A Lysine-rich Protein from Spermatozoa of the Mollusc Mytilus edulis*

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SUMMARY

A small basic protein φ3II has been purified from spermatozoa of Mytilus edulis. It was extracted from chromatin by ethanol-HCl and further purified by chromatography on Bio-gel P-10 and Sephadex G-100. The protein has a molecular weight of about 5000. Half its residues are lysine and its is also rich in alanine, proline, and serine. It shows some compositional similarities to protamines if lysines are considered homologous with arginines. The composition of φ3II is also somewhat similar to that of the carboxyl region of lysine-rich histone.

A search is being made to determine how restricted variations are in amino acid sequence in lysine-rich histones compared to the severe restrictions in arginine-rich histone (1). Structural variations have been found among the lysine-rich histones of different species (4) and raise the possibility that a broad spectrum of structural variations will be found in lysine-rich histones, perhaps even eliminating the clear distinction now apparent between the lysine-rich class (F1) and the moderately lysine-rich class (F2a). Indeed the F2c histone from avian erythrocytes (5, 6), histone T (7), and a new family of lysine-rich histones in Holothuria tubulosa (3) could all be looked upon as intermediates in a series of structures with characteristics ranging from those of lysine-rich histones, such as calf thymus F1, to those of moderately lysine-rich histones, such as F2b, from the same tissue. In somewhat the same vein a spectrum of structural variation might be looked for bridging protamines and histones, and Stellwagen and Cole (8) pointed out an intriguing compositional analogy between protamine and the carboxyl half of lysine-rich histone. More generally, Bloch (9) included in his classification of sperm histones a type that was intermediate in size and in amino acid composition between histones and protamines, although he made cautionary note that the compositions were determined on mixtures of proteins. Such “intermediate” basic proteins have been found in the spermatozoa of echinoderms (10, 11) and molluscs (12). In particular, recent studies by Subirana et al. (12) in Mytilus edulis revealed a component that was very rich in lysine and had an electrophoretic mobility characteristic of protamine. Further comparison to protamine is encouraged by the observation that this very lysine-rich component occurs only in ripe gonads. Since its properties are intermediate between those of histones and the previously studied protamines from fish (13, 14) and bull (15), we felt led to purify and characterize it further as described in the present paper.

MATERIALS AND METHODS

Electrophoresis was conducted on 15% polyacrylamide gel at pH 4.5 according to Panyim and Chalkley (16). Amino acid analyses were performed according to Moore and Stein (17) on a Beckman-Spinco amino acid analyzer after hydrolysis in 6 N HCl for 22 hours at 110°.

NH₂-terminal residues were determined by the 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) method according to Gray (18) and the hydrolysates were chromatographed on polyamide layers according to Woods and Wang (19). The phenylisothiocyanate method was also used as described by Konigsberg and Hill (20).

COOH-terminal residues were determined with diisopropylphosphorofluoridate-treated carboxypeptidase A and B obtained from Worthington. The digests were prepared at 37° in 0.2 M NaCl in 0.05 M sodium barbital, pH 8.0. The enzyme to substrate ratio was 1:100 by weight. The samples were applied directly to a Beckman-Spinco analyzer in citrate buffer, pH 2.2.

Peptide maps were prepared by tryptic hydrolysis of approximately 2 mg of histone in 250 μl of 0.02 M ammonium bicarbonate using 1% trypsin by weight. The mixture was frozen after 16 hours and freeze-dried. The freeze-drying was usually repeated after redissolving in 250 μl of distilled water. The peptides were dissolved in 25 μl of 5% formic acid and the entire sample was applied to Whatman No. 3MM filter paper sheets for electrophoresis at pH 3.5 in pyridine-acetic acid-water (1:10:69 by volume) for 50 min at 5000 volts. Chromatography was used

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RESULTS AND DISCUSSION

A general description of the basic proteins in the spermatozoa of mussels has been presented elsewhere (12). Of the total basic protein 72% is a protamine-like protein containing 30% arginine and 24.3% lysine. Ripe spermatozoa also contain somatic histones comprising 15% of the basic proteins. The remaining 13% of the basic proteins was prepared by Method 2 of Johns (24) which yields histone F3 from most tissues; for M. edulis the fraction was called φ3. This fraction was shown by gel electrophoresis to contain some somatic histones and a component with a very high mobility resembling that of protamines.

When φ3 was submitted to chromatography on Amberlite IRC-50 under the conditions used for histones (25), the great majority of the amino acid residues was irreversibly adsorbed. This observation was not surprising since salmine requires 40% guanidinium chloride for elution (26). Columns of Bio-Gel P-10 (27) were then used for chromatography of φ3 with the results presented in Fig. 1 and Table I. Three peaks were evident: amino acid analysis demonstrated that little peptide material was present in the third peak and that the second peak represented the lysine-rich component. The first peak probably represents the somatic histone F3, as shown by the presence of cystine, the high amounts of glutamic acid and alanine, and the low content of glycine.

The material represented by the second peak was termed φ3II and was next chromatographed on Sephadex G-100 yielding a single slightly skewed peak (Fig. 2). Amino acid composition was constant (Table I) across the peak, suggesting that the material represented by the peak was essentially homogeneous. The material showed a single band when tested by polyacrylamide gel electrophoresis.

The truly remarkable feature of the composition of φ3II is that 50% of the amino acid residues are lysine, compared to 25 to 30% in the case of lysine-rich histones and compared to about 67% arginine in the case of the protamines (e.g., clupeine). High alanine, serine, and proline contents, along with low levels of hydrophobic amino acids, are reminiscent of both protamine and lysine-rich histone. The composition is also intriguingly similar to the carboxyl half of a lysine-rich histone (28). This might be taken to suggest that φ3II is an artifact derived from proteolysis of lysine-rich histone during isolation, especially since proteases are known to be present in chromatin (29). Proteolysis is not a likely explanation, however, since the numerous fast-running electrophoretic bands which apparently (30) result from this sort of degradation were not detected in the histone preparations from which φ3 was isolated. If proteolytic degradation did occur, it must have been far more specific than observed previously, in which case it might well be an important step in the maturation of the sperm cells in the mussel.

It was clearly important to estimate the molecular weight of φ3II, and this was first studied using the method of sodium dodecyl sulfate polyacrylamide gel electrophoresis. The material was pooled for chromatography on Sephadex G-100. The material showed a single band when tested by gel electrophoresis to contain some somatic histones and a component identical but separate peptide maps by dipping the paper in a phenylalanine-cadmium reagent (22). Arginine peptides were detected on identical but separate peptide maps by dipping the paper in a mixture of equal volumes of 0.02 M, phenanthrenequinone in anhydrous ethanol and 10% sodium hydroxide in 60% ethanol and allowing the paper to dry at room temperature in a hood (23).
TABLE II
Edman degradation of $\phi$III

Amino acid compositions before and after removal of NH$_2$ terminus by Edman degradation are presented as moles of amino acid per mole of protein; figures in parentheses are nearest integers. Molecular weight calculated from integers is 5015.

| Amino acid      | Untreated protein | Edman-degraded protein |
|-----------------|-------------------|------------------------|
| Lysine          | 22.8 (23)         | 19.2 (--)              |
| Histidine       | 0.08              | 0.09                   |
| Arginine        | 2.03 (2)          | 2.05 (2)               |
| Aspartate       | 0.19              | 0.20                   |
| Throneine$^b$   | 1.05 (1)          | 1.05 (1)               |
| Serine$^b$      | 4.71 (5)          | 4.65 (5)               |
| Glutamate       | 0.25              | 0.23                   |
| Proline         | 4.89 (5)          | 4.79 (5)               |
| Glycine         | 0.29              | 0.49                   |
| Alanine         | 9.13 (9)          | 8.28 (8)               |
| Half-cystine    | 0.00              | 0.00                   |
| Valine          | 0.76 (1)          | 0.77 (1)               |
| Methionine      | 0.03              | 0.00                   |
| Isoleucine      | 0.10              | 0.18                   |
| Leucine         | 0.27              | 0.22                   |
| Tyrosine        | 0.03              | 0.03                   |
| Phenylalanine   | 0.03              | 0.03                   |

$^a$ Some internal lysine is lost in Edman degradation.
$^b$ Not corrected for hydrolytic loss.

The molecular weight was confirmed by COOH-terminal analysis using carboxypeptidase A + B digestions. As shown in Fig. 3, carboxypeptidase B released 2 moles of lysine per mole of protamine (mol wt = 5015) before carboxypeptidase A was added. Ultimately 3.5 moles of lysine and 1.2 mole of alanine were released indicating the COOH-terminal sequence (---(Ala,Lys)Lys-Lys).

The amount of alanine released (about 1 mole for a molecular weight of 5000) confirms the assumed molecular weight because the next integral value would be most difficult to reconcile with the NH$_2$-terminal result.

The molecular weight was further confirmed by the tryptic peptide map shown in Fig. 4. The distribution of tryptic peptides by paper electrophoresis and paper chromatography revealed about 10 spots (compare to clupeines (31) which would be expected to yield about 7 tryptic peptides from their 30 to 33 residues). Two of these spots were found to contain arginine (apparently in equal amounts) when analyzed with phenanthrenequinone (23). Since the number of arginine residues was calculated at 2 per molecule on the basis of the amino acid composition and an assumed molecular weight of about 5000, the latter assumption is confirmed.

Further consideration of the peptide map leads to the speculation that $\phi$III may have some homology with a portion of lysine-rich histone. A comparison of Fig. 4 with similar maps of the...
| Amino acid | Clupeine | Iridine | Salmine | φ311 peptide* |
|------------|----------|---------|---------|----------------|
|            | Y | II | Z | b | H | I |
| Lysine     | 20| 21| 22| 21| 21| 23| 18|
| Arginine   | 2 | 1 | 2 | 2 | 2 | 2 | 2 |
| Threonine  | 3 | 2 | 3 | 4 | 4 | 5 | 1 |
| Serine     | 2 | 3 | 2 | 3 | 2 | 3 | 5 |
| Proline    | 1 | 2 | 2 | 2 | 2 | 2 | 2 |
| Glycine    | 1 | 2 | 2 | 1 | 1 | 0 | 14|
| Alanine    | 2 | 2 | 2 | 2 | 2 | 2 | 2 |
| Isoleucine | 1 | 1 | 1 | 1 | 1 | 1 | 1 |

* This peptide has been reported to give an approximate analysis of the COOH-terminal 44 residues of a lysine-rich histone from rabbit thymus (27).

lysine-rich histones of *H. tubulosa* (3) allows every spot in Fig. 4 to be matched to a spot in the earlier peptide maps. Of course, the chance for coincidence is significant when the composition of φ311 is compared (see Table III) to the COOH-terminal region (e.g. the COOH-terminal 44 residues of rabbit thymus lysine-rich histone peak 3 (28)). Whether or not there is such a homology awaits the determination of the amino acid sequence of both the histone and φ311, both of which are under way in our laboratories. When the sequence of φ311 is known, it will also be of interest to look for homology with protamines such as clupeines, iridines, and salmine since the comparison of their amino acid composition in Table III shows intriguing differences as well as similarities.

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