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Enzymatic hydrolysis of chicken viscera and bones: Rest raw material characterization and evaluation of industrially relevant process parameters on product yields

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ABSTRACT

Enzymatic hydrolysis is an efficient processing method for valorizing chicken rest raw material by generating new food- or feed ingredients. This paper characterizes two types of chicken rest raw material: viscera and a mixture of bones, skin and remaining meat, and evaluates how industrially relevant hydrolysis process designs affect overall yield of all products, including hydrolysate, sediment, lipid, and emulsion. Eleven hydrolyses were performed using viscera and bone materials, endogenous and commercial enzymes, pre-treatments, variations in water addition and hydrolysis times. Compared to bone materials, viscera had higher proteolytic activity, more readily water-soluble components, and lower effect of adding commercial enzymes on product yields. The process of heating the raw material to hydrolysis temperature greatly impacted product yields, representing about 50 % of the overall hydrolysate production from viscera during hydrolysis. Pre-inactivation of endogenous enzymes reduced initial, but not final, hydrolysate yields. Adding commercial enzymes to pre-inactivated viscera had no effect on yields compared to not adding enzymes. Reducing water addition lowered initial hydrolysate yield from bone material. Lipid yield reflected lipid content in the raw material, and thermal pre-separation of lipids did not increase the total lipid recovery. In general, hydrolysis of viscera generated more emulsion than the bone material.

1. Introduction

Adding value to rest raw materials is crucial for improving the sustainability of food production sectors. Substantial volumes of chicken rest raw materials are produced globally, but remain an underutilized resource, possibly because of strict European Union regulations on the use and processing of animal by-products [1,2]. These materials are often called by-products, co-streams, side-streams, and rest raw materials, and are referred to as the latter in this study to underline their potential as a promising nutritional resource. Poultry accounted for almost 40 % of the global meat production in 2020 [3]. Between 37 % and 67 % of the live weight of chicken is considered rest raw material [4,5], depending on what anatomical parts are regarded as suitable for direct human consumption. Improved utilization and valorization of these materials may therefore influence the sustainability of chicken production. Valuable and nutritious biomolecules such as proteins and lipids can be recovered and recycled from chicken rest raw materials, and these can be used as food and feed ingredients in the food chain [6,7]. The proximate composition and properties of these proteins and lipids vary and may affect their suitability for further processing [5,7].

Enzymatic hydrolysis is an efficient processing method for adding value to protein-rich rest raw materials by cleaving the proteins into water soluble peptides and free amino acids [6,8]. Extensive research on a wide range of raw materials have shown that proteases can be applied to solubilize proteins and separate them from lipids to generate new products. The main product is considered a high value protein-rich hydrolysate consisting of peptides and free amino acids with a wide range of functional, bioactive, and nutritional properties [6,8]. Insoluble proteins are collected in the sediment phase, which also consists of minerals in addition to lipids and can be used as feed ingredients. The

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hydrolysis also releases lipids from the raw material that can be separated into a lipid product. Finally, hydrolysis often generates an emulsion phase made up from a mixture of lipids and proteins with emulsifying properties, which may have fewer direct application possibilities, and are often hard to separate from the other products industrially. Even though the protein hydrolysate is often considered the main product of highest value, it can represent only a small portion of the total dry matter among all product fractions. How the solids in the raw material distribute into these four product fractions are of great industrial and economic importance when evaluating the overall efficiency of a hydrolysis process. The yields depend on several factors, such as the composition of the raw material, pre-treatment before hydrolysis, enzyme type, enzyme and substrate concentration, hydrolysis time, and water addition [7].

Many studies on enzymatic hydrolysis of poultry rest raw materials mainly focus on the effect of commercial enzymes on protein recovery in the hydrolysate, and its functional and nutraceutical properties [5,9–15]. Several of the studies include screenings of proteases, optimization of temperature and investigation of different water- and enzyme concentrations to achieve these properties. In many of the published studies, the process parameters such as enzyme concentrations, the amount of water added, or hydrolysis times are not necessarily industrially relevant as they are associated with high processing costs. For example, more water than raw material (v/w) is often added to the hydrolyses [9,13–16]. This should however be as low as possible to reduce energy use and costs associated with heating the water to hydrolysis temperature and inactivation, followed by evaporation and drying to create stable end products. At the same time, the water content should be high enough to allow optimal working conditions for the enzymes and support solubilization of proteins [9]. Pre-processing such as inactivating endogenous enzymes in the raw material by exposure to high temperatures before hydrolysis can be used in marine raw materials to avoid autolysis and increase process control by subsequently adding specific, commercial enzymes depending on the desired properties of the hydrolysate [17]. However, the heat may cause conformational changes within the protein, reducing solubility making it resistant to enzymatic breakdown, and reduce yield and nitrogen recovery in hydrolysates [18–21].

Lipids in marine raw materials can be recovered as an oil product, as well as in the sediment, emulsion, and hydrolysate [22,23]. Separating lipids from marine raw material before hydrolysis improve stability and reduce oxidation of the oil product [22–24] and reduce the lipid contents in hydrolysates [23]. Lipids may reduce the hydrolysate quality by oxidation and reducing the sensory properties [8]. Since marine lipids generally have a lower melting point and different fatty acid composition compared to poultry lipids [25], conditions for separating lipids from chicken rest raw material may therefore require different processing conditions than what is currently known for marine materials. Chicken fat is frequently used in pet food due to its high levels of the essential fatty acid linoleic acid, and its commercial value depends on maintaining high stability and low oxidation [4]. Thermal separation of lipids from the raw material before hydrolysis may increase the hydrolysis capacity of the processing facility by reducing the processing volume [22] and shielding the lipid from prolonged thermal exposure during hydrolysis and possible degradation [20,23]. Autolysis of rest raw material is a low-cost method that may have high efficiency, but less reproducibility and process control than using commercial enzymes [26]. The composition of poultry rest raw material hydrolyzed industrially can vary, and this can in turn affect the quality of the protein hydrolysate products [13]. Hydrolysis time may have a large influence on yield development of hydrolysate. Reducing the time can increase the process efficiency and have a large economic and industrial impact. Lipids, emulsion, and the insoluble components recovered from hydrolysis of chicken rest raw material represent large volumes of valuable proteins and lipids with commercial potential. It can be processed further to recover specific components [23] or used directly as feed ingredients [27] and should be included when evaluating the viability of the process designs.

The aim of this study was to explore chemical characteristics of chicken rest raw materials such as proteolytic activity and proximate composition that are relevant for further hydrolysis. The aim was also to investigate the effect of industrially relevant hydrolysis process designs such as pre-inactivation of endogenous enzymes, thermal pre-separation of lipids, autolysis, use of commercial enzymes, low water addition and hydrolysis time, on all four hydrolysis product yields. The research parameters were chosen to be industrially relevant.

2. Material and methods

2.1. Raw material

Fresh chicken rest raw material from Hubbard chickens was collected at a local slaughterhouse (Trondelag, Norway). The raw material consisted of 12 separate fractions: viscera, head, neck, wish bone, upper back, lower back, carcass, wing tip, breast skin, thigh skin, thigh bone and feet. The fractions were stored separately in plastic bags on ice and transported to Trondheim within 2 h after slaughter. The separate raw material fractions were weighed, minced (Savioli meat grinder, 32 Classic, 5 mm holes), distributed in plastic bags (1 kg in each), immediately frozen at −20 °C, and stored at −80 °C. During the hydrolyses, the viscera was treated separately from the 11 remaining fractions that were mixed and collectively called bone materials.

2.2. Determination of general proteolytic activity of raw material

To determine the proteolytic activity, crude extracts were prepared by homogenizing raw material in distilled water (1:2 w/w) using an Ultra Turrax for 20 s before centrifuging (10,400*g*, 20 min, 4 °C). The proteolytic activity of the extracts was determined as described by Barret [28] with modifications according to Stoknes [29]. Proteolytic activity of viscera and bone material was determined at natural pH (6.1 and 7.0 respectively) at 30, 40, 50, 60, 70 and 80 °C, and at pH 3 at 30 and 40 °C. The proteolytic activity is presented as cut % of wet weight, representing the increase in acid soluble peptides.

To find the required temperature for inactivation of proteolytic enzymes in viscera, a second approach was also tested. Minced viscera was distributed in plastic bags (2 mm thickness), heated to, and kept at target temperature (50, 60, 70, 80 and 90 °C) for 10 min, and immediately cooled on ice. The proteolytic activity of the viscera after heat treatment was analyzed as described above, with incubation with bovine hemoglobin at 55 °C. The analyses were performed in triplicate.

2.3. Acid soluble peptides in raw material

The amount of acid soluble peptides in the raw material was determined as described by Hoyle and Merritt [30]. 2 ml enzyme extract (prepared as described above for general proteolytic activity) was mixed with 2 ml 20 % TCA, incubated for 30 min, and then filtered (Schleicher & Schull 70 mm filter paper). The content of acid soluble peptides in the extracts was determined according to Lowry [31].

2.4. Proximate composition of raw material

The proximate composition of the raw materials was determined, as well as dry matter content of hydrolysis products hydrolysate, sediment, and emulsion. Total nitrogen content in the raw material was determined according to the Kjeldahl method [32]. Crude protein was estimated by using the standard protein conversion factor 6.25. Total lipid content of the raw material was determined according to Bligh and Dyer [33]. Dry matter content was determined gravimetrically by drying at 105 °C for 24 h and ash content was determined by heating at 550 °C for 12 h [34]. All analyses were performed in triplicate.

2.5. Enzymatic hydrolysis

To evaluate the influence of various process conditions on enzymatic hydrolysis product yields, a series of hydrolyses were performed with varying raw material, pre-treatments, enzyme type and water addition as shown in Table 1.

Two different raw materials were used: 1) chicken viscera and 2) bone materials (a homogenous mixture of the remaining 11 fractions, weights of each fraction corresponding to the weight from one chicken). For both viscera (V) and bone materials (B), two different commercial enzymes, Endocut02L (EC) (bacterial alkaline endoprotease, Tailorzyme, Denmark) and Protamex (Pr) (mix of microbial endoproteases, Novozymes, Denmark) were added in separate hydrolyses (V-E+EC, V-E+Pr, B-E+EC, B-E+Pr) (Table 1). For viscera, an autolysis with only naturally present endogenous enzymes was also performed (V-E). Thermal pre-inactivation (INACT) of endogenous enzymes in viscera before hydrolysis was also investigated. In hydrolyses V-INACT, V-INACT-EC and V-INACT-EC-LW (LW referring to low water addition) the viscera were heated to 80 °C and kept for 10 min before hydrolysis. To study mild thermal separation of lipids from viscera before hydrolysis (V-T-E+EC), the viscera was vacuum packed (1 cm thickness) and heated to and kept at 66 °C for 15 min in a water bath, before lipids were removed by centrifugation (9000*g*, 10 min, 20 °C). After the lipid layer was removed by pipetting, the remaining raw material was then hydrolyzed. The temperature and time for thermal separation were determined in a pre-screening experiment (data not shown). The amount of water added to the hydrolysis mixture was also studied. For most hydrolyses, the reaction mixture consisted of 50 % raw material and 50 % added water (w/w). In hydrolyses B-E+EC-LW, V-E+EC-LW and V-INACT-EC-LW however, the reaction mixture consisted of 90 % raw material and 10 % water. All hydrolyses were performed at 55 °C at natural pH without pH-control. All hydrolyses were performed in duplicate. The hydrolysis process was performed as illustrated in Fig. 1. Frozen raw material (-80 °C) was thawed overnight at 4 °C. Raw material was mixed with preheated water (55 °C) in a 2-liter closed batch reactor (Syrris Atlas vessel reactor) with heating cap. The reaction mixture was stirred at 100 rpm using a half-moon shaped electrical impeller to ensure homogenous mixture. The reaction mixture was heated to set temperature (55 °C).

The hydrolysis start was defined as when the reaction mixture reached set temperature (0 min), and commercial enzyme (0.1 % w/w) was added to hydrolyses marked EC or Pr (Table 1). To study the kinetic development of product yields throughout the hydrolyses, samples (600 g) of the reaction mixture were collected at 0 min, and after 60 and 120 min of hydrolysis. In addition, a sample representing the reaction mixture before heating (00 sample) was prepared outside the reactor by mixing raw material (300 g) and water.

Enzymatic activity in samples were inactivated immediately after sampling by microwave heating at 90 °C for 15 min. The inactivated reaction mixtures were cooled, transferred to 50 ml centrifugation tubes, and centrifuged at 4500*g* at 40 °C for 15 min. The samples were frozen standing upright at −80 °C. The frozen samples were taken out of the tube and the product phases (lipid, emulsion, hydrolysate, and sediment) were separated by cutting. Dry matter content of hydrolysate, sediment and emulsion was determined. The yields were calculated as grams dry product per 100 g raw material (wet weight). Microsoft Excel and R Studio (R version 4.2.1) were used for data processing and statistical analysis. Analysis of variance (ANOVA, TukeyHSD) was performed to investigate significant differences between hydrolyses and hydrolyses time, and significance level was set to $p < 0.05$.

3. Results and discussion

3.1. Raw material weight and composition

3.1.1. Weight

Substantial volumes of nutritional components with high potential for further valorization are available in chicken rest raw material. The total amount of rest raw material generated from one chicken with an approximate live weight of 2 kg, was 1.2 kg (Table 2). The rest raw material is made up of 11 bone fractions and viscera, blood and feathers excluded. Viscera was the single largest fraction, accounting for ¼ of the total rest raw material weight. The individual weights of the different rest raw material fractions are similar to those reported by Henry [35]. About 40 % of the overall combined raw material from one chicken was dry matter, which consisted of approximately 55 % lipid, 37 % protein and 10 % ash. From the rest raw material of a chicken, one could potentially extract 180 g protein, 268 g lipid and 49 g ash.

3.1.2. Protein

Due to the variations in the amount of meat, bones and fat remaining on the rest raw material, proximate composition varied somewhat. The protein content ranged from 9.7 % (skin) to 21.0 % (feet) (Table 2), which is similar to earlier publications [36–39]. High protein content in wishbone, feet, and carcass (19.1–21.0 %) was expected due to the high amounts of remaining meat, connective tissue, and bones in these fractions. Pure chicken bone has a high protein content, which is mainly collagen [40,41], in addition to ash. Chicken breast meat has a higher protein content than mechanically deboned chicken meat, the latter containing cartilage, bones, and skin [42]. This suggests that high levels of pure meat in the rest raw material fractions may increase the protein content of the rest raw material overall. The protein content in viscera (12.6 %) is also comparable to other studies, with values which ranged from 12.8 % to 15.1 % depending on which organs were included [26,36,38]. Due to its large mass, viscera is the single biggest contributor to the protein content (35 g per chicken).

3.1.3. Ash

Ash content ranged between 0.4 % and 9.4 % (Table 2) depending on the amount of remaining bone structure in the fraction. Thigh bone had

Table 1
Overview of hydrolyses and process parameters. B = bone material. V = viscera. E = endogenous enzymes. EC = commercial enzyme Endocut02L. Pr = commercial enzyme Protamex. INACT = pre-inactivated raw material. LW = low water (10 %). T = thermal separation of lipids before hydrolysis.

Hydrolysis	Raw material	Pre-treatment	Endogenous enzymes	Commercial enzyme	Water addition (solid:liquid)
B-E+EC	Bone material		x	Endocut02L	50:50
B-E+Pr	Bone material		x	Protamex	50:50
B-E+EC-LW	Bone material		x	Endocut02L	90:10
V-E	Viscera		x		50:50
V-E+EC	Viscera		x	Endocut02L	50:50
V-E+Pr	Viscera		x	Protamex	50:50
V-E+EC-LW	Viscera		x	Endocut02L	90:10
V-INACT	Viscera	Inactivation endogenous enzymes			50:50
V-INACT-EC	Viscera	Inactivation endogenous enzymes		Endocut02L	50:50
V-INACT-EC-LW	Viscera	Inactivation endogenous enzymes		Endocut02L	90:10
V-T-E+EC	Viscera	Thermal separation of lipids	x	Endocut02L	50:50

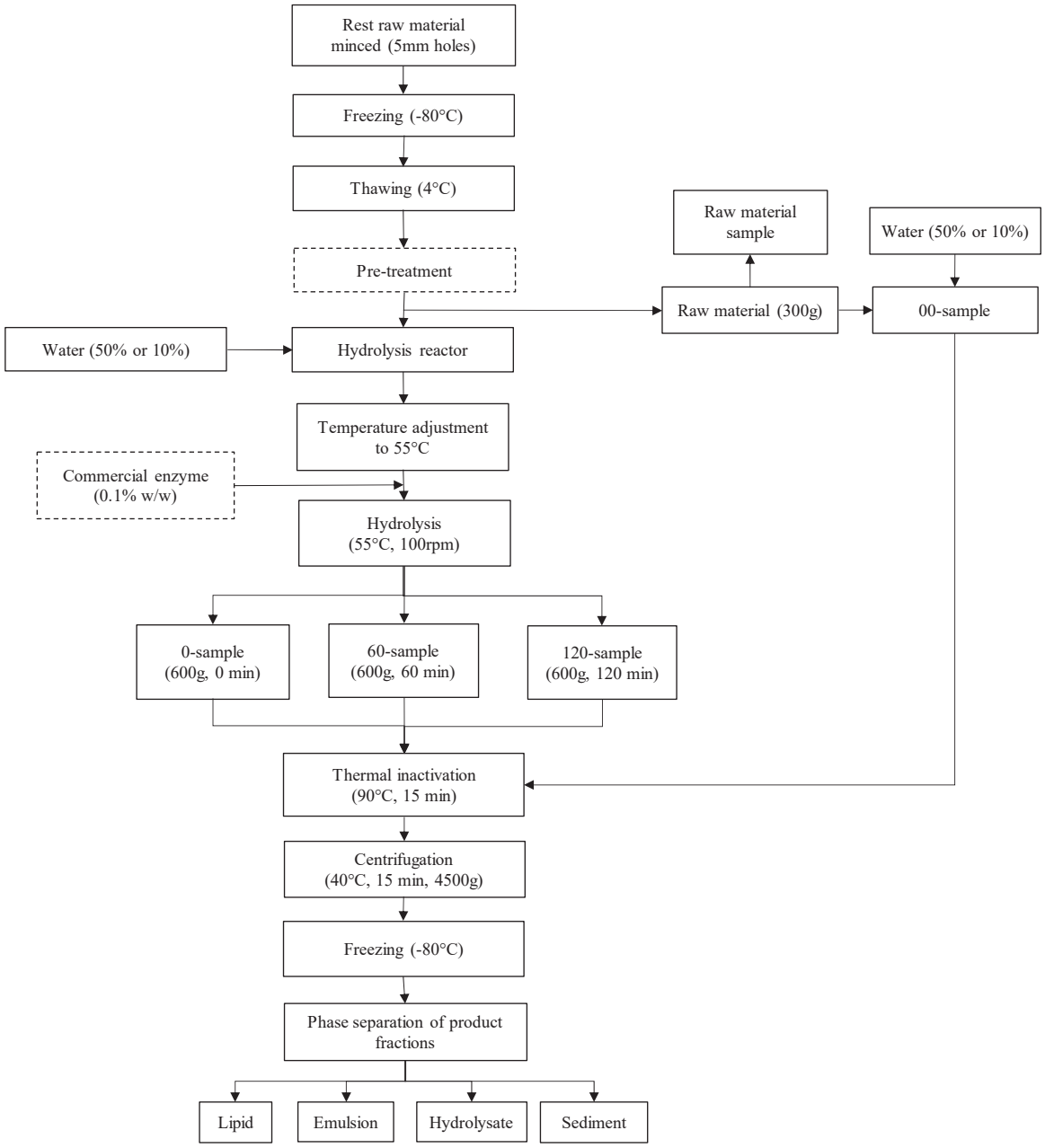

Fig. 1. Flow chart of hydrolysis process.

the highest ash content (9.4 %) and contributed to 26 % of the overall ash content found in the rest raw material. As expected, viscera and skin had low ash content (0.4–0.9 %).

3.1.4. Lipid

Lipid content ranged between 4.8 % and 62.8 % (Table 2). Breast and thigh skin were analyzed together and had the highest lipid content (62.8 %). This was in strong contrast to other studies which reported a lipid content of 38–40 % [43]. The breast- and thigh skin, and the wing tip which contained a lot of skin, were especially difficult to mince properly. Despite reanalysis, the standard deviation remained high. The sum of the lipid, protein and ash content of these fractions is substantially higher than the dry matter content, indicating that the lipid content is too high. The lipid content in viscera was 18.8 % and is reported to vary from 4.53 % to 15.8 % [26,36,38] depending on which organs are included. Despite having a lower lipid content than other fractions, the high mass of viscera makes it the highest contributor to overall lipid content (19 %).

3.2. Proximate composition and proteolytic activity of raw material used in enzymatic hydrolyses

3.2.1. Proximate composition

The 12 raw material fractions (Table 2) were separated into two main fractions before hydrolysis: viscera and bone materials (the remaining 11 fractions). The proximate composition of viscera and bones fractions used in the hydrolyses are shown in Table 3.

The bone material had a higher dry matter, ash, protein, and lipid content than viscera. Other studies on chicken rest raw material have reported similar or higher protein contents (12.9–23.5 %) depending on which fractions were included [13,26,36,37,40]. The same studies also found a large variation in lipid content (6.9 %-38.3 %) and ash content (1.4–11.8 %). As expected, the bone material has higher ash content (4.3 %) than viscera (0.9 %).

Table 2
(1) Proximate composition of chicken rest raw material fractions, g/100 g (mean ± SD, analyses performed in triplicate). Breast skin and thigh skin were analyzed together. (2) Weight of each fraction (g) (n = 15–121) from one chicken. The percentage of each fraction based on total amount of rest raw material from one chicken (%). The percentage of each fraction based on live weight of one chicken (%). (3) Percentage of ash, protein, and lipid of dry matter (DM) for each fraction. Parts of the data have previously been presented by Helgeland-Rossavik [26].

	Proximate composition of each fraction				Weight of fractions			Percentage of DM		
	Dry matter (g/100 g)	Ash (g/100 g)	Protein (g/100 g)	Lipid (g/100 g)	Weight per chicken (g)	Percentage of total rest raw material (%)	Percentage of live weight (%)	Ash (% of DM)	Protein (% of DM)	Lipid (% of DM)
Viscera	34.0 ± 2.8	0.9 ± 0.0	12.8 ± 0.9	18.2 ± 3.0	275.6	23	14	3	38	54
Head	30.5 ± 0.5	4.1 ± 0.2	16.2 ± 0.1	7.6 ± 3.3	58.7	5	3	14	53	25
Feet	38.8 ± 0.9	6.1 ± 0.7	21.0 ± 0.4	14.7 ± 1.0	83.4	7	4	16	54	38
Wish bone	36.3 ± 0.4	2.7 ± 0.2	20.5 ± 0.3	14.2 ± 0.4	18.8	2	1	7	56	39
Neck	39.4 ± 1.5	3.1 ± 1.4	12.5 ± 0.3	22.8 ± 0.6	85.5	7	4	8	32	58
Wing tip	41.4 ± 0.9	5.6 ± 0.4	15.2 ± 0.2	30.5 ± 4.9	17.0	1	1	13	37	74
Thigh bone	39.0 ± 1.1	9.4 ± 0.8	16.7 ± 0.6	11.6 ± 0.3	135.2	11	7	24	43	30
Upper back	38.2 ± 0.8	4.8 ± 1.1	17.4 ± 0.8	17.1 ± 0.1	110.3	9	6	13	46	45
Lower back	44.9 ± 5.4	5.1 ± 1.8	13.7 ± 2.5	26.7 ± 0.2	180.6	15	9	11	31	60
Carcass	35.3 ± 0.8	6.1 ± 0.9	19.1 ± 0.3	4.8 ± 0.1	111.5	9	6	17	54	14
Thigh skin	61.2 ± 1.4	0.4 ± 0.2	9.7 ± 0.5	62.8 ± 9.7	75.0	6	4	1	16	103
Breast skin	61.2 ± 1.4	0.4 ± 0.2	9.7 ± 0.5	62.8 ± 9.7	58.8	5	3	1	16	103
Total					1210.3		62.3			

Table 3
Chemical composition of wet chicken rest raw material used in hydrolyses, g/100 g (mean ± SD, n = 19, analyses performed in triplicate).

	Dry matter (g/100 g)	Ash (g/100 g)	Protein (g/100 g)	Lipid (g/100 g)
Bone material	40.8 ± 1.4	4.3 ± 0.7	15.1 ± 0.6	24.4 ± 2.7
Viscera	34.0 ± 2.8	0.9 ± 0.0	12.8 ± 0.9	18.2 ± 3.0

High protein and low lipid content is generally desirable for raw material that will be hydrolyzed, as more protein is available for cleavage to increase hydrolysate yield, and low lipid may reduce lipid content in hydrolysate and avoid unwanted lipid oxidation. Both bone material and viscera contain about 37–38 % protein and 55–58 % lipid on dry matter basis, while bones contain a higher ash content (11 %) than viscera (3 %) on dry matter basis.

3.2.2. *Proteolytic activity of raw material*

The proteolytic activity of enzyme extracts from viscera and bone materials were analyzed at different temperatures and pH to evaluate their possible contribution to enzymatic cleavage of proteins during hydrolysis. The proteolytic activity of viscera and bone material varied with pH and temperature. The general proteolytic activity at natural pH (6.1 ± 0.05 and 7.0 ± 0.05 for viscera and bone material respectively) is shown in Fig. 2.

Viscera has a very high proteolytic activity compared to bone material. This was expected as bone material mostly contains structural proteins such as collagen, elastin and keratin, and no digestive enzymes, which are normally found in internal organs. Viscera and bone material show a similar temperature dependence pattern at natural pH with maximum activity at 60 °C, and slightly lower at 50 °C and 70 °C. This pattern is in accordance with other studies on chicken intestinal enzymes [45]. Little to no activity was detected at 30 °C, 40 °C and 80 °C. This suggests that endogenous enzymes naturally found in the raw material are active and may contribute to enzymatic hydrolysis during the heating of the raw material to hydrolysis temperature, during the hydrolysis, and even above hydrolysis temperature before final inactivation temperature is reached. pH appears to have a big impact on proteolytic activity, as the activity increased at lower pH (Table 4), from non-detectable for viscera at natural pH 6.1–89.98 (cut % of wet weight) at pH 3. Viscera and bone material also showed similar temperature dependency at lower pH, as the activity increased from 30 °C to 40 °C. At natural pH, no proteolytic activity was detected for viscera at 40 °C. In contrast, at pH 3 the activity at 40 °C was over 40 times more than the highest proteolytic activity detected at any temperature at a natural pH. Even though 40 °C is close to the physiological temperature of chicken, the pH seems crucial, and the conditions are likely closest to the physiological parameters at a pH of 3. These results are in accordance with previous studies, as Jamdar [45] found optimal degradation of chicken intestines to occur at a pH of 2.5 and temperature of 60 °C, probably due to a range of proteases, including pepsin and cathepsin D. Acid proteases are well known and efficient enzymes associated with the acidic conditions in the digestive system of animals [46–49]. This suggests that for the endogenous enzymes to contribute to the hydrolysis, pH reduction of the rest raw material could be an alternative.

3.3. *Thermal pre-inactivation of endogenous enzymes in viscera before hydrolysis*

Inactivating the endogenous enzymes in the raw material before hydrolysis is a method to control the enzyme activity during the hydrolysis and improve reproducibility. To investigate the necessary temperature at which endogenous enzymes could be pre-inactivated, the viscera was exposed to a series of increasingly higher temperatures at a natural pH of 6.1. This was done prior to the remaining proteolytic activity being analyzed by preparation of enzyme extracts and incubation

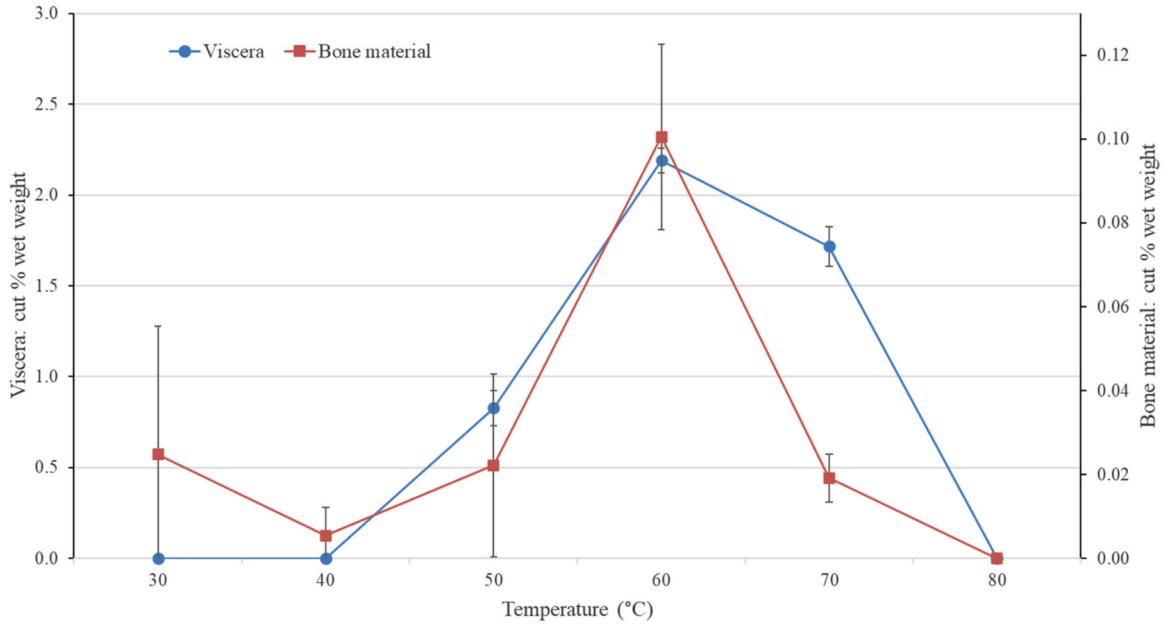

Fig. 2. Proteolytic activity of viscera and bone material at natural pH (6.1 and 7 respectively) at temperatures 30–80 °C. The values for viscera at 30, 40 and 80 °C were negative, and are set as zero in the graph. Parts of the data have previously been presented in Hals [44].

Table 4
Proteolytic activity of viscera and bone material at pH 3 at temperatures 30 °C and 40 °C (soluble proteins, % of wet weight). Parts of the data has previously been presented by Hals [35].

	30 °C	40 °C
Bone material	1.38 ± 0.07	2.07 ± 0.17
Viscera	51.73 ± 1.77	89.98 ± 1.76

with hemoglobin. This approach is slightly different from the one shown in Section 3.2. In the current section, the aim was to investigate the stability of the proteases within the raw material source, rather than in the enzyme extracts.

The highest enzyme activity was recorded at 50 °C, in contrast to the previous section where the highest activity was recorded at 60 °C. The remaining proteolytic activity in viscera after heating to different temperatures is shown in Fig. 3. Proteolytic activity is high after heating at 50 °C and 60 °C, and low at 70 °C. No activity was detected at 80 °C and 90 °C, and the enzymes are considered thermally inactivated at these temperatures. Similar results were found for proteolytic activity in salmon muscle [50], where optimal conditions were pH 8 and temperature 65 °C, probably due to the presence of heat-stable alkaline proteases. The activity declined above 70 °C.

In the subsequent hydrolyses where the effect of thermal inactivation of endogenous enzymes on product yield and quality was affected, the raw material was thermally treated at 80 °C for 10 min before hydrolysis (hydrolyses V-INACT, V-INACT-EC and V-INACT-EC-LW).

3.4. Yield of hydrolysate, sediment, emulsion, and lipid

Enzymatic hydrolysis and pre-heating conditions of chicken rest raw material resulted in four product fractions: hydrolysate, sediment, emulsion and lipid. The dry matter yield of each fraction indicates how

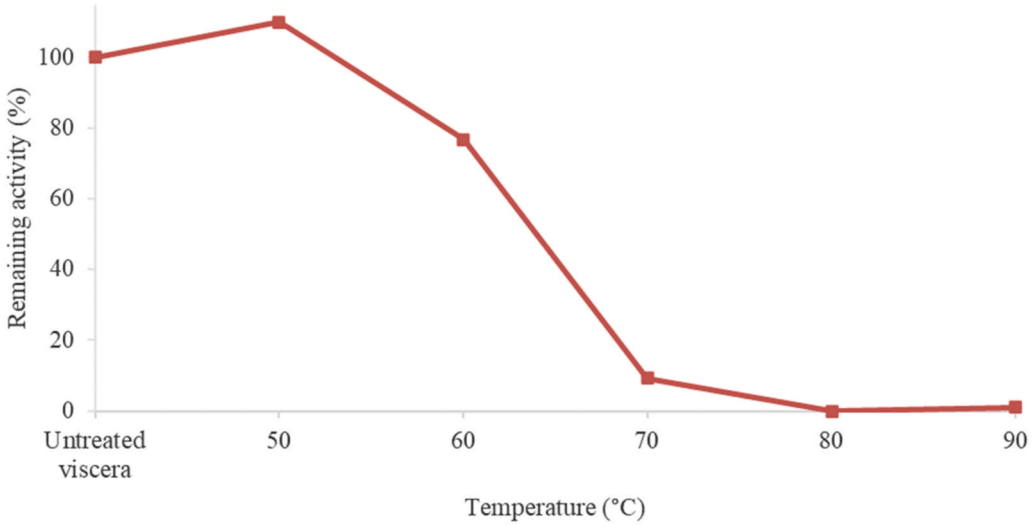

Fig. 3. Proteolytic activity from viscera at natural pH 6 after incubation at 50–90 °C, shown as the percentage of enzyme activity of untreated viscera. The data have previously been presented by Helgeland-Rossavik [51].

the solids in the raw material were distributed during the hydrolysis,. Figs. 4–7 show the product yields as grams of dry hydrolysate, sediment, emulsion and lipid per 100 g wet raw material for all 11 hydrolyses at all four sampling times (00, 0, 60, 120 min. The data is also presented in Table 5 (Supplementary material). The statistical data is presented in Table 6–9 in Supplementary material.

3.4.1. Development of hydrolysate and sediment yields during hydrolysis

3.4.1.1. The impact of different commercial enzymes and hydrolysis time on viscera. The hydrolysate yield from autolysis using only endogenous enzymes in viscera (V-E) was only slightly lower than the yield achieved by adding commercial enzymes (V-E+EC and V-E+Pr, Fig. 4). The difference in the mean between V-E (8.7 g dry hydrolysate/100 g raw material) and V-E+EC (10.7 g dry hydrolysate/100 g raw material) after 120 minutes was not statistically different but is still of commercial interest. Hydrolyzing untreated salmon and cod viscera with commercial enzymes did not result in higher hydrolysate yields than autolysis [23,55], suggesting that adding commercial enzymes to raw materials rich in endogenous enzymes has limited impact on hydrolysate yield. Continuing the hydrolysis beyond 60 min did not influence the hydrolysate yield for autolysis, and this was also the case when adding commercial enzymes. The development of increased hydrolysate yields was mirrored by a subsequent decrease in sediment yields (Figs. 4–5), which is to be expected as more proteins in the raw material are cleaved to smaller, soluble peptides during hydrolysis and recovered in the hydrolysate, while insoluble materials are recovered in the sediment. These results are similar to those reported on hydrolysis of other raw materials [23]. There was no significant decrease in sediment yield from 60 to 120 min, indicating that it may not be necessary to continue the hydrolysis for 120 min. For marine raw material, it also appears that optimal hydrolysis time with regards to hydrolysate yield is shorter than 120 min, as hydrolysate yield can stabilize earlier depending on the raw material and enzymes present [22,23]. Reducing hydrolysis time by ensuring a high rate of hydrolysis and high yield of solubilized material is an important economic driver for industrial hydrolysis [23].

It is worth noting that for untreated viscera the largest increase in hydrolysate yield and decrease in sediment yield appears to be during the heating period from 00 to 0 min. The amount of solubilized material (shown as hydrolysate yield) increased 52–96 % during heating, which represents about 50 % of the total increase in yield during the whole hydrolysis. This suggests that the use of temperature and time to solubilize proteins from the viscera raw materials is an important contributor to product yields, and the actual start time of hydrolysis is somewhere between 00 and 0 min. To optimize control, the start could be defined at an earlier point, the heating method could be tailored and made more efficient, and the enzyme could be added sooner. Untreated viscera generally had a higher initial amount of solubilized material (hydrolysate), higher emulsion yield and lower sediment yield compared to bone material (00-samples, Figs. 4–6). The amount of acid soluble peptides in the viscera was about four times higher than in bone material (data not shown), indicating that viscera raw material is initially more degraded than bone material. Higher enzyme concentrations and pH adjustments to achieve optimal conditions for enzymes may increase the hydrolysate yields and protein recoveries, but also represents a higher production cost.

3.4.1.2. The impact of different commercial enzymes and hydrolysis time on bone material. Bone material (B-E+EC, B-E+Pr) did not have a statistically different final hydrolysate yield or higher sediment yield compared to untreated viscera hydrolyzed with commercial enzymes (V-E+EC, V-E+Pr), while it had similar yields to viscera hydrolyzed with endogenous enzymes (V-E) (Figs. 4, 5 and 8). The increase in hydrolysate yield and decrease in sediment yield was highest between 0 and 60 min, after which it stabilized. Whether a lower hydrolysis time could be applied to reach the same yield should be studied further.

Bone material generally reached a lower final hydrolysate yield and higher sediment yield than viscera. These differences are possibly due to the low initial amount of solubilized material (hydrolysate) at both 00 and 0 minutes for bone material (Fig. 4). Parts of the bone material solids do not appear to be easily solubilized and hydrolyzed, possibly due to the rigid structure and high collagen content [12,56]. The bone material also has a higher ash and mineral content, which are partly insoluble in water and are recovered in the sediment. In addition, viscera has a higher proteolytic activity (Fig. 2) around 50 °C where the temperature increase during heating was slow and holding time was therefore long.

Heating the raw material and water in the reactor to a hydrolysis

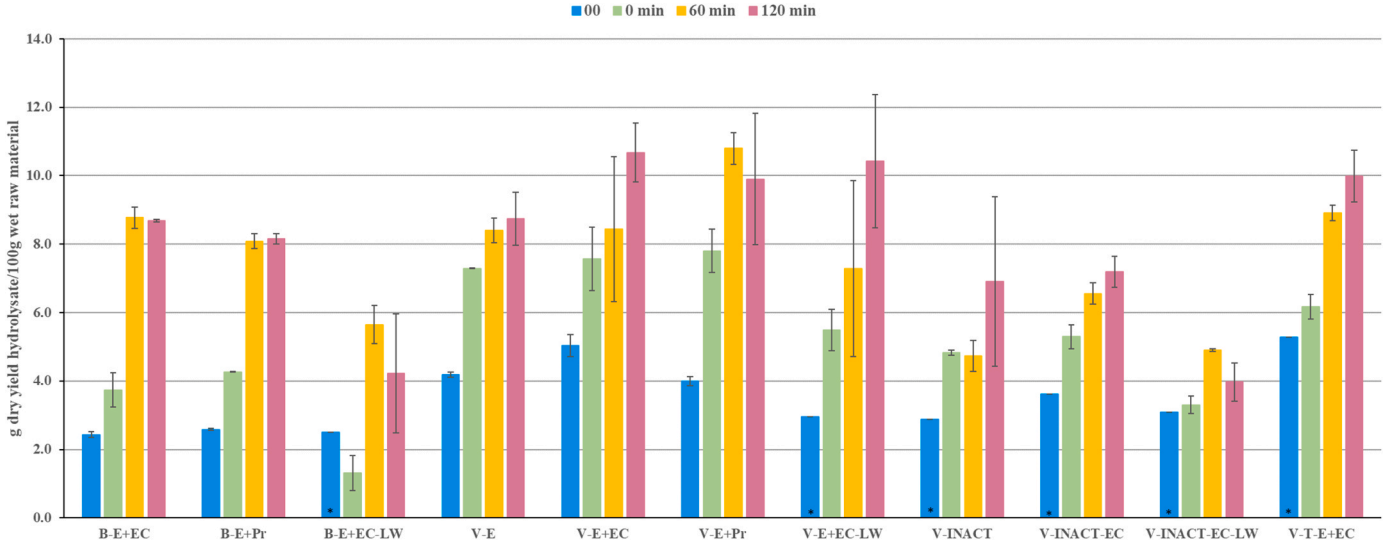

Fig. 4. Hydrolysate yield for all hydrolyses at all hydrolyses times (00, 0, 60, and 120 min) as dry yield (g) per 100 g wet raw material. Values are given as means with standard deviation as error bars. B = bone material. V = viscera. E = endogenous enzymes. EC = commercial enzyme Endocut02L. Pr = commercial enzyme Protamex. LW = low water (10 %). INACT = pre-inactivated raw material. T = thermal separation of lipids before hydrolysis. Parts of the data have previously been presented by Fålun, Roland and Forshaug [52–54]. * For some of the samples, there was not enough sample to measure the dry matter content gravimetrically for both replicates. In these cases, the dry matter content of the same fraction of the other replicate was used to calculate the yield. In case no dry matter of the other replicate was available, the average dry matter of all fractions at the same time point were used.

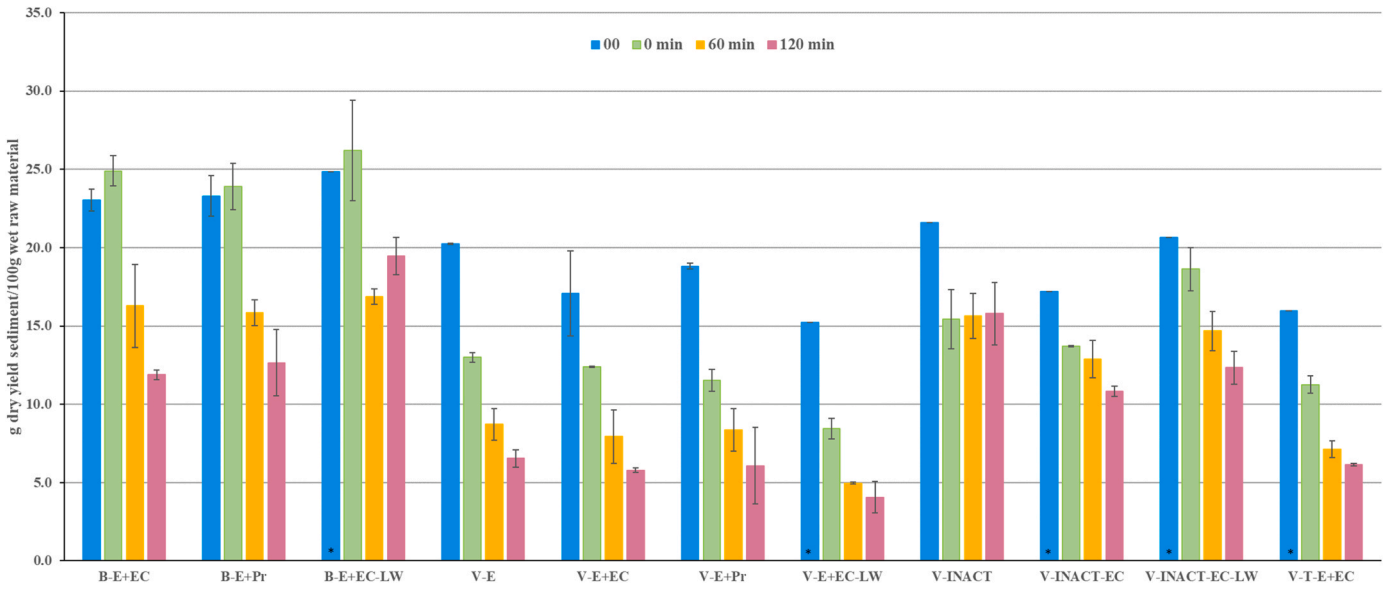

Fig. 5. Sediment yield for all hydrolyses at all hydrolyses times (00, 0, 60, and 120 min) as dry yield (g) per 100 g wet raw material. Values are given as means with standard deviation as error bars. B = bone material. V = viscera. E = endogenous enzymes. EC = commercial enzyme Endocut02L. Pr = commercial enzyme Protamex. LW = low water (10 %). INACT = pre-inactivated raw material. T = thermal separation of lipids before hydrolysis. Parts of the data have previously been presented by Fålun, Roland and Forshaug [52–54]. * For some of the samples, there was not enough sample to measure the dry matter content gravimetrically for both replicates. In these cases, the dry matter content of the same fraction of the other replicate was used to calculate the yield. In case no dry matter of the other replicate was available, the average dry matter of all fractions at the same time point were used.

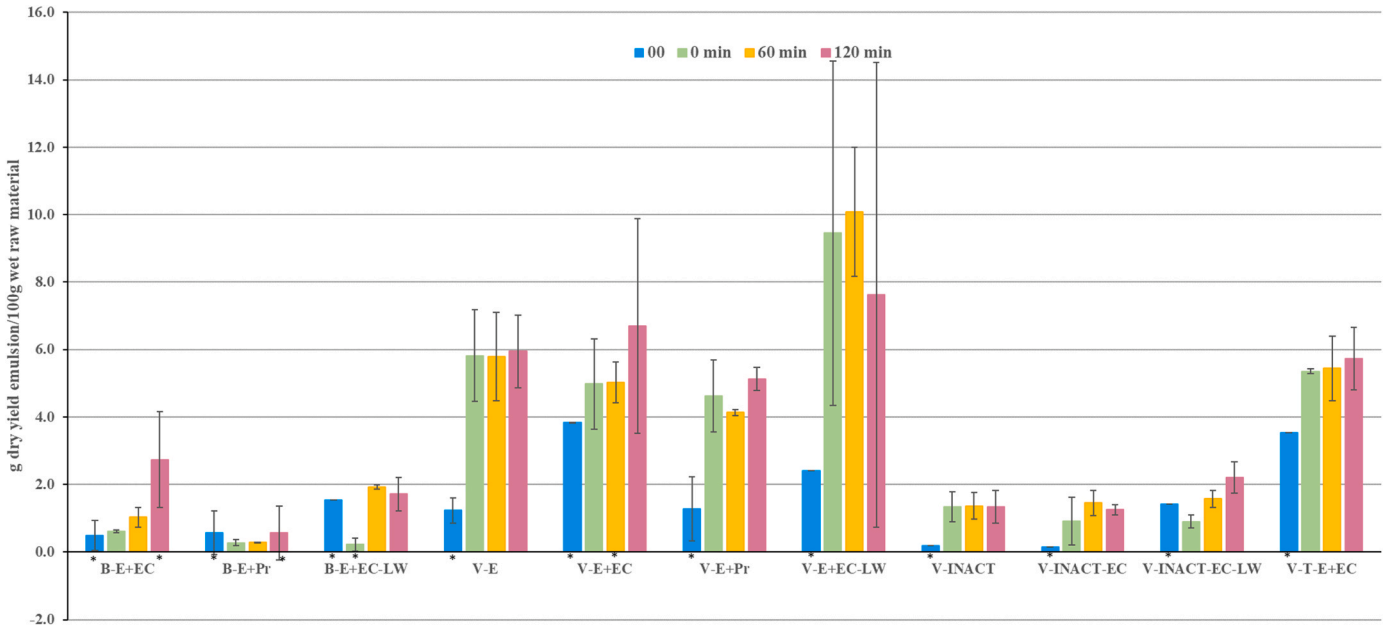

Fig. 6. Emulsion yield for all hydrolyses at all hydrolyses times (00, 0, 60, and 120 min) as dry yield (g) per 100 g wet raw material. Values are given as means with standard deviation as error bars. B = bone material. V = viscera. E = endogenous enzymes. EC = commercial enzyme Endocut02L. Pr = commercial enzyme Protamex. LW = low water (10 %). INACT = pre-inactivated raw material. T = thermal separation of lipids before hydrolysis. Parts of the data have previously been presented by Fålun, Roland and Forshaug [52–54]. * For some of the samples, there was not enough sample to measure the dry matter content gravimetrically for both replicates. In these cases, the dry matter content of the same fraction of the other replicate was used to calculate the yield. In case no dry matter of the other replicate was available, the average dry matter of all fractions at the same time point were used.

temperature of 55 °C (00 to 0 min) lasted from 35 to 80 min. There was still a 59 % increase in hydrolysate yield for bone material from 00 to 0 minutes despite low endogenous enzyme activity, indicating that the amount of solubilized protein may have increased due to exposure to higher temperatures over time, which can help dissolve materials containing high levels of collagen [56].

Contrary to viscera, only 20 % of the total yield increase is attributed to the heating period from 00–0 min. This indicates that adding commercial enzymes is more important for producing a high hydrolysate yield for bone material than viscera. The type of commercial enzyme tested in this study does not appear to affect the hydrolysate and sediment yields throughout the hydrolyses. However, other commercial enzymes may have caused higher yields. For example, Lindberg [12] showed that a range of different enzymes resulted in varying hydrolysate

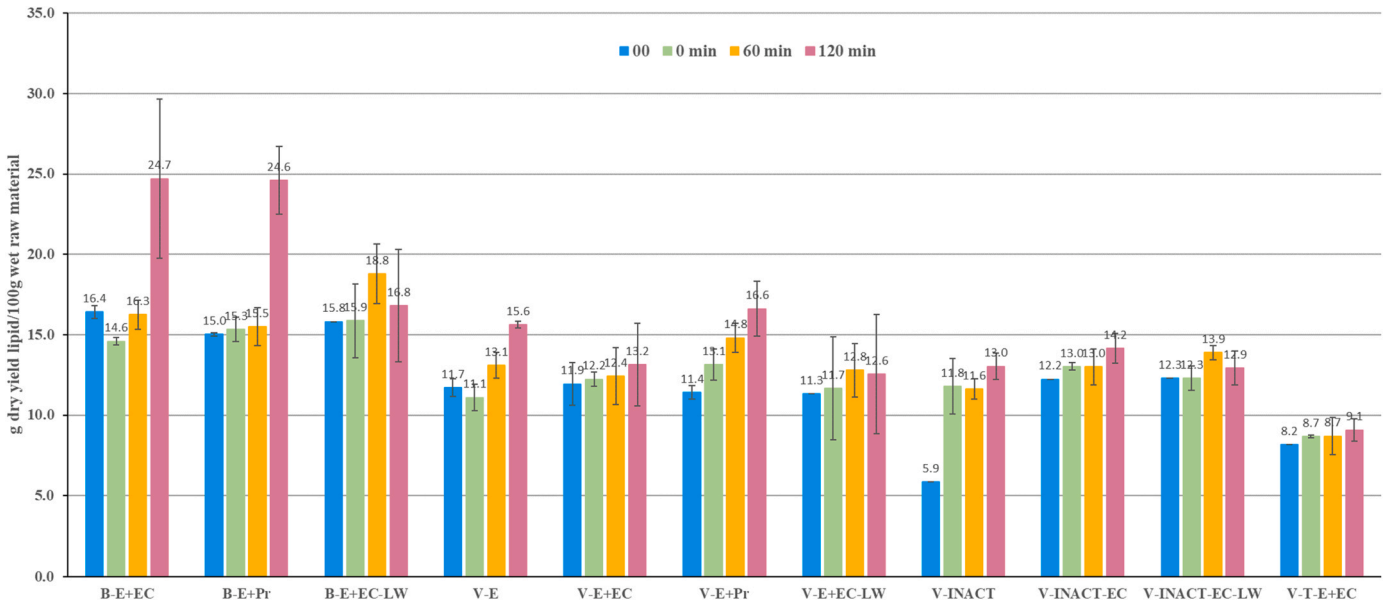

Fig. 7. Lipid yield for all hydrolyses at all hydrolyses times (00, 0, 60, and 120 min) as dry yield (g) per 100 g wet raw material. Values are given as means with standard deviation as error bars. B = bone material. V = viscera. E = endogenous enzymes. EC = commercial enzyme Endocut02L. Pr = commercial enzyme Protamex. LW = low water (10 %). INACT = pre-inactivated raw material. T = thermal separation of lipids before hydrolysis. Parts of the data have previously been presented by Fålun, Roland and Forshaug [52–54].

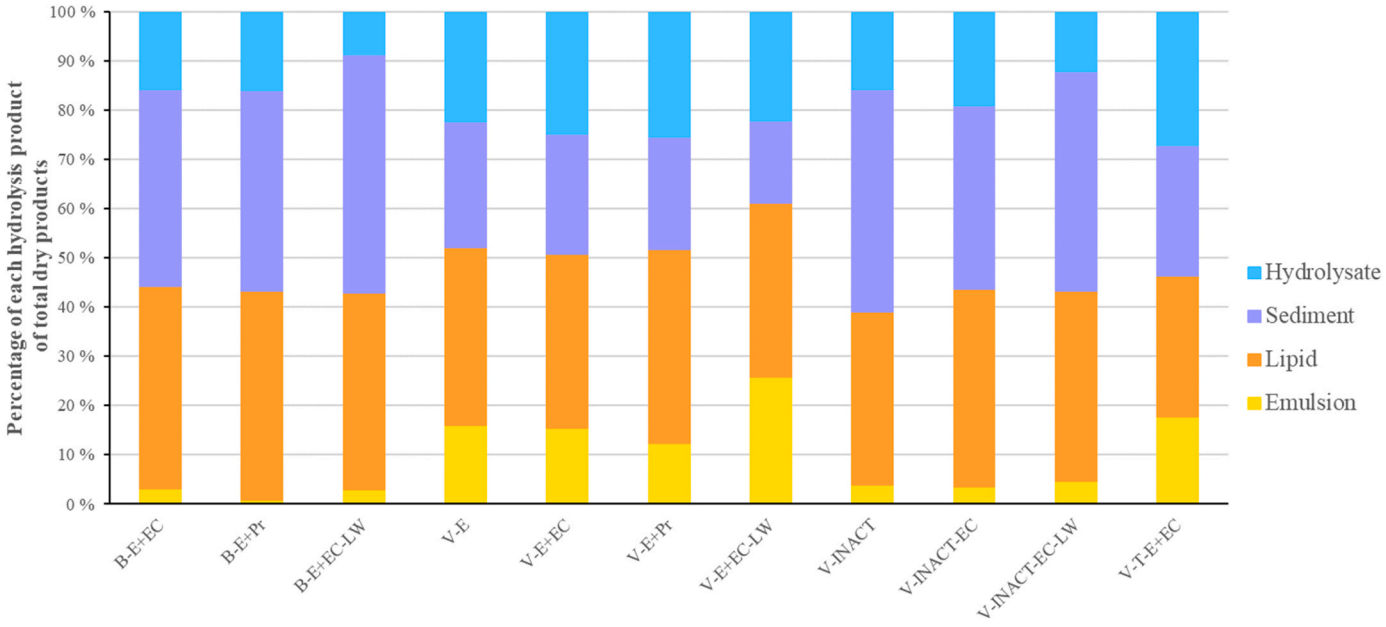

Fig. 8. Distribution of product yields for each hydrolysis. The data is based on g dry yield product/100 g dry raw material for 0-, 60-, and 120-min sampling combined. B = bone material. V = viscera. E = endogenous enzymes. EC = commercial enzyme Endocut02L. Pr = commercial enzyme Protamex. LW = low water (10 %). INACT = pre-inactivated raw material. T = thermal separation of lipids before hydrolysis. The data have previously been presented by Fålun, Roland and Forshaug [52–54].

yields depending on the type of poultry raw material used. In addition, all hydrolyses in this study were performed at a natural pH without pH adjustment, which may also have affected the enzyme efficiency. Endocut02L and Protamex have efficient pH ranges from 6 to 10 and 6–9 depending on the substrate according to the manufacturers. In addition to applying commercial enzymes, Lindberg [12] also suggested that a large part of collagen in poultry raw material can be solubilized from the sediment phase during high temperature inactivation, releasing proteins of different molecular weights into the hydrolysate phase.

3.4.2. *Pre-treatments*

Pre-inactivation of endogenous enzymes in the raw material by heating (V-INACT, V-INACT-EC, V-INACT-EC-LW) changed the raw material and decreased the hydrolysate yield at 0 min, but no significant difference was found at 60 and 120 min (Figs. 4, 5 and 8). Interestingly, adding commercial enzymes to pre-inactivated viscera (V-INACT-EC) showed a similar yield development throughout the hydrolysis to when commercial enzyme was not added (V-INACT), with the exception of the 60 min sampling, suggesting that enzymes may have a limited effect on pre-inactivated viscera. In addition, pre-inactivated viscera hydrolyzed

with Endocut02L (V-INACT-EC) did not reach statistically higher hydrolysate yields than autolysis (V-E). These are important points in an industrial context, as based on yield, the added cost of pre-inactivation and addition of enzyme does not appear to enhance the hydrolysate yields.

Pre-inactivated viscera initially seemed less susceptible to further enzymatic cleavage. This indicates that the high temperature in pre-inactivation reduces the raw materials' potential for hydrolysis, which is also reported on marine raw materials [17,19,23,57]. The heat may cause the proteins to denature and aggregate, leading to conformational changes or association into larger peptides making the proteins less susceptible to hydrolysis and solubilization [58–60]. Physical changes in the raw material were visually observed at increasing temperatures, such as reduced viscosity, a more rigid texture and change in color from red to brown [51]. Protein denaturation is known to occur below 80 °C in chicken breast and thigh muscle [61], and protein solubility in chicken breast decreases when increasing the temperature from 23 to 80 °C [62]. Granulation and gel formation has been observed in beef and chicken meat above 50 °C [63,64], which may negatively influence subsequent hydrolysis. Pre-inactivation in marine raw materials may be necessary to increase process control [17], since proteolytic activity in fish vary with e.g. season and fishing ground [65,66]. Characterization of similar, natural variations of endogenous enzymes activity among chicken rest raw material may reveal that these variations are not as prominent, and the need to pre-inactivate may not be necessary to achieve sufficient process control. Several studies on marine raw materials have also reported the possible formation of insoluble lipid-protein complexes during exposure to high temperatures as an explanation to reduced hydrolysate and increased sediment yields [17, 20,23,67].

It is also worth noting that the hydrolysate yield for inactivated viscera (V-INACT) increased by 139 % throughout the hydrolysis without the presence of endogenous enzymes or addition of commercial enzymes. The increase of solubilized material can therefore be attributed to solubilization of proteins without further enzymatic cleavage, as other cellular structures such as membranes are degraded during the heating that can lead to a release of soluble compounds. The increase in hydrolysate yield for pre-inactivated viscera and commercial enzymes may therefore also not only be due to the presence of Endocut02L, but to general solubilization as observed with no addition of commercial enzymes.

Thermal separation of lipids before hydrolysis (V-T-E+EC) resulted in the highest initial hydrolysate yields (00 min) among the raw materials (Fig. 4), probably due to active endogenous enzymes during thermal treatment (Fig. 2). However, this initial advantage did not affect the final yields, as the hydrolysate and sediment yield development throughout the hydrolyses were similar to hydrolyses with untreated viscera with both endogenous and commercial enzymes (V-E+EC, E-E+Pr). This is in accordance with a study on hydrolysis of salmon raw material where hydrolysate yield was unaffected by mild thermal treatment [20]. This may indicate that the proteins available for enzymatic cleavage are hydrolyzed rather quickly by endogenous or commercial enzymes, and that the insoluble proteins remaining in the raw material or sediment are not susceptible to further hydrolysis by the enzymes, hence reaching a maximum yield.

3.4.3. Water addition

Reducing the water addition can have several economic and industrial benefits, such as decreasing the reaction volume through the process and minimizing the energy required to heat the water before the hydrolysis and subsequent removal of water by evaporation and drying to create a stable product. However, adding too little water may have negative effects on hydrolysate yields depending on raw material input and process design [9]. Adding sufficient water appears to be critical to maximize hydrolysate yields from bone materials, as adding only 10 % water (B-E+EC-LW) significantly reduced the hydrolysate yield and increased the sediment yields compared to adding 50 % water (B-E+EC) at 0 min (Figs. 4–5). Despite a large difference in the mean values of hydrolysate yields, there was no statistically significant difference between 10 % and 50 % water addition on hydrolysates yields, but a significant difference between sediment levels after 120 min was observed. Hydrolysis of salmon backbones with no addition of water halved the hydrolysate yield compared to when adding water [22], similar results were found when hydrolyzing cod rest raw material [17]. A possible explanation was that the high viscosity of the reaction mixture could prevent hydrolysis as insufficient water addition can prevent the raw material from mixing properly and the enzymes to access the proteins in the raw material [6]. The bone material with 10 % water added was very viscous, resulting in increased heating time and difficulties in taking representative samples. Moreover, this highlights that water addition is also important to provide a uniform raw material mixture that is easy to handle in a processing facility.

By contrast to bone material, untreated viscera had more similar hydrolysate yields after 120 min irrespective of water addition (V-E+EC and V-E+EC-LW), despite having a slower hydrolysate yield development. Adding only 10 % water to the raw material (V-E+EC-LW) decreased the initial amount of solubilized material (hydrolysate) for viscera (00-samples, Fig. 4), indicating that the water amount affects how much of the available water-soluble compounds that are able to move from the raw material to the water phase. However, this pattern was not observed when adding 10 % water to pre-inactivated viscera (V-INACT-EC-LW) or the bone material (B-E+EC-LW), possibly due to a lower content of easily water-soluble components in these raw materials. Adding less water to untreated viscera did not significantly affect the sediment yield.

Reducing the water addition for pre-inactivated viscera (V-INACT-EC-LW) resulted in low hydrolysate yields, with no development throughout the hydrolyses, comparable to bone material with low water addition (B-EC-LW). As previously shown, adding commercial enzymes to pre-inactivated viscera (V-INACT-EC) did not increase the hydrolysate yield compared to not adding commercial enzymes (V-INACT). The observed increase in hydrolysate yield was attributed to the solubilization of already soluble material into the water phase. By reducing the water addition (V-INACT-EC-LW), a significant increase in hydrolysate throughout the hydrolysis from 00 min to 120 min was not observed.

Water addition appears to be important for solubilizing the soluble components from the raw material and sediment when hydrolyzing bone material and inactivated viscera. By adding more water the hydrolysate yields for these raw materials can be increased. This is in accordance with studies by Šližytė and Liaset on the effect of hydrolysis conditions on yield and nitrogen recovery from marine raw materials [19,68,69]. They suggested that the negative influence of low water addition is caused by increasing product inhibition of the enzymes due to high substrate concentrations, which inhibits furthers hydrolysis of raw material, or the formation of insoluble protein-lipid complexes.

3.4.4. Development of emulsion and lipid yields during hydrolysis

3.4.4.1. Lipid yield. The lipid yield was more stable throughout the hydrolyses compared to other product yields, with a slight increase observed at higher hydrolysate times (Fig. 7). Bone material generally had a higher lipid yield than viscera, which was expected due to the higher lipid content in the raw material. A very high lipid yield was observed at 120 min for bones hydrolyzed with commercial enzymes (B-E+EC, B-E+Pr), and lipid recovery for B-E+EC apparently exceeded 100 % (Fig. 9). Even if care was taken during sampling, the lipid tended to separate and float to the top of the reactor causing difficulties in sampling. As previously mentioned, the reaction mixtures containing bone material generally appeared to have a higher viscosity than viscera, which makes separating the fractions after hydrolysis more difficult [22].

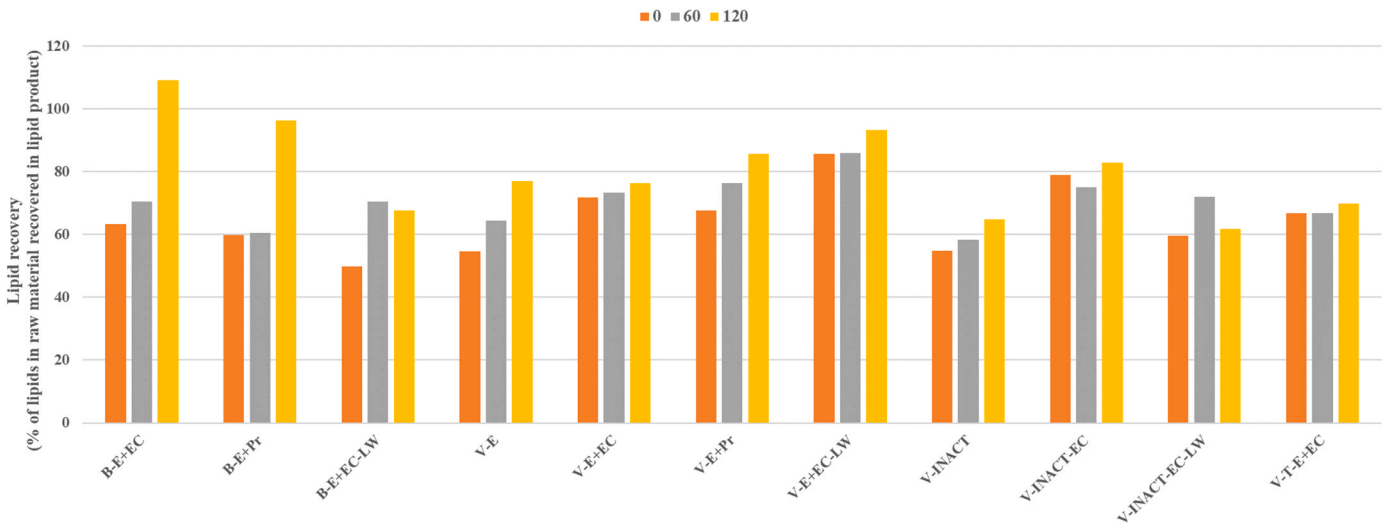

Fig. 9. Percentage of lipids in raw material recovered in the lipid product (lipid recovery) after 0-, 60- and 120-min hydrolysis. B = bone material. V = viscera. E = endogenous enzymes. EC = commercial enzyme Endocut02L. Pr = commercial enzyme Protamex. LW = low water (10 %). INACT = pre-inactivated raw material. T = thermal separation of lipids before hydrolysis. Parts of the data have previously been presented by Fålun, Roland and Forshaug [52–54].

Untreated viscera hydrolyzed with commercial or only endogenous enzymes (V-E, E-E+EC, V-E+Pr) had similar oil yields throughout the hydrolyses. Pre-inactivation or low water addition for viscera did not appear to influence lipid yield. Other studies on marine raw material have found that reduced water addition had different effects on lipid yield [17,19,22,68]. Šližytė found that no water addition reduced the oil yield during hydrolysis of defatted salmon backbones [22] and suggested this was due to the high viscosity of the reaction mixture which may limit hydrolysis and trap the oil in the reaction mixture, hence making it difficult to separate by centrifugation. This highlights that water is also important for oil separation in addition to protein hydrolysis and solubilization.

The lipid yield from thermally separated viscera was the lowest, which is expected as 38 % of the lipid was separated from the raw material before the hydrolysis (data not shown). Combined with the subsequent hydrolysis, thermal separation gave a total lipid recovery of 76 %, and did not affect the overall lipid recovery compared to only performing hydrolysis (Fig. 9). Even if thermal separation of lipids before hydrolysis increases the overall lipid recovery, the process may still be of industrial interest. The rest raw material contains significant amount of lipids (18–25 %, Table 3) and removing 38 % before hydrolysis will substantially reduce the raw material volume for hydrolysis. Studies on thermal separation of oil from marine raw materials have also shown that the pre-separated oil has higher stability and less oxidation [22–24].

3.4.4.2. Development of emulsion yield. The amount of emulsion appeared to depend more on the raw material than hydrolysis time for most hydrolyses. Bone material generally produced a lower emulsion yield than viscera. Bone materials also tended towards having a slightly lower hydrolysate yield and may have generated less peptides with emulsifying abilities. Untreated viscera and thermally separated viscera both had high hydrolysate and high emulsion yields. Conversely, pre-inactivated viscera had low emulsion yields. Thermally treated cod rest raw material was also found to produce less emulsion compared to untreated raw material [17]. The thermal stress of the inactivation may cause protein-lipid complexes, which can resist enzymatic hydrolysis and end up in the sediment or emulsion layer between the oil and hydrolysate, or cause changes within the protein structures resulting in loss of emulsifying capacity [23]. Some samples have high standard deviations (Figs. 4–7) probably due to mechanical difficulties in separating the emulsion from the hydrolysate and lipid phases.

3.5. Industrial relevance and implications

Viscera shows a higher proteolytic activity than bone materials, and the endogenous enzymes in both fractions are significantly more active at lower pH. This suggests that lowering the pH may improve the hydrolysis of the raw material, but will simultaneously require downstream handling to remove salts from the products, and the use of process equipment that tolerates low pH.

All of the different hydrolysis process designs investigated in this study did not necessarily result in statistically different product yields. This may be due to the low replication (n = 2), or that the differences between the chosen parameters are small. However, the differences in mean and standard deviations may still be industrially relevant and indicate the direction for further, in-depth research. The hydrolyses showed that different raw materials have different capacities for autolysis, and that bone material is more dependent on addition of commercial enzymes than viscera to reach high hydrolysate yields. In an industrial context, the enzyme should not only be adapted to the origin of the raw material, such as fish or chicken, but also the specific fraction to be hydrolyzed. Pre-inactivation of endogenous enzymes in viscera limited the development of hydrolysate yield throughout the hydrolyses, also when adding commercial enzymes. This suggests that thermal inactivation caused irreversible changes in the raw material. The necessity for pre-inactivation should be considered in each specific case and should be performed carefully in order to avoid preventing further hydrolysis.

Adding less water to the hydrolyses influenced the yields of the different hydrolysis products. Water represents a large cost industrially, for heating and subsequent removing downstream. For the industry, this means that the optimal water addition for the specific raw material needs to be studied in detail. This may have a large influence on the total flow through the process system as many hydrolysis industries operate with high or varying water additions to accommodate proper pumping or flow. Reducing water addition may therefore have a large impact on overall processing capacity and cost, and the dimensions of downstream process equipment such as evaporators and driers.

The hydrolyses in this study also showed that hydrolysate only represents 10–25 % of the overall dry matter of the products (Fig. 8). This means that most of the product fractions are other products, such as sediment, lipid, and emulsion. In order to industrially investigate the viability and the optimal process conditions, the quality needs and potential markets for these other products should also be investigated.

4. Conclusion

This study shows that different chicken rest raw material fractions have different proximate compositions, proteolytic activities, and susceptibilities for solubilization and hydrolysis. Industrially relevant process design variations such as enzyme choice, pre-treatment, water addition, and hydrolysis time affect the product yields. The autolytic capacity of the raw material could be utilized and explored alongside addition of commercial enzymes, as viscera showed high proteolytic activity at 50–60 °C at a natural pH, and even higher at pH 3. The proteins liberated into solution during heating of raw material to hydrolysis temperature represented 20–50 % of the total hydrolysate yield increase during the whole process. Heating time and temperature are powerful tools in solubilizing material and creating high hydrolysate yields and could be further optimized to improve process control. Viscera and bone materials responded differently to addition of commercial enzymes, highlighting that enzymes should be chosen based on the specific raw material, its composition and desired products. Thermal pre-inactivation may cause irreversible changes in the raw material resulting in lower yields, and the necessity of this process step should be evaluated by considering the product specifications needs and yields. Water addition influences solubilization of the raw material and should be optimized to increase yields and reduce processing costs. Improving the overall utilization of all fractions generated from chicken production will increase the sustainability of the meat production sector. Therefore, the yields of all hydrolysis products should be evaluated as potentially valuable products for the food or feed industry.

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CRediT authorship contribution statement

Kathrine Kjos Five: Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Ingvild Fålun**: Writing – review & editing, Methodology, Investigation, Formal analysis. **Gabriel J. Roland**: Writing – review & editing, Investigation, Formal analysis. **Daniel Forshaug**: Writing – review & editing, Investigation, Formal analysis. **Martin-Kristofer Helgeland-Rossavik**: Writing – review & editing, Investigation, Formal analysis. **Ragnhild Hals**: Writing – review & editing, Investigation, Formal analysis. **Ingrid Schafroth Sandbakken**: Writing – review & editing, Formal analysis. **Turid Rustad**: Writing – review & editing, Supervision, Methodology, Conceptualization.

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.

Data availability

Data will be made available on request.

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