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Review Article

AN OVERVIEW ON PURIFICATION OF MICROBIAL AGARASES

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ABSTRACT

Extra cellular agarases are in great demand in lipid peroxidation, hydroxyl radical scavenging activity by generating agaro oligosaccharides and protoplast isolation. Most of the marine microbial flora have evolved to produce extra cellular agarases to utilize agar as a carbon source. In order to produce these enzymes in industrial scale, researchers were exploring non marine sources like soil, lake, hot spring to screen potent agarolytic enzyme producing microbial flora and cloning agarose promising gene into industry feasible microorganisms. Purification of extra cellular agarase has led to the determination of its 3D structure, which gives a better sight and understanding about its properties, and function. Purification is a multistage process involving the use of various separation techniques. Current review helps to give a wider view on the methods employed by different researches for the purification of extracellular agarase, produced by non-marine and marine environment microbiota.

Keywords: Extra cellular, agarase, purification, marine and microbiota.

INTRODUCTION

Enzymes are also known as biocatalysts, involved in various metabolic pathways in plants, animals and various microorganisms. Extracellular agarases holds the capability to hydrolyze agar into 3,6-anhydro-Lgalatose and D-galactose by cleaving linear chain linked by alternating β-1,4- and α-1,3-glycosidic bond. Agar is mainly derived from the cell walls of a particular family of red algae species (*Rhodophyceae*), which includes *Gelidium* and *Gracilaria.*[1,2] Agaro oligosaccharides have been applied in various areas, including antioxidative activity, inhibition of lipid peroxidation, scavenging free radicals, moisturizing skin and the whitening effect on melanoma cells.[3]

In this review we summarized various purification techniques adapted for the purification of extra cellular agarases produced from marine and non maine microbiota viz., *Agarivorans* sp., *Alteromonas* sp., *Pseudoalteromonas* sp., *Thalasomonas* sp., *Vibrio* sp. and *Paenibacillus* sp.[4-9]

Pre-Purification Techniques

For every extraction process, enzyme nature plays a vital role as it gives information on how to proceed with the purification process. Enzymes are manufactured in bioreactors for commercial purposes, thus has to be purified for further use, as they are generally in crude form. Depending on the source, agarase can be produced extracellularly or intracellularly, most of the agarase found till date are extracellular in nature, thus they are separated by centrifugation process after fermentation in large scale. They are further purified depending on the nature and property of enzyme. Most common method used after centrifugation was the precipitation method by various inorganic salts (ammonium sulphate), or organic solvents (acetone or alcohols), or non-ionic hydrophilic polymers (polyethylene glycol). A large amount of purification methods which have been used so far comprises the use of ammonium sulphate (inorganic salt) followed by different chromatography methods like that of anion exchange chromatography followed by gel filtration chromatography.

Precipitation by ammonium sulphate also called as salting out process which mainly works on the principle that, most of the proteins are less soluble in solutions which contain high salt concentration, ammonium sulphate is especially used for this process because its solubility is very high, the solubility of any protein in this case enzyme totally depends on the ionic strength of the solution, thus when the

ionic strength is effectively high enough the protein precipitates out of the solution . The next process involves the removal of the salts, which has been added to the enzymes this is done by either dialysis, or the use of buffer containing mixture of SDS, Tris-HCl and phenol.

Jiang Li et *al* used about 70% ammonium sulphate for salting out the enzyme and then used dialyses process for purifying it further to obtain beta agarase from *Pseudoalteromonas sp.* NJ21.[10] Tao Song *et al* was isolated seven agarolytic bacteria from plant by taking supernatant and adding solid ammonium sulphate and then 1.5 ml of 20 mMTris–HCl was used followed by and dialysis against the same buffer at 4℃ for 2-3 days.[2] Whereas Wandong et *al* was isolated two agarases from the cultural supernatant of *Vibrio sp*. F-6 using solid ammonium sulphate to 80% saturation and the precipitated crude agarase was then treating it with 40 ml of 20Mm Tris-HCl buffer, at pH 7.8.[8] Ravichand Jonnadula *et al* obtained 103-fold extracellular agarase enzyme from *microbulbifer* strain CMC-5 by dialysis against 20mM Tris-Cl and several times centrifugation.[11] Extracellular agarase of *Bacillus cereus* ASK202 was purified 32-fold by using freeze dryer and the use of sodium phosphate buffer, followed by dialysis at 4℃, by Jo Kim *et al*.[12]

Kohtaro *et al* used about 60-80% ammonium sulphate for salting out the enzyme and then it was dialyzed against Tris-HCl buffer for purifying it further to obtain beta Agarase from *Alteromonas* sp.[13] Philippe *et al* purified α-agarase from *Alteromonas agarylticus,* strain GJ1B by centrifuging the culture media. The supernatant was concentrated by ultracentrifugation, the ultracentrifuge was adjusted to 70% saturation with ammonium sulphate, and the precipitate was pelleted and suspended in Tris/HCl buffer.[5] Leon *et al* purified extracellular agarase from *Alteromonas* sp. strain C-1 by using 75% of ammonium sulfate and the pellet was resuspended in Tris-HCl-EDTA buffer containing phenyl methyl sulfonyl fluoride and dialyzed.[14] Xiao *et al* purified novel β-agarase from *Agarivorans albus* YKW-34 by ultrafiltration followed by dialysis against Tris–HCl buffer.[15] Yasmin *et al* subjected the cell-free supernatant to 40~60% ammonium sulphate precipitation followed by dialysis against Tris-HCl buffer to in order to purify agarose enzyme from *Bacillus megaterium.*[16] *Vibrio* sp JT0107 was isolated from marine organisms like that of *Laminaria sp*. and *Undaria pinnatijida* and then these was able to provide 45-fold endo type β agarase by the use of ammonium sulphate precipitation method followed by the use of Tris-HCl buffer and dialysis process.[17]

Chromatographic Steps

To study the characteristics and properties of different agarases, further purification is a must, this is mainly done with the help of various chromatographic methods. Enzymes can be separated depending on their characteristic features like size, shape, charge, hydrophobic group present and binding capacity from a mixture of solution. The chromatography types are mainly of two categories one based on the molecular characteristics and interaction type, which includes ion exchange, affinity chromatography, and another, based on stationary bed e.g. column, thin layer and paper chromatography. Affinity chromatography is one of the most used type for agarose purification.

In affinity chromatography, a ligand is used which can make a complex with a specific protein and binds with the filling material of the column. The specific protein is bound to the solid support whereas the free protein leaves the column. If the bound proteins have to be extracted then they can leave on addition of some salt solution or by changing ionic strengths. Up till date three kinds of affinity chromatography media have been used in agarase purification. The first kind of medium is the cross-linked agarose such as agarose CL-6B, which results in a marked increase of agarase specific activity based on the specific affinity of enzyme and substrate.[4] The affinity chromatography method was adapted for *Alteromonas agarlyticus* strain GJ1B,[5] The dialyzed solution obtained from *Agarivorans albuns* YKW-34 was applied to a DEAE Sepharose FF column and Sephacryl S-100 HR column.[15] For the purification of rAgaD which was cloned from *Vibrio* sp. PO-303 The second kind of medium is Ni^{2+} Sepharose is used.[18]

Anion exchangers are another type, which is widely used for agarose purification, mostly single step chromatography, does don't give the required result thus a combination of chromatographic methods is used for better results. The use of Hydroxyapatite for the purification of a few agarases has been a success as well.[7] A strain of *Thalassomonas* sp JAMA-A33 has been purified using hydroxyapatite followed by two chromatography technique one being anion exchange and another column purification by hydroxyapatite and then again by gel filtration chromatography.[7] *Thalassomonas* sp NJ21 was also used to purify agarase (Aga21), using Q-Sepharose F.F. column and two chromatography via anionic exchange chromatography and gel chromatography for

purification.[10] *Microbulbifer* strain CMC-5 was used and about 103-fold purification of the enzyme was obtained by ultrafiltration, ion exchange chromatography, using diethyl aminoethyl sepharose FF, and gel filtration, using sephacryl S-300HR, with a yield of 6.7%. [11]Ghazi *et al* used ammonium sulphate fractionation as well as two chromatographic methods that being the gel filtration and anion exchange for the purification of agarase enzyme up to 49.7 fold from *Bacillus* sp., H12.[19]The same techniques were even used by Hisashi Suzuki, to extract and purify β-Agarase from *Bacillus* sp. MK03 but was able to obtain 129-fold from the culture,[20] *Acinetobacter* sp. PS12B was used as a source for the extraction of agarase by the use of ammonium sulphate precipitate which was further followed by DEAE-cellulose ion-exchange chromatography which gave about 10-fold of the original crude extract.[21]

β-agarase I and II was purified from *Pseudomonas Atlantica*, β-agarase I was purified 670-fold from the bacterial medium by a new method, using ammonium sulphate precipitation and gel filtration on Sephadex G-100 whereas β-agarase II was partially purified (5-fold) from the soluble fraction of disrupted cells by chromatography on Sephadex G-100, hydroxyapatite and DEAE-Spharose CL-6B.[22] A hydrophobic column chromatography using a column of Toy pearl Phenyl-650 M was used to purify beta-agarase from a halophilic archaeon *Halococcus* sp. 197A as well as for *Vibrio* sp. JT0107 but then it was chromatographed on Mono Q chromatography.[17,23] DEAE Sepharose CL-6B column that was used to get extracellular agarase of *Bacillus cereus* ASK202 that was purified up to 32-fold.[12] Agarase was also obtained from *Bacillus megaterium*, which was loaded on Sepharose CL-4B column containing 0.02% sodium azide.[16] For *Vibrio sp. AP-2* with the use of column chromatography as well as some amount of DNase and RNase the authors were able to get 328-fold of the original amount.[24]

Non-Marine Source

Since most of the microorganism that are capable of producing agarase enzymes are mainly found in marine environment, researches are working hard to get hold of non-marine recourses. Few papers were able to provide information regarding new source and their purification method such as the work of Tao Song *et al* who was able to extract and purify 30.2 fold of β-agarase(SSG-1a) from *Paenibacillus* sp.SSG-1 which was isolated from soil, using ammonium sulphate precipitation and DEAE FF chromatography.[9] Another agar degrading bacteria (*Bacillus* and *Cocci*) was isolated and agarolytic activity was seen thus the enzyme was purified using ammonium salt precipitation followed by the use of column chromatography (DEAE-cellulose).[25] *Agarivorans albus* YKW-34 is a type of agarolytic microorganism which was taken from the gut of turban shell to extract β-agarase, Xiao *et al* was able to recover about 30% with 10 fold of the original extract by using DEAE Sepharose FF column.[15]

S.No	Organism Name	Types	Molecular Weight	Purification Methods	Reference
1	*Vibrio* sp.F 6	Alpha and Beta agarase	Alpha-54KDa Beta-34.5KDa	Ammonium Sulphate Salting Out Column Chromatography	8
2	*Thalassomonas* BCRC17492	Extracellular	70KDa	Ammonium Sulphate Salting out Column Chromatography	3
3	*Bacillus cereus* ASK202	Beta agarase	90KDa	Ammonium Sulphate Salting Out Dialysis	12
4	*Agarivorans albus* YKW-34	Beta agarase	50KDa	Ultrafiltration	15
5	*Pseudoaltinomonas sp. AG4*	Beta agarase	33KDa	Ammonium Sulphate Salting Out Dialysis	6
6	*Halococcus* 197A.	Beta agarase	55KDa	Column Chromatography	23
7	*Microbulbifts sp. CMC5*	Beta agarase	59KDa	Ultrafiltration, Ion Exchange Chromatography Gel Filtration and Dialysis	11
8	*Paenibacillus sp.SSG-1*	Beta agarase	77KDa	Ammonium Sulphate Salting Out Ion Exchange Chromatography	2
9	*Pseudoalteromonas* sp. JYBCL 1	Beta agarase	60kDa	Ammonium Sulphate Salting Out Gel Filtration	25
10	*Vibrio sp JT107*	Beta agarase	107KDa	Ammonium Sulphate Salting Out Ion Exchange Chromatography	17
11	*Vibrio sp AP-2*	Beta agarose extracellular	20KDa	Ammonium Sulphate Salting Out Affinity Chromatography Column Chromatography	24
12	*Alteromonas E-1*	Extracellular Beta agarase	82KDa	Ammonium Sulphate Salting Out Ion Exchange Chromatography	13

				Gel Filtration Chromatography	
13	*Agarivorians sp.HZ105*	Extracellular Beta agarase	58KDa	Ammonium Sulphate Salting Out	4
14	*Agarivorians YKW-39*	Extracellular Beta agarase	50KDa	Ammonium Sulphate Salting Out Ion Exchange Chromatography	15
15	*Bacillus MK03*	Extracellular Beta agarase	112KDa	Ammonium Sulphate Salting Out	20
16	*Alteromonas GJ1B*	Intracellular Beta agarase	180KDa	Ammonium Sulphate Salting Out Affinity Chromatography	5
17	*Thalasomonas JAMDA33*	Alpha agarase	85KDa	Ammonium Sulphate Salting Out Ultrasonication, Gel Filtration	7
18	*Alteromonas sp. C1*	Extracellular	52KDa	Ammonium Sulphate Salting Out Anion Exchange Chromatography Gel Filtration	14
19	*Bacillus magaturium*	Extracellular	12-15KDa	Ammonium Sulphate Salting Out Gel Filtration	16
20	*Acinotobacter sp. PS12B*	Extracelullar	24KDa	Ammonium Sulphate Salting Out Ion Exchange Chromatography	21
21	*Alteromonas SY3712*	Extracelullar Beta agarase	35.5KDa	Ammonium Sulphate Salting Out Ion Chromatography, Gel Filtration	27
22	*Cellvibrio sp. WU-0601*	Alpha agarase	84KDa	Ammonium Sulphate Salting Out Dialysis, Gel Filtration	28
23	*Rhodococcus sp. Q5*	Extracellular	54KDa	Ammonium Sulphate Salting Out Gel Electrophoresis	29
24	*Pseudomonas atlantica*	Agarase (I,II)	I -210KDa II -63KDa	Affinity chromatography Molecular Sieve Chromatography	22

CONCLUSION

Agarase enzyme has a broad application in various sectors, whether it be food or medical or cosmetic. They are also used as biological tools for various research, thus the production and purification of the agarase enzyme has been an area of interest of many scientists. Till now the purification process of agarase consists of multistage techniques such as, the use of ammonium sulphate precipitation or the use of acetone, followed by gel filtration and other chromatographic methods. Researches have also shown sufficient yield of agarase using these processes. But there is as such no common methodology to extract and get the greatest yield which can be cost effective as well. Agar in itself is highly costly thus making the enzyme costlier, researchers are trying to overcome this problem, various recombinant technology are also been practised which have shown good results. Although the isolation and purification of agarase is an old process but new cost-effective methods are to be developed for easier use thus are still on experimental stage and not commercially available.

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