Process Biochemistry 46 (2011) 1847–1852

doi:10.1016/j.procbio.2011.06.014

Primary recovery of lipase derived from Burkholderia sp. ST8 with aqueous micellar two-phase system

Introduction

Aqueous two-phase system (ATPS) has gained widespread applications for the separation, purification and concentration of various species, ranging from organic compounds [1], metal ions [2,3] to biomolecules like cells, organelles and proteins [4–6]. AMTPS, which is also termed as surfactant-mediated phase separations or cloud-point extraction [7], has been employed as an alternative to the traditional ATPS for the purification of biomolecules [8–11]. Binary phase micellar system can be generated by using single surfactant compound at relatively low concentration [12]. The commonly used phase-forming surfactants include Triton X-114 (polyoxyethylene detergent, $C_{14}E0_6$) and different detergents from alkyl polyoxyethylene family (C_mEO_n) like n-decyltetraethylene oxide ($C_{10}EO_4$) [13]. Micelles are formed in the aqueous solution when the addition of the surfactant is above the minimum concentration of surfactant or known as critical micelle concentration (CMC) [14]. The formation of two coexisting phases in the AMTPS is induced when the temperature of the surfactant solution was shifted above a lowest critical point, which is defined as cloud-point temperature [7]. The homogeneous surfactant solution will become turbid and undergo macroscopic phase separation upon raising the temperature above the cloud-point. The surfactant molecules will self-aggregate to form a coacervate (micelle-enriched) phase, in equilibrium with an aqueous (surfactant-depleted) phase [14].

The clouding phenomenon is resulted from the dehydration of the polyoxyethylene chain and low solubility of micelles at above the cloud-point temperature [7]. The intermicellar attraction and the mixing of micellar clusters are responsible for the appearance of turbidity and a micellar phase can be then separated out from the solution due to a small difference in density. This clouding phenomenon is reversible upon cooling the solution below the cloud-point temperature. The cloud-point temperature of the surfactant is highly dependent on the surfactant's conformational structure and it can also be controlled by the addition of different compounds [7]. For example, addition of inorganic salts such as sodium chloride and ammonium sulfate can depress the cloud-point temperature of surfactant as a result of salting-out

effect [7,15]. Application dealing with protein purification and isolation in AMTPS was mainly focused on a variety of amphiphilic biomolecules, integral membrane protein extraction and some extracellular proteins [16,17]. Affinity of proteins for the surfactant is mainly based on their surface hydrophobicity [7,18], size [19] or charge [20]. In AMTPS, micellar structure (e.g. lamellar, spherical, cubical, planar) and size could be modulated and optimized to entrap target protein, owing to the self-assembly character and labile nature of micelles [12]. Target proteins can be then solubilized and embedded in the micelles, thus permitting them to be selectively extracted and concentrated into the surfactant-rich phase.

Lipases (E.C. 3.1.1.3) are hydrolases that catalyze the hydrolysis of carboxyl ester bonds presents in triacylglycerols to liberate fatty acids and glycerol [21,22]. Lipase has been widely used in the pharmaceutical and chemical industries [22–24]. Different strategies for microbial lipase purification have been developed, including multi-step precipitation and chromatography [25]. These conventional purification methods in general are time-consuming, low scalability and cost effectiveness, and sometimes may offer an unsatisfied purification yield of desired product [25]. The objective of the present study is to demonstrate the use of the AMTPS constituted of nonionic surfactant for the primary recovery of lipase from microbial source. The phase behavior (cloud-point temperature) and the partitioning behavior of lipase were investigated in different micellar systems formed by the widely available surfactants such as triblock copolymer (trade name Pluronic) and Triton. Moreover, removal of surfactant from the purified lipase is also studied by an introduction of new salt solution (potassium thiocyanate) into the micellar phase.

2. Materials and methods

2.1. Materials

The nonionic surfactant Triton X-114 (TX-114), Pluronic L31 (Mn~1100), Pluronic L61 (Mn~2000), Pluronic L81 (Mn~2800), Pluronic L121 (Mn~4400), ferric chloride ($FeCl_3$), ammonium thiocyanate (NH_4SCN), potassium thiocyanate (KSCN), potassium chloride (KCl), p-nitrophenyl (pNP) as well as bicinchoninic acid (BCA) protein assay kit were purchased from Sigma–Aldrich (St. Louis, USA). Potassium nitrate (KNO_3), potassium sulfate (K_2SO_4), potassium acetate (CH_3CO_2K), potassium phosphate dibasic (K_2HPO_4) and potassium monobasic (KH_2PO_4) were obtained from Merck (Darmstadt, Germany). p-Nitrophenyl laurate (pNPL) was delivered by Fluka Co. (USA). The nonionic surfactants and salts were used without further purification.

2.2. Cultivation of Burkholderia sp. ST8

Burkholderia sp. ST8 cells were cultivated in a 500 ml shake flask. The working

volume of the cultivation was 100 ml. The production medium consisted of nutrient broth, 0.325% (w/v); Tween 80, 1% (v/v); $CaCl_2$, 0.1% (w/v) and gum arabic, 1% (w/v) [5]. The pH of the medium was adjusted to 9 before sterilization. The inoculum was pre-grown in the 1% (w/v) nutrient broth for 16 h. 5% (v/v) inoculum was added to the medium and the cultivation was carried out at 30 °C and agitation speed of 200rpm in an incubation shaker. After 72 h of incubation, the fermentation culture was harvested and applied for the protein partitioning studies.

2.3. Analytical procedures

2.3.1. Lipase activity assay

Lipase activity was measured by the spectrophotometric quantification of pNP formed by the cleavage of substrate pNPL [26,27]. The procedure of lipase activity assay has been described previously [5]. The results were expressed as a mean of triplicate readings with an estimated error of ±5%.

2.3.2. BCA assay

The concentration of protein was determined by the BCA protein assay [28] following the manufacturer's instructions. The samples were subjected to acetone precipitation in order to eliminate the interference from surfactant. The pellets were then resolubilized by dissolving in a 5% (w/v) solution of sodium dodecylsulfate (SDS). The procedure of BCA assay has been described in a previous publication [5]. The results were expressed as amean of triplicate readings with an estimated error of ±5%.

2.3.3. Determination of the Pluronic concentration

The concentration of nonionic Pluronic surfactant was determined as reported elsewhere [29,30]. A solution of 0.1M ammonium ferrothiocyanate was first prepared by dissolving anhydrous $FeCl_3$ and NH_4SCN in deionized distilled water. The sample solution (0.5 ml) was mixed with ammonium ferrothiocyanate solution (0.5 ml) in a 2.0 ml microcentrifuge tube, followed by addition of chloroform (0.5 ml). The mixturewasvigorously shaken for 30 minand centrifuged at 3000rpmfor 2min. The bottom chloroform phase was withdrawn and the solution's absorbance was measured at 510nm using spectrophotometer. The concentration of Pluronic surfactant in the sample was then calculated with the calibration curve of Pluronic standard solution, which was generated by using the serially diluted standard solutions of Pluronic.

2.3.4. Coexistence curve of the AMTPS

The coexistence curve of the nonionic surfactant in buffer was constructed by the visual observation on the change of solution's opacity (cloud-point method) [31]. First of all, different concentrations of surfactant solutions were prepared and subsequently heated in a water bath (Model F12-ED, JULABO Labortechnik GmbH, Seelbach, Germany) until the transparent solutions became turbid. The water bath has a temperature stability of±0.03%. The solution was continuously stirred in order to maintain thermal and chemical equilibrium. At a cooling rate of approximately 0.5 °C per min, the cloud-point temperature was monitored and recorded as the average temperature at which the solution exhibited single and transparent phase. The measurement of cloud-point temperature was repeated twice and the precision was within 0.5 °C.

2.4. Experimental procedure

AMTPS was formed by weighing appropriate quantities of surfactant solution, buffer and salt into the 2ml microcentrifuge tube, in terms of the percentage by weight (%, w/w). Weighed crude feedstock was loaded at 50% (w/w) concentration to generate AMTPS with total weight of 2 g. The resulting solution was thoroughly vortexed and then incubated in an isothermal water bath at a fixed temperature of 2 ◦C above its cloud-point temperature. After at least 30 min of equilibration, the phase separation was facilitated by centrifugation at 10,000rpm and temperature of 2 ◦C above their respective cloud-point temperatures for 10 min. After thermal phase separation, volume of the top (V_T) and bottom (V_B) phases was read and determined from the calibrated lines on the centrifuge tube. The aliquot from each phase (150_l) was collected with the use of pipette and was appropriately diluted with buffer, before it was analyzed for lipase activity and total protein concentration.
For experiments involving the back-extraction of lipases into aqueous phase and the removal of surfactant, lipase was first partitioned in the primary partition step. In the second step, the lipase-depleted top aqueous phase was removed and replaced by an equal volume of salt solution. The solution was then mixed thoroughly and separated into two distinct phases by centrifugation at 10,000rpm and room temperature for 10 min. The lipase activity and concentration of surfactant in both phases were then analyzed. The process scheme of AMTPS for the recovery of lipase is shown in Fig. 1.

The distribution of a solute (i.e. lipase, protein and surfactant) between the phases is described by partition coefficient (K), which is defined as the ratio of equilibrium concentration of solutes in the top phase and the bottom phase, i.e. K = solute (top phase)/solute (bottom phase). Selectivity of the lipase over the contaminant proteins in AMTPS is given as Selectivity = K_L/K_P, where indices L and P are the partition coefficients of the lipase and total proteins, respectively. The volume ratio of the AMTPS is determined as $V_R = V_T/V_B$. The purification fold of lipase is calculated according to the following equation, based on the bottom phase for which the lipase has shown preference: $P_{FB} = (E_B/P_B)\times(P_C/E_C)$, where E_B and E_C are the concentrations of lipase in the bottom phase and crude feedstock, respectively; whereas P_B and P_C are the concentrations of total protein in the bottom phase and crude feedstock, respectively. The yield (%) of the lipase in bottom phase is given as the function of V_R and K_L: Y_B (%) = $100/[1 + (V_R \times K_L)]$. The results reported here are the mean of two independent experiments with an estimated error of ±5%.

2.5. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Analysis

SDS-PAGE analysis [32] was carried out using polyacrylamide gel made of a 12% (v/v) resolving gel and a stacking gel of 4.5% (v/v). The gels were run in a Mini-Vertical SE250 electrophoresis unit (Amersham Biosciences, USA) at 110V and 36mA for 75 min. The resulting gel was then stained with Coomassie® Brilliant Blue. Protein bands were visualized and analyzed after destaining.

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