Golden Research Thoughts
Volume 2, Issue. 7, Jan. 2013
ISSN:-2231-5063
Available online at www.aygrt.net

ORIGINAL ARTICLE

PURIFICATION OF GLUTAMINASE ENZYME PRODUCED FROM ERWINIA

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Abstract:

The purpose of this study was to do Purification of the Glutaminase enzyme produced from free cells of Erwinia species at flask level. Glutaminase can be isolated from a number of sources such as plants, animals and microorganisms. Glutaminase is an important enzyme that serves many functions. It plays a key role in the energy and nitrogen metabolism of mammalian cells. Glutaminase is very important food enzyme used in food industries for flavor enhancement. Glutaminase, in combination with or as an alternative to asparaginase could be of great significance in enzyme therapy for cancer especially acute lymphocytic leukemia. Glutaminase enzyme was produced from free cells of Erwinia under optimized conditions such as Temperature, pH, Time, Inducer concentrations etc. After production of Glutaminase enzyme, Partial purification of enzyme was done with Ammonium Sulphate precipitation method. After isolation, the Glutaminase enzyme was purified with Gel filtration Chromatography & Ion Exchange chromatography. After purification by both methods, Purified samples were analyzed for enzyme activity & protein content. Enzyme activity was determined by Nessler's method & protein content was determined by Bradford method. It was found that after purification of crude sample by both methods, Gel Filtration chromatography shows maximum enzyme activity and specific activity than the samples purified with Ion Exchange Chromatography. Also %age recovery (97.59%) & purification fold (1.70) obtained was found maximum from the samples purified with Gel Filtration Chromatography. From above results it was concluded that Gel filtration method is Better method for the purification of Glutaminase enzyme than Ion exchange Chromatography.

KEYWORDS-

Glutaminase, Enzyme purification, Gel Filtration Chromatography etc.

MATERIALS AND METHODS:

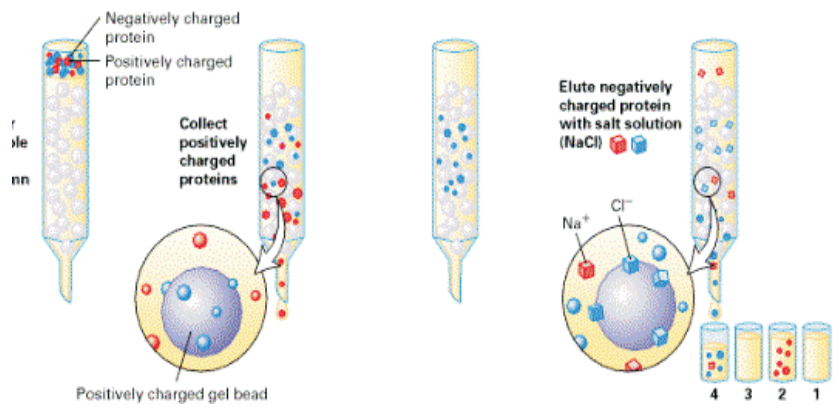
ION EXCHANGE CHROMATOGRAPHY:

It was done by following procedure of Pharmacia LKB Biotechnology, 1991 and Roe, 1989). The separation of proteins by Ion exchange chromatography requires differential binding of the proteins to the ion exchange matrix. After proteins are applied to an ion exchange column, the proteins which have no affinity for the matrix are removed during the washing of column. Then the adsorbed proteins are removed in an elution step. Since different proteins generally bind to ion exchange matrix with different affinities, it is usually possible to separate them by gradually increasing the salt concentration during the elution step.

Title :PURIFICATION OF GLUTAMINASE ENZYME PRODUCED FROM ERWINIA
Source:Golden Research Thoughts [2231-5063] PUSHPINDER PAUL yr:2013 vol:2 iss:7.

For example, proteins which are negatively charged near neutral pH would stick to a positively charged anion exchange matrix. The act of binding would displace a counter ion (such as Cl- with a loading buffer containing 0.1M chloride) from the ion exchanger. If a higher concentration of the counter-ion were applied (0.3M Cl-), a protein with low net charge might be displaced. To remove another protein with greater net charge, 0.5M chloride may be required. By progressively raising the counter ion concentration, clean separations of the two proteins can be achieved. Based on the ion exchange principle, cation exchange chromatography was performed:

The column was packed by dissolving 2g of CM-cellulose in 40 ml of 50mM Potassium phosphate buffer having pH7.5. The column was equilibrated with 10mM Tris HCl buffer having pH 9.5 for 20 minutes. 1ml of crude enzyme with 50mg of glycine was loaded on the column. Tris HCl buffer (10mM) with equal volume of 0.1 M NaCl and 0.5M NaCl was then loaded for 12 minutes and fractions were collected 3 ml each in 4 test tubes respectively. Again, the equal volume of 1.0M NaCl was loaded for 12 minutes and fractions were collected. Finally the elution buffer having 10mM Tris HCl buffer with equal volume of 0.02M NaCl, having pH8 was loaded to collect final fractions.



Ion Exchange Chromatography

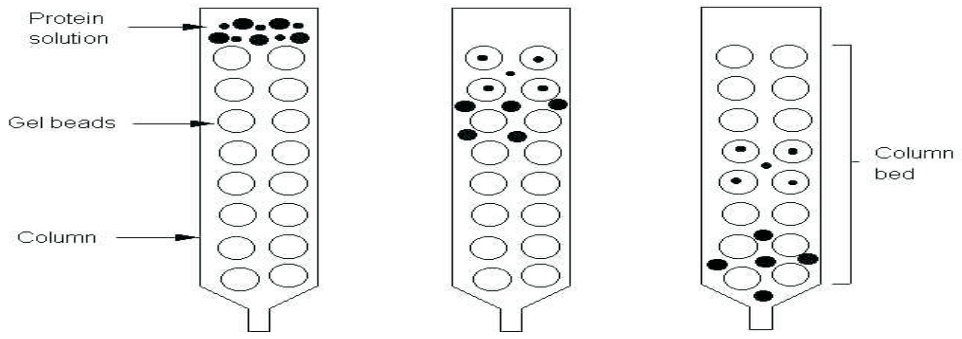
GEL FILTRATION CHROMATOGRAPHY:

It was done by the following procedure of (Ackers, 1973 and Andrews, 1965). A disposable pipette served as the chromatography column. Put a small wad of glass wool, tamped firmly but not too tightly, at the bottom to have flat surface. Filled the column about half with water and checked the flow to be maintained as ~2 drops per sec.The flow can be slowed by tamping glass wool more firmly. Swirl the Sephadex suspension and added it to the column. Allowing the water to flow until the volume of the settled gel comes to about 10 ml. Sucked off clear liquid as gel settles and replaced it with more gel suspension. Avoid air bubbles! Put a small flat layer of glass wool on top. Allowed the surface of the glass wool to dry –

Do not let air enter the bed! – then stopped the flow. Filled 25 small test tubes with 3.0 ml of water each and set the rack aside. Added into a small test tube 0.90 ml of the blue dextran solution and 0.30 ml of the bromophenol blue solution. Mixed them well. Placed an empty 10 ml graduated cylinder under the column outlet to catch drops temporarily. Carefully loaded 0.50ml of blue mixture onto the dry surface of the column. Allowed the sample to run into the gel until the surface was dry and then washed it with a few drops of water. Now, filled the column with water to the top. Keep on adding water as necessary to maintain the continuous flow. As soon as the colored molecules start migrating down the column, determined the number of drops in 1.0 ml using a graduated cylinder. Collected effluent in the graduated cylinder upto the first blue band started to emerge. From this point, started collecting 6-drop fractions into 25 test tubes. Collected the fractions of 6 drops until the entire trailing blue band was eluted. Mixed each collected fraction well, read its absorbance at 590 nm by taking water as blank. If reading is > 1.1, dilution can be made appropriately. By trial and error, prepared the separate dilutions of Blue Dextran and bromophenol blue stock solutions so as to give individual absorbance at 590 between 0.4 and 0.9. Recorded the dilutions and its absorbance . Pooled the fractions containing Blue Dextran in a graduated cylinder. Mixed the contents well and recorded the pooled volume. Took its absorbance at 590nm. Repeated the same process

for the bromophenol blue fractions. Some points were noted carefully:

The kind of Sephadex used, the dimensions of the column, plot of A590 vs. fraction number, pooled fractions, the amount of Blue Dextran and bromophenol blue loaded (A590 x volume loaded) and amounts of (a) Blue Dextran and (b) bromophenol blue recovered: i.e.[A590 of pool] x [ml of pool] OR [sum of A590 for all fractions containing desired compound] x [volume of one fraction] Note that the volume of one fraction = 3.0 ml + volume of 6 drops. After calculations, calculated the recovery of each compound



Gel Filtration Chromatography

RESULT & DISCUSSION

PURIFICATION BY ION EXCHANGE CROMATOGRAPHY:

The enzyme was purified with the help of Ion exchange chromatography. 1ml of enzyme with 50mg of glycine was loaded in the column. Then, Tris HCl buffer (10mM) with equal volume of 0.1 M NaCl and 0.5M NaCl was loaded for 12 minutes and 3 ml each were collected in 4 test tubes. Tris HCl buffer with equal volume of 0.5 M NaCl was then loaded for 12 minutes and fractions were collected. Finally, elution buffer having 10mM Tris HCl buffer with equal volume of 0.02M NaCl at pH8 was run. The sixteen purified samples were collected for crude enzyme. All the samples were assayed for enzyme activity by Nesseler's and Bradford methods. The last four samples showed maximum enzyme activity. The fraction showing maximum activity were pooled and used for SDS analysis.

Table 4.3.2 Results of the estimation of enzyme activity and specific activity of purified samples by Ion exchange chromatography

Enzyme sample S.No.	Enzyme activity(U/ml)	Protein content mg/ml	Specific activity(U/mg)
Standard	2414.32	8.1	298.06
1	0.0	0.0	0.0
2	0.0	0.0	0.0
3	0.0	0.0	0.0
4	0.0	0.0	0.0
5	0.0	0.0	0.0
6	0.0	0.0	0.0
7	0.0	0.0	0.0
8	0.0	0.0	0.0
9	0.0	0.0	0.0
10	0.0	0.	0.0
11	499.56	2.15	232.35
12	499.56	2.15	232.35
13	**583.07**	**2.25**	**259.14**
14	416.06	1.5	277.37
15	249.05	0.8	311.31

Table4.3.3 Table for the estimation of enzyme activity and specific activity of crude and purified enzyme

Sr. No.	Sample	Enzyme Activity (U/ml)	Protein (mg/ml)	Specific Activity (IU/mg)	Purification Fold	Recovery (%)
1	crude	2414.32	8.1	298.06	-	-
2	purified	2247.31	8.85	253.93	0.85	93.08

PURIFICATION BY GEL FILTRATION CHROMATOGRAPHY:

The enzyme was purified with the help of gel filtration chromatography. The fifteen purified samples were collected by passing the crude enzyme through 2×10cm Sephadex G-100 column. The flow rate was maintained 1ml/min. All the samples were assayed for enzyme activity by Nesseler's and Bradford method. Fifth, sixth, seventh, eighth and ninth samples showed maximum enzyme activity. 8th and 9th sample were used for SDS for molecular weight determination.

Table 4.3 showing the estimation of enzyme activity and specific activity of purified samples after gel filtration chromatography.

Enzyme sample S.No.	Enzyme activity(U/ml)	Protein content mg/ml	Specific activity(U/mg)
Standard	2685.34	11.17	240.40
1	0.0	0.0	0.0
2	0.0	0.0	0.0
3	0.0	0.0	0.0
4	0.0	0.0	0.0
5	0.0	0.2	0.0`
6	373.57	0.52	718.40
7	416.06	0.4	1040.15
8	832.12	2.9	286.93
9	**999.13**	**3.37**	**296.47**
10	0.0	0.0	0.0
11	0.0	0.0	0.0
12	0.0	0.0	0.0
13	0.0	0.0	0.0
14	0.0	0.0	0.0
15	0.0	0.0	0.0

Table4.3.1 Table for the estimation of enzyme activity and specific activity of crude and purified enzyme.

Sr. No.	Sample	Enzyme Activity (U/ml)	Protein (mg/ml)	Specific Activity (IU/mg)	Purification Fold	Recovery (%)
1	crude	2685.34	11.17	240.40	-	-
2	purified	2620.88	6.39	410.15	1.70	97.59

CONCLUSION:

It was found that after purification of crude sample by Gel Filtration chromatography maximum enzyme activity and specific activity was achieved. Also %age recovery (97.59%) & purification fold (1.70) obtained was found very well. From above results it was concluded that Gel filtration method is useful method for the purification of enzymes.

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