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Design of a new, twelve-channel electrophoretic apparatus based on the Gradiflow technology

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Abstract

The Gradiflow technology, originally designed to carry out binary, size-based and charge sign-based electrophoretic protein separations, has been extended to simultaneously obtain multiple protein fractions from a single electrophoretic separation. The separation unit of the new apparatus houses the anode and cathode compartments and up to twelve shallow separation compartments through which the background electrolyte solution that contains the separated protein fractions is recirculated. The separation compartments are formed from grids as thin as 1.2 mm and polyacrylamide membranes as thin as 0.15 mm, all with corresponding multiple inlet and outlet ports. The average pore size of the polyacrylamide membranes can be varied to permit passage of proteins in the 5000–800 000 molecular mass range. The electric field, orthogonal to the flow paths of the recirculated background electrolyte, selectively moves the sample components across the polyacrylamide separation membranes. Selective protein transport can be achieved by exploiting differences in either the relative size of the proteins or the charge sign of the proteins. The advantages of the new apparatus stem from the synergistic combination of the short electrophoretic transfer distances, high electric field strength, large effective surface areas of the separation membranes, and the great flexibility with which apparati containing one to twelve separation compartments can be created.

Keywords: Instrumentation; Preparative electrophoresis; Proteins

1. Introduction

Eight years ago, a membrane-mediated electrophoretic technology, the Gradiflow technology [1] was introduced to effect preparative-scale, electrophoretic, binary separations of protein mixtures. The essence of the Gradiflow technology is the combination of the recirculating hydraulic flow of the protein mixture through two shallow separation compartments with an orthogonal electrophoretic transport of the different proteins across a single separation membrane between the separation compartments. Separation results when proteins are selectively transported from the anodic separation compartment into the cathodic separation compartment (or vice versa) across the separation membrane [1–10]. Migration of the target proteins out from the separation compartments and into the electrode compartments is prevented by restriction membranes that act as interfaces between the separation compartments and the electrode compartments, and whose pores are small enough to prevent passage of the target proteins but large enough to permit passage of the background electrolyte ions. Selective protein transport across the separation membrane has been achieved by exploiting differences in either the size of the proteins (more properly, differences in the hydrated ion radii of the like-charged proteins) or the

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charge sign of the proteins (i.e., whether the proteins are cationic or anionic at a selected pH in a selected background electrolyte) or both [1–10].

In order to realize size-based selective protein transport across the separation membrane, a background electrolyte is chosen in which all proteins of the mixture are either cationic or anionic. Then, a separation membrane is chosen that has an average pore size that is slightly larger than twice the gyration radius of the hydrated ion of the protein that is expected to cross the membrane. The separation membranes have narrow pore size distributions. The protein mixture is passed through (or recirculated through) one of the separation compartments: when the processed proteins are cationic, they are fed into the anodic separation compartment, when the processed proteins are anionic, they are fed into the cathodic separation compartment. Upon application of an electric field orthogonal to the hydraulic flow paths, all proteins migrate toward the separation membrane but only the proteins whose effective size is smaller than the effective size of the pores in the separation membrane pass through the membrane. By virtue of their small pore size, the restriction membranes prevent passage of the target proteins out from the proper separation compartments into the respective electrode compartments. Thus, a binary separation is achieved: proteins larger than a certain size remain trapped in the feed stream while those smaller than that certain size end up trapped in the receiving stream. The selectivity of the separation does not depend on the magnitude of the mobility of the proteins; proteins smaller than the target size will all eventually go through the separation membrane: those with a smaller mobility will take longer to go through, those with a higher mobility will go through faster.

In order to realize charge sign-based selective protein transport across the separation membrane, a background electrolyte is chosen in which the target protein is cationic while all other proteins (or some of the other proteins) are anionic or vice versa. This is accomplished by selecting the pH of the background electrolyte according to the isoelectric points (p*I* values) of the target protein and the contaminant proteins. Then, a separation membrane is chosen that has an average pore size that is much larger than the effective size of the target protein (or the target protein and some of the other proteins or all the proteins). Once again, in order to realize the separation, the protein mixture is passed through (or recirculated through) one of the separation compartments: if the protein to be moved is a cation, the feed stream is directed into the anodic separation compartment. If the protein to be moved is an anion, the feed stream is directed into the cathodic separation compartment. Upon application of an electric field orthogonal to the hydraulic flow paths, the oppositely charged proteins migrate toward the respective electrodes, cross the large-pore separation membrane as needed, but are prevented from leaving their destined separation compartment and entering the respective electrode compartment by the narrow pores of the restriction membranes. Thus, once again, a binary separation is achieved: anionic proteins end up trapped in the anodic separation compartment, cationic proteins end up trapped in the cathodic separation compartment. Also, just as in the size-based separations, separation selectivity does not depend on the magnitude of the mobility of the proteins: anionic proteins eventually all end up in the anodic separation compartment, cationic proteins end up in the cathodic separation compartment. The only difference is that proteins with a smaller mobility take longer to get into their destination compartment than those with a higher mobility. Separation selectivity is tuned by changing the pH of the background electrolyte with respect to the p*I* values of the proteins to be separated.

The Gradiflow technology can offer high throughput, because the separation compartments are shallow and the membranes are thin (both promote short electrophoretic transfer distances and high electric field strengths), the surface area of the separation membrane is large, and heat removal is efficient.

The current, commercial implementation of the Gradiflow technology shares a limitation inherent to any binary separation: when a single protein needs to be isolated from a complex mixture of proteins, two sequential binary separations have to be carried out under slightly different conditions. For example, to isolate a M_r 50 000 protein from a mixture of proteins that contains M_r 30 000, 50 000 and 100 000 proteins, the first separation could use a separation membrane that permits passage of the undesired M_r 30 000 protein. Then, the mixture of the M_r 50 000

and 100 000 proteins left in the feed stream would be re-separated with a membrane that permits passage of the M_r 50 000 target protein but retains the M_r 100 000 contaminant protein. For a charge sign-based separation to isolate, for example, a pI=5 protein from a mixture of proteins that contains pI=3, pI=5 and pI=8 proteins, the first separation could use a background electrolyte with a pH of 4. In this background electrolyte the pI=3 protein is anionic and remains trapped in the anodic separation compartment. The pI=5 and pI=8 proteins are cationic in the pH 4 background electrolyte and move through the wide-pore separation membrane into the cathodic separation compartment, where they are trapped. Then, the mixture of the pI=5 and pI=8 proteins could be re-separated using another background electrolyte with a pH of, e.g., 6, rendering the pI=5 target protein anionic and thus trapping it in the anodic separation compartment while sending the pI=8 contaminant protein through the wide pore separation membrane into the cathodic separation compartment.

The objective of the present paper is to describe a new Gradiflow apparatus that eliminates the binary separation limitation of the current Gradiflow BF200 instrument and permits the simultaneous recovery of multiple fractions from the same separation. Additionally, by using independent anolyte and catholyte reservoirs and independently fed multiple separation compartments, the new instrument can also serve as a platform for electrophoretic separations that require the use of nonhomogeneous background electrolyte systems.

2. Experimental

2.1. Chemicals

Trishydroxymethylaminomethane (Tris), boric acid, disodium salt of ethylenediaminetetraacetic acid, sodium dodecylsulfate (SDS), acrylamide, *N*,*N*′-methylenebisacrylamide, ammonium persulfate and tetramethylethylenediamine were obtained from Aldrich (Milwaukee, MI, USA).

All solutions were freshly prepared using deionized water from a Milli-Q unit (Millipore, Milford, MA, USA). Polyacrylamide membranes with different nominal pore sizes were from Gradipore (French's Forest, Australia). Native polyacrylamide gel electrophoresis (PAGE) analysis in pH 8.3 Tris–glycine buffer was carried out on 4–20% iGels (Gradipore) according to the manufacturer's instructions. The molecular mass standards (all from Gradipore) for the native PAGE separations were equine α2-macroglobulin tetramer (M_r 800 000), equine α2-macroglobulin dimer (400 000), phycoerythrin (250 000), bovine serum albumin (67 000), ovalbumin (45 000) and lactalbumin (14 700).

Bench Mark Protein Ladder molecular mass standards (Invitrogen) were used for SDS–PAGE. The protein markers consisted of 15 engineered proteins with the following molecular masses, 10 000, 15 000, 20 000, 25 000, 30 000, 40 000, 50 000, 60 000, 70 000, 80 000, 90 000, 100 000, 120 000, 160 000, and 220 000.

2.2. Preparative electrophoretic equipment

All preparative-scale protein separations were completed with the new, twelve separation-compartment Gradiflow apparatus (Gradipore). The schematic of the system is shown in Fig. 1. The main elements of the apparatus are the separation unit, the anolyte and catholyte reservoirs, the anolyte and catholyte pumps (centrifugal pumps producing a flow-rate of 2 l/min) and their associated tubing, twelve sample reservoirs and their associated tubing,

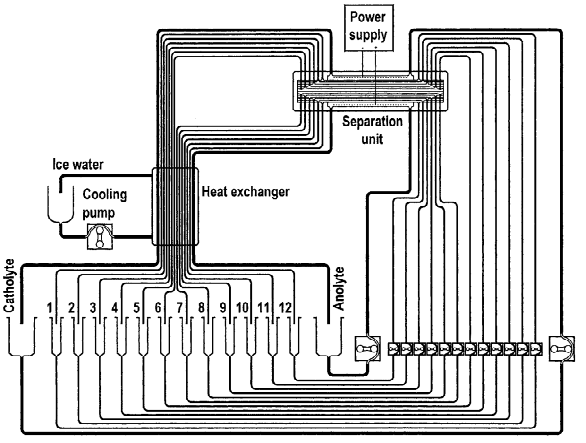

Fig. 1. Schematic of the new Gradiflow preparative electrophoretic apparatus configured with 12 separation compartments.

a twelve-channel, variable-speed peristaltic pump, a cooling facility, and a Consort E 861, 600 V, 1000 mA d.c. power supply (Turnhout, Belgium). The power supply is connected to the anode and cathode through safety switches which automatically disconnect the power supply from the separation unit when the cover of either electrolyte reservoir is opened.

The separation unit, shown in a partially exploded view in Fig. 2, houses the anode, the anode compartment, the anodic gasket, the twelve separation compartments formed by the separation membranes and the grids, the cathodic gasket, the cathode compartment and the cathode. For the sake of clarity, only the membranes and the grid of the first separation compartment are shown separately, the grids and membranes for compartments 2–12 are shown as a single block. The anode and cathode are made of platinum-coated titanium mesh. The active depth of both the anode and cathode compartments is about 2 mm. The thickness of both the anodic and cathodic gaskets (made of silicon rubber) is 1 mm.

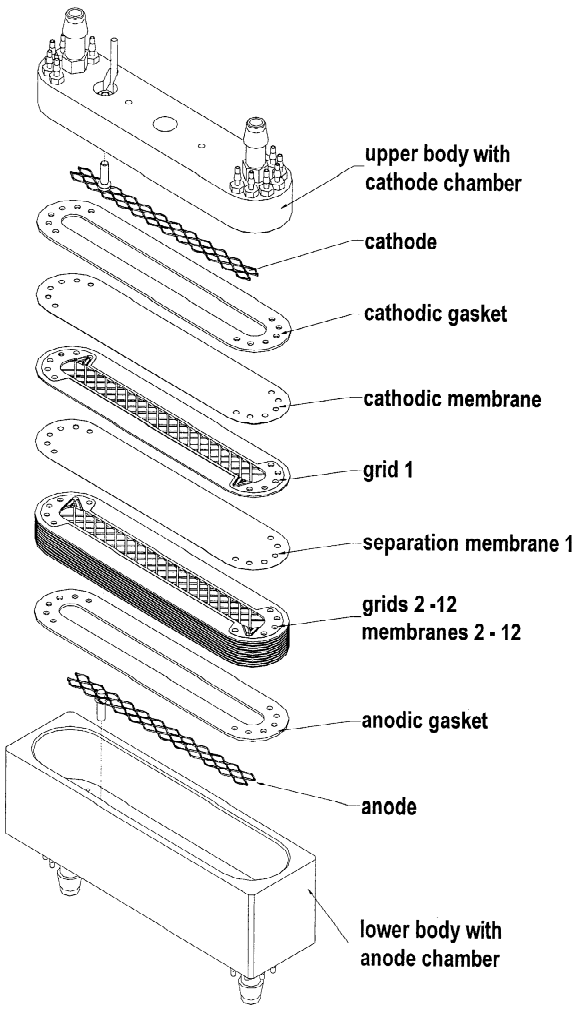

Fig. 2. Exploded view of the separation unit of the new Gradiflow preparative electrophoretic apparatus.

The twelve separation compartments are formed by twelve separation compartment grids and thirteen polyacrylamide membranes. Six of the twelve separation compartments have their inlet and outlet connections at the anodic side of the separation unit and are formed by the first set of six different grids. The other six separation compartments have their inlet and outlet connections at the cathodic side of the separation unit and are formed by the second identical set of six different grids. Three of the six different separation compartment grids that make up one set are shown in frontal view in Fig. 3, the other three are mirror images of those shown. Each grid is 1.2-mm thick, each membrane is about 0.15-mm thick.

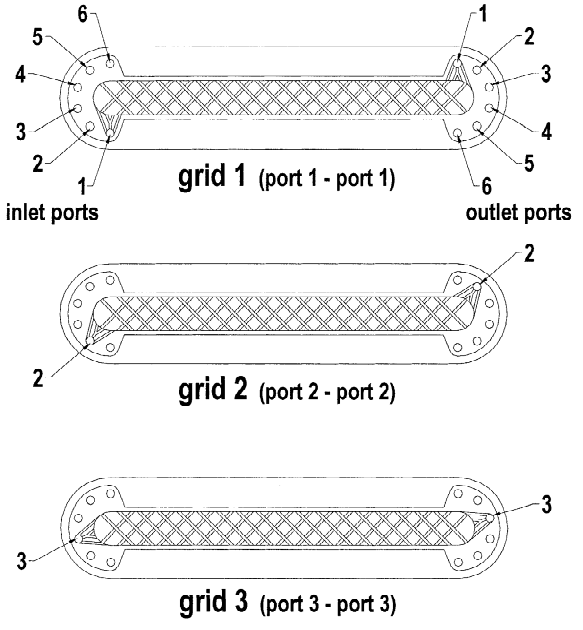

Fig. 3. Frontal view of the three grids that are used to form the separation compartments in the separation unit of the new Gradiflow preparative electrophoretic apparatus.

There are six entry holes and six exit holes at the opposite ends of each grid. There is also a lattice of ribs in the middle of the grid that defines the actual separation compartment space. The lattice is formed by two sets of vertically offset ribs that run at 45° and 145° with respect to the long axis of the grid. The objective of the lattice is to support the adjacent polyacrylamide membranes and provide a weaving flow path to minimize membrane fouling. Liquid is brought into and out of each of the six grids of a set slightly differently. In the first grid, recessed channels connect the first entry hole and the sixth exit hole to the lattice, the other holes provide flow-through paths between the inlet and outlet lines at the anodic side of the separation unit and the second, third, fourth, fifth and sixth separation compartments. In the second grid, recessed channels connect the second entry hole and the fifth exit hole to the lattice, the others provide flow-through paths between the inlet and outlet lines at the anodic side of the separation unit and the third, fourth, fifth and sixth separation compartments. In the third grid, recessed channels connect the third entry hole and the fourth exit hole to the lattice, the others provide flow-through paths between the inlet and outlet lines at the anodic side of the separation unit and the fourth, fifth and sixth separation compartments. In the fourth grid, recessed channels connect the fourth entry hole and the third exit hole to the lattice, the others provide flow-through paths between the inlet and outlet lines at the anodic side of the separation unit and the fifth and sixth separation compartments. In the fifth grid, recessed channels connect the fifth entry hole and the second exit hole to the lattice, the others provide flow-through path between the inlet and outlet lines at the anodic side of the separation unit and the sixth separation compartment. Finally, in the sixth grid, recessed channels connect the sixth entry hole and the first exit hole to the lattice. Since the polyacrylamide membrane above the sixth separation compartment does not have any inlet and outlet holes, the upper six separation compartments can be formed using an identical grid and membrane set as in the lower six separation compartments, except that their inlet and outlet holes are connected to the inlet and outlet lines at the cathodic side of the separation unit.

A major advantage of this design is that, depending on the requirements of the separation at hand, different separation apparati having one to twelve separation compartments in various configurations can be formed from the same elements by selecting the appropriate grids and membranes.

Size-selective polyacrylamide membranes with characteristic average pore sizes and pore-size distributions serve as anti-convective, ion-permeable barriers between the separation compartments. The average pore size and pore size distribution of the membranes are chosen to facilitate *trans*-membrane transport of certain constituents and substantially prevent *trans*-membrane transport of other constituents. Size-selective polyacrylamide membranes (Gradipore) which permit passage of proteins in the M_r 5000–800 000 range were produced by copolymerizing, on a polyethylene terephthalate substrate (Mitsui, Melbourne, Australia), acrylamide and *N,N′*-methylenebisacrylamide. The polyacrylamide membranes had an approximate thickness of 0.15 mm and a geometric surface area of 12 cm^2.

3. Results and discussion

The new Gradiflow apparatus containing all twelve separation compartments was used to separate, under non-denaturing conditions, the proteins in chicken egg white according to their size. Prior to the preparative electrophoretic separation, the separation unit was assembled using the separation compartment grids and polyacrylamide membranes with different nominal molecular mass cut-offs (NMM). The membrane between the anode compartment and the first separation compartment had an NMM of 5000. The membrane between the first and second separation compartments had an NMM of 10 000, the membrane between the second and third separation compartments had an NMM of 50 000, the membrane between the third and fourth separation compartments had an NMM of 65 000, the next membrane between the fourth and fifth separation compartments had an NMM of 100 000, the next membrane between the fifth and sixth separation compartments had an NMM of 125 000. The membrane between the sixth and seventh separation compartments had an NMM of 150 000, the next membrane between the seventh and eighth separation

compartments had an NMM of 200 000. The eighth and ninth compartments were separated by an NMM 300 000 membrane. The ninth and tenth separation compartments were separated by a 500 000 NMM membrane. The tenth and eleventh separation compartments and the eleventh and twelfth separation compartments were separated by 800 000 membranes. The twelfth separation compartment was separated from the cathode compartment by a membrane with an NMM of 5000.

Following assembly of the separation unit, the anolyte, catholyte and sample reservoirs were filled with deionized water and the pumps were turned on to check the system for leaks. Then, the anolyte, catholyte and sample reservoirs were filled with the same background electrolyte: 90 m*M* Tris, 90 m*M* borate, 1 m*M* EDTA at pH 8.51 (TBE). In the anode and cathode reservoirs and in each sample reservoir the volume of background electrolyte was 2 l and 20 ml, respectively. The feed sample was prepared by diluting 15 ml egg white, at a rate of 1–10, with the background electrolyte. The egg white solution was filtered through polyethylene terephthalate paper prior to use, and 15 ml of this solution was loaded into the sample reservoir connected to the tenth separation compartment. The separation was conducted at 500 V for 4 h. The initial current was 172 mA, the final current was 50 mA. After 4 h, the potential and the pumps were turned off and the protein fractions were harvested. The separation unit was disassembled and the system was sanitized with a 0.1 *M* NaOH solution.

Aliquots of the fractions harvested at the end of the separation (after 4 h of electrophoresis) were analyzed by native PAGE. The composite image of the destained gels is shown in Fig. 4. The original egg sample was applied to the lane on the left and a mixture of the respective molecular mass standards to the lane on the right (lane 14). Proteins were observed in the feed solution between molecular masses as low as about 40 000, and as high as about 800 000. Under native PAGE conditions, a signifi-

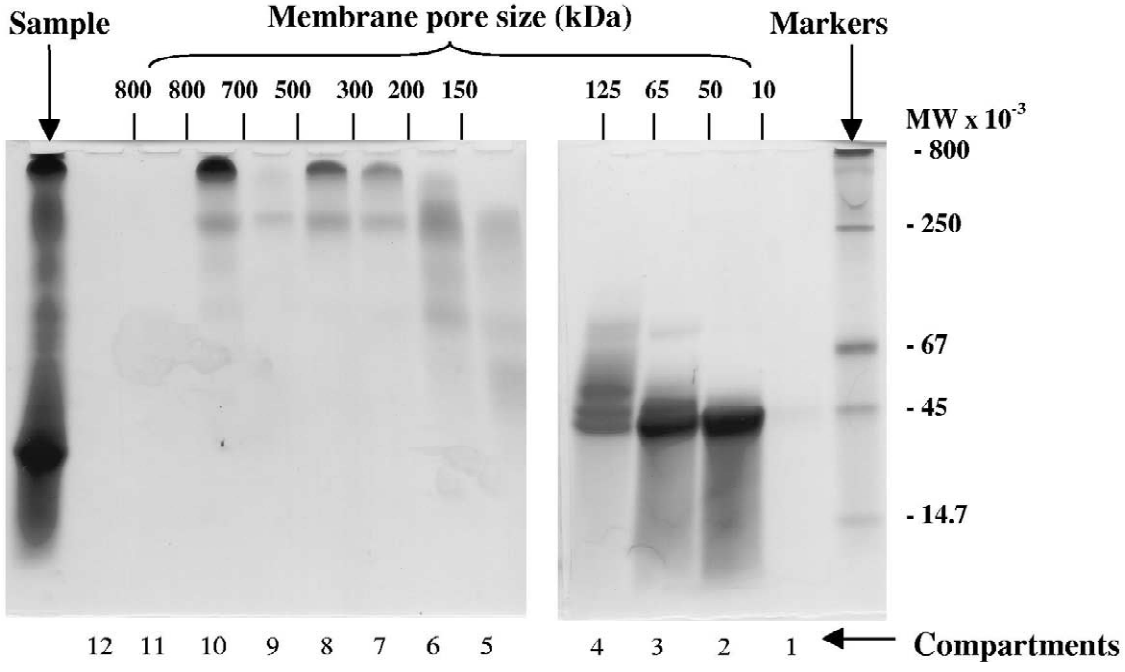

Fig. 4. Composite image of native PAGE analysis of the contents of the separation compartments after 4 h of electrophoresis of chicken egg white. First lane: feed sample. Then follow: compartments 12–1. Final lane, mixture of molecular mass standards. The nominal pore sizes (in kilodaltons) of the membranes separating the compartments are also shown. For clarity the M_r 5000 membranes separating anodic and cathodic electrolytes from compartments 1 and 12 are not shown. MW, molecular mass (M_r).

cant proportion of the protein was present in the high-molecular mass region of the gel indicating that the original sample contained aggregated protein (Fig. 4, sample lane). The separation profile shows there was progressive fractionation through the separation compartments from higher-molecular mass aggregates to lower-molecular mass proteins: the farther away the separation compartment is from the feed compartment (tenth compartment), the lower the apparent molecular mass of the bands. This separation profile was consistent for separations carried out with sample loading at 1 in 10 dilution or 1 in 4 dilution (data not shown).

The predominant proteins present in egg white can be identified by their abundance and molecular mass on SDS gels where the monomeric polypeptides are resolved under denaturing conditions [11]. The higher-molecular mass fraction (retained by the 700 000 membrane) composed a 70 000 protein on SDS–PAGE (Fig. 5, compartment 10) (ovotransferrin, p*I* 6.2–7.2) and a second group of proteins (molecular masses ca. 50 000 by SDS–PAGE) (ovoinhibitors, p*I* 5.1) and the third group that is poorly resolved (ovomucoid, p*I* 4.1). Ovotransferrin was also the predominant protein in a number of other high-molecular mass fractions (compartments 7–9 bounded by membranes NNM 700 000–200 000). The data indicate that ovotransferrin forms complexes under these separation conditions; The low relative mobility of ovotransferrin may be attributed in part to the lower net charge on this protein with its p*I* between 6 and 7.0 when the majority of egg white proteins have much lower pI<5.0. However, the presence of other lower p*I* proteins within the aggregate fraction (Fig. 4, compartment 10) indicates that size was the most significant contributing factor to the fractionation. The majority of ovalbumin (the most abundant protein in egg white) migrated down

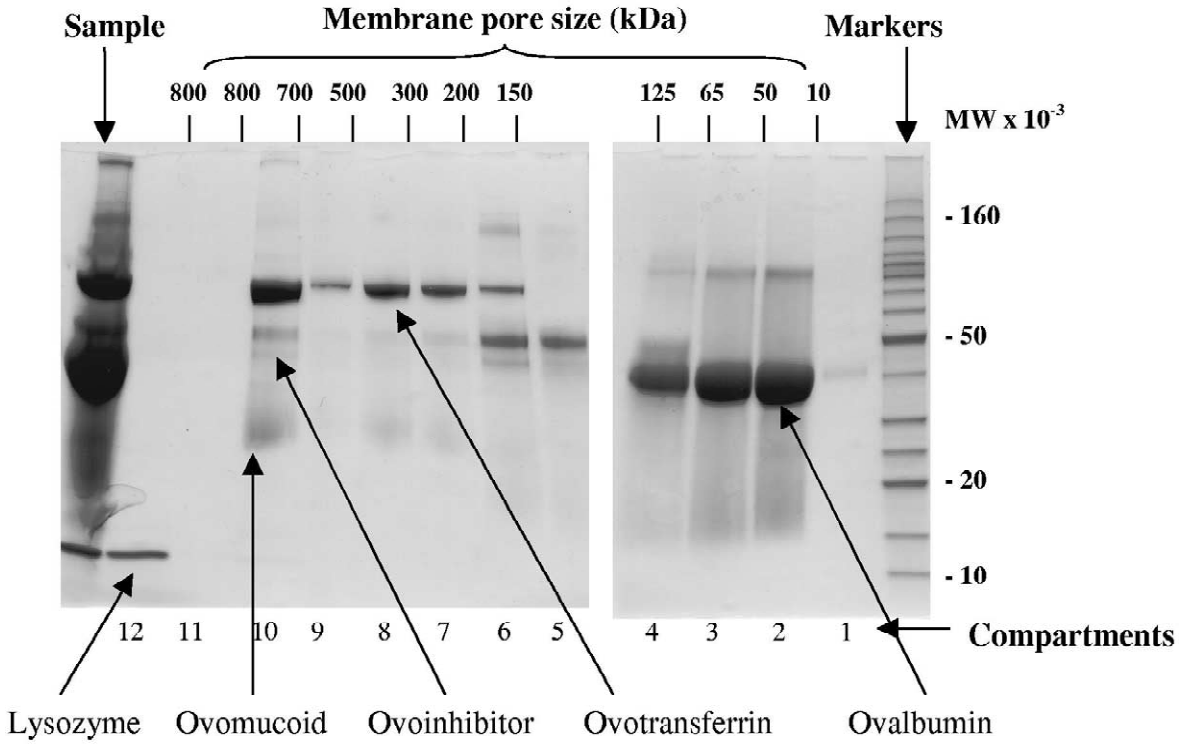

Fig. 5. Composite image of SDS native PAGE analysis of the contents of the separation compartments after 4 h of electrophoresis of chicken egg white. First lane: feed sample. Then follow compartments 12–1. Final lane, mixture of molecular mass standards. The nominal pore sizes (in kilodaltons) of the membranes separating the compartments are also shown. For clarity the M_r 5000 membranes separating anodic and cathodic electrolytes from compartments 1 and 12 are not shown. MW, molecular mass (M_r).

to compartments 2 and 3. On the other hand the positively charged protein lysozyme (15 000, p*I* 10.5) migrated to compartment 12 where it was retained by the 5000 restriction membrane.

4. Conclusions

This paper describes the design of the new Gradiflow preparative electrophoretic apparatus whose separation unit contains one to twelve separation compartments. The separation compartments are formed from thin grids and thin polyacrylamide membranes with different average pore sizes. The hydraulic flow paths of the recirculating background electrolyte through the shallow separation compartments is orthogonal to the electric field. This arrangement permits selective, size-based or charge sign-based transport of proteins across the membranes and the trapping of proteins according to their sizes in the respective separation compartments.

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