Yellow Pigments of Pearl—II. HCl-methanol Soluble Yellow Pigments

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In the previous paper¹⁾ existence of a carotenoid as one of the components of yellow nacre was reported. However, by all accounts the authors assumed that the carotenoid pigment might be a common component of pearls regardless of their color.

It has not been cleared how far the carotenoid pigment participates in the coloration of yellow pearl though, main pigment of the yellow nacre seems to exist besides the carotenoid pigment.

In the present experiment, inquiring into the SAWADA's iron complex of peptide²⁾, the authors isolated relatively large amount of yellow pigment which mainly consisted of conchiolin-like proteinous substance.

Experimental and Results

The iron complex of peptide by SAWADA. SAWADA reached a conclusion that the yellow pigment obtained from the nacre of yellow pearl by extraction with HCl-MeOH had to be an iron complex of peptide consisting several kinds of amino acids, since he detected Ala., Gly., Leu., Asp., and Glu. by means of paper chromatography on an acid hydrolyzate of the pigment, and observed relatively high content of iron in the nacre of yellow pearl in comparison with that of pink pearl.

In pursuit of the SAWADA's yellow pigment, a lot of yellow nacles were crushed in a mortar and submitted to extraction with 1 N HCl-MeOH (2 N HCl: MeOH, 1:1). The procedure is essentially identical with SAWADA's. After concentrated in vacuo, the extract was mixed with HCl-acidic n-butanol. The mixture was washed with 1 N HCl and then concentrated in vacuo. A small amount of reddish brown residue was obtained. The residue was hydrolyzed with conc. HCl in a sealed tube at 110°C for 40 hours. The hydrolyzate was concentrated in vacuo and passed through an ion exchange column of Amberlite IR-120. After the column was washed with water, amino acids were eluted with 2 N NH₄OH. The amino acids solution was concentrated to dryness in vacuo and further dried over P₂O₅ for 12 hours so as not to retain trace of moisture, and then converted into N-trifluoroacetyl n-butyl ester derivatives followed GEHRKE *et al.*³⁾ for gas chromatographic analysis.

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In Fig. 1, resulting gas chromatogram and conditions for the analysis are shown. In the case of neopentyl glycol succinate column Ala., Val., Gly., Ileu., Leu., The., Pro.,

Fig. 1. Gas chromatogram of amino acids in the acid hydrolyzate of yellow pigment. 1.5% neopentyl glycol succinate on 60/80 mesh Shimalite, 2.43 m $\times$ 3 mm i.d. glass tube. Injection temp. 200°C, initial temp. 120°C, final temp. 225°C, 5°C/min.

Table 1. Amino acid constituents of the pigment of yellow pearl and pearl conchiolin.

Amino acids	Pigment of yellow pearl*	Pearl conchiolin**
Alanine***	++	11.063%
Valine	+	1.748
Glycine***	+++	12.996
Isoleucine	+	1.453
Leucine***	+++	5.614
Threonine	+	0.776
Proline	+	0.291
Methionine	+	0.924
Phenylalanine	++	2.290
Aspartic acid***	+++	7.419
Glutamic acid***	++	2.122
Lysine	+	2.909
Serine†	±	3.902
Tyrosine†	±	2.820
Arginine†	±	0.625
Histidine††		0.335
Ammonia		0.155
Cysteic acid		2.273

* Amount of amino acids presumed from peak area on the gas chromatogram shown in Fig. 1.

** Reported by TANAKA *et al.*⁽¹⁾

*** Reported by SAWADA⁽²⁾ as the constituent amino acids of iron complex.

† Detected by means of DC-550 column.

†† Histidine, cysteine, and cystine could not be analyzed by this method.

Met. (and/or Hypro.), Phe., Asp., Glu., and Lys. were separately eluted in the mentioned order. When the sample was analyzed with DC-550 column, three separated peaks of Ser., Tyr., and Arg. appeared among peaks of the other amino acids. It was not clear whether His., CySH., and (CyS)₂ were contained in the sample, because they could not be identified as independent peaks by either column. In Table 1, amounts of the constituent amino acids of the pigment were estimated from each chromatogram peak area and arranged so as to facilitate comparison with the data on pearl conchiolin analyzed by TANAKA *et al.*⁴⁾ The amino acid composition of the pigment of yellow nacre and that of pearl conchiolin seems to coincide rather well with each other.

Extraction of yellow pigments. The extraction procedure is outlined in Fig. 2. The yellow nacles were decalcified with 5% acetic acid in the same way as described in the previous paper¹⁾. Crude conchiolin, thoroughly decalcified nacles, was treated with MeOH followed by treatment with 1 N HCl-MeOH (conc. HCl: MeOH, 1:11). The MeOH extract colored yellow and contained some carotenoid pigments as was reported before, and 1 N HCl-MeOH extract colored deep gold. The deep gold colored extract was concentrated to dryness in vacuo and dried in vacuum desiccator overnight. The reddish brown residue was dissolved in a small amount of MeOH, and colorless insoluble matters were filtered off. The filtrate was concentrated to dryness in vacuo and dried in vacuum desiccator. After the procedure was repeated once more, finally a small amount of reddish brown residue was obtained. The final product was insoluble both in ether and

Fig. 2. Extraction of yellow pigments from the nacre of yellow pearl.

chloroform but soluble in acetone and several sorts of alcohols acidified with HCl.

Paper chromatography. The reddish brown residue was chromatographed on Toyo No. 51A filter paper (40×40 cm), using 2 N HCl-n-BuOH (1:1) as solvent. As shown in Fig. 3, five colored bands were separately noticed on the chromatogram, i.e. a relatively weak yellow band at around Rf 0.15 (B-1), a deep yellow-brown band with Rf between 0.43 and 0.60 (B-2), a weak yellow-brown band with Rf between 0.60 and 0.75 (B-3), a grey-orange band with Rf between 0.80 and 0.85 (B-4), and a grey-orange band with Rf between 0.95 and 1.0 (B-5).

The five kinds of pigments were separately extracted with 1 N HCl-MeOH from their corresponding bands on the chromatogram and then rechromatographed on Toyo No. 51 A filter paper (40×40 cm), using 1 N HCl-n-BuOH (1:1) as solvent. In the Fig. 3 resulting chromatogram is shown. The B-3 was completely separated into two fractions, B-2 and B-4. After well dried at room temperature, the chromatogram paper was cut lengthwise into strips of 1 cm wide, which were separately sprayed with various kinds of spary reagents⁵⁾. The results obtained are shown in Table 2. The B-1 revealed intense prussian blue color with ferric chloride potassium ferricyanide reagent, green-brown color with ammoniacal silver nitrate reagent, intensified yellow color with 2,4-dinitrophenylhydrazine reagent, and orange tint with ben-zidine reagent. However, it was negative to

Fig. 3. Diagrammatic paper chromatograms of the pigments of yellow pearl. Left: The chromatogram of the pigments, solvent: 2-N HCl-n-BuOH (1:1), Right: Each fraction (B-1-B-5) on the left diagram was submitted to rechromatography using 1 N HCl-n-BuOH (1:1) as solvent. Most of them were further divided into two fractions on the right diagram as directed by arrows.

Table 2. Color development of the bands on the chromatogram with several spray reagents.⁵⁾

Reagents	Bands			
	B-1	B-2	B-4	B-5
Ferric Chloride Potassium Ferricyanide	Prussian blue +++	Prussian blue ++	Prussian blue +	Prussian blue +++
Ammoniacal Silver Nitrate	Green-brown +	Purple-brown +	Purple-brown ±	—
Antimony Trichloride	—	Orange-brown +	Orange-brown ±	Orange-brown +++
2,4-Dinitrophenyl- hydrazine	Yellow ±	—	—	Orange +++
Ben-zidine	Orange ±	—	—	Orange +++

antimony trichloride reagent. The B-2 and B-4 revealed prussian blue with ferric chloride potassium ferricyanide reagent, purple-brown with ammoniacal silver nitrate reagent, and orange-brown color with antimony trichloride reagent, but showed any color change neither with 2,4-dinitrophenylhydrazine nor benzidine reagents. On the contrary, the B-5 was positive to above mentioned all reagents except ammoniacal silver nitrate reagent. According to the results, all of the four pigments seem more or less to bear reducing property in common.

Absorption spectra in the ultraviolet region. The pigments which correspond to those B-1, B-2, B-4, and B-5 fractions of paper chromatogram were extracted separately with 1 N HCl-MeOH and submitted to absorption spectra analysis in 1 N HCl-MeOH with Shimadzu Multipurpose Recording Spectrophotometer MPS-model 50 L. Four of the pigments had no absorption band within the visible region though, as is shown in the Fig. 4, in the ultraviolet region, fraction B-1 and fractions B-2 and B-4 revealed three and two absorption bands, respectively. However, the pigment of fraction B-5 did not reveal any absorption band. According to the shape of spectra of the four pigments, their spectral characteristics are unable to deciphered though, they seem to be classified into three types, i.e. fraction B-1, fractions B-2 and B-4, and fraction B-5.

It may easily be noticed that the above mentioned classification well coincides with that based on color reactions of the pigments to various kinds of spary reagents.

Constituent amino acids of the pigments. Fractions B-1, B-2, B-4, and B-5 were separately extracted with 1 N HCl-MeOH from the paper chromatogram, concentrated, and hydrolyzed with 6 N HCl at 110°C for 24 hours in a sealed tube. After concentrated in vacuo, the hydrolyzate was submitted to two dimensional thin-layer chromatography according to the procedure of FAHMY *et al.*⁽⁶⁾ When sprayed with ninhydrin, fraction B-1 did not give any spot on the chromatogram but fraction B-5 gave two positive spots. As shown in Fig. 5, seven spots were detected with ninhydrin on the chromatogram of fraction B-2, of which R_f values were corresponded to those of following amino acids:

Fig. 4. Absorption spectra of each fraction of the pigments separated by means of paper chromatography. Solvent: 1 N HCl-MeOH.

Fig. 5. Two dimensional thin-layer chromatograms of amino acids in the acid hydrolyzates of the pigments.

Left: Fraction B-2, Right: Fraction B-4.

Ala., Val., Gly., Leu. (and/or Ileu.), Phe., Asp., and Try. In the case of fraction B-4 in addition to the above seven amino acids another five ninhydrin-positive spots were noticed, of which Rf values were corresponded to those of Thr., His., Pro., Lys., and Arg.

Examination of sugars in the pigments. To prevent possible contamination of carbohydrate of filter paper origin, the reddish brown pigment was newly prepared from the yellow nacre without using any filter paper. The pigment was hydrolyzed with 3 N H_2SO_4 at 100°C for 6 hours and then submitted to examination of sugars.

The hydrolysate was neutralized with $\text{Ba}(\text{OH})_2$, submitted to centrifugation for removal of resulting BaSO_4 , concentrated in vacuo, and then thin-layer chromatographed using silica gel G layer and isopropanol-acetic acid-water (3:1:0.7) as solvent. The sugars were detected by naphthoresorcline sulfuric acid reagent.⁷⁾ With the reagent one purple-blue spot appeared at around Rf 0.3 which was lower than those of glucose and galactose and closely resembled those of glucuronic acid and galacturonic acid. The true entity of this spot, however, could not be examined because of its trifling amount.

Discussion

Fifteen kinds of amino acids were detected on the hydrolyzate of the yellow pigment of pearl which corresponds to SAWADA's iron complex of peptide. The composition of the amino acids rather resembled those of pearl conchiolin reported by TANAKA *et al.* Both the pigment and pearl conchiolin contained relatively large amount of Ala., Gly., Leu., Asp., and Glu., which were namely reported as constituents of the iron complex of peptide by SAWADA. From these results, it may be deduced that the major constituent of the yellow pigment is not peptide but proteinous substance originated in pearl conchiolin.

The pigment, which corresponds to SAWADA's, was separated into four fractions by means of repeated paper chromatography using acidified n-BuOH as solvent. Among them, fraction B-1 was the smallest in quantity and fraction B-2 the largest.

With respect to spectral characteristics in the ultraviolet region and the behaviors to several spray reagents for paper chromatography, these fractions were classified into three types; fraction B-1, fractions B-2 and B-4, and fraction B-5. The classification seemed to be supported by the results of analysis on amino acids constitution of these pigments. The acid hydrolyzates of the both fractions B-2 and B-4 gave many ninhydrin positive spots on the thin-layer chromatogram but that of fraction B-1 gave none. In the case of fraction B-5 only two spots appeared.

The kinds of amino acids found in fraction B-2 were less than those of fraction B-4. However, the amino acids of relatively small quantity seemed to be overlooked in the fraction B-2 as postulated from chromatograms shown in Fig. 5, and the amino acid composition of the fraction B-2 was considered to be essentially identical with that of fraction B-4. The amino acids detected both in fractions B-2 and B-4 coincide with those contained in relatively large amount in pearl conchiolin with the exception of Glu., Ser., and Lys. From these results, it seems to be concluded that the pigments both in the fractions B-2 and B-4 may consist mainly of pearl conchiolin-like proteinous substance.

The shell conchiolin in molluscs, according to TANAKA *et al.*⁽⁸⁾, presumed to be formed from mucoprotein and mucopolysaccharide which were secreted from mantle tissues in the course of shell formation. On the other hand, HORIGUCHI⁽⁹⁾, who analyzed the shell constituents of shellfish, found hexosamine in the shell conchiolin. If the pearl conchiolin is formed in same mechanism as in the shell conchiolin, it may contain the like of sugars in the shell conchiolin.

To examine the supposition, the crude yellow pigments were newly prepared from the decalcified yellow nacre without using filter paper in order to avoid any carbohydrate contamination of the paper origin. The acid hydrolyzate of the pigment gave a characteristic purple-blue color reaction of sugar with naphthoresorcine sulfuric acid reagent on a thin-layer chromatogram. It seems likely, therefore, the pigment consist mainly with conchiolin-like proteinous substance having chemically combined sugar in its construction and may play a major role in the yellow coloration of the pearl.

The pigments of fractions B-1 and B-5 seemed to be nonproteinous nature though, intrinsic nature of them were remained unexamined because of their minor importance.

Summary

The pigments responsible for the coloration of the yellow nacre of pearl were investigated. Yellow pigments were extracted with HCl-MeOH, fractionated by paper chro-

matographic procedure, and analyzed on their constituents. The yellow pigments consist of four kinds of pigments which were classified into three types based on their spectral characteristics in the ultraviolet region and color reactions with various kinds of spray reagents for paper chromatography. Among them, major yellow pigment, after acid hydrolyzed, gave rise to many amino acids and the substance giving color reaction characteristic of sugars.

From these results, it was deduced that conchiolin-like proteinous substance bearing some sugar derivatives as its constituent might be a major yellow pigment of the yellow pearl.

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