



PHYSICO-CHEMICAL STUDIES OF MOLYBDENUM (VI)- β-AMYLASE INTERACTION

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ABSTRACT

The binding of molybdate ions has been studied with β -amylase using polarography and equilibrium dialysis techniques. The extent of binding has been determined by means of Tanford's method and the value of average association constant ($\log K$) and maximal number of binding site (n) have been calculated from Scatchard plot at pH 5.57. These have been found to be 3.3600 and 17, respectively. The non linear plots from polarographic and equilibrium dialysis at pH 5.57 are analysed for primary and secondary sites as well as their apparent association constants. Their $\log K_1$ and $\log K_2$ have been found to be 3.9884 and 2.9348 from polarographic and 4.0355 and 3.2907 from equilibrium dialysis, respectively. The number of primary (n_1) and secondary (n_2) sites have been found to be 4 and 13, respectively. The involvement of single primary sites has also been confirmed from absorption spectra of Mo (VI) - β -amylase mixture, which revealed absorption peak at pH 5.57 and not at other higher pH values. In higher pH range, where polarographic is not applicable, so Mo (VI) binding to β -amylase is determined by dialysis equilibrium method at higher pH-range. These have been found to be 3.4070, 2.2788, 2.7980, 2.7568 and 17, 16, 14, 7 at pH values 5.57, 7.50, 9.50 and 11.50, respectively. A good correspondence among the data obtained from the two techniques supported that electrostatic effects are negligible and the alterations of binding constants is solely due to molybdate ion- β -amylase combination. The number of sites available for binding with molybdate ions is much less than the actual number of cationic groups on β -amylase molecule. The qualitative binding results of pH-metry and spectroscopy are also strongly support the results of diffusion current and dialysis methods. The pH dependence of Mo (VI) - β - amylase interaction could be explained by assuming an interaction with the positively charged groups such as ϵ -ammonium, imidazolium and guanidinium groups, which are ionized at different pH levels. The involvement of fewer such groups in binding processing may be due to the in availability of all cationic groups due to folded structure of β -amylase molecule. However, owing to the complexity of the macromolecular structure, it was difficult to predict the nature of exact groups that were involved in the molybdate β -amylase interaction. The free energies of molybdenum (VI) - β - amylase complexes were also determined for the support of binding of Mo (VI) with β -amylase.

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Key words : Molybdenum, β -amylase, Equilibrium dialysis, Polarography.

INTRODUCTION

β -Amylase (1,4- β -glucan maltohydrolase) is a hydrolytic enzyme, which digests 1,4- β -glucosidic linkages of starch-type substrates and yields β -maltose. The β -amylase exists as isoforms in several plants such as barley¹⁻³, Canadian hard red spring wheat⁴ and soybean⁵. Visuri and Numi² purified β -amylase from barley and identified four isoforms with different isoelectric points. Recently, four major forms of the barley β -amylase with different molecular masses were separated⁶. In barley and wheat seeds, β -amylase exist in two forms : free form and bound form⁷⁻¹⁰. The free form of the enzyme can be extracted in saline solution, whereas the bound-form is known to convert into free-form during germination⁸⁻¹¹. In germinating leguminous seeds, such as soybean and lentils, β -amylase activities decrease during germination^{12,13}.

In alfalfa, Doehlert et al.¹⁴ have reported the occurrence of β -amylase in their amylase preparation obtained by gel filtration on Sephadex G-75. Kohno et al.¹⁵ have also purified the alfalfa β -amylase by gel filtration and ion-exchange chromatography and suggested the existence of several isoforms of this enzyme. Kohno et al.¹⁶ reported the purification of alfalfa β -amylase to homogeneity by chromatofocusing and cation-exchange chromatography, and the identification of five isoforms of the enzyme with different isoelectric points, but the same molecular mass (61000).

On account of increasing significance of various electrolytes in the biological systems, considerable attention has been paid to investigate the binding of electrolytes to biopolymers¹⁷. Although majority of these investigations deal with cations, the presence of anions greatly affects the binding properties^{18, 19}. Previous studies on anion binding have usually employed large hydrophobic anions²⁰⁻²² and simple inorganic anions^{23, 24}. However, not much is known on the binding of metaloxoanions with proteins^{25, 26}. Arora et al.²⁷ have compared the vanadate anion binding by bovine serum albumin (BSA) and bovine pancreatic trypsin (BPT) employing diffusion current measurement and equilibrium dialysis method²⁸. These workers have also reported the binding of vanadate ion to soluble ovalbumin (OAS) and denatured ovalbumin (OAD)²⁹ employing polarography and equilibrium dialysis methods. Malik and Arora^{30, 31} made a quantitative study on the binding of molybdate ion with fibrillar and globular proteins employing physico-chemical methods. Arora et al.^{32, 33} have also reported its binding to gelatin and OAS and OAD. They also reported its binding to BSA in soluble range³⁴ as well as in insoluble range³⁵.

In spite of the several biological^{36, 37} and biochemical³⁸⁻⁴³ functions of molybdenum, its linking in the form of molybdate anion to β -amylase has never been reported. In this paper, the binding between β -amylase and molybdate anion has been reported employing polarographic and dialysis equilibrium methods. The results are also supported by pH-metric and ultra-violet spectroscopy. The effect of pH and protein concentration on the binding has been discussed and a probable mechanism of interaction has been proposed.

EXPERIMENTAL

Enzyme : β -Amylase was a Sigma Chemical Products (Mol. wt. 61,000). Its stock solution was prepared in doubly distilled water. The concentration of β -amylase solution was determined by colorimetric biuret method. It was stored in a refrigerator and purified toluene was added to check its surface denaturation.

Reagents : Sodium molybdate (E. Merck) was dissolved in double distilled water and estimated gravimetrically by oxine method. Sodium acetate and acetic acid (BDH) were used for the preparation of acetate buffer of pH 5.57 while phosphate and carbonate buffers were prepared from reagent grade chemicals. KCl (BDH) solutions were used for the adjustment of ionic strengths at 0.15 M.

Polarographic measurements : These were made on a Toshniwal polarograph in conjunction with an Osaw galvanometer in the external circuit. A polarographic cell as recommended by Tanford⁴⁴ was used. Purified nitrogen was passed through the reaction mixtures to remove the dissolved oxygen and triply distilled mercury was used for the dropping mercury electrode (DME). The polarographic cell was immersed in a water thermostat at 25°C. The capillary used had flow rate of about 2.2 mg/sec. with a drop time of 3.5 seconds. The rate of flow of mercury drops was determined by the Lingane's method. Since the capillary characteristics (m.t.) have a marked effect on the diffusion current (which is directly proportional to $m^{2/3}t^{1/6}$), these factors were, therefore, controlled carefully through out these studies. The following mixtures were prepared for diffusion current measurements –

- (i) Different amounts of β -amylase (2.0 to 25 g/L) were taken in different tubes and 1.0×10^{-4} M sodium molybdate solution was added to each of them. The total volume was made 10 mL. by adding buffer of pH 5.57, distilled water and KCl solution to have an ionic strength at 0.15 M. The results are expressed in the form of protein concentration vs. i_d/i_{d_0} where i_{d_0} and i_d are the diffusion current Mo (VI) in the absence and presence of β -amylase.

- (ii) A fixed amount of (β -amylase (5.0g/L) was taken in different tubes and varying amounts of sodium molybdate (1.0 to 50×10^{-4} M) were added in a total volume of 10 mL made so by buffer, water and KCl.
- (iii) Varying amounts of sodium molybdate (1.0 to 50×10^{-4} M) were taken as in (ii) in the absence of β -amylase. The results of all the above sets are reported in the form of current voltage curves (polarograms).

Dialysis equilibrium method : In this method, a solution of known β -amylase concentration (5.0 g/L) at the desired ionic strength (0.15) in KCl was placed inside a cellophane tubing, which had been properly rinsed and finally soaked in KCl solution of the desired ionic strength. The dialysis tubing was inserted in a glass stoppered boiling tube having the outside solution KCl and sodium molybdate. The desired pH of the 'inside' and 'outside' solutions was maintained with the help of buffers of pH's 5.57, 7.50, 9.50 and 11.50, respectively. Several similar sets of mixtures having fixed 'inside' β -amylase and varying 'outside' sodium molybdate (1.0 to 50×10^{-4} M) solutions (total volume 5.0 mL 'inside' and 'outside') were prepared. The tubes were mechanically shaken at 25°C for a time just sufficient to attain the equilibrium. The outside solution was then separated and analysed for its molybdenum content colorimetrically by oxime method⁴⁵.

In a condition, when no precipitation occurred, the average number of moles of molybdate ion bound per mole of β -amylase (mol. wt. 61,000)⁴⁶, (V_M) may be determined directly as follows:

$$V_M = \frac{C_O - C_F}{[P]}$$

Where C_O and C_F represent the total and free molar concentrations of molybdate and $[P]$ is the total molar concentration of β -amylase (1.0×10^{-4} M). Control experiments indicated that molybdate ion binding by the dialysis tubing was essentially negligible, but a small loss of protein always occurred, when the bag was tied off and the initial volume of β -amylase was always less than the outside solution.

pH-Metric measurements : These measurements were made on an Elico pH-meter using a wide range combined electrode. The instrument was calibrated against 0.05 M potassium hydrogen phthalate solution (pH 4.0) and 0.05 M borax solution (pH 9.20) for the acidic and basic ranges, respectively.

Procedure

(i) (a) Varying amounts of 0.10 M KOH or HCl were taken in different tubes and 5.0 g/L of β -amylase solution was added to each of them. The total volume was made 15 mL by adding distilled water and KCl solution to maintain the ionic strength at 0.15 M.

(b) Varying amounts of 0.10 M KOH or HCl were mixed with 0.002 M sodium molybdate and 5 g/L β -amylase and the total volume was made 15 mL as in (a).

(ii) 0.002 M sodium molybdate were mixed with different amount of KOH or KCl in a total volume of 15 mL with and without β -amylase. The pH-values were recorded immediately after preparing different mixtures. Purified nitrogen was passed through alkaline solutions to ensure complete inert atmosphere. The results are shown in the form of pH vs. volume of HCl or KOH and as Δ pH plotted against pH of mixtures or volume of acid or alkali added.

Spectrophotometric measurements: These were made on an Elico U.V. visible spectrophotometer. For these measurements, the spectra of 1 g/L β -amylase in the presence and absence of 0.0001 M molybdate were recorded at five different pH values viz., 4.50, 5.57, 7.9, 9.2 and 11.7, respectively. These spectra are given in Figures 1 to 6 in the form of absorbance (OD) plotted against wavelength (λ). The shift in the curve of β -amylase in the presence of molybdenum was taken as a qualitative evidence of binding. The molar extinction coefficient (ϵ) was calculated by the following relation:

$$\epsilon_{\text{Mo}} = \frac{\text{Absorbance Mo} + \text{Absorbance } \beta\text{-amylase}}{C \times d}$$

and

$$\epsilon_{\beta\text{-Amylase}} = \frac{\text{Absorbance } \beta\text{-amylase}}{C \times d}$$

When, $d = 1$ cm, then

$$\epsilon = \frac{\text{Absorbance (O. D.)}}{\text{Molar concentration (C)}}$$

The different values were calculated at 210 nm because β -amylase and molybdate exhibited maximum absorbance at this wave length.

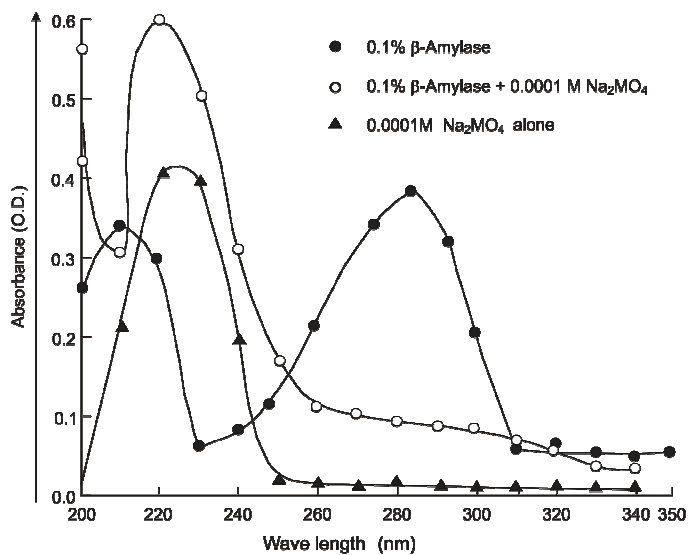


Fig. 1 : Plots of absorbance against wavelength (nm) in β -amylase-molybdate system at pH = 5.57 and 25°C

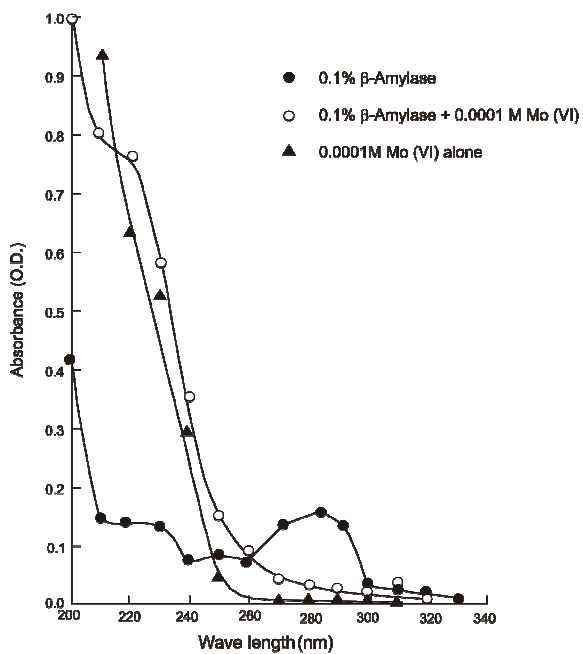


Fig. 2 : Plots of absorbance against wavelength (nm) in β -amylase-molybdate system at pH = 4.50

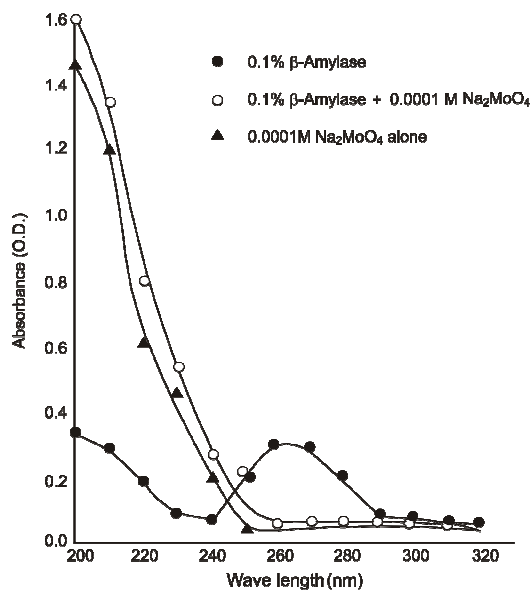


Fig. 3 : Plots of absorbance against wavelength (nm) in β -amylase-molybdate system at pH = 7.90

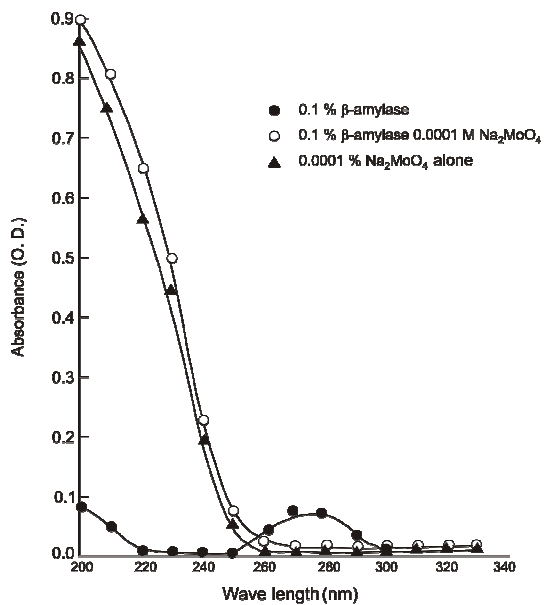


Fig. 4 : Plots of absorbance against wavelength (nm) in β -amylase-molybdate system at pH = 9.20

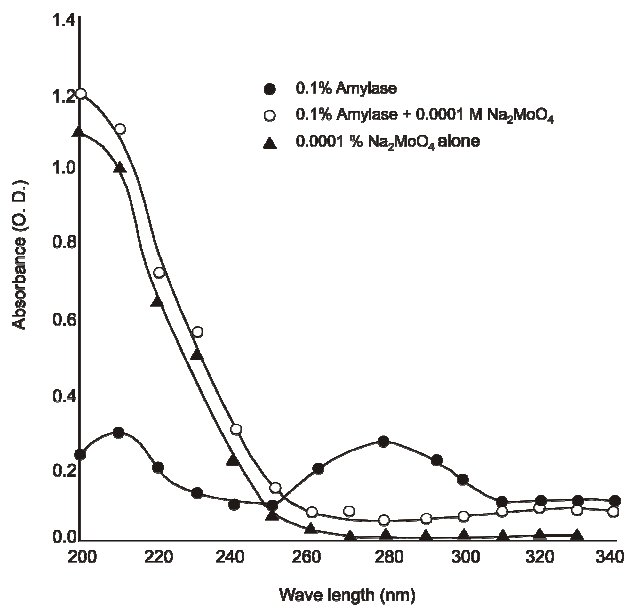


Fig. 5 : Plots of absorbance against wavelength (nm) in β -amylase-molybdate system at pH = 11.72

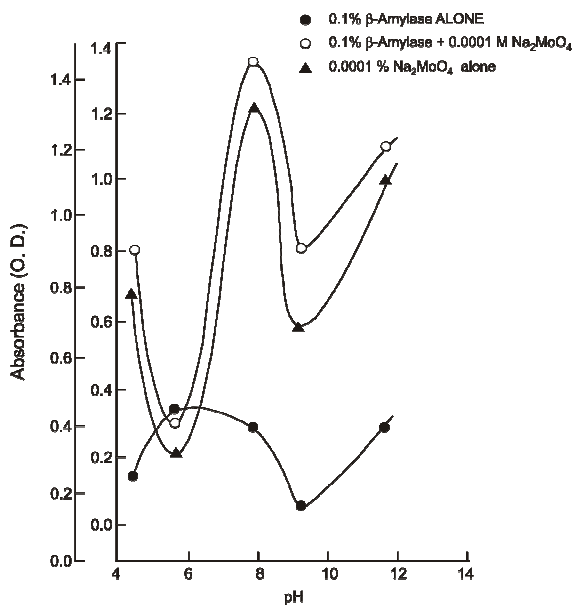


Fig. 6 : Absorbance v/s pH at 210 nm in β -amylase-molybdate system

RESULTS AND DISCUSSION

Diffusion current measurements

On the addition of β -amylase to buffered molybdate solutions caused the depression of the polarograms, however, their shape remains unaffected. If there was no change in the height of polarogram then there could not be interaction between molybdenum and β -amylase. Since the general shape of all polarograms was identical in presence of added β -amylase, hence no change in molybdenum species could be predicted. Although the diffusion current of molybdate ion decreases upon the addition β -amylase, no shift in half-wave potential ($E_{1/2}$) was found in the present investigation. A similar reduction in the diffusion current was also observed by Malik et al.^{30, 47} and Arora et al.^{32, 34} in the interaction of dyes and molybdate ions with proteins, and hence, the decrease in the diffusion current of molybdate anion in the presence of increasing amounts of β -amylase must be attributed to binding. These observations are in agreement with those of Tanford⁴⁴, who believed that when a reducible substance is bound to a protein, then there is a reduction in the diffusion current and this decrease may be used to calculate the linkage constants. Assuming i_d and i_{do} as the diffusion currents of molybdate ion in the presence and absence of β -amylase, and C_O , C_F and C_B as the molar concentration of total, free and bound molybdate ion in the system, we may calculate the results as follows –

$$C_O = C_F + C_B \quad \dots (i)$$

$$\text{or, } C_B = \frac{C_O - i_d/i_{do} \cdot C_O}{(1 - k)} \quad \dots (ii)$$

Equation (ii) gives the value of bound molybdate directly. The value of k may be obtained by plotting i_d/i_{do} against protein or molybdate concentration. It is the limiting value of diffusion current ratio, when so much protein has been added that all the molybdate is protein bound. This value of k is obtained by an extrapolation method.

$$\frac{i_d}{i_{do}} = k \quad \text{at limit } C_F \longrightarrow 0 \quad \dots (iii)$$

The value of k was found to be 0.30. Further evaluation of the binding data will prove that the value of k is independent of the moles of molybdate ion bound/mol of β -amylase.

The average number of mole of molybdate ion bound/mol. of β -amylase (mol. wt.

61,000) (V_M) was calculated by the relation, $V_M = C_B / [P]$, if β -amylase concentration is quite high, then the decrease in diffusion current of molybdate in presence of β -amylase may be ascribed to probable complex formation between molybdenum and enzyme or adsorption of enzyme on mercury drop or viscosity change of the system. Since the studies were carried out at pH 5.57, every care was taken to keep the enzyme in its native state, the influence of factors like adsorption and viscosity can be neglected and the decrease in diffusion current can be taken as a true index for ligand - enzyme interaction. It is evident from the data that V_M values are relatively higher at lower concentration of β -amylase than at its higher concentration. From the concentration dependence of V_M , it appears that the kinetics of equilibration may be slower in case of concentrated enzyme solution⁴⁸. Klotz and Urquhart⁴⁹ reported that the binding of methyl orange by protein was dependent on the concentration of protein. Ray et al.⁵⁰ also observed that 10g/1 BSA solution bound less sodium dodecyl sulphate SDS than did 1 g/L protein solution.

The results of polarographic measurement are plotted according to Scatchard equation⁵¹ in Figures 5 and 6. However, the plot exhibited deviation from linearity on higher V_M values. The non-linear nature of the plot goes to show that the linkage sites are not indential. The association constants ($\log K_1$, $\log K_2$) and their respective linkages sites (n_1 , n_2) were determined by means of modified Scatchard equation⁵² and are compiled in Table 1. The primary binding site (n_1) was found to be 4 with an association constant K_1 equal to 9750. This is also supported from U.V. absorption spectrum of β -amylase in absence and presence of molybdate ion. This site may be the histidine residue as this also absorbs at 210 nm as that of β -amylase. The secondary binding sites (n_2) are found to be 13 with weaker association constant (K_2) equal to 861.5. These secondary sites may be lysyl and guanidinyl groups of β -amylase. The existence of two type of sites are also confirmed by the dialysis equilibrium method discussed below.

Dialysis equilibrium results

This technique possesses an additional advantage, since like diffusion current measurements, it does not suffer from the presence of other competing ions in the solution. The binding data obtained from this method, the Scatchard plot is plotted at pH 5.57, 2 and revealed non-linear nature while a linear relationship was found to exist at other pH values, which indicated the nonexistence of primary binding sites (n_1). The number of linkage sites ($n = n_2$) of pH 5.57 and their association constant ($K = K_2$ of pH 5.57) are compiled in Table 1. The lesser value of association constants than n_1 indicates a stronger combination with primary site than that of other sites. From pH dependence of interaction, it is likely to assume that the cationic groups on (β -amylase molecule are the major molybdate anion

binding sites. It may be also concluded that the difference in pK of molybdate acid and the reactive cationic sites may account, at least in part, for the difference in the binding behaviour.

The differentiation of the linkage sites viz., imidazolium, ϵ -ammonium and guanidinium, can be made from the non-linear Scatchard plot. This plot has been considered as consisting of two straight line regions more than one type of linkage sites⁵³. The intercept of the upper part with the abscissa gives the value of n_1 for the number of linkage sites in the first class, corresponding to the slope K_1 . To determine the number of sites in the second class and their binding constant, one plots V_M^1/C_F against V_M^1 , where $V_M^1 = V_M -$ number of primary binding sites (n_1). This method subtracts the contribution of the primary sites to the experimental data. This is valid when the two classes of sites are non-interacting and it appears to be the case at pH 5.57. The number of primary binding site (n_1) i.e. 4 are indicative that molybdate ion has greater affinity for this group. Assuming the stepwise dissociation of side chain groups, the n_1 site must correspond to the imidazolium group of histidyl residue (pK = 7.50) where n_1 must correspond to ϵ -ammonium groups (pK = 9.87) and guanidinium groups (pK > 12). Among 13 sites are pH 5.57, the value at pH 7.50 remains 17, which indicates ϵ -ammonium groups (pK = 9.87) and guanidinium groups. On the other hand at pH 9.50, the 14 sites include guanidinium and a few ϵ -ammonium but at pH 11.50, 7 sites are solely the guanidinium groups.

Although β -amylase contains a total of 43 cationic sites, but only a fewer of these are involved in the linkage process. The reason for the involvement of lesser sites may be a conformational one. β -Amylase, being an enzyme protein, possess a significant structure brought about by hydrophobic interactions, hydrogen bonding and other intersegmental cohesive forces. However, since the folded core of a protein is known to be inaccessible to water, a rough estimate of 40-60% of the total sites may be available to interact with the anions⁴⁷. The results obtained in this study pointed to the significance of attraction between positively charged protein sites and the negative charge on molybdate ion, as well as bonding between nitrogen and molybdenum atom^{38, 39}. The amino acid composition of β -amylase was reported by Kohno and Coworker⁴⁶ (Table 2).

Ultraviolet spectroscopy

The absorption spectra of β -amylase in absence and presence of molybde ion (Figs. 1 to 5) at different pH values also supported the pattern of β -amylase molybdate linkage constants.

Table 1. Binding constants of Mo(VI) β -amylase system at different pH values by polarographic and equilibrium dialysis methods at Temp.=25°C and $\mu = 0.15M$

pH	Technique	Log K	Log K ₁	Log K ₂	ΔG^0 (K.cal/mole)	ΔG^0_1 (K.cal/mole)	ΔG^0_2 (K.cal/mole)	η	η_1	η_2
5.57	Pol.	3.3600	3.9884	2.9348	-4.6118	-5.4744	-4.0287	17	4	13
5.57	Eq. dialysis	3.4070	4.0355	3.2907	-4.6764	-5.539	-4.5167	17	4	13
7.50	Eq. dialysis	3.2788	--	--	-3.9438	--	--	16	--	--
9.50	Eq. dialysis	2.798	--	--	-0.38405	--	--	14	--	--
11.50	Eq. dialysis	2.7568	--	--	-3.8405	--	--	7	--	--

Table 2. Amino acid composition of β -amylase

Amino Acid	Number per mole	Amino acid	Number per mole
Aspartic acid	67	Methionine	14
Threonine	19	Isoleucine	34
Serine	39	Leucine	55
Glutamic acid	71	Tyrosine	24
Proline	35	Phenylalanine	25
Glycine	59	Lysine	22
Alanine	37	Histillime	8
Valine	38	Arginine	13

The spectra in the absence and presence of the molybdate ion at pH 5.57 is different than at other pH values. The absorption peak at 210 nm may be due to histidine amino acid residue, which binds the molybdate anion. This is supported by a single stronger linkage site determined from polarography and dialysis equilibrium methods. The spectra at all other pH values are devoid of this absorption peak at 210 nm indicating the absence of stronger site in molybdate ion interaction. A plot of absorbance (OD) vs. pH at 210 nm exhibited (Fig. 6) greatest absorbance at pH 5.57 and least at pH 9.20. It again increases at pH 11.70, which may be due to deprotonation of nitrogen loci and unfolding of β -amylase structure. This unfolding may be responsible for decreased molybdate ion binding, which is also supported by the lesser number of linkage sites from dialysis equilibrium method. Again at pH 5.57, the shift in absorption maxima of β -amylase in presence of molybdate from 210 to 220 nm (red shift) pointed to a stronger combination between β -amylase and the molybdate ion. Since the maxima is not shown at other pH values; hence, the stronger binding site (n_1) is not present under these conditions. The qualitative results of uv spectroscopy strongly supported the linkage constants of pH 5.57 obtained from polarographic and dialysis equilibrium methods.

pH-Metric evidence of molybdate linkage

β -amylase (5 g/L) was titrated in the presence (2×10^{-3} M) and absence of molybdate using 0.1 M HCl and 0.1 M KOH solutions, respectively. The curves are shown in the form of acid or alkali added vs. pH values of the resulting mixtures (Fig. 7 and 8). These curves of β -amylase and molybdate- β -amylase mixture are quite apart, hence valuable information regarding qualitative binding was obtained. There was a slight precipitation in the acidic range and hence, more hydrogen ions were consumed most probably due to formations of polynuclear complexes as was proposed by Malik and Arora³¹ and Arora et al.³⁵ in the binding of molybdate ions with proteins. Some useful information has also been obtained from the curves of Δ pH vs. pH or volume of acid or alkali added (Fig. 9). The Δ pH of molybdate + β -amylase minus β -amylase vs. volume or pH of β -amylase-molybdate mixtures indicated that the change was minimum near the isoelectric point for β -amylase isoelectric pH 4.85 whereas it increased upto pH 9.50 and then decreased. These observations also show that the complexation of molybdate ion takes place upto 9.50 and then decreases. The appearance of maxima at pH 4.25 in the Δ pH vs. pH curves shows that binding is maximum upto this pH and on both sides of this pH, it decreases. Thus, on both the sides, the decrease in Δ pH values pointed to a change of the ionic species of molybdenum. It is difficult to correlate pH-metric results with those obtained from polarographic and dialysis results.

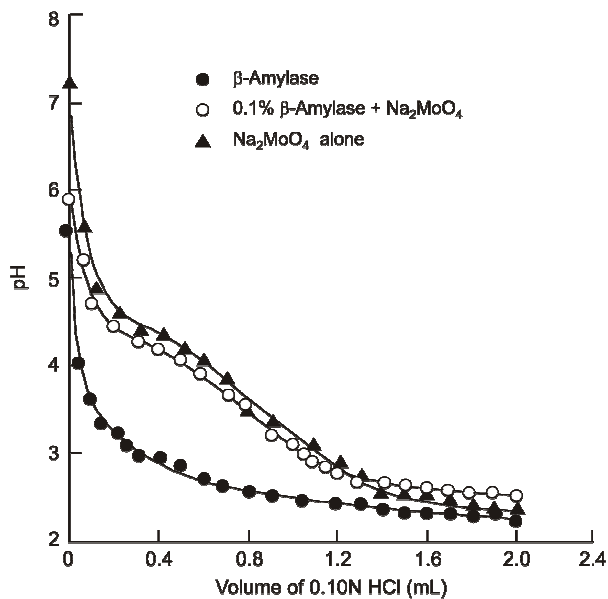


Fig. 7 : pH titration of Mo (-▲-▲-), Mo (VI) + β -amylase (-o-o-) and β -amylase (0.66%) (-●-●-) against 0.10 N HCl (TV = 15.0 mL) $\mu = 0.15$ M

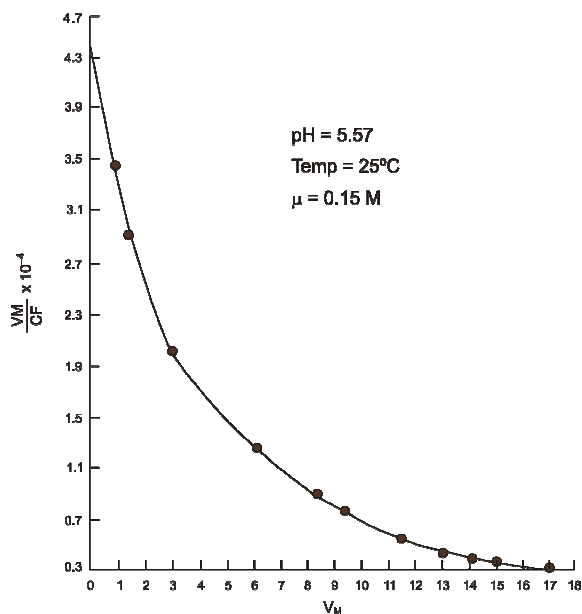


Fig. 8 : Scatchard plot of data at 5.57 by dialysis method

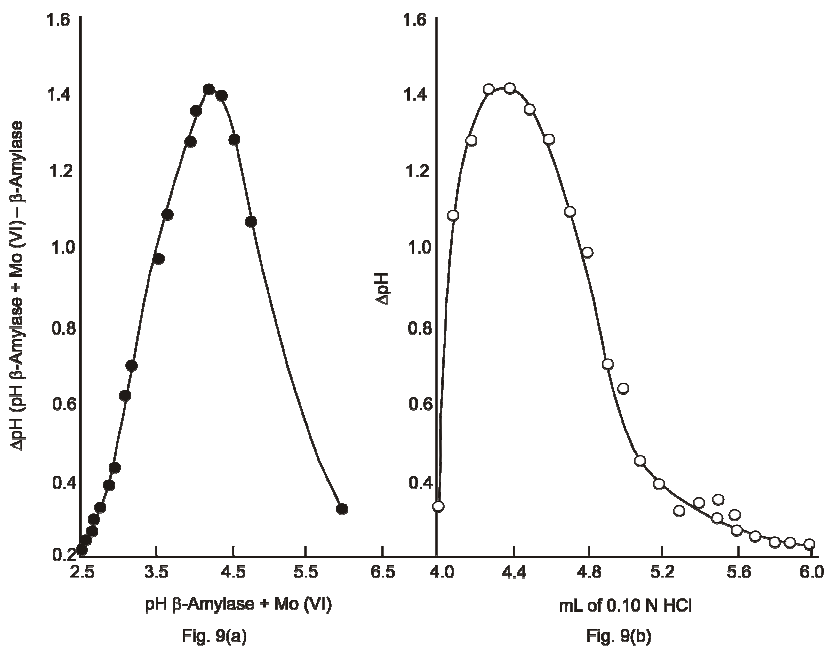


Fig. 9: (a) – Δ pH (β -amylase–Mo (VI)– β -Amylase) plotted against pH of mixture (b) – Δ pH plotted against mL of 0.10 N HCl added in β -Amylase–Mo (VI) system

CONCLUSION

From the results obtained from different methods, a tentative hypothesis can be proposed regarding the molybdate - β -amylase interaction. The pH-dependent linkage constants suggest that cationic groups are the main sites for linking the molybdate anions. β -Amylase deprotonation and increased negative charge would cause such pH-dependent results. The persistence of linkage above pH 9.50 may be ascribed to the guanidinium groups of arginine residues (13 are present in β -amylase), which have a higher value of ionization constant ($pK > 12$). The total number of linkage sites show that all the 43 cationic sites are not available for linkage. The smaller number of sites at all pH values; thus, does not allow us to determine, which cationic sites are involved in linking with molybdenum. These results are in agreement with those reported by Malik et al.³⁰ and Arora et al.³² in the interaction of molybdate ions with proteins. Exactly similar results were obtained by Craig et al.⁵⁴ in the interaction of ovalbumin with chloroaurate ions in the acidic solutions.

The present pH-dependent results between molybdate species and β -amylase could

thus be explained by assuming an interaction with the positively charged groups. The complex formation between Mo (VI) and histidine⁵⁵ as well as guanidine⁵⁶ further goes to support our results in lower pH range. Among the protonated groups, the possibility of the carboxyl groups is excluded since N-acetyl histidine was incapable of interaction with Mo (VI) while histidine methyl ester is being capable of complexation with Mo (VI)⁵⁷. In most of the cases, the postulation involved a direct bonding between Mo (VI) and nitrogen atoms, which is akin to enzyme-Mo (VI) linking, since enzyme nitrogenase⁵⁷ contained molybdenum-nitrogen bonds in the native state.

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