Inorganic Constituents of Bone of Fish

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The inorganic constituents of fish bone ash were examined by both X-ray diffraction analysis and elemental analysis. Fifteen species of fish were selected from Teleostomi and Elasmobranchii. Cattle and swine as Eutheria and fowl as Neornithes were also investigated to compare with the Teleostomi and Elasmobranchii. All ashed bone samples were classified in to three groups composed mainly of either hydroxyapatite (HAP), β type $\text{Ca}_3(\text{PO}_4)_2$ (TCP), or their mixture; HAP type, TCP type, and HAP-TCP type. Sample species of the HAP type were sea bream, horse mackerel, carp, shark, cattle, swine, and fowl. The TCP type species was Japanese anchovy. HAP-TCP types were sardine, mackerel, tilefish, croaker, triggerfish, lizard fish, Spanish mackerel, flying fish, conger eel, and flat fish. Molar ratios of Ca with or without either Mg or Na to P for the examined bone ashes coincided with the theoretical values of HAP and TCP.

Key words: bone, mineral, hydroxyapatite, ash, element, calcium phosphate, X-ray diffraction analysis

The inorganic constituents of bone are well known as calcium phosphates, more exactly hydroxyapatite, $[(\text{Ca}_{10-x})(\text{H}_3\text{O}^+)_{2x}(\text{PO}_4)_6(\text{OH}^-)_2]$, (x is 0 to 2, HAP in abbreviation) with extremely complicated crystal structure.¹⁻⁷⁾ HAP is a kind of apatites such as fluorapatite and chloroapatite, being replaced by a hydroxy group in the halogen position of apatites. The inorganic constituents of fish bone, however, have scarcely been investigated to date. This investigation was aimed to make clear the crystal structure and elemental composition of the inorganic constituents of fish bone, and to compare these with land animals.

Materials and Methods

Bone Samples of Fishes

Fifteen species of fish were selected from Teleostomi and Elasmobranchii, and their bones were examined for X-ray diffraction analysis and elemental analysis after ashing. As Teleostomi, sea bream *Pagrus major*, horse mackerel *Trachurus japonicus*, carp *Cyprinus carpio*, Japanese anchovy *Engraulis japonica*, sardine *Sardinops melanostictus*, mackerel *Scomber japonicus*, tilefish *Branchiostegus japonicus*, croaker *Nibea mitsukurii*, triggerfish *Stphanolepis cirrhifer*, lizard fish *Saurida undosquamis*, Spanish mackerel *Scomberomorus niphonius*, flying fish *Prognichthys agoo*, conger eel *Anago anago*, and flat fish *Paralichthys olivaceus* all were used for the experiments. Gummy shark *Mustelus manazo* as an Elasmobranchii was chosen. Cattle *Bos taurus* var. domestics and swine *Sus scrofa* var. domestics as Eutheria and fowl *Gallus gallus* domestics as Neornithes were also examined to compare

these with Teleostomi and Elasmobranchii.

Preparation of Bone Ash

Bone samples were boiled in hot water at first and washed again with water after removal of all remaining flesh. The cleansed bones were then dried at room temperature, and ashed at 600°C for 3 h using an electric furnace. Cattle bone, however, was ashed for more than 3 h at the same temperature because it remains too hard to be ground in a mortar even after ashing for only 3 h. After cooling in the furnace, the bone ash was finely ground in a mortar to a powder of 145 mesh. These powdered samples were submitted for X-ray diffraction analysis and elemental analysis.

X-ray Diffraction Analysis

X-ray diffraction analysis of powdered bone samples were carried out using a Rigaku Diffractometer (Rigaku Electric Instrument, Type RAD- γ A) with a monochromatic $\text{CuK}\alpha$ radiation. Measuring conditions were the same as those previously reported.⁸⁾ The X-ray diffraction patterns, that is, the relation of angle of diffraction 2θ and intensity of the diffracted beam, for three strong peaks of the samples were compared with those of authentic chemicals and Powder Diffraction File's data,⁹⁾ and the crystal structure of the ash was examined for identification.

Elemental Analysis

Twelve of elements (Ca, Na, Mg, K, Fe, Zn, Mn, Cu, Pb, Ni, Co, and Cd) were determined by atomic absorption spectrometry. P was determined spectrophotometrically by the Fiske-Subbarow's method. Atomic absorption

analysis were carried out using a Hitachi atomic absorption spectrophotometer, Type Z-8000. The ash samples were dissolved in 6 N HCl and dried up on a water bath. The residue was dissolved again in 0.1N HCl and the elements were determined after appropriate dilutions.

Authentic Chemicals for X-ray Diffraction Analysis

HAP (Sigma Chemical Company) was used as an authentic chemical in the X-ray diffraction analysis. Beta type $\text{Ca}_3(\text{PO}_4)_2$ (TCP in abbreviation, Nacalai Tesque Co.) was another authentic sample, because HAP changes to TCP with heating at a high temperature on the basis of X-ray diffraction pattern.¹⁾ That is, the hydroxy group of HAP is lost at high temperature (more than about 600°C), and then the crystal structure of HAP is partly destroyed. This destroyed HAP resembles TCP very closely in X-ray diffraction pattern.

Results and Discussion

Comparison of the X-Ray Diffraction Patterns between Bone Ash Samples and Authentic Chemicals

In the X-ray diffraction patterns of eighteen ash samples, six strong peaks appeared predominantly at angles of diffraction of 49.5°, 34.4°, 31.8°, 31.0°, 27.8°, and 25.9°. The peaks at 31.8° and 31.0° were far stronger than others. The peak at 31.8° corresponds to HAP, while those at 31.0° to α and β types of TCP and β types of $\text{Ca}_2\text{P}_2\text{O}_7$, according to Powder Diffraction File in calcium phosphate compounds. HAP peaks at 31.8°, 25.9° and 49.5°, and TCP likewise at 31.0°, 34.4°, and 27.8°. These six peaks were confirmed in many bone ash samples, and consequently the inorganic constituents of the fish bone were thought to be the mixture of HAP and TCP. But, TCP might have artificially produced from HAP during ashing at 600°C. The presence of TCP was also found in sardine scale ash with heating at 600°C for 3 h as reported.⁸⁾

In order to further clarify the above estimation that the ash may be composed of HAP and TCP, relative intensities of the diffracted beams for two groups of individual three angles were calculated. The results are summarized in Table 1. The figures represent relative intensity in percent of second and third peaks against the first, or the strongest peak for either corresponding HAP and TCP. As shown in Table 1, characteristic peaks of HAP were confirmed in sea bream, horse mackerel, carp, shark, cattle, swine, and fowl, while the peak of TCP was confirmed only in Japanese anchovy. Both peaks of HAP and TCP were verified in sardine, mackerel, tilefish, croaker, triggerfish, lizard fish, Spanish mackerel, flying fish, conger eel, and flat fish.

From these results, all the samples were divided into three groups: The first group was characteristic of HAP (sample No. 1–7); the second group corresponded to TCP (No. 8), and the third to both HAP and TCP (No. 9–18). These three groups were named HAP type, TCP type, and HAP-TCP type, respectively. They are summarized in Table 2, and typical X-ray diffraction patterns of the fish bone ash were shown in Fig. 1 for sea bream, Japanese anchovy, and mackerel as the representatives of HAP, TCP, and HAP-TCP types of ash samples, respectively.

Table 1. Relative intensities of major X-ray diffraction peaks of bone ashes (%)

Sample	Diffracted angle, 2θ	31.8°	25.9°	49.5°	31.0°	34.4°	27.8°
Hydroxyapatite* ¹		100* ²	40* ²	40* ²	—	—	—
β - $\text{Ca}_3(\text{PO}_4)_2$ * ¹		—	—	—	100* ²	40* ²	50* ²
1. Sea bream		100	35	27	—	—	—
2. Horse mackerel		100	32	24	—	—	—
3. Carp		100	34	29	—	—	—
4. Shark		100	38	30	—	—	—
5. Cattle		100	37	28	—	—	—
6. Swine		100	31	27	—	—	—
7. Fowl		100	39	30	—	—	—
8. Japanese anchovy* ³		—	—	—	100	44	40
9. Sardine		100	40	27	100	56	58
10. Mackerel		100	40	28	100	58	65
11. Tilefish		100	50	29	100	56	57
12. Croaker		100	43	31	100	53	50
13. Triggerfish		100	41	28	100	59	59
14. Lizard fish		100	35	29	100	56	68
15. Spanish mackerel		100	80	33	100	58	55
16. Flying fish		100	47	29	100	49	48
17. Conger eel		100	42	29	100	50	56
18. Flat fish		100	38	29	100	57	61

*¹ Authentic chemicals.

*² Values from Powdered Diffraction File⁹⁾ (relative intensity against the most highest peak).

*³ Boiled in a salt solution and dried fish product (*niboshi*, usually used as soup stock in Japan).

Table 2. Classification of the bone ashes

Type of crystal structure* ¹	Corresponding species
HAP type	Sea bream, horse mackerel, carp, shark, cattle, swine, fowl
TCP type	Japanese anchovy* ²
HAP-TCP type	Sardine, mackerel, tilefish, croaker, triggerfish, lizard fish, Spanish mackerel, flying fish, conger eel, flat fish

*¹ HAP, hydroxyapatite; TCP, β type $\text{Ca}_3(\text{PO}_4)_2$.

*² *Niboshi* product (see Table 1).

Elemental Contents of Bone Ash

Thirteen elements were determined, as summarized in Table 3. In all samples, Ca was the most abundant element, ranging from 34 to 36 g/100 g ash (% in abbreviation) except for Japanese anchovy, where it occurred at 24.4%. The next abundant element after Ca was P. The content was about a half of the Ca content.

Sodium content occurred in range from 0.38 to 2.22%, except that an extreme higher content of Na appeared only in Japanese anchovy (*niboshi* product). The high Na content in Japanese anchovy were partly due to its processing method. Because *niboshi* is produced by boiling raw Japanese anchovy in a salt solution and drying.

Magnesium ranged from 0.42 to 0.99%, with a mean and standard deviation of $0.65 \pm 0.13\%$, and was accepted as almost constant in the sample species. The potassium

Fig. 1. Typical three different X-ray diffraction patterns of the fish bone ash of 15 samples. HAP, hydroxyapatite; TCP, β type $\text{Ca}_3(\text{PO}_4)_2$.

Table 3. Element contents of bone ashes

Sample species	Ca	P	Na	Mg	K	Fe	Zn	Mn	Cu	Pb	Ni	Co	Cd
HAP type													
1. Sea bream	35.4%	17.5%	0.81%	0.54%	0.215%	47 ppm	109 ppm	41 ppm	1.4 ppm	0.4 ppm	<0.1 ppm	<0.2 ppm	<0.1 ppm
2. Norse mackerel	35.8	17.3	0.38	0.52	0.657	117	85	16	1.2	1.0	0.4	<0.2	<0.1
3. Carp	35.6	16.9	1.01	0.61	0.199	39	280	21	0.9	0.5	0.6	<0.2	<0.1
4. Shark	34.9	16.9	1.55	0.42	0.373	27	91	48	2.0	0.6	0.4	<0.2	<0.1
5. Cattle	36.5	17.2	0.85	0.81	0.340	267	209	1.4	0.7	0.8	<0.1	<0.2	<0.1
6. Swine	36.9	17.0	0.77	0.66	0.364	114	218	1.1	1.2	0.2	<0.1	<0.2	<0.1
7. Fowl	36.1	17.4	0.84	0.70	0.490	376	340	4.8	0.9	0.2	<0.1	<0.2	<0.1
TCP type													
8. Japanese anchovy*	24.4	16.3	6.27	0.99	3.07	434	252	158	10.3	4.8	<0.1	0.4	<0.1
HAP-TCP type													
9. Sardine	35.8	17.3	0.42	0.47	0.363	236	280	113	2.5	3.1	<0.1	<0.2	<0.1
10. Mackerel	33.9	17.0	2.22	0.67	0.299	158	150	11	0.9	0.5	<0.1	<0.2	<0.1
11. Tilefish	35.1	18.4	0.55	0.66	0.317	45	119	52	1.4	2.0	0.4	<0.2	<0.1
12. Croaker	35.1	17.8	0.60	0.53	0.372	825	104	53	1.4	0.3	0.2	<0.2	<0.1
13. Triggerfish	34.4	17.8	0.46	0.59	0.194	163	273	74	69.0	1.7	12.1	<0.2	<0.1
14. Lizard fish	35.7	17.8	1.48	0.67	0.209	23	196	148	2.1	0.5	<0.1	<0.2	<0.1
15. Spanish mackerel	34.0	18.6	0.81	0.77	0.997	407	219	43	1.8	0.6	0.9	<0.2	<0.1
16. Flying fish	34.4	17.8	0.47	0.85	0.313	297	129	29	9.2	0.9	2.9	<0.2	<0.1
17. Conger eel	35.7	17.7	0.52	0.55	0.329	147	265	218	2.1	1.6	1.0	<0.2	<0.1
18. Flat fish	35.9	18.1	0.70	0.54	0.454	20	339	94	0.2	0.6	<0.1	<0.2	<0.1

* *Niboshi* product (see Table 1).

content was $0.39 \pm 0.22\%$ except for the high content in Japanese anchovy at 3.07%. Thus potassium varied widely by different species. Iron ranged from 20 to 825 mg/kg of ash (corresponding to ppm in abbreviation; 252 ± 268 ppm), ranging from one tenth to one hundredth of K content. Zinc was almost constant at 197 ± 79 ppm, being lower than that of iron. Manganese of fish bone was at 11–

218 ppm (55 ± 58 ppm), showing a wide scattering of content by different sample species. The Mn contents of cattle, swine, and fowl ash were as low as one tenth of those of fish, compared with the other elements. Copper was 0.2–69 ppm with a great difference in the sample species, but no relation was found between the content and the species. The content of Pb, Ni, Co, and Cd was very

low in all samples, although a few exceptions were found.

The Molar Ratio of Ca to P

In Table 2, the bone ash samples were clarified as consisting of either HAP, TCP, or a mixture of them. Moreover, the results of the X-ray diffraction analysis should be ascertained by molar ratio of Ca to P, or Ca/P from the previous elemental analysis data. Since, the chemical formula of HAP is $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ if crystal structure is complete, the molar ratio of Ca/P comes to 1.67. That of TCP likewise comes to 1.50. Therefore, the ratio should theoretically come to 1.50–1.67 for the mixture of HAP and TCP. On the basis of the contents of Ca and P as shown in Table 3, the molar ratio of Ca/P was calculated and summarized in Table 4.

The Ca of HAP can be easily substituted by other elements,^{1,3-7)} and average composition of bone minerals is written as $[(\text{Ca}_{10-x}^{2+}(\text{H}_3\text{O}^+)_{2x}(\text{PO}_4^{3-})_6(\text{OH}^-)_2)][\text{Ca}^{2+} \cdot \text{Mg}_{0.3}^{2+} \cdot \text{Na}_{0.3}^+ \cdot \text{CO}_3^{2-} \cdot \text{citrate}_{0.3}^{3-}]$.¹⁾ The sum of Ca and the subsequently major divalent element, magnesium, was thought to indicate the relative total Ca content. The molar ratio of the sum of Ca and Mg against P was also calculated, and

shown in Table 4.

The molar ratio of Ca/P for the HAP type species was 1.54–1.68, slightly lower than the theoretical value of 1.67. The sum of Ca and Mg against P was 1.60–1.73, being closer to the theoretical value. This indicates that a portion of the Ca of HAP was replaced by other elements.

In Japanese anchovy, which belongs to the TCP type, the molar ratio of Ca/P was 1.20, and even $(\text{Ca} + \text{Mg})/\text{P}$ was as low as 1.23. Either ratio was extremely low as compared to the theoretical value. The lower value of Ca/P might have risen in extremely low Ca content as compared with other samples. As stated above, the Japanese anchovy sample used in this study was a *niboshi* product, and consequently Ca of TCP may be replaced by Na from the NaCl solution. Assuming that the total Ca content of Japanese anchovy may be the sum of Ca and Na, $(\text{Ca} + \text{Na})/\text{P}$ in molar ratio was calculated at 1.67, a theoretical value of HAP. From this result, it seemed that a part of Ca of bone ash of Japanese anchovy was replaced by Na. In HAP-TCP type samples, the molar ratio of Ca/P was 1.41–1.60, and the ratio was approximately the same with a theoretical value for TCP. Presuming the Ca content as the sum of Ca and Mg, $(\text{Ca} + \text{Mg})/\text{P}$ in molar ratio was calculated to be 1.47–1.63, more closer to the theoretical value.

Table 4. Molar ratios of calcium to phosphorus of the bone ash of fishes, mammal, and bird

Samples analyzed	Ca/P* ²	(Ca + Mg)/P* ²
Hydroxyapatite* ¹	1.67* ³	—
β type $\text{Ca}_3(\text{PO}_4)_2$ * ¹	1.50* ³	—
HAP type		
1. Sea bream	1.54	1.60
2. Horse mackerel	1.60	1.64
3. Carp	1.63	1.67
4. Shark	1.60	1.63
5. Cattle	1.64	1.70
6. Swine	1.68	1.73
7. Fowl	1.60	1.65
TCP type		
8. Japanese anchovy* ⁴	1.20	1.23
HAP-TCP type		
9. Sardine	1.60	1.63
10. Mackerel	1.54	1.59
11. Tilefish	1.47	1.52
12. Croaker	1.52	1.56
13. Triggerfish	1.49	1.54
14. Lizard fish	1.55	1.60
15. Spanish mackerel	1.41	1.47
16. Flying fish	1.49	1.55
17. Conger eel	1.56	1.60
18. Flat fish	1.53	1.57

*¹ Authentic chemicals.

*² Molar ratio.

*³ Theoretical values.

*⁴ *Niboshi* product (see Table 1).

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