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Purification of acetohydroxy acid synthase by separation in an aqueous two-phase system

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Abstract

Extraction in a polyethylene glycol (PEG)–phosphate aqueous two-phase system was considered as a primary step in purification of the acetohydroxy acid synthase III large catalytic subunit from an *E. coli* extract. Extraction optimization was achieved by varying the system parameters. Two systems with the following weight compositions were chosen for purification: PEG-2000 (16%)–phosphate (6%) and PEG-4000 (14%)–phosphate (5.5%)–KCl (8%), both at pH 7.0 and 1 mg total protein per 1 g system. Significant purification was achieved by a single extraction step with 70% recovery of the enzyme. After an additional ion-exchange chromatography step, pure enzyme was obtained in a 50% overall yield.

Keywords: Aqueous two-phase systems; Partitioning; Enzymes; Acetohydroxy acid synthase

1. Introduction

Acetohydroxy acid synthase (AHAS) is an enzyme that catalyzes the first common step in the biosynthesis of the branched-chain amino acids in plants, yeast and bacteria [1,2]. AHAS is of considerable biotechnological interest, because it is responsible for production of essential amino acids [3] and is the target of major herbicides [4].

The current conventional methods for purification of AHAS and its subunits are elaborate and slow, and include the use of an expensive hydrophobic column [5,6]. For the separate subunits of this protein, which are relatively labile, an alternative method is desirable. In the present work, an extraction method based on aqueous two-phase system (ATPS) [7] was considered for use in separation of AHAS catalytic subunits from bacterial extracts. This method features are simplicity, mildness, rapidity and low cost [8,9]. There are many factors, which can be manipulated to obtain the desired partition of the target protein in the extraction system [7,10]. Polyethylene glycol (PEG)–salt systems are preferred for practical application in protein separation [10,11].

Partition experiments were carried out with the pure catalytic subunit of the wild type *Escherichia coli* AHAS isozyme III (62 kDa) and with background proteins, in order to find optimal conditions for their maximal separation. The partition coefficients of both the target enzyme and background proteins were compared for different parameters of ATPS. Based on the partition observations, a two-step purification process was designed, that combines

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ATPS extraction and an ion-exchange chromatography step.

2. Experimental

2.1. Materials

PEGs with average molecular masses of 1000, 2000, 4000 or 6000 were obtained from Fluka (Buchs, Switzerland). K_2HPO_4, NaH_2PO_4 and KCl were obtained from Sigma (St. Louis, MO, USA). Fractogel TSK DEAE-650M was purchased from Merck (Darmstadt, Germany).

2.2. Aqueous two-phase systems

Two-phase systems were prepared from stock solutions of PEG-1000, 2000, 4000 or 6000 (60%, w/w), phosphate buffer as a mixture of K_2HPO_4 and NaH_2PO_4 (40%, w/w) and KCl (23%). Phosphate was chosen as constituent of the salt phase because of its minimal adverse effect on the enzymatic activity. The total system weight was 2 g. After 2 min of gentle mixing of the system components, low-speed centrifugation (300 *g*, 5 min) was used to speed up phase separation. The phase volume ratio was held near one. All the partition experiments were carried out at 20°C.

2.3. Ion-exchange chromatography

Ion-exchange chromatography was performed according to Vyazmensky et al. [5] on a 7×0.5 cm column of Fractogel TSK DEAE-650M. The column was eluted at 0.5 ml min^{-1} with a 30 ml linear gradient from 0 to 0.25 *M* of KCl in buffer solution.

2.4. Enzyme production and assay

The large, catalytic subunit of AHAS isozyme III was expressed after induction with isopropyl thiogalactoside in *E. coli* XLMRF carrying plasmid pUI, which encodes AHAS III large subunit gene *ilvI* [12]. Bacteria were grown and crude extract was prepared as described [12]. Enzyme for the partition experiments was purified according to the method described [5]. A crude extract of the same strain grown without expression of the target protein was used as background proteins.

AHAS activity was measured by determination of acetolactate production from pyruvate [5].

2.5. Other analyses

The phase compositions of PEG and phosphate were determined in a manner similar to that reported by Merchuk et al. [13]. The binodals of the aqueous two-phase systems were obtained using the turbidity method [7].

Total protein concentration was measured by the method of Bradford [14], using bovine serum albumin as standard. The partition coefficient *K* is defined as the protein concentration in the upper phase divided by that in the lower phase.

Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on 13% polyacrylamide gel [15] and stained with Coomassie Blue.

3. Results and discussion

3.1. Partition behavior of pure AHAS and the background proteins

3.1.1. Effect of PEG molecular mass

The partition coefficient of AHAS showed a stronger dependence on the PEG molecular mass than that of the background proteins (Fig. 1); a lower PEG molecular mass resulted in a higher partition coefficient. This behavior is common for many proteins in PEG–dextran and PEG–salt systems [16,17]. In the PEG-1000 system, the AHAS partition coefficient had a maximal value. However, the partition selectivity was low, and separation of the enzyme from the contaminating proteins was poor. Poor partition selectivity of proteins at low molecular masses of PEG is a commonly observed phenomenon [8,17]. Maximal separation of AHAS from background proteins was achieved in the PEG-2000 system. The separation in the initial PEG-4000 system was not satisfactory, but it could be improved by manipulation of the system parameters (see below). The high viscosity complicates handling of PEG-6000 system in protein purification. Therefore,

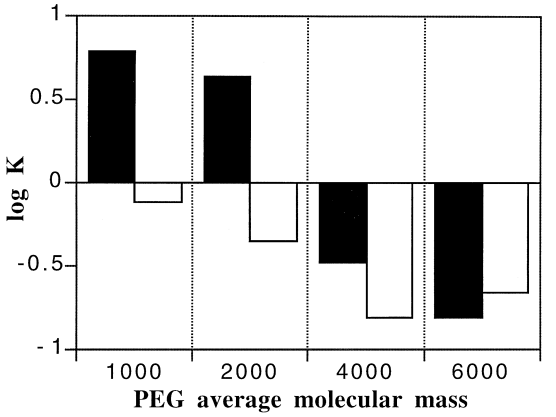

Fig. 1. Partition behavior of AHAS III catalytic subunit (black bars) and background proteins (white bars) in PEG–phosphate systems with different PEG average molecular masses. The systems used were (weight composition): PEG-1000 (19%)–phosphate (6.5%), PEG-2000 (16%)–phosphate (6%), PEG-4000 (12%)–phosphate (6%), PEG-6000 (11%)–phosphate (5.5%), all at pH 7.0.

intermediate values of PEG molecular mass (PEG-2000 and 4000) were chosen for further work.

3.1.2. Effect of stability ratio

The stability ratio (SR) defines the distance of a system from the binodal [18]. In the range studied, increasing SR did not change the partition coefficient *K* of pure AHAS in the PEG-2000 system (Fig. 2). The increase in *K* for PEG-4000 at high values of SR was associated with precipitation of the protein at the interface. System compositions near the binodal curve (SR=0.1 for PEG-2000 system and SR=0.14 for PEG-4000 system) were chosen to minimize the precipitation.

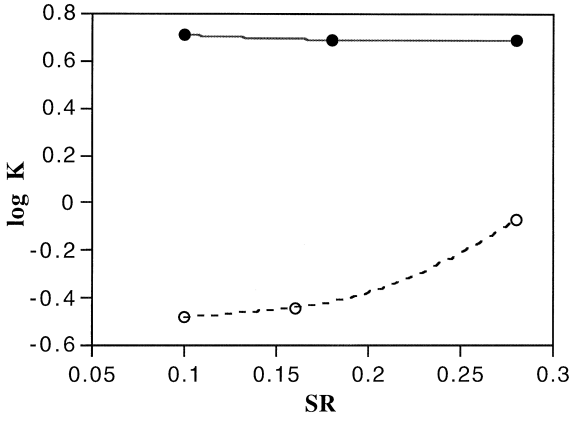

Fig. 2. Partition behavior of AHAS in PEG-2000–phosphate (filled circles) and PEG-4000–phosphate (open circles) systems as a function of stability ratio (SR) at pH 7.0.

3.1.3. Effect of KCl concentration

It has been shown in PEG–dextran systems, that ions of added salt distributed unequally between the phases lead to an electrostatic potential difference between the phases [7,16]. This raises the possibility of changing the partition coefficient of specific proteins according to their charge [19]. It appears, however, that in PEG–salt systems, a selective increase in the partition coefficient for certain proteins at high KCl concentrations is caused mostly by surface hydrophobicity differences between the proteins [20,21], and not by charge differences between them. The AHAS large subunit apparently has a fairly hydrophobic character, as revealed by hydrophobic interaction chromatography [5,6], and therefore prefers the more hydrophobic PEG phase when the salt concentration in the system increases. This feature can be used for a selective enhancement of the AHAS partition coefficient by addition of KCl.

Fig. 3 shows that in both PEG-2000 and PEG-4000 systems the partition coefficient of AHAS is maximal at high KCl concentrations. The best separation of the enzyme from the background proteins was obtained in the PEG-4000 system containing 8% (w/w) KCl. In PEG-2000 system addition of KCl did not improve the net separation.

3.1.4. Effect of pH

As a general rule, negatively charged proteins prefer the upper PEG-rich phase and positively charged proteins partition to the lower phase [17]. As the pH increases above the isoelectric point (p*I*) of a protein, it becomes negatively charged, its interaction with PEG becomes stronger [22], and the partition coefficient increases. Fig. 4 shows the expected effects of pH. The values of *K* for both AHAS and background proteins were similarly dependent on pH in PEG-2000– and PEG-4000–phosphate systems. The majority of the indigenous proteins from *E. coli* are acidic [23,24] and the p*I* of the AHAS III catalytic subunit is neutral [25]. Therefore at high pH both AHAS and background proteins are negatively charged and prefer the PEG-rich phase. Thus, a

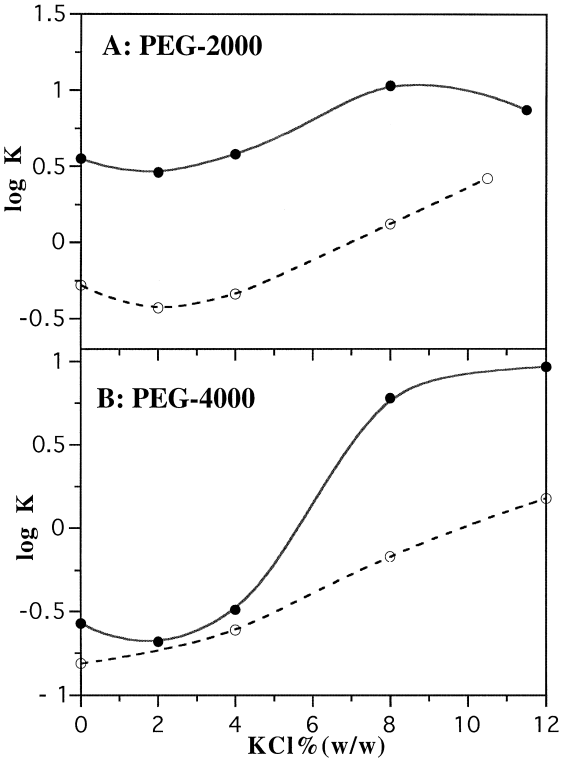

Fig. 3. Partition behavior of AHAS and background proteins in the PEG-2000 (16%)–phosphate (6%) system (A) and the PEG-4000 (14%)–phosphate (5.5%) system (B), as a function of added KCl concentration at pH 7.0. Filled circles: AHAS; open circles: background proteins.

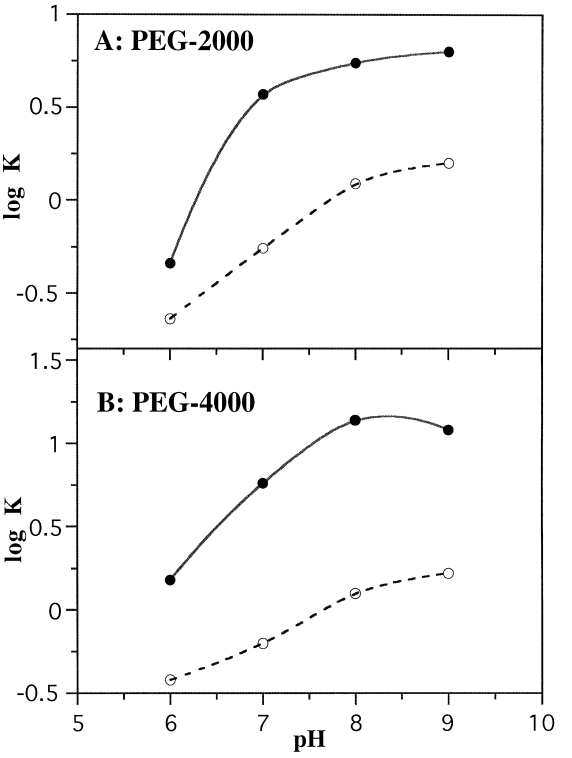

Fig. 4. Partition behavior of AHAS and background proteins in the PEG-2000 (16%)–phosphate (6%) system (A) and in the PEG-4000 (14%)–phosphate (5.5%)–KCl (8%) system (B), as a function of pH. Filled circles: AHAS; open circles: background proteins.

change in pH from pH 7.0 does not improve the separation.

3.1.5. Effect of protein concentration

As the protein concentration comes close to the solubility limit, saturation behavior is observed. Further increase in the protein concentration caused the protein to precipitate at the interface and lowered its recovery. A final total protein load of 1 mg per 1 g system was chosen as optimal.

3.2. Purification of AHAS III catalytic subunit by ATPS

Taking the above results into account, two extraction systems that allowed for a good separation between AHAS and the background proteins were chosen, system I: PEG-2000 (16%)–phosphate (6%) and system II: PEG-4000 (14%)–phosphate (5.5%)–KCl (8%), both at pH 7.0.

The two systems were used for purification of AHAS from *E. coli* XLMRF/pUI crude extract. The proteins were analyzed by SDS–PAGE after each purification step (Fig. 5).

Analysis by SDS–PAGE showed that in both systems AHAS is entirely confined to the top phase and most of the contaminant proteins partition to the bottom phase. The presence of background protein contaminants did not have a strong effect on the partition behavior of AHAS. The specific enzymatic activity in the PEG phase was >70% of that of pure enzyme. However, AHAS recovery was about 70%, because of the enzyme tendency to precipitate at the interface.

An additional step of back extraction from the PEG phase into the phosphate phase with a low

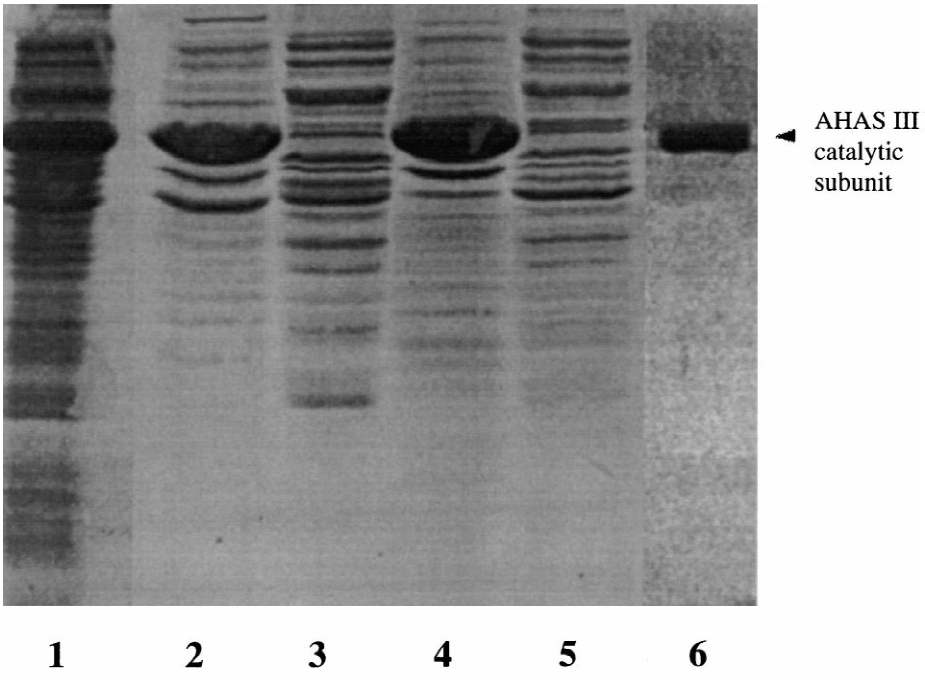

Fig. 5. Reducing SDS–PAGE gel of the purification steps. 1, Crude *E. coli* extract (50 μg protein); 2, upper phase of extraction system I (30 μg protein); 3, lower phase of extraction system I (30 μg protein); 4, upper phase of extraction system II (30 μg protein); 5, lower phase of extraction system II (30 μg protein); 6, protein from ion-exchange chromatography of the upper phase of extraction system I (20 μg protein).

concentration of KCl and lower overall concentration of PEG and phosphate did not improve the purification (not shown). An attempt to recover the precipitated enzyme by back extraction also was not successful. Apparently, the enzyme undergoes irreversible denaturation upon the precipitation.

A DEAE ion-exchange column was chosen for further purification of the protein. Although system II yielded a slightly better purification (as judged by the SDS–PAGE gel picture), it was decided to further purify the top phase from system I, because of its low salt concentration and viscosity. This phase was directly applied to the column, and the bound enzyme was released with a gradient of KCl. By this step most of the remaining contaminants, as well as PEG, were removed (Fig. 5).

The two-step process described above allowed one to obtain an enzyme with a specific activity as high as that obtained by the conventional, more complicated procedure [5,6]. The yield of the overall process was 50%, the same as the yield achieved by the conventional procedure. Use of ATPS can be considered as a rapid, simple and effective first step in AHAS purification procedure.

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References

[1] H.E. Umbarger, in: F.C. Neidhardt, J.L. Ingraham, K. Low, B. Magasanik, M. Schaechter, H.E. Umbarger (Eds.), *Escherichia coli* and *Salmonella typhimurium* – Cellular and Molecular Biology, Vol. 1, American Society for Microbiology, Washington, DC, 1987, p. 352.
[2] D.M. Chipman, Z. Barak, J.V. Schloss, Biochim. Biophys. Acta 1385 (1998) 401.

[3] L. Eggeling, S. Morbach, H. Sahm, J. Biotechnol. 56 (1997) 167.
[4] G.M. Kishor, D.M. Shah, Annu. Rev. Biochem. 57 (1988) 627.
[5] M. Vyazmensky, C. Sella, Z. Barak, D.M. Chipman, Biochemistry 35 (1996) 10339.
[6] Z. Barak, J.M. Calvo, J.V. Schloss, Methods Enzymol. 166 (1988) 455.
[7] P.A. Albertsson, Partition of Cell Particles and Macromolecules, Wiley, New York, 1986.
[8] H. Walter, D.E. Brooks, D. Fisher, Partitioning in Aqueous Two-Phase Systems – Theory, Methods, Uses, and Applications to Biotechnology, Academic Press, London, 1985.
[9] A. Carlson, Sep. Sci. Technol. 23 (1988) 785.
[10] O. Cascone, B.A. Andrews, J.A. Asenjo, Enzyme Microb. Technol. 13 (1991) 629.
[11] J.A. Asenjo, A.S. Schmidt, F.M. Hachem, B.A. Andrews, J. Chromatogr. 668 (1994) 47.
[12] M. Vyazmensky, T. Elkayam, D.M. Chipman, Z. Barak, Methods Enzymol. Branched Chain Amino Acids, part B, in press.
[13] J.C. Merchuk, B.A. Andrews, J.A. Asenjo, J. Chromatogr. B 711 (1998) 285.
[14] M. Bradford, Anal. Biochem. 72 (1976) 248.
[15] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning – A Laboratory Manual, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989.
[16] P.A. Albertsson, G. Johansson, F. Tjerneld, in: J.A. Asenjo (Ed.), Separation Processes in Biotechnology, Marcel Dekker, New York, 1990, p. 287.
[17] A.S. Schmidt, A.M. Ventom, J.A. Asenjo, Enzyme Microb. Technol. 16 (1994) 131.
[18] A. Kaul, Rationale for the Selection of Aqueous Two-Phase Systems for the Purification of Recombinant Proteins Expressed in *Escherichia coli*, Ph.D. Thesis, University of Reading, Reading, 1995.
[19] G. Johansson, in: H. Walter, D.E. Brooks, D. Fisher (Eds.), Partitioning in Aqueous Two-Phase Systems – Theory, Methods, Uses, and Applications to Biotechnology, Academic Press, London, 1985, p. 161.
[20] T.T. Franco, B.A. Andrews, A.J. Asenjo, Biotechnol. Bioeng. 49 (1996) 300.
[21] B.Y. Zaslavsky, L.M. Miheeva, P.Y. Aleschko, A.U. Mahmudov, T.O. Bagirov, E.S. Garaev, J. Chromatogr. 439 (1988) 267.
[22] J.G. Huddleston, K.W. Ottomar, D.M. Ngonyani, A. Lyddiatt, Enzyme Microb. Technol. 13 (1991) 24.
[23] A.T. Andrews, I. Noble, S. Keeratipibul, J.A. Asenjo, Biotechnol. Bioeng. 43 (1994) 1.
[24] P.W. Woolston, A Physicochemical Database for an Expert System for the Selection of Recombinant Protein Purification Processes, Ph.D. Thesis, University of Reading, Reading, 1994.
[25] C.H. Squires, M. DeFelice, J. Devereux, J.M. Calvo, Nucleic Acids Res. 11 (1983) 5299.