Effect of pretreatment on enzymatic hydrolysis of bovine collagen and formation of ACE-inhibitory peptides

Yuhao Zhang a,b, Karsten Olsen b, Alberto Grossi b, Jeanette Otte b,*

a College of Food Science, Southwest University, No. 2 Tiansheng Road, Beibei District, Chongqing 400716, PR China
b Department of Food Science, Faculty of Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

A R T I C L E   I N F O

Article info
Received 8 January 2013
Received in revised form 21 March 2013
Accepted 2 May 2013
Available online 24 May 2013

Keywords:
Collagen
Boiling
High pressure
Enzymatic hydrolysis
ACE-inhibition
DH

A B S T R A C T

Bovine collagen was pre-treated (boiled or high pressure (HP)-treated) and then hydrolysed by 6 proteases. The degree of hydrolysis (DH) and the angiotensin-converting enzyme (ACE)-inhibitory activity of hydrolysates were measured. All enzymes used were able to partly degrade collagen and release ACE-inhibitory peptides. The highest ACE-inhibitory activity was obtained with Alcalase. Pretreatment significantly influenced the DH and ACE-inhibition. For most enzymes, boiling for 5 min resulted in a significantly higher DH and ACE-inhibitory activity. With Alcalase and collagenase, hydrolysis and release of ACE-inhibitory peptides occurred without any pretreatment, but HP-treatment significantly improved the DH and ACE-inhibitory activity. HP did not markedly affect the hydrolysis with the other enzymes. The major peptides obtained with Alcalase were identified; all were released from the triple helix structure of collagen. Many of these peptides had C-terminal sequences similar to known ACE-inhibitory peptides. The present results suggest that collagen-rich food materials are good substrates for the release of potent ACE-inhibitory peptides, when proper pre-treatment and enzymatic treatment is applied.

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1. Introduction

Large quantities of by-products from livestock, such as horn, hoof, bone and skin, or fish, such as bone and scales, are produced during food and meat processing. Presently, a major part of these by-products are discarded or used as feed. However, these waste products contain a large amount of collagen and thus can be utilised as protein resources for novel food materials.

Many types of collagen have been identified, but about 90% of the collagen in the body is of type I (Reed, Vernon, Abrass, & Sage, 1994; Risteli, Eslamaa, Niemi, Novamo, & Risteli, 1993). Collagen molecules are composed of three α-chains intertwined in the so-called collagen triple-helix. This particular structure, mainly stabilized by intra- and inter-helix hydrogen bonds, is the product of an almost continuous repetition of the Gly-X-Y- sequence, where X is mostly proline (Pro) and Y is mostly hydroxyproline (Hypro) (Ichikawa et al., 2010). Only the very short N- and C-terminal regions (15–26 amino acid residues), do not form triple helical structures, as they are largely made up of lysine and hydroxyllysine (Hyly) residues, as well as their aldehyde derivatives, engaging in intra- and inter-molecular covalent cross-links (Belitz, Grosch, & Schieberle, 2009; Bruckner & Vanderrest, 1994). Four to eight collagen molecules in cross-section are stabilized and reinforced by covalent bonds to constitute the basic unit of collagen fibrils (Gomez-Guillen, Gimenez, Lopez-Caballero, & Montero, 2011).

Major applications of collagen include leather products, cosmetics (due to their good moisturising property) and biomedical products such as wound dressings, implants, and drug carriers, as well as gelatin production in food-related industries (Nam, You, & Kim, 2008). Gelatin is a soluble protein compound, obtained by partial hydrolysis of collagen. Extraction of gelatin is normally done by a chemical pre-treatment with an acid or alkali, which breaks non-covalent bonds so as to disorganise the collagen structure, thus producing adequate swelling and collagen solubilisation (Djabourov, Lechaire, & Gaill, 1993). Subsequent heating at temperatures above 45 °C cleaves the hydrogen and covalent bonds to destabilise the triple-helix and causes a helix-to-coil transition and thus conversion into soluble gelatin (Gomez-Guillen et al., 2002).

Collagen-rich animal by-products can be added value by enzymatic modification (Kida et al., 1995; Morimura et al., 2002), and over the past decade, collagen-rich discarded materials from the meat and food processing industries, have been found to be valuable sources of hydrolysates and peptides with bioactive properties such as antioxidant, antihypertensive/ACE-inhibitory activity, antimicrobial and immunomodulatory activities (Aleman, Gimenez, Montero, & Gomez-Guillen, 2011; Aleman, Gimenez, Perez-Santin, Gomez-Guillen, & Montero, 2011; Byun & Kim, 2001; Ding et al., 2011; Ichimura, Yamanaka, Otsuka, Yamashita, & Maruyama, 2009; Kim, Byun, Park, & Shahidi, 2001; Molinero, Julia, Erro, Robert, & Infante, 1988; Saiga et al., 2003, 2008; Yang et al., 2009).
Most studies concerning collagen-derived peptides, have focused on antihypertensive/ACE-inhibitory activity, since ACE-inhibitory properties seem to be associated with the unique amino acid composition of collagen. It is known that peptides containing a Pro or Hyp residue at the C-terminal position, such as Gly-Pro-Hyp-Gly-Thr-Asp-Gly-Ala-Hyp, Gly-Pro-Pro-Gly-Ala-Hyp, Gly-Pro-Pro-Gly-Ala-Hyp and Gly-Phe-Hyp-Gly-Pro, have strong ACE-inhibitory activity (IC50 from 8.6 to 200 μM) (Ichimura et al., 2009; Oshima, Shimabukuro, & Nagasawa, 1979).

However, due to the triple-helical structure, collagen is not accessible for enzymatic cleavage, except for enzymes belonging to the matrix metallo protease (MMP) family, including various collagenases, which contain both a catalytic site and a hemopoxin domain (Chung et al., 2004). Actually, the substrate used in all previous reports about release of ACE-inhibitory peptides from collagen was not native collagen but gelatin or an extract of collagen obtained by boiling for >3 h (Saiga et al., 2008) which is probably also gelatin. From these gelatinous substrates ACE-inhibitory peptides have been released by the use of Alcalase, pronase E, Aspergillus protease, trypsin, pepsin and other proteases (Byun & Kim, 2001; Ichimura et al., 2009; Kim et al., 2001; Nam et al., 2008; Saiga et al., 2008; Zhao et al., 2007). It would, however, be advantageous to find a less energy consuming way to pretreat native insoluble collagenous materials to destabilise the triple-helix and expose inner sites of collagen for enzymatic attack, or to find some enzymes able to attack the triple-helix structure of collagen effectively.

High hydrostatic pressure (HP) in the range of 100–1000 MPa is used for processing, in order to inactivate microorganisms and improve functional properties of proteins (Grossi, Gkarane, Otte, Erdhjerg, & Orlien, 2012; Knudsen, Otte, Olsen, & Skibsted, 2002). Upon HP processing, the balance of intramolecular and solvent–protein interactions is changed, probably by disturbing the balance of the non-covalent interactions that stabilize the native conformation of proteins (Torrezan, Tham, Bell, Frazier, & Cristianini, 2007). HP treatment has been shown to reduce the thermal stability of bovine collagen, by dissociating the collagen fibres into fibrils and fibres into molecules (Ichinoseki, Nishiumi, & Suzuki, 2006), and vine collagen, by dissociating the collagen fibres into fibrils and fibrils into gelatin (Gomez-Guillen, Gimenez, & Montero, 2005). Heat treatment is often used before enzymatic hydrolysis of proteins to unfold the peptide chain and expose internal cleavage sites (Considine, Patel, Anema, Singh, & Creamer, 2007; Otte, Schumacher, Ipson, Ju, & Qvist, 1999). However, there is little work addressing the influence of short-term boiling as a pre-treatment to enzymatic hydrolysis of collagen.

The aim of the present study is to determine the effect of HP and boiling as pre-treatments on the hydrolysis of collagen by six commercial proteases and release of ACE inhibitory peptides. Furthermore, peptide profiles were revealed and peptides in the most accessible hydrolysates were identified, and the contribution of these to the bioactivity discussed.

2. Materials and methods

2.1. Materials and chemicals

Bovine collagen from achilles tendon (Type I, Insoluble, C9879), and Alcalase® from Bacillus licheniformis (P4860), proteinase K from Trirarachium album (P6556), thermolysin from Bacillus thermoproteolyticus rokko (P1512), pepsin from porcine stomach mucosa (P7000), trypsin from bovine pancreas (T9201), ACE (EC 3.4.15.1, rabbit lung, 0.25 units), o-aminobenzoyleglycyl-p-nitro-L-phenylalan-aryl-proline (ABZ-Gly-Phe(NO2)-Pro) and o-phthaldialdehyde (OPA) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Collagenase from Clostridium histolyticum (P1510), NuPAGE 10% Bis–Tris gels, LDS sample buffer and MOPS SDS running buffer were obtained from Invitrogen A/S, Carlsbad, CA. Precision plus Protein Standard All Blue marker was obtained from Bio-Rad Laboratories, Inc., Hercules, CA. Dithiothreitol (DTT) was obtained from Applichem GmbH, Darmstadt, Germany. All chemicals were of analytical grade or of the highest available purity. Highly purified water (Milli-Q PLUS, Millipore Corporation) was used for preparation of all buffers and solutions.

2.2. Samples preparation and pretreatment

Collagen (0.07 g) was suspended in 7 ml of phosphate buffer with a different pH according to the optimal pH of the enzyme (Table 1), and kept at 4 °C overnight for hydration. Three samples were made for each enzyme, one was left untreated and two were subjected to boiling and HP, respectively, as pretreatments. The samples to be boiled were immersed in a boiling water bath for 5 min, then immediately cooled for 10 min by tap water. The samples for HP treatment were quantitatively transferred into polyethylene bags and sealed under vacuum. HP treatment was performed in the pressurising chamber of a QUINTUS Food Processing Cold Isostatic Press QFP-6 (Aureve Technologies AB, Västerås, Sweden) at 600 MPa for 15 min. Then, the samples were completely transferred back into tubes and subjected to hydrolysis. The time interval between the end of the HP treatment and the start of the hydrolysis was 2 h.

2.3. Hydrolysis of samples

The samples were pre-incubated at the optimal temperature for the enzyme (Table 1) for 10 min before addition of the enzyme solution. The enzyme–substrate ratio was 1:100 (w/w) in all experiments. Sample aliquots (1 ml) were withdrawn after 1, 3, 60, 120, and 240 min of hydrolysis and immediately heated at 90 °C for 15 min to inactivate the enzyme. After cooling (20 min on ice) and centrifugation (11,000 g for 5 min; Microcentrifuge 154.RF, Ole Dick Instrumentmakers ApS, Hvidovre, Denmark), the supernatant was filtrated through a cellulose acetate filter (0.45 μm pore size, Sartorius, Götttingen) and frozen at –18 °C. The pH of pepsin hydrolysates was modified to 7.0–7.5 using 2 M NaOH before freezing. Blank samples without hydrolysis were prepared in phosphate buffer pH 7.5 and pH 2.0, respectively, and kept at 4 °C for 12 h. These were then incubated at 37 °C for 4 h and treated as the hydrolysate samples.

2.4. Determination of DH

The OPA method used to measure the degree of hydrolysis (DH) was a modification of that described by Spellman, McCvoy, O’Cuinn, and FitzGerald (2003). The OPA reagent (5 mM) was

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Optimal condition</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcalase</td>
<td>pH 8.0, 55 °C</td>
<td>0.1 M phosphate buffer</td>
</tr>
<tr>
<td>Collagenase</td>
<td>pH 7.5, 37 °C</td>
<td>0.1 M phosphate buffer</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>pH 8.0, 37 °C</td>
<td>0.1 M phosphate buffer</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>pH 7.5, 37 °C</td>
<td>0.1 M phosphate buffer</td>
</tr>
<tr>
<td>Pepsin</td>
<td>pH 2.0, 37 °C</td>
<td>0.05 M phosphate buffer</td>
</tr>
<tr>
<td>Trypsin</td>
<td>pH 8.0, 37 °C</td>
<td>0.1 M phosphate buffer</td>
</tr>
</tbody>
</table>
prepared according to the description of Spellman et al. on the day
of use. The assay was carried out by transferring an adequate vol-
ume of the hydrolysate sample into a 10 ml tube and adding 2.4 ml
of OPA reagent. Sample volumes between 20 and 140 µl were
used to give an absorbance below 1.0. The reaction was allowed to pro-
ceed for 10 min to reach a plateau, and then the reaction mixture
was transferred to 3 ml disposable cuvettes (Cat. No. 7590 05,
BRAND GMBH + CO KG) and the absorbance was measured at
340 nm (Varian Cary 3 UV–visible spectrophotometer, Mulgrave,
Australia). A blank containing only 2.4 ml of OPA reagent was also
made. All determinations were made in duplicate. The DH was cal-
culated according to the formula:

\[ DH (\%) = 100 \times \frac{n/N}{A_{\text{sample}} - A_{\text{blank+sample}}} \times \frac{M_d}{\varepsilon c} \]

where \( A_{\text{sample}} \) is the absorbance of the samples at 340 nm. \( M \) is the
molar mass of the protein (Da), and \( d \) is the dilution factor and cal-
culated according to the amount of sample used. \( \varepsilon \) is the molar
extinction coefficient of OPA at 340 nm (6000 M\(^{-1}\) cm\(^{-1}\)) and \( c \) is
the protein concentration (10 g/l). A molar mass of bovine type I
collagen of 406.940 Da and the number of peptide bonds per colla-
gen molecule (N) of 4287 were used for the calculation. These were
found from the absorbance measurements according to the formula:

\[ n = \frac{(A_{340\text{sample}} - A_{340\text{unhydr sample}}) \times M_d}{\varepsilon c} \]

2.6. SDS–PAGE analysis of solubilised proteins and fragments

Four control samples, unhydrolysed but subjected to the vari-
ous pre-treatments, and the proteinase K hydrolysates made with
HP pre-treatment were analysed by SDS–PAGE. The control sample
with pH 2 was prepared in 0.05 M phosphate buffer, pH 2.0, and
left at 4 °C for 12 h to evaluate the degradation of collagen by acid.
Samples (25 µl) were prepared with 65 µl of LDS sample buffer,
and 10 µl of 1.0 M DTT, and boiled for 5 min. The samples were
immediately cooled in an ice bath and centrifuged (15,000g for
5 min). Forty microlitres of unhydrolysed samples and 15 µl of
hydrolysed samples were loaded on the gel, together with 5 µl of
standard markers (Precision plus Protein Standard All Blue mark-
er). NuPAGE 10% Bis–Tris gels were used according to the manu-
facturer’s instructions in a XCell II Mini-Cell (El 9001, Novex,
San Diego, CA). Electrophoresis was run at 200 V for 55 min in cassettes
containing the MOPS SDS running buffer. The gel was stained with
0.1% Coomassie brilliant blue R-250 (2% concentrated phosphoric
acid, 15% ammonium sulphate, 17% ethanol, 0.1% Coomassie brilli-
ant blue) for at least 3 h on a rocking table and destained in
Mill-iQ water for at least 1 h. The gels were photographed by a
charge-coupled device (CCD) camera (Raytest, Camilla II, Strauben-
hardt, Germany). The intensity of the bands was quantified using
Phoretix 1D software, version 2003.02. A minimum profile back-
ground was subtracted. The concentration of protein in the bands
was determined as the peak area of the selected bands in the lane
profile.

2.7. Characterisation and identification of peptides by LC–MS

Selected samples from all the enzymatic hydrolysates and un-
treated samples were analysed by LC–MS/MS as described by Otte,
Shalaby, Zakora, Pripp, and El-Shabrawy (2007) using an Agilent
1100 MSD Trap with Chemstation for LC 3D systems Rev. B.01.03
(Agilent Technologies 2001–2005) and Trap Control software ver-
sion 5.3 (Bruker Daltonics GmbH 1998–2005). Sample volumes of
25 µl were injected and eluted in 75 min using a gradient with 100% A
(0.1% TFA in water) for 5 min followed by a linear increase to 60% B
(0.1% TFA in 90% acetonitrile) over the next 70 min. MS spectra were recorded using the range 50–2000 m/z and the target
mass 1521, and Auto MS (2) spectra were recorded from two pre-
cursor ions with the Smart Parameter Setting on.

Data was processed by Bruker Daltonics Data Analysis version
3.3. All MS(n) compounds from each hydrolysate were searched
for matching proteins using Mascot Version 2.1 (Matrix Science
Ltd., London, UK) with selection of trypsin or no enzyme. Further-
more, Biotools software (Version 3.0, Bruker Daltonics, Bremen,
Germany) was used to assign peptide masses to particular frag-
ments of collagen. Bovine type I collagen α1(1) chains and one
α2(1) chain (P02453 and P02465, respectively, from the UniProt database).

The theoretical maximum DH obtainable with some enzymes was
calculated from the specificity of the enzyme and the number of
such residues present in the collagen α-chains mentioned above
as an average from two α1(1) chains and one α2(1) chains. This was performed
using the Peptide Cutter software (http://web.expasy.org/pep-
tide_cutter/).

2.5. Determination of ACE-inhibitory activity

The ACE-inhibitory assay was performed in microtitre plates
using ABZ-Gly-Phe(NO2)-Pro as the substrate, as described by Sent-
andreau and Toldra (2006) with some modifications. Aliquots
(50 µl) of hydrolysates and of ACE solution (50 µl; 6 µM/ml in
150 mM Tris-buffer, pH 8.3) were added into the well. The micro-
titre plate was shaken for 10 s and incubated at 37 °C for 10 min.
The reaction was started by addition of 200 µl of preheated
(37 °C) substrate solution (0.45 mM ABZ-Gly-Phe(NO2)-Pro in
150 mM Tris–Base buffer, pH 8.3, containing 1.125 mM NaCl). Fluo-
rescence measurements were taken every min for 30 min (Tecan
Genios Plus, Tecan US, Durham, North Carolina, USA) using excita-
tion and emission wavelengths of 360 and 415 nm, respectively.
The control sample (100% ACE activity) contained Tris–Base buffer
instead of hydrolysate. Blank samples containing only substrate, or
substrate and sample (or buffer instead of ACE) were also made.
All samples were assayed in triplicate.

The slope of the fluorescence vs time curve was taken as a mea-
sure of the ACE activity, and the ACE inhibition (%) was calculated
as:

\[ \text{ACE – inhibition} = \frac{100 - 100 \times \left( \frac{x_{\text{sample}} - x_{\text{blank+sample}}}{x_{\text{control}} - x_{\text{blank}}} \right)}{100} \]

where \( x_{\text{control}} \) and \( x_{\text{sample}} \) are the slopes for the control and sam-
ple, respectively, and \( x_{\text{blank}} \) and \( x_{\text{blank+sample}} \) are the slopes from
the blanks with ACE replaced by 150 mM Tris–Base buffer.
3. Results and discussion

3.1. Overall effects of pre-treatment and enzymatic hydrolysis on DH and ACE-inhibitory activity

As expected, the control samples without enzyme showed a DH below 0.1% and displayed no ACE-inhibitory activity, except for the sample incubated at low pH which showed a low DH and ACE-inhibitory activity. This might be due to breakage of some bonds, including covalent bonds and hydrogen bonds, under the acid conditions (Kolodziejska, Skierka, Sadowska, Kołodziejski, & Niecikowska, 2008). After hydrolysis, with all the enzymes used, the DH increased and a high ACE-inhibitory activity was obtained (Figs. 1–3). The reason that all the enzymes, and not only matrix metalloproteinases (MMP) (collagenase), were able to degrade collagen might be that part of the collagen had been broken down into gelatin, during manufacture or subsequent incubation, making it more accessible to other enzymes.

Taking all results together, there was a significant effect of all parameters tested, i.e. the pre-treatment used before hydrolysis, the enzyme used, and the time of hydrolysis, on the DH and ACE-inhibitory activity of hydrolysates (Table 2).

3.1.1. Overall effect of pre-treatment

When considering all enzymes and hydrolysis times, boiling was the pre-treatment giving rise to the highest DH and ACE-inhibitory activity of the collagen hydrolysates (Table 2). This could be due to some inner sites in the triple-helix structure being exposed during boiling resulting in cleavage of part of the hydrogen bonds and covalent bonds, resulting in helix-coil transition of collagen (Zhang, Li, & Shi, 2006). Overall, the HP pretreatment significantly improved the DH of collagen hydrolysates in comparison to the untreated samples, however the release of ACE-inhibitory peptides was significantly reduced (Table 2). The reason that a HP treatment (600 MPa, 15 min), performed less than 2 h prior to hydrolysis, did lead to a general increase in the release of ACE-inhibitory peptides might be that the triple-helix structure of collagen was not weakened by this treatment. The helical collagen structure is saturated with intramolecular hydrogen bonds, and there is one (or two) hydrogen bond between the –CO and –NH groups of the main chains per every three amino acid residues of collagen (Ramachandran & Chandras, 1968). During HP, some intramolecular hydrogen bonds may be disrupted and some new hydrogen bonds with water molecules formed. However, the new hydration structure by hydrogen bonding still could stabilize the triple-helix structure of collagen (Potekhin et al., 2009). Consequently, the inner Pro-rich part of collagen, which gives rise to ACE-inhibitory peptides (Minkiewicz, Dziuba, & Michalska, 2011), were not exposed after the HP treatment. Alternatively, the structural changes caused by the HP treatment were partly reversed before hydrolysis. On the other hand, the significantly higher DH in the HP pretreated samples than in the untreated samples might be due to HP causing some stable changes in the non-triple helix structure of collagen, making them more readily accessible to the enzymes.

3.1.2. Overall effect of hydrolysis time

When all the enzymes were considered together, there was a significant effect of hydrolysis time from 1 to 240 min on the DH, which increased significantly with time (Table 2). The ACE-inhibitory activity of hydrolysates also increased with hydrolysis time, but the maximum was reached after 60 min in most cases. In general, peptides were quickly released during hydrolysis to reach half of the maximum DH and ACE-inhibitory activity after 30 min of hydrolysis (Figs. 1–3), however this varied with the enzyme used.

3.1.3. Overall effect of the enzyme used

When all pre-treatments are taken into account, Alcalase was the most effective enzyme in hydrolysing collagen and giving rise to ACE-inhibitory peptides, followed by collagenase and then proteinase K (Table 2). The lowest DH was found in the hydrolysates...
made with pepsin, but the ACE-inhibitory activity of these hydrolysates was higher than of those made with trypsin (Table 2). These results show that even though a high DH was obtained with some proteases, the specificity of the enzyme was contributing to determining the ACE-inhibitory activity of the released peptides.

3.2. Effects of pre-treatment and hydrolysis time on DH and ACE-inhibitory activity for each enzyme

3.2.1. Alcalase and collagenase

The hydrolysates made with Alcalase and collagenase showed the highest DH of all hydrolysates (Fig. 1), and the DH and ACE-
Table 2
Effects of pre-treatment, enzyme, and hydrolysis time on DH and ACE-inhibitory activity of collagen hydrolysates. Overall results.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Significance level (p)</th>
<th>Effect on DH</th>
<th>Effect on ACE-inhibitory activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>&lt;0.0001</td>
<td>Boiled &gt; HP-treated &gt; Untreated</td>
<td>Boiled &gt; Untreated &gt; HP-treated</td>
</tr>
<tr>
<td>Enzyme</td>
<td>&lt;0.0001</td>
<td>Alcalase &gt; collagenase &gt; Proteinase K &gt; trypsin &gt; thermolysin &gt; pepsin</td>
<td>Alcalase &gt; Proteinase K &gt; collagenase &gt; thermolysin &gt; pepsin &gt; trypsin</td>
</tr>
<tr>
<td>Hydrolysed time</td>
<td>&lt;0.0001</td>
<td>240 &gt; 120 &gt; 60 &gt; 30 &gt; 1 min</td>
<td>240 &gt; 120 &gt; 60 &gt; 30 &gt; 1 min</td>
</tr>
<tr>
<td>Pretreatment-hydrolysis time</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrolysis time + enzyme</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme + pretreatment</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment + hydrolysis + time + enzyme</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Analysis based on type III sum of squares.

b Compared by Tukey’s studentized range test.

c >Significantly higher than (p < 0.05).

d >Higher than, but not significantly different (p > 0.05).

Inhibitory activity were significantly affected by the pretreatment (Table 3).

The DH increased quickly from 1 to 30 min of hydrolysis, with Alcalase and reached a DH of around 12% after 60 min. Visually, the insoluble collagen in the hydrolysis mixture disappeared, and an almost clear solution resulted within 10 min. This shows that the Alcalase preparation is able to degrade collagen, although it does not belong to the MMP family of proteases. Other studies have shown similar results (Cheng, Liu, Fan, & Sakata, 2008; Liu, Chen, Su, & Zeng, 2011). Alcalase is a member of the serine S8 endoproteinase family, which has a broad specificity, and therefore, degraded the triple-helix structure of collagen within short time. After 1 min of hydrolysis, the boiled sample showed the highest DH and best ACE-inhibitory activity compared to the HP treated and the untreated sample (Fig. 1). Boiling causes damage to the triple-helix structure of collagen (Zhang et al., 2006), thus, this sample was hydrolysed faster and the ACE-inhibitory peptides released more rapidly. Interestingly, the HP pretreatment improved the DH significantly from 30 to 240 min of hydrolysis (Fig. 1). The ACE-inhibitory activity was also slightly higher after HP treatment in hydrolysates taken after 30 min of hydrolysis with Alcalase, and reached 88.5%. However, from 60 min of hydrolysis, pre-treatment had no significant influence on the ACE-inhibitory activity (80.8–90.4%) of the Alcalase hydrolysates, which implies that Alcalase had completely degraded the triple-helical structure and released the ACE-inhibitory peptides in all the samples. This level of activity is slightly better than those obtained by Liu et al. (2011) and Cheng et al. (2008) who used Alcalase for the production of ACE inhibitory peptides from sea cucumber collagen and chicken bone protein, respectively.

Collagenase belongs to MMPs, which initially cleave collagens I, II and III at specific Gly-Leu or Gly-Ile bonds located approximately three quarters along the chain from the N-terminus (Chung et al., 2004). This cleavage is critical for the initiation of further collagen breakdown since it causes damage to the triple-helix and exposure of more peptide bonds. In this way collagenase unwinds the triple-helical structure first, before hydrolysing the peptide bonds. Since this is a slow process, the hydrolysis reaction is slow, which is also reflected in the DH which increases throughout hydrolysis (Fig. 1c). Reports on the use of collagenase to hydrolyse collagenous material and produce active peptides are scarce. Oshima et al. (1979) hydrolysed gelatin by collagenase for various times up to 24 h, and the ACE inhibitory activity of hydrolysates was time-dependent with the maximum obtained after 24 h of hydrolysis. Their results confirm that the hydrolytic process with collagenase is slow, and we therefore expect, that an even higher DH and ACE-inhibitory activity might be obtained if the hydrolysis time is prolonged.

Pre-treatment by boiling significantly improved the hydrolysis, and also increased the ACE-inhibitory activity of hydrolysates taken from 1 to 120 min (Fig. 1d). After 240 min of hydrolysis, a DH of 18.1% was obtained, and the corresponding ACE-inhibitory activity was 80.5%. This suggests that the triple-helical structure was damaged by boiling and collagenase could hydrolyse the peptide bonds directly without unwinding first. Interestingly, the HP treatment before hydrolysis also increased the DH compared to the untreated samples. However, this was not reflected in a significantly higher ACE-inhibitory activity, which implies that triple-helical structure still persisted after the HP pretreatment. The pretreatment had no significant influence on the ACE-inhibitory activity of collagenase hydrolysates after 240 min of hydrolysis, which suggests that collagenase had completely unwound the triple-helical structure and released the ACE-inhibitory peptides in all the samples at that time.

For both enzymes, Alcalase and collagenase, the DH of the HP-treated sample was significantly lower than that of the untreated sample when hydrolysis started, which means that, after HP treatment, the conformation of collagen did not allow collagenase to unwind or Alcalase to degrade the triple-helix as efficiently. However, the DH increased for the HP treated samples, indicating that after HP, the unwinding and degradation, respectively, of the triple-helix structure during incubation was facilitated. This might be due to HP causing aggregation of some parts of collagen, and once these were damaged by initial hydrolysis, they were quickly hydrolysed.

3.2.2. Thermolysin and trypsin

The ability of thermolysin and trypsin to hydrolyse collagen was very limited, the DH reaching only about 1% in the untreated and HP treated samples (Fig. 2). The advantage of boiling as a pretreatment was most evident for these enzymes, with significant effects on DH obtained with both enzymes (Fig. 2). During hydrolysis with these enzymes, the DH increased with time, especially in the boiled sample, however, it remained low. The highest DH in boiled samples was around 7%, which is close to the theoretical DH (8.3%) obtainable with trypsin but much lower than expected.
for thermolysin (14.8%), which has a much broader specificity. A study using a sequence analogue to the collagen x1-chain 772780 (Gly-Pro-Gln-Gly-Nph-Ala-Gly-Gln-Val, where Nph is 4-nitrophenylalanine) showed that the rate of thermolysin hydrolysis of the Nph-Ala bond was reduced 15-fold in the native peptide compared with the heat denatured peptide, demonstrating that the triple-helical conformation inhibits general proteolysis of susceptible sequences (Fields, 1995). Another study (Zhang et al., 2006) confirms that native collagen is hardly digested by trypsin due to its stable triple helix, whereas denatured products such as gelatin is easily attacked by trypsin, explaining why boiling was a better pretreatment than the others.

The higher amount of peptides released from the boiled collagen sample was also reflected in a significantly higher ACE-inhibitory activity of these samples (Fig. 2), reaching quite high values, with a maximum around 70% for the trypsin hydrolysates and around 75% for the thermolysin hydrolysates. The specificity of trypsin is mainly towards basic amino acids, and the presence of a positively charged amino acid at the ultimate C-terminal position may contribute significantly to the inhibitory potency of peptides to ACE (Otte et al., 2007). Thermolysin, which is specific for many hydrophobic amino acid residues, should generate peptides with the most favourable amino acids (hydrophobic) at the C-terminal position, explaining the slightly higher ACE-inhibitory activity obtained with this enzyme.

Although HP pre-treatment, before hydrolysis with thermolysin resulted in a significantly higher DH than for the untreated sample (Table 3), the ACE-inhibitory activity of the hydrolysates obtained with both enzymes from the HP treated collagen samples was significantly lower than for the untreated sample (Table 3, Fig. 2b and d). HP in this case obviously was not beneficial, maybe because it lead to aggregation of parts of the collagen, which were accessible to these enzymes, and thus caused a different composition of peptides in the hydrolysates.

3.2.3. Proteinase K and pepsin

As expected, these two enzymes also hydrolysed collagen to a limited degree (Fig. 3), but the effect of pre-treatment, especially boiling, was not as marked as for thermolysin and trypsin. The ACE-inhibitory activity of these two enzymes was also not much affected by the pre-treatment (Fig. 3).

The DH of hydrolysates, made with proteinase K, increased with time for the untreated and HP treated samples, whereas the boiled sample reached a maximum (around 5%) after 60 min. The ACE-inhibitory activity only increased significantly from 1 to 30 min, and reached a value close to 80%. This value is close to the value obtained by Arihara, Nakashima, Mukai, Ishikawa, and Itoh (2001) for hydrolysates of porcine skeletal muscle proteins (major components were myosin, actin and collagen) made with proteinase K (82.1% after 18 h of hydrolysis). So proteinase K seems to cleave quickly what can be cleaved, followed by a very slow increase in DH, which does not affect ACE-inhibitory activities. This might be due to preferential cleavage of the non-helical parts of collagen by this enzyme, since this part also has more susceptible peptide bonds than the helical part. For example, the theoretical DH of proteinase K upon hydrolysis of the x1-chain from bovine collagen is 23.94%, while the values are 18.85% and 35.27% for hydrolysis of the triple-helix and the non-triple-helix regions, respectively.

Boiling of the collagen sample, before hydrolysis, did increase the DH significantly, but the ACE-inhibitory activity was only slightly affected. Interestingly, the HP treatment improved the DH, but the ACE-inhibitory activity of these samples was slightly lower (not significant) than of the untreated samples (Table 3). This might be because the triple helix region of collagen was not hydrolysed by proteinase K and therefore the strong ACE-inhibitory peptides were not released after the HP pretreatment. The proteinase K hydrolysates were further investigated by SDS–PAGE analysis.

During hydrolysis with pepsin, the DH increased with time for all samples, but remained below 3%. From 1 to 30 min, the ACE-inhibitory activity of all samples also increased significantly, then non-significantly afterwards. The final ACE-inhibitory activities were below 70%. The effect of boiling on DH and ACE-inhibitory activity was not significant, whereas the effect of HP treatment was to lower the DH (significantly) and the ACE-inhibitory activity (non-significantly). This might be due to the acid extracting some of the collagen or gelatine, especially in combination with boiling. In the HP treated sample, these proteins do not seem to be extracted to the same degree. Maybe HP in the acidic conditions leads to formation of aggregates that are not dissolved or not easily degraded by pepsin. The control sample, kept in phosphate buffer with pH 2.0 overnight at 4 °C, had a DH around 0.7%, showing that part of the collagen was degraded by the acid during this process. The DH of the peptic hydrolysates was the lowest of all hydrolysates. This is consistent with the theoretical DH obtainable with pepsin on collagen of 6.88%, which is also the lowest within all enzymes. Thus, very few sites in collagen seem to be susceptible to pepsin.

3.3. Relation between DH and ACE-inhibitory activity

In many cases, the values for DH and ACE-inhibition both increased with time of hydrolysis or with a certain pre-treatment. However, as discussed above, this was not always the case. To explore the relationship between DH and ACE-inhibitory in more detail, results from all hydrolysates and hydrolysates with selected enzymes are plotted in Fig. 4. The ACE-inhibitory activity of all hydrolysates increased with DH up to around 4%, after which it seemed almost unaffected by further increases in DH (Fig. 4a). This trend could partly stem from the fact that 90% ACE-inhibition was near the maximum measurable with this method. A similar trend has been observed in the studies by Walsh et al. (2004) and Nielsen, Martinussen, Flambard, Sørensen, and Otte (2009) for milk protein-derived ACE-inhibitory peptides. The correlation between DH and ACE-inhibition could best be described by a logarithmic function with the equation \( y = a - (bc)^x \), where \( y \) is the ACE-inhibition, \( x \) is the DH (%), and \( a, b, \) and \( c \) are the regression parameters. Using this equation it was also possible to calculate the DH at which half of the maximal ACE-inhibition was obtained (DH\(_{50}\)), in this case 1.1%. The same trend was seen for most of the individual hydrolysates, i.e. those made with collagenase (with a DH\(_{50}\) of 2.5%; Fig. 4b), thermolysin, trypsin, and proteinase K (not shown). In contrast, a more linear relationship was seen between the DH and ACE-inhibitory activity for the hydrolysates made with Alcalase and pepsin (Fig. 4c and d), which reached the highest and the lowest DH, respectively. As discussed before, the ACE-inhibitory peptides were quickly released during hydrolysis with Alcalase, and the values for DH and ACE-inhibition of these hydrolysates were beyond 10% and 80%, respectively, after 30 min and were not significantly increased after 60 min. Therefore, most of the points in Fig. 4c are in the upper part, giving a shallow linear relation (with DH\(_{50}\) around 4.3%). Pepsin hydrolysis, on the other hand, resulted in only low DH and the points were at the lower end (Fig. 4d) where both the DH and ACE-inhibitory activity still increased, giving a steep linear correlation, and in this case DH\(_{50}\) was around 1%.

3.4. Characterisation of collagen molecules and degradation products in pretreated collagen samples and hydrolysates

3.4.1. Polypeptides detected by SDS–PAGE analysis

The effect of the various pre-treatments on the solubilisation of individual collagen molecules and primary degradation products
was investigated by SDS–PAGE (Fig. 5). Two \( \alpha \)-chains and a \( \beta \)-component (dimer of \( \alpha \)-chains) were found in all unhydrolysed samples, since they were heated at 90 °C for 15 min to inactivate the enzyme (lanes 1–3). The boiled sample had slightly less \( \beta \)-component and more components with smaller molecular weights (below 100 kDa), indicating that boiling promotes degradation of collagen chains. Interestingly, the sample that had been subjected to HP treatment was quite similar to the others. However, this sample contained less of the lower molecular weight protein fragments (below 100 kDa), showing that less of the subunits (\( \alpha \)- and \( \beta \)-components) of collagen were degraded by heating after HP pretreatment. In contrast, the control sample that had been incubated overnight in phosphate buffer, pH 2.0, (lane 4) contained almost no \( \alpha \)- and \( \beta \)-components and in turn contained several components with a molecular weight below 75 kDa, and even as low as 15–20 kDa. This shows that the acid had cleaved part of the covalent bonds and agrees with the fact that treatment of collagen with acid causes extraction of gelatin (Gomez-Guillen et al., 2005).

The hydrolysates taken after 30 min and 120 min of hydrolysis with proteinase K were particularly interesting; the HP-treated sample had a lower DH but a higher ACE-inhibitory activity than the boiled samples. SDS–PAGE analysis of the HP treated samples hydrolysed with proteinase K is shown in Fig. 5, lanes 5–9. Similar bands were seen at all hydrolysis times (B1 to B8). All the proteins with a MW > 100 kDa (compare to untreated samples) had been degraded after 1 min of hydrolysis. Data analysis by Phoretix 1D showed that only two bands (\( B_5 \) and \( B_6 \)) increased with time of hydrolysis. This might be due to the band intensity measured, resulting from a balance between the formation, by degradation of native collagen, and further degradation into peptides with a smaller molecular weight. The DH of this sample was significantly increased from 120 to 240 min (Fig. 3), so some of the small peptides, that could not be displayed in this gel must have been further degraded during this period. The discrepancy with respect to effect of HP on DH and ACE-inhibitory activity must be sought in the composition of the low molecular weight peptides.
3.4.2. Peptide profiles of hydrolysates

All peptide profiles, except those from the pepsin-hydrolysates, showed the presence of a multitude of small peptides (eluting between 10 to 50 min). Peptide profiles of selected hydrolysates are shown in Fig. 6. There was a very good agreement between the amount of peptides in the profiles and the DH in the hydrolysates as a function of pre-treatment. The hydrolysates obtained after boiling, except with Alcalase and pepsin, contained a much higher concentration of peptides than those from the untreated and HP-treated samples, as shown for the thermolysin hydrolysates in Fig. 6a, confirming the higher DH in these samples. In fact, for the Alcalase hydrolysates, a slightly higher peptide concentration was seen in the hydrolysates made after HP treatment (Fig. 6b), in accordance with the higher DH in these hydrolysates (Fig. 1a). Conversely, for hydrolysis with pepsin, the HP-treated sample gave the lowest amount of peptide material (Fig. 6c), in accordance with a lower DH in these samples (Fig. 3c). Apart from the different amounts, the profiles were quite similar, indicating that in general the same peptide bonds were accessible to Alcalase irrespective of the pre-treatment. The peptides released after pepsin hydrolysis eluted between 50 and 65 min, suggesting that these were much bigger than those released by the other enzymes. Furthermore, a slightly different peptide profile for the HP-treated sample (Fig. 6c) suggests that some parts of collagen (giving rise to peptides with the longest elution time) were less accessible to pepsin after the HP treatment.

The peptide profiles of the collagenase and proteinase K hydrolysates were practically identical (except for the amount) for the three pre-treatments, meaning that these enzymes hydrolysed the same peptide bonds, just at a different rate. Interestingly, the

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Fig. 6. Peptide profiles of collagen hydrolysates made with (a) thermolysin (60 min), (b) Alcalase (30 min), and (c) pepsin (60 min) after various pre-treatments. Upper panel: no pre-treatment; middle panel: boiled for 5 min; lower panel: HP treated.

Fig. 7. Detailed peptide profile (reversed-phase HPLC) of the collagen hydrolysate with the highest ACE-inhibitory activity, which was obtained after pre-treatment with HP and hydrolysis with Alcalase for 30 min. Major masses in peaks are given above peaks.
Table 4  
Masses and tentative identification of major peptides in the Alcalase hydrolysate (30 min) of collagen pre-treated with HP.

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<th>Mass obs. [M+H]</th>
<th>Suggested peptide (score)</th>
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<th>Mass calc. [M+H]</th>
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* P with boldface means Hyp (hydroxylated proline).

* This sequence could not be confirmed.
peptide amount in the hydrolysate made with thermolysin from the HP treated collagen was higher than that from the untreated sample (Fig. 6a), in accordance with the higher DH in these samples (Fig. 2a). The peptide profiles for the thermolytic and tryptic hydrolysates made with Alcalase (30–240 min of hydrolysis), so one of these hydrolysates was characterised in more detail. The peptide profile of the hydrolysate obtained after 30 min of hydrolysis is shown in Fig. 7, with the masses of the major peptides indicated.

A tentative identification of these peptides was undertaken with the results, as shown in Table 4. All identified sequences were from the triple helix structure of collagen. This shows that Alcalase can degrade the triple helix structure of collagen effectively, probably due to its broad specificity. Twenty-nine of the peptides identified from the Alcalase hydrolysate contained Pro or Hyp at one of the three C-terminal positions (Table 4). And many of the peptides contained hydrophobic amino acids at one or more of the three C-terminal positions. These could all have high ACE-inhibitory activity, since the C-terminal tripeptide plays a predominant role in competitive binding to the active site of ACE especially if this has a high hydrophobicity (Byun & Kim, 2002; Gomez-Guillen et al., 2011) and especially in the presence of proline. This is further substantiated by the fact that the peptide GPGP (retention time 35.8 min, Table 4) has the same C-terminal as GPV, which has been isolated from bovine gelatin and shown to have an IC50 of 4.67 μM (Kim et al., 2001). Furthermore, the peptides EPHYP/GPAGLP/GP (retention time 21.6 min) and LGPSGP/GPSP/GP (retention time 32.4 min) share the three C-terminal amino acids with the peptide GGPP/GP (IC50 = 91 μM) obtained from porcine skin gelatin by a protease from Aspergillus oryzae (Ichiimura et al., 2009) and with GAP/GP/GP (IC50 = 29 μM) obtained from chicken collagen by protease FP (Saiga et al., 2008). The peptide RGAGP/G (Retention time 16.6 min) just has an additional N-terminal Arg residue in comparison to GAP/GP, which possesses ACE-inhibitory activity (Ichiimura et al., 2009). The peptides mentioned may thus contribute to the ACE-inhibitory activity of this hydrolysate.

Several of the peptides identified further contained Lys or Arg at the ultimate C-terminal position, which should contribute to the inhibitory potency (Hernandez-Ledesma, Contreras, & Recio, 2011). Therefore, at least 40 of the peptides released by Alcalase, from bovine collagen, are expected to have good ACE-inhibitory activity. Among them nine of the peptides identified seem particularly interesting, i.e. GDRGDAGPK, GVAGPK, MGPR, GEGGPGP, GSPGPGPK, GLPGPK RGPGPM PGGPAGPK and GPAGPPGP, since they contain both Lys, Arg or a very hydrophobic amino acid at the ultimate C-terminal position and Pro at the penultimate position. None of these 9 peptides have previously been reported in the literature, and the ACE-inhibitory activity remains to be determined.

4. Conclusions

The present results confirm that collagen is a good substrate for release of potent ACE-inhibitory peptides. With Alcalase and collagenase the ACE-inhibitory peptides can be released without any pretreatment. When other enzymes are used, a pretreatment, such as boiling for 5 min, is necessary to obtain a good yield of active peptides. Interestingly, HP-treatment, which is receiving increasing interest for protein denaturation, also significantly influenced the outcome of the subsequent hydrolysis, but the effect was not the same for all enzymes. HP-treatment significantly improved the DH and ACE-inhibitory activity of hydrolysates made with Alcalase and collagenase, whereas the opposite was the case for hydrolysates made with trypsin and pepsin. With proteinase K and thermolysin, a HP pre-treatment increased DH but not the ACE-inhibitory activity. The highest ACE-inhibitory activity was obtained from HP-treated collagen subsequently hydrolysed with Alcalase for 30 min, and all identified sequences from this hydrolysate were from the triple helical structure of collagen. These results imply that collagen rich by-products from animal processing plants might be turned into valuable bioactive peptides by proper pre-treatment and enzymatic treatment.

Acknowledgements

We gratefully acknowledge the financial support from the Chinese Government through the scholarship under the State Scholarship Fund for Yuhao Zhang, and the support from the Danish Strategic Research Council through the NOVENIA project. We thank Cristian De Gobba for skilful help with the ACE assay. We are also thankful to Kirsten Sjøstrøm, Jie Yin and Sishe Jongberg, Department of Food Science, for their technical assistance and advice.

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