MULTIPLE MOLECULAR FORMS OF AVIAN ACROSIN: DIFFERENCES IN THEIR KINETIC PROPERTIES

Jenny J. L. HO* and Stanley MEIZEL**

Department of Human Anatomy, School of Medicine, University of California, Davis, California 95616

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1. Introduction

The trypsin-like enzyme acrosin (EC 3.4.21.10) has been reported to exist in the spermatozoa of several species [1]. Multiple molecular forms of bull acrosin [2.3], boar acrosin [4] and avian acrosin [5] have been previously demonstrated. This paper describes the partial purification and partial characterization of multiple forms of avian acrosin and presents the first data indicating differences between the catalytic properties of multiple molecular forms of an acrosin.

2. Materials and methods

2.1. *Materials*

Hyamine 2389 was a gift from Rohm and Haas Co. Amido Schwartz was obtained from Allied Chemicals. Benzamidine·HCl, acrylamide (for electrophoresis), *N,N*-dimethyl formamide and bromphenol blue were obtained from Eastman Kodak Co. Sigma Chemical Co. was the source of DEAE-Sephadex, 2-mercaptoethanol, Coomassie brilliant blue, egg albumin (ovalbumin, Grade V), leucine aminopeptidase (Type V), carboxypeptidase A (Type I), *p*-aminobenzamidine HCl, sodium dodecyl sulfate (SDS) and *N*-α-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA). Soybean trypsin inhibitor (5X crystallized), bovine serum albumin (crystalline), conalbumin (5X crystallized), *N*-α-*p*-tosyl-L-arginine methyl ester· HCl (TAME), *N*-α-benzoyl-L-arginine ethyl ester·HCl (BAEE) and *N*-α-benzoyl-D,L-arginine-β-naphthylamide·HCl (BANA) were obtained from Nutritional Biochemical Corp. Fox Chemical Co. was the source of *N*-α-carbobenzoxy-D, L-arginine-β-naphthylamide·HCl (CANA).

2.2. *Sperm homogenization*

Washed ejaculated spermatozoa from *Gallus domesticus* were used (Ho and Meizel, manuscript in preparation). The sperm extract was produced by freezing and thawing a 12% suspension (wet wt/vol) of spermatozoa five times in 0.05% hyamine (in 0.05 M Tris–HCl, pH 7.4 buffer). The suspension was then centrifuged at 30 000 *g* for 1 hr and dialyzed against the starting buffer of the ion exchange columns.

2.3. *Enzymatic assays*

BANA hydrolysis was measured at 25°C by a modification (Ho and Meizel, manuscript in preparation) of a fluorometric procedure [6]. The hydrolysis of BAPNA, TAME and BAEE was measured by spectrophotometric assays at 25°C [7–9]. Assays of the hydrolysis of all substrates were carried out in pH 8.0, 0.1 M Tris–HCl buffer. The assay mixture for BANA contained 0.05 M $CaCl_2$. The inhibitors, dissolved in dimethyl formamide, were added to the reaction mixture without pre-incubation with enzyme. A unit of enzymatic activity is that amount of enzyme which hydrolyzed 1 μmol of the substrate per min at 25°C. The Michaelis–Menten constant (K_M) and the inhibitor dissociation constant (K_i) determined for several substrates and inhibitors [10,11]. These K_M's and K_i's are apparent values.

* Present address: Department of Zoology, University of California, Berkeley, California 94720 USA.

** To whom reprint requests should be addressed.

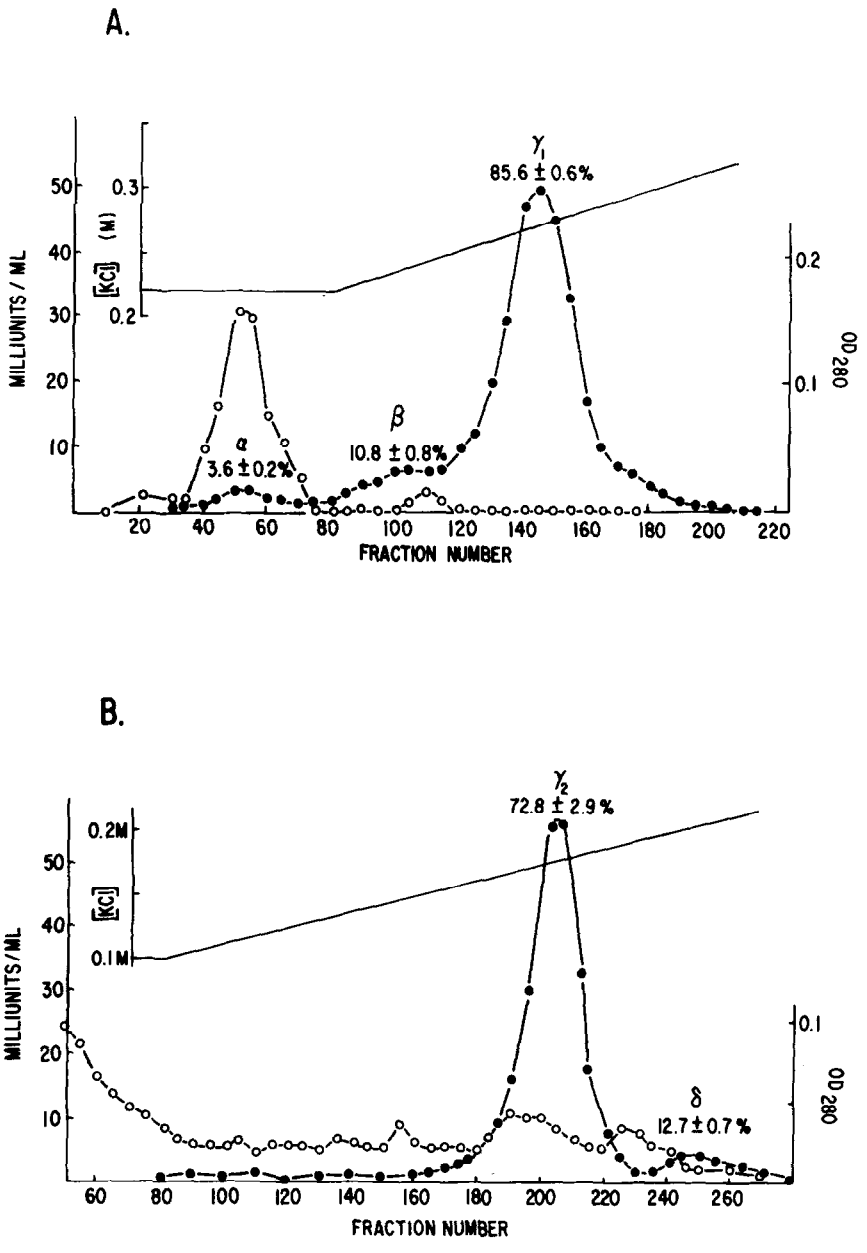

Fig.1. The elution patterns of avian acrosin from two 1.5 × 26 cm DEAE-Sephadex ion exchange column. 8–10 ml of enzyme preparation (approx. spec. act. of 0.2 U/mg protein) previously dialyzed against the starting buffer was applied to each column. One ml fractions were collected. The elution rate was 7.5 – 8 ml/hr. Protein containing fractions were detected by measurement of their OD_{280} (○—○) and acrosin activity was assayed with BANA at pH 8 in the presence of 0.05 M $CaCl_2$ (●—●). Four separate chromatographic experiments were done with each column and the percentage of total activity eluted in each acrosin fraction is shown in the figs (mean ± standard error of the mean). (A) The buffer used was 0.0625 M Tris-borate buffer, pH 9.0, containing 0.22 M KCl. The gradient was from 0.22 M to 0.44 M KCl and the total vol of the gradient was 400 ml. (B) The buffer used was 0.05 M Tris–HCl, pH 8.5, containing 0.1 M KCl. The gradient was from 0.1M KCl to 0.3 M KCl with a total gradient volume of 400 ml.

2.4. *Protein determination*

Protein was measured by the method of Lowry et al. [12] with bovine serum albumin as the standard.

2.5. *DEAE-Sephadex column chromatography*

The conditions for the chromatography are described in fig.1. Enzymatic activity was measured with BANA as the substrate (5×10^{-4} M). Protein was measured at a wavelength of 280 nm, and the KCl concentration was determined with a Radiometer Conductivity Meter.

2.6. *Molecular weight determination by polyacrylamide gel electrophoresis*

The flatbed electrophoresis solutions described earlier [5] were modified for use with Hoeffer disc acrylamide gel equipment and a Heathkit Model IP-15 power supply. Concentrations used for the 7.0 cm long running gels were 6%, 8%, 10% and 12% acrylamide. The spacer gel was omitted. Electrophoresis was begun at 6 mA/gel and run until the bromphenol blue tracking dye had almost reached the end of the gel, usually 1.5 hr. Enzymatic activity was detected as previously reported [5] except that CANA was used as the substrate (5×10^{-4} M). Proteins were stained with 1% Amido Schwartz. The apparent molecular weights of the sperm enzymes were calculated [13] with the following proteins as standards: conalbumin, the major protein band of soybean trypsin inhibitor, ovalbumin, and bovine serum albumin.

SDS gel electrophoresis [14] was performed with the following standard proteins: ovalbumin, bovine serum albumin, carboxypeptidase A, leucine aminopeptidase, and bovine trypsin for apparent molecular weight determination of the sperm enzymes. Proteins were stained with Coomassie blue [14].

Three determinations of molecular weight were made with each method.

3. Results

3.1. *DEAE-Sephadex chromatography*

Figs.1A and 1B show the elution patterns obtained when the sperm extract was chromatographed. Recovery from columns at both pH's was 80–90%. The crude extract had a specific activity of approx. 0.15 U/mg protein. The specific activities for peaks α,β, and δ are listed in table 1. γ_2 had the highest specific activity of 2.9 U/mg protein, almost a thirty-fold purification whereas γ_1 had a specific activity of 1.9 U/mg protein.

As indicated in fig.1, most of the activity was eluted in γ_1 and γ_2. Both of these two fractions, when electrophoresed on 8% acrylamide and stained for activity (not shown), contained two of the electrophoretic multiple forms, b and c, that characterized the crude sperm extracts [5].

Electrophoretic band a [5], the fastest moving band anodally, occured only occasionally in sperm

Table 1
Comparison of several catalytic properties of acrosin ion-exchange fractions

	DEAE-Sephadex, pH 9.0[a]		DEAE-Sephadex, pH 8.5[a]	Analysis of data[b]	
	α	β	δ	α vs β	β vs δ
Specific activity (BANA U/mg protein)	0.01	0.2	0.5		
K_M (10^{-3} M)					
BAEE	0.032 ± 0.001	0.042 ± 0.004	0.009 ± 0.001	P>0.01	P<0.001
TAME	0.024 ± 0	0.037 ± 0.004	0.006 ± 0	P>0.01	P<0.001
BANA	0.103 ± 0.009	0.107 ± 0.004	0.071 ± 0.006	P>0.01	P<0.01
K_i (10^{-4} M)					
Benzamidine	0.242 ± 0.003	0.202 ± 0.010	0.088 ± 0.026	P<0.01	P<0.01
p-Aminobenzamidine	0.018 ± 0.002	0.011 ± 0.003	0.050 ± 0	P>0.01	P<0.001

[a]Mean ± standard error of the mean (3 to 4 determinations were made of K_M or K_i for each substrate or inhibitor).
[b]Students t test.

extracts and did not appear in the preparations used for chromatography. The electrophoretic mobility of ion exchange fraction δ appears to be identical to that of band b whereas the mobility of α and β appear to be identical to that of crude extract band c.

3.2. *Kinetic comparison of fractions*

Table 1 lists the kinetic constants of three of the ion exchange fractions as well as the analysis of significance of the differences between the data for the fractions. Since γ_1 and γ_2 were shown to contain two multiple forms, no kinetic data is presented for them. In almost every case, the kinetic constants of α and β were not significantly different. However, there were statistically significant differences between the data for β and that for δ.

Each of the individual multiple forms exhibited the substrate activation type of plot for BAPNA when V was plotted against $V/(S)$ [15]. Therefore the K_M for BAPNA is not given in table 1.

3.3. *The apparent molecular weight*

The three electrophoretic bands of enzymatic activity (a,b and c) gave parallel lines when the logarithm of their relative mobilities in different concentrations of acrylamide were plotted as a function of acrylamide concentration. This result indicates that all three enzymes have identical or nearly identical molecular weights [13] and thus differ mainly in charge. The mol. wts determined for each band of enzymatic activity were as follows: (a) 49 500 ± 2100; (b) 43 300 ± 2200; and (c) 47 400 ± 2500.

Fraction δ contained more than one protein band after electrophoresis in the presence of SDS. The major protein band of δ had an apparent mol. wt of 25 600 ± 1100. Fraction β consisted of only one protein band. Its apparent mol. wt was 27 700 ± 1100. Mol. wts were not determined by SDS electrophoresis for α, γ_1 and γ_2.

4. Discussion

The kinetic properties of fractions α and β were quite similar (table 1) and α migrated with the same mobility as β, suggesting that α and β may be the same enzyme which eluted in different fractions as a result of some artifact of the ion exchange technique. Under our experimental conditions only a small percentage of the total applied activity was separated by chromatography into the multiple forms. This together with the observation that ordinary acrylamide gel electrophoresis gave molecular weight values that were almost twice those obtained from SDS electrophoresis or from Sephadex G-100 column chromatography (Ho and Meizel, manuscript in preparation), indicates that acrosin aggregation may occur under certain conditions.

Assuming that α is identical to β, the results presented in table 1 suggest that there are differences between the kinetic properties of two molecular forms of avian acrosin, β and δ. However, future studies utilizing substrates with more than one amino acid will be necessary in order to determine whether the multiple forms have different specificities for particular amino acid sequences and thus whether they may hydrolyze different proteins in their natural substrate (probably the hen egg vitelline membrane [16]).

The multiple forms may be a result of the hydrolysis of slightly different bonds during the process of activation from a zymogen precursor as is true for bovine pancreatic trypsin [17–19]. It is of interest that rabbit sperm acrosin does appear to be derived from a zymogen [20].

The conditions for preparation and the subsequent storage of the active enzymes may be important in the formation of the multiple forms. Under our conditions of extraction and purification, the proportion of activity in each of the ion exchange fractions (fig.1) is consistent from preparation to preparation.

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