BIOTECHNOLOGICAL POTENTIAL
OF
IMMOBILIZED ENZYMES

BY

MOHAMMAD YAQQOUB

DEPARTMENT OF CHEMISTRY
UNIVERSITY OF BALOCHISTAN
QUETTA.
1997
BIOTECHNOLOGICAL POTENTIAL OF IMMOBILIZED ENZYMES

by

MOHAMMAD YAQOOB

DEPARTMENT OF CHEMISTRY
UNIVERSITY OF BALOCHISTAN
QUETTA
1997
THE UNIVERSITY OF BALOCHISTAN

Biotechnological Potential of Immobilized Enzymes

being a Thesis submitted for the Degree of

Doctor of Philosophy

in the University of Balochistan

by

Mohammad Yaqoob, M. Phil. (BALN)

August, 1997.
<table>
<thead>
<tr>
<th>SECTION</th>
<th>1.</th>
<th>1.1</th>
<th>1.2</th>
<th>1.2.1</th>
<th>1.2.1.1</th>
<th>1.2.1.2</th>
<th>1.2.1.3</th>
<th>1.2.2</th>
<th>1.2.3</th>
<th>1.3</th>
<th>1.3.1</th>
<th>1.3.2</th>
<th>1.4</th>
<th>1.5</th>
<th>1.5.1</th>
<th>1.5.2</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>INTRODUCTION</td>
<td></td>
<td></td>
<td>Enzymes</td>
<td>Immobilized enzymes</td>
<td>Methods of enzyme immobilization</td>
<td>Carrier binding method</td>
<td>Covalent binding method</td>
<td>Adsorption method</td>
<td>Cross-linking method</td>
<td>Entrapment method</td>
<td>Matrix entrapment</td>
<td>Microencapsulation</td>
<td>Changes in enzyme properties after immobilization</td>
<td>Bioanalytical potential of immobilized enzymes</td>
<td>Immobilized lipases</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
SECTION 2.

Isolation and purification, immobilization of bovine pancreatic lipase for the production of biosurfactants and measurement of lipase activity on FIA.

2
2.1
2.2
2.3
2.4
2.4.1
2.4.2
2.4.3
2.4.4
2.4.5
2.4.6
2.4.7
2.4.8
2.5
2.5.1
2.5.2
2.5.2.1
2.5.3
2.5.3.1
2.5.3.2

Introduction
Lipases
biosurfactants
flow injection assay for lipase
Experimental
Materials and methods
Lipase assay
protein assay
Precipitation procedure
SDS polyacrylamide gel electrophoresis
Phenolic resin preparation and immobilization
Procedure for transesterification
Instrumentation
Results and discussion
Purification and some properties of bovine pancreatic lipase
Lipase catalyzed transesterification between sugar alcohol and oils
Yield if immobilized enzymes by cross-linking procedure
Emulsified substrates in a flow system:
The measurement of lipase activity
Optimization of flow system
Calibration data
2.6 Conclusions
References

SECTION 3

Production of phosphatidate by immobilized phospholipase-D

3. Introduction
3.1 Phospholipids
3.2 Phospholipases
3.3 Experimental
3.3.1 Materials and methods
3.3.2 Assay method for phosphatidic acid production from lecithin
3.3.2.1 Separation by thin layer chromatography
3.3.2.2 Assay of choline released by choline oxidase
3.4 Results and discussion
3.4.1 Optimization studies
3.4.2 Production of phosphatidic acid
3.5 Conclusions
References

SECTION 4

Chemiluminescent and spectrophotometric determination of glycerol-3-phosphate and glycerophosphorylcholine using soluble and immobilized enzymes

4. Introduction
4.1 Experimental
and

4.1.1 Materials / methods 64
4.1.2 Immobilization procedure 65
4.1.3 Instrumentation 66
4.1.4 Procedures 66
4.1.4.1 FIA-CL procedure for glycerol-3-phosphate (GP) 66
4.1.4.2 FIA-CL procedure for glycerophosphorylcholine (GPC) 68
4.1.4.3 Spectrophotometric procedure for GP 68
4.1.4.4 Spectrophotometric procedure for GPC 69
4.2 Results and discussion 69
4.2.1 Yield of immobilized enzyme by cross-linking procedure 69
4.2.2 Optimization of FIA-CL manifold 69
4.2.3 Calibration date for GP and GPC 71
4.2.4 Spectrophotometric determination of GP and GPC 71
4.2.4.1 Optimization 71
4.2.4.2 Calibration date for hydrogen peroxide, GP and GPC 73
4.3 Conclusions 73

References 78

SECTION 5.

Chemiluminescent and spectrophotometric assays for choline, phospholipase-D and phosphorylcholine using immobilized enzymes

5. Introduction 80
5.1 Experimental 82
5.1.1 Materials and methods 82
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1.2</td>
<td>Immobilization procedure</td>
<td>83</td>
</tr>
<tr>
<td>5.1.3</td>
<td>Instrumentation</td>
<td>83</td>
</tr>
<tr>
<td>5.1.4</td>
<td>Procedures</td>
<td>83</td>
</tr>
<tr>
<td>5.1.4.1</td>
<td>Chemiluminescent procedure for choline</td>
<td>83</td>
</tr>
<tr>
<td>5.1.4.2</td>
<td>Chemiluminescent procedure for choline via phospholipase-D</td>
<td>85</td>
</tr>
<tr>
<td>5.1.4.3</td>
<td>Spectrophotometric procedure for phosphorylcholine</td>
<td>85</td>
</tr>
<tr>
<td>5.2</td>
<td>Results and discussions</td>
<td>86</td>
</tr>
<tr>
<td>5.2.1</td>
<td>Optimization of FIA-CL manifold</td>
<td>86</td>
</tr>
<tr>
<td>5.2.2</td>
<td>Calibration data</td>
<td>88</td>
</tr>
<tr>
<td>5.2.3</td>
<td>Spectrophotometric determination of phosphorylcholine using Alkaline phosphatase reactor</td>
<td>88</td>
</tr>
<tr>
<td>5.2.3.1</td>
<td>Optimization of FIA manifold</td>
<td>88</td>
</tr>
<tr>
<td>5.2.3.2</td>
<td>calibration data</td>
<td>93</td>
</tr>
<tr>
<td>5.3</td>
<td>Conclusions</td>
<td>93</td>
</tr>
<tr>
<td>References</td>
<td></td>
<td>97</td>
</tr>
<tr>
<td>Conclusions</td>
<td></td>
<td>99</td>
</tr>
<tr>
<td>Future Trends</td>
<td></td>
<td>101</td>
</tr>
<tr>
<td>Publications</td>
<td></td>
<td>103</td>
</tr>
</tbody>
</table>
CERTIFICATE

This is to certify that Mr. Mohammad Yaqoob who was registered in Ph.D. (Reg. No. 1979/BU-93/R-19) in the Department of Chemistry, University of Balochistan, under my supervision in December has successfully completed advance courses and research work on "Biotechnological Potential of Immobilized Enzymes". Now he may be allowed to submit the thesis on the above mentioned topic to the University of Balochistan for the fulfilment of Ph.D. degree.

Prof. Dr. Mohammad Masoom
Research Supervisor
Institute of Biochemistry
University of Balochistan
Quetta..

Prof. Dr. Naim M. Hassan
Chairman
Department of Chemistry
University of Balochistan
Quetta.
ACKNOWLEDGEMENT

The author would like to thank Prof. Dr. Mohammad Masoom, Institute of Biochemistry, for his guidance and constant interest during the course of this work, Dr. Abdul Nabi, Associate Professor, Department of Chemistry, for his help and encouragement and Prof. Dr. Naim M. Hassan, Dean Faculty of Science and Chairman, Department of Chemistry for providing the facilities at the Department of Chemistry, University of Balochistan.

We gratefully acknowledge the financial support provided by Pakistan Science Foundation for the conduct of this work. Without the generous support of PSF in the form of grant (B-BU/CHM (211)), this work could not be materialized. The partial support provided by Third World Academy of Sciences (Grant No. BC. 92-087) for the conduct of part of the work is also highly appreciated.
Summary of the Thesis submitted for Ph.D. degree

by

Mohammad Yaqoob

on

"Biotechnological Potential of Immobilized Enzymes"

The analytical and biotechnological applications of enzymes are well known for the selective transformation of substrate to products. Immobilization of enzymes offers the advantage of these catalysts to be separated physically from the mixture of substrate and product for reuse, mechanical strength microbial resistance, thermal stability, chemical functionality, hydrophobic/hydrophilic character, ease of regeneration, loading capacity and cost. These solid state catalysts may prove novel for the production of many compounds of interest.

The work presented in this thesis is divided into five sections. The first section is devoted to an up to date knowledge on the biotechnological potential of immobilized enzymes, in analysis and synthesis, backed up by a very recent and vast survey of literature. The second section describes experimental work. Followed by an introduction, the isolation and purification to electrophoretic homogeneity of bovine pancreatic lipase, the utilization of this enzyme in an immobilized form for the production of biosurfactants and a flow injection procedure for lipase activity measurement using emulsified substrates is given. The third section deals with the application of phospholipases, in particular phospholipase-D in an immobilized for the production of phosphatidate. The remaining sections i.e., fourth and fifth, phospholipase-D in soluble form coupled with other immobilized enzymes is used for the analysis of substrate of clinical importance, including glycerol-3-phosphate, glycerophosphorylcholine, phosphorylcholine and choline etc. using spectrophotometric and chemiluminescent detection systems.

In the end a brief discussion on general conclusions and suggestions for future work are given.
SECTION 1

INTRODUCTION
1. **INTRODUCTION**

1.1 **ENZYMES**

Enzymes are defined as 'protein with catalytic properties due to their power of specific activation'. They catalyze the reactions which take place in the living cell, e.g., the hydrolysis of fats, sugars and proteins and their resynthesis. They also catalyze many form of redox reactions which provide energy to the cell.

Enzymes occupy an important place in analytical biochemistry and many investigations require their detection and quantitation. Studies of the enzymes content of blood plasma are particularly useful in clinical biochemistry; both in the monitoring of normal metabolic processes and in the detection of abnormal levels of enzyme production or release. Enzymes are valuable analytical tools and offer sensitive and specific methods of quantitation for many substances. The increasing availability of high purified enzyme preparation, both in solution and immobilized forms, permits the development of a wide range of methods. Enzymes often need for their activity the presence of a non-protein portion, which may be closely combined with the protein, in which case it is said to be prosthetic group or more loosely associated, in which case it is said to be coenzyme. The presence of certain metals, including iron, magnesium, copper, zinc, manganese or cobalt, is often necessary for their action. One outstanding feature of enzymes is that many of them are highly specific. A slight change in the stereochemical configuration of the molecule is sufficient to make a particular enzyme incompatible and unable to effect hydrolysis.

Enzymes on the basis of their catalytic activity are divided in various classes. These include, i) oxidoreductases, ii) transferases, iii) hydrolases, iv) lyases, v) isomerases and vi) ligases.
1.2 IMM O B I L I Z E D E N Z Y M E S

Immobilized enzymes can be defined as "the localization of enzymes to water insoluble matrices by physical or chemical means or both with retention of their catalytic activities and which can be utilized repeatedly or continuously". This major breakthrough in enzyme technology occurred in 1916 [1], immobilizing invertase on animal charcoal and observed that enzyme retained most of its activity over a long period of time. Their work in this regard was ignored until mid 1950s. Since then much attention has been paid to the preparation and utilization of these enzyme derivatives; their science and technology have been reviewed [2-6].

In comparison, soluble enzymes are potent catalyst, but there are still disadvantages associated with these enzymes. These disadvantages are; i) extremely high cost of some enzymes and their unavailability. This high cost is even more of a factor when continuous flow, automated analysis systems are employed or when the enzyme requires a coenzyme for its function and ii) instability, due to denaturation, has also restricted their analytical involvement.

The advent of immobilized enzymes have overcome these difficulties. Immobilized enzymes have solved the problem of cost, secondly, there is an increase in the stability of an enzyme and because of this, a single aliquot of immobilized enzyme can be utilized repetitively and continuously for thousand of assays. Thirdly, enzymes can then be stored at room or at some elevated temperature for months or years without any loss in activity. Finally, immobilized enzymes provide the possibility of a greater variety of engineering designs for continuous processes.

1.2.1 Methods of Enzyme Immobilization

The technology of immobilized enzymes started initially with some applications in the area of analysis of certain common compounds, that provided the methodological know-how and the stimulus for further research into the area. Attempts for many year's since their introduction have been focused to find out; a) the best procedure for immobilization that can give a more stable and active product, b) a more reliable support
in terms of its low susceptibility to organic solvents and no swelling upon packing and c) the cost of the support material has also been the matter of consideration in such studies. Many of the shortcomings have been reduced and the field of application of these catalysts have been broadened. Today these catalysts are not only used for analytical purposes but also for the synthesis of certain important compounds. Not only enzymes but whole cells and cell organelles have been immobilized and utilized for the purpose. Immobilized enzymes are classified on the basis of the method adopted for their immobilization including; carrier binding method and entrapping method.

1.2.1.1 Carrier Binding Method

This method involves the binding of the enzyme to water insoluble carriers. As the carrier is directly involved in binding to the enzyme, its chemical composition, particle size and surface area play an important role. The carrier binding method can be further classified into three major categories.

1.2.1.1.1 Covalent Binding Method

This method involves the formation of covalent bonds between the enzyme and its support, has been the most widely used technique for insolubilization of enzymes. Although the method is often tedious, it provides an immobile enzyme firmly bound to its supports, other advantages include flexibility of the method and the ability of the immobilized enzyme to achieve good flow properties, especially inorganic material and many organic polymers [7-9] are used as a support matrix for enzyme immobilization because of their insolubility, mechanical stability, surface area, the functional groups they possess and also does not swell on packing. During the operation the bound enzyme does not leak from support even if factors such as pH, solvent, substrate and temperature are changed. The only drawback of covalent coupling in certain cases is the low initial yield of the active enzyme, probably due to the blockage of some of active sites by involvement of its group/s in covalent bond formation. The functional groups in enzyme protein which are normally involved in covalent bond formation include the α and ε-amino groups, β-
and γ-carboxyl groups, the phenolic group of tyrosine, the sulfhydryl group of cysteine, the hydroxyl group of serine and the imidazole group of histidine.

Three main kinds of carrier have been used as a matrix for enzyme immobilization because of their insolubility, mechanical stability, swelling properties, surface area and the functional groups they possess. There are inorganic supports such as glass, natural polymers and synthetic polymers.

1.2.1.1.2 Adsorption Method

This is the easiest method to performed, based on physical adsorption of enzyme protein on the surface of a water insoluble carrier. Protein molecules may interact with the carrier by ionic, hydrophobic, hydrogen bonding and van der Waal’s interactions but only the first two of these are likely to lead to a greater attraction of the enzyme for the carrier than for water and so to its binding. Practically such enzyme derivatives can be obtained just by stirring the enzyme with the matrix for some time. A wide variety of solids has been used to adsorb enzymes, a list of which is given elsewhere [10]. Although the method is cheap, easily carried out, gives a high initial yield and there are less chances of disruption of enzyme protein, a significant problem is the leakage of the enzyme from the carrier during use as the binding force between the carrier and the enzyme is very weak [11].

1.2.1.1.3 Cross-linking Method

Immobilization of enzymes to supports via cross-linking agents has rapidly emerged within the last decade and is one of the most popular methods nowadays. Bifunctional reagents have been used as cross-linking agents among which glutaraldehyde is most frequently used. Among the carriers, controlled porosity glass particles (CPG) is gaining much acceptance. The method involves three steps; i) activation of the carrier, mainly by silanization, ii) treatment of activated carrier with cross-linking agent and iii) linking of enzyme to carrier via cross-linking agent. The sequence of reactions involved and experimental details of this method of enzyme immobilization are given elsewhere [12].
1.2.1.2 **Entrapping Method**

Enzymes have been immobilized by entrapment in polymers of synthetic or natural origin. The method is further subdivided into matrix entrapment and microencapsulation.

1.2.1.2.1 **Matrix Entrapment**

In this method the enzymes are physically entrapped within a cross-linked lattice type water insoluble polymer. This is a sort of gel entrapment in which the polymer is allowed to form cross links in the presence of enzymes. The most popular matrices for gel entrapment include polyacrylamide, polyvinyl alcohol and silica gel, among which the polyacrylamide appears to be the best [13], and someone has investigated suitable conditions for immobilizing enzymes by this method [14]. This method has the advantage of being chemically simple, having a high initial yield and the product can be stored dry up to 80 days with little appreciable loss of activity [13], but the drawback is the loss in enzyme activity due to slow leaching during operation [2]. Other disadvantages include poor flow properties and low reactivity for high molecular weight substrates.

1.2.1.2.2 **Microencapsulation**

In the mid 1960s, a new approach to enzyme immobilization based on the encapsulation of enzyme solution in semipermeable membranes of various polymers [15-17] was introduced. The semipermeable membrane prevents the enzyme from leaking out while permitting the exchange of substrate and products. Chemically the process is very simple and involves three steps; i) aqueous microdroplets of an enzyme solution are formed by emulsifying with water immiscible organic solvent, ii) addition of a polymer solution to the stirred emulsion, resulting in the formation of polymer membrane on the surface of microdroplet and iii) dispersion of resulting microcapsules into an aqueous phase. The diameter of the microcapsules varying from 10 m to 1 mm. Although these
enzyme preparations have limited analytical applications [18] and someone has studied
their biomedical applications [19].

1.2.2 Changes in Enzyme Properties after Immobilization

Immobilization of an enzyme results in a change in many of the physical and kinetic
properties of the corresponding soluble counterpart. This may be due to a considerable
change in the micro-environment of the enzyme. Among the changes in physical
properties, the storage and temperature stabilities are most prominent. Numerous enzymes
have been reported with an increase in long-term storage and thermal stability. Sulphite
oxidase, which in soluble form loses its activity rapidly (20% after six h) at room
temperature, when immobilized on controlled pore glass showed no loss in activity for
months in spite of continuous use at 25°C [20]. This increase in stability may be due to
the stabilization of the native structure of enzyme. Immobilization also has a profound
effect on the optimal pH. Depending on the nature of the carrier, the pH may shift towards
a higher or a lower value [21]. If the carrier used for immobilization is negatively charged,
then more positively charged ions mainly H⁺, will accumulate at the boundary layer
between the carrier and the surrounding solution. To compensate for that, the pH of the
bulk solution has to be displaced towards more alkaline pH and vice versa.

Enzyme kinetics and therefore reactivity are changed after immobilization, possibly
due to the placement of enzyme in a new environment. The charge on the carrier may also
contribute to a change in the rate constants. Excellent discussions on this aspect are
available [22, 23].

1.2.3 Bioanalytical Potential of Immobilized Enzymes

With the development of potent immobilization methods for enzymes during the
past decade, these enzymes modifications have evolved from a research curiosity into an
entire branch of biotechnology and are replacing their soluble counterparts in nearly every
field of application. A tremendous amount of research has been carried out in this area.
The analytical, clinical and biomedical applications of devices based on immobilized
enzymes can be divided into three major categories; i) Immobilized enzyme used in
conjunction with ion-selective electrodes provide very convenient methods of analysis. The immobilized enzyme is held in a gel or membrane around the electrode and the substance to be measured diffuses into the enzyme gel and its conversion to the product alters the ionic equilibrium across the ion-selective membrane. The main advantage of enzyme electrode lies in the simplicity of the method of analysis although the presence of interfering ions may present a major problem, particularly when using cation selective electrodes [24-26]. ii) Immobilized enzyme reactors are the examples of the use of immobilized enzymes in flow systems. This combination of immobilized enzymes with flow system has emerged recently as a powerful technique. The reactors are simply columns holding immobilized enzymes on appropriate supports. Solutions carrying analyte can be made to pass through and react with enzyme, the enzyme being retained for use over and over again, while the product is carried to the detector. The advantages of reactors is that almost any detector system can be used which can record the concentration of substrate, a product or coenzyme. Principally, there are two types of reactors, a) Packed-bed reactors, receiving much attention. Their use was previously avoided because of one disadvantage, the pressure drop across the reactor, but nowadays with introduction of inorganic supports like glass beads, CPG etc., that problem has disappeared as these supports maintain their integrity and do not swell on packing, so that the pressure drop is minimized. The unique advantage of the packed-bed reactors is its large surface area, thus it has the potential of providing a relatively larger number of immobilized enzyme molecules, therefore making possible they almost complete conversion of substrate into product and b) Open-tubular reactor are comparatively difficult to construct. They are prepared by covalently immobilizing enzymes onto the inner wall of a tubing made of nylon or polyacrylamide. The advantages of open-tubular reactors are; low pressure drop, well suited for air-segmented analyzers, blood samples do not block the reactor and their commercial availability. The problem associated with these reactors is the small amount of enzyme that can be attached to the inner wall of the tube owing to the small surface area. Therefore, to achieve reasonable conversion of substrate to product, a very long reactor would be required, which in turn would increase the dispersion, an important factor in flow injection analysis. The packed-bed and open-tubular reactors have been used
successfully in flow systems [27-30] and iii) Immobilized enzyme stirrers is a versatile application of immobilized enzymes. The enzyme is held on a stirring bar with a polymer net over it. These devices were introduced and utilized for the determination of urea and glucose [31, 32]. The advantages of these reactors is their stability, economy and applicability to a wide variety of detection systems. Enzymes have also been immobilized in the form of a membrane and used on the surface of an electrode for substrate analysis, but besides this a number of other interesting applications have appeared which explore other properties of enzyme reactors. An elegant application of membranes of immobilized enzymes has been a compact sensor for pesticides [33].

1.3 **IMMOBILIZED LIPASES**

Increased interest in the chemistry and biotechnology of fats and oils has merged in recent years. This trend can mainly attributed to the fact that oleochemicals are derived from renewable sources (e.g., vegetable oils and animal tallow). Hence they may be produced in virtually every country of the world. In addition, the increasing surplus of fats and oils in the more developed countries has bolstered both fundamental and applied research aimed at the manufacture of alternative lipid derived products on an industrial scale [34, 35]. By virtue of their economic and ecological advantages, oleochemical compete successfully with long chain acids, alcohols and their derivatives of petrochemical origin [36, 37].

With recent advances in bio-reactor technology and genetic engineering, many new and interesting ideas for employing biotechnology to produce oleochemicals from fats have been investigated. Among the most promising chemical routes of industrial interest are the hydrolysis, ester synthesis and interesterification reactions of lipids brought about by lipases [38]. Lipases selectivity lower the activation energies of the chemical reactions they catalyze [39]. With these enzymes, one can achieve much higher specificities and major enhancements in reaction rates relatives to non-enzymatic reactions [40].

Esterification reactions between polyhydric alcohols and free fatty acids are, in essence, the reverse of the hydrolysis reaction of the corresponding glyceride. The equilibrium between the forward and the reverse reactions is usually controlled by the
water content of the reaction mixture. Examples of high value chemicals obtained via use of lipases include the synthesis of oleic acid esters of primary and secondary aliphatic and terpenic alcohols [41] and the production of geranyl and menthyl esters from butyric acid and geraniol, or lauric acid and menthol respectively [42].

The term interesterification refers to the exchange of acyl radicals between an ester and an acid (acidolysis), an ester and an alcohol (alcoholysis), or an ester and another ester (transesterification). The lipase catalyzed interesterification reaction of triglycerides and fatty acids has been investigated intensively for the last two decades. This reaction is promoted when the concentration of water in the reaction system is very low so that the equilibrium is shifted towards synthesis, but the water present is sufficient to retain the enzyme activity [43]. The reaction is currently of great potential industrial interest. In this process, the physico-chemical properties of oils and fats of high economical values [44].

The use of membrane bioreactors for the enzymatic processing of fats and oils is becoming an increasing alternative field of research to substitute stirred-tank and fixed bed reactors [45]. Therefore, the lipase catalyzed interesterification and hydrolysis reactions have been performed either in a two phase membrane reactor system or in a membrane emulsion reactor [46].

Lipase-catalyzed glycerolysis of fats has proved to be an interesting alternative for the high yield industrial production of monoglycerides [47]. Lipases from several origins - in soluble form or immobilized on solid supports of different aquaphilicity have been used for the production of mono- and di-oleylglycerol by hydrolysis of triolein. The porcine pancreatic lipase adsorbed on celite was found to be the most effective biocatalyst tested using a microemulsion system [48]. High activity and excellent selectivity towards the formation of intermediate acylglycerols were achieved (the conversion to diolein plus monoolein was 79% per 5 h of reaction).

Fatty acids and glycerin, which are important raw materials in the oil industry, have been produced by the hydrolysis of triglycerides at high pressure and temperature (50 atm, 250°C). However, recently, the possibility of industrial applications of microbial lipase has been investigated as a means of energy conversion. In particular, a thermostable lipase that hydrolyzes oil and fat with a high melting point is important for commercial use.
A research group has investigated a new thermostable lipase and a bioreactor suitable for commercial use due to cost savings [49]. Palm stearin oil, which has a high melting point and is generally used as the commercial raw material in oil chemistries, was hydrolyzed by a thermostable lipase produced from Pseudomonas sp. The initial reaction rate of oil hydrolysis was controlled by the interfacial area of the water/oil emulsion, where water was emulsified in oil using a new bioreactor of a draft type which has the capability of functioning as an efficient continuous reactor for the separation of oil and water and the recovery of the enzyme was introduced. By using the single-stage reactor, the hydrolysis ratio of palm stearin oil was mentioned at 80% up to 230 h of operation and percentage recovery of the enzyme was 89.8%. A countercurrent multistage reactor (three-stage reactor) was developed with the aim of improving the hydrolysis ratio. In the continuous hydrolysis of palm stearin oil using this reactor, the retention time of which was 36 h at each reactor, a high percentage hydrolysis of 95% and an increased glycerin concentration up to 30% were observed.

1.3.1 Immobilized Lipases in Organic Solvents

Conventional biochemical wisdom holds that enzymes inactivate when exposed to organic solvents. The inactivation indeed occurs (via protein denaturation) when water-miscible organic solvents are added to aqueous protein solution [50, 51]. In recent years much research has centered on the conduct that many enzymes can work in organic solvents containing little or no added water [52-54]. Specifically, when an enzyme powder is placed in an organic solvent, it displays acceptable catalytic activity, especially if certain rules and guidelines are followed [54]; i) hydrophobic solvents are preferable to hydrophilic ones (although some enzymes remain catalytically active even in most hydrophilic organic solvents); ii) some enzymes require exogenous water addition to the dry organic solvents; iii) enzyme powders are prepared (e.g., freeze-dried) from aqueous solution at the pH optimal for enzymatic activity; and iv) since enzymes are insoluble in nearly all organic solvents, the enzyme particles should be sufficiently small and their suspension should be continuously agitated in order to minimize diffusional limitations.
The realization that enzymes can function in nonaqueous media has eliminated many misgivings about using enzymes as practical catalysts.

Most of the enzymes used as catalysts of preparative conversions in organic solvents to date have been hydrolases, namely, lipases and proteases. In addition to their catalytic versatility, these enzymes are readily available commercially in sizable quantities and at relatively low cost. In addition to expanding the range of reactions feasible via biocatalysis, enzymatic catalysis in organic solvents has revealed some unexpected and beneficial phenomena. For instance, the stability of enzymes in nonaqueous media may be greatly enhanced compared to that in water [55], substrate specificity of enzymes can be regulated by the solvent [56], the recovery of products and biocatalyst can be achieved, less risk of microbial contamination, undesirable side reaction promoted by water can be suppressed, organic substrates often have greater solubility in organic solvent and even the enzyme property of particular interest enantioselectivity can be controlled by the solvent [57].

With recent advances in enzyme technology, novel enzyme materials have been developed by coating an enzyme with surfactants [58, 59]. Among them, surfactant-coated lipase is soluble in anhydrous organic solvents and shows 2-100 times higher catalytic activity compared with other enzymatic systems, including lipase in organic/aqueous emulsion [60] dispersed powdered lipase [61, 62] and poly(ethylene glycol)-grafted lipase in organic solvents [63]. The lipid-coated lipase can prepare esters directly from acid and alcohol without requiring between activated esters and alcohols.

1.3.2 Immobilized Lipases in Supercritical Fluids

A relatively new technique to obtain pure products and to integrate the separation process is the use of supercritical fluids (SCF) as solvents. The use of SCF as solvents for a variety of extractive applications have been introduced in the last few years. Recently, SC solvents also have been applied as solvent in non extractive applications such as high pressure micronization and chromatography and also as a chemical reaction medium as well. Since the first reports on the use of SCF as a reaction medium [64,65], several studies on oxidation, hydrolysis, transesterification, esterification and interesterification
[66-73] have proven the feasibility of enzymatic reactions in SCF. The advantages of using supercritical carbon dioxide as a medium for enzymatic catalyzed reactions have been well documented [74, 75]. Frequently, the temperature range used for SC carbon dioxide in processing is compatible with the use of enzymes as catalysts. An additional benefit of using SCF along with enzymatic catalysis is that it provides a medium for the recovery of products or reactants. However, a limitation of the process may arise from the nonpolarity of carbon dioxide, which preferentially dissolves hydrophobic compounds.

Esterification between oleic acid and oleyl alcohol, catalyzed by immobilized lipase in a batch-stirred tank reactor with SC carbon dioxide as solvent has been investigated [76], which produced higher reaction rates at supercritical conditions than in the solvent free system. A continuous fixed-bed reactor was also designed based on the results obtained from batch experiments. At 150 bar, 40°C, and with water activity 0.46% w/w, the activity of the preparation is practically unchanged when carbon dioxide was used as solvent. The addition of small amounts of water increased the conversion rate. The higher conversion also was observed at longer residence time, but using n-butane as reaction medium, a decrease in conversion was observed [77].

1.4 LUMINESCENCE

The term luminescence describes the emission of ultraviolet/visible and infrared radiations from an excited electronic state of a molecule. The various types of luminescence can be classified according to the means by which energy is supplied to excite the luminescence molecule [78]. If the excitation energy is obtained from a chemical energy of the reaction, the process is termed “chemiluminescence” (CL). The de-excitation process is accompanied by the emission of photon which is generally represented by;

\[
A + B \rightarrow C^* \\
C^* \rightarrow C + hv
\]

where \(C^*\) = excited state product.
Chemical light producing system can also take place in living organisms and the term "bioluminescence" (BL) is used for such emission [79, 80]. Chemiluminescence is observed in solid, gas and liquid phase reactions and all have been used analytically in biochemical, toxicological and environmental areas covering various aspects of applications [81-83], in liquid phase, biochemicals, carcinogens and drugs in a variety of samples [84]. There are many inorganic and organic chemical reactions that produce CL in the liquid phase. However, only few different systems have used for analytical purposes [85, 86], for e.g., luminol (5-amino-2,3-dihydrophathalazine-1,4-dione), lucigenin (N,N-dimethyl-9,9-biacridinium nitrate), lophone (2,4,5-triphenyl-imidazole), gallic acid (3,4,5-trihydroxybenzoic acid), morphine, codeine, bis(2,4,5-trichlorophenyl (oxalate), pyrogallol, inorganic siloxanes and acridinium esters. The luminol followed by lucigenin are the most commonly employed CL reactions [87].

Recent studies involves luminol electrogenerated chemiluminescence (ECL) which is much more attractive as compared to the conventional luminol reactions. ECL is a generic term that describes the stimulation of CL by electrode processes [88]. ECL is a technique by which a CL reaction is produced in the vicinity of an electrode surface when certain potential is applied to it [89]. The reaction can then be controlled and manipulated by alteration to the applied potential. Existing flow CL methods can be simplified by reducing the number of reagents as reagents can be produced on line.

1.5 FLOW INJECTION ANALYSIS

The term flow injection analysis (FIA) was originated in 1974 [90], has now gained widespread acceptance within the scientific community and several reviews have been published [91-96] and the inventors of the technique have written a book on FIA [97].

Flow injection analysis is based on the injection of a liquid sample into a moving nonsegmented continuous carrier stream of a suitable liquid. The injected sample forms a zone, which is then transported toward a detector that continuously records the absorbance, electrode potential or other physical parameter as it continuously changes due to the passage of the sample material through the flow cell [97].
1.5.1 Basic Components of FIA

An FIA system can easily be assembled from the components already present in an analytical laboratory. The basic requirement are a pump, an injection valve, flexible plastic tubes to connect the injection valve to the detector equipment with a flow through small volume cell and a chart recorder shown in Fig. 1.1.

Peristaltic pumps which can easily accommodate 2-4 channels (streams) are most commonly used to propel the carrier stream at a constant steady flow rate in the range from 0.5-3 ml min⁻¹. Although this function can be performed by liquid chromatography pumps, or even gravitation.

An injection valve which allows the reproducible insertion or introduction of an accurately measured sample volume (10-200 µl) into the flow stream without stopping it. By far the commonest means are Rheodyne by-pass rotary valves of the type use in HPLC [98].

The manifold consists of flexible polyethylene tubing with uniform inner diameter. Reaction coils are made by winding the appropriate lengths of tubing around small methacrylic cylinders. These and other manifold components are glued to small Lego blocks which in turn are then attached to a Lego board.

Almost any flow-through detector for wet chemical reactions can be used in FIA. The flexibility of FIA with regard to the detection system is one of the main reasons for the broad applicability of FIA. A wide range of detection methods has been used with FIA among which are uv/visible spectrophotometry (most widely used), fluorimetry [99], chemiluminescence [100, 101], electrochemiluminescence [102-104], atomic absorption [105], electroanalytical detectors [106, 107], diode array detectors [108] and others [109-112].

Measurement of peak height is the most common one followed by peak area evaluation and peak to peak measurements. The microcomputer may be used to collect
Fig. 1.1 (a): The simple single line FIA system utilizing a carrier stream of reagent, R, S, FC and W are the sample injection, detector and waste respectively.

(b): The analog output has the form of a peak. The recording starts at S (time of injection) H, is the peak height, T is the residence time corresponding to the peak height measurement.
and process the data and to control various instrument modules such as the transport
system, the injection system and the detector.

1.5.2 Applications of Immobilized Enzymes in Flow Injection Analysis (FIA)

Flow injection analysis involves the injection of sample in discrete manner into an
un-segmented carrier or reagent stream. The technique has the clear advantages of high
sampling rate, low reagent consumption, simplicity and versatility as compared to air-
segmented continuous flow analysis and batch procedures. Coupling of the enzyme
technology with FIA has resulted in a very rapid and sensitive analytical system.
Nowadays, most of the enzymatic determinations are carried out by making use of
immobilized enzyme reactors. These reactors have been used with sample injection for a
large number of substances [113-117].
REFERENCES


33. L. H. Goodson, W. B. Jacobs in Ref. 19.


98. Products for liquid chromatography, Rheodyne, Cat. No. 1, P.O. Box 996, Cotati, California, USA., (1983).


SECTION 2

INTRODUCTION TO LIPASES AND BIOSURFACTANTS, ISOLATION AND PURIFICATION OF BOVINE PANCREATIC LIPASE, ITS IMMOBILIZATION FOR THE PRODUCTION OF BIOSURFACTANTS AND MEASUREMENT OF LIPASE ACTIVITY BASED ON FIA
2. INTRODUCTION

2.1 LIPASES

Lipases are soluble enzymes that bind to lipid/water interfaces where they catalyze hydrolysis of the water-insoluble lipids to more polar products which can be incorporated into cell membranes and be used in further metabolic processes. Lipases differ from other esterases in that their substrates are insoluble and therefore aggregated. It follows that lipase carry out catalysis in a heterogeneous system. Their activity is usually maximal only when the enzyme is adsorbed to a lipid/water interface. This catalytic property, known as "interfacial activation" was described [1] and proposed a conformational change of the enzyme at the interface. The theoretical treatment of lipase kinetics at the interface is reviewed [2].

Pancreatic lipase is the major lipolytic enzyme involved in the digestion of triacylglycerols starts in the small intestine into which the zymogen prolipase is secreted by the pancreas, there it is converted into active lipase, having a molecular weight of 50 kDa and consisting of 449 amino acid residues [3, 4]. The most peculiar property of lipase is that the activity is strongly inhibited by surface active agents named bile salts [5]. It was proposed that the bile salt coating of triglyceride globules presents a negatively charged surface that inhibits pancreatic lipase adsorption and activation. To counteract this effect a specific lipase anchoring pancreatic protein called colipase, which has no lipolytic activity by itself, having molecular weight of around 12 kDa, binds to lipase 1:1 molar ratio, restores the activity of lipase in the presence of bile salt and shift the pH optimum from 9 to 6. Lipase acts on bile salt covered triacylglycerol droplets interface at slightly acidic conditions and catalyzes the hydrolytic removal of one or both of the fatty acid residues to yield a mixture of free fatty acids and 2-monoacylglycerols with a small fraction of the triacylglycerols that remain unhydrolyzed [6, 7].

The lipase family also includes two other vertebrate proteins that show very similar hydrolytic functions but distinct enzymes are lipoprotein lipase and hepatic lipase.
Lipoprotein lipase catalyze the same chemical reaction as classical pancreatic lipases, hydrolysis of the primary ester bonds in tri- and diglycerides to form free fatty acids and 2-monoglycerides, but there are at least two major differences. Lipoprotein lipase hydrolyzes phospholipids to about 10% of the rate of triglycerides hydrolysis. Furthermore, lipoprotein lipase is active on simple, partly soluble esters like p-nitrophenyl acetate while classical pancreatic lipases have little if any activity on soluble substrates and the physiological substrates for lipoprotein lipase are the triglyceride rich plasma lipoproteins [8].

Hepatic lipase is also important in lipoprotein and phospholipid metabolism [9] and through its action on high density lipoproteins may mediate delivery of cholesterol from peripheral tissues to the liver [10]. It may also be involved in the metabolism of intermediate density lipoprotein to low density lipoprotein in the liver [9].

Lipases are widely distributed in animals, plants and micro-organisms and purified with characterization [11-20]. In particular, extracellular lipases from micro-organisms have received much attention for their potential use in biotechnology, mainly because of their easy availability and high stability [21].

The purification of enzyme/s from easily available sources, in the laboratory, where they are to be utilized further is a necessity in The Third World Countries, where the availability of commercial enzymes in the active form can not be guaranteed. The purification of lipase/s from bovine pancreas reported in the present study is a good example. The method is very facile and in a couple of chromatographic steps provides a homogeneous enzyme preparation as confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.2 BIOSURFACTANTS

Biosurfactants are surface active compounds (detergents, surfactants, amphiphiles) consisting of both hydrophilic and hydrophobic moieties. They possess the capability to reduce the surface tension (air-water) of aqueous system, to reduce the interfacial tension of liquid-liquid (oil-water) or liquid-solid systems and to effect changes in the foaming properties of aqueous mixtures [22].
These surface active compounds are able to facilitate reactions and mass transfer at the interface. The economic importance of synthetic surfactant is reflected by the increasing number of publications and patent claims as well as by increasing consumption on a world-wide scale [23]. Some of these synthetic surfactants are toxic and not readily biodegradable. No single surfactant is suitable for all required applications. Therefore, alternative sources of surfactants or surfactant precursors are a necessity [24].

During recent years a considerable number of studies on surfactants produced by a variety of microorganisms including bacteria, yeasts and filamentous fungi have been reported [25-27]. Besides chemicals surfactants, biosurfactants represent a promising group for attempts to enlarge the present range of the product palette for different uses. Most of the biologically produced tensides show outstanding advantages, such a biodegradability and low toxicity, as compared to common synthetic surfactants. Successful applications of biosurfactants were reported for in-situ microbial enhanced oil recovery [28, 29], for the handling of oils spills [30, 31], for the use as food additive [32], for the applications as emulsifiers in agricultural systems [33] and in cosmetics [34].

Basically, there are six major classes of biosurfactants including; i) glycolipids, ii) lipopeptides/lipoproteins, iii) phospholipids, iv) neutral lipids, v) substituted fatty acids and vi) lipopolysaccharides. Structure, function and the physiological role of these biological surface-active agents have already been described [35-37].

The common hydrophobic (lipophilic) moiety in biosurfactants is the hydrocarbon chain of a fatty acid, where as the polar of hydrophilic group is derived from the ester or alcohol functional groups of neutral lipids, from the carboxylate group of fatty acids or amino acids, or in the case of glycolipids, from carbohydrates. When the surfactants are extracellular, they cause the emulsification of the hydrocarbon. When they are cell wall associated, they facilitate the penetration of hydrocarbons to the periplasmic space. Many of the biosurfactants known today have been investigated with a view toward possible technical applications. Because biosurfactants are readily biodegradable and can be produced in large amounts by microorganisms and thus are not dependent on petroleum derived products, they might well be able to replace, in some instances, the traditional synthetic surfactants [38, 39].
Extracellular microbial biosurfactants are usually glycolipids [35], i.e., they contain a sugar moiety as a hydrophilic segment and a fatty acid moiety as a lipophilic one. An alternative to the production of such biosurfactants by fermentation would be the in vitro enzymatic synthesis from the constituent parts. From the economic standpoint, it would be particularly advantageous to prepare them from sugar and vegetable oils both, surplus agricultural products.

Lipases are ubiquitous biocatalysts whose biological function is to catalyze the hydrolysis of triacylglycerol to yield fatty acids, reformation of fats, synthesis of various classes of glycerides and the optical resolution of chemically synthesized racemic acids or alcohols [40, 41]. These catalysts are capable of catalyzing a variety of alternative enzymatic reactions with considerable commercial biotechnological potential including the transesterification reactions in anhydrous non-aqueous systems [42, 43], the interesterification of oils and fats to modify the composition of triglyceride mixture [44], the synthesis of esters with application in the food, cosmetic, drug, detergent, paint and other industries or as surfactants [45, 46, 42] and the synthesis and selective acylation of peptides [47].

The authors have prepared phenolic resin in the laboratory, utilized the resins for immobilization of lipases from bovine and porcine pancreas powder by crosslinking with glutaraldehyde. These immobilized enzymes were used for the production of biosurfactants in dry pyridine between sugar alcohol and various plant oils.

2.3 FLOW INJECTION ASSAY FOR LIPASE

Olive oil is a natural substrate for the assay of lipase [48, 49] requires much time and is insensitive while the synthetic substrates including laurates of phenols are simple and in general fairly sensitive assay systems for lipase.

In the present investigation a FIA procedure for the quantitative determination of lipase is also described. The need to develop this fast assay system arose due to the fact that in our laboratory purification of lipases from various sources is in progress and such fast assay procedures play a vital role in purification procedures. The method is based on
the hydrolysis of p-nitrophenyl laurate by soluble lipase and the rate of formation of p-nitrophenol is measured spectrophotometrically at 405 nm using the following reaction scheme:

\[
\text{NO}_2^{-} \text{O-C-(CH}_2\text{)}_{10}\text{CH}_3 \xrightarrow{\text{Lipase}} \text{NO}_2^{-} \text{O-C-(CH}_2\text{)}_{10}\text{COOH} + \text{H}_3\text{C-(CH}_2\text{)}_{10}\text{OH}
\]

\[p\text{-Nitrophenyl laurate, } p\text{-Nitrophenol, } \lambda_{\text{max}} 405 \text{ nm.}\]

2.4 EXPERIMENTAL

2.4.1 Materials and Methods

DEAE-Sephadex, p-nitrophenyl laurate, crude lipase preparation (type II) from porcine pancreas, lipase from Rugosa Pseudomonas, triolein, olive and soybean oils, sugar alcohol (D-sorbitol), chemically prepared esters of sorbitols and fatty acids like sorbitan monolaurate, monopalmitate, monostearate and monooleate and pyridine from Sigma (St Louis, MO), Sephadex G-100 from Pharmacia (Sweden); electrophoresis reagents and molecular weight markers from Bio-Rad (USA), precoated silica gel plates from Aldrich and silica gel for column chromatography (60 um particle size) from Merck were obtained. All other compounds and solvents used in the present study were of reagent grade.

2.4.2 Lipase Assay

The enzyme was assayed spectrophotometrically using p-nitrophenyl laurate (pNPL) emulsified with polyvinylalcohol (PVA) [50]. An emulsion of pNPL as a substrate was prepared as follows. pNPL solution (20 mM, 1 ml) in acetone was added to a mixture of 8 ml of 0.1 M acetate buffer (pH 3.6) and 4 ml of 1% PVA solution and emulsified. The emulsion was diluted with water to a total volume of 20 ml. The resulting emulsion is stable for 3 days when stored at 4°C.
The assay medium consisting of 2.3 ml of barbital buffer (0.1 M, pH 9.0) and 0.6 ml of substrate emulsion were taken in the cuvette and the solution mixed thoroughly and incubated at 30°C for 5 min. At 0 time 0.1 ml of enzyme solution (from each fraction) was added and the reactants were mixed gently. The progress of the reaction was followed at 405 nm. In the blank the enzyme was replaced by 0.1 ml water.

One unit of lipase activity was defined as the amount of enzyme that liberated 1 µmol of p-nitrophenol per min from pNPL at optimum conditions.

2.4.3 Protein Assay

Protein was estimated by the method of Lowry [51], using bovine serum albumin as a standard.

2.4.4 Precipitation Procedure

The pancreatic tissue was collected from the slaughterhouse immediately after the slaughter of the animals and frozen on ice. The glands were defatted, cut into small pieces and minced. About 800 g of the frozen minced tissue was homogenized in 1500 ml of Tris-Cl buffer [50 mM, pH 8.0 containing 50 µM phenylmethyl-sulphonylfluoride (PMSF) in dry isopropanol] in a Minohum Omni Mixer for 2 - 4 times of 10 s. The concentration level of PMSF was maintained (50 µM) in homogenate solution. The tissue debris was removed by centrifugation at 13000 * g for 30 min at 4°C. An equal volume of acetone chilled to -20°C was added to the centrifuged supernatant and stirred at 300 rpm with the TRI-R overhead stirrer for 10 min at -10°C by sitting the beaker in a salt-ice bath. The protein precipitate was collected by centrifugation at 6000 * g for 5 min and resuspended in 800 ml of chilled acetone and stirring for a further 10 min. The precipitate was filtered through Whatman 42 filter paper under vacuum in a Buchner funnel, dried under vacuum and obtained 49 g of powder, stored at -20°C.

2.4.5 SDS Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in denaturing conditions in the presence of sodium dodecyl sulfate was performed as described [52], using 15% polyacrylamide gel.
Electrophoresis was performed on a vertical slab mini gel apparatus (Model Mini-protean II cell, Bio-Rad, USA). The proteins were stained with Coomassie Brillant Blue R-250.

2.4.6 Phenolic Resins Preparation and Immobilization Procedure

A 0.33 M solution of hydroquinone was prepared in 4.2 M boiling HCl and refluxed. In the refluxing solution 40 ml of aqueous formaldehyde solution (38%) was added dropwise. The resultant brown gelatinous suspension was further refluxed for 180 min with constant stirring before the addition of cold water. Brown gelatinous mass settled, the upper layer of supernatant was removed by filtration, washed with cold water and then the gel was dried under vacuum. This method produced 34 g dried source of the brown coloured resin with active -OH groups on the surface.

The enzymes used in the present study were immobilized on phenolic resins according to established methods [53, 54] by crosslinking with glutaraldehyde. Phenolic resins (4 g) was boiled in 5% HNO₃ for 30 min, washed with water and dried in an oven. The dried resin was derivatized with an aqueous solution of 3-aminopropyltriethoxysilane at pH 3.45 for 150 min at 80°C with constant stirring. The alkylamino phenolic resin was filtered off, washed with water and dried. This silanization process was repeated to ensure maximum activation of the resin and treated with 30 ml of 2.5% glutaraldehyde in phosphate buffer (0.1 M, pH 7.0) for 60 min at room temperature. The resin was washed thoroughly with water and then with phosphate buffer twice.

Crude lipase preparations (1.0 g of porcine pancreas powder and 1.0 g of bovine pancreas powder (lab. isolated)) were separately suspended in 40 ml of phosphate buffer (0.01 M, pH 7.0) with gradual stirring for 30 min at room temperature, centrifuged at 13000 * g for 20 min. The supernatants were dialyzed overnight against the same buffer at 4°C to remove low molecular weight substances and then centrifuged. The enzyme solutions were concentrated by burying the dialysis bag with solution in a jar containing sucrose and kept it in a refrigerator.

Porcine and bovine pancreatic lipases 37500 and 30600 Units were separately immobilized in 2 g aliquots of the derivatized resins and the suspensions were stirred at
4°C for 180 min at 150 rev min⁻¹ and left overnight in a refrigerator. The buffer phase was recovered and the resins were washed with cold water followed by phosphate buffer (0.1 M, pH 6.0), dried under vacuum, stored at 4°C and utilized whenever required. The protein contents of the residues were measured [51] to evaluate the yield of the immobilization procedure.

2.4.7 Procedure for Transesterification

Transesterification between triolein, olive and soybean oils and sugar alcohol catalyzed by immobilized pancreatic lipases were carried out in 100 ml glass flasks separately. Reactions were initiated by adding 250 mg of immobilized lipases into 25 ml pyridine containing 325 mg sugar alcohol (D-sorbitol, 71.3 mmol/l) and 1.8 ml triolein, olive and soybean oils (81.3 mmol/l). The temperature of the reaction system was controlled at 40°C by immersion in a water bath (Kottermann Labortechni, Karl Kolb, Germany) with shaking (150 rev min⁻¹). The reactions were stopped after 48 hrs by filtering the enzyme. The solvent containing the products and un-reacted materials was removed by vacuum evaporation. The residues were first washed with toluene and then dissolved in methanol (10-15 ml). The resultant methanol solution was passed through a column (10 x 150 mm) packed with dry silica gel, eluted with chloroform-methanol-water (64:10:1, v/v/v) at a flow rate of 40 ml h⁻¹. The effluent was concentrated at low temperature (35-40°C) and the products formed were identified by thin layer chromatography using precoated silica gel plates. After developing in the mobile phase chloroform-methanol-water (64:10:1), the plates were dried, sprayed with 2.5% H₂SO₄ in ethanol followed by heating at 60°C for 20 min.

2.4.8 INSTRUMNTATION

The flow injection manifold used for assay of lipase activity is shown in Fig. 2.7, which consists of Ismatec (Reglo 100) peristaltic pump, Model 5020 Rheodyne injection valve equipped with a 90 µl loop and a spectrophotometer (LKB, Novaspec II) with a flow-through cell (30 µl) connected to a chart recorder (Kipp & Zonen, BD
40), Standard size teflon tubings (0.5 mm, i.d.) were used to connect the components of flow injection system.

2.5 RESULTS AND DISCUSSION

2.5.1 Purification and Some Properties of Bovine Pancreatic Lipase

All solvents and buffers used during the purification were supplemented with 10 mM 2-mercaptoethanol. 4.0 g of solvent extracted powder was suspended in 40 ml of Tris-Cl buffer [50 mM, pH 8.0, 4 mM CaCl₂·2H₂O, 40 μl of PMSF (50 μM in dry isopropanol)] and was gently stirred for 90 min at 4°C. An equal volume of PMSF solution was added 2 times during the extraction mentioned above. Insoluble material was removed by centrifugation at 18000 * g for 30 min and the supernatant was precipitated with 60% (NH₄)₂SO₄. The resulting suspension was allowed to stand for 30 min and then was centrifuged at 18000 * g for 10 min at 4°C. The precipitate containing lipase activity was dissolved in a minimum volume of Tris-Cl buffer with 3 further additions of PMSF (15 μl) at 20 min intervals and processed further.

The clear solution obtained was loaded on a DEAE-Sephadex column (2.0 x 25 cm), equilibrated with Tris-Cl buffer (20 mM, pH 8.0, 4 mM CaCl₂·2H₂O and eluted at 4 °C with a flow rate of 50 ml h⁻¹. Fractions of 3 ml were collected measured the absorbance at 280 nm for protein activity and at 405 nm for lipase activity using pNPL emulsified as a substrate. After elution of unbound material without activity, one peak with lipase activity was eluted with a linear gradient of concentration of 0-400 mM NaCl in buffer (Fig. 2.1). The fractions with high activity were pooled and concentrated.

The concentrated solution of lipase was loaded on a Sephadex G-100 column (1.5 x 60 cm), equilibrated with Tris-Cl buffer (20 mM, pH 8.0, containing 4 mM CaCl₂·2H₂O and 400 mM NaCl) and eluted with the same buffer (Fig 2.2). Elution was performed at a flow rate of 20 ml h⁻¹. Fractions of 3.0 ml were collected.

Figure 2.3 shows the gels after SDS-PAGE of crude and purified lipase. The molecular weight was calculated to be 47 kDa with Coomassie staining, with a single
Fig. 2.1: Chromatography on DEAE-Sephadex. Ammonium sulphate precipitated proteins were dissolved in Tris-Cl buffer (20 mM, pH 8.0), loaded on a DEAE-sephadex column (2 x 25 cm) and equilibrated with the same buffer. The flow rate was adjusted to 50 ml h⁻¹ and 3 ml fractions were collected. Bound fraction was eluted with a linear gradient of NaCl (0 - 400 mM). The chromatography was carried out at 4°C.
Fig. 2.2: Chromatography on Sephadex G-100. The active fractions of lipase from the DEAE-sephadex column were pooled, concentrated and loaded on sephadex G-100 column (1.5 x 60 cm), equilibrated with Tris-Cl buffer (20 mM, pH 8.0) containing 400 mM NaCl. The flow rate was adjusted to 20 ml h⁻¹ and 3 ml fractions were collected. The chromatography was carried out at 4°C.
Fig. 2.3: SDS-PAGE of purified lipase from bovine pancreas at different stages. Lanes: 1 & 6: Marker proteins, phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and soybean trypsin (21 kDa); 2: aqueous extract; 3: ammonium sulphate fraction; 4: DEAE-sephadex and 5: gel filtration.
protein band found to be associated with activity. Table 2.1 summarizes the result of the specific activities and yields during the purification of 4 g of acetone powder.

As shown in Fig. 2.4, the effect of pH on the activity of lipase investigated by using Tris-Cl (0.1 M) of varying pH range. The catalytic activity of lipase increases with increasing pH up to 9.0. Above this pH, the pNPL starts self hydrolysis without lipase addition. The optimum temperature for the enzyme reaction was 50°C.

The response of varying concentration of calcium ions was investigated. As shown in Fig. 2.5, the lipase activity rose gradually in pNPL emulsified system as the calcium concentration increased and was maximum at 4.0 mM and on further addition, the conversion of substrate into product was decreased.

The rate of pNPL as a function of substrate concentration up to 3 mM was studied. The plot of the rate of pNPL hydrolysis catalyzed by lipase against substrate concentration showed saturation after 0.25 mM substrate concentration (Fig. 2.6).

The procedure described is very simple, economical in terms of time and cost, as the purification steps are greatly reduced. A single protein band with a molecular weight 47 kDa was found to be associated with lipase activity. This value is similar to that reported for bovine pancreatic lipase [20]. But the specific activity value of 114 U mg⁻¹ in this case is higher than reported previously. The use of PMSF to all precolumn buffers to inhibit serine proteases was necessary as PMSF decays in aqueous solutions and reacts only with active protease. The addition of 2-mercaptoethanol in buffers during the purification was found to increase the yield of the purified enzyme. Its utilization in an immobilized form for the production of biosurfactants will certainly be fastered by this purification and characterization.

2.5.2 Lipase Catalyzed Transesterification between Sugar Alcohol and Oils

2.5.2.1 Yield of the Immobilized enzymes by Cross-linking Procedure

The recovery of porcine and bovine pancreatic lipases incubated with the glutaraldehyde-treated phenolic resins were 43 and 55% respectively. The immobilized enzymes were utilized for about three times without any appreciable change in their
### TABLE 2.1

Purification of Bovine Pancreatic Lipase.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total activity (Units)</th>
<th>Protein (mg)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous extract</td>
<td>17955</td>
<td>1890</td>
<td>9.5</td>
<td>100</td>
</tr>
<tr>
<td>((\text{NH}_4)_2\text{SO}_4) fraction</td>
<td>14445</td>
<td>926</td>
<td>15.6</td>
<td>80</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>7006</td>
<td>113</td>
<td>62.0</td>
<td>39</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>3534</td>
<td>31</td>
<td>114.0</td>
<td>19</td>
</tr>
</tbody>
</table>

*\(\text{p}-\text{nitrophenyl laurate was used as substrate.}\)*
Fig. 2.4: Effect of Tris-Cl on the lipase activity, purified from bovine pancreas.
Fig. 2.5: Effect of calcium ions concentration on the lipase activity.
Fig. 2.6: Effect of p-Nitrophenyl laurate hydrolysis by lipase purified.
activity. The enzymatic activity was completely preserved after three months storage at 4°C.

Transesterification reactions of sugar alcohol with triolein, olive and soybean oils were carried out in dry pyridine using immobilized pancreatic lipases. TLC analysis of the enzymatic reaction products giving a single spot indicated the formation of monoesters of equimolar mixture of sorbitol to oleic acids. 13C NMR studies [55] of these monoesters formed by porcine pancreatic lipase in soluble form revealed that sorbitol monooleates are acylated at C-1 and C-6 position indicating the selective transesterification reaction acylating only on primary hydroxyl groups. Table 2.2, shows the acylation of sorbitol by triolein, olive and soybean oils catalyzed by immobilized pancreatic lipases in pyridine.

Transesterification of carboxylic acid with an alcohol in aqueous medium, catalyzed by lipases has a considerable influence, both on the product and substrate. In non-aqueous solvent as the reaction medium, the situation is completely changed [42]. But sugars are insoluble in many of non-aqueous solvents and are therefore, not feasible to use [56]. An organic solvent like pyridine dissolve sugars and some lipases can easily function as catalysts [57] but have slow reaction rate with porcine pancreatic lipase. However, the enzymatic reaction rate was significantly raised when sugar alcohols were used and are nowadays utilized industrially for the preparation of surfactants by chemical means [58] and therefore used in the present investigation.

Oils are triglycerides of saturated and un-saturated fatty acids [59]. In olive oil the fraction of oleic acid is approximately 83.5% and soybean oil contains oleic acid to about 26%. Porcine pancreatic lipase transfer fatty acid moieties from an oil to a primary hydroxyl group of sorbitol without a striking preference for the nature of the fatty acid and its position in the glycerides, but in the case of bovine pancreatic lipase, the specificity of transesterification reaction has not yet been reported. Although chemical methods for the production of monoesters of sorbitol and fatty acids exist [60], they are not economically feasible, and the corresponding esters are not commercially available. The industrially utilized alternative method [61] is very harsh and involves high temperatures and concentrated H2SO4 as a catalyst; consequently, sorbitol undergoes
**TABLE 2.2**

Sorbitol acylation by triolein, olive and soybean oils catalyzed by immobilized pancreatic lipases in pyridine.

<table>
<thead>
<tr>
<th>Oils</th>
<th>Triolein</th>
<th>Olive oil</th>
<th>Soybean oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of sorbitol (mg)</td>
<td>325</td>
<td>325</td>
<td>325</td>
</tr>
<tr>
<td>Yield obtained of sorbitol monoester (mg) by IPPL</td>
<td>32</td>
<td>25</td>
<td>29</td>
</tr>
<tr>
<td>Yield obtained of sorbitol monoester (mg) by IBPL</td>
<td>74</td>
<td>80</td>
<td>123</td>
</tr>
</tbody>
</table>

*Conditions: 325 mg sorbitol + 1.8 ml of triolein, olive and soybean oils + 25 ml of anhydrous pyridine and 250 mg of immobilized porcine and bovine pancreatic lipases (IPPL and IBPL) added. Suspension shaken (150 rev min⁻¹) at 40°C for 48 hrs and the product recovered and purified as described in procedure for transesterification.*
dehydration before to acylation and the resultant products are sorbitan monoesters. Enzymatically prepared sugar alcohol monoesters possess superior surface-active properties compared to chemically produced sorbitan monoesters. This is most likely due to higher hydrophilicity of sorbitol vs. sorbitan and consequently, a more optimal hydrophilic lipophilic balance value [62].

2.5.3 Emulsified Substrates in a Flow System: The Determination of Lipase Activity

2.5.3.1 Optimization of Flow System

In order to obtain a system with high accuracy, various parameters were studied including, reagents concentration, buffers pH values, temperature, flow rate, and mixing coil length.

Preliminary experiments were carried out to find the pH optimum for the activity of soluble enzyme using Tris-Cl buffers (0.05 M) whose pH ranged from 7.8 - 9.0. The results are shown in Table 2.3. Maximum activity is found at pH 8.6. The effect of Barbital buffer (0.05 M) was also investigated and a maximum activity was found at pH 8.2. But in the present study, Tris-Cl buffer 0.05 M, pH 8.6 was selected and used subsequently.

To select the concentration level of pNPL for hydrolysis, different concentrations (0.1 - 1.5 mM) of it were used. As shown in Table 2.3, the peak height absorbance increased gradually up to 0.9 mM and decreased as the concentration was further increased. The pNPL solution of 0.9 mM was selected for further studies. The effect of emulsifying agent PVA was also investigated using various concentrations from 0.01 - 0.2% (w/v). As shown in Table 2.3, highest absorbance was obtained with 0.15% (w/v) which was selected and used for subsequent investigations.

The temperature dependency of lipase activity on the hydrolysis of pNPL was investigated using circulating water bath at various temperatures around the mixing coil. The results are shown in Table 2.3. There is an increase in response with increase in temperature up to 50°C and on further increase in temperature the hydrolysis of pNPL
Fig. 2.7: FIA manifold for the determination of lipase activity.

A = Mixing coil 30 cm, B = Mixing coil 340 cm, R = Recorder,
W = Waste.
TABLE 2.3

Effect of various parameters on the rate of hydrolysis of p-nitrophenyl laurate by lipase.

<table>
<thead>
<tr>
<th>Tris-Cl buffer (0.1 M)</th>
<th>pH</th>
<th>7.8</th>
<th>8.2</th>
<th>8.6</th>
<th>9.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance*</td>
<td></td>
<td>0.020</td>
<td>0.025</td>
<td>0.03</td>
<td>0.02</td>
</tr>
</tbody>
</table>

| p-nitrophenyl laurate (mM) | 0.30 | 0.60 | 0.90 | 1.20 | 1.50 |
| Absorbance*                | 0.04 | 0.05 | 0.06 | 0.05 | 0.04 |

| Polyvinylalcohol (%)       | 0.01 | 0.05 | 0.10 | 0.15 | 0.20 |
| Absorbance*                | 0.20 | 0.04 | 0.05 | 0.05 | 0.04 |

| Temperature (°C)           | 20   | 30   | 40   | 50   | 60   |
| Absorbance*                | 0.02 | 0.03 | 0.04 | 0.07 | 0.04 |

*Mean of three readings.
was decreased. The temperature of the mixing coil was maintained at 30°C for further optimization.

The effect of flow rate and mixing coil length were also investigated in order to obtain the best overall response of the system in terms of sensitivity and rapidity. The stream of emulsion gave a significant response at a flow rate of 0.1 ml min\(^{-1}\), while the stream of Tris-Cl Buffer (0.05 M, pH 8.6) gave maximum absorbance at a flow rate from 0.1 ml min\(^{-1}\) with gradual decrease in absorbance to 0.32 ml min\(^{-1}\) having effect on sample dispersion, nonsteady baseline, dual peak response and long time consumption (10 - 12 samples h\(^{-1}\)) and therefore a flow rate of 0.64 ml min\(^{-1}\) was used throughout the experiment which gave a steady baseline and single sharp peak. There was a slight gradual increase in peak height absorbance when the mixing coil length was increased from 90 - 440 cm. A mixing coil length of 340 cm was found to be suitable and therefore was selected. The effect of sample loop was also optimized and a sample loop of 90 µl was chosen for all further studies.

2.5.3.2 Calibration Data

A stock solution of lipase (ex. Rugosa Pseudomonas) with an activity of 44.0 U mg\(^{-1}\) was prepared by dissolving 0.7 mg in 1.4 ml of Tris-Cl buffer (0.05 M, pH 8.6). From this stock solution a series of standard solutions covering the range 1.1, 2.2, 3.3, 4.4 and 5.5 U, diluted up to 1 ml with Tris-Cl buffer (0.05 M, pH 8.6) were injected using optimized conditions. The calibration data is given in Table 2.4. The detection limit (2 x blank noise) was 0.1 U ml\(^{-1}\). The sampling rate was 25 h\(^{-1}\), and the rsd for 10 injections of 92.5 U ml\(^{-1}\) was 1.5%.

2.6 CONCLUSION

The purification procedure of lipase from bovine pancreas is simple, economical in terms of times and costs. A single protein band with a molecular weight of 47 kDa which is similar to the reported values but having high specific activity value than reported previously.
**TABLE 2.4**

Calibration data for lipase.

<table>
<thead>
<tr>
<th>(U ml⁻¹)</th>
<th>1.10</th>
<th>2.20</th>
<th>3.30</th>
<th>4.40</th>
<th>5.50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance*</td>
<td>0.016</td>
<td>0.033</td>
<td>0.045</td>
<td>0.057</td>
<td>0.06</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>0.70</td>
<td>0.40</td>
<td>1.30</td>
<td>1.70</td>
<td>2.30</td>
</tr>
</tbody>
</table>

*Mean of three readings.*
Bovine and porcine pancreatic lipases in immobilized form are more economical with the practical standpoint when dry pyridine is utilized as reaction medium for transesterification process. The yield of sorbitol monoester was higher with immobilized bovine pancreatic lipase as compared to the immobilized porcine pancreatic lipase.

The FIA system reported has a reasonable sample throughput flexible and can easily be adopted for the analysis of biological samples. The sensitivity of the assay can be increased by stopping the enzyme with the reagent in the mixing coil for 2 - 4 min. but the sampling rate will be decreased.
REFERENCES


28. E.C. Donaldson and J. B. Clark, Proceedings: Int. Conf. on Microbial Enhancement of oil recovery, May 16- 21, (1982), Shangri-La, Afton, Oklahoma, USA.


SECTION 3

PRODUCTION OF PHOSPHATIDATE BY IMMobilized PHospholipase-D
3 INTRODUCTION

3.1 PHOSPHOLIPIDS

Phospholipids are the constituents of all biological membranes. Due to their superior emulsification properties, phospholipids and their partial hydrolysis products, lysophospholipids, have numerous applications in food, cosmetics, pharmaceutical and other industries [1]. Their properties depend on the fatty acid components and the polar component bound to the glycerol backbone. By changing the hydrophilic/lipophilic balance of phospholipids using lipases or phospholipases, it is possible to produce tailor made lecithins for specific applications.

3.2 PHOSPHOLIPASES

The enzymes catalyzing the deacylation of phospholipids involve phospholipase A₁, A₂ and B and lysophospholipase. These enzymes catalyze the specific hydrolysis of fatty acids ester bonds at position 1 and 2, of 3-sn-phosphoglycerides, diacylphospholipids, monoacylphospholipids or lysophospholipids [2]. Lipases, which have broad substrate specificity are also capable of hydrolyzing fatty acid ester bonds in phospholipids [3].

Fig. 3.1. Site of action of some enzymes acting on phospholipids molecules.
Phospholipase-A₁: A fungal enzyme.

Phospholipase-B: Also acts at this site but its substrate is a lysophospholipid and not a phospholipid.

Phospholipase-A₂: a) This enzyme removes the acyl group at C-2 for prostaglandin and leukotriene synthesis. This enzyme is also present in snake venom. Large amount of lysolecithin is produced after snake bite and acts as a potent haemolytic agent.

b) The enzyme lecithin cholesterol acyl transferase (LCAT) uses this acyl group for esterification of cholesterol.

c) The acyl group at C-2 is mostly unsaturated and prone to oxidative change.

Phospholipase-C: This is a membrane bound enzyme. It is activated by certain hormones, generating inositol-P₃ and diacylglycerol which modify activities of certain enzymes.

Phospholipase-D: Basically a plant enzyme is getting the attention of biotechnologists due to its very interesting applications.

Phospholipase-D (PL-D, EC 3.1.4.4) is an important enzyme of the phospholipids metabolism. It catalyzes the hydrolytic cleavage of the terminal phosphate diester bond of glycerophospholipid containing choline, ethanolamine, serine or glycerol yielding 1, 2 diacylglycerol-sn-3 phosphate (PA) and a free alcohol polar head group. This enzyme very efficiently catalyzes a transphosphatidylation reactions [4-6], which in the presence of primary short chain alcohols, leads to the formation of the corresponding phosphatidylalcohol [7]. PL-D was first described in carrot roots and spinach leaves [8, 9] and has since then been found to exist in many plants [10-14] which are dependent on calcium ions (25-40 mM) [15, 16]. PL-D has also been reported to be present in bacteria, fungi, human placenta and porcine brain and lung [17-22]. In a variety of animal cells and organs, the enzyme has been reported to play an important role in signal transduction cascades and appears to be activated by various hormones, neurotransmitters and some growth factor [23].
Phosphatidic acid is a useful starting material for chemical synthesis of phospholipids [24]. The chemical acylation of sn-glycerol-3-phosphate is the simplest way for preparing desaturated phosphatidic acid species [25]. The most common methods for the preparation of phosphatidic acid containing unsaturated fatty acids are the extraction from wheat germ [15] and the enzymatic degradation of phospholipids [16]. This last reaction is catalyzed by phospholipase-D.

Applications of immobilized enzymes for the synthesis of important compounds is rapidly growing [26, 27]. These enzyme derivatives offer advantages such as repeated and continuous use of the catalyst and the production of compounds in the pure form. In this study we report the immobilization of phospholipase-D on porous glass beads their use in a closed loop system for the production of phosphatidic acid from lecithin.

3.3 EXPERIMENTAL

3.3.1 Materials and Methods

Phospholipase-D (PL-D, EC 3.1.4.4, was isolated from cabbage), controlled porosity glass (CPG, 200 - 400 mesh), 3-aminopropyltriethoxysilane and commercial soybean lecithin were obtained from Fluka Chemicals (Buchs, Switzerland). All other chemicals were analytical grade. For immobilization of the enzyme 100 Units of PL-D were taken per 1.0 g of the CPG [28]. Almost 80% of the enzyme was bound to the support. No deterioration in the enzyme activity was noticed after usage for 6 months. When not in use, the columns were stored at 4°C.

The circulatory system containing the immobilized enzyme column is shown in Fig. 3.1(a). The incubation mixture (4.0 ml was circulated through the immobilized enzyme column) consisted of 1.0 mM suspension of phosphatidylcholine in 20 mM diethylbarbitone buffer (pH 6.5) containing 30 mM calcium chloride and 0.2% Triton X-100. The circulating system was composed of a peristaltic pump connected with the reservoir and the phospholipase-D column (5.0 x 50 mm). The flow rate was maintained at 0.1 ml min⁻¹. All experiments were carried out at 25°C. Aliquots (10 μl) of the suspension were taken at timed intervals from the reservoir for estimating the release of choline from
lecithin. The production of phosphatidic acid formed during the reaction was estimated using 100 µl aliquots of the suspension taken at different time intervals for thin layer chromatography.

3.3.2 Assay Method for Phosphatidic Acid Production from Lecithin

Two approaches were used to assay the production of phosphatidic acid from lecithin.

3.3.2.1 Separation by Thin Layer Chromatography (TLC)

Phosphatidylcholine and phosphatidic acid from circulating solution were extracted according to established method and quantitation of the lipid phosphate was performed accordingly [29], using a two step development of the silica plates (Mackerey & Nagel, Duren, Germany). The plates were developed 9.0 cm with a chloroform/methanol/4.0 M NH₄OH mixture (65:25:4, v/v/v), dried under vacuum and developed in the same direction (8.0 cm) with chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v). The lipid phosphate was determined according to established method [30].

3.3.2.2 Assay of Choline released by Choline Oxidase

The rate of choline released was also used to assess indirectly the production of phosphatidic acid using the immobilized enzyme mini-column in a flow system as described earlier [28].

3.4 RESULTS AND DISCUSSION

3.4.1 Optimization Studies

Phopholipase-D from cabbage is a calcium ion dependent enzyme. Soluble enzyme requires about 30 mM calcium ions [15, 16]. To optimize the calcium ions concentration for the immobilized enzyme, the circulating suspension contained of 1.0 mM phosphatidylcholine, 0.2% Triton X-100, 20 mM diethylbarbitone buffer (pH 6.5) and
1.0 - 50 mM calcium chloride. No detector response was obtained when calcium was omitted, whereas the maximum activity of the PL-D was occurred at about 30 mM calcium ions concentration, which was used in all subsequent experiments.

For the optimization of pH for this bioconversion process diethylbarbitone buffer in the circulating suspension was used in the range 5.5 - 7.5. The maximum activity of the immobilized PL-D was obtained at pH 6.5, as opposed to pH 5.6 for the soluble enzyme. Phosphate buffer could not be used because of the presence of calcium ions. PIPES buffer (Piperazine-N, N-bis[2-ethanesulfonic acid]), which is best in this pH range, strongly depressed the production of phosphatidic acid.

When solubilized PL-D is used to convert phospholipids into phosphatidic acid, diethyl ether is usually added to the incubation mixture in order to increase the interaction between lipid substrate and enzyme. Triton X-100 has also been used for same purpose [31]. The use of Triton X-100 rather than an organic solvent in the present circulating system was preferred as dispersing agent since a peristaltic pump was used to circulate the reservoir contents through the column. Substrate suspensions were prepared in Triton X-100 in the range of 0.1 - 0.6%. The optimal detergent concentration was 0.2% and this was used in all further experiments.

3.4.2 Production of Phosphatidic Acid

The time course of the reaction is shown in Fig. 3.2(a). The conversion of phosphatidylcholine to phosphatidic acid is shown when the progress of the reaction was followed by two different methods, viz enzymic determination of choline released (rsd 1.2% (n = 3)) and also by the determination of phosphate (rsd 2.5% (n = 3)) after TLC separation of phosphatidylcholine and phosphatidic acid. As can be seen the TS 50 (Time required for 50% substrate conversion to products) is about 2 h. Figure 3.2(b) shows the amount of phosphatidic acid formed during the course of reaction, when 100 µl aliquots were separated by TLC.

The residual phosphatidylcholine and phosphatidic acid produced after 3 h of incubation when separated by TLC were also subjected to the analysis of their fatty acid composition. The gas chromatography of the fatty acid methyl esters prepared by
Fig. 3.1 (a): Schematic representation of the circulating system used for the production of phosphatidic acid from lecithin. The percentage conversion of lecithin to phosphatidic acid was calculated both measuring the choline produced (o--o) using the choline oxidase system (not shown) and quantitating the phosphatidic acid formed (■■). (b): Chromatographic analysis on silica gel plates of 100 µl sample of the circulating solution taken after various time intervals. The first development with chloroform/methanol/4 M ammonium chloride (65:25:4, v/v/v) was needed for removing the Triton X-100. The second developing system was chloroform/methanol/acetic acid/water mixture (25:15:4:2, v/v/v/v). Lanes 1 & 7, phosphatidylcholine and phosphatidic acid standards, respectively. Aliquots of the circulating solutions were taken for analysis at 0 incubation time (lane 2), after 30 min (lane 3), 1 h (lane 4), 2 h (lane 5) and 3 h (lane 6).
transesterification with 0.3% methanolic sulphuric acid (4 h at 70°C under N₂ atmosphere) is shown in Table 3.1. It is evident that under the conditions used the immobilized enzyme did not show any selectivity for the unsaturated molecular species. In fact the same fatty acid composition is found in both lipids.

Phospholipase-D from cabbage was immobilized by cross-linking with glutaraldehyde on to porous glass beads and packed in micro-columns for the production of phosphatidic acid. This compound, although the simplest member of the phosphoglyceride family is one of the most difficult phospholipids to prepare. Isolation on a preparative scale is hampered by the low content of this compound in natural sources. Egg lecithin hydrolysis by PL-D appears to be the most convenient procedure for the preparation. Immobilization of the partially purified enzyme and its utilization initially for the production of phosphatidic acid, has the advantages of repeated use of same batch of enzyme preparation and rapid termination of the reaction. This also provides evidence that packed columns of larger dimensions should be suitable for converting phosphatidylcholine into phosphatidic acid on a preparative scale. The other point of interest noted is the non-selectivity of the immobilized enzyme PL-D for the fatty acid groups attached. This will be more helpful in the preparation of phospholipids containing selected fatty acid groups. The same immobilized enzyme system can be used for the transphosphatidylolation of phospholipid.

3.5 CONCLUSIONS

The results obtained using the immobilized choline oxidase and the electrochemical detection of the hydrogen peroxide produced are completely superimposable to those obtained using the conventional phosphate determination, following the TLC separation of phosphatidic acid. Simplicity and rapidity are the advantages of the enzymatic method over the chemical quantitation of the phosphate.
TABLE 3.1.

Fatty acid composition of phosphatidic acid produced by partial hydrolysis of soybean phosphatidylcholine.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Phosphatidylcholine</th>
<th>Phosphatidic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>16 : 0</td>
<td>3.4 ± 0.6</td>
<td>3.3 ± 0.4</td>
</tr>
<tr>
<td>18 : 0</td>
<td>1.2 ± 0.2</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>18 : 1</td>
<td>16.4 ± 1.3</td>
<td>15.3 ± 0.9</td>
</tr>
<tr>
<td>18 : 2</td>
<td>72.1 ± 2.2</td>
<td>74.2 ± 3.1</td>
</tr>
<tr>
<td>18 : 3</td>
<td>6.9 ± 1.6</td>
<td>6.0 ± 3.2</td>
</tr>
</tbody>
</table>

A 1.0 mM solution of phosphatidylcholine (4.0 ml) in diethylbarbitone buffer (pH 6.5) containing 30 mM CaCl₂ and 0.2% Triton X-100 was made to circulate in the manifold shown in Fig. 3.1(a) (flow rate 0.1 ml min⁻¹). After 3 h, phosphatidic acid and residual phosphatidylcholine were isolated chromatographically and their fatty acid composition was evaluated by gas chromatography. Data are expressed as percent in mol.
REFERENCES


SECTION 4

CHEMILUMINESCENT AND SPECTROPHOTOMETRIC DETERMINATION OF GLYCEROL-3-PHOSPHATE AND GLYCEROPHOSPHORYLCHOLINE USING SOLUBLE AND IMMOBILIZED ENZYMES
4. **INTRODUCTION**

In phospholipids, glycerol-3-phosphate is esterified with fatty acids and phosphate is linked to the polar head group (choline, serine etc.). Phospholipases cleave a phospholipid molecule, releasing glycerol-3-phosphate (GP), fatty acids and a polar head group. Glycerophosphorylcholine (GPC, a glycerol molecule with a phosphate at C-3 is linked to choline) is a water soluble phosphodiester derived from hydrolysis of phosphatidylcholine catalyzed by phospho-lipase A₂ and lysophospholipase or phospholipase B [1]. GPC is present in seminal plasma of various mammalian species including man [2], where its role still remains to be determined. It accumulates in epididymal fluids upon conversion of phospholipids derived from plasma lipoprotein by epididymis epithelial cells [3, 4]. GPC is considered a good biochemical index of epididymal function, because epididymal secretion seems to represent 55 to 90% of GPC concentration found in ejaculates from fertile males [5].

Several methods have been reported for the determination of GP and GPC based on precipitation and instrumental techniques [6-8]. An enzymatic batch procedure involves phosphodiesterase hydrolysis of GPC into GP and choline. Choline is then measured spectrophotometrically by choline oxidase method [9]. A similar enzymatic procedure for phospholipids involving immobilized enzyme mini-reactors with flow injection analysis based on amperometric detection system has also described [10]. Chemiluminescence (CL) in conjunction with flow injection analysis (FIA) has the advantages of sensitivity, reproducibility and high sample throughput [11]. The use of immobilized enzymes in FIA-CL technique provides the additional benefits of economy and reliable quantitation of clinical and biochemical substances [12, 13].

In the present study we report two different methods based on FIA-CL and spectrophotometric detections for the estimation of GP and GPC. FIA-CL method involves the reaction of GPC with phospholipase-D releasing GP and choline. GP is, in turn oxidized by glycerol-3-phosphate oxidase into dihydroxyacetone phosphate and
hydrogen peroxide. Luminol under alkaline conditions produces chemiluminescence when catalyzed by Co(II) in the presence of H₂O₂. The enzymatic reaction scheme can be shown as:

\[
\text{PL-D} \\
\text{Glycerophosphorylcholine} + \text{H}_2\text{O} \rightarrow \text{Glycerol-3-phosphate} + \text{Choline}
\]

\[
\text{G-3-PO} \\
\text{Glycerol-3-phosphate} \rightarrow \text{Dihydroxyacetone PO}_4 + \text{H}_2\text{O}_2
\]

\[
\text{Co(II)} \\
2\text{H}_2\text{O}_2 + \text{Luminol} + \text{OH}^- \rightarrow \text{hv} + 3\text{-aminophthalate} + \text{N}_2 + 3\text{H}_2\text{O}.
\]

The spectrophotometric method involves the same enzymatic reaction as mentioned using phospholipase-D and glycerol-3-phosphate oxidase enzymes in soluble form. The enzymatically produced hydrogen peroxide which oxidizes iodide into triiodide measured at 352 nm. The reaction scheme involved is:

\[
\text{Mo(VI)} \\
\text{H}_2\text{O}_2 + 3\text{I}^- + 2\text{H}^+ \rightarrow \text{I}_3 + 2\text{H}_2\text{O}
\]

4.1 EXPERIMENTAL

4.1.1 Materials and Methods

Glycerol-3-phosphate oxidase (G-3-PO; EC 1.1.3.21, ex. Streptococcus thermophilus) solution was prepared by dissolving 2.5 mg (50 Units) in 5 ml of barbital buffer (0.05 M, pH 7.0) or succinate buffer (0.05 M, pH 6.0), phospholipase-D (PL-D; EC 3.1.4.4, ex. streptomycyes species, 250 Units/0.8 mg solid), solution was prepared by dissolving 0.1 mg (31.25 Units) in 5.0 ml of succinate buffer (0.1 M, pH 6.0), stored at 4°C and used whenever required, glycerol-3-Phosphate (disodium salt, hexahydrate), glycero-phosphorylcholine (from egg yolk, free), luminol (5-amino-2,3,-dihydro-1,4-phthalazinedione), glutaraldehyde, controlled-pore glass (CPG, 120-200, pore diameter
116 °A or 200-400, 1280 °A), 3-aminopropyltrietoxysilane were obtained from Sigma Chemical Co., St. Louis, MO. (USA).

GPC stock solution was prepared from a 5 mg/ml GPC solution in methanol by taking an appropriate volume in a tube and evaporating on a rotary evaporator at 40°C. The residue was dissolved in succinate buffer (0.1 M, pH 6.0) to give a final concentration required and the standards were prepared by appropriate dilution with succinate buffer (0.05 M, pH 6.0) containing 30 mM CaCl₂·2H₂O.

GP stock solution (10 mM) was prepared by dissolving 82 mg of GP in 25 ml of succinate buffer (0.1 M, pH 6.0) or barbital buffer (0.05 M, pH 7.0) and the standards were prepared by appropriate dilution with the same buffers.

Luminol and cobalt(II) stock solutions (1 mM) were prepared separately by dissolving 4.43 mg of luminol in 25 ml of carbonate buffer (0.1 M, pH 10.5) and 6.0 mg of cobalt chloride in 25 ml of water. Working standards of each covering the range 1x10⁻⁹ - 1x10⁻⁴ M were prepared by serial dilution of the stock solutions with carbonate buffer (0.1 M, pH 10.5).

Hydrogen peroxide (30%, v/v, 10 mM) solution was prepared by diluting 0.1 ml of H₂O₂ in 100 ml of water and standards were prepared by appropriate dilution. Potassium iodide solution (0.2 M) was prepared by dissolving 3.32 g of KI in 100 ml of water, stored in the dark and prepared freshly after every several days. Ammonium molybdate solution (4.0 mM) was prepared by dissolving 0.247 g of (NH₄)₆Mo₇O₂₄·4H₂O in 50 ml of water. All other chemicals used were AnalaR (E. Merck, Darmstadt., Germany) and deionised/distilled water was used throughout.

4.1.2 Immobilization Procedure

The enzyme used in the present study was immobilized on controlled pore glass (CPG), according to previous procedures [14, 15]. Glycerol-3-phosphate oxidase (80 Units) was immobilized in 0.5 g aliquot of the derivatized glass beads. After the immobilization reaction, the beads were washed twice with 10 ml of phosphate buffer (0.1 M, pH 6.0). The protein content of washings was measured according to the reported method [16], to evaluate the yield of the immobilization on glass beads. The
immobilized enzyme was packed in glass column (2.5 x 30 mm) plugged with glasswool at both ends. The column was washed with a stream of cold phosphate buffer (0.1 M, pH 7.0) and utilized as needed. When not in use, the column was stored at 4°C in succinate buffer (0.1 M, pH 6.0).

4.1.3 INSTRUMENTATION

The flow injection system shown in Fig. 4.1, consists of a peristaltic pump (Ismatec Reglo 100, 4 channel), a rotary injection valve (Rheodyne 5020, 30 µl sample loop) and a purpose-built flow through chemiluminescence detector [17]. The light emission was recorded on a strip chart recorder (Kipp & Zonen BD 40). The immobilized enzyme column was thermostated using a circulating water bath (Erweka, Germany) at 30°C when in operation. Standards sized 0.5 mm i.d. teflon tubing was used to assemble the components.

For the spectrophotometric determination of GP and GPC the absorbance was measured at 352 nm with a Hitachi U-1100 uv/vis spectrophotometer, using 10 mm silica cuvettes. A thermostated water bath was used to maintain the temperature during incubation.

4.2.4 PROCEDURES

4.1.4.1 FIA-CL Procedure for GP

The activity of the immobilized glycerol-3-phosphate oxidase was investigated by incorporating the packed column in the flow injection system shown in Fig. 4.1. A stream of succinate buffer (0.1 M, pH 6.0) was pump through the system at a flow rate of 0.7 ml min⁻¹. Aliquots (30 µl) of the same buffer containing 1 mM GP were allowed to pass through mini-column. Hydrogen peroxide produced was merge at a T-piece with another stream of carbonate buffer (0.1 M, pH 10.5) containing luminol (1X10⁻⁵ M) and cobalt(II) (1x10⁻⁵ M). The two streams were allowed to travel 2.2 cm before passing into a glass coil (1.0 mm i.d. x 100 mm length) placed directly in front
Fig. 4.1: A purpose built flow injection CL analyzer for GP and GPC determination, equipped with peristaltic pump, injection valve, immobilized glycerol-3-phosphate oxidase column, photomultiplier tube enclosed in a light tight housing, power supply and chart recorder.
of an end-window photomultiplier tube (PMT) (Thorn EMI 9789 QB). The PMT output was fed to a strip chart recorder.

4.1.4.2 FIA-CL Procedure for GPC

For the determination of GPC, the column of PL-D was placed prior to glycerol-3-phosphate oxidase column. PL-D requires calcium ions for activity [18], but when used on-line in the buffer stream calcium ions formed a precipitate with carbonate buffer causing blockage of the flow cell, and this system had to be abandoned. The production of GP from GPC catalyzed by PL-D was performed off-line using soluble PL-D and the production of GP was monitored using the manifold described for GP determination.

The off-line production of GP was carried out with PL-D (31.25 Units/5 ml in succinate buffer, 0.1 M, pH 6.0), 50 µl was introduced into a series of tubes containing GPC standards prepared in succinate buffer (0.1 M, pH 6.0, containing 30 mM CaCl₂, 2H₂O). The reaction mixture was incubated at 30°C for 10 min and then injected into the manifold. All other reaction conditions for CL emission were the same as described above.

4.1.4.3 Spectrophotometric Procedure for GP

From each standard solution of GP in barbital buffer (0.05 M, pH 7.0); 0.5 ml was mixed with 0.4 ml of the same buffer and 0.1 ml of glycerol-3-phosphate oxidase solution (50 Units/5 ml) in a tube. The reaction mixture was incubated for selected time periods (e.g. 10 min) in the thermostated water bath at 37°C. The hydrogen peroxide liberated was determined as follows: A 0.5 ml portion of the incubation mixture was mixed with 0.97 ml of barbital buffer, 1.5 ml of potassium iodide solution (0.2 M) and 0.03 ml of ammonium molybdate solution (4 mM). Addition of ammonium molybdate to neutral or buffered iodide solution after the addition of hydrogen peroxide sample catalyzes their reaction and results in rapid conversion of iodide ion to triiodide [19]. The solution was left for 5 min at room temperature and then the absorbance of triiodide was read at 352 nm. In blanks the enzyme was replaced by 0.1 ml of buffer.
4.1.4.4 Spectrophotometric Procedure for GPC

From each standard solution of GPC in succinate buffer (0.05 M, pH 6.0); 0.5 ml was mixed with 0.3 ml of the same buffer containing 30 mM CaCl₂, 2H₂O, 0.1 ml of Phospholipase-D solution (31.25 Units/5 ml) and 0.1 ml of glycerol-3-phosphate oxidase (50 Units/5 ml) in a tube. The reaction mixture was incubated for 10 min at 37°C in a thermostated water bath. Free hydrogen peroxide was determined as described for GP.

4.2 RESULTS AND DISCUSSION

4.2.1 Yield of the Immobilized enzyme by Cross-linking Procedure

In the crosslinking procedure more than 90% of the enzyme incubated with glutaraldehyde-treated beads was covalently bound to the glass. Only 5-9% of the protein incubated with the glutaraldehyde derivatized glass beads was detected in the supernatant after the reaction. The immobilized enzymes packed in glass column was utilized for about 300 h during a 3 month period (stored at 4°C) without any deterioration in their activity.

4.2.2 Optimization of the FIA-CL Manifold

The FIA-CL manifold for GP and GPC was optimized by investigating the effect of various parameters including reagent concentrations, flow rate, pH, and temperature.

The effect of luminol and cobalt(II) concentrations were studied over the range of 1x10⁻⁹ - 1x10⁻⁴ M as shown in Table 4.1. The maximum chemiluminescence (CL) obtained was at 1x10⁻⁵ M of luminol; above this concentration no significant increase in emission was observed. CL signal with respect to cobalt(II) was also studied and the maximum emission found was at a concentration of 1x10⁻⁵ M.

The effect of pH on the activity of immobilized glycerol-3-phosphate oxidase was investigated, using succinate buffer (0.1 M) of various pH values (5.0 - 7.0) as a carrier stream (Table 4.1). The maximum amount of hydrogen peroxide produced enzymatically was observed at pH 6.0 and used in further investigation of conditions.

The effect of flow rate on the CL signal was characterized over the range 0.3 - 2.0 ml min⁻¹. As shown in Table 4.1, the emission increased from 0.3 - 0.7 ml min⁻¹ and further increase in flow rate decreased the CL signal. The optimum flow rate was
**TABLE 4.1.**

Effect of various parameters on the analytical response for the determination of GP and GPC.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1x10^-9</th>
<th>1x10^-8</th>
<th>1x10^-7</th>
<th>1x10^-6</th>
<th>1x10^-5</th>
<th>1x10^-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminol (M)</td>
<td>0.20</td>
<td>1.00</td>
<td>3.00</td>
<td>4.60</td>
<td>7.00</td>
<td>7.50</td>
</tr>
<tr>
<td>intensity* (mV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cobalt(II) (M)</td>
<td>1x10^-9</td>
<td>1x10^-8</td>
<td>1x10^-7</td>
<td>1x10^-6</td>
<td>1x10^-5</td>
<td>1x10^-4</td>
</tr>
<tr>
<td>intensity* (mV)</td>
<td>0.05</td>
<td>0.25</td>
<td>0.55</td>
<td>1.50</td>
<td>2.50</td>
<td>2.70</td>
</tr>
<tr>
<td>Succinate buffer (0.1 M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>5.0</td>
<td>5.5</td>
<td>6.0</td>
<td>6.5</td>
<td>7.0</td>
<td></td>
</tr>
<tr>
<td>intensity* (mV)</td>
<td>0.40</td>
<td>0.90</td>
<td>1.50</td>
<td>1.20</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Flow rate (ml min^-1)</td>
<td>0.3</td>
<td>0.6</td>
<td>0.9</td>
<td>1.2</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>intensity* (mV)</td>
<td>0.50</td>
<td>1.00</td>
<td>0.81</td>
<td>0.53</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>intensity* (mV)</td>
<td>0.92</td>
<td>1.31</td>
<td>1.44</td>
<td>0.75</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>

*Mean of three readings.
0.7 ml min\(^{-1}\) for both channels. The decrease in signal above 0.7 ml min\(^{-1}\) was due to shorter residence time of the substrates within the immobilized enzyme column.

The effect of temperature on the activity of immobilized enzyme was investigated over the range 30-60\(^\circ\)C by pumping water through water jacket around the immobilized enzyme column. As shown in Table 4.1, there was an increase in response with increase in temperature up to 40\(^\circ\)C. The column was maintained at 30 \(^\circ\)C for all subsequent studies.

4.2.3 Calibration Data for GP and GPC

The results obtained from three injections of GP and GPC standards covering the range 1.5x10\(^{-4}\) - 7.5x10\(^{-3}\) M and 2x10\(^{-5}\) - 10x10\(^{-5}\) M under the optimized conditions are shown in Table 4.2. The relative standard deviation is generally < 2\% over the whole range and the detection limits (2 x background signal) are 5x10\(^{-7}\) M and 1x10\(^{-6}\) M respectively. This could be improved further by the use of enhancers for the luminol CL system [20]. The sample throughput was 40 h\(^{-1}\) for each analyte.

4.2.4 Spectrophotometric Determination of GP and GPC

4.2.4.1 Optimization

Three different buffer solutions were examined for the determination of GPC using Phospholipase-D and glycerol-3-phosphate oxidase. Barbital and citrate buffers were not suitable for such combined system giving low absorbances. Succinate buffer (0.05 M) was used in the pH range 5.0 - 6.5. Maximum absorbance is found at pH 6.0 and was used in further studies.

The effect of temperature on the activity of phospholipase-D and glycerol-3-phosphate oxidase was investigated for GPC determination by incubating the reaction mixture in a thermostated water bath for 10 min at various temperatures. The results are shown in Table 4.3. There is an increase in absorbance with an increase in temperature up to 50\(^\circ\)C, however for all subsequent studies reaction mixtures were maintained at 37\(^\circ\)C.
TABLE 4.2.

Calibration data for chemiluminescent determination of GP and GPC.

<table>
<thead>
<tr>
<th></th>
<th>GP</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (x 10(^{-4}) M)</td>
<td>1.50</td>
<td>3.00</td>
<td>4.50</td>
<td>6.00</td>
<td>7.50</td>
</tr>
<tr>
<td>intensity(^\text{*}) (mV)</td>
<td>0.20</td>
<td>0.35</td>
<td>0.45</td>
<td>0.54</td>
<td>0.66</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>2.00</td>
<td>1.00</td>
<td>1.20</td>
<td>2.00</td>
<td>3.41</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>GPC</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (x 10(^{-4}) M)</td>
<td>2.00</td>
<td>4.00</td>
<td>6.00</td>
<td>8.00</td>
<td>10.00</td>
</tr>
<tr>
<td>intensity(^\text{*}) (mV)</td>
<td>0.14</td>
<td>0.30</td>
<td>0.45</td>
<td>0.62</td>
<td>0.80</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>1.42</td>
<td>1.00</td>
<td>2.30</td>
<td>3.00</td>
<td>2.00</td>
</tr>
</tbody>
</table>

\(^\text{*}\)Mean of three readings.
The enzyme catalyzed reaction under optimum conditions in the assay procedure for GPC is shown in Table 4.3. A reaction mixture time of 10 min was optimum and selected for all further studies.

The effect of potassium iodide concentration (0.1-0.3 M) on the process of oxidation of iodide to triiodide was examined (not shown). The highest absorbance was obtained with 0.2 M potassium iodide, which was subsequently used.

4.2.4.2 Calibration Data for Hydrogen peroxide, GP and GPC

Standards solutions of hydrogen peroxide, GP and GPC were treated according to the method described under the procedures (Table 4.4). The calibration graphs shown in Fig. 4.2 and 4.3 are linear covering the range 0.02 - 0.1 mM for hydrogen peroxide and 0.2 - 1.0 mM for GP and GPC. The correlation coefficient for hydrogen peroxide is 0.9993 (n=5) and regression equation \( y = 0.0007x + 2.025 \) \( [y = \text{absorbance}; \ x = \text{concentration (mM)}] \). Similarly the correlation coefficients for GP and GPC are 0.9993 and 0.9991 (n=5 of each) with regression equations \( y = 0.0135x + 0.0445 \) and \( y = 0.027x + 0.003 \) respectively. The limits of detection are lower than \( 2 \times 10^{-6} \) M for GP and GPC, with relative standard deviation generally 1.2% over the range investigated.

4.3 CONCLUSION

The results obtained demonstrate that FIA-CL is an excellent tool for the study of GP and GPC using immobilized enzymes. The technique is economical and has considerable potential for the development of highly selective and sensitive assays of clinical and biochemical analytes. The stability of immobilized enzymes and rate of analysis shows that the method could be applied to real samples. The detection limit for GP \( 5 \times 10^{-7} \) M and \( 1 \times 10^{-6} \) M for GPC is very sensitive as compared with the previously reported results [9].

The described spectrophotometric procedures for hydrogen peroxide, GP and GPC determination are feasible for analysis of these clinically important analytes without using any sophisticated instrument. The detection limits for GP and GPC are comparatively higher than the previously reported method [9].
**TABLE 4.3.**

Effect of temperature and incubation time on the activity of enzymes.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absorbance*</td>
<td>0.02</td>
<td>0.028</td>
<td>0.032</td>
<td>0.036</td>
<td>0.025</td>
</tr>
<tr>
<td>Incubation time</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(min)</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Absorbance*</td>
<td>0.018</td>
<td>0.030</td>
<td>0.037</td>
<td>0.037</td>
<td>0.036</td>
</tr>
</tbody>
</table>

*Mean of three readings.
**TABLE 4.4.**

Calibration data for spectrophotometric determination of hydrogen peroxide, GP and GPC.

<table>
<thead>
<tr>
<th></th>
<th>Hydrogen peroxide</th>
<th>GP</th>
<th>GPC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Conc. (mM)</strong></td>
<td>0.02  0.04  0.06  0.08  0.1</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Absorbance</strong></td>
<td>0.04  0.084 0.123 0.159 0.205</td>
<td>0.09</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>RSD (%)</strong></td>
<td>1.1  0.9  1.2  1.4  1.6</td>
<td>1.5</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*Mean of three readings.*
Fig. 4.2: Calibration graph for hydrogen peroxide.
Fig. 4.3: Calibration graph: (●) glycerol-3-phosphate and (□) glycerophosphorylcholine.
REFERENCES


SECTION 5

CHEMILUMINESCENT AND SPECTROPHOTOMETRIC ASSAYS FOR CHOLINE, PHOSPHOLIPASE-D AND PHOSPHORYLCHOLINE USING IMMOBILIZED ENZYMES
5. **INTRODUCTION**

Phospholipids are important structural components of membranes and have many biological functions in living organisms [1]. Their properties depend on the fatty acid and polar component bound to the glycerol backbone. By changing the hydrophilic/lipophilic balance of phospholipids using lipases and phospholipases, it is possible to produce tailor-made lecithins for specific applications [2]. Furthermore, choline analysis in food products and drugs is of immense importance [3].

The most widely used method for the determination of phospholipids in membranes and in the body fluids involves chromatographic isolation and spectrophotometric determination of organic phosphate [4]. Non-chromatographic methods for determining choline, phosphatidylcholine and sphingomyelin have been introduced using specific enzymes [5-7]. Amperometric methods for choline and PC determination based on immobilized enzymes in flow injection systems have also been described [8-10] with a limit of detection of $5 \times 10^{-5}$ M. The chemiluminescent methods based on batch procedures allow these compounds to be measured at picomolar levels in various biological samples [11-13].

Phosphorylcholine is an important intermediate in the biosynthesis of phosphatidylcholine. The compound is also formed in human body as a result of hydrolysis of lecithin and sphingomyelin by enzymes i.e. lecithinase (PL-C) and sphingomyelinase respectively. Lecithin to sphingomyelin ratio in amniotic fluid is routinely determined in clinical laboratories to assess the fetus lung maturity [14]. Though the determination of phosphorylcholine levels for clinical diagnosis is a very rare test, however, this has attracted much attention as a result of increasing interest in phosphatidylcholine metabolism [3]. The previously published procedures [15] for phosphorylcholine determination involve the use of HPLC which is comparatively slow and the use of sophisticated instrument, generally is not available in most of the laboratories in Pakistan.
In the present study we report two methods using chemiluminescent and spectrophotometric detectors for the determination of choline and phosphorycholine respectively. In the chemiluminescent method a purpose-built flow injection chemiluminescence detector for the determination of choline is described based on the oxidation of luminol by hydrogen peroxide. Hydrogen peroxide is produced by the hydrolysis of choline by using immobilized choline oxidase. Choline is enzymatically produced via cleavage of phosphatidylcholine by phospholipase-D. The sequence of enzymatic and chemiluminescent reactions is as follows:

\[ PL-D \]
\[ \text{Phosphatidylcholine} + \text{H}_2\text{O} \rightarrow \text{Phosphatidic acid} + \text{Choline} \]

\[ C\text{HOx} \]
\[ \text{Choline} + 2\text{O}_2 + \text{H}_2\text{O} \rightarrow \text{Betaine} + 2\text{H}_2\text{O}_2 \]

\[ Co(II) \]
\[ 2\text{H}_2\text{O}_2 + \text{Luminol} + \text{OH}^- \rightarrow \text{Light} + 3\text{-aminophthalate} + \text{N}_2 + 3\text{H}_2\text{O} \]

While the spectrophotometric method based on immobilized alkaline phosphatase column in a flow injection system is developed for the rapid determination of phosphorylcholine by using the following reaction scheme:

\[ Alk. \text{ Phosphatase} \]
\[ \text{Phosphorylcholine} \rightarrow \text{Choline} + \text{Phosphate} \]

\[ \text{Phosphate} + \text{Amm. molybdate} \rightarrow \text{Amm. phosphomolybdate} \]

**reducing agent**
\[ \text{Amm. phosphomolybdate} \rightarrow \text{Molybdenum blue, \( \lambda_{max.} = 660 \text{ nm.} \)} \]
5.1 EXPERIMENTAL

5.1.2 Materials and Methods

Choline oxidase (CHOx, EC 1.1.3.17, ex. alcaligenes species), choline hydroxide, dimyristoyl phosphatidylcholine (PC), Triton X-100, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), glutaraldehyde, controlled pore glass (CPG, 120-200, pore diameter 116 Å), 3-aminopropyltri-ethoxysilane from Sigma Chemical Co., St. Louis, MO,(USA), Alkaline phosphatase (EC 3.1.3.1, ex. from calf intestine, 500 Units/mg) from Boehringer (Mannheim, Germany and phosphorylcholine from Fluka (Buchs, Switzerland) were obtained. All other chemicals used were AnalAR (BDH).

Choline stock solution (1 mM) was prepared from a 45% choline hydroxide in methanol by taking an appropriate volume of choline hydroxide in a tube and evaporated on a rotary evaporator at 40°C. The residue was dissolved in phosphate buffer (0.1 M, pH 8.0). Fresh standards of choline were prepared by appropriate dilution with the same buffer.

Phosphatidylcholine stock solution (10 mM) was prepared from PC isolated from Ox brain or from synthetic dimyristoyl PC by dissolving the required amounts (mg) of PC in 1 ml of ether and evaporated the solvent. The residue was dissolved in the buffer containing 0.2% of Triton X-100 to a final concentration of 10 mM in acetate buffer (0.1 M, pH 5.6) and fresh standards of PC were prepared from stock whenever required.

Luminol and cobalt(II) stock solutions (1 mM) were prepared separately by dissolving appropriate amounts (mg) of luminol and cobalt chloride in 25 ml of carbonate buffer (0.1 M, pH 10.5) and in 25 ml of water respectively. Working standards of each covering the range 1x10⁻⁸ - 1x10⁻⁴ M were prepared by serial dilution of the stock solutions with carbonate buffer (0.1 M, pH 10.5).

Phosphorylcholine stock solution (0.1 M) was prepared by dissolving 3.29 g phosphorylcholine in 100 ml Tris-Cl buffer (0.1 M, pH 9.0) and working standards were prepared by further dilution.
Ammonium molybdate (hepta) solution (0.005 M) was prepared by dissolving 1.54 g of the compound in 250 ml of water containing 3.5 ml of nitric acid (0.2 M). Ascorbic acid solution 0.9% (w/v) was prepared in water containing 1% glycerol (v/v) which prevents the precipitation inside the flow cell.

5.1.2 Immobilization Procedure

Choline oxidase (28 Units) and alkaline phosphatase (200 units) were immobilized separately in 0.5 g aliquot of controlled pore glass (CPG) by crosslinking with glutaraldehyde, following the procedure described previously [8]. The resulting immobilized enzymes were packed in glass columns (2.5 x 30 mm). The supernatant liquid from the immobilization procedure was measured for protein content according to the reported method [16]. More than 80-85% of the enzymes were covalently bound to the glass and only 9-20% of the protein was detected in the supernatant, after the reaction.

5.1.3 INSTRUMENTATION

The flow injection manifolds shown in Fig. 5.1 and 5.3 were constructed with an Ismatec Reglo 100, 4 channel peristaltic pump, a Rheodyne 5020 injection valve (30 μl sample loop), a purpose-built flow through chemiluminescence detector [17] and a spectrophotometer (LKB Novaspec II, Bromma, Sewden) with a flow through cell (30 μl). Analytical readout was obtained on a kipp & Zonen chart recorder. Immobilized enzyme packed glass columns were thermostated at 30°C when in operation and kept at 4°C when not in use. The manifold tubing was standard sized 0.5 mm i.d. teflon.

5.1.4 PROCEDURES

5.1.4.1 Chemiluminescence Procedure for Choline

The activity of the immobilized choline oxidase was investigated by inserting the packed column in the flow injection manifold shown in Fig. 5.1. A stream of phosphate buffer (0.1 M, pH 8.0) was made to flow through the manifold at a flow rate of 0.6 ml min⁻¹. Aliquots of choline (1 mM) prepared in phosphate buffer were injected to pass
Fig. 5.1: FIA-CL analyzer for choline determination.
through packed column. The hydrogen peroxide produced enzymatically was merged at a T-piece with another stream of carbonate buffer (0.1 M, pH 10.5) containing luminal and cobalt(II) (1x10⁻⁴ M) each. The two streams were allowed to travel 2.2 cm before passing through a glass coil (1 mm i.d. x 100 mm length) placed directly in front of end-window photomultiplier tube (PMT, Thorn EMI 9789 QB), and reflective mirror was placed behind the coil. The PMT, glass coil and T-piece were enclosed in a light tight housing. Analytical readout was obtained on a chart recorder. Immobilized enzymes glass columns were thermostated at 30°C by flowing water through water jacket around the enzyme column designed [18] when in operation and kept at 4°C when not in use.

5.1.4.2 Chemiluminescence Procedure for Choline via Phospholipase-D

Phospholipase-D (PL-D) from savoy cabbage was purified by the methods for the transphosphatidyl transfer process [19, 20]. The activity of this purified PL-D was investigated off-line by taking 0.2 ml of PC stock solution (10 mM) in acetate buffer (0.1 M, pH 5.6, containing 0.2% Triton X-100 and 30 mM CaCl₂. 2H₂O), was mixed with 1.7 ml of acetate buffer and 0.1 ml of enzyme solution. The reaction mixture was incubated at 30°C for 30 min. Further increase of the incubation time did not show any improvement in the intensity. Free choline was determined by injecting a 30 μl portion of the incubation mixture into the stream of phosphate buffer consisting immobilized choline oxidase packed column. The hydrogen peroxide produced was measured on-line according to the procedure as described for choline.

5.1.4.3 Spectrophotometric Procedure for phosphorylcholine

The flow injection manifold used for the determination of phosphorylcholine is schematically shown in Fig. 5.3. A peristaltic pump was used to propel the Tris-Cl buffer (0.1 M, pH 9.0) carrier stream at 0.9 ml min⁻¹, ammonium molybdate and reducing agent streams through PTFE tubing (0.8 mm, i.d.) at 0.6 ml min⁻¹. Phosphorylcholine standards (90 ul) were injected into the Tris-Cl buffer carrier stream via a rotary valve, passed through the immobilized alkaline phosphatase column and merged with ammonium molybdate and reducing agent in a 80 cm mixing coil length. The absorbance of
molybdenum blue was monitored at 660 nm using a spectrophotometer with a flow through cell connected to a chart recorder.

5.2 RESULTS AND DISCUSSION

5.2.1 Optimization of FIA-CL manifold

The CL-FIA manifold was optimized by investigating the effect of various parameters by univariate approach as shown in Table 5.1. The effect of luminol and cobalt(II) concentrations were studied over the range of 1×10⁻⁸-1×10⁻⁴ M. The maximum signal obtained was at 1×10⁻⁵ M of luminol; above this concentration no appreciable increase in emission was observed. The CL signal with respect to cobalt(II) was also studied and optimum emission found was at a concentration level of 1×10⁻⁵ M. Further increase in cobalt(II) concentration had little effect on the CL emission because of saturation of luminol with respect to cobalt(II).

The effect of pH on the activity of immobilized enzyme, choline oxidase was studied by using phosphate buffer (0.1 M) of various pH values (7.0 - 9.0) as a carrier stream. The maximum activity was found at pH 8.0 as reported previously [21] and used for further investigation of conditions.

The effect of flow rate on the CL signal was characterized over the range 0.3 - 1.5 ml min⁻¹. The CL emission increased from 0.3 - 0.6 ml min⁻¹ and further increase in flow rate decreased the CL signal. The maximum flow rate obtained was 0.6 ml min⁻¹, due to the suitable residence time of substrate within the immobilized enzyme column.

The effect of temperature on the activity of choline oxidase packed column was studied over the range 30 - 60°C. There was an increase in response with increase in temperature upto 50°C. The column was maintained at 30°C to protect the enzyme from denaturation and to increase the life time of the enzyme column.
### TABLE 5.1

Effect of various parameters on the determination of choline.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1x10⁻⁸</th>
<th>1x10⁻⁷</th>
<th>1x10⁻⁶</th>
<th>1x10⁻⁵</th>
<th>1x10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminol (M) intensity (mV)</td>
<td>1.00</td>
<td>3.00</td>
<td>4.60</td>
<td>7.00</td>
<td>7.50</td>
</tr>
<tr>
<td>Cobalt(II) (M) intensity (mV)</td>
<td>0.25</td>
<td>0.55</td>
<td>1.50</td>
<td>2.50</td>
<td>2.70</td>
</tr>
<tr>
<td>H₂O₂ (M) intensity (mV)</td>
<td>0.25</td>
<td>1.50</td>
<td>2.60</td>
<td>4.10</td>
<td>4.30</td>
</tr>
<tr>
<td>Phosphate buffer (0.1 M) pH intensity (mV)</td>
<td>7.0</td>
<td>7.5</td>
<td>8.0</td>
<td>8.5</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>0.27</td>
<td>0.30</td>
<td>0.25</td>
<td>0.17</td>
</tr>
<tr>
<td>Flow rate (ml min⁻¹) intensity (mV)</td>
<td>0.70</td>
<td>1.10</td>
<td>0.85</td>
<td>0.50</td>
<td>0.30</td>
</tr>
<tr>
<td>Temperature (°C) intensity (mV)</td>
<td>0.92</td>
<td>1.12</td>
<td>1.30</td>
<td>1.40</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*Mean of three injections.
5.2.2. **Calibration Data**

The results obtained from triplicate injections of choline standards covering the range 2 - 10×10⁻⁵ M under the optimized conditions is shown in Fig. 5.2. The relative standard deviation was 1.8-2.8% over the range investigated (Table 5.2) and the limit of detection was 1×10⁻⁷ M, with sampling throughput of 30 h⁻¹. The method was applied to the determination of egg yolk phosphatidylcholine, and the results compared with the analysis carried out by the enzymatic-amperometric detection [8]. The value of PC in egg yolk (% by weight) obtained was 34.2±1.5 (n=6) with this method as compared to 32.4±1.0 (n=6) when analyzed by the enzymatic-amperometric system. The method was also applied to the determination of PC in whole brain extract of male rats. The value obtained by the established method was 4.35±0.20 μmole (CV = 2.80, n = 6), as compared to 4.24±0.14 μmole (CV = 3.30, n = 10) of PC per brain by the enzymatic-amperometric method [9].

Standard solutions of PL-D (lab. isolated from savoy cabbage) covering the range of 0.2 - 1.0 U ml⁻¹ were prepared in acetate buffer (0.1 M, pH 5.6) and treated with PC accordingly. Free choline was injected in the optimized FIA-CL manifold. A linear calibration graph was obtained shown in Fig. 5.2, with relative standard deviation 1.6 -3.0% in the whole range investigated (Table 5.2).

5.2.3 **Spectrophotometric Determination of Phosphorylcholine using Alkaline Phosphatase Reactor**

5.2.3.1 **Optimization of FIA manifold**

Preliminary experiments were carried out to find the optimized pH for the activity of the immobilized enzyme. Phosphorylcholine solution 1×10⁻³ M was prepared in 0.1 M Tris-Cl buffer of varying pH values ranging from 8.0 - 9.5. The peak absorbance obtained at the detector was plotted against pH shown in Fig. 5.4. The immobilized alkaline phosphatase exhibited its highest activity at pH 9.0 and was therefore used subsequently. The optimized pH for soluble alkaline phosphatase is rather 9.8 [22, 23]. The immobilized enzyme packed in glass column was utilized for about 300 h without
Fig. 5.2: Calibration graph for choline and phospholipase-D.
TABLE 5.2

Calibration data for choline and PL-D determination.

<table>
<thead>
<tr>
<th>Choline</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. ( \times 10^{-5} ) M</td>
<td>2.0</td>
<td>4.0</td>
<td>6.0</td>
<td>8.0</td>
<td>10.0</td>
</tr>
<tr>
<td>intensity (^*) (mV)</td>
<td>0.17</td>
<td>0.30</td>
<td>0.47</td>
<td>0.64</td>
<td>0.80</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>1.80</td>
<td>2.70</td>
<td>2.00</td>
<td>2.80</td>
<td>2.40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PL-D</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (U ml(^{-1}))</td>
<td>0.2</td>
<td>0.4</td>
<td>0.6</td>
<td>0.8</td>
<td>1.0</td>
</tr>
<tr>
<td>intensity (^*) (mV)</td>
<td>0.2</td>
<td>0.30</td>
<td>0.57</td>
<td>0.78</td>
<td>1.02</td>
</tr>
<tr>
<td>RSD (%)</td>
<td>1.60</td>
<td>2.24</td>
<td>1.90</td>
<td>2.50</td>
<td>3.00</td>
</tr>
</tbody>
</table>

\(^*\) Mean of three injections.
Fig. 5.3: Flow injection manifold for phosphorylcholine determination, equipped with peristaltic pump, immobilized Alkaline phosphatase reactor, injection valve, mixing coil and spectrophotometer (660 nm).
Fig. 5.4: Effect of pH of the carrier stream (Tris-Cl buffer) on the detector response.
any appreciable change in their activity. The enzymatic activity was completely preserved after three months storage at 4°C.

Other experimental parameters for the manually operated three channel manifold were optimized by univariate approach. The effect of ammonium molybdate and ascorbic acid on the absorbance of the molybdenum blue were examined. The results shown in Fig. 5.5, gave the most suitable response at 5 x 10⁻³ M and 0.9% (w/v) respectively and therefore were used.

The effect of flow rate and mixing coil length were also calibrated in order to obtain the best overall response of the system in terms of speed, and sensitivity. The channel having immobilized enzyme packed reactor (Tris-Cl buffer stream) gave a significant response at a flow rate of 0.9 ml min⁻¹, while in the other two channels maximum absorbance was achieved at a flow rate of 0.6 ml min⁻¹. The mixing coil length of 80 cm which gave the optimum response was used to produce steady baseline. A sample volume of 90 μl was chosen for adequate response and less dispersion.

5.2.3.2 Calibration Data

Standard solutions of phosphorylcholine in Tris-Cl buffer (0.1 M, pH 9.0) were injected in the manifold, to assess its suitability for the quantification of Phosphorylcholine. The calibration data is given in Table 5.3. The detection limit (at S/N = 2) was 10 μM, [RSD 1.8% (n = 3)]. The resulting calibration data had a correlation co-efficient of 0.99 (n = 6). The sampling rate was 45 h⁻¹ and the RSD for 10 injections of 1 x 10⁻³ M Phosphorylcholine was lower than 1.0%.

5.3 CONCLUSION

The use of a purpose-built CL detector in conjunction with a flow injection analysis provides a sensitive and selective procedure for the determination of choline. The limit of detection for choline is improved at least 500 fold compared to the enzymatic-amperometric procedure. The method could easily be applied indirectly for the assay of PL-D in various samples. The immobilized enzyme columns used on-line further added to
Fig. 5.5: Effect of ammonium molybdate (■) and ascorbic acid (●) on molybdenum blue formation.
<table>
<thead>
<tr>
<th>Phosphorylcholine</th>
<th>Conc. (x10⁻⁴ M)</th>
<th>1.00</th>
<th>3.00</th>
<th>6.00</th>
<th>9.00</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absorbance*</td>
<td>0.03</td>
<td>0.06</td>
<td>0.12</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>RSD(%)</td>
<td>0.45</td>
<td>0.40</td>
<td>0.44</td>
<td>0.37</td>
</tr>
</tbody>
</table>

*Mean of three readings.
the stability and reliability of FIA-CL system. The correlation between the two methods was acceptable.

The automated system reported for the determination of phosphorylcholine has a high sampling capacity and is very flexible. The method presented can easily be adopted for the analysis of biological and food samples.
CONCLUSIONS

The work presented in this dissertation explains a facile procedure for enzyme isolation and purification from bovine pancreas, the preparation of phenolic resin and commercially available support such as controlled porosity glass and their use for immobilization of commercial and laboratory isolated enzymes; glycerol-3-phosphate oxidase, choline oxidase, phospholipase-D and lipases and the use of chemiluminescence and spectrophotometric detection techniques in conjunction with flow injection analysis incorporating immobilized enzymes for the assays of clinically important analytes are reviewed and the following findings are made.

1. The present work is a good example of the broader applicability for isolation and purification of enzymes in a couple of steps provided a homogeneous enzyme preparation as confirmed by SDS-PAGE. It is difficult to get the commercial enzymes in the laboratory, because of their high price and denatured form when received. The only remedy is that enzyme be isolated and purified and then utilized for the purpose. In the present study it has been proved, that isolation and purification in the laboratory is the better alternative.

2. Immobilization of enzymes is believed to stabilize the enzyme because of the chemical and physical interaction between enzyme molecule and supporting materials. Clinical chemistry finds important applications of immobilized enzymes. We took this advantage immobilizing various enzymes for hydrolysis and synthesis of important compounds.

3. The commercially available supports for immobilization of enzymes are very expensive which makes the process very expensive especially in large scale. We have attempted to prepare a support (phenolic resin) in the laboratory which has proved to be the excellent in terms of its cost, giving an active product and does not swell on packing.
4. In the present work, the phenolic resin was utilized for immobilization of pancreatic lipase’s by cross-linking with glutaraldehyde and these immobilized lipases were used for the production of biosurfactants in dry pyridine between sugar alcohol and oils. Biological surfactants possess a number of potential advantages over the chemical manufactured counterpart, including low toxicity, biodegradability and ease of synthesis from inexpensive and have applications numerous area, particularly for enhanced oil recovery and in food, beverages, cosmetics and pharmaceutical preparations.

5. Phosphatidic acid is a useful starting material for chemical synthesis of phospholipids. In the present work the immobilization of phospholipase-D on porous glass beads is described, used in a closed loop system, initially for the production of phosphatidic acid from lecithin. The use of immobilized enzymes for the synthesis of important compounds is rapidly growing. The enzyme derivative offer advantages such as repeated and continuous use of the catalyst and the production of compounds in the pure form.

6. The conventional instrumentation for monitoring chemical reactions involving soluble enzymes is a batch procedure. Reagents are added to a cuvette containing the sample is measured spectrophotometrically. This approach is less convenient when using immobilized enzymes and can give poor reproducibility with low detection limits. In the present study various continuous flow manifolds incorporating immobilized enzymes have been reported using flow through chemiluminescence and spectrophotometer detectors. The species analyzed using these continuous flow manifolds include; glycerol-3-phosphate, glycerophosphorylcholine, phosphorylcholine, choline and phospholipase-D activity from cabbage. These methods are attractive in terms of economy and sensitivity. The rapid and reproducible mixing achieved by flow injection manifold presented in the thesis detected clinically important substrates in picomole level.
REFERENCES


FUTURE TRENDS

1. Phospholipase-D also catalyzes the transfer reaction by which the phosphatidic acid moiety is transferred to an acceptor alcohol (transphosphatidylation). We could extend our studies using PL-D from various species in immobilized form and applied it as the transphosphatidylation catalyst of egg yolk phosphatidylcholine with alcohols to produce various phospholipids derivatives in aqueous as well as in organic solvents. Pure products are very difficult and expensive to prepare. Enzyme gave almost 100% of pure product which saves the cost of purification and minimizes loss of expensive raw materials.

2. Glycerol oxidase acts predominantly on glycerol. This enzyme also oxidize dihydroxyacetone to glycerosone but does not oxidize glycerol-3-phosphate. Glycerol oxidase is not available commercially, we could make our attempts to culture and isolate this enzyme from microorganism using established methods of culture, isolation and purification and can be immobilized/coimmobilized on suitable supports for the purpose. The enzyme assay based on the oxidation of glycerol producing hydrogen peroxide which can easily be determined amperometrically as well as by chemiluminescent detection. Glycerol oxidase in combination with lipoprotein lipase could also be employed in immobilized form for the determination of triglyceride in biological samples.

3. The biosynthesis of phosphatidylserine in mammalian tissues has been reported to depend solely on an exchange reaction between exogenous L-serine and endogenous phospholipids present in particulates possessing this activity. This reaction is characterized as being a non-energy requiring and calcium ions stimulated incorporation of bases into their corresponding phospholipids. Serine dehydratase could be isolated and purified from mammalian source and utilized for the assay of phosphatidylserine in combination with phospholipase-D in immobilized form.
4. Chemiluminescence (CL) and more recently electrochemiluminescence (ECL) is very attractive in terms of selectivity, sensitivity, rapidity and economy. The use of CL compounds can further be used to analyze a wide variety of clinically important species. Quantitative analysis based on CL rely on reproducible measurement of light. Flow injection analysis in conjunction with CL can overcome the disadvantages associated with batch procedures. The use of ECL as an analytical technique has got potential covering various aspects. For e.g., FIA-ECL could be used to investigate the analytical potential of tris (2,2-bipyridine) ruthenium (II) [Ru (bpy)$_3$]$_2^+$ ECL system by developing methodologies for the analysis of nicotinamide adenine dinucleotide (NADH) and NAD-NADH converting enzymes dehydrogenases and their substrates.
PUBLICATIONS


