecules depending on the pressure range, exposure time, rate of compression, and the overall balance of parameters responsible for maintaining the structure of biomolecules (Mozhaev et al., 1996; Perrett and Zhou, 2002).
In this chapter, we demonstrate a novel use of pressure energy in immobilization technique. We have used horseradish peroxidase as a model enzyme to demonstrate pressure-induced covalent ligation onto polymer material. Horseradish peroxidase is used as a biocatalyst for organic synthesis and biotransformation as well as in coupled enzyme assays, chemiluminescent assays, immunoassays and the treatment of waste waters and in several biotechnological applications (Adam et al. 1999; Veitch and Smith, 2001; Azevedo et al. 2003). We have for the first time immobilized biomolecules by pressure energy instead of conventional thermal incubation.

3.2 EXPERIMENTAL METHODS

3.2.1 Photochemical activation of polystyrene microtiter plate

To optimize time of irradiation and amount of the photolinker reagent for activation of polystyrene microtiter plates, each well of microtiter plates were loaded with varying concentration (2.5, 5, 7.5, 10, 15 and 20 μmol) of FNAB dissolved in 50 μL methanol. The plates were kept in a fume hood in dark and the solvent was evaporated completely. The FNAB coated polystyrene microtiter plates were then exposed to UV light of 365 nm for 3, 6, 9, 12, 15 and 20 min, respectively, in an UV Stratalinker. After UV irradiation, plates were washed with methanol to remove excess unbound photolinker. A control experiment was performed similarly without using FNAB. The plates were dried in air and further used for enzyme immobilization to check the efficacy of the activation. The functionalized polystyrene microtiter plates were loaded with horseradish peroxidase (0.25 μg/well dissolved in 100 μL of 0.01 M PBS, pH 7.2) and incubated at 37°C for 2 hours. After incubation, the wells were washed with washing buffer (0.01 M PBS, pH 7.2 and 0.1% Tween-20) and the HRP-immobilized plates were used for enzymatic assay to observe the extent of activation by the photolinker.

3.2.2 Horseradish peroxidase assay

The HRP was assayed by adding 100 μL of substrate-dye buffer (4 mg o-phenylenediamine and 4 μL H₂O₂ in 12 mL citrate buffer of 0.2 M, pH 5) into the
wells of microtiter plate. After color development, the reaction was stopped by adding 20 μL of stop solution (5% H₂SO₄). After stopping the enzymatic reaction, 100 μL solutions from each well were transferred into new microtiter plate for measuring absorbance at 490 nm in an ELISA reader (SpectraMax 190, Microplate Reader, Molecular Devices, USA.

3.2.3 Designing of apparatus to carry out pressure-induced ligation

Effect of pressure on immobilization of enzyme into the wells of a polystyrene microtiter plate was examined by performing the immobilization experiment in a closed chamber by applying pressure. For applying the pressure inside the closed chamber (pressure cooker), the pressure regulator from the lid of the pressure cooker was removed and a vacuum tube was inserted through the vent pipe. The other end of the vacuum tube was passed through a pressure gauge and connected to a cylinder filled with argon. Enzyme was loaded into the wells of polystyrene microtiter plate and kept inside a 5-litre closed chamber (pressure cooker). The pressure inside the chamber was regulated by the argon cylinder and the applied pressure was measured by a pressure gauge connected with the argon cylinder.

3.2.4 Optimization of pressure for immobilization of HRP

Effect of pressure on immobilization of enzyme to the polymer surface was examined by immobilizing HRP into the activated and untreated wells of polystyrene microtiter plate. A microtiter plate, loaded with HRP (0.125 μg/well diluted in 100 μL of PBS) into the activated and untreated wells was kept inside the chamber and a pressure of 1.0 x10⁵ Pa was applied for 10 minutes. Similarly, four other HRP loaded microplates were kept inside the chamber separately and pressure of 1.5 x10⁵, 2.0 x10⁵, 2.5 x10⁵ and 3.0 x10⁵ Pa, was applied for 10 min, respectively. In the same way, a control experiment was performed without applying any additional pressure. After the stipulated time period, pressure was released and the wells were washed vigorously five-times with washing buffer. After washing the plate the immobilized HRP was used for enzymatic assay as described in section 3.2.2.
3.2.5 Optimization of time for immobilization of HRP by pressure

For optimization of time required for pressure-induced immobilization of enzyme, 0.125 μg/well of HRP diluted in 100 μL PBS was loaded into the activated and untreated wells of the polystyrene microtiter plate. The HRP loaded plates were kept inside the chamber and subjected to an optimized pressure of $2.0 \times 10^5$ Pa for different time period of 5, 15, 25, 35 and 45 min, respectively. Similarly, another set of experiments were performed using same reagent and microtiter plate having activated and untreated well, without applying additional pressure. After releasing the pressure, the plates were washed with washing buffer and immobilized enzyme was assayed as described in section 3.2.2.

3.2.6 Concentration-dependent immobilization of HRP by pressure

Optimum amount of HRP required for immobilization by pressure was determined by loading variable amount of HRP (31.2, 62.5, 125, 250, 500 and 1000 ng/well, diluted in 100 μL of PBS) into the activated and untreated wells of a polystyrene microtiter plate. The HRP loaded plates were kept inside the chamber and optimum pressure of $2.0 \times 10^5$ Pa was applied for 25 minutes. After 25 min, pressure was released slowly and the wells were washed with washing buffer. The immobilized enzyme was assayed colorimetrically as described in section 3.2.2.

3.2.7 Comparative study of pressure-induced immobilization

Pressure-mediated covalent immobilization of enzyme was compared with other established method of immobilization using same reagents. For conventional method, 125 ng/well of HRP diluted in 100 μL of PBS was immobilized into the activated and untreated wells of the polystyrene microtiter plate by overnight (12 h) incubation at 4°C. Another experiment was performed by 3 h incubation at 37°C. Further, for comparison HRP (125 ng/ well/ 100 μL of PBS) was loaded into the activated and untreated wells of a polystyrene microtiter plate and incubated for 25 min at the optimized pressure of $2.0 \times 10^5$ Pa at room temperature (25°C). Similarly, a control experiment was carried out in 25 min at room temperature without applying pressure. After incubation in different conditions, the plates were washed with washing buffer and enzyme was assayed as described in section 3.2.2.

Chapter 3

Pressure – A Novel Tool for Immobilization of Protein
3.2.8 Stability of immobilized system

3.2.8.1 Thermal stability

Thermal stability of immobilized enzyme was investigated after immobilizing the HRP into the activated wells of polystyrene microtiter plate at an optimized pressure of 2.0x10^5 Pa in 25 minutes. The plate with immobilized-HRP was incubated with PBS at 45°C, 60°C and 75°C, respectively in a thermal incubator. Similarly, free enzyme was also incubated. The activity of immobilized-HRP was measured after 15, 30, 45, 60, 75 and 90 min, respectively. After the thermal treatment the buffer was removed and the plate was immediately cooled and enzyme was assayed. The residual activity of the immobilized and free HRP was calculated by relating to the initial absorbance value as 100% activity.

3.2.8.2 Storage stability

Storage stability of pressure-induced immobilized-HRP was investigated by measuring the activity after being stored at 4°C for 25 days. The activity measurement was performed at regular intervals. The residual activity was determined with respect to absorbance recorded at first day, which was taken as 100% activity.

3.2.8.3 Reusability

The enzymatic activity of pressure-induced immobilized-HRP was measured fifteen times by colorimetric assay. After each assay, the wells were washed with PBS and fresh substrate-buffer was poured into the HRP immobilized wells for activity measurement. The enzymatic activity after first assay was taken as 100% activity.

3.2.9 Studies on kinetic parameters

The kinetic parameters were determined by measuring HRP activity against increasing concentration of hydrogen peroxide as substrate. Michaelis–Menten constant (Km) and the maximum reaction velocity (Vmax) of free and immobilized HRP were calculated by plotting the Lineweaver–Burk plot. The Km and Vmax values of free and immobilized-HRP were determined by analyzing the slope and intercept of the Lineweaver–Burk plot of the reciprocals of the reaction velocity versus hydrogen peroxide concentration.
3.2.10 Immobilization of glucose oxidase by pressure energy

Glucose oxidase (15 µg/100 µL of 0.01 M PBS/well) loaded plate was subjected to an optimized pressure of 2.0x10^5 Pa for 25 minutes. After washing, the immobilized GOD was assayed by adding 100 µL of 10% glucose and 20 µL peroxidase (0.1 mg/mL in PBS) followed by adding 20 µL of o-dianisidine (3 mg/mL in phosphate buffer, pH 7.2). Reaction was terminated by adding 20 µL of H_2SO_4 and absorbance was recorded at 490 nm.

3.2.11 Immobilization of alkaline phosphatase by pressure energy

Alkaline phosphatase (30 µg/100 µL of bicarbonate buffer, pH 9.6/well) was immobilized in the activated wells of a polystyrene microtiter plate in 25 min at a pressure of 2.0x10^5 Pa. ALP was assayed by adding 100 µL of pNPP (p-Nitrophenyl Phosphate) to each well from a stock solution of 1 mg of pNPP dissolved in 1 mL of bicarbonate buffer containing 0.02% MgCl_2, pH 9.6. Reaction was terminated by adding 20 µL of 2 M NaOH and absorbance was taken at 405 nm.

3.2.12 Immobilization of invertase by pressure energy

Immobilization of invertase was carried out by loading 100 µL of invertase from a stock solution of 1 mg/mL invertase in to the activated wells of a microtiter plate and subjected to a pressure of 2.0x10^5 Pa for 25 minutes. The activity of immobilized-invertase was checked by adding 100 µL of 0.5 M sucrose solution into the wells and incubating at 55°C for 60 minutes. After incubation, hydrolyzed products were transferred to a separate well. Glucose was assayed by adding 200 µL of assay buffer (0.5 mg of glucose oxidase, 0.12 mg of HRP and 0.24 mg of o-dianisidine in 2 mL of PB) to 50 µL of hydrolyzed sugar. After 30 min, color development was stopped by adding 50 µL of 5% H_2SO_4 and absorbance was recorded at 490 nm.

3.3 RESULTS AND DISCUSSIONS

In this chapter, we are demonstrating an unconventional approach of covalent immobilizing enzymes by applying pressure. For immobilization of enzyme onto solid matrix by pressure, we have used HRP as a model enzyme. HRP is a
holoenzyme of molecular weight 40 KDa and contains one heme group and two calcium atoms. The enzyme converts hydrogen peroxide to water and nascent oxygen in a catalytic reaction. The nascent oxygen oxidizes a dye (colorless compound) to a colored compound. The measurement of absorbance of the colored compound directly gives an estimation of the enzyme activity.

To facilitate covalent binding we have used activated polystyrene microtiter plate. Activation of wells of the polystyrene microtiter plate was carried out by a simple photochemical reaction involving a photolinker, FNAB. On exposure to UV light of 365 nm, FNAB molecule generates a highly reactive nitrene radical which inserts to the C–H bond of polystyrene by nitrene insertion mechanism and forms an activated surface. Activated surface contains labile fluoro group which is displaced by the primary amine group of a protein and forms covalent linkage. Activation of polystyrene matrix by FNAB in a photochemical process is shown in Scheme 3.1.

Scheme 3.1: Photochemical activation of polystyrene surface by 1-fluoro-2-nitro-4-azidobenzene followed by covalent ligation of protein.
The influence of UV irradiation time and amount of FNAB on the extent of activation of the wells of polystyrene microtiter plate was studied by carrying out the reaction by varying the amount of FNAB and irradiation time. The activation of the polystyrene plates was tested by immobilizing HRP. It was observed that the degree of activation of polystyrene surface increases with increasing the amount FNAB and time of UV irradiation (Figure 3.1). From the figure, it can be seen that the optimum amount of FNAB required for photochemical activation of a well of polystyrene microtiter plate was 10 μmole and the time of UV irradiation was 12 minutes.

In previous chapter we have shown that polymer surface, activated by photochemical method using the photolinker, FNAB, can be used for covalent ligation of proteins by thermal incubation. Here, we employed pressure energy instead of thermal incubation to carry out immobilization of biomolecules on FNAB-activated polystyrene surface. Pressure-induced covalent immobilization was carried out in a pressure cooker and pressurized argon or nitrogen gas filled in a laboratory cylinder was used as a pressure source.

In a typical experiment, effect of pressure on immobilization of HRP was investigated by carrying out experiment in the activated and untreated wells of polystyrene microtiter plate at different pressure ranging from 1.0×10^5 Pa to 3.0×10^5 Pa in 10
minutes (Figure 3.2). Result shows that immobilization of enzyme increases with pressure and gets declined with further increase in pressure. Maximum HRP immobilization onto the activated surface is observed when $2.0 \times 10^5$ Pa of pressure is applied. Decrease in absorbance at higher pressure might be due to inactivation of enzyme. Further, more than five-fold increase in immobilization by pressure was observed in comparison to control experiment performed similarly onto activated surface without applying any additional pressure. On the contrary, untreated surface does not show any appreciable immobilization.

![Figure 3.2: Pressure-induced immobilization of HRP in 10 min into the activated (blue column) and untreated (orange column) wells of a polystyrene microtiter plate. Control experiment was carried out without applying pressure.](image)

Figure 3.3 shows time-dependent immobilization of HRP by pressure. It can be seen from the figure that immobilization of HRP increases with increase in time of applied pressure. Maximum immobilization of HRP onto activated polystyrene surface achieved in 25 min at an optimum pressure of $2.0 \times 10^5$ Pa, beyond which no further significant change is observed. On the other hand, experiment carried out without applying pressure, shows less immobilization onto the activated surface in 25 min, indicating that pressure indeed accelerates immobilization onto the FNAB-activated surface. In contrast, untreated surface shows negligible immobilization with or without pressure even after incubation for longer duration.
Further, concentration-dependent pressure-induced immobilization was studied at an optimized pressure of $2.0 \times 10^5$ Pa in 25 minutes. A dose dependent experiment shows an increase in HRP immobilization by applying pressure onto the activated surface with increase in concentration of the enzyme and after attaining the maximum binding it get saturated (Figure 3.4). This suggests that the entire activated surface available for covalent linkage has been utilized in binding of enzyme and no further increase in binding can take place. Moreover, untreated surface does not show any significant immobilization of enzyme with or without applying pressure in such a short time period.

**Figure 3.4:** Dose-dependent immobilization of HRP into the activated (closed symbols) and untreated (open symbols) wells of a polystyrene microtiter plate by applying a pressure of $2.0 \times 10^5$ Pa (square) and without pressure (circle).
Immobilization of HRP by pressure onto polymer surface is schematically represented in Scheme 3.2. To find out whether pressure accelerates covalent binding of enzyme on an activated surface, we first immobilized BSA onto activated surface prior to immobilization of HRP by pressure. The results show insignificant absorbance value, indicating that HRP immobilization does not occurred; this is expected as the labile fluoro group of the activated surface might have been exhausted by NH$_2$-group of BSA and no further reactive moieties are available for covalent binding of HRP. On the other hand, untreated surface does not showed any binding of enzyme which suggests that pressure is indeed responsible for covalent linkage between protein and the activated surface (Scheme 3.2).

The increase in immobilization by mild pressure is due to the covalent ligation of protein or enzyme to the activated surface. This might be because pressure accelerates chemical reaction between the fluoro group of the activated surface and the NH$_2$-group of the protein, which forms stable covalent linkage with the removal of HF, and thus accelerates the immobilization procedure.

Scheme 3.2: Schematic representation of pressure-induced immobilization of proteins onto an activated and untreated polystyrene surface.
To quantify the amount of immobilized HRP to the solid matrix, a standard curve is prepared by plotting absorbance values obtained from an assay of increasing concentration of free enzyme (0.02–40 ng). The amount of immobilized-HRP was calculated by correlating the absorbance value, obtained by assay of immobilized-HRP, with the standard curve. The amount of HRP immobilized in the activated well by pressure in 25 min is 12 ng.

Thereafter, we compared pressure-induced immobilization of HRP onto activated and untreated surface with other methods (Figure 3.5). The results show that pressure-induced immobilization of HRP carried out in 25 min gives comparable results (0.642 ± 0.031) with that of experiment performed by 3 h incubation at 37°C (0.655 ± 0.029) in a thermal incubator and over-night incubation at 4°C (0.672 ± 0.036). Thus, pressure-induced immobilization is not only rapid but the result is also akin to that of conventional method. Therefore, pressure-induced immobilization gives stable covalent binding of biomolecules which is prerequisite in several techniques including preparation of microarray chips.

![Figure 3.5: Comparison of immobilization of HRP onto the activated (blue column) and untreated (orange column) surface (A) at 25°C in 25 min without applying pressure; (B) at a pressure of 2.0 x 10^5 Pa in 25 min; (C) at 37°C in 3 h incubation; and (D) at 4°C in over-night incubation.](image)

In order to investigate thermal stability, the activity of pressure-immobilized and free HRP was studied at 45°C, 60°C and 75°C for 90 minutes. Result shows that at 45°C the immobilized and free HRP are stable which is evident from Figure 3.6; the residual activity in both the cases almost remains unchanged. At 60°C, the
immobilized enzyme retains 64% residual activity while free enzyme retains only 33% of initial activity after 90 min of incubation. This demonstrates better stability of immobilized enzyme towards thermal incubation than free enzyme. However, at 75°C both the immobilized and free enzyme lose almost all its activity after 30 min of incubation (Figure 3.6).

![Figure 3.6: Thermal stability of HRP over time at different temperature. Closed symbol indicates immobilized enzyme and open symbol represents free enzyme.](image1)

Time-dependent stability of the covalently immobilized enzyme was also investigated by storing the immobilized and free enzyme at 4°C for 25 days. The immobilized HRP shows 80% retention of activity after 25 days whereas free enzyme retains only 21% of its initial activity, indicating better stability of immobilized system (Figure 3.7).

![Figure 3.7: Storage stability of HRP over time. Closed symbol indicates immobilized enzyme and open symbol represents free enzyme.](image2)
Reusability is another crucial parameter for immobilized system in practical applications. To evaluate the operational stability, the immobilized HRP was assayed 15 times repeatedly. The covalently immobilized enzyme retained almost full activity after three successive uses and after ten cycles of repeated use it retained more than 50% of its initial activity (Figure 3.8).

![Figure 3.8: Reusability of immobilized-HRP used for enzyme assay for multiple times.](image)

The effect of the substrate concentration on the reaction rate, catalyzed by free and immobilized HRP, was studied using varying concentration of $\text{H}_2\text{O}_2$ substrate. Michaelis–Menten constant ($K_m$) and the maximum reaction velocity ($V_{\text{max}}$) of free and immobilized HRP are calculated from the Lineweaver–Burk plot (Figure 3.9).

![Figure 3.9: Kinetics of immobilized (square) and free (circle) HRP using Lineweaver–Burk plot.](image)
The $K_m$ values are 34.7 $\mu$M for free and 55.3 $\mu$M for pressure-induced immobilized-
HRP, respectively. The $V_{max}$ values are calculated as 1.089 mM/min for free and
0.957 mM/min for immobilized-HRP, respectively. It was observed that the values of
kinetic parameters of the immobilized enzyme are different from those of the free
enzyme. Decrease in activity of an enzyme upon immobilization may be due to
conformational change of the enzyme caused by the immobilization process. Also,
once an enzyme has been immobilized, it finds itself in a micro-environment that may
be significantly different from that existing in free solution. The diffusion of substrate
from the bulk solution to the micro-environment of an immobilized enzyme can limit
the rate of the enzyme reaction.

Finally, to examine whether pressure-induced method of immobilization really work
for other proteins, we immobilized glucose oxidase, alkaline phosphatase and
invertase, separately into the activated wells of polystyrene microtiter plate at $2.0 \times 10^5$
Pa of pressure in 25 minutes (Figure 3.10). In control experiment, immobilization of
these enzymes was carried out without applying any additional pressure at the same
time and temperature. All enzymes shows approximately five-fold higher absorbance
value when the immobilization is carried out by pressure than without applying
pressure onto activated surface, similar to that of HRP immobilization.

![Figure 3.10: Immobilization of glucose oxidase, alkaline phosphatase and
invertase separately into the activated wells of polystyrene microtiter plates
in 25 min at a pressure of $2.0 \times 10^5$ Pa (blue column) and without pressure
(orangle column).]
3.4 CONCLUSIONS

A novel method for covalent immobilization of proteins onto activated polymer matrix by pressure has been described. The heterofunctional photolinker provides great advantage of activating a variety of polymers. The optimum amount of FNAB was 10 μmol for activation of a well of a polystyrene microtiter plate and the time of UV irradiation was 12 minutes. The optimum pressure and time required for covalent binding of protein to the activated surface was $2.0 \times 10^5$ Pa and 25 min, respectively. The immobilized enzyme by pressure shows better kinetic parameters, thermal and storage stability with respect to free enzyme and can be used for repeated use. The method is found to be equally effective for immobilization of other enzymes including glucose oxidase, alkaline phosphatase and invertase. The newly described method for protein immobilization by pressure is simple, rapid and efficient, and can be used for immobilization of biomolecules onto activated surface by manipulating the pressure energy and duration of applied pressure.