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Post-enzymatic hydrolysis heat treatment as an essential unit operation for collagen solubilization from poultry by-products

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ABSTRACT

Enzymatic protein hydrolysis (EPH) is an invaluable process to increase the value of food processing by-products. In the current work the aim was to study the role of standard thermal inactivation in collagen solubilization during EPH of poultry by-products. Hundred and eighty hydrolysates were produced using two proteases (stem Bromelain and Endocut-02) and two collagen-rich poultry by-products (turkey tendons and carcasses). Thermal inactivation was performed with and without the sediment to study the effect of heat on collagen solubilization. A large difference in molecular weight distribution profiles was observed when comparing hydrolysate time series of the two proteases. In addition, it was shown that 15 min heat treatment, conventionally used for inactivating proteases, is essential in solubilizing collagen fragments, which significantly contributes to increasing the protein yield of the entire process. The study thus demonstrated the possibility of producing tailored products of different quality by exploiting standard heat inactivation in EPH.

1. Introduction

Enzymatic protein hydrolysis (EPH) has in recent years been recognized as a versatile technology for improved utilization and valorization of proteins from food processing by-products such as cuts from poultry and fish fillet processing (Aspevik et al., 2017; Esteban & Ladero, 2018). During EPH, proteins from by-products are digested and solubilized by the catalytic action of proteases. After protease inactivation at near-boiling temperatures, the EPH process includes downstream recovery of three crude fractions: peptides, lipids, and a collagen- and mineral-rich low value sediment. Depending on by-product composition, choice of protease and processing settings, the peptide products of EPH processes can have very different composition and hence exhibit differences in both functional and biological properties (Oliveira, 2021; Wouters, Rombouts, Fierens, Brijs, & Delcour, 2016). By-products from the poultry industry are typically very complex and consist of different tissues, i.e., connective and muscle tissue. Connective tissues contain large amounts of collagen proteins. Muscle tissue, on the other hand, is rich in actin and myosin, which are the main myofibrillar proteins. Myofibrillar and collagen proteins have very different amino acid composition and structural characteristics rendering different physical stability, and nutritional properties of the proteins. While many industrial EPH processes mainly focus on achieving a high protein yield, there is a growing interest in exploiting the possibilities related to increased protein quality and composition. In this context, there is an unrealized potential in designing multi-step EPH processes to separate and extract the different components from complex poultry by-products. In this way, production of protein products with different functional, nutritional, and biological properties can potentially be achieved (Lindberg et al., 2021).

Collagen proteins can have various forms and the structures are highly specialized for their respective functions (Cen, Liu, Cui, Zhang, & Cao, 2008; Schmidt et al., 2016; Shoulders & Raines, 2009). The unique properties of collagen and collagen-derived peptides, such as excellent physical properties, biocompatibility, biodegradability and low allergenicity, have been widely utilized in for example food products, pharmaceuticals, medical products, and cosmetics (Chattopadhyay, Raines, & Glick, 2014; Gomez-Guillen, Gimenez, Lopez-Caballero, &

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Montero, 2011; Hashim, Ridzwan, Bakar, & Hashim, 2015; Hong, Fan, Chalamaiah, & Wu, 2019; Liu, Nikoo, Boran, Zhou, & Regenstein, 2015; Avila Rodríguez, Rodríguez Barroso, & Sánchez, 2018; Silva et al., 2014). In traditional extraction and production of collagen-rich products, including partially degraded and denatured collagen (i.e., gelatin), the collagen-rich parts of the animals, like the skin, hoofs, tendons, bones, and cartilage, are separated and processed. Separating collagen-rich parts from larger animals such as bovine and porcine is relatively easy and is done manually in the industry. For small animals such as chicken and fish, this separation is a labor-intensive procedure. At the same time, using machines is difficult since the collagen-rich parts are small and mixed with muscle tissue. Thus, for the latter, an alternative strategy for separation of proteins with different properties is to use selective proteases in such a way that different proteins are digested and solubilized at different stages in the EPH process.

Collagen proteins in their native form have a characteristic triple helix structure formed by three α-chains held together by hydrogen bonds. The α-chains consist of repeating triplets of the amino acids Gly-X-Y, where X and Y frequently are proline (Pro) and hydroxyproline (Hyp), respectively (Shoulders & Raines, 2009). The structure is stabilized by intramolecular hydrogen bonds between glycine (Gly) in adjacent chains resulting in high thermal stability. Collagen-rich materials are therefore generally resistant to enzymatic degradation. However, it is well-known that heat energy, with or without pH adjustment, can be used to extract collagen by disrupting hydrophobic interactions and hydrogen bonds in the collagen structure by increasing the kinetic energy (Liu et al., 2015; Schmidt et al., 2016). This causes the molecules to vibrate so rapidly and vigorously that the secondary bonds maintaining the collagen structure are disrupted (Shoulders & Raines, 2009). Studies have also demonstrated that ultrasonic treatment, which increases the kinetic energy in the system, can be used to improve collagen extraction yields from EPH processing dramatically (Li, Mu, Cai, & Lin, 2009; Yu, Zeng, Zhang, Liao, & Shi, 2016). In a recent study, Lindberg et al. selected and studied the activity of two specific proteases (i.e., stem Bromelain and Endocut-02) towards collagen-rich and myofibrillar protein-rich poultry materials (Lindberg et al., 2021). Using nitrogen content measurements and size exclusion chromatography (*SEC*), the study indicated that liberation of larger collagen fragments and collagen peptides from poultry by-products could be related to the heat inactivation step of the EPH process. In addition, it was shown that the molecular weight distribution profiles of the hydrolysates from collagen-rich by-products were dependent on the type of protease used.

The protein yield after EPH is typically attributed to the efficiency of the protease and other process conditions, such as hydrolysis time, but usually not to the thermal inactivation step. In industrial EPH, heat treatment is a standard unit operation to inactivate proteases and to pasteurize hydrolysates (Hou, Wu, Dai, Wang, & Wu, 2017; Tavano, 2013). This is commonly done without removing the non-dissolved fraction, hereby referred to as the sediment. However, to the knowledge of the authors, the specific role of heat treatment in solubilization of proteins and peptides following EPH has not been studied systematically. In the current work, the aim was to study the role of the thermal inactivation step in collagen solubilization during the EPH process of poultry by-products. Moreover, by exploiting differences in the physical properties between the two major protein classes (i.e., myofibrillar proteins and collagens), the possibility of tailoring the product composition of poultry protein hydrolysates was explored. This was studied by comparing EPH samples which were produced using two different processing strategies, i.e., thermal inactivation of the entire EPH reaction mixture, and thermal inactivation of the EPH reaction mixture after removal of the sediment.

2. Materials and methods

2.1. Materials

The poultry raw materials, i.e., turkey tendons (TT) and turkey carcasses (TC), were provided by Nortura (Hærland, Norway). The tendons were manually separated from turkey by-products. Both TT and TC were ground using a Seydelmann SE130 grinder (Stuttgart, Germany), vacuum packed in 350 g packages and stored at −20 °C until use. Protein content was measured using combustion analysis (Dumas, ISO 16634–1). Ash analysis was performed according to ISO 5984, water content was determined according to ISO 6496, and fat content analysis was performed according to EC 152/2009. Amino acid composition analysis was performed according to ISO 13903:2005, EU 152/2009 for TC and Hong Ji Liu et al. for TT (Liu, Chang, Yan, Yu, & Liu, 1995). The protease product stem Bromelain (B) powder 1200 GDU was provided by Bromelain Enzyme (Jakarta, Indonesia) and Endocut-02 (E) by Tailorzyme AsP (Herlev, Denmark). Chemicals without further specified origin were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Enzymatic protein hydrolysis

EPH experiments were run employing two poultry raw materials (i.e., TT and TC) and two protease preparations (i.e., B and E). For all raw materials and protease combinations, the EPH processes were run for 0, 5, 15, 30, 45, 60, 90 and 120 min, respectively. Two processing approaches for each of the enzymatic hydrolysis experiments were performed post hydrolysis: *i*) thermal inactivation of the entire reaction mixture (i.e., including the sediment); and *ii*) thermal inactivation of the reaction mixture after removal of the sediment. This was done to show and compare how heat facilitates the release of protein fragments and peptides to the water phase. In addition, all EPH experiments were performed in triplicates. This resulted in a total of 180 individual EPH processes. A summary of the 180 EPH conditions is presented in Table 1.

The EPH reactions were performed, six at a time, in randomized order. Prior to each run, a 350 g raw material package was heated in a

Table 1
Overview of the 180 samples collected and analyzed from enzymatic hydrolysis of poultry by-products.

Sample name	By-product material	Protease	Heat treatment	Total number of samples, (hydrolysis time in minutes)*
TTBS	Turkey tendons	Bromelain	With sediment	21, (5, 15, 30, 45, 60, 90 and 120)
TTES	Turkey tendons	Endocut-02	With sediment	21, (5, 15, 30, 45, 60, 90 and 120)
TTB	Turkey tendons	Bromelain	Without sediment	21, (5, 15, 30, 45, 60, 90 and 120)
TTE	Turkey tendons	Endocut-02	Without sediment	21, (5, 15, 30, 45, 60, 90 and 120)
TCBS	Turkey carcasses	Bromelain	With sediment	21, (5, 15, 30, 45, 60, 90 and 120)
TCES	Turkey carcasses	Endocut-02	With sediment	21, (5, 15, 30, 45, 60, 90 and 120)
TCB	Turkey carcasses	Bromelain	Without sediment	21, (5, 15, 30, 45, 60, 90 and 120)
TCE	Turkey carcasses	Endocut-02	Without sediment	21, (5, 15, 30, 45, 60, 90 and 120)
TTS	Turkey tendons	–	With sediment	3, (0)
TT	Turkey tendons	–	Without sediment	3, (0)
TCS	Turkey carcasses	–	With sediment	3, (0)
TC	Turkey carcasses	–	Without sediment	3, (0)
Total number of samples				**180**

*All experiments were performed in triplicates.

water bath holding 50 °C for 60 min. To 500 mL of preheated Erlenmeyer flasks, 50 g of raw material was added. The flasks were placed in an Innova 40 incubator (Eppendorf, Hamburg, Germany) at 50 °C, and 95 mL preheated water (50 °C) was added to each flask. The mixtures were stirred (300 rpm shaking) for 5 min, followed by the addition of 0.5 g protease dissolved in 5 mL of water. The EPH processes were performed at 50 °C under 300 rpm shaking. The zero-minute samples were treated the same way without added protease. All the reaction mixtures were subjected to centrifugation (5200*g* for 5 min) at the set time points. After centrifugation, the reaction mixtures, with or with the sediment removed, were transferred to 400 mL beakers and heated to 95 °C in a Menumaster microwave oven (ACP, IA, USA) and mixed. The respective reaction mixtures were then kept at 95 °C for 15 min in a water bath. Following the heat treatment, the reaction mixtures inactivated using processing strategy *i* were immediately subjected to centrifugation (5200*g* for 5 min). For the reactions where the sediment was removed prior to heat inactivation (strategy *ii*) there was no need for an extra centrifugation step. The fat was separated from the hydrolysate using a separation funnel (200 mL) for all TC samples at temperatures above 70 °C. All the hydrolysates and zero-minute water phases were vacuum filtrated while above 70 °C using T2600 Pall filters (Pall Corporation, NY, USA) and transferred to plastic containers. The filtrates were stored at −40 °C before lyophilization, performed using a Gamma 1–16 LSCplus freeze dryer (Martin Christ Freeze Dryers, Osterode am Harz, Germany).

2.3. Dumas analysis

The protein yields were calculated based on total nitrogen recovery from the substrate and proteases added to the reaction mixtures. Nitrogen content for all the 180 hydrolysis samples and proteases were measured by combustion analysis, i.e., the Dumas method. The lyophilized hydrolysates and proteases were analyzed using a Vario EL cube instrument (Elementar, Langenselbold, Germany), using sulfanilamid as a correction standard and 5 mg samples packed in tin foil, as described by Rieder et al. (2021). Different nitrogen-to-protein conversion factors are generally used for different proteins, and while 5.55 is commonly used for collagen, 6.25 is the usual conversion factor for muscle proteins (Mariotti, Tomé, & Mirand, 2008). Since all materials in the current study were based on mixtures of these two types, and not purified collagen or myofibrillar tissues, the conversion factor 6.25 was chosen for all samples. This was considered satisfactory since the protein yield first and foremost was used for study relative changes during the EPH processes.

2.4. Hydroxyproline analysis

The amino acid Hyp was used to quantify collagen indirectly (Cissell, Link, Hu, & Athanasiou, 2017; Stoilov, Starcher, Mecham, & Broekelmann, 2018). The collagen yields were calculated based on Hyp recovery. Collagen content was calculated based on the assumption that collagen contains 13.5% Hyp. The Hyp content for the 180 hydrolysates was determined using a hydroxyproline assay kit (MAK008-1KT) from Sigma-Aldrich (St. Louis, MO, USA). The analysis was performed following the Sigma-Aldrich and Offengenden et al. descriptions, with minor modifications (Offengenden, Chakrabarti, & Wu, 2018). Solutions of 1 mg/mL of each hydrolysate in 1.0 M HCl were mixed 1:1 with concentrated HCl (~12 M) in 2 mL Wheaton sample vials with PTFE lined solid caps from Sigma-Aldrich. The samples were hydrolyzed for 3 h at 110 °C in a heating block from VWR (Radnor, PA, USA). After hydrolysis, 10 μL of the samples were transferred to a Pierce 96-Well Polystyrene Plate (Thermo Fisher Scientific, Waltham, MA, USA) and dried at 60 °C before 100 μL of Chloramine T in oxidation buffer was added to each sample and standard well and mixed. After 5 min, 100 μL of dimethylamine borane (DMAB) reagent was added and the plate was incubated in an oven at 60 °C for 90 min. The absorbance was measured at 560 nm using a BioTek Synergy H1 spectrophotometer (BioTek Instruments, VT, USA). The hydroxyproline content was then determined using the standard curve of hydroxyproline as described in the Sigma-Aldrich protocol.

2.5. Degree of hydrolysis

The samples were prepared by dissolving 10 mg/mL lyophilized hydrolysates in 0.21 M sodium phosphate buffer (pH 8.2). The samples were then left to rehydrate overnight. Before analysis, the samples were subjected to sonication at 50 °C for 30 min, followed by a dilution to 0.5 mg/mL in 1% w/v SDS-solution. The degree of hydrolysis (DH%) of the samples were determined using the TNBS method. (Kristoffersen et al., 2020). All measurements were performed in triplicates and the DH% values were then calculated using h_{tot} values calculated from the amino acid composition of the by-product materials.

2.6. Size exclusion chromatography

The chromatographic separation was performed using a Thermo Scientific Dionex UltiMate 3000 Standard System (Thermo Fisher Scientific) and a BioSep-*SEC*-s2000 column (300 × 7.8 mm, Phenomenex, Torrence, CA, USA) at 25 °C following a previously published method with minor modifications (Wubshet et al., 2017). The injection volume was 10 μL for the standards (2 mg/mL in Milli-Q) and 10 μL for the sample solutions (10 mg/mL lyophilized hydrolysate in 0.1% (v/v) acetic acid). The mobile phase consisted of 30% acetonitrile and 0.05% trifluoroacetic acid in ultrapure water (v/v). Chromatographic runs were controlled from the Chromeleon Chromatography Data System software V 7.2 SR4 (Thermo Fisher Scientific). From chromatographic runs of both the standards and hydrolysates, a UV trace of 214 nm was used. The retention times of the standards, presented in supporting information (SI) Table S-1, were used to construct a third polynomial fitted calibration curve (Vander Heyden, Popovici, & Schoenmakers, 2002). Finally, the chromatograms were subjected to area analysis, and mass-average molar masses were calculated using PSS winGPC UniChrom V 8.00 (Polymer Standards Service, Mainz, Germany).

2.7. Data analysis

Effects of the four experimental factors “by-product material”, “protease”, “heat treatment” and “hydrolysis time” were evaluated by *N*-way ANOVA, including main effects and 2-factor interactions. The explained variance, also denoted as η^2 or effect size, was also calculated for each effect.

3. Results and discussion

A total of 180 protein hydrolysates were prepared for the presented study. The hydrolysates were produced using two raw materials from poultry processing (i.e., turkey tendons (TT) and turkey carcass (TC)) and two proteases (i.e., stem Bromelain (B) and Endocut-02 (E)). The study was designed to focus primarily on the effect of the thermal inactivation step following enzymatic hydrolysis, with an emphasis on collagen solubilization. Hence, two processing approaches for each of the enzymatic hydrolysis experiments were performed post hydrolysis: *i*) thermal inactivation of the entire reaction mixture (i.e., including the sediment); and *ii*) thermal inactivation of the reaction mixture after removal of the sediment (Fig. 1). Products from these two sets of reactions were systematically compared in terms of protein and collagen yields, DH%, and molecular weight distribution profiles.

3.1. Analysis results

The amino acid composition and proximate composition of the two raw materials are presented in SI Table S-2. Generally, the amino acid

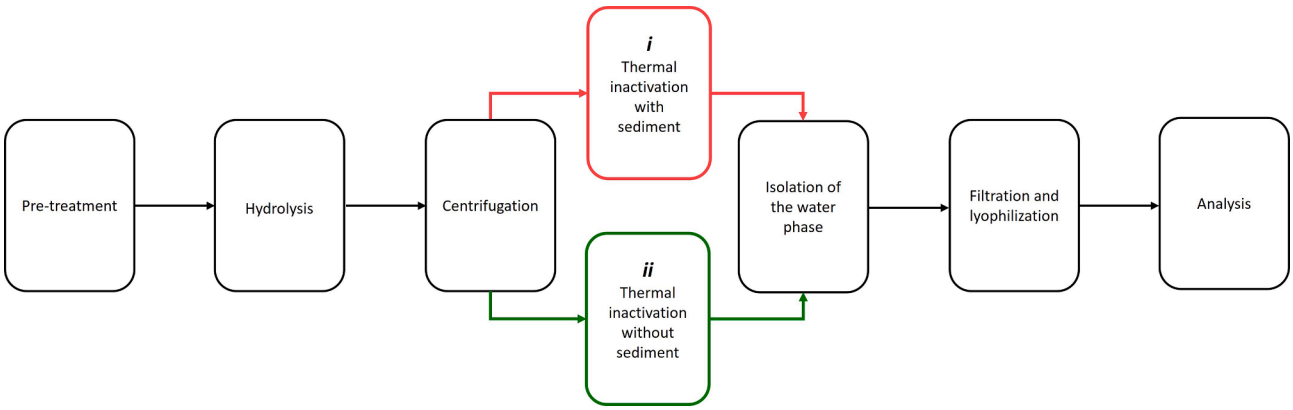

Fig. 1. Flowchart overview of the EPH processes: processing strategy *i*) thermal inactivation with the sediment (indicated in red), and processing strategy *ii*) thermal inactivation without sediment (indicated in green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

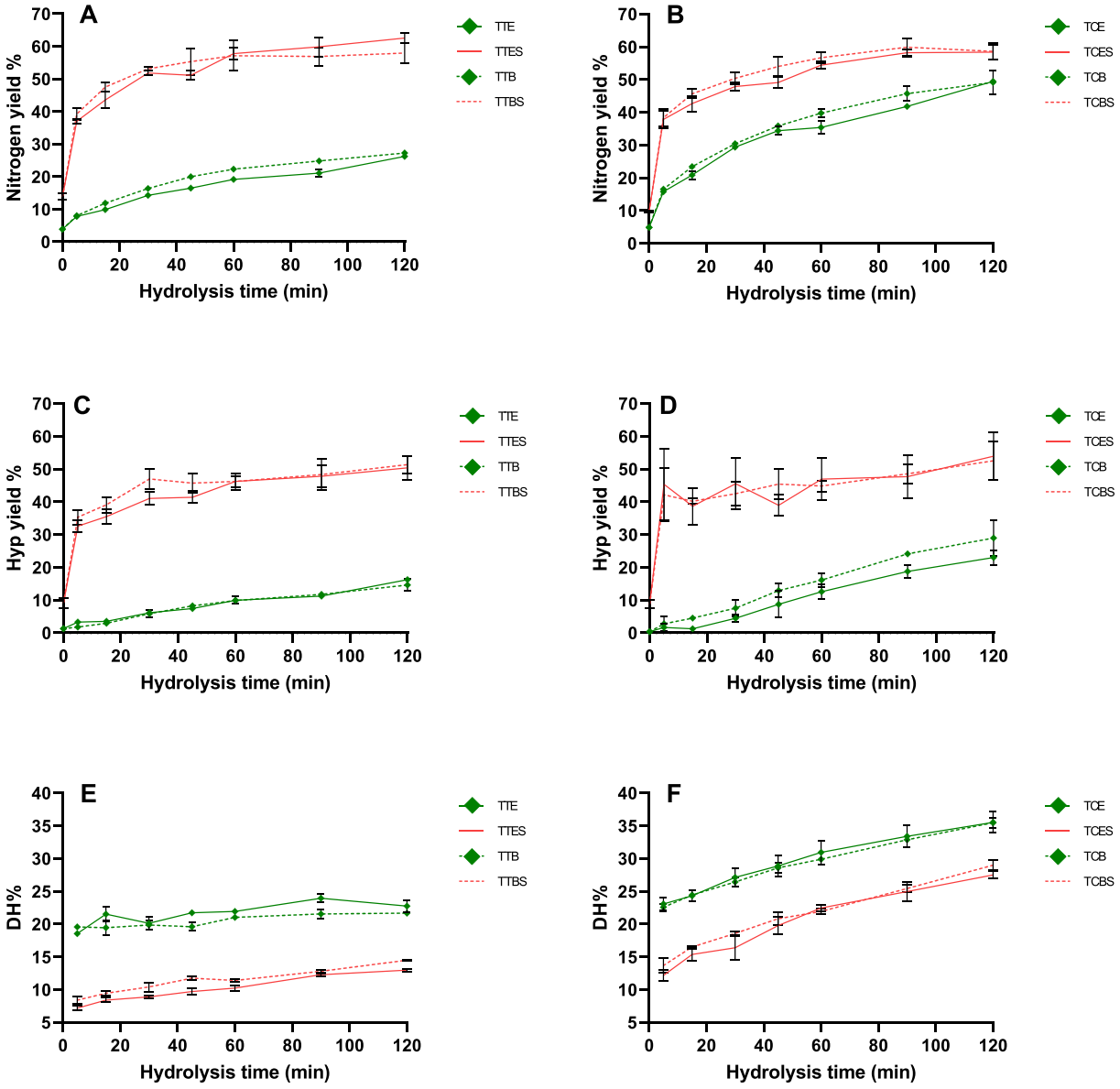

Fig. 2. The average nitrogen and Hyp yields, and DH% values of the triplicate hydrolysis series. A) nitrogen yields from TT, B) nitrogen yields from TC, C) Hyp yields from TT, D) Hyp yields from TC, E) DH% of the TT samples and F) DH% of the TC samples. B and E indicate the protease used and S indicates heat inactivation with sediment. DH% measurements of the zero-minute samples are not included.

composition of the two materials was comparable. The main difference is seen in the amino acids associated with collagen, i.e., Gly, Pro, Hyp, Ala, and Arg (Shoulders & Raines, 2009). Based on the proximate composition analysis, using 6.25 as the nitrogen-to-protein conversion factor, TT and TC contained 31.8% and 18.8% protein, respectively. Assuming that collagen contains about 13.5% Hyp, TT and TC consisted of 67.1% and 33.4% collagen, respectively (Neuman & Logan, 1950). Expectedly, the by-product material mainly constituting connective tissue (i.e., TT) was found to contain significantly higher collagen levels compared to turkey carcass (TC).

The relative nitrogen and Hyp yields and DH% values of the EPH products of the two poultry by-product materials are presented in Fig. 2. Effects of the experimental factors on nitrogen yield, Hyp yield and DH% values were studied by *N*-way ANOVA analysis. The results are presented in SI Table S-3. Note that the power of the experimental design is high, meaning that some effects might be statistically significant even if the effect size is negligible. The nitrogen yields, i.e., protein yields, for the different hydrolysis conditions ranged from 3.8% to 64.3% over the 120 min hydrolysis time (Fig. 2A and 2B). The nitrogen yield for each of the 180 EPH processes are presented in SI Table S-4. Comparing the results shown in Fig. 2A and 2B, major differences in protein yield were observed between the two processing strategies studied, i.e., thermal inactivation of the entire reaction mixture (processing strategy *i*, red time series) and inactivation of the reaction mixture after removal of the sediment (processing strategy *ii*, green time series). The difference was especially large in the case of the collagen-rich TT material (Fig. 2A). After 120 min of hydrolysis of TT, the protein yield was more than doubled when thermal inactivation was performed with sediment. Using stem Bromelain, the increase was 113%, and for Endocut-02 139% compared to the same reaction where sediment was removed before the inactivation step. These results clearly showed that the standard 15 min heat inactivation step contributes more to protein solubilization than the 120 min enzymatic hydrolysis reaction alone. This was also reflected in the *N*-way ANOVA results presented in SI Table S-3. The increase in yield after 120 min of hydrolysis of TC as a result of heat inactivation with sediment was relatively lower, i.e., 19.2% increase for stem Bromelain and 18.0% for Endocut-02, compared to TT. The collagen content difference of the two raw materials may explain the observed difference as collagens are known to be less accessible for EPH digestion compared to muscle proteins. As described earlier, the collagen content of TC was significantly lower compared to TT. By comparing the increase in yield due to heat inactivation with sediment between TT and TC (Fig. 2A and 2B) it can be suggested that a major contributor to increase in yield using processing strategy *i* may be linked to heat induced collagen solubilization.

To study differences in actual collagen yields between the two processing strategies, a Hyp assay was used. Following the amount of Hyp released into the water phase during an EPH process and downstream processing steps serves as a parameter to monitor solubilization of collagen-derived protein fragments and peptides. Relative Hyp yields are provided in Fig. 2C and 2D, whereas the calculated collagen yield for each of the 180 samples is presented in SI Table S-5. As shown in Fig. 2C and 2D, the relative Hyp yields from TT and TC ranged from 0.2% to 58.5% during the time series. Collagen solubilization was also observed in the zero-minute samples, prepared the same way without addition of the protease, with a yield ranging from 0.2% to 10.8%. After five minutes of EPH, the collagen yields were increased at least three-fold when the reactions were terminated with the sediment compared to inactivation without sediment, verifying a rapid release of collagen as a result of EPH in combination with thermal inactivation (processing strategy *i*). When the reaction mixture was thermally inactivated without the sediment, on the other hand, there was a gradual linear increase of Hyp yields. The highest Hyp yield in this case was observed after 120 min of hydrolysis in the TC series with stem Bromelain (i.e., 34.7 %). The Hyp yields were slightly higher, but not significantly higher at all time points, for the TCB series compared to the TCE series. A similar difference in relative Hyp yields between the protease time series TTB and TTE was not observed. Higher relative yields of Hyp from TC compared to TT using processing strategy *ii* can partially be explained by lower protein content and the difference in the collagen part of all proteins of the TC relative to the TT material. The composition difference results in a relatively higher enzyme-to-collagen ratio in EPH of TC than for TT. In addition, differences in the structural network and composition of collagen types in the two raw materials are known to have an impact (Cen et al., 2008; Schmidt et al., 2016; Shoulders & Raines, 2009). Nevertheless, the Hyp yield results were consistent with the increase in protein yields as a result of heat inactivation and showed that, in addition to inactivating the protease, the heat treatment can serve as an extraction step for collagen fragments and peptides in EPH processes. Processing strategy was also identified as the major contributor to collagen yields in the *N*-way ANOVA results, see SI Table S-3. Heat-induced solubilization of collagen due to loss of molecular order of the triple helix is well described in the literature. However, heat induced collagen solubilization following enzymatic digestion was studied in depth here for the first time.

A classical approach to study EPH reactions is to follow the development in percentage cleaved peptide bonds, i.e., DH%, during the reaction or products (Wubshet et al., 2019). In order to study the role of the proteases in the degradation of the two raw materials, DH% values were measured. The results are presented in Fig. 2E and 2F, and individual DH% values are available in SI Table S-6. The results showed major differences between the processing strategies, but only minor differences between the proteases. This is in resemblance with the trends seen in protein yields and are supported by the *N*-way ANOVA results presented in SI Table S-3. Processing strategy *i* resulted in lower DH% values than processing strategy *ii*. This is most likely due to introduction of high molecular weight collagen fragments liberated from the sediment as a result of the heat treatment. Hence, since DH% is inversely proportional to molecular weight, products with presumably large collagen fragments (i.e., higher Hyp) were also associated with lower DH%. Another notable observation was the relatively small change in DH% within the 120-minute reaction time of the TT material. The difference in DH% between 5- and 120-minutes samples was notably lower than for TC. This may indicate that there is a difference in how the proteases digest and liberate proteins from collagen-rich raw material (i.e., TT) compared to myofibrillar-rich material (i.e., TC).

Molecular weight distribution profiles and mass-average molar mass values measured using *SEC* are commonly used as a quality parameter to monitor EPH processes (Cho, Unklesbay, Hsieh, & Clarke, 2004; Kristoffersen et al., 2020; Van Beresteijn et al., 1994; Wubshet et al., 2017). The molecular weight distribution profiles have, in addition to serving as a quality measure, the potential to unveil more information about the proteases' mode of action towards collagen and muscle proteins. In the present study, *SEC* was therefore used to evaluate the effects of the different processing conditions on the resulting hydrolysates. The applied *SEC* method was optimized to separate proteins and peptides in the molar mass range between 204 and 66,000 g/mol. The retention times of molar mass standards and the calculated mass-average molar mass values for all the hydrolysates are provided in SI Table S-1 and S-7.

In contrast to the previous results shown in Fig. 2, the molecular weight distribution profiles unveiled clear differences also between the proteases used (Figs. 3 and 4). Overall, hydrolysates produced using processing strategy *i* contained a larger share of early eluting (5.5 – 7.5 min) higher molecular weight proteins and protein fragments as compared to those produced using processing strategy *ii*. This once again reinforces our previous observation that a significant amount of high molecular weight protein fragments and peptides are solubilized after thermal inactivation of the hydrolysis reaction, and that a significant share of the heat-dissolved protein is collagen fragments.

As expected, for the TTBS series, a general increase in smaller peptides eluting after nine minutes was observed, while protein fragments and peptides eluting earlier gradually decrease until 60 min of

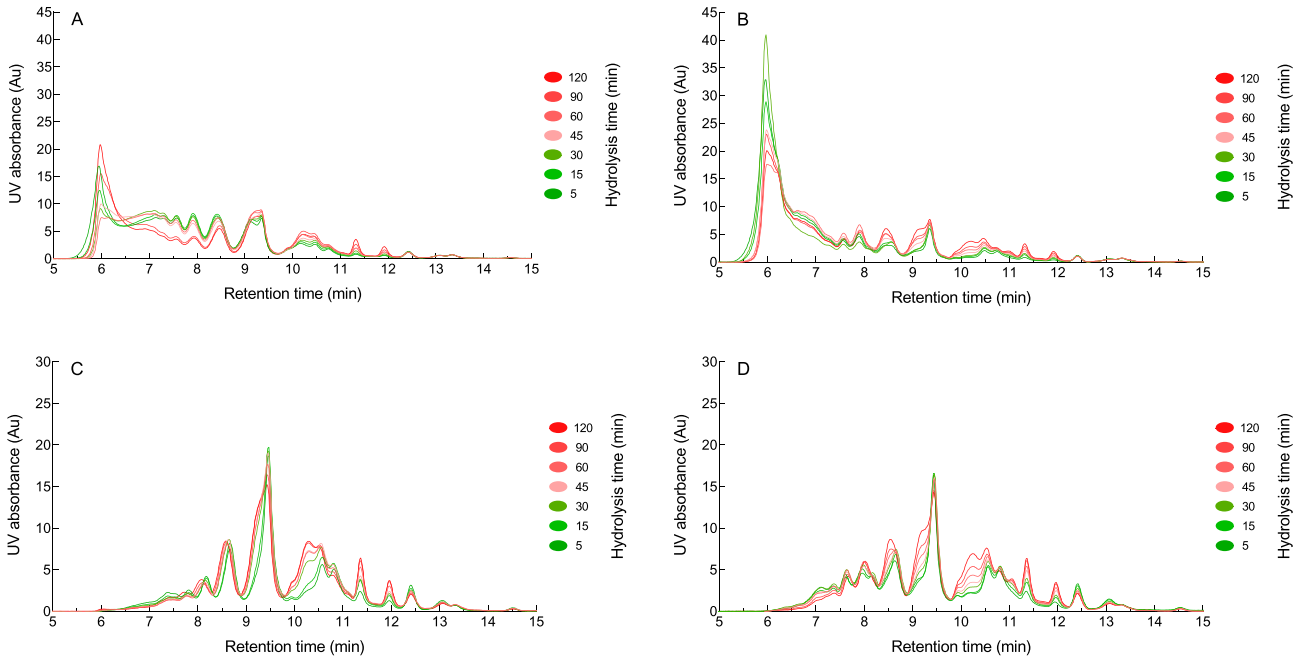

Fig. 3. Molecular weight distribution profiles of the four different TT EPH time series. A) TTBS heat treated with sediment, B) TTES heat treated with sediment, C) TTB heat treated without sediment, and D) TTE heat treated without sediment.

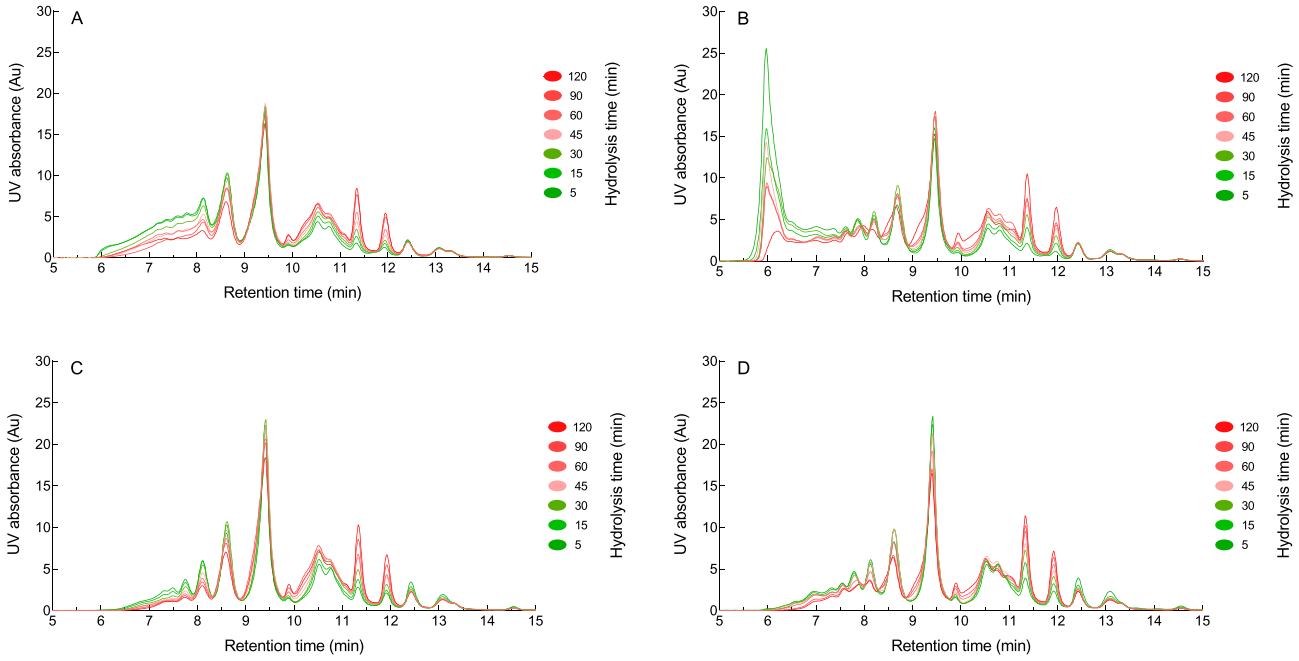

Fig. 4. Molecular weight distribution profiles of the four different TC EPH time series. A) TCBS heat treated with sediment, B) TCES heat treated with sediment, C) TCB heat treated without sediment, and D) TCE heat treated without sediment.

hydrolysis (Fig. 3A). However, an unexpected sudden increase in the larger protein fragments and decrease in mid-size protein fragments were observed in the 90- and 120-minutes samples, i.e., protein fragments eluting from 5.5 to 6.5 min and from 6.5 to 9 min, respectively. This effect was observed in all three replicates of the TTBS time series. A possible explanation is the composition and size range of the protein fragments and peptides in these hydrolysates. The TTBS samples after 60 min of hydrolysis are among the most collagen-rich in this study. Protein hydrolysates, especially from collagen, are known to aggregate, and changes in aggregation behavior can occur as a result of the EPH reaction (Meyer & Morgenstern, 2003). For the TTES hydrolysates, a gradual decrease in protein fragments eluting between 5.5 and 7.5 min and an increase in peptides and free amino acids eluting after 7.5 min was observed (Fig. 3B). The samples produced using processing strategy *ii* on TT are shown in Fig. 3C and 3D. A clear difference in the molecular weight distribution profiles for the protease chosen was observed in the time series, similar to those reported by Lindberg et al. (Lindberg et al., 2021). Hydrolysates produced using processing strategy *ii* and TT with Endocut-02 resulted in hydrolysates containing increased levels of high molecular weight protein fragments and peptides compared to the stem Bromelain time series.

The molecular weight distribution profiles of the TC hydrolysate

time series are presented in Fig. 4. Compared to the TT hydrolysate time series, the most striking differences were seen in the time series inactivated with sediment. In the TCES series, the amount of high molecular weight fragments eluting between 5.5 and 7.5 min was high at the first time points, greatly decreasing throughout the reaction. This was comparable to the molecular weight distribution profile evolution of the TTES series. The same was not observed for the hydrolysate time series produced using stem Bromelain and processing strategy *i* (TTBS and TCBS). In TCBS, only a small fraction of high molecular weight fragments was eluted between 5.5 and 7.5 min from the start of the reaction with a small reduction happening over time. This shows that the mode of action of the two proteases used in the current study indeed affects the resulting composition of the hydrolysates. Stem Bromelain has a preference towards cleaving peptide chains with an Arg or Lys residue in the *P*1 position, while the subtilisin type Endocut-02 prefers hydrophobic amino acids (Trp, Phe, Tyr) (Ahmad et al., 2017; Bhagwat & Dandge, 2018; Hale, Greer, Trinh, & James, 2005; Johanning et al., 1998). In collagen the preferred cleaving sites are mostly found in the telopeptides, i.e., the ends of the triple helix structure, and not in the triple helix core. The collagen derived protein fragments should, thus, be of higher molecular weight than protein fragments and peptides derived from muscle proteins. In addition, the preferred cleaving sites and location of these in the triple helix structure of collagen are different for the two proteases, with stem Bromelain's intrinsic selectivity including more of possible cleaving sites within the core triple helix structure than Endocut-02. This explains the differences observed between the molecular weight distribution profiles of the eight different EPH time series shown in Figs. 3 and 4.

3.2. General discussion

In the current study, the results of two different EPH processing strategies of collagen-rich by-products are compared. The standard processing strategy used by industry, i.e., strategy *i* in the current study, resulted in similar protein yields to those reported for poultry collagen by Offengenden et al. using a single protease and two-hour reaction time (Offengenden et al., 2018). Collagen extraction from turkey by-products has also been reported using heat treatment and pH-shift as extraction strategies. However, these processes included a longer processing times and lower yields (i.e., 6.36% and 13.51%, respectively) compared to the combined enzymatic hydrolysis and heat treatment strategy *i* reported in the current study (i.e., 45.6% to 58.5% yield after two hours EPH) (Du, Keplová, Khiari, & Betti, 2014). Heat assisted collagen extraction under acidic conditions, with or without pepsin, or under alkali conditions, are well-known and established practices in industry (Liu et al., 2015; Schmidt et al., 2016). Yet, thermally facilitated solubilization of collagen fragments and peptides following a standard enzymatic protein hydrolysis at neutral pH is not extensively studied. The current study clearly shows that at the end of EPH and prior to thermal inactivation, the reaction mixture constitutes of solubilized collagen-derived peptides and intact and partly hydrolyzed collagen not liberated into the water phase. The latter was only solubilized when heat was added to the system, i.e., energy was added to interrupt the strong intramolecular interactions.

The molecular weight distribution profiles of the hydrolysates produced using processing strategy *ii* were not substantially different using the two proteases and reaction conditions studied. Higher relative Hyp and protein yields were observed from TC compared to TT, but this can also be explained by differences in protein content and the protein composition of the raw materials. The findings suggest that both larger collagen-derived protein fragments as well as smaller peptides are to a high degree kept in the sediment until heat inactivation. This can be explained by several factors such as the strong intermolecular interactions in collagens and their tightly packed, less accessible, fiber structures, resulting in high thermostability (Fu, Therkildsen, Aluko, & Lametsch, 2019; Gomez-Guillen et al., 2011; Lasekan, Abu Bakar, & Hashim, 2013; Shoulders & Raines, 2009). The calculated collagen content in percent of dry weight of all protein hydrolysates in the study are presented in Fig. 5. The results show that if the sediment is removed prior to inactivation, i.e., process strategy *ii*, it is possible to produce hydrolysates containing significantly reduced amounts of collagen fragments and peptides compared to process strategy *i*. This was observed using both proteases and raw materials. The results thus support the hypothesis that differences in solubility of collagen versus myofibrillar proteins during EPH processing can be exploited to produce protein hydrolysates with different composition and qualities using the same by-product material and protease. In addition, it is evident that a better understanding of how and when the collagen fraction of complex by-product materials is solubilized during EPH processes can have implications for the development of industrial processes for tailoring hydrolysates with specific composition and quality.

Specialized and tailored hydrolysates for specific applications represent an important step forward for the EPH industry as it allows for easy product differentiation. Hydrolysates containing collagen-derived protein fragments and peptides can exhibit very different biological and functional properties dependent, compared to hydrolysates rich in myofibrillar proteins, on factors such as molecular weight distribution, degree of hydrolysis and composition (Lindberg et al., 2021). The results of the current study show that solubilization of collagen can be controlled in EPH processes of complex by-products. This feature can be utilized to retrieve fractions enriched in myofibrillar and collagen peptides on one hand, and gelatin or even more hydrolyzed collagen fractions as a separate product on the other. This could potentially all be achieved in one process by including a size-separation step by e.g., ultrafiltration, in downstream processing when sediments are included during inactivation. Another possible solution to achieve this is to perform a decanter-based separation of sediment and hydrolysate before inactivation. After adding new water to the sediment fraction, heat inactivation of the hydrolysate and the sediment/hydrolysate fractions are performed separately to allow for the collagen proteins to be solubilized separately from EPH-derived myofibrillar and collagen peptides. Further possibilities in tailor-making hydrolysates applying this strategy include using other proteases, combinations of proteases and reaction conditions, e.g., pH, temperature and stirring.

4. Conclusion

In the current work, the aim was to study the role of the standard thermal inactivation step in collagen solubilization during EPH of poultry by-products. The results show that collagen fragments are solubilized at a slower pace compared to myofibrillar proteins resulting in a gradual shift in the composition of the water dissolved protein fragments and peptides. The results also clearly show that at the end of the enzymatic hydrolysis and prior to thermal inactivation, the reaction mixture constitutes of solubilized collagen-derived peptides and intact and partly hydrolyzed collagen not liberated into the water phase. The latter group was only solubilized when heat was added to the system, i.e., energy was added to interrupt the strong intramolecular interactions. The study demonstrated that in collagen-rich by-products, a 15 min heat treatment, conventionally used for inactivating the protease, is a vital unit operation in solubilizing collagen fragments and contributes to significantly increasing protein yield of the entire process. A large difference in molecular weight distribution profiles was observed when comparing stem Bromelain hydrolysate time series with Endocut-02. This shows that the two proteases have very different modes of action towards the main protein components in by-products under the conditions studied. Furthermore, the results support the hypothesis that separation of the sediment prior to thermal inactivation can be a useful strategy to produce protein hydrolysates with tailored composition and quality from poultry by-products.

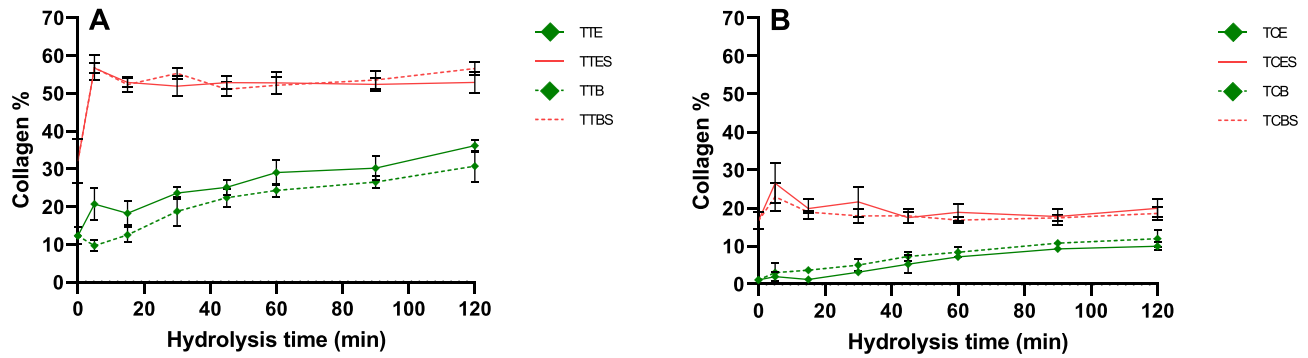

Fig. 5. Average collagen content of the triplicate hydrolysis series. A) relative collagen content in the TT samples, and B) relative collagen content in the TC samples.

CRediT authorship contribution statement

Kenneth Aase Kristoffersen: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Data curation, Visualization. **Nils Kristian Afseth:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Ulrike Böcker:** Writing – original draft, Writing – review & editing. **Katinka Riiser Dankel:** Investigation, Writing – original draft, Writing – review & editing. **Mats Aksnes Rønningen:** Investigation, Writing – original draft. **Andreas Lislelid:** Investigation, Writing – original draft. **Ragni Ofstad:** Writing – original draft, Writing – review & editing. **Diana Lindberg:** Conceptualization, Writing – original draft, Writing – review & editing. **Sileshi Gizachew Wubshet:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Data curation, Visualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.132201.

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