Effect of enzymatic hydrolysis on surface activity and surface rheology of type I collagen

Aleksandra Kezwoń1, Ilona Chromińska1, Tomasz Frączyk2, Kamil Wojciechowski\*1

1Faculty of Chemistry, Warsaw University of Technology, Noakowskiego 3, 00-664 Warsaw, Poland

2Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland

\*corresponding author kamil.wojciechowski@ch.pw.edu.pl

Abstract

We describe the adsorption behaviour and rheological properties of a calf skin type I collagen, and of its hydrolysates obtained using a Clostridium histolyticum collagenase (CHC) under moderate conditions (pH 7, 37 oC). The effect of CHC concentration (2·10-9 M - 2·10-6 M) and incubation time (35 min – 85 min) was studied and optimized to achieve the highest decrease of surface tension and the highest dilational surface viscoelasticity of the adsorbed layers. SDSPAGE electrophoresis and reverse-phase high performance liquid chromatography (RP-HPLC) were used to characterise the hydrolysis products. The results show that even simple modifications (heat treatment, pH change, partial hydrolysis) of collagen enhances its surface properties, especially in terms of surface dilational elasticity modulus. The use of low enzyme concentration (CHC-to-collagen molar ratio of 4·10-3) and short incubation time (<45 min) results in moderately hydrolysed products with the highest ability to lower surface tension (γ=53.9 mN/m) forming highly elastic adsorbed layers (surface dilational elasticity, E’=74.5 mN/m).

Keywords: Clostridium histolyticum; calf skin collagen; surface tension; surface dilational rheology; collagen hydrolysate

1. Introduction

An increasing interest in food and cosmetic products with low content of synthetic additives calls for searching of sustainable and biocompatible surfactants of natural origin (biosurfactants). In view of the limited oil resources, at longer timescales biosurfactants could help to reduce costs. Even more importantly, should have less negative impact on the environment than their synthetic counterparts. So far, the milk and eggs proteins as well as biosurfactants from the groups of rhamnolipids and saponins show the highest promise in this respect1-3. In the context of foam and emulsion stabilization, globular proteins (β-lactoglobulin2,4-6 and lysozyme2,7,8) and random-coil β-casein2,9,10 are most often studied. Despite a huge availability as a waste by-product from meat and fabrics industries, only few reports describe surface activity of fibrous proteins such as collagen, keratin or fibroin. For example, a native silk protein, fibroin, was shown to adsorb strongly at the air/water interface 11. The structure of the adsorbed fibroin layers was proposed on the basis of surface tension and surface dilational rheological studies at low and high bulk protein concentration. Ozdemir et al. reported on interactions of keratin from chicken feather with synthetic (sodium dodecyl sulfate, SDS) and natural (rhamnolipids) surfactants at the air/water interface. They concluded that keratin is capable of forming surface complexes with both surfactants. A more hydrophobic SDS-keratin complex was found to be more surface active compared to the rhamnolipid–keratin one 12. Zaitsev et al. described the effect of denaturants (urea and thiourea) on surface activity of collagen 13. They noticed rapid and irreversible changes of the collagen monolayers, especially for thiourea. Li et al. analysed the effect of acylation on surface tension of aqueous collagen solutions 14,15. A reduction from 71 mN∙m-1 down to 56 mN∙m-1 was achieved by reacting collagen with lauryl chloride and succinic anhydride. Even though it is not clear whether the excess of reagents was effectively removed from the reaction mixture, the works by Li et al. nicely show the potential of collagen to act as an amphiphile after a relatively straightforward modification. Chi et al. were able to enhance surface activity of a pig skin collagen by hydrolysing it using alcalase and subsequently grafting with oleoyl groups 16. Also our recent work on the effect of temperature on surface tension and surface rheology of type I collagen from calf skin shows that even simple temperature treatments can improve its surface activity. Collagen solutions (1.3∙10−5 M) subjected to heating at 90 oC for 60 min followed by cooling down to 21°C produced highly elastic adsorbed layers. Their dilational surface elasticity modulus, E’, increased from 58 mN.m-1 (for native collagen) to 71 mN.m-1 (for the heat-activated collagen) at the highest frequency of oscillation (0.1 Hz) 17. Currently about 28 types of collagen of different structure, amino acid composition and biological role are known 18. A collagen molecule consists of three left-handed helical polypeptide chains, rolled into a right-handed triple helix with molecular weight of about 300 kDa. The common feature of all collagens is a repeating Gly-X-Y amino-acid sequence 19, where X and Y can be any amino acid, although proline (Pro) or hydroxyproline (Pro-OH) residues are the most often encountered 20,21. Collagen contains both polar (aspartic acid (Asp), glutamic acid (Glu), arginine (Arg), lysine (Lys)), and apolar (proline (Pro), serine (Ser), glicyne (Gly)) amino acids 14. Hence, despite the fact that in native collagen the helices are rolled in a way to expose the hydrophilic segments to the solution, these fibrous proteins have a potential to develop an amphiphilic character. The latter can be unleashed by at least partial denaturation and/or hydrolysis of the triple helix, using chemical 22,23 or enzymatic methods 3. The latter usually require milder conditions (close to physiological ones) 24 and allow for obtaining products with well-defined properties 25 free of residual organic solvents or toxic chemicals 26. For these reasons, enzymatic hydrolysis of proteins from soymilk 23,27, fish byproducts 24,28,29 or milk proteins (casein 30-32, β-lactoglobulin 33,34) is more and more often employed in food and pharmaceutical industries 24,29,33,35,25,28,31,34. In this paper we present a study of the effect of hydrolysis of a calf skin type I collagen with a collagenase from Clostridium histolyticum (CHC) on surface tension and surface dilational viscoelasticity. CHC is a collagen-specific enzyme, effective at near-room temperatures and pH values close to neutral. It belongs to endopetidases 36 and operates by recognising a -R-Pro-XGly-Pro-R- sequence (where X is most often a neutral amino acid) and subsequently cleaving the peptide bonds between X and Gly to release a Gly-NH2 terminus 37,38. Collagen type I from bovine calf skin was chosen as the most widely available potential source of biocompatible amphiphiles. Axisymmetric Drop Shape Analysis (ADSA) method was used to determine dynamic surface tension and surface dilational storage and loss moduli (E’ and E”). The molecular weight of the partial hydrolysis products was assessed using SDS-PAGE, and their apolar character was probed using RP-HPLC.

2. Experimental

2.1. Chemicals

Type I collagen from bovine calf skin was obtained from Biochrom AG (Collagen G, 4 g∙L-1, solution in 0.015 M HCl) and was used as received. Other chemicals for the enzymatic hydrolysis: hydrochloric acid (84428), collagenase from Clostridium histolyticum (CHC) type I (C0130), enzyme activator: calcium chloride (21097), tris(hydroxymethyl)aminomethane, TRIS (T6066) were purchased from Sigma Aldrich. Milli-Q water (Millipore) was used to prepare all solutions. Surface purity of water and all chemicals (HCl, CaCl2, enzyme) used in this work was verified by monitoring their dynamic surface tension for 1h. Similar tests were run for all the glassware, by measuring surface tension of the last water used for rinsing the glassware. All glassware was cleaned with Hellmanex II solution (Hellma, Worldwide) and acetone prior to rinsing with Milli-Q water.

2.2. Temperature-denatured collagen solution at different pH

Collagen solution (pH=1.8) at a concentration of 5.0·10-6 M was prepared by diluting the stock collagen solution (4 g∙L-1 in 0.015 M HCl) using hydrochloric acid solution (0.015 M). 0.1 M NaOH and 0.015 M HCl solutions were used for preparing collagen solutions of different pH (pH=3.0, pH=7.0 and pH=9.0). The prepared solutions were heated at 90 °C for 10 min in order to partially denature collagen, and then cooled down to 21 °C. This procedure hinders the collagen fibril formation at neutral pH. Dynamic surface tension of such freshly prepared solutions was measured as described in 2.6.

2.3. Enzymatic hydrolysis

The procedure for enzymatic hydrolysis was adapted from refs 37,38. A 5.0∙10-6 M solution of collagen (pH 7.5) containing 5.0·10-2 M TRIS, 1.0·10-3 M CaCl2 and 0.015 M HCl was prepared by diluting the stock collagen solution (4 g∙L-1 in 0.015 M HCl). All samples were first heated for 10 minutes at 90 °C and cooled down to 21 °C before addition of the required amount of CHC solution. The samples with CHC (S1 – 2.0·10-9 M; S2 – 2.0·10-8 M; S3 – 1.7·10-7 M; S4 – 2.0·10-7 M; S5 – 3.3·10-7 M; S6 –2.0·10-6 M;) were heated to 37 °C for a defined period of time, after which a 0.1 M HCl solution was added to reach pH 3.0 and quench the enzymatic reaction 38.

2.4. SDS-PAGE electrophoresis

In order to estimate molecular masses of the partial hydrolysates of collagen, the latter were subjected to electrophoretic separation using SDS-PAGE with a 12% running gel in a running buffer containing 3.02 g∙L-1 TRIS, 14.40 g∙L-1 glycine, and 1.00 g∙L-1 sodium dodecyl sulphate (SDS). Electrophoresis was performed at constant voltage of 200 V. The gels were washed with an aqueous solution containing 50% (v/v) MeOH and 7% (v/v) acetic acid. Next, the gels were stained for 1h with Coomassie Brilliant Blue G-250 (0.006% (w/v)) in aqueous solution of 10% (v/v) acetic acid and washed overnight with distilled water. Images of the stained products in gels were documented with the use of E-gel Imager (Life Technologies).

2.5. Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

Chromatographic analyses were carried out on a High-Performance Liquid Chromatograph (Breeze, Waters) equipped with a binary pump and an autosampler. An absorbance detector (Waters) with detection wavelength of 220 nm was employed to track the protein and its hydrolysates profiles in the analysed samples. An analytical C18 column (ACE, 5 μm particle size, 250  4.6 mm) was used. The mobile phase consisted of 0.1% (v/v) trifluoroacetic acid (TFA) in water (A), and 0.1% (v/v) TFA, 90% (v/v) acetonitrile in water (B).

2.6. Dynamic surface tension and surface dilational rheology using Axisymmetric Drop Shape Analysis (ADSA) method

All measurements were performed at constant temperature (21 °C, controlled with a thermostatic bath) using a PAT-1 tensiometer (Sinterface Technologies, Germany), as described in ref. 39. A drop of the collagen or of its hydrolysate solution was formed at the tip of a steel capillary immersed in a glass cuvette (20 ml) filled with air. Each measurement using the Axisymmetric Drop Shape Analysis (ADSA) method consisted of two parts. In the first part (1 s – 3600 s) the drop volume was kept constant (21 μl), providing a dynamic surface tension measurement (i.e., surface tension as a function of time). The equilibrium surface tension (γeq) for surface tension isotherm was obtained by extrapolating the dynamic surface tension to the infinite time following the approach by Joos and Hansen 40,41, as an intercept with the ordinate in γ vs t-1/2 coordinates

(1)

where γ is the dynamic surface tension, γeq is the equilibrium surface tension, T is the temperature, R is the gas constant, D is the diffusion coefficient, C0 is the bulk concentration, t is the time. In the second part of the measurement (3600 s – 5000 s), harmonic (sinusoidal) modulations of the volume of the drop were applied, using the following frequencies of oscillations: 0.005, 0.01, 0.02, 0.05, 0.1 Hz and the relative amplitude of 4.4 %. The sinusoidal oscillations of the drop volume lead to a sinusoidal response of surface tension. The surface dilational rheological parameters: imaginary - loss modulus (E”), and real - elastic modulus (E’) of the adsorbed layer were calculated from the amplitude and the phase shift of the surface tension response. E’ and E” constitute the complex surface dilational viscoelastic modulus E 39:

(2)

where γ is the interfacial tension, A is the area subjected to deformation, and i=(-1)1/2. Each measurement of surface tension and surface dilational rheology was repeated at least three times (typically four-six times).

3. Results

3.1. Effect of pH on surface properties of temperature-denatured collagen In the first part, the effect of pH on hydrolysis as well as surface activity and surface rheological parameters of temperature-denatured collagen was studied. Four values of pH were chosen, corresponding to: a) the starting collagen solution (pH 1.8), b) optimum conditions for the enzymatic hydrolysis (pH 7.0), c) pH of the reaction mixture after quenching the enzymatic hydrolysis reaction with HCl (pH 3.0), and d) proximity of the isoelectric point of collagen (pH 9.0). In order to gain insight into the extent of temperature denaturation and the hydrophobic character of the products obtained under different pH conditions, a reverse phase high performance liquid chromatography (RP-HPLC) analysis was first performed. The technique allows to separate components of a given mixture (e.g., denaturation or hydrolysis products) according to their polar-apolar character 42-44. The RP-HPLC chromatograms of the collagen solutions subjected to thermal denaturation at different pH are shown in Figure 1A. In all cases, the products maintain their character, as proved by the lack of significant changes of retention times. A double-peak pattern in the chromatograms arises from the presence of two polypeptide chains with different amino-acid sequences 44, α1 and α2, characteristic for type I collagen. This confirms that the employed short temperature denaturation (10 min, 90 oC) does not result in extensive collagen hydrolysis, even though some changes in the composition can be observed above pH 1.8. The beneficial effect of temperature denaturation on surface activity of collagen type I at pH=1.8 has been described in our previous work17. The results described here extend this study to the pH range up to 9.0. The dynamic surface tension of collagen solutions (5.0·10-6 M) of pH 1.8, 3.0, 7.0 and 9.0 after a short temperature treatment (10 min, 90 oC) are shown in Fig. 2A, and the corresponding extrapolated equilibrium surface tension vs pH dependency is shown in Fig. 2B. Even though after 1 hour adsorption, all the curves for pH above 1.8 almost overlap, the extrapolated equilibrium values show clear variation with the pH. The deepest reduction of surface tension, associated with the highest surface activity, can be observed around pH 9.

5 10 40 50 60 70

0.0

0.2

0.4

0.6

0.8

1.0

5 10 40 50 60 70

0.0

0.2

0.4

0.6

0.8

1.0

5 10 40 50 60 70

0.0

0.2

0.4

0.6

0.8

1.0

T5 T4

T3

T2

T1

C

S1

S2

S4

S4

S5

S6

C

pH 9.0

pH 7.0

pH 3.0

tretention /min

Absorbance Absorbance Absorbance

pH 1.8

A

C

B

Fig. 1. RP-HPLC chromatograms for: (A) native collagen solutions (5.0·10-6 M) at different pH; (B) hydrolysis mixtures with different CHC/collagen molar ratios: 4·10-4 (S1), 4·10-3 (S2), 3·10-2 (S3), 4·10-2 (S4), 7·10-2 (S5), 4·10-1 (S6).; incubation time 60 min; (C) hydrolysis mixtures with different incubation time: 35 min (T1), 45 min (T2), 60 min (T3), 75 min (T4), 85 min (T5). CHC/collagen molar ratio: 4·10-3. C – control (collagen without CHC, incubation time 60 min).

0 1000 2000 3000

55

60

65

70

2 4 6 8

50

52

54

56

pH 7.0

pH 3.0

pH 9.0

mN.m-1

t /s

pH 1.8

A B

eq /mN.m-1

pH

Fig. 2. Effect of pH on (A) dynamic and (B) equilibrium interfacial tension of collagen solutions (5.0·10-6 M).

The surface dilational rheological parameters, described by the storage (E’) and loss (E”) moduli, characterise mechanical properties of the adsorbed layers. The latter are believed to play crucial role in kinetic stabilization of foams and emulsions, hence the need to evaluate E’ and E”. The measurements were performed in a frequency range 0.005-0.1 Hz by harmonically oscillating the drop volume, as described in the experimental part. The results for 5.0·10-6 M collagen solutions at pH of 1.8, 3.0, 7.0 and 9.0 as a function of oscillation frequency are shown in Fig. 3 A and B.

0.00 0.05 0.10

30

40

50

60

70

0.00 0.05 0.10

6

9

12

15

18

2 4 6 8

0

10

20

30

40

50

60

70

B

E' /mN.m-1

f /Hz

A C

E'' /mN.m-1

f /Hz

E'' / mN.m-1

E' / mN.m-1

pH

0

10

20

30

40

50

60

70

Fig. 3. Effect of pH (pH 1.8 (), pH 3.0 (), pH 7.0 (), pH 9.0 ()) on surface dilational rheological parameters: (A) elastic modulus, E’ and (B) loss modulus, E” for the layers of temperature-denatured collagen adsorbed at the water/air interface. (C) E’ () and E’’ () as a function of pH value for the highest frequency of oscillation (0,1 Hz). Collagen concentration in all solutions was fixed at 5.0·10-6 M.

For all samples, the storage modulus, E’, describing elastic properties of the layers increases with increasing frequency of oscillation (f). With increasing pH, the E’ vs f curves maintain their character but shift to lower values, suggesting that the layers become less elastic at higher pH (Fig. 3A). For the loss modulus, E”, describing viscous properties of the layers, the values are much smaller than for E’ and no clear trends can be noticed. Overall, a small decrease of E” with increasing frequencies can be observed, especially for pH 1.8 (Fig. 3B). Both moduli tend to their respective plateaux at high oscillation frequencies, suggesting that the values for f=0.1 Hz approach those of the high-frequency limit (equilibrium Gibbs elasticity, E0 45). The E’ and E” vs pH dependency for f=0.1 Hz shown in Fig. 3C confirm that indeed the adsorbed thermally denatured layers of collagen type I become less surface elastic with increasing pH, while above pH 3.0 their surface viscous properties do not change significantly with pH. For all pH the layers remain predominantly elastic (E’>E”).

3.2. Effect of collagenase concentration on enzymatic hydrolysis of collagen

The effect of collagen-specific enzyme, collagenase from Clostridium histolyticum (CHC) was analysed for a fixed collagen concentration of 5.0·10-6 M. Six collagen samples containing between 2·10-9 M and 2·10-6 M of CHC (S1-S6) were prepared as described in the experimental section with pH adjusted to 7.0. After one hour incubation at 37 °C, the enzymatic reaction was quenched with 0.1 M hydrochloric acid (final pH 3.0) prior to further analyses. The samples were first loaded onto a 12% polyacrylamide gel for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in order to probe the extent of hydrolysis. The technique allows for separating components of a given mixture according to their relative masses. For comparison, the unhydrolysed collagen (C) and CHC (E), together with a “Low Range Protein Ladder” as a molecular weight standard were also loaded on the same gel (Fig. 4).

Fig. 4. SDS-PAGE electropherogram of CHC-hydrolysed collagen: L - Low Range Protein Ladder, C – control (collagen without CHC, incubation time 60 min). CHC/collagen molar ratio are as follows: S1 – 4·10-4; S2 – 4·10-3; S3 – 3·10-2; S4 – 4·10-2; S5 – 7·10-2; S6 – 4·10-1. E – CHC at a concentration 1·10-5 M. Initial collagen concentration: 5.0·10-6 M.

In agreement with the literature data, a high-molecular weight fraction of the native collagen (top of the lane C in Fig. 4) shows a pattern typical for the polypeptide chains constituting collagen type I: two α chains (α1 and α2 chain), their dimer (β), and small amounts of γ components (trimer) 46-48. The pattern for CHC, consisting of four subunits49 with the overall molecular weight of 109 kDa also agrees with the literature data 38,50. Because of its low content in the hydrolysed samples S1-S6 (CHC/collagen molar ratio varying between 4·10-4 and 4·10-1, the signals from CHC are not seen in lanes S1-S6. Sample S6, with the highest CHC/collagen ratio 0.4, shows almost complete hydrolysis of the protein to short polypeptide chains with molecular weights below 15 kDa. Samples S3-S5 show similar patterns pointing to an extensive hydrolysis with formation of a significant fraction of 25 kDa polypeptides. Further reduction of the CHC/collagen ratio to 4·10-3 (S2) results in a completely different pattern in SDS-PAGE (lane S2 in Fig. 4). A wide range of hydrolysis products with molecular weights between 15 and 50 kDa are observed, suggesting only weak hydrolysis of collagen under these conditions. On the other hand, for S1, where CHC/collagen ratio is as low as 4·10-4, practically no hydrolysis products could be observed, and the pattern is similar to the native collagen (lane C in Fig. 4). The RP-HPLC results (Fig. 1B) agree very well with those from SDS-PAGE. The samples S3-S6 (with CHC/collagen ratio between 3·10-2 and 4·10-1) are devoid of native collagen, as can be judged from the absence of long retention peaks in the range of 65-75 min. Instead, new fractions with retention times 7-14 min, and 35-65 min appear. Thus, it can be expected that the S3-S6 samples contain products significantly more polar than the native collagen. Similarly to SDSPAGE, the chromatogram of sample S2 shows distinct peak pattern. Even though the native collagen peaks are not present (as for S3-S6), the hydrolysis products appear at longer retention times (in the range 48–70 min) suggesting that they are less polar than for samples S3-S6. As it could be anticipated from the SDS-PAGE results, S1 contains mostly native collagen, with only minor extent of hydrolysis, as proven by a slight reduction of the peak at 68 min, associated with α2 polypeptide chain 44 of type I collagen. Figure 5 presents the dynamic and equilibrium surface tension results for the native collagen and its hydrolysis mixtures obtained with CHC concentration in the range 2·10-9 M to 2·10-6 M, corresponding to the CHC/collagen ratio of 4·10-4 - 4·10-1 (S1-S6). It should be stressed that CHC alone exhibits very low ability to reduce surface tension. Below a concentration of 2.5·10-7 M (corresponding approximately to the composition of sample S4), the dynamic surface tension curves coincide with those for the pure solvent (see Supplementary Materials, Fig. SM1). For the samples with high CHC content (S3-S6), the initial rate of surface tension decay is increased as compared with the native collagen. Nevertheless, after one hour, the decrease of surface tension for these samples is lower than for the native collagen. To illustrate the detrimental effect of extensive hydrolysis on surface activity of hydrolysates, dynamic surface tension of a sample with high CHC/collagen ratio was followed during 3 h (see Supplementary Materials, Fig SM2).

The measurement started immediately after addition of CHC, and the reaction was purposely not quenched by addition of HCl. The data collected in Fig. SM2 clearly shows a gradual decay of the ability to lower surface tension in the following measurements.

The extrapolated equilibrium values shown in Fig. 5B confirm the trend observed in dynamic surface tension measurements: decrease of the CHC/collagen ratio favours reduction of surface tension. Both the dynamic and equilibrium results point to S2 as the most efficient one in lowering surface tension. Interestingly, reduction of surface tension can be observed even for the sample with as little as 2·10-9 M of CHC (S1), where both SDS-PAGE and RP-HPLC showed only slight hydrolysis.

0 1000 2000 3000

55

60

65

70

1E-3 0,01 0,1

54

56

58

60

62

64

C

S1

S3

S2

S4

S5

eq /mN.m-1

 /mN.m-1

t /s

S6

C

CCHC/Ccollagen

Fig. 5. (A) Dynamic and (B) equilibrium interfacial tension for collagen hydrolysis mixtures with different CHC/collagen molar ratios: S1 – 4·10-4; S2 – 4·10-3; S3 – 3·10-2; S4 – 4·10-2; S5 – 7·10-2; S6 – 4·10-1; (open symbols). In all solutions initial collagen concentration was fixed at 5·10-6 M, incubation time 60 min. C (solid symbol/dashed line) – control (collagen without CHC, incubation time 60 min). pH in all solutions was fixed at 3.0.

The rheological parameters for the layers formed by hydrolysed collagen at the air/water interface as a function of frequency of oscillation are shown in Fig. 6. Similarly to the native collagen at the same pH (3.0), all E’ values increase with increasing frequency, while E” do not show any clear trends and are always lower than E’. In comparison with the native collagen, the adsorbed layers formed in samples with high CHC content (S3-S6) display lower storage moduli.

The hydrolysis products are thus not capable of forming as elastic adsorbed layers as the native protein. On the other hand, the layers formed in the slightly hydrolysed samples (S1-S2) display exceptionally high surface dilational elasticity: E’ increases from 36 mN∙m-1 for the control (native collagen) to 69 mN∙m-1 for S2 (all values obtained for the highest probed oscillation frequency of 0.1 Hz, see Fig. 6C). Interestingly, also the loss moduli (E”) values are clearly higher for S1 and S2 than for the other samples (including the native sample). The results for f=0.1 Hz (Fig. 6C) confirm the presence of a maximum in both rheological moduli of the adsorbed layers for CHC/collagen ratio of 4·10-3, followed by a shallow minimum. This points to a rather complex effect of hydrolysis on mechanical properties of the adsorbed layers.

0.00 0.05 0.10

10

20

30

40

50

60

70

0.00 0.05 0.10

3

6

9

12

15

18

1E-3 0.01 0.1 1

0

20

40

60

E' /mN.m-1

f /Hz

A C

E'' /mN.m-1

f /Hz

E'' / mN.m-1

E' / mN.m-1

CCHC/Ccol

0

20

40

60

B

Fig. 6. Surface dilational rheological parameters: (A) elastic modulus, (B) loss modulus of adsorbed layer collagen hydrolysis mixtures with (open symbols) and without CHC (solid symbol) as a function of oscillation frequency. CHC/collagen molar ratio S1 – 4·10-4 (), S2 – 4·10-3 (), S3 – 3·10-2 (), S4 – 4·10-2 (), S5 – 7·10-2 (); S6 – 4·10-1 (); collagen without CHC, incubation time 60 min (); (C) E’ () and E’’ () of adsorbed layer collagen hydrolysis mixtures with and without CHC (E’ – dotted line, E” dashed line) as a function of CHC/collagen molar ratio. In all solutions initial collagen concentration was fixed at 5·10-6 M. pH in all solutions was fixed at 3.0.

3.3. Effect of incubation time on enzymatic hydrolysis of collagen

The degree of enzymatic hydrolysis depends not only on the enzyme concentration, but also on the duration of the enzymatic reaction (the time of incubation of a protein with an enzyme). Based on the results from the CHC concentration dependence of the collagen hydrolysates’ surface activity, a fixed CHC/collagen ratio of 4·10-3 was chosen for studying the effect of incubation time.

Five collagen samples, all with CHC concentration of 2∙10-8 M, were prepared as described in the experimental part (T1-T5) and were incubated at 37°C for 35, 45, 60, 75 and 85 min, respectively. After the given time, the enzyme was inactivated by adding 0.1 M hydrochloric acid (final pH 3.0). The samples were first loaded on a 12% polyacrylamide gel, together with the unhydrolyzed collagen (C), the enzyme (E), as well as the “Low Range Protein Ladder”, and subjected to a SDS PAGE analysis (Fig. 7).

Fig. 7. SDS-PAGE electropherogram of CHC-hydrolysed collagen: L - Low Range Protein Ladder, C – control (collagen without CHC, incubation time 60 min). T1-T5 collagen hydrolysis mixture with CHC/collagen molar ratio fixed at 4·10-3. Incubation time respectively T1 – 35 min; T2 – 45 min; T3 – 60 min; T4 – 75 min; T5 – 85 min.

According to expectations, increasing the incubation time leads to the products of decreasing molecular weight. For incubation times exceeding 75 min (T4-T5) the products with molecular weights above 30 kDa were no longer observed. The high molecular weight hydrolysates (60-80 kDa) can only be observed in the samples exposed to CHC for the shortest time (T1-T2). Thus, as far as the incubation time is concerned, the shorter it is, the higher is the fraction of high molecular mass hydrolysis products in the mixture. The RP-HPLC results (Fig. 1C) agree well with those from SDS-PAGE. The hydrolysis products are slightly more polar than the starting collagen, eluting between 45 and 75 min. Their polar character increases with increasing incubation time, as judged by a slight shift of the peaks towards shorter elution times. The low intensity of a characteristic double peak at the retention times between 65 and 70 min suggests that in all cases the fraction of unmodified native protein in the mixture is low. Fig. 8 compares the dynamic and equilibrium surface tension obtained for native collagen and for the partially hydrolysed samples T1-T5. Despite clear differences in the SDS-PAGE patterns, both dynamic and equilibrium surface tension are rather insensitive to the duration of enzymatic hydrolysis. In all cases the initial rates of surface tension decays are much higher than for the native protein. Eventually, the surface tension stabilises faster and at lower values than for the control.

0 1000 2000 3000

54

57

60

63

66

40 50 60 70 80

53

54

55

56

57

C

T3

T5

T4

T2

 /mN.m-1

t /s

T1

C

eq /mN.m-1

t /s

A B

Fig. 8. (A) Dynamic and (B) equilibrium interfacial tension for collagen hydrolysis mixtures with different incubation time: 35 min (T1), 45 min (T2), 60 min (T3), 75 min (T4), 85 min (T5), (open symbols). In all solutions initial collagen concentration was fixed at 5·10-6 M. CHC concentration was fixed at 2·10-8 M (CHC/collagen molar ratio 4·10-3); C – control sample, collagen without CHC, incubation time 60 min (solid symbol/dashed line). pH in all solutions was fixed at 3.0 Similar conclusions concerning the insensitivity of surface properties of the reaction products on the duration of enzymatic reaction can be drawn from the surface dilational rheology results. The comparison of E’ and E” (Fig. 9) shows that only the sample subjected to the longest hydrolysis are slightly less capable of forming highly elastic adsorbed layers. The high-frequency limit data (f=0.1 Hz) show slight decay of both E’ and E”, but the trend is much smaller than one might expect from the differences in the SDS-PAGE results for samples T1-T5. The highest values of the storage and loss moduli were observed for the shortest incubation times: 35 and 45 min (E’=74.5 and E”=17 mN∙m-1).

0.00 0.05 0.10

20

30

40

50

60

70

0.00 0.05 0.10

5

10

15

20

40 60 80

0

20

40

60

E' /mN.m-1

f /Hz

E'' /mN.m-1

f /Hz

E'' / mN.m-1

E' / mN.m-1

t /min.

0

20

40

60

A B C

Fig. 9. Surface dilational rheological parameters: (A) elastic modulus, (B) loss modulus of adsorbed layer collagen hydrolysis mixtures with (open symbols) and without CHC (solid symbol) as a function of oscillation frequency. Incubation time: 60 min (without enzyme) (), T1 – 35 min (), T2 – 45 min (), T3 – 60min (), T4 – 75 min (), T5 – 85 min (); (C) E’ () and E’’ () of adsorbed layer collagen hydrolysis mixtures with and without CHC (E’ – dotted line, E” dashed line) as a function of CHC/collagen molar ratio. In all solutions initial collagen concentration was fixed at 5·10-6 M and CHC concentration - at 2·10-8 M (CHC/collagen molar ratio 4·10-3). pH in all solutions was fixed at 3.0

4. Discussion

A supramolecular structure of collagen is strongly affected by pH and temperature. This peculiarity of fibrous proteins is employed by nature for construction of collagen fibres, the major component of extracellular matrix. On the other hand, extreme pH and prolonged heating might even lead to unfolding and partial hydrolysis of collagen fibres and transformation into gelatin 51. So far, major research focus was put on studying the effect of pH on collagen self-assembly, its microstructure, or structure of collagen fibrils 52-56. The effect of temperature and pH on surface properties of collagen was not extensively studied, because of a generally acknowledged poor surface activity of collagen. Nevertheless, we have shown in our previous paper that even a mild temperature treatment at pH 1.8 affects the surface tension and surface dilational rheology of the adsorbed layers 17. A more extensive study described in this paper confirmed that heating for 10 min at 90 oC in the pH range from 1.8 to 9.0 does not result in any significant hydrolysis of calf skin collagen type I (note the persistence of the characteristic collagen double peak from α1 and α2 polypeptide chains 44 in Fig. 1A). Despite it, the surface tension shows a monotonic dependency on pH, with the highest reduction for pH 9.0. Under conditions employed in this study, most likely only a separation of the triple helices into individual ones (α1 and α2) followed by unfolding of the individual helices into random coils takes place during sample preparation (in the absence of CHC). This shows that an increase of surface activity can be achieved even without hydrolysing collagen, only by means of partial unfolding of the triple helix 57. The increasing ability to reduce surface tension with increasing pH is probably related to approaching the isoelectric point 58,59 (pI= 8.3 for the native collagen 60). Screening of electrical charges near pI of globular proteins was often reported to enhance their surface activity 59. However, if the unfolding (or minimisation of the net charge) of the collagen molecule favours its adsorption, the mechanical properties of the adsorbed layer generally worsen with increasing pH, as shown by a slight decrease of the dilational surface storage modulus, E’ (Fig. 3C). In contrast to the simple temperature treatment, the presence of a collagen-specific enzyme (collagenase, CHC) clearly leads to partial hydrolysis of the protein at close-to-physiological conditions (pH 7, 37 oC). The degree of hydrolysis is dependent on both the enzyme concentration and incubation time. The highest reduction of surface tension and the highest surface dilational storage modulus increase was observed for only slightly hydrolysed collagen samples (S1, S2). For the CHC/collagen molar ratio 4·10-3 (S2), increasing the incubation time did not affect significantly surface activity of the products, even though the SDS-PAGE and RPHPLC proved that the degree of hydrolysis was continuously increasing (T1-T5). This shows that the enzyme concentration needs to be controlled better than the actual duration of the enzymatic reaction. Collagenase is known to attack fragments between Pro-X and Gly-Pro-Y (where Y can be any amino acid residue and X is most often a neutral amino acid 37,38), eventually hydrolyzing collagen to very short and hydrophilic tripeptide fragments. If too much enzyme is present, the population of short peptides in the reaction mixture gets too high and the most amphiphilic highmolecular mass fraction disappears, resulting in reduced surface activity of the mixture. Hydrolysis of collagen has a positive effect also on the initial rate of surface tension decay, which is probably related to the reduction of molecular weight and hence the size of the adsorbing molecules. One could speculate that all rearrangements within the adsorbed layer are also faster for smaller molecules. The best surface activity in terms of surface tension reduction and dilational surface storage modulus was obtained for the hydrolysate mixture containing mostly intermediate hydrolysis products (S2) obtained during incubation time of 45 min (T2). It is worth noting that E’ for this layer (74.5 mN·m-1) is not only higher than for the native (36 mN·m-1), or temperature-denatured collagen (69.8 mN·m-1 at pH 9.0), but also higher than for many globular proteins of comparable molecular weights, e.g., β-lactoglobuline with molar weight of 18.4 kDa, E’= 60 mN·m-1 (5·10-6 M) 4,5 or β-casein with molar weight of 23.8 kDa, E’= 55 mN·m-1 (1·10-6 M) 9,10,61 .

5. Conclusions

The effect of pH and enzymatic hydrolysis using collagenase from Clostridium histolyticum (CHC) on surface activity of collagen was studied by measuring surface tension and surface dilational rheology. All measurements were performed at room temperature at a collagen concentration of 5.0·10-6 M (1.5 mg/mL) The results were complemented with SDS-PAGE and RP-HPLC analysis of the products, which allowed us to correlate the degree of denaturation and enzymatic hydrolysis with their surface activity. The method presented in this paper allows for obtaining hydrolysates under relatively mild conditions (pH 7, 37 oC). Both the enzyme concentration and incubation time were optimised, in the range of 2·10-9 M - 2·10-6 M and 35 – 85 min, respectively. The best results were obtained for the incubation time 45 min and the CHC concentration of 2·10-8 M, corresponding to the enzyme/protein molar ratio of 4·10-3. The products allowed for lowering the air/water surface tension to 53.9 mN/m, due to formation of a highly visco-elastic adsorbed layers. At the high frequency limit (f=0.1 Hz), the layers were shown to be predominantly elastic, with storage modulus, E’= 74.5 mN·m-1, and loss modulus, E”=17.5 mN·m-1. In the absence of CHC, increasing pH to 9.0 allows for further reduction of surface tension to 49.8 mN.m-1, but at the expense of E’ and E”. The results show that the enzymatic hydrolysis with CHC under well controlled conditions (low enzyme concentration) results in moderately hydrolysed products. These products display significantly improved surface properties. Further studies on employing other enzymes are in progress in our laboratory.

Acknowledgements

This work was financially supported by the Warsaw University of Technology and COST CM1101 Action.

References

1. Youssef NH, Nguyen T, Sabatini DA, McInerney MJ. Basis for formulating biosurfactant mixtures to achieve ultra low interfacial tension values against hydrocarbons. Journal of Industrial Microbiology and Biotechnology. 2007;34(7):497-507.

2. Kezwon A, Wojciechowski K. Interaction of quillaja bark saponins with food-relevant proteins. Adv Colloid Interface Sci. 2014;209:185-195.

3. Dexter AF, Middelberg APJ. Peptides as functional surfactants. Industrial and Engineering Chemistry Research. 2008;47(17):6391-6398.

4. Lad MD, Birembaut F, Matthew JM, Frazier RA, Green RJ. The adsorbed conformation of globular proteins at the air/water interface. Physical Chemistry Chemical Physics. 2006;8(18):2179-2186.

5. Noskov BA, Grigoriev DO, Latnikova AV, Lin S-, Loglio G, Miller R. Impact oi globule umolding on dilational viscoelasticity of ß-lactoglobulin adsorption layers. J Phys Chem B. 2009;113(40):13398-13404.

6. Perriman AW, Henderson MJ, Holt SA, White JW. Effect of the air-water interface on the stability of ß-lactoglobulin. J Phys Chem B. 2007;111(48):13527-13537.

7. Alahverdjieva VS, Grigoriev DO, Ferri JK, et al. Adsorption behaviour of hen egg-white lysozyme at the air/water interface. Colloids Surf Physicochem Eng Aspects. 2008;323(1-3):167-174.

8. Ruso JM, González-Pérez A, Prieto G, Sarmiento F. Study of the interactions between lysozyme and a fully-fluorinated surfactant in aqueous solution at different surfactant-protein ratios. Int J Biol Macromol. 2003;33(1-3):67-73.

9. Maldonado-Valderrama J, Fainerman VB, Gálvez-Ruiz MJ, Martín-Rodriguez A, Cabrerizo- Vílchez MA, Miller R. Dilatational rheology of ß-casein adsorbed layers at liquid-fluid interfaces. J Phys Chem B. 2005;109(37):17608-17616.

10. Wojciechowski K, Kezwon A, Lewandowska J, Marcinkowski K. Effect of ß-casein on surface activity of quillaja bark saponin at fluid/fluid interfaces. Food Hydrocoll. 2014;34:208-216.

11. Yang Y, Dicko C, Bain CD, et al. Behavior of silk protein at the air-water interface. Soft Matter. 2012;8(37):9705-9712.

12. Özdemir G, Sezgin OE. Keratin-rhamnolipids and keratin-sodium dodecyl sulfate interactions at the air/water interface. Colloids and Surfaces B: Biointerfaces. 2006;52(1):1-7.

13. Fadeev AS, Yampolskaya GP, Levachev SM, Zaitsev SY. Collagen denaturation in spread monolayers at the air-water interface: Experiments and a possible model of the process. Biochemistry (Moscow) Supplement Series A: Membrane and Cell Biology. 2008;2(1):62-72.

14. Li C, Tian H, Duan L, Tian Z, Li G. Characterization of acylated pepsin-solubilized collagen with better surface activity. Int J Biol Macromol. 2013;57:92-98.

15. Li C, Liu W, Duan L, Tian Z, Li G. Surface activity of pepsin-solubilized collagen acylated by lauroyl chloride along with succinic anhydride. J Appl Polym Sci. 2014;131(14).

16. Chi Y-, Zhang Q-, Liao X-, Zhou J, Shi B. Physicochemical properties and surface activities of collagen hydrolysate-based surfactants with varied oleoyl group grafting degree. Industrial and Engineering Chemistry Research. 2014;53(20):8501-8508.

17. Kezwon A, Wojciechowski K. Effect of temperature on surface tension and surface dilational rheology of type I collagen. Colloids Surf Physicochem Eng Aspects. 2014;460:168-175.

18. Kadler KE, Baldock C, Bella J, Boot-Handford RP. Collagens at a glance. J Cell Sci. 2007;120(12):1955-1958.

19. Hulmes DJS. Vertebrates collagens - structures, functions and biomedical applications. In: Scheibel T, ed. Fibrous proteins. 1st ed. CRC Press; 2008:12-29.

20. Punitha V, Raman SS, Parthasarathi R, et al. Molecular dynamics investigations on the effect of D amino acid substitution in a triple-helix structure and the stability of collagen. J Phys Chem B. 2009;113(26):8983-8992.

21. Persikov AV, Ramshaw JAM, Brodsky B. Prediction of collagen stability from amino acid sequence. J Biol Chem. 2005;280(19):19343-19349.

22. Cabra V, Arreguin R, Vazquez-Duhalt R, Farres A. Effect of alkaline deamidation on the structure, surface hydrophobicity, and emulsifying properties of the Z19 a-zein. J Agric Food Chem. 2007;55(2):439-445.

23. Chan W-, Ma C-. Acid modification of proteins from soymilk residue (okara). Food Res Int. 1999;32(2):119-127.

24. Caessens PWJR, Gruppen H, Visser S, Van Aken GA, Voragen AGJ. Plasmin hydrolysis of ß-casein: Foaming and emulsifying properties of the fractionated hydrolysate. J Agric Food Chem. 1997;45(8):2935-2941.

25. Zambrowicz A, Timmer M, Polanowski A, Lubec G, Trziszka T. Manufacturing of peptides exhibiting biological activity. Amino Acids. 2013;44(2):315-320.

26. Agyei D, Danquah MK. Industrial-scale manufacturing of pharmaceutical-grade bioactive peptides. Biotechnol Adv. 2011;29(3):272-277.

27. Jung S, Murphy PA, Johnson LA. Physicochemical and functional properties of soy protein substrates modified by low levels of protease hydrolysis. J Food Sci. 2005;70(2):C180-C187.

28. Jeon Y-, Byun H-, Kim S-. Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes. Process Biochemistry. 1999;35(5):471-478.

29. Thiansilakul Y, Benjakul S, Shahidi F. Compositions, functional properties and antioxidative activity of protein hydrolysates prepared from round scad (decapterus maruadsi). Food Chem. 2007;103(4):1385-1394.

30. Girardet J-, Debomy L, Courthaudon J-, Miclo L, Humbert G, Gaillard J-. Viscoelastic properties of oil-water interfaces covered by bovine ß-casein tryptic peptides. J Dairy Sci. 2000;83(11):2410-2421.

31. Kilara A, Panyam D. Peptides from milk proteins and their properties. Crit Rev Food Sci Nutr. 2003;43(6):607-633.

32. Panyam D, Kilara A. Emulsifying peptides from the tryptic hydrolysis of casein. J Food Sci. 2004;69(3):FCT154-FCT163.

33. Elias RJ, Bridgewater JD, Vachet RW, Waraho T, McClements DJ, Decker EA. Antioxidant mechanisms of enzymatic hydrolysates of ß-lactoglobulin in food lipid dispersions. J Agric Food Chem. 2006;54(25):9565-9572.

34. Groleau PE, Morin P, Gauthier SF, Pouliot Y. Effect of physicochemical conditions on peptide-peptide interactions in a tryptic hydrolysate of ß-lactoglobulin and identification of aggregating peptides. J Agric Food Chem. 2003;51(15):4370-4375.

35. Klompong V, Benjakul S, Kantachote D, Shahidi F. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 2007;102(4):1317-1327.

36. Mookhtiar KA, Van Wart HE. Clostridium histolyticum collagenases: A new look at some old enzymes. Matrix (Stuttgart, Germany).Supplement. 1992;1:116-126.

37. Nimptsch A, Schibur S, Ihling C, et al. Quantitative analysis of denatured collagen by collagenase digestion and subsequent MALDI-TOF mass spectrometry. Cell Tissue Res. 2011;343(3):605-617.

38. Sugasawara R, Harper E. Purification and characterization of three forms of collagenase from clostridium histolyticum. Biochemistry (N Y ). 1984;23(22):5175-5181.

39. Loglio G, Pandolfini P, Miller R, et al, eds. Drop and bubble shape analysis as a tool for dilational rheological studies of interfacial layers. ; 2001Studies in Interface Science; No. 11.

40. Makievski AV, Fainerman VB, Miller R, Bree M, Liggieri L, Ravera F. Determination of equilibrium surface tension values by extrapolation via long time approximations. Colloids Surf Physicochem Eng Aspects. 1997;122(1-3):269-273.

41. Fainerman VB, Makievski AV, Miller R. The analysis of dynamic surface tension of sodium alkyl sulphate solutions, based on asymptotic equations of adsorption kinetic theory. Colloids Surf Physicochem Eng Aspects. 1994;87(1):61-75.

42. Deyl Z, Mikšík I, Eckhardt A. Preparative procedures and purity assessment of collagen proteins. Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences. 2003;790(1-2):245-275.

43. Hofman KA, Hall BI. Ion exchange HPLC of a marine collagen. Journal of Liquid Chromatography and Related Technologies. 2009;32(17):2512-2529.

44. Van Der Rest M, Fietzek PP. A comprehensive approach to the study of collagen primary structure based on high-performance liquid chromatography. European Journal of Biochemistry. 1982;125(3):491-496.

45. Lucassen-Reynders EH, Cagna A, Lucassen J. Gibbs elasticity, surface dilational modulus and diffusional relaxation in nonionic surfactant monolayers. Colloids Surf Physicochem Eng Aspects. 2001;186(1-2):63-72.

46. Banerjee I, Mishra D, Das T, Maiti S, Maiti TK. Caprine (goat) collagen: A potential biomaterial for skin tissue engineering. Journal of Biomaterials Science, Polymer Edition.

2012;23(1-4):355-373.

47. Ogawa M, Moody MW, Portier RJ, Bell J, Schexnayder MA, Losso JN. Biochemical properties of black drum and sheepshead seabream skin collagen. J Agric Food Chem. 2003;51(27):8088-8092.

48. Yan M, Li B, Zhao X, et al. Characterization of acid-soluble collagen from the skin of walleye pollock (theragra chalcogramma). Food Chem. 2008;107(4):1581-1586.

49. Merkel JR. Purification and characterization of a marine bacterial collagenase. Biochemistry (N Y). 1978;17(14):2857-2863.

50. Wolters GHJ, Vos-Scheperkeuter GH, Van Deijnen JHM, Van Schilfgaarde R. An analysis of the role of collagenase and protease in the enzymatic dissociation of the rat pancreas for islet isolation. Diabetologia. 1992;35(8):735-742.

51. Bozec L, Odlyha M. Thermal denaturation studies of collagen by microthermal analysis and atomic force microscopy. Biophys J. 2011;101(1):228-236.

52. Dehsorkhi A, Castelletto V, Hamley IW, Adamcik J, Mezzenga R. The effect of pH on the self-assembly of a collagen derived peptide amphiphile. Soft Matter. 2013;9(26):6033-6036.

53. Noitup P, Morrissey MT, Garnjanagoonchorn W. In vitro self-assembly of silver-line grunt type I collagen: Effects of collagen concentrations, pH and temperatures on collagen selfassembly. J Food Biochem. 2006;30(5):547-555.

54. Tien W-, Chen M-, Yao P-. Effects of pH and temperature on microstructure and morphology of hydroxyapatite/collagen composites synthesized in vitro. Materials Science and Engineering C. 2012;32(7):2096-2102.

55. Yan M, Li B, Zhao X, Qin S. Effect of concentration, pH and ionic strength on the kinetic self-assembly of acid-soluble collagen from walleye pollock (theragra chalcogramma) skin. Food Hydrocoll. 2012;29(1):199-204.

56. Silva CC, Pinheiro AG, Thomazini D, et al. Effect of the pH on the piezoelectric properties of collagen films. Materials Science and Engineering B: Solid-State Materials for Advanced Technology. 2001;83(1-3):165-172.

57. Guo L, Colby RH, Lusignan CP, Whitesides TH. Kinetics of triple helix formation in semidilute gelatin solutions. Macromolecules. 2003;36(26):9999-10008.

58. Wierenga PA, Gruppen H. New views on foams from protein solutions. Current Opinion in Colloid and Interface Science. 2010;15(5):365-373.

59. Pezennec S, Gauthier F, Alonso C, et al. The protein net electric charge determines the surface rheological properties of ovalbumin adsorbed at the air-water interface. Food Hydrocoll. 2000;14(5):463-472.

60. Zhang Z, Li G, Shi B. Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes. Journal of the Society of Leather Technologies and Chemists. 2006;90(1):23-28.

61. Dan A, Gochev G, Krägel J, Aksenenko EV, Fainerman VB, Miller R. Interfacial rheology of mixed layers of food proteins and surfactants. Current Opinion in Colloid and Interface Science. 2013;18(4):302-310.