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REVIEW

Utilization and prospect of purification technologies in natural proteins, peptides and recombinant proteins

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Abstract
Protein, being a fundamental biological macromolecule, assumes a critical function in various vital activities of cellular growth, heredity, reproduction, and other biological processes. Moreover, the utilization of recombinant proteins is extensive in the advancement of pharmaceutical materials, enzyme industrial implementation, and fundamental proteomics investigation. Effective purification methods are necessary for the production of recombinant proteins as well as natural proteins and peptides to ensure their efficient production. Various methods have been developed to improve protein purification, incorporating the recent advancements in protein separation technology. This review is focused on the sophisticated approach to the purification techniques of protein, peptide, and recombinant protein. The system known as the multi-column plate adapter (MCPA) proves to be highly cost-effective in the field of protein purification methods. The assessment has determined that the method of employing an aqueous two-phase system (ATPS) is an economically sound, and highly efficient approach in protein purification. Thus, the ATPS might represent a feasible approach to single-stage purification, deviating from the complex chromatography procedure. The chromatography strategies have been improved for the purification of peptides including batch liquid, continuous and semi-continuous chromatography. In the realm of recombinant protein purification, affinity purification presents several advantages compared to alternative methods. To produce, identify, and purify recombinant proteins from their host systems in large quantities with high efficiency, affinity strategies—particularly fusion strategies—have been developed. Hence, the purpose of this review is to implement the various purification technologies, their potential employments, fundamentals, advantages, and limitations.

Keywords Advanced techniques · Recombinant protein · Natural protein · Peptide · Protein purification

Introduction

It is essential to purify proteins to properly understand their functions, interactions, and structures. The isolation of high-quality proteins plays a crucial role in protein purification. Additionally, purified proteins are vital in the development of novel therapies and biotechnological products. Challenges persist with the ineffective process of expressing and purifying recombinant proteins specifically in the field of biotechnology. The determination of the interconnection between a protein and a ligand holds significant importance in the realm of biochemistry and the discovery of novel

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pharmaceuticals (Salleh et al. 2022; Selmi et al. 2019). Consequently, the process of protein and peptide purification assumes a crucial role in both academic and industrial sectors (Liu et al. 2020). Diverse approaches have been implemented to streamline the purification protocol, encompassing the affinity-based methodology (Colgrave et al. 2022; Salleh et al. 2022; Mahmoodi et al. 2019). The expression of proteins from different species is an essential procedure to investigate the biological functionalities of genes, explore the industrial employment of enzymes, and pharmaceutical proteins conduct fundamental research in proteomics (Tripathi et al. 2019; Banki and Gerngross, 2005; Healthcare 2007). The multiplicity of diverse purification methods has expanded owing to the distinct characteristics and quantities of recombinant and natural proteins and peptides. The efficient purification methods and high-yielding expression hosts are imperative for achieving cost-effective production of recombinant proteins (Klopp et al. 2018). The process of isolating, separating, and purifying a variety of proteins, peptides, and specific molecules is utilized in a wide range of fields within the realms of bioscience and biotechnology. Consequently, the progress in understanding and utilizing separation techniques is a crucial aspect of research and development in the field of biology. New methods for separation are required to effectively handle solutions with low concentrations of desired molecules, along with various other compounds present, including particles (Liu et al. 2020). In an optimal strategy for purification, it is essential to achieve equilibrium among several factors that impact the ultimate outcome. These factors encompass the velocity of purification, the rate of recovery, the capability, and the distinctiveness. The attainment of a critical objective for emerging bioseparation technologies is the advancement of uncomplicated, dependable, expandable, and expeditious techniques for the isolation of proteins. Natural proteins or proteins engineered through genetic modification are frequently found in combinations, and a significant number of essential proteins exhibit low quality in biological materials. Hence, the extraction of proteins, while ensuring that their structure, composition, and functionality remain unaltered, has consistently posed a challenge in the field of protein research (Liu et al. 2020). With the advancements in the field of technological progress, numerous novel methods have been employed for the purpose of purifying proteins. Particularly, the appeal lies in the platform techniques that can be readily employed on novel protein targets with limited optimization (Liu et al. 2020; Dominguez et al. 2018; Zhou et al. 2020a, b; Arshad et al. 2017; Li et al. 2020; Ma et al. 2019). These comprehensive techniques possess the capability to expedite the process of investigation and improvement for new innovations and diminish the duration necessary for commercial distribution. Concerning the significance of protein purification, this review intends to discuss recent developments in improving techniques for separating natural proteins peptides and the recombinant proteins.

Novel methods in natural protein purification

System of multi-column plate adapter

Many investigations pertaining to protein structure and functionality necessitate the utilization of various techniques to establish effective and efficient methods for purifying proteins. The phenomenon of purifying therapeutic proteins, including antibodies, is denoted. Subsequently, various technologies have emerged to fulfill the requisite. Nevertheless, attaining greater protein output and purity in a single process typically involves a higher cost that may not be practical for small-scale research purposes (Cha and Kwon 2018). The present instruments employed in this field encompass automated protein maker, fast protein liquid chromatography (FPLC) and immobilized metal affinity chromatography (IMAC). Some scientists posit that it may be possible to devise a more cost-effective technique for purifying proteins that offers enhanced purity and a reduced time frame by utilizing a collection plate with multiple wells and employing the force of gravity. This concept serves as the fundamental principle underlying the multi-column plate adapter (MCPA) system. The MCPA system offers the flexibility to be configured in diverse manners and under varying conditions, encompassing buffer systems, imidazole concentration in wash buffer, and volume of lysate load and alternative resin types (Matte 2020). The MCPA system is comprised of an array of elongated drip filter plates that are equipped with a sealing mat, along with additional customary laboratory materials. Subsequent to the process of cleansing, this system can be utilized repeatedly for the purpose of protein purification. The utilization of affinity chromatography is implemented as a technique for purifying proteins within the system. It has been observed that certain researchers have employed ion-exchange column chromatography in conjunction with the aforementioned method (Kineavy et al. 2021). A research investigation on the efficiency of a protein purification method utilizing affinity chromatography and the exclusion of vacuum pressure, which has a relatively affordable price, yielded satisfactory results. The investigation proficiently isolated yeast AbpSH3 mutants by means of nickel (Ni) resins during the process of denaturing and native purification circumstances. This observation was deduced through the utilization of SDS–polyacrylamide gel electrophoresis (SDS-PAGE) examination. In the circumstance wherein the samples were subjected to native purification states, it was discovered that the existence of common impurities at

25 kDa, which were initially identified under denaturing predicament (Dominguez et al. 2018), were successfully eliminated through the purification process. The method of purifying proteins has been expanded from utilizing one column for each protein across 24 various specimens to employing 12 columns for each protein across two distinct samples. The samples exhibited a considerable degree of purity, harboring identical impurities to those obtained through protein purification carried out on a small scale. Researches (Dominguez et al. 2018) unveiled that the MCPA system employed for purification of protein likewise yielded an adequate quantity of protein suitable for biophysical examination, including circular dichroism (CD) spectroscopy. The use of MCPA system comes with certain limitations. Purifying antibodies using this technique remains a difficult task. The challenges in developing antibody therapeutics vary between the early and later stages. At the start, numerous constructs need to be produced and purified in small microgram to medium milligram quantities to assess product characteristics and detect manufacturing risks through various in vitro and in vivo tests. Although it is relatively easy to parallelize purification processes on a small scale (milligram) using MCPA method, scaling up automation and parallelization for larger antibody production volumes presents increasing difficulties as the quantity of products being captured increases (Matte 2020).

System of aqueous two-phase

The aqueous two-phase system (ATPS) is a method of separating liquids that has demonstrated significant promise in the restoration, extraction and refinement of a diverse range of biological substances (Singla and Sit 2022; Asenjo and Andrews 2011). ATPs provide a chance to apply the principles of liquid–liquid extraction from traditional chemical engineering to cleanse biological products. The existence of organic solvents and extreme process conditions like high temperature or pressure may limit the use of liquid–liquid extraction, posing a risk of harming biological products (Grilo et al. 2015). ATPS can be created with environmentally friendly materials in a gentle water-based setting. Studies have shown that the key characteristics of ATPS for biomolecule purification are minimal tension at the interface and a high water concentration, which helps maintain the biological activity of molecules. ATPS can arise through the combination of different substances in a water-based solution. Among these combinations, dual-polymer systems like PEG/dextran have shown significant success in separating various products. These systems are commonly employed in initial studies due to their extensive research and well-documented effectiveness. Soon after, dextran alternatives began to gain popularity. PEG alternatives, specifically thermoseparating EOPO copolymers, emerged as more cost-effective options compared to dextran. Polymer/salt systems also proved to be effective alternatives with impressive results at a reduced cost. Citrate, phosphate, and sulfate were identified as the most commonly used salts in these systems (Singla and Nandam Sit 2022). The employment of this method is frequently observed in the process of isolating and refining enzymes and proteins. Menegotto et al. (2021) utilized ATPS for the purification of the *Arthrospira platensis* protein. They applied a step-by-step approach in their experimental design to assess how much protein could be recovered from the *A. platensis* extract using ATPS consisting of varying molecular weights of polyethylene glycol (PEG: 1500, 4000, 6000, and 8000 Da) and a saline solution containing potassium phosphate and sodium citrate. Optimal conditions for the recovery of A. platensis protein were determined through the use of 16% sodium citrate and 18% PEG 1500 Da, resulting in a purification factor of 1.02 and a protein recovery rate of 75%. The beneficial of using ATPS technique in the purification of proteins is evident in the uncomplicated and rapid separation process, while simultaneously minimizing the denaturation of enzymes (Ahmed 2016; Gupta et al. 2007). The component in both stages that holds utmost significant is water which accounts for approximately 80–90% of the composition. Additionally, a majority of polymers possess the ability to stabilize the structure of proteins, as indicated in the investigation (Rosa et al. 2007). There exist a variety of ATPS, including micellar aqueous two-phase systems (ATPS-M) as well as polymeric aqueous two-phase systems (ATPSP) (Vicente et al. 2019). Furthermore, there are ionic liquid-based aqueous two-phase systems along with reverse aqueous two-phase micellar systems (ATPS-RM) (Nie et al. 2022; Lopez 2014). This method has its drawbacks, including a lack of comprehensive knowledge at the molecular level and constraints in effectively modeling and enhancing partitioning processes. One of the main obstacles hindering the wider adoption of ATPS in industrial settings is the absence of reliable models to predict the partition of desired products (Torres-Acosta et al. 2018). Despite the current lack of specific models, there is ongoing research dedicated to their creation in this field. The effectiveness of predictive models in predicting outcomes has been demonstrated, yet a major drawback is the significant variability observed when the characteristics of any given ATPS are altered. ATPS can consist of diverse phase components and additives, and the operation conditions can vary, such as using different pH levels or temperatures. Another disadvantage of this method is its constraints when implemented in industrial settings, such as issues with phase separation and the expense of polymers (Zimmermann et al. 2017). Systems with elevated salt levels can also experience corrosion of metal pumps,

lines, or other equipment due to the corrosive properties of the phases. The cost of employing ATPS in industry might also be limited by the expenses related to managing and disposing of certain components used for forming phases. Following the separation process, handling components of the phase system can be made easier through various methods, like using polymers that can be easily biodegraded and recovered. Expenses related to polymer-conjugated affinity ligands could be minimized by substituting dextran with more affordable options such as crude dextran, polyacrylic acid, or hydroxypropyl starch (Mc Queen and Lai 2019; Torres-Acosta et al. 2018).

System of cell-surface display based on sumo–ulp1

The technique of cell-surface display offers the possibility to specifically direct proteins towards the outer membrane of microbial cells. This is achieved through the fusion of an anchoring motif, which ultimately results in the completion of the cell-surface display process. This particular method, widely recognized as a pragmatic approach, has gained considerable acclaim for its versatility in a multitude of fields, such as the advancement of immunizations, the fabrication of eco-friendly bio-absorbents, and the engineering of biocatalyst complete-cells (Salleh et al. 2022; Lee et al. 2003). Recently, the proposition was made that this particular method possesses the capability to streamline the process of protein purification. Zhou et al. (2020a, b) documented the utilization of cell-surface display within the realm of protein purification. This involved the utilization of Ulp1 protease, which facilitated the cleavage of a SUMO-fused target protein that had been exposed on the exterior membrane of *Escherichia coli*, as depicted in Fig. 1. Following the initiation of protein synthesis, the cells are collected, suspended in a solution for cleavage, and allowed to incubate together. The bacterial cells carrying SUMO and Ulp1 on their surfaces can be conveniently separated using a centrifuge. Following protein induction, the cells are collected, mixed with cleavage buffer, and incubated for the cleavage process. Centrifugation allows for efficient removal of bacterial cells carrying SUMO and Ulp1 on their exteriors. The purification technique was assessed by utilizing red fluorescent protein (mCherry). Following just 30 min of incubation, a yield of purified mCherry protein (7.72 ± 1.05 mg from 1 L of bacterial culture) was achieved. The protein purity exceeded 80%, and there is potential for enhancement through basic ultrafiltration to achieve purity levels exceeding 90%.

The utilization of SUMO, a protein analogous to ubiquitin, has been employed to enhance the stability and solubility of the target protein via fusion at the N-terminus (Bird 2011). The Ulp1 protease cleaves the SUMO tag

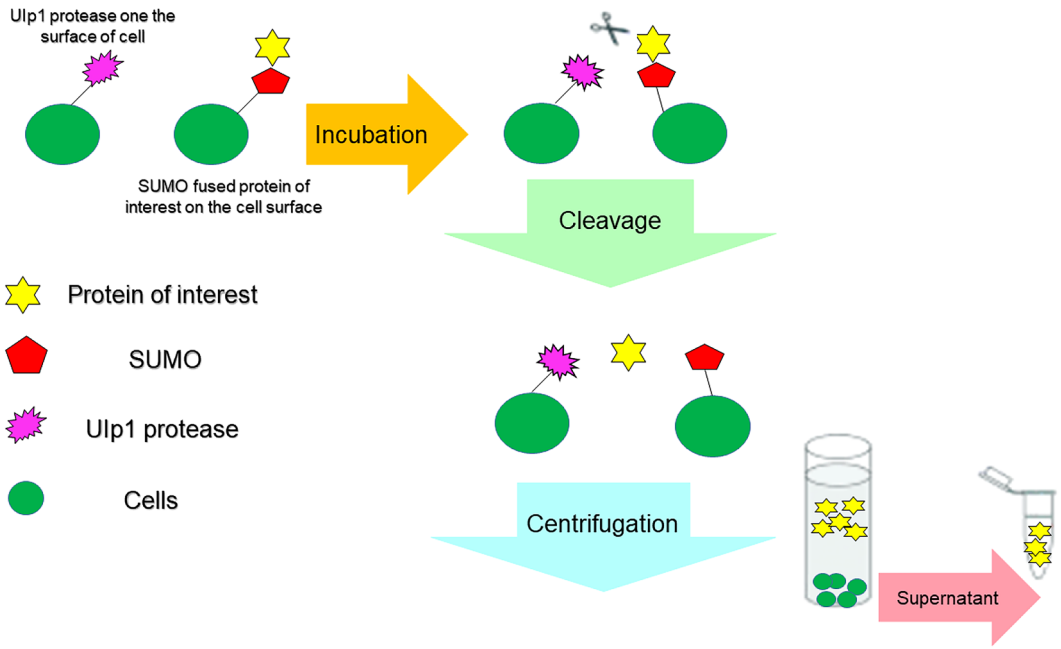

Fig. 1 Illustration of the protein purification strategy based on cell-surface display of SUMO-fused recombinant protein and Ulp1 protease. The SUMO tag can be removed from the chimeric protein using Ulp1 protease, which identifies the specific SUMO structure for producing the original protein without additional amino acids. The Ulp1 enzyme, which is displayed on the surface, cleavage the N-terminal fusion protein of SUMO, causing the release of the original target protein in the buffer solution. Since both SUMO and Ulp1 are attached to the cell surface, they can be conveniently eliminated through centrifugation

located at the N-terminus, causing a disruption in the SUMO tertiary structure. This prevents incorrect cleavage within the target protein and results in the generation of a natural target protein without extra amino acids (Li et al. 2018). The efficacy of the SUMO-Ulp1 configuration in the purification of proteins can be achieved through the utilization of two distinct vehicles: (i) the induction of the target protein fused with SUMO on the surfaces of cells and (ii) the induction of the Ulp1 protease on the surfaces of cells (Zhou et al. 2020a, b). In this particular system, the N-terminus of a target protein that is fused with SUMO will undergo cleavage through the action of the Ulp1 protease that is displayed on the surface. This phenomenon results in the liberation of endogenous target proteins within the solution of the buffer. The surface-expressed SUMO and Ulp1 protease can be eliminated concurrently with the cells via the process of centrifugation. Consequently, the desired protein can be gathered in the configuration of a supernatant subsequent to the centrifugation, and enhanced to a higher degree of purity through the uncomplicated technique of ultrafiltration (Zhou et al. 2020a, b).

Chromatography methods

Chromatography comprises a collection of separation methodologies that encompass the deceleration of molecules in relation to the mobile phase, leading to the establishment of a quantifiable segment (Coskun 2016; Chigome and Torto 2011). Chromatographic methods employed for the separation of protein mixtures have proven to be highly efficacious and have been extensively employed for the purification of distinct proteins (Garland et al. 2023; Ye et al. 2007). The process of purification relies on the dimension of protein, electrical charge, its hydrophobic nature, as well as its interaction with specific biological compounds. The specific attributes of the protein are depicted in Fig. 3 (Geng 2022; Hedhammar 2005). The initial step of the chromatography process commonly involves a stage of capture, during which the target protein is bound to the adsorbent while the impurities remain unbound (Baur et al. 2016). Furthermore, proteins with weak binding affinity will be rinsed out, allowing the target protein to be released (Hedhammar 2006). Various chromatography techniques exist including affinity and ion exchange chromatography (Gulhane et al. 2022; Lee and Lee 2004).

Advancement in the chromatography techniques

Chromatography methodologies have exhibited extraordinary advancements, presenting elevated precision, discernment, and effectiveness. Different forms of chromatography, such as size exclusion, affinity chromatography, hydrophobic interaction, and ion exchange chromatography, have attained the status of conventional techniques in the process of protein purification. Advancements in the field of genetic manipulation and the development of novel expression systems have facilitated the process of synthesizing large quantities of recombinant proteins. Expression in various cell systems, including mammalian, bacteria, plant, insect and yeast cells, has become a customary practice, offering a wide range of alternatives for the synthesis of proteins. Protein purification has changed dramatically with the transformation of flexible protein tags like MBP-tag, His-tag, and GST-tag. This makes the purification process easier by enabling quick and targeted binding to affinity resins. By addressing the difficulties posed by the hydrophobic nature of membrane proteins, techniques for isolating them have greatly improved. Among the methods that have been successful in producing functional membrane proteins are nanodiscs, detergent solubilization, and amphipols (Salvatore 2023).

Types of chromatography, their advantages and obstacles in protein purification

Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is a highly adaptable method that can separate protein molecules based on variations in hydrophobic properties, similar to how reversed-phase separations function. HIC uses non-denaturing solvents and is commonly used for purifying proteins, in various stages such as capturing, intermediate purging, and refining. Additionally, due to its capacity for distinguishing between substances with slight differences in hydrophobicity, HIC offers benefits in isolating variations with subtle modifications that may reveal a greater or lesser hydrophobic surface (Ewonde 2022). HIC employs a stationary phase that is somewhat resistant to water, often with shorter ligands compared to those used in reversed-phase chromatography. Popular ligands include C4, and the binding density of the ligands is usually lower. A strong salt solution is incorporated to encourage protein bonding with the hydrophobic regions on the bonded phase. Gradual reduction of the salt concentration during the process enables the protein to be released, with elution typically occurring from the least hydrophobic to the most hydrophobic molecules in HIC. When the eluent salt concentration is high enough, the protein's hydrophobic regions are more likely to bind with the hydrophobic stationary phase (Lothert and Wolff 2023). This results in sharp chromatographic peaks as the protein molecules concentrate in that region. Ammonium sulfate is commonly employed as the salt in HIC due to its high efficiency in precipitating proteins. This helps enhance the interaction between the protein and

the stationary phase without compromising the protein's structure. Water-soluble substances like detergents are able to decrease protein binding at low levels by competing with proteins for binding spots on the surface of the stationary phase (Edwonde 2022). One of the key benefits of HIC is its ability to offer a level of selectivity that cannot be achieved with IEX as well as affinity resins. Another benefit of this method is that separation happens under mild-in most cases non-denaturing circumstances (Fekete and Murisier 2021). HIC has a number of drawbacks, such as experiencing noticeable alterations in baseline when gradient elution is used and needing mobile phases that are not easily evaporated, making the process of isolating peptides more complicated. However, this method is still employed for purification of small peptides as well as antibodies (Chen et al. 2022).

Liquid chromatography

Liquid chromatography (LC) is a method of separation where substances are divided depending on their adsorption to a stationary phase and a moving liquid phase. In LC, the sample is inserted into a column containing a static phase like silica gel or polymer. The sample (crude peptide) is then pushed through the column with a moving solvent known as the mobile phase. Different components are sorted depending on how much they are attached to either the stationary or mobile phases while passing through the column and coming into contact with the stationary phase. Various techniques, such as UV–visible spectroscopy and mass spectrometry, are employed in discerning the individual components that have been separated (Fekete et al. 2023). Utilizing the LC has various advantages. The popularity of LC comes from its wide array of separation modes, the ability to adjust selectivity through various parameters, as well as its durability, dependability, and ability to work with different detectors. Nevertheless, LC's ability to separate compounds is often restricted when analyzing intricate samples with numerous components (Burlet-Parendel and Faure 2021).

Ion chromatography

Ion chromatography (IC) is a method of chromatography that sorts elements based on their electrical charge (El Rassi 2017). IC generally involves a resin-based stationary phase with charged groups like sulfonic acid or quaternary ammonium. The specimen is inserted into the column, and a fluid known as the mobile phase, usually consisting of an electrolyte solution, is employed to propel the specimen along the column. The stationary phase influences the movement of the components in the column, separating them based on their charge. Various techniques such as conductivity or UV–visible spectroscopy are employed to identify the individual components that have been separated (Rybakova 2023). IC has a key benefit in being able to identify and measure a diverse array of ions, including inorganic and organic compounds, even in intricate mixtures. An advantage of IC is its capability to analyze numerous ions at once during a single operation, which helps in conserving both time and resources. This method is very sensitive, able to identify ions in extremely low concentrations, sometimes even at levels as minute as parts-per-trillion (ppt) or parts-per-billion (ppb) (Nesterenko 2023). IC also offers the benefit of selectivity. By utilizing various stationary phases like anion exchange and cation exchange, specific ions can be targeted for analysis. This targeted approach helps in minimizing interference from other ions in the sample, leading to more precise results (Jensen 2023). IC is a technique that is easily operated, allowing analysts of all levels of experience to use it effectively. Ion Chromatography has its benefits, but it also has some drawbacks. One significant limitation is the absence of a universal detection method. Although conductivity detection is widely utilized for its sensitivity to ions, it is unable to offer details about the structure of the separated substances. The capacity of IC to analyze non-ionic species or molecules that do not effectively interact with ion-exchange resins is limited. Furthermore, smaller laboratories and research facilities may encounter obstacles due to the expenses related to equipment and supplies. The need for specialized training and expertise can also make maintenance and troubleshooting difficult (Diederich et al. 2023).

High-performance liquid chromatography (HPLC)

The mobile phase in high-performance liquid chromatography (HPLC) is pushed through the column using increased pressure. HPLC is commonly employed as a fast and efficient technique for separating and identifying the components of mixtures. Stationary phases such as silica gel, polymer beads, or protein-based materials are utilized in HPLC for this purpose (Aseem 2023). Numerous techniques, such as UV–visible spectroscopy and mass spectrometry, are employed to determine the individual elements within the sample. One of the main advantages is its applicability to a wide range of analytes, encompassing small molecules as well as large biomolecules and polymers (Nakov et al. 2023). HPLC is becoming the preferred technology for bioanalytical testing, including drug analysis in natural fluids, residue detection in food, analysis of samples in scientific and environmental fields, and research in life sciences. Finally, the unparalleled precision and power of HPLC coupled with UV detection technology establish it as an essential tool for Quality Control purposes. Implementing

this approach could prove to be expensive as it involves acquiring numerous expensive materials, continual power supply, and regular maintenance. HPLC is not as effective as GC. HPLC is difficult to operate and certainly more expensive compared to other similar instruments in terms of cost (Timchenko 2021).

Size-exclusion chromatography

Size-exclusion chromatography (SEC) is a method of liquid chromatography that sorts components based on their sizes. The stationary phase in SEC is usually a resin made from polymers that have pores of a particular size. After the sample is injected into the column, it is carried through by the mobile phase (Kumari et al. 2023). As the components move through the column, they separate based on their sizes. Porous structures effectively separate larger components from smaller components as they pass through, allowing larger components to be the first to exit the column. In contrast, smaller components are able to be retained for a longer period of time within the pores. SEC is a widely utilized method for the purification of proteins and various biomolecules (Peukert et al. 2022). The main benefits of SEC lie in its simplicity both in concept and practice, particularly when using a single detector that can sense changes in concentration like a DRI or UV/V is spectrophotometer (Wu et al. 2023). The majority of SEC experiments are carried out using a single solvent continuously to prevent issues related to preferential analyte solvation in mixed solvents. This approach enables the precise connection of different types of detectors for enhanced sensitivity and detection capabilities. One of the difficulties in utilizing SEC is the tendency to overlook that SEC separates molecules based on their size rather than their molar mass. Another obstacle is due to the Over-reliance of peak-position and ease of concept and of incorrect execution. The last drawback of this method is related to the assumptions with respect to column recovery (Striegel 2022).

Affinity chromatography

Affinity chromatography involves the separation of biomolecules through their interaction with a ligand that is attached to a stationary phase, using liquid chromatography as the method of separation (Kohoutova and Brabcova 2019). A protein, nucleic acid, or small molecule known as a ligand can attach specifically to a target biomolecule like receptor, enzyme, or antibody. As the sample flows through the column, the affinity matrix captures the desired biomolecule while allowing unwanted molecules to pass through without being affected (Rodriguez et al. 2020). Changing the conditions, including pH, salt concentration, or temperature, is used to release the target biomolecule when performing affinity chromatography. This method is commonly utilized in pharmaceutical research, biochemical and biotechnological approaches to separate and clean proteins and other biomolecules for analysis or medical applications (Swaetz et al. 2023; Lacki and Riske 2019). AC represents a highly efficient method that is readily accessible for recombinant protein purification through a single-step process. This technique is known for its remarkable degree of selectivity and typically possesses a significant capacity (Dutta and Bose 2022; Hage et al. 2017; Khoury et al. 2015). Affinity tags consist of exogenous residues that exhibit specificity in their ability to bind to a chemical as well as biological ligand that is situated on a solid support (for example, his-tags that are connected to an immobilized protein partner or an immobilized Ni) (Fig. 2). AC has a key benefit in its ability to specifically and selectively purify the target protein to a high level of purity. Moreover, it is possible to enhance the purification of the desired protein or isolate it from other proteins in the sample by incorporating affinity chromatography with other chromatography methods. Optimizing the binding conditions for a specific molecule may pose a challenge. The interaction strength between the ligand and the target molecule may vary due to factors such as pH levels, temperature, and the concentration of salt. Hence, finding the ideal conditions for a specific affinity chromatography experiment can pose difficulties (Lortie 2023; Heydari et al. 2023).

In terms of the purification process, chromatography that relies on interactions between metal-affinity tags is referred to as immobilized metal ion affinity chromatography (IMAC) (Irankunda et al. 2022). Some proteins can be purified in a targeted manner through the utilization of the interaction between various cations including $Co2+$, $Ni2+$, $Zn2+$, $Cu2+$, and the polyhistidine tag (Kimple et al. 2013). The employment of IMAC is widely regarded as an indispensable instrument in the process of purifying fusion proteins and achieving in a streamlined manner that requires only a single step. An illustration of purification entails the green fluorescent protein at its N-terminus section, chloramphenicol acetyl transferase as well as the dehydrofolate reductase being fused to a naturally occurring poly histidine affinity tag consisting of 19 amino acids as an example of recombinant protein purification. The outcomes based on carboxymethyl aspartate cross-linked agarose immobilized on cobalt(II) ions demonstrated that all three proteins were successfully purified under circumstances close to physiological features. With a recovery rate of more than 77% for every separated protein, the purification was completed in less than an hour using one step of chromatography method (Lin et al. 2015; Yang et al. 2018). The C-terminal of human urogastrone was fused to a various amino acid tag—polyarginine—in a different

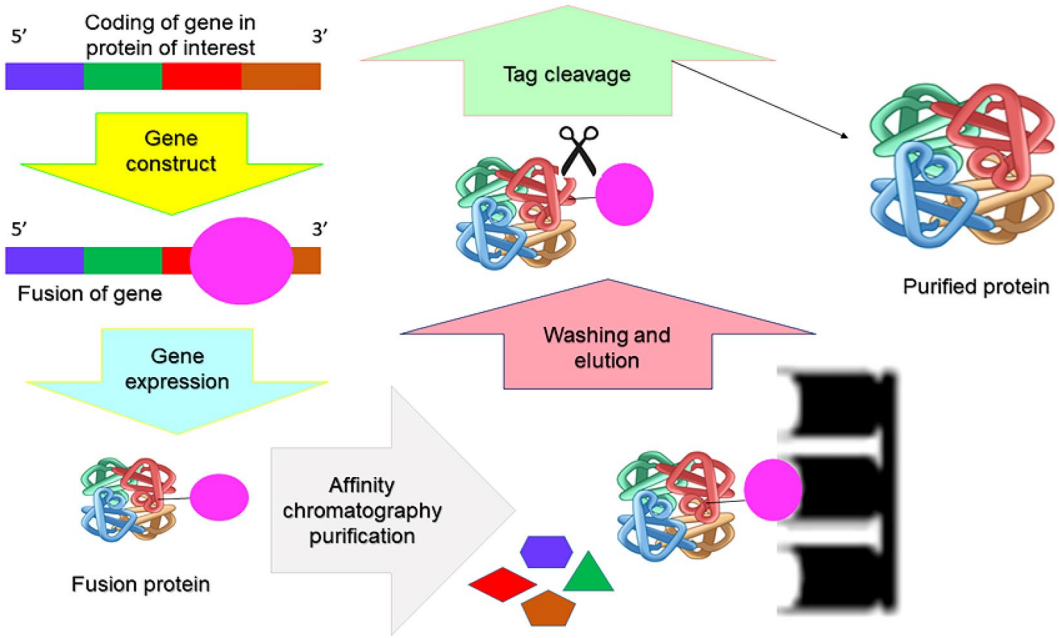

Fig. 2 Procedures involved for recombinant protein purification by employment of affinity chromatography. Initially, a unique tag is attached to proteins at the DNA level to produce tagged fusion proteins. These proteins are then gathered and introduced into a column that holds particular ligands for the protein that is being targeted. Unnecessary substances are removed through washing before a protease is introduced to isolate the desired protein from the affinity tag

investigation to aid in the target's purification. The tail with positive charged produces at pH of around 5.5 results in occurrence of strong binding to the sulfated ion exchanged Sephadex composed of negative charged. Due to its positive charge below pH of approximately 12, the arginine residue connects to any negative charge of ion exchanger. Nevertheless, above the pH 5 the majority of the native bacterial proteins represent negatively charged which prevents them from attaching to the exchanger containing negative charge (Pina et al. 2014). Moreover, carboxypeptidase B has the ability to eliminate the tag, as it is an exopeptidase that selectively breaks down arginine and lysine amino acids at the C-terminal end in a specific order. In the present investigation, subsequent to the elimination of polyarginine via carboxypeptidase B, an additional degree of refinement was attained utilizing the iterative SP-Sephadex column chromatography. The urogastrone that underwent purification employing this approach exhibited an exceptionally high level of purity, surpassing 95%. Furthermore, the technique discussed herein possesses the capacity for scalability due to its inherent simplicity and the cost-effectiveness associated with ion-exchange chromatography (Akparov et al. 2015; Fang et al. 2018; Porodko et al. 2018).

Chiral chromatography

Chiral chromatography, a form of liquid or gas chromatography, is used to distinguish enantiomers, which are molecules that are mirror images of one another (Zhang et al. 2019). Enantiomers are indistinguishable in their physical and chemical attributes except for their ability to rotate plane-polarized light in opposite directions. In the field of chiral chromatography, the stationary phase is usually a chiral choosing agent, like a cyclodextrin or a protein-derived resin, that exhibits a distinct preference for one enantiomer compared to the other. The column receives the sample injection, and the enantiomers are distinguished according to their unique interactions with the stationary phase (Wahab et al. 2018). Chiral chromatography finds application across different industries such as flavors, and fragrances, pharmaceuticals, and agrochemicals with the crucial role of ensuring enantiomeric purity for biological effectiveness, safety, or sensory attributes. Predicting the appropriate combination of CSP and mobile phase for separating a pair of enantiomers can be a challenging task in chiral chromatography. This difficulty arises due to the complexity of the system and the numerous variables involved. It is impossible to predict the next course of action from failed experiments, making each attempt unique and presenting the main obstacle (Tarafder and Miller 2021).

This method offers improved convenience, precision, and reliability in separating and analyzing substances (Liu et al. 2023).

Advanced techniques in peptide purification

Batch liquid chromatography

In the context of batch liquid chromatography, it is imperative that the injected sample undergoes full elution prior to the subsequent injection, while simultaneously ensuring that the column is meticulously cleansed and regenerated between two consecutive injections. The selection of the quantity of sample injected is made to exceed the capacity of the column until a balance is reached between the level of impurities present, the amount of desired product obtained, and the overall efficiency achieved. An excessive increase in the load of column results in a deterioration from the perspective of the differentiation between the desired peak and the closely eluting species, thus this phenomenon directly impacts the purity of the product. Frequently a compromise must be made between purity and yield in the field of preparative single-column chromatography. This situation arises due to the presence of impurities that bear a strong chemical resemblance to the desired product, thereby leading to similar chromatographic characteristics (Dienstbier et al. 2020; Müller-Späth et al. 2013).

This phenomenon leads to an inadequate level of resolution, as the baseline of the peaks is not effectively distinguished. Moreover, it exacerbates when the loadings increase. The anterior and/or posterior regions of the principal peak, consequently, intersect with the peaks of impurities that elute in close proximity, and as a result, they are typically not accumulated in the reservoir for collection, which must adhere to exceedingly uncompromising standards of purity. The window exclusion that overlap leads to an inevitable decline in the recovery. Conversely, expanding the window for collection would result in a favorable outcome for the recovery, though at the expense of purity. In this particular eventuality, it is not possible to achieve both high purity and high yield simultaneously. This phenomenon indicates a fundamental constraint inherent in single-column chromatography, commonly known as the "purity-yield trade-off" (De Luca et al. 2021). To mitigate this trade-off, it is feasible to diminish the volume of the sample loaded or employ less steep gradients; however, this alternative would result in reduced productivity and increased consumption of solvent (Vogg et al. 2019). De Luca et al. (2020) developed this method for the purification of a therapeutic peptide from a crude synthetic mixture. The yield-purity trade-off often seen in traditional batch preparative chromatography can be reduced using this approach. Additionally, automation can streamline the purification process. The purification of a therapeutic peptide using this technique was approximately 90%.

Continuous and semi-continuous chromatography

The regions that overlap and result in the trade-off between yield and purity, as demonstrated earlier, can be either eliminated, although this would involve a consistent and unjustifiable expenditure of valuable resources, or alternatively, they can be reused through the process of reprocessing and purification. The reprocessing of the product volume that contains impurities can be performed in two ways. Firstly, it can be done by the operator in a manual way. Alternatively, it can be done automatically using a recycling valve that allows the eluent flow to be directed back into the same column. This process is similar to what occurs in closed-loop steady-state recycling chromatography (SSR) (Dienstbier et al. 2020; Grill and Miller 1998). In the initial scenario, the fractions comprising impure product, which are in close proximity to eluting impurities, are injected again into the identical column by the operator, regardless of whether or not some fresh feed is added. If there is no addition of fresh feed, the batch process consisting of two steps results in a significantly reduced level of productivity due to the repetition of the processing of the same portion of feed. However, within the context of closed-loop systems, the fresh sample from the SSR is injected into the inner part of the chromatographic profile that is in circulation, precisely at a designated time. Due to the utilization of a solitary column, the act of injecting the feed into the apparatus does not occur in a continuous manner. Consequently, it is not feasible to classify SSR as a continuous procedure. In the early 1960s the 4-zone simulated moving bed (SMB) (Guest 1997) was patented which adheres to the same principles as SSR however operates in a continuous manner. This is made possible by utilizing four or more columns to create a circuit that features with each two outlets and inlets (Xie et al. 2002). The SMB technique relies on the fundamental concept of countercurrent chromatography, wherein the motion of the stationary phase is virtually contrary to that of the mobile phase (Pfister et al. 2018). Due to technical constraints, it is not feasible to directly transfer the packed bed into the system or the chromatographic column. Instead, an artificial system comprising of multiple columns and valves is employed to replicate this motion. As a result, the compound that elutes faster (mobile phase) and the compound that elutes slower (stationary phase) can be excluded into two separate streams, namely the raffinate and the extract, respectively (Fig. 3) (Chernev et al. 2016).

This characteristic undoubtedly renders this methodology exceptionally attractive for the refinement of binary combinations. This particular mode of chromatography

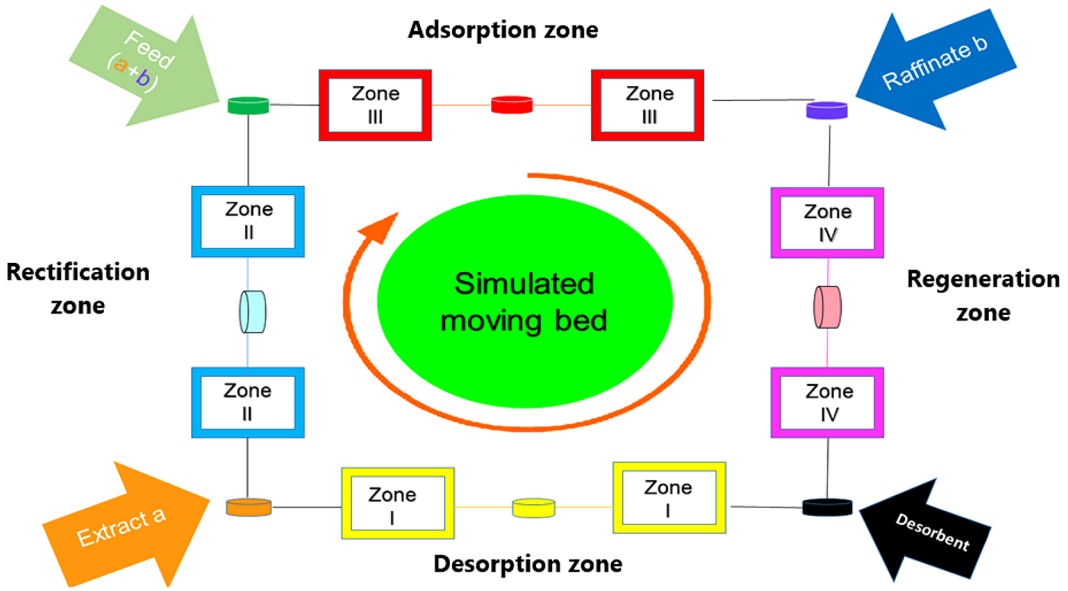

Fig. 3 Schematic figure for simulated moving bed (SMB) technique in protein purification process. In this particular method, every zone was equipped with just a single chromatography column. In SMB, multiple columns of the same type are linked together in a sequential manner. In this figure, the desorbent flows into the initial column while the feed is introduced into the fifth column. This particular feed consists of a combination of two different components, labeled as a and b. The fast-moving element a is extracted in the raffinate stream that exits from the base of column number 4. Component b, which adheres firmly to the stationary phase, is collected from the lower part of column number 3, referred to as the extract. Each zone in a SMB has different flow rate. The loop continuously shifts ports to track the movement of solute bands. Whenever the SMB port changes, the desorbent and feed transfer to the next column inlet downstream

enables the enhancement of the utilization of the stationary phase, thus leading to a significantly greater throughput in comparison to the batch method. Additionally, it has the potential to conserve up to 90% of the mobile phase, which is a remarkable outcome in terms of green chemistry, particularly when applied at an industrial field (Zhang et al. 2023; Imamoglu 2002). The principal drawback of SMB resides in the obligatory upfront capital expenditure due to the substantially higher cost of the equipment in comparison to batch LC systems possessing comparable production capacity. In the commercial sector with a large scale, the reduction in the usage of solvents and the augmentation of efficiency achieved through SMB validate the notable initial expenditure, whereas in laboratory settings, alternative approaches are frequently favored. However, the closed-loop SSR apparatus is a preparative chromatograph utilized in liquid chromatography. It is connected to a recycling and injecting valve, which is necessary for the recycling of the unresolved portion. Continuous chromatography has been utilized for the purpose of handling ternary separations, resulting in significant reductions in the utilization of solvents throughout the purification of peptides (Zydney 2016). In regard to alternative chromatographic methods that rely on the automated recycling of impure components, and in the context of continuous separation of the fractions, the distinctive characteristic is not the continuous aspect itself, but rather the opposing movement of the stationary phase in relation to the mobile stage (Pfister et al. 2018). Two or more columns are interconnected in a sequential manner, whereby the positions of the streams entering and exiting the columns are shifted periodically with high precision. The act of manipulating the valves enables individuals to obtain appropriately refined product windows, to reuse contaminated portions, and to eliminate impurities, as would be elucidated subsequently. By employing the tandem SMB technique, a notable enhancement in productivity by over fivefold and a substantial reduction in solvent consumption by over threefold were observed. These favorable outcomes were accompanied by the attainment of a remarkably high yield of 99% under stable operating conditions. To bridge the gap that exists between the separation of batches in solvent gradient conditions and the practice of continuous chromatography in the realm of ternary mixtures, a technique known as Multicolumn Countercurrent Solvent Gradient Purification (MCSGP) was developed (Aumann and Morbidelli 2006; Herneandez and Juaristi 2010; de Luca et al. 2020; Kim et al. 2021). In the MCSGP methodology, it is possible to utilize a range of 2–6 columns. The variant of MCSGP that employs six columns stands as the most aged and intricate one in terms of the fluidics aspect. In this particular instance, the chromatographic process can be categorized as continuous due to the uninterrupted injection

of the feed into the system, be it in a single column or an alternate one (Kim et al. 2022). In situations where there are only two columns used, such as in the latest iteration of MCSGP, the system exhibits a significantly reduced level of complexity and operates in a cyclical and continuous manner (Steinebach et al. 2017).

The continuous injection of the sample into the unit is not feasible due to the necessity of technical periods for the retrieval of the purified product and disposal of the impurities window. Consequently, the twin-column MCSGP represents a process that is characterized by a semi-continuous nature. The capacity to internally reintegrate the impurity segments of the chromatogram into the apparatus affords three notable benefits. The initial aspect concerns the mechanization of the entire procedure, thereby enabling individuals to reduce the required duration for the purifying and prevent possible mistakes induced by the operators (Catani et al. 2020). The second approach aims to surmount the trade-off between yield and purity that is commonly observed in batch processes. This is accomplished by ensuring that the unit only releases the target peptide once it has been purified. The last advantages rely on a substantial decrease in solvent consumption is ultimately accomplished as the solvent, which carries impure product, persists in circulation throughout the system in the overlapping areas. The MCSGP methodology is an effective procedure that is highly compatible with intricate purification processes involving various types of biomolecules such as proteins, monoclonal antibodies, peptides and, more recently, oligonucleotides. In numerous instances, this technique has demonstrated favorable outcomes (approximately 99%) in terms of achieving a balance between yield and purity, as well as enhancing productivity and minimizing solvent usage (Tarafder et al. 2008). Chen et al. (2023) utilized the SMB technique to effectively separate three components, which involves eliminating tightly bound impurities from the desired substance. A demonstration was conducted to show how the SMB approach can effectively remove strongly bound host cell proteins (HCP) from the target monoclonal antibody (mAb) in a non-affinity cation exchange capture step, serving as a proof of concept. The overall recovery was around 89%.

Supercritical fluid chromatography (SFC) method

Liquid chromatography is frequently considered to be the most optimal choice within the realm of peptide separations, it nonetheless encompasses certain restrictions. As previously researched, substantial quantities of organic solvents are essential in this procedure. Furthermore, it is typically necessary to employ multiple purification processes. To enhance the sustainability and ecological compatibility of the entire procedure, the utilization of Supercritical Fluid Chromatography (SFC) for purifications may serve as a prospective resolution. This is attributable to the fact that supercritical CO2, serving as the chief constituent of the mobile phase, is deemed as an environmentally friendly solvent. Additionally, this solvent is deemed non-hazardous, economical, non-corrosive, non-flammable, and poses no harm for utilization (West 2018). The utilization of supercritical carbon dioxide, owing to its gentle critical temperature of 31 °C, renders SFC exceptionally well-suited for thermally unstable substances. In conjunction with carbon dioxide, the environmentally friendly substance ethanol has the ability to substitute methanol or even acetonitrile. Preparative SFC offers an additional benefit compared to preparative LC in terms of enhanced efficacy, attributable to the utilization of elevated flow velocities (Felletti et al. 2019). Supercritical fluids, in fact, demonstrate elevated diffusivities and diminished viscosities in comparison to liquids, thereby bearing striking resemblance to gases in terms of their characteristics. This leads to a 3- to 4-fold increase in velocity, resulting in optimal efficiency in comparison to HPLC, which offers distinct benefits in terms of productivity and time (Guiochon and Tarafder 2011). Studies have documented the viability of large-scale purification of biomolecules such as peptides and proteins using SFC techniques. This particular process has recently been explored for preparative applications of this nature. Govender et al. (2020) explored the use of SFC techniques in the purification of a set of peptides, including a tetrapeptide (LYLV), octapeptide (DRVYIHPF), and nonapeptide (LYLVCGERG), using readily accessible columns at a small scale. The 2-ethyl pyridine column was found to be the best choice due to its consistency, quality of peaks, effectiveness in separating compounds, and retention rates, resulting in peptide recoveries of 80–102%. The purification process for these peptides was made more efficient, environmentally friendly, and cost-effective by reducing the run times to only 13 min compared to the traditional RP-HPLC methods that took 50 min.

Purification of the recombinant proteins using the novel techniques

Purification with tandem affinity method

The employment of a pair of distinct affinity tags, denoted as dual affinity tags, in the process of separation and protein purification is known as tandem affinity process, which yields substantial and efficient purification of the recombinant protein (Fig. 4) (Dantas et al. 2016; Rubio et al. 2005; Gingras et al. 2005).

In a particular investigation, the purification process involved the utilization of protein A and calmodulin-binding

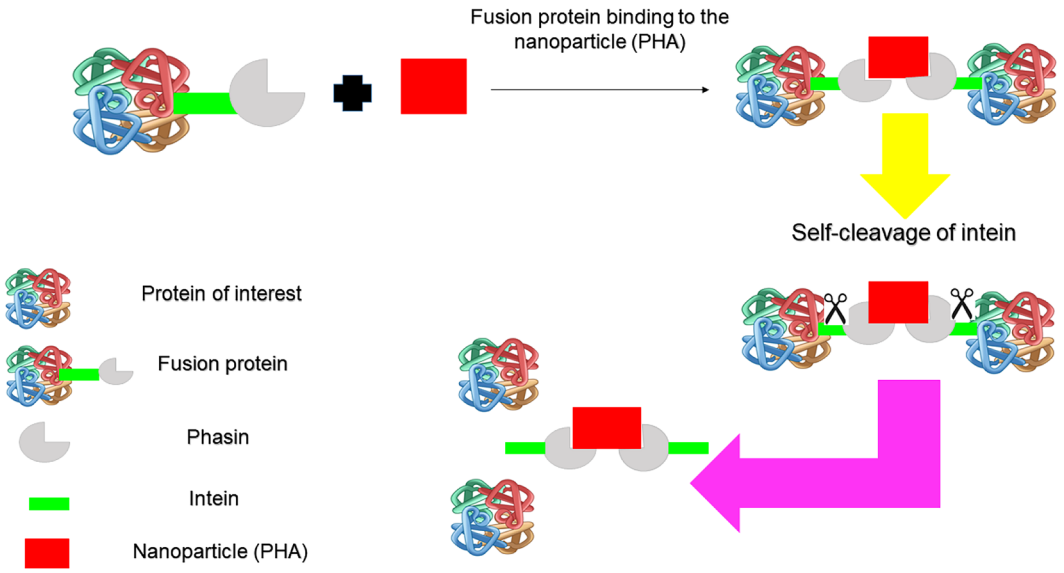

Fig. 4 Dual affinity tag fusion technique in protein purification process. A fusion protein is created by attaching two different affinity tags (intein and Phasin) to a target protein at the DNA level. Each fusion construct contains two affinity tags positioned at the C-terminal and N-terminal of the protein, as well as specific sequences for protease cleavage. In this method fusion proteins binding to the nanoparticles such as Polyhydroxyalkanoates (PHA), then self-cleavage occurs by temperature and pH. Finally, the protein of interest separated

peptide in conjunction. The (tandem affinity purification) TAP-tagged proteins were immobilized onto the surface of IgG Sepharose through the mediation of protein A, followed by their exposure to tobacco etch virus (TEV) protease with the aim of protein complex separation. Subsequently, the protein that had been tagged with TAP was rendered immobile by being affixed onto calmodulin-Sepharose, utilizing its calmodulin-binding peptide, all while calcium was present. Consequently, the removal of calcium ions leads to the releasing of the protein complex that is marked with the TAP tag (Fraseur and Kinzer-Ursen 2018; Gong-Hong et al. 2004; Gingras et al. 2005; Rubio et al. 2005). Therefore, the target protein was subjected to purification through the implementation of two affinity chromatography techniques. The utilization of a combination of dual tags, in conjunction with fluorescent proteins, offers the potential to effectively observe and monitor the various stages involved in the expression procedure. The utilization of individual tags for the purpose of one-step purification presents certain limitations in relation to the overall output, level of purity, and rate of recovery. In a separate investigation, the positioning of His8 and Strep-tag-II was observed at the terminal C of *Discosoma* sp. vascular endothelial growth factor, and red fluorescent protein. Following this, these proteins underwent a series of purification steps involving IMAC and StrepTactin affinity, resulting in a remarkably swift and effective purification process with a level of homogeneity exceeding 99%. The yields obtained from this purification method ranged from 29 to 81%. Using IMAC in primary purification effectively removes a significant proportion of biotinylated proteins and biotin, while simultaneously concentrating the recombinant protein. This enhanced concentration of the target protein facilitates stronger binding to the StrepTactin column, resulting in a more efficient purification process (Du et al. 2022; Cass et al. 2005).

Method of affinity precipitation

The method of affinity precipitation merges the specificity provided by an affinity ligand with the benefits associated with traditional precipitation techniques, including the straightforward equipment needs and the potential for expansion in size. The technique of affinity precipitation has been utilized as a promising approach for the isolation and refinement of proteins from substantial quantities of diluted solution substances by means of reversible soluble-insoluble polymers that are connected to an affinity ligand (Morris 2019; Hilbrig and Freitag 2003).

Chen and Hoffman (1990) conducted a study where they designed a copolymer composed of N-isopropyl acrylamide and N-acryloxysuccinimide, which was employed for the immobilization of Protein A on the copolymer matrix for the purpose of purifying IgG through an affinity-precipitation method. Additionally, Zhou et al. (2010) successfully employed the synthesis of a thermo-responsive polymer, with hydrophobic butyl groups acting as the ligand, to conduct the purification of lipase. In furtherance, Ling and

Zhu (2012) conducted an application of a copolymer that is responsive to heat comprising N-vinyl-2-caprolactam (NVCL) and methacrylic acid, in conjunction with copper serving as the ligand for the purpose of purifying Bovine Serum Albumin (BSA). In furtherance, Ling and Zhu (2012) carried out an application of a copolymer that is responsive to heat comprising N-vinyl-2-caprolactam (NVCL) and methacrylic acid, in conjunction with copper serving as the ligand for the purpose of purifying Bovine Serum Albumin (BSA).

Inverse transition cycling (ITC) purification process

Elastin-Like polypeptides (ELPs), which are synthetic biopolymers that exhibit thermal responsiveness, consist of Valine-Proline-Glycine-Xaa-Glycine (VPGXG) as the repetitive sequences, where X represents any amino acid other than Proline. ELPs possess complete solubility in aqueous solutions below their critical temperature. However, in the aqueous solutions they gradually aggregate to form the insoluble proteins upon surpassing this temperature (Sarvestani et al. 2021; Ge et al. 2005; Trabbic-Carlson et al. 2004). The technique known as Inverse Transition Cycling (ITC) utilizes the thermally responsive characteristic of ELP to perform the purification of recombinant proteins (Sarvestani et al. 2021; Meyer and Chilkoti 1999). Generally, the phenomenon of reversible solubility and environmental sensitivity is observed in recombinant proteins when they are combined with ELP. Consequently, in ITC, a recombinant protein fused with an ELP can be isolated from other proteins with a high degree of purity through repeated processes of aggregation, centrifugation, and dissolution of the fusion protein (Coolbaugh et al. 2017). This methodology is characterized by its simplicity, speed, scalability, and independence from specialized equipment or compounds. It is well-suited for accomplishing high-throughput expression and purification of proteins, and has the added benefit of markedly enhancing protein solubility and preserving its functional activity. In spite of the fact that it is a cost-effective technique for the purification of proteins which does not involve chromatography, this method has limitations in its applicability to all categories of proteins (Omoumi et al. 2018; Floss et al. 2009; Conley et al. 2009). One specific investigation provided evidence that the presence of ELP tags consisting of 30 pentapeptide repetitions has a beneficial effect on the purification and the recovery of genetically engineered proteins. The recovery rates of fusion proteins are observed to be higher in the case of C-terminal ELP fusion tags, as opposed to N-terminal ELP fusions, which is indeed an interesting finding. Consequently, the final yield of recombinant proteins and the quality of their purification are influenced by the size and orientation of the ELP tag (Conley et al. 2009). Recently, a fusion protein comprising an Elastin-Like polypeptide (ELP) and a Z-domain, derived from the B domain in Protein A, has been developed for the purpose of affinity precipitation in multiple applications. ITC purification resulted in achieving an impressive purity level of 88% with effective removal of nucleic acids (Haas et al. 2022; Swartz 2018).

Affinity partitioning procedure

The efficacy of the affinity partitioning procedure lies upon the propensity of certain solute molecules to dissolve within a specific liquid phase of an immiscible liquid−liquid system, commonly known as an aqueous two-phase system (ATPS) (Fig. 5). The utilization of this strategy in ATPS is widely employed in the biomolecules purification process.

The aqueous two-phase systems affinity extraction/partitioning (ATPAP) demonstrates a notable level of specificity inherent in affinity techniques, while simultaneously incorporating the robust conditions characteristic of conventional liquid–liquid extraction systems (ATPS) to efficiently recover and purify a diverse range of biological molecules. The ATPAP approach offers a substantial recovery output and can be readily expanded and established. Furthermore, this approach is cost-effective, eco-friendly and selective. To achieve the optimization of ATPAP, it is imperative to thoroughly analyze and understand the impact of various factors including the strength of the ions in the system, the concentration and nature of the affinity ligand, the polymers chemical behavior in the active groups, and the polymer's coupling of the post-activation ligand (Ruiz-Ruiz et al. 2017). Several molecules have the ability to form connections with ATPS polymer constituents as affinity ligands. Trypsin as well as thaumatin were separated and purified through ATPAP method that were specifically devised using PEG 8000 and DEX T500. The findings of the study demonstrated that the enhancement of the retrieval and refinement of the desired proteins can be significantly augmented through the utilization of modified ATPS involving biospecific ligands connected to PEG molecules, all while maintaining the fundamental integrity of the system (Silva et al. 2021; Andrews et al. 1990). It is important to acknowledge that polymers must exhibit chemical activity through chemical reactions to achieve a high degree of ligand coupling. In fact, numerous strategies for activation have been implemented to accomplish this goal. In the instance of PEG and DEX, the methodologies depend on the functional hydroxyl groups of the macromolecules. The Affinity ligand has the capability to exist in two different states, such as a free entity in the solution phase or as a component that is chemically bound to form a phase. Jiang et al. (2015) utilized an affordable and uncomplicated aqueous two-phase affinity partitioning system using metal ligands to enhance the specificity of

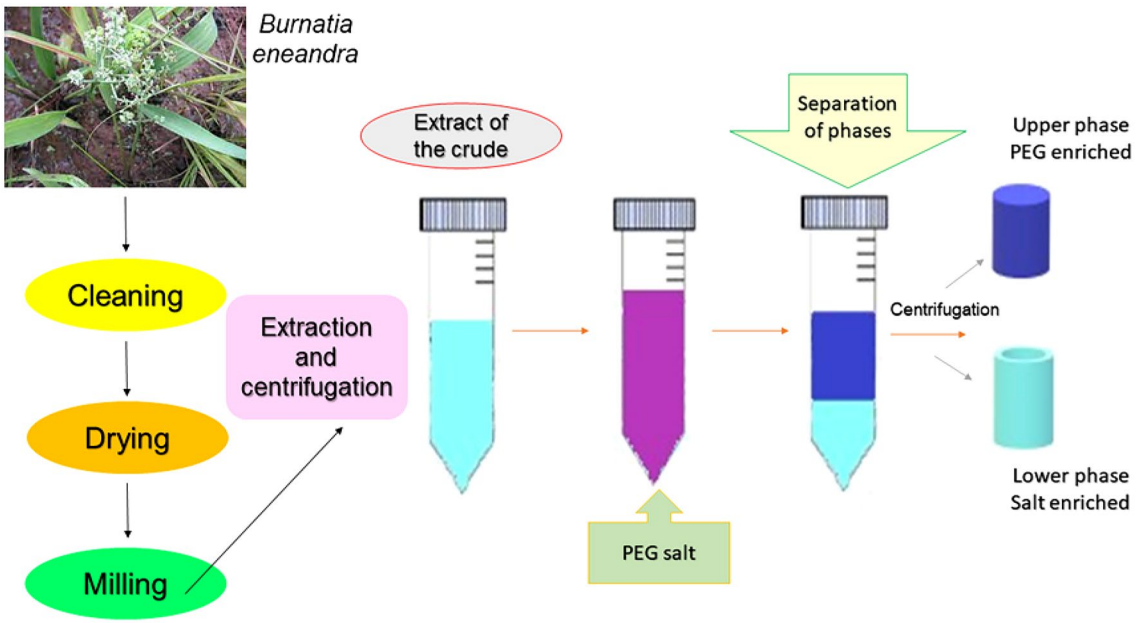

Fig. 5 Separation and purification of enzyme using the aqueous-two phase system (ATPS). Under ideal circumstances, the alpha-amylase enzyme from *Burnatia enneandra* was isolated and purified through the utilization of an aqueous two-phase extraction system (ATPS). Furthermore, the process of separation was thoroughly examined. The concentration of Polyethylene glycol (PEG) and ammonium sulfate (AS), as well as the pH level, were adjusted to enhance the purification of the alpha-amylase enzyme from *B. enneandra*. Additionally, investigations into enzyme characteristics revealed that the alpha-amylase produced was classified as an acidic enzyme

extracting commercial papain. The initial step involved the activation of polyethylene glycol 4000 with epichlorohydrin, followed by its bonding with iminodiacetic acid through covalent bonding. The polyethylene glycol-iminodiacetic acid was ultimately linked to the unique metal ligand Cu2+. The system achieved a high yield of 90.3% and a purification level of 3.6 fold when operating under ideal conditions, consisting of 18% w/w sodium sulfate, 18% w/w polyethylene glycol 4000, 1% w/w polyethylene glycol-iminodiacetic acid-Cu2+, and maintaining a pH of 7. Recent investigations have emphasized the significance of employing the ligand in its free form within the solution phase to prevent the polymers chemical activity. The primary benefits of utilizing ligands without activation and coupling in a solution include the absence of ligand coupling and activity of polymer, as well as the time and reduction in resource employment (Barbosa et al. 2008; Maestro et al. 2008). Currently, there exists a significant focus on the utilization of natural polymers and affinity tags such as Reppal PES starch, Guar Gum Galactomannans, and carbohydrate-binding modules (CBMs) (Antov et al. 2006).

Method using hydrophobin fusion in purification

Certain fungi produce small proteins known as hydrophobins that have the ability to secrete onto surfaces and control interfacial forces. One unique characteristic of hydrophobins is that a specific region of their surface contains hydrophobic aliphatic side chains, which in turn leads to the creation of a distinct "hydrophobic patch" on the protein's surface. The amphipathic characteristic exhibits a strong resemblance to the surfactants' behavior, which consists of a hydrophobic and a hydrophilic component. The hydrophobicity of their fusion partner can be modified by hydrophobins as a result of this characteristic, thus enabling their application in the efficient purification through the implementation of a surfactant-induced aqueous biphasic system. In this particular system, a surface-active agent effectively separates fusion proteins along the surface-active agent phase, while the larger portion of the specimen persists in the aqueous stage (Jugler et al. 2020). Isobutanol as the nondenaturing organic solvents, may subsequently be effortlessly segregated from the surfactant phase, thereby facilitating the separation of fusion protein. ATPS represents a straightforward, expeditious, high-capacity, cost-effective, and expandable technique. Furthermore, the proteins are protected from denaturation through the utilization of this methodology (Conley et al. 2011). The utilization of hydrophobin I (HFBI) derived from *Trichoderma reesei* in conjunction with GFP has been employed in the streamlining of the purification procedure of GFP from extraction of the plant leaf through the application of the ATPS technique. This experimental investigation has conclusively demonstrated the inherent capability of this particular approach to specifically

retrieve as much as 91% of the GFP-HFBI, despite the fact that the fusion of HFBI did not impede the fluorescence characteristics of GFP (Joensuu et al. 2010). The technology of hydrophobin fusion possesses specific benefits, including its suitability for the expression of problematic and harmful proteins, and the absence of any inhibitory effects from the hydrophobin component on the functionality of the protein of interest. Furthermore, hydrophobins exhibit a resilient and precise affinity towards polymeric surfactants, thus offering potential for enhancing the process of protein purification. Consequently, these findings propose hydrophobins as advantageous tags in the advancement of industrial, research endeavors and clinical pertaining to the purification of recombinant proteins (Berger and Sallada 2019; Conley et al. 2011).

Protein purification using self-cleaving affinity method

The elimination of the affinity tags through proteolytic means poses a challenge to the further development of the affinity purification technique. This is due to the potential lack of specificity exhibited by the protease employed, which increases the likelihood of undesired cleavage of the fusion protein occurring at locations other than the intended target sites. In addition, the majority of cleavage reactions take place under conditions of elevated temperature, potentially leading to the denaturation of the resulting protein. In addition, it is possible that the cleavage site may not be accessible for all fusion proteins. In recent times, affinity tags that possess the ability to self-cleave have been created using inteins, which are protein elements that are known for their self-splicing properties. These self-cleaving affinity tags have the ability to efficiently eradicate the necessity for protease intervention in the process of purifying the fusion protein (Prabhala et al. 2023; Shah and Muir 2014). The cleavage function of inteins is clearly associated with the genetic mobility of the sequences that code for inteins. Inteins commence with either Serine or Cysteine and terminate with Asparagine. The residues frequently perform as nucleophiles. Inteins, serving as intron-like components, have the capability to facilitate the process of protein splicing by means of organizing a splicing precursor that possesses a domain with homing endonuclease part (Amaranto et al. 2021; Wood and Camarero 2014). The splicing of protein is an intermolecular reaction occurring after the translation of proteins, wherein four nucleophilic dispersions are guided by the intein as well as the initial C-extein residue. This procedure may serve as a technique for manipulating proteins. The protein of interest is covalently attached to either the amino or carboxyl end of the intein. Dithiothreitol (DTT), free cysteine, and β-mercaptoethanol, as the thiols, induce the hydrolysis of peptide bonds at either the N-terminus or C-terminus of the intein, thereby enabling the separation of the target protein from the remaining portion of the fusion protein that is attached to the column (Shah and Muir 2014; Volkmann and Mootz 2013). The process of self-cleavage takes place in conditions of low reactivity, thereby protecting the purified proteins from undergoing denaturation and the subsequent loss of their functional properties (Prabhala et al. 2022; Xu and Evans 2001). In an investigation, the designated protein is connected to the C-terminal region of an altered intein; conversely, a diminutive affinity tag is incorporated within a loop segment of the intein's endonuclease domain for the purpose of affinity purification (Prabhala et al. 2022). In another study, Amaranto et al. (2021) studied different self-cleaving affinity tags based on intein technology as a method for expressing and purifying recombinant proteins in *Escherichia coli* using single-step affinity chromatography. The protein targeted for research was human growth hormone (hGH), which has two internal disulfide bridges and a phenylalanine residue at the beginning. By attaching an Sce VMA1 intein affinity tag containing a thiol group at the N-terminus, researchers were able to isolate hGH that lacked disulfide bonds. Research was conducted on inteins that can self-cleave in response to changes in pH or temperature. It was found that using an N-terminal Ssp DnaX intein resulted in a fully cleaved cytosolic protein, while using an N-terminal Ssp DnaB intein resulted in a cytosolic fusion protein that could not release hGH. Analysis was conducted on the production of the target protein in the periplasm using a signal peptide at the beginning and a pH-responsive self-cleaving affinity tag at the end. It became feasible to express and purify hGH protein with disulfide bonds and no extra amino acids by expressing hGH in the periplasm, where it was fused to a novel C-terminal DnaX intein-based self-cleaving affinity tag.

Affinity separation using the nanoparticles

In the process of recombinant protein purification, the surface-modified nanoparticles can be employed in the affinity separation technique specifically the protein of his-tagged. Immobilized metal ion affinity chromatography through the metal ion immobilization applies diverse solid matrices to capture an affinity ligand. Polymeric beads, which have nickel ions (Ni2+) that are covalently immobilized, are frequently employed for the purpose of purifying recombinant proteins with a 6xHis tag. However, this methodology possesses certain limitations, including the necessity for pre-treatment to eradicate colloidal contaminants present within the cellular structure. Furthermore, it entails a significant usage of solvents and is characterized by a time-demanding process (Vedarethinam et al. 2023). To address these challenges, the proposition

of utilizing Magnetic-beads for segregation has been put forward as a viable substitute. Hydrophilically modified iron oxide nanoparticles exhibit a superior binding rate when compared to other types of Magnetic-beads due to their elevated ratio of surface area to volume and superior ability to disperse in a medium composed of water. Recently, the employment of Fe-Pt nanoparticles modified with nitrilotriacetic acid was undertaken for the purpose of purifying 6xHis-Tagged protein through IMAC method (Zhao et al. 2021; Minker et al. 2020; Xu et al. 2004). However, this approach is unsuitable for extensive purification endeavors as a result of its exorbitant expense and intricate synthetic process. In a separate investigation, nanoparticles with a core–shell structure consisting of superparamagnetic Fe3O4@silica and functionalized with iminodiacetate were employed to chelate Ni2 + ions for the purpose of purifying proteins tagged with 6xHis (Do-Le et al. 2023; Mohapatra et al. 2007a). The enhancement in the rate of purification with an increase in hydrophilicity has led to the adoption of silica as a prospective approach for the encapsulation of magnetic nanoparticles. This is attributed to the inert surface provided by silica to the nanoparticulate systems. This research exhibited that superparamagnetic silica nanoparticles, which have been charged with Ni2+, possess a distinctive affinity towards recombinant proteins that are tagged with 6xHistidine. Additionally, these nanoparticles are proven to be remarkably efficient, cost-effective, biocompatible, and adaptable in their approach (Vedarethinam et al. 2023; Mohapatra et al. 2007a, b). Seyedinkhorasani et al. (2022) implemented affinity nanoparticle separation to isolate a recombinant protein in inclusion bodies. They expressed EGFP-His and SK-His proteins with His-tags in soluble and inclusion body states, respectively, in *E. coli* BL-21 (DE3). MNPs consisting of a Fe3O4 magnetic core, SiO2 coating, and Ni2 + on the exterior were produced using sol–gel and hydrothermal processes before being examined through XRD, VSM, and SEM imaging. Synthesized Fe3O4@NiSiO3 and Fe3O4@NixSiOy MNPs were used to isolate EGEP-His and SK-His proteins in their natural and altered states. The purified proteins were measured for amount and quality using micro-Bradford assay and SDS-PAGE. Fe3O4@NiSiO3 and Fe3O4@NixSiOy were used to produce 192 and 188 µg/mg of SK-His, respectively.

Nanoparticles of the hydrophobic polymer

Polyhydroxyalkanoates (PHA) are a group of microorganisms that produce hydrophobic biopolyesters (Zhu et al. 2022). Numerous proteins, such as phasing proteins, PHA depolymerase (PhaZ), repressor protein (PhaR), and PHA synthase (PhaC) can be found on the surface of PHA granules. Moreover, a number of studies illustrate the proposition that these proteins have the capacity to serve as viable affinity tags in the process of recombinant protein purification (Zhu et al. 2022; Malhotra 2009; Wang et al. 2008). For instance, polyhydroxybutyrate (PHB) represents the most basic iteration of PHA, which, when combined with a binding protein that is specific to PHB, leads to the development of a self-contained technique for the expression and purification of proteins. In this approach, the desired protein efficiently interacts with the granules through the PHB-binding tag, Subsequently, this compound can be readily retrieved employing uncomplicated mechanical procedures and instruments. This technique has proven to be effective in laboratory settings for purifying proteins with a satisfactory yield while maintaining a high level of functionality. Additionally, it serves as a straightforward, affordable, and dependable approach for purifying the proteins under gentle circumstances. This methodology is suitable in numerous alternative expression systems and has the capability to be readily expanded for the purpose of producing large quantities of recombined proteins (Banki et al. 2005). A recent investigation presents a novel purification approach that incorporates a pH-inducible self-cleaving intein, PHA nanoparticles, and PHA granule-linked phasin (Brown et al. 2022). In this particular approach, the fusion proteins that are connected to nanoparticles were concentrated by employing the technique of centrifugation. Subsequently, the protein was released into the solution by means of self-cleavage of intein. Lastly, the proteins that were released were gathered by pelleting the nanoparticles through an additional step of centrifugation. The utilization of this particular strategy proves to be highly efficient, uncomplicated, and dependable in the process of recombinant protein purification. Furthermore, the act of preparing nanoparticles is remarkably effortless and cost-effective (Trabbic-Carlson et al. 2004). The application of affinity matrices and proteases is not necessary for the implementation of this approach, thereby resulting in significant advantageous economically. This particular methodology solely comprises of uncomplicated cleansing and centrifugation procedures. Additionally, it diminishes the likelihood of deterioration and denaturation of desired proteins by inducing the involvement of self-cleaving peptides. To broaden the use of this method for diverse proteins the researchers divided the protein production and purification stages by utilizing hydrophobic polymer nanoparticles into the in vitro and in vivo procedures. PHAs, being an expensive resource, are frequently inaccessible to a multitude of research facilities. In an effort to tackle these challenges, the researchers have successfully demonstrated that the phasin exhibits a non-discriminatory affinity towards three distinct types of nanoparticles, namely PCL, PHBHHx, and PLA (Wang et al. 2008). Han et al. (2023) created magnetic microspheres made of polyhydroxyalkanoate

(PHA) for the purpose of purifying and immobilizing a new carbonyl reductase (RLSR5) from a recombinant *Escherichia coli* lysate in a single step. The inner part of this microsphere, which repels water, was made up of a polymer called poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), known for its biocompatibility. Magnetic Fe_3O_4 particles were incorporated into the polymer while the solvent was evaporating. The fusion protein consisting of PHA particle-binding protein (PhaP) and RLSR5 (PR) was produced in recombinant *E. coli*, with a hydrophobic outer layer. The Fe3O4@PHBHHx magnetic core efficiently separated the hydrophilic shell from the *E. coli* lysate. By self-assembling, the Fe3O4@PHBHHx core and the hydrophilic shell combined to form Fe3O4@PHBHHx-PR, thus avoiding the need to separate the fusion protein due to interactions both hydrophobic and hydrophilic. The quantity of RLSR5 attached to PHA microspheres was measured at 121.9 mg/g. Using Fe3O4@PHBHHx-PR, (R)-tolvaptan was produced with 99% enantiomeric excess and 97% bioconversion efficiency. Even after 10 recovery cycles, the catalyst retained 78.6% of its activity. Similarly, a multitude of additional ordinary and low-cost hydrophobic polymers possess potential for using in creating of nanoparticles that can effectively employ in the scope of protein purification objectives. Premature cleavage of intein, which refers to the cleavage occurring before the designated time, may potentially manifest throughout the process of protein expression and the subsequent incubation of cell lysate with nanoparticles. For instance, the employment of highly controllable thiol-inducible inteins can address this issue (Perler 2002). Another approach to manage this undesired cleavage is by engineering inteins structurally, with the removal of certain residues to solve this complication. Another technical concern arises from the nanoparticle aggregation phenomenon throughout the process of centrifugation, which leads to a reduction in the overall yield. Hence, it is of utmost significance to utilize innovative and sophisticated methodologies to facilitate the dissemination of nanoparticles and mitigate the occurrence of aggregation. A viable strategy to inhibit aggregation is the utilization of magnetic beads that are coated with hydrophobic polymers. These magnetic nanoparticles, which are not coated, are comparatively less costly and easier to manufacture than the protein-modified magnetic beads that are presently employed for purification purposes (Wood and Camarero 2014; Sadeghi et al. 2023).

Oleosin fusion method

The plant organelles including the seed oil bodies serve as reservoirs for lipids, essential for the process of germination. These organelles are composed of a matrix of triacylglycerol (TAG), which is encased by a monolayer consisting of proteins and phospholipids. As a result of the presence of distinct structural proteins known as oleosins, these entities demonstrate exceptional stability within both isolated preparations and host cells. This is attributable to the constraints imposed by electronegative repulsion and steric restrictions (Qiang et al. 2020; Frandsen et al. 2001). Seeds that encompass oleosin-fused proteins, derived from genetically modified plants, have demonstrated remarkable efficacy as carriers for the synthesis and purification of recombinant proteins (Qiang et al. 2020). In a comprehensive investigation, a novel technique was enhanced to achieve optimal production of the recombinant proteins in *E. coli*. Furthermore, this method offers the advantage of facile isolation of these proteins through the utilization of artificial oil bodies (AOB) (Lojewska et al. 2016). It is worth noting that this approach bears resemblance to the protein purification method employing recombinant oil bodies obtained from genetically modified plants. In this study, a substantial amount of GFP was successfully generated and purified with exceptional quality (Peng et al. 2004). AOB is generated through the joint effort of three entities: oleosin, TAG, and phospholipids (PLs). In contrast to ligand affinity purification techniques, this approach proves to be relatively economical as it eliminates the necessity for the column of costly ligand coupled (Peng et al. 2003; Sorgan et al. 2002). The utilization of the AOB technique results in a significant enhancement of the output through a streamlined and expedited procedure, in contrast to the seed oil-body system. In the context of the AOB expression/purification process, the utilization of soluble and self-folding polypeptides is employed to specifically identify the protein of interest with the intention of obstructing the aggregation of recombinant proteins. By doing so, the functionality of these proteins is protected, thereby preserving their intended purpose. In this particular investigation, to begin with, the oleosin-Xa-GFP protein was produced with the context of *E.coli*. Subsequently, the combination of phospholipid, oleosin-Xa-GFP, and triacylglycerol was employed to generate synthetic oil bodies. After undergoing centrifugation, the oleosin-Xa-GFP complex was found to be situated on the exterior of AOB. Subsequently, the soluble GFP molecule was liberated from the oleosin protein, upon Xa factor cleavage, resulting in the collection of GFP in a final supernatant with remarkably high yield (85%) and purity, achieved through the process of concentrating (Peng et al. 2004).

Obstacles and future perspectives in purification of proteins

Advancements in biotechnology and thorough research on the structure and functions of different proteins have led to rapid advancements in protein separation and purification technology. Purifying and separating functional recombinant

proteins, natural proteins and peptides is a difficult task in biotechnology. Recently, progress has been made in addressing this challenge through the utilization of various methods including physicochemical purification, affinity purification, and fusion technology (Table 1) (Du et al. 2022; Liu et al. 2020). Purifying proteins often presents inevitable obstacles. Proteins frequently exist in complicated mixtures in cells or tissues, making the purification a delicate and difficult process. Other factors that contribute to this complexity include biological protein activity, structure integrity, host contamination, purity, and solubility of samples (Aguiar and Domingues 2023). As such, the foundation and essential component of protein-related research is effective method in purification of proteins. There have been various difficulties with protein production. The process of establishing a protein purification scheme involves a number of laborious steps, continuous exploration, lengthy cycles, and repeated trials, all of which raise the possibility of protein activity loss (Liu et al. 2020). In addition, obtaining the protein of interest with high purity necessitates the collaboration of several devices, thereby raising the expense of research. Furthermore, the protein of interest are not the only biological macromolecules that are frequently regarded as impurities. Ultimately, the field has always struggled with the membrane protein's isolation and purification so the question of how to effectively separate and purify different membrane proteins remains intriguing (Du et al. 2022).

There are a variety of sophisticated techniques available for purifying proteins, however despite their benefits, certain constraints may hinder their effectiveness. Tag-mediated protein purification and immobilization techniques are now being recognized as environmentally friendly and economical technologies compared to traditional chemical immobilization methods. These strategies enable proteins and peptides to be purified and immobilized onto natural, synthetic, or hybrid materials in a single step, streamlining the fabrication process for protein-engineered materials (Tang et al. 2024). Covalently binding tags are considered the best choice for permanently immobilizing proteins, however, they are limited in their ability to bind to different materials without requiring any chemical modifications, only being compatible with protein-based materials. On the other hand, affinity tags can attach to various substances (both synthetic and natural), making them better suited for purifying proteins rather than immobilizing them for an extended period (Gupta et al. 2023). Downstream protein purification methods present various obstacles such as reducing protein levels, capturing protein, handling unstable proteins, eliminating protein aggregates, removing HCPs, and ensuring protein recovery. Furthermore, there are certain limitations associated with the advanced methods implemented in this study. For instance, in batch liquid chromatography, not all of the adsorbent present in the column is effectively utilized as well as a significant quantity of eluent is required to extract the isolated components, leading to a decrease in product concentration (Salvatore 2023). Continuous chromatography often faces inherent limitations when it comes to balancing yield and purity, particularly for separations that involve numerous impurities related to the product. One of the primary obstacles encountered by process developers handling a recombinant protein that is not able to be purified with affinity chromatography is the issue of low purity during the initial chromatography capture step. The use of aqueous two-phase systems (ATPS) on a large scale has faced challenges due to various factors, including difficulties in obtaining the required level of selectivity in protein extraction, the cost of the phase-forming components, and the need for wastewater treatment in ATPS processes (Torres-Acosta et al. 2018).

According to these challenges, the best course for future development should focus on streamlining the process of production, lowering the cost and building the recycling system that can reuse the profitable materials without compromising the functionality and yield of the protein of interest (Colgrave et al. 2021). Majority of the molecules used in various industries are proteins, therefore, the cost of production and the efficiency in creating appropriate dosage formulations for these proteins are typically dependent on the progress of the respective investigation and development (Salleh et al. 2022). The current methods for purifying proteins are not adequate in terms of purity. Therefore, more research and effort are required to create novel protein purification techniques including continues chromatography methods that are showing potential for improving both the speed and effectiveness of protein purification processes, development of affinity ligands and nanomaterials holds potential to improve the precision and exclusivity of purification methods, implementing automation and robotics in protein purification processes can minimize the need for manual labor, enhance consistency in results, and boost the overall efficiency of the products (Decker et al. 2023). The miniaturizing of purification methods through the use of microfluidics and lab-on-a-chip devices could allow for quicker and more effective purification at a reduced size as well as innovative approaches to membrane protein manipulation are important for the progression of drug discovery and structural biology. Ongoing research in this area is vital for the development of new techniques (Salvatore 2023).

Conclusion

With the continuous progress in biotechnology and comprehensive investigation on the configuration and operation of diverse proteins, alongside the technological enhancements for the separation and purification of proteins, the MCPA

Table 1 Summary of some purification methods, their advantages, limitations, and applications

Purification method	Advantages	Limitations	Applications
Multi-column plate adapter	Effective, Efficient, Flexible	Small scale proteins	Recombinant and natural protein, antibody
Aqueous two-phase	Friendly-environment, cost effective, rapid separation	High temperatures and pressure have negative effect on the product, significant variability in predicted models	Enzyme, recombinant protein, natural protein
SUMO-UIp1	Eco-friendly, Rapid, Simple, high efficiently	Low productivity	Natural protein, peptide
Liquid chromatography	Wide array of samples, adjust selectivity through various parameters, durability, dependability	Restriction in analyzing intricate samples with numerous components	Peptides purification
Ion chromatography	Identify and measure various array of ions, high selectivity, easy operate	Limitation for identifying the non-ionic specimens, expensive	Enzyme purification
High performance liquid chromatography	Applicability to wide range of analytes, high precision, small amount of sample is needed	Difficult operation, expensive	Mostly in purification of protein, peptides and therapeutic drugs
Affinity chromatography	Highly efficient, high selectivity	Interaction strength between ligand and target is vary due to the temperature and pH	Recombinant protein, natural protein and peptide, nucleic acids
Simulated moving bed	Highly efficient, high purity, high yield, large feet treatment, simple process control	Need to improve for more complex separation, low productivity	Protein, peptide
Supercritical chromatography	Short analysis time, possibility of using a high flow rate, low viscosity of the mobile phase, high efficiency, low cost	Does not work for water soluble compounds, limited for highly polar compounds, heavy and large CO2 tank	Peptide
Tandem affinity method	Quantitatively determines the protein without prior knowledge of complex composition, high yield, simple to execute	Limitation of up to 22% of isolation and identification of purified tagged protein, low efficiency of protein, competition of endogenous protein with tagged protein	Protein, recombinant protein
Inverse transition cycling	Inexpensive, efficient, high stability of recombinant protein, high purity	Low yield, high purification cost, aggregation and precipitation of product	Recombinant protein
Affinity partitioning	Specificity, simplify, easy to scale up	Low selectivity, low yield	Recombinant protein
Self-cleavage affinity	Save time, save labor, low cost	Extra purification steps for separation of the protein of interest, The used protease are not fully specific, expensive	Recombinant protein

system presents an exceptionally cost-effective approach for conducting research on protein treatment by means of sample purification. The cells containing surface-bound SUMO and Ulp1 would be effectively eliminated through a gentle centrifugation process, resulting in the isolation of the purified target protein exclusively in the supernatant. For the most part, the SUMO-Ulp1 approach to the cell surface offers a simple method for purifying proteins in their localized form, which only depend on the ideal cleavage and centrifugation procedures. Target peptides are most frequently separated and purified using regular liquid column chromatography. Strategies for magnetic separation are still relatively novel and are therefore, constantly being refined. The primary goal of upstream procedure is, in fact, to produce natural peptide as pure as possible to aid in purification chromatography. Conversely, automating process, and raising the yield as well as the chromatographic step reduction are the downstream goals. ACN and water, which are still the primary eluents, can be used in smaller amounts by employing the semi-continuous chromatography, as it recycles the fractions with mixed forms in the subsequent column instantly.

In the field of biotechnology, the separating and purifying of the recombinant protein is one the most difficult effort. Several approaches including physicochemical purification, affinity and fusion purification strategies have been employed a partial solution to this issue. Fusion technology offers a powerful approach for purification with high-throughput, as the researches have demonstrated. It is evident that the integration of the fusion strategy with nanotechnology holds significant potential in this particular realm of scientific investigation. Moreover, a multitude of inventive methods can be formulated by incorporating diverse nanomaterials in conjunction with self-cleaving peptides to achieve a streamlined, efficient, cost effective, and extensive purification process for recombinant proteins. To ensure the economic and ecological viability of these emerging methods with improved or advanced features, it is anticipated that innovative and remarkably effective protein purification and immobilization methods that can be accomplished will emerge in the near future.

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