Electronic Supplementary Information for

A Green Process for the Specific Decomposition of Chicken Feather Keratin into Polythiol Building Blocks

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2 General Section

2.1 Sequence Alignment of the four Chicken Feather Keratins

Figure S1 shows a sequence alignment of the Chicken Feather Keratins 1–4 (CFKs 1-4) with the UniProt accession numbers P02450 (CFK1), P04458 (CFK2), P20307 (CFK3), and P20308 (CFK4).

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Feather Keratin 1 P02450   MSCFDLCRPCGPTPLANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAAGSS          60
Feather Keratin 2 P04458   MSCYDLCRPCGPTPLANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAVGSS          60
Feather Keratin 3 P20307   MSCFDLCRPCGPTPLANSCNEACVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTLVGSS          60
Feather Keratin 4 P20308   MSCYDLCRPSAPTPLANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAVGSS          60
                           ***:*****..********** ********************************* .***

Feather Keratin 1 P02450   TSAAVGSILSEEGVPISSGGFGISGLGSRFSGRRCLPC           98
Feather Keratin 2 P04458   TSAAVGSILSEEGVPISCGGFGISGLGSRFSGRRCLPC           98
Feather Keratin 3 P20307   TSAAVGSILSEEGVPISSGGFGISGLGSRFSGRRCLPC           98
Feather Keratin 4 P20308   TSAAVGSILSEEGVPISSGGFGISGLGSRFSSRRCLPY           98
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Figure S1. Sequence alignment of the Chicken Feather Keratins 1-4. The sequences were taken from the UniProt database[1], and the alignment was performed using Clustal Omega.[2]

2.2 Fragmentation Patterns of Chicken Feather Keratin

Figure S2 shows the theoretical fragmentation of Chicken Feather Keratin 1 after cleavage by selected proteases.

CFK1 fragments after cleavage with chymotrypsin

M | SCF | DL | CRPCGPTPL |
ANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPIL |
SSFPQNTAAGSSTSAAVGSIL |
SEEGVPISSGGF | GISGL | GSRF |
SGRRCLPC

CFK1 fragments after cleavage with trypsin

MSCFDLCRPCGPTPLANSCNEPCVR | QCQDSR |
VVIQPSPVVVTLPGPILSSFPQNTAAGSSTSAAVGSILSEEGVPISSGGFGISGLGSR |
FSGR | RCLPC

CFK1 fragments after cleavage with papain

MSCFDLCRPCGPTPLANSCNEPCVR |
QCQDSRVVIQPSPVVVTLPGPILSSFPQNTAAGSSTSAAVGSILSEEGVPISSGGFGISGLGSRFSGRRCLPC

CFK1 fragments after cleavage with pepsin

MSC | F | D | L |
CRPCGPTP |
LANSCNEPCVRQCQDSRVVIQPSPVVVTL |
PGPIL | SSF |
PQNTAAGSSTSAAVGSI | L | SEEGVPISSGG |
F | GISG | L | GSRF | SGRRCLPC

Figure S2. Cleavage sites of selected proteases at Chicken Feather Keratin 1 (CFK1).

2.3 Information on the used enzymes

All enzymes used are commercially available preparations with the following properties (manufacturer´s information):

Trypsin (CAS 9002-07-7) was obtained as a lyophilised powder derived from porcine pancreas with an activity of 1,000-2,000 BAEE units mg^{-1} solid in a "suitable for cell culture" quality.

Chymotrypsin (CAS 9004-07-3) was obtained as a lyophilised powder derived from bovine pancreas with an activity of ≥ 1,000 USP-U mg^{-1} solid in a "suitable for biochemistry" quality. It had a trypsin activity of ≤ 25 USP-U $mg^{-1,}$ and the optimal pH is around pH8.

Papain (CAS 9001-73-4) was obtained as a lyophilised powder derived from *Caprica papaya* with an activity of > 30,000 USP-U mg^{-1} in a "suitable for biochemistry" quality.

Pepsin (CAS 9001-75-6) was obtained as a lyophilised powder derived from pigs' stomachs with an activity of ≥ 0.5 E mg^{-1}, Ph. Eur. in a "suitable for biochemistry" quality.

Protease A-01 (CAS 9014-01-1) was obtained as a brown liquid derived from a genetically modified *Bacillus sp.* strain with an activity of 1,100 U mL^{-1}. It is an alkaline protease for splitting insoluble protein-containing residues into easily soluble or dispersible peptides and has a pH optimum of pH9.0.

Protease S-02 (CAS 9025-49-4) was obtained as a brown liquid derived from *Aspergillus niger* with an activity of > 50 U mL^{-1}, with a pH optimum of pH3.0. It is an acid protease typically used to mash flour-containing material.

Alcalase (CAS 56-81-5) was obtained as a liquid derived from *Bacillus subtilis* with an optimal pH between pH7.0 and pH8.5 and an activity of ≥5 U/g. It is often used in dehairing, bating leather or food processing or to improve the digestibility of animal feeds.

Savinase (CAS 9014-01-1) was obtained as a liquid, derived from a *Bacillus sp.* strain, with an activity of ≥ 16 U g^{-1} with a working pH range from pH7 to pH11. It is typically used for the extraction of animal proteins.

Esperase (CAS 9014-01-1) was obtained as a liquid derived from a *Bacillus sp.* strain, with an activity of ≥ 8 U g^{-1} with a working pH range from pH7 to pH11.

It has to be noted that the above-listed enzyme activities are determined using standardised substrates and cannot be readily transferred to keratin. Determination of the specific activities towards feather keratin of the enzymes investigated was not the focus of this study but is possible, e.g. after conversion of the feather keratin substrate to azokeratin as described by Gonzalo *et al.*[3]

3 Experimental Procedures

3.1 Enzymatic Hydrolysis of Chicken Feathers

In our study, the proteases alcalase, protease A-01, savinase, esperase, trypsin, chymotrypsin, papain, pepsin, and protease S-02 were tested. 0.5 g of pretreated chicken feathers were mixed with 8.5 mL of a selected aqueous buffer containing 10 mg of solid freeze-dried enzyme or 100 µL of the liquid enzyme. Table S1 summarises the respective buffers and reaction conditions.

Table S1. Buffers and reaction conditions for enzymatic hydrolysis of chicken feathers.

Enzyme	Buffer	pH	Hydrolysis Temperature [°C]	Additives
Alcalase	0.1 mol L^{-1} potassium phosphate[a]	8.0	60	
Protease A01	0.1 mol L^{-1} potassium phosphate	8.0	60	
Protease S02	0.05 mol L^{-1} citrate[b]	3.0	60	
Savinase	0.1 mol L^{-1} potassium phosphate	8.0	60	
Esperase	0.1 mol L^{-1} potassium phosphate	8.0	60	
Chymotrypsin	0.1 mol L^{-1} potassium phosphate [c]	7.8	50	10 mmol L^{-1} $CaCl_2$
Trypsin	0.1 mol L^{-1} potassium phosphate	7.8	50	10 mmol L^{-1} $CaCl_2$
Papain	0.1 mol L^{-1} potassium phosphate[d]	6.0	65	5 mmol L^{-1} L-cysteine
Pepsin	0.05 mol L^{-1} phosphate citrate[e]	2.2	37	
Control	0.1 mol L^{-1} potassium phosphate	7.8	50	

[a] 0.0935 mol L^{-1} dipotassium hydrogen phosphate, 0.0065 mol L^{-1} potassium dihydrogen phosphate. [b] 0.0094 mol L^{-1} sodium citrate dihydrate, 0.0906 mol L^{-1} citric acid. [c] 0.0855 mol L^{-1} dipotassium hydrogen phosphate, 0.0145 mol L^{-1} potassium dihydrogen phosphate. [d] 0.0138 mol L^{-1} dipotassium hydrogen phosphate, 0.0862 mol L^{-1} potassium dihydrogen phosphate. [e] 0.004 mol L^{-1} disodium hydrogen phosphate, 0,098 mol L^{-1} citric acid.

All buffers were prepared using desalted water with a specific resistance ≤ 18.2 MΩ/cm at 25 °C. Initially, a 100 mmol L^{-1} potassium phosphate buffer was used, as indicated in the table. During the study, the buffer strength was reduced to 50 mmol L^{-1}, and it was shown that adding the additives listed in the table could be omitted without affecting the hydrolysis result. The reaction batches were incubated in 50 mL polypropylene screw cap tubes under agitation (150 rpm) for up to 48 h at enzyme temperature optimum (see Table S1). The solutions were centrifuged at 4,142xg at 15 °C for 20 min to remove insoluble particles. Insoluble fractions were washed with desalted water, dried at 50 °C *in vacuo,* and weighed. The supernatants were freeze-dried, weighed, and used for analytics or subjected to reduction or film formation experiments. Negative controls were performed without the addition of enzymes. This workflow was used throughout the study and is illustrated in Figure S3.

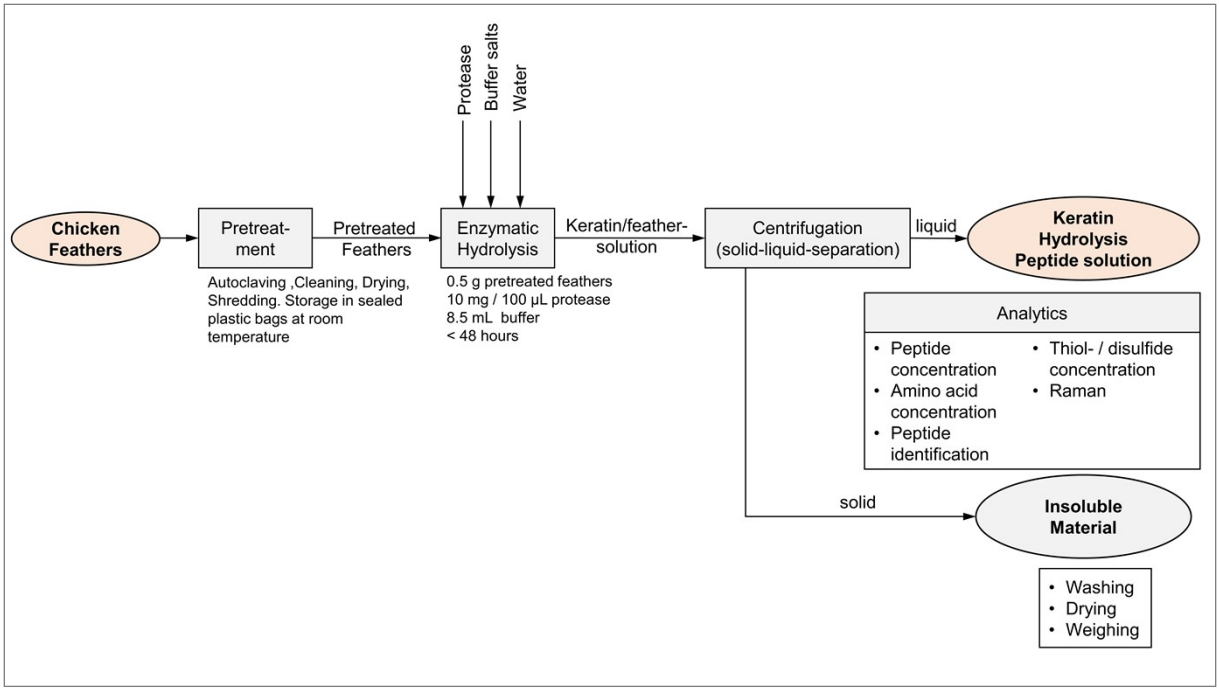

Figure S3. Workflow for the enzymatic hydrolysis of chicken feathers.

Combined hydrolysis approaches were performed by chemical hydrolysis, followed by preparing keratin hydrolysis peptides (KHPs) as described in this section.

Figure S4 summarises the workflow for the enzymatic hydrolysis of chicken feathers in a setup scaled to 25 L and the subsequent preparation of KHP films.

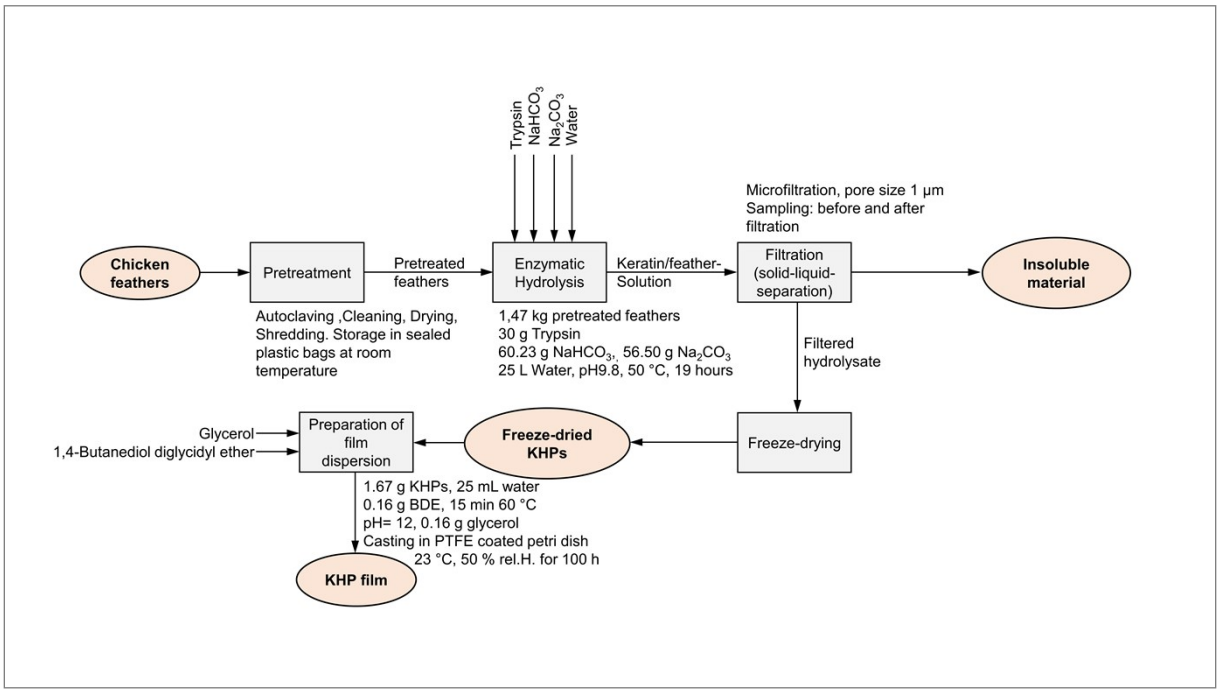

Figure S4. Workflow for the enzymatic hydrolysis of chicken feathers on a 25 L scale and preparation of KHP films.

3.2 Analytical Methods

3.2.1 Analysis of Protein Concentration

Peptide concentrations had to be measured in solutions with very different compositions. The biuret method was used after we could show that in the relevant protein concentration range around 20 mg mL^{-1}, substances such as urea, amino acids, or reducing agents only lead to an acceptable change in the results. For this purpose, 20 mg mL^{-1} bovine serum albumin (BSA), 50 mmol L^{-1} L-cysteine, 50 mmol L^{-1} L-serine, 50 mmol L^{-1} sodium sulfite or 4 mol L^{-1} urea, or combinations of the substances were dissolved in desalted water, and the protein concentrations were determined by the Biuret method. Table S2 summarises the results.

Table S2. Protein concentration measurements in different solutions

	Measured protein concentration [mg mL^{-1}]		Measured protein concentration [mg mL^{-1}]
Water	0.4	4 mol L^{-1} urea	0.4
20 mg mL^{-1} BSA	20.1	4 mol L^{-1} urea, 20 mg mL^{-1} BSA	19.9
20 mg mL^{-1} BSA, 50 mM L-cysteine	21.9	4 mol L^{-1} urea, 20 mg mL^{-1} BSA, 50 mmol L^{-1} L-cysteine	21.3
20 mg mL^{-1} BSA, 50 mM sodium sulfite	21.0	4 mol L^{-1} urea, 20 mg mL^{-1} BSA, 50 mmol L^{-1} sodium sulfite	21.4
20 mg mL^{-1} BSA, 50 mM L-serine	19.7	4 mol L^{-1} urea, 20 mg mL^{-1} BSA, 50 mmol L^{-1} L-serine	21.2

To determine protein concentrations, 50 mL of Biuret reagent[4] was prepared by first dissolving 0.30 mmol copper (II) sulfate pentahydrate in 25 mL desalted water. Under stirring, 1.1 mmol of potassium sodium tartrate tetrahydrate was added and dissolved. Then, 15 mL 3% (w/v) sodium hydroxide solution was added dropwise. Then, 0.90 mmol potassium iodide was added and filled to 50 mL with desalted water. 500 µL Biuret reagent was added to 250 µL of protein sample and mixed well. After 20 min at room temperature, 200 µL of each sample was added to a microtiter plate, and the absorbance at 540 nm was measured in triplicates. Solutions of bovine serum albumin in desalted water with concentrations between 0.5 g L^{-1} and 4.0 g L^{-1} were used for calibration, and the protein samples were diluted to an appropriate concentration before measurements.

3.2.2 Determination of Soluble and Insoluble Contents of Hydrolysis Solutions

From the protein concentrations, the masses of the respective dissolved peptides and amino acids were determined by multiplication with the volume of the hydrolysis mixture and set in relation to the originally weighed-in feather biomass and multiplied by 100. Thus, the percentage yields of soluble peptides and dissolved amino acids were determined. The insoluble portion of the chicken feathers remaining was weighed and also set in relation to the initial weight to determine the percentage of insoluble material.

3.2.3 Determination of Free Thiol and Disulfide Moieties

Based on the publications by Hansen *et al.*[5] and Kurz *et al.*[6], a modified HPLC method for detecting thiols and disulfides was established. This enabled us to determine the number of free thiols and disulfide bonds in aqueous peptide mixtures via reaction of thiols with 4,4´-dithiodipyridine (DTDP) to form 4-thiopyridine (4-TP), which could be quantified by measuring its absorption at 324 nm. Since we could not achieve sufficient separation of the analyte molecules by applying RP-HLPC methods described in the above publications, we identified HILIC (hydrophilic interaction liquid chromatography) as a suitable separation technology.

First, a 40 mmol L^{-1} DTDP solution was prepared as described by Kurz *et al.*[6] To prepare L-cysteine standards, 10 mmol L^{-1} L-cysteine hydrochloride monohydrate was dissolved in desalted water and diluted to concentrations between 5.0 mmol L^{-1} and 5.0 µmol L^{-1} with desalted water.

Sample Preparation for Thiol Quantification

100 µL of the sample or standard solution was mixed with 1.85 mL of a buffer solution containing 6 mol L^{-1} guanidine HCl, 100 mmol L^{-1} phosphate, and 0.2 mmol L^{-1} EDTA at pH = 5.0. 50 µL DTDP solution was added, mixed well, and incubated for 10 min at room temperature in the dark. The solution was filtered through a 0.2 µm syringe filter, and 1 mL was transferred to an amber HPLC glass vial and subjected to HPLC analysis.

Sample Preparation for Disulfide Quantification

A 30%(w/v) sodium borohydride ($NaBH_4$) solution in 1 mol L^{-1} NaOH was prepared. 150 µL of a buffer solution containing 6 mol L^{-1} guanidine HCl and 0.5 mol L^{-1} tris(hydroxylmethyl)aminomethane were transferred in a 1.5 mL reaction tube, and 10 µL of the sample solution was added. Subsequently, the solutions were incubated for 30 min at 45 °C and gentle shaking (200 rpm) to ensure proper protein denaturation. After 20 µL $NaBH_4$ solution was added, the reaction mixture was incubated for 60 min at 65 °C with shaking at 150 rpm. 50 µL n-octanol was added as an anti-foam agent. 76 µL of 6 mol L^{-1} HCl was added and mixed well by pipetting the entire volume up and down five times. 94 µL of a 750 mmol L^{-1} sodium phosphate buffer at pH = 5.0 was added. 100 µL of this solution was mixed with 1.85 mL of a buffer solution containing 6 mol L^{-1} guanidine HCl, 750 mmol L^{-1} sodium phosphate buffer, and 0.2 mmol L^{-1} EDTA at pH5.0. 50 µL DTDP solution was added, mixed well, and incubated for 10 min at room temperature in the dark. The solution was filtered through a 0.2 µm syringe filter, and 1 mL was transferred to an amber HPLC glass vial and subjected to HPLC analysis.

HPLC Analysis

HPLC measurements were performed on a Nexera X2 instrument (Shimadzu, Japan) with a Phenomenex Luna HILIC column (2 µM, 200 Å, 150 x 4.6 mm). The oven temperature was 40 °C, and the injection volume was 10 µL. Gradient elution was performed according to Table S3 at a 1 mL min^{-1} flow rate.

Table S3. The gradient used for the elution of 4-TP. Solvent A: 10 mM ammonium formate, pH = 3.00, adjusted with formic acid. Solvent B: Acetonitrile.

Time [min]	Solvent A [%]	Solvent B [%]
0	10	90
2	10	90
10	50	50
12	50	50
13	10	90
15	10	90

The reaction product 4-thiopyridine (4-TP) was detected at 2.47 min using a wavelength of 324 nm (Figure S5).

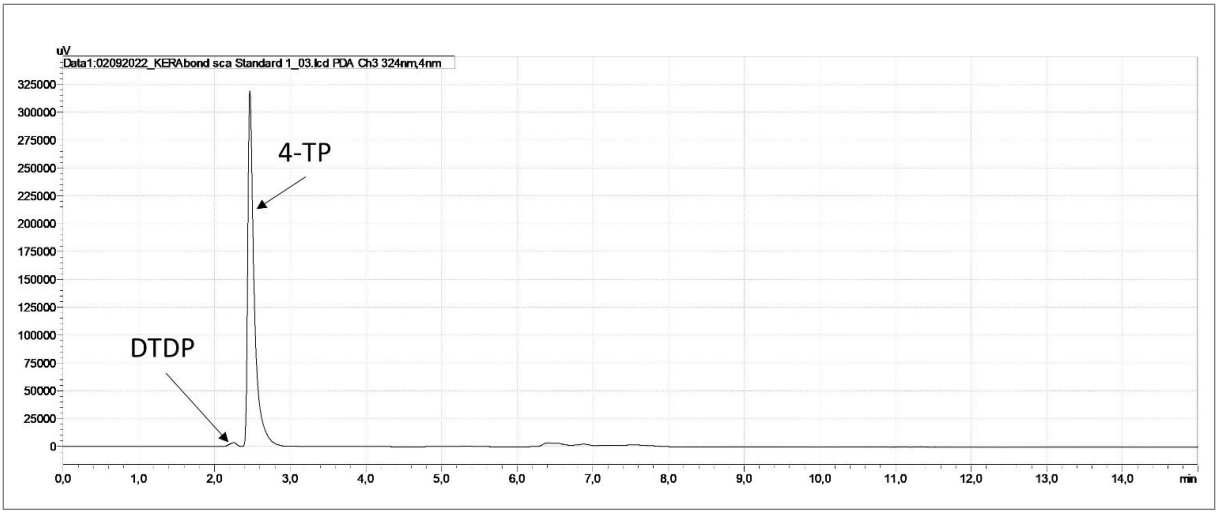

Figure S5. Chromatogram of a reaction mixture for thiol quantification containing a 10 mmol L^{-1} L-cysteine hydrochloride monohydrate and DTDP. The reaction product 4-TP was detected at 2.47 min using a wavelength of 324 nm.

The peak area monitored at 2.47 min was proportional to the 4-TP concentration in the standard samples. The peak areas were integrated using the Shimadzu LabSolutions software (version 5.99), and a reference curve, as shown in Figure S6, was obtained with the analysed L-cysteine hydrochloride monohydrate standards.

Sample solutions were analysed accordingly, and the 4-TP amount was calculated using the reference curve from analysing the L-cysteine standards.

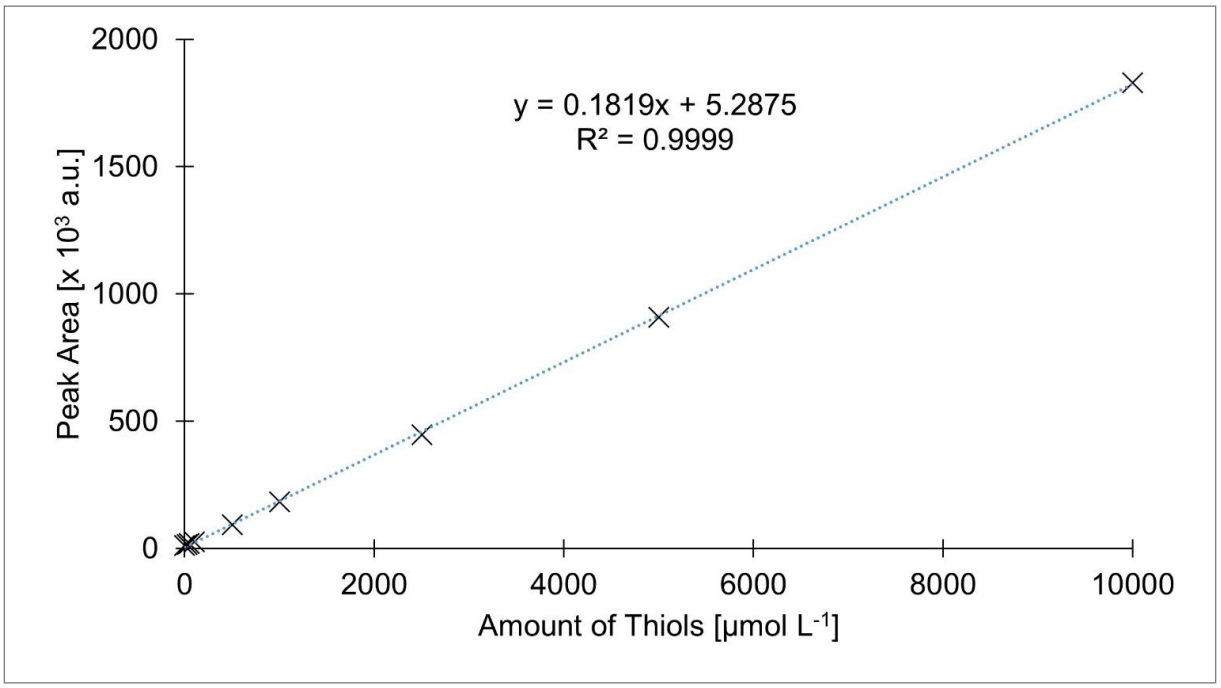

Figure S6. Peak Area as a function of the 4-TP amount in L-cysteine hydrochloride monohydrate standard solutions.

The number of thiols in the original sample was determined from the measured 4-TP amount, considering dilution during sample preparation.

The number of thiols present that were initially buried in disulfide bridges could be calculated by subtracting the measured free thiols from the total number of thiols measured after reduction with $NaBH_4$, according to equation (1).[6]

$$c_{RSSR}\left[\mu mol_{SH}\, g_{protein}^{-1}\right] = (c_{total\ thiols} - c_{RSH}) \quad (1)$$

3.2.4 SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970).[7] A denaturing and discontinuous procedure was used. The acrylamide gels were produced in-house and consisted of a stacking gel with 5% acrylamide and a 16% acrylamide separation gel, each with a degree of crosslinking of 2.6%. For electrophoresis, a polyacrylamide gel was clamped into the vertical electrophoresis unit. The buffer chamber was filled with 1xSDS running buffer (25 mmol L^{-1} Tris base, 0.192 mol L^{-1} glycine, 0.1 % (w/v) SDS). Liquid samples were diluted 1:2 in 2xSDS loading dye (30 % (v/v) glycerol, 15% (v/v) β-mercaptoethanol, 1.2 % (w/v) SDS, 150 mmol L^{-1} Tris base, pH6.8, 20 mg L^{-1} bromophenol blue) and heated to 95 °C for 5 min. Solid residues (20 mg) from hydrolysis experiments were mixed with 100 µL 2xSDS loading dye and heated to 95 °C for 30 min with constant mixing at 1,100 rpm. 5 to 10 µL of each sample and 10 µL protein standard (unstained protein standard, broad range (10-200 kDa), New England Biolabs) were loaded onto a gel. SDS-PAGE was performed with 40 mA per gel for about 45 min. After electrophoresis, the gel was transferred to a container with Coomassie Brilliant Blue staining solution (0.2% (w/v) Coomassie Brilliant Blue G250, 0.2% (w/v) Coomassie Brilliant Blue R250, 50% (v/v) ethanol, 10% (v/v) pure acetic acid) and shaken for up to 2 h. Destaining was achieved by shaking the gel in desalted water. A digital picture was taken for evaluation with the gel documentation system.

3.2.5 Sequence Analysis of Chicken Feather Keratins

Chicken feathers were pretreated and hydrolyzed as described above using the following conditions: 5 g chicken feathers, 85 mL desalted water, 8 mol L^{-1} urea, 50 mmol L^{-1} L-cysteine, pH10.5 for 19 h at 70 °C while shaking at 300 rpm. After centrifugation (4,000xg, 20 min, room temperature), discontinuous SDS-PAGE was performed as described in section 3.2.4. After Coomassie-staining, bands at 10 kDa were visible (Figure S7).

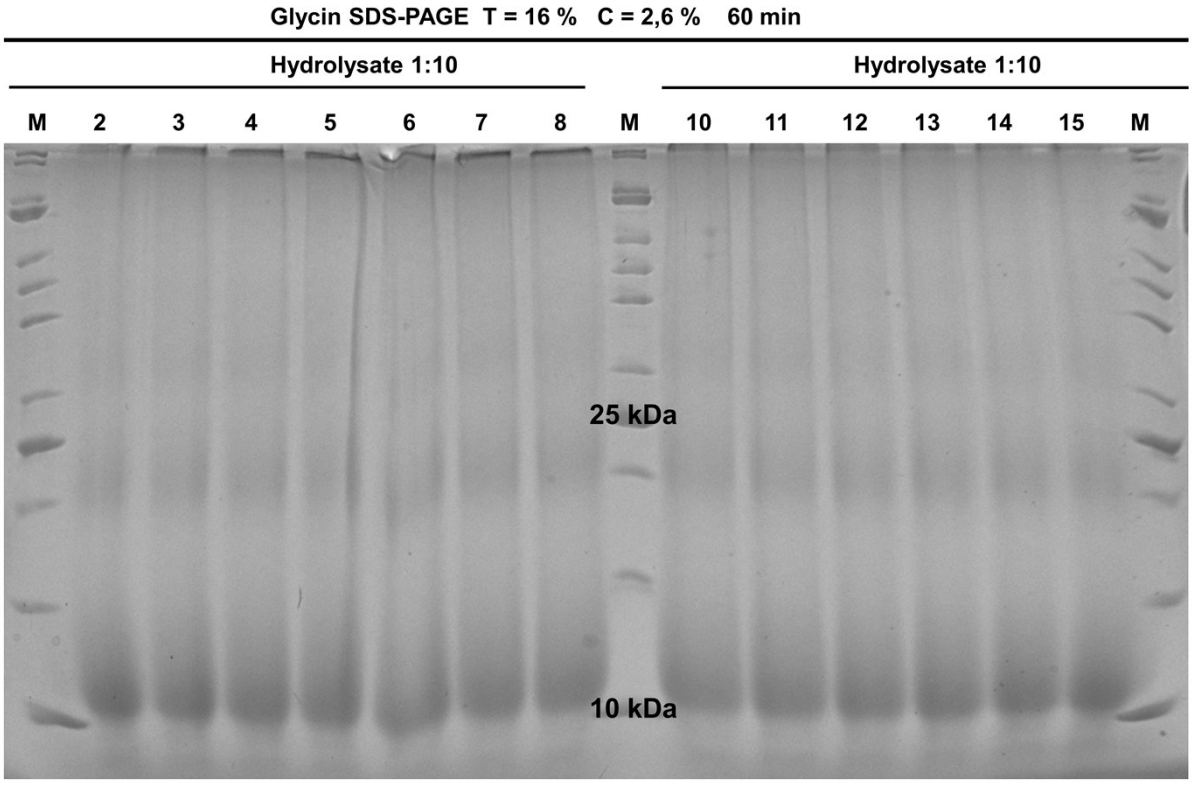

Figure S7. SDS-PAGE of chicken feathers dissolved with 8 mol L^{-1} urea and 50 mmol L^{-1} L-cysteine. M: Molecular weight standards. Lanes 2-8 and 10-15: Identical samples of the dissolved chicken feathers.

The stained 10 kDa bands were cut out of the gel, destained, and after a tryptic in-gel digest, the underlying 10 kDa proteins were identified as Chicken Feather Keratins 1 and 4 via nano liquid chromatography-electrospray ionisation tandem mass spectrometry (nanoLC-ESI-MS/MS). (Tryptic in-gel digest and all following analytical steps were performed by Proteome Factory AG, Berlin, Germany.)

3.2.6 Peptide screening after enzymatic hydrolysis of chicken feathers

Lyophilized chicken feather keratin hydrolysate was dissolved in desalted water, and a reduction with Tris-(2-carboxyethyl)-phosphine (TECP) was performed, followed by alkylation of free thiols with iodoacetamide. The peptide mixture was separated, and peptide masses and sequences were identified with nano-liquid chromatography-electrospray ionisation tandem mass spectrometry (nanoLC-ESI-MS/MS). Peptide identification was performed using the software PEAKS with a database containing the chicken feather keratin sequences and allowing the following modifications: Carbamidomethylation (57.02), deamidation (N, Q): 0.98), oxidation (M): 15.99, acetylation (N-term): 42.01. Peak areas from nanoLC-ESI-MS/MS were used to calculate the abundances of identified peptides (all steps were performed by Proteome Factory AG, Berlin, Germany).

4 Results and Discussion

4.1 The Influence of Autoclaving on the Hydrolysis Results

It was investigated whether the autoclaving of feathers influences the yields of soluble material during hydrolysis. Since unautoclaved feathers could not be used due to the microbial load, cleaned goose down was used. These were autoclaved and subjected to enzymatic hydrolysis with trypsin. In parallel, a hydrolysis without prior autoclaving and a negative control without enzyme were carried out.

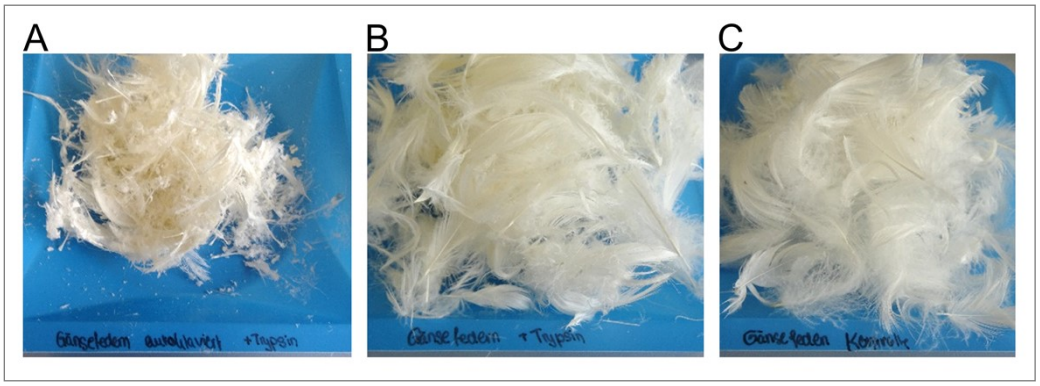

Figure S8. Residues after hydrolysis of autoclaved (A) or non-autoclaved (B) goose feathers with trypsin. Conditions: 50 mmol L-1 potassium phosphate buffer, pH7.8, hydrolysis time: 16 h at 50 °C, protease used: trypsin. Negative control (C) without the addition of the protease.

As can be seen in Figure S8, significant decomposition occurred when using autoclaved goose feathers, whereas no decomposition was seen in the non-autoclaved feathers or the control without trypsin. The yield of soluble material was 31.1% for A (autoclaved feathers) and only 6.6% for (B) non-autoclaved feathers.

4.2 Specific Proteases

The degradation of feathers by conventional serine and cysteine proteases, including subtilisin, papain, chymotrypsin, pepsin, and trypsin, was already shown by Ramnani & Gupta (2007)[8] in the presence of a suitable reducing agent (subtilisin, chymotrypsin, papain) or following pretreatment by any of the proteases (trypsin, pepsin).

In our study, we could demonstrate the hydrolysis of chicken feathers by trypsin, chymotrypsin, and papain with yields of soluble material of up to 47% (w/w) and without adding reducing agents (Figure S9).

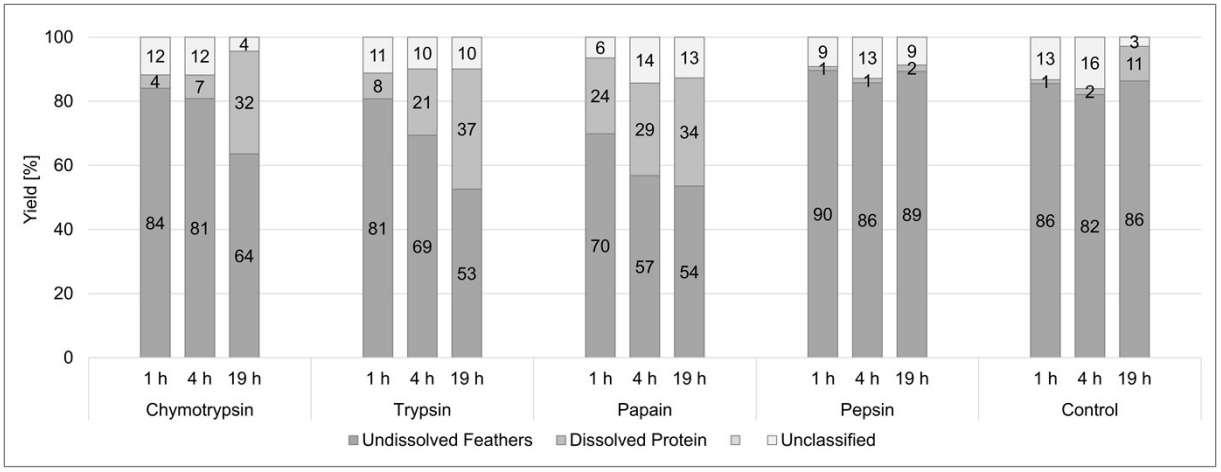

Figure S9. Specific enzymatic hydrolysis of chicken feathers. The percentages of soluble proteins or amino acids and insoluble residues are given in relation to the biomass used. The values given are mean values from duplicate determinations.

The addition of reducing agents and urea was also investigated, leading to an increased amount of soluble material of up to 89% (w/w) (Figure S10).

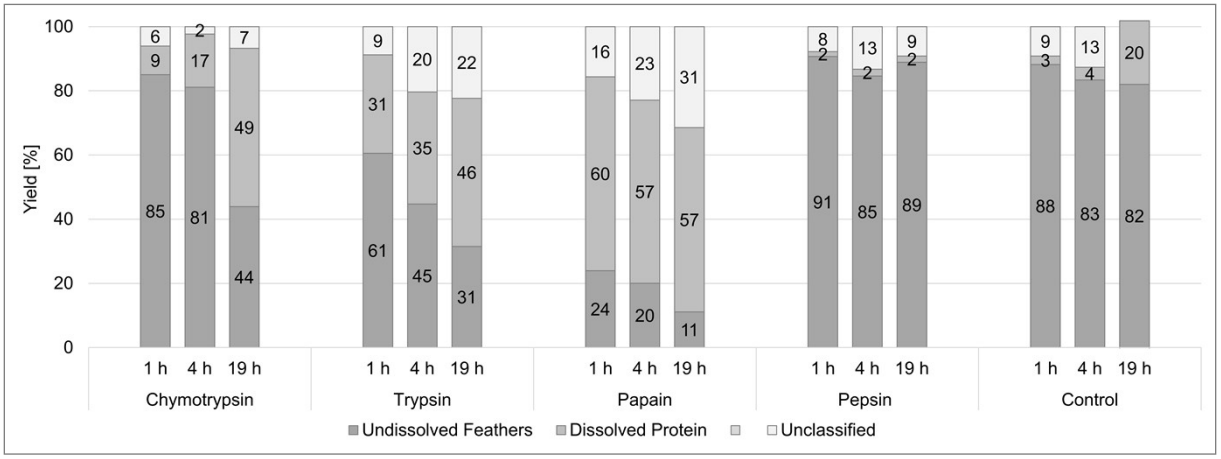

Figure S10. Specific enzymatic hydrolysis of chicken feathers with reducing conditions. The reaction mixtures contained 50 mmol L^{-1} sodium sulfite. The percentages of soluble proteins or amino acids and insoluble residues are given in relation to the biomass used. The values given are mean values from duplicate determinations.

4.3 Trypsin

Trypsin is known to catalyse the hydrolysis of peptide bonds at the carboxyl side of lysine and arginine, except if there is a proline C-terminal to these amino acids.[9] Although evidence shows cleavage also occurs before proline[10], trypsin is known for its high specificity.[11] An *in-silico*-cleavage of CFK1 with trypsin was performed using the Expasy peptide cutter tool.[12] This led to an N-terminal 2,714 Da fragment after cleavage at Arg25, containing five L-cysteines (Figure S1). Thus, trypsin should be well suited for controlled hydrolysis of chicken feathers to generate polythiol peptides.

Exemplarily, the maximum amount of thiol groups introduced to the hydrolysates through the addition of trypsin was calculated according to Table S4 below:

Table S4: Origin of the thiol groups in tryptic chicken feather keratin hydrolysates. A CFK1 content of 90 % CFK1 was assumed for chicken feathers, and that for trypsin, we calculated with a purity of 100%.

	UniProt ID	Mass [g]	MW [Da]	Cys	Purity [%]	Quantity [µmol]	Percentage
CFK1	P02450	0.5	9972	8	90	361.01	98.6%
Trypsin, porcine	P00761	0.01	23300	12	100	5.15	1.4%

We demonstrated that trypsin has keratinolytic activity. Depending on the reaction time, it was possible to decompose 47% (w/w) of the chicken feather biomass used without adding other substances, such as urea or reducing agents (Figure S11). The protein concentration in the final hydrolysate was determined to be 21 mg mL^{-1}. A thiol concentration of 11.3 mmol L^{-1} was measured after reduction with $NaBH_4$. No thiols were detected without reduction, indicating a substantial cross-linking via disulfide bridges. Adding the reducing agent sodium sulfite to the hydrolysis reaction increased the dissolved amount of chicken feathers from 47% (w/w) to 69% (w/w) (Figure S11).

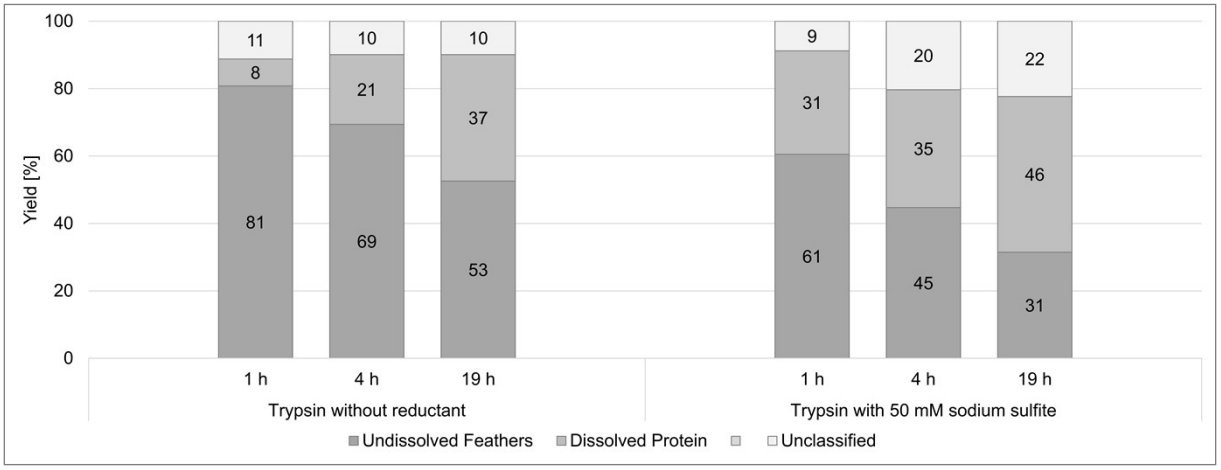

Figure S11. CFK-hydrolysis with trypsin with and without adding the reducing agent sodium sulfite.

Savinase showed a higher keratinolytic activity, with a yield of 68% (w/w) after 19 h, compared to trypsin, with a yield of 53% (w/w) (Figure S12).

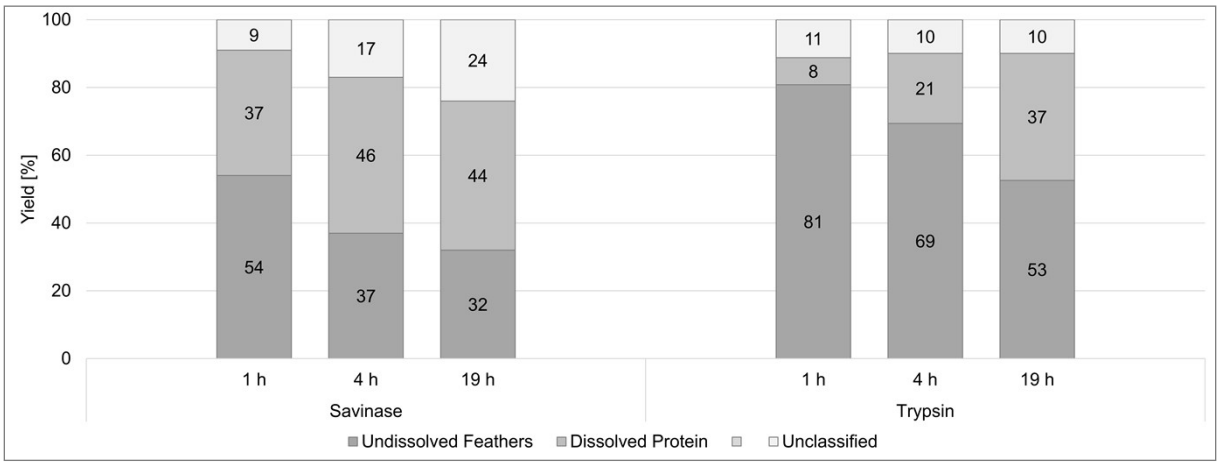

Figure S12. Comparison of enzymatic CFK-hydrolysis approaches using the unspecific savinase and the specific trypsin.

By using a combined chemical and enzymatic degradation approach followed by SDS-PAGE, it was possible to show the efficient hydrolysis of chicken feathers by trypsin: The chemical hydrolysis resulted in the formation of

a clearly visible 10 kDa band in SDS-PAGE and after addition of trypsin to the hydrolysate, the 10 kDa band disappeared. This indicated the hydrolysis of the respective protein. Already 0.5 h after addition, total hydrolysis of the keratin was accomplished. The 10 kDa band faded and disappeared completely after 19 h incubation and subsequent SDS-PAGE analysis (see Figure S13).

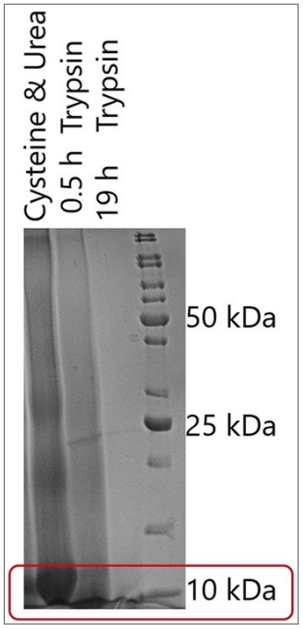

Figure S13. Keratinolytic activity of trypsin.

4.4 Optimisation of chicken feather hydrolysis with trypsin

The following figures and tables show further results in which pre-treated chicken feathers could be decomposed with trypsin. The conditions were optimised and the hydrolysates were characterised.

Table S5. Yields of soluble peptides in hydrolysis experiments with renewed addition of trypsin after 24 h.

Reaction Time	24 h	32 h	32 h + trypsin after 24 h	48 h	48 h + trypsin after 24 h
Soluble Peptides Yield [%]	56.7 ± 0.2	61.9 ± 1.2	63.0 ± 0.9	63.4 ± 0.8	67.5 ± 0.3

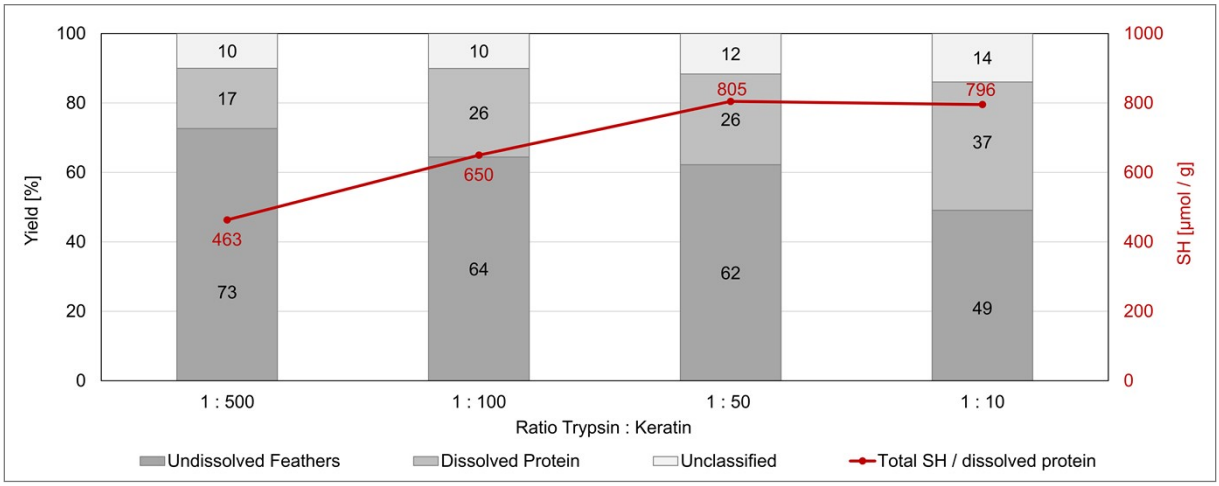

Figure S14. Hydrolysis of Chicken Feather Keratin with Trypsin, using different Trypsin: Keratin ratios. Reaction conditions were: 8.5 mL, 500 mg chicken feathers, 50 mM potassium phosphate buffer, pH7.8, for 16 h. The highest SH content per g of solubilised peptides was received with a 1:50 ratio of trypsin:keratin.

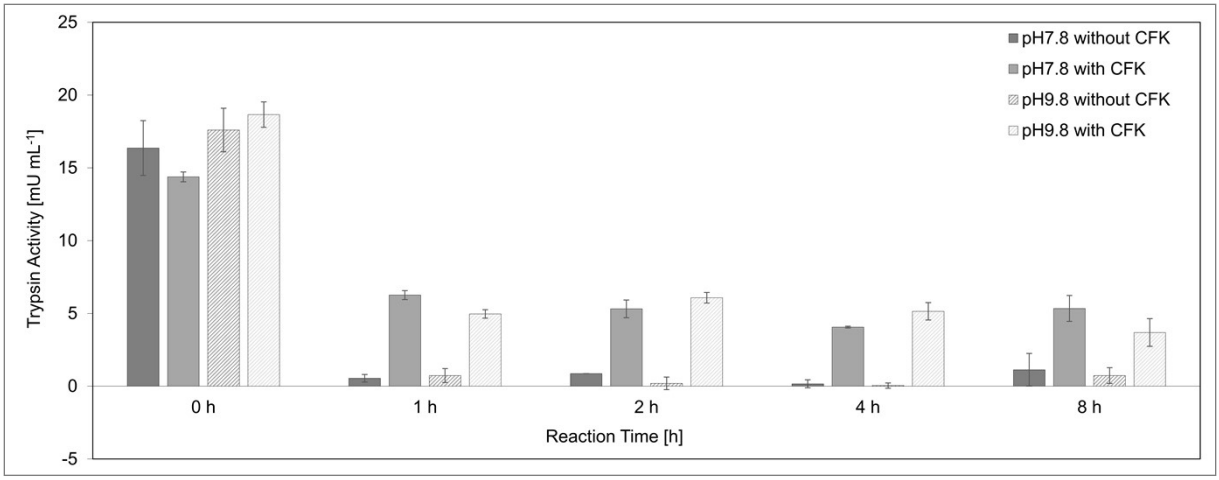

Figure S15. Trypsin activity in CFK hydrolysis reactions in dependence of reaction time. The activity measurements were carried out using a Trypsin Activity Colorimetric Assay Kit (Sigma Aldrich, MAK290) according to the manufacturer's instructions with *p*-nitroaniline as substrate. Values are averages of two measurements; error bars are standard deviations.

Table S6. The most abundant peptides in a tryptic feather keratin hydrolysate.

>sp|P02450|KRFC_CHICK Feather keratin 1 OS=Gallus gallus OX=9031 PE=3 SV=2 **MSCFDLCRPCGPTPLANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAAGSSTSAAVGSILSEEGVPISSGGFGISGLGSRFSGRRCLPC** >sp|P20308|KRFA_CHICK Feather keratin 4 OS=Gallus gallus OX=9031 PE=3 SV=2 **MSCYDLCRPSAPTPLANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAVGSSTSAAVGSILSEEGVPISSGGFGISGLGSRFSSRRCLPY**	% of total peak area	MW [Da]
....DLCRPCGPTPL.....	45.8	1,285
.SCFDLCRPCGPTPL.....	8.4	1,721
.....AVGSILSEEGVPISSGGF.....	5.1	1,705
.....SEEGVPISSGGF.....	3.2	1,165
....DLCRPCGPTPLAN.....	2.2	1,470
.....STSAAVGSILSEEGVPISSGGF.....	1.7	2,051
....DLCRPCGPTPLANSCNEACVR.....	1.5	2,447
.....SFPQNTAVGSSTSA.....	1.5	1,353
.....VVIQPSPVVVTLPGPILS.....	1.5	1,814
.....ILSEEGVPISSGGF.....	1.4	1,391
.....SFPQNTAVGSSTSAAVGSILSEEGVPISSGGF.....	1.4	3,039
.....TAVGSSTSAAVGSILSEEGVPISSGGF.....	1.4	2,466
.....VVIQPSPVVVTLPGPILS.....	1.4	1,815
.....AAVGSILSEEGVPISSGGF.....	1.0	1,776
.....IQPSPVVVTLPGPILS.....	1.0	1,616
.....SAAVGSILSEEGVPISSGGF.....	1.0	1,863
.....TAVGSSTSAAVGSIL.....	0.9	1,320
.....FGISGLGSR.....	0.9	892
.....GISGLGSRF.....	0.9	892
......CRPCGPTPL.....	0.9	1,056
.....VVIQPSPVVVTLPGPILSSFPQN.....	0.8	2,387
.....SEEGVPISSGGFGISGLGSR.....	0.8	1,892
.....SFPQNTAVGSSTSAAVGSIL.....	0.7	1,893
.....AVGSSTSAAVGSILSEEGVPISSGGF.....	0.7	2,365
.....IQPSPVVVTLPGPILSSFPQN.....	0.7	2,189
.....SEEGVPISSGGFGIS.....	0.6	1,422
....DLCRPCGPTPLANSCNEACVR.....	0.6	2,446
.....VVIQPSPVVVTLPGPILSSFPQN.....	0.6	2,388
.....SILSEEGVPISSGGF.....	0.6	1,478
.SCFDLCR.....	0.6	998
.....LCRPCGPTPL.....	0.5	1,170
...FDLCRPCGPTPL.....	0.4	1,432
.....AVGSSTSAAVGSIL.....	0.4	1,219
.....VVIQPSPVVVTLPGPIL.....	0.4	1,727
.....AVGSILSEEGVPISSGGFGIS.....	0.4	1,962
.SCFDLCRPCGPTPLAN.....	0.4	1,906
.....IQPSPVVVTLPGPILSSFPQNTAVGSSTSA.....	0.3	2,951
..... SSTSAAVGSILSEEGVPISSGGF.....	0.3	2,138
.SCFDLCRPCGPTPLANSCNEACVR.....	0.3	2,883

.....SSFPQNTAVGSSTSAAVGSIL.....	0.3	1,980
.....IQPSPVVVTLPGPIL.....	0.3	1,529
.....SFPQNTAVGS.....	0.3	1,006
.....VVIQPSPVVVTLPGPILSSFPQNTAVGS.....	0.3	2,803
.....SSFPQNTAVGSSTSA.....	0.3	1,440
.....IQPSPVVVTLPGPILSSFPQNTAVGS.....	0.3	2,604
.....VGSILSEEGVPISSGGF.....	0.2	1,634
.....VVIQPSPVVV.....	0.2	1,036
.....VVIQPSPVVVTLPGPIL.....	0.2	1,728
.....VVIQPSPVVVTLPGPILSSFPQNTAVGS.....	0.2	2,804
.....SPVVVTLPGPILS.....	0.2	1,278
.....TSAAVGSILSEEGVPISSGGF.....	0.2	1,964
.....TAVGSSTSAAVGSILSEEGVPISSGGFGIS.....	0.2	2,723
.....LANSCNEPCVRQCQDSR.....	0.2	2,078
.....VIQPSPVVVTLPGPILS.....	0.1	1,715
.....SFPQNTAVGSSTS.....	0.1	1,282
.....VVIQPSPVVVTLPGPILSSFPQ.....	0.1	2,273
.....IQPSPVVVTLPGPILSSFPQNTAVGSST.....	0.1	2,792
..CYDLCRPSAPTPLANSCNEPCVR.....	0.1	2,779
.....VVIQPSPVVVTLPGPILSSFPQNTAVGSST.....	0.1	2,991
.....NTAVGSSTSAAVGSIL.....	0.1	1,434
.....VVIQPSPVVVTLPGPILSSFPQ.....	0.1	2,274
.....IQPSPVVVTLPGPILSSFPQ.....	0.1	2,075
.....AAVGSILSEEGVPISSGGFGIS.....	0.1	2,033
.SCFDLCRPCGPTPL.....	0.1	1,679
.....IQPSPVVVTLPGPILSSFPQNTAVG.....	0.1	2,517
.....ILSEEGVPISSGGFGIS.....	0.1	1,648
.....NTAVGSSTSAAVGSILSEEGVPISSGGF.....	0.1	2,580
.....VVIQPSPVVVTLPGPILSSFPQNTAVG.....	0.1	2,716
.....VVIQPSPVVVTLPGPILSSFPQNTAVG.....	0.1	2,717
.....VVIQPSPVVVTLPGPILSSFPQNT.....	0.1	2,488
....DLCRPCGPTPLANSCN.....	0.1	1,832
.....SEEGVPISSGGFGISGL.....	0.1	1,592
.....VVIQPSPVVVTLPGPILSSFPQNT.....	0.1	2,489
.....IQPSPVVVTLPGPILSSFPQNTAVGSSTS.....	0.1	2,880
........PCGPTPLANSCNEACVR.....	0.1	1,903
.....IQPSPVVVTLPGPILSSFPQNT.....	0.1	2,290
.....VVIQPSPVVVTLPGPILSSFPQNTAVGSST.....	0.1	2,992
.....TLPGPILSSFPQNTAVGSSTSA.....	0.1	2,131
.....ILSEEGVPISSGGFGISGLGSR.....	0.1	2,118
.....VIQPSPVVVTLPGPILS.....	0.1	1,716
..... STSAAVGSIL.....	0.1	904
.....IQPSPVVVTLPGPILSSFPQNTAVGSS.....	0.1	2,691
.....VVIQPSPVVVTLPG.....	0.1	1,404
.SCFDLCRPCGPTPLANSCNEACVR.....	0.1	2,882
....DLCRPCGPTP.....	0.1	1,172
.....VVIQPSPVVVTLPGPILSSFPQNTAVGSS.....	0.0	2,890
.....GSILSEEGVPISSGGF.....	0.0	1,535
.....VIQPSPVVVTLPGPILSSFPQN.....	0.0	2,288
.....IQPSPVVVTLPGPILSSFPQNTAVGSSTSA.....	0.0	2,952
.....SPVVVTLPGPIL.....	0.0	1,191
.....VIQPSPVVVTLPGPIL.....	0.0	1,628
.....VVIQPSPVVV.....	0.0	1,037
.....SSTSAAVGSIL.....	0.0	992
.....SPVVVTLPGPILSSFPQN.....	0.0	1,851
.....IQPSPVVVTLPGPILSSF.....	0.0	1,850
.....VIQPSPVVVTLPGPILSSFPQNTAVGSSTSA.....	0.0	3,050
.....SILSEEGVPISSGGFGIS.....	0.0	1,735
.....PSPVVVTLPGPILS.....	0.0	1,375
.....SPVVVTLPGPILSSFPQNTAVGSSTSA.....	0.0	2,612
.....TPLANSCNEPCVR.....	0.0	1,517
......CRPCGPTPLAN.....	0.0	1,242
.....QCQDSR.....	0.0	792
.....SFPQNTAVGSSTSAAVGSILSEEGVPISSGGF.....	0.0	3,040

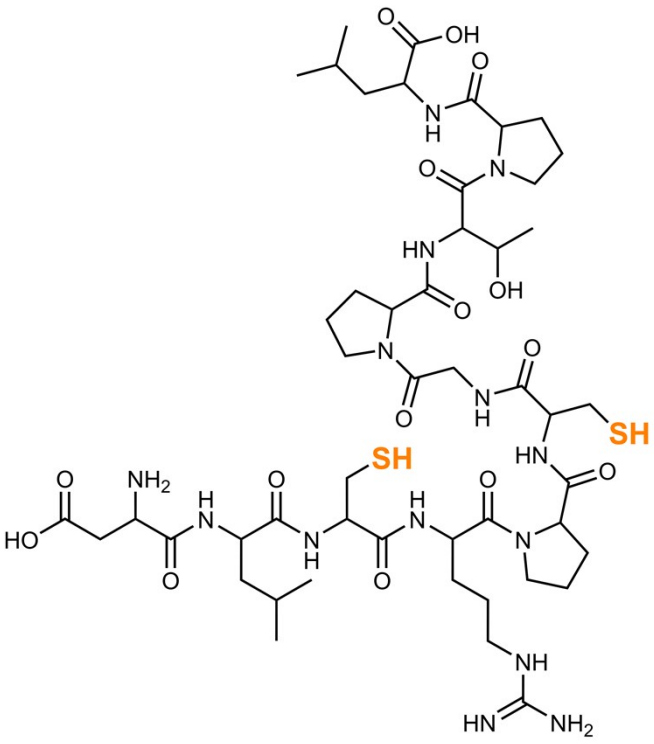

Figure S16. Structure of the most abundant peptide with the sequence DLCRPCGPTPL.

Table S7. Differential Scanning Calorimetry of KHP-films. Values are mean values from two measurements ± standard deviations.

Film	A (KHPs)	B (KHPs + BDE)	C (KHPs + Glycerol)	D (KHPs + BDE + Glycerol)
T_g [°C]	172.0 ± 2.1	152.2 ± 0.1	106.5 ± 0.0	104.2 ± 1.8

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