THE INTERACTION OF CEREAL α-AMYLASE WITH CYCLOHEPTAAMYLOSE

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Randall Joseph Weselake

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ABSTRACT

Weselake, Randall Joseph, M.Sc., The University of Manitoba, October 1981. The Interaction of Cereal α-Amylase With Cycloheptaamylose. Major Professor: R. D. Hill.

There is strong evidence that cycloheptaamylose is a competitive inhibitor of amylases in general. A column of immobilized cycloheptaamylose has been shown to bind cereal α-amylase, however, the mechanism of interaction remained unclear.

The enzyme-ligand interaction was investigated using both kinetic and equilibrium binding methods. High specific activity cereal α-amylase was prepared free of β-amylase from germinated wheat and triticale kernels by affinity chromatography of polyvinylpolypyrrolidone treated and dialyzed crude extracts. The effect of cycloheptaamylose on the interaction of α-amylase with both gelatinized and raw starch was studied. Equilibrium dialysis was conducted to assess the binding parameters of the enzyme-ligand interaction. Radioactive cycloheptaamylose prepared from commercially available cycloheptaamylose was used for this purpose.

It was found that cereal α-amylase interacted with the cycloheptaamylose at a non-catalytic site and the dissociation constant for binding was 3.0×10^{-5} M. Cycloheptaamylose had no effect on the enzyme's ability to hydrolyze starch in solution. In contrast cycloheptaamylose inhibited binding to and hydrolysis of starch granules. The

results confirmed that the enzyme had a non-catalytic site that appeared to be responsible for binding to starch granules so as to facilitate hydrolysis. The same non-catalytic site also appeared to be the site of cycloheptaamylose binding.

INTRODUCTION

Cycloamyloses or cyclodextrins are cyclic oligomers of glucose linked α(1→4) and possess neither a reducing nor a non-reducing end group. Because of their similarity to portions of the α-helix of amylose their interaction with amylases has been studied extensively. Both cyclohexaamylose (α-cyclodextrin) and cycloheptaamylose (β-cyclodextrin) have been found to be inhibitors of amylases in general.

During studies to develop purification procedures for cereal α-amylase it was found that the enzyme would bind to an affinity column of cycloheptaamylose linked to epoxy-activated Sepharose 6B (Silvanovich and Hill, 1976). Inhibition studies with cycloheptaamylose and cereal α-amylase have been inconclusive (Silvanovich, 1977) and the mechanism by which the enzyme interacted with the ligand still remains unclear. It is possible that cycloheptaamylose is bound at the active site of cereal α-amylase or at a non-catalytic site on the enzyme. Some α-amylases, including the cereal enzyme, are believed to have such a non-catalytic site that allows for binding to starch granules.

The interaction between cycloheptaamylose and cereal α-amylase was investigated using both gelatinized starch and starch granules. Kinetic studies with gelatinized starch were conducted to see if cereal α-amylase was competitively inhibited by cycloheptaamylose. Kinetic studies with starch granules were done to focus on the effect of cycloheptaamylose on the interaction of the enzyme with starch granules at its non-catalytic site. Equilibrium binding studies were also done

in order to quantitatively assess the strength of binding and the stoichiometry of binding of cereal α-amylase to cycloheptaamylose. A sensitive assay for cycloheptaamylose was developed in order to effectively perform the binding studies.

LITERATURE REVIEW

Fundamental Properties of Cereal α-Amylase

Alpha-amylases (α, 1→4 glucan 4-glucano hydrolase, E. C. 3.2.1.1.) are endo-enzymes which hydrolyze internal segments of a starch chain to produce smaller glucose oligomers. The products of hydrolysis assume the α-configuration at the anomeric carbon atom of glucose (Banks and Greenwood, 1975). Alpha-amylases are found in bacterial, plant and animal systems (Thoma et al., 1971). In cereal seeds α-amylase is responsible for the mobilization of insoluble starch reserves in the endosperm thus serving as an initial step in the provision of available carbohydrate for the germination process (Banks and Greenwood, 1975). The cereal enzyme has important technological relevance in both the baking and brewing industries. The level and control of α-amylase is important in obtaining high quality end products.

Electrophoresis of extracts of wheat kernels at various stages of development has established the involvement of two major groups of α-amylase isozymes (Olered and Jonsson, 1970). The more acidic or α-I group was found to be present throughout cereal kernel development while the more neutral α-II group became apparent only upon germination. The levels of both isozyme groups rose sharply during germination. Alpha-II isozymes were found to constitute a much larger proportion of the total α-amylase activity in the germinated seed.

Isoelectricfocusing, a high resolving technique, showed that each isozyme group was composed of a number of other isozyme bands (Nishikawa and Nobuhara, 1971; MacGregor, 1976). Silvanovich and Hill (1977) have reported isoelectric points ranging from 4.6 to 5 for α-I isozymes and isoelectric points near 6.2 for α-II isozymes prepared from germinated triticale seeds. MacGregor et al. (1974) and Marchylo et al. (1976) have found similar isoelectric points for both barley and wheat α-amylase isozymes. For the longest time the multiplicity of isozyme bands was attributed to either artifacts induced by the purification procedure or to some other post-translational effect (Marchylo et al., 1976). However the multiple bands have still been found to exist in freshly prepared crude extracts (MacGregor and Ballance, 1980a). Recent evidence from experiments using isolated aleurone tissues has suggested that α-amylase isozymes may be a result of differential gene expression (Jacobsen, 1980). Hare et al. (1978) have observed that multiple bands can also be induced by ampholytes binding to macromolecules during isoelectricfocusing.

The cereal α-amylases are monomeric enzymes with reported molecular weights ranging from 40,000 to 58,000 (Greenwood and Milne, 1968; MacGregor et al., 1974; Tkachuk and Kruger, 1974; Marchylo et al., 1976; Silvanovich and Hill, 1977; MacGregor, 1978). There appears to be no trend that indicates that the α-I group is significantly different from the α-II group in molecular weight. However it has been reported for immature hard red spring wheat and immature barley that the α-I isozymes are larger than α-II isozymes of germinated seeds (Marchylo et al., 1976; MacGregor, 1978). In one instance where a purified

wheat α-amylase fraction was assessed by analytical ultracentrifugation, a species of 86,000 was determined (Tkachuk and Kruger, 1974). Molecular weights using gel filtration on Sephadex have shown anomolous values below 40,000 which have been attributed to a mild interaction between α-amylase and the carbohydrate of Sephadex (Manners and Marshall, 1972; Tkachuk and Kruger, 1974).

The amino acid composition of wheat α-amylase fractions have been found to be quite similar (Tkachuk and Kruger, 1974). Large amounts of glutamyl, aspartyl and glycyl residues were found indicative of the more anodic tendencies of the isozymes. Arginine was the only amino acid that showed significant fluctuation between isozyme groups.

The tertiary structure and activity of cereal α-amylase like other α-amylases is preserved by calcium (Greenwood and Milne, 1968). A very low concentration of calcium in the buffer solution is required to maintain full enzyme activity indicating that the affinity of the enzyme for calcium is high.

The pH activity profiles and pH stability profiles of cereal α-amylases from various sources have been found to be quite similar (Greenwood and MacGregor, 1965; MacGregor et al., 1974; Tkachuk and Kruger, 1974; Marchylo et al., 1976; MacGregor, 1978). The pH optimum for activity in each case was about 5.5. The pH stability profiles indicated the need for a slightly higher pH to maintain the activity of the enzyme over extended time periods. Alpha-I isozymes appeared to be more acid stable than α-II isozymes (MacGregor, 1978). Silvanovich and Hill (1977) have demonstrated that the presence of β-limit dextrin has a marked effect on increasing the stability of both

α-I and α-II isozyme groups at all pH points studied. The α-I isozymes have also been found to be much less heat stable than the α-II isozymes (Silvanovich and Hill, 1977 ; MacGregor, 1978).

Hao et al. (1977) have described the stabilizing effects of bovine serum albumin (BSA) during the measurement of α-amylase activity. MacGregor and Ballance (1980b) have used BSA to stabilize barley α-amylase isozymes in experiments where the enzyme must maintain full activity at elevated temperatures over long time periods. Cereal α-amylase has been found to withstand temperatures as high as 70°C for short time periods as long as the enzyme remained in crude extract (Kneen et al., 1943; MacGregor, 1978). The presence of BSA was found to confer stability to homogeneous α-amylase preparations such that they could be frozen in solution without loss of activity upon thawing. Quantitative extraction of barley α-amylase from polyacrylamide gels after isoelectricfocusing has recently been made possible by using BSA in the extracting medium (MacGregor and Ballance, 1980a).

Purification of Cereal α-Amylase

Most procedures for the purification of α-amylase rely on the enzyme's strong affinity for its substrate at low temperature. Schwimmer and Balls (1949a) used a column of wheat starch granules to adsorb malt α-amylase from an alcoholic solution at low temperature prior to crystallizing the enzyme. Precipitation of α-amylases from various sources using glycogen in the presence of ethanol at 0 to 4°C has been routinely used as an intermediate fractionation step (Schramm and Loyter, 1966). The precipitation step is based on the formation

of an enzyme-dextrin complex "where many molecules of both reactants are linked to each other" (Levitzki et al., 1964). Pancreatic α-amylase has been shown to have at least two binding sites that would facilitate the formation of such a precipitate (Loyter and Schramm, 1966). Rabbit muscle phosphorylase a and b (α, 1→4 glucan orthophosphate glucosyl transferase, E. C. 2.4.1.1.) have also been shown to form similar aggregates with glycogen (Selinger and Schramm, 1963).

More refined purification procedures for cereal α-amylases have been developed which usually included the ethanol-glycogen step along with a number of other separation techniques (Botes et al., 1967; Kruger and Tkachuk, 1969; MacGregor et al.,1971; MacGregor et al., 1974; Marchylo et al., 1976; Warchalewski and Tkachuk, 1978). However the procedures were lengthy and usually resulted in a low yield of homogeneous enzyme. Much of the effort in the procedures was directed toward the removal of β-amylase (α, 1→4 glucano maltohydrolase, E. C. 3.2.1.2.); an enzyme present at high levels in many germinating seeds (Thoma et al., 1971). This exo-enzyme liberates maltose sequentially from the non-reducing end of a glucose chain leaving the anomeric carbon atom of maltose in the β-configuration (Banks and Greenwood, 1975). The specific activity of this enzyme is extremely high and trace amounts present in α-amylase preparations can lead to misinterpretations of kinetic data. One of the most effective ways of removing β-amylase activity is by heat treatment of the crude extract (Kneen et al., 1943). Beta-amylase is denatured along with some other proteins while α-amylase remains active. The heat step has been used with much success but extensive chromatography is still required to remove trace amounts of β-amylase.

In recent years affinity chromatography of enzymes and proteins has served to abbreviate complex purification procedures to a few simple steps. The ethanol-glycogen step is essentially an affinity step but its application still results in co-precipitation of impurities (Schramm and Loyter, 1966). Therefore the precipitated material usually requires further processing before the impurities are effectively removed. Tkachuk (1975) extended the idea of the ethanol-glycogen step by using a column of glycogen linked to Sepharose to purify wheat α-amylase from crude extracts of germinated wheat kernels in one step. The enzyme was eluted with solubilized glycogen in the moving phase. The procedure was high yielding and resulted in α-amylase of 90% purity. However further processing of the eluted enzyme by ion-exchange chromatography was required before a homogeneous preparation was obtained. In addition, Silvanovich (1977) found that the method was not readily reproducible.

Silvanovich and Hill (1976) have introduced a method for the isolation of high specific activity α-amylase from a solubilized ethanol-glycogen precipitate of an extract of germinated triticale kernels. They used an affinity column which consisted of cycloheptaamylose-epoxy-Sepharose 6B. Cycloheptaamylose or β-cyclodextrin is a seven membered cyclic glucose oligomer that has neither a reducing nor a non-reducing end group (French, 1957). The cyclic dextrin appeared to act as a substrate analogue for cereal α-amylase. Gibbons (1979a, 1979b) has used this affinity method to isolate α-amylase from germinated barley.

The technique for preparing the affinity column was based on a

procedure introduced by Vretblad (1974a, 1974b) where cyclohexaamylose was attached to epoxy-activated Sepharose 6B to produce a gel with immobilized cyclohexaamylose. This in turn was used to purify sweet potato β-amylase. Sweet potato β-amylase had a high affinity for cyclohexaamylose (Thoma et al., 1963a), whereas cycloheptaamylose did not appear to have a high affinity for β-amylase (Silvanovich and Hill, 1976). Furthermore Vretblad's affinity column did not retain Bacillus subtilis α-amylase (1974b). It was this observation that prompted Silvanovich (1977) to think that a subtle change in ligand would serve to retain cereal α-amylase.

Alpha-amylases from sources other than higher plants have also been purified by using affinity chromatography (Buonocore et al., 1975; Buonocore et al., 1977). In these cases the immobilized ligand consisted of an albumin fraction from the wheat kernel that was found to inhibit α-amylases from many sources (Silano et al., 1975). The albumin affinity columns have not been used on higher plant α-amylases because there was no inhibition of the plant enzyme by wheat albumins.

The two major cereal α-amylase isozyme groups (α-I, α-II) have been routinely separated using ion-exchange chromatography (MacGregor et al., 1971; Silvanovich and Hill, 1977). Silvanovich (1977) has reported a preferential release of α-I isozymes from the cycloheptaamylose affinity column when using the β-limit dextrin of waxy maize to elute the affinity column. However trace amounts of α-II were still apparent in the α-I eluted component. The remaining α-II isozymes could then be eluted with cycloheptaamylose in the moving phase. In contrast Gibbons (1979a, 1979b) has found preferential elution of α-II

isozymes using β-limit dextrin. The elution differences observed by by both investigators still remain to be resolved. Sargeant et al. (1978) have used preparative isoelectricfocusing to separate α-I and α-II isozymes of germinated wheat.

Solution Kinetics and Action Pattern of Cereal α-Amylase

Kinetic studies on α-amylases have been almost entirely restricted to systems using soluble substrates which is not surprising when one considers the difficulties encountered in dealing with heterogeneous systems (McLaren and Packer, 1970). Cereal α-amylases have been found to hydrolyze high molecular weight substrates more rapidly than low molecular weight substrates (Thoma et al., 1971). Internal segments of the starch chain are hydrolyzed rapidly to form dextrins from 6 to 8 glucose units in length and α-limit dextrin. The presence of α-limit dextrin is caused by the presence of α(1→6) linkages in the amylopectin portion of starch (Greenwood and Milne, 1968). Very large α-limit dextrins have been obtained when using glycogen as a substrate for pancreatic α-amylase (Heller and Schramm, 1964). The larger size of glycogen limit dextrins was attributed to the more frequent appearance of interfering α(1→6) branch points in the glycogen molecule.

The affinities of α-I and α-II isozyme groups for both soluble starches and β-limit dextrin have been found to be very similar. Marchylo et al. (1976) have reported Km values of 2.5, 5.33 and 2.35 x 10^{-4} gm/ml for three α-I components of immature hard red spring wheat. Silvanovich and Hill (1977) have reported a Km value of 2.5 x 10^{-4} gm/ml for α-I isozymes and a Km of 2.9 x 10^{-4} gm/ml for α-II isozymes

in work with triticale α-amylase. These findings were in good agreement with the early work of Botes et al. (1967) where a Km of 2.88 x 10^{-4} gm/ml was found for total malt sorghum α-amylase.

The smaller dextrin products of cereal α-amylase action are often referred to as achroic dextrins. Their formation has been attributed to the number of subsites at the active site of the enzyme where each subsite accomodates one glucose moiety (Greenwood and Milne, 1968). Thoma et al. (1971) have suggested that analysis of product distribution was indicative of the size of the binding site. The product patterns were related only to productive modes of binding and therefore were more meaningful than data based on changes in Km and Vmax as a function of glucose oligomer length.

The action pattern of cereal α-amylase is different from the mammalian α-amylases. The mammalian α-amylases form dextrins, smaller than achroic dextrins at a similar rate regardless of substrate size whereas cereal α-amylase action is severly impeded by the use of shorter substrate chains (Greenwood and Milne, 1968; Thoma et al., 1971).

The period of attack from initial substrate structure to achroic dextrin accumulation is known as the random phase of attack, however, the bonds near the ends of the chains are non-randomly attacked (Greenwood and Milne, 1968). If achroic dextrins are incubated with cereal α-amylases for extended time periods, smaller dextrins will eventually become apparent. Extremely large amounts of enzyme are required in order to drive this secondary reaction effectively (MacGregor, 1978). Very little is known of this sluggish reaction and it is often referred to as the non-random phase of attack (Greenwood and Milne, 1968).

French and Abdullah (1966) have demonstrated that α-amylase may be reversible. They attributed the reverse behavior to contamination by a transferase which seemed to accompany malt α-amylase through the various stages of purification. However Robyt and French (1970) have provided evidence for a synthetic reaction in the case of purified pancreatic α-amylase which was not believed to be due to transferase contamination.

Three major modes of attack could be operating in the degradation of α-amylase substrates (Banks and Greenwood, 1975). In the case of single chain degradation the entire substrate molecule is degraded before the enzyme dissociates to attack another substrate molecule. Secondly there is the possibility of multichain degradation where the enzyme dissociates from the substrate after cleaving a single bond. The intermediate form of attack is referred to as multiple attack and has been proven to be the mode of attack in the case of pancreatic α-amylase (Robyt and French, 1967; 1970). It was also shown that the polarity of attack was toward the non-reducing end of the chain. Greenwood and Milne (1968) were unable to provide an unequivocal statement as to the operation of multiple attack in the case of data based on germinated wheat α-amylase.

The Interaction of α-Amylases With Starch Granules

The action of α-amylase on starch *in vitro* can be studied with raw starch in suspension as well as with starch in solution (McLaren and Packer, 1970). It remains to be explained, however, why the rate of hydrolysis *in vitro* is so much slower than *in vivo* (Walker and Hope, 1963). Therefore factors affecting the susceptibility of starch

granules to enzymic degradation do not appear to be clearly understood.

Early studies on starch granule digestion indicated that the extent of hydrolysis was dependent on the type of amylase and the source of the starch granules (Stamberg and Bailey, 1939). At this time it was also firmly established that plant β-amylase alone had virtually no effect on starch granule degradation. Experiments with starches from various sources indicated that potato starch was almost totally resistant to α-amylase attack (Balls and Schwimmer, 1944). Waxy type starch which was highest in amylopectin content was found to be more susceptible than normal starch which contained approximately 30% amylose (Leach and Schoch, 1961; Banks and Greenwood, 1975).

Schwimmer and Balls (1949a) had made observations on the affinity of malt α-amylase for wheat starch granules at low temperature. The adsorption phenomenon was studied in detail in a following paper (Schwimmer and Balls, 1949b). Malt α-amylase appeared to interact with wheat starch granules via a site different from the site responsible for catalysis. This was inferred because maltose non-competitively inhibited the action of malt α-amylase on starch in solution and that maltose desorbed α-amylase from starch granules. Surprisingly α-dextrin, which was apparently achroic dextrin, was found to stimulate adsorption of malt α-amylase. The efficiency of adsorption was found to be proportional to the surface area of the granules. The adsorption process obeyed the Freundlich relation $Ea = kAE^n$, where Ea is the amount of enzyme adsorbed, E is the equilibrium concentration of the enzyme, A is the surface area of the starch granule and k and n are constants (Banks and Greenwood, 1975).

Similar conclusions were later reached by Walker and Hope (1963) but the study encompassed α-amylases from different sources. Salivary and pancreatic α-amylase adsorbed to maize starch granules most efficiently at low temperature at a pH of 7.4. The rate of hydrolysis of maize starch granules by the mammalian enzyme increased at higher temperatures. Bacillus subtilis α-amylase adsorbed very weakly to the starch granules at $0^{o}C$ and degraded them very slowly at higher temperatures. Aspergillus oryzae α-amylase neither adsorbed to nor degraded the granules.

More recently MacGregor (1979) found that barley α-amylase adsorbs most effectively to isolated barley starch granules at pH 5.5 and $4^{o}C$. Barley α-I isozymes were also found to be more efficient in the degradation of starch granules than α-II isozymes which suggested that the two groups may have different modes of attack (MacGregor and Ballance, 1980b). In contrast Sargeant et al. (1978) found that wheat α-I isozymes had little affinity for fully developed wheat starch granules whereas wheat α-II isozymes adsorbed quite strongly. A differential physiological role for the two groups of isozymes was then implied.

Sandstedt and Mattern (1960) had previously correlated initial rapid phases of starch granule digestion with the degree of physical damage. The slow phase of hydrolysis thereafter was believed to represent the digestion of intact granules. Walker and Hope (1963) have suggested that minor mechanical damage was of no consequence in susceptibility to enzymic degradation. Their digestion profiles were based on data that represented hydrolysis of the intact starch granules

or the slow phase of digestion. However minor damage has been found to have an important bearing on granule hydrolysis measurements taken within the first few minutes of granule degradation (MacGregor and Ballance, 1980b).

McLaren and Packer (1970) have made a detailed study on enzyme reactions in heterogeneous systems. In 1963 McLaren provided a kinetic model to explain the hydrolysis of starch granules by α-amylase. The model assumed that the initial velocity of granule hydrolysis was proportional to only that portion of the enzyme that was adsorbed to the granules. Adsorption to starch granules was known to be an equilibrium phenomenon. Deviations from the model were attributed to the fact that the adsorption of the enzyme onto the starch granule was not as rapid as compared to the rate of hydrolysis. Furthermore the site of adsorption had been shown to include others besides the site responsible for hydrolysis (Schwimmer and Balls, 1949b; Walker and Hope, 1963).

Both the light and electron microscope have been used extensively in understanding the starch granule and its degradation. Starch granules of wheat have been found to range in size from 5 microns to 30 microns whereas barley granules appeared to consist of two discrete populations (Bartgate and Palmer, 1972). Small spherical granules comprised about 90% of the starch and were near 5 microns in diameter. The remaining larger "lenticular" granules were about 25 microns in diameter. Large wheat starch granules were found to be most susceptible to amylase degradation at the equitorial groove whereas other regions appeared to be randomly atacked (Evers and McDermott, 1970; Dronzek et al., 1972). Large granules exposed to α-amylase for long time periods had

surface pinholes suggesting preferential surface digestion followed by digestion from within (Bathgate and Palmer, 1973; MacGregor and Ballance, 1980b). Smaller granules appeared to have been degraded only from the outside. Recent observations on fractionated and enzymically degraded barley granules have suggested that there are significant structural differences between large and small granules (MacGregor and Ballance, 1980b). Recent studies on the possibility of structurally different large and small wheat granules have been inconclusive (Meredith, 1981).

Binding Analysis of Enzyme-Ligand Interactions

Equilibrium Binding

The study of protein -ligand systems offers the advantage that in most cases the protein is much larger than the ligand. This is the basis for most procedures used to investigate binding strength and stoichiometry of binding. Klotz et al. (1946) used a cellulose membrane which was permeable only to small molecules to separate two solution compartments. One compartment had a fixed concentration of protein. The affinity of the protein for a binding ligand was studied by dialyzing the macromolecule against different concentrations of ligand. The technique eventually became known as equilibrium dialysis and is now the most classical method for studying protein-ligand interactions. In most cases a sensitive assay for the ligand is required. Radioactively labelled ligands are usually used for this purpose.

Equilibrium dialysis data are usually expressed in the form of a Scatchard (1949) plot where the concentration of ligand bound per mole of enzyme ($\bar{\upsilon}$) divided by the concentration of free ligand (c) is

plotted against $\bar{\upsilon}$. A linear relationship is obtained from such a plot when there is only one class of equivalent and independent binding sites. This relationship can be described by the following equation:

$$\frac{\bar{\upsilon}}{c} = \frac{1}{K_d} n - \frac{1}{K_d} \bar{\upsilon}$$

(Scatchard, 1949; eg. Mora et al., 1974)

The dissociation constant (K_d) describes the strength of the interaction and can be obtained from the reciprocal of the slope of the line. The number of moles of ligand bound specifically per mole of protein (n) can be derived from the intercept of the line on the $\bar{\upsilon}$ axis.

Deviations from linearity are often found in Scatchard plots. Statistical methods are usually used to obtain "best fits" to such data (Thakur et al., 1980). Common causes of deviations from linearity are often the result of negative cooperativity and non-specific binding. Negative cooperativity involves multiple classes of sites where initial binding of a ligand causes a decrease in affinity for further binding of the ligand (Preiss and Kosuge, 1976; Thakur et al., 1980). Non-specific effects have been found to involve further binding of a ligand under saturating conditions but not necessarily at the site on the protein which is most specific for the binding of the ligand (Mora et al., 1974). Large differences in charge between proteins and ligands have been found to lead to electrostatic effects. This difficulty is usually overcome by the inclusion of high salt concentrations in the equilibrium dialysis compartments (Scatchard et al., 1950).

Kinetic Methods

Many enzymes can be assayed very rapidly. The Lineweaver and Burk (1934) method is well established and universally used to determine the affinity of an enzyme for its substrate. The equilibrium constant (K_m) describes the reversible combination of an enzyme with its substrate. To determine stoichiometry of binding one must resort to equilibrium binding studies under conditions that will stop the formation of product. A good example is equilibrium dialysis of pancreatic α-amylase against maltotriose which was conducted at pH 10 to prevent hydrolysis (Loyter and Schramm, 1966).

The Lineweaver-Burk (1934) method can also be used to determine inhibitor constants. The double reciprocal plot of 1/v versus 1/s is represented by the reciprocal form of the Michaelis equation:

$$\frac{1}{v} = \frac{K_m}{V}\frac{1}{s} + \frac{1}{V} \qquad (1)$$

where v is initial velocity of reaction, V is maximum velocity at saturating substrate concentrations and s is substrate concentration If an inhibitor is studied where K_i is the equilibrium constant for the reversible combination of an enzyme with a competitive inhibitor then the equation becomes:

$$\frac{1}{v} = \frac{K_m}{Vs} + \frac{1}{V} + \frac{K_m}{Vs}\frac{i}{K_i} \qquad (2)$$

where i is the inhibitor concentration. In competitive inhibition the enzyme competes for both substrate and inhibitor at the catalytic site. Plots obtained at different inhibitor concentrations by the Lineweaver-Burk analysis of competitive inhibition all intersect at

the ordinate at 1/V. The slope is defined by $K_m/V\ (1 + i/K_i)$.

Non-competitive inhibition is the situation in which the "inhibitor binds to a site on the enzyme other than the active site so the enzyme-substrate complex does not form at a normal rate and once formed the complex does not decompose to form product at the normal rate " (Lehninger, 1975). The slope of non-competitive inhibition is described by the same relation as for competitive inhibition (equation 2) except the lines all intersect at $-1/K_m$ on the abscissa. At different inhibitor concentrations the ordinate intercepts are defined by $1/V\ (1 + i/K_i)$.

Lineweaver-Burk calculations used to determine K_i involve the effect on the velocity by varying both s and i independently. Dixon (1953) devised a shorter method to obtain competitive inhibitor constants where only i was varied in the presence of a fixed amount of substrate (s_1). The reciprocal of the initial velocity then fluctuated as a function of inhibitor concentration. The same experiment conducted in a second substrate concentration (s_2) yielded another line. The intersection of the two lines to the left of the vertical axis was found to lie at $-K_i$ according to the following proof. Each substrate line was represented by equation (2). At the point of intersection to the left of the vertical axis 1/v and i were found to be the same for both lines. Therefore

$$\frac{K_m}{s_1} + \frac{1}{} + \frac{K_m}{s_1}\frac{i}{K_i} = \frac{K_m}{s_2} + \frac{1}{} + \frac{K_m}{s_2}\frac{i}{K_i}$$

which simplified to

$$\frac{1}{s_1}\left(1+\frac{i}{K_i}\right)=\frac{1}{s_2}\left(1+\frac{i}{K_i}\right)$$

This was only true if either $s_1 = s_2$ or $i = -K_i$.

Dixon (1953) further indicated that the method could be used to study non-competitive inhibition. In this case the lines met at a common point on the abscissa to the left of the ordinate to give $-K_i$. This was verified by substituting $1/v = 0$ into equation (2).

Purich and Fromm (1972) have suggested that the Dixon method cannot be used with assurance to distinguish between competitive and non-competitive inhibition. In certain cases of non-competitive inhibition it was shown that the binding of inhibitor to the enzyme could affect substrate binding and vice versa. This mixed inhibition at times manifested itself as competitive inhibition when plotted according to the Dixon method. Therefore the authors suggested that the safest approach to the study of inhibition was the Lineweaver-Burk method.

Cycloamyloses

Properties

The cycloamyloses or cyclodextrins are among the products of action of Bacillus macerans amylase [1, 4-α-D-glucan 4-α(1,4α- glucano)-transferase, E. C. 2.4.1.19.] (French, 1957; Kitahata et al., 1978). Starch serves as a substrate for this bacterial enzyme. Cycloamyloses vary in size but mainly occur as 6 to 8 membered cyclic glucose oligomers that are designated cyclohexa-, cyclohepta- and cyclooctaamylose (α-, β- and γ-cyclodextrin) respectively. The glucose units are linked α(1→4) as in amylose and the ring structure does not allow the molecules

to display reducing activity (French, 1957). Cycloamyloses were first unknowingly observed by Villiers in 1891 in his bacterial cultures which were apparently contaminated with Bacillus macerans. Later Schardinger correctly identified Bacillus macerans as the source of cyclodextrin formation. He then proceeded to characterize the cyclic molecules.

It was soon found that cycloamyloses displayed a high degree of reactivity with organic molecules by accomodating them in the center hydrophobic region of the donut shaped structure (Cramer and Hettler, 1967). This property of inclusion made the cycloamyloses excellent models for enzyme catalysis (Bender and Komiyama, 1978). The reactivities of the cavity were found to vary with the type of cycloamylose and provided a basis for the early fractionation of cyclodextrins by organic precipitation (French, 1957). Table 1 illustrates some fundamental physical properties of cycloamyloses (Bender and Komiyama, 1978). Cyclohexa- and cycloheptaamylose are particularly resistant to chemical hydrolysis compared to starch (Swanson and Cori, 1948). Only more concentrated acids are capable of effectively hydrolyzing cyclohexa- and cycloheptaamylose. At pH 0.133 at 40^{o}C the half-life of cycloheptaamylose was found to be 48 days (Bender and Komiyama, 1978).

Susceptibility To Enzymic Hydrolysis

Cyclohexa- and cycloheptaamylose are also resistant to attack by most starch degrading enzymes. Exo-enzymes such as β-amylase are unable to degrade cyclodextrins because there is no non-reducing end available (Thoma and Koshland, 1960). French found that cyclohexaamylose was totally resistant to attack by salivary α-amylase and that

TABLE 1. Physical properties of the cycloamyloses

Cycloamylose	Number of glucose residues	Molecular weight (calculated)	Water solubility (gm/100 ml)	Specific rotation $[\alpha]_D^{25}$	Cavity dimensions (Angstroms)	
					Internal diameter	Depth
cyclohexaamylose	6	972	14.5	150.5 ± 0.5	4.5[a]	6.7[a]
cycloheptaamylose	7	1,135	1.85	162.5 ± 0.5	≃7.0[b]	≃7.0[b]
cyclooctaamylose	8	1,297	23.2	177.4 ± 0.5	≃8.5[b]	≃7.0[b]

[a]From x-ray analysis
[b]Estimated from Courtald molecular model

cyclohepta- and cyclooctaamylose were only slightly hydrolyzable. Later Abdullah et al. (1969) found that pancreatic α-amylase degraded cyclooctaamylose by the multiple attack mechanism. Mammalian α-amylases appeared to be unable to effectively hydrolyze the cyclohexa- and cycloheptaamyloses (French, 1957; Mora et al., 1974). However it was known that certain fungal and bacterial amylases could effectively break open the cyclic structures (French, 1957; Svetsugu et al., 1974). A thorough investigation of the action of Taka-amylase A (E. C. 3.2.1.1.) indicated that the fungal enzyme could degrade cyclodextrins by the multiple attack mechanism (Svetsugu et al., 1974).

Separation and Detection

The preparative separation of cycloamyloses from each other and from other oligosaccharides has been done by using various chromatographic methods. Cyclohexa- and cycloheptaamylose were shown to be easily separable on charcoal columns (Lammers, 1969). Morerecently gel filtration at high temperature has proven to be an adequate means of separating cyclodextrins from each other as well as from other non-cyclic oligosaccharides (Heyraud and Rinaudo, 1978; Zsadon et al., 1978). Heyraud and Rinaudo (1978) used a refractometer to monitor the appearance of the sugars from column effluents. Separation of cycloamyloses for analytical purposes has been done using thin layer chromatography and more recently by high pressure liquid chromatography (Wiedenhof, 1964; Hokse, 1980).

The specific detection of cyclodextrins has mainly been based on the optical properties (Table 1) and on the ability of the cyclo-

dextrins to form inclusion complexes with certain fluorescent dyes. A polarimeter has been used successfully to detect varying amounts of one type of cycloamylose at a time (Mora et al., 1974; Zsadon et al., 1978). Flow-through polarimeters have been used, like refractometers, to monitor cycloamyloses in column effluents (Zsadon et al., 1978). Kondo et al. (1976) have used 2-p-toluidinylnaphthalene-6-sulphonate (TNS) to analyze mixtures of cyclohexa- and cycloheptaamylose. The fluorescent dye showed a different increase in fluorescence with the same level of each cycloamylose. Kondo et al. (1976) have also been able to accurately assess the concentration of cycloamylose in the presence of starch by pre-hydrolyzing the starch with glucoamylose (E. C. 3.2.1.3.). The main disadvantage of both the optical and fluorescent method is that they are not very sensitive to low concentrations of cyclodextrin.

Compounds are routinely prepared in radioactive form to allow their detection at low concentrations. Andersen et al. (1963) prepared ^{14}C-cycloamyloses by feeding developing potatoes with $^{14}CO_2$. The ^{14}C-starch was isolated and treated with Bacillus macerans amylase to form ^{14}C-cycloamyloses. The ^{14}C-amylose of choice then had to be fractionated from the mixture. The method of preparation was obviously quite involved. Thoma et al. (1963a) have used the method successfully to prepare ^{14}C-cyclohexaamylose for use in equilibrium dialysis experiments with β-amylase.

Cycloamyloses As Substrate Analogues

The ring structure of cyclohexa- and cycloheptaamylose has been found to conform closely to the helical structure of the amylose chain (Thoma and Koshland, 1960; Mora et al., 1974; Simon et al., 1974). Many starch synthesizing and degrading enzymes have been found to possess a strong affinity for these cycloamyloses but were incapable of hydrolyzing them. The cyclic structure which lacked a reducing end group proved ideal because there was no interference with subsequent analysis of reducing activity when enzymes were exposed to substrates in the presence of the cycloamylose. These major attributes have rendered cyclohexa- and cycloheptaamylose as excellent substrate analogues.

Interaction of Cycloamyloses With Phosphorylases

Green and Stumpf (1942) initially found that cycloamyloses were capable of competitively inhibiting the synthetic activity of potato phosphorylase. Much later a more detailed study revealed that the inhibitor constants for potato phosphorylase were 1.6, 2.9 and 9.8 x 10^{-5} M for cyclohexa-, cyclohepta- and cyclooctaamylose respectively (Staerk and Schlenk, 1967). On the contrary Smith (1971) found a very mild non-competitive inhibiton of rabbit muscle phosphorylase by cycloheptaamylose which was less than 15% regardless of substrate concentration. The failure of the cyclic dextrin to compete with the substrate for the active site in the mammalian enzyme contrasted with the strong inhibiton observed at the active site in the case of the plant enzyme. Fletterick et al. (1976) have used x-ray diffraction

to analyze enzymatically active crystals of rabbit muscle phosphorylase to demonstrate that a ligand binding site exists about 25 Angstroms from the active site. Maltoheptose, a linear chain of seven $\alpha(1\rightarrow4)$ linked glucose molecules, was used as a glycogen analogue. Extremely high concentrations of maltoheptose did not cause the 7-membered chain to bind at the catalytic site indicating the existence of a separate binding site. The binding site is believed to allow phosphorylase to remain harnessed to a glycogen particle during degadation of the glycogen (Fletterick and Madsen, 1977).

More recently Shimomura and Fukui (1980) have reconfirmed the existence of a non-catalytic site on rabbit muscle phosphorylase by using affinity electrophoresis of chemically modified phosphorylase. This time cycloheptaamylose was used as a substrate analogue and once again binding was distant from the active site. Experiments with modified potato phosphorylase showed that cycloheptaamylose bound much closer to the active site (Shimomura and Fukui, 1980).

Interaction of Cycloamyloses With β-Amylase

Cycloamyloses have also been used to obtain important kinetic insights into the action of β-amylase and the results provided strong evidence for the "Induced-fit Theory" (Thoma and Koshland, 1960). The theory suggested that some enzymes were capable of altering their conformation to accomodate the substrate in question. Thoma and Koshland studied the inhibition of sweet potato β-amylase by cyclohexa- and cycloheptaamylose and found inhibitor constants of 5.00×10^{-4} and 4.80×10^{-4} M respectively. The Dixon (1953) method of analysis revealed that the inhibition was competitive. Results of equilibrium

dialysis experiments with ^{14}C-cyclohexaamylose showed a slightly higher affinity as depicted by the dissociation constant (K_d) of 1.7×10^{-4} M (Thoma et al., 1963a). One molecule of cyclohexaamylose was bound per subunit of β-amylase. Marshall (1973) later questioned the early findings with β-amylase and found that cyclohexaamylose was a much more potent inhibitor of sweet potato β-amylase than cycloheptaamylose. Inhibitor constants of 3.10×10^{-4} and 2.0×10^{-3} M were found for cyclohexa- and cycloheptaamylose respectively showing that cyclohexa-amylose was more than 6 times as inhibitory as cycloheptaamylose. The two contrasting reports may be due to different substrates. Thoma and Koshland (1960) used a 24-unit amylopectin while Marshall (1973) used amylopectin as a substrate. Nevertheless Marshall has argued that the "Induced-fit Theory" did not apply to β-amylase and stated that ring distortion of cycloamyloses was taking place instead. In response to Marshall's criticisms Thoma stated that "the arguments were not soundly based on enzyme kinetics and were not convincingly supported by Marshall's own data". Thoma (1974) further indicated that the entire problem had to be re-evaluated since the Dixon (1953) method used by both investigators had been shown to have its limi-tations (Purich and Fromm, 1972). Whatever the final outcome of this controversy the lower affinity of sweet potato β-amylase for cyclo-heptaamylose, reported by Marshall(1973), does support the findings of Silvanovich and Hill (1976) where cereal β-amylase appeared to pass through the cycloheptaamylose affinity column unretarded.

Interaction of Cycloamyloses With α-Amylases

Studies regarding α-amylases were found to be even more puzzling. Table 2 shows inhibitor and dissociation constants obtained for various α-amylases in their interaction with cycloamyloses. The data are based on kinetic and physical methods.

Thoma et al. (1963b) found that 8.0×10^{-3} M cyclohexaamylose did not detectably inhibit pancreatic α-amylase. In contrast Hoschke et al. (1976) found that pancreatic α-amylase could be inhibited by cyclohexaamylose with a $K_i = 10^{-4}$ M. The findings of Hoschke et al.(1976) were in close agreement with those of Mora et al. (1974) where a K_i of 2.00×10^{-4} M was reported for cycloheptaamylose in the inhibition of pancreatic α-amylase. Dissociation constants determined by other methods were in close agreement with the kinetic data of Mora et al. (1974), (Table 2).

Ohnishi (1971) has also reported that Bacillus subtilis α-amylase was competitively inhibited by cycloheptaamylose, but very weakly, with a $K_i = 1.8 \times 10^{-2}$ M. Ohnishi et al. (1973) have further shown that the binding of cycloheptaamylose to the bacterial α-amylase involves nearly 3 subsites. An exposed tryptophyl residue was believed to be associated with the subsites because the interaction of the enzyme with cycloheptaamylose caused a change in adsorption at 393 nm. Mora et al. (1974) have implied that a tryptophyl residue was also involved in the binding of cycloheptaamylose to pancreatic α-amylase.

Pancreatic α-amylase was found to bind 3 moles of cycloheptaamylose specifically, however, there was an apparent non-specfic binding at

TABLE 2. Inhibitor and dissociation constants for the interaction of α-amylases with cycloamyloses

α-Amylase	Cycloamylose	Constant (M)	Method	References
pancreatic	cyclohexaamylose	no inhibition	kinetic	Thoma et al. (1963b)
pancreatic	cyclohexaamylose	10^{-4} (K_i)	kinetic	Hoschke et al. (1976)
pancreatic	cyclohexaamylose	6.3×10^{-4} (K_d)	difference spectroscopy	Mora et al. (1974)
pancreatic	cycloheptaamylose	2.0×10^{-4} (K_i)	kinetic	Mora et al. (1974)
pancreatic	cycloheptaamylose	1.4×10^{-4} (K_d)	ultracentrifugation	Mora et al. (1974)
pancreatic	cycloheptaamylose	2.2×10^{-4} (K_d)	difference spectroscopy	Mora et al. (1974)
Bacillus subtilus	cycloheptaamylose	1.8×10^{-2} (K_i)	kinetic	Ohnishi (1971)

higher concentrations of cycloheptaamylose (Mora et al., 1974). Simon et al. (1974) supported these findings using small angle x-ray scattering techniques. The active site of pancreatic α-amylase was found to be a trough that was capable of accomodating 3 cyclohepta-amylose molecules. It was also suggested that the helical substrate, amylose, could bind in this trough, too.

Evidence concerning the nature and strength of the interaction of cereal α-amylase with cycloamyloses is sparse. However, as discussed previously, an effective affinity method to purify cereal α-amylase using immobilized cycloheptaamylose has been developed by Silvanovich and Hill (1976). It was also recently reported that both cyclohexa- and cycloheptaamylose inhibited the germination of barley seeds (Szejtli et al., 1980). Barley seeds germinated in the presence of 10^{-2} M cyclohexaamylose showed 79.4% inhibition of shoot growth and 66.4% inhibiton of root growth after 2 days. The investigators have implicated the possible inhibiton of α-amylase by cyclohexa- and cycloheptaamylose eventually in combination with linear dextrins.

MATERIALS AND METHODS

Biological Material

Triticale 6A190 (X Triticosecale Wittmack) and wheat (Triticum aestivum Neepawa) were used. Germinated and freeze dried triticale was prepared as described by Silvanovich and Hill (1976). The wheat was germinated for 3 to 4 days at 21°C, freeze dried and stored at 4°C.

Determination of α-Amylase Activity

Activity based on the disappearance of substrate was determined by the method of Briggs (1961) with the exception that β-amylase was not incorporated into the assay medium. The β-limit dextrin of waxy maize starch was used as a substrate at a concentration of 0.075%. An IDC unit is defined as the amount of enzyme required to change the absorbance of an iodine complexed-β-limit dextrin solution from 0.6 to 0.4 in 100 minutes. Absorbance measurements were done at 540 nm using a Zeiss PM QII spectrophotometer. All other absorbance measurements were done with the same instrument. At all times the degree of hydrolysis was kept such that the normalized values for optical density never fell below 0.4 absorbance. The assay was conducted at 35°C in 0.05 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$).

An appearance of product assay was used to establish initial velocity The velocity determinations were done by measuring the appearance of

reducing activity from a 0.5% solution of gelatinized waxy maize starch at 30°C in 0.05 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$). Reducing activity was determined using the Nelson (1944) adaptation of the Somogyi method for the determination of glucose using reagents recommended by Robyt and Whelan (1968). This system was also used to assay β-amylase.

The concentration of protein in solution was determined by the method of Lowry et al. (1951).

Total carbohydrate was determined by the phenol-sulphuric acid method of Dubois et al. (1956) and was used to accurately assess substrate concentrations as well as the carbohydrate content of enzyme preparations.

Preparation and Characterization of Affinity Gel

Preparation of Cycloheptaamylose-epoxy-Sepharose 6B

The affinity gel was prepared according to the method of Silvanovich and Hill (1976) which was adapted from the method of Vretblad (1974a) for immobilizing cyclohexaamylose.

Six grams of lyophilized epoxy-activated Sepharose 6B (Pharmacia Chemical Company) was swollen in 100 ml of distilled water overnight. The swollen gel was then washed on a glass sintered funnel for a period of one hour using gentle suction. The gel was then washed with 100 ml of 0.1 N NaOH and excess liquid removed by suction. The gel was transferred to a 150 ml erlenmeyer along with 500 mg of cycloheptaamylose (Sigma Chemical Company) in 20 ml of 0.1 N NaOH. The flask was sealed

and placed in a shaker bath at 45°C for 19.5 hours. The reacted gel was then cooled down to room temperature and washed on a glass sintered funnel with about 400 ml of 25 mg/ml glucose followed by 400 ml of distilled water. The gel was then equilibrated with one liter of 0.02 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$). The gel was stored in 0.02% sodium azide in the presence of equilibrium buffer at 4°C when not in use.

Determination of Degree of Substitution

Five hundred milligrams of epoxy-activated Sepharose 6B was reacted with ^{3}H-cycloheptaamylose by a scaled down procedure of the above method to prepare the affinity gel. Two x 0.1 ml portions of sedimented ^{3}H-cycloheptaamylose-epoxy-Sepharose 6B were then assayed for radioactivity using a Searle Analytical Mark II Liquid Scintillation spectrometer. Aqueous samples were prepared for counting by mixing with 3 ml of Aquassure (New England Nuclear Corporation). Determination of radioactivity in other experiments was conducted in a similar fashion.

Procedures For Isolation of Cereal α-Amylases

Extraction, Polyvinylpolypyrrolidone Treatment and Dialysis

The entire purification of α-amylase was carried out at 0-4°C. Enzyme activity and protein were monitored throughout the preparation. Germinated and freeze dried kernels of either wheat or triticale were ground to flour in a microanalytical mill. Thirty grams of the ground material was extracted with 0.2 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$). The extracting medium was stirred magnetically for one hour

and centrifuged at 10,000 x g for 20 minutes.The pellet was discarded. Ten milligrams of insoluble polyvinylpyrrolidone (PVP) (Sigma Chemical Company) was introduced for every milliliter of supernatant. The crude extract was stirred in the presence of insoluble PVP for 30 minutes. The insoluble PVP was then filtered out with glass wool and washed with an additional 10 ml of extraction buffer to retrieve residual enzyme. The wash was then combined with the major portion of the treated extract. Absorbance measurements at 280 nm were taken before and after insoluble PVP treatment. The extract was then dialyzed overnight against 8 liters of 0.02 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$). After dialysis the extract was centrifuged at 10,000 x g for 20 minutes to remove any protein that precipitated as a result of the decrease in ionic strength.

The dialysis procedure was based on a solubility study where 3 ml aliquots of triticale crude extract were dialyzed against various concentrations of buffer ranging from 0.005 to 0.2 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$). After 16 hours the extracts were removed from dialysis and centrifuged at 10,000 x g for 20 minutes.

Affinity Chromatography

Preparative Affinity Chromatography. A peristaltic pump, set to deliver 10 ml/hr, was used to apply the low ionic strength supernatant to a 1.6 cm x 7.6 cm column of cycloheptaamylose-epoxy-Sepharose 6B pre-equilibrated with 0.02 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$). After application of the extract, the column was washed with approximately 6 bed volumes of equilibrating buffer. The column was then

eluted with 30 ml of 0.3 M NaCl in equilibrating buffer to remove any protein bound through ionic interactions. A 30 ml wash of equilibrating buffer followed. The α-amylase was then eluted with 8 mg/ml of cycloheptaamylose in the moving phase. The fractions were analyzed for absorbance at 280 nm and assayed for enzyme activity by the Briggs (1961) assay. The enzyme peak was pooled immediately after assaying to prevent formation of an insoluble precipitate. The cycloheptaamylose was removed from the enzyme by gel filtration on a 2.5 cm x 32 cm column of BioGel P-4 (200-400 mesh) pre-equilibrated with 0.05 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$). The enzyme peak was pooled. In preparations where wheat extracts were used the affinity column was cleaned with 6 M urea intermittently and equilibrated with several bed volumes of buffer before reuse.

Elution With Glucose and Glucose Oligomers. The following substances were tested for their effectiveness in releasing α-amylase from the affinity gel: 44 mM glucose, 22 mM maltose, 16 mM maltotriose, 8 mg/ml gelatinized glycogen and 8 mg/ml β-limit dextrin. Glucose, maltose and maltotriose were obtained from the Sigma Chemical Company. Glycogen was obtained from the Fisher Scientific Company and β-limit dextrin was prepared from gelatinized wɞxy maize starch.

Columns of 0.5 cm x 4 cm were filled with one milliliter portions of sedimented affinity gel. Each column was equilibrated with 0.02 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$) at 4°C. Fifty microliters of purified triticale α-amylase (α-I and α-II) containing 2500 IDC units was applied to each column. A 3 ml wash with equilibrating buffer followed. The effluents were collected in test tubes

and analyzed for unbound activity. Three milliliters of the appropriate glucose oligomer was then applied to each column and the effluents were collected in another series of test tubes. The percent release of total activity applied was determined in each case.

Another experiment was designed on a larger scale to test for preferential release of isozymes by β-limit dextrin. Triticale α-amylase purified by the ethanol-glycogen precipitation method (Silvanovich and Hill, 1976) was applied to a 1.6 cm x 7.6 cm column of affinity gel and then eluted at 10 ml/hr with 7.5 mg/ml of β-limit dextrin. Five milliliter fractions were collected which were monitored for absorbance at 280 nm. The fractions were then analyzed for their isozyme content by isoelectricfocusing.

Determination of β-Amylase Behavior On Affinity Gel. A 1 ml column of cycloheptaamylose-epoxy-Sepharose 6B was tested for its ability to retain sweet potato β-amylase (Sigma Chemical Company) under the same conditions used to process α-amylase. One milliliter of sweet potato β-amylase with an activity of 3.6 μmoles/min/ml was applied to the affinity column followed by 3 ml of equilibrating buffer. The unbound material was collected in a test tube and assayed for enzyme activity. A similar study was conducted using purified barley β-amylase provided by Dr. A. W. MacGregor of the Canadian Grain Commission.

Separation of α-I and α-II Isozyme Groups

The α-I group was prepared by passing the enzyme, eluted from the affinity column, onto a 2.5 cm x 26 cm column of carboxy-methyl (CM) cellulose (Whatman CM-32) equilibrated with 0.02 M sodium acetate

(pH 5.5, 0.001 M $CaCl_2$). An upward flow of 20 ml/hr was used. Approximately 2,500,000 IDC units were applied to the column. Five milliliter fractions were collected and the absorbance of each fraction was determined at 280 nm. The unretained fractions were pooled and assayed for enzyme activity and protein. The pooled enzyme was concentrated to a small volume using a 50 ml Amicon concentrator cell equipped with a YM 10 Membrane. One mg/ml of BSA was incorporated and the enzyme was then frozen in one ml aliquots for future use.

Alpha-II isozymes were prepared by fractionating purified wheat α-amylase (obtained from the affinity step) on a 0.5 cm x 10 cm column of N, N-diethylamino-ethyl (DEAE) cellulose (Whatman DE-32) equilibrated with 0.05 M imidazole-Cl (pH 7.4, 0.001 M $CaCl_2$). A volume of 0.6 ml containing 72,000 IDC units of isozyme mixture was applied to the column. The enzyme was eluted from the column with a linear gradient consisting of 20 ml eqilibration buffer versus 20 ml equilibration buffer with 0.2 M NaCl. Fractions of 1.5 ml were collected and the absorbance of each fraction was determined at 280 nm. The elution profile was divided into 4 major fractions each of which were pooled. The pooled fractions were dialyzed against 0.05 M sodium acetate (pH 5.5, 0.001 M$CaCl_2$). One mg/ml of BSA was incorporated into each fraction after dialysis. The fractions were then frozen for future analysis of activity and isozyme content.

Electrophoretic Methods

Sodium Dodecylsuphate Electrophoresis

Electrophoresis was performed according to the method of Weber and Osborne (1969) in 10% polyacrylamide gels. Samples were disrupted in the presence of 1% sodium dodecylsulphate (SDS) and 1% β-mercaptoethanol by boiling for 10 minutes. The sample gel contained a 30 µg load of purified α-amylase from the affinity step. The reference gel contained 10 µg portions of the following proteins: bovine serum albumin, ovalbumin and α-chymotrypsinogen A (Sigma Chemical Company).

Isoelectricfocusing

Flat-bed isoelectricfocusing in polyacrylamide gel was performed on triticale α-amylase from the affinity step using a pH gradient of 4 to 11. Alpha-amylase bands were detected comparatively by starch and β-limit dextrin zymograms by a method adapted from Doane (1967). After the zymograms were prepared the gel was stained non-specifically for protein with Coomassie blue R-250.

The crude extract of triticale α-amylase and the unbound fraction of the affinity column were separated by isoelectricfocusing in a pH gradient of 4 to 8 according to the method of MacGregor (1976). The current was maintained at 14 milliamps till the voltage reached 600 volts. The run was terminated when the current stabilized at 5 milliamps. The duration of the run was about 3 hours. Amylase bands were detected using starch zymograms.

All other isoelectricfocusing runs were conducted using commer-

cially available pre-cast gels (Fisher Scientific Company) with ampholytes ranging from 3.5 to 9.5. Focusing was carried out for 1.5 hours using a constant power of 30 watts and an initial current of 30 milliamps. The runs were terminated when the current stopped dropping. This system was used to monitor the preparative separation of α-I and α-II isozyme groups as well as the types of isozymes that were eluted from the affinity column by β-limit dextrin. The enzyme was applied in a volume of about 20 µl usually containing about 500 IDC units. The samples were applied on adsorptive pads near the cathode. Amylase bands were detected using starch zymograms. Reaction times to prepare the zymograms ranged from 15 to 30 minutes at room temperature depending on the amount of enzyme that was present.

Inhibition Studies With Gelatinized Starch

Inhibition studies using gelatinized waxy maize starch were done with triticale α-amylase from the affinity step, wheat α-I isozymes, wheat α-II isozymes and barley β-amylase. The barley enzyme was a gift from Dr. A. W. MacGregor. The assays were done at 30°C in the presence of 0.05 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$). The substrate concentrations ranged from 0.015% to 0.07%. Cycloheptaamylose concentrations of up to 10 mM were used. The release of reducing activity was monitored according to the method previously described for assaying α-amylase by the appearance of product. All reactions were terminated after 10 minutes or less. Alpha-amylase activities, determined under saturating conditions, ranged from 0.02 to 0.12 µmoles/min/ml.

Beta-amylase activity was 0.138 μmoles glucose/min/ml when determined under saturating substrate conditions. The data were plotted according to the method of Dixon (1953) for the analysis of competitive inhibition. The least squares fit method was used to draw a line through data points that showed an apparent slope.

Inhibition Studies With Starch Granules

Procedure For Studying Inhibition of Binding

Isolated cereal α-amylases, previously frozen in the presence of BSA, were thawed for use. Similar enzyme activities were mixed with 25 mg of starch granules in 5 ml of 0.05 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$) in separate test tubes containing concentrations of cycloheptaamylose ranging from 0 to 10 mM. All operations were carried out at 0-4°C to minimize hydrolysis. The test tubes were sealed and their contents tumbled slowly for 30 minutes at 4°C on a rotary shaker to allow for optimum enzyme adsorption (MacGregor, 1979). The tubes were then centrifuged at 2000 x g for 5 minutes and the supernatants asssayed by the method of Briggs (1961) or by the appearance of reducing sugar. The amount of enzyme bound was determined from the difference between the amount of enzyme added and the amount that was present in the supernatant. The percent of enzyme remaining bound was plotted as a function of the cycloheptaamylose concentration.

Three major studies were conducted. Triticale α-amylase from the affinity step (α-I and α-II), containing 645 IDC units, was introduced into each tube of a series with waxy maize starch granules.

The systems with wheat α-I isozymes (510 IDC units) and wheat α-II isozymes (585 IDC units) were set up with wheat starch granules.

Waxy maize starch granules (amioca pearl starch) were obtained from American Maize Products. Wheat starch granules were obtained from the British Drug House and contained 0.19% protein and 10.8% moisture.

Procedure For Studying Inhibition of Granule Hydrolysis

The hydrolysis experiments were done at 35°C and 15°C. The enzymes and starch used were from the same batches used in the desorption experiments. In the 35°C experiment 50 mg of waxy maize starch granules were suspended in 10 ml of 0.05 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$) containing 1 mg/ml of BSA. An identical system was prepared that contained 1 mg/ml of cycloheptaamylose by weight. The tubes were then warmed to 35°C in a water bath and a small volume of triticale α-amylase mixture containing 3300 IDC units was added. The tubes were mixed and a 1 ml aliquot was removed while mixing. The reaction was stopped by adding 0.02 ml of 5 N HCl. The tubes were sealed and gently rotated in a 35°C water bath. At intervals, 1 ml aliquots were taken while gently mixing the suspension. The aliquot was mixed with 0.02 ml of 5 N HCl to stop the reaction. Ater enzyme deactivation the granules were spun down using a Beckman Microfuge. The supernatants were retained at 4°C for analysis of soluble carbohydrate released.

Similar experiments were conducted at 15°C by studying wheat α-I and α-II isozyme groups independently in the hydrolysis of wheat starch granules. An enzyme activity of 2300 IDC units was used in each study.

A control system without enzyme was used to check for spontaneous release of soluble carboydrate. The extent of hydrolysis at each time interval was then corrected for spontaneous release of carbohydrate. Reducing activity released in the soluble fractions was also determined. The 5 hour supernatants were also analyzed for products of hydrolysis by thin layer chromatography on Kieselguhr G using the method of Weill and Hanke (1962). Ten microgram loads were applied to 20 cm x 20 cm plates of Kieselguhr G and eluted 10 cm past the point of application with n-butanol : ethanol : water (5: 3 : 2). Five μg each of glucose, maltose and maltotriose were also chromatographed in separate lanes. After drying the plates were developed using the detection method of Stahl and Kaltenbach (1961).

Preparation of ^{3}H-Cycloheptaamylose

Tritiated cycloheptaamylose was prepared from a commercial preparation of non-radioactive cycloheptaamylose (Sigma Chemical Company). The procedure was based on the oxidation of the carbon-6 of the glucose monomers using chromic oxide (Harding et al., 1975).

The oxidizing solution was prepared by dissolving 2.7 gms of chromic oxide in 2.3 ml of concentrated sulphuric acid followed by dilution to 10 ml with distilled water. One hundred milligrams of cycloheptaamylose was dissolved in 10 ml of distilled water. The carbohydrate level when re-evaluated by the phenol-sulphuric acid method (Dubois et al., 1956) was 93 mg of glucose. The oxidizing solution (0.15 ml) was added to the cycloheptaamylose solution and the mixture was left at room temperature for 18 hours. Excess $BaCO_3$ was then added to

precipitate sulphate, chromate and any organic acid formed during the reaction. Further deionization was carried out after barium carbonate precipitation by passing the supernatant through three columns of mixed bed ion-exchanger containing approximately 5 ml each of Rexyn 300 (Fisher Chemical Company) with a wet exchange capacity of 0.82 meq/ml. Reducing activity of the column effluent was estimated by the Nelson (1944) adaptation of the Somogyi method for the determination of glucose using reagents recommended by Robyt and Whelan (1968).

The partially oxidized carbohydrate (15 ml) was reduced with tritiated sodium borohydride according to a modification of the method of McLean et al. (1973). Tritiated sodium borohydride (New England Nuclear) was added to a solution containing 26 mg of non-radioactive sodium borohydride, prepared in 5 ml of 0.08 N KOH, to give a final specific activity of 1.27×10^7 dpm/μmole. The radioactive sodium borohydride was added gradually to the oxidized cycloheptaamylose. The reaction was carried out in a fume hood. After 18 hours the solution was treated with 2.5 ml of 0.6 N HCl to terminate the reaction. The slightly acidic solution was then adjusted to neutrality with 0.1 N KOH. The neutral solution was evaporated to dryness using a rotary evaporator. The dried material was dissolved in 30 ml of distilled water and evaporated to dryness a second time. The material was dissolved in 5.75 ml of distilled water and applied to a 2.5 cm x 28 cm column of BioGel P-4 (200-400 mesh) equilibrated with distilled water. An upward flow of 20 ml/hr was used to elute the column and 6 ml fractions were collected. The fractions were assayed for radioactivity and carbohydrate. The radioactive carbohydrate peak was pooled and

lyophilized for storage.

Radiochemical purity was assessed by thin layer chromatography on Silica gel G based on a method by Wiendenhof (1964) for separating cyclohexaamylose, cycloheptaamylose, glucose and maltose. Radioactive cycloheptaamylose, standard cyclohexa- and cycloheptaamylose were spotted on Silica gel G plates of 20 cm x 20 cm (Fisher Scientific Company) using 5 to 10 µg loads. The plate was first eluted with n-butanol : glacial acetic acid : water (6 : 3 : 1). After the solvent front had migrated 12.5 cm beyond the origin, the plate was removed and dried. The plate was eluted a second time in the same direction using n-butanol : glacial acetic acid : water : pyridine : dimethyl-formamide (6 : 3 : 1 : 2 : 4) to a point 6 cm past the origin. The plates were dried and developed with iodine vapor (Greenway et al., 1953). The lane containing the radioactive cycloheptaamylose was collected in 1 cm sections, suspended in a gel prepared from Aquassure and water, and assayed for radioactivity. Recovery of radioactivity was greater than 95 percent. In another experiment designed to check for contamination by linear dextrins only the first solvent system was used. The plate was then dried and the same solvent system used a second time in the same direction. Radioactive cycloheptaamylose was compared to radioactive cycloheptaamylose that had been exposed to sweet potato β-amylase (Sigma Chemical Company). Both maltose and maltotriose were used as standards in separate lanes and were visualized using the detection reagent of Stahl and Kaltenbach (1961) which contained 9 ml 95% ethanol, 0.5 ml of anisaldehyde and a few drops of glacial acetic acid. The plate was developed by placing it in an oven at 100°C for 30 minutes.

Binding Analysis Procedures

Test For Reversibility of Interaction

Purified wheat α-amylase containing both isozyme groups (3.5 mg) was applied to the preparative affinity column and partially eluted with 15 ml of ^{3}H-cycloheptaamylose which had a specific activity of 1.32×10^{5} dpm/mg. The eluted enzyme was pooled (12 ml) and applied to a 2.5 cm x 28 cm column of BioGel P-4 (200-400 mesh) which was equilibrated with 0.05 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$). Fractions of 6 ml were collected and the protein detected by absorbance at 280 nm. Radioactivity was determined throughout the elution and carbohydrate was analyzed in the region where cycloheptaamylose would be expected to appear.

Equilibrium Binding Studies

Binding studies were conducted at 4^{o}C in the presence of 0.05 M sodium acetate (pH 5.5, 0.001 M $CaCl_2$) using equilibrium dialysis cells with two compartments of 1 ml. The compartments were separated by a 10,000 molecular weight cut-off dialysis membrane. Experiments were done with α-amylase purified from both triticale and wheat. Freshly purified enzyme from the affinity step was used after processing on BioGel P-4 to remove cycloheptaamylose. The enzyme was then concentrated to about 1 mg/ml in an Amicon concentrator cell. A molecular weight of 41,000 was assumed since α-II was the major component of the total enzyme in the isozyme mixture (Silvanovich and Hill, 1977). In each experiment a fixed concentration of enzyme was dialyzed against

at least 6 different concentrations of ^{3}H-cycloheptaamylose. In one experiment the protein concentration was 8.3 μM and ^{3}H-cycloheptaamylose ranged from 1.2 to 31.4 μM. In the other experiments the protein concentration was about 20 μM and ^{3}H-cycloheptaamylose ranged from 5 to 280 μM. Two batches of ^{3}H-cycloheptaamylose were used which had specific activities of 7.84×10^{5} and 1.08×10^{6} dpm/μmole. The dialysis cells were never completely filled so as to allow optimum movement of the ligand through the membrane during equilibrium dialysis. A control cell with ^{3}H-cycloheptaamylose, originally introduced into one side of the dialysis unit, was used to determine when equilibrium was established. The cells were placed on a horizontal shaker at 4^{o}C in a cold room. Both compartments of the control cell had equal radioactivity within 66 to 110 hours. The time to reach equilibrium appeared to be highly dependent on the volume in the cells and the degree of shaking.

After equilibrium was reached duplicate samples of each compartment were assayed for radioactivity. Protein concentration was re-estimated because minor volume changes were found to occur in the compartments after dialysis. The concentration of cycloheptaamylose bound was determined from the difference between the ligand concentration in the protein chamber and the free ligand concentration (c) in the protein-free chamber. A "best fit" to the Scatchard (1949) plot was obtained by using the General Linear Models Procedure from the Statistical Analysis System (Helwig and Council, 1979).

RESULTS AND DISCUSSION

Enzyme Purification

Purification From Crude Extracts

In the past cereal α-amylase has been purified from crude extracts using a combination of involved procedures (Kruger and Tkachuk, 1969; MacGregor et al., 1974; Warchalewski and Tkachuk, 1978). The use of cyclopheptaamylose-epoxy-Sepharose 6B as an affinity gel for retaining cereal α-amylase has greatly reduced the purification task involved in obtaining homogeneous α-amylase (Silvanovich and Hill, 1976 ; 1977). The task of purification has now been even more abbreviated by the elimination of the ethanol-glycogen precipitation step (Schramm and Loyter, 1966; Silvanovich and Hill, 1976).

Polyvinylpyrrolidone is frequently used in preparative plant enzymology because of its ability to complex deleterious phenolic compounds (Loomis, 1974). Crude extracts treated with insoluble PVP prevented the affinity column from discoloring with time thus reducing the necessity for using 6 M urea to clean the gel each time a new preparation was initiated. Extracts pre-treated with insoluble PVP absorbed 14% less at 280 nm than they did prior to treatment indicating some component(s) was being removed by the PVP. In the past oxidation of phenolics have been implicated with regard to affinity gel discoloration (Tkachuk, 1975; Silvanovich and Hill, 1976).

Low ionic strength dialysis followed by centrifugation resulted in a supernatant that retained the enzyme activity of the previous step (Table 3). More than one-half of the protein was in the pellet without apparent co-precipitation of enzyme. However the low ionic strength step has only been used with complete success with triticale extracts. Wheat extracts (Table 4) appeared to contain full activity upon centrifuging the low ionic strength material but within a short time turbidity appeared in the extract which seemed to be associated with a drop in α-amylase activity. Thus much of the wheat enzyme appeared to be lost through precipitation during application to the affinity column in the next step. On the otherhand low ionic strength triticale extracts maintained their clarity for over a week at $4^{o}C$ indicating that the composition of the two kernel extracts must have had distinct differences at this stage of purification. It therefore appears that the dialysis step as such was of limited usefulness in preparing wheat α-amylase. After the affinity step less than 38% of the wheat enzyme was recovered in pure form (Table 4). Perhaps by dialyzing against a slightly higher ionic strength, a more stable extract could be obtained.

The dialyzing step was based on pilot studies conducted at various sodium acetate concentrations (Figure 1). Triticale α-amylase was almost totally recovered from dialysis of crude extracts against buffer concentrations as low as 5 mM. Twenty millimolar sodium acetate was chosenbecause it was apparent that adequate buffering capacity could be maintained in exchange for the removal of a substantial portion of extraneous material.

TABLE 3. Purification of α-amylase from germinated and freeze dried triticale kernels

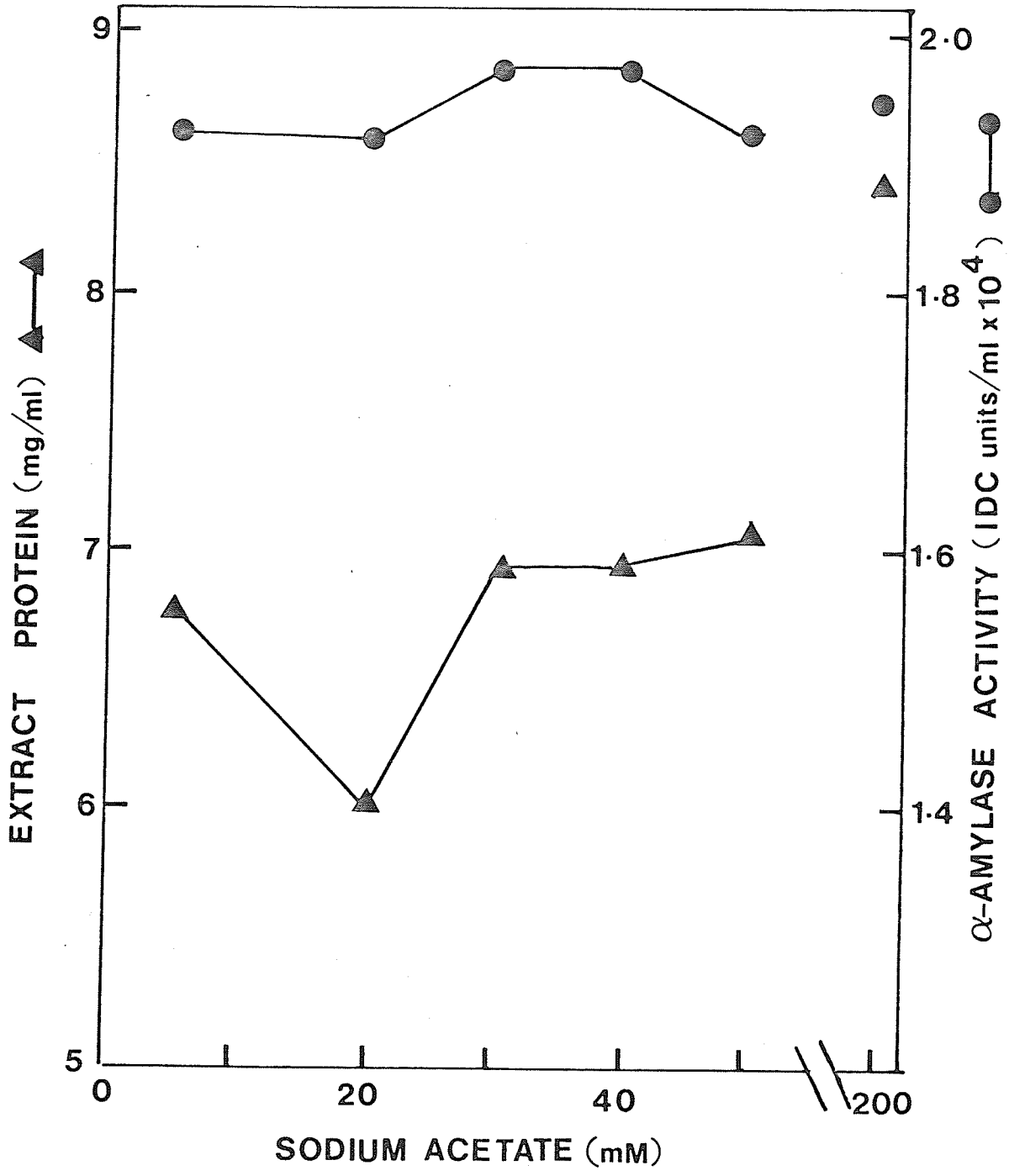
Fraction	Volume (ml)	Protein (mg/ml)	Activity (IDC units/ml)	Specific Activity (units/mg)	Total Activity (units)	Purification Fold	Recovery %
crude extract	74	24	24,800	1,033	1,835,200	1	100
PVP treatment	81.5	23	19,200	835	1,564,800	0.8	85
dialysis step	138	5.6	11,400	2,036	1,573,200	1.97	85
affinity peak	54	0.135	38,500	285,185	2,079,000	276	113
unretained by affinity column	284	n. d.*	1,160	-	329,440	-	5.6

* not determined

TABLE 4. Purification of α-amylase from germinated and freeze dried wheat kernels

Fraction	Volume (ml)	Activity (units/ml)	Total Activity (units)	Recovery %
crude extract	166	40,000	6,640,000	100
PVP treatment	148	39,330	5,821,000	88
dialysis step	200	29,000	5,800,000	87
affinity peak	57	43,614	2,486,000	37.5
unretained by affinity column	405	1,435	581,000	8.8

Figure 1. Effect of dialyzing crude extracts of triticale against sodium acetate buffers of different ionic strengths at pH 5.5 at $4^{o}C$.

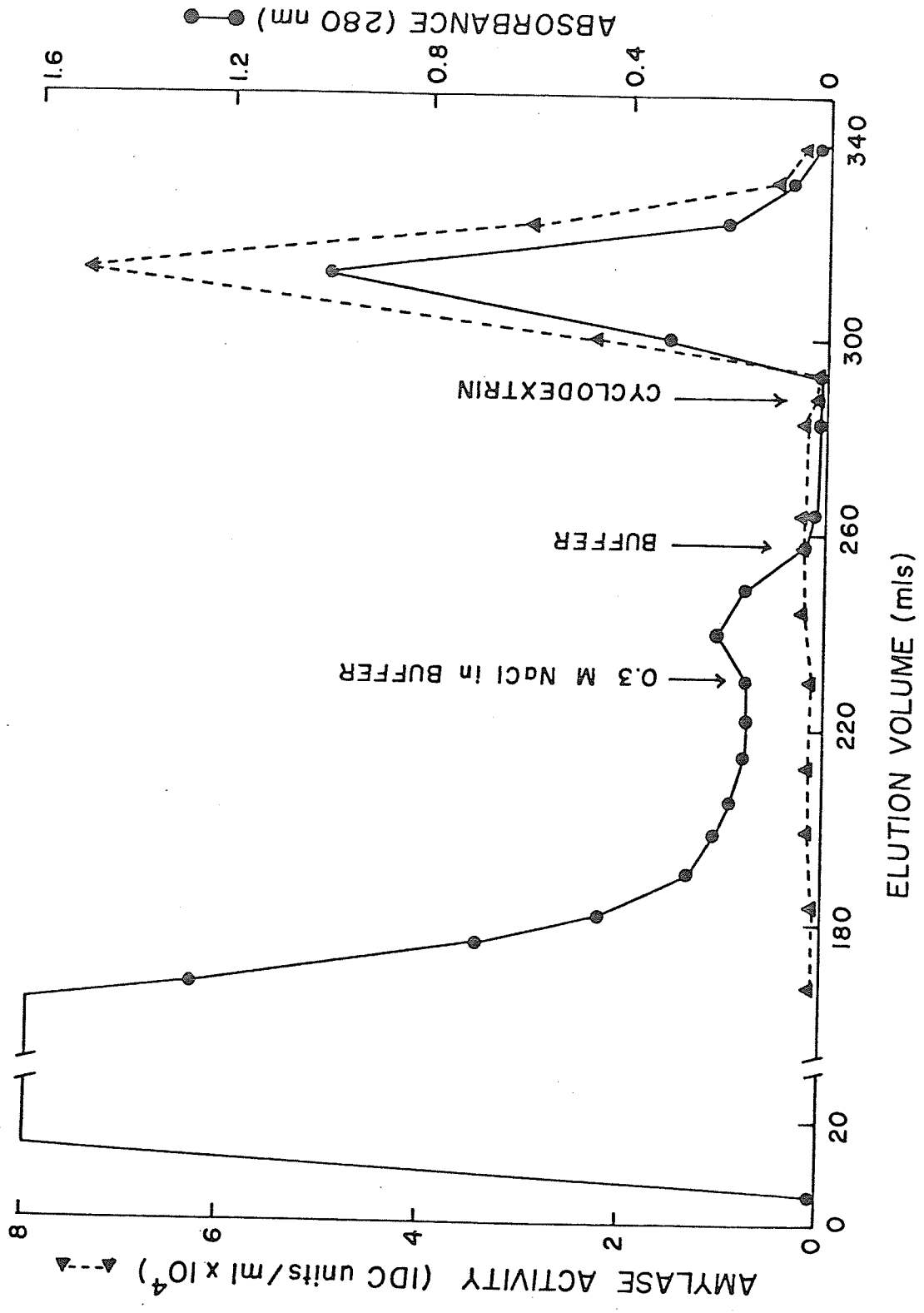

9
8
7
6
5
EXTRACT PROTEIN (mg/ml)
2·0
1·8
1·6
1·4
α-AMYLASE ACTIVITY (IDC units/ml x 10^4)
0
20
40
200
SODIUM ACETATE (mM)

The α-amylase was purified 276 fold over the crude extract. Yields as high as 113% have been found. At this point there is uncertainty as to the reason for yields in excess of 100%. One possibility is that α-amylase inhibitors, other than those studied by Silano et al. (1975), could have been separated from the α-amylase during the affinity step. In the case of wheat (Table 4) the much lower yield was attributed to the precipitation phenomenon which probably would serve to mask any increased yield.

Silvanovich and Hill (1976, 1977) have reported a specific activity of 230,000 IDC units/mg for triticale α-amylases where the solvent step was included. From Table 3 it can be seen that even higher specific activity was obtained by the new method. The triticale enzyme had a specific activity of almost 290,000 IDC units/mg whereas wheat α-amylase had a specific activity of 260,000 IDC units/mg. Protein was only determined on the end product of the wheat enzyme preparations. The similarity in specific activity would be expected since wheat and triticale are genetically related. The specific activities of different preparations fluctuated somewhat but usually were around 250,000 units/mg. From the appearance of reducing activity assay, activities of the enzyme processed on the affinity column ranged from 175 to 212 μmoles glucose/min/mg of protein.

Figure 2 depicts an affinity chromatography profile for the purification of triticale α-amylase. A large amount of material absorbing at 280 nm passed through the column unretarded. The application of 0.3 M NaCl in equilibrating buffer did not cause any significant release of α-amylase but did have an effect in removing slightly retarded

Figure 2. Affinity chromatography of triticale α-amylase.


AMYLASE ACTIVITY (IDC units/ml x 10^4)
ABSORBANCE (280 nm)
ELUTION VOLUME (mls)
0.3 M NaCl in BUFFER
BUFFER
CYCLODEXTRIN
0
2
4
6
8
0
20
180
220
260
300
340
0.4
0.8
1.2
1.6

impurities more quickly from the column. However when the total α-amylase unretained by the affinity column was assayed, it was found that a significant portion of the enzyme passed through the affinity column unretarded by immobilized cycloheptaamylose. The phenomenon was common to both triticale and wheat enzyme preparations. Gibbons (1979a) has reported similar findings during the purification of barley α-amylase on cycloheptaamylose-epoxy-Sepharose 6B. He has attributed the occurrence of unbound enzyme to either a difference in the α-amylase or to an overloading effect. The overloading effect could be answered by reapplying the unbound α-amylase to the affinity column. In the triticale preparation (Table 3) the unbound enzyme amounted to 21% of the total α-amylase that was applied to the affinity column. In the case of the wheat preparation (Table 4) this value was only 10% but was probably much higher since the precipitation phenomenon, discussed earlier, could have had a profound effect on decreasing the level of unbound α-amylase as well.

The highly purified enzyme was obtained when the column was eluted with cycloheptaamylose in the moving phase (Figure 2). If the enzyme was not pooled shortly after elution from the affinity column, the peak tubes became turbid within a few hours. Cycloheptaamylose appeared to cause an irreversible precipitation of the enzyme where high concentrations of both ligand and enzyme were present. However when the enzyme remaining in solution was assayed for activity and protein, specific activities were found to be either the same or higher than enzyme that was freshly eluted from the affinity column. The precipitation appeared to be an "all or none" event that left the α-amylase

in the supernatant in a highly active form. Microscopic observation of the precipitate revealed a mixture of amorphous material and fine needle-like crystals.

Extraneous cycloheptaamylose was effectively removed from the pooled enzyme by chromatography on BioGel P-4 (Figure 3). However the sieved α-amylase preparation still has approximately 5 to 10% carbohydrate by weight that appeared strongly associated with the enzyme. Dr. A. W. MacGregor (personal communication) has observed that freshly purified cereal α-amylases always have carbohydrate associated with them which can only be removed after a number of passes through DEAE-cellulose ion-exchange columns. Studies with radioactive cycoheptaamylose, that will be discussed later, indicated that there was no cycloheptaamylose that was so tightly bound to the enzyme that gel filtration would not remove it.

The theoretical capacity of the affinity column was very high. After reaction with ^{3}H-cycloheptaamylose, 3.6 μmoles of cycloheptaamylose was found to be immobilized per ml of swollen gel. Commercially available epoxy-activated Sepharose 6B used in this synthesis was described as having 15 to 20 μmoles of spacer arm per ml of swollen gel. This indicated that as much as 24% of the available spacer arms were substituted with cycloheptaamylose in the preparation of the affinity gel.

The purified triticale enzyme appeared homogeneous upon electrophoresis in the presence of SDS. The molecular weight of triticale α-amylase when compared to standards was slightly smaller than ovalbumin (45,000) (Figure 4) which was in agreement with the findings of

Figure 3. Separation of cycloheptaamylose from α-amylase on BioGel P-4.

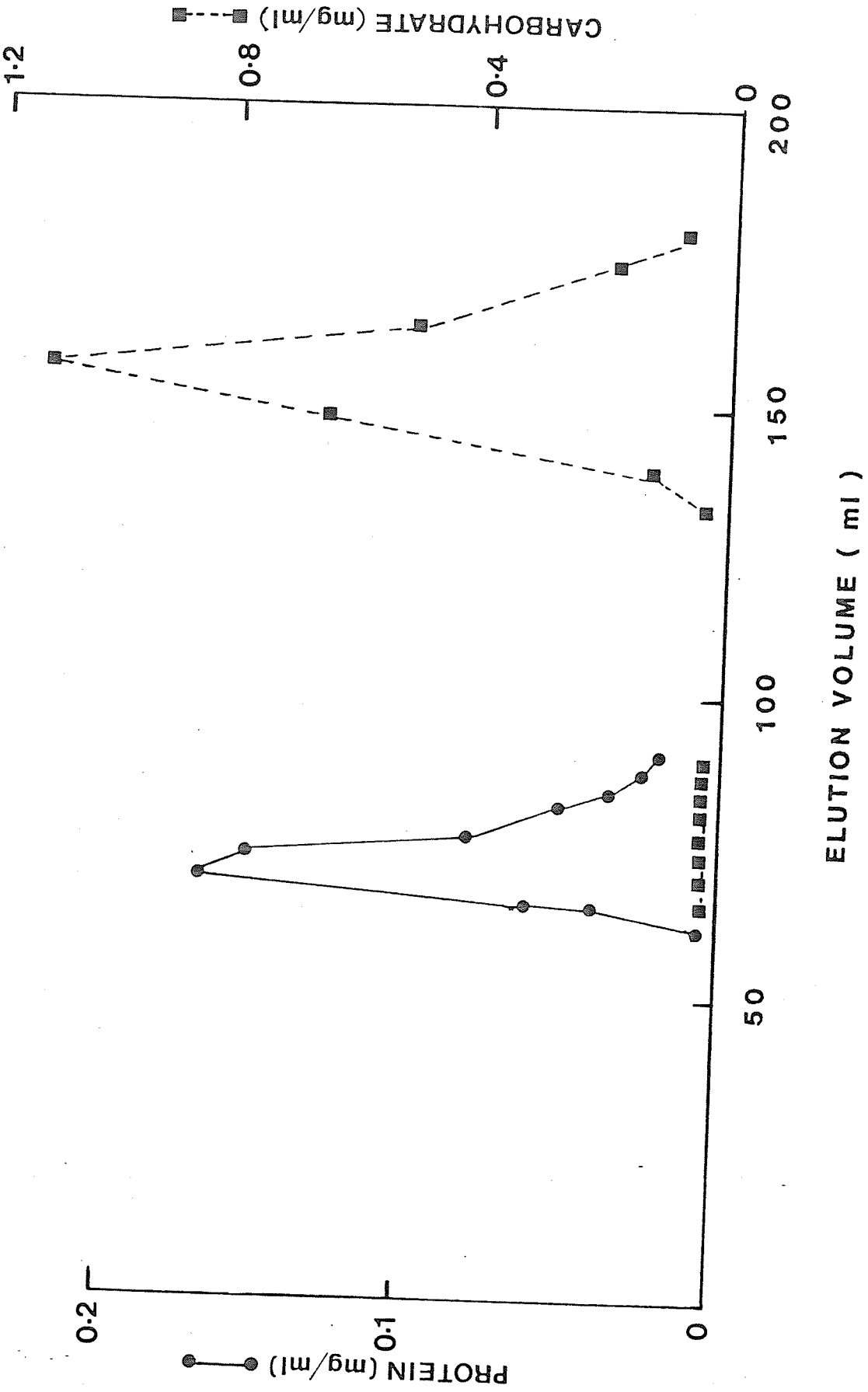
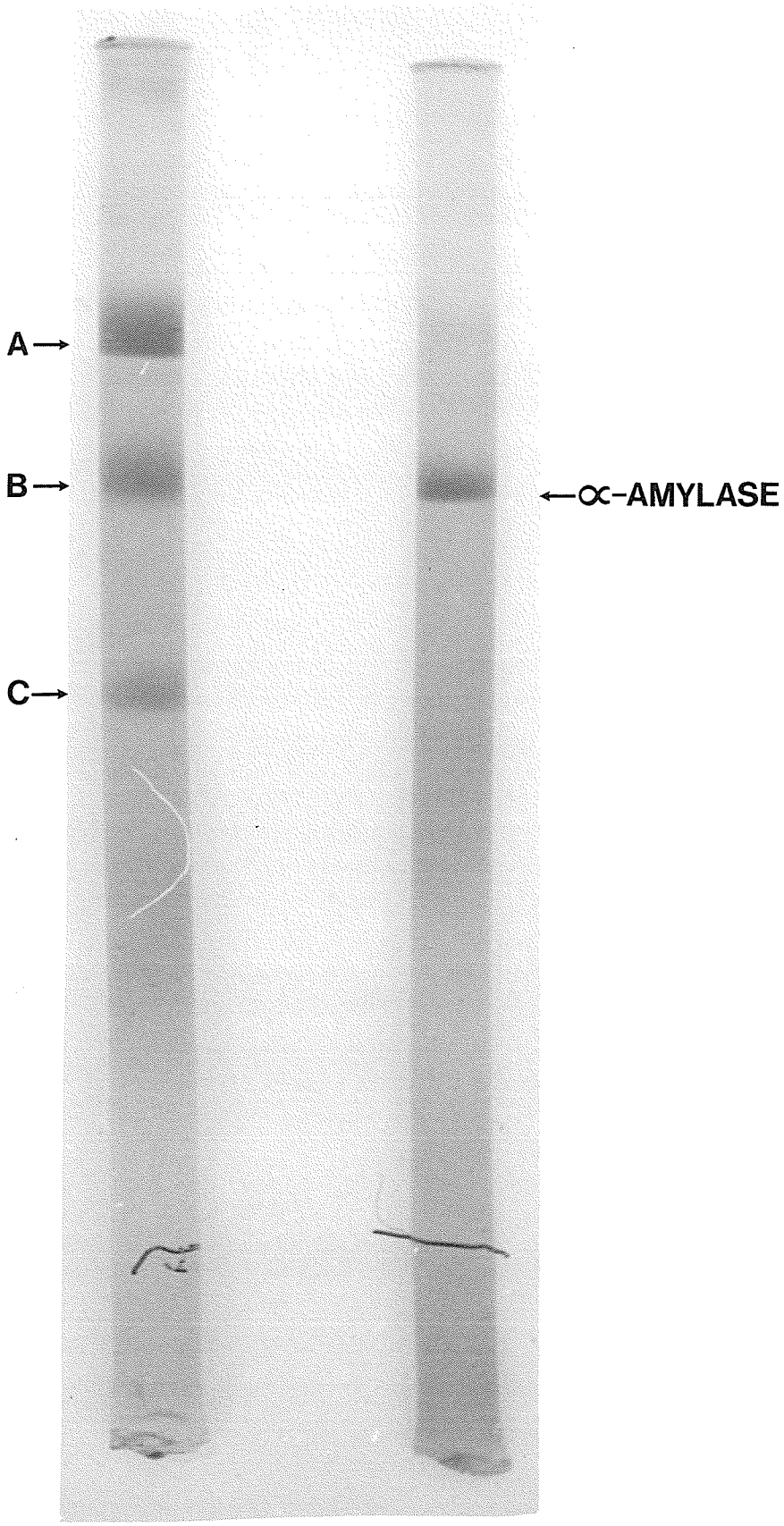

CARBOHYDRATE (mg/ml)
1·2
0·8
0·4
0
200
150
100
50
ELUTION VOLUME (ml)
0·2
0·1
0
PROTEIN (mg/ml)

Figure 4. Sodium dodecylsulphate gel electrophoresis of purified triticale α-amylase compared to three standards.

Legend: A = Bovine serum albumin (68,000)

B = Ovalbumin (45,000)

C = α-Chymotrysinogen A (25,000)


A
B
C
←∝-AMYLASE

Silvanovich and Hill (1977) where molecular weights of 40,000 and 41,000 were reported for triticale α-I and α-II isozyme groups respectively.

Comparative zymograms obtained after isoelectricfocusing of the triticale α-amylase showed similar patterns (Figure 5). Since β-limit dextrin will not react with β-amylase but starch will react, identical zymogram patterns strongly implied that the preparation was free of β-amylase (MacGregor et al., 1974). The non-specific stain failed to show all the isozyme bands indicating that it was of limited usefulness in assessing the homogeneity of the preparation.

Starch zymograms obtained upon isoelectricfocusing of material unretarded by the affinity column had a majority of bands which focused between the α-I and α-II isozyme groups and were probably caused by β-amylase isozymes which were not held back by the affinity gel (Figure 6). Thin layer chromatography of the products of starch hydrolysis of separated α-I and α-II isozyme groups showed no maltose present. Furthermore small columns of cycloheptaamylose-epoxy-Sepharose 6B did not retain preparations of either sweet potato β-amylase or barley β-amylase. This was in good agreement with the apparent lack of retention of wheat and triticale β-amylase by the affinity column.

Separation of α-I and α-II Isozyme Groups of Wheat

In order to study the α-I and α-II isozyme groups separately it was necessary to fractionate the α-amylase after affinity chromatography. Wheat α-I isozymes prepared by negative CM-cellulose chromatography (pH 5.5) had a specific activity of 162,000 IDC units/mg of

Figure 5. Comparative zymograms of triticale α-amylase on β-limit dextrin and starch after isoelectricfocusing.

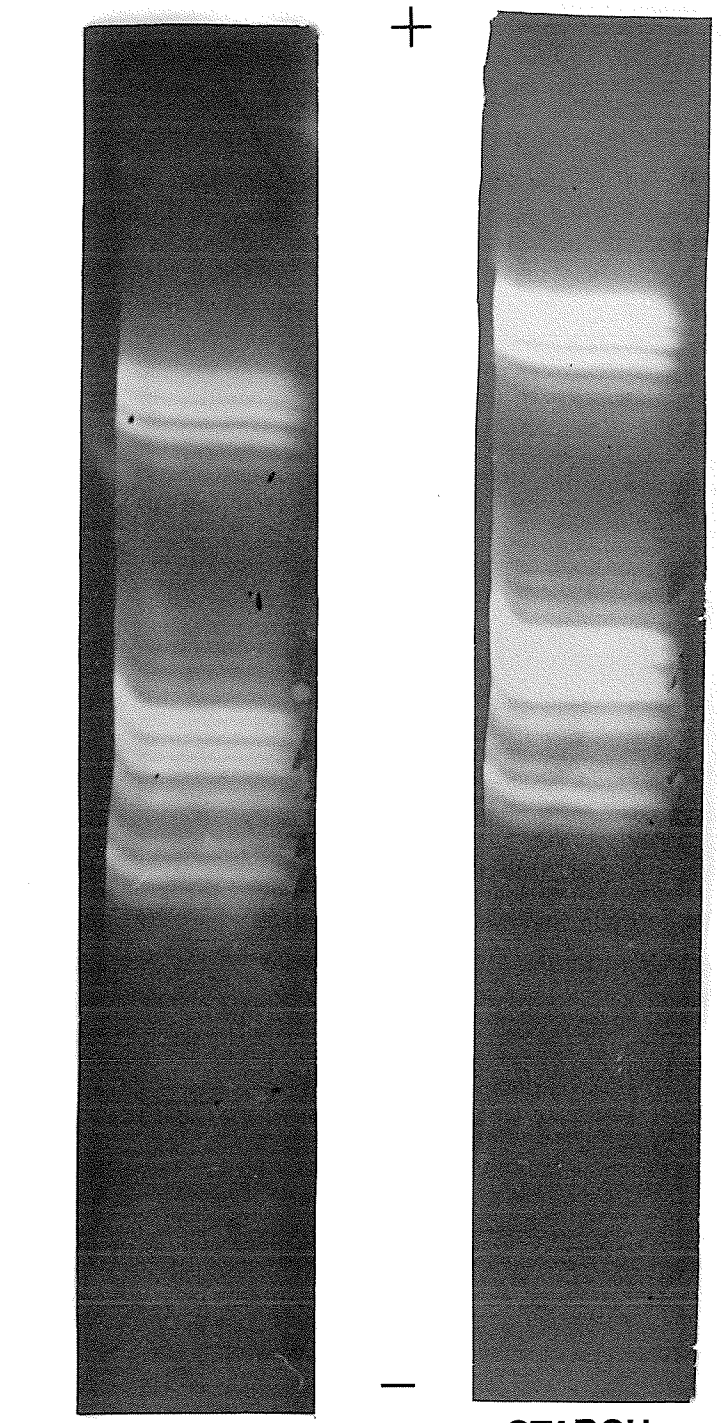
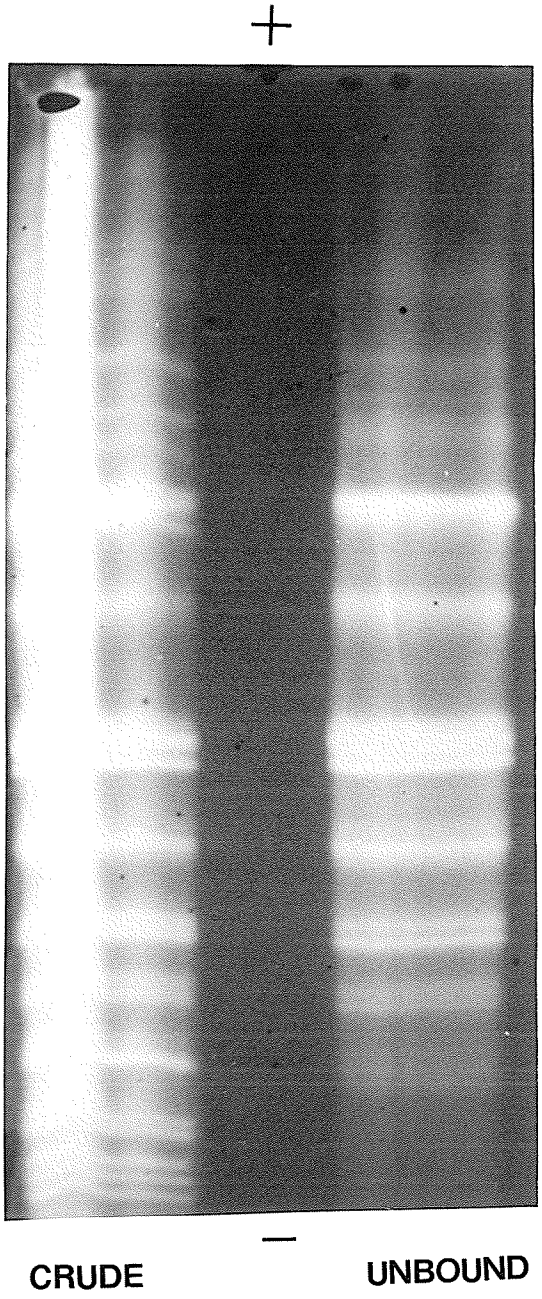

+
—
β-LIMIT DEXTRIN
STARCH

Figure 6. Isoelectricfocusing of triticale extract before and after passing through the affinity column.


+
—
CRUDE
UNBOUND

protein. The Briggs value was in good agreement with Silvanovich and Hill (1977) who reported a specific activity of 169,000 units/mg protein for α-I isozymes of triticale after CM-cellulose chromatography. Figure 7 illustrates zymogram patterns of purified wheat α-I isozymes after passing through CM-cellulose and the original unfractionated mixture. The α-I activity recovered from the CM-cellulose column represented 11% of the enzyme applied to the column. Attempts to elute α-II isozymes of wheat from CM-cellulose with salt gradients were unsuccessful regardless of whether the ion-exchange step was placed before or after the affinity step. Wheat α-II eluted from the CM-cellulose column was always inactivated and the solution was turbid. The ethanol-glycogen precipitation step used by Silvanovich and Hill (1977) may have conferred stability to α-II isozymes when in the presence of high salt because they successfully obtained high specific activity triticale α-II in high yield by elution of CM-cellulose with salt gradients.

Wheat α-II isozymes were purified under conditions where the isozymes were not exposed to high salt. The isozymes were eluted from a DEAE-cellulose column at an earlier stage in the salt gradient at pH 7.4. The absorbance profile for the elution of α-amylase from DEAE-cellulose is shown in figure 8. The eluted peak was segregated into 4 fractions. Isoelectricfocusing of each fraction showed that fraction B was exclusively composed of α-II isozyme components (Figure 9) and constituted 25% of the enzyme activity applied to the column. However certain components of the α-II group appeared to be enriched in fraction B whereas fraction C displayed the enrichment of other isozyme

Figure 7. Starch zymogram after isoelectricfocusing of wheat α-amylase fractionated by CM-cellulose chromatography.

UNBOUND UNFRACTIONATED

Figure 8. DEAE-cellulose chromatography of wheat α-amylase at pH 7.4 at 4°C.

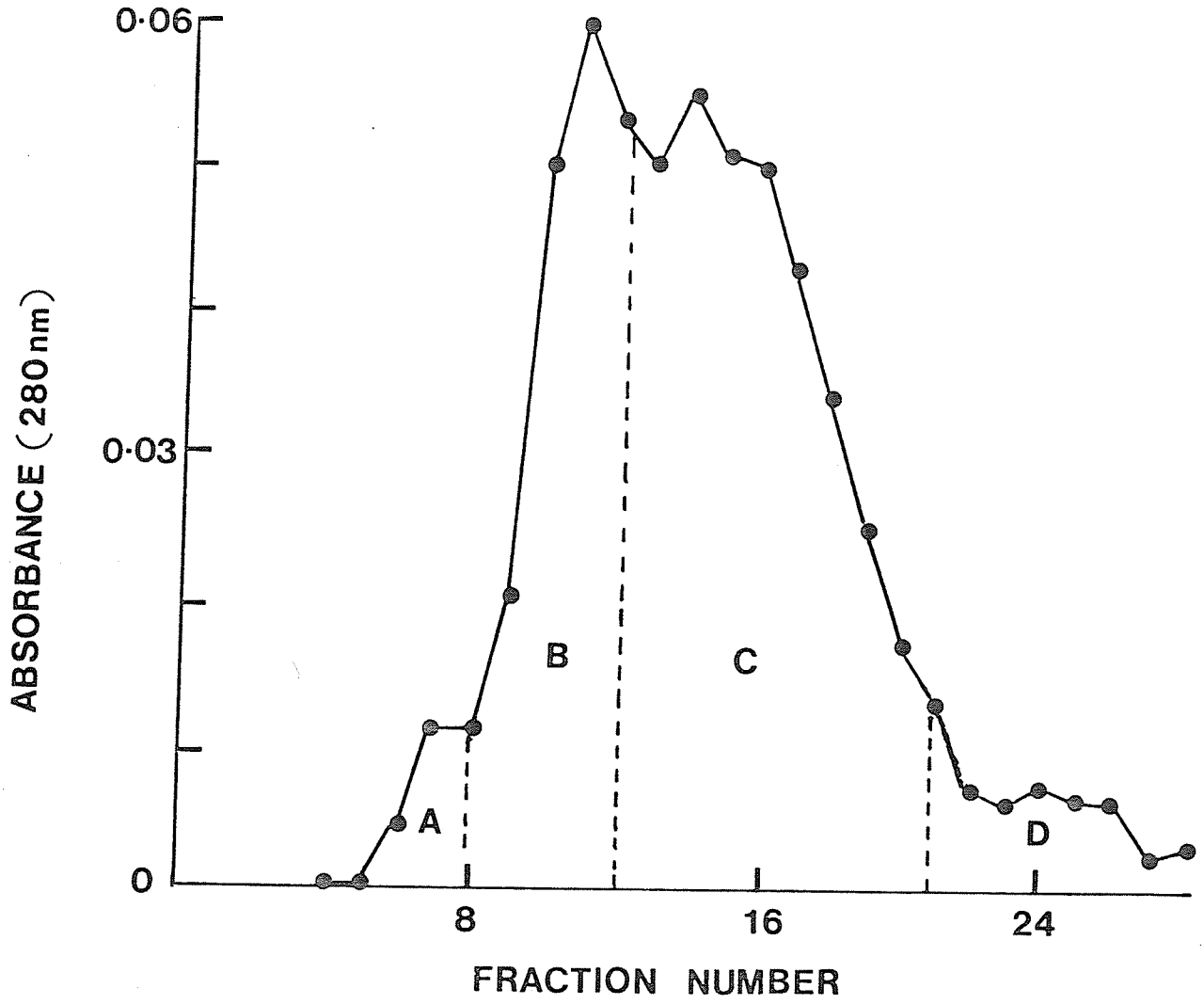
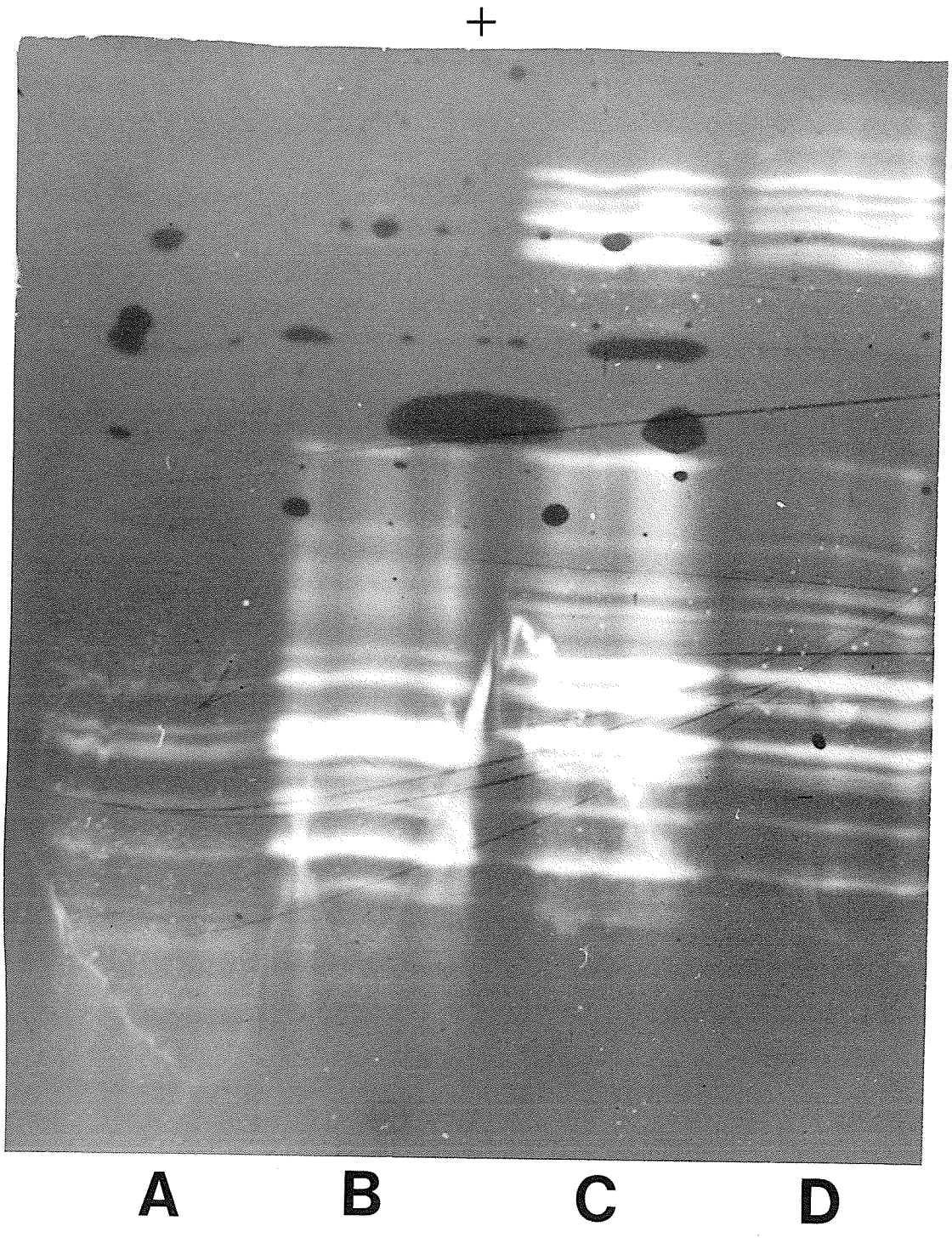

0·06
0·03
0
ABSORBANCE (280 nm)
A
B
C
D
8
16
24
FRACTION NUMBER

Figure 9. Starch zymogram after isoelectricfocusing of wheat α-amylase fractionated by DEAE-cellulose chromatography.


+
A
B
C
D
–

components within the α-II group (Figure 9). But fraction C also contained some α-I isozymes and therefore was rejected on this basis. The specific activity of fraction B was not assessed due to the presence of BSA. Both α-I and α-II isozymes were stabilized for freezing by incorporating 0.1% BSA (MacGregor and Ballance, 1980a).

Elution By Compounds Other Than Cycloheptaamylose

An attempt was made to see if α-amylase could be eluted from the affinity gel by glucose and various glucose oligomers in the moving phase. It has been reported that the β-limit dextrin of waxy maize starch, a branched glucose polymer, can elute either α-I or α-II cereal α-amylases preferentially from the affinity gel (Silvanovich, 1977; Gibbons, 1979a; 1979b). The types of isozymes eluted by β-limit dextrin were re-examined in this series of experiments.

Studies on the influence of glucose and glucose oligomers on the release of bound triticale α-amylase isozymes indicated that longer chain lengths were more effective in releasing the enzyme (Table 5). Three ml portions of either glucose or maltose were almost totally ineffective while an equivalent volume of maltotriose was partially effective. Both β-limit dextrin and glycogen solutions eluted all the bound α-amylase.

The results of the elution studies implied that a certain critical chain length might be necessary for effective elution of α-amylase isozymes. The study could be more systematically conducted if the enzyme-bound affinity columns were treated with glucose oligomers of increasing degree of polymerization to a point beyond the achroic

TABLE 5. Effect of glucose and glucose oligomers on the release of cereal α-amylase from the affinity gel

Treatment	Percent Release
44 mM glucose	4%
22 mM maltose	4%
16 mM maltotriose	14%
8 mg/ml β-limit dextrin	100%
8 mg/ml glycogen	100%

dextrin length. It is also possible that very high concentrations of maltose may be effective in removing all the α-amylase from the affinity gel. Buonocore et al. (1975) found that 0.5 M maltose was necessary to effectively elute non-plant α-amylases from wheat albumin affinity columns. The experiments conducted with triticale α-amylase used glucose and glucose oligomers at concentrations that were comparable on a weight per volume basis to elutions effected by 8 mg/ml cycloheptaamylose.

Elution of the affinity column with β-limit dextrin did not appear to preferentially release one isozyme group over the other when the eluted fractions were analyzed by isoelectricfocusing. Both isozyme groups were also apparent in the fractions from the same column after continued elution with cycloheptaamylose. This is in contrast to the findings of Silvanovich (1977) who reported that β-limit dextrin eluted α-I isozymes preferentially over α-II isozymes. The α-I isozymes had trace amounts of α-II isozymes associated with them. The majority of α-II isozymes were then eluted with cycloheptaamylose. To further confuse the issue Gibbons (1979a, 1979b) has found preferential release of α-II isozymes from the affinity column when he used β-limit dextrin as an eluant prior to cycloheptaamylose application. He attributed the difference to the possibility that barley α-amylase isozyme groups may behave differently from triticale isozyme groups with respect to their interaction with the affinity gel. As they stand the differences in isozyme elution with β-limit dextrin still remain unclear.

Silvanovich (1977) has shown that cyclohexaamylose is just as effective as cycloheptaamylose in removing the bound enzyme from the

affinity column. This suggested that the nature of the interaction between cyclohexaamylose and cereal α-amylase might be quite similar to the interaction involving cyclohepatamylose. The elution with cyclohexaamylose also suggests that cereal α-amylase is quite different from bacterial α-amylase because Vretblad's (1974b) column of cyclohexaamylose did not retain bacterial α-amylase.

Inhibition Studies With Gelatinized Starch

Studies With Cereal α-Amylase

The possibility that cereal α-amylase may be immobilized on the cycloheptaamylose-epoxy-Sepharose 6B at the active site prompted an investigation on the effect of cycloheptaamylose on the action of the enzyme on starch in solution. Earlier investigators had used similar kinetic methods to show that both Bacillus subtilis and pancreatic α-amylase were inhibited by cycloheptaamylose at the active site (Ohnishi, 1971; Mora et al, 1974).

Inhibiton studies using a mixture of α-I and α-II isozymes from triticale revealed no effect on the hydrolysis of gelatinized starch using concentrations as high as 6 mM (Figure 10). The Dixon plots were parallel to the x-axis. The same held true for α-I and α-II isozyme groups isolated from wheat using concentrations of cycloheptaamylose as high as 10 mM (Figure 11).

Mora et al. (1974) have used concentrations of cycloheptaamylose as high as only 1.6 mM to effectively inhibit pancreatic α-amylase. They used substrate concentrations ranging from 0.02 to 0.1% to produce a family of Dixon plots intersecting in the fourth quadrant to show competitive inhibition.

Figure 10. Dixon plots for the hydrolysis of gelatinized starch by triticale α-amylase in the presence of increasing concentrations of cycloheptaamylose.

S = starch concentration

Enzyme activity = 0.12 μmoles glucose/min/ml

Reaction time = 10 minutes

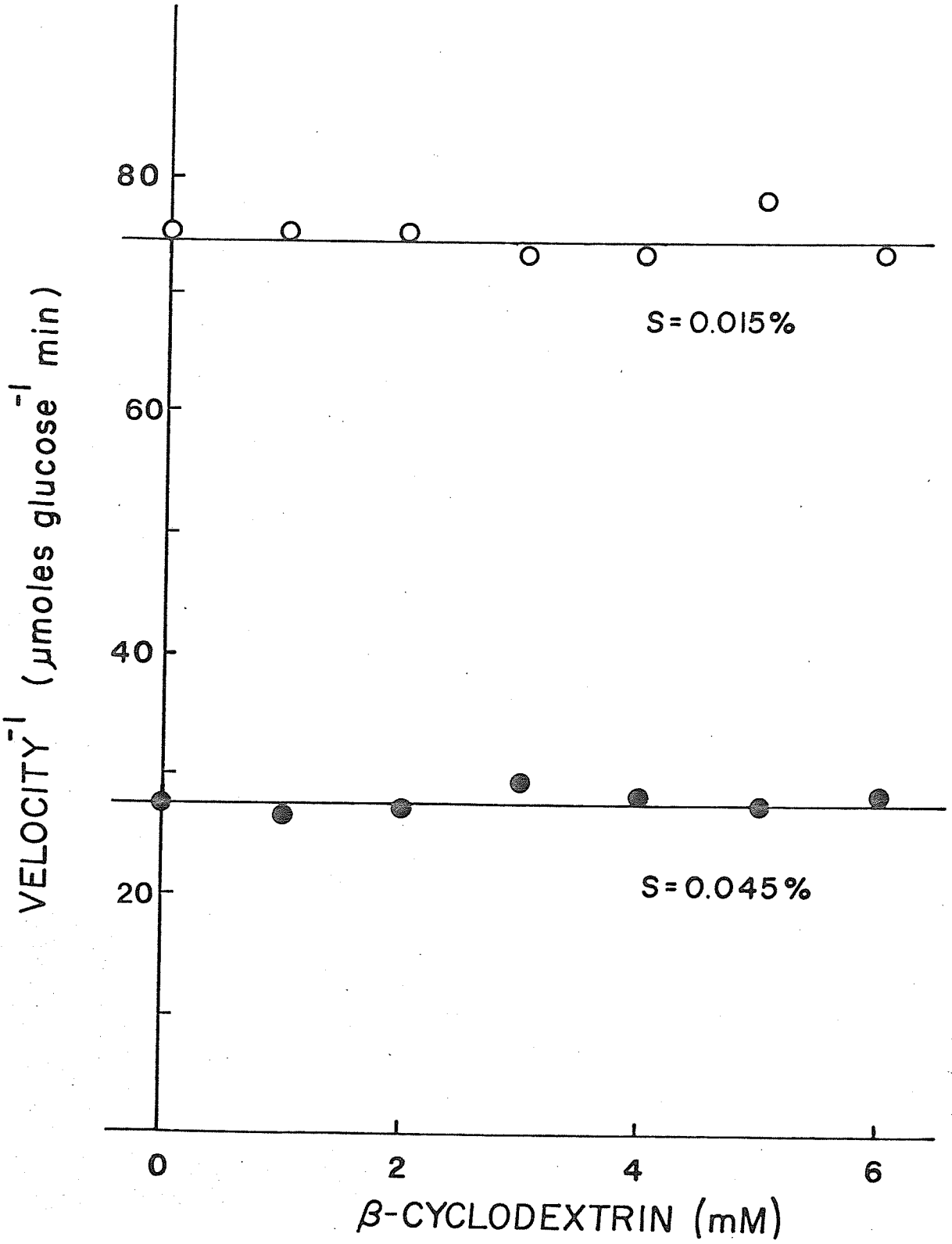
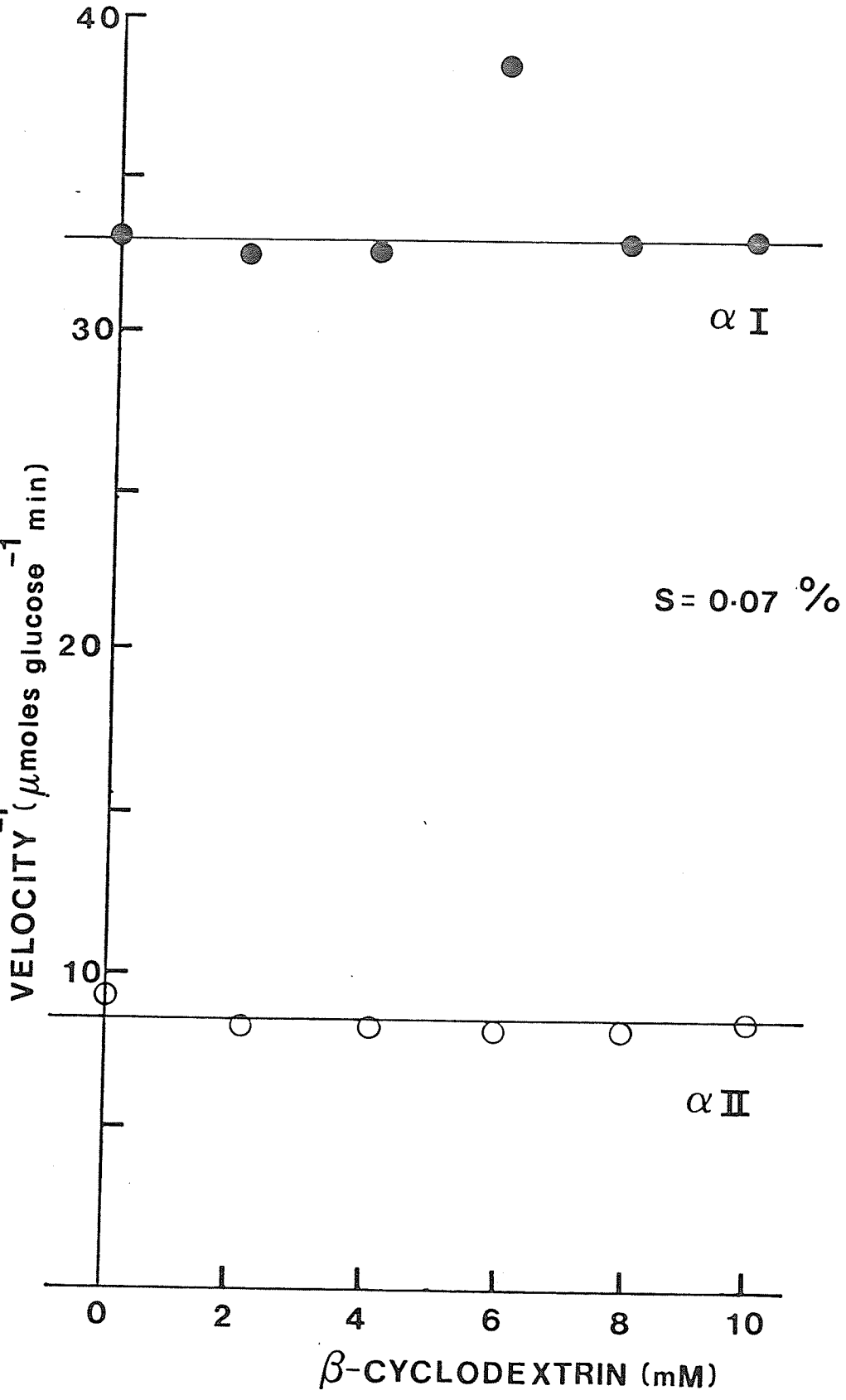

80
60
40
20
VELOCITY^{-1} (μmoles glucose^{-1} min)
S = 0.015%
S = 0.045%
0
2
4
6
β-CYCLODEXTRIN (mM)

Figure 11. Dixon plots for the hydrolysis of gelatinized starch by wheat α-amylases in the presence of increasing concentrations of cycloheptaamylose.

S = starch concentration

Enzyme activities: 0.13 μmoles glucose/min/ml α-I
0.02 μmoles glucose/min/ml α-II

Reaction time = 3 minutes


40
30
20
10
0
2
4
6
8
10
α I
α II
S = 0·07 %
VELOCITY^{-1} (μmoles glucose^{-1} min)
β-CYCLODEXTRIN (mM)

Studies With Barley β-Amylase

The lack of retention of triticale, wheat, barley and especially sweet potato β-amylase by the affinity column warranted a separate kinetic study of the inhibition of cereal β-amylase by cycloheptaamylose. The negative behavior of the enzyme on the cycloheptaamylose affinity column was quite surprising when viewed in contrast to Vretblad's (1974a,1974b) findings using the cyclohexaamylose column where sweet potato β-amylase was strongly retained under similar conditions.

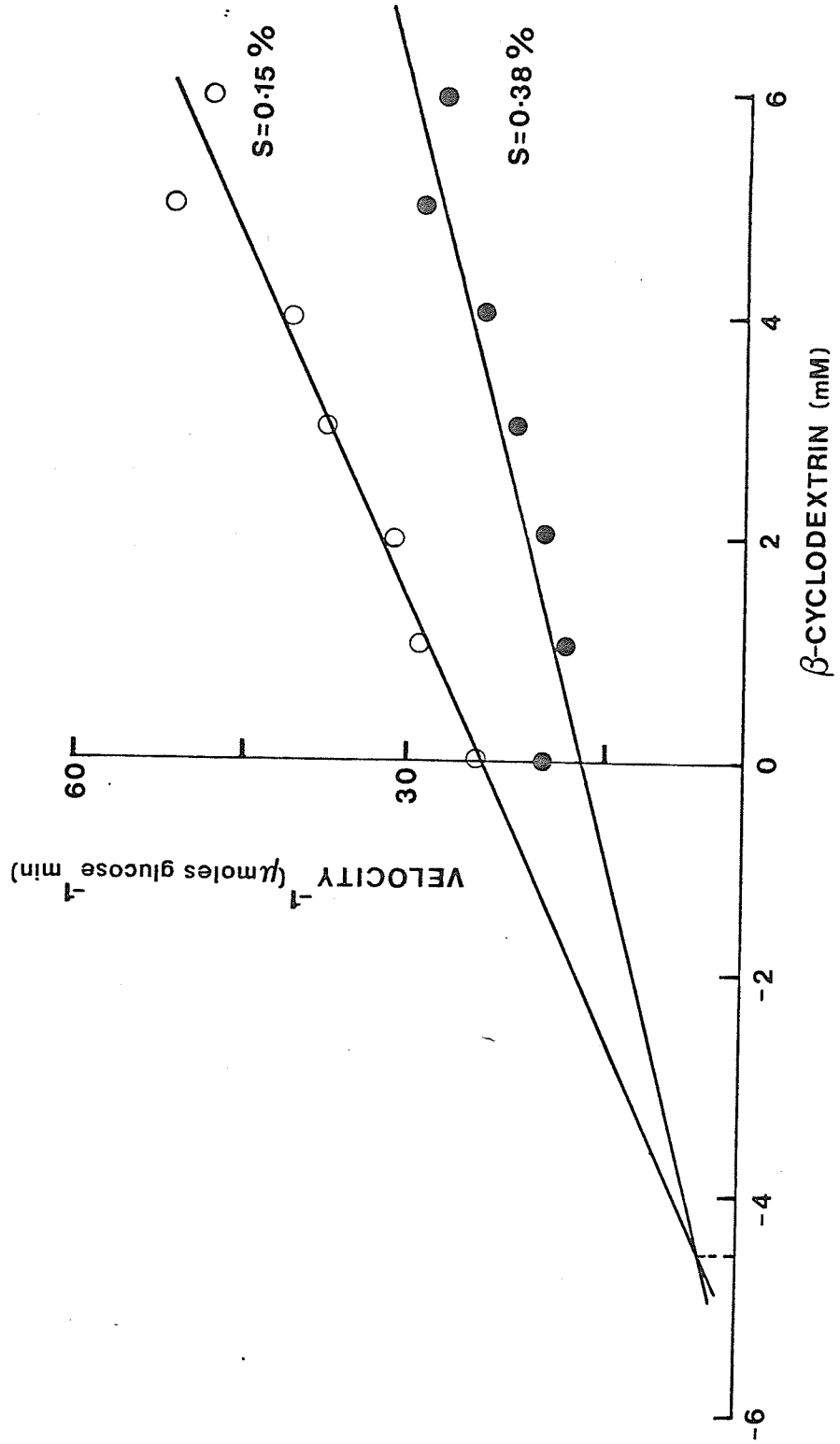
Barley β-amylase was inhibited by cycloheptaamylose with an apparent K_i = 4.5 mM which was estimated from the Dixon plots in figure 12. The results were not peculiar because other investigators have reported inhibition of sweet potato β-amylase by cyclohexa- and cycloheptaamylose (Thoma and Koshland, 1960; Marshall, 1973). Based on the inhibition data on cereal α-amylase and barley β-amylase, the affinity column should have held back cereal β-amylase preferentially to cereal α-amylase. The paradox could be explained on the basis that cereal α-amylase interacted with the affinity column at a site on the enzyme different from the active site. The behavior of cereal β-amylase on the cyclodextrin affinity columns as well as inhibition of cereal β-amylase by cycloheptaamylose is in need of further study.

Figure 12. Dixon plots for the hydrolysis of gelatinized starch by barley β-amylase in the presence of increasing concentrations of cycloheptaamylose.

S = starch concentration

Enzyme activity = 0.138 μmoles glucose/min/ml

Reaction time = 5 minutes


S=0·15 %
S=0·38 %
VELOCITY $^{-1}$ (μmoles glucose $^{-1}$ min)
60
30
0
β-CYCLODEXTRIN (mM)
6
4
2
-2
-4
-6

Effect of Cycloheptaamylose On the Interaction Between Cereal α-Amylase and Starch Granules

Inhibition of Cereal α-Amylase Binding

The evidence for an adsorptive, non-catalytic site on cereal α-amylase prompted an investigation to determine if the cycloheptaamylose would interfere with the binding of α-amylase to starch granules and thus indicate a functional role for the non-catalytic site. Initially the effect of cycloheptaamylose on the adsorptive relation between α-amylase and starch granules at low temperature was studied. In this way adsorption would be maximized and hydrolysis minimized (MacGregor, 1979) thus putting the focus on the non-catalytic site.

Both α-I and α-II isozymes of wheat could be adsorbed to wheat starch granules at $4^{o}C$ at pH 5.5. The fact that adsorption did occur was in agreement with the findings of different investigators (Schwimmer and Balls 1949b; Walker and Hope, 1963; MacGregor, 1979).

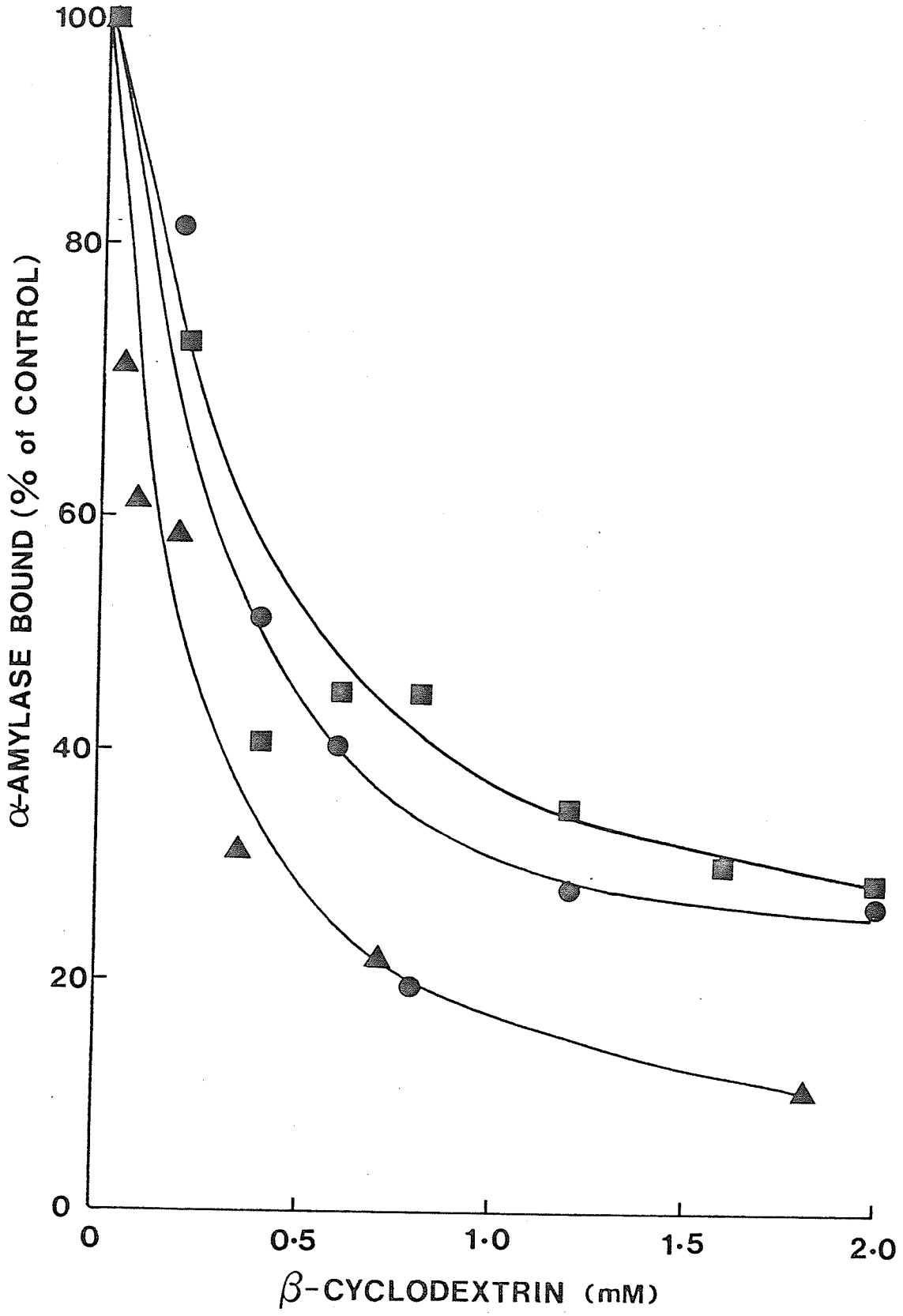
Cycloheptaamylose was found to inhibit the binding of cereal α-amylases to starch granules (Figure 13). Low concentrations of the cyclic dextrin were effective in inhibiting the binding of nearly 50% of the enzyme that would be expected to be bound if cycloheptaamylose were not present. Cycloheptaamylose, therefore, had a positive role in shifting the equilibrium away from starch granule binding. Only 10% of the triticale α-amylase remained bound to waxy maize starch at 1.8 mM cycloheptaamylose. In the case of wheat α-amylase and wheat starch, about 25% of each isozyme group remained bound at 2 mM cycloheptaamylose. The fact that cereal α-amylase was more inhibited

Figure 13. Effect of cycloheptaamylose on the binding of cereal α-amylase to starch granules at 4°C.

Legend: ■= wheat α-I and wheat starch

●= wheat α-II and wheat starch

▲= triticale α-amylase and waxy maize starch


100
80
60
40
20
0
α-AMYLASE BOUND (% of CONTROL)
0
0·5
1·0
1·5
2·0
β-CYCLODEXTRIN (mM)

in binding to waxy maize starch than wheat starch is suggestive of significant structural differences on the surfaces of the different types of starch granules used.

In contrast to this study Sargeant et al. (1978) found that wheat α-I isozymes did not adsorb to mature wheat starch granules. However their analysis of the products of hydrolysis from the action of α-I on starch was shown to contain maltose in addition to large oligiosaccharides suggesting contamination by β-amylase.

Five hour hydrolyzates of wheat starch granules (15°C) by the α-I and α-II wheat isozymes used in the present study were found to be free of maltose when analyzed by thin layer chromatography. Only achroic dextrins and α-limit dextrin were apparent indicating that the preparations were free of β-amylase.

Inhibition of Release of Soluble Carbohydrate

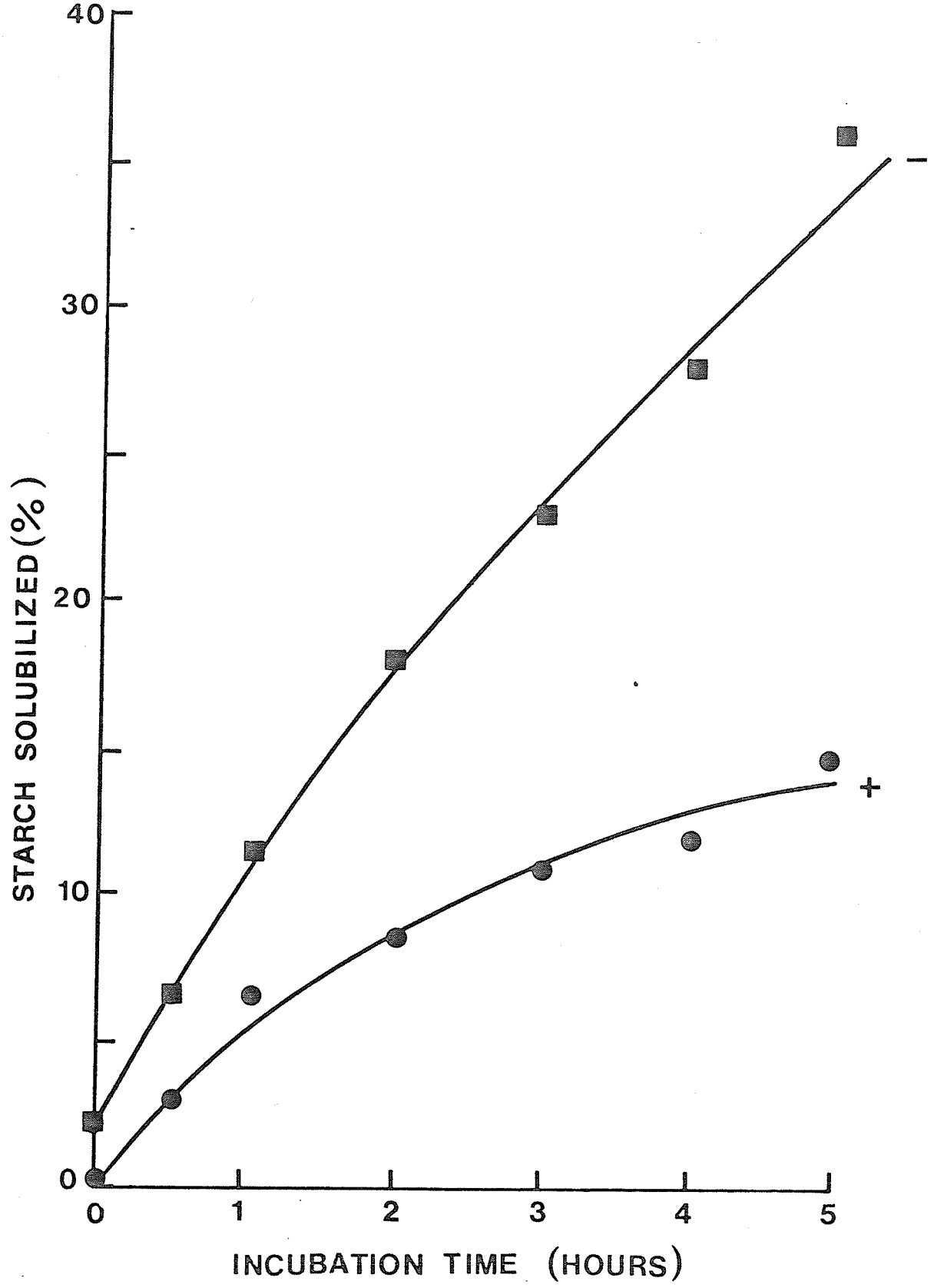
The effect of cycloheptaamylose on the hydrolysis of starch granules by cereal α-amylase was studied at temperatures of 35 and 15°C. Since cycloheptaamylose has been shown to interfere with α-amylase adsorption to starch granules, the next logical step was to determine if inhibition of binding would interfere with the enzymic hydrolysis of the granules.

A time course of the effect of cycloheptaamylose on the release of soluble carbohydrate from waxy maize starch by triticale α-amylase is shown in figure 14. There was approximately a 60% decrease in the release of soluble starch by the cyclodextrin. The solubilization was non-linear both in the presence and absence of cycloheptaamylose.

Figure 14. Effect of cycloheptaamylose on the release of soluble carbohydrate from waxy maize starch granules by triticale α-amylase at 35°C.

Legend: ■ = control hydrolysis

● = hydrolysis in the presence of 0.82 mM cycloheptaamylose


40
30
20
10
0
STARCH SOLUBILIZED (%)
0
1
2
3
4
5
INCUBATION TIME (HOURS)
−
+

The biphasic nature of the curves was similar to that reported by Sandstedt and Mattern (1960) and MacGregor and Ballance (1980b) who considered the more rapid phase of the reaction to represent release due to damaged starch while the slower portion was considered hydrolysis of intact granules. Extrapolation of the slower phase of the curves to the y-axis gave an estimate of starch damage which was less than 5% for waxy maize starch.

The hydrolysis of wheat starch granules by both groups of wheat isozymes was conducted at 15°C in order to simulate conditions that might exist at germination. Once again the extent of starch damage was less than 5% which should have had no significant effect on the extended hydrolysis observed. Figure 15 shows release of soluble carbohydrate as a function of time and Figure 16 shows appearance of reducing activity as a function of time. Alpha-I isozymes were slightly more efficient in the digestion of the wheat starch granules than α-II isozymes which was in agreement with the findings of MacGregor and Ballance (1980b). There was approximately 30% inhibition of hydrolysis of both α-I and α-II isozyme groups in the presence of 0.82 mM cycloheptaamylose. The spontaneous release of soluble carbohydrate from wheat starch granules incubated without enzyme never exceeded 7% of the total soluble carbohydrate released over the 5 hour incubation period (Figure 15). Spontaneous release of soluble carbohydrate was not detectable in the form of reducing activity.

Cycloheptaamylose was about twice as effective an inhibitor of α-amylase catalyzed hydrolysis in waxy maize as opposed to wheat starch. Furthermore 1.4 times more enzyme was used in the waxy maize study

Figure 15. Effect of cycloheptamylose on the release of soluble carbohydrate from wheat starch granules by wheat α-amylase isozyme groups at 15°C.

Legend: ○= control hydrolysis

●= hydrolysis in the presence of 0.82 mM cycloheptaamylose

□= spontaneous release of soluble starch without enzyme present

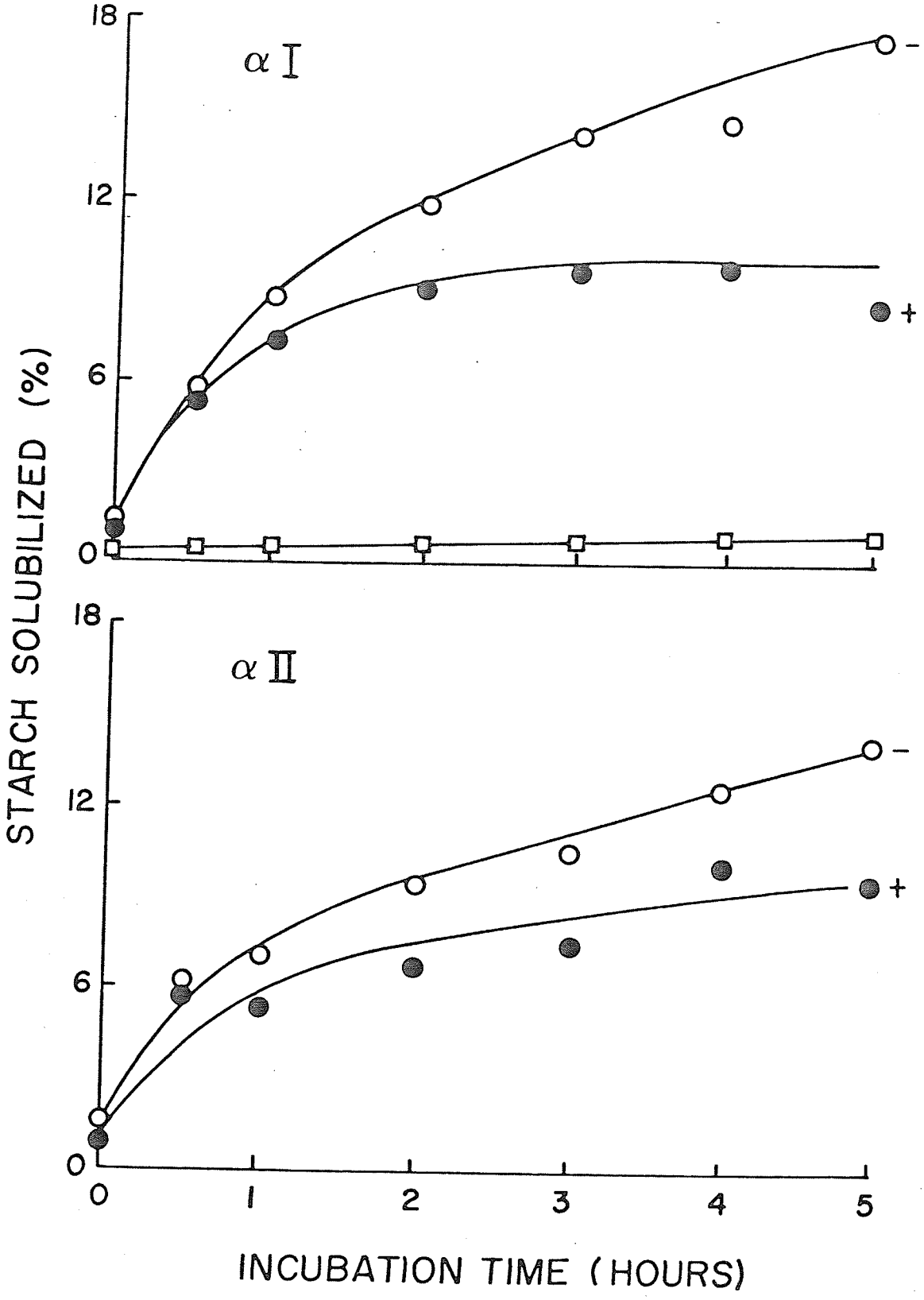
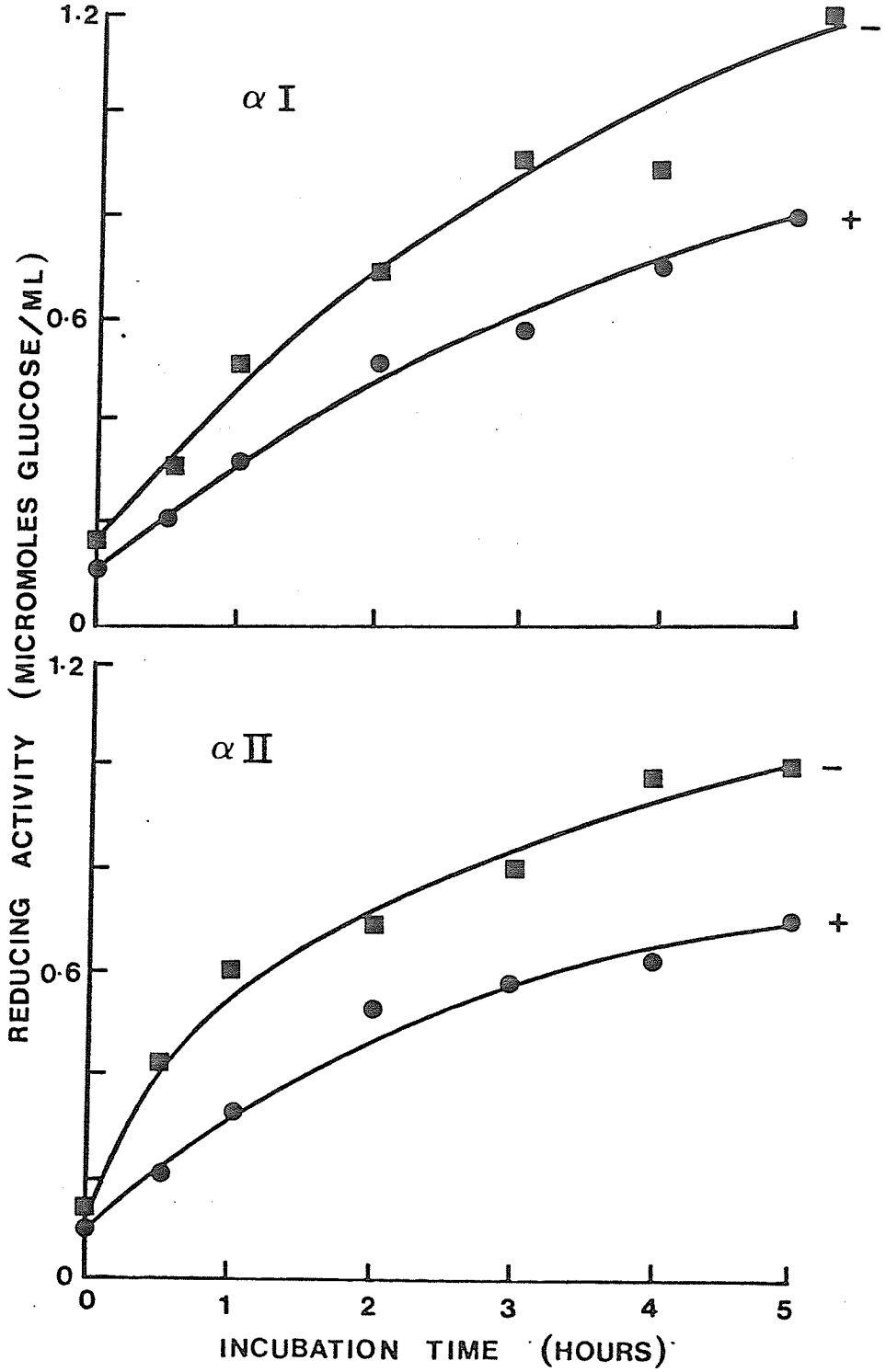

αI
αII
18
12
6
0
STARCH SOLUBILIZED (%)
0
1
2
3
4
5
INCUBATION TIME (HOURS)
−
+

Figure 16. Effect of cycloheptaamylose on the appearance of reducing activity from wheat starch granules hydrolyzed by wheat α-amylase isozyme groups at 15°C.

Legend: ■ = control hydrolysis

● = hydrolysis in the presence of 0.83 mM cycloheptaamylose


1·2
αI
0·6
0
−
+
REDUCING ACTIVITY (MICROMOLES GLUCOSE/ML)
1·2
αII
0·6
0
−
+
0
1
2
3
4
5
INCUBATION TIME (HOURS)

than in the wheat study. The type of starch granules used seemed to have a great effect on the amount of inhibition caused by the presence of the cyclodextrin. The findings were in agreement with cycloheptaamylose being a more effective inhibitor of α-amylase binding to waxy maize starch than to wheat starch at $4^{o}C$ (Figure 13).

Binding of Cycloheptaamylose To Cereal α-Amylase

Radioactive Cycloheptaamylose

Before successful binding studies could be conducted it was necessary to find a sensitive assay for cycloheptaamylose. A method had been developed by Andersen *et al.* (1963) for preparing ^{14}C- cyclodextrins. However the method of preparation was very laborious and involved a number of different fractionation procedures. Instead a simple method was developed using a modification of the Jones oxidation for allylic alcohols followed by reduction with tritiated sodium borohydride.

The Jones oxidation using chromic acid (Bowden *et al.*, 1946) is frequently used for the oxidation of allylic alcohols. It is performed under relatively mild conditions that would not be expected to hydrolyze the α(1→4) linkages in cycloheptaamylose (Swanson and Cori, 1948).

The yield for this stage of the reaction was 38 percent (Table 6) based on the determination of glucose in the effluent from ion-exchange chromatography. It was apparent, therefore, that a consderable portion of the reaction proceeded beyond the aldehyde stage to form the corresponding glucuronic acid. It is also possible that some of the cyclodextrin rings could have broken open to form charged or non-charged

TABLE 6. Recovery of products during the reactions forming tritiated cycloheptaamylose

	CrO_3 oxidation	$NaBT_4$ Reduction
Yield (mg)	35	33.2
%	38	95
Reducing activity (μmoles glucose)	4.68	n. d.*
Specific Activity (dpm/μmole)	-	1.08×10^6

* Not detectable

linear dextrins. The charged products would be removed through precipitation with barium carbonate and ion-excange chromatography. The amount of reducing activity was 4.68 μmoles glucose indicating that about 2.5% of the available gluose equivalents in the product were oxidized. The yield of the borohydride reduction was 94% and the product had no detectable reducing activity. The specific activity of the final product was about one-half of the theoretical specific activity assuming one tritium incorporated per reducing equivalent. The tritium did not appear to be incorporated in a position that was easily exchangeable as conditions that would be expected to exchange tritium had little effect on the activity of the product.

There are a number of potential impurities that could contaminate the final product. Deionization after chromic acid oxidation removed the majority of charged species as the conductivity of the solution after ion-exchange chromatography was 7 μmhos. Saccharides ranging from sorbitol to G-7 linear dextrin could be present after sodium borohydride reduction. The tritiated cycloheptaamylose eluted as a single peak of constant specific activity on BioGel P-4 chromatography. Radioactive contaminants of smaller molecular weight and salt ions eluted after ^{3}H-cycloheptaamylose. Thin layer chromatography of the purified ^{3}H-cycloheptaamylose gave a single spot (Figure 17) having an R_f value identical to commercial cycloheptaamylose. Treatment of radioactive cycloheptaamylose with β-amylase followed by thin layer chromatography showed no spots other than radioactive cycloheptaamylose (Figure 18)indicating that the preparation did not contain linear dextrins. Reduced linear dextrins are known to be easily

Figure 17. Profile of radioactivity of prepared ^{3}H-cycloheptaamylose after thin layer chromatography.

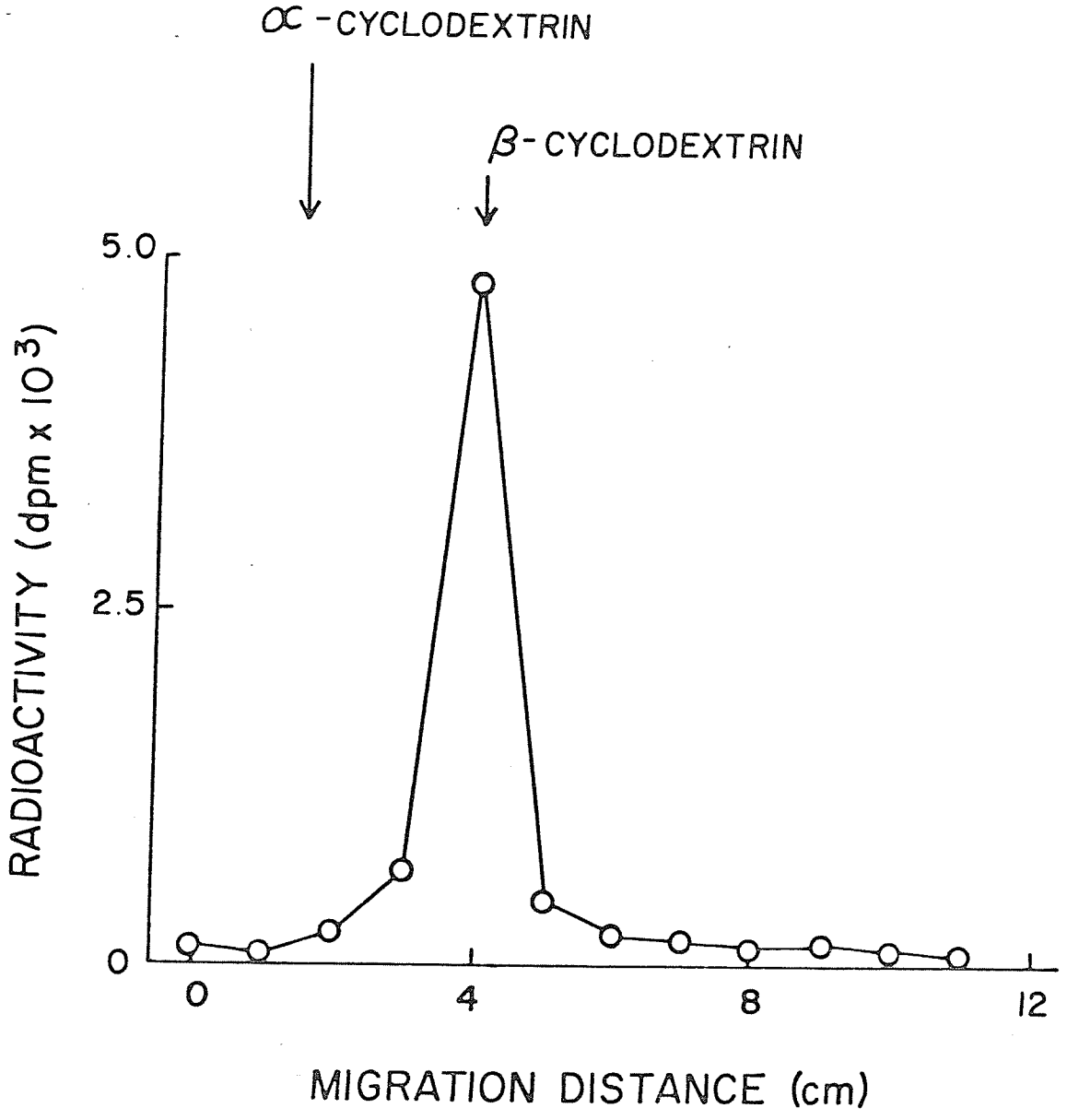
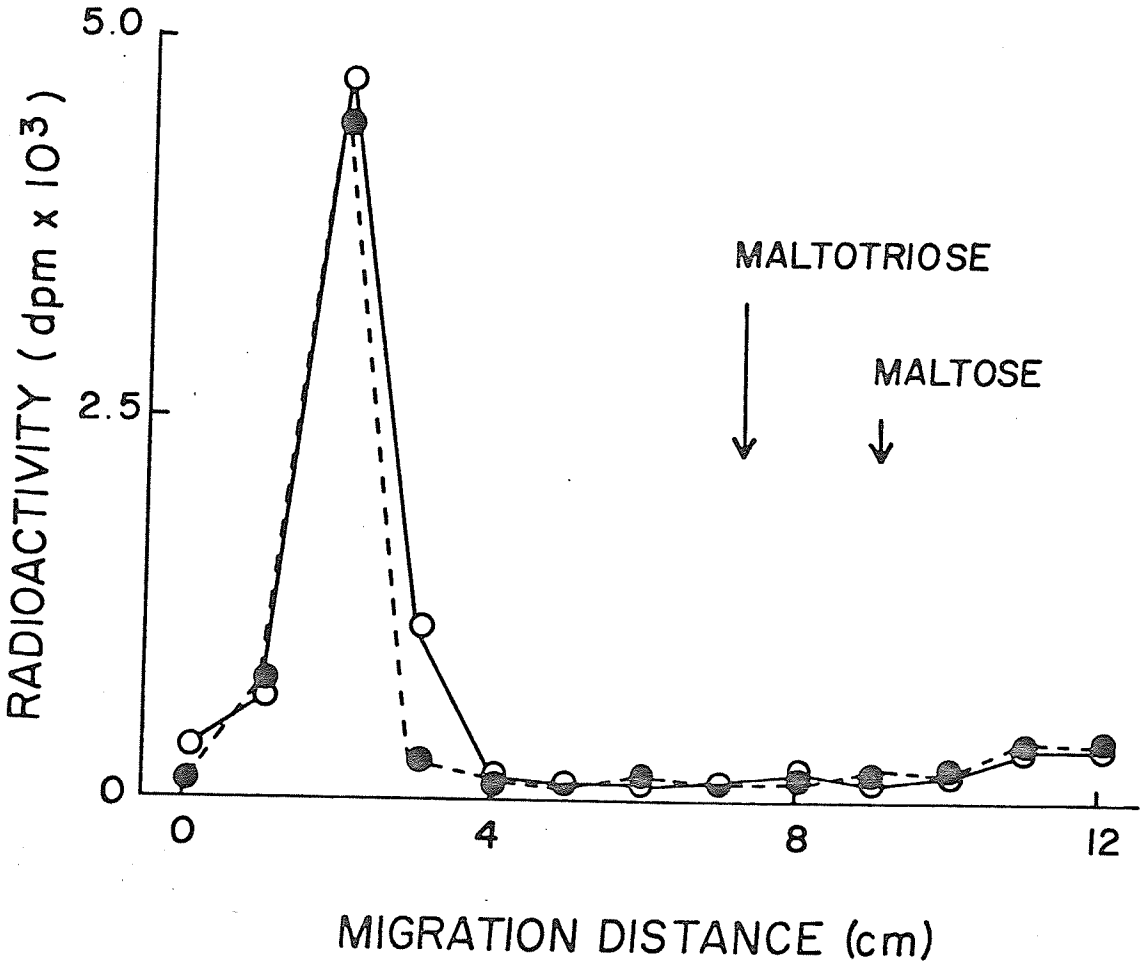

α-CYCLODEXTRIN
β-CYCLODEXTRIN
5.0
2.5
0
RADIOACTIVITY (dpm x 10³)
0
4
8
12
MIGRATION DISTANCE (cm)

Figure 18. Profile of radioactivity in thin layer chromatography of ^{3}H-cycloheptaamylose before and after exposure to sweet potato β-amylase.

Legend: O—O = Before β-amylase treatment

●—● = After β-amylase treatment


5.0
2.5
0
RADIOACTIVITY (dpm x 10³)
MALTOTRIOSE
MALTOSE
0
4
8
12
MIGRATION DISTANCE (cm)

hydrolyzable by β-amylase (Parrish et al., 1970).

The specific activity of the final product can be greatly increased, if necessary, by using higher specific activity NaB^3H_4. It should also be possible to prepare ^{3}H-cyclohexaamylose in a similar fashion.

Determination of Binding Parameters

Cereal α-amylase appeared to be retained quite strongly to the cycloheptaamylose affinity column and attachment of the ligand appeared to be at a non-catalytic site. Radioactive cycloheptaamylose was used to assess the strength of this interaction and to determine the stoichiometry of the interaction.

Enzyme eluted from the affinity column with ^{3}H-cycloheptaamylose was readily separated from the tritiated cycloheptaamylose by chromatography on BioGel P-4 (Figure 19). This experiment clearly demonstrated that the enzyme could be effectively prepared free of cyclodextrin thus allowing it to be used in equilibrium binding studies with the same ligand.

The results of three equilibrium dialysis experiments were plotted (Figure 20) according to the method of Scatchard (1949). At lower concentrations of cycloheptaamylose the relation between $\bar{\upsilon}/c$ versus $\bar{\upsilon}$ appeared to be linear. However, as relatively high concentrations were reached the curve tended to flatten out displaying what seemed to be non-specific binding. A tangent drawn from the initial portion of the curve intersected the abscissa at about 0.25 which should be the binding capacity (n). A fractional capacity was not likely since

Figure 19. Separation of ^{3}H-cycloheptaamylose from α-amylase on BioGel P-4.

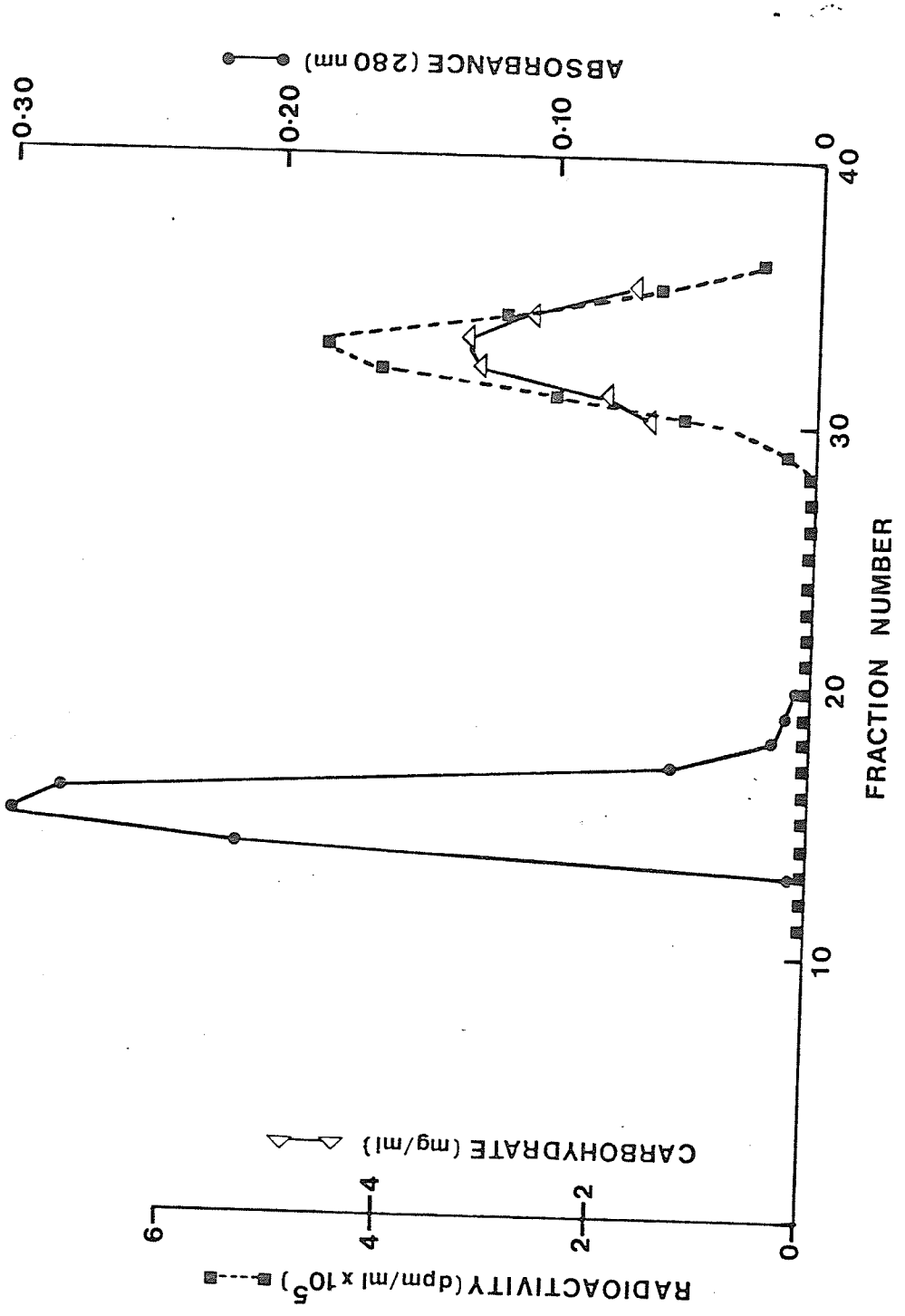

ABSORBANCE (280 nm)
0·30
0·20
0·10
0
40
30
20
10
FRACTION NUMBER
CARBOHYDRATE (mg/ml)
RADIOACTIVITY (dpm/ml x 10⁵)
6
4
2
0

Figure 20. Scatchard plot for binding of ^{3}H-cycloheptaamylose to α-amylase at 4°C.

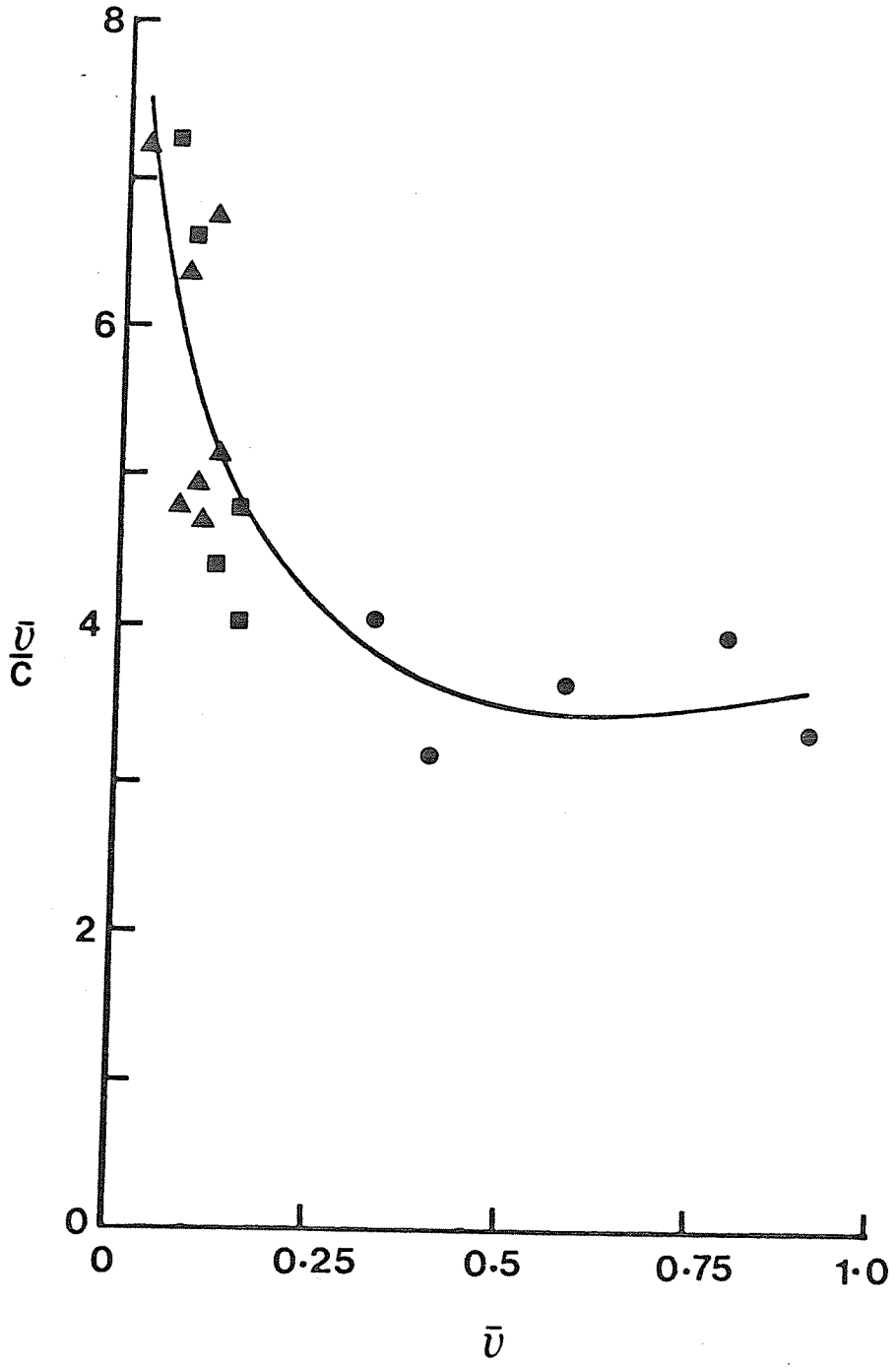
Legend: $\bar{\upsilon}$ = μmoles cycloheptaamylose bound per μmole of α-amylase

c = Free concentration of cycloheptaamylose in μmoles/ml

● = Wheat α-amylase

■ = Triticale α-amylase

▲ = Triticale α-amylase


8
6
4
2
0
$\frac{\bar{v}}{c}$
0
0·25
0·5
0·75
1·0
$\bar{v}$

cereal α-amylase was known to exist as a monomer (Greenwood and Milne, 1968). However Tkachuk and Kruger (1974) have reported a molecular weight of 86,000 for one ultracentrifugation experiment and have suggested that wheat α-amylase may be capable of dimerizing upon standing. The enzymes used in the present series of experiments remained in equilibrium dialysis for at least 66 hours. If dimerization were considered as a possiblity, a fractional capacity of 0.50 would still have been apparent. When the enzyme was assayed after equilibrium dialysis there was no significant loss of activity but one cannot be be certain that a portion of the non-catalytic binding sites had not deteriorated in this time frame. Furthermore enzyme preparations used in these studies had 5 to 10% undefined carbohydrate associated with them and one can only speculate on the involvement of this carbohydrate under conditions of high protein concentration in the presence of cycloheptaamylose. Another possibility that might explain the fractional capacity would be if one molecule of cycloheptaamylose accommodated 4 molecules of monomeric α-amylase. If dimerization was taking place then cycloheptaamylose might bind the two dimers at their non-catalytic sites. Once placed in a dilute form in an assay environment the entire complex could dissociate leaving the monomers to display their true activity. At this point there is uncertainty as to the reason for having observed fractional binding capacity. Perhaps a more appropriate answer could be obtained by using a method where equilibrium is established in a shorter time period.

Nonetheless the initial slope could still be used to obtain a valid dissociation constant (K_d). From 1/ slope a $K_d = 3.0 \times 10^{-5}$ M

was determined indicating that cereal α-amylase had a high affinity for cycloheptaamylose thus accounting for the strong retention of the enzyme on the affinity column. The dissociation constant for the apparent non-specific binding was probably much higher and at this point could not be accurately assessed. The non-specific binding may be associated with the earlier observations of enzyme precipitation where high concentrations of both α-amylase and cycloheptaamylose were present. Mora et al. (1974) have also reported non-specific binding of cycloheptaamylose to the pancreatic α-amylase at relatively high levels of cycloheptaamylose. Perhaps a more linear relationship could be established if the protein concentrations in the equilibrium dialysis cells were reduced but then the $\bar{\upsilon}$ value determination would become subject to error. The answer here would be to prepare very high specific activity ^{3}H-cycloheptaamylose.

GENERAL DISCUSSION

The strong affinity of cereal α-amylase for immobilized cycloheptaamylose has enabled the purification of the enzyme from PVP treated and dialyzed crude extracts of both germinated wheat and triticale kernels. Beta-amylase passed through the affinity column unretarded which was surprising since barley β-amylase appeared to be competitively inhibited by cycloheptaamylose with a $K_i = 4.5 \times 10^{-3}$ M. In contrast Vretblad (1974a, 1974b) has shown that a column of cyclohexaamylose will retain sweet potato β-amylase and the potato enzyme has been shown to be competitively inhibited by both cyclohexa- and cycloheptaamylose (Thoma and Koshland, 1960; Marshall, 1973). It seems therefore that immobilization of cycloheptaamylose on epoxy-activated Sepharose 6B may have decreased the affinity of β-amylase for the ligand or that the change from a 6-membered cyclic dextrin to a 7-membered cyclic dextrin may have had a marked effect with respect to the retention of β-amylase.

The interaction between cycloheptaamylose and cereal α-amylase did not appear to exist at the catalytic site. Rather a non-catalytic site was implicated because cycloheptaamylose did not interfere with the hydrolysis of gelatinized starch at the active site yet the enzyme was retained by the affinity column. Cereal α-amylase, in this respect, appeared quite different from Bacillus subtilis and pancreatic α-amylase where the enzymes were shown to compete for both substrate and cyclo-

heptaamylose at the active site (Ohnishi, 1971; Mora et al., 1974). Figure 21 is a schematic representation of what may be occurring when cereal α-amylase hydrolyzes starch in solution with and without the presence of cycloheptaamylose. When the cyclodextrin is present it might occupy a non-catalytic site without inhibiting the action of the enzyme on its soluble substrate. It is also possible that the achroic dextrins generated during hydrolysis may also be capable of interacting with the non-catalytic site. This contention is strongly supported by the elution studies done with glucose oligomers of increasing size where it was found that progressively longer dextrin chain lengths were necessary for effective release of the enzyme bound to cycloheptaamylose. Achroic dextrins are likely to be the most eligible oligomers for interacting with α-amylase in a similar fashion to cycloheptaamylose at the non-catalytic site. However a more definitive study is required here using dextrins of defined length ranging from G-4 to G-8.

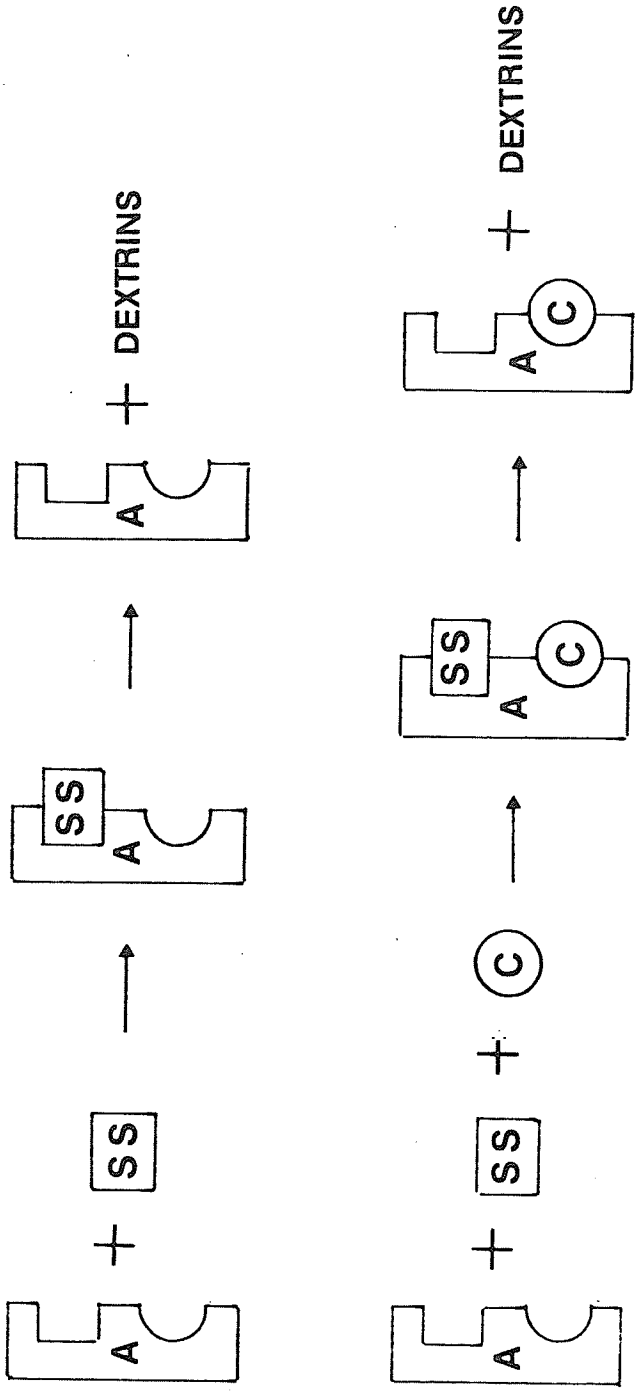
Studies with starch granules as substrate showed that the cereal enzyme could be inhibited in binding to and hydrolyzing the granules. From the results of these experiments, the gelatinized starch experiments and the affinity chromatography experiments it was concluded that cereal α-amylase was likely interacting with starch granules via the non-catalytic site. The binding process at this adsorptive site would likely be a prerequisite for efficient catalysis which may allow the enzyme to remain harnessed to the starch granule before and after making a hydrolytic scission. This "foothold" type behavior was suggested by other investigators but the evidence has thus far been indirect

Figure 21. Schematic representation of the hydrolysis of starch in solution by cereal α-amylase in the absence and presence of cycloheptaamylose.

Legend: **S S** = Starch in solution

A = α-Amylase

C = Cycloheptaamylose


A
+
SS
A
SS
+ DEXTRINS
A
+
C
A
SS
C
A
C
+ DEXTRINS

(Schwimmer and Balls, 1949a; Walker and Hope, 1963). Figure 22 is a schematic representation of what may be occurring at the surface of the starch granule in the absence and presence of cycloheptaamylose. Without the cyclodextrin starch granule degradation proceeds efficiently facilitated by adsorption via the binding site on the enzyme. With cycloheptaamylose present the binding site is occupied and the enzyme rendered inefficient in binding to the starch granule which in turn renders the catalytic site inefficient in hydrolysis.

The existence of a non-catalytic site on cereal α-amylase can be likened to the situation of rabbit muscle phosphorylase. Fletterick and Madsen (1977) have provided evidence for a non-catalytic binding site on the phosphorylase which may allow the enzyme to remain harnessed to a glycogen particle while degrading it.

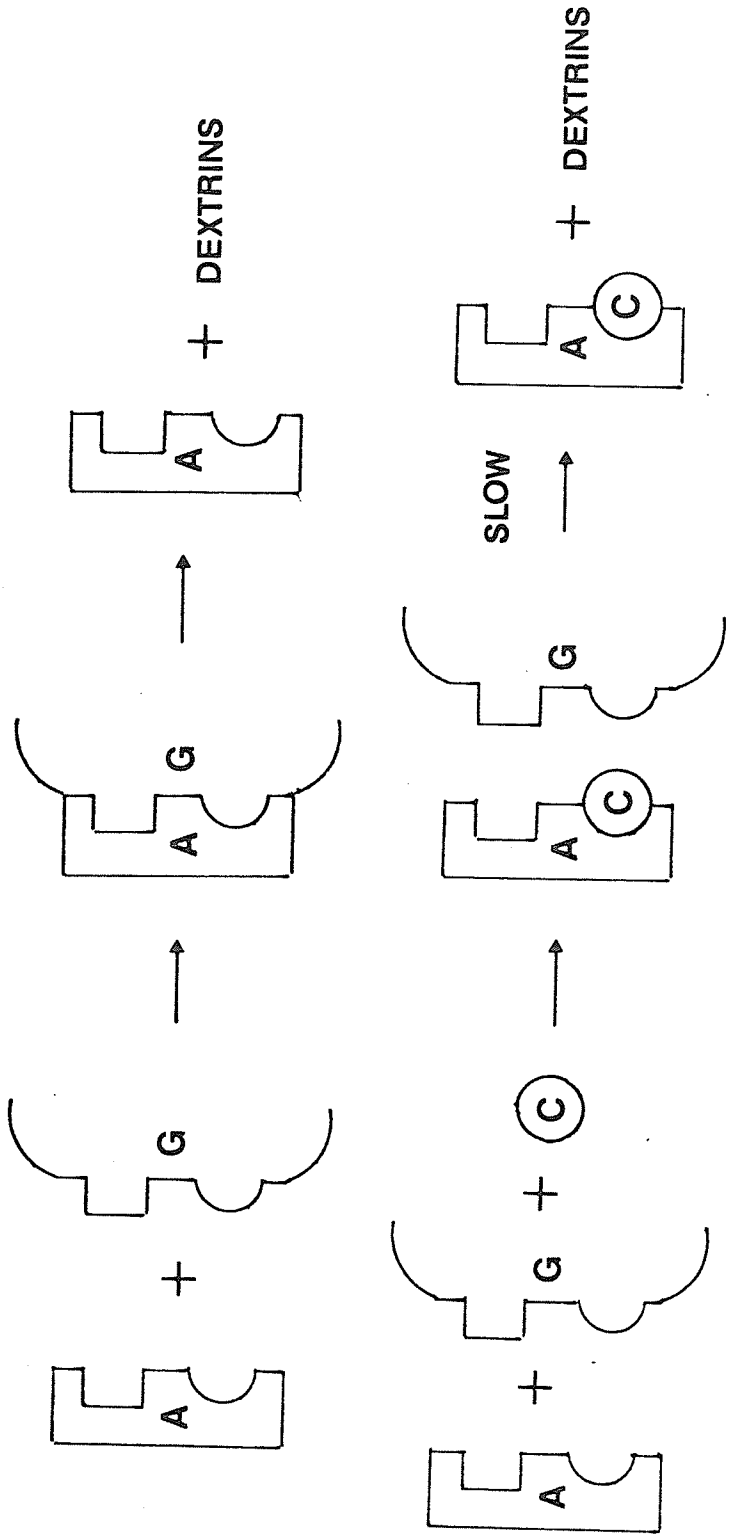
No major differences were found in the way that cycloheptaamylose inhibited binding to and hydrolysis of wheat starch granules by either α-I or α-II isozyme groups. However their appeared to be a difference in the affinity of α-amylase for waxy maize starch when compared to wheat starch. Cycloheptaamylose was found to be more efficient in inhibiting binding to and hydrolysis of the waxy maize granules. The results suggested that waxy maize starch granules may have more regions on their surface to accommodatethe binding of more enzyme. The studies clearly emphasized the importance of cycloheptaamylose as a tool in probing the relation between α-amylase and starch granules. It would be of interest to see how the affinity of α-amylase for the starch granules changes at different stages of degradation and how the effect of cycloheptaamylose differs at each stage of degradation.

Figure 22. Schematic representation of the hydrolysis at the surface of a starch granule by cereal α-amylase in the absence and presence of cycloheptaamylose.

Legend: **G** = Starch granule

A = α-Amylase

C = Cycloheptaamylose


A
G
+
G
A
+ DEXTRINS
C
+
G
A
+
A
C
SLOW
+ DEXTRINS

The process of understanding how the rate of starch granule degradation can be increased is an important industrial concern.

The procedure for preparing ^{3}H-cycloheptaamylose that was used in the binding studies was simple and straightforward since it was based on a simple oxidation step with chromic acid followed by a reduction step using $NaB^{3}H_{4}$. High specific activity ^{3}H-cycloheptaamylose was obtained and was comparable to the specific activity of ^{14}C-cyclodextrins prepared by the more involved method of Andersen et al. (1963). There is no reason to doubt that the simple procedure developed could be used with success to prepare ^{3}H-cyclohexaamylose.

Tritiated cycloheptaamylose was easily separated from cereal α-amylase indicating that the interaction was reversible. A quantitative investigation of the relation between the cyclodextrin and the cereal enzyme by equilibrium binding studies revealed a dissociation constant of 3.0×10^{-5} M. However the reason for a fractional capacity of 0.25 remained unclear. The curvature of the Scatchard plot was suggestive of non-specific binding. Alpha-amylase isozyme mixtures were used in the study (α-I and α-II). The curvature may in part be suggestive of differing affinities of the various α-amylase isozymes, within the mixture, for cycloheptaamylose. If this last speculation is true then it may be possible to preferentially elute α-amylase isozymes bound to the affinity column by using a cyclodextrin or achroic dextrin gradient. However the inhibition of α-I and α-II iso zymes in the hydrolysis of starch granules was similar indicating that the affinities of the two isozyme groups for cycloheptaamylose were probably similar. Any differences in

in affinity for cycloheptaamylose existing among the isozymes would then have to lie among the many other isozymes that comprise the two major groups.

BIBLIOGRAPHY

ABDULLAH, M., FRENCH, D. and ROBYT, J. F. 1966. Multiple attack by α-amylases. Arch. Biochem. Biophys. 114: 595-598.

ANDERSEN, G. H., ROBBINS, F. M., DOMINGUES, F. J., MOORES, R. G. and LONG, C. L. 1963. The utilization of Schardinger dextrins by the rat. Toxicol. Appl. Pharmacol. 5: 257-266.

BALLS, A. K. and SCHWIMMER, S. 1944. Digestion of raw starch. J. biol. Chem. 156: 203-211.

BANKS, W. and GREENWOOD, C. T. 1975. Starch and Its Components. Edinburgh University Press, Great Britain, pp. 191-306.

BATHGATE, G. N. and PALMER, G. H. 1972. A reassessment of the chemical structure of barley and wheat starch granules. Die Stärke 24: 336-341.

BATHGATE, G. N. and PALMER, G. H. 1973. The in vivo and in vitro degradation of barley and malt starch granules. J. Inst. Brew. 79: 402-406.

BENDER, M. L. and KOMIYAMA, M. 1978. Cyclodextrin Chemistry. Springer-Verlag, New York.

BOTES, D. P., JOUBERT, F. J. and NOVELLIE, L. 1967. Kaffircorn malting and brewing studies XVII-Purification and properties of sorghum malt α-amylase. J. Sci. Food Agric. 18: 409-415.

BOWDEN, K., HEILBRON, I. M., JONES, E. R. H. and WEEDON, B. C. L. 1946. The preparation of acetylenic ketones by oxidation of acetylenic carbinols and glycols. J. Chem. Soc. pp. 39-45.

BRIGGS, D. E. 1961. A modification of the Sandstedt, Kneen and Blish assay of α-amylase. J. Inst. Brew. 67: 427-431.

BUONOCORE, V., DEPONTE, R., GRAMENZI, F., PETRUCCI, T., POERIO, E. and SILANO, V. 1977. Purification and properties of α-amylase from chicken (Gallus gallus L.) pancreas. Molec. Cell. Biochem. 17: 11-16

BUONOCORE, V., POERIO, E. C., GRAMENZI, F. and SILANO, V. 1975. Affinity column purification of amylases on protein inhibitors from wheat kernel. J. Chromatog. 114: 109-114.

CRAMER, F. and HETTLER, H. 1967. Inclusion compounds of cyclodextrins. DieNaturwissenschaften 54: 625-632.

DIXON, M. 1953. The determination of enzyme inhibitor constants. Biochem. J. 55: 170-171.

DOANE, W. W. 1967. Quantitation of amylases in Drosophila separated by acrylamide gel electrophoresis. J. Exp. Zool. 164: 363-378.

DRONZEK, B. L., HWANG, P. and BUSHUK, W. 1972. Scanning electron microscopy of starch from sprouted wheat. Cereal Chem. 49: 232-239.

DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. and SMITH, F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350-356.

EVERS, A. D. and McDERMOTT, E. E. 1970. Scanning electron microscopy of wheat starch II. Structure of granules modified by α-amylolysis - Preliminary Report. Die Stärke 22: 23-26.

FLETTERICK, R. J. and MADSEN, N. B. 1977. X-rays reveal phosphorylase architecture. TIBS July: 145-148.

FLETTERICK, R. J., SYGUSCH, J., SEMPLE, H. and MADSEN, N. B. 1976. Structure of glycogen phosphorylase A at 3.0 Å resolution and its ligand binding sites at 6 Å. J. Biol. Chem. 251: 6142-6146.

FRENCH, D. 1957. The Schardinger dextrins. Adv. Carbohyd. Chem. 12: 189-260.

FRENCH, D. and ABDULLAH, M. 1966. Transferase activity in malt amylase preparations. Cereal Chem. 43: 55-562.

GIBBONS, G. C. 1979a. On the localization and transport of α-amylase during germination and early seedling growth of Hordeum vulgare. Carlsberg Res. Commun. 44: 353-366.

GIBBONS, G. C. 1979b. Screening for α-amylase in cereals. Improved gel-diffusion assay using a dye-labelled starch substrate. Carlsberg. Res. Commun. 44: 21-25.

GREEN, D. E. and STUMPF, P. K. 1942. Starch phosphorylase of potato. J. Biol. Chem. 142: 355-366.

GREENWAY, R. M., KENT, P. W. and WHITEHOUSE, M. W. 1953. Detection of non-reducing carbohydrates in paper electrophoresis and chromatography. Research 6: 6S-7S.

GREENWOOD, C. T. and MILNE, E. A. 1968. Starch degrading and synthesizing enzymes: A discussion of their properties and action patterns. Adv. Carbohyd. Chem. 23: 281-366.

HAO, K., TAKEUCKI, T., SATO, S. and SUGIMURA, T. 1977. Effects of albumin and other proteins during assay of amylase activity. Clin. Chem. Acta 79: 75-80.

HARDING, K. E., MAY, L. M. and DICK, K. F. 1975. Selective oxidation of allylic alcohols with chromic acid. J. Org. Chem. 40: 1664-1665.

HARE, D. L., STIMPSON, D. I. and CANN, J. R. 1978. Multiple bands produced by interaction of a single macromolecule with carrier ampholytes during isoelectric focusing. Arch. Biochem. Biophys. 187: 274-275.

HELLER, J. and SCHRAMM, M. 1964. α-Amylase limit dextrins of high molecular weight obtained from glycogen. Biochim. Biophys. Acta 81: 96-100.

HELWIG, J. T. and COUNCIL, K. A. 1979. SAS User's Guide. SAS Institute Inc., North Carolina.

HEYRAUD, A. and RINAUDO, M. 1978. Gel permeation chromatography of glucose oligomers on polyacrylamide gels - Thermodynamic and steric partition mechanisms. J. Chromatog. 166: 149-158.

HOKSE, H. 1980. Analysis of cyclodextrins by high-performance liquid chromatography. J. Chromatog. 189: 98-100.

HOSCHKE, A, LASZLO, E. and HOLLO, J. 1976. Application of cyclohexaamylose ligand affinity chromatography for the analysis of amylolytic enzymes. 27th Starch Convention, Detmold, W. Germany, April 28 to 30, Lecture No. 1.5.

JACOBSEN, J. V. 1980. Biochemical and physiological aspects of aleurone behavior associated with barley germination. The Australian Biochemical Society and The Royal Australian Chemical Institute Cereal Chemistry Division: Workshop on biochemical, nutritional and industrial aspects of starch and other cereal carbohydrates. Melbourne, Australia, July 2 and 3.

KITAHATA, S., OKADA, S. and FUKUI, T. 1978. Acceptor specificity of the transglycosylation catalyzed by cyclodextrin glycosyltransferase. Agric. Biol. Chem. 42: 2369-2374.

KLOTZ, I. M., WALKER, F. M. and PIVAN, R. B. 1946. The binding of organic ions by proteins. J. Amer. Chem. Soc. 68: 1486-1490.

KNEEN, E., SANDSTEDT, R. M. and HOLLENBECK, C. M. 1943. The differential stability of the malt amylases - separation of the alpha and beta components. Cereal Chem. 20: 399-423.

KONDO, H. NAKATANI, H. and HIROMI, K. 1976. Analysis of mixtures of α- and β-cyclodextrins using fluorescent dyes. Carbohyd. Res. 52: 1-10.

KRUGER, J. E. and TKACHUK, R. 1969. Wheat α-amylases. I. Isolation. Cereal Chem. 46: 219-226.

LAMMERS, J. N. J. J. 1969. Reproducible separation of α- and β-cyclodextrin on charcoal columns. J. Chromatog. 41: 462-466.

LEACH, H. W. and SCHOCH, T. J. 1961. Structure of the starch granule. II. Action of various amylases on granular starches. Cereal Chem. 38: 34-46.

LEHNINGER, A. L. 1978. Biochemistry. Worth Publishers Inc., New York, pp. 197-201.

LEVITSKI, A., HELLER, J. and SCHRAMM, M. 1964. Specific precipitation of enzyme by its substrate: The α-amylase-macrodextrin complex. Biochim. Biophys. Acta 81: 101-107.

LINEWEAVER, H. and BURK, D. 1934. The determination of enzyme dissociation constants. J. Amer. Chem. Soc. 56: 658-666.

LOOMIS, W. D. 1974. Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. In Meth. Enzymol. 31: 528-544.

LOWRY, O H., ROSEBROUGH, H. J., FARR, A. L. and RANDALL, R. J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

LOYTER, A. and SCHRAMM, M. 1966. Multimolecular complexes of α-amylase with glycogen limit dextrin. J. Biol. Chem. 241: 2611-2617.

MacGREGOR, A. W. 1976. A note on the formation of α-amylase in de-embryonated barley kernels. Cereal Chem. 53: 792-796.

MacGREGOR, A. W. 1978. α-Amylase I from malted barley - physical properties and action pattern on amylose. Cereal Chem. 55: 754-765.

MacGREGOR, A. W. 1979. Isolation of large and small granules of barley starch and a study of factors influencing the adsorption of barley malt α-amylase by these granules. Cereal Chem. 56: 430-434.

MacGREGOR, A. W. and BALLANCE, D. 1980a. Quantitative determination of α-amylase enzymes in germinated barley after separation by iso-electricfocusing. J. Inst. Brew. 86: 131-133.

MacGREGOR, A. W. and BALLANCE, D. 1980b. Hydrolysis of large and small granules from normal and waxy barley cultivars by alpha-amylases from barley malt. Cereal Chem. 57: 397-402.

MacGREGOR, A. W., THOMPSON, R. G. and MEREDITH, W. O. S. 1974. α-Amylase from immature barley: purification and properties. J. Inst. Brew. 80: 181-187.

MANNERS, D. J. and MARSHALL, J. J. 1972. Studies on carbohydrate metabolizing enzymes. Part XXVIII. Preparation and some properties of α-amylase isoenzymes from malted rye. Die Stärke 24: 3-8.

MARCHYLO, B., KRUGER, J. E. and IRVINE, G. N. 1976. α-Amylases from immature hard red spring wheat. I. Purification and some chemical and physical properties. Cereal Chem. 53: 157-173.

MARSHALL, J. J. 1973. On the interaction of β-amylase with substrate and inhibitors with comments on Koshland's induced fit hypothesis. Eur. J. Biochem. 33: 494-499.

McLAREN, A. D. 1963. Enzyme reactions in structurally restricted systems IV. The digestion of insoluble substrates by hydrolytic enzymes. Enzymologia XXVI: 237-246.

McLAREN, A. D. and PACKER, L. 1970. Some aspects of enzyme reactions in heterogeneous systems. Adv. Enzymol. 33: 245-308.

McLEAN, C., WERNER, D. A. and AMINOFF, D. 1973. Quantitative determination of reducing sugars, oligiosaccharides and glycoproteins with (^{3}H) borohydride. Anal. Biochem. 55: 85-92.

MEREDITH, P. 1981. Large and small starch granules in wheat - are they really different? Starch/Stärke 33: 40-44.

MÓRA, S., SIMON, I. and ELÖDI, P. 1974. Studies on the active center of pancreatic amylases. I. Binding of β-cyclodextrin. Molec. Cell. Biochem. 4: 205-209.

NELSON, N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153: 375-380.

NISHIKAWA, K. and NOBUHARA, M. 1971. Genetic studies of α-amylase isozymes in wheat. I. Location of genes and variation in tetra- and hexaploid wheat. Jap. J. Genetics 46: 345-353.

OHNISHI, M. 1971. Studies of the interaction of substrate analogues with bacterial liquefying α-amylase by means of spectrophotometry and steady state kinetics. J. Biochem. 69: 181-189.

OHNISHI, M., HATANO, H. and HIROMI, K. 1973. Difference-spectrophotometry of the interaction of cycloheptaamylose with saccharifying α-amylase from Bacillus subtilis. J. Biochem. 74: 519-524.

OLERED, R. and JÖNSSON, G. 1970. Electrophoretic studies of α-amylase in wheat II. J. Sci. Food Agric. 21: 385-392.

PARRISH, F. W., SMITH, E. E. and WHELAN, W. J. 1970. Actions of starch carbohydrases on chemically modified maltodextrins. Arch. Biochem. Biophys. 137: 185-189.

PREISS, J. and KOSUGE, T. 1976. Regulation of enzyme activity in metabolic pathways. In Plant Biochemistry. Ed. by J. Bonner and J. E. Varner, Academic Press, New York, pp. 277-336.

PURICH, D. L. and FROMM, H .J. 1972. Limitations in the use of Dixon plots to evaluate enzyme inhibition. Biochem. Biophys. Acta 268: 1-3.

ROBYT, J. F. and FRENCH, D. 1967. Multiple attack hypothesis of α-amylase action: action of porcine pancreatic, human salivary and Apergillus oryzae α-amylases. Arch. Biochem. Biophys. 122: 8-16.

ROBYT, J F. and FRENCH, D. 1970. Multiple attack and polarity of action of porcine pancreatic α-amylase. Arch. Biochem. Biophys. 138: 662-670.

ROBYT, J. F. and WHELAN, W. J. 1968. In Starch and Its Derivatives. Ed. J. A. Radley, Chapman Hall Ltd., London, p. 432.

SANDSTEDT, R. M. and MATTERN, P. J. 1960. Damaged starch. Quantitative determination in flour. Cereal Chem. 37: 379-390.

SARGEANT, J. G. and WALKER, T. S. 1978. Adsorption of wheat alpha-amylase isoenzymes to wheat starch. Starch/Stärke 30: 160-163.

SCATCHARD, G. 1949. The attractions of proteins for small molecules and ions. N. Y. Acad. Sci. 51: 660-672.

SCATCHARD, G., SCHEINBERG, I. H. and ARMSTRONG, G. H. Jr. 1950. Physical chemistry of protein solutions. IV. The combination of human serum albumin with chloride ion. J. Amer. Chem. Soc. 72: 535-540.

SCHRAMM, M. and LOYTER, A. 1966. Purification of α-amylases by precipitation of amylase -glycogen complexes. In Meth. Enzymol. 8: 533-537.

SCHWIMMER, S. and BALLS, A. K. 1949a. Isolation and properties of crystalline α-amylase from germinated barley. J. Biol. Chem. 179: 1063-1075.

SCHWIMMER, S. and BALLS, A. K. 1949b. Starches and their derivatives as adsorbents for malt α-amylase. J. Biol. Chem. 180: 883-894.

SELINGER, Z. and SCHRAMM, M. 1963. An insoluble complex formed by the interaction of muscle phosphorylase with glycogen. Biochem. Biophys. Res. Commun. 12: 210-214.

SHIMOMURA, S. and FUKUI, T. 1980. A comparative study on α-glucan phosphorylase from plant and animal: interrelationship between the polysaccharide and pyridoxal phosphate binding sites by affinity electrophoresis. Biochemistry 19: 2287-2294.

SILANO, V., FURIA, M., GIANFREDA, L., MACRI, A., PALESCANDOLO, R., RAB, A., SCARDI, V., STELLA, E. and VALFRE, F. 1975. Inhibition of amylases from different origins by albumins from the wheat kernel. Biochim. Biophys. Acta 391: 170-178.

SILVANOVICH, M. P. 1977. α-Amylase from triticale 6A190: isolation and characterization. Ph.D. Thesis. University of Manitoba.

SILVANOVICH, M. P. and HILL, R. D. 1976. Affinity chromatography of cereal α-amylase. Anal. Biochem. 73: 430-433.

SILVANOVICH, M. P. and HILL, R. D. 1977. α-Amylases from triticale 6A190: purification and characterization. Cereal Chem. 54: 1270-1281.

SIMON, I., MÓRA, S. and ELÖDI, P. 1974. Studies on the active centre of pancreatic amylase. II Small angle x-ray scattering investigations. Molec. Cell. Biochem. 4: 211-216.

STAERK, J. and SCHLENK, H. 1967. Interaction of potatoe phosphorylase with cycloamyloses. Biochim. Biophys. Acta 146: 120-128.

STAHL, E. and KALTENBACH, U. 1961. Dunnschicht-chromatographie. VI Mitteilung. Spurenanalyse von zukergemischen duf kieselgur g-schichten. J. Chromatog. 5: 341-350.

STAMBERG, O. E. and BAILEY, C. H. 1939. Studies on wheat starch. II The action of amylases on raw wheat starches. Cereal Chem. 16: 319-329.

SUETSUGU, N., KOYAMA, S., TAKEO, K. and KUGE, T. 1974. Kinetic studies on the hydrolysis of α-, β- and ϒ-cyclodextrins by Taka-amylase A. J. Biochem. 76: 57-63.

SWANSON, M. A. and CORI, C. F. 1948. Studies on the structure of polysaccharides. I Acid hydrolysis of starch-like polysaccharides. J. Biol. Chem. 172: 797-804.

SZEJTLI, J., TETENYI, M. and TETENYI, P. 1980. Agent for effecting the germination of seeds and growth of seedlings. Chemical Abstracts. 92: 207.

THAKUR, A. K., JAFFE, M. L. and RODBARD, D. 1980. Graphical analysis of ligand-binding systems: evaluation by Monte Carlo studies. Anal. Biochem. 107: 279-295.

THOMA, J. A. 1974. Interaction of β-amylase with substrates and inhibitors with comments on Koshland's induced fit theory. A reply. Eur. J. Biochem. 44: 139-142.

THOMA, J. A. and KOSHLAND, D. E. Jr. 1960. Competitive inhibition by substrate during enzyme action. Evidence for the induced fit theory. J. Amer. Chem. Soc. 82: 3329-3333.

THOMA, J. A., KOSHLAND, D. E. Jr., RUSCICA, J. and BALDWIN, R. 1963a. The number of binding sites of sweet potato beta-amylase. Biochem. Biophys. Res. Commun. 12: 184-188.

THOMA, J. A., SPRADLIN, J. E. and DYGERT, S. 1971. In The Enzymes, Ed. by P. D. Boyer, Academic Press, New York. Vol. 5, pp. 115-189.

THOMA, J. A., WAKIM, J. and STEWART, L. 1963b. Comparison of the active sites of alpha- and beta-amylases. Biochem. Biophys. Res. Commun. 12: 350-355.

TKACHUK, R. 1975. Competitive affinity chromatography of wheat α-amylase. FEBS Letters 52: 66-68.

TKACHUK, R. and KRUGER, J. E. 1974. Wheat α-amylases. II. Physical characterization. Cereal Chem. 51: 508-529.

VRETBLAD, P. 1974a. Immobilization of ligands for biospecific chromatography via their hydroxyl groups. The cyclohexa-amylose-β-amylase system. FEBS Letters 47: 86-89.

VRETBLAD, P. 1974b. Biospecific affinity chromatography of sweet potato β-amylase. Biochem. Soc. Trans. 2: 1327-1328.

WALKER, G. J. and HOPE, P. M. 1963. The action of some α-amylases on starch granules. Biochem. J. 86: 452-462.

WARCHALEWSKI, J. R. and TKACHUK, R. 1978. Durum wheat α-amylases: isolation and purification. Cereal Chem. 55: 146-156.

WEBER, K. and OSBORN, M. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244: 4406-4412.

WEILL, C. E. and HANKE, P. 1962. Thin-layer chromatography of malto-oligiosaccharides. Anal. Chem. 34: 1736-1737.

WIEDENHOF, N. 1964. Thin-layer chromatography of cyclodextrins and some other sugars using microchromatoplates. J. Chromatog. 15: 100-102.

ZSADON, B., SZILASI, M., SZEJTLI, J., SERES, G. and TÜDÖS, F. 1978. Chromatography of alpha-, beta- and gamma-cyclodextrin on dextran gel columns. Starch/Stärke 30: 276-279.