Review

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Polyelectrolytes–protein complexes: a viable platform in the downstream processes of industrial enzymes at scaling up level

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Abstract

Complexation between proteins and polyelectrolytes through electrostatic interactions gives either soluble or non-soluble complexes, leading to phase separation. The formation and the stability of these complexes are influenced by pH, ionic strength, ratio between protein and polyelectrolyte and charge density of protein and polyelectrolyte. This review presented the advantages on the protein precipitation by adding a polyelectrolyte solution to a natural enzyme source (microbiological, vegetal or animal homogenate), over the classical precipitation methods. An enzyme can be precipitated like non-soluble complex, separated and then re-dissolved, thus the method works, at the same time, like concentrative and pre-purification steps together.

Keywords: polyelectrolytes; enzyme purification; downstream processes, bioseparation

INTRODUCTION

The world demand for enzymes is forecast to rise by 6.4% per annum to reach US$6.9 billion in 2017. Increasing per capita incomes in developing countries will help strong gains in consumer-related industrial applications such as food and beverages, animal feed, cleaning products, biofuel production, wastewater treatment chemicals, paper and pulp and other applications in research, biotechnology and diagnostics. In the last 60 years, enzymes have traditionally been purified in a variety of ways such as precipitation with organic solvents and salts or by different chromatographic techniques.[1–4] These methods involve a large number of steps, which make them time-consuming and difficult to scale up. Production of proteins by genetically engineered microorganisms, yeasts, fungi and animal cells has become a very important technique for the preparation of pharmaceuticals and other molecules used in biotechnology.

In recent years, the field of protein isolation and purification in scaling up has grown significantly as result of the need for large amounts of enzymes to be used in biotechnological processes. Bioseparation steps for the recovery of the final product can account for 50–80% of overall production costs. Thus, many traditional methods are no longer used because of their high cost, short life and polluting effects on the environment.

In the enzyme scaling up purification process, the main problem is the use of large volumes of biomass where the target enzyme is present (homogenates or microbial suspensions) and the need to reduce these volumes immediately. The homogenates are formed by suspensions of cells, cell debris, membranes and proteases released from the destruction of tissues.[5,6] All these give homogenates with low stability and high degradation rate within hours, so isolation methods are necessary to reach a concentrated extract of the main enzyme in a short time.[7]

About 90% of the protein purification methods use traditional ammonium sulfate as precipitant, in the range 40–70% of saturation level[8–10] which corresponds to about 400 g L^{-1} of this salt, so, this amount must be multiplied by the thousands of litres forming a homogenate, producing at the end of the process a great amount of waste to be treated due to the toxicity of ammonium cation.

In order to establish a competitive biotechnological process for enzyme purification some research groups have developed bioseparative methodologies based on the use of polyelectrolytes (PE) using their capacity to form non-soluble PE complexes with proteins.[11–14] The basis of this technique has been known for many years, with first reports published about the interaction between a protein and synthetic PE in the 1950s.[15–17] This methodology has great advantages over classical precipitation methods using solvents and salts because it can be easily scaled up, uses environment friendly PE and has low cost.

The paper reviews the present situation of protein isolation and purification using the precipitation method with protein–PE non-soluble complexes with special attention to scaling up the production of industrial enzymes.

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POLYELECTROLYTES

Polyelectrolytes (PE) are macromolecules that have a relatively large number of functional groups that are electrically charged or, under certain conditions, acquire electric charge. They can be polycations, polyanions or polyampholytes, since the net charge depends on possessing functional groups, which may be charged negatively or positively.[18] The principle groups present in the PE are the amines, amides, carboxylic and sulphonic groups. The importance of this classification is to know when the PE has the necessary conditions to interact with the molecule of interest, like proteins. The solubility of PE in water depends on the state of charge of said groups and, therefore of the environmental conditions affecting this state, as the pH. The pH variations in the medium produce changes in the ionization state of the hydrophilic groups and, therefore, to the entire PE. When the PE is electrically charged intra- and inter-chain repulsions are produced, which maintain the macromolecule in an extended conformation and, therefore, water soluble. When the PE is neutral, the PE chains can interact with each other and eventually form insoluble aggregates. At the same time the PE can be synthetic or natural but when the secondary structure is not present, there are no behavior differences among them. This is important to the development of methods that use natural and 'no contaminants' PE.[19] Examples of synthectic PEs are polyacrylic acids and polystyrene, among others. They are obtained by industrial synthesis and cannot be discarded in the environment, because they are not degradable; also some are toxic and carcinogenic. On the other hand we have natural PEs like chitosan, alginate, carrageenan, arabic gum, pectin, etc. They are electrically charged polysaccharides with strong or weak acid or basic groups; they are friendly to the environment because they can be discarded without producing any negative changes.

PEs having sulphonic groups lose their protons at pH values as low as 1.5, so the PE will have a negative net electrical charge and it will be soluble in aqueous media at pH higher than 1.5.[20,21] PE with carboxylic or amine will be soluble only when the groups have a net electrical charge because the charges will repel each other favoring interaction with the solvent molecules.[22,23] PE with carboxylic groups will be protonated at pH values lower than 3.5 (which is the average value of pKa of this group); the absence of electrical charge in the PE chain favors the interaction between them, so an aggregation process results, decreasing the PE solubility.

COMPLEX FORMATION BETWEEN PE AND PROTEINS

The macroscopic and microscopic path of the PE–proteins interaction

It is known that PE strongly interacts with proteins of opposite electrical charge to form complexes according to the experimental conditions of the medium.[18,24–27] The result of the interaction is the formation of water soluble complexes, coacervates or non-soluble complexes. The earliest reports studied the interaction of synthetic PE such as polystyrene sulfonate with β-lactoglobulin, poly (dially1-dimethylammonium chloride) and potassium poly (viny1 alcohol sulfate) with bovine serum albumin.[28–30] Later, the introduction of polysaccharides having electrically charged groups increased the number of PEs, thus increasing the possibilities of PE–protein complex (PE-P) formation.[31,32]

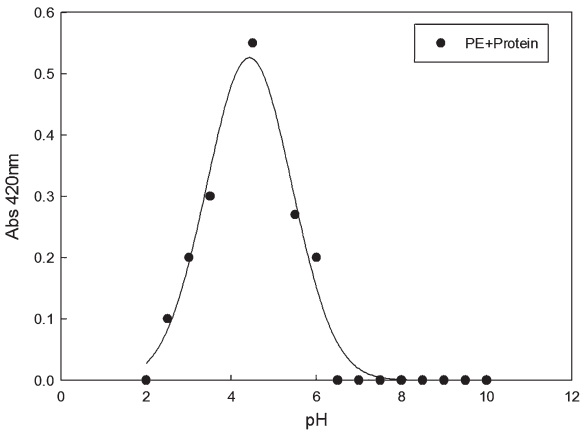

Figure 1. Phase diagrams: example of dependence of the absorbance at 420 nm varying the medium pH at a constant protein/polymer molar ratio for a basic protein and an acid PE.

The basic way to follow the formation of a non-soluble PE-P is to measure the turbidity (absorbance at 420 nm) of the medium vs. the pH. From these data it is easy to determine the pH zone over which the PE-P is non-soluble. Figure 1 shows an example of turbidimetry acid-base titration curves for the complex formation between a basic protein and an acidic PE. The maximum non-solubility of the complex can be seen between pH 4.0 and 4.5. Above pH 5.0, turbidity decreases probably because acidic groups of PE are protonated, losing their negative electric charge, so they cannot interact with the protein. Finally, at pH 6.0, the protein loses its positive electric charge, so there are fewer positive electric charges available to interact with the negatively charged PE groups. The best condition for PE-P non-soluble complex formation is at pH 4.5, where the turbidity is higher, as can be seen in Fig. 1. It is necessary, to make turbidity measurements of the protein and the polymer separately in order to check their behavior against pH.

A turbidimetry titration curve of the protein (at constant concentration, and pH of higher precipitation) with increasing PE concentration is carried out as shown in Fig. 2. When the PE concentration in the medium is increased the turbidity reaches a plateau which is the condition where all the protein is forming the complex. Turbidimetric titrations show sigmoid or hyperbolic behavior (Fig 2(a) and (b)). Examples of these types of system can be found in several published articles.[33,34]

The turbidimetry titration curve gives useful information about the ratio of PE-P formation, or the minimal amount of PE necessary to precipitate all the protein present in a desired solution volume, as is shown in Fig. 2. The point of intersection of the two hatched lines, one of them being the slope of the graph and the other the maximum absorbance obtained, and corresponds to a given concentration of PE, if the inverse of this value is multiplied by the concentration of protein, the result is the mass of protein per unit mass of PE (expressed in g because the molecular weight of a PE is generally not known). This value has been termed by some authors as 'e'[34-36] and it is interpreted as the pseudo-stoichiometric ratio of PE-P formation. PE-P formation is driven by a chemical equilibrium in which the following species coexist in equilibrium: protein, free PE and the complex formed. According to the strength of the interaction forces involved in complex formation, this balance will be more or less shifted to the complex formation as follows:

$$\text{Protein}_{(\text{soluble})} + \text{PE}_{(\text{soluble})} <=> \text{P} - \text{PE}_{(\text{non}-\text{soluble})} \quad (1)$$

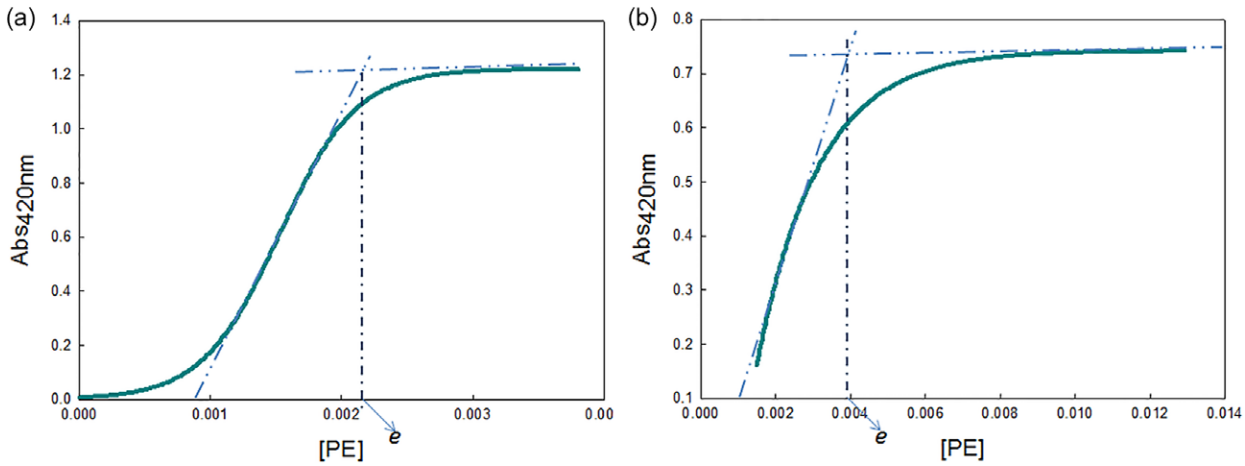

Figure 2. Example of turbidimetric titration curves of a constant protein concentration with PE at a fixed pH. The ***e*** parameter represents the experimental ratio protein/polyelectrolyte necessary to precipitate all the protein in solution.

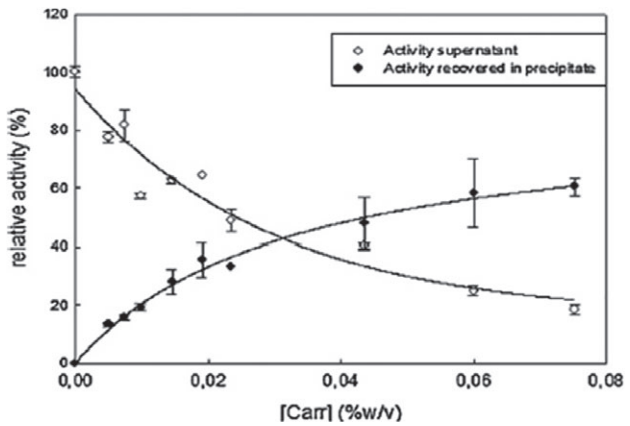

Figure 3. Recovery of the Chi activity in the precipitate and in the supernatant at different initial concentrations of precipitant agent (Carr). Medium 10 mmol L^{-1} acetate buffer pH 4.5. Enzyme activity was measured at pH 8.2.Temperature 25 °C. Taken from ref. 22.

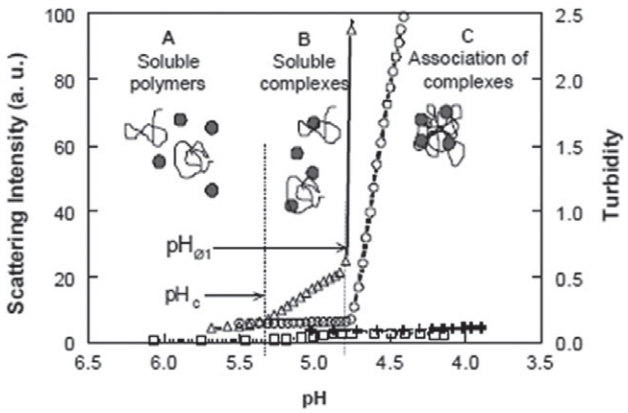

Figure 4. Turbidimetry and light scattering acid base titration using whey protein (0.1%) and gum Arabic mixtures (0.2%). A, B and C stability and instability regions (A, B, C); (Δ) scattering intensity measurements, (○) turbidity measurements, (□) scattering intensity of whey protein and (+) scattering intensity of arabic gum. [NaCl] 12.5 mmol L^{-1}. Temperature: 25 °C. Taken from ref. 36.

It has been determined experimentally[21,22] that the free protein concentration (Protein$_{(soluble)}$) expressed in the above equation is an adequate parameter to determine the PE capability of PE-P formation. Thus, when the PE interacts in a stronger way with a protein, it shifts the equilibrium to the right, the free protein concentration in solution being very low.

Figure 3 shows experimental equilibrium curves,[21] according to Equation (1). The relative Protein$_{(soluble)}$concentration in solution after precipitating the complex by addition of a PE is plotted vs. the total PE concentration added. It may be noted that as PE concentration increases, the percentage of free protein decreases to reach a constant value, depending on the type of protein and PE. From these equilibrium curves it can be seen that Protein$_{(soluble)}$ is not less than 20% and may reach 30% for the example of a chymotrypsin–carrageenan system (Fig. 3). Thus, the value of ´*e*´ calculated from Fig. 3 is valid for systems in which equilibrium is shifted largely to the formation of non-soluble complex. It can be see that 20 to 30% of the total protein was lost in the supernatant and so, it did not precipitate with PE addition. The ´*e*´ value calculated from the turbidimetric curve does not reflect the amount of complex formed relative to the initial amount of soluble protein; therefore, it should be taken as a pseudo-stoichiometry ratio. It will be an approximation of the true protein/PE mass ratio forming the complex. The true mass ratio protein/PE should then to be calculated from the experimental curve shown in Fig. 3.[21]

The examples presented in the above reports do not allow obtaining information about the microscopic behavior of PE-P formation. Light scattering has been used by several authors to obtain information about the interaction between proteins and PE.[37,38] Figure 4 shows an acid-base titration curve at constant protein/PE ratio varying the medium pH[38] which is similar to Fig. 1; the curves obtained from turbidimetry and light scattering measurements are shown. Light scattering measurements give the pH value at which the protein forms a soluble complex with the PE, this pH is $pH_{c.}$ Turbidity measurements show the pH value of macroscopic phase formation which corresponds to the formation of non-soluble complex this being pH_{ϕ}. According to Dubin *et al.*[39–42] the value of pH_{ϕ} is dependent on the experimental medium conditions, such as ionic strength, by which the protein PE complex formation may be modulated, while pH_c depends on the nature of the protein and PE.

In a number of cases it was found that at low ionic strength and at pH values for which the proteins and PE carry the same

net electrical charge the non-soluble complex is formed at pH below pH_c the phenomenon has been ascribed to the attraction between PE charges and oppositely charged 'patches' on the protein surface.[43]

PE-P AS TOOL FOR THE DOWNSTREAM PROCESSING OF PROTEINS AND ITS POTENTIAL USE IN SCALING UP

Criterion for the choice of an adequate PE for the protein precipitation

The interaction between proteins and synthetic polymers has been studied extensively, in particular for the modulation of living processes, immobilization or stabilization of enzymes, modification of substrate affinity, changing properties of food products, and for the development of many pharmaceutical applications.[36,44–48] Precipitation finds a place in most protein purification protocols and has traditionally been applied as a simple and rapid technique to protein concentration and purification at the beginning of downstream processing.[49]

A wide variety of synthetic and natural PE have been used to precipitate proteins that result in non-soluble complex formation.[41,50,51] The non-soluble complex can be easily separated by centrifugation or simple decantation. Precipitation as a product concentration step has several advantages, such as easy scale up, simple equipment and can be based on a large variety of alternatives.

An ideal PE for protein precipitation must:[40,42,44,52–54]

- contain free electrically charged groups for ligand coupling;
- not interact strongly with the impurities to prevent non-specific co-precipitation;
- preserve the secondary and tertiary structures of the protein without adversely affecting their biological activity;
- give good yield and purification;
- be commercially available and cheap:

Besides, the precipitate formed should be:

- easily solubilized: the precipitation–solubilization process should be carried out by pH changes or by ionic strength;
- non-toxic so that its use may be allowed by food regulations.

In general, enzyme purification using a PE involves essentially the following steps, as is shown in Fig. 5.

First step: Selection of a PE having an opposite charge to the target enzyme that will be separated. In order to do this, it is necessary to know the protein isoelectric pH and the pKa of the PE. Figure 6 shows the general strategy for this selection, taking as example a protein with isoelectric pH 5.0 and 9.0

A protein with an isoelectrical point (pI) of 5.0 was selected since 60% of the proteins have a pI close to this value. The most common acid base groups present in the PE are sulphonic (pK_a 1.0), carboxylic (pK_a 4.0) and weakly basic (NH_2^+) (pH 6.5). The interaction of a protein with the SO_3 groups occurs over a wide range until the medium pH reached the isoelectric point enzyme where the protein passes from positive electric charge to negative electric charge. In the case of using a PE with weak acid groups (COOH), the protein–PE interaction begins at a pH where the acid groups lose their proton until the pI of the protein. In this case the interaction zone is greatly reduced with respect to the above example. In the case of a PE with weak basic groups, the interaction zone will have a pH lower limit of the PI of the protein and an upper limit of pK_b of PE; as shown in Fig. 6 the pH interval of interaction is reduced, in this example, to pH 5.0 to 6.5.

Protein with extremely basic pI; as is the case of Try (pI 10.5) the pH zone of the interaction increases significantly with the two acid PE assayed, but does not interact with a weak basic PE (Fig. 6).

Second step: The next step is to verify in an experimental manner the protein–PE formation conditions; so the curves shown in Figs 1 and 2 are obtained assaying the complex formation using the pure target protein and the selected PE, to determine the pH interval over which the complex is formed and the minimum concentration of PE needed to fully precipitate the protein.

After selecting the PE, we must verify that it does not affect the secondary and tertiary structures of the target enzyme and thus its biological activity. This condition is important to validate the method to be applied. This is accomplished at the pH wherein the complex is soluble and using the pure form of the target enzyme. Then the pH value at which precipitation PE-P is higher should be selected, and a turbidimetric titration curve at a constant enzyme amount with increasing PE concentration is carried out. It is well documented that the presence of a PE should produce a modification in the biological activity of an enzyme due to a change induced by PE on the secondary and tertiary structures.[55] This may be caused by interaction due to either the electrostatic forces or the hydrophobic effect involved in the complex formation. Consequently, PE can break interactions between groups of the protein necessary for the structure or the PE may interfere with the active site of the enzyme. So, it is necessary to assay the effect of PE on the target enzyme. The recommended studies are:

- Biological activity of the enzyme in the absence and presence of PE at increasing concentrations.
- Circular dichroism spectra of the enzyme in the absence and presence of PE at increasing concentration. This technique is very useful because it shows modifications to the secondary and tertiary structures of the protein induced by the PE.
- Enzyme thermal stability in the presence of PE. This technique allows us to know the melting temperature of a macromolecule (T_m), the temperature where 50% is in native form and 50% is in its denatured form. The information given by this technique is useful in the sense that it allows us to determine whether the presence of PE increases or decreases the thermal stability of an enzyme.[55]

Picó *et al.*[55] reported that some polymers decreased the biological activity of Chymotrypsin, suggesting a modification of the tertiary structure in the catalytic site. Synthetic PE having strong acid or basic groups interacts with high affinity with the protein by coulombic strength, also the presence of hydrophobic chains interacts with the hydrophobic patches in the protein surface; together, these induce a loss of water structure which contribute to the denaturalization of the macromolecule with loss of biological activity.

Third step: Having determined the best variables in the PE-P formation, the precipitation of the target macromolecule from its natural sources (animals and plant homogenates or microbiological suspension) should be assayed. Obviously, the great concentration of biomass (which represents around 99.9% of the total protein concentration with respect to the concentration of target protein present) has a negative influence on the experimental variables determined for pure enzyme precipitation in buffer solution. In a complex system such as a homogenate, there is an interaction between the PE and cellular wastes, such as membrane and other

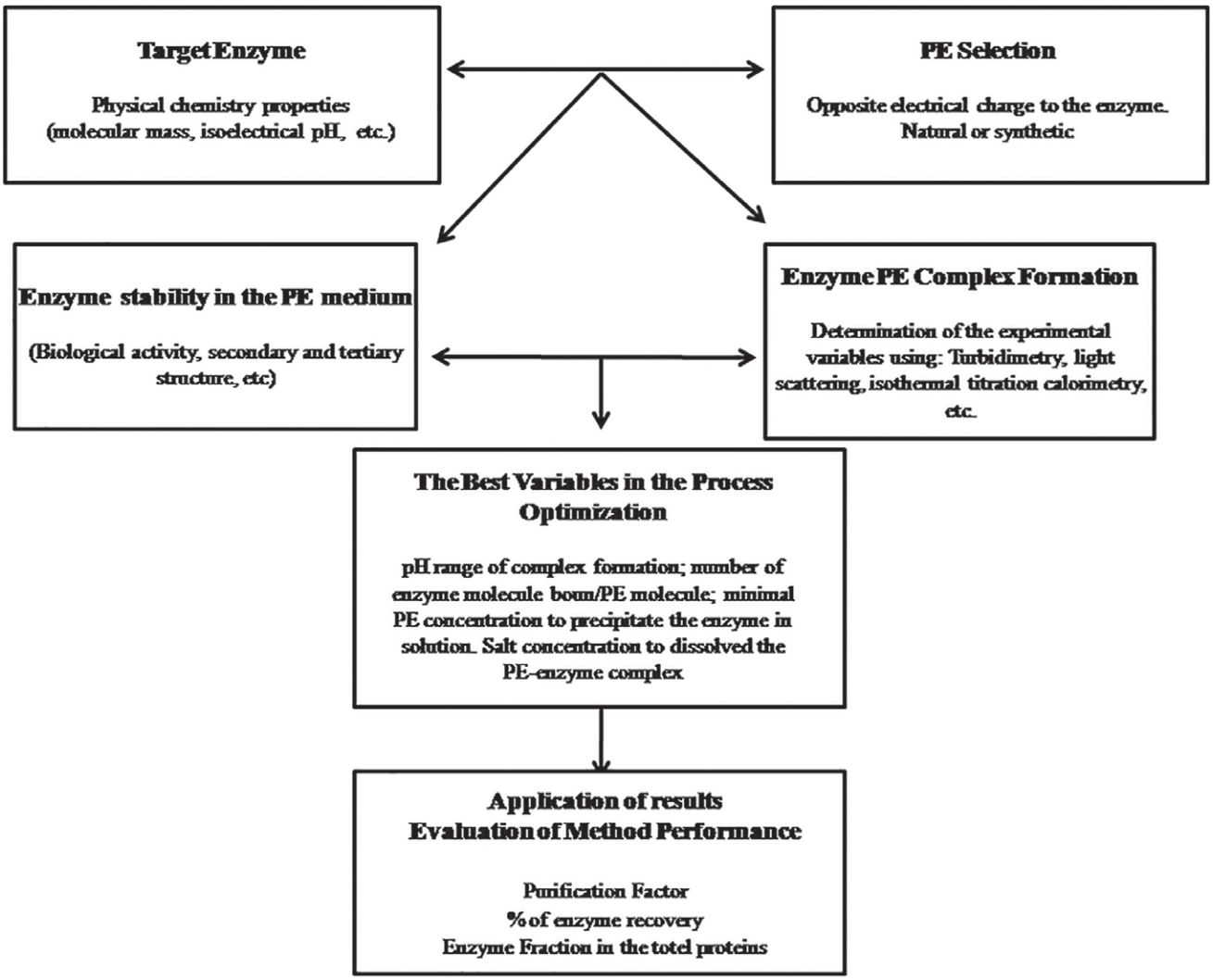

Figure 5. Practical strategy for the enzyme isolation process using PE–enzyme complex formation development and scale-up for the downstream processing of industrial enzymes.

proteins, also protein–protein interaction are produced. One consequence is that part of the PE reacts with other macromolecules and cellular debris, thus producing a greater requirement of the PE mass than the value calculated from the experiment using the target pure protein, so part of the impurities precipitate with the target protein, contributing to decrease the yield of the process significantly.

A great number of synthetic PEs have been used for the precipitation of target enzymes with a significant yield. As is the case of polyacrylate, which was used to isolate amylase from a fungi culture, a purification factor around 2 and recoveries of 70% were found.[44] Also, proteases from fresh pancreas homogenate was isolated with a purity of 5-fold and a recovery of 33% under the best conditions tested.[56] Woitovich *et al.*[57] using Eudragits ® L100 and S100 (a polyacrylate derivate) purified peroxidase from a fresh extract of *Raphanus sativus L*, recovery of biological activity was 50% of the initial activity in the homogenate with 1.5-fold increase in its specific activity. The total Eudragit® concentration to precipitate the enzyme was very low, about 2×10^{-3}% w/v with a reduction of the volume of the final product of 10 times.

Natural PEs are ideal as precipitate agents for proteins, because they are non-toxic and environment friendly. The cationic PE chitosan interacts with lipase from *Candida rugosa*, which allows separation of this enzyme from a crude extract of fungi with an enzyme recovery > 90%.[51] β-lactoglobulin forms complexes with chitosan, and can be recovered with a yield of 90% and protein purity of 95%.[58]

The above examples show that the yield of the precipitation process by PE addition is in the order of 50–90%, with purification factor around 1.5 to 4.0. Analyzing both variables, the precipitation process of an enzyme by PE could be considered of moderate performance, but it must be taken into account that a single operation is being applied (precipitation) to a system which has not previously been treated. On the other hand, the second variable, the purification factor with values of 1.5 to 4.0, would seem to be very low. However, given the very low concentration at which the target enzyme is present in its original source, this value may be considered as acceptable.

Proteins–salts precipitation vs polyelectrolytes–protein non-soluble complex precipitation

The precipitation technique of a macromolecule by forming a poorly soluble PE-P was quickly introduced as a viable alternative to compete with precipitation using salts such as ammonium

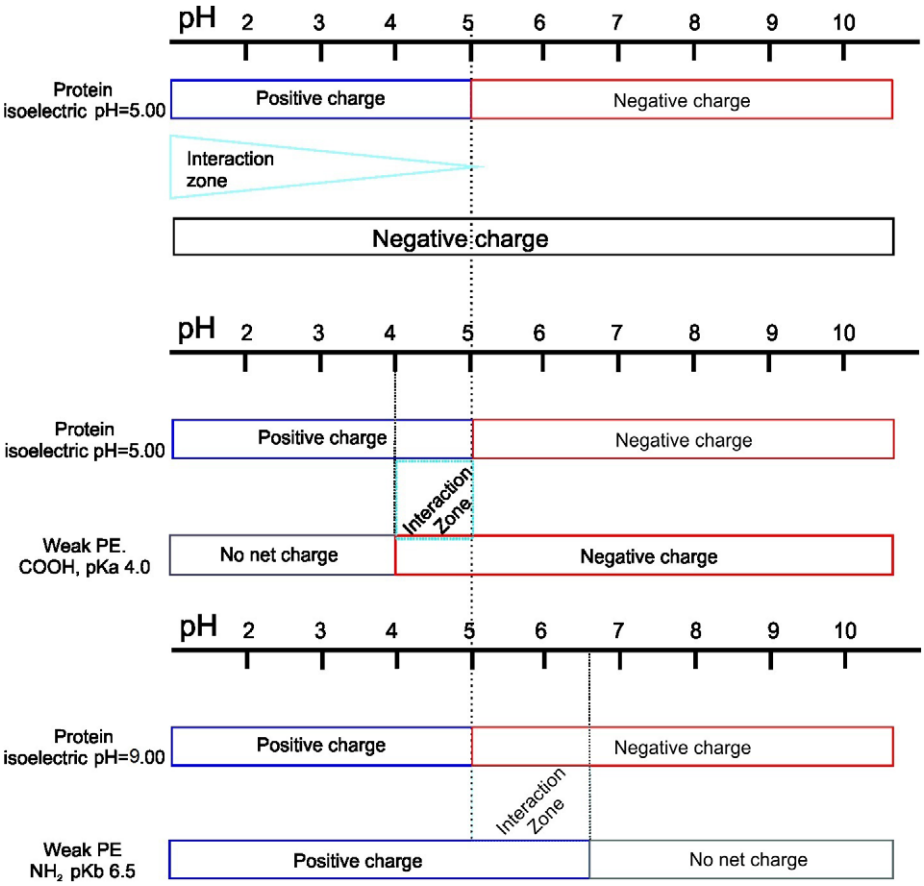

Figure 6. The possibilities of interaction between a protein of pI 5.0 and a protein with pI 9.0 with weak and strong acid polyelectrolytes.

sulfate, or synthetic not electrically charged polymers, like polyethylenglycol.[59] But, there are important advantages and disadvantages to both techniques. Precipitation with PE uses a total concentration in the order of 0.001 to 0.15% w/v, against values of 20 to 50% w/w of salts and non-electrically charged polymers. Besides, as PEs are polysaccharides, they are capable of thermodynamically stabilizing macromolecules in solution. Their low cost and the low concentration required for precipitation makes the process inexpensive. They do not interfere with the process for which the enzyme is being used and do not have negative effects on the final product, since most of them meet the standards of the FDA regulations.

Table 1 summarizes the advantages and disadvantages of precipitation with PE as a downstream process for protein in general.

Protein precipitation with PE combines several operations in only one: separation, enrichment and concentration of a target protein, avoiding the addition of other operations such as the removal of part of the biomass by centrifugation and the elimination of high amounts of salts by dialysis. It also promotes a reduction of the end volume in a significant manner. All this leads to an easy application of a scaling up procedure. A general platform for the pre-purification of industrial enzymes using PE precipitation usually includes the following steps: the target enzyme is produced by the biomass (microorganism suspension, animal or vegetal), filtration for the retention of cell and cell debris is necessary if the biomass amount is high. However, at the same time, it is possible to add the PE concentrated solution directly on the biomass suspension which is then are mixed (at pre-established conditions) in a stirred tank. A decantation process follows, allowing separation of the solid phase (the protein-PE rich phase) from the rest of the solution. The above decrypted step includes several unit operations such as centrifugation of a large biomass volume, a significant volume reduction of the

Table 1. Advantages and disadvantages of precipitation using PE in comparation with other precipitation methods

Polyelectrolytes	Salt or solvent precipitation
Low concentration of the precipitant agent 10^{-3} to 10^{-5} % P/V	Higher concentration: 50% P/V (ammonium sulfate). 20% P/V (ethanol)
No modification of the secondary and tertiary structure of the macromolecule	A modification of the secondary and tertiary structure may occur
Non-toxic if natural PE are used They can be discarded in the environment	Salts in high concentrations are toxic. They cannot be discarded in the environment
No recycling process is necessary	Recycling process is more likely
Same time PE may be more expensive than salt, this is compensated by the small amounts of PE used	Salt like ammonium sulfate is not expensive, but the higher amount used represents a significant total cost of the process
Yields are in the range 50–90%	Yields higher > 80%
It is not necessary to eliminate the PE	Requires removal of salt using dialysis

initial volume of biomass (a reduction of 10 to 50 times in the same time), and the production of a solid phase rich in the target protein. Because the target protein is present in a solid phase and bound to a PE, this structure has high thermodynamic stability, allowing the protein to be stored in the form of PE-P.

The effect of PE on enzyme stabilization and stability

PE-P formation induces a more ordered structure, so the thermodymic stability of the complex is greater than that of PE or protein alone. As result, an elevation of the protein melting temperature upon complexation with PE is observed. Complex stability is also influenced by the hydrophobicity and electric charge density of PE and protein. Because natural PEs are polysaccharides, these are excluded from the protein domain, increasing the hydration of the macromolecule, so a thermal stability is observed. Also, PE induces preservation of enzymatic activity through time, especially when the enzyme is stored in solution for long times.[34,60]

CONCLUSIONS AND FUTURE CHALLENGES

During past years, the production of industrial enzymes has been boosted by remarkable progress in the upstream processing. However, the platforms that are being used currently are limited in terms of performance and scalability in downstream processing, which generates a bottleneck in this area. In addition, downstream processing costs form a major component of the final cost of enzymes. For this reason, in recent decades laboratories around the world have focused their efforts on developing new methods of purification and concentration of macromolecules.

The proteins may interact with PE resulting in soluble or insoluble complexes and this interaction can be used as a method of purification and concentration of proteins. The development of the chemistry of natural PE from the 1980s, enabled provision of non-toxic, environmental friendly PE (most were polysaccharides), which were easily degradable by microorganisms. PE-P are a valuable alternative for the design of platforms to isolate and purify protein due to their relatively easy scalability, capacity of continuous operation and high capacity. The possibility of using natural PE is an important tool for the protection of the environment. Studies of the last 10 years have contributed to knowledge of the molecular mechanisms of complex formation and the behavior of the physiochemical variables which drive this process. Validation of the method by which the complex formation is produced, is proved by the number of patents published not only in the enzymes industrial production area, but also in the purification of antibodies, judging by its use in animals or humans.

In the last 5 years, there has been a significant increase in the number of addressing the formation, knowledge and molecular mechanism of these complexes, especially when involving natural PE or when the target protein has a biotechnological application. All the experience gained has allowed the development of scaling methodologies applicable to macromolecules, especially enzymes, with application in biotechnological processes. This technology contributes to solving a few important items: first during the bioseparation process, the way to concentrate and eliminate the cellular debris and other proteins from a fresh homogenate of a microbiological suspension, animal or vegetal and at last, the improvement over the currently established methods in terms of economic and environmental sustainability.

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