The history of crude peroxidase extraction biology essay

Science, Biology

INTRODUCTION

The isolation and purification of a biotechnological product to a suitable form for its use is popularly known as downstream processing. In most cases, this will mean product recovery from dilute aqueous state, process complexity depends on the product purity. Main aim is to minimize the number of required steps and increase its yield to the maximum possible value. Downstream processing implies manufacturing purified product fit for specific use, generally in marketable quantities. Downstream processing also refers to the recovery and purification of biosynthetic products, particularly pharmaceuticals, from natural sources such as animal or plant tissue, including the recycling of salvageable components and the proper treatment and disposal of waste. It is a very important step in the manufacture of pharmaceutical products such as antibiotics, hormones, antibodies and vaccines; industrial enzymes; and natural fragrance and flavor compoundsDownstream processing operations can be divided them into four groups which can be applied to bring a product from its natural state by component of a tissue, cell or fermentation broth through progressive improvements in concentration and purity. Insoluble removal is generally the first step involving the capture of the products as a solute in a liquid free of particulate, for example separation of cells, cell debris and other particulate matter from an antibiotic containing fermentation broth. Common methods to achieve this

are filtration, centrifugation, sedimentation, precipitation, flocculation, electro-precipitation, and gravity settling. Additional operations such as grinding, homogenization, or leaching, required recovering products from

solid sources such as plant and animal tissues are also included in this group. Isolation of products can be explained as the removal of components whose properties differ markedly from the desired product. In most cases, water is the chief impurity and isolation steps are designed especially to remove it, reducing the material volume to be handled and concentrating product. Solvent extraction, adsorption, ultra filtration, and precipitation are few of the unit operations involved. Product’s purification is for separating the contaminants resembling the product very closely in physical and chemical properties. Generally steps involved during these stages are expensive to carry out and require sophisticated and sensitive equipment. This stage contributes significantly to the entire downstream processing expenditure. Examples of operations include affinity, size exclusion, reversed phase chromatography, crystallization and fractional precipitation.

Polishing of product consists of the final processing steps ending with packaging of the product in a stable form and conveniently transportable. Crystallization, desiccation, lyophilisation and spray drying are typical unit operations. Depending on the products intended use, polishing may include operations to sterilize the product and remove or deactivate trace contaminants which might compromise product safety. Sometimes recovery methods may be considered to combine two or more stagesEnzymes can be defined as complex organic protein compounds which are located in every living cell, and they are produced in them. They accelerate organic processes such as the breakdown of starch, protein, fat or sugar as catalysts, Peroxidases [E. C. 1. 11. 1. 7] a oxidoreductase produced by a number of plants and micro organisms generally has iron porphyrin ring

catalyzing oxidation of many organic substrate of aromatic compound.(1) They mostly have a molecular weight ranging from 30, 000 to150, 000 Da. (14) It is considered as one of the most heat stable enzyme.(3) It is a ubiquitous enzyme. (5) Peroxidase a widely distributed compound in nature, catalyzes the reduction of hydrogen peroxide to water, making it harmless. H2O2 being a common end product during oxidative metabolism, as it is a strong oxidizing agent, could be toxic upon accumulation. But peroxidase helps plant to get rid of excess H2O2. (4) It is mainly found as haenoprotein, although there are some unusual peroxidises containing metal ion such as selenium, valadium or flavin prosthetic groups. (4) In plants they mainly participate in the lignification process as well as the mechanism of defence in physically damaged or infected tissues. Peroxidase has been implicated in many metabolic processes some of which are cell development, membrane integrity and ethylene biogenesis. (9)According to amino acid homology and metal binding capabilities, these are classified into three classes. Class1 consists of intracellular peroxidises e. g. cytochrome c peroxidises. Class 2 consists of secondary fungal enzymes e. g. manganese peroxidises and Class 3 consisting of secretary plant peroxidise e. g. seedcoat soybean peroxidise (7) On the basis of sequence similarity, peroxidises can be categorized into two superfamilies: the mammalian peroxidises superfamily and plant peroxidises superfamily and they are named after there sources e. g. horseradish peroxidises, soybean peroxidises or their substrate e. g. lignine peroxidases, cytochrome c peroxidises. (4) It is divided into three classes which differ in their molecular weight absorbtion spectra, 1. ferriprotoporphyrin peroxidises which are brown in colour and their main

source is plant, animal and microorganisms 2. verdoperoxidases which are green in nature and are found in animals 3. Flavoprotein peroxidises having FAD as prosthetic group and found in animals and microorganisms. (5)The enzyme is found to exist in both membrane-bound and soluble forms and is found in plasmalemma, vacuoles, tonoplast, and in and outside the cell wall and has a variety of functions. It is involved in plant hormone regulation, defense mechanisms, indoleacetic acid degradation during maturation and senescence of fruits and vegetables , and lignin biosynthesis . Because of its multiple functions, the enzyme is commonly found as several iso enzymes in plants.(15)Peroxidases differ significantly from one another in their primary structures, even though the crystal structure and catalytic centres are quite similar. Plant peroxidases are known to be composed of a range of isoenzymes. It is accepted that the pattern of peroxidase isoenzymes changes during plant development. (8)Because of its oxidative nature, peroxidase could replace current chemical oxidant techniques in several areas. (14) Enzyme investigation may be of interest not only for its negative effects on colour and flavour, degradation of pigments and nutritive value but also for its positive effects. Peroxidases from soybean were considered to be involved in defence of plant against pathogens. Increased peroxidase activity is sometimes reported as a defensive response to most if not all metals, which may cause damage to the normal functions of plant cells. (11) The most frequently cited cause of seed deterioration is lipid peroxidation, which begins due to free radicals production, leading to cell damage and seed death. (12) Being the most heat resistant enzyme, it is used in food industry these days as an index of blanching procedures.(3) There is an

empirical relationship between residual peroxidise activity and the development of flavours and odour in food.(5)It is preferred for preparing enzyme conjugated antibodies due to its high specificity towards certain substances. (3) It has several bioremediation applications. Its activity is seen in several species of plant, thus there is a demand for continuous search of novel peroxidises for various applications.(4) Due to its extensive bioactivation properties and potential application in clinical, biochemical, biotechnological and related areas, it is receiving high attention. (7) it is applied as a component of diagnostic clinic reagent, mainly ELISA for detecting hepatitis diseases B, C and G, HIV and also quantification of uric acid, glucose, cholesterol and lactose. (1) HRP has also been used in several diagnostic applications in medicine such as the detection of 8-hydroxydeoxyguanosine and its analogues in urine, to identify bladder and prostate cancer risks.(14)It is used for the treatment of waste water containing phenolic compounds, synthesizing aromatic compound, eliminating peroxidises from food industrial waste. (1)) It is used for the synthesis of various aromatic chemicals and removal of peroxidise from food stuffs and industrial waste.(7) It has a potential for soil detoxification and bioremediation of waste water.(2) Reduction of peroxides at the expense of electron donating substrates, make peroxidases useful in a number of biotechnological applications which can be successfully used for biopulping and biobleaching in the paper industry, and can produce oxidative breakdown of synthetic azo dyes, bioremediation of wastewater and have also found use in biosensors. (13)Production of peroxidises from microorganism has also attracted researcher’s interest and Coprinus

cinereus and Coprinus macrorhizus are known to produce it. These basidiomycetes are quite sensitive to shear stress, hence making its production in stirred tank fermentor, not suitable. (5) Oxidation of wide varieties of organic compounds has led to this speculation that the enzyme can be associated with loss of colour, flavour, and nutritional value of raw and processed foods. (15)Recently, enzymatic approach has attracted much interest in the removal of dye from aqueous solutions because enzymes can act on specific recalcitrant pollutants and the catalytic action of enzymes is extremely high as compared to chemical catalysts due to higher reaction rates and milder reaction conditions. They can catalyze reactions at relatively low temperature and in the entire aqueous phase pH range with low retention time. From a practical point of view the use of peroxidases in vitro for that purpose may represent a more feasible system, provided that a number of conditions are fulfilled:(a) a simple and applicable system; (b) minimal requirements of compounds; (c)low cost and stable enzyme; and (d) short treatment periods. (13)Odorant compounds such as phenols, indoles, fatty acids, ammonia, hydrogen sulfide and mercaptans are either initially present in manure or result from anaerobic transformation of animal waste. Some methods which are expensive and require specialist knowledge were used but recently HRP has been proven an effective alternative for deodorization of manures.(14)Electrochemical biosensors can offer a potential for peroxidase application. Recently, it has found great amount of use in analytical systems, especially for the determination of hydrogen peroxide and organic hydroperoxides. When co-immobilized with a hydrogen peroxide-producing enzyme they can be exploited for determination of

glucose, alcohols, glutamate and choline.(14)Phenol occurs mostly in the waste water of industries such as high temperature coal conversion, petroleum refinery, plastic, wood and dye industries. Phenol, a carcinogen can be of major health concern, even in low concentrations.(18) Short-term effects may include respiratory irritation, burning eyes and headaches. Chronic effects of high level of exposures include weakness, weight loss, anorexia, muscle pain and fatigue. Many technologies have been determined for the removal and degradation of phenolic compounds in these wastewaters (16). Current trend of methods for phenol removal from waste water include microbial degradation, use of oxidizing agent including ozone and UV, solvent extraction, adsorption etc [17]. Enzymatic method can be safe and easy way of phenol removal. Phenolic waste water is a major issue when disposed untreated as phenol, the most widely used organic compound is found in the waste water of various industries like that of petroleum refining, coal conversion, plastic, resin, textiles, iron and steel manufacturing, pulp and paper manufacturing is toxic in nature, and has a water purification standard of <1 ug/lt. Enzymatic removal with peroxidises of phenolic compound is being investigated.(2) Recently it has been used for the decolourization and removal of textile dyes from polluted water.(7) The novel applications of peroxidase include treatment of waste water contaminated with dye because textile industry effluents are major source of water pollution due to stable nature of dyes and mutagenic and carcinogenic nature. Conventional wastewater treatment systems, such as activated sludges, physicochemical treatments (flocculation, ozonationor membrane filtration) and treatment with microbes are ineffective because of high cost,

low applicability and production of metabolites.(13)They are versatile biocatalysts with ever increasing number of applications thus researchers still investigate for new peroxidises of elevated stability and properties suitable for different applications. (4) Although relevant reviews on peroxidises activity in plants are available broad effort for their characterization are very limited.(4) This enzyme is poorly studied compared to its wide use. (4)

Materials and Methods

Materials and Reagents

Radish, tomato, cabbage, turnip, pH7 phosphate buffer, pH6 citrate buffer, 3, 3', 5, 5' tetra methyl benzidine from sigma, H_2O_2, phenol, $FeCl_3$, SDS-PAGE kit,

Crude peroxidase extraction

To prepare the crude extract, vegetable sources were washed properly, 200gms of vegetable source was cut, blended using chisel and mixed with the help of 200gms of pH7 phosphate buffer. The enzyme was filtered using chisel cloth and all the waste thrown, enzyme along with buffer was centrifuged at 8000rpm for 10 minutes to remove other small impurities to be filtered. Supernatant was taken and filtered with whatmann filter paper and was stored as crude extract in a beaker. To remove impurities like catalase, it was heated at 65°C for 3 minutes. The crude enzyme was then stored in a refrigerator and was brought to room temperature before every experiment.

Peroxidase and Protein Assay

The enzyme activity was measured using a chemical that does not occur in plants, but that changes colour, making the reaction easy to monitor, 50ul of 5% 3, 3', 5, 5' tetra methyl benzidine was taken and mixed along with 100ul of pH6 citrate buffer and 100ul enzyme extract, the test tube was shaken and 100ul of H_2O_2 was added and then enzyme activity was monitored by measuring the OD at 620nm. Whereas total protein estimation of each enzyme extract was determined using Lowry's et al.

Ammonium sulphate precipitation

Precipitation of protein was carried out using ammonium sulphate method, in which different concentration of salt of ammonium sulphate was added to the sample. The concentration varied from 30% to 80%, amount of salt required was calculated and added to the sample, which was then vortexed, till the salt is solubilised, this was followed by centrifugation at 8000rpm for 10 minutes. due to centrifugation, supernatant and pellet were separated, Then the reading for protein and enzyme assay from the pellet and supernatant was noted separately.

Aqueous two-phase extraction

ATPS was prepared using different concentrations of PEG, ammonium sulphate along with crude extract. After addition, sample was vortexed and then allowed to settle down, till the two phases were formed. The top and bottom phases were separated and the enzyme activity and protein concentration of both phases were measured. The partition coefficient and specific activity of the enzyme were calculated.

Chromatography

1ml of purified peroxidase enzyme obtained, was subjected to gel filtration chromatography with the help of sephadex-G-100 column. The column was packed to the height of 15 cm in a glass column with an internal diameter of 1. 5 cm. Sample was poured in the column and eluted with phosphate buffer of pH 7. Fractions of purified enzyme were collected and the enzyme activity as well as the protein content was determined, as mentioned in the previous section.

SDS PAGE

The partial purified peroxidase was used for protein electrophoresis. For SDS-PAGE; 100µg from each sample were boiled in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol then separated on a 12% separating gel. Electrophoresis was run at room temperature at 50 V for 120 min.

Effect of various parameters

Various parameters were monitored to check the effect of these parameters on the activity of peroxidase and then all the monitored parameters were optimized.

Reaction time

The selected enzyme concentration (0. 5ml) was added to 1ml of buffer substrate and absorbance values which determine the enzyme activity were recorded at 1 minute interval for 10 minutes.

Temperature

Enzyme extract was incubated at various temperatures ranging from 40 to 90°C for 10 minutes in a water bath, and then they were checked for OD and enzyme activity was determined for each temperature.

pH

5ml of enzyme extract was taken in each test tube and adjusted for pH ranging between 4 to 9 by the addition of 1M HCl or 1M NaOH. Each sample was then kept at that particular pH for 30 minutes at room temperature, after which enzyme activity is monitored by finding the OD.

Inhibitor

Different concentration of inhibitor (0. 2, 0. 4... 1) which in this case is sodium azide was prepared and mixed with same concentration of enzyme extract (0. 5), it was kept for 10 minutes in this state and enzyme activity in each case was determined by determining the OD of the sample.

Role of phenol

First the standard graph for purified phenol sample was plotted, then purified peroxidase from all the sources was taken and various concentration of enzyme extract was added to a particular concentration of phenol. The OD was monitored at 580nm for the activity of phenol. The peroxidase source which gives the lowest OD for phenol activity was found and was considered as the best source of peroxidase for the removal of phenol.

Results and Discussion

Crude peroxidase extraction

The crude extracted from all the four sources were checked for enzyme activity after which they were denatured by heating. It was found that turnip gave the best yield for crude among all the sources available and the value was found to be 1. 5U/ml, whereas cabbage gave the least enzyme activity of 0. 9U/ml. It was found that enzyme activity of all the sources was quite similar for crude, but the difference between enzyme activity is commonly attributed to the variation in enzyme activity with respect to anatomical location, plant’s age and state of being fresh.

Ammonium sulphate precipitation

Ammonium sulphate precipitation helped to improve peroxidase purification and concentrate for the crude extract. The specific activity and purification fold following ammonium sulphate treatment were found to be twice that of the crude extract. It is the most commonly used reagent for salting out, because its high solubility permits achievement of solubility for highly volatile solutions. In this precipitation process, as no precipitation was observed from 0-20%, Peroxidase began to precipitate when at least 30% ammonium sulphate was added, reaching the highest specific activity at 80% saturation, so it was concluded that, as the value of salt increased, enzyme activity also started increasing and maximum yield was obtained when the salt concentration was 80%, this was the case for all the vegetable sources.

Aqueous two-phase extraction

Different concentrations of PEG and salts were tried and Phase compositions were varied in such a way that the volume of the bottom phase (which needs to be subjected to further processing) is reduced. System having significant effect on partition coefficient was studied for further use. With PEG2000, peroxidase prefers the top phase, however at low molecular weight of PEG, many proteins having high k value lead to poor separation from the contaminant protein, whereas high molecular weight PEG prevents successful extraction of peroxidase in the top phase and therefore separating it from bulk precipitate.

Chromatography

Gel permeation chromatography was performed by sephadex G-100. Fraction collected, were tested for enzyme activity and total protein value, which proved very efficient as fraction showed up to 9. 43 fold purification with maximum specific activity of 10. 94 U/mg with highly reduced protein contents. This technique provides higher degree of purification appreciating this method and encouraging its further use for purification purposes.

Reaction time

As can be seen from the graph, rise in the OD value was recorded at uniform interval, enzyme activity value increased up to 5 or 6 minutes, based on the source, which was followed by a decline in the activity telling us that maximum activity of peroxidase is around 5 minutes in given conditions, and the activity starts decreasing thereafter. The result revealed that initially rate of reaction increased rapidly as peroxidase had high turnover number,

but when substrate started getting exhausted, peroxidase activity with the passage of time decreased. Therefore, during all the experiments, the enzyme activity value was calculated at 5 minutes.

Temperature

Peroxidase being one of the most heat stable enzyme can tolerate very high range of temperature without losing its activity, but it was found from the experiment that the optimum temperature where the enzyme showed maximum activity was around 55-65°C, after which, though the enzyme is active, its activity starts decreasing. After 60°C, with a further increase in temperature the activity decreased. A sharp decline in the relative activity was observed after 80°C.

pH

Optimum pH value of the enzyme was determined by measuring its enzyme activity at various pH. Peroxidase was found to be stable over wide range of pH, but was found to be more active in the acidic environment when compared to the basic one. The optimum pH was found to be around 6-6. 5, the value of enzyme activity decreased before and after that pH.

Inhibitor

Sodium azide was found to be the inhibitor for peroxidase, and the relation between enzyme activity and concentration of peroxidase was found to be inversely proportional, i. e. on increasing the concentration of inhibitor, the activity of enzyme decreased. This can be seen clearly in the graph

Role of phenol

Activity of peroxidase was inversely proportional to the amount of phenol i. e. amount of phenol decreased on increasing the concentration of enzyme extract. Phenol’s maximum degradation was seen with high concentration of enzyme, and source which degraded maximum phenol was found to be turnip. As the concentration of phenol increased, reduction in phenol degradation efficiency was observed. Enzymatic treatment using peroxidase is a viable option for the degradation of phenol.