STABILIZATION OF HISTONES FROM RAT LIVER

L.D. NOODEN*, H.W.J. VAN DEN BROEK**
and J.S. SEVALL
Division of Biology, California Institute of Technology, Pasadena, Calif. 91109, USA

Received 20 November 1972

1. Introduction

The existence of protease(s) which rapidly and preferentially degrades histones is now well documented (see [1–4], and literature cited). As the reconstitution of chromatin and the biological role of histones are studied further, this protease will be of even greater concern that it is now. Thus far, the best methods for blocking this activity employ a very high concentration of $NaHSO_3$, 50 mM, and/or low pH's, 5–6 [3] either of which may have undesirable effects. In this report, we describe a new way of inhibiting the histone protease, stabilizing the histones of rat liver, and some improved conditions for storage in solution.

2. Materials and methods

Chromatin and thence histones were prepared from frozen male rat livers (Pel-Freeze Biologicals, Rogers, Ark.) as described elsewhere [5]. The proteins in this chromatin were dissociated in 3 M NaCl, 10 mM Tris pH 8, separated from DNA on BioGel A-50 m and then precipitated with 95% saturated $(NH_4)_2SO_4$. Finally, the histones were removed from the other chromosomal proteins by adsorption onto BioRex 70 from 0.4 M NaCl, 10 mM Tris pH 7 and collected by elution with 1 M NaCl, 1 mM $NaHSO_3$, 10 mM Tris pH 7 to give a solution with about 1.2 mg/ml histone. For studies on storage conditions, aliquots of this solution were modified in composition by additions or by dialysis.

PMSF (phenylmethylsulfonyl fluoride) from Cal Biochem was dissolved initially in isopropanol and thence diluted with H_2O to give a stock solution with 1 mM PMSF and 10% isopropanol. Histone solutions (eluants from BioRex described above) were adjusted to the appropriate concentration of PMSF and dialyzed 24 hr against 10 mM Tris pH 7 with PMSF at 4°. PMSF was also removed from some of these samples by dialyzing against 2000 vol of 10 mM Tris pH 7 for 24 hr.

Samples were taken from the various stored histone solutions and dialyzed against 7 M deionized urea at 4° for at least 24 hr. Shortly before electrophoresis, about 1/20 vol of mercaptoethanol was added to the histone solution.

All of the gels (about 0.6 × 7 cm) in this report were prepared and run according to the method of Panyim and Chalkley [6]. These contained 0.9 M acetic acid, 2.5 M urea and 15% acrylamide. After 3 hr of preelectrophoresis, the gels were loaded with sample (75 μl were used in gels shown in figs. 1A and B), run for 3 hr at 2 mA per gel, stained with 1% Amidoschwartz in 40% ethanol–7% acetic acid and destained electrophoretically in the ethanol–acetic acid solution alone.

3. Results

Histones prepared by adsorption onto the anion

* Present address: Department of Botany, University of Michigan, Ann Arbor, Mich. 48104, USA.
** Present address: Department of Genetics, Agricultural University, Wageningen, The Netherlands.

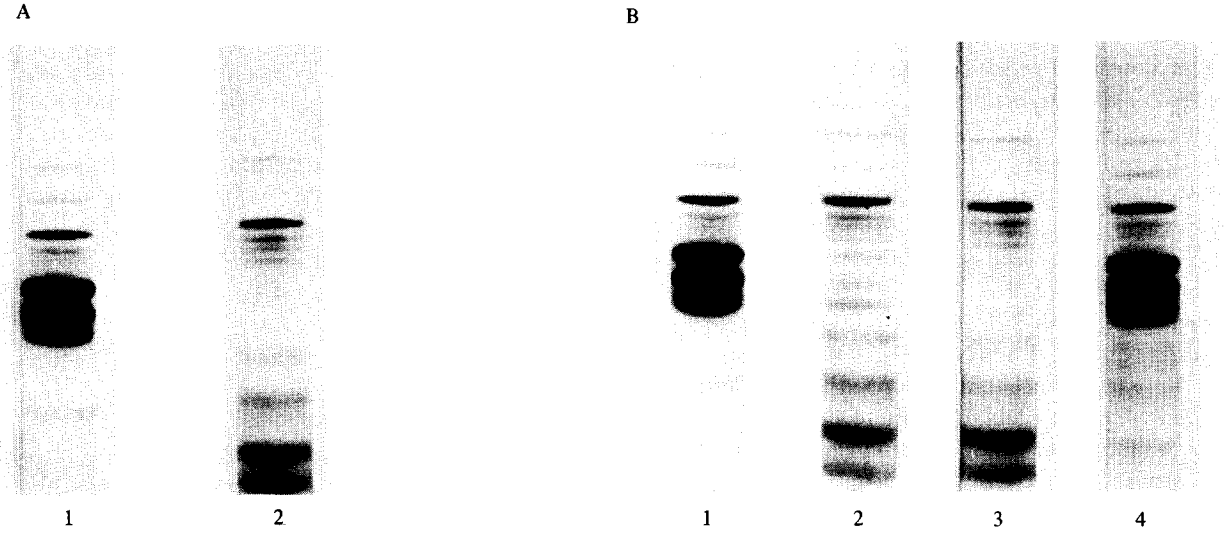

Fig. 1. Analytical polyacrylamide gels with acidic urea (method of Panyim and Chalkley [5]). Direction of movement downward. A) Left to right: (1) Reference – Histones after elution from BioRex 70. (2) Same histones after dialysis against 10 mM Tris pH 7 for 24 hr. B) Left to right: (1) Reference – Histones treated with 100 μM PMSF (pH 8) for 24 hr. (2) Histones treated with 10 μM PMSF (pH 8) for 24 hr. (3) Histones dialyzed against 10 mM Tris pH 8 for 24 hr. (4) Histones treated with 100 μM PMSF (pH 8) for 24 hr then dialyzed against 10 mM Tris pH 8 for 24 hr.

exchange resin BioRex 70 followed by elution with 1 M NaCl contain the histone proteolytic activity even though they are loaded in 0.4 M NaCl which prevents adsorption of most nonhistone proteins [5]. As a result, the histones prepared from BioRex 70 are highly unstable and do not store well.

In order to stabilize the histones for storage and eventual use in other studies, we tested a variety of storage conditions. Freezing, of course, halted the proteolytic activity but only while the samples were frozen, otherwise it did not seem to affect the histones in any way which could be detected on acidic urea polyacrylamide gels. After some guesswork and testing, we found that these histones were stable at 4° in a combination of 1 mM $NaHSO_3$, Tris at pH 7 and NaCl at 1 M or greater, and thereafter, this solution was used to elute the histones from BioRex. Histones could be stored in this solution for at least 2 weeks at 4° without changes that can be detected with acidic urea gel electrophoresis. Not only does removal of $NaHSO_3$ and NaCl or any substantial decrease in their concentrations destabilize these histone preparations (fig. 1A), but even removal of the NaCl alone results in a similar degradation of the histones at pH 7. Fig. 1A also illustrates the differential susceptibility of the different histones to degradation; histone I (the uppermost dark band in the gels) is relatively resistant, while the other histones are degraded relatively rapidly and at roughly the same rate (a detailed study might well show some differences). As the histone bands decrease in stainability, other bands which run ahead of the histones increase. These are probably large fragments derived from the histones.

The proteolytic inhibitor, phenylmethylsulfonyl fluoride (PMSF) [7], blocks the histone hydrolyzing activity when used at 100 μM with similar results at pH 7 and pH 8 (fig. 1B). At 10 μM, degradation is retarded somewhat; however, it still occurs. The 100 μM PMSF can be dialyzed out of this histone solution and the solution stored at pH 8 in the absence of $NaHSO_3$ of NaCl for about 24 hr without noticeable degradation. Histones handled in this way without PMSF treatment show extensive degradation.

Unlike the 50 mM $NaHSO_3$ commonly used, the inhibitory level of PMSF (100 μM) does not strongly absorb UV at 230 nm, a wavelength which is useful in estimating protein contents especially in column eluants.

4. Discussion

The protease(s) which degrade the histones present a serious obstacle to many types of studies on the histones themselves and also studies of their interaction with nonhistones and DNA as in reconstitution. Not only is this activity present in histones prepared without acid extraction (e.g., adsorption onto BioRex 70), but it has even been noted in HCl extracts of chromatin [2].

We found that these histones could be stabilized for storage at 4° in 1 mM $NaHSO_3$, 1 M NaCl, Tris pH 7. Very high concentrations of $NaHSO_3$ such as 50 mM were not needed. Contrary to earlier reports [3, 4], we found the histone hydrolyzing enzyme(s) to be very active at low salt concentrations. Furlan and Jericijo [1] also noted substantial activity at low salt concentrations. The lower salt concentration could cause the histones to precipitate thereby becoming less accessible to the protease [4]. We did not observe any significant precipitation in our histone solutions at low salt concentrations; however, it must be noted that our histones were prepared and handled in a different way. Up to now, the principal method for blocking this activity has been with $NaHSO_3$ at high concentrations (50 mM), which is undesirable not only because it may interfere with chromatin isolation [8] and measurement of the protein but contributes to ionic strength not to mention other possible effects on histones, other proteins, and even DNA [9]. Furthermore, the inhibitory effect of $NaHSO_3$ is readily reversible. Thus we sought to find a better method for inhibiting the protease(s) and tested PMSF, a proteolytic inhibitor which functions much like the highly toxinc diisopropyl phosphorofluoridate (DPF) by binding serine in the active site [7]. It turned out to be a powerful inhibitor of histone degradation, and it is not reversible within a period of 24 hr; however, since the phenylmethylsulfonyl group is slowly released from α-chymotrypsin [7], this group is probably eventually lost from the histone hydrolyzing enzyme too. Thus the proteolytic activity would probably eventually be reactivated, but the time required would depend on the conditions. Pharmacological studies suggest that PMSF should not be as toxic as DPF [10]. Not only is PMSF useful in stabilizing histones in solution during studies on chromatin reconstitution, etc., but it may be helpful in the preparation of histones by milder procedures such as that described by us elsewhere [5].

Acknowledgements

This investigation was supported in part by U.S. Public Health Service Research grant no. GM-13762. LDN was supported by National Institutes of Health Special Research Fellowship No. 1-F03-GM-51264-01, HWJB by a research fellowship from the Netherlands Organization for the Advancement of Pure Research and JSS by a Damon Runyon Memorial Fund Research Fellowship.

References

[1] M. Furlan and M. Jericijo, Biochem. Biophys. Acta 147 (1967) 135.
[2] M. Furlan and M. Jericijo, Biochem. Biophys. Acta 147 (1967) 145.
[3] J. Bartley and R. Chalkley, J. Biol. Chem. 245 (1970) 4286.
[4] J.I. Garrels, S.C.R. Elgin and J. Bonner, Biochem. Biophys. Res. Commun. 46 (1972) 545.
[5] H.W.J. van den Broek, L.D. Noodén and J.S. Sevall, Biochemistry, in press.
[6] S. Panyim and R. Chalkley, Arch. Biochem. Biophys. 130 (1969) 337.
[7] A.M. Gold and D. Fahrney, J. Amer. Chem. Soc. 3 (1964) 783.
[8] L. Kleiman and R.C. Huang, J. Mol. Biol. 64 (1972) 1.
[9] M. Hayatsu and R.C. Miller, Biochem. Biophys. Res. Commun. 46 (1972) 120.
[10] P. Turini, S. Kurooka, M. Steer, A.N. Corbascio and T.P. Singer, J. Pharmacol. Exptl. Therap. 167 (1969) 98.