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PREPARATION OF THE MULTIENZYME SYSTEM GRAMICIDIN S-SYNTHETASE 2 WITH AN AQUEOUS THREE-PHASE SYSTEM

ANGELA KIRCHNER, MARIANNE SIMONIS and HANS VON DÖHREN*
Institut für Biochemie und Molekulare Biologie, Technische Universität Berlin, Franklinstrasse 29, D-1000 Berlin 10 (F.R.G.)
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SUMMARY

The distribution of gramicidin S-synthetase activity from disrupted cells suspended in aqueous two- and three-phase systems was investigated. An optimized three-phase system containing 5% dextran, 8% Ficoll, 11% PEG and 6.7% disrupted cells was found to be effective in extracting gramicidin S-synthetase activity. The activity yield achieved was higher in comparison to other preparation methods, and the subsequent purification steps were greatly facilitated. The time needed for the preparation of the labile gramicidin S-synthetase was considerably reduced. The combination of the aqueous phase extraction with chromatographic methods yielded 19 mg gramicidin S-synthetase 2 in essentially pure form from 30 g (wet weight) of cells.

INTRODUCTION

The synthesis of the cyclic decapeptide gramicidin S cyclo(D-Phe-Pro-Val-Orn-Leu)$_2$ known to be produced by *Bacillus brevis* ATCC 9999 is catalyzed by gramicidin S-synthetase (GS). This enzyme system is comprised of two multifunctional polypeptide chains: gramicidin S-synthetase 1 (GS 1) and gramicidin S-synthetase 2 (GS 2) of molecular weight (MW) 120 000 and 280 000, respectively. Several procedures have been employed to prepare both synthetases in essentially pure form by using conventional purification methods[1–5].

Although these methods proved successful they were relatively time-consuming. Much care was needed to avoid degradation especially of the multienzyme GS 2 as the ability of GS 2 to catalyze peptide formation may easily be lost due to partial proteolysis or cleavage of the 4′-phosphopantetheine-containing entity[6,7].

In recent years new methods for the purification of biomaterials have been developed. One of these is the partition of cells, particles and macromolecules, etc., in aqueous phase systems[8,9]. Usual aqueous phase systems comprise water-soluble polymers such as dextran, and poly(ethylene glycol). The small differences in the interfacial tension and dielectric constant of aqueous phases and the possible stabilizing effect of the polymers render these systems very suitable for the preparation of labile enzymes.

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In the present study the possibility of using phase partition to purify the relatively labile multienzyme GS 2 was investigated. Besides using the frequently employed water–dextran–poly(ethylene glycol) two-phase system, three-phase systems consisting of water–dextran–Ficoll–poly(ethylene glycol) were examined.

MATERIALS AND METHODS

Chemicals

Dextran 5126 (7265) of average molecular weight 252000, industrial grade, was obtained from Sigma and poly(ethylene glycol) (PEG) 6000 from Serva. DE-52 was obtained from Whatman and Ultrogel AcA-34 from LKB. Ficoll was obtained from Pharmacia Fine Chemicals and [^{3}H]-L-ornithine (5 Ci/mol) from New England Nuclear. Glass beads of diameter 0.1 mm were obtained from W. A. Bachhofen.

Cultivation of cells

Bacillus brevis ATCC 9999 was grown according to the method described by Chiu[10] using a synthetic medium with fumarate plus glycerol as carbon sources. Alternatively, these bacteria were cultivated in a medium containing glutamate as described by Aust[11]. The cells were harvested by centrifugation and stored frozen at −20°C.

Cell disintegration

For small-scale preparation, 5 g (wet weight) of cells were thawed and suspended in 30 ml phosphate buffer [0 m*M* sodium phosphate, 0.25 m*M* EDTA and 2 m*M* dithioerythritol (DTE); pH 7.2] at 4°C and disrupted with an ultrasonic cell disrupter at a medium intensity and amplitude "2" for 2 min. For larger preparations of crude extract, 35 g (wet weight) of cells were suspended in 105 ml phosphate buffer (see above), and disrupted in a Dyno-mill equipped with a 300-ml batch glass chamber. The chamber was filled with 0.1-mm glass beads up to 80% of its total volume. The chamber and bearings were cooled by circulating ethanol at −15°C. Homogenization of cells was carried out for 10 min at an agitation speed of 2000 rpm and 10 m/s. The glass beads were removed by filtering through a fritted glass column and the resulting cell suspension was used in subsequent purification steps.

Preparation of crude extract with ammonium sulphate

The disrupted cell suspension was centrifuged at 15000 *g* for 10 min. The supernatant was treated with Streptomycin sulphate at a final concentration of 2% (w/v) and the precipitated nucleic acids were removed by centrifugation at 15000 *g* for 10 min. Then the protein in the supernatant was precipitated by ammonium sulphate at 55% saturation. After stirring for 30 min, the precipitated protein was collected by centrifugation at 20000 *g* for 20 min. The sediment was resuspended in a small volume of phosphate buffer and dialyzed against phosphate buffer before carrying out further purifications. All procedures were performed at 4°C.

Partition of protein in two-phase systems

Stock solutions of 20% (w/w) dextran and 30% (w/w) PEG were prepared and added to 35 g of disrupted cell suspension to final concentrations as indicated in

Results. The mixtures were then stirred at 4°C for 30 min and centrifuged at 400–3000 *g*, 2°C for 5–20 min. Aliquots from the top and bottom phases were taken for the assay of enzyme activities and protein contents.

Partition of protein in three-phase systems

Various amounts of the polymers dextran, Ficoll, PEG and phosphate buffer (20 m*M* sodium phosphate, 0.25 m*M* EDTA) were added to 35 g disrupted cell suspension to give final concentrations as indicated in Results and discussion. The mixture was stirred at room temperature for 30 min and then centrifuged at 10000 *g* for 20 min, at 10°C. Aliquots from the three phases were taken for the determination of enzyme activities and protein concentrations.

Preparation of gramicidin S-synthetase

A 35-g (wet weight) amount of cells was prepared and disrupted as described above. The suspension was then added to an optimized three-phase system. After phase separation the Ficoll-rich phase was with drawn and gently mixed with an appropriate amount of DE-52 pre-equilibrated with phosphate buffer. The mixture was gently stirred for 5 min at room temperature. After centrifugation at 50 *g* at 4°C for 30 min, the supernatant containing Ficoll was removed. The ion exchanger DE-52 was resuspended in phosphate buffer and the resulting slurry was packed in a column and washed with ten bed volumes of buffer. Then the protein was eluted with phosphate buffer containing 1 *M* sodium chloride. Five fractions of 5 ml were collected and assayed for enzyme activities and protein concentrations.

Gel permeation chromatography on Ultrogel AcA-34

GS 1 and GS 2 were separated by gel filtration on an Ultrogel AcA-34 column (60 cm × 2.5 cm) equilibrated with phosphate buffer. A 10-ml volume of the highly active DEAE-cellulose fraction was applied and eluted with equilibration buffer at a flow-rate of 50 ml/h. Fractions of 12 ml were collected and assayed for GS 1 and GS 2 activities, respectively.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Samples from all purification steps were characterized by electrophoresis on 7.5% polyacrylamide gels in the presence of SDS using the discontinuous buffer system described by Laemmli[12]. After electrophoresis, gels were stained with Coomassie Brilliant Blue R 250.

Enzyme assay

The activity of gramicidin S-synthetase was estimated according to the Millipore filter assay of Gevers *et al.*[13]. Each assay medium contained 10 m*M* sodium phosphate, pH 7.2, 5 m*M* ATP (pH 7.0 with sodium hydroxide), 25 m*M* magnesium chloride, 5 m*M* dithioerythritol, 5 m*M* L-Phe, L-Pro, L-Val, L-Leu, 50 n*M* [^{3}H]-L-Orn (specific activity 50 Ci/mol) and 6.25 n*M* EDTA in a volume of 0.2 ml. Incubation was carried out for 10 min at 37°C.

Protein determination

Protein was determined by measuring the absorbance at 260 and 280 nm as described by Warburg and Christian[14].

RESULTS AND DISCUSSION

Partition of gramicidin S-synthetase activity in dextran–PEG two-phase systems

The partition of the gramicidin S-synthetase (GS) in two-phase systems was evaluated from the ratio of the activities of GS in the top to that in the bottom phase. The dependence of the partition of the multienzyme system on the concentration of sodium chloride present in the two-phase systems is shown in Fig. 1. A minimum of activity was partitioned to the top phase at a salt concentration around 0.5 *M*. Similar behaviour was observed with other proteins[8]. Although higher salt concentrations increased the partition of GS to the top phase, such conditions were not adopted for the preparation of crude extract because of the adverse effects of high salt concentrations on subsequent purification steps. Taking these considerations into account, systems containing no salt were then investigated by varying the concentrations of dextran and PEG, respectively. Small variations in the partition were achieved by changing the concentration of PEG, whereas increasing concentrations of dextran caused more of the enzyme to be partitioned to the bottom phases.

A comparison of the two-phase system for the preparation of crude extracts with the ammonium sulphate purification method shows that a better activity yield could be achieved by the former method in fewer steps (Fig. 2). Yet, there was no significant improvement in the specific activity. A better partition system giving rise to an higher purification factor was then sought.

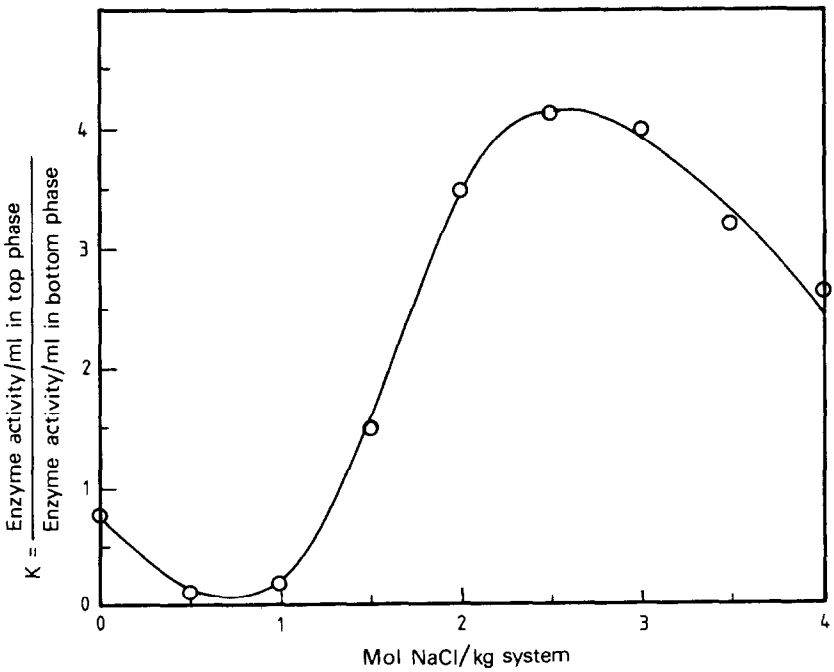

Fig. 1. Dependence of the partition coefficient of GS on the sodium chloride concentration in the dextran–PEG two-phase system. Phase composition: 1.64% dextran, 7.0% PEG.

Partition of GS in three-phase systems

By mixing more than two polymers aqueous polyphase systems can be formed. Three-phase systems consisting of dextran, Ficoll and PEG have been employed for the study of partition of haemoglobin, bovine serum albumin, enzymes from a lysate of baker's yeast and for the preparation of a membrane-bound phospholipase[15,16]. These systems have certain advantages over the two-phase systems in that impurities might be extracted into two phases instead of one.

Our preliminary investigations indicated that the use of a three-phase system might be very promising for the extraction of GS. To optimize the three-phase system for enzyme extraction, the polymer concentrations and the biomass loading of the three-phase system were varied. With increasing concentration of dextran, the percentage of activity in the middle Ficoll-rich phase decreased, whereas the activity in the bottom dextran-rich phase increased (Fig. 3). This trend is similar to that in the two-phase system. On the other hand, increasing the PEG concentration led to an increase in activity in the middle and a decrease in activity in the bottom phase (Fig. 4).

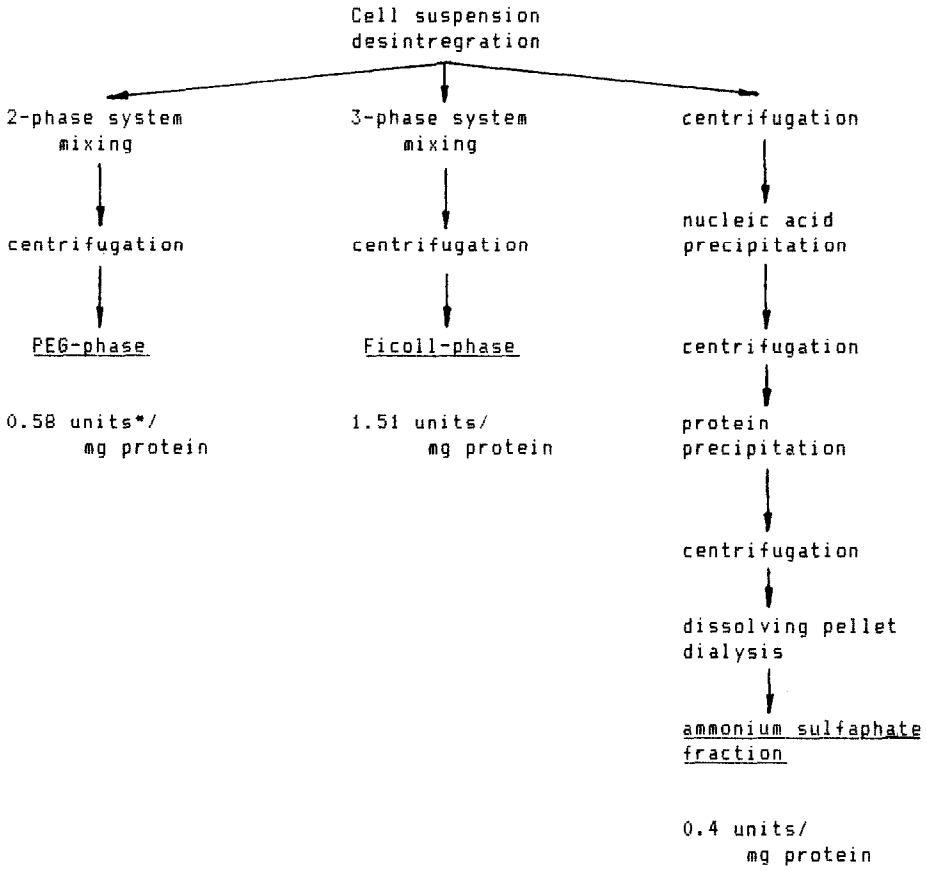

Fig. 2. Comparison of three extraction procedures for gramicidin S-synthetase. *Unit: 1 nmol product min^{-1}.

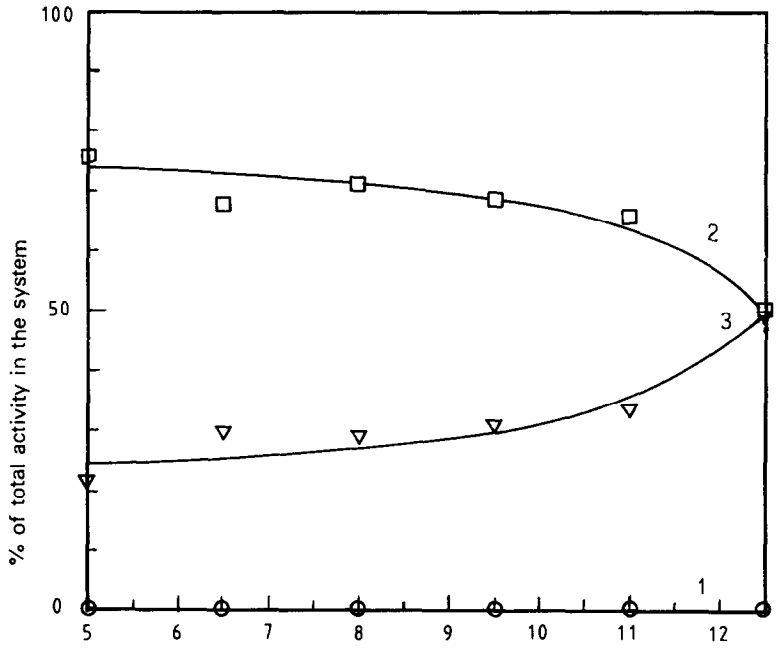

Fig. 3. Influence of increasing concentration of dextran on the distribution of GS in the dextran–Ficoll–PEG three-phase system. Phase composition: 8% Ficoll, 11% PEG and % dextran as shown. Relative activities: 1, in the top phase; 2, in the middle phase; 3, in the bottom phase.

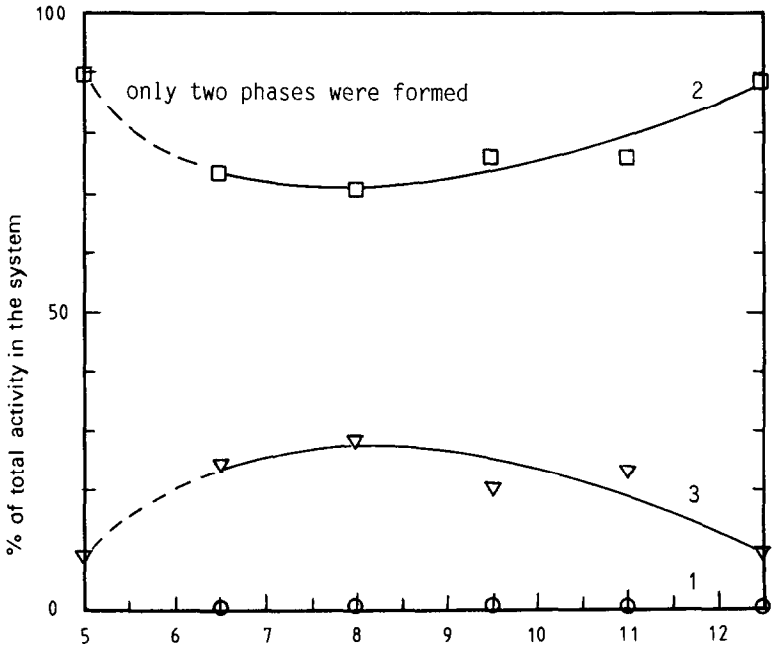

Fig. 4. Influence of increasing concentration of PEG on the distribution of GS in the dextran–Ficoll–PEG three-phase system. Phase composition: 8% Ficoll, 7.3% dextran an PEG as shown. Relative activities as in Fig. 3.

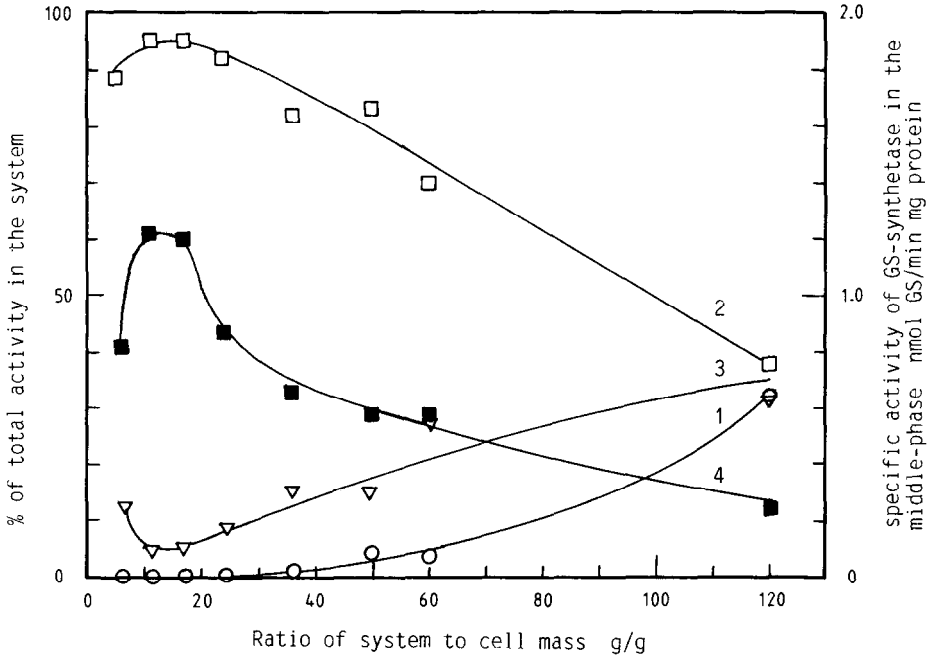

Fig. 5. Optimization of the cell loading on the three-phase system for the extraction of GS. Phase composition: 5% dextran, 8% Ficoll and 11% PEG. Relative activities as in Fig. 3. 4, Specific activity in the middle phase.

Based on these results, a system consisting of 5% dextran (average molecular weight 252000), 8% Ficoll and 11% PEG was chosen. A higher PEG concentration was not employed because the system was too viscous.

The optimum biomass loading of the system was then examined. Fig. 5 shows that the optimum loading is about 5–7% (w/w).

Preparation of GS using a three-phase system

A crude enzyme extract was prepared by using an optimized three-phase system of the following composition: 6.7% disrupted cells (wet weight), 5% dextran, 8% Ficoll and 11% PEG. The procedure was as described in the previous section. For further purifications, the enzyme was extracted from the Ficoll-rich phase using DE-52. A batch process was employed to obtain a more concentrated sample. The optimum amount of DE-52 for extraction of the enzyme was 0.4–0.5 g per g Ficoll-rich phase (about 10 mg protein). After separating the adsorbent, the polymer could be used again. This may be more economical for large-scale purification of enzymes, as the costs of such materials might be reduced considerably.

After desorbing the enzyme with 1 *M* sodium chloride, the fraction was then applied to an AcA-34 column and eluted as described. The highest GS 2 activity was found in two fractions. The purity of GS 2 was examine by SDS-PAGE. Fig. 6 shows that the enzyme in these fractions was nearly pure. The yield and purification factor are summarized in Table I.

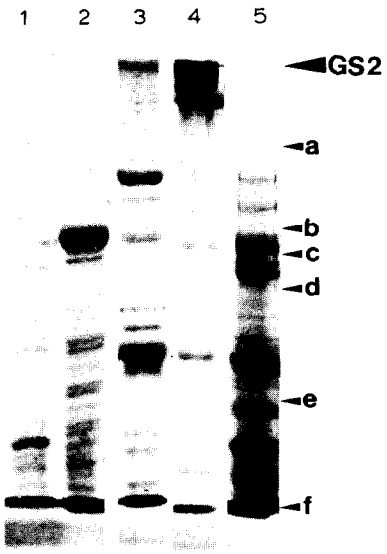

Fig. 6. Control of purification steps by SDS-PAGE. Lanes of protein: 1, in the top phase; 2, in the bottom phase; 3, in the middle phase; 4, in the eluate from AcA-34. 5, ammonium sulphate fraction from conventional preparation. Arrows indicate positions of gramicidin S-synthetase 2 (GS 2), myosin (MW 205 000) (a), β-galactosidase (116 000) (b), phosphorylase (95 000) (c), bovine serum albumin (67 000) (d), ovalbumin (43 000) (e) and carbonic anhydrase 29 000 (f).

Comparison of the three-phase system with other methods

It is seen from Fig. 2 (see also Fig. 6) that the preparation of crude extract using three-phase partitioning is more advantageous than other methods. The specific activity of GS and the enzyme yield were much higher. In the subsequent purification steps no difficulties were experienced. On the contrary, the high purifying effect of the three-phase system facilitated the subsequent purification to such an extent that a nearly pure GS 2 fraction was obtained after the gel chromatography on AcA-34.

The reason for the better yield and higher specific activity achieved in the three-phase system is still unknown. The stabilizing effect of the polymers and the low interfacial tensions between the phases might be among the factors contributing to this result. For a better understanding of the phase partitioning of solutes and

TABLE I

PURIFICATION OF GRAMICIDIN S-SYNTHETASE 2

Fraction	*Protein (mg)*	*Total activity (nM GS min^{-1})*	*Specific activity (nM GS $min^{-1}mg^{-1}$ protein)*	*Yield (%)*
Middle Ficoll-rich phase	637.5	963	1.51	100
Eluate from DE-52	296.8	644	2.2	67
Ultrogel AcA-34	18.9	450	23.8	47

polyelectrolytes, a characterization of the phase systems employed is helpful as suggested by Zaslavsky *et al.*[17,18]. Unfortunately, this has been done with only a few of the frequently used two-phase systems[19,20]. As few examples using three-phase systems to extract enzymes have been documented, it is too early to draw any conclusions concerning the potential use of such systems for purifying enzymes.

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REFERENCES

1 M. Yamada and K. Kurahashi, *J. Biochem. (Tokyo)*, 66 (1969) 529–540.
2 T. L. Zimmer and S. G. Laland, *Methods Enzymol.*, 43 (1975) 567–579.
3 H. Koischwitz and H. Kleinkauf, *Biochim. Biophys. Acta*, 429 (1976) 1041–1051.
4 C. Christiansen, K. Aarstad, T. L. Zimmer and S. G. Laland, *FEBS Lett.*, 81 (1977) 121–124.
5 R. J. Fleischaker, Jr., *M.S. Thesis*, Massachusetts, Institute of Technology, Cambridge, MA, 1977.
6 H. Koischwitz and H. Kleinkauf, *Abstr. Commun. 9th Meet. Fed. Europ. Biochem. Soc.*, Hungarian Biochem. Soc., Budapest, 1974, p. 89.
7 H. Koischwitz and H. Kleinkauf, *Biochim. Biophys. Acta*, 429 (1976) 1052–1061.
8 P. A. Albertsson, *Partition of Cell Particles and Macromolecules*, Wiley-Interscience, New York, 1971.
9 M.-R. Kula, K. H. Kroner and H. Hustedt, *Adv. Biochem. Eng.*, 24 (1982) 73–118.
10 C.-W. Chiu, *Doctor Thesis*, Technische Universität Berlin, Berlin, 1984.
11 H.-J. Aust, *Doctor Thesis*, Technische Universität Berlin, Berlin, 1984.
12 U. K. Laemmli, *Nature (London)*, 227 (1970) 680–685.
13 W. Gevers, H. Kleinkauf and F. Lipmann, *Proc. Natl. Acad. Sci. U.S.A.*, 60 (1968) 269–276.
14 O. Warburg and W. Christian, *Biochem. Z.*, 310 (1941) 384–421.
15 P. A. Albertsson, *Biochemistry*, 12 (1973) 2525–2530.
16 A. Hartmann, G. Johansson and P. A. Albertsson, *Eur. J. Biochem.*, 46 (1974) 75–81.
17 B. Yu. Zaslavsky, M. M. Larisa and S. V. Rogzhin, *Biochim. Biophys. Acta*, 510 (1978) 160–167.
18 B. Yu. Zaslavsky, N. M. Mestechkina and S. V. Rogozhin, *J. Chromatogr.*, 260 (1983) 329–336.
19 B. Yu. Zaslavsky, L. M. Miheeva, N. M. Mestechkina, L. G. Shchyukina, M. A. Chlenov, L. I. Kudrjashov and S. V. Rogozhin, *J. Chromatogr.*, 202 (1980) 63–73.
20 B. Yu. Zaslavsky, L. M. Miheeva and S. V. Rogozhin, *J. Chromatogr.*, 212 (1981) 13–22.