Preparation and Properties of Co-Reticulated Invertase Supported by an Ultrafiltration Membrane

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Yeast invertase was co-reticulated with glutaraldehyde to bovine serum albumin to give a soluble bound enzyme that was immobilized as a tightly adhering layer on the active surface of an ultrafiltration membrane. The Michaelis constant and stability of this immobilized-enzyme system are compared with those of the enzyme either in the native form or immobilized as a dynamically formed gel layer on an ultrafiltration membrane, as previously described by us [Drioli, Gianfreda, Palescandolo & Scardi (1975) *Biotechnol. Bioeng.* 17, 1365–1367].

In a previous paper (Drioli *et al.*, 1975) we suggested a new physical method for enzyme immobilization based on the dynamic formation of an enzyme–gel layer on the active surface of an ultrafiltration membrane by taking advantage of concentration polarization, i.e. a phenomenon peculiar to the ultrafiltration of macromolecular solutions (Blatt *et al.*, 1970). Thus, when the substrate solution is ultrafiltered through such an enzyme–gel composite membrane, chemical change is followed by a mass-transfer process involving only product(s) and unchanged substrate (if present). The kinetic behaviour of two hydrolytic enzymes, namely acid phosphatase (EC 3.1.3.2) and invertase (EC 3.2.1.26), immobilized by the suggested method has been described (Drioli *et al.*, 1975, 1976). Subsequently, to make the enzyme–gel layer firmer, we tried several cross-linking procedures with glutaraldehyde, but the results were not very satisfactory, probably because of the exiguous amount of enzyme present as a gel layer on the membrane surface (generally not exceeding $15\mu\text{g}/\text{cm}^2$ with a calculated thickness of about $1.5\mu\text{m}$). A tightly adhering enzyme layer was obtained when, instead of the native enzyme, we used a soluble form of the enzyme prepared by co-cross-linking it to an inert protein.

We present here, by way of example, a detailed procedure for preparing a co-reticulated enzyme firmly attached to an ultrafiltration membrane and some properties of this immobilized-enzyme system.

Materials and Methods

Yeast invertase was from Boehringer Biochemia (Milan, Italy); sucrose and glutaraldehyde (25%, w/v, solutions) were from Serva Feinbiochemica (Heidelberg, Germany); bovine serum albumin was from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

All other chemicals were from major suppliers. The ultrafiltration membrane used as an enzyme support was type DDS 600 from the Danish Sugar Corp. (Nakskov, Denmark).

Soluble co-reticulated invertase was prepared by the following procedure, which is a modification of that described by Paillot *et al.* (1974). Invertase (5 mg) was dissolved in 2.4 ml of 0.02 M-potassium phosphate buffer, pH 6.8, containing 158 mg of bovine serum albumin; then 1.6 ml of a 1.25% (w/v) glutaraldehyde solution freshly made up in the same buffer was added, and the mixture was kept for 18 h at 4°C. The cross-linking process was stopped by the addition of glycine up to 1.0% (w/v) final concentration. The soluble co-reticulated invertase thus obtained was dialysed for 24 h against several changes (a total of 2 litres) of 1.0% (w/v) glycine solution in 0.02 M-potassium phosphate buffer, pH 6.8. To assay the activity of this preparation, samples (5 μl) were incubated at 30°C with 3.0 μmol of sucrose in 1 ml of 0.1 M-sodium acetate/acetic acid buffer, pH 4.65; after 10 min the reaction was stopped by adding 1 ml of the copper alkaline reagent (Nelson, 1944), and the reducing products of the sucrose hydrolysis were determined by Nelson's (1944) method and expressed as glucose.

To immobilize the invertase on the ultrafiltration membrane, 0.5 ml of the soluble co-reticulated enzyme was diluted to 10 ml with 0.02 M-potassium phosphate buffer, pH 6.8, and ultrafiltered to dryness by using a pressure of 100 kPa (1 atm) of N_2 at room temperature (18–20°C). This operation was carried out in a water-jacketed ultrafiltration cell (57 ml capacity, 4 cm inner diameter), which was used throughout all experiments.

The enzyme reaction was started by pressurizing [200 kPa (2 atm)] the stainless-steel reservoir containing the substrate solution in 0.1 M-sodium acetate/acetic acid buffer, pH 4.65, after connexion to the

ultrafiltration cell with polyethylene pressure tubing. The temperature was kept at $30 \pm 0.1^\circ\text{C}$ by circulating thermostatically controlled water through the outer jacket of the ultrafiltration cell. Fractions of permeated solution were automatically collected at 3 min intervals, and the content of reducing sugars in each fraction was determined by Nelson's (1944) method on appropriate samples. The flow rate of the permeated solution was measured with a capillary-flow meter. After each run, the enzyme composite membrane was washed by ultrafiltering 0.1M-sodium acetate/acetic acid buffer, pH4.65, until no reducing sugar could be detected in the permeated solution. When not in use, the enzyme composite membrane was stored in the assembled cell at $4-5^\circ\text{C}$ to avoid possible microbial contamination.

Results and Discussion

The catalytic activity of the soluble co-reticulated invertase prepared as described above was about 50% of that of the native enzyme. The activity loss may be explained in terms of a decreased number of active sites accessible to substrate, either because their functional groups are directly involved in the covalent binding to glutaraldehyde or because they are situated in inaccessible pockets or segregated regions

of the complex three-dimensional structure of the co-reticulated enzyme.

The immobilization of the co-reticulated invertase on the active surface of the ultrafiltration membrane is an irreversible process. The layer uniformly coating the membrane, visible to the naked eye because of its reddish-brown colour, cannot be removed mechanically without damaging the active surface of the membrane. In addition to this macroscopic evidence, the immobilized enzyme appears to be insoluble, as we found no activity in solution after 10 ml of 0.1M-sodium acetate/acetic acid buffer, pH4.65, had been poured into the depressurized ultrafiltration cell and left overnight at room temperature with magnetic stirring.

The irreversible formation of an enzyme layer tightly bound to the ultrafiltration membrane may be explained by assuming that the soluble bound enzyme, once ultrafiltered to dryness as described in the Materials and Methods section, behaves as a proteic adhesive. Its co-reticulated macromolecules unite to form a sort of intricate network interacting strongly with the supporting membrane, so that when the enzyme layer is placed in aqueous solutions it merely swells up to a definite volume (depending on its degree of reticulation), whereas if the same enzyme

Fig. 1. Kinetic behaviour of co-reticulated invertase immobilized on a DDS 600 ultrafiltration membrane (a) The sucrose concentration (mm) of the solutions ultrafiltered through the immobilized-enzyme system was: Δ , 1.0; $\circ$, 1.5; $\blacksquare$, 2.0; $\square$, 3.0; $\bullet$, 4.0. Other experimental conditions are described in the text. P , Concentration (mm) of the hydrolysis products (expressed as glucose) present in the permeated fractions. (b) Double-reciprocal plot of P_{∞} (the steady-state value of P for each sucrose concentration) against sucrose concentration.

layer had been obtained dynamically, i.e. by ultrafiltration without drying, it would tend to swell indefinitely until complete dissolution.

Fig. 1(a) shows the time course of the sucrose hydrolysis catalysed by the co-reticulated invertase-ultrafiltration-membrane system, for five different substrate concentrations. After about 60 min, the reaction attained a steady state; the concentration of the hydrolysis products in the permeated solution (P) remained constant (P_{∞}). Since the flow rate was practically constant throughout all the runs (4.6 ± 0.1 ml/h), P_{∞} represents the hydrolysis rate of the immobilized-enzyme system. From the double-reciprocal plot of P_{∞} against sucrose concentration (Fig. 1b), an apparent Michaelis constant of 10.5 mM was calculated. This $K_{m_{app}}$ value is in fairly good agreement with the K_m value (16.7 mM) found for the native enzyme under the same conditions of temperature and pH, and with the $K_{m_{app}}$ value (16.2 mM) previously reported for the enzyme immobilized as a dynamically formed gel layer (Drioli *et al.*, 1976).

Although we made no direct measurement of the storage stability of the co-reticulated invertase immobilized on the ultrafiltration membrane, an indirect knowledge of this property can be obtained if we consider that no activity decrease was detected after the 2-week period required for carrying out the set of experiments reported in the present paper. Furthermore the native invertase and that immobilized as a dynamically formed gel layer showed half-

lives of 5.67 and 160 h respectively (Cantarella & Ragosta, 1975).

Apart from any considerations of efficiency, possible practical applications etc., we think that the immobilized-enzyme system described here may represent a valuable tool for investigating the kinetic behaviour of a cofactor-dependent enzyme or an enzyme in the presence of inhibitors or other chemical agents.

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