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TECHNOLOGICAL APPROACHES TO THE EXTRACTION AND PURIFICATION BY ULTRAFILTRATION TECHNIQUES OF TARGET PROTEIN MOLECULES FROM ANIMAL TISSUES: A REVIEW

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Abstract

Effective isolation and purification of protein is a great challenge nowadays. The key aspect is protein stability and solubility, which primarily depend on protein structure and its amino acid sequence. Manipulations with pH and ionic strength are the first attempts to increase protein stability and solubility. Different additives that are allowed or prohibited in the food industry are applied for overcoming protein aggregation. Sugars, polyhydric alcohols and amino acids are the most attractive among them. Trehalose, glycerol, arginine, glycine and proline demonstrated outstanding properties that make them perspective for application during isolation and purification of proteins singly or in combination with each other or other compounds. However, the algorithm of effective isolation and purification of protein could be significantly varied depending on its structure.

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Introduction

Specific proteins are in great demand in laboratory practice, pharmacy and food industry. Most protein therapeutics currently on the market are recombinant and developed to treat a wide variety of clinical indications, including cancers, autoimmunity/inflammation, exposure to infectious agents, genetic disorders and other diseases [1,2]. The standard proteins, enzymes, antibodies, etc. are amongst the most widely used research reagents but often their quality is inadequate and can result in poor data reproducibility, including due to non-sufficient purification or loss of structure or activity during these processes [3]. Plant and animal (dairy, egg, and meat) proteins are widely used in the food industry [4], including therapeutic food additives based on tissue-specific proteins [5].

Proteins are polypeptide structures consisted of unique sequences of amino acids. Side amino acid chains could be positively or negatively charged, form four different levels of complexity (primary, secondary, tertiary, and quaternary structure) by hydrogen, ionic and hydrophobic bonds or disulfide bridges, which also contribute to stabilization of protein structure [6, 7, 8]. Amino acids could be hydrophobic or polar, basic or acidic, forming net charge and solubility of protein. The acid/base properties of proteins are essential in biochemistry [9], as well as isoelectric point value prediction [10]. Various methods of isolation exist and have been developed for certain purposes based on the unique characteristics of each protein, such as the amino acid composition, sequence, subunit structures, size, shape, net charge, isoelectric point, solubility, heat stability and hydrophobicity [11]. The aim of the article is to review the technological approaches to the extraction and purification by membrane techniques of protein molecules from animal tissues.

Isolation of target protein molecules from animal tissues

Tissue homogenization is a key step for molecular biology studies [12], where chemical or mechanical approaches are chosen depending on a purpose or type of a target biomolecule. Mechanical/physical methods for disrupting samples include grinding, shearing, beating, and shocking, which could be combined with chemicals for process intensification [13]. A wide range of laboratory, semi- and industrial equipment is successfully used, but the final approach is based on the properties and further use of a target biomolecule. Most proteins are sensitive to high temperature and aggressive chemicals. Moreover, for some purposes it is necessary to obtain proteins with the preserved biological activity, and a lot of chemicals are not permitted in pharmaceuticals or food additives.

Innovative techniques for protein extraction are intensively developing, including the aqueous two-phase system, subcritical water extraction, enzyme-, microwave- and ultrasound-assisted extraction, pulsed electric field and high voltage electrical discharge extraction, high hydrostatic pressure-assisted extraction, and supercritical carbon dioxide techniques [14]. However, water-based extraction remains the cheapest one, where the most important is knowledge of the value of the isoelectric point (pI) of target proteins. The pI is the pH of a solution, at which the net charge of a protein becomes zero, the negative and positive charges are balanced, reducing repulsive electrostatic forces, and the attraction forces predominate, causing aggregation and precipitation [15]. Modification of pH by alkali or acid leads to proteins become negatively or positively charged, resulting in electrostatic repulsions between molecules and hydration of charged residues, contributing to the solubility of proteins [16]. Moreover, salts could also stabilize protein molecules [17]. Based on the known pI value of a target protein group, as well as on predominance of acidic or basic amino acid residues in protein structure, it is possible to predict the advisable pH and ionic strength of solution for intensification of the extraction process. Summarizing, if pI > buffer pH, lower the pH by 1 unit, if pI < buffer pH, raise the pH by 1 unit, if pI=buffer pH, try both ways [18].

Purification of animal proteins by membrane techniques

Membrane technologies represent an efficient and environmentally friendly option for the separation, fractionation, and purification of bioactive compounds from different animal tissues [19]. The most widespread use of membrane technologies is in the dairy industry [20]. Membrane processes are extremely diverse; various types of filtration processes, membranes, polymers for membrane manufacture are used depending on purposes. The ultrafiltration is a commonly used approach for protein separation, fractionation, and purification. However, adsorption, molecule aggregation, and denaturation are the main problems that a scientist faces during the ultrafiltration process of proteins in the native form [21,22]. Aggregation is a general term that encompasses several types of interactions or characteristics. Protein aggregates can be a result of various mechanisms and can be classified in several ways, including soluble/insoluble, covalent/noncovalent, reversible/irreversible, and native/denatured. For protein solutions, the presence of aggregates of any type is usually considered undesirable for the reason that aggregates can reduce the efficiency of purification and separation of protein-peptide mixtures or protein solutions [23]. Aggregation, as well as formation of a highly concentrated layer at the border of the filtration membrane or adsorption to it significantly interferes with filtration (Figure 1).

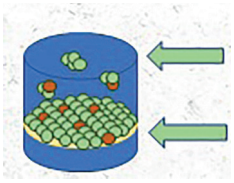

Figure 1. The main challenges during protein ultrafiltration

The easiest way to prevent aggregation and adsorption during ultrafiltration is the dilution, followed by implementation of diafiltration (feeding solution with the same pH and ionic strength) in order to maintain constant pH and ionic strength [24–26]. Cromwell et al. noted that the selection of the membrane material, the optimal pore size (throughput) and the rate of transmission of the protein solution through it are important aspects in preventing adsorption of aggregates on the membrane surface and affect the efficiency of purification or separation of target proteins [23].

Another way is to use agents that may promote protein solubility, such as kosmotropes, weak kosmotropes, chaotropes, amino acids, sugars and polyhydric alcohols, detergents [27].

Kosmotropic salts have a higher salting-out effect according to the Hofmeister series. They act as a protein stabilizer (usually small ions, low polarizability), and as polar water-structure makers [28]. For weak kosmotropic salts, such as NaCl and KCl, the recommended initial concentration is 300mM and 200mM, respectively, the recommended concentration range is 0–1M [27,28]. Strong kosmotropic salts are $MgSO_4$, $(NH_4)_2SO_4$, Na_2SO_4, Cs_2SO_4, with the recommended concentration range from 0–0.2M to 0–0.4M [27, 28]. It was also reported that potassium citrate in a concentration of 0.1M was effective in solubilizing four proteins, such as proline-rich antigen 2, C2 domain-containing protein (in combination with mannitol), unnamed apical complex protein, which previously appeared totally insoluble [29].

Chaotropic salts have a higher "salting-in" effect according to the Hofmeister series, but they can reduce protein–protein interactions by shielding charges and by preventing the stabilization of salt bridges [28]. The recommended concentration range for such salts as $CaCl_2$, $MgCl_2$, LiCl, RbCl, NaSCN, NaI, $NaClO_4$ and NaBr varies from 0–0.2 M to 0–0.8 M [27], the recommended initial concentration for $CaCl_2$ and $MgCl_2$ is 10–50 mM, while for NaI — 0.2 M [28]. Urea, guanidine HCl, N-Methylurea, N-Ethylurea and N-Methylformamide belong to mild chaotropes. Guanidine HCl and urea are the most common denaturing agents for protein denaturation and then renaturalize the protein to its active form [30]. To form β-sheets, the protein–protein interaction must be larger than the hydrogen bond interaction formed between urea and protein, which slows down the aggregation process in urea. The two different behaviors of urea indicate that it can affect the aggregation in a nonmonotonic way [31]. Therefore, the low concentration of urea in many cases has also been used to

solubilize inclusion body aggregates, while the use of the high concentration of chaotropes like urea and guanidine hydrochloride results in complete denaturation of these existing secondary structures and often leads to aggregation of protein molecules during the refolding process [32]. The recommended initial concentration for urea and guanidine HCl is 0.5M, the recommended concentration range is 0–2M [28]. However, urea or guanidine HCl addition is a hard denaturation step [33] that is usually applied in proteomic studies, such as electrophoresis [34].

Non-ionic (triton X-100, tween 80 or 20, n-dodecyl β-d-maltoside (DDT), polyoxyethylene cetyl ether (Brij 56), n-octyl-β-d-glucoside (OG)), ionic (cetyltrimethylammonium bromide (CTAB), sodium lauroyl sarcosinate (Sarkosyl), sodium dodecyl sulfate) and zwitterionic (non-detergent sulfo betaine (NDSB), 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), zwittergent 3–14, lauryldimethylamine N-oxide (LDAO)) detergents are also used for prevention of protein aggregation [28]. The use of non-ionic or zwitterionic detergents at low concentrations (no more than 1%, usually recommended 0.1%) helps solubilize protein aggregates without denaturing the proteins [27,28,35].

Trimethylamine N-oxide (TMAO) forms direct attractive interactions with polypeptides, stabilizes collapsed conformations via a mechanism that is distinct from glycine and betaine. It was also proposed that TMAO stabilizes proteins by acting as a surfactant for the heterogeneous surfaces of folded proteins [36]. The recommended initial concentration for TMAO is 0.5M, the recommended concentration range is 0–1M [28]. Glycine betaine (GB) is a naturally occurring osmolyte that has been widely recognized as a protein protectant preventing protein aggregation, but it may have opposite effects on protein stability [37–39]. 1M glycine betaine was effective in solubilizing four proteins, for e. g. lanosterol 14-alpha demethylase [29].

Sugars and polyhydric alcohols are also widely used for prevention of protein aggregation, including glucose, sucrose, trehalose, lactose, glycerol, sorbitol, mannitol, xylitol, inositol [27,28]. Polyol and sugar osmolytes can perturb protein h-bonds to affect protein function [40] and stabilize the lattice structure of water, thus increasing surface tension and viscosity. They stabilize hydration shells and protect against aggregation by increasing the molecular density of the solution without changing the dielectric constant, the usually recommended amount varies from 10 to 40% [41].

A number of articles report on the stabilization of various biomolecules by trehalose (and in some cases, sucrose) [42]. Trehalose inhibits aggregation of lysozyme, insulin [43], as well as 0.75 M trehalose was effective in solubilizing 21 proteins, such as 14-alpha sterol demethylase Cyp51B, sensory transduction histidine kinase, putative (in combination with mannitol), sensor proteins, metallopeptidase domain protein, cytochrome P450 51, proline-rich antigen 5, etc. [29]. It is hypothesized that trehalose prevents the inactivation and aggregation of proteins at lower temperature and stabilizes the cell membrane by delaying the onset of phase shift from liquid crystal to gel state [42]. Both trehalose and sucrose induce a well-defined protein–protein distance, which could explain why these inhibit protein–protein interactions and associated protein aggregation, but superior anti-aggregation effect of trehalose could be also explained by the fact that local solvent structures are highly important for explaining the protein stabilization mechanism [44]. The structure-stabilizing effect of sucrose is conferred on the protein by the increase in the solvent cohesive force when sucrose is added to water in the solvent system [45]. Sucrose has been shown to inhibit IL-1ra dimer formation [46]. The recommended initial concentration for trehalose and sucrose is 0.5M, the recommended concentration range is 0–1M; for glucose and lactose, the recommended concentration range is 0–2M and 0.1–0.2M, respectively [27,28].

It was reported that glycerol prevented protein aggregation by inhibiting protein unfolding and by stabilizing aggregation-prone intermediates through preferential interactions with hydrophobic surface regions that favor amphiphilic interface orientations of glycerol [47]. It was also found that the preferential hydration of proteins in glycerol-water mixture minimized the surface of contact between proteins and glycerol to stabilize those native structures [48]. The recommended initial concentration for glycerol is 10%, the recommended concentration range is 5–40% [27,28]. Sorbitol in a concentration of 0.5M-2M was shown to demonstrate a negative influence on the unfolded form of lysozyme, thereby, stabilizing the native form [49], and 0.3 M sorbitol increased recombinant bovine sex determining region Y protein solubility [50]. Sorbitol has also been shown to reduce aggregation of nucleocapsid protein of rhabdovirus after its expression in *Escherichia coli*, which is likely due to exert its effect on folding by altering the structure and properties of water around the folding protein molecule [51]. It is also commonly used as an additive to promote refolding of solubilized proteins [32]. The recommended initial concentration for sorbitol is 0.5M, the recommended concentration range is 0.2–1M or 0–40%w/v [27,28]. The addition of 10% (w/v) mannitol to the buffer matrix resulted in a 4.2-fold decrease in the IgG4-N1 aggregation rate constant compared to that for the control condition [52]. Mannitol in a concentration of 0.5M could increase the solubility of seven proteins, such as sensory transduction histidine kinase, putative (in combination with trehalose), Hsp20/alpha crystallin domain-containing protein and unnamed apical complex protein (as component of complex buffer), etc. [29]. The recommended initial concentration for sorbitol is 2%, the recommended concentration range is 0–15%w/v [27,28]. Xylitol in a concentration of 0.1M could increase the solubility of metalloprotease 1 and lanosterol 14-alpha-demethylas [29]. The recommended initial concentration for xylitol is 0.5M, the recommended concentration range

is 0.2–1M or 0–30%w/v [27,28]; while for inositol, the recommended concentration range is 0–10% w/v [27].

The application of amino acids as anti-aggregation agents is in demand in the food industry and bioactive additives production. It has also been reported that after a compound is combined with an amino acid, the pharmacological activity of the compound is enhanced, water solubility is improved, and cytotoxicity is reduced [53]. Amino acids and derivatives thereof increase the surface tension of water in a concentration of 20–500 mM [41]. The summarizing information about amino acid application is presented in Table 1.

Table 1. Amino acids used to stabilize proteins and to prevent aggregation [27,28]

Amino acid and derivatives thereof	Recommended initial concentration	Recommended concentration range
Glycine	250 mM	0.5–2 M/0.5–2%
Arginine L-HCl	125 mM	0–2 M
Arginine ethylester	250 mM	0–500 mM
Proline	250 mM	0–1 M
Potassium glutamate	250 mM	0–500 mM
Arginine L	50 mM	0–5M

Among 15 amino acids tested, arginine exhibited the best results in preventing the formation of aggregates [54]. The hydrophobic surfaces present on the proteins interact with the hydrophobic surface presented by the arginine clusters. The masking of hydrophobic surface inhibits protein-protein aggregation [55]. Arginine in a concentration of 0.1 to 1 M is customarily included in solvents used for refolding the proteins by dialysis or dilution. In addition, arginine at higher concentrations, e. g., 0.5–2 M, can be used to extract active, folded proteins from insoluble pellets obtained after lysing *Escherichia coli* cells. It was shown that interactions between the guanidinium group of arginine and tryptophan side chains may be responsible for suppression of protein aggregation by arginine [56]. In general, arginine is found to interact with the aromatic and charged side chains of surface residues. In particular, arginine interacts with aromatic and charged residues due to the cation–π interaction and salt-bridge formation, respectively, to stabilize the partially unfolded intermediates. The self-interaction of arginine leads to the formation of clusters which, due to their size, crowd out the protein–protein interaction [57]. Arginine is also shown to form stacking and T-shaped structures with aromatic amino acids, the types of cation–p and N–H...p interactions, respectively, known to be important contributors to protein stability. The analysis also shows that arginine-arginine interactions lead to stable clusters, with the stability of the clusters arising from the stacking of the guanidinium part of arginine. The results show that the unique ability of arginine to form clusters with itself makes it an effective aggregation suppressant [58]. Arginine in a concentration of 10–500 mM demonstrated the inhibitory effects on the initial aggregation kinetics of bovine insulin [59], in a concentration of 0.75 M used for renaturation of lysozyme from hen egg white and the prevention of aggregation resulted in 94% recovery yield [43]. L-Arginine in a concentration of 0.375 M increased the solubility of six proteins, such as pentapeptide repeat family protein, LPPN Rv2270, membrane skeletal protein IMC1, TgDCX, unnamed apical complex protein in mixture with trehalose or as a component of the complex buffer [29]; in a concentration of 400 mM, it was the key player in the refolding of human glucose 6-phosphate dehydrogenase, preventing the aggregation of folding intermediate [60]. Arginine (in the form of hydrochloride salt Arg·HCl) is often used in formulations exhibiting high RSA (reversible self-association) and a propensity for aggregation; glutamate salt of arginine (Arg·Glu) was able to decrease the propensity of the mAbs (monoclonal antibodies) to aggregate, particularly at pH values closer to their pI [61]. It was also demonstrated that addition of L-Arg and L-Glu at 50 mM to the buffer could dramatically increase the maximum achievable concentration of soluble protein, preventing protein aggregation and precipitation, increasing the long-term stability and protecting from proteolytic degradation [62]. It was found that the protein solubility enhancement is related to the relative increase in the number of arginine and glutamic acid molecules around the protein in the equimolar mixtures due to additional hydrogen bonding interactions between the excipients on the surface of the protein when both excipients are present. The presence of these additional molecules around the protein leads to enhanced crowding, which suppresses the protein association [63]. It was also proposed that below 100 mM arginine acts like glycine, above 100 mM it shows destabilizing effects similar to guanidinium hydrochloride [64].

Glycine alone demonstrates two stages of stabilization. The first effect (at concentrations below 100 mM) is protein specific and is probably due to multiple direct interactions with the polar or charged side chains and the partial charges on the peptide backbone of the protein. The second stage (at concentrations above 100 mM) is similar to high charge density anions where it was ascribed to competition for water between the unfolding protein and the cosolute [64]. Glycine in a concentration of 100 mM is often used for preparing elution buffers [65–67]. Glycine stabilizes collapsed conformations of hydrophobic elastin-like polypeptides via a classical preferential depletion mechanism [36].

Glycine and proline showed a certain ability to stabilize hemoglobin [68]. It was proposed that proline with a concentration of >3 M behaves as an enzyme stabilizer as well as a protein solubilizing solute and forms an amphipathic supramolecular assembly and successfully thwarts the aggregation associated with the refolding of bovine carbonic anhydrase [43]. Experimental evidence suggests that proline inhibits protein aggregation by binding to folding intermediate(s) and trapping the folding intermediate(s) into enzymatically inactive, "aggregation-insensitive"

state(s) [69]. Proline contains a closed ring structure in its side chain which has a hydrophobic surface, which enables it to interact with proteins through hydrophobic interactions [43]. It has been suggested that multimeric forms proline may be responsible for its aggregation inhibitory effects [55]. Proline in a concentration of 0.5 M increases the solubility of four proteins, such as lanosterol 14-alpha demethylase, TgDCX, unnamed apical complex protein as a component of the complex buffer [29], it effectively inhibits protein aggregation during the refolding of bovine carbonic anhydrase [70]. Proline in a concentration of 0.2 M inhibits aggregation of Alzheimer's amyloid beta 1–42 (Aβ1–42) peptide; the effect of 0.5 M and 1.0 M concentrations was also studied [55]. Besides arginine, a positively charged amino acid (such as histidine and lysine) can inhibit aggregation [71], and arginine stabilized all three domains of IgG [72].

Many researchers use a comprehensive approach, preparing complex buffers with compounds that serve as a ligand to allow the protein to remain in a soluble conformation (metal or an amino acid); additives that reduce protein-protein interactions (chaotropic agents) or stabilize intra-molecular bonds (kosmotropic agents); compounds known to affect protein stability (charged amino acids, reducing agents, polyols and sugars); and, additives that significantly altered buffer or salt conditions [29].

Conclusion

Proteins are complex biomolecules, each certain protein is a unique sequence of amino acids. During isolation and purification of proteins, its structure should be taken into account. When facing challenges accompanied isolation and purification of proteins, the first steps are changing the pH of the solution, the salt concentration or the salt. The easiest way to prevent protein aggregation and adsorption during ultrafiltration is the dilution, followed by implementation of diafiltration. However, it is often not enough. Another way is to use agents that may promote protein solubility, such as kosmotropes, weak kosmotropes, chaotropes, amino acids, sugars and polyhydric alcohols, detergents, as well as use combinations of these compounds.

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