Starch Phosphorylase: An Overview of Biochemical Characterization, Immobilization and Peptide Mapping

Ritu Jain[1], Sarika Garg[2] and Anil Kumar[1*]

[1]*School of Biotechnology, Devi Ahilya University, Khandwa Road, Indore-452001, India.*
[2]*Department of Neuroscience, The University of Montreal Hospital Research Centre (CRCHUM),
Bureau/Office: CRCHUM R09.476, Tour Viger, 900, rue St-Denis, Montréal, Québec H2X 0A9,
Canada.*

Authors' contributions

This work was carried out in collaboration between all authors. Authors RJ and SG have equal contribution in this manuscript. Author RJ prepared the first draft of the manuscript. Authors RJ and SG both searched literature. The manuscript has been finalized by authors SG and AK. Author AK also designed the study and managed the analyses of the study. All authors read and approved the final manuscript.

Review Article

ABSTRACT

Starch phosphorylase (EC 2.4.1.1) is the key enzyme which catalyzes the reversible conversion of starch and inorganic phosphate into glucose-1-phosphate (G-1-P) and has been reported in many higher plants. It can be exploited for industrial purposes for tailoring starch and for the synthesis of biopolymers which may be useful in food industry and are also of clinical importance. Starch in the form of insoluble granules accumulates in chloroplasts as the primary product of photosynthesis. Multiple forms of the enzyme have been reported in different plant tissues which have been predicted to have different roles in starch metabolism. Here, various biochemical properties have

Corresponding author: Email: ak_sbt@yahoo.com;

been reviewed. Various techniques for enzyme immobilization have been discussed. Besides, reports on immobilization of starch phosphorylase from different sources and on different solid matrices have also been reviewed. Peptide mapping reports of various proteins have also been assessed in this review.

1. INTRODUCTION

The glucans are the polysaccharides which have monomers of D-glucose linked by glycosidic bond in their structure. There are two types of glucans- α and β. The α – glucans include dextran, glycogen, starch and pullulan and β-glucans include cellulose, curdlan, laminarin, chrysolaminarin, lentinan, pleiran and zymosan. The family Glycosyl transferases, is the largest family of the enzymes of carbohydrate metabolism. Polyglucans (starch and glycogen) are the main source of carbon and energy for many organisms and α-glucan phosphorylase plays an important role in glucan metabolism and has wide distribution in plants, animals, and prokaryotes. Its mode of action is degrading polyglucans by phosphorolysis and is mostly active in the homodimeric form with vitamin cofactor, pyridoxal 5'-phosphate [1,2]. Starch acts as primary energy source or reserve in higher plants. Starch in the form of insoluble granules accumulates in chloroplasts as the primary product of photosynthesis.

Amylose and Amylopectin are the basic components of starch (Fig. 1). ADP-glucose pyrophosphorylase, starch synthase and branching enzyme play a major role in starch synthesis. Amyloplast is the organelle in plant storage cells/tissues where starch is synthesized and deposited. Biosynthesis of starch or starch like polymers in plants with functional properties can be obtained by using different techniques like knockout, antisense RNA techniques and by expressing heterologous genes [3]. In developing seeds, starch production takes place and this starch acts as a carbon reservoir or storage for long term usage [4]. At the simplest level, the process of starch degradation requires an initial hydrolytic attack on the intact starch granule followed by de-branching (hydrolysis) of α - (1, 6)-linkages to produce linear glucan chains, and finally, the degradation of the linear chains to glucosyl monomers. There is a range of plastidial enzymes with starch-degrading capabilities which may participate in the process of starch degradation and turnover. The α -amylase (EC

3.2.1.1) and other endo-amylases hydrolytically cleave α -(1-4)-glucosyl bonds resulting in the production of a mixture of linear and branched moieties and, ultimately, glucose, maltose, malto-triose, and a range of branched α- limit dextrins. In addition, β-amylase (EC 3.2.1.2) catalyzes the hydrolysis and removal of successive maltose units from the non-reducing end of α - glucan chain. Alternatively, α- (1, 4) - glucosyl bonds may be cleaved phosphorolytically by starch phosphorylase to produce glucose-1-phosphate from successive glucosyl residues at the non-reducing end of the α - glucan chain.

Tetlow et al. [4] reported the way of process of starch degradation as depicted in Fig. 2.

2. STARCH PHOSPHORYLASES AND STARCH METABOLISM

Starch phosphorylase is an important enzyme for future studies and research. Starch phosphorylase (EC 2.4.1.1) catalyzes the reversible conversion of starch and inorganic phosphate into glucose-1-phosphate (G-1-P) and has been reported in many higher plants [5,6]. Glucose-1-phosphate can be used as an antibiotic in circulatory system therapy and in cardio-pathy therapy as it acts as a cytostatic compound [7].

The reaction can be described by the following equation:

$$(\alpha\text{-1, 4-D-glucosyl})_n + \text{Pi} \leftrightarrow (\alpha\text{-1,4-D-glucosyl})_{n-1} + \text{G- 1- P}$$
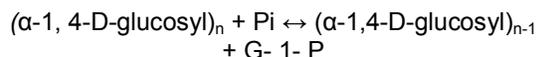
In plants, starch phosphorylase has been found in both cytosolic and chloroplastic forms. Starch phosphorylase has been found to be widely distributed in plant kingdom. It has been studied from pea seeds and leaves [8], potato tubers [9], maize [10], barley [11], germinating pea seeds [12], spinach [13], sweet potato [14], banana leaves [15], seaweed [16] and sweet corn [17]. It has also been studied from number of other plants. Role of starch phosphorylase has been

described to be in starch degradation. The starch phosphorylase present in plastids is responsible for starch degradation due to the low glucose-1-phosphate and high Pi/g-1-p ratio found *in vivo* [1,18]. Contrarily, few scientists have described role of starch phosphorylase in primer synthesis which is used in starch synthase reaction [4,19-22]. Earlier, our laboratory illustrated the possible roles of phosphorylase in plants in starch metabolism. In plastid, starch granule may be attacked by glucan water dikinase, phosphoglucan water dikinase and α-amylase to form branched glucan and thereafter branched glucan is attacked by starch debranching enzyme to form linear glucan. The linear glucan is attacked by Pho1 to form glucose-1-phosphate which is either used in degradation route to form triose phosphate and triose phosphate comes out in the cytoplasm or linear glucan is acted upon by β-amylase to form maltose which is transported in the cytoplasm and there, it may be used in the formation of heteroglycan using disproportionating enzymes. The heteroglycan in the cytoplasm is degraded by Pho2 to form glucose-1-phosphate [23]. Rathore et al. [23] also described the role of Pho1 and Pho2 in the formation and degradation of glucose-1-phosphate in plastid and cytoplasm, respectively and also reported synthetic and degradative pathway/route in plastid for glucose -1-phosphate. In plastid, Pho 1 is responsible for glucose-1-phosphate formation from linear glucan as a substrate and this glucose-1-phosphate can be utilized in degradation route to form triose phosphate or can be utilized in synthetic route to form starch stored in starch granules with the help of starch synthase, starch debranching enzyme and ADP-glucose pyrophosphorylase enzymes. Rathore et al. [23] also proposed that maltose sugar in plastid can be produced from linear glucans by the action of β amylase.
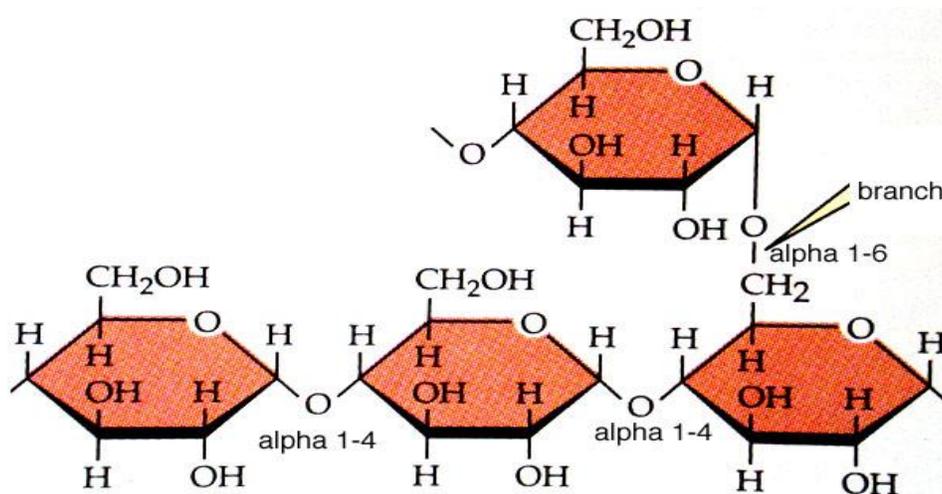
Fig. 1. Structure of starch (polymer of α-D- glucose)- Amylose, a constituent of starch has α-1,4 glycosidic bonds whereas, amylopectin, another constituent of starch has both α- 1, 4 glycosidic bonds and α- 1, 6 glycosidic bonds (source:http://academic.brooklyn.cuny.edu/biology/bio4fv/page/starch.html)
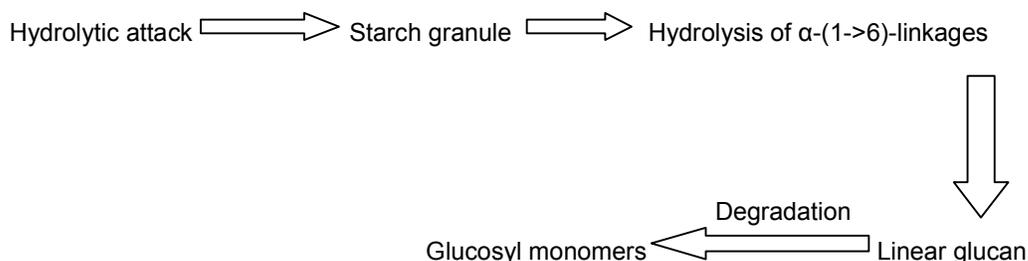
Fig. 2. The process of starch degradation in higher plants (Tetlow et al. [4])

Presence of multiple forms of starch phosphorylase has been reported in number of plant tissues. Post translational modifications viz. glycosylation, proteolytic modification and aggregation may be the reasons for multiplicity of starch phosphorylase [24]. In spite of multiple forms, reaction catalyzed by starch phosphorylase is same reflecting the characteristic feature of all starch degrading enzymes [23,25,26]. Synthesis of substrate starch for ADP glucose starch synthase has been favored by some multiple forms of starch phosphorylase [27]. In spinach leaves, multiple forms of pho1 in chloroplast and pho2 in cytoplasm have been reported and comparison has been done with respect to glycogen as substrate. As compared to Pho1, Pho2 has high affinity for glycogen [13].

Tsai and Nelson [10] reported different multiple forms pattern of starch phosphorylase during different developmental stages in maize endosperm and different multiple forms were assigned to have role in starch synthesis and degradation. Singh and Sanwal [28] reported presence of multiple forms in banana fruit pulp and showed role of different multiple forms in starch synthesis and degradation. Gerbrandy and Verleur [29] reported presence of multiple forms in growing and sprouting potato tubers and assigned role of different multiple forms in starch synthesis and degradation. However, it is considered that it has much limited role in degradation of starch *in vivo* since starches as well as starch phosphorylase both are impermeable to plastidial membrane. It is well known that starch synthesis occurs inside the plastid [23]. It has been shown that extra-plastidial enzymes are involved in novel glucan metabolism in the cytoplasm [30,31]. From so many studies, now it may be said that presence of multiple forms of starch phosphorylase is of physiological importance. Different multiple forms have different physiological functions. Intracellular localization of multiple forms also varies; a few are present in the cytoplasm and others in the plastids. It is well known that starch is not present in the cytoplasm and the multiple forms and starch phosphorylase as well as starch are not permeable through plastidial membrane. Also, starch is not known as a physiological substrate of cytoplasmic starch phosphorylase. More studies are required to unequivocally prove that heteroglycan present in the cytoplasm is the physiological substrate as has been reported by Fettke et al. [31].

Starch phosphorylase is regulated by pH, temperature, redox potential, oligosaccharides level and covalent modification [32,33]. Potato phosphorylase is stable at 30ºC at pH 6 to 8 [34]. Lee and Kamogawa et al. [34,35] reported the inactivation of potato's starch phosphorylase at a temperature above 55ºC.

3. STUDY OF STARCH PHOSPHORY-LASE IN DIFFERENT SOURCES

Mateyka and Schnarrenberger [36] compared starch phosphorylases of spinach leaves and corn leaves and reported that the two multiple forms of corn leaf phosphorylase are homologous to the spinach phosphorylase I and II. They reported that in corn phosphorylase, one multiple form having homology with spinach phosphorylase I is present in the cytoplasm of mesophyll cells whereas the other multiple form having homology with spinach phosphorylase II is present in the chloroplasts of bundle sheath cells. They reported that affinity of corn leaves starch phosphorylase and spinach starch phosphorylase I for phosphate and soluble starch is same, whereas affinity of corn leaves phosphorylase for glycogen is 15 times more compared to spinach phosphorylase [37,38].

Hsu et al. [39] purified and characterized the cytosolic form of starch phosphorylase from etiolated rice seedlings. Pho1 is inactivated by cyclodextrins and maltotetrose. However, thiol-reagents increased enzyme activity by 10-20% and metal ions viz. Zn^{+2}, Hg^{+2}, and Ag^+ reduced the enzyme activity. They also reported that there is no effect of sugar phosphate on Pho 1 enzyme activity in the absence of g- 1-p. Alignment was also done of partially sequenced enzyme purified from rice seedling with the peptide sequence of Pho 1 of *Oryza sativa* and *Triticum aestivum* and enzyme matched with four peptide sequences as shown in the Table 1

Witt and Sauter [40] reported partial purification of amylase and starch phosphorylase from poplar wood. They wanted to separate the multiple forms of starch degrading enzymes that were detected in extracts from poplar wood on native electrophoresis gels. They reported that endoamylase from poplar wood is not dependent on Ca^{2+} unlike other plant endoamylases. They also reported two multiple forms of starch phosphorylase from poplar wood, one present in the cytosol (type I) and it was retarded by immobilized amylopectin in acrylamide gels and it does not attack native starch granules, and the

other form present in the amyloplast (type II) which was eluted at higher salt concentration from the anion exchanger and was hardly retarded on amylopectin containing gels. Weinhausel et al. [41] reported the isolation and characterization of starch phosphorylase from *Corynebacterium callunae*, a gram positive bacteria. This glucan phosphorylase depends on pyridoxal 5-phosphate for enzyme activity and has an important role in carbohydrate metabolism in bacteria. Glucan phosphorylase from *C. callunae* is induced by maltose. The enzyme from *C. callunae* is not catabolically repressed by D-glucose unlike glycogen phosphorylase from *Escherichia coli* which is controlled by catabolite repression. They also reported biochemical properties like effect of pH and temperature on the enzyme activity, cofactor analysis, effect of metabolites etc. They also determined the N-terminal sequence (Pro-Glu-Lys-Gln-Pro-Leu-Pro-Ala-Ala-Leu) and total amino acid composition of α- glucan phosphorylase. They reported that N-terminal region is entirely different compared to other glucan phosphorylases. However, total amino acid composition was similar to the enzyme isolated from *E. coli*, *Solanum tuberosum* and rabbit muscle. They explained the contribution of substrate chain length to binding energy by assuming two glucan binding sites, an oligosaccharide binding site composed of five subsites and a high affinity polysaccharide site separated from the active site. They gave a structural model of the molecular shape obtained with the help of small angle X-ray scattering. They found that model of *C. callunae* phosphorylase differs from rabbit muscle phosphorylase in size and axial dimension. They showed no inhibition of *C. callunae* phosphorylase by glucose, nucleotides, caffeine which distinguishes it from other regulated phosphorylases. All the α-glucan phosphorylases including glycogen phosphorylase from rabbit muscle, potato phosphorylase*, E. coli* glycogen phosphorylase have been reported to have pyridoxal 5'-phosphate (PLP) as a cofactor. Removal of PLP causes loss of the enzyme activity which may be restored on incubation of the enzyme with an excess of PLP. Reduction of the enzyme with sodium borohydride (NaBH$_4$) resulted in a material which is still 60% enzymatically active. However, all other PLP containing enzymes where PLP is involved in catalysis are totally inactivated by reduction with NaBH$_4$. It binds as an imine through its C4 formyl group to an epsilon amino group of a lysine residue. There are reports where PLP has been shown to be involved in structural maintenance and/or acts as allosteric effector molecule [42]. Kumar and Sanwal [43-45] found absence of PLP in case of banana leaf and tapioca leaf phosphorylases. Kumar [42] reviewed role of PLP in phosphorylases in length.

Characterization of plastidial starch phosphorylase isolated from *Triticum aestivum* L. endosperm has been done by Tickle et al. [46]. They observed a single band of Pho activity using zymogram analysis in wheat endosperm crude extract and also at each stage of development and intensity of this band increased throughout the development stage until approximately to that 18 dpa. They studied the role of amyloplast localized starch phosphorylase isoform in starch synthesis in the developing wheat endosperm. Hukman et al. [47] showed that in endosperm, starch accumulation is highest between 10 to 30 dpa. Based on the results, they concluded that throughout this period, Pho 1 protein and activity in the endosperm support the role in synthesis of starch for Pho1. They presented the direct correlation between the expression of Pho 1 isoform in wheat endosperm and starch synthesis.

Kumar and Sanwal [48] reported that banana leaf phosphorylase amino acid composition is different from rabbit and potato phosphorylase, as they are poor in acidic and aromatic amino acids but enriched in basic and heterocyclic amino acids contents. Although starch phosphorylase is being studied since last more than 30 years, it has a special space in research area and now direction of research has changed from its purification and characterization to its immobilization, structural studies and its bioinformatics analysis. Precise methods for separation and purification of phosphorylases are required. Till date, people have concentrated on starch as a substrate for cytoplasmic phosphorylase despite the fact that starch is not present in the cytoplasm. Therefore, there is a strong need of characterization of the enzyme from a different angle.

Table 1. Matched amino acid sequences of Pho1 of *Oryza sativa and Triticum aestivum*

Significant hit	Species	Accession number	Match amino acid sequence
Pho1	*Oryza sativa*	gi|12025466	EGQEEIAEDWLEK TDQWTSNLDLLTGLR QLLNILGAVYR SGAFGTYDYAPLLDSLEGNSGFGR
Pho1	*Triticum aestivum*	Q9LKJ3	TDQWTSNL DLLTGLR

4. EXTRACTION OF STARCH PHOSPHORYLASE FROM DIFFERENT PLANT SOURCES

Purification of starch phosphorylase from young banana leaves was done long time [44]. The enzyme was extracted from young leaves by grinding in a waring blender and buffer employed was 0.01 M Tris-HCl, pH7.5 containing 0.02 M 2-mercaptoethanol and 0.05% Triton x-100. The enzyme has also been isolated from spinach leaves using 100 mM imidazole-HCl buffer, pH 7.0 containing 0.5% polyvinylpyrrolidone, 20 mm 2-mercaptoethanol and 0.1mM phenyl methyl sulfonyl fluoride (PMSF) [37]. The same buffer has been used for the extraction of the enzyme from corn (*Zea mays* L.) [36]. The HEPES-NaOH buffer (100 mM, pH 7.0) containing 10% glycerol, 1 mM EDTA, 1 mM dithioerythritol (DTE) and 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) have been used for extraction of the enzyme from spinach [49]. The enzyme has been isolated from poplar wood by using 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$ and 10 mM 2-mercaptoethanol [40]. The enzyme has also been isolated from etiolated rice seedlings by using 50 mM imidazole-HCl buffer, pH 7.0 having 1 mM PMSF, 1 mM benzamidine, 1 mM EDTA and 1 mM DTT [39]. Cabbage leaves starch phosphorylase has been extracted using 0.01 M Tris-HCl buffer, pH 7.5 containing 20 mM 2-mercaptoethanol and 50 mM EDTA [50]. The enzyme from wheat endosperm has been extracted using 100 mM Tris- HCl buffer, pH 7.5 [46]. The enzyme from potato tuber has been extracted using extraction buffer containing 100 mM HEPES buffer with 5 mM EDTA, 5 mM dithiothreitol, 0.2% (w/v) polyvinylpyrrolidone and 0.5% (w/v) bovine serum albumin [9]. Shatters and West [51] reported extraction of starch phosphorylase from the leaves of *Digitaria eriantha* using LCS buffer containing 0.33 mM sorbitol, 50 mM Tricine-NaOH, pH 8.0, 2 mM $NaNo_3$, 2 mM EDTA, 1 mM $MnCl_2$, 5 mM $MgCl_2$ and 5 mM K_2HPO_4. Srivastava et al. [52] reported extraction of starch phosphorylase from *Cuscuta reflexa* filaments using 0.05 M Tris – HCl, pH 7.6 containing 0.02 M 2-mercaptoethanol and 0.2% Triton X-100. Garg and Kumar [53] reported isolation of starch phosphorylase from seeds of Indian millet using 50 mM Tris – HCl buffer, pH 7.6 containing 10 mM 2- mercaptoethanol, 20 mM EDTA and 1% Triton –X -100. Kumar and Sanwal) [45] reported extraction of starch phosphorylase from tapioca leaves using 0.01 M Tris-HCl buffer , pH 7.5, 0.02 M 2- mercaptoethanol and 0.2% Triton X-100. Venkaiah and Kumar [54] reported extraction of the enzyme from sorghum leaves and also reported presence of multiple forms. They extracted the enzyme from leaves by using 0.05 M Tris HCl buffer, pH 7.6 containing 0.02 M EDTA, 0.02 M 2-mercaptoethanol, 1 % Trition-X-100 and 1 mM PMSF. Extraction of starch phosphorylase from germinating Bengal gram seeds has also been done using 50 mM Tris-HCl buffer, pH 7.6 containing 20 mM 2-mercaptoethanol, 10 mM EDTA and 1% Tritin-X-100 [55]. From the studies, it is clear that isolation medium of plant starch phosphorylases is more complex. In most of the cases, a non-ionic detergent, Triton-X-100 has been used which protects the enzyme from phenolics by dissociating protein quinone complex. Besides, it also ruptures the membranes of the organelles including that of plastids where one multiple form is localized. Depending on the stiffness of the tissue, grinding technique is selected. Most often and not, Waring blender is used, while some people also make use of pestle and mortar.

5. IMMOBILIZATION OF STARCH PHOSPHORYLASE

The term "Immobilized enzyme" refers to "enzyme physically confined or localized in a certain defined region of space with retention of catalytic activity and can be used repeatedly and continuously. The system of immobilization is consisted of the enzyme to be immobilized, the matrix for the support and the mode of attachment viz. physical adsorption, ionic linkages or through covalent bonding [56]. There are various methods of immobilization of enzymes summarized in Fig. 3.

An immobilized enzyme should have two essential functions namely catalytic function (CF) and non-catalytic function (NCF). Role of catalytic function is to drive the substrate conversion into the product within the given time and space and role of non-catalytic function is to make easy separation of the catalyst from the process, its reusability and process control [57]. Tischer and Kasche [58] reported some specific parameters of immobilized enzymes mentioned in Table 2.

Interaction of the enzyme and properties of carrier material control the characteristic features of the immobilized enzyme. After immobilization, measurement must be done for the yield of the enzyme activity, its efficiency, and operational stability in stirred tank fermenter and in number of cycles. With these data, one can determine the performance that is the product formed per unit enzyme or the amount of the enzyme required per unit product formed [59]. For industrial use of an immobilized enzyme, all the above mentioned parameters should be considered to make the process economically satisfied.

There are many advantages and disadvantages of immobilized enzymes over soluble enzymes (described in Table 3) [60].

Sheldon [61] described various techniques of enzyme immobilization. He critically reviewed various methods of immobilization especially in context of industrial processes particularly for use in non-aqueous media. He also emphasized on the use of novel supports viz. hydrogels, mesophorous silicas and smart polymers.

Immobilization of starch phosphorylase from different sources has been carried out on various matrices resulting in change of its physico-chemical properties. Starch phosphorylase from seeds of Indian Millet variety KB 560 has been immobilized on brick dust [53]. They reported the various characteristic features of immobilized enzyme with respect to soluble enzyme, like immobilized enzyme showed 3.5 fold purification compared to soluble enzyme, the temperature range was also different for both the enzymes. The optimum temperature for the enzyme activity has been found to be 37ºC with two half temperature optima at 34ºC and 40ºC, while half life of immobilized enzyme was 9 hours at 30ºC and 1 hour at 50ºC. In case of soluble enzyme the half life was 5 hours at 30ºC where as 30 minutes at 50ºC. They also reported that the

enzyme can be reused many times and can be useful for glucose-1-phosphate production Kumar and Sanwal [62] reported immobilization of starch phosphorylase from mature banana leaves on polyacrylamide matrix. Immobilized enzyme retained about 80% activity and was more stable and could be reused several times. Garg and Kumar [50] immobilized starch phosphorylase from cabbage leaves for production of glucose -1-phosphate. They immobilized partially purified enzyme on hen egg shell and two fold purification was shown on immobilization compared to soluble enzyme and retention of enzyme activity upon immobilization was nearly 56%. Venkaiah and Kumar [63] immobilized starch phosphorylase from sorghum leaves using egg shell as solid support and showed retention of 46% enzyme activity. Srivastava et al. [64] reported immobilization of starch phosphorylase from *Cuscuta reflexa* on egg shell and reported retention of 50% enzyme activity upon immobilization. They reported that after ion exchange column chromatography, 95% pure glucose-1-phosphate was obtained as tested using phosphoglucomutase and glucose -6-phosphate coupled enzyme assay. Conrath et al. [65] designed an enzyme electrode using immobilized acid phosphatase, glucose oxidase, mutarotase and phosphorylase with bovine serum albumin by glutaraldehyde cross linked dialysis membrane. It has been observed that after immobilization, there is increase in the specific activity of phosphorylase which is due to selective binding of phosphorylase and some other proteins on the solid supports. It is pertinent to mention that in most of the cases partially purified phosphorylase has been used for immobilization from the point of economics for production of glucose-1-phosphate. In most cases, there is increased optimum temperature and thermo-stability for the immobilized enzyme compared to soluble enzyme which may be exploited for industrial use. The increased thermo-stability and optimum temperature have been explained due to change in the conformation of the enzyme after immobilization.

There are number of other reports on immobilization of enzymes and microbial cells of commercial importance. Kourkoutas et al. [66] reviewed various immobilization technologies and matrices used in alcohol beverages. They described basic methods of cell immobilization and effect of immobilization on microbial cells. They also described various advantages of immobilized cells over free cell systems viz. prolonged activity and stability of biocatalyst,

higher cell densities per unit bioreactor volume, increased substrate uptake and yield improvement, feasibility of continuous processing, increased tolerance to high substrate concentration and reduced end product inhibition, feasibility of low temperature fermentation leading to improved product quality, easier product recovery, regeneration and reuse of biocatalyst, reduced risk of microbial contamination, reduced maturation time for some products. Bakoyianis et al. [67,68] described various matrices used for immobilization of cells for wine making viz. inorganic supports namely mineral kissiris, a cheap, porous volcanic mineral found in Greece containing nearly 70% SiO_2, and organic supports namely alginates, cellulose, carrageenan, agar, pectic acid and chitosan.

Loukatos et al. [69] used porous g-alumina as support for cell immobilization for use in continuous wine making. Ogbonna et al. [70] used glass pellets covered with membrane of alginate as support for immobilization of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* cells and used

immobilized cells for making wine. Otsuka [71] used cellulose covered with calcium alginate as support for immobilization of yeast and showed wine preparation using immobilized yeast. Lommi and Advenainen [72] used DEAE cellulose covered with an anion exchange resin for immobilization of yeast. Besides, enzymes have been immobilized on different solid supports for enhancing their activity and reusability.

Datta et al. [73] gave an overview of enzyme immobilization techniques and support materials used for immobilization. They mentioned that enzyme immobilization provides an excellent base for increasing availability of the enzyme to the substrate with greater turnover over a period of time. They assessed several natural and synthetic supports for their efficiency in enzyme immobilization. They reviewed natural supports viz. Alginate, chitosan and chitin, collagen, carrageenan, gelatine, cellulose, starch, pectin, sepharose, and synthetic polymers viz. Zeolites, ceramics, celite, silica, glass, activated carbon, charcoal used for enzyme immobilization.
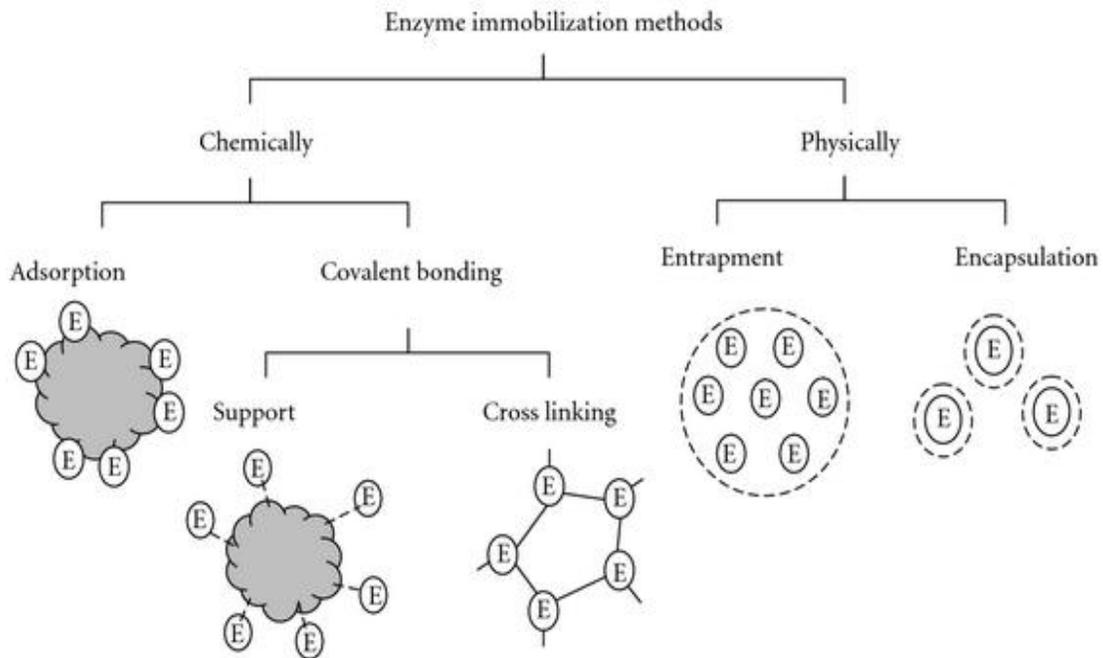
**Fig. 3. Different methods of immobilization of the Enzymes
(http://www.hindawi.com/journals/er/2011/468292/Fig1/)**

Table 2. Selected characteristic parameters of immobilized enzymes

Enzyme	**Biochemical properties**
	Molecular mass, prosthetic groups, functional groups on proteinsurface, purity (inactivating/protective function of impurities)
	Enzyme kinetic parameters
	Specific activity, pH-, temperature profiles, kinetic parameters for activity and inhibition, enzyme stability against pH, temperature, solvents, contaminants, impurities.
Carrier	**Chemical characteristics**
	Chemical basis and composition, functional groups, swelling behavior, accessible volume of matrix and pore size, chemical stability of carrier
	Mechanical properties
	Mean wet particle diameter, single particle compression behavior, flow resistance (for fixed bed application), sedimentation velocity (for fluidized bed), abrasion (for stirred tanks)
Immobilized enzyme	**Immobilization method**
	Bound protein, yield of active enzyme, intrinsic kinetic parameters (properties free of mass transfer effects)
	Mass transfer effects
	Consisting of partitioning (different concentrations of solutes inside and outside the catalyst particles), external and internal (porous) diffusion; this gives the effectiveness in relation to free enzyme. Determined under appropriate reaction conditions.
	Stability
	Operational stability (expressed as activity decay under working conditions), storage stability
	Performance
	Productivity (amount of formed product per unit or mass of enzyme) enzyme consumption (e.g. units kg–1 product, until half-)life

Table 3. Advantages and disadvantages of immobilized enzymes (Dicosimo et al. [60])

Advantages	Disadvantages
Amenable to continuous and batch formats	Loss of enzyme activity upon immobilization
Reuse over multiple cycles possible	Unfavorable alterations in kinetic properties
Improved stability over soluble enzyme forms	Cost of carrier and fixing agents
Favorable alterations in pH and temperature optima	Cost of immobilization process
Sequester enzyme from product stream	Mass transfer limitations
Co-immobilization with other enzymes possible	Subject to fouling

Chloroperoxidase, a heme-containing enzyme which exhibits catalase, peroxidase and cytochrome P450 enzyme activities in addition to halogenations reaction, has been covalently immobilized on to amino groups containing magnetic beads. For that, acrylate based magnetic beads were prepared by co-polymerization of monomers namely glycidylmethacrylate, methylmethacrylate and ethyleneglycol dimethacrylate via suspension polymerization. Thereafter, epoxy groups of the magnetic beads were converted into amino groups in the presence of ammonia during thermal precipitation iron oxide crystal in the beads' structures [74]. Kadima and Pickard [75] showed immobilization of chloroperoxidase on aminopropyl glass. Garg et al. [76] reported immobilization of urease on carboxyl terminated surface of glutathione capped gold nanoparticles and showed that immobilized enzyme can be exploited for biosensor fabrication, immunoassays and as *in vivo* diagnostic tools. Sahare et al. [77] reported immobilization of peroxidise enzyme onto porous silicon

functionalized with 3-aminopropyldiethoxysilane and showed enhanced enzyme activity and stability of the enzyme upon immobilization. Nisha et al. [78] reviewed methods, applications and properties of immobilized enzymes. They reported that method of immobilization and solid support must be selected based on the type of application. They described various methods for enzyme immobilization and also discussed applications of immobilized enzymes in various industries viz. food industry, biodiesel industry, waste water treatment, textile industry, detergent industry etc. Yu et al. [79] reported non-covalent immobilization of cellulases using reversibly soluble polymers, Eudragit S-100 and Eudragit L-100 and showed use of immobilized enzyme in bio-polishing of cotton fabric. They also studied the characteristics of cellulose-Eudragit S-100 (CES) and cellulose Eudragit L-100 (CEL) using Fourier transform infrared (FTIR) spectra, circular dichroism (CD) spectra, and fluorescence spectra. The CES retained 51% enzyme activity whereas CEL retained only 42% enzyme activity after three cycles of repeated uses indicating that CES exhibits more stability compared to CEL. Endoglucanase has been immobilized using the technique of microencapsulation with gum Arabica as support, a natural polymer having biodegradable property. Encapsulation helps in retaining enzyme activity in the presence of detergents. Endoglucanase has been used in detergent and textile industries [80]. Kirby et al. [81] reported a simple method for high yield drug entrapment in liposomes using the technique of dehydration and rehydration. The same group reported that on encapsulation of cheese ripening enzymes in liposomes, there is acceleration of the rate of ripening of Cheddar cheese by nearly 100 fold over previously reported methods. They reported that objective measurement of cheese ripening by proteolytic indices, and subjective evaluation of flavor quality and intensity by trained taste panels indicated that cheeses are ripened by the microencapsulated enzyme in half the normal time thus making the process economical [82].

In food industry, enzyme inulinase is used for the production of fructose syrups. Fructose and fructo-oligosaccharides are important constituents in food, beverages and pharmaceutical industries due to their beneficial effects in diabetic patients, low carcinogenicity, in increasing iron absorption in children and higher sweetening property compared to sucrose. Besides, fructose also enhances flavour and color and product stability in food and bevarages

[83-85]. Yewale et al. [86] reported immobilization of inulinase from *Aspergillus niger* on chitosan and showed its usefulness in continuous inulin hydrolysis. Garlet et al. [87] immobilized inulinase non-covalently on multi-walled carbon nanotubes. They showed fast adsorption in about six minutes with a loading capacity of 51,047 units per gram support using 1.3% (v/v) inulinase concentration and 1: 460 adsorbent adsorbate ratio. They also reported 100% retention of immobilized enzyme activity during five weeks incubation at room temperature. Another example of carrier matrix for the support of enzyme immobilization is the amino functionalized $Fe_3O/SBA15$ composite used for *Candida rugosa* lipase, act as a biocatalyst for the interesterification of soyabean oil and methyl stearate [88].

6. PEPTIDE MAPPING

Peptide mapping called as peptide mass fingerprinting is a technique used to identify and characterize a purified protein. Here, protein is converted into peptide fragments either by chemical or enzymatic treatment and these fragments are separated and identified by comparing with the standard reference material. However, the known standard is not absolutely required if the protein is either unknown or uncharacterized previously. First two dimensional gel electrophoresis is carried out followed by the isolation and elution of the protein bands, which are subjected to treatment with a panel of proteases. The partial or complete digestion of the intended peptide generates number of fragments having different charge to mass ratio. The number of peptide fragments along with their respective size provides a peculiar pattern of peptide fragments which is compared with the peptide finger database of previously known proteins. The unknown protein may be characterized on the basis of similarities and dissimilarities with the earlier characterized proteins. Sometimes it is also used in conjunction with multidimensional HPLC for better resolution, if initial sample is not so pure. This method is preferable over the other methods available due to high sensitivity, specificity and versatility [89]. The magnitude of sensitivity is so much that even a single amino acid difference among peptides or proteins may be deduced using this technique. A specific map is developed through peptide mapping for a unique protein known as peptide map and it is the fingerprint of protein and is the end product of several chemical processes that provide a comprehensive understanding of the

protein being analyzed. However, this technique may be simplified, if the protein of interest is previously well characterized as in our study on starch phosphorylase isoforms Pho1 and Pho2, which are well reported in number of other plants, but till date have not been studied using this technique.

There are four major steps in peptide mapping viz. isolation and purification of the protein, enzymatic or chemically selective cleavage of peptide bonds, separation of peptides by chromatography or electrophoresis and analysis and identification of peptides. Isolation and purification is much crucial step since contamination and impurities substantially hinder the resolution and consistent pattern of peptide mapping. It is the most tedious step because it needs lot of hit and trials. For starch phosphorylase, we have used two different approaches in our laboratory: (i) Classical chromatography techniques and (ii) Ultra filtration technique followed by native and SDS-PAGE electrophoresis. For selective cleavage of peptide bonds, there are two methods available viz. enzymatic method and chemical method. There are number of cleavage agents with their specificity of cleavage site(s). However, choice of cleavage agent depends upon the type of protein to be digested. For example, trypsin cleaves a peptide bond where carboxyl group has been contributed by either arginine or lysine, chymotrypsin cleaves a peptide bond where carboxyl group has been contributed by phenylalanine, tyrosine or tryptophan. The chemical agent, cyanogen bromide cleaves a peptide bond where carboxyl group has been contributed by methionine, whereas 2-nitro-5-thio-cyanobenzoic acid cleaves a peptide bond where amino group has been contributed by cysteine (http://www.nihs.go.jp/dbcb/Bio-Topic/peptide.pdf). Jeno et al. [90] reported a method where protein eluted in picogram quantity from Coomassie Blue stained two dimensional gel, may generate internal sequence data without blotting step. In the procedure, they reduced and alkylated the stained proteins with dithiothreitol and iodoacetamide in the presence of 1% sodium dodecyl sulfate and then digested with endoproteinase LysC.

Peptides generated after digestion of proteins from silver stained gels can be sequenced and analyzed by mass spectrometry. Shevchenko et al. [91] analyzed silver stained one dimensional gel of yeast protein by nano-electrospray tandem mass spectrometry which covered more than 1000 amino acids without any chemical modifications. They also mentioned that this silver staining procedure is preferable over Coomassie staining due to consumption of less time for sample preparation and also low level sequence analysis of proteins. Before digestion, number of parameters viz. pretreatment given to the sample and optimization of conditions like pH, temperature, time and amount of cleavage agent to be used must be taken into consideration (http://www.nihs.go.jp/dbcb/Bio-Topic/peptide.pdf).

For separation of peptides, RP-HPLC, ion exchange chromatography (IEC), capillary electrophoresis, and electrophoresis can be used. Among chromatographic separation techniques, HPLC and RP-HPLC are used very commonly. To use these techniques, selection of column, solvent, mobile phase, selection of the gradient, isocratic selection, temperature of the column, flow rate are very important. Intestinal calcium binding proteins after proteolytic digestion have been separated through HPLC and are considered as efficient techniques for the mixture of smaller peptides [92]. Cleveland et al. [93] reported peptide mapping by limited proteolysis and analysis by gel electrophoresis. After proteolytic digestion, peptides can be easily separated on 15% polyacrylamide gel having SDS due to large and different molecular weights of peptides. The generated band pattern is the characteristics feature of the protein with the particular proteolytic enzyme. Compared to different types of HPLC techniques, capillary electrophoresis technique has been proved to be better in recent few years. A very small size of sample say about 5 pico-mole quantity of protein can also be separated with capillary electrophoresis. This technique can separate proteins with high efficiency and in shorter analysis time [94]. Vaclav [95] reported improvement in the methodology of capillary electrophoresis including different separation modes viz. isoelectric focusing, isotachophoresis, affinity electrophoresis, multi-dimensional separations, zone electrophoresis, electro-chromatography.

On the basis of properties of peptides viz. difference in size, charge, hydrophobicity, shape and specific binding capacity, one can easily separate these peptide molecules through the above mentioned techniques. All the methods involving electro-migration depend on different properties of peptide molecule listed in Table 4.

6.1 Analysis and Identification of Peptides

After generation and separation of peptides, characterization of peptide fragments is done by Mass Spectroscopy (MS) either by inserting directly the peptide fragment or by online LC-MS for structure analysis. To determine the type of amino acid modification and to sequence a modified protein, tandem MS is used. To assign the disulphide bonds to various sulfhydryl containing peptides, a method has been developed by comparing the mass spectra of the digest before and after reduction. Major tools for the analysis and characterization of peptide finger prints or mapping are:

6.1.1 Mass spectroscopy

By fragmenting the sample in the instrument with multiple analyzers used in tandem mass spectrometer, structural information can be generated. Mass spectrometer has number of applications in biotechnology, pharmaco-genomics, clinical chemistry, environmental biology, geological analysis etc. due to abundant data of DNA sequencing and proteomics. Work has been enhanced to manage those data and with development in proteomics. After MS, protein directly can be compared with these databases and can be identified [96]. Ashcroft [97] reported number of ionization methods viz. electron impact (EI), electro spray ionization (ESI), matrix assisted laser desorption ionization (MALDI) etc. Among all the methods, MALDI and ESI are used widely for ionizing the bio-molecules. Karas et al. [98] reported that the use of matrix in MS yields ions without any fragmentation and this can be applicable to thermo- labile bio-molecules. Electro spray ionization has recently been upgraded with a powerful advancement of making complete ions of large and flimsy polar molecules which plays an important role in biological arrangement [99]. Gooley and Packer [100] reported that separation of proteins having high and low iso-electric points and membrane should be done by one dimensional gel electrophoresis instead of two dimensional gel electrophoresis.

6.1.2 MALDI- MS

In this technique, matrix absorbs the laser light as sample is embedded in it [101]. MALDI TOF is the commonly used technique which has reflector, post source decay capability delayed extraction. Here, matrix plays an important role. There are acidic and basic matrices according to their efficiency of generating mass spectrum. Selection of the matrix is important and depends upon the sample to be analyzed. For smaller sized proteins, basic matrix, 2-amino 4- methyl-5-nitro pyridine has been reported to be suited well and for oligonucleotides of short length and homo-polymers of thymidine, 2-amino-5-nitropyridine matrix is reported to give good results [102]. Gobom et al. [103] reported sample preparation and purification through nano-scale reverse phase column to improve the quality of spectrum of complex samples and contaminated mixtures by MALDI. They also reported that DHB (2,5 dihydroxy-benzoic acid) acts as more efficient matrix compared to CHCA (α-cyano 4 hydroxy cinnamic acid) which is generally used for fragile bio-molecules. It is necessary that peptide ions generated should be stable which is possible by using DHB.

In ESI, sample or analyte should be in solution form and ions from this solution are converted into gaseous phase for the mass spectrum analysis with the help of electrical energy and with increased sensitivity. ESI plays an important role in analyzing neutral compounds by converting them into ions by protonation or cationisation. The complete path of an ionic conversion of sample from solution to gaseous phase is a multistep procedure. Initially charge droplets in the form of fine spray are released and then evaporation of solvent takes place and finally from highly charged droplet, ion is ejected and enters into the triple quadrapole mass analyzer and then the spectrum generated by processing in data system or computer. From each peptide, ESI produces number of multiple charged ions. For online mass and sequence determination, triple quadrapole spectrometer with ion source is commonly used [104].

6.1.3 Protein databases

Protein databases play an important role in identifying the proteins from the peptide finger printing map of proteolytically digested protein sample. The availability of these databases exponentially enhances the use of proteomics that is the computational analysis of proteins. Due to the development in bioinformatics tools or homology search programs like BLAST and FASTA, it is possible to generate data of proteins which are modified or differ in the sequence from the database as these homology search programs display the sequences which are closely related to the sample peptide map [105]. Comparing the peptide map and computational data of proteins, one can study and screen the

known proteins, detect translation errors, characterize post translational modification, locate disulphide bonds [106]. There are reports on the identification and characterization of microorganism proteins [107]. Dworzanski et al. [108] studied taxonomical classification and identified bacteria using mass spectrometric based proteomics. 'Profound' is an Bayseian algorithm based search engine which can be used for comparing the map with protein databases in order to identify proteins on the basis of their properties. On generation of low quality or if there is no sufficient data or if the sample contains mixture of proteins then also Profound identifies the protein reflecting the advantageous feature of the search engine [109]. Another database named as 'ProMEX' is a useful source for the study of plant system biology. It is a database for proteins and protein phosphorylation sites [110]. Brendt et al. [111] and Hummel et al. [110] reported an algorithm which depends upon number of parameters to identify true protein from the database. They also reported some possibilities to generate additional data because sometimes data collected by mass finger printing is not sufficient. To get more data, one can use another protease with different specificity or to improve spectrum quality, one should re-measure the spectrum with different parameters & to obtain important sequence information. For that, PSD MS/-MS peptide fragmentation technique can be used. Searching partial mass spectrometric peptide map of protein gives more specificity and error tolerance in mass spectrometric and database information. For map generated from four to five peptides, one can also use search engine like Protein Identification Resource (PIR) [112]. Yates [113] reported that development in computer algorithm is based on two different types of data generated, one from mass spectrometers and other from tandem mass spectrometer. It is possible to determine the molecular weight of

digested peptides to identify the protein through peptide mass finger printing using mass spectrometer and the databases. Another method is based on the information which is specific in fragmentation pattern and in sequence information generated from tandem mass spectrometer. This information is used in databases based on translated protein sequences and expresses tag sequences (EST) and it is very useful when the complete sequencing of genome of experimenting organism is not done but a large amount of expressed tag sequences are available. If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterization of a protein through peptide mapping is to reconcile and accounts for at least 95% of the theoretical composition of the protein structure. (http://www.nihs.go.jp/dbcb/Bio-Topic/peptide.pdf).

Peptide mapping or peptide fingerprinting is a plausible technique having huge potential for characterization of proteins previously unknown for their biochemical, biophysical and functional properties. However, much lesser literature is available on plant proteins especially on enzymes originated from plant sources. Yoon et al. [114] reported that botanical origin of commercial starches has been used for quality control of starch based products. Isolation of starch and its purification is considered quite cumbersome due to the fact that number of starch granules associated proteins (SGAPs) are available in plants such as soluble starch synthase I (SSI), starch branching enzyme II (SBEII), and granule-bound starch synthase I (GBSSI) which are irreversibly entrapped within the starch matrix and cannot be completely removed from starch granules by ordinary starch purification processes [115,116]. Cho et al. [117]

Table 4. Relationship between the properties and techniques for separation of peptide molecules

Properties of peptide molecule	Electro-migration Separation technique
Electrophoretic mobility (reflecting charge, size, and shape simultaneously.	Zone electrophoresis and isotachophoresis in a non sieving, free solution media.
Size	Zone electrophoresis in sieving media
Charge	Isoelectric .focusing, electrokinetic chromatography with ion – exchange pseudophase and by ion exchange electrochromatography
Hydrophobicity	Electrokinetic chromatography with micellar, microemulsion and br reverse phase chromatography.
Specific interaction with other biomolecules	Bio affinity electrophoresis.

reported identification of botanical origin of starch by using peptide mass fingerprinting of granule bound starch synthase. Starch is used as an ingredient in large number of products viz. Papers, foods, cosmetics, and drugs. This enzyme is an important enzyme of carbohydrate metabolism. Therefore, to identify any such protein including GBSSI is very important to conclude whether starch is originated from plant source. As these granules bound proteins or enzymes are the hallmark of starch originated from plant source, Cho et al. [117] emphasized that to analyze starch, number of methods such as microscopy, rheological measurements, X-ray diffractometry, FT-IR spectroscopy, and differential scanning calorimetry are available but for identifying the botanical origin of starches or starch based products, no reliable tool has been developed. However, for proteins, peptide mass fingerprinting (PMF) using MALDI-TOF-MS is one of the fastest and cheapest method.

Badgujar and Mahajan [118,119] reported peptide mass fingerprinting and N-terminal amino acid sequencing of cysteine protease named as Nivulian-II from *Euphorbia nivulia*. They also used peptide mass fingerprinting to elucidate details of the enzyme. They showed that this protease is a glycoprotein. Their mass fingerprinting analysis revealed peptide matches to Maturase K of *Banksia quersifolia.* The N-terminal sequence had partial homology with other cysteine proteases of biological origin and this work has very high impact in current trend of market where the requirement of cost effective proteases is increasing for various products such as innovative detergents. Yadav et al. [120] reported the characterization of a protein 'milin' from *Euphorbia milli,* a mollusc. Using the techniques of electrophoresis, gel filtration chromatography, mass spectrometry and other tests, they identified it as a homodimeric plant subtilisin like serine protease. The subunits of milin are differentially glycosylated affecting dimer association, solubility and proteolytic activity. The dimeric dissociation is SDS – insensitive and strongly temperature dependent but does not appear to be linked by disulphide bridges. They used peptide mass fingerprinting and *de novo* sequencing of the tryptic fragments but could not identify putative domains in the protein. The association studies of the milin suggested that it might play a role in the physiological function and in controlling Schistosomiasis (A mollusc originated disease). Singh and Dubey [121] studied a protease, procerain b from *Calotropis procera* and

characterized it using peptide mass fingerprinting and N-terminal sequencing. This protease is shown to be involved in milk clotting and because of this property it may be exploited in cheese industry. It has also been mentioned that this protease may be a diet additive to stimulate digestion. N-terminal sequencing and PMF revealed that it is 80% similar to the Asclepain, which is a cysteine protease and functions like papain. Abdel Rahman et al. [122] analyzed this protein using electrospray ionization and matrix assisted laser desorption/ionization (MALDI) using hybrid quadruple time of flight tandem mass spectrometry. They showed presence of a signature peptide in the protein which may be useful for quantitative approach of the allergen prorein.

7. CONCLUSION

Starch in the form of insoluble granules accumulates in chloroplasts as the primary product of photosynthesis and acts as a primary energy source or reserve in higher plants. Starch phosphorylase (EC 2.4.1.1) is the key enzyme which catalyzes the reversible conversion of starch and inorganic phosphate into glucose-1-phosphate and has been reported in many higher plants. Multiple forms of the enzyme have been reported from many plant tissues which have been predicted to have different roles in starch metabolism. Here, various biochemical properties have been reviewed. Various techniques for enzyme immobilization have been discussed and reports on immobilization of starch phosphorylase from different sources and on different solid matrices have also been reviewed. Advantages of immobilized enzymes over soluble enzymes have been discussed. Disadvantages of immobilized enzymes viz. possible loss of enzyme activity upon immobilization, cost of carrier and fixing agent and immobilization process which affects the economics of the process have also been discussed. Peptide mapping technique for proteins and reports on peptide mapping of various proteins have also been illustrated in this review. In future research, there is need to elucidate the physiological role(s) of cytoplasmic phosphorylase (Pho 2). More research is required in exploiting the industrial uses of starch phosphorylase. Work should be carried out to search more useful immobilization techniques to prepare more thermo-stable enzyme which may be important for industrial purposes. Biopolymers can be prepared by exploiting starch phosphorylase which may be useful in food

industry and can be of clinical importance. Besides, there is requirement of developing rapid and cheaper ways for purification of the enzyme. Research work must be carried out to construct chimeric starch phosphorylase having higher affinity for its substrates.

ACKNOWLEDGEMENTS

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Newgard CB, Hwang PK, Fletterick RJ. The family of glycogen phosphorylases: Structure and function. Crit Rev Biochem Mol Biol. 1989;24:69-99.
2. Buchbinder JL, Rath VL, Fletterick RJ. Structural relationships among regulated and unregulated phosphorylases. Annu Rev Biophys Biomol Struct. 2001;30:191-209
3. Buleon A, Colonna P, Planchot V, Ball S. Starch granules: Structure and biosynthesis. Int. J. Biol. Macromol. 1998;23:85-112.
4. Tetlow IJ, Morell MK, Emes MJ. Recent developments in understanding the regulation of starch metabolism in higher plants. J. Exp. Bot. 2004b;55:2131-2145.
5. Fukui T. Plant Phosphorylases: Structure and Function. In: Akazawa T, Imaseki H (eds.), Martinus Nijhuff/ Dr. W. Junk Publishers, the Hague. N. Front. Plant Biochem. 1983;71-82.
6. Schachtele C, Steup M. α-1,4-Glucan phosphorylase from leaves of spinach (*Spinacia oleracea* L.). In situ localization by indirect immunofluorescence. Planta. 1986;167:444-451.
7. Weinhausel A, Nidetzky B, Rohrbach M, Blauensteiner B, Kulbe KD. A new maltodextrin-phosphorylase from Cornybacterium Callunae for the production of glucose 1- phosphate. Appl. Microbiol. Biotechnol. 1994;41:510-516.
8. Hanes CS. The breakdown and synthesis of starch by an enzyme system from pea seeds. Proc Roy Soc (London) B. 1940a;128:421–500.
9. Hanes CS. The reversible formation of starch from Glucose-1-phosphate catalyzed by potato phosphorylase. Proc R Soc (London) B. 1940b;129:174–208.
10. Tsai CS, Nelson OE. Two additional phosphorylases in developing maize seeds. Plant Physiol. 1969;44:159-167.
11. Baxter ED, Diffus CM. Enzymes of carbohydrate metabolism in developing Hordeum distichum grain. Phytochemistry. 1973;12:1923-1928.
12. Matheson NK, Richardson RH. Starch phosphorylase enzymes in developing and germinating pea seeds. Phytochemistry. 1976;15:887-892.
13. Steup M, Latzko E. Intracellular localization of phosphorylases in spinach and pea leaves. Planta. 1979;145:69-75.
14. Chang TC, Lee SC, Su JC. Sweet potato starch phosphorylase- purification and characterization. Agric Biol Chem. 1987;51:187-195.
15. Kumar A, Sanwal GG. Multiple forms of starch phosphorylase from banana leaves. Phytochemistry. 1977;16:327-328.
16. Yu S, Pederson M. Purification and properties of α-1,4- glucan phosphorylase from the red sea weed (*Gracilaria sordida* Harvey). Physiol Plant. 1991;81:149-155.
17. Lee EY, Braun JJ. Sweet corn phosphorylase:purification and properties. Arch. Biochem. Biophys. 1973;156:276–286.
18. Preiss, J, Levi C. Starch Biosynthesis and degradation. The biochem of plants. 1980;3:3371-3417.
19. Brisson N, Giroux H, Zollinger M, Camirand A, Simard C. Maturation and subcellular compartmentation of potato starch phosphorylase. Plant Cell. 1989;1:559-566.
20. Albrecht T, Koch A, Lode A, Greve B, Schneider-Mergener J, Steup M. Plastidic (Pho1- type) phosphorylase isoforms in potato (*Solanum tuberosum* L.) plants: expression analysis and immunochemical characterization. Planta. 2001;213:602-613.
21. Dauvillée D, Chochois V, Steup M, Haebel S, Eckermann N, Ritte G, Ral JP, Colleoni C, Hicks G, Wattebled F, Deschamps P, d'Hulst C, Liénard L, Cournac L, Putaux JL, Dupeyre D, Ball SG. Plastidial

phosphorylase is required for normal starch synthesis in *Chlamydomonas reinhardtii*. Plant J. 2006;48:274-285.

22. Satoh H, Shibahara K, Tokunaga T, Nishi A, Tasaki M, Hwang SK, Okita TW, Kaneko N, Fujita N, Yoshida M, Hosaka Y, Sato A, Utsumi Y, Ohdan T, Nakamura Y. Mutation of the plastidial. α -glucan phosphorylase gene in rice affects the synthesis and structure of starch in the endosperm. Plant Cell. 2008;20:1833-1849.

23. Rathore RS, Garg N, Garg S, Kumar A. Starch phosphorylase: Role in starch metabolism and biotechnological applications. Crit Rev Biotechnol. 2009;29:214-224.

24. Yu S, Pederson M. One step purification to homogeneity and isoforms of α -1,4-glucan phosphorylase of red sea weeds. Plant Physiol. Biochem. 1991a;29:9-16.

25. Smith AM, Zeeman SC, Thorneycroft D, Smith SM. Starch mobilization in leaves. J Exp. Bot. 2003;54:577-583.

26. Li Z, Morell MK, Rahman S. Rice and products thereof having starch with an increased proportion of amylase. US patent application 20070300319; 2007.

27. Frydman RB, Slabnik E. The role of phosphorylase in starch biosynthesis. Ann. New York Acad. Sci. 1973;210:153-169.

28. Singh S, Sanwal GG. Multiple forms of α-glucan phosphorylase in banana fruits. Phytochemistry. 1976;15:1447-1451.

29. Gerbrandy SJ, Verleur JD. Phosphorylase isoenzymes: Localization and occurrence in different plant organs in relation to starch metabolism. Phytochemistry. 1971;10:261-266.

30. Fettke J, Poeste S, Eckermann N, Tiessen A, Pauly M, Geigenberger P, Steup M. Analysis of cytosolic heteroglycans from leaves of transgenic potato (*Solanum tuberosum L.*) plants that under- or overexpresses the Pho 2 phosphorylase isozyme. Plant Cell Physiol. 2005;46:1987-2004.

31. Fettke J, Eckermann N, Kötting O, Ritte G, Steup M. Novel starch-related enzymes and carbohydrates. Cell. Mol. Biol. 2007;52:OL883–OL904.

32. Smith AM, Zeeman SC, Smith SM. Starch degradation. Annu. Rev. Plant Biol. 2005;56:73-98.

33. Orzechowski S. Starch metabolism in leaves. Acta Biochem Polonica. 2008;55:435-445.

34. Lee YP. Potato phosphorylase. I. Purification, physico-chemical properties and catalytic properties. Biochem. Biophys. Acta. 1960;43:18-24.

35. Kamogawa A, Fukui T, Nikuni A. Potato α-glucan phosphorylase: Crystallization, amino acid composition and enzymatic reaction in the absence of primer. J. Biochem. 1968;63:361-369.

36. Mateyka C, Schnarrenberger C. Purification and Properties of Mesophyll and Bundle Sheath Cell α-Glucan Phosphorylases from *Zea mays* L. Equivalence of the Enzymes with the Cytosol and Plastid Phosphorylases from Spinach. Plant Physiol. 1988;86:417-422.

37. Preiss J, Okita TW, Greenberg E. Characterization of the spinach leaf phosphorylases. Plant Physiol. 1980;66:864-869.

38. Steup M, Schachtele C, Latzko E. Purification of a non-chloroplastic α- glucan phosphorylase from spinach leaves. Planta. 1980;48:168-173.

39. Hsu JH, Yang CC, Su JC, Lee PD. Purification and characterization of a cytosolic starch phosphorylase from etiolated rice seedlings. Bot. Bull. Acad. Sin. 2004;45:187-196.

40. Witt W, Sauter JJ. Partial Purification of Amylases and Starch Phosphorylases from Poplar Wood. Plant Physiol. 1994;146:15-21.

41. Weinhausel A, Griessler R, Krebs A, Zipper P, Haltrich D, Kulbe KD, Nidetzky B. α -1,4-D-glucan phosphorylase of gram-positive Corynebacterium callunae: Isolation, biochemical properties and molecular shape of the enzyme from solution X-ray scattering. Biochem J. 1997;326:773-783.

42. Kumar A. Starch phosphorylase in plants. J. Sci. Ind. Res. 1989;48:568-576.

43. Kumar A, Sanwal GG. Characterization of purified starch phosphorylase from mature banana (*Musa paradisiacal*) leaves. Indian J. Biochem. Biophys. 1981;18:421-424.

44. Kumar A, Sanwal GG. Purification and physicochemical. properties of starch phosphorylase from young banana leaves. Biochemistry. 1982a;21:4152-4159.

45. Kumar A, Sanwal GG. Starch phosphorylase from tapioca leaves: Absence of pyridoxal phosphate. Arch Biochem Biophys.1982b;217:341-350.

46. Tickle P, Burrell MM, Coates SA, Emes MJ, Tetlow IJ, Bowsher CG.

Charecterization of plastidial starch phosphorylase in *Tritcium aestivum* L. endosperm. J. Plant Physiol. 2009;166:1465-1478.

47. Hurkman WJ, McCue KF, Altenbach SB, Korn A, Tanaka CK, Kothari KM, Johnson EL, Bechtel DB, Wilson JD, Anderson OD, DuPont FM. Effect of temperature on expression of genes encoding enzymes for starch biosynthesis in developing wheat endosperm. Plant Science. 2003;164:873-881.

48. Kumar A, Sanwal GG. Amino acid composition of starch phosphorylase from banana (*Musa paradisiaca*) leaves. Ind. J. Biochem. Biophys. 1982d;19:62-63.

49. Hammond JB, Preiss J. Spinach Leaf Intra and Extra Chloroplast Phosphorylase Activities during Growth. Plant Physiol.1983;73:709–712.

50. Garg N, Kumar A. Immobilization of starch phosphorylase from cabbage leaves: Production of glucose-1-phosphate. Braz J Chem Eng. 2008;25:229-235.

51. Shatters RG Jr, West SH. Purification and characterization of non-chloroplastic α-1, 4-glucan phosphorylases from leaves of Digitaria eriantha Stent. J. of plant physiol. 1996;149:501-509.

52. Srivastava S, Nighojkar A, Kumar A. Purification and chatrecterization of starch phosphorylase from *Cuscuta reflexa filaments.* Phytochemistry. 1995;39:1001-1005.

53. Garg S, Kumar A. Immobilization of starch phosphorylase from seeds of Indian millet (*Pennisetum typhoides*) variety KB560. Afr J Biotechnol. 2007;6:2715-2720.

54. Venkaiah B, Kumar A. Multiple forms of starch phosphorylase from Sorghum leaves. Phytochemistry. 1996;41:713-717.

55. Upadhye SP, Kumar A. Immobilization of starch phosphorylase from Bengal gram seeds: Production of glucose-1-phosphate. The Genet Eng Biotechnol. 1996;16:145-151.

56. Brena BM, Viera FB. Immobilization of enzyme and cells. Methods in Biotec. 2006;22:15-30.

57. Cao L. Carrier-bound Immobilized Enzymes: Principles, Application and Design. Wiley-VCH Verlag GmbH & Co; 2006. ISBN:9783527607662. DOI:10.1002/3527607668.

58. Tischer W, Kasche V. Immobilized enzymes: Crystals or carriers? Trends Biotechnol. 1999;17(8):326-335.

59. Tischer W, Frank Wedekind. Immobilized Enzymes: Methods and Applications. Top. curr. chem. 1999;200:96-118.

60. DiCosimo R, McAuliffe J, Poulose AJ, Bohlmann G. Industrial use of immobilized enzymes. Chem. Soc. Rev. 2013;42:6437-6474.

61. Sheldon RA. Enzyme immobilization: The quest for optimum performance. Adv. Synth. Catal. 2007;349:1289-1307.

62. Kumar A, Sanwal GG. Immobilized starch phosphorylase from mature banana (*Musa paradisiaca)* leaves. Indian J. Biochem. Biophysics.1981;114-119.

63. Venkaiah B, Kumar A. Egg shell bound starch phosphorylase packed bed reactor for the continuous production of glucose-1-phosphate. J. Biotechnol. 1994;36:11-17.

64. Srivastava S, Nighojkar A, Kumar A. Immobilization of *Cuscuta reflexa* starch phosphorylase: Production of glucose-1-phosphate using bioreactors. J Ferment Bioeng. 1996;81:355-357.

65. Conrath N, Griindig B, Hiiwel St, Cammann K. A novel enzyme sensor for the determination of inorganic phosphate. Anal. Chim. Acta. 1995;309:47-52.

66. Kourkoutas Y, Bekatorou A, Banat IM, Marchant R, Koutinas AA. Immobilization technologies and support materials suitable in alcohol beverages production. Food Microbiol. 2004;21:377-397.

67. Bakoyianis V, Kanellaki M, Kalliafas A, Koutinas AA. Low temperature wine-making by immobilized cells on mineral kissiris. J. Agric. Food Chem.1992;40: 1293–1296.

68. Bakoyianis V, Kana K, Kalliafas A, Koutinas AA. Low temperature continuous wine-making by kissiris supported biocatalyst. J. Agric Food Chem. 1993; 41:465–468.

69. Loukato P, Kiaris M, Ligas I, Bourgos G, Kanellaki M, Komaitis M, Koutinas AA. Continuous wine-making by g-alumina-supported biocatalyst. Quality of the wine and distillates. Appl. Biochem. Biotechnol. 2000;89:1–13.

70. Ogbonna JC, Amano Y, Nakamura K, Yokotsuka K, Shimazu Y, Watanabe M, Hara SA. Multistage bioreactor with replaceable bioplates for continuous wine fermentation. Am. J. Enol. Vitic.1989;40: 292–297.

71. Otsuka K. Wine making. Jpn. Kokkai Tokkyo Koho. 80, 159, 789. Tita, O, Jascanu V, Tita M, Sand C. Economical

comparative analysis of different bottle fermentation methods. Proceedings of the AVA, International Conference on Agricultural Economics, Rural Development and Informatics in the New Millennium, Hungary. 2003;1980.

72. Lommi H, Advenainen J. Method using immobilized yeast to produce ethanol and alcoholic beverages. European Patent Application EP. 1990;361:165.

73. Datta S, Christena LR, Rajaram YRS. Enzyme immobilization: an overview on techniques and support materials. Biotech. 2013;3:1-9.

74. Bayramoğlu G, Kirlap S, Yilmaz M, Topaare L, Arıca MY. Covalent immobilization of chloroperoxidase onto magnetic beads: Catalytic properties and stability. Biochem. Eng. J. 2008;38:180–188.

75. Kadima TA, Pickard MA. Immobilization of chloroperoxidase on aminopropyl-glass. Appl. Environ. Microbiol. 1990;56:3473–3477.

76. Garg S, De A, Mozumdar S. pH dependent immobilization of Urease on glutathione capped gold nanoparticles. J Biomed Material Res. Epub ahead of print; 2014.

77. Sahare P, Ayala M, Duhalt RV, Vivechana A. Immobilization of peroxidase enzyme onto theporous silicon structure for enhancing its activity and stability. Nanoscale Research Letters. 2041;9:409.

78. Nisha S, Arun K S, Gobi N. A Review on Methods, Application and Properties of Immobilized Enzyme. Chem. Sci. Rev. Lett. 2012;1:148-155.

79. Yu Y, Yuan J, Wang Q, Fan X, Wang P, Cui L. Non-covalent immobilization of cellulases using the reversibly soluble polymers for bio-polishing of cotton fabric. Biotechnol. Appl. Biochem; 2014. DOI: 10.1002/bab.1289. [Epub ahead of print].

80. Ramakrishnan A, Pandit N, Badgujar M, Bhaskar C, Rao M. Encapsulation of endoglucanase using a biopolymer Gum Arabic for its controlled release. Bioresour. Technol. 2007;98:368-372.

81. Kirby CJ, Gregoriadis G. Dehydration – rehydreation vesicles: Simple method for high yield drug entrapment in lisosomes. Biotechnol. 1984;2:979.

82. Kirby CJ, Brooker BE, Law BA. Accelerated ripening of cheese using liposome encapsulated enzyme. Int J of Food Sci and Tech. 1987;22:355-375.

83. Roy I, Sardar M, Gupta MN. Exploiting unusual affinity of usual polysaccharides for bioseparation of enzymes on fluidized beds. Enzyme. Microb. Technol. 2000;27:53-65.

84. Hanover LM, White JS. Manufacturing, compositon, and aplications of fructose. Am. J. Clin. Nutr. 1993;58:724S-732S.

85. Treichel H, Mazutti M, Maugeri Filho F, Rodrigues MI. Technical viability of the production, partial purification and characterisation of inulinase using pretreated agroindustrial residues. Bioproc Biosyst Eng. 2009;32:425-433.

86. Yewale T, Singhal RS, Vaidja AA. Immobilization of inulinase from Aspergillus niger NCIM 945 on chitosan and its application in continuous inulin hydrolysis. Biocatal Agric Biotechnol. 2013;2:96–101.

87. Garlet TB, Weber CT, Klaic R, Foletto EL, Jahn SL, Mazutti MA, Kuhn RC. Carbon Nanotubes as Supports for Inulinase Immobilization. Molecules. 2014;19:14615-14624.

88. Zang X, Xie W. Enzymatic Interesterification of Soybean Oil and Methyl Stearate Blends Using Lipase Immobilized on Magnetic Fe3O4/SBA-15 Composites as a Biocatalyst. Oleo Sci. [Epub ahead of print]; 2014.

89. Primrose S, Twyman R. In Principles of gene manipulation and genomics. Blackwell Publishing press; 2006.

90. Jenö P, Mini T, Moes S, Hintermann E, Horst M. Internal sequences from proteins digested in polyacrylamide gels. Anal.Biochem. 1995;224:75-82.

91. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver stained polyacrylamide gels. Anal. Chem. 1996;68:850-858.

92. Fullmert CS, Wasserman RH. Analytical Peptide Mapping by High Performance Liquid Chromatography .APPLICATION TO Intestinal calcium binding proteins. J. Biol. Chem. 1979;254:7208-7721.

93. Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK. Peptide Mapping by Limited Proteolysis in Sodium Dodecyl Sulfate and Analysis by Gel Electrophoresis. J. Biol. Chem. 1977;252:1102-1106.

94. Cobb KA, Novotny MV. Peptide mapping of complex proteins at the low -picomole

level with capillary electrophoretic separations. Anal. chem. 1997;64:879-886.

95. Vaclav K. Recent advances in capillary electrophoresis of peptides Electrophoresis. 2001;22:4139-4162.

96. Yates JR. 3rd. Mass spectrometry from genomics to proteomics. Trends Genet. 2000;16:5-8.

97. Ashcroft AE. In Ionization Methods in Organic Mass Spectrometry. The Royal Society of Chemical Sciences press, London. 1997;1-26.

98. Karas M, Bachmann D, Bahr U, Hillenkamp F. Matrix-assisted ultraviolet laser desorption of non – volatile compounds. Int. J. mass spectrom. 1987;78:53-68.

99. Fenn JB, Mann M, Meng CK, Wong SF, Whitehouse CM. Electrospray ionization for mass spectrometry of large biomolecules. Science. 1989;24:64-71.

100. Gooley AA, Packer NH. The importance of co- and post-translational modifications in proteome projects. Proteome Research: New Front, Functional Genomics Principles and Practice. Wilkins W, Wilhams K, Appei R. Hochstrasser D. Springer. 1997;65-91.

101. Rusconi F, Schmitter JA. Sample preparation for analysis by MALDI-TOF mass spectrometry. Analysis. 1998;26:M13-M17.

102. Fitzgerald MC, Parr GR, Smith LM. Basic matrices for the matrix-assisted laser desorption/ionization mass spectrometry of proteins and oligonucleotides. Anal. Chem. 1993;65:3204-3211.

103. Gobom J, Nordhoff E, Migorodskaya E, Ekman R, Roepstorff P. Sample purification and preparation techniques based on nano scale reversed –phase columns for the sensitive analysis of complex peptide mixtures by matrix assisted laser desorption/ ionization mass spectrometry. J. Mass Spectrom. 1999;34:105-116.

104. Lahm HW, Langen H. Mass spectrometry: A tool for the identification of proteins separated by gels. Electrophoresis. 2000;21:2105-2114.

105. Johnson RS, Davis MT, Taylor JA, Patterson SD. Informatics for protein identification by mass spectrometry. Mass spectrometry in proteomics. 2005;35:223-236.

106. Pappin DJ, Horjup P, Bleasby AJ. Rapid identification of proteins by peptide – mass fingerprinting. Curr. Biol. 1993;3:327-32.

107. Demirev PA, Fenselau C. Mass spectrometry for rapid characterization of microorganisms. Annu. Rev. Anal. Chem. 2008;1:71-93.

108. Dworzanski JP, Snyder AP. Classifiation and identification of bacteria using mass spectrometry-based proteomics. Expert Rev Proteomics. 2005;2:863-878.

109. Zhang W, Chait BT. Profound: An expert system for protein identification using mass spectrometric peptide mapping information. Anal. Chem. 2000;72:2482-2489.

110. Hummel J, Niemann M, Wienkoop S, Schulze W, Steinhausr D, Selbig J, Wather D, Weckwerth W. Pro MEX: A mass spectral reference databases for proteins and protein phosphorylation sites. BMC. 2007;8:216.

111. Brendt P, Hobohm U, Langen H. Reliable automatic protein identification from matrix-assisted laser desorption/ionization mass spectrometric peptide fingerprints. Electrophoresis. 1999;20:3521-3526.

112. Mann M, Hojrup P, Roepsorff P. Use of mass spectrometric molecular weight information to identify protein sequence databases. Biol. Mass Spectrom. 1993;22:338-345.

113. Yates JR. 3rd. Databases searching using mass spectrometry data. Electrophoresis. 1998;19(6):893-890.

114. Yoon JW, Jung JY, Chung HJ, Kim MR, Kim CW, Lim ST. Identification of botanical origin of starches by SDS –PAGE analysis of starch granule-associated proteins. J of cereal Sci. 2010;52:321-326.

115. Koziol AG, Marquez BK, Huebsch MP, Smith JC, Altosaar I. The starch granule associated proteomes of commercially purified starch reference materials from rice and maize. J proteomics. 2012;75:993-1003.

116. Mu-Forster C, Huang R, Powers JR, Harriman RW, Knight M, Singletary GW, Keeling PL, Wasserman BP. Physcial association of starch biosynthetic enzymes with starch granules of maize endosperm. Plant Physiol. 1996;111:821-829.

117. Cho DH, Han JA, Lim ST. Identification of botanical origin of starch by using peptide mass fingerprinting of granule- bound starch synthase. J Cereal Sci. 2013;57:264-266.

118. Badgujar SB, Mahajan RT. Peptide Mass Fingerprinting and N-Terminal Amino Acid equencing of Glycosylated Cysteine Protease of *Euphirbia nivulia* Buch.-Ham. J Amino Acids. 2013;1-6.
119. Badgujar SB, Mahajan RT. Charecterization of Thermo- and Detergent Stable Antigenic Glycosylated Cysteine Proteases of *Euphorbia nivulia* Buch.-Ham. and Evalution of Its Ecofriendly Applications. Scientific World J. 2013;1-12
120. Yadav SC, Jagannadham MV, Kundu S, Jagannadham MV. A kinetically stable plant substilase with unique peptide mass fingerprints and dimerization properties. Biophys. Chem. 2009;139:13-23.
121. Singh AN, Dubey VK. Exploring applications of procerain b, a novel protease from Calotropis procera, and characterization by N-terminal sequencing as well as peptide mass fingerprinting. Appl. Biochem. Biotechnol. 2011;164:573-80.
122. Abdel Rahman AM, Lopata AL, O'Hehir RE, Robinson JJ, Banoub JH, Helleur RJ. Charecterization and de novo sequencing of snow crab tropomyosin enzymatic peptides by both electrospray ionization and matrix-assisted laser desorption ionization QqToF tandem mass spectrometery. J. Mass Spectrom. 2010;45:372-81.

_____