CHARACTERIZATION OF THE INTERACTION OF BARLEY ALPHA-AMYLASE II WITH AN ENDOGENOUS ALPHA-AMYLASE INHIBITOR FROM BARLEY KERNELS

by

© Andrew John Halayko

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ABSTRACT

Halayko, Andrew John, MSc., The University of Manitoba, March, 1987. Characterization of the Interaction of Barley α-Amylase II With an Endogenous α-Amylase Inhibitor From Barley Kernels. Major Professor: Dr. R.D. Hill

Binding of purified endogenous inhibitor of α-amylase with the group II isozymes of α-amylase purified from barley kernels was determined to be optimal at pH 7.0 in low salt media. Anti-amylolytic strength of the inhibitor at optimum pH conditions was magnified approximately 8-fold from that exhibited at pH 5.5, the pH optimum for α-amylase II activity. Modification of the α-amylase/subtilisin inhibitor with N-bromosuccinimide at pH 5.0 under non-denaturing conditions indicated that one of the protein's 3 tryptophan side-chains was accessible to external media. Oxidation of all inhibitor tryptophans was effected in denaturing media, 8.0 M urea (pH 4.0), resulting in complete loss of amylolytic suppression capabilities. This loss of inhibitory strength was not inflicted upon inhibitor which only had its exposed tryptophan oxidized, suggesting a negligible role for this residue in anti-amylolytic activity. Dimethylsulfoxide and ethylene glycol perturbed the spectra of exposed tryptophanyl side-chains of α-amylase II about 4 nm towards longer

wavelengths. The magnitude of ultraviolet difference absorption spectra and quenching of fluorescence emission spectra in solvent perturbation studies on *a*-amylase II indicated that 6-9 of the enzyme's 16 tryptophan residues are exposed on the surface of the molecule. The binding interaction of *a*-amylase II from barley malt with purified *a*-amylase/subtilisin inhibitor was probed using difference spectroscopy, fluorescence titration and gel filtration. The environment around a single enzymic tryptophan was more hydrophobic in the complex. This was suggested by the difference spectrum of *a*-amylase II induced by inhibitor or by inhibitor that had previously had its only exposed tryptophan side-chain oxidized with N-bromosuccinimide. Spectral characteristics showed a major peak at 294 nm, maximum molar extinction difference of about 1700 $M^{-1}cm^{-1}$, and minor peaks at 284 nm and 275-277 nm. Binding affinity at pH 7.0 was increased by at least 7-fold from pH 5.5. A concentration of 0.3 M NaCl decreased complex association by 50-fold. All methods used indicated that the inhibitor and *a*-amylase II combine at a molar ratio of 2:1. However, the fluorescence titration curve of *a*-amylase II with inhibitor also showed a quenching plateau at equimolar concentrations. Thus, 1:1 stoichiometric complexes might exist and the binding of a second inhibitor intensifies hydrophobic shifts in the environment of an enzymic tryptophan side-chain. It appears that when the inhibitor binds with *a*-amylase II it affects an enzymic tryptophan residue which may be essential for productive enzyme-substrate binding.

1. INTRODUCTION

Mature and sprouting grains from various cereals have been shown to possess a proteinaceous inhibitor of endogenous α-amylase II, the major group of carbohydrate hydrolases secreted during germination (Weselake et al., 1983a, 1985a; Mundy et al., 1983). Its presence poses some potential for practical significance in baking technology, where excess amylolytic activity is undesirable, and for brewing technology, where retarded amylolysis during initial stages is unwanted. Though its actual physiological role is not yet clear, the inhibitor may be important in damping rapid endosperm hydrolysis and attenuating germination by allowing the embryo to develop to a stage where it can use mobilized starch reserves (Hill et al., 1987).

The mechanism of inhibition is undetermined. However, several aspects of the interaction which takes place between α-amylase II iso-enzymes and the inhibitor have been investigated. The inhibitor acts by forming a complex with α-amylase II (Weselake et al., 1983a,b; Mundy et al., 1983). There is also an indication that the affinities of the macromolecules for each other are considerably greater at slightly basic pH (Weselake et al., 1983b). Inhibitory ability is decreased as the ionic strength of the buffering system is increased (Weselake et al., 1983a), suggesting that binding involves ionic interaction. Though several characteristics of inhibitor-enzyme binding are known,

little is known about the quantitative aspects of the specific interactions involved.

The determination of some features of protein structure involved in complex formation and quantification of these features is achievable using the technique of difference spectroscopy (Herskovits, 1967; Ohnishi, 1971). The technique has been used primarily for protein-ligand binding studies (Dahlquist et al., 1966; Elodi et al., 1972; Clarke and Svensson, 1984a) but Buonocore et al. (1980, 1984) have successfully investigated the complexes of wheat albumin inhibitors with insect and chicken pancreatic α-amylases. Fluorescence titration has also been used successfully to elucidate specific binding interactions between proteins and ligands (Hiromi et al., 1982; Clarke and Svensson, 1984a). Selective oxidation of tryptophan residues using N-bromosuccinide (Spande and Witkop, 1967a,b) has been used in conjunction with difference spectroscopy (Hayashi et al., 1963; Gibson and Svensson, 1986) and fluorescence studies (Clarke and Svensson, 1984a) to yield extremely specific information regarding tryptophan involvement in protein binding interactions. The specific interaction of α-amylase II with its inhibitor from barley kernels has been examined with these techniques in this thesis.

2. LITERATURE REVIEW

2.1. Alpha-Amylases

2.1.1. Introduction

Alpha-amylases (α,1-4 glucan 4-glucanohydrolase, E.C. 3.2.1.1.) are an extensively studied group of enzymes found in mammalian, fungal, bacterial, crustacean and higher plant systems (Greenwood and Milne, 1968). They preferentially hydrolyze most internal α, 1-4 glycosidic bonds of long chain glucose polymers to yield smaller oligosaccharide products. The glucose unit at the reducing end of each product is left in the α-D configuration, hence, the naming of the enzyme (Thoma et al.,1971).

In cereals, α-amylase is the major hydrolase secreted by the aleurone during germination (Gibbons, 1981). Its importance is of the first order in mobilization of endosperm starch reserves for the growing embryo. Cereal α-amylase is synthesized de novo by the aleurone layer in response to gibberellic acid (Varner, 1964; Chrispeels and Varner, 1967) produced by the developing embryo (MacLeod and Palmer, 1966). The embryo itself has also been reported as a prominent site for α-amylase synthesis (Gibbons, 1981). Gibberellic acid and abscisic acid regulate synthesis of the enzyme by aleurone tissue by controlling transcriptional levels of messenger RNA (mRNA) coding for α-amylase (Ho and Varner, 1974; Higgins et al., 1982; Jacobsen and Beach,

1985; Rogers, 1985). There is evidence that the aleurone's rough endoplasmic reticulum is the site of α-amylase synthesis and that it plays a role in transporting the protein to the Golgi complex where it is glycosylated and subsequently secreted (Jacobsen and Knox, 1973; Jones and Jacobsen, 1982; Akazawa and Hara-Nishimura, 1985).

Germinated and malted barley or wheat have been broadly studied because of the technological significance of α-amylases in malting and baking. Of primary interest in this report are the cereal α-amylases. However, pertinent data about amylases from other thoroughly studied sources will also be considered.

2.1.2. Heterogeneity of Alpha-Amylases

Numerous studies of the characterization of malt α-amylases have been conducted since they were isolated (Schwimmer and Balls, 1948). In early investigations using agar gel electrophoretic separation techniques Frydenberg and Nielsen (1965) demonstrated that at least 5 α-amylase isozymes existed in germinating barley seeds. Later studies using ion exchange chromatography (MacGregor et al., 1971), polyacrylamide gel isoelectric focusing (MacGregor, 1977), and chromatofocusing (Marchylo and MacGregor, 1983) confirmed that malt α-amylase was a heterogeneous enzyme assemblage. Olered and Jonsson (1970) defined two major groups of α-amylase isozymes on the basis of agar gel

electrophoretic mobility of malted wheat extracts. Isoelectric focusing studies have shown these two groups exist in most cereals including different varieties of wheat, barley and triticale (Silvanovich and Hill, 1977; MacGregor, 1978; Sargeant, 1980). One of the two groups, called "green" amylase, predominates in growing seed while the second group is actively synthesized only in germinating seed and is called "malt" amylase (Olered and Jonsson, 1970). The former group has since been designated as α-I and the latter group as α-II on the basis of their isoelectric points (MacGregor, 1977). The two groups have now been further differentiated on the basis of peptide mapping, immunochemical characterization (Jacobsen and Higgins, 1982) and amino acid composition (Svensson et al., 1985).

A third group of α-amylase isozymes has also been seen for malted barley in isoelectric focusing studies (MacGregor and Ballance, 1980a). However, this group has proved to be a group of complexes formed exclusively between α-amylase II isozymes and an endogenous, proteinaceous inhibitor found in barley endosperm (Mundy, et al., 1983; Weselake et al., 1983a). The inhibitor is a heat labile factor (Weselake et al.,1983a). The presence of this inhibitor in malted barley extracts accounts for the reported phenomena of heat activation of α-amylase II fractions and the heat facilitated conversion of α-amylase III to α-amylase II in

several studies (Frydenberg and Nielsen, 1965; Tanaka and Akazawa, 1970; MacGregor and Ballance, 1980a). Conversion of *a*-amylase III to *a*-amylase II has also been facilitated during the kilning process (MacGregor and Daussant, 1981), chromatofocusing (Marchylo and MacGregor, 1983), ion exchange chromatography and isoelectric focusing (MacGregor and Ballance, 1980a) of amylase extracts from barley malt.

Studies with wheat and barley indicate that the basis for disparity between the two major groups of isozymes is that they are each coded for by separate genes or families of genes (Jacobsen and Higgins, 1982; Rogers and Milliman, 1983; Lazarus et al., 1985). Notable differences have been found in the nucleotide sequences of several full and partial length complementary-DNA (cDNA) clones prepared from barley aleurone mRNA which codes for *a*-amylases (Rogers and Milliman, Chandler et al., 1984; Rogers, 1985). These differences probably indicate that heterogeneity within the major *a*-amylase isozyme groups is the result of the expression of a family of genes which code for each group (Lazarus et al., 1985). Jacobsen and Higgins (1982) have suggested that heterogeneity within the *a*-amylase groups might be an attribute arising from differential control of the genes by gibberellic acid at the transcriptional level. Post-translational modification of synthesized amylases has also been suggested as a possible means for internal group heterogeneity (Motojima and Sakaguchi, 1982).

2.1.3. Properties of Alpha-Amylases

Consistent molecular weight determinations for cereal *a*-amylases have been achieved using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. A value of 40,000 to 50,000 is most commonly obtained using this method (Tkachuk and Kruger, 1974; Silvanovich and Hill, 1977; Jones and Carbonell, 1984). Tkachuk and Kruger (1974) reported a value of 45,100 using sedimentation diffusion. Gel filtration chromatography studies using Bio-Gel, a polyacrylamide material, have generated molecular weight values around 45,000 (Rodaway, 1978) but a value of 23,000 has been reported by Tkachuk and Kruger (1974) using Bio-Gel P-150. Sephadex gels can give anomalously low molecular weight values due to the carbohydrate nature of the matrix (Tkachuk and Kruger, 1974).

The most convenient method of differentiating between *a*-amylase I and *a*-amylase II is on the basis of isoelectric point (pI) using polyacrylamide electrofocusing. The *a*-I group is more acidic with a reported pI ranging from 4.5-5.1 while the *a*-amylase II group ranges between 5.9 and 6.4 depending on the source (Silvanovich and Hill, 1977; MacGregor and Ballance, 1980a; Jacobsen and Higgins, 1982).

Activity of cereal *a*-amylases is optimal around pH 5.5 (MacGregor et al., 1974; Tkachuk and Kruger, 1974). The *a*-amylases are rapidly denatured at pH's less than 3 but

retain their integrity over a range from pH 4 to pH 9 (Greenwood and Milne, 1968).

The *a*-amylases are Ca^{2+} containing metalloenzymes which form a tight metal-chelate structure (Vallee et al., 1959; Fischer and Stein, 1960). The interaction confers the stability and structural rigidity necessary for the catalytic activity of the enzymes (Hsiu et al., 1964). Removal of Ca^{2+} by chelation with ethylenediaminetetraacetic acid (EDTA) inactivates the *a*-amylase II isozymes but does not affect those of the *a*-I group (Jacobsen et al., 1970). Removal of Ca^{2+} by electrodialysis was responsible for a reversible loss of *a*-amylase activity (Stein et al., 1964). However, the enzymes may become susceptible to proteolytic attack in the absence of Ca^{2+} (Chrispeels and Varner, 1967). The *a*-amylases show strong resistance to proteolysis when Ca^{2+} is bound (Greenwood and Milne, 1968; Jones and Jacobsen, 1982). Plant *a*-amylases have a low affinity for Ca^{2+} compared to those from other sources (Fischer and Stein, 1960). In fact, when working with cereal *a*-amylases, a concentration of at least 1 mM $CaCl_2$ has been suggested for all buffering systems in order to maintain stable, optimally active preparations (Tkachuk and Kruger, 1974). In a more recent study, Jones and Jacobsen (1983) reported that a lack of Ca^{2+} in the incubation media for barley aleurone layers prevented the secretion of *a*-amylase II isozymes but not those of the *a*-amylase I group. These

workers found no evidence of proteolytic degradation of *a*-amylases in media devoid of Ca^{2+}

Bovine serum albumin (BSA) bestows a stabilizing effect on cereal *a*-amylase isozymes under extreme conditions of temperature (MacGregor and Ballance, 1980b). Purified *a*-amylase extracts can be stored frozen for long periods of time in the presence of 0.1% BSA with little loss of enzyme activity upon thawing (Weselake and Hill, 1983). Homogeneous preparations of *a*-amylase have also proved to be stable for some time when stored at $2^{o}C$ in the presence of an antibacterial agent (Tkachuk and Kruger, 1974).

The ultraviolet absorption spectra of *a*-amylases are typical of most proteins. Maximum absorption of 279 nm was seen for wheat and insect *a*-amylases in water (Tkachuk and Kruger, 1974; Buonocore et al., 1976b). A double absorption peak at 275 nm and 286 nm was seen for wheat *a*-amylase in alkaline solution (Tkachuk and Kruger, 1974). The fluorescence emission and excitation spectra of *a*-amylases are also typical of a number of proteins with maxima at 340 nm (Buonocore et al., 1976b; Clarke and Svensson, 1984a) and 280 nm (Clarke and Svensson, 1984a) respectively.

The amino acid compositions of *a*-amylases from various sources differ widely (Greenwood and Milne, 1968). However, a comparison of wheat and barley *a*-amylase compositions indicates that the cereal enzymes are probably similar in

their amino acid contents (Rodaway, 1978). Svensson et al. (1985) showed α-amylase I and α-II fractions from Himalaya barley malt to be quite similar in amino acid composition (Table 1). They differed only in that the more acidic isozyme group contained considerably higher contents of serine, alanine and methionine residues. Of the aromatic amino acids, tyrosine and phenylalanine were represented by 15 and 16 residues respectively per molecule of both α-I and α-amylase II (Table 1). The number of tryptophan residues for α-amylase II only, was determined to be 16 by second-derivative ultraviolet-spectroscopy. These results closely resemble those obtained in analyses of gibberellic acid treated Himalaya barley (Rodaway, 1978) and malted wheat amylase samples (Tkachuk and Kruger, 1974).

Amino acid sequences for α-amylase I and α-amylase II, deduced from nucleotide sequences of the corresponding cDNA clones (Chandler et al., 1984; Rogers and Milliman, 1983), show about 77% homology. Svensson et al. (1985) have published partial sequences for both major groups of α-amylase from Himalaya barley malt extracts. These results correlate highly with the inferred sequences from Chandler et al. (1984) and Rogers and Milliman (1983) for cDNA clones that encode α-amylase I and α-amylase II respectively. It has been suggested that there is no difference in the sequences of different α-amylase II isozymes (Jacobsen and Higgins, 1982; Svensson et al., 1985). However, Rogers and

TABLE 1

Amino acid composition of barley malt α-amylases[1]

Amino Acid	Residues per molecule α-amylase I	α-amylase II
Aspartic acid (Asp)	50	52
Threonine (Thr)	18	17
Serine (Ser)	25	14
Glutamic acid (Glu)	35	30
Proline (Pro)	18	19
Glycine(Gly)	44	46
Alanine (Ala)	46	35
Valine(Val)	28	25
Methionine (Met)	12	6
Isoleucine (Ile)	23	23
Leucine (Leu)	32	28
Tyrosine (Tyr)	15	15
Phenylalanine (Phe)	16	16
Histidine (His)	13	16
Lysine (Lys)	21	22
Arginine (Arg)	15	17
Tryptophan (Trp)	-	16
Half-cystine (1/2cys)	-	3
TOTAL	411	400

[1] Svensson et al., (1985).

Milliman (1984) have provided evidence that different mRNA's exist for at least two α-amylase II isozymes.

Studies on the crystal structure of Taka-amylase A (Matsuura et al., 1980, 1984) and pancreatic α-amylase (Payan et al., 1980) by x-ray diffraction provide information on the relationship between structure and function for amylolytic enzymes. These proteins feature a deep cleft, in which the active site is situated, that separates the molecule into two unequal globular units.

There seem to be several specific binding sites for substrates on most α-amylases, those of cereals included (Loyter and Schramm, 1966; Ohnishi et al., 1973a; Payan et al., 1980; MacGregor and MacGregor, 1985). One is situated in the active site region of characteristic groove (Payan et al., 1980) while one or two more are located on the molecule surface at a distance from the active centre (Jolley and Gray, 1976; Ohnishi et al., 1973b; Matsuura et al., 1984; MacGregor and MacGregor, 1985; Gibson and Svensson, 1986). Glutamic acid and aspartic acid residues are thought to be most important in the reactive site for catalysis of glycosidic bond hydrolysis (Greenwood and Milne, 1968; Jolley and Gray, 1976; Matsuura et al., 1984). Tryptophan, tyrosine and histidine have been implicated in substrate binding at the active site of many carbohydrases (Elodi et al., 1972; Kita et al., 1982; Clarke and Svensson, 1984a, 1984b; Jimbo et al., 1984; Matsuura et al., 1984).

Furthermore, modification of specific exposed tryptophan residues on bacterial liquefying α-amylase (Fujimori et al., 1974), glucoamylase of various fungal origins (Ohnishi and Hiromi, 1976; Inokuchi et al., 1982; Clarke and Svensson, 1984a, 1984b) and fungal Taka-amylase A (Kita et al., 1982) greatly reduces or destroys enzymatic activity. Various studies have shown tryptophanyl residues to be important components at the subsites of substrate binding (Ohnishi et al., 1973b; Kita et al., 1982; Clarke and Svensson, 1984b). These residues and possibly some of tyrosine as well have been cited as important in the maintenance of the three-dimensional structure which facilitates productive enzyme-substrate binding (Greenwood and Milne, 1968; Matsuura et al., 1984). X-Ray crystallography studies have also confirmed the role of Ca^{2+} as a stabilizer of enzyme integrity for effective catalytic ability (Matsuura et al., 1980; Payan et al., 1980).

2.2. Endogenous Inhibitors of Cereal Alpha-Amylase

2.2.1. Introduction

Cereal protein inhibitors of α-amylase from a variety of sources are well documented and have been the subjects of several reviews (Deponte et al., 1976; Buonocore et al., 1977; Warchalewski, 1983). The biophysical properties and characteristics of inhibitor/ α-amylase interaction have been most thoroughly studied for two inhibitory wheat

albumin components having molecular weights of 12,000 and 24,000 daltons (Deponte et al., 1976; Buonocore et al., 1976a, 1980, 1984; Petrucci et al., 1978). The inhibitors have been designated as "0.28" and "0.19" respectively on the basis of their electrophoretic mobilities relative to bromophenol blue (Deponte et al., 1976). However, these inhibitors are not active against endogeneous *a*-amylases. They are, instead, active against mammalian, avian and insect amylases and probably serve as a measure of insect resistance for cereals (Buonocore et al., 1977).

There have been scattered reports of the existence of endogenous protein inhibitors of cereal *a*-amylases. Blanco-Labra and Iturbe-Chinas (1981) and Warchalewski (1976, 1977a, 1977b, 1983) have given accounts of heat resistant, low molecular weight inhibitors from maize, durum and winter wheats respectively. In addition, wheat purothionins have been shown to inhibit wheat *a*-amylase activity in vitro by controlling the availability of Ca^{2+} to serve as the enzyme's cofactor (Jones and Meredith, 1983).

An endogenous *a*-amylase inhibitor, possessing some physical properties of the soybean trypsin inhibitor (Kunitz) family (Hejgaard, et al., 1983) has been characterized. The protein inhibitor is biospecific for the group II isozymes of malt *a*-amylase and has been shown to exist in barley (Hejgaard et al., 1983; Mundy et al., 1983; Weselake et al., 1983a, 1983b), wheat (Mundy et al., 1984;

Weselake et al., 1985a), rye and triticale (Weselake et al., 1985a). The inhibitor is not present in sorghum, millet or corn and probably not in oats (Weselake et al., 1985a). The inhibitors show endogenous and, in general, cereal anti-amylase activity (Mundy et al., 1983; Weselake et al., 1985a) but are inactive against malted sorghum, mammalian, fungal, bacterial and insect α-amylases (Mundy et al., 1983, 1984). This inhibitor also strongly suppresses the activity of the bacterial serine proteinase subtilisin (Mundy et al., 1983, 1984) and is probably the same subtilisin inhibitor previously isolated from barley by Yoshikawa et al. (1976). Furthermore, it is highly likely that this inhibitor was recognized as "band 2 protein" which co-purified with barley malt α-amylase (Rodaway, 1978). The α-amylase/subtilisin inhibitor, ASI, has been isolated from barley (Hejgaard et al., 1983; Mundy et al., 1983; Weselake et al., 1983a, 1983b, 1985a, 1985b) and wheat (Mundy et al., 1984) and designated by the acronyms BASI and WASI respectively (Hejgaard et al., 1983; Mundy et al., 1984; Munck et al., 1985).

2.2.2. Properties of ASI

Purification schemes for BASI are well established (Mundy et al., 1983; Weselake et al., 1983a, 1983b, 1985b). Isolated inhibitor preparations are homogeneous as assessed by SDS gel electrophoresis, analytical isoelectric focusing

(Mundy et al., 1983; Weselake et al., 1983a) and amino acid sequencing (Hejgaard et al., 1983; Weselake et al., 1983b; Maeda, 1986; Svendsen et al., 1986). However, immunochemical studies indicate the existence of three isoforms of inhibitor (Sadowski et al., 1986). Monomeric molecular weight estimates of the inhibitor by SDS electrophoresis have been between 20,000 and 21,000 daltons (Yoshikawa et al., 1976; Mundy et al., 1983; Weselake et al., 1983b). The complete amino acid sequence of a single peptide chain of BASI has been reported (Svendsen et al., 1986) and was used to calculate a molecular weight of 19,865 daltons. Using gel filtration on Bio-Gel P100, Weselake et al. (1983b) determined a molecular weight of 20,000 daltons and reported that the inhibitor did not appear to self associate under the chromatographic conditions used. However, Rodaway (1978), looking at gel filtration of "band 2 protein" on Bio-Gel P60, suggested that the native form was that of a dimer because the protein gave a molecular weight similar to that of α-amylase, which is approximately 45,000 daltons.

The isoelectric point (pI) of the inhibitor has been estimated by analytical isoelectric focusing and by electrophoresis at various pH's. Reported values have been 7.0 (Yoshikawa et al., 1976; Sadowski et al., 1986), 7.2 (Mundy et al., 1983), and 7.3 (Weselake et al., 1983b).

The inhibitor is heat labile. It loses anti-α-amylase activity at a linear rate as temperature is raised from 50°C to 70°C (Munck et al., 1985). After 15 minutes at 70°C only a small fraction of the original activity remains (Mundy et al., 1983; Weselake et al., 1983a).

Weselake et al. (1983b) and Mundy et al. (1983) have published nearly identical amino acid compositions for BASI. Mundy et al. (1984) have analyzed the amino acid compositions for WASI; inhibitors from barley and wheat appear to be comprised of extremely similar proportions of primary amino acid residues (Table 2). Comparable parallelism in amino acid composition exists between the α-amylase/subtilisin inhibitors, "band 2 protein" from barley malt (Rodaway, 1978; Mundy et al., 1983) and the subtilisin inhibitor from barley (Yoshikawa et al., 1976; Mundy et al., 1983). The aromatic amino acids, tryptophan, tyrosine and phenylalanine, are represented by 3, 6 and 6 residues respectively per 20,000 dalton inhibitor molecule (Table 2). The amino acid composition of the inhibitor is not related to those published for a purothionin homolog from barley flour (Ozaki et al., 1980) or for the "0.19" and "0.28" inhibitory wheat albumins (Buonocore et al., 1977).

The complete amino acid sequences of BASI and WASI are known (Maeda, 1986; Svendsen et al., 1986), the single peptide chains contain 181 and 180 residues respectively, and both have two disulfide bridges. Partial sequences

TABLE 2

Amino acid compositions of the barley and wheat α-amylase/subtilisin inhibitors (BASI and WASI)

Amino Acid	Residues per monomeric polypeptide[1]		
	BASI Weselake *et al.* (1983b)	BASI Mundy *et al.* (1983)	WASI Mundy *et al.* (1984)
Aspartic Acid (Asp)	19.0	16.9	18.6
Threonine (Thr)	10.0	8.4	6.8
Serine (Ser)	8.4	9.6	9.3
Glutamic Acid (Glu)	14.7	15.3	15.0
Proline (Pro)	13.4	15.0	15.5
Glycine (Gly)	17.5	20.2	20.1
Alanine (Ala)	20.8	16.4	16.7
Valine (Val)	14.0	12.8	14.3
Methionine (Met)	nil	2.2	2.0
Isoleucine (Ile)	8.2	6.9	6.9
Leucine (Leu)	9.9	11.9	11.6
Tyrosine (Tyr)	6.0	6.4	5.9
Phenylalanine (Phe)	5.3	7.0	6.5
Histidine (His)	4.6	9.1	7.5
Lysine (Lys)	7.0	6.6	6.1
Arginine (Arg)	13.1	11.6	13.6
Tryptophan (Trp)	2.6	2.9	2.8
Half-cystine (1/2Cys)	8.8	4.1	4.0
TOTAL	183.3	183.4	183.2

[1] Compositions adjusted by Mr 20,000 for all proteins.

determined for N-terminal residues of BASI (Hejgaard et al., 1983; Weselake et al., 1983b) and WASI (Mundy et al., 1984) show 96% homology. An α-amylase/trypsin inhibitor from Indian finger millet seed has also been isolated and sequenced (Shivaraj and Pattabiraman, 1981; Campos and Richardson, 1983) but it differs from BASI and would probably fall into another family of serine proteinase inhibitors (Mundy et al., 1984). In addition, millet seed does not contain any protein that shares immunochemical identity with BASI (Weselake et al., 1985a). There is very little sequence homology between either of the two major inhibitory albumins from wheat with BASI or WASI (Hejgaard et al., 1983; Maeda, 1986). A subtilisin inhibitor isolated from rice grains (Kato et al., 1972) shows 75% homology with BASI and WASI (Mundy et al., 1984). However, Weselake et al. (1985a) have reported that they were unable to detect anti-barley-amylase activity or an immunochemically similar BASI protein from rice.

Protein inhibitors of serine proteases can be classified into 10 or more families based on non-homology of primary structure, reactive site(s) identity and positioning and numbers of intrachain disulfide bridges (Laskowski and Kato, 1980). The soybean trypsin inhibitor (Kunitz), designated STI (Kunitz), has been extensively characterized and is representative of the proteinase inhibitor family of the same name (Kunitz, 1946, 1947; Koide and Ikenaka, 1973a,b;

Koide et al., 1973; Sweet et al., 1974). Yoshikawa's et al. (1976) subtilisin inhibitor from barley has been included in this family (Laskowski and Kato, 1980). The subtilisin inhibitor and BASI are now known to be one and the same as confirmed by their identical N-terminal primary sequences (Hejgaard et al., 1983). In fact, BASI exhibits varying degrees of sequence homology with at least 5 leguminous STI (Kunitz) type proteinase inhibitors which are similar in molecular weight as well (Hejgaard et al., 1983). BASI and WASI possess 26% and 31% sequence homology respectively, with soybean trypsin inhibitor (Kunitz) (Maeda, 1986; Svendsen et al., 1986). The three proteins are strikingly similar in that all have approximately 180 amino acid residues, two disulfide bridges and almost identical molecular weights around 20 Kd (Koide and Ikenaka, 1973a; Svendsen et al., 1986). Maeda (1986) reports that WASI and STI (Kunitz) have the same arrangement and positions of their two intramolecular disulfide bridges. The secondary structure of BASI and WASI has been predicted to be a β-sheet structure segregated by 4 segments of α-helical domain (Maeda, 1986; Svendsen et al., 1986). At this level, STI (Kunitz), BASI and WASI generally correspond to each other (Maeda, 1986; Svendsen et al., 1986). However, the molecules do not appear to be completely superimposable (Svendsen et al., 1986). The barley and wheat amylase/subtilisin inhibitors are believed to belong to the STI (Kunitz) family (Hejgaard et al., 1983; Maeda, 1986).

2.2.3. The BASI/Alpha-Amylase Interaction

Analytical isoelectric focusing has revealed that the barley α-amylase/subtilisin inhibitor specifically inhibits the α-amylase II isozymes of germinating cereal grains by forming a complex with the enzyme (Mundy et al., 1983: Weselake et al., 1983a). Moreover, using gel filtration, the formation of a new molecular weight species has been demonstrated by applying pre-incubated α-amylase II/BASI mixtures to Bio-Gel P100 columns (Weselake et al., 1983b; Halayko et al., 1986). The complexes formed gave a new peak with a 41,000 dalton molecular weight which was lower than would be expected but higher than individual values for the inhibitor and α-amylase II in the same experiment. Mosolov and Shul'gin (1986) reported a similar low molecular weight value for the complex between subtilisin and a 20,000 dalton inhibitor from wheat.

The heat labile α-amylase III isozyme group seen in isoelectric focusing experiments on barley (MacGregor and Ballance, 1980a) has proved to be a BASI/α-amylase II complex (Weselake et al., 1983a). The isoelectric point of the α-amylase II/inhibitor complex, approximately 6.9, is intermediate to those for α-amylase II and BASI (Weselake et al., 1983a, 1985b).

Recently, Munck et al. (1985) have described an extremely sensitive and specific enzyme-linked immunosorbent assay for

BASI. However, most measurements of the anti-α-amylase activity for BASI have been accomplished using modified α-amylase activity assays (Weselake et al., 1983a, 1983b). The interaction of the enzyme and inhibitor is not instantaneous and a 5 minute incubation period is needed prior to assay (Weselake et al., 1983b). BASI appears to be "double-headed" because anti-α-amylase activity is not affected by the presence of subtilisin in assay solutions (Mundy et al., 1983).

The inhibitor/α-amylase II interaction is sensitive to pH. Weselake et al. (1983a) have reported that inhibition is greater at pH 8.0 than pH 5.5. In the presence of soluble substrate an equimolar amount of inhibitor to α-amylase II suppressed 40% of enzyme activity at pH 8.0 whereas similar antagonism at pH 5.5 was only achieved with an 1100 molar excess of BASI over enzyme (Weselake et al., 1983b). Using insoluble starch granules for substrate and with a 10-fold molar excess of BASI over barley α-amylase II, no inhibitory activity could be detected at pH 4.6 whereas inhibition of enzyme activity increased to 30% and 65% at pH's 5.2 and 6.0 respectively (Weselake et al., 1985b). Some studies on α-amylase isozymes have shown a more elevated enhancement of α-amylase II activity following BASI heat inactivation at pH 7.5 compared to at pH 5.0 (Tanaka and Akazawa, 1970; MacGregor and Ballance, 1980a). Optimum pH for inhibition of α-amylase II activity by BASI

appears to be around 7.0 (Weselake et al., 1985b; Halayko et al., 1986).

The inhibitory ability of BASI is decreased as the ionic strength of the buffering milieu is increased (Weselake et al., 1985b; Halayko et al., 1986). At least a 10-fold decrease in BASI anti-amylase activity was seen when sodium chloride concentration was increased from 0 to 0.2 M at pH 5.5 in the presence of insoluble substrate (Weselake et al., 1985b). Similarly, a 50-fold decrease in complex affinity was measured when sodium chloride concentration was increased from zero to 0.2M at pH 7.0 (Halayko et al., 1986).

Dissociation of BASI from enzyme-inhibitor complexes immobilized on cycloheptaamylose-conjugated epoxy-Sepharose 6B was accomplished in the presence of 0.5 M sodium chloride at pH 5.5 (Weselake et al., 1985b). The results of such investigations, in conjunction with pH effect data, clearly indicate that ionic interaction is the stabilizing force for the α-amylase/BASI complex.

Apparent inhibition constants, Ki, between 4.0×10^{-8} and 5.7×10^{-7} M for BASI with α-amylase II at pH 6.0 have been reported by Mundy et al. (1983) following preliminary kinetic studies. Results suggested a mixed type mechanism of inhibition and the authors concluded that BASI/α-amylase II binding did not show the same "tight" binding characteristic of many protease/protein inhibitor

interactions. Halayko et al. (1986) have reported inhibitor-α-amylase II dissociation constants around 1 μM and 7 μM for pH's 7.0 and 5.5 respectively.

2.2.4. Physiological Role of ASI

Currently the role(s) that the amylase/subtilisin inhibitor might play in the developing or germinating seed are speculative. In the mature cereal caryopsis, ASI is found throughout the endosperm and is also present in the aleurone and embryo (Munck et al., 1985 ; Weselake et al., 1985a ; Mundy et al., 1986 ; Hill et al., 1987). The inhibitor may comprise up to 0.5% of the total seed protein in barley (Munck et al., 1985) and levels in wheat, rye and triticale seem to be comparable (Weselake et al., 1985a).

Time course studies of grain filling detect active synthesis of BASI at 20 days post-anthesis and termination of synthesis 10 days later (Munck et al., 1985; Lauriere et al., 1985). This is the same time period in which large amounts of storage proteins are synthesized. However, the inhibitor is an unlikely storage protein due to its resistance to proteolytic degradation (Weselake et al., 1985a). It is synthesized only in the endosperm during development and levels remain unchanged in quiescent and germinating mature seeds (Munck et al., 1985 ; Weselake et al., 1985a). Abscisic acid (ABA), and gibberellic acid (GA_3), affect the synthesis and/or accumulation of BASI in

embryoless half-seeds and cultured aleurone layers (Mundy, 1984; Mundy et al., 1986). Abscisic acid induces concomitant accumulation of inhibitor and its mRNA and suppression of levels of α-amylase and its mRNA. The reverse effect is induced with GA_3 (Mundy, 1984; Mundy et al., 1986).

The possibility that the inhibitor may act as a source of resistance to pre-harvest sprouting in cereals is an attractive one. The levels of inhibitor in the endosperm are sufficient to quench the α-amylase II formed in the first 4-6 days of germination (Hill et al., 1987). However, it has been observed that the levels of ASI and immunity to precocious germination are uncorrelated in several barley and wheat varieties (Munck et al., 1985; Weselake et al., 1985a; Munck and Mundy, 1987).

The inhibitor is probably one of many factors which control endosperm degradation. Amylase/subtilisin inhibitor is available around the starch granules of the endosperm in mature grains (Hill et al., 1987; Lecommandeur et al., 1987; Munck and Mundy, 1987). Weselake et al. (1985b) have shown that BASI anti-α-amylase II activity is not lost in the presence of high concentrations of starch granules. Hence, in the germinating seed, functional inhibitor is found where α-amylase II is secreted.

It has been suggested that the inhibitor's function may actually be that of a germination attenuator which acts by damping the activity of limited levels of α-amylase II (Hill et al., 1987). Since BASI is an ABA-inducible protein it is likely that it plays a regulatory role during kernel filling when ABA levels are elevated (Mundy, 1987; Hill et al., 1987). The presence of a modulator of amylase action in the endosperm may serve to fine tune the germination process. Soluble saccharide levels would be suppressed for a time such that the embryo could mature and utilize them, thus avoiding low osmotic potentials and rapid kernel swelling (Hill et al., 1987). Environmental conditions during seed development may cause changes in ASI synthesis (Hill et al., 1987). However, no studies have endeavored to answer this question. The activity of BASI toward α-amylase II is independent of temperature changes within normal environmental ranges (Weselake et al., 1985b).

2.3 Ultraviolet Difference Absorption Spectroscopy

The absorption of light in the ultraviolet (UV) range (250-300 nm) by proteins is a characteristic attributable to the aromatic side chains of the amino acid residues, tryptophan, tyrosine and phenylalanine (Herskovits, 1967). Shifts or perturbations can occur in the UV spectra of the chromophoric residues if some physical properties (e.g. refractive index, polarity, solvent-solute interaction) of

their aqueous media are altered. The technique of difference spectroscopy exploits these spectral shifts to gain information regarding the structure of proteins in solution. The changes in UV absorption are measured for a test solution against a native reference solution of identical concentration to produce difference spectra. These spectra have been used quantitatively and qualitatively to specify the environment, location and involvement of aromatic amino acids in enzyme-substrate or protein-protein interactions (Elodi et al., 1972; Kunikata et al., 1978; Buonocore et al., 1980; Gibson and Svensson, 1986).

2.3.1 Solvent Perturbation

In this technique, difference spectra are produced from proteins while parameters such as pH, temperature and ionic strength are kept constant, but solvent composition is varied. Mild or inert substances which do not change polypeptide conformation but do induce measurable shifts in aromatic side-chain spectra are added to aqueous protein samples (Herskovits, 1967). Examples of the perturbants used are dimethylsulfoxide, glycerol, sucrose, ethylene glycol and polyethylene glycol (Herskovits and Sorensen, 1968b; Ananthanarayanan and Bigelow, 1969). These substances tend to decrease the polarity of aqueous solvents causing shifts in absorbance maxima of the aromatic amino

acids to longer wavelengths (red shifts) (Herskovits, 1967). Therefore, when the solvent perturbed spectrum of a test solution is measured against an unperturbed reference, a solvent perturbation difference spectrum is produced.

Characteristic difference spectra have been obtained from solvent perturbation studies of N-acetylethyl esters of aromatic amino acids (Herskovits and Sorensen, 1968a; Ananthanarayanan and Bigelow, 1969). Tryptophan and proteins in which absorbance is dominated by tryptophan show 2 major perturbed peaks, one at 290-294 nm and a smaller one at 281-284 nm, with a small shoulder around 278 nm. Tyrosine and tyrosine-rich proteins show two perturbation maxima, 286-288 nm and 278-281 nm, in their difference spectra. Phenylalanine shows no characteristic peaks above 270 nm (Herskovits, 1967).

Ultraviolet solvent perturbation difference spectra are the sum of the perturbations of the spectra of aromatic side chains (Herskovits, 1967). Only those residues which are exposed to the solvent changes caused by the added perturbant contribute to the recorded difference spectra. Those chromophoric side chains which are buried in folds or cavities of the protein's tertiary or quarternary structure are inaccessible to perturbants. Thus, their absorbance characteristics are not affected by solvent changes (Herskovits and Laskowski, 1960). The number of exposed residues of trytophan or tyrosine in a protein has been

estimated from solvent perturbation difference spectra (Ohnishi, 1971; Kunikata et al., 1978). Model compounds of either residue have been used as 100% exposed references (Herskovits, 1967; Gibson and Svensson, 1986). The number of exposed aromatic residues on a protein is calculated from the ratio of the perturbation or molar absorptivity difference, $\Delta\epsilon$, of the protein to the model compound at the wavelength specific for the residue of interest, λ max, eg. 290-294 nm for tryptophan (Ohnishi, 1971; Ohnishi et al., 1975).

2.3.2. Binding Interactions

Protein interactions with other molecules can be studied by difference spectroscopy when binding causes changes in the environment of chromophoric residues. A large volume of such work exists (Hayashi et al., 1963; Dahlquist et al., 1966; Elodi et al., 1972; Mora et al., 1974; Ohnishi et al., 1975; Nitta et al., 1983). Qualitatively, the aromatic residue specific difference spectra produced by binding interactions are the same as those produced by solvent perturbation (Herskovits, 1967). Hence, as in solvent perturbation studies, the identity of the residue(s) contributing to the measured difference spectrum of a protein binding another molecule can be obtained by comparing the shape of the experimental spectrum with known residue-specific difference spectra (Clarke and Svensson, 1984a; Gibson and Svensson, 1986).

If a protein (P) reversibly binds with another molecule (X) to give a stable complex (PX),

$$P + X \leftrightharpoons PX \qquad (1)$$

and that interaction produces a measurable difference spectrum, the magnitude of $\Delta\epsilon$ at λmax for that spectrum has been reported to be proportional to the concentration of PX (Dahlquist et al., 1966; Elodi et al., 1972; Mora et al., 1974). When the concentration of X is progressively increased, equilibrium will shift right and P becomes saturated (Ohnishi, 1971). The difference molar absorptivity at λmax for the protein becomes $\Delta\epsilon$max in this situation and it represents the additive contributions of the perturbations of all affected chromophores (Hayashi et al., 1963; Ohnishi, 1971). Values for $\Delta\epsilon$max around 1,600 $M^{-1}cm^{-1}$ and 600 $M^{-1}cm^{-1}$ at 290 nm and 284 nm respectively have been attributed to the masking of one tryptophan or tyrosine residue of a polypeptide backbone (Donovan, 1968; Ohnishi, 1971; Ohnishi et al., 1973a). The ratio of the measured $\Delta\epsilon$max produced when a protein binds another molecule to the equivalent wavelength, single-residue $\Delta\epsilon$max value has been used as an estimate of the number of specific chromophores affected in binding interactions (Ohnishi, 1971; Ohnishi, 1973a). For example, Ohnishi (1971) reported that the binding of cycloheptaamylose to bacterial α-amylase produced a difference spectrum attributable to one tryptophan and one tyrosine residue on the basis of the spectrum's shape and magnitude.

Quantitative difference absorption measurements involve the titration of proteins with appropriate substrates or other ligands. Difference spectra have been used to draw mole-ratio plots of $\Delta\epsilon$ at λmax as a function of protein:ligand molar ratios (Nitta et al., 1983). If complex formation is favorable, two straight lines of different slope are obtained and the intersection of them occurs at a mole ratio corresponding to the binding stoichiometry of the complex formed (Skoog and West, 1982). Binding stoichiometry has been successfully calculated in this manner for many protein-ligand interactions (Ohnishi, 1971; Nitta et al., 1983) including those between wheat albumin inhibitors and insect and avian α-amylase (Buonocore et al., 1980).

The dissociation constants, Kd, of protein-ligand complexes (PX) have been calculated from the ligand-concentration dependence of $\Delta\epsilon$ at λmax for binding interactions which produce measurable difference spectra (Ohnishi, 1971; Elodi et al., 1972; Mora et al., 1974; Kunikata et al., 1978; Nitta et al., 1983). For reversible binding described by the equilibrium indicated in Equation 1, absorption difference, $\Delta\epsilon$, at λmax is assumed to be proportional to the concentration of PX and $\Delta\epsilon$max is reached when P is saturated with X (Elodi et al., 1972; Nitta et al., 1983). Then by Michaelis-Menten-type formula,

$$\frac{1}{\Delta\epsilon} = \frac{Kd}{\Delta\epsilon max} \cdot \frac{1}{[X]} + \frac{1}{\Delta\epsilon max} \qquad (2)$$

where Kd= the apparent dissociation constant of the formation of the PX complex and [X]= the concentration of ligand (Ohnishi, 1971; Kunikata et al., 1978). If $1/\Delta\epsilon$ is plotted against 1/[X], Lineweaver-Burk plot, a straight line is obtained having an abscissa intercept equal to -1/Kd if [X] is greatly in excess over the protein (Dixon and Webb, 1967; Ohnishi, 1971). When solved for, Kd is the reciprocal of the affinity of the protein for ligand (Dixon and Webb, 1967). This method of analysis of difference spectra has been used to estimate dissociation constants in order to quantify complex formation between various α-amylases and substrates or substrate analogues (Ohnishi, 1971; Elodi et al., 1972; Nitta et al., 1983).

3. MATERIALS AND METHODS

3.1. Plant Material

Barley (Hordeum vulgare cv. Argyle) grain was used as the starting material for all studies. Mature seed was stored at 4°C, kernels were germinated by placing them on water soaked absorbent paper for 4 days at 22°C. Germinated grain was lyophilized and stored at 4°C. All seed material was later ground to a fine meal in a Udy Cyclone Mill prior to extraction procedures.

3.2. Purification of Alpha-Amylase II

Alpha-amylase II was purified from 4-day germinated barley kernels (Hordeum vulgare cv. Argyle) essentially as described by Weselake et al. (1983b).

All steps were performed at 4°C unless otherwise indicated. Five hundred grams of ground green malt were extracted in 1 L of 200 mM sodium acetate buffer (1 mM $CaCl_2$, pH 5.5), centrifuged, supernatant heated at 70°C for 15 minutes and centrifuged again. The supernatant was dialyzed overnight in several changes of 20 mM sodium acetate (1 mM $CaCl_2$, pH 4.75), centrifuged and then the supernatant was slowly pumped through an affinity column of cycloheptaamylose-epoxy-Sepharose 6B equilibrated with dialysis buffer (Silvanovich and Hill, 1976). The column was washed with 5 bed volumes of 80 mM sodium acetate (1 mM

$CaCl_2$, pH 4.75) followed by 2 bed volumes of the same buffer containing 300 mM NaCl to elute non-specifically bound proteins and inhibitor bound to *a*-amylase II. Finally, the column was washed with 2 more bed volumes of equilibration buffer. Amylase was then eluted directly onto a column of carboxymethyl cellulose (CMC) with 80 mM sodium acetate (0.2% cycloheptaamylose, 1 mM $CaCl_2$, pH 4.75). The CMC column was extensively washed with 80 mM sodium acetate (1 mM $CaCl_2$, pH 4.75) to remove *a*-amylase I iso-enzymes. The *a*-amylase II was eluted from the column using a linear gradient of 80 mM sodium acetate (1 mM $CaCl_2$, pH 4.75) and 200 mM sodium acetate (1 mM $CaCl_2$, pH 5.5). The eluted enzyme peak was concentrated by pressure ultrafiltration through YM5 Amicon filters and assessed for purity by silver staining (Merril et al., 1981) of sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE (Laemmli, 1970) and analytical isoelectric focusing (MacGregor, 1976). Purified *a*-amylase II was either stored longterm at -20°C in the presence of 0.1% bovine serum albumin (Weselake and Hill, 1983) or at 4°C with 0.02% sodium azide for short durations of 2 weeks or less (Tkachuk and Kruger, 1974).

3.3. Purification of Inhibitor

Amylase/subtilisin inhibitor was prepared from pearled barley kernels (Hordeum vulgare cv. Argyle) by the methods of Weselake et al. (1985b).

All steps were done at 4°C and, briefly, consisted of the following. Four hundred grams of mature seed meal were extracted in 2 L of 20 mM sodium acetate (1 mM $CaCl_2$, pH 5.5). The mixture was centrifuged and the supernatant subjected to ammonium sulfate fractionation. The protein precipitating between 40 and 70% saturation was retained by centrifugation, resuspended and dialyzed exhaustively against 5 mM Tris-HCl (1 mM $CaCl_2$, pH 8.0). The dialysate was passed through a diethylaminoethyl (DEAE)-Sephacel ion exchange column, washed with dialysis buffer and subjected to a linear salt gradient up to 150 mM NaCl in 5 mM Tris-HCl (1mM $CaCl_2$, pH 8.0). Absorbance at 280 nm and inhibitor anti-amylase activity of eluted fractions were measured. Active fractions were pooled, concentrated by pressure ultrafiltration through YM5 Amicon filters and then passed through a Bio-Gel P-30 (100-200 mesh) gel filtration column twice. Inhibitor fractions were identified by zymogram staining of analyical isoelectric focusing gels (Doane, 1967; MacGregor, 1976) and by silver staining of SDS-PAGE runs (Laemmli, 1970; Merril et al., 1981). Concentrated samples of purified inhibitor were stored at -20°C.

3.4. Protein and Activity Assays

3.4.1. Protein Determination

Molar conversion absorbtivity factors, calculated for α-amylase II and inhibitor in 1,4-piperazinediethanesulfonic acid (Pipes) buffer, were used to convert absorbance at 280 nm into concentration. The constants were obtained by dividing molar protein concentrations, determined using the method of Lowry et al. (1951), by the corresponding absorbances at 280 nm for given samples. Bovine serum albumin was used as a standard. Molar determinations were based on molecular weights of 20 and 45 kilodaltons for inhibitor and α-amylase respectively. In general, the protein content of stock concentrates of enzyme and inhibitor were determined directly by the method of Lowry et al. (1951) and absorbance at 280 nm was used to detect protein in column effluents during purification procedures.

3.4.2. Inhibitor and Alpha-Amylase Activity Determination

Anti-amylolytic activity of the inhibitor and the hydrolytic activity of α-amylase II were measured using the method of Briggs (1961) as modified by Weselake et al. (1983b). Reactions for amylase or inhibitor assays were done at 35°C in low ionic strength buffers of 20 mM sodium acetate (1 mM $CaCl_2$) or 10 mM Pipes-NaOH (1 mM $CaCl_2$) at various pH's. The substrate used was β-limit dextrin prepared from waxy maize starch.

For anti-amylase activity measurements, 500 μLs of appropriately diluted inhibitor were added to equal volumes of diluted α-amylase II. Inhibitor was added in a 3-fold molar excess over amylase. After a 15 minute incubation, 1 ml of substrate was added and the reaction was stopped 10 to 20 minutes later by the addition of 5 mls of acidified I_2-KI solution (0.05% KI, 0.005% I_2, 0.05 N HCl). Color loss of undigested substrate was measured as Iodine Dextrin Color (IDC) units at 540 nm on a Zeiss PM QII spectrophotometer. An IDC unit is defined as that amount of α-amylase that diminishes absorbance of iodine-β-limit dextrin complex from 0.6 to 0.4 in 100 minutes (Briggs, 1961). Anti-α-amylase II activities of inhibitor samples were calculated from the relative differences between IDC units measured for amylase in the presence and absence of inhibitor. Amylase activity was determined by substrate disappearance without inhibitor.

3.5. Equilibration of Aqueous Protein Samples

Aqueous protein samples were equilibrated to experimental pH and ionic strength by diluting a 1 ml aliquot to 10 mls with appropriate buffer then reconcentrating to 1 ml using pressure ultrafiltration through YM5 Amicon filters. Dilution and concentration steps were repeated four times for each protein sample.

To confirm the reliability of the equilibration procedure 10 mls of 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) were

filtered through a YM5 ultrafiltration membrane to a volume of 1 ml. The concentrated buffer was then diluted back to 10 mls with deionized water. Concentration and dilution steps were each repeated 6 times. The conductance of the original buffer and of the re-diluted concentrates was measured using a Markson #4402 Electromark Analyzer to assess the effectiveness of the ultrafiltration cell as a rapid dialysis chamber.

3.6. Effect of pH on Inhibitor Activity

The anti-α-amylase II potential of BASI at various pH's was examined. Both α-amylase II and inhibitor were pre-equilibrated with buffer which was suitable for the pH's of interest. A 10 mM Pipes-NaOH (1 mM $CaCl_2$) medium was used for pH's from 6.1 up to pH 7.5 while 20 mM sodium acetate (1 mM $CaCl_2$) was appropriate for study at pH 5.5. Enzyme (3.39×10^{-3} μmol) and inhibitor (1.04×10^{-2} μmol) were pre-incubated for 15 minutes and a further 15 minutes of β-limit dextrin hydrolysis followed. Amylase activity was measured for the same time course at each pH.

3.7. Gel Filtration Studies

Experiments were performed generally as described by Buonocore et al. (1980). Inhibitor-α-amylase II binding was studied using gel filtration on a column (1.5 x 60 cm) of Bio-Gel P100 (100-200 mesh) equilibrated with 10 mM Pipes-

NaOH (1 mM $CaCl_2$, pH 7.0). All protein samples applied to the column were pre-equilibrated in the same buffer as the column. Mixtures of inhibitor and α-amylase II, 2 ml total, were applied to the bottom of the column and eluted with an upward flow rate of 10 ml/hour. Molar ratio mixtures of 1:1, 8.89×10^{-3} μmol of each protein, and 5:1, 6.44×10^{-2} μmol of inhibitor with 1.29×10^{-2} μmol of α-amylase II, were pre-incubated for 15 minutes before being applied to the column. Inhibitor (6.73×10^{-2} μmol) and α-amylase II (1.56×10^{-2} μmol) were also applied individually to the column to determine elution yields. Fractions of 1.7 ml were collected and tested for absorbance at 280 nm. Peaks of absorbance were pooled and concentrated by pressure ultrafiltration through YM5 Amicon membranes. Protein content was determined by the method of Lowry et al. (1951) and the identity of each peak was confirmed by SDS-PAGE using the Laemmli (1970) system.

3.8. Spectrophotometric Techniques

3.8.1. Modification of the Inhibitor

Irreversible oxidation of specific tryptophan residues of the inhibitor or α-amylase II with N-bromosuccinimide (NBS) was performed as described by Spande and Witkop (1967a, 1967b). Inhibitor or enzyme (40-55 μM) were equilibrated in either 50 mM sodium acetate (1mM $CaCl_2$, pH 5.0) or 8.0 M urea (1mM $CaCl_2$, pH 4.0 with glacial acetic acid) and

filtered through 0.45 μm nylon filters prior to titration with 10 mM NBS in the same buffer. The titration was monitored by recording the decrease in absorbance at 280 nm, A_{280}, corrected for dilution, using a Zeiss PM QII spectrophotometer. Titrations were performed directly in quartz spectrophotometer cuvettes. When decreases in A_{280} ceased, NBS addition was stopped and an aliquot of N-acetyl-L-tryptophan ethyl ester was added to use up any unreacted NBS (Clarke and Svensson, 1984a).

The number of tryptophan residues modified per molecule of inhibitor or amylase was calculated using the equation described by Spande and Witkop (1967a):

$$n = \frac{\Delta A_{280} \times 1.31 \times M.W.}{A_{280} \times a.f. \times 5500} \qquad (3)$$

Where the factors represented are, n=number of oxidized tryptophan residues per mole of protein; A_{280}= initial absorbance of the protein sample at 280 nm; ΔA_{280}= corrected absorbance decrease at 280 nm; 1.31=an empirical factor which corrects for the decline in absorbance at 280 nm of the oxidized tryptophan chromophore, oxindole, from that of the native tryptophan chromophore, indole; M.W.=molecular weight of the protein being tested; a.f = absorptivity factor converting A_{280} to mg/ml of protein; 5500 = molar extinction coefficient at 280 nm for tryptophan. Absorptivity factors for inhibitor and *a*-amylase II were calculated by dividing known protein concentration in mg/ml,

as determined by the method of Lowry et al. (1951), by absorbance at 280 nm of the same samples.

In a related study, the anti-amylolytic activities of equal amounts of inhibitor and oxidized inhibitor were measured. Native inhibitor, inhibitor modified by NBS oxidation in acetate buffer, inhibitor oxidized in urea solution and α-amylase II were each equilibrated in 10 mM Pipes (1 mM $CaCl_2$, pH 7.0) prior to assay. Enzyme (3.10 x 10^{-3} μmol) and inhibitor samples (1.16 x 10^{-2} μmol) were pre-incubated for 15 minutes at 35°C and subsequent substrate digestion was allowed to proceed for 15 minutes.

3.8.2. Ultraviolet Difference Absorption Spectroscopy

The absorption of ultraviolet (UV) light (260 nm-320 nm) by BASI and α-amylase II and the relative contributions of their aromatic side chains (tryptophan, tyrosine and phenylalanine) to this absorption were studied under various conditions by the technique of difference spectroscopy (Herskovits and Laskowski, 1960; Herskovits, 1967). Changes induced in the UV spectra of the proteins by the addition of perturbing agents, were detected using a "double-blanking" procedure which involved the use of dual-compartment optical cells. "Double-blanking" was attained by adding equal volumes of perturbant to the protein or "test" chamber of the sample cell and to the buffer or "blank" chamber of the

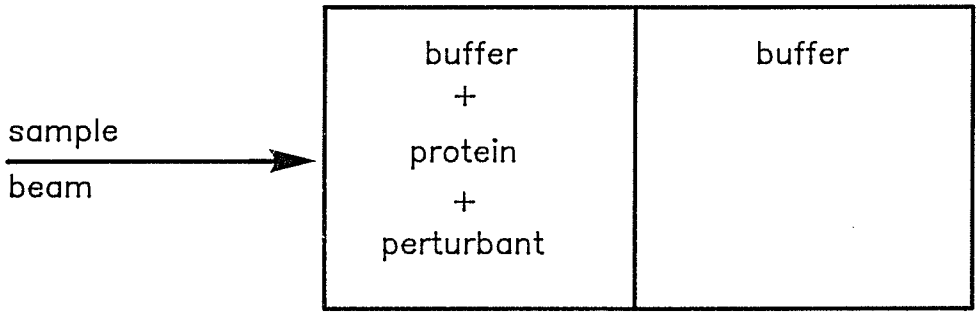
reference cell. This arrangement is represented schematically in Figure 1. All spectra, 260 nm to 320 nm, were measured on a Cary Model 15 recording spectrophotometer at 20 - 23°C using 20 mm pathlength double-chamber quartz cuvettes. All protein samples were filtered through 0.45 μm nylon filters prior to use.

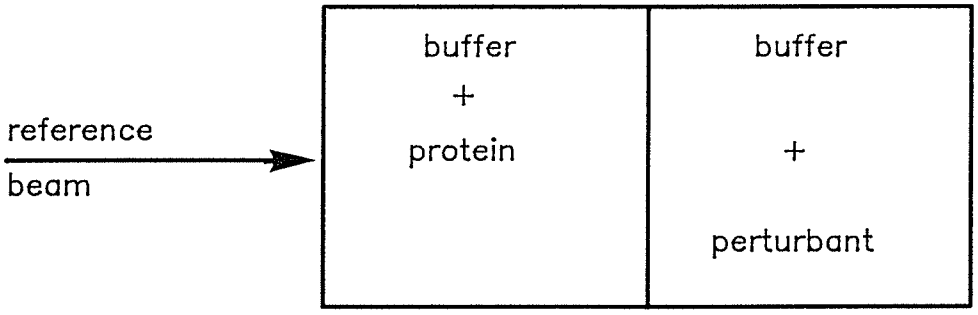
3.8.2.1. Solvent Perturbation of α-Amylase II

Aqueous α-amylase II samples were perturbed by the addition of the solvents dimethylsulfoxide (DMSO) or ethylene glycol. These perturbants were chosen because they are inert with respect to causing conformational changes in protein structure and are known to produce measurable spectral shifts in accessible tryptophanyl side chains by decreasing the polarity of the aqueous milieu (Herskovits, 1967; Ohnishi, 1971).

Aqueous α-amylase II samples were equilibrated in 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) and passed through 0.45 μm nylon filters prior to spectroscopy. A volume (1.5 mls) of α-amylase (11.5 μM) was added to the front "test" chambers of the sample and reference cuvettes. An equal volume of 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) was added to each cuvette's back chamber and a baseline spectrum was measured. Difference spectra were induced by adding 10 to 50 μL of either perturbant to the "test" chamber of the sample cell

Figure 1. Schematic representation of the arrangement of the double chamber cuvettes used in difference spectroscopy.


sample
beam
buffer
+
protein
+
perturbant
buffer


reference
beam
buffer
+
protein
buffer
+
perturbant

and the "blank" chamber of the reference cell. An equal volume of buffer was added to the "test" chamber of the reference cell. New spectra were recorded 3 minutes later. Scans were repeated three times at a rate of 15 nm/min using a full scale absorbance of 0.1 units. Addition of perturbants continued until their concentration reached 10-20%.

In order to estimate the number of solvent accessible tryptophan side chains on *a*-amylase II, the intensities of changes in the difference spectra of the enzyme were compared to those induced with equal concentrations of perturbant on the model compound, N-acetyl tryptophan ethyl ester (Ac-Trp-OEt). The free amino acid model was chosen as a 100% exposed reference because it exhibits maximum spectral changes at the same wavelengths (290-293 nm) as tryptophan residues in polypeptide chains (Herskovits and Sorensen, 1968). Aqueous solutions of Ac-Trp-OEt (300 μM) in 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) were perturbed with DMSO or ethylene glycol as described for *a*-amylase II.

3.8.2.2. Inhibitor Induced *a*-Amylase Difference Spectra, pH Effects

Inhibitor/*a*-amylase II binding properties were studied by difference spectroscopy as described by Herskovits (1967) and Clarke and Svensson (1984a). Difference spectra of *a*-

amylase II (9.5-12.9 μM) were induced with inhibitor (59-70 μM) in 10 mM Pipes (1 mM $CaCl_2$, pH 7.0) or with inhibitor in 20 mM sodium acetate (1 mM $CaCl_2$, pH 5.5). The front chambers of the sample and reference cuvettes were loaded with 500 μL of α-amylase II sample. An equal volume of appropriate buffer was added to the back chamber of each cell. The baseline spectrum produced was used to correct subsequent difference spectra. To induce difference spectra, inhibitor (20-40 μL) was added to the front chamber of the sample cell, which contained enzyme, and to the back chamber of the reference cell. Similarly, an equal volume of buffer was added to the front chamber of the reference cell. After a 5 minute incubation, spectra were recorded three times for each addition at a scan rate of 15 nm/min using a full-scale absorbance of 0.1 units. Additions were continued until no further spectral perturbation, corrected for dilution, was evident.

3.8.2.3. Inhibitor Induced α-Amylase Difference Spectra, Salt Effects

The effect of increasing NaCl concentration on inhibitor/α-amylase II binding in 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) was examined. Aliquots of 2.0 M NaCl were added to sample chambers containing α-amylase II that was maximally perturbed by inhibitor and to the corresponding reference samples. After 3 minutes, three spectra were recorded for each addition and corrected for dilution.

3.8.3. Fluorescence Titration

Estimation of the number of exposed tryptophanyl side chains and binding of inhibitor with *a*-amylase II were investigated further using fluorescence titration (Brand and Witholt, 1967; Clarke and Svensson, 1984a). Fluorescence spectra of *a*-amylase II were recorded with a Perkin-Elmer LS-5 fluorescence spectrophotometer. The enzyme, in either 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) or 20 mM sodium acetate (1 mM $CaCl_2$, pH 5.5) shows emission and excitation maxima at 336 and 280 nm respectively. Therefore, changes in the enzyme's fluorescence emission at 336 nm, with excitation at 280 nm, caused by solvent perturbation or by titration with inhibitor were measured.

3.8.3.1. Solvent Perturbation of *a*-Amylase II

Purified enzyme was equilibrated in either 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) or 20 mM sodium acetate (1 mM $CaCl_2$, pH 5.5) prior to fluorescence titration with either dimethylsulfoxide or ethylene glycol. A sample of *a*-amylase (2.0 mls, 0.4-0.9 μM) was placed in a quartz cuvette and an equal volume of appropriate buffer was placed in a second, reference, cell. Small volumes (20-100 μL) of perturbant were added to both sample and reference cells. After 3 minutes the fluorescent intensity of the enzyme, corrected for dilution, was recorded using the reference cell as a

zero. Addition of perturbants continued until their concentration reached 10 to 20%. Fluorescence changes were expressed as the percentage difference in fluorescence intensity (ΔF%) relative to that of the original enzyme solution, i.e.:

$$\Delta F\% = \frac{Fi - Ff}{Fi} \times 100\% \qquad (4)$$

where Fi and Ff are initial and final fluorescence intensities respectively (Gibson and Svensson, 1986).

The number of exposed tryptophan residues on α-amylase II was estimated at both pH 5.5 and 7.0. The ΔF% values obtained for the enzyme were compared to those seen for N-acetyl tryptophan ethyl ester at both pH's from both perturbants. Aqueous solutions of Ac-Trp-OEt (10-20 μM) were perturbed with DMSO or ethylene glycol as described for α-amylase II.

3.8.3.2. Titration of α-Amylase with Inhibitor

Amylase and inhibitor were equilibrated in 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) and filtered through 0.45 μm nylon filters prior to use. To start, the sample cell contained 2 ml of α-amylase II (0.2-0.5 μM) and the reference cell held 2 ml of buffer. Emission of the enzyme was measured using the reference cell as a zero. Aliquots (15-40 μL) of inhibitor (2.0-3.5 μM) were added to both sample and

reference cells. After 5 minutes, fluorescence emission of the sample, corrected for dilution, was recorded six times using the reference cell containing inhibitor as zero emission. Samples were titrated until no further quenching of emission occurred. Fluorescence changes were expressed as percent difference relative to free enzyme ($\Delta F\%$).

4. RESULTS AND DISCUSSION

4.1. Protein Purification and Estimation

The purification scheme for *a*-amylase II afforded the recovery of 39 mgs of protein from 500 grams of ground 4-day germinated barley (Table 3). The protein recovered was purified about 109-fold and represented about 25% of the original amylolytic activity in the crude extract. In preliminary purification attempts much of the *a*-amylase fractionated from the affinity column tended to precipitate. This was probably due to the high concentration of enzyme and the presence of cycloheptaamylose (CHA) in the active fractions eluted. For this reason the affinity column was connected in sequence with the ion exchange (CMC) column just prior to the elution of *a*-amylase with CHA. The affinity ligand could be separated from *a*-amylase II on the ion exchange column by extensive washing with column equilibration buffer. In addition, purified *a*-amylase II eluted in a slightly larger volume from this column than from the affinity column. Consequently, no precipitation problems were incurred using this purification scheme.

From 400 grams of mature barley meal, 5379 mgs of crude protein were extracted (Table 4). The final yield of inhibitor (6 mgs) indicates that it must have comprised at least 0.1% of the soluble proteins in the crude extract. This value is of the same order of magnitude previously

TABLE 3

Purification of ∝-amylase II from germinated barley

Fraction	Volume (ml)	Total Protein (mg)	Total Activity (IDC units x 10^{-6})	Specific Activity (units/mg)	Purification (fold)	Recovery (%)
crude extract	641	17,218	61.8	3589	1.0	100
heat treatment	600	10,105	40.6	4021	1.1	66
dialysis	745	4,781	36.5	7630	2.1	59
carboxymethyl cellulose peaks	29	39	15.3	392,257	109.3	25

TABLE 4

Purification of the ∝-amylase/subtilisin inhibitor from barley

Fraction	Volume (ml)	Total Protein (mg)	Total Activity (anti-amylase units, x 10^{-5})	Specific Activity (anti-units/mg)	Purification (fold)	Recovery (%)
crude extract	1,630	5,379	20.9	390	1.0	100
40-70% ammonium sulfate cut, after dialysis	151	966	7.7	800	2.1	37
DEAE-Sephacel concentrate	15	30	5.1	16,833	43.2	24
Bio-Gel P30 concentrate	10	6	3.1	51,666	132.5	15

reported for inhibitor content in mature cereal grain (Munck et al., 1985). The yields and specific activities of both protein were similar to those obtained in previously reported purifications (Weselake and Hill, 1982; Weselake et al., 1983b).

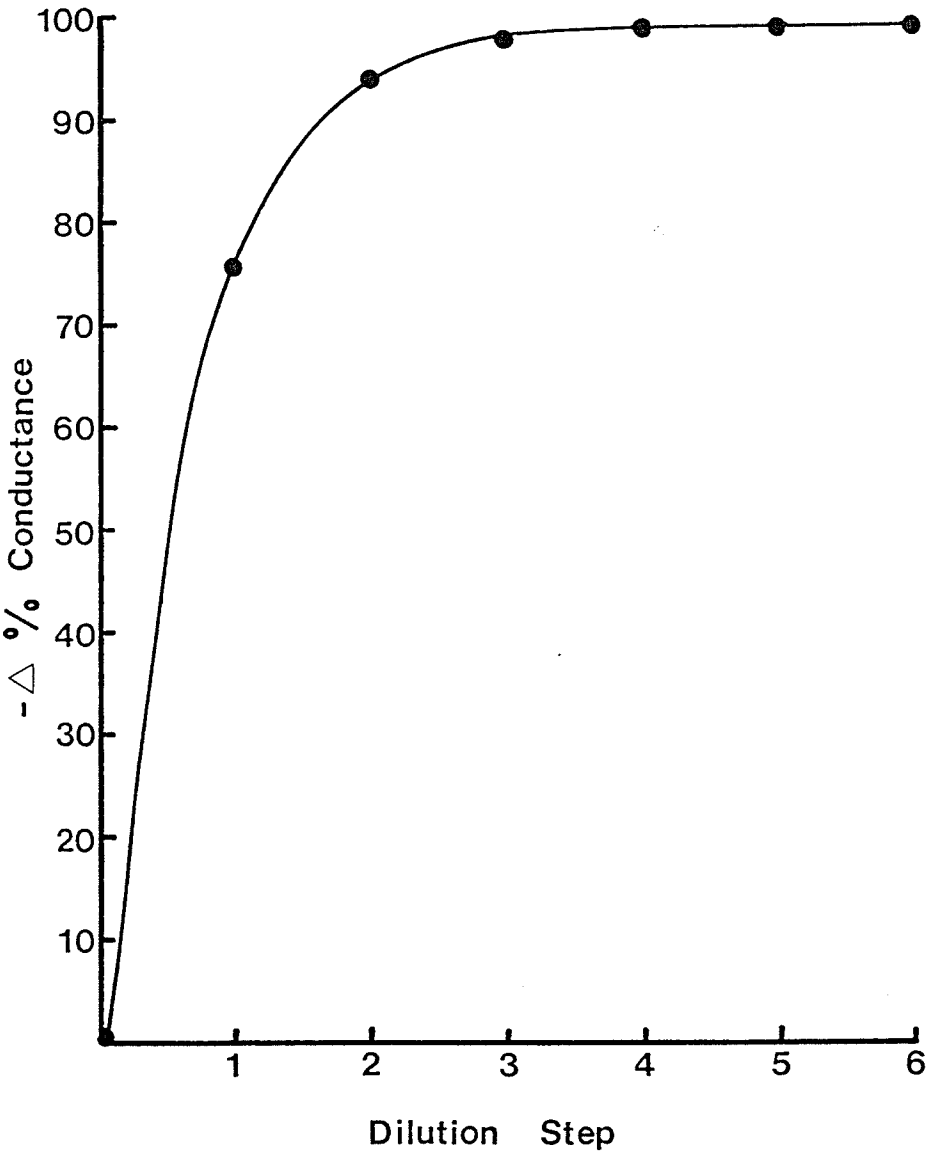
The mean factors calculated for extinction coefficients, to convert absorbance at 280 nm to molar concentrations of purified protein were $1.16 \times 10^5 M^{-1}cm^{-1}$ for α-amylase II and $2.27 \times 10^4 M^{-1}cm^{-1}$ for the inhibitor. Both values are for absorbance in 1,4-piperazine-diethanesulfonic acid (Pipes) buffer and have standard errors of less than 3.5%. These values were of invaluable use for fast and accurate protein determinations of samples being used in spectrophotometric studies.

4.2. Sample Equilibration

Utilization of a 10 ml Amicon pressure ultrafiltration cell as a rapid equilibration chamber for sample preparation was efficient and effective. A single concentration-dilution step of 10 mls of 10 mM Pipes buffer, conductivity 1020 μmho, with water, 1.5 μmho, resulted in a 76% reduction in conductance of the buffer (Figure 2). The time required to complete one step was approximately 15-20 minutes depending on the pressure used. By completion of the fourth dilution step conductance of the buffer was 3.1 μmho, corresponding to a 99.7% equilibration to water (Figure 2).

Figure 2. Rapid dialysis of Pipes buffer with water by pressure ultrafiltration.

Starting buffer was 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0), conductance = 1020 μmho's. Water conductance = 1.5 μmho. 1 dilution step = concentrating of 10 mls of buffer to 1 ml and diluting back to 10 mls with water.


100
90
80
70
60
50
40
30
20
10
-△ % Conductance
1
2
3
4
5
6
Dilution Step

Subsequent steps failed to increase equilibration significantly. Hence, four dilution steps were utilized in equilibrating protein samples for use in other studies.

4.3. Effect of pH on Inhibitor Activity

The anti-amylolytic activity of the inhibitor appears to be optimal from pH 6.5-7.0 for starch granule substrate (Weselake et al., 1985b). A study of inhibitor activity from pH 5.5 to 7.5 was undertaken to approximate the pH at which inhibition of soluble starch hydrolysis is maximized (Figure 3). Anti-activity of inhibitor at each pH was calculated against the hydrolytic activity of α-amylase II at the same pH. Maximum inhibition of soluble starch hydrolysis was seen at pH 7.0 where 89% of α-amylase II activity was inhibited (Figure 3). In accordance with previous reports, the maximum α-amylase II activity was seen at pH 5.5 (MacGregor et al., 1974). The ionic strengths of the sodium acetate buffer used at pH 5.5 and the Pipes buffering medium employed for pH's from 6.1 to 7.5 were quite similar (Table 5). The Pipes system was chosen because of its buffering range (pH 5.8-7.8) and because it has a negligible calcium binding constant (Good et al., 1966).

Figure 3. Effect of pH on the anti-amylolytic activity of the inhibitor

Conditions:
20 mM sodium acetate (1 mM $CaCl_2$) was used at pH 5.5, 10 mM Pipes-NaOH (1 mM $CaCl_2$) was used at all other pH's. In each test tube 3.39×10^{-3} μmol α-amylase II, 1.04×10^{-2} μmol inhibitor and 0.025% soluble β-limit dextrin as substrate. Reaction times were 15 minutes at 35°C.

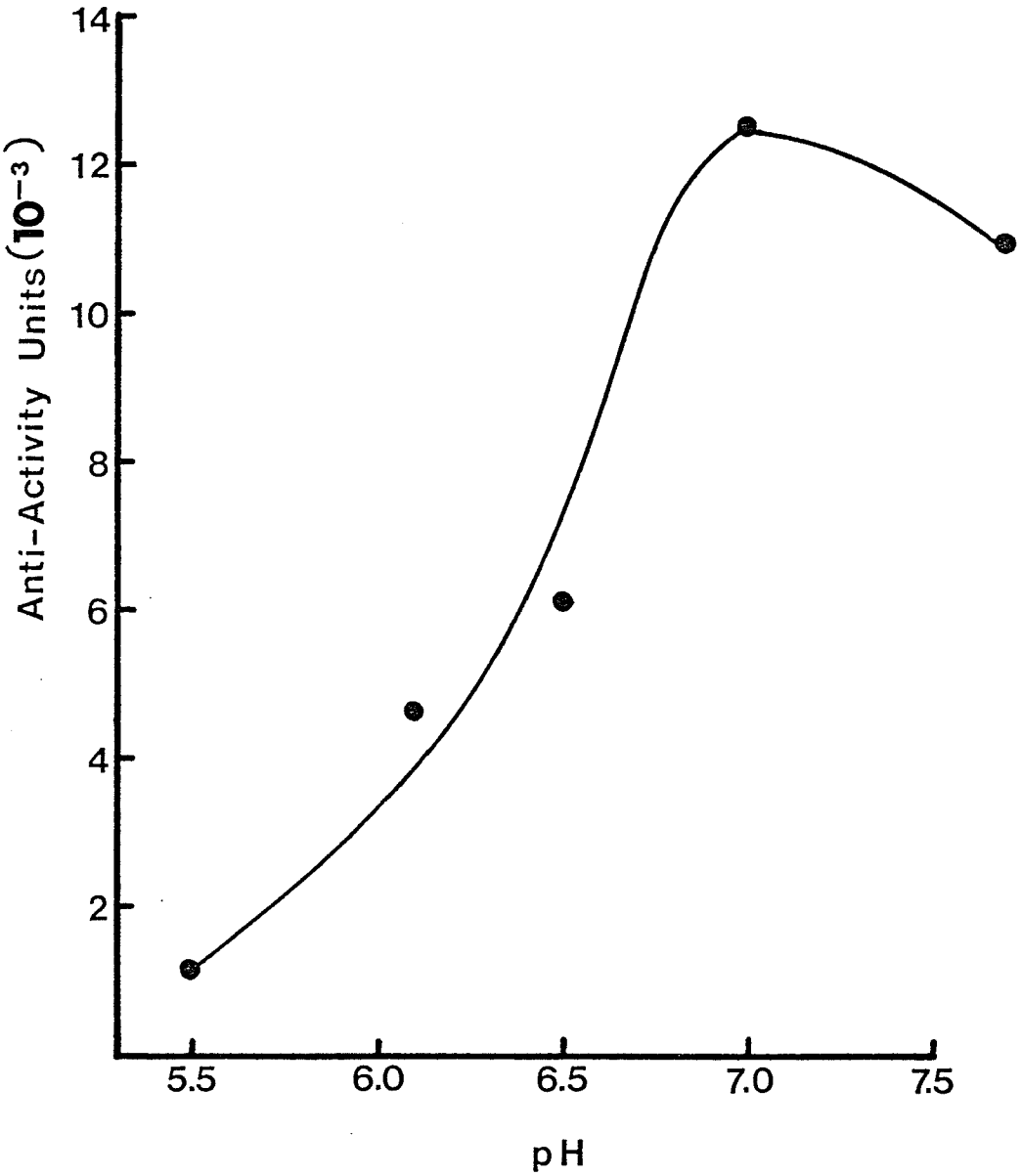

14
12
10
8
6
4
2
Anti-Activity Units (10^{-3})
5.5
6.0
6.5
7.0
7.5
pH

TABLE 5

Conductivity measurements of the buffer systems employed in estimating pH effect on inhibitor activity

pH	Buffering medium[1]	Conductance (μmho)
5.5	20 mM sodium acetate	1100
6.1	10 mM Pipes-NaOH	720
6.5	10 mM Pipes-NaOH	850
7.0	10 mM Pipes-NaOH	1020
7.5	10 mM Pipes-NaOH	1140

[1] All media contained 1 mM $CaCl_2$

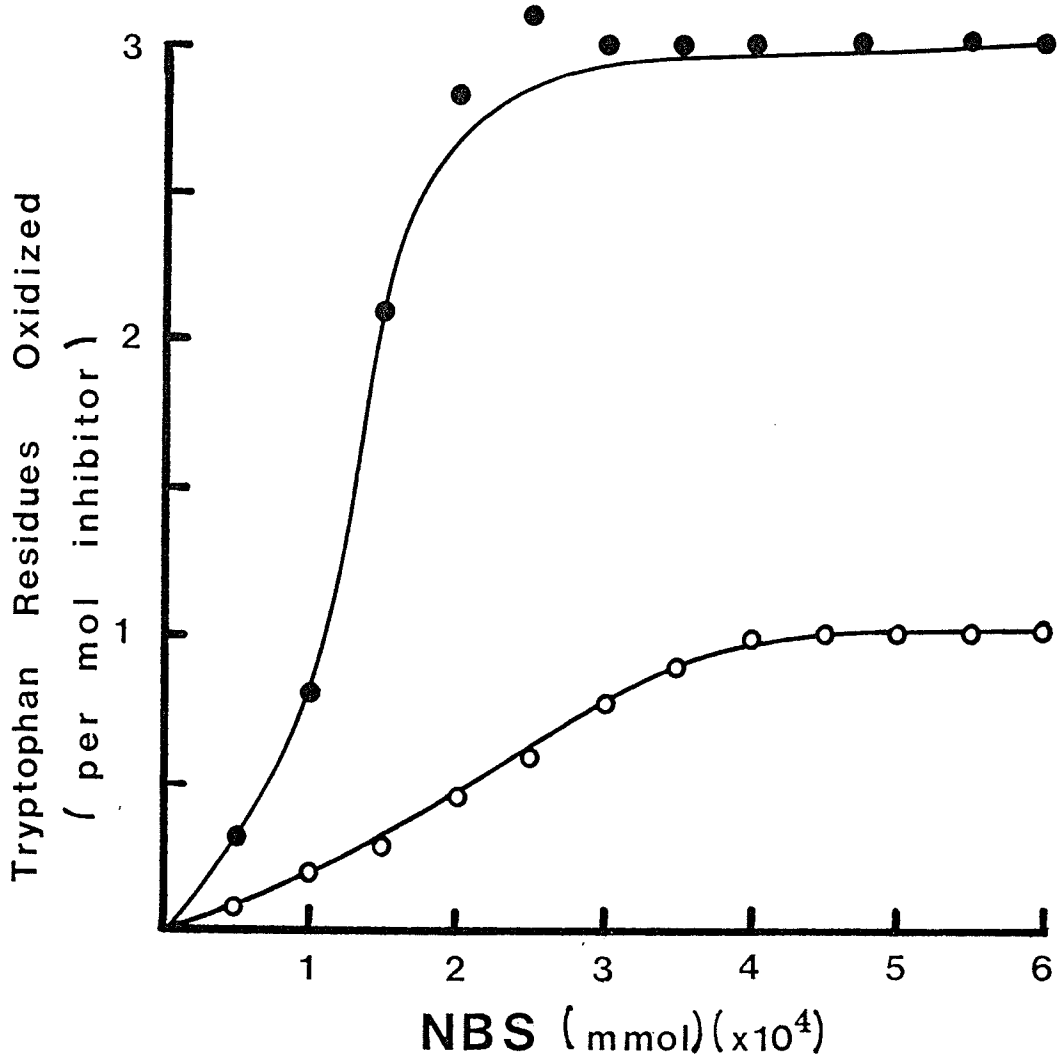
4.4. Modification of Inhibitor and α-Amylase II

The numbers of inhibitor tryptophan residues irreversibly oxidized by N-bromosuccinimide (NBS) in 50 mM sodium acetate (1 mM $CaCl_2$, pH 5.0) and in 8.0 M urea (1 mM $CaCl_2$, pH 4.0) are demonstrated in Figure 4. In the presence of urea the inhibitor is unfolded, making all three of its tryptophan residues susceptible to oxidation. However, only one of these residues is modified when the inhibitor is in its native conformation in acetate buffer. This is indicative of two inhibitor tryptophan side-chains being "buried", making them inaccessible to NBS, while one is accessible or "exposed" in the folded state (Spande and Witkop, 1967b). The molar consumption of NBS in oxidizing each mole of tryptophan from the inhibitor was 3.3 and 3.4 in the acetate and urea media respectively. This relatively low consumption of the oxidizing agent normally indicates that only tryptophan residues were modified, as oxidation of other residues would have revealed a much higher consumption of NBS by the inhibitor (Spande and Witkop, 1967a).

Oxidation of α-amylase II at pH 5.0 was inconclusive as it was marred by a rapid precipitation of protein after one tryptophan residue had been modified. This result has also been recorded elsewhere (Gibson and Svensson, 1986). Based on a prior study (Okada et al., 1963), reactions were also attempted at pH 6.0 at room temperature, but no success was achieved. Gibson and Svensson (1986) have been able to

Figure 4. Oxidation of inhibitor with N-bromosuccinimide

Conditions:
Buffer = 50 mM sodium acetate (1 mM $CaCl_2$, pH 5.0) (O) or 8.0 M urea (1 mM $CaCl_2$, pH 4.0) (●).
Inhibitor concentration = 5.4×10^{-5} M.
Aliquots (5 or 10 μL) of 10 mM NBS were added to 1 ml of inhibitor, samples stirred for 2 minutes and absorbance measured at 280 nm before subsequent additions.


Tryptophan Residues Oxidized
(per mol inhibitor)
3
2
1
1
2
3
4
5
6
NBS (mmol)(x10⁴)

reduce the amount of protein precipitation that occurs with barley α-amylase II oxidation by carrying the reactions out at 4°C in 50 mM 2(N-morpholino)ethanesulphonic acid (MES), pH 6.7, half saturated in $CaSO_4$. However, the enzyme was still prone to precipitation at higher NBS concentrations, so this procedure was not attempted.

Inhibitor, partially oxidized inhibitor and completely oxidized inhibitor were re-equilibrated in Pipes buffer and their anti-amylolytic activities at pH 7.0 were determined (Table 6). Modification of the inhibitor's "exposed" tryptophan residue with NBS does not appear to reduce its activity. It has been shown that α-amylase denatured by urea can be reactivated by removing the denaturing agent (Imanishi et al., 1963). However, complete loss of activity was seen for inhibitor which had all three of its tryptophan side-chains oxidized under denaturing conditions. Apparently the one exposed inhibitor tryptophan side-chain is not crucial to the protein's ability to inhibit α-amylase II activity.

TABLE 6

Anti-amylolytic activity of inhibitor and oxidized inhibitor

Activity measured at pH 7.0 (10 mM Pipes-NaOH, 1 mM $CaCl_2$). Results represent means ± SE of five replications.

Inhibitor sample	Relative anti-activity units ($\times 10^{-3}$)[1]
Native	7.7 ± 1.0
Oxidized[2]	7.6 ± 1.1
Oxidized[3]	0.0 ± 0.0

[1] Determined using Briggs (1961) assay as modified by Weselake et al (1983b).
[2] Inhibitor oxidized in 50 mM sodium acetate (1 mM $CaCl_2$, pH 5.5).
[3] Inhibitor oxidized in 8.0 M urea (1 mM $CaCl_2$, pH 4.0).

4.5. Characterization of *a*-Amylase II Tryptophan Residues

The tendency of *a*-amylase II to precipitate during oxidation reactions with NBS was high. Therefore, ultraviolet (UV) and fluorescence solvent perturbation spectrophotometric techniques were employed to determine what fraction of the 16 enzyme tryptophan side chains are exposed.

4.5.1. Ultraviolet Difference Spectroscopy Solvent Perturbation

The difference spectra of *a*-amylase II produced by DMSO and ethylene glycol at pH 7.0 each showed a major peak at 293 nm, a smaller peak around 284 nm and a shoulder between 274 and 279 nm (Figure 5a). This is very characteristic of the perturbed spectra of tryptophan residues in polypeptides (Herskovits, 1967). The model tryptophan compound, N-acetyl tryptophan ethyl ester (Ac-Trp-OEt), showed this same characteristic difference spectrum when exposed to the same perturbants (Figure 5b). One will notice that the wavelength maxima for perturbed *a*-amylase tryptophan residues are approximately 3 to 4 nm higher than the corresponding peaks for the model compound (Figure 5). This phenomenon is not uncommon as incorporation of chromophoric residues into polypeptide chains is known to produce a slight shift in the difference spectra toward longer wavelengths (Wetlaufer, 1962; Donovan, 1964).

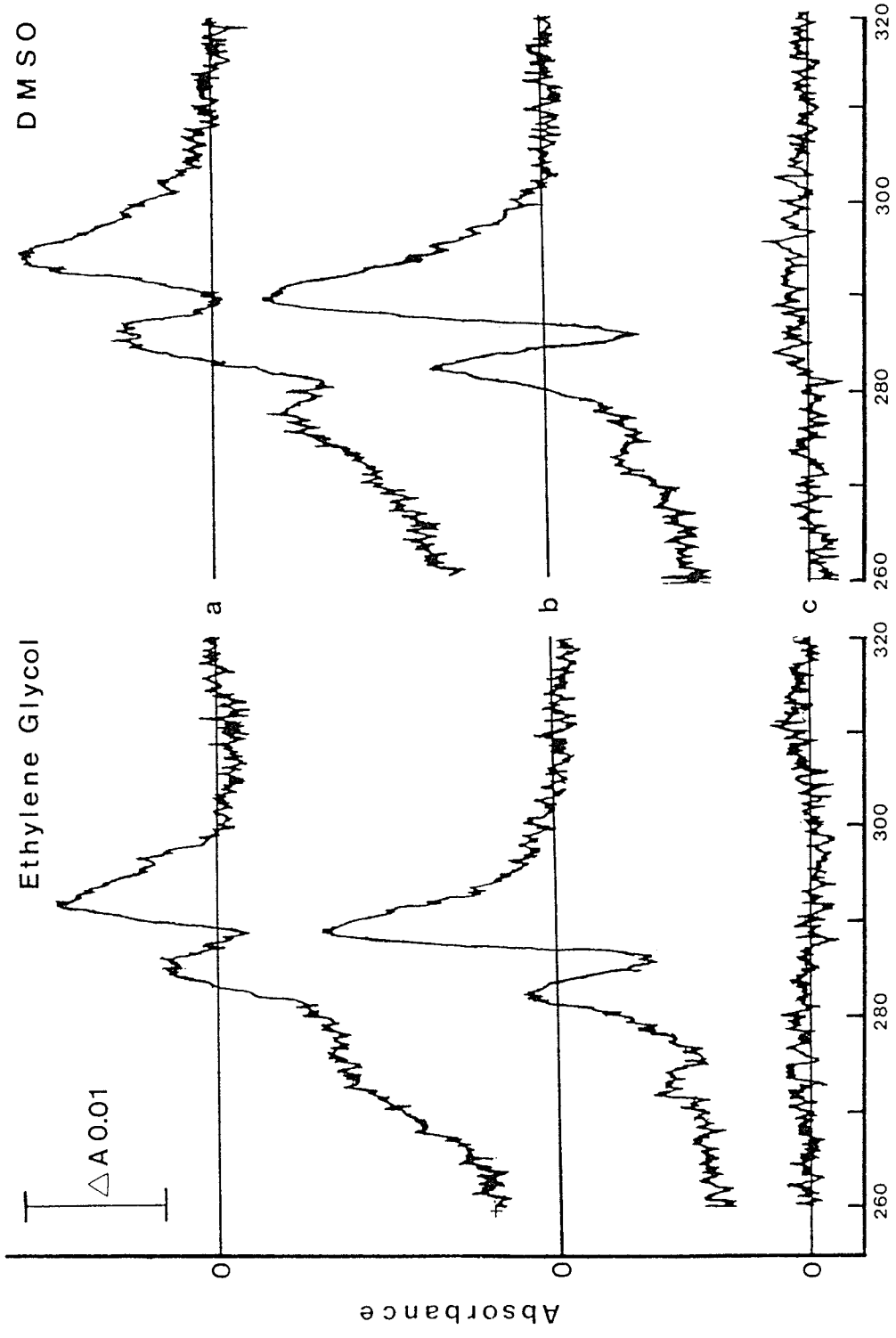
Figure 5. Solvent perturbation difference spectra of α-amylase II and N-acetyl tryptophan ethyl ester.

a. α-amylase II; concentration = 11.5 μM; spectra recorded for 10% v/v of ethylene glycol or DMSO

b. N-acetyl tryptophan ethyl ester; concentration = 300 μM; spectra recorded for 10% v/v of ethylene glycol

c. baseline spectrum for α-amylase II recorded prior to addition of perturbants

Conditions:
buffer = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0); temperature = 20-23°C; scan rate = 15 nm/minute; full scale absorbance = 0.1 units.


Ethylene Glycol
DMSO
△A 0.01
a
b
c
0
0
0
Absorbance
260
280
300
320
260
280
300
320
Wavelength (nm)

The magnitude of the major characteristic peak of the *a*-amylase II tryptophan difference spectra at 293 nm increases linearly as perturbant concentration is raised up to 20% (v/v) (Figure 6). The same holds true at 290 nm for the model tryptophan compound, Ac-Trp-OEt (Figure 6). The slope of the plot of molar absorptivity difference, $\Delta\epsilon$, at 293 nm for the enzyme or at 290 nm for the model, against perturbant concentration is characteristic of the total contribution of all tryptophan residues accessible to solvent (Herskovitz, 1967). In the case of the model compound, it is a single tryptophan residue and it is 100% exposed to solvent. Therefore, the ratio of the slope of the plot for *a*-amylase II to that for Ac-Trp-OEt gives the number of enzyme tryptophan residues accessible for solvent (Herskovits and Sorensen, 1968b; Ohnishi, 1971). Plots of the effect of DMSO or ethylene glycol on the difference spectra maxima of *a*-amylase and Ac-Trp-OEt at pH 7.0 are shown in Figure 6. The respective slopes and ratios of same are summarized in Table 7. The UV solvent perturbation data indicate that the number of *a*-amylase II tryptophan residues accessible to ethylene glycol or DMSO is 8 to 9. The fact that both perturbants give the same numbers is not surprising as their mean molecular diameters are nearly the same, 4.3 and 4.0 respectively (Herskovits, 1967).

Figure 6. Effect of perturbant concentration on the difference molar absorptivity of *a*-amylase II and Ac-Trp-OEt at pH 7.0.

A. Effect of increasing ethylene glycol concentration.

B. Effect of increasing DMSO concentration.
● = Ac-Trp-OEt
O = *a*-amylase II

Conditions:
a-amylase concentration = 11.5 μM;
Ac-Trp-OEt concentration = 300 μM;
buffer = 10 mM Pipes-NaOH (1 mM $CaCl_2$,
pH 7.0); temperature = 20-23°C.

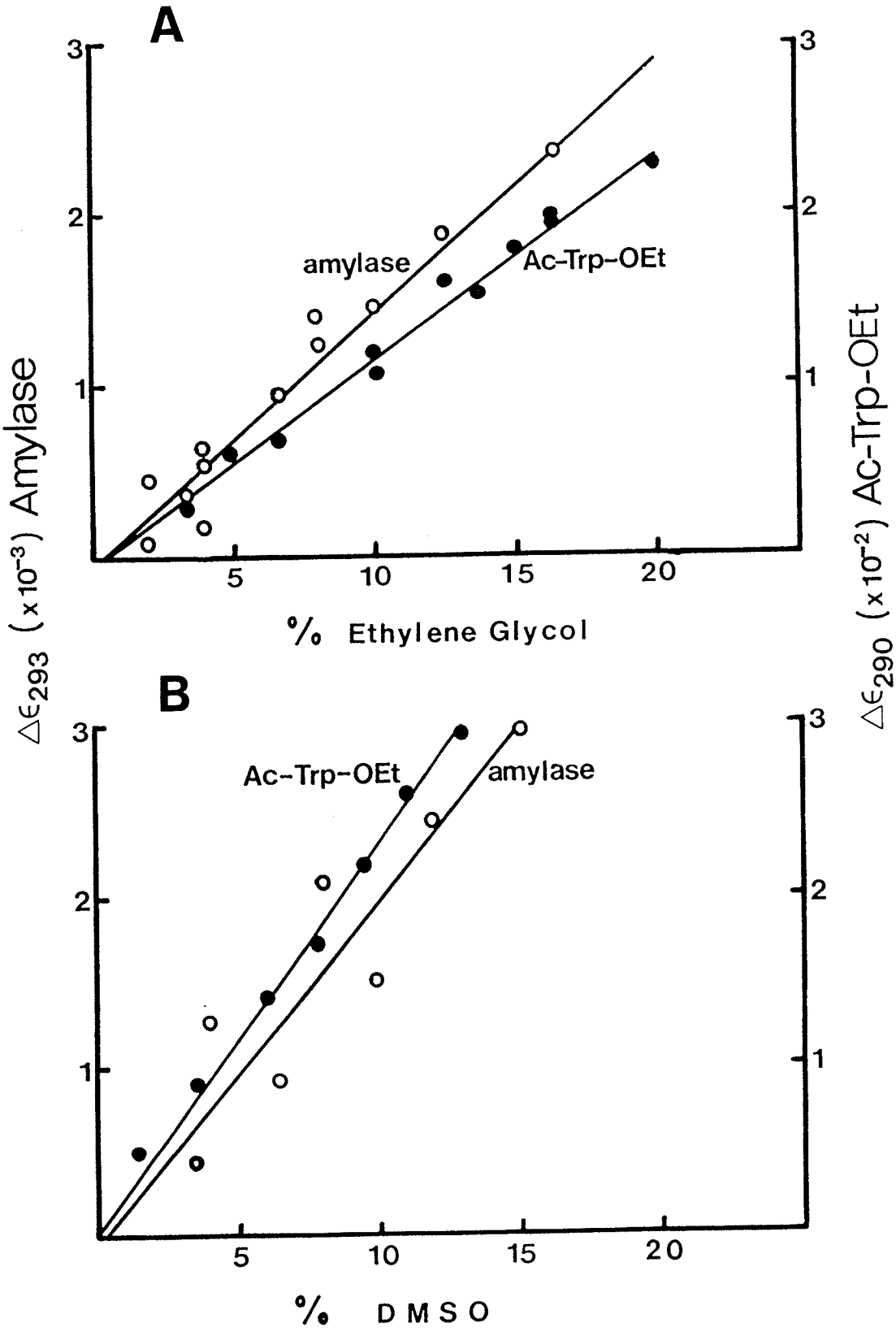

A
amylase
Ac-Trp-OEt
% Ethylene Glycol
$\Delta\epsilon_{293}$ ($\times 10^{-3}$) Amylase
$\Delta\epsilon_{290}$ ($\times 10^{-2}$) Ac-Trp-OEt
B
Ac-Trp-OEt
amylase
% DMSO

TABLE 7

The slope relationships between perturbant concentration and molar absorptivity difference or percent fluorescence emission difference for ∝-amylase II and Ac-Trp-OEt

Method of Analysis	pH	Perturbant	Slope for ∝-amylase II	Slope for Ac-Trp-OEt	Slope Ratio
U.V. Difference Spectroscopy	7.0	DMSO	198.3[a]	23.2[b]	8.5
	7.0	ethylene glycol	139.2[a]	14.8[b]	9.4
Fluorescence Titration	7.0	DMSO	2.21[c]	0.30[c]	7.4
	7.0	ethylene glycol	2.02[c]	0.35[c]	5.8
	5.5	DMSO	2.10[c]	0.30[c]	7.0
	5.5	ethylene glycol	2.63[c]	0.35[c]	7.5

a. $\text{slope} = \frac{d\,\Delta\epsilon_{293}}{d\ \%P}$ b. $\text{slope} = \frac{d\ \Delta\epsilon_{290}}{d\ \%P}$ c. $\text{slope} = \frac{d\ \Delta F\%}{d\ \%P}$

where: $\Delta\epsilon_{293}$ = molar absorptivity difference for ∝-amylase; $\Delta\epsilon_{290}$ = molar absorptivity difference for Ac-Trp-OEt, $\Delta F\%$ = percent fluorescence emission difference at 330 nm, and %P = perturbant concentration.

4.5.2. Fluorescence Spectroscopy Solvent Perturbation

Fluorescence emission and excitation maxima for α-amylase II at pH 7.0 occurred at 336-340 nm and 280-282 nm respectively (Figure 7). These values correspond well with those previously reported for amylases from various sources (Buonocore et al, 1976b; Clarke and Svensson, 1984a; Gibson and Svensson, 1986). Coincidently, it is well established that the typical emission fluorescence maximum of tryptophan residues in protein is about 340 nm whereas tyrosine residues show an intensity maximum around 300 nm (Teale, 1960; Brand and Witholt, 1967). Fluorescence emission, also known as intensity, is sensitive to chemical reactions or solvent perturbations, and, thus many of the spectral effects detectable by difference spectroscopy can also be observed and measured by means of fluorescence measurements. Therefore, comparison of the changes in fluorescence emission at 336 nm of α-amylase to those for Ac-Trp-OEt during titration with either DMSO or ethylene glycol should reveal information regarding the number of exposed enzymatic tryptophan residues.

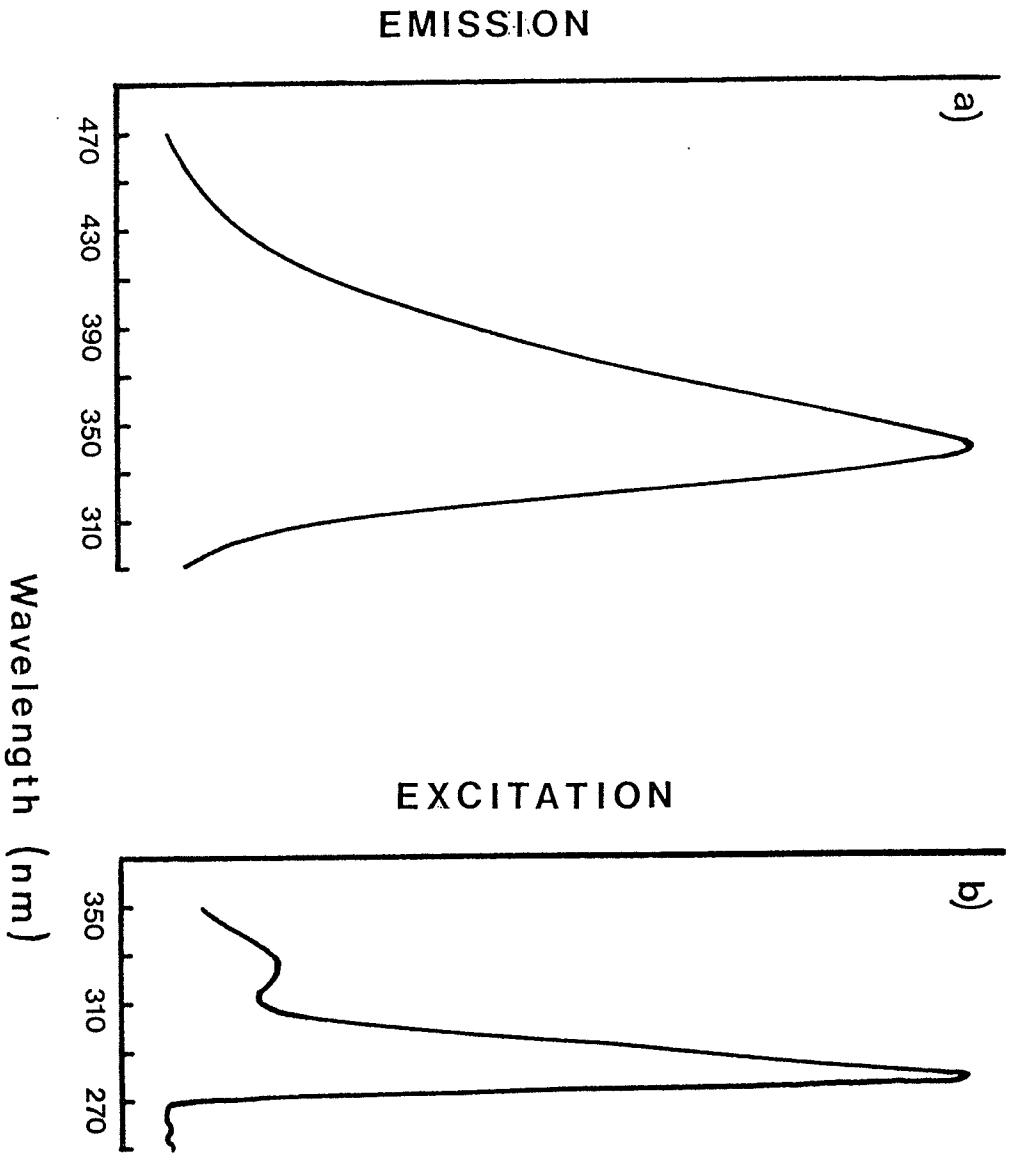
Titrations of α-amylase II at pH 7.0 with DMSO or ethylene glycol reduced the enzyme fluorescence intensity at 336 nm (Figure 8A). Similar titrations of amylase in pH 5.5 sodium acetate buffer produced extremely similar changes in emission spectra. Excitation of Ac-Trp-OEt with 280 nm

Figure 7. Emission and excitation fluorescence spectra of α-amylase II.

a) Fluorescence emission intensity recorded with excitation by while light as a function of emission wavelength.

b) Fluorescence emission intensity as a function of the wavelength of exciting light.

Arbitrary units are represented on the ordinate-axis. Enzyme concentration = 0.91 μM in 1.0 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0). Temperature = 20-23°C, Scan rate = 60 nm/min.


EMISSION
a)
470
430
390
350
310
Wavelength (nm)
EXCITATION
b)
350
310
270

wavelength UV-light effected emission maxima between 344 and 346 nm at pH 7.0 and 5.5 (Figure 8B). This is in agreement with the previously reported emission maxima for non-protein incorporated tryptophan residues of about 350 nm (Brand and Witholt, 1967). As was the case for α-amylase, the fluorescence intensity of Ac-Trp-OEt at its emission maximum was attenuated by titration with perturbants (Figure 8B).

Plots of percent decreases in fluorescence intensity against perturbant concentration showed a positive, linear relationship for both α-amylase II and Ac-Trp-OEt at pH 5.5 and 7.0 (Figures 9 and 10). Similar plots of molar absorptivity differences, $\Delta\epsilon$, for enzyme and model compound were used to estimate the number of solvent accessible enzymic tryptophans in difference spectrophotometric studies. In this case, the ratio of the slope of the plots of perturbant effect on fluorescence intensity for α-amylase II to that for Ac-Trp-OEt is indicative of the number of enzyme tryptophan residues accessible to solvent (Brand and Witholt, 1967; Herskovits and Sorensen, 1968b). These values are summarized in Table 7 along with the difference spectroscopy results.

Fluorescence measurements indicate that the number of exposed α-amylase II tryptophanyl residues is from 6 to 8 and that there is no difference in the solvent accessible numbers of these residues at pH 5.5 and 7.0 (Table 7). Estimates from difference spectroscopy measurements are up

Figure 8. Fluorescence emission spectra of α-amylase II and Ac-Trp-OEt with increasing concentrations of ethylene glycol or DMSO at pH 7.0.

A. Solvent perturbation emission spectra of α-amylase II.

B. Solvent perturbation emission spectra of Ac-Trp-OEt.

Enzyme and Ac-Trp-OEt pre-equilibrated with 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0).

a. spectra in equilibrating buffer
b. spectra induced with 5% ethylene glycol
c. spectra induced with 10% DMSO

Concentration, α-amylase = 0.45 μM; Ac-Trp-OEt = 6.1 μM; Temperature = 20-23°C.; Scan rate = 60 nm/min.

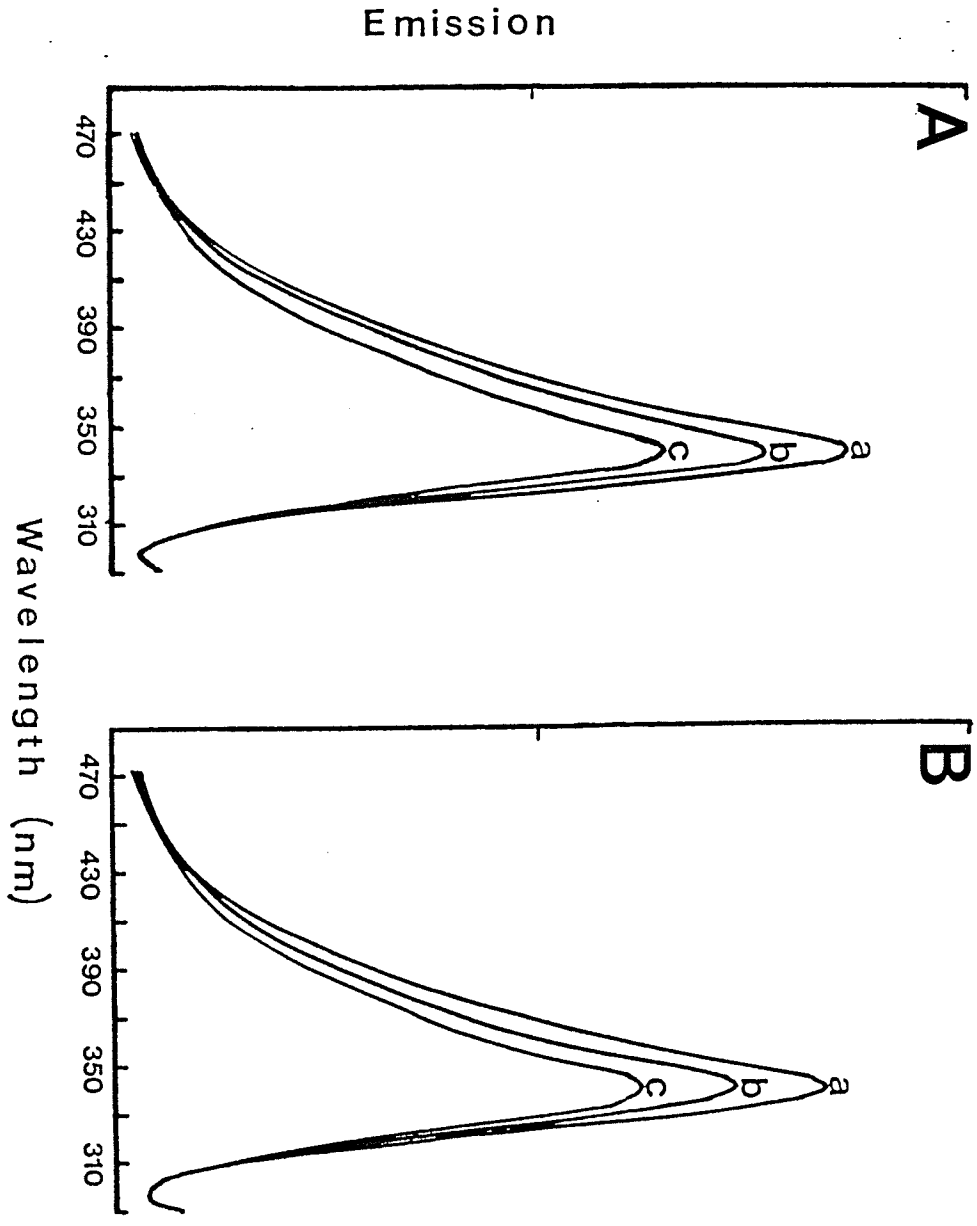

Emission
A
B
a
b
c
470
430
390
350
310
Wavelength (nm)

Figure 9: The effect of ethylene glycol concentration on the percent decrease in fluorescence emission ($-\Delta F\%$) at 336 nm in pH 5.5 or 7.0 media.

■ = α-amylase, pH 5.5
○ = α-amylase, pH 7.0
● = Ac-Trp-OEt, pH 7.0

Conditions:
Buffers = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) or 20 mM sodium acetate (1 mM $CaCl_2$, pH 5.5). Enzyme concentration = 0.52 μM, Ac-Trp-OEt = 10.51 μM. Temperature = 20-23°C, Scan rate = 60 nm/min.

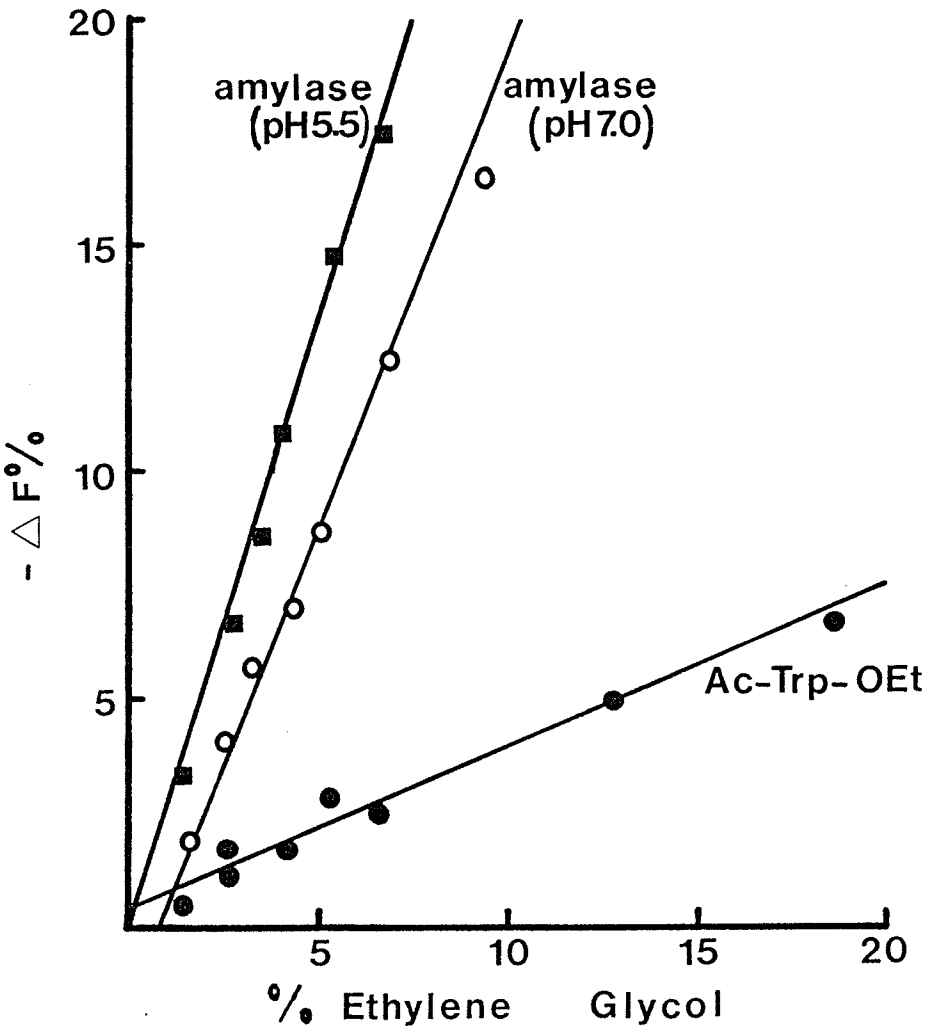
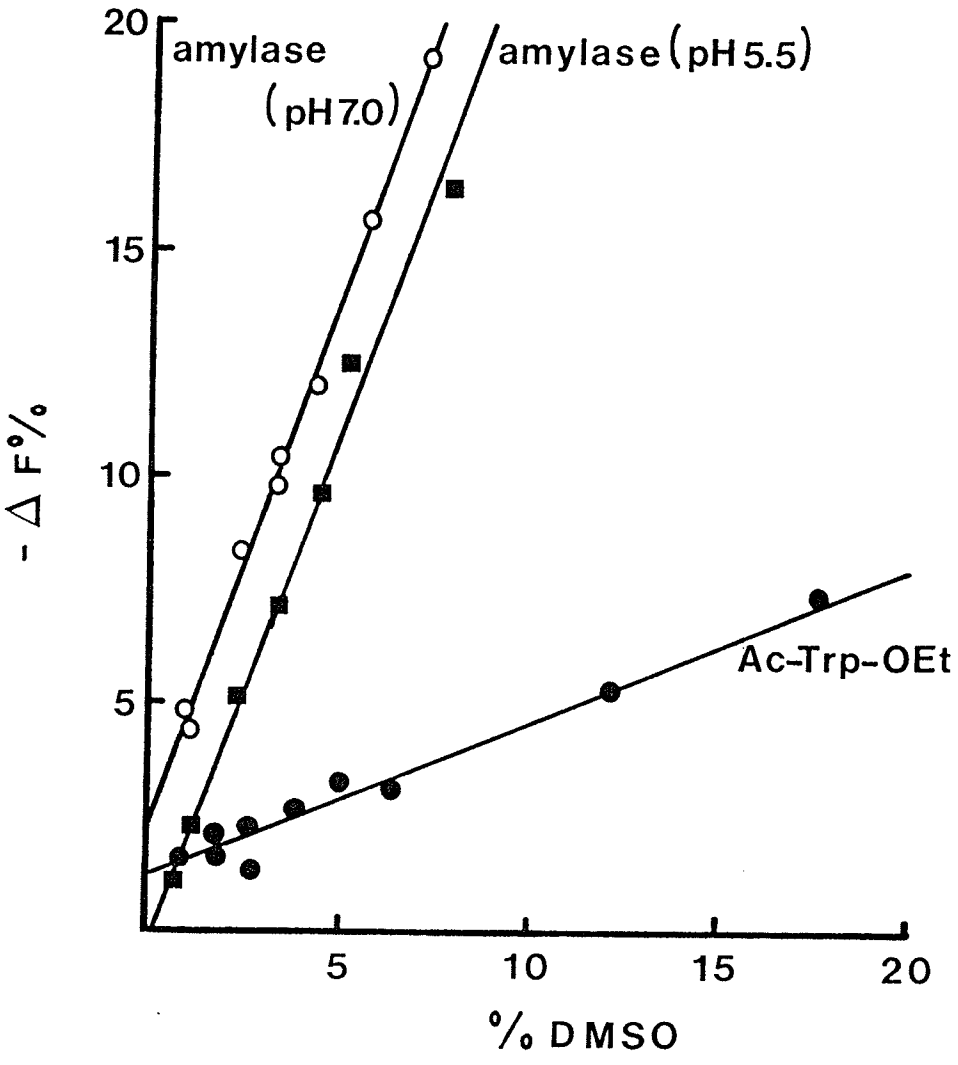

20
15
10
5
- △ F%
amylase
(pH5.5)
amylase
(pH7.0)
Ac-Trp-OEt
5
10
15
20
% Ethylene Glycol

Figure 10: The effect of DMSO concentration on the percent decrease in fluorescence emission (-ΔF%) at 336 nm in pH 5.5 and 7.0 media.

■ = α-amylase, pH 5.5
○ = α-amylase, pH 7.0
● = Ac-Trp-OEt, pH 7.0

Conditions
Buffers = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) or 20 mM sodium acetate (1 mM $CaCl_2$, pH 5.5). Enzyme concentration = 0.52 μM, Ac-Trp-OEt= 10.51 μM. Temperature = 20-23°C.
Scan rate = 60 nm/min.


20
15
10
5
- Δ F%
amylase
(pH7.0)
amylase (pH5.5)
Ac-Trp-OEt
5
10
15
20
% DMSO

to 2 residues higher than those for fluorescence studies. However, it appears as if 6-9 or approximately 40 to 55% of all *a*-amylase II tryptophan residues are exposed to the solvent environment. Similar numbers of exposed tryptophans have been estimated for bacterial liquefying *a*-amylase (Ohnishi, 1971). Gibson and Svensson (1986) have estimated that 4-6 tryptophanyl residues are exposed to solvent for barley *a*-amylase II using difference spectroscopy. However, they accounted for tyrosine contribution to the overall perturbed spectrum of the enzyme but failed to show an example of a typical difference spectrum. The difference spectra measured here were strictly dominated by tryptophanyl characteristics and no tyrosine-specific contributions were evident. Therefore, the latter was not considered in difference molar absorptivity measurements. The fluorescence intensity measured at 336 nm in these studies is specific for tryptophan and no tyrosine interference needs to be considered.

4.6. Inhibitor/*a*-Amylase II Binding

4.6.1. Gel Filtration Studies

4.6.1.1. Complex Stoichiometry

Binding stoichiometry of the inhibitor/*a*-amylase II complex was investigated by gel filtration at pH 7.0. Pre-equilibrated mixtures of the two proteins were filtered

through a sieving matrix and the amount of each free protein and the amount of each involved in complexation were indicative of the protein binding ratio. When α-amylase II and inhibitor samples were applied separately their respective elution volumes from the Bio-Gel P100 filtration column were 45.5 and 58.5 mls (Figure 11). Only two peaks were eluted when a preincubated equimolar mixture of enzyme and inhibitor was applied to the column (Figure 11, Table 8). The first peak consisted of the inhibitor-enzyme complex as identified by its SDS-electrophoresis profile (Figure 12). The second peak consisted of free α-amylase II as indicated by its elution volume and SDS-electrophoresis mobility (Figure 12, Table 8). It appears that all of the inhibitor from the original mixture has combined to form a complex with approximately half of the enzyme that was present. This indicates a molar binding ratio of between 1.5 and 2.0 inhibitor to one α-amylase II (Table 8). When inhibitor and enzyme, 5:1 molar ratio, was filtered through the column, peaks corresponding to the complex and to free inhibitor were eluted (Figure 11, Table 8). The peaks were positively identified on the basis of their elution profiles and SDS-electrophoretic mobilities (Figure 12). Assuming that the inhibitor protein that didn't elute as a part of the second peak had bound and eluted with all of the enzyme present as a part of the complex, a molar binding ratio with α-amylase II of 2:1 was obtained (Table 8). Buonocore et al. (1980, 1984) have similarly reported a 2:1 stoichiometry

for the binding of the 0.28 wheat albumin inhibitor with yellow mealworm and chicken pancreatic α-amylase.

Figure 11. Gel filtration chromatography of α-amylase II and inhibitor on Bio-Gel P100.

(a) Superimposed elution profiles of inhibitor (60 nmol) and α-amylase II (16 nmol) applied separately.

(b) Elution profile of 1:1 molar ratio mixture of inhibitor (9 nmol) and enzyme (9 nmol).

(c) Elution profile of 5:1 molar ratio mixture of inhibitor (64 nmol) and α-amylase (13 nmol).

Conditions:
Equilibration buffer = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0); Column dimensions = 1.5 x 60 cm; Fraction volume = 1.7 mls; Flow rate = 10 mls/hour; Sample volumes = 2.0 mls.

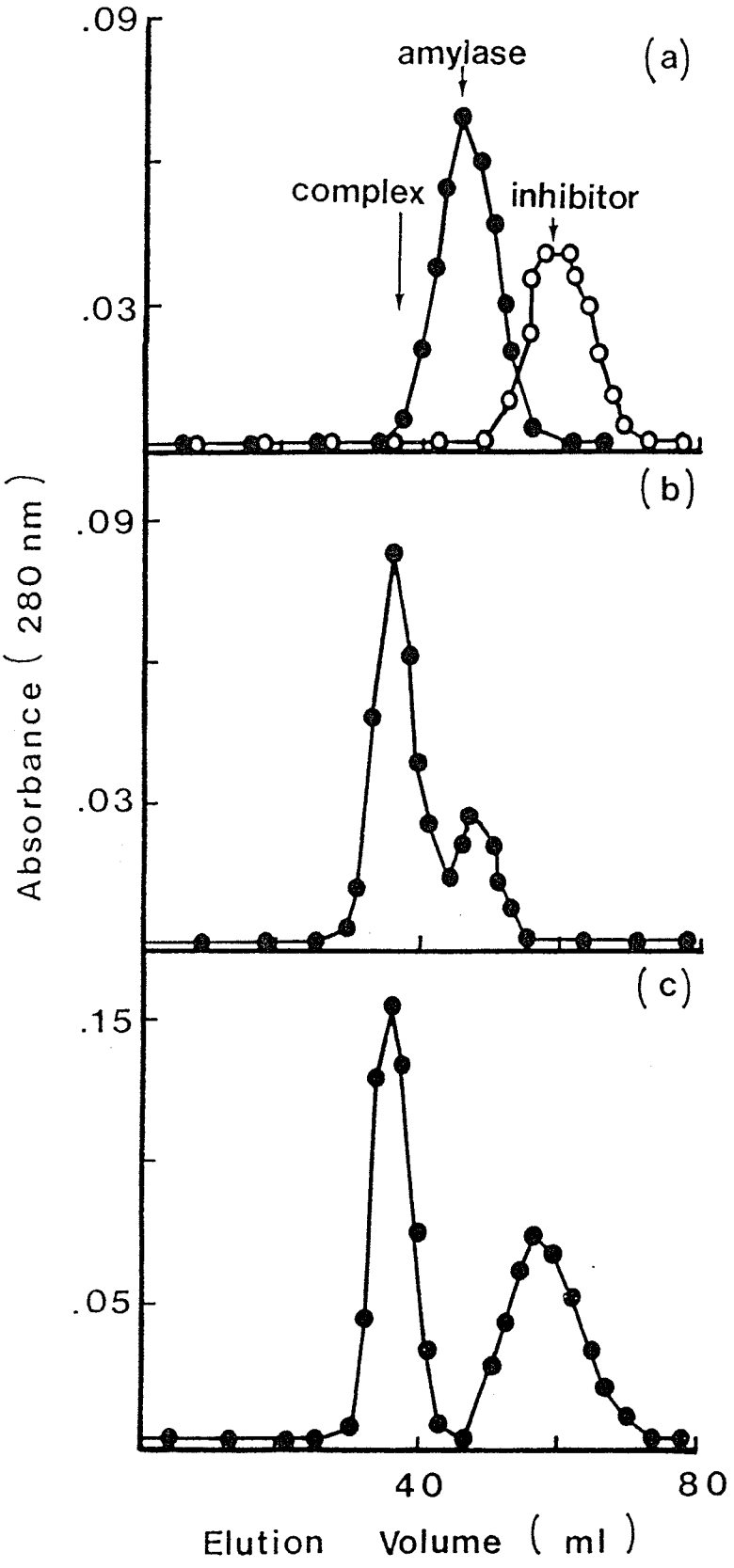

.09
amylase
(a)
complex
inhibitor
.03
(b)
.09
.03
(c)
.15
.05
Absorbance (280 nm)
40
80
Elution Volume (ml)

TABLE 8

Determination of inhibitor/amylase binding ratios by gel filtration

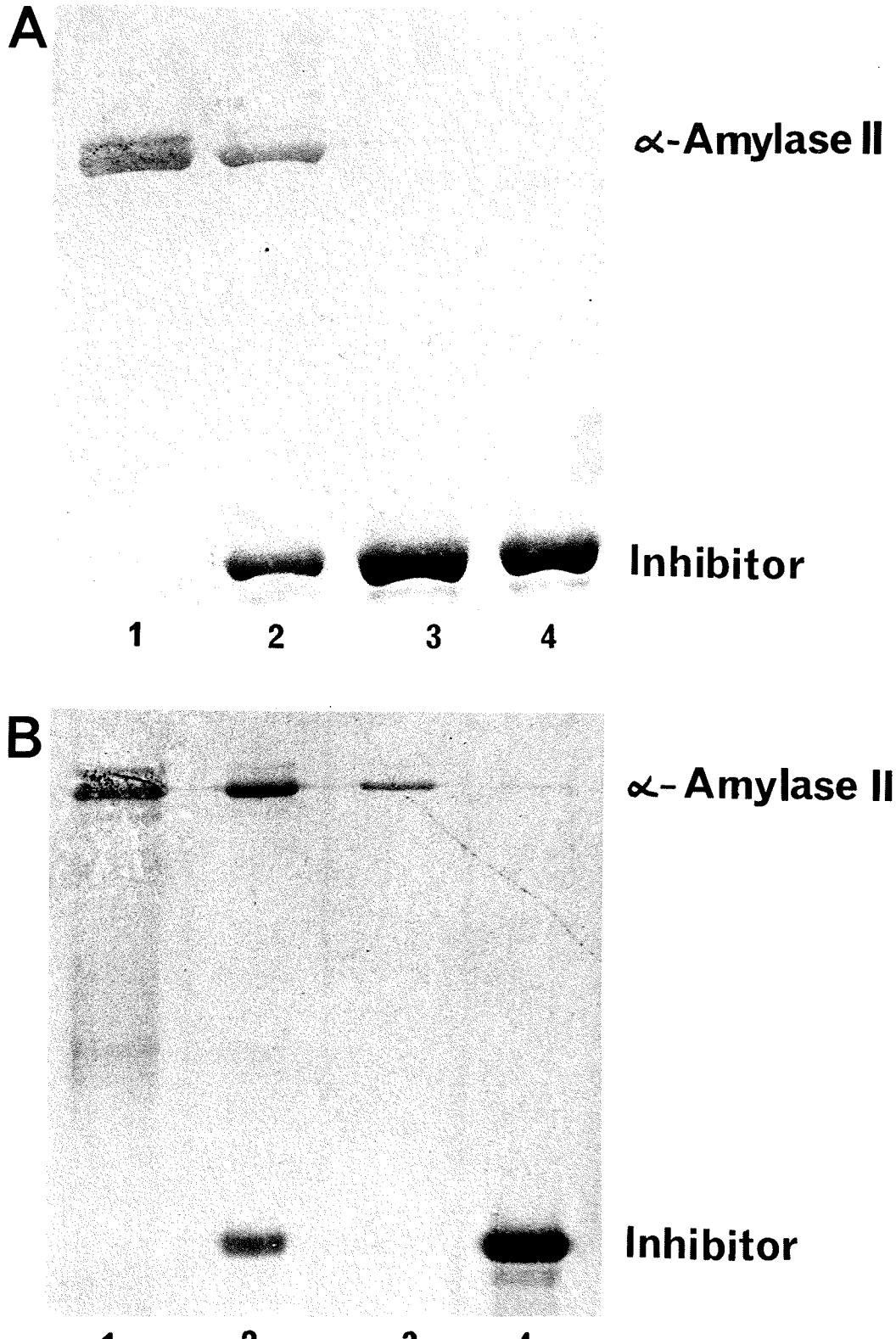
Mixture Applied		Molar Ratio	Free Protein Eluted		Binding Ratio
Inhibitor (nmol)	Amylase (nmol)		Inhibitor (nmol)	Amylase (nmol)	
8.9	8.9	1:1	0	3.3	1.6:1
64.4	12.9	5:1	39.1	0	2.0:1

Figure 12. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of protein peaks eluted from the Bio-Gel P100 column in gel filtration studies.

A. Gels stained to reveal the constituent proteins of the peaks eluted for the 5:1 inhibitor-amylase sample.

B. Gel showing the constituent proteins of the peaks eluted for the 1:1 inhibitor-amylase sample.

Lane 1 = α-amylase II standard
Lane 2 = 1st eluted peak for respective experiments
Lane 3 = 2nd eluted peak for respective experiments
Lane 4 = barley α-amylase/subtilisin inhibitor standard


A
α-Amylase II
Inhibitor
1
2
3
4
B
α-Amylase II
Inhibitor
1
2
3
4

4.6.2. Ultraviolet Difference Spectroscopy

4.6.2.1. Involvement of Chromophoric Residues

Difference spectra were produced for *a*-amylase II by titration with inhibitor at pH 7.0 and pH 5.5. In order to eliminate the possibility that the inhibitor's exposed tryptophanyl side chain was contributing to the *a*-amylase difference spectrum, inhibitor which had previously had its exposed side chain oxidized by NBS was also used in some studies at pH 7.0. Difference spectra of *a*-amylase II induced by native or partially oxidized inhibitor at pH 7.0 and by native inhibitor at pH 5.5 were qualitatively identical. Representative spectra are shown in Figure 13. All of the spectra showed maximum perturbation at 294 nm with an additional peak at 284 nm and a prominent shoulder at 275-277 nm. The difference spectra presented are quite characteristic of tryptophan involvement in inhibitor/*a*-amylase II binding.

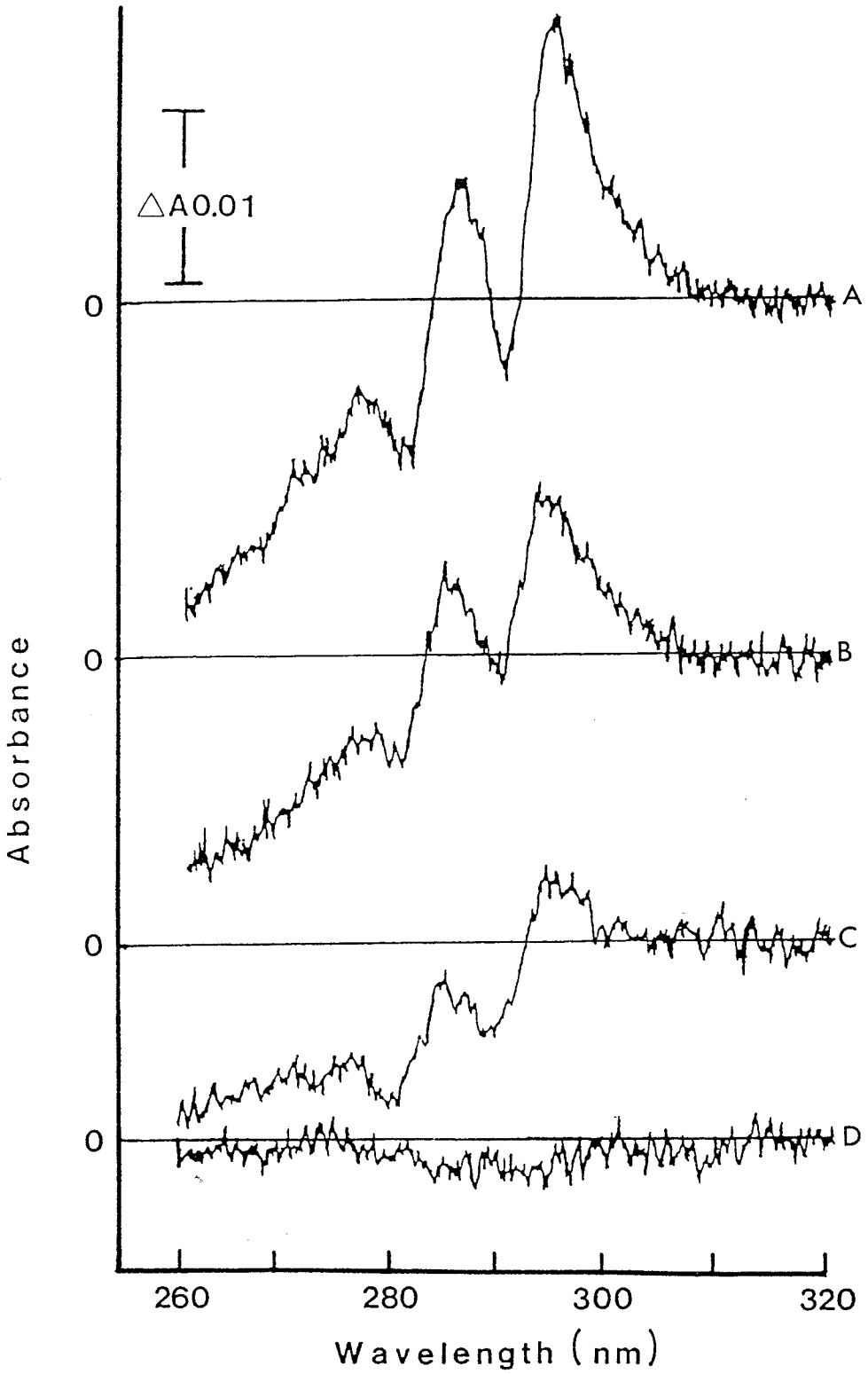
Positive differences around 290 nm seen in the difference spectra induced by inhibitor/*a*-amylase II binding indicate that the spectra originate from a red shift of at least one tryptophan residue (Ananthanarayanan and Bigelow, 1969; Elodi et al., 1972; Nitta et al., 1983). Buonocore et al. (1980, 1984) have previously shown difference spectra indicating the involvement of tryptophan side chains in the binding of wheat albumin inhibitors with *a*-amylase from yellow mealworm and chicken pancreas.

Figure 13. Difference absorption spectra of α-amylase II produced by titration with inhibitor.

Spectra represent different molar ratios of inhibitor to α-amylase II:

A. 2:1
B. 1.2:1
C. 0.5:1
D. baseline spectrum

Conditions:
Buffer = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0); temperature = 20-23°C; scan rate = 15 nm/minute; α-amylase = 9.7 μM.


△A0.01
0
0
0
0
A
B
C
D
Absorbance
260
280
300
320
Wavelength (nm)

4.6.2.2. Binding Stoichiometry

Increased concentrations of inhibitor or partially modified inhibitor relative to *a*-amylase II resulted in proportional increases in spectral perturbations at 294 nm. Maximum spectral perturbation at 294 nm was achieved at inhibitor *a*-amylase II ratios that were greater than or equal to 2:1 for both partially oxidized inhibitor and native inhibitor (Figure 14). To induce maximal difference spectra the enzyme must be completely saturated with inhibitor.

4.6.2.3. Binding Affinity

Difference molar extinction coefficients at 294 nm, $\Delta\epsilon_{294}$, are proportional to the concentrations of the inhibitor-enzyme complex (Ohnishi, 1971; Elodi et al., 1972; Nitta et al., 1983). Michaelis-Menten formulae allow apparent dissociation constants for the complex to be extrapolated from double reciprocal plots of $\Delta\epsilon_{294}$ versus inhibitor concentration (Ohnishi, 1971; Elodi et al., 1972). Representative plots, made from data depicted in Figure 14, for a single titration with inhibitor at pH 5.5 and pH 7.0 are drawn in Figure 15. Estimated apparent dissociation constants and maximum molar extinction coefficient differences at 294 nm, $\Delta\epsilon$max, for the titrations of *a*-

Figure 14. Dependence of spectral perturbation at 294 nm on the inhibitor:α-amylase II ratio.

Curves represent titrations of α-amylase with:
● = native inhibitor, pH 7.0
○ = native inhibitor, pH 5.5
■ = partially oxidized inhibitor, pH 7.0

Conditions:
buffers = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) or 20 mM sodium acetate (1 mM $CaCl_2$, pH 5.5); conductance = 1020 μmho's at pH 7.0 and 1100 μmho's at pH 5.5; temperature = 20-23°C; α-amylase = 9.7 μM; molar extinction differences, $\Delta\epsilon_{294}$, are based on a molecular weight of 45 kDa for amylase.
weight of 45 kDa for amylase.

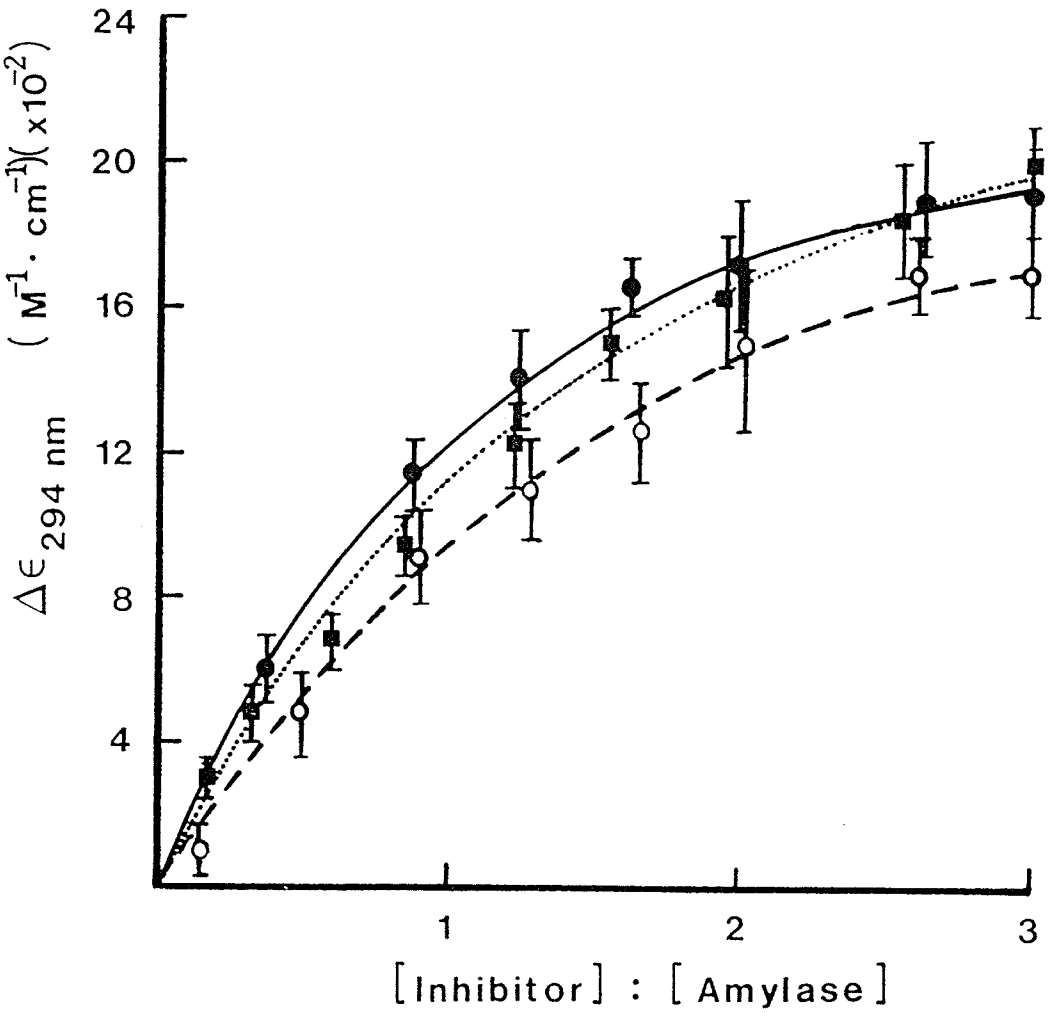
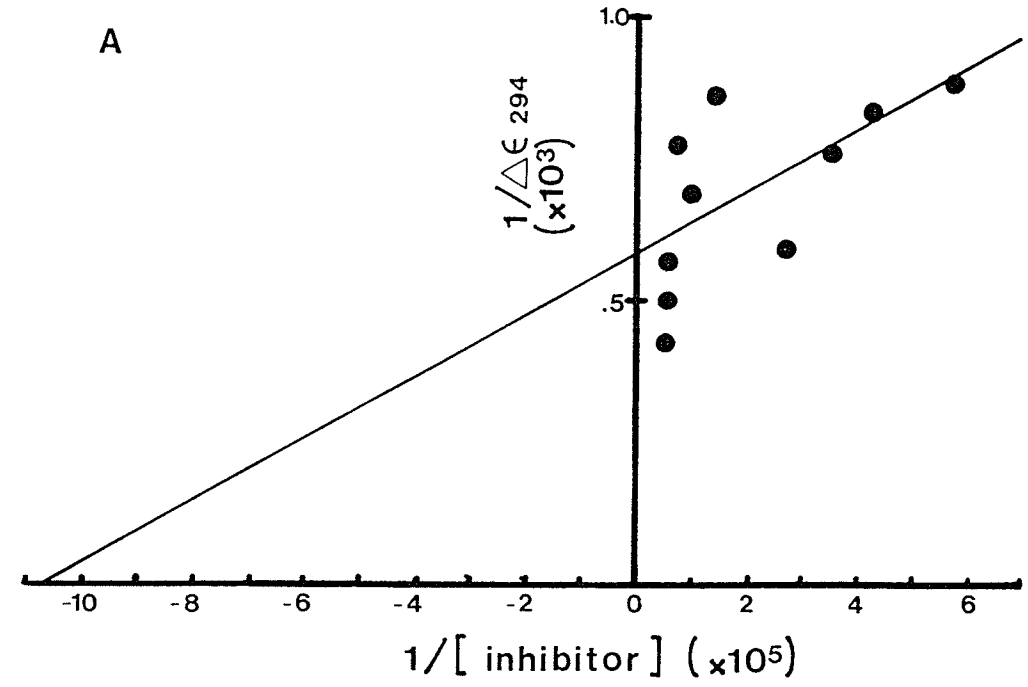

24
20
16
12
8
4
$\Delta\epsilon_{294\ nm}$ ($M^{-1} \cdot cm^{-1}$)($x10^{-2}$)
1
2
3
[Inhibitor] : [Amylase]

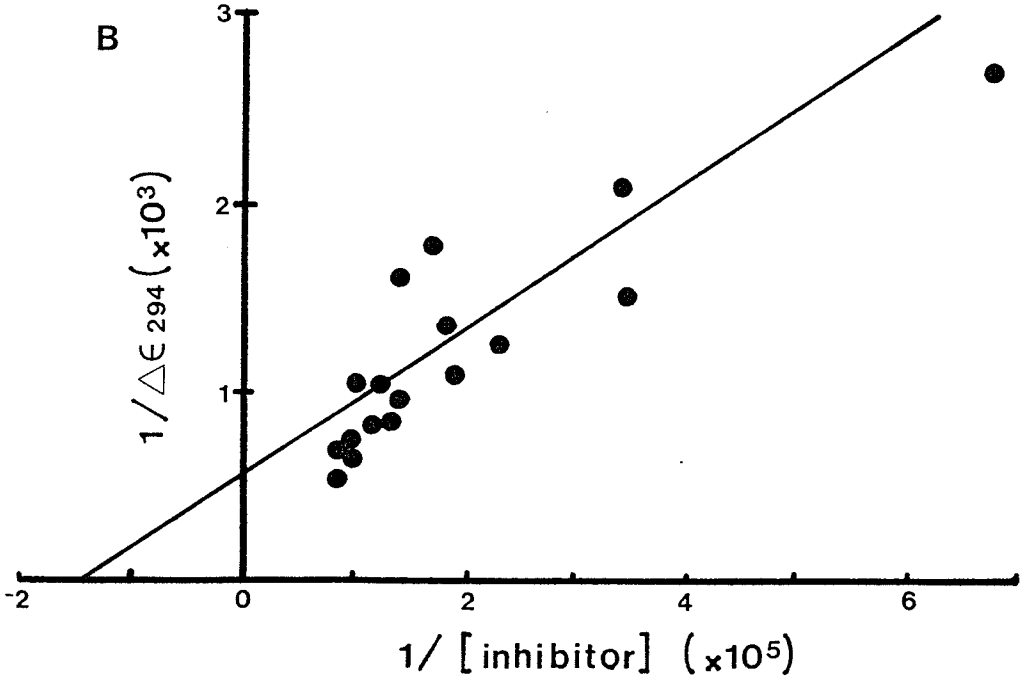
Figure 15. Lineweaver-Burk double reciprocal plots of molar extinction differences, $\Delta\epsilon_{294}$, of α-amylase II as a function of inhibitor concentration at pH 7.0 and 5.5.

A. pH 7.0

B. pH 5.5

Conditions:
buffers = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0) or 20 mM sodium acetate (1 mM $CaCl_2$, pH 5.5); temperature = 20-23°C; α-amylase II = 9.7 μM.


A
1.0
.5
$1/\Delta\epsilon_{294}$ ($\times 10^3$)
-10
-8
-6
-4
-2
0
2
4
6
1/[inhibitor] ($\times 10^5$)


B
3
2
1
$1/\Delta\epsilon_{294}$ ($\times 10^3$)
-2
0
2
4
6
1/ [inhibitor] ($\times 10^5$)

amylase II with inhibitor samples are listed in Table 9. Inhibitor-enzyme affinity appears to be approximately 7 fold greater at pH 7.0 than at pH 5.5. This difference in binding affinities was apparent in earlier studies of pH effects on inhibitory activity where an 8-fold decrease in anti-amylolysis was seen at pH 5.5 from pH 7.0. Weselake et al. (1983b) have reported inhibitor activity and apparent affinity for α-amylase II increases as pH is increased from 5.5 to 8.0. Tanaka and Akazawa (1970) reported that upon heating at 70°C for 15 minutes the activity of barley (cv. Mushashino mugi) α-amylase II was enhanced. The increased activity with heating was probably due to denaturation effects on inhibitor bound to enzyme. A more pronounced increase in enzyme activity was seen at pH 6.9 than at pH 5.0. This seems to indicate that complex association at pH 6.9 was greater than at pH 5.0.

A double-reciprocal plot of α-amylase II titration with partially oxidized inhibitor at pH 7.0 is shown in Figure 16. Inhibitor/enzyme complex stability seemed to be somewhat reduced when inhibitor with a modified exposed tryptophan residue was a constituent but that stability was still greater than that seen for unmodified inhibitor-enzyme binding at pH 5.5 (Table 9). In contrast to the apparent loss of its α-amylase binding affinity the modified inhibitor did not lose any anti-amylolytic activity at pH 7.0 (Table 6). The apparent loss of affinity detected in

TABLE 9

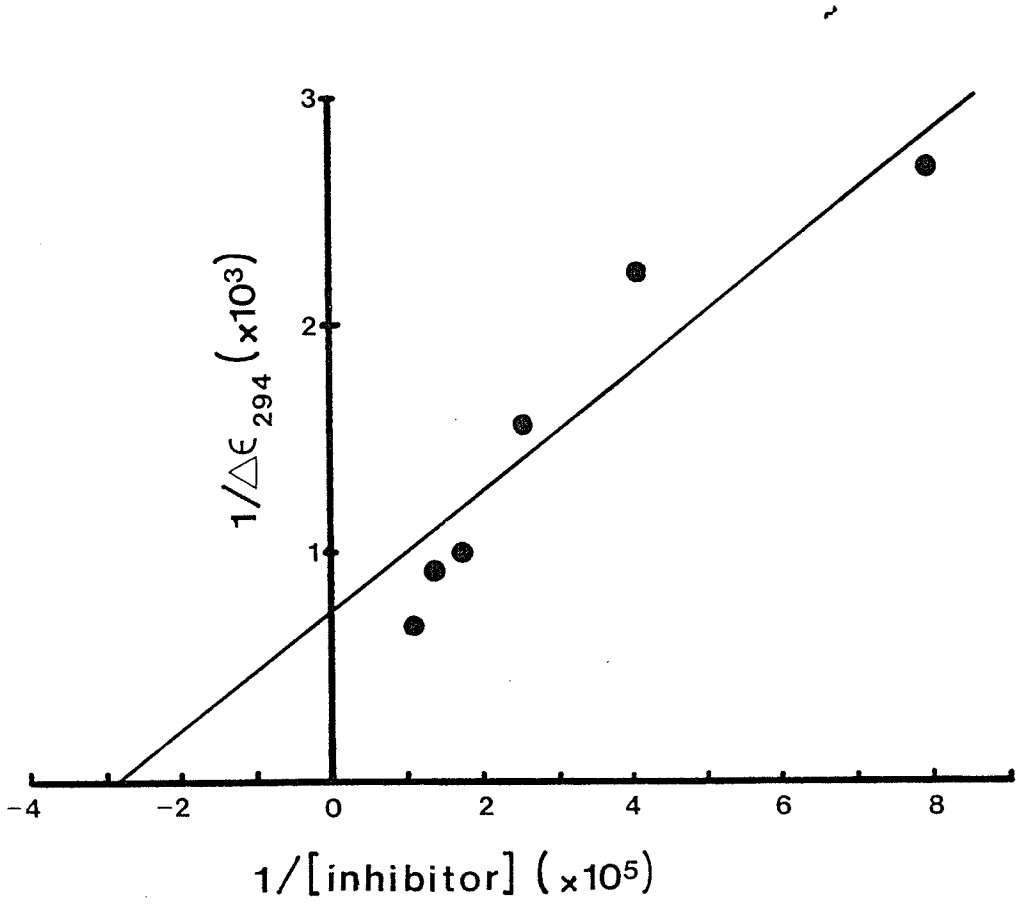
Apparent dissociation constants, Kd, and difference molar extinction coefficient values, $\Delta\epsilon_{294}$, based on spectrophotometric titrations of α-amylase II with inhibitor and partially oxidized inhibitor.

pH	Inhibitor sample	Kd (μM)	$\Delta\epsilon$max ($M \times cm^{-1}$)
7.0	native	0.9±0.3	1700±140
7.0	oxidized	3.5±0.7	1400±200
5.5	native	6.7±1.3	1750±160

Values represent means ± S.E. of at least three replications.

Figure 16. Lineweaver-Burk double reciprocal plot of molar extinction differences, $\Delta\epsilon_{294}$, of α-amylase as a function of partially oxidized inhibitor concentration at pH 7.0.

Conditions:
Buffer = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0); temperature = 20-23°C; α-amylase II = 10.1 μM.


3
2
1
$1/\Delta\epsilon_{294}$ ($\times 10^3$)
-4
-2
0
2
4
6
8
1/[inhibitor] ($\times 10^5$)

difference spectroscopic studies may be due to a small conformational difference in the modified inhibitor which does not affect its attraction to α-amylase but does affect the expression of binding-induced changes observed in the spectrum of the enzyme.

When spectral perturbations produced by specific enzyme-ligand binding are analyzed using Michaelis-Menten formulae, information regarding the affinity and aromatic participation in the interactions is obtained (Ohnishi et al., 1973a; Nitta et al., 1983; Clarke and Svensson, 1984a). A basic assumption of the Michaelis-Menten theory is that bound substrate should constitute an insignificant proportion of the total substrate concentration. Therefore, in analyses of binding data, total ligand concentrations are considered and are assumed to be the same as "free" ligand concentrations (Ohnishi, 1971). In difference spectroscopic studies, the inhibitor concentration was not significantly greater than that of the enzyme because absorbance at 280 nm had to be kept below 1.8 in order for Lambert-Beer's law to hold (Nitta, et al., 1983). Consequently, total inhibitor concentration does not represent free inhibitor concentration because the ratio of "free" to "bound" inhibitor can not approach infinity. This situation throws a degree of error into the analyses employed in this study because total inhibitor concentration has still been used to represent "free" inhibitor concentration. Values for Kd of

the inhibitor-enzyme complex are therefore probably not precisely what would be expected under ideal conditions. However, they are of the same order of magnitude as those reported by Mundy et al. (1983) and probably represent relative differences in the Kd values calculated.

In depth analysis of α-amylase II difference spectra indicates that only one of two amylase-bound inhibitors may be responsible for the shifts induced in the enzyme's ultraviolet spectrum. By example, Figure 14 shows, for a single inhibitor-amylase titration experiment at pH 7.0, a $\Delta\epsilon$max value of 1810 $M^{-1}cm^{-1}$. Amylase concentration was 9.7 μM, therefore when fully saturated should, assuming a 2:1 binding ratio, interact with 19.4 μM of inhibitor. At 50% of saturation (1:1 molar ratio of inhibitor to α-amylase), the measured $\Delta\epsilon_{294}$ was 1125 $M^{-1}cm^{-1}$ Theoretically, this value should be 50% of $\Delta\epsilon$max, but in fact, it represents 62% of $\Delta\epsilon$max in this case. Accordingly, if both inhibitor molecules binding to each α-amylase II molecule are responsible for inducing spectral shifts, then 12 μM of inhibitor (62% of saturation) must be bound to the enzyme. Clearly this is not possible as only 9.7 μM of inhibitor had been added at this point. Alternatively, if one assumes that only a single enzyme-bound inhibitor induces amylase difference spectra, then only 9.7 μM of inhibitor need be bound to saturate the perturbation of the α-amylase ultraviolet spectrum. On this basis, when the 1:1 molar

ratio of inhibitor to α-amylase II was reached (and 62% of $\Delta\epsilon$max was measured) (Figure 14), only 6.0 μM of inhibitor was responsible for the measured spectral perturbation. This seems quite possible as 9.7 μM of inhibitor was actually present. Previous inhibitor activity studies suggest 2:1 binding with α-amylaseII (Mundy et al., 1983; Weselake et al., 1983a) and the same was confirmed by gel filtration here (Table 8). It seems then, that only one of the bound inhibitor molecules actually induces the spectral shifts detected during difference spectroscopy.

There is no significant difference between the estimated $\Delta\epsilon$max values induced by any of the inhibitor samples (Table 9). Qualitative and quantitative similarities between difference spectra induced by the inhibitor and by inhibitor which had its exposed tryptophan residue modified seem to indicate that a common enzymic tryptophan is involved. The $\Delta\epsilon$max of the inhibitor-enzyme interaction was found to be between 1400 and 1700 $M^{-1}cm^{-1}$ (Table 9). A denaturation blue shift of a single tryptophan residue in protein produces a $\Delta\epsilon$ between -1500 and -1600 $M^{-1}cm^{-1}$ at 290 nm (Donovan, 1964). It has been assumed that specific red-shifts caused by binding interactions are opposite to denaturation blue-shifts (Ohnishi, 1971; Ohnishi et al., 1973a). The specific difference spectra induced by inhibitor/α-amylase II binding are the result, therefore, of the masking of one tryptophan residue.

There was no loss of inhibitory activity by modified inhibitor (Table 6). Also, inhibitor with modified exposed tryptophan residues can induce molar extinction differences equal to those induced by native-state inhibitor on the spectrum of *a*-amylase II. Consequently, the functional tryptophan appears to be a residue of the enzyme constituent of the complex. The red-shift difference spectrum of *a*-amylase II induced by the binding of the inhibitor indicates that a more hydrophobic region is being formed around an enzymic tryptophan residue (Hayashi et al., 1963; Dahlquist et al., 1966). Whether the tryptophan directly interacts with the inhibitor or whether it is affected by a conformational change of the enzyme away from the interaction site during binding is unknown at this point.

The effects of increasing salt concentration in the form of NaCl at pH 7.0 on inhibitorenzyme complex stability were studied by difference spectroscopy and analyzed by a Michaelis-Menten-type formula (Kunikata et al., 1978; Nitta et al., 1983):

$$\Delta\epsilon_{294} = \Delta\epsilon\text{max} \times I/(Kd + I) \qquad (5)$$

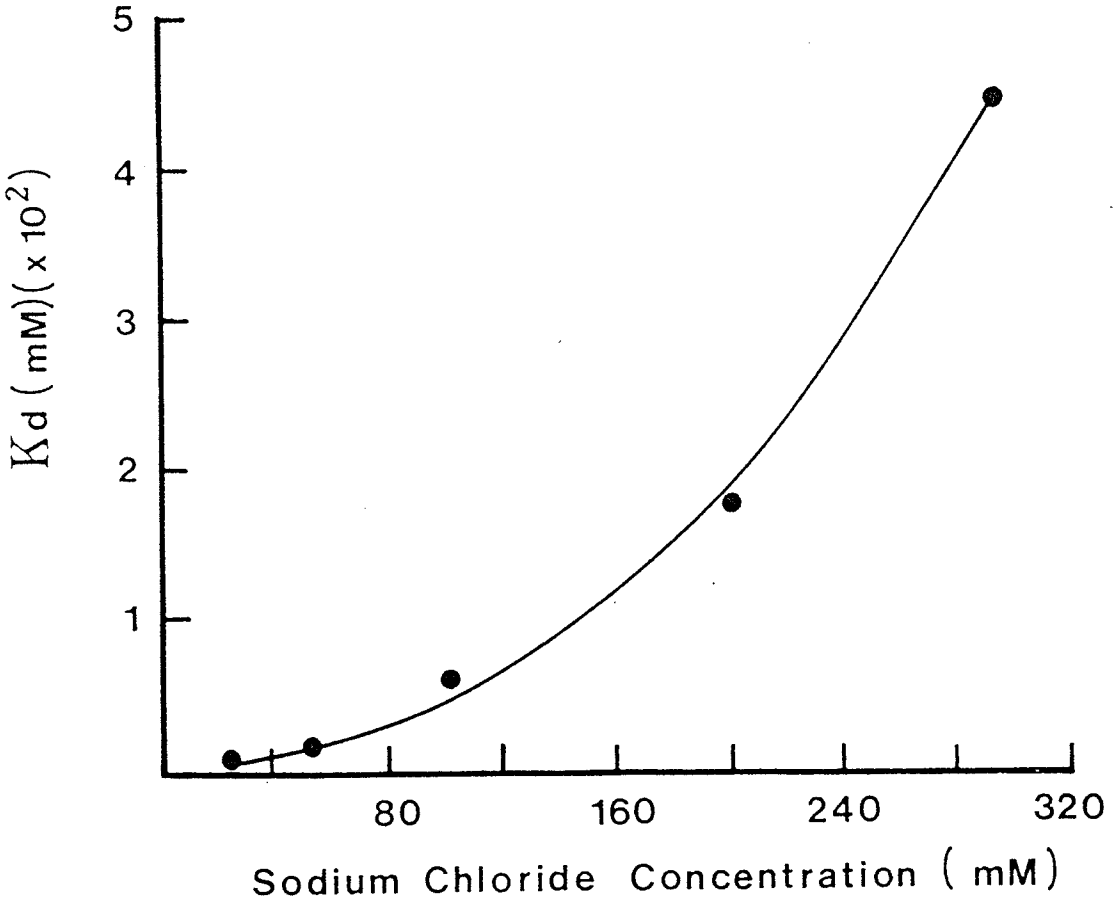
where $\Delta\epsilon_{294}$ is the measured spectral difference, corrected for dilution, at each salt concentration, I is inhibitor concentration at each measurement and Kd is the apparent dissociation constant for the inhibitor-enzyme complex. A value of 1700 $M^{-1}cm^{-1}$ (Table 9) was used for $\Delta\epsilon$max in all calculations. Rearranging Equation 5 to solve for Kd at each salt concentration, one obtains,

$$Kd = [(\Delta\epsilon max \times I)/\Delta\epsilon_{294}] - I \qquad (6)$$

By gradually increasing the concentration of NaCl up to 300 mM, complex association steadily declined to approximately 50-times less than was seen when no NaCl was present in the buffered medium (Figure 17). This seems to confirm the postulated ionic nature of the inhibitor-enzyme interaction (Weselake et al., 1983b).

Figure 17. Effect of sodium chloride concentration on the apparent dissociation constant, Kd, of the α-amylase II/inhibitor complex.

Conditions:
starting buffer = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0); temperature = 20-23°C.


5
4
3
2
1
Kd (mM)(x 10^2)
80
160
240
320
Sodium Chloride Concentration (mM)

4.6.3. Fluorescence Spectrophotometry

4.6.3.1. Binding Stoichiometry

Fluorescence titration of *a*-amylase II with native-inhibitor at pH 7.0 was used to verify some of the difference spectroscopic binding studies. The fluorescence method is considerably more sensitive and up to 100-fold lower protein and ligand concentrations have been used as compared to UV spectrophotometry (Donovan, 1964; Brand and Witholt, 1967; Hiromi et al., 1982; Tanaka et al., 1982).

Fluorescence emission spectra of *a*-amylase II at pH 7.0 in the absence and presence of increasing amounts of inhibitor are shown in Figure 18. The intrinsic emission fluorescence of the free enzyme at 336 nm, the characteristic wavelength for tryptophan, was quenched in proportion to the amount of inhibitor added. Maximum fluorescence quenching was achieved at ratios of approximately 2-3 mol inhibitor per mol *a*-amylase II (Figure 19). However, at a ratio of about 1:1 a plateau in quenching exists, so the titration of the enzyme's tryptophan residue by inhibitor appears to be biphasic. As amylase was titrated, the amount of inhibitor fluorescence which had to be corrected for increased to approximately 20% of total enzyme emission. Consequently, quenching measured at higher inhibitor concentrations may have been subject to some anomalous deviations from the direct effects of inhibitor binding *a*-amylase II.

Figure 18. Fluorescence emission spectra of α-amylase II with increasing concentrations of inhibitor at pH 7.0.

Spectra represent different molar ratios inhibitor to α-amylase II:

a. α-amylase II emission spectrum
b. 0.8:1
c. 1.3:1
d. 2.0:1

Conditions:
buffer = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0); temperature = 20-23°C; α-amylase II = 0.31 μM; excitation wavelength = 280 nm; scan rate = 60 nm/minute.

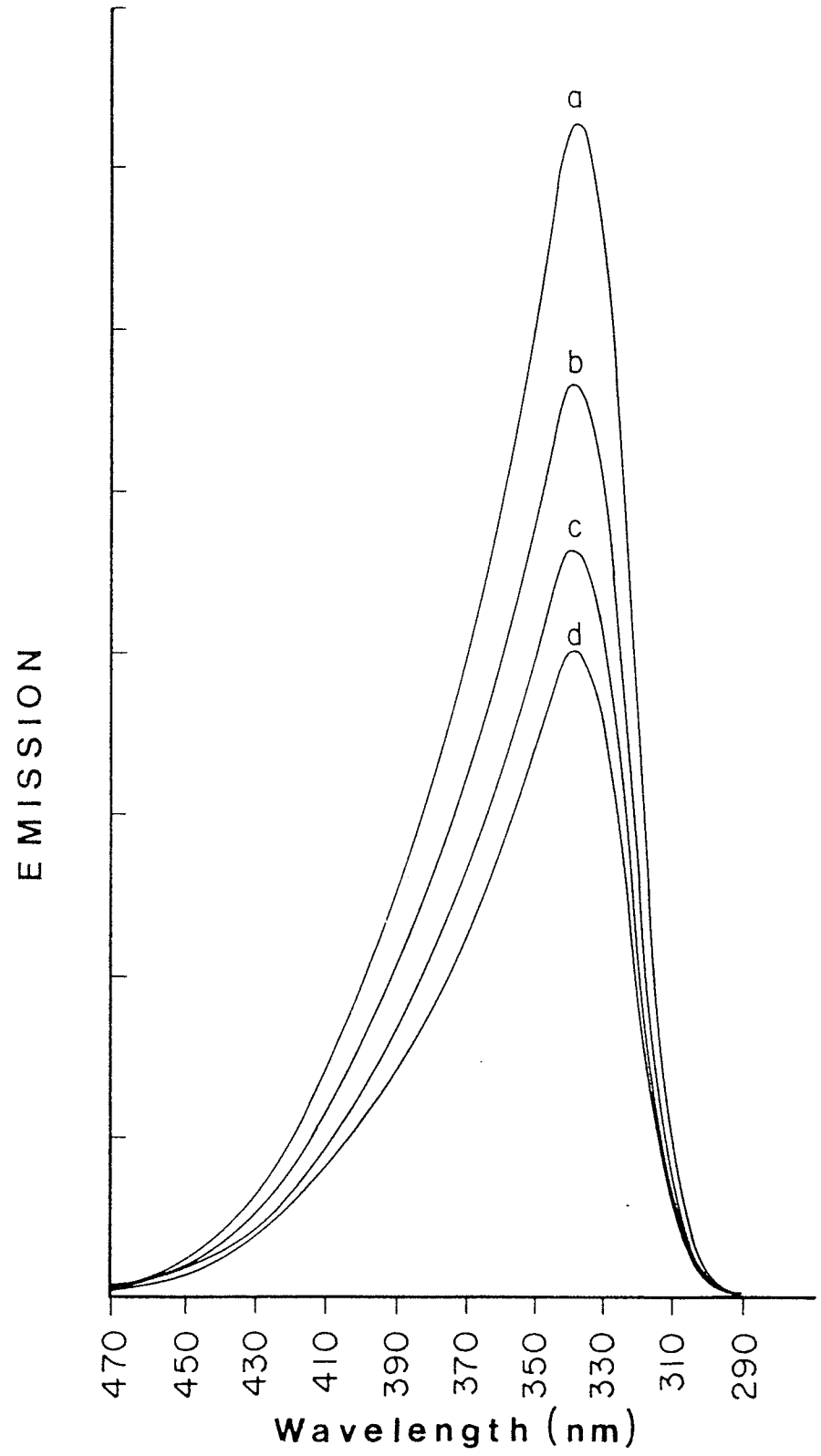
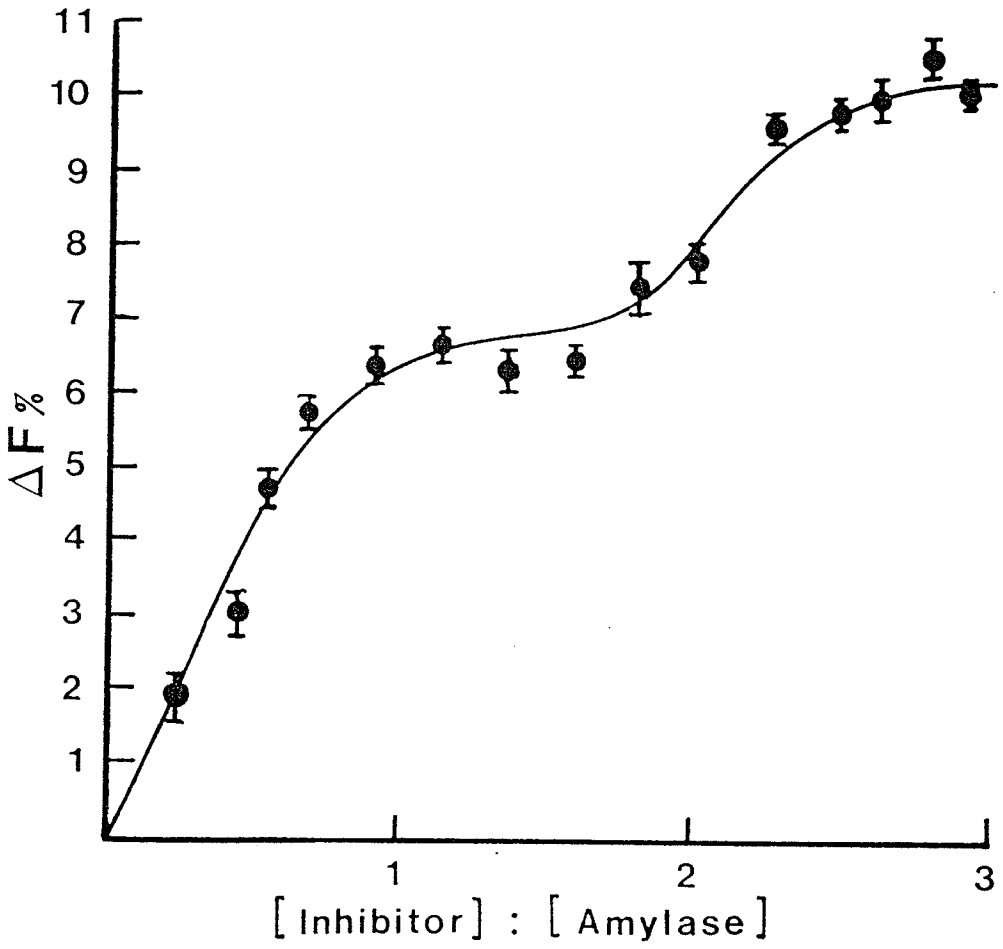

a
b
c
d
EMISSION
470
450
430
410
390
370
350
330
310
290
Wavelength (nm)

Figure 19. Percent fluorescence quenching at 336 nm, $\Delta F\%$, of α-amylase II by inhibitor at pH 7.0.

Conditions:
buffer = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0);
temperature = 20-23°C. Error bars illustrate means ± S.E. of six measurements.


11
10
9
8
7
6
5
4
3
2
1
ΔF%
1
2
3
[Inhibitor] : [Amylase]

Gel filtration, difference spectroscopy and fluorescence titration studies all indicate a stoichiometric binding ratio of 2:1 for inhibitor with *a*-amylase II. Weselake et al. (1983b) showed that an equimolar amount of inhibitor suppresses 40% of *a*-amylase II activity at pH 8.0. This indicates that the enzyme was not fully saturated under these experimental conditions. Mundy et al. (1983) plotted increasing BASI:*a*-amylase II molar ratios against percent *a*-amylase inhibition at pH 6.0 in the presence of a soluble starch substrate. They reported a 40% decrease in amylolytic activity as the inhibitor:enzyme ratio was increased to 2:1 but a further decrease of only 15% as the ratio reached 10:1. The inhibition data were approximated by two lines intersecting at an inhibitor:amylase II molar ratio of about 2:1. Similarly, Buonocore et al. (1980, 1984) have reported a 2:1 stoichiometry for binding of the dimeric 0.28 wheat albumin inhibitor with yellow mealworm and chicken pancreatic *a*-amylase.

Rodaway (1978) has reported the existence of a protein that copurifies with *a*-amylase from barley aleurone layers. Designated "band-2 protein", it has an amino acid composition strikingly similar to the inhibitor and, based on gel chromatography, appears to exist as a dimer. Therefore, it is possible that the inhibitor exists as a dimer in some instances including during *a*-amylase II binding. If this is the case then the exposed tryptophan on

each inhibitor moeity must not play a role in the protein's dimerization, otherwise partially oxidized inhibitor would not bind to *a*-amylase II in the same ratio as native-state inhibitor does.

The evidence supporting the existence of a 1:1 stoichiometric complex between *a*-amylase II and the inhibitor is tenuous. Sensitive fluorescence measurements showed the existence of a quenching plateau at equimolar concentrations of enzyme and inhibitor during titration experiments (Figure 19). The existence of the plateau might suggest an abundance of 1:1 complexes. However, gel filtration and difference spectroscopy studies showed no indication of such complexes.

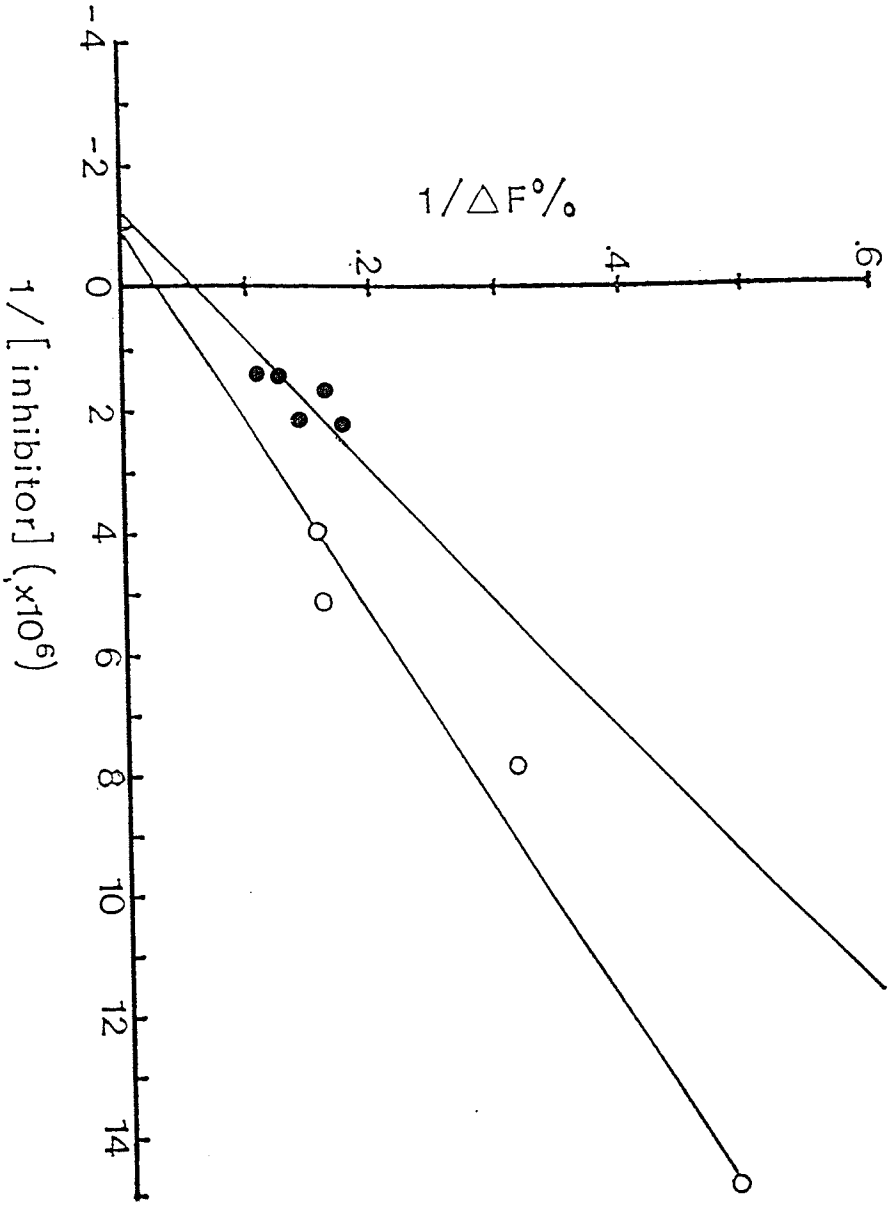
4.6.3.2. Complex Stability

Each quenching phase of the fluorescence titration curve for *a*-amylase II by inhibitor was analyzed separately using double reciprocal plots of the change in fluorescence emission intensity versus inhibitor concentration (Figure 20). Apparent dissociation constant, Kd, for the first phase of the inhibitor-*a*-amylase II mole-ratio plot was 1.1 μM and for the second phase, 0.8 μM. These Kd values correspond well with the apparent dissociation constant estimated for inhibitor-enzyme binding at pH 7.0 using difference spectrophotometry (Table 9).

Figure 20. Lineweaver-Burk double reciprocal plot of the first and second phases of percent fluorescence differences at 336 nm, $\Delta F\%$, of α-amylase II as a function of inhibitor concentration at pH 7.0.

O= phase 1
●= phase 2

Conditions:
buffer = 10 mM Pipes-NaOH (1 mM $CaCl_2$, pH 7.0);
temperature = 20-23°C; α-amylase = 0.4 μM.


1/ΔF%
.2
.4
.6
-4
-2
0
2
4
6
8
10
12
14
1/[inhibitor] (x10⁶)

5. GENERAL DISCUSSION AND CONCLUSIONS

To summarize the major findings of the experiments herein described, the optimal pH for inhibition of barley α-amylase II by an endogenous inhibitor was 7.0. The inhibitor was shown, by NBS oxidation, to possess one exposed and 2 buried tryptophanyl residues whereas solvent perturbation techniques indicated that α-amylase II had 6-9 of its 16 tryptophan side-chains exposed to external media. The exposed inhibitor tryptophan was not important in the maintenance of anti-amylolytic activity. The inhibitor formed complexes with α-amylase II by binding in a 2:1 molar ratio as determined by gel filtration, ultraviolet difference spectrophotometry and fluorescence spectroscopy. The fluorescence titration curve of α-amylase II with inhibitor was biphasic in nature and exhibited a plateau which may have corresponded to the existence of a 1:1 molar ratio complex of inhibitor and amylase. Difference spectra induced in α-amylase II by the inhibitor revealed that the environment around one enzymic tryptophanyl residue was made more hydrophobic during the binding interaction. The same technique indicated that the association of inhibitor/amylase complexes was about 7 times stronger at pH 7.0 (Kd = 0.9 μM), than at pH 5.5 (Kd = 6.7 μM). Also, complex association declined about 50-fold as salt concentration was increased from zero to 300 mM NaCl, confirming the previously postulated ionic nature of the binding interaction.

Since only one *a*-amylase II tryptophan residue is affected when two molecules of inhibitor bind to it, the gross physical structure of the complex formed could exist in one of two schemes. In the first scheme there could be a direct interaction of two inhibitor molecules with each enzyme molecule. This arrangement would either mask an exposed enzymic tryptophan directly or induce conformational changes in the *a*-amylase moiety forcing a tryptophanyl residue into a more hydrophobic region. In the second scheme the inhibitor could act as a dimer during enzyme association. As with the first scheme this form of binding could result in direct masking of an exposed enzymic tryptophan or could cause enzyme conformational shifts and the subsequent introduction of a tryptophan side chain into a more hydrophobic region. The biphasic nature of the fluorescence titration curve of *a*-amylase II with inhibitor could indicate that the binding of one inhibitor molecule results in a substantial increase in hydrophobicity around a single enzymic tryptophan. Similarly, modified saturation expansion of *a*-amylase II difference spectra data indicates that only one of the two amylase-bound inhibitors may, in fact, be responsible for the shifts induced in the enzyme's ultraviolet spectrum. The presence of the second molecule of inhibitor may or may not than serve to intensify the hydrophobic shift on the important *a*-amylase II tryptophan regardless of which binding scheme is followed.

In light of what is known about the amylolytic control exhibited by the inhibitor and with regard to the specifics of the binding interaction pursued in this work, a rudimentary mechanistic model for *a*-amylase II inhibition by the *a*-amylase/subtilisin inhibitor may be formulated. Barley *a*-amylases are known to possess substrate binding sites at the active centre and on the surface of the molecule located at some distance from the active site (Loyter and Schramm, 1966; Weselake and Hill, 1982, 1983; Gibson and Svensson, 1986). Tryptophan involvement has been implicated in substrate binding at the active site of various carbohydrases (Ohnishi and Hiromi, 1976; Nitta et al., 1983; Buonocore et al., 1984; Clarke and Svensson, 1984a; Jimbo et al., 1984). In numerous cases tryptophan residues have been shown to be important components at the subsites of substrate binding (Ohnishi et al ., 1973b; Kita et al., 1982; Clarke and Svensson, 1984b; Gibson and Svensson, 1986). The residues appear to be important in the maintenance of the threedimensional structure which facilitates productive substrate binding. Modification of specific tryptophan residues on bacterial *a*-amylases (Ohnishi et al., 1973b), saccharifying *a*-amylase (Fujimori et al., 1974), glucoamylase of Aspergillus niger (Clarke and Svensson, 1984a, 1984b), and Taka-Amylase A of A. oryzae (Kita et al., 1982) greatly reduces or destroys the enzyme's catalytic ability. On the basis of these studies it seems reasonable to propose that barley *a*-amylase II/inhibitor

binding alters the environment of a specific enzymic tryptophan residue so that productive enzyme-substrate binding might be made less favorable. Therefore, inhibition of glucosidic bond hydrolysis is effected.

To thoroughly confirm the characteristics of inhibitor/*a*-amylase II binding more specific research is required. Difference and fluorescence spectrophotometric techniques could be used to see how the binding of substrates or their analogues might affect enzyme/inhibitor association. These studies would result in more precisely localizing the position of the affected enzymic tryptophan. Structural analysis of both inhibitor and *a*-amylase II from barley by X-ray crystallography would provide information useful in understanding complex formation. In fact, the structural rearrangements which occur during binding could also be observed for either constituent under X-ray crystal structure analysis. In addition, circular dichroism analysis of individual complex components and of the complex itself would produce information regarding the local environments of chromophores as well as the changes of the backbone conformation of the proteins during binding. The information obtained from these studies could be used effectively in conjunction with kinetic binding data to confidently predict a detailed mechanism of inhibition for the *a*-amylase/subtilisin inhibitor. If such a mechanism could be elucidated it would likely shed considerable light

upon the role that the inhibitor plays in seed physiology and its possible usefulness as an attenuator of unwanted amylolytic activity in baking.

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