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Tricine–Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis for the Separation of Proteins in the Range from 1 to 100 kDa

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A discontinuous sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) system for the separation of proteins in the range from 1 to 100 kDa is described. Tricine, used as the trailing ion, allows a resolution of small proteins at lower acrylamide concentrations than in glycine–SDS–PAGE systems. A superior resolution of proteins, especially in the range between 5 and 20 kDa, is achieved without the necessity to use urea. Proteins above 30 kDa are already destacked within the sample gel. Thus a smooth passage of these proteins from sample to separating gel is warranted and overloading effects are reduced. This is of special importance when large amounts of protein are to be loaded onto preparative gels. The omission of glycine and urea prevents disturbances which might occur in the course of subsequent amino acid sequencing.

KEY WORDS: SDS–PAGE; proteins; peptides.

The SDS–PAGE[1] system, in part already published (1), was developed because a separation of the 11 protein subunits of the bc_1 complex from beef heart was not achieved with any other SDS–PAGE method tested. The excellent SDS–PAGE systems of Neville and Glossmann (2), Laemmli (3), and a modification of the latter according to Douglas *et al.* (4) all have an insufficient resolving power below 10 kDa. In the SDS–PAGE systems using urea according to Merle and Kadenbach (5) and Swank and Munkres (6) not all subunits could be detected and either the reproducibility or the resolution was not sufficient for our purposes.

The new SDS–PAGE system for small proteins had to be a discontinuous method, so that dilute protein samples could be applied directly without prior concentration. Thus the well-established and reproducible method of Laemmli (3), based on the results of Davis (7) and Ornstein (8), was chosen as the basis for the method described.

The stacking limit for large proteins in the Laemmli system lies far beyond 100 kDa, but proteins below 20 kDa are only partly or not at all separated from the bulk of SDS, as can be verified experimentally in quickly stained stacking gels. This property causes the streaking of small proteins if one tries to use higher acrylamide concentrations in the separating gel. We therefore tried to find new ways to separate small proteins from the SDS before these proteins are separated at higher acrylamide concentrations. During optimization, the rules for finding the optimal stacking conditions in electrophoresis and in isotachophoresis (8–12) proved to be very helpful.

MATERIALS AND METHODS

Chemicals. SDS, Tricine p.A., urea p.A., glycerol p.A., acrylamide 2× cryst., bisacryl-

[1] Abbreviations used: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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TABLE 1

STOCK SOLUTIONS FOR SDS-PAGE

Buffer	Tris (M)	Tricine (M)	pH	SDS (%)
Anode buffer	0.2	—	8.9[a]	—
Cathode buffer	0.1	0.1	8.25[b]	0.1
Gel buffer	3.0	—	8.45[a]	0.3

Acrylamide-bisacrylamide mixture	Percentage acrylamide (w/v)	Percentage bisacrylamide (w/v)
49.5% T, 3% C	48	1.5
49.5% T, 6% C	46.5	3.0

[a] Adjusted with HCl.

[b] No correction of the pH, which is around 8.25.

amide 2× cryst., Pyranin (8-hydroxy-1,3,6,-pyrenetrisulfonic acid), and Serva blue G were obtained from Serva. TEMED was purchased from Sigma, and Tris was from Boehringer-Mannheim. Ammonium persulfate and bromphenol blue were purchased from Merck (Darmstadt).

Gel preparation. The stock solutions prepared for gel electrophoresis are given in Table 1. The composition of these acrylamide mixtures and of all gels is defined by the letters T and C according to Hjerten (13). T denotes the total percentage concentration of both monomers (acrylamide and bisacrylamide). C denotes the percentage concentration of the crosslinker relative to the total concentration T. All solutions were kept at room temperature with the exception of the acrylamide:bisacrylamide mixtures, which were stored at 4°C. The composition of separating, "spacer," and stacking gels is given in Table 2.

The 10% T, 3% C gel was used as a uniform separating gel, only overlaid by a 4% T, 3% C stacking gel (2 cm). Separating and stacking gels were polymerized together, without prior degassing, by addition of 150 μl of a 10% ammonium persulfate solution and 15 μl TEMED/30 ml. It is essential that the separating gel polymerizes first.

All other gels (cf. Table 2) were composed of a small-pore gel (16.5% T) overlaid by a 10% T, 3% C spacer gel (2–3 cm) that again was overlaid by the 4% T, 3% C stacking gel (1–2 cm). The small-pore gel and the short 10% T spacer gel (without glycerol) were polymerized together within 15 min by addition of 100 μl of 10% ammonium persulfate and 10 μl TEMED to 30 ml of each mixture. The 10% T gel again was overlaid with water. Some minutes after termination of the polymerization the water was replaced by the 4% T stacking-gel mixture polymerized by 100 μl of 10% persulfate and 10 μl TEMED per 12.5 ml.

The 10% T gel between the stacking and small-pore gels was omitted if the resolution of proteins below 5 kDa was of minor interest.

Sample application. The protein samples were incubated for 30 min at 40°C in 4% SDS, 12% glycerol (w/v), 50 mM Tris, 2% mercaptoethanol (v/v), 0.01% Serva blue G adjusted with HCl to pH 6.8 Serva blue G was used as the tracking dye, although it migrated as a broad band. It ran only a little faster than the smallest proteins in all gels, whereas the commonly used bromphenol blue ran behind the smallest proteins in 10% T, 3% C gels but before them in high-percentage acrylamide gels.

Usually the filling height of the sample was 2.5–10 mm. The sample, 0.5–2 μg per protein band, was laid under the cathode buffer using a microliter syringe. In the case of small proteins (1–3 kDa) an amount of 2–5 μg should be applied because of partial loss during the staining–destaining procedure. The sample load for 1.6-mm (3.2-mm) preparative gels amounted to 1 (2) mg per band, depending on the distance of the bands to be separated and on the molecular masses of the proteins.

Electrophoresis conditions. Slab gels were used in a vertical apparatus similar to that

described by Studier (14), except that the support of the upper buffer tank was displaced to the rear side so that the central part of the glass plates could be covered with aluminium plates for a better distribution of locally produced heat. This prevents the bending of the bands in preparative gels. The aluminium plates were never in contact with the electrode buffers. A very similar apparatus (No. 30442) is available from Renner GmbH (Dannstadt, FRG).

Usually either long slab gels (total length, 27 cm) with small-pore-gel dimensions of 22 × 15.5 × 0.07 (0.16; 0.32) cm or short gels (total length, 14.5 cm) with small-pore-gel dimensions of 10 × 14 × 0.07 (0.16) cm were used. The electrophoresis was performed at room temperature, cooled only by the ambient air. All electrophoresis runs started at 30 V. After about 1 h, when the sample had completely entered the stacking gel, the running conditions were set as listed in Table 3.

Fixing, staining, and destaining. The protein bands were fixed in a solution containing 50% methanol and 10% acetic acid for 30 min (0.7-mm gels) or for 60 min (1.6-mm gels), before they were stained with 0.025% Serva blue G in 10% acetic acid for 1–2 h, depending on the thickness and on the composition of the gels. A complete background destaining of the 0.7-mm gels was achieved by shaking the gels in 10% acetic acid for 2 h; the destaining solution was renewed every 30 min. Rapid "fixing," staining, and destaining was essential in the case of small proteins to avoid their elution. It should be noted that not all small proteins could be stained, because they either eluted completely and/or did not bind the dye. Methanol was omitted from the staining solution for two reasons: the time needed for staining was reduced due to an increased swelling of the gels, and the dye even bound to proteins that were only badly stained in methanolic solution.

RESULTS

Resolution with Different Gel Types

We used 31 proteins and protein fragments, listed in Table 4, as standards to test the electrophoresis system. The migration distances of all the proteins, standardized to the migration distance of a 1.0-kDa protein, were used to construct the calibration curves of Fig. 1.

The calibration curves, beginning with the upper curve, correspond to the following gels (see also "Gel preparation" and Table 2 for gel composition and Table 1 for electrode buffers).

The 10% T, 3% C gel overlaid by a 4% T, 3% C stacking gel has a calibration curve that

TABLE 2

COMPOSITION OF SEPARATING, "SPACER" AND STACKING GELS

			Separating gels			
	Stacking gel 4% T, 3% C	"Spacer" gel 10% T, 3% C	10% T, 3% C	16.5% T, 3% C	16.5% T, 6% C	16.5% T, 6% C with 6 M urea
49.5% T, 3% C solution	1 ml	6.1 ml	6.1 ml	10 ml	—	—
49.5% T, 6% C solution	—	—	—	—	10 ml	10 ml
Gel buffer	3.1 ml	10 ml	10 ml	10 ml	10 ml	10 ml
Glycerol	—	—	4 g	4 g	4 g	—
Urea	—	—	—	—	—	10.8 g
Add water to a final volume of	12.5 ml	30 ml	30 ml	30 ml	30 ml	30 ml

TABLE 3

ELECTROPHORESIS CONDITIONS FOR DIFFERENT GEL TYPES

Gel type	Small-pore-gel dimensions (cm)	Voltage at		Current at		Time (h)
		Start	End	Start	End	
10% T, 3% C	11.5 × 14 × 0.07	150 V const.		(70 mA	30 mA)	4
	11.5 × 14 × 0.16	110 V const.		(90 mA	45 mA)	5
	24 × 15.5 × 0.07	140 V const.		(40 mA	12 mA)	16
	24 × 15.5 × 0.16	130 V const.		(80 mA	25 mA)	16
	24 × 15.5 × 0.32	120 V const.		(115 mA	40 mA)	20
16.5% T, 3% C	10 × 14 × 0.07	90 V const.		(30 mA	8 mA)	16
	10 × 14 × 0.16	85 V const.		(45 mA	15 mA)	16
	22 × 15.5 × 0.07	(120 V	380 V)	30 mA const.		16
	22 × 15.5 × 0.16	(80 V	250 V)	50 mA const.		23
16.5% T, 6% C	10 × 14 × 0.07	105 V const.		(38 mA	10 mA)	16
	10 × 14 × 0.16	95 V const.		(55 mA	13 mA)	16
	22 × 15.5 × 0.07	(130 V	440 V)	30 mA const.		19

Note. Underlined values for voltage or current were held constant. As a result the corresponding values given in parentheses could be measured. All electrophoresis runs started at 30 V constant for about 1 h. Voltage or current was only raised to the values given in the table when the sample had completely left the sample pocket.

is linear down to the inflection point that occurs in all gels at 5.0–5.5 kDa. Besides its usefulness for the analytical separation of proteins in the range from 5 to 100 kDa, it is the gel type preferentially used for preparative purposes, because the heat production is lower and the elution of proteins is better compared to the other gel types. The resolution below 5 kDa is poor, but it still allows a decision whether any complex mixture of proteins contains a protein in the range from 1 to 5 kDa at all.

The 16.5% T, 3% C gel without urea is useful for the range from 1 to 70 kDa. It is overlaid with a 10% T spacer gel and a 4% T stacking gel. The spacer gel between stacking and small-pore gels can be omitted if the resolution below 5 kDa is of minor interest.

The 16.5% T, 6% C gels, with and without 6 M urea, have their best resolution between 5 and 20 kDa. The resolution below 5 kDa is increased by urea as described by Swank and Munkres (6).

Major differences in resolution between the gels with and without urea, observed with some multiprotein complexes, were due to special properties of the proteins rather than to the differences in the slopes of the calibration curves. The bc_1 complex of beef heart mitochondria, for example, was resolved better without urea, whereas the cytochrome oxidase from the same source needed urea for optimal resolution. In all gels where urea was used instead of glycerol, more deviations from the standard curves were observed, especially of small proteins. The B chain of insulin (3.5 kDa), for example, migrated like a 7-kDa protein in gels with urea and Bradykinin could only be detected as a very broad band at much higher apparent molecular mass.

Figure 2 shows the resolution of a selection of standard proteins on the gel types described by the calibration curves in Fig. 1. The MW-SDS-17 kit from Sigma (2.51–17.0 kDa) and the PMW kit from Pharmacia (2.55–17.2 kDa) (CNBr fragments of myoglobin from horse heart and from sperm whale) should comprise five proteins each, but were, in fact, resolved into six bands.

Moreover, four of the six bands appeared as doublets. Taking into account the existence of an additional CNBr fragment "1 + 3" not mentioned in the information leaflets of the two kits, the six proteins fit the calibration curves, although with some deviation of the 8.2-kDa fragments and the 2.51-kDa oligopeptide of the MW-SDS-17 kit. In the case of doublets, the average migration of the two bands was used. An explanation for the appearance of these doublets cannot yet be given. A heterogeneity within the myoglobins themselves cannot be excluded, but no doublets of the uncleaved myoglobins could be detected. On the other hand, the appearance of doublets was dependent neither on

TABLE 4

MOLECULAR MASSES OF PROTEINS AND PEPTIDES

	kDa	Protein	Source
1.	97.4	Phosphorylase *b*, rabbit muscle	[a]
2.	68	Bovine serum albumin	[b]
3.	45	Egg albumin	[a]
4.	36	Glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle	[a]
5.	29	Carbonic anhydrase, bovine	[a]
6.	27.9	Cytochrome c_1 of bc_1 complex, bovine	s
7.	21.5	Fe_2S_2 Protein of bc_1 complex, bovine	s
8.	17.2	Myoglobin, sperm whale	PMW
9.	17.0	Myoglobin, horse heart	MW-SDS-17
10.	14.6	CNBr fragment "1 + 2" from 8.	PMW
11.	14.4	CNBr fragment "1 + 2" from 9.	MW-SDS-17
12.	13.4	Subunit 6 of bc_1 complex, bovine	s
13.	12.4	Cytochrome *c*, horse heart	[c]
14.	10.8	CNBr fragment "1 + 3" from 8.	PMW
15.	10.7	CNBr fragment "1 + 3" from 9.	MW-SDS-17
16.	9.4	CNBr fragment 1-87 from 7.	s
17.	8.2	CNBr fragment 1 from 8.	PMW
18.	8.2	CNBr fragment 1 from 9.	MW-SDS-17
19.	8.0	Subnit 9 of bc_1 complex, bovine	s
20.	7.6	CNBr fragment 1-71 from 7.	s
21.	7.2	Subunit 10 of bc_1 complex, bovine	s
22.	6.7	CNBr fragment 1-62 from 7.	s
23.	6.4	Subunit 11 of bc_1 complex, bovine	s
24.	6.4	CNBr fragment 2 from 8.	PMW
25.	6.2	CNBr fragment 2 from 9.	MW-SDS-17
26.	3.5	Glucagon, bovine and porcine	[a]
27.	3.5	Insulin B-chain, bovine	[a]
28.	2.55	CNBr fragment 3 from 8.	PMW
29.	2.51	CNBr fragment 3 from 9.	MW-SDS-17
30.	1.45	Bacitracin	[d]
31.	1.06	Bradykinin	[d]

Note. s, prepared according to (2). PMW, peptide molecular-weight standards from Pharmacia. MW-SDS-17, molecular-weight marker kit from Sigma.

[a] Sigma.

[b] Behring Werke.

[c] Boehringer-Mannheim.

[d] Serva.

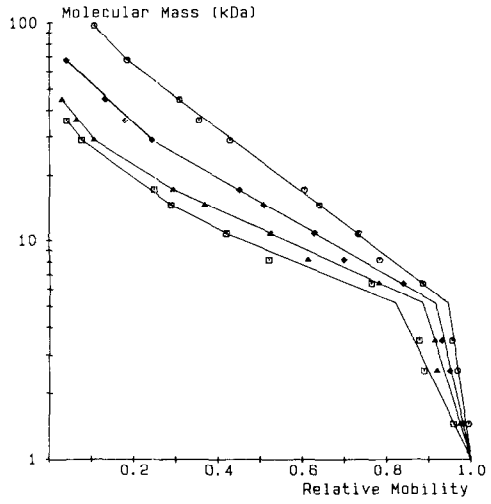

FIG. 1. Calibration curves for different types of separating gels. 10% T, 3% C (○); 16.5% T, 3% C (●); 16.5% T, 6% C (▲); 16.5% T, 6% C plus 6 M urea (□). The composition of the gels is given in Table 2 and the electrode buffers in Table 1. The mobilities of the proteins relative to that of a 1-kDa protein are plotted versus their molecular masses, given on a logarithmic scale. All proteins listed in Table 4 were used to construct the calibration curves. For the sake of clarity, the positions of only some selected proteins are indicated. For the lowest curve these are the proteins of the PMW molecular-weight marker kit (17.2, 14.6, 10.8, 8.2, 6.4, and 2.55 kDa), bacitracin (1.45 kDa), glucagon (3.5 kDa), carbonic anhydrase (29 kDa), and glyceraldehyde-3-phosphate dehydrogenase (36 kDa). In the curves above one extra protein is indicated each time. These are egg albumin (45 kDa), bovine serum albumin (68 kDa), and phosphorylase *b* (97.4 kDa).

the concentration of mercaptoethanol nor on the incubation time. The CNBr cleavage of all other proteins tested so far did not cause any doublets.

In Fig. 3 the discontinuous Tricine system with and without 8 M urea is compared to the continuous system of Swank and Munkres at the same acrylamide and crosslinker concentrations. The denotation 13.3% T, 6% C is equivalent to the denotation 12.5% acrylamide and 1:15 crosslinkage according to Swank and Munkres. The slopes of the calibration curves of all gels are roughly the same above 15 kDa, but in the range from 15 to 5 kDa the resolution by the discontinuous method is better, even when urea is omitted. On the other hand, the slope below 5 kDa is lower in the continuous system. However, as the bands, especially of small proteins, are considerably broader in a continuous system, the sharpness of the bands in the discontinuous system compensates for the steeper slope.

Stacking and Destacking of Proteins

The purpose of stacking gels is to concentrate the proteins of the sample to a stack of very thin protein bands before they reach the separating gel. All proteins of interest should be in the stack and, as a second prerequisite, all proteins have to be separated from the bulk of stacked SDS.

The stacking and destacking was tested empirically by comparing the positions of bromphenol blue, Serva blue G, Pyranin (8-hydroxy-1,3,6,-pyrenetrisulfonic acid), SDS, and suitable standard proteins within stacking (and spacer) gels (Figs 4A–4D). The "upper" stacking limit for large proteins, i.e., the molecular mass of the largest protein that was still in the stack, was easily determined this way. SDS was localized by precipitation with $BaCl_2$ according to Wyckoff *et al.* (9). It is unnecessary to test if there is a "lower" stacking limit that could exclude the SDS complexes of small proteins from the stack, as even SDS itself is stacked (9). However, in agreement with Kyte and Rodriguez (15), we think it is essential to test whether the small proteins run within the stacked SDS or separate from it, because all proteins that are not completely separated from the bulk of SDS give rise to a streaking of the corresponding bands.

In Fig. 4A the stacking within a 4% T, 3% C gel, run under Laemmli conditions (125 mM Tris–HCl, pH 6.8), is shown. Even the dimer of bovine serum albumin (136 kDa) was in the stack (lane 8), but the proteins below 20 kDa (lanes 4 and 5) extended into the zone of SDS

and proteins below 12.4 kDa (lanes 1–3) were completely within the SDS.

The separation of small proteins from the SDS was improved when the 4% T, 3% C stacking gel described in Table 2 (0.75 M Tris–HCl, pH 8.45) was used in combination with the glycine cathode buffer according to Laemmli (Fig. 4B). Myoglobin (17 kDa) and cytochrome *c* (12.4 kDa) no longer extend into the zone of SDS (lanes 4 and 5), but the proteins below 12.4 kDa still migrate with the SDS (lanes 1–3). Due to the higher pH, compared to the stacking gel of Laemmli, the upper stacking limit was shifted down to 100 kDa. Bovine serum albumin (68 kDa), for example, was in the

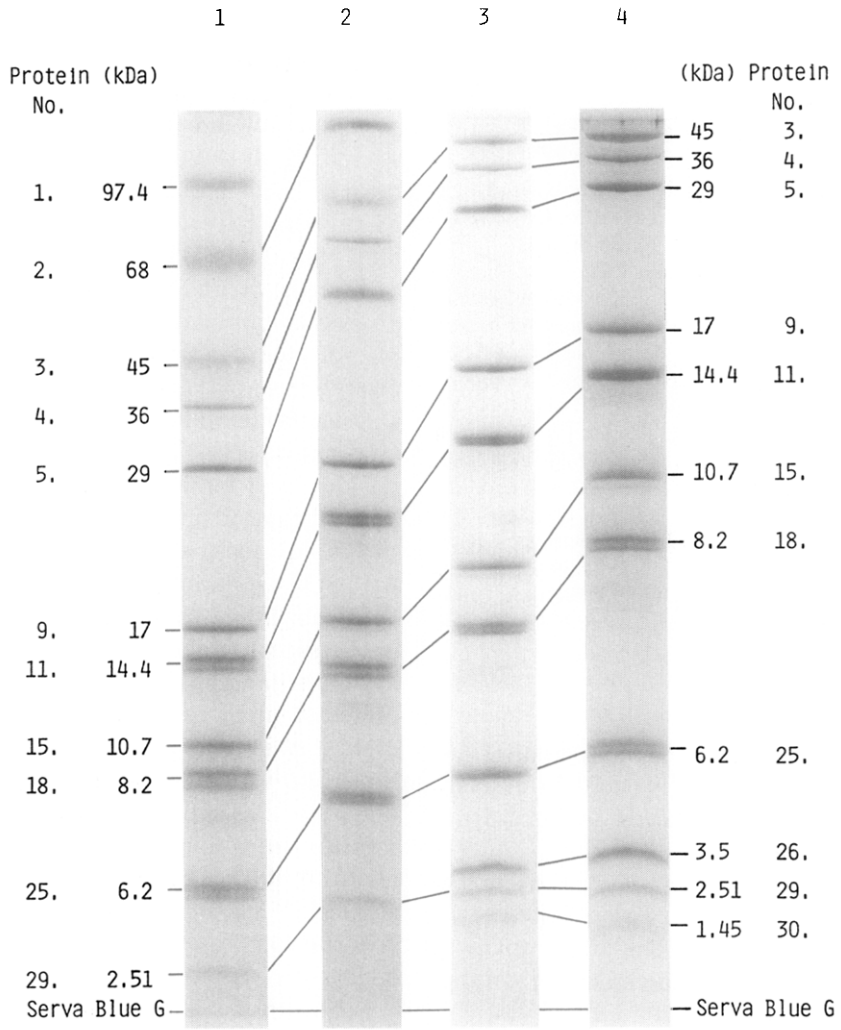

FIG. 2. Separation of selected standard proteins on the four gel types, characterized by the calibration curves in Fig. 1. Lane 1, 10% T, 3% C; lane 2, 16.5% T, 3% C; lane 3, 16.5% T, 6% C; lane 4, 16.5% T, 6% C plus 6 M urea. The numbering of the protein bands is the same as in Table 4. The low-molecular-weight marker kit MW-SDS-17 (proteins No. 9, 11, 15, 18, 25, and 29) is applied to all gels to allow a direct comparison.

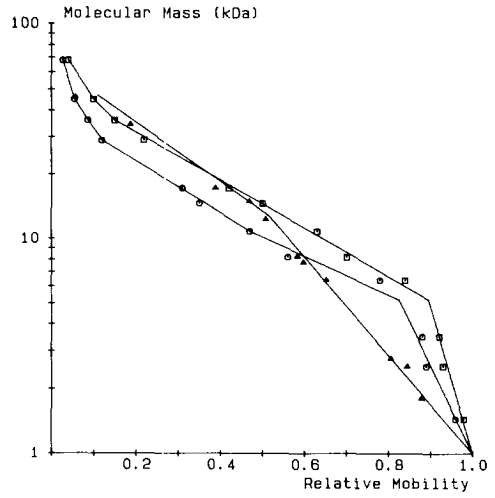

FIG. 3. Comparison of the calibration curve of the 13.3% T, 6% C gel with 8 M urea according to Swank and Munkres (adapted from (6)) (▲) with those of our 13.3% T, 6% C gels without (□) or with (○) 8 M urea. The positions of the same proteins as in Fig. 1 are indicated.

stack, while its dimer was already destacked (lane 8).

A further improvement was achieved by substituting the glycine cathode buffer for the Tricine cathode buffer (Fig. 4C). The upper stacking limit was shifted down to 30 kDa and all proteins above that limit were destacked as sharp bands (lanes 7 and 8). In line with this reduced stacking range, even a 6.4-kDa protein (lane 3) was separated from the SDS. Glucagon with a molecular mass of 3.5 kDa (lane 2) was concentrated at the trailing edge of the SDS, but part of it still extended into the SDS zone. Bradykinin (1.06 kDa) migrated with the SDS, which was concentrated by a factor of 2 compared to Figs. 4A and 4B.

A separation even of the 1-kDa proteins from the SDS could be achieved at acrylamide concentrations between 8 and 10%, but with a view to the sharpness of protein bands up to 20 kDa we preferred to use a 10% T, 3% C spacer gel, overlaid by the 4% T, 3% C stacking gel described above (Fig. 4D). On entering the 10% T gel, this group of proteins close to 1 kDa was destacked in such a way that the proteins stayed very close to the SDS, i.e., the mobility of these small proteins had to be comparable to that of Tricine in order to get sharp bands.

The analysis of the stacking gels described showed that a separation of small proteins from the SDS is only possible at the cost of a reduced stacking range within the stacking gel. This does not mean, however, that other proteins of interest with higher molecular masses necessarily give rise to broad bands.

Within the sample pocket, large proteins, up to at least 100 kDa, were concentrated at the trailing edge of the moving boundary since Tricine has a lower effective mobility than even the SDS complexes of 100-kDa proteins in free solution. The existence of this stacking effect for large proteins within the sample pocket was deduced from the appearance of narrow bands of the respective proteins in the 4% T stacking gel (Fig. 4C). In order to test the efficiency of this concentration effect, we compared the resolving properties of a uniform 10% T, 3% C gel with that of a 10% T, 3% C gel that had a 3% T, 3% C stacking gel (pH 7.5) on top. Within this stacking gel all proteins below 100 kDa were in the stack (Table 5). We observed no difference in bandwidth of the proteins above 20 kDa. However, the protein bands below 20 kDa broadened with falling molecular mass when the stacking gel was omitted. This empirical approach showed that there is no need for a broad stacking range within the stacking gel.

For optimal resolution of proteins, especially of the small ones, three groups of proteins were consecutively concentrated and resolved. Large proteins from 30 to 100 kDa were concentrated within the sample pocket and separated in the 4% T, 3% C stacking gel at pH 8.45. At the same time the group of proteins between 6 and 30 kDa was stacked and separated from the SDS in this 4% T, 3% C stacking gel. The smallest proteins down to

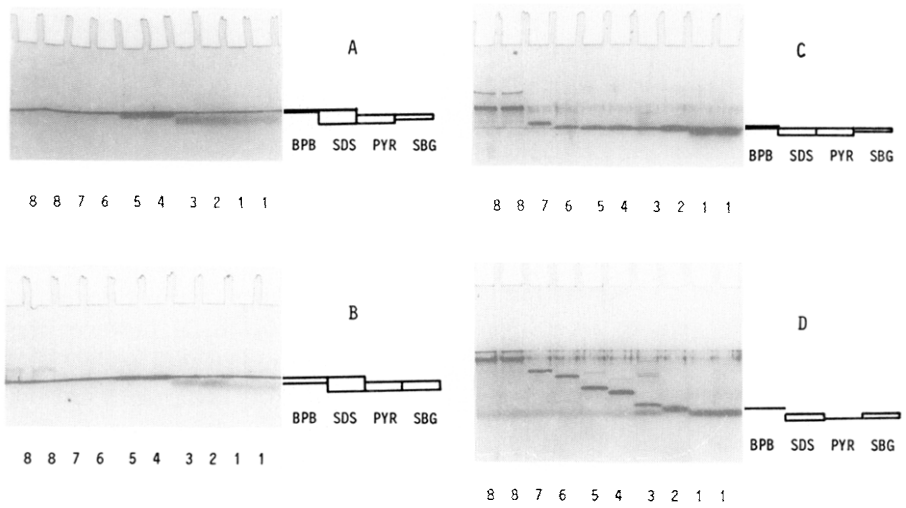

FIG. 4. Stacking and destacking of standard proteins in different 4% T, 3% C gels. (A) Stacking gel according to Laemmli (3), i.e., 125 mM Tris–HCl, pH 6.8; glycine cathode buffer according to Laemmli. (B) Stacking gel as described in Table 2, i.e., 0.75 M Tris–HCl, pH 8.45; glycine cathode buffer according to Laemmli. (C) Same stacking gel as in (B), but Tricine cathode buffer as described in Table 1. (D) Same gel as in (C) combined with an additional 10% T, 3% C "spacer" gel. The filling height of the sample was 5 mm. The standard proteins applied were lane 1, Bradykinin (1.06 kDa); lane 2, glucagon (3.5 kDa); lane 3, subunit 11 of bc_1 complex (6.4 kDa); lane 4, cytochrome *c* (12.4 kDa); lane 5, myoglobin (17.0 kDa); lane 6, carboanhydrase (29 kDa); lane 7, glyceraldehyde-3-phosphate-dehydrogenase (36 kDa); lane 8, dimeric and monomeric bovine serum albumin (136/68 kDa). Additionally, the positions of bromphenol blue (BPB), Serva blue G (SBG), Pyranin (PYR), and SDS are indicated.

1 kDa were then separated from the SDS in 10% T, 3% C gels.

Shifting the stacking limit to lower molecular masses also means that the proteins are separated at lower acrylamide concentration. Figure 5 shows the resolution of the PMW kit on gels with the same acrylamide–bisacrylamide composition (10% T, 3% C), but the electrophoresis was performed with different methods. The separation distance between myoglobin (17.2 kDa) and its 2.55-kDa CNBr fragment was 40% of the total running dis-

TABLE 5

COMPOSITION OF STACKING GELS FOR DIFFERENT STACKING RANGES

Stacking range up to (kDa)	Gel type	Tris (M)	pH[a]	Cathode buffer according to
100	3% T, 3% C	0.41	7.5	This paper
60	4% T, 3% C	0.41	7.5	This paper
30	4% T, 3% C	0.75	8.45	This paper
6	8% T, 3% C	0.75	8.45	This paper
100	4% T, 3% C	0.75	8.45	Laemmli (3)

[a] Adjusted with HCl.

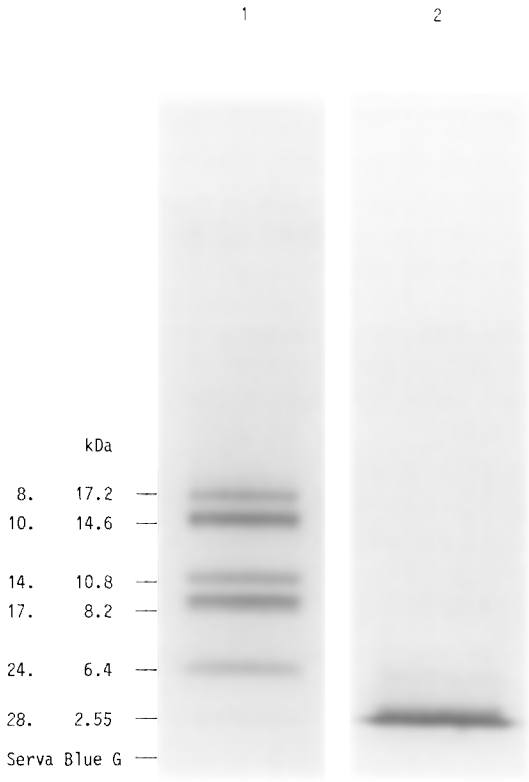

FIG. 5. Resolution of the PMW standard protein kit in 10% T, 3% C gels achieved with our method (lane 1), compared to that of the Laemmli system (lane 2).

tance with our method (lane 1), whereas in the electrophoresis according to the original Laemmli procedure with an acidic sample gel all proteins with a molecular mass of 10 kDa and below migrated with the buffer front. This difference between the Tricine system and the glycine system could also be observed when our method was compared to that of Merle and Kadenbach; the resolution of their 19.3% T, 3% C gel with 6 M urea was achieved in our system by a 16.5% T, 3% C gel without urea. The calibration curves of the two gels were almost identical down to 5 kDa (not shown), but in our system even proteins down to 1 kDa could be resolved.

Discussion

The electrophoresis system described is the only one that proved to be able to resolve all the proteins of beef heart bc_1 complex, where six of the 11 proteins are crowded within the range from 6.4 to 13.4 kDa. The superiority of this method for resolving proteins, especially in the range from 5 to 30 kDa, is based mainly on the introduction of Tricine as the trailing ion and on the tailoring of the stacking limits as narrow as possible within the low-molecular-mass range.

The stacking of small proteins in the presence of SDS is difficult, because small peptides all form complexes of protein and detergent of the "same" size and charge (16) as the SDS micelle itself. Wyckoff *et al.* (9) therefore recommended using minimal amounts of SDS, but often this requirement cannot be met, especially when protein samples have a high content of lipid or detergent. When 4% SDS (140 mM) is used, the situation resembles that of the isotachophoresis, where instead of the high SDS load the protein load is high (10) and the resolution within the stack, i.e., the separation of small proteins from the bulk of SDS, becomes a problem. In contrast to isotachophoresis conditions, however, the extension of the stack should be minimized by performing the electrophoresis at relatively high ionic strength.

Glycine (pK 9.6) and Tricine (pK 8.15) behave quite differently in the stacking of proteins. Glycine, as used in the Laemmli system, leads to a stacking even of very large proteins, because it migrates very slowly in the acidic stacking gel; however, proteins below 20 kDa are only partly or not at all separated from the bulk of SDS. Comparing the modified Laemmli procedures that nevertheless resolve proteins smaller than 20 kDa we found that a shift of the upper stacking limit into the lower molecular-mass range was the general principle. There are several different possibilities allowing that shift. Mostly the ratio of the effective mobility of glycine compared to the effective mobility of the proteins is raised. Merle and Kadenbach (5), for example, slowed down the

mobility of the proteins more than that of glycine by raising the acrylamide concentration of the stacking gel to 8%. Cabral and Schatz (17) made the stacking gel alkaline so that the effective mobility of glycine was raised and additionally the acrylamide concentration of the sample gel was raised to 10%. In a similar way, a separation of small proteins from the SDS may be achieved within the alkaline, low acrylamide part of gradient gels before these small proteins are separated at higher acrylamide concentrations (4). Bothe *et al.* (18) decreased the effective mobility of the proteins by using a linear gradient starting with 10% acrylamide and enhnaced the effective mobility of glycine by raising the pH to 8.96, using the base ammediol, 2-amino-2-methyl-1,3-propanediol (pK 8.8). This base was introduced by Wyckoff *et al.* (9) instead of Tris (pK 8.3).

Another possibility for changing the ratio of the relative mobilities of trailing ions and proteins is to change the trailing ion itself. Kyte and Rodriguez (15) introduced 2-morpholineethanesulfonic acid as the trailing ion, whereas Anderson *et al.* (19) used only a temporary acetate phase. Tricine was suggested as a trailing ion in some of the buffer systems of the "Jovin output" (11), but conditions for a separation of small proteins from the bulk of SDS were not elaborated.

With Tricine, a stacking and destacking of small proteins could be achieved at ideal conditions, i.e., at the same pH as in the separating gel and at low acrylamide concentrations. At the usual pH values between 6.8 and 8.8, Tricine migrated much faster than glycine in a stacking gel, despite its higher molecular mass, because much more Tricine was in the migrating, anionic form. As a consequence, the stacking limit was shifted to the low-molecular-mass range. The stacking and destacking of proteins therefore could be achieved at lower acrylamide concentrations than those in the glycine systems.

There are three reasons why the stacking range within the stacking gel was not extended beyond 30 kDa by making the sample gel acidic:

(i) As stated by Ornstein (8), the pH of the stacking gel should be the same as in the separating gel "to be near ideality."

(ii) In order to improve the stacking of the proteins of interest, the stacking limits should be tailored as narrow as possible (10,12). The resolution within the stack, especially the separation of the smallest proteins from the stacked bulk of SDS, is better the lower the stacking limit is set (Figs. 4A–4C).

(iii) The fact that the proteins above 30 kDa are already separated from the stack of smaller proteins before reaching the separating gel prevents gel damaging or other overloading effects.

Summarizing, the Tricine electrophoresis system presented here offers the following advantages:

(i) A separation of proteins in the range from 1 to 100 kDa is achieved at acrylamide concentrations as low as 10% and low crosslinkage. By using higher, but still moderate, acrylamide concentrations (16.5% T, 6% C), a resolution of proteins in the range from 5 to 20 kDa is achieved, which is superior to all other electrophoretic separations published. The effective resolution below 5 kDa is also superior to that achieved by the Swank and Munkres system, despite the steeper slope, because the protein bands are much sharper.

(ii) The gels can be run with and without urea and there is no need for casting gradient gels.

(iii) Because of the destacking of proteins larger than 30 kDa already within the 4% T gel and due to the low acrylamide concentrations the system needs, it is ideal for preparative tasks. The omission of glycine and urea prevents disturbances which might occur in the course of subsequent amino acid sequencing.

(iv) The electrophoresis system works reproducibly even at some deviation in ionic strength and pH. It tolerates the application of high amounts of protein without overloading effects and the application of protein samples in high-ionic-strength media, e.g., 2 M NaCl.

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