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Development of a novel ampholyte buffer for isoelectric focusing: electric charge-separation of protein samples for X-ray crystallography using free-flow isoelectric focusing

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The purification of biological macromolecules has remained a severe bottleneck in three-dimensional structure determination in the field of protein crystallography. By the use of the Free Flow Electrophoresis (ProTeam FFE; TECAN Group Ltd) apparatus, target protein samples can be purified in a high yield, while a preparative separation is performed in solution without using solid matrices such as polyacrylamide gel. A novel ampholyte buffer suitable for crystallization in the use of the ProTeam FFE apparatus has been developed. This buffer is able to generate pH gradients owing to the use of low-molecular-weight electrolytes. The effect of the flow rate on the pH gradient using the novel ampholyte buffer has been elucidated. When the flow rate was lowered to 78 ml h^{-1}, the pH gradient in the electrophoretic chambers was generated in an almost linear fashion.

1. Introduction

The determination of the structure of biological macromolecules by X-ray crystallography consists of five major steps: gene cloning and expression, purification, crystallization, phase determination and refinement. Due to recent technological advances in the measurement of X-ray diffraction data and in structure-analysis techniques in this field, it is now possible to solve phase problems and to rapidly refine crystal structures. On the other hand, the so-called 'wet work' such as gene cloning and expression, purification and crystallization, remain time-consuming. In particular, the crystallization of proteins still depends on trial-and-error efforts. To achieve the straightforward crystallization of biological macromolecules, the purification of target samples to a degree of highly homogeneous states is indispensable. Column chromatography is usually employed for performing purification with high homogeneity, not only to a single-band state examined by SDS–PAGE, but also to a state of a single dispersive electric charge. However, column chromatography has known disadvantages such as the loss of target proteins during the procedure. In addition, it is often laborious to optimize the buffer type and flow rate to achieve the most effective separation of target proteins. The isoelectric focusing technique is an alternative means of obtaining electrically uniform protein samples. According to the conventional procedure followed when applying this technique, pH gradients are essential for additional separation steps. The target protein migrates to the position that is equivalent to its isoelectric point (p*I*) under the influence of an electric field. Each ampholyte focuses on a different pH position, due to the variety of properties associated with different p*I* values. Isoelectric focusing with support media such as a gel matrix has been widely used as an analytical method in the field of biochemistry, since it has high resolving ability. However, the development of isoelectric focusing for preparative separation has remained difficult due to the effects of natural convection, which cause a reduction in the separating resolution (Hanning, 1961; Egen *et al.*, 1984; Bier *et al.*, 1989; Balmann & Sanchez, 1991; Balmann *et al.*, 1991; Yonemoto *et al.*, 1995; Baygants *et al.*, 1997). Commercially available equipment used for preparative isoelectric focusing (*e.g.* Isoprime; Hoefer) uses p*I*-selective membranes to achieve pH gradients (Righetti & Gelfi, 1984; Righetti *et al.*, 1987, 1989; Wenger *et al.*, 1987). The p*I*-selective membranes

that contain polyacrylamide gel possess a great capacity for separation. However, the preparation of such membranes remains a time-consuming and difficult task. The free-flow type of isoelectric focusing equipment, such as the Free Flow Electrophoresis (ProTeam FFE) apparatus, which is commercially available from TECAN, can make use of the fractions in the 96-well plates under native or denatured conditions. The ProTeam FFE is able to afford high-yield separation because it is free from solid matrices such as polyacrylamide gel. In fact, the ProTeam FFE can be employed for studies involving the preparative separation of proteins from organelles and membrane vesicles, as well as those from lipophilic membranes (Kuhn *et al.*, 1990; Kasicka *et al.*, 1994; Weber & Bocek, 1996).

However, the ProTeam FFE uses 'Prolyte' reagents consist of individual low-molecular-weight acids and bases to achieve pH gradients (Weber & Bocek, 1996; Weber & Bauer, 1998). Since the Prolyte reagents also contain viscous hydroxypropylmethylcellulose, it is difficult to completely remove the Prolyte reagents from the purified proteins. The contamination of Prolyte reagents in protein solutions may negatively affect crystal growth. In addition, the complete composition of the Prolyte reagents is unknown, as is that of other commercially available reagents used for preparative isoelectric focusing such as Ampholine (Amersham Pharmacia) and Biolyte (Bio-Rad). There also remains the possibility that some ingredients included in the ampholytes could affect the biological activity of the target proteins.

For these reasons, we developed a novel ampholyte buffer that is useful for separation by isoelectric focusing and is also suitable for experiments involving further crystallization. This ampholyte can generate pH gradients consisting of natural and synthesized electrolytes of low molecular weight. In the present report, we demonstrate that our novel ampholyte can form a pH gradient that is useful for protein separation. Moreover, we have performed isoelectric focusing experiments using this novel ampholyte together with a detergent that is useful for the separation of membrane proteins.

2. Construction of the ProTeam FFE

Fig. 1 schematically depicts an overview of the ProTeam FFE apparatus (Burggraf *et al.*, 1995; Weber & Bocek, 1996). The electrophoretic device consists of an electrophoretic chamber and an anodic and a cathodic chamber. Cation- and anion-exchange membranes are used for the separation of the electrophoretic chamber from the electrode chambers. The size of the electrophoretic chamber is 500 mm high, 100 mm wide and 0.4 mm thick. The electrode length is 500 mm high. The electrophoretic chambers are created by the insertion of a polyvinylchloride spacer between the separation plates. The ampholytes are introduced into the seven inlets of the apparatus from the running buffer reservoir and the protein-sample inlets. Applying a potential difference, cation and anion species migrate toward the cathodic and anodic sides, respectively. At the same time, protons in the anolyte and hydroxide ions in the catholyte permeate through the cation and anion-exchange membranes, respectively, and into the electrophoretic chamber in order to satisfy the condition of electroneutrality. The pH gradient is then formed in the electrophoretic chamber. Each protein gradually focuses at a different pH position according to its respective p*I* value. Focused protein samples were collected into 96-well plates *via* an in-line multi-channel outlet.

Here, the electrode solutions used were 50 m*M* H_2SO_4 (anode) and 100 m*M* NaOH (cathode). The counterflow solution and the electrolyte solution were 20%(*v*/*v*) glycerol and 5 m*M* KCl, respectively. The ampholytes were 2–5 m*M* L-aspartic acid (p*I* 2.77), 0.5–5 m*M* L-glutamic acid (p*I* 2.82), 1–5 m*M* *m*-aminobenzoic acid (p*I* 3.93), 1–15 m*M* L-cysteine (p*I* 5.07), 1 m*M* pentaglycine (p*I* 5.32), 1–15 m*M* glycylglycine (p*I* 5.60), 1–5 m*M* D-cycloserine (p*I* 5.9), 5 m*M* histidylglycine (p*I* 6.81), 5–10 m*M* L-histidine (p*I* 7.47), 5–10 m*M* L-carnosine (p*I* 8.17), 5 m*M* anserine (p*I* 8.27), 1–5 m*M* lysine (p*I* 9.74) and 2–10 m*M* L-arginine (p*I* 10.76). In addition, 2%(*v*/*v*) Tween-20 was used as a non-ionic detergent.

3. Development of a novel ampholyte buffer for crystallization

3.1. Criterion for choosing an ampholyte buffer

In the 1960s, Svensson investigated whether or not it was possible to use amino acids and other simple ampholytes to establish a pH gradient (Svensson, 1962). In addition, he determined which ampholytes would be suitable for this purpose. The degree of ionization of the ampholyte and its buffering ability at or near its own p*I* was found to depend on the value obtained by $pI - pK_1$. Recognizing them as poor ampholytes, Svensson excluded all those with the property $pI - pK_1 > 2.5$.

In the present study, we selected suitable amino acids by the following criteria. We initially chose aspartic acid, the most acidic ampholyte, and arginine, the most basic ampholyte, in Svensson's list of acceptable ampholytes (Svensson, 1962). Both amino acids were always included as the content of two inlets among seven inlets in all further combinations. Then, amino acids with p*I* values between the pH value of aspartic acid and that of arginine were chosen five

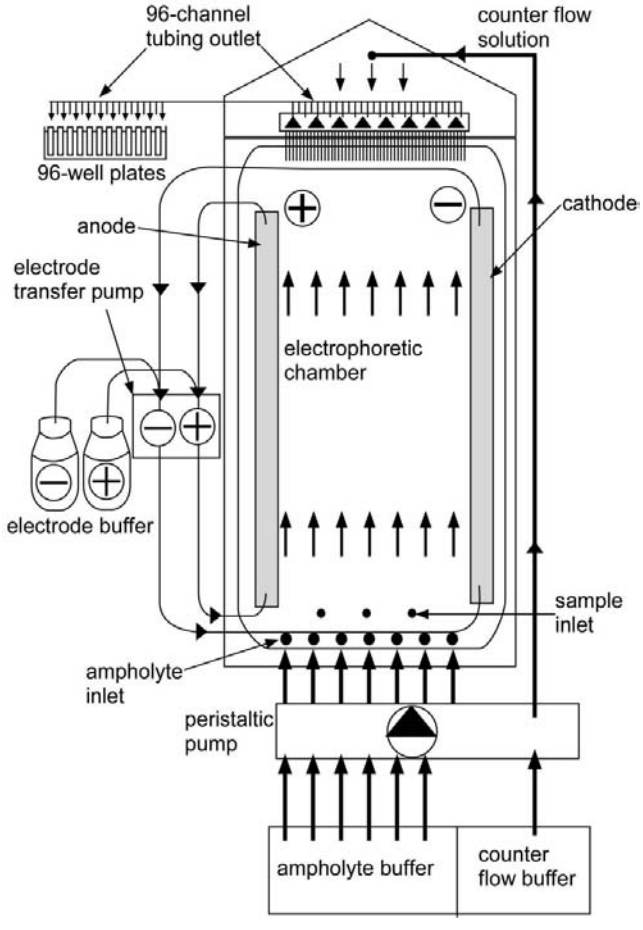

Figure 1
Schematic overview of the ProTeam free-flow electrophoresis apparatus.

Table 1
The combinations of amino acids used in this experiment.

The values in parentheses are data for the isoelectric point.

No. 1	No. 2	No. 3	No. 4	No. 5
L-Aspartic acid (2.77)	L-Aspartic acid (2.77)	L-Aspartic acid (2.77)	L-Aspartic acid (2.77)	L-Aspartic acid (2.77)
L-Glutamic acid (2.82)	L-Glutamic acid (2.82)	*m*-Aminobenzoic acid (3.93)	*m*-Aminobenzoic acid (3.93)	*m*-Aminobenzoic acid (3.93)
Pentaglycine (5.32)	D-Cycloserine (5.90)	D-Cycloserine (5.90)	L-Cysteine (5.07)	Glycylglycine (5.60)
L-Histidine (7.47)	L-Histidine (7.47)	L-Histidine (7.47)	L-Histidine (7.47)	Histidylglycine (6.81)
L-Carnosine (8.17)	L-Carnosine (8.17)	L-Carnosine (8.17)	L-Carnosine (8.17)	Anserine (8.27)
L-Lysine (9.74)	L-Lysine (9.74)	L-Lysine (9.74)	L-Lysine (9.74)	L-Lysine (9.74)
L-Arginine (10.76)	L-Arginine (10.76)	L-Arginine (10.76)	L-Arginine (10.76)	L-Arginine (10.76)

remaining inlets. As regards these latter amino acids, they were chosen such that their pH values were in approximately equal pH intervals among the seven amino acids. Finally, we chose amino acids with $pI - pK_1$ values that were as small as possible. The selected combinations of amino acids used in this experiment are shown in Table 1. After the examination of these five combinations of amino acids in Table 1, it was found that combination No. 3 reflected the best performance and this combination was employed for additional experiments (data not shown).

Isoelectric focusing was performed on the ProTeam FFE apparatus in the standard manner, according to the manufacturer's instructions. The ampholyte buffers were introduced into the FFE apparatus at 78–225 ml h^{-1} through all of the seven inlets at the bottom of the electrophoretic chamber. The maximum value of the electric power is set to 50 W. The current and the applied voltage were drifted by the influence of the components in the used buffer. The upper limit of the applied voltage was then set to 1500 V. The retention time in the electrophoretic chamber for the sample was about 30–10 min, which corresponded to the flow rates of 78–225 ml h^{-1}. After stabilizing the separation for the retention time, the separated ampholytes were collected *via* the counterflow solution through the 96-channel tubing outlet. The volume of the collected fraction was approximately 1.5 ml. The pH levels of the fractions were measured with a pH electrode.

3.2. Effect of flow rate on the pH gradient

Fig. 2 illustrates the relationship between the flow rate and the pH gradient. The ampholytes contained 2 m*M* L-aspartic acid, 1 m*M* *m*-aminobenzoic acid, 1 m*M* D-cycloserine, 5 m*M* L-histidine, 5 m*M* L-carnosine, 1 m*M* lysine and 2 m*M* L-arginine. The collectors were numbered from the anode side. When the flow rate was set to 225 ml h^{-1}, a mild gradient of pH 1.5–3.0 was generated in the range of fractions 1–50, whereas the gradient of pH 3.0–12.5 in the range of fractions 51–96 was steep. When the flow rate was set to 158 ml h^{-1}, the pH gradient in the range of fractions 1–96 was similar to that obtained when the flow rate was set to 225 ml h^{-1}. Moreover, when the flow rate was set to 78 ml h^{-1}, the pH gradient in the range of fractions 1–96 was almost linear. This result was due to the fact that the corresponding flow rate became smaller when the retention time increased. Therefore, when the retention time increased, each ampholyte under the influence of an electric field moved to its own p*I* position more precisely. However, both the applied voltage (315 V) and the current (38 mA) were insufficient for protein separation. It was concluded that more than 500 V should be applied for protein separation.

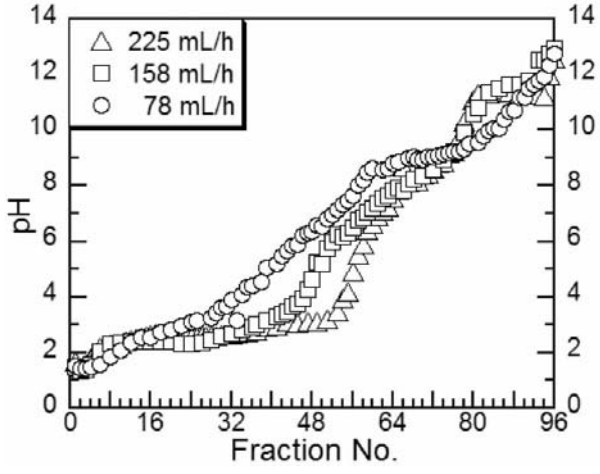

Figure 2
Effect of flow rates on pH gradient.

3.3. Effect of the ampholyte concentration on the pH gradient

The concentration of the ampholyte was increased in order to raise the applied voltage. The flow rate was set to 78 ml h^{-1}, which generated a sufficient retention time. The ampholytes contained 5 m*M* L-aspartic acid, 5 m*M* *m*-aminobenzoic acid, 5 m*M* D-cycloserine, 10 m*M* L-histidine, 10 m*M* L-carnosine, 5 m*M* lysine and 10 m*M* L-arginine. Moreover, in order to separate membrane proteins that were used for crystallization, some detergents were added to each ampholyte. A non-ionic detergent, Tween-20, was employed because it was not ionized in electrolysis. The concentration of Tween-20 was set at 2%(*v*/*v*), which was more than the critical micelle concentration (CMC; 0.08%). Fig. 3 shows how the concentration of

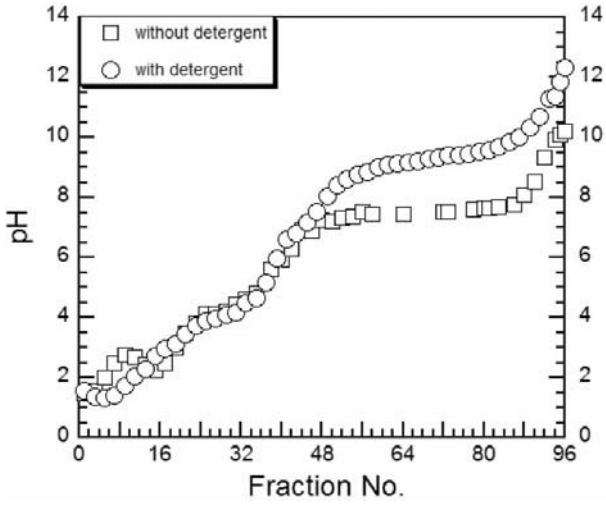

Figure 3
Effect of the ampholyte buffer concentration, with or without detergent, on the pH gradient.

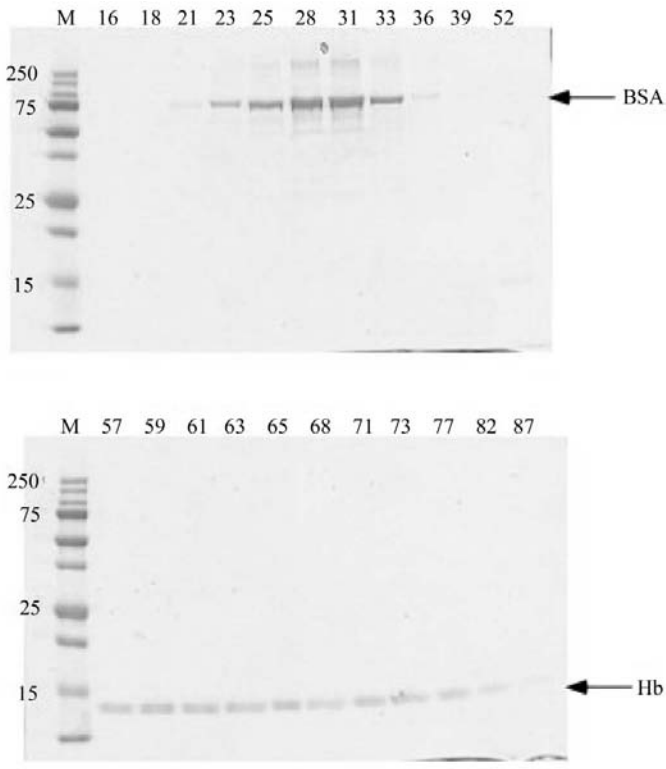

Figure 4
SDS–PAGE analysis of the binary mixture proteins. The lane number shows the channel number.

ampholytes in the presence and absence of detergent affected the pH gradient. When the detergents were used, the pH gradient of fractions 1–96 was almost linear. In the absence of detergent, the pH gradient of fractions 1–48 was similar to that observed when the detergent was used, whereas the pH value in fractions 48–88 remained neutral, *i.e.* approximately pH 7.5. Moreover, the pH values of fractions 89–96 became lower when it was used with detergent. pH 7.5 is close to the isoelectric point of L-histidine (p*I* 7.47) and L-carnosine (p*I* 8.17). Therefore, this result may be due to the higher concentration of L-histidine and L-carnosine.

3.4. Validation of the novel ampholyte buffer

We evaluated the performance of the novel ampholyte buffer under the same experimental conditions without detergents described in §3.3. The binary mixture consisting of 5 mg ml^{-1} bovine serum albumin (BSA) and 5 mg ml^{-1} bovine hemoglobin (Hb) was used as a test sample. The binary mixture was introduced into the apparatus from the third protein sample inlet and the flow rate was then set to 0.77 ml h^{-1}. After running using FFE, the two proteins in the mixture were successfully separated as shown in the SDS–PAGE analysis (Fig. 4). BSA was separated in fractions 21–36, whereas Hb was in fractions 52–87.

4. Conclusions

In this study, we developed a novel ampholyte buffer for isoelectric focusing that was found to be useful for use with the ProTeam FFE apparatus. Since the present ampholytes consisted only of low-molecular-weight amino acids, they were easily removed from the target proteins after separation. According to the aims of a particular study, linear or stepwise pH gradients can be generated for the apparatus using these ampholytes by adjusting the concentration of the ampholytes. This ampholyte buffer is also applicable for the purification of membrane proteins with detergent and for zone electrophoresis using a homogenous buffer system.

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