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Extraction of xanthine oxidase from milk by counter-current distribution in an aqueous two-phase system

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ABSTRACT

By comparing the partitioning of total milk protein with that of pure xanthine oxidase, it was established that a two-phase system containing 7% (w/w) Dextran T 500 and 5% (w/w) polyethylene glycol 6000 in the range of 5–25 m*M* sodium phosphate is suitable for the extraction of the enzyme from milk. In this system, the bulk of milk protein partitioned in the upper phase, whereas the pure enzyme showed a preferential affinity for the lower phase. The enzyme appears also to be released from the membrane of the milk fat globules as a consequence of the phase partitioning itself, as the enzyme activity (undetectable in the boud form) increased when successive partitionings of milk were carried out. After 57 transfers, centrifugal counter-current distribution (CCCD) of milk in this two-phase system allowed the separation of the xanthine oxidase from the bulk of milk protein. The enzyme thus isolated was mostly resolved as an unique peak, identified densitometrically after sodium dodecyl sulphate polyacrylamide gel electrophoresis. The results obtained show that CCCD of milk in a two-phase system is an useful procedure to achieve the release and isolation of xanthine oxidase from milk.

INTRODUCTION

Xanthine oxidase (EC 1.2.3.2) is a complex enzyme abundant in the milk fat globule membrane, containing flavin adenine dinudeotide (FAD), molybdenum and iron–sulphur cofactors [1]. The purification of the enzyme from milk or butter requires a previous step involving the release of the enzyme from membrane material. Solvent extraction and protease or detergent treatment have been used for this purpose [2]. Diverse procedures have been employed for its isolation [3–6] and, more recently, an affinity chromatographic method using Sepharose 4B–folate [7] has been described.

The extraction of biological material using aqueous two-phase systems prepared from solutions of two water-soluble polymers is a powerful technique for the separation of macromolecules [8,9]. One of the systems most employed for the purification of proteins consists of dextran and polyethylene glycol (PEG) [10]. PEG is a water-soluble synthetic polymer with interesting properties. In addition to its use for the preparation of two-phase systems, it has applications as a precipitating or fusing

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agent [11]. The influence of PEG on the partitioning of membrane components between the external phase and the membrane has already been described [12], and this effect has been discussed as a smooth detergent-like behaviour [13].

The aim of this work was to apply the dextran–PEG two-phase system as a single procedure to achieve the purification of xanthine oxidase from whole cow milk. The results obtained show that the enzyme is both released and isolated from the bulk of the milk protein by counter-current distribution in a Dextran 500–PEG 6000 two-phase system.

EXPERIMENTAL

Chemicals

Dextran (relative molecular mass, M_r = 500 000) was obtained from Pharmacia and PEG 6000 (M_r 6000–7500) from Serva. Pure xanthine oxidase from buttermilk (X-4500) and all other chemicals were obtained from Sigma.

Milk

Fresh dairy milk was kept at 4°C (2–3 h). Any kind of physical treatment was completely avoided before phase partitioning.

Xanthine oxidase assay

Xanthine oxidase was assayed by the method of Avis *et al.* [14] in experiments in which commercial pure enzyme was employed. This method consisted in the spectrophotometric measurement at 290 nm of the uric acid formed in the enzymatic reaction. However, this method is inadequate for the determination of the enzyme in samples of milk with a high protein content. In these instances, the enzymatic activity was determined by measuring the hydrogen peroxide formed in the enzyme reaction. This was carried out by means of two coupled enzymatic reactions. The first involved catalase (E.C. 1.11.1.6) plus ethanol, producing acetaldehyde. The second coupled reaction was accomplished by aldehyde dehydrogenase (E.C. 1.2.1.5) plus NADP [15]. Reduction of NADP by acetaldehyde was then determined spectrophotometrically at 340 nm. The activity was linear during the first 4–5 min. When this coupled reaction was used (high milk protein content in batch experiments, see Fig. 3), different sample dilutions were performed, and the activity was determined in the range where the absorbance increase was proportional to the protein concentration.

Two-phase systems

The systems were prepared from stock aqueous solutions of the polymers, 40% (w/w) PEG and 20% (w/w) dextran, made as described previously [8]. The polymer solutions and stock solutions of 1.2 *M* phosphate buffer, 2 m*M* EDTA, milk and water were weighed and mixed to give the final concentrations described below. When batch experiments were carried out, 5.0-g two-phase systems containing 0.5 g of milk were prepared and mixed by 20 inversions. Phase separation was speeded up by centrifugation at 1000 *g* for 1 min. All operations were carried out at 0–4°C either in batch or in counter-current distribution experiments. When batch experiments were carried out, protein and/or xanthine oxidase were determined in the mixed two-phase system and in the upper phase after separation of the batch had occurred.

Centrifugal counter-current distribution

The counter-current distribution system used was constructed on the basis of that described by Akerlund [16]. With this device, the time for the separation of the two phases is shortened by centrifugation. The apparatus contains 60 chambers arranged in a circle, allowing transfer of the upper phases relative to the lower phases. To carry out centrifugal counter-current distribution (CCCD) experiments, 100 g of a two-phase system containing 7% (w/w) dextran, 5% (w/w) PEG, 0.2 m*M* EDTA and 10 m*M* sodium phosphate (pH 7.0) were prepared and mixed, and 1.55 ml of this two-phase system were loaded in chambers 3–59. A 8.0-g two-phase system was prepared with the same composition but also containing 3.5 g of milk, and 1.55 ml of this mixture were then loaded in chambers 0–2. The shaking and centrifugation time were 45 and 30 s, respectively, and 57 transfers were performed. After the run, the systems were transformed into homogeneous solutions by the addition of 1.55 ml of 50 m*M* sodium phosphate pH 7 in each cavity. The fractions were then collected and analysed. Protein was determined by the method of Bradford [17], by which no interference with the two-phase-forming polymers is produced. Results are presented as the percentage in every chamber of the total enzyme activity and protein determined after the run, as described previously [18].

Electrophoresis

Samples collected from each CCCD chamber were lyophilized and dissolved in 0.75 ml of 50 m*M* sodium phosphate (pH 7.5), then 0.4 ml was incubated with 50 μl of 30% mercaptoethanol and 50 μl of 45% sodium dodecyl sulphate (SDS) at 100°C for 5 min. SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of 5 μl of this treated sample was carried out in a 10–15% PhastGel gradient (Pharmacia PhastSystem). Staining was carried out by the optimized silver method for SDS–PAGE gradient gel media (PhastGel TM) and densitometric tracing was performed on an LKB Ultroscan XL laser densitometer.

RESULTS

By comparing the partitioning of the total milk protein with that of xanthine oxidase, the usefulness of phase partitioning for enzyme purification could be evaluated. A number of parameters were first investigated to establish the optimum conditions for the extraction of the enzyme by phase partitioning. Fig. 1 shows that an increase in the concentration of dextran and PEG results in an enhanced preference of the bulk of milk protein for the lower, dextran-rich phase. The greatest extent of milk protein partitioning in the upper, PEG-rich phase was achieved in a two-phase system containing 7% dextran and 5% PEG in the range of 5–25 m*M* sodium phosphate. At higher polymer concentrations, the maximum partitioning of the milk protein in the upper phase occurred when the sodium phosphate concentration exceeded 25 m*M*.

The partitioning behaviour of pure xanthine oxidase (0.001% protein) in a 7:5 dextran–PEG two-phase system is shown in Fig. 2a. The maximum partitioning of the enzyme in the upper phase also occurred in the range 5–20 m*M* sodium phosphate, whereas higher salt concentrations promoted a stronger enzyme preference for the lower phase. It is remarkable that the percentage distribution in the upper phase of the bulk protein is much higher than that the enzyme. This effect made this poly-

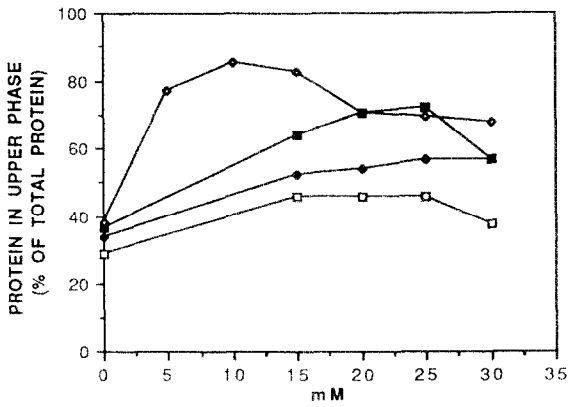

Fig. 1. Partitioning of milk protein as a function of sodium phosphate concentration at pH 7. The two-phase system contained (○) 7 and 5, (■) 7.5 and 5.5, (◆) 8 and 5.5 and (□) 8 and 6% dextran and PEG, respectively, and 0.2 m*M* EDTA.

mer concentration a promising variable for achieving the enzyme purification. In the presence of 10 m*M* sodium phosphate, an increasing concentration of potassium chloride promoted an enhanced affinity of the xanthine oxidase for the lower phase (Fig. 2b), and the partition behaviour was very similar between pH 5.5 and 8 (Fig. 2c). It is noteworthy that increasing amounts of enzyme in the two-phase system enhanced the partitioning in the lower phase (Fig. 2d).

However, when the xanthine oxidase activity was determined after partitioning of milk in a two-phase system of the composition established previously (Fig. 2b), an impaired behaviour of the enzyme was found. The enzyme activity was barely detected and the distribution of the scarce activity found was preferential for the upper phase. A study of the effect of successive extractions on the activity and partitioning of the xanthine oxidase from milk was therefore carried out. The results obtained after two further extractions of a first initial two-phase system are shown in Fig. 3. It can be observed how each re-extraction accounts for an increase in the xanthine oxidase activity. This increase was substantial in the upper phase, although it was also important in the lower phase after the second re-extraction. These results clearly indicated that an adequate extraction of the enzyme from milk by phase partitioning could only be achieved by a multi-step extraction process, such as the counter-current distribution can perform.

Fig. 4 shows the CCCD diagrams obtained from (a) pure xanthine oxidase and (b) milk. In both instances, the enzymatic activity was mostly located in the first ten chambers, whereas the protein bulk mostly appeared in the last 20 chambers (Fig. 4b) with another peak in the middle of the run.

The distribution of 1 molecule in a two-phase system is represented by the partition ratio, G. The G value is defined as the percentage of the molecule in the mobile part of the system (upper phase in counter-current distribution) divided by the percentage in the stationary part (lower phase). From the positions of the peaks in the CCCD diagram, the G value can be calculated using the approximate equation $G = r$

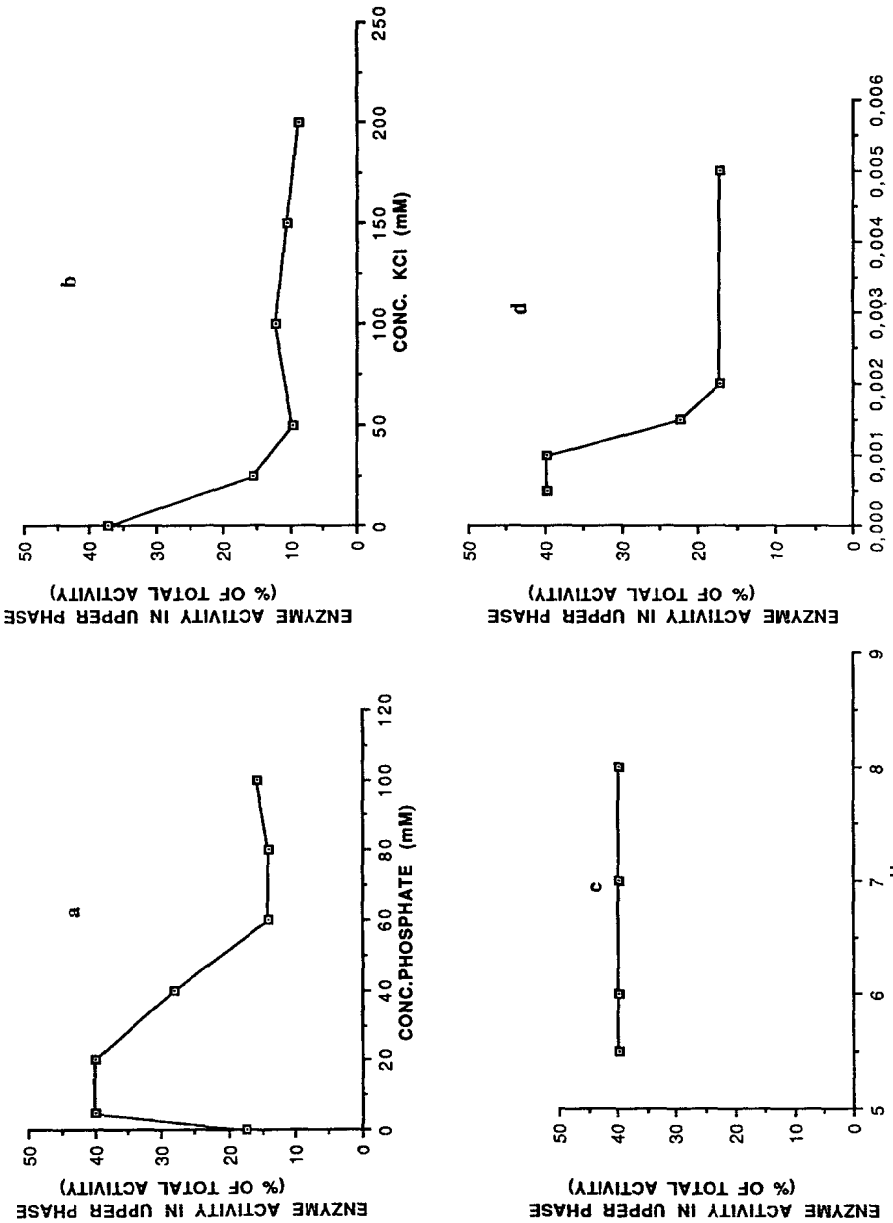

Fig. 2. Partitioning of pure xanthine oxidase as a function of (a) sodium phosphate concentration at pH 7 in a two-phase system containing 7% dextran, 5% PEG and 0.2 m*M* EDTA; (b) KCl concentration in the presence of 7% dextran, 5% PEG, 0.2 m*M* EDTA and 10 m*M* sodium phosphate (pH 7); (c) pH with a two-phase system containing 7% dextran, 5% PEG, 0.2 m*M* EDTA and 10 m*M* sodium phosphate; and (d) xanthine oxidase concentration, expressed as % (w/w), in a two-phase system with the same composition as (c) to pH 7.

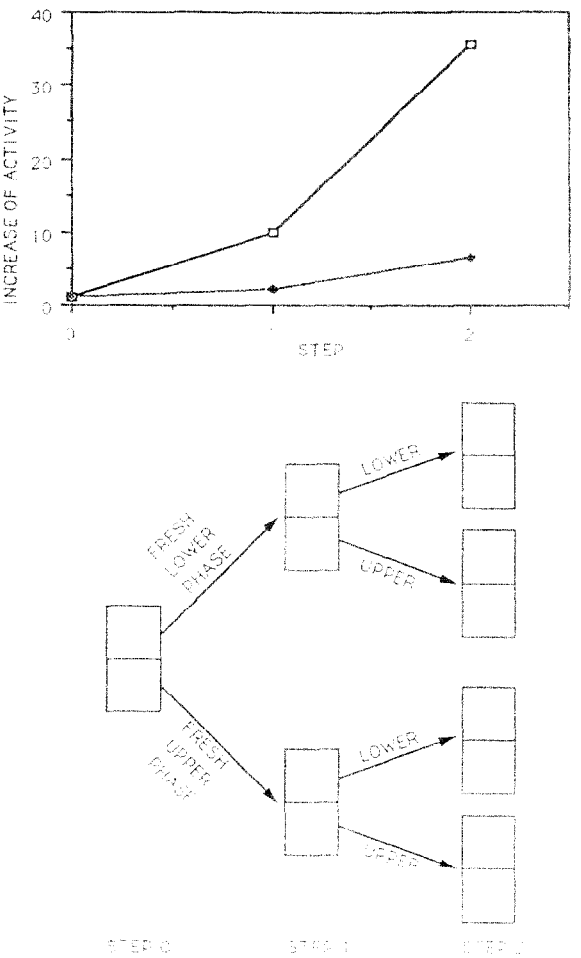

Fig. 3. Increase in the xanthine oxidase activity in the (■) upper and (◆) lower phases of successive phase partitionings of milk, in a two-phase system containing 7% dextran, 5% PEG, 0.2 m*M* EDTA and 10 m*M* sodium phosphate (pH 7). A diagram of the experimental procedure is shown underneath. The initial upper and lower phases of a first two-phase system (step 0) were re-extracted with fresh upper and lower phases respectively, obtained from a new two-phase system of the same composition. Then, each of the four phases thus obtained (step 1) was newly re-extracted with the corresponding opposite fresh phase of a two-phase system of the same composition (step 2). The increase in the enzyme activity shown is then the ratio of the sum of the activities found in all the upper and lower phases obtained in each of step 1 or 2 from the initial upper or lower phase, divided by the activity found in the upper or lower phase of the initial step 0 two-phase system, respectively.

($n = i$), where i is the number of the chamber into which the enzyme peak has moved and n is the number of transfers [18]. The G values of xanthine oxidase and the bulk protein were calculated from the positions of the peaks in the CCCD diagram (Fig. 4). The protein peaks with G values of 1.33 and 6 correspond to the whey protein bulk (including β-lactoglobulin and α-lactalbumin) and caseins, respectively (data not shown).

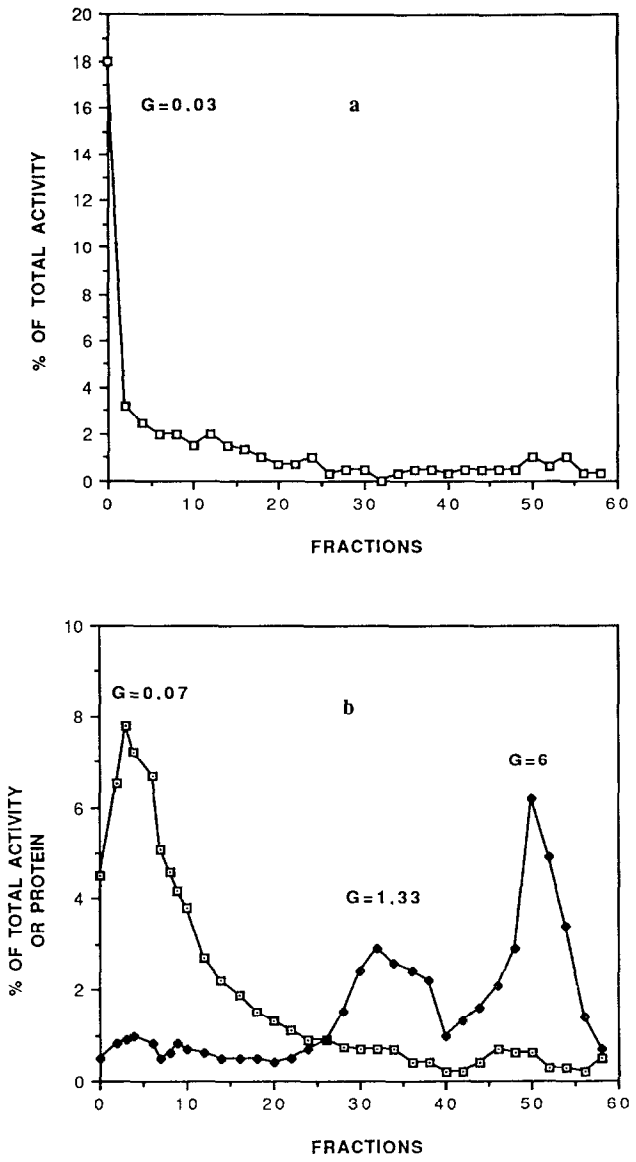

Fig. 4. CCCD diagrams of (a) pure xanthine oxidase (0.005% of protein in the loading chambers) and (b) milk. Results show the percentage in each chamber of the total (□) enzyme activity and (◆) protein obtained and determined after the run.

Protein extracted from milk and obtained from chambers 0–10 in CCCD (Fig. 4b) was resolved as an apparently unique electrophoretic band. This band can be easily made visible, but densitometric tracing was carried out to acheive a more precise and quantitative analysis of the gel obtained. The material thus detected was almost entirely located in a major peak, although some other very minor peaks ap-

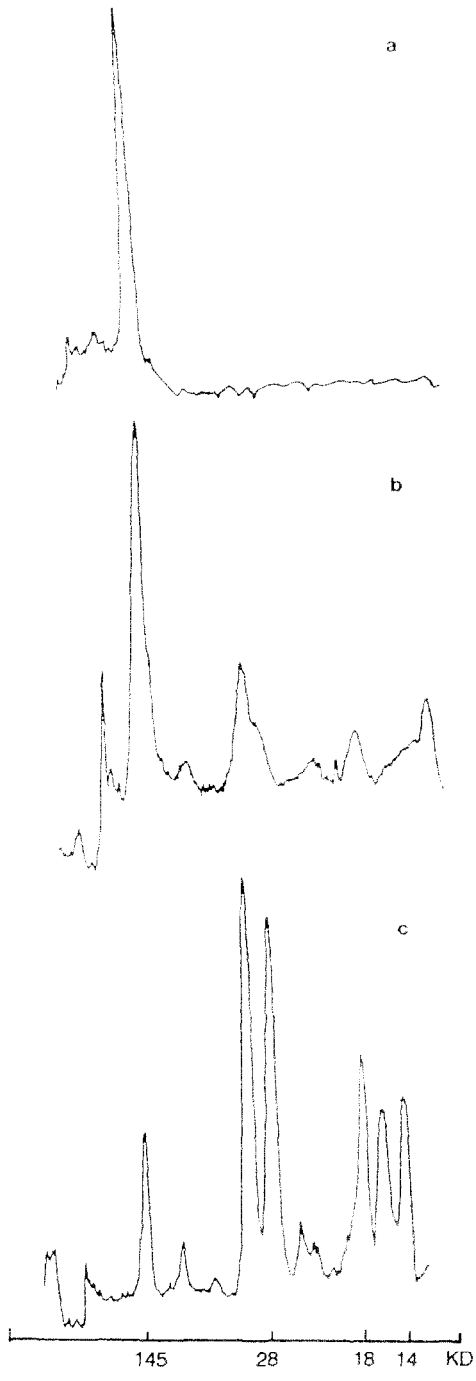

Fig. 5. Densitometric traces of material obtained from chambers 0–10 after CCCD of (a) milk commercial xanthine oxidase; (c) trace for whole milk. KD = kilodalton.

peared in the upper part of the gel (Fig. 5a). Commercial pure xanthine oxidase used as a control showed a main densitometrically determined peak (Fig. 5b) of the same mobility as that of the milk xanthine oxidase obtained from CCCD chambers 0–10 (Fig. 5a). The other milk proteins showing a higher mobility than xanthine oxidase (Fig. 5c) were not detected in the first ten CCCD chambers.

As a precise determination of the enzyme activity cannot be performed in whole milk, the increase in the specific activity of the purified xanthine oxidase with respect to the milk xanthine oxidase cannot be determined. However, quantification of the densitometric tracing of the SDS-PAGE of the material extracted from milk and obtained from chambers 0–10 (Fig. 4b) shows that more than 80% of this material appeared in the major peak, which has the same electrophoretic mobility as the major component of the commercial pure enzyme (Fig. 5b). This percentage is *ca.* fifteen times higher than that of the corresponding peak (with the same electrophoretic mobility) obtained from whole milk.

DISCUSSION

The results obtained show that analytical CCCD of milk in a two-phase system containing 7% dextran, 5% PEG, 0.2 m*M* EDTA and 10 m*M* sodium phosphate (pH 7) is an adequate technique for extracting milk xanthine oxidase. The enzyme thus obtained appeared to be substantially free from other contaminant proteins detectable by SDS-PAGE under the experimental conditions used.

The high efficiency of this method for purifying xanthine oxidase from milk is due to two different effects. First, the enzyme is probably extracted from the membrane of the milk fat globules as a consequence of the phase partitioning itself. It is well known that xanthine oxidase bound to the fat globules shows very low activity, and that enzyme determination requires previous release of the enzyme [2]. Thus, when milk was partitioned in a single-batch two-phase system (Fig. 3; step 0), the enzyme activity was almost undetactable. However, successive extraction gave a substantial increase in the activity in the upper phase (Fig. 3; steps 1 and 2). As extractions proceed (Fig. 3; step 2), the enzyme appears to show a moderate increase in its affinity for the lower phase. These results can be interpreted as a partial and progressive release of the enzyme from the fat globules, with the consequent detection of the activity. As release is finally accomplished, the enzyme increases its partitioning in the lower phase, showing a very low G value in a multi-step extraction procedure (CCCD of milk; Fig. 3b). Such a releasing action of the two-phase system could be due to a combined effect of the mentioned ability of PEG for membrane extraction [11–13] together with the likely preference of the milk lipids for the upper PEG-rich phase in opposition to the preferential partitioning of the xanthine oxidase in the upper phase (Fig. 2).

In addition, the very different partition ratios of the milk bulk protein (Figs. 1 and 4b) and xanthine oxidase (Figs. 2 and 4a) is an important complementary advantage of this phase partition extraction of xanthine oxidase from milk. The great affinity of the xanthine oxidase for the lower phase is showed by its constant partition behaviour over wide ranges of pH (Fig. 2c) and temperature (4–25°C; data not shown), and even rises to 90% enzyme partitioning in the lower phase at a potassium chloride concentration higher than 25 m*M* (Fig. 2c). The slight difference in the G

values obtained for the pure enzyme and for the enzyme separated from milk (Fig. 4) could be due to the fact that the enzyme has first to be released from the fat globule membrane during the first transfers, inducing a lag effect in its preferential partitioning for the lower phase.

In the CCCD experiments using pure commercial xanthine oxidase, almost all the total initial activity (more than 90%) was found in the first ten chambers. However, this estimation cannot be made in the case of CCCD with whole milk, owing to the difficulty in detecting the activity in the sample, as stated above.

The G values obtained for the xanthine oxidase in the CCCD experiments (Fig. 4), both for the commercial pure form and for the enzyme separated from milk, were lower than those which could be expected from the percentage partitioning of the enzyme in the upper phase in the batch experiments (Figs. 1 and 2). This discrepancy could be explained by the different amount of enzyme present in each instance. In fact, we have found that increasing concentrations of xanthine oxidase in a two-phase system account for a higher partitioning of the enzyme in the lower phase (Fig. 2d).

Xanthine oxidase extracted from milk by CCCD appears as an almost unique band (Fig. 5b) in SDS-PAGE under the experimental conditions used here. This band showed the same electrophoretic mobility as the main component of the commercial enzyme (Fig. 5b).

The main protein components of milk are casein, α-lactalbunin and β-lactoglobulin [1,2]. Casein corresponds to the major peaks electrophoretically separated in the middle of the SDS-PAGE of milk, and α-lactalbunin and the two isoforms of β-lactoglobulin are the three major fastest components found at the end of the trace in Fig. 5c (data not shown). Therefore, the two minor peaks appearing in the xanthine oxidase extracted from milk by CCCD (Fig. 5a) do not correspond to any of these major milk proteins. It cannot be ruled out that they could represent some oligomeric forms of the enzyme.

These results clearly show that CCCD in a dextran–PEG two-phase system can be an advantageous single method for purifying xanthine oxidase from milk. Therefore, further studies on the use of different (cheaper) two-phase-forming polymers and on large-scale techniques could be justified in order to achieve the purification of the enzyme in the most convenient way for preparative and or commercial purposes.

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