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Collagen from common minke whale (*Balaenoptera acutorostrata*) *unesu*

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ABSTRACT

Collagen was prepared from common minke whale *unesu* and characterised. The yield of collagen was high, about 28.4% on a wet weight basis. By SDS–PAGE and CM-Toyopearl 650 M column chromatography, the collagen was classified as type I collagen. The denaturation temperature of the collagen was 31.5 °C, about 6–7 °C lower than that of porcine collagen. Attenuated total reflectance-FTIR analysis indicated that acid-soluble collagen from common minke whale *unesu* held its triple helical structure well, but the structures of porcine skin collagen and pepsin-solubilized collagen from common minke whale *unesu* were changed slightly, due to the loss of N- and C-terminus domains.

1. Introduction

Collagen is the most abundant protein of animal origin and comprises about 30% of total animal protein. At present, nineteen variants of collagen (namely type I-XIX) have been reported (Bailey, Paul, & Knott, 1988). These are widely distributed in skins, bones, cartilage, tendons, ligaments, blood vessels, teeth, cornea, and all other organs of vertebrates (Senaratne, Park, & Kim, 2006). Particularly, type I collagen is found in all connective tissue, such as skins and bones. For industrial applications as functional foods, biomedical materials, and cosmetics collagen has been extracted from the skins of vertebrate species, such as pig, calf and cow. The outbreak of bovine spongiform encephalopathy (BSE), transmissible spongiform encephalophathy (TSE), and foot-and-mouth disease (FMD) have resulted in anxiety among users of collagen and collagen-derived products from these land animals (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). Moreover, collagen obtained from pig cannot be used as a component of some foods for religious reasons (Sadowska, Kolodziejska, & Niecikowska, 2003).

Recently, alternative collagen sources have become desirable. Until now, as part of a study looking at the effective use of underutilised resources, we have tried to prepare and characterise the collagens from aquatic organisms (Nagai, Araki, & Suzuki, 2002; Nagai, Nagamori, Yamashita, & Suzuki, 2002; Nagai & Suzuki, 2000a; Nagai & Suzuki, 2000b; Nagai, Yamashita, Taniguchi, Kanamori, & Suzuki, 2001; Nagai et al., 1999, 2000, Nagai & Suzuki, 2002). As a result, a great amount of collagen could be obtained from many marine vertebrates and invertebrates, although the physical and chemical properties of these collagens were different from those of land animals.

In Japan, whale fishing has existed since the early twentieth century. Traditionally, Japanese eat the whale meat as an important source of protein. Presently, the meat, skins, and fins of common minke whale (*Balaenoptera acutorostrata*) are on sale as a by-product of investigative whaling. Minke whales have 50–70 ventral grooves, extending from the jaw to the navel. *Unesu* is a Japanese delicacy, a bacon made from these ventral grooves. The objective of this study was to extract and characterise the collagen from common minke whale *unesu*.

2. Materials and methods

2.1. Samples and pretreatment

Common minke whale (*Balaenoptera acutorostrata*) *unesu* was obtained from Marukou Inc. Co. in Shimonoseki City, Yamaguchi Prefecture, Japan. The *unesu* was stored at −85 °C until used. Before use, the *unesu* was cut into small pieces (0.5 × 1.0 cm) by a scalpel. The following procedures were carried out at 4 °C. The pieces of *unesu* were extracted with 10 volumes of chilled 99.5% ethanol to remove fat for 2 days, changing the solvent once a day. The *unesu* was washed with chilled, distilled water for 1 day, changing the solution four times a day, and the solid material was treated with 10 volumes of 0.1 M NaOH with gentle stirring to remove non-collagenous proteins for 2 days, changing the liquid twice a day. Then the *unesu* was washed with chilled, distilled water for 2 days, changing the solution daily, and lyophilised.

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2.2. Extraction of collagen

All preparative procedures were at 4 °C. The lyophilised *unesu* was extracted with 20 volumes of 0.5 M acetic acid with gentle stirring for 3 days. The extract was centrifuged at 50,000*g* for 1 h. The supernatants were pooled and salted out by adding NaCl to a final concentration of 0.8 M to obtain type I collagen effectively. This was followed by precipitation of the collagen by the addition of NaCl at a final concentration of 2.3 M at neutral pH in 0.05 M Tris–HCl (pH 7.5). The resultant precipitate was obtained by centrifugation at 50,000*g* for 1 h, and dissolved in 0.5 M acetic acid, dialysed against 0.1 M acetic acid for 1 day, distilled water for 3 days, changing the solution twice a day, and then lyophilised (acid-soluble collagen). The residue from acetic acid extraction was washed with distilled water, suspended in 0.5 M acetic acid, and was digested with 1.0% (w/w) pepsin (EC 3.4.23.1; 2 × crystallized; 3,085 U/mg protein, Sigma-Aldrich Co., St. Louis, MO) by continuous stirring. The viscous solution was centrifuged at 50,000*g* for 1 h, and the supernatants were pooled and dialysed against 0.02 M Na_2HPO_4 (pH 7.2) for 3 days, with a change of solution twice a day, to inactivate the pepsin. After centrifugation at 50,000*g* for 1 h, the precipitate was dissolved in 0.5 M acetic acid and salted out by adding NaCl at a final concentration of 0.8 M, followed by precipitation of the collagen by addition of a final concentration of 2.3 M NaCl in 0.05 M Tris–HCl (pH 7.5). The resultant precipitate was obtained by centrifugation at 50,000*g* for 1 h, dissolved in 0.5 M acetic acid, dialysed against 0.1 M acetic acid for 1 day, distilled water for 3 days (changing the solution twice a day), and then lyophilised (pepsin-solubilized collagen).

2.3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) analysis

SDS–PAGE was performed as described previously (Nagai et al., 2002). The collagen sample was dissolved in a sample buffer (0.5 M Tris–HCl, pH 6.8, containing 2% SDS and 25% glycerol) with 2-mercaptoethanol. After the electrophoresis (an electrical field of 0.4 V/min was applied to the gel, and increased to 0.75 V/min once the dye fronts reached the separating gel), the gel was stained with 10% acetic acid/25% ethanol containing 2.5% Coomassie brilliant blue R-250 (Fluka Fine Chemical Co. Ltd., Tokyo, Japan) for 30 min and destained with 7.5% acetic acid/5% methanol for 1 day, changing the solution three times a day until the bands were clear. Molecular weight markers [myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa)] were purchased from Sigma-Aldrich Co., and were used as marker proteins.

2.4. Peptide mapping

Collagen samples (0.5 mg) were dissolved in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5% SDS and heated at 100 °C for 5 min to allow effective enzyme digestion. After cooling in ice, denatured collagen samples were digested at 37 °C for 30 min using lysyl endopeptidase (0.24 amidase activity) from *Achromobacter lyticus* M497-1 (EC 3.4.21.50; Wako Pure Chemical Industries Ltd., Osaka, Japan). After addition of SDS to a final concentration of 2%, the digestion was stopped by boiling for 5 min. SDS-PAGE was performed by the method of Laemmli (1970) using 15% gels.

2.5. CM-Toyopearl 650 M column chromatography

Twenty milligrams of collagen sample were dissolved in 5 ml of 20 mM sodium acetate buffer (pH 4.8) containing 6 M urea, at 4 °C for 1 day by continuous stirring. The solution was denatured at 45 °C for 30 min, and was centrifuged at 50,000*g* at 20 °C for 1 h. The supernatants were pooled and applied to a CM-Toyopearl 650 M column (1.0 cm × 6.0 cm), previously equilibrated with the same buffer. The elution was performed by a linear gradient of 0-0.15 M NaCl in the same buffer at a flow rate of 0.8 ml/min. The subunit components were detected by absorbance at 230 nm and the fractions indicated by the numbers were examined by SDS–PAGE using 7.5% gels.

2.6. Determination of denaturation temperature (T_d)

Denaturation temperature was measured by the method of Nagai (2004a, 2004b). A Canon-Fenske type viscometer with an average shear gradient of 400 s^{-1} containing 5 ml of 0.03% collagen in 0.1 M acetic acid was immersed in a water bath at temperatures of 20–50 °C. After incubation at each temperature for 30 min, the viscosity of collagen solution was measured and the fractional change was calculated as follows:

Relative viscosity (η_r) = flow time of sample solution/flow time of 0.1 M acetic acid

$$\text{Specific viscosity}(\eta_{sp}) = (\eta - \eta_0)/\eta_0 = \eta_r - 1$$

assuming the densities of the solutions were the same: η = efflux time of the sample solution and η_0 = efflux time of acetic acid.

The denaturation temperature was determined as the temperature at which 50% reduction of viscosity was obtained. Each point is the mean of triplicate determinations.

2.7. Amino acid analysis

Collagen samples were hydrolysed under reduced pressure in 6 M HCl at 110 °C for 24 h, and the hydrolysates were analysed on a JASCO liquid chromatography system by on-line precolumn derivatisation with *o*-phthalaldehyde. This system consisted of a JASCO PU-2080 Plus intelligent HPLC-pump, a JASCO FP-2020 Plus intelligent fluorescence detector, a JASCO CO-2060 Plus intelligent column thermostat, a JASCO DG-2083-53 3-line degasser, a JASCO LG-2080-02 ternary gradient unit, a JASCO AS-2057 Plus intelligent sampler, and a JASCO CrestPak C18 S (4.6 mm × 150 mm) reversed-phase column (JASCO Co., Tokyo, Japan). The excitation and emission wavelengths were set at 345 mm and 455 nm, respectively. Eluents were filtered through Millipore membrane filters (pore size: 0.45 μm; Millipore Corporation, Billerica, MA).

2.8. Attenuated total reflectance-Fourier transform infra red (ATR-FTIR) spectroscopy

ATR-FTIR spectra were collected at 20 °C and 40% relative humidity, by coupling the ATR accessory (ATR PRO410-S: JASCO Co.) to a JASCO FT/IR-4100 type A system. The IR spectrometer bench was equipped with a globar source, a KBr beam splitter, and a triglycine sulfate detector. The ATR sampling device utilised a diamond internal reflection element embedded into a ZnSe support/focusing element in a single reflection configuration. Spectra were obtained over the range of 4000–650 cm^{-1} at 4 cm^{-1} resolution. The resultant spectra were analysed using the IR Protein Secondary Structure Analysis program (JASCO Co.).

3. Results and discussion

3.1. Extraction of collagen from common minke whale unesu

The *unesu* of common minke whale, was treated using 0.5 M acetic acid, was hardly solubilized. The yield of acid-soluble collagen was very low about 0.9% on a wet weight basis. On the contrary, pepsin-solubilized collagen was readily solubilized from

the residue from the acetic acid extraction, and was then purified by differential salt precipitation. The yield of pepsin-solubilized collagen was very high, about 28.4% on a wet weight basis.

We have tried to prepare collagens from marine organisms and a great amount of collagen can be obtained from: fish skin (Japanese sea bass, 51.4%, chub mackerel, 49.8%, and bullhead shark, 50.1% (Nagai & Suzuki, 2000a); ocellate puffer fish, 44.7% (Nagai et al. 2002), rhizostomous jellyfish mesogloea (35.2%); (Nagai et al., 2000), *C. arakawai* arm (62.9%); (Nagai et al., 2002), paper nautilus outer skin (50.0%); (Nagai & Suzuki, 2002), and cuttlefish outer skin (35.0%); (Nagai et al., 2001), respectively. Other researchers have reported the yields of fish skin collagen as follows: channel catfish acid-soluble (25.8%) and pepsin-soluble collagens (38.4%); (Liu, Li, & Guo, 2007), deep-sea redfish acid-solubilized (47.5%) and pepsin-solubilized collagens (92.2%); (Wang, An, Xin, Zhao, & Hu, 2007), and grass carp pepsin-soluble collagen (46.6%); (Zhang et al., 2007). In comparison with these reports, the yield of collagen from common minke whale *unesu* was slightly low.

3.2. SDS–PAGE patterns of collagen

The pepsin-solubilized collagen from common minke whale *unesu* was examined by SDS–PAGE, using a 7.5% gel. There were two distinct species in the α region (Fig. 1). This collagen existed as trimers consisting of two distinct α chains, α1 and α2. The existence of an α3 chain was not identified under the electrophoretic conditions. A small amount of β chain was obtained in this collagen. In electromobility, the positions of the chains of collagen from common minke whale *unesu* were similar to those of porcine skin. Hence, a major component of collagen extracted from common minke whale *unesu* is type I collagen. Acid-soluble collagen from common minke whale *unesu* showed the same electrophoretic patterns as those from pepsin-solubilized collagen from common minke whale *unesu* under these conditions (data not shown).

3.3. Peptide mapping of collagen

The denatured pepsin-solubilized collagen from common minke whale *unesu* and porcine skin one were examined by SDS–PAGE using 15% gel to directly compare the pattern of peptide fragments with porcine skin collagen. The collagens were hydrolysed by lysyl endopeptidase to some extent, giving peptide fragments of molecular weights ranging from about 80 to 14 kDa (Fig. 2). The electrophoretic pattern of pepsin-solubilized collagen from common minke whale *unesu* was different from that of porcine one, suggesting the peptide fragments of collagen from common minke whale *unesu* were fairly different from those of porcine skin.

3.4. Subunit composition of collagen

The denatured collagen was applied to a CM-Toyopearl 650 M column to determine the subunit composition of pepsin-solubilized collagen from common minke whale *unesu*. The chromatographic fractions were identified by SDS–PAGE using 7.5% gel. Two protein fractions contained an α chain as a major component (Fig. 3): this collagen consisted of two α chains such as α1 (fraction numbers 6–7) and α2 (fraction numbers 15–19) in the order of their elution. Several fractions were analysed by SDS–PAGE. As a result, the collagen from common minke whale *unesu* had a chain composition of $(\alpha 1)_2\alpha 2$ heterotrimer, similar to that of mammalian collagen, such as porcine skin.

3.5. Thermal behaviour of collagen

The heat transformation of collagen is interpreted as disintegration of the collagen triple helical structure into random coils. This is accompanied by a change in physical properties, such as viscosity, sedimentation, diffusion, light scattering, and optical activity (Usha & Ramasami, 2004). Fig. 3 shows the changes of fractional viscosity with increasing temperature in pepsin-solubilized collagen from common minke whale *unesu* and in collagen from porcine skin. T_d of pepsin-solubilized collagen from common minke whale *unesu* was calculated to be 31.5 °C, which was lower by about 6–7 °C than that of porcine skin collagen (T_d = 37.0 °C). This value was fairly high, compared to those from other aquatic organisms reported by Nagai et al. (1999), Nagai et al. (2000, Nagai et al. (2001), Nagai et al. (2002), Nagai et al. (2002), Nagai and Suzuki (2000b), Nagai and Suzuki (2002) From these reports, the stability of collagen appears to be correlated with body temperature and environmental temperature (Fig. 4).

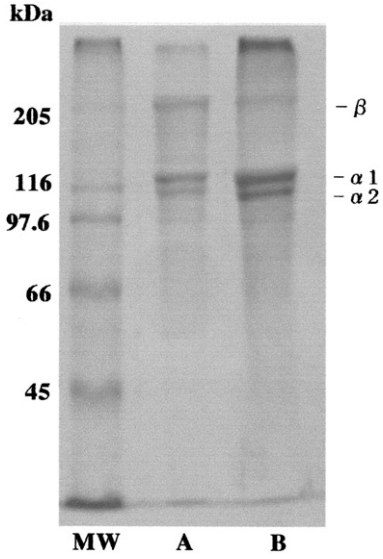

Fig. 1. SDS–polyacrylamide gel electrophoresis of porcine skin type I collagen and common minke whale *unesu* collagen on 7.5% gel containing 3.5 M urea. (MW) high molecular marker: (A) porcine skin collagen; (B) pepsin-solubilized collagen from common minke whale *unesu*.

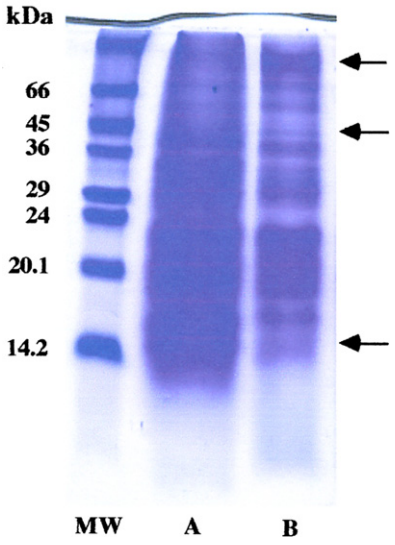

Fig. 2. Peptide mapping of lysyl endopeptidase digests from collagen samples using 15% gel. (MW) low molecular marker; (A) porcine skin collagen; (B) pepsin-solubilized collagen from common minke whale *unesu*.

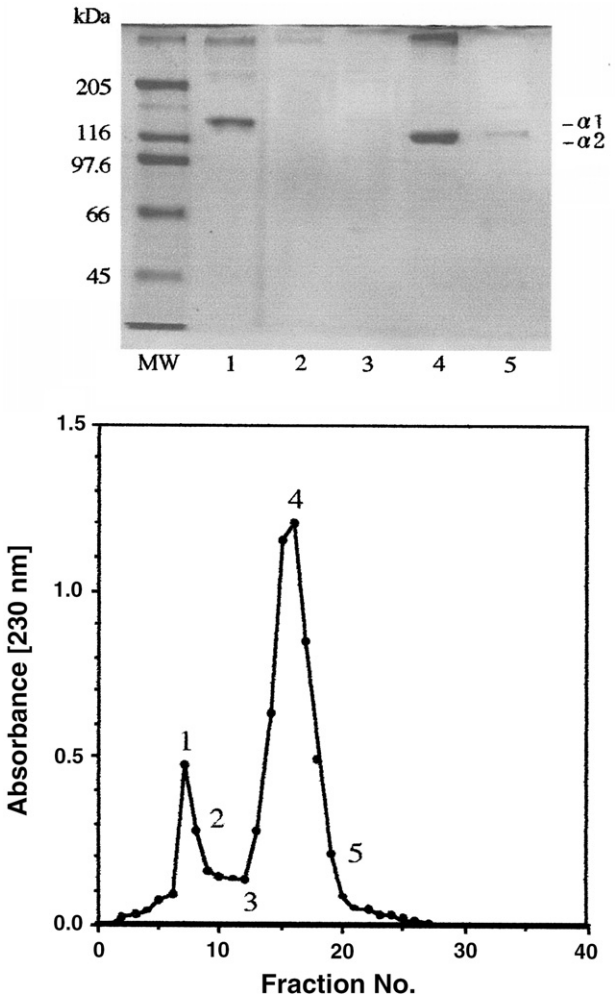

Fig. 3. CM-Toyopearl 650 M column chromatography of denatured pepsin-solubilized collagen from common minke whale *unesu*. A 1.0 × 6.0 column of CM-Toyopearl 650 M was equilibrated with 0.02 M sodium acetate buffer (pH 4.8) containing 6 M urea, and maintained at 37 °C. The collagen sample (20.0 mg) was dissolved in 5 ml of the same buffer, denatured at 45 °C for 30 min, and then eluted from the column with a linear gradient of 0–0.15 M NaCl at a flow rate of 0.8 ml/min. The fractions indicated by the numbers were examined by SDS-PAGE using 7.5% gel.

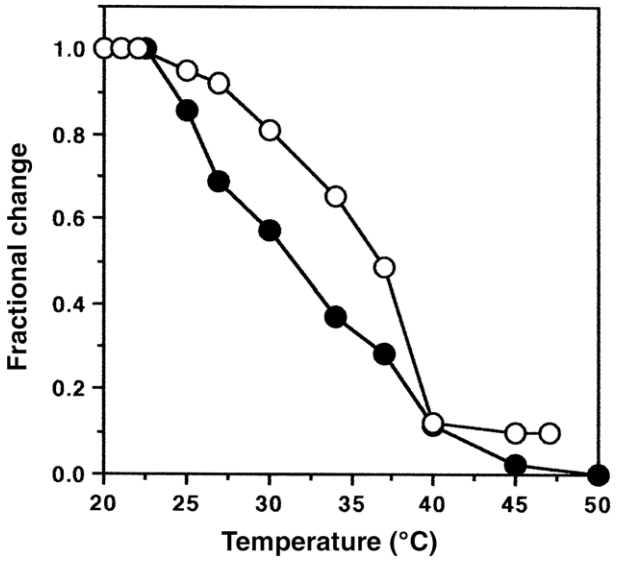

Fig. 4. Thermal denaturation curve of pepsin-solubilized collagen from common minke whale *unesu*. The denaturation temperature was measured by viscosity in 0.1 M acetic acid. The incubation time at each temperature was 30 min. Collagen concentration 0.03%; (○) porcine skin collagen; (●) pepsin-solubilized collagen from common minke whale *unesu*.

Table 1
Amino acid composition of common minke whale *unesu* pepsin-solubilized collagen

Amino acid	Residues/1000
Hydroxyproline	78
Hydroxylysine	7
Aspartic acid	52
Threonine	27
Serine	38
Glutamic acid	78
Proline	121
Glycine	316
Alanine	104
Valine	26
Methionine	7
Isoleucine	12
Leucine	30
Tyrosine	6
Phenylalanine	16
Lysine	26
Histidine	6
Arginine	50
Total	1000

3.6. Amino acid composition of collagen

The amino acid composition of collagen from common minke whale *unesu*, expressed as residues per 1000 total residues, is shown in Table 1. Glycine (316 residues) was the most abundant amino acid in collagen from common minke whale *unesu*, and there were relatively high contents of proline, alanine, glutamic acid, and hydroxyproline, while isoleucine, hydroxylysine, methionine, tyrosine, and histidine were very low. Tryptophan and cysteine were not detected. It is well known that imino acids, such as proline and hydroxyproline exist in collagen. The higher the imino acid content, the more stable are the helices of collagen. The molecular structure of collagen is maintained mainly by restrictions on changes in the secondary structure of the polypeptide chain, imposed by the pyrrolidine rings of proline and hydroxyproline, and also maintained partially by the hydrogen bonding ability of the hydroxy group of hydroxyproline. The total contents of imino acid were 19.9%, similar to those in collagens from fish scale such as sardine, red sea bream, and Japanese sea bass (19.3–19.7%; Nagai, Izumi, & Ishii, 2004), Japanese sea bass caudal fin (19.3%; Nagai, 2004a), and bigeye snapper skin (19.3%; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka 2005), but significantly higher than those of collagens from edible jellyfish exumbrella (12.2%; Nagai et al., 1999), cuttlefish outer skin (18.8%; Nagai et al., 2001), ocellate puffer fish skin (17.0%; Nagai et al., 2002), diamondback squid outer skin (18.6%; Nagai, 2004b), channel catfish skin (17.0%; Liu et al., 2007), and walleye pollock skin (18.4%; Yan et al., 2008).

Hydroxyproline is derived from proline by post-translational hydroxylation, mediated by prolylhydroxylase (Li, Liu, Gao, & Chen, 2004). It is known that the degree of hydroxylation of proline influences the thermal stability of collagen: a higher degree of hydroxylation is associated with a higher denaturation temperature of collagen. The degree of hydroxylation of proline residues in collagen from common minke whale *unesu* was 39.2%. Previous papers have measured the degree of hydroxylation of proline in collagen samples from 32.8% to 51.7% (Kittiphattanabawon et al., 2005; Liu et al., 2007; Nagai, 2004a, 2004b; Nagai & Suzuki 2000b; Nagai

et al., 1999; Nagai et al., 2001; Nagai et al., 2002; Wang et al., 2007; Zhang et al., 2007; Nagai et al., 2002, Yan et al., 2008). It appears that it was the lower imino acid content rather than the extent of hydroxylation that seems to be the reason for the lower denaturation temperature observed for collagens from aquatic organisms.

3.7. ATR-FTIR spectroscopy analysis of collagen

The ATR-FTIR spectrum of collagen from common minke whale *unesu* is shown in Fig. 5. It is a similar spectral pattern to those of other species collagens (Liu et al., 2007; Muyonga, Cole, & Duodu, 2004; Wang et al., 2007; Yan et al., 2008). That is, the amide A band position was found at 3306 cm^{-1} associated with N–H stretching vibration and shows the existence of hydrogen bonds. According to Doyle, Bendit, and Blout (1975), a free N–H stretching vibration occurs in the range 3400 to 3440 cm^{-1}, and when the NH group of a peptide is involved in a hydrogen bond, the position is shifted to lower frequencies, usually around 3300 cm^{-1}. From the result, it indicated that many of the NH groups of this collagen were involved in hydrogen bonding. Amide B band of the collagen was observed at 2928 cm^{-1}; that is related to asymmetrical stretch of CH_2 (Muyonga et al., 2004).

The amide I band with characteristic frequencies in the range from 1600 to 1700 cm^{-1} was mainly associated with the stretching vibrations of the carbonyl group (C=O bond) along the polypeptide backbone (Payne & Veis, 1988), and was a sensitive marker of the peptide secondary structure (Surewicz & Mantsch, 1988). The amide I band of this collagen was found at 1640 cm^{-1}. The amide II and III bands were observed at 1541 cm^{-1} and 1235 cm^{-1}, resulting from N-H bending vibrations and C-H stretching, respectively (Payne & Veis, 1988). It suggests the existence of helical arrangements of collagen from common minke whale *unesu*. Moreover, the resultant spectra were analysed using IR Protein Secondary Structure Analysis Program (JASCO Co.). As a result, the percentage of secondary structural components such as α-helix, β-sheet, β-turn, and others (random coil structure) of sample species could be calculated. For porcine skin collagen the components were, α-helix, 9%; β-sheet, 51%; β-turn, 13%; and others, 15%, for common minke whale *unesu* acid-soluble collagen the components were, α-helix, 15%;, β-sheet, 45%; β-turn, 16%; and others, 18%, and for common minke whale *unesu* pepsin-solubilized collagen: α-helix, 8%; β-sheet, 50%; β-turn, 15%; and others, 17%. It suggests that the triple helical structure well held in acid-soluble collagen from common minke whale *unesu* is different from porcine skin collagen and common minke whale *unesu* pepsin-solubilized collagen. In this way, ATR-FTIR spectroscopy was one of the most versatile techniques for the quick identification and characterisation of common minke whale *unesu* collagen because it gathers information from any type of sample.

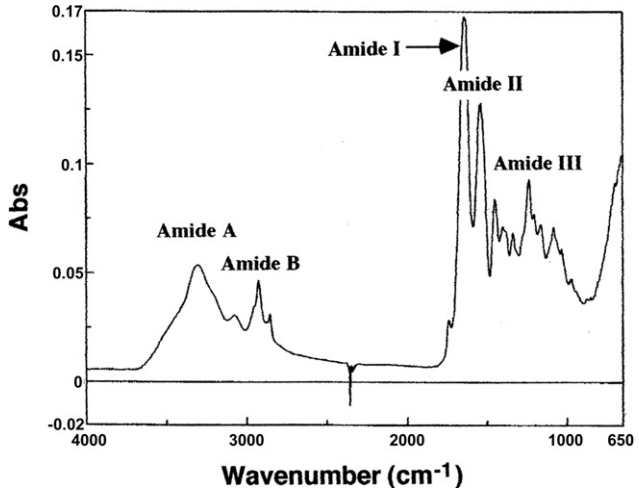

Fig. 5. Fourier transform infrared spectra of pepsin-solubilized collagen from common minke whale *unesu*.

4. Conclusions

A large amount of collagen was obtained from common minke whale *unesu* by pepsin treatment. By SDS–PAGE and CM-Toyopearl 650 M column chromatography, this collagen was classified as type I collagen, with slight differences in terms of thermal stability and molecular structure, in comparison with porcine skin collagen. Unless the problem of BSE infection in land animals is resolved, aquatic materials, as an alternative source of collagen, will attract much attention in the foods, cosmetics, and medical fields in future.

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