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Use of compact, porous units with immobilized ligands with high molecular masses in affinity chromatography and enzymatic conversion of substrates with high and low molecular masses[1]

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Abstract

Different ligands with high molecular masses are immobilized on compact, porous separation units and used for affinity chromatography. In subsequent experiments different enzymes are immobilized and used for converting substrates with low and high molecular masses. Disk or tube with immobilized concanavalin A (ConA) are used as model systems for lectin affinity chromatography. The enzyme glucose oxidase is used as a standard protein to test the ConA units. Subsequently glycoproteins from plasma membranes of rat liver are separated, using units with immobilized ConA. The enzyme dipeptidyl peptidase IV, which is used as a model protein in the experiments, is enriched about 40-fold in a single step, with a yield of over 90%. The results are only slightly better than those obtained with ConA when it is immobilized on bulk supports. The important improvement lies in the reduction of separation time to only 1 h. Experiments concerning the isolation of monoclonal antibodies against clotting factor VIII (FVIII) are carried out on disks, combining anion-exchange chromatography and protein A affinity chromatography as a model for multidimensional chromatography. Both IgG (bound to the protein A disk) and accompanying proteins (bound to the anion-exchange disk) from mouse ascites fluid are retarded and eluted separately. With the immobilized enzymes invertase and glucose oxidase (GOX) the corresponding substrates with low molecular masses, saccharose and glucose, are converted. It is shown that the amount of immobilized enzyme and the concentration of the substrate are responsible for the extent of the conversion, whereas the flow-rates used in the experiments have no effect at all. The influence of immobilization chemistry was investigated with GOX. Indirect immobilization with ConA as spacer proved to be the best alternative. With trypsin, immobilized on a disk, substrates with high molecular masses are digested in flow-through. For optimal digestion the proteins have to be denatured in the buffer for sodium dodecyl sulfate–polyacrlyamide gel electrophoresis prior to application. In contrast to the conversion of substrates with low molecular masses, flow-rates play an important part in conversion of substrates with high molecular masses. With lower flow-rates a higher degree of digestion is achieved.

Keywords: Affinity adsorbents; Disks; Immobilized proteins; Enzymes; Immunoglobulins; Carbohydrates; Proteins

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1. Introduction

The use of membranes and compact, porous units such as disks and monoliths has a 10-year-long history [1–4]. The method has been increasingly well established, especially on an analytical scale for fast analysis of proteins and other biopolymers with high molecular masses [5–8]. However, scale-up and preparative use of such units has proved to be more complicated than expected. The problems that presented themselves were, above all, sample distribution and therefore the construction of the separation unit, but also the question of capacity [9,10]. Recently these problems have come closer to a solution, and membranes as well as compact, porous tubes and monoliths have been used for preparative isolation of proteins from complex mixtures [9–12]. The most common uses of preparative separation units are ion-exchange chromatography [9–11] and affinity chromatography with immobilized ligands with low molecular masses [2,12,13].

While the use of affinity chromatography with immobilized ligands with low molecular masses became quite widespread from the very beginning [12–16], the same mode with immobilized ligands with high molecular masses on membranes and other compact, porous supports is hardly used at all. This is surprising considering that one of the first applications of membranes in chromatography was carried out in affinity mode, with immobilized ligands with high molecular masses [1,2]. The affinity chromatographic methods described so far involve immobilized protein A and protein G [1,17–19], antibodies [19], receptors [20] and other immobilized proteins with different biological functions [2,6,21].

The immobilization of enzymes to compact, porous supports and the use of such devices as reactors for the conversion of substrates with either low or high molecular masses is regarded as a method of its own [22,23]. Preliminary experiments have shown that such enzyme reactors have much better rates of conversion than the reactors, in which enzymes have been immobilized to bulk, porous supports [22]. Despite its potential importance, both in theoretical and practical terms, very little has so far been done to prove it.

This report deals with further applications of compact, porous separation devices with immobilized ligands with high molecular masses, concerning both affinity chromatography and enzymatic conversion of different substrates.

2. Experimental

2.1. Instrumentation

The HPLC system consisted of two pumps, a programmer, a spectral photometer with a deuterium lamp and a loop injection valve (all from Knauer Gerätebau, Berlin, Germany) and a fraction collector (Bio-Rad, Munich, Germany). In the case of ion-exchange chromatography the salt gradient was controlled by measuring the osmotic pressure (Halbmikro-Osmometer, Typ Dig. L., Knauer Gerätebau). For semi-preparative chromatography a BioPilot system (Pharmacia, Vienna, Austria) was used.

2.2. Chemicals

Chemicals of analytical-reagent grade were purchased from Merck (Darmstadt, Germany), Serva (Heidelberg, Germany) or Sigma (Munich, Germany).

2.3. Plasma membranes and monoclonal antibodies

Rat liver plasma membranes were isolated by zonal centrifugation using a Kontron centrifuge (Kontron Analytik, Munich, Germany) as described elsewhere [24]. Membrane purity was routinely checked by electron microscopy and by assaying of marker enzymes as described by Tauber and Reutter [24]. Monoclonal antibodies against human coagulation factor VIII were produced using the method of Koehler and Milstein [25]. Protein content of the samples was determined according to the procedure of Lowry et al. [26].

2.4. Preparation of separation units

2.4.1. Units for ion-exchange and affinity chromatography

The poly(glycidyl–methacrylate) disks and tubes were a product from BIA d.o.o. (Ljubljana,

Slovenia). The thickness of the layers was between 2 and 5 mm, the diameter was 10 mm or 25 mm respectively. The tubes were 50 mm long with a bed volume of 8.8 ml. The preparation of separation units used for ion-exchange chromatography has been described elsewhere [4,27]. For affinity chromatography the ligand was positioned on the disk or compact, porous tube in epoxy form in situ. The following protocol was used: after installing the 25-mm disk in the appropriate cartridge, any remaining nonpolymerized components were washed out with 20 ml of methanol. The disk was then rinsed with 40 ml of bidistilled water and 40 ml of 0.1 *M* sodium boronate buffer (pH 8.0). The ligand (in this case either protein A or concanavalin A (ConA) or the enzymes glucose oxidase (GOX), trypsin or invertase), dissolved in boronate buffer, was added in quantities of about 5–40 mg/g of support. The protein concentration of the ligand solution was 0.5–2 mg/ml, the sample volume between 5 and 50 ml. The ligand solution was pumped through the disk at a flow-rate of 2 ml/min and then left to circulate on the disk for at least 2 h. Ligand immobilization was monitored by a corresponding in-process system, as described elsewhere [9,10]. Subsequently the disk was rinsed with 50 ml of boronate buffer. Any remaining free epoxy groups were blocked with 20 ml of 0.2 *M* Tris–HCl buffer (pH 8.0). The disk was then rinsed with boronate buffer or Tris-buffered saline (TBS) (pH 7.4), and stored at 4°C for further usage. In the case of the 10-mm disk, the procedure was linearly down-scaled respectively. For tubes linear upscaling was carried out.

2.4.2. Immobilization of glucose oxidase

The flow injection analysis (FIA) glucose analyser (ASIA, Ismatec, Zurich, Switzerland) is based on the enzymatic oxidation of β-D-glucose to D-gluconolactone by immobilized enzyme GOX [28]. The cosubstrate oxygen is simultaneously converted by hydrogen peroxide. In the next step, hydrogen peroxide reacts with 1-phenyl-2,3-dimethyl-4-amino-5-pyrazolone and phenol (both from Kemika, Zagreb, Croatia). The second reaction is catalyzed by enzyme peroxidase (horseradish peroxidase, HRP, Sigma). The reaction scheme is:

$$\beta\text{-D-glucose} + O_2(\text{GOX}) \Rightarrow \text{gluconicacid} + H_2O_2$$

$$H_2O_2 + \text{pyrazolone} + \text{phenol(HRP)}$$

$$\Rightarrow \text{chromogenic product detection at 505 nm}$$

According to the first protocol, GOX is immobilized on the epoxy-activated disk as described above. According to the next protocol, a spacer built of ethylenediamine and glutaraldehyde was first introduced. For these purposes, the original epoxy groups were treated with 100% ethylenediamine for 24 h at room temperature. Afterwards, the amino groups were modified with 10% (v/v) glutaraldehyde in water for 24 h at room temperature. According to the alternative protocol, GOX is bound to the disk, which already contains immobilized ConA.

Briefly, in the first step 10 ml of a solution of 5 mg of ConA/ml in 0.1 *M* Na-acetate buffer (pH 5.0) with 5% (w/v) mannose, 1 m*M* Ca^{2+} and 1 m*M* Mn^{2+}, is pumped over an epoxy-activated disk (10-mm diameter and 3-mm layer thickness) for 24 h. The flow-rate is 0.5 ml/min. The free epoxy groups are blocked with 20 ml of glycine in 0.1 *M* Na-acetate buffer (pH 5.0). Reaction time in this case is 2 h, flow-rate 0.5 ml/min. To the disk with immobilized ConA, 20 ml of solution with 5 mg/ml of GOX in 0.1 *M* Na-acetate buffer (pH 5.0) with 1 m*M* Ca^{2+} and 1 m*M* Mn^{2+}, are subsequently applied. Binding time for GOX is 24 h, the flow-rate is 0.5 ml/min. The amount of immobilized enzyme is determined in-process, using a QA-anion-exchange compact porous disk, as described in [7].

2.5. Buffers

The buffers used for anion-exchange chromatography were the following: buffer A was 10 m*M* Tris–HCl (pH 7.8); buffer B was Buffer A containing 1 *M* sodium chloride.

For isolation of antibodies buffer A (application buffer) was 50 m*M* sodium phosphate (pH 8.0) with 0.15–0.20 *M* sodium chloride. The bound antibodies were eluted with 0.1 *M* sodium citrate (pH 2.4) (buffer B).

For ConA affinity chromatography a buffer was used, which contained 10 m*M* Tris–HCl (pH 8.0) with 0.155 *M* sodium chloride, 1 m*M* calcium and magnesium each, 0.1% (v/v) Triton X-100, reduced,

and 0.01% (w/v) sodium azide (buffer A). Elution buffer for ConA chromatography was buffer A with 0.2 *M* (α-D-methyl-mannopyranoside).

2.6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

The dialysed and freeze-dried samples were dissolved in 62.5 m*M* Tris–HCl buffer (pH 6.8) containing 3% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol and 0.001% (w/v) bromophenol blue. In other experiments, 10–30 μl of sample were taken from the collected fractions after chromatographic separation and mixed with buffer containing five times higher concentrations of the above-mentioned substances. The amount of buffer taken for the experiments was measured in such a way as to yield the original concentration after dilution by the sample. SDS–PAGE was carried out by the Laemmli method [29], using a mini system (Protean, Bio-Rad, Munich, Germany). The amount of applied protein was between 5 and 20 μg per line.

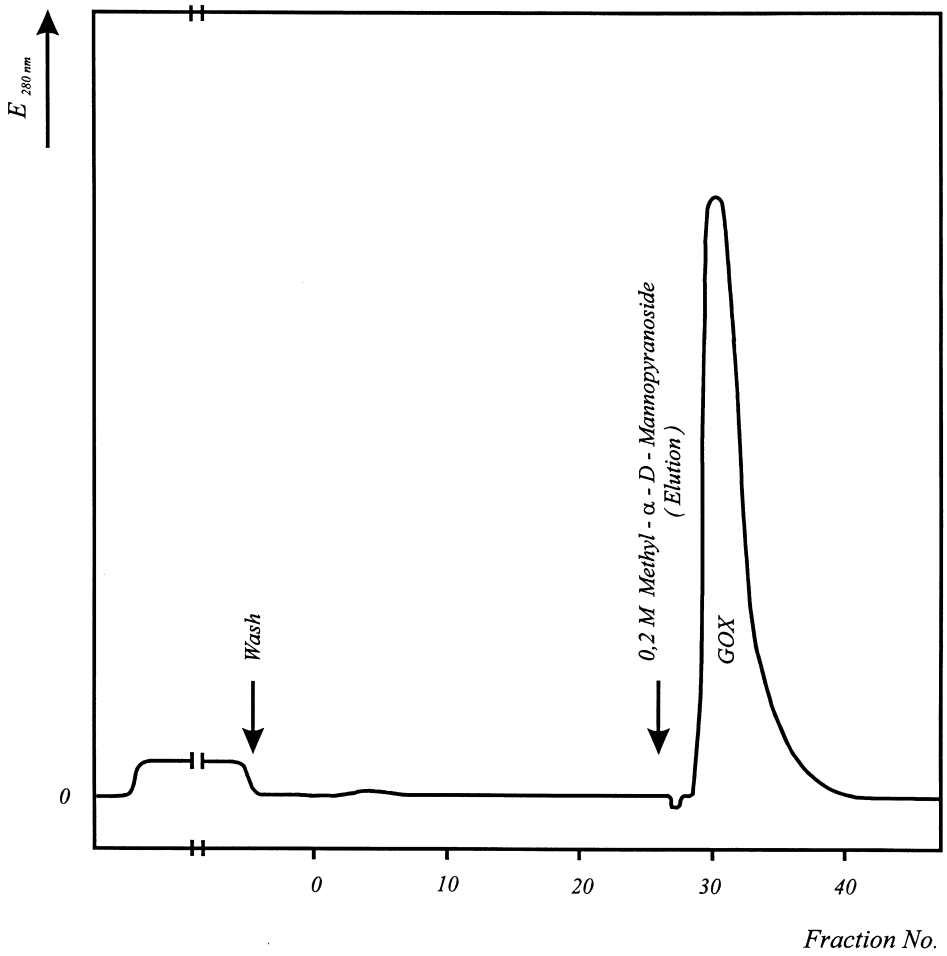

Fig. 1. Affinity chromatography with a compact, porous unit with the immobilized lectin concanavalin A (ConA). Test chromatogram with the enzyme glucose oxidase (GOX). A 10-ml volume of GOX solution (0.1 mg/ml) was applied to a tube (8.8-ml bed volume). The tube was subsequently rinsed with 1% Triton X-100 in Tris-buffered saline (TBS). The bound enzyme can be eluted with 0.2 *M* methyl-α-D-mannopyranoside. For conditions see Section 2.

2.7. Determination of enzymatic activity

Enzyme units are given as micromoles per minute. Dipeptidyl peptidase IV (DPP IV) activity was determined according to the method of Nagatsu et al. [30], using tosyl-glycine-proline-*p*-nitroanilide (Bachem, Bubendorf, Switzerland) as substrate. Between 10 and 100 μl of sample was added to 90 and 180 μl of 0.1 *M* Tris–HCl buffer (pH 8.0) and 10 μl of substrate, and incubated at 37°C for 30 min. The reaction was stopped by adding 800 μl of 1 *M* sodium acetate (pH 4.5) and the optical density was measured at 405 nm, using an Eppendorf photometer (Eppendorf, Hamburg, Germany).

The enzymatic conversion of saccharose with invertase immobilized on a disk was carried out in a 0.1 *M* sodium phosphate buffer (pH 4.6), according to the following protocol: A 5% (w/v) or 10% (w/v) saccharose solution was applied to a disk (diameter 25 mm, layer thickness 4 mm) with immobilized invertase. The flow-rate at conversion was between 0.5 and 3 ml/min. The concentrations of saccharose,

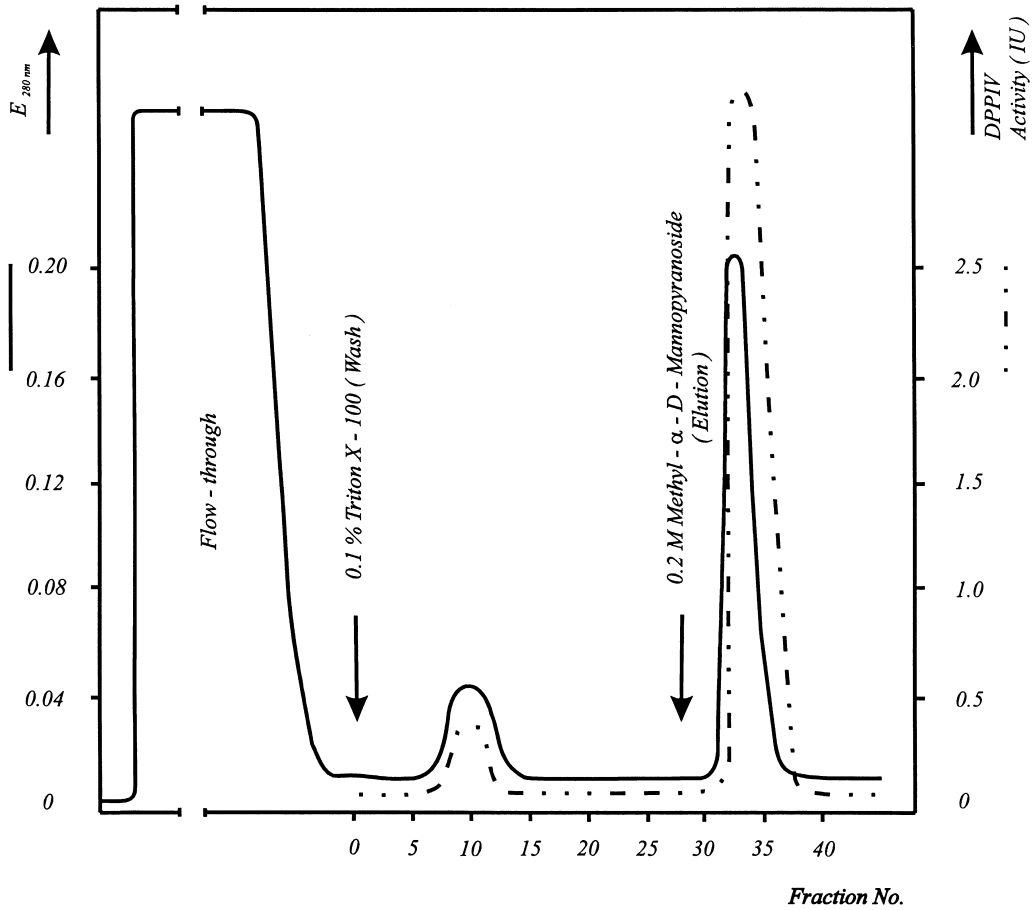

Fig. 2. Affinity chromatography with a compact, porous unit with immobilized ConA. A 10-ml volume of membrane protein solution from rat liver was applied to a tube (8.8-ml bed volume) with immobilized ConA. The protein concentration in the sample was 1.1 mg/ml, the activity of the enzyme dipeptidyl peptidase IV (DPP IV) was 0.68 U/ml (specific activity is 0.62 U/mg of protein). The unit was then rinsed with 1% Triton X-100 in TBS. The bound proteins were eluted with 0.2 *M* methyl-α-D-mannopyranoside. Protein concentration and DPP IV activity were measured in each fraction. Chromatographic conditions; flow-rate 2 ml/min during application and rinsing of sample at a back pressure of 0.2 MPa and 1 ml/min during elution (back pressure 0.1 MPa), room temperature. For other conditions see Section 2. (Fig. 2 continued on next page.)

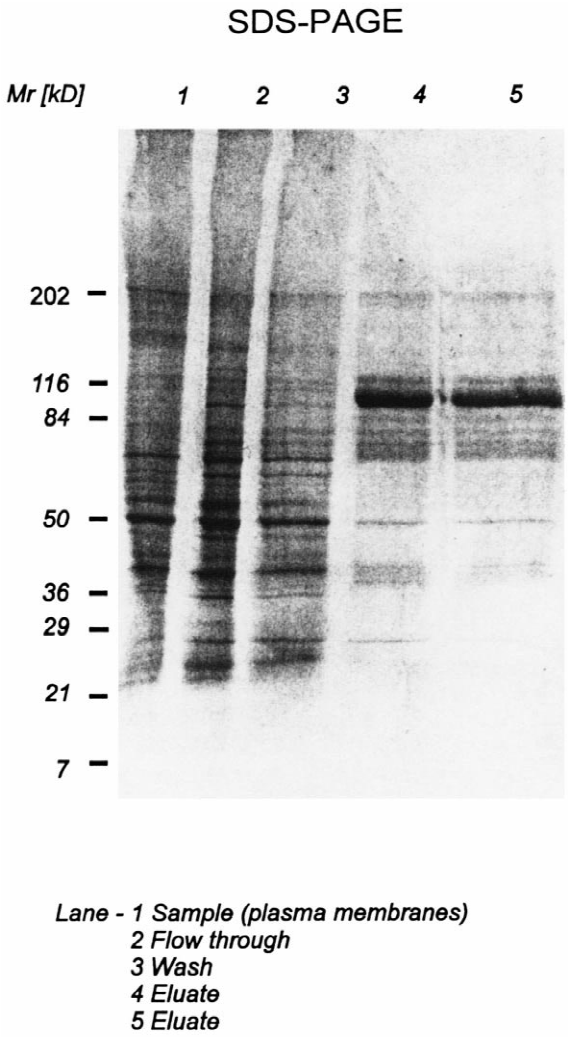

Fig. 2. (*continued*)

glucose and fructose were measured with a Dionex HPLC system.

For digestion with immobilized trypsin, the proteins transferrin, bovine serum albumin and ovalbumin with a concentration of 1 mg/ml were solved in the sample buffer for SDS–PAGE and incubated at 95°C for 1 min. The protein solution was pumped through a disk (diameter 10 mm, layer thickness 3 mm), at flow-rates of 0.1 ml/min and 0.5 ml/min. Its enzymatic conversion was documented by SDS–PAGE.

3. Results and discussion

3.1. Affinity chromatography

Immobilization of ConA to the epoxy-activated tube was carried out with a maximum quantity of 30 mg/g of support. As is seen in Fig. 1, the tube bound GOX. This glycoprotein was chosen as a model substance in order to test the functioning of the unit with immobilized ConA.

A number of membrane glycoproteins from rat liver, among them the enzyme dipeptidyl peptidase IV (DPP IV) and several adhesion proteins such as cell-CAM bind to the column and can then be enriched in the eluate [31,32]. Here DPP IV was measured as model protein. The results of the measurements are shown in Fig. 2 and Table 1. This glycoprotein binds fully to the column and is satisfactorily enriched in the eluate. Specific activity in the eluate was about 21.3 U/mg and therefore slightly higher than the 20 U/mg obtained with ConA–Toyopearl or ConA–Sepharose [31,32]. Enrichment was also slightly higher in the case of the disk, 38 times compared with 32 times. An important advantage of chromatography with the ConA disk was the speed of separation, which was achieved in less than 1 h. Recovery of enzymatic activity of DPP IV is about 91%, cf. Table 1.

Table 1
ConA affinity chromatography of DPPIV from solubilised rat liver plasma membranes

Sample	Total protein (mg)	DPPIV			
		Activity (I.U.)	Specific activity (U/mg)	Yield (%)	Enrichment
Solubilized plasma membranes	12.2	6.8	0.56	100	–
Eluate	0.29	6.2	21.3	91	38×

3.2. Two-dimensional chromatography

Because of their low back pressure and the option to carry out fast separations, membrane-based and disk-based systems are particularly useful for so-called multidimensional chromatography. Such a separation system combines several units with different ligands in a tandem operation. This allows separation by several interactions of the sample component in the system. So far ion-exchange membranes and membranes with immobilized dye–ligand in a sequence [12] or as a mix, packed on top of one another in a holder [13], have been used for isolating proteins. With another application, monospecific polyclonal antibodies against the calcium-binding protein annexin VI were isolated [19]. The antibodies which crossreact with other, similar proteins, were removed from the antiserum by passing through disks with the corresponding immobilized antigens (annexins with low molecular masses). Fig. 3 shows another example for the successful use of such techniques. Monoclonal antibodies from ascites fluid were purified in a tandem consisting of a QA disk and a disk with immobilized protein A. Contaminations from the ascites fluid, above all mouse serum albumin, bind to the first disk with its anion-exchange ligand, whereas the monoclonal antibodies flow through the first disk and bind only to the second with its immobilized protein A. Elution of the bound proteins from the anion-exchange disk is carried out with a salt gradient. The monoclonal antibody is eluted from the protein A disk using a buffer with low pH. An amount of 2.4 mg of IgG was isolated from 1 ml of ascites fluid. In SDS–PAGE no discernible impurities were found (not shown here). During sample application as well as

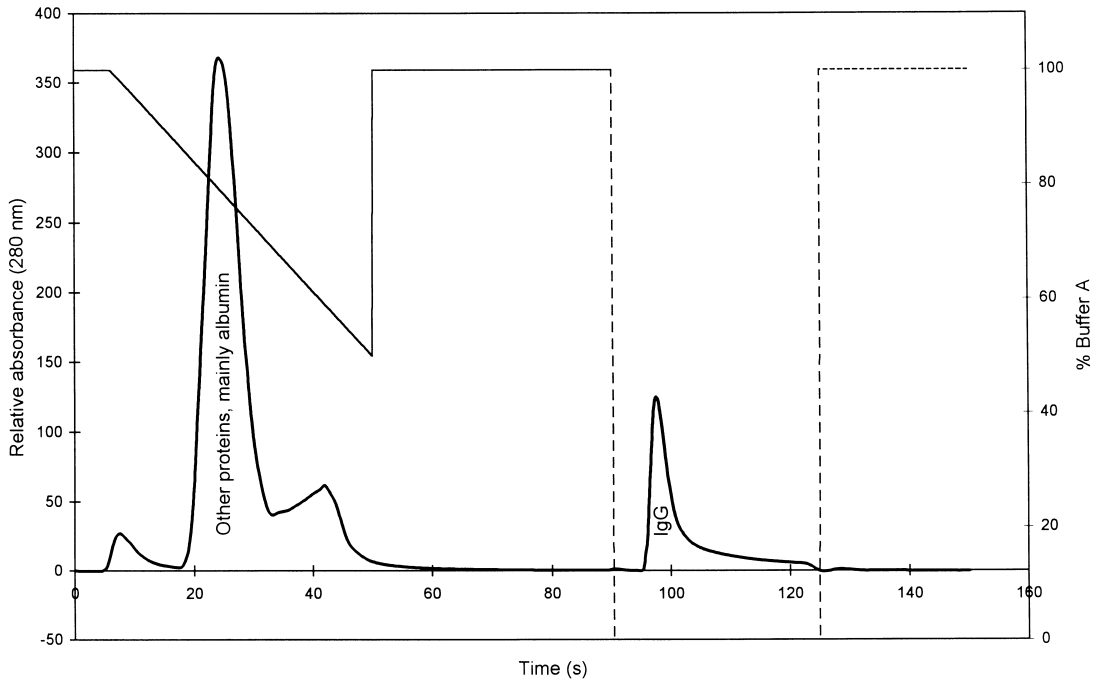

Fig. 3. Separation of IgG from other proteins in mouse ascites fluid with two-dimensional chromatography, consisting of anion-exchange chromatography and protein A affinity chromatography. A 1-ml volume of ascites fluid with anti-clotting factor VIII (FVIII) monoclonal antibody no. 69.31 was applied to a tandem of a QA and a protein A disk. A large part of the proteins binds to the QA disk, except the IgG, which is subsequently captured by the protein A disk. The accompanying proteins were eluted from the QA disk with a salt gradient. Then the IgG was eluted from the protein A disk (retention time, 100 s). Chromatographic conditions: flow-rate 2 ml/min, pressure 0.3–0.5 MPa, room temperature. The gradient is shown in the figure. For other conditions see Section 2.

during elution with the buffers, both separation units were in the system. It is theoretically possible to switch either unit out of the system. But in this case it was not necessary, since the second unit with immobilized protein A is not sensitive to the salt concentrations required for elution from the first unit.

3.3. Conversion with immobilized enzymes of substrates with low or high molecular masses

Fig. 4 shows hydrolysis of sucrose to glucose and fructose by means of immobilized invertase. A 5% (w/v) sucrose solution was fully converted in a disk with 25-mm diameter and 4-mm layer thickness with immobilized invertase in flow through. Complete conversion was achieved in the experiment at flow-rates of 0.5, 1.0, 2.0 and 3.0 ml/min. Higher flow-rates did not impair the performance of the unit. However, when a 10% saccharose solution was used, no complete conversion of the substrate was achieved by a single flow through the invertase disk (cf. Fig. 4C). In this case the substrate solution had to be pumped several times through the enzyme reactor.

The disk with immobilized trypsin can be used for conversion of substrates with both high and low molecular masses [7,23]. In the case of substrates with low molecular masses the flow-rate is no limiting factor (cf. Ref. [7,22,23] and Fig. 3). However, the extent of conversion depends on the flow-rate in the case of substrates with high molecular masses. As can be seen in Fig. 5, the proteolytic digestion is more effective with lower flow-rates. In order to make the corresponding peptide bonds in the protein accessible to the immobilized enzyme, it had to be denatured. This was achieved by adding the reducing buffer for SDS–PAGE and heating up the sample before digestion.

Under these conditions the digestion experiments were highly reproducible. The disks with immobilized proteases can therefore be used for the conversion of investigated proteins and subsequent peptide-mapping.

3.4. Determination of glucose with immobilized glucose oxidase

Analysis of glucose concentration in fermentation

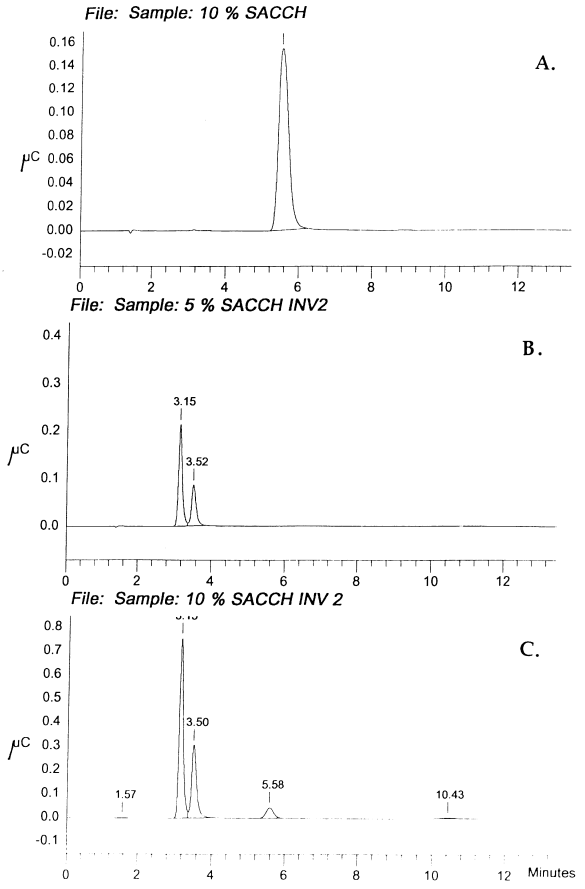

Fig. 4. Enzymatic conversion of saccharose with a disk with immobilized invertase. (A) Sample before enzymatic conversion with 10% (w/v) saccharose; (B) enzymatic conversion of a 5% (w/v) saccharose solution. Conditions: 2 ml of saccharose solution were pumped through a disk with immobilized invertase (25-mm diameter, 4-mm layer thickness), flow-rate, 2 ml/min, pressure 0.1 MPa, room temperature. Subsequently a random sample was taken and analysed. First peak = fructose, retention time = 3.15 min; second peak = glucose, retention time = 3.52 min; saccharose could not be detected. (C) Enzymatic conversion of a 10% (w/v) saccharose solution, conditions as in (B). In this case nonconverted saccharose was detected (third peak, retention time = 5.58 min). For other conditions see Section 2.

broths with an FIA analyser with immobilized GOX [28] was carried out in order to test the application of a disk with immobilized enzyme for such in-process measurements, as compared to other tools. The results of such a comparison are shown in Fig. 6. If the enzyme is immobilized directly at the epoxy groups of the support (curve 4 in Fig. 6), the

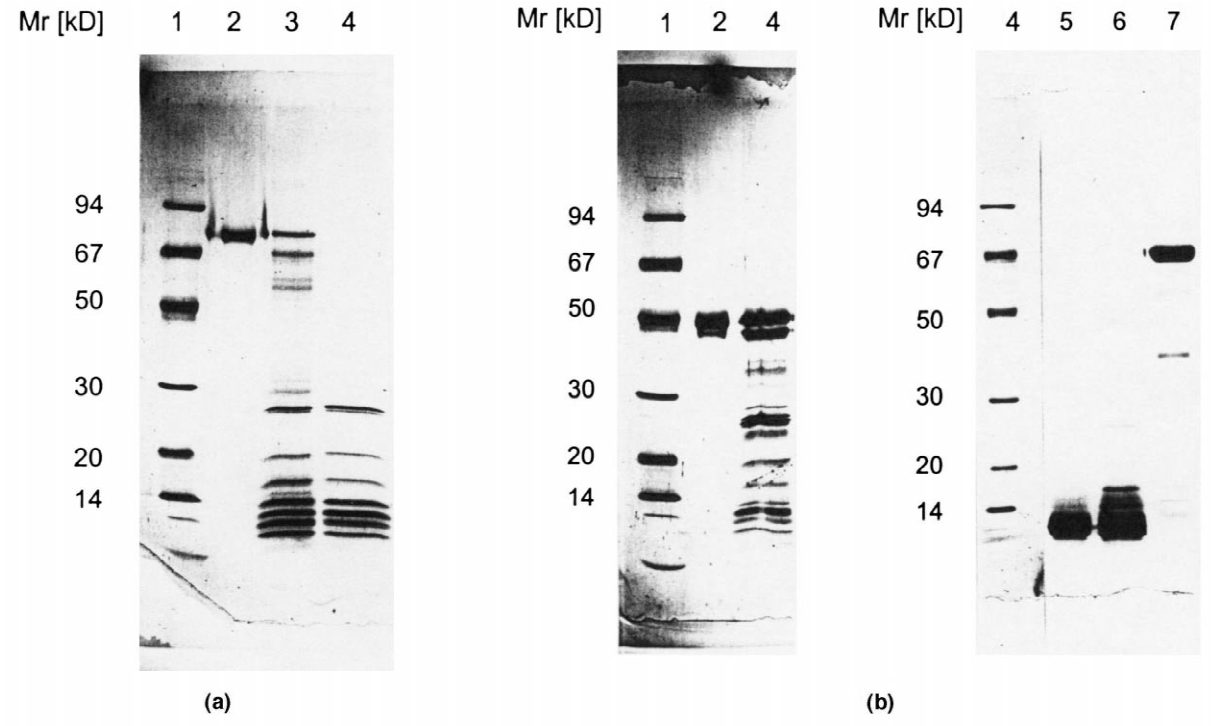

Fig. 5. Enzymatic conversion of substrates with high molecular masses on a disk with immobilized trypsin. (a) Conversion of transferrin. Lane 1=calibration proteins; lane 2=sample before enzymatic conversion; lane 3=sample (5 ml, conc. 1 mg/ml), boiled in the sample buffer for SDS–PAGE under denaturing conditions and pumped through the disk (trypsin) at a flow-rate of 0.5 ml/min (pressure 0.1 MPa); lane 4=the same sample as in lane 3, but pumped through the disk (trypsin) at a flow-rate of 0.1 ml/min (no back pressure). (b) Conversion of ovalbumin and bovine serum albumin under different conditions. Lane 1=calibration proteins; lane 2=ovalbumin, sample before enzymatic conversion; lane 3=ovalbumin, sample after enzymatic conversion; the sample with nondenatured ovalbumin (5 ml, conc. 1 mg/ml) was pumped through the disk (trypsin) at a flow-rate of 0.1 ml/min; lane 4=calibration proteins; lane 5=BSA sample after enzymatic conversion, conditions flow-rate through the reactor was 0.1 ml/min; lane 6=BSA sample after enzymatic conversion; before application to a disk (trypsin) it was boiled in the sample buffer under denaturing conditions; flow-rate through the reactor, 0.5 ml/min; lane 7=BSA, sample was applied after enzymatic conversion, nondenatured; for other conditions see lane 3 and Section 2.

enzymatic conversion of glucose is rather low. The corresponding detector response is about 55–60% of that of the commercially available detector. The response is improved, if an appropriate spacer is used. By adding ethylenediamine and glutaraldehyde (EDA–GA) as spacer between GOX and the surface of the support, the conversion is made more effective, but it is still unsatisfactory (curve 3 in Fig. 6). If the enzyme is immobilized at its oligosaccharide part to the previously immobilized lectin ConA, the necessary space between it and the support is established. As the active center is found in the protein part of the molecule, it becomes by this way of immobilization fully accessible to the substrate. The optimized immobilization yielded a detector response which was comparable to that of the commercially available detector (curves 2 and 1 in Fig. 6).

Acknowledgements

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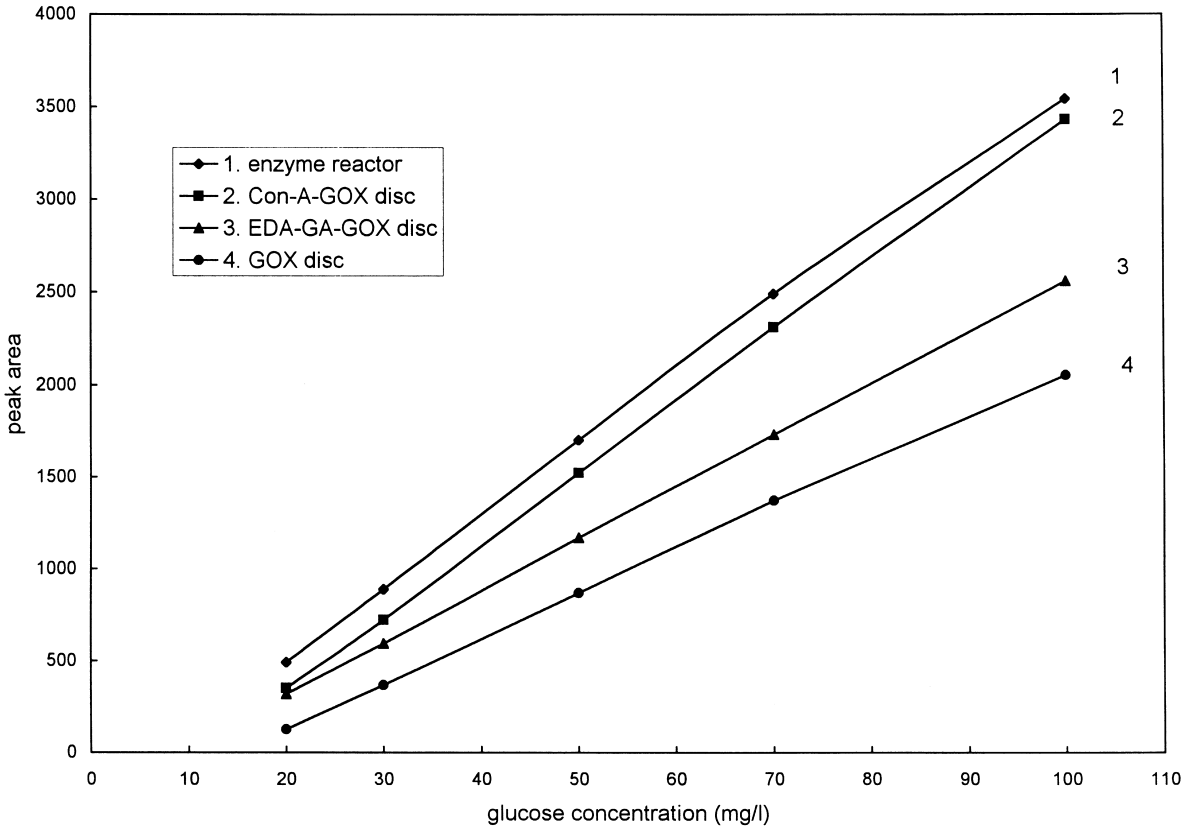

Fig. 6. Calibration curves for glucose standard solution for different disks and the commercially available enzyme reactor. Curve 4=GOX, immobilized on an epoxy-activated disk; curve 3=GOX, immobilized on a disk with ethylendiamine–glutaraldehyde as spacer; curve 2=GOX, immobilized on a disk with ConA; curve 1=commercial enzyme reactor.

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