DEEP EUTECTIC SOLVENTS FOR EXTRACTION AND PURIFICATION OF PLANT PROTEINS

Data de aceite: 02/05/2024

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ABSTRACT: Recently, diverse methods for plant protein extraction have been studied due to the potential applications that proteins have, either for obtaining food, or for the treatment and prevention of diseases. Various plant species and even agro-industrial wastes have been used, however, the extraction and purification of these compounds involve challenges including energetical and economical costs, environmental impact, etc., hence it is important to find new strategies to carry out this process. In recent years, deep eutectic solvents (DES) have been studied in order to substitute common solvents used for protein extraction and, besides they follow the green chemistry principles. DES are able to extract proteins at with high yields, nevertheless, in spite of their multiple advantages, some challenges need to be explored to improve extraction and purification of plant proteins. The aim of this chapter is to show the multiple advantages of DES as emerging mixtures in both extraction and purification of plant proteins, as well as the opportunity areas and the challenges, that actually, can limit their large-scale application.

PALABRAS-CLAVE: green solvents, eutectic mixture, large-scale

INTRODUCTION

In recent years, the extraction of plant proteins (isolates and concentrates) has received considerable attention due to their potential of applications in 1) food, such as the obtention of emulsifiers (by legume proteins), obtainment of dietary proteins and preparation of flours derived from plant protein (Sim et al., 2021; Kim et al., 2020), as well as, 2) treatment and prevention of diseases in human and animals, because several plant protein have shown antimicrobial, antioxidant, anticancer, neuro-modulatory activities, among others (Wani et al., 2020; Kianfar et al., 2021; Navaf et al., 2023; Münch et al., 2024). Proteins are commonly extracted by standardized processes from various plant species (Navaf et al., 2023; Münch et al., 2024). Plant proteins are known to have great benefits when are included in the human diet, not only because obtaining animal protein generates a major negative impact on the environment, but also because plant proteins can be extracted from numerous agro-industrial residues (Segatto et al., 2022). Due to the increased need of obtaining plant proteins, conventional methods that involve organic solvents (methanol, acetone, benzene, chloroform, hexane, etc.) have been used on a large scale but these solvents have the properties of being flammable, explosive, poorly biodegradable and toxic, so the use of these kind of chemicals cause damage to the environment and to the human health, for this reason, environmentally friendly solvents, such as the deep eutectic solvents (DES) represent an approach to protein extraction that seeks to reduce the disadvantages of traditional organic solvents (Vigier et al. , 2015; Benvenutti et al., 2019; Socas et al., 2021; Kumar et al., 2021).

The term “eutectic” is derived from the greek word meaning low melting point and can be applied for different systems like alloys and also in liquid medium (Liu et al., 2018). In recent decades, DES have been used as unconventional solvents and they can be defined as “Mixtures of two or more pure compounds for which the eutectic point temperature is lower than that of an ideal liquid mixture, presenting significant negative deviations from ideality” and, due to their multiple properties, they have been used for the extraction of bioactive compounds, stabilization of biomolecules, enhancers of drug delivery, as well as, for the treatment of food industry waste (Molnar et al., 2024). The advantage of DES consists in their simple and inexpensive preparation, which involves only mixing and stirring at temperature below 150°C (Botelho Junior et al., 2022). Some generalities of DES are shown in Figure 1.

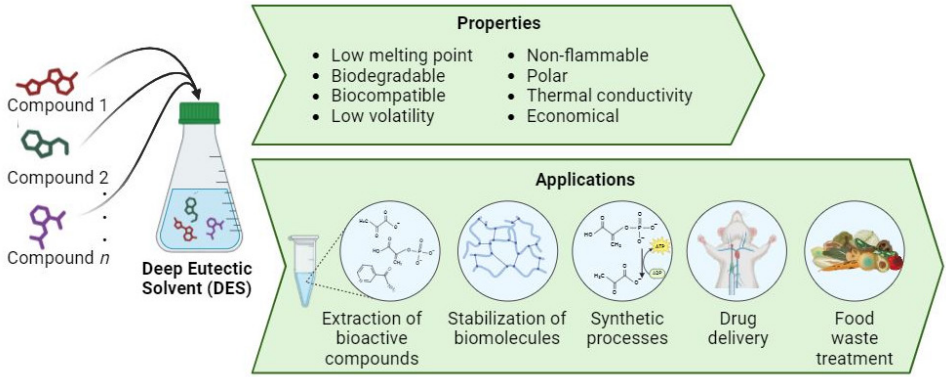

Figure 1. Properties of DES and their principal application areas

DES are formed by a mixture of quaternary ammonium salts that act as hydrogen bond acceptors (HBA) and a contra-compound, which acts as a hydrogen bond donor (HBD), these may consist of biomolecules such as sugars, carboxylic acids, amino acids, as well as some ionic molecules (Bowen et al., 2022). In Figure 2, we show examples of DES compounds as well as their classification. The properties of DES depend on the intermolecular interactions between their HBA and HBD components, as well as the nature of their hydrogen bonds, which are known to decrease their melting point, leading to the obtention of a liquid eutectic solvent mixture without requiring any additional processing or purification (Paul & Gotor-Fernandez, 2022).

DES have been classified into 5 groups: type 1 is formed by the combination of quaternary ammonium salt and metallic chloride; type 2, quaternary ammonium salt and metallic chloride hydrate; type 3, quaternary ammonium salt and HBD (organic acids, amides or polyols); type 4, metallic salts and HBD; and type 5, which consists of a new class of nonionic HBA and HBD (Figure 2). Other authors classify them according to the nature of their components into NADES (natural deep eutectic solvents), in other words, composed of natural components (biomolecules such as organic acids, amino acids, polyols, sugars or choline derivatives), THEDES (therapeutic deep eutectic solvents), those prepared of pharmaceutically active components, and TDES (ternary deep eutectic solvents), those prepared of three components (Kumar et al., 2017; Hansen et al., 2020; Sun et al., 2023).

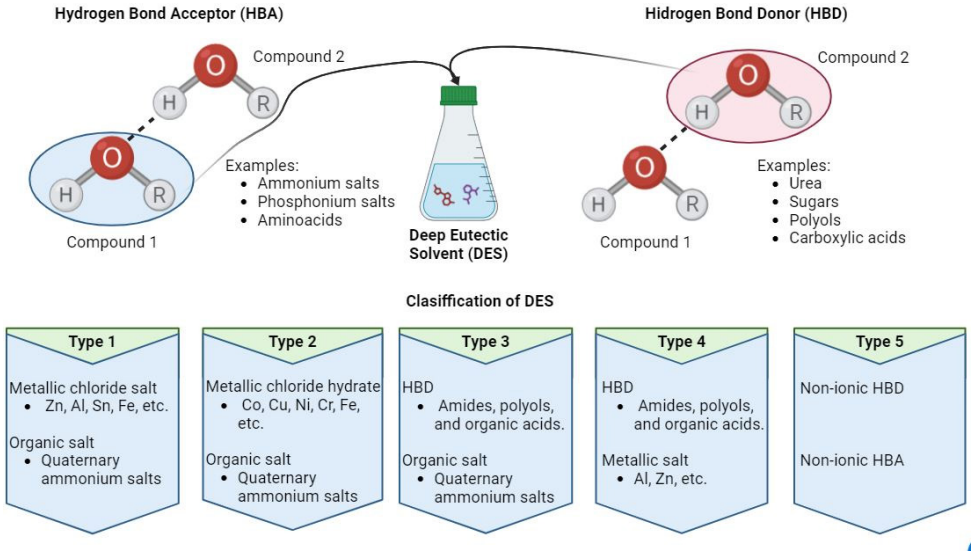

Figure 2. Composition of DES and their classification

Due to the use of DES for bioactive compounds extraction, some properties, such as viscosity, must be evaluated, since this property affects both hydrogen bonds and van der Waals interactions; at high viscosity values, the diffusion coefficients of analytes can be reduced causing low mass transfer and long term extraction (Hikmawanti et al., 2021), the viscosity depends on the composition of DES and its preparation temperature, then, it is important to carefully choose a suitable DES and evaluate the whole extraction conditions. Thus, the aim of the present chapter is to show the advantages of DES use for the extraction of plant proteins and expose some benefits in the purification of these biomolecules, as well as to argue the opportunity areas and challenges to overcome their large-scale application.

ADVANTAGES OF USING DES FOR PROTEIN EXTRACTION OVER CONVENTIONAL ORGANIC SOLVENTS

Ferreira & Sarraguça (2024) report that the selection of the ideal solvent for protein extraction not only has to be focused on obtaining the molecules of interest but also must be a sustainable, biodegradable, and innocuous compound with no negative impact on both the environment and human health. The only solvent that possesses these characteristics is water, however, it cannot be used for all types of extractions, since it does not dissolve hydrophobic compounds due to its high dielectric constant, hence the use of solvents that, at least decrease this environmental impact and are more biodegradable must follow some of the 12 principles of green chemistry (Ferreira & Sarraguça, 2024). It has been demonstrated that DES are more effective for protein extraction than classical organic solvents and, in addition the extraction efficiency is higher; DES have shown higher selectivity (Molnar et al.,

2024). At industrial scale, the most commonly methods used for protein extraction involve organic solvents, as well as alkaline and acid solutions or enzyme-based extraction (Figure 3). Previous procedures corrode the industrial equipment, generating massive industrial effluents of acid and alkaline solutions. Therefore, alternatives are being sought to find a more environmentally friendly and sustainable approach to replace existing protein extraction methods (Zhou et al., 2022; Patra et al., 2023). It has been reported that DES have been employed in a diverse range of industrial and commercial applications; on the other hand, they have also been employed in research in specific areas such as Chemistry and Biology sectors (Hansen et al., 2020). Such research has shown DES as promising candidates to be applied for the extraction of bioactive compounds, although these mixtures need to be evaluated for safety application (Mbous et al., 2017).

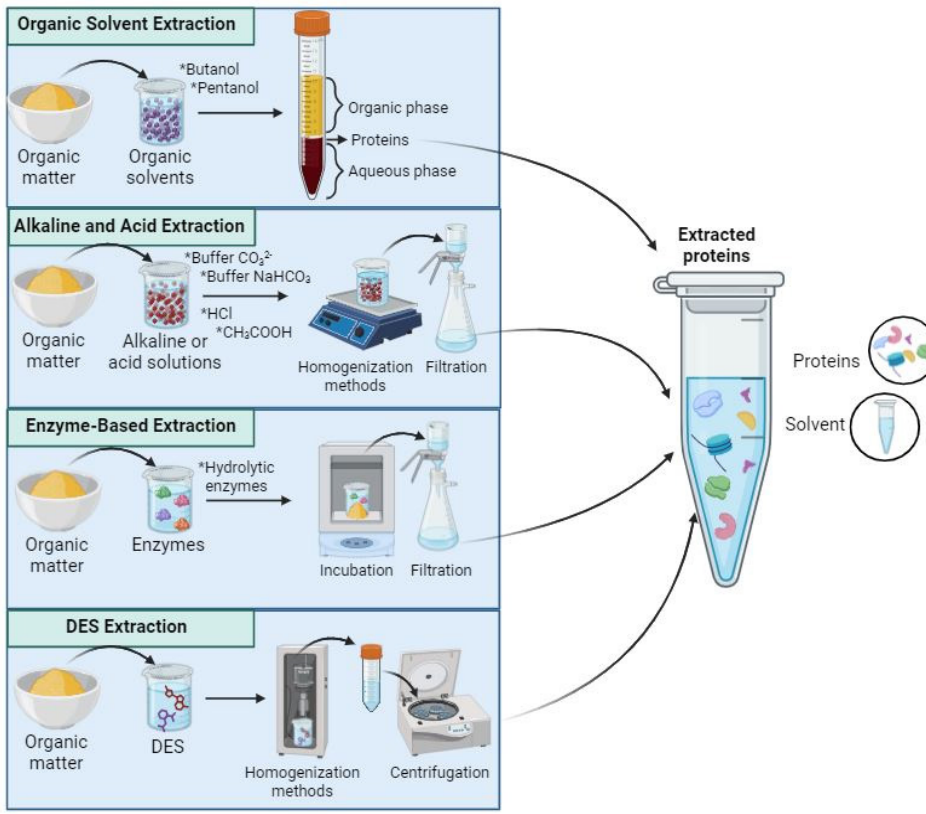

Figure 3. Most common methods for protein extraction

PROTEIN EXTRACTION AND PURIFICATION WITH DES

Regardless of the many advantages of DES, some recovery issues of the dissolved solutes after extraction have been reported such as solvent distillation but techniques are being explored: use of macroporous resins, solid phase extraction (SPE), antisolvent method and back extraction (Molnar et al., 2024), as shown in Figure 4, however, these methodologies still require the use of organic solvents such as ethanol and methanol (Palos-Hernández et al., 2022). However, the most recent techniques in protein extraction and purification involve the use of DES (Lin et al., 2022; Karimi et al., 2024); processes based on these solvents have reported higher extraction efficiencies up to 62-90% for plant proteins and aminoacids, higher results than other techniques, such as acid-based method (Li et al., 2021; Bowen et al., 2022; Zhou et al., 2022).

The purification of proteins extracted by DES can be carried out by precipitating them or by forming an insoluble layer using solvents such as ethanol, in this way, the elimination by evaporation of the EtOH components that dissolve the DES, allows the recovery of the initial compounds. Another option, is using a DES initially as an extraction medium and then use it as a formulation system, thus obviating the problem of solute recovery. Additionally, it is known that the addition of antisolvents (ethanol or water) is probably the best method for solute recovery, as it does not require specific equipment, additional costs and allows the development of sustainable processing (Ruesgas-Ramón et al., 2017).

The use of DES for the extraction and purification of plant proteins from agricultural and agro-industrial wastes has a great potential to be scalable due to the large amounts of raw materials that are generated (Bowen et al., 2022) and because DES are cheaper than common organic solvents. The advantages of using DES in protein extraction is based on the performance of the following three conditions: affinity, solubility, stability as well as the low costs (Landa-Castro et al., 2020; Hewage et al., 2024).

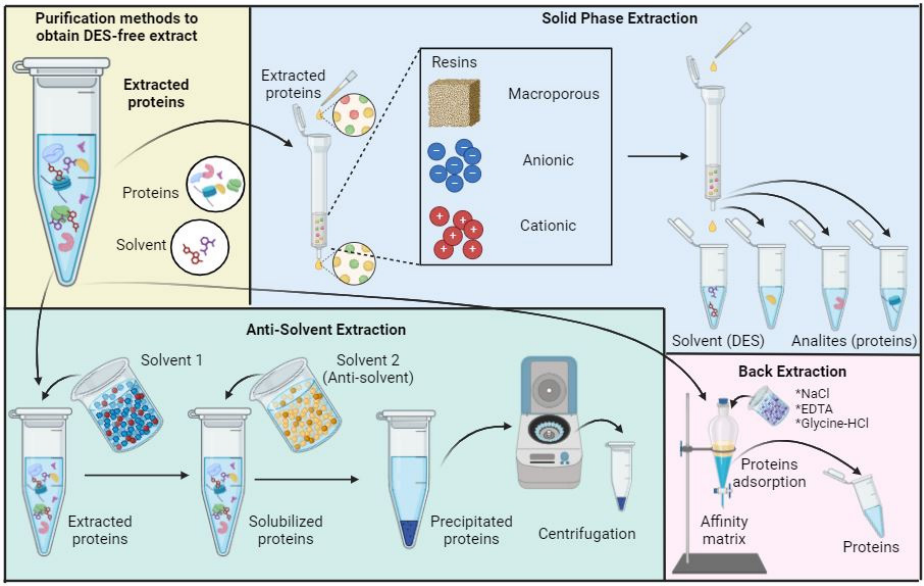

Figure 4. Purification methods for proteins extracted with DES

In general, in methods of solid-liquid extraction, water is used to reduce the viscosity of DES and, therefore, the efficiency of the extraction process is improved. It is known that higher viscosity in DES is related to the presence of a large amount of hydrogen bonds, which, in some cases, can avoid protein solubilization. However, it is necessary to use an acceptable volume of water that prevents disruption of hydrogen bond interactions between the components of DES since an excessive volume of water can negatively affect the beneficial properties of DES (Ruesgas-Ramón et al., 2017; Ling et al., 2020).

The purification process of proteins is fundamental for research as well as for industrial sectors ie. in-depth study or to commercialize safe products. Generally, purification process of proteins include precipitation using water or aqueous buffers in combination with organic solvents (acetone), bases (ammonium sulfate) or salts such as ion exchange, also electrophoresis or affinity chromatography can be used (Adhikari et al., 2010; Gómez-García et al., 2022). But these methods have multiple disadvantages such as negative effects on proteins including denaturation or complexation and high costs. That is the reason why viable alternatives are being studied suggesting the use of DES for liquid-liquid extraction due to shorter separation time and enhanced extraction efficiency (Xu et al., 2015).

Some DES most commonly used for extraction and purification of proteins include betaine (HBA) and urea, glucose, sorbitol, and glycerol (HBD). Protein extraction can be done also by using aqueous two-phase systems (ATPS), i.e., mixtures of compounds such as betaine:urea, which has been demonstrated as one of the most outstanding DES, due to its capacity to purify bovine serum albumin (BSA) up to 99.82% (Li et al., 2016). On the other hand, DES mixtures with choline chloride:glycerol have also been used for BSA purification obtaining efficiently of, 98.71% (Xu et al., 2015).

OPPORTUNITIES AND CHALLENGES OF USING DES TO EXTRACT PLANT PROTEINS

Bellow we will mention some advantages and opportunities of using DES, as well as the challenges faced by its application for the extraction and purification of proteins at large scale.

Some of the advantages of using DES for the extraction of plant proteins is that solvents have been viewed as mixtures rather than pure compounds, hence, no chemical reaction occurs and there is no need to eliminate residues (Moldes et al., 2022). Additionally, the non-flammable property of DES makes synthesis safer at high temperature and ambient pressure with less energy costs (Ge et al., 2017).

The protein extraction principle by DES relies on the phenomenon of aggregation and envelopment, which is non-destructive of the protein structure, making it particularly suitable for the nondestructive extraction of bioactive compounds. Most studies have reported that DES present high efficiency of protein extraction (Zhou et al., 2022), then DES can be applied to multiple areas (electrochemistry, organic and inorganic chemistry) and their properties (phase behavior, melting temperature, density, conductivity, surface tension and polarity) can be modified based on the chemical nature of compounds and their molar ratio (Ling & Hadinoto, 2022).

It has been suggested that the use of DES for specific applications such as food and pharmaceutical approaches might not require purification steps (Ling & Hadinoto, 2022). Rico et al. (2021), reported that DES can be adapted for the extraction of several valuable compounds from diverse raw material sources exceeding, in many cases, the organic solvents efficiency but obviously depends on the target compound (Palos-Hernández et al., 2022). Due to the wide variety of DES, the selection of an ideal mixture for a given group of compounds is crucial, and even though this selection can influence in the extraction of the compounds, their evaluations are usually not complicated (Palos-Hernández et al., 2022).

However, some consideration should be taken for DES use because they can extract other compounds different of proteins such as peptides, polar lipids, sugar residues, etc. Most of the literature, do not consider their low selectivity, therefore, it is necessary a better understanding of the parameters that could affect or favor DES interactions with the proteins (Ruesgas-Ramón et al., 2017).

The low efficiency of back extraction methods is another weakness that can be highlighted about the use of DES (Zhou et al., 2022), as well as their volatilization and incompatibility with chromatographic and detection systems, in which, the reliable evaluation of the target compounds is usually complicated (Socas-Rodríguez et al., 2021).

An important challenge for protein extraction with DES is that their density and pH can affect the extraction efficiency; the selection of HBA and HBD components, as well as their molar ratio are also important due to their influence on solvation interactions (Abbasi

et al., 2022; Patra & Pandiselvam, 2023). Moreover, conditions as solvent recyclability and thermal stability of DES, is known to be involved in the protein purification and is often complex due to it can consume large amounts of energy making industrial scale-up difficult (Vigier et al., 2015). Furthermore, the inherent bioactivity of DES might be a limitation for assessing the activity associated with the extracted proteins as well as for other bioactive compounds (Ling & Hadinoto, 2022).

Despite the promising future of using biocompatible solvents, there is an urgent need to develop environmentally friendly, reliable, and simple methods that allow us to recover and purify proteins and that allow proper recycling and regeneration of the solvent used, so that the environmental impact is minimal or negligible. Nevertheless, the state of the art is still in its infancy; there are few studies that address protein purification or solvent recovery (Cannavacciuolo et al., 2022; Moldes et al., 2022) Specifically, by employing innovative extraction techniques that enhance the extraction process such as the ultrasonic sonication technique based on liquid-liquid extraction improves dispersion in viscous DES and enhances mass transfer. Similarly, microwave irradiation is also used in liquid-liquid extraction, improving the extraction of proteins and polar compounds. In the case of other methodologies that improve protein extraction with DES, the use of electrochemical techniques through magnetic composites of metallic nanoparticles in solid phase can be mentioned (Vigier et al., 2015; Kist et al., 2021).

CONCLUSION

The advantages to use deep eutectic solvents for plant proteins is noteworthy mainly because they are environmentally friendly, consist of low-cost components, present low toxicity and are biodegradable, characteristics that make them safe for food and pharmaceutical applications. It is important to consider that DES preparation must be a priority to facilitate the development of sustainable purification of proteins. Nevertheless, the selection of a solvent for a specific protein extraction is still a challenge, therefore, it is vitally important to carry out research to get them at industrial scale.

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