Technical paper

Biological Availability of Docosahexaenoic Acid from Fish Oil Encapsulated in Zein-Coated Porous Starch Granules in Rats

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The investigation was designed to study whether DHA in fish oil encapsulated in porous starch granules coated with water-insoluble corn protein, zein, can be utilized during passage through the gastrointestinal tract in rats. The *in vitro* digestion trial showed that zein used for coating starch granules was decomposed by proteolytic enzymes. After feeding porous starch granules containing fish oil, coconut oil, or corn oil for 3 weeks, serum triglycerides and phospholipids were significantly lower in the rats on fish oil than in the other rats. The proportion of DHA in total fatty acid of the fish oil group was significantly higher in both liver and brain lipids than in the other two groups. These results clearly show that fish oil encapsulated in porous starch granules coated with zein could be digested and absorbed. Moreover, it was confirmed that the granules had been changed morphologically by scanning electron microscopic analysis of the contents of the alimentary sections, showing that the film of zein had been digested in stomach and small intestine. The digestibility of fish oil in the capsules was shown to be 96.8%.

Keywords: zein, capsule, *in vivo* digestion, DHA, lipid metabolism, rat

The idea of porous starch granules was first proposed by Whistler *et al.* (1984). Monma *et al.* (1990) reported that starch granules produced many circular small holes after action of amylolytic enzyme on native cornstarch granules. Thereafter, research to make effective use of porous starch granules began (Monma, 1991; Franco *et al.*, 1987, 1988; Fannon *et al.*, 1992; Yamada *et al.*, 1995a). The porous starch granule has small holes of 1 μ m in diameter after the action of amylolytic enzyme on cornstarch, and its porosity is higher than 50%. The granules are useful to protect unstable products from oxidation, light-decomposition, or to confine an unpleasant odor or bitter taste, etc. When these substances are buried in the holes of porous starch granules and coated with a film, oxidation, decomposition, and release of the peculiar smell can be effectively prevented.

It is well known that n-3 unsaturated fatty acids (UFAs) such as docosahexaenoic acid (DHA) have preventive and curative effects on cardiovascular diseases such as liver infarction and arteriosclerosis (Anderson & Connor, 1989; Lakshman *et al.*, 1988; Sanchez-Muniz *et al.*, 1999; Engler *et al.*, 1999). A main source of DHA is fish oil. It is a type of highly polyunsaturated fatty acid, is easily oxidized and has a peculiar smell. Accordingly, preservation of foods which contain such kind of unsaturated fatty acid has been a serious problem. The zein-coated fish oil-encapsulated porous starch granules (capsules) could overcome the difficulties in preserving fish oil containing DHA. The main purpose of the present research was to examine whether fish oil in the capsules could be utilized by animals efficiently.

Materials and Methods

Design and preparation of zein-coated starch granules (capsules) Zein-coated fish oil-encapsulated porous starch granules were produced by adding porous starch granules to purified fish oil and then coating them with a water-insoluble corn protein, zein. The capsules contained 50% refined fish oil which included 20% DHA, and were small spheroid granules 20–30 μ m in diameter. The process is shown in Fig. 1 and photographs of the granules in Fig. 3 (A). Zein-coated coconut oil-encapsulated porous starch granules were prepared as a control, in order to test whether the purified fish oil coated with zein can be absorbed in a digestion trial with rats. Table 1 shows the compositions of these two products.

The porous starch granules were produced by treating cornstarch with the enzyme of *Aspergillus* sp. K-27 (Abe *et al.*, 1985). They have high strength and their porosity is 50%.

The zein for coating was prepared by the following method. Corn gluten meal was defatted with a hexane : ethanol=1 : 1 mixture at 55°C for 1 h (4 times), followed by drying with air. The defatted corn gluten meal was extracted with 70% ethanol at 55°C for 5 h. The extract was kept at 4°C for 24 h, and centrifuged (3000 rpm, 10 min) at 4°C. The supernatant was spray-dried.

Proteolytic digestion of the capsules The capsules or the zein used for coating was suspended in 5ml of hydrochloric acid solution (pH2.2) for pepsin, 0.1 M phosphate buffer (pH 7.7) for trypsin, or 0.1M phosphate buffer (pH 8.3) for α -chymotrypsin, respectively. Ten milligrams of pepsin (Sigma Chemical Co., 3200 units/mg solid), trypsin (Sigma Chemical Co., bovine pancreas, 10400 units/mg solid), or α -chymotrypsin (Wako Pure

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Fig. 1. Production of zein-coated starch capsules.

Chemical Industries, Ltd., bovine pancreas, 1000 units/mg) were added (ratio of enzyme to zein was 1 : 100), respectively, and the reactions were carried out at 37°C for 5 h. The hydrolyzed products of the capsules and the zein were analyzed with SDS-PAGE (Laemmli, 1970).

Animals Male Wistar rats aged 3 weeks (Japan SLC Inc., Shizuoka) were used. The animal room was maintained at $22 \pm 2^\circ\text{C}$ and 12 h-light/dark cycle (light: 8:00–20:00). After the rats were acclimated to the facility on a laboratory chow (Type MF, Oriental Yeast, Co., Tokyo) for one week, they were subjected to the experiments.

The animal experiments were conducted in accordance with the guidelines of the Committee on Animal Research of Mie University.

Electron microscopic analysis of the capsules in rat diges-

tive tract To observe the morphological change of zein film in the digestive tract, the experimental diet contained 10% capsules, 20% casein, and 70% sucrose. The control diet contained 20% casein and 80% sucrose. Two groups of 3 animals each were given the experimental diets *ad libitum* for one week, then were anesthetized deeply with diethyl ether and killed. The contents of stomach, upper and lower sections of the small intestine, caecum and colon were collected with a small amount of water, centrifuged, and washed with distilled water 2–3 times. The residues were freeze-dried and examined in a scanning electron microscope (SEM) (S-4000, Hitachi, Ltd., Tokyo).

Lipid analysis Three groups of 5 animals each were given the experimental diets *ad libitum* for 3 weeks. The diets were made to contain 20% (w/w) dietary fat with corn oil (control group), purified DHA-rich fish oil, or coconut oil (Table 2). On

Table 1. Compositions of zein-coated fish oil-encapsulated porous starch granules and coconut oil-encapsulated porous starch granules (%).

Composition	Zein-coated fish oil-encapsulated porous starch granules	Zein-coated coconut oil-encapsulated porous starch granules
Type of oil	Purified fish oil containing 20% DHA	Coconut oil
	50	50
Porous starch	14	14
Zein	30	30
Vitamin C	5	5
Emulsifying agent ^(a)	0.5	0.5
Antioxidants ^(a)	0.5	0.5

^(a)Product of Taiyo Kagaku Co., Ltd.**Table 2.** Compositions of the experimental diets (%).

	Control group	Fish oil encapsulated group	Coconut oil encapsulated group
Casein	20	20	20
Oil or granules	Corn oil	Zein-coated fish oil-encapsulated porous starch granules ^(c)	Zein-coated coconut oil-encapsulated porous starch granules ^(c)
	10	20	20
Mineral mixture ^(a)	4	4	4
Vitamin mixture ^(a)	1	1	1
Cellulose ^(b)	2	2	2
Cornstarch	63	53	53
kcal/g (calculated)	4.22	4.00	4.00

^(a)According to Harper; A. E. Harper: *J. Nutr.*, (1959), **68**, 405., ^(b)Type E, Toyo Roshi Co., ^(c)Shown in Table 1. **Fig. 2.** SDS-PAGE profiles of enzyme-treated capsules. Electrophoresis was carried out on 15% gel. Lane 1, molecular weight standard; 2, zein; 3, pepsin; 4, zein treated with pepsin; 5, capsules treated with pepsin; 6, trypsin; 7, zein treated with trypsin; 8, capsules treated with trypsin; 9, α -chymotrypsin; 10, zein treated with α -chymotrypsin; 11, capsules treated with α -chymotrypsin.

the last day of the feeding term, the rats were deprived of the food for 4 h (9:00–13:00) and blood was withdrawn from the abdominal aorta under diethyl ether anesthesia. The blood was centrifuged at $1700\times g$ for 15 min to separate serum. The liver, perirenal adipose tissue, and brain were excised immediately and frozen at -30°C for lipid analysis.

Total hepatic lipids were extracted according to the method of Folch *et al.* (1957). Serum and hepatic lipids were analyzed for total cholesterol (TC), triglycerides (TG), phospholipids (PL),

and high-density lipoprotein cholesterol (HDL-C), using commercial diagnostic kits (“Cholesterol C2 Test-wako” for TC, “Triglyceride Test-wako” for TG, and “Phospholipid B Test-wako” for PL from Wako Pure Chemical Industries, Osaka, and “HDL-C2 Daiich” for HDL-C from Daiichi Pure Chemicals Co., Tokyo). All organic solvents contained 0.01% butylated hydroxytoluene (Takamura *et al.*, 1987).

Fatty acid compositions of the serum and tissues were determined according to the following method. Lipids were extracted from serum or homogenized tissue samples according to the method of Folch *et al.* (1957) and the extracts were evaporated to be dried under nitrogen gas. Methyl-esterified fatty acids were analyzed by a gas-liquid chromatograph (GC-14B, Shimadzu Co., Ltd., Kyoto) equipped with a $30\text{ m}\times 0.32\text{ mm (i.d.)}$ HR-SS-10 capillary column (Shinwa, Japan), data processor (Chromato pac C-R7A, Shimadzu Co., Ltd., Kyoto), and a flame-ionization detector (Ikeda *et al.*, 1989). Fatty acids were identified based on the relation between the carbon number of a homologue and retention time or by comparing the retention time with that of a reference standard.

Digestibility of fish oil in the capsules Five 4-week old rats were fed the capsule diet (the diet for the fish oil group in Table 2) mixed with 0.5% chromium oxide as an indicator for 10 days. Feces were collected from the 5th to the 7th and the 8th to the 10th day, then dried at 100°C to a constant weight and ground.

The fat in diet and feces was measured gravimetrically by Soxhlet extraction with ether. Chromium oxide in diet and feces was measured by the modified dry method of Hesford and Buhner (1978).

Digestibility of fat was calculated according to the following equation.

Digestibility (%) =

$$100 - 100 \times \frac{\text{Cr}_2\text{O}_3 \text{ in diet (\%)}}{\text{Cr}_2\text{O}_3 \text{ in feces (\%)}} \times \frac{\text{fat in feces (\%)}}{\text{fat in diet (\%)}}.$$

Statistical analysis The results were expressed as means with their standard errors (SE). The data were analyzed by means of analysis of variance, using the Microsoft Excel 98 (Microsoft Corporation). Differences between means were assessed by Student's *t*-test (Snedecor & Cochran, 1976), and significant differences in the means were inspected at $p < 0.05$.

Results

Proteolyzability of the capsules The results of SDS-PAGE showed that the capsules could be completely decomposed with pepsin and α -chymotrypsin. The molecular weight of the zein in capsules treated with these two enzymes was approximately less than 14kDa, respectively (Fig. 2), while that in capsules treated with trypsin was approximately 14–21kDa.

Morphological changes of the capsule in rat digestive tract By observing the insoluble residues in the stomach with a SEM, some morphological changes were found on the surface of the capsules: there were many pores visible as shown in Fig. 3 (B).

SEM observation of the capsules in the upper and lower sections of the small intestine showed that their surface portion, zein film, could be decomposed by proteinases, resulting in many pores on the surface of the granules. These pores enabled the fish oil to be released. The shapes of the capsules in the upper small

(A)

(D)

(B)

(E)

(C)

(F)

Fig. 3. Scanning electron micrographs of the capsules in rat digestive tract. Photographs of the original capsules (A), and capsules in stomach (B), upper small intestine (C), lower small intestine (D), caecum (E), and colon (F).

intestine are shown in Fig. 3(C). The contents of the lower small intestine were also observed by SEM but no complete capsules were observed; photographs of the capsules in the lower small intestine are shown in Fig. 3(D). No complete capsules were also observed in the contents of caecum and colon as shown in Fig. 3 (E, F). The photographs indicated that the capsules could have been digested by the digestive proteolytic enzymes.

Effects of the capsule feeding on lipid metabolism in rats Body weight, food intake, and liver weight of the rats are shown in Table 3. In comparison with the control group, liver weight of the fish oil group was significantly higher.

Serum lipid concentrations are shown in Fig. 4. There were no significant differences in total cholesterol among the three groups. Phospholipid and triglyceride levels in the fish oil group

Table 3. Body weight, food intake, and liver weight of the rats.

Dietary group	Control	Fish oil-encapsulated porous starch granules	Coconut oil-encapsulated porous starch granules
Initial body weight (g)	80±2.3	81±2.6	81±1.8
Final body weight (g)	232.5±11.62	236.8±11.46	227.5±5.74
Food intake (g/day)	15.5±0.3	14.3±0.4	14.9±0.3
Liver weight (g/100g B.W)	3.81±0.07 ^{a)}	4.85±0.07 ^{b)}	4.11±0.25 ^{a)}
Brain weight (g/100g B.W)	0.76±0.04	0.75±0.03	0.79±0.02

Values are expressed as the mean±S.E. (n=5).

^{a)(b)}: Values bearing different letters are significantly different ($p<0.05$).

Fig. 4. Serum lipid concentrations in the rats fed zein-coated fish oil-encapsulated porous starch granules, zein-coated coconut oil-encapsulated porous starch granules, and corn oil diets. Values are expressed as the means±S.E. (n=5). a,b: columns bearing different letters are significantly different ($p<0.05$). TC, Total cholesterol; HDL-C, High-density lipoprotein cholesterol; PL, Phospholipids; TG, Triglycerides.

Fig. 5. Hepatic lipid contents in the rats fed zein-coated fish oil-encapsulated porous starch granules, zein-coated coconut oil-encapsulated porous starch granules, and corn oil diets. Values are expressed as the means±S.E. (n=5). a,b: columns bearing different letters are significantly different ($p<0.05$). TL, Total lipid; TC, Total cholesterol; PL, Phospholipids; TG, Triglycerides.

were found to be significantly lower than those of the other two groups. Hepatic lipid contents shown in Fig. 5: the hepatic contents of total lipids, cholesterol, triglycerides, and phospholipids of the fish oil group were significantly lower than the corn oil group. The hepatic contents of total lipids and triglycerides of the fish oil group were significantly lower than the other two groups.

The fatty acid compositions of the serum lipids are shown in Table 4. The proportion of n-3 fatty acids in the total fatty acid of serum of the fish oil group was higher. However, n-3 fatty acids were not detected in the control group or the coconut oil group. Fatty acid compositions of the brain lipids are shown in Table 5; except for DHA, there were no great differences among the 3 groups. The proportion of DHA in total fatty acids in the brain lipids of the fish oil group was significantly higher than those of the other two groups. Higher DHA was also detected in the hepatic lipids of the fish oil group (Table 6). Furthermore, in the perirenal adipose tissue, DHA was found only in the fish oil group (data not shown).

Digestibility of fish oil in the capsules The digestibility of fish oil in the capsules was $96.8\% \pm 0.15$ in the rats indicating that the fish oil was fully absorbed *in vivo*.

Table 4. Fatty acid compositions of the serum lipids (%).

Fatty acid	Control group	Fish oil encapsulated group	Coconut oil encapsulated group
12:0	1.6	Tr.	1.35±0.53
14:0	0.57±0.26 ^{a)}	0.37±0.01 ^{a)}	2.46±0.23 ^{b)}
16:0	16.18±1.01	13.10±0.98	15.10±0.39
16:1	0.74±0.03 ^{b)}	0.41±0.05 ^{a)}	2.50±0.13 ^{c)}
18:0	10.28±1.19	10.95±0.56	11.43±1.00
18:1(n-9)	11.36±2.21 ^{b)}	6.66±0.48 ^{a)}	14.12±1.35 ^{b)}
18:2(n-6)	18.04±2.67 ^{b)}	4.43±0.42 ^{a)}	10.51±1.59 ^{b)}
18:3(n-6)	ND	ND	ND
18:3(n-3)	ND	ND	ND
20:4(n-6)	18.63±2.10	15.08±0.30	14.74±1.69
20:5(n-3)	ND	3.45±0.22	ND
22:6(n-3)	ND	7.86±0.45	ND
Total n-3	ND	11.31±0.66	ND
Total n-6	36.66±4.56 ^{b)}	19.51±0.68 ^{a)}	23.72±4.01 ^{a,b)}

Values are expressed as the mean±S.E. (n=5). ^{a)(b)(c)}: Values bearing different letters are significantly different ($p<0.05$), ND: Not detected, Tr.: Trace amount (less than 0.1%).

Table 5. Fatty acid compositions of the brain lipids (%).

Fatty acid	Control group	Fish oil encapsulated group	Coconut oil encapsulated group
12:0	ND	ND	ND
14:0	0.23±0.02	0.20±0.04	0.23±0.03
16:0	21.25±1.38	20.28±3.06	21.25±3.07
16:1	ND	ND	ND
18:0	19.55±2.98	19.75±3.41	19.55±3.02
18:1(n-9)	20.10±3.05	19.70±4.31	20.10±3.01
18:2(n-6)	1.20±0.01	1.09±0.30	1.19±0.01
18:3(n-6)	ND	ND	ND
18:3(n-3)	ND	0.10±0.01	ND
20:4(n-6)	9.00±1.01	8.15±1.21	9.00±2.02
20:5(n-3)	ND	ND	ND
22:6(n-3)	9.84±0.04 ^{a)}	18.03±0.14 ^{b)}	9.87±0.03 ^{a)}
Total n-3	9.84±0.04 ^{a)}	18.01±0.09 ^{b)}	9.87±0.03 ^{a)}
Total n-6	10.17±0.07	9.26±0.09	10.20±0.07

Values are expressed as the mean±S.E. (n=5).

^{a)(b)}: Values bearing different letters are significantly different ($p<0.05$), ND: Not detected.

Table 6. Fatty acid compositions of the liver lipids (%).

Fatty acid	Control group	Fish oil encapsulated group	Coconut oil encapsulated group
12:0	0.45±0.18	0.42±0.31	0.58±0.14
14:0	ND	1.25±1.27	2.84±0.38
16:0	18.15±1.54	18.25±1.65	18.28±1.61
16:1	0.85±0.45	0.81±0.45	0.82±0.48
18:0	6.31±0.42 ^{a)}	18.60±0.37 ^{c)}	12.37±0.42 ^{b)}
18:1(n-9)	19.43±0.80 ^{b)}	9.59±0.20 ^{a)}	24.82±0.08 ^{c)}
18:2(n-6)	26.87±0.98 ^{c)}	4.23±0.09 ^{a)}	4.91±0.11 ^{b)}
18:3(n-6)	0.15±0.03	ND	Tr
18:3(n-3)	ND	ND	ND
20:4(n-6)	11.32±0.71	10.80±0.24	10.04±0.30
20:5(n-3)	ND	1.81±0.55	ND
22:6(n-3)	1.38±0.10 ^{a)}	19.53±0.34 ^{c)}	1.91±0.01 ^{b)}
Total n-3	1.38±0.10 ^{a)}	21.35±0.34 ^{c)}	1.91±0.01 ^{b)}
Total n-6	38.34±0.73 ^{b)}	15.40±0.36 ^{a)}	14.95±0.40 ^{a)}

Values are expressed as the mean ± S.E.(n=5).

^{a)b)c)}: Values bearing different letters are significantly different ($p<0.05$).

ND: Not detected, Tr.: Trace amount (less than 0.1%).

Discussion

n-3 Unsaturated fatty acids such as DHA are relatively rich in fish oil (Ackman, 1976). Feeding n-3 unsaturated fatty acid-deficient oils to rats decreased the DHA level in brain phospholipids (Yamamoto *et al.*, 1987; Philbrick *et al.*, 1987). Fish oil has an unpleasant odor and is easily oxidized; accordingly, preserving and transporting foods which contain fish oil is difficult. The odor of the fish oil can be disguised and the oil's oxidation containing DHA prevented by microcapsulization as described in this report.

Whether zein can be readily decomposed by the digestion system is a key factor in the utilization of fish oil contained in the capsules. One of the purposes of the present investigation was to test whether the capsules can be decomposed by proteolytic enzymes. It has been shown that the molecular weight of a native zein is 24 kDa (Abe *et al.*, 1981). The results of SDS-PAGE showed that digestibility of the capsules with pepsin or α -chymotrypsin is higher and that the capsules cannot be decomposed satisfactorily by trypsin (Fig. 2). These results indicate that the capsules may be degraded by proteinases in the gastrointestinal tract.

By examining using SEM whether the capsules exist in the colon, it was sought to prove whether they were digested in the digestion system. As had been expected, no complete capsules were observed in the intestinal contents, indicating that the capsules were utilized during passage through the digestive tract.

Feeding DHA decreased triglyceride and cholesterol levels in the serum of rats (Harris *et al.*, 1984; Kobatake *et al.*, 1984). Lipid analysis showed that serum triglycerides and phospholipids were significantly lower in the fish oil group than in the other two groups. The proportion of DHA in total fatty acid in the brain lipids of the fish oil group was significantly higher than those of the other two groups (Table 5). Higher DHA level was also observed in the hepatic lipid of the fish oil group (Table 6).

The digestibility of the fish oil in the capsules was shown to be very high (96.8%) in the present research. Thus, substances confined in holes of the porous starch can be easily subjected to digestion and absorption.

From the results of the proteolytic digestion, morphological changes of the capsules, lipid analysis, and the digestibility of fish oil, it can be concluded that the capsules are easily digested

by the alimentary system.

Zein is a by-product in the production of cornstarch from grain corn. It is a protein obtained from corn gluten meal by extraction with 60–90% ethanol. A zein film forms after an ethanol solution of zein is dried. Suzuki (1995) reported that a 0.1 mm-thick zein film could completely prevent oxygen from permeating. Zein has been proposed to be used as a coating material in the food industry (Parris *et al.*, 1998; Yamada *et al.*, 1995b).

A number of coating materials are now used in the chemical industry, although there are few in the medicinal or food industries. Most foods are generally in an aqueous state or moist, so water-insoluble substances are more suitable as a coating. Zein will be applied in many areas of the medicinal and food industries as a coating material.

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