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Extraction of Vitamin B2 from Bovine Colostrum using Surfactant Based ATP System

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Abstract: Riboflavin, also known as vitamin B2, is an easily absorbed micronutrient with a key role in maintaining health in humans and animals. The objective of the present study was to compare the extraction and partition coefficient of Riboflavin present in bovine colostrum using various surfactants based aqueous two phase (ATP) system. A new efficient technique, surfactant based ATPs were used for the separation and purification of high value bio-molecules Here the Triton X- 100 / Tween 80 based ATP system was used and it is the best alternative for the conventional organic solvent extraction and polymer based ATP systems. The resulting protein was purified and confirmed on gel filtration chromatography column. From the results it was found that Tween 80 based ATP system is highly selective and yield higher than the Triton X- 100 based ATP system.
Keywords Surfactants, Bovine Colostrum, Riboflavin, Aqueous Two phase.

Introduction

Vitamins are an essential group of food, which are essential in the diet for maintenance of body cell and normal metabolic functions. They are divided into water-soluble and fat soluble vitamins [1][3]. The well-known B-complex belongs to the first group. Vitamin B2 (riboflavin) has a well-defined process in metabolism of fats, carbohydrates, and respiratory proteins [4]. So it is important to develop a simple, fast, and inexpensive method for the determination of this compound. Several analytical methods have been developed for the determination of vitamin B2 concentrations including flurometric [5], liquid chromatographic [6], and capillary electrophoretic methods [7]. The standard method of determination of B1, B2, and B6 in medicine is spectrophotometry. Riboflavin (Vit.B2" C17 H2O N4 °6" Mol.Wt.376.36) is greenish yellow substance present in all body fluid especially blood and milk which play a significant biochemical function.

A mutual incompatibility between two phase components results in the formation of aqueous biphasic system [2, 3]. It is a Liquid-Liquid extraction technique which has significant advantages over other conventional system such as short time consumption, quick phase separation, selective extraction, improved purity and yield[4-7]. The flexibility of the ABS has already been reported for the extraction and purification of biological products just as plant proteins, enzymes, antibodies and drug molecules by polymer/polymer or polymer/salt system [8-10]. Despite, the characteristic features of polymers like high viscosity, density and restricted polarity interfere with the effective phase formation. In this regard, it is essential to develop a new and efficient ABS system. [11, 12]. In addition to that Surfactants have together lipophilic and hydrophilic character for extensive partitioning of biological materials. Besides, the presence of high water content in aqueous phases

in Surfactant–salt ATPS provides additional advantages than other form of ATPs[13]. Various biomolecules such as, proteins, vitamins , monoclonal antibodies, antibiotics, cell organelles and growth factors has been fractionated by aqueous two phase system for preparative reasons. Detergents based Aqueous two-phase systems can also be formed by use of salt/polymer in addition to the polymer/polymer and polymer/salt systems[14]. Hence, the present work was performed with an objective of designing new surfactant based AB system of Tween80/Triton X-100 +K_2HPO_4 for partitioning of Riboflavin. The resulting undergoes gel filtration chromatography technique for purity range. This study moreover investigates a specific activity of the aqueous two phase extraction of Tween 80/Triton X-100 based ATP system.

Experimental Details

Materials and Purity

Tween 80 and Triton X-100 (98%) were purchased from Sigma Aldrich and Di potassium hydrogen phosphate (99.5%) was purchased from Hi media and used without further purification. Double distilled water was used for solution preparations throughout the experiment.

Apparatus

Analytical balance (BL-220H, Shimadzu Corporation, Japan). Hot Plate (Technical made in SUNBIM, India). Magnetic stirrer (Remi Laboratory, India). PC based Double beam spectrometer (Model2202 Systronics, India).

Crude preparation and Characterization

Crude bovine Colostrum was collected from the well healthy bovine in local dairy farms of Thanjavur. The collected Colostrum was then skimmed by centrifuged at 10000 rpm for 3o minutes at 4 °C. Casein was precipitated by adjusting the pH between 4.6-4.8 with 0.5 M HCl under stirring. The precipitated casein was discarded and the whey supernatant was centrifuged at 17,902 g at 4 °C for 20 min using an Eppendorf Centrifuge Model 5810R. Final whey filtration was achieved with a 0.45 µm membrane filter. The whey was adjusted to the desired pH using 0.5 M NaOH[15].

Purification of Riboflavin in Triton X 100+K_2HPO_4 system

Riboflavin partitioning was carried out with Triton X 100 and K_2HPO_4 ATP system. Crude extract was added to the aqueous two phase mixture and mixed gently. Centrifugation was performed at 6000rpm for 30 mins at 4◦C for effective phase separation. Two phases formed were separated carefully. Backward extraction was done with top phase separated by adding 150mM NaCl and allowed for phase separation. Then, the resulting bottom phase was subjected for gel filtration chromatography.

Purification of Riboflavin in Tween 80+K_2HPO_4 based ATP system

Forward extraction

Colostrum whey protein isolate which were stored in phosphate buffer pH 6.5 were mixed with Tween 80 and inorganic salt K_2HPO_4. The concentrations of surfactant and salt used for the forward extraction were considered from the binodal curve. At defined concentration, the Tween 80 and salt were mixed in a graduated centrifuge tube with clarified whet isolate. The temperature of the system was adjusted to 25◦C .And the two phase separation was carried out by performing centrifugation at 6000 rpm for 10 minutes, and then the resulting top phase was carefully separated to perform backward extraction.

Backward extraction

In the backward extraction process, aqueous two phase system was formed by blending 20% of NaCl salt with the top phase separated from the forward extraction. The centrifugation of the mixture at 8000 rpm for 10 minutes resulted in two phase formation and the salt rich bottom phase containing the target riboflavin was separated by micropipette and the Gel filtration chromatography analysis was carried out to perform purification studies.

Riboflavin assay

Riboflavin concentrations were assayed by measuring absorbance of Top and bottom phases obtained after phase separation, at 510 nm using an UV-Visible spectrophotometer (Model Ultra spec 2100 Pro, Amersham Biosciences, England). An absorbance-concentration standard curve was developed from six standard concentrations from 0 to 1 mg mL-1. Samples were diluted with reference buffer to within the absorbance range of the standard curve.

Gel Filtration Chromatography

Whey component was identified by using Gel Filtration Chromatography (GFC) or size exclusion chromatography. A Sephadax G -250 column (AKTA Prime Plus, GE, and Sweden) was used for protein purification with the column size of 5ml. The column was previously equilibrated with pH 6.8 buffer solution at flow rate of 2.5ml/min. Sodium phosphate buffer was prepared with 0.05% sodium azide to identify the occurrence of riboflavin. The samples isolated from the backward extraction were injected to remove the ions present in the salt solution. A sample volume of 0.5 ml was introduced into the column through an injection port. The sample elution occurred at the same flow rate were collected separately with fraction collector. The elution was monitored by UV-Vis spectrophotometer at 510nm and the data obtained were interpreted with prime view software. The final fraction was collected and the presence of riboflavin was confirmed by assay.

Results and Discussion

The extraction of riboflavin from crude sample was carried out through Triton X 100 ATP system and compared with Tween 80 ATP system. The results show that the maximum extraction was found at Tween 80 based ATP system. The parameters like pH and temperature were analyzed against specific activity.

Triton X 100 ATP system Vs Tween 80 based ATP system

The partition coefficient (K_R) was found to be 25 in Triton X 100 ATP system where Tween 80 based ATP system gives maximum K_R of about 48 were shown in Table 1 and 2. The gel filtration chromatography results gives purity factor about 80.4 and 87% yield from the Tween 80 based system.

Table 1 Riboflavin Extraction – Triton X 100 ATP system

System	Partition Coefficient, K_R	% yield	Purity factor
Crude	25	100	1
Triton X 100 + KH_2PO_4	30	56	2.80
GFC	-	45	4.0

Table 2 Riboflavin Extraction –Tween 80 based ATP system

System	Partition Coefficient, K_R	% yield	Purity factor
Crude	25	100	1
Tween 80 +K_2HPO_4	48	95	35.5
GFC	-	87	80.4

Effect of pH on Specific activity

Figure 1 depicts the interrelationship between the pH and Partition coefficient of the Tween 80 based ATP system. The highest partition coefficient was found to be at pH 6.5.At higher and lower pH conditions there is tremendous decrease in the K value and there was no appreciable distribution observed at pH below 4. The partition coefficient shows sharp decrease which depicts the acidic or alkaline strength which denatures the vitamin property.

Effect of Temperature on specific activity

In a Tween 80 based ATP system, Figure 2 shows that the effect of temperature towards the partition coefficient. The K_R value was found to be higher at 30ºC which reveals the high extraction of riboflavin at

optimum temperature shows the endothermic nature of the process. Further increase in temperature affects the partition coefficient which underwent irreversible instability of the vitamin property. The study depicts the unfavour phase formation at low temperature range and denaturation at higher ranges of temperature which reduces the protein interactions towards surfactant.

Purity Study on Gel Filtration Chromatography

The resulting fractions were undergone purification on Gel Filtration Chromatography column. Based on the molecular weight the target molecules were eluted. The resulting peak from the GFC chromatogram confirms the purity range of the systems is shown in Figure 3 and 4. From the GFC fractions, Tween 80 based ATP system achieved high selective separation for riboflavin extraction.

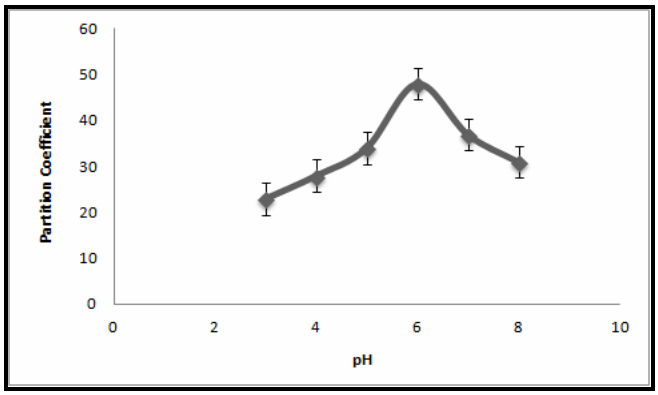

Fig 1: Effect of pH on partition coefficient of Riboflavin

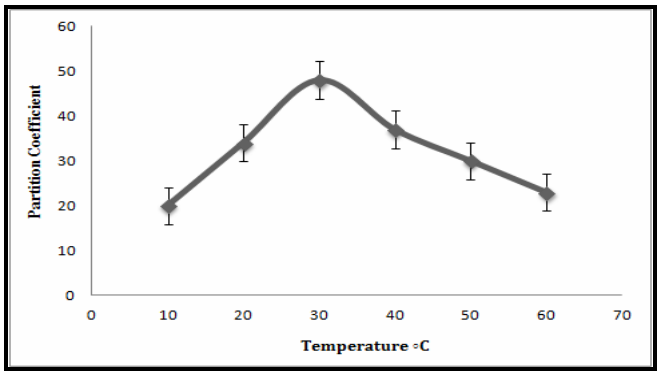

Fig 2: Effect of temperature on partition coefficient of Riboflavin.

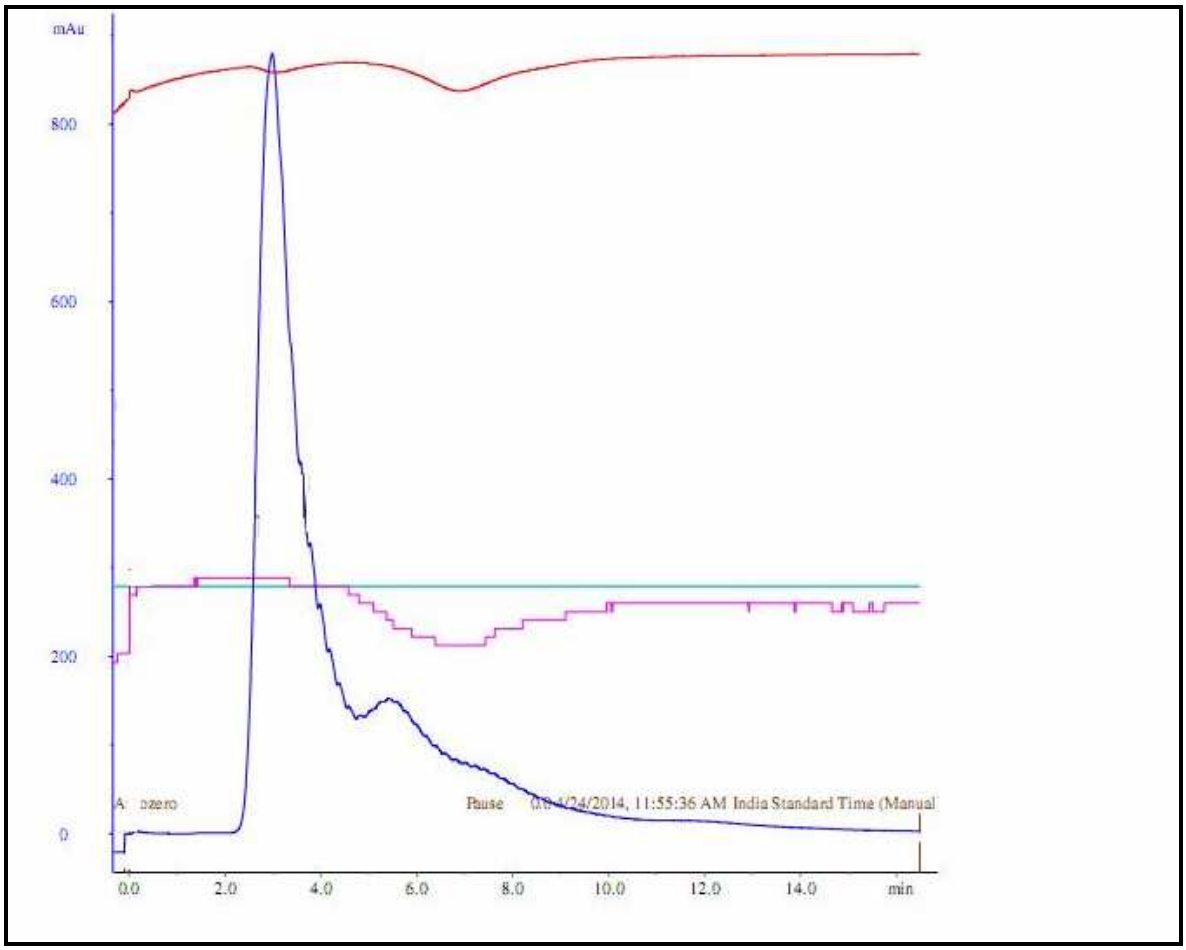

Fig 3: GFC for riboflavin from backward extraction on Sephadex G 100 column, 5ml @ pH 7.5 in Triton X 100 ATP system with a flow rate of 2.5ml/min.

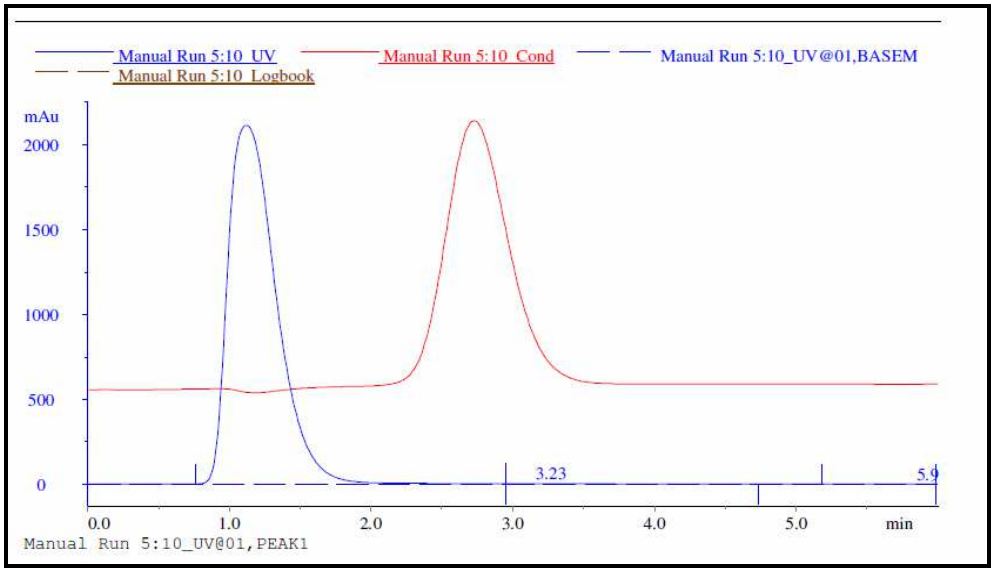

Fig 4: GFC for riboflavin from backward extraction on Sephadex G 100 column, 5ml @ pH 7.5 in Tween 80 based ATP system with a flow rate of 2.5ml/min.

Conclusion

This work reveals the advantages of surfactant based ATP system for the purification of riboflavin from Bovine colostrum. This method provides a convenient method for pure enzyme extraction. The extraction of riboflavin through Tween 80 based ATP system proves a simple and rapid process compared with other conventional methods. Surfactant based ATP system shows high selectivity and easy scalability of the system favours the large scale application of riboflavin purification in industries.

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