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Journal of Molecular Catalysis B: Enzymatic 3 (1997) 121–125

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

A convenient activity assay for immobilized lipases

Gerard F.H. Kramer *, Lawrence Batenburg, Johannes T.P. Derksen, F. Petrus Cuperus

Agrotechnological Research Institute (ATO-DLO), P.O. Box 17, 6700 AA Wageningen, The Netherlands

Abstract

A convenient assay is presented for testing the activity of lipases immobilized on hydrophobic supports. In one phase (2-ethylhexanol:ethyl caprylate:hexadecane, 5:5:1 w/w/w), both substrate and internal standard are stirred with 1–10 mg immobilized lipase. The interesterification product, 2-ethylhexyl caprylate, is monitored by GC. The reproducibility is well within the 10% range, which is very good considering the heterogeneity of the observed system.

Keywords: Activity assay; Immobilized lipase; Interesterification; Alcoholysis; Gas chromatography; Controlled wateractivity (a_w); Inactivation

1. Introduction

The performance of enzymes in non-aqueous reactions is improved by immobilization on support materials. An equal distribution of the enzyme on the surface of the material eliminates mass transfer barriers within clusters of non-dissolved enzyme molecules and facilitates the recovery of the biocatalyst after the reaction. On the other hand, immobilization may also imply the introduction of new mass transfer barriers [1].

While studying the activity and stability of immobilized lipases, one will find that the generally used lipase assays, like the tributyrine test [2,3], are not giving satisfying results, due to several mass transfer limitations. All methods use emulsified substrates to ensure the interfacial activation of lipases [4].

To overcome the difficulties associated with the heterogeneous character of immobilized lipases, a non-aqueous, one phase assay systems seem more appropriate. As a consequence, the thermodynamic water activity (a_w) has to be taken into consideration when a non-aqueous system is chosen [5]. The water activity determines the amount of water bound to the enzyme molecule. Part of this water is essential for keeping the active conformation. Water activity equilibration on a lab-scale is possible by employing saturated salt buffers. For making an accurate comparison between enzyme samples, they have to be equilibrated at the same a_w, and remain constant during the assay.

A uniform, one-phase method for testing the activity of immobilized lipase preparations that considers both mass transfer and water activity is not known in literature. But many reaction systems, some described as industrial applications, could be used for this purpose. For in-

* Corresponding author. E-mail: g.f.h.kramer@ato.dlo.nl.

PII S1381-1177(96)00056-2

stance the interesterification of myristic acid with olive oil [6] and the esterification studied by Valivety et al. [5]. It should be noted that methods which use triglyceride substrates [7] may be mass transfer limited, due to the high molecular mass of these substrates and the viscosity of the medium. Also, the number of sample preparation steps required in the Novo method [7] is impractical when more than one sample has to be analyzed at the same time.

Here, an assay is presented, based on an alcoholysis reaction in a one phase system. An alcoholysis reaction was chosen, as at lower values the water activity remains relatively stable at the setpoint. Partial hydrolysis of the ester at higher a_w will cause a decrease in this value [5]. On the other hand, esterification reactions give an inherent increase in water activity, even intensified by the more hydrophobic nature of the ester product.

The present assay reaction, the alcoholysis of ethyl caprylate with 2-ethylhexanol is known for its slow kinetics, thus decreasing the significance of the mass transfer rate.

Several unnecessary errors that could otherwise be introduced during sampling are excluded by adding the internal standard for the GC-analysis, hexadecane, to the assay medium. The easy sampling procedure also allows the management of several assay reactions at the same time.

A low a_w value was selected as a setpoint for the immobilized enzyme preparations. During preliminary experiments it was found that equilibration of the assay medium on the same water activity as the immobilized enzyme was not necessary. Drying the medium with molecular sieves (3 Å) to $a_w = 0.02$ gave reproducible results. It was reasoned that stripping of water from the enzyme did not take place to an extent that influenced the linearity of the conversion rate during the observed time.

Obviously one has to be cautious when translating the results of one assay to another, or to a specific application, since substrate specificity and reaction conditions can cause large differences in rates (see for instance Ref. [8]). Activity assays, such as described here, are especially helpful to compare changes in activity of an enzyme as a function of time or exposure to denaturing conditions.

The method proved to be very useful for making accurate and independent studies of the immobilized lipase activity in several applications. Here, this is illustrated by results of a model inactivation experiment with *C. antarctica* lipase B and a chemical modifier, that is known to react with second order kinetics in a homogeneous system.

2. Materials

Freeze-dried *C. antarctica* lipase B (CALB) and Accurel^R EP 100 (macroporous polypropylene (PP)) were kind gifts of Novo Nordisk (Bagsvaerd, DK) and Akzo Faser AG (Obernburg, FRG), respectively. The enzyme was immobilized according to the procedure of Macrae et al. [9]. The reaction mixture included ethyl caprylate > 98%, hexadecane (SAF, Bornem) and 2-ethylhexanol (Merck, Darmstadt). All other chemicals were also purchased from Merck. 2-Ethylhexyl caprylate was made by sulphuric acid catalysed esterification of 2-ethylhexanol with caprylic acid. Samples of immobilized lipase were weighed with a Sartorius R200D analytical balance (accurate to 5 decimal places). The alcoholysis reactions were performed in 4 ml sample vials with teflon lined screw caps and stirred with magnetic micro stirring bars (2 × 7 mm). Samples were analyzed on a Varian 3400 GC (FID detector) equipped with an 8200 autosampler and a CP Sil 5CB column (Chrompack, NL).

Determination of the water concentration in the dried medium was performed by a Karl–Fisher titration. The water activity of the medium was obtained by extrapolation of the value for water saturated medium to zero, as a linear relation between water concentration and water

activity may be assumed for non-polar media [10].

3. Procedure

Immobilized CALB was dried in vacuo. Samples from an inactivation experiment were washed extensively with ether prior to drying. An amount of dried material (2–10 mg) was weighed into a reaction vial and then equilibrated through the headspace with the appropriate saturated salt buffer for 3 days (Valivety, 1992). A micro stirring bar was added after weighing. A stock solution of reaction medium was prepared by mixing 2-ethylhexanol, ethyl caprylate and hexadecane (5:5:1 w/w/w) and subsequent drying with molecular sieves (3 Å).

At the start of the assay reaction, 2 ml of the reaction medium was transferred into the vial containing the equilibrated immobilized lipase. The vial was then closed and stirred at 300 rpm and 20°C. About 5 vials were managed simultaneously. At intervals, samples of 50 μl were taken from the vial, diluted with 1 ml of chloroform and analyzed by GC. The reaction product is quantified on basis of its response relative to the internal standard i.e. hexadecane. The response factor was determined with pure hexadecane and 2-ethylhexyl caprylate.

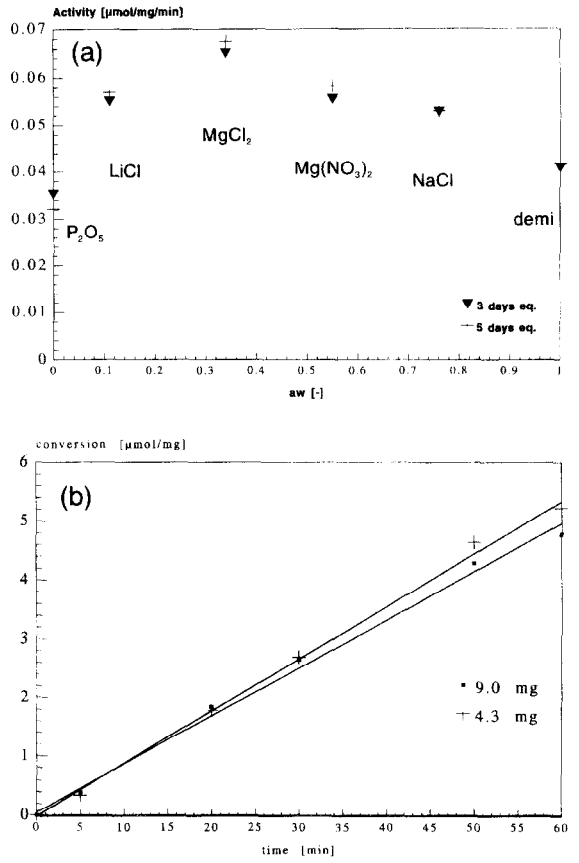
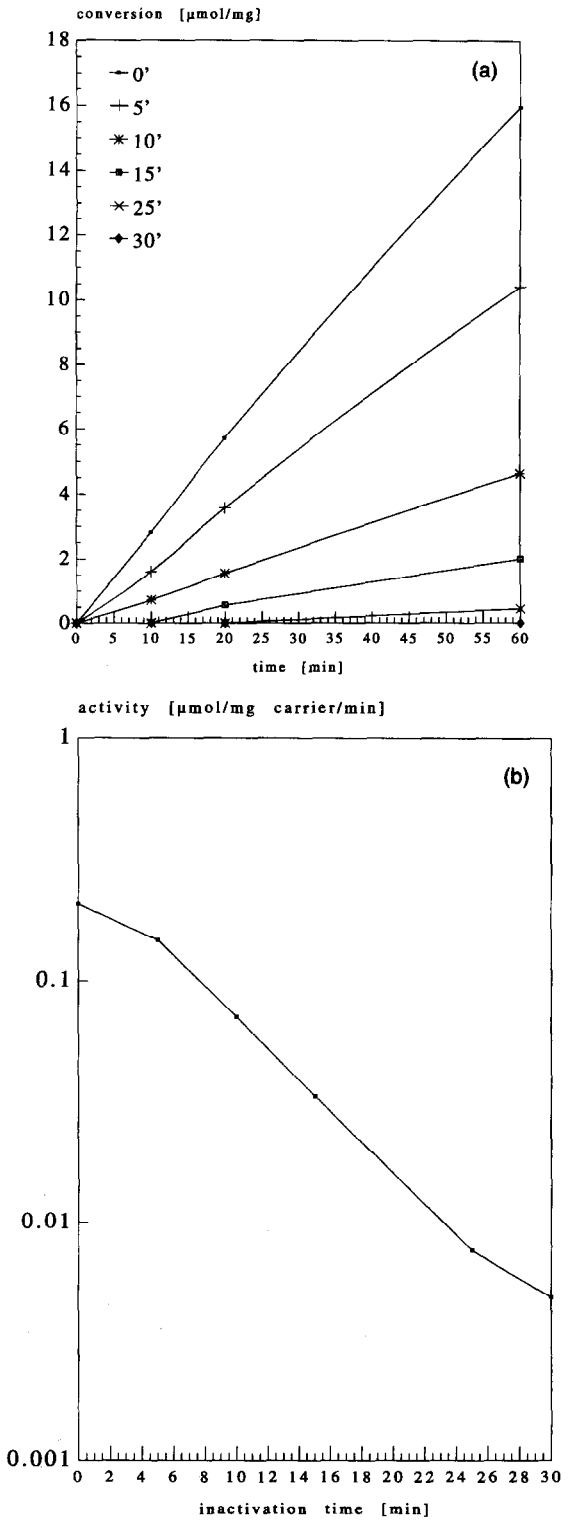


Fig. 1. (a) Activity of *C.antarctica B* on macroporous PP as a function of its a_w setpoint. (b) Regression lines of the conversion (μmol/mg carrier) to 2-ethylhexyl caprylate for two different amounts of sample.

4. Results

For equilibration of the immobilized enzyme on a certain water activity (a_w), an adequate time has to be taken. Comparing the determined activities after 3 and 5 days equilibration with salt buffers indicates that 3 days is sufficient (Fig. 1a). At high water activities it was observed that the carrier particles adhere to each other, which decreases the mass transfer. Also, partial hydrolysis of ethyl caprylate was detected at these water activities. It should be noted that the samples dried over phosphorous pentoxide can not have reached zero water activity, as might be suggested in the graph, because the enzyme is still partially active. The observed optimum for this combination of enzyme, carrier and medium is 0.34. For the interesterification assay, a setpoint for the water activity of the immobilized enzyme between 0.02 and the optimum might be chosen. The lithiumchloride buffer (0.12) was chosen because its a_w is closest to the setpoint of the medium and also the amount of hydrolysis will be negligible. Probably, the activity at this point is very similar to the actual activity at $a_w = 0.02$. This procedure resulted in a good reproducibility of the assay, as is shown in Fig. 1b. Here, the assayed activity of a double amount of



immobilized enzyme is well within a 10% error range.

The performance of the assay as a tool for studying the effect of immobilization procedures and the stability of immobilized lipases, is illustrated by a denaturation experiment with a chemical modifier. This compound is chemically modifying one of the essential amino acids of CALB in a bimolecular reaction. During the inactivation process, samples of the immobilized enzyme are removed and washed thoroughly with ether to stop the inactivation. Assaying the activity of these samples with the alcoholysis method gave a set of straight lines, representing the relative activity, with slopes decreasing as a function of the contact time (Fig. 2a). When the slopes (rate per weight amount of immobilized enzyme) are plotted in a logarithmic scale as a function of the inactivation time, an almost straight line is obtained, as might be expected for a 2nd order reaction.

5. Conclusions

The present method proves to be a useful tool for studying the activity in time of immobilized lipase preparations. It is also accurate enough to study inactivation kinetics. The linearity of the conversion during 1 h (see for instance Fig. 2a) seems to indicate that equilibration of the assay medium at the same water activity as the immobilized enzyme is not necessary. Probably, the difference in activity of CALB on PP between a_w-values of 0.02 and 0.12 is not very big. This is very convenient since equilibration of pure substrate medium through the vapour phase is time consuming [5]. An additional advantage is that at this low water activity, the stock solution can be stored for a longer period of time be-

Fig. 2. Inactivation of CALB by a chemical modifier (a) interesterification assays of samples with different inactivation times (b) interesterification rate versus inactivation time (contact time chemical modifier).

cause the rate of spontaneous interesterification is low.

The method might be further improved by finding an efficient way to equilibrate the assay medium on the same water activity as the immobilized enzyme. A faster equilibration may be obtained by using a co-solvent with a high log P, which decreases the water saturation point of the medium. Another possibility might be to dry the immobilized lipase preparation with molecular sieves to $a_w = 0.02$ as well. But for use of the assay with other lipases, this may cause an unacceptable decrease in activity.

By using a component of the assay medium as an internal standard for the GC-analysis possible errors are limited to the weighing procedure, the addition of assay medium to the reaction vial and the GC analysis. The error caused by the GC analysis is minimized by taking several samples during 1 h and using the regression coefficients for the slopes. Data from similar samples are normally well within a 10% error range, which is well acceptable considering the heterogeneous system that is studied.

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