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Novel chitosan membranes as support for lipases immobilization: Characterization aspects

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ABSTRACT

Membranes of chitosan (QS), chitosan treated with glutaraldehyde (QGA) and chitosan crown ether (QCE) were utilized as carriers for immobilization of *Candida antarctica* and *Candida rugosa* lipases. Membrane supports were characterized by several techniques (Raman spectroscopy, elemental analysis by CHN determination and Energy Dispersive X-ray (EDX), water sorption isotherms, and surface area from nitrogen sorption data). To verify the presence of enzymes, some of these techniques were also used for lipase on chitosan biocatalytic systems. Measurements of protein load from Biuret assays and catalytic activity in esterification in nonaqueous media were also made for the immobilized enzymes. Sorption isotherms at 20, 30, 40 and 50 °C for QS, QGA and QCE supports were fitted to the Guggenheim, Anderson and Böer model. GAB monolayer moisture parameter, Xm, varied between 0.029 and 0.051 for QS, 0.039 and 0.058 for QGA and 0.039–0.075 g of water g^{-1} s.s. for QCE membranes. Elemental analysis and Raman spectra measurements of the lipase, supports and immobilized lipase systems gave evidence of the presence of enzymes on supports. Chitosan supports with internal surface area (m2 g^{-1}) among 3.31 and 1.26 were obtained. Regardless of these low values, acceptable protein load (0.61 to 3.21%) and esterification initial rates were achieved (0.88–2.75 mmol min^{-1} g of $protein^{-1}$).

1. Introduction

Enzymatic catalysis in nonaqueous solvents has gained considerable interest for the preparation of natural products, pharmaceuticals, fine chemicals and food ingredients (Carrea & Riva, 2000; Faber & Franssen, 1993; Margolin, 1993; Ru et al., 2000). Improved thermostability, favored synthesis over hydrolysis, tunable enzyme selectivity via medium engineering, and the simplicity of product recovery owing to the more easily evaporated reaction solvent, are reasons why nonaqueous bioprocessing is so attractive (Lee & Dordick, 2002; Turner & Vulfson, 2000; van Unen, Engbersen, & Reinhoudt, 2001; Vermuë & Tramper, 1995). Lipases (glycerol esters hydrolase, E.C.3.1.1.3) have been widely used to produce organic chemicals, biosurfactants, oleochemicals, agrochemicals, paper, cosmetics, fine chemicals and pharmaceuticals (Sharma, Chisti, & Banerjee, 2001). Lipases can also catalyze ester synthesis reactions in organic solvent systems. Among these esters, the 1-butyl oleate is used to decrease cloud point and pour point of diesel and biodiesel during winter, as poly vinyl chloride plasticizer, water-resisting agent and hydraulic fluid (Ghamgui, Karra-Chaábouni, & Gargouri, 2004; Linko et al., 1998).

Chitosan is an amine polysaccharide obtained from alkaline deacetylation of chitin, an unelastic and nitrogenated polysaccharide, which is found on the walls of the fungi and outer skeleton of arthropodes such as insects, crustaceans and beetles. Amino groups make chitosan one of the few found in nature cationic polyelectrolyte ($pK_a \approx 6.5$). Chitosan is known for its biocompatibility allowing its use in various medical applications (Chandy & Sharma, 1990; Okamoto et al., 1993), the production of value-added food products (Muzzarelli, 1996; Shahidi, Arachchi, & Jeon, 1999) and can be considered as biodegradable (Berger, Reist, Mayer, Felt, & Gurny, 2004).

Chitosan has been used as a matrix for immobilization of lipases (Alsarra, Betigeri, Zhang, Erans, & Neau, 2002; Betigeri & Neau, 2002) and many other enzymes (Krajewska, 2004; Spagna, Barbagallo, Casarini, & Pifferi, 2001). Enzymes bound to sugars, or sugar-based polymers like chitosan are stabilized during

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lyophilization and in nonaqueous environments. This may be due to a reduction of autolysis, that is a multipoint attachment limiting enzyme distortions or microenvironmental effects (Pandey, Benjamin, Soccol, Nigam, & Soccol, 1999; Wang et al., 1992).

The water activity (a_w) of a medium is an important factor in lipase catalyzed synthesis (Graber, Bousquet-Dubouch, Sousa, Lamare, & Legoy, 2003; Malcata, Reyes, Garcia, Hill, & Amundson, 1992). The retention (or release) of water on a solid carrier in which an enzyme is immobilized affects enzyme action in various ways: by influencing enzyme structure via noncovalent bonding and disruption of hydrogen bonds; by facilitating reagent diffusion. The relationship between total moisture content and the water activity of the chitosan support, over a range of values at constant temperature yields a moisture sorption isotherm. This type of isotherms gives information about the water sorption mechanism, interactions between the biopolymer and water, and also helps to establish the final moisture content on dehydration (Rodríguez-Aragón & López-Fidalgo, 2007; Telis, Kohayakawa, V.S., Pedro, & Gabas, 2005).

In previous studies we evaluated the results of the combination of freezing and thawing cycles and the addition of glutaraldehyde and 18-crown-6 ether on superficial chitosan membranes characteristics (Orrego & Valencia, 2009). Particularly we study the modulation of chitosan crystallinity derived by those treatments and the effect of biopolymer crystallinity on protein load. We also studied kinetic aspects for the immobilized *Candida rugosa* lipase in the esterification reaction of 1-butyl oleate in iso-octane (Orrego, Valencia, & Zapata, 2009). The aim of the present work is to compare and expand the results of the above approaches with the characterization by solid state techniques of three different chitosan membranes intended now as supports for two enzymes (*Candida rugosa* lipase and *Candida antarctica* lipase). Measurements of protein load and catalytic activity in butyl oleate synthesis in iso-octane were also made for the immobilized lipase.

2. Materials and methods

2.1. Reagents and materials

Chitosan flakes (high molecular weight 602 kDa, degree of deacetylation 76.5%), *Candida rugosa* lipase (with a nominal specific lipolytic activity of 1104 U mg^{-1} solid and containing ~18.26% protein based on the Biuret protein assay) were obtained from Sigma Chemical Co. (St. Louis, MO, United States), *Candida antarctica* lipase (with a nominal specific lipolytic activity of 910 U mg^{-1} solid and containing ~9.02% protein based on the Biuret protein assay) was obtained from Novo Nordisk A/S (Bagsvaerd, Denmark); *iso*-octane and *n*-butanol were purchased from Panreac (Barcelona, Spain) and oleic acid from Carlo Erba (Milan, Italy). All other organic and inorganic reagents were of analytical grade.

2.2. Support production

2.2.1. Cryogelled chitosan support (QS)

The procedure for support production was described in a previous work (Orrego & Valencia, 2009).

2.2.2. Supports treated with glutaraldehyde (QGA) and with 18-crown-6 ether (QCE)

The procedure for support production was described in a previous work (Orrego & Valencia, 2009).

2.3. Moisture sorption isotherms

Moisture sorption isotherms of QS, QGA and QCE membrane supports were made by static gravimetric method (Rockland, 1960) in a laboratory set up consisted of eight glass hermetic flasks, six of them with different saturated salt solutions in their base (LiCl, $MgC1_2$, K_2CO_3, NaCl, KCl and $BaCl_2$). These salts have a range of relative humidity from 5% to 90% (Greenspan, 1977; Bizot, 1987). In the upper side of each flash was placed ca. 0.2 g of dried membranes obtained after 3 days of dehydration under air at 60 ± 0.7 °C in a forced convection oven PJTECH Thermolab Kryoven (Medellín, Col). The dried membranes enclosed under the humidity controlled environment of the solutions into the sealed flasks were placed in the oven at four different temperatures (20, 30, 40 and 50 °C). The gain of water was measured with an Ohaus Adventurer Balance (Pine Brook, NJ, USA, 65–310 g, precision, ±0.1 mg). When the weight of the sample stayed constant for 3 consecutive days it was considered that membrane moisture equilibrium was accomplished. This equilibrium was reached between 7 and 20 days.

2.4. Surface area measurements

Surface areas assays of QS, QGA and QCE supports were made in a porosity and surface area analyzer ASAP 2020 (Micromeritics Instrument Corporation, Gosford, New South Wales, Australia). As calibration standards were used α-alumin and kaolinite, with 0.52 ± 0.03 $m^2 g^{-1}$ and 15.8 ± 0.09 $m^2 g^{-1}$, of surface area, respectively. Samples were degasified at 100 °C with a heating rate of 10 °C/min for 24 h, under evacuation rate of 10 mmHg/s, until 7 μmHg of stable pressure was reached. After this procedure, sorption isotherms were obtained at 77 K. Adsorbed volume data at standard conditions of temperature and pressure were mathematically transformed according with the BET method (Brunauer, Emmett, & Teller, 1938).

2.5. Immobilization of lipase on chitosan supports

Lipases from *C. rugosa* (E.C.3.1.1.3) and *C. antarctica* were immobilized onto QS, QGA and QCE film supports to obtain the systems showed in Table 1.

During immobilization, the enzymes were dissolved (final protein content in lipase solutions: 1.2 ± 0.1 mg ml^{-1}) and pre-incubated at 35 °C in 50 ml of phosphate buffer (pH 7.2) under gentle stirring for 2 h. After that; 1 g of chitosan film was submerged into the enzyme solution for 20 h at 20 °C under agitation (120 rpm). Subsequent to immobilization, films were taken out, washed thoroughly with deionized water and rinsed with phosphate buffer solution. The resultant immobilized lipase on chitosan films were taken out and stored at 4 °C. During storage the films dehydrated and the water activity of the films were measured periodically at 4 °C in a thermoconstanter NOVASINA analyzer until their water activity were between 0.5 and 0.6. Before that they were packed in high barrier plastic bags. After immobilization the catalytic supports were placed in controlled humidity chambers until the equilibrium water activity (0.53 ± 0.04) was reached.

2.5.1. Protein loading assay

The amount of immobilized enzyme on the membrane was determined by measuring the initial and final concentrations of protein within the enzyme and washing solutions using the Biuret

Table 1
Lipase on chitosan immobilized systems.

Support or membrane	Immobilized system	
	C. rugosa lipase	*C. antarctica* lipase
QS	QSR	QSA
QGA	QGAR	QGAA
QCE	QCER	QCEA