ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 2, pp. 162-164. © Pleiades Publishing, Ltd., 2009.
Original Russian Text © V. I. Surovtsev, V. M. Borzenkov, K. V. Detushev, 2009, published in Biokhimiya, 2009, Vol. 74, No. 2, pp. 201-204.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM08-196, December 28, 2008.

Adsorption Chromatography of Proteins

V. I. Surovtsev, V. M. Borzenkov*, and K. V. Detushev

State Research Center of Applied Microbiology and Biotechnology, Ministry of Health and Social Development of the Russian Federation, 142279 Obolensk, Moscow Region, Russia; fax: (4967) 36-0010; E-mail: vmborzenkov@mail.ru

Received June 5, 2008
Revision received September 10, 2008

Abstract—A method for adsorption chromatography of proteins is proposed. A protein solution is passed through a cellulose column at a pH value corresponding to an isoelectric point of the protein. Depending on the charge of unwanted proteins, they either remain at the origin (if charges of protein and ion-exchanger are opposite) or are released from the column (if charges of protein and ion-exchanger coincide). Elution volume of the purified protein is higher than for the second group of unwanted proteins because movement of the uncharged protein of interest includes its adsorption on cellulose followed by subsequent desorption caused by the elution buffer. Problems of optimization of buffers and adsorbents are discussed. Applicability of the method of adsorption chromatography is illustrated using purification of horseradish peroxidase as an example.

DOI: 10.1134/S0006297909020060

Key words: adsorption chromatography, adsorbent-ion exchanger, cellulose, buffer, peroxidase, amine

During adsorption chromatography of a mixture of solutes, separation of its components occurs due to their different affinity to the solid adsorbent. Adsorption chromatography was discovered by Tsvet in 1903 [1]. Now various variants of this method are widely used in both laboratory and industrial conditions for purification and separation of low molecular weight organic compounds. The only version of the method of adsorption chromatography used for separation of proteins, the most complex organic substances, is chromatography on hydroxyapatite. It has been used for purification of some acidic and basic proteins. However, chromatographic fractionation and purification of proteins on hydroxyapatite is basically empiric and it is hard to predict results and recommend optimal elution conditions [2]. In this study, we have employed another method, which is in our viewpoint more effective in solving the abovementioned problem. Efficiency of this method is demonstrated using one of the most widely used enzymes in biochemistry and biotechnology—horseradish peroxidase.

Abbreviations: MOPS, 3-(N-morpholino)propanesulfonic acid; MOPSO, 3-(N-morpholino)-2-hydroxypropanesulfonic acid; TEA, triethanolamine; TES, N-tris(hydroxymethyl)methyl-2-aminosulfonic acid.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Triethanolamine (TEA), N-tris(hydroxymethyl)methyl-2-aminosulfonic acid (TES), DEAE-cellulose 52, CM-cellulose 52, and 4-aminoantipyrine (4-amino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one) were from Sigma-Aldrich (USA). Horseradish peroxidase with RZ of 0.3 was from Reanal (Hungary). Other reagents were purchased from domestic suppliers.

Crude lyophilized preparation of peroxidase with RZ of 0.3 (15 g) was suspended in 300 ml of 0.02 M TEA-HCl buffer, pH 7.2, centrifuged (at 3000*g* for 20 min), and applied onto a DEAE-cellulose (Cl^--form) (10 × 20 cm) equilibrated with the same buffer. The enzyme solution (300 ml) was carefully layered onto the column until it was adsorbed onto the sorbent (~15 min). Solvents were applied in the similar way; it was then added during free movement of a brown band (of the enzyme) to maintain the solvent at a constant level. Enzyme movement along the column was monitored visually. The enzyme was eluted from the column within 50-60 min in the volume of 450-500 ml. This enzyme solution was then applied onto the CM-cellulose column (Na^+-form) (of the same volume) equilibrated with 0.02 M TES-NaOH buffer, and slow movement of the enzymatic band along the column was monitored. Final volume was 850-900 ml. This pro-

cedure yielded 1.5 g of purified peroxidase with RZ of 3.35 and activity of 1000-1100 U/mg of enzyme.

Peroxidase activity of the purified enzyme was assayed using 4-aminoantipyrine in phenolic solution as substrate. Changes in light absorption of the reaction medium were measured at 510 nm and 25°C. The reaction rate v (in U/mg) was determined from the linear part of the curve using the ratio: $v = \Delta A_{510}/(6.58\ [E])$, where ΔA_{510} is the rate of change of light absorption per minute and [E] is enzyme concentration in U/mg [3].

Concentration of crude enzyme was determined by the Bradford method, and concentration of purified enzyme was estimated by absorbance at 403 nm: 1 mg of enzyme per ml corresponds to $A_{403} = 0.44$.

RESULTS AND DISCUSSION

Adsorption of solutes (including proteins) to sorbents involves various forces of noncovalent interactions (dipole–dipole, hydrophobic, van der Waals–London forces, and hydrogen and ion bonds) [2]. Nevertheless, the total energy of these bonds is less than the binding energy (in the case of affinity chromatography, e.g. enzyme–inhibitor, antigen–antibody) or ion interaction on an ion exchanger and so adsorption interactions either are not realized or are realized during binding of the unwanted proteins in the sample (thus decreasing purification factor).

Protein purification by means of adsorption chromatography requires protein movement along the adsorbent column due to adsorption–desorption processes whereas unwanted proteins would remain at the origin (due to ion exchange) or rapidly go down due to interaction with the ion-exchange matrix of the same sign of charge. This can be achieved in the case of equilibration of an adsorbent-ion exchanger with buffer at pH that corresponds to pI a protein of interest. In this case a part of the unwanted proteins remains at the origin, the another part of these protein goes down the column depending on their particular charges, whereas the protein of interest will be adsorbed at each particles of the sorbent and then desorbed due to competition of the buffer component to the same sorbent. Only unwanted proteins characterized by pI values close to that of the protein of interest (within several tenths of a pH unit) can move due to adsorption–desorption, and therefore decrease the degree of purification. Thus, the ultimate preconditions required for successful use of adsorption chromatography include lack of unwanted proteins, or presence of their minor quantities or their removal at previous or subsequent stages as well as good solubility of proteins of interest at their pI (some proteins, such as luciferase, are basically insoluble at their pI).

An adsorbent-ion exchanger has to meet several criteria. It should provide effective adsorption of proteins; exhibit chemical stability (because of introduction of ionogenic groups into it), hydrophilicity; it should be free of the sieve effect; it should have a large specific adsorption capacity (referred to 1 g of the adsorbent), high ratio of surface to volume, and large ion-exchange capacity. Due to these criteria, DEAE- and CM-cellulose are convenient adsorbent ion exchangers. Adsorption–desorption of proteins occurs on cellulose (uncharged protein should move competing with buffer both on anion and cation exchangers), whereas ionogenic groups are required for binding and elution of unwanted proteins.

The elution buffer should provide desorption of the adsorbed protein and movement of some proportion of unwanted proteins (others are retained due to ion exchange). The rate of movement of the desired protein along a column should be less than that for ballast proteins. These conditions can be achieved using a buffer containing monobasic aliphatic or sulfonic acids (RCOOH, RSO_3H), as an acidic part (alternatively, the latter may be charged as R_3NH^+). Radicals (uncharged parts) of buffer components provide competition with protein adsorbed on cellulose and its desorption, whereas buffer ions on the surface of a macromolecule (it has a charge of opposite sign versus the ion exchanger) bind counterions (Cl^- or Na^+, respectively), thus providing ion exchange of unwanted proteins. The buffer should be used at rather low concentration (0.02-0.03 M) because increasing buffer concentration increases its competition with groups of the adsorbed protein; this results in rapid movement of the desired protein and its mixing with unwanted proteins. On the other hand, the buffer should provide stable pH value and, consequently, should have pK_a close to the pI of the adsorbed protein. Ion-exchange properties of DEAE- and CM-cellulose are realized within the range of pH 4-9. In some ion-exchange celluloses, e.g. DEAE-Sephacel, ion exchange can occur at pH of 2-12 [2], and this extends the range of proteins that can be purified by this method.

The pK_a values for unsubstituted aliphatic acids are about 4-5, whereas sulfonic acids are strong acids. Required pK_a value can be achieved by insertion of large electron donor groups into these acids, and they should provide effective competition of the radical formed with protein groups for binding to the adsorbent. On the other hand, pK_a values for amines, very weak acids, are about 10-11, and so insertion of groups decreasing pK_a to the required values is needed. Having a set of buffers (most of them are well known) with the interval of about 0.5 pH unit, it is obviously possible to purify many proteins by means of this method of adsorption chromatography.

Let us to consider purification of horseradish peroxidase. Peroxidase is an enzyme (or several isoenzymes characterized by similar isoelectric points) with pI at 7.2 and molecular mass of 40 kDa. Highly purified enzyme has RZ $\geqslant$ 3.1. The most effective method for its purification is ion-exchange chromatography. However, the latter

Purification of peroxidase by adsorption chromatography

Stage of purification	Volume, ml	Total activity × 10^{-6}, U	Protein content, g	Specific activity, U/mg	Yield, %
Initial preparation	300	1.97	13.3	148	100
Adsorption chromatography on DEAE-cellulose	475	1.81	1.85	979	92
Adsorption chromatography on CM-cellulose	875	1.58	1.50	1050	80

method cannot provide required purity, and so purification is a multistage process. For example, purification of peroxidase from a crude preparation (RZ ~ 0.2) includes ion-exchange chromatography on DEAE- and CM-celluloses and gel filtration on Sephadex G-100. Using 1 g of the crude preparation, only 50 mg of highly purified peroxidase is obtained [4].

Table shows results of peroxidase purification by adsorption chromatography on DEAE- and CM-celluloses. Use of the adsorption method resulted in a 2-fold increase in yield of peroxidase; using this method, it is possible to work with large amounts of protein without additional stages. The purification procedure takes about 2 h, whereas ion-exchange chromatography requires much longer (several days).

For protein purification by the adsorption method, the enzyme solution with RZ ~ 0.3 was loaded onto the DEAE-cellulose column equilibrated with 0.02 M TEA-HCl buffer, pH 7.2 (pK value of 7.7 for the buffer was determined by the Debye–Huckel equation from pK_a^0, the pK_a value at zero ionic strength [5]). During elution, a part of the unwanted proteins remains at the origin (due to ion exchange), whereas the other part rapidly goes down due to repulsion from the sorbent particles of the same sign of charge. Peroxidase movement in 0.02 M buffer occurs due to adsorption on the sorbent followed by subsequent desorption induced by the elution buffer. The rate of its movement is slower than that of the unwanted proteins and therefore the enzyme is purified from both the unwanted proteins remaining at the origin and also from moving proteins. After slow passage of the brown band of peroxidase (monitored visually) through the DEAE-cellulose column, the resulting enzyme solution is loaded onto the CM-cellulose column equilibrated with TES-NaOH buffer, pH 7.2 (buffer pK_a of 7.4). Other buffers can also be used: MOPS-NaOH buffer based on 3-(N-morpholino)propanesulfonic acid with pK_a^0 of 7.2 and MOPSO-NaOH buffer based on 3-(N-morpholino)-2-hydroxypropanesulfonic acid with pK_a^0 of 7.0.

For 0.02 M TES-NaOH buffer, radical concentration exceeds molar concentration of the purified peroxidase (50 mg/ml) by a factor of ~17; this is probably sufficient for effective competition with enzyme groups for the adsorbent. At 0.01 M concentration, movement of the enzyme band significantly decreases due to ineffective competition with superficial groups of peroxidase for the adsorbent. At 0.05 M concentration, movement significantly increases and purification does not occur. Unwanted proteins (if any) passed through the sorbent are separated from the enzyme, and uncharged peroxidase slowly passes through the cation exchanger. Only unwanted proteins with pI near 7.2 can compete with peroxidase for adsorbent binding, thus decreasing the purification factor. However, under our experimental conditions they are either absent or present in minor quantities. Thus, this procedure yields highly purified enzyme with average RZ value of 3.35 and specific activity 1000-1100 U/mg (assayed with 4-aminoantipyrine as substrate).

Results of the present study suggest that this method of adsorption chromatography can be applicable for protein purification.

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