A RAPID AND HIGHLY RESOLVING METHOD FOR PROTEIN SUBUNIT SEPARATION

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1. Introduction

SDS–gel electrophoresis for the separation of protein subunits [1–3] is based exclusively on molecular weight differences of the polypeptides and requires relatively long electrophoresis time.

For separations of large protein subunits which possess very similar molecular weights (e.g., the subunits of yeast fatty acid synthetase) SDS–gel electrophoresis in our hands gave no satisfactory results. Furthermore, shorter separation times are highly desirable in order to avoid the cleavage of labile covalent protein–substrate bonds in active site labelling studies. Therefore, a new method for the rapid and highly resolving separation of protein subunits has been devised which depends not only on molecular weights, but also on the different amino acid composition of the polypeptides in question.

2. Materials and methods

2.1. *Commercial materials*

Sephadex G-50 (Pharmacia, Frankfurt), Soluene 350 and Dimilume 30 (Packard Instruments, Frankfurt), citraconic anhydride and guanidinium–HCl (Fluka, Buchs), Coomassie brilliant blue G-250, SDS, 2,4,6-trinitrobenzene sulfonic acid, acrylamide, Bis and Temed (Serva, Heidelberg), TEA and 2-mercaptoethanol (Roth, Karlsruhe), iodo-1-[^{14}C]-acetamide (57 μCi/μmol) (The Radiochemical Centre, Amersham), all other chemicals (Merck, Darmstadt). All reagents were of the highest purity available. Iodoacetic acid was recrystallized from pentane/heptane, 1:1 (v/v).

Abbreviations: CU electrophoresis, citraconylation–urea electrophoresis; Bis, *N,N'*-methylene bisacrylamide; Temed, *N,N,N',N'*-tetramethylethylenediamine; TEA–HCl, triethanolamine–hydrochloride; FAS, fatty acid synthetase from yeast; IAA, iodoacetic acid; SDS, sodium dodecylsulphate

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2.2. *Proteins*

Yeast FAS was isolated according to [4]. The other proteins were generously provided by the following colleagues at the Max-Planck-Institut für Biochemie, Martinsried: Collagens (Dr P. Müller), *Escherichia coli* RNA polymerase (Dr W. Zillig), *E. coli* RNA polymerase β and β' subunits (Dr P. Palm), *E. coli* RNA polymerase σ subunit (Dr B. Rexer), porcine hemoglobine (Dr T. Kleinschmidt), human fibrinogen α, β and γ subunits (Dr A. Henschen-Edman). Beef kidney pyruvate dehydrogenase was kindly provided by Dr G.-B. Kresze, University of Munich.

Specific labelling of the yeast FAS α subunit with iodo-1-[^{14}C]acetamide was performed according to [5].

2.3. *Pretreatment of proteins for electrophoresis*

2.3.1. Carboxymethylation

The protein solution (60 μg/ml to 15 mg/ml) was poured into double the volume of denaturation buffer: 7 M guanidine–HCl, 0.7 M TEA–HCl adjusted to pH 8.4 with NaOH, 0.12 M 2-mercaptoethanol. Denaturation and reduction was allowed to proceed for $\geqslant$ 30 min at 40°C, followed by carboxymethylation, essentially according to [6]: to 600 μl of the reduced samples, 50 μl of a solution of 2.68 g IAA in 1 ml 1 N NaOH was added and maintained at pH > 8.0 by occasional addition of small amounts of 1 N NaOH.

After 15 min, the samples were treated with citraconic anhydride.

2.3.2. Citraconylation

To 600 μl aliquots of the carboxymethylated samples 60 μl of a 1:1 mixture of citraconic anhydride/tetrahydrofuran (freshly prepared) was added in 10 portions over a period of 10 min at 0°C or room temperature. The pH was kept at about 8.2 (glass electrode) by 9–11 subsequent additions of 60 μl portions of 1 N NaOH. The samples were dialysed for 2 h with stirring against 500 vol. Maurer system 1 electrophoresis buffer (0.6 g Tris and 2.88 g glycine/l, pH 8.3) [7] containing 6 M urea at room temperature.

2.4. *Electrophoresis*

The dialysed samples (5–300 μl) were applied to 60 × 5 mm polyacrylamide gels (system Maurer 1 [7]) and run of 4°C for 1–3 h at 200–300 V (stabilized). Staining was performed with Coomassie brilliant blue G-250.

Titration of free amino groups with 2,4,6-trinitrobenzene sulfonic acid was performed according to [8].

3. Results and discussion

In contrast to most of the untreated proteins which precipitated on top of the urea containing gels, all proteins modified as above, including the collagens, readily migrated in urea containing gels during electrophoresis.

Figure 1 shows the resolution of yeast FAS into two distinct bands and the exclusive incorporation of radioactivity of an α-specific marker into only one band, indicating complete separation into homogeneous polypeptides. Unlike in SDS–gel electrophoresis, where the β subunit (mol. wt 180 000) [11] is the faster migrating polypeptide, the α-chain (mol. wt 185 000) [11] migrates faster with the method described here than the β subunit. This indicates that the separation largely depends upon the amino acid composition [18] of the proteins. The third faint band probably resembles a proteolytic product of chain α (α′) that has also been observed on SDS–gels [11].

Further evidence for the homogeneity of the separated polypeptides comes from:

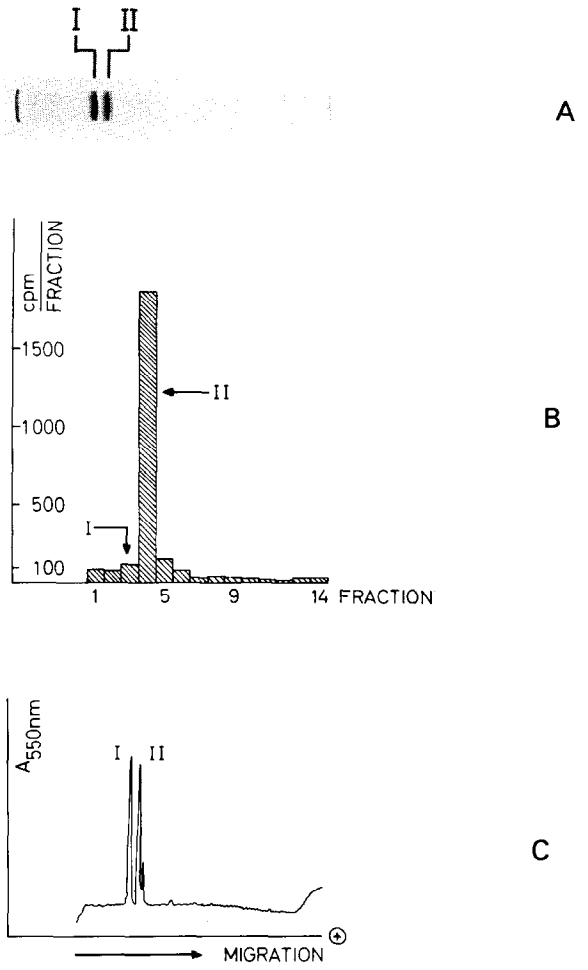

Fig.1.(A) Separation of FAS–polypeptide chains. FAS (6 mg/ml) was treated with denaturation buffer, 2-mercaptoethanol, IAA and CA as in section 2. The dialyzed sample 10 μg was applied to the urea-containing gel (0.5 × 6 cm, 3.4% acrylamide) and run as in section 2. (B,C) Localization of FAS α subunit. FAS was treated with iodo-1-[^{14}C]acetamide, a specific label for the α subunit [5], modified and run as in section 2. (B) Distribution of radioactivity along the gel. (C) Densitomeric scanning profile of the gel after staining.

(i) Titration of free amino groups [8] after gel filtration on Sephadex G-50 which showed complete acylation indicating a high specificity of citraconic anhydride for the ε-amino groups of lysine in accordance with [12,17]. (Reaction with protein thiol groups was prevented by prior carboxymethylation [6].)

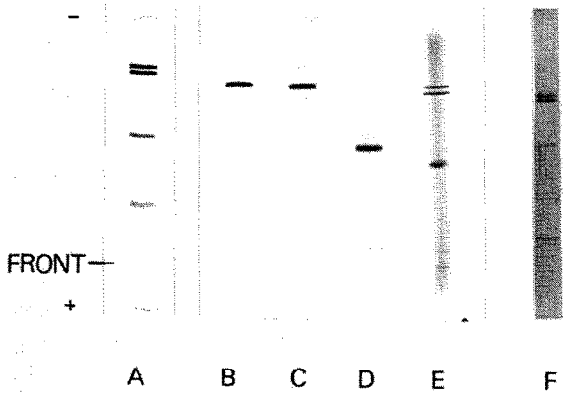

Fig.2. Separation of *E. coli* RNA polymerase subunits. RNA polymerase and its subunits β', β and σ were treated and run on gels as in section 2. Acrylamide concentration of the gels A–E was 3.4%. A complete RNA polymerase, B subunit β', C subunit β, D subunit σ, E subunit β', β and σ mixed, F complete RNA polymerase on a SDS–acrylamide gradient gel (5–15% acrylamide, Laemmli system [3]), kindly provided by Professor Zillig.

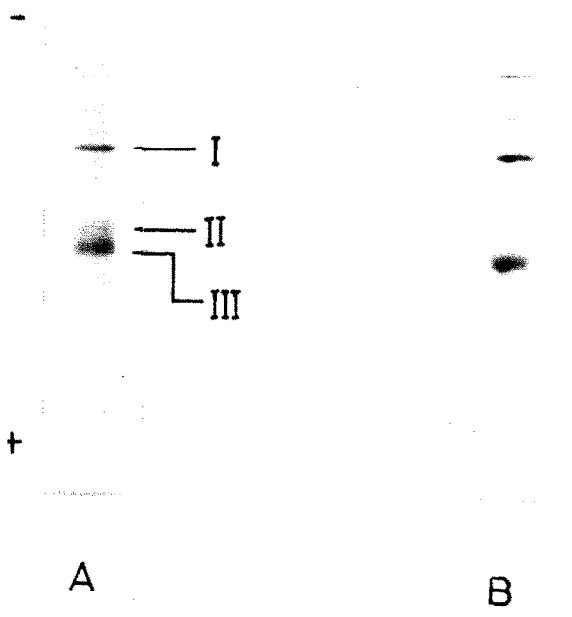

Fig.3. Separation of different collagen subunits. The collagens were treated and run on gels as in section 2. Acrylamide concentration was 3.4%. A type I collagen from chicken, B type III collagen from calf.

(ii) The separation of *E. coli* RNA polymerase which leads to a gel pattern (fig.2) comparable with that obtained by SDS gradient gel electrophoresis (W. Zillig, personal communication). The isolated subunits β', β and σ migrated essentially as single bands (fig.2).

Other examples for the applicability of CU-electrophoresis are shown in fig.3,4.

As fibrous proteins often display anomalous electrophoretic behavior type I and type II collagens were examined: Type I collagen is known to have the composition $(\alpha_1 I)_2\alpha_2$ and to contain dimeric and trimeric forms [14]. The intensity of the band II and III suggests the faster moving band to be α_1I, slightly separated from α_2 (intensity relationship 2:1) (fig.3A). The much more slowly running fraction should resemble oligomeric material. Type III collagen (fig.3B), in agreement with its subunit structure, gave a strong main band. Again some slower moving oligomeric material is visible.

As expected aldolase (a tetramer with four identical subunits) yielded one single band (fig.4A).

The resolution of fibrinogen into three main bands, I, II and III (fig.4B–F), resembling the subunits α, β and γ, was in agreement with SDS–gel electrophoretic studies (fig.4L). Band I shows some heterogeneity due to a known proteolytic degradation of fibrinogen subunit α. It may be noted that with our method (fig.4F) the protein bands appear more concentrated than they do on SDS–gels (fig.4L). This may result from the low diffusion due to relatively high field strength and short migration times.

Fig.4. CU-electrophoresis of human fibrinogen, rabbit muscle aldolase, beef kidney pyruvate dehydrogenase, and porcine hemoglobin. The proteins were treated and run on gels as in section 2. Acrylamide was 7.5% in A–G, L and 10% in H, K. A, rabbit muscle aldolase; B, human fibrinogen (complete); C, human fibrinogen, subunit α; D, human fibrinogen, subunit β; D, human fibrinogen, subunit γ; F, human fibrinogen, subunits α, β and γ mixed; G, pyruvate dehydrogenase from beef kidney; H, porcine hemoglobin; K, SDS–gel of beef pyruvate dehydrogenase, system [2], kindly provided by Dr Kresze; L, SDS–gel of human fibrinogen system [1], kindly provided by Dr Henschen-Edman.

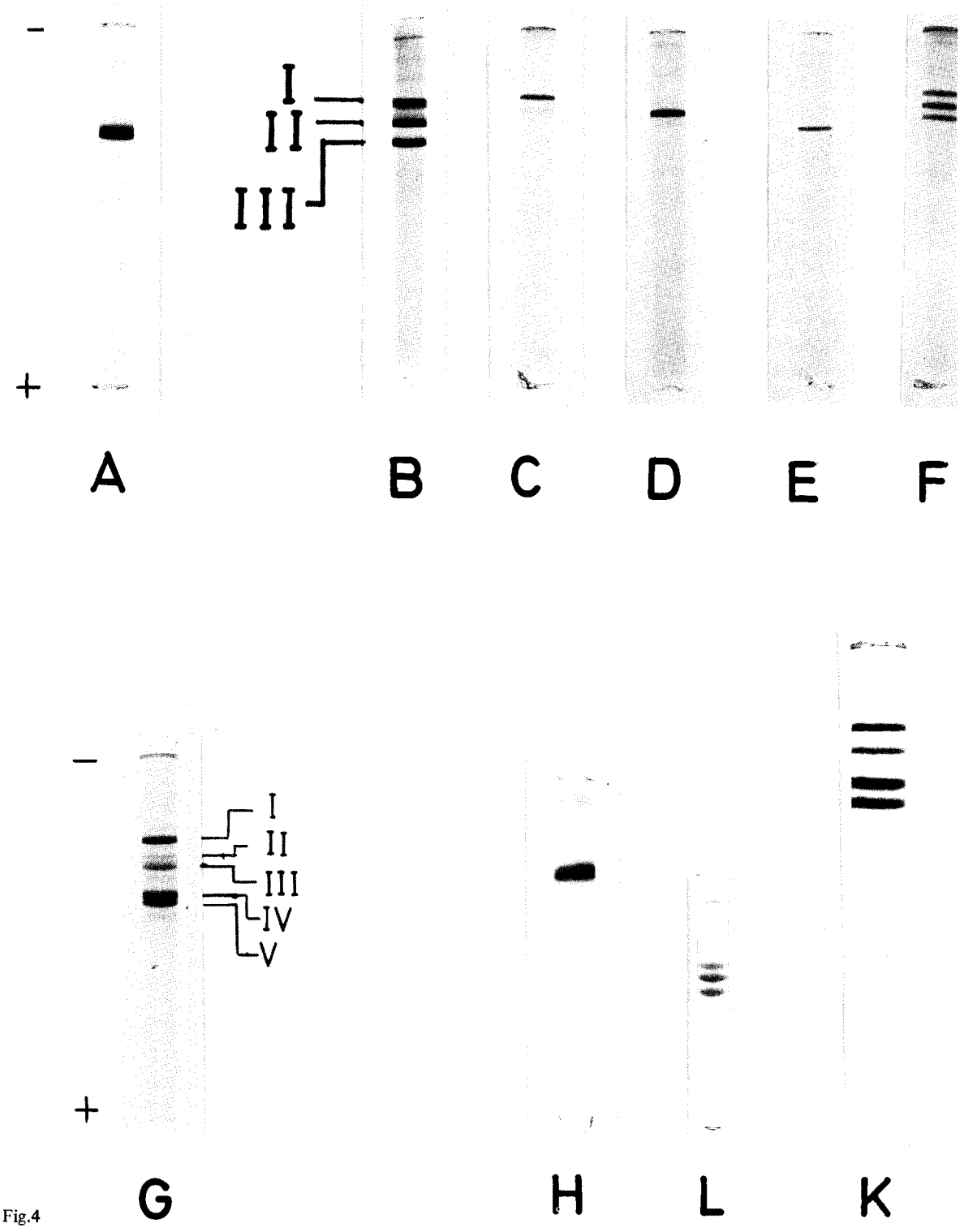

Fig.4

Pyruvate dehydrogenase complex from beef kidney (fig.4G) was resolved into the subunits known from SDS–gels (fig.4K): transacetylase (I), dihydrolipoyl dehydrogenase (III), and the dehydrogenase α and β subunits (IV, V). The faint band (II) could resemble the pyruvate dehydrogenase kinase, one of the regulatory subunits of this complex.

In the case of porcine hemoglobin (fig.4H) no separation into two chains could be achieved. The almost identical molecular weights of the α and β chains together with only minute differences in their acidic amino acid and lysine content [15] might explain this failure.

In conclusion CU-electrophoresis appears as a useful tool for subunit structure investigation: Homogeneous large-pore gels could be used for separation of proteins within a wide molecular weight range; for instance, RNA polymerase subunits with mol. wt 165 000 and 155 000 (β' and β, respectively), of 89 000 (σ) and 40 000 (α) all appeared as sharp zones on homogeneous 5% or 3.4% gels.

The short separation time raises the possibility of using the method for quantitative labelling studies with labile enzyme substrate complexes. Thus, it was possible to assign the three transferases of FAS from yeast to its β subunit [16]. If, as in labelling experiments, a more rapid procedure is required, equilibration with urea-containing sample buffer can be accomplished by gel filtration instead of dialysis.

Citraconic anhydride was used because its aminopeptidyl derivatives are stable enough to avoid artifacts and labile enough to allow for reactivation of proteins to biological activity.

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References

[1] Shapiro, A. L. and Maizel, J. V. (1969) Anal. Biochem. 29, 505–514.
[2] Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412.
[3] Laemmli, U. K. (1970) Nature 227, 680–685.
[4] Lynen, F. (1969) Methods Enzymol. 14, 17–33.
[5] Kresze, G. B., Oesterhelt, D., Lynen, F., Castorph, H. and Schweizer, E. (1976) Biochem. Biophys. Res. Commun. 69, 893–899.
[6] Crestfield, A. M., Moore, S. and Stein, W. H. (1963) J. Biol. Chem. 238, 622–627.
[7] Maurer, H. R. (1971) Disc Electrophoresis, Walter de Gruyter, Berlin, New York.
[8] Habeeb, A. F. S. A. (1966) Anal. Biochem. 14, 328–336.
[9] Spiess, J. (1976) PhD Thesis, University of Munich.
[10] Friedrich, J. (1978) PhD Thesis, University of Munich.
[11] Schweizer, E., Kniep, B., Castorph, H., Holzner, U. (1973) Eur. J. Biochem. 39, 353–362.
[12] Atassi, M. Z. and Habeeb, A. F. S. A. (1972) Methods Enzymol. 25B, 546–553.
[13] Gibbons, I. and Perham, R. N. (1970) Biochem. J. 116, 843–849.
[14] Fietzek, P. P. and Kühn, K. (1976) Int. Rev. Connect. Tiss. Res. 7, 1–60.
[15] Dayhoff, M. O. ed (1976) Atlas of Protein Sequence and Structure vol. 5, suppl. 2, pp. 198–204, The National Biomedical Research Foundation, Washington.
[16] Engeser, H., Wieland, F. and Lynen, F. (1977) FEBS Lett. 82, 139–142.
[17] Dixon, H. B. F. and Perham, R. N. (1968) Biochem. J. 109, 312–314.
[18] Wieland, F., Renner, L., Verfürth, C. and Lynen, F. (1979) Eur. J. Biochem. in press.