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Determination of Sulphydryl and Disulphide Groups in Lysozyme

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Abstract -- Proteins contain several actual or potential sulfhydryl groups. These groups are very important for cellular respiration. A remarkable method of altering many proteins is to dissolve them in urea or other amide solutions. When a protein is partially denatured, that means only part of it is converted into a form insoluble under conditions under which the native protein is soluble, the insoluble fraction has the number of reactive SH and S-S groups characteristic of completely denatured protein, whereas the soluble fraction has the number characteristic of protein which has not been denatured at all. Finally, when a protein is converted by urea into a form which has an increased number of S-S groups, that form is insoluble in a medium in which native protein is soluble. In denaturation, formation of insoluble protein and increase in detectable SH and S-S groups are closely related aspect. The sulphide groups in the protein with DTNB in the tubes of 1, 2 and 3 having 1.365moles, 6.588moles and 0.158 moles respectively. Disulphide fluorescence quenching assay gives the number of moles of disulphide groups per mole of protein of the lysozyme was 10.4nmoles.

Index terms – Lysozyme, Cary Eclipse, Fluorescence Quenching Assay and Bovine Serum Albumin.

I. INTRODUCTION

Lysozyme is a commercially important enzyme and is currently used as a cell disrupting agent for extraction of bacterial intracellular products, as a food additive in milk products, as an antibacterial agent for food storage and as a drug for treatment of ulcers and infections [1]. Lysozyme is widely distributed among eukaryotes and prokaryotes, and could be classified into three major types: chicken type (c-type), goose type (g-type) and invertebrate type (i-type). Thus far, the c-type lysozyme has naturally occurred in most organisms, including viruses, bacteria, plants, insects, reptiles, birds and mammals, and it was exceptionally abundant in chicken egg whites [2]. Proteins may contain several actual or potential sulfhydryl groups. The importance of these groups for cellular respiration had been recognized early by deRey Pailhade and has been since extensively investigated by Hopkins and his co-workers [3, 4 and 5]. Such groups appear to be of importance not alone for purely oxidation-reduction mechanisms but for the enhanced functioning of certain hydrolytic biocatalysts such as urease [6 and 7], papain [8 and 9], kathepsin [10], and cerebrosidase [11].

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On the other hand, the presence of intact disulfide linkages appears to be necessary to the physiological behaviour of other active principles such as insulin [12 and 13], the appearance of free -SH groups coin- ciding with loss in activity. A remarkable method of altering many proteins is to dissolve them in urea or other amide solutions, whereby, in certain proteins, as Hopkins first pointed out, free -SH groups make their appear ance [5]. No estimation of the number of -SH groups liberated by this reagent has yet been made. In all proteins investigated, with the possible exception of egg albumin, the appearance of -SH groups in urea solution is coincident with dissociation of the protein molecule [14 and 15]. The objective of this paper is to ddetermine the number of sulfhydryl groups in a protein with DTNB (5, 5'-Dithio-bis (2-nitrobenzoic acid) and also determine the number of disulphide groups in Lysozyme by using fluorimetric method.

II. METHODOLOGY

A. Isolation of ovalbumin

1ml of the egg white was transferred into a tube and labelled and stored at 4^0C for protein assay. 2ml of the egg white was taken and placed into a centrifuge tube. 2ml of saturated ammonium sulphate solution was added. It was kept for 30min. The globulin was centrifuged at 3500rpm for 20min. The supernatant was transferred into a centrifuge tube. 2ml of 0.25M H2SO4 was filled in a 10ml cylinder and then added the same volume of saturated ammonium sulphate solution and left it for 5min to precipitate. It was centrifuged at 3500rpm for 20min and recovered the precipitate of ovalbumin and dissolved the pellet of ovalbumin and added equal volume of saturated ammonium sulphate and allowed to stand for 10min. Again centrifuged this at 3500rpm for 15min and discarded the supernatant. The precipitate was suspended in a small volume of water and transferred into small dialysis tubing.

B. Isolation of Lysozyme

3 gm of carboxymethylcellulose (CMC) was placed in a conical flask and dissolved it with the 1ml of sodium bicarbonate. 2ml of the egg white was taken and 8ml of sodium bicarbonate was added to it. It was then mixed thoroughly and labelled it as 'Lysozyme A' and stored it at 4^0C. The diluted egg white in bicarbonate buffer was added to the CMC in the conical flask. It was then mixed and the flask was placed in an ice bath.

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This suspension was transferred into a 10 ml centrifuge tube and centrifuged it at 3500 rpm for 10min. 1.5ml of supernatant was separated and labelled it as 'Lysozyme B' and stored it at 4^0C. CMC was washed in centrifuge tube by resuspending it in 6ml of bicarbonate buffer. Then this was centrifuged at 3500 rpm for 10min. The supernatant was separated and labelled it as 'Lysozyme C' and stored it at 4^0C. To the washed CMC pellets, 2ml of sodium carbonate buffer was added and stirred it 5min and centrifuged it at 3500rpm for 10min. Dialysed the supernatant which was recovered against running tap water for 2 hours to remove the alkali. Collected the 1.5ml of this solution and labelled it as 'Lysozyme D' and stored it at 4^0C.

C. Determination of total protein concentration

Standard BSA (Bovine Serum Albumin) dilutions were prepared in test tubes. The absorbencies of those dilutions were observed at 540nm and those were recorded. A graph was plotted between Concentration and Absorbance.

D. Activity and assay of Lysozyme

The bacterial culture was taken. Few ml of 0.066M phosphate buffer was added to bacterial culture. The buffer was swirled for remove the bacteria from the surface. The dilutions were made to obtain 15ml of bacterial suspension from that resulting suspension. 2ml of bacterial suspension was taken and added 0.5ml of 'Lysozyme A' solution. The solution was thoroughly mixed. Recorded the absorbencies at 450nm and continued it for every 30sec till 5min. same thing was repeated for Lysozymes B, C and D. Some graphs were plotted for these 4 samples between absorbance and time.

E. Measurement of Sulphide and Disulphide groups in a protein with DTNB

Lysozyme solution at a concentration of 0.5 mg/ml was used here. Four test tubes were set up and they were labelled as 1, 2, 3 and 4. 1.44gms were added into the test tubes of 2, 3 and 4. 0.1ml of 0.1M EDTA was added to the each tube. Then 1.0 ml of lysozyme solution was added to test tubes of 1, 2 and 3 and mixed them well. 50gms of Sodium borohydride was weighed out and it was carefully dissolved in 2 ml of distilled water. A care was taken when hydrogen gas was slowly evolved. 1 ml of sodium borohydride was added to test tube 2. 1 ml of distilled water was added to test tubes of 1 and 3. The volume of each tube was made up to 3.0 ml by adding the water. And then one drop of Octyl alcohol was added to each tube. This was mixed well and shook to dissolve the urea. Tubes were placed in a water bath at 37-38ºC and allowed the reduction reaction to proceed for 30 min. Then 0.5ml of 1M KH_2PO_4 which contained 0.2M HCl was added to each tube. Wetted the sides of the tubes of destroy the borohydride. 2 ml of acetone was added to each tube after 5 min mixed well. Then bubble nitrogen was passed through each tube for 2 min. 0.5ml of 10mM DTNB in 0.05 M phosphate buffer pH 8 was added immediately after purging with nitrogen. Finally, bubble nitrogen was passed through each tube for 2 min to fill the gas space. Stopper the tubes and allowed to stand for 15 mins at 37ºC. 3ml glass cuvettes and Varian spectrophotometer were used for reading the absorbance at 412 nm. Then the instrument was zeroed on water. The tubes were taken straight from the water bath to the cuvette keeping the contents warm to prevent phase separation which will cause cloudiness and prevent a true absorbance reading.

F. Determination of disulphide groups in lysozyme using a fluorometric method

1ml solution of crystalline RNase was diluted in distilled water. This diluted solution was contained 31.5 microgms/ml of RNase of Molecular weight is 12,640. The mole of disulphide groups per ml of this solution was calculated. A standard curve of RNase solution using dilutions ranging from 1 to 10 nmoles disulphide per ml was prepared. And then all tubes were made to 1 ml. A solution without any disulphide was used as a blank. Including this blank there was needed 11 tubes. Duplicate tubes of 1:32 dilution of the lysozyme solution (0.5 mg/ml) were prepared. Diluted the 10^{-4} M fluorescein mercuric acetate (FMA) in 0.01M NaOH (solution provided) in the 1:10 dilutions with 1 M NaOH. 1 ml of this diluted Fluorescein mercuric acetate (FMA) is added to all test tubes. 8 ml of 1 M NaOH was added to all tubes and mixed well. Tubes were left to stand for 1 hour.

G. Spectrophotometer using the Cary Eclipse

Cary eclipse fluorescence spectrophotometer was turned **on**. Cary Eclipse software was selected and opened it for the various utilities to determine the spectral properties of the FMA so that optimum settings were used for disulphide assay. Scan utility was selected and opened. The reagent blank of the standard curve tubes were taken and filled the cell about 2/3 full with carefully for not to any contamination. The outside of the sample cell was wiped gently with tissue and placed in the cell holder of the instrument. Then Prescan option was selected. Removed the popup window by clicked on **OK** and started the scan. The scan was allowed a minute to complete. The trace for excitation (red) and emission (black) were drawn on the graph window. The values of the Excitation wavelength, Emission wavelength and the PMT (photomultiplier tube) voltage of the used instrument were recorded. The graph was saved in the word file.

H. Disulphide Fluorescence Quenching Assay

The **Simple Reads** utility was opened and went to **Setup** at left top and changed the Excitation (Ex) and Emission (Em) wavelength setting to the optimum settings. The slit width was left at 5 nm. Then went to the Options tab of the popup window and selected Manual for the PMT voltage and set the voltage at the value that was obtained in the scan. OK was pressed to effect this change. After PMT settings was completed, the fluorescence reading into the onscreen report was recorded by pressing the **READ** at top of screen. This was the zero disulphide (blank). Then the relative fluorescence of each tube of the standard curve and the duplicate lysozyme sample were READ with the same settings by carefully replacing the sample in the sample cell. After completion of readings washed out the cell with water thoroughly and dried carefully. Disulphides quench the fluorescence of FMA in alkaline solution so the protein standards and samples had less fluorescence than the blank.

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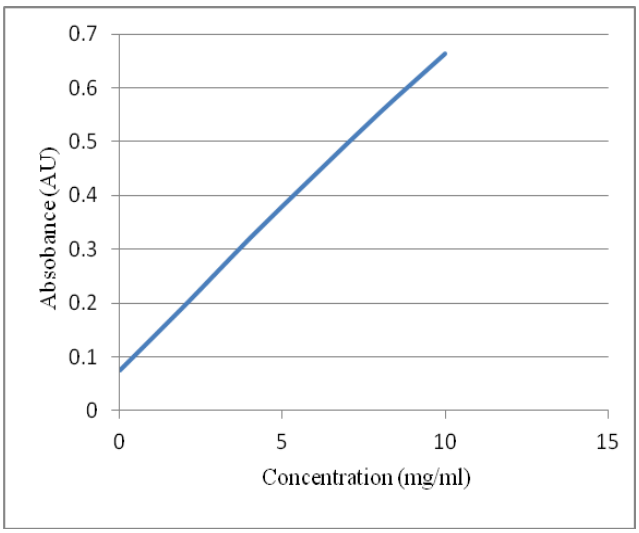
A graph was plotted between the fluorescence reading and against the number of disulphide groups (in nmole) for the RNase standards. From the fluorescence reading of lysozyme on the standard curve was worked back via dilution factors to the number of moles of disulphide groups per ml of original solution. The number of moles of disulphide groups per mole of protein was calculated.

III. RESULTS

A. *Lysozyme activity*



Graph 1: Standard curve of BSA

From the graph1
€= (A2-A1)/ (C2-C1)=0.06
Concentration of diluted egg white solution C=A/€L=5.1
A is Absorbance of that solution.
L is Width of the cuvette.
€ is Beer's law coefficient=1cm.
Concentration of Lysozyme A=3.08
Concentration of Lysozyme B=3.21
Concentration of Lysozyme C=1.73
Concentration of Lysozyme D=1.48
Absorbance and Concentrations:

Solutions	Absorbance (AU)	Concentration (mg/ml)
Diluted egg white	0.306	5.1
Lysozyme A	0.185	3.08
Lysozyme B	0.193	3.21
Lysozyme C	0.104	1.73
Lysozyme D	0.089	1.48

From the table 1 of Lysozyme A:
The gradient of tangent is at t=0,dA/dt=0.089/2.3= 0.038AU/min.
The amount of lysozyme A activity in the absorbance change of 0.001AU/(min.U)=38.
Lysozyme A in the terms of volume in units= 38/0.5=76U/ml.
(Because we added 0.5ml of Lysozyme A solution)
Specific activity of Lysozyme A=76/1.48=51.3U/mg.
(Protein content of the Lysozyme A is 1.48mg/ml)
Total activity of Lysozyme A activity=76*3.1= 235.6U.
(Multiplying the activity in terms of volume by the volume of that sample)

Activity, specific activity and total activities of Lysozyme A, B, C and D:

Samples	Gradient of Tangent (AU/min)	Lysozyme activity (U/ml)	Protein content (mg/ml)	Specific activity (U/mg)	Total activity (U)
Lys A	0.038	76	1.48	51.3	235.6
Lys B	0.021	42	1.73	24.27	130.2
Lys C	0.020	40	3.21	12.46	124
Lys D	0.019	38	3.08	12.3	117.8

B. *Calculation of sulphide groups in the tubes*

The absorbencies at 412nm of Tube 1, 2, 3 and blank (no protein) were 0.270, 0.655, 0.193 and 0.182 respectively. Absorbance values of the tubes of 1, 2 and 3 were subtracted from the tube 4 (blank). After subtraction, the absorbance values were 0.098, 0.473 and 0.011.

- Molar absorptivity is 12,000/ M, cm.
- Molecular weight of the lysozyme is 13, 930.
- Weight of protein (M) is 0.5 mg from the given data.

$$\frac{\text{MW protein X Asorbance X vol (6ml)}}{\text{12,000 X M (Wt. of protein in mg)}}$$

- Volume is 6 ml.

The number of sulphyde groups (N) in each tube were calculated from this formula

N=

- For Tube 1: N = 1.365.
  Similarly ,
- For TUBE 2: N= 6.588.
- For TUBE 3: N= 0.153.

Calculation of moles of disulphide groups per ml
The weight of RNase in the 1 ml of diluted solution = 31.5 X 10^{-6} gm/ml.
Molecular weight of the RNase= 12, 640.
Formula for number of moles: No. of moles= Weight/ Molecular weight.
Number of moles of RNase in 1ml = $31.5X10^{-6}/12,640$
$=2.5 X 10^{-9}$ moles/ml
No. of molecules of RNase per ml =No. of moles of RNase X Avogadro's number (N)
$=2.5X10^{-9} X 6.022 X 10^{23}$ molecules/ml
There are 4 disulphide groups per molecule of RNase,
Hence,
No. of disulphide groups per molecule of RNase
$=2.5X10^{-9} X 6.022 X 10^{23}$
X4 disulphide groups/ml
Therefore,
Moles of disulphide groups = $2.5X10^{-9} X 6.022 X 10^{23}$ X4 /Avogadro's number
= 10X10-9 moles/ml
Moles of disulphide groups = 10 nmoles/ ml.

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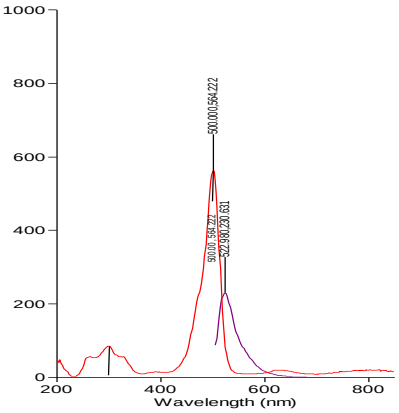
C. Spectrophotometry Using Cary Eclipse Fluorescence Spectrophotometer (Spectrofluorimeter)

Spectrum of Fluorescein Mercuric Acetate (FMA) The spectral properties of the FMA was determined to obtain the optimum settings for the disulphide assay



Graph 2: Graph obtained in Cary eclipse Fluorescence

Spectrophotometer showing the spectrum of Fluorescein Mercuric Acetate (FMA).

D. Disulphide Fluorescence Quenching Assay

The relative fluorescence of all the 11 tubes of the standard curve and the 2 tubes with the duplicate lysozyme sample were read using the spectrum settings and the PMT voltage was taken in such a way that the scale was between 800-1000 relative fluorescent units. The result was obtained as follows.

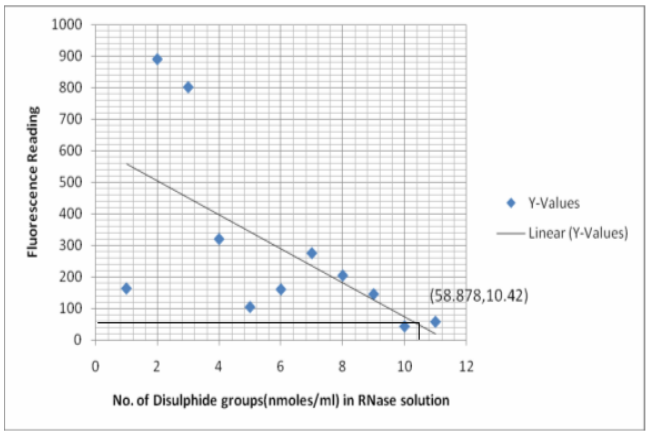
Simple Reads Report
Report Time: Wed 06 May 03:23:26 PM 2009
Software Version: 1.1(132)
Instrument Parameters

Instrument	Cary Eclipse
Instrument Serial Number	EL04094118
Data mode	Fluorescence
Ex. Wavelength (nm)	500.00
Em. Wavelength (nm)	522.98
Ex. Slit (nm)	5
Em. Slit (nm)	5
Ave Time (sec)	0.1000
Excitation filter	Auto
Emission filter	Open
PMT Voltage (V)	550

Results Flags Legend
@ = Over-range

Tubes	Read Int. (a.u.)
1	826.843 (blank)
2	164.508
3	890.787
4	802.139
5	321.150
6	105.825
7	161.704
8	276.381
9	205.634
10	146.579
11	44.380 (lysozyme)
12	58.878 (lysozyme)

A graph was plotted between the obtained fluorescence readings against the number of disulphide groups (in nmole) for the RNase standards.



Graph 3: shown No. of disulphide in RNase solution on x-axis vs Fluorescence readings on y- axis.

From the graph,
The corresponding values for the absorbance of the duplicate lysozyme solutions are 10.4 nmol/ml when converted comes to 10.4 X 10^{-3} µmol/ml.
The duplicate lysozyme solution was prepared in the 1:32 ratio,
The volume of the lysozyme used= 31.25 µl,
The concentration of the lysozyme from stock was 0.5 mg/ml,
Hence,
The weight of the lysozyme in the 1000µl lysozyme solution
= (31.25 µl/1000 µl) X 500 µg/ml
= 15.625 µg/ml
Therefore,
No. of Moles of lysozyme = Weight/Molecular weight
= 15.625X10^{-6}gm/13,930
= 1.12167 X 10^{-9} mol
= 1.12167 nmol
Therefore,
The number of moles of disulphide groups per mole of protein,
The number of moles of disulphide groups in lysozyme solution one
= 10.4 nmoles /ml.

IV. DISCUSSION

From the table 2, Lysozyme activities of four samples Lys A, B, C, D were 38U, 21U, 20U, 19U respectively. The specific activities of four samples Lys A, B, C, D were 51.3, 24.27, 12.46, 12.3U/mg respectively. But the specific activity values could not give expectable results. From the observation of these values, the values are decreased progressively. The total activities of these four samples Lys A, B, C, D are 235.6, 130.2, 124, 117.8U respectively.

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These values are increased progressively. The Total activity values could have given the good and expectable results. From the analysis of measurement of sulphide and disulphide groups in a protein with DTNB, tube 2 contained the urea and sodium borohydrate, tube 1 did not contain urea and sodium borohydrate. From the table 3, tube 1 had 1.365 sulphide groups and tube 2 had 6.588 sulphide groups in their protein sample. When a protein is partially denatured, that means only part of it is converted into a form insoluble under conditions under which the native protein is soluble, the insoluble fraction has the number of reactive SH and S-S groups characteristic of completely denatured protein, whereas the soluble fraction has the number characteristic of protein which has not been denatured at all. Finally, when a protein is converted by urea into a form which has an increased number of S-S groups, that form is insoluble in a medium in which native protein is soluble. In denaturation, formation of insoluble protein and increase in detectable SH and S-S groups are closely related aspect. From that tube 2 had increased sulphide groups than tube 1. Coming to Sodium borohydride (NaBH4), it can be destroyed by acidification or with acetone. Reduction of the --SS-- linkage in pure chemical compounds with this agent Acetone alone destroyed the excess NaBH4, but after treatment with DTNB a yellow colour developed slowly even in solutions free of --SH groups, and absorbance of solutions with --SH present gradually increased with time For this reason a mixture of HCl with acetone was adopted. Acetone also served as an antifoam agent, but too much acetone precipitated protein which no longer dissolved at neutrality. From the table 3, the no. of sulphide groups in tube 2, are 6.588 moles and the no. of sulphide groups in tube 3 are 0.158 moles. So tube 2 which had sodium borohydrate has increased sulphide groups because it reduction the sulphide linkage and encourage to precipitate the protein. That's why the protein insoluble which contain increased sulphide groups. Where as tube 3 which had no sodium borohydrate has decreased sulphide groups when compare with the tube 2. From the graph 3, which was shown the no. of disulphide groups in RNase solution on x-axis and Fluorescence readings on y- axis, the mole of disulphide groups per ml of protein is 10.4 nmoles which is obtained from the fluorimetric method.

V. CONCLUSION

The sulphide groups in the protein with DTNB in the tubes of 1, 2 and 3 having 1.365moles, 6.588moles and 0.158 moles respectively. There is much difference in number of free Sulphide groups in tubes 1 and 2 will be 5.223moles. So the tube which has urea has more sulphide groups than other. At the same time and similarly there is much difference in number of free sulphide groups in tubes 2 and 3 will be 6.430. So the tube which has sodium borohydrate has increased sulphide groups than other tube. From the second technique i.e by disulphide fluorescence quenching assay gives the number of moles of dilsulphide groups per mole of protein of the lysozyme was 10.4nmoles.

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