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PURIFICATION OF BOVINE FERRITIN

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ABSTRACT

The iron storage protein ferritin with a high molecular weight of about 450.000 was isolated from the spleen of cattle. Confirmation of ferritin in solutions was made by showing that it had a high content of iron relative to the protein concentration. The purity of the isolated ferritin was tested by 5.0 % polyacrylamide disc gel electrophoresis. In the gels multiple ferritin bands stained for both iron and protein were observed.

ÖZET

Yaklaşık molekül ağırlığı 450.000 olan yüksek molekül ağırlıklı demir depo proteini ferritin sığır dalağından ayrıldı. Çözeltilerde ferritin varlığı protein yoğunluguna oranla yüksek demir içerikli oluşu ile saptandı. Ayrılan ferritinin saflığı % 5.0'lik polyacrylamide jel elektroforezi ile gösterildi. Jellerde hem demir hem de protein için boyanan çoklu (multiple) ferritin bantları gözlendi.

INTRODUCTION

In 1937 Laufberger /1/ isolated a crystallizable protein from horse spleen which contained over 20 % by dry weight of iron. Much earlier Schmiedeberg /2/ reported that there was an unknown iron containing component of liver that was heat stable. Later, Granick /4/ has extensively studied the physical and chemical properties of horse spleen ferritin. Conditions for the crystalization with $CdSO_4$ were also described /4/. The occurence of ferritin in tissues from man, dog, guinea pig, mouse, rat, pig, rabbit, and cat was also shown by immunoprecipitin reaction /3/.

In early studies the determinations of ferritin protein and iron have been based on chemical assays that need high yield. Since the first development of an

immunoradiometric assay for ferritin by Addison et al., /5/, in 1972, it has been possible to measure the levels of ferritin in human serum. Serum ferritin levels are clearly useful in the diagnosis of simple iron deficiency /10/.

The objective of our present study is to improve a radioimmunoassay technique to measure the serum ferritin levels of cattle and to estimate the amount of storage iron at various physiological states. For this, the first step was to isolate bovine ferritin in a pure form.

MATERIALS AND METHODS

Horse spleen ferritin, acrylamide and bisacrylamide were purchased from Sigma Chemical Company. Sepharose 6B ready to use and Sephadex G200 in a powder form were also obtained from Sigma. All other chemicals used were reagent grade. Bovine spleen used for ferritin isolation was obtained from freshly slaughtered animals and was frozen and stored at - 20°C until use.

The isolation methods used were those of Linder and Munro /6/ and Penders et al., /7/. Gel filtration on Sepharose 6B and Sephadex G200 was done for further purification of the isolated ferritin. Protein determinations have been carried out by the method of Lowry et al., /8/. Horse spleen ferritin was used as a standard. Iron was measured by ICSH method /14/. Polyacrylamide disc gel electrophoresis (PAGE) was achieved according to the method of Davis /9/. Gels were stained for protein using Coomassie brilliant blue and for iron by immersing the gels in a solution of 2 % potassium ferrocyanide. Gels were fixed in the 12,5 % TCA (trichloracetic acid) solution for 15 min before staining. The staining solution for protein contained 50 ml of % 1 Coomassie brilliant blue, 250 ml of methanol, 35 ml of acetic acid and 165 ml of water in a total volume of 500 ml. Destaining solution contained 350 ml of methanol, 80 ml of acetic acid and 570 ml of water in a total volume of 1000 ml. Gels stained for iron washed with distilled water.

Two methods were applied for isolation. In the first one /6/, isolation of ferritin from bovine spleen was started with homogenizing the tissue by an ULTRA TURRAX, 18/2N type homogenizer for 10 minutes. About 100 g of spleen tissue were weighed and cut into small pieces. Then it was placed in a homogenizer baloon of 500 ml capacity. To this 200 ml of 0.15 mM NaCl solution was

added. Also, PMSF (Paramethyl sulphonyl floride) was added to the solution at a concentration of 0.1 mM to prevent the action of proteases. Homogenate obtained was heated to 75°C for 5 min with constant mixing in a hot water bath at 90°C. Then a clear red - brown supernatant was obtained by centrifugation at 1.500 g for 20 min. At this stage of isolation NaN_3 (sodium azide) was added to the supernatant at a concentration of 0.02 % (w/v) to prevent bacterial activity. Then the pH of the solution was adjusted to 4.8 by dropwise addition of 7.5 % acetic acid solution. MCE (mercaptoethanol) was also added to the solution for the prevention of the disulfide bond formation between protein molecules. After leaving the solution at 4°C overnight, acid denatured proteins were precipitated by centrifugation and the sediment was discarded. After readjustment of the pH of the supernatant to 6.5 by 0.1 M NaOH, ammonium sulphate saturation to 50 % (300 g per liter) was achieved by adding small amounts of ammonium sulphate salt to the solution. The pellet obtained by centrifugation was redissolved in the smallest necessary volume of 0.05 M borate buffer, pH 8.6. This isolated crude ferritin was further purified by gel filtration with Sepharose 6B and Sephadex G 200. The 7 ml fractions collected from the Sepharose 6B column (4,5x64 cm) were read at 280 nm (Fig.1.) and those containing ferritin were pooled into a flask. This dilute ferritin fraction was concentrated by 50 % ammonium sulphate saturation and the pellet obtained by centrifugation was redissolved in a volume of borate buffer and dialysed against the same buffer overnight. The same procedure was repeated for the pooled ferritin fraction from the Sephadex G 200 column (1,7x35 cm).

In the second method /7/ used for isolation, the spleen was homogenized, heated and centrifuged as in the first method. The filtrate was ultracentrifuged at 78,000 g for 1 h by a Spino L 2 type ultracentrifuge. The pellet obtained was redissolved in 0.15 mM NaCl solution containing 0.02 % NaN_3 and centrifuged at 7,000 g for 1 h. Then the supernatant fraction was centrifuged at 95,000 g for 1 h. The sedimented ferritin was dissolved in the same solution.

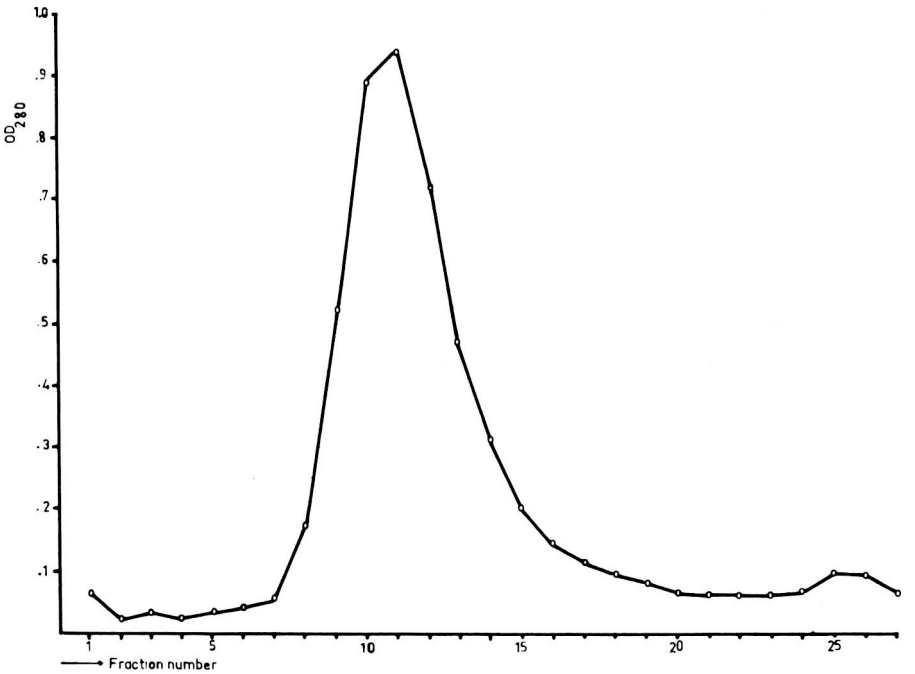

Fig. 1. Fractions from Sepharose 6B 100 column were read at 280 nm and only one peak has been observed.

RESULTS AND DISCUSSION

The presence of ferritin in solutions has shown that it contains a high content of iron relative to protein concentration. The iron to protein ratio of the purified ferritin was estimated to be 22 %. Any protein other than ferritin does not contain so much iron in the body.

In order to test the ferritin preparations for contaminant proteins, bovine spleen ferritin samples together with commercial horse spleen ferritin were run on 5.0 % polyacrylamide disc gel electrophoresis using the standard method of Davis /9/. The gels showed multiple ferritin bands stainable for both iron and protein. The bands observed with decreasing electrophoretic mobilities were corresponded to the monomer, dimer, trimer, etc. The number of bands formed by horse and bovine ferritins in 5 % polyacrylamide

gels were drawn and presented in Fig.2. The electrophoresis of bovine ferritin purified by ultracentrifugation yielded four ferritin bands whereas another preparation purified by gel filtration gave only two ferritin bands. Commercial horse ferritin formed three bands with faster electrophoretic mobilities than those of bovine ferritin (Table-1). No bands were observed that stained for protein but not iron. The presence of multiple bands after disc gel electrophoresis had been reported earlier /11,12/. The origin and the physiological significance of the formation of oligomers are not clear but they have been considered to be the initial steps in the conversion of ferritin to hemosiderin /13/.

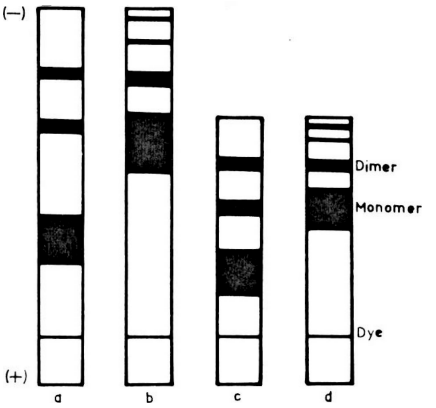

Fig. 2. 5 % Polyacrylamide gel electrophoresis of horse and bovine spleen ferritins. a,c: horse, b,d: bovine spleen ferritins stained for iron and protein respectively.

Attempts were also made to crystallize bovine ferritin with 5 % $CdSO_4$ treatment. Only round edged spheres were observed with bovine ferritin while crystals of horse ferritin with its conformational characteristics were apparent under the microscope.

Consequently we may conclude that these ferritin preparations purified by either method were pure enough for the production of antibody against bovine ferritin.

Table-1. Relative mobilities of bovine and horse spleen ferritins on 5 % polyacrylamide gel electrophoresis with 0.05 M Tris-glycine buffer at pH 8.3. Gels were 9 cm long. For each gel 5 mA was applied for 5 hours.

Species	R_f Values for ferritin polymers			
	Monomer	Dimer	Trimer	Tetramer
Bovine	.50±.01	.225±.005	.10±0	.035±.005
Horse	.79±0	.395±.005	.20±0	

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