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Isolation and Characterization of a Novel Nuclear Protein from Pollen Mother Cells of Lily

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ABSTRACT

Pollen mother cells of the lily (*Lilium speciosum*) were found to have a histone-H1-like protein (PMCP) not detected in other tissues. The PMCP appears from the late S-G_2 period of premeiosis and is present in mature pollen. PMCP and H1 were extracted from pollen mother cells with 5% perchloric acid and isolated by reverse-phase high-performance liquid chromatography. The amino acid composition of PMCP differs from that of somatic H1. However, PMCP is similar to H1t in mammalian testis with regard to amino acid composition.

The process of microsporogenesis in higher plants can be divided into two stages: (a) meiosis of the pollen mother cell; and (b) microspore maturation. Meiosis is the fundamental type of cell division for gamete generation in all sexual organisms. After meiosis, microspores develop into mature pollen by plant-characteristic haploid mitosis, giving rise to the vegetative nuclei and generative nuclei present in single mature pollen grains.

The G_2 period of premeiosis is a critical point at which mitotic division changes to meiotic division (the so-called G_2 commitment). Cytological analysis using cultures of pollen mother cells has revealed that this stage is essential for establishment of bivalent chromosome pairing and chiasma formation (9).

At this stage, the appearance of a new histone has been reported (3, 21, 24). However, the question arises as to whether this protein really is formed *de novo* (that is not a posttranslational product) and whether in fact it is a histone. No biochemical evidence has yet been obtained to clarify this aspect, although similar proteins have been reported in spermatogenesis in other species (1, 12, 14, 18, 19, 23). H1t is a specific variant of H1 histone, being detected only in the mammalian testis (12), and Sp H1 is specific only to the sperm of sea urchin (23).

In the present study, a new specific protein occurring during microsporogenesis was isolated and identified by two-dimensional gel electrophoresis. Isolation of this protein and H1 histone, and determination of their amino acid contents were also done using HPLC with a reverse-phase column.

MATERIALS AND METHODS

Plant Material

Bulbs of *Lilium speciosum* were purchased from Takii Seed Co., Ltd., and grown in a greenhouse and experimental field at the National Institute for Environmental Studies, Tsukuba, Japan. Flower buds were harvested and pollen mother cells were collected by squeezing the anthers. The cells were frozen with liquid nitrogen and stored in a deep freezer (−80°C) until use.

Isolation of Nuclei

Nuclei from leaf and stem cells were obtained by homogenization with a Polytron (13), and those from mature pollen using a Teflon-glass homogenizer. Pollen mother cells were rinsed with buffer to remove tapetal cells and their nuclei. The nuclei of the pollen mother cells were isolated using a slight modification of the method of Hotta and Stern (7), employing a YEDA press instead of a French press. The nuclear suspension was centrifuged at 2,000*g* for 2 min. The pellet was rinsed two or three times with a buffer composed of 0.5 M hexylene glycol, 25% glycerol, 5 mM $CaCl_2$, 0.25 M sucrose, 1 mM PMSF, and 10 mM Tris-HCl (pH 7.4). The pellet was added to 2.0 M sucrose, and then centrifuged at 10,000*g* for 15 min. It was finally rinsed with 0.5% Nonidet P-40 in the buffer and then three times with the buffer alone.

Histone Preparation

Histones were extracted from isolated nuclei with 0.4 N H_2SO_4. The H1[1] fraction was extracted directly from tissue with 5% $HClO_4$ and the supernatant was precipitated with 25% TCA. The precipitate was rinsed with 1% HCl-acetone, and it was dried under vacuum.

Gel Electrophoresis

For histone analysis, acetic acid-urea (8 M) PAGE (16) was used for the first dimension and SDS-PAGE (11) for the second. Gels were stained with 0.25% Coomassie brilliant blue R-250 (w/v) in 50% methanol and 10% acetic acid.

Isolation of PMCP and H1

Pollen mother cells were collected from about 300 buds by squeezing. Their nuclei were isolated, and PMCP and H1 histone were extracted with 5% $HClO_4$. Thereafter, the procedure used was the same as that described previously for histone extraction. About 200 to 900 μg of 5% $HClO_4$ was

[1] Abbreviations: H1, H1 histone; PMCP, pollen mother cell protein.

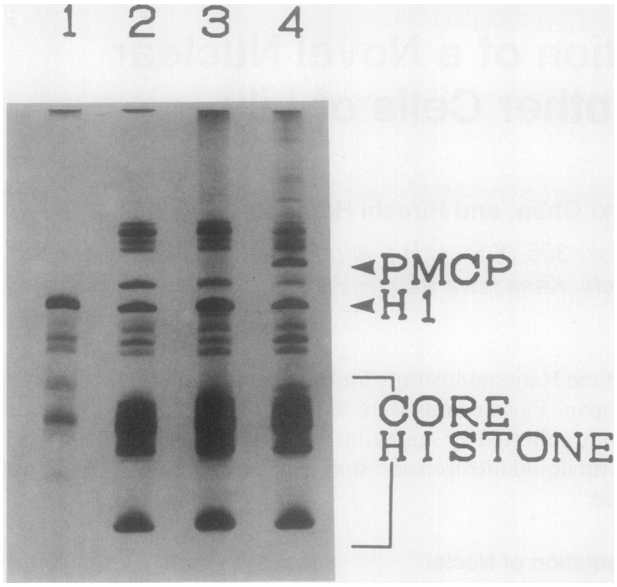

Figure 1. Acid urea PAGE of extracts from various tissues. H_2SO_4 extracts (0.4 N) from isolated nuclei were prepared, except those of root tip. From root tip, a 5% $HClO_4$ extract was used. Approximately 5 to 8 μg of protein was loaded except for A (0.5–1 μg) and gels were stained with Coomassie brilliant blue. Lane 1, root tip; Lane 2, stem; Lane 3, leaf; Lane 4, pollen mother cell.

dissolved in distilled water and applied to a reverse-phase column (C18-300; Nakarai) for HPLC. Ten mM $NaClO_4$, 100 mM H_3PO_4, and a step-wise gradient of acetonitrile were used at a flow rate of 1 mL/min. The following a stepwise scheme was employed: 33% acetonitrile for the first 5 min, 33 to 43% for 5 to 15 min, 43% for 15 to 20 min, and 43 to 44% for 20 to 35 min. Each fraction was collected, freeze-dried, and examined by SDS-PAGE. The desired fraction was subjected to a second cycle of HPLC using the same conditions as those for the first cycle.

Amino Acid Composition

Purified samples were hydrolyzed in 6 N HCl in an evacuated tube at 100°C for 24 h and analyzed with a Hitachi automatic amino acid analyzer.

RESULTS

Determination of PMCP in Various Tissues

Figure 1 shows the acid-soluble nuclear protein profiles of lily tissues. Comparison of the proteins in various tissues revealed little difference among them in the band patterns on acid urea gel. However, PMCP was found to be specific to pollen mother cells. All histones were prepared from a 0.4 N H_2SO_4 extract of isolated nuclei. However, from root tip tissue, 5% $HClO_4$-soluble protein was prepared because only a small amount was isolated from this tissue. PMCP was also extractable with 5% $HClO_4$ (data not shown).

Association of PMCP with Microsporogenesis

The appearance of PMCP at different stages of microsporogenesis is shown in Figure 2. In lily plants, bud length is correlated with the developmental stage of meiosis. PMCP was found initially in preparations from buds 13 mm long (about the S period of premeiosis), and continued to be present until leptotene. PMCP was also present in mature pollen.

The band intensites of PMCP and H1 stained with Coomassie brilliant blue were estimated using a densitometer after separation by SDS-PAGE. Percentages of PMCP and H1 in a pachytene nuclear preparation as measured by absorption at 600 nm were 67 and 33%, respectively, on SDS-PAGE (data not shown).

Two-Dimensional Gel Electrophoresis of Acid-Soluble Proteins

It is possible that PMCP is a posttranslationally modified product of another nuclear protein, which could be investigated by two-dimensional gel electrophoresis. Therefore, histones were electrophoresed using a combination of acid urea PAGE and SDS-PAGE. Figure 3 represents the migration patterns of pollen mother cell and leaf histones. From the zymogram, it is suggested that PMCP is a new nuclear protein occurring in microsporogenesis, and not a modified or multimer protein. The mol wt of lily PMCP and H1 estimated by the method of Brandt *et al.* (4) were 27,000 and 23,000, respectively. Two-dimensional gel analysis showed some differences between pollen mother cell and leaf cell nuclear proteins by use of only acid urea PAGE or SDS-PAGE. No significant differences could be observed for H1, core histone (14 kD protein, and 18 kD protein, etc.), or a group of proteins migrating between H1 and core histone (probably a high mobility group). However, there was another group of proteins (Fig. 3, arrowheads on the right) that was not observed in pollen mother cell nuclei.

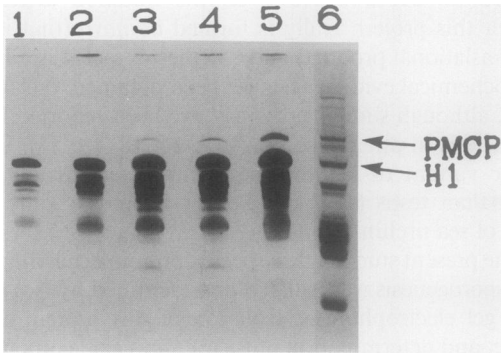

Figure 2. Appearance of PMCP by analysis of acid urea PAGE. Lanes 1 to 6 are 5% $HClO_4$ extracts from whole anther: each fraction includes a constant cell number of pollen mother cells. Lane 6 is a 0.4 N H_2SO_4 extract from isolated nuclei. Gels were stained with Coomassie brilliant blue. Lane 1, 10-mm bud; Lane 2, 13 mm-bud; Lane 3, 15-mm bud; Lane 4, 17-mm bud; Lane 5, leptotene; Lane 6, mature pollen.

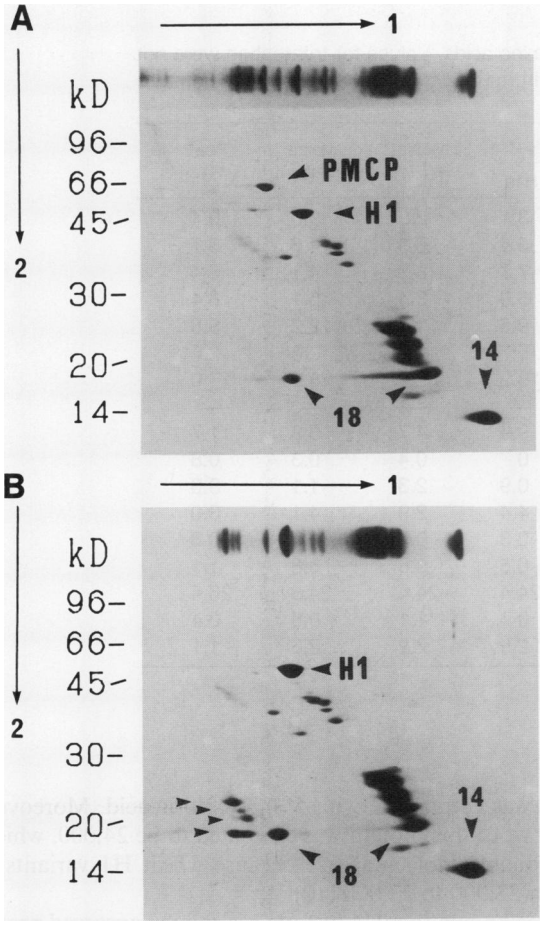

Figure 3. Two-dimensional gel electrophoretic profiles of pollen mother cell and leaf nuclear proteins. Migration in the first dimension is from left to right (acid urea); migration in the second dimension (SDS 15% polyacrylamide gel) is from top to bottom. Each panel's protein content is about 5 to 8 μg. Gels were stained with Coomassie brilliant blue. A, acid-soluble nuclear proteins of pollen mother cells; B, acid-soluble nuclear proteins of leaf. The numbers 14 and 18 represent 14-kD and 18-kD proteins, respectively. Proteins for which arrowheads point to the right could not be observed in pollen mother cell nuclei.

Isolation of PMCP and H1

PMCP and H1 were separated using an octadodecyl reverse-phase column. The elution profile of the 5% $HClO_4$ extract from pollen mother cells is shown in Figure 4. Four major peaks were obtained. After SDS-PAGE analysis of all fractions, fraction 3 was found to correspond to PMCP and fraction 4 to H1 (Fig. 5).

Amino Acid Composition of PMCP

PMCP appears to be highly basic in nature. The pH values of PMCP and H1 were 9.8 and 10.0, respectively (data not shown).

The amino acid compositions of PMCP and H1 isolated by reverse-phase HPLC are shown in Table I. The composition of lily H1 was found to be similar to that of rat H1, having high levels of lysine (22.3%) and alanine (25.4%), and a low level of arginine (2.0%). The lysine/arginine ratio was 11.2. However, a slight difference was observed in proline content: that of lily was about 15% and that of rat about 10%. The amino acid composition of PMCP also showed similarity to H1. PMCP showed a high content of lysine (13%), alanine (17.2%), and proline (10.2%), and a low content of arginine (2.0%). The lysine/arginine ratio was 6.5. Therefore, it is not a modified product of H1. PMCP showed a lower proline content than H1, whereas the levels of asparagine, serine, and glycine were higher than in H1. A similar correlation has been seen between testis-specific H1t and other subtypes of H1 (20).

DISCUSSION

It is possible that the present nuclear protein associated with microsporogenesis (PMCP) was a modified product of a protein such as H1. However, in this experiment, it became clear that PMCP is in fact a novel protein (Table I). The PMCP of *L. speciosum* was observed around the S to G_2 period of premeiosis (Fig. 2). This stage is critical for determination of meiosis in the lily, and is known as the G_2 commitment (20). According to cytological studies of cultured meiocytes, G_2 commitment is classified into certain categories, such as the mitotic, asynaptic, achiasmatic, and meiotic types (9). One of these is the abnormal meiotic division type, in which pairing stability cannot be maintained. Bivalent chromosome pairing accompanies formation of the synaptinemal complex in lily microsporogenesis. Thus, it is speculated that PMCP is a component of the synaptinemal complex or a preceding stage that ensures stability of pairing, since PMCP is synthesized just before chromosome pairing.

Two-dimensional gel electrophoresis of lily acid-soluble proteins presented some difficulty with histone identification. We identified 43-kD protein as lily H1 because the protein contained high lysine (22.3%) and low arginine (2%) levels,

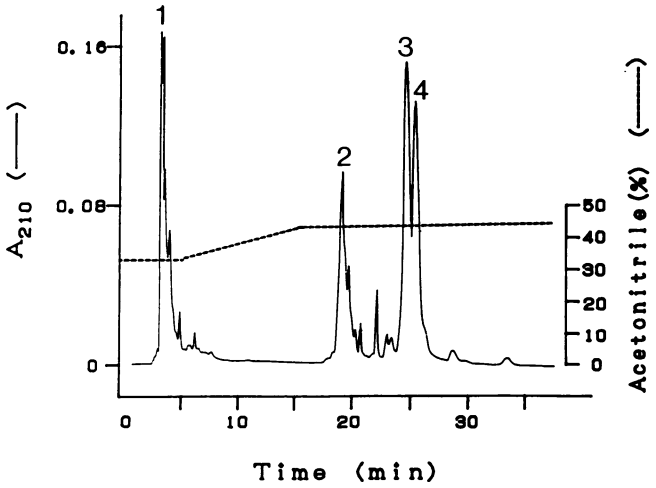

Figure 4. Elution profiles of PMCP and H1 isolation. Approximately 200 μg of a 5% $HClO_4$ extract was loaded on the Nakarai C-18 column and developed as described in "Materials and Methods." Absorbance was monitored at 210 nm. This elution profile represents the first cycle of HPLC.

Table I. *Amino Acid Component of Lily PMCP and H1*

The values expressed as moles per 100 moles of total amino acids. Values for tryptophan were not determined. Values from H1t are from Ref. 4; H1a and H1c, from Ref. 2; wheat H1, from Ref. 10; maize H1, from Ref. 5; and pea H1, from Ref. 8.

	Lily		Rat			Wheat	Maize	Pea
	PMCP	H1	H1t	H1c	H1a	H1 (1)	H1	H1
Asp	7.3	2.7	5.0	2.2	3.1	2.3	3.0	0.4
Thr	6.3	5.2	5.5	4.6	6.4	5.3	4.6	5.7
Ser	9.8	4.6	10.1	7.5	7.7	3.7	4.1	6.4
Glu	8.5	5.7	5.3	4.4	5.0	3.4	5.1	6.4
Pro	10.2	14.2	5.7	8.8	9.6	11.7	12.2	9.8
Gly	8.6	4.0	9.7	6.8	5.7	2.2	3.3	2.6
Ala	17.2	25.4	15.7	24.6	19.2	32.2	28.2	17.0
Cys	2.4	0.19	—	—	—	—	—	—
Val	4.5	3.4	5.1	5.2	9.9	4.7	3.9	11.7
Met	0.94	0.46	1.5	0.4	0	0.4	0.3	0.8
Ile	4.7	2.2	1.1	1.7	0.9	2.3	1.1	3.0
Leu	0.26	4.0	7.3	4.7	4.4	2.9	4.1	3.0
Tyr	1.4	0.83	0.6	0.5	0.3	0.9	1.1	0.8
Phe	1.1	1.5	1.2	0.5	0.5	0.8	0.8	1.1
Lys	13.0	22.3	19.2	25.3	24.4	24.4	24.5	26.4
His	1.9	1.0	0	0	0	1.1	0.8	0.4
Arg	2.0	2.0	7.2	2.7	2.9	2.2	2.6	1.1

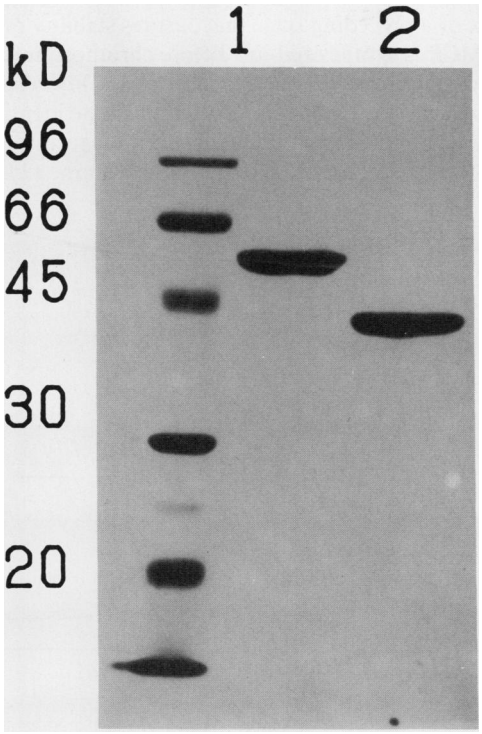

Figure 5. SDS-PAGE of isolated PMCP and H1. Approximately 10 μg of protein from the second cycle of isolation was electrophoresed on SDS 13% polyacrylamide gel. Lane 1, PMCP; Lane 2, H1.

and was extractable with 5% perchloric acid. Moreover, the mol wt of this protein was estimated to be 24,000, which is a reasonable molecular size because wheat H1 variants range from 23,000 to 27,000 (10).

It is known that H4 histone is a much conserved protein in all organisms, and that mammalian H4 migrates to a 14 kD position on SDS-PAGE. Thus, the 14 kD protein of lily nuclear protein might also be lily H4. An 18 kD protein migrated above H1 on acid urea gels, not only among leaf histones but also among pollen mother cell histones, and also migrated above H4. However, these proteins migrated to the same position on SDS-PAGE. Therefore, this 18 kD protein, which migrates above H1 on acid urea gel, may be a dimer of H3 histones. H2 histones can be ruled out, because they would migrate in the very high-mol-wt region on SDS-PAGE in comparison with animal histones. In fact, plant H2 histones are quite different from those of animals (for review, see ref. 22).

Some differences seem to exist between core histone of pollen mother cells and that of leaves. However, no clear difference could be demonstrated here. In rat testis, the nucleosome core is organized not only by somatic core histone but also by testis-specific TH2A histone (25). The pachytene nucleosome core of rat testis is thought to be more loosely organized than the somatic one due to the presence of this core histone variant (17). Moreover, in lily, at least five kinds of core histones seem to exist. Characterization of lily core histones will be necessary in order to study its chromatin organization.

The amino acid composition reported by Sheridan and Stern (21), who compared the leaf H1 fraction with the pollen mother cell H1 fraction by stepwise acetone precipitation, was not from a purified sample. Thus, the fractions would

have contained many kinds of protein. The purities of PMCP and H1 isolated by octadodecyl reverse-phase HPLC were more than 90% (determined by densitometry after Coomassie brilliant blue staining of the SDS-PAGE). Clear differences in the amino acid compositions of PMCP and H1 were observed. The lysine content of PMCP was slightly low level compared with H1, although PMCP has a high lysine content (13%) and a low arginine content (2.0%) similar to H1t, which is a mammalian testis-specific H1 variant. Thus, PMCP could be classified as a lysine-rich histone.

An unusual H1 different from somatic H1 has been reported to be associated with spermatogenesis in many species. From the present study it is suggested that PMCP is similar to male gamete-specific histones of other species, which are associated with male gametogenesis (1, 12, 14, 18, 19, 23). Species- and tissue-specific subtypes of H1 are localized at nucleosome linker regions, and thought to change this linker DNA length (2). From these reports, it can be speculated that these proteins might cause a change in chromatin organization, bringing about specific gene expression during male gamete development. Thus, it is important to know whether PMCP also exists in megasporogenesis. However, it is technically difficult to obtain the required amount of megaspore mother cells for biochemical experiments. Therefore, for such studies, *in situ* assays may be needed using a specific anti-PMCP antibody.

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