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Immobilization of plant peroxidases in cellulose–ionic liquid films

Svetlana V. Muginova, Dina A. Myasnikova, Aleksei E. Polyakov and Tatiana N. Shekhovtsova*

Department of Chemistry, M. V. Lomonosov Moscow State University, 119991 Moscow, Russian Federation.
Fax: +7 495 939 4675; e-mail: tnshekh@yandex.ru

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The optically transparent cellulose–1-butyl-3-methylimidazolium chloride films with immobilized cationic and anionic plant peroxidases were found catalytically active in the oxidation reactions of aryldiamines and catecholamines.

Hydrophilic ionic liquids (ILs) are promising solvents for cellulose,[1] which can be used for the formation of cellulose fibers, films, sponges, beads, *etc*.[1,2] Cellulose films with impregnated organic reagents were successfully applied to the sorption of transition metal ions with the subsequent colorimetric determination[3] and to the development of fluorescent sensors for the determination of O_2.[4] Haloid ILs, especially, 1-butyl-3-methylimidazolium chloride ([bmim][Cl]) were used for obtaining cellulose films with noncovalently and covalently immobilized enzymes.[5–9] Based on the activity of laccase,[5,6] esterase,[7] cellulase[8] and β-galactosidase[9] in the cellulose–hydrophilic IL films, it is impossible to predict a success of the immobilization of other biocatalysts. The prospects of the application of cellulose films in combination with enzymes obtained with the help of ILs to chemical analysis, biotechnology and bioremediation are unclear.

The aim of this work was to develop a procedure for the noncovalent immobilization of plant peroxidases into cellulose–[bmim][Cl] films and to test these films in the enzymatic transformation of organic substrates. Considering commercial availability, high catalytic activity and stability of horseradish peroxidase (HRP) and soybean peroxidase (SBP), they were chosen as test materials. In spite of the similar molecular weights, a high degree (57%) of the structure homology, and the same quantity of disulfide bridges, HRP and SBP differ in the degree of glycosylation (9 and 4 glycosylation sites, respectively) and isoelectric points (pI 8.2–9.0 and 4.1, respectively).[10]

When HRP or SBP was added to a supercooled cellulose–[bmim][Cl] melt according to recommendations for laccase immobilization,[5] the melt crystallized to make impossible the further casting of a film.

HRP was used as a model enzyme to optimize the conditions of cellulose dissolution in [bmim][Cl] and cellulose film preparation. The cellulose contents of 2.0–4.75 wt% were used for obtaining transparent, thin, sturdy and flexible films with other enzymes.[5] Among the two ways to dissolve cellulose in IL (thermostatic heating[3] and microwave heating[11,12]), the former was chosen, as microwave irradiation often led to cellulose destruction.[3] The temperature of a cellulose–IL mixture varied from 65 °C (the melting point of [bmim][Cl]) to 90 °C (at higher temperature the cellulose destruction began) in increments of 5 °C. The criterion of fullness of the cellulose dissolution in IL was the heating time after which the cellulose–IL melt became visually transparent. The optimum conditions were the following: cellulose : IL weight ratio of 3.5 : 100, 85 °C (the temperature of the melt) and 6 h (the time of the cellulose dissolution in IL). Note that a commercial sample of [bmim][Cl] should be dried to obtain transparent, thin, flexible and sturdy cellulose films.

The optimum enzyme concentration in the cellulose–[bmim][Cl] mixture was 2.8 wt%. The films obtained at this enzyme concentration exhibited the highest catalytic activity, which can be controlled either visually or spectrophotometrically (see Online Supplementary Materials). Surprisingly, SBP dissolved in the cellulose–[bmim][Cl] mixture more slowly than HRP; this may be caused by a difference in their pI values. To test this hypothesis, we studied peanut peroxidase (PNP) and bovine serum hemoglobin (Hb). The time (h) of enzyme/protein dissolution in the cellulose–[bmim][Cl] melt increased in the order 6 (HRP) < < 7 (Hb) < 10 (SBP) < 96 (PNP), in which the pI values decreased: 8.2–9.0 > 7.0 > 4.1 > 3.89. Thus, the dissolution of the cationic peroxidases was more rapid than that of the anionic enzymes. This conclusion is consistent with published data[5] on cationic laccase (pI 8.2).

The procedure for the immobilization of plant peroxidases in the cellulose–[bmim][Cl] film included the following steps: 0.6 mg of a solid peroxidase, 38.5 mg of cellulose, and 1.1 g of dried [bmim][Cl] were successively added to a plastic test tube with a stopper. The mixture was thermostated for 6 h at 85 °C with stirring. The hot melt was spilled on hot glass slides (4.5×2.6 μm) and casted quickly with a glass stick to form a cover with a thickness of ≈0.3 mm. Three films with surface areas of 11.7 cm^2 were cast (Figure 1). Irregular film edges were neatly cut out and cast away. A leftover film was cut into 20 squares of 7×7 mm^2. The film thickness was calculated as a mass-to-square ratio of the film based on the assumption that the film density was approximately equal to the density of [bmim][Cl] (1.1 g cm^{-3}).

The cellulose films were transparent and sturdy only when the glass slides were placed into a refrigerator (+4 °C) in a sealed container immediately after 1 h casting. After that, the slides were placed successively into two 4-ml portions of water for 10–15 min to remove an excess of IL. In the course of washing, the films detached from the glass slides. The enzyme leaching during this procedure was evaluated by determining the catalytic activity of wash water in the reaction of 3,3′,5,5′-tetramethylbenzidine (TMB) oxidation with H_2O_2 (see S3, Online Supplementary Materials). The degree of the biocatalyst leaching from the cellulose film (%) increased in the order HRP (0.04) < SBP (1.8) < Hb (1.9). Taking into account the amount of the enzymes

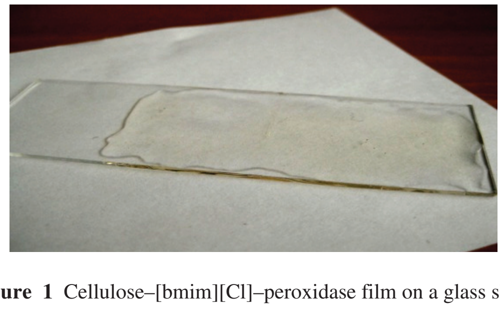

Figure 1 Cellulose–[bmim][Cl]–peroxidase film on a glass slide.

leached from the film as a result of its washing with water, the test form contained 2×10^{-10} mol of both peroxidases.

Film transmittance was spectrophotometrically controlled by measuring the absorbance of the film at 200–700 nm (A_λ was almost constant in this range). The transmittance of the films casted from the same melt was 94±3%; $n = 3$, $P = 0.95$.

The catalytic activity of the enzyme-containing films was estimated visually in the oxidation of aryldiamines [TMB, *o*-dianisidine (*o*-DA) and *o*-phenylendiamine (*o*-PDA)] and catecholamines (dopamine, adrenaline, α-methyldopa and dobutamine) by H_2O_2.[†] The rate of the reaction of the immobilized HRP increased in the same sequence of the substrates as in the case of the native enzyme (the logarithms of octanol–water partition coefficient, log P, are given in parentheses): *o*-PDA (0.32) < < *o*-DA (1.65) ≈ TMB (4.02); adrenaline (–1.37) < α-methyldopa (–1.7) < dopamine (–0.98) < dobutamine (3.6).[13,14] The same sequence was observed for SBP, with the exception that adrenaline and dobutamine were not oxidized with this enzyme.

The most important result is that HRP and SBP noncovalently immobilized in cellulose films retain at least 54 and 58%[‡] of their initial catalytic activity, respectively, at high IL content of the cellulose mixture (96.6 wt%) and high temperature (85 °C) of the melt. The cellulose film may be reused at least once if properly washed by water just after the rate of the indicator reaction is fixed. However, after washing step the catalytic activity of HRP in the cellulose film decreases by a factor of almost 2.5.

Note that both peroxidases included into the cellulose films exhibited their oxidase activity in the oxidation of aryldiamines: a film became pale blue (pale red or pale yellow) after the addition of a drop of TMB (*o*-DA or *o*-PDA, respectively) solution on it, *i.e.*, in the absence of H_2O_2. Thus, immobilized HRP and SBP keep all their catalytic properties, which are intrinsic to native plant peroxidases in the studied indicator reactions.

Among the three ways to store films (in water; in a dry state in air; and in a swollen state in a sealed container), the last one was chosen (see Online Supplementary Materials). The cellulose films with immobilized HRP and SBP, which were stored in a swollen state in a sealed container at room temperature (20–25 °C), retained their initial catalytic activity for a week. After 35 days of storage, the residual activities of HRP and SBP in the films were 80 and 70%, respectively.

Table 1 Residual activity of plant peroxidases in cellulose films in the reaction of TMB oxidation by H_2O_2.

Enzyme[a]	Time when a cellulose film attained the fixed intensity of solid brown colour / s		Residual activity (%)
	Water solution of an enzyme	Immobilized enzyme	
HRP	6	11	54
SBP	10	17	58

[a] Cellulose films with immobilized Hb showed no catalytic activity, which could be related to a lower thermostability of Hb in comparison with HRP and SBP.[15]

Thus, the optically transparent, thin, flexible and sturdy cellulose–[bmim][Cl]–peroxidase films demonstrate high catalytic activity, stability and substrate specificity typical of native peroxidases in the oxidation of aryldiamines and catecholamines. The proposed procedure is more appropriate for the immobilization of cationic peroxidases. The films are promising as sensitive elements of optical biosensors for the determination of organic substrates of plant peroxidases.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.mencom.2013.03.005.

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[†] *Visual determination of the catalytic activity of the biocatalysts in a cellulose film.* First, a 20 µl portion of 0.1 mM solution of aryldiamine (or catecholamine) and a 10 µl portion of 0.1 mM solution of H_2O_2 were successively added to the same point of a film. Timer was turned on at the moment when H_2O_2 was added. In the oxidation reaction of TMB, the catalytic activity of HRP and SBP in the film was characterized by the time of the appearance of solid brown colour of the film (S6, scale 1, Online Supplementary Materials). In the oxidation reactions of other reducing substrates (with the exception of adrenaline and dobutamine), the catalytic activity of the biocatalysts was characterized by the time when the intensities of colours in the films with HRP and SBP became equal by visual inspection. Fixed colours are marked with circles on the scales 4–7 (S6).

[‡] The residual catalytic activity (%) of plant peroxidases in a cellulose film sized 7×7 mm^2 (in other words, in a test form) was calculated as $(t_0/t)\times100$, where t_0 and t were the times (s) when cellulose films prepared without enzyme (the solution of the biocatalyst was later added onto the film) and with enzyme, respectively, attained solid brown colour in the reaction of TMB oxidation by H_2O_2 (Table 1). The catalytic activity of plant peroxidases immobilized into a cellulose film was determined as described above. In the blank experiment, a 30 µl portion of 0.136 µM solution of the biocatalyst was added on an enzyme-free film. After 10 min (this time was needed to absorb the enzyme solution by the film) a 20 µl portion of 0.1 mM solution of TMB and a 10 µl portion of 0.1 mM solution of H_2O_2 were successively added to the same point of a film.