Preparation, Processing, and Rheology of Thermoplastic Collagen

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ABSTRACT: This article describes a technology for the thermoplastic treatment of collagen. Based on limed, untanned cattle pelts a fine powder, termed Thermoplastic Collagen (TC) is produced, which can be processed using conventional thermoplastic techniques. Central step in the production of TC is the partial denaturation of collagen, which was achieved by four different methods. Extrusion of TC at temperatures below 100°C is possible after addition of 30–60% water as plasticizer. In order to maintain permanent flexibility and reduce tackiness, glycerol and stearic acid are used as additives. Shear and elongational viscosities of TC-water-mixtures decrease with the shear rate which is consistent with the behavior of thermoplastic materials. The power law can be applied, yielding power law indices of 0.15–0.35. The viscosities are more distinctly influenced by raw material and water content than by temperature or glycerol content. Material properties of the extrudates are described and compared with the starting material. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 128: 4201–4211, 2013

INTRODUCTION

Collagen is the most common structural protein of animals, constituting about 30% of the whole protein content. It is part of the extracellular matrix of connective tissues, such as bone, cartilage, tendon, ligament, and skin. Today, 28 types of collagen are known and type I being the most important.[1,2] Fibers of collagen type I are the major constituent of skin, tendons, and ligaments. Its basic structural unit is a heterotrimer consisting of three helical protein chains (α-chains), which are twisted among each other in form of a triple helix. These rod-like molecules are ordered in a parallel and staggered way forming fibrils and fibers. In animal tissues, the collagen protein chains are cross-linked with each other, mainly via the side chains of lysine or hydroxylysine.[1,3] The cross-linking stabilizes the tissue. It increases with animal age and is the reason for the general insolubility of collagen in water.

Collagen containing tissues (mainly skin and bone) serve as raw material for different industrial products, such as leather, sausage casings, adhesives, gelatin, or medical devices. The technologies, which lead to these products, make use of collagen at different stages of processing and degradation. Thus the production of leather uses the complete and intact collagen matrix of the skin. On the other end of the scale, we find gelatin, which is a highly degraded product of collagen. The reduction of protein chains by hydrolyzation and damage of cross-links cause its high water solubility.

As a protein, collagen can be regarded as a biopolymer. It is known from other biopolymers, such as plant proteins, starch, or cellulose, that it is possible to process them in analogy to synthetic polymers by thermoplastic techniques, such as extrusion or injection molding, in order to obtain products of different forms (e.g., cast or molded pieces, sheets, films, or coatings).[4–14]

For such processes neither intact, undenatured collagen fibers nor highly degraded gelatin are suitable. For the use of collagen as a thermoplastically processable biopolymer, a collagen variant between these two extremes must be produced, which maintains long protein chains but reduces their cross-linking and intertwining. Such a collagen can be described as partially denatured and because it can be transformed into a thermoplastic state under appropriate conditions, is termed as "Thermoplastic Collagen (TC)." The production technology of TC is established in our institute and in some aspects mentioned in an international patent.[15] It is described in this report, along with the conditions of thermoplastic processing of TC in a conventional extruder and the properties of TC in comparison with the corresponding extrudates. A great part of the report consists in the rheological characterization of the protein melt. Viscosity curves were measured using an extensional die at the extruder exit, which allows the determination of shear and elongational viscosities by pressure measurement under extrusion conditions. The shear viscosity is commonly used to characterize the processing performance of polymer melts. The dependence of TC shear viscosity on the

shear rate resembles the curve progression of thermoplastic materials. In regard to future technical applications of TC, it is of great interest, how the viscosity of TC melts can be influenced and regulated. Thus, the dependence of the viscosity on the main process parameters (plasticizer content, temperature, raw material) were investigated. Beside the shear viscosity, the elongational viscosity of TC melts was determined and included in the investigation for two reasons: (1) it is known from the literature that polymers with identical shear flow properties may differ in their technological behavior which is reflected in the extensional flow properties,[16–19] and (2) in many thermoplastic forming processes, like film blowing, thread spinning, or injection molding, extensional deformation plays an important role.

Possible applications of TC-based products can be imagined as packaging material (food or non-food), in the agricultural sector (e.g., mulching films), as material for pharmaceutical or medical purposes (e.g., biocompatible threads) or in the pet food sector (e.g., chewing bones or functional food). The fact that collagen is biodegradable can be a great advantage concerning the waste management after use. The possibilities of the production of differently formed TC products by various post-extrusion treatments, like film-blowing or thread spinning, are discussed in this report. Mechanical properties of TC extrudates are evaluated in regard to their potential technical applications.

EXPERIMENTAL

Materials

TC powder was produced at FILK from cattle hides according to the procedure described below. Glycerol (technical grade, 99%) was purchased from AppliChem (Darmstadt, Germany), gelatin (bovine gelatin B) was from Gelita AG (Eberbach, Germany), and trypsin (from bovine pancreas) was from Sigma-Aldrich (Steinheim, Germany).

Technological Processes

Production of TC. Cattle hide was used as raw material for the recovery of collagen. The raw hides were treated in a beamhouse according to established standard techniques in tanneries. After soaking and liming with $Ca(OH)_2$, the hair was removed using sodium sulfide. The resulting pelt was delimed with ammonium sulfate and the pH adjusted to 8.5. After neutralization with formic acid to a final pH between 6.5 and 7.5, a bleaching step with 0.5% H_2O_2 followed. The pelts were flaked and in some preparations, they were splitted. The resulting material was partially denatured by one of three different methods.[20,21]

Denaturation by Heating in Excess Water. As raw material were used salt-preserved bovine hides and different splits (grain, middle, flesh) of cattle hide after liming times of 50 and 120 h, respectively. Water was heated to 80°C. Pelts were added and treated for 20 min, after the temperature of 80°C was reached anew. The moist material was spanned on a frame and air dried under ambient conditions for 48 h. In total, 38 batches in varying amounts were produced. For further characterization and rheology experiments, two identically produced batches were selected, which were based on grain split of adult cattle hide and provided sufficient amount of material for all investigations.

Denaturation by Extrusion. Raw material was cattle hide, using the whole profile as well as different splits (grain, middle, flesh) after a standard liming process. Air dried pelts with a moisture content of 15–22% were roughly ground in a cutting mill and processed in a co-rotating twin-screw extruder (Multi Process Compounder, MPC 37 × 36 D, 11 heating zones, L/D = 36, Krauss Maffei Berstorff GmbH, München, Germany) with varying parameters: extrusion temperature (over the last nine heating zones) 90–130°C, screw speed 60–100 rpm, and end pressure 40–95 bar. The extrudate was pressed through a die plate (three holes with 3 mm diameter). In total, 20 batches were produced, many of them in small scale to optimize the processing range. It appeared that extrusion temperatures above 120°C led to increased darkening of the material, probably due to partial combustion of collagen. For further characterization and rheology experiments a batch, based on grain split of adult cattle hide and produced under moderate conditions (T = 115°C, screw speed 60 rpm), was used.

Denaturation by Microwave Treatment. Exposure to microwave radiation was performed on an industrial microwave equipment at Dornier GmbH (Lindau, Germany). Wet pelts based on grain splits of adult cattle hide passed a microwave rod antenna by a conveyor. In several preliminary experiments, the technical parameters (microwave power, antenna height, conveyor speed, and cycle number) were optimized. The following parameters were used in the final experiment: antenna height – 50 mm above conveyor, microwave power – 15 kW, transport speed – 0.25 m/min, number of cycles – 3. It appeared that the pelts were not evenly heated, therefore the material was additionally air dried before grinding.

Dry partially denatured collagen material was ground with a cutting mill into crude pellets and then into a fine powder using a turborotor mill (type G-35, Görgens Engineering GmbH, Dormagen, Germany). The resulting powder was subjected to sieve classification, because it was observed that crude particles did not melt in the following extrusion process and may act as defects or points of rupture in the final product. The fraction with a grain size below 355 μm was used as TC. Four batches, which were used for further characterization and rheological investigations, are termed as follows, indicating the method of denaturation by indices: TC_{HW1} and TC_{HW2} (denaturation in hot water, identical production conditions for both batches), TC_{EX} (denaturation by extrusion), and TC_{MW} (denaturation by microwave).

Thermoplastic Processing of TC. TC powder was mixed manually with varying amounts of water and, when indicated, with glycerol. The following TC/water/glycerol ratios were used for the different TC batches. For rheological measurements with TC and water only (indication of the water amount as percentage of the whole TC/water mass): TC_{HW1}, TC_{HW2}, TC_{MW}: 40, 50, and 60% water, TC_{EX}: 30, 40, and 50% water; for rheological measurements with TC, water, and glycerol: mass fractions 1.0 TC : 0.67 water : 0.83 glycerol; for the production of TC films: mixtures with 55% water (of whole TC/water mixture), stearic acid (2% in regard to TC mass) and varying amounts of glycerol (0-35% of the TC mass). The particular mixture was then

homogenized using a 25 l laboratory mixer (type MSHK25, Trusioma, Greiz, Germany) and stored overnight in a closed bag to equilibrate. The TC-mixture was processed using a twin-screw extruder ($L/D = 40$, eight heating zones, type MP19TC, APV Baker, Newcastle, UK) with a screw speed of 120 rpm and the following temperature profile: 70-80-90-90-90-90-90-90°C. The mixture was added using a gravimetric feeder with a dosing rate of 15 g/min. A pelletizing die with a nozzle diameter of 4 mm was used to produce strands which were pelletized after cooling. The pellets were used for material characterization. TC films were produced using a ring-shaped die (diameter 30 mm, gap width 0.8 mm) with air hose connection for film blowing. All dies were heated by an external device, and the temperature was 90°C.

Material Characterization

Moisture content. A sample of TC powder or extrudate was weighed before and after drying (102°C, 5 h). The moisture content was calculated from the mass difference in relation to the initial sample weight.

Protein content. The total nitrogen content of the sample (powder or extrudate) was determined by titration of ammonia after acidic hydrolization. The sample (ca. 3 g) was treated with 30 ml sulfuric acid (98%) and 5 g catalyzer in a Kjeldahl flask. After dilution with 170 ml water, the solution was transferred into a distillation flask and alkalized with 70 ml sodium hydroxide (32% solution). Resulting ammonia was distilled with water vapor into 100 ml of a boric acid solution and then titrated with 0.5 N sulfuric acid. The total nitrogen content (in % regarding the sample weight) was calculated based on the titration volume and the initial sample weight. In order to convert the total nitrogen content into protein content, the factor 5.62 was used, according to the standard method DIN 53308.

Content of amide nitrogen. A sample of TC powder or extrudate (ca. 500 mg) was hydrolyzed with 20 ml 6 N HCl for 16 h at 105°C. The solution was then diluted with distilled water and 10 ml thereof were transferred to the distillation flask of a Parnas-Wagner apparatus. After addition of 2.8 g Ca(OH)$_2$ and 40 ml water, the resulting ammonia was distilled with water vapor into a flask, containing 10 ml 0.01 M sulfuric acid and methyl red as indicator. Neutralization with 10 ml 0.02 M ammonium hydroxide was followed by titration with 0.01 M sulfuric acid. Glutamine was used as a control standard and water for a blank value. The content of amide nitrogen N_{amide} in the sample was calculated based on the titration volume.

DCM soluble substances. A sample of TC powder or extrudate (ca. 5 g) was subjected to extraction with dichloromethane (DCM, 40°C, 6 h) in a Soxhlet apparatus. After removal of DCM from the extract, the residue was dried at 102°C overnight and weighed after cooling down in an exsiccator. The content of DCM soluble substances was calculated from the ratio of initial sample weight and residue weight.

Ash content. A sample of TC powder or extrudate (0.5–2 g) was annealed in a platinum crucible for 2 h at 600°C in a muffle furnace. After cooling down in an exsiccator, the residue was weighed. The ash content was calculated in relation to the initial sample weight.

pH value. A sample of TC powder or extrudate (ca. 5 g) was agitated in 100 ml distilled water for 6 h at 20°C. The supernatant was decanted and the pH measured using a glass electrode.

Water solubility. A sample of TC powder or extrudate (ca. 1 g) was agitated in an Erlenmeyer flask with deionized water (50 ml) for 16 h at 4°C or for 6 h at 60°C, respectively. Insoluble parts were filtrated and the dry mass of the filtration residue was determined. The water solubility was calculated as the ratio of the dry masses of filtration residue and weighed sample. All measurements were performed in duplicates.

Proteolytic degradation. TC powder samples (ca. 25 mg each) were incubated in 0.2 M NH$_4$HCO$_3$ buffer (pH = 7.8) at 23.5 or 37°C after addition of 0.01% aqueous trypsin solution. The reaction was interrupted and the residue after centrifugation dried at 60°C and weighed. The solubility of TC under the given conditions was calculated as the ratio of the dry masses of residue and initial weight. Samples were taken after 1, 2, 4, 8, and 24 h, and measurements were performed in duplicates. As control, the solubility in buffer alone without addition of trypsin was determined.

Amino acid profile. A protein sample (TC powder or extrudate) (ca. 1.5 mg) was treated with 1.5 ml 6 N HCl for 20 h, then dried and the residue dissolved in lithium citrate buffer (pH 2.2). Thirty microliters of the resulting amino acid solution were injected into an amino acid analyzer "Biochrom 30 plus" (Onken, Gründau, Germany), where the amino acids were separated chromatographically, derivatized with ninhydrin, and detected at wave lengths 440 and 570 nm. The concentration of each amino acid was calculated from the corresponding peak areas at 440 nm, according to calibration with an amino acid standard. The method does not allow the distinction between Asn and Asp (denoted as Asx) or Gln and Glu (denoted as Glx), and Trp is not detected.

DSC measurements. The sample (TC powder or extrudate) was soaked in phosphate buffer (pH 7) for 2–5 h. DSC measurement was performed in a differential scanning calorimeter "DSC7" (Perkin Elmer, Waltham, MA) using an aluminum crucible in a temperature interval from 15 to 100°C with a heating rate of 5°C/min. Onset and maximum of peaks were determined from the graphical display of heat flow vs. temperature. For comparison, bovine gelatin B was used.

Viscosity of TC melts. Pellets of TC-based mixtures with varying water content were processed using a single screw extruder "Rheomex 302" ($L/D = 33$, four heating zones), combined with a melt pump and a slit die provided with pressure sensors (PolyLab OS system, ThermoFisher Scientific, Karlsruhe, Germany). Two different dies were applied. A single slit die (slit dimension 18 × 0.8 mm, three pressure sensors) allowed the determination of shear viscosity only, whereas an extension die ("X-die," slit dimension 20 × 2 mm to 5 × 0.5 mm, four pressure sensors) was used in order to record shear and elongational viscosity simultaneously.[22] If not mentioned otherwise, the extrusion temperature was 90°C. The shear rate was controlled by the

melt pump (from 0.1 to max. 50 rpm in logarithmic steps), covering a shear range from 0.16 to 5238 s^{-1} (exception: measurement of TC$_{MW}$ with 40 and 60% water was performed in linear steps in the range from 10.2 to 512.3 s^{-1}). Each mixture was measured at least five times. The shear and elongational viscosities were calculated from the measured pressure differences using the integrated software "PolySoft OS 2.2.0.20" (Thermo-Fisher Scientific, 2006). Multiple measurements were averaged and displayed graphically using double logarithmic diagrams.

Mechanical properties of TC films. Tensile strength and elongation at break were determined using a tensile test equipment LRX5K (Ametek GmbH, Germany). Film samples were equilibrated in standard climate (23°C, 50% RH) for 24 h. Dumbbell-shaped test specimens (free clamping length 50 mm, width 10 mm) were cut out of the films along and across the extrusion direction. Strain was applied with a speed of 100 mm/min until break, and the stress-strain diagram was recorded. Results of five measurements were averaged.

RESULTS AND DISCUSSION

Production of TC

Raw Material. Although the experiments which are reported here were performed with TC, based on cattle hide of different origin, the method is not limited to bovine material. Production of TC and thermoplastic processing were just as successfully accomplished with porcine skin. Because cattle hide was easier to obtain, we concentrated on this material. Generally, the reported technology should be applicable to collagen of any species.

Denaturation of Collagen. The preparation of TC powder and its subsequent thermoplastic treatment are subject of an international patent (US 20090226557).[15] TC was prepared from untanned bovine pelts. The preparation of pelts from cattle hide is a standard beamhouse process and well established in the tanning industry. With regard to possible technical applications of TC in the future, tanneries can serve as a convenient source of raw material for the TC production. Therefore, the beamhouse process was adopted and not altered. The crucial step in the conversion of collagen from a fibrillar constituent of a pelt into a thermoplastically processable biopolymer is a denaturation step. It consists in a treatment in which collagen is partially denatured by energy input. In our experiments, three different denaturation methods were used which differ in type and origin of the energy input. Method A (heating in excess hot water) is characterized by the use of thermal energy, method B (extrusion) combines thermal and mechanical energy, whereas method C (microwave radiation) is also mainly a thermal process. In the patent US 20090226557, denaturation is achieved by drying at high temperature (80°C) in a rack dryer or furnace. One of our purposes in the development of the TC production process was the separation of drying and denaturation. Treatment in a rack dryer does not meet this demand, because both processes proceed simultaneously. Therefore, we considered this method less suitable for our intention. We refer to this method as denaturation method D but do not include corresponding TC batches in our report. Scheme 1 shows an overview of the methods. Within these groups of variously denatured TC, differ-

ent batches were produced with varying technological parameters, as described in the Experimental Section.

Characterization of TC Powder

Four batches of TC powder were characterized regarding the following parameters: composition (moisture, protein, fat, and ash content), pH, degree of degradation (DSC profile, amide nitrogen content), and water solubility (at 4 and 60°C, respectively). Table I shows the determined values.

Composition. In contrast to commercially available isolates of, for example, plant proteins, TC powder is a material of high purity. Approximately, 93-98% of the dry matter consist of collagen. The only minor constituents are fat (determined as DCM soluble substances) and salts or mineral compounds (determined as ash). Batch TC$_{HW2}$ differs slightly from the others in a higher mineral content. Besides natural fluctuations in the raw hide, the liming process with its use of different salts is the main source of varying mineral content. Regardless of several drying steps in the course of TC production, each TC powder contains between 7 and 11% water. Although the different denaturation steps stress the protein material in a different kind, the amino acid composition is not affected. The percentage of the amino acids is identical for all investigated batches with deviations only in the first decimal place. Table II shows the averaged values. They are consistent with earlier literature data.[23,24]

Amide Nitrogen Content. The overall content of amide nitrogen is a measure for the degradation of collagen. Treatment of hide in the beamhouse process, especially intensive liming at basic pH, leads to the deamidation of glutamine and asparagine. The maximum value for amide nitrogen content, based on the amino acid composition of bovine collagen,[24] is 6.53 mg nitrogen per g protein, lower values indicate the degree of deamidation. This degree increases in a basic environment, which is confirmed by the data of Table I, showing lower amide content for slightly basic TC$_{HW2}$ and TC$_{MW}$, whereas the other TC batches with slightly acidic pH show amide values close to 6 mg N/g protein. However, the amide nitrogen content depicts only the state of the glutamine and asparagine side chains and the determination can be influenced by other, unspecific nitrogen sources. This value therefore reflects only a part of different possible degradation mechanisms. The effect can not be detected in the standard amino acid analysis (Table II), where the pairs Glu/Gln and Asp/Asn are not distinguished.

DSC Measurements. Differential scanning calorimetry profiles are an indicator for the existence of triple-helical fibrils in the material. Intact triple-helical fibrillar collagen shows denaturational transitions in the range of 53–60°C.[25,26] Transitions between 30 and 37°C, which were observed for all TC batches (Table I), indicate that the triple-helical structure is destroyed. The measurement can be used as an evidence of denaturation. For comparison, DSC profiles of bovine gelatin showed a peak at 35.1°C.

Water Solubility. For identical amino acid composition, the water solubility of a protein is correlated with the chain length
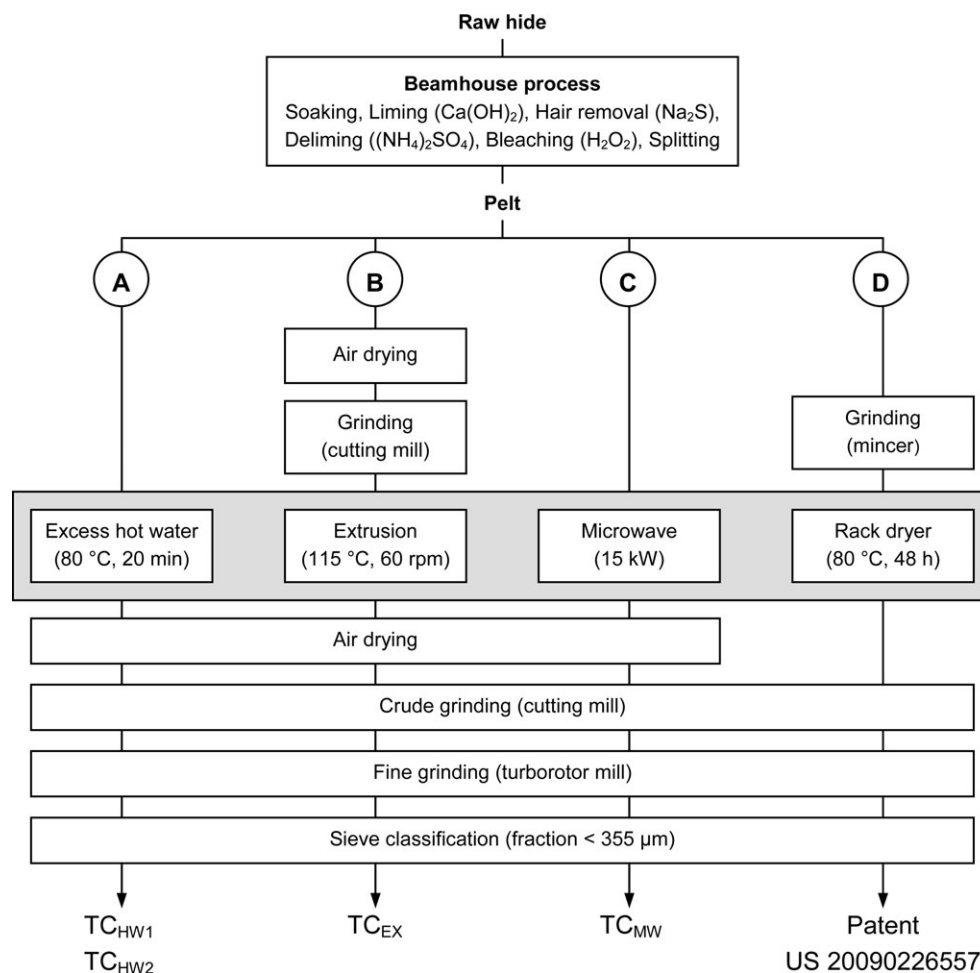
Scheme 1. Process for the production of TC powder (designation of the batches as described in the text); the different denaturation methods are highlighted in grey. Method D is described in the cited patent.[15]

and amount of cross links and can therefore be used as a rough measure for the degree of degradation. It has been shown earlier that thermo-mechanical treatment of collagen increases the solubility.[20] The data of Table I show that, in this respect, the degradation of TC_{EX} is most pronounced among the TC batches based on adult hide. However, it should be considered that during the denaturation process A, a part of the collagen already dissolves in the hot water. Thus the degradation of batches TC_{HW} may be higher than the water solubility suggests.

Proteolytic Degradation. The solubilities of TC batches in ammonium carbonate buffer showed the same tendency compared with the solubilities in pure water. TC_{EX} and TC_{HW1} are better soluble than TC_{HW2} and TC_{MW}. Generally, the solubility in buffer is lower than in pure water, which may be due to the slightly higher pH. The addition of trypsin significantly increases the solubility of collagen up to values above 90% in all cases. The reaction appeared to proceed fast, the final values being already reached after 1 h (data not shown). Only the

Table I. Characterization of Thermoplastic Collagen Powders

Batch no.	pH	Water (%)	Protein[a] (% d.m.)	Fat[a] (% d.m.)	Ash[a] (% d.m.)	N_{amide}[a] (mg/g d.m.)	Water solubility[a] (% d.m.) at 4°C	at 60°C	DSC[b] (°C)
TC_{HW1}	6.2	7.0	98.3	0.6	0.8	5.6	12.1	33.5	31.3
TC_{HW2}	8.7	10.3	93.2	0.9	4.0	4.9	9.6	20.4	33.4
TC_{EX}	6.9	11.2	98.6	0.7	1.0	6.0	35.4	44.2	31.6
TC_{MW}	7.5	10.8	96.5	0.3	1.9	4.0	6.5	31.7	33.4

[a]Value in relation to dry matter, [b]Onset of peak in DSC measurement (gelatin for comparison: 35.1°C).

Table II. Amino Acid Composition (in %) of Bovine TC Before and After Extrusion

Amino acid[a]	TC powder	Extrudate
Ala	11.1 (0.1)	10.7 (0.1)
Arg	5.1 (0.1)	5.0 (0.1)
Asx	4.7 (0.1)	4.7 (0.1)
Glx	7.1 (0.2)	7.3 (0.1)
Gly	33.1 (0.2)	33.1 (0.3)
His	0.5 (0.0)	0.5 (0.0)
Ile	1.3 (0.0)	1.3 (0.0)
Leu	2.6 (0.1)	2.6 (0.1)
Lys	2.7 (0.0)	2.7 (0.0)
Hyl	0.7 (0.1)	0.8 (0.0)
Met	0.6 (0.1)	0.7 (0.0)
Phe	1.3 (0.1)	1.2 (0.1)
Pro	12.6 (0.1)	12.8 (0.2)
Hyp	8.8 (0.2)	8.8 (0.2)
Ser	3.4 (0.1)	3.5 (0.2)
Thr	1.9 (0.2)	1.7 (0.1)
Tyr	0.4 (0.1)	0.4 (0.1)
Val	2.1 (0.0)	2.1 (0.1)

[a]Asn/Asp and Glu/Gln are not distinguished, Cys and Trp are absent in collagen.Hyl, hydroxylysine; Hyp, hydroxyproline.
Average of all investigated batches, deviations from average in parentheses.

batch TC$_{HW1}$ shows a further slight increase of the solubility up to 24 h. The final averaged values after 24 h (at 23.5/37°C) are TC$_{HW1}$ − 93.7/97.3%, TC$_{HW2}$ − 91.4/92.7%, TC$_{EX}$ − 98.2/99.1%, TC$_{MW}$ − 95.4/97.2%, showing only marginal differences between the batches. The values demonstrate that TC is generally well accessible for proteases like trypsin. Figure 1 compares the solubilities of the different TC batches in pure water (4°C, 16 h and 60°C, 6 h) and in ammonium carbonate buffer (23.5 and 37°C, 8 h) with and without addition of trypsin.

Thermoplastic Processing of TC
Plasticizer and Additives. TC can be plastified in a conventional extruder only after addition of a considerable amount of water as a plasticizer. TC based on adult hide requires quantities from 30 to 60% (in relation to the total mass of the TC/water mixture), depending on the individual batch. At lower water content, the thermal-mechanical energy input of the extruder is insufficient for plastification, at higher water content, the TC/water mixture resembles a suspension which can not be plastified. Water is the most effective plasticizer for collagen and indispensable for thermoplastic processing. However, extrudates of TC/water mixtures desiccate fast at storage under ambient conditions and become stiff and brittle. The addition of glycerol as a permanent plasticizer overcomes this problem. In order to avoid the subsequent migration of glycerol to the surface of the extrudate, its amount should not exceed 35% (in relation to the TC dry matter). Extruded mixtures of TC/water/glycerol tend to be sticky and adhesive that interferes with following processing

steps like calendering or film blowing. The addition of a small quantity of stearic acid appeared to avoid this disadvantage. About 1–2% stearic acid (in relation to TC dry matter) effectively act as a lubricant and separating agent. Mixtures of TC with water, glycerol, and stearic acid were successfully used in the preparation of collagen sheets and films.

Viscosity of TC Melts. In order to reduce the number of parameters, viscosity measurements of collagen melts were performed with simple mixtures of TC and water only, apart from one series with addition of glycerol. The results of these measurements are presented as the dependence of shear viscosity on shear rate and elongational viscosity on elongation rate in Figures 2, and 4–6. Viscosities were calculated based on measured pressure differences in the die using the software of the PolyLab system (see Experimental Section). This program provides the apparent viscosities. Because apparent and true viscosity differ only by a small proportionality factor and because the data were not used for the determination of absolute values, but only for comparative purpose, the apparent values were used throughout. The curves are not as smooth as comparable curves for synthetic polymers, but the run of the shear viscosity of TC is consistent with the behavior of a thermoplastic material. The nearly linear decrease of the viscosity with increasing shear rate in a double logarithmic display resembles the well known linear part of viscosity curves of conventional polymers, which can be mathematically described by the Cross or Carreau models.[27,28] A linear fit of the double logarithmic curves according to the power law ($\eta = K \cdot \gamma^{n-1}$) leads to indices n of 0.15–0.35, which is somewhat lower than for conventional polymers and comparable with biopolymers like soy protein or cellulose derivatives.[28–31] Therefore, it is appropriate to characterize melts of partially denatured collagen as thermoplastic material in a certain moisture and temperature range. The elongational viscosity is between 20 and 180 times higher than the shear viscosity, which is in a range known from other polymers, like polyethylene, polypropylene, polystyrene, or modified cellulose.[17,31,32]

TC enters the thermoplastic state only with a considerable amount of plasticizer. Water is the most effective additive in
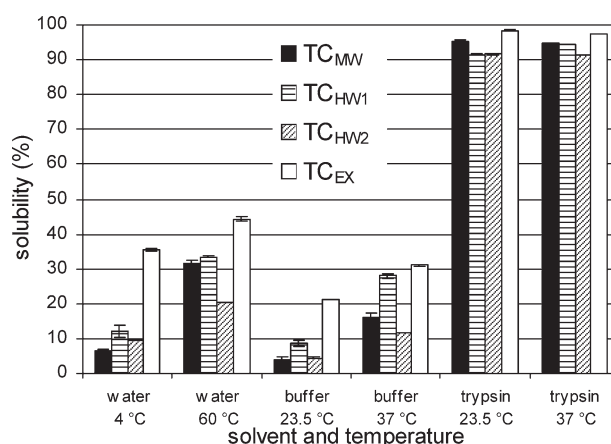

Figure 1. Solubilities (in % of dry mass) of TC powder in ammonium carbonate buffer with and without trypsin (23.5 and 37°C, 8 h) and in water (4°C, 16 h and 60°C, 6 h).
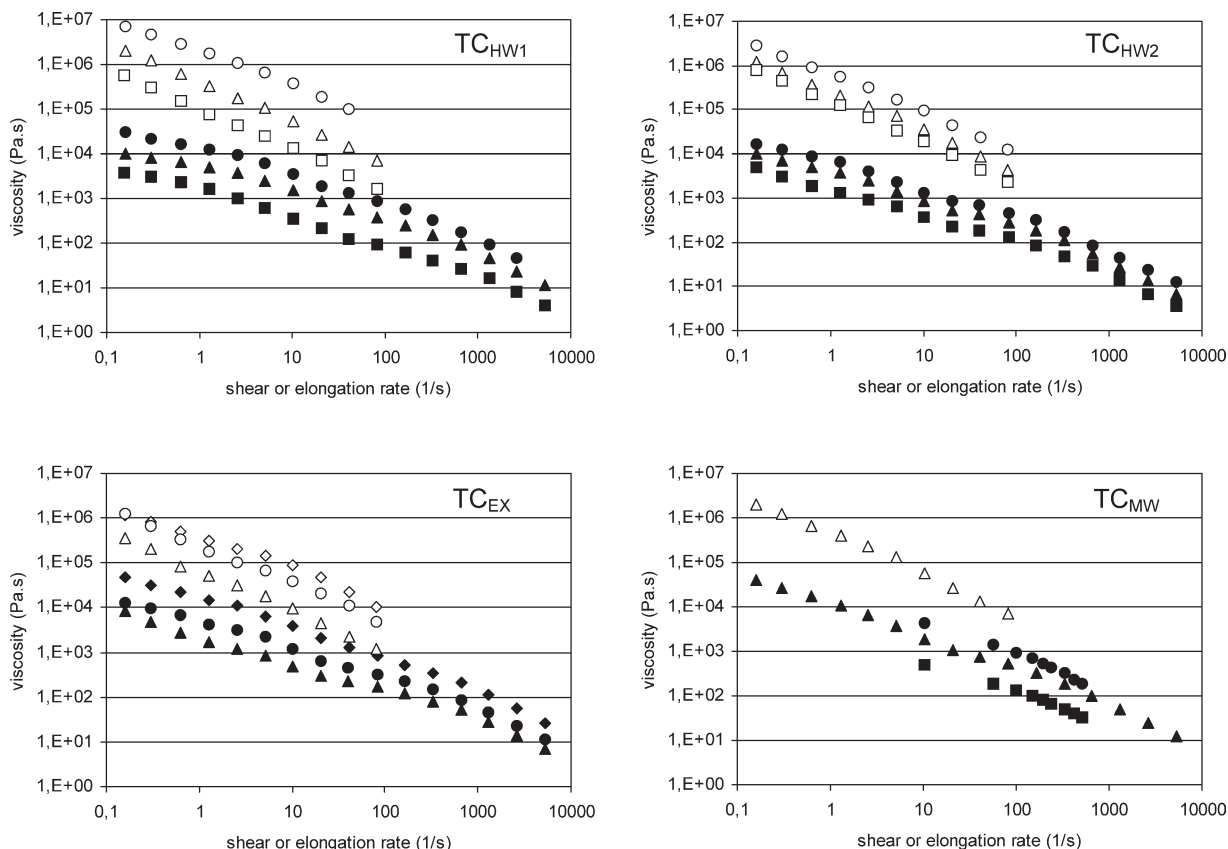
Figure 2. Shear viscosity (filled symbols) and elongational viscosity (empty symbols) of TC_{HW1}, TC_{HW2}, TC_{EX}, and TC_{MW} for different water contents at 90°C: 30% water (diamonds), 40% water (circles), 50% water (triangles), and 60% water (squares).

this respect. Figure 2 shows the influence of various amounts of water on the viscosity of TC melts. By adjusting the water content, the melt viscosity can be changed over a range of at least one order of magnitude. The particular influence of the water content on the viscosity values varies considerably with the individual TC batches. Figure 3 shows the shear viscosities of
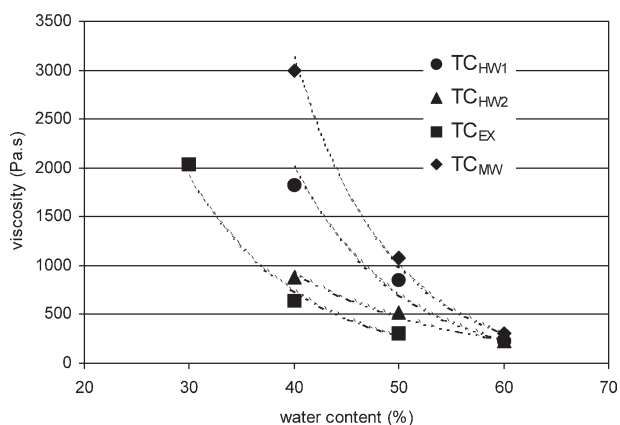

Figure 3. Influence of water content on the shear viscosity of different TC batches at 90°C at a shear rate of 20 s^{-1}: TC_{HW1} (circles), TC_{HW2} (triangles), TC_{EX} (squares), and TC_{MW} (diamonds). The dashed lines represent an exponential fitting of the measured data. The viscosity values for TC_{MW} with 40% and 60% water are interpolated from Figure 2.

the different measured TC/water mixtures at a shear rate of $20°s^{-1}$. This value complies approximately with the shear rate expected in an extruder under the conditions used here (screw diameter 18.5 mm, screw speed 120 rpm).[33] The data for TC_{MW} at a water content of 40 and 60% were interpolated according to Figure 2 because they had not been measured directly. The moisture dependence of the shear viscosity can be approximated by an exponential function, which resembles the temperature or pressure dependence known for other thermoplasts.[31,32] When comparing the batches TC_{HW1}, TC_{HW2}, and TC_{MW}, which were processed in the same moisture range, it is obvious that there is a distinct difference in the water content dependence. Although these three batches show similar shear viscosities with 60% water content, they clearly differ at 40%. This different behavior is not due to the denaturation method in the TC production process, because even the identically produced batches TC_{HW1} and TC_{HW2} differ considerably.

Glycerol which is often reported to be used as plasticizer for proteins, such as wheat gluten,[4,34–36] is less effective with collagen. Figure 4 shows the effect of glycerol addition in comparison with the addition of the same amount of water. There is almost no change in the shear viscosity, indicating that glycerol does not improve significantly the fluidity of the protein melt. In maintaining permanent flexibility of the extrudates, it acts rather as a hygroscopic agent than as a plasticizer itself. This fact is supported by the observation that it is impossible to
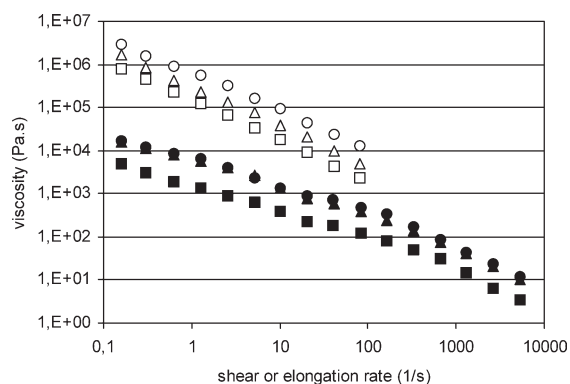
Figure 4. Influence of water and glycerol content on the shear viscosity (filled symbols) and elongational viscosity (empty symbols) of TC_{HW2} at 90°C. TC/water/glycerol mass ratios of the mixtures are 1/0.67/0 (circles), 1/0.67/0.83 (triangles), and 1/1.5/0 (squares).

Figure 6. Shear viscosity (filled symbols) and elongational viscosity (empty symbols) for different TC batches with equal water content (50%) at 90°C: TC_{HW1} (circle), TC_{HW2} (diamond), TC_{EX} (square), and TC_{MW} (triangle).

plastify collagen with glycerol alone, water being essential for the thermoplastic process. It is known that water plays a crucial role in the stability of the collagen triple helix by formation of water bridges and hydration networks between the protein chains, and the amount of water significantly influences the collagen denaturation temperature and enthalpy.[37–39] The shear viscosity dependence of TC on the water content and the low effectivity of glycerol, which is not involved in hydration networks, are consistent with these observations. However, there is a distinct decrease in the elongational viscosity after glycerol addition, albeit less than in the case of water.

The temperature influence is little pronounced. Due to the high water content of the investigated TC mixtures. the possible temperature range of thermoplastic processing is rather limited. Thus, viscosity measurements have been made only between 90 and 110°C in order to avoid abrupt water evaporation and combustion of collagen. Figure 5 shows that the shear viscosity is influenced by temperature to a much lesser extent in comparison with varying water content.

A certain influence of the TC powder itself can be seen when comparing the viscosity curves of different TC batches. Figure 6 shows the shear and elongational viscosities of four TC batches
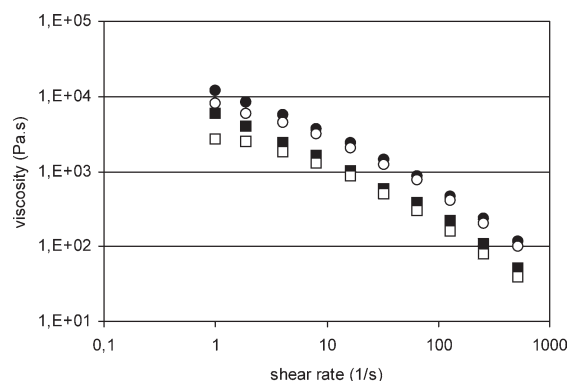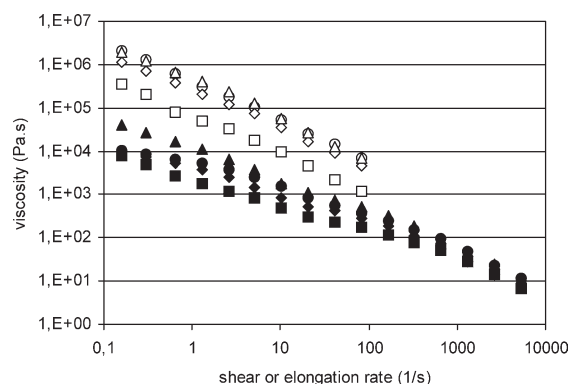

Figure 5. Influence of temperature on the shear viscosity of TC_{HW1}: mixtures containing 40% (circles) and 50% water (squares) were measured at 90°C (filled symbols) and 110°C (empty symbols).

with identical water content of 50%. While TC_{MW} yields the most viscous melt under these conditions, the TC_{EX} melt is the most fluid, the curves of the TC_{HW} batches lying between them. The viscosity differences between the two outer batches cover a range of nearly one order of magnitude, which is similar to the effect of varying water content. To put it another way, in order to reach the shear viscosity of 50% water containing TC_{EX}, TC_{MW} requires 60% water (Figure 3). The origin of these differences is not clear. The most probable approach assumes the degree of degradation as the underlying parameter. An interpretation of the viscosity measurements in this respect leads to the conclusion that batch TC_{EX} is more degraded than the other batches, whereas TC_{MW} is the batch with the lowest degree of degradation. Hence, TC_{EX} is easier to plastify and shows lower viscosity at a given water content. Because the raw material is the same in all cases (grain split), the difference should be mainly due to the denaturation process. Obviously extrusion is more effective in denaturation of collagen than treatment in excess hot water or by microwave. This can be explained by the simultaneous input of thermal and shear energy during extrusion in comparison to merely thermal energy in the other processes. This conclusion agrees with the above mentioned results concerning the water solubility. However, even the two TC_{HW} batches, based on the same raw material and denatured under identical conditions, are not completely congruent. That means that there are structural differences in the TC material, which can not be ascribed to the denaturation process but must be sought in the raw material. Cattle hide is a natural product. Its properties depend on a lot of parameters, such as age, race, geographical background, and nutrition of the cattle, so that it is impossible to standardize the material in a way that the resulting TC gives exactly the same results. This natural deviation has to be considered when using TC as a starting material. The comparison of the elongational viscosities shows the same tendency as the shear viscosities, but the difference between TC_{EX} and the other batches is more expressed. For polymers like polyethylene variations in the elongational viscosity are usually connected with the degree of branching.[17] This concept hardly applies to collagen chains, which are generally linear except for potential cross-linking. Further research concerning the

Table III. Characterization of TC Pellets After Extrusion

Batch	Ratio TC : water (calculated) before extrusion	pH	Water (%)	Protein[a] (% d.m.)	Fat[a] (% d.m.)	Ash[a] (% d.m.)	N_{amide}[a] (mg/g d.m.)	Water solubility (% d.m.)[a] at 4°C	Water solubility (% d.m.)[a] at 60°C	DSC[b] (°C)
TC_{HW1}	40 : 60	6.3	58.6	97.9	0.2	1.0	4.2	20.4	37.9	35.4
TC_{HW2}	60 : 40	8.8	39.2	93.6	0.2	3.8	3.7	3.2	9.9	32.0
TC_{HW2}	40 : 60	8.8	59.5	90.6	0.3	3.9	3.0	1.5	10.5	31.7
TC_{EX}	50 : 50	6.9	50.4	96.4	0.1	1.0	4.0	5.4	25.2	33.0
TC_{MW}	50 : 50	7.0	49.8	93.4	0.0	1.8	3.7	0.7	11.7	36.1

[a]Value in relation to dry matter, [b]Onset of peak in DSC measurement (gelatin for comparison: 35.1°C).

molecular collagen structure in the melt is necessary in order to explain differences in the elongational viscosities and technological behavior in forming processes like film-blowing.

Characterization of TC Extrudates

Composition. The comparison of the analytical data of the extrudates (Table III) with the starting TC powders (Table I) shows only small differences. It can be seen that the protein and fat contents decrease after the extrusion, but this effect is hardly significant. The relatively moderate extrusion temperature obviously does not induce severe modifications in the protein material. This feature is confirmed by the comparison of the collagen amino acid composition before and after extrusion (Table II). The percentage of amino acids is not changed by the process. As with the starting TC powders, there is no significant difference in composition between the particular extrudates, irrespective of TC origin or water content, so that Table II contains the averaged values. The determined water content of the extrudates (Table III) decreased in regard to the total water content of the mixtures before extrusion (i.e., moisture of TC powder plus added water amount). That means that a part of the water evaporated during the extrusion. This amount increases with decreasing water content of the pre-extrusion mixture. It comprises in average 6.9% for mixtures with 60% added water (TC_{HW1}, TC_{HW2}), 9.8% for mixtures with 50% added water (TC_{EX}, TC_{MW}), and 15.1% for the mixture with 40% added water (TC_{HW2}).

Amide Nitrogen Content. Amide side chain degradation which has been already observed in the TC powders, increased by extrusion. The extent of this effect differs, covering a range of 10-40% in regard to the starting value, but no dependence on material or water content can be deduced. As with the TC powders, this effect can not be seen in the amino acid profile (Table II). In summary, it can be stated that thermal-mechanical treatment slightly increases the deamidation of collagen.

Water Solubility. The extrusion process obviously induces structural modifications which are not detectable on the level of chemical composition. The water solubility of the extrudates is in most cases lower than that of the corresponding TC powders. The effect of decreasing protein solubility after heat or pressure treatment is well known for other proteins, such as wheat gluten.[40–43] It is reported to be due to the formation of additional cross-links via hydrophobic interactions or disulfides or even

peptide bond formation. Although in wheat gluten, the disulfide route plays a major role, in cysteine-free collagen other mechanisms should be dominant. An exception of this tendency presents TC_{HW1}, which shows a slightly increased solubility after extrusion. The reason for this different behavior is not clear. Further investigations are needed to evaluate the underlying processes.

Mechanical Strength. The mechanical properties of extruded TC are inferior to synthetic polymer materials. Data concerning the tenacity and elongation of threads, produced from mixtures of TC, water, and glycerol, were presented in the literature.[21] Without further treatment like cross–linking, these threads are mechanically not durable. The tensile properties of TC films confirm these data. Films with thicknesses ranging from 65 to 130 μm, were produced from different TC batches, denatured in hot excess water (TC_{HW}), because extrudates of TC denatured by extrusion could not be blown to large films. A small amount of stearic acid (2% in relation to TC mass) in the starting mixture served as a separating agent in order to reduce the stickiness of glycerol containing TC films. The mechanical stability of pure TC films depends on the starting material. When using different TC batches with identical production conditions (denaturation in hot excess water), the tensile strength of resulting films varied from 7 to 40 MPa. This limited reproducibility makes the evaluation of mechanical properties of TC films difficult. Table IV shows typical results, but one must have always in mind that the use of another raw material may lead to different values. The data show that the tensile strength of glycerol free films is comparable with that of synthetic polymers, like polyethylene or polypropylene, whereas the elongation is much lower.[44] There is a slight difference in the mechanical parameters depending on the measuring direction: tensile strength along the extrusion direction is lower than across, and vice versa for the elongation. Addition of glycerol significantly decreases the tensile strength (except a slight increase at low concentrations below 10%), whereas the elongation increases. Glycerol is an important additive in TC-based mixtures for films, because it serves as permanent plasticizer and maintains the flexibility of the otherwise fast desiccating film. Commonly, amounts of 25% glycerol (in relation to TC mass) are used. Films with this composition have a low tensile strength, and the elongation is comparable with that of polyvinylchloride.[44] Another drawback is the high sensitivity to moisture. The

Table IV. Tensile Strength and Elongation at Break of TC$_{HW}$-Based Films with Different Amounts of Glycerol

Glycerol content (% of TC mass)	Climate[a]	Measuring direction in relation to extrusion direction	Tensile strength (MPa)[b]	Elongation at break (%)[b]
0	Standard	Along	23.0 (5.5)	1.8 (0.5)
		Across	31.3 (2.5)	1.6 (0.4)
10	Standard	Along	26.2 (1.9)	1.7 (0.1)
		Across	32.9 (3.4)	2.1 (0.4)
20	Standard	Along	11.9 (1.0)	23.9 (6.6)
		Across	19.0 (0.8)	12.4 (1.2)
25	Standard	Along	9.3 (1.0)	41.2 (5.2)
		Across	15.0 (1.3)	16.8 (5.2)
30	Standard	Along	6.8 (0.7)	55.9 (8.6)
		Across	9.1 (1.0)	30.7 (2.9)
25	Saturated	Along	0.4 (0.1)	82.6 (12.1)

[a]Standard climate is 23°C, 50% RH, [b]Standard deviations in parentheses.

tensile strength of TC films is almost completely lost in a water saturated atmosphere.

Thermoplastic Forming. Independent on the starting material or the denaturation method TC is suitable for injection molding. Differently formed parts can be produced by this technique, for example collagen-based chewing bones for dogs. The extrusion technique makes higher demands on the starting material and offers a great variety of extrudate forms depending on the used die. The extrusion of endless strands by pressing the protein melt through a nozzle is the simplest way of forming plasticized collagen. Independent on the used TC batch within the particular moisture and temperature range strands were smoothly extruded. After cooling down, the strands were pelletized and these pellets used for further characterization. By decreasing the nozzle diameter down to 0.5 mm and less, flexible collagen threads were extruded which can be spun to fibers. This technique is reported elsewhere.[21] To obtain large sized polymer films, film blowing is the method of choice and well established in the processing of synthetic polymers. However, in the case of collagen, only a small part of all produced TC batches could be successfully blown to large sized films after extruding the collagen melt through a ring-shaped die. Obviously, the suitability of a TC melt for film blowing strongly depends on the preceding denaturation method. Thus, it was in no case possible to blow extrudate tubes of TC batches, denatured by extrusion. Extrudates of TC batches, denatured in hot excess water, appeared to be blowable in 50% of cases. This fact demonstrates that even identically produced TC batches differ significantly in this respect. Addition of up to 10% gelatin to the mixture before extrusion considerably improved the ability of film blowing. The reason of the different behavior of the various TC batches is not clear and requires more detailed investigation and material characterization in the future.

Possible Applications of TC. The results of the mechanical testing show that pure TC films are only partially suitable for applications under mechanical stress or in a moist environment. However, TC can be combined with synthetic polymers, which increase the mechanical strength and decrease the moisture sensitivity (data not shown). For example, in recent trials, we demonstrated that large sized films, produced from a blend of TC with the biodegradable polyester Ecoflex® (BASF), can be successfully used in the agricultural sector as mulching films for plants with a short growth period, such as field salad. Threads of TC may be used in medical applications as filaments or textiles after additional treatment, which increases the mechanical and biological stability.[21] Further potential applications are packaging material with short time use, where mechanical stress and moisture exposure are marginal, or products in the pet food industry, e.g., chewing bones for dogs, made of TC by injection molding.

CONCLUSION

The partial denaturation of limed, but untanned pelts with subsequent fine grinding led to a collagen powder called TC, which can be processed by established thermoplastic technologies, such as extrusion. Although the experiments reported here were based on cattle hide, the technique is not limited to bovine material. The process has also been successfully performed with porcine TC and can be probably expanded to other species. Viscosity measurements of the melt under extrusion conditions show that TC can be considered as a thermoplastic material. The properties of the protein melt and its plasticity mainly depend on the raw material and the type of the denaturation process. It appears that denaturation by extrusion degrades the material to a great extent such that the extrudate shows decreased mechanical stability and can not be blown to large-scaled films. Denaturation in hot excess water is an energy consuming batch process with high amounts of waste water, which is coupled with loss of collagen by dissolution and can not be completely controlled, yielding products of varying quality. The optimal degradation technique appears to be microwave treatment, which can be easily performed as a continuous process. Its efficiency is comparable with the hot excess water process. Plastification of TC can be achieved only by addition of a considerable amount of water as plasticizer. Other useful additives

are glycerol as permanent plasticizer and stearic acid as lubricant. Collagen melts can be processed into various forms, like strands, threads, bands, or films. The low mechanical strength and high moisture sensitivity of pure TC products demand additional treatment, like cross linking or blending with synthetic polymers, in order to improve these properties. Potential applications of TC-based products can be imagined in different technical sectors, like packaging, agriculture, medicine, or pet food, where biocompatibility and biodegradability of TC are advantageous.

ACKNOWLEDGMENTS

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