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AN INVESTIGATION OF ACTIVE FRAGMENTS AND THE ACTIVE SITE OF SWEET POTATO BETA-AMYLASE

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
Rene Evard
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AN INVESTIGATION OF ACTIVE FRAGMENTS AND THE ACTIVE SITE OF SWEET POTATO β-AMYLASE

By

Rene Evard

A THESIS

Submitted to the School of Advanced Graduate Studies of Michigan
State University of Agriculture and Applied Science
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

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AN INVESTIGATION OF ACTIVE FRAGMENTS AND THE ACTIVE SITE OF SWEET POTATO β-AMYLASE

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AN ABSTRACT

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Approved ____

ABSTRACT

The reaction of sweet potato β-amylase with a number of proteolytic enzymes was investigated with the hope of producing active gragments. Isolation and characterization of the active fragments should provide valuable information concerning the nature of the amino acids at or near the active site, and lead to a mechanism of enzymatic catalysis for hydrolysis of acetal linkages.

Nevertheless, under ordinary conditions, sweet potato β-amylase is resistant to proteolysis catalyzed by trypsin, chymotrypsin, pepsin and subtilisin, as indicated by total retention of β-amylase activity and identical electrophoretic patterns before and after incubation with these proteases.

Papain appears to catalyze rapid hydrolysis of sweet potato β-amylase, but no active fragments could be detected in these mixtures by paper electrophoresis experiments. The action of carboxypeptidase on sweet potato β-amylase increases the activity of the latter.

Experiments were also conducted in urea solutions which could conceivably unfold the β-amylase molecule and render it more susceptible to proteolysis. No digestion occurred with trypsin or chymotrypsin under such conditions; however, pepsin degraded the sweet potato β-amylase quite rapidly in 6 or 8 M urea solutions. The presence of active fragments in these mixtures could not be detected by paper electrophoresis experiments either.

Although active in 8 M urea solutions, sweet potato β-amylase was rapidly and irreversibly denatured, as shown by loss of activity and precipitation upon removal of the urea.

A complete amino acid analysis of sweet potato β-amylase indicated the presence of 10 to 11 cystine residues and 20 histidine residues per molecule. Aspartic and glutamic acids were present in the largest amounts. No tryptophan is present in sweet potato β-amylase.

The inhibition of sweet potato β-amylase was investigated with p-chloromercuribenzoate, iodoacetamide and *N*-ethylmaleimide. The fastest rate of inhibition was obtained with p-chloromercuribenzoate, and the slowest with iodoacetamide. The rate of inhibition by N-ethylmaleimide was intermediate. The inhibition of sweet potato β-amylase with N-ethylmaleimide was followed spectrophotometrically at 300 mμ. After reaction of one μmole of N-ethylmaleimide with one sulfhydryl group of the enzyme the inactivation was complete. However N-ethylmaleimide still reacted with other sulfhydryl groups of the molecule.

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I. HISTORICAL INTRODUCTION

Very little is known about the intimate structure of enzymes, the nature of active fragments of minimal size, or the amino acids sequences in the neighborhood of active sites. Much valuable information about the mechanism of enzyme action can be obtained by experiments carried out with native proteins; however it seemed reasonable to assume that information concerning active sites and the mechanism of enzyme action could be obtained more readily in an investigation of enzyme fragments still possessing some catalytic activity. Using specific endopeptidases one should be able to split an enzyme under mild, controlled conditions allowing the formation of fragments which conceivably could retain some activity. A crystalline β-amylase has been chosen for such a study.

Amylases are enzymes which catalyze the hydrolysis of starches and glycogens. In nature they act with other carbohydrases in carrying out the rapid breakdown of starches to sugars which can be utilized by the living cell. They occur in higher plants, molds, yeasts, bacteria and are also present in many secretions and tissues of the animal body. Moreover, they have been used as tools for elucidation of the structure of the substrates upon which they act. Much of the early information concerning the action of the amylases is contradictory, mainly because of impure preparations. However, as these enzymes have been crystallized, it has been possible to study their properties much more quantitatively.

The amylases of malted barley and wheat have been extensively studied by many of the early investigators. One of the first reports is that of Payen and Persoz (1) who noted that the action of an infusion of malted barley grain upon starch is attributable to the presence of a particular transforming agent which they named diastase (from its supposed property of separating the interior of the starch granules from the outer envelop and producing dextrins and sugars). The investigations of O'Sullivan (2, 3) gave information regarding the formation of glucose, maltose and dextrins when malt extracts reacted with starch. O'Sullivan's results and those of Marker (4), and Brown and Heron (5) showed that possibly two diastic ferments were present in extracts of malted barley: one producing maltose, and little dextrin, destroyable at low temperature, the other producing more dextrin and less maltose and destroyable at a higher temperature. Subsequently, these two ferments were called saccharogenic or β-amylase, and dextrinogenic or α-amylase. The saccharogenic amylases are characterised by their ability to liberate maltose rapidly without disrupting the remainder of the starch molecule. This maltose, in the β form exhibits a high dextrorotatory rotation, hence the name β-amylase (6, 7, 8).

An investigation (9) for the presence of amylases in a number of systems indicated that α-amylases are abundant in animals, plants, bacteria, fungi, but no β-amylase could be found in tissues of animal origin. Gore and Jozsa (10) tested a number of plants for the presence of "liquifying" and "saccharifying" diastases. They found α-amylase in soybean, peas, beans, lentils, corn, rice, rye, barley, wheat, potatoes, turnips, and carrots, whereas β-amylase was only present in soybeans, rye, barley, potatoes, and oats (11). At the present time it is recognised that β-amylases predominate in seeds (both germinating and ungerminated).

The increase in amylase activity which occurs during germination was attributed by early investigators to an actual synthesis of the enzyme. Baker (12) found two different diastases in ungerminated barley, and their action was different before and after germination. Ugrunow (13) observed that both α-amylase and β-amylase were present in the early stages of development of wheat kernels, and as maturation progressed α-amylase disappeared whereas β-amylase decreased. Upon germination, the reverse was noted, α-amylase developed early, and β-amylase increased to a maximum later on. Blish and Sandstedt (14) thought that amylases were not proteins, but associated with the latter, and liberated through the action of proteolytic enzymes. Myrback and Ortenblad (15) found that the amylase of grain is present under two different forms: one β-amylase active, and another inactive. The latter can be activated by the action of proteolytic enzymes, such as papain which doubles the activity. Hills and Bailey (16) confirmed the observation that papain increased the activity of ungerminated barley by 100 percent. This increase in β-amylase activity was due to proteolytic losses associated with a water insoluble material. Chrzasscz and Janicki (17) postulated the presence of an amylase inhibitor "sito-amylase." On germination of barley, the protein degradation products counteract the effect of "sito-amylase," and restore the full activity of the amylase. Several other attempts have been made (18) in order to explain this increase in amylase activity upon germination. From the more reliable information, it would appear that β-amylase exists in seeds and grains and is activated by the action of proteases, much in the manner of chymotrypsin and trypsin.

1. Isolation and Purification of β-Amylase

The β-amylases from different sources have been purified by many procedures. First of these was alcohol precipitation suggested by Osborne (19) in 1895, and also carried out by Sherman and Schlesinger (20), van Klingenburg (21) and Naylor (22). By repeated alcohol fractionations of extracts of barley malt, and fractionations with ammonium sulfate followed by dialysis, Sherman et al. (23) and Caldwell and Doebbeling (24) were able to obtain a preparation which had reasonable β-amylase activity. However, no α-amylase was present. Kneen and Hollenbeck (25) studied the differential stability of malt amylases, and observed that the α-amylase could be precipitated with a 56-68 percent alcohol concentration; nevertheless, an overlapping of solubility prevented a clear-cut separation of the enzymes. More useful was the technique of Waldenschmidt-Leitz and Purr (26) who found that the malt β-amylase could be selectively adsorbed on an alumina column at pH 3.8. One of the easiest methods to separate malt α-and β-amylases was noted by Ohlsson (27) who found that the α-amylase is completely destroyed by treatment of an acidified extract of malt, at pH 3.3 and 0° for fifteen minutes. Ohlsson also observed that malt β-amylase could be destroyed in the presence of the α-amylase by heating the neutral malt extract for fifteen minutes at 70°. Blum, Bak and Brae (28) used less drastic conditions for inactivation by heat and low pH and low temperature. These workers found that acidification of a malt amylase preparation at 20° and pH 4.2 resulted in an almost complete destruction of the α-amylase in four hours. Heating the neutral enzyme suspension at 60° for one hour destroyed the β-amylase activity of the preparation. Olson, Burkart and Dickson (29) completely inactivated the α-amylase of an aqueous malt extract at pH 3.3 and 0°; heating the same extract at 70° for fifteen minutes completely removed the β-amylase activity.

Using a combination of these techniques, Meyer, Fischer and Piguet (30) were able to crystallize malt β-amylase. As a starting material, these workers used a commercial malt extract, which was first treated at 0° and pH 3.6 to remove the α-amylase. This operation was followed by ammonium sulfate precipitation and acetone fractionation which finally gave a crystalline material having a constant activity, and possessing all the characteristics of a pure protein. However, prior to Meyer's crystallization of malt β-amylase, Balls, Walden, and Thompson (31, 32) had already obtained a crystalline preparation of a β-amylase from sweet potatoes. These workers fractionated an extract of sweet potatoes with ammonium sulfate and obtained a crystalline protein exhibiting a high activity.

Since α-amylases appear during germination, use of a nongerminated seed seemed to be more reasonable for the preparation of β-amylases.

Mayer, Spar and Fischer (33) prepared a crystalline β-amylase from freshly ground wheat flour by essentially the same procedure as that used for the preparation of malt β-amylase.

A comparison of the properties of the crystalline β-amylases from three different sources shows interesting relationships (33) (Table I).

TABLE I

PROPERTIES OF CRYSTALLINE β-AMYLASES

	Wheat	Malt	Sweet Potato
Percent Nitrogen	14.3	14.1	15.1
Sulfhydryl Groups	+	+	+
Optimum pH	5.3	5.2	4.8
Stability (pH Range)	4.5-9.2	4.5-8.0	
Isoelectric Point	6.0	6.0	4.8
Absorption Max. (m)	280	280	280
Inactivation by Metals	+	+	+

Soybeans have also been investigated for the presence of β-amylase. Martin and Newton (34) obtained a crude preparation from this source which, after precipitation with alcohol, gave a powder having the catalytic activity of a β-amylase. The same group further purified this preparation (35), and, recently, other workers obtained a crystalline β-amylase from soybean (36).

2. Methods for Determining β-Amylase Activity

The methods for the determination of β-amylases, are based upon the increase in reducing power of a solution of starch. Widely used is the method devised by Sandstedt (37) and modified by Kneen and Sandstedt (38) and later by Schwimmer (39). Basically, this method involves the reduction of ferricyanide by maltose, followed by treatment with potassium iodide and titration of the liberated iodine with thiosulfate. More recently, Noelting and Bernfeld (40) assayed the β-amylase activity by measuring colorimetrically the extent of reduction of 3,5-dinitrosalicylic acid by maltose. Polarimetric methods (41) and copper reduction (42) have also been proposed, but they do not appear to have come into wide use.

3. Mechanism of β-Amylase Action

Much of the early work with β-amylases was done to elucidate the structure of the starches. Crude preparations were used before the advent of crystalline enzymes. Such crude enzyme preparations contained varying traces of α-amylases and maltases. The results and conclusions from early investigations of the mechanism of β-amylase action are open to question. In the following discussion, no attempt will be made to distinguish between the specific β-amylases, since they all operate in essentially the same fashion. The mode of action of β-amylase can be summarized as follows: Only α 1,4-glucosidic linkages are attacked. Beta maltose is the only low molecular weight product of the action of β-amylases (6, 7, 43). Several lines of evidence indicate that the attack by β-amylases starts at the nonreducing end of the starch molecule. Maltodextrins oxidized by hypoiodite (44, 45) or by mercuric oxide (46) can be hydrolysed by β-amylases, to give maltose, and a dextrinic acid of lower complexity. Methylation studies (47) show that β-amylase limit dextrin of amylopectin contains all of the end groups and all branching points found in the original amylopectin molecule.

Pertinent to this is the work of French and Summer (48) who studied the action of β-amylase as it approaches a 1,6-linkage. Branched oligosaccharides of low molecular weight were prepared and subjected to the action of β-amylase. The qualitative study of the action of β-amylase in various oligosaccharides analogous to the branch points in amylopectin led to the conclusion that the β-amylase action stops before the glucose unit next to a 1,6 branch. These results are summarized in Table II.

TABLE II

ACTION OF β-AMYLASE ON BRANCHED OLIGOSACCHARIDES

Non-Resistant Arrangements	Resistant Arrangements
0-0 0-0—0	0 - 0 - 0 - 0
0-0-0- 0-0	0 - 0 - 0 - 0 - 0
0-0- 0-0-0-0	0 - 0 - 0 - 0 - 0
0-0-0- 0	0 - 0 - 0 -
0- Glucose with its reducing group 0-0- 2 glucoses linked 1,4.	0 - 0 - 0
0- - 0 2 glucoses linked 1,6.	

This investigation confirms the earlier observation that maltotriose is not attacked by β-amylase (49, 50). However, as French pointed out, model substrates of low molecular weight do not necessarily react in the same way as the naturally occurring products of high molecular weight.

Another aspect of the mode of action of β-amylase has been in dispute. Considered for the amylose kind of starch, the mechanism may be of a "single-chain" type, where the enzyme attacks one amylose molecule and degrades it completely before attacking another. On the other hand, it may be a "multiple-chain" type in which all the amylose molecules are shortened in a random attack. After careful review of the evidence available, it appears that in cases where crystalline β-amylases are used the action may be intermediate between these two mechanisms. French et al. (51) observed that the complete hydrolysis of amyloheptaose liberates two molecules of maltose and one of maltotriose. No amylopentaose found during the course of the hydrolysis. Kerr and Cleveland (52) also obtained data which they consider as favoring the "single-chain" mechanism. No dextrins of intermediate size could be found in hydrolysates of amylose; the partially hydrolyzed samples had the same degree of polymerization as the original amylose. Greenwood and co-workers (53) also presented good evidence of a "single-chain" action. No intermediate fragments could be identified in the reaction mixtures on examination of the iodine affinity of the amylose and its sedimentation constant. This action is constant with the remarkably high turn-over number (250,000) reported for this enzyme (54). However, Bailey and Whelan (55) investigated the action pattern of β-amylase under a variety of conditions using as substrates two maltodextrins and a synthetic amylose of average chain length of 49 units. With all the substrates and under all conditions the action pattern was always intermediate between single and multichain. Their results show that the enzyme removes several maltose units during each encounter with a substrate molecule. These authors suggest either that the enzyme molecule contains several active centers or that multiple reaction can occur at a single active center. A polysaccharide having an average chain length of 44 glucose units was synthesized by Bailey and French (56), and they labelled the nonreducing end with C^{14}. Brief action by β-amylase on the synthetic polysaccharide gave radioactive maltose, and their results indicate that the enzyme removes 4.3 maltose units per effective enzyme-substrate encounter. The possibility of multiple active centers acting simultaneously on a single substrate molecule was eliminated by use of an interiorly labelled glucan of the amylose type. From this work it follows that the action pattern of β-amylase depends upon the relative values of the kinetic constants which govern the formation, dissociation and reaction of the intermediate enzyme-substrate complex. Therefore completely "multichain" or completely "single-chain" action are extreme cases of a common action pattern.

Another aspect of the action of β-amylase is the stereospecificity. Using O^{18} as a tracer, it has been possible (57, 58) to demonstrate that both α- and β-amylase cleave a α 1,4-glucosidic bond of starch or glycogen on the C1 side. The O^{18} content of the product, maltose, corresponded to approximately 90 percent of the theorical incorporation from the medium. In order to account for the inversion of configuration of the products with β-amylase, Koshland (59) proposes that β-amylase facilitates a direct displacement at the potential aldehyde carbon with a SN_2 type reaction. An alternate mechanism could be as follows: the initial step in hydrolysis is protonation of the bridge oxygen, forming an oxonium ion which is cleaved at the C1 side, and the resulting carbonium ion is stereospecifically hydrated. This mechanism is in agreement with acetal hydrolysis (60).

4. Active Cores of Enzymes

The integrity of protein structure for biological activity of enzymes has been a puzzling question for many years. However, at the present time, there appears to be some evidence which indicates that proteins can be modified in several ways and still retain some of their biological activity. So far, the best tools to modify proteins have been the proteolytic enzymes. Acting under mild and controlled conditions, one should be able to split specifically certain bounds in the protein molecule with these reagents and be able to isolate fragments possessing at least part of the activity of the original protein.

That active enzyme fragments can be isolated was first claimed by Bresler *et al*. (61). These workers autolyzed a crystalline trypsin solution and separated the peptides produced by ultracentrifugation. These fragments, possessing a molecular weight between 2000 and 3000, retained five to ten percent of the original proteolytic activity. The same group of workers was also able to demonstrate that active fragments can be obtained upon trypsin digestion of aldolase. Perlmann (62) reported the formation of enzymatically active, dialyzable fragments during the autodigestion of pepsin. However, Williamson (63) in attempting to repeat this work found the active fragments in the nondialyzable fraction. Chernikov (64) also reported finding dialyzable active peptides from the autolysis of trypsin, chymotrypsin and pepsin. Recently, Hess and Wainfan (65) found some enzymatically active components in trypsin autolysate, showing that there was produced in small quantities a proteolytic enzyme smaller than trypsin.

The digestion of cytochrome c with pepsin has been studied (66). From this a product of relatively low molecular weight was obtained. The "pepsin modified" cytochrome c contains the prosthetic group of the enzyme linked to a peptide which represents part of the original protein moiety of cytochrome c. The hemopeptide exhibits interesting enzymatic properties, different from those of native cytochrome c. The fragment is inactive in both the succinic oxidase and the cytochrome oxidase systems. But it strongly catalyzes the oxidation of ascorbic acid. Catalase (67) has also been digested with pepsin. This treatment results in a progressive inactivation of the catalytic activity of catalase; however, the peroxidatic activity toward pyrogallol increases. The "pepsin-modified" catalase was isolated and further identified as a protein of fairly high molecular weight. It is interesting to note that even though the specificity toward hydrogen peroxide is lost through digestion, some of the more general oxidative properties appear.

As a result of proteolysis with subtilisin, Richards (68) has been able to remove a peptide from the N-terminal end of the ribonuclease molecule. The peptide and the reduced protein separately have less than five percent of the original activity of the enzyme. Under certain conditions, recombination of the two fragments results in full restoration of ribonuclease activity. A distinction is usually made between enzymes composed solely of amino acids and those containing a nonamino acid prosthetic group. The separation of the ribonuclease-subtilisin system into an inactive protein and a dialyzable highly specific "cofactor" suggests that this distinction may not be so sharp as supposed. It should be of interest to examine the possibility that the peptide fragment of ribonuclease contains the active site and the remainder of the protein serves as an "apoenzyme."

Although not as striking as enzyme modifications by endopeptidases, enzymes may be modified through the use of exopeptidases. This further illustrates that the integral protein is not always necessary for biological activity. Carboxypeptidase (69) liberates three amino acids from the chymotrypsin molecule without altering its activity. However, more significant is the work of Smith with leucine aminopeptidase (70) on papain. The N-terminal portion of mercuripapain is extensively degraded by leucine aminopeptidase, and, although about one-half to two-thirds of the 180 amino acids can be removed, there is no loss in proteolytic activity after reaction with cysteine and versene. The substrate specificity does not change, and the sum of the amino acids removed and those in the residual, degraded enzyme is in essential agreement with the composition of the native protein. Further experiments indicate that some of the fragments produced during autolysis of papain are active (71).

The above experiments indicate the possibility of degrading enzymes while conserving part of their activity in fragments of minimal size. Isolation and characterization of the active fragments should provide valuable information concerning the nature of the amino acids at the active site and lead to the synthesis of model enzymes. With these ideas in mind, sweet potato β-amylase was digested with several endopeptidases of known and varied specificity with the hope that it might be possible to isolate some fragments still possessing some amylolytic activity.

II. EXPERIMENTAL

1. Apparatus

Spectrophotometers.-- The Beckman B model spectrophotometer and 1-cm Corex cells were used for absorbance measurements in the visible range. Absorbance measurements in the ultraviolet were made using the Beckman model DU spectrophotometer with 1-cm quartz cells.

Electrophoresis.-- The Tiselius electrophoresis apparatus (Model 138, Perkin Elmer Corp.) was used for moving boundary electrophoresis studies. For the conductivity measurements, an Industrial Instruments Co. conductivity bridge (Model RC-1 B) equipped with a cell of 0.4893 constant was used.

For paper electrophoresis, the type LKB 3276 Paper Electrophoresis Equipment was used, (supplied by Ivan Sorvall Inc.). The paper strips were from Schuster and Schuell, 40 by 410 mm.

pH Meter.-- A Beckman Model H2, glass electrode, line operated pH meter was used.

Sterile Apparatus.-- All proteolytic digestions of longer than six hours duration were carried out in a sterile apparatus and the digestion mixtures were sterilized by filtration. The apparatus consisted of a Pyrex bacterial filter attached to a 35-ml filter tube which was fitted with a capillary to allow the withdrawing of samples during digestion without contamination. The apparatus was sterilized at 15 lbs. pressure for 15 minutes in the autoclave before every run.

Dialysis.--A Visking Cellophane tubing was used.

Paper Chromatography.-- The Chromatocabs Model B (Research Equipment Corp.) was used for descending runs. Ascending chromatograms were developed in the chromatography apparatus manufactured by University Apparatus Co. The chromatograms were dried in the Research Equipment Corp. oven constructed for this purpose.

2. Reagents

Amylase.-- The sweet potato β-amylase was a twice crystallized preparation obtained from Worthington Biochemical Corp.

Proteolytic Enzymes.-- Crystalline chymotrypsin, (salt-free), trypsin, twice crystallized (containing 50 percent magnesium sulfate), pepsin (twice crystallized) were all obtained from Nutritional Biochemical Corp. The crystalline papain suspension, in versene-cysteine buffer, and the carboxypeptidase (twice crystallized in 10 percent lithium chloride) were obtained from Worthington Biochemical Corp. Subtilisin was a gift from Eli Lilly Co.

The potato amylopectin was prepared from potato starch according to the procedure of Th. Schoch (69) and Noelting et al. (40).

Veronal Buffer pH 8.6.-- This buffer used in the paper electrophoresis experiments was prepared by dissolving:

8.712 g. Veronal

1.872 g. Sodium hydroxide

6.476 g. Sodium acetate

60 ml. 0.1 M Hydrochloric acid

and the total volume made up to 1 l. with water.

Staining solution for Proteins.— Into 500 ml. of ethanol saturated with mercuric chloride is dissolved 0.5 g. of Bromphenol Blue.

Reagent for Detection of Maltose.— The reagent used to detect maltose in the paper electrophoresis experiments was prepared according to Bachan and Savage (81).

4 Percent Aniline in 95 percent ethanol	5 vol.
4 Percent diphenylamine in 95 percent etha.	5 vol.
85 Percent phosphoric acid	1 vol.

All other reagents used were of the C.P. or reagent grade quality, unless otherwise specified.

3. Assay of β-Amylase Activity

The β-amylase activity was determined by the method of Noelting and Bernfeld (40, 82), wherein the colored reduction product from the reaction of maltose and 3,5-dinitrosalicylic acid is measured spectrophotometrically at 540 mμ.

Reagent.— Ten grams of 3,5-dinitrosalicylic acid was moistened with a few drops of water, then 200 ml. of 2 N sodium hydroxide was added and diluted to 500 ml. with water, after complete solution at room temperature, 300 g. of Rochelle salt was added and the volume brought to 1 l. with water. This solution was stable in the absence of carbon dioxide from the air.

Procedure.-- For all experiments, unless otherwise specified, the assay procedure was carried out in the following manner: 0.1 ml. of the enzyme solution to be assayed and 0.9 ml. of redistilled water were placed into a 25-ml. glass-stoppered volumetric flask which was equilibrated in a water bath at 20° C. One milliliter of a 1 percent amylopectin solution was added to this, and the reaction was allowed to proceed for 180 seconds. The reaction was then stopped by addition of 2 ml. of the 3,5-dinitrosalicylic acid reagent. Following this, the flasks were placed in a boiling water bath for 5 minutes. The resulting solutions were then cooled and diluted to 25 ml. with water. The absorbance was read at 540 mu against that of a blank prepared in a similar fashion, but in which the enzyme solution was replaced with water.

4. Maltose Standardization

A maltose stock solution was prepared by dissolving 101.6 mg. of maltose hydrate in 100 ml. of water. One ml., 2 ml., 3 ml., and 5 ml. aliquot respectively were diluted to 10 ml. and one ml. of each diluted solution was used for the standardization of the reagent.

5. Tryptic Digestion of Sweet Potato β-Amylase

The crystalline β-amylase was removed by means of a spatula, and 0.234 g. placed into a test tube. This was dissolved in 7.5 ml. of phosphate buffer (pH, 7.0, 0.01 M) and placed in a dialyzing membrane. Dialysis was then allowed to proceed against the same buffer as was used for dissolving the sample, for 18 hours at 4°C. At the end of this time the volume was made up to 10 ml. with the 0.01 M phosphate buffer, and a 5-ml. sample was removed for determination of the electrophoretic pattern of the β-amylase preparation. To the remainder of the solution was added 0.01 g. of crystalline trypsin. The tryptic digestion was allowed to proceed in the sterile apparatus for 20 hours at 30°C. At the end of the digestion period, a sample was removed for assay of activity and the remainder was dialyzed in the cold room against 300 ml. of phosphate buffer and used for the electrophoretic analysis.

6. Chymotryptic Digestion of Sweet Potato β-Amylase

A 0.212 g. sample of sweet potato β-amylase was dissolved and made up to 5 ml. with phosphate buffer (pH 7.0, 0.1 M). To this solution was added 0.01 mg. of chymotrypsin. A sample of this mixture was removed immediately for assay of β-amylase activity and the remainder of the solution was placed in the sterile apparatus and incubated at 30°C for 12 hours. Another sample was then removed for assay of β-amylase activity, and the remainder was equilibrated against phosphate buffer for the electrophoretic analysis.

7. Peptic Digestion of Sweet Potato β-Amylase

A 0.175 g. sample of sweet potato β-amylase was dissolved into 6.5 ml. of acetate buffer of pH 5.10 (0.05 M) and a sample was removed for assay of activity. After addition of 0.010 g. of pepsin, the solution was placed in the sterile apparatus. Digestion was allowed to prosecd for 12 hours at 30°C. After removal of a sample for assay, the remainder of the solution was dialyzed against 300 ml. of phosphate buffer (pH 7.0, 0.1 M) for use in the electrophoretic analysis.

Experiments were also performed at a lower pH where pepsin is more active. At pH 3.0, the sweet potato β-amylase was inactive and precipitated from the mixture, but, upon digestion with pepsin, the precipitate dissolved. No activity could be detected in this mixture, and paper electrophoretic experiments indicated that the protein had been broken down considerably.

8. Papain Digestion of Sweet Potato β-Amylase

Preliminary experiments with papain indicated that digestion of sweet potato β-amylase might take place as indicated by a decrease in activity. However these experiments were not always reproducible. A new sample of papain was obtained, and more precise determinations were made. The preparation of the samples was carried out according to the procedure of Kimmel and Smith (83).

A citrate buffer solution was prepared by dissolving 2.94 g. of sodium citrate dihydrate in a few ml. of 0.1 N HCl until the pH became 6.0, and then diluting to 100 ml. A cysteine-versene solution was also made up from 0.605 g. of cysteine and 1.46 g. of di-sodium ethylenediamine tetraacetate diluting to 100 ml. The digestion mixture was prepared by mixing 2.5 ml. of the citrate buffer, approximately 5 mg. of the β-amylase, 1 ml. of the versene-cysteine solution, 0.3 ml. of the papain suspension and 1 ml. of redistilled water. A sample of this mixture was removed before the addition of the papain, and used as a control in the assay and paper electrophoresis experiments. The solution was then incubated at 30°C in the sterile apparatus. The results of these experiments are reported in Tables IX and X.

In order to study the effect of the addition of fresh papain to a sample which had already been digested with papain, the following experiment was carried out as described above, but after some digestion had taken place, as indicated by a decrease in activity, 0.1 ml. of fresh papain suspension was added after 24 hours, and the β-amylase activity of the mixture determined. The results of these experiments are reported in Table VII.

9. Paper Electrophoresis Experiments

Paper electrophoresis was utilized in a number of experiments because it affords a means of separating protein and peptide components of such mixtures on a very small scale. It is also possible to determine the enzymatic activity of the components directly on the paper.

Procedure for Paper Electrophoresis.— Six hundred ml. of veronal buffer pH 8.6 (0.125 M) was placed into each electrode vessel and a liquid junction established between the vessels by means of a siphon to equilibrate the liquid levels. The paper strips were immersed in the buffer, allowed to dry in the air for 5 minutes, then placed on the casset. The cover was then placed on the apparatus and the system allowed to stand for 15 minutes in order to bring it to equilibrium. Twenty- to 30-ml. samples were applied on each strip by means of the sample applicator provided with the instrument. Usually a current of 3 mA per strip was applied for 12 to 16 hours for the electrophoresis.

Protein staining.-- After removal of the strips, they were cut in half lengthwise and one section dried in the oven at 110°C for 30 minutes. After this, the strips were placed in the bromphenol blue staining solution for 10 minutes and stirred occasionally. The strips were then washed in a 2 percent acetic acid solution and dried in the air. The protein appeared as a blue band.

Assay of Activity on Paper.-- The other section of the strip usually was used for assay of the β-amylase activity. The paper strip was first allowed to dry at room temperature. It was then sprayed with a 1 percent starch solution and allowed to dry in the air. Following this, the strip was sprayed lightly with the diphenylamine-aniline reagent. After drying in an oven for 2 to 3 minutes at 110°, a brown band, indicating the presence of maltose, appeared where active β-amylase was present.

10. Experiments with Subtilisin

The procedure used here was that of Sinn, Behrens and Bromer (84). Approximately 5 mg. of sweet potato β-amylase was dissolved in 10 ml. of acetate buffer (pH, 7.7, 0.05 M), and a sample removed for assay of activity. Two mg. of subtilisin was then added to the mixture and the solution incubated at 30°C. Samples were removed from time to time and assayed according to the usual procedure. The results are reported in Table X.

11. Experiments with Carboxypeptidase

Carboxypeptidase is an exopeptidase from the pancreas which catalyzes the hydrolysis of peptide bonds at the carboxyl terminus of proteins and peptides. Its action on sweet potato β-amylase was studied utilizing the procedure of Fraenkel-Conrat et al. (85).

Preparation of the Carboxypeptidase Solution.-- One tenth ml. of the crystalline carboxypeptidase suspension was dissolved in a few ml. of 0.01N NaOH, and phosphate buffer (pH, 7.5; 0.1 M) was added until the volume of the solution was 25 ml. One ml. of the resulting solution contained approximately 0.2 mg. of enzyme.

Amino Acids Determination.-- For the amino acid determination 1 ml. aliquots were removed and 2 ml. of trichloroacetic acid added. The precipitated protein was centrifuged from the mixture. One ml. of the supernatant solution was placed in a 25-ml. volumetric flask and 1 ml. of the ninhydrin-hydrindantin reagent (90) described elsewhere was added to this. The flasks were then placed in a boiling water bath for 15 minutes. After cooling, they were diluted to the mark with 50 percent ethanol and the optical density read at 570 uu against that of a blank.

Test of Carboxypeptidase Activity.-- The activity of the carboxypeptidase was tested on casein in the following manner. Into a tube were placed these solutions:

3 ml. of a 6 percent casein solution in phosphate buffer pH 7.4.

2 ml. phosphate buffer pH 7.5.

1 ml. carboxypeptidase solution.

This was incubated at 30°, and one ml. aliquots were removed after 30, 60, and 140 minutes. Amino acid determinations were carried out on these samples as indicated above. The results are given in Table XI and they indicate that, under these conditions, the carboxypetidase is active.

Action of Carboxypeptidase on β-Amylase.-- Prior to treatment with carboxypeptidase, the β-amylase sample was extensively dialyzed against phosphate buffer (pH, 7.5; 0.1 M) at 2°, in order to remove most of the ammonium ion which interferes with the amino acid determination. To the dialyzed β-amylase solution was added 1 ml. of the diluted carboxypeptidase and the volume was made up to 10 ml. with phosphate buffer. This mixture was then incubated at 30°. Samples were removed at regular intervals for assay of activity and amino acid determination. Also, in order to check whether or not carboxypeptidase autolyses, a control was set up in the following manner: 1 ml. of the carboxypeptidase solution was made up to 10 ml. with the phosphate buffer and allowed to stand under the same conditions as the reaction mixture. The results of the experiments with carboxypeptidase are reported in Table XII.

12. Proteolysis and Denaturation of Sweet Potato β-Amylase in Urea Solutions

Denatured proteins are usually more susceptible to the attack of proteolytic enzymes than are native proteins (86). For this reason, the effect of urea on sweet potato β-amylase during proteolysis was investigated with the hope that under these conditions the β-amylase might be less resistant to proteolytic attack. The effect of urea solutions alone on this β-amylase was also investigated.

Preliminary experiments in 8 M urea indicated that sweet potato β-amylase is active under these conditions but that it is denatured in approximately 6 to 8 hours, depending upon the concentration of the enzyme. The reversibility of the reaction was studied in the following manner: approximately 5 mg. of sweet potato β-amylase was dissolved in a solution which was 8 M in urea and 0.05 M in acetate. The pH of this mixture was 5.0. The β-amylase activity of this solution was determined immediately after mixing and again at intervals during a period of standing at 30° of a number of hours. The remainder of the solution was placed in a membrane and exhaustively dialyzed against acetate buffer (pH 5.0, 0.05 M) at 2° to remove the urea. The outside solution was changed several times during the course of dialysis. A white precipitate appeared at the bottom of the dialysis bag indicating that the denatured protein is insoluble in buffer. The results of this experiment are reported in Table XXIII.

Peptic Digestion in 8 M Urea.-- To 5 ml. of 8 M urea was added approximately 5 mg. of sweet potato β-amylase. One ml. of this solution was removed for control, and 1 mg. pepsin was added to the remainder. Both the digestion mixture and the control were incubated at 30°C. Samples were removed for assay of activity, and 1 ml. aliquots were added to 2 ml. of 7.5 percent trichloroacetic acid. The supernatant solutions from the trichloroacetic acid precipitation mixtures were for absorption measurements at 280 mu in order to check for proteolytic action. The results of this experiment are reported in Table XUV.

Experiments in 6 M Urea.-- Since experiments in 8 M urea indicated a rapid denaturation of sweet potato β-amylase, the effect of 6 M urea was studied.

To a 10 ml. solution of 6 M urea was added approximately 5 mg. of sweet potato β-amylase, and the activity determined at different intervals to study the denaturation under these conditions. The results are reported in Table XXV.

Peptic Digestion in 6 M Urea.-- As indicated by the previous experiments, there is a slow denaturation of β-amylase upon treatment with 6 M urea. Consequently, the urea denaturation along with peptic digestion were studied at this urea concentration also. To 10 ml. of a 6 M urea solution was added approximately 5 mg. of sweet potato β-amylase. A 4 ml. aliquot of the urea-β-amylase solution was removed, and to this sample was added 2 ml. of a pepsin solution containing 6 mg. of pepsin. Both solutions were incubated at 30°C. and samples were removed at regular intervals to determine the β-amylase activity. Several runs carried out in this manner gave the results shown in Table XVII.

Peptic Digestion in 4 M Urea.-- Since the actual urea concentration in the previous experiments was 4 M several experiments in 4 M urea were carried out. To 2 ml. of 4 M urea was added approximately 5 mg. of sweet potato β-amylase, 1 ml. was taken out for control, and to the remainder was added 1 mg. of pepsin. Both solutions were incubated at 30°C, and samples taken out at different periods of time. The results are given in Table XXVII. Paper electrophoresis was also carried out on a number of these samples.

Chymotryptic Digestion in 6 M Urea.-- To 5 ml. of a solution of 6 M urea was added approximately 5 mg. of sweet potato β-amylase, and one ml. taken out for assay and control. One mg. chymotrypsin was added to the remainder, and both solutions were incubated at 30°C. Again, samples were removed for β-amylase assay at regular intervals. The results of this experiment are shown in Table XVIII.

Tryptic Digestion in 6 M Urea.-- An experiment similar to the one described for chymotryptic digestion was performed using trypsin instead of chymotrypsin. This gave the results recorded in Table XIX.

Heat Stability of Sweet Potato β-Amylase.-- An experiment was performed in order to determine the stability of sweet potato β-amylase to heat, and the possibility of combining a heat treatment in conjunction with proteolysis.

Approximately 5 mg. of sweet potato β-amylase was dissolved in 5 ml. of acetate buffer pH 5.0 (0.1 M). The activity of this solution was determined. A sample of the mixture was then heated in boiling water for 5 minutes after which the β-amylase activity was determined again. The remainder of the unheated mixture was placed in a water bath at 70°C for 6 minutes, and this solution was also assayed for β-amylase activity after cooling. To this solution, preheated at 70°, was added 1 mg. trypsin. The resulting mixture was incubated at 30°C for 18 hours. The results of this experiment are given in Table XX.

13. Paper Chromatography

In order to account for some of the results obtained from the action of proteolytic enzymes on sweet potato β-amylase, it became necessary to study the composition of this enzyme. This was done first qualitatively by paper chromatography.

Preparation of the Sample.-- The sample of sweet potato β-amylase was hydrolyzed for paper chromatography according to the method of Cowgill and Pardee (87). About 5 mg. of sweet potato β-amylase was dissolved in 0.1 ml of constant boiling, glass-distilled hydrochloric acid and placed in a Pyrex tube (internal diameter = 4 mm.). The tube was placed in ice-water, evacuated at the water pump and sealed. Hydrolysis was allowed to proceed for 12 hours at 115° in an oven. After this treatment, the hydrolysate was light yellow with very small traces of black material. The hydrolysate was then evaporated to dryness in a vacuum desiccator over sodium hydroxide. One ml. of water was added to the residue, and this solution was again dried in the desiccator. The dried sample was taken up in 1 ml. of 10 percent isopropyl alcohol, and stored in the refrigerator until needed for paper chromatography.

Solvent Systems.-- After a few experiments with various solvents, the phenol-water and butanol-acetic acid-water systems were found to give the best results in terms of resolution and general quality of the chromatograms. The phenol-water mixture was prepared according to Berry *et al*. (88). In order to obtain the best results it was necessary to redistil the phenol at reduced pressure. One hundred g. of phenol and 20 ml. of an aqueous solution containing 6.3 percent sodium citrate and 3.7 percent sodium dihydrogen phosphate were then mixed to prepare the aqueous solution. The butanol-acetic solution was prepared by mixing redistilled n-butanol, water, and glacial acetic acid in a 4:5:1 ratio, respectively. The aqueous phase of this mixture was used to equilibrate the chamber, and the organic phase used for resolution. This solvent was prepared fresh before every run.

Resolution.-- Whatman No. 1 filter paper sheets were cut to form a square 18x18 inches. Lines were drawn 1 inch from one of the edges, and 3 inches from an adjacent edge. The sample was applied at the junction of the lines. For the hydrolysates, a 10 sample was applied in several portions, maintaining the spot 4 mm. in diameter. Solid mixtures of 0.5 mg. of each amino acid were dissolved in 10 percent isopropyl alcohol, and run on separate chromatograms in order to compare the Rf values.

Usually the chromatograms were first placed in the phenol solvent and allowed to run in ascending fashion for 24 to 30 hours. In this manner, the solvent travelled about 25 cm. from the origin. After this treatment, the papers were dried in the draft oven at room temperature for 12 hours. Then, they were placed in the other cabinet for descending chromatography in butanol-acetic acid for 10 to 12 hours. After drying the chromatograms, they were sprayed with a 0.2 percent solution of ninhydrin in 95 percent ethanol with 5 percent collidine. The spots for the amino acids appeared after heating for 5 minutes in the draft oven at 90°C.

In later experiments, it was found preferable to run the chromatograms in butanol-acetic acid first in a descending fashion, allowing the solvent run to the end of the paper. After drying, the resulting chromatograms were run in phenol water. The best chromatograms were obtained by this latter method.

Attempts were made to use other solvent systems, such as collidine, lutidine but were not found satisfactory because of poor resolution and streaking. The results of the paper chromatography are given in Table XXI.

14. Chromatography of Amino Acids

Apparatus.— A Technicon Fraction Collector was used to collect the effluents from the columns. A Beckman B Spectrophotometer equipped with an adapter for test tubes was used for absorbance determinations.

Test Tubes.— The standardization of the test tubes was carried out according to the procedure of Stein and Moore (89). A solution of methyl red in 3 N hydrochloric acid was prepared to give an optical density of 0.6 to 0.7, using water as a blank. Three hundred test tubes, soft glass, 150x16 mm. were obtained and checked so that they would not

vary by more than ± 0.005 optical density readings.

Circulating Bath.— A Precision Scientific Co., (Chicago, Ill.) circulating water bath was used to maintain the columns at 50°C during the course of a run.

Columns.— A jacketed column 165 cm. long, with an inner diameter of 0.9 cm. was used for the chromatography of the neutral and acidic amino acids. For the determination of the basic amino acids, a condenser with an inner diameter of 0.9 cm. and fitted with sintered plate was used. It was packed with resin up to the desired height.

Reagents.— The Amberlite CG-120, type 2 (200 mesh) and the ethylene glycol monomethyl ether were purchased from Fischer Scientific Co., Thiodiglycol was obtained from Pierce Chemical, Rockford, Ill. The BRIJ 35 (polyoxyethylene lauryl alcohol) wetting agent was obtained from the Atlas Powder Co. Ninhydrin was purchased from the Nutritional Biochemical Corp.

The hydrindantin was prepared according to the procedure of Stein and Moore (90). To 20 g. of ninhydrin in 500 ml. of water at 90° was added 20 g. of ascorbic acid in 100 ml. of water at 90°, with stirring. Crystallization was then allowed to proceed for 30 minutes without further heating. After this, the solution was cooled and the crystalline hydrindantin was collected on a glass filter and dried over phosphorus pentoxide *in vacuo*. The yield was 18.75 g.

The ninhydrin reagent used for amino acids determination was prepared by dissolving 2 g. of ninhydrin and 0.3 g. hydrindatin in 75 ml. of methyl cellosolve. To solution was added 25 ml. of 4 M acetate buffer (pH, 5.25). This reagent was usually freshly prepared each day.

Preparation of the Resin.-- In order to obtain the best results, only resin particles of the appropriate size were employed for the chromatography. The procedure used for classification of the resin particles was that reported by Moore et al. (91) with a few modifications. One lb. of resin was suspended in 10 l. of distilled water, stirred and allowed to settle for 5 to 6 hours. This treatment was repeated three times. Each time the supernatant liquid was removed by siphoning. The resin was then transferred to a large Buchner funnel and rinsed slowly with 2 l. of 4 N hydrochloric acid. This treatment was followed by washing with 500 ml. of distilled water. The resin was then suspended in 2 l. of 4 N sodium hydroxide and heated on a steam bath for one hour. Finally, the resin was collected on a Buchner funnel and rinsed with distilled water until the washings were neutral. For the separation of the resin, a 2-l. separatory funnel was attached to a stop-cock which was in turn joined to a distilled water tap. The resin was poured into the funnel and a piece of glass tubing, fitted with a stopper, was placed on top of the funnel. Water from the tap was allowed to run through the funnel from the bottom and out the top, the flow rate being controlled by means of the stop-cock. The suspension of resin in the overflow was collected on a Buchner funnel. The conditions for separation are tabulated below.

Flow Rate	Time of Flow, Hours	Use
113 ml./min.	3	Discarded
280 ml./min.	2	Used this fraction for the 15-cm. column.
580 ml./min.	2	Used this fraction for the 150-cm. column.

Both the 280 and the 580 fraction were run through this procedure again, and then each was washed on a Buchner funnel with 1 l. of 4 N sodium hydroxide until the filtrate was alkaline. This operation was followed by washing with 2 l. of water. All resin fractions were stored in the refrigerator under 0.2 N sodium hydroxide.

Buffers.— Each of the buffers (pH, 3.25 and 4.25) were prepared in 18 l. quantities and stored in the cold room. The pH 5.28 buffer was made in 5 l. quantity. The BRIJ 35 wetting agent and thiodiglycol were added in the amounts required just before use.

Preparation of the Ion Exchange Columns.— The sodium salt of the resin was washed first with water and then with several hundred ml. of 0.2 M buffer at pH 4.25. Both columns were poured with a suspensions of resin in this buffer. In preparing the 150-cm. column, 90 ml. of the suspension was poured and 2 cm. of resin allowed to settle under gravity. After this, air pressure was applied at the top of the column (6 lbs./sq. in.). This gave a section of resin approximately 30 cm. in length. This procedure was then repeated, removing the supernatant liquid each time by siphoning. When the column had reached 150 cm., 0.2 N sodium hydroxide was allowed to flow through the column under gravity overnight. Then the buffer of pH 3.25 was substituted and allowed to flow until the column had reached equilibrium. The column was then ready for use.

The 15-cm. column was poured in a similar manner in one section. Before use it was equilibrated with buffer at pH 5.28.

Operation of the Column.— In order to check the flow rate and the resolving power of the column, a synthetic mixture of amino acids was prepared by weighing out one millimole each of fourteen amino acids. These were dissolved together in 3 ml. of 6 N hydrochloric acid, and the solution was diluted to 10 ml. with water. This stock solution was kept in the refrigerator. When ready for use, 1 ml. of the stock solution was diluted to 100 ml. with buffer (pH, 3.25) and 1 ml. of the resulting solution placed on the column. The circulating bath was turned on and the temperature of the column kept at 50°. One ml. of the amino acid mixture containing 1 um. of each amino acid was placed on the resin and allowed to settle under gravity. Then 2 ml. of buffer pH 3.25 was also added and again allowed to settle under gravity, after which the column was connected with the separatory funnel containing the buffer, and air pressure of 3 pounds per square inch was applied to the system. The effluent was then collected in 2-ml. fractions. At first a flow rate of 17 to 18 ml. per hour was obtained, but in the later runs, after the resin had settled the rate of flow dropped to 12 to 14 ml. per minute. This diminution in flow rate did not seem to affect the resolution of amino acids by the column.

An eluent at pH 4.25 was introduced in time to allow valine to emerge with the new buffer. According to Stein and Moore (91) this change is to be made at an effluent volume 2.15 times that at which the aspartic peak has emerged. Stein and Moore also noted that the position of cystine is extremely sensitive to pH and that it is necessary to determine empirically the exact pH value of the initial buffer so that cystine will be eluted about midway between alanine and valine. In previous work (92) Moore and Stein suggested that at a higher pH cystine is eluted with alanine and at a lower pH cystine appears nearer valine. a pH range from 4.0 to 4.5 was tried but without success. Later in runs with hydrolysates it was found desirable to elute with the buffer pH of 3.25 for a longer period than that recommended by Stein and Moore. The best separations between cystine and valine were obtained when the buffer at pH 4.25 was introduced at an effluent volume 2.15 times the peak of aspartic acid plus 30 to 40 ml. When this was done, valine was eluted slowly before cystine, and a good separation of valine and cystine was obtained. This procedure did not affect the resolution of the other amino acids.

After the elution of phenyl alanine, 0.2 N sodium hydroxide was allowed to run through the column overnight under gravity at room temperature. Then buffer at pH 3.25 was again introduced. After equilibrium was established the column was again ready for use.

Leucine Standard.-- A leucine standard solution was prepared by dissolving 0.131 g. of L-leucine in redistilled water and making up the volume to 10 ml. One ml. of this stock leucine solution was diluted to 200 ml. and 0.5 to 2 ml. aliquots were taken for preparation of the standard curve. Similarly, aliquots of 1 to 1,000 dilution of the stock solution were used for L-leucine samples in the 0.05 to 0.2 mole range. Each aliquot was made up to 2 ml. volume, and 1 ml. of the ninhydrin-hydrindantin reagent was added to each. The test tubes (calibrated curvettes) were then placed in a boiling water bath for 15 minutes. After cooling, 10 ml. of 50 percent ethanol was added to each tube, and the absorbance was read at 570 m against that of a blank. For tubes having an optical density reading over 1.0, an extra 5 ml. of 50 percent ethanol was added. The standard leucine curves are shown in Figure 5.

The amount of amino acid present in each tube of effluent was determined in a similar manner as described above, except that proline was read at 440 m . In the runs with protein hydrolysates, 80 to 120 tubes were run simultaneously every evening and every morning. When working with the 15-cm. column, all tubes were analyzed at the end of the run. The amount of amino acids present was calculated in terms of leucine equivalents which were divided by the color yield of each amino acid as reported for this reagent (90).

Preparation of the Samples.— The sweet potato β-amylase was thoroughly dialyzed against distilled water for 48 hours, in the refrigerator, changing the outside solution several times. The sample was then dried in the vacuum desiccator over phosphorus pentoxide, and stored in this manner until needed for hydrolysis. The hydrolysis was carried out with a mixture of 10 to 20 mg. of dried protein and 4 ml. of constant boiling hydrochloric acid, in an evacuated, sealed tube. The temperature for hydrolysis was 105°, and the times were either 13 or 72 hours. Separate samples were prepared for the basic amino acid determinations. At the end of the hydrolysis, the hydrolysates were dried under vacuum over sodium hydroxide. One ml. of water was then added to the residue, and the drying process was repeated in order to insure complete removal of the hydrochloric acid. The hydrolyzed samples were taken up in 10 ml. of either pH 3.25 or pH 5.28 buffer, and either used immediately or stored in the deep freeze. The results of the amino acid analysis of sweet potato β-amylase are reported in Table XXIII.

Performic Acid Oxidation.-- In order to ascertain the amount of cystine present, the protein was subjected to performic acid oxidation. Ten to 20 mg. of a dried sample of sweet potato β-amylase was mixed with 0.9 ml. of 90 percent formic acid and 5 drops of 30 percent hydrogen peroxide. The reaction mixture was allowed to stand at room temperature overnight. At first, the protein turned black, but after a few hours it seemed to dissolve. The solution was evaporated to dryness in a vacuum desiccator, and, then, the sample was hydrolyzed as described above. Two samples were run to check the amounts of cystine and valine. No attempt was made to determine the other amino acids in these samples, since it appeared that some of them had been destroyed to a considerable degree.

Tryptophan Determination.-- Since tryptophan is destroyed during acid hydrolysis, basic hydrolysis was carried out in order to determine whether or not this amino acid is present in sweet potato β-amylase. A dried sample of protein was placed in a Pyrex tube and 1 ml. of 0.38 M barium hydroxide was added. The tube was then evacuated at the water pump and sealed. Hydrolysis was carried out in an oven at 110°C for 17 hours. The hydrolysate was then neutralized with dilute sulfuric acid, and the precipitated barium sulfate removed by centrifuging and washed. The hydrolysate was then dried in the vacuum desiccator and made up to a volume of 10 ml. with water when needed for the chemical determination of tryptophan. The procedure followed was that of Spies and Chambers (93).

Eight ml. of 23.8 N sulfuric acid, and 1.0 ml. of 2 N sulfuric acid, containing 30 mg. of recrystallized p-dimethylaminobenzaldehyde, were mixed and cooled to 25°C. To this mixture was added 1.0 ml. of either a solution of tryptophan of known concentration or the hydrolysate. After shaking the resulting mixture, it was kept in the dark for one hour. One-tenth ml. of 0.04 percent sodium sulfite was then added to the mixture, which was again mixed by shaking, and the color allowed to develop for 30 minutes at room temperature in the dark. The optical density was read at 600 mu against that of a blank. Standards containing 0.20 mg., 0.10 mg., and 0.04 mg. of tryptophan were run in order to establish a standard curve. The results of these experiments are reported in Table XXIII.

15. Inhibition Studies

The importance of sulfhydryl groups in the action of β-amylase has long been recognized (77, 73). Accordingly, experiments were performed to study the inhibition of sweet potato β-amylase by iodoacetamide, p-chloromercuribenzoate, and N-ethylmaleimide.

Stock solutions of iodoacetamide, p-chloromercuribenzoate and N-ethylmaleimide ($1x10^{-2}$ M) were prepared. For use in preliminary experiments these solutions were diluted to $1x10^{-4}$ M.

Approximately 25 mg. of sweet potato β-amylase suspension was dissolved in 30 ml. of phosphate buffer at pH 7.5 (0.1 M). In each of three 10-ml. volumetric flasks was placed 9 ml. of the β-amylase solution and 1 ml. of $1x10^{-4}$ M solution of inhibitor. The solutions were incubated at 30°C and assayed from time to time. After a certain period of time, 1 ml. of $1x10^{-2}$ M of each inhibitor was added respectively to each flask.

Another β-amylase solution was prepared under the same conditions as above, and after 2 hours of incubation with 10^{-4} M inhibitor, 1 ml. of $1x10^{-2}$ M inhibitor solution was again added to the reaction flasks. When the β-amylase was completely inhibited, 1 ml. of $1x10^{-2}$ M glutathione was added in order to determine whether or not this inhibition could be reversed by this substance.

The inhibition by N-ethylmaleimide was also investigated, following the reaction of the sulfhydryl groups of β-amylase with this substance spectrophotometrically, along with the decrease in activity of the β-amylase solution. The method used here was that described by Alexander (74). Alexander showed that when present in excess, N-ethylmaleimide reacts stoichiometrically with sulfhydryl compounds, and the decrease in absorption at 300 mu can be used as an assay method for sulfydryl groups.

A 0.0015 M N-ethylmaleimide stock solution was prepared, and 2 ml. of this solution was added to 1 ml. of a β-amylase solution. A blank was prepared, replacing the N-ethylmaleimide solution with distilled water, and the absorbance read at 300 mu. The reaction of N-ethylmaleimide with the sulfhydryl groups of the β-amylase was allowed to proceed directly in the quartz cells used for spectrophotometric measurements. At regular intervals, 0.1-ml. samples were removed for assay of β-amylase activity. The protein concentration in the reaction mixture was determined on the blank by the Rosenthal-Cundiff biuret procedure (102). The results of this experiment are recorded in Table XXVII.

16. Photooxidation of Sweet Potato β-Amylase

The photooxidation of sweet potato β-amylase was carried out according to the procedure of Weill and Seibles (101). Approximately 5 mg. of sweet potato β-amylase was dissolved in a few ml. of phosphate buffer (pH 7.5, 0.1 M) and 0.5 ml. of a 0.02 percent solution of methylene blue added. The reaction mixture was incubated at room temperature 30 cm. away from a 15 watts lamp. The activity of the β-amylase was checked at the start of the experiment and after 6 hours. The results of this experiment are reported in Table XXVIII.

III. RESULTS AND DISCUSSION

1. Action of Proteolytic Enzymes on Sweet Potato β-Amylase

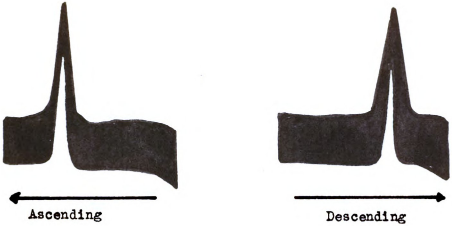
a. Tryptic Digestion.— After being allowed to react with trypsin, sweet potato β-amylase showed no decrease in activity as indicated in Table III. That the protein was not modified to any extent is demonstrated by a comparison of the electrophoretic pattern before and after trypsin treatment, as shown in Figures 1 and 2.

TABLE III

RESULTS OF TRYPSIN DIGESTION

Sample	Activity (Absorbance at 540 mu.)	
Original solution	.500	.490
After trypsin digestion	.500	.495

b. Chymotryptic Digestion.— Results similar to those observed with trypsin were obtained upon treatment with chymotrypsin as indicated in Table IV and Figure 3, which represents the electrophoretic pattern after chymotryptic action.



Ascending

Descending

FIGURE 1.-- Electrophoretic Pattern of Sweet Potato -Amylase.

(In Phosphate Buffer , pH 7.0, 0.1 M, for 7200 sec. at 14 mA)

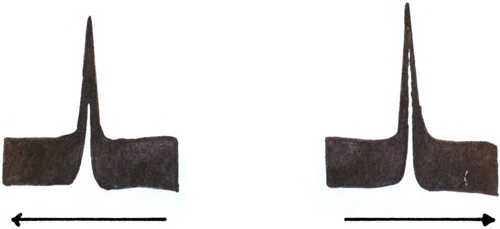


FIGURE 2.--ELECTROPHORETIC PATTERN OF SWEET POTATO -AMYLASE AFTER DIGESTION WITH TRYPSIN.

(In Phosphate Buffer, pH 7.0, o.1 M, for 7200 sec. at 14 mA)

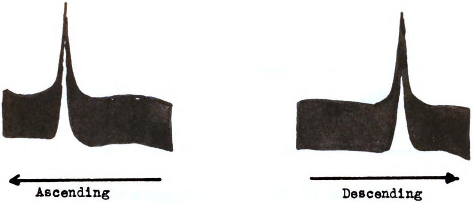


FIGURE 3.-- ELECTROPHORETIC PATTERN OF SWEET POTATO -AMYLASE

AFTER DIGESTION WITH CHYMOTRYPSIN.

(In Phosphate Buffer, pH 7.0, 0.1 M, for 7200 sec. at 14 mA)

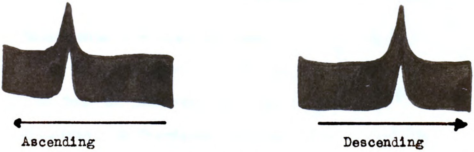


FIGURE 4.--ELECTROPHORETIC PATTERN OF SWEET POTATO -AMYLASE

AFTER DIGESTION WITH PEPSIN.

(In Phosphate Buffer, pH 7.0, o.1 M, for 7200 sec. at 14 mA)

TABLE IV

RESULTS OF CHYMOTRYPSIN DIGESTION

Sample	Activity (Absorbance at 540 mμ.)	
Original solution	.501	.502
After digestion	.510	.510

c. Peptic Digestion.— Pepsin also is inactive on sweet potato β-amylase at pH 5 as shown by the results reported in Table V. As in the case of chymotrypsin and trypsin, no changes either or electrophoretic pattern could be detected upon treatment of this endopeptidase. (Figure 4.)

TABLE V

RESULTS OF PEPSIN DIGESTION

Sample	Activity (Absorbance at 540 mμ.)	
Original solution	.950	.950
After digestion	.950	.955

Experiments were also performed at a lower pH where pepsin could conceivably be more active. At pH 3.0, the sweet potato β-amylase precipitated from the solution, and, upon digestion with pepsin, the precipitate dissolved. No β-amylase activity could be detected in this mixture, and paper electrophoresis experiments indicated that the protein had been digested, since no band appeared upon treatment with bromophenol blue.

d. Proteolysis by Papain.— Papain is proteolytic enzyme from the latex of the papaya (*Carica papaya*) which has been obtained in crystalline form (98). Preliminary experiments indicated that there is a decrease in activity of the β-amylase upon treatment with papain. Further experiments indicated a decrease in activity up to a certain point at which proteolysis seemed to stop. Typical of these results are those reported in Tables VI and VII.

TABLE VI

ACTION OF PAPAIN ON SWEET POTATO b-AMYLASE

Sample	Digestion Period (Hours)	Activity (Absorbance at 540 mu.)	
	(Run No. 1)		
Control	0	.480	.485
Digested	6	.385	.385
Digested	17	.350	.365
Digested	24	.350	.340
	(Run No. 2)		
Control	0	.235	.237
Digested	6	.150	.155
Digested	48	.128	.122

TABLE VII

PAPAIN ACTION ON SWEET POTATO β-AMYLASE

Control			Digested		
Period (Hours)	Activity (Absorbance at 540 mu.)		Period (Hours)	Activity (Absorbance at 540 mu.)	
0	.210	.210	0	.210	.210
13	.200	.200	13	.141	.141
24	.200	.200	24	.140	.137

These data indicate that papain acts to bring about proteolysis of sweet potato β-amylase, and that most of the action takes place in a short period of time. Thus, after 6 hours of digestion, there is no further decrease in activity. An explanation for these data is either that the β-amylase was modified in such a way as to resist further hydrolysis under the influence of papain, or that papain itself was inactivated. In order to check this, fresh papain was again added to a digested sample and a further loss in activity occurred as indicated in Tables VIII and IX.

TABLE VIII

ADDITION OF PAPAIN TO AN ALREADY DIGESTED SAMPLE
(Run No. 1)

Sample	Period (Hours)	Activity (Absorbance at 540 mu.)	
Control	0	.485	.480
Digested	6	.380	.370
Digested	24*	.310	.318
Digested	3	.320	.330
Digested	5	.230	.230

*0.1 ml. fresh papain added here.

TABLE IX

ADDITION OF PAPAIN TO AN ALREADY DIGESTED SAMPLE
(Run No. 2)

Sample	Period (Hours)	Activity Absorbance at 540 mμ.)	
Control	0	.580	.580
Digested	2	.580	.580
Digested	5	.570	.575
Digested	6	.510	.520
Digested	8*	.440	.440
Digested	3	.410	.420
Digested	8	.320	.320

*0.1 ml. fresh papain added here.

In examining the final mixtures by paper electrophoresis, only a band corresponding to unmodified β-amylase appeared. No evidence for active fragments was detected. In these experiments partially digested samples of sweet potato β-amylase were run along with a control. No significant differences could be detected. Control and digested samples were also run on the same strip, and only one band appeared.

e. Action of Subtilisin.-- Subtilisin is a bacterial protease from *Bacillus subtilis*. It has been crystallized, and its action is of the endopeptidase type (99). The action of subtilisin on sweet potato β-amylase is reported in Table X.

f. Carboxypeptidase.-- Since, with the exception of papain, the endopeptidases seemed to have little or no effect on sweet potato β-amylase, action of the exopeptidase carboxypeptidase was tried. Although the action of carboxypeptidase on sweet potato β-amylase--if it were to catalyse any hydrolysis--would probably be not as useful as that of an

TABLE X

ACTION OF SUBTILISIN ON SWEET POTATO β-AMYLASE

Period of Digestion (Hours)	Activity (Absorbance at 540 mμ.)	
0	.660	.670
2.5	.665	.660
14	.660	.660
24	.635	.629
36	.600	.610

endopeptidase in general terms of solving this problem, it should effect certain modifications in the integral structure of β-amylase, and give information about its composition.

That the carboxypeptidase used in these experiments, under these conditions was active was demonstrated by its action on casein, (shown in Table XI).

TABLE XI

ACTION OF CARBOXYPEPTIDASE ON CASEIN

Digestion Period (Minutes)	Non Protein Nitrogen (Absorbance at 570 mμ.)
0	.330
30	.780
60	.860
140	1.10

TABLE XII

ACTION OF CARBOXYPEPTIDASE ON SWEET POTATO β-AMYLASE

Digestion Period (Minutes)	Activity (Absorbance at 540 mu.)		Non Protein Nitrogen (Absorbance at 570 mu.)	
	(Run No. 1)			
0	.580	.580	.297	.305
30	.615	.620	.310	.315
60	.620	.620	.315	.315
120	.640	.640	.315	.310
	(Run No. 2)			
0	.310	.315	.035	
30	.338	.345	.035	
60	.375	.365	.045	
120	.370	.355	.040	
420	.390	.400	.040	

These results indicate a striking increase in β-amylase activity upon carboxypeptidase treatment. The absence of a significant increase in nonprotein nitrogen, as determined by this method, might be attributed to liberation of amino acids at levels below the sensitivity of the reagent. This proteolysis by carboxypeptidase warrants further investigation.

The resistance of sweet potato β-amylase to proteolysis with trypsin, chymotrypsin and pepsin, even though a little surprising, may indeed be more common than was suspected at first. Stein and Fischer (78) report that a number of α-amylases from various sources are also resistant to proteolytic attack by trypsin and chymotrypsin. These enzymes perform their physiological functions for the most part in the presence of a number of proteases and seem to be protected from their action through binding with metal ions. This was demonstrated by occurrence of proteolysis of the α-amylases when they were incubated with either trypsin or chymotrypsin in the presence of complexing agents such as ethylenediamine tetraacetic acid. Proteolysis under these circumstances could be stopped by the addition of calcium ion or a number of other divalent ions. In a subsequent publication these authors report that all α-amylases investigated contain at least 1 g. atom of firmly bound calcium ion per mole. Stein and Fischer propose the following scheme to explain the resistance of α-amylase to proteolysis:

$$\begin{array}{c} \text{Native amylase} \\ \text{(active} \end{array} \underset{\text{metal ion}}{\overset{\text{metal ion binding agent}}{\rightleftarrows}} \begin{array}{c} \text{"metal poor" amylase} \\ \text{(active)} \end{array}$$

protease

break down products (inactive)

A similar observation was reported by Azari and Feeney (79) in the case of conalbumin and transferrin. These iron binding proteins form very similar stable complexes with 2 atoms of iron. They were found to be more resistant to hydrolysis by chymotrypsin or trypsin than the metal-free proteins.

Although no metal ion has been reported to be associated with β-amylase,(Balls (32) found traces of lead in the ash of crystalline β-amylase), its stability toward proteolysis could be explained in a similar manner. This point warrants further investigation, and may lead to the solution of this problem.

2. Experiments in Urea Solutions

Sweet potato β-amylase is active in 8 M urea, but is very rapidly inactivated in this medium. That this is an irreversible reaction, is indicated in Table XIII.

TABLE XIII

IRREVERSIBLE DENATURATION OF SWEET POTATO β-AMYLASE BY 8 M UREA

Sample	Period (Hours)	Activity (Absorbance at 540 mμ.)	
Original	0	.085	.090
Original	5	.000	.000
After dialysis against acetate buffer	18	.000	.000

The inactive sweet potato β-amylase is soluble in 8 M urea, but during the course of dialysis, it precipitates out as the urea concentration decreases.

A few experiments in 8 M urea were carried out in the presence of pepsin, and the results (reported in Table XV) indicate that in 8 M urea proteolysis took place. However, denaturation was too rapid to obtain satisfactory hydrolysis.

TABLE XIV

ACTION OF PEPSIN IN 8 M UREA

Sample	Digestion Period (Minutes)	Activity (Absorbance at 540 mμ.)		Non Protein Nitrogen (Absorbance at 280 mμ.)	
Control	0	.160	.158	—	—
Digested	30	.012	.015	.493	
Digested	42	.000	.000	.635	
Digested	60	.000	.000	.630	
Control	60	.082	.082	.000	

The denaturation in 6 M urea is not nearly as rapid as in 8 M urea as the results shown in Table XV indicate.

TABLE XV

DENATURATION OF SWEET POTATO β-AMYLASE IN 6 M UREA

Incubation Period (Hours)	Activity (Absorbance at 540 mμ.)	
0	.545	.535
13	.430	.440
23	.255	.265
25	.200	.210
50	.000	.000

As indicated above, there is a slow denaturation in 6 M urea, and this could render the protein more susceptible to proteolysis. Consequently, the urea denaturation along with peptic digestion has been studied. The results of this type of experiments are reported in Table XVI.

TABLE XVI

PEPTIC DIGESTION OF SWEET POTATO β-AMYLASE IN 6 M UREA

Digest			Control		
Digestion Period (Minutes)	Activity (Absorbance at 540 mμ.)		Period (Hours)	Activity (Absorbance at 540 mμ.)	
0	.500	.510	0	.600	.601
15	.325	.350	4	.525	.540
45	.200	.200	10	.540	.540
60	.185	.190	19	.501	.510
75	.120	.140	25	.300	.305
120	.095	.105	28	.220	.230
180	.055	.050	33	.080	.082
550	.033	.025	34	.072	.075

Since the actual urea concentration in the previous experiments was 4 M, after the addition of the pepsin solution, determination of the peptic action on sweet potato β-amylase was carried out starting with a 4 M urea solution.

In this instance, the interesting observation was made that treatment of sweet potato β-amylase in 6 M urea affects the molecule in a different manner, even though the actual concentration at the time of proteolysis is the same. Paper electrophoresis of the digested samples did not reveal any significant changes in the protein structure. The activity remained with the main component which migrated within experimental error, at the same velocity as did the control.

TABLE XVII

ACTION OF PEPSIN ON SWEET POTATO β-AMYLASE IN 4 M UREA

Digested Sample (Hours)	Activity (Absorbance at 540 mμ.)		Control (Hours)	Activity (Absorbance at 540 mμ.)	
0	.128	.125	0	.128	.125
3.5	.084	.085	3.5	.129	.125
8	.065	.071	8	.124	.125
24	.070	.070	24	.115	.110

The results obtained with trypsin and chymotrypsin, reported in Tables XVIII and XIX seem to indicate little change in activity beyond that which can be attributed to denaturation under these conditions. Experiments were also performed in 6 M urea with papain and subtilisin. These enzymes did not seem to act upon sweet potato β-amylase under these conditions either.

TABLE XVIII

ACTION OF CHYMOTRYPSIN IN 6 M UREA

Sample	Period of Digestion (Hours)	Activity (Absorbance at 540 mμ.)	
Control	0	.365	.360
Digested	4.5	.212	.209
Digested	20	.218	.221
Control	20	.265	.261

TABLE XIX

ACTION OF TRYPSIN IN 6 M UREA

Sample	Period of Digestion (Hours)	Activity (Absorbance at 540 mμ.)	
Control	0	.110	.100
Digested	3.5	.110	.110
Digested	18	.065	.065
Control	18	.065	.065

3. Heat Stability of Sweet Potato β-Amylase

Since heat acts much in the same manner as urea (80), one experiment was carried out to investigate the stability of sweet potato β-amylase toward heat and the possibility of proteolysis with trypsin after a short heat treatment.

TABLE XX

EFFECT OF HEAT ON SWEET POTATO β-AMYLASE

Sample	Activity (Absorbance at 540 mμ.)	
Original	.160	.170
After 5 minutes at 100°	.000	.000
After 6 minutes at 70°	.105	.115

A sample of the β-amylase which had been treated at 70° for 6 minutes, was incubated with trypsin and the activity checked after 1 and 18 hours of digestion. No decrease in activity was found.

4. Results of Paper Chromatography of Sweet Potato β-Amylase Hydrolysates

Two dimensional paper chromatography of sweet potato β-amylase hydrolysates revealed the presence of 16 amino acids. No attempt was made to separate and distinguish between leucine and isoleucine. These experiments indicated that the presence of histidine was doubtful. However, subsequent experiments showed that it is present in small amounts. The results of the paper chromatography experiments are reported in Table XXI. In this Table are also given Rf values of amino acids obtained with a synthetic mixture of the amino acids believed to be present in the hydrolysate. The Rf values reported in two different sources from the literature for these amino acids in the solvents employed in these experiments are also included. It should be noted that, the literature records wide differences in the results of paper chromatography of amino acids, so that one must run known standards for identification purposes instead of relying on reported Rf values.

5. Results of the Amino Acids Analysis

The results of the amino acids analysis are reported in Tables XXII and XXIII and Figures 5 and 6 represent a typical chromatographic fractionation of the amino acids of sweet potato β-amylase hydrolysate.

TABLE XXI

PAPER CHROMATOGRAPHY OF HYDROLYSATES OF SWEET POTATO β-AMYLASE

Amino Acid	Color of Spot	Rf in Phenol-Water System			
		Mixture	Hydrolysate	Refe. A (88)	RefB (94)
Leucine	Purple	0.80	0.80	0.79	0.84
Phenylalanine	Brownish	0.80	0.80	0.78	0.85
Valine	Purple	0.75	0.75	0.64	0.78
Proline	Yellow	0.89	0.92	0.85	0.88
Tyrosine	Brown	0.51	0.49	0.52	0.51
Alanine	Purple	0.50	0.51	0.60	0.55
Methionine	Purple	0.79	0.76	0.73	0.81
Threonine	Reddish	0.43	0.38	0.39	0.50
Arginine	Purple	0.41	0.38	0.41	0.89
Glycine	Reddish	0.32	0.29	0.30	0.41
Serine	Purple	0.24	0.22	0.24	0.36
Lysine	Purple	0.24	0.20	0.39	0.81
Cystine	Grey	0.13	0.09	0.08	—
Aspartic	Blue	0.11	0.09	0.07	0.19
Glutamic	Purple	0.24	0.25	0.16	0.31
Histidine	Greyish	0.53	?	0.55	0.69

TABLE XXIII

AMINO ACID COMPOSITION OF SWEET POTATO β-AMYLASE

Amino Acid	Grams of Amino Acid Residues per 100 grams of Protein										Average Values
	13-Hour Hydrolysate No. 1			13-Hour Hydrolysate No. 2		13-Hour Hydrolysate No. 3		72-Hour Hydrolysate No. 1	72-Hour Hydrolysate No. 2		
	Run 1	Run 2	Run 3	Run 1	Run 2	Run 1	Run 2	Run 1	Run 1	Run 2	
Aspartic	---	14.25	13.85	14.50	14.31	(12.35)	13.80	13.50	14.60	13.35	14.02
Threonine	3.38	3.58	3.42	3.50	3.24	3.39	3.49	3.18	3.82	2.60	3.55
Serine	3.03	3.58	3.17	3.18	3.18	2.96	3.42	2.48	(2.96)	2.62	3.37
Glutamic	(10.14)	11.10	11.60	11.11	11.05	10.58	10.32	11.00	11.10	(9.45)	10.98
Proline	5.07	6.59	4.92	4.87	5.10	5.30	6.47	5.21	5.22	4.60	5.34
Glycine	3.86	4.24	4.35	3.89	4.24	3.82	4.75	4.34	3.70	3.53	4.08
Alanine	4.56	5.09	5.39	5.35	5.06	5.41	5.63	5.30	(4.49)	4.75	5.11
Valine	---	5.31	4.86	4.64	5.72	5.06	5.25	6.95	5.92	5.50	5.47
Cystine	---	1.33	1.39	1.64	(6.0)	---	1.71	1.28*	1.41*	1.28	1.44
Methionine	4.23	3.82	3.72	4.34	3.98	(3.21)	3.87	3.39	3.40	3.65	3.83
Isoleucine	4.21	3.87	4.17	3.80	4.16	(3.05)	4.54	4.15	5.12	3.96	4.22
Leucine	8.20	7.26	8.10	8.18	7.97	6.70	(6.51)	6.95	6.57	6.18	7.50
Tyrosine	6.87	5.61	5.72	6.83	6.06	5.46	5.87	4.80	(5.48)	5.04	6.33
Phenylalanine	6.65	(3.65)	5.74	6.46	5.77	6.13	(4.87)	6.22	(5.08)	(5.05)	6.16
Lysine	6.0	6.25	6.50	6.12	5.83	6.40	6.35	6.29	6.14		6.21
Histidine	1.63	2.18	1.55	1.59	1.73	1.56	(0.79)	1.71	1.73		1.71
Arginine	6.15	4.67	4.87	4.75	4.30	(3.66)	4.99	4.34	4.53		4.83
Ammonia	1.33	1.33	1.32	1.78	1.64	1.32	---	---	---		1.45

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*Determined as Cysteic acid after performic acid oxidation.

TABLE XXIII

NUMBER OF AMINO ACIDS RESIDUES PER MOLECULE OF SWEET POTATO β-AMYLASE

Amino Acid	Grams of Amino Acid Residues Per 100 g. Protein	Number of Residues For a Molecular Weight of 152,000 (54)
Aspartic	14.0	185
Threonine	3.6	54
Serine	3.4	60
Glutamic	11.0	130
Proline	5.3	83
Glycine	4.1	109
Alanine	5.1	109
Valine	5.5	85
Half-cystine	1.44	21
Methionine	3.8	44
Isoleucine	4.2	57
Leucine	7.5	101
Tyrosine	6.3	62
Phenylalanine	6.2	62
Lysine	6.2	74
Histidine	1.7	19
Arginine	4.8	47

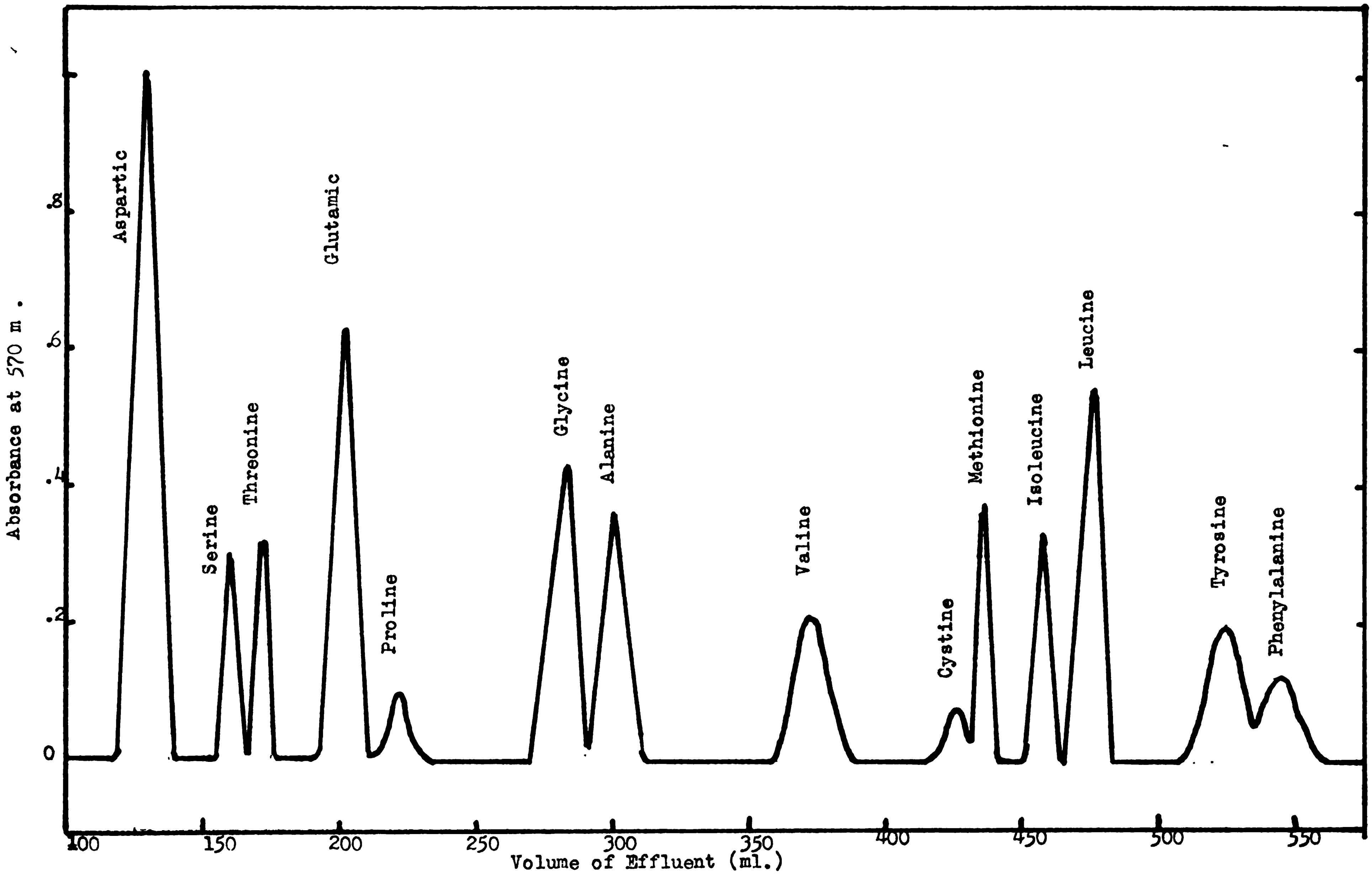

Figure 5.— Effluent Curve for the Neutral and Acidic Amino Acids from a Hydrolyzate of Sweet Potato -Amylase. The separation was done on a 0.9x150 cm. column of IRC-120 Resin, for a 1.31 mg. sample.

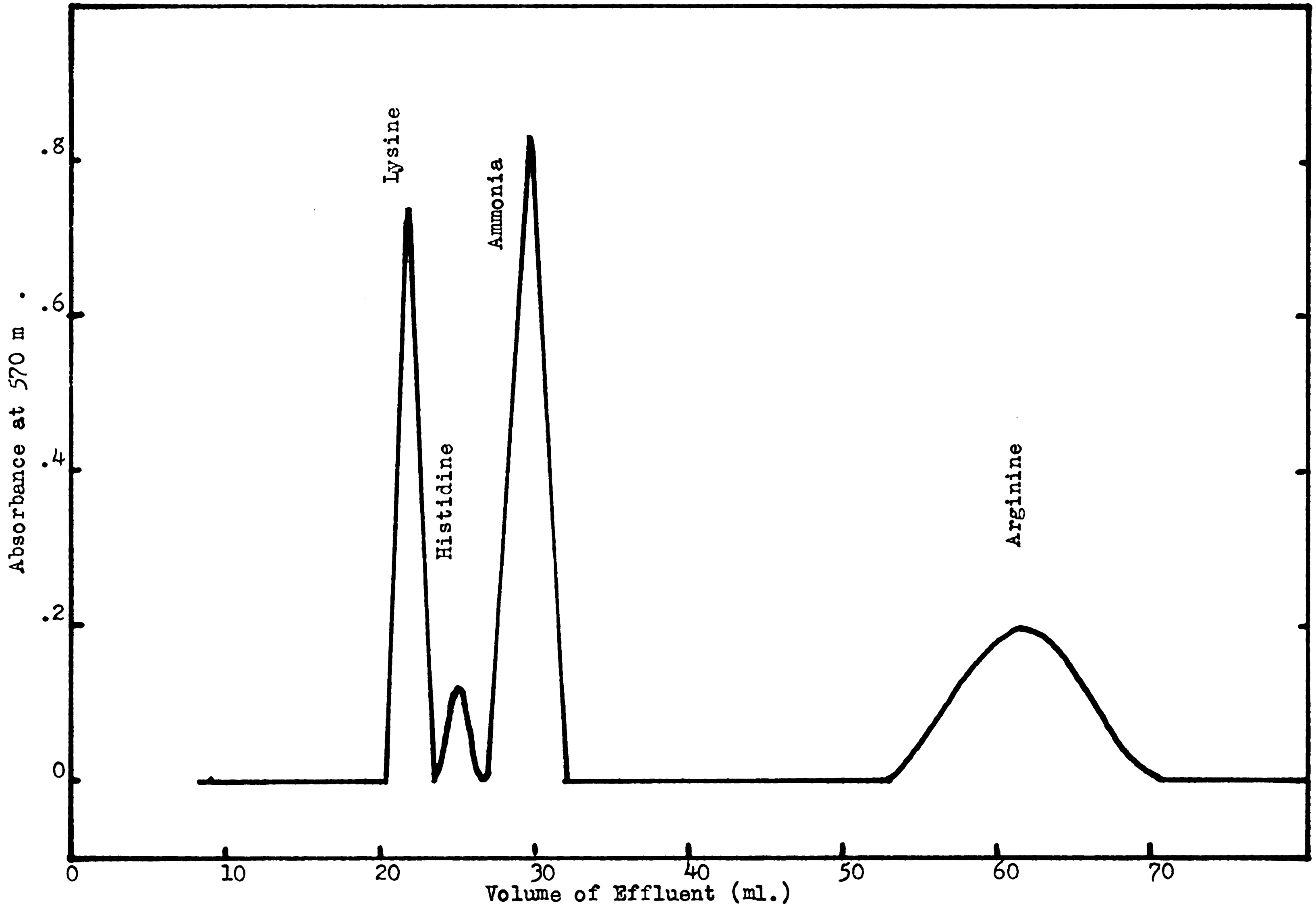

FIGURE 6.-- Effluent Curve of the Basic Amino Acids from a Hydrolyzate of Sweet Potato -Amylase.

The separation was done on a 0.9x15 cm. column of IRC-120 Resin, for a 1.47 mg. sample

Seventy-two-hour hydrolysates indicated that serine, threonine and tyrosine undergo the most decomposition under these acidic conditions. Therefore, the original amounts of these substances were calculated by extrapolation according to the method of Hirs *et al.* (95). As previously mentioned, the main difficulty in the column chromatography of these hydrolysates was in separation of cystine and valine. Varying the pH of the second buffer between 4.0 and 4.5 did not seem to influence the separation of these two amino acids. However, upon holding the pH 3.25 buffer past the recommended volume for changing, valine underwent elution when the buffer of pH 4.25 was applied. Although valine did not give a sharp peak, the separation was satisfactory. That the order of elution was reversed under these conditions, was proved by employing performic acid oxidized samples. Figure 7 is typical of the separation obtained on a performic acid oxidized sample. Only cysteic acid and valine were calculated in this case, since other amino acids such as threonine, serine and tyrosine showed considerable destruction under this treatment.

Histidine and cystine are the amino acids present in the lowest amounts. Assuming a molecular weight of 152,000 (54) for sweet potato β-amylase, there would be 19 to 20 histidine residues and 21 half cystine residues per molecule. In all determinations, equivalent amounts of glycine and alanine were found, as well as a 2 to 1 ratio of leucine to isoleucine. Aspartic and glutamic acids residues make up approximately 25 percent of the sweet potato β-amylase.

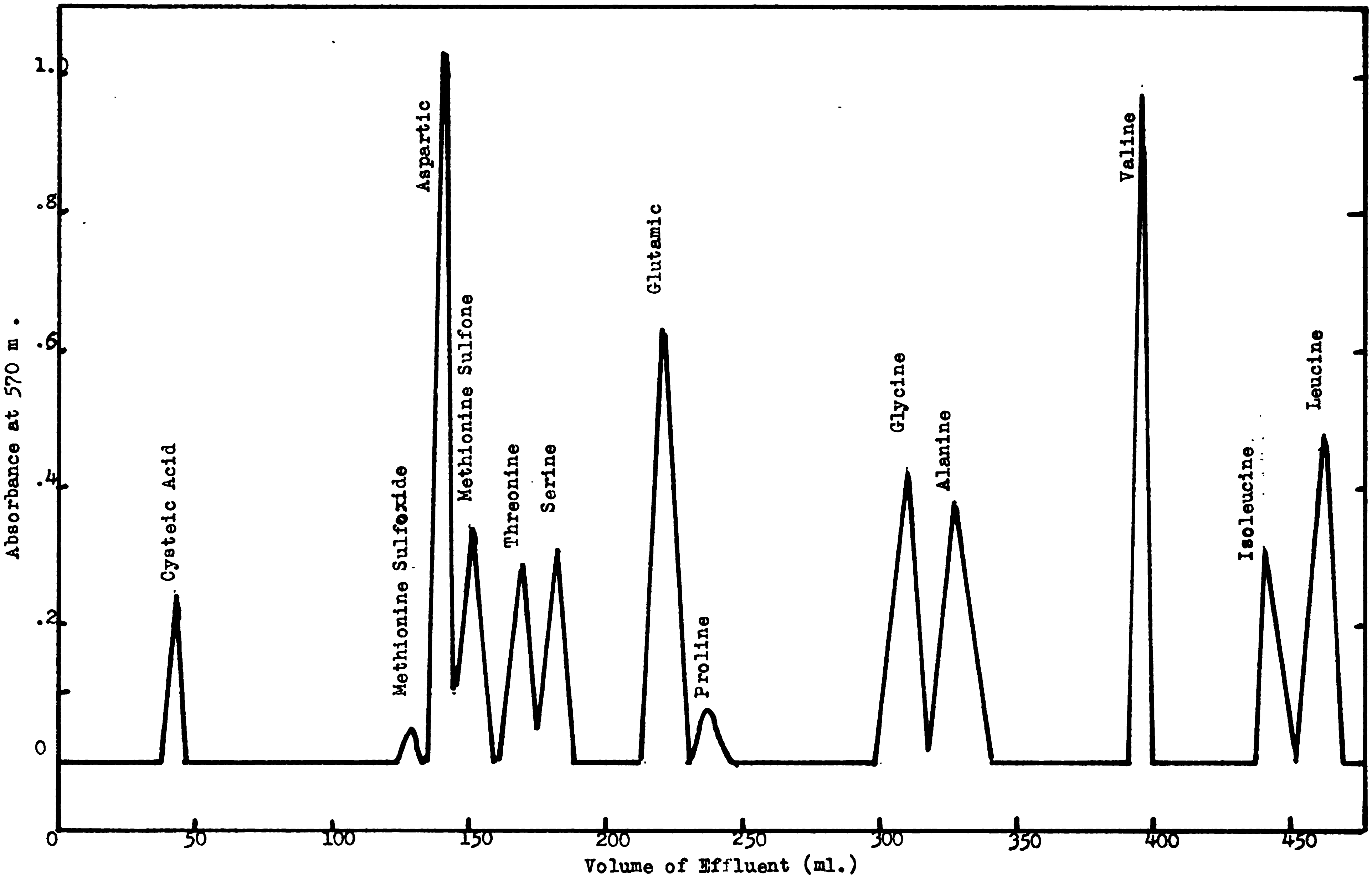

Figure 7.— Effluent Curve of a Performic Acid-oxidized Sample (1.61 mg.) of Sweet Potato -Amylase Hydrolyzate.

In general, the values obtained for the amino acids of sweet potato β-amylase agree with those of Balls *et al.* (32), as determined separately for individual amino acids by specific reactions. Balls' values are as follows:

Arginine	6 percent
Tyrosine	7.0 percent
Cystine	0.76 percent
Methionine	4.32 percent.

The absence of tryptophan was indicated by the results of the basic hydrolysis and colorimetric determination. This is reported in Table XXIV.

TABLE XXIV

RESULTS OF TRYPTOPHAN DETERMINATION

Sample (mg. Tryptophan)	Absorbance at 600 mμ	
0.20	.900	.910
0.10	.415	.425
0.04	.150	.145
Basic hydrolysate	.015	.012
Basic hydrolysate--0.02 mg. Trypt. added	.095	.090

This also checks with the results of Balls(32) who did not find any tryptophan in sweet potato β-amylase.

6. Results of Inhibition Studies

Since our first approach to this problem did not give the results hoped for, it was decided to examine the effect of inhibitors on β-amylase. Sulhydryl groups have been recognised as essential for the activity of β-amylase (72). Singer et al. (73) studied the action of a number of sulfhydryl reagents in connection with the oxidation of the sulfhydryl groups of this enzyme. In the present approach, an attempt was made to find a reagent which would form an adduct with the enzyme and which later could be used as a "tag" for the active site. The inhibitory properties of N-ethylmaleimide, p-chloromercuribenzoate and iodoacetamide were examined. The results obtained are reported in Table XXV.

TABLE XXV

ACTION OF INHIBITORS (At $1x10^{-5}$ M) ON SWEET POTATO β-AMYLASE

Sample	Activity (Absorbance at 540 mμ.) at Different Reaction Times			
	30 Minutes		4 Hours	
Control	.540	.530	.540	.530
N-Ethylmaleimide	.540	.530	.530	.540
Iodoacetamide	.600	.610	.600	.590
p-Chloromercuribenzoate	.325	.305	.300	.305

The effects of a higher concentration (0.01 M) of these inhibitors on the same samples are shown in Table XXVI.

TABLE XXVI

EFFECT OF ADDITION OF INHIBITOR ON SWEET POTATO β-AMYLASE

Sample	Activity (Absorbance at 540 mu.) at Different Reaction Times			
	30 Minutes		90 Minutes	
N-Ethylmaleimide	.190	.200	.065	.063
Iodoacetamide	.540	.530	.510	.505
p-Chloromercuribenzoate	.000	.000	----	----

These inhibition experiments were repeated with N-ethylmaleimide and iodoacetamide. The β-amylase was first incubated at concentrations of 1x10^{-6} M of each inhibitor respectively, and, after 2 hours, 1 ml. of 1x10^{-2} M inhibitor added. The results are shown in Table XXVII. Complete inhibition of β-amylase activity with N-ethylmaleimide took place after a period of 5 hours, whereas after 24 hours of incubation with iodoacetamide, approximately 30 percent of the β-amylase activity still remained.

The number of sulfhydryl groups necessary for β-amylase activity was determined according to the procedure of Alexander (74). The difference in absorbance between reacted and unreacted N-ethylmaleimide is divided by the molar extinction coefficient (620) of this substance, and the resulting quotient is equal to the molar sulfhydryl concentration of the sample. As determined by the biuret reaction, the sample contained 2 umoles of sweet potato β-amylase. These results are reported in Table XXVIII.

TABLE XXVII

RESULTS OF INHIBITION WITH IODOACETAMIDE AND N-ETHYLMALEIMIDE

Sample	Activity (Absorbance at 540 mu.)					
	After 2 Hours in 10^{-5} M		After 5 Hours in 0.01 M		After 24 Hours	
N-Ethylmaleimide	.610	.600	.010	.010	.000	.000
Iodoacetamide	.620	.625	.470	.480	.215	.220

Control at 0 time gave readings of .660 and .670

TABLE XXVIII

NUMBER OF SULFHYDRYL GROUPS NECESSARY FOR ACTIVITY OF β-AMYLASE

Period of Incubation with N-ethylmaleimide (Hours)	Activity (Absorbance at 540 mu.)		Activity (Absorbance at 300 mu.)	Absorbance	Calculated umoles SH Groups
0	.560	.570	.980		
5	.015	.020	.845	.135	2.18
17	.000	.000	.678	.302	4.88

As a result of these experiments, N-ethylmaleimide seems to be a most promising inhibitor for sweet potato β-amylase. However, the total number of sulfhydryl groups in a protein molecule could not be determined by reaction with this reagent, since Roberts and Rouser (103) found that N-ethylmaleimide reacted with only 60 percent as many sulfhydryl groups in serum albumin as did p-chloromercuribenzoate. Nevertheless, with sweet potato β-amylase, the reaction with the sulhydryl group essential for activity is preferred, and it may well be possible to label an active site in this manner.

7. Results of Photooxidation of Sweet Potato β-Amylase

Photooxidation with methylene blue was used by Weill and Seibles (101) to demonstrate the importance of histidine in the catalytic activity of ribonuclease. Total inhibition was observed at an uptake of 3 moles of oxygen per mole of this enzyme, at which point the only significant change in amino acid composition was the destruction of 3 moles of histidine out of a total of 4. That histidine is not necessary for activity of sweet potato β-amylase was indicated by complete retention of the activity of this amylase after 6 hours illumination in the presence of oxygen and methylene blue.

TABLE XXIX

RESULTS OF ILLUMINATION OF SWEET POTATO β-AMYLASE IN THE PRESENCE OF METHYLENE BLUE AND OXYGEN

Period (Hours)	Activity (Absorbance at 540 mμ.)	
0	.280	.275
6	.280	.285

8. Recapitulation

The behavior of sweet potato β-amylase toward proteolytic enzymes cannot be satisfactorily explained on the basis of the amino acid composition. Trypsin catalyzes the hydrolysis of peptides bonds involving arginyl and lysyl groups, whereas chymotrypsin with a few exceptions, catalyses the hydrolysis of bonds involving tyrosyl and phenylalanyl groups. Since these amino acids were found to be present in sweet potato β-amylase, the resistance to proteolysis by these enzymes is probably brought about by the conformation of the substrate.

Although pepsin is a relatively nonspecific protease [resembling papain in this respect (80)], it, nevertheless, favors catalysis of hydrolysis of bonds involving glutamyl and aspartyl groups. The high content of these amino acids in sweet potato β-amylase might explain proteolysis of this protein by papain under normal conditions, as well as the action of pepsin in urea solutions.

The increase in β-amylase activity upon treatment with carboxypeptidase may be caused by removal of an amino acid residue masking the active site.

The active conformation of the sweet potato β-amylase may be retained through hydrogen bonding involving mostly glutamic, aspartic acids and tyrosine. The irreversible denaturation in urea possibly occurs through rupture of these hydrogen bonds, and there may not be sufficient disulfide bridges to restore the original shape of the molecule after removal of the urea. It is significant that trypsin (97) is reversibly inactivated in 8 M urea solutions. As long as the disulfide linkages of trypsin remain intact, the unfolded molecule retains the capacity to refold to the active conformation on lowering the urea concentration. Rupture of the disulfide linkage by appropriate reducing agents, leads to a more extensive unfolding of the molecule which then becomes so distorted that the secondary structure essential for activity can no longer be regenerated by simple dilution.

The work of Singer (73) demonstrated that there are a number of sulfhydryl groups present in sweet potato β-amylase, and that upon intramolecular oxidation of these groups to disulfide linkages, the activity of the β-amylase was lost. Rowe and Weill (96) showed that the inhibition of β-amylase with ascorbic acid is non-competitive. These authors suggest that ascorbic acid inhibition and its reversal by cysteine shows that ascorbic acid is acting by some mechanism other than a direct reaction with the essential sulfhydryl groups of the enzyme. From these investigations it appears that 1) a number of disulfide linkages are present in β-amylase; 2) there are also some sulfhydryl groups, and 3) reaction of one sulfhydryl group with N-ethylmaleimide is sufficient to completely inhibit β-amylase activity.

Since the original approach to this problem did not give the results hoped for, the nature of the active site of β-amylase could be investigated through the use of inhibitors. This type of approach has already been successfully carried out for a number of enzymes [phosphoglucomutase (75), chymotrypsin (76), thrombin (77) and papain (100)].

IV. SUMMARY

1. Under ordinary conditions for proteolysis, sweet potato β-amylase showed a resistance to hydrolysis by trypsin, chymotrypsin pepsin and subtilisin, as indicated by total retention of activity and no changes in the electrophoretic pattern before and after incubation with these enzymes.

2. Papain degrades β-amylase rapidly to small fragments which do not retain any amylolytic activity.

3. The action of carboxypeptidase on sweet potato β-amylase increases the activity of the latter.

4. Sweet potato β-amylase is irreversibly denatured in 8 M urea.

5. In 8 M urea and in 6 M urea pepsin degrades sweet potato β-amylase as indicated by a rapid loss in activity, but no fragments possessing any β-amylase activity could be detected by paper electrophoresis experiments.

6. No proteolysis by trypsin or chymotrypsin could be detected in urea solutions.

7. A complete amino acid analysis of sweet potato β-amylase has been performed. Seventeen amino acids are present. The results indicate a high amount of glutamic and aspartic acid, equimolar amounts of glycine and alanine, and approximately a two to one ratio of leucine to isoleucine. No tryptophan is present. Between 10 and 11 cystine residues and 20 histidine residues are present per molecule of sweet potato β-amylase.

8. The inhibition of sweet potato β-amylase with *N*-ethylmaleimide iodoacetamide and p-chloromercuribenzoate has been studied. A complete inhibition of sweet potato β-amylase is obtained with N-ethylmaleimide in 10^{-3} M concentrations, in 5 hours at which time only one sulfhydryl group appears to have reacted.

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