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Optimisation of isolation and purification of the jack bean enzyme urease by extraction and subsequent crystallization

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ABSTRACT

A detailed investigation into the isolation by extraction and the purification by crystallization of the enzyme urease from jack bean meal is presented with a view to developing a large-scale process. The effect of different extraction solvents and additives upon the resulting crystals is characterised in terms of crystal size, protein yield and protein purity. The enzymatic activity of urease is exploited in order to quantify the amount and purity of the protein obtained. The extraction procedure was optimised and all stages of the process were monitored in order to provide as detailed a picture as possible of the impact of different process stages upon the intermediate products. Almost all protein is extracted in a single extraction step. Further extraction steps using the same meal but fresh solvent yield only small additional protein yields. Less than 10% of total protein is extracted in a second step, less than 3% in a third extraction. Different solvents lead to clear differences in product quality, with no single solvent optimising all quality criteria. In addition, the point at which the acetone precipitant is introduced into the process has a clear influence on the product. Judicious choice of extraction conditions can significantly increase the size of the urease crystals at the cost of product purity, quantified by specific activity. High product purity (a maximum 138-fold increase in purity was observed with a maximum yield of 8.4%), in contrast, leads to smaller crystals. Comparison to the purest commercially available urease revealed a similar urease content.

1. Introduction

Protein products are of growing importance in the chemical, biochemical and pharmaceutical industries. They find use in medical applications (therapeutics and diagnostics), the chemical, cosmetics and food industry and, to a significant extent, in detergent production, amongst others. Enzymes, proteins with specific catalytic properties, represent a considerable fraction of all proteins and protein products. At the end of the 1990s industrial enzymes alone had a sales value of $1.5 billion (US) in the Unites States (Walsh, 2002). As a result protein purification is an issue that needs to be addressed. For high-value products, in particular for therapeutic proteins, purification *via* chromatographic methods is commonly employed. Despite the effectiveness of chromatography, the costs of the processes are significant and only justifiable for high-added value products. Crystallization for the purpose of purification is potentially a cost-saving alternative to chromatographic purification, but has not found wide application on the industrial scale. Although fractional precipitation is used to concentrate and isolate protein fractions from a raw material, examples of industrial crystallization implemented as the sole method for large-scale purification of a protein product are rare (Visuri, 2000).

The most important criteria for industrial crystallization are obtaining maximum product purity and recovery in a minimum of purification steps, a short process duration as well as low-environmental impact (Ulrich and Jones, 2004, 2006). In the case of technical enzymes a further factor has to be considered, namely the catalytic activity of the product (Jones and Ulrich, 2005; Weber et al., 2005): when purifying an enzyme its activity must be retained, otherwise the product is

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rendered worthless. The purification of urease from raw jack bean meal by means of extraction followed by crystallization will serve to show that crystallization is a sufficiently rapid process that yields a high purity, active product with sufficient yield. A significant body of knowledge with respect to the fundamentals of protein crystallization has been accumulated by that part of the crystal growth community interested in protein crystallization as a route to high-quality single crystals for structure elucidation *via* X-ray diffraction (Sawyer and Turner, 2000). In these studies, micro-methods such as vapour diffusion, low-volume dialysis, or micro-batch crystallization are usually employed (Ducruix and Giegé, 2000). Apart from the technical differences between the individual methods, all require highly purified protein and crystallization is initiated by means of precipitating agents, generally inorganic salts or poly(ethers). In order to optimise a crystallization process, the solubility of the desired protein with respect to the temperature, pH, precipitant concentration and type, as well as other relevant factors (pressure, the presence of buffer salts and their chemical nature, for example), should be known together with relevant information on the kinetics of nucleation and crystal growth (Burke et al., 2001; Galkin and Vekilov, 2001; Bhamidi et al., 2002; Gorti et al., 2005; Rosenberger et al., 1996). However, with the exception of a small number of proteins such as hen egg white lysozyme (Howard et al., 1988; Forsythe et al., 1999; Retailleau et al., 2002; Benas et al., 2002), ovalbumin (Judge et al., 1996) glucose isomerase (Chayen et al., 1988) or α-amylase (Veesler et al., 1996), limited information is available for solubilities compared to the wealth of data available for small molecule and ionic crystals.

Additional complications arise due to the nature of the raw materials, generally stemming from renewable resources or biotechnological processes, from which protein products are obtained. Apart from proteins they contain a significant number of other substances with varying concentrations. In addition, the number of different proteins and their individual concentration can vary depending on protein type and provenance of the raw material. Concentrations of ovalbumin in hen egg white can reach 65–70% (Wagner, 1954), but there are a large number of proteins, the amount of which in the source material can be very low, such as the *lac* repressor of wild-type *E. coli* with about 0.002% of the total protein content (Voet and Voet, 1995). Due to the complexity of feed materials, interactions between individual components can be expected and may result in the inhibition of crystal nucleation and growth. Due to the fact that proteins are very large molecules (typically 10–100 kDa), slow crystal growth can be expected. Solubility measurements using a micro-batch technique (Howard et al., 1988; Judge et al., 1996) show that equilibration of a solid–liquid system can take weeks. However, it is known that crystals of lysozyme and some other proteins can be obtained within a few hours (Judge et al., 1995). A further problem is the instability of proteins in general, resulting in the loss of catalytic activity in enzymes. Most proteins are chemically and conformationally stable only within a narrow temperature and pH range and tend to degrade with time at room temperature and at temperatures and pH values beyond their natural range. Enzymatic activity can also be affected detrimentally by the presence of contaminants. In terms of further processing of protein crystals it is important to note that they typically integrate 30–80% water (Lorber and Giegé, 2000) and are therefore extremely soft, which is critical for solid–liquid separation.

The enzyme employed in this study is urease, obtained from jack bean meal (*Canavalia ensiformis*). Its crystallization was first reported by Sumner in 1926 (Sumner, 1926a). Urease is a nickel enzyme and is found in plants and in many bacteria, algae and fungi (Hausinger and Karplus, 2001). Due to their high-urease content, jack beans are the preferred source of this enzyme. Jack bean urease (urease in following descriptions) exists as a hexamer of six identical monomeric subunits, each having a molecular weight of 91 kDa (Hausinger and Karplus, 2001). The isoelectric point of urease is at pH 5.0–5.1 (Sumner and Hand, 1929) and it is here that the lowest solubility is expected. The total protein content of jack bean meal (JBM in following descriptions) is approximately 22% (Jones and Johns, 1916–1917) and the urease content is varies between 0.07% and 0.14% of total mass, depending upon type and provenance of the jack bean meal (Sumner and Hand, 1928).

Urease catalyses the decomposition of urea. It is therefore of use in medical diagnostics and is employed for the measurement of urea content in human blood (Walsh, 2002). Catalytic activity is used in this work in order to quantify both the urea yield in different processing steps as well as the enzymatic purity. Two measures are employed, the total activity and the specific activity. The former quantifies the amount of *active* enzyme present, the latter is a measure for purity and is calculated by normalising the total activity to the total protein content. It is important to stress that the total activity is *not* a measure for the *total amount* of urease present, as deactivated enzyme is not detected. To our knowledge, the crystal structure of jack bean urease has not yet been solved to high resolution, although the lattice parameters and space group symmetry are known (Jabri et al., 1992). Crystals of urease have cubic symmetry with octahedral crystal habit. The crystal size achievable using standard methods of crystallization is around 5 μm (Sumner, 1951).

In addition to urease three other major jack bean proteins, precanavalin, concanavalin A and concanavalin B, are well characterised. Precanavalin (Smith et al., 1982) (M_w = 49 kDa for the monomer) is a seed storage protein and is the major protein component of the jack bean. Originally named canavalin, the name precanavalin is used here (as used in Smith et al., 1982) in order to distinguish this protein from the modified protein obtained by crystallization using a trypsin digest (Sumner and Howell, 1936). Precanavalin has not yet been crystallized without conversion to canavalin, rather it precipitates in amorphous form from a protein extract. The concanavalin A monomer (M_w = 26 kDa) (Olson and Liener, 1967) is known to crystallize as bisphenoids with rhombic symmetry. These can reach dimensions of *ca.* 100 μm (Sumner, 1919). Concanavalin B is a monomer (M_w = 33 kDa; Hennig et al., 1995) and crystallizes as large (up to 2 mm) needles (Sumner and Howell, 1936). Other known jack bean proteins are the toxic enzyme canatoxin, possibly a modified form of urease (Follmer et al., 2001), the enzymes *N*-glycanase (Sheldon et al., 1998), chitinase (a modified form of concanavalin B; Schlesier et al., 1998; Hahn et al., 2000), and α-mannosidase (Kimura et al., 1999).

There is a significant body of work on the crystallization of jack bean urease (Hanabusa, 1961; Mamiya and Gorin, 1965; Blakeley et al., 1969, amongst others) and almost all based their work on the method of Sumner (1951). In his work urease was extracted at 28 °C using a 32% (v/v) acetone solution in water and then crystallized by cooling the extract to 4 °C. An improved method for *re*crystallization of urease was suggested 1941 by Dounce (1941) and was used by most authors cited above. Since the 1960s, chromatographic methods have