The Physicochemical and Biopharmaceutical Properties of Fragmented Keratin as a New Drug Carrier

Junichiro NODA,^a Teruko IMAI,^a Kenji KIDA,^b and Masaki OTAGIRI^{*.a}

Department of Pharmaceutics, Faculty of Pharmaceutical Science, Kumamoto University,^a 5-1 Oe-honmachi, Kumamoto 862, Japan and Department of Applied Chemistry, Faculty of Engineering, Kumamoto University,^b 2-39-1 Kurokami, Kumamoto 860, Japan. Received November 1, 1995; accepted December 27, 1995

Two types of fragmented keratin were prepared from buffalo horn and hoof using savinase and Na₂S, and their physicochemical and biopharmaceutical properties were examined in mice. The number-average molecular weight of enzymatically fragmented keratin (E-FK), chemically fragmented keratin (C-FK), and fragmented gelatin (FG) were 8000, 33000, and 6600, respectively. The systematic acute toxicity of FKs was significantly low. Moreover, the immunogenicity of FKs was significantly lower than that of superoxide dismutase. FKs and FG were partially hydrolyzed by trypsin. FKs were digested easily by α -chymotrypsin, but FG underwent less hydrolysis under the same conditions. FKs were bound to plasma proteins, including albumin, and also to some proteins in liver and kidney homogenates. In plasma, E-FK was hydrolyzed slowly, but in liver and kidney homogenates it showed slightly faster hydrolysis. In contrast, FG was not hydrolyzed in any of the media used here. After intravenous administration of FKs and FG to mice, these molecules were rapidly eliminated from the plasma. E-FK and C-FK were taken up into the kidneys ($CL_{\text{uptake, kidney}}$: 10400, 11600 $\mu\text{l/h/g}$), and then gradually excreted in urine. FG was excreted rapidly into urine (CL_{urine} : 6360 $\mu\text{l/h}$). Interestingly, C-FK was also taken up into the liver (CL_{liver} : 4820 $\mu\text{l/h}$). These results indicated that fragmented keratins are biodegradable materials and might be used as new types of liver- and kidney-specific targeting carriers.

Key words enzymatically fragmented keratin; chemically fragmented keratin; fragmented gelatin; biodegradation; drug carrier; tissue distribution

Due to recent developments in biotechnology, bioactive peptides such as cytokines and hormones have been used as clinical therapeutic agents. However, these molecules have some disadvantages such as: 1. very short biological half-life, 2. antigenicity and immunotoxicity, and 3. physicochemical instability. To minimize these disadvantages, many trials have been performed to examine the suitability of chemical modification and/or liposome modification of bioactive peptides using drug carriers. It is very important to select suitable drug carriers for this purpose. Numerous natural and synthetic polymers have been used as drug carriers. For example, polyethylene glycol (PEG),¹⁾ poly styrene-*co*-maleic acid anhydride (SMA),²⁾ divinyl ether-maleic anhydride (Pyran),³⁾ polyvinyl alcohol (PVA),⁴⁾ dextran,⁵⁾ albumin,⁶⁾ galactose,⁷⁾ lecithin,⁸⁾ and fragmented gelatin⁹⁾ have been used as drug carriers. Desirable characteristics of polymers for use as drug carriers are biocompatibility, biodegradability, and no toxicity.

Gelatin, which is the hydrolyzed structural protein collagen constituent of cartilage, tendon and corium, is a good candidate as such a polymer and has already been used as a medicinal material and food additive. It is possible to obtain rapid or slow drug release by using low or high molecular weight gelatin, respectively, as carriers.¹⁰⁾ Recently, we reported the possible utility of fragmented gelatin as a drug carrier for superoxide dismutase (SOD).⁹⁾ Succinyl gelatin-conjugated SOD (Suc-gel-SOD) showed a longer half-life, lower immunogenicity, greater accumulation in the liver, and better therapeutic effect against ischemic foot pad than native SOD. Moreover, a lubricating activity on red blood cells during passage through microcapillaries and a better suppressive effect on the development of collagen-induced

arthritis were observed in Suc-gel-SOD.^{9,11)}

Keratin is another animal structural protein, a common constituent of skin, hair, horn, hoof, and feather. Keratin is an insoluble filamentous or protean structural protein, and it is related to intercellular junctions and the cytoskeleton. However, unlike gelatin, keratin was not available until recently for use in pharmaceutical medicine. This is because keratin contains a large number of cysteine residues and so has many S–S bonds, making its crystals so hard that they can not be fragmented. Keratin is the most abundant of the proteins of the intermediate filament (IF) family; for example, human epithelial cells contain at least 19 types of keratin, while nail and hair contain around 8.¹²⁾ Keratin intermediate filament (KIF) are composed of a pair of polymers of acidic and basic types of keratin subunits. Grosenbaugh *et al.* found the presence of two kinds of components, low and high sulfur fractions, in equine hoof.¹³⁾ The low sulfur fraction contained KIF components (40 to 80 kDa) and reacted with monoclonal antibodies which were specific for the acidic and basic keratin subtype. The other high sulfur fraction contained keratin intermediated filament associated protein (KIFAP, 10 to 30 kDa). Therefore, a fragmented keratin might target specific cells though interaction between KIF and KIFAP. In our previous study,¹⁴⁾ we found that buffalo horn and hoof heated under steam pressure were easily hydrolyzed by proteases. Furthermore, we found a new method for chemical fragmentation using Na₂S.

In this study, we prepared both enzymatically and chemically fragmented keratin from buffalo horn and hoof, waste materials from the processing of livestock which have high protein content (more than 90%). The potential for the use of fragmented keratin as a new drug carrier was evaluated by studying its physicochemical and

* To whom correspondence should be addressed.

biological properties and tissue distribution after intravenous administration into mice.

MATERIALS AND METHODS

Animals Male ddY mice (27–30 g) were obtained from SLC (Shizuoka, Japan).

Materials The buffalo horn and hoof were generous gifts from Nishida Shokai (Nara, Japan). Savinase (from *Bacillus subtilis*) was purchased from Novo-Nordisk A/S (Bagsvaerd, Denmark). Fragmented gelatin (FG) with a mean molecular weight of 6600 was obtained from Nitta Gelatin Co., Ltd. (Osaka, Japan). Sephadex G-50, Sephacryl S-300, S-500, DEAE Sephacel, and CM Sepharose CL-6B were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Guanidine hydrochloride and chloramine T were purchased from Nakalai Tesque (Kyoto, Japan). Trypsin (from porcine pancreas, trypsin activity: 4000–5000 USP units/mg, chymotrypsin activity: less than 25 USP chymotrypsin units/mg) and α -chymotrypsin (from bovine pancreas, chymotrypsin activity: min. 1000 USP chymotrypsin units/mg, trypsin activity: less than 10 USP units/mg) were purchased from Wako Pure Chemicals Co., Ltd. (Osaka, Japan). Na^{125}I , $^{51}\text{CrCl}_3$, aprotinin, and chymotrypsinogen were obtained from ICN Biochemicals, Inc. (Costa Mesa, CA). Cytochrome c, ovalbumin, bovine serum albumin (BSA), BSA dimer, and BSA trimer were purchased from Sigma Chemical Co. (St. Louis, MO). All other materials used in this study were of analytical grade. Deionized distilled water was used throughout the study.

Methods for Preparation of Fragmented Keratin from Buffalo Horn and Hoof The horn and hoof of buffalo were enzymatically and chemically fragmented.

Enzymatically fragmented keratin (E-FK): The horn and hoof of buffalo were fragmented by savinase treatment according to a previous report.¹⁴⁾ The buffalo horn and hoof were heated at 160 °C for 1.5 h under 6 kgf/cm² steam pressure and crushed. Two liters of water and 125 g of substrate were added into jar fermenters (Tokyo Rikakikai Co., Ltd., Tokyo, Japan), which were then heated to 50 °C, followed by the addition of 6.25 g of savinase. The reaction was carried out at pH 8.3 and 50 °C for 1 h. After boiling, the reaction mixture was centrifuged (8000 $\times$ g, 20 min) and the residual substrate was measured after drying at 90 °C for 8 h. The enzymatic digestion yield of buffalo horn and hoof was 67.7%. E-FK was dialyzed against deionized water using dialysis membranes (Spectrapore 6, MW 1000, W. R. Grace & Co., Danvers, MA). Keratin was precipitated by lowering of the ionic strength, removed by centrifugation (10000 $\times$ g, 20 min) and then lyophilized. Over 99% of Na^+ ions in the original solution were removed (atomic absorption spectrometry analyzer, Z-8000, Hitachi, Tokyo, Japan).

Chemically fragmented keratin (C-FK): One liter of water and 50 g of untreated buffalo horn and hoof were put into a jar fermenter, then $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ (12.3 g, final concentration of Na_2S : 4 g/l) was added and allowed to react at 40 °C for 16 h. As a result of H_2S and OH^- liberated by the hydrolysis of Na_2S , the chemical digestion yield of buffalo horn and hoof was 72%. After

centrifugation, C-FK was dialyzed to remove low molecular weight fragmented keratin and Na_2S , and then lyophilized. More than 99% of the Na^+ ions were removed.

Estimation of E-FK, C-FK, and FG Chain Molecular Weight by Gel Filtration in 6M Guanidine Hydrochloride The molecular weights of E-FK, C-FK, and FG were determined by gel filtration using 6M guanidine hydrochloride as an eluent.¹⁵⁾ Sephacryl S-300 and S-500 columns (1.5 $\times$ 100 cm) were equilibrated with 6M guanidine hydrochloride. Ten milligrams of E-FK, C-FK, or FG were dissolved in 1 ml of 1.5M Tris-HCl buffer (pH 8.5) containing 7M guanidine hydrochloride, and then 4.5 mg of dithiothreitol was added to reduce disulfide bonds, followed by reaction for 1 h at 25 °C. Then, 10.6 mg of monoiodoacetic acid was added to introduce carboxymethyl groups onto the SH groups. After reaction for 1 h, the mixture was placed on the column. Eluted fractions (4 ml each) were analyzed at 280 nm. The standard molecular markers were as follows: aprotinin (6500), cytochrome c (13000), chymotrypsinogen (25000), ovalbumin (45000), BSA (66000), BSA dimer (132000), and BSA trimer (198000). Standard proteins were used after treatment by the same procedure. The number-average molecular weight can be described as follows:

$$\overline{M}_n = \frac{\sum_{i=1}^{\infty} N_i M_i}{\sum_{i=1}^{\infty} N_i}$$

where N_i is the number of molecules with M_i .

Analytical Methods Protein concentrations were determined by the method of Lowry *et al.* using BSA fraction V as a standard.¹⁶⁾ The concentrations of free amino groups were determined by measuring free amino groups with trinitrobenzene sulfuric acid (TNBS) using a calibration curve of alanine.¹⁷⁾ The net electric charges of E-FK, C-FK, and FG were measured in 1/15M sodium phosphate buffer (pH 7.4) by a batch method using DEAE Sephacel, an anion exchanger, and CM Sepharose CL-6B, a cation exchanger, as described previously.¹⁸⁾ The surface tensions of E-FK, C-FK, and FG were determined using a Du Noüy surface/interfacial tensionmeter at 25 °C (Shimadzu Co., Ltd., Kyoto, Japan).

Circular dichroism (CD) spectra of 0.1 mg/ml FKs were measured using a JASCO J-720 (Japan Spectroscopic Co., Ltd., Tokyo, Japan) spectropolarimeter in 1 mm path length cells at 25 °C. The relative contents of individual secondary structures in FKs were determined from the CD spectra according to the method of Chen *et al.*¹⁹⁾ Fourier transform infrared spectra (FTIR) of buffalo horn and hoof, heated buffalo horn and hoof, and FKs were obtained by the KBr tablet method using a JEOL JIR-6500 (Japan Electron Optic Laboratory, Tokyo, Japan) FTIR spectrophotometer under dry N_2 . The dried raw material and KBr were mixed to give a 1 : 200 ratio on a weight basis.

The amino acid compositions of buffalo horn and hoof, E-FK, C-FK, and FG were determined using a JEOL JLC-300 (Japan Electron Optic Laboratory, Tokyo, Japan) amino acid analyzer after hydrolysis in 6N HCl at 110 °C for 24 h *in vacuo*. Only Trp was determined after hydrolysis in 2N NaOH at 110 °C for 16 h *in vacuo*.

Systematic Acute Toxicity of FKs Male ddY mice

weighing about 25 g were kept in wire mesh cages, fed with standard pelleted chow and allowed tap water *ad libitum*. The animal room temperature was maintained at $24 \pm 2^\circ\text{C}$, with a relative humidity of $55 \pm 10\%$, on a 12 h light/dark cycle. E-FK or C-FK (30 mg/ml), dissolved in 1/15 M sodium phosphate buffer (pH 7.4), was intravenously administered at doses of 300 mg/kg. The body weight of each mouse was measured every 2 d for 14 d. The control mice received 1/15 M sodium phosphate buffer (pH 7.4) only. After 14 d, mice were sacrificed, and their lung, liver, kidney, and spleen were excised and weighed. Tissues were stained with hematoxylin and eosin and the sliced tissues were observed using a microscope.

Immunological Experiment The immunogenicity of FKs was evaluated by measuring the anti-E-FK, anti-C-FK, and anti-SOD IgG antibody production in rabbits after immunization. Every two weeks, three rabbits for each group received a subcutaneous injection of 0.25 mg of FKs (three times), 0.5 mg of FKs (twice), and 1 mg of FKs (twice) with Freund's complete adjuvant. Serum samples were collected on the 7th day after the last immunization. The passive cutaneous anaphylaxis (PCA) reaction of anti-E-FK and anti-C-FK IgG antibody in the serum was evaluated in guinea pig skin as described previously.²⁰⁾ Six hours after subcutaneous injection of diluted rabbit sera, the degree of the PCA reaction was quantified by measuring the diameter of Evans blue-bound albumin that had entered the skin from the vascular lumen.

Radiolabeling of E-FK, C-FK, and FG FKs were radiolabeled with Na¹²⁵I by the chloramine T method.²¹⁾ Fragmented keratin had the following specific radioactivity after labeling with ¹²⁵I: E-FK, 5.8×10^5 and C-FK, 8.3×10^5 cpm/mg. Fragmented gelatin was labeled with ⁵¹Cr by attaching the chelating agent, DTPA anhydride.²²⁾ FG had a specific radioactivity of 7.5×10^5 cpm/mg after labeling with ⁵¹Cr. The radioactivities were measured with an autowell γ -system (ARC-2000, Aloka, Japan).

Protein Binding of E-FK, C-FK, and FG to Plasma and HSA The protein bindings of E-FK, C-FK, and FG were estimated by ultrafiltration using an Amicon MPS-1 micropartition system with a YMT ultrafiltration membrane (molecular weight cutoff: 30000, W. R. Grace & Co., Danvers, MA). ¹²⁵I-Labeled E-FK, C-FK, or ⁵¹Cr-labeled FG was added to mouse plasma or 4% HSA (1 ml), and the mixtures were centrifuged at 25°C at 3000 rpm for 20 min. The radioactivity in the permeated fractions was then measured.

Stability of E-FK, C-FK, and FG in Buffer and Biological Media The stabilities of E-FK, C-FK, and FG in buffer and enzyme solutions were evaluated from the increases in free amino groups after digestion.²³⁾ Five milligrams of E-FK, C-FK, or FG were dissolved in 1 ml of 1/15 M sodium phosphate buffer (pH 7.4). To this solution, 0.2 ml of freshly prepared 5 mg/ml trypsin or α -chymotrypsin solution was then added. The reaction mixtures were incubated at 37°C . The reaction was stopped after 0, 10, 20, 30, 60 min by heating for 20 min at 90°C and the amounts of free amino groups were determined by the TNBS method.¹⁷⁾

The stabilities of FKs in plasma and tissue homogenates were evaluated using E-FK. Three hundred microliters of

¹²⁵I-labeled E-FK (0.2 mg/ml) were added to 500 μl of 1/15 M sodium phosphate buffer (pH 7.4), mouse plasma, liver, or kidney homogenate (10 mg protein/ml), and the reaction was carried out at 37°C for 1 h. The reaction mixture was applied to a Sephadex G-50 column (1 $\times$ 30 cm) and eluted on 1/15 M sodium phosphate buffer (pH 7.4). Fractions were collected and radioactivities were measured.

Tissue Distributions of E-FK, C-FK and FG, after Intravenous Administration to Mice ¹²⁵I-Labeled E-FK, C-FK, or ⁵¹Cr-labeled FG was injected *via* the tail vein into ddY mice at a dose of 2 mg/kg. The mice were sacrificed by taking blood from the jugular vein into heparinized tubes at appropriate times after i.v. injection. The various tissues were excised and weighed, and then radioactivities were counted.

Kinetic Analysis The plasma concentrations of ¹²⁵I-labeled E-FK, C-FK, or ⁵¹Cr-labeled FG, as a function of time, were analyzed using a nonlinear least squares program, MULTI.²⁴⁾ The tissue distribution data were evaluated according to tissue uptake clearance.²⁵⁾ When the tissue distribution within a short time period after intravenous administration is considered, its efflux processes may be negligible. As based on plasma concentration, the changes in the amount of radioactivity in a tissue with time can be described as follows:

$$dX(t)/dt = CL_{\text{uptake}} \cdot C(t) \quad (1)$$

where $C(t)$ (% of dose/ml) is the plasma concentration and $X(t)$ (% of dose/g) is the amount in 1 g of the tissue at time t , CL_{uptake} (ml/h/g) is the tissue uptake clearance from the plasma to the tissue. Equation 1 integrates to

$$X(t_i) = CL_{\text{uptake}} \cdot \int_0^{t_i} C(t) dt = CL_{\text{uptake}} \cdot AUC_{0-t_i} \quad (2)$$

where AUC_{0-t_i} is the area under the plasma concentration curve from time 0 to t_i . Equation 2 divided by $C(t_i)$ gives

$$K_p(t_i) = X(t_i)/C(t_i) = CL_{\text{uptake}} \cdot AUC_{0-t_i}/C(t_i) \quad (3)$$

According to Eq. 3, the CL_{uptake} value can be estimated from the initial slope of $K_p(t)$ vs. $AUC_{0-t}/C(t)$ at any specific time t . Then, the organ clearance (CL_{org}) is expressed as

$$CL_{\text{org}} = CL_{\text{uptake}} \cdot W \quad (4)$$

where W (g) is the total weight of the organ. Urinary clearance (CL_{urine}) was calculated by Eq. 2 using the accumulated amount excreted in urine. The total body clearance (CL_{total}) is expressed as

$$CL_{\text{total}} = \text{Dose}/AUC_{0-\infty}$$

where $AUC_{0-\infty}$ is calculated by the trapezoidal rule and extrapolation to infinite time.²⁴⁾

Statistical Analysis The results are expressed as mean with standard deviation. Statistical evaluation of the data was carried out using the unpaired Student's t -test.

RESULTS AND DISCUSSION

Amino Acid Analysis of Buffalo Horn and Hoof, E-FK, C-FK, and FG Table 1 summarizes the amino acid com-

Table 1. Amino Acid Compositions of Buffalo Horn and Hoof, E-FK, C-FK, and FG

% of amino residues									
	Buffalo horn and hoof	E-FK	C-FK	FG		Buffalo horn and hoof	E-FK	C-FK	FG
Asp	6.7	7.5	8.2	6.4	Ile	3.0	3.5	2.9	1.8
Thr	5.6	6.0	5.1	3.0	Leu	8.2	9.0	9.6	4.0
Ser	10.8	9.5	9.6	3.8	Tyr	5.9	4.2	4.3	0.4
Glu	12.6	14.8	15.0	10.1	Phe	2.9	2.3	2.5	2.6
Gly	12.3	12.1	12.6	27.6	His	0.6	0.5	0.9	0.8
Ala	6.3	6.5	7.4	9.9	Lys	2.7	3.0	3.0	5.7
Val	4.1	4.9	4.4	3.3	Trp	0.4	0.2	0.4	0.0
Cys	3.7	0.4	0.8	0.2	Arg	6.8	7.5	6.4	10.6
Met	0.6	0.4	0.8	1.0	Pro	6.8	7.8	6.1	27.5

The amino acid compositions were determined after hydrolysis in 6N HCl at 110°C for 24 h *in vacuo*, except that of Trp, which was determined by hydrolysis in 2N NaOH at 110°C for 16 h *in vacuo*.

positions of buffalo horn and hoof, C-FK, E-FK, and FG. The buffalo horn and hoof contained 3.7% cysteine and 12.3% glycine, more similar to the amino acid composition of soft keratin (stratum) than hard keratin (hair, nail).²⁶⁾ Since the FKs were obtained from the supernatant fractions, their cysteine contents were markedly reduced. The other amino acids showed slight changes in relative amounts after fragmentation. No differences in amino acid composition were observed between E-FK and C-FK. Compared to the extracted keratin from equine hoof reported by Grosenbaugh *et al.*, the amino acid compositions of C-FK and E-FK were very similar to the low sulfur fraction containing KIF components which were interfibrillar in the cells.¹³⁾ FKs contained more than 7% aromatic amino acid residues. On the other hand, FG contained a high proportion of glycine and proline (27.6 and 27.5%) residues because collagen, which is the source of gelatin, has a glycine every three amino residues and contains a high level of proline (more than half of which is hydroxylated).

Physicochemical Properties of E-FK, C-FK, and FG

Table 2 summarizes the physicochemical properties of E-FK, C-FK, and FG. The molecular weights of FKs and FG were estimated by gel filtration. To minimize the differences in effective molecular radius of each protein, the proteins were denatured to random coils with 6M guanidine hydrochloride. The S-S bonds of the proteins were reduced with dithiothreitol, and rebinding was prevented by the introduction of an acetyl group into SH groups.

The molecular range of E-FK was 30000–1000; maximum molecular weight was 6000, and number-average molecular weight was 8000. The molecular range of C-FK was 100000–1000; maximum molecular weight was 10000, and number-average molecular weight was 33000. The molecular range of FG was 30000–1000; maximum molecular weight was 3000, and number-average molecular weight was 6600. E-FK showed a similar molecular weight to FG.

To determine the net electric charge under physiological conditions, the binding of FKs and FG to the ion exchange region in 1/15M sodium phosphate buffer (pH 7.4) was measured. As shown in Table 2, FKs were adsorbed onto

Table 2. Physicochemical Properties of E-FK, C-FK, and FG

	E-FK	C-FK	FG
Molecular distribution ^{a)}	1000–30000	1000–100000	1000–30000
Maximum molecular weight ^{a)}	6000	10000	3000
Number-average molecular weight ^{a)}	8000	33000	6600
Absorption at ion exchange region (%)			
DEAE Sephacel	30.4	53.1	20.3
CM Sepharose CL-6B	0	0	0
Relative number of free amino groups after reaction			
90°C for 1 h	1.0	1.0	1.0
1N HCl for 1 h	1.0	1.0	1.0
0.1M Na ₂ CO ₃ (pH 11.5) for 1 h	1.1	1.1	1.0
1N NaOH for 1 h	1.5	1.5	1.2
Solubility (% at 25°C)			
In water	1	0.1	>30
In pH 7.4 buffer	6	4	>30
Surface tension ^{b)} (mN/m)	40.3	45.1	48.7
pH ^{c)}	6.5	6.3	6.1

a) Molecular weights were determined by gel chromatography in 6M guanidine hydrochloride. b) 4% solution in 1/15M sodium phosphate buffer (pH 7.4) at 25°C. c) 0.1% solution at 25°C.

DEAE Sephacel, an anion exchanger, but not onto CM Sepharose CL-6B, a cation exchanger, indicating that FKs had a negative charge under physiological conditions. FG had a weaker negative charge than FKs under physiological conditions. In addition, the solubilities of FKs were low in water (4% of C-FK and 6% of E-FK) were dissolved in 1/15M sodium phosphate buffer, pH 7.4, due to their negative charge. The solubility of FG was more than 30%.

The chemical stabilities of FKs and FG were evaluated under various conditions. As shown in Table 2, both FKs and FG were stable against heat and exposure to acidic conditions, but they were hydrolyzed under alkaline conditions. The surface tensions of all solutions were low, indicating that FKs and FG were surface-active substrates, like other proteins.

To obtain information on the secondary structure composition of buffalo horn and hoof and FKs, CD and FTIR spectra were measured. The FTIR spectra of buffalo horn and hoof showed five peaks, at 1683, 1652, 1558, 1540, and 1521 cm⁻¹. From the analysis of FTIR spectra according to Miyazawa *et al.*,²⁷⁾ the five peaks in the

series, from beginning with the high wave number, were attributed to amide I for antiparallel β -sheet, amide I for random coil, amide II for α -helix, amide II for random coil, and amide II for parallel β -sheet, respectively. In contrast, in heated buffalo horn and hoof, 1683, 1558, and 1521 cm^{-1} peaks were decreased, indicating that the α -helix and β -sheet structures were specifically destroyed and changed to a random coil conformation by heating. The structural changes in buffalo horn and hoof following heating facilitated subsequent enzymatic fragmentation. The FTIR spectra of FKs showed only two peaks, at 1652 and 1540 cm^{-1} , which were attributed to a random coil structure, the same as that seen in heated buffalo horn and hoof. From the results of analysis of CD spectra according to Chen *et al.*,¹⁹⁾ the α -helix and β -sheet contents of E-FK were 3.7 and 7.1%, respectively, the rest being composed of random coil and β -turn. C-FK contained 12.0% α -helix and 9.8% β -sheet. It was demonstrated that the secondary structure composition of FKs were almost entirely random coil from the results of FTIR and CD spectra.

Systematic Acute Toxicity of FKs Body weight change following 14 d in the groups administered 300 mg/kg of FKs remained the same as those of the control group. Fourteen days after intravenous administration, the pathological diagnosis of lung, liver, kidney, and spleen and the tissue weight of the groups administered FKs were not significantly different from the control group. These results indicated that the toxicity of FKs was significantly low. Moreover, FKs were administered at doses of 300 mg/kg to mice and on 14th, 21th, 28th, and 35th d, 100 mg/kg of FKs were repeatedly administered. Body weight change following 42 d of the groups administered FKs remained the same as those of the control group, and all mice were alive.

Immunological Properties of FKs Antigenicity and immunogenicity are important parameters for drug carriers. As shown in Table 3, five diluted anti-E-FK rabbit sera did not induce any PCA reaction, suggesting that E-FK was not recognized by the immune system at all. Besides, five diluted anti-C-FK rabbit sera induced a PCA reaction, but the intensity of that induced by anti-SOD rabbit sera was much higher than that by anti-C-FK (perhaps due to low IgG production). These results show that the immunogenicity of FKs is significantly low.

Stabilities of E-FK, C-FK, and FG to Trypsin and α -Chymotrypsin The enzymatic stabilities of E-FK, C-FK, and FG were evaluated using trypsin and α -chymotrypsin. Figure 1 shows the temporal profiles of an increasing relative number of free amino groups after enzymatic digestion. As shown in Fig. 1, all of the substrates were hydrolyzed by trypsin and α -chymotrypsin. Trypsin is a serine protease which catalyzes cleavage at arginine and lysine residues in the peptide amide bond from the carboxyl side.²⁸⁾ The arginine and lysine residue contents of FKs were 7 and 3%, and those of FG were 10.6 and 5.7%, respectively. Therefore, the susceptibilities of FKs and FG to hydrolysis by trypsin were almost the same. On the other hand, α -chymotrypsin is a serine protease which mainly catalyzes hydrolysis at aromatic amino acid or leucine residues in the peptide amide bond

Table 3. PCA Reaction of Immunized Rabbit Sera in Guinea Pig Skin

Antisera	Dilution	Degree of PCA reaction and positive percentage ^{a)}				
		—	+	++	+++	++++
Anti-E-FK	$\times 100$	100	0	0	0	0
	$\times 25$	100	0	0	0	0
	$\times 5$	100	0	0	0	0
Anti-C-FK	$\times 100$	100	0	0	0	0
	$\times 25$	50	33	17	0	0
	$\times 5$	0	17	17	50	16
Anti-native SOD	$\times 100$	0	0	0	8	92

The immunized rabbit (see text for details) sera were injected into guinea pigs subcutaneously. a) Diameter of vascular permeability shown by Evans blue dye in the skin: —, $<5\text{ mm}$; +, $5\text{--}10\text{ mm}$; ++, $10\text{--}15\text{ mm}$; +++, $15\text{--}20\text{ mm}$; +++, $>20\text{ mm}$. Percentages indicate the results for a group of three rabbit sera.

Fig. 1. Effects of Trypsin and α -Chymotrypsin on the Hydrolysis of E-FK, C-FK, and FG

—○—, E-FK; —△—, C-FK; —●—, FG.

from the carboxyl side.²⁹⁾ Since FKs have a high aromatic amino residue content (about 7%) compared with FG (about 3%), FKs were hydrolyzed more extensively than FG by this enzyme. Therefore, FKs might be more easily hydrolyzed by several proteases in the body compared with FG.

Stabilities of E-FK in Plasma and Tissue Homogenates

To estimate the *in vivo* fragmentation of FKs, the molecular weight of ^{125}I -labeled E-FK was evaluated after incubation in plasma, liver, and kidney homogenates for 1 h at 37°C . Figure 2 shows the elution profiles of ^{125}I -labeled E-FK on Sephadex G-50 after incubation. As shown in Fig. 2, the elution profile of E-FK in buffer showed only one peak with high radioactivity in fractions 20—25. On the other hand, in all biological media, the new peaks corresponding to blue dextran fractions appeared at 10—15 fractions, and the 20—25 fraction radioactivities were decreased. The radioactivities of the 10—15 fractions may have been due to the fraction of E-FK bound with some proteins in biological fluids, including plasma, liver and kidney homogenate. The bound fractions in plasma and liver homogenate were greater than that in kidney homogenate. Furthermore, radioactivities in small molecular weight fractions (25—35) were increased in liver and kidney homogenates,

suggesting that E-FK was fragmented in these media. Conversely, the small molecular weight fractions of E-FK showed no increases in plasma. It was demonstrated that E-FK was bound to biological components in plasma and tissue and then fragmented in the liver and kidney. On the other hand, FG was not bound to any biological component and was not fragmented in any of the biological media examined under the same conditions (data not shown).

To demonstrate the possibility that FKs bind to some proteins in a biological component, the binding of E-FK, C-FK, and FG to plasma and HSA were estimated *in vitro*. As shown in Table 4, three kinds of peptides, especially C-FK, were bound to a plasma component. Moreover,

Fig. 2. Gel Chromatogram on Sephadex G-50 of ^{125}I -Labeled E-FK after Incubation in Buffer and Biological Media for 1 h at 37°C

—○—, buffer; —●—, plasma; —▲—, liver homogenate (10 mg protein/ml); —■—, kidney homogenate (10 mg protein/ml).

Table 4. *In Vitro* Binding Percentage of ^{125}I -Labeled E-FK, C-FK and ^{51}Cr -Labeled FG to Mouse Plasma and HSA

	Binding fraction (%)		
	E-FK	C-FK	FG
Plasma	72.9 ± 2.8	92.2 ± 4.5	71.9 ± 2.6
4% HSA	59.9 ± 6.5	72.5 ± 2.2	44.1 ± 1.6

Each value represents the mean $\pm$ S.D. of three experiments.

Fig. 3. Temporal Profiles of ^{125}I -Labeled E-FK, C-FK, and ^{51}Cr -Labeled FG in Plasma and Urine after Intravenous Administration to Mice

—○—, E-FK; —△—, C-FK; —●—, FG. Data represent mean $\pm$ S.D. Test compounds were administered at dose of 2 mg/kg.

FKs were bound to plasma and HSA more strongly than FG. It was clear that 80% of the binding of FKs in plasma depended on binding with albumin. Therefore, the binding component of FKs in liver and kidney must be protein.

Keratin is known to play an important role as a KIF in the intracellular junctions and cytoskeleton of cells like vimentin and desmin¹²⁾ which connect the nuclear surface to the cytoplasm, terminating at the plasma membrane.³⁰⁾ KIF is known to have high affinity to a specific protein, named KIFAP.¹²⁾ KIFAP are expressed in various cells such as paranemin,³¹⁾ synemin,³²⁾ trichohyalin,³³⁾ plectin,³⁴⁾ spectrin,³⁵⁾ ankyrin,³⁶⁾ desmoplakin,³⁷⁾ and lamin B.³⁸⁾ As the amino acid compositions of FKs are similar to the KIF component of equine hoof, FKs might interact with cellular KIFAPs. As shown in Fig. 2, the higher affinity of E-FK to liver than kidney might reflect the differences in KIFAP protein content between liver and kidney.

Biodisposition of E-FK, C-FK, and FG after Intravenous Administration to Mice Figure 3 shows the plasma and urine concentration profiles of ^{125}I -labeled C-FK, E-FK, and ^{51}Cr -labeled FG with time, and Fig. 4 shows the K_p values of various organs at 30 min after intravenous administration (dose 2 mg/kg) to mice. The $T_{1/2\alpha}$ and $T_{1/2\beta}$ of FG was small, suggesting that FG was eliminated more rapidly from plasma than FKs. As shown in Table 4, FKs were strongly bound to plasma protein, so these were eliminated from plasma more slowly. The excretion rate of FG in urine was higher than those of FKs. The molecular weights of C-FK and FG excreted in urine compared to their respective initial molecular weights (data not shown), suggesting that the excreted C-FK was more fragmented than that of the initial molecular weight, but FG was not changed. In fact, the enzyme sensitivities of FKs and FG were different; C-FK was more easily fragmented than FG in the body, as shown in Fig. 1. In general, the biodistributions of proteins are provided mainly by their molecular weight. Some proteins with molecular weights smaller than 50000 are rapidly excreted in urine. As shown in Fig. 4, the K_p values of FG in various organs were less than 1 except in the kidney, suggesting a low affinity for the tissues and rapid excretion in urine. On the other hand, the K_p values of E-FK were large in

the kidney. In addition, the K_p values of C-FK were large in the liver, kidney, and spleen (Fig. 4). The large K_p value in the spleen implies increasing reticuloendothelial system (RES) uptake with increasing molecular weight. E-FK and C-FK were accumulated in a low amount in the gastrointestinal tract; similar results were obtained from PVA and PEG.³⁹⁾

Figure 5 shows the liver and kidney accumulation time profiles of ^{125}I -labeled FKs and ^{51}Cr -labeled FG after i.v. injection into mice, and Table 5 summarizes the pharmacokinetic parameters of E-FK, C-FK, and FG. As shown

Fig. 4. K_p Values of ^{125}I -Labeled E-FK, C-FK, and ^{51}Cr -Labeled FG at 30 min after Intravenous Administration to Mice

■, E-FK; □, C-FK; ▨, FG. Data represent mean $\pm$ S.D. Test compounds were administered at dose of 2 mg/kg.

Fig. 5. Temporal Profiles of ^{125}I -Labeled E-FK, C-FK, and ^{51}Cr -Labeled FG in Liver and Kidney after Intravenous Administration to Mice
—○—, E-FK; —△—, C-FK; —●—, FG. Data represent mean $\pm$ S.D. * $p < 0.05$ vs. FG. Test compounds were administered at dose of 2 mg/kg.

Table 5. Pharmacokinetic Parameters of ^{125}I -Labeled E-FK, C-FK, and ^{51}Cr -Labeled FG after Intravenous Administration to Mice

Material	$AUC_{0-\infty}$ (% of dose h/ml)	$T_{1/2\alpha}$ (min)	$T_{1/2\beta}$ (min)	Clearance ($\mu\text{l/h}$)			Tissue uptake index ($\mu\text{l/h/g}$)	
				CL_{total}	CL_{urine}	CL_{liver}	Liver	Kidney
E-FK	9.7	2.4	51.0	10300	5950	1080	649	10400
C-FK	12.3	0.7	56.3	8120	1900	4820	3190	11600
FG	6.8	1.6	19.6	14700	6360	260	172	4210

Test compounds were administered at dose of 2 mg/kg. See text for details. Results are calculated as means, $n = 3-4$.

in Fig. 4, C-FK showed a much higher accumulation in liver than FG and E-FK. Moreover, FKs showed higher accumulation in the kidney than FG. From Table 5, for C-FK, CL_{liver} was large, suggesting that C-FK was predominantly taken up by the liver. As shown in Fig. 2, E-FK interacted with some proteins in liver and kidney homogenates and C-FK had a higher affinity to plasma protein than E-FK (Table 4), suggesting that C-FK had a high affinity to some proteins in the liver and kidney. Blouin *et al.*, showed that keratin was expressed on liver epithelial cells using anti-keratin antibody.⁴⁰⁾ It is considered that a KIF-KIFAP network was expressed on liver cells. Moreover, the liver possesses a sinusoidal vascular structure, with large interendothelial cell junctions (fenestrations up to 150 nm) and the complete absence of a basement membrane.⁴¹⁾ Therefore, the access of macromolecules to the surface of hepatocytes and Kupffer cells is particularly easy, suggesting that FKs could access KIFAPs easily on liver cells and accumulate to the liver. Moreover, as for the RES uptake with increasing molecular weight, C-FK was accumulated in the liver and spleen but more studies are needed to confirm this. With an increase in dose to 10 mg/kg, the pharmacokinetic parameters of C-FK were not changed (data not shown), suggesting that the tissue uptake processes were linear, at least at doses between 2 and 10 mg/kg.

Recently, we reported that Suc-gel-SOD showed a longer half-life, lower immunogenicity, greater accumulation in the liver, and a better therapeutic effect against ischemic foot pad than native SOD.⁹⁾ Suc-gel-SOD was accumulated in liver at 24.6%/g at 1 h after intravenous administration, suggesting that gelatin might be a useful liver-specific targeting carrier for SOD. In the above-

mentioned work the gelatin was accumulated in the liver at 3.8%/g at 1 h after i.v. injection. The fragmented keratin used in this report (especially C-FK) had high affinity to liver, so FKs might be more effective liver targeting carriers than fragmented gelatin.

In conclusion, fragmented keratins are new biodegradable polymers which might be useful as liver- and kidney-specific carriers.

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REFERENCES

- 1) Katre N. V., *Adv. Drug Deliv. Rev.*, **10**, 91—114 (1993).
- 2) Ogino T., Inoue M., Ando Y., Awai M., Maeda H., *Int. J. Pept. Protein Res.*, **32**, 153—159 (1988).
- 3) Oda T., Akaike T., Hamamoto T., Suzuki F., Hirano T., Maeda H., *Science*, **244**, 974—976 (1989).
- 4) Kojima Y., Maeda H., *J. Bioact. Compat. Polym.*, **8**, 115—131 (1993).
- 5) Munechika K., Sogame Y., Kishi N., Kawabata Y., Ueda Y., Yamanouchi K., Yokoyama K., *Biol. Pharm. Bull.*, **17**, 1193—1198 (1994).
- 6) Wong K., Cleland L. G., Pozmansky M. J., *Agent Action*, **10**, 231—239 (1980).
- 7) Fujita T., Nishikawa M., Tamaki C., Takakura Y., Hashida M., Sezaki H., *J. Pharmacol. Exp. Ther.*, **263**, 971—978 (1992).
- 8) Igarashi R., Hoshino J., Takenaga M., Kawai S., Morizawa Y., Yasuda A., Otani M., Mizushima Y., *J. Pharmacol. Exp. Ther.*, **262**, 1214—1219 (1992).
- 9) Kojima Y., Haruta A., Imai T., Otagiri M., Maeda H., *Bioconjugate Chem.*, **4**, 490—498 (1993).
- 10) Imai T., Kimura S., Iijima T., Miyoshi T., Ueno M., Otagiri M., *J. Pharm. Pharmacol.*, **42**, 615—619 (1990).
- 11) Kakimoto K., Kojima Y., Ishii K., Onoue K., Maeda H., *Clin. Exp. Immunol.*, **94**, 241—246 (1993).
- 12) Steinert P. M., Roop D. R., *Ann. Rev. Biochem.*, **57**, 593—625 (1988).
- 13) Grosenbaugh D. A., Hood D. M., *Am. J. Vet. Res.*, **53**, 1859—1863 (1992).
- 14) Kida K., Morimura S., Noda J., Nishida Y., Imai T., Otagiri M., *J. Ferment. Bioeng.*, **80**, 478—484 (1995).
- 15) Wayne W. F., Kenneth G. M., T. C., *J. Biol. Chem.*, **244**, 4989—4994 (1969).
- 16) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
- 17) Fields R., *Methods Enzymol.*, **25**, 464—468 (1972).
- 18) Ross C. F., Matsumoto S., Takakura Y., Hashida M., Sezaki H., *Int. J. Pharm.*, **22**, 75—87 (1984).
- 19) Chen Y. H., Yang J. T., Chau K. H., *Biochemistry*, **13**, 3350—3359 (1974).
- 20) Garvey J. S., Cremer N. E., Sussdorf D. H., "Methods in Immunology," W. A. Benjamin, Inc., Reading, MA., 1977, pp. 455—458.
- 21) Hunter W. M., Greenwood F. C., *Nature*, **194**, 495—496 (1962).
- 22) Hnatowich D. J., Layne W. W., Childs R. L., *Int. J. Appl. Radiat. Isot.*, **33**, 327—332 (1982).
- 23) Bos O. J. M., Fischer M. J. E., Wilting J., Janssen L. H. M., *Biochem. Biophys. Acta.*, **953**, 37—47 (1988).
- 24) Yamaoka K., Tanigawara Y., Nakagawa T., Uno T., *J. Pharmacobio-Dyn.*, **4**, 879—885 (1981).
- 25) Takakura Y., Fujita T., Hashida M., Sezaki H., *Pharm. Res.*, **7**, 339—346 (1990).
- 26) Yu J., Yu D. W., Checkla D. M., Freedberg I. M., Bertolino A. P., *J. Invest. Dermatol.*, **101**, 56S—59S (1993).
- 27) Miyazawa T., Blout E. R., *J. Am. Chem. Soc.*, **83**, 712—719 (1961).
- 28) Smyth D. G., *Methods Enzymol.*, **11**, 216—222 (1967).
- 29) Smyth D. G., *Methods Enzymol.*, **11**, 222—224 (1967).
- 30) Osborn M., Weber K., *Lab. Invest.*, **48**, 372—394 (1983).
- 31) Price M. G., Lazarides E., *J. Cell Biol.*, **97**, 1860—1874 (1983).
- 32) Granger B. L., Lazarides E., *Cell*, **22**, 727—738 (1980).
- 33) Lee S. C., Kim I. G., Marekov L. N., O'Keefe E. J., Parry D. A., Steinert P. M., *J. Biol. Chem.*, **268**, 12164—12176 (1993).
- 34) Herrmann H., Wiche G., *J. Biol. Chem.*, **262**, 1320—1325 (1987).
- 35) Mangeat P. H., Burridge K., *J. Cell Biol.*, **98**, 1363—1377 (1984).
- 36) Georgatos S. D., Marchesi V. T., *J. Cell Biol.*, **100**, 1955—1961 (1985).
- 37) Jones J. C. R., Goldman R. D., *J. Cell Biol.*, **101**, 506—517 (1985).
- 38) Georgatos S. D., Blobel G., *J. Cell Biol.*, **105**, 117—125 (1987).
- 39) Yamaoka T., Tabata Y., Ikada Y., *J. Pharm. Pharmacol.*, **47**, 479—486 (1995).
- 40) Blouin R., Blouin M. J., Royal I., Grenier A., Roop D. R., Loranger A., Marceau N., *Differentiation*, **52**, 45—54 (1992).
- 41) Seymour L. W., *Crit. Rev. Ther. Drug Carrier Syst.*, **9**, 135—187 (1992).