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DISSOLVABLE DISULPHIDE–POLYACRYLAMIDE GELS FOR THE ELECTROPHORETIC ANALYSIS OF CHROMOSOMAL PROTEINS AND FOR AFFINO-ELECTROPHORESIS OF THIOL-PROTEINS

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SUMMARY

A discontinous gel system is described that is composed of the dissolvable, disulphide-containing acrylamide gel developed by Hansen and the acetic acid–urea gel as introduced by Panyim and Chalkley. A cleavable SDS gel is used for the identification and an initial separation of proteins according to molecular weight; it is then solubilized by tris–mercaptoethanol and layered on the acidic gel, where it functions as a stacking gel with a pronounced concentrating capacity. The final resolution obtained by this technique permits the identification of single protein species according to the degree of enzymatic modification (acetylation and phosphorylation). Side reactions of the disulphide gel matrix with thiol proteins are discussed and other possible applications are considered.

INTRODUCTION

During our studies on the post-synthetic modification of histones we have developed a routine procedure for quantifying the number of serine phosphates or lysine acetyl groups incorporated per histone molecule. The conventional method used to this end consists of selective extractions of histone species by multi-step applications of salts, acids and organic solvents, followed by gel electrophoresis[1,2]. Unfortunately, modifications may seriously affect the extraction behaviour or solubility of the parent histone[3,4] and, in addition, the procedure is difficult to scale down to the minute amounts of protein obtained by *in vivo* labelling techniques. Two-dimensional gel electrophoresis with isoelectric focussing in the first direction and sodium dodecyl sulphate (SDS) electrophoresis in the second has been used successfully for the analysis of the acidic group in nuclear non-histone proteins[5], but attempts to adapt this system to histones have failed owing to their high isoelectric points (> 11).

Recently, Hansen has prepared polyacrylamide gels with the cleavable cross-linking agent bisacryloylcystamine for the electrophoresis and recovery of RNA[6] and of DNA restriction fragments[7]. We have investigated the utility of this gel for the

separation of chromosomal proteins according to molecular weight and have considered the further processing of single protein species for subfractionation according to charges.

MATERIALS AND METHODS

Bisacryloylcystamine was synthesized from cystamine dihydrochloride (Aldrich, Milwaukee, Wisc., U.S.A.) and acryloyl chloride (Fluka, Neu-Ulm, G.F.R.) as described by Hausen[6]. 1,1,1-Trifluoro-3-bromoacetone (3-bromo-1,1,1-trifluoropropanone) was a product of Peninsular Chemresearch (Gainsville, Fla., U.S.A.).

Polyacrylamide–SDS separation gels (13 or 15%) were prepared according to Laemmli[8] from stock solutions of 30% acrylamide and 0.8% of N,N'-methylenebisacrylamide (1.2% of bisacryloylcystamine in the case of the cleavable gel variant). A slab gel (13 × 7 × 0.2 cm) was polymerized from the respective materials and overlayed with a 13 × 3 × 0.2 cm stacking gel[8]. Samples (1 mg/ml of each protein) were applied in a solution of 0.06 *M* tris·HCl (pH 6.8), 2% SDS, 10% glycerol and 0.001% bromphenol blue (no β-mercaptoethanol). Electrophoresis was carried out at 80 V for 3–6 h.

For subfractionation purposes, 12% acrylamide gels containing 0.9 *M* acetic acid and 6 *M* urea were prepared in glass tubes (15 cm × 4 mm I.D.) according to Panyim and Chalkley[9]. Samples were applied in 8 *M* urea, 5% β-mercaptoethanol and 0.001% crystal violet and run at 140 V (0.7 mA per gel) for 10–14 h. Alternatively, a sample transfer was performed from the cleavable SDS gel as detailed in the text. All gels were stained overnight in a solution of 0.06% Coomassie Brillant Blue G250 in 10% trichloroacetic acid and diffusion destained in 7.5% acetic acid. Densitograms from the gels were obtained with a Gilford Model 240 spectrophotometer, supplemented with a linear transport attachment and a 0.05 × 2.33 mm slit plate.

RESULTS AND DISCUSSION

Optimizing the gel system

The acetic acid–urea containing polyacrylamide system proposed by Panyim and Chalkley[9] was the first electrophoretic method to be applied systematically to histone identification. While these gels have an excellent capacity for the separation of histone subfractions, there is frequent overlap between the bands and no single set of experimental conditions sufficies to resolve maximally all species present[10]. Therefore, we have adapted the system of Laemmli [8], by using a 3% polyacrylamide stacking gel (60 m*M* tris·HCl, pH 6.8, 0.15% SDS) and a 13% polyacrylamide separation gel (380 m*M* tris·HCl, pH 8.8, 0.2% SDS).

Our modification of the Laemmli system involves the replacement of methylenebisacrylamide with the cleavable analogue bisacryloylcystamine in the separation gel while the stacking gel was maintained. As the disulphide cross-link is longer and more flexible than the conventional one[6], it was expected that the acrylamide concentration would have to be increased in order to achieve a separation comparable to that with the original system. In an exploratory experiment, a gel (13 × 7 × 0.2 cm) was prepared in which the acrylamide concentration was increased linearly

from 13 to 20% in the horizontal direction. Under standard conditions (3 h at 80 V and 36–16 mA), histone H4 exhibited R_F values of 0.9 (13%), 0.75 (15%) and 0.4 (19%) if refered to bromphenol blue as the tracking dye. On this gel, the sample run at a 15% acrylamide concentration displayed R_F values comparable to but with a separation superior to the normal system. Fig. 1 shows the densitograms obtained from a histone mixture. Owing to the low concentration of histone H2a, this fraction is barely visible as a shoulder adjacent to the H2b band in the standard gel. In the dissolvable gel variant, on the other hand, it is clearly resolved. Specific pre-treatments of histone samples required to achieve such a separation will be dealt with later.

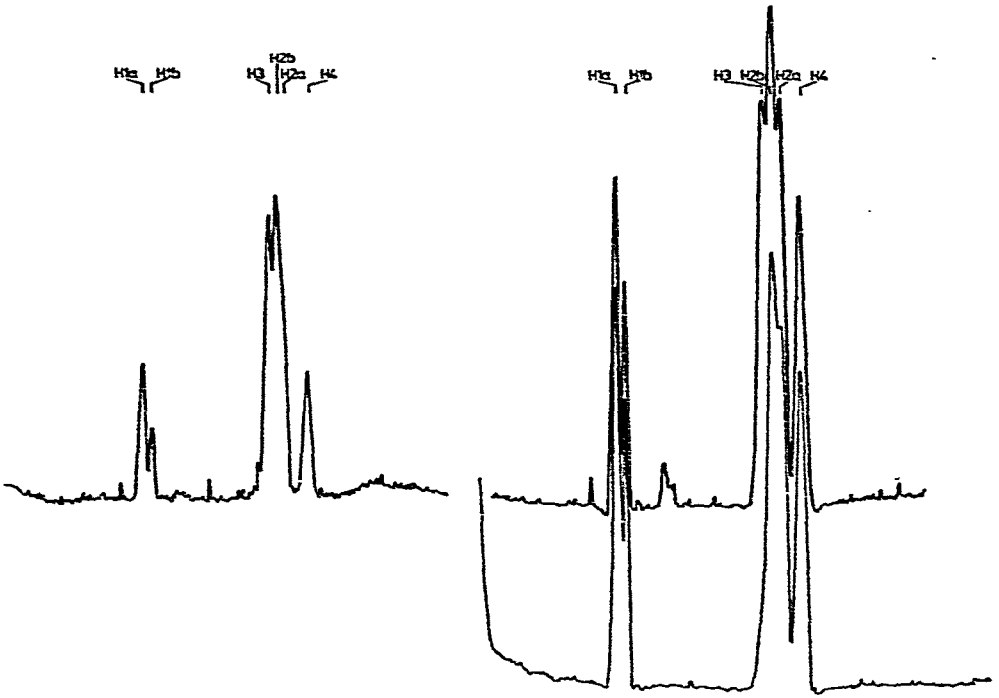

Fig. 1. Electrophoretic separation of an H2a deficient histone mixture on standard SDS gel according to Laemmli[8] (left) and the corresponding SDS–disulphide gel (right). Each gel was supplied with 20 μg of calf thymus histones in 5 μl of sample buffer. Proteins were either applied directly (bottom trace) or after the addition of 0.4 μg of trifluorobromoacetone (TFBA) (top trace, right). The right-hand traces were derived from gels A and D in Fig. 2, respectively.

Preparation of samples

The matrix of a cystamine-containing gel bears similarities to those affinity resins which are in use for the retardation of thiol-proteins via thiol–disulphide interchange reactions[11]. In a histone mixture, the H3 fraction is the only species to contain a cysteine residue[12] and this quality has repeatedly been utilized for its isolation[10,13]. If subjected to electrophoresis on a disulphide-containing gel, only 12% of an H3 histone from calf thymus appears in the expected position while 88% is locked at the interface between the stacking and separation gels (Figs. 1 and 2, traces F and G). As the use of a reducing agent such as mercaptoethanol is precluded in this system

(*cf.*, legend to Fig. 3), it is conceivable that the 12% of H3 which is not retarded represents species with an intramolecular S–S bridge between cysteines 96 and 110[12]. The absence of S–S-linked dimeric H3 forms in traces C to G should be noted, as these will be shown to be part of the band at the origin.

While the retardation of thiol-proteins may offer specific advantages, it will frequently be preferable to detect them in the fractionation range of the gel. 1,1,1-Trifluoro-3-bromoacetone is a neutral reagent with the capacity to mask specifically thiols under mild conditions[14]. If a whole histone mixture is dissolved in 1 ml of sample buffer (30 m*M* tris–hydrochloric acid, pH 6.8, 5% glycerol, 1% SDS + bromophenol blue) and 2 μl of this reagent are added 10 min prior to electrophoresis, a complete histone pattern, including the H3 monomer and the two dimeric forms, is seen to emerge (Fig. 2, traces A and B).

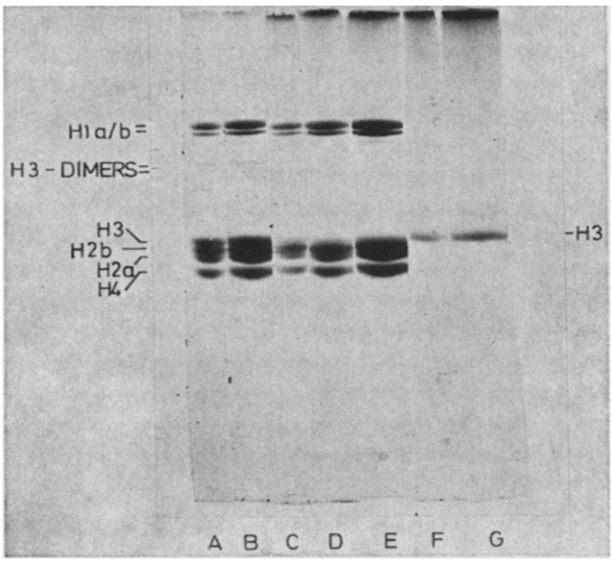

Fig. 2. Electrophoretic separation of a calf-thymus histone mixture and of histone H3 on disulphide gels. Slots were supplied with 10–30 μg of histones in 5 μl of the sample buffer. The histones were either applied directly (sections C–G) or after incubation for 15 min with 0.4 μg of trifluorobromoacetone (sections A and B).

This analysis scheme has been extended to the so-called "high mobility" group (HMG) of proteins present in chicken erythrocyte nuclei. All four main fractions (HMG 1, 2, 14 and 17) have been reported to contain cysteines[15]. Although a complete pattern of these proteins is obtained after the suggested alkylation procedure (Fig. 3, trace C), only HMG 1 and 2 are missing in the absence of that step (Fig. 3, trace B). It must therefore be concluded that thiols are either absent in HMG 14 and 17, or masked in the same way as in the minority fraction of histone H3. Labelling experiments using the thiol-specific compound difluorescein disulphide[16] or the selective alkylating reagent [1-^{14}C]iodoacetamide are in agreement with this interpretation (*cf.*, Fig. 3). Iodoacetamide has been suggested as a stabilizing reagent for cysteine residues during SDS gel electrophoresis[17], but protein bands at the gel

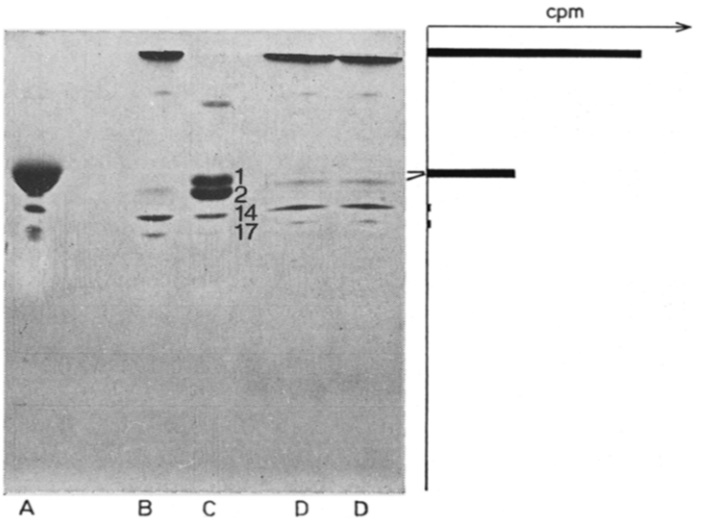

Fig. 3. Electrophoresis of HMG proteins on disulphide gels. Each slot was supplied with 20 μg of a protein mixture in 5 μl of sample buffer. For slot C, protein was pre-treated with 0.4 μg of trifluorobromoacetone, and for slot D with 0.9 μg (0.25 μCi) of [^{14}C]iodoacetamide. The detection of radioactivity was achieved after solubilizing the stained bands in tris–mercaptoethanol and adding the slurry obtained (300 μl) to the scintillator (solid bars). With a blank prepared from [^{14}C]iodoacetamide in the absence of protein, no radioactivity was found at the start of the separation gel. Section A shows that mercaptoethanol must not be used in sample buffers as the stacking gel does not provide a sufficient barrier against its diffusion.

origin proved that it is far less effective than fluorobromoacetone under our conditions (*cf.*, traces C and D in Fig. 3).

Sample transfer from the dissolvable gel to acetic acid–urea gels

Our objective in initiating a study on cleavable SDS slab gels was the recovery of the five principal histones from cell cultures in amounts that would allow subfractionation on a different gel. As a first step, we describe some model experiments on calf thymus whole histone which demonstrate that stained SDS gel slices can be processed in a way that allows an undisturbed protein pattern to be developed upon re-electrophoresis.

The gel strips in Fig. 2 are parts of a cleavable SDS slab gel; from section E, five stained protein bands were cut, *i.e.*, origin, H1b, H2b, H2a and H4. To remove excess of trichloroacetic acid, each band was soaked in 1 ml of 0.4 *M* Tris·HCl (pH 8.2) in a micro test-tube and shaken vigorously for 1 h. The liquid was then withdrawn and replaced with 25 μl of 8 *M* urea, 0.4 *M* tris·HCl (pH 8.2) and 75 μl of β-mercaptoethanol. Gel pieces were homogenized in this medium with the aid of a glass bar and left on a vortex mixer overnight for complete dissolution.

The homogeneous blue slurry obtained was then layered on the surface of 20% polyacrylamide gels containing 6 *M* urea, which had been prepared in glass tubes (11 × 0.4 cm I.D.). Any space remaining in the tubes was carefully supplied with the electrode buffer (0.9 *M* acetic acid), and electrophoresis was performed at 0.7 mA per gel for 5 h. After the usual staining and destaining sequence, all histone bands

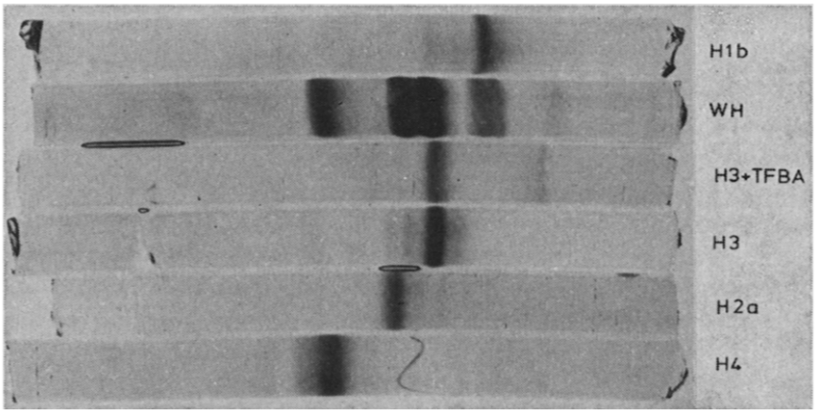

Fig. 4. Re-electrophoresis of Fig. 2 proteins on 11-cm 20% acrylamide–acetic acid–urea tube gels. Stained bands were cut from the disulphide gel and processed as described in the text. H3 was recovered both from the top of gel D (Fig. 2) and from gel B (Fig. 2); the latter fraction is marked "H3 + TFBA" as its thiols had been alkylated prior to its application to the Fig. 2 gel.

were found to have R_F values resembling a whole histone reference that had undergone no comparable pre-treatments (Fig. 4).

The H3 band regenerated from the origin of gel section E (Fig. 2) illustrates that cleavage of S–S bonds is efficient under these conditions; it is indistinguishable from alkylated H3 derived from section B (Fig. 2), suggesting that no positive charges have been masked by side reactions involving trifluorobromoacetone.

To improve the resolution, the length of the gel was extended to 15 cm and the acrylamide concentration finally reduced to 12% (Fig. 5). After the usual sample transfer technique, electrophoresis revealed that all calf thymus histones except H1a and H1b are composed of subfractions, while the acetylated forms of histones H3 and H4[18] are particularly well resolved. Again, subfractionation is not a consequence of the alkylating reagent as the most lysine-rich histone, H1a, behaves identically whether or not it has been exposed to trifluorobromoacetone (Fig. 5). It is particularly evident from Fig. 5 that proteins derived from the cleaved SDS gel are resolved better than the counterparts present in the whole histone (WH) reference, although sample sizes of up to 600 μl were applied in the former instance. This apparent improvement is ascribed to the fact that the cleaved acrylamide not only acts as a sample gel but also as a stacking gel with a very pronounced concentrating capacity: on applying the electric field, histones start to migrate to the anode due to the negative charges imposed by the SDS carried over from the disulphide gel. This initial migration is apparent from the residual Coomassie Blue stain, which forms a very sharp, upwards moving band during the first hour. At the interface of the sample medium (tris·HCl, pH 8.2) and the anode buffer (0.9 *M* acetic acid, pH 2.4) the histones will become recharged, *i.e.*, follow their intrinsic positive charges in the downwards direction while the dye is visibly lost. SDS is either also stripped off (the presence of urea weakens the affinity to proteins[19]) or at least has no further influence at the lower pH value.

Fig. 5 includes two representative examples of *in vivo* modifications of the histones. (i) If a cell line from chinese hamster ovaries (CHO) is arrested in the

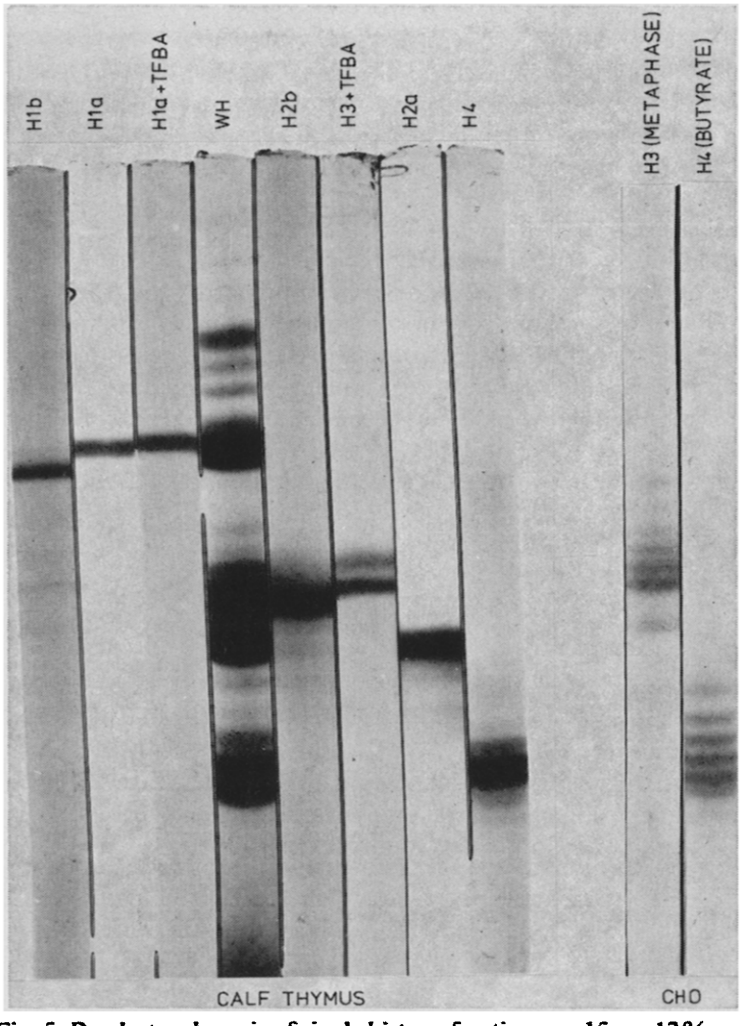

Fig. 5. Re-electrophoresis of single histone fractions on 15-cm 12% acrylamide–acetic acid–urea gels. Histone fractions were isolated via disulphide–SDS gels from calf-thymus whole histone that had been alkylated ("TFBA") or had not been pre-treated (left-hand gels). Specifically modified histones H3 and H4 were similarly isolated from a CHO cell line that had been treated as described in the text (right-hand gels).

metaphase by the addition of colcimide, the H3 histone becomes specifically phosphorylated[1]. We are at present trying to isolate chromatin subunits (nucleosomes) from metaphase cells, which is complicated by the high phosphatase activities at this stage of the cell cycle[1]. The gel depicted shows the H3 fraction of a partially degraded chromatin in which part of the unmodified histone has recurred while species carrying one to five phosphate groups are still visible. (ii) If the CHO cell line is grown in a medium containing sodium butyrate, the deacetylation of histones is suppressed, as described for HeLa cells[20]. Fig. 5 shows a variety of acetylated H4 subfractions that were obtained by processing the single H4 band on the cleavable SDS gel as described above.

CONCLUSION

Polyacrylamide gels containing the reversible cross-linker bisacryloylcystamine may be adapted to the separation of proteins. Thiol-proteins are recovered from the origin of such a gel while the other species exhibit normal electrophoretic behaviour. This property appears promising for the convenient identification of reduced thiol groups or the separation of otherwise similar proteins; in instances where it could cause complications, it can be readily eliminated with a mild alkylating reagent such as 1,1,1-trifluoro-3-bromoacetone.

For cleaving a gel contaning up to 20% of acrylamide, the conditions of Hansen[6] had to be modified, mainly by adding an aqueous alkaline component to the solubilizing agent, β-mercaptoethanol. By this procedure even concentrated gels are converted into a homogeneous slurry that can be transferred to the next electrophoretic system where it may be repolymerized by air oxidation if desired[6]. During our study, a discontinous gel system was constructed from the solubilized material containing the protein sample and the acid–urea gel first applied by Panyim and Chalkley[9]. In this combination, the separating power of the acidic gel is greatly increased and it is feasible both to identify proteins and to characterize them according to their modifications during the suggested two-step procedure.

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