Isolation, Purification and Characterization of Proteins from the Meat of the Bird G. *Centropus sinensis sinensis*

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Summary: The protein from the meat of the bird *G. Centropus sinensis sinensis* was extracted with different solvent system. It was found that the yield of protein with distilled water was maximum (1.43 mg/ml, having O.D. 1.0 at 280 nm). The isolated protein was purified in natural form. The purification process includes gel filtration on Sephadex G-75 and ion exchange chromatography on DEAE-cellulose. The protein was fractionated in six fractions which appeared homogeneous as judged by electrophoresis. Molecular weights of the protein fractions determined by SDS-polyacrylamide gel electrophoresis were 2,50,188; 2,10,251; 1,90,242; 1,25,871; 35,314 and 17,159, respectively. Each of the proteins F_1a, F_1b and F_2a having molecular weight 2,50,188; 2,10,250 and 1,25,871 respectively was found to contain four identical subunits. The molecular weight of the sub-units in the proteins F_1a, F_1b and F_2a were respectively 62,540; 52,500 and 31,450. Protein F_1c have molecular weight 1,90,242 contained two pair of identical subunits of molecular weight 54,213 and 41,025 respectively. The other proteins F_2b and F_2b contained no subunit. The proteins were non specific in hemagglutination for goat and human 'O' type red blood cells but specific in agglutination for rat and bovine red blood cells. All the proteins were glycoproteins, having sugar content of about 2.77%.

Introduction

The bird "Crow pheasant" belongs to the genus *Centropus sinensis* of the family *Cuculidae* [1]. Its vernacular name is "Kuyo" or "Kuku". The bird is well known to the native physicians of some parts of Bangladesh for its recognized usefulness in the treatment of rheumatic fever and rheumatism. This edible wild bird is familiar with the people of different parts of the country. It is found in plenty, specially in the spring, in the districts of Khulna, Bagerhat, Jessore and Kushtia. Inspite of its recognized curative effects on rheumatism, no such work on isolation, purification and characterization of meat protein from the bird has so far been reported in literature. Studies of cytoagglutinating properties of meat protein on red blood cell may enable us to express the nutritional significance of the protein. Studies on mucoproteins and glycoproteins along with their characterization and identification of monosaccharide unit/units may form an integral part of the research to explain the importance of glycoprotein [2].

In view of the recognized usefulness of the meat of this bird in oral administration in the treatment of rheumatism and rheumatic fever, the present work has been undertaken and the results have been reported in this communication. Amino acid profile in meat protein and fatty acid components in lipid from the bird will form a separate study in subsequent research.

Results and Discussion

The crude protein, obtained by 50% ammonium sulphate precipitation, was applied to Sephadex G-75 column at 4°C, which was previously equilibrated with the 5 mM sodium phosphate buffer (pH 7.6). The protein was eluted from the column with the same buffer. As shown in Fig. 1, the crude protein was separated into three fractions - F_1, F_2 and F_3. The protein in fraction F_1 and F_2 were pooled as indicated by solid lines and were precipitated with ammonium sulphate, dissolved in water, dialyzed against water and finally against 10 mM Tris-HCl buffer, pH 8.4. After removal of the insoluble material produced during dialysis, the clear protein solution was loaded on to the DEAE-cellulose column, previously equilibrated with 10 mM Tris-HCl buffer, pH 8.4. The protein in the column was eluted with a linear gradient of NaCl from 0 to 0.3 M in the same buffer. As shown in Fig. 2, the protein was eluted mainly as a single but broad peak, indicating the presence of more than one components. In order to separate these

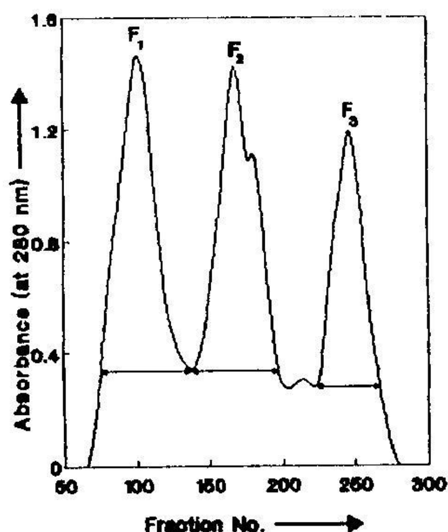

Fig.1: Gel filtration pattern of 50% saturated fraction on Sephadex G-75 column. The crude extract was applied to the coumn (125 x 2.29 cm) of Sephadex G-75 pre-equilibrated with 5 mM sodium phosphate buffer saline, pH 7.6 at 4°C and eluted with the same buffer at a flow rate of 20 ml/hr.

components, the elution was carried out stepwise with increasing concentrations of NaCl in the same buffer. As demonstrated in Fig. 3, the F_1 fraction was separated as three sharp main peaks, F_1a, F_1b, F_1c and a small peak F_1d. On the other hand, fraction F_2 was also fractionated in the same manner, where the sharp peaks were designated by F_2a, F_2b, F_2d and the small one by F_2c. As fractions F_1d and F_2c contained small amount of protein, they were not used for further study. The purity of these fractions were checked by polyacrylamide disc gel electrophoresis at pH 8.5. It was observed that all the fractions gave single band.

Molecular weight of the purified proteins were determined by SDS-polyacrylamide gel electrophoresis according to the method of Weber and Osborn [7] at pH 7.2. Ureas, β-galactosidase, α-amylase, egg albumin, carbonic anhydrase, lysozyme were used as reference proteins. Molecular weight was calculated by comparing the mobility of the purified proteins with that of the standard proteins (Fig. 4). The molecular weight of the proteins F_1a, F_1b, F_1c, and F_2a, F_2b, F_2d were found to be 2,50,188; 2,10,251; 1,90,242 and 1,25,871; 35,314; and 17,159 respectively.

For subunit determination, the protein samples were treated with 1% β-mercaptoethanol and then subjected to SDS-electrophoresis. Although the fractions F_1a, F_1b and F_2a gave single band but their molecular weight were calculated to be 62,540; 52,500; and 31,450 respectively. So, these three pure proteins contained 4 subunit of equal size. On the other hand, the fraction F_1c gave two bands corresponding to molecular weight of about 54,213 and 41,025. It was evident that this protein also contained 4 subunits of two pair; one with molecular weight 54,213 and the other pair with molecular weight 41,025. The other two proteins F_2b did not change in mobility when subjected to SDS-electrophoresis upon treatment with β-mercaptoethanol. So these proteins did not posses any subunit structure.

Hemagglutination study of fraction F_1a, F_1c, F_2a, F_2b and F_2d were performed using goat, bovine, albino rat and human 'O' type red blood cells. All the proteins specifically agglutinated albino rat and

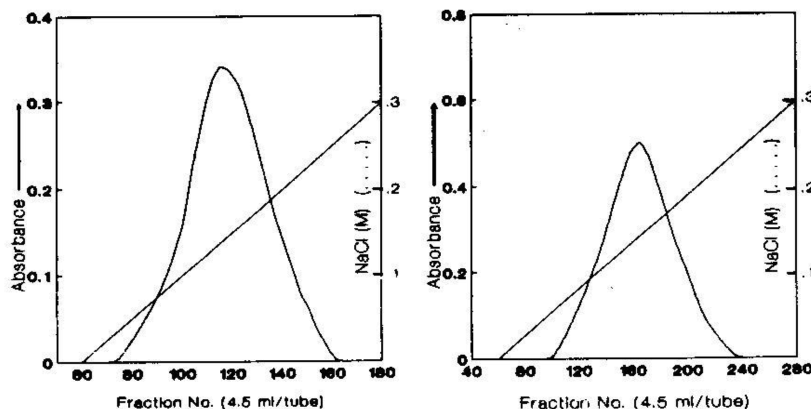

Fig.2: Gradient ion exchange chromatography of F_1 and F_2 fraction (A and B) of DEAE-cellulose column. Fraction F_1 and F_2 obtained from gel filtration (Fig. 1) was applied to DEAE-cellulose column (30 x 2.22 cm) prewashed with 10 mM Tris-HCl buffer, pH 8.4, at 4°C and eluted by a linear gradient of NaCl (0-0.3 M) in the same buffer.

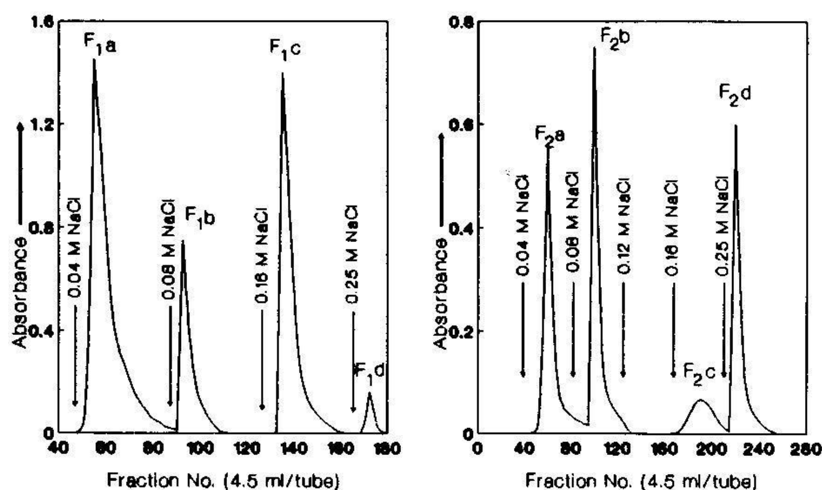

Fig. 3: Stepwise ion exchange chromatography of F_1 and F_2 fractions (A and B) on DEAE-cellulose column. Fraction F_1 and F_2 obtained from gel filtration Fig. 1) was applied to DEAE- cellulose column (30 x 2.22 cm) prewashed with 10 mM Tris-HCl buffer, pH 8.4 at 4°C and eluted by stepwise increases of NaCl concentration in the same buffer.

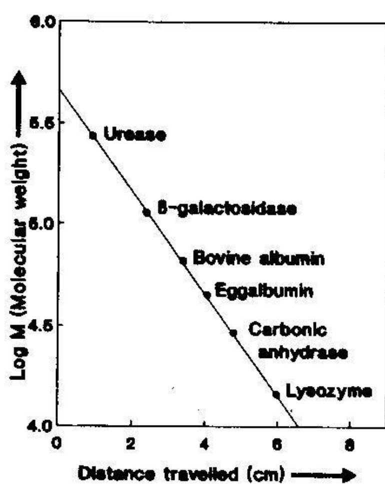

Fig. 4: Standard curve for the determination of molecular weight of protein by SDS-polyacrylamide gel electrophoresis.

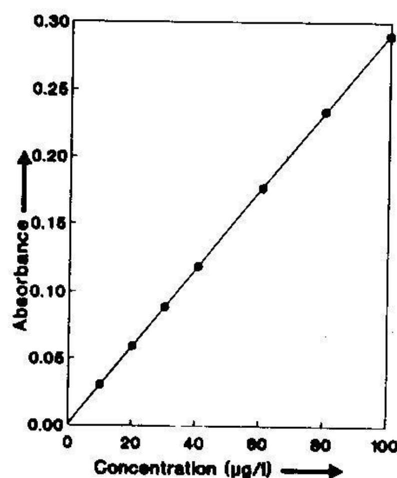

Fig.5: Standard curve of glucose for estimation of percentage of sugar present in glycoprotein.

bovine blood cells. Human "O" type and goat blood cells were agglutinated by none of these proteins.

The minimum hemagglutination dose (MHD) for the three proteins determined by mixing 0.2 ml. 4% washed rat RBC (in 5 mM PBS containing 0.9% NaCl pH 7.2) and 0.2 ml. protein solutions of varying concentration. Hemagglutination was observed after 1-2 hours incubation at 37°C. The sedimented cells were resuspended and examined under microscope. Table 1 shows the agglutination of rate RBC by the five purified proteins. The degree of hemagglutination was scored as 4+, 3+, 2+, 1+ and ± was described in materials and method.

All the purified proteins gave yellow-orange color in the presence of phenol sulphuric acid, indicating that the protein contain sugar. The percentage of sugar present in the glycoproteins were calculated from the standard graph of glucose (Fig. 5). It was found that the protein F_1a, F_1c, F_2a, F_2b and F_2d contained about 3.41%, 2.89%, 1.41%, 4.01% and 2.42% sugar, respectively.

Table 1: Hemagglutination of different purified proteins with RBC from male albino rat.

Protein samples	Concentration (O.D. at 280 nm)	Hemagglutination
	0.15	4+
	0.12	3+
F_1a	0.09	2+
	0.06	1+
	0.04	±
	0.10	4+
	0.09	3+
F_1c	0.08	2+
	0.07	1+
	0.06	±
	0.08	4+
	0.06	3+
F_2a	0.04	2+
	0.02	1+
	0.009	±
	0.05	4+
	0.04	3+
F_2b	0.03	2+
	0.02	1+
	0.01	±
	0.10	4+
	0.009	3+
F_2d	0.008	2+
	0.007	1+
	0.005	±

From the foregoing evidence, it has been found that meat protein under investigation contains five individual proteins having different molecular weights. Each of these proteins containing sugar has the ability of agglutinating the bovine and rat RBC. These specific glycoproteins may act as antibodies to inhibit the specific virus particles that cause rheumatic fever.

Experimental

Extraction of crude protein

In order to extract protein from the meat of the bird in natural form all the operations were performed at 2-4°C. The meat was blended into a slushy mass using a homogenizer (Model AM-5, OGAWA SEIKI Co., Ltd. Japan) with cold distilled water [3-4]. The process was repeated again in cold and frothing was avoided. The homogenate was filtered through a double fold muslin cloth to remove any lumps. The homogenate was centrifuged at 12 x 10^3 r.p.m. for 15 minutes and the supernatant was made 100% saturation with ammonium sulphate. The resulted precipitate was collected by centrifugation and dialyzed against deionized water for 24 hour. After centrifugation, the optical densities of the clear supernatant were measured at 280 nm. Finally, solid ammonium sulphate solution was added to the clear supernatant and the precipitate obtained from 50% ammonium sulphate saturation was used as crude extract [5].

Gel filtration

Gel filtration was performed through a Sephadex G-75 column (for details of column size, flow rate etc., are stated in the legends to relevant figures) with 5 mM sodium phosphate buffer saline, pH 7.6.

DEAE-cellulose column chromatography

A column of DEAE-cellulose was prepared and equilibrated with 10 mM Tris-HCl buffer, pH 8.4. After dialysis against the same buffer, the protein was applied to the column and eluted stepwise with increasing concentrations of NaCl in the same buffer.

Polyacrylamide disc gel electrophoresis.

This was carried out at pH 8.5, using 7.5% gel as described by Ornstein [6]. The protein in the gel was detected by staining with 1% amido black.

Molecular weight determination

Molecular weight of the proteins were determined by polyacrylamide gel electrophoresis in sodium dodecylsulphate (SDS) according to Weber and Osborn [7], using 10% gel. The protein was treated with or without 1% β-mercaptoethanol at 100°C for 3 minutes. Ureas, β-galactosidase, α-amylase, egg albumin, carbonic anhydrase, lysozyme were served as calibration proteins for the molecular weight determination. The gels were stained with Coomassic brilliant blue R 250.

Hemagglutination test

To a siliconized test tube (0.5 x 7 cm) containing 0.2 ml of 4% washed human 'O' type red blood cells in 5 mM sodium phosphate buffer (pH 7.2), various concentrations of protein solution were added. The mixture was incubated at 37°C for 1 hour, The degree of hemagglutinating activity was graded according to the pattern formed by the agglutinated cells on the bottom of the tubes [8-9] and the results

were recorded as 4+, 3+, 2+, 1+ and ± [10]. A 4+ reaction indicate a complete aggregation of cells involving almost all the erythrocytes present in the suspension, whereas a ± indicated a null point where aggregation of cells was not identified. Tests were also performed with rat, goat and bovine red blood cells.

The minimum hemagglutinating dose (MHD) was measured as the smallest concentration of protein that gave the visible hemagglutination. For this protein solution were diluted serially (starting from 1.0 O.D/ml to 0.001 O.D/ml) and then used for hemagglutination test.

Test for glycoprotein and estimation of reducing sugar

Phenol in the presence of sulphuric acid can be used for the quantitative colorimetric microdetermination of sugars and their methyl derivatives, oligosaccharides and polysaccharide [11]. This method was employed for detecting the presence of sugar in protein. Quantitative estimation was performed by taking glucose as standard.

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