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Aqueous two-phase systems as an alternative process route for the fractionation of small inclusion bodies

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Abstract

Aqueous two-phase protocols have been established which successfully generate highly purified preparations of small inclusion bodies (IBs) from whole cell homogenates. Particle size analysis of disruptates confirmed that intense disruption (concomitant with maximal product release) was compromised by the corelease of contaminating solutes and the micronisation of cell debris yielding a similar particle size range to the IBs (100–200 nm). PEG 300–phosphate systems enabled partial recovery of IBs in the top phase of ATPS. In contrast, PEG 8000–phosphate systems partitioned IBs more efficiently as a discrete sediment within the lower phase, whilst the majority of micronised debris remained in the interphase. The α-glucosidase IB yield and purity in ATPS was bettered only by analytical sucrose density gradient centrifugation, which is not readily scaleable for application in process operations. The successful recovery of such small IBs from complex homogenates highlights a generic role that ATPS techniques might play in the recovery and purification of new bioparticulate products (viral and plasmid gene therapy vectors, particulate protein vaccines etc.).

Keywords: Aqueous two-phase systems; Inclusion bodies

1. Introduction

Overexpression of foreign genes in prokaryotes such as *E. coli* commonly leads to the production of intracellular aggregates of the recombinant protein defined as inclusion bodies (IBs) [1,2]. Primary recovery of the product in the form of IBs has some attractive process features which can be exploited in biotechnology processes. For example, the product is sequestered intracellularly in a near homogenous state in dense granules characterised by a discrete size distribution (generally 400–1200 nm) [3]. Such product definition invites obvious routes for purification and recovery.

The location of the product has a profound influence upon subsequent downstream processing and product recovery. The access and release of intracellular proteins can be achieved by a variety of means and the selection of a specific separation system will depend upon the product concentration and the nature of the contaminating debris particles and proteins. In some cases recombinant proteins expressed intracellularly (e.g., α-2b interferon [4]) can be released by a process involving partial solubilisation of the cell wall and membrane, thus

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enabling extraction of the protein without full disruption.

The removal of cellular debris from IBs has traditionally been accomplished by fractional centrifugation using continuous-flow centrifuges. In terms of scale-up these centrifuges are less efficient than laboratory centrifuges [5] so that efficient debris removal may become unpredictably compromised. An alternative approach to IB processing at large scale has been pioneered by researchers and process engineers at Genentech (San Francisco, CA, USA) [6,7], to successfully achieve high yields of human insulin-like growth factor I (IGF-I) sourced from IBs by the integration of in situ solubilisation with PEG–salt aqueous two-phase extraction. Aqueous two-phase systems (ATPS) provide a viable technique for large scale cell and protein fractionation [8], and have the potential advantage of providing selective fractionation not possible by centrifugation and microfiltration processes operated to recover IBs. However, historically the empirical nature of ATPS [9] has necessitated the development of new process parameters for each individual product. In contrast, centrifugation and filtration processes developed for a certain cell type and product can be generally applied generically without significant modification. The current rapid development of a mechanistic understanding of the operation of ATPS will benefit the predictive design and operation of systems to achieve desired separation objectives with complex, particulate feedstocks.

The IB system under investigation in the present study expresses yeast α-glucosidase PI (EC 3.2.1.20) derived from Bakers' yeast in *E. coli*. The native form of the enzyme (M_r 67 000) is a soluble, monomeric protein, isoelectric point (p*I*) 5.6, containing five free cysteine residues and no disulphide bonds. However, the IB form is an enzymatically inactive, insoluble aggregate of recombinant protein accumulated within the cytoplasm as small (150 nm) dense particles. These insoluble particles uniquely provide a vehicle for studying and optimising the fractionation processes applicable for recovery of small particles from obdurate heterogeneous feedstocks. Their inherent size range (100–200 nm) is particularly relevant to that possessed by contemporary bioparticulate products such as viral and gene therapy vectors and vaccines currently in development.

2. Experimental

2.1. Standard expression conditions

Fermentations were conducted at working volumes of 1, 4 and 15 l in 20 l LSL Biolafitte fermenters. Cells (*E. coli* K12 R/182 harbouring plasmids pKK177-3 GLUCPI, pREM6677) were grown in Luria broth supplemented with 40 mg/l ampicillin at 37°C. The pH was maintained at 6.8 controlled by the addition of 2 *M* H_2SO_4, whilst pO_2 was maintained above saturation by cascade control of agitation speed (350 rpm), pressure and air flow-rate (1.5 vessel volumes per minute). Inoculation was performed with 10% volumes of overnight cultures grown in M9 minimal medium supplemented with 0.5% casamino acids, 40 mg/l ampicillin and 10 mg/l trimethoprim. Upon reaching an absorbance at 550 nm of 1.5–2.0, protein synthesis was induced by addition of 5 m*M* IPTG (isopropyl-β-D-thiogalactopyranoside). Nutrient feed was supplied by an initial charge of 0.2% (w/v) D-glucose. Whole broths were harvested aseptically and the biomass concentrated by centrifugation (10 000 *g*, 30 min, 10°C, Beckman JA-10 rotor) thereby eliminating pigments, unmetabolised growth media and extracellular materials. Solid cellular material was stored at −20°C.

2.2. Homogenisation

Cells were resuspended at 2% and 25% wet mass per volume of intact cells (2 and 25%, wet w/v) from 1, 4 and 15 l fermentations in deionised water, and homogenisations undertaken with a 15MR APV Manton Gaulin (APV Products, Crawley, Sussex, UK) high-pressure homogeniser operated at a pressure of 55.2 MPa. Repeated cycles (up to a maximum of 10) were performed to ensure complete disruption of intact cells. The feed temperature was maintained at 10±2°C for each pass. Following homogenisation, the disruptate was used directly in aqueous two-phase studies or clarified by centrifugation (39 000 *g*, 45 min, 10°C, JA-17 rotor), where

soluble protein released at cell rupture was discarded in supernatants. The insoluble debris and IBs harvested as the solid fraction were stored at −20°C until required.

2.3. Density gradient centrifugation protocols

Cell disruptates derived from 2% wet weight/volume of intact cells (referred to hereafter as 2% eww/v) was layered onto the upper surface of a preestablished discontinuous sucrose gradient (the densities screened ranged from 1.11 to and 1.30 g/ml in increments of 0.02 g/ml). Centrifugation was conducted in a Beckman L80 ultracentrifuge for 30 min at 40 000 *g*, 20°C using a SW-28 rotor.

2.4. Standard centrifugation protocols

Two established protocols (A) adapted from Marston et al. [1] and (B) according to Schoner et al. [10] were investigated. An initial common step exploited centrifugation of the 2% (eww/v) cell lysate (12 000 *g*, 15 min, 4°C, JA-17 rotor). The supernatants were discarded and the pellets resuspended in (A) 50 m*M* Tris–HCl, pH 8.0, 50 m*M* NaCl, 10 m*M* EDTA, 0.1 m*M* PMSF and 0.5% (v/v) Triton X-100 or (B) deionised water. Following a second centrifugation as above, supernatant and pellet from protocol (A) was analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), whilst the solid fraction from protocol (B) was resuspended in 0.1 *M* Tris–HCl (pH 8.5) containing 8 *M* urea and centrifuged once more. The resultant solid and supernatant fractions were then analysed by SDS–PAGE.

2.5. Tangential-flow microfiltration

A Minitan (Millipore) unit fitted with polysulphone membranes of pore size 0.45 μm and membrane area of 0.48 m^2 was operated as a tangential-flow microfiltration at 20°C and 0.07 MPa transmembrane pressure yielding a permeate flux of 30 l h^{-1} m^{-2}.

2.6. ATPS

ATPS were formed directly in cell lysates (equivalent to 2% eww/v intact cells) by addition of solid poly(ethylene glycol) (PEG, of average molecular mass of 300 or 8000; Sigma, St. Louis, MO, USA) and a mixture of potassium dihydrogenorthophosphate (KH_2PO_4) and dipotassium hydrogenorthophosphate (K_2HPO_4; Sigma) to yield the appropriate weight percent system composition (%, w/w). Control of system pH was achieved by manipulation of the ratio of dibasic to monobasic phosphate (e.g., 18:7 to yield system pH 6.8). After 10 min vigorous mixing to achieve solid dissolution, phase separation and definition was enhanced by a brief low speed centrifugation step (860 *g*, 10 min, 20°C, Beckman JA-17 rotor).

2.7. Phase contrast microscopy

Samples taken from the top, bottom and the interphase (a third discrete phase analogous to those described by Boland and coworkers [11,12]) of the ATPS were observed through a film of oil on an Olympus BH-2 microscope at 1000-fold magnification.

2.8. SDS–PAGE

SDS–PAGE analysis of isolated fractions was conducted according to the discontinuous method of Laemmli [13]. Samples were resuspended in denaturing buffer, 3.25% SDS and 5% β-mercaptoethanol in 62.5 m*M* Tris–HCl, pH 6.8, and boiled at 100°C for 5 min prior to electrophoretic analysis on 7.5% T–2.65% C polyacrylamide separating gel and a 4% stacking gel operated in a Mini Protean vertical electrophoresis cell (Bio-Rad Labs.). Protein bands were visualised by staining with 0.1% Coomassie Brilliant Blue R-250, 40% methanol, 10% acetic acid. Laser densitometry (Pharmacia LKB Ultroscan XL and BioSoft Quantiscan) was used to quantify the intensity of discretely stained protein bands relative to known bovine serum albumin (BSA) standards electrophoresed within a defined range of concentrations (25–250 μg/ml). Unless stated otherwise, SDS–PAGE analysis was performed only upon

samples of recovered insoluble materials. Any residual insoluble material in samples prepared for SDS–PAGE was eliminated by centrifugation at 11 600 *g* in a MSE Minifuge prior to electrophoretic analysis.

2.9. Protein assay

The protein content of isolated phase samples was estimated using a bicinchoninic acid assay (BCA; Pierce, Rockford, IL, USA) and the results expressed relative to a calibration plot derived from the assay of standard concentrations of BSA.

3. Results and discussion

3.1. Productive fermentation

The fermentation conditions were developed to maximise the production of the insoluble IB form (>90%) of α-glucosidase relative to the soluble counterpart. This was achieved by regulation of the growth temperature (37°C) and pH (pH range 6 to 8) as detailed by Kopetzki et al. [14,15]. Fig. 1, depicts the progress of the fermentation after induction with IPTG. As the protein synthesis is fully induced (at about 180 min) a rapid decrease in detectable soluble α-glucosidase was accompanied by the appearance of phase bright, α-glucosidase IBs within the cytoplasm of the *E. coli* host cells (see Fig. 1 photographic insert).

3.2. Cell disruption

Efficient cell breakage is required if high process yields of intracellular products are to be achieved. The most frequently employed methods at industrial scale are mechanical and are based upon liquid shear using high pressure homogenisers such as the Manton Gaulin (15MR APV) [16]. All disruption techniques investigated herein were normalised in terms

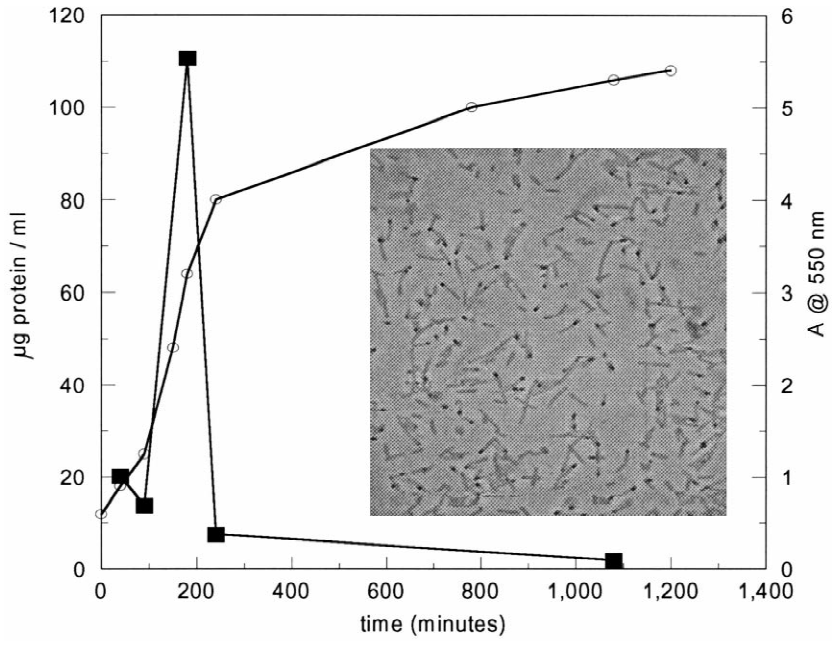

Fig. 1. Growth profile of an α-glucosidase IB fermentation. The growth profile (○; OD 550 nm) and the α-glucosidase activity (■) obtained using optimal conditions for IB production in *E. coli* at 37°C, pH 6.8 after induction with 5 m*M* IPTG (*t*=0; full details given in Section 2.1). The photographic insert was derived from the phase-contrast microscopy of an aliquot withdrawn 10 h post induction (magnification ×1000).

of protein release from 2% (eww/v) intact cell slurry against data obtained using such a device, operated at 55.2 MPa for five passes (see Fig. 2). The cytoplasmic location of the IB product meant that the application of mild lysis techniques of osmotic shock, freeze–thaw or treatment with alkali, often employed for the extraction of periplasmic proteins, were inefficient. Sonication (40 W output) and enzyme lytic methods were included as laboratory benchmarks, but would have proved unsuitable for process scale applications. Enzymatic lysis using lysozyme (0.2 mg/ml) introduces a further contaminant and may prove to be economically unacceptable at large scale. Disruption efficiency and particle sizes for all non-mechanical methods were screened by phase-contrast microscopy and analysis by Malvern Mastersizer (data not shown).

Multiple passes through the homogeniser were investigated to reduce the number of intact cells and attempt the micronisation of debris to a particle size range less than that of the IBs [16]. Such particles have previously been reported as chemically and physically robust entities in such process environments [1,8,17]. Particle size analysis of the disruptate (see Fig. 3) indicated that, after three sequential homogenisations, the disruptate contained both debris and IB particles falling within an identical mean range of diameters (100–200 nm). This served to compound the difficulty anticipated for product isolation from debris. The physical and chemical properties of the homogenate (particle size, density and shape) are critical in determining the degree of separation possible with such a mixture [3]. The small size differences between IBs and resultant debris potentially compromises the application of size driven separation processes conventionally employed for the recovery of IBs and other bioparticulates. It is important to note that in the present study varied biomass concentrations (2 to 25%, eww/v)

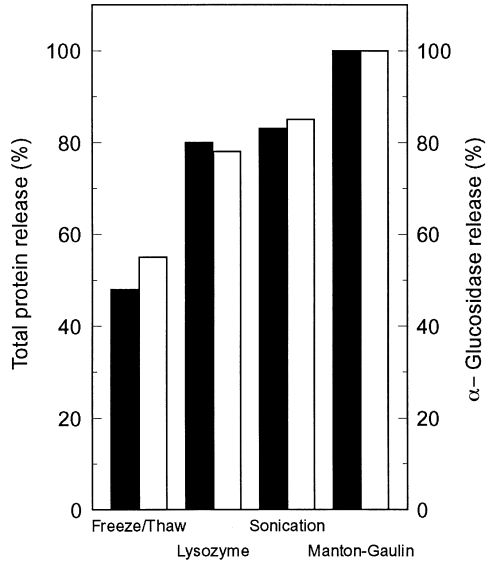

Fig. 2. Comparison of cell disruption techniques. Equivalent aliquots of intact cells at 2% (wet w/v) were subjected to (i) freeze–thaw (three consecutive cycles), (ii) incubation with lysozyme at 0.2 mg/ml for 1 h and (iii) sonication (40 W output) on ice. All values for total protein release (■) and α-glucosidase release (□) are expressed as percentages relative to those attained by high-pressure homogenisation with the Manton Gaulin homogeniser (five passes, 55.2 MPa, 10°C).

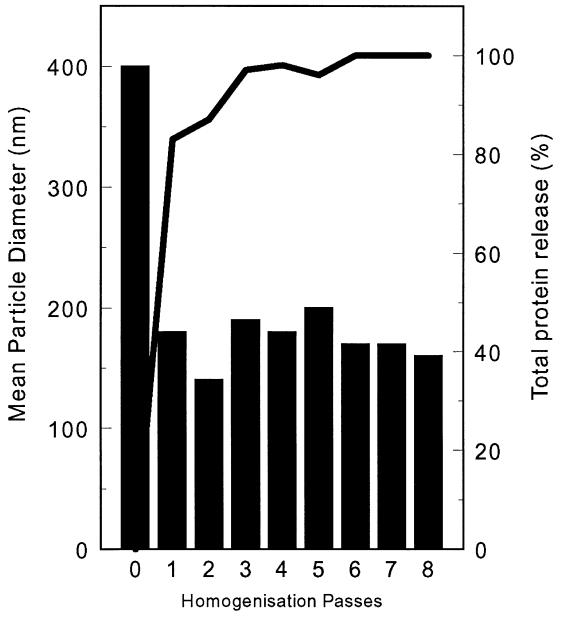

Fig. 3. Optimisation of high-pressure homogenisation. Intact cells at 2% (wet w/v) were passed through the homogeniser (55.2 MPa, 10°C). Samples removed after each cycle were analysed for mean particle size (Malvern Mastersizer; solid bars) and for cell wall integrity by phase-contrast microscopy. Release of total soluble protein (line) was determined by BCA assay and is expressed relative to the value obtained after eight cycles of homogenisation.

did not significantly alter the quality of the subsequent homogenate (data not shown).

3.3. Fractionation of IB particles from cell debris

Primary fractionation processes were screened to evaluate their relative efficiency in excluding contaminating material whilst maximising the recovery and minimising the loss of the target product. The main contaminants in all IB preparations were identified as peptidoglycans, lipids, nucleic acids, lipopolysaccharides, insoluble cell debris and associated membrane bound proteins (data not shown). The comparative performance of density gradient centrifugation, standard centrifugation, tangential flow microfiltration and ATPS were evaluated by densitometric quantitation of SDS–PAGE analyses undertaken with samples of recovered IB products.

3.4. Density gradient centrifugation

The analytical technique of sucrose density gradient centrifugation enabled fractionation of IB particles from similarly sized debris contaminants at laboratory scale. It also facilitated an estimation of the buoyant density of the IB (1.22 g/ml) whilst acting as a benchmark in method comparisons. High density is a common characteristic of IB products [2,3], reflected in the phase bright, refractile nature observed under phase contrast microscopy. The sedimentation behaviour of IB was similar to that of a non-enveloped capsid of hepatitis C virus [18], and in both cases cell debris migrated under centrifugal forces to a density range of 1.11 to 1.13 g/ml.

3.5. Standard centrifugation

IBs have been conventionally recovered at laboratory and industrial scales by processes of centrifugation augmented by various washing procedures [1,10]. In most circumstances, the IBs and debris are first sedimented and then resuspended in mild conditions exploiting either a chaotrope (8 *M* urea) or a detergent, such as 0.5% SDS or 0.5% Triton X-100, to preferentially solubilise contaminant debris rather than product. Solubilised components are therefore eliminated in the supernatant of a second centrifugation step.

3.6. Tangential-flow microfiltration

Bacterial extracts have traditionally been regarded as unsuitable for depth filtration (exploiting pore sizes up to 0.45 μm) due to their tendency to block the filtration medium. Tangential-flow microfiltration, where elevated shear forces at the filter surface are employed to offset fouling, provide a scaleable clarification regime applicable to particulate recovery. This size based particle fractionation was assessed using cell lysate [equivalent to 2% (eww/v) of intact cells] applied to a Minitan microfiltration rig operated at 0.07 MPa transmembrane with 0.45-μm polysulphone membranes (eight arranged in series yielding a total area of 0.48 m^2). Preliminary studies of the retentate evaluated by SDS–PAGE indicated that neither micronised debris or IB particles significantly traversed the membrane. The device served only to provide a 10-fold concentration of the process feedstock with both IB product and debris accumulating on the retentate side of a heavily fouled membrane. Microfiltration regimes may be optimised by manipulation of buffer conditions and back flushing operations [19,20], but the abject failure of preliminary screening invited assessment of an alternative process.

3.7. ATPS

Based upon the successful fractionation and recovery of Prp and scrapie associated fibrils (10–100 nm largest dimension) from bovine brain homogenates [21], PEG 300–potassium phosphate systems operated at pH 6.8 were evaluated in the recovery of IBs. The first intention was to implement a two-stage extraction step whereby the top phase from a primary extraction would be subjected to challenge by a fresh bottom phase [22] with the intention of backstripping the product. Systematic evaluation of systems at long tie line length (TLL>25%, w/w, <59%, w/w) and volume ratio (V_r~1.0), showed insufficient selectivity to warrant application for the fractionation of IB product to a specific phase. The consistent formation of a significant third phase (the interphase) in the formerly ATPS studied served to entrap both product and debris components. This reduced the maximum recoverable product in the PEG-rich top phase to less than 30% of the total input. Phase-contrast micro-

scopy of top and interphase fractions indicated a small proportion of IBs free from debris within the top phase, whilst the majority of IB and debris remain within the interphase (see Fig. 4).

Reassessment of the literature for the fractionation of particulates (cell debris [22], virus like particles [23] and viral vectors [24,25]) prompted the investigation of PEG 8000–phosphate systems, where particle and molecular rejection was expected. Systems compositions, predominantly in the upper quadrant of the binodal as specified by Hustedt et al. [26] and Bamberger et al. [27], were systematically assessed. Phase forming chemicals were added as solids to *E. coli* disruptates derived by homogenisation from the equivalent of 2% wet weight intact cells per unit volume (2%, eww/v). Centrifugation at 860 *g* assisted phase separation. Systems (TLL 20.5%, w/w, V_r 0.7) composed of PEG 8000 and the dibasic potassium phosphate salt at pH 8.9 achieved the best recoveries, where IBs were harvested as a discrete sediment within the lower salt-rich phase, whilst the majority of micronised debris remained in the interphase (see Fig. 5). Both top and bottom phases remained relatively particle free. It should be noted that control separations based on gravity settling over a 24-h period at room temperature confirmed that observed fractionation was due to bona fide partitioning phenomena and not simply the influence of centrifugal forces. The densities of the top and bottom phases were determined as 1.07 and 1.12 g/ml, respectively i.e., values lower than the estimates of buoyant density derived for IB by analytical ultracentrifugation. In contrast, the less efficient PEG 300–potassium phosphate system had a top phase density of 1.11 g/ml and a bottom phase density of 1.32 g/ml.

More critical evaluation of the partition characteristics were made by preparing a discontinuous ''pseudo ATPS'' using two sucrose solutions having the same density as the top and bottom phases of the successful PEG 8000–phosphate system. Harvested

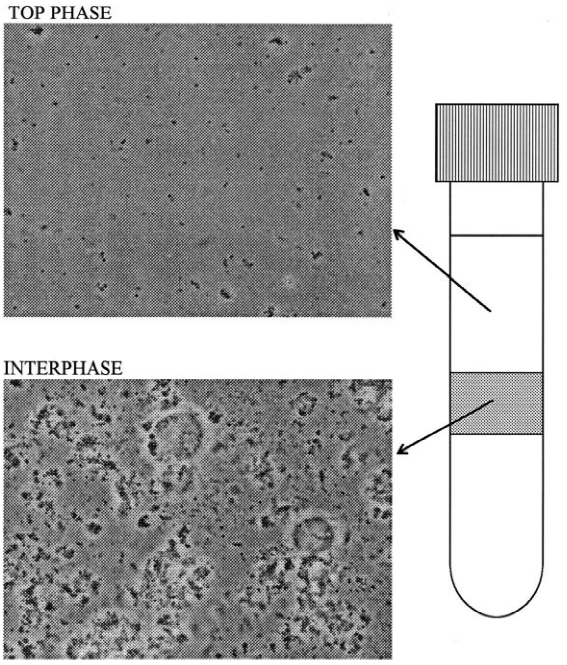

Fig. 4. Schematic representation of PEG 300–phosphate ATPS (20:20, w/w), pH 6.8, TLL=44.5% (w/w) and volume ratio=1.0. The interphase is defined as a third discrete phase within the ATPS analogous to those described by Boland and coworkers [11,12]. Phase-contrast microscopy was used to visualise the isolated phase fractions as described in Section 2.7 at a magnification of ×1000.

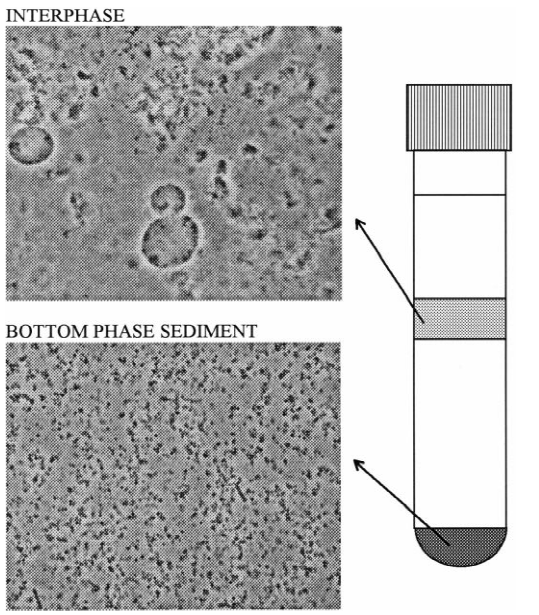

Fig. 5. Schematic representation of PEG 8000–phosphate ATPS (10:10, w/w), pH 8.9, TLL=20.5% (w/w) and volume ratio=0.7. Phase-contrast microscopy was used to visualise the isolated phase fractions as described in Section 2.7 at a magnification ×1000. Top phase samples were free of particles (data not shown).

samples were analysed by SDS–PAGE in tandem with ATPS controls. In the sucrose based systems, both IB and debris material remained dispersed at the interface and within the dense phase. The absence of the sediment in these ''pseudo ATPS'' highlights other, as yet unknown physicochemical driving forces as being significant for particle fractionation in the PEG–phosphate system. The present poor understanding of these mechanisms remain an impediment to the predictive establishment and operation of particle fractionation in ATPS (highlighted in Ref. [9]).

Since the IB product was recovered here as a sediment, attempts were made to minimise the phase volumes. Movement along the tie line enabled a controlled variation in phase volume ratio (V_r). From a starting composition of PEG 8000–phosphate (10:10, w/w), movement up the tie line with PEG concentrations exceeding 10% (w/w) yielded volume ratios >0.7. This reduced the volume of the bottom phase until the interphase merged with the sedimented material and thus made IB fractionation very difficult. The mass of sediment harvested did not significantly change with increasing volume ratio. However, the interphase increased in mass and occupied an increasing percentage volume of the whole system. Similar observations were made when investigating systems with PEG 8000<10% (w/w) and phosphate>10% (w/w), ($0.01<V_r>0.7$) whereby a reduction in the top phase volume was accompanied by an increase in the volume of the interphase and a reduction in the mass of recovered sediment.

SDS–PAGE analysis of isolated fractions from this systematic screening indicated that the PEG 8000–phosphate system (10:10, w/w) (TLL=20.5%, w/w; V_r=0.7) provided the best compromise between operational V_r and maximum partition and recovery of product in the bottom phase sediment.

3.8. Comparative performance of IB fractionation regimes

Quantitation of the recovered IB fractions, and an assessment of the selectivity of recovery, was made by laser densitometric analysis of SDS–PAGE (Fig. 6). Equivalent loads of disrupted cells were processed by each method (density gradient centrifugation, standard centrifugation tangential flow microfiltration and PEG 300, PEG 8000–phosphate ATPS) and the recovered product streams were prepared for analysis as described in Section 2. The purity of the recovered IB was assessed relative to that recovered for analytical, density gradient centrifugation data. Fig. 7 indicates that the ATPS provides the best selective fractionation of α-glucosidase in IB form, contributing approximately 80% of the protein staining with Coomassie blue in the bottom phase sediments. The urea washed particles from the centrifugation protocol (A) yielded a cleaner preparation than that recovered from the detergent washing (protocol B). However, both contained more low-molecular-mass protein impurities (14 000–60 000 Da) than the fractions recovered from ATPS. Analysis of the retentate from the microfiltration experi-

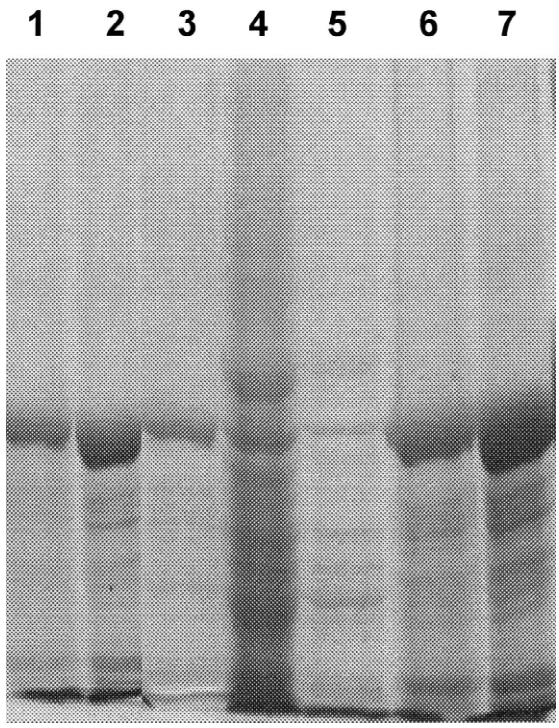

Fig. 6. SDS–PAGE analysis of methods screened for the isolation of IBs from cell debris. Samples prepared as described in Section 2.8. Lane identities (1) density gradient centrifugation, (2) bottom phase sediment PEG 8000–phosphate ATPS, (3) standard α-glucosidase (M_r 67 000 Da), (4) tangential-flow microfiltration retentate, (5) top phase of PEG 300–phosphate ATPS, (6) centrifugation protocol (B) 8 *M* urea, (7) centrifugation protocol (A) 0.5% (v/v) Triton X-100.

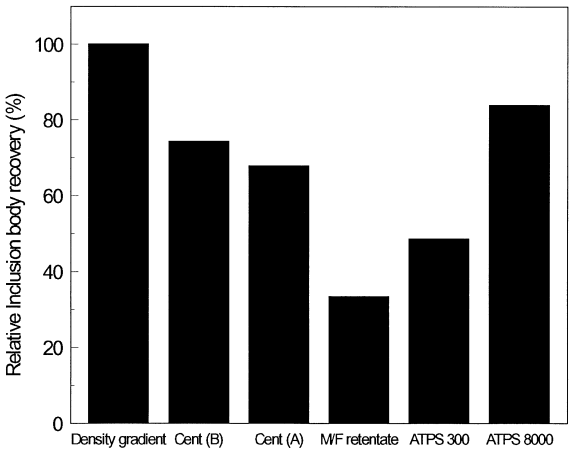

Fig. 7. Laser densitometry of SDS–PAGE analysis of methods screened for the isolation of IBs from cell debris. IB recovery (represented by the intensity of gel staining for α-glucosidase at M_r 67 000) is expressed relative to that recorded for analytical density gradient centrifugation used as a benchmark in these studies.

ment confirmed the dominant effect of concentration for this process. The sequestration of dense IBs as a sediment, in the PEG 8000–phosphate ATPS, free from the bulk micronised cellular debris, provides a valuable means of selective fractionation of the target particulate which is not possible when employing conventional, size based purification techniques.

4. Conclusions

A simple aqueous two-phase system has been identified and successfully implemented for the fractionation of small IBs from fine debris in cell homogenates, thereby, solving many of the problems imposed by processing fine particulates. The best IB recoveries were obtained with PEG 8000–phosphate systems where IBs could be isolated as a discrete sediment within the lower phase, whilst the majority of micronised debris remained in the discrete interphase. The α-glucosidase IB yield and purity in ATPS was only bettered by scale limited analytical density gradient centrifugation in sucrose media. The successful recovery of such small IB particulates from complex homogenates highlights a generic role that simple ATPS might play in the purification of new bioparticulate products which are characterised by nanometre scale dimensions (e.g., viral or plasmid gene therapy vectors and particulate protein vaccines).

Acknowledgements

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