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Purification of G-Protein Coupled Receptor from Membrane Cell of Local Strain of *Saccharomyces cerevisiae*

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Abstract: *The aim of this study to purify GPCR from a local strain of S. cerevisiae using gel filtration chromatography techniques , by packing materials for columns which will be chosen of low cost comparing to the already used in published researches, which depend on the costly affinity chromatography and other expensive methods of purification. Local strain of S. cerevisiae chosen for extraction and purification of G-protein coupled receptor (GPCR) .The strains were obtained from biology department in Al- Mosul University, Iraq. The isolated colony was activated on Yeast Extract Pepton Dextrose Broth (YEPDB) and incubated at 30 C˚ for 24 h .Loop fully of the yeast culture was transferred to (10ml) of yeast extract peptone glucose agar (YEPGA) slant , then incubated at 30C˚for 24h , after that it was stored at 4C˚ ,the yeast cultures were reactivated and persevered after each two weeks period. S.cerevisiae was identified by morphological, microscopic characterization and biochemical test . The GPCR that extract from membrane of S.cerevisiae was purified by gel filtration chromatography in two steps using Sepharose 6B. The optical density for each fraction was measured at 280 nm by UV-VS spectrophotometer then the GPCR concentration was determined by using ELISA Kit . The fractions which gave the highest absorbance and concentration of GPCR were collected .The molecular weight of GPCR was determined by gel filtration chromatography using blue dextrin solution. Standard curve was plotted between log of molecular weight for standard protein and the ratio of Ve/Vo of GPCR . The purity of the GPCR that extracted and purified from whole cell of S, cerevisiae were carried out by using SDS-PAGE electrophoresis In the first step 5ml of crude extract was applied on sepharose 6B column (1.6x 96 cm) which previously equilibrated with 50 mM phosphate buffer saline pH= 7.4 . Multiple proteins peaks appeared after elution with elution buffer (PBS PH= 7.4 containing 0. 5 % DDM). One peak only give positive result with GPCR assay, fractions representing GPCR were collected , pooled and concentrated by sucrose. In the second step five active fractions from the previous step were collected and applied once again on the same column and same conditions. This step gave a single peak that was identical with the peak of GPCR concentration ,maximum concentration of GPCR that observed in the fractions (34-38) was* 18.541 *(ng/ml) . The specific activity for these fractions was* 261.14 *(ng/mg) protein with yield of 47.717%. The present study a chive a relatively high purification of GPCR from membrane fraction of a local strain S. cerevisiae with fold purification* 5.094 *and a yield of 47.717%. and molecular weight about~55KD.*

Key words: *GPCR purification, S.Cerevisea, membrane cell*

I. Introduction

G-protein coupled receptors (GPCRs) are integral membrane proteins characterized by seven transmembrane helices and comprise one of the largest known superfamilies of receptors with in excess of 2000 genes identified across taxa [1]. They are attractive and proven drug targets in a wide range of therapeutic areas due to their involvement in signaling and response to diverse external stimuli in nearly all human cell types [2].

Bacterial, yeast, and insect hosts have successfully been implemented for high-level expression of soluble proteins, and similar approaches have been applied toward the expression of membrane proteins[3] .

Although all of these systems have proven useful for expression of some heterologous membrane proteins, each host has advantages and disadvantages associated with its use. For instance, microbial hosts such as E. coli and yeast offer well-understood genetics, low cost of culture, and relatively easy scale up. Insect and mammalian cells can perform many more complicated protein processing and post-translational modifications that may be necessary for proper function, but prove costly and time-intensive. However, even with several host systems available, heterologous expression has not yet systematically allowed for highlevel expression of any given GPCR of interest, and typically relies on trial-and-error methods. [4]

Even though all GPCRs share a commonality in their seven transmembrane domain segments and in their ability to couple to trimeric G-proteins, they also display great diversity in their overall function, ligand preference, tissue location, and physiological prevalence [5] .

Furthermore, significant differences exist in how proteins are expressed and processed in various heterologous systems, which may have a direct impact upon folding and activity of heterologously expressed

GPCRs. Given its ease of genetic manipulation, rapid growth, and eukaryotic secretory pathway, yeast are an attractive host system for the development of a robust GPCR expression system. Yeast have been successfully used for the heterologous expression of membrane proteins, specifically GPCRs. [6]

Three GPCRs are known in S. cerevisiae included a-factor receptor (Ste2), ∝- factor receptor (Ste3) and Gpr1 . Although Ste2 and Ste3 are both coupled to Gpr1 and activate the mating pathway ,the sequence similarity between them is limited [7]

Both haploid yeast cells types (∝ and a) express mating -type specific gene products such as the ∝ -factor pheromone and the Ste2 in a- cells , and the ∝- factor and Set3 in ∝- cells. Pheromone binding to either receptor stimulates the exchange of GDP to GTP on the protein Gpa1 , which in turn dissociates from the dimmer , consisting of Ste4 and Ste18 The Ste 4 - Ste 18 dimmer transmits the signal to Ste 20 , the first member of the activated protein kinase family , which activates a MAP -kinase cascade [8]

This process that is similar to hormone desensitization in mammalian cells . At the level of the receptor Set2 and Set 3 are down regulated by hyperphosphorylation of several C-terminal residues , followed by ubiquitylation , internalization and degradation . At the level of the G∝ protein ,GPa1, desensitization depends on the GTPase stimulating protein which is a member of the RGS -protein family[9].

Astudy using a native S. cerevisiae expression system ,1 mg quantities of His-epitope tagged α-factor receptor was purified on a Ni-NTA column and reconstituted in lipid vesicles. The binding activity of the reconstituted receptor indicated that only 6 % of the receptor was capable of ligand binding, but the addition of solubilized membranes from S. cerevisiae to the artificial membrane restored most of expected ligand binding activity (at least 80 %). Nevertheless, the co-factors for this effect were not identified despite extensive efforts. In another study, α-factor receptor was purified using a transient expression system in HEK293 EBNA cell line . About 1 mg of Ste2p was purified per liter of culture with relatively high affinity to a fluorescently-labeled α-factor. However, for large scale purification a stable expression system is required, and the heterogeneous glycosylation of the recombinant protein in a mammalian system may interfere with the formation of diffractable crystals. As almost all human GPCRs do not exist naturally in high abundance, heterologous expression systems are required to achieve sufficient protein yields for structural characterization, generally at the mg/L scale or greater. [10].

Recent expression of the human adenosine A2a receptor (hA2aR) in S. cerevisiae has yielded active protein at greater than 10 mg/L of culture, which has facilitated its purification[11] Other studies have also cited improper trafficking of recombinant membrane proteins in yeast [12]

The aim of this study to purify GPCR from a local strain of S. cerevisiae using gel filtration chromatography techniques , by packing materials for columns which will be chosen of low cost comparing to the already used in published researches, which depend on the costly affinity chromatography and other expensive methods of purification.

II. Materials and Methods

Local strain of S. cerevisiae obtained from department of Biology / College of Science / AL Mosul University. The isolated colony was activated on YEPGB and incubated at 30 C˚ for 24 h.

Loop fully of the yeast culture was transferred to (10ml) of YEPGA slant , then incubated at 30 C˚ for 24h , After that it was stored at 4C˚ .The yeast cultures were reactivated and persevered after each two weeks period[13].

S.cerevisiae was identified by morphological, microscopical characterization and biochemical test.

The pellet cells were thawed and resuspended in solubilization buffer (50 mM PBS buffer , PH = 8.0 , 100mM NaCl , 5mM MgCl2 , 1mM AEBSF) and protease inhibitor cocktail (us 10 µl for 1ml) with n-Dodecyl-β-D-maltoside (DDM) (1%) and 5mM β-mercaptoethanol were added to the lysed sample with gentle swirling on ice [10] . and agitation with sterilized beads (0.4-0.5 mµ) by using vortex mixer several cycles of agitation (30-60 sec) was interspersed with cycles of cooling on ice[14] ,

The suspension was removed by pasteur pipte and suspension examined under light microscope (100X) , the disruption of the cell wall was observed, the suspension was centrifuged again at 5000 rpm to remove debris and remaining suspension was centrifuged at 25000 rpm for 120 min by ultra cooling centrifuge.

The Final membrane pellet was washed and resuspended in solubilization buffer (50 mM PBS buffer , PH = 8.0 , 100mM NaCl , 5mM MgCl2 , 1mM AEBSF) and stirred on ice for 1 hour and centrifuged at 14000 rpm([10] .

Gel filtration Chromatography was used in purification of GPCR from membrane fraction in two steps.

Sepharose 6B which washed several times with 50mM PBS (PH = 7.4) , degassed by vacuum pump to remove the air bubbles and poured gradually in a column by using glass rod to avoid forming bubbles . Gel was left to settle down and packed well to give column with (96 x 1.5) cm. The gel was equilibrated with 50 mM PBS (PH = 7.4) .

The GPCR crude that prepared by solubilization of membrane was applied to the sepharose 6B column as a second step of purification that was pre equilibrated with 50 mM PBS PH = 7.4 , the GPCR crude was eluted from the column by elution buffer(PBS PH=7.4 with 0.5%DDM). Aliquot of 5ml for each fraction was collected with flow rate (0.5 ml/min).The fractions that gave the highest absorbance at 280 nm and GPCR concentration were collected. Then ,these fractions are concentrated by sucrose using dialysis tube.

The concentrated GPCR from first step was applied to the sepharose 6B column that pre equilibrated by 50 mM PBS (PH = 7.4) , and eluted with elution buffer . Aliquot of fraction were collected in each tube with flow rate of 0.5 ml/min and optical density were determined for each collected fraction at 280 nm by UV-VIS spectrophotometer. The fractions that gave the highest absorbance were collected , protein concentration measured by using Bradford method . The GPCR concentration was determined by using ELISA Kit from BlueGene Biotech, Shanghai, China for all steps of purification. The fractions which gave the highest absorbance and concentration of GPCR were collected.

The molecular weight of GPCR was determined by gel filtration chromatography using blue dextrin solution .Standard curve was plotted between log of molecular weight for standard protein and the ratio of Ve/Vo of GPCR [15].

The purity of the GPCR that extracted and purified from cell membrane of S. cerevisiae were carried out by using SDS-PAGE electrophoresis[16].

III. Result and discussion

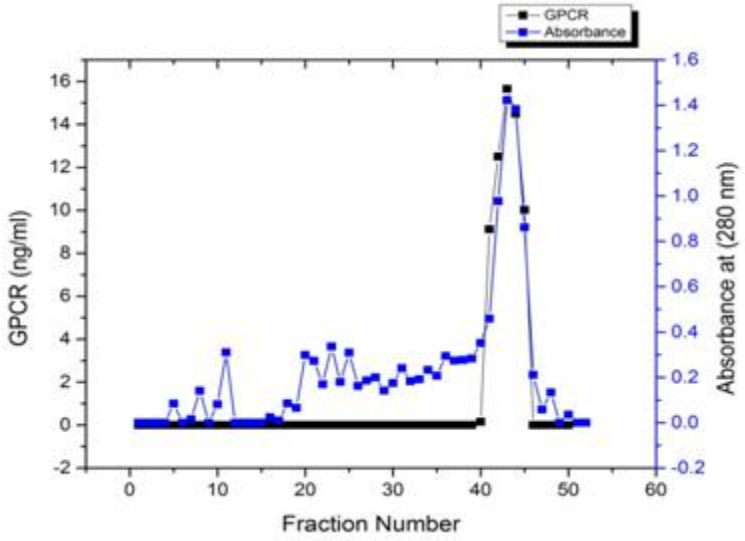
The local strain of S.cerevisiae were identified by studying specific microscopically, morphological and biochemical characterization[17].

The ability of isolate S.cerevisea for fermentation and assimilation was examined ,which glucose, fructose, sucrose, galactose, lactose, maltose, raffinose were used. The results illustrated that glucose, fructose, sucrose, galactose, , maltose, raffinose) were fermented and assimilated by the isolate strain while lactose was not fermented and assimilated by this isolate. The isolate also show un ability to hydrolyzed urea and produce ammonia. The characters are inagreement with previous study[18].

Yeast cells were thawed and resuspended in solubiziation buffer and transferred to tube containing acid – washed , dried , and the cells were lyses by shaking with glass beads. All steps performed at 4C˚and butters were supplemented with protease inhibitors cocktail in phosphate buffer[19].

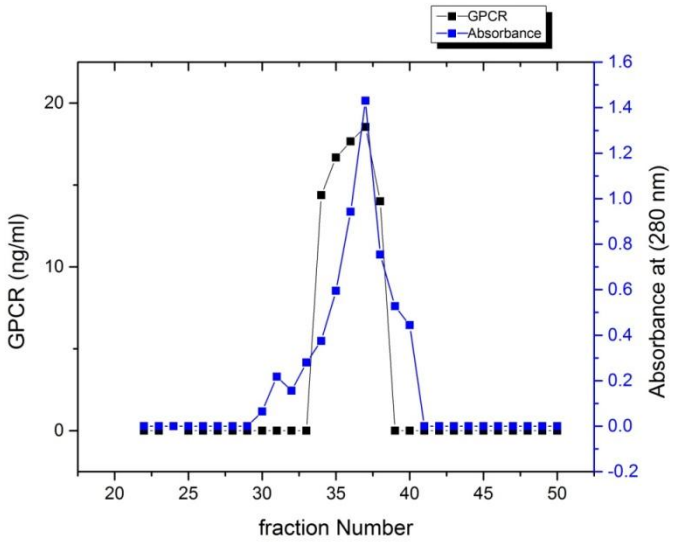
GPCR extracted from membrane of S. cerevisiae was solubilized and purified by gel filtration chromatography in two steps. In the first step 5ml of crude extract was applied on sepharose 6B column (1.5x 96 cm) which previously equilibrated with 50 mM phosphate buffer saline pH= 7.4.

Results in figure (1) illustrates multiple proteins peaks appeared after elution with elution buffer (PBS PH= 7.4 containing 0. 5 % DDM). One peak only give positive result with GPCR assay. Fractions representing GPCR were collected , pooled and concentrated by sucrose.



Figure(1):Purification of GPCR by Gel Filtration Chromatography , first step.

In the second step five active fractions from the previous step were collected and applied once again on the same column and same conditions. This step gave a single. peak that was identical with the peak of GPCR concentration as shown in figure (2)



Figure(2):Purification of GPCR by Gel Filtration Chromatography, second step.

Table (1) represented volume ,protein concentration, GPCR concentration , specific activity ,purification fold and yield for all purification steps of GPCR from membrane of isolated *S. cerevisiae* The maximum concentration of GPCR that observed in the fractions (34-38) was 18.541 (ng/ml) . The specific activity for these fractions was 261.14 (ng/mg) protein with yield of 47.717%.

Table (1): Volume ,protein concentration, GPCR concentration , specific activity ,purification fold and yield for all purification steps of GPCR from membrane of isolated *S. cerevisiae*

Steps	volume (ml) (	Conc. Protein mg/ml)(	GPCR Conc. (ng/ml)	Specific Activity (ng/mg)	Purification fold	yield (%)
Crude GPCR	10	0.69	35.37	51.26	1	100
Gel filtration First step	16	0.131	19.428	148.30	2.893	87.88
Gel filtration Second step	8	0.071	18.541	261.14	5.094	47.717

The molecular weight is determined by gel filtration in sepharose -6B based on the standared curve made by standard protein figure (3). The Blue Dextran 2000 was used as a guide for assessing the correct column packaging and the astimation of void volume ,In this study the curve plotted between absorbance at 600 nm fraction number and the resolution of one identical peak indicated the correct column packaging . Five ml of the concentrated active fraction by sucrose obtained in the gel filtration monitored for absorbance at 280 and for GPCR concentration was used for estimation of molecular weight of GPCR by gel filtration chromatography with the aid of fraction of standard protein, showed that the resolute protein(~54.950KD) for GPCR extract from membrane of *S.cerevisiae*.

The molecular weight and purity of GPCR was determined by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) as shown in the figure (3). The molecular weight of GPCR that extracted from solubilization membrane fraction of *S.cerevisiae* was(~ 55 KD) with single band**.**

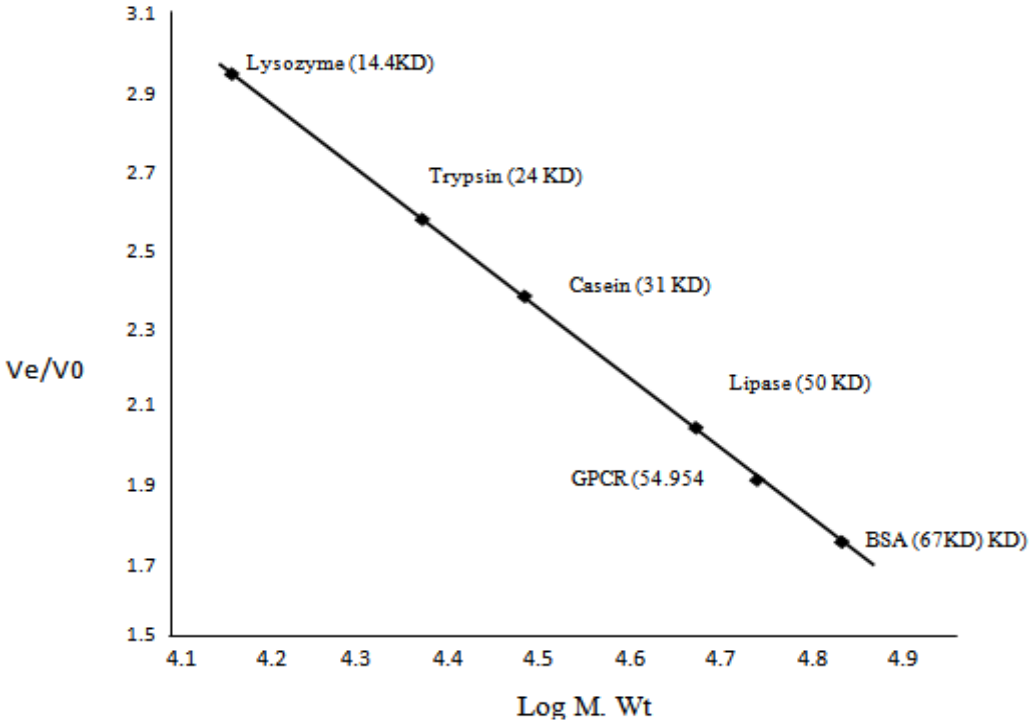


Figure (3): Estimation of molecular weight of GPCR by gel filtration

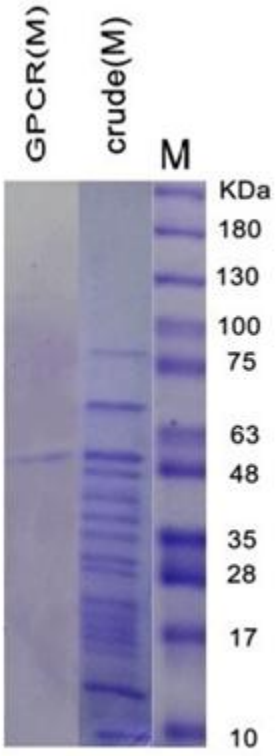


Figure (4): Polyacrylamide gel electrophoresis of purified GPCR. crude(M): Membrane fraction

From previous reports, the isolated of membrane protein from S, cerevisiae cloned in eukaryotic cell found to be easy extracted due to membrane proteins produced in S. cerevisiae is challenging, and the limited number of membrane protein structure is due to difficulties encountered with production, solubilization and purification of appropriate amounts of membrane proteins that are able to from crystals diffracting at a high resolution [20,21].

In previous study, the alfa – factor , which is a 13- amino acid residue- long peptide agonist of the yeast pheromone response pathway, was successfully displayed on the yeast plasma membrane. Signal activation was observed by employing a fluorescent reporter gene assay. Extended application of this system to human GPCRs which comprise one of the most important types of drug targets[22].

The range of application of technology for displaying peptide ligands on yeast plasma membranes namely" pep display" was extended in a recent study to activation human GPCR that was heterologous produced in S. cerevisiae. The methodology presented in that study could be useful for identifying novel peptide ligands for both liganded and orphan mammalian GPCRs .The authors suggest that plasma membrane is more suitable than cell wall in terms of peptide- ligand display. The higher accessibility of membrane- displayed ligands to GPCRs may be important for efficient activation[23]..

Other recent study demonstrated the production of large quantities of high quality eukaryotic membrane proteins in S. cerevisiae , a high- copy vector was modified to express membrane proteins C-terminally – fused to a tobacco protease detachable[24].

The conclusion from this study is The present study a chive a relatively high purification of GPCR from cell membrane of S. cerevisiae with fold purification 5.094 and a yield of 47.717%.and molecular weight about ~55KD .

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