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Comparison of pig and chicken pepsins for protein hydrolysis

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Abstract — The aim of the present study was to compare the degree of proteolysis with pig (PP) and chicken (CP) pepsins in order to find out whether PP can be used instead of CP to simulate gastric hydrolysis in the chicken. First, the pH activity profile of the two pepsins was compared using three substrates. For haemoglobin, CP showed a slightly higher optimal pH than PP, 2.5–3 and 2, respectively. For two plant protein sources (peas, wheat), the optimal pH was similar for the two enzymes, about pH 1.5. For the three substrates tested, CP exhibited a high level of activity over a broader pH range than PP. Second, the susceptibility of the two plant proteins to hydrolysis by each of the two pepsins was studied at pH levels near the chicken gastric pH (1.5–3.5). For PP, pea proteins were hydrolysed more than wheat ones, while, for CP, the hydrolysis was dependent on pH. Therefore, the classification of the two studied protein sources was dependent on the enzyme species and pH. The results of this study show that the choice of in vitro hydrolysis conditions to assess the digestibility of proteins must be made with great care. © Inra/Elsevier, Paris.

in vitro protein hydrolysis / pepsin / pig / chicken

Résumé — Comparaison de l'aptitude des pepsines de porc et de poulet à hydrolyser les protéines. L'objectif de cette étude a été de comparer le degré de protéolyse par les pepsines de porc (PP) et de poulet (CP) pour savoir si la PP peut être utilisée à la place de la CP pour simuler l'hydrolyse gastrique chez le poulet. Dans un premier temps, le profil d'activité en fonction du pH de ces deux protéases a été comparé sur trois substrats. Sur l'hémoglobine, le pH optimal de la CP s'est avéré légèrement plus élevé que pour la PP, 2,5–3 et 2 respectivement. Avec deux sources de protéines végétales (pois, blé), le pH optimal d'hydrolyse s'est révélé similaire, soit pH 1,5. Avec les trois substrats, la CP présente une hydrolyse importante sur une plus large gamme de pH que la PP. Dans un deuxième temps, l'hydrolyse des deux protéines végétales par chacune des deux pepsines a été étudiée à des pH voisins de ceux rencontrés dans le gésier de poulet (1,5–3,5). Alors qu'avec la PP les protéines de pois ont été mieux hydrolysées que celles du blé, dans le cas de la CP le résultat dépend du pH. La classification des deux sources de protéines étudiées dépend donc de l'origine animale de

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l'enzyme utilisée, et du pH. Les résultats de cette étude montrent que les méthodes de prédiction de la digestibilité des protéines par hydrolyse in vitro doivent porter une attention particulière quant au choix des conditions d'hydrolyse utilisées. © Inra/Elsevier, Paris.

hydrolyse in vitro de protéines / pepsine / porc / poulet

1. INTRODUCTION

In order to formulate diets for animal nutrition, it is necessary to know the amino acid digestibility of feeds. Tables of amino acid digestibility coefficients for a wide variety of common feedstuffs have been published [24, 25]. There is, however, a great variation among different samples of the same feedstuff. Thus, in vitro assays are needed in order to predict the protein digestibility of feed ingredients. Numerous studies have been conducted for this purpose.

In most cases, the conditions used, such as temperature, pH and time, for predicting digestibility are those met in the digestive tracts of mammals and not in birds [9, 11, 26]. But these two phyla show differences in digestive physiology. Body temperature is slightly higher in the chicken, about 40 °C instead of 37 °C in pig. The pH values along the digestive tract are not the same [5, 15, 21] and depending on the pH, proteins have various structures, which can lead to various susceptibilities of hydrolysis. The transit time to the end of the small intestine is shorter in chickens, about 4 h for soluble compounds and 6 h for insoluble compounds [32], than in pigs, about 5 h 30 min for soluble compounds and 14 h 30 min for insoluble compounds [7]. Moreover, the characterization of avian enzymes reveals differences with respect to activities and inhibitor sensitivities as compared to mammalian enzymes [3, 17–19, 28].

Among the various one-enzyme or multienzyme in vitro methods, the pepsin hydrolysis assay seems to be acceptable as a rapid test to predict animal protein quality in vivo [2, 26]. It is also used with plant proteins [11, 16]. But, porcine pepsin (composed mainly of pepsin A) is used whatever the species despite some studies have shown that pepsins from different species show different enzymatic properties [3, 22, 28]. These studies are not taken into account probably because of some discrepancies in the literature. For example, according to some studies, avian pepsins (probably only one species in the chicken, named pepsin A [18]) seem to show optimum activities for haemoglobin at pH values near 3 as compared with 2 for pig pepsins [3, 33, 35]. In contrast, Levchuk and Orekhovich [22] and Pletschke et al. [28] observed similar optimum pH values for haemoglobin with avian and porcine pepsins. Thus, more studies are needed to clarify this controversy. Another matter of consideration is the pH of the stomach, which varies according to the species and feed intake: in chickens fasted or fed commercial feed ad libitum, the gizzard pH is about 2.5 [12, 15] and in the pig stomach the pH after a meal is between 1.8 and 5.8 [21].

The present work was undertaken to compare the pH activity profile of pig and chicken pepsins using haemoglobin as a typical protease substrate [1]. Moreover, contrary to previous studies which used simple protein sources (casein or haemoglobin) or synthetic compounds as substrates, we studied pepsin hydrolysis at various pH levels using plant proteins commonly used in monogastric feeding (peas and wheat). These two protein sources were chosen for their very different protein composition. Whereas legumes are composed mainly of albumins and globulins, cereals are com-

posed of gliadins and glutenins. The consequences of the results observed to assess protein digestibility by in vitro hydrolysis are discussed.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals used were of analytical grade. Sodium pentobarbital was obtained from Sanofi (Marne-la-Coquette, France). Tris sodium acetate, NaOH, potassium cyanide and ninhydrin were obtained from Merck (Nogent-sur-Marne, France). Ammonium sulphate, ammonium hydrogenocarbonate and sodium acetate salts were obtained from Prolabo (Fontenay-sous-bois, France). Acetic acid and 2-methoxyethanol were provided by Prolabo. Trichloroacetic acid (TCA) was supplied by Sigma (Saint-Quentin-Fallavier, France), as well as haemoglobin (H-2625) and bovine serum albumin (A-4503). Pepsin from porcine gastric mucosa was obtained from Merck (7190).

2.2. Plant protein sources

Pea concentrate, obtained by air classification, and vital wheat gluten were kindly supplied by GEMEF and Roquette, respectively. They were in the form of fine powders.

The protein contents of these two products were estimated from the nitrogen content determined by the Kjeldahl method. The nitrogen to protein conversion factor used was 6.25.

2.3. Biological materials

Glandular stomachs were obtained from broiler chickens (*Gallus gallus*, strain JV 915 and white Ross) of 24 days of age. Birds were fasted in order to obtain a higher pepsin activity in the final product [3]. After overnight fasting, the birds were killed with an intracardiac injection of sodium pentobarbital (1 mL per bird). Proventricula were recovered immediately after death. Visible fat was removed but the mucosae were too fragile to strip. The organs were washed with cold distilled water and were kept on ice in plastic boxes for 30 min maximum and frozen at –20 °C where they were stored until required.

2.4. Preparation of chicken pepsinogen

Crude extract of chicken pepsinogen was obtained using the stepwise salting-out methods of Yasugi and Mizuno [35] and Pichova and Kostka [27] with some modifications. Because the mucosae were too fragile to strip, the whole proventricula were used as the source of pepsinogen. All subsequent steps were performed from 0 to 4 °C. Frozen glandular stomachs (80 g) were cut into pieces and were homogenized with a mixer (Ultraturrax IKA) in 160 mL of 0.4 M Tris acetate buffer pH 8.6 in a 250 mL beaker, in ice. The homogenate was centrifuged at 11 000 *g* for 30 min at 4 °C, and the zymogen was salted-out stepwise from the supernatant by saturated ammonium sulphate solutions at 25, 45 and 80 %. After centrifugation at 11 000 *g* for 10 min, the precipitates from the 2nd and 3rd salting-out steps were pooled and dissolved in 80 mL of 10 mM NH_4HCO_3 at pH 7. The solution was dialysed against the dissolving buffer at 4 °C for 48 h with two changes of buffer per day. The solution was then freeze-dried and stored at –20 °C.

2.5. Activity of pepsins on haemoglobin at various pH levels

Pepsin activity was determined according to the method of Anson [1] with some modifications. Hydrolyses were performed using 2.5 mL of acid-denatured haemoglobin (2 %) in 10 mL tubes. The pH of the reaction mixtures was adjusted to a range of pH levels from 1 to 5, by steps of 0.5, with 0.1 M NaOH or 1 M HCl without the use of a buffer [3] and the volumes were adjusted so that each tube would be identical. The volume of the required acid or base was determined in a preliminary experiment. Just before use, the enzymatic solution was prepared in 0.01 M HCl, and for the chicken pepsinogen, the solution was let to stand at room temperature for 10 min to activate the zymogen [3, 27, 35]. The enzymatic solutions were neutralized with 0.01 M NaOH and diluted with water. The final pH values of these solutions were about 5 and never over 6.5 because of the inactivation of pig and chicken pepsins at pH values over 6–6.5 and 7.5–8, respectively [3]. Enzymatic solutions (0.5 mL, 120 $\mu g \cdot mL^{-1}$) were added to haemoglobin solutions (final weight ratio E/S: 1/820) and the final pH values were controlled. The hydrolyses were allowed to proceed for 10 min in a thermostatically controlled water

bath at 40 °C, which corresponds to the body temperature of the chicken. The reaction was stopped by adding 5 mL of 10 % TCA (final concentration: 2.7 %), as in the standard assay, and let to stand at 4 °C. Blanks were made by adding TCA before the enzymatic solution. The TCA precipitate was discarded after centrifugation at 10 000 *g* for 10 min at 4 °C. The absorbance of the TCA-soluble products was measured at 280 nm. It was assured that the absorbance differences at 280 nm between the test and the blank samples were inferior to 0.8, for the linearity of the assay [4]. The activity for each pH was calculated as follows: one proteolytic unit was defined as the amount of enzyme which produced an increase in absorbance at 280 nm of 0.001 per minute under the assay conditions. For the pH activity profile, it was expressed as a percentage of the maximum activity of each enzyme. Each hydrolysis at one pH was performed in triplicate.

2.6. Colorimetric protein assay

The determination of protein content in solution was performed according to a modified method of Landry and Delhaye [20]. This assay avoids the underestimation of compounds of low molecular weight. Fifty microlitres of 8 M NaOH were added to 150 µL of the sample in polypropylene threaded tubes (2 mL) with installed O-ring screwcaps provided by Starsted (Nümbrecht, Germany). They were tightly sealed then heated to 130 °C (± 0.5 °C) in a dry block heater (Pierce) for 120 min. After cooling, they were centrifuged (3 000 *g*, 3 min) to spin down the water condensed at the cap level. The following was added to each of a series of tubes: 100 µL 40 % (v/v) acetic acid, 400 µL 60 % (v/v) 2-methoxyethanol and 150 µL 0.2 mM potassium cyanide in acetate Rosen buffer [31] diluted two times. The potassium cyanide solution prepared from a stock solution of 0.01 M. Ninhydrin (150 µL of a 3 % (p/v) solution in 2-methoxyethanol) was also added. Each addition was followed by vortexing. Then the tubes were sealed and heated for 15 min at 100 °C in a dry block heater. After cooling at room temperature and waiting less than 1 h after the end of heating, the absorbance of the solution was measured at 570 nm. A blank was prepared by solubilizing the sample medium and reagents only. Bovine serum albumin (in a range 0–50 $\mu g \cdot mL^{-1}$) was used as a standard protein.

2.7. Pepsin hydrolyses of proteins from peas and wheat, at various pH levels

Hydrolyses were performed on protein suspensions. One hundred millilitres of erlenmeyer containing magnetic stirring bars (350 rpm) were used. To each erlenmeyer, 20 mL of 0.01 M HCl were added. They were incubated in a thermostatically controlled water bath (40 °C) and closed with rubber plastic. When the correct temperature was reached, an equivalent of about 200 mg of proteins (weighed with an accuracy of 0.1 mg) was added. Pea concentrate or wheat gluten were suspended for 10 min and the pH was adjusted as described above (see section 2.5). The enzymatic solution (4 mL, 0.6 $mg \cdot mL^{-1}$) was prepared as described above (see section 2.5) and added to the protein suspension (final weight ratio E/S: 1/80). Then, the hydrolyses were allowed to proceed for 10 min at 40 °C. The reactions were stopped by adding TCA to a final concentration of 10 % instead of 2.7 %, as previously used for haemoglobin because of an inefficiency of low TCA concentration in precipitating the proteins tested, especially pea proteins. Blanks were prepared by adding TCA before the enzymatic solution. The samples were let to stand at 4 °C. The TCA precipitates were discarded after centrifugation at 10 000 *g* for 10 min at 4 °C. The absorbance at 280 nm could not be used because of substances interfering particularly with the pea concentrate (nucleic acids, polar pigments such as flavonoïds, saponins, vitamins). TCA-soluble products were measured by the protein assay described above (see section 2.6) after dilution with distilled water to 1/20 and 1/10 for pea concentrates and wheat gluten, respectively (TCA final concentration of 0.5 and 1 %, respectively). The degree of hydrolysis was measured by liberated TCA-soluble products. Data were expressed as the percentage of maximum hydrolysis. Each hydrolysis at one particular pH was performed in duplicate.

2.8. Protein solubility in acid conditions of plant proteins

An equivalent of about 200 mg of protein was suspended in 22.5 mL of HCl, pH 1.5, 2.5 or 3.5, in 100 mL erlenmeyer and was incubated at 40 °C. The mixtures were homogenized for 10 min by magnetic stirring at 350 rpm. The pH values were adjusted with HCl. The samples

were centrifuged for 10 min at 9 000 *g* at ambiant temperature and the supernatants were assayed as described above, after a 1/50 dilution. Data were expressed as milligrams of solubilized protein per 100 mg of initial protein.

2.9. In vitro pepsin hydrolyses kinetic profiles of proteins from peas and wheat

An equivalent of about 200 mg of proteins, weighed to an accuracy of 0.1 mg, were incubated at 40 °C in a water-bath, in a 100 mL erlenmeyer with 22.5 mL of HCl pH 1.5, 2.5 or 3.5. The mixtures were homogenized for 10 min by magnetic stirring at 350 rpm. The pH values were adjusted with HCl as described before and 2.5 mL of porcine or chicken pepsin/HCl (550 U·mL^{-1}, see section 2.5) were added (final weight ratio E/S was about 1/80 as for pH hydrolysis profiles). For each pH, the hydrolysis kinetics were performed for 60 min. Moreover, other hydrolyses for 20 and 50 min were also performed at each pH, in triplicate. Prior to the addition of the enzyme (time 0) and at various time intervals thereafter, 400 µL samples of the suspension were removed in duplicate from the erlenmeyer and the reaction was stopped by precipitating proteins in 10 % final TCA. Samples were let to stand at 4 °C and centrifuged for 10 min at 9 000 *g*. The supernatants were assayed as described above, after a 1/50 dilution. Data were expressed as milligrams of TCA-soluble oligopeptides liberated per 100 mg of initial protein.

2.10. Statistical analysis

The results were analysed by one-way and two-way analysis of variance using Statview software. The statistical significance of differences between means was evaluated using the Scheffe F Test.

3. RESULTS

The protein contents (N × 6.25) of pea concentrate and wheat gluten were 52.3 and 85.5 % of dry matter, respectively.

3.1. Crude extract of chicken pepsinogen

Crude extract (3.46 g) of chicken pepsinogen was obtained from 80 g of proventriculus. The results of the enzymatic activity assay on haemoglobin at the optimum pH of chicken pepsin after filtration on a 0.22 µm membrane was 665 U·mg^{-1} (see section 2.5.). Porcine pepsin preparation obtained by Merck had an activity of 550 U·mg^{-1} at the optimum pH determined in our study.

3.2. Optimum pH of the two pepsins

Activities of pepsins on haemoglobin at various pH, expressed in percentage of activity at the optimum pH, are shown in *figure 1*. The optimum pH level of the activity

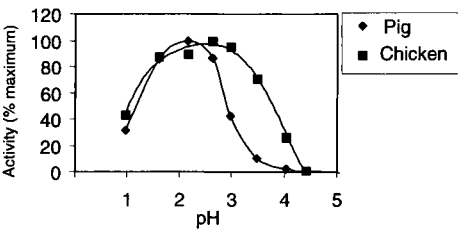

Figure 1. pH activity profiles of pig and chicken pepsins with haemoglobin as the substrate. Hydrolysis was performed on 2.5 mL of acid-denatured haemoglobin (2 %). The pH of the reaction mixture was adjusted to a pH range 1–5, by steps of 0.5, with 0.1 M NaOH or 1 M HCl, and the final volume was adjusted. Enzymatic solutions (0.5 mL, 120 µg·mL^{-1}) were added to haemoglobin solutions (final weight ratio E/S: 1/820) and the final pH values were controlled. Hydrolyses were allowed to proceed for 10 min at 40 °C. The reactions were stopped by adding TCA to a final concentration of 2.7 %. Blanks were prepared by adding TCA before the enzymatic solution. Absorbances of the TCA-soluble products were measured at 280 nm. The results are expressed as the percentage of the maximum activity (see section 2.5). Each hydrolysis at one pH was performed in triplicate.

Table I. Optimum pH of pig and chicken pepsins on various substrates. For details, see *figures 1* and *2*.

Substrates	Pepsins	
	Pig	Chicken
Haemoglobin	2	2.5–3
Pea concentrate	1–1.5	1–1.5
Wheat gluten	1.5	1.5

was slightly lower for pig pepsin than for chicken pepsin, 2 and 2.5–3, respectively (*table I*). The activity of chicken pepsin was less sensitive to pH change than that of the pig pepsin: the activity remained above 40 % from pH 1.0 to 3.9 for chicken pepsin, while this was observed in a more narrow range of pH (1.1–3.0) for the pig pepsin.

Pepsin hydrolyses of pea concentrate and wheat gluten at various pH are shown in *figure 2*. The optimum pH level of hydrolysis was lower with these two protein sources when compared to haemoglobin. Moreover, it was similar for pig and chicken pepsins, pH 1–1.5 for pea concentrate and pH 1.5 for wheat gluten (*table I*). As with haemoglobin, however, it was also observed that chicken pepsin was less sensitive to pH change than pig pepsin.

3.3. Protein solubility in acid conditions

Protein solubility at pH 1.5–3.5, measured by amounts of soluble nitrogen compounds in these media and expressed as the percentage of total protein, are presented in

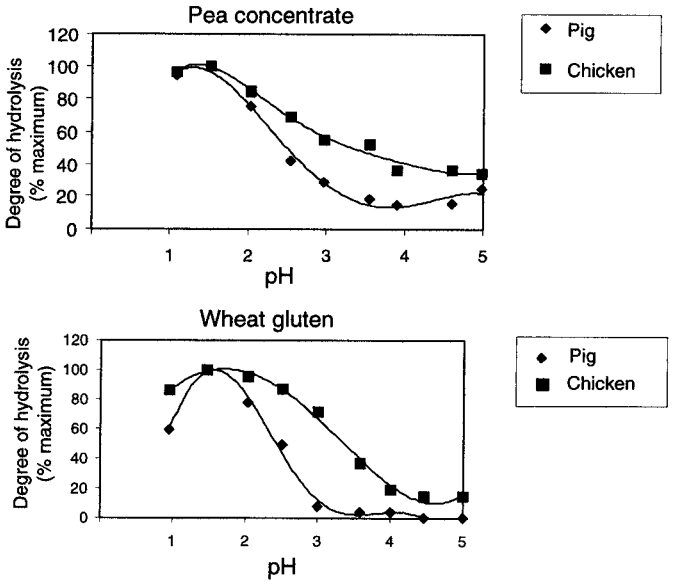

Figure 2. pH activity profiles of pig and chicken pepsins with vegetable protein sources as substrates (pea concentrate, wheat gluten). The hydrolyses were performed on protein suspension, on an equivalent of about 200 mg of protein in 20 mL 0.01 M HCl. After the adjustment of the pH, the enzymatic solution (4 mL, 0.6 mg·mL^{-1}) was added (final weight ratio E/S: 1/80). Hydrolysis was allowed to proceed for 10 min at 40 °C. The reaction was stopped by adding TCA to a final concentration of 10 %. Blanks were prepared by adding TCA before the enzymatic solution. TCA-soluble products were measured by performing a colorimetric assay of protein as described in the text (see section 2.6). The degree of hydrolysis was measured by liberated TCA-soluble products and expressed as the percentage of maximum hydrolysis. Each hydrolysis at one pH was performed in triplicate.

Table II. Protein solubility (%) of pea concentrate and gluten proteins in acid conditions.

pH	Pea concentrate	Wheat gluten
1.5	33.5 ± 0.08 c	33.0 ± 0.05 c
2.5	30.4 ± 0.03 b	33.0 ± 0.02 c
3.5	10.0 ± 0.29 a	33.0 ± 0.08 c

An equivalent of about 200 mg of proteins was suspended in 22.5 mL of HCl, pH 1.5, 2.5 or 3.5, and incubated at 40 °C. pH was adjusted with HCl. After centrifugation, the supernatants were assayed by the colorimetric protein assay as described in the text (see section 2.6). Data were expressed as milligrams of solubilized protein per 100 mg of initial protein. The values are means of duplicate (mean ± SE, $n = 3$).
Values with different letters are significantly different ($P < 0.05$).

table II. For pea concentrate proteins, solubility was different with different pH. It decreased as the pH increased, with a sharp decrease between pH 2.5 and 3.5, from 30.4 to only 10.0 % of soluble compounds. For wheat gluten, protein solubility is similar whatever the pH of the suspension (33.0 %).

3.4. Pepsin hydrolysis at various acid pH of the two plant proteins (pea concentrate and wheat gluten)

The percentage of low molecular weight compounds liberated from pea or wheat proteins during hydrolysis with pig or chicken pepsin at pH 1.5–3.5 are represented in *figure* 3. At the optimum pH (1.5) these time course hydrolysis profiles showed fast hydrolysis during the first 10 min, followed by slow hydrolysis until 60 min. At pH 2.5 and 3.5, a slow hydrolysis was observed.

Considering triplicate determinations of pepsin hydrolyses at 20 and 50 min, the differences of degree of hydrolysis were statistically analysed (*table III*). With porcine pepsin, pea proteins were more hydrolysed than wheat proteins whatever the pH values. On the contrary, with chicken pepsin, the order of the hydrolysis of the two proteins was dependent on pH. At pH 1.5, the degree of hydrolysis was similar. At pH 2.5, pea proteins were less hydrolysed than wheat proteins. At pH 3.5, as with pig pepsin, pea proteins were hydrolysed more than wheat ones. The difference in the hydrolysis between the two protein sources was higher with pig pepsin than with chicken pepsin. For example, after 50 min of hydrolysis at pH 1.5, 2.5 and 3.5, differences between pea and wheat hydrolysis were 10.5, 12.7 and 9.9 for pig pepsin, respectively, instead of 0.0, 8.4 and 8.5 for chicken pepsin.

In this experiment, the effects of pepsin origin, type of proteins, pH and interactions could be statistically tested (*table IV*). The main effects that were observed were those of protein and pH. Many interactions were also observed, mainly between protein source and enzyme and between enzyme and pH. There was also an interaction between the three studied parameters, protein sources, enzymes and pH. According to the interactions, it appeared that the pepsin origin affected the results in different ways depending on pH and proteins.

4. DISCUSSION

Pepsinogen was extracted in an alkaline buffer in order to neutralize the acid of the gland, and not in acid or a neutral buffer in order to avoid the autolytic digestion of pepsinogen [18]. Acetone precipitation and the stepwise salting-out with saturated ammonium sulphate solutions are among the various precipitation procedures used to obtain pepsinogen from the supernatant of

the homogenate of glandular stomachs. We chose to use the stepwise salting-out of pepsinogen, because according to Pichova and Kostka [27], a loss of activity occurs during the precipitation of zymogen from the homogenate by acetone. The one step salting-out method of Yasugi and Mizuno [35] was not chosen because of contamina-

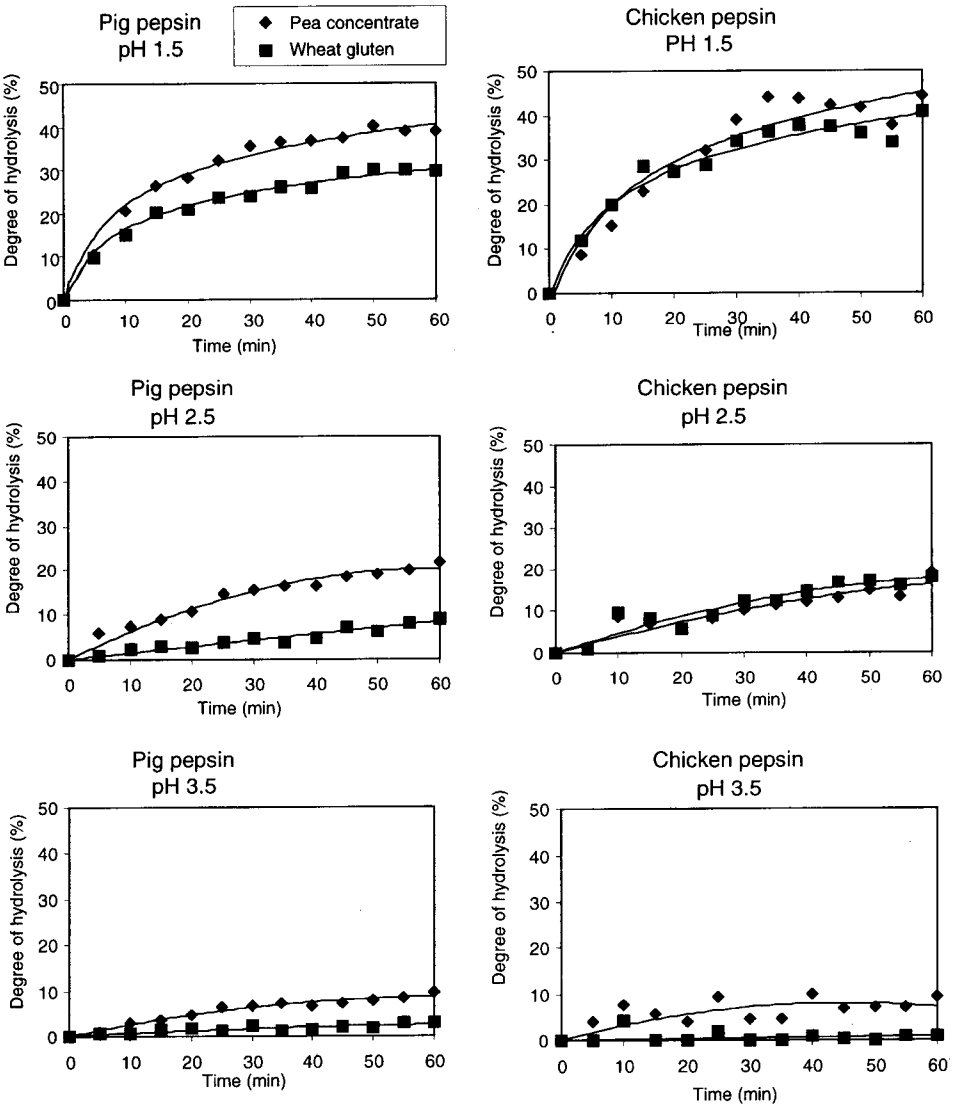

Figure 3. Hydrolysis kinetic profiles of proteins from peas and wheat with pig and chicken pepsins. An equivalent of about 200 mg of proteins was incubated at 40 °C in 22.5 mL of HCl pH 1.5, 2.5 or 3.5. The pH was adjusted with HCl and 2.5 mL of porcine or chicken pepsin/HCl (550 U·mL^{-1}) were added. The proteins were precipitated in 10 % final TCA at various intervals of incubation. TCA-soluble products were assayed by a colorimetric assay of protein as described in the text (see section 2.6). Data were expressed as milligrams of oligopeptides liberated per 100 mg of initial protein.

Table III. The degree of hydrolysis with pepsins in acid conditions, of proteins from peas and wheat (mean ± SE, $n = 3$).

Species	pH	Protein source	Hydrolysis time	
			20 min	50 min
Pig	1.5	pea concentrate	35.2 ± 2.07 b	50.2 ± 1.18 b
		wheat gluten	26.5 ± 0.52 a	39.7 ± 0.88 a
	2.5	pea concentrate	10.3 ± 0.94 b	19.6 ± 0.53 b
		wheat gluten	2.6 ± 0.63 a	6.9 ± 0.96 a
	3.5	pea concentrate	6.9 ± 0.64 b	12.5 ± 0.20 b
		wheat gluten	0.9 ± 0.93 a	2.6 ± 1.33 a
Chicken	1.5	pea concentrate	28.0 ± 1.70 a	45.5 ± 0.79 a
		wheat gluten	29.9 ± 1.22 a	45.5 ± 2.00 a
	2.5	pea concentrate	11.1 ± 0.82 a	18.6 ± 0.75 a
		wheat gluten	13.5 ± 0.22 b	27.0 ± 2.53 b
	3.5	pea concentrate	4.5 ± 0.43 b	9.3 ± 0.63 b
		wheat gluten	0.0 ± 0.00 a	0.8 ± 0.75 a

For details, see *figure* 3. Each hydrolysis was performed in triplicate.
Values in the same column with different letters (for the same species and pH values) are significantly different ($P < 0.05$).

tion of the preparation with large amounts of mucin-like materials and a loss of activity observed by Bohak [3]. Our results showed a final product with haemoglobin activity similar to that of porcine pepsin usually used in in vitro studies, 665 and 550 U·mg^{-1} for chicken and pig pepsins, respectively.

Some studies have shown that pepsins of various species, in particular mammals and birds, do not have the same enzymatic properties [3, 22, 28]. Concerning the controversy about the optimum pH of pepsins from avian species, our results confirmed those of studies that showed a higher optimum pH of pepsins in birds than in pigs [3, 33, 35]. These optimum pHs are defined for haemoglobin. The optimum pH of an enzyme depends, however, on its substrates, as do those of gastric proteases [28]. In our study, we observed that while the optimum pH was 2 and 2.5–3 for haemoglobin hydrolysis with porcine and chicken pepsins, respectively, they were lower for the two plant proteins tested. This optimum pH (1.5) was similar despite the pepsin and plant protein source. It is slightly lower than the mean pH (about 2.5 [12, 15]) found in the gizzard of broiler chickens fed a commercial feed, or in the stomach of the pig after a meal

Table IV. Effect of protein source, enzyme and pH on the degree of hydrolysis.

Effect (p)	Hydrolysis time	
	20 min	50 min
Protein source (PS)[1]	< 0.0001	< 0.0001
PH[2]	< 0.0001	< 0.0001
Enzyme (E)[3]	0.2003	0.0013
PS × E	< 0.0001	< 0.0001
E × pH	< 0.0001	< 0.0001
PS × pH	0.1897	0.0017
PS × E × pH	0.0059	< 0.0001

[1] Pea concentrate, wheat gluten.
[2] pH 1.5, 2.5, 3.5.
[3] Pig, chicken pepsins.

(between 1.8 and 5.8 [21]). Thus, it seems that in vivo pepsin hydrolysis does not proceed under optimal conditions.

It may be remarked that chicken pepsin exhibited a broader pH range for a higher activity than did porcine pepsin which had a more specific pH activity. This was particularly evident with wheat proteins (*figure 2*). Contamination of chicken pepsin with cellular enzymes was probably not at the origin of the larger pH activity range. A larger pH range of avian pepsin activity compared to pig pepsin was also observed with purified chicken [3] or ostrich [28] pepsins. Avian pepsins seem less sensitive to non-optimal pH values, especially the highest pH values, than the pig pepsin. These proteases seem to be more adapted to a large pH range in the stomach than pig pepsin. Although ad libitum feeding does not lead to an increase in gizzard pH in chickens [12], the pH value varies greatly between individuals, from 0.4 to 5.4 [15]. Moreover, in some cases such as in laying hens, a high variation of gizzard pH is observed because of calcium salts for shell formation [23]. Avian pepsins may be less sensitive to pH change because of their stability at higher pH values [3, 27, 28]. In contrast, pig pepsin has a narrow pH range, whereas the pig stomach shows an important pH variation due to its feeding by meal; an increase of 3 and even 4 pH units after a standard growing feed can be observed, followed by a decrease to reach the initial pH of about 1.8 [21]. An increase in pH is also observed in birds (such as geese) fed by tube feeding [8].

Use of the two pepsin preparations led to different degrees of hydrolysis of wheat and pea proteins. This confirmed the results of Keilova and Kostka [17] that showed different digestion products of the B-chain of oxidized insulin by chicken and pig pepsins.

The degree of hydrolysis depended on several factors (*table IV*). The protein sources were implicated because they have different protein compositions. Whereas legumes are composed mainly of albumins and globulins, cereals are composed of gliadins and glutenins. The different structures of these proteins may lead to different susceptibilities to hydrolysis. The pH effect was important because of a change of protein structure and enzyme activity with pH (*figures 1* and *2*). The pepsin source effect appeared only with long time hydrolysis (50 min) and not with short time hydrolysis (20 min) probably because the consequences of different enzymatic properties appeared only after long hydrolyses. Various interactions were shown in the degrees of hydrolysis (*table IV*). With short time hydrolysis no enzyme effect was observed; it did, however, occur in interaction with protein source and pH. The interaction between the protein source and the enzyme, is probably due to the specificity of the proteases for their substrates. Indeed, chicken pepsin is more specific than pig pepsin: in contrast with pig pepsin, avian pepsins are unable to cleave low molecular substrates [3, 10, 22, 28]. Chicken pepsin is more efficient in hydrolysing typical substrates such as haemoglobin and less active in clotting milk than pig pepsin [10, 22]. The interaction between the enzyme and pH is due to different pH profiles of each pepsin as observed in our study. The interaction between the protein source and pH appeared with long hydrolysis times. This is probably due to a change in protein structure with pH. The interaction between the three factors studied was due to the interactions explained above.

The comparison of the degrees of hydrolysis of the two protein sources showed more similar results with chicken pepsin than with porcine pepsin. It can be deduced that the chicken pepsin is less sensitive to changes in the environment such as pH or protein source. This would mean that chicken pepsin is more adapted to change than the pig pepsin. This adaptation may be due to shorter transit time in birds, which therefore need to have more efficient enzymes. Indeed, the mean transit time in the chicken gizzard is about 20 and 50 min for soluble and insoluble markers, respectively [32],

whereas it is about 3 and 13 h, respectively, in the pig stomach [7].

Solubility can be considered to influence protein hydrolysis: a higher solubility is supposed to increase hydrolysis by increasing the exposure of peptide bonds to enzymes. Indeed, Woodward and Carroll [34] observed that changes in the relative hydrolysis of casein and soya-bean proteins by pepsin for those pH values varying from 2.6 to 4.6 or by pancreatin from pH 5.6 to 7.6 paralleled the substrate solubility. They also observed that when these proteins were heated, both the hydrolysis and solubility decreased. In our study, the decrease in pea concentrate protein solubility with pH increase, which is particularly important between pH 2.5 and 3.5, is due to an isoelectric pH at about 4–4.5 [14]. Wheat gluten proteins have the same solubilities between pH 1.5 and 3.5, about 30 %, as they are principally composed of prolamins which are basic proteins with an isoelectric pH higher than 5 [29]. At pH 1.5, pea and wheat proteins have similar solubilities and classification of these two protein sources by hydrolysis depends on the enzymatic species. At pH 2.5, pea proteins were slightly less soluble than wheat ones, and the classification of these two protein sources was different between the two pepsins. At pH 3.5, pea proteins were markedly less soluble than wheat ones, and were better hydrolysed whatever the enzyme species used. Thus, even if solubility may be implied in protein hydrolysis, it is not the only factor involved. In the same way, numerous studies have shown no simple relationship between the susceptibility to hydrolysis and solubility [6, 30].

5. CONCLUSION

Studies which attempt to classify proteins with in vitro methods often use pig pepsin whatever animal is concerned. But proteases of different species have various enzymatic properties. With the two tested protein sources, erroneous classification is obtained if pig pepsin is used instead of chicken pepsin. In order to find out whether the observed results are due to particular structures of pea and wheat proteins or can be generalized, other protein sources must be tested. In practice, our results suggest that the conditions of in vitro hydrolysis used to assess the digestibility of proteins may be defined for each species as has already been done in fish [13]. The appropriate enzyme (derived from the species of interest) should be employed, and the pH of the digestive tract and digestion time should be taken into consideration.

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