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Influence of Ionic Strength, pH, and SDS Concentration on Subunit Analysis of Phycoerythrins by SDS-PAGE

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Abstract Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is often used for subunit analysis of proteins, but it is not efficient to make the α- and β-subunits of phycoerythrins separated by normal SDS-PAGE. In this research, subunit components and subunit molecular weights of four purified phycoerythrins were analyzed by SDS-PAGE. Four factors including Tris concentration, pH, ammonium persulfate (APS), and SDS concentration were studied for their effects on SDS-PAGE of phycoerythrins. It showed that these factors can influence the separation of α- and β-subunits, electrophoresis effect of γ-subunits, apparent molecular weights of subunits, and mobility of marker proteins. The α- and β-subunits separated better in the case of lower SDS concentration, lower Tris concentration, higher pH, and/or lower APS addition in separating gels. The molecular weights of α- and β-subunits increased when Tris concentration increased in a certain range. It can be concluded that factors critical to subunit analysis by SDS-PAGE are SDS concentration and ionic strength, both of which are related to critical micelle concentration of SDS and ratio of SDS monomer to micelle in SDS-PAGE system. The ratio is postulated to influence SDS-PAGE by influencing the amount of SDS bound to polypeptides and shapes of polypeptide–SDS complexes.

Keywords Red alga · Phycoerythrin · SDS-PAGE · Subunit · Ionic strength · pH · SDS concentration

Abbreviations

PE	Phycoerythrin
PC	Phycocyanin
PEC	Phycoerythrocyanin
APC	Allophycocyanin
PEB	Phycoerythrobilin
PUB	Phycourobilin

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PCB Phycocyanobilin
PXB Phycobiliviloin
APS Ammonium persulfate

Introduction

Phycobiliproteins are water-soluble proteins and major light-harvesting pigments found widely in red algae and cyanobacteria [1, 2]. Phycobiliproteins are divided into four classes based on their absorption properties: phycoerythrins (PE, λ_{max} ~565 nm), phycocyanins (PC, λ_{max} ~620 nm), phycoerythrocyanin (PEC, λ_{max} ~575 nm), and allophycocyanins (APC, λ_{max} ~650 nm) [2–6]. Phycoerythrins can be divided into three main classes depending on their absorption spectrum: B-phycoerythrin (peaks at 545 and 565 nm and a shoulder at 499 nm), R-phycoerythrin (peaks at 499 and 565 nm and a shoulder/peak at ~545 nm), and C-phycoerythrin (peak at 565 nm) [7]. R-PE and B-PE are found in red algae, while C-PE is found in cyanobacteria. R-PE and B-PE are usually composed of three subunits, α, β and γ, and their structures are generally described as hexameric aggregates $(\alpha\beta)_6\gamma$ or $(\alpha\beta)_3\gamma(\alpha\beta)_3$ [2, 6, 8]. R-PE may have a linker polypeptide or a second subunit γ [9, 10].

Each subunit of phycoerythrins commonly contains one or more chromophores (phycobilins) bound to specific cysteines in apoprotein chains by thioether bonds. Outstanding absorption and fluorescence properties of phycobiliproteins in visible region originate from phycobilins and their interactions within polypeptide chains [11, 12]. Eight different phycobilins were found in phycobiliprotiens from red algae and cyanobacteria [13]. The most representative phycobilins are phycoerythrobilin (PEB), phycourobilin (PUB), phycocyanobilin (PCB), and phycobiliviloin (PXB). PEB is found in C-PE, R-PE, B-PE, and R-phycocyanin (R-PC), PUB is found in R-PE and B-PE, PCB is found in PC, AP, and PEC, and PXB is only found in PEC [11, 12]. Types of phycobiliproteins in organisms and phycobilins they carry depend on the species of origin, but the phycobilin-binding sites are quite conserved in phycobiliprotein evolution [11–13].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is often used for the subunit analysis of proteins, and the separating gel commonly used contains 0.375 M Tris–HCl (pH 8.8) and 0.1% SDS [14, 15]. But this SDS-PAGE system is not efficient for the subunit analysis of phycoerythrins; for example, the α- and β-subunits cannot be satisfactorily separated, and they often appeared in a broad band [12, 16, 17]. In papers reporting better separating effects, SDS-PAGE was usually performed with separating gels of high T=16.5% or gradient concentrations [10, 18]. Our research was to optimize SDS-PAGE for the subunit analysis of phycoerythrins by evaluating five influence factors. Satisfying results had been found by modulating these factors, and critical factors and possible mechanisms for the factors to influence SDS-PAGE were discussed. Because these factors can influence mobility of marker proteins, this research was supposed to offer references to SDS-PAGE applied to analyzing polypeptide component of protein complexes.

Materials and Methods

Materials

Macroalgae were obtained from tidelands of Yantai in spring (March) and stored at −70°C after contaminants were removed.

Chemicals

Acrylamide and *N*,*N*′-methylenebisacrylamide were produced by Bio Basic Inc. Other SDS-PAGE chemicals were purchased from Shanghai Sangon Biological Engineering Technology Co. Ltd. Low-molecular-weight protein markers (14,400–97,400 Da) were produced by Shanghai Institute of Biochemistry Academia Sinica.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Phycoerythrins were prepared and purified according to the methods described by Sun et al. [19], and the purified phycoerythrins were concentrated with a 30-kDa filter tube by centrifugation at 5,000×*g* for 20 min at 4°C. The concentrated solution was merged with the same volume of 2× SDS-PAGE sample buffer which contained the following: SDS (4%, *m*/*v*), b-mercaptoethanol (10%, *v*/*v*), bromophenol blue (0.005%, *m*/*v*), glycerol (20%, *v*/*v*), and Tris–HCl (0.1 M, pH 6.8). Then, the mixture was kept in boiling water for 5 min and centrifuged at 12,000×*g* for 15 min at room temperature before loading.

Electrophoresis was carried out in a vertical slab gel apparatus (Beijing Liuyi Instrument Factory, DYCZ-24DN) with gel volume of 82×64×1 mm. Separating gels had a gel concentration of 13% (*C*=3%) and pH 8.8–9.9, and they contained 0.05–0.8% (*m*/*v*) SDS, 0.0417–1.5 M Tris–HCl, 0.067% (*v*/*v*) TEMED, and 0.016–0.07% APS. Stacking gels had a gel concentration of 5% (*C*=3%) and pH 6.7, and they contained 0.1% (*m*/*v*) SDS, 0.0625 M Tris–HCl, 0.125% (*v*/*v*) TEMED, and 0.05–0.225% (*m*/*v*) APS. Electrode buffer contained 50 mM Tris, 0.384 M glycine (pH 8.3) and 0.1% (*m*/*v*) SDS.

SDS-PAGE was preformed at room temperature (27°C) or in a refrigerator (14–16°C) with constant current of 20 mA in stacking gels and 40 mA in separating gels.

Staining of Gels

Imidazole–SDS–zinc reverse staining of separating gels described by Fernandez-Patron et al. [20] was performed after electrophoresis, and then the gels were put in UVP Bioimaging systems (Biospectrum-AC) to be observed of fluorescence induced by UV (365 nm). Afterwards, the reverse stained gels were soaked in acetic acid for 30 s to be clarified, then the gels were stained with 0.2% (*m*/*v*) Coomassie Brilliant Blue G-250 containing 40% methanol (*v*/*v*) with 7.5% (*v*/*v*) acetic acid for 2 h, and finally, the gels were destained in 0.5 M NaCl solution.

Data Processing

Standard curves and their linear correlation coefficient (R^2) were gained through Microsoft Excel software.

Results

Subunit Components of Phycoerythrins

Phycoerythrins used in this research are R-PE. R-PE is known to consist of three kinds of subunits, designated α, β, and γ, in structures of $(\alpha\beta)_6\gamma$ and may have some other

polypeptides, such as linker polypeptides [9, 10]. In gels of imidazole–SDS–zinc reverse staining, subunits of phycoerythrin which have phycobilins can interact with Zn^{2+} and emit clear fluorescence under UV irradiation (365 nm) [21]. The molecular weights of α- and β-subunits are about 17–22 kDa, while the molecular weight of γ-subunit is about 30 kDa [2]. If marker proteins were not considered, the image of Coomassie Brilliant Blue staining exhibited the same band patterns as the image of imidazole–SDS–zinc reverse staining (Fig. 1), which is to say that all subunits of phycoerythrins used in this research shown in SDS-PAGE were chromophore-containing polypeptides. It demonstrated that the phycoerythrin samples used in this research contained no colorless polypeptides because colorless polypeptides, such as linker polypeptides of phycobiliprotein complexes, contain no chromophore and cannot emit fluorescence by interacting with Zn^{2+} in gels of imidazole–SDS–zinc reverse staining [21].

Subunit components of phycoerythrins can be conveniently identified according to the fluorescent bands and their corresponding molecular weights. R-PE may have two kinds of γ-subunit (γ_1 and γ_2) or linker polypeptides [17]. The subunits of R-PE from *Polysiphonia urceolata*, as shown in Fig. 1, lane 1, exhibited six bands as follows: two main bands whose molecular weights were below 20 kDa were α- and β-subunits (the lower was α); two bands whose molecular weights were about 30 kDa were γ-subunits, and one of them was strong (γ_1) while the other was weak (γ_2); two weak bands at about 40 and 50 kDa were believed to be the products of incompletely dissociated R-PE, but these two bands still need further researches to testify. Similarly, the subunits of R-PE from *Heterosiphonia japonica* (Fig. 1, lane 2) and *Callithamnion corymbosum* (Fig. 1, lane 3) also exhibited six bands; two γ-subunits of the former showed equal amount, and those of the latter were also judged to be of equal amount as from Fig. 3, although they could not be exactly determined from Fig. 1, lane 3. *Callithamnion* was reported to have two γ-subunits before [17].

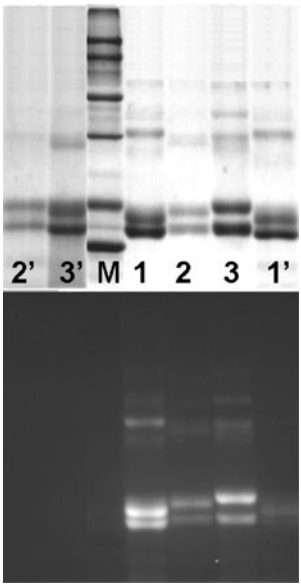

Fig. 1 Imidazole–SDS–zinc reverse staining (*bottom*) and Coomassie Brilliant Blue G-250 staining (*top*) of the same gel of SDS-PAGE of phycoerythrins (pH 8.9, 27°C; 0.292 M Tris). *M* marker, *1*, *1′ P. urceolata*, *2*, *2′ H. japonica*, *3*, *3′ C. corymbosum*. *Samples 1–3* were newly prepared, *samples 1′–3′* were prepared for several days and boiled several times

Effects of Four Factors on Subunit Separation of PE by SDS-PAGE

pH Influence on Separation of α- and β-Subunits

The α- and β-subunits of phycoerythrins have close molecular weights and are not easy to be efficiently separated by SDS-PAGE. Figure 2 showed influences of pH of separating gels on SDS-PAGE of four phycoerythrins. It can be found that higher pH had better effects on the separation of α- and β-subunits of all the three phycoerythrins; the R-PE from *P. urceolata* needed higher pH than the other two R-PEs. The proper pH to make α- and β-subunits separated well in the condition of Fig. 2a–c was higher than 9.3 for R-PE from *P. urceolata* and higher than 8.90 for R-PE from *H. japonica* and *C. corymbosum*.

On the other hand, the influence of pH on subunit separation was related to some other factors. For example, when there were more APS in separating and stacking gels (Fig. 2d–f) or when pH values were measured at a lower temperature (Fig. 2g–i), higher pH was needed to make α- and β-subunits well separated.

Ion Concentration Influence on Separation of α- and β-Subunits

Figure 3 showed the influences of different Tris–HCl concentrations in separating gels (pH 8.90) on SDS-PAGE of three phycoerythrins. It showed that lowering Tris concentration in separating gels had similar effects to increasing pH on separation of α- and β-subunits. The α- and β-subunits cannot be separated when Tris concentration was too high (higher than 0.563 M, Fig. 3d), and they can be well separated when Tris concentration was lowered (0.208–0.375 M, Fig. 3a–c).

From Fig. 3, we can find that lowering Tris concentration in separating gels when pH was low (8.90) had similar effects to increasing pH when Tris concentration was not changed (0.375 M). Influences of increasing Tris–HCl concentration and NaCl addition of separating gels when pH was high (9.50) were shown in Fig. 4. It can be found that both increasing Tris–HCl concentration and NaCl addition can make the α- and β-subunits difficult to be separated and had similar effects as lowering pH. Tris–HCl concentration was more sensitive than NaCl addition: the ionic strength of the separating gel in Fig. 4a was obviously larger than those in Fig. 4c (the conductance of 0.035 M NaCl (Fig. 4a) was 40.5 ms/cm at 25°C; the conductance of 0.375 M Tris–HCl (pH 9.5; the excess of Fig. 4c over Fig. 4a) was 27.8 ms/cm at 25°C), but the α- and β-subunits were separated well in Fig. 4a and could not be efficiently separated in Fig. 4c.

APS Concentration Influence on Separation of α- and β-Subunits

Figure 5 showed the influence of different APS concentrations in stacking gels and separating gels (pH 8.9): APS concentration was 0.0667% (*m*/*v*) in separating gel and 0.225% in stacking gel in Fig. 5a (the usual doses used in winter and the separating gel were polymerized in 30–60 min), 0.0167% in separating gel, and 0.05% in stacking gel in Fig. 5b (the separating gel was polymerized in about 40 min at 27°C). Results showed that the amount of APS addition in stacking and separating gels had influence on the separation of α- and β-subunits: high APS addition had poor effects either in stacking gel or in separating gel (Fig. 5a), but increasing pH of separating gels can get satisfying effects (Fig. 2d–f). Results also showed that the influence of pH on subunit separation in Fig. 2d–f (higher APS additions) had similar trend as that in Fig. 2a–c (lower APS additions), but exhibited hysteresis.

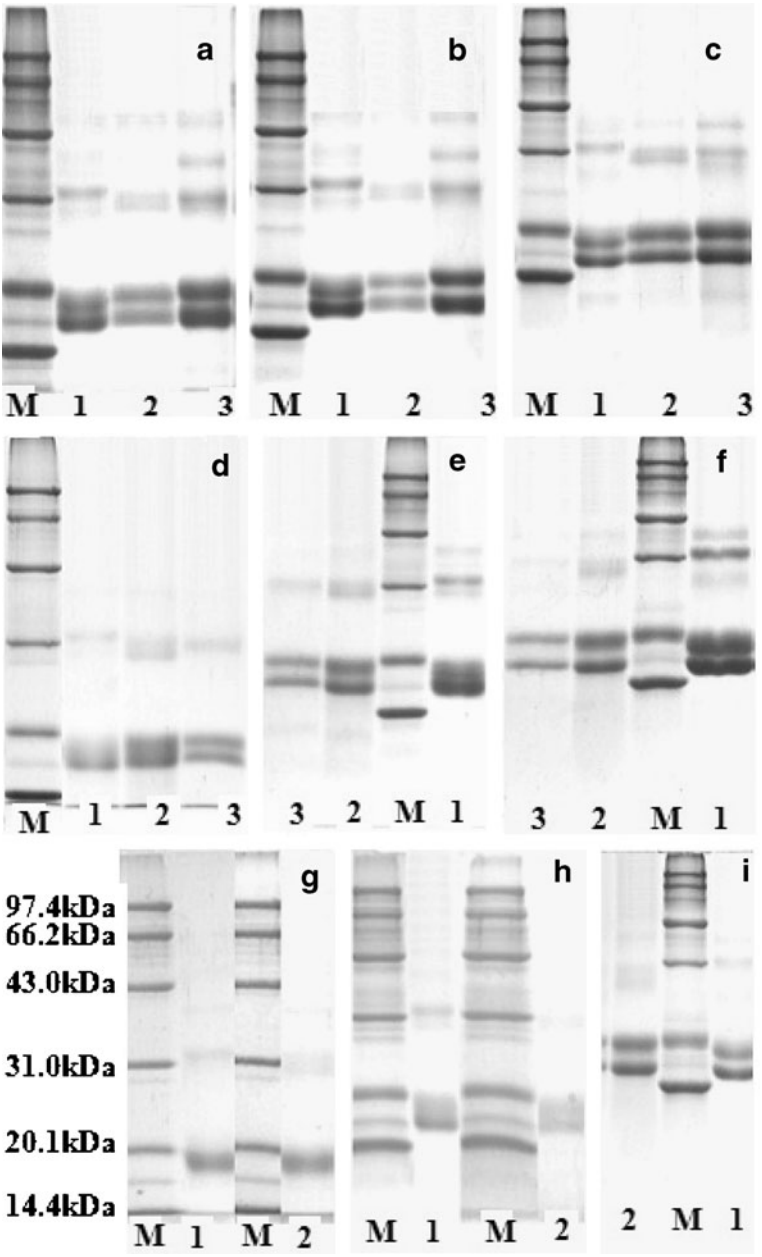

Fig. 2 Influence of pH in separating gels on SDS-PAGE of phycoerythrins (Tris concentration, 0.375 M). *M* markers, *1 P. urceolata*, *2 H. japonica*, *3 C. corymbosum* (the same as Figs. 3, 4, 5, and 6). SDS-PAGE of **a**–**f** were performed at 27°C, and the pH was also measured at 27°C. **a**–**c** were performed with APS concentration of 0.017% in separating gels and 0.05% in stacking gels, while **d**–**f** were performed with APS concentration of 0.067% in separating gels and 0.225% in stacking gels (the gels were prepared in beakers on ice to avoid polymerizing too fast). SDS-PAGE of **g**–**i** were performed at 14°C with APS concentration of 0.067% in separating gels and 0.225% in stacking gels., and the pH was also measured at 14°C. pH of separating gel: **a** 8.90, **b** 9.09, **c** 9.31, **d** 8.62, **e** 8.90, **f** 9.23, **g** 8.82, **h** 9.30, **i** 9.51

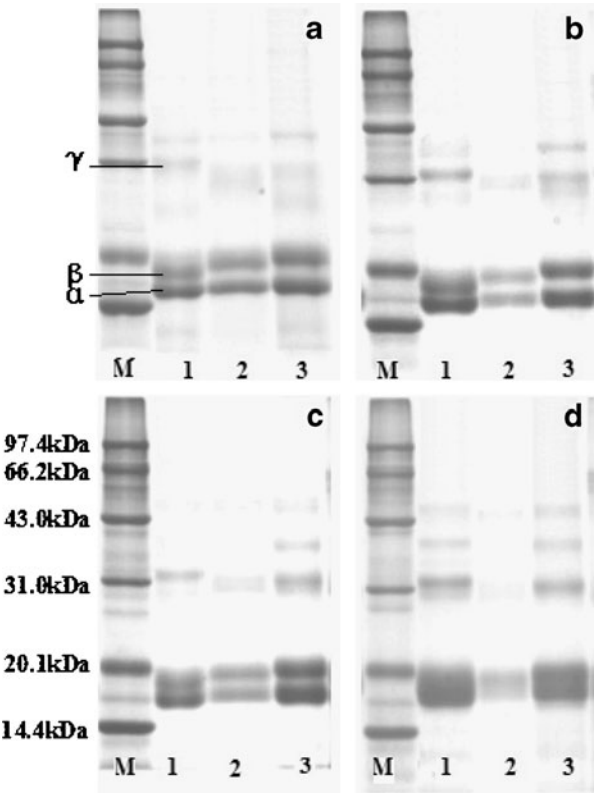

Fig. 3 Influence of Tris–HCl (pH 8.90, 27°C) concentration in separating gels on SDS-PAGE of phycoerythrins. Tris concentration (*M*) were 0.208 (**a**), 0.292 (**b**), 0.375 (**c**), and 0.563 (**d**)

SDS Concentration Influence on Separation of α- and β-Subunits

SDS concentration in SDS-PAGE system, including separating gel, stacking gel, and electrophoretic buffer, can influence the separation of α- and β-subunits. It can be seen from Fig. 6 that decreasing SDS concentration in a certain range was in favor of the separation of α- and β-subunits. When Tris concentration in separating gel was 0.375 M (pH 8.8), the α- and

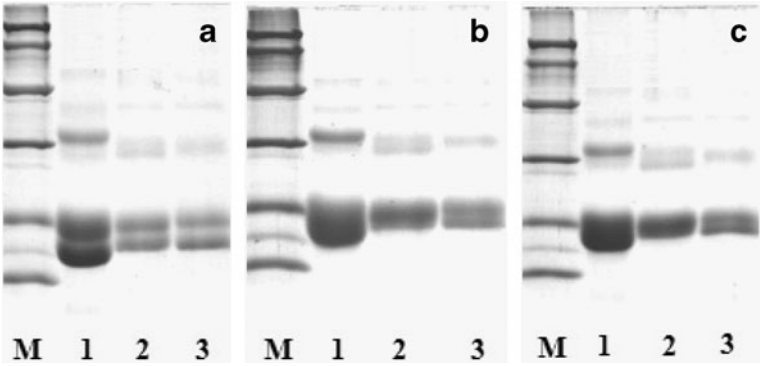

Fig. 4 Influence of Tris–HCl (pH 9.50, 27°C) concentration and NaCl addition in separating gels on SDS-PAGE of phycoerythrins. **a** 0.25 M Tris, 0.035 M NaCl; **b** 0.25 M Tris, 0.05 M NaCl; **c** 0.625 M Tris, 0 M NaCl

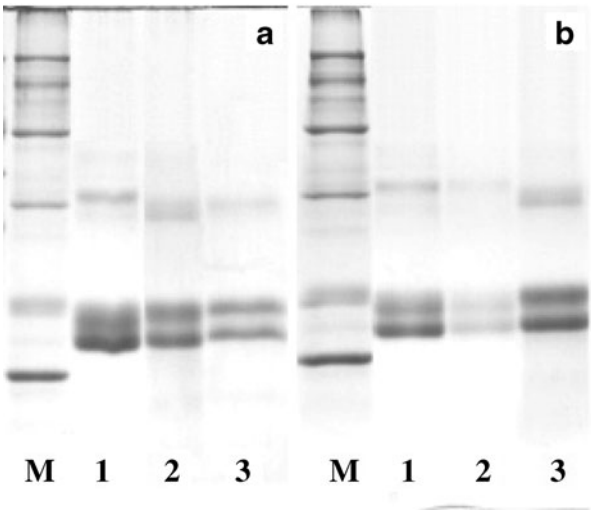

Fig. 5 Influence of APS concentration in stacking and separating gels (pH 8.9) on SDS-PAGE of phycoerythrins. APS concentration was 0.0667% (*m*/*v*) of separating gel and 0.225% of stacking gel in **a** (the usual doses used in winter and the separating gel was polymerized in 30–60 min) and 0.0167% of separating gel and 0.05% of stacking gel in **b** (the separating gel was polymerized in about 40 min at 27°C)

β-subunits were separated well when SDS concentration was 0.05% (Fig. 6d), but they could not be separated when SDS concentration was 0.1% (Fig. 6c). When Tris concentration in separating gel was 0.25 M (pH 8.8), the α- and β-subunits were separated well when SDS concentration was 0.1% (Fig. 6a), but could not be separated when SDS concentration was 0.2% (Fig. 6b) or higher. In the case of pH 9.5 in separating gels (Tris concentration 0.375 M), the α- and β-subunits were separated well when SDS concentration was 0.1% or 0.2% (Fig. 6e), but could not be separated when SDS concentration was 0.3% (Fig. 6f) or higher. Moreover, SDS concentration can also obviously influence the mobility of marker proteins.

pH and Ion Concentration Influences on γ-Subunits

Influences of pH and Tris concentration in separating gels on γ-subunits were the reverse of those on α- and β-subunits: higher Tris concentration and lower pH had better effect (thin and clear band). The γ_1-subunits of three phycoerythrins were shown as a thin and clear band when Tris concentration was over 0.292 M (Fig. 3) or pH was below 9.3 (Fig. 2) in the separating gels and was shown as a broad and faint band when Tris concentration was lowered or pH was increased (Figs. 2 and 3). The bands of γ_2-subunits tended to be broad and down away from γ_1-subunits with a decrease of Tris concentration or increase of pH in separating gels (Figs. 2 and 3).

Molecular Weight of Subunits

Standard curves of lgMw versus relative electrophoretic mobility (mR) were used to tell molecular weights of phycoerythrin subunits, and linear correlation coefficient (R^2) can tell the accuracy of standard curves. Results showed that Tris concentration had no obvious influence on R^2 of standard curves, but the R^2 of standard curves made with four marker proteins were obviously higher than R^2 of standard curves made with six marker proteins (Table 1). R^2 of six marker proteins varied much with a change of Tris concentration, but the changes could not be explained by any rules, whereas R^2 of four marker proteins was found as having some correlations with the Tris concentration: when the Tris concentration

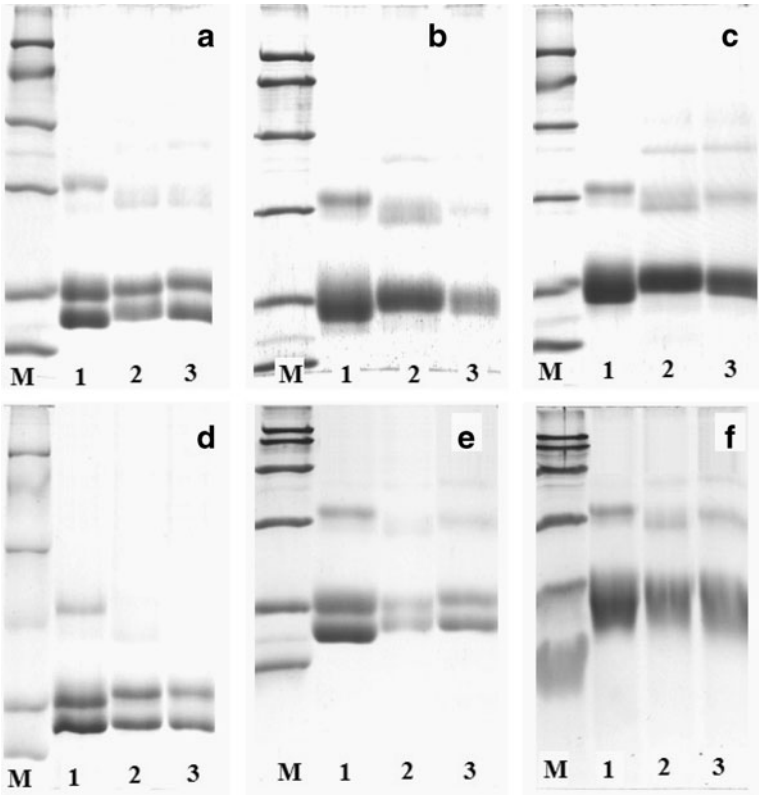

Fig. 6 Influence of SDS concentration on SDS-PAGE of phycoerythrins. **a**, **b** were performed with separating gels of pH 8.8 (25°C) and 0.25 M Tris. **c**, **d** were performed with separating gels of pH 8.8 (25°C) and 0.375 M Tris. **e**, **f** were performed with separating gels of pH 9.5 (25°C) and 0.375 M Tris. SDS concentration: **a** 0.1%, **b** 0.2%, **c** 0.1%, **d** 0.05%, **e** 0.2%, **f** 0.3%

was 0.375 M, R^2 of four marker proteins was maximized, and a higher or lower Tris concentration resulted in lower R^2, though the changes were not obvious (Table 1).

SDS concentrations had an obvious influence on the mobility of subunits and marker proteins; R^2 of standard curves of different SDS concentrations are shown in Table 2. It is shown that low SDS concentration (e.g., 0.05% and 0.075% with pH 9.5) resulted in higher R^2 of six marker proteins but lower R^2 of four marker proteins; high SDS concentration (e.g., 0.2% and 0.3% with pH 9.5) resulted in lower R^2 of six marker proteins but higher

Table 1 Linear correlation coefficient (R^2) of standard curves of lgMw versus mR for SDS-PAGE using separating gels of different Tris concentration (pH 8.90, 27°C).

Tris concentration	0.125	0.167	0.292	0.375	0.5	0.625	0.729
Six marker proteins	0.9772	0.9687	0.9705	0.9713	0.9746	0.9755	0.9770
Four marker proteins	0.9921	0.9920	0.9977	1	0.9994	0.9990	0.9986

Six marker proteins: standard curves made with six marker proteins. Four marker proteins: standard curves made with four marker proteins whose molecular weights are relatively lower

Mw molecular weight, *mR* relative electrophoretic mobility

Table 2 Linear correlation coefficient (R^2) of standard curves of lgMw versus mR for SDS-PAGE using separating gels of different SDS concentration (25°C).

SDS concentration (%)	Tris concentration 0.25 M, pH 8.8	Tris concentration 0.375 M, pH 8.8	Tris concentration 0.375 M, pH 9.5
0.05	0.9786 (0.9589)	0.9849 (0.9909)	0.9764 (0.9689)
0.075		0.9721 (0.9992)	0.9728 (0.9479)
0.1	0.9674 (0.995)	0.968 (0.9952)	0.9714 (0.9938)
0.2	0.97 (0.9987)		0.9263 (0.9984)
0.3	0.9826 (0.9862)		0.9223 (0.9966)
0.5	0.976 (0.9997)		0.962 (0.9994)
0.8	0.984 (0.9531)		

The data outside the parentheses are R^2 gained by six marker proteins; the data in brackets are R^2 gained by four marker proteins

R^2 of four marker proteins; middle SDS concentration resulted in middle R^2 of six marker proteins but high R^2 of four marker proteins.

It can be seen from Table 3 that molecular weights of phycoerythrin subunits obtained by SDS-PAGE can be influenced by Tris concentration in separating gels. For α- and β-subunits, their molecular weights increased when Tris concentration increased in a certain range and were on terraces when Tris concentration was high or low enough (≥0.625 or ≤0.292 M; Table 3). As for subunit γ, there was a peak when Tris concentration was about 0.292 M (Table 3).

Discussion

Essential Factors Influencing SDS-PAGE of Phycoerythrins

Because SDS concentration in gels and buffers can obviously influence the mobility of subunits and marker proteins, it should be an essential factor. Though the optimum SDS

Table 3 Molecular weights (kDa) of phycoerythrin subunits got by SDS-PAGE with separating gels having different Tris concentration (pH 8.90) according to standard curves made with four marker proteins.

Subunits		Tris concentration (M)						
		0.125	0.167	0.292	0.375	0.5	0.625	0.729
1	α	16.0	15.7	16.2	16.5	17.9[a]	18.9[a]	19.0[a]
	β	18.0	17.7	18.0	18.5			
	γ_1	30.0	32.9	32.8	32.1	31.9	31.5	31.3
2	α	16.6	16.3	16.7	17.2	17.9	19.1[a]	19.4[a]
	β	19.5	18.8	19.2	19.3	19.3		
	γ_1	29.6	31.8	32.7	31.7	31.4	30.7	31.0
3	α	16.7	16.3	16.8	17.1	17.9	19.1[a]	19.4[a]
	β	20.5	19.3	19.5	19.6	19.5		
	γ_1	27.2	32.1	32.5	31.4	31.3	30.6	30.4

1 P. urceolata, *2 H. japonica*, *3 C. corymbosum*

[a] Average molecular weight of the α- and β-subunits when they could not be separated

concentration varied with ion concentration and pH, a certain range of SDS concentration with which the α- and β-subunits were separated well can be found whether ion concentration or pH was high or low. SDS concentration also can obviously influence the mobility of marker proteins, especially when it was low or high enough, so its influence should be universal.

The other three factors having effects on subunit separation were ion concentration, pH, and APS concentration. Lowering Tris (or NaCl) and/or APS concentration or increasing pH can bring good effects on the separation of the α- and β-subunits of phycoerythrins. Essentially, these three factors may be attributed to ion concentration in separating gel, especially concentration of positive ions. Furthermore, these three factors can also influence mobility of marker proteins; this will be discussed in the following.

In this research, high pH showed similar effects on phycoerythrin SDS-PAGE as low Tris concentration (buffers with higher pH and higher Tris concentration had the same Cl^- concentration as buffers with lower Tris concentration and lower pH according to the amount of HCl addition). For example, the separating gels having the same Cl^- concentration but different pH were used in the experiments of Figs. 2b and 3b, and they had similar effects on the subunit separation except for relative electrophoretic mobility. Similar things occurred in Figs. 2c and 3a. That higher pH bringing lower relative electrophoretic mobility should be due to the increase of the negative charges of bromophenol blue under higher pH. No matter pH was high (9.50) or low (8.90), changing Tris (or NaCl) concentration in separating gels can determine the subunit separation. Temperature can obviously influence pH of Tris–HCl buffers, but the electrophoresis temperature had no obvious influence on SDS-PAGE of phycoerythrins in our research. So pH itself may not be an essential factor influencing subunit separation, but ion concentration might be (gels of different pH have different ion concentrations). When Tris concentration was not changed, buffers having higher pH need lower HCl addition and has lower Cl^- and $Tris^+$ concentration, which can be deduced from Eq. 1, so the influence of pH on subunit separation influences ion concentration essentially:

$$pH = pK_a + log[Tris/Tris^+]. \quad (1)$$

High pH in separating gel (e.g., 9.5) could have fine effects on the separation of α- and β-subunits, but both increasing Tris–HCl concentrations and NaCl addition can make the α- and β-subunits difficult to be separated. Moreover, Tris–HCl concentration and NaCl addition had different sensitivities: ionic strength of the separating gel in Fig. 4a was obviously larger than those of the separating gels in Fig. 4c (by measuring the conductance of solution), but the α- and β-subunits were separated well in Fig. 4a but could not be efficiently separated in Fig. 4c. Ionic strength (conductance) can represent the amount of ions in solution, and because the main negative ions in separating gels are Cl^-, ionic strength can represent its concentration. So the difference in sensitivity between Tris–HCl and NaCl should not be due to Cl^- but the positive ions ($Tris^+$ and Na^+). Tris–HCl being more sensitive than NaCl may be due to two aspects: $Tris^+$ is larger than Na^+ and Tris can constantly be dissociated into $Tris^+$ and $Tris^+$ concentration in gel is maintained steadily during electrophoresis, while Na^+ concentration in gel is gradually decreased during electrophoresis. The latter should be more important. So the most possible essential factors influencing subunit separation of phycoerythrins are the concentration of positive ions in separating gel and their buffering capability.

More APS addition resulted in poor separating effects. This may have two reasons: increasing ion concentration in gels and oxidation of a part of subunits.

It is known that pH of Tris–HCl buffer is related to temperature: lower temperature results in higher pK_a of Tris and higher pH, and the temperature coefficient is about −0.026 pH unit per degree Celsius [22]. So Tris–HCl buffers having the same pH but measured at different temperatures have different ion strengths; this should be considered especially in winter.

In conclusion, the essential factors considered above should be SDS concentration in gels and buffers and ionic strength in separating gels. Decreasing the SDS concentration had good effect on the separation of α- and β-subunits when ionic strength of the separating gel is high. Similarly, decreasing ionic strength had good separation effect when SDS concentration is normal (0.1%) or higher (0.2%), and factors which can lower ionic strength (ion concentration) have better effects. To lower ion concentration in separating gel, we can decrease Tris–HCl concentration in a certain range or increase pH (decrease HCl addition), and increasing pH can gain low ionic strength as well as sufficient buffering capability of pH.

Influence of SDS Concentration and Ion Concentration on Mobility of Marker Proteins

SDS concentration had an obvious influence on the mobility of marker proteins, and the R^2 of standard curves for SDS-PAGE using separating gels of different SDS concentration varied much (Table 2). Influences of SDS concentration on mR of marker proteins and subunits of R-PE from *P. urceolata* (pH 8.8 at 25°C, Tris concentration 0.25 M in separating gels) were shown in Fig. 7. It was shown that mR of marker proteins and subunits compared with the 14.4-kDa marker protein changed obviously when the SDS

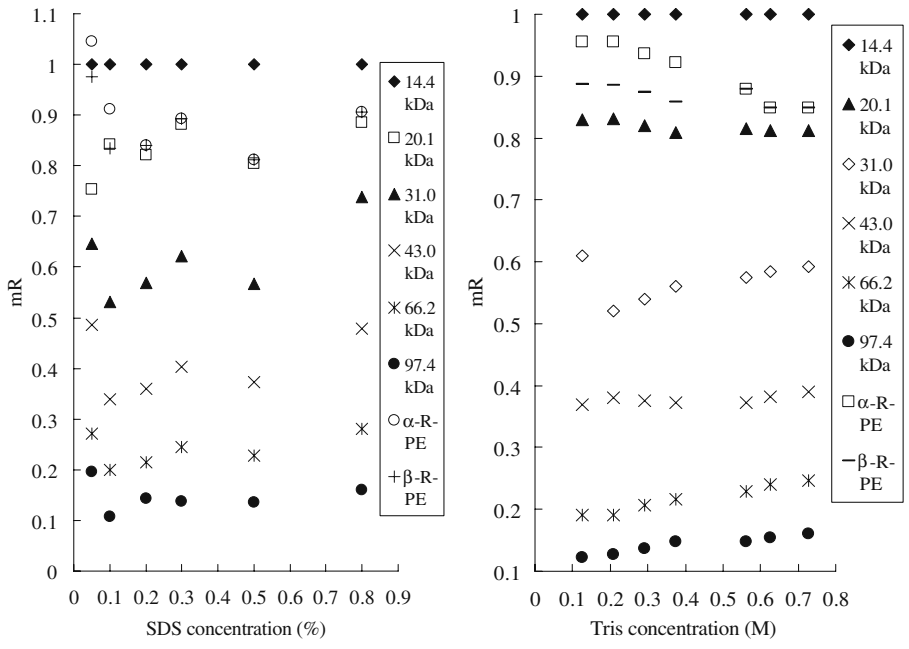

Fig. 7 Influence of SDS (*left*, pH 8.8 at 25°C, Tris concentration 0.25 M of separating gels) and Tris (*right*, pH 8.9 at 27°C, SDS concentration 0.1% of separating gels) concentration on relative electrophoretic mobility (*mR*) of marker proteins and the subunits of *P. urceolata*. The mR of the 14.4-kDa marker protein was 1

concentration changed. The change curves of 31.0-, 43.0-, 66.2-, and 97.4-kDa marker proteins were similar, and they were different from those of the 14.4- and 20.1-kDa marker proteins and subunits. This implied that the 31.0-, 43.0-, 66.2-, and 97.4-kDa marker proteins had similar characteristics in composition and alignment of amino acids, but differed in the 14.4- and 20.1-kDa marker proteins and subunits.

Ion concentration also can influence the mobility of marker proteins, though R^2 changed little (Table 1). For example, the 31.0-kDa protein between 20.1- and 43.0-kDa proteins was closer to the 20.1-kDa protein when Tris concentration increased (Fig. 3), and decreasing pH had similar influence (Fig. 2). Therefore, the influence of these factors should be popular to other proteins besides phycoerythrins. When Tris concentration changed, R^2 changed little, but the molecular weights of subunits obviously changed. It is difficult to judge which molecular weights we obtained were closer to the real ones, but changing these factors can be useful in subunit analysis. Influences of Tris concentration on mR of marker proteins and subunits of phycoerythrins from *P. urceolata* (pH 8.9 of separating gels at 27°C, SDS concentration 0.1%) were shown in Fig. 7. It showed that mR of the 31.0-, 66.2-, and 97.4-kDa marker proteins compared with the 14.4-kDa marker protein increased obviously when Tris concentration increased in a certain range (Tris concentration, 0.2–0.8 M), while mR of the 20.1- and 43.0-kDa marker proteins changed little when Tris concentration changed; this can explain why the 31.0-kDa marker protein changed obviously in images of SDS-PAGE. It also showed that mR of subunits decreased when Tris concentration increased in a certain range. All these above testified that different polypeptides had different responses to the change of SDS and ion concentration.

Possible Mechanisms

Critical micelle concentration (cmc) of SDS is about 8.0 mM (0.23%) at 25°C [23] or 8.1 mM (0.233%) [24]; when the ionic strength of separating gels was low (pH 9.5), α- and β-subunits can be separated well when SDS concentration was 0.2% and cannot be separated when SDS concentration was 0.3%. The cmc of SDS can be influenced by ionic strength, pH, and temperature, but ionic strength has obviously a larger influence than the other two [23–25]. The cmc of SDS were 3.8 mM (0.11%) in 0.02 M NaCl and 0.83 mM (0.024%) in 0.2 M NaCl, while cmc of SDS in 0.1 M NaCl under different temperatures was 0.04% at 17°C and 18°C, 0.041% at 20°C, and 0.048% at 30°C [25]. All these showed that the turning points of SDS concentration for subunit separation were close to the cmc of SDS in separating gels and should be related to the ratio of monomer to micelle or the amount of monomeric SDS in nature. When the ratio or amount of monomeric SDS was high enough, α- and β-subunits can be separated well. High ionic strength can lower the cmc of SDS and can increase the unfolding rates of proteins in SDS mainly because there are more positive ions in or around the SDS micelles and they can screen the repulsion between the sulfate headgroups of SDS [24, 25]; this is in accordant with the conclusion we have drawn ahead that the concentration of positive ions is an essential factor. Increasing ionic strengths (NaCl) could gradually shift the conformation of initial protein–SDS complex or the transition state toward more compact and structured states [25]. One monomeric SDS may be bound to one hydrophobic amino acid residue when the ratio of monomer to micelle was high enough or there was enough monomeric SDS. When SDS concentration was largely higher than cmc, SDS may be assembled into micelle around proteins especially around big hydrophobic amino acid residues (several SDS bound to one residue, e.g., phenylalanine), which may increase SDS binding amount, make the structure of polypeptides tight, and reduce difference between polypeptides.

Compared with bands of marker proteins, the subunit bands were obviously different in shape and breadth, and the calculated molecular weights varied obviously with different Tris concentrations in separating gels. So the aforementioned phenomena should be associated with the composition and alignment of amino acids and chromophores of phycoerythrins subunits.

There are two chromophores (PEB) in the α-subunits of both R-PE and B-PE, and the chromophores are linked to specific cysteine residues in the polypeptide chains by single thioether bonds [26]. The β-subunits of both B-PE and R-PE have three chromophores: two of them are PEB and are linked to cysteine residues by single thioether bonds and the other one is PEB in B-PE but PUB in R-PE, and it is linked to two cysteine residues as a circle by two thioether bonds. PEB and PUB are isomeric compounds and each has two carboxylic groups. Killilea et al. [27] postulated that there was a linkage between the ring C of phycourobilin and apoprotein which involves the hydroxy side chain group of serine in an ester linkage with the carboxylic group of the propionic side chain of PUB. Chromophores may influence the mobility of subunits (especially β-subunits) during electrophoresis under different conditions.

On the other hand, the α-subunits of B-PE and R-PE have the same chromophore composition and alignment [26], but the α-subunits of B-PE and R-PE had different sensitivity to the change of ionic strength (according to our unpublished research); moreover, samples which were boiled more times had no fluorescence with imidazole–SDS–zinc reverse staining, but their bands had no changes with Coomassie Brilliant Blue staining (Fig. 1), and the mobility of marker proteins can obviously be influenced by SDS and ion concentration. These imply that composition and alignment of chromophores may not be the essential cause, but those of amino acids.

This research was about SDS-PAGE of phycoerythrins. The α- and β-subunits behaved to be one or two bands under different SDS concentrations or ion concentrations in separating gels. The α- and β-subunits can be separated well with about 0.2–0.3 M Tris concentration (pH 8.9) in separating gel and 0.1% SDS in SDS-PAGE system, but the pH must be measured at about 25°C. The optimal condition was not unalterable; for example, if there was higher Tris concentration in separating gel, we could increase pH or decrease SDS concentration to get good separation effects. The influencing factors considered here are universal in SDS-PAGE of other proteins; modulating these factors may make polypeptides whose molecular weights are close separated well and could testify whether a band contains one or two polypeptides during subunit or proteome analysis by SDS-PAGE.

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