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Effects of poultry raw material variation and choice of protease on protein hydrolysate quality

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ABSTRACT

The aim of this study was to investigate the role of compositional variations in side-streams from the chicken and turkey industry on product characteristics after enzymatic protein hydrolysis (EPH). Variation was obtained using four raw materials, namely chicken and turkey carcasses, and mechanically deboned chicken and turkey residues. Alcalase 2.4 L, Corolase 2TS and Flavourzyme, three commercial proteases with different specificities, were used to investigate impact of protease choice on product characteristics. Besides protein yield, effects on product characteristics such as nutrient and amino acid composition, size distribution of peptides, degree of hydrolysis (DH%), rheological properties, and angiotensin-I-converting enzyme (ACE) inhibition, were investigated. Results show that choice of protease and industrially relevant variations in poultry raw material composition both have major effect on product composition and the other quality parameters studied. Protein yield, proximate composition, and bioactivity were more affected by protease than raw material variation. Raw material variation had a bigger impact on quality parameters such as peptide size distribution and amino acid composition, exemplified by the sum of essential amino acids. This means that raw material variation should be handled and accounted for to realize the true potential that exists in tailor-making EPH products with specified properties for targeted applications.

1. Introduction

Currently underutilized and often edible side-streams from the fish and meat industry, also known as by-products or co-streams, represent a viable source for nutrient recycling into food and feed ingredients [1,2]. In this context, EPH is a well-established method for valorization, resulting in protein and lipid ingredients for feed, pet food and human markets [2]. In EPH, protein-rich raw materials are solubilized and digested by proteases into peptides and single amino acids. The product quality of the final hydrolysate is influenced by several factors that can be grouped into process-specific, substrate-specific, and protease-specific factors [3].

The current EPH-based industry is mainly established for processing of raw materials and side-streams from the marine, aquaculture, or the dairy industries. However, side-streams from the meat industry are generally not utilized to the same extent. One reason for this might be the regulations restraining the use of edible and non-edible side-streams from animals by the European Union, e.g., the hygiene, the animal by-product, and the Transmissible Spongiform Encephalopathies (TSE) regulations [4–6]. In this context, poultry raw materials are of extra interest for the EPH industry as poultry are not susceptible to TSE diseases. Also, based on global meat production, poultry is one of the largest sources of edible side-streams, most of which can be used for human consumption. Poultry is interesting also from a sustainability context as it is suggested to be one of the most sustainable sources of meat protein on a CO_2-eq per kg protein basis [7].

Many poultry slaughterhouses facilitate processing of different types of poultry species at the same facility, hence generating side-streams from more than one species available for valorization by EPH processing. For EPH facilities, it would be of extra interest to be able to process raw materials from e.g., chicken and turkey without intermediate downtime in processing for shift between species. This would also allow for cost-cutting by a reduced need for cold-room storage of raw materials in between batches. However, studies show that the composition of side-

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streams from chicken and turkey differ [3,8]. Therefore, it might be hypothesized that when aiming towards markets with strict product specifications, mixing of raw materials from different species might result in products failing to meet defined product specifications. Similar studies on e.g. salmon show that differences in raw material compositions indeed affect end product qualities, and hence, their intended applications [9]. Furthermore, raw material quality and composition from a single species varies, being dependent on e.g. physiological factors, post-slaughter storage temperature and time of storage, as well as deboning settings [3,10]. To circumvent this substrate variation during EPH processing, Wubshet et al. studied the potential of using raw material characterisation and process settings to predict yield and product quality of protein hydrolysates from a range of poultry side-streams [11].

Many studies have been published on the use of EPH for utilisation of side-streams from either chicken or turkey, also addressing intended applications of the hydrolysates [12–18]. However, to the authors' knowledge, no studies have been targeted on understanding how industrially relevant raw material variations in, and between, poultry species affect end-product qualities in EPH processing. The aim of this study was thus to investigate the role of industrially relevant variability of chicken and turkey raw materials on product characteristics after EPH. Four different raw materials, namely, chicken carcass (CC), mechanical deboned chicken residue (MDCR), turkey carcass (TC) and mechanical deboned turkey residue (MDTR), were subjected to EPH. To compare the possible raw material effect with that of protease choice on product characteristics, three different commercial proteases with different specificities were selected. Two of the selected commercial proteases displays mainly endo-peptidase activity (Alcalase 2.4 L and Corolase 2TS), and one exhibits mainly exo-peptidase activity (Flavourzyme). The effects of changes in raw materials and proteases were characterised by means of general properties, i.e., protein yield, general nutrient composition, DH%, rheological properties, and molecular weight distribution of peptides, as well as more market-specific EPH properties. The latter was examined using amino acid composition to calculate nutritional quality parameters and by studying the ability of each hydrolysate to inhibit the blood-pressure regulating enzyme ACE.

2. Materials and methods

2.1. Chemicals and hydrolysis materials

The poultry raw materials CC, MDCR, TC, and MDTR were supplied from a Norwegian slaughterhouse (Nortura, Hærland, Norway). "Carcass" was in this study defined as raw material after removal of head, feathers, neck, intestinal content, breast filet, legs (both thigh and drumstick) and tail. The other two raw materials (MDCR, MDTR) were residues after mechanical deboning at the day of collection at the industrial plant. At the day of collection, raw materials were ground using a Seydelmann SE130 meat grinder (Seydelmann, Stuttgart, Germany) and a mesh size of 1 cm. The material was vacuum packed in individual packages and stored frozen at -20 °C until the day of hydrolysis. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except Hip-His-Leu (Bachem, Bubendorf, Switzerland) and TNBSA (Thermo Scientific, Waltham, MA, USA). Water used for HPLC analyses was purified by a deionization and 0.22 μm membrane filtration system (MilliporeSigma, Burlington, MA, USA).

2.2. Enzymatic protein hydrolysis and sampling

All raw materials were hydrolysed by commercially available food-grade enzyme preparations: Flavourzyme and Alcalase 2.4 L (Alcalase) (both from Novozymes A/S, Denmark), and Corolase 2TS (Corolase) (AB enzymes, Darmstadt, Germany). Controls for the four raw materials were prepared by running the EPH process without adding proteases. All reactions were performed in duplicates. The hydrolysis reactions were run at 50 °C in a Reactor-Ready™ jacketed reaction vessel (Radleys, Saffron Walden, United Kingdom), using circulating water for heating (Julabo GmbH, Seelbach, Germany). Before hydrolysis, 500 g of the raw materials were thawed at room temperature before mixing with 1 L of water (22 ± 1 °C). The reaction mixtures were heated under stirring for approximately 40 min until reaching 50 ± 1 °C. At t = 0, the commercial protease (1 % w:w protease: raw material) was added. The reaction mixtures were sampled at set timepoints following the description by Wubshet et al. [19]. After a reaction time of 80 min, the reaction mixtures were thermally inactivated in two steps: 1) initial heating using a Menumaster commercial microwave oven (ACP, IA, USA); and 2) continued heating in a water bath where samples were kept at a minimum of 90 °C for 15 min. The samples were centrifuged using a Multifuge 4 KR (Thermo Scientific) for 15 min. (5200 *g*, 25 °C) to separate the sediment from the liquid phases. Subsequent fat and water phase separation was performed using a separator funnel. The weight of all three phases was recorded, the water phase was aliquoted in 250 mL plastic containers and stored frozen at −40 °C until lyophilized using a Gamma 1–16 LSC plus freeze dryer (Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany).

2.3. Nutrient composition and amino acid analysis

Protein, fat, and ash content as well as amino acid composition of all raw materials and hydrolysates were determined. Total nitrogen of each sample was measured according to the Nordic Committee on Food Analysis method NMKL 6 [20], based on the Kjeldahl method. The values were converted to percentage protein using the standard protein conversion factor 6.25. These results from raw material and hydrolysates were used to calculate protein recovery. Fat content was determined according to NMKL 131 [21], and ash measurements were performed according to NMKL 173 [22]. All raw materials and hydrolysates were analysed for amino acid composition based on the method ISO 13903:2005 as stated by Commission Regulation EC 152/2009. The essential amino acid index (EAAI) was calculated according to Eq. (1),

$$EAAI = \sqrt[n]{\frac{Lys_p}{Lys_s} \, x \, \frac{His_p}{His_s} \, x \, \frac{Trp_p}{Trp_s} \, x \ldots x \, \frac{Ile_p}{Ile_s}} \quad (1)$$

with the subscript *p* referring to the hydrolysate protein, and *s* referring to values from the standard whole egg protein [23,24].

2.4. Size exclusion chromatography

The SEC analysis was carried out as described by Wubshet et al. [19]. Lyophilized hydrolysate was dissolved in water to a final concentration of 25 mg/mL. After filtration through a Millex-HV PVDF 0.45 μm 33 mm filter (MilliporeSigma), samples were used as injection solutions. Chromatographic separation was performed using an Agilent 1200 series instrument (Agilent Technologies, Santa Clara, CA, USA) consisting of a quaternary pump, a degasser, a thermostatic column compartment, a photodiode-array detector, and an auto sampler. Using an injection volume of 10 μL, separation was performed at 25 °C using BioSep-SEC-s2000 column (Phenomenex, 300 × 7.8 mm). The mobile phase consisted of a mixture of acetonitrile and ultrapure water in a proportion of 30:70 (v:v), containing 0.05 % trifluoroacetic acid. Chromatographic runs were controlled from OpenLAB CDS Rev. C. 01.07 (Agilent Technologies). From chromatographic runs of both standards and hydrolysates, a UV trace of 214 nm was monitored. The molecular weight, average retention time, and standard deviation (SD) of the analytical standards are found in Table S-2. Weight average molecular weights from the time series samples were calculated using molecular weight calibration standards according to a previously developed method (see SI, Tables S-3 and S-4) [19]. The area under the curve of each chromatogram was normalized and divided into four sections based on estimated peptide molecular weight ranges (see SI,

Table S-5).

2.5. Degree of hydrolysis measurements

The DH% was measured using a modified version of the TNBS method [25,26]. The measurements were performed in triplicates as described in Kristoffersen et al. [27].

2.6. Bioactivity measurements

The ACE inhibitory activity assay used was based on the liberation of hippuric acid from Hip-His-Leu substrate catalysed by ACE. The samples were prepared by dissolving the dried hydrolysates to a concentration of 1.0 mg/mL in Milli-Q, followed by a serial dilution to obtain a range of sample concentrations directly in 96 well plates. After the on-plate dilution, each well contained 25 μL sample solution with a concentration range from 1.0 down to 0.03 mg/mL. This serial dilution was performed in duplicate, and for the two serial dilutions, the subsequent treatment was different: 1) For the first series, 25 μL Milli-Q was added. 2) For the second series, 25 μL 5 mM Na_2EDTA was added to inhibit the ACE enzyme immediately when added to the mixture. Furthermore, all samples in both serial dilutions were mixed with 25 μL of 5 mM Hip-His-Leu (Bachem, Bubendorf, Switzerland) and 25 μL of 20 mU/mL rabbit lung ACE in 0.1 M borate buffer (pH 8.3) containing 300 mM NaCl. After 2 h incubation at 37 °C, the reaction was stopped by adding 25 μL of 5 mM Na_2EDTA solution to the first dilution series and 25 μL Milli-Q to the second to compensate for the volume. After stopping the reaction, 15 μL of 0.25 M NaOH, 35 μL of 0.5 M Bicine (pH 9.1) containing 1.00 M NaCl and 25 μL of 1.5 % TNBSA in 31 mM Na_2HPO_4 was added. After 10 min incubation at 37 °C, the yellow colour developed was measured at 405 nm in a spectrophotometer, and IC_{50} (the concentration of inhibitor that inhibits 50 % of the activity) was calculated. All measurements were performed in triplicates.

2.7. Rheology measurements

Freeze dried hydrolysate samples were dissolved in phosphate buffered saline (PBS) at a protein concentration of 50 mg/mL, approximately equivalent to final protein concentration in samples during hydrolysis, by stirring with a magnetic stirring bar in 50 mL glass bottles with lid. The bottles were placed in a water bath at 80 °C for 30 min. Samples were cooled to 20 °C for 30 min and filtered through 0.8 μm syringe filters. Viscosity of the reconstituted hydrolysates was measured using a Physica MCR 301 rheometer (Anton Paar, Stuttgart, Germany) fitted with a double gap geometry (DG26.7). The DG geometry was used with overfill (approximately 10 mL) to avoid undesirable surface tension and capillary effects. After a temperature equilibration of 60 s, apparent viscosity was measured at 22 °C at constant shear rate of 10 s^{-1}. All measurements were performed in duplicate with a time difference of one year between the replications.

3. Results and discussion

The raw material variation in the present study was obtained by use of four raw materials: two based on carcasses before deboning (CC, TC), and two constituted residuals after mechanical deboning (MDCR, MDTR). Raw materials before and after mechanical deboning can thus be regarded as representing the extremes in side-stream raw material variation during industrial EPH processing in respect to for example meat and collagen content.

3.1. Raw material composition

The gross composition and the amino acid composition of the raw materials are presented in Table 1. The studied raw materials varied in both gross chemical and amino acid composition. The protein content, as estimated by the Kjeldahl method using a protein conversion factor of 6.25, indicates approximately the same protein content. However, there was an expected higher amount of bone and collagen-rich connective tissue in the mechanically deboned residues, evident by higher ash and hydroxyproline (Hyp) contents, respectively (Table 1). The protein content of MDCR and MDTR were on par with earlier reports, although the present raw materials showed a higher moisture and lipid content, and less ash [28,29]. The amount of collagen in the different raw materials can be estimated from the content of hydroxyproline (Table 1). Based on the assumption that collagen consists of 13.5 % Hyp, the collagen contents in CC, MCDR, TC and MDTR were 12, 28, 27 and 53 % of the total protein content, respectively [30,31]. As seen in Table 1, the sum of essential amino acids (EAA), for the chicken raw materials were on par with the reference value set by FAO for whole egg proteins, while for the turkey raw materials, the values were close, or slightly lower [24,32]. The higher fat contents observed in Table 1 of turkey raw materials were in accordance with data from a previous study on Norwegian poultry raw materials [11].

Table 1
Raw material, amino acid and proximate analysis data (g/100 g raw material). The table also includes calculations of the amount of collagen in the raw materials and sum of EAA of raw materials in comparison with whole egg protein using FAO/WHO values [24,32].

Amino acids (g/100 g)	Egg	CC	MDCR	TC	MDTR
Arg	6.2	6.5	6.8	7.0	7.1
His	2.4	2.8	2.4	2.2	2.0
Lys	5.3	8.8	7.4	6.9	5.8
Asp		9.4	8.6	8.4	7.1
Glu		15	14	13	12
Ser		4.1	3.9	3.7	3.6
Thr	4.0	4.5	4.1	3.7	3.3
Cys + Cys ox		1.0	0.74	0.98	0.79
Gly		7.0	10	11	14
Pro		4.8	6.8	7.0	8.4
Hyp		1.6	3.7	4.4	7.1
Ala		6.5	6.8	7.0	7.8
Val	7.2	4.9	4.5	4.1	3.6
Ile	6.6	4.5	3.8	3.5	2.9
Leu	8.8	7.6	6.8	6.4	5.5
Met	3.2	2.3	1.7	2.4	1.9
Phe	5.8	4.6	4.2	4.1	3.4
Tyr		3.3	2.7	2.7	2.3
Trp	1.7	1.2	0.97	0.98	0.67
Collagen*(g)		12	28	33	53
EAAs† (g)	28	32	28	27	24
Protein		19	19	17	18
Fat		9.9	8.4	19	15
Ash		3.8	6.8	6.4	8.8

* Assuming a 13.5 % Hyp content of total amino acids in poultry collagen.
† EAAs: essential amino acids.

3.2. Product composition

Corolase, Alcalase, and Flavourzyme were used for EPH of the four different raw materials in the study. Although these proteases have different pH and temperature optima, the hydrolysis reactions were run at ambient pH (6.2 ± 0.2 for all raw materials) using the same temperature, irrespective of protease and raw material. These settings were selected to mimic those typically used by the hydrolysis industry in Norway.

3.2.1. Chemical composition of hydrolysates

The protein, ash, and fat composition of the hydrolysates is provided in SI, Table S-1. For all raw materials, hydrolysis with Alcalase or Corolase resulted in hydrolysates with slightly higher protein content (84–88 g/100 g) than hydrolysates generated by Flavourzyme (78–82

g/100 g). Flavourzyme hydrolysis resulted in hydrolysates with relatively higher ash contents, regardless of raw material. Hydrolysis of MDTR resulted in the highest fat content in hydrolysates (0.76–1.4 g/100 g), regardless of protease, reflecting the higher fat content in the corresponding raw material.

3.2.2. Nitrogen yield and protein content

Nitrogen yield and protein content of all hydrolysates are presented in Fig. 1, being compared to their respective product controls (i.e., raw materials with no proteases). Although nitrogen is present in many different molecules within living organisms, the nitrogen yield indicates the amount of protein recovered from the raw materials. The product protein content, on the other hand, indicates the relative protein purity of the dried protein hydrolysates.

Compared to studies where e.g. viscera and intestines are included, a low autolytic activity can be expected for the current raw materials since these major protease-containing organs are excluded [12]. The results from the control products in Fig. 1 verified this, evident by the low total nitrogen yield in product controls (7.9–13 %). Comparing the nitrogen yield resulting from hydrolysis with the three proteases, products from Alcalase and Corolase reactions generally result in high nitrogen yields (72–91 %) and high product protein contents (87−90 g/100 g), with Alcalase being the most efficient protease. Contrary to this, Flavourzyme hydrolysis results in substantially lower nitrogen yields (32–50 %) while retaining high relative protein contents in the protein hydrolysate (77−83 g/100 g). Comparing raw materials, hydrolysis of turkey raw materials seemingly resulted in higher nitrogen yields than the chicken raw materials. Considering the relatively higher amounts of fat, ash, and non-soluble collagen in the turkey materials, this was a bit surprising. It might potentially be explained by problems associated to Kjeldahl protein measurements on materials with high fat contents, such as that of the current turkey raw material. It is known that Kjeldahl protein measurements on materials with high amounts of fat can lead to an underestimation of the protein content due to incomplete digestion of the start sample, leading to a slight overestimation of the final nitrogen yield in the turkey hydrolysates [33]. A hydrolysate containing 78 % protein and a 46 % protein recovery starting from turkey deboned residue was seen resulting from a 2 h hydrolysis at 60 °C using 0.4 % Papain (w:w) [28]. That is a significantly lower yield than seen here, with 80 % and 84 % nitrogen yields resulting from Corolase and Alcalase hydrolysis, respectively. As discussed in the Fonkwe publication, the relatively low yield might be a result of that raw materials containing high amounts of bones, which are harder to hydrolyse than meat and tendons. Nevertheless, this difference in yield seen resulting from hydrolysis of two similar raw materials, albeit at different conditions, indicates the importance of raw material composition on resulting yield. To the authors' knowledge, apart from these two studies, there is a surprising lack of published work on use of only EPH as a method to valorise MDTR raw materials. In that respect, both studies indicate that from a yield perspective, much can still be achieved using EPH to extract proteins from MDTR.

A protein recovery of 91 % was reported by Kurozawa et al. to be the maximum achievable using Alcalase on chicken breast meat at optimum conditions: 52.5 °C, 4.2 % (w/w) enzyme preparation to substrate ratio, and a pH value of 8.00 [34]. The relatively high yield seen from poultry side-streams has also been verified in a study by Rossi et al. After a 2 h EPH of heat-denaturated mechanically deboned poultry residue (MDPR), an 89 % nitrogen recovery was seen after use of 2.5 % Alcalase concentration at 50 °C, pH 7.5. Also, in the Rossi study, use of 3.5 % Flavourzyme during a 120 min hydrolysis performed at 50 °C, pH 6.0, resulted in a nitrogen recovery of 67 %. The higher yields in the Rossi study were probably an effect of higher protease concentrations and a longer hydrolysis time. Also, Alcalase hydrolysis was performed at a pH closer to the stated optimal pH of the protease. In the current study, lower Alcalase concentrations were used while still achieving a respectable 80 % nitrogen yield without use of pH adjustments and a shorter time than used in the Rossi study. This means that there is a potential for cost-reduction by use of less pH-adjusting chemicals, in turn resulting in a less elaborate downstream processing to reduce salt concentration, and by use of a lower amount of protease. The optimal compromise between these factors will be dependent on existing infrastructure and production investments (CAPEX) and operational costs (OPEX) of each EPH processing plant [35].

Although Flavourzyme results in relatively lower yields than the endoproteases in this study, for specific applications, Flavourzyme could still be a preferred protease. Wang & Shahidi reported on Flavourzyme resulting in higher yields of antioxidant peptides than Alcalase, Neutrase, trypsin and pepsin in hydrolysis of turkey meat. Using 3 % Flavourzyme concentration, the optimal conditions for maximising production of antioxidant peptides from a defatted turkey meat powder were 50.09 °C, pH 5.42, and a hydrolysis time of 1.08 h [36].

3.2.3. Degree of hydrolysis

DH%-values indicate the number of broken peptide bonds per total peptide bonds resulting from hydrolysis reactions. The results are provided in Fig. 2, and show that type of raw material seems to have only a minor influence on resulting DH% values, as compared to effect of protease type. There was less than 8 % difference in the Corolase to

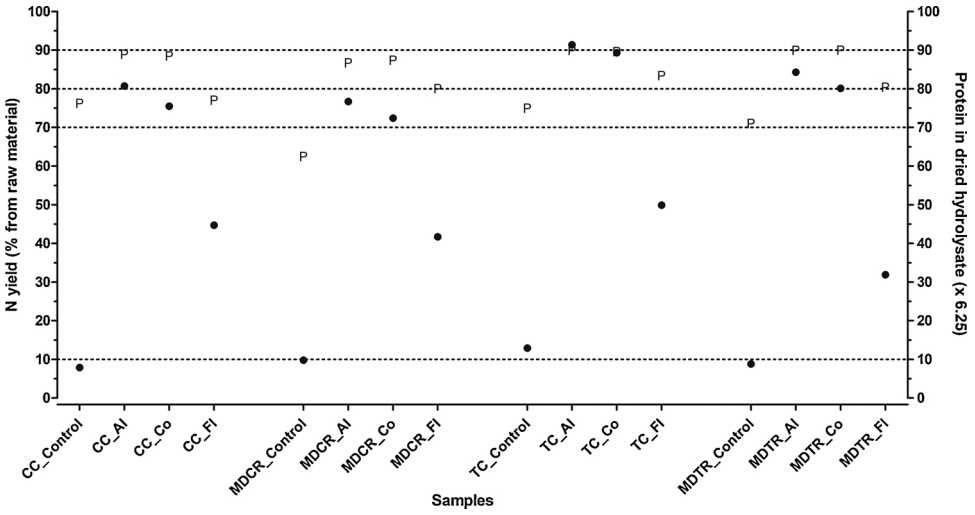

Fig. 1. Comparison of hydrolysate nitrogen yield from raw materials (•) (% nitrogen extracted from raw material) and protein content (P) (Kjeldahl protein, g/100 g dry weight) in hydrolysates, in control (without proteases) and after enzymatic protein hydrolysis of all raw materials (measurement uncertainties 10 %).

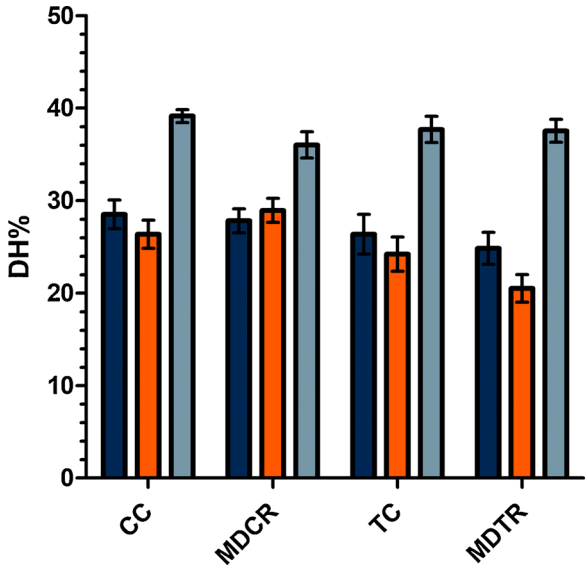

Fig. 2. The DH% values of the resulting hydrolysates. The values are based on triplicate measurements using the TNBS method (error bars: ±SD). The Alcalase hydrolysates are visualized in dark blue, Corolase hydrolysates in orange, Flavourzyme hydrolysates in light blue.

Alcalase DH% values for all raw materials, except for MDTR where an approximately 20 % difference was observed. The differences in DH% values from use of exopeptidases as compared to endopeptidases, i.e., Flavourzyme to Alcalase, were higher. For CC and TC, the Flavourzyme DH% values were ca. 40 % higher than the Alcalase values, while for MDCR and MDTR, they were ca. 30 % and 50 % higher, respectively. This also holds for a comparison in Corolase vs. Flavourzyme values. The relatively higher DH% of Flavourzyme compared to an endopeptidase such as Alcalase has also been reported in other cases, for example in hydrolysis of salmon raw materials using pH-stat for DH% measurements [37]. Although choice of protease had more significant effect on resulting DH% values, hydrolysis of turkey raw materials generally resulted in slightly fewer cleaved peptide bonds than hydrolysis of chicken raw materials.

The relative differences in bond-cleaving between the protease preparations corresponds well with results reported by Merz and co-workers [38]. In the study, Corolase, Alcalase and Flavourzyme were used in hydrolysis of lupin proteins resulting in low, medium, and high DH%, respectively, albeit with generally lower resulting DH% values than those seen in the present study. The DH%-values resulting from any hydrolysis are dependent on both process settings, exemplified by enzyme concentration and time, as well as the amount of easily accessible proteins within the material [39]. Hence, the DH%-values reported by Merz and co-workers cannot be directly compared with the DH %-values obtained in the present study. It is still interesting to note that the observed trend in DH% between proteases from the Merz study was seen also in the present study for all materials, except for MDCR.

3.2.4. *Peptide size characterization*

To gain further insight into the inherent properties of the hydrolysate peptides, all hydrolysates were subjected to SEC analysis. The notable variations in molecular weight distribution profiles shown in Fig. 3 results from both raw material and protease preparation differences. The F1 region was the region with the largest signal differences seen in peak profiles between protease preparations and raw materials. For all protease preparations, the high molecular weight F1 fraction resulting from turkey hydrolysates was higher than F1 of chicken hydrolysates. High peaks in the early eluting F1 region are potentially a result from thermal extraction of non- or partly digested collagen molecules from the raw materials in the final heat-inactivation step of the process. It is well-

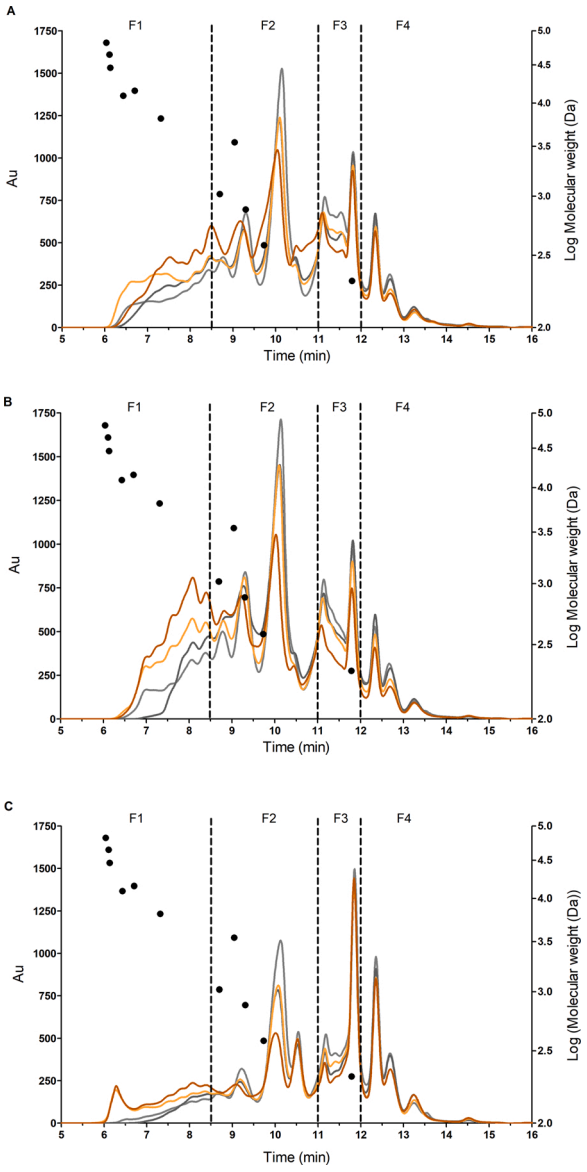

Fig. 3. The resulting SEC chromatograms for all hydrolysates measured at 214 nm. From top to bottom, A) Alcalase, B) Corolase, C) Flavourzyme hydrolysates with CC in light and MDCR in dark grey colour, TC in light orange, and MDTR in brown. The chromatograms have been divided into four fractions, F1-F4. The $\log_{10}$ values (right y-axis) of the Mw standards used for calibration (SI, Table S-2) have been included as black dots in the chromatograms.

known that heat, often in combination with other treatments such as enzymatic hydrolysis, dissolves collagenous material into solution [40]. Interestingly, the order of peak area in the F1 fraction, from highest to lowest peaks, i.e. MDTR > TC > MDCR > CC, corresponds to the order of collagen content in the raw materials as presented in Section 2.1. It is also worth noticing that CC, the raw material displaying the lowest signal in the F1 fraction, displayed the highest peaks in the F2 and F3 fractions, and vice versa. This shows that the relative distribution of peptide sizes changes with raw materials. This is also reflected in the weight average molecular weights of the hydrolysates after 80 min hydrolysis time (SI, Table S-3). Comparing the result of using different proteases on individual materials, the SEC profiles in Fig. 3 show that the Alcalase and Corolase hydrolysates (both with mainly endo-protease activity), are more alike than Flavourzyme hydrolysates (mainly exopeptidase activity). The large peak at 10 min was highest for hydrolysates from CC raw material for all protease preparations.

3.2.5. Rheology

To investigate if there were raw material- or protease-related effects on viscosity, all hydrolysates were subjected to rheology measurements. Most hydrolysates had a very low viscosity in the range of 1–3 mPas when re-dissolved at 50 mg/mL protein content in PBS (Fig. 4). This is close to the viscosity of water at 20 °C which is 1.002 mPas. Hydrolysates prepared with Flavourzyme showed generally higher viscosities than hydrolysates prepared with Alcalase or Corolase. Especially the collagen-rich turkey raw materials resulted in hydrolysates with a higher viscosity in combination with Flavourzyme. This was most pronounced for MDTR, which showed an average viscosity of approximately 10 mPas with Flavourzyme and was thus the only hydrolysate to reach viscosities in the semi-dilute solution regime. While this may have an impact in thin liquid food applications, it is unlikely to contribute to viscosity of thick liquid or solid foods. However, when MDTR was dissolved at higher protein concentrations (100 and 200 mg/mL), the liquid was gelled during cooling to room temperature (results not shown). This indicates that MDTR, and to a lesser extent TC-hydrolysates, prepared with Flavourzyme contain large enough collagen peptides to potentially form a three-dimensional gel-network like it is found in gelatine gels. If so, it might be possible to capture these high molecular weight compounds using e.g. ultrafiltration under optimized conditions [41]. The potential to form gelatine was also reflected in the higher content of collagen amino acids (Gly, Pro, Hyp) in the turkey hydrolysates (Table 2). The hydrolysates prepared with Flavourzyme had lower contents of collagen amino acids than those prepared with Alcalase or Corolase (Table 2). The SEC chromatograms of the different hydrolysates did in general not indicate a higher relative proportion of large peptides (F1 and F2) in the MDTR and TC samples hydrolysed with Flavourzyme compared to the other samples. However, there was a small peak at 6 min, only seen in these two samples, which might consist of partly digested or undegraded dissolved collagen molecules.

The viscosity values in Fig. 4 are average values of two separately dissolved sample duplicates. The values obtained for TC and MDTR hydrolysates prepared with Flavourzyme were significantly lower at the second measurement after one year storage of the freeze dried hydrolysate powders at −20 °C. Frozen storage has been reported to decrease the solubility of macromolecules such as beta-glucans, presumably due to the formation of inter-molecular aggregates [42,43]. The lower viscosity values after storage may therefore be the result of a reduced protein/peptide solubility.

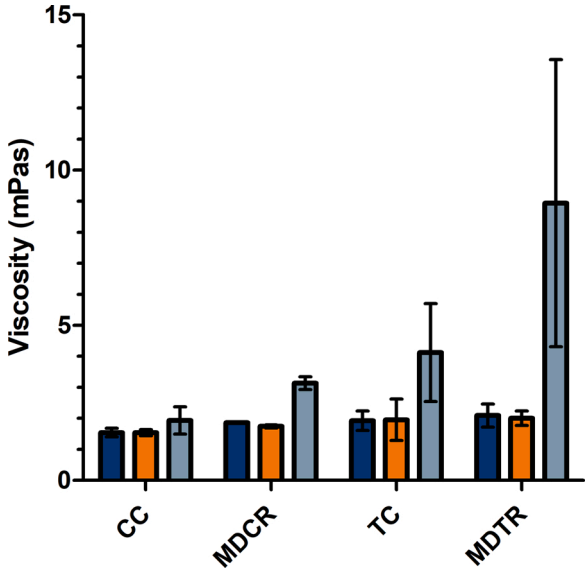

Fig. 4. The resulting viscosity divided by raw material. A total of 5 % of the hydrolysates were mixed in PBS buffer to a final protein concentration of 50 mg/mL. Each hydrolysate was mixed at 80 °C for 30 min, followed by cooling for 30 min at 20 °C. The measurements were performed in duplicates (error bars: ±SD). The Alcalase hydrolysates are visualized in dark blue, Corolase hydrolysates in orange, and Flavourzyme hydrolysates in light blue.

3.2.6. Amino acid composition

To investigate the nutritional values of the hydrolysates, the amino acid composition of the hydrolysates was investigated and shown to vary first of all depending on raw materials, but also to a certain degree on protease used (Table 2). The largest differences in amino acid composition, based on raw material differences using the same protease, was seen in collagen-associated amino acids, i.e., Gly, Pro, Hyp. In more detail, for Hyp which showed the largest difference, there is a 3-fold difference between the hydrolysates with the highest Hyp content (MDTR) and the lowest (CC), independent of protease used. Trp also showed interesting concentration differences between materials, especially resulting from Alcalase and Corolase hydrolysis. The material yielding overall highest Trp amounts were CC and the lowest MDTR. This is in accordance with Trp being essentially non-existent in collagen and MDTR being the most collagen-rich of the studied raw materials [44]. The percentwise difference in amount of Trp after hydrolysis for both Alcalase and Corolase (highest/lowest value) are approximately 1.5 times.

The hydrolysate nutritional properties and hence, properties related to certain industrial applications can be linked to different sums of amino acids. The bottom rows in Table 2 present three different sums of amino acids, whereof “collagen” reports on the percent of collagen in the respective hydrolysates, based on Hyp content. As indicated by the differences in Hyp presented above, use of the two endoproteases in hydrolysis of collagen-rich MDTR raw material resulted in hydrolysates with high final collagen content. Although Flavourzyme has been reported to contain three endoproteases [45,46], the low collagen yield can probably be attributed to the Flavourzyme exopeptidases being less effective in hydrolysing the more inaccessible collagenous materials.

Knowledge in the sum of EAAs in protein sources is another important element for evaluations of the protein hydrolysate contribution to achieving nitrogen balance [32]. As seen in Table 2, the highest overall EAA values were observed after hydrolysis of the CC raw material, especially using Flavourzyme. This was dependent on higher relative amount of Lys, but also Thr, Ile, Leu, and Phe. Many of mentioned amino acids are good substrates for the LAPA, LAP2, DPP4, and DPP5 exopeptidases in Flavourzyme [45–47]. Hence, although Flavourzyme hydrolysates resulted in lower total protein yield, addition of Flavourzyme together with endopeptidases during hydrolysis might help to achieve better overall EAA values. From a human nutritional standpoint, it can also be interesting to compare the resulting amounts of EAAs to recommended human adult requirements. Recommended EAA values are incorporated in Table 2 in the column named “FAO” and are given as a requirement pattern, calculated as the individual amino acid requirements divided by the total protein requirements (mean protein requirements 0.66 g protein/kg per day) [32]. Although Cys and Tyr are not EAAs, these amino acids were included due to the closely linked metabolism of these amino acids to Met and Phe, respectively [32]. For the studied poultry hydrolysates, the individual EAA values in almost all cases met the adult requirements according to FAO. The nutritional quality of most foods is to a large degree dependent on the amounts of Lys and Trp. From that aspect, the high values of Lys, coupled to high values of Trp in the chicken and TC hydrolysates were of extra interest. This indicates that use of poultry raw materials with higher relative amounts of meat (CC, TC) is important for production of nutritionally relevant hydrolysates. Another chemical method to evaluate the nutritional quality is the EAAI, highly correlated to the biological value of proteins [23]. Oser described the EAAI as the geometric mean of “the egg ratios”, i.e. the ratio of each EAA compared with their amount in whole egg protein. The studied hydrolysates differ markedly in their respective EAAI value, ranging from a value of 0.87 in CC hydrolysates down to the lowest values of around 0.7 in the collagen-rich MDTR

Table 2
Amino acid composition of the resulting hydrolysates in g/100 g protein. The table also includes the calculated amount of collagen in the hydrolysates, and relevant nutritional parameters given as EAA, and EAAI in comparison with whole egg protein and FAO/WHO values [24,32].

			Alcalase				Corolase				Flavourzyme			
Amino acids (g/100 g protein)*	FAO	Egg	CC	MD CR	TC	MD TR	CC	MD CR	TC	MD TR	CC	MD CR	TC	MD TR
Arg		6.2	6.6	6.8	6.6	7.0	6.7	6.8	6.9	7.1	6.5	6.4	6.4	6.5
His	1.5	2.4	2.8	2.3	2.3	1.9	2.8	2.4	2.3	2.0	3.0	2.5	2.6	2.3
Lys	4.5	5.3	8.6	7.1	7.3	6.0	8.6	7.4	7.4	6.2	9.5	8.2	8.2	7.2
Asp			9.4	8.6	8.5	7.9	9.4	8.7	8.7	7.9	9.5	9.0	8.9	8.5
Glu			15	14	14	13	15	14	15	13	17	16	16	15
Ser			4.0	3.8	3.8	3.6	4.0	3.8	4.0	3.7	4.2	4.0	4.1	3.9
Thr	2.3	4.0	4.5	4.0	3.8	3.4	4.5	4.1	4.0	3.5	4.5	4.3	4.1	3.9
Met + Cys	2.2		3.8	3.6	4.2	3.2	3.5	3.7	3.8	3.0	3.8	3.9	3.7	3.5
Cys + Cys ox	0.6		1.1	1.2	1.3	1.1	1.1	1.1	1.2	1.1	1.3	1.4	1.2	1.3
Met	1.6	3.2	2.7	2.5	2.9	2.1	2.4	2.5	2.6	1.9	2.5	2.5	2.5	2.2
Gly			7.3	11	10	14	7.6	10	9.8	14	6.0	8.3	8.3	10
Pro			4.9	6.7	6.5	8.1	5.3	6.3	6.4	8.1	4.3	5.3	5.8	6.5
Hyp			1.9	4.0	3.9	6.0	2.0	3.6	3.5	5.9	1.0	2.4	2.7	3.4
Ala			6.8	7.3	7.0	7.7	6.8	7.2	7.0	7.6	6.7	7.1	7.0	7.3
Val	3.9	7.2	4.9	4.4	4.3	4.0	4.8	4.5	4.3	3.9	4.9	4.7	4.5	4.4
Ile	3.0	6.6	4.3	3.7	3.8	3.2	4.2	3.8	3.8	3.2	4.5	4.2	4.1	3.9
Leu	5.9	8.8	7.5	6.7	6.6	5.8	7.4	6.8	6.7	5.9	7.9	7.2	7.2	6.8
Phe + Tyr	3.8		7.4	6.6	6.4	5.6	7.1	6.6	6.6	5.7	7.2	6.8	6.5	6.4
Phe		5.8	4.4	4.0	3.8	3.5	4.3	4.0	3.9	3.5	4.5	4.3	4.0	3.9
Tyr			3.0	2.5	2.6	2.1	2.8	2.6	2.7	2.2	2.7	2.5	2.6	2.5
Trp	0.6	1.7	0.75	0.66	0.65	0.52	0.77	0.66	0.65	0.50	0.62	0.58	0.56	0.50
Collagen**(g)			14	30	29	44	15	26	26	44	7.7	18	20	25
FAO sum	28		44	39	39	33	43	39	39	33	45	42	41	39
EAA†		51	38	33	33	29	37	34	33	29	39	36	35	33
EAAI†		1.0	0.87	0.77	0.77	0.67	0.85	0.79	0.78	0.67	0.87	0.81	0.79	0.74

* Measure uncertainty between 6–20 %, Eurofins.
** Value calculated from assumption of 13.5 % Hyp content of total amino acids in poultry collagen.
† EAAs: essential amino acids. EAAI: essential amino acid index.

hydrolysates. Hence, raw material variability has the largest effect on the resulting EAAI, although results indicate that Flavourzyme resulted in slightly higher overall EAAI values than the other proteases. Results indicate that processing of very collagen-rich material, such as MDTR, in separate batches from CC and TC would result either in collagen-enriched products relevant for collagen applications, or products enriched in EAAs for nutritional applications, respectively. One can also argue for the importance of mixing meat- with collagen-rich materials from a nutritional standpoint, as mixing will increase the EAAs values to acceptable levels in hydrolysates containing the otherwise EAA-deficient collagen-rich mechanically deboned residues.

3.2.7. *Bioactivity*

The markets with the highest potential for hydrolysate peptides are functional foods or pharmaceuticals [48,49]. One of the most frequently reported bioactivity assays used for screening of bioactive peptides is the ACE assay, based on inhibition of the angiotensin-I-converting enzyme [50]. An earlier study showed that hydrolysis of chicken legs using Alcalase resulted in peptide mixtures with ACE inhibition activity [51,52]. The hydrolysates that produced a measurable inhibition activity, $IC_{50,}$ below 1.0 mg/mL in the current study are presented in Fig. 5. Alcalase hydrolysis of CC and MDTR resulted in peptides mixtures with moderate ACE inhibition. The best ACE inhibition in this study was seen with hydrolysates originating from Corolase proteolysis, where all hydrolysates resulted in IC_{50} values below 1.0 mg/mL. The IC_{50} values originating from hydrolysis using either Flavourzyme or control samples (without enzymes) were all above 1.0 mg/mL, suggesting that these samples have low to no ACE inhibition potential. Taken together, these results imply that when aiming at producing ACE inhibiting hydrolysates, both choice of protease preparation and raw material are important parameters.

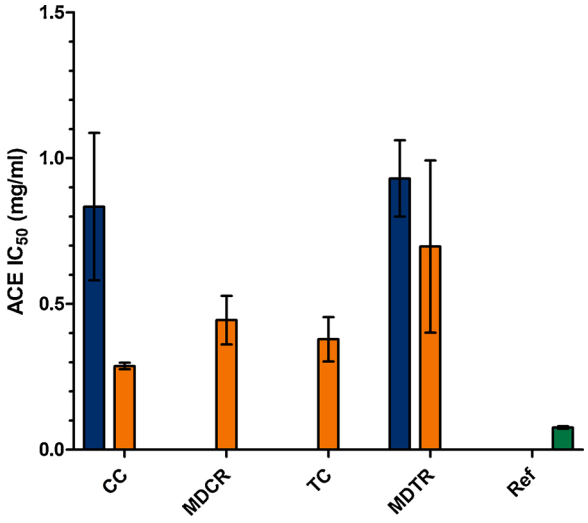

Fig. 5. The inhibitory concentration needed to achieve a 50 % reduction of rabbit lung ACE activity, restricted to hydrolysates performing a mean IC_{50} value resulting from triplicate measurements within the assay measurement limit, 1.0 mg/mL (error bars: ±SD). Alcalase values in dark blue, Corolase values in orange. Also included to the right in green, are the IC_{50} value for a reference hydrolysate obtained from digestion of egg white lysozyme with Alcalase [52].

3.3. *Industrial implications of the results*

The present study evaluated four poultry raw materials representing "extreme" samples, thus spanning industrially relevant chemical variations in poultry side-streams. The study shows that both raw material and protease type affect the end properties of the hydrolysates. For the

EPH industry, this means that care should be taken to investigate which of these factors that has the biggest impact on the final application. However, the complexity also arises already in industrial processing of poultry, where, to a varying degree, these raw materials will be mixed dependent on what kind of poultry are slaughtered at any given moment. Thus, the properties of the hydrolysates produced could vary significantly from day to day, and even from hour to hour. As shown in this study, the need to adjust process settings related to raw material variation will depend on the targeted application for the hydrolysates. The question is then how to account for the raw material variability in EPH processes based on complex raw materials. One strategy to handle this would involve better "sorting" of the raw materials, for instance not allowing raw materials of different species to be mixed. This could involve practical challenges related to having enough storage capacity, alongside possible microbial and oxidative degradation. Also, one should remember that e.g., feed regimen used in breeding and the age at slaughter also induce intra-species raw material variations. Wubshet et al. provided another solution for handling raw material variations. By applying vibrational spectroscopy measurements of raw materials, they were able to predict and optimize product properties of the hydrolysates [11]. Still, the question remains if the analytical tools studied have the chemical specificity needed to address the key quality parameters of the raw materials influencing relevant product properties. The present study presents a range of complimentary analytical techniques giving a good overview of the physical and chemical properties of the hydrolysates and the results could give important indications on which relevant parameters should be analysed in an industrial setting. Detailed analytical tools like NMR and LCMS would be needed to complement this picture even further and would most likely reveal even greater differences in product qualities related to raw material variation [3]. Given the relevant raw material variability, overall, such characterisation would be needed to fully understand industrial EPH, and to maximise its potential towards relevant applications.

4. Conclusion

This study shows that both choice of enzymes and industrial relevant variations in poultry raw material composition have a major effect on product composition and product qualities in EPH. The choice of protease showed to have the largest effect on the ACE inhibition measured. Raw material in combination with choice of protease influenced yield, DH%, rheology, and peptide size distribution. However, although choice of protease had an effect, the relative distribution of collagen-to-meat in the raw materials were shown to have a larger effect on the nutritional value of the peptides produced, with meat-rich materials providing the higher nutritional values. On the other hand, the results indicate that acceptable EAA values from collagen-rich materials for food applications can be reached if collagen-rich raw materials are mixed with meat-rich raw materials. In conclusion, depending on the targeted market for a hydrolysate from a given EPH process, raw material variation should be accounted for. Only then can the true potential of using enzymes for tailor-making EPH products with specific properties be realized.

Transparency document

The Transparency document associated with this article can be found in the online version.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.procbio.2021.07.014.

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