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Biophysical Interaction of Cationic Detergent Dodecyl Trimethylammonium Bromide with α-Amylase Inhibitory Activity for Targeting Drug Delivery

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ABSTRACT
Surfactants are known to play a vital role in many processes of interest in both fundamental and applied science. Amylases are important hydrolase enzymes which have been widely used since many decades. The interaction of dodecyl trimethyl ammonium bromide (DTAB) as a cationic surfactant with α -amylase has been investigated through analysis of acid-base titration curves of enzyme at various concentrations of DTAB. In this regard the acid-base titration curves of enzyme in various concentrations of DTAB have been measured and analyzed using Tanford theory. The intrinsic ionization constant (pk_{int}) and work function (w) of ionized groups were calculated. The trend of variations of pk_{int} and w shows that, the maximum unfolding of enzyme occurs in concentration range of 3-5mM of DTAB. The binding of DTAB ions to the carboxylic groups has been occurred more than other groups. Moreover the enzyme removed from globular form after this concentration range of DTAB.
Keywords: α-Amylase; Inhibitory; Dodecyl Trimethylammonium Bromide

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INTRODUCTION

Amylase is a general name that represents a group of effective enzymes on the starch and glycogen. The most important kinds of amylase are α and β that can be obtained from different sources such as bacillus and mammals body. The used α -amylase in this research has been obtained from bacillus sub tiles with molecular mass of 69000 Dalton that contains 588 amino acid tails and it's enzyme commission E.C.3.2.1.1 [1].

The recent constructional studies on the α -amylase of bacilli and mammals, specify interesting features of catalytic properties and constructional form of these series of enzymes and indicate that α -amylase belongs to a great set of glycogen hydrolyses. About physical properties, α -amylase has been investigated in group of compact globular proteins and optimum conditions of enzyme operation are the temperature of 25 c and pH=6 [2].

Bacillus α -amylase has been used vastly in industrial processes. Measurement of α -amylase activity is carried out based on decreasing of sugar amount which is exposed to the enzyme. Because of importance cases of α -amylase, today, study on this enzyme is carried out in scientific and industrial centers [3].

Titration of protein is one of the important informational tools about titrated groups on the protein surface. All proteins contain various acidic and basic sites and because of proteins difference in kind and order of amino acids, each protein has special acidic and basic properties. In proteins titration, tyrosine and cytosine are highly important. Side chain of these amino acids terminate to the COOH, NH_2, guanidine, imidazole, phenol and – SH groups, so they have acidic and basic properties. Also two titrated agents of final amine and final carboxyl cause that proteins have acidic and basic property [4]. The first purpose of titration studies is extraction of information about quality of acidic and basic groups of side chains in proteins and also extraction of ideas about shape and size of protein molecule in solution through interpreting the electrostatic interactions values.

In this report our interest is at first investigation of the α -amylase variations in presence of different concentrations of DTAB and presentation of a mechanism for Justification of variations kind and attainment to the role of different part of surfactant in this kind of interaction. Then with determining the

titration curves of α -amylase in presence of DTAB and analyzing it through existence theories, the role of titrated groups in this interaction will be investigated.
The other purpose of this research is development of existence theories in analysis of DTAB binding data to the α -amylase and totally we can obtain more information about kind of interaction of surfactants with proteins through analysis of obtained results [5].

MATERIAL AND METHODS

Materials

Chloridric acid, sodium hydroxide, dodecyl trimethyl ammonium bromide (DTAB), sodium bromide and α -amylase E.C.3.2.1.1 with bacillus sub tiles were obtained from Merck. All solutions were prepared using double distilled water and were freshly prepared before using. Experiments were carried out at 25°c and in ionic strength of 1mM NaBr.
All pH-metric determinations were done on a Horriba F-12 pH-meter. Because of pH-meter sensitivity to the temperature, all experiments were carried out under the temperature controlling of pH-meter and thermostat. Magnetic stirrer on the model of HM-101 was used to homogenize the solutions.

Method

At first the electrolyte solution of NaBr (1mM) was prepared, then 1ml of NaBr solution was transferred to the titration cell. The Nitrogen gas was passed over the solution surface for 5 minutes in order to emitting the CO_2 from solution and emitting the oxygen from surface of solution. In order to regulate the PH at pH=7, NaOH (0.01M) or HCl (0.01M) was added to the solution, and was titrated with NaOH (0.01M) until pH=12.
In all experiment stages, temperature was controlled through water rotation around the solution cell and regulation of thermostat temperature of water bath at 25°c. In second stage, 1ml of the same electrolyte solution was titrated with HCl (0.01M) until pH=2.5, after traversing the initial stages in first stage, (passing the Nitrogen gas, temperature regulation and pH regulation at pH=7).
In each stage, pH of solution was recorded after each addition of a specified amount of NaOH or HCl. In order to trust in equilibration in all experiments, pH was recorded 2 minutes after adding the acid or base solution. These data have been obtained for solutions with volume of 1ml and surfactants with different concentrations of ($1*10^{-3}$, $3*10^{-3}$, $5*10^{-3}$ and $7*10^{-3}$M) at mentioned conditions in previous stages in presence of α -amylase and in absence of α -amylase.
All solutions were prepared using electrolyte solutions of NaBr. Considering the obtained results we can determine the number difference of added acid or base moles for reaching to a specified pH, between a solution that contains protein and a solution without protein. If we assume that all carboxylic groups on the α -amylase molecule have been protonated at pH=2.5, we can calculate the average number of dissociated H^+ of an α -amylase molecule (r) at each specified pH. With drawing the plot of dissociated H^+ moles number (r) versus pH, the titration curve is obtained. The results of experimental calculations of r at various pH acid-base titrations with α -amylase in concentration of 0.003 M of DTAB are listed in table 1.

Table1. The results of experimental calculations of r at various pH, [DTAB] =0.003 M

pH	ΔV	V	r	pH	ΔV	V	r
3.79	10.00	6.90	200.10	2.50	300	207.00	0.00
4.14	3.98	2.75	204.25	2.53	270	186.30	20.70
4.23	3.78	2.61	204.39	2.55	250	172.50	34.50
4.88	0.20	0.13	206.90	2.57	222	153.18	53.80
5.48	2.90	2.00	205.00	2.60	180	124.20	82.80
6.31	2.73	1.88	205.12	2.63	129	89.15	117.80
7.00	0.00	0.00	207.00	2.73	114	79.00	128.00
7.36	1.40	0.97	207.97	2.74	116	80.04	126.70
7.50	2.33	1.60	208.60	2.78	107	73.60	133.40
7.66	2.72	1.90	208.90	2.80	105	72.45	134.55
7.85	5.00	3.45	210.45	2.82	108	74.17	132.80
8.04	7.29	5.04	212.04	2.88	80	55.20	151.80
8.05	7.39	5.10	212.10	2.94	66	45.54	161.46
8.20	9.00	6.21	213.20	2.98	58	40.02	166.98
8.44	11.00	7.60	214.60	3.04	40	27.60	179.40
8.50	10.78	7.44	214.44	3.17	32	22.20	184.80
9.21	12.10	8.30	215.30	3.18	31	21.56	185.44
9.40	13.45	9.30	216.30	3.30	26	18.11	188.90
9.76	13.7	9.40	216.40	3.33	27	19.11	187.90
9.84	16.10	11.11	218.11	3.56	20	13.80	193.20

RESULTS AND DISCUSSION

The results of first site set calculations in concentration of 0.003M of DTAB are listed in table 2. And the results of second site set calculations in concentration of 0.003 M of DTAB are listed in table 3.

Table2. The results of calculations for first site set, [DTAB] =0.003M

r_1	pH	$\log(\frac{r_1}{n_1 - r_1})$	$pH - \log(\frac{r_1}{n_1 - r_1})$	Z
15.00	2.51	-0.69	3.20	263.48
20.70	2.53	-0.52	3.05	257.78
27.00	2.52	-0.35	2.87	251.48
34.50	2.55	-0.19	2.74	243.98
53.80	2.57	0.19	2.38	224.68

Table3. The results of calculations for second site set, [DTAB] =0.003 M

r_2	pH	$\log(\frac{r_2}{n_2 - r_2})$	$pH - \log(\frac{r_2}{n_2 - r_2})$	Z
117.80	2.63	0.34	2.29	264.77
123.80	2.70	0.42	2.28	258.80
126.70	2.73	0.46	2.27	255.87
130.00	2.75	0.50	2.25	252.57
134.55	2.80	0.56	2.24	248.02

Analysis of α -amylase titration curves in presence of DTAB

Titrated groups in α -amylase enzyme and their pka are listed in table 4.The titration curve is obtained from constructing the plot of dissociated H^+ ions number (r) versus pH. Different part of calibration curve is related to the titration of different sites of molecule. Based on tables 5 and 6 and considering the pka of titrated groups, it is obvious that carboxyl groups have the most acidity, imidazole and α -amine groups are intermediate and phenol and side chain amine groups have the least acidity (Guanidine groups are titrated at pH>12 that aren't considered according to the investigation range).

Table4. Titrated groups of α -amylase

Titrated groups	The number of groups from amino acid succession	Expected pKa from small molecules data
α-COOH	10.00	3.75
β, γ - COOH	99.00	4.60
Imidazole	17.00	7.00
α - NH2	10.00	7.80
ε - NH2	59.00	9.60
Phenolic	19.00	10.20
Guanidine	23.00	>12.00

Table5. Calculated amounts of w and pk_{int} of α -amylase in different concentrations of DTAB for first site set

[DTAB]	W	pk_{int}
0	0.0180	-2.2362
1	0.0180	0.2778
3	0.0240	2.3118
5	0.0210	-1.5324
7	0.0150	-0.5993

Table6. Calculated amounts of w and pk_{int} of α -amylase in different concentrations of DTAB for second site set

[DTAB]	W	pk_{int}
0	0.0054	-10.7700
1	0.0110	1.1776
3	0.0040	1.4486
5	0.0050	1.6673
7	0.0008	2.9852

For analyzing the titration curves, in Tanford theory it has been assumed that all titrated groups of one kind in protein molecule have equal intrinsic dissociation constant (pk_{int}). Considering α -amylase as a polyelectrolyte, if n_i group of one kind were in α -amylase molecule, and r_i was the number of ionized groups of i kind in a specified pH we have:

$$\frac{r_i}{n_i - r_i} = \frac{x_i}{1 - x_i} = \frac{k_i}{a_{H^+}} \qquad x_i = r_i / n_i$$

Considering the electrostatic interactions between sites, k_i is obtained based on the equation below mentioned:

$$K_i = (k_{int})_I e^{2wz}$$

Where w is work function, z is the charge on the protein surface and $(k_{int})_i$ is intrinsic dissociation constant for titrated group of i. Using titration curve, r (the total number of dissociated H^+ groups) that is the sum of r_i for all groups, is determined and considering the approximate amount of pka of groups, we can estimate the r_i from titration curve. In other hand in absence of information about distribution quality of charged groups on the protein, amount of 0.8680 wz was considered independent of group kind from which protein is dissociated.

The Z charge at first is due to the number of dissociated or bound proteins, that its amount is zero at isoelectric pH (IpH) and in other hand is influenced by binding of ions in the solution. So in calculation of z, the average amount of bound surfactants ions to the protein should be considered. In this system, ions of Na^+ and Br^+ exist in addition to the surfactant ions, that with considering the results of Scatchard and his colleagues studies and slight amount of binding of these ions to the α -amylase in studied concentrations, we can ignore their contribution in the charge value on the α -amylase [6].

So in presence of DTAB, the charge on the protein surface (z) at each specified pH is obtained from equation below mentioned:

$$Z = r_{(IpH)} - r + V_{DTAB}$$

Where, $r_{(IpH)}$ is the average number of dissociated H^+ of an α -amylase molecule at its isoelectric pH. IpH=5.6 was considered according to the reference. V_{DTAB} is the average number of bound surfactants to the α -amylase molecule. Considering their independence from pH, their amounts have been extracted from binding isotherms. we can determine amounts of $(pk_{int})_i$ and w for that group in different concentration of surfactant.

Analysis of the α -amylase titration curves in presence of DATB

Titration curves of α -amylase in presence of different concentrations of DTAB are shown in Figure 1.The calculated amounts of pk_{int} and w of ionized groups for first and second site sets of α -amylase are presented in tables 5 and 6. The total pk_{int} variations depend on the environmental variations around the titrated groups. For interpreting these results, we can claim that the environmental variations around these groups in effect of interaction with DTAB are due to the two factors:

Binding of hydrophobic tails of surfactant in adjacent sites of titrated groups that causes to decrease regional dielectric coefficient of these groups environment and also decrease their solution amount that causes to decrease the ionization amount and increase the groups' pk_{int}.Unfolding of protein that causes to increase the solution and increasing of ionization amount of titrable groups causes to decrease pka of these groups.

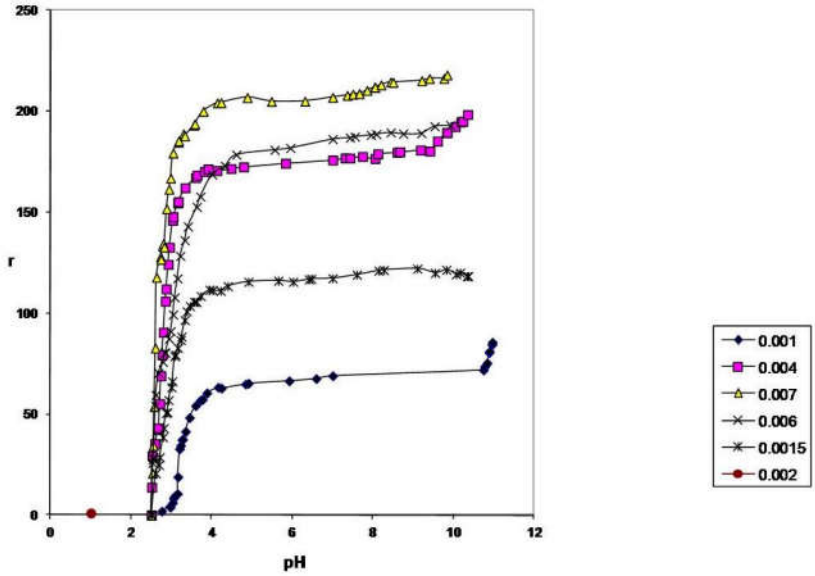

Figure1. Titration curves of α -amylase in presence of different concentrations of DTAB

According to the tables 5 and 6, amounts of pk_{int} of first site set are positive up to concentration of 3mM of DTAB and in concentrations of 5 and 7mM, amounts of pk_{int} become unusually negative, indicates that applied approximations aren't correct.

These approximations are globularity of proteins and equality of titrable groups, so we can claim that the most unfolding occurs in DTAB concentrations, which are more than 3mM. In second site set, this issue occurs in DTAB concentrations more than 5 mM. This issue indicates that these groups are not highly exposed to the solution and follow the related approximations after protein unfolding.

The observed changes of w, based on different concentrations of DTAB can be justified according to the equation below mentioned:

$$W = \frac{\varepsilon^2}{2D\ \mathrm{Re}\ KT}\left(1 - \frac{K\ \mathrm{Re}}{1 - Ka}\right)$$

This equation is obtained based on the Debye-Huckel theory. Re is the radius of globular molecule, a, is effective radius of globular molecule (radius of hydrated molecule), D is dielectric constant, and k is Debye -Huckel constant. Depending on the protein radius (Re) and environment dielectric constant (D), increasing of Re or D cause to decrease w and decreasing of these two factor cause to increase w.

About w variations in presence of different concentration of DTAB, (Table 5), according to the discussion about pk_{int} variations of carboxyl groups we can claim that interaction of DTAB with α -amylase and binding of its hydrophobic tail, around the titrable groups, cause to decrease the dielectric coefficient and increase the w according to the equation.

The trend of increasing about w_1 (first site set) is also due to this reason. With increasing the DTAB concentration and increasing the binding amount of DTAB to the protein, amount of D decreasing and w increasing continue until α -amylase be unfolded and it's effective radius be increased because of denaturation, that causes to decrease the w.So reciprocity of two mentioned factor and their relative amount in different concentrations of DTAB, causes that w_1 in DTAB concentration of 3mM be the most amount and we can claim that in this concentration the most unfolding of α -amylase has been occurred. About w_2 amount, we observe the same trend and in concentration range of 3-5mM, w has the least amount. According to the obtained results we can claim that, up to DTAB concentration of 3mM, α -amylase has it's globular shape and then in concentration range of 3-5 mM the globular shape changes because of unfolding and denaturation in effect of binding of DTAB, and analysis of titration curves according to the Tanford theory, doesn't represent acceptable results. Binding of DTAB before unfolding around the titrable carboxylic groups is much more than imidazole and amine groups. Because the variations amount of pk_{int} and w for carboxyl groups is much more than other groups

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