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β amylase purification from sweet potato (*Ipomoea batatus*): Reverse micellar extraction versus ammonium sulphate precipitation

Santhosh Ramakrishnan and Senthil K. Rathnasamy*

Bio Separation Engineering laboratory, School of Chemical and Biotechnology, Sastra University, Thanjavur, India

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ABSTRACT

Extraction of β Amylase from sweet potato (Ipomoea batatus) in reverse micellar system was studied and compared with the conventional procedure using ammonium sulphate precipitation. The β Amylase recovered using latter method was generally contaminated with other proteins, and its purity was dependent on the crude protein concentration in the source used for separation. Highly pure β Amylase was obtained in two steps from its aqueous extract of sweet potato by the use of reverse micellar extraction system of AOT(Sodium bis-2 ethylhexyl sulphosuccinate)/Iso octane. The process parameters such as surfactant concentration, pH in the forward extraction and Ionic strength, pH in the backward extraction are varied to optimize the RME system efficiency. The resulting fractions were studied for purity using gel filtration chromatography with73% protein recovery with 33 purity fold in RME system, where as in the precipitation method it is 42% recovery with 9 folds.

Keywords: β Amylase, Sweet potato, Reverse micellar extraction, Ammonium sulphate precipitation

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INTRODUCTION

Bioseparation processing is developing as a significant and separate discipline in bioprocess industry which involves the separation and purification of high value bio active molecules that are derived from various crude sources. It is a set of unit operations performed step by step which depends upon the feed status inorder to improve the yield and purity of the desired product. In this process sequence, the core step acts as the deciding unit operation which has to be optimized and characterized for the target compound.

Organic solvent extraction dominated in extraction process industry till the previous decade with inherent disadvantages such as poor selectivity and protein denaturation. The major disadvantages in organic extraction like protein functional loss and intermediate complex formations are overcome by biological extraction methods viz. aqueous two phase extraction, reverse micellar extraction and super critical fluid extraction[1]. Among these methods, reverse micellar extraction applications are emerging highly popular and used because of its high selectivity and ease of scale up facility[2, 3]. Reverse micellar extraction involves the formation of micelles within an organic solvent which attracts the target protein into it[4, 5]. In reverse micelle, the surfactant molecules with hydrophilic head project towards the centre surrounded by organic liquid whereas the hydrophobic tail faces outwards[6] and it facilitates mild separation conditions with high selectivity.

Amylase is an enzyme which breaks down starch into sugar molecules. β amylase is one of the forms of amylase which is also synthesized by bacteria, fungi and plants. Sweet potato, cereal, grains and wheat are the main sources

of β amylase. When compared to other sources sweet potatoes have the major β amylase enzyme. β amylase is used in the production of maltose syrup. Sweet potatoes belong to convolvulaceae, which have high nutritive value.

Sweet potato (*Ipomoea batatus*) is high nutritive and good source of sugar, carbohydrate, calcium, iron, minerals and vitamins. It is another rich source of β amylase. It is also investigated for many pharmacological and medical applications like anti-inflammatory, antimicrobial, antihypertensive, and antioxidant activity. Sweet potatoes have been selected for this study since it is cheap and also a rich source of β amylase. The present study is about the design and development of bioseparation sequence for the β amylase enzyme from its crude source of sweet potato. The reverse micellar extraction is opted as the core step of this sequence, to be optimized and characterized. The purity and yield estimation is also studied and evaluated for process selectivity.

MATERIALS AND METHODS

2.1 Materials

Sweet potatoes used in this work were bought from the local market. AOT (sodium bis-2 ethylhexyl sulphosuccinate) was procured from SIGMA and Iso-octane (AR GRADE) was purchased from Merck, Mumbai, India All other chemicals used for the estimations were of AR grade.

2.2 Methods

2.2.1 Crude preparation and pretreatment

Sweet potato was peeled off, weighed for 50 grams, washed, cuts into small pieces and soaked in sodium sulphite solution (1%) for 3 hours in order to remove impurities. Over a period of 24 hours the pieces were dried in hot air oven at 40ºC and then crushed. By centrifugation at 10,000 rpm for about 10 minutes, the solid particles in the crushed mixture were separated.The supernatant was collected separately and stored at 4ºC.

2.2.2 Protein fractionation by Ammonium sulphate precipitation

Clarified enzyme solution was used for this step by dissolving it in the 50mM phosphate buffer and the pH was adjusted to 6.5. Previously the protein content and enzyme activity was determined for the crude enzyme solution. 10 ml of enzyme solution was saturated by ammonium sulphate from 30%w/v to 80%w/v. The salt was mixed thoroughly and then the salt enriched solution was incubated at 4^oC for 8hr. The saturated solution was centrifuged at 10000rpm for 20minutes. Supernatants were discarded and the precipitates were dissolved in the phosphate buffer at pH of 6.5. The precipitates were estimated for protein content and enzyme activity.

2.2.3 Reverse Micellar extraction (RME): Forward extraction system

β amylase enzyme was extracted from its clarified crude source using anionic surfactant based Reverse micellar extraction (RME) system. The RME system was formed by dissolving AOT in the iso-octane. In forward extraction, 3 ml of pretreated enzyme sample was mixed with equal volume of Reverse micellar solution and incubatedfor 30 minutes at room temperature. The above mixture was centrifuged at 6000 rpm for 10 minutes. The phases were separated without interference with each other and the phase volumes were measured.

2.2.4 Reverse Micellar extraction (RME): Backward extraction system

The backward extraction was performed for enzyme recovery and simultaneously with surfactantseparation. The top micellar phase from forward extraction was used for this extraction.2 ml of micellar phase (β amylase bounded) was mixed with an equal volume of fresh aqueous phase containing NaCl in order to recover the enzyme from the micellar phase to this aqueous phase.The above mixture was incubated at room temperature for 40 minutes and then centrifugedat 8000 rpm for 10 minutes. The phases were separated and werecheckedfor β amylase enzyme activity and total protein estimation.

2.2.5 Total protein estimation

By using Lowry total protein estimation, the protein content of the sample was assessed. The calibration chart was constructed using BSA as standard [11].

2.2.6 β amylase assay

Enzyme activity of the samples was estimated for β amylase as one enzyme unit converts 1mg of maltose per minute from the substrate starch. With dilutions ranging from 0.2 to 1 mg/ml, a maltose standard curve was set up. Samples were buffered with 0.0016M sodium acetate maintained at pH 4.8. 2ml sample of enzyme extract was added with

1ml starch solution was kept for incubation for about 15minutes at 37^{o}C. Di-nitro salicylic acid reagent was added to all the samples. The reaction mixtures were boiled at 95^{o}C for 5 minutes and then cooled. After zeroing the spectrophotometer, the absorbance was read at 540 nm.

2.2.7 Optimization of Surfactant concentration, pH in Forward extraction:
Reverse micelle formation is highly dependent on the surfactant concentration. Concentrations of AOT are varied from 50 to 200mM with an increment unit of 25. The pH of the sample should be varied from 3 to 5 with increment units of 0.5.The pH of the sample should be below the pI value for the better extraction. The above conditions are applied in the forward extraction of RME system and the parameters are studied for maximum enzyme activity.

2.2.8 Optimization of pH and KCl in backward extraction:
Phosphate buffer was used for the pH variation in backward extraction. The pH of the RME system was varied from 3.5 to 5.5. KCl dissolved aqueous phase was used in the backward extraction for enzyme migration. KCl was added with distilled water for ion migration in the solution. Since the removal of enzyme was favored when divalent concentration is more, the negative ion migration is enhanced. The concentration of KCl was varied from 0.25 to 1.25M.

2.2.9 Purity analysis on Gel filtration chromatography(GFC) column
Purity analysis of the β amylase obtained from the salt precipitation and RME backward stage were performed on the gel filtration chromatography on a Histrap 5cm, 5ml Sephadex G100 column /AKTA Prime plus FPLC system (GE life sciences, SWEDEN) pre-equilibrated with phosphate buffer at pH 6.5 about 10 column volumes (CV) at a rate of 3ml/min. β amylase enzyme sample obtained from the salt precipitation and RME systems was preconditioned with equilibration buffer.2ml of enzyme sample was injected in to the column and eluted with the same equilibration buffer at a flow rate of 1.5ml/min. Fractions were collected when UV (278nm)absorbance showed peak in the PC interface by fraction collector. Protein content and the β amylase assay were carried out for the fractions.

RESULTS AND DISCUSSION

The present investigation deals with selectivity of the β amylase extraction from sweet potato and the comparison study of using salt precipitation and Reverse micellar extraction. The conventional salt precipitation method was employed using ammonium sulphate and the enzyme solution was precipitated at 45% saturation. There were many protocols reported for the β amylase enzyme purification both from the microbial source and natural plant source. Amongst plant sources sweet potato was found to be a cheap source with rich β amylase content, which was yet again proved in this investigation. An attempt has been made to develop a highly selective separation operation using the minimal number of treatments. In our study, the pre treated crude enzyme was utilized for β amylase enzyme extraction using reverse micellar extraction system. The system comprised of AOT/Isooctane in the presence of clarified enzyme solution in forward extraction. The β amylase enzyme bound top phase was treated using backward extraction by the addition of NaCl. The RME system developed has a core unit operation and the same was optimized for the maximum enzyme separation. The system also characterized with respect to pH, surfactant concentration in the forward extraction and salt concentration in the backward extraction for the β amylase maximum enzyme activity. The fractions were studied for purity analysis on the gel filtration chromatography column (Sephadex G100).The final results were compared with the conventional ammonium sulphate precipitation method and listed in Table. I&II. The resulting β amylase activity was found to be 3452Units with purity fold 9 and the protein recovery was 45% in salt precipitation. 12659Units in reverse micellar extraction and the yield was found to be 73 and protein fold is 33.

Table I: β amylase separation from sweet potato using Ammonium sulphate precipitation

Steps	Protein content, µg	Enzyme activity, U	Specific enzyme activity, U/µg	% Yield	Purity factor
Crude Sample	248	95	383	100	1.0
Ammonium sulphate precipitate	112	318	2839	45	7.4
GFC	104	359	3452	42	9

Table II: β amylase separation from sweet potato using AOT based Reverse Micellar extraction

Steps	Protein content, µg	Enzyme activity, U	Specific enzyme activity, U/µg	% Yield	Purity factor
Crude	248	95	383	100	1.0
Sample	218	1289	5940	88	15.5
RME: Forward	195	1500	7692	79	20
RME: Backward	182	2304	12659	73	33
GFC					

3.1 Protein fractionation with ammonium sulphate precipitation:

The pretreated enzyme solution was used for the investigation. The resulting clarified enzyme solution was treated for the enzyme extraction and purification. To the enzyme solution ammonium sulphate was saturated from 30% to 80%. The sample solutions were kept under controlled temperature with continuous mixing. The hydrophobic content in the enzyme decided the time and amount of the precipitation. The enzyme solution was centrifuged and the pellet was separated from the supernatants. The pellets were re-suspended in the phosphate buffer with a pH of 6.5. Total protein and β amylase enzyme activity was estimated in the pellet from and elution of the gel filtration chromatography.

3.2 Effect of AOT concentration in RME forward extraction system

AOT concentration was studied at the range of 50mM to 200mM in presence of Iso-octane for the maximum enzyme activity present in a sample volume of about 5ml. Reverse micellar extraction is majorly influenced by surfactant concentration and it serves as platform for the extraction. There is no micelle developed when concentration of anionic surfactant is below 25mM.Protein solubilisation strongly depends on the concentration of surfactant and hydrophobicity content of the micelle. An increase in the concentration of AOT resulted in an increase in hydrophobicity with anionic charge. The resulting protein interactions favor high affinity towards the β amylase. The enzyme was encapsulated inside the hydrophilic heads and the remains were retained in the aqueous phase. It was observed from the Fig. I that the enzyme activity increases from 870U/ml to 1225U/ml along with concentration of AOT and the maximum was found at 100mM. β amylase activity started to decrease after this AOT concentration and it declined to 960U/ml at the AOT concentration 200mM due to the higher hydrophobic charges which reduced the electrostatic interaction of β amylase with AOT micelle. β amylase enzyme recovery was also found less at higher concentration of AOT. High enzyme recovery was suppressed due to unfavorable electrostatic interaction in forward extraction.

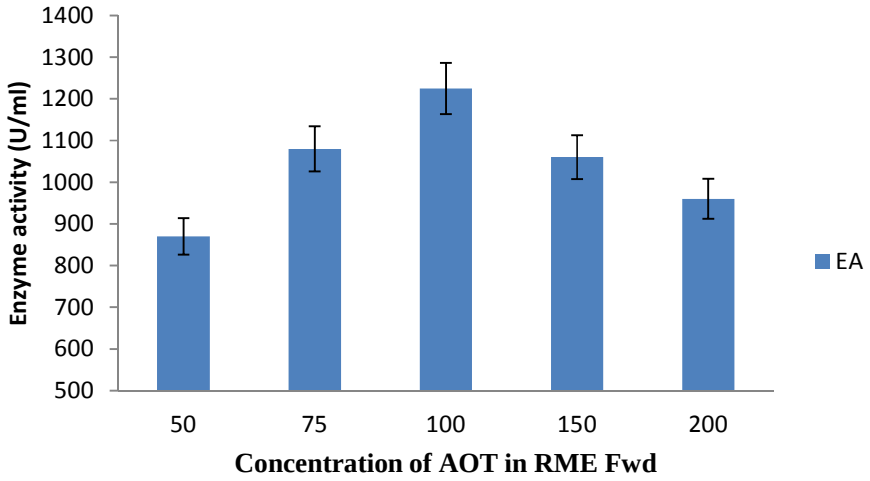

Fig. I- Effect of AOT concentration in RME forward extraction

3.3 Effect of pH in RME forward extraction

The effect of RME system pH was studied on β amylase activity in the forward extraction at 100mM AOT concentration in Iso octane. The system pH was studied from 3 to 5 with an interval of 0.5 against the enzyme activity. The system pH determines the protein net charge. Enzyme activity increased rapidly when the system pH

increased from 3 to 4 and the maximum enzyme activity (1260U/ml) was found at pH 4 from the Fig. II. The pH of the system was still raised to pH 5 in two intervals, but the enzyme activity decreased slightly to 1060U/ml. This was due to the net charge of protein which did not favor enzyme extraction. Thus the pH of the sample was optimized to 4.0 for the forward RME extraction. The pH of the sample was maintained below the pI value of the sample. When the pH was increased the ions got distracted which did not result in any active recovery[7, 8]. There was no appreciable enzyme recovery beyond pH 4.0 as due to the protein hydrophobic patches were exposed to highly anionic surfactant hydrophilic tails thus minimize the extraction.

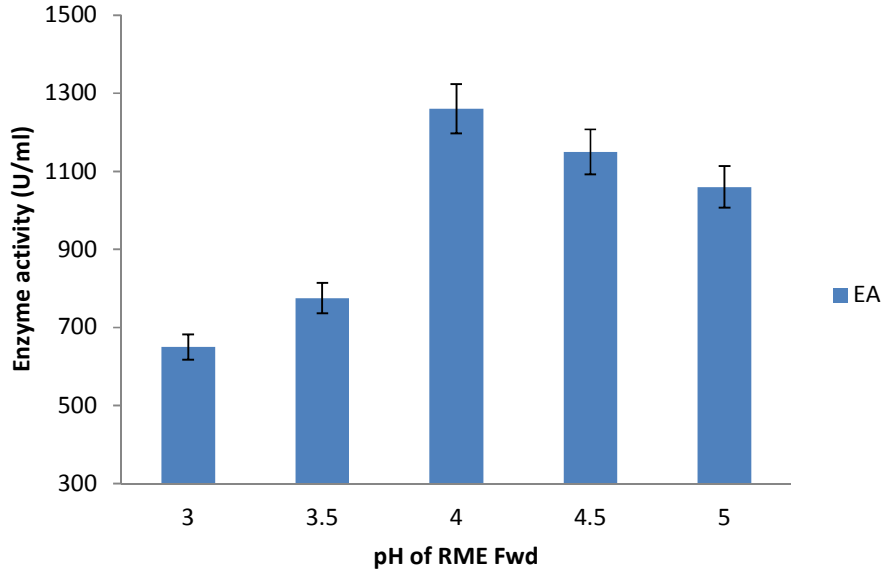

Fig.II- Effect of pH in RME forward extraction

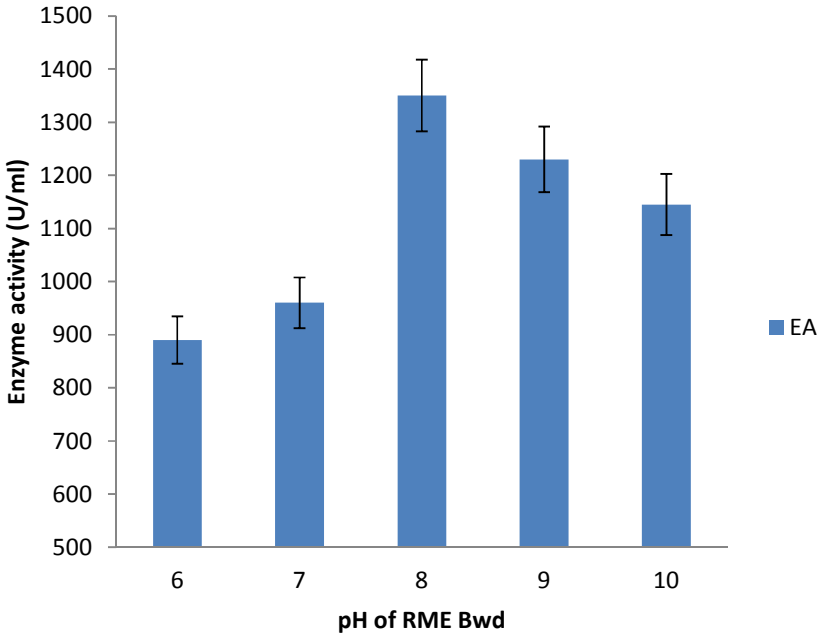

Fig.III- Effect of pH in RME backward extraction

3.4 Effect of pH in RME backward extraction

The pH of the aqueous solution in backward extraction should be kept above the pI value of target protein. The pH of the backward system was studied from pH 6 to 10 for the β amylase enzyme activity. The enzyme locked in the reverse micellar phase has to be released back to the aqueous solution. When pH was maintained above pI of β amylase[5, 9], the negative charge was induced in the sample which favored the electrostatic attraction[8]. The high enzyme activity 1350U/ml was found at pH8 and it declined to1145U/ml when pH is exceeded the optimal value 8 which is shown in the Fig.III.

3.5 Effect of salt concentration in backward extraction

The ionic strength in the backward extraction is another major factor which decides the enzyme recovery of the RM extraction. There was no salt addition in RME forward extraction to ensure high micelle formation. Salt concentration also influences the micelle size and distribution in the forward extraction. Increases in salt concentration decrease the micelles size and it influences the enzyme encapsulation and thus the protein recovery reduced[1, 2]. In the backward extraction NaCl concentration was added at the range from 0.25- 1.25M. From the graph it was found that the enzyme activity increased with salt concentration at the initial ranges, AOT surfactant micelles size decreased and the enzymes are released to the salt aqueous phase. However the maximum β amylase activity 1500U/ml was obtained at 0.75M NaCl as shown in the Fig.IV. Beyond the optimal NaCl concentration, the enzyme activity decreased drastically as high ionic strength started denaturing the enzyme recovery. The optimal salt concentrated enzyme trapped phase was studied for further purity analysis on Gel filtration chromatography column.

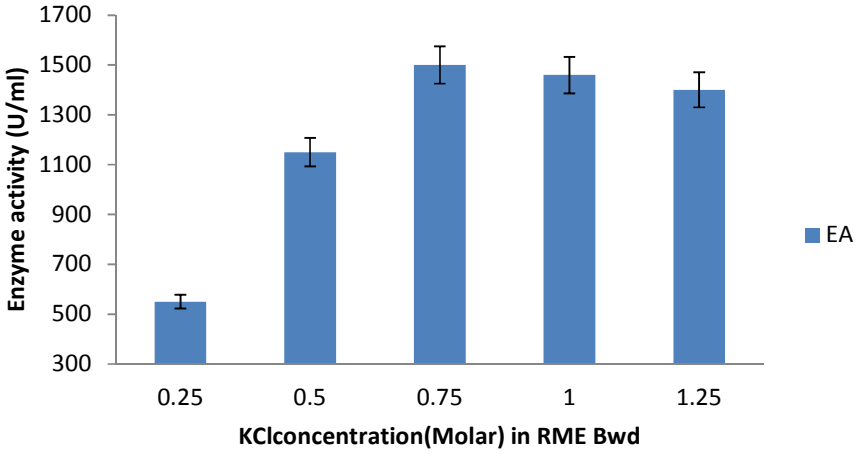

Fig.IV- Effect of Ionic strength in RME backward extraction

3.6 Purification Analysis on Gel Filtration Chromatography (GFC)

Gel Filtration Chromatography (GFC)column permeating the protein based on the molecular weight reflects in the retention time and also the purity level of the protein fraction. The UV absorbance is monitored at 278nm and normally the individual protein content was observed by a single peak in UV scale in mille absorbance unit (mAU)against retention time in minutes. When enzyme fractions were loaded into sephadex column, the gel beads permeate the protein that depends upon the molecular weight and it is inversely proportional to the retention time of the individual protein. The present study was carried out to confirm the purity level of the β amylase enzyme and also the selectivity of the RME system. GFC chromatography peaks shown in the Figure5 & 6 for the β amylase enzyme fractions were obtained from the salt precipitation and RME system respectively. In Fig.V there are two peaks observed one with 63mAU on 39mins and another one 48mAU on 40min. The accompanying peak may be due to unclarified crude protein. This shows that a fraction of salt precipitation is not much pure and selective for β amylase[10].From Fig.VI it was observed that there was a single chromatography peak of the fraction from the RME system. The chromatogram had a single peak and there by ensured the fact that the fraction contained a single protein. The peak absorbance of 960mAufurther confirmed that the fraction loaded was highly pure. Thus the process parameter once again revealed that the RME system applied forβ amylase extraction is highly selective and the physio-chemical conditions maintained are optimum.

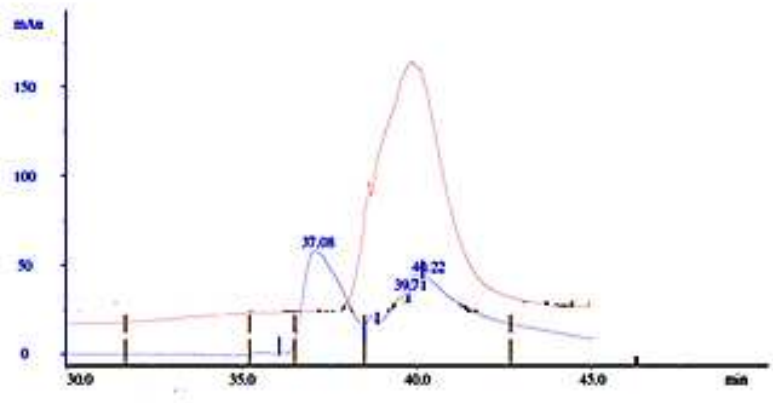

Fig.V- GFC purification of Ammonium sulphate precipitation sample

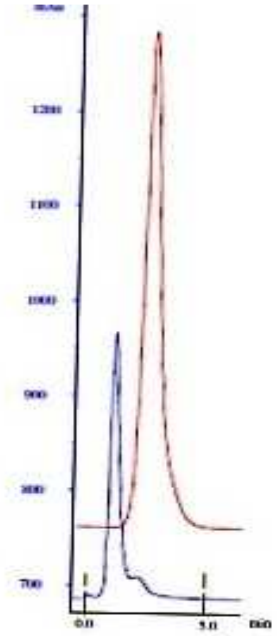

Fig.VI- GFC purification of Reverse micellar extraction sample

3.7 SDS-Page Analysis

Purified β amylase enzyme fractions of salt precipitation and RME backward system from the gel filtration chromatography was analyzed in SDS-Page for the final confirmation along with marker. The bands indicated that the RME fraction lies exactly close to the standard marker range and the single band shows the high purity of the system shown in the Fig.VII.

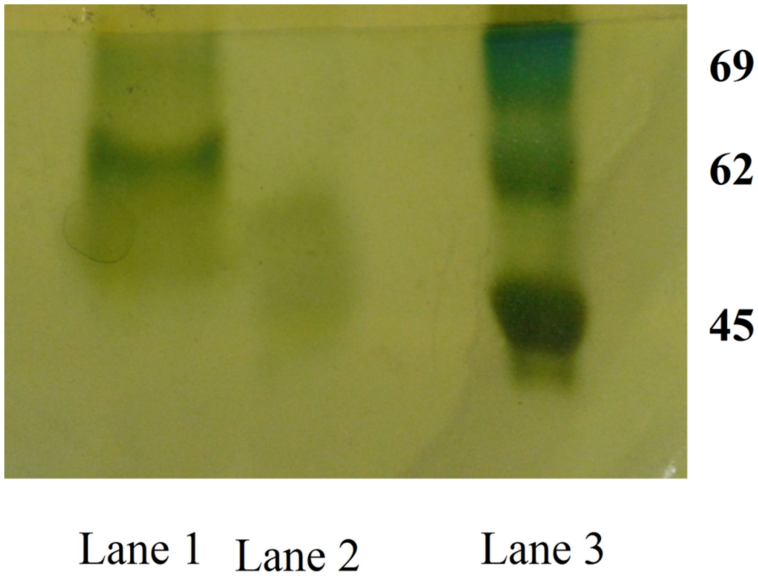

Fig.VII- SDS Page analysis:
Lane 1: β Amylase fraction from RME system
Lane 2: β Amylase fraction from ammonium sulphate precipitation
Lane 3: Standard molecular weight marker

CONCLUSION

β amylase was extracted by anionic surfactant (AOT) based reverse micellar extraction system with iso-octane as solvent in forward extraction and the same was recovered from reverse micelle by the addition of NaCl in backward extraction resulting in increased selectivity of 20 purity fold with 79% enzyme recovery. Purity study of β amylase enzyme was carried out on gel filtration chromatography(sephadex G) and the elution fractions were found to be highly pure of 33 purity fold with 73% enzyme recovery. The AOT based RME system of β amylase was found to be dependent on surfactant concentration, pH in the forward extraction and pH, NaCl concentration in the backward extraction. From this observation RME extraction can be used as core unit operation and can be applied for the crude enzyme separation with high purity. The present study illustrated that anionic surfactant (AOT) based reverse micellar extraction could be used as single step purification for the purification of β amylase from sweet potato.

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