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A COMBINED RECYCLING AFFINITY COLUMN–FILTRATION TECHNIQUE

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SUMMARY

A new preparative purification technique for biological macromolecules is developed by combining the affinity column chromatography and the membrane filtration technique. A unique feature of the affinity column–filtration apparatus is that the filtrate from the filtration unit is recycled back to the inlet of the affinity column, thus decreasing considerably the total amount of ligands required for the affinity column. A second important feature is that the pump which connects the affinity column and the filtration unit is monitored by an infrared sensor device on the filtration cell; consequently, the flow of the column eluent into the filtration unit is controlled automatically by the solution height in the filtration cell. Highly homogeneous regulatory subunits of type I cAMP-dependent protein kinase from rabbit skeletal muscle are demonstrated to be obtainable by the new purification technique. Thus, this apparatus could have important applications in the purification of a wide range of biological macromolecules. A test for estimating affinity bound proteins is also discussed.

INTRODUCTION

Affinity chromatography involves the covalent attachment of a ligand onto an insoluble polymer (*e.g.* agarose) which can be packed as the stationary support into a chromatographic column. Because of the specific binding that occurs between the covalently attached ligand and a particular enzyme, the affinity column can absorb selectively the particular enzyme from a mixture of proteins passed through it. Subsequent elution of the adsorbed enzyme can be achieved by eluting the affinity column with buffer solutions of high ionic strength or denaturants which can perturb the affinity of the enzyme for the covalently attached ligand resulting in dissociation of the enzyme–ligand complex. Alternatively, elution of the tightly adsorbed enzyme from insoluble supports can be achieved by using a solution containing a high concentration either of the ligand or a ligand analogue which can compete with the covalently attached ligand for the enzyme.

The importance of using a ligand analogue in affinity chromatography is most

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evident in cases where the binding affinity of protein to ligands is particularly strong. For example, the affinity of the regulatory subunit of cAMP-dependent protein kinase for cAMP is extremely high[1], and the ligand analogue, 1,N^6-ethenoadenosine 3′,5′-cyclic monophosphate, has been successfully employed for eluting the regulatory subunit from an affinity column packed with cAMP-Sepharose[2]. A drawback of this method is that, in order to wash off the adsorbed enzymes for a reasonably good yield, a large volume of the ligand analogue containing eluant is sometimes needed. This can be particularly painful, if the ligand analogue has to be synthesized chemically or if it is very expensive. In this communication, a method is described to circumvent this need of a large amount of ligand analogue by recycling the eluant in a closed system.

EXPERIMENTAL PROCEDURES

Materials

Epoxy-activated Sepharose 6B was obtained from Pharmacia. ACA-44 ultragel was supplied by LKB. Phenylmethylsulfonylfluoride (PMSF), cAMP and its analogue, 1,N^6-etheno-cAMP, were products of Sigma. Other chemicals and solvents were of reagent grade. Filtration cell (Model 10) and microporous filters (YM-10) were purchased from Amicon.

Methods

8-Bromo-cAMP and 8-(β-hydroxyethylthio)-cAMP were prepared following the procedure of Muneyama *et al.*[3], and 8-(β-hydroxyethylthio)-cAMP was subsequently used together with epoxy-activated Sepharose 6B to synthesize the cAMP affinity matrix[4].

The cAMP-dependent protein kinase I from rabbit skeletal muscle was purified to the stage of DEAE-cellulose chromatography using the procedure of Huang and Huang[5].

The affinity column–filtration apparatus

A new affinity column filtration apparatus, shown diagrammatically in Fig. 1, was constructed for the continuous depletion of adsorbed enzymes, the regulatory subunit of cAMP-dependent protein kinase I in this case, from the cAMP affinity matrix by the recycled ligand analogue, 1,N^6-etheno-cAMP, containing eluent. The apparatus consisted of an affinity column packed with cAMP-affinity matrix. A pump connects the column outlet to the filtration unit, and the on/off switch of the pump is controlled automatically through an amplifier by an infrared sensor attached to the filtration unit, shown in detail in Fig. 1. The column eluant is pumped into the filtration unit and it is stirred constantly by a magnetic bar. The solvent and small molecules including the free ligand analogue in the filtration unit are forced to pass through the filter membrane by a constant pressure (*ca.* 35 p.s.i.) provided by a nitrogen tank equipped with a regulator valve; hence, the regulatory subunit-cAMP analogue complex was separated and concentrated in the filtration unit. The solution in the filtration cell is monitored by an infrared sensor which, in turn, regulates the on/off switch at the pump connecting the column and the filtration unit. When the solution is lowered to below the level where the infrared sensor is positioned, the

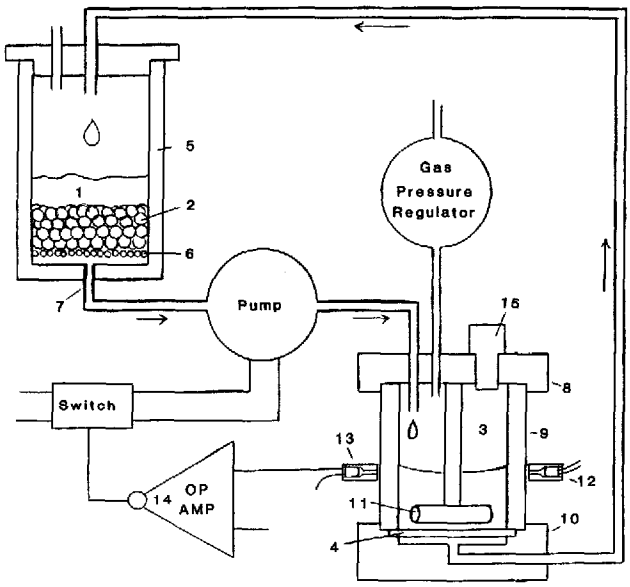

Fig. 1. Affinity column–filtration apparatus. The solvent (1) passes through a bed of affinity beads (2) complexed with the macromolecules for which they are specific. The solvent is pumped into a pressurized chamber (3) where the solvent and other small molecules, such as ligands, are forced by the pressure through a molecular filter (4). Larger biological macromolecules are retained by the filter. The solvent, depleted of macromolecules, is returned to the bed of beads where more macromolecules are dissolved. 5 = Column vessel, 6 = nylon screen, 7 = column outlet, 8–10 = pressure chamber components, 11 = the magnetic stir bar, 12 = the infrared emiting diode and collimator, 13 = the receiving collimator and infrared triggered transistor, 14 = an electrical operational amplifier, and 15 = a plug in the opening used for the removal of the macromolecule concentrate. The flow-rate of solvent in the system is limited by the permeability of and the area of the filter. The area of the filter in the design of the apparatus is limited because small volumes cannot efficiently be collected from the surface of a large filter. Macromolecules will plug the filter if a certain pressure is exceeded. This pressure is dependent on, among other things, the concentration and the character of the macromolecule. The stir bar keeps the concentration of the macromolecule from going high at the surface of the membrane and allows the filter to be operated at a higher pressure without stopping up. The filter cannot be forced by a pump to pass a higher flow-rate than that which is optimal for a given macromolecule. In the apparatus shown, the variations of the pump-rate is cushioned by the gas in the pressure cell. The pressure in the cell is held constant by a source of pressurized gas and a gas pressure regulator. The pump has a higher flow-rate than that of the filter and is switched on and off by a switch which is controlled by the level of solution in the pressure cell. An infrared beam from the diode senses the presence of the solution in the cell by the solution deflecting the beam off of the infrared phototransistor which activates the amplifier which operates the switch and pump.

solution does not refract the incident infrared beam into, and hence ceases to activate, the phototransistor in the sensor. Consequently, the pump is switched on and the column eluant is pumped into the filtration unit. The filtrate from the filtration unit is recycled back to the inlet of the affinity column as illustrated in Fig. 1.

Purification of the regulatory subunit of cAMP-dependent protein kinase I from rabbit skeletal muscle

Prior to the mixing of cAMP-Sepharose affinity beads with crude protein kinase I preparation after the DEAE-cellulose fractionation step, the cAMP-Sepharose

beads were washed extensively with distilled water. Pilot experiments were first carried out in the cold room at 0°C as follows. To a series of known volume of crude protein kinase solution, a fixed amount of washed cAMP-Sepharose affinity beads was added. After rotating the mixture in a mechanical rotator for 15 min, the beads were allowed to sediment. Aliquots from the supernatant in various tubes were assayed for protein kinase activity in the presence and absence of cAMP, and the sedimented Sepharose affinity beads also were subjected to protein assay. If the supernatant in one of the pilot tubes showed the same enzymatic activity of protein kinase in the presence and absence of cAMP, and the volume of crude solution was also maximal, this volume was also minimal where the affinity beads were shown to have a maximum of bound proteins. The ratio of the mass of Sepharose affinity beads to the volume of crude protein kinase solution in this tube was determined and used subsequently for the preparative separation of the regulatory subunit of cAMP-dependent protein kinase I.

For a large scale purification, an appropriate amount of slurried Sepharose affinity beads was added to the crude DEAE-cellulose fraction of protein kinase with rapid mixing at 0°C. After rotating for 30 min, the Sepharose affinity beads were then allowed to sediment, and the supernatant was removed. The chromatographic column (3 cm in diameter) of the affinity column–filtration apparatus described earlier was then loaded, in the cold room at 0°C. The sedimented beads were washed, at 0°C, exhaustively over a fine nylon mesh, which had been pretreated with silicone, with 0.5 *M* sodium chloride and with PMSF in buffer R consisting of 10 m*M* morpholinoethanesulfonic acid (MES)–10 m*M* Tris–1 m*M* EDTA–1 m*M* dithiothreitol–10 m*M* methylamine at pH 6.5. A 14-ml aliquot of 0.5 *M* sodium chloride buffer R containing 5 m*M* cAMP analogue, 1,N^6-etheno-cAMP, at pH 6.5, was then applied to elute the regulatory subunit, and the column's outlet was coupled to the pump which, in turn, was connected to the inlet of the filtration unit. The pump, infrared beam, the sensor and the magnetic bar were switched on, and the affinity purifications were performed automatically at 0°C. In the apparatus, the regulatory subunit-cAMP analogue complex was constantly collected and concentrated on top of the filtration membrane. In addition, the unbound cAMP analogue in the filtrate was recycled back to the inlet of the chromatographic column to further elute the regulatory subunit. This type of affinity column–filtration apparatus thus provided a novel means for a combined powerful separation method for enzymes and the recycling of the expensive ligand analogues.

The concentrated regulatory subunit-cAMP analogue complex (15–30 mg in 1 ml) collected in the filtration cell was removed and then subjected to gel-filtration chromatography on an ACA-44 column (115 × 1.5 cm I.D.) which had been washed and equilibrated with buffer R containing 0.1 *M* sodium chloride. Following application of the sample, regulatory subunits were eluted from the ACA-44 column with the same buffered sodium chloride solution, and the eluant was collected drop-wise in a fraction collector.

Protein kinase assay. Protein kinase activity was assayed radioisotopically. The assay solution (total volume 90 μl) contained 5 μmol glycylglycine buffer (pH 7.0), 0.05 mg histone type IIA substrate, 3 nmol [γ-^{32}P]ATP (specific radioactivity 50–100 c.p.m. per pmol), 0.5 μmol magnesium dichloride, and cAMP (0.1 nmol) if added. The enzymatic reaction was initiated by adding a portion (10 μl) of protein kinase

solution. Following incubation at 30°C for 10 min, an aliquot (75 μl) was precipitated onto a piece of filter paper and washed in 10% (w/v) trichloroacetic acid; the paper was dried and counted for radioactivity (^{32}P) as described by Huang and Huang[5].

Other techniques. Protein was determined spectroscopically by the method of Bradford[6]. The presence of bound regulatory subunit on the Sepharose affinity bead was detected as follows: An aliquot (5–10 mg) of the damp caked, washed regulatory subunit containing Sepharose affinity beads was loaded into a small disposal plastic column (3 ml, Quik-sep column, Isolab). The column was washed first with buffer R containing 0.5 *M* sodium chloride and then with distilled water. The washed Sepharose affinity beads were washed rapidly with 0.3 ml freshly prepared Bradford reagent two times. The Sepharose affinity beads were then stirred gently in the presence of another volume of Bradford reagent for at least 2 min, and the Sepharose affinity beads were allowed to drain. The beads were washed several times with 1 ml of distilled water followed by draining off the water. At this stage, the blue color of the column bed indicated the presence of bound regulatory subunit on the Sepharose affinity beads. In fact, the blue dye can be eluted off the column with 95% ethanol, and the absorbance of the dye-ethanol eluant at 595 nm can be determined. From the final results obtained with the affinity column–filtration apparatus, one can estimate the total amount of protein originally adsorbed on a given weight of Sepharose affinity beads. Based on the amount of bound protein per mg of Sepharose affinity beads and the absorbance, at 595 nm, of the dye eluted off the small plastic column packed with a known amount of Sepharose affinity beads, one can estimate empirically the amount of bound protein absorbed per mg of Sepharose affinity beads in subsequent experiments simply by determining the absorbance of the dye-ethanol eluant at 595 nm.

Silicone treatment. A 2–5 ml of silicone solution, a 95% ethanol solution saturated with high-vacuum silicone grease (Dow Corning), was used to rinse the glass or plastic surface as described earlier.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in 0.2 *M* Tris–0.2 *M* acetate buffer, pH 6.4, containing 0.1% SDS on 5.3% polyacrylamide gel as described previously[2].

RESULTS AND DISCUSSION

The regulatory subunits of cAMP-dependent protein kinase I from rabbit skeletal muscle prepared before and after the affinity column–filtration step were analyzed by SDS–PAGE as shown in Fig. 2. Before the affinity column–filtration step, the preparation is seen to contain heterogenous collections of many polypeptide bands, whereas the sample after the purification step using the affinity column–filtration apparatus shows a single, intense electrophoretic band of apparent molecular weight 48 000, a band that has been observed for the regulatory subunit of cAMP-dependent protein kinase I[5]. This result demonstrates that the affinity column–filtration apparatus has a high separation efficacy for the enzyme or protein purification.

The elution profile from the affinity column–filtration apparatus for homogenous regulatory subunits of the type 1 rabbit skeletal muscle protein kinase is presented in Fig. 3. Complete depletion of the bound regulatory subunits (13 mg) from the Sepharose affinity beads (5 ml) by the recycled 1,N^6-etheno-cAMP (initial

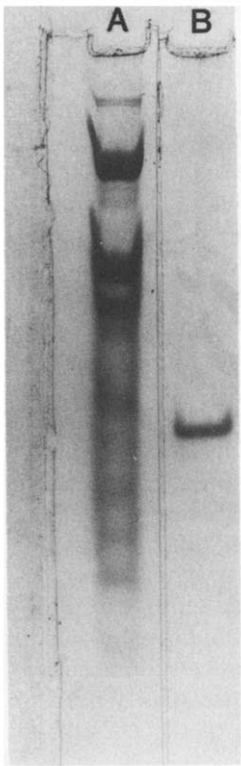

Fig. 2. Lane A, SDS–PAGE of cAMP dependent protein kinase type I from rabbit skeletal muscle purified through the DEAE step. Lane B, Purification of the material in lane 1 by the affinity column–filtration apparatus showing a single intense staining band of protein with an apparent molecular weight of 48 000 daltons. This band is the cAMP binding regulatory subunit of the protein kinase which was present at a low concentration in the material shown in lane 1.

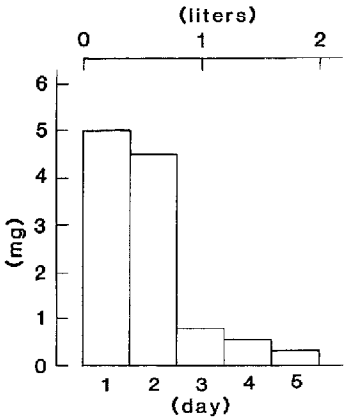

Fig. 3. A typical separation. An amount of 2.55 kg of rabbit muscle was purified through the DEAE step. Wet cake cAMP affinity beads (5 g) were added to the 600 ml DEAE fraction and the cAMP binding protein was purified as in the text. The bar graph shows the yield per day in milligrams. Also shown is an approximate volume of solution recirculated on to the column.

concentration 2 mM) is observed to require more than a week with an approximate flow-rate of 0.3 ml/min at 0°C. However, more than 80% of total bound protein are eluted off during the first 4-day period.

From theoretical considerations one can predict the aspects of a given elution. The rate of production of an enzyme in moles per unit of time, R, is related to the saturation of the immobile ligand by the formula

$$R = K\frac{[\mathrm{EL}]}{[\mathrm{L}]}$$

where [EL] is the concentration of the insoluble ligand complexed with the enzyme, and [L] is the concentration of the insoluble ligand that is unoccupiedby the enzyme. Furthermore, the proportionality constant, K, is related to the flow-rate as follows

$$K = [\mathrm{L}_1]\frac{K_1}{K_2}(\mathrm{d}v/\mathrm{d}t)$$

where $[\mathrm{L}_1]$ is the concentration in moles per liter of the free soluble ligand or ligand analogue in the solution of the system; K_1 is the dissociation constant for the enzyme from the immobile ligand; K_2 is the dissociation constant of the enzyme from the soluble ligand; $\mathrm{d}v/\mathrm{d}t$ the flow-rate in liters per unit of time.

The rate of production R can also be shown, theoretically, when the beads are only fractionally loaded, to decay exponentially with time. Experimentally, shown in Fig. 3, an exponential decay in the production of the regulator subunit of protein kinase can be seen from days 2 through 5.

To obtain the most rapid recovery of the macromolecule, the immobilized ligand should be nearly saturated with the macromolecule. If the macromolecule bound to the immobilized ligand [EL] is a fraction of the immobilized ligand [L] the rate of production will be correspondingly small. The elution shown in Fig. 3 was from initially saturated affinity beads.

To keep recovery times as short as possible the flow-rate $\mathrm{d}v/\mathrm{d}t$ should be as large as possible for the amount of the immobilized ligand. Short columns with relatively large diameters should be used and large area filters should be used consistent with the more limited recoverability of the enzyme off of a large filter.

The amount of bound protein on the cAMP-Sepharose affinity beads can be increased by adding 1 mM of MgATP into the crude enzyme preparation after the DEAE-cellulose fractionation step and by incubating the crude enzyme preparation and the Sepharose affinity bead overnight, at 0°C, in the presence of PMSF, a protease inhibitor. It is well established that MgATP is required for the dissociation and association of the cAMP-dependent protein kinase and its subunits[2,7]. The association–dissociation equilibria of protein kinase and its subunits may be necessary prior to the binding of the regulatory subunit to the cAMP-Sepharose affinity bead.

The main emphasis of this communication is to report on the development of an affinity column–filtration apparatus for the separation of biological macromolecules such as enzymes or proteins. Although only one type of affinity matrix to purify a particular enzyme subunit is discussed here, the range of potential applications of

the affinity column–filtration apparatus can be greatly extended, if different types of affinity matrix are used in the apparatus under various conditions. An important feature of the new apparatus involves the recycling of the ligand analogues; hence, this apparatus is particularly useful for preparative fractionations of proteins or enzymes involving the use of expensive ligands.

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