PIROCROCIN HYDROLYSIS BY IMMobilIZED $\beta$-GLUCOSIDASE

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SUMMARY.
$\beta$-Glucosidase from sweet almond was immobilized onto a nylon support and used to hydrolyze picrocrocin, the glycoside precursor of the saffron essential volatile oil, safranal. The nylon support was derivatized as hydrazide and the enzyme attached through Schiff base to bonds. The coupling efficiency was 46.8%, the immobilization yield 29.5%, and the derivative showed 24.2 and 4.0 U/mg activity for p-nitrophenylglucoside and picrocrocin, respectively, as substrates. Kinetic parameters of the immobilized derivative were determined, with picrocrocin as substrate, showing $K_M = 7.2$ mM and $V_{max} = 4.0$ U/mg. Glucose behaved as a competitive inhibitor ($K_i = 95.0$ mM). The immobilized derivative was thermally stable up to 45°C; from that temperature onwards thermoinactivation occurred. The operational deactivation showed a biphasic pattern, $t_{1/2}$ being 4.2 days for the first four days of continuous operation, and 20.1 days from that point on. The immobilized enzyme lost a 50% of its initial activity after 30.7 days of storage at 4°C.

INTRODUCTION.
In recent work $\beta$-glucosidase has been applied for aroma liberation (Vasserot et al., 1989), since several natural aromatic compounds from plants are synthesized as non-volatile and odourless terpenyl-$\beta$-glycosides. The extraction of these precursors and subsequent hydrolysis with exogenous $\beta$-glucosidases could be a procedure to obtain the desired aroma by a controlled process. Safranal is included among these compounds; it is the essential volatile oil responsible for the characteristic saffron odour and aroma, and represents a highly valuable ingredient for the flavouring of certain foods. Its glycosylated precursor, picrocrocin, could be hydrolysed by $\beta$-glucosidase yielding D-glucose and 2,2,6-trimethyl-4-hydroxy-1-carboxaldehyde-1-cyclohexene (HTCC), this later yielding safranal after dehydration (Madan et al., 1966; Mouseron Canet et al., 1966; Zarghami and Heinz, 1971).

$\beta$-Glucosidase immobilization would allow continuous picrocrocin hydrolysis to be carried out. Immobilization of enzyme $\beta$-glucosidase has been described using a broad range of support media, such as polyamid, (Rao et al., 1983), nylon (Hsuanyu and Laidler, 1986), alumina (Fadda et al., 1989). Among these supports nylon is an interesting material due to its chemical and physical properties, possibility of presenting different physical forms (films, membranes, powder, pellets, hollow fibers, tubes, etc) and chemical modifications, high stability and relatively low cost (Hornby and Golstein, 1976).
Thus, considering the potential use of immobilized β-glucosidase for aroma liberation from their glycosylated precursors, and bearing in mind the importance of safranal as additive in the food industry, the present work reports on picrocrocin hydrolysis with β-glucosidase immobilized onto nylon and the properties of the immobilized enzyme: kinetic parameters, optimum operational conditions and thermal, storage and operational stabilities.

MATERIALS AND METHODS

Materials. β-Glucosidase (EC 3.2.1.21) from sweet almond (5 units/mg, with salicin as substrate at pH 5.0 and 37°C) and the synthetic substrate p-nitrophenyl-β-D-glucopyranoside (PNPG) were from Sigma. The natural substrate, picrocrocin, was extracted and purified from saffron as previously described (Iborra et al., 1992). Nylon (NY 300 HD, 20 yards/cm, 0.20 mm thread diameter, 36 % open surface, 170 g/m²) was purchased from Züricher Beuteltuchfabrik (ZBF). All other reagents were analytical grade.

Immobilization procedure. Immobilization procedure was carried out by a modified method of Hornby and Goldstein (1976), as follows: a) Nylon pellets were O-alkylated with dimethyl sulphate (4 min at 100°C) and washed with cold methanol to render imidate-activated nylon; b) to obtain the derivatized nylon-hydrazide, the support was mechanically stirred (2 h at 25°C) with 3 % adipic acid dihydrazine and 3 % N-ethylmorpholine in methanol solution, and then washed with 0.2 M bicarbonate buffer pH 9.2; c) the modified support was activated with 12.5 % glutaraldehyde (in 0.1 M bicarbonate buffer pH 9.2, for 30 min at 25°C) and then the enzyme was immobilized on its active surface. The enzyme was added in a 0.1 M phosphate buffer solution pH 7.5. The obtained suspension was gently stirred for 2 h at 25°C and finally for 12 h at 4°C. In order to reduce the Schiff bonds, 0.5 mg of sodium borohydride were added before and after treatment at 4°C. When the immobilization process finished the immobilized derivative obtained was washed with 0.1 M acetate buffer pH 5.5 and stored at 4°C until use. Protein concentration was determined by the Lowry method (Lowry et al., 1951) in both the supernatant and washed fractions, to obtain the amount of protein immobilized onto the solid support.

Activity was defined as units per mg of immobilized protein, coupling efficiency as the ratio between the enzymatic activity shown by the immobilized enzyme and the activity removed from the solution during the immobilization process, and the immobilization yield as the ratio between the activity shown by the immobilized enzyme to that of the soluble enzyme used for immobilization.

Enzymatic assay. Standard enzymatic assays were performed in a discontinuous thermostatted reactor at 37°C, pH 5.5 (0.1 M acetate buffer) and 1 ml reaction volume. Substrate concentration was 40 mM or 15 mM for picrocrocin or PNPG, respectively, while the concentration for the immobilized derivative was 3.5 mg/ml. Samples of 80 µl (including immobilized derivative) were withdrawn at regular intervals during the first 10 min. From this volume 50 µl, after removing the immobilized derivative, was collected. For the synthetic substrate assays aliquots were diluted to 3 ml with 0.5 M NaOH and their absorbance measured a 400 nm with a Spectronic 2000 spectrophotometer, while for picrocrocin aliquots were diluted to 500 µl with cold water and stored at -20°C until analysis by high performance liquid chromatography (HPLC), using a Shimadzu LC6A liquid chromatograph equipped with a 150 mm length x 4 mm ID Lichrosorb RP-C18 (5 µm particle size) column and a spectrophotometric detector, according to the method described by Iborra et al. (1992). One unit of enzymatic activity was defined as the amount of enzyme which produces 1 µmol of product within one minute at 37°C and pH 5.5.

For enzyme thermal stability assays, 62 mg/ml of immobilized derivative was incubated at different temperatures ranging from 35°C to 70°C in acetate buffer (0.1 M pH 5.5). Aliquots were withdrawn during 2 h at 30 min intervals for temperatures below 50°C, and at 15 min intervals for those higher than 50°C. Residual activity was measured using PNPG as substrate.