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Purification by reflux electrophoresis of whey proteins and of a recombinant protein expressed in *Dictyostelium discoideum*[1]

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Abstract

Protein purification that combines the use of molecular mass exclusion membranes with electrophoresis is particularly powerful as it uses properties inherent to both techniques. The use of membranes allows efficient processing and is easily scaled up, while electrophoresis permits high resolution separation under mild conditions. The Gradiflow apparatus combines these two technologies as it uses polyacrylamide membranes to influence electrokinetic separations. The reflux electrophoresis process consists of a series of cycles incorporating a forward phase and a reverse phase. The forward phase involves collection of a target protein that passes through a separation membrane before trailing proteins in the same solution. The forward phase is repeated following clearance of the membrane in the reverse phase by reversing the current. We have devised a strategy to establish optimal reflux separation parameters, where membranes are chosen for a particular operating range and protein transfer is monitored at different pH values. In addition, forward and reverse phase times are determined during this process. Two examples of the reflux method are described. In the first case, we describe the purification strategy for proteins from a complex mixture which contains proteins of higher electrophoretic mobility than the target protein. This is a two-step procedure, where first proteins of higher mobility than the target protein are removed from the solution by a series of reflux cycles, so that the target protein remains as the leading fraction. In the second step the target protein is collected, as it has become the leading fraction of the remaining proteins. In the second example we report the development of a reflux strategy which allowed a rapid one-step preparative purification of a recombinant protein, expressed in *Dictyostelium discoideum*. These strategies demonstrate that the Gradiflow is amenable to a wide range of applications, as the protein of interest is not necessarily required to be the leading fraction in solution. © 1997 Elsevier Science B.V.

Keywords: *Dictyostelium discoideum*; Reflux electrophoresis; Proteins; Whey proteins; Recombinant proteins

1. Introduction

The electrophoretic separation of proteins is based on their differences in size, net charge (isoelectric point, p*I*) and mobility (net surface charge/M_r ratio)

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under non-denaturing or denaturing conditions. High resolution protein separation can be conducted in two dimensions, where sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) is combined with isoelectric focusing [1,2]. In two-dimensional electrophoresis (2-DE) thousands of proteins are separated on a single gel. Currently the 2-DE technology is capable of separating up to μg amounts of single proteins that, in combination with sensitive analysis techniques, make it a powerful tool in protein identification and characterisation studies [3,4]. The development of technologies that adopt a two-dimensional approach to scale up protein purification are therefore powerful.

Membrane filtration combined with electrophoresis is potentially a powerful separation technology, since it fractionates by both the size and charge properties of the analytes. Membrane filtration allows for high throughput and is used for both small-scale laboratory applications and large-scale industry processes, whereas PAGE provides a high resolution in a short time frame (<2 h).

A combination of membrane and electrophoretic technologies for preparative protein purification is therefore a powerful synergy, as this combines the advantages of both techniques resulting in rapid fractionation, high sample loading and high resolution. The focus of this study was to develop a strategy for protein purification by reflux electrophoresis, allowing purification of proteins at both μg and mg levels under non-denaturing conditions. Previously we have described the Gradiflow and how fractionation can be controlled for a mixture of proteins using both continuous and reflux electrophoresis [5,6].

While working with complex protein mixtures, it was apparent that proteins could be divided into three groups, depending on the polyacrylamide based membrane pore size and buffer pH of the Gradiflow system. First, there is a group of proteins that are excluded from entering the membrane, which defines the membrane's practical pore limit [7,8]. Second, there is a group of proteins that are retarded in their migration through the membrane, and separation occurs based on differences in relative mobility. Finally, there is a group of proteins that have a similar relative mobility and rapidly migrate through the membrane as a single leading fraction.

Here we describe the method development for fractionation of proteins from complex protein mixtures by reflux electrophoresis using the Gradiflow. We present results on the purification of milk whey proteins from the tammar wallaby (*Macropus eugenii*). Following this separation, a reflux strategy was developed and applied to purify a recombinant glycoprotein expressed and secreted by *Dictyostelium discoideum.*

2. Materials and methods

2.1. Chemicals

2.1.1. Gradiflow buffers:

Tris–EDTA–boric acid (TEB; Gradipore, Sydney, Australia), stock solution 800 m*M* Tris–boric acid, 17 m*M* EDTA (pH 8.4); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; BDH, Poole, UK), stock solution 250 m*M* (pH 7.0); 2-(N-morpholino)ethanesulfonic acid (MES: Sigma, St. Louis, MO, USA), stock solution 250 m*M* (pH 5.5).

Reducing agent was β-mercaptoethanol (β-ME) (Sigma). Liquid acrylamide containing cross linking agent (N,N′-methylene bisacrylamide) were at 29:1 ratio (Bio-Rad, Hercules, CA, USA). Native sample buffer contained 12% (v/v) glycerol, 0.01% (w/v) bromophenol blue in 80 m*M* TEB (Gradipore). Reducing SDS sample buffer contained 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-ME and 0.01% (w/v) bromophenol blue in 100 m*M* Tris–HCl, pH 6.8. Acetonitrile was HPLC grade (Mallenckrodt, Paris, KY, USA) and trifluoroacetic acid (TFA) was peptide synthesis grade (Auspep, Melbourne, Australia).

2.2. Gradiflow apparatus

Three parallel membranes are used in the Gradiflow apparatus and protein separation takes place in the central membrane- separation membrane. The separation membrane is a thin supported polyacrylamide membrane, which lies perpendicular to the sample flow (15 ml min^{-1}). Two outer membranes – restriction membranes – separate sample from cooled running buffer. The membranes are inserted as a cartridge into a perspex separation unit parallel to the flow and electrodes. As sample enters the orthogonal electric field, proteins migrate to-

wards an electrode of opposite charge. Hence, it is possible to fractionate samples by both size and charge simultaneously. Fractionation only occurs within the central separation membrane, as the restriction membranes do not allow passage of proteins larger than M_r 4000. For an overview of operation of the Gradiflow see Ref. [9].

2.3. Gradiflow membranes

Reinforced polyacrylamide membranes were supplied by Gradipore. Separation membranes with apparent pore limits M_r of 25 000, 45 000, 60 000, 80 000 and 200 000 were used.

2.4. Preparation of samples for the Gradiflow

2.4.1. Wallaby whey samples

Tammar wallaby milk samples were kindly provided by Professor Des Cooper (Macquarie University). Milk samples were centrifuged at 12 000 *g* in an Eppendorf 5416 C microfuge (Hamburg, Germany) at 4°C, to separate the lipid, whey and casein components. Whey was collected after 15 min and re-centrifuged for 15 min. A 400-μl volume of whey was added to 3 ml 40 m*M* TEB (pH 8.4) prior to separation in the Gradiflow.

2.4.2. Recombinant PsA samples

A recombinant form of the *D. discoideum* cell surface antigen, PsA, was expressed in *D. discoideum* [10]. The molecule contains the first 95 amino acids of the native sequence commencing with Tyr–Asp–Tyr at the *N*-terminus and ending at the *C*-terminus with Ala–Pro–Thr [11]. For PsA1–95 expression, $2.5 \cdot 10^8$ transformed *D. discoideum* cells were added to 500 ml starvation buffer (20 m*M* MES buffer, pH 6.5) in a 2-l flask and rotated at 150 r.p.m. at 21±1°C. Following 24 h starvation, during which the PsA1–95 was secreted, cells were removed by centrifuging for 30 min at 8000 *g*, 4°C. Prior to loading onto the Gradiflow, supernatant was concentrated 10-fold to 50 ml using a Sartorius Easyflow unit (Göttingen, Germany) with a nominal M_r cut-off of 10 000.

The pH was adjusted by adding stock buffer to the PsA1–95 concentrate. Dialysis of samples was not necessary. For experiments carried out at pH 8.4, stock TEB buffer was added to a final concentration of 80 m*M*, 40 m*M* TEB (pH 4) was used as running buffer. For experiments carried out at pH 7.0, stock HEPES buffer was added to a final concentration of 50 m*M*; 50 m*M* HEPES (pH 7.0) was used as running buffer. For experiments carried out at pH 5.5, stock MES buffer was added to a final concentration of 50 m*M*; 50 m*M* MES (pH 5.5) was used as running buffer.

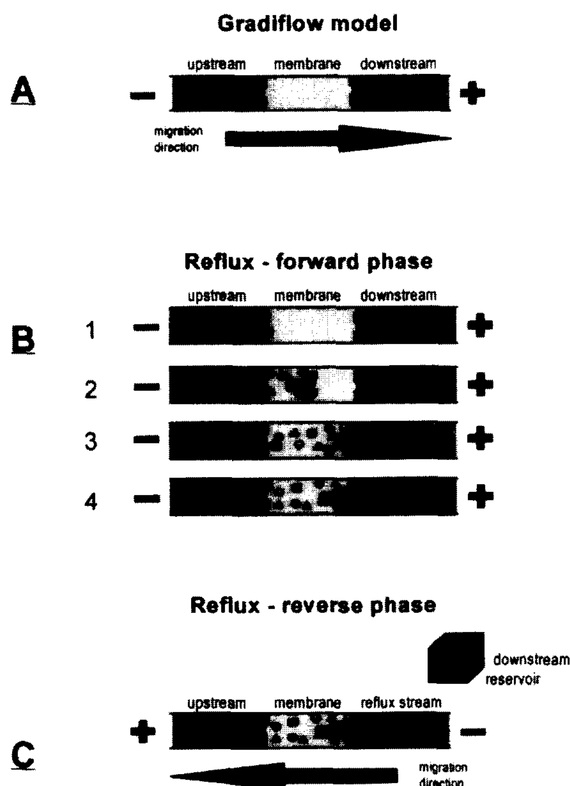

Fig. 1. (A) Normal mode of operation with the Gradiflow, where some proteins on one side of the membrane are required to migrate to the other side of the membrane, the downstream. The proteins required to migrate to the downstream may represent a single protein species or a group of different proteins. (B) In the forward phase proteins with the highest mobility migrate first into the downstream (steps 1–4). The forward phase is stopped before trailing, undesired, proteins enter the downstream. While the leading fraction is retained in a downstream reservoir, an alternate Reflux reservoir containing running buffer is temporarily connected to the downstream flow path. (C) The current is subsequently reversed to clear the membrane of trailing proteins. This is the reverse phase. To completely clear the membrane the reverse phase must always be longer than the forward phase. The next cycle is started after reconnecting the downstream reservoir.

2.5. Reflux electrophoresis

A detailed description of the reflux concept is shown in Fig. 1. For the separation of the wallaby whey proteins the buffer conditions were kept constant and two different membranes were used. The initial migration of candidate whey proteins was monitored using a continuous electrophoresis mode (CEM) of 250 V for 30 min. Samples (10 μl) were taken at fixed time intervals and fractions were analysed by 1-D PAGE. Following the analysis of fractions collected during the CEM, the reflux cycles were established for the forward phase and a reverse phase.

For the separation of recombinant PsA, a series of experiments were performed where membranes of different pore size were used with different buffer conditions. These experiments were performed under CEM, where 10-μl samples were taken at fixed time intervals and fractions were analysed as described above. From these results optimal reflux parameters were defined for a particular membrane and buffer, and times were selected for the reflux forward and reverse phases.

2.6. Analytical PAGE and gel staining

2.6.1. Wallaby whey samples

Samples were separated on a precast 10–30% SDS gradient gel under reducing conditions (100°C, 5 min) and on precast 5–40% native gradient gels, supplied by Gradipore. Prior to SDS-PAGE, samples were diluted 1:1 with reducing SDS sample buffer and boiled for 5 min. A 10-μl sample was loaded onto each lane. Native sample was diluted 1:1 with native sample buffer and 10 μl was directly loaded onto each lane. SDS gels were run with in 50 m*M* Tris–tricine–SDS (pH 8.5). Native running buffer was 80 m*M* TEB (pH 8.4). All gels with milk whey samples were run in a Micrograd electrophoresis apparatus from Gradipore.

2.6.2. Recombinant PsA samples

Samples were separated on a SDS-polyacrylamide gel under reducing conditions essentially as previously described [12], except using a 15% separating gel, which resolves PsA (M_r~10 000) well. Gels were cast and run on a Mini-Protean II apparatus from Bio-Rad, at constant voltage of 150 V. Prior to gel loading, samples were diluted 1:1 with reducing SDS sample buffer and boiled for 5 min. A 10-μl portion of reduced sample was loaded onto each lane.

Proteins were visualised by a silver stain method as described previously [13].

2.7. Chromatography

The purity of recombinant PsA separated by reflux electrophoresis was established using reversed-phase high-performance liquid chromatography (RP-HPLC) monitored on a SMART HPLC System

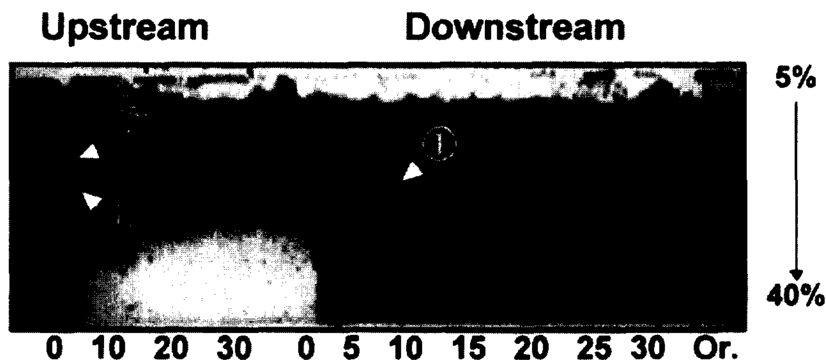

Fig. 2. Silver stained native 5–40% gradient PAGE of a time course fractionation of tammar whey. Conditions: continuous forward phase fractionation using TEB buffer (pH 8.4) with a 80 000 membrane. Key: numbers below gel indicate time (min) at which fraction was taken. Left 0–30, upstream fractions. Right 0–30, downstream fractions. Or, original sample. Proteins (1) and (2) were purified. Note that protein (1) migrates to the downstream within 10 min.

(Pharmacia Biotech, Uppsala, Sweden). Fractions (100 μl) of samples separated by the Gradiflow were chromatographed on a Sephasil C_8 SC2 1/10 column at a flow-rate of 100 $\mu l\ min^{-1}$. The separation was monitored at 214 nm and eluted with a gradient of 0–85% (v/v) acetonitrile in 0.05% (v/v) trifluoroacetic acid (TFA) over 60 min.

2.8. Protein sequencing

Native gels were electroblotted onto a PVDF (Bio-Rad) according to Ref. [14]. Protein bands were stained with 0.1% amido black and destained in water. The candidate bands were excised and sequenced using a LF3000 Protein Sequenator from Beckman Instruments (Fullerton, CA, USA).

3. Results

The protein complement of wallaby whey represents a challenging separation for the Gradiflow as several proteins have similar mobilities under native electrophoretic conditions. Two proteins were select-

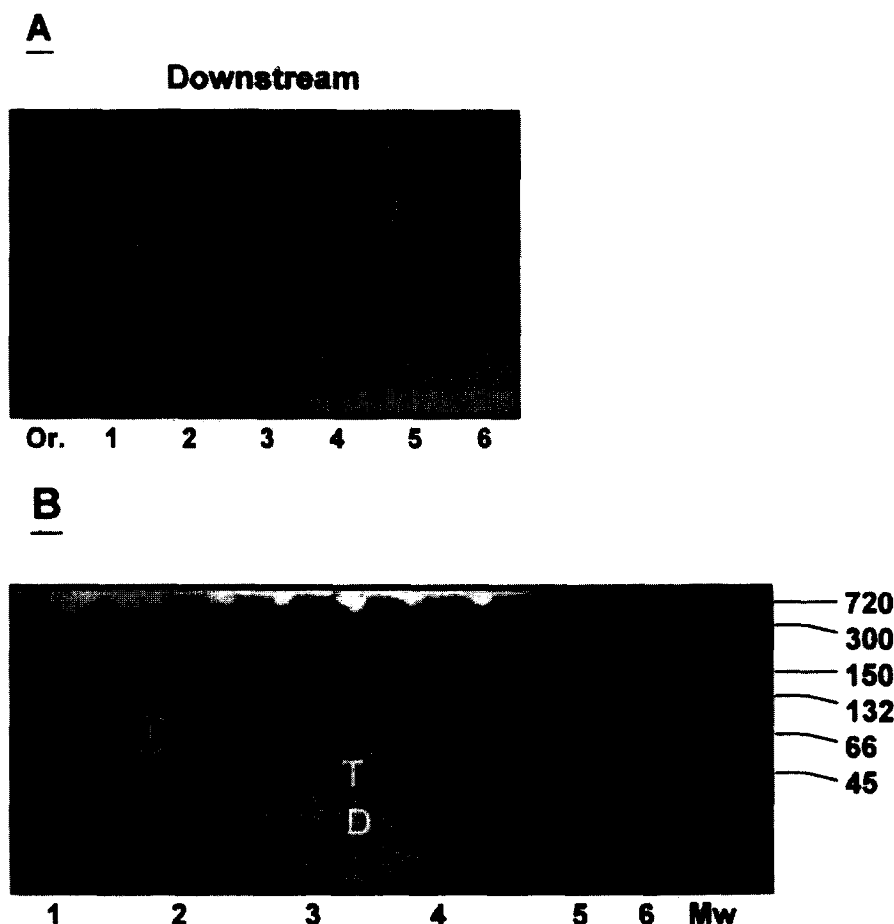

Fig. 3. Silver stained native 5–40% gradient PAGE. (A) First step in purification of protein (1) removal of lower molecular weight wallaby whey proteins. Repetitive forward phase collection in the downstream reservoir. Numbers 1–6 indicate forward phase. Or, original sample. (B) Second step in purification of target protein (1). 1 upstream at t_0 (retained in the upstream after 6 reflux cycles), 2 downstream at t_0, 3 upstream after 10 min forward phase, 4 downstream after 10 min forward phase, 5 downstream after second forward phase, 6 third reflux forward phase collected as a separate fraction. M_r markers (Gradipore), from top to bottom; α2-macroglobulin, α2-macroglobulin subunit, phycoerythrin, BSA dimer, BSA and ovalbumin. T and D on gel indicate the tetramer and the dimer of protein (1), see text for details.

ed to develop a reflux strategy [indicated as (1) and (2) in Fig. 2]. Both proteins were separated using a two-step procedure described in Section 2.5. First, all proteins with a faster mobility than the candidate protein are removed, and in the second step the candidate protein is collected as a product. Both steps use the reflux method, although the first step is on a group of proteins and the second step is only on the candidate protein.

The initial conditions of the Gradiflow were 40 m*M* TEB (pH 8.4), 250 V, and M_r 80 000 membrane. After 5 min the migration of a group of proteins was observed in the downstream that had an apparent lower M_r than protein (1) (Fig. 2). However, protein (1) was also observed to enter the downstream after 5 min. The optimal reflux conditions for removal of the apparently lower M_r proteins was therefore selected as a 4-min forward phase and a 6-min reverse phase (4F–6R). In Fig. 2 a significant transfer of protein (1) was observed in the downstream within 10 min. Hence, for the second step of the fractionation the forward phase was extended to 10 min and a reverse phase of 12 min (10F–12R) to capture the protein (1).

Fig. 3A shows the proteins that had migrated into the downstream after each forward phase, using the parameters discussed above for protein (1) (4F–6R). With each reflux cycle less protein was collected in the downstream and it was assumed that the amount of proteins with an apparent low M_r had diminished from the upstream compartment. Indeed, after 6 reflux cycles most of the proteins with a lower apparent M_r than protein (1) had been removed from the upstream (Lane 1, Fig. 3B). This allowed protein (1) to be collected in the downstream in the second reflux procedure (10F–12R, see Fig. 3B, lanes 4 and 5). Even after one forward phase most of protein (1) had entered the downstream. However, Fig. 3B (lanes 4 and 5) suggests that not all proteins with an apparent lower M_r than protein (1) had been separated in the first reflux procedure. This was shown not to be the case. When upstream and downstream samples equivalent to Fig. 3B lanes 3 and 4 (i.e. a single 10F phase) were run on denaturing SDS-PAGE, only one dominant band of apparent M_r 20 000 was identified in the downstream (Fig. 4 lane 4). This suggested that the M_r 20 000 protein was a monomeric form of the two bands labelled T and D

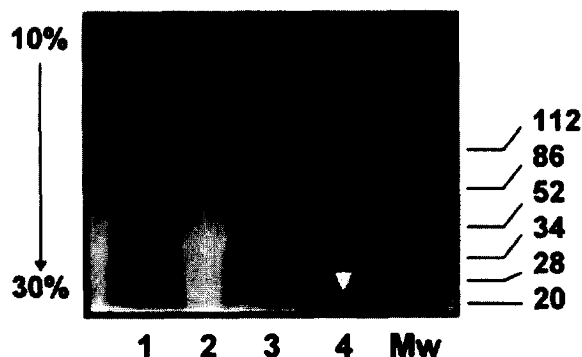

Fig. 4. Silver stain analysis of whey purification by reducing SDS-PAGE on a 10–30% gradient gel. Key: 1 upstream (ups) sample prior to fractionation of protein (1) (corresponding to lane 1 in Fig. 3B); 2 downstream (dos) at t_0; 3 ups after t_{10}; 4 dos final after 10 min forward phases (protein (1)). Note reduced apparent mass of major protein (1) in lane 4 is 18 000. Low range M_r markers from Bio-Rad.

in lane 4, Fig. 3B. β-Lactoglobulin (BLG) is known to occur in multiple oligomeric forms under native electrophoretic conditions [15], which suggested that protein (1) is a BLG tetramer (Fig. 3B, T) which had partially dissociated into a dimer (Fig. 3B, D).

Following purification of protein (1) we attempted to purify protein (2). As it was not possible to monitor fractionation in real time during Gradiflow operation, we could not assume that all proteins with an apparent lower M_r than protein (2) had migrated out of the upstream. Therefore a preliminary reflux run was performed to remove the proteins left in the sample that had an apparent lower M_r than protein (2) using a 100 000 membrane. The larger pore size membrane was preferred to the smaller membrane (80 000) in order to maintain a rapid run time; i.e., an 80 000 membrane would retard proteins more than the 100 000 membrane. Reflux cycles were set at 10F–12R, and were repeated four times. As a result, proteins of an apparent lower M_r than protein (2) were collected in the downstream [Fig. 5, A and B, group (3)]. The second reflux procedure was extended to 12.5F–14R and protein (2) was purified after four forward reflux cycles (Fig. 6). Protein (2) had an apparent mass of 100 000 (Fig. 6) under native conditions and 88 000 under reducing conditions (Fig. 7).

The overall run time for the purification of both proteins (1) and (2) was within 5 h: for protein (1)

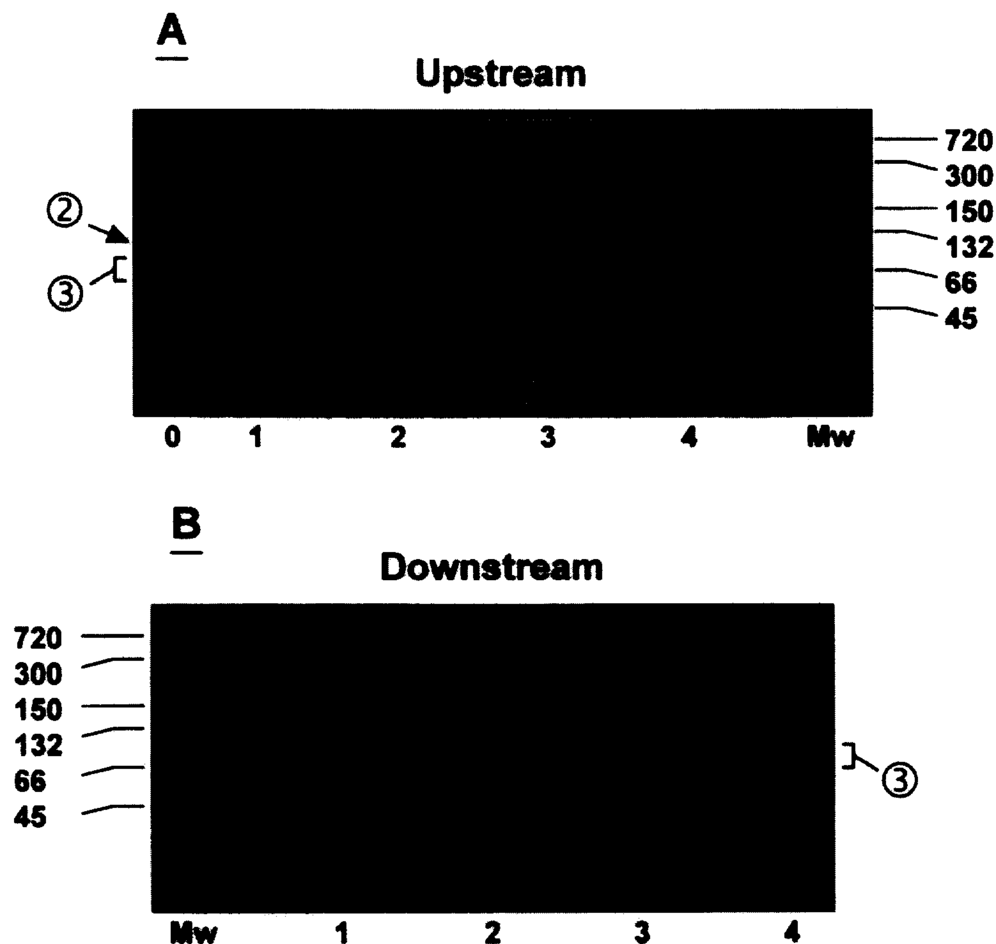

Fig. 5. Silver stained native 5–40% gradient PAGE of partially fractionated wallaby whey proteins. Removal of smaller proteins before purification of protein (2). The group of smaller proteins have been indicated as (3). The gels show that by the second forward phase, the smaller contaminating proteins have been removed, lane 2 gel A. A+B Key: numbers 1–4 indicate fractions after forward phases 1–4 respectively. Unmarked lanes are the following reverse phase fractions. M_r markers, as in Fig. 3. 0 upstream at t_0, which is the same as upstream at end of run from previous step (Fig. 3B, lane 3).

104 min was needed and for protein (2) an extra 194 min. The purified samples were subsequently run on native PAGE and electroblotted onto a PVDF membrane and subjected to Edman degradation. Protein (1) was shown to have a dominant signal V E N I R which matched to BLG from tammar wallaby [16], while a minor signal of D A P K S E matched to tammar wallaby albumin [17]. It was not surprising to see two signals as both proteins migrated the same distance under the native-PAGE conditions (compare protein (1) in lane 4 and BSA in lane 'M_r markers' in Fig. 3B). Indeed, SDS-PAGE revealed that three other proteins were co-purified with BLG, one of which was tammar albumin; for the other two no sequence data was obtained (Fig. 4). Protein (2) had an amino terminal sequence of N D I G P G F W for which no match was found against any database entries (Swiss-Prot rel. 34 or PIR rel. 49). This is not unexpected as the marsupial genome is poorly defined.

The separation of whey proteins demonstrated the potential of reflux electrophoresis where the reflux cycle time and membrane type are varied to collect different proteins. This approach suggested to us that

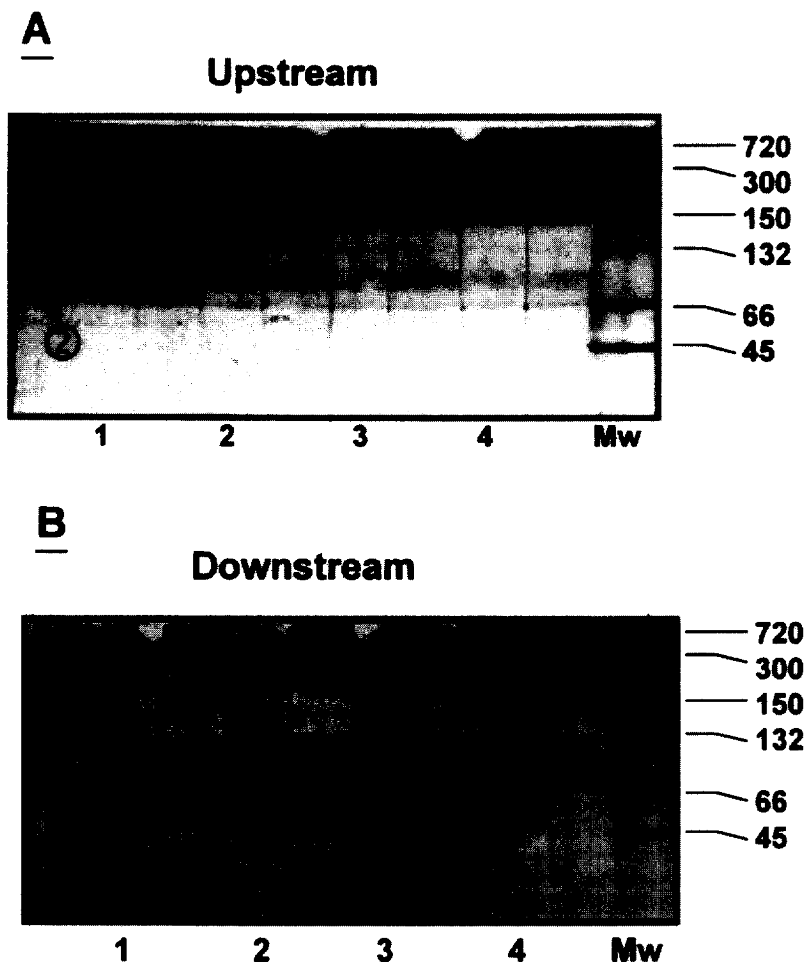

Fig. 6. Silver stained native 5–40% gradient PAGE of the purification of target protein from wallaby whey after removal of smaller contaminating proteins, shown in Fig. 5. (A) Decrease of protein (2) after each forward phase in upstream, numbers 1–4 indicate cycle. M_r markers, as in Fig. 3. (B) Accumulative collection of target protein (2) in downstream reservoir. Key: numbers 1–4 indicate the forward phase. M_r markers, as in Fig. 3.

the strategy to rapidly define optimal reflux conditions (i.e., membrane type, pH and cycle times) would be to monitor sample fractionation during CEM with a range of different pore size membranes at different pH values.

This strategy was then applied to purify recombinant PsA1–95. Samples (6 ml) of ten-fold concentrated MES buffer supernatant containing PsA1–95 were used to define optimal reflux conditions (separation conditions are summarised in Table 1). CEM was performed and the migration of PsA1–95 from the upstream to the downstream was monitored by SDS-PAGE, where fractions were collected at 1-min intervals for 15 min. PsA1–95 showed moderate to poor transfer at pH values less than 8.4, which indicated a relatively low charge-to-mass ratio. Rapid transfer into the downstream was observed with three membranes at pH 8.4 (Table 1). The greatest difference in PsA1–95 transfer, relative to other trailing proteins, was observed with an M_r 45 000 membrane (Table 1). The reflux forward phase was selected at 6 min as trailing components started to enter the downstream after this stage. Hence for PsA1–95 purification the optimal reflux

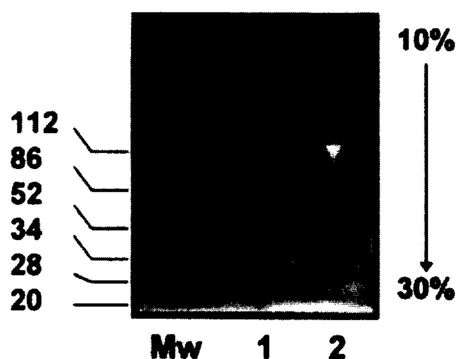

Fig. 7. Silver stained 10–30% gradient SDS-PAGE. Analysis of whey purification by reducing SDS-PAGE on a 10–30% gradient gel. Key: 1 ups prior to fractionation of protein (2) at t_0; and 2 purified protein (2). Apparent molecular mass of protein (2), lane 2 is 90 000. Low range M_r markers from Bio-Rad.

electrophoretic conditions were chosen as reflux cycles of 6-min forward phase and 7-min reverse phase (6F–7R) using a 45 000 membrane at pH 8.4.

Having established reflux conditions PsA1–95 was purified from 500 ml of starvation buffer supernatant which had been concentrated to 50 ml. Samples were taken after each forward phase and analysed by SDS-PAGE. Fig. 8 shows the transfer of PsA1–95 into the downstream. In addition, immuno-dot assays, using a PsA-specific monoclonal antibody, MUD1, indicated that approximately 70% had migrated to the downstream after 20 reflux cycles (data not shown). Quantitation by amino acid analysis [18] showed that approximately 0.5 mg PsA1–95 was recovered, which was consistent with expression levels of 1.5 mg l^{-1}. RP-HPLC was used on an analytical scale to assay the purity of PsA1–95 (see Fig. 8, lane P) (Fig. 9). Due to partial glycosylation (Collins et al. in preparation), the Gradiflow product appears as two bands on the SDS gel.

4. Discussion

Previously we have introduced the Gradiflow and described its use in continuous mode protein fractionation [5,9]. In addition, we have reported the reflux electrophoresis concept and demonstrated that time-dependent separation can be achieved by controlling forward and reverse phases [6]. Here we have applied reflux electrophoresis to two samples.

We have purified two milk whey proteins from tammar wallaby. The tammar whey proteins demonstrate that the method is applicable to proteins which are not the smallest protein, or protein with the highest mobility, from a complex mixture of proteins. We have also shown the purification of a recombinant glycoprotein, secreted from the eukaryotic expression system *D. discoideum*, in a single pass. The recombinant form of (PsA is 10 000 and has a theoretical p*I* of 4.18 [19]. PsA1–95 appears to be the smallest abundant protein in the starvation buffer (Fig. 8) and is secreted at approximately 1.5

Table 1
Optimisation of conditions for reflux electrophoresis of PsA1–95

Membrane operating range (M_r)	Operating pH 8.4		7.0		5.5	
	PsA1–95	Other	PsA1–95	Other	PsA1–95	Other
25 000	+	–	nd		nd	
45 000	+++	–	–	–	–	–
60 000	+++	++	++	+	–	–
80 000	+++	++	++	+	–	–
200 000	nd		nd		+++	+++

The effect of different pH and membrane porosity on PsA1–95 mobility relative to trailing larger 'other' proteins was monitored by running concentrated supernatant continuously for 15 min and visualising fractionation by SDS-PAGE.
+=slow, ++=medium and +++=rapid transfer rate across membrane, –=no transfer and nd=not determined.
Transfer was qualitatively analysed by silver stained gels, where transfer between PsA and larger proteins was compared. +++ indicates dark protein band, + faint band and – no bands visible.

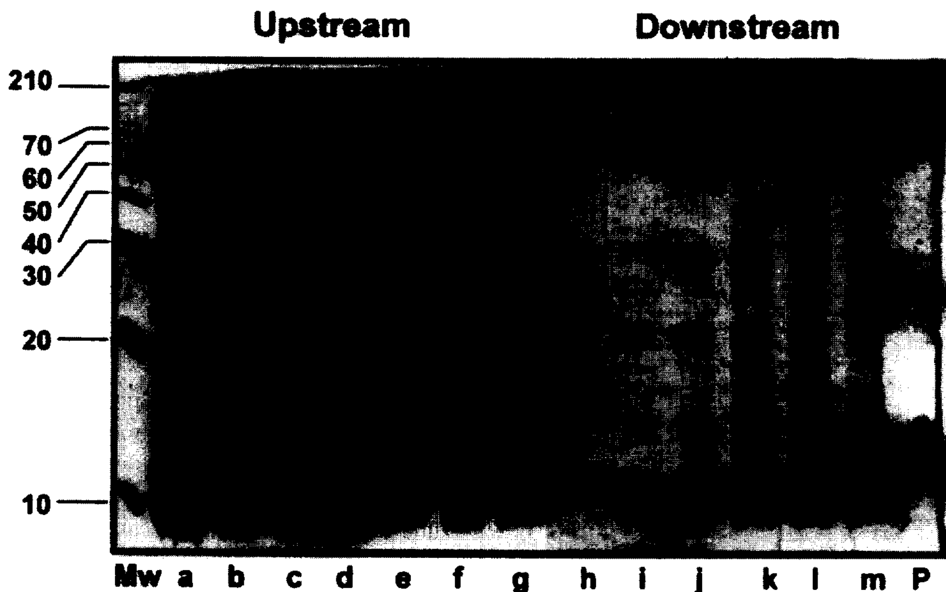

Fig. 8. Silver stained 15% SDS gel showing results from reflux electrophoresis of PsA1–95. Reflux electrophoresis was performed at pH 8.4 using a 45 000 membrane and twenty 67–FR cycles. Key: M_r 10 000 protein ladder (Gibco BRL); a=original sample; then samples after b one, c six, d eleven, e sixteen, f nineteen and g twenty cycles; and h downstream after one, i six, j eleven, k sixteen, l nineteen and m twenty cycles; P pure PsA1–95 obtained after further RP HPLC of Gradiflow product (Fig. 9) appears as two bands, due to partial glycosylation [11].

mg l^{-1}, which is between 2–4% of the total secreted protein concentration (data not shown). Therefore, it was a good candidate to test our reflux method for research scale preparative purification. Based on this work we propose a general operation strategy for purification of proteins reflux electrophoresis.

To successfully purify a protein, knowledge of the protein of interest must first be gained. Generally,

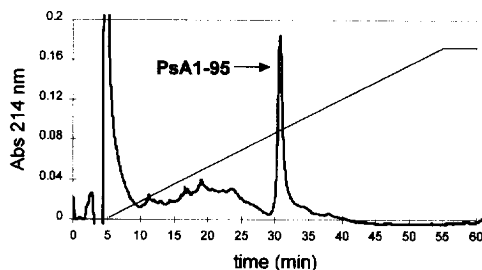

Fig. 9. Reverse-phase chromatogram of Gradiflow sample of PsA1–95 after final cycle of reflux electrophoresis (as described in Fig. 9). Chromatography performed on a SMART HPLC system with a Sephasil C_8 SC21/10 column using 100 μl of Gradiflow product. PsA1–95 was eluted with a linear gradient of 0–85% (v/v) acetonitrile in 0.05% (v/v) TFA. Flow-rate was 100 μl min^{-1}.

information describing physical characteristics of a protein is useful, as is information about other proteins in the preparation which are likely to frustrate attempts at purification. However, data generated by techniques other than native electrophoresis may be ambiguous. While molecular mass estimations from SDS-PAGE, size-exclusion chromatography, or gene sequence information are useful, they do not represent the equivalent size of a protein in native electrophoretic conditions. Likewise, p*I* estimates from isoelectric focusing (IEF) using immobilised pH gradients, ampholytes or amino acid sequence information may also be misleading. The surface-charge density of a protein is not apparent from the p*I* while it is an important parameter in native electrophoresis [20,21], as it is indicative of the rate of transfer through Gradiflow membranes. Therefore, we use p*I* and M_r only as a general indicators in our purification methods. Recombinant PsA is a good example of how proteins migrate unexpectedly, i.e., a 45 000 membrane at pH 8.4 was needed for successful purification of a 10 000 protein, p*I* 4.18. Hence, to rationalise protein parameters for the Gradiflow, we suggest performance of a

series of simple experiments where optimal conditions are determined by monitoring protein migration at different pH values with a set of membranes varying in pore size.

Although the Gradiflow separation membranes are only 0.1-mm thick, proteins in simple mixtures may still separate into discrete parallel bands while migrating through the separation membranes (zonal separation). However, zonal separation of proteins from complex biological mixtures is a difficult task as proteins of similar electrophoretic mobility can frustrate purification attempts by entering the downstream before the target protein has been completely fractionated. We expect this to be partially due to the orthogonal flow path of the sample to the membrane and electric field; i.e., not all the sample can enter the membrane at the same time. To prevent comigration of proteins reflux electrophoresis has been proposed [6].

In summary, we suggest the following strategy for Gradiflow operation. First, perform pore limit electrophoresis (PLE) in a native gradient gel [7,8] and perform a time-dependent analysis of the protein migration in a gel of low acrylamide concentration (<10%T; %T=acrylamide cross linking agent in g/100 ml [22]). From PLE and low concentration gels, information on approximate native M_r and relative mobility can be obtained, respectively. With the size and mobility known, a useful series of membranes can be chosen to monitor protein migration at a range of pH values.

Yield, resolution and speed are important in any purification process. The applications described here demonstrate the feasibility of the reflux method for preparative protein purification. In addition, we have been able to purify β-lactoglobulin multimers, indicating the ability to purify proteins in their native conformation. A Gradiflow apparatus with Windows based software is under development providing automated control. This system will overcome the task of manual reflux, allowing rapid method development for defining optimal conditions and longer processing times, resulting in higher yields.

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