ISOLATION OF PROTHROMBIN FROM BOVINE PLASMA BY ION EXCHANGE CHROMATOGRAPHY AND GEL FILTRATION

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Abstract

Prothrombin among several plasma proteins is the most important in blood clotting process. Bovine blood was collected in sodium citrate, centrifuged to obtain plasma. The plasma was assayed for prothrombin using prothrombin time test assay method. The clotting time for the crude plasma was 56 seconds. The plasma was then adsorbed on barium sulphate and separated by anion exchange chromatography. 30 fractions were collected and assayed for prothrombin. Some fractions had complete coagulation within 45 - 50 minutes, while some fractions had partial coagulation within 60 - 65 minutes. These fractions were pooled together and further separated based on molecular weight by gel filtration. 20 fractions were collected and assayed for prothrombin. Some fractions had complete coagulation within the time range of 50 – 60 minutes, while some had partial coagulation within 65 - 70 minutes. From the result obtained prothrombin was successfully isolated from the bovine plasma.

Key words: Plasma, prothrombin**,** anion exchange chromatography, gel filtration

Introduction

Prothrombin (factor II) is a glycoprotein present in plasma that forms one of the indispensable factors of coagulation, with molecular weight 68,000-72000 and alanine amino terminal [1,2,3]. It could be converted to thrombin by extrinsic thromboplastin during the second stage of blood clotting. It is a vitamin K-dependent plasma protein which is synthesised in the liver. Prothrombin among several plasma proteins is the most important in blood clotting process. It contains elements of thrombin molecule which acts to convert fibrinogen to fibrin. This converting activity is a step in blood clotting in which factor Xa and factor V reacts with phospholipids in the presence of calcium to activate prothrombin to form thrombin [4].

The deficiency of prothrombin is known as hypoprothrombinemia. Subjects with a more severe prothrombin deficency have severe brusing, bleeding from the nose and mouth, menorrhagia, muscle bleeds and head bleeds after trauma. Levels of prothrombin deficiency can range from 2.0% - 50.0% of normal blood. Correction of prothrombin deficiency could be achieved with the use of prothrombin complex concentrates (PCCs). It is an enzyme and the amount formed is a function of the amount of precursor substance [5].

The pioneering work of Schmidt in 1872 and Morawitz in 1905 brought out classical theory of blood coagulation. Further support came from Mellanby in 1909 and 1933 respectively who isolated from blood plasma a substance which although had no coagulant activity, but could be converted into an active coagulant resembling thrombin [6].

In classical theory of coagulation, prothrombin was considered to be zymogene form of thrombin, it could be activated and previous studies indicated that the process involves cleavage of the large glycopeptide from the amino terminal end of the molecule [2]. After activation, thrombin and two nonthrombin forming fragments were identified in the reaction mixture[7]. These three activation products account for the total mass of prothrombin molecule. This identification was based on sephadex ion exchange chromatography and Sodium dodecyl sulphate (SDS) electrophoresis [8,9]. The chemistry of prothrombin and mechanism of conversion had been investigated [5]. Human prothrombin was purified from the plasma by coupling barium citrate adsorption, hydroxyapatite chromatography and DEAE sephadex chromatography [10].

The aim of this work is to isolate prothrombin from bovine plasma using anion exchange chromatography and gel filtration. Further work is ongoing to quantify the amount of

prothrombin that could be isolated from a given amount of blood and carry out the electrophoresis of the isolated fractions.

Experimental Methods

The materials for this work were bovine plasma, sephadex G100, sephacel cellulose, tris buffer (0.10 M chloride, tris buffer pH 7.5), 15.00 millimolar chloride, tris buffer pH 7.5, sodium citrate, sodium chloride, barium sulphate, sodium hydroxide, Dade^{R} Actin^{R} FSL Reagent, and calcium chloride. All reagent used for this work were of analytical grade and prepared with distilled water.

Sample Collection

Fresh bovine blood was collected from abattoir Zango, Zaria in Kaduna State from the vein of a three year old male bovine as it was being bled in 0.10 M sodium citrate in the ratio of 9:1 in a test tube. From previous history the bovine had no record of any kind of infection. The blood sample was immediately centrifuged for 15 min at 4630 g using model J-21B centrifuge to obtain bovine plasma. The supernatant obtained (plasma) was stored in the deep freezer at a temperature of 4^{o}C until use for a maximum of 30 hours [11]. The plasma (1.00 ml) was assayed for prothrombin using prothrombin time test (Dade^{R} Actin^{R} FSL Activated PTT Reagent).

Prothrombin Assay

Calcium chloride was prewarmed at 37^{0}C for 2 min while 0.10 ml of Dade^{R} Actin^{R} FSL reagent was prewarmed for 1 min at 37^{0}C on a water bath respectively. Then 0.10 ml of the prewarmed Dade^{R} Actin^{R} FSL reagent and citrated plasma were pippetted respectively into a plastic tube. The mixture was incubated at 37^{0}C for 3 min and the prewarmed calcium chloride

was added simultaneously and shaken well. The crude plasma clotted within 56 sec. Which was an indication that prothrombin was present in the plasma.

Equilibration of Columns

Two columns (height 30 cm, diameter 2 cm) were clamped vertically with a stopper. Glass wool was introduced into the column near the tap with a long rod. The column was packed by pouring the DEAE Sephacel and G-100 Sephadex respectively into each column and allowing them to settle by gravity [12]. The taps were opened for the gel to be properly packed. Then the eluting solvent (0.14 M NaCl in 0.10 M chloride- tris buffer pH 7.5) was introduced to equilibrate the column. The glass wool which is porous allows only the eluting solvent to pass down the column and serve as a support for the gel. It was ensured that the level of the solvent was not allowed to fall below the level of the gel to prevent disruption of the gel bed. The concentration ranged of 0.20 M-1.00 M NaCl were used to wash down the column. Then the prepared buffer 0.14 M NaCl in 0.10 M tris chloride was introduced into the column to elute all the NaCl in the gel. The pH of the eluting solvent was used as an indicator to ensure that all the NaCl has been washed out of the gel.

Preparation of $BaSO_4$ Elute

Exactly 5.00 ml of citrate plasma was stirred for 20 min in 0.4 ml of distilled water containing 0.1 g of $BaSO_4$. The mixture was centrifuge at 4630g for 3 min, after which 1 ml of 1 mM-trisodium citrate containing 0.45 % NaCl was added, then centrifuge for another 3 min, it was stopped and 5 ml of 0.1M citrate NaOH buffer pH 6.8 was added and centrifuged for 30

min. The centrifuge was stopped and the supernant was filtered off. The filtrate obtained is the prepared $BaSO_4$ elute [13].

DEAE Sephacel Chromatography

Exactly 5.00 ml of the $BaSO_4$ elute was carefully layered in the packed DEAE sephacel column. This was eluted at a flow rate of 20 ml/hr with a concentration gradient of 0.10 M-0.50 M NaCl in 0.10 M chloride –tris buffer pH 7.5 [13]. 30 fractions were collected, 3.00 ml each and were assayed using prothrombin time test assay method. The fractions containing prothrombin were pooled together and further separated using gel filtration.

Gel Filtration using G-100 Sephadex

Exactly 6.00 ml of the pooled fractions containing prothrombin was placed on the column and the column eluted at a flow rate of 20 ml/hr with a concentration gradient of 0.10 M NaCl in 0.10 M chloride tris buffer to 0.50 M NaCl in 15.00 mM chloride tris buffer pH 7.5. 20 fractions were collected 2.00 ml each [13]. These fractions were assayed for prothrombin using prothrombin time test assay method.

Results

Table 1: Fractions collected from DEAE Sephacel Chromatography			
Fractions	**Complete coagulation**	**Partial coagulation**	**None**
1	✓		
2			✓
3			✓
4			✓
5		✓	
6		✓	
7		✓	
8	✓		
9	✓		
10	✓		
11			✓
12			✓
13			✓
14	✓		
15	✓		
16			✓
17			✓
18			✓
19			✓
20		✓	
21			✓
22			✓
23			✓
24			✓
25			✓
26			✓
27	✓		
28	✓		
29			✓
30			✓

Table 2: Fractions collected from Gel Filtration			
Fractions	**Complete coagulation**	**Partial coagulation**	**None**
1			✓
2			✓
3			✓
4	✓		
5	✓		
6			✓
7			✓
8		✓	
9	✓		
10			✓
11			✓
12			✓
13		✓	
14			✓
15			✓
16			✓
17			✓
18	✓		
19	✓		
20			✓

Discussion

Citrated plasma was obtained from bovine blood by centrifugation using a J – 21B model centrifuge for 15 min. Prothrombin was isolated from 5 ml of the citrated plasma by barium sulphate adsorption. Prothrombin was isolated from this adsorbate/adsorbent composition based on charge by DEAE sephacel anion exchange chromatography. The fractions containing prothombin were pooled and further purification based on its molecular weight by G – 100 sephadex. The fractions collected from these separation techniques were assayed using

prothrombin time test assay method also known as “one stage assay method” this is a qualitative test carried out to ascertain the presence of prothrombin.

Table 1 is the result of prothombin test on the fractions collected from DEAE sephacel column chromatography. Fractions 8, 9, 10, 14, 15, 27 and 28, showed complete coagulation within 45-50 min while fractions 1, 5, 6, 7 and 21 showed partial coagulation within 60-65 min. Table 2 is the result of prothrombin test on the fractions collected from gel filtration. Fraction 4, 5, 18 and 19 showed complete coagulation within 50-60 min, while fractions 14 and 8 showed partial coagulation within 65-70 min.

Conclusion

The results obtained, indicated that prothrombin could be isolated from bovine blood based on its charge by anion exchange chromatography and molecular weight by gel filtration.

Recommendation

The amount of prothombin that could be isolated from a given amount of blood should be determined and electrophoresis of the isolated fractions performed.

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