Self-organization of Oligopeptides Obtained on Dissolution of Feather Keratins in Superheated Water

Jie Yin,[†,‡,||] Sanjay Rastogi,*[,†,||,#] Ann E. Terry,[†,||,⊥] and Crisan Popescu[§]

Department of Chemical Engineering, Eindhoven University of Technology, P.O. Box 513, 5600MB Eindhoven, The Netherlands, Department of Macromolecular Science, Fudan University, 220 Handan Road, 200433 Shanghai, People's Republic of China, DWI at the RWTH Aachen, Aachen 52056, Germany, The Dutch Polymer Institute, P.O. Box 902, 5600AX Eindhoven, The Netherlands, ISIS Facility, Rutherford Appleton Laboratory, Chilton, Didcot, Oxfordshire, OX11 0QX, United Kingdom, and IPTME, Loughborough University, Leicestershire, LE11 3TU, United Kingdom

Received August 21, 2006; Revised Manuscript Received December 5, 2006

Keratins are self-organized proteins that are abundantly available in wool, feather, human hair, etc., making them a potential cheap feedstock for the modification of amino acids. This paper explores the hydrolysis of keratin in water under specific pressure−temperature conditions where the hydrolysis through scission of the protein chain yields oligopeptides. Here we report for the first time that, under appropriate conditions, these oligopeptides self-assemble into a hierarchical architecture, the process being followed in time by optical microscopy. Birefringent needle-like crystals are observed which tend to nucleate heterogeneously. When given sufficient time, these needles become tens of microns in length and act as further nuclei, developing a highly repetitive structure of several hundreds of microns in size. Micro-focus X-ray diffraction studies supported by in situ microscopy reveal that these needles have a crystal structure similar to that of the native protein, although better organized along the *ab*-plane. Spectroscopic studies on these structures show crystalline bands that disappear above 150 °C, coinciding with an endothermic peak in DSC. Amino acid analysis shows that the self-assembled birefringent entities are indeed oligopeptides, consisting of sequences of approximately 40 amino acids. The proposed ecofriendly route provides an effective route for obtaining oligopeptides that can be used as important building blocks for the synthesis of a range of novel polymers. The oligopeptides obtained from the sustainable source can be used as important building blocks for the synthesis of a range of novel polymers.

Introduction

Proteins consist of amino acids sequences, which in most cases give rise to three-dimensional structures due to intermolecular interactions between amino acids. In particular, fibrous proteins consist of extensive regions of regular secondary structures and amino acid sequences, hydrogen bonding being one of the main sources of attraction between backbone peptide links. The type of amino acids along a molecular strand may serve as both hydrogen bond acceptors and donors over a certain range of pH, and as acceptors or donors (but not both) at other pH values. It is this hydrogen bonding in proteins, and equally in natural polymers in general, which is of particular interest in this paper, especially how it is strongly influenced by the presence of a solvent, for example, water.

The polarity and hydrogen-bonding capabilities of water make it highly interactive with natural polymers. In aqueous solutions of proteins, water disrupts the electrostatic and hydrogen bond interactions between several molecules and is a very effective competitor to intermolecular polar interactions. In particular for proteins the hydrogen atoms of water may replace the amide group as hydrogen bond donors and the oxygen atom of water may replace the carbonyl oxygen as acceptors.

Keratin is abundantly present in nature and is the major component of hair, wool, feather, nail, and horns, the amino acid content and sequence of which may vary slightly depending upon the source. Fiber-forming keratins are distinguished from other structural proteins such as silk, fibrin, collagen, and muscle proteins by their amino acid composition and in particular by the number of cystine units in the keratin molecule. It is these cystine units, forming intermolecular S−S cross-links between the peptide chains[1] which lead to the hardness and thus usefulness of keratins. However, the same cystine amino acids also lead to the insolubility of keratins and thus limit their recyclability. If one considers that keratins from feathers account for 90% of all structural keratinous proteins, and that the waste product from the poultry industry amounted worldwide to more than 4 million tons of feathers annually at the end of the last century, the reuse of this cheap potential feedstock of amino acids, after separation and purification, could also yield a very valuable source of amino acids for the synthesis of other proteins or polymers. For such an approach to be effective, routes of dissolution involving the addition of foreign compounds, e.g., either enzymatic degradation, alkaline or acidification or the addition of a reducing agent, should be avoided. The dissolution route chosen should also have the potential to be scaled up into an industrial process. Therefore, we have explored the use of water hydrolysis to break down the keratins. At present, the only recycling of feather keratins via partial hydrolysis is to reduce the proteins into relatively long, digestible amino acids

* To whom correspondence should be addressed. E-mail: s.rastogi@tue.nl or s.rastogi@lboro.ac.uk.
† Eindhoven University of Technology.
‡ Fudan University.
§ DWI at the RWTH.
|| The Dutch Polymer Institute.
⊥ Rutherford Appleton Laboratory.
Loughborough University.

10.1021/bm060811g CCC: $37.00
Published on Web 01/26/2007

Table 1. Influence of Temperature and Time on the Dissolution of Feather Barbs in Distilled Water[a]

temp (°C)	time (min)	W_{before} (mg)	W_{after} (mg)
180	30	20.6	18.7
180	120	22.1	5.6
200	60	22.7	8.6
200	120	20.9	3.4
220	30	23.2	12.2
220	60	24.5	5.6
220	90	24.3	4.0
220	120	23.4	0.4

[a] W_{before} is weight of feather barbs before dissolution. W_{after} is weight of feather barbs left after the dissolution time.

for animal feeds, although this application is of limited nutritional benefit due to the absence of certain vital amino acids. Instead, we are much more interested in exploiting the potential of feather keratins to provide a cheap and renewable source of amino acids for further synthesis. By exploring further the $p-T$ phase diagram of water, we can show that it is possible to yield much shorter oligopeptides, consisting of sequences of between 10 and 18 amino acids in length. The supercritical region of water exists above 374 °C, 22.10 MPa where water becomes a good solvent for many materials.[2] Yet, even above the ambient boiling point and below the supercritical point, water is in the superheated state where the hydrogen bonding between water molecules weakens considerably, increasing the mobility of the water molecules.[3] This superheated state of water is highly effective in the hydrolysis/dissolution of keratins to obtain oligopeptides. In the initial study reported here, we explore the potential for the thus-obtained oligopeptides to self-assemble into crystals which we believe will be an important aid for the purification and separation of these oligopeptides and thus enhance their applicability as building blocks for future materials.

Method Used

In superheated water the hydrolysis of keratin is possible. The material used for our studies is the barb of chicken feathers. The feathers are pretreated by cleaning, cutting, degreasing, and drying;[4] details are provided in the Supporting Information. The barbs from dry feathers are sealed in a steel pressure cell[5] with water at a concentration of 20 mg/mL and placed in a preheated oven. Since the steel vessel is sealed, the pressure in the cell will increase with temperature due to the partial pressure of the water within the cell. At ~220 °C, the pressure in the vessel will be ~22 bar. The degree of dissolution of the barbs varies within the examined temperature window of 180−220 °C for different dissolution times, Table 1; for example, almost complete dissolution occurs after heating at 220 °C for 120 min and after cooling of the pressure vessel under flowing water to room temperature a suspension with only a small amount of residue (0.4 mg) is obtained. The residue is probably composed from non-amino acid components within the starting feather, for example, fatty acids. Formation of H_2S gas is noticed on opening of the pressure cell. Subsequent analysis and experiments reported in this paper are performed on a water solution of the barbs of chicken feathers obtained on dissolution at 220 °C for 120 min. The amino acid analysis for the solution reveals the presence of amino acid residues as summarized in Table 2.

Table 2, column 3, gives the ratio of amino acids in the keratin solution in water, showing the absence of cystine and cysteine. The absence of these amino acids and the smell, and thus the presence, of H_2S can be associated with the breaking of the S−S bond of cystine. For comparison the published amounts of amino acids of feather keratin is shown in columns 4 and 5 of Table 2. The last two columns in the table give the proportions of amino acids in g/100 g as found in the feathers before its dissolution. The last column gives amino acids in the feathers corrected for the case of zero cystine, cysteine, and asparaginine and 1% of threonine and serine, and adjustment of the relative percentages of the remaining amino acids to have 100% amino acids. This correction allows a direct comparison of the relative proportions of each amino acid before and after the dissolution, columns 5 and 3, respectively, which shows that although arginine diminishes, the remainder of the amino acids is not largely affected by the hydrolysis of the barbs. Additionally, it should be noted that, oddly, alanine and ornithine and, to a lesser extent, glycine increase in amount upon hydrolysis as compared to the amounts found in the feather barbs, prior to their dissolution. This may highlight a very complex process occurring on the hydrolysis of feather keratins or perhaps amino acid extraction from small amounts of the feather shaft which may be included in the starting materials but not in the feather prior to hydrolysis as shown in column 5.

Feather keratins appear to be small proteins, which are uniform in size, with a molar mass around 10 kDa. Assuming an average molecular weight of 100 for each amino acid, this would yield on average a sequence length of 100 amino acids.[6] To determine the molar mass range of the oligopeptides in the water solution, matrix-assisted laser

Table 2. Amino Acid Composition

amino acid	symbol	amino acid composition of keratin solution in water (g/100 g solution)	amino acids before dissolution (g/100 g feather)	amino acids before dissolution corrected for cystine,cysteine, and asparaginine[a] (g/100 g feather)
cysteine	Cys	0.00	0.688	0.000
asparaginine	Asp	0.483	8.057	0.00
threonine	Thr	1.243	5.204	1.447
serine	Ser	1.371	11.829	1.447
glutamic acid	Glu	17.185	12.178	17.622
proline	Pro	15.203	10.619	15.409
glycine	Gly	10.410	5.487	7.94
alanine	Ala	11.815	3.103	4.49
valine	Val	9.937	7.218	10.445
cystine	(Cys)2	0.000	7.111	0
methionine	Met	0.308	0	0
isoleucine	Ile	5.433	4.588	6.639
leucine	Leu	10.950	8.408	12.167
tyrosine	Tyr	3.523	3.018	4.367
phenylalanine	Phe	4.863	3.771	5.457
ornithine	Orn	3.356	0.183	0.265
lysine	Lys	0.915	0.832	1.204
histidine	His	0.036	0.172	0.249
arginine	Arg	2.967	7.506	10.853

[a] Correction of amounts on considering that all cystine and cysteine are decomposed.

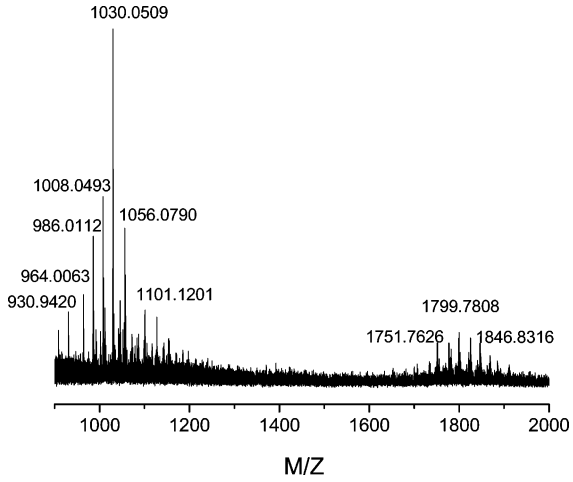

Figure 1. MALDI-TOF data on water solution of feather barbs (20 mg/mL) prepared at 220 °C for 120 min. The graph shows the presence of discrete molar masses of oligopeptides around 1 and 1.8 kDa.

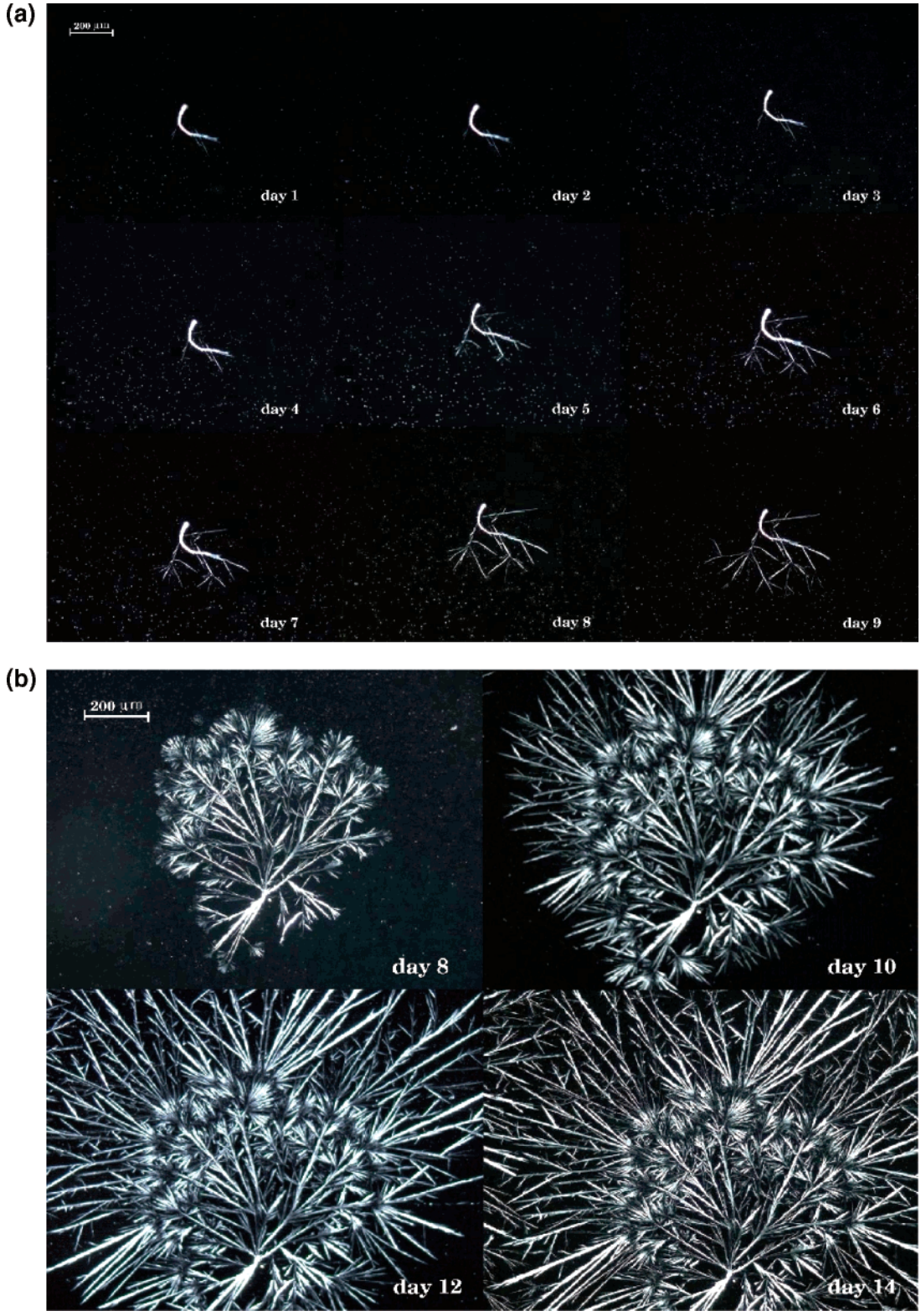

Figure 2. Birefringent entities as viewed between cross polars are the crystals of oligopeptides nucleated on a dust particle present on a glass substrate, Figure 2a. The oligopeptides are obtained on the dissolution of chicken feather in water at 220 °C for 120 min in a closed pressure vessel. The crystals grow with time (days) when left at room temperature. Crystal growth appears to be dendritic in nature and becomes more apparent in Figure 2b. Crystals at the edge tend to act as nucleation sites for further crystal growth.

desorption ionization time-of-flight mass spectrometry (MALDI-ToF-MS) analysis is performed on a Biflex III, Fa. Bruker Daltonics equipped with a 337 nm nitrogen laser. An acceleration voltage of 148 kV is applied and all measurements are performed in the positive mode. The matrix used is *trans*-3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA) at a concentration of 20 mg/mL in demineralized water. A feather solution is prepared by the usual method of dissolution in superheated water at 220 °C for 120 min at a concentration of 20 mg/mL. After cooling of these samples to room temperature, the (supernated) solution (20 mg/mL) is mixed with a premixture of acidified water (0.1 vol % TFA) and acetonitrile in a ratio of 2:1 vol %. The volume of sample solution taken is such that the concentration

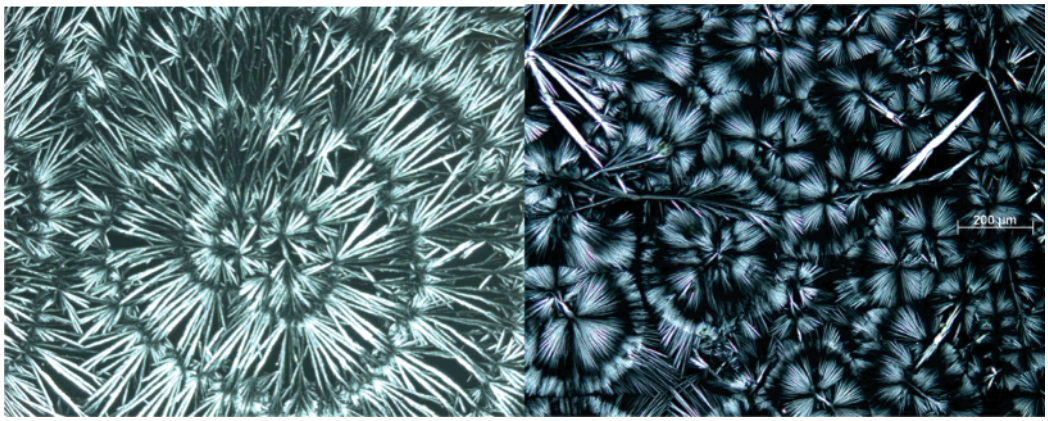

Figure 3. Crystal growth from the same solution on the glass slide after 2 weeks (left) and on the rubber substrate after 2 days (right). Crystallization is performed at room temperature. For the same magnification it is evident that the number of nuclei on the rubber substrate is much higher than that on the glass slide. It is evident that crystallization strongly depends on the substrate.

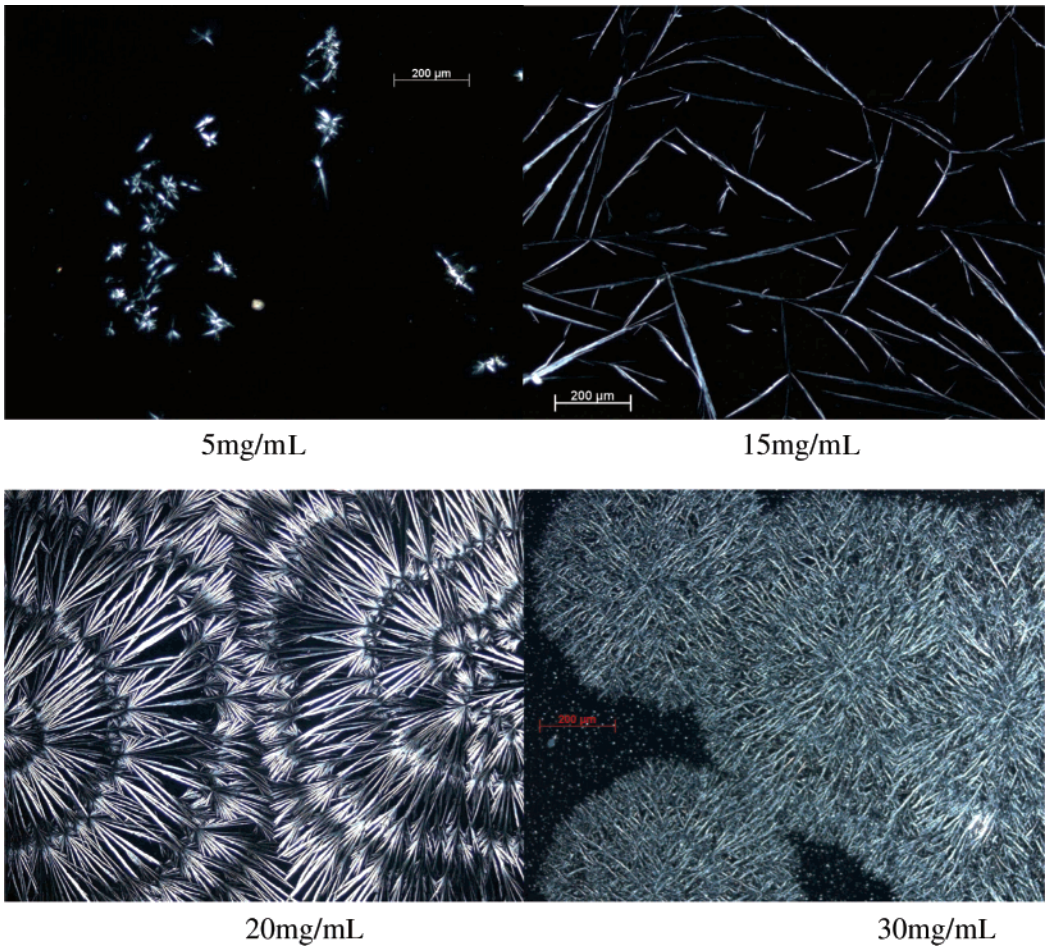

Figure 4. Chicken feather barbs of varying amounts in water (ranging from 5 to 30 mg/mL) are dissolved (or hydrolyzed) at 220 °C for 120 min. A drop of solution placed on a glass slide is left to crystallize in a fume hood at 25 °C, for 2 weeks. Optical micrographs show self-assembly of the oligopeptides with increasing concentration of the oligopeptides in the water solution. Crystal density increases with the increasing concentration.

of the feather solution is about 100 pmol/μL. Solutions of the matrix and the sample are mixed in a ratio of 1:1 vol %. The mixed solution is hand-spotted on a stainless steel MALDI target and left to dry.

MALDI-ToF analysis of the solutions indicates the presence of peptide sequences in two discrete molar mass ranges around 1.0 and 1.8 kDa (see Figure 1). Thus, assuming an average molecular weight of 100 for each amino acid, the obtained peptides are between a sequence of 10 or 18 amino acids in length, suggesting that on hydrolysis a considerable proportion of the amino acid sequence is conserved.

Self-assembling Process of Oligopeptides

When a drop of hydrolyzed solution of concentration 20 mg/mL is left to dry on a clean glass substrate, needle-like birefringent entities are observed to grow by optical microscopy between crossed polars. These entities are embedded in a non-birefringent matrix which is waxy in appearance. Typical optical micrographs taken between crossed polars are shown in Figure 2. The nucleation of the crystals occurs from any heterogeneity present on the substrate. Crystallization appears to be diffusion-

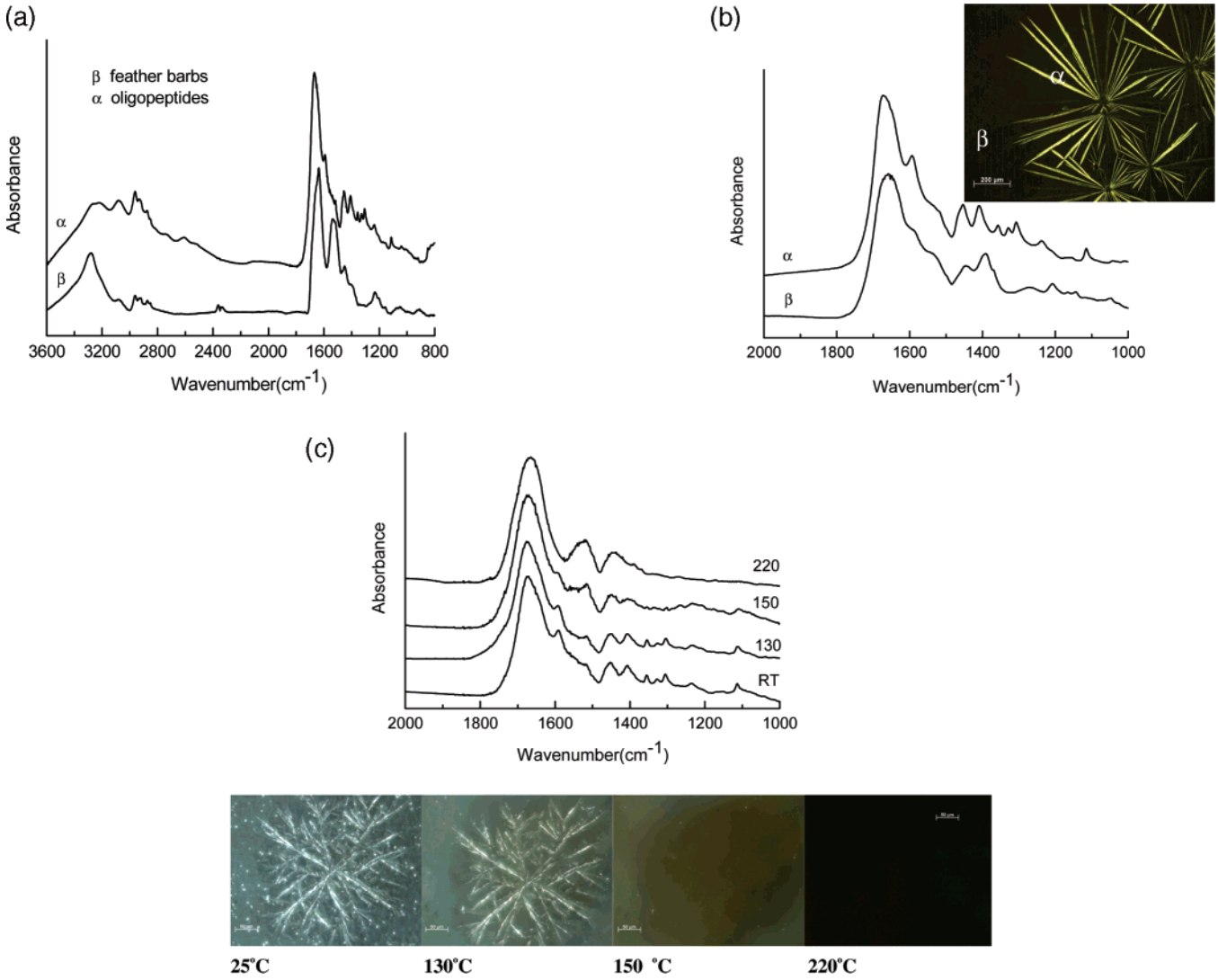

Figure 5. Figure 5a refers to FTIR spectra recorded on native keratin from chicken feather barb (β), and oligopeptides obtained on crystallization of the feather−water solution (α). Figure 5b shows FTIR spectra of crystal (α) and amorphous (β) regions in the same sample as shown in the optical micrograph recorded from the microscope attached to the FTIR spectrometer. Figure 5c is a composite picture of FTIR spectra recorded in situ on crystals while heating from room temperature to 220 °C. Micrographs are recorded subsequently while heating the sample on a hot stage.

controlled, leading to the formation of dendritic-like crystals, where the crystal tip acts as a nucleation site for further growth. Ultimately, big dendritic crystals originating from single nuclei fill the field of view of the optical microscope.

Crystals are observed to grow with time (days) at room temperature and the crystal growth is strongly dependent on the substrate, which has included glass, PTFE, and rubber. The growth rate increases with increasing surface roughness, although the overall crystal morphology does not appear to change (see Figure 3 for the influence of the surface roughness on crystal growth). If a droplet of the solution is dried on a glass substrate under vacuum at room temperature or in an oven at 50 °C for several days, no dendritic crystals appear. This suggests that the evaporation rate of water influencing the diffusion rate of the keratin molecules to the growing crystal interface has an important consequence on the crystal growth.[7]

Crystal growth is also strongly influenced by feather concentration in the water solution. Figure 4 shows the self-assembly process of oligopeptides after the dissolution/hydrolysis of varying amounts of chicken feather barbs hydrolyzed in water (ranging from 5 to 30 mg/mL) at 220 °C for 120 min. A drop of solution when left to crystallize in a fume hood at 25 °C, for 2 weeks, shows self-assembly of the oligopeptides. From the optical micrographs it is evident that crystal density strongly depends on the oligopeptide concentration, influencing the overall crystal growth.

In contrast, upon complete hydrolysis in 6 M/L HCl solution at 100 °C for 22 h, single amino acids can be obtained and the crystal morphology changes from dendritic- to plate-like.

Conformational and Structural Analysis of the Self-assembled Oligopeptides

To investigate the conformation and structure of the crystals grown from solution, spectroscopic and micro-focus X-ray diffraction techniques have been used. For FTIR studies, a droplet of the suspension is placed on a zinc selenium disk and dried in a fume hood at room temperature for several days. FTIR spectra with a spatial resolution of 2 cm^{-1} are averaged over 200 scans collected on a Bio-Rad FTS 6000 spectrometer with attached microscope. Figure 5a shows FTIR spectra at room temperature (RT) of feather before dissolution (β) and of crystals grown from the feather−water solution (α). Both spectra show

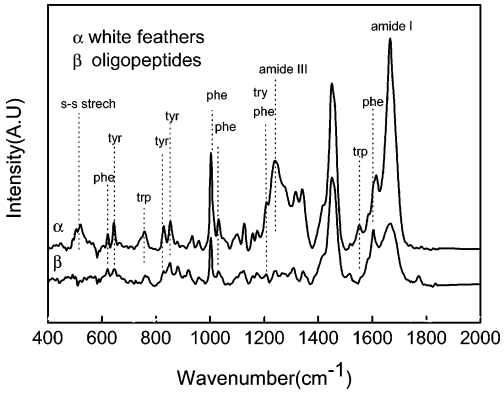

Figure 6. Raman spectra of chicken feather (barbs) before dissolution (α) and of crystals grown from the feather−water solution (*β*).

the presence of NH (∼3300 cm^{-1}) and CO (∼1660 cm^{-1}) groups in the crystals, suggesting that a similar conformation and local molecular environment is retained in the crystals grown from solution.[8,9] Figure 5b shows FTIR spectra of two different regions in the optical micrograph as viewed between crossed polars in the FTIR microscope: a birefringent region encompassing needle-like crystals (spectrum α) and an isotropic region surrounding the birefringent region (spectrum *β*). Compared to spectrum *β*, spectrum α shows crystallinity-sensitive absorption bands mainly between 1300 and 1360 cm^{-1},[10] related to CH deformation and wagging of CH_2. Upon heating of the crystals in situ, from room temperature to 220 °C, the crystals tend to decrease in birefringence around 130 °C with a reduction in the intensity of the crystalline bands in the FTIR spectra, Figure 5c. Ultimately, the crystals disappear at 150 °C with the disappearance of the crystalline bands in the FTIR spectra. By DSC, a broad endotherm is observed in the region of 130−150 °C and can be associated with the denaturation process. On cooling of the sample, no birefringent entities reappear, and a spectrum recorded at room temperature is similar to the spectrum *β* in Figure 5b, i.e., a spectrum from the amorphous region.

The absence of cystine (see Table 2) in the amino acid analysis of dissolved keratin is suggested. To confirm these findings, Raman spectra are recorded on the crystals at room temperature. Figure 6 shows room-temperature Raman spectra of feathers (α) and crystals grown from the solution (*β*). When compared with the feather, bands associated with amide I, phenylalanine, are present in the crystal whereas the S−S band at 400 cm^{-1} and amide III bands in the region 1200−1400 cm^{-1} are absent in the crystals. The absence of the bands must be due to the hydrolysis of the feathers.

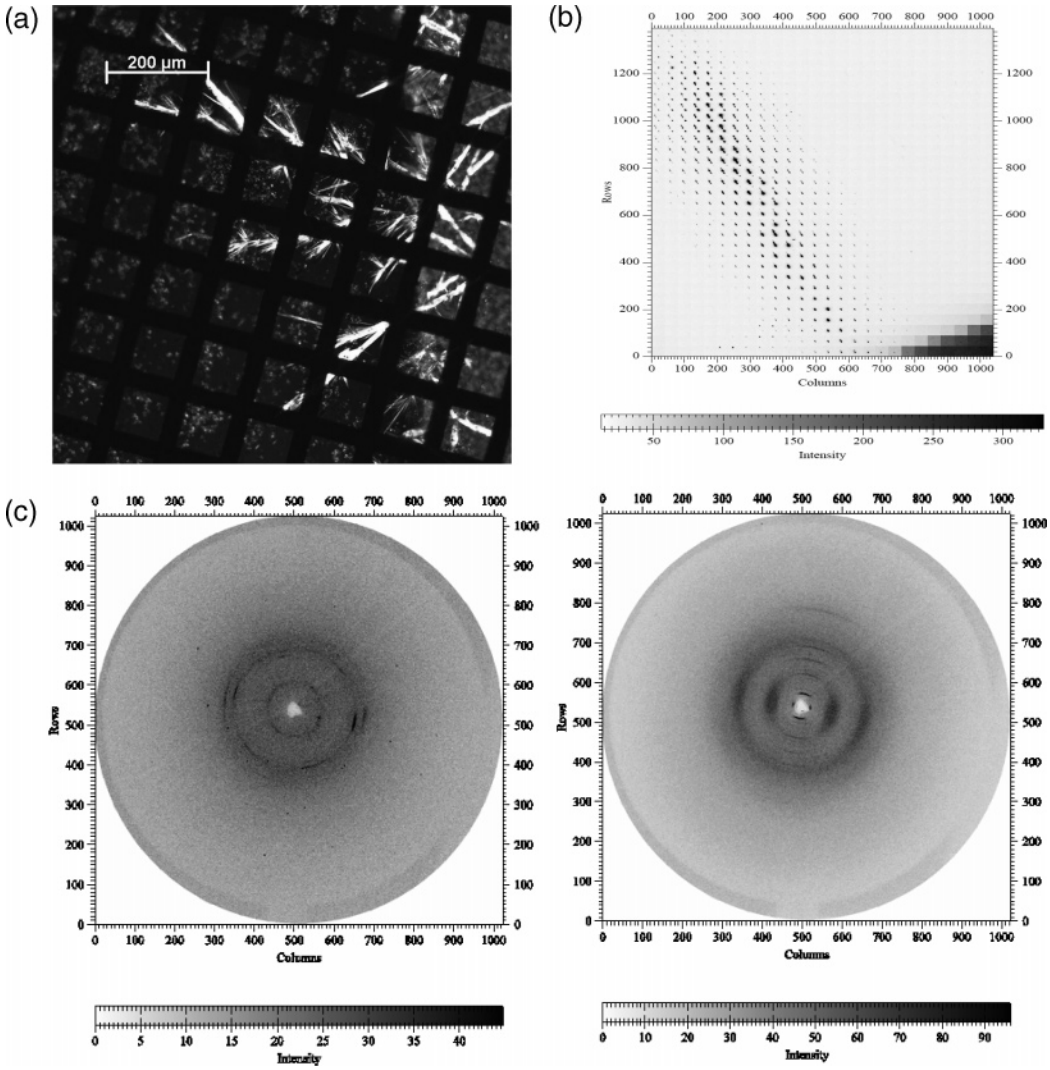

Figure 7. (a) Crystals grown from the oligopeptide solution on a carbon-coated Cu grid. (b) shows a series of an X-ray diffraction peak obtained on scanning of a selected crystal region within one square of the Cu grid. (c) 2D X-ray diffraction pattern of the crystals. (c) 2D X-ray diffraction pattern of the chicken feather barbs prior to dissolution.

To investigate the crystal structure, oligopeptide crystals are grown on a carbon-coated Cu grid, Figure 7a. The microfocus X-ray diffraction beamline ID13 at ESRF, Grenoble, is used to determine the crystal structure. A square in the Cu grid with crystals is selected and scanned in a mesh of steps of 2 μm using a monochromatic X-ray beam of size 1×1 μm^2. 2D diffraction patterns from the crystal show arcs and/or spots, with a characteristic diffraction angle which repeats across the sample. If a region of interest (ROI) within the 2D pattern is chosen which contains a diffraction reflection which appears in a number of patterns and a composite pattern is constructed of this ROI plotted for every step point in the mesh scan, the resulting pattern mirrors the optical image of the chosen scan area, taken with crossed polars, Figure 7b. Diffraction patterns from outside the birefringent area on the Cu grid show no crystalline reflections; i.e., the material is amorphous.

Figure 7c is a typical X-ray diffraction pattern recorded from a crystalline region. For comparison, the barbs from a cleaned and degreased white chicken feather are also scanned. A typical 2D pattern, Figure 7d, is highly oriented, revealing the fibrous nature of the feather. On the meridian, several orders of diffraction layer lines are observed. Two broad arcs can be seen on the equator which occur at the same scattering angle as the two innermost arcs in Figure 7c, suggesting that the crystallization of the oligopeptides occurs on the same underlying *ab*-plane as the β-sheet structure of the feather keratins. Along the *ab*-plane, the sharp reflections of the oligopeptides compared to the diffuse reflections of the barb suggest that large crystals can be obtained which is supported by the sizes of the birefringent entities shown in the optical micrographs. Detailed FTIR, Raman spectroscopy, and XRD analysis on the self-assembled oligopeptides suggest the conservation of the conformational and crystallographic order of their source, in this case, a chicken feather. This would be only feasible for oligopeptides with an amino acid composition similar to the parent protein.

Conclusions

It is known that water shows an unusual pressure−temperature phase behavior such that above the boiling point, but below the supercritical temperature, at elevated pressures, the water is in the superheated state. In this state hydrogen bonding between the water molecules weakens and the diffusion permeability increases. We have been able to show that the hydrolysis of keratin, for example, from feathers, can occur in superheated water. Depending on the dissolution temperature and pressure, it is feasible to obtain oligopeptides which range in sequence length from a few to several tens of amino acids. These oligopeptides are able to self-assemble into crystalline structures as evidenced by the beautiful hierarchical structures shown in the optical micrographs in this paper obtained upon drying a solution of the hydrolyzed feather keratin. The structures exhibit features revealing the conservation of the conformational and crystallographic order of native keratins. An important conclusion from the structural analysis is that the amino acid sequences from the parent protein prior to hydrolysis are largely preserved in the oligopeptides. These findings are not limited to chicken feather keratin only. Keratin obtained from different sources such as wool, stretched wool, and human hair of different origin also shows similar crystal growth from hydrolyzed solution. The oligopeptides tend to denature around 130−150 °C, whereas the denaturation of amino acids occurs at around 50 °C; a difference in the denaturation temperature may arise due to the different sequence lengths of the amino acids.

The importance of the self-assembly process is that since crystallization occurs, it should be feasible to extract, fractionate, and purify the oligopeptides in a similar manner to that done in the processing of conventional polymers. Thus, there is potential for this ecofriendly recycling of a waste product from, an example, the poultry industry to yield a renewable, cheap feedstock which can form the "building blocks" of amino acid sequences for further use, perhaps even the synthesis of novel materials.

Acknowledgment. The authors thank the Dow Chemical and the Dutch Polymer Institute (DPI) for financial support. The authors also thank Mr. Otto van Asselen for helping in FTIR studies, Dr. Guido Heunen, Mr. Jules Harings. and Dr. Christian Riekel for X-ray studies performed on beamline ID13 at ESRF, Grenoble.

Supporting Information Available. Sample preparation, amino acid analysis, Raman spectroscopy, and denaturation and crystallization of amino acids. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Fraser, R. D. B.; MacRae, T. P.; Rogers, G. E. *Keratins. Their composition, structure and biosynthesis*; Thomas: Springfield, IL, 1972.
(2) Haar, L.; Gallargher, J. S.; Kell, G. S. *NBS/NRC Steam Tables*; Hemisphere Publishing Corp: New York, 1984.
(3) Truskett, T.; Dill, K. A. *Biophys. Chem.* **2003**, *105*, 449−459.
(4) Schrooyen, P. Feather Keratins: Modification and film formation. Ph.D. Thesis, Universiteit Twente, Enschede, The Netherlands, 1999; ISBN 9036513022.
(5) Rastogi, S.; Terry, A. E.; Vinken, E. *Macromolecules* **2004**, *37*, 8825−8828.
(6) Marshall, R. C.; Gillespie, J. M. *Aust. J. Biol. Sci.* **1977**, *30*, 389−400.
(7) Yang, C. H.; Brown, J. N.; Kopple, K. D. *Int. J. Peptide Protein Res.* **1979**, *14*, 12−20.
(8) Chalmers, J. M.; Hannah, R. W.; Mayo, D. W. Spectra-Structure Correlations: Polymer Spectra. In *Handbook of Vibrational Spectroscopy*; Chalmers, J. M., Griffiths, P. R., Eds.; John Wiley & Sons: Chester, UK, 2002; Vol. 4, pp 1893−1918.
(9) Koenig, J. L. *Spectroscopy of Polymers*; American Chemical Society: Washington, DC, 1992.
(10) Naik, V. M.; Krimm, S. *Int. J. Peptide Protein Res.* **1984**, *23*, 1−24.

BM060811G