SOME KINETIC STUDIES WITH α-AMYLASE FROM *ASPERGILLUS ORYZAE*

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A THESIS SUBMITTED FOR THE DEGREE OF M.Sc.

OF THE UNIVERSITY OF GLASGOW

September, 1973.

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ACKNOWLEDGEMENTS

It gives me pleasure to thank Professor B. Capon who made it possible for me to undertake the work described in this thesis, and whose interest and support were always a great encouragement to me.

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CONTENTS

Abstract

The chemical constitution and structure of amylases and the kinetics of reactions catalyzed by amylases are reviewed.

The kinetics of hydrolyses of maltotetraose and maltotetraitol catalyzed by the α-amylase from Aspergillus Oryzae (Taka-amylase A) were measured under controlled conditions. At low concentrations (2×10^{-3} M) maltotetraose is split into two maltose molecules and maltotetraitol into maltose and maltitol, but at higher concentrations (20×10^{-3} M) maltotetraose forms maltotriose along with maltose and a very little glucose is also formed, while maltotetraitol is split into maltose, maltitol and maltotritol. These were thought to arise from transglycosylation reactions. Because of these tranglycosylation reactions it was not possible to measure the Michaelis parameters k_{cat} and K_m for the hydrolyses, but the second-order constants k_{cat}/K_m were measured at low concentrations of maltotetraose at several pHs while for maltotetraitol only at optimum pH. The rates for maltotetraose were about ten times greater than maltotetraitol measured under the same conditions at optimum pH.

Attempts to study the active centre of α-amylase from A. oryzae by perturbation difference spectroscopy were made but no specific interaction of maltose with the enzyme could be observed.

INTRODUCTION

1. Introduction

A. What are Amylases?

It is true to state that amylases are among the earliest known enzymes. At the beginning of the 18th century factors responsible for starch digestion were reported in wheat extracts[1], saliva[2], malt[3] and Aspergillus oryzae[4].

Marker[5] in 1877 first suggested that there might be two types of diastic ferments, one producing more dextrin and less maltose and the other producing much maltose and a little dextrin. Nowadays these enzymes are well distinguished from each other and known as α- and β-amylases or Endo- and Exo- amylases respectively.

Kuhn[5] discovered that hydrolytic product of starch by pancreatic amylase mutarotates downwards and has the α- configuration. He named it as α-amylase [α, 1→4 - glucan glucanohydrolase EC 3.2.1.1]: α-Amylases attack in a random manner on linear substrates and are called Endoamylases.

In 1930 Ohlsson[7] discovered another amylase, the hydrolytic product of which was only maltose having the β- configuration. He named this enzyme as β-amylase [α, 1→4 - glucan maltohydrolase EC 3.2.1.2]: β-Amylases are exoamylases as they hydrolyze the substrate by attacking the alternate linkages from the non-reducing

outer chain ends. Both α- and β- amylases catalyze the hydrolysis of α,1 → 4 - glucosidic linkages of polysaccharides such as starch, glycogen or their degradation products, but the hydrolytic products are different due to difference in their mode of action.

The interest in the reaction mechanism is not new. The gradually increasing industrial utilisation of amylolytic enzymes requires more thorough knowledge about their reaction mechanism and their catalytic properties. Therefore the catalytic properties of amylases have been studied not only for their academic interest but also for their application to industry. At present time several mechanisms for amylase action have been proposed, but no satisfactory quantitative account of carbohydrase catalysis is at hand. Some reasons for this are that amino acid sequences and structures are unknown and there is no knowledge yet of the structure of any active centre.

In the following sections the physical properties, chemical structure and general properties of amylases which have been isolated from different sources will be reviewed extensively.

B. Occurrence

Amylases can be isolated from several sources. α-Amylases are present in living organisms such as animals

and plants. Culture media of bacteria and moulds are rich sources of amylases, while β-amylases appear to be found only in plants.

Plant and animal α-amylases are isolated from the following sources: hog pancreas[8], human pancreas[9], rat pancreas[10], malted sorghum[11], soya bean[12], broad bean[13], malted barley[14], saliva[15], malted wheat[16], pigeon pancreas[17], and shore crab[18], while bacterial and mould α-amylases have been isolated in the crystalline form from various sources such as: Aspergillus oryzae[19,20], Bacillus subtilis[21,22], Aspergillus candidus[23], Bacillus coagulans[24], Aspergillus niger[25], Bacillus mercerans[26], Bacillus Polymyxa[27].

β-Amylases can be isolated from sweet potato[28], malted barley[29], wheat[30], barley[31], and soya beans[32].

II Physical Properties, Chemical Composition and Structural Characteristics of Amylases

A. Molecular Weight of Amylases

The molecular weights of most of the amylases are in the range of 45,000 to 60,000 as shown in the table 1.

Table I

Molecular Weights of Amylases[33]

Source of α-amylase	Molecular Weight	Source of β-amylase	Molecular Weight
Hog pancreas	45,000[a]	Germinated sorghum	55,900[a]
Human saliva	69,000[a]	Soya beans	61,700[a]
	55,000[a]	Sweet potato	197,000[d] (tetramer)
Soya beans and broad beans	45,000[b]	Sweet potato	47,500[c] (monomer)
		Wheat	64,200[a]
Aspergillus oryzae	51,000[d]		
	52,600 ±2600[d]		
Bacillus subtilis	48,900 (monomer)		
	96,900 (dimer)		

(a) Calculated from velocity sedimentation
(b) Determination by gel permeation
(c) Determined in 8M guanidinium chloride by sedimentation equilibrium
(d) Calculated from sedimentation equilibrium

The molecular weight of Taka-amylase A, kinetic properties of which were studied in our present work, was determined by Lee and McKelvey[34] by sedimentation equilibrium method. The sedimentation equilibrium run was performed at a rotor speed of 31410 rpm in Kel-F-double sector with quartz window and with Rayleigh interference optical system at 19.2°. The plot of logarithm of concentration against the square of the distance from the centre of rotation is linear. The molecular weight determined by this method, using value of $\bar{\nu} = 0.7$ is $52,600 \pm 2600$.

Amylases generally have a sedimentation coefficient equal to 4.5s approximately but B. Subtilis is an exception in having a sedimentation constant equal to 6s, which would correspond to a molecular weight of the order of 100,000. Fischer and Stein observed[35] that the sedimentation pattern changes with the addition of zinc or EDTA. They found that when a metal binding agent such as EDTA was added to the enzyme another form of it is produced having sedimentation constant equal to 4s approximately. Viscosity measurements showed that the change in sedimentation velocity was due to depolymerisation of the molecule, complete reconversion of which to the original native form can be achieved by the dialysis of the former against a zinc containing buffer. It was believed that the two form exists as monomer and dimer.

B. Amino Acid Composition

The amino acid composition of amylases from different sources are given in table II[33]. It can be seen that there are no striking similarities between these amino acid compositions although all amylases catalyse the hydrolysis of α, 1 → 4 - glucosidic linkages in polysaccharides. Bacillus Subtilis liquefying α-amylase differs from most of the amylases by the fact that it neither contains cysteine nor cystine residues, but Bacillus Subtilis saccharifying α-amylase was found to contain cystine residues. Aspergillus oryzae α-amylase (Taka-amylase A) is poor in basic amino acid composition which might contribute to its acidic character. α-Amylases appear to be rich in tyrosine and tryptophan which accounts for their high extinction coefficient at 280 nm. Their different tyrosine to tryptophan ratio provides them with distinctive absorption spectra.

End group analysis has been carried out by only a few groups. Determination of end groups with dinitrofluorobenzene by Sanger's method showed that Taka-amylase A has single alanyl residue at the amino terminal of the molecule[36]. Similar results were obtained by Tsugita[37] and Narita and Akabori[38] independently. They determined the amino acid sequence of an α-DNP* substituted peptide isolated from the partial acid hydrolyzate of the DNP-

* DNP: dinitrophenyl)

Table II

Amino Acid Composition of Amylases[33]

Amino Acid	Human Saliva α	Hog Pancreas α	Sweet Potato β	Wheat β	Aspergillus Oryzae α-amylase		Bacillus Subtilis amylase saccharifying	
Glycine	6.8	6.7	4.7	5.6	5.6	6.59	6.01	4.82
Alanine	4.4	6.9	5.4	6.0	5.92	6.80	5.29	6.11
Valine	6.9	7.8	5.9	7.3	6.03	4.69	6.14	4.61
Leucine	5.8		7.5	9.7	7.64	8.3	6.10	6.09
Isoleucine	5.8	11.5	4.6	4.3	6.34	5.2	4.55	6.15
Serine	7.8	4.1	3.0	3.5	6.04	6.48	5.21	8.46
Threonine	4.5	3.9	2.8	3.1	8.38	10.86	5.59	6.24
½-Cystine	4.4	2.3	1.3	1.5	2.09	1.6	0	0.32
Methionine	2.4	2.1	4.1	2.9	2.14	2.2	1.47	1.80
Proline	3.6	3.6	5.4	6.0	4.22	4.18	3.37	2.60
Phenylalanine	7.2	10.1	5.7	6.1	3.99	4.25	6.01	4.36
Tyrosine	5.5	5.3	6.2	6.9	10.88	9.55	9.05	5.84
Tryptophan	7.2	6.7	4.0	4.4	3.78	3.97	6.22	
Histidine	3.2	3.9	2.0	4.8	1.82	2.02	3.90	3.69
Lysine	6.3	4.9	7.4	4.7	4.77	5.94	7.42	4.87
Arginine	8.7	5.8	5.4	8.0	2.97	2.74	6.09	5.29
Aspartic Acid	19.3	14.5	12.7	12.4	15.91	16.53	14.49	16.44
Glutamic Acid	9.6	10.5	9.9	13.8	8.09	6.95	12.94	11.02
Amide	1.3	1.6	-	1.3	1.68	1.50	1.74	1.98

Compositions are expressed in grams per 100 g of protein.

amylase as follows:

alanyl-glycyl-aspartyl-glutamyl-seryl-alanyl-leucyl-threonine

Hydrazinolysis[39] of the amylase from Aspergillus oryzae showed that serine, alanine and glycine are the C-terminal of the enzyme, but this was not proved upon reinvestigation by the same worker using carboxypeptidase A and modified hydrazinolysis methods[40]. They incubated the enzyme with carboxypeptidase and the DNP-derivatives of the released amino acids were estimated by an amino acid analyser. Seven moles of amino acids were observed but serine was the predominant one throughout the digestion. After 24 hours of incubation no decrease of amylase activity was observed, indicating that the C-terminal amino acid is not essential for the activity of the amylase. They also confirmed by the hydrazinolysis technique that the C-terminal acid of the amylase was serine. The difference was compared with the previous hydrazinolysis method and was explained by the possible hydrolysis of the amino acid hydrazides to the corresponding free amino acids. It is clear from the above observations that Taka-amylase A has a single peptide chain with an alanyl residue at its N-terminal and seryl residue at its C-terminal.

Sugae[41] determined the N-terminal of Bacillus subtilis α-amylase by the dinitrofluorobenzene method, as valine. He also determined the amino acid sequence of an α-DNP-peptide isolated from the pronase digestion of DNP-amylase, which was proposed to be

Val-Asp[or Asp(NH_2)-Gly-Glu[or Glu(NH_2)]-Ser-

The C-terminal residue of B. subtilis α-amylase was determined by the hydrazinolysis method but more conclusive results were obtained by ^3H-label technique[42]. Lysine was identified as the main radioactive amino acid in the 24 hours hydrolysate of "^3H-labelled" B. subtilis α-amylase by paper chromatography.

It is interesting that the amino acid compositions of the N-terminal penta peptides of B. subtilis α-amylase and α-amylase from Aspergillus oryzae resemble one another, except for the following two points:

(i) the N-terminal amino acid of B. subtilis α-amylase is valine while that of Taka-amylase A is alanine.

(ii) the amino acid sequence (-Asp-Gly-) next to N-terminus in B. subtilis is reverse of that (-Gly-Asp-) in Taka-amylase A.

C. Functional Groups

Reactions of enzymes with selective reagents is of course intended to specify the role of side chain residues

in enzymic action.

Caldwell and Co-workers used nitrous acid, ketene and acetic anhydride for determining the role of free amino groups and came to the conclusion that these groups are not essential for the catalytic activity of pancreatic- and Taka-amylase. Phenylmercuric chloride[44], iodoacetamide and p-mercuribenzoate did not inactivate the pancreatic amylase suggesting that the sulphydryl group was not essential for the catalytic activity of the enzyme.

Alkylation of the sulphydryl group in β-amylase[45] from sweet potato caused the partial inactivation of the enzyme. Activity loss appears to be influenced by the size of the alkylating agent. Small but significant changes are attributed to minor conformational changes on modification. It was speculated that thiol group is not essential for catalytic activity but plays a regulatory role.

Toda et al[46] determined that Taka-amylase A lost its activity upon incubation with iodoacetamide or iodoacetate at pH 8 at 50° in presence of EDTA. Sulphydryl groups were alkylated. It was also determined that about 15% activity of s-carboxymethyl Taka-amylase A could be recovered, but not with s-carboxyamidomethyl Taka-amylase A, by the addition of calcium. It was assumed that the carboxylate group in s-carboxymethylated

Taka-amylase A could replace the sulphydryl groups as a chelating site for calcium. They suggested that although a sulphydryl group seems not to be involved in the active site of the amylase, its modification caused alteration of the activity. Therefore the sulphydryl group will stabilise the active conformation of the amylase molecule by chelating with calcium.

Ikenaka[47] found out that Taka-amylase A lost its activity when 2 moles of tyrosine and 5 moles of lysine residues were dinitrophenylated, while only 40% activity was lost when 11 out of 22 lysine residues were dinitrophenylated with 2,4-dinitrobenzene-1-sulphonate. It was thought that some specific phenolic group of tyrosine residues may be closely related to the enzymic activity and also ε-amino groups of lysine may not be essential for amylase activity. These results are in agreement with those of Tamaoki et al[48]. They found out that Taka-amylase A lost 63% of its activity when incubated with 2-methoxy-5-nitrotropane and some activation of maltosidase activity. In this case 11 out of 22 lysine residues reacted with the reagent. From these results they concluded that ε-amino groups of the lysine residues in Taka-amylase A may not be essential for amylase activity, and the decrease of the amylase activity of nitrotropanyl-Taka-amylase A may be due to steric hindrance by nitrotropanyl residue combined with the amino group of a lysine

residue situated close to the active site.

The effect of polyalanylation on Taka-amylase A has been studied by a group of workers[49]. Poly-DL-alanyl Taka-amylase A was prepared by the reaction of N-carboxy-DL-alanine anhydride with Taka-amylase A and purified by chromatography on DEAE-cellulose. Three samples containing 25, 45 and 64 residues of alanine per mole of Taka-amylase A were prepared and in all three cases it was found that 7 - 8 among the 23 amino groups in the enzyme had served as initiators for poly-alanylation. Using amylose as the substrate the enzymic activity of the three polyalanylated enzymes was compared. The activity decreased with the increase of average length of polyalanylated side chain but the activity towards phenyl α-maltoside (low molecular weight substrate) remains unaffected. They suggested that there is a steric hindrance for the interaction of substrate of high molecular weight such as amylose, but the active site of the amylase remains unimpaired on polyalanylation.

There has been much speculation on the nature of the catalytic groups of amylases due to variation of the kinetic parameters with pH. The pK_a's of chloride free pancreatic amylase are 4.8 and 6.0 - 6.4 and the chloride activated enzyme are 5.6 - 5.9 and 8.8 - 9.0[50,51]. Similar results were obtained with β-amylase from sweet

potato having pK_a's of 3.75 and 7.0[52]. The possibility that the more basic pK_a's were associated with the ionization of a phenolic group of tyrosine was excluded by showing the absence of ionization of such a group spectrophotometrically and intervention of phosphate was excluded by its absence. It was also shown that sulphydryl and ε-amino groups are not essential for the catalytic activity. Therefore carboxylate and imidazolium groups are identified as catalytic groups in agreement with the earlier proposal of Ono and his Co-workers[53] for bacterial amyloliquefaciens α-amylase.

Zherebtsove[54] interpreted the pK data on much firmer grounds by measuring the heats of ionization of ionizing side chain. For β-amylase from barley the pK_b and pK_a values were equal to 3.4 and 8.1 respectively in .06 M phosphate citrate buffer at 30°. The larger values of pK_b gave a basis for believing that either the imidazole group is situated next to another ionogenic group which intensifies its basicity or some other group functions instead of the imidazole. Photooxidation with methylene blue caused inactivation of the enzyme confirming the presence of imidazole in the catalytic site of β-amylase. Heats of ionization of acidic and basic groups were found to be +2565 and +6970 cal/mole respectively. These heats of ionization are characteristic of carboxylic and imidazole side chains. From

the kinetic data and photooxidation experiments the catalytic groups for porcine pancreatic α-amylase are also thought to be imidazole and carboxyl [55,56,50]. It has also been suggested that carboxylate and imidazolium are catalytic groups of soya bean[12] and broad bean[13] α-amylases.

D. Carbohydrate Components

The glycoprotein nature of Taka-amylase (EC 3.2.1.1) had been shown by a positive Molish Reaction[57]. The carbohydrate components were found to consist of 8 moles of mannose, 1 mole of xylose and 2 moles of hexosamine[58], but the nature of hexosamine was not confirmed at that time. Tsugita and Akabori[59] had isolated a glycopeptide from the enzymic digest of Taka-amylase A and suggested that the carbohydrate moiety was linked to the enzyme through a hydroxyl group of serine but they were unable to isolate hexosamine in the peptide.

In recent years increasing attention has been given to the composition and structure of carbohydrates in proteins and the nature of the protein carbohydrate linkages in several glycoproteins[60,61,62]. The carbohydrate prosthetic groups of almost all the known glycoproteins contain hexosamine and in some cases it was proved that the hexosamine linked the carbohydrate and protein parts. This information led Ikenaka[63] to

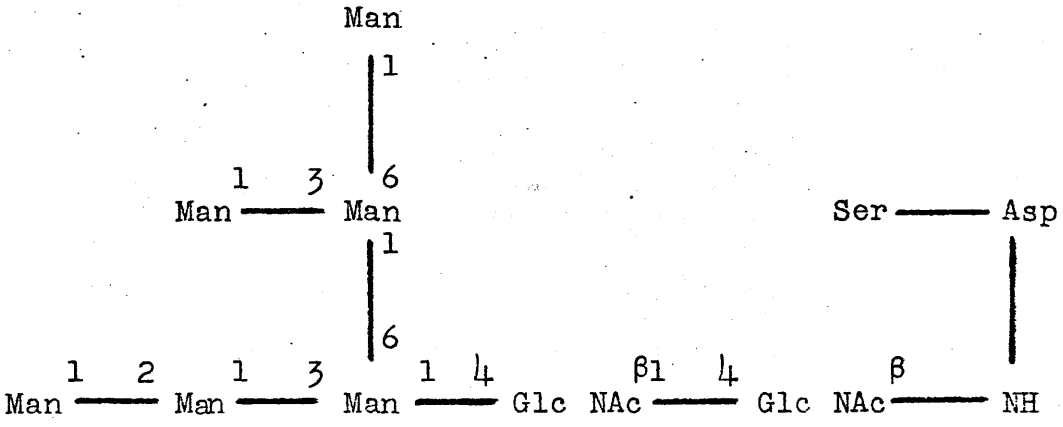
reinvestiage the nature of carbohydrate linkage in glycopeptide of Taka-amylase A. By the proteolytic digestion of the enzyme with pronase P, a glycopeptide of Taka-amylase A was prepared, the composition of which was found to be aspartic acid, serine, N-Acetyl glucosamine, ammonia, mannose, galactose and xylose in the molecular ratio of 1:1:2:1:6:0.8:0.5. Results of dinitrophenylation, hydrazinolysis and periodate oxidation of the glycopeptide led to conclusion that the carbohydrate prosthetic group is linked at the reducing group of one of the glucosamine molecule to β-carboxylic group of aspartic acid via an amide linkage as:

Ser-Asp NH-Carbohydrate.

Periodate oxidation and hydrazinolysis of the glycopeptide showed that amino-terminal serine possessed a free hydroxyl and that the β-carboxyl group of aspartic acid was not free.

Recently the complete sequence of the glycopeptide of Taka-amylase A has been published by the same group of workers[64]. They used O-methylation, partial acetolysis and periodate techniques. By combining these results and the previous results[65] in which α- and β-mannosidases were used for the elucidation of anomeric configuration of mannose and N-acetyl-β-glucosaminidase was used to

determine the configuration of N-acetyl glucosamine, the complete sequence of glycopeptide of Taka-amylase A is determined as shown below.



E. Structure of Amylases

Amylases have received a very little attention as compared to other enzymes, as the amino acid sequence of a single amylase has not yet been determined, contradictory results for covalent intermediates have been recorded and little information of the active centre is available.

The investigation of the number of binding sites per molecule of sweet potato β-amylase by Thoma and Spadlin[45] showed that there were four binding sites per molecular weight of 197,000. They observed that the molecular

Weight dropped to 47,500 in 8M guanidinium chloride suggesting that sweet potato β-amylase is tetrameric. From equilibrium dialysis against cyclohexaamylose a competitive inhibitor, the number of moles of inhibitor bound per active site of the enzyme came out to be 0.93. Since one mole of inhibitor is effective in blocking one catalytic site, it appears likely that there is one catalytic site per 50,000 daltons of β-amylase or four independent binding sites per 197,000 molecular weight. These results are in agreement with the results of Thoma and Koshland[66] who showed that there is one binding site per 50,000 of β-amylase.

Schrumm and Loyter[67] determined the number of binding sites of Hog pancreatic α-amylase by equilibrium dialysis at pH 10 and showed that 1 mole of enzyme specifically bound 2 moles of maltotriose. The number of binding sites appeared to be two. Ability of the enzyme to form multimolecular complexes with limit dextrin accounts for two independent binding sites. Robyt and French[68] studied the action pattern of Porcine pancreatic α-amylase in relationship to the substrate binding site of the enzyme. Using labelled maltodextrin (specifically labelled in the reducing glucose unit with C^{14}) the initial rates of formation of the labelled products were determined for each substrate. The data are consistent with a five-glucose binding site, in which the catalytic groups are

located at bond 2.

Taka-amylase A is one of the amylases whose structure has been studied most extensively chemically and physiochemically. It has been known for a long time that most α-amylases from different sources contain at least one atom of calcium which is firmly bound and required for their activities[69]. Toda et al showed that the essential calcium in Taka-amylase A [EC 3.2.1.1.] could be removed reversibly by incubating the enzyme with EDTA at pH 8 and 50°C, while the masked sulphydryl group became available to sulphydryl reagent under these conditions. Modification of the resultant sulphydryl group caused alteration in the state of the active site and affinity for calcium atom. Probably the sulphydryl group of the sole cysteine residue is one of the calcium-binding sites in Taka-amylase A. The sulphydryl group does not seem to be in the active site of the amylase, but plays a key role in maintaining the active conformation by chelating with the essential calcium atom. As Taka-amylase has a relatively high molecular weight, it is rather difficult to determine its primary and tertiary structures; that is why little information of the active site has been obtained. Recently Ono and his Coworkers[70a] studied the influence of molecular structure of linear maltodextrin on Taka-amylase A catalyzed hydrolysis. As the turnover number $V_{max}/(E)_o$ increased with chain length

up to n = 7 and became practically constant for n more than 7, they suggested that the specificity region of Taka-amylase A spans about 7 glucose units. These workers[70b] also studied the inhibition by analogues for the hydrolysis of synthetic substrate. They concluded from their results that equatorial orientation of OH groups is favorable for binding and that the CH_2OH group at C^5 in association with the OH group at C^2 or the methyl group at C^1 plays some crucial roles in the specificity of this enzyme towards substrate analogues. Studying the partial binding affinities of glycosides, they suggested that there is a region with hydrophobic character near the binding site, which is acceptable for a glycone in α-conformation.

III The Catalytic Action of Amylases

A. Specificity

The specificity of amylases is to catalyse the hydrolysis of α, 1 → 4 - glucosidic linkages in polysaccharides or their degradation products. α-Amylases act on polysaccharides in a random manner while β-amylases act from the non-reducing end of the chain to break every alternate glucoside linkages.

Taka-amylase A from aspergillus oryzae shows quite high specificity in relation to glycon requirements.

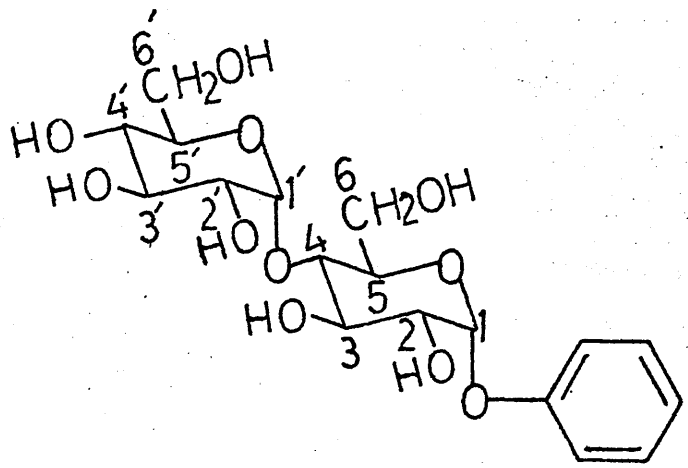


Fig. 1.

Phenyl α-maltoside (fig 1) is used as a substrate for Taka-amylase A[71] and it is also known that this substrate librates phenol by the action of the enzyme. Modified phenyl α-maltoside has been used by a number of workers for studying the glycon specificity of the Taka-amylase A. Partially O-methylated amylose[72] and O-methyl derivatives of phenyl α-maltoside[73] were hydrolyzed by Taka-amylase A and it was found that 2'-O methyl maltoside was not hydrolyzed by Taka-amylase A, whereas 4'-O, 6'-O, and 4', 6' - di-O-methyl maltosides (see fig 1 for numbering) were hydrolyzed to produce the corresponding maltose

derivatives. But in all these cases the identified products were unsubstituted at the reducing end of the substrates. However glycosides in which either the C-2 or C-6 hydroxyl was methylated were not attacked by the Taka-amylase A. These results confirm Ikenaka's[74] work which showed that monoacetyl maltoside in which the hydroxyl group at C-6 was acetylated, was found not to be hydrolyzed by Taka-amylase A. Weil and Bratt[75] also reported that the hydroxyl on C-6 replaced by methoxy group as in 6-O-methyl amylose was resistant to hydrolysis by several maylases. Isemure and Co-workers[76] studied the hydrolysis of a number of modified phenyl α-maltosides. Substitution of 6'- hydroxyl group with halogen lowers the rate of hydrolysis while with methoxy group enhances the rate. It means the existence of an electronegative atom such as oxygen or halogen at 6' - position played an important role. On the other hand replacement of -OH group on C-6 by electro-negative group made the substrate immune to enzymatic reaction. Substitution of hydrogen at C-6' hydroxyl decreased the rate of hydrolysis. The Taka-amylase A catalysed hydrolysis of 6-deoxy-6-fluoro-α-maltoside was slower than that of phenyl maltoside, suggesting that electronegativity is necessary at position -6 and also position -6 has steric requirements[77]. Hydroxyl groups on C-2 in both the glucose residues of the substrate are

important for the enzymic action[72,73]. Evidence for the necessity of the 2'-hydroxyl group on the non-reducing end glucose residue of the substrate was obtained in the investigation of phenyl 2'-O-methyl-α-maltoside which is completely immune to enzyme[78]. While the importance of 2-hydroxyl group on the reducing end has been shown by studying the hydrolysis of phenyl 2-O-methyl-α-maltoside, phenyl 2-deoxy-α-maltoside and phenyl 4-O-gluosyl-α-maltoside, all of these compounds are not hydrolyzed by Taka-amylase A including 4-O-glucosyl-α-mannoside, which has axial hydroxyl at position C-2, suggesting steric requirements at position -2.[79] Action of Taka-amylase A on phenyl α-maltoside in which the terminal C-4' was modified showed that the hydroxyl group on C-4' in phenyl α-maltoside was almost indifferent to enzymatic reaction. But inversion of configuration of the C-4' considerably retards the enzyme catalyzed reaction[80].

Aglycon specificity of Taka-amylase A is not rigid. Matsubara et al[81] studied the effect of aglycon specificity on the rate of hydrolysis of maltosides. Taka-amylase A catalyzes the maltosidic cleavage of various α-maltosides such as methyl-, ethyl-, phenyl, and p-nitrophenyl α-maltoside, suggesting that this enzyme has a broad aglycon specificity. Recently aglycon specificity of Taka-amylase A has been studied utilising transfer reactions[82]. Maltitol, phenyl α-D-glucoside, phenyl

β-D-glucoside, phenyl-2-O-methyl-α-D-glucoside and 6-O-methyl-α-D-glucoside were effective as acceptors.

B. Kinetics of Hydrolytic Reactions

a. The Effect of Ions on Rate of Hydrolysis

Most, possibly all, α-amylases are metalloenzymes requiring calcium for their catalytic activity and its removal results in both reversible and irreversible inactivation[83]. All the calcium may be removed from the α-amylases of Bacillus Subtilis and human saliva most conveniently by electrodialysis and the resulting calcium-free proteins have lost most of their activity. It was suggested that "By forming a tight metal chelate structure, the metal produces intramolecular cross-links similar in function to disulfide bridges, which confer to the α-amylase molecule the structural rigidity required for effective catalytic activity". Fischer and Stein noted that inactivation due to loss of Ca^{2+} could be ascribed to proteolytic degradation or to exposure to the unfavorable conditions used for the release of metal rather than spontaneous unfolding of apoenzyme. Human saliva α-amylase requires one gram atom of calcium for its full activity while bacterial amylase at least four gram atoms of calcium per mole. Taka-amylase A binds 10 gram atoms of calcium per mole. Nine of them can be removed by dialysis against 0.02 M sodium acetate without any effect

on the enzymic activity, suggesting that they only stabilise the enzyme molecule against denaturation. But the undialyzable one was not removed after six days of dialysis against EDTA, and it has been claimed that this firmly bound calcium cannot be removed without denaturation. However Tanaka[84] showed that EDTA caused only inactivation and the activity could be recovered by the addition of calcium. Kato et al[46] also confirmed that this firmly bound calcium could be removed reversibly by incubating the enzyme with EDTA at pH 8.0 at 50°. The activity lost could be recovered by the addition of calcium. The firmly bound calcium atom in Taka-amylase A can be replaced by other divalent cations such as magnesium, strontium and barium, without causing appreciable change in the amylase activity[85]. But zinc and cadmium could not reactivate Taka-amylase A inactivated with EDTA. It appears that these metals can replace calcium only when the active conformation of the enzyme molecule is preserved, and consequently the binding site is intact.

Halides[35] ions also strongly enhance the catalytic efficiency of mammalian α-amylases; their effectiveness decreases in the order of $Cl^- > Br^- > I^-$. Thoma and Wakim[51] studied the effect of Cl^- ion on the pH- V_{max} profile. As V_{max} for Cl^- free hog pancreatic α-amylase depends on the ionization of two groups of $pK_a = 4.8$ and 6.0 - 6.4, but in the presence of 0.25 M potassium

chloride these are changed to 5.6 - 5.9 and 8.8 - 9.0. The separation of the apparent ionization constants of porcine pancreatic α-amylase although contributing to enhancement of activity can account for only a small fraction of it. Mayer and Fischer[86] suggested that the optimum sodium chloride concentration for the porcine pancreatic α-amylase is 10 mM, with higher concentration an inhibitory effect is produced.

Heavy metal ions[87] have been shown to inhibit amylases such as mercury, copper, lead and silver. Mercury is more effective in its inhibitory action than copper or lead. Ascorbic acid and ammonium molybdate also have an inhibitory effect on α-amylases from higher plants. The mechanism of inhibition is not known yet, but generally sulfhydryl group modification is thought to be the cause of it.

b. Effect of pH

Catalytic activity and enzyme stability both depend upon the pH and have been studied by several workers.

The optimum pH for β-amylases[88] lies between pH 4 and 6 and they are relatively stable at pH 3.6.

α-Amylases[33] from mammals and higher plants are generally stable from pH 5.5 to 8.0. Animal amylases exhibit maximum activity around neutral pH, but if chloride is removed, pH shifts downwards. Thoma and Wakim[55] studied the stability of α-amylase from porcine pancreas.

They incubated 1.4×10^{-7} M solution of α-amylase (10^{-8} M Cl^- conc.) in 0.03 M calcium acetate buffer at various pH values for 40 minutes at 25°. Enzyme samples were diluted with 0.03 M phosphate buffer at pH 7 and tested for enzyme activity by standard assay. The pH of all the solutions after dilution was 7.0 ± 0.1. It was seen that α-amylase is stable in the presence of Cl^- over most of pH range 4 - 10.5 as well as in the absence of Cl^- ion. Activity is 100% around pH 6.9.

Ikenaka and Akabori[71] studied the pH dependence of amylase activity and α-phenyl-maltosidase activity. The optimum pH of both activities was between pH 5 and 5.5, and the shapes of the activity curves were similar. Ono and his co-workers[88] reported the optimum pH for B Subtilis α-amylase as 5.8 to 6.0 at 25°.

The effect of calcium salts on the stability of amylases has been described by Fischer and Stein[35]. They found that removal of calcium from the amylase molecule results in a remarkable labilization of the enzyme. The pH ranges of stability were greatly reduced by hog pancreatic and B Subtilis α-amylase. Both amylases had lost their resistance towards alkaline pH's and also readily coagulated in contrast to native enzymes.

The change in activity of enzyme as a function of hydrogen ion concentration is often employed to measure the pK_a values of the catalytic groups. Wakim et al[55]

showed the influence of pH on V_{max} and K_m for porcine pancreatic α-amylase. The slopes of acidic and basic limbs of the curves were experimentally less than unity, indicating that simple mode of pH dependence of enzymatic reaction does not describe the system. The divergence of V_{max} from unit slopes may arise from electrostatic perturbation of ionization constants, multiple intermediates or conformational effects[89]. The pK_a values in the presence of chloride are approximated to be 5.6 - 5.9 and 8.8 - 9.0, while in the absence of chloride are estimated to be 4.7 - 4.9 and 6.0 - 6.4. Change in the rate-limiting step might have shifted the pH rate profile upon addition of chloride ions. From the ionization constants it is thought that carboxyl and imidazole residues are present in the catalytic group.

Action Mechanism of Amylases

Amylases may be divided into two classes according to the hydrolysis or transfer reactions which they catalyze with retention or inversion of configuration at the anomeric centre of the carbohydrates. The former class includes α-amylases[6,50,51] and the latter β-amylases and gluc-amylases[91,90]. For many years it was believed that enzymes which invert configuration were single displacement catalyst and enzymes that retain configuration were double displacement catalyst[92]. A double displacement

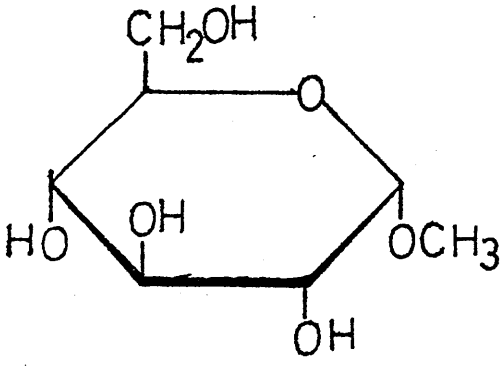
reaction giving rise to an even number of Walden inversions would normally be expected to proceed through a covalent glycosyl intermediate. Actually the reaction involves nucleophilic participation by a group in the enzyme which would yield a glycosyl enzyme of inverted configuration which on reaction with water or another hydroxylic compound would yield a product with the same configuration as the starting glycoside. It must be emphasised, however, that at present there is no corroborating evidence for the intervention of a glycosyl enzyme in any glycosidase-catalysed reaction[93], although covalent catalysis has been conclusively demonstrated for a related enzyme, sucrose phosphorylase[94].

Thoma[51,55] suggested an enzyme directed approach of the hydrolytic water molecule to the reaction centre as an alternative explanation of the stereochemistry of amylases. For the enzymes that retain configuration direct front-side approach of attacking water molecule on the oxycarbonium ion was postulated; for inverting enzymes back-side solvation of an oxycarbonium ion was postulated. If the glucopyranoside undergoing the attack achieves a half-chain configuration along the reaction coordinate, solvent approach from above or below the ring is clearly possible. Because of steric crowding, the front-side attack by water on a glycoside in the C-1 conformation is probably impossible, but steric congestion is greatly relieved when the glucoside is distorted to a half-chair

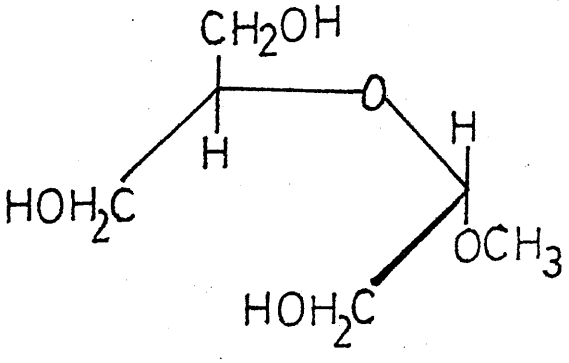
configuration. Enzymes that retain configuration must permit some translocation of the aglycon from the binding site to allow the approach of solvent, but this is not necessary for the enzymes that invert configuration. As a common intermediate is proposed for both α- and β-amylase, the stereochemistry of the product is not a useful guide to the timing of water attack along the reaction coordinate. The X-ray crystallographic studies of a lysozyme inhibitor complex furnish support for reasonableness of the distortion postulate[95]. The diffraction pattern of an inhibitor enzyme complex strongly suggests that the substrate lies in a crevice in the enzyme and that substantial distortion of the ring undergoing hydrolysis is required to accommodate the substrate into the cleft. Two carboxyl groups straddle the reactive centre, one acting as a general acid catalyst and the other as an anion involved in ion pair formation or related action.

Acid hydrolysis of acetals is believed to be a model for the enzyme catalyzed hydrolysis reactions by many workers, as the transition states of both possess carbonium ion character. At least two factors, distortion and side chain functional catalysis, are thought to be important contributing agents to catalysis, assuming no interaction mechanism between ring distortion and functional group catalysis, which is questionable.

A substantial number of nonbonded interactions and tortional strains are introduced into a glucopyranoside when it is twisted to half-chair conformation[97], hydrolysis of these compounds is relatively more difficult as compared with structurally similar open-chain acetals. The depressing effect of strain on the rate of hydrolysis[51,96] can be estimated by comparing the hydrolysis rates of α-methyl-D-glucoside (I) with that of an open chain analogue (II).



(I)



(II)

At 25°C (II) is cleaved approximately 10^6 times faster than (I) as computed from the energies of activation for acid-catalyzed hydrolysis. This rate enhancement, according to transition state theory, is achieved by lowering the energy difference between the ground state and transition state by 8.7 k cal mol^{-1}. The activation barrier for interconversion of the cyclohexane chair-form estimated at approximately +10 k cal $mole^{-1}$, approximates this energy

difference[97]. Minimum strain energy of binding N-acetyl glucosamine into the distortion subsite on lysozyme is estimated to be +6 kcal/mole, which is composed of the actual distortion energy and the energy release on binding the distorted monomer[98]. This strain energy is still 2.5 kcal/mole less than required to compensate for the increase in the activation barrier introduced by cyclizing an acetal. Distortion of the pyranoside ring towards the half-chair conformation may be used to compensate for the increase in the activation barrier caused by cyclizing the acetal. This energy used for substrate distortion is acquired at the expense of favourable enzyme-substrate contacts. The difficulty is overcome in case of polymeric substrate, as endothermic distortion of one monomer residue can be compensated for by exothermic interaction of adjacent monomers. But for glucosidase the distortion problem is quite severe, because the lack of adjacent groups in substrate for compensating exothermic interactions.

Carboxyl and imidazole groups have been implicated as the catalytic agents in a variety of amylases[50,51,53]. The probable mode of participation of these side chains is based upon the behaviour of the model system. Extensive data on acetal hydrolysis have been reviewed and discussed by Capon[98] and BeMiller[96]. Since the same catalytic groups appear to be participating in catalysis by α-,

and β-amylases, they operate by a common mechanism and "the different steric courses result from 'specific guidance' of the water molecule to the reaction centre by the enzyme". The protonated imidazole acts as a general acid catalyst and the carboxylate group stabilises the developing glycosyl cation electrostatically through ion-pair formation.

Capon[98] used solvent isotopic effects to argue against general base catalysis by a carboxyl group with β-amylase and nucleophilic catalysis for α-amylases. The solvent isotope effect, at pH or pD 7.0, $V_{max}(H_2O)/V_{max}(D_2O) = 1.25$, is the same for α- and β-amylases. This value is very similar to solvent isotopic effect for the intramolecular general acid-catalyzed hydrolysis of 2-carboxyphenyl β-D-glucoside $\underline{k}_{(H_2O)}/\underline{k}_{(D_2O)} = 1.57$. "In summary, general acid catalysis acting in concert with some sort of nucleophilic participation (electrostatic solvation or covalent bond formation) seems a reasonable process to invoke for amylase catalysis"[33].

EXPERIMENTAL AND RESULTS

Taka-amylase A Catalyzed Hydrolysis of Maltotetraitol

The rates of hydrolysis of maltotetraitol at different concentrations have been studied by potentiometric titration method, based on the reduction of alkaline potassium ferricyanide. This method was first used by Hagedon and Jensen,[99] but the procedure described below has been modified to take advantage of modern equipment, namely automatic potentiometric titrator.

Calibrations were made for glucose, maltose, equimolecular mixture of glucose and maltose, and maltotetraose. It was clear from the calibrations that maltotetraose cannot be used as a substrate in this method, because the difference between the titre of a certain molarity of maltotetraose and the titre of double the molarity of maltose was not large enough to measure correctly. For this reason reduced maltotetraose is tried for the kinetic study.

Reduction of Maltotetraose

Maltotetraose was reduced according to Nakamura and Tamura's method.[100] To 100 mg of maltotetraose dissolved in 50 ml of distilled water, 50 ml of 2% sodium borohydride in distilled water was added. The solution was allowed to stand for one hour at room temperature and the excess of sodium borohydride was destroyed by adding 80 ml

of Amberlite IR - 120(H). The resin was removed by a sintered glass filter and was washed with 100 ml of MeOH. The filtrate and the washing were combined and evaporated to dryness below 40° in vacuo in a rotary evaporator. To the residue 400 ml of MeOH was added and evaporated to dryness to remove the borates as trimethyl borate. The final treatment was repeated three times.

It was found that the large scale reduction of maltotetraose did not give reproducible results, and also the zero reading is too high to follow the reaction, so small scale reductions of the samples required for each kinetic run were carried out each time.

Reagents

A. Potassium Iodate

An $\frac{N}{10}$ solution was made by dissolving 0.8918 g of 'Analar' potassium iodate (previously dessicated at 145°C for 5 hours) in 250 ml of distilled water. This solution was used as the primary standard for the estimations and diluted to make an $\frac{N}{5000}$ solution for titration. It was found that both $\frac{N}{10}$ and $\frac{N}{5000}$ solutions of potassium iodate could be stored for several months in a brown bottle, without deterioration, but as a precaution the $\frac{N}{10}$ solution was stored in a refrigerator and then warmed to room temperature for dilution purposes.

B. Alkaline Potassium Ferricyanide

An $\frac{N}{500}$ solution of potassium ferricyanide was made by dissolving 0.33 ± 0.001 g of 'Analar' potassium ferricyanide and 5.3 g of anhydrous sodium carbonate in 500 ml of distilled water. This was stored in a brown bottle, but was not used more than two consecutive days, since the method requires the solution to be very nearly $\frac{N}{500}$.

C. Sodium Thiosulphate

An $\frac{N}{5000}$ solution of sodium thiosulphate was prepared by diluting a stock solution of $\frac{N}{10}$ sodium thiosulphate. The stock solution was prepared from a 'B.D.H.' concentrated volumetric solution, and when freshly prepared was used to check the standard potassium iodate solution. Sufficient solution for two days was usually found convenient.

D. Citric Acid-Zinc Sulphate Reagent

This reagent was prepared in bulk from citric acid (400 g of monohydrate) and zinc sulphate (100 g of the heptahydrate) and made up to 1 litre with distilled water.

E. Potassium Iodide

A fresh solution, 3 g of 'Analar' potassium iodide in 100 ml of water was made daily.

Titration Assembly

The titration assembly was based on a Radiometer Titrator model TTT 1C with a Radiometer combined platinum/calomel electrode. The solution to be titrated was placed in a beaker with a magnetic stirrer and the titrant (KIO_3 solution) delivered through a small diameter glass tube fitted with the Radiometer combined electrode with its exit placed between and just below the two electrodes. The delivery tube was connected by means of rubber tubing, via a magnetically operated valve, to a 10 ml burette. The burette could be filled readily from a reservoir, by means of a three-way stop-cock which greatly facilitated routine analyses.

Ancillary Equipment

Reagents B and C were dispensed from 1 ml and 10 ml bulb pipettes respectively. The accuracy of ordinary 1 ml pipettes was found by weighing the delivered amount of alkaline ferricyanide, and it was found to deliver 1 ml samples respectively with an accuracy of $\pm 1\%$.

Reagents D and E were dispensed from 5 ml and 1 ml dispensers respectively. The actual volumes of these reagents were not required to a greater accuracy than the dispensers (probably no better than $\pm 2\%$).

The Principle of the Estimation for Reducing Sugar.

The reducing compound (maltose) in alkaline solution will reduce a given quantity of ferricyanide. The reduced ferricyanide is complexed with Zn^{++} to prevent back oxidation, and the residual ferricyanide, in acid solution, oxidises iodide ions to free iodine which is then reduced by adding an excess of sodium thiosulphate. The residual thiosulphate is then titrated with potassium iodate, using a potentiometric method.

Detailed Procedure for the Estimation of Reducing Sugars

1. Determination of equivalent point for titration

To 10 ml of distilled water, in a beaker, was added 1 ml of potassium iodide (E), 5 ml of zinc sulphate/ citric acid reagent (D) and 10 ml of thiosulphate (C). The beaker was placed in the titration assembly and stirred by means of a magnetic follower, then the electrodes and delivery tube from burette was inserted, the titrator was set to the appropriate scale ('Endpoint' + 300 mV) and the titration started. The titration was stopped at appropriate points before and after the equivalence point and a note made of the millivolt reading of the instrument. The curve of volume added against millivolt reading was drawn to determine the equivalence potential. The equivalence potential was

taken as the mid-point of the large and abrupt (i.e. for 0.01 ml of potassium iodate) change in meter reading at the end-point of the titration. This was usually of the order of +285 millivolts.

2. Determination of titre of the thiosulphate

The titration solution was prepared as for Section I and the titrator set as follows:

a. The end-point dial set at +300 millivolts, i.e. above the equivalence point.

b. Proportional band dial to 1.0, i.e. the instrument is set so that the magnetic valve from the burette remains open until the meter reading is 1 pH unit from the end-point, when it is actuated by the instrument to add small volumes of titrant till the end-point is reached. (Actually it is arranged so that the volume added is smaller as the end-point is approached).

c. Delay of shut off dial set to 10 seconds. Thus when the magnetic valve has been shut for more than 10 seconds, i.e. end-point reached, the magnetic stirrer is switched off automatically.

d. Turning the selector knob from 'Reading' to 'Up-Scale' opens the magnetic valve and starts the titration.

Three estimations of the titre for thiosulphate were made daily, titre = T ml.

3. Determination of titre of the ferricyanide.

To 10 ml of distilled water was added 10 ml of ferricyanide (B), 1 ml of (E), 5 ml of (D) and 20 ml of (C) in a beaker. The titration was carried out as before, the average of three estimations is = F ml.

4. Determination of the titre for sugar solutions

Solutions of sugars (i.e. glucose, maltose, mixture of equimolar glucose and maltose and maltotetraose) in distilled water were prepared of various concentrations ($0.05 - 0.5 \times 10^{-4}$ molar) to calibrate the method.

10 ml of sugar solution was pipetted into a dry, clean boiling tube, followed by 1 ml of (B). The solution was mixed rapidly and placed in a boiling water bath for 15 minutes, then withdrawn and cooled under running tap water for 2 minutes. To the cooled solution was added 1 ml of (E) with mixing, and the mixture was then set aside at room temperature for 3 minutes.

At the end of this time 5 ml of (D) was mixed into the solution, followed by 10 ml of (C), the contents of the boiling tube were then washed carefully into a beaker, and titrated as already described. Titre = P ml.

When boiling tubes were in the boiling water bath, 'glass beads' were placed in the necks of the tubes to prevent loss of contents and splashing and contamination from the water bath.

The titre obtained for ferricyanide was corrected:

The added ferricyanide is equivalent to (2T - F) ml of potassium iodate solution, thus if no reduction takes place the volume of the iodate needed to obtain equivalence would be

(2T - F) - T = T - F ml.

Thus the corrected titre for the reduced solution is:

P - (T - F) ml of potassium iodate.

This method was used for glucose, maltose and maltotetraose, a separate calibration being necessary for each of them. Straight line calibrations were found for them, except for concentrations that caused almost complete reduction of the available potassium ferricyanide. From calibrations it was seen that the difference between the titre of certain molarity of maltose was not large enough to be measured correctly. For this reason reduced maltotetraose was tried as a substrate in the kinetic experiments.

Thus samples obtained from kinetic runs were analysed as described above.

Solutions for Kinetic Run

1. Buffer Solutions

'Analar' grade reagents were used for the preparation of buffer solutions. Solutions were prepared using degassed, distilled water.

pH measurements

The pH of the buffer solutions was measured at the temperature of the experiment using a Radiometer Model 26 pH Meter having an external temperature compensator. The pH meter incorporated a Radiometer type G202 C glass electrode and a type K401 calomel electrode. The meter was standardised against commercial standard buffers complying to BS 1947, 1961.

2. Enzyme Solution

Enzyme used was Taka-amylase A (α-amylase from Aspergillus oryzae). Enzyme solutions of different concentrations were made in buffers.

3. Maltotetraitol Solution

Maltotetraitol solution of different concentrations (viz 2 - 20 x 10^{-4} molar) was prepared in buffer solutions.

The Kinetic Procedure Used Was:

The enzyme and substrate solutions were thermostated before starting the reaction. Then 1 ml of enzyme solution was added to 10 ml of maltotetraitol solution and starting time was noted. Before adding enzyme 1 ml of maltotetraitol was taken out to check the zero reading. On the addition of 1 ml of enzyme the solution became diluted, presumed allowance was made for this dilution.

Samples were removed after every 30 minutes with a 1 ml bulb pipette, and transferred to the boiling tube.

followed by 1 ml of N/500 alkaline potassium ferricyanide. The solution was mixed rapidly and placed in a boiling water bath for 15 minutes, then withdrawn and cooled under running tap water for 2 minutes. To cooled solution was added 1 ml of potassium iodide with mixing and allowed to stand for 3 minutes. At the end of this time 5 ml of (D) was mixed into the solution, followed by 10 ml of (C), the content of the boiling tube was then washed carefully into a beaker and titrated as previously described.

Duplicate samples were always analysed in separate batches to maintain a check on the reproducibility of the method.

Taka-amylase A Catalyzed Hydrolysis of Maltotetraose

The rates of hydrolysis of maltotetraose at different concentrations and at different pH were determined on a Perkin Elmer 141 polarimeter fitted with a transmitting potentiometer which allowed the optical rotation to be continuously monitored on a strip chart recorder. Since there was always trouble with this potentiometer, the change in optical rotation was directly read from the digital counter. The reaction cell of the polarimeter was equipped with a water jacket through which water at a constant temperature was circulated from a Lauda electronic thermostating bath. The temperature in the bath was measured with a National physical laboratory calibrated thermometer.

The initial slopes were determined using a linear least square program, written by Prof. B. Capon.

Buffers solutions

All chemicals used for the preparation of buffered and other solutions were of the highest grade commercially available. The distilled water used for making all kinds of buffer solutions was originally boiled under vacuum in order to remove all the carbon dioxide.

pH measurements

The pH of all the buffer solutions was measured at

the temperature of the kinetic experiment with a Radiometer model 26 pH meter, with an external temperature compensator. A radiometer type G 202C glass electrode was used together with a type K401 calomel electrode. The pH meter was standardised against commercial standard buffers complying to BS 1947, 1961.

Procedure.

The enzyme and substrate solutions were equilibrated in thermostating temperature bath for half an hour. To 1.2 ml solution of maltotetraose of certain concentration, 1 ml of enzyme solution (having concentration 2×10^{-6}M) was added. This solution was pipetted with a fine pipette into the reaction cell whose volume was just less than 1 ml. Any air bubbles trapped in the cell were removed by shaking. The optical rotation changes were normally followed at a wavelength of 365 nm (mercury vapour lamp) to obtain the largest possible rotational change. This rotational change taken directly from the digital counter was used to calculate the initial slopes. These slopes were in degree per second and were converted to M sec^{-1} on the assumption that one mole of maltotetraose yielded two moles of maltose.

How the concentration of the enzyme was checked.

Every day the concentration of the enzyme was checked by measuring the initial slope for the substrate's lowest concentration. In the case of maltotetraitol it was 2×10^{-4}M and in the case of maltotetraose it was 2.5×10^{-3}M. It was noted that enzyme solution in buffer could safely be used for three days.

U.V. Difference Spectrophotometric Study of Taka-Amylase A

Difference spectral measurements were carried out in a Cary Model 14 recording spectrophotometer. Zeiss PMQ 11 spectrophotometer was also used for some measurements. For both spectrophotometers measurements the temperature of the cell block was maintained at 25.0°C. The cell block of the Zeiss was kept constant to within 0.005°C by an electronic relay system. The Cary Model 14 was fitted with an automatic five cell compartment which was maintained at a constant temperature along with the reference block by a Lauda electronic thermostatting bath to within 0.03°C. The temperature was measured in the cell block before and after each run.

(a) A pair of matched tandem double cells designed to substract the solvent contribution to the difference spectrum directly in a single operation was employed to measure the difference spectra, having path length 1 cm.

(b) Slit width was tried to keep as near as possible to

0.2 mm.

(c) Dyno setting for Cary Model 14 was kept at position 2.

(d) A 0 - 0.1 absorbance unit slide wire was used, but for measuring the absorbance of enzymes a 0 - 1.0 slide wire was used.

(e) Before each set of measurements, the spectrophotometer is compensated to give a satisfactory base line in the wavelength region of interest.

Procedure

15.6 μ M amylase in .01M acetate buffer pH 5.3 was placed in one compartment of each reference and sample cell. In the other compartment of each cell was placed maltose solution of different concentrations in the same acetate buffer pH 5.3. These cells were placed in the reference and sample compartments, and a base line was recorded. Then the amylase and maltose solution in the sample compartments were mixed together and the spectrum was recorded. The difference between the two spectra taken before and after mixing, should be the actual difference spectrum produced due to interaction of maltose with the amylase.

The procedure is the same for Zeiss PMQ 11 spectrophotometer but in that case there was no automatic scanning. So we have to change the wavelength manually and the absorbence is read from the scale. Then the difference spectra were plotted.

TLC Experiments for the Study of the Hydrolytic Products of Maltotetraose and Maltotetraitol

Preparation of Chromatoplates

30 g of Kieselgel nach Stahl (Type 60) and 70 ml of distilled water were manually shaken in a glass stoppered flask for about one minute. The slurry was transferred to an adjustable applicator and layers 0.25 mm thick were deposited on clean glass plates. The plates were allowed to dry overnight at room temperature, and then heated for half an hour before using in order to activate the silica.

Procedure

20 x 10^{-3}M maltotetraose (13 mg/1 ml) was hydrolyzed by Taka-amylase A at pH 5.3 and at 25°C. Aliquots were taken out after 15 minutes and applied to the plates with small capillary tubes and applications were made in small increments under a stream of warm air from a drier to facilitate rapid drying, and thus to minimise spot diffusion.

Ascending chromatography was conducted in closed glass tanks lined with Whatman No 1 paper saturated with the developing solvent to a distance of 10 cm from the origin. The plates were run twice in order to achieve clear separation of the spots. After a run the plates were dried with the aid of a drier then were returned to the glass tank for another ascension.


G
17
173
174
A
B

Solvent system

Best solvent system used for the separation of maltooligosaccharides[101] was used. The system produced distinctly superior separations and was chloroform-acetic acid-water (10:79:11, V/V).

Detection of spots

Spraying agent used was a ceric sulphate spray. The reagent was made by adding 50 ml of concentrated sulphuric acid to 450 ml of distilled water, and then dissolving 5 g of solid ceric ammonium sulphate. The sprayed plate was heated for 10 min. at 130°. The oligosaccharides appeared to be as black spots, as seen in the photographs of the chromatograms.

Description of the photograph of the TLC plate.

The photograph shows the thin-layer chromatogram of maltotetraose (20 x 10^{-3}M) hydrolyses by Taka-amylase A at pH 5.3 on Kieselgel layers:-

Lane G, Rf = 0.49, reference glucose

Lane M, Rf = 0.39, reference maltose

Lane M^3, Rf = 0.31, reference maltotriose

Lane M^4, Rf = 0.22, reference maltotetraose

Lanes A → B Enzyme hydrolytic products of maltotetraose taken out at different intervals of time.

Hydrolysis of maltotetraose

Results of the hydrolysis of maltotetraose (20 x 10^{-3}M) are shown in the photograph of the TLC plate. It is seen that at higher concentration of maltotetraose (20 x 10^{-3}M) the products are maltose and maltotriose while only a small amount of glucose could be seen after one and a half hour reaction. While at lower concentration of maltotetraose, the only product found was maltose.

Hydrolysis of maltotetraitol

Same procedure was used for the identification of hydrolytic products of maltotetraitol hydrolysis. Same solvent and spraying agent was used.

At lower concentration (2 x 10^{-3}M) of maltotetraitol the products were identified as maltose and maltitol, while at higher concentrations (20 x 10^{-3}M) the products were maltose, maltitol and maltotriose and maltotritol, while glucose could be seen only under uv lamp.

Table 1

The Taka-amylase A Catalyzed Hydrolysis of Maltotetraitol at pH 5.2 and 25.0°C.

10^4 x Substrate Concn, M.	10^{10} x Initial Slope, M Sec^{-1}.
2	1.117
4	1.962
8	3.720
12	5.753
16	7.498
20	10.079
26	11.646
30	11.835

k_{cat}/K_m = 2.3 M^{-1} Sec^{-1}

Sd = 2.9%

Enzyme concn. = 2×10^{-7}M

Table 2

The Taka-amylase A catalyzed Hydrolysis of Maltotetraose at 25.0°C.

pH 4.2

10^{-3} x Substrate Concn., M.	10^{-7} x Initial Slope, M Sec^{-1}
2.5	0.657
5.0	1.50
7.5	1.914
10.0	3.007
15.0	3.114
20.0	2.597

k_{cat}/K_m = 14.9 M^{-1} Sec^{-1}

Sd = 11.7%

Enzyme Concn. = 2×10^{-6}M

Table 3

The Taka-amylase A catalyzed Hydrolysis of Maltotetraose at 25.0°C.

pH 4.8

10^{3} x Substrate Concn., M.	10^{7} x Initial Slopes, M Sec^{-1}
2.5	1.372
5.0	1.914
7.5	2.374
10.0	3.281
15.0	4.648
20.0	4.922

k_{cat}/K_m = 12.9 M^{-1} Sec^{-1}

Sd = 10.1%

Enzyme Concn. = 2×10^{-6} M

Table 4

The Taka-amylase A catalyzed Hydrolysis of Maltotetraose at 25.0°C.

pH 5.3

10^{-3} x Substrate Concn., M	10^{-7} x Initial Slopes, M Sec^{-1}
2.5	2.050
5.0	3.021
7.5	4.58
10.0	5.414
15.0	8.121
20.0	7.738
25.0	9.433

k_{cat}/K_m = 23.3 M^{-1} Sec^{-1}

Sd = 8.1%

Enzyme Concn. = 2 x 10^{-6}M

Table 5

The Taka-amylase A catalyzed Hydrolysis of Maltotetraose at 25.0°C.

pH 5.8

10^{-3} x Substrate Concn., M	10^{-7} x Initial Slopes, M Sec^{-1}
2.5	2.365
5.0	3.554
7.5	4.511
10.0	6.562
15.0	7.834
20.0	7.136

k_{cat}/K_m = 27.0 M^{-1} Sec^{-1}

Sd = 1.2%

Enzyme Concn. = 2×10^{-6} M

Table 6

The Taka-amylase A catalyzed Hydrolysis of Maltotetraose at 25.0°C.

pH 6.5

10^{-3} x Substrate Concn., M	10^{-7} x Initial Slopes, M Sec^{-1}
2.5	2.406
5.0	4.101
7.5	5.605
10.0	6.986
15.0	8.599
20.0	9.447

k_{cat}/K_m = 30.4 M^{-1} Sec^{-1}

Sd = 3.2%

Enzyme Concn. = 2×10^{-6} M

DISCUSSION

In enzyme reactions whenever a process depends upon a simple association, the plot of the initial velocity against substrate concentration will be a rectangular hyperbola. In 1902 Henry suggested that the enzyme first forms a complex with its substrate and this subsequently breaks down giving the free enzyme and the products of the reaction. Michaelis and Menten in 1913 put forward a theory involving an association of this type, which has been the foundation of the greater part of the enzyme kinetics[102]. If the process is written as:

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

where E is the enzyme, S is the substrate, ES is enzyme-substrate complex and P is the product, then according to Michaelis the rate of the enzyme reaction is determined by the rate of breakdown of the enzyme-substrate complex given by

$$v = \frac{V}{1 + \frac{K_s}{S}} \qquad \text{equ. (1)}$$

V = maximum velocity

K_s = substrate constant

S = substrate concentration

This is the well known Michaelis equation and can be

rearranged

$$(V - v)\ (K_s + S) = VK_s$$

when $S = K_s$, v will be equal to V/2 (half the maximum velocity).

The value of S which is experimentally found to give half the maximum velocity (the Michaelis constant) is written K_m; so that under these conditions $K_m = K_s$. The equality depends on the truth of assumption that equilibrium is maintained between ES, E and S. Many enzymes show a very high catalytic activity so an alternative treatment applicable to such cases was put forward by Briggs and Haldane in 1925. This is based on the postulate that at any moment the rates of formation and breakdown of ES complex are essentially equal, so that its concentration can be regarded as constant and the system may be treated as being in a steady state.

To calculate the steady state parameters the initial rates and respective substrate concentration are fitted to Michaelis Menten equation (equ. 4), which relates the initial reaction rate to the enzyme concentration according to Scheme 1, as follows.

Scheme 1

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + \text{product}$$

$$\text{rate} = k_{cat}\,[E]\,[S] \,/\, (K_m + S) \qquad \text{equ.(2)}$$

$$V_{max} = k_{cat} \cdot E \qquad \text{equ.(3)}$$

$$\text{rate} = V_{max} \,/\, \left(1 + \frac{K_m}{S}\right) \qquad \text{equ.(4)}$$

where V_{max} is experimental maximum velocity, k_{cat} the rate constant for the breakdown of the Michaelis complex and K_m the experimental Michaelis constant.

In order to get accurate values of K_m and V_{max}, it is necessary to obtain some experimental points at substrate concentrations where V approaches V_{max}.

An attempt to determine the kinetic parameters V_{max}, K_m for Taka-amylase A catalyzed hydrolysis of maltotetraose and maltotetraitol has failed due to lack of enough experimental points at high enough substrate concentrations. However the k_{cat}/K_m values have been calculated from the rates for the first four lowest substrate concentrations. In both polarimetric and potentiometric methods there was a restriction in using the higher substrate concentrations. With the

polarimetric method, there was a regular decrease in rotation at the low concentrations of the substrate as the reaction proceeds, but at the higher concentrations of the substrate there was first an increase and then a decrease in the rotation. While with the potentiometric method at higher concentrations it was difficult to measure the concentration of reducing sugar accurately. Further at higher concentrations, the reaction is probably not a simple one. In order to check this, the products of the hydrolytic reaction were examined by TLC as described in the experimental section. The results showed that at lower concentrations maltotetraose gives only maltose, while maltotetraitol gives maltose and maltitol. But at higher concentrations different behaviour was found (Fig 1 in experiment section). Hydrolysis of 20 mM maltotetraose catalyzed by Taka-amylase A (conc. 2×10^{-6}M) in an acetate buffer having pH 5.3 at 25°C showed the formation of maltose and maltotriose on TLC plate. Only a small amount of glucose could be seen after one and a half hours reaction. This showed that a large amount of maltotriose is formed but very little glucose, suggesting that maltotetraose is not hydrolyzed directly to give maltotriose, for if this were so an equal amount of glucose should also be present at the same time. Similarly malto-

tetraitol at higher concentrations (20 mM) forms maltose, maltitol, maltotriose and maltotritol, and a glucose band could only be seen under the UV lamp at the end of the reaction.

A similar phenomenon of concentration dependence of the action pattern of maltotriose and maltotetraose by pancreatic α-amylase was observed by Robyt and French[68], which was explained as due to condensation reaction.

The action pattern of Taka-amylase A on homologous linear maltodextrin of DP 4 to 7 has been studied by oligosaccharides mapping method using labelled substrates[103]. (Concentrations of the maltodextrins not quoted.) They showed that for maltotetraose only one mode of cleavage is predominant as indicated by the arrow in Fig 1.

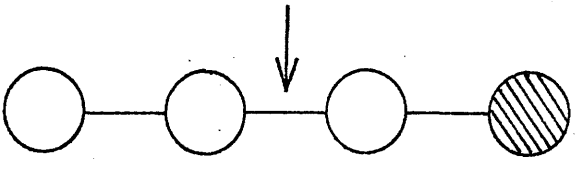


Fig 1

○ glucose unit, ◉ reducing end glucose,

_____ , α, 1__4 glucosidic linkage.

Recently Ono et al[70a] studied the effect of chain length

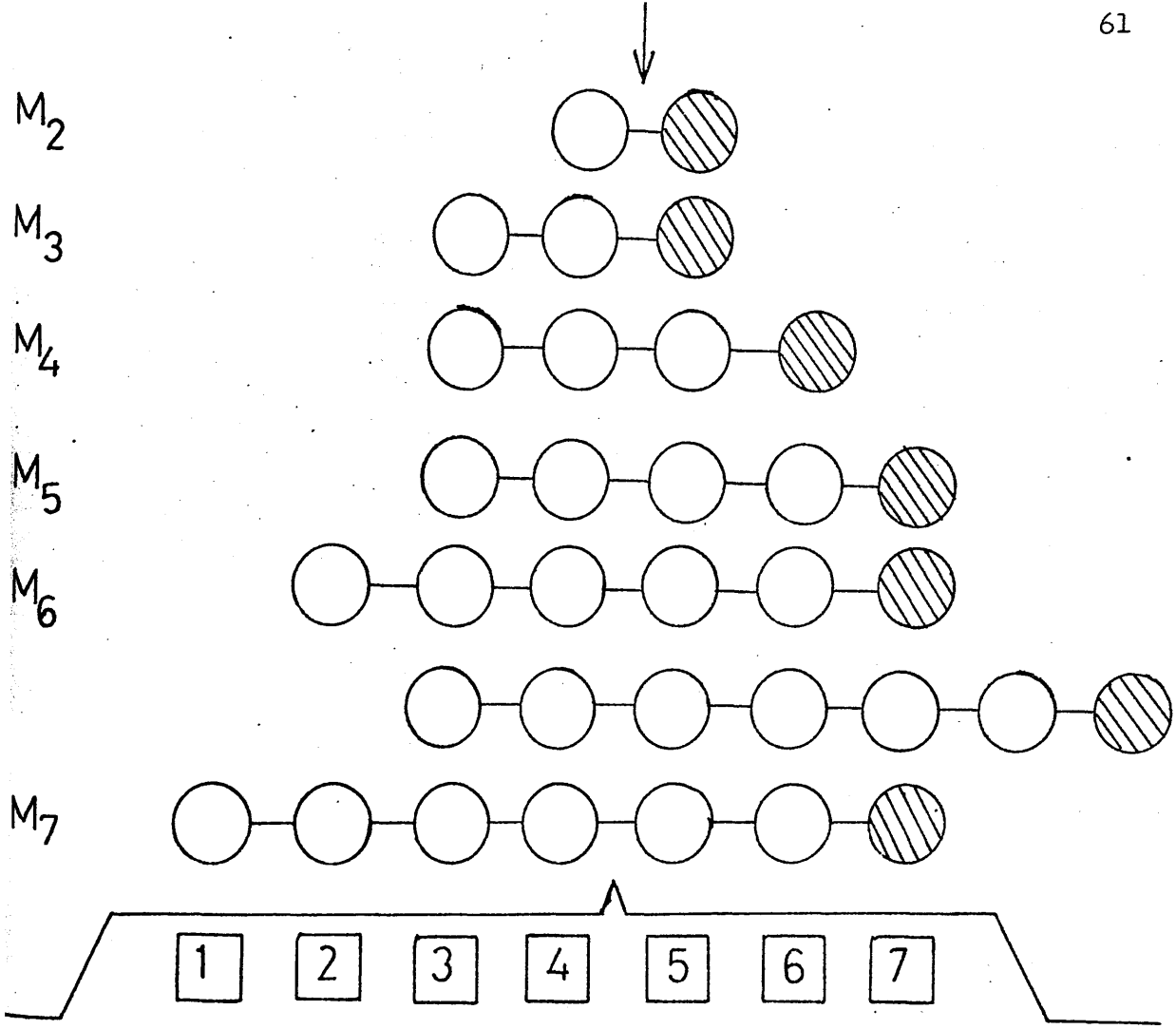


Fig 2

Schematic model for the predominant productive ES complexes for various maltooligosaccharides. The wedge represents the subsite in the specificity region of Taka-amylase A, which are numbered as indicated. O D-glucose residues; ⊖ reducing end glucose; ——, α,1→4 glucosidic linkage. M_2 maltose, M_3 maltotriose, M_4 maltotetraose, M_5 maltopentaose, M_6 maltohexaose, M_7 maltoheptaose.

of linear maltodextrin on the kinetic parameters, which was interpreted in terms of the probability of forming productive and non-productive ES complexes. Only one predominant productive ES complex has been suggested for maltotetraose as shown in Fig 2 (concentration range of maltotetraose is not quoted only $K_m = 8 \times 10^{-3}M$ is given). The Fig 2 shows that only one mode of cleavage is predominant for substrate of DP 2 — 6 indicated by the arrow, while two modes of cleavage are predominant with DP = 7. The catalytic site is located between the third and fourth subsite counted from the terminal one, where the reducing end of maltopentaose in the predominant productive complex is to be situated and the specificity region of the Taka-amylase A was suggested to be 7 glucose units on the basis of kinetic parameters.

Three distinct hypothetical bi-mechanisms can be considered in the explanation of the change in the action pattern of maltotetraose with the increase in the substrate concentration. The more likely involves a transfer reaction. Transfer reactions have been reported for several α-amylases[104,105], although these reactions were not shown to be dependent on relatively high concentrations

of substrate. Transfer reactions have also been reported for lysozyme when incubated with its products[106,107].

The hydrolyzing enzymes can be regarded as acting like transferring enzymes because hydrolysis is the transfer of a part of the substrate molecule to a hydroxyl group from water. Hydrolysis of maltotetraose by Taka-amylase A at low concentrations yields only maltose because of the large amount of water compared to substrate. But at higher concentrations, the situation is different. There is a large amount of substrate present and the reaction can be written as

$$\text{Maltotetraose} + E \rightleftharpoons \text{Maltose} + \text{Maltosyl enzyme}$$

$$\text{Maltosyl enzyme} + \text{Maltotetraose} \rightleftharpoons \text{Maltosyl enzyme - maltotetraose complex}$$

$$\text{Maltosyl enzyme - Maltotetraose complex} \longrightarrow \text{Maltohexaose} + E$$

$$\text{Maltohexaose} + E \longrightarrow 2\ \text{Maltotriose}$$

The concentration of the complex between the matosyl enzyme and maltotetraose will be increased with increasing concentration of maltotetraose. Hence transfer of the maltosyl unit to the maltotetraose forming maltohexaose is more likely to occur. The maltohexaose will then be attacked readily by the enzyme to give two

molecules of maltotriose.

The possibility of the transfer of a glucosyl unit from maltotetraose has been ruled out because there is very little formation of glucose during the reaction. The results for maltotetraitol hydrolysis also confirmed this supposition that transfer of a maltosyl unit (in this case from non-reducing end) to maltotetraitol forms the maltohexaitol which upon hydrolysis gives maltotriose and maltotritol. The very little amount of glucose (visible only under UV lamp) might be due to the hydrolysis of maltotriose by Taka-amylase A. The reason for the even smaller amount of glucose formed from maltotetraitol hydrolysis may be that only one molecule of maltotriose is formed upon maltohexaitol hydrolysis, so the amount of glucose formed is reduced.

Condensation of two molecules of substrate is also not a likely mechanism because in case of maltotetraitol, the two molecules could not condense to give maltooctaitol. In the case of maltotetraose, if condensation occurs then the mode of cleavage of maltooctaose will not be as straightforward as that of maltohexaose-one.

The involvement of a shifted molecular enzyme substrate complex is also unlikely, because in that case the second molecule of the substrate would push the first into a position at the active site that promotes the hydrolysis of bond 3 (Fig 3) instead of bond 2 (Fig 1 and 2). This would lead to equimolar amount of glucose and maltotriose.

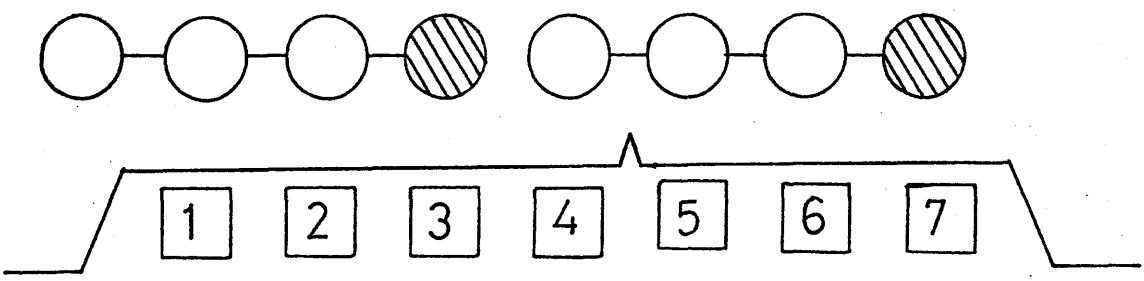


Fig. 3.

The wedge represents the subsite in the specificity region of Taka-amylase A, which are numbered as indicated 0, glucose unit ∅, reducing end glucose, ———, α, 1 —— 4 glucosidic linkage.

On the basis of TLC results and these hypothetical conclusions, a transfer reaction of a maltosyl unit is the most likely explanation for the bi-reaction of maltotetraose and maltotetraitol hydrolysis.

An attempt has been made to study the kinetics of hydrolysis of maltotetraose and maltotetraitol by Taka-amylase A at different pHs. As there was a difficulty in measuring the initial rates of the reaction at higher concentrations, the Michaelis parameters K_m and V_{max} could not be evaluated. Only k_{cat}/K_m are calculated from the rates for the four lowest substrate concentrations. (Tables 1 - 6 Experimental and Results section.) In the case of maltotetraitol, no hydrolysis was observed below or above the optimum pH of enzyme (concentration 2×10^{-7}M), so k_{cat}/K_m was evaluated only at pH 5.2. By comparing the k_{cat}/K_m values of maltotetraose and maltotetraitol at optimum pH and 25°C as below:

Substrate	k_{cat}/K_m M^{-1} Sec^{-1}
Maltotetraitol	2.3
Maltotetraose	23

it could be suggested that maltotetraose is better substrate for Taka-amylase A than maltotetraitol as k_{cat}/K_m

for maltotetraose is ten times higher than that for maltotetraitol. Also the reduction of the reducing-end glucose unit (modification at C-1) does not make it immune to enzymatic hydrolysis, but only lowers the rate of hydrolysis. We may therefore conclude that for hydrolysis of α, 1→4-glucopyranosidic bonds by Taka-amylase A, a tetrasaccharide is the minimum size of substrate required but that the reducing end unit can be rendered acyclic as in maltotetraitol. However, at the present time it is impossible to say more about the influence of molecular structure of substrate maltotetraose and its analogue maltotetraitol on Taka-amylase A catalyzed hydrolysis.

A comparison with the k_{cat}/K_m value calculated by Ono *et al*[70a] with the present calculated value was tried, but there is a large difference in both values expressed in moles per second. From the results, it is not clear how they have converted the initial slopes in degrees per minute into moles per second. There is much confusion about this conversion because if we do not convert the initial slopes into moles per second, our k_{cat}/K_m value is three times less than theirs, but if the conversions are done, our value is 3×10^2 times less than their values as shown in Table A. Therefore, although Ono *et al*[70a]

gave the value of V_{max}/E as min^{-1}, it seems probable that this is not correct and that they have not carried out the conversion of the rates from degrees per minute to moles per minute.

Table A

k_{cat}/K_m values of maltotetraose hydrolysis catalyzed by Taka-amylase A at different pHs at 25°C.

pH	k_{cat}/K_m	
	M^{-1} Sec^{-1}	deg. per min M^{-2}
4.2	14.9	1.0×10^4
4.8	12.9	0.9×10^4
5.3	23.3	1.2×10^4
5.8	27.0	1.8×10^4
6.5	30.4	2.2×10^4

According to Ono *et al* the k_{cat}/K_m value for the hydrolysis of maltotetraose catalyzed by Taka-amylase A at pH 5.3 and 25°C.

4.2×10^5 M^{-1} min^{-1} or 7×10^3 M^{-1} Sec^{-1}

The k_{cat}/K_m increases with the increase of pH as shown in Table A. If the ionic state of maltotetraose is considered to be unchanged over the pH range 4.2 - 6.5, the increase is k_{cat}/K_m with increasing pH may be considered to reflect the presence in the enzyme of a catalytically important prototropic group that is effective in its dissociated form. Imidazole and carboxylic groups are supposed to be present in the side chain as catalytic groups in amylases[50,51]. One possible function of such a carboxylate group would be to form an ion pair with the cationic group of the substrate in a manner analogous to that postulated for the interaction of amylases[51] and lysozyme[98] for its specific substrates. The protonated imidazole group was visualised as acting as a general acid catalyst and the carboxylate group stabilising the developing glucosyl cation electrostatically through ion pair formation. However it is difficult to identify any catalytic group from the variation of k_{cat}/K_m with pH since a sufficiently large pH range was not studied.

An attempt was made to study the active centre of α-amylase from Aspergillus oryzae (Taka-amylase A) by the difference spectrum characteristic of enzyme-product

interaction between Taka-amylase A and the end product of its catalytic action, i.e. maltose, at the optimum pH of the enzyme 5.3 and at 25°C. With the change in maltose concentration (0.2 - 2%) no regular change in the absorbance at any wavelength was observed. Thus we were unable to see any characteristic perturbation difference spectrum due to tryptophan. It was attempted to see the maltose-induced difference spectra of pancreatic α-amylase and N-acetyl tryptophan methyl ester under exactly the same conditions as described by Elodi et al[108]. It is interesting that we observed three maxima at 291, 283.7 and 273 coinciding fairly well with those of the perturbation difference spectrum of N-acetyl tryptophan ethyl ester measured in 20% maltose, reported by Elodi et al, but a negative difference was observed above 298 to 330 nm in contrast to the positive difference reported by the latter. However, with pancreatic α-amylase our results were the opposite of Elodi et al[108]. A positive difference was observed above 291 nm (they observed a negative difference above 295 nm), while a negative difference was seen below 291 to 260 nm with minima around 271 nm; while these other people observed three positive maxima at 290, 284 and 279 nm with a relatively wide trough, between 295 and 310 nm. with a minima centred around 297 nm. The

difference in the results could be due to the difference in enzyme preparation, but nothing more could be said about it.

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* ABB Archives Biochemistry Biophysics
BBA Biochimica Biophysica Acta
JACS Journal American Chemical Society
JBC Journal Biological Chemistry
JCS Journal Chemical Society

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