A C T A U N I V E R S I T A T I S L O D Z I E N S I S
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AMINO ACID COMPOSITION AND N-TERMINAL RESIDUES OF GOOSE, DUCK AND HEN PROTHROMBIN

Prothrombin obtained from oxylated goose, duck and hen plasma was purified by filtration on Sephadex G-25, chromatography on hydroxyapatite and DEAE-Sephadex A-50. Specific activities of isolated preparations were found to be 610-650 NIH/mg. Goose as well as duck and hen prothrombin preparations displayed alanine as N-terminal residue similarly to mammalians. Amino acid analysis of these proteins showed a higher content of aspartic and glutami acids in all the three kinds of prothrombin in comparison with mammalian one.

INTRODUCTION

Prothrombin is an inactive form of thrombin (E.C.3.4.21.5) which plays a pivotal role in the final stages of blood coagulation by converting fibrinogen to fibrin. In the past few years very extensive studies on mammalian prothrombin have been performed, Seegers [17-18] and Magnusson [13-14]. Human or bovine prothrombin is a glycoprotein [15-16] consisting of a single polypeptide chain with molecular weight of about 70 000 [1, 6, 11, 12]. Alanine is the N-terminal residue for prothrombin of so far investigated mammalians [man, ox, horse, sheep, dog, rat] and serine constitutes the C-terminal amino acid [4, 20]. Dichroism spectrum analysis indicates that 75% of the molecule is of a randomcoil structure and 5% is an α-helix [3,

7]. The studies of physicochemical and biological properties of avian prothrombin are by far less advanced. Therefore it appeared interesting to identify the N-terminal residues and amino acid composition of goose, duck and hen prothrombin.

MATERIALS AND METHODS

Isolation of prothrombin

Prothrombin preparations were obtained from goose, duck and hen blood according to Cox and Hanahan method [5]. 5 l of fresh blood were centrifuged and $BaSO_4$ was added to plasma (10 g/ /100 ml plasma) to adsorb prothrombin. Protein was eluted from the complex with 0.06 M sodium citrate. The supernatant obtained after centrifugation was added to powder cellulose to adsorb the accompanying proteins, especially factor X. The remaining protein contaminations were precipitated by ammonium sulfate. 209 g $(NH_4)_2SO_4$ were added to 1000 ml of the supernatant, stirred for one hour and centrifuged for 30 min. at 440 g. The appropriate protein fraction containing prothrombin was salted out by adding 2 g/10 ml ammonium sulfate to the supernatant. The precipitated proteins were dissolved in the minimal volume of 0.01 M trisodium citrate (pH 6.5) and purified chromatographically.

Purification of the prothrombin preparation

Purification of protein fraction was carried out according to Benarous, Labie and Josso method [2] using gel filtration and followed by chromatography on hydroxyapatite and DEAE-Sephadex A-50. Crude protein was filtrated on Sephadex G-25 column (2.5 x x 100 cm) using 0.15 M potassium phosphate buffer at pH 6.8 for elution. In the course of separation one main peak containing prothrombin and 2-3 peaks most probably the degradation products of this protein were obtained. Afterwards the main peak was purified by hydroxyapatite chromatography. The eluate from

Sephadex G-25 (100 mg protein) was applied to a column (2.4 x x 20 cm) with hydroxyapatite and elution was performed in a linear gradient of 0.2-0.5 M potassium phosphate buffer (500 ml in each chamber) at a flow rate of 60 ml/hr. DEAE-Sephadex A-50 chromatography was the final stage carried out in the linear gradient of 0.0-0.7 M NaCl in potassium phosphate buffer (350 ml in each chamber). 20-25 mg of protein was applied to a column (2.5 x 30 cm). Pure protein was eluted with salt solution at the concentration 0.4-0.5 M NaCl. The obtained fractions containing prothrombin were pooled together and thickened until final concentration 1 mg/ml.

Determination of the activity of thrombin preparations

The activity of thrombin preparations was determined by two-stage (Jackson, Thomas and Hanahan) method [10]. Prothrombin was converted into thrombin by incubation (37°) with thromboplastin and calcium ions for 20 min. and next its activity was determined on the basis of clotting ability of 0.1% fibrinogen solution.

Identification of N-terminal amino acids

Coose, duck and hen preparations of prothrombin were subject to DNS procedure [8, 9]. 50 nmoles of lyophilized protein were dissolved in 0.3 ml of water and 0.15 ml of 0.4 M phosphate buffer and 250 mg urea. Afterwards 0.25 ml of dimethylformamid and 0.25 ml of 5-dimethylaminonaphtalinsulfonsaure-1-chlorid in acetone were added to the above solution. Next it was carefully stirred and left in a dark room for 30 min. at 20°C. Then the mixture was diluted four times by adding 3 ml of water and 4 ml of 10% TCA. The formed sediment was centrifuged (10 min., 2000 g), washed twice with acetone and ether, and dried. Finally 0.25 ml of 5.5 M HCl were added to the sediment. Hydrolysis was carried out in flooded tubes for 6 h at 110°C. The hydrolyzate was diluted with water and evaporated until the elimination of HCl. The rest was dissolved in 0.1 ml of acetone: 1 M HCl mixture 1 : 1 v/v.

Thinlayer chromatography according to Sthal [19]

Glass plates (20 x 20) covered with 0.1 mm silica gel layer were activited for 1 h at 110°C. Hydrolyzate and amino acid samples were applied to each plate. Separation was carried out in two systems : 1) benzene : piridine : acetic acid (16 : 4 : 1), 2) toluene : 2-chloroethanol : ammonia 28% (6 : 10 : 4). After developing the chromatogram in the first system the plates were dried at 110°C and fluorescence was evoked by UV to identify N-terminal amino acid of the investigated proteins.

Automatic amino acid analysis

The analysis of the proteins was preceeded by acid hydrolysis. 0.25 ml of 5.5 M HCl were added to 1 mg protein. Hydrolysis was done in flooded glass tubes for 24, 48 and 72h at 110°C. The hydrolyzates were evaporated until dry under the same conditions. The procedure was repeated several times to remove HCl. Amino acid composition of the protein hydrolyzate was determined on Joel automatic analyzer of amino acids.

RESULTS AND DISCUSSION

Isolated prothrombin preparations from goose, duck and hen plasma after filtration on Sephadex G-50 yielded always one main peak corresponding to the prothrombin fraction and 2-3 other small ones representing protein contaminations (Fig. 1).

Chromatography of the prothrombin fraction on hydroxyapatite resulted in four distinct peaks. The last one, eluted with phosphate buffer at a concentration 0.3-0.5 M, represented more purified goose prothrombin fraction (Fig. 2).

Afterwards the prothrombin peak was further separated on DEAE-Sephadex A-50 column. The fraction eluted in the range of 0.3-0.4 M NaCl contained pure and active prothrombin preparation, moving in polyacrylamide gel as a single band (Fig. 3, 4 A). Analogical patterns were obtained for duck and hen pro-

thrombins. Their electrophoretical mobilities are shown in Fig. 4 A and 4 B.

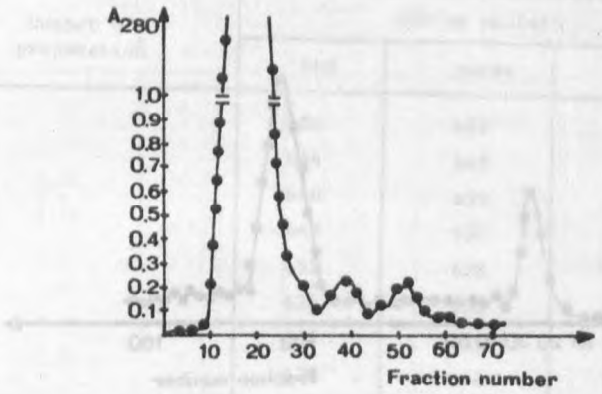

Fig. 1. Purification of crude goose prothrombin on Sephadex G-25 (for details see Materials and methods)

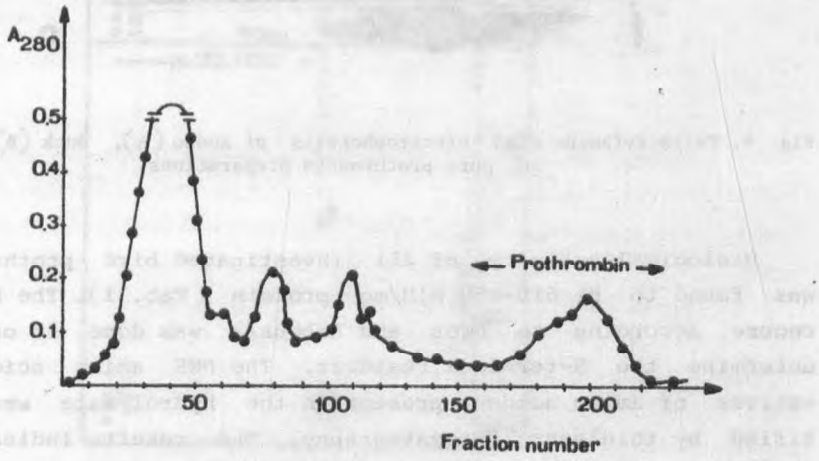

Fig. 2. Separation of goose prothrombin on hydroxyapatite

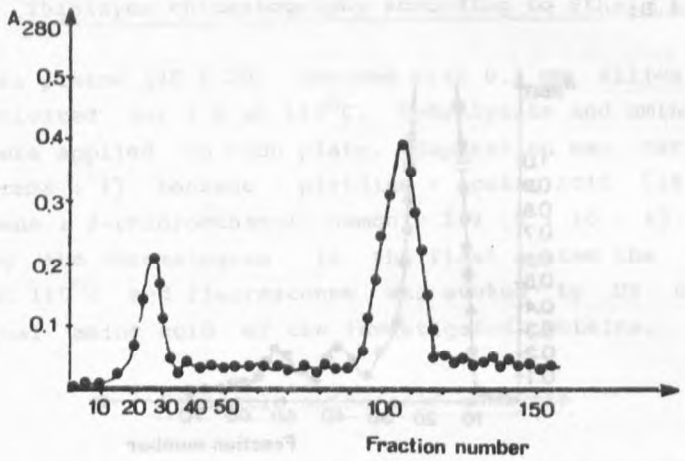

Fig. 3. Chromatography of goose prothrombin on DEAE-Sephadex A-50

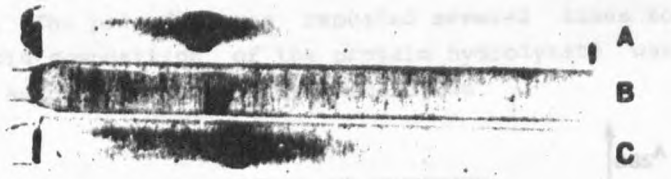

Fig. 4. Polyacrylamide disc electrophoresis of goose (A), duck (B) and hen (C) pure prothrombin preparations

Biological activity of all investigated bird prothrombins, was found to be 610-650 NIH/mg protein (Tab. 1). The DNS procedure according to Cros and Laboussa was done in order to determine the N-terminal residues. The DNS amino acid derivatives of amino acids present in the hydrolyzate were identified by thinlayer chromatography. The results indicate that alanine is the N-terminal amino acid for all three kinds of prothrombin (Fig. 5). Comparative amino acid analysis of all kinds of prothrombin preparations carried out by automatic analyser following acid hydrolysis is shown in Tab. 2.

Table 1

Specific activity of prothrombin preparations measured by two stage text according to Jackson, Thomas and Hanahan method

Number of preparation	NIH/mg protein		
	hen	goose	duck
1	656	623	610
2	644	648	602
3	648	630	620
4	649	636	598
5	652	628	626
6	636	650	612
7	649	623	600
8	678	646	628
9	650	628	596
10	652	626	620
Average	651.4	634.1	611.2
Standard deviation	10.7	9.9	11.9

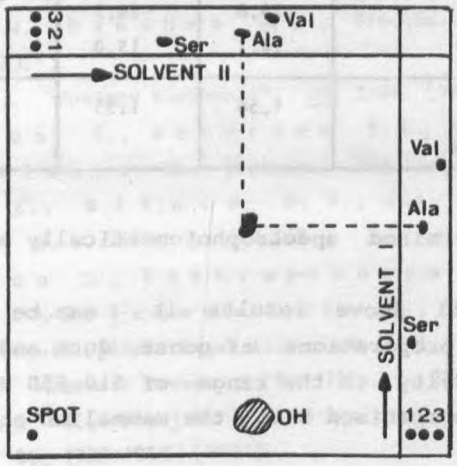

Fig. 5. Bidimensional thin layer chromatography of goose dansyl amino acid

Table 2

Amino acid composition of avian prothrombin

Amino acids	amino acids/1 mol protein		
	hen	goose	duck
Asp	59.2	61.8	60.7
Tre	32.1	29.9	31.5
Ser	27.8	28.9	26.9
Glu	68.6	69.1	67.4
Pro	32.0	29.5	30.0
Gli	39.8	40.4	39.5
Ala	30.2	29.6	29.4
Wal	31.0	29.8	29.1
Met	ślad	ślad	ślad
Ile	20.4	21.2	19.8
Leu	39.8	37.9	39.0
Typ	18.9	21.3	22.6
Fen	19.0	18.8	17.9
His	13.8	14.2	14.0
Liz	44.1	43.5	45.1
Arg	24.8	26.3	27.0
Trp	14.1	15.0	-
$\frac{\text{Asp + Glu}}{\text{Liz+His+Arg}}$	1.54	1.55	1.49

Tryptophan was determined spectrophotomertically after alkaline hydrolysis.

Summarizing all above results it can be stated that:

1. Prothrombin preparations of goose, duck and hen possess the biological activity in the range of 610-650 NIH/mg proteins that is lower in comparison with the mammalian one (about 3000 NIH/mg protein).

2. Alanine is a common N-terminal amino acid.

3. Prothrombins of investigated birds are quite comparable in respect of amino acid composition as well as the ratio of acid and base amino acid (1.49 - 1.55).

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SKŁAD AMINOKWASOWY I N-KOŃCOWE AMINOKWASY PROTROMBINY GĘSI, KACZEK I KUR.

Protrombinę otrzymywano z plazmy szczawianowej gęsi, kaczek i kur metodą Cox'a i Hanahan (1970), oczyszczano metodą Benarous, Labie i Josso (1973), stosując filtrację na żelu Sephadex G-25, chromatografię na hydroxyapatycie oraz chromatografię na żelu DEAE-Sephadex A-50. W jednorodnych pod względem elektroforetycznym białkach, wykazujących aktywność 610-650 NIH/mg protrombiny oznaczono N-końcowe aminokwasy. Stwierdzono, że N-końcowym aminokwasem w preparatach protrombiny gęsi, kaczek i kur jest alanina, podobnie jak u ssaków (człowiek, koń, wół, owca, pies, szczur). Analiza składu aminokwasowego wspomnianych białek ujawniła wysoką zawartość kwasu asparaginowego i glutaminowego we wszystkich rodzajach protrombin.