Polypeptide/Layered Silicate Nanocomposites Using Fish-Based Collagen Peptide: Effect of Crosslinking and Chain Extension of the Collagen Peptide

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ABSTRACT: Collagen peptide (ColP), which was extracted from fish scale with hot water was mixed with glutaraldehyde (GA) in water in the presence of sodium montmorillonite (MMT), and subsequent heat treatment gave the ColP crosslinked by GA (GA-ColP)/MMT composite films. Also, the ColP was chain extended by the use of a water-soluble carbodiimide and 1-hydroxybenzotriazole to give a chain-extended collagen peptide (CE-ColP). The composite films based on the CE-ColP and MMT were prepared by solution-casting method. The XRD and TEM analyses of their composites revealed that the exfoliated nanocomposites whose silicate layers are finely dispersed are formed for CE-ColP/MMT, and that the degree of exfoliation decreases with the increasing feed amount of GA for GA-ColP/MMT. Although GA-ColP/MMT films were very brittle, CE-ColP/MMT films were relatively tough. Storage moduli of the CE-ColP/MMT films were higher than that of CE-ColP film, and increased with increasing inorganic content. The 10% weight loss temperature of GA-ColP (325°C) was higher than that of CE-ColP (302°C), and the addition of MMT to GA-ColP and CE-ColP resulted in a slight rise of the degradation temperature. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 4024–4030, 2007

Key words: clays; nanocomposites; peptides; collagen; montomorillonite

INTRODUCTION

Polymer–clay nanocomposites have attracted great interests since they often exhibit greatly improved mechanical, thermal, barrier, and frame retardant properties at low clay content in comparison with more conventional microcomposites. Furthermore, recently, the nanocomposites based on biodegradable and biocompatible polymers and layered silicate have been attracting attention because the layered clay is environmentally friendly, naturally abundant and economic, and the improvement of various properties in comparison with the control polymers can enlarge the application fields of the polymers. There have been many literatures on the nanocomposites based on biodegradable polyesters such as poly(L-lactide),[1-5] poly(ε-caprolactone),[6,7] and poly (butylene succinate)[8–10], and the nanocomposites based on biocompatible poly(amino acid)'s such as polylysine[11–15] and poly(glutamic acid).[15] Regarding collagen-based nanocomposites, many literatures have been reported on collagen/hydroxyl apatite nanocomposites.[16–20] However, few literatures are known regarding collagen or a similar polypeptide/layered silicate nanocomposites.[21,22] Fish scale is a natural nanocomposite composed of collagen and hydroxyl apatite. Most of fish scales are discarded and are not effectively used at present. Collagen peptide (ColP) can be obtained from fish scales by the extraction with hot water under a high pressure. Because the molecular weight of the ColP is much lower than that of native collagen because of the hydrolysis, the film obtained from the ColP is very brittle and cannot be applicable to many biodegradable and biocompatible materials. Therefore, the crosslinked ColP (GA-ColP) with gultaraldehyde (GA)[19,20,23] and the chain-extended ColP (CE-ColP) by use of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC-HCl) and 1-hydroxybenzotriazole (HOBt)[24–26] were used in this study. This article describes the morphologies and properties of the bio-based nanocomposites of GA-ColP and CE-ColP with sodium montmorillonite (MMT) prepared by the solution-casting method.

EXPERIMENTAL

Materials

ColP (M_n, 21,300 g/mol, and M_w, 29,100 g/mol, measured by GPC with light scattering), which was extracted from scale of a red bream with hot water

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was supplied by Kanemoto Kaisan (Chiba, Japan). Sodium (Na^+) montmorillonite (MMT, Kunipia F, cation exchange capacity (CEC): 115 meqiv/100 g) was supplied by Kunimine Industries (Tokyo, Japan). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC-HCl) was purchased from Aldrich (Milwaukee, USA). 1-Hydroxybenzotriazole (HOBt) and 50 wt % aqueous solution of glutaraldehyde (GA) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). All the other chemicals used in this work were reagent grade and used without further purification.

Preparation of GA-ColP/MMT composites

A suspension of 0.15 g of MMT in 10 mL of water was added to a solution of 4.85 g of ColP in 85 mL of water. After stirring for 24 h at room temperature, 0.24 g of 50 wt % aqueous solution of GA (1.21 mmol) was added and stirred at 40°C for 10 min. The mixture was poured on a poly(tetrafluoroethylene)-coated plate, dried at 50°C for 27 h, and then heated at 120°C for 2 h and finally at 190°C for 3 h to give a GA-ColP/MMT composite with feed GA content 0.25 mmol/g-ColP and MMT content 3 wt % (abbreviation: GA0.25-ColP/M3). In a similar procedure, GA-ColP/MMT film with feed GA content 0.50 (or 1.00) mmol/g-ColP and MMT content 3 (or 5) wt % (abbreviation: GA0.50 (or 1.00)-ColP/M3 (or 5)) was also prepared.

Preparation of CE-ColP

The reaction scheme for the chain extension of ColP is shown in Figure 1. To optimize the reaction condition, feed amounts of ColP, HOBt, and EDC-HCl were changed. A typical reaction condition was as follows: to a solution of 4.0 g of ColP in 20 mL of water were added 0.507 g (3.75 mmol) of HOBt and 0.383 g (2.00 mmol) of EDC-HCl. After stirring for 48 h at room temperature, the solution was freeze dried for 24 h. The obtained solid was dissolved in DMSO, reprecipitated with ethanol, filtered, and dried *in vacuo* for 24 h at room temperature to give CE-ColP as a white powder in a quantitative yield.

Preparation of CE-ColP/MMT composites

A suspension of 0.15 g of MMT in 10 mL of water was added to a solution of 4.85 g of CE-ColP in 85 mL of water, and stirred for 24 h at room temperature. The mixture was poured on a poly(tetrafluoroethylene)-coated plate, dried at room temperature for 2 days, and further dried at 120°C for 4 h to give a CE-ColP/MMT composite with MMT content 3 wt % (abbreviation:CE-ColP/M3). In a similar procedure, CE-ColP/MMT film with MMT content 5 wt % (CE-ColP/M5) was also prepared.

Measurements

Amino acid analysis of ColP was carried out by the conventional method using a Hitachi L-8800 amino acid analyzer.

Degree of swelling (S_w) of GA-ColP and GA-ColP/M was measured according to the equation, $S_w = 100(W_a - W_b)/W_b$, where W_a is the weight after dipping a sample in phosphate buffered saline (PBS, 0.14*M* NaCl, 0.01*M* Na_2HPO_4, 0.002*M* NaH_2PO_4, pH 7.4) at room temperature for 24 h, and W_b is the weight after drying the swelled sample *in vacuo* at 40°C for 24 h.

Molecular weight of CE-ColP was measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel permeation chromatography (GPC). The CE-ColP were dissolved in the loading buffer containing 0.63*M* Tris–HCl, 2 w/v % SDS, 10 w/v % glycerol, 0.001 w/v % bromophenol blue, and 5 v/v % 2-mercaptoethanol. The CE-ColP solution and a molecular marker solution (Bio-Rad Precision Plus Protein Standards; 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa) were applied to the SDS-PAGE, and the gel was stained with Coomasie Brilliant Blue using a Rapid CBB kit (Kanto Chemical, Tokyo, Japan). The gel was scanned with a Canon 8200F scanner and analyzed on a personal computer using Scion Image software (available at http://www.scioncorp.com/). M_w of CE-ColP was calculated using a calibration curve generated from the protein marker profile data. GPC of CE-ColP was carried out at room temperature on a Shodex GPC analysis apparatus equipped with two SB-806M HQ GPC columns (Showa Denko K. K., Tokyo, Japan) and a DAWN EOS multiangle light scattering detec-

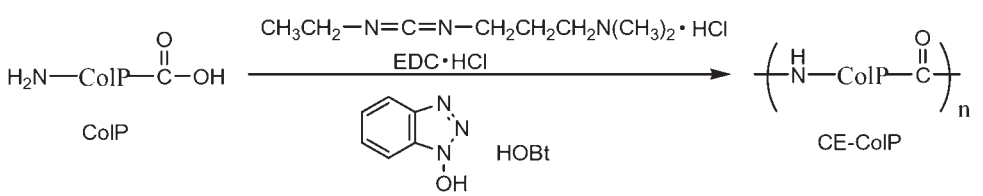

Figure 1 Reaction scheme for the chain extension of ColP.

tor (Wyatt Technology, CA) and a Shodex RI-101 reflective index detector. About 0.2*M* Sodium chloride aqueous solution was used as an eluent at a flow rate of 0.5 mL/min.

Circular dichroism (CD) spectra were measured on a Jasco J-720 spectropolarimeter in the range of 190–250 nm (scanning speed: 20 nm/min) at a temperature of 10 or 25°C. Each sample was measured three times and the data were averaged. The sample concentration of aqueous solution was 0.025 mg/mL.

Transmission electron microscopy (TEM) was performed on an H-500 TEM (Hitachi, Tokyo, Japan) with a 75 kV accelerating voltage. The films were sectioned into roughly 100-nm thin sections at −70°C using an ultramicrotome with a diamond knife and then mounted on 200-mesh copper grids.

X-ray diffraction (XRD) analysis was performed at ambient temperature on a Rigaku RINT-2100 X-ray diffractometer (Tokyo, Japan) at a scanning rate of 2.0°/min, using Cu Kα radiation (wavelength, λ = 0.154 nm) at 40 kV and 14 mA. Sodium montmorillonite was analyzed as powders. The blended materials were prepared in films of 0.40-mm thickness by compression molding.

Dynamic viscoelastic measurements of the rectangular plates (length 15 mm, width 5 mm, thickness 0.5 mm) cut from the composites films were performed on a DMA7 dynamic mechanical analyzer (PerkinElmer, MA) on a three-point bending platform of 5 mm at a frequency of 10 Hz and a heating rate of 2°C/min. Samples were dried *in vacuo* for 24 h prior to the measurement. The 5% weight loss temperature was measured on a TGA7 thermogravimetric analyzer (PerkinElmer) under nitrogen atmosphere at a heating rate of 20°C/min.

RESULTS AND DISCUSSION

Characterization of ColP

The amino acid composition of the ColP extracted from scales of red bream is summarized in Table I. When compared with a typical animal-based collagen where proline (Pro) and hydroxyproline (Hyp) are about 13 and 9 mol %, respectively, the fish-based ColP contains less amount of Hyp (2.3 mol %). This result suggests that the helix-coil transition of fish scale-based collagen occurs at a lower temperature than that of animal collagen. Also, the fish-based collagen contains aspartic acid (Asp), glutamic acid (Glu), and lysine (Lys), suggesting that the side-chain carboxylic acid group and amino group can crosslink by the condensation reaction. The M_w's of ColP measured by SDS-PAGE and light scattering-GPC were 45,000 and 29,100, respectively. Considering that the original collagen has molecular weight of over 100 kDa, it is apparent that the hydrolysis of the fish-based collagen occurred during the extraction with hot water.

TABLE I
Composition of Constituent Amino Acids of ColP

Amino acid	Mol %
Asp	4.71
Thr	2.53
Ser	3.54
Glu	7.61
Gly	35.68
Ala	14.49
Val	2.05
Met	1.18
Ile	0.79
Leu	2.05
Typ	0.36
Phe	1.40
Lys	2.83
His	0.65
Arg	5.67
Hyp	2.30
Pro	12.13

Crosslinking of ColP by GA

The pendant amino groups of Lys residues of collagen can react with the two aldehyde groups of GA to form Schiff bases. It is known that the formed Schiff bases are converted to complex crosslinked compounds containing pyridinium, unsaturated carbonyl, and dihydropyridine moieties via Mannich-type and aldol condensation reactions.[23] It is expected that similar crosslinking reactions also occur in the reaction of ColP and GA. Although we could not characterize the detailed crosslinked structure between ColP and GA, the degree of crosslinking was estimated from the degree of swelling (Sw) in phosphate-buffered saline (PBS) for GA-ColP and GA-ColP/M (Table II). Although ColP was soluble in PBS, all the GA-ColP's were insoluble and swelled in PBS, suggesting the occurrence of crosslinking reaction. The Sw of the GA-ColP's decreased with increasing feed amount of GA, indicating an increase of the degree of crosslinking. Although the obtained GA-ColP film was stiffer than the ColP film, both the films were too brittle to measure mechanical properties.

Chain-extention of ColP

Because ColP and GA-ColP films were very brittle materials, the chain extension of ColP by the condensation reaction without crosslinking was investigated. It is known that the collagen crosslinks by the treatment with a water soluble carbodiimide, EDC-HCl.[18] Recently, it is also reported that collagenlike polypeptide with high molecular weight is synthe-

TABLE II
Degree of Swelling for GA-ColP and GA-ColP/M

Sample	Degree of swelling (%)
GA0.25-ColP	464
GA0.50-ColP	351
GA1.00-ColP	318
GA0.25-ColP/M3	420
GA0.50-ColP/M3	291
GA1.00-ColP/M3	250
GA0.25-ColP/M5	300
GA0.50-ColP/M5	209
GA1.00-ColP/M5	201

sized by the reaction of chemically synthesized oligopeptide of Pro, Hyp, and Gly with EDC-HCl and HOBt.[26] This type of reaction was applied to the fish-based ColP (Fig. 1). Table III shows the SDS-PAGE-determined M_w of CE-ColP obtained under various reaction conditions. Although the M_w generally increases with the increasing amount of EDC-HCl, further addition of EDC-HCl caused crosslinking of the ColP to yield a gel insoluble in any solvent. As a result of many trials, the gelation of product due to crosslinking was suppressed by an increase of HOBt. The effect of HOBt is not clear, but the following factor may contribute: The coordination of basic HOBt with carboxylic groups on the side chain of Asp and Glu residues can depress crosslinking reaction of the carboxylic groups. The attained maximum M_w of CE-ColP determined by SDS-PAGE was 146,000, which was considerably higher than that of ColP (45,000). The M_w's determined by GPC light scattering method for the CE-ColP and ColP were 114,200 and 29,100, respectively. Although there is some difference between the two methods, the tendency of molecular weight was in good agreement.

CD spectrum of CE-ColP in water was measured to confirm the ability to form a triple-helical structure. Figure 2 shows the CD spectra of CE-ColP in water at 10 and 25°C and calf skin collagen in water

TABLE III
SDS-PAGE-Determined M_w of CE-ColP Obtained Under Various Reaction Conditions

Run	ColP (wt %)	HOBt (m*M*)	EDC-HCl (m*M*)	M_w
1	10	9.38	15.6	72,000
2	10	18.75	62.5	77,000
3	10	125.0	15.6	91,000
4	20	125.0	56.3	102,000
5	20	125.0	75.0	(gelation)
6	20	125.0	93.8	(gelation)
7	20	187.5	75.0	125,000
8	20	187.5	100.0	146,000
9	20	187.5	112.5	113,000
10	20	192.5	131.3	(gelation)

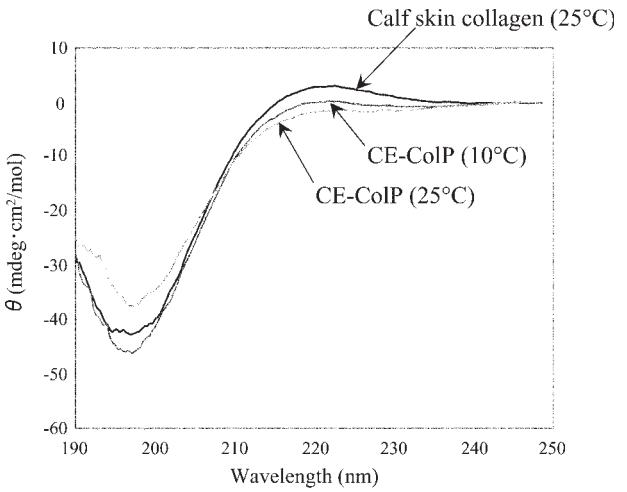

Figure 2 CD spectra of CE-ColP (10 and 25°C), and calf skin collagen (25°C) in water.

at 25°C as a reference sample. The calf skin collagen with triple-helical structure exhibits CD spectrum, which is characterized by a negative peak around 200 nm and a small positive peak around 220 nm.[27] The positive peak around 220 nm of CE-ColP at 10°C is weaker than that of calf skin collagen at 25°C, and stronger than that of CE-ColP at 25°C. This result is closely linked to the fact that a triple-helical structure of fish collagen is unstable at a room temperature. Although CD spectra of ColP in water are not shown in the figure, the spectra were very similar to those of CE-ColP, suggesting ColP and CE-ColP represent a transition between the weak triple-helical structure and the nontriple-helical conformations in water.

Clay dispersion for GA-ColP/M and CE-ColP/M composites

Because the crosslinked GA-ColP was insoluble in water, GA-ColP/M composite films were prepared by mixing ColP and GA in water in the presence of MMT, subsequent heating finally at 190°C. On the other hand, CE-ColP/M composite films were simply prepared by mixing CE-ColP and MMT in water, and subsequent drying. As is shown in Table II, GA-ColP/M composite films showed lower S_w than the corresponding GA-ColP films. If the crosslinking reaction is disturbed by the presence of MMT, the decrease of crosslinking density would result in an increase of S_w. Therefore, it is apparent that the dispersion of MMT in the matrix polymer does not disturb the crosslinking reaction. Also, S_w of GA-ColP/M decreased with increasing MMT content, indicating that the permeation of PBS is disturbed by the MMT.

Figure 3 shows the XRD patters of various ColP-based composites. The XRD peak at $2\theta = 6.62^\circ$ (1.33

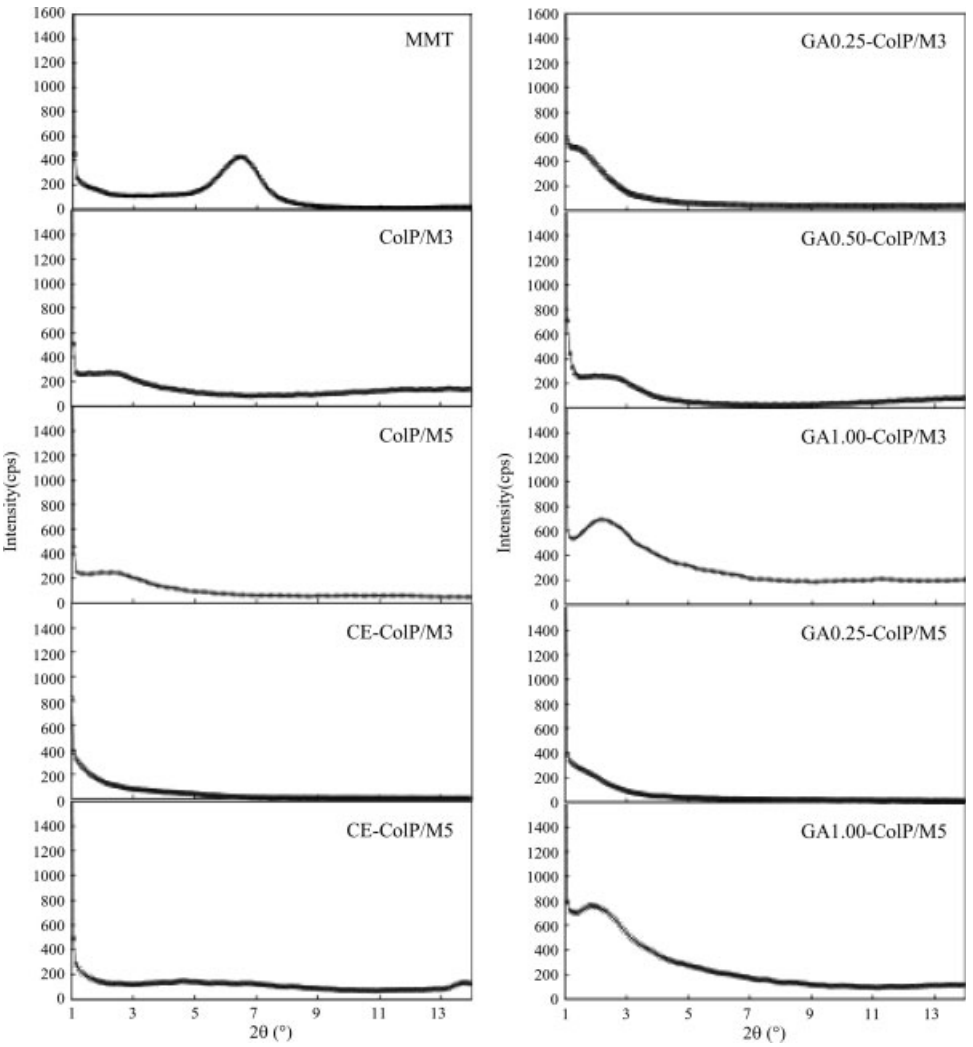

Figure 3 XRD patterns of MMT and ColP-based composites.

nm) observed for MMT itself disappeared and no clear peak was appeared for ColP/M and CE-ColP/M composites, indicating that the formation of exfoliated nanocomposites. Regarding GA-ColP/M composites, the XRD peak corresponding to interlayer spacing (d) of clay platelets gradually became clear with the increase in feed GA amount. The GA1.00-ColP/M composites showed a clear XRD peak around at 2°, indicating the presence of the intercalated clay platelets with d = 4 nm. These results indicate that the degree of exfoliation decreased with the increasing degree of crosslinking. Because the silicate platelets should be exfoliated in the mixture of ColP, MMT, and water before the addition of GA, it is suggested that some aggregation of the clay occurs during the crosslinking reaction and evaporation of water.

The TEM images of the ColP-based composite films with MMT content 3 wt % are shown in Figure 4, where the dark lines represent the silicate layers in the collagen-based matrix (bright). It is apparent that a considerable degree of exfoliation occurs for ColP/M3 and CE-ColP/M3, and that the aggregation of clay platelets increases with the increase in feed amount of GA in agreement with the result of the XRD. The collagen-based peptides are very hydrophilic. The ammonium and guanidyl groups at the peptide side chain based on Lys and Arg units can exchange with the sodium ions in the clay interlayer. These factors contribute to the insertion of the collagen-based peptide chain into the clay platelets. However, the crosslinking of the peptide chain with GA physically disturbs the insertion.

Thermal and mechanical poperties of GA-ColP/M and CE-ColP/M composites

Figure 5 shows the dynamic viscoelastic curves of CE-ColP and CE-ColP/M nanocomposites. Storage modulus of CE-ColP/M nanocomposites was higher than that of the control CE-ColP over the temperature range from 20 to 100°C, and an increase of

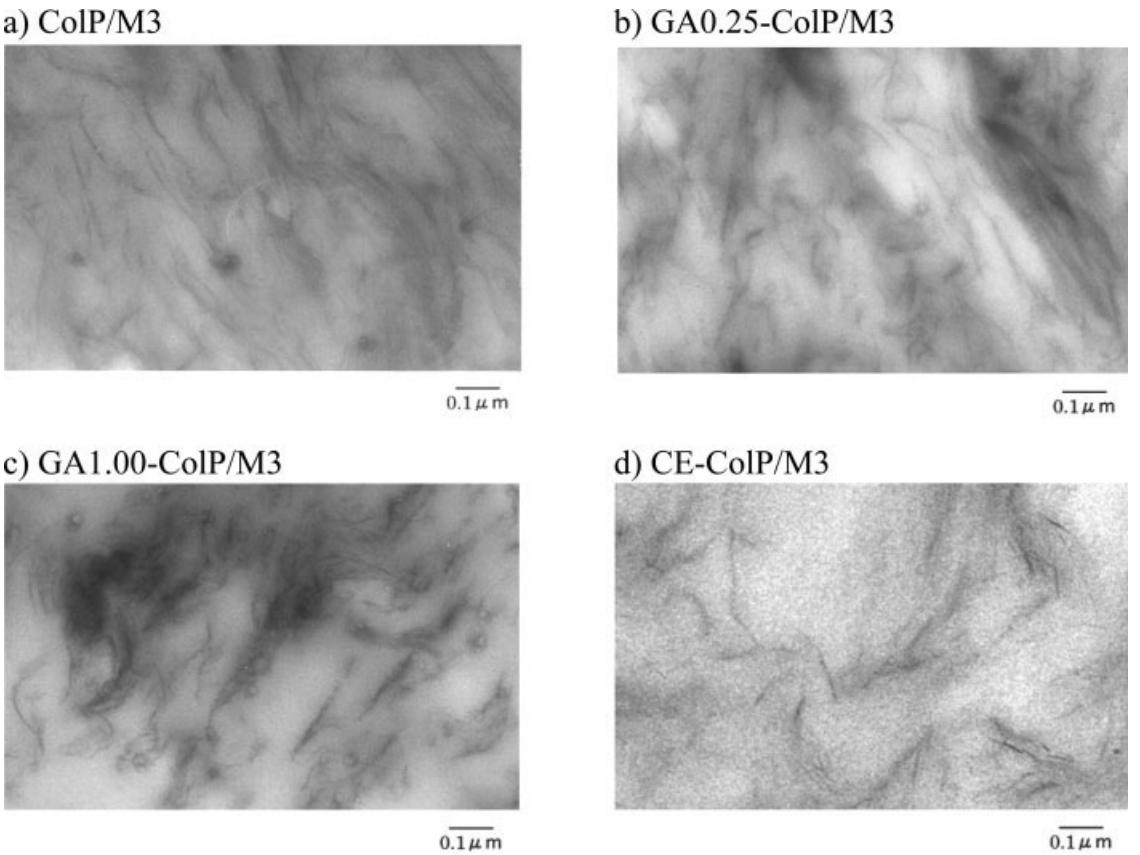

Figure 4 TEM images of (a) ColP/M3, (b) GA0.25-ColP/M3, (c) GA1.00-ColP/M3, and (d) CE-ColP/M3.

MMT content led to an increase of storage modulus. It is apparent that the finely dispersed clay platelets reinforce the matrix CE-ColP. We could not measure the dynamic viscoelastic properties of ColP, GA-ColP, ColP/M, and GA-ColP/M, because the obtained films were too brittle to prepare the rectangular samples by cutting. The brittleness of the ColP-based films is related to the fact that the molecular weight of ColP is relatively low. Therefore, the films using CE-ColP with a higher molecular weight than ColP have good flexibility. Both the crosslinking of ColP with GA and the formation of nanocomposite with MMT are not effective to improve the brittleness.

Figure 6 shows the TGA curves of various ColP-based films. The 10% weight loss temperatures are

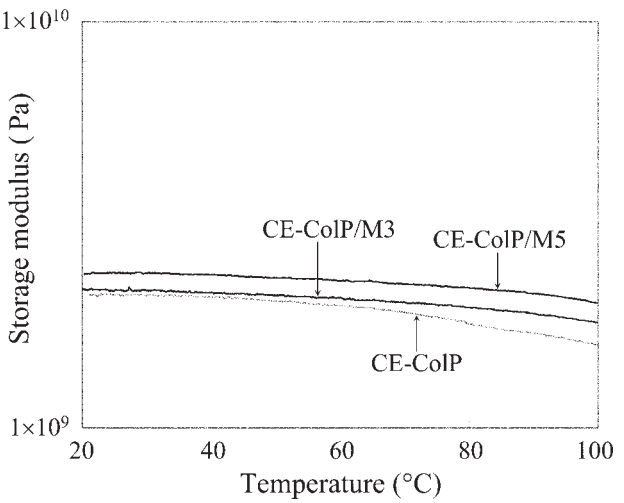

Figure 5 Dynamic viscoelastic curves of CE-ColP, CE-ColP/M3, and CE-ColP/M5 films.

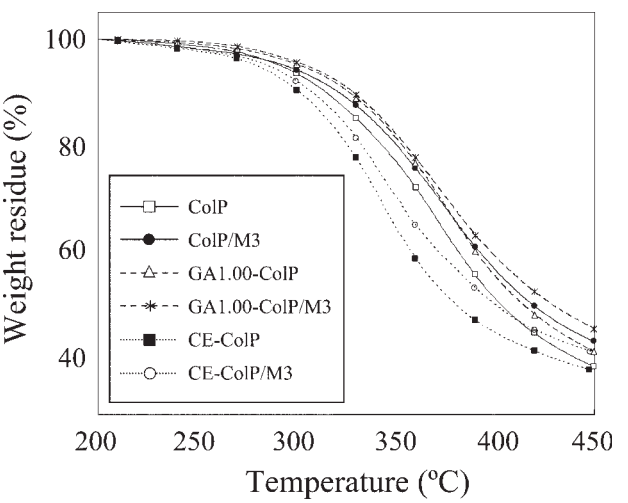

Figure 6 TGA curves of ColP, ColP/M3, GA1.00-ColP, GA1.00-ColP/M3, CE-ColP, and CE-ColP/M3.

TABLE IV
10% Weight Loss Temperature of ColP-Based Films

Sample	10% weight loss temperature (°C)
ColP	315
ColP/M3	322
ColP/M5	324
GA0.25-ColP	325
GA0.50-ColP	326
GA1.00-ColP	326
GA0.25-ColP/M3	327
GA0.50-ColP/M3	330
GA1.00-ColP/M3	329
GA0.25-ColP/M5	326
GA0.50-ColP/M5	328
GA1.00-ColP/M5	330
CE-ColP	302
CE-ColP/M3	308

summarized in Table IV. All the GA-ColP films showed higher 10% weight loss temperature than ColP, suggesting that the crosslinking of ColP by GA suppresses the weight loss by the thermal fragmentation. The reason why the 10% weight loss temperature of CE-ColP was a little lower than that of ColP is not clear, but it is thought that the DMSO used in the preparation may be remaining in the film. All the ColP-based composites showed a little higher 10% weight loss temperature than the corresponding peptides without MMT, suggesting that the finely dispersed clay platelets suppress the thermal degradation of matrix peptides and/or the evaporation of the degraded low molecular weight compounds.

CONCLUSIONS

To improve the mechanical and thermal properties of ColP extracted from fish scale, crosslinking of ColP by GA and chain extension of ColP using EDC and HOBt were first investigated, and their nanocomposites with MMT were next investigated. Although GA-ColP was insoluble in water due to crosslinking, the film was very brittle. The chain extension of ColP without crosslinking was attained by the condensation reaction of ColP using controlled feed amounts of EDC and HOBt. Although exfoliated nanocomposites with fine dispersion of MMT were formed in case of ColP/M and CE-ColP/M, the clay dispersion became worse with increasing degree of crosslinking for GA-ColP/M. The storage modulus of CE-ColP/M was higher than that of CE-ColP, and increased with the increasing MMT content. Regarding thermal degradation temperature, all the nanocomposites with MMT showed higher thermal degradation temperature than the corresponding films without MMT, and GA-ColP/M showed a higher degradation temperature than CE-ColP/M.

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