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Concentration and purification of β-glucosidase from *Aspergillus niger* by using aqueous two-phase partitioning

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Abstract

The extracellular enzyme β-glucosidase, present in a culture filtrate and produced by *Aspergillus niger*, was concentrated up to 700 times by two-phase partitioning. The two-phase systems were achieved by dissolving dextran and poly(ethylene glycol) in the culture filtrate in such proportions that the lower phase, containing the enzyme, consisted of a very small volume compared with the upper phase. The enzyme had high affinity for the lower phase when the system contained 100 m*M* KSCN at pH 8.0, and the recoveries of β-glucosidase were in the range of 85–95% with a concentrating factor of 60–720 times. At the same time, the enzyme was purified 2–3 times.

Keywords: *Aspergillus niger*; Aqueous two-phase systems ; Partitioning; β-Glucosidase ; Protein ; Enzyme

1. Introduction

The downstream processes in which microbially produced enzymes are purified and concentrated are in most cases costly and time-consuming. In the present work, we show how an enzyme can be concentrated up to 700 times by using the techniques of aqueous two-phase partitioning [1,2] with a system composed of dextran and poly(ethylene glycol) (PEG). These two-phase systems have earlier been used for concentration of viruses (up to 300 times) [3] and proteins have, during purification by extraction, been concentrated severalfold (up to 10 times) judged from partitioning data [4]. A large portion of enzyme can be recovered in concentrated form by choosing partitioning conditions so that the enzyme has an extremely high affinity for the lower (dextran-rich) phase and that this phase composes only a small fraction (1.2–0.06%) of the system. By this method the enzyme can be concentrated a great number of times and, at the same time, partly purified from other proteins (which have a lesser affinity for the lower phase) and from substances of low molecular weight which are partitioned with equal concentrations between the two phases. The process has been investigated, in the present work, by using a solution (culture filtrate) of β-glucosidase obtained by fermentation of *Aspergillus niger*, which produces this enzyme. The concentrating process is simple, cheap, and reliable and it can be carried out in a short time (1 h or less) even with large quantities of enzyme solution.

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2. Experimental

2.1. Chemicals

Dextran 500 [relative molecular mass (M_r)= 500 000] was purchased from Pharmacia (Uppsala, Sweden). PEG 8000 (M_r=8000) was obtained from Union Carbide (New York, NY, USA) as Carbowax 8000. Hydroxypropyl starch (HPS), Reppal 100, was purchased from Carbamyl (Kristianstad, Sweden). All chemicals were of analytical grade.

2.2. Fermentation

Fermentation was performed in a 25 l fermenter with 18 l working volume (BIOSTAT U, Braun, Germany) at 30°C and mixing at 350 rpm. Medium was based on Mandels salts [5] and the carbon source was 10 g l^{-1} glucose. Inoculum was prepared in shake flasks in 5% (w/w) malt extract. A 10% (w/w) 48 h old inoculum was used. The initial pH was in the range 5.5–6.0 and pH was adjusted during the fermentation with 10% (w/w) H_2SO_4 and 10% (w/w) NaOH to 5.5. Foam was controlled by the addition of Silicon Antifoam (BDH, Poole, UK). The fermenter was aerated at a flow-rate of 18 l min^{-1}. Enzyme production was continued for four days and samples were withdrawn every 24 h and analysed for β-glucosidase activity. After terminating the process, the fermentation broth was filtered through a glass filter and the culture filtrate was stored at 3°C.

2.3. Enzyme assay

The β-glucosidase activity was determined according to Nordkrans [6] but using 5 m*M* substrate instead of the recommended 1 m*M* concentration. A 0.1 ml of sample was mixed with 1 ml of 5 m*M* 4-nitrophenyl-β-D-glucopyranosid in 50 m*M* sodium citrate buffer pH 4.8 and kept at 50°C for 10 min. The reaction was stopped by addition of 2 ml of 1 *M* Na_2CO_3 followed by 10 ml of water and the absorbance was measured at 400 nm. In the blank 0.1 ml water was used instead of the enzyme sample. The activity in units (U) was calculated as mmol *p*-nitrophenol produced per min. One absorbance unit corresponds to 1.32 enzyme units per ml. The assay was done in duplicate or triplicate on each sample and the mean of the obtained values was used.

2.4. Protein assay

Protein content was determined according to Bradford [7] at 595 nm using correspondingly diluted phases or systems without sample as blanks. Bovine serum albumin was used as a standard. The determination was done in triplicate on each sample and the mean was used.

2.5. Phase diagram

Two-phase systems were prepared from PEG and dextran (containing 4.0% water) by dissolving the calculated masses of the polymers in water to a final weight of 10 g. After separation of the phases by centrifugation for 10 min at 1000 *g*, the content of dextran was determined by polarimetry and that of PEG from the refractive index (using corrections due to the presence of the dextran) using diluted phase samples as described earlier [8].

2.6. Two-phase partitioning

Calculated amounts of PEG, dextran, and salt (normally KSCN) were dissolved in culture filtrate to which had been added 1–2 m*M* phosphate buffer and whose pH had been adjusted with 3.6% ammonia. The dextran was dissolved before the addition of PEG. To dissolve the polymers the mixture was carefully mixed with a glass rod or, for larger quantities, with a magnetic stirrer. The systems were centrifuged at low speed using a fixed angel rotor. In some cases graduated and calibrated 10 ml centrifuge tubes were used which allowed determination of the phase volumes. Samples were taken by using an adjustable pipette (Gilson, Villiers-le-Bel, France), but their accurate masses were determined by using an analytical balance giving four decimals. After dilution the phases were analysed for enzyme and protein content.

2.7. Calculations

The amount of partitioned material, *m*, (enzyme or total protein) in the two phases (upper, u, and lower, l) gives the partition ratio *G* defined as $G=m_u/m_l$

[9]. The partition coefficient, $K=C_u/C_l$, where C is the concentration of material in upper and lower phases, respectively, is calculated from the determined concentrations but may also be obtained from the volumes of the two phases, V_u and V_l, and the G-value via $K=GV_l/V_u$. The percental partitioning into the upper phase is $100\cdot G/(1+G)$ and into the lower phase $100/(1+G)$.

2.8. Counter-current distribution

The distribution was carried out manually as described by Johansson and Andersson [10] using nine transfers. After equilibration of the first system (number 0) the main portion of the upper phase (leaving 0.1 ml) was transferred to a pure lower phase (the same volume as in the initial tube) supplemented with 0.1 ml of upper phase (forming system number 1). After addition of a fresh upper phase to tube number 0 the two systems were again equilibrated. This procedure was repeated until ten systems were obtained (number 0–9). To each system 15 ml of 5 m*M* sodium citrate buffer pH 4.8 were added to convert them into the single phase region. The contents of β-glucosidase and total protein were determined in these diluted systems. The percents of recovered enzyme and protein in each tube were calculated.

3. Results

3.1. Phase diagram

The phase diagram at 25°C is shown in Fig. 1a. When high polymer concentrations are used the upper phase consists of an essentially pure PEG solution while the lower phase besides the dextran (and water) also contains a low concentration of PEG. From the tie-lines, connecting the points representing the polymer compositions of top and bottom phase, the ratio between the masses of the phases can be calculated [1]. This ratio, mass of top phase divided by mass of bottom phase, is equal to the ratio of the two segments of the tie-line to the right and to the left, respectively, of the point representing the total composition of the phase system. The slope of the tie-lines in Fig. 1a increases with the PEG concentration in the top phase. The tie-line can be seen as a section of a straight line and therefore be described by the equation

$$C_{PEG} = k \cdot C_{dextran} + C_{PEG,top} \quad (1)$$

where C_{PEG} and $C_{dextran}$ are the concentration pairs along the line, $C_{PEG,top}$ is the PEG concentration in the top phase, and k is the slope varying from -0.49 (at $C_{PEG,top}=10\%$) to -0.65. The compositions of the lower phases (for polymer-rich systems) lay on a straight line with the equation

$$C_{PEG} = -0.01 \cdot C_{dextran} + 0.6 \quad (2)$$

Combination of these two equations (giving the intercept of the two lines) yields the concentration of dextran in the lower phase, $C_{dextran,bottom} = (C_{PEG,top}-0.6)/(-k-0.01)$.

For a system with the total composition of PEG, C^*_{PEG}, and of dextran, $C^*_{dextran}$, the tie-line is divided into the two segments x and y (see Fig. 1a) and $y/x=(C_{dextran,bottom}-C^*_{dextran})/(C^*_{dextran}-C_{dextran,top})$=mass ratio (top/bottom). Introducing the relation for the dextran concentration in the bottom phase and that $C_{dextran,top}$ is practically zero gives

$$y/x = \frac{C_{PEG,top}-0.6}{C^*_{dextran}(-k-0.01)} - 1 \quad (3)$$

The mass ratios calculated for various low concentrations of dextran are shown in Fig. 1b when the top phase contains a high concentration (10–26.4%) of PEG. The volume ratio (=mass ratio multiplied with the ratio of densities for bottom and top phase) is in this interval 1.05 to 1.10 times the mass ratio.

3.2. Effects of salt on the partitioning of β-glucosidase

Table 1 shows the dependence of the partition coefficient on the pH value in the presence of different salts. The steering effect of the salts is more significant with increasing pH due the increased negative net charge of the enzyme molecule. The K-values are especially low, $K<0.0005$, with KSCN, $KClO_4$ and KCl and the recovery in the bottom phase is >99.8%. The results also show that the lowest K-values (equal to the highest affinity for the

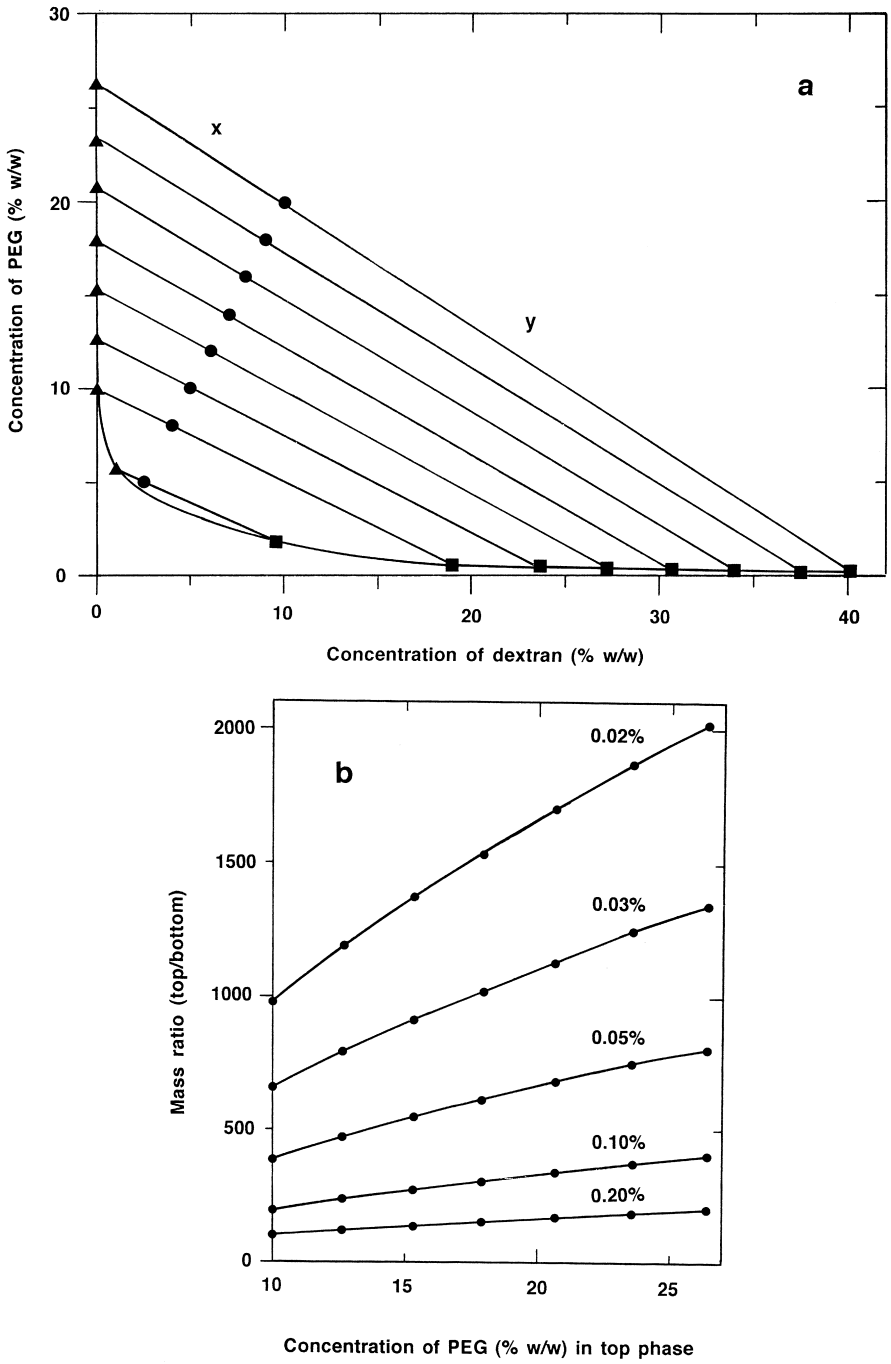

Fig. 1. (a) Phase diagram for the system dextran 500, PEG 8000, and water at 25°C. (●) Total polymeric compositions of systems studied; (▲) determined composition of top phases; (■) determined composition of bottom phases. The tie-line connecting the points representing top and bottom phase of the same system is divided in the parts *x* and *y* by the point for the total composition. (b) Estimated mass ratios (top phase/bottom phase) as function of PEG concentration in top phase for various total concentrations (0.02–0.10% w/w) of dextran. The volume ratios are 5% (at 10% PEG) to 10% (at 26% PEG) greater than the mass ratios.

Table 1
Partition coefficient, *K*, and percent of enzyme recovered in the bottom phase when partially purified β-glucosidase was partitioned at various pH values in a 12% (w/w) PEG and 6% (w/w) dextran system containing the indicated salt; temperature, 25°C

pH	*K*/% In bottom phase				
	50 m*M* Li_2SO_4	50 m*M* K_2SO_4	100 m*M* KCl	100 m*M* KSCN	100 m*M* $KClO_4$
3.9	0.0094/96.6	0.0049/98.2	0.0040/98.5	0.0030/98.9	0.0033/98.8
4.6	0.0053/98.0	0.0024/99.1	0.0007/99.8	<0.0005/>99.8	<0.0005/>99.8
6.4	0.0142/94.9	0.0020/99.3	<0.0005/>99.8	<0.0005/>99.8	<0.0005/>99.8
8.1	0.0258/91.1	0.0025/99.1	<0.0005/>99.8	<0.0005/>99.8	<0.0005/>99.8

lower phase) will most probably be obtained with either KSCN or $KClO_4$ at high pH values. Extrapolation to lower pH values indicate that the partition coefficient should be independent of the salt somewhere in the pH region 3.0–3.5, which should also be the isoelectric point of β-glucosidase [11].

3.3. Repeated extractions with upper phase

Culture filtrate was included (80% w/w) in a 10 g system containing 16% (w/w) PEG, 3% dextran (w/w), 50 m*M* KSCN, and 1 m*M* sodium phosphate buffer pH 8.5. The resulting two-phase system had 9.3 ml upper phase containing 4% of the enzyme and 0.6 ml lower phase with 96% of enzyme. The lower phase was then extracted four times by equilibrating with the same volume (9.3 ml) of fresh upper phases (from a system with water instead of culture filtrate). The volume of the lower phase was slightly reduced during these steps and was 0.5 ml after the last two extractions. The recovery of enzyme in the five upper phases and the final lower phase is shown in Fig. 2. The final lower phase contained 94.5% of total enzyme and 35% of total protein. Thus, this repeated extraction gave a 2.7-fold purification of the enzyme. The partition coefficient of the enzyme, except for the first extraction step, was in the range of 0.001–0.005.

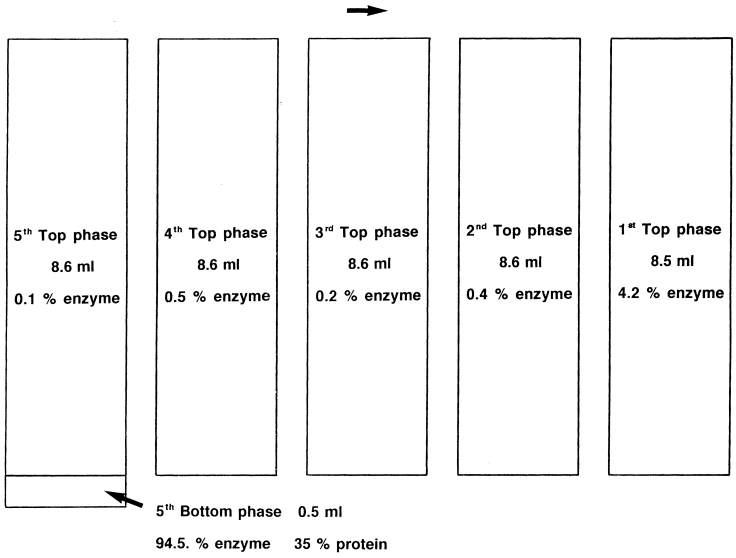

Fig. 2. Purification of β-glucosidase by partitioning into a bottom phase which then was equilibrated four times with pure top phases. The phase systems contained 16% (w/w) PEG, 3% (w/w) dextran, 50 m*M* KSCN and 1 m*M* Na phosphate buffer pH 8.5. The initial system also contained culture filtrate, 80% (w/w) of total system. Temperature, 25°C.

3.4. Counter-current distribution of culture filtrate

The result of a nine transfer counter-current distribution [9,10] of culture filtrate using a system composed of 12% (w/w) PEG, 12% dextran (w/w), 50 m*M* KSCN, and 1 m*M* sodium phosphate buffer pH 8.5 is shown in Fig. 3. Most of the enzyme activity, nearly 98%, remained in the first system (number 0) due to the high affinity of β-glucosidase for the lower phase. Proteins, on the other hand, were found in all ten tubes but 38% was recovered in the first tube. This gives a 2.6-fold purification of the enzyme in this tube similar to what was found after the repeated extractions above (2.7-fold). The G-value can be calculated by using the relation:

$$\frac{T_{i+1}}{T_i} = \frac{i+1}{n-i}G \qquad (4)$$

T_i and T_{i+1} are the relative amounts of enzyme in tube i and $i+1$, respectively, and n is the number of transfers [9]. Applying this equation on the ratio of percent enzyme in tubes 0 and 1 gives a G-value of 0.002. The volume ratio of the two-phase system was 1.8:1 which gives a K-value of 0.001 for the enzyme.

3.5. Effect of PEG concentration on recovery in bottom phase

The effect of concentration of PEG on enzyme recovery in the lower phase and the degree of purification can be seen in Fig. 4. The concentration of dextran was kept relatively constant and changed from 0.49 to 0.41% (w/w) while PEG increased from 15 to 26%. Good recovery, more than 90%, was obtained between 11 and 22% (w/w) PEG. When 16% (w/w) PEG or higher was used the upper phases could, after centrifugation for 10 min at 700 *g* easily be removed by pouring them out of the centrifuge tubes since the bottom phase was so small and viscous that it remained in the bottom of the tube. The observed degree of purification varied between 2.0- and 3.5-fold.

3.6. Partition of β-glucosidase in various systems with lower phase of medium volumes

Extractions of β-glucosidase with bottom phases of measurable volumes are demonstrated in Table 2. The recovery was very good (>95%) for all systems between 14 and 20% (w/w) PEG. Increasing PEG concentration also favoured the recovery of proteins

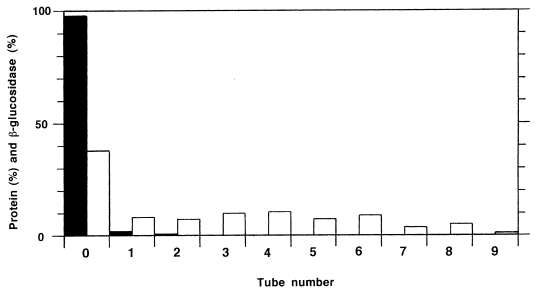

Fig. 3. Counter-current distribution of culture filtrate using nine transfers. The two-phase systems contained 12% (w/w) PEG, 12% (w/w) dextran, 50 m*M* KSCN and 1 m*M* Na phosphate buffer pH 8.5. The initial system (number 0) also contained culture filtrate (75% w/w). Filled bars, amounts of β-glucosidase; empty bars, amounts of protein, both in percent of total recovered enzyme and protein, respectively. The size of each system was 10 g. Temperature, 25°C.

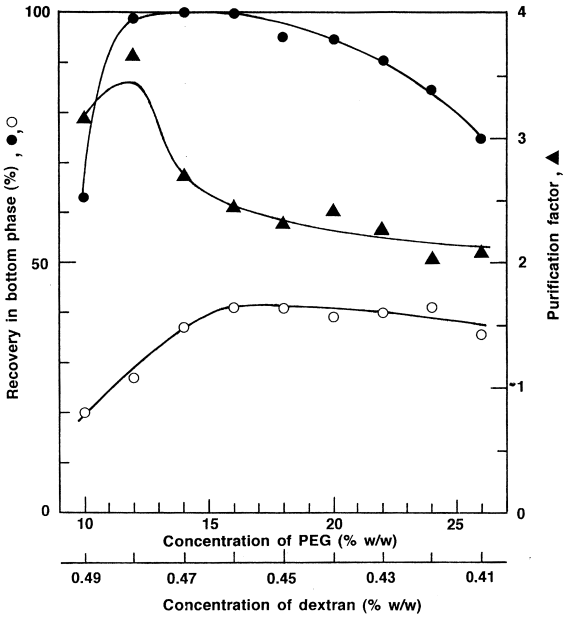

Fig. 4. Recovery of β-glucosidase, (●), and of protein, (○), in the bottom phase of systems containing various concentrations of PEG and the purification factor (=times increase in specific activity) for the enzyme in the bottom phase, (▲). The two-phase systems contained PEG, dextran, 100 m*M* KSCN, 1 m*M* Na phosphate buffer and culture filtrate (maximum possible amount), pH 8.5. Temperature, 25°C.

Table 2
Partitioning of β-glucosidase in two-phase systems which besides PEG and dextran also contained 100 m*M* KSCN, 1 m*M* Na phosphate buffer and culture filtrate (74–86% depending on amount of polymers): the pH was adjusted to 8.0; temperature, 25°C.

System composition		Volume ratio (top/bottom)	Enzyme			Protein			Purification factor in bottom phase (times)	Increase in enzyme concentration in bottom phase (times)	Separatorion capacity log ($K_{protein}$/ K_{enzyme})
PEG (% w/w)	Dextran (% w/w)		Recovery in bottom phase (%)	*K*	Concentration in bottom phase (U ml^{-1})	Recovery in bottom phase (%)	*K*	Concentration in bottom phase (mg ml^{-1})			
10.80	2.31	8.3	81	0.028	16.8	39	0.18	16	2.1	6.0	0.81
12.60	2.70	8.9	93	0.0084	17.4	46	0.13	18	2.0	6.7	1.19
14.52	3.08	11.8	97	0.0029	22.5	52	0.08	20	1.9	8.8	1.44
16.20	4.50	6.6	98	0.0038	14.4	61	0.10	23	1.6	4.7	1.42
18.00	4.90	7.3	96	0.0059	13.2	60	0.09	22	1.6	4.8	1.18
19.80	5.40	7.5	96	0.0051	14.9	71	0.05	25	1.4	5.0	0.99

in the bottom phase and therefore decreased the degree of purification from 2.1- to 1.4-fold. The relatively low purification factor is also due to the modest volume ratios, (<12, Table 2), which also is the reason for the low concentration factor (4.7–8.8 times) of enzyme in the bottom phase. The separatory capacity of the systems can measured as the logarithm of the ratio between the partition coefficients of protein and enzyme, respectively, log ($K_{protein}/K_{enzyme}$) [12]. The best value are obtained for the two systems in the middle of series. These two systems have upper phases containing 16.2 and 18.8% PEG (Fig. 1a).

3.7. Time for collection of phases by centrifugation

Fig. 5 shows the recovery of enzyme in small centrifuge tubes, 0.429×5 cm when 2 g of extract with 18% (w/w) PEG was centrifuged for various time at 310 *g* in a fixed angel rotor and the liquid was drained from the tube as completely as possible. The eventual pellet or bottom phase was dissolved in buffered water of known volume and the activity was determined. The experiment was carried out with 0.2% (w/w) dextran as well without any dextran. Without dextran the enzyme activity was gradually recovered with time in the bottom of the tube. After

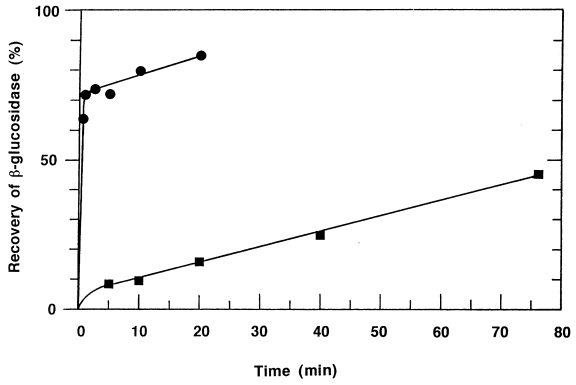

Fig. 5. Effect of centrifugation time on recovery of sedimented β-glucosidase from culture filtrate provided with 18% (w/w) PEG with 0.2% (w/w) dextran, (●); without dextran, (■). The mixtures also contained 100 m*M* KSCN and 1 m*M* Na phosphate buffer, pH 8.0. The centrifugation was carried out at 400 *g* for 5 min. Temperature, 25°C.

76 min of centrifugation as much as 45% of the activity was pelleted. When the low concentration of dextran was included about 70% of the enzyme was recovered in the bottom of the tube within 1 min of centrifugation, while longer centrifugation time slowly increased the yield. The high concentration of PEG probably precipitates the enzyme and some other proteins, but at the relatively low protein concentration present no voluminous precipitate is formed but instead small protein particles which will sediment fairly slowly through the highly viscous PEG solution. The bottom phase droplets on the other hand will sediment much more quickly and in a short time move the extracted enzyme to the bottom of the tube. The sedimentation constant for the droplets can be estimated, from the values in Fig. 5, to be 100–150 times larger than that for protein particles.

3.8. Recovery in lower phase as function of dextran concentration

The influence on the recovery of β-glucosidase as function of dextran concentration, but with constant PEG concentration, 18% (w/w) is shown in Fig. 6. Two conditions were investigated. In one case the culture filtrate with pH 5.5, was used with no addition of salt. In the other case KSCN was included in such amount that the concentration in the final system was 0.12 *M* and pH was adjusted with 3.6% ammonia to pH 8.0. The high pH and the addition of salt in the latter case gives better recovery in the bottom phase. The recovery is reduced with dextran concentrations below 0.05% (w/w) and under 0.024% (w/w) the recovery of enzyme in percent is linearly dependent on the concentration.

3.9. Preparative extraction

Based on the above findings the following extractions were carried out on a larger scale using a minute bottom phase. The size of the systems was chosen so that the lower phase had a measurable volume. Each system had a mass of 255 g and a volumes of 249 ml. Bottom phases with volumes ranging from 2.84 to 0.155 ml were obtained by

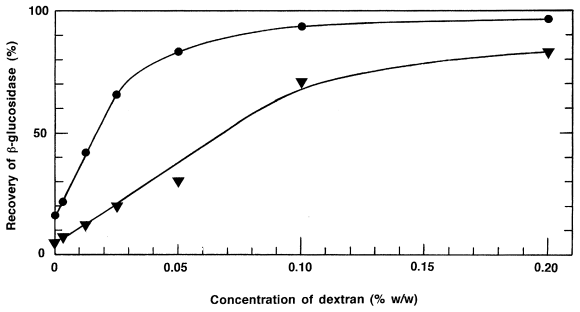

Fig. 6. Recovery of β-glucosidase in lower phase as function of dextran concentration. The systems contained 18% (w/w) PEG, various amounts of dextran, and culture filtrate: (▼), with culture filtrate, pH 5.5 with no addition of salt; (●), culture filtrate with 0.12 *M* KSCN and pH adjusted to 8.0. Temperature, 25°C.

using various concentrations of dextran. The extract was first supplemented with KSCN and adjusted to pH 8.0 with ammonia followed by addition of a calculated amount of solid dextran which was dissolved under mixing. To 209 ml of this solution 46 g of solid PEG were added and when all polymer was dissolved (with aid of careful mixing) the systems were left at room temperature for 1 h. Each system was centrifuged in 25 ml portions at a low speed (310 *g*) for 5 min, the majority part of the upper phase was then removed and new portions of uncentrifuged system were added to the tubes. Finally, the bottom phases in all tubes were collected and transferred into a calibrated graduated tube. Small portions of upper phase were used to transfer the lower phase as completely as possible. The volume of the total bottom phase was determined both by weighing the tube+bottom phase after careful removal of top phase and by weighing out the same volume of water in the tubes. The β-glucosidase activity as well as the total protein concentration were determined. The results of the extractions are summarised in Table 3. While the culture filtrate used had an enzyme concentration of 1.98 U ml^{-1}, the concentration in the bottom phase was 64 times higher in the largest bottom phase and 722 times higher in the smallest bottom phase. The recoveries in the systems, except for the one with the smallest bottom phase, were 83–88% (calculated using the added amount of enzyme) or 94% (calculated on recovered activity in top+bottom phase). The discrepancy may be due to incomplete recovery of the small lower phase (6–9% or 0.06–0.21 ml). In the system with the smallest lower phase the corresponding recoveries are 54% and 88%, respectively, and this would be due to a loss of 39% of bottom phase or only 0.1 ml. The purification factor was in the range of 2.3–2.5 except for the smallest bottom phase where the purification factor was close to 3.3.

3.10. Alternative bottom phase polymer

HPS (Reppal 100) was tested as an alternative to dextran as a bottom phase polymer. When systems (without culture filtrate) contained 18% (w/w) PEG and 0.12 *M* KSCN, the HPS concentrations 2.00, 1.00, 0.41, and 0.164% (w/w) gave the volume ratios of 32, 58, 140, and 210, respectively. In comparison, the volume ratios obtained when corresponding concentrations of dextran are used (Table 3 and from the phase diagram in Fig. 1a) are 16, 30, 83, and 220, respectively. The recovery of enzyme in the bottom phases of the four PEG–HPS systems, when culture filtrate was included, were >99, >99, 97 and 96%, respectively, showing that this polymer works as well as (or better than) dextran.

3.11. Settling time

The time for settling of the phases, not using centrifugation, was studied for the systems containing 18% (w/w) PEG, 0.12 *M* KSCN and 1.00 and 0.41%, respectively, of either dextran or HPS. The systems were mixed in plastic bottles with flat walls (culture flasks) and the bottles were placed in position such that the height of system was 1.0–1.2 cm. The time for settling of the phases was 50–80 h for both the PEG–dextran and the PEG–HPS system. By slowly raising the bottle into an upright position, allowing the bottom phase to slide along the wall, the bottom phase can be collected at the bottom of the bottle.

4. Discussion and conclusion

The separation of proteins by partitioning in aqueous two-phase systems based on dextran and

Table 3
Extraction of β-glucosidase from 209 ml of culture filtrate by formation of a two-phase system (255 g, 249 ml) containing PEG and dextran; temperature, 25°C

System composition		Volume ratio	Bottom phase							
PEG (% w/w)	Dextran (% w/w)	(top/bottom)	Volume (ml)	Enzyme concentration (U ml^{-1})	Enzyme recovery		Increase in enzyme concentration (times)	Protein concentration (mg ml^{-1})	Specific activity (U mg $protein^{-1}$)	Purification factor (times)
					of added (%)	of totally recovered (%)				
18.0	0.409	83	2.84	127	87	94	64	1.05	121	2.3
18.0	0.164	220	1.08	338	88	94	170	2.83	119	2.3
18.0	0.082	449	0.53	651	83	94	328	5.08	128	2.5
18.0	0.037	1540	0.155	1430	54	88	721	8.37	171	3.3

PEG has in general been carried out at moderate concentrations of the two polymers [1]. Too high concentrations of polymers will force practically all soluble proteins into the lower dextran-rich phase as a consequence of their low solubility in concentrated PEG solutions and practically no separation will be achieved. In this work we have taken advantage of this extreme partitioning into one phase to use two-phase systems with high polymer content and great differences between the volumes of the two phases to concentrate the protein of interest. A large phase volume ratio (top to bottom phase) was obtained by using a small amount of dextran and a large amount of PEG resulting in a small lower phase in which the enzyme was collected.

To avoid any unnecessary dilution of the enzyme solution the dry polymers were dissolved directly into the enzyme containing culture filtrate which first was supplemented with KSCN (added in dry form) and the pH was adjusted with 3.6% (1.9 *M*) ammonia. The dextran was added first and allowed to dissolve completely before the PEG was added. By increasing of the PEG concentration a relatively large bottom phase containing a large portion of the protein was formed. The bottom phase volume was continuously reduced and the affinity of the proteins, and especially of the enzyme, for this phase increased at the same time.

The repeated extractions and the counter-current distribution experiments, Figs. 2 and 3, show that not only can 95% or more of the enzyme be retained in the lower phase (partition coefficient approximately 0.001) but also that the enzyme can be purified 2.6–2.7 times due to less extreme partition coefficients for some of the contaminating proteins. At the same time most of the dark brown components of the culture filtrate were removed.

The concentrations of PEG and dextran have been optimized, and the results in Figs. 4 and 6 and Table 2 indicate that the best recoveries are obtained using 12–20% (w/w) of PEG. Higher PEG concentrations give lower recovery of the enzyme, probably because of slow sedimentation of bottom phase droplets in the highly viscous upper phase. The minimal dextran concentration which gives good recovery depends of course on the concentration of PEG but can easily be determined by experiments like that in Fig. 6.

The large scale extraction was not carried out in one centrifugation step due to the available rotor design and the need to be able to measure the volume of the bottom phase. This may explain the partial loss of activity in contrast to earlier experiments where the centrifugal force (g-value$\times$time) was six times higher. It is therefore reasonable to assume a recovery of 95% or more is possible in this case under optimal conditions. The appearance of the four systems in Table 3, if these had been centrifuged in single tubes, is shown in Fig. 7. The observed volume ratios, Table 2, (83, 220, 449, and 1540) are higher than those calculated from the phase diagram (80, 200, 401, and 890), using a density ratio of 1.075. If, however, we assume that not all bottom phase has been recovered, a 100% yield of enzyme corresponds to larger bottom phases which then should have given the volume ratios 75, 202, 389, and 868, respectively.

Because of the low cost of PEG (around 2.5–3 £ or 4–5 US$ per kg) its contribution to the cost will

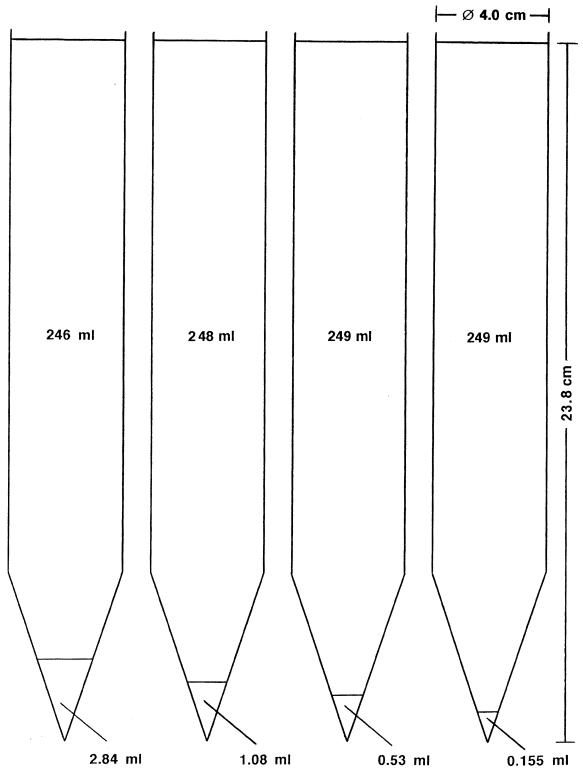

Fig. 7. Dimensions of phase systems in Table 3 if centrifuged in tubes with conical lower part.

be (for 0.22 kg) 0.6 £ or 1 US$ per litre culture filtrate. The cost for fractionated dextran (0.8 g) will be roughly the same. The latter cost can be reduced to $\frac{1}{5}-\frac{1}{10}$ by exchanging dextran for a cheaper bottom phase polymer, such as HPS. The cost of KSCN and ammonia together is less than $\frac{1}{10}$ of the cost of PEG. The use of low speed centrifugation seems to be necessary for this concentration method due to the very slow settling of the phases which is a consequence of the low concentration of bottom phase droplets in the system after mixing.

The high concentration factor is a consequence of the very low initial enzyme concentration. When more concentrated solutions are used the solubility limits will be exceeded and part of the enzyme (and other proteins) will be precipitated. Also under such circumstances the concentration process may still work while protein precipitates often are found collected at the interface between the two phases.

In conclusion, aqueous two-phase systems with extreme ratios of the volumes of the phases can be used for several hundredfold concentrating of enzymes in combination with severalfold purification in a single step or in a multistep process.

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