Developing a Process for Serum Albumin Recovery from Goat (*Capra hircus*) Blood using Polyethylene Glycol by Optimization Strategy

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Abstract—*Polyethylene glycol (oxy-1,2-ethane diol) used as a precipitating agent to enhance separation efficiency of crude serum albumin by precipitating other heavy molecular weight proteins of blood. Goat (Capra hircus) blood was taken & firstly it was fractionated by addition of different PEG's concentration successively to the supernatant obtained after the centrifugation. The main purpose of this layman approach to study the supernatant phase after the addition of PEG (4000, 6000) & parameters like total protein, serum albumin, separation efficiency, absolute yield & dynamic yield were evaluated separately for respective PEG. The purpose of this study to fit the experimental results into non- ionic polymer based precipitation model developed by juckes. Sugars like glucose, sucrose and trehalose and polyols such as glycerol and sorbitol are known to stabilize biological macromolecules in solution by stabilizing hydrophobic interaction of protein. For optimization purpose parameters considered are polyethylene glycol amount, pH of PEG's solution, & glycerol percentage to maximize albumin fraction in supernatant. These three parameters are optimized by using minitab-16 statistical software to design an experimental data based on response surface methodology in order to find best suitable condition for the polyethylene glycol based blood fractionation in order to give separation efficiency with respect to albumin.*

Keywords*: Absolute yield, Dynamic yield, Minitab-16, Polyethylene glycol, polyol, separation efficiency*

1. INTRODUCTION

Blood is a rich source of protein, about 6 to 8 % protein primarily consisting of albumin, globulins, and fibrinogen as well as more than 100 smaller proteins present in plasma. Plasma is liquid part of blood & when fibrinogen protein taking out from it then it refers as serum. For normal Adult Goat: Blood volume (ml/kg) - 60-70 & Total protein-(55–70 g/L) have albumin (25-40 g/L), α –globulins (4-7g/L) β-globulins(6-12 g/L) & γ-globulins (15-20 g/L) [1].

Albumin is a single non glycosylated polypeptide chain of 585-609 amino acids having molecular weight ranges as 65 kDa, 66 kDa, or 67 kDa. The polypeptide has alpha-helical structures & no β-sheet. There are three structurally similar helical domains (I, II, III), each divided into subdomains A and B. X-ray crystallographic studies have led to albumin being described as a "heart-shaped molecule" while in solution it looks like a flexible ellipsoid shape [2]. A major function of albumin is to provide most of the natural osmotic pressure (oncotic pressure) of plasma. It also has a major role in transportation function like fatty acids, drug molecules, toxins, hormones etc. and is especially important in transporting substances in aqueous media which are sparingly soluble.

Today there are two routes to get albumin protein either from conventional plasma fractionation approach (more common) or using recombinant DNA technology (recently developed). Plasma fractionation approach generally employs precipitation & chromatography strategy in which plasma fractionated into different fractions containing blood proteins. Cold ethanol fractionation [3] is based on the differential solubility of plasma proteins; albumin has the highest solubility and lowest isoelectric point of the major plasma proteins and hence is precipitated last. Industrial scale processes for the manufacture of albumin based on the above protein separation methods have evolved from those primarily based on cold ethanol fractionation to highly automated chromatographic processes and to integrated hybrid processes involving elements of both of these technologies.

Recombinant DNA technology for the production of heterologous proteins involves cloning the gene of a given protein by inserting the gene into a host organism & growing the transformed organism in fermenters of various capacities followed by harvesting and purification steps. The methylotrophic yeast, *Pichia pastoris*, is the most attractive of the alternative yeast expression hosts because systems with this yeast as host usually possess a high production level. The high cell density fermentation of recombinant *Pichia pastoris* for human serum albumin (HSA) production is a high oxygen demand process & this oxygen demand is usually met by increased agitation rate or use of oxygen-enriched air. However, fermentation using microbubble dispersion (MBD) can supply adequate oxygen to the microorganisms at

relatively low agitation rates because of improved oxygen mass-transfer of the microbubbles used for the sparging [4].

2. MATERIALS AND METHODS

2.1 Blood collection & processing

Goat blood was collected from nearby butcher's shop in 50ml sterile centrifuge tube without any anticoagulant agent. Coagulation was permitted to 40min & centrifuged at 6000rpm for 30min with temperature 10°C. Supernatant was collected in fresh tube & further subjected to 0.25micron filter. Filtrate was stored at 4°C & used as source for albumin.

2.2 Chemical reagent preparations

Polyethylene glycol (PEG) 4000 & 6000, carbowax grade was purchased from Srl chemicals. PEG's solutions were prepared in phosphate buffer of desired pH which further sterilized by filtration mode & stored at 4°C.

Bradford Reagent: The assay reagent prepared by dissolving 100 mg of Coomassie blue G250 (CBG) in 50 mL of 95% ethanol then solution is mixed with 100 mL of 85% phosphoric acid and make up the volume to 1 L using distilled water[5].

The reagent should be filtered through Whatman No. 1 filter paper and then stored in an amber bottle at room temperature. It is stable for several weeks. However, during this time dye may precipitate from the solution and so the stored reagent should be filtered before use [5].

Preparation of BCG assay's reagent [6]

1-Succinate acid (0.1M pH-4 prepared by 11.9g in 800ml distilled water & adjust pH using 0.1N NaOH & make it volume upto 1L & stored at 4°C).

2 -BCG dye stock solution (0.60mM, by dissolving 0.420gm of dye in 10ml 0f 0.1N NaOH in 1L volumetric flask & dilute upto its volume using distilled water & stored at 4°C).

3 -Working solution preparation (0.15 mmoles BCG per liter). 1 volume of bromcresol green stock solution is added to 3 volumes of succinate buffer (0.10 moles per liter). Brij-35, 30%, is added (4 ml per liter) and the pH adjusted to 4.2 ±0.1. Addition of 5ml of working solution for determination of protein contain in solution using standard curve.

Surfactant (Brij- 35) is included in the working dye solution to minimize the absorbance of the reagent blank, to prevent turbidity and gave linearity in calculation however tween 20 may be used as an alternative.

2.3 Protein Assay

Bradford Assay (for total protein)

The CBG dye binds more specifically to the cationic residues, lysine and arginine hence it implies that the response of the assay would depend on the amino acid composition of protein, this consider major drawback of the assay [7]. The original method designed by Bradford remains the most convenient and widely adopted assay for fast determination of protein after 2 min and before 1hr in 3 ml cuvettes against a reagent blank prepared from 0.1 ml of the appropriate buffer and 5 ml of protein reagent (standard assay). Two types of assay are described here: the standard assay, which is suitable for measuring between 10-100µg protein, and the microassay for detecting between 1-10 µg protein [7].

BCG (Bromocresol green) Assay (for serum albumin)

The BCG method introduced in 1965 specifically to measure albumin protein in a solution containing other proteins. This method is rely on the specific binding of BCG, an anionic dye, to the protein at acid pH about 4.2 led to produce a color change of the indicator from yellow-green to blue-green by shifting in the absorption wavelength of formed complex (Dye + albumin) & absorbance noted at 630nm. The intensity of the color formed deciphered as the concentration of albumin in the sample. This approach enable to monitor the absorbance change from about 5 s after mixing BCG reagent and serum but albumin concentrations determined from this showed no significant differences from those obtained by Laurell rocket immune-electrophoresis when absorbance recorded within 10 second otherwise prolong time it will overestimate [8].

2.4 Procedure of fractionation & optimization

For stepwise fractionation-

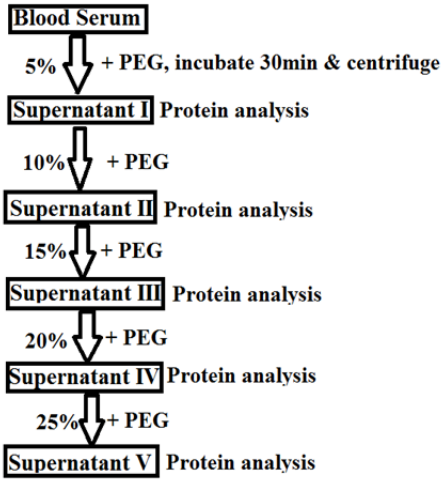

Fig. 1: Simplified approach for fractionation of blood serum by addition of different PEG conc. to successive supernatant & each steps supernatant go through protein analysis.

Parameters were evaluated at each step-

Separation efficiency is the ratio of crude albumin protein per total protein.

$$Separation\ efficiency, SE = \frac{crude\ serum\ albumin}{total\ proten} \times 100$$

Absolute yield is the ratio of crude protein amount to the actual albumin's amount protein present in sample before precipitation.

Dynamic yield is the ratio of crude albumin to the amount of crude albumin present in the preceding supernatant.

Juckes model [9]-

When non-ionic polymers added to the solution resulted in steric exclusion of protein molecules from the volume of solution occupied by the polymer. A model developed by Juckes model for this phenomenon by considering the protein molecule as a solid sphere and polymer molecule as rod form, which gave the following equation for S, the solubility of the protein:

$$\ln S = \beta' - K' C_p$$

$$K' = \frac{\bar{V}}{2.303}\left(\frac{r_s + r_r}{r_s}\right)^3$$

S =solubility of the protein; β′ is a constant & r_s and r_r the radius of the protein solute and polymer rod, respectively, V is the partial specific volume of the polymer, c_p is the polymer concentration.

For optimization-

Three parameters like PEG's amount, PEG's solution pH & Glycerol amount were taken for optimization. The optimized values were used in minitab-16 software to design an experiment based on response surface methodology. The values obtained from designed experiment were used to get best result with respect to separation efficiency.

3. RESULTS & DISCUSSION

3.1 For stepwise fractionation

When PEG (6000 & 4000) were added to blood serum separately in successive manner (Fig-1) means after addition of respective PEG concerned parameters were evaluated using supernatant, obtained after centrifugation. This supernatant further subjected for the addition of higher concentration of respective PEG & same parameters were evaluated from supernatant & this process was done for five steps as shown in fig.2 & fig.4 (Total protein-mean±6mg/ml; albumin mean±3mg/ml; separation efficiency-mean±2% & yield-mean±2%).

The results obtained in both cases were fitted into juckes model equation for total protein & serum albumin for respective PEG. For PEG-4000 referred to Fig.3, r^2=0.9556 for total protein (mg/ml) & r^2=0.9659 for serum albumin (mg/ml). While for PEG-6000 referred to fig.5, r^2=0.98 & r^2=0.99 for total protein (mg/ml) & serum albumin (mg/ml) for respectively.

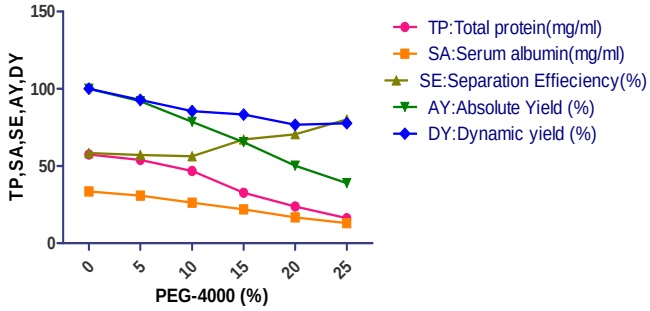

Fig. 2: Stepwise fractionation resultant graph with parameters for PEG-4000 at pH-7.4 & 37°C.

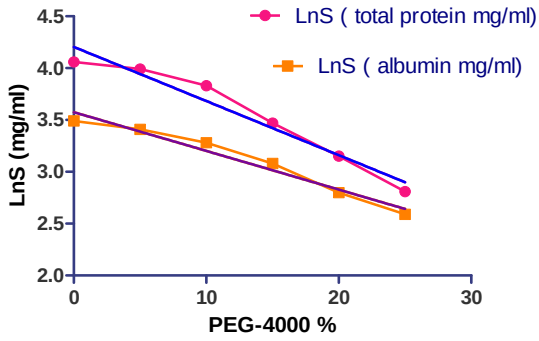

Fig. 3: Juckes Model fitted experimental data semi-log graph generated by stepwise addition of PEG-4000 at pH-7.4 & room temperature.

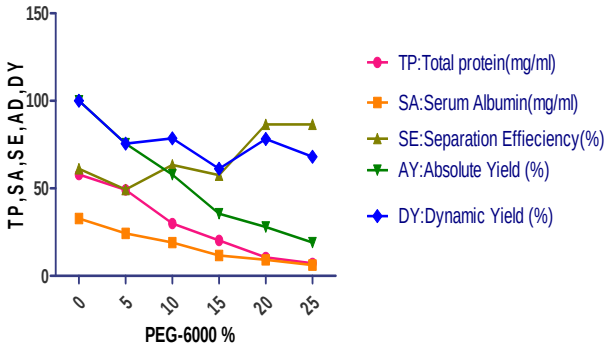

Fig. 4: Stepwise fractionation resultant graph with parameters for PEG-6000 at pH-7.4 & 37°C.

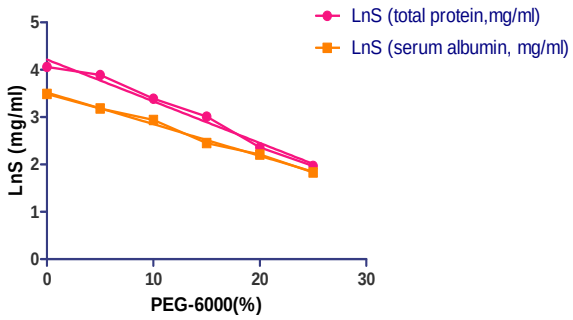

Fig. 5: Juckes Model fitted experimental data semi-log graph generated by stepwise addition of PEG-6000 at pH-7.4 & room temperature.

3.2-For optimization of PEG-4000

When we added PEG's flakes directly to distilled water then it cause alteration in pH of PEG's solution so all PEG solution were prepared in buffer. For optimization, three parameters were considered like Glycerol amount, PEG's amount & pH of PEG's solution.

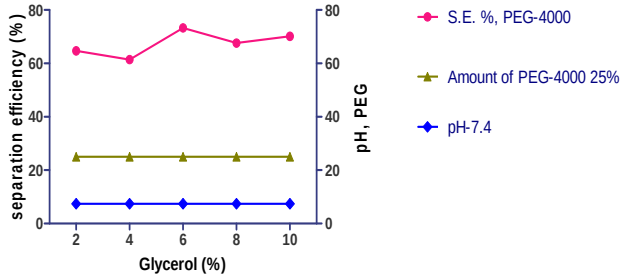

Fig. 6: Graph showing optimization of glycerol amount (%) by keeping PEG-4000 % & pH of solution constant.

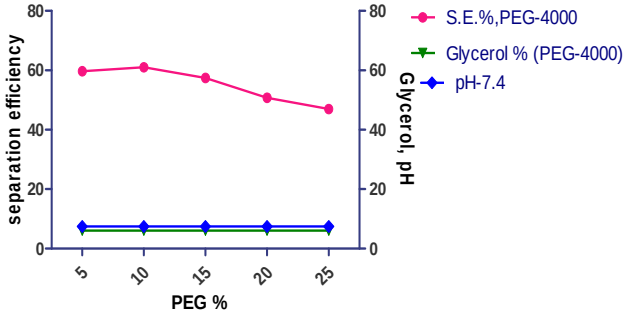

Fig. 7: Graph showing optimization of PEG-4000 amount (%) by keeping Glycerol % & pH of solution constant.

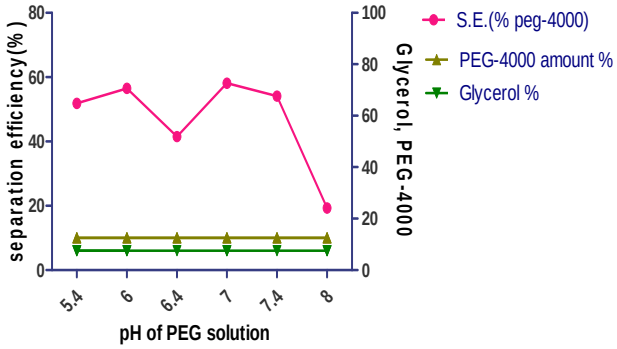

Fig. 8: Graph showing optimization of pH of PEG-4000 solution by keeping PEG-4000 amount % & Glycerol %.

Optimized parameters; 6% glycerol from Fig.6, 10% PEG-amount from Fig.7 & pH-7 from fig.8 gave separation efficiency: 60.24%±2%, 72.48%±2% & 58.12%±2% respectively. So these values are considered in minitab-16 tool for designing an experiment.

3.3. Designed conditions for separating serum albumin from Goat blood serum using polyethylene glycol 4000 by response surface methodology using minitab-16 tool.

PEG-4000 %	pH	Glycerol %
10	6.9	6
15	6.4	4
10	7.4	6
10	6.9	6
10	6.9	8
15	7.4	8
5	6.4	4
15	6.4	8
10	6.4	6
10	6.9	6
5	7.4	4
10	6.9	6
5	6.4	8
10	6.9	4
10	6.9	6
15	6.9	6
15	7.4	4
5	7.4	8
10	6.9	6
5	6.9	6

4. CONCLUSION

To filter out best approach of fractionation for respective PEGs one must consider the process by two ways; firstly in terms of Absolute yield & separation efficiency, number of fractionation steps should be minimum without compromising above two parameters. For purity conformation SDS-PAGE & HPLC can be used & residual PEG present in protein solution

can be removed by DEAE cellulose based approach & ethanol based precipitation.

Once optimization of precipitating agent is done then for large scale process precipitation design system may be design.

5. ACKNOWLEDGEMENTS

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REFERENCES

[1] Alberghina D, Casella S, Vazzana I, Ferrantelli V, Giannetto C, Piccione. Analysis of serum proteins in clinically healthy goats (*Capra hircus*) using agarose gel electrophoresis. *Vet Clin Pathol*. **2010** *Sep;39(3):317-2*1

[2] *He XM, Carter DC.* " Atomic structure and chemistry of human serum albumin". Nature Nature 358, 209 - 215 (16 July 1992)

[3] Cohn EJ, Strong LH, Hughes WL, Mulford DJ, Ashworth JN, Merlin M, Taylor HL. (1946) "Preparation and properties of serum and plasma proteins IV. A system for the separation into fractions the protein and lipoprotein components of biological tissues and fluids". J Am Chem Soc, 68, 459–475.

[4] KOBAYASHI K., KUWAB S., OHYA T. etal "Addition of Oleic Acid Increases Expression of Recombinant Human Serum Albumin by the AOX Promoter in *Pichia pastoris* " JOURNAL OF BIOSCIENCE AND BIOENGINEERING Vol. 89, No. 5, 479-484. 2000

[5] Bradford, M. M. (1976) "A rapid and sensitive method for the quantitation of microgram quantittes of protein utilizing the principle of protein-dye binding". Anal. Biochem.

[6] Rodkey, F. L., "Binding of bromcresol green by human serum albumin." Arch. Biochem. And Biophys. 108, 510 (1964Biochem Tech Bull No 11, 1967.

[7] Compton, S. J. and Jones, C. G (1985) "Mechanism of dye response and interference in the Bradford protein assay". Anal. Biochem.

[8] Corcoran RM, Duman SM. "Albumin determination by a modified bromcresol green method". Clin Chem 1977

[9] Juckes, I. R. M. (1971)." Fractionation of proteins and viruses with polyethylene glycol". Biochem. Biophys. Acta, vol. 229, p. 535