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AN ENDOGENOUS α-AMYLASE INHIBITOR FROM BARLEY KERNELS

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

Randall Joseph Weselake

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

Department of Plant Science

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submitted by Randall Joseph Weselake

in partial fulfilment of the requirements for the Ph.D. degree.

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AN ENDOGENOUS a-AMYLASE INHIBITOR FROM BARLEY KERNELS

BY

RANDALL JOSEPH WESELAKE

A thesis submitted to the Faculty of Graduate Studies of the University of Manitoba in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure

Page

Figure

Page

ABSTRACT

Weselake, Randall Joseph, Ph.D., The University of Manitoba, October 1984. An Endogenous α-Amylase Inhibitor from Barley Kernels.

Major Professors: Dr. A.W. MacGregor and Dr. R.D. Hill.

An inhibitor of malted barley α-amylase II and germinated wheat α-amylase II was purified 125-fold from a crude extract of barley kernels by $(NH_4)_2SO_4$ fractionation, ion exchange chromatography on DEAE-Sephacel and gel filtration chromatography on Bio-Gel P-60. The inhibitor was a protein with a molecular weight of 21,000 daltons as determined by sodium dodecyl sulphate gel electrophoresis. A molecular weight of 20,000 daltons was determined for the inhibitor by gel filtration chromatography. The inhibitor had an isoelectric point of 7.3. Heat treatment of the inhibitor at 70°C for 15 minutes resulted in considerable loss of inhibitor activity. Amino acid analysis indicated the presence of about 9 half-cystine residues per mole. The neutral isoelectric point of the inhibitor suggested that some of the apparently acidic residues (glutamic and aspartic) existed in the amide form. The first twenty N-terminal amino acids were sequenced. Some homology appeared to exist between α-amylase II inhibitor and trypsin inhibitor from barley. Isoelectric focusing of a mixture of α-amylase II and inhibitor resulted in the formation of a complex with an isoelectric point different from that of either inhibitor or enzyme alone. Complex formation between α-amylase II and the inhibitor was detected,

also, by the appearance of a new molecular weight species after gel filtration chromatography on Bio-Gel P-100. The enzyme-inhibitor complex was retained on a column of cycloheptaamylose-epoxy Sepharose 6B suggesting that the site of inhibitor binding for enzyme was different than the site of cycloheptaamylose binding on the enzyme.

Enzyme and inhibitor had to be pre-incubated for 5 minutes before maximum inhibition was attained. Inhibition increased with an increase in pH values from 5 to 8 and decreased when the salt concentration was raised from zero to 200 mM NaCl, suggesting that the interaction between α-amylase II and inhibitor was due to charge effects. Inhibition of starch granule hydrolysis was affected by pH and salt concentration but temperatures ranging from 15°C to 35°C had no effect. Inhibition was independent of starch granule concentration suggesting that the inhibitor could be effective in inhibiting α-amylase in the endosperm where starch concentration is high. Starch granules hydrolyzed by α-amylase II in the presence of inhibitor were not degraded as extensively as granules hydrolyzed without inhibitor. The inhibitor did not appear to alter the way in which linear amylose or starch granules were hydrolyzed by α-amylase II, but the main effect was a decrease in the rate of hydrolysis. The inhibitor had no effect on α-amylase I from either malted barley or germinated wheat, nor did the inhibitor have any effect on mammalian α-amylases, thus showing the biospecificity of the inhibitor for cereal α-amylase II.

Pearling studies indicated that inhibitor activity was distributed throughout the endosperm of barley kernels. All cultivars of barley, wheat, rye and triticale that were examined contained inhibitor activity, but activity was not found in sorghum, oats, millet, rice or

maize. Immunochemical studies showed that barley cultivars contained a protein that shared complete immunochemical identity with the purified inhibitor. Wheat, rye and triticale cultivars contained proteins that shared partial immunochemical identity with the purified inhibitor. Sorghum, oats, millet, rice and maize did not elicit an immunochemical response with antibody directed against purified inhibitor. These studies suggested that endogenous α-amylase inhibitors were not restricted to barley but were present, also, in other cereals.

INTRODUCTION

It has been known for some time that cereals contain proteins with inhibitor activity against animal α-amylases (Kneen and Sandstedt, 1943, 1946; Shainkin and Birk, 1970) but endogenous inhibitors of cereal α-amylase have been reported only recently (Blanco-Labra and Iturbe-Chinas, 1981; Meredith and Jones, 1982; Warchalewski, 1977a). These endogenous inhibitors could regulate starch degradation in the germinating seed by controlling α-amylase activity.

Isoelectric focusing experiments have shown that an extract of green malt contained three major groups of α-amylase, designated α-amylases I, II and III, in order of increasing isoelectric point (MacGregor and Ballance, 1980a). Determination of enzyme activity in the three α-amylase groups, showed that group III contained most of the activity, but if the extract was heat treated prior to focusing, the activity appearing as α-amylase III was reduced substantially and reappeared in the form of α-amylase II. Furthermore, preliminary investigations indicated that the endosperm of barley kernels contained a factor which could convert α-amylase II into α-amylase III. The present study describes the purification and characterization of a heat labile protein from barley kernels that converts α-amylase II into α-amylase III and biospecifically inhibits α-amylase II. The fundamental physicochemical properties of the inhibitor were determined. In attempting to elucidate a physiological role for the inhibitor, experiments were designed to show formation of an enzyme-inhibitor complex and the

effect of various experimental conditions on the enzyme-inhibitor interaction were studied in vitro using both solubilized starch and starch granules. Both an inhibitor assay and antibodies, raised against purified inhibitor, were used to detect the inhibitor in a number of different cereals.

LITERATURE REVIEW

Cereal α-Amylases

Alpha-amylase plays a major role in the mobilization of starch reserves in germinating cereal kernels. Most of the studies on biosynthesis of cereal α-amylase have been confined to barley because of the technological importance of this enzyme system in malting. It is well established that α-amylase is synthesized de novo by barley aleurone layers (Briggs, 1963, 1964; Filner and Varner, 1967) and that this process can be stimulated by application of gibberellic acid (Chrispeels and Varner, 1967; Paleg, 1960). In germinating barley, however, both the aleurone layer and embryo have been shown to contain α-amylase (Briggs, 1964). The relative amounts of α-amylase synthesized by the aleurone and embryo tissues has been a subject of controversy. For example, MacLeod and Palmer (1966) reported that the embryo did not produce α-amylase whereas Gibbons (1979; 1981) suggested that the embryo was a major contributor of the enzyme. Scanning electron microscopy studies of germinating barley kernels have shown that starch degradation started in an area of the endosperm close to the embryo (MacGregor, 1980). This conclusion was reached, also, in similar studies with germinating durum wheat (MacGregor and Matsuo, 1982). Studies on the biosynthesis of rice α-amylase have shown that the embryo was a major contributor of α-amylase in early stages of rice germination (Miyata et al., 1981; Okamoto and Akazawa, 1979). In

germinating sorghum, however, the embryo was found to be the only producer of α-amylase (Aisien and Palmer, 1983; Aisien et al., 1983).

Schwimmer and Balls (1948) were the first investigators to prepare malt α-amylase in crystalline form and since then the enzyme has been characterized in detail. Frydenberg and Nielson (1965) made an in-depth study of the polymorphism of germinated barley α-amylase using zone electrophoresis in agar gels. Following electrophoresis of extracts, activity staining revealed a number of α-amylase components. Heat treatment of the extracts prior to electrophoresis resulted, however, in disappearance of some enzyme bands and subsequent enrichment of other bands. Jacobsen et al. (1970) have used a similar electrophoretic procedure to examine α-amylase components produced by isolated aleurone layers, but fewer components were resolved when the banding patterns were compared to the results of Frydenberg and Nielson (1965).

The resolution of α-amylase components has been improved considerably by analytical isoelectric focusing in flat-bed polyacrylamide gels (MacGregor, 1976). Studies using this technique demonstrated that a green malt of barley contained three main α-amylase groups, designated α-amylases I, II and III in order of increasing isoelectric point (MacGregor and Daussant, 1979). Alpha-amylases II and III were found to be related immunochemically (MacGregor and Daussant, 1981). Quantitative experiments showed that a large proportion of the total activity, extractable from gels after isoelectric focusing, was in the form of α-amylase III, and heat treatment of extracts prior to focusing resulted in conversion of a large proportion of α-amylase III into α-amylase II (MacGregor and Ballance, 1980a). More recently, it has

been demonstrated that α-amylase III is a complex of α-amylase II and a heat labile proteinaceous inhibitor of α-amylase II (Mundy et al., 1983; Weselake et al., 1983a). Barley α-amylase I has been shown to make up only a very small proportion of the total α-amylase activity in an extract of green malt (MacGregor and Ballance, 1980a). This enzyme component was not affected by heat treatment.

Alpha-amylases purified from aleurone layers of Himalaya barley and from kernels of germinated wheat and triticale have been shown, by isoelectric focusing, to consist of two major groups of enzymes separated by about one pH unit (Jacobsen and Higgins, 1982; Sargeant, 1980; Sargeant and Walker, 1978; Silvanovich and Hill, 1977). The presence of two major groups of α-amylase in malted wheat was demonstrated originally by Olered and Jonsson (1970) using agar gel electrophoresis. The α-amylase I group of wheat, however, has been shown to constitute a considerably larger proportion of the total α-amylase activity when compared to the proportion of α-amylase I in barley malts (MacGregor and Ballance, 1980a; Sargeant and Walker, 1978).

It has been shown that the two major groups of α-amylase in germinated wheat are under independent genetic control (Gale, 1983; Nishikawa and Nobuhara, 1971; Nishikawa et al., 1981). A similar explanation has been provided for the heterogeneity of barley α-amylase based on experiments with isolated aleurone layers and embryoless half seeds (Callis and Ho, 1983; Jacobsen and Higgins, 1982). Furthermore, the barley experiments appeared to indicate that the expression of two α-amylase genes or groups of genes was controlled differentially at the transcription level, by gibberellic acid (GA_3). This process was dir-

ected, probably, by two or more different populations of mature messenger ribonucleic acid (mRNA) molecules.

Each α-amylase group has been shown to consist of a number of minor components (Jacobsen and Higgins 1982; Nishikawa and Nobuhara, 1971; Sargeant and Walker, 1978; Tkachuk and Kruger, 1974). This micro-heterogeneity may have resulted from differential expression of one gene series during germination (Nishikawa et al., 1981). Jacobsen and Higgins (1982) have suggested that minor components of major enzyme groups may represent different polypeptides derived from different mRNAs that are transcribed from a single gene.

Post-translational effects also might account for the differences within major α-amylase groups. Motojima and Sakaguchi (1982) have found differences in the degree of trimethylation of lysyl residues in wheat α-amylase and have suggested that the minor components of α-amylase could result from differential trimethylation of the enzyme following translation. These authors concluded that studies of α-amylase genes, using zymogram analysis, were questionable because isoelectric points of minor α-amylase components might not reflect differences related to gene expression.

Cereal α-amylases are monomeric proteins with a generally accepted molecular weight ranging from about 40,000 to 45,000 daltons (Greenwood and Milne, 1968c; Tkachuk and Kruger, 1974; Rodaway, 1978). Gel permeation chromatography in both Sephadex and Bio-Gel matrices has often resulted in determination of anomalously low molecular weights for cereal α-amylases (Manners and Marshall, 1972; Marchylo et al., 1976).

Calcium is believed to stabilize the tertiary structure of

α-amylases and thereby preserve enzyme activity (Stein et al., 1964). Plant α-amylases were found to have a relatively low affinity for calcium when compared to α-amylases from other sources (Fischer and Stein, 1960). The two major groups of α-amylase produced by isolated aleurone layers have been shown to differ in their sensitivity to chelating agent (Jacobsen et al., 1970). The low isoelectric point group was insensitive to ethylenediaminetetraacetic acid (EDTA) whereas the more cathodal enzyme group was inactivated by chelating agent. Furthermore, calcium was required for the secretion of the more cathodal α-amylase group from the aleurone layer (Jones and Jacobsen, 1983).

In the starchy endosperm, cereal α-amylase interacts with both starch granules and solubilized starch components released by the enzyme from the granules. The optimum pH for this hydrolytic process has been shown to be about pH 5.5 (Greenwood and MacGregor, 1965). Kinetic studies have been conducted using linear amylose as substrate since all products of hydrolysis are linear and relatively easily analyzed. Starch components in solution were hydrolyzed in two distinct phases by the cereal enzyme (Greenwood and MacGregor, 1965; Greenwood and Milne, 1968a, 1968b, 1968c). Initially, there was a rapid phase of hydrolysis where α(1→4) linkages were hydrolyzed randomly. The second, slow phase of hydrolysis, was more selective or non-random because small products formed during the initial phases were hydrolyzed with more difficulty in the second phase (Bird and Hopkins, 1954). With linear amylose as substrate, glucose oligomers of 2 and 6 glucose units in length eventually predominated in the reaction mixture (Greenwood and Milne, 1968a). The difficulty with which smaller maltodextrins (less than 8 glucose units in length) were hydrolyzed has been attributed to a differential susceptibility to hydrolysis of certain α(1→4)

linkages near the reducing and non-reducing ends of the starch chain (Bird and Hopkins, 1954; Greenwood et al., 1965).

Hydrolysis of starch granules by cereal α-amylase is a considerably slower process than hydrolysis of solubilized starch. Starch granules are known to adsorb α-amylases (McLaren, 1963; Walker and Hope, 1963). Evidence has been presented showing that the cereal enzyme was adsorbed to starch granules by a non-catalytic site which facilitated starch granule hydrolysis (Schwimmer and Balls, 1949b; Weselake and Hill, 1983). The degree of adsorption of barley α-amylase decreased with an increase in temperature and the pH optimum for enzyme adsorption was similar to the pH optimum for the hydrolysis of starch in solution (Greenwood and MacGregor, 1965; MacGregor, 1979). Alpha-amylase II from both germinated wheat and malted barley has been shown to hydrolyze starch granules, but there have been conflicting reports on the ability of α-amylase I to hydrolyze starch granules (MacGregor and Ballance, 1980b; Sargeant, 1980; Sargeant and Walker, 1978; Weselake and Hill, 1983).

The efficiency of hydrolysis of starch granules has been related to the structural components of the granule. Starches with high amylopectin content were hydrolyzed faster than normal starches by α-amylase (Goering and Eslick, 1976; Leach and Schoch, 1961; MacGregor and Ballance, 1980b). Barley starch was shown to consist of two distinct populations of granules (MacGregor et al., 1971a; May and Buttrose, 1959; Palmer, 1972). Although large granules comprised about 90% of the total starch by weight, the small granules outnumbered the large granules (Bathgate and Palmer, 1972). Small starch granules were degraded faster than large granules by barley α-amylases (MacGregor and

Ballance, 1980b; Palmer, 1972). In wheat, however, small starch granules were hydrolyzed more slowly than large granules by wheat α-amylase (Lineback and Ponpipom, 1977; Palmer, 1972).

Inhibitors of α-Amylase

Naturally occurring compounds with inhibitor activity against α-amylase have been reported in plants and cultures of micro-organisms. Early investigations by Chrzaszcz and Janicki (1934) suggested that buckwheat malt contained insoluble components which inhibited α-amylase activity by adsorbing the enzyme from solution. The nature of this adsorbent, however, was not characterized or investigated further.

Products of α-amylase-catalyzed hydrolysis of starch have been shown to inhibit α-amylase activity (Elodi *et al.*, 1972; Schwimmer, 1950). Presumably, maltodextrins, resembling internal segments of the starch molecule, accumulate in enzymatic digests of starch and interfere eventually with the ability of α-amylase to interact with the substrate. Cycloamyloses or cyclic oligomers of maltodextrins, produced by *Bacillus macerans* amylase, have been shown to inhibit various α-amylases by behaving as substrate analogues (French, 1957; Mora *et al.*, 1974; Ohnishi, 1971; Weselake and Hill, 1983). The high affinity of α-amylases for cycloamyloses has allowed the cyclic oligomers to be used effectively as immobilized ligands for purification of α-amylases (Hoschke *et al.*, 1976; Silvanovich and Hill, 1976).

Polyphenolic compounds or tannins from certain varieties of sorghum and legume seeds have been reported to inactivate α-amylases as

well as a number of other enzymes (Daiber, 1975; Griffiths, 1981; Miller and Kneen, 1947; Strumeyer and Malin, 1969). Tannins have been shown to inactivate enzymes required during brewing of sorghum beer (Daiber, 1975). Furthermore, high levels of polyphenolics in cereals and legumes could reduce the nutritional value of such seeds as animal feed (Chang and Fuller, 1964; Griffiths, 1981). Presumably, the polyphenolics decrease the effectiveness of digestive enzymes.

Inhibitors of intestinal α-glucosidases have been found in microbial cultures of the genera Actinoplanes and Streptomyces (Omoto et al., 1981; Schmidt et al., 1977). These inhibitors were shown to be complex oligosaccharides containing some nitrogen. Small molecular weight polypeptides, with anti-amylase activity, have been isolated from cultures of the genera Streptomyces, also (Aschauer et al., 1981; Murao et al., 1980, 1981). Inhibitors from microbial sources have been considered as possible therapeutic agents for the treatment of diabetes, obesity and related metabolic disorders (Puls et al., 1977; Sachse and Williams, 1979).

The cereal kernel contains proteins with inhibitor activity against α-amylases from various sources. Kneen and Sandstedt (1943, 1946) reported that a heat stable component of wheat flour inhibited α-amylases from some mammalian and bacterial sources, but fungal and cereal α-amylases were not affected by this component. Since then heat stable inhibitor proteins of various animal α-amylases have been found in the albumin fraction of wheat flour and the inhibitors constituted about 1% of the flour (Shainkin and Birk, 1970; Silano et al., 1973; Silano, 1978). Other inhibitor proteins have been isolated from non-cereal plant sources such as kidney bean (Marshall and Lauda, 1975),

peanut cotyledons (Inshad and Sharma, 1981), Colocasia antiquorum tubers (Sharma and Pattabiraman, 1980) and yam tubers (Sharma and Pattabiraman, 1982). The inhibitor proteins of α-amylase and their characteristics have been the subject of a number of reviews (Buonocore et al., 1977; Dreher et al., 1984; Marshall, 1975; Silano, 1978; Warchalewski, 1983; Whitaker, 1983).

Deponte et al. (1976), using gel filtration chromatography, separated three α-amylase inhibitors from wheat flour. These inhibitors had molecular weights of 60,000, 24,000 and 12,000 daltons. The most thoroughly studied of these inhibitors were the 24,000 and 12,000 dalton components and they were coded 0.19 and 0.28, respectively, based on their electrophoretic mobility relative to bromophenol blue at pH 8.5 (Deponte et al., 1976, Silano et al., 1973). Sodium dodecyl sulphate treatment disrupted the 24,000 dalton species (0.19) into two monomers which were quite similar to the 12,000 dalton (0.28) native monomer (Deponte et al., 1976). One mole of reducing sugar (glucose equivalent) was bound covalently to each mole of inhibitor subunit and these sugars have been implicated as being recognition sites for the binding of α-amylase (Buonocore et al., 1980; Petrucci et al., 1978). Studies have shown that the inhibitors (0.19 and 0.28) have a high affinity for α-amylases from chicken pancreas and yellow mealworm (Buonocore et al., 1980, 1984). Complex formation between 0.19 inhibitor albumin and mealworm α-amylase was demonstrated visually by gel electrophoresis, at pH 8.5, of enzyme-inhibitor mixtures (Buonocore et al., 1976). The complex had an electrophoretic mobility which was different from the mobilities of the two contributing species. Enzyme-

inhibitor complexes were detected also after gel filtration chromatography of enzyme-inhibitor mixtures (Buonocore et al., 1976; 1980). The binding ratios of inhibitor to enzyme were 1:1 and 2:1 for the 0.19 and 0.28 inhibitors, respectively, and it has been suggested that the enzyme-inhibitor complexes are stabilized by ionic bonding (Buonocore et al., 1976; 1980).

Other inhibitor proteins of mammalian α-amylase have been isolated from cereals. Granum and Whitaker (1977) isolated from wheat an inhibitor with an electrophoretic mobility of 0.55, based on gel electrophoresis at pH 8.5. The inhibitor had a molecular weight of 30,000 daltons and an isoelectric point of 4.2. It was effective against human α-amylase but not porcine pancreatic α-amylase.

Maeda et al. (1982) have found an inhibitor in wheat (coded 0.53) that was 500 times more effective in inhibiting human salivary α-amylase than pancreatic α-amylase. The native molecular weight of this inhibitor was 24,000 daltons, but following sodium dodecyl sulphate treatment, the inhibitor dissociated into two subunits.

Other investigators (O'Connor and McGeeney, 1981a; O'Connor and McGeeney, 1981b; O'Donnell and McGeeney, 1976) have isolated inhibitor proteins, from wheat flour, similar to those described by Deponte et al. (1976). Studies were conducted with those inhibitors and human α-amylases in an attempt to develop methods to differentiate between salivary and pancreatic α-amylases in human serum. The binding of inhibitor to human α-amylase did not appear to occur at the active site of the enzyme and the inhibitor did not exert its effect by chelating calcium (O'Connor and McGeeney, 1981b).

Granum (1978) has isolated from rye flour a protein which inhibited pancreatic and salivary α-amylases. The inhibitor was shown to have an isoelectric point of 5.8 and a native molecular weight of 28,000 daltons. Under denaturing conditions, the inhibitor dissociated into two subunits of identical size. This inhibitor was similar to the 0.19 inhibitor from wheat flour (Granum and Whitaker, 1977; Silano, 1978).

Indian finger millet has been shown to contain a bifunctional inhibitor of pancreatic α-amylase and trypsin (Shivaraj and Pattabiraman, 1981). The inhibitor was a basic protein with a molecular weight of 14,300 and formed a ternary complex with α-amylase and trypsin. The sites of interaction, on the inhibitor, were found to be different for each enzyme.

Sequencing studies have shown a high degree of homology among inhibitor proteins of α-amylase and this homology has been found to extend to trypsin inhibitors, as well. Both 0.19 and 0.28 inhibitors showed a high degree of homology in their N-terminal sequences (Petrucci et al., 1978; Redman, 1976) with barley trypsin inhibitor and the bifunctional α-amylase/trypsin inhibitor from Indian finger millet (Campos and Richardson, 1983; Odani et al., 1982, 1983). Kashlan and Richardson (1981) have sequenced the 0.28 inhibitor and found a single polypeptide chain of 123 residues which corresponded to a molecular weight of 13,400 daltons. Maeda et al. (1983a) have sequenced the subunit of the 0.53 inhibitor and found 124 residues with 9 cysteine residues per subunit. Comparison of the sequences of 0.53 and 0.28 inhibitors showed a high degree of homology in the cysteine regions (Maeda et al., 1983a). Maeda et al. (1983b) have shown, also, that

the subunit of the 0.53 inhibitor contained 4 disulfide bonds. In contrast, Petrucci et al. (1978) found that subunits of the 0.19 inhibitor and the monomeric 0.28 inhibitor had 5 intramolecular disulfide bonds that were essential for inhibitor activity.

Inhibitor proteins of the wheat kernel that inhibit mammalian α-amylases may have nutritional importance and it has been suggested that such inhibitors should be considered when assessing diets for patients with digestive disorders and for infants (Marshall, 1975; Silano, 1978). Puls and Keup (1973) have suggested that inhibitor proteins of α-amylase have potential therapeutic value in the treatment of diabetes and obesity. Inhibitor proteins that vary greatly in their specificity for human salivary and pancreatic α-amylase have been used as agents in determining the contributing levels of each enzyme in human serum thus demonstrating the practical value of such inhibitors in disease diagnosis (O'Donnell et al., 1977). The inhibitor albumins of the wheat kernel have been found, also, to be effective against α-amylases of insects that feed on grain. These inhibitors might confer some resistance to the cereal kernel against insect infestation (Silano et al., 1973; Yetter et al., 1979). With the possibility of a number of applications, it is understandable why these inhibitors have been so thoroughly characterized.

Research on endogenous α-amylase inhibitors in cereals has appeared only recently in the literature. Warchalewski (1977a, 1977b, 1977c, 1983) has used DEAE-cellulose chromatography to separate a number of heat stable inhibitors of wheat α-amylase from durum wheat, winter wheat and malted winter wheat. These inhibitors, however, have not been purified or characterized completely. Warchalewski (1977c)

has suggested that the appearance of some α-amylase activity, during germination of wheat, may result from dissociation of α-amylase-inhibitor complexes, initially present in the ungerminated seed and that such a mechanism of α-amylase release might supplement de novo synthesis of the enzyme.

An endogenous inhibitor of α-amylase has been isolated from the endosperm of maize (Blanco-Labra and Iturbe-Chinas, 1981). Gel filtration chromatography indicated a molecular weight of about 30,000 daltons. The inhibitor had activity against a number of insect α-amylases as well as Bacillus subtilis α-amylase, but the inhibitor did not inhibit α-amylases from other plant sources tested nor did it have any effect on mammalian α-amylases.

Jones and Meredith (1982) reported that wheat purothionins had inhibitor activity against endogenous α-amylase in extracts of germinated wheat. Addition of calcium to extracts of sprouted wheat flour nullified the inhibitor effect of β-purothionin, suggesting that purothionins may act by controlling calcium availability to cereal α-amylase. The authors suggested that purothionins might be effective physiologically, only if they were compartmentalized around α-amylase in the seed.

More recently, an endogenous inhibitor of malted barley α-amylase was purified and characterized, independently, by two research groups. Weselake et al. (1983a, 1983b) used ungerminated barley as the source of inhibitor whereas Mundy et al. (1983) isolated the inhibitor from green malt. Amino acid sequencing studies (Hejgaard et al., 1983; Weselake et al., 1983b) along with other properties of the inhibitors indicate, strongly, that the two proteins are identical. A similar

inhibitor has now been isolated from wheat (Mundy et al., 1984). A detailed account of the purification and properties of the α-amylase inhibitor from barley is the topic of this thesis.

MATERIALS AND METHODS

Purification of α-Amylase

Purification of α-Amylase I

Alpha-amylase I was purified from green barley malt (Hordeum vulgare cv Conquest) as described by MacGregor (1977), and from germinated wheat (Triticum aestivum cv Neepawa) by the method of Weselake and Hill (1983). The enzymes were stored frozen in small aliquots in the presence of 0.1% bovine serum albumin (BSA).

Purification of α-Amylase II

One kilogram of ground green malt was extracted with 2000 ml of 200 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5). After centrifugation (10,000 x g, 10 minutes), the extract was heated at 70°C for 15 minutes to remove β-amylase (MacGregor et al., 1971b), cooled, centrifuged and dialyzed against 20 mM sodium acetate buffer (1 mM $CaCl_2$, pH 4.75). The extract was added to a column (4.3 x 36 cm) of carboxymethyl cellulose (CMC) equilibrated with the same buffer and, after thorough washing with starting buffer, the column was eluted sequentially with 80 mM sodium acetate buffer (1 mM $CaCl_2$, pH 4.75) to remove α-amylase I (MacGregor, 1977) and 200 mM sodium acetate buffer (1 mM $CaCl_2$, pH 4.75) to remove α-amylase II. This enzyme was dialyzed against 100 mM sodium acetate (1 mM $CaCl_2$, pH 4.75) and purified

further on a CMC column (2 x 90 cm) using a linear gradient of 1000 ml of 100 mM sodium acetate buffer and 1000 ml of 200 mM sodium acetate buffer (both at pH 4.75 and containing 1 mM $CaCl_2$). Affinity chromatography (Silvanovich and Hill, 1976) was used as a final purification step to yield an enzyme of high purity as assessed by isoelectric focusing followed by protein and enzyme activity staining. The enzyme was stored frozen in small aliquots in the presence of 0.1% BSA. Alpha-amylase II was purified from germinated wheat as described by Weselake and Hill (1983).

Purification of Inhibitor

Barley (Hordeum distichum cv Klages) kernels were dehusked for 20 seconds in a pearling machine. Eighty-five grams of pearled kernels were ground to flour in a Udy Mill. The meal was extracted with 420 ml of 20 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5) at 4°C for 60 minutes. Subsequent purification procedures were performed at 4°C, also. After centrifugation of the slurry for 30 minutes at 13,000 x g, the supernatant solution was subjected to $(NH_4)_2SO_4$ fractionation. Material precipitating between 40 and 70% $(NH_4)_2SO_4$ was resuspended in 15 ml of 5 mM Tris-HCl buffer (1 mM $CaCl_2$, pH 8.0). The suspension was dialyzed at pH 8.0 and then centrifuged at 18,000 x g for 20 minutes to remove insoluble material. The supernatant solution was put on a DEAE-Sephacel (Pharmacia Fine Chemicals AB, Box 175, S-75104, Uppsala 1, Sweden) column (2 x 44 cm) equilibrated with dialysis buffer. After sample application, the column was washed with approximately one bed volume of equilibration buffer at a flow rate of 40 ml/hr. A linear gradient consisting of 300 ml equilibration buffer and 300 ml of the

same buffer containing 150 mM NaCl then was applied. The same flow rate was maintained and 5.3 ml fractions were collected. Conductivity measurements were made at room temperature on every tenth fraction using a YSI model Conductivity Bridge (Yellow Springs Instrument Co., Yellow Springs, Ohio, 45387, USA) equipped with a type CDC 314 conductivity probe (Radiometer, Copenhagen, Denmark). Inhibitor activity and absorbance at 280 nm were determined. The inhibitor solution, eluting in a volume from 405 to 450 ml, was pooled and concentrated by pressure ultrafiltration on a UM2 membrane (Amicon Corp., 182 Conant St., Danvers, MA, 01923, USA). The concentrate (10 ml) was applied to the bottom of a Bio-Gel P60 (100-200 mesh, Bio-Rad Laboratories, 2200 Wright Ave., Richmond, CA, 94804, U.S.A.) gel filtration column (2.6 x 77 cm) equilibrated with 5 mM Tris-HCl buffer (1 mM $CaCl_2$, pH 8.0). The column was eluted with the same buffer using an upward flow rate of 15 ml/hr and 5.6 ml fractions were collected. Active fractions, eluting in the range of 220 to 270 ml, were pooled, concentrated (Amicon PM10) and frozen at -15°C for subsequent analysis.

Purification of the inhibitor was scaled-up and modified during the course of the project. Five hundred grams of 10 second pearled kernels of barley were used as starting material and the flour was extracted with 2500 ml of 20 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5). After ammonium sulphate fractionation the pellet containing inhibitor was resuspended in approximately 100 ml of 5 mM Tris-HCl buffer (1 mM $CaCl_2$, pH 8.0). After dialysis at pH 8.0 and centrifugation, the supernatant solution was put on a DEAE-Sephacel column (2.6 x 82 cm). The inhibitor was eluted with a gradient formed from 1000 ml of equilibration buffer and 1000 ml of buffer containing 150 mM NaCl.

Fractions (22 ml) containing inhibitor, eluting in volume from approximately 1230 to 1610 ml, were pooled and concentrated to 14 ml (Amicon PM10). One half of the inhibitor solution was then applied to the bottom of a Bio-Gel P-30 (100-200 mesh) column (2.6 x 82 cm) equilibrated with 50 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5). The gel filtration column was eluted with an upward flow rate of 15 ml/hr. and 5 ml fractions were collected. Pooled inhibitor peaks (145 ml) from both Bio-Gel P-30 runs were concentrated to 11.5 ml and subjected to gel filtration on Bio-Gel P-30 a second time. Active fractions eluting in a volume from approximately 190 to 265 ml were pooled, concentrated to 15 ml and stored frozen in small aliquots.

Detection of Protein, α-Amylase and Inhibitor

Protein Determination

The protein content of enzyme and inhibitor solution was determined by the method of Lowry et al. (1951) using BSA as a standard. Protein standards were prepared in 5 mM Tris-HCl buffer (1 mM $CaCl_2$, pH 8.0) for analysis of protein solutions in the same buffer. Absorbance measurements at 280 nm were used to detect protein in fractions eluted from chromatography columns.

Quantitative Detection of α-Amylase and Inhibitor Activity

Unless indicated otherwise, enzyme and inhibition assays were performed at 35°C. A modification of the Briggs (1961) assay was used to determine α-amylase activity at pH 5.5. The enzyme was diluted into 2 ml of 50 mM sodium acetate buffer containing 1mM $CaCl_2$ and 100 µg of

BSA. Two ml of β-limit dextrin solution (0.65 mg/ml) were added to the enzyme solution and the reaction was stopped after 5 to 20 minutes by adding 10 ml of acidified I_2-KI solution (0.05 N HCl, 0.5 mg KI/ml, 0.05 mg I_2/ml). Loss of iodine-binding-capacity was determined at 540 nm. Alpha-amylase activity was expressed as Iodine Dextrin Color (IDC) units.

The modified Briggs (1961) assay was adapted to measure inhibitor activity at pH 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$) during purification steps and for analysis of inhibitor content in crude extracts of cereals. One ml of appropriately diluted inhibitor was pre-incubated for 15 minutes with 1 ml of appropriately diluted barley α-amylase II, containing 100 µg of BSA. Control digests without inhibitor were prepared. After pre-incubation, β-limit dextrin (2 ml) was added to start the reactions.

In other experiments with purified inhibitor a reducing sugar assay (Nelson, 1944; Robyt and Whelan, 1968) was used to determine α-amylase and inhibitor activity. One hundred µl of inhibitor solution were pre-incubated with 100 µl of α-amylase II, containing 1 mg/ml of BSA. The reaction was started by adding 200 µl of 1% soluble starch and terminated by adding alkaline copper reagent. One unit of α-amylase activity liberated 1 µmole glucose equivalent/minute.

Under the conditions of the assay:

Inhibitor Activity = (Amylase Activity Without Inhibitor) -
(Amylase Activity With Inhibitor)

One unit of inhibitor activity nullified 1 unit of α-amylase activity. In some cases the degree of inhibition was expressed as:

Percent Inhibition =

$$\frac{\text{(A.A. Without I.)} - \text{(A.A. With I.)}}{\text{(A.A. Without I.)}} \times 100$$

In other cases α-amylase activity remaining in the presence of inhibitor was expressed as:

Percent Maximum Amylase Activity =

$$\frac{\text{(Amylase Activity With Inhibitor)}}{\text{(Maximum Amylase Activity Without Inhibitor)}} \times 100$$

Oualitative Detection After Isoelectric Focusing

Analytical isoelectric focusing was carried out at 1°C according to MacGregor (1976). The effect of inhibitor on isoelectric focusing patterns of α-amylase was analyzed in a pH 4 to 8 ampholine gradient. Thirteen µg of malted barley α-amylase II were incubated with 56 µg of inhibitor in 0.5 ml of 20 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5), containing 1 mg/ml BSA, for at least 15 minutes at room temperature. Thirty µl of incubation mixture were applied to an adsorptive pad on the gel surface and then focused. Zymograms were prepared with β-limit dextrin substrate as described by MacGregor (1976). Isoelectric focusing was conducted, also, using pre-cast polyacrylamide gels (LKB-Producter AB, Box 305, S-161 26 Bromma, Sweden). The following samples were applied to a pH 3.5 to 9.5 focusing gel: (lane 1) 6 µg inhibitor, (lane 2) 8 µg inhibitor, 10 µg α-amylase II, and 10 µg BSA; and (lane 3) 10 µg α-amylase II and 10 µg BSA. Focusing was carried out according to manufacturer specifications and protein bands were visualized using the silver stain procedure of Merril et al. (1981).

Two adjacent applications of purified inhibitor were focused in a pH 3.5 to 9.5 ampholine gradient. One lane of focused inhibitor was visualized for protein by the silver stain procedure; the other lane was removed in a 2 x 11 cm section of gel and the surface was flooded with 200 µl of barley α-amylase II (18 µg) in 5 mM Tris-HCl buffer (1 mM $CaCl_2$, pH 8.0) containing 1 mg/ml BSA. The gel was incubated at room temperature for 30 minutes, sandwiched with a starch substrate plate for 15 minutes at 35°C (pH 8.0) and then stained with acidified I_2-KI solution.

Physico-chemical Characterization of Inhibitor

Molecular Weight Estimation

Electrophoresis Under Denaturing Conditions. The molecular weight of the inhibitor was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli (1970) system. Marker proteins from Pharmacia and 10 µg of purified inhibitor were used. In addition, SDS-PAGE for molecular weight estimation was conducted using a gradient gel system developed by Marchylo (in preparation).

Gel Filtration on Bio-Gel P-100. Molecular weights of inhibitor, α-amylase II and enzyme-inhibitor complex were estimated by gel filtration on a column (1.6 x 54 cm) of Bio-Gel P-100 (100-200 mesh) at pH 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$, 4°C). Samples were applied to the bottom of the column in 0.50 ml of equilibration buffer. The column was eluted with an upward flow rate of 6 ml/hr and 1.93 ml fractions were collected. Calibration proteins were obtained from the Sigma Chemical Co. (P.O. Box 14508, St. Louis, MO, 63178, USA).

Twenty µg of inhibitor in 0.50 ml of buffer were applied to the column and fractions were assayed for inhibitor activity at pH 8.0. In a separate run, α-amylase II (9 µg) was applied to the column and fractions were assayed for α-amylase at pH 5.5 (200 mM sodium acetate, 1 mM $CaCl_2$). A mixture of inhibitor (76 µg) and α-amylase II (9 µg) was equilibrated for at least 15 minutes prior to column application. Elution of α-amylase II-inhibitor complex was detected by assaying fractions for α-amylase activity at pH 5.5 and elution of excess inhibitor was detected by assaying fractions at pH 8.0 for inhibitor activity.

Isoelectric Point Determination

Analytical polyacrylamide gel isoelectric focusing was conducted with purified inhibitor in a pH 3.5 to 9.5 gel. The gradient of pH in the gel was determined using a surface electrode (Multiphor pH Surface Electrode, LKB catalogue No. 2117-111) and the position of inhibitor in the gradient was established by protein staining (Merril et al., 1981).

Heat Stability

Heat treatment of purified inhibitor, in the presence of 500 µg/ml BSA and 10 mM $CaCl_2$, was conducted at pH 5.5 (200 mM sodium acetate) and pH 8.0 (40 mM Tris-HCl) at 70°C for 15 minutes. Inhibitor activity remaining was then assayed at pH 8.0.

Amino Acid Analysis and Sequencing

The method for amino acid determination has been described by Duckworth and Bell (1982). Tryptophan was determined as described by

Edelhoch (1967) and half-cystine was determined after oxidation to cysteic acid by the method of Moore (1963). Amino-terminal sequencing was performed using a Beckman 890C Sequencer, with 2 mg Polybrine (Pierce Chemical Co., P.O. Box 117, Rockford, Illinois, 61105, USA) in the spinning cup. A 0.1 M Quadrol program was used, Beckman catalogue No. 030176. Phenylthiohydantoins of amino acids were identified by HPLC on a Perkin-Elmer HS-3 reversed-phase column, using a Perkin-Elmer System 4 Liquid Chromatograph and the elution system recommended by the manufacturer.

Behavior of α-Amylases II and III on Cycloheptaamylose-epoxy-Sepharose 6B

Cycloheptaamylose (CHA), purchased from the Sigma Chemical Company, was linked covalently to epoxy-activated Sepharose 6B (Pharmacia) as described by Silvanovich and Hill (1976). One ml aliquots of CHA-epoxy-Sepharose 6B were loaded at 4°C into small disposable columns (Bio-Rad) and equilibrated with 20 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5).

Flour (500 mg), prepared from green malt (Hordeum vulgare cv Conquest), was extracted with 2.5 ml of equilibration buffer at 22°C for 15 minutes on a Labquake shaker (Labindustries, Berkeley, CA, USA). The slurry was centrifuged at 13,000 x g for 20 minutes and the supernatant solution was retained at 4°C. One ml aliquots of extract were added to each of two affinity columns. Both columns were washed with 5 ml of equilibration buffer. One column was eluted with a further 4 ml portion of equilibration buffer and the other column was eluted with 2 ml of equilibration buffer containing 0.3 M NaCl followed by 2 ml of

equilibration buffer. Each column was then eluted with 2 ml of equilibration buffer containing 8 mg/ml of CHA.

The extract and the two fractions eluted by CHA solution were analyzed for α-amylase activity by the modified Briggs (1961) assay at pH 5.5. Equal activities (130 IDC units) from each sample were then subjected to isoelectric focusing analysis using pre-cast gels (pH 5.5 to pH 8.5). Zymograms of α-amylase banding patterns were prepared after focusing.

This experiment was repeated, using 1 ml mixtures of purified α-amylase II (8.7 µg) and purified inhibitor (37.3 µg). The mixtures were allowed to equilibrate for 15 minutes at 4°C prior to column application.

Inhibition Studies with Soluble Substrates

Pre-incubation Requirement

Pre-incubation time required for maximum inhibition was studied at pH 5.5 (200 mM sodium acetate, 1 mM $CaCl_2$) and pH 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$). Solutions of enzyme (100 µl) and inhibitor (100 µl) were pre-incubated for different time periods prior to the addition of soluble starch (200 µl of a 1% solution). At pH 8.0, 0.30 µg of α-amylase and 0.20 µg of inhibitor were used and at pH 5.5, 0.15 µg of α-amylase and 1.90 µg of inhibitor were used. Enzyme solution was used to initiate the reaction where the effect of zero pre-incubation time was studied. Enzyme reactions in these time-dependent studies were allowed to proceed for 7 minutes.

Hydrolysis of Starch in Solution

Release of reducing power with time was determined for α-amylase II at pH 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$) in the absence and presence of inhibitor. Two ml solutions each of enzyme (6.0 µg) and inhibitor (3.8 µg) were pre-incubated for 15 minutes prior to addition of 4 ml of 1% starch solution. Aliquots of 0.4 ml were removed into alkaline copper reagent, every 30 seconds, and subsequently analyzed for reducing activity. A control digest was set up without inhibitor.

Biospecificity of Inhibition

Similar activities of α-amylases I and II purified from malted barley and germinated wheat were pre-incubated for 15 minutes with a fixed concentration of inhibitor at pH 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$). After addition of soluble starch, reactions were allowed to proceed for 7 minutes.

Diluted human saliva (800-fold) and appropriately diluted porcine pancreatic α-amylase (Sigma Chemical Company) were tested with inhibitor at pH 8.0, also.

Effect of Inhibitor Concentration

The effect of different concentrations of inhibitor on a fixed amount of α-amylase II (0.10 µg) was studied at pH 5.5 (200 mM sodium acetate, 1 mM $CaCl_2$) and pH 8.0 (40 mM Tris-HCl, 1 mM $CaCl_2$). Enzyme and inhibitor were pre-incubated for 15 minutes prior to addition of soluble starch. Enzyme reactions were allowed to proceed for 8 minutes at pH 5.5 and 22 minutes at pH 8.0.

Effect of pH

Soluble starch hydrolysis by α-amylase II (0.10 μg) in the absence and presence of inhibitor (0.10 μg) was examined as a function of pH. A citrate-phosphate buffer system (Elving et al., 1956) of 0.05 μ ionic strength containing 1 mM $CaCl_2$ was used from pH 5.0 to 7.5. A 50 mM Tris-HCl buffer (1 mM $CaCl_2$) was used from pH 7.2 to 9.1. Both pre-incubation (15 minutes) and enzyme reaction were conducted at the same pH. Enzyme reactions were allowed to proceed for 7 minutes for the pH range 5.0 to 6.7 and 20 minutes for the pH range 7.1 to 9.1.

Effect of Sodium Chloride Concentration

Soluble starch hydrolysis by α-amylase II (0.14 μg) in the absence and presence of inhibitor (3.20 μg) was examined as a function of NaCl concentration. A 5mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5) was used with NaCl concentrations ranging from 0 to 200 mM NaCl. Both pre-incubation (15 minutes) and enzyme reaction (8 minutes) were conducted at the same ionic strength.

Effect of Temperature

Soluble starch hydrolysis by α-amylase II (0.14 μg) in the absence and presence of inhibitor (0.64 μg) was examined at 35°C and 18°C, in the presence of 200 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5). Both pre-incubation (15 minutes) and enzyme reaction were conducted at the same temperature. Enzyme reactions were allowed to proceed for 6 minutes at 35°C and 18 minutes at 18°C.

Inhibition of Amylose Hydrolysis

Amylose Digestion. Linear amylose was prepared from potato as described by Banks et al. (1959). Hydrolysis of amylose by α-amylase II in the absence and presence of inhibitor was conducted at 35°C in 5 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5). Enzyme (10.3 µg) and inhibitor (48.0 µg) were pre-incubated for 15 minutes in 2 ml of buffer containing 1.25 mg of BSA. Eight ml of a 0.10% amylose solution (equilibrated at the appropriate temperature) were added to initiate enzyme reactions. One ml aliquots were removed at different time intervals and added to micro-centrifuge tubes containing 10 µl of 2N HCl. Portions of the acidified aliquots were assayed for reducing power and the remainder was lyophilized.

Thin Layer Chromatography. Aliquots, taken after 10, 60 and 180 minutes of hydrolysis, were lyophilized and then reconstituted with distilled water to an approximate carbohydrate concentration of 10 mg/ml. Thin layer chromatography (TLC) was performed according to Wursch and Roulet (1982) using 20x20cm Silica Gel 60 (silanised) TLC plates (BDH Chemicals, 350 Evans Avenue, Toronto, Ontario, M8Z 1K5, Canada). Samples of 1 µl were applied in 7 mm bands with a Linomat III Applicator (Camag, Basle, Switzerland). The plate was irrigated three times with 1-propanol-acetone-water (45:30:25) and the carbohydrates were visualized as described by Hansen (1975). The resulting spots were scanned with a dualwavelength scanner (Shimadzu CS-910, Shimadzu Corp., Kyoto, Japan) at 540 nM with a slit height of 5 mm, slit width of 0.1 mm and scanning speed of 1 cm/minute. The signal was processed by an integrator (Reporting Integrator, Model 3390A, Hewlett Packard, 1000 N.E. Circle Blvd., Corvallis, OR, 97330, USA) and the dextrin profile was printed out at a chart speed of 1 cm/minute.

Inhibition Studies with Starch Granules

Preparation of Starch Granules

Large starch granules were prepared from normal barley (Hordeum vulgare cv Manchurian) as described by MacGregor (1979). Starch granule damage was assessed by an enzymatic method (Sandstedt and Mattern, 1960; MacGregor and Ballance, 1980b).

The Hydrolysis System

Hydrolysis experiments were performed in a temperature-controlled growth cabinet (Blue M Electric Company, Blue Island, IL, 60406, USA), using procedures similar to those described previously (MacGregor and Ballance, 1980b; Weselake and Hill, 1983). All buffers contained 1 mM $CaCl_2$ and 1 mg/ml BSA. Starch granules were hydrated in 1 ml of buffer for 30 minutes, at the appropriate temperature. Enzyme controls and enzyme-inhibitor mixtures were pre-incubated in 5 ml of buffer at the same temperature for 30 minutes. Hydrolysis of granules was initiated by adding 4 ml of the pre-incubated solutions to the hydrated starch granules. Experiments were conducted at 18°C with 10 mg/ml of starch granules and approximately 5 µg of α-amylase II (approximately 3000 IDC units), unless indicated otherwise. The reaction vials were sealed and rotated on a Labquake shaker. Aliquots (0.50 ml) were removed from the reaction mixture after 1, 2, 3, 4 and 5 hours while gently mixing the suspension. Enzyme reaction in the aliquots was stopped by adding enough HCl to lower the pH to approximately 2.5. Suspensions were then centrifuged for 5 minutes in a micro-centrifuge (Eppendorf centrifuge 5413, Brinkman Instruments Inc., Cantiague Road, Westbury, NY, 11590, USA) and supernatant solutions were analyzed for carbohydrate content

(LaBerge et al., 1973). Control digests without enzyme were used to check for spontaneous release of carbohydrate. The rate of release of soluble carbohydrate from starch granules was determined by linear regression analysis of the analytical data.

Effect of Inhibitor Concentration

The effect of inhibitor concentration (10.5, 21.5, 53.0, 106.5 and 213.0 µg per 5 ml of buffer) on α-amylase II activity was studied in the presence of 50 mM sodium acetate buffer (pH 5.5).

Effect of pH

Starch granule hydrolysis by α-amylase II in the absence and presence of inhibitor (19.2 µg) was examined as a function of pH. A citrate-phosphate buffer system (Elving et al., 1956) of 0.005 µ ionic strength was used for pH values ranging from 4.4 to 6.3 and Tris-HCl (5 mM) buffer was used for pH values 6.8 and 7.9.

Effect of Sodium Chloride and Buffer Concentration

Sodium chloride concentrations ranging from 20 to 200 mM NaCl were prepared in 5 mM sodium acetate buffer (pH 5.5). Sodium acetate buffer (pH 5.5) concentrations ranged from 5 to 200 mM. Total inhibitor in the reaction vessel was 19.2 µg.

Effect of Temperature

Temperatures ranging from 15°C to 35°C were used with 19.5 µg of inhibitor in 50 mM sodium acetate buffer (pH 5.5).

Effect of Starch Granule Concentration

Starch granule concentrations ranging from 5 to 80 mg/ml were used with 19.2 µg of inhibitor in a 50 mM sodium acetate buffer (pH 5.5).

Effect of Inhibitor on α-Amylase I from Malted Barley

Starch granule hydrolysis by α-amylase I (13.5 µg) in the absence and presence of inhibitor (300 µg) was examined at pH 5.5 (50 mM sodium acetate).

Examination of Hydrolysis Products and Degraded Starch Granules

Hydrolysis of starch granules by α-amylase II (4.8 µg) at pH 5.5 (5 mM sodium acetate) and 35°C was conducted in the absence and presence of inhibitor (20 µg). Aliquots were removed at time intervals ranging from 1 to 53.5 hours for the control and 14 to 68.5 hours for the inhibited reaction. Time course release of soluble carbohydrate was determined in each case. Control aliquots (5, 28.5 and 53.5 hours) and aliquots from the enzyme-inhibitor system (20, 43.5 and 68.5 hours) were lyophilized. The samples were reconstituted with a small volume of distilled water and then made up in a solution of 90% ethanol to a final carbohydrate concentration of approximately 0.5 mg/ml. Products of hydrolysis were analyzed by TLC. Ten µl of each sample were applied to the plates.

Another study was conducted at 18°C with 5.5 µg of α-amylase II and 128 µg of inhibitor. The enzyme and enzyme-inhibitor systems were allowed to react with starch granules for 15 and 64 hours, respectively. Supernatant solutions from these two digests were lyophilized and then reconstituted in a small volume of distilled water. A portion

of these solutions was then treated with a high concentration of purified barley β-amylase (MacGregor and Morgan, 1984) overnight at 35°C. Thin layer chromatography was performed on the untreated and β-amylase-treated samples.

Starch granules remaining in the 18°C and 35°C digests were washed several times with distilled water, lyophilized and examined by scanning electron microscopy (MacGregor and Ballance, 1980b).

Distribution of Inhibitor Activity in the Barley Kernel

Analysis of Pearling Fractions

Pearling fractions were prepared from 25 gm of barley (Hordeum distichum cv Klages) kernels. Fractions were removed at 20 second intervals up to 120 seconds and the remaining core was ground to meal in a Udy mill. Samples of 500 mg from each fraction, including the core, were extracted with 2.5 ml of 20 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5) at room temperature for 60 minutes on a Labquake shaker. Supernatant solutions were retained after centrifugation at 13,000 x g for 20 minutes. The extracts were analyzed for protein content and inhibitor activity at pH 8.0.

Another series of extractions was performed with distilled water. The pH of the flour suspensions was determined prior to centrifugation. Supernatant solutions, obtained after centrifugation, were diluted 10-fold and then analyzed for conductivity using a YSI Model Conductivity Bridge (Yellow Springs Instrument Co.) equipped with a type CDC 314 conductivity probe (Radiometer).

Removal of the Husk

Barley kernels were soaked in 70% sulphuric acid for 90 minutes,

filtered over glass wool and washed thoroughly with distilled water. Remaining portions of husk were removed by hand and the naked kernels were allowed to dry at room temperature overnight. Both intact and dehusked kernels were finely ground in a coffee mill (Braun AG, Type KSM 1, Frankfurt/M, W. Germany) and assayed for inhibitor activity.

Detection of Inhibitor in Various Cereals

Immunochemical Methods

Antibody Production. Two young female rabbits, weighing approximately 2.5 kg each, were bled at the ear for control serum which was kept frozen at -15°C until required. A stock solution of purified inhibitor (the antigen) containing 1.74 mg/ml of protein was prepared in 5 mM Tris-HCl buffer containing 0.9% NaCl (1 mM $CaCl_2$, pH 8.0). Freund's complete adjuvant (0.40 ml) was mixed thoroughly with an equal volume of stock inhibitor solution. The mixture was administered intradermally in a shaved area on the lower back. Each rabbit received 300 µg of protein in the primary injection. Subsequent booster shots of inhibitor (100 µg), in 0.50 ml of 0.9% NaCl, were administered subcutaneously, in the loose skin beneath the neck. Booster shots were administered at 14, 31, 70, 107 and 146 days after the primary injection. Bleedings of 5 to 10 ml were taken between injections and the serum retained at -15°C for future analysis. On day 161 the rabbits were bled, exhaustively, by cardiac puncture. The rabbits weighed about 5 kg each at this time and approximately 90 ml of anti-serum was recovered from each animal. The anti-sera were lyophilized separately and stored desiccated at 4°C.

Isolation of γ G-Immunoglobulin. The γ G-immunoglobulin (IgG) fraction was isolated from the immune serum (2.5 ml) of one rabbit from a bleeding taken at day 54. The anti-serum was diluted to 7.5 ml with distilled water and treated with 1.84 gm of ammonium sulphate. The mixture was equilibrated overnight at room temperature and then centrifuged at 10,000 x g for 20 minutes. The pellet was resuspended in 5 ml of 10 mM sodium phosphate buffer (pH 8.0) and the IgG component was isolated by anion-exchange chromatography, essentially as described by Fahney (1967) except that DEAE-Sephacel was substituted for DEAE-cellulose. The unretained peak, containing the IgG fraction, was concentrated to 2.5 ml (Amicon PM10), dialyzed against 0.9% NaCl and then kept frozen at -15°C.

Analysis of Cereal Extracts by Ouchterlony Double-Diffusion. Barley cultivars (Klages, Bonanza and Himalaya), wheat cultivars (Neepawa, Columbus and Northstar) durum wheat, rye, triticale, sorghum, oats, millet, rice and maize were surveyed for inhibitor by an immunochemical method. Approximately 10 gm portions of cereal kernels were ground finely in a coffee mill. Samples of 500 mg of the meal were extracted with 2.5 ml of 0.9 % NaCl at room temperature for 15 minutes on a Labquake shaker. Supernatant solutions were retained after centrifugation at 13,000 x g for 20 minutes. One ml of each extract was concentrated approximately 7-fold in a B-15 Minicon (Amicon) concentrator unit. The remainder of each extract was frozen (-15°C) and retained for determination of inhibitor activity. The concentrated extracts were analyzed, qualitatively, for inhibitor using the Ouchterlony (1967) double diffusion system and apparatus purchased from the Gelman

Instrument Company (600 South Wagner Rd., Ann Arbor, MI, 48106, USA).

The diffusion matrix consisted of 1% Agarose A (Pharmacia), 0.9% NaCl, 1% polyethylene glycol 8000, and 0.02% sodium azide. Control serum or purified IgG (6 µl of 11.6 mg/ml protein) was placed in center wells. Outer wells received 6 µl aliquots of purified inhibitor (1 µg) or concentrated extracts. Diffusion was allowed to proceed for 24 hours at room temperature. Diffusion plates were then washed for 2 days with three changes of 0.9% NaCl. After a final wash with distilled water for 1 hour, the agarose was dried to a thin film on the microscope slides with a hair dryer. Slides were removed from the immunodiffusion tray and stained individually for protein in plastic trays using the silver stain procedure of Willoughby and Lambert (1983).

Analysis of Inhibitor Activity in Cereal Extracts

Frozen extracts were thawed and assayed for inhibitor activity at pH 8.0. Diluted solutions of inhibitor were prepared in 1 ml of buffer (40 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.0). Extracts of Klages and Bonanza barley were diluted 500-fold. Extracts of Himalaya barley, all wheat cultivars, durum wheat, rye and triticale were diluted 200-fold. The remaining cereal extracts were diluted 100-fold. Diluted extracts were pre-incubated (15 minutes, 35°C) with 1 ml of α-amylase II (0.043 µg) in buffer containing 100 µg of BSA. Reactions were allowed to proceed for 15 minutes after the addition of β-limit dextrin solution. The coefficient of variation for the determination of inhibitor activity in extracts was determined by extracting six portions (500 mg) of ground Klages barley and assaying each for inhibitor activity.

RESULTS AND DISCUSSION

Purification and Physico-Chemical Properties of α-Amylase Inhibitor

Preliminary investigations indicated that crude extracts of barley kernels contained a component which could inhibit malted barley α-amylase II. Maximum inhibition was attained after a short pre-incubation of inhibitor and enzyme, prior to addition of substrate. Because inhibition was substantially higher at pH 8.0 than at pH 5.5 inhibitor activity was monitored during purification procedures at pH 8.0 using the modified Briggs (1961) assay for α-amylase. A reducing power assay could not be used to monitor inhibitor activity because of interfering components in the barley extract.

The purification scheme followed for isolating the inhibitor is shown in Figure 1. Polyphenolics are present in barley husks (Harris, 1962) and there is evidence that some polyphenolics can inhibit cereal α-amylase (Strumeyer and Malin, 1969; Daiber, 1975). Therefore, the husk was removed by pearling prior to extraction. After flour extraction and ammonium sulphate fractionation of the extract, the inhibitor was isolated by ion-exchange chromatography, followed by gel filtration chromatography.

The inhibitor was purified 125-fold from the crude extract with an overall recovery of 19% (Table 1). These results indicate that the pearled kernels contained approximately 0.01% inhibitor protein, assuming that the extraction (Figure 1) was efficient and that the inhibition observed was caused by inhibitor protein, only. Almost 60% of the

Figure 1. Outline of purification scheme for α-amylase inhibitor.

PURIFICATION SCHEME

BARLEY KERNELS (cv. Klages)

↓

PEARLED BARLEY (20 sec pearl)

↓

GRINDING (Udy cyclone mill)

↓

MEAL EXTRACTION
(60 min, 5°C, 20mM sodium acetate, 1mM $CaCl_2$, pH 5.5)

↓

AMMONIUM SULPHATE FRACTIONATION
(40 - 70%)

↓

DIALYSIS AT pH 8.0 (5mM Tris-HCl, 1mM $CaCl_2$)

↓

DEAE-SEPHACEL CHROMATOGRAPHY (pH 8.0)

↓

BIO-GEL P-60 GEL FILTRATION

total inhibitor activity present in the crude extract was lost, however, after ammonium sulphate fractionation and dialysis. Therefore, it is possible that part of the α-amylase inhibitor activity present in the crude extract was caused by factors other than the inhibitor protein. Development of a specific assay for the inhibitor would answer this question.

During ion-exchange chromatography on DEAE-Sephacel (Figure 2), inhibitor activity emerged as one peak at a conductivity between 400 and 500 μmho in the salt gradient. The peak fraction had a salt concentration of approximately 70 mM NaCl and the peak of inhibitor activity coincided with a peak of absorbance at 280 nm. Some starch hydrolyzing activity, detected by the reducing power assay, was eluted later in the salt gradient and was due probably to β-amylase. Overall, ion-exchange chromatography was the most effective purification step, giving a purification of about 25-fold (Table 1).

The inhibitor peak from DEAE-Sephacel chromatography was separated into two major protein peaks by gel filtration chromatography on a column of Bio-Gel P-60 (Figure 3) but only one of these had inhibitor activity against α-amylase II. Total inhibitor activity of both chromatographic steps was determined after concentration of the active fractions. In each series of chromatography and concentration, a 10% loss of inhibitor was incurred (Table 1).

To prepare sufficient quantities of inhibitor for characterization studies, the purification was scaled-up and modified. About 6 times more starting material (500 gm of 10 second pearled kernels) was used in the new procedure. Both bed volume and total elution volume were

TABLE 1. Purification of α-amylase inhibitor.

Fraction	Volume (ml)	Total Protein (mg)	Total Inhibitor Activity[a] (anti units)	Specific Activity (anti units/mg)	Purification (fold)	Recovery (%)
crude extract	353	1272	561,100	440	1	100
40-70% $(NH_4)_2SO_4$ (after dialysis)	15	240	235,500	980	2	42
DEAE-Sephacel (after concentration)	10	7.8	167,000	21,410	49	30
Bio-Gel P60 (after concentration)	5	1.9	105,000	55,260	125	19

[a] Based on modified Briggs (1961) assay for α-amylase.

Figure 2. Ion-exchange chromatography on DEAE-Sephacel of the 40 to 70% $(NH_4)_2SO_4$ fraction of the barley extract.

Equilibration buffer was 5 mM Tris-HCl (1 mM $CaCl_2$, pH 8.0). A linear gradient consisting of 300 ml equilibration buffer and 300 ml of the same buffer containing 150 mM NaCl was used to elute the ion-exchange column. Column dimensions = 2 x 44 cm. Flow rate = 40 ml/hr. Fraction volume = 5.3 ml.

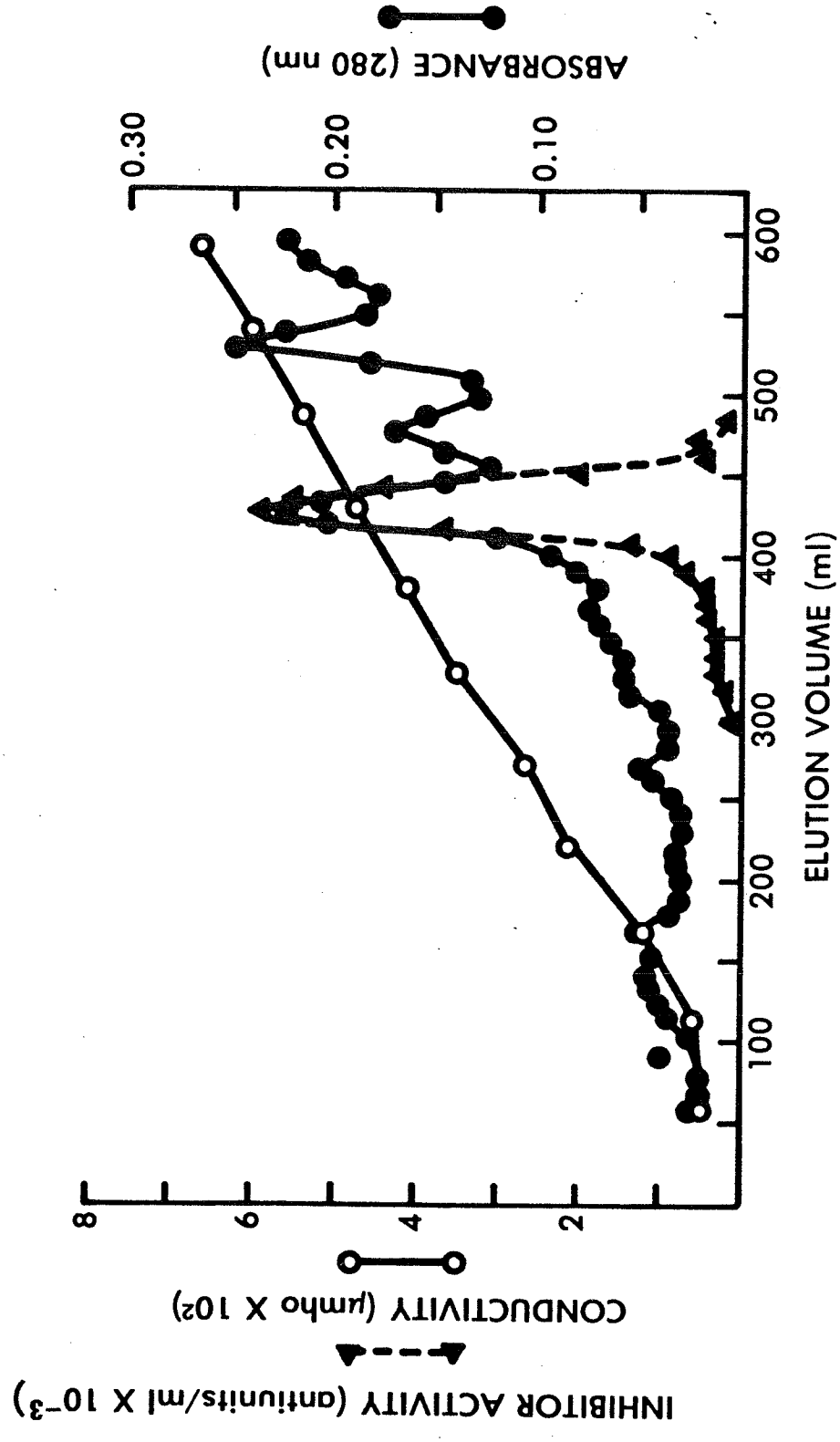
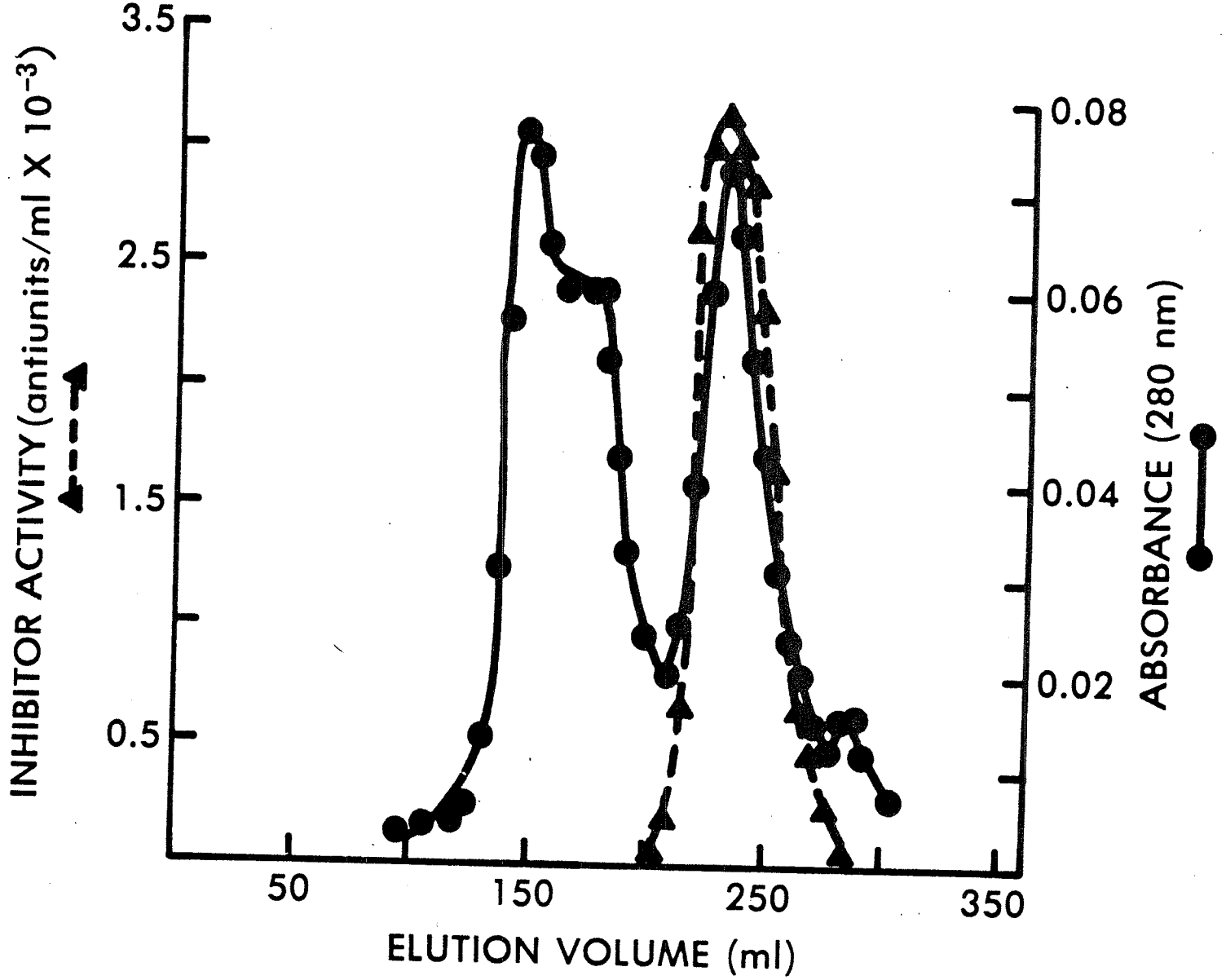

INHIBITOR ACTIVITY (antiunits/ml X 10⁻³)
CONDUCTIVITY (μmho X 10²)
ABSORBANCE (280 nm)
ELUTION VOLUME (ml)
0.30
0.20
0.10
8
6
4
2
100
200
300
400
500
600

Figure 3. Gel filtration chromatography on Bio-Gel P-60 (100-200 mesh) of the inhibitor peak eluted from the the DEAE-Sephacel column.

Equilibration buffer was 5 mM Tris-HCl (1mM $CaCl_2$, pH 8.0). Column dimensions = 2.6 x 77 cm. Flow rate = 15 ml/hr. Fraction volume = 5.6 ml. Volume of sample applied = 10 ml.


3.5
2.5
1.5
0.5
INHIBITOR ACTIVITY (antiunits/ml X 10^{-3})
0.08
0.06
0.04
0.02
ABSORBANCE (280 nm)
50
150
250
350
ELUTION VOLUME (ml)

increased about 3-fold during ion-exchange chromatography on DEAE-Sephacel. Gel filtration chromatography on Bio-Gel P-30 replaced chromatography on Bio-Gel P-60. The absorbance profile at 280 nm of the eluent from chromatography on Bio-Gel P-30 was similar to the profile obtained from the Bio-Gel P-60 column (Figure 3) in the original purification procedure. Two major peaks of protein were apparent but the high protein concentration present resulted in considerable overlap of peak edges. A second gel filtration chromatography on Bio-Gel P-30 was required to separate the inhibitor from contaminating protein.

The modified procedure for purification of inhibitor was considerably better. In the original purification, 1.9 mg of inhibitor were isolated from 85 gm of pearled kernels (Table 1) but in the modified procedure 24 mg of inhibitor were isolated from 500 gm of pearled kernels. Therefore, the yield of isolated inhibitor was increased from 22 to 48 µg/gm of pearled kernels. This increased yield might be attributed to the higher protein concentrations prevailing throughout the scaled-up purification procedure. At low protein concentration, the inhibitor was adsorbed by surfaces of dialysis bags, concentrator membranes and, possibly, column-packing materials. Adsorption of inhibitor to surfaces may have been responsible for some of the yield losses in the original purification (Table 1).

Before characterizing the properties of the inhibitor, homogeneity of the preparation was evaluated. This was accomplished by analytical isoelectric focusing, SDS-gel electrophoresis and amino acid sequencing. Purified inhibitor gave a single band of protein after isoelectric focusing (Figure 4a). The ability of the focused protein to inhibit α-amylase II was assessed, also. Following isoelectric focusing,

the gel was flooded with a solution of α-amylase II at pH 8.0. Presumably, under these conditions, hydrolysis of starch by α-amylase II would be inhibited at a position in the gel where the inhibitor was focused. The corresponding band on the zymogram in Figure 4b was the area where α-amylase II was inhibited. Undegraded starch in this region gave a blue iodine color on an otherwise clear background. The positions of the protein-stained band and inhibitor activity band were identical, giving direct evidence that the purified protein had inhibitor activity against α-amylase II. The homogeneity of the isolated inhibitor was demonstrated further by the presence of only one protein band after SDS-gel electrophoresis (not shown). High purity of the preparation was supported, also, by the sequencing of the first 20 N-terminal amino acids. A unique amino acid was found with each cycle of the sequenator.

The molecular weight of the inhibitor was determined by three methods. Sodium dodecyl sulphate gel electrophoresis indicated a molecular weight of 21,000 daltons for the inhibitor (Figure 5). A similar molecular weight was determined by SDS gradient-gel electrophoresis (20,400 daltons) and by gel filtration chromatography on Bio-Gel P-100 (20,000 daltons). Results of all molecular weight determinations were in good agreement. The inhibitor did not appear to self-associate to form a dimer or larger polymer under the conditions used during gel filtration chromatography. These values were similar to the molecular weight of an α-amylase II inhibitor isolated from green malt (Mundy et al., 1983).

Amino acid analysis (Table 2) showed that the inhibitor contained about 9 half-cystine residues/mole but no detectable methionine. At

Figure 4. Isoelectric focusing of purified α-amylase inhibitor.

a. Gel section stained for protein.

b. Gel section showing inhibitor activity.

Arrow indicates sample application point.

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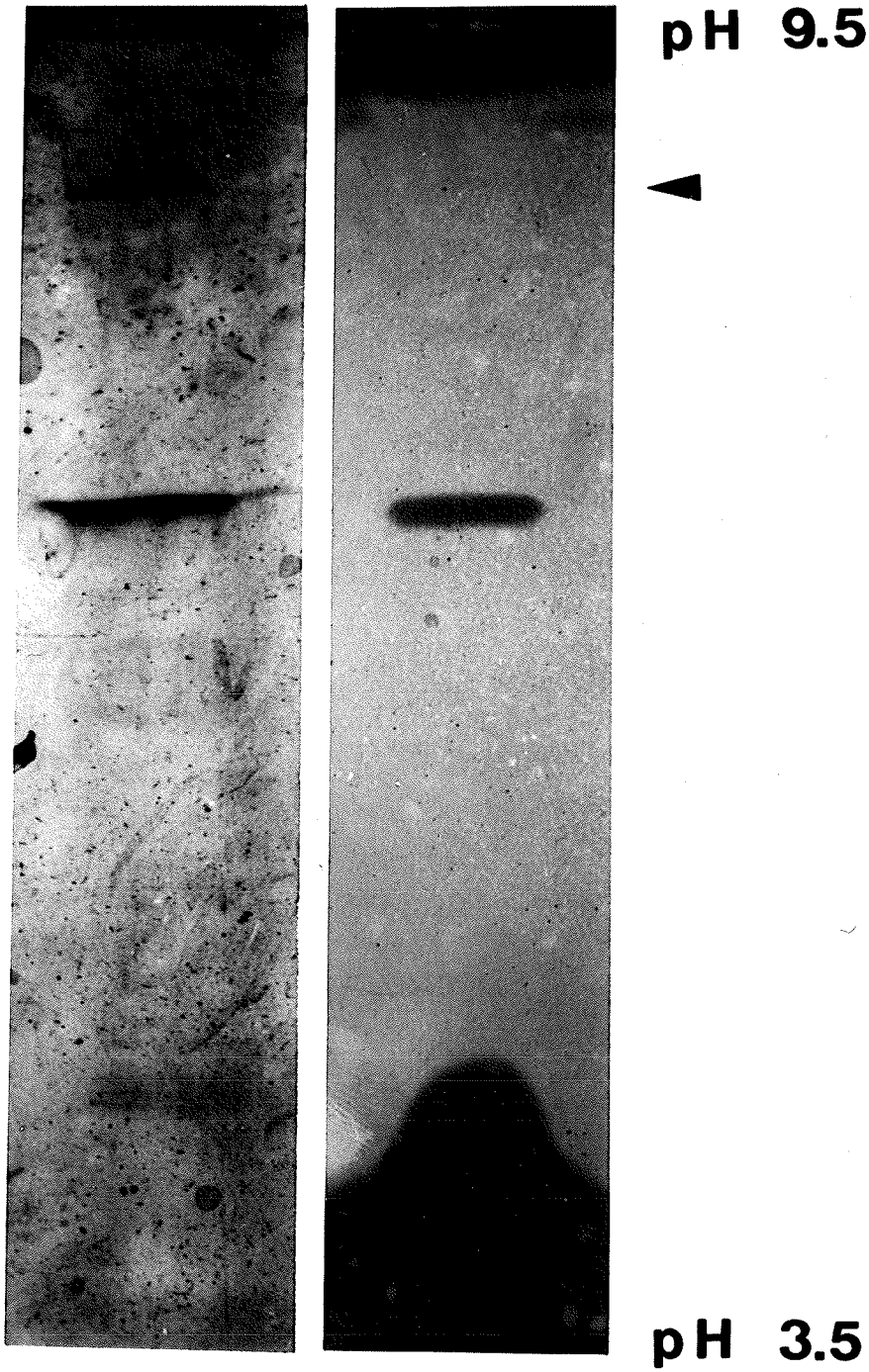
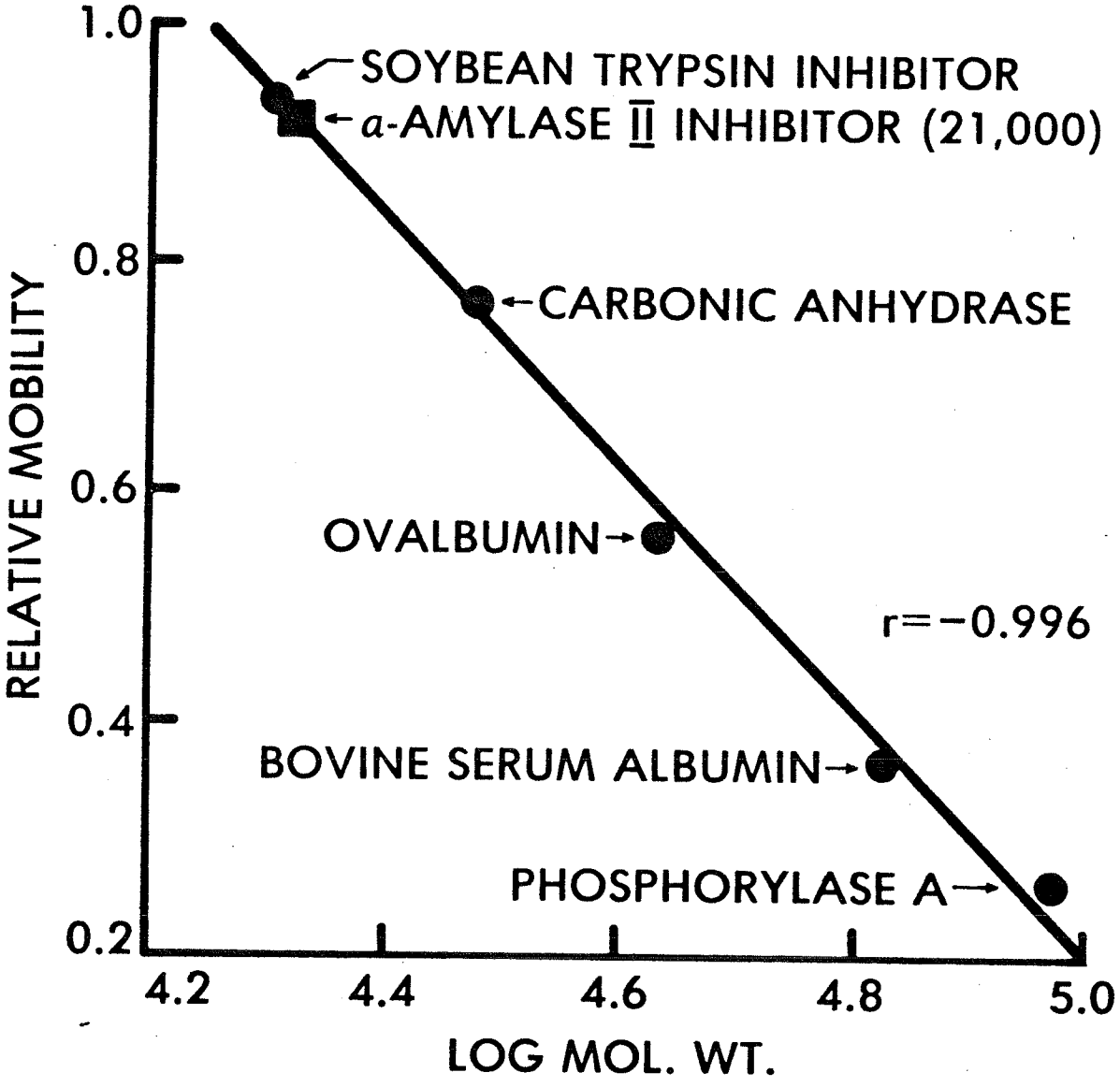

pH 9.5
pH 3.5
a
b

Figure 5. Molecular weight of α-amylase inhibitor estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Molecular weights of marker proteins:

Soybean Trypsin Inhibitor	20,100
Carbonic Anhydrase	30,000
Ovalbumin	43,000
Bovine Serum Albumin	67,000
Phosphorylase A	94,000


1.0
0.8
0.6
0.4
0.2
RELATIVE MOBILITY
SOYBEAN TRYPSIN INHIBITOR
a-AMYLASE II INHIBITOR (21,000)
CARBONIC ANHYDRASE
OVALBUMIN
r=−0.996
BOVINE SERUM ALBUMIN
PHOSPHORYLASE A
4.2
4.4
4.6
4.8
5.0
LOG MOL. WT.

this stage, the degree of disulfide bonding has not been determined. The presence of a relatively high proportion of aromatic amino acids accounts for the absorbance of the inhibitor at 280 nm. The ratio of aspartic and glutamic acid residues to basic residues was 1.4. This would suggest that some of the apparently acidic residues exist as amides since the isoelectric point of the inhibitor was found to be 7.3 by isoelectric focusing analysis. A similar isoelectric point was found for the α-amylase II inhibitor from green malt (Mundy et al., 1983).

The information obtained from amino acid analysis allowed comparisons to be made with other known enzyme inhibitor proteins from cereals. The relatively high content of half-cystine is a characteristic shared by barley trypsin inhibitor (Mikola and Suolinna, 1969) and inhibitor albumins from the wheat kernel that inhibit animal α-amylase (Buonocore et al., 1977). α-Amylase II inhibitor, however, has a greater proportion of basic amino acid residues than does inhibitor albumins from wheat. Jones and Meredith (1982) have shown that purothionins may act as endogenous α-amylase inhibitors in wheat but the amino acid composition of the α-amylase II inhibitor (Table 2) indicates clearly that this inhibitor is not a purothionin analogue (Ozaki et al., 1980).

The sequence of the first 20 N-terminal amino acids of the inhibitor was determined (Table 3). Amino acid residues were numbered sequentially from the N-terminal amino acid. Despite the high half-cystine content of the inhibitor (Table 2), no half-cystine was found in the first 20 N-terminal residues. Some homology appears to exist between the α-amylase II inhibitor and trypsin inhibitor from barley

TABLE 2. Amino acid composition of α-amylase inhibitor.

Amino Acid	Residues per 20,000 g[a]
Aspartic acid (Asp)	19.0
Threonine (Thr)	10.0
Serine (Ser)	8.4
Glutamic acid (Glu)	14.7
Proline (Pro)	13.4
Glycine (Gly)	17.5
Alanine (Ala	20.8
Valine (Val)	14.0
Methionine (Met)	nil
Isoleucine (Ile)	8.2
Leucine (Leu)	9.9
Tyrosine (Tyr)	6.0
Phenylalanine (Phe)	5.3
Histidine (His)	4.6
Lysine (Lys)	7.0
Arginine (Arg)	13.1
Tryptophan (Trp)	2.6
Half-cystine ($\frac{1}{2}$ Cys)	8.8

[a] Based on molecular weight determined by gel filtration chromatography.

TABLE 3. N-terminal sequence of α-amylase inhibitor compared to barley trypsin inhibitor.

α-Amylase Inhibitor	Barley Trypsin Inhibitor[a]
1 Ala[b]	1 Phe[b]
Asp	Gly
Pro	Asp
Pro	5 Ser
5 Pro	Cys
Val	Ala
His	Pro
Asp	Gly
Thr	10 Asp
10 Asp	Ala
Gly	Leu
His	Pro
Glu	His
Leu	15 Asp
15 Arg	Pro
Ala	Leu
Asp	Arg
Ala	Ala
Asn	Cys
20 Tyr	20 Arg
	Thr
	Tyr

[a]From Odani et al (1982)
[b]N-terminal amino acid

A single sequenator run on 0.46 mg of α-amylase inhibitor (23 nmol assuming 20,000 D) gave 112% coupling (26 nmol alanine in cycle 1) and a repetitive yield of 91.4%.

(Table 3). Residues 14 to 16 (Leu-Arg-Ala) of the inhibitor coincided with residues 16 to 18 of the barley trypsin inhibitor. Once this degree of homology was established, proline 5 and tyrosine 20 of the inhibitor coincided with proline 7 and tyrosine 22 of the barley trypsin inhibitor. There was no strong homology in the N-terminal region between inhibitor albumins from wheat (Kashlan and Richardson, 1981; Odani et al., 1982) and α-amylase II inhibitor. The N-terminal sequence of the α-amylase II inhibitor from barley kernels, however, is in complete agreement with the N-terminal sequence described for the α-amylase II inhibitor from green malt (Hejgaard et al., 1983; Mundy et al., 1983), indicating that they are probably the same protein. These two α-amylase II inhibitors appear, also, to be homologous with a number of protease inhibitors from cereal and legume sources (Hejgaard al al., 1983).

The α-Amylase II - Inhibitor Complex

Green malt contains 3 major groups of α-amylase as assessed by isoelectric focusing and the groups are designated I, II and III, in order of increasing isoelectric point (MacGregor and Ballance, 1980a). Furthermore, α-amylase groups II and III share immunochemical identity (MacGregor and Daussant, 1981). Preliminary investigations suggested that extracts of barley endosperm contained a factor which converted purified α-amylase II into α-amylase III. A mixture of inhibitor and α-amylase II resulted in a similar conversion when the mixture was assessed by isoelectric focusing and activity staining. Focusing of purified α-amylase II revealed one group of α-amylase (Figure 6, lane 1), whereas focusing of a mixture of α-amylase II and inhibitor

revealed α-amylase II and α-amylase III (Figure 6, lane 2). The focusing pattern of the enzyme-inhibitor mixture was similar to the focusing pattern of α-amylases in a green malt extract (Figure 6, lane 3). Presumably, α-amylase III in green malt was a result of the formation of an α-amylase II-inhibitor complex. During development of the inhibitor purification scheme, fractionated extracts of barley kernels were assessed, qualitatively, for inhibitor content, by following the ability of barley fractions to convert α-amylase II into α-amylase III. Appearance of α-amylase III activity in the zymograms (Figure 6, lanes 2 and 3) suggested that the enzyme-inhibitor complex might have dissociated under the conditions used to prepare the zymogram. It is possible, also, that under these same conditions the enzyme-inhibitor complex might be capable of hydrolyzing substrate.

Formation of an α-amylase II-inhibitor complex was shown, more directly, by isoelectric focusing of a mixture of α-amylase II and inhibitor followed by protein staining. The focused inhibitor is shown in lane 1 of Figure 7 and focused α-amylase II is in lane 3. The complex, in lane 2 of Figure 7, corresponded to α-amylase III and had an isoelectric point which was intermediate to the isoelectric points of the two contributing species. When enzyme-inhibitor mixtures were applied to different positions, above pH 5.0, on a gel with a preformed pH gradient, similar isoelectric focusing patterns were observed, following protein staining (not shown). Other workers have demonstrated the formation of a similar complex using a mixture of α-amylase II and an α-amylase II inhibitor isolated from green malt (Mundy et al., 1983).

Figure 6. Isoelectric focusing zymograms demonstrating the effect of inhibitor on α-amylase II.

1. α-Amylase II.

2. α-Amylase II plus inhibitor.

3. Green malt extract.

Arrow indicates sample application point.

Approximately equal enzyme activities were applied in each lane.

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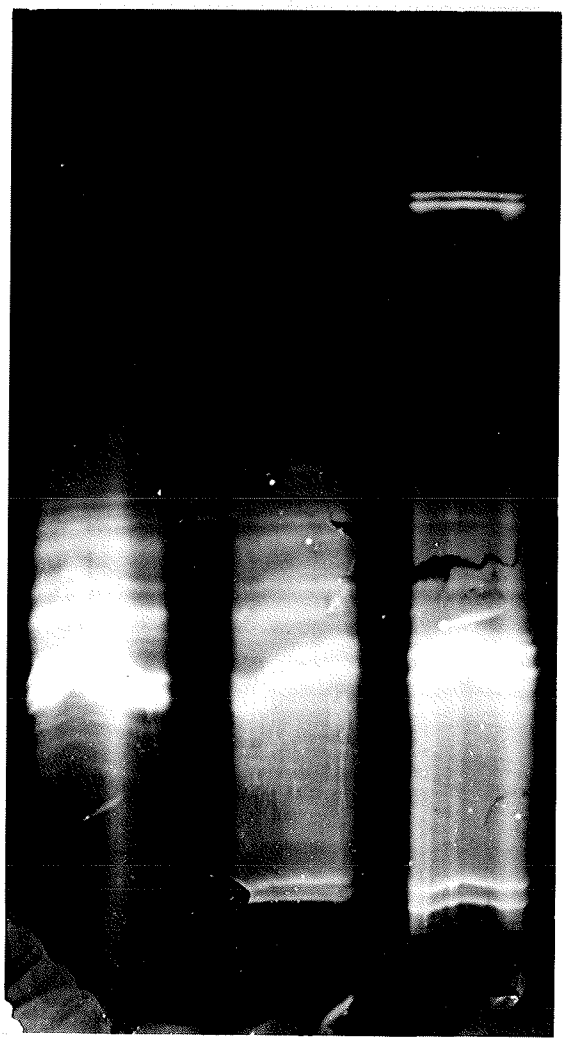
pH 4.0
α-AMYLASE I
α-AMYLASE II
α-AMYLASE III
pH 8.0
1
2
3

Figure 7. Isoelectric focusing gel, stained for protein, demonstrating the effect of inhibitor on α-amylase II.

1. Inhibitor.

2. Inhibitor, α-amylase II and BSA.

3. α-Amylase II and BSA.

Arrow indicates sample application point.

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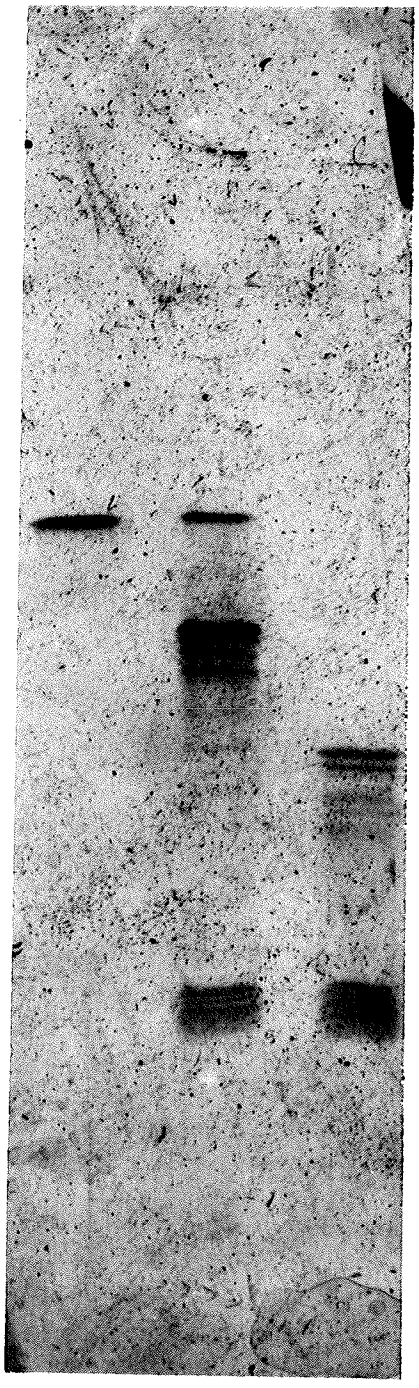

pH 9.5
INHIBITOR
α-AMYLASE III
α-AMYLASE II
BSA
pH 3.5
1
2
3

Formation of the α-amylase II-inhibitor complex was studied, also, by gel filtration chromatography on Bio-Gel P-100. This was conducted at pH 8.0 because the affinity of enzyme for inhibitor was high at this pH. Elution volumes of the inhibitor, α-amylase II and the α-amylase II-inhibitor complex were all different (Figure 8). Molecular weights of the inhibitor and α-amylase II were apparently approximately 20,000 and 32,000 daltons, respectively (Figure 9). This molecular weight for α-amylase is anomalously low, compared to the generally accepted value of about 45,000 daltons (Greenwood and Milne, 1968c), but similar low molecular weights have been reported for wheat α-amylases using Bio-Gel as a sieving matrix (Marchylo et al., 1976). Gel filtration chromatography of a mixture of α-amylase II and excess inhibitor, yielded a new species (Figure 8) having a molecular weight of 41,000 daltons (Figure 9), and a protein peak that corresponded to a molecular weight of 20,000 daltons which presumably was excess inhibitor. The 41,000 dalton species was due to formation of an enzyme-inhibitor complex and would correspond to the α-amylase III observed after isoelectric focusing (Figure 7, lane 2). A molecular weight of about 52,000 daltons would be expected for the complex but the lower value obtained suggests that the complex may not behave like a globular protein during gel filtration chromatography.

Having shown that α-amylase III is a complex of α-amylase II and inhibitor, other properties of the complex were then investigated. Isoelectric focusing experiments demonstrated previously that an extract of green malt, when heated at 70°C for 15 minutes, resulted in the conversion of a large proportion of α-amylase III into α-amylase II (MacGregor and Ballance, 1980a). Malt α-amylase in a crude extract is

Figure 8. Gel filtration chromatography of inhibitor, α-amylase II and α-amylase II-inhibitor complex on Bio-Gel P-100 (100-200 mesh).

Equilibration buffer was 40 mM Tris-HCl (1mM $CaCl_2$, pH 8.0). Column dimensions = 1.6 x 54 cm. Volume of sample applied = 0.50 ml. Flow rate = 6 ml/hr. Fraction volume = 1.93 ml. Alpha-amylase activity was assayed at pH 5.5 (200 mM sodium acetate buffer, 1 mM $CaCl_2$, 250 µg/ml BSA). The enzyme-inhibitor complex was detected by assaying for α-amylase activity at pH 5.5. Inhibitor activity was assayed at pH 8.0 (40 mM Tris-HCl buffer, 1mM $CaCl_2$, 250 µg/ml BSA).

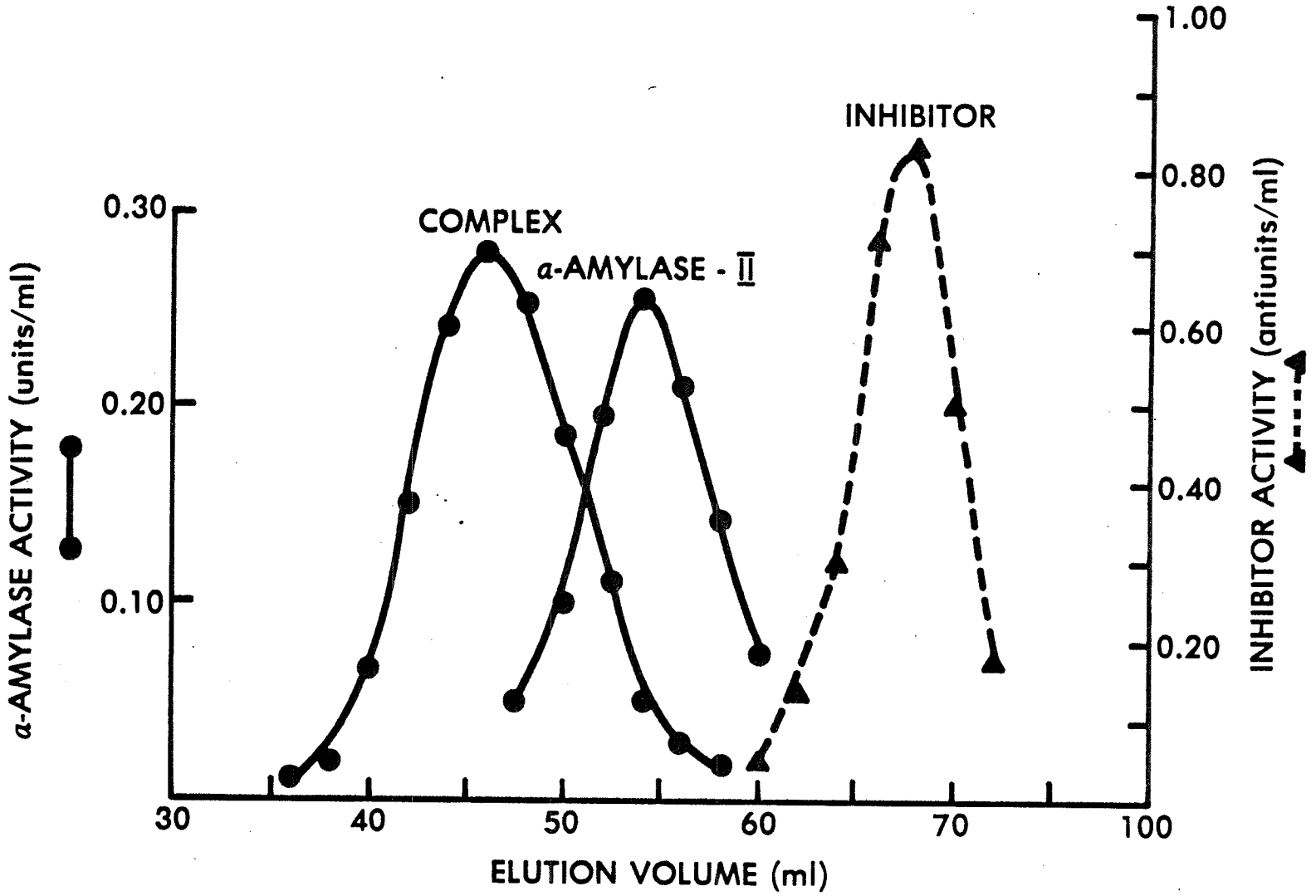
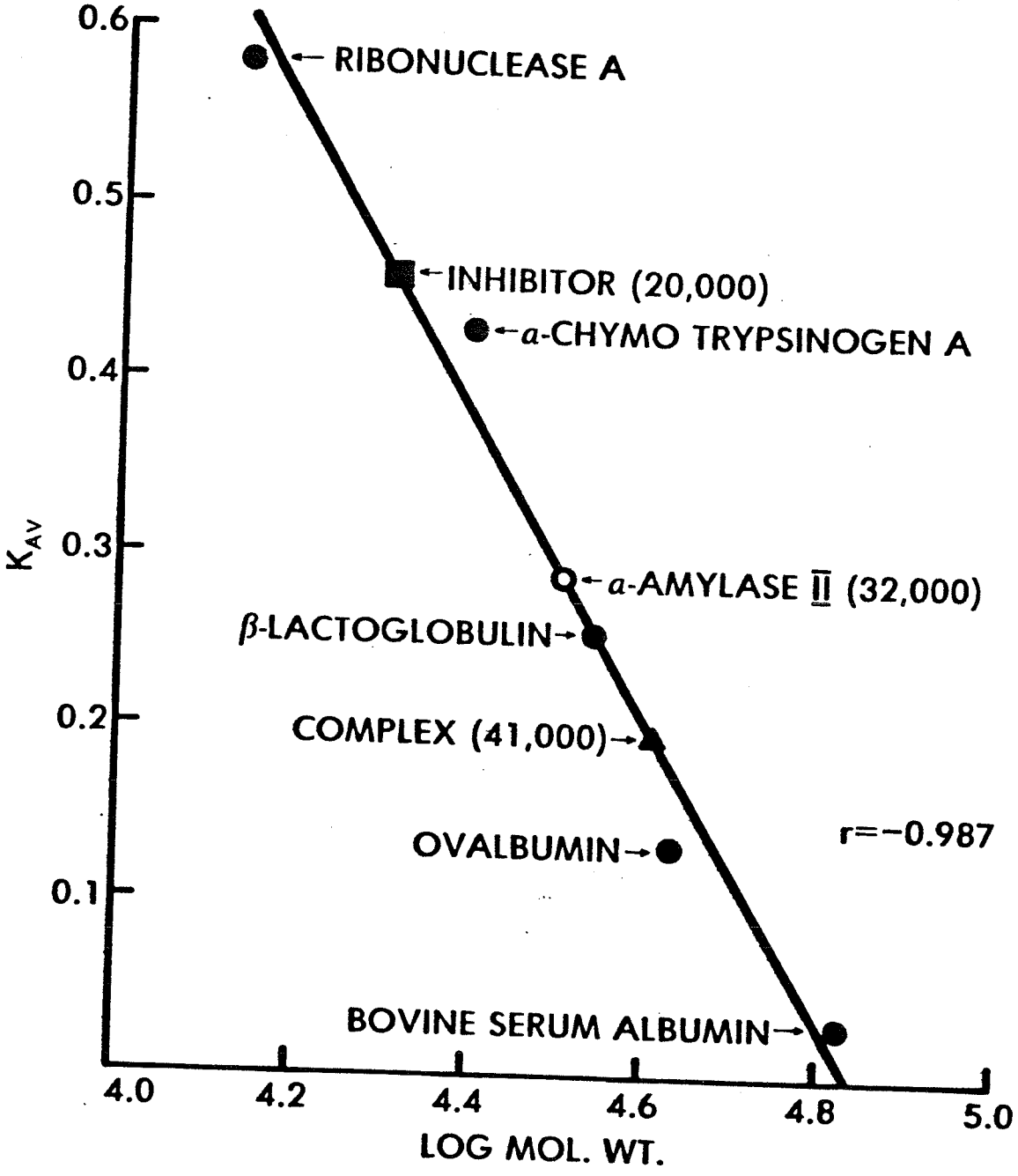

INHIBITOR
COMPLEX
a-AMYLASE - II
0.30
0.20
0.10
1.00
0.80
0.60
0.40
0.20
30
40
50
60
70
100
a-AMYLASE ACTIVITY (units/ml)
INHIBITOR ACTIVITY (antiunits/ml)
ELUTION VOLUME (ml)

Figure 9. Molecular weights of inhibitor, α-amylase and the α-amylase-inhibitor complex estimated by gel filtration chromatography on Bio-Gel P-100 (100-200 mesh).

Molecular weights of marker proteins:

Ribonuclease A	13,700
α-chymotrypsinogen A	25,000
β-lactoglobulin	35,000
Ovalbumin	43,000
Bovine Serum Albumin	67,000


0.6
0.5
0.4
0.3
0.2
0.1
K_{AV}
RIBONUCLEASE A
INHIBITOR (20,000)
a-CHYMO TRYPSINOGEN A
a-AMYLASE II (32,000)
β-LACTOGLOBULIN
COMPLEX (41,000)
OVALBUMIN
BOVINE SERUM ALBUMIN
r=−0.987
4.0
4.2
4.4
4.6
4.8
5.0
LOG MOL. WT.

known, however, to be relatively stable to heating at 70°C (Kneen et al., 1943; MacGregor et al., 1971b). Assuming α-amylase III is a complex of enzyme and inhibitor, it would appear that the inhibitor was the heat labile component of the complex. This was verified by heating inhibitor solutions, buffered at pH 5.5 and pH 8.0, at 70°C for 15 minutes. Considerable diminution of inhibitor activity occurred (Table 4), indicating that the inhibitor was heat labile under these conditions. Early studies by Frydenberg and Nielsen (1965), using zone electrophoresis, indicated that when germinated barley extracts were heated at 70°C for 15 minutes, some forms of α-amylase disappeared but other forms of the enzyme were enriched. Presumably, the heat-induced α-amylase conversions observed by these early investigators might be linked, also, to the heat lability of the α-amylase II inhibitor.

Previous experiments in which malt extracts were heated at pH 5.5 for 15 minutes, indicated nearly complete conversion of α-amylase III to α-amylase II, but at pH 8.0, approximately 30% of the α-amylase III was still present (MacGregor and Ballance, 1980a). A stronger complex would be expected for α-amylase II and the inhibitor at pH 8.0 than at pH 5.5 since enzyme inhibition was considerably higher at pH 8.0 than at pH 5.5. It is possible that the thermal stability of the inhibitor increased when it was associated with α-amylase II at pH 8.0. Previous experiments by MacGregor and Daussant (1981) indicated that only 15% of the α-amylase III was converted to α-amylase II during kilning.

Properties of the α-amylase II-inhibitor complex were studied also by affinity chromatography on cycloheptaamylose-epoxy-Sepharose 6B. Isoelectric focusing analysis of a green malt extract indicated the presence of both α-amylases II and III in the extract (Figure 10A, lane

TABLE 4. Inhibition of barley α-amylase II at pH 8.0 before and after heat treatment of the inhibitor at 70°C for 15 minutes.

pH During Heating	Percent Inhibition	
	Before Heating	After Heating
5.5	93	22
8.0	97	14

1). Alpha-amylase I was excluded from the pH gradient (pH range 5.5 to 8.5) since the isoelectric point of this enzyme group was below 5.5 (MacGregor, 1977; 1978). When a portion of this extract was loaded onto a column of affinity gel, washed with buffer and then with a solution of cycloheptaamylose (CHA) both α-amylases II and III were eluted (Figure 10A, lane 2). If a 0.3 M NaCl wash of the gel preceded elution with CHA, however, only α-amylase II was released from the column (Figure 10A, lane 3). Presumably, the enzyme-inhibitor complex dissociated in the NaCl solution thereby releasing the inhibitor from the CHA-epoxy-Sepharose 6B column. Enzyme activity released by CHA solution was similar in both cases, indicating that salt solution did not disrupt the CHA-α-amylase II interaction. Mixing of α-amylase II and inhibitor resulted in the formation of α-amylase III (Figure 10B, lane 1). When the mixture was loaded onto the affinity column, washed with buffer and then with CHA solution, both α-amylases were eluted (Figure 10B, lane 2). Again, a 0.3 M NaCl wash of the affinity gel prior to enzyme elution with CHA solution, resulted in the release of only α-amylase II (Figure 10A, lane 3). Therefore, the salt solution appeared to be equally effective in disrupting the α-amylase II-inhibitor complex, whether the source of inhibitor was an extract of green malt or an enzyme-inhibitor mixture. The α-amylase II inhibitor from green malt was shown to co-purify with α-amylase II during affinity chromatography when a salt wash was not used prior to elution of enzyme with CHA solution (Mundy et al., 1983). These experiments suggest that at least part of the interaction between α-amylase II and inhibitor is due to ionic bonding. Furthermore, the site of inhibitor binding on the enzyme molecule did not appear to be the same as the site of CHA

Figure 10. Isoelectric focusing zymograms of a malt extract (A), and a mixture of α-amylase II and inhibitor (B), before and after elution from a column of cycloheptaamylose-epoxy-Sepharose 6B.

1. Prior to column application.

2. Eluted with cycloheptaamylose.

3. Eluted with cycloheptaamylose after the column was washed with NaCl solution.

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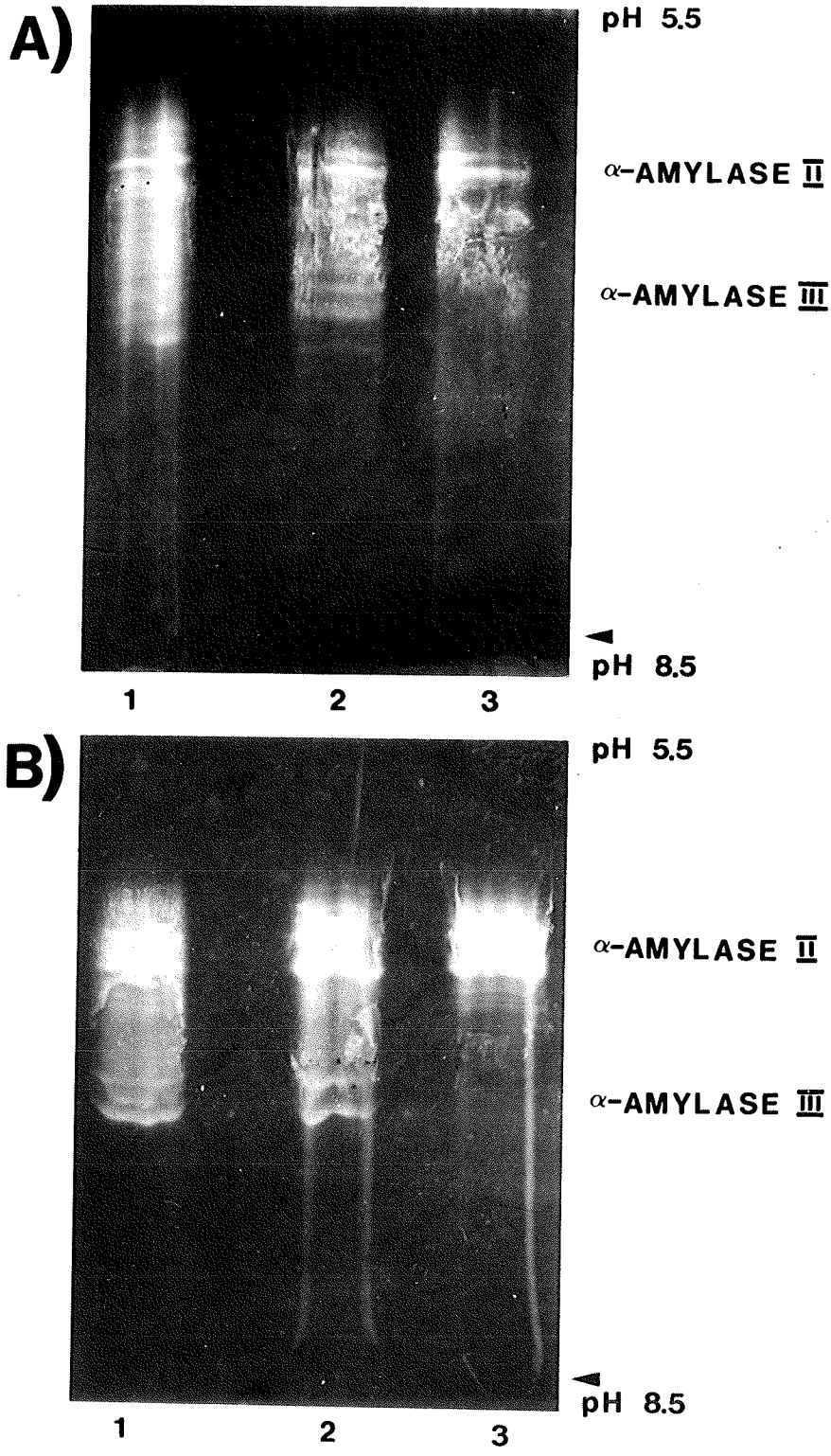

A)
pH 5.5
α-AMYLASE II
α-AMYLASE III
pH 8.5
1
2
3
B)
pH 5.5
α-AMYLASE II
α-AMYLASE III
pH 8.5
1
2
3

binding (Weselake and Hill, 1982) because the inhibitor could be removed from CHA-epoxy-Sepharose 6B without removing α-amylase II from the column.

Effect of Inhibitor on α-Amylase Catalyzed Starch Hydrolysis

The interaction between α-amylase II and inhibitor was characterized further by inhibition studies using starch in solution as a substrate for the enzyme. Inhibition of α-amylase II did not occur instantaneously upon addition of inhibitor. Maximum inhibition was attained at both pH 8.0 and pH 5.5 only after 5 minutes of pre-incubation of enzyme and inhibitor and no further changes were observed even after extended preincubation of up to 60 minutes (Figure 11). The interactions of animal α-amylases with inhibitor proteins from the wheat kernel are known to be time-dependent processes also (Buonocore et al., 1977). Presumably, this time requirement was necessary before enzyme and inhibitor were maximally associated, under the experimental conditions used. Therefore, all enzyme and inhibitor mixtures were pre-incubated for at least 15 minutes prior to the addition of starch solution and subsequent determination of residual α-amylase activity.

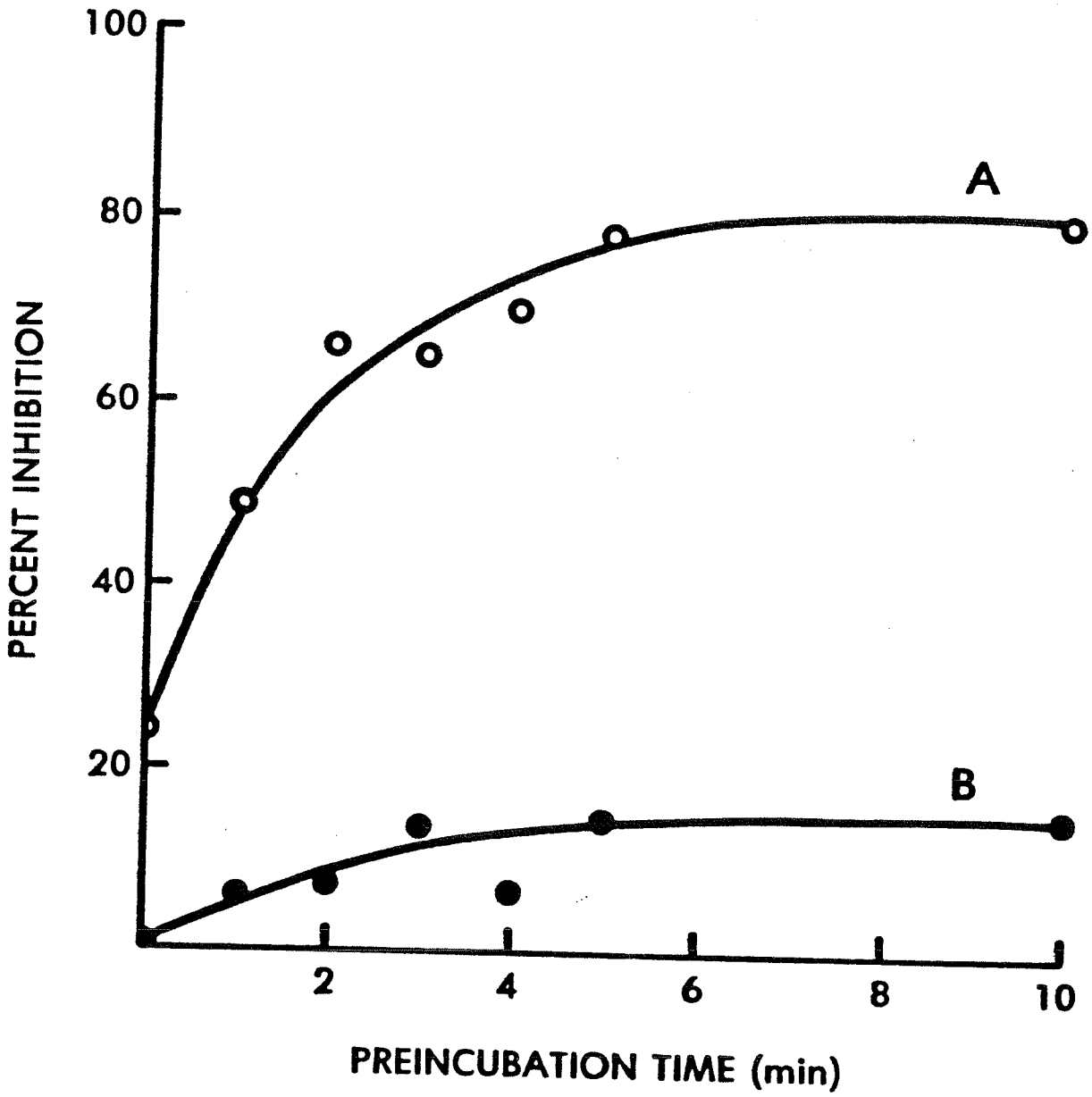
Following pre-incubation of α-amylase II and inhibitor, starch hydrolysis was monitored by the appearance of reducing activity. Figure 12 illustrates the hydrolysis, at pH 8.0, of starch solution by α-amylase II in the absence and presence of inhibitor. After 7 minutes the control hydrolysis resulted in the release of 3% of the potential reducing activity of the substrate. Routinely, enzyme reactions were not allowed to proceed beyond 3% hydrolysis of the substrate. Starch

Figure 11. Effect of pre-incubation time on inhibition of α-amylase activity at pH 8.0 (A) and pH 5.5 (B).

Digest conditions:

A. 40 mM Tris-HCl buffer (1mM $CaCl_2$, 250 µg/ml BSA, pH 8.0) at 35°C. Enzyme concentration = 0.75 µg/ml. Inhibitor concentration = 0.50 µg/ml. Substrate was 0.5% starch in solution. Reaction time = 7 minutes.

B. 200 mM sodium acetate buffer (1mM $CaCl_2$, 250 µg/ml BSA, pH 5.5) at 35°C. Enzyme concentration = 0.38 µg/ml. Inhibitor concentration = 4.75 µg/ml. Substrate was 0.5% starch in solution. Reaction time = 7 minutes.


100
80
60
40
20
PERCENT INHIBITION
A
B
2
4
6
8
10
PREINCUBATION TIME (min)

hydrolysis in the presence of inhibitor was linear during the duration of the reaction (Figure 12). Once the pre-incubation requirement was fulfilled, addition of solubilized starch to the α-amylase II-inhibitor mixture did not result in an exponential liberation of reducing power. This suggests that the substrate did not disrupt the α-amylase II-inhibitor complex, under the experimental conditions used. The possibility that the substrate induced a very rapid and undetectable partial dissociation of the complex, however, cannot be excluded. Other investigators have reported partial dissociation of α-amylase-inhibitor complexes at lower pH values following addition of solubilized starch to equilibrated mixtures of enzyme and inhibitor (Buonocore et al., 1980; Mundy et al., 1983).

In the first section, dealing with purification and properties of the inhibitor, it was stated that inhibition of α-amylase II by the inhibitor was substantially higher at pH 8.0 than at pH 5.5. A detailed study on the effect of pH on α-amylase II inhibition was conducted and the results are shown in Figure 13. Citrate-phosphate buffers of constant ionic strength were used for the pH range 5.0 to 7.5. This buffer system could not be used at higher pH values because of precipitation of calcium salts. Therefore, Tris-HCl buffer was used from approximately pH 7.0 to pH 9.0, thus allowing an overlap with the citrate-phosphate buffer system. The pH optimum for α-amylase II activity was about pH 5.5, in agreement with previous results (Greenwood and MacGregor, 1965). In the presence of inhibitor, the relative activity of α-amylase II was lowered (Figure 13). An approximately 2-fold molar excess of inhibitor over enzyme was used, assuming molecular

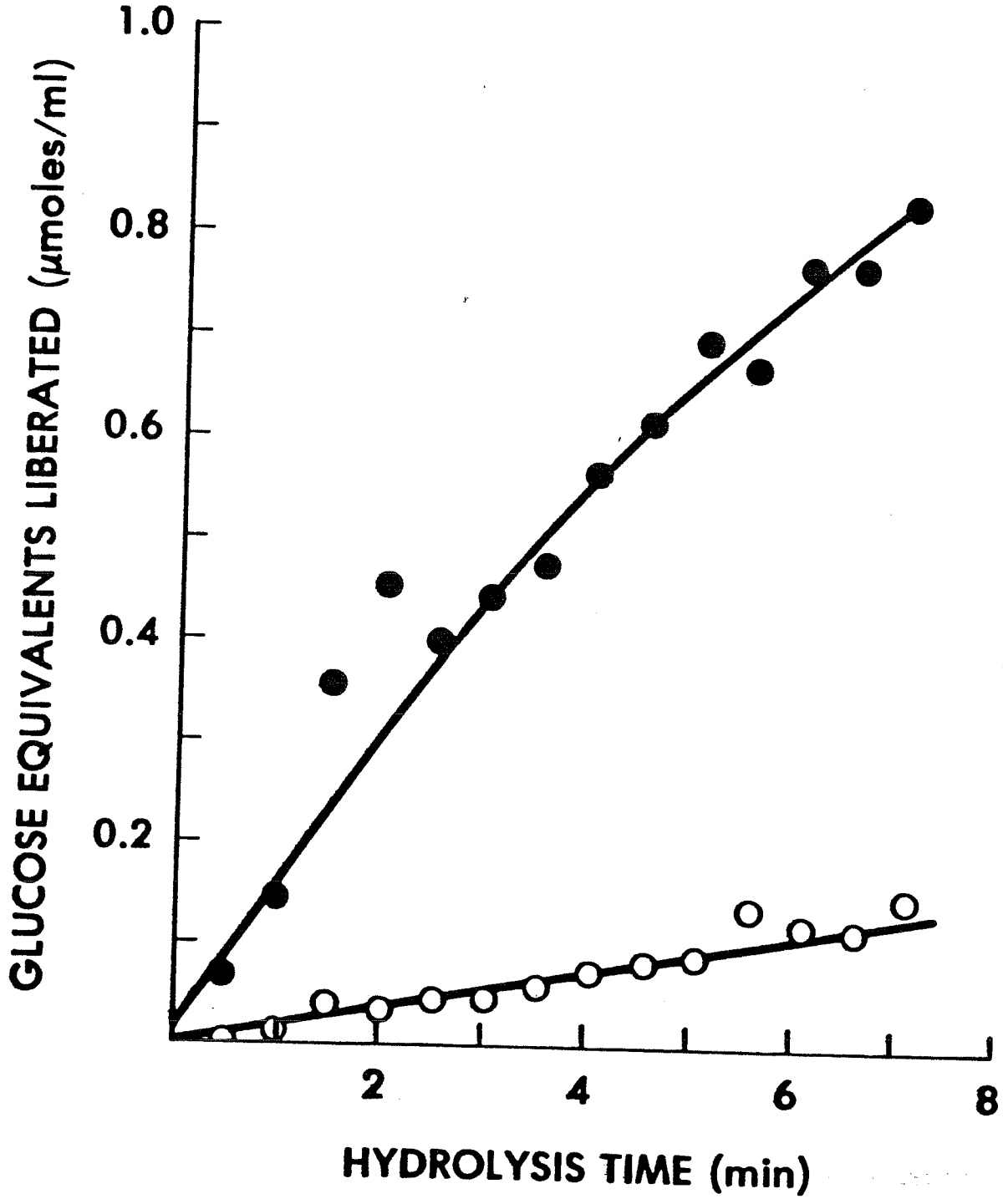
Figure 12. Hydrolysis of starch in solution by α-amylase II in the absence and presence of inhibitor.

● = control hydrolysis.

○ = hydrolysis in the presence of inhibitor.

Digest conditions:

40 mM Tris-HCl buffer (1 mM $CaCl_2$, 250 µg/ml BSA, pH 8.0) at 35°C. Enzyme concentration = 0.75 µg/ml. Inhibitor concentration = 0.48 µg/ml. Substrate was 0.5% starch in solution.


1.0
0.8
0.6
0.4
0.2
GLUCOSE EQUIVALENTS LIBERATED (μmoles/ml)
2
4
6
8
HYDROLYSIS TIME (min)

weights of 45,000 and 20,000 for α-amylase II and inhibitor, respectively. In the presence of inhibitor, enzyme activity at pH 5.5 was slightly lower than the maximum enzyme activity. At pH 8.0, although the activity of the control reaction decreased to 40% of the maximum enzyme activity, the inhibited reaction decreased to 15% of the maximum activity. Differences between control and inhibited reactions indicated that the degree of inhibition increased from 5% to 63% inhibition at pH 5.5 and pH 8.0, respectively. The increased affinity of inhibitor for α-amylase II at higher pH values might be linked to de-protonization of amino acid residues at the site(s) of interaction between the two macromolecules.

The large difference in the degree of inhibition at pH 5.5 compared to pH 8.0 prompted a detailed study on the effect of inhibitor concentration on the hydrolysis of starch solution by α-amylase II, at these two pH values. Under the experimental conditions used, an approximately 1100 molar excess of inhibitor over α-amylase II was required to attain 40% inhibition at pH 5.5 (Figure 14A), but at pH 8.0 only 1 mole of inhibitor per mole of enzyme was required to achieve the same inhibition (Figure 14B). Furthermore, inhibition at pH 8.0 was linear up to about 70% inhibition, but, large excesses of inhibitor were required, thereafter, to produce only small increases in inhibition. Therefore, when quantifying inhibitor activity at pH 8.0, the inhibition was maintained below 70% to stay within the linear range of the inhibitor assay. The high concentration of inhibitor required to cause inhibition at pH 5.5 suggests that the enzyme and inhibitor exist in a free and combined form. Mundy et al. (1983) have reached the same conclusions in studies on the effect of inhibitor (from green malt)

Figure 13. Effect of pH on hydrolysis of starch in solution by α-amylase II in the absence and presence of inhibitor.

(●) citrate-phosphate buffer system and (▲) Tris-HCl buffer without inhibitor.

(○) citrate-phosphate buffer system and (△) Tris-HCl buffer with inhibitor.

Digest conditions:

Citrate-phosphate buffer system of 0.05μ ionic strength and 50 mM Tris-HCl buffer at 35°C. Each buffer contained 1 mM $CaCl_2$ and 250 μg/ml BSA. Enzyme concentration = 0.25 μg/ml. Inhibitor concentration = 0.25 μg/ml. Substrate was 0.5% starch in solution. Hydrolysis times were 7 minutes for pH range 5.0 to 6.7 and 20 minutes for pH range 7.1 to 9.1.

All enzyme activities are relative to the control activity which equals 100 at pH 5.4.

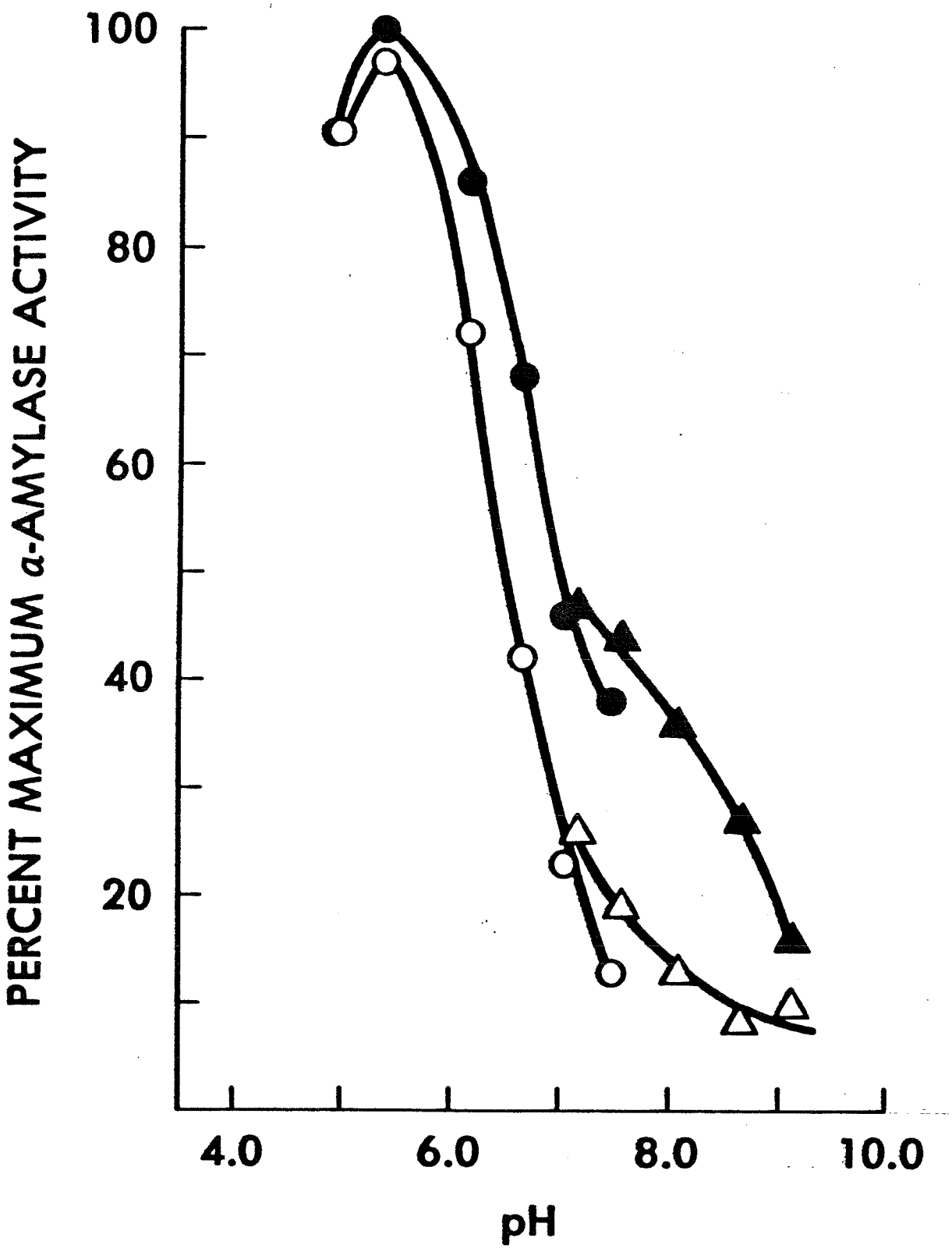
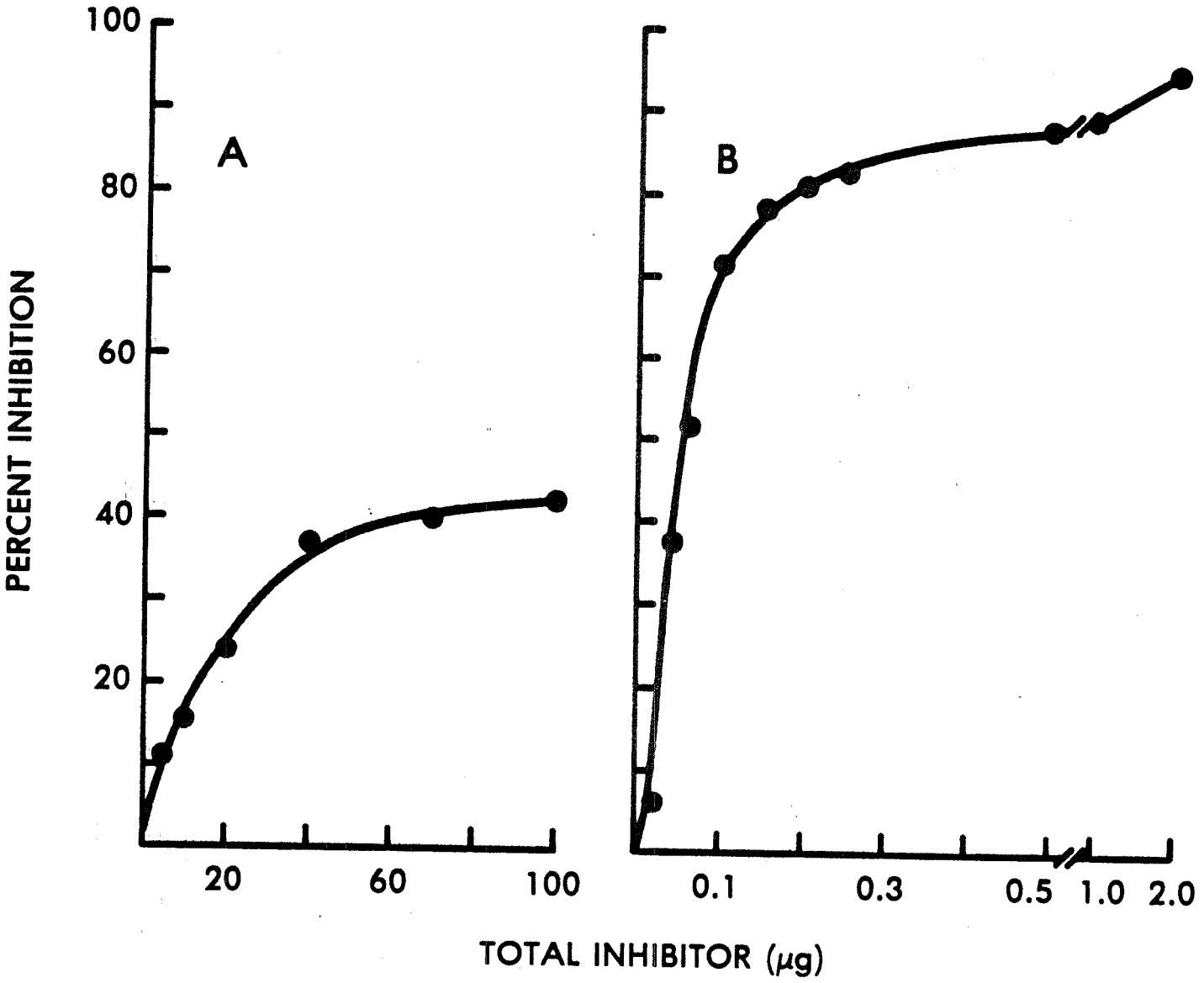

PERCENT MAXIMUM α-AMYLASE ACTIVITY
100
80
60
40
20
4.0
6.0
8.0
10.0
pH

Figure 14. Effect of inhibitor concentration on α-amylase II activity at pH 5.5 (A) and pH 8.0 (B).

Digest conditions:

A. 200 mM sodium acetate buffer (1 mM $CaCl_2$, 250 µg/ml BSA, pH 5.5) at 35°C. Enzyme/0.40 ml of reaction mixture = 0.10 µg. Substrate was 0.5% starch in solution. Hydrolysis time = 8 minutes.

B. 40 mM Tris-HCl buffer (1 mM $CaCl_2$, 250 µg/ml BSA, pH 8.0) at 35°C. Enzyme/0.40 ml of reaction mixture = 0.10 µg. Substrate was 0.5% starch in solution. Hydrolysis time = 22 minutes.


100
80
60
40
20
PERCENT INHIBITION
A
B
20
60
100
0.1
0.3
0.5
1.0
2.0
TOTAL INHIBITOR (μg)

concentration on α-amylase II activity at pH 6.0.

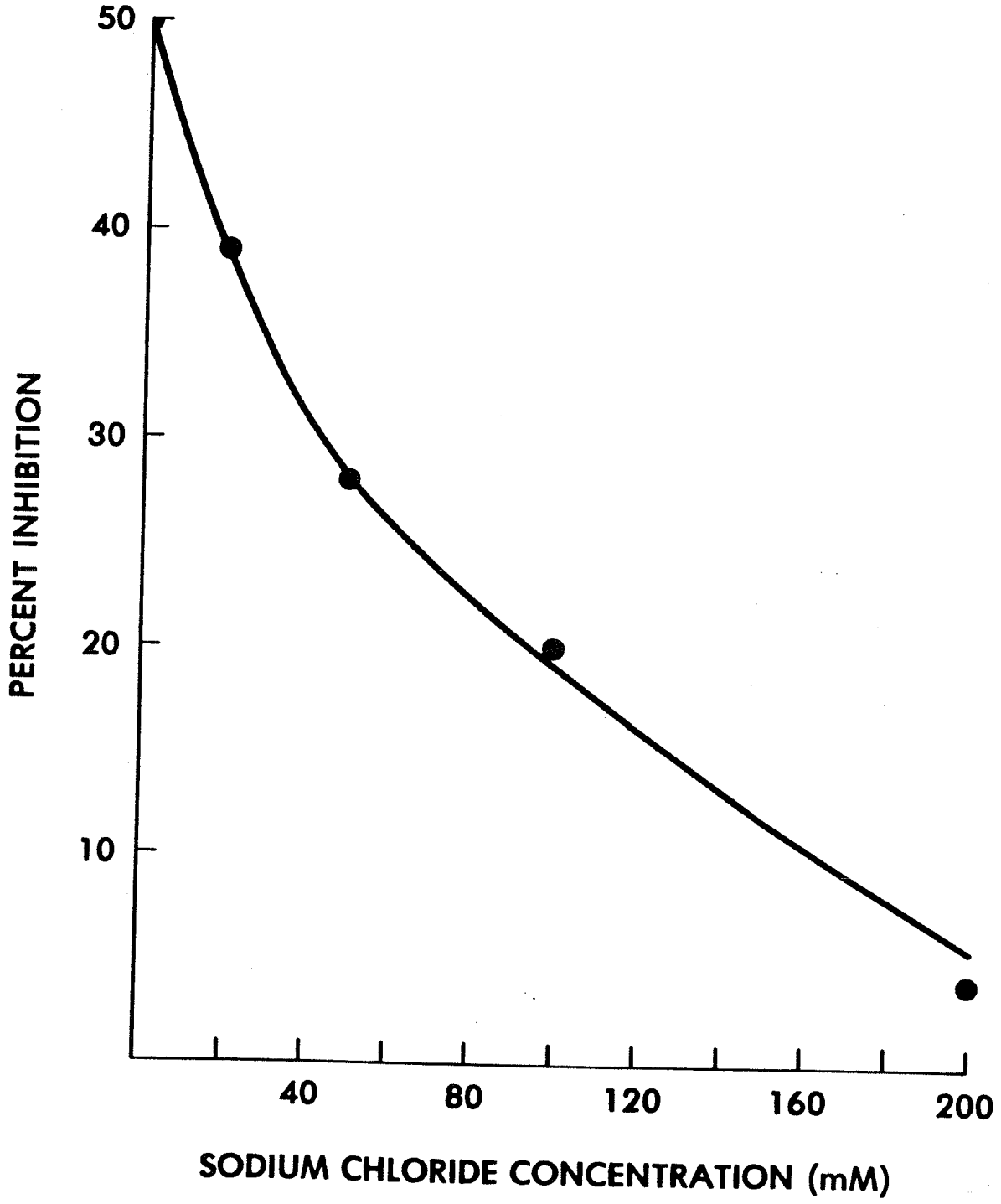
The inhibition curves in Figure 14 offer an explanation for the α-amylase activity exhibited by α-amylase III (α-amylase II-inhibitor complex) after isoelectric focusing (Figure 6, lanes 2 and 3) or after gel filtration chromatography carried out at pH 8.0 (Figure 8). Although a strong complex between α-amylase II and inhibitor can be formed at pH 8.0 (forming α-amylase III), when the pH is lowered to 5.5 for α-amylase activity analysis, the complex is weakened. Thus, some α-amylase II is liberated and its activity then may be detected.

In the previous section, affinity chromatography studies with mixtures of α-amylase II and inhibitor showed that 0.3 M NaCl had a dissociating effect on the enzyme-inhibitor complex (α-amylase III) at pH 5.5. Inhibition studies were conducted to determine the effect of NaCl concentration on the inhibition of α-amylase II by the inhibitor. In the absence of inhibitor, concentrations as high as 200 mM NaCl, in the presence of 5 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5), had no effect on the activity of α-amylase II (not shown). As salt concentrations were increased, the effectiveness of the inhibitor decreased (Figure 15), but this decrease was not linear. A concentration of 50 mM NaCl decreased the inhibition to 28% whereas 200 mM NaCl was required to decrease the inhibition to 5%. The results of this study suggested that the α-amylase II-inhibitor interaction was due, largely, to charge effects at pH 5.5 and that the affinity of enzyme for inhibitor was higher at low ionic strength. The concentration of sodium acetate buffer appeared to affect the degree of inhibition, also. A 50-fold molar excess of inhibitor over α-amylase II resulted in 50% inhibition of enzyme activity when the experiment was conducted in a 5 mM

Figure 15. Effect of sodium chloride concentration on inhibition of α-amylase II by the inhibitor.

Digest conditions:

5 mM sodium acetate buffer (1 mM $CaCl_2$, 250 µg/ml BSA, pH 5.5) at 35°C. Enzyme concentration = 0.35 µg/ml. Inhibitor concentration = 8.00 µg/ml. Substrate was 0.5% starch in solution. Hydrolysis time = 8 minutes.


50
40
30
20
10
PERCENT INHIBITION
40
80
120
160
200
SODIUM CHLORIDE CONCENTRATION (mM)

sodium acetate buffer, in the absence of NaCl (Figure 15). In contrast, a 50 molar excess of inhibitor resulted in less than 10% inhibition of enzyme activity when the experiment was performed in a 200 mM sodium acetate buffer (Figure 14A). Presumably, buffer concentration had a similar effect to NaCl concentration, on the degree of inhibition of α-amylase II by the inhibitor.

The interaction between α-amylase II and inhibitor has been shown to be affected by pH, salt concentration and inhibitor concentration. Presumably, inhibitor or dissociation constants, derived from kinetic studies with solubilized starch could fluctuate enormously, depending on the experimental conditions used. Any attempt to analyze the α-amylase II-inhibitor interaction, using classical kinetic procedures (Lineweaver and Burk, 1934; Dixon, 1953) would be questionable. In rapid-equilibrium kinetics, substrates and inhibitors are assumed to interact rapidly and reversibly with the enzyme. Secondly, the total inhibitor in the system must be in considerable excess over the total enzyme such that the combination of enzyme and inhibitor does not reduce significantly the free inhibitor level. A pre-incubation period of approximately 5 minutes was required for α-amylase II and the inhibitor before maximum inhibition was achieved, when assaying for residual α-amylase activity (Figure 11). Therefore, equilibrium between α-amylase II and inhibitor was not reached rapidly, thereby invalidating the use of classical kinetic procedures. At pH 8.0, a significant portion of the total α-amylase II inhibitor in the system is bound by the enzyme (Figure 14B), once again invalidating the use of classical kinetic procedures. Furthermore, starch in solution, as a substrate, poses problems in kinetic analysis of α-amylase inhibition. Starch is

a poorly defined substrate and during hydrolysis, the nature of the substrate changes continuously. Hydrolytic products, varying in size, serve as substrates for further enzymatic digestion, but these products may be hydrolyzed further at different rates by α-amylase (Greenwood and Milne, 1968b). Therefore, kinetic parameters derived from classical kinetic procedures describing α-amylase II catalyzed hydrolysis of starch in solution may not be meaningful.

Effect of Inhibitor on Hydrolysis of Amylose

The types of products formed during hydrolysis of linear dextrins by α-amylase can yield valuable information on the action pattern and reaction mechanism of this type of enzyme (Greenwood and Milne, 1968a, 1968b; Thoma et al., 1971). Hydrolysis of linear amylose by barley α-amylase II, in the absence and presence of inhibitor, was performed to determine if the inhibitor altered the way in which the enzyme hydrolyzed the substrate. Linear amylose does not contain α(1→6) branch points and so yields only linear products on enzymatic digestion. These products can be analyzed more easily than the mixture of linear and branched products that would be obtained from starch hydrolysis. α-Amylase II catalyzed hydrolysis of linear amylose consisted of a rapid phase of hydrolysis followed by a considerably slower phase (Figure 16). Within the first 10 minutes of enzyme reaction 0.9 μmole/ml glucose equivalent was liberated, but an additional 170 minutes of hydrolysis were required to liberate a further 0.3 μmole/ml of glucose equivalent. The shape of the hydrolysis curve was in agreement with previous work on the hydrolysis of linear amylose by cereal α-amylases (Greenwood and Milne, 1968a). The rapid phase of hydrolysis is caused

by a random attack of the enzyme to produce a mixture of smaller dextrins. The slower phase of hydrolysis is due to non-random hydrolysis of these dextrins. Non-random hydrolysis has been attributed to the difficulty with which progressively smaller dextrins are hydrolyzed by the enzyme (Bird and Hopkins, 1954; Greenwood et al., 1965).

In the presence of a 10-fold molar excess of inhibitor, the extent of hydrolysis of amylose was reduced (Figure 16). Reducing power, liberated after 10 and 180 minutes of enzymatic hydrolysis, was about 0.6 and 0.9 μmole/ml glucose equivalent, respectively. After 180 minutes, the inhibited α-amylase had liberated 27% less reducing power than was released in the control reaction. Although the extent of hydrolysis, in the presence of inhibitor, was less than the extent of the control hydrolysis, the shapes of both hydrolysis curves were similar (Figure 16).

The small products of amylose hydrolysis by α-amylase II were examined by thin layer chromatography (TLC), to gain an insight into the reaction products produced by the enzyme. Aliquots from both control and inhibited digests were taken after 10 minutes of hydrolysis, shortly after completion of the rapid stage of amylose hydrolysis. Additional aliquots were taken after 60 and 180 minutes of hydrolysis, when the enzyme reaction was in the slower, non-random stage of hydrolysis. These time intervals were chosen to allow for an effective study of the distribution of small products in the digests, as a function of hydrolysis time. A photograph of the TLC plate, visualized for dextrins, is shown in Figure 17. Good separation was obtained for maltodextrins containing up to 15 glucose units (Figure 17, lane c). Corresponding densitometer tracings for the control and inhibited digests

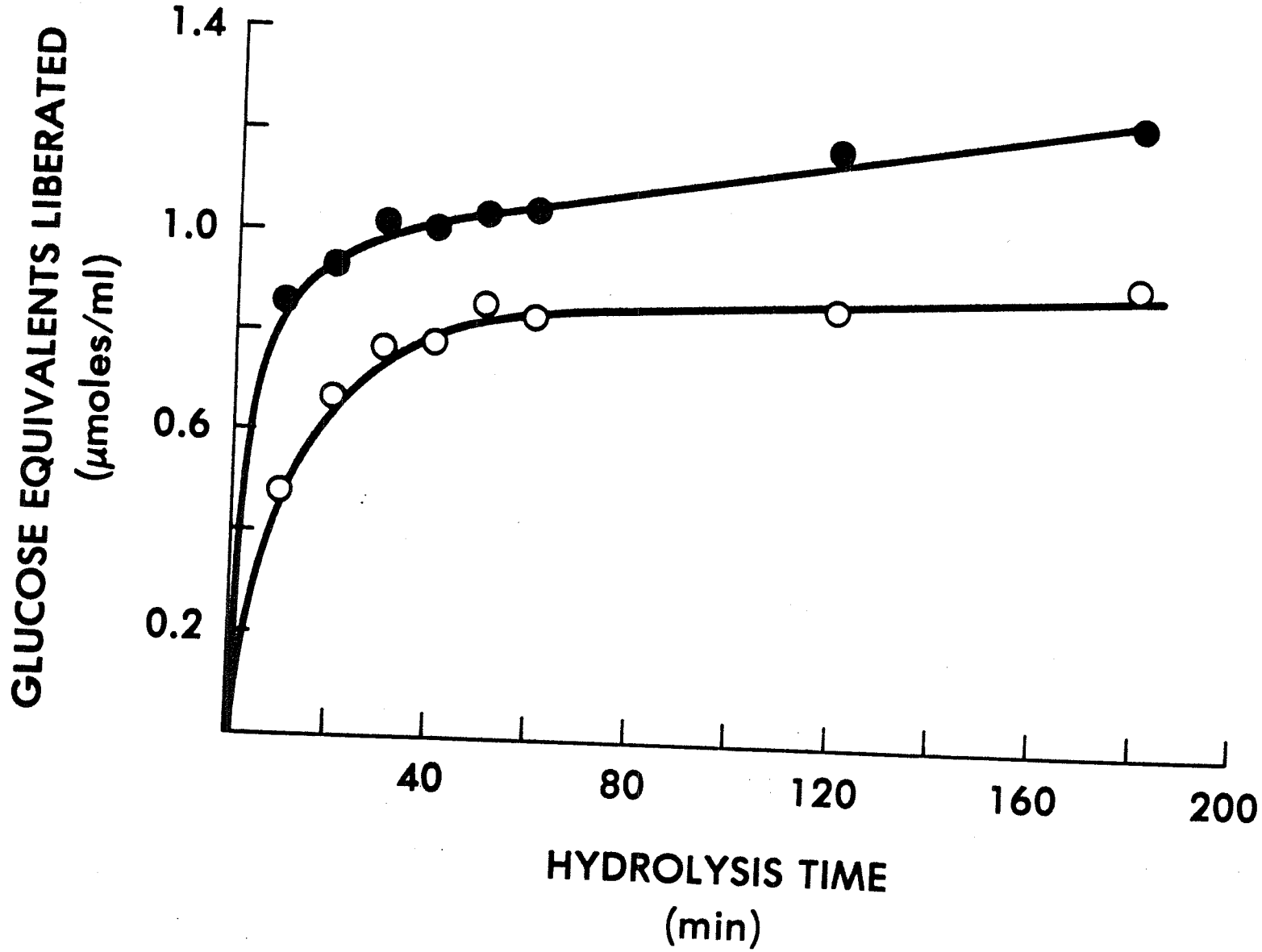
Figure 16. Hydrolysis of amylose by α-amylase II at pH 5.5 in the absence and presence of inhibitor.

● = control hydrolysis.

O = hydrolysis in the presence of inhibitor.

Digest conditions:

5 mM sodium acetate buffer (1mM $CaCl_2$, 125 µg/ml BSA, pH 5.5) at 35°C. Enzyme concentration = 1.0 µg/ml. Inhibitor concentration = 4.8 µg/ml. Substrate was 0.08% linear amylose.


GLUCOSE EQUIVALENTS LIBERATED
(μmoles/ml)
1.4
1.0
0.6
0.2
40
80
120
160
200
HYDROLYSIS TIME
(min)

are shown in Figures 18 and 19, respectively. After 10 minutes of hydrolysis, there were no products larger than 8 glucose units (G 8) in length in the control digest, except for a very small quantity of carbohydrate at the origin (Figure 18A). At this time the main products of hydrolysis were G 7 and G 6. After 60 minutes, G 8 had disappeared and G 6 had become the predominant maltodextrin (Figure 18B). After 180 minutes, G 7 had nearly disappeared whereas G 6 and G 2 were now predominant (Figure 18C). The accumulation of relatively high proportions of G 6 and G 2 after extended hydrolysis of linear amylose by cereal α-amylases has been described, previously (Greenwood and Milne, 1968a).

Digests with inhibitor, had a greater proportion of larger glucose oligomers when compared to control digests after the same times of hydrolysis. After 10 minutes, the hydrolyzate of the inhibited reaction contained maltodextrins ranging from G 1 to G 15 (Figure 19A). A relatively high proportion of unresolved dextrin, however, remained at the origin. After 60 minutes of inhibited hydrolysis, there was only a very small quantity of carbohydrate at the origin, whereas G 7, G 6 and G 2 predominated in the hydrolyzate (Figure 19B). After 180 minutes, G 8 had disappeared and G 6 began to predominate over G 7 (Figure 19C).

Similar reducing power was released by the control and inhibited reaction after 10 and 60 minutes of hydrolysis, respectively (Figure 16). A comparison of densitometer tracings of the control digest at 10 minutes (Figure 18A) and the digest of the inhibited reaction at 60 minutes (Figure 19B) indicated that the dextrin profiles were similar. Under the experimental conditions used, the inhibitor did not appear to alter the way in which α-amylase II catalyzed the depolymerization of

Figure 17. Thin layer chromatography of products of amylose hydrolysis by α-amylase II in the absence and presence of inhibitor.

Lane a: maltose.

Lanes b, d and f: control hydrolysis after 10, 60 and 180 minutes, respectively.

Lanes c, e and g: inhibited hydrolysis after 10, 60 and 180 minutes, respectively.

NOTICE/AVIS

PAGE(S) 92 IS/ARE EST/SONT black + white photo

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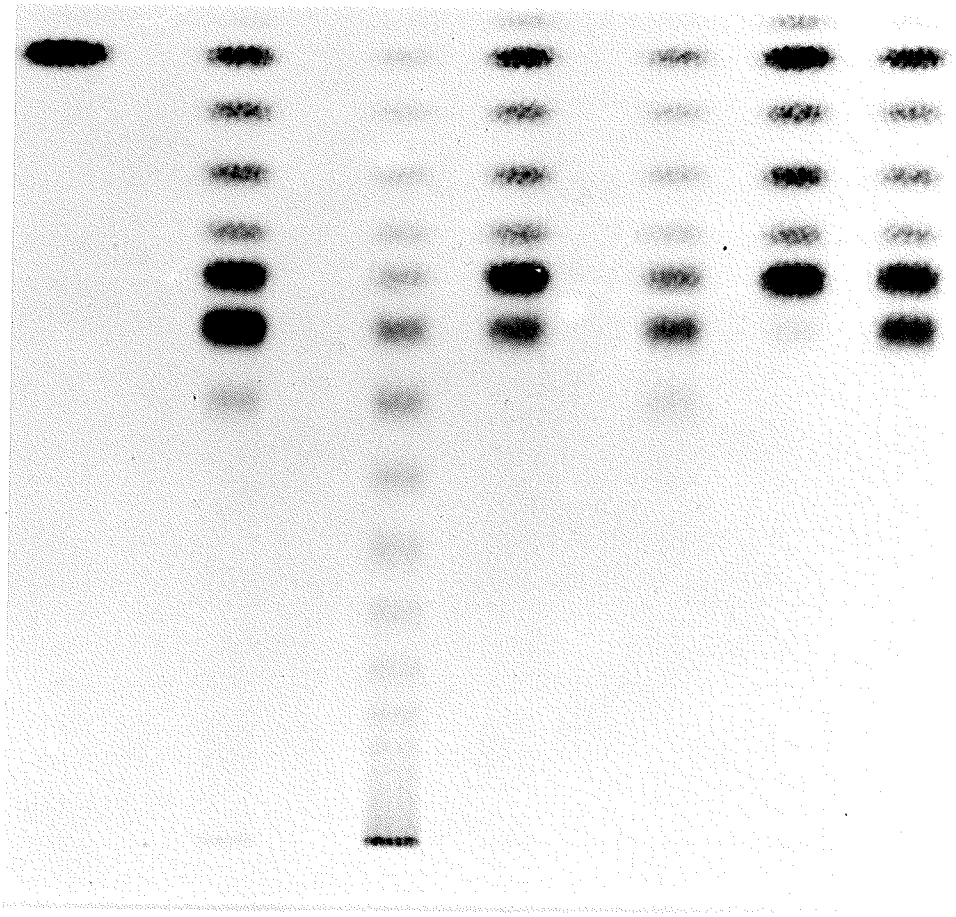
VEUILLEZ ECRIRE A L'AUTEUR POUR LES RENSEIGNEMENTS OU VEUILLEZ CONSULTER L'EXEMPLAIRE DONT POSSEDE LE DEPARTEMENT DES ARCHIVES ET DES COLLECTIONS SPECIALES, BIBLIOTHEQUE ELIZABETH DAFOE, UNIVERSITE DU MANITOBA, WINNIPEG, MANITOBA, CANADA, R3T 2N2.



a b c d e f g

Figure 18. Dextrin profiles of products after hydrolysis of amylose by α-amylase II.

Densitometer tracings of TLC separated hydrolysis products. Peaks 1 to 8 are considered to be glucose oligomers ranging from one glucose unit to 8 glucose units in length.

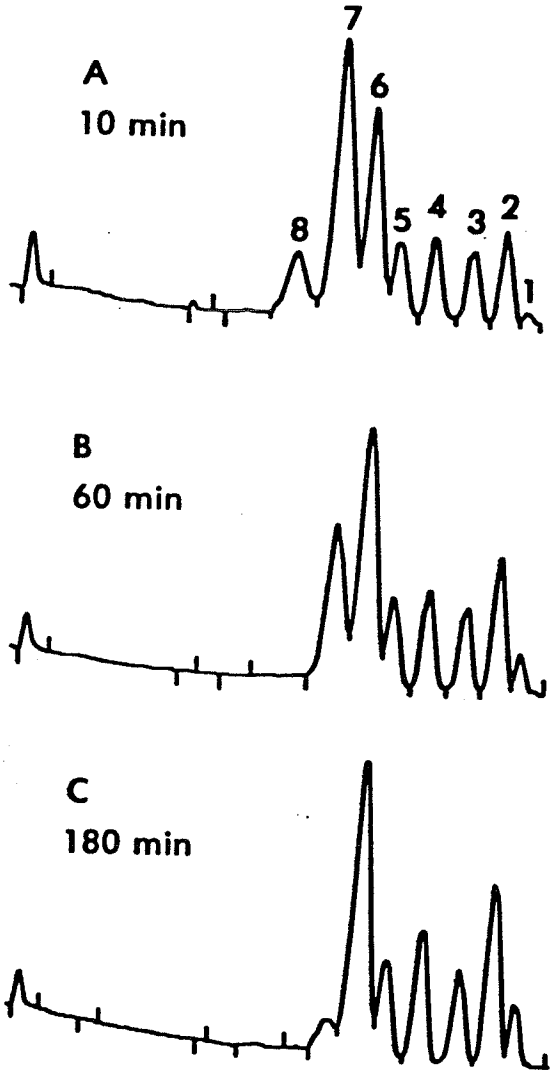
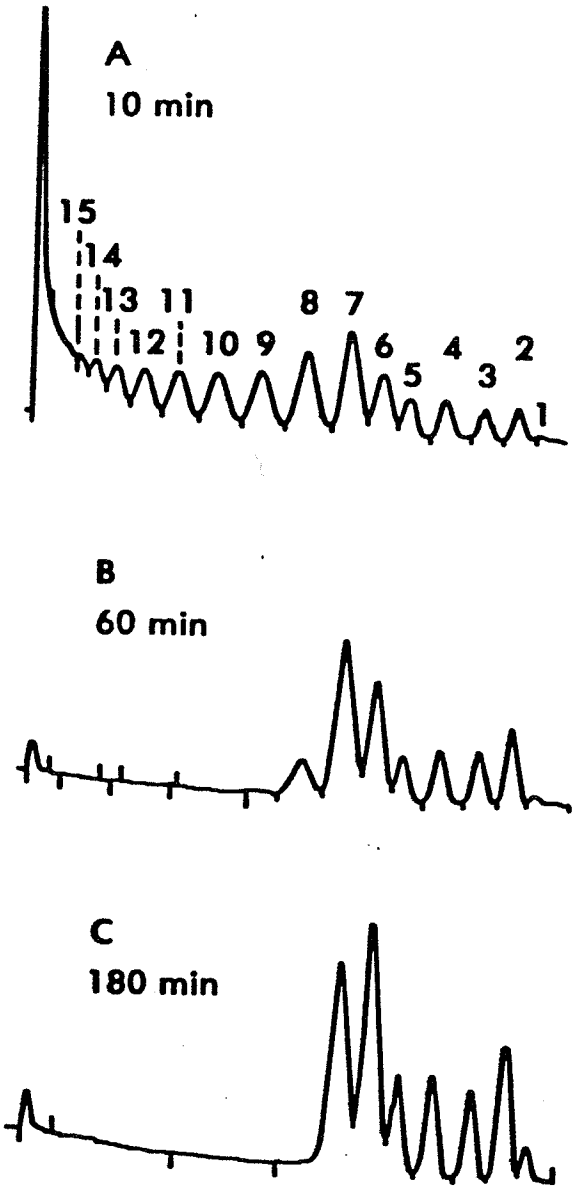

A
10 min
7
6
8
5
4
3
2
1
B
60 min
C
180 min

Figure 19. Dextrin profiles of products after hydrolysis of amylose by α-amylase II in the presence of inhibitor.

Densitometer tracings of TLC separated hydrolysis products. Peaks 1 to 15 are considered to be glucose oligomers ranging from one glucose unit to 15 glucose units in length.


A
10 min
15
14
13
11
12
10
9
8
7
6
5
4
3
2
1
B
60 min
C
180 min

linear amylose. Instead, the main effect of the inhibitor was in delaying the rate at which linear amylose was depolymerized by α-amylase II.

Effect of Inhibitor on Hydrolysis of Starch Granules

Inhibition studies with solubilized substrates have provided important information on the nature of the interaction between α-amylase II and the inhibitor. But, under *in vivo* conditions, α-amylase is responsible for the initial hydrolysis of starch granules. Therefore, a study of the effect of inhibitor on starch granule degradation by α-amylase might be more meaningful than using starch in solution, when attempting to elucidate a physiological role for the inhibitor.

Parameters affecting the α-amylase II-inhibitor interaction were studied by observing changes in the rate of hydrolysis of large starch granules from barley. In these experiments, the term hydrolysis refers only to enzymatic digestion resulting in the release of soluble carbohydrate (expressed as soluble starch) from the granule. For example, α-amylase may catalyze the hydrolysis of some α(1→4) glycosidic bonds in the starch granule without the corresponding release of a soluble fragment. A temperature of 18°C was used, in most cases, because it was similar to temperatures used during the steeping and germination phases of malting and a pH of 5.5 was used to ensure optimal enzyme activity (Greenwood and MacGregor, 1965). Starch granule experiments were conducted with BSA in the reaction mixture to stabilize the enzyme during the relatively long reaction periods used (MacGregor and Ballance, 1980b). At pH 5.5 and 18°C, BSA was found to stabilize α-amylase II, completely, for at least 20 hours.

Figure 20 illustrates how the rate of starch granule hydrolysis by

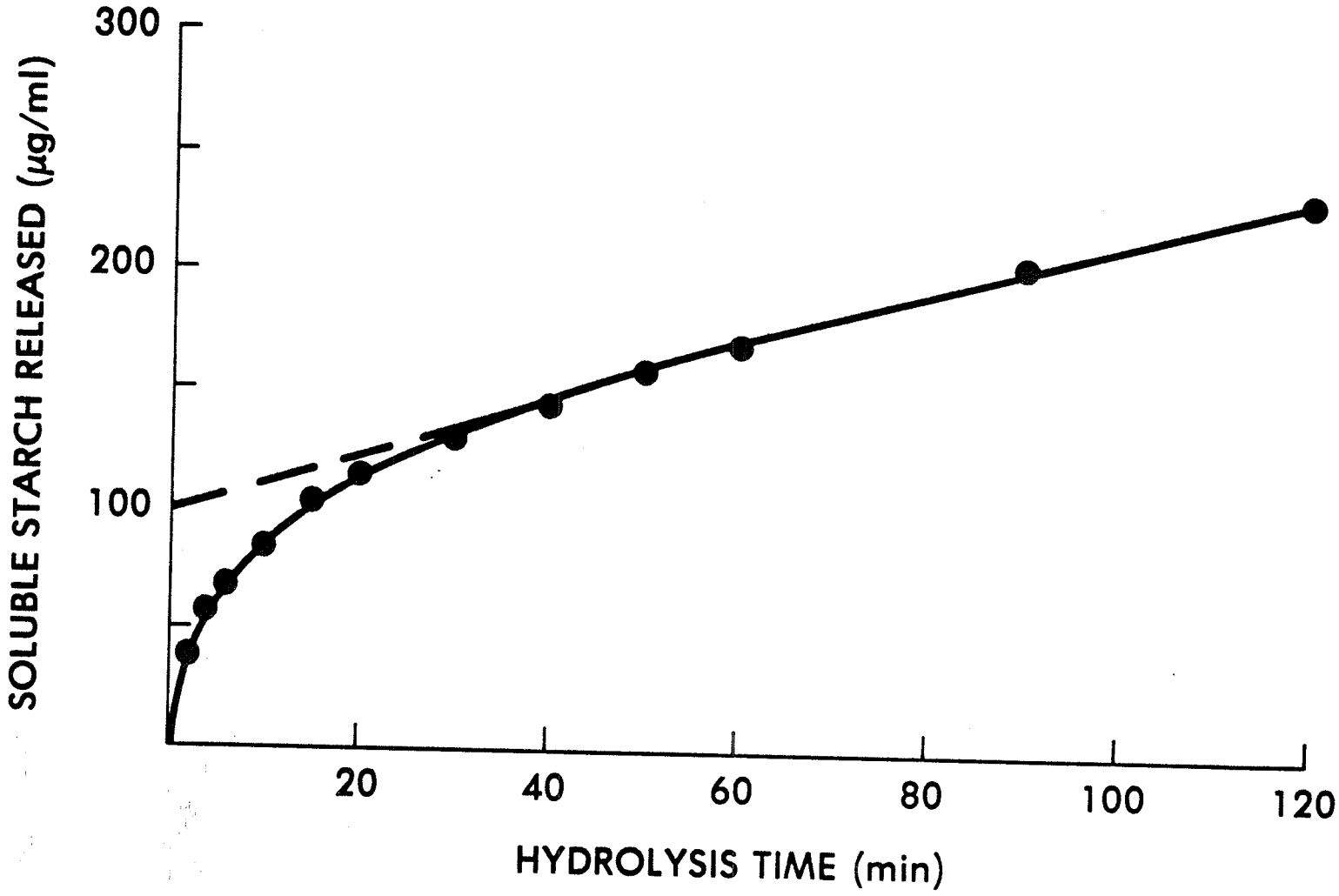
α-amylase was established. Initially, the release of soluble starch was rapid and curvilinear with reaction time, but eventually the rate of hydrolysis decreased and the release of soluble starch became linear with reaction time. The rapid phase of starch granule hydrolysis is believed to be related to starch damage (Sandstedt and Mattern, 1960). During isolation of starch granule fractions from cereal kernels, some granules become physically damaged and the extent of damage can vary, depending on the preparative conditions. Damaged granules are more susceptible to enzymatic digestion than are intact granules. The degree of starch granule damage can be estimated by extrapolating a line from the linear portion of the hydrolysis curve to the y-axis (Figure 20). The intercept is an indication of the extent of starch damage (Sandstedt and Mattern, 1960; MacGregor and Ballance, 1980b). With the batch of starch used in this study the damage was estimated to be approximately 1% of the starch granules. The slow phase of starch granule hydrolysis is believed to reflect degradation of intact starch granules by the enzyme and has been shown to be almost linear for relatively long time periods, when using large starch granules as substrate for barley α-amylases (MacGregor and Ballance, 1980b). In the inhibition studies, the slope of the slow phase was used to express the rate of starch granule hydrolysis. Spontaneous release of soluble starch, without enzyme present, never amounted to more than 2% of the soluble starch released at any time by the enzyme and, therefore, was considered negligible when determining the rate of starch hydrolysis (Figure 21A).

The effect of various concentrations of inhibitor on starch granule hydrolysis by α-amylase II is shown in Figure 21. Increasing

Figure 20. Hydrolysis of starch granules by α-amylase II.

Digest conditions:

50 mM sodium acetate buffer (1 mM $CaCl_2$, 1 mg/ml BSA, pH 5.5) at 18°C. Enzyme concentration = 0.9 µg/ml. Starch granule concentration = 10 mg/ml.


300
200
100
SOLUBLE STARCH RELEASED (μg/ml)
20
40
60
80
100
120
HYDROLYSIS TIME (min)

concentrations of inhibitor decreased the rate of starch granule hydrolysis (Figure 21A). Slopes of the reaction lines were taken as rates of hydrolysis and converted to percent inhibition based on the rate of the control reaction. Figure 21B shows changes in inhibition with an increasing molar excess of inhibitor over α-amylase II. Between zero and 20-fold molar excess of inhibitor, there was a large increase in inhibition but, thereafter, the inhibition appeared to reach a plateau. With a 50-fold molar excess of inhibitor, the inhibition was approximately 70%. The inhibition curve was similar to that obtained for the inhibition of starch solution hydrolysis by α-amylase II at pH 5.5 (Figure 14A). Inhibition in the starch solution system, however, levelled out at 40% inhibition, with an 1100-fold molar excess of inhibitor. It is difficult to make meaningful comparisons between the two systems because the substrates are in different physical states. Furthermore, the buffer concentrations used in each experiment were different and ionic strength has been shown to have a profound effect on the inhibition (Figure 15).

Hydrolysis of large starch granules by α-amylase I, at pH 5.5, was linear with time also (Figure 22), agreeing with previous work (MacGregor and Ballance, 1980b). In the presence of a 50-fold molar excess of inhibitor there was no change in the rate of granule hydrolysis (Figure 22). Evidence is presented, in a later section, showing that the inhibitor had no effect on the hydrolysis of starch solution by α-amylase I at pH 8.0. Furthermore, the inhibitor had no effect on the isoelectric focusing pattern of α-amylase I. Therefore, the inhibitor has no effect on α-amylase I and is biospecific for α-amylase II.

Figure 21. Effect of inhibitor concentration on hydrolysis of starch granules by α-amylase II.

A. Release of soluble starch by α-amylase with: no inhibitor (●—●); and with 5 (○—○), 10 (■—■), 25 (□—□), 50 (▲—▲) and 100 (△—△) fold molar excesses of inhibitor.

●···● = release of soluble starch without enzyme.

B. Degree of inhibition at different molar excesses of inhibitor over α-amylase.

Digest conditions:

50 mM sodium acetate buffer (1 mM $CaCl_2$, 1 mg/ml BSA, pH 5.5) at 18°C. Enzyme concentration = 0.9 µg/ml. Starch granule concentration = 10 mg/ml.

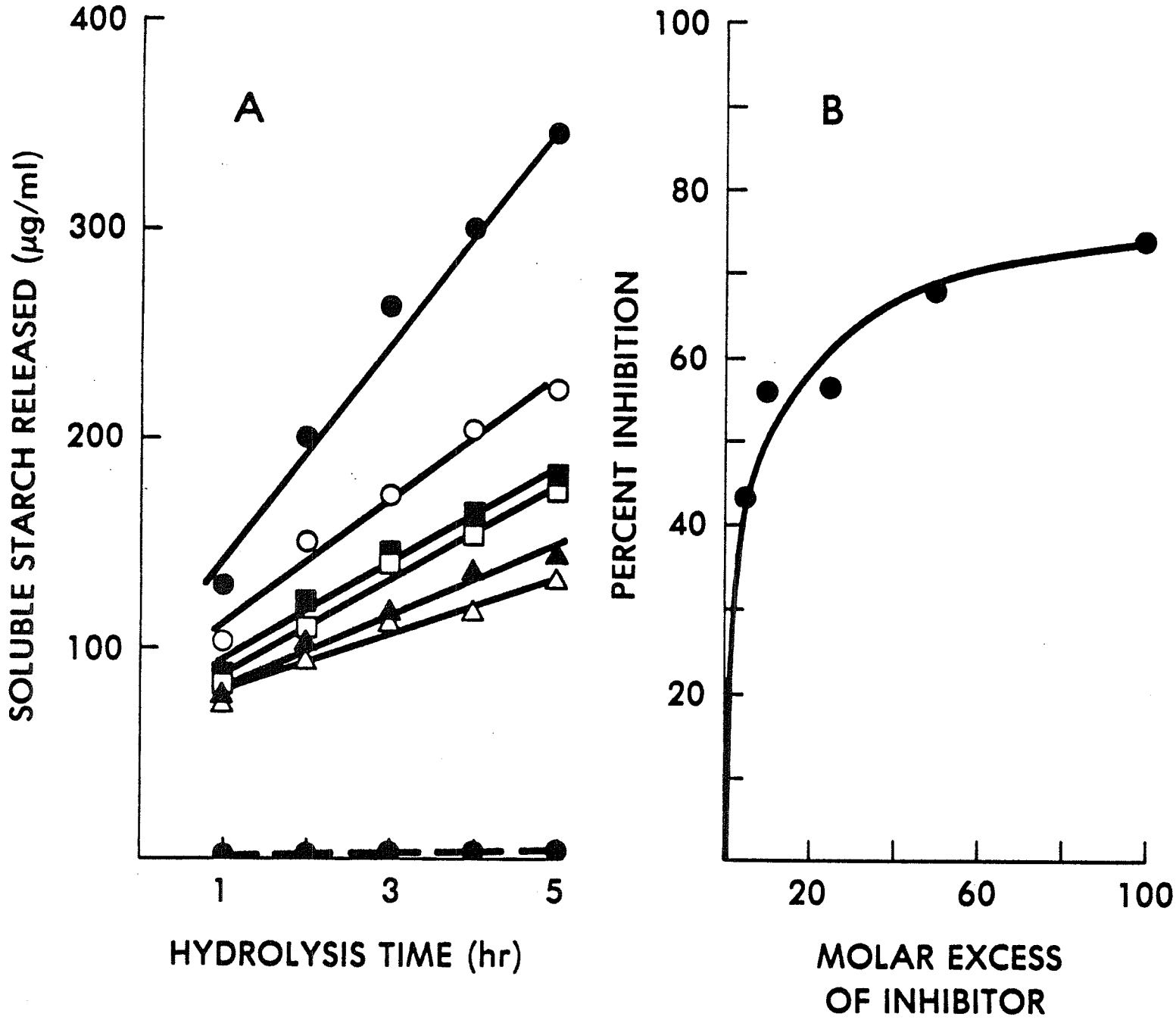
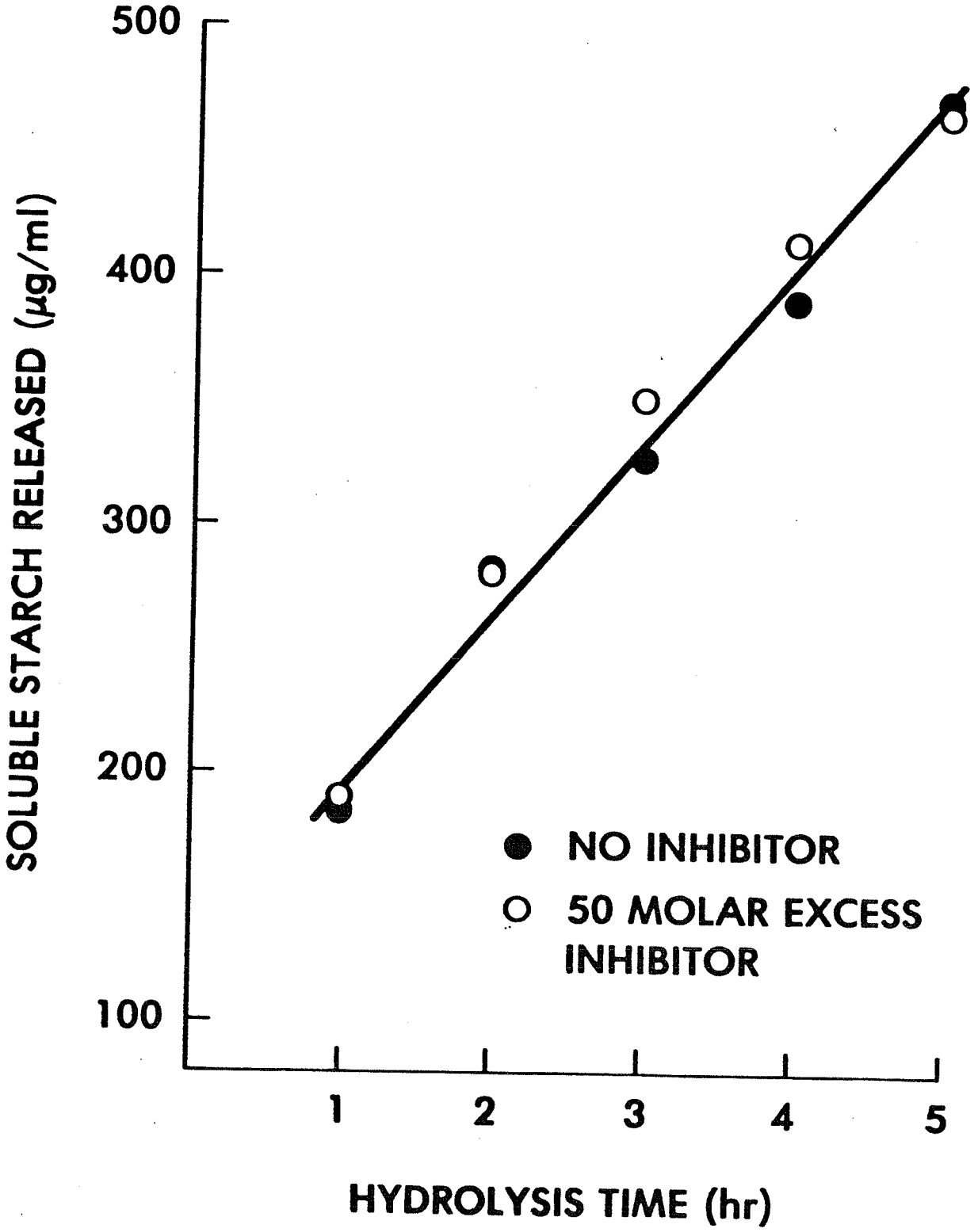

A
400
300
200
100
SOLUBLE STARCH RELEASED (μg/ml)
1
3
5
HYDROLYSIS TIME (hr)
B
100
80
60
40
20
PERCENT INHIBITION
20
60
100
MOLAR EXCESS
OF INHIBITOR

Figure 22. Hydrolysis of starch granules by α-amylase I in the absence and presence of inhibitor.

Digest conditions:

50 mM sodium acetate buffer (1 mM $CaCl_2$, 1 mg/ml BSA, pH 5.5) at 18°C. Enzyme concentration = 2.7 µg/ml. Starch granule concentration = 10 mg/ml.


500
400
300
200
100
SOLUBLE STARCH RELEASED (µg/ml)
NO INHIBITOR
50 MOLAR EXCESS INHIBITOR
1
2
3
4
5
HYDROLYSIS TIME (hr)

The pH activity profile for hydrolysis of large starch granules by α-amylase II (Figure 23) was similar to that observed for solubilized starch (Figure 13). In both cases the pH optimum was close to pH 5.5, which is the generally accepted value for malt α-amylase activity (Greenwood and MacGregor, 1965). The effect of inhibitor on starch granule hydrolysis by α-amylase II was similar to that observed previously, with solubilized substrate. In the presence of a 10-fold molar excess of inhibitor, the activity of α-amylase II was lowered and the pH optimum of the inhibited enzyme shifted to below pH 5.0 (Figure 23). There was no inhibition at pH 4.6, but at pH 5.2 only 70% of maximum enzyme activity was obtained. At pH 6.0, control and inhibited activities were 67% and 23% of maximum activity, respectively. The differences between the control and inhibited reactions showed that inhibition increased from 30% at pH 5.2 to 65% at pH 6.0, indicating the sensitivity of the enzyme-inhibitor interaction to hydrogen ion concentration.

In the previous section, an increase in NaCl concentration from zero to 200 mM was shown to decrease the effect of inhibitor on hydrolysis of starch solution by α-amylase II at pH 5.5 (Figure 15). Inhibition was shown, also, to decrease when the sodium acetate buffer concentration was raised from 5 to 200 mM at pH 5.5. Therefore, the effects of salt and buffer concentration on inhibition were examined using the starch granule system. α-Amylase II activity decreased as NaCl concentration was increased (Figure 24). Maximum enzyme activity was based on starch granule hydrolysis conducted in 5 mM sodium acetate buffer. At 200 mM NaCl, the α-amylase activity decreased to about 60% of the maximum activity. This decrease in activity was related, presumably, to an inhibition in the adsorption of α-amylase to starch

Figure 23. Effect of pH on the hydrolysis of starch granules by α-amylase II in the absence and presence of inhibitor.

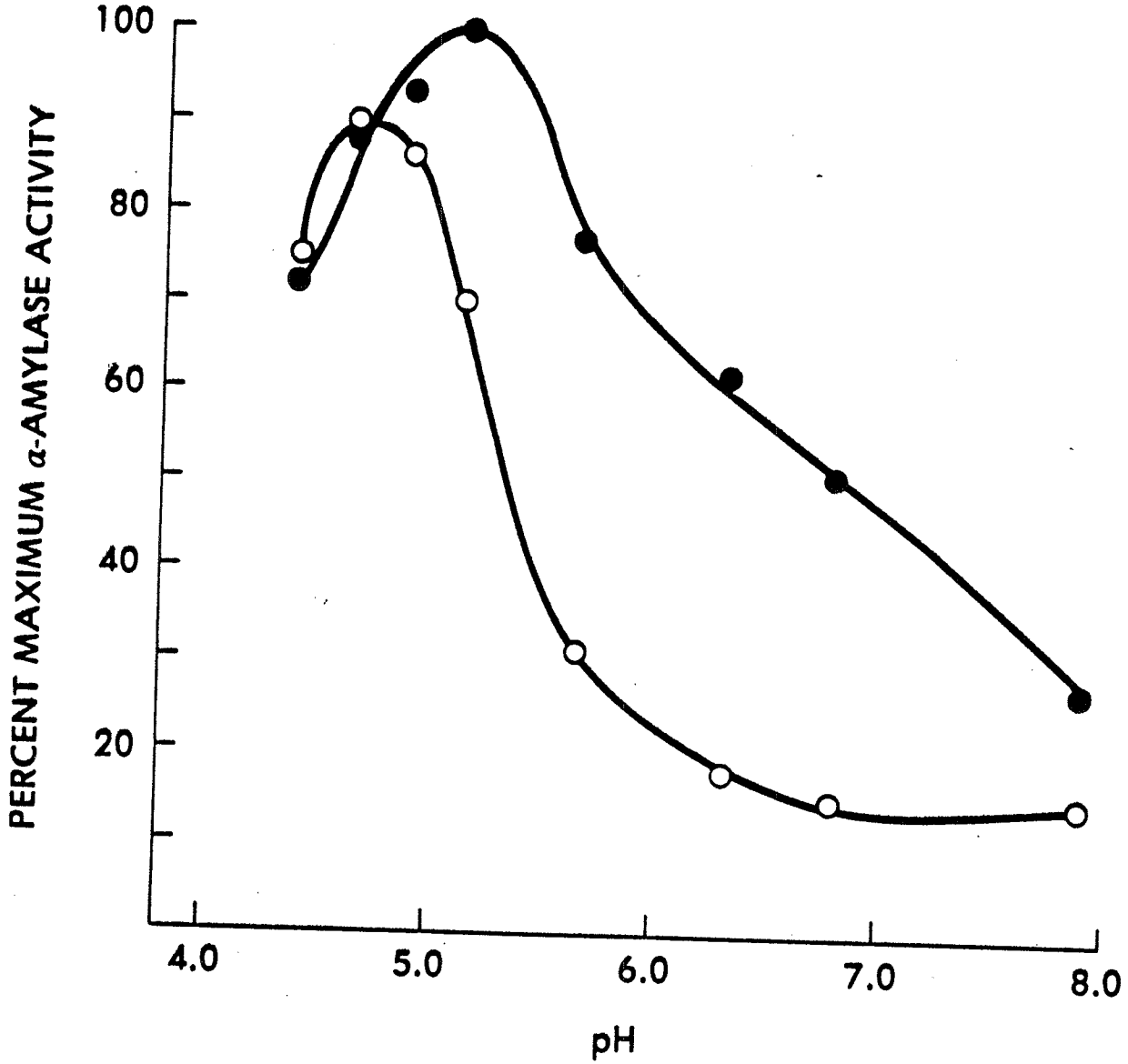
● = control activity.

○ = activity in the presence of inhibitor.

Digest conditions:

Citrate-phosphate buffer system of 0.005μ ionic strength for pH range 4.4 to 6.3 and 5 mM Tris-HCl buffer for pH values 6.8 and 7.9. All buffers contained 1 mM $CaCl_2$ and 1 mg/ml BSA. Temperature = 18°C. Enzyme concentration = 0.9 μg/ml. Inhibitor concentration = 3.8 μg/ml. Starch granule concentration = 10 mg/ml.

All enzyme activities are relative to the control activity which equals 100 at pH 5.2.


PERCENT MAXIMUM α-AMYLASE ACTIVITY
100
80
60
40
20
4.0
5.0
6.0
7.0
8.0
pH

granules, because the same concentrations of salt did not affect enzymatic hydrolysis of starch in solution. Hockenhull and Herbert (1945) found that adsorption of Clostridium acetobutylicum amylase to starch granules was inhibited by salts.

Increasing concentrations of NaCl did not have an appreciable effect on inhibition of α-amylase by the inhibitor. Enzyme activity remained about 50% of the maximum over the salt concentrations used (Figure 24). In contrast, a 50 molar excess of inhibitor was shown to have little effect on the enzymatic hydrolysis of solubilized starch in the presence of 200 mM NaCl (Figure 15).

Increasing the concentration of sodium acetate buffer, from 5 to 200 mM, had a similar effect on decreasing α-amylase II activity but little change was observed in the degree of enzyme inhibition (Figure 25). The net effects of both NaCl and sodium acetate concentration were comparable. More experiments would be required to clarify whether these effects were attributable simply to an increase in ionic strength or if they were more causally related to the concentration of the sodium ion.

During germination, the temperature of a seed may vary considerably and so the effect of temperature on inhibition of starch granule hydrolysis was examined over a range of temperatures (Figure 26). In the control hydrolysis, enzyme activity at 35°C was designated as 100%. At 15°C the relative activity of α-amylase II was 30% of the maximum value, indicating that the rate of starch granule hydrolysis increased more than 3-fold over a temperature span of 20°C. Although adsorption of the enzyme to the substrate decreases with increasing temperature (MacGregor, 1979), the increased catalytic activity of α-amylase more

Figure 24. Effect of sodium chloride concentration on hydrolysis of starch granules by α-amylase II in the absence and presence of inhibitor.

● = control activity.

○ = activity in the presence of inhibitor.

Digest conditions:

5 mM sodium acetate buffer (1 mM $CaCl_2$, 1 mg/ml BSA, pH 5.5) at 18°C. Enzyme concentration = 0.9 µg/ml. Inhibitor concentration = 3.8 µg/ml. Starch granule concentration = 10 mg/ml.

All enzyme activities are relative to the control activity which equals 100 at a buffer concentration of 5 mM sodium acetate without NaCl.

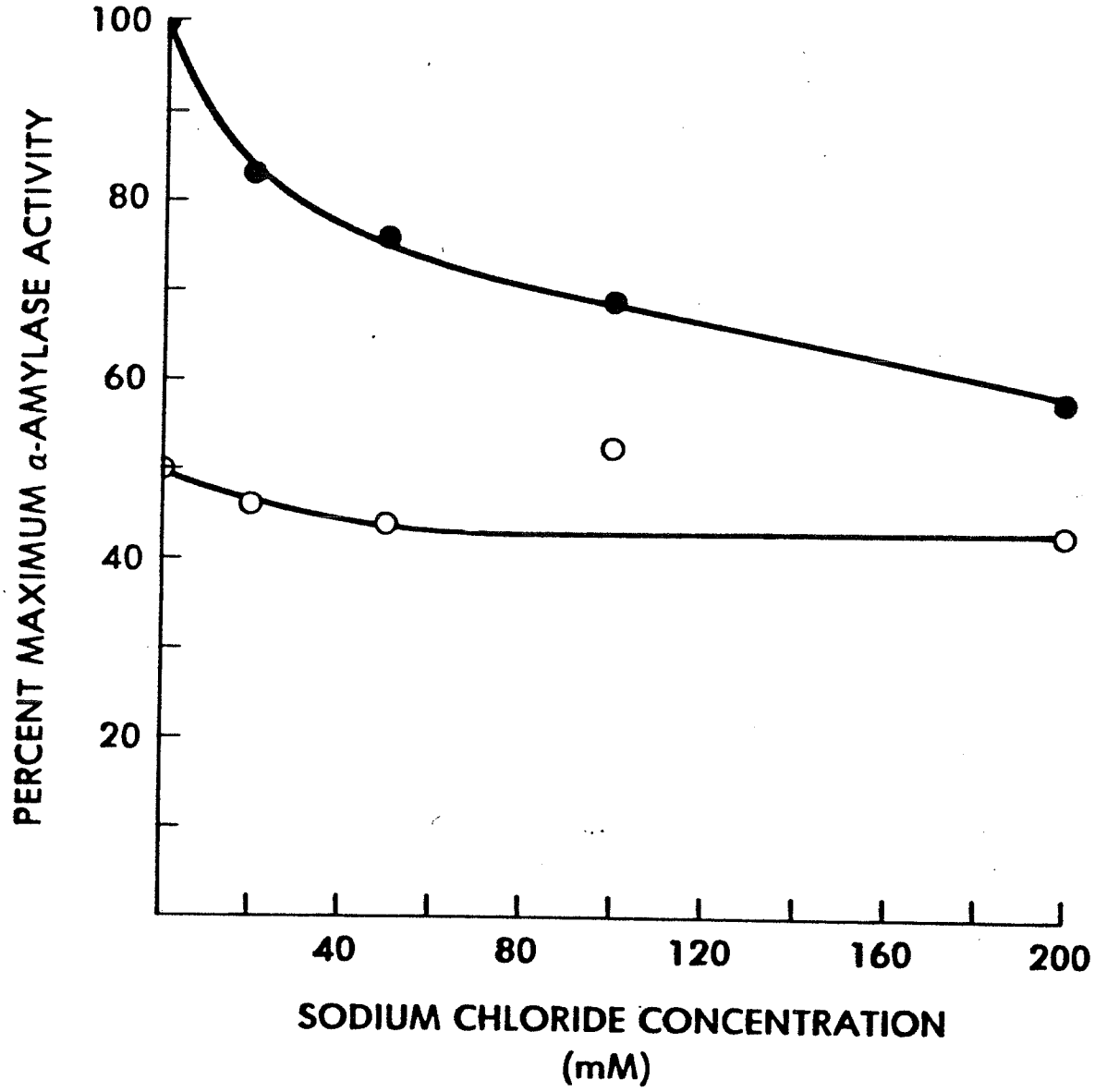

100
80
60
40
20
PERCENT MAXIMUM α-AMYLASE ACTIVITY
40
80
120
160
200
SODIUM CHLORIDE CONCENTRATION
(mM)

Figure 25. Effect of buffer concentration on hydrolysis of starch granules by α-amylase II in the absence and presence of inhibitor.

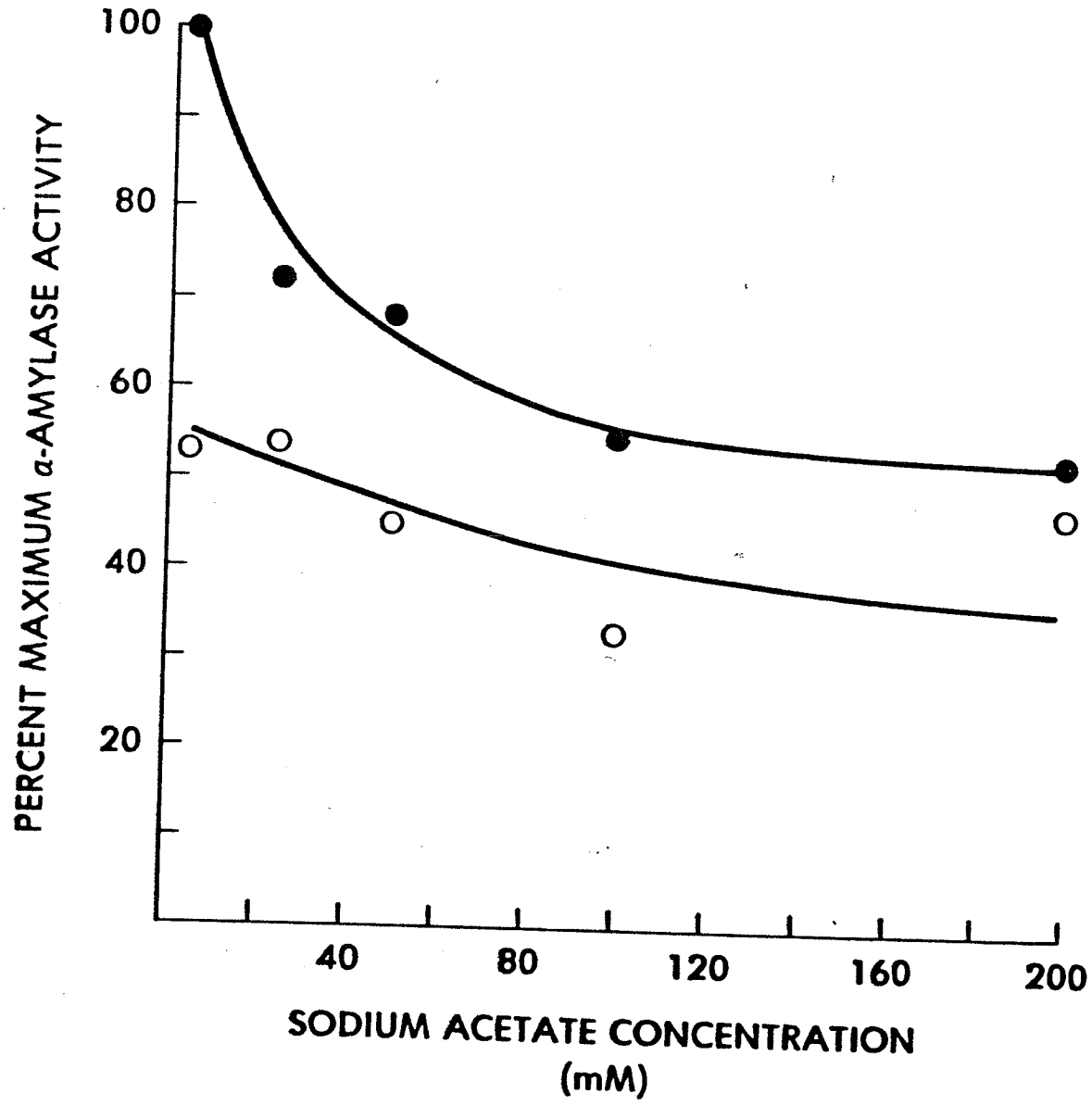
● = control activity.

○ = activity in the presence of inhibitor.

Digest conditions:

Sodium acetate buffer (1 mM $CaCl_2$, 1 mg/ml BSA, pH 5.5) at 18°C. Enzyme concentration = 0.9 µg/ml. Inhibitor concentration = 3.8 µg/ml. Starch granule concentration = 10 mg/ml.

All enzyme activities are relative to the control activity which equals 100 at a buffer concentration of 5 mM sodium acetate.


100
80
60
40
20
PERCENT MAXIMUM α-AMYLASE ACTIVITY
40
80
120
160
200
SODIUM ACETATE CONCENTRATION
(mM)

than made up for the decrease in enzyme adsorption (McLaren, 1963). In the presence of a 10-fold molar excess of inhibitor, the activity of α-amylase II was lowered at 35°C and 15°C to 64% and 20% respectively, of maximum control activity (Figure 26). The differences between control and inhibited enzyme activities at each temperature indicated about 35% inhibition of α-amylase II activity in each case. Therefore, inhibition was independent of temperature from 15°C to 35°C. Similar results were obtained in the starch solution system (not shown).

Increasing the substrate concentration usually increases the velocity of an enzyme reaction until the enzyme becomes saturated with substrate and the reaction rate then becomes constant. In attempting to ascribe a physiological role to the inhibitor it was important to consider the effect of starch granule concentration on the α-amylase II-inhibitor interaction since this substrate is present at an extremely high concentration in the endosperm of germinating barley. Therefore, inhibition of α-amylase II, as a function of starch granule concentration, was examined (Figure 27). All enzyme activities were relative to the activity of the control reaction with a substrate concentration of 80 mg/ml. The rate of production of soluble products increased rapidly up to a starch concentration of 60 mg/ml and then appeared to level out. In contrast, the reaction rate of α-amylase II became maximal at about 3 to 4 mg/ml of substrate concentration when using starch in solution to assay enzyme activity at pH 5.5 (not shown). The difference in substrate concentrations required to saturate the enzyme is attributable, obviously, to the difference in the nature of the two substrates.

The presence of a 10-fold molar excess of inhibitor decreased the

Figure 26. Effect of temperature on hydrolysis of starch granules by α-amylase II in the absence and presence of inhibitor.

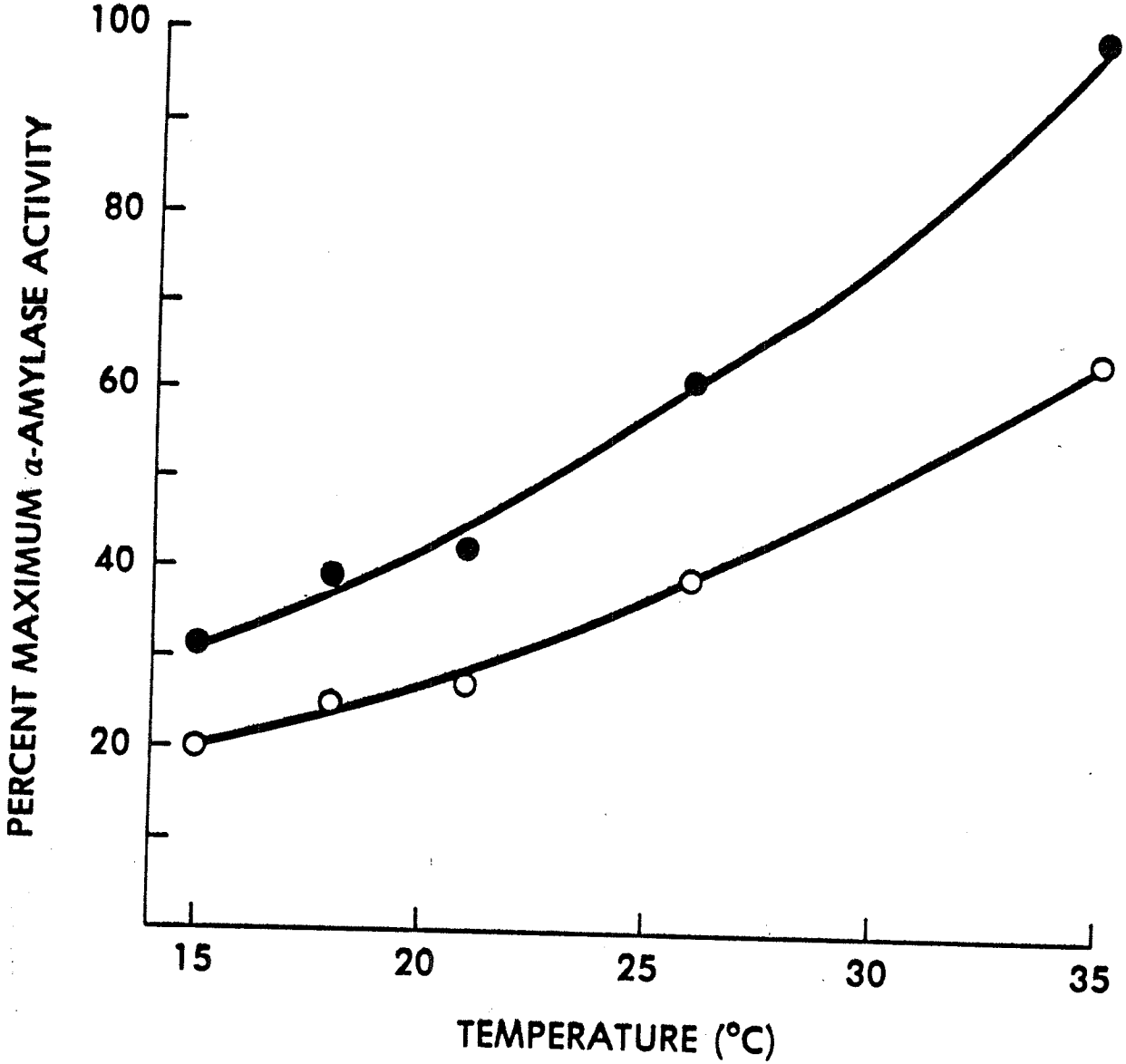
● = control activity.

O = activity in the presence of inhibitor.

Digest conditions:

50 mM sodium acetate buffer (1 mM $CaCl_2$, 1 mg/ml BSA, pH 5.5). Enzyme concentration = 0.9 µg/ml. Inhibitor concentration = 3.8 µg/ml. Starch granule concentration = 10 mg/ml.

All enzyme activities are relative to the control activity which equals 100 at a temperature of 35°C.


100
80
60
40
20
PERCENT MAXIMUM α-AMYLASE ACTIVITY
15
20
25
30
35
TEMPERATURE (°C)

rate of hydrolysis of starch granules by α-amylase II at each starch concentration studied (Figure 27). At a starch concentration of 60 mg/ml, the inhibited α-amylase had 70% of the maximum enzyme activity. The differences between control and inhibited enzyme activities, using the actual experimental points, indicated inhibition ranging from 23% to 37%. Inhibition was relatively constant at all substrate concentrations. Therefore, the inhibitor could be effective in regulating α-amylase II activity in the endosperm where starch concentrations are high.

Other studies were conducted to determine if the presence of inhibitor affected the appearance of enzymatically-degraded starch granules and the types of soluble products formed during digestion of granules. Large starch granules from barley are degraded slowly by barley α-amylases (MacGregor and Ballance, 1980b). It was necessary, therefore, to allow hydrolysis of granules by α-amylase II to proceed for relatively long time periods to obtain significant granule degradation. Results from such a hydrolysis are shown in Figure 28. Initially, the release of soluble starch was linear with reaction time, but after about 5 hours of hydrolysis release of product was curvilinear with time. After 50 hours of reaction time, 50% of the starch granules were digested. The eventual curvature of the hydrolysis process with time might be attributed to factors such as increasing resistance of starch granules to hydrolysis, inhibition of enzyme activity by soluble products or enzyme deactivation. For example, dextrins have been shown to inhibit binding of cereal α-amylase to starch granules (Schwimmer and Balls, 1949b; Weselake and Hill, 1983). In the presence of a 10-fold

Figure 27. Effect of starch granule concentration on the hydrolysis rate of starch granules by α-amylase II in the absence and presence of inhibitor.

● = control activity.

○ = activity in the presence of inhibitor.

Digest conditions:

50 mM sodium acetate buffer (1mM $CaCl_2$, 1 mg/ml BSA, pH 5.5) at 18°C. Enzyme concentration = 0.9 µg/ml. Inhibitor concentration = 3.8 µg/ml.

All enzyme activities are relative to the control activity which equals 100 at a substrate concentration of 80 mg/ml.

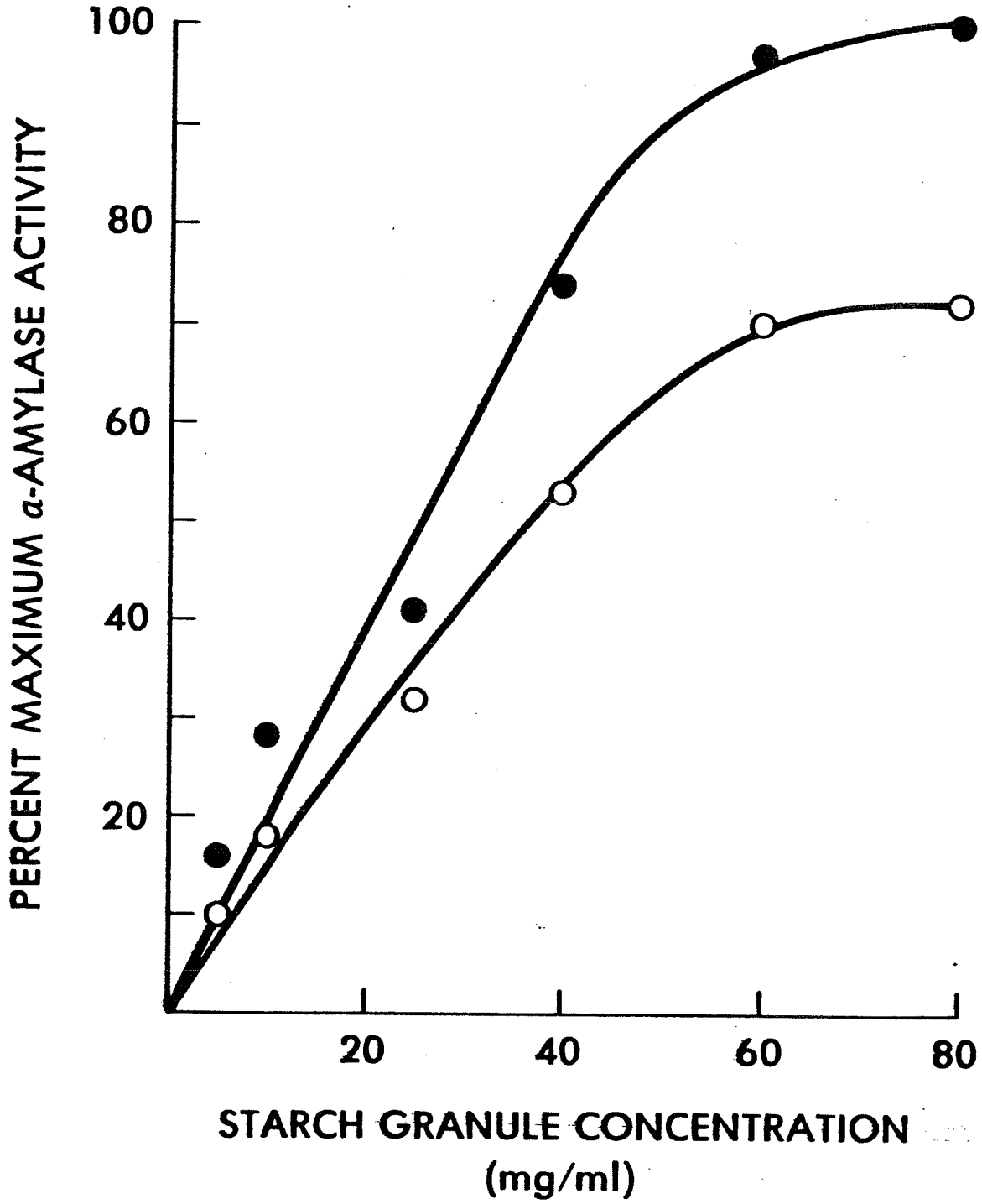

100
80
60
40
20
PERCENT MAXIMUM α-AMYLASE ACTIVITY
20
40
60
80
STARCH GRANULE CONCENTRATION
(mg/ml)

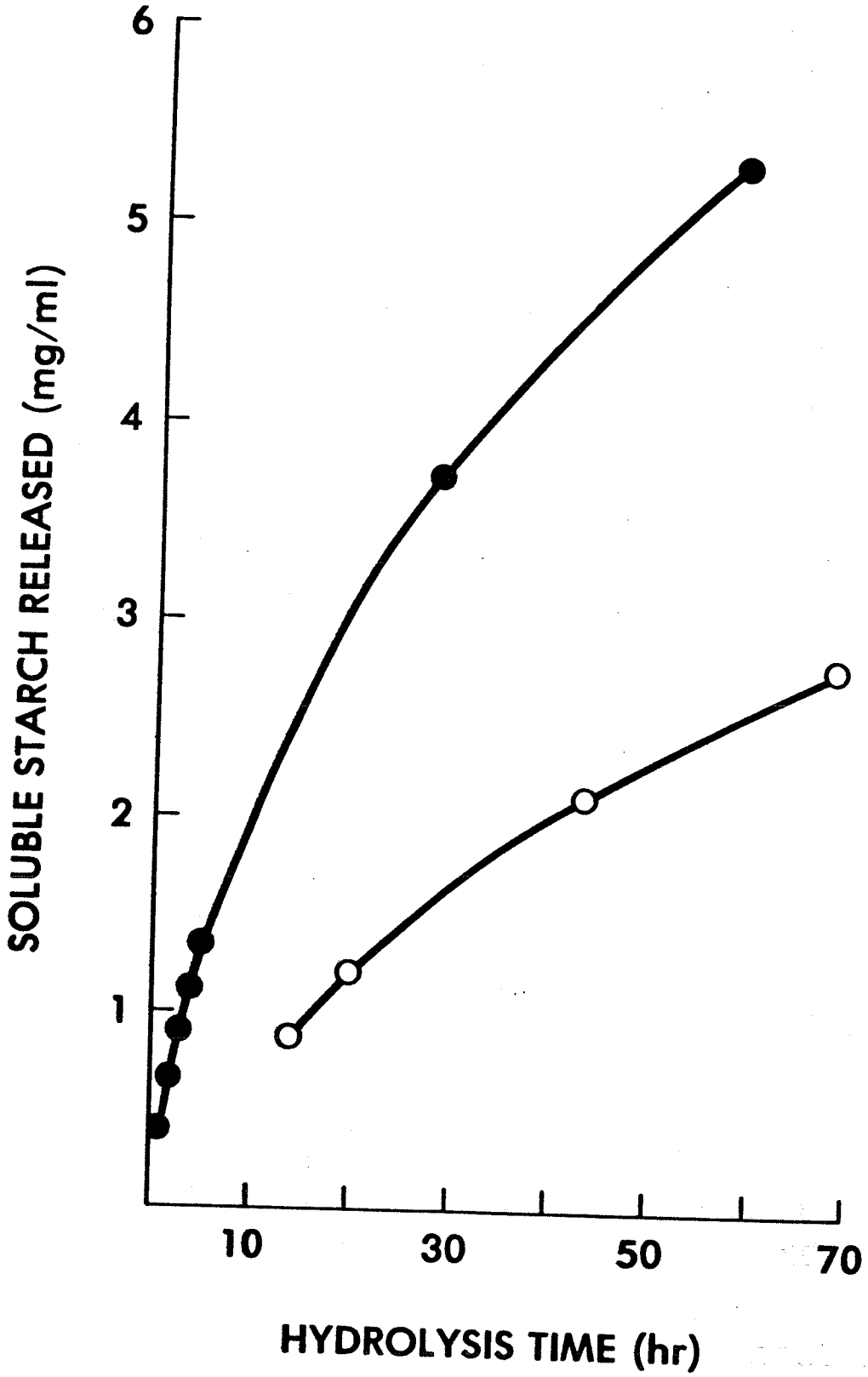
Figure 28. Extended hydrolysis of starch granules by α-amylase II at pH 5.5 and 35°C in the absence and presence of inhibitor.

● = control hydrolysis.

○ = hydrolysis in the presence of inhibitor.

Digest conditions:

5 mM sodium acetate buffer (1 mM $CaCl_2$, 1 mg/ml BSA, pH 5.5). Enzyme concentration = 1.0 µg/ml. Inhibitor concentration = 4.0 µg/ml. Starch granule concentration = 10 mg/ml.


6
5
4
3
2
1
SOLUBLE STARCH RELEASED (mg/ml)
10
30
50
70
HYDROLYSIS TIME (hr)

molar excess of inhibitor, the hydrolysis of starch granules was curvilinear with reaction time, also, and after 50 hours of reaction time 23% of the granules were digested. Therefore, degradation of large starch granules by α-amylase II was suppressed about 2-fold, by the inhibitor, after 50 hours of hydrolysis.

Samples degraded for 58.5 hours, in the absence of inhibitor, and 68.5 hours, in the presence of inhibitor were examined by scanning electron microscopy. Starch granules, hydrolyzed without inhibitor present, clearly showed visual signs of degradation (Figure 29A). Some granules were pitted while others were more extensively digested. Similar observations for digestion of large normal starch granules of cereals have been well documented in the past (Dronzek et al., 1972; Evers and McDermott, 1970; Kiribuchi and Nakamura, 1973; MacGregor and Ballance, 1980b; Palmer, 1972). Granules degraded by the inhibited enzyme were much more intact, indicating that the inhibitor was very effective in delaying degradation of starch granules by α-amylase II (Figure 29B). This result was predictable since only 28% of the granules were solubilized during hydrolysis in the presence of inhibitor (Figure 28). These results suggest that the inhibitor only delayed the process of enzymatic digestion and did not alter the way in which the granules were digested.

Hydrolysis experiments were conducted at 18°C, as well, because this temperature was used predominantly throughout the starch granule studies. Digestion of granules, with an enzyme concentration similar to that used at 35°C, resulted in 11% release of soluble starch after 15 hours of reaction time. In the presence of a 50-fold molar excess of inhibitor only 7% of the granules were solubilized after 64 hours of

Figure 29. Scanning electron micrographs of large normal starch granules digested by α-amylase II in the absence (A) and presence of inhibitor (B).

Digest conditions:

5 mM sodium acetate buffer (1mM $CaCl_2$, 1 mg/ml BSA, pH 5.5) at 35°C. Enzyme concentration = 1.0 µg/ml. Inhibitor concentration = 4.0 µg/ml. Starch granule concentration = 10 mg/ml. Starch granules digested without inhibitor (A) for 53.5 hours. Starch granules digested with inhibitor (B) for 68.5 hours.

NOTICE/AVIS

PAGE(S) 124 IS/ARE EST/SONT black + white photo

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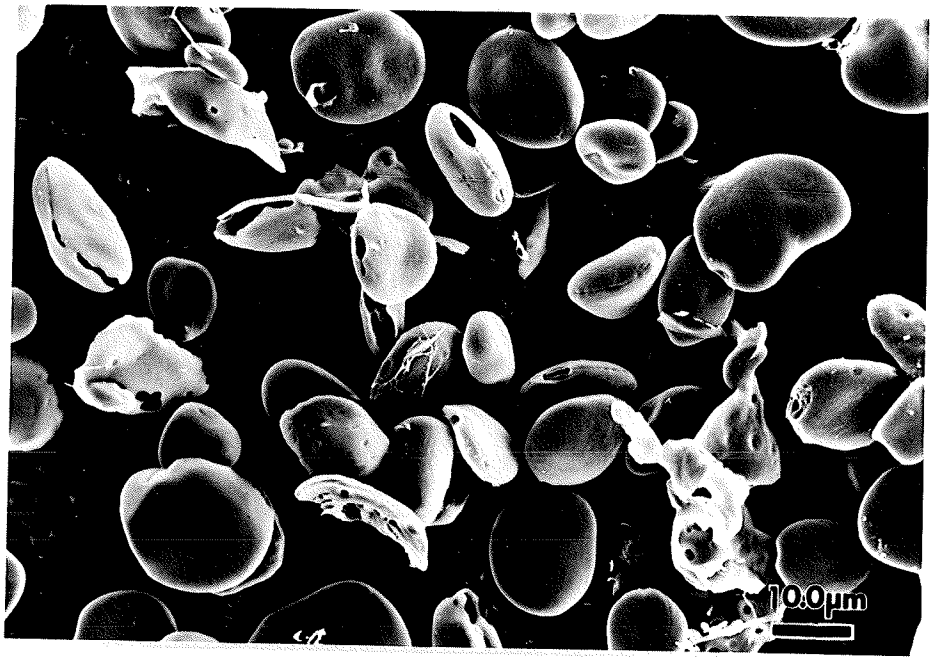
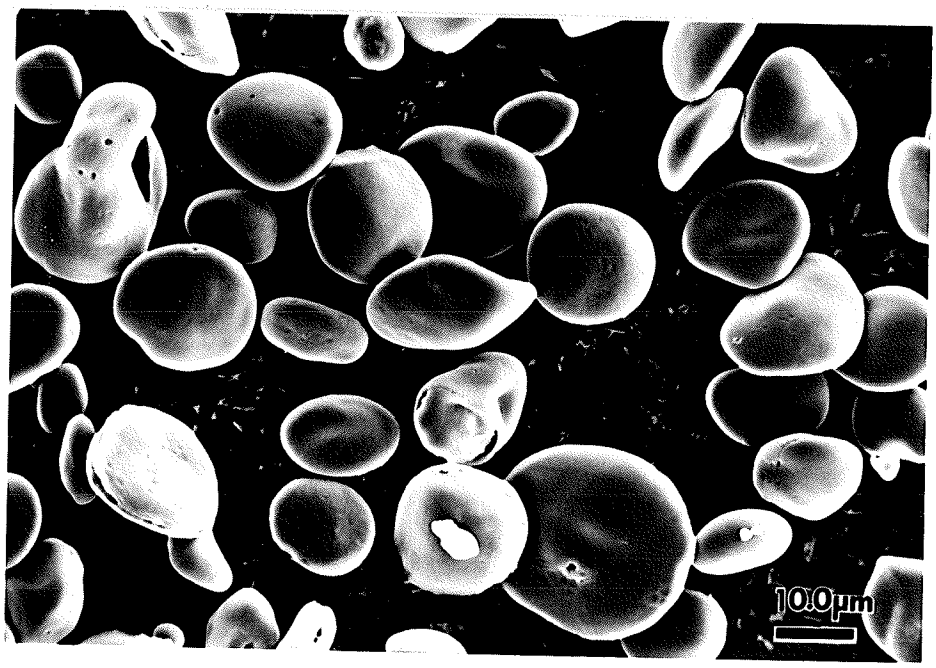
VEUILLEZ ECRIRE A L'AUTEUR POUR LES RENSEIGNEMENTS OU VEUILLEZ CONSULTER L'EXEMPLAIRE DONT POSSEDE LE DEPARTEMENT DES ARCHIVES ET DES COLLECTIONS SPECIALES, BIBLIOTHEQUE ELIZABETH DAFOE, UNIVERSITE DU MANITOBA, WINNIPEG, MANITOBA, CANADA, R3T 2N2.

A)



B)



hydrolysis. No conclusions could be drawn, however, from scanning electron micrographs of these granules since there were no visible signs of degradation (not shown).

Products formed from starch granules after 5, 28.5 and 53.5 hours of hydrolysis by α-amylase II at 35°C are shown in Figure 30. After 5 hours of reaction time, dextrins ranging from G 1 to G 7 were apparent in the profile but G 6 and G 2 were the main products of hydrolysis (Figure 30A). There was, however, a relatively high proportion of carbohydrate at the origin for all samples examined. This component appeared to be highly resistant to hydrolysis by α-amylase II. After 28.5 hours, G 7 had almost disappeared, whereas G 4 and G 2 became the main products of hydrolysis (Figure 30B). After 53.5 hours, G 2 was the major product of hydrolysis (Figure 30C). The extensive reduction in size of smaller dextrins so that G 2 ultimately predominated suggests that the linear soluble products were subjected to a high degree of non-random enzymatic hydrolysis.

Densitometer tracings of the products from granules digested by inhibited enzyme are shown in Figure 31. After 20 hours of reaction time, G 6 and G 2 were the main products of hydrolysis along with a high proportion of carbohydrate at the origin (Figure 31A). After 43.5 hours, G 6 and G 2 were still the main products of hydrolysis, but G 7 was greatly diminished (Figure 31B). There was little change in the dextrin profile after 68.5 hours of hydrolysis (Figure 31C). The quantity of soluble starch released from the granules after 20 hours of inhibited hydrolysis was similar to the soluble starch released after 5 hours in the control system (Figure 28). Dextrin profiles for these

Figure 30. Dextrin profiles of products after hydrolysis of starch granules by α-amylase II.

Densitometer tracings of TLC separated hydrolysis products. Peaks 1 to 7 are considered to be glucose to maltoheptaose.

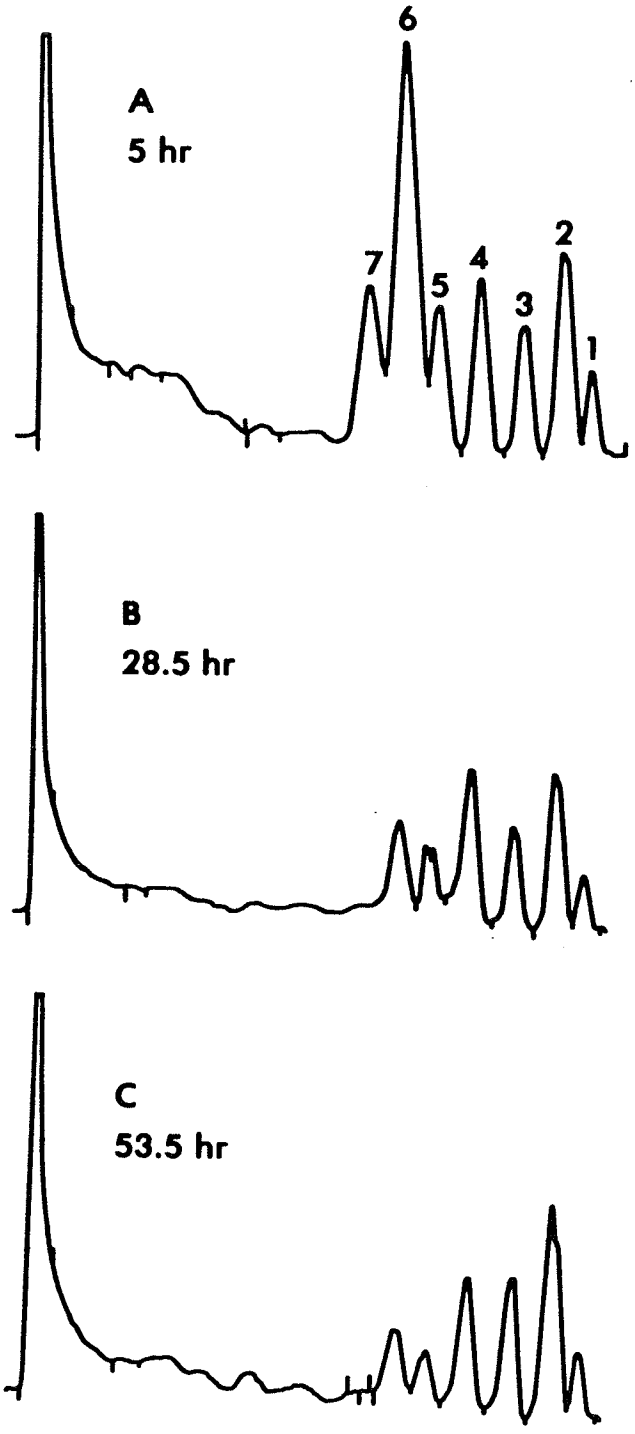
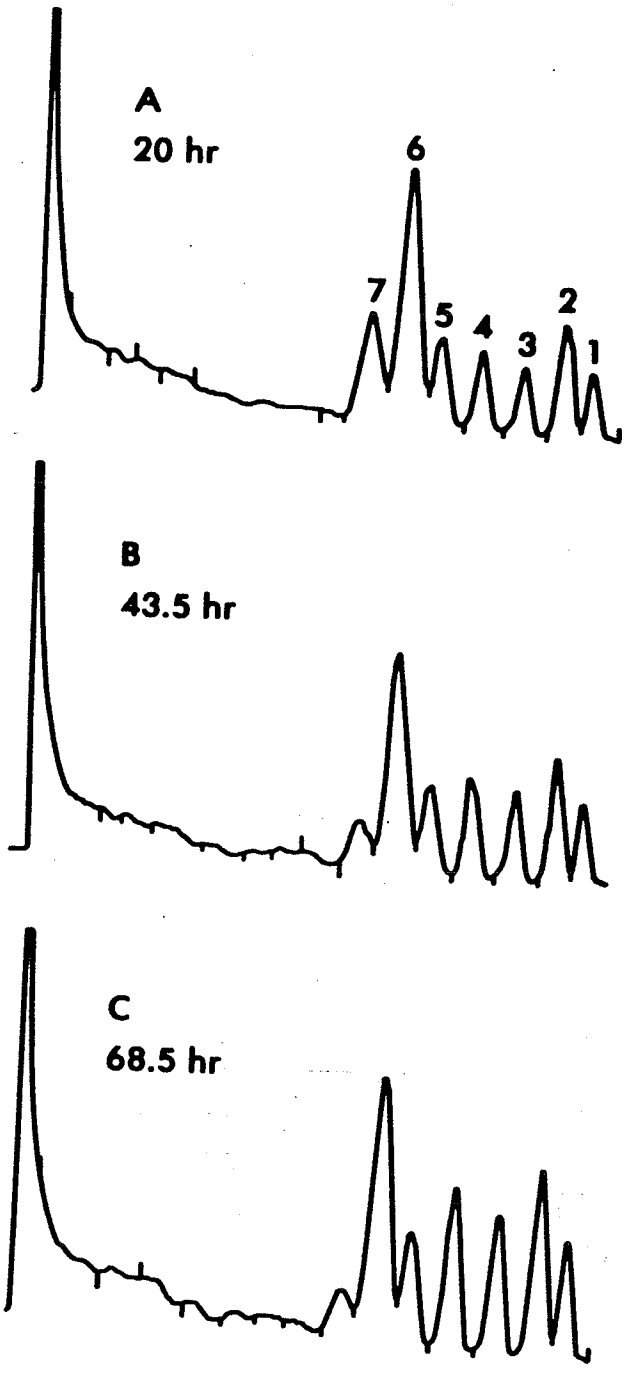

A
5 hr
6
7
5
4
3
2
1
B
28.5 hr
C
53.5 hr

Figure 31. Dextrin profiles of products after hydrolysis of starch granules by α-amylase II in the presence of inhibitor.

Densitometer tracings of TLC separated hydrolysis products. Peaks 1 to 7 are considered to be glucose to maltoheptaose.


A
20 hr
6
7
5
4
3
2
1
B
43.5 hr
C
68.5 hr

two samples were similar, also (Figure 30A, 31B). Based on this comparison, the inhibitor did not appear to alter the overall enzymatic reaction pattern. The result was in agreement with observations on the depolymerization of linear amylose by α-amylase II in the absence and presence of inhibitor. Once again, the main effect of the inhibitor was in delaying substrate breakdown rather than changing the mode of depolymerization.

Similar results were obtained after TLC analysis of products of starch granule hydrolysis at 18°C (not shown). These hydrolyzates were treated with β-amylase and subsequent TLC analysis revealed glucose and maltose along with some carbohydrate at the origin. The results indicated that the small products of enzymatic digestion, ranging from G 1 to G 7, were linear and that the carbohydrate remaining at the origin was probably branched.

There is a major difficulty inherent in the analysis of products from enzymatic starch granule digestion. It is technically difficult to isolate initial products of hydrolysis before these soluble products are hydrolyzed further by the enzyme. Therefore, in the studies conducted, the reaction products represented the net hydrolytic effect of both starch granule degradation and further hydrolysis of soluble products.

Very little published information is available on the effect of inhibitor proteins on α-amylase catalyzed hydrolysis of starch granules. The results reported in this section suggest that the α-amylase II inhibitor may not have an effect on the action pattern of the enzyme. Perhaps inhibition of starch granule degradation could be investigated further by determining the chemical structure of both soluble products and remaining degraded starch granules.

Distribution of Inhibitor Activity in the Barley Kernel and Possible Physiological Relevance

In attempting to determine a possible physiological role for the inhibitor, it was important to gain some insight on the distribution of inhibitor in the barley kernel. Pearling fractions of barley kernels were collected, sequentially, after different degrees of abrasion and after 120 seconds of pearling, the remaining core fraction constituted approximately 40% of the starting material. Equal portions of each pearling fraction were extracted with buffer and the extracts were analyzed for inhibitor activity and protein content. Inhibitor activity of the extracts remained relatively constant at 2000-2500 anti IDC units/ml of extract from the 20-40 second pearling fraction right through to the remaining core fraction (Figure 32). Inhibitor activity of the first pearling fraction (0-20 seconds) was considerably lower (1400 anti IDC units/ml) than the activity in other fractions. This reduction in activity was attributable, probably, to the presence of a high proportion of abraded husk in the first pearling fraction. Removal of the husk by sulphuric acid treatment did not affect the total inhibitor activity per barley kernel, indicating that husks did not contain inhibitor activity.

Protein content of extracts of pearling fractions decreased as the core of the seed was approached (Figure 32). This would be expected because the highest concentration of protein in cereal kernels is localized in the outer regions of the kernel (Morris *et al.*, 1946; Novacek *et al.*, 1966). Extracts of the 20-40 second pearling fraction and the remaining core fraction contained 6 and 2 mg/ml of protein,

respectively. Based on this extractable protein, the specific activity of the inhibitor was 340 and 1250 anti IDC units/mg of protein in these two fractions, respectively, indicating that the inhibitor was not localized where soluble protein concentration was highest in the kernel. A more precise localization of inhibitor in the endosperm would require analysis by more elaborate techniques such as immunohistochemistry.

During germination, barley α-amylase is produced by the aleurone layer and the embryo, and the enzyme diffuses from these tissues into the starchy endosperm (Briggs, 1964; Gibbons, 1979; MacLeod and Millar, 1962; MacLeod and Palmer, 1966; Varner, 1964). During diffusion, α-amylase would then encounter inhibitor in the outer regions of the endosperm. It is not known if the total inhibitor content per kernel changes during germination, but the presence of a high proportion of α-amylase III relative to α-amylase II in green malt (MacGregor and Ballance, 1980a) indicates that the inhibitor remains active during germination. Therefore, the inhibitor does not appear to be degraded by proteolytic enzymes during germination, indicating that it probably does not serve as a reserve protein.

In the previous section, it was demonstrated that the α-amylase II-inhibitor interaction was sensitive to both pH and salt concentration. Therefore, an attempt was made to determine if pH and salt concentration varied throughout the seed. Pearling fractions were extracted with water and assayed for pH and conductivity. The pH was determined on slurries of pearling fractions and conductivity was determined on 10-fold dilutions of extracts. Both pH and conductivity

Figure 32. Inhibitor activity and protein content of extracts of barley pearling fractions.

Inhibition assays were performed at pH 8.0 (40 mM Tris-HCl buffer, 1 mM $CaCl_2$, 50 µg/ml BSA) and 35°C.

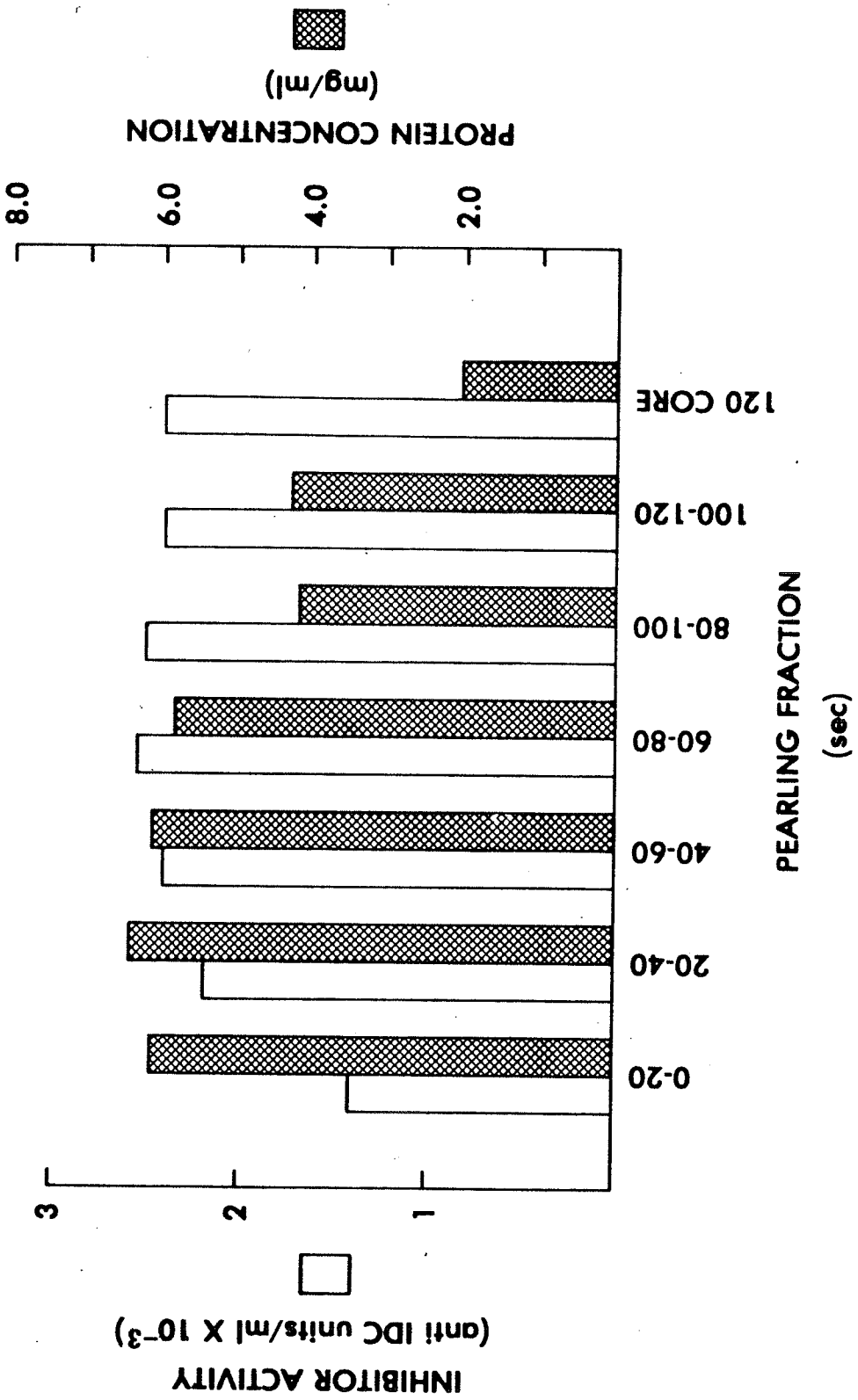
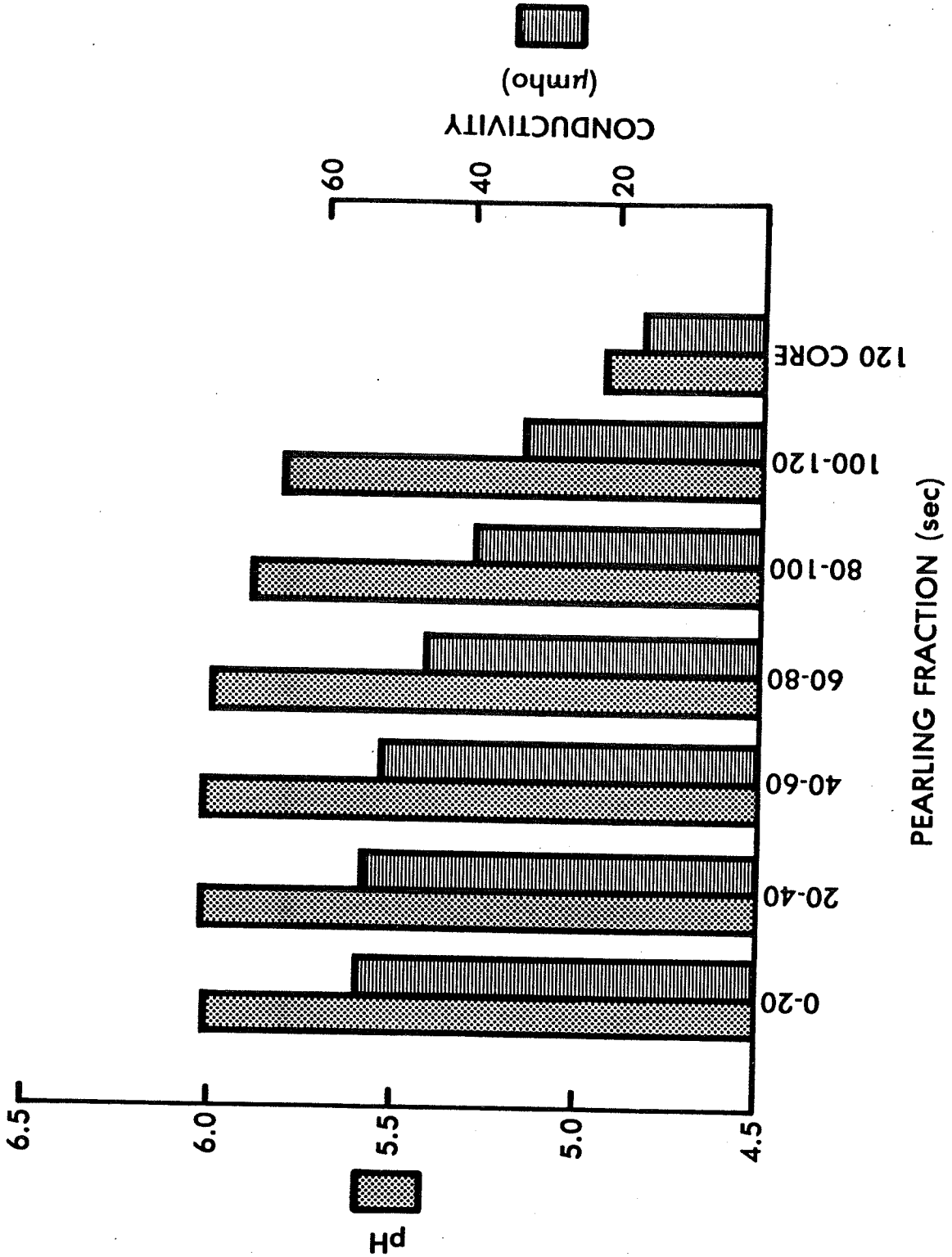

PROTEIN CONCENTRATION
(mg/ml)
8.0
6.0
4.0
2.0
120 CORE
100-120
80-100
60-80
40-60
20-40
0-20
PEARLING FRACTION
(sec)
3
2
1
INHIBITOR ACTIVITY
(anti IDC units/ml X 10⁻³)

Figure 33. pH and conductivity of extracts of barley pearling fractions.


pH
6.5
6.0
5.5
5.0
4.5
CONDUCTIVITY
(μmho)
60
40
20
PEARLING FRACTION (sec)
0-20
20-40
40-60
60-80
80-100
100-120
120 CORE

decreased towards the core of the seed (Figure 33). Slurries of pearling fractions, ranging from the 0-20 seconds fraction to the 60-80 seconds fraction, had a pH value close to 6. Thereafter the pH decreased, with the remaining core fraction having a pH of about 5. The conductivity decreased from 53 µmho for the first pearling fraction (0-20 seconds) to 16 µmho for the remaining core. Presumably, the high mineral content in outer regions of the barley endosperm (Pomeranz, 1973; Liu et al., 1974) accounts for higher conductivity of extracts prepared from outer pearling fractions and these minerals could contribute to the soluble ion pool in the endosperm. The effects of these endogenous ions on α-amylase II activity and the α-amylase II-inhibitor interaction would have to be investigated before making predictions on ionic strength effects in the endosperm. Physiologically, the enzyme-inhibitor interaction could be influenced by conditions such as specific ion effects, endosperm pH, insoluble components of the endosperm, limited water availability or localization of inhibitor within specific regions of the endosperm.

Inhibitor Activity in Various Cereal Grains

During characterization of the properties of the α-amylase II inhibitor, the source of inhibitor was always Klages barley. Previous isoelectric focusing studies indicated, however, that all green malts examined contained α-amylase III, and so must have contained α-amylase II inhibitor (MacGregor, 1980; MacGregor and Ballance, 1980a; MacGregor and Daussant, 1981; Marchylo and MacGregor, 1983). Therefore, it seemed reasonable to assume that the inhibitor would be present in all

types of barley and possibly in other cereals. This hypothesis was tested in two ways, using extracts of different types of barley and of other common cereals. In one series of experiments, antibodies, raised against purified inhibitor, were tested for their ability to form an insoluble antigen-antibody complex when allowed to diffuse against cereal extracts in an agarose matrix. A second approach involved determination of inhibitor activity in cereal extracts. Assays were carried out with appropriately diluted extracts at pH 8, under conditions where the affinity of the inhibitor for α-amylase II was high.

Specific cultivars of barley, wheat, durum wheat and rye were used but complete identifying information was not available for the other cereals examined. Extracts, prepared using 5 parts of 0.9% NaCl to one part of ground seed, were concentrated (about 7-fold) to intensify precipitin reactions during immunodiffusion experiments. These concentrated extracts were tested using the Ouchterlony (1967) double-diffusion system. The results are shown in Figure 34. Purified antibody, applied to a central well in the agarose matrix, was allowed to diffuse against the extract in question, which was applied to a peripheral well. Another well, adjacent to the test extract, received inhibitor purified from Klages barley. The precipitin line produced by the antibody-inhibitor interaction served as a criterion of identity when characterizing the precipitin reactions that occurred when related antigens in the test extracts reacted with the same antibodies. Following diffusion, washing and drying of immunodiffusion plates, the precipitin lines were visualized by silver staining for protein. Pre-immune serum (Figure 34, well A) failed to show a precipitin reaction with purified inhibitor (C), thereby serving as a control to eliminate

the possibility of artifacts. Antibody was used in the form of an isolated IgG fraction. The absence of other serum proteins reduced the washing time required to remove non-reacted proteins from the system. This was important, especially when using the highly sensitive silver stain. In all cases the antibody fraction (B) and the purified inhibitor (C) formed a precipitin line in the matrix between the two wells. Extracts of Klages (1), Bonanza (2) and Himalaya (3) barleys formed precipitin lines with purified antibody (B). These lines fused with the lines formed from the precipitin reaction of purified inhibitor and antibody fraction. This observation indicated that all of these barley cultivars contained an antigen that shared complete immunochemical identity with the purified inhibitor, thus providing evidence that the same inhibitor was present in each cultivar of barley examined. Extracts of Neepawa (4), Columbus (5) and Northstar (6) hexaploid wheats reacted with the antibody, as well. Precipitin lines generated by the wheat extracts fused with the precipitin line formed by the purified inhibitor (C), but a part of the precipitin line, due to the purified inhibitor, extended further as a spur. Therefore, the wheat cultivars contained a component which shared only partial immunochemical identity with the purified inhibitor. These results suggest that there is an inhibitor protein in wheat which shares a high degree of amino acid sequence homology with the α-amylase II inhibitor from barley, but the sequences of the two proteins are not identical. Partial immunochemical identity was found, also, for extracts of durum wheat (7), rye (8) and triticale (9), indicating that these cereals probably contain inhibitor also. The presence of a precipitin line for durum wheat extract suggests that the inhibitor is not restricted to hexaploid wheats.

Extracts of sorghum (10), oats (11), millet (12), rice (13) and maize (14) failed to show immunochemical precipitin lines, indicating, strongly, that these cereals did not contain a similar inhibitor or even a closely related protein.

Results of the immunochemical analysis are summarized in Table 5, along with determinations of extractable inhibitor activity. The coefficient of variation of inhibitor activity was less than 5% for 6 extractions of Klages barley, indicating that the overall procedure of extraction and subsequent determination of inhibitor activity was highly reproducible. Inhibitor activity was found only in extracts that showed an immunochemical reaction with the antibody and, therefore, the two analyses were correlated in this respect. There was considerable variation in extractable inhibitor activity among barley cultivars examined but the values were higher than those obtained for the other grains. Bonanza barley had the highest inhibitor activity per gm of ground seed and this activity was more than twice the activity of Himalaya barley. Inhibitor activity for wheat cultivars, rye and triticale ranged from 3330 to 4330 anti IDC units per gm of ground seed, indicating that the level of inhibitor in these seeds was comparable. The wheat cultivar, Columbus, is known to be highly resistant to sprouting (Campbell and Czarnecki, 1981; Noll, 1983), but inhibitor levels in this cultivar were not abnormally high when compared to Neepawa and Northstar wheats. Therefore, it is unlikely that sprouting resistance of Columbus wheat can be attributed to the type of endogenous inhibitor characterized in this thesis. It is important to mention, however, that inhibitors from the wheat cultivars, rye and triticale, may be more or less effective against their own endogenous

Figure 34. Ouchterlony double-diffusion analysis of extracts of cereal kernels.

A. Pre-immune serum.

B. Anti-inhibitor IgG.

C. Purified inhibitor.

1. Hordeum distichum cv Klages (barley).

2. Hordeum vulgare cv Bonanza (barley).

3. Hordeum vulgare cv Himalaya (barley).

4. Triticum aestivum cv Neepawa (wheat).

5. Triticum aestivum cv Columbus (wheat).

6. Triticum aestivum cv Northstar (wheat).

7. Triticum turgidum cv Wakooma (durum wheat).

8. Secale cereale cv Puma (rye).

9. Triticosecale wittmack (secondary hexaploid triticale)

10. Sorghum bicolor (white sorghum).

11. Avena sativa (oats).

12. Pennisetum americanum (millet).

13. Oryza spp. (rice).

14. Zea mays (maize).

NOTICE/AVIS

PAGE(S) 142 IS/ARE/EST/SONT black + white photo

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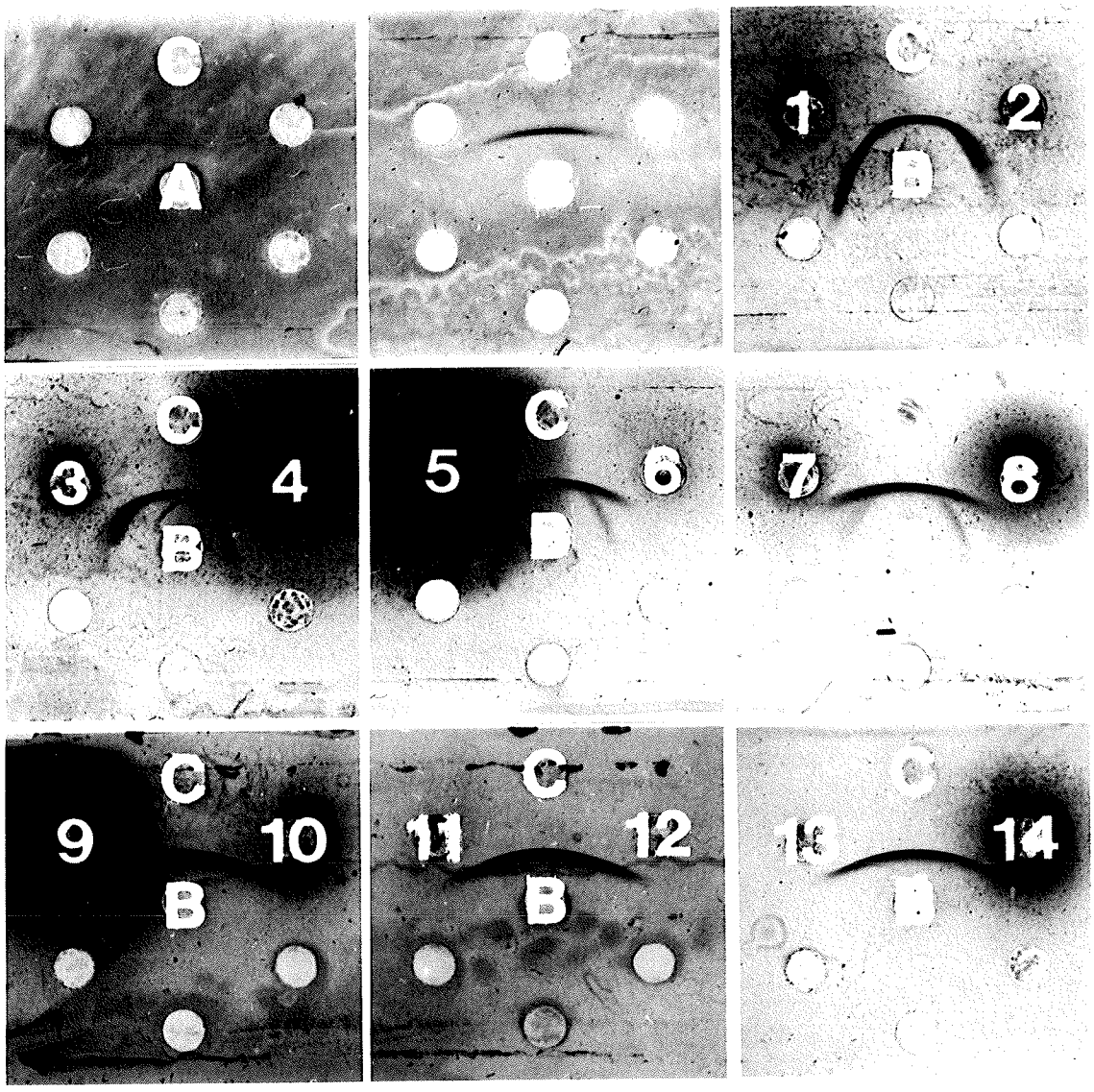
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TABLE 5. Immunochemical reactivity and inhibitor activity of cereal kernel extracts.

Cereal	Immunochemical Identity	Inhibitor Activity (anti IDC units/ gm of seed)
Hordeum distichum cv Klages (barley)	complete	8,325*
Hordeum vulgare cv Bonanza (barley)	complete	13,325
Hordeum vulgare cv Himalaya (barley)	complete	5,330
Triticum aestivum cv Neepawa (wheat)	partial	3,795
Triticum aestivum cv Columbus (wheat)	partial	3,330
Triticum aestivum cv Northstar (wheat)	partial	4,330
Triticum turgidum cv Wakooma (durum wheat)	partial	3,665
Secale cereale cv Puma (rye)	partial	3,330
Triticosecale wittmack (secondary hexaploid triticale)	partial	4,000
Sorghum bicolor (white sorghum)	n.p.	n.d.
Avena sativa (oats)	n.p.	n.d.
Pennisetum americanum (millet)	n.p.	n.d.
Oryza spp. (rice)	n.p.	n.d.
Zea mays (maize)	n.p.	n.d.

* Coefficient of variation = 4.5 percent.

n.p. no precipitin

n.d. not detectable

α-amylases. Therefore, the study could be somewhat misleading because barley α-amylase II provides the reference enzyme activity upon which all inhibitor activity determinations were based. Results of both the immunochemical study and inhibitor activity survey do suggest, however, that endogenous α-amylase inhibitor proteins are not restricted to barley.

Germinated wheat contains two major groups of α-amylases having isoelectric points similar to the two α-amylase groups of a green barley malt (MacGregor, 1983; Sargeant and Walker, 1978). A comparative study was conducted to determine if the barley α-amylase II inhibitor was effective in inhibiting these α-amylases from germinated wheat. A fixed quantity of inhibitor was tested against similar enzyme activities of α-amylases I and II from both malted barley and germinated wheat (Table 6). Alpha-amylase I from both cereals was not affected, in agreement with results from starch granule experiments in which a 50 molar excess of inhibitor had no effect on granule hydrolysis by α-amylase I from barley malt (Figure 22). In contrast, α-amylase II from both malted barley and germinated wheat was inhibited strongly by the barley α-amylase II inhibitor (Table 6). The α-amylase II groups from both malted barley and germinated wheat appear to have structural similarities because both enzyme groups interacted with the same inhibitor. Furthermore, it has been shown that the major α-amylase groups from both germinated barley and wheat shared immunochemical identity (Daussant, 1978). Under the same experimental conditions, the inhibitor had no effect on porcine pancreatic or human salivary α-amylases, once again showing the biospecificity of the inhibitor for cereal α-amylase II.

TABLE 6. Effect of inhibitor on the activity of α-amylases from a green malt of barley and germinated wheat[a].

	Enzyme Activity		
	No Inhibitor	Inhibitor	Percent Inhibition
	µmoles glucose/min/ml		
Malted barley α-amylase I	0.067	0.069	0
Malted barley α-amylase II	0.109	0.013	88
Germinated wheat α-amylase I	0.100	0.104	0
Germinated wheat α-amylase II	0.094	0.018	81

[a] Studies were carried out at pH 8.0 and 35°C.

Approximately equal amounts (protein) of inhibitor and α-amylase were used.

This biospecificity could be important during early stages of starch mobilization in the barley kernel endosperm. The inhibitor might preferentially reduce α-amylase II activity thereby allowing α-amylase I to be more effective in substrate degradation at this time. Extracts of sorghum, oats, millet, rice and maize had no effect on barley α-amylase II activity (Table 5). Germinated forms of sorghum,rice and maize appear to contain relatively high proportions of α-amylases which are similar to the α-amylase I groups of malted barley and germinated wheat (MacGregor, 1983; Mundy, 1982). Therefore, the inhibitor would not be expected to interact with these α-amylases.

CONCLUSION

In summary, an inhibitor of α-amylase II from malted barley and germinated wheat has been purified from barley kernels. The inhibitor had molecular weights of 21,000 and 20,000 daltons when determined by SDS gel electrophoresis and gel filtration chromatography, respectively. The isoelectric point of the inhibitor was 7.3. N-terminal sequence analysis indicated that the inhibitor was different from other known cereal proteins with inhibitor activity against animal α-amylases, but identical to an endogenous barley α-amylase inhibitor purified recently from green malt. Isoelectric focusing and gel filtration chromatography indicated the formation of an enzyme-inhibitor complex when mixtures of α-amylase II and inhibitor were subjected to analysis by these methods. Inhibition of α-amylase activity increased when the pH was increased from pH 5 to 8 and decreased when salt concentration was increased from zero to 200 mM NaCl, suggesting that the enzyme-inhibitor complex was stabilized by ionic bonding. The effect of the inhibitor was independent of starch granule concentration suggesting that the inhibitor could be effective in inhibiting starch granule hydrolysis by α-amylase II in the endosperm. Inhibitors of cereal α-amylase were not restricted to barley but were found, also, in wheat, rye and triticale.

Much research remains to be carried out with the endogenous α-amylase inhibitor. For example, the mechanism of inhibition has to

be determined as well as the precise localization of the inhibitor in the endosperm during barley kernel development, maturation and germination. Proteolytic treatment of the inhibitor and subsequent analysis of fragments for inhibitor activity should allow determination of the specific regions of the inhibitor molecule that are responsible for inhibiting cereal α-amylase. The biospecificity of the inhibitor for α-amylase II may permit development of a specific assay for α-amylase I. By differentially inhibiting α-amylase II activity, α-amylase I might then be detected in mixtures of the two enzymes.

Analysis of enzyme banding patterns, following isoelectric focusing or electrophoresis of cereal extracts, has been used to make predictions on gene expression. The α-amylase II inhibitor was shown to cause major changes in barley α-amylase banding patterns following isoelectric focusing. The possibility of a similar phenomenon occurring in other enzyme systems can not be overlooked. Therefore, the interpretation of zymograms, to make predictions on gene expression, may be questionable in some cases.

A number of practical implications become apparent from this study. Kilned malt contains a large proportion of α-amylase III indicating that the inhibitor survives kilning. It is possible, therefore, that the inhibitor could inhibit some of the α-amylase activity in the initial stages of brewing. Pre-harvest sprouting of cereals can result in serious problems associated with technological processing. In bread making, a relatively low level of α-amylase in the flour can lead to excessive breakdown of starch during fermentation and subsequent baking, resulting in a product with a sticky crumb and inferior loaf volume. Addition of barley fractions, containing α-amylase inhibitor, to

sprouted wheat flour might result in a dough with more suitable baking characteristics. Furthermore, if inhibitor content of grains could be correlated with sprouting resistance then it might be useful to breed cereal grains with increased inhibitor content.

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