DETERGENTLESS MICROEMULSIONS AS MEDIA FOR ENZYMATIC REACTIONS: CATALYTIC PROPERTIES OF LACCASE IN THE TERNARY SYSTEM HEXANE–2-PROPANOL–WATER

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The catalytic activity and stability of laccase in the detergentless ternary system hexane – 2-propanol – water were studied. In the microemulsion region of the phase diagram the enzyme exhibited the highest catalytic activity and stability, which were comparable to those in aqueous solution. The character of the microenvironment inside microemulsion droplets was studied using nitrate anion as spectral probe and its polarity was found to correspond to that of 75% v/v aqueous 2-propanol. In principle, laccase can be recovered many times from the microemulsion without loss of catalytic activity.

Recently we observe a considerably growing interest in biocatalytic systems working in organic solvents with low water content (for reviews see refs[1–10]). Use of such systems has several advantages over traditionally used aqueous solutions. Firstly, they solve the problem of solubility of hydrophobic substrates. Secondly, in organic solvents the thermodynamic equilibrium of many enzymatic reactions is shifted towards the desired products: this concerns reactions in which water is one of the products, particularly synthesis of ester, peptide or amide bonds. Thirdly, in many cases the use of organic solvents contributes to protection against bacterial contamination of technological equipment which represents a serious problem when working with aqueous solutions.

A wide practical utilization of non-aqueous biocatalytic systems is considerably limited by the fact that organic solvents usually denaturate the enzyme. To avoid the denaturation, several approaches have been developed, such as the use of two-phase systems, immobilization, entrapment of the enzyme into reversed micelles of detergents, etc. (for reviews see refs[1–10]). Recently we have suggested a new approach to biocatalytic systems in non-aqueous solvents, based on utilization of

detergentless microemulsions[11,12]. Detergentless microemulsions[13,14] are formed in ternary systems consisting of a hydrocarbon, 2-propanol (or 1-propanol) and water and represent thermodynamically stable and optically transparent dispersions of aqueous microdroplets in the hydrocarbon solvent. These droplets are stabilized by alcohol molecules, adsorbed on their surface, and have spherical symmetry. We have shown earlier that various enzymes, such as trypsin[11,12], chymotrypsin[11], cholesterol oxidase and catalase[15] retain their activity and stability when dissolved in detergentless microemulsions consisting of hexane, 2-propanol and water. The catalytic activity is preserved due to inclusion of enzyme molecules into aqueous microdroplets in which they are protected by a 5–7 Å thick aqueous layer against denaturation by the organic solvent[16]. In this respect, detergentless microemulsions are analogous to systems of reversed micelles, widely used as media for enzymatic reactions[8–10]. However, in contrast to reversed micelles, these microemulsions are entirely free of detergents: this substantially simplifies product separation and enzyme recovery[15].

Our present communication investigates possible use of detergentless microemulsions as a medium for reactions catalysed by laccase, an enzyme capable of oxidizing a wide variety of organic compounds. Since the enzyme also oxidizes compounds sparingly soluble in water[17], the use of a non-aqueous reaction medium appears particularly attractive. We also studied the microenvironment of the enzyme inside the microemulsion droplets, the possibility of recovering laccase from the microemulsions, and its repeated use.

RESULTS AND DISCUSSION

Phase Diagram

The phase diagram of the ternary system hexane – 2-propanol – water ($2 \cdot 10^{-3}$ mol l^{-1} sodium citrate, pH 4·5) at 20°C is given in Fig. 1. According to Smith and Barden[13], the phase diagram contains following regions: Region A corresponds to an unstable non-transparent macroemulsion which spontaneously separates into two layers on standing. The other three regions of the diagram (B, C, and D), correspond to stable and transparent phases. In the region D we encounter normal ternary solution of hexane, 2-propanol and water, without any microstructure. Region B corresponds to a microemulsion, consisting of water microdroplets, dispersed in a medium of high hexane content. The existence of a dispersion phase in region B, possessing properties of bulk water, has been confirmed by NMR spectroscopy[14]. The region C which is intermediate between regions B and D, corresponds to hydrogen-bonded aggregates of water and 2-propanol molecules, dispersed in the organic phase.

Catalytic Activity and Stability of Laccase in the Ternary System Hexane – 2-Propanol – Water

The most important result of our study consists in the finding that in a detergentless microemulsion of low water content laccase retains its catalytic activity. Data on the catalytic activity of the enzyme ($V/[E]_0$) in the ternary hexane – 2-propanol – water systems of various compositions are given in Table I (the numbering of the systems in the table corresponds to that of the points in Fig. 1). As seen from Table I, the enzyme is most active in the microemulsion region. The maximum catalytic activity observed in our medium (point 5 in Fig. 1 and No 5 in Table I) represents about 20% of that found in an aqueous solution, although the total water content in this microheterogeneous system does not exceed 7 vol.%.

In the microemulsion region laccase not only shows maximal catalytic activity compared with the other phase diagram regions, but also has the highest stability as shown by comparison of first-order inactivation rate constants for various hexane – – 2-propanol – water systems (Table I).

Apparently, the catalytic activity and stability of laccase are preserved because the enzyme molecules are situated inside water microdroplets suspended in the organic solvent medium. The droplets are stabilized by molecules of 2-propanol adsorbed on the surface, as shown in Fig. 2. Such structures have been described by us previously[16] for detergentless microemulsions containing dissolved trypsin.

Catalytic Activity and Stability of Laccase in Binary Water – 2-Propanol Mixtures

In order to ascertain whether introduction of a third component (hexane) into the system represents any advantage concerning the catalytic activity and stability of

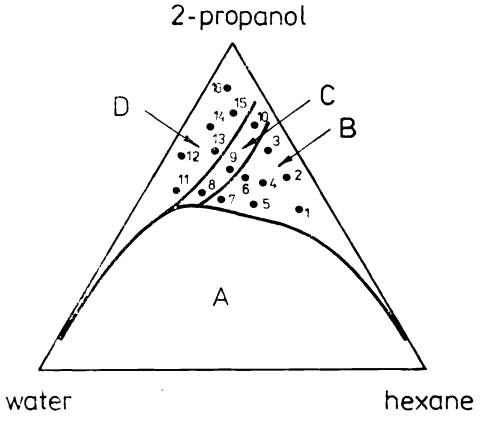

FIG. 1

Phase diagram of the ternary mixture hexane – 2-propanol – water ($2 \cdot 10^{-3}$M sodium citrate, pH 4·5) at 20°C. Concentrations of the components are expressed in molar fractions. The numbered points represent composition of the studied systems (cf. Table I). Regions: A separated phases, B microemulsions, C hydrogen bond-associated molecules of water and 2-propanol, D normal ternary solutions. The boundaries of the regions were determined by conductometric titration[13]

laccase, we studied this enzyme in binary mixtures 2-propanol – water. Results are summarized in Fig. 3 which plots the relative catalytic activities (*a*) and the first-order rate constants of inactivation (*b*) against concentration of 2-propanol in the mixture. Comparison of the data with those in Table I shows that at comparable concentrations of water the catalytic activity and stability of laccase are much higher in the ternary mixtures than in aqueous 2-propanol. The use of ternary mixtures is thus much more advantageous.

TABLE I

Catalytic activity and stability of laccase, dissolved in ternary systems hexane – 2-propanol – water using pyrocatechol as substrate

System[a]	Composition, vol%			$V/[E]_0$ min^{-1}	$k_{in} \cdot 10^{2c}$ h^{-1}
	hexane	2-propanol	water[b]		
			Microemulsions		
1	59·8	38·6	1·6	1 398	—
2	49·9	49·0	1·1	413	—
3	40·4	58·1	1·5	784	3·2
4	47·5	49·8	2·7	1 544	—
5	49·2	46·5	4·3	2 140	5·1
6	40·3	55·7	4·0	1 362	—
7	39·2	54·1	6·7	2 067	—
			Intermediate region		
8	30·0	61·7	8·3	1 642	12·9
9	33·5	61·5	5·0	1 350	9·2
10	30·3	68·1	1·6	711	—
			Normal ternary solutions		
11	20·2	68·7	11·1	1 501	—
12	9·0	82·2	8·8	421	—
13	23·6	70·8	5·6	761	—
14	14·7	80·6	4·7	625	—
15	19·6	77·8	2·6	299	22·3
16	8·9	89·1	2·0	96	—
—	—	—	100	10 600	0·5

[a] The number corresponds to the number of point in Fig. 1; [b] $2 \cdot 10^{-3}$M sodium citrate, pH 4·5; [c] first-order rate constant of inactivation.

Microenvironment of Laccase Dissolved in the Detergentless Microemulsion

The character of the microenvironment inside microemulsion droplets containing dissolved enzyme (Fig. 2) was studied using nitrate anion as a low-molecular probe whose spectral properties depend on the polarity of its environment[18]. In microemulsion, potassium nitrate is located exclusively inside water microdroplets because, as we found in a separate experiment, the nitrate is insoluble in binary hexane – 2-propanol mixtures whose composition corresponds to that of the dispersion medium in microemulsions. Consequently, spectral characteristics of nitrate anion in the microemulsion afford information on the character of the medium inside the microemulsion droplets, and do not reflect the average polarity of the system as a whole.

In the microemulsion of composition described by point **7** (Fig. 1) the nitrate anion

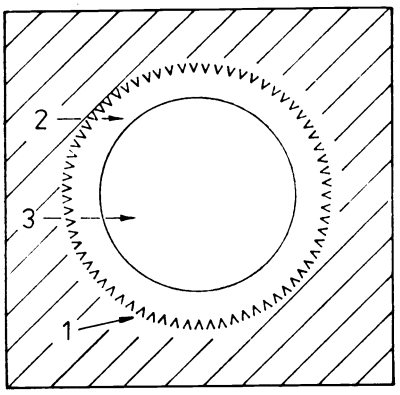

FIG. 2

Schematic representation of an aqueous microemulsion droplet with enzyme molecule. 1 2-propanol molecules, 2 water molecules, 3 enzyme molecule. Hatched area represents the organic solvent

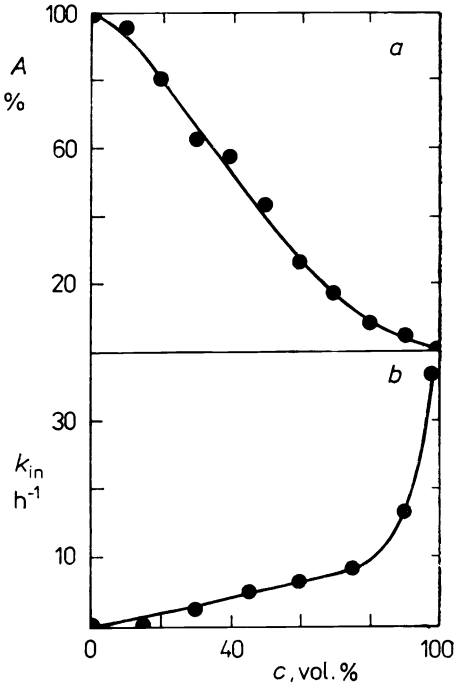

FIG. 3

Dependence of *a* relative catalytic activity, *A* (%), and *b* first-order inactivation rate constant, k_{in} (h^{-1}), of laccase in binary mixtures water ($2 . 10^{-3}$M sodium citrate, pH 4·5) – 2-propanol on concentration of 2-propanol, *c* (vol.%)

absorbs at 303·2 nm. In order to relate the observed wavelength to the microenvironment polarity it was necessary to study the spectral properties of the nitrate in some standard medium of known composition. As such suitable standard medium we used binary mixtures water – 2-propanol because this alcohol, being present in the detergentless microemulsion in high concentration and being capable of unlimited mixing with water, should be present inside the aqueous microemulsion droplets[16]. In other words, water – propanol mixtures can approximate the medium inside water microdroplets existing in microemulsion. The dependence of the nitrate absorption wavelength in water – 2-propanol mixtures on their composition is depicted in Fig. 4. As seen, in the microemulsion the nitrate ion absorbs at the same wavelength (303·2 nm) as in a binary mixture containing about 75 vol.% of 2-propanol. We can thus conclude that the polarity of the medium inside the microemulsion droplets corresponds to that of 75 vol.% aqueous solution of 2-propanol. This agrees with our previous polarity determinations using a fluorescent probe[16]. It is worth while to notice that the actual content of 2-propanol in droplets amounts to about 20 vol.% (ref.[16]) i.e. much less than found by the polarity determination. This seemingly contradictory observation can be explained by the fact that the polarity of the medium in microemulsion droplets is invariably much lower than that of the liquid from which they are formed[19,20]. On the whole, we can conclude that the microenvironment surrounding the enzyme molecule in a microemulsion droplet differs substantially from the aqueous phase. Obviously, particularly this is the reason why catalytic properties and stability of laccase in a detergentless microemulsion differ from those in water (see Table I).

Recovery of the Enzyme from the Microemulsion

The sequence of operations used for the recovery of laccase from the microemulsion is schematically depicted in Fig. 5 (see also Experimental). The method is based

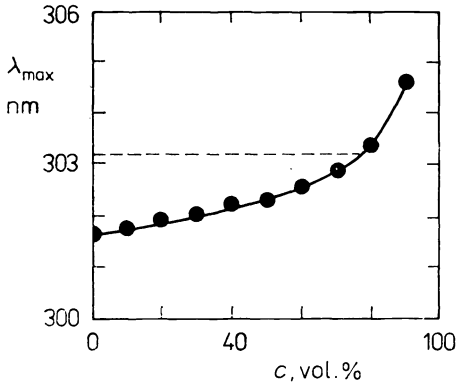

FIG. 4

Dependence of the absorption maximum wavelength, λ_{max} (nm), of nitrate anion on concentration of 2-propanol, c (vol.%), in binary mixtures water ($2 . 10^{-3}$M sodium citrate, pH 4·5) - 2-propanol. Broken line denotes the wavelength found for the microemulsion of composition corresponding to point 7 in the phase diagram (Fig. 1). Concentration of the nitrate anion was 0·1 mol l^{-1}, based on the volume of water present in the system

on the fact that addition of an excess of hexane to a ternary mixture, described by any point in the region B, C or D of the phase diagram, shifts this point into the region A (Fig. 1), representing unstable ternary mixtures. Such mixtures easily separate into two immiscible phases composed essentially of water and hexane[13]. Laccase remains in the aqueous phase and can be used again. As shown in Fig. 6, the enzyme retains significant activity even after several recovery cycles. Principally, it is thus possible to use the enzyme many times if the reaction is carried out batchwise in detergentless microemulsions.

EXPERIMENTAL

Materials: Laccase (E.C. 1.10.3.2) was isolated from culture medium of *Coriolus versicolor* in the Enzyme Laboratory, Section of Biocatalysis, Armenian branch of the "IREA" enterprise.

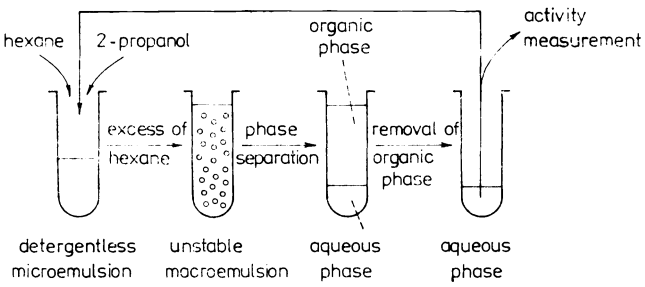

FIG. 5
Sequence of operations in recovering laccase from a microemulsion

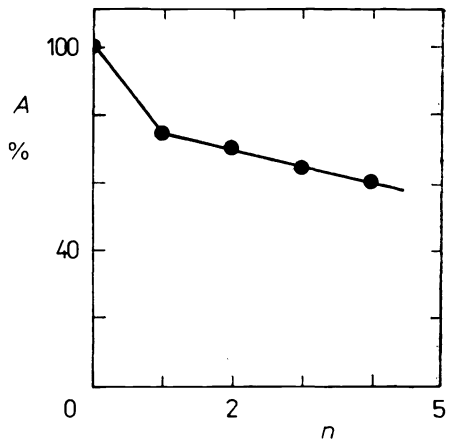

FIG. 6
Dependence of relative catalytic activity of laccase, A (%), on the number of recoveries, n, from microemulsion, corresponding to point 5 in Fig. 1

Enzyme concentration was determined spectrophotometrically at 610 nm, taking the molar extinction coefficient as 4·6 mol^{-1} (ref.[17]). Pyrocatechol was purchased from Kharkov Chemical Works and was purified by distillation. Distilled hexane ("Reakhim") and 2-propanol (spectroscopic grade, "Chemapol") were used as solvents. In all experiments, $2 . 10^{-3}$M sodium citrate buffer, pH 4·5, was employed. Potassium nitrate ("Reakhim") was used without purification.

Preparation of the medium: The ternary systems were prepared by mixing calculated amounts of hexane, 2-propanol and aqueous buffer. The mixture was vigorously shaken until a stable transparent solution was obtained. No additional equilibration of the systems was necessary. Laccase and pyrocatechol were introduced into the system as concentrated solutions in water and 2-propanol, respectively.

Determination of catalytic activity of laccase: A solution of the enzyme was added to a mixture of calculated amounts of hexane, 2-propanol and aqueous buffer solution, so as the enzyme concentration in the whole volume was $2 . 10^{-8}$ mol l^{-1}. The reaction was started by addition of pyrocatechol in 2-propanol, the substrate concentration being $3 . 10^{-2}$ mol l^{-1} (for the whole volume of the system). Under the given conditions, this concentration represented a saturated solution. The reaction was followed spectrophotometrically at 385 nm on a Beckmann-25 instrument, equipped by thermostated (25°C) cells. The reaction rate was calculated using the extinction coefficient of liberating benzoquinone as $515 . 10^{3}$ mol^{-1} (determined separately). The catalytic activity of the enzyme in binary mixtures 2-propanol – water was determined in an analogous manner.

Determination of stability of laccase: The enzyme was incubated at 20°C in a ternary system hexane – 2-propanol – water ($2 . 10^{-3}$M sodium citrate, pH 4·5). Content of the active enzyme in the mixture was determined by withdrawing samples at given time intervals, and measuring catalytic activity of laccase in the oxidation of pyrocatechol as described above. The stability of laccase in binary mixtures 2-propanol – water was determined analogously.

Study of absorption spectra of nitrate ion: A mixture of hexane, 2-propanol and aqueous solution of potassium nitrate, corresponding to the given point in Fig. 1, was placed into a spectrophotometer cell (Hitachi U-3400). The reference cell was filled with the same ternary solvent mixture without the nitrate. Spectra in the binary mixtures 2-propanol – water were obtained analogously. In all cases the concentration of potassium nitrate was 0·1 mol l^{-1} based on the volume of water present in the system. For all systems studied, the extinction coefficient of the nitrate anion amounted to about $7 . 10^{3}$ mol^{-1}.

Recovery of laccase from the microemulsion: Calculated amounts of hexane and 2-propanol were added to aqueous solution of laccase to make a microemulsion of composition corresponding to point 5 in Fig. 1 (see Fig. 5). The system obtained was mixed with a fivefold excess of hexane and the non-transparent macroemulsion formed was centrifuged (3 000 rpm) for 5 min. The aqueous (enzyme-containing) phase was separated from the organic one and the cycle was repeated. Prior to the experiment, and each cycle, a sample of the aqueous phase was taken and the enzyme activity was determined (Fig. 6) according to the above-described method, using the aqueous buffer as the reaction medium.

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