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# Purification of histone H1 polypeptides by high-performance cation-exchange chromatography

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## SUMMARY

Calf thymus histone 1 (H1) was cleaved by chemical and enzymatic methods and the resulting polypeptides were fractionated by high-performance cation-exchange. Up to 1 mg of H1 polypeptides were loaded onto a 50 × 5 mm I.D. cation-exchange column and fractionated to greater than 95% purity in less than 30 min. This is the first report on the separation of H1 polypeptides by a strong cation-exchange matrix. In addition, the high-performance cation-exchange chromatography protocol represents a significant decrease in fractionation time when compared to conventional ion-exchange and gel filtration chromatography. The utility of this procedure is shown when the H1 peptides purified by the protocol were used to define antigenic domains of H1 band by procainamide-induced lupus and idiopathic systemic lupus erythematosus. The majority of the sera tested by enzyme-linked immunoassay (ELISA) reacted to the C-terminal peptides of H1 indicating this to be the major antigenic domain of H1.

## INTRODUCTION

Histones are low-molecular-weight nuclear proteins with a high content of arginine or lysine. Histone 1 (H1) is the most variable of the histones and the number of H1 subfractions varies from tissue to tissue and species to species[1]. Although the precise cellular role of H1 remains unknown, an important adjunct to these studies is the ability to isolate and purify various domains of the native protein. These types of studies, pioneered by Crane-Robinson and co-workers[2,3], have shown that H1 can be cleaved into three structural domains. The purification of these peptides has provided an important approach to determine the primary structures and antigenic domains of H1 and its variants[4–6]; to study their role in chromatin structure[7] and the transport of newly synthesized H1 from the cytoplasm to the nucleus[8].

Using H1 peptides prepared by enzymatic or chemical cleavage, it has been demonstrated that the central region, made up of approximately 75 amino acids, contains hydrophobic residues and under physiological conditions assumes a globular structure. This domain locates H1 in the nucleosome at the point where DNA

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strands exit the chromatosome[7]. The cationic N-terminal domain consists of approximately 33 amino acid residues while the randomly coiled C-terminal domain makes up the other half of the intact H1 molecule.

Most protocols call for the separation and purification of the cleavage products of H1 by gel filtration and weak cation-exchange chromatography. Because gel filtration and ion-exchange chromatography of the cleaved H1 polypeptides normally requires extensive fractionation time, other approaches such as high-performance cation-exchange chromatography (fast protein liquid chromatography, FPLC) have been evaluated.

We describe an FPLC protocol that provides a rapid and quantitative method for purifying H1 polypeptides. This method can be used to fractionate milligram quantities of H1 polypeptides by employing a short, cation-exchange column and a linear gradient of sodium chloride in phosphate buffer. The polypeptides can be fractionated to more than 95% purity in 20–30 min. The utility of this approach in preparing peptides that can be used to determine immunodominant domains of H1 that react with systemic lupus erythematosus (SLE) and procainamide induced lupus sera is demonstrated.

## MATERIALS AND METHODS

### *Cleavage of calf thymus H1*

H1 was extracted from calf thymus with 5% aqueous trichloroacetic acid and purified by chromatography on a Bio-Gel P-60 column[9]. A schematic representation of H1 and the different peptides derived by the following chemical and proteolytic cleavage is shown in Fig. 1.

*N-Bromosuccinimide cleavage of H1.* N-bromosuccinimide (NBS) cleaves on the C-terminal side of tyrosine residue 72 of calf thymus H1[10]. An amount of 2.5 mmol of H1 in 10 ml of 50% aqueous acetic acid was digested with 25 mmol of freshly prepared NBS. After 3 h 25 mmol of fresh NBS was added and the reaction terminated after 4 h by lyophilization. This resulted in the production of peptides 1–72 and 73–220 (see Fig. 1).

*Chymotryptic digestion of H1.* A 50-mg amount of purified calf thymus H1 in 5 ml of 50 mM Tris–HCl (pH 8.0) was cleaved with four units of $\alpha$-chymotrypsin at 24°C for 15 min to yield peptides corresponding to residues 1–106 and 107–220, and residual intact H1[11]. After the reaction was terminated with 5 m*M* phenylmethylsulfonyl fluoride, the peptides were dialysed (Spectra Por 3, molecular weight cut-off 3500) overnight at 4°C against 5 m*M* acetic acid, lyophilized and the dried powder stored at −20°C in a desiccator.

*Thrombin digestion of calf thymus H1.* Thrombin cleaves on the C-terminal side of residue 122 of calf thymus H1 at the sequence -(Pro)-Lys-Lys-Ala to produce peptide residues 1–22 and 123–220[12]. Digestion of 50 mg of H1 in 5 ml of 50 m*M* Tris–HCl (pH 8.0) buffer containing 2 m*M* calcium chloride was achieved with 50 units of thrombin for 6 h at 36°C. The reaction was terminated by the addition of 5 m*M* diisopropyl fluoride and the peptides were dialysed against 5 m*M* acetic acid and lyophilized.

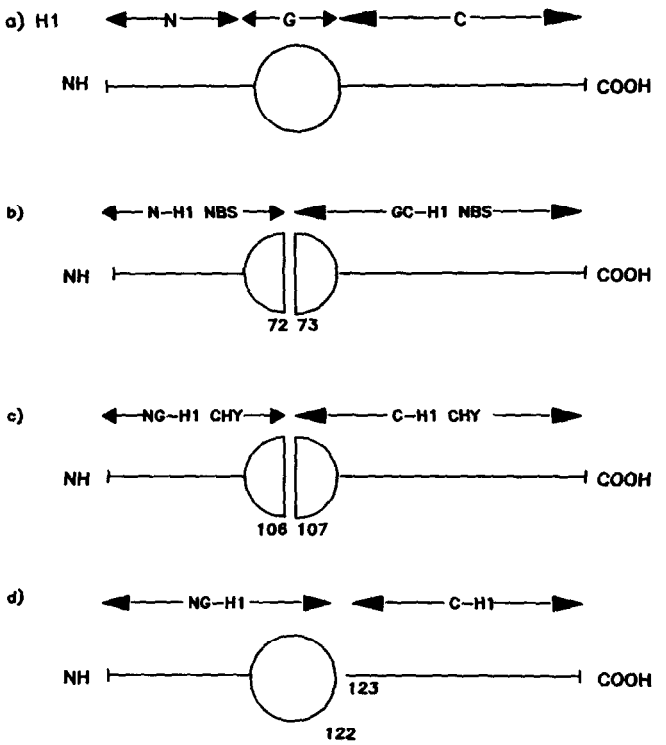


Fig. 1. Calf thymus H1 and peptides obtained by chemical and enzymatic cleavage. (a) Intact calf thymus H1 represented as a tripartate structure with the N-terminus (N), the central globular domain (G) and the C-terminus (C). (b) Peptides generated by NBS cleavage, GC-H1 NBS (residues 73–220) and N-H1 NBS (residues 1–72). (c) Peptides generated by chymotrypsin, C-H1 CHY (residues 107–220) and NG-H1 CHY (1-106). (d) Peptides generated by thrombin digestion C-H1 (residues 123–220) and NG-H1 (residues 1–122).

*FPLC of H1 polypeptides*

Calf thymus H1 polypeptides generated by digestion with NBS, chymotrypsin and thrombin were separated by FPLC. Lyophilized H1 polypeptides were dissolved at a concentration of 5 mg/ml in 0.1 *M* phosphate buffer (pH 7.0) and 200 ml of sample loaded onto a Mono Q column (HR5/5, 50 × 5 mm I.D., Pharmacia). The progress of chromatography was monitored and controlled by the Pharmacia FPLC LCC-500 computer. The peptides were eluted with a linear gradient of 0 *M* NaCl–0.1 *M* phosphate buffer (pH 7.0) to 1.0 *M* NaCl–0.1 *M* phosphate buffer (pH 7.0) over 20–30 ml. The eluate was monitored at 214 nm and 1-ml fractions were collected. The peptides were precipitated with $\frac{1}{5}$ volume of 100% trichloroacetic acid and washed three times with 100 volumes of acetone–25 m*M* hydrochloric acid. The powder was vacuum-dried and stored at −20°C in a desiccator.

*Enzyme-linked immunoassay (ELISA) of H1 polypeptides*

ELISA was performed as previously described[5]. The polystyrene plates were coated with 2 nmol of intact H1 and its polypeptides and subsequently tested with patient sera at a dilution of 1:100.

*Polyacrylamide gel electrophoresis (PAGE)*

Polyacrylamide gels (18%) in sodium dodecyl sulfate (SDS) were cast at a dimension of 120 × 170 × 15 mm and electrophoresis was performed at 30 mA per gel for 6 h[13].

*Amino acid analysis*

Amino acid analysis of H1 polypeptides purified by FPLC was performed on a Beckman 119 LC amino acid analyzer after hydrolysis in 6 *M* hydrochloric acid (1 ml per mg protein) at 110°C for 24 h *in vacuo* with 1% phenol to avoid excessive degradation of tyrosine. The amino acid composition of the H1 peptides was compared to published reports and has been previously published (see Table I)[8].

## RESULTS

*FPLC and identification of N-bromosuccinimide-digested H1*

Digestion of H1 with NBS produces two major polypeptides which are represented as three bands on the polyacrylamide gels (see Fig. 2B, lane b). The C-terminus of H1 (GC-H1 NBS) is characterized by microheterogeneity and therefore it appears as a doublet (Fig. 2B, lanes b and c). The N-terminal peptide appears as a faint Coomassie blue stained single band (Fig. 2B, lane b).

When H1 was digested with N-bromosuccinimide and fractionated on a Mono S column with a linear gradient of increasing sodium chloride concentration, three peaks were observed. The first peak between 0–0.08 *M* sodium chloride beginning at 1 ml and ending at 4 ml. No polypeptides were identified in this peak. A second peak started eluting at 0.3 *M* sodium chloride at 8 ml, finishing at approximately 13 ml and sodium chloride concentration of 0.55 *M* (Fig. 2A). The second peak, containing N-H1 as determined by amino acid analysis, was more than 95% pure as demonstrated by SDS-PAGE (Fig. 2B, lane d; Table I). A third peak, which eluted between 14 and 21 ml and at a salt concentration between 0.65 and 0.95 *M* sodium chloride, contained GC-H1 NBS (Table I). The purity of this peak was more than 95% as determined by SDS-PAGE (Fig. 2B, lane c). Two bands in the gel (Fig. 2B, lane c) reflect the microheterogeneity in the C-terminal half of the H1 molecule. This doublet was demonstrated in all the C-terminal polypeptides produced by chemical or enzymatic cleavage whereas the N-terminal H1 polypeptide appeared as a single band (see Figs. 2B, 2B and 4B). Because the FPLC fractionation was highly reproducible and performed in less than 30 min, it was possible to chromatograph several aliquots of NBS-digested H1 to obtain milligram amounts of pure GC-H1 NBS and H-H1 NBS.

*FPLC and identification of chymotrypsin-digested H1*

Chymotrypsin digestion of calf thymus H1 results in three major bands and other minor ones visualized in SDS-polyacrylamide gels (Fig. 3B, lane b). These bands were identified by amino acid analysis (Table I) as C-H1 CHY (residues 107–220) and NG-H1 CHY (residues 1–106).

The FPLC profile of chymotrypsin-digested H1 was similar to that obtained with NBS-digested H1 (Fig. 2A and 3A). Three peaks were observed and the first narrow peak (1 ml) between 0.0 and 0.1 *M* sodium chloride is the solvent front and no

TABLE I

AMINO ACID ANALYSIS OF HISTONE 1 PEPTIDES PRODUCED BY CHEMICAL OR ENZYMATIC CLEAVAGE

Results are expressed as mol % of recovered amino acids.

| *Amino acid* | *N-H1 NBS (1–72)* | | *GC-H1 NBS (73–220)* | | *NG-H1 CHY (1–106)* | | *C-H1 CHY (107–220)* | | *NG-H1 (1–122)* | | *C-H1 (123–220)* | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Calc.* | *Obs.* | *Obs.*[a] | *Obs.* | *Calc.* | *Obs.* | *Obs.*[b] | *Obs.* | *Calc.* | *Obs.* | *Obs.*[c] | *Obs.* |
| Aspartic acid | 0 | 0.5 | 2.2 | 3.0 | 1.9 | 1.1 | 0.9 | 1.0 | 2.4 | 2.0 | 0.2 | 1.0 |
| Threonine | 4.2 | 4.6 | 5.1 | 5.4 | 5.7 | 5.3 | 4.5 | 5.0 | 4.9 | 4.3 | 4.8 | 4.9 |
| Serine | 9.7 | 9.1 | 5.0 | 5.0 | 11.3 | 9.4 | 3.3 | 4.7 | 10.7 | 11.3 | 2.7 | 2.4 |
| Glutamic acid | 5.6 | 5.2 | 2.3 | 2.7 | 5.8 | 5.8 | 1.0 | 1.6 | 5.7 | 5.7 | 1.2 | 1.9 |
| Proline | 12.5 | 12.0 | 8.7 | 9.3 | 8.5 | 8.0 | 12.7 | 11.2 | 9.0 | 8.8 | 10.0 | 9.3 |
| Glycine | 5.6 | 6.0 | 7.6 | 8.1 | 8.5 | 9.1 | 3.2 | 4.9 | 8.2 | 7.6 | 4.4 | 3.7 |
| Alanine | 29.2 | 29.0 | 25.5 | 25.9 | 20.8 | 21.3 | 32.1 | 29.6 | 20.5 | 20.9 | 30.9 | 29.1 |
| Cystine | 0 | – | – | – | 0 | – | 0 | – | 0 | – | – | – |
| Valine | 5.6 | 5.0 | 4.5 | – | 6.6 | 7.2 | 1.7 | 2.9 | 5.7 | 5.1 | 2.5 | 2.1 |
| Methionine | 0 | – | – | – | 0 | – | 0 | – | 0 | – | – | – |
| Isoleucine | 1.4 | 0.9 | 0.7 | 0.9 | 1.9 | 1.2 | 0 | 0.4 | 1.6 | 2.2 | 0 | 0.3 |
| Leucine | 5.6 | 5.6 | 3.5 | 3.9 | 7.5 | 6.8 | 0.9 | 0.9 | 7.4 | 7.8 | 0.1 | 0.6 |
| Tyrosine | 0 | – | – | – | 0.9 | – | 0 | – | 0.8 | – | 0 | – |
| Phenylalanine | 0 | – | 0.7 | – | 0.9 | – | 0 | – | 0.8 | – | 0 | – |
| Histidine | 0 | 0.6 | – | 0.5 | 0 | 0 | 0 | 0 | 0 | 0.6 | – | 0.6 |
| Lysine | 16.7 | 17.5 | 32.9 | 33.6 | 16.0 | 21.7 | 39.8 | 37.6 | 18.9 | 19.5 | 43.2 | 42.7 |
| Arginine | 4.2 | 4.1 | 0.7 | 1.3 | 3.8 | 2.8 | trace | 0.2 | 3.3 | 3.5 | 0 | 0.4 |

[a] Ref. 10.
[b] Ref. 11.
[c] Ref. 12.

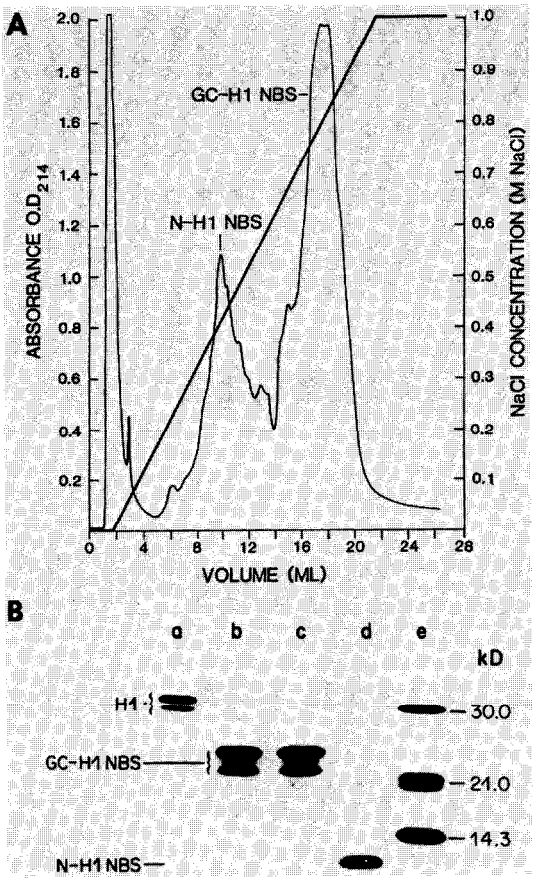


Fig. 2. (A) FPLC profile of histone H1 peptides digested with NBS. A 1-mg amount of histone H1 peptide was loaded on a Mono S column HR5/5 (50 × 5 mm I.D., Pharmacia) and eluted with increasing salt concentration (solid line) in 0.1 *M* phosphate buffer, pH 7.0 at a flow-rate of 1 ml/min. (B) SDS-polyacrylamide gel profile of purified NBS-digested histone H1 peptides. (a) Purified intact calf thymus H1. (b) Total NBS digest of H1. (c) C-Terminal peptide, residues 73–220. (d) N-Terminal peptide, residues 1–72. (e) Molecular weight markers. kD = kilodaltons.

polypeptides were identified. The second peak that eluted between 0.2 and 0.32 *M* sodium chloride and 7–10 ml (Fig. 3A) contained NG-H1 CHY and was represented as a single band on the gel (Fig. 3B, lane d). The third peak, containing C-H1 CHY, eluted between 0.5 and 0.65 *M* sodium chloride in a 2-ml volume between 13.5 and 15.5 ml (Fig. 3A). This peak was greater than 90% pure as assessed by an overloaded Coomassie blue stained gel (Fig. 3B, lane c).

*FPLC and identification of thrombin-digested H1*

H1 digested by thrombin resulted in three protein bands as seen by SDS-PAGE (Fig. 4B, lane b). These bands were identified as the singlet NG-H1 (residues 1–106) and the doublet C-H1 (residues 107–220).

Fractionation of thrombin-digested H1 by FPLC was slightly different from

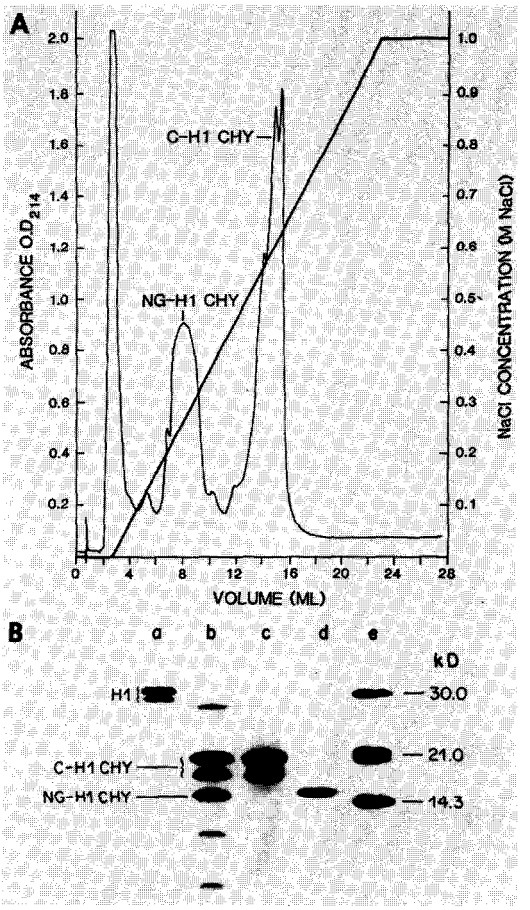


Fig. 3. (A) FPLC profile of histone H1 peptides digested with chymotrypsin. A 1-mg amount of histone H1 peptide was loaded on a Mono S column HR5/5 (50 × 5 mm I.D., Pharmacia) and eluted with increasing salt concentration in (solid line) 0.1 *M* phosphate buffer, pH 7.0 at a flow-rate of 1 ml/min. (B) SDS-polyacrylamide gel profile of purified chymotrypsin-digested histone H1 peptides. (a) Purified calf thymus histone H1. (b) Total chymotryptic digest of H1. (c) C-Terminal peptide 107–220. (d) N-Terminal peptides 1–106. (e) Molecular weight markers.

that of chymotrypsin and NBS-digested H1. Three peaks were seen but the last two large peaks were not homogeneous peaks in that each peak was characterized by one or two shoulders (Fig. 4A). When each of the peaks were analyzed by SDS-PAGE and amino acid analysis, no differences were noted in the peptide profile (Table I). This may reflect different degrees of acetylation or phosphorylation. Therefore, the shoulders in the second peak between volumes of 8–12 ml contained NG-H1 and the two shoulders in the third peak between volumes 12–15 ml contained C-H1 (Table I). SDS-PAGE analysis showed that the polypeptides NG-H1 and C-H1 were fractionated to greater than 95% purity (Fig. 4B, lanes c and d). NG-H1 eluted between 0.3 and 0.4 *M* sodium chloride in 2 ml beginning at 9 and ending at 11 ml. C-H1 eluted between 13–15 ml at a salt concentration of 0.52 and 0.62 *M* sodium chloride (Fig. 4A).

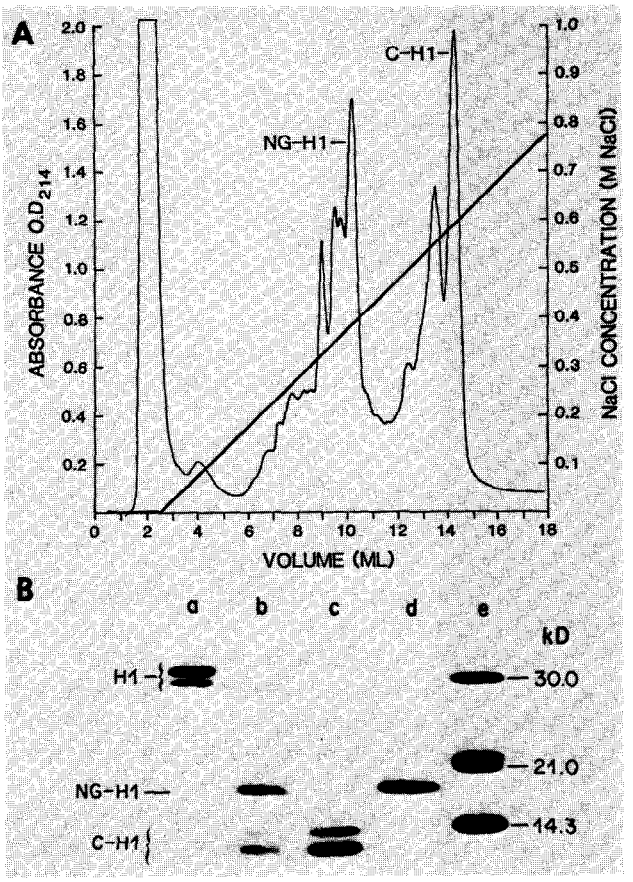


Fig. 4. (A) FPLC profile of histone H1 peptides digested with thrombin. A 1-mg amount of histone H1 peptide was loaded on a Mono S column HR5/5 (50 × 4 mm I.D., Pharmacia) and eluted with increasing salt concentration (solid line) in 0.1 *M* phosphate buffer, pH 7.0 at a flow-rate of 1 ml/min. (B) SDS-polyacrylamide gel profile of purified thrombin digest of H1 peptides. (a) Purified calf thymus histone H1. (b) Total thrombin digest of H1. (c) N-Terminal peptides of H1, residues 1–22. (d) C-Terminal peptides of H1, residues 123–220. (e) Molecular weight markers.

*ELISA of H1 polypeptides*

The H1 peptides generated by the various chemical and enzymatic digestions and purified by FPLC were used to identify antigenic domains of H1. SLE sera and procainamide-induced lupus sera were reacted with H1 and the H1 peptides bound to Immunolon II microtiter plates (Fig. 5). All six SLE sera (median O.D. 1.59) and all six drug-induced lupus (DIL) sera (median O.D. 1.45) tested bound to intact H1. Predominant reactivity with the H1 peptides was noted only in the C-terminal peptides (73–220, 107–220 and 123–220). The N-terminal peptides were not found to be reactive with DIL sera and minimally reactive with SLE sera. Control sera exhibited no reactivity toward either intact H1 or any of its peptides.

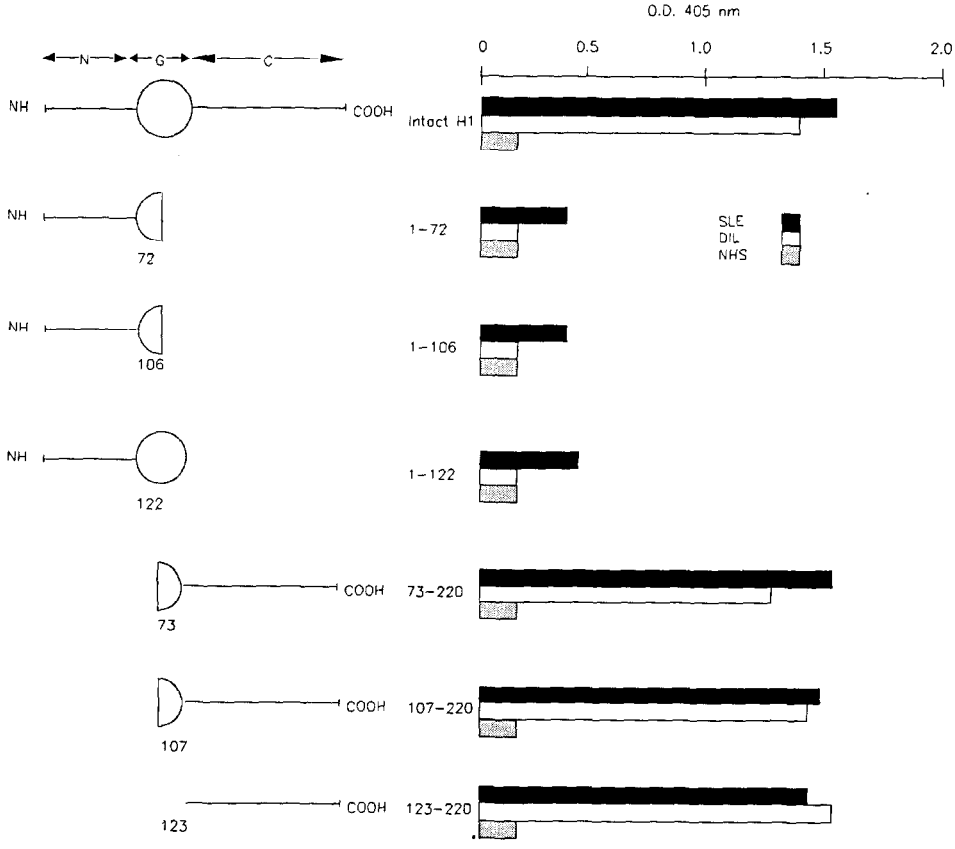


Fig. 5. ELISA of H1 and the cleavage peptides with SLE, DIL and normal human serum (NHS). Of each peptide 2 nmol were coated onto the plates and ELISA was performed as described in Materials and methods.

## DISCUSSION

In the past, the fractionation and purification of histones and histone polypeptides has generally relied on gel filtration and ion-exchange chromatography. Two types of gel filtration matrices have been used for fractionating these basic, low-molecular-weight proteins —a cross-linked dextran matrix (Sephadex, Pharmacia) and a polyacrylamide sieve (Bio-Gel P, Bio-Rad).

*Cleavage products of H1 have been purified by gel filtration chromatography* for various studies. When NBS-digested H1 polypeptides were separated on a Bio-Gel P-60 column (40 × 1.9 cm I.D.) at a flow-rate of 6 ml/h, 6 h were required to separate the amino and carboxy terminal fragments and upon rechromatography of the two peaks, SDS-PAGE showed no cross-contamination[14]. Similarly, 6 h were required to fractionate α-chymotrypsin digested H1. Chromatography on different Sephadex matrices has also been used to separate and purify various H1 polypeptides[15,16]. Like other gel filtration techniques, the time required to resolve the peptides were long ranging from 20 to 50 h. However, despite these drawbacks, gel

filtration is technically quite simple, special buffers and gradients are not required and quantitative amounts of polypeptide can be fractionated.

Ion-exchange chromatography is technically more difficult, requires a buffer gradient and complicated washing steps but quantitative amounts of proteins and peptides can be fractionated. Histones and their polypeptides have generally been fractionated on weak cation-exchange material such as carboxymethyl cellulose (CM-cellulose) or Amberlite IRC-50, a weakly acidic cationic copolymer of methacrylic acid and divinyl benzene. Cellulose ion-exchange material such as CM-cellulose provides a good matrix for ion-exchange since proteins and peptides do not bind tightly to it. Histone fractionation on weak ion-exchange columns has been performed at a pH near neutrality in order to ionize the carboxylate groups. H1 fragments resulting from thrombin digestion have been fractionated on carboxymethyl cellulose columns (Whatman CM-32) at a flow-rate of 30 ml/h but up to 33 h were required to resolve the different peptides[12]. Ishimi *et al.*[17] decreased the separation time by 50% using similar ion-exchange media (Whatman CM-52) and by decreasing the size of the column (12 × 1 cm I.D.). H1 peptides derived from cyanogen bromide digestion have also been separated on Whatman CM-52 columns but once again the elution times were long[18].

We have devised a fast and efficient protocol for the fractionation of H1 polypeptides using a strong cation-exchange matrix and elution with a linear salt gradient. The Mono S (Pharmacia) matrix is a beaded hydrophilic resin with a pore size of 10 m*M*. The charged group on the gel ($-CH_2SO_3^-$) has an ionic capacity of 0.13–0.18 mmol/ml. The elution time for the H1 polypeptides was constantly less than 30 min, and the profile was highly reproducible. With the LCC-500 controller (Pharmacia) the samples are easy to load, elute and collect. The linear salt gradient is automatically created by two pumps controlled by the computer.

In summary, the FPLC protocol described here provides a unique technique for fractionating H1 polypeptides that has advantages of speed and reproducibility when compared to other gel filtration and ion-exchange techniques. We have successfully used the H1 polypeptides purified by the FPLC technique to determine the antigenic domains of H1 bound by SLE and DIL sera. This purification protocol will be useful for investigators wishing to examine the primary structure of H1 variants, the role of H1 and its domains on chromatin structure, and the effect of H1 peptides on enzyme activity.

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REFERENCES

1 J. D. McGhee and G. Felsenfeld, *Annu. Rev. Biochem.*, 49 (1980) 1115.
2 L. Bohm and C. Crane-Robinson, *Biosci. Rep.*, 4 (1984) 365.
3 J. Allan, P. G. Hartman, C. Crane-Robinson and F. X. Aviles, *Nature (London)*, 288 (1980) 675.
4 J. Gohill and M. J. Fritzler, *Mol. Immunol.*, 24 (1987) 275.

5 J. Gohill, P. D. Cary, M. Couppez and M. J. Fritzler, *J. Immunol.*, 135 (1985) 3116.
6 C. Von Holt, W. N. Strickland, W. F. Brandt and M. S. Strickland, *FEBS Lett.*, 100 (1979) 201.
7 J. Allan, T. Mitchell, N. Harborne, L. Bohm and C. Crane-Robinson, *J. Mol. Biol.*, 187 (1986) 591.
8 C. Dingwall and J. Allan, *EMBO J.*, 3 (1984) 1933.
9 E. L. Bohm, W. N. Strickland, M. Strickland, B. H. Thwaits, D. R. Van Der Westhuizen and C. Von Holt, *FEBS Lett.*, 34 (1973) 217.
10 M. Bustin and R. D. Cole, *J. Biol. Chem.*, 244 (1969) 5291.
11 E. M. Bradbury, G. E. Chapman, S. E. Danby, P. G. Hartman and P. L. Riches, *Eur. J. Biochem.*, 57 (1975) 521.
12 G. E. Chapman, P. G. Hartman and E. M. Bradbury, *Eur. J. Biochem.*, 61 (1976) 69.
13 J. O. Thomas and R. D. Kornberg, *Methods Cell. Biol.*, 18 (1978) 429.
14 D. S. Singer and M. F. Singer, *Nucleic Acids Res.*, 3 (1976) 2531.
15 S. C. Rall and R. D. Cole, *J. Biol. Chem.*, 246 (1971) 7175.
16 M. Bustin and R. D. Cole, *J. Biol. Chem.*, 245 (1970) 1458.
17 Y. Ishimi, H. Yasuda, Y. Ohba and M. Yamada, *J. Biol. Chem.*, 256 (1981) 8249.
18 T. C. Mitchell, P. Sautiere, C. Turner and L. Bohm, *Biochim. Biophys. Acta*, 832 (1985) 235.