AN ANALYTICAL FLOW SYSTEM BASED ON REVERSIBLE IMMOBILIZATION OF ENZYMES AND WHOLE CELLS UTILIZING SPECIFIC LECTIN–GLUCOPROTEIN INTERACTIONS

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1. Introduction

The use of immobilized enzymes in enzyme-based analysis offers many advantages. Enzyme electrodes [1,2], continuous spectrophotometer systems based on reactors with immobilized enzymes [3,4] and enthalpimetric methods, e.g., the enzyme thermistor [5,6], are some of the analytical systems used.

A common problem in all analytical work is the tendency for enzyme denaturation leading to a low reproducibility of the system. One way of circumventing this problem has been to add high excess of enzyme so that initially only a small part of the potentially active enzyme molecules are exposed to substrate, whereas the majority are hidden in areas of the support isolated from the bulk solution by diffusional restrictions [7,8]. On denaturation of part of the enzyme molecules, substrate will come in contact with previously latent molecules. This results in a high operational stability of the system. An alternative method of improving the operational stability is to co-immobilize the enzyme with bacteria degrading enzymes, thereby eliminating the denaturation caused by bacterial activity [9].

A further method for improvement of stability was recently presented where a small amount of enzyme was reversibly immobilized utilizing the interaction between antigen and antibody. In the course of 10–15 min the enzyme could be washed off the system and replaced by fresh enzyme [10].

The present paper describes the application of another reversible group-specific immobilization procedure to analytical flow systems, e.g., enzyme thermistors and continuous spectrophotometric devices. The procedure employs immobilized lectins as 'anchoring' units for the enzyme molecules to be used in the analytical system.

2. Materials and methods

Glucose oxidase (EC 1.1.34) from *Aspergillus niger* type V, 1250 U/ml, peroxidase (EC 1.11.1.7) type II from horse radish and catalase (EC 1.11.1.6) type C-30 from beef liver and α-methylglucoside were purchased from Sigma, St. Louis, MO. Dextran T-40, Sepharose CL-4B and Con-A–Sepharose were obtained from Pharmacia Fine Chemicals AB, Uppsala, *Lens culinaris* hemagglutinin type A from Miles-Yeda, Rehovot, and α-methylmannoside from Koch-Light England. All other chemicals used were of analytical grade.

2.1. *Immobilization of lectin*

Sepharose CL-4B, 5 g wet wt, was activated with 250 mg BrCN in 5 ml 1 M Na_2CO_3 for 8 min following conventional procedures [11]. The coupling proceeded in 0.1 M $NaHCO_3$ with 5–6 mg lectin/5 g Sepharose, for 24 h at 4°C.

2.2. *Enzymatic analysis*

2.2.1. Thermistor assay

The lectin–Sepharose was packed in the insulated glass column (total vol. 0.5 ml) of the thermistor unit, with a thermistor immersed in the top of the bed. A continuous flow of buffer with intermittent pulses of

sample (1 min) or washing solution was pumped through the column (flow rate 0.75 ml/min). Heat changes due to enzyme reaction were registered as a change in the resistance of the thermistor, amplified and recorded on a strip chart recorder.

2.2.2. Photometric assays

A small column was packed with the sorption material and connected to a flow cuvette (total vol. 18 μl) in a spectrophotometer (Beckman 24). Catalase activity was registered as a change in A_{240} [12] and glucose oxidase was assayed at 550 nm using co-immobilized peroxidase and in the perfusing sample 14 mM phenol and 0.8 mM 4-aminoantipyrine [13]. One minute samples with a flow rate of 0.75 ml/min were usually applied.

2.3. *Immobilization of the enzyme/cells*

An appropriate amount of enzyme was dissolved in 1 ml buffer and introduced into the flow stream. During passage over the lectin bed, enzyme was bound and could later be used in analysis. To eliminate the risks of enzyme adsorption to the walls of the tubing, a special arrangement with valves close to the reactor bed was used. This furthermore reduced the washing step required after a new enzyme addition. Human red blood cells (RBC) were also immobilized on concanavalin A–Sepharose utilizing an identical procedure.

2.4. *Dissociation procedure*

When the enzyme on the column was to be replaced either by fresh enzyme of the same kind or of another species, a pulse of dissociating solution was introduced. Dissociation was carried out with 0.1–1.0 M α-methylmannoside or α-methylglucoside dissolved in the perfusing buffer. Washing with 0.2 M glycine–HCl, pH 2.2, was also used.

2.5. *Glycosylation of catalase*

The procedure used followed in principle that published [14]. Dextran T-40, 500 mg, was dissolved in 5 ml 0.1 M acetate buffer, pH 5.60. $NaIO_4$, 100 mg, was added and the reaction mixture was kept in darkness at room temperature for 2 h before 500 μl ethyleneglycol was added. After 30 min the pH was adjusted with 2 M Na_2CO_3 to 8.5 and 10 mg catalase was added. Coupling proceeded for 18 h at 4°C. To destroy unreacted groups 1 ml 1 M glycine, pH 6.5, was added.

3. Results and discussion

The capacity of lectins to bind various carbohydrate residues has been recognized for several years [15]. The best-known species of this class of proteins is concanavalin A [16], but several other lectins are now commercially available. As the specificity for carbohydrate differs from one lectin to another, these systems offer group-specific sorbents [15].

In the present study, concanavalin A was mainly used: glucose oxidase and peroxide were found to bind very firmly to this immobilized lectin.

Usually, when enzymes are immobilized and applied in analysis, a change in catalytic properties of the enzyme does not markedly influence the final analysis

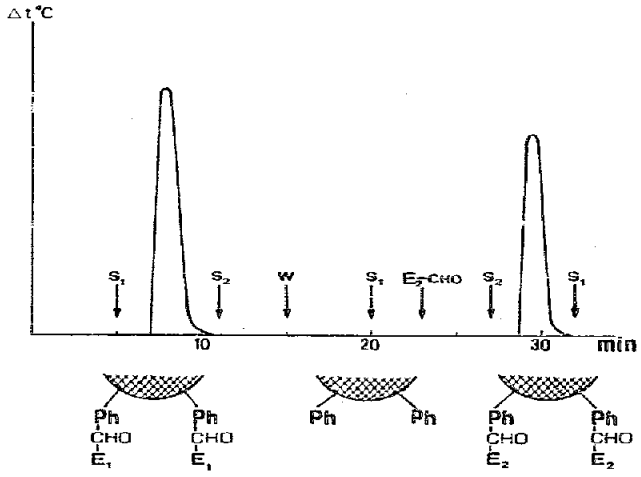

Fig.1. Schematic presentation of an assay cycle. The arrows indicate changes in the perfusion medium, normally 0.1 M Tris–HCl, pH 7.0, with 1 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. Flow rate 0.75 ml/min. The cycle starts with glucoprotein (E_2–CHO) bound to the phytohemagglutinin (Ph)-containing support material. At the arrows marked S_1 and S_2, substrate is introduced for enzymes E_1 and E_2, respectively. The heat signals obtained upon substrate pulses are represented by peaks. At the arrow W a pulse of 0.2 M glycine–HCl, pH 2.2, is introduced in order to split the complex and to wash the system. A new enzyme E_2–CHO is then introduced and substrate S_2 can be assayed.

results, since high excess of enzyme molecules camouflages such a change. However, in low-substituted systems such changes might play an important role. It should in this context be stressed that the immobilization procedure applied here is very gentle and that no covalent bonds are formed.

The dissociation of the enzyme from the lectin support is a crucial point in this analytical system, see fig.1. As judged from the literature, dissociation by pulses of free carbohydrate is in many cases sufficient to break the interaction between the glucoprotein and the immobilized lectin. A more thorough control however reveals that the dissociation step is often very time-consuming and also in most cases does not go to completion. Instead a glycine–HCl wash, known to be efficient in breaking antigen–antibody interactions [17,18], was applied and as can be seen from fig.3, no enzymic activity reamained on the column. It was also shown that the lectin column retained its capacity to bind glucoproteins, and the sorption material could be used over and over again without any marked effect on the analytical results obtained.

Glucose oxidase was used for assay of glucose either with the enzyme thermistor unit or with a spectrophotometric assay using co-immobilized peroxidase (also a glucoprotein) [19]. The results of glucose oxidase and peroxidase assays using the thermistor are shown in fig.2.

The concanavalin-A system was used with preparations of varying degree of substitution. When dealing with the proteins studied here, no difference with respect to the glycine wash used could be detected. When using α-methylmannoside or α-methylglucoside in the perfusing buffer 10% enzyme activity still remained, in the case of highly-substituted preparations.

The results are consistent with literature data concerning binding and elution of cells from lectin–Sepharose of varying degree of substitution [20].

To facilitate the washing step lectin from *Lens culinaris* was used instead of concanavalin A. The

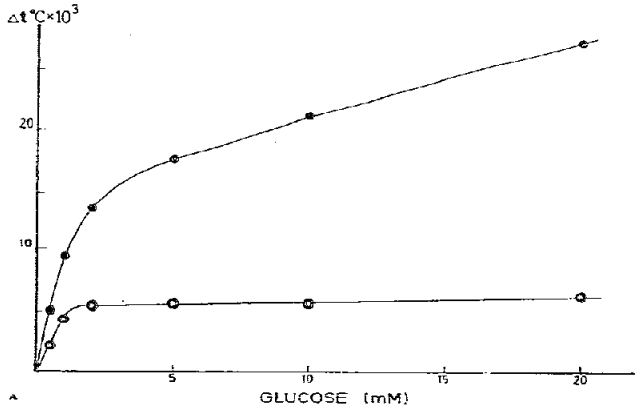

Fig.2. A. Measured peak height (Δt°C) obtained from a glucose oxidase–concanavalin A–Sepharose column (●) or a glucose oxidase–red blood cell–concanavalin A–Sepharose column (•) as a function of the concentration of glucose dissolved in 0.1 M Tris–HCl buffer pH 7.0, being 1 M in NaCl, 1 mM in $MgCl_2$, $MnCl_2$ and $CaCl_2$ (1 min pulse, flow rate 0.75 ml/min).

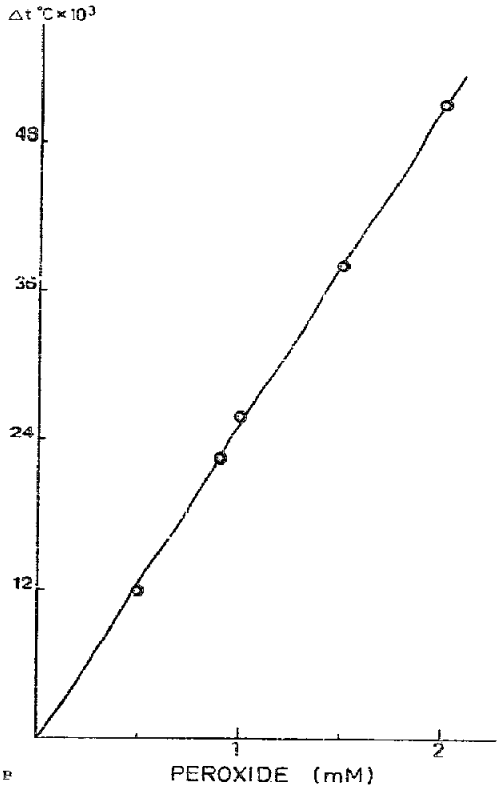

Fig.2. B. Measured peak height (Δt°C) obtained from peroxidase–concanavalin A–Sepharose column as a function of the concentration of hydrogen peroxide dissolved in the same buffer as A, with 0.8 mM 4-amino-antipyrine and 14 mM phenol.

specificity is reported to be the same but the affinity between glucoprotein and the lectin is 50-times lower [21].

In studies with peroxidase it turned out that the enzyme was bound to the sorption material in high yields, but during the subsequent analysis a constant leakage took place. Under the experimental conditions used, this lectin is not useful.

The concanavalin A system was tested with various enzymes, glucoproteins as well as pure proteins, and it was found that the binding to the column only took place with the glucoproteins whereas pure proteins were washed through the system. This was also so with native catalase, but after the enzyme had been modified, by attaching carbohydrate chains to the protein backbone, binding did take place. It was also shown that elution processes as described for native glucoproteins were also applicable to artificially prepared glucoproteins (fig.3).

When red blood cells (RBC) and glucose oxidase were co-immobilized, an increase in heat response was obtained, as well as in the concentration region giving linear relationships between concentration and heat signal.

This fact was interpreted in terms of a better oxygen supply from oxygenated hemoglobin in the red blood cells.

Any heat derived from the metabolism in the immobilized RBC was avoided by blocking the glycolytic pathway by addition of 10 mM fluoride to buffer and substrate solutions.

Immobilized whole cells, earlier applied in large scale processes [22], may also be used in analytical systems [23]. The potential of the reversible immobilization approach applied in analysis is very promising, especially when dealing with labile species, e.g., whole cells or membrane-bound enzymes.

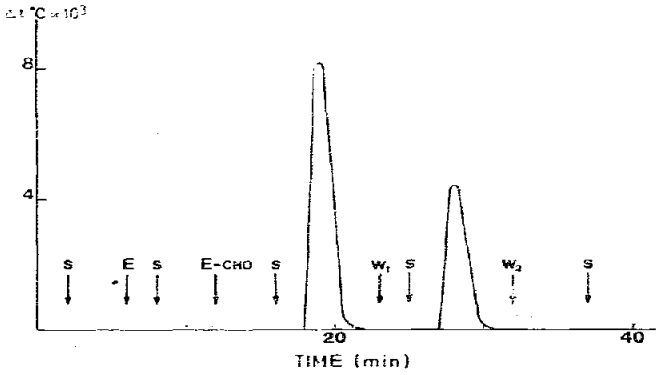

Fig.3. Schematic presentation of an assay and washing cycle for catalase and glucosylated catalase. The arrows indicate changes in the perfusion medium, normally 0.1 M Tris–HCl, pH 7.0, with 1 M NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ (flow rate, 0.75 ml/min). At the arrows marked 'S' 2 mM hydrogen peroxide is introduced as a 1 min pulse, at the arrows 'E' and 'E–CHO', catalase and glycosylated catalase are introduced, respectively. W_1 indicates a pulse of 0.5 M α-methylmannoside in the perfusing buffer whereas 'W_2' stands for a 5 min wash with 0.2 M glycine–HCl, pH 2.2.

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