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Purification and partial characterization of the pancreatic proteolytic enzymes trypsin, chymotrypsin and elastase from the chicken

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Abstract

The purpose of this work was to isolate, purify and partially sequence trypsin, chymotrypsin and elastase from the chicken pancreas. The extraction of the pancreatic zymogens with 0.5 *M* $CaCl_2$ at pH 7.5 for 9 h appeared to be most effective in obtaining maximum recovery of the three enzymes. The sequential *Cucurbita maxima* trypsin inhibitor I/bovine pancreas trypsin inhibitor/soybean trypsin inhibitor affinity chromatography gave the best result for the isolation of trypsin, chymotrypsin and elastase, respectively, from the same extract. For each proteinase, multiple form of enzymatic activity could be observed after gel electrophoresis and each form was further purified on an ion-exchange column. The N-terminal amino acid sequence of trypsin and chymotrypsin showed homologies with the bovine enzymes whereas elastase showed homologies with the porcine enzyme. The molecular mass of trypsin, chymotrypsin and elastase were estimated to be 23 500, 25 700 and 25 000, respectively, which are values close to those in mammalian species. Although some kinetic constants (K_m and k_{cat}/K_m) appeared different from those observed in other species, the pH dependent enzymatic activities were similar to those reported in other animal species. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Affinity chromatography; Enzymes; Trypsin; Chymotrypsin; Elastase

1. Introduction

The importance of chicken pancreatic proteolytic enzymes has been well recognized in studies on various vegetable components of the chicken diet which are rich in proteolytic enzymes inhibitors [1–4]. However, in spite of their importance for the nutritionists, very little is known about the nature of pancreatic proteolytic enzymes of avian species. Ryan [5] purified turkey trypsin and chicken chymotrypsin from an 0.125 *M* sulfuric acid extract of chicken and turkey pancreas after activation of the zymogens with either trypsin or intestinal extracts from the chicken. However, he failed to separate chymotrypsin and trypsin from turkey and chicken pancreas, respectively. Using extraction at alkaline pH, activation with enterokinase and trasylol-Sepharose affinity chromatography for purification, Hartley et al. [6] partially characterized a cationic trypsin from the ostrich pancreas. More recently, Smith et al. [7] isolated and partially characterized trypsinogen, and multiple forms of chymotrypsinogen and trypsin from the pancreas of the same species.

The present paper gives the results on the optimization of zymogen activation of chicken pancreatic homogenates under different conditions and the

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simultaneous purification of trypsin, chymotrypsin and elastase by serial affinity column chromatography on *Cucurbita maxima* inhibitor I (CMTI-I), bovine pancreas trypsin inhibitor (BPTI) and soybean trypsin inhibitor (STI) bound to Sepharose 4B followed by ion-exchange chromatography on a monodisperse cation-exchange column.

2. Experimental

2.1. Materials

Pancreas was removed from freshly killed four-week-old broiler chickens and stored frozen at −25°C until used. *N*-α-Benzoyl-DL-arginine *p*-nitroanilide (BA*p*NA), *N*-succinyl–Ala–Ala–Pro–Phe *p*-nitroanilide ($SucAla_2ProPheNA$), *N*-succinyl–Ala–Ala–Ala *p*-nitroanilide ($SucAla_3NA$), soybean trypsin inhibitor Kunitz type (STI), *p*-nitrophenyl *p*-guanidinobenzoate–HCl (NPGB), Sephadex G-75-120, the molecular mass (M_r) standard proteins (cytochrome *c* from horse heart, bovine trypsin, carbonic anhydrase from bovine erythrocytes, ovalbumin and bovine albumin) were all purchased from Sigma (St. Louis, MO. USA). Sepharose 4B was obtained from Pharmacia (Uppsala, Sweden), the BPTI from Bayer (Wuppertal, Germany) and CMTI-I as previously described [8]. All other reagents were of analytical grade.

2.2. Extraction and activation of proteolytic enzymes

Pancreatic tissues were thawed, cut in small pieces and homogenized for 5 min in a glass homogenizer with either three volumes of 0.5 *M* $CaCl_2$ containing 0.02% sodium azide or three volumes of ice-cold 0.125 *M* H_2SO_4. The calcium chloride homogenate was divided into four portions and the pH adjusted with 1 *M* acetic acid or 1 *M* Tris to 4.5, 5.5, 6.5 and 7.5, respectively. The pH was corrected three times during the first 2 h and remained constant thereafter. All portions of homogenate were left at room temperature (25°C) up to 27 h. At various times, small aliquots were taken, centrifuged at 13 000 *g* for 15 min and proteolytic activities in the supernatant were assayed. Usually, the activation process was continued until the proteolytic activities decreased.

The 0.125 *M* sulfuric acid homogenate was stirred for 2 h in an ice-bath and centrifuged at 15 000 *g* for 30 min. Solid ammonium sulfate was added to the pooled supernatant fractions to reach 0.5 saturation at 4°C. After 1 h, the precipitate was recovered by centrifugation, dissolved in distilled water, and dialyzed against distilled water for 24 h. The dialysate was brought to 50 m*M* $CaCl_2$ concentration and porcine enterokinase (0.25 mg/ml) added to facilitate zymogen activation.

2.3. Enzyme assays

All enzymatic activities were measured at 25°C in a final volume of 1 ml. Trypsin and chymotrypsin were assayed with the chromogenic substrates BA*p*NA [9] and $SucAla_2ProPheNA$ [10], respectively in 0.2 *M* Tris–HCl buffer, pH 8.0 containing 20 m*M* $CaCl_2$. Elastase activity was determined with $SucAla_3NA$ according to Bieth et al. [11].

2.4. Protein determination

The protein concentration was determined by the microbiuret method [12], bovine serum albumin being used as a standard.

2.5. Preparation of the columns for affinity chromatography

BPTI, STI and CMTI-1 were coupled to cyanogenbromide-activated Sepharose 4B according to March et al. [13]. The binding capacity of the immobilized inhibitors, when assayed with bovine trypsin, was found to be 5.7, 5.5 and 7.0 mg/ml for BPTI, STI and CMTI, respectively.

2.6. Affinity chromatography

Extract from 20 g of chicken pancreata, activated at pH 7.5 for 9 h, was adjusted to pH 8.0 with 1 *M* Tris and passed through a series of three affinity columns at a flow-rate of 0.5 ml/min. The size of the columns was such that the binding capacity of each exceeded by 40% the enzyme content in the extract. The first column used (CMTI-I-Sepharose 4B, 7×1.0

cm), was washed with 0.2 *M* Tris–HCl, pH 8.0 buffer and the flow-through kept for the next step. The column was further washed with 0.2 *M* Tris–HCl, pH 8.0 with 1 *M* NaCl until the absorption at A_{280} fell below 0.01. Bound trypsin was eluted with 0.001 *M* HCl. The flow-through from the CMTI column was immediately applied to a BPTI affinity column (10×1.5 cm). A similar procedure as described above for trypsin isolation was followed to wash and elute chymotrypsin from the column. The non-adsorbed material from the BPTI column was loaded onto the STI column (7.0×1.0 cm), washed at pH 8.0 and the elastase eluted with 0.001 *M* HCl.

2.7. Ion-exchange chromatography

The enzymes were further purified by fast protein liquid chromatography (FPLC) using a monodisperse cation-exchange column (Mono S HR 5/5, Pharmacia). Lyophilized trypsin, chymotrypsin and elastase were resuspended in formate buffer (50 m*M*, pH 3.0), acetate buffer (50 m*M*, pH 5.0) and acetate buffer (50 m*M*, pH 4.7), respectively. After equilibration and loading the samples, the column was eluted with NaCl gradient.

2.8. Gel electrophoresis

Electrophoresis was performed at pH 4.5 in 7.5% polyacrylamide gel according to Reisfield et al. [14]. The gels were stained with 0.2% Amido Black B and destained in 7% acetic acid or with silver staining [15]. Protein bands stained with Amido Black B were checked for activity by incubation of gels in 0.2 *M* Tris–HCl, pH 8.0 buffer with the appropriate synthetic substrates. Sodium dodecyl sulfate (SDS) electrophoresis was performed according to Wyckoff et al. [16] in gradient gel ranging from 8 to 20%.

2.9. Determination of active enzyme concentration

The concentration of active trypsin in the preparation was determined by active-site-titration with NPGB [17]. The standardized trypsin solution was used to titrate the human α_1 proteinase inhibitor which, in turn was used for determining the activity of purified chicken proteinase.

2.10. Determination of molecular mass

Molecular masses were determined by use of a Sephadex G-75-120 column equilibrated with 0.1 *M* acetic acid and by SDS–polyacrylamide gel electrophoresis (PAGE). The column (150×1.5 cm I.D.) was standardized with cytochrome *c* from horse heart (12 400), bovine trypsin (23 800), carbonic anhydrase from bovine erythrocytes (29 000), ovalbumin (45 000), bovine albumin (66 000) and edestin (300 000).

2.11. N-Terminal amino acid sequencing

N-Terminal sequence analysis of trypsin (three forms), chymotrypsin (one form) and elastase (two forms) were performed with an Applied Biosystem 470A gas-phase sequencer, using the program designed by the manufacturer. Cystein was derivatized to *S*-β-(4-pyridylethyl) cystein with 4-vinylpyridine [18].

2.12. Determination of kinetic constants

All tests were performed at 25°C, using 0.2 *M* Tris–HCl, pH 8.0 buffer, added with 20 m*M* $CaCl_2$ in the case of trypsin. K_m and k_{cat} values were calculated from an Eddie–Hofstee plot using a wide range of substrate concentrations. The best fit obtained by least-squares method.

2.13. Effects of pH on enzymatic activities

The activities of trypsin, chymotrypsin and elastase were determined spectrophotometrically as described above. The following 0.2 *M* buffers were used: acetate (pH 4.0–5.0), Tris–malate (pH 6.0–8.6), and carbonate (pH 9.0–11.2).

3. Results

3.1. Effect of pH and extraction time on the activation of enzymes

The autoactivation of trypsinogen and chymotrypsinogen appeared to give the best result at pH 7.5 (Fig. 1). Both enzymes reached maximum activity

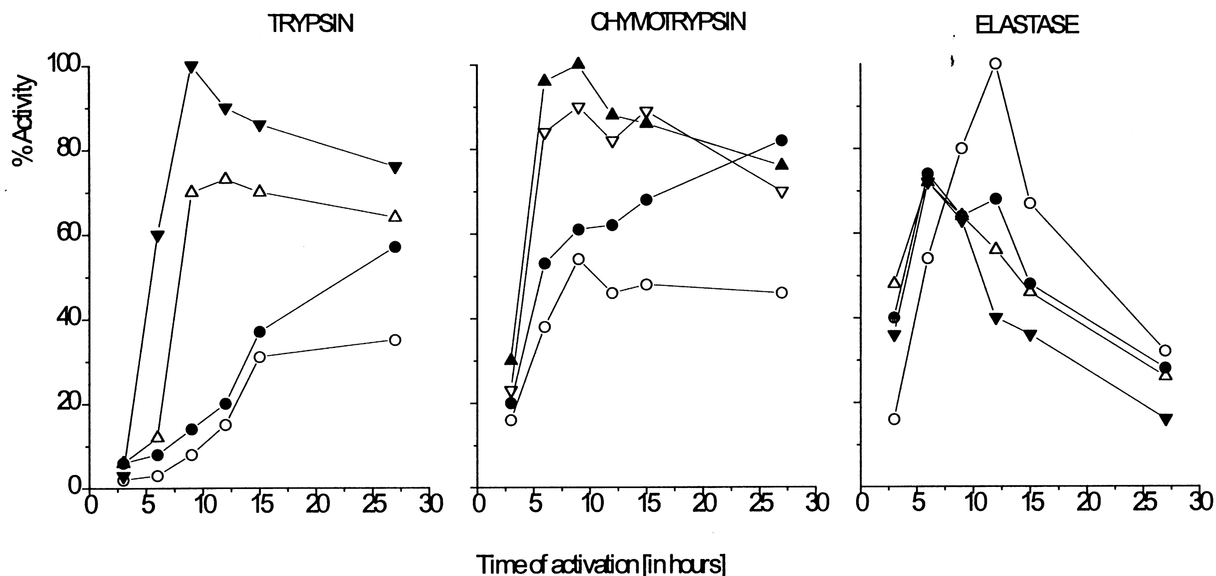

Fig. 1. Activation of zymogens: trypsinogen, chymotrypsinogen and proelastase at pH 4.5 (○), 5.5 (●), 6.5 (△) and 7.5 (▲).

after a 9-h incubation time. Thereafter, activity gradually decreased to about 75% after 27 h. At pH 6.5, the autoactivation proceeded similarly; however, the yield was lower by about 25% for trypsin and 10% for chymotrypsin. Under acidic conditions, the activation process proceeded much slower. After the same time of incubation, trypsin and chymotrypsin, respectively, reached only 10% and 55% of the activity detected at pH 7.5. Contrary to trypsinogen and chymotrypsinogen, the proelastase activation process was more effective under acidic conditions. The highest elastase activity was reached at pH 4.5 after 12 h of incubation. Afterwards, elastase was rapidly inactivated, losing about 70% of activity within the next 15 h. At pH 7.5, elastase activity increased slowly up to 65% activity after 9 h and decreased steadily thereafter.

0.125 *M* sulfuric acid is commonly used for the extraction of proteolytic enzymes from the pancreas. However, extracts of chicken pancreata obtained by this procedure had only 10% of the chymotrypsin activity found in 0.5 *M* $CaCl_2$ extracts and only traces of trypsin and elastase activity, even after a 24-h activation with enterokinase.

3.2. Separation of enzymes by affinity chromatography

The elution profiles of the enzymes originally bound to immobilized inhibitors but released after HCl treatment, were all similar, with a single symmetrical protein peak with coinciding activity being obtained in each case (not shown). All preparations were free of the other two potentially contaminating proteinases.

3.3. Electrophoresis after affinity chromatography

Fig. 2 presents the electrophoregram of purified trypsin, chymotrypsin (after activation under different conditions) and elastase activated at pH 7.5. All trypsin preparations consisted of two forms, an anionic and a cationic. After sulfuric acid extraction and activation with enterokinase, the trypsin preparation contained approximately the same amount of cationic and anionic form (Fig. 2b) whereas in the preparations after autoactivation at pH 7.5 and 5.5 (Fig. 2a and c), we noted three bands of trypsin, one predominant anionic and two cationic forms.

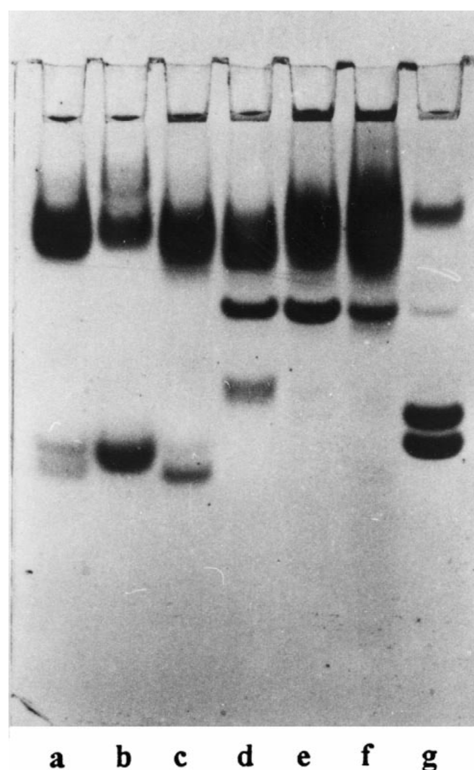

Fig. 2. Polyacrylamide gel electrophoresis at pH 4.5 of the chicken trypsin [(a) after autoactivation at pH 5.5, (b) separated from the debris after sulfuric acid extraction and activated with porcine enterokinase at pH 5.5, (c) after autoactivation at pH 7.5], chymotrypsin [(d) after autoactivation at pH 5.5, (e and f) after autoactivation at pH 7.5] and elastase [(g) autoactivated at pH 7.5].

Chymotrypsin preparations after autoactivation at either basic (Fig. 2e and f) or acid pH (Fig. 2d) consisted of two or three active forms. Elastase preparation showed two distinct active forms migrating close to each other under those conditions and two trace of inactive bands with slow mobility.

3.4. Separation of enzymes by ion-exchange chromatography

The elution profiles of trypsin, chymotrypsin and elastase from a Mono S column are presented in Fig. 3. For trypsin, three main peaks were observed at the NaCl concentration of 0.23 *M*, 0.25 *M* and 0.27 *M*, respectively. The first peak (T1) was corresponding to the anionic form of trypsin whereas the other two (T2 and T3) were the cationic forms. A part of chymotrypsin did not bind to the Mono S column and was recovered from the flow-through. The bound chymotrypsin resolved onto two smaller peaks eluted at 0.05 and 0.15 *M* NaCl and the main peak (C) eluted at 0.1 *M* NaCl concentration. Only peak C presented one single band on electrophoresis whereas the other peaks appeared to contain more than one enzyme form. The elution of elastase with increasing concentration of NaCl gave two well separated peaks (E1 and E2) at 0.11 *M* and 0.13 *M*, NaCl respectively. For trypsin and elastase, each peak corresponded to a pure form of the enzyme, as confirmed by electrophoresis at pH 4.5 and SDS–PAGE (not shown).

3.5. Molecular mass and enzyme concentrations

SDS–PAGE under reducing conditions revealed that except for T-3 preparation, all preparations after treatment with DFP migrated as a single band. The molecular masses were calculated to be 23 500, 24 000 and 25 000 for trypsin, chymotrypsin and elastase, respectively. Trypsin T-3 had a main band of M_r 16 500 and one minor band of about 7000. When the molecular masses of trypsin, chymotrypsin and elastase were estimated by Sephadex G-75 chromatography the values obtained were 24 500, 23 800 and 21 600, respectively. Active enzyme content in the lyophilized preparations was 85% for trypsin, 84% for chymotrypsin and 73% for elastase, with an overall recovery of over 70% for trypsin and chymotrypsin and 60% for elastase (Table 1).

3.6. N-Terminal amino acid sequences

For each form of trypsin, 23 to 24 residues were recovered (Fig. 4). The anionic form (T1) and one cationic form (T2) gave one N-terminal sequence each whereas two sequences were obtained from T3, one being identical to that of T-2 form. For chymotrypsin (C), two N-terminal amino acid sequences

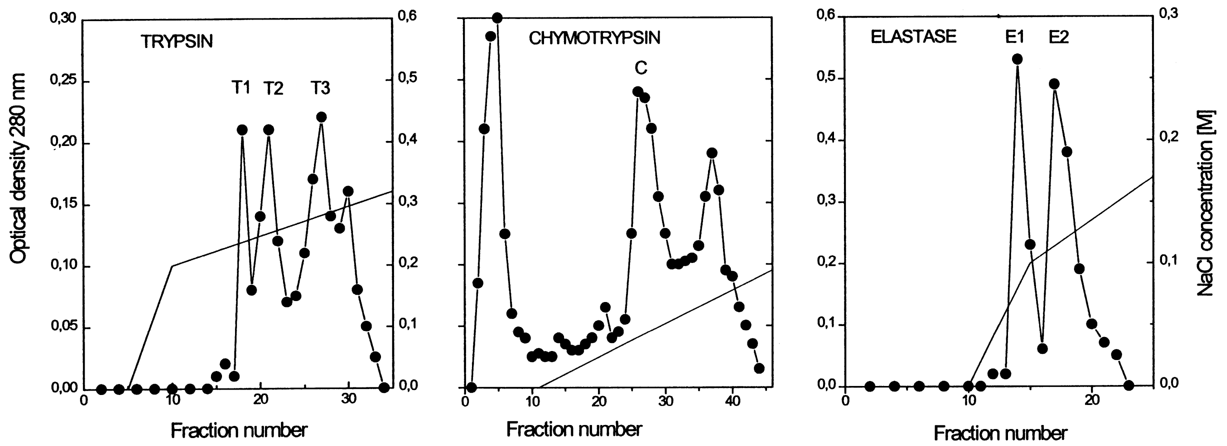

Fig. 3. Cation-exchange chromatography of trypsin, chymotrypsin and elastase on a Mono S FPLC column. Lyophilized trypsin, chymotrypsin and elastase obtained after affinity chromatography were dissolved in formate buffer (50 m*M*, pH 3.0), acetate buffer (50 m*M*, pH 5.0) and acetate buffer (50 m*M*, pH 4.7), respectively. After equilibration and loading the samples, the column was eluted with increasing gradient concentration NaCl.

Table 1
Recovery of proteolytic enzymes from 20 g of chicken pancreas

Enzyme	Activity (in units $\cdot 10^{-3}$)[a]		Yield (%)
	In extract	In lyophilized preparation	
Trypsin	20.68	15.10	73
Chymotrypsin	3410.00	2560.00	75
Elastase	27.10	16.80	62

[a] One unit=increase by 0.01/min of the optical density at 405 under used conditions.

were read, with 16 and 24 residues, respectively (Fig. 5). No differences in amino acid at the N-terminal were detected between the two forms of elastase up to the 23rd residue (Fig. 6).

3.7. Determination of kinetic constant

The Michaelis constants and the catalytic rate constants of trypsin, chymotrypsin and elastase for a

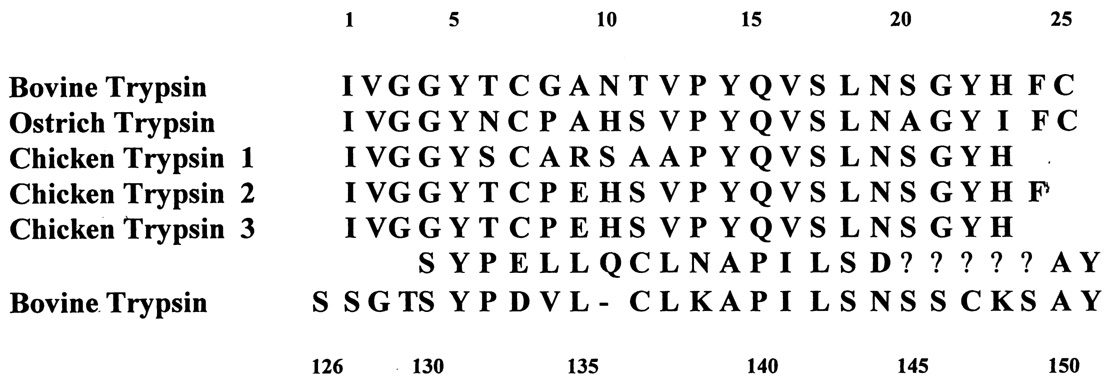

Fig. 4. N-Terminal amino acid sequence of the chicken trypsin T1, T2 and T3 as compared to the bovine trypsin and ostrich trypsin.

					1				5					10				↓			20					25					30					35				
BC					**C**	**G**	**V**	**P**	**A**	**I**	**Q**	**P**	**V**	**L**	**S**	**G**	**L**	**S**	**R**	**I**	**V**	**N**	**G**	**E**	**E**	**A**	**V**	**P**	**G**	**S**	**W**	**P**	**W**	**Q**	**V**	**S**	**L**	**Q**	**D**	**K**
CC	**S**	**L**	**P**	**S**	**C**	**G**	**V**	**P**	**A**	**I**	**T**	**P**	**V**	**I**	-	-	-	**R**	**R**	**I**	**V**	**N**	**G**	**E**	**P**	**A**	**V**	**P**	**G**	**S**	**W**	**P**	**W**	**Q**	**V**	**S**	**L**	**Q**	**Y**	**G**

BC: C G V P A I Q P V L S G L S R I V N G E E A V P G S WP WQ V S L Q D K T G F

CC: S L P S C G V P A I T P V I - - - R R I V N G E P A V P G S WP WQ V S L Q Y G N F F

Fig. 5. N-Terminal amino acid sequence of the chicken chymotrypsin as compared to the bovine chymotrypsin. BC=Bovine chymotrypsin; CC=chicken chymotrypsin.

	1				5					10					15					20					25
Porcine Elastase	**V**	**V**	**G**	**G**	**T**	**E**	**A**	**Q**	**R**	**N**	**S**	**W**	**P**	**S**	**Q**	**I**	**S**	**L**	**Q**	**Y**	**R**	**S**	**G**	**S**	**S**
Chicken Elastase 1	**V**	**V**	**G**	**G**	**T**	**E**	**A**	**K**	**T**	**H**	**A**	**W**	**P**	**S**	**Q**	**I**	**S**	**L**	**Q**	**Y**	**Y**	**S**	**G**		
Chicken Elastase 2	**V**	**V**	**G**	**G**	**T**	**E**	**A**	**K**	**T**	**H**	**A**	**W**	**P**	**S**	**Q**	**I**	**S**	**L**	**Q**	**Y**	**Y**	**S**	**G**	**G**	**S**

Fig. 6. N-Terminal amino acid sequence of the chicken elastases as compared to the porcine elastase.

specific substrate are summarized in Table 2. The kinetic constants showed that both cationic forms of the chicken trypsin are very similar and differ significantly from the anionic form, showing a K_m value three-times lower and a k_{cat}/K_m four-times higher than those of the anionic form.

3.8. Effects of pH on enzymatic activities

For the three trypsin forms, enzymatic activity was recorded above pH 5.0 and the maximum activity was observed at pH 8.0–8.2. The activity curve for chymotrypsin showed a similar pattern with a maximum activity between pH 8.6 and 10. With elastase, activity was noticed above pH 5.0 with a maximum activity at pH 8.6 and 8.2 for elastase 1 and elastase 2, respectively.

4. Discussion

It was already observed by Ryan [5] that a sulfuric acid extract of chicken pancreata was almost completely free of trypsin due to, as he suggested, the lability of its zymogen to acid. We found, however, that trypsinogen is not acid-soluble and could be

Table 2
Kinetic constants of the chicken trypsin, chymotrypsin and elastase as compared to those of other animal species

Enzyme	Species	Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	Ref.
Anionic trypsin	Chicken	BApNA	0.619	0.355	0.573	This paper
	Dog	BApNA	0.99	–	–	[24]
Cationic trypsin 1	Chicken	BApNA	0.197	0.473	2.401	This paper
Cationic trypsin 2	Chicken	BApNA	0.244	0.500	2.409	This paper
Cationic trypsin	Dog	BApNA	1.12	–	–	[24]
Trypsin	Carp	BApNA	0.039	3.10	79.49	[35]
	Bovine	BApNA	0.939	0.611	0.650	[9]
Chymotrypsin	Chicken	$SucAla_2ProPheNA$	0.109	44.2	405.5	This paper
	Bovine	$SucAla_2ProPheNA$	0.093	35.0	380.0	[10]
Elastase 1	Chicken	$SucAla_3NA$	3.149	7.69	2.44	This paper
Elastase 2	Chicken	$SucAla_3NA$	2.862	6.89	2.40	This paper
Elastase 1	Human	$SucAla_3NA$	1.40	0.55	0.39	[28]
Elastase 2	Human	$SucAla_3NA$	13.00	0.11	0.008	[28]
Elastase	Pig	$SucAla_3NA$	1.15	16.6	15.09	[11]
	Carp	$SucAla_3NA$	2.24	3.47	1.55	[35]

recovered from the debris by reextraction at basic or slightly acidic pH and porcine enterokinase was required for its activation. Chicken chymotrypsin was divided between the sulfuric acid-soluble fraction and the debris. However, the total activity was 80% lower than that recovered after extraction under neutral conditions. The highest trypsin and chymotrypsin activities were observed after extraction and autoactivation at pH 7.5 (Fig. 1). Under the same conditions, elastase had 65% of its maximal activity. However, in compliance with the aim of our work, i.e., the simultaneous isolation of trypsin, chymotrypsin and elastase, those conditions were chosen for activation. Similar results were reported with chicken trypsin and chymotrypsin [19] and with trypsin from the ostrich [6]. Affinity chromatography with bound inhibitors is widely used to purify proteolytic enzymes, notably from the pancreas. However, inhibitors like BPTI, STI, LBI are polyvalent and bind more than one single proteolytic enzyme, making the purification process more difficult. In our studies, we were able to separate highly purified trypsin, chymotrypsin and elastase by taking advantage of both the specificity of the inhibitors used and certain properties of chicken proteolytic enzymes. CMTI has been shown to bind only trypsin [8] and was therefore used in the first affinity column. This very selective inhibitor allowed us to obtain trypsin without destroying chymotrypsin. It is an advantage over the acid destruction of chymotrypsin described by some authors [6,20] to obtain pure trypsin. BPTI has a relatively broad inhibitory specificity, acting on trypsin, chymotrypsin, plasmin and kallikreins [21]. Although it is used to purify human leukocyte elastase [22], BPTI does not inhibit pancreatic elastase [23]. Therefore, after the BPTI affinity chromatography, only chymotrypsin was bound and eluted with 0.001 *M* HCl. STI is known to inhibit trypsin, chymotrypsin but not pancreatic elastase [23]. However, we found that chicken pancreatic elastase was inhibited by STI. Because of the particularity of this enzyme, we used a STI-affinity column for our final isolation step. According to our results, the sequential CMTI/BPTI/STI affinity chromatographies gave the best and fastest results in simultaneous isolation of trypsin, chymotrypsin and elastase from the chicken pancreas. The active-site titration showed that the activity of our preparations were similar to that reported for commercial products. Alike trypsin in dog [24], pig [25], rat [26] and human [27] chicken trypsin seems to have both cationic and anionic forms. Chicken chymotrypsin also consisted of two or three active forms depending on the activation conditions. Pancreatic elastase gave two close bands with elastolytic activity. A similar feature was previously reported for human pancreatic elastase [28]. The upper band appeared to be contaminated with a protein with neither elastase, trypsin nor chymotrypsin activity. Little information is published on molecular mass of pancreatic proteolytic enzymes in birds. With Sephadex G-75, the molecular masses for trypsin, chymotrypsin and elastase were found to be 24 500, 23 800 and 21 600, respectively. SDS–PAGE under reducing conditions gave higher values for elastase (25 000), lower for trypsin T-1 and T-2 (23 500). Trypsin T-3 consisted of a 16 500 main band and a 7000 minor band suggesting that this is a two-chain molecule. This was confirmed by the N-terminal amino acid sequence analysis. The M_r for trypsin was similar to that reported in ostrich (24 500) [6] and higher than that of the turkey (18 600) [29,30]. Chymotrypsin (C) M_r value obtained by SDS–PAGE (24 000) was close to that calculated by gel filtration chromatography. However, as shown by the N-terminal sequence analysis, this is a two-chain molecule with the shorter chain made of 16 amino acid residues. This fragment was missing in the computation of the M_r by SDS–PAGE and the 1700 corresponding to this fragment should be added, making our estimation for M_r of chymotrypsin (C) 25 700 similar to that reported by Ryan et al. [29]. No previous work on M_r of avian pancreatic elastase was reported. The M_r of the chicken pancreatic elastase appeared to be similar to that of the pig [31], carp [32] and human [28,33,34]. The N-terminal amino acid sequence analysis showed some homologies for trypsin and chymotrypsin with the bovine enzymes and for elastase with the porcine enzyme. For trypsin, the percentage of identity between the bovine enzyme and the chicken enzyme in the N-terminal portion was 74 and 83% for the anionic and cationic form, respectively. Moreover, the chicken cationic trypsin compared favorably (over 90% identity) with the ostrich trypsin. The cationic form T2 gave one N-terminal sequence whereas T3 gave two sequences, one of them identical to the N-terminal of trypsin T-2 and second one representing

virtually the complete sequence of the second chain of the molecule. This second sequence also presented a high homology (59%) with the portion of bovine α-trypsin from Ser130 to Tyr151. According to these data, we consider that T2 corresponds to the β form and T3 to the α form of the cationic trypsin. The N-terminal sequence of the chymotrypsin C gave also two sequences. The shorter chain corresponded to the first 15 amino acid residues of the bovine chymotrypsinogen, showing a 50% homology. It is worth noting that the chicken chymotrypsin differs from the bovine enzyme by a tetrapeptide (SLPS) which extends beyond cystein. The longer chain corresponded to the segment Ile16 to Gln34 of the bovine chymotrypsinogen, showing a 79% homology. The data indicated that we have purified and partially sequenced the π-like form of the chicken chymotrypsin. The N-terminal sequence of the chicken elastases showed high homologies with the porcine elastase, with 74% and 71% homologies for elastase 1 and elastase 2, respectively. The kinetic constants showed that both cationic forms of the chicken trypsin are very similar and differ significantly from the anionic form. The constants were also different from those reported in other species [9,24,35]. For chymotrypsin, the kinetic constants were comparable between chicken and bovine species [10]. Both forms of the chicken elastase had similar kinetic constants which differ markedly with the constants observed in other species [11,28,35]. The effects of pH on enzymatic activities revealed no major difference between the three forms of trypsin and the trypsins from other species [6,24,25,27,36]. The same observation was made for the two elastase forms [34,35]. Like chymotrypsins of other species [37], the chymotrypsin of the chicken showed a broader pH dependent activity with 80% activity or more between pH 7.4 and 11.0.

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