THE UNIVERSITY OF MANITOBA

THE α-AMYLASE FROM IMMATURE HARD RED SPRING WHEAT; PURIFICATION AND CHARACTERIZATION

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

DEPARTMENT OF PLANT SCIENCE

WINNIPEG, MANITOBA

MAY, 1975

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ACKNOWLEDGEMENTS

I am pleased to gratefully acknowledge the excellent guidance and advice extended to me by Dr. J.E.Kruger. During the course of this research and in the preparation of this thesis, he made many pertinent observations and suggestions which increased the quality of this work immensely. The completion of this research and thesis, without his guidance, would have been a much more difficult task.

Thanks are also in order for Dr.G.N.Irvine, Director, Grain Research Laboratory, Canadian Grain Commission, for the use of laboratory facilities; Dr.A.W.MacGregor for his interest, helpful suggestions and discussion, and Dr.W.Bushuk for his time and effort spent on my program.

Grateful acknowledgement is extended to the National Research Council for financial assistance.

ABSTRACT

Marchylo, Brian Alexander, M.Sc., The University of Manitoba, May 1975. The α-Amylase from Immature Hard Red Spring Wheat; Purification and Characterization.

Major Advisors: Dr. G. N. Irvine and Dr. R. Tkachuk.

The α-amylase present in an immature Canadian Hard Red Spring (HRS) wheat (T. aestivum cv. Neepawa) was isolated and characterized. The steps involved in isolating the enzyme consisted of an extraction of wheat kernels at 20 days post-anthesis, heat treatment for 15 minutes at 70°C, acetone fractionation between 25%-54% (v/v), and formation of a glycogen-amylase complex. A yield of 18.4% with a 1902-fold purification was obtained. The α-amylase was then separated into three isozymes by ion-exchange chromatography on DEAE-cellulose. The isozymes were characterized in terms of pH optimum and stability, change in action pattern with pH, Km, thermal stability, activation energy, effects of SH reagents, molecular weight and isoelectric point. The properties of the three isozymes were similar with the main difference being in their isoelectric points and chromatographic and electrophoretic properties.

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INTRODUCTION

α-Amylase is an enzyme present in animals, plants and lower microorganisms which hydrolyzes the α-1,4 glucosidic linkages present in starch and glycogen. In wheat, the enzyme is of considerable practical importance because it is an integral part of the processes involved in breadmaking. Wheat α-amylase may affect dough properties such as gassing power and dough consistency; and if in excess will result in extensive liquefaction and dextrinization, yielding a bread with a wet sticky crumb (Bloksma, 1971).

The enzyme is present in the wheat kernel from early growth through maturation, and during germination. The greatest level of the enzyme is found in the germinated wheat kernel. Elevated levels of α-amylase sometimes found in the sound wheat kernel are usually attributable to the occurrence of a small amount of sprouting. Because of this, most of the research on wheat α-amylase has been with germinated kernels (Kruger and Tkachuk, 1969; Olered and Jönsson, 1970; Kruger, 1972b; Tkachuk and Kruger, 1974).

Some recent research has indicated that the α-amylase present in immature wheat also may affect the overall resultant level present in the final crop (Olered and Jönsson, 1970). Thus, during the growth and maturation of a wheat kernel, the level of α-amylase rises and then falls as the kernel matures. With certain wheat cultivars, the final level of α-amylase may still be quite substantial. In addition, if wheat is harvested with a high incidence of immature wheat kernels present, this also will result in an elevated level of α-amylase. In fact, this appears to be the case with a substantial amount of the wheat harvested in 1974.

It is known that the α-amylase present in immature wheat consists of 3 isozymes (Kruger, 1972a). The objective of this investigation was the purification and characterization of these isozymes from the immature HRS wheat, Neepawa. The α-amylases were characterized in terms of such properties as pH optimum and stability, change in action pattern with pH, Km, thermal stability, activation energy, inhibition by SH reagents, molecular weight and isoelectric point. It was felt that this knowledge would aid in the future assessment of the affect of these immature α-amylase isozymes on wheat quality.

LITERATURE REVIEW

Historically, α-amylase is one of the earliest enzymes to be discovered and studied in cereals. As far back as 1811, Kirchoff noted that wheat extracts possessed a digestive action on starch. In 1883 Payen and Persoz discovered a starch digesting substance in barley malt which they designated diastase. Lintner, in 1887, concluded that although barley malt contained a starch liquefying and a starch saccharifying enzyme, the latter component was the only one present in ungerminated barley.

Further studies on these starch digesting substances in cereals definitely showed that more than one type of amylase enzyme was present. The two amylases involved were termed α-and β-amylases by Kuhn (1924, 1925) because the hydrolytic products of the degradation were in the α-form, with downward mutarotation, or in the β form, with upward mutarotation, respectively. Recently, this has been verified by Semenza et al (1969) using the more sensitive technique of gas-liquid chromatography. An alternate means of referring to the two enzymes was introduced by Ohlsson (1930) who called α-amylase the dextrinizing amylase and β-amylase the saccharifying amylase.

Since these early studies, extensive work has been carried out on α-amylase. It is now known that α-amylase (α-1, 4-glucan 4-glucano-hydrolase, EC 3.2.1.1.) specifically catalyses the hydrolysis of α-1, 4 glucosidic linkages of starch, glycogen and their degradation products (Allen and Spradlin, 1974). α-Amylases have been classed as endo-amylases for they can cleave the α-1, 4 glucosidic linkages interior to the substrate chain. By contrast, β-amylase, an exoamylase-hydrolyses the α-1, 4 glucosidic linkages sequentially from the non-reducing end of a substrate chain, but cannot cleave interior linkages. A comparison of these closely associated hydrolytic enzymes is given in Table I.

Table I

A Comparison of α and β Amylases

Characteristic	α-Amylase	β-Amylase
Cleavage point	α-1, 4 Glucosidic bond Cleave C_1-O_4 bond	β-1, 4 Glucosidic bond Cleave C_1-O_4 bond
Configuration of new reducing unit	α	β
Mechanism	Endo-attack	Exo-attack
End products	Oligosaccharides mixture	Maltose
Decrease in viscosity and iodine staining	Rapid	Slow
Action at branch point	Can bypass	Cannot bypass
Transferase activity	Insignificant	Insignificant
Origin	Plant and animal	Plant

Extracted from Thoma et al (1971)

Sources Of α-Amylase

α-Amylase may be found throughout the plant and animal kingdom. The sources of α-amylase may be classed into four main groups: a) the higher starch containing plants; b) mammals; c) bacteria and d) fungi (Greenwood and Milne, 1968b). It has become apparent that the properties and modes of action of α-amylases are peculiar to their sources (Greenwood and Milne, 1968b) although some properties are common to all. The α-amylases from all sources are slightly acidic, water-soluble proteins with an approximate molecular weight of 50,000 and contain at least 1 gram atom of calcium, which is essential for their activity (Fischer and Stein, 1960). By contrast, most other properties, such as pH optima, are different between groups. For example, the pH optimumof various plant α-amylases falls between pH 4.7 - 6.0 (Greenwood and Milne, 1968b). In comparison, it is found that the mammalian α-amylases have a pH optimumof about 6.9 (Bernfeld et al, 1948; Bernfeld et al, 1950).

In recent years α-amylases have been intensively studied, and as a result, the body of knowledge concerning this enzyme has grown tremendously. α-Amylases have been crystalized or purified from many sources including higher plants. Of particular interest to this discussion are those α-amylases purified from cereals. Schwimmer and Balls (1949b) were probably the first to crystalize a cereal α-amylase (barley malt). Since then the enzyme has been purified from other cereal sources such as barley (Greenwood and MacGregor, 1965), Triticale (Lee and Unrau, 1969), malted wheat (Kruger and Tkachuk, 1969), malted rye (Manners and Marshall, 1972) and immature barley (MacGregor et al, 1974). In most cases, standard purification procedures such as heat treatment, acetone or salt fractionation, followed by glycogen complexing were employed.

Because of the immense body of knowledge now available even on plant α-amylases, this review will limit itself to the discussion of the cereal α-amylases. Some references may be made to α-amylases present in other groups for comparative purposes.

α-Amylase From Cereal Seeds

Cereals, in common with other plants, synthesize starch as a reserve polysaccharide. The storage area for this starch is the endosperm of the seed. Some mechanism must be available for the breakdown of the stored starch back to simple sugars which can be subsequently utilized by the growing plant. This is carried out by carbohydrate metabolizing enzymes located in the seed, with α-amylase being one of the major enzymes responsible.

The level and anatomical location of the α-amylase depends on the natural state of the seed, that is, whether the seed is in the immature, resting or germinating state. The α-amylase present in the resting seed will be discussed first, followed by the α-amylase of the germinating seed and finally the immature seed.

A. The α-Amylase of the Resting or Dormant Seed

Upon reaching maturity the α-amylase activity of the cereal seed is very low. Originally it had been felt that the mature seed contained no α-amylase prior to germination (Blish et al, 1937; Grabbar and Daussant, 1964). This has since been proved false by Greenwood and MacGregor (1965), MacGregor et al (1971) and Olered and Jonsson (1970), who have definitely shown that α-amylase activity is present in ungerminated wheat, barley and other cereals.

In wheat and most other cereals, the α-amylase activity level present in the mature resting seed will vary from variety to variety and from year to year. In most instances, the activity is not very high and is not significant when compared to the activity present in the germinated seed. However, an elevated level of α-amylase activity

is sometimes present. Although this may be a varietal characteristic, it is usually due either to a small amount of sprouting or to incomplete maturation.

B. Germinated Cereal α-Amylase

Much of our present day knowledge of cereal α-amylase stems from studies of the α-amylase present in the germinating cereal. A great body of work has been collected concerning the synthesis of α-amylase during germination, as well as the chemical and physical properties of the germinated cereal α-amylase. Each of these topics will be considered in turn in the following discussion:

1. Biosynthesis of germinated α-amylase: A concerted effort to determine the site of α-amylase synthesis during germination has been carried out in recent years, especially in the barley seed. Originally it was felt that most hydrolytic enzymes were released directly into the endosperm by the scutellum epithelium of the embryo. This has since been disproved and it is now conceded that the α-amylase of germinating barley is synthesized in the aleurone layer in response to gibberellic acid or a natural hormone of the gibberellin type (Briggs, 1964; Varner, 1964; Varner et al, 1965). It was observed visually that the digestion of the starchy endosperm began adjacent to the aleurone layer and proceeded centripitally until the reserve material of the endosperm had been digested (Varner et al, 1965). Dronzek et al (1972) noted that starch granules in sprouted wheat were more severely attacked near the aleurone layer than those in the interior. This suggested that α-amylase in wheat is also synthesized in the aleurone and then secreted into the endosperm. Recently Jacobsen and Knox

(1973) have localized the site of α-amylase synthesis even more precisely. Employing immunofluorescence techniques, they found that the aleurone grains and aleurone grain membranes were implicated in α-amylase synthesis.

Of related interest is the finding of Gibson and Paleg (1972) that in wheat and probably barley aleurone cells, the α-amylase was lysosomal. This implies that after synthesis, the α-amylase is separated from its substrate by a limiting membrane and is not allowed to migrate free within the aleurone cell.

2. De Novo synthesis of α-amylase: For many years it was not known if the α-amylase was reactivated from a pre-existing protein. It has since been ascertained that the majority of the α-amylase is synthesized de novo. The first proof of de novo synthesis was by Varner and Ram Chandra (1964). Using ^{14}C labelling, they proved that the label was incorporated into the α-amylase of barley during germination. Subsequently, Chrispeels and Varner (1966) found that α-amylase synthesis was halted by metabolic inhibitors. This eliminated the possibility of any part of the α-amylase existing as an enzymatically inactive precursor and being subsequently converted into active enzyme. Finally, density labelling studies by Filner and Varner (1967) again indicated that essentially all of the α-amylase arose by de novo synthesis from free amino acids derived from pre-existing aleurone proteins. In the case of germinated wheat α-amylase, Daussant and Renard (1972) used immunochemical methods to show that about 98% of the α-amylase was synthesized de novo. Combined, these results clearly indicate that the major portion of germinated α-amylase is synthesized de novo.

3. Chemical and physical properties of germinated cereal α-amylase: Extensive chemical and physical characterization studies have been carried out on the α-amylase in germinating cereal seeds in order to gain a better understanding of this important enzyme. Some of these properties have been briefly mentioned in a preceding section, but will now be expanded upon, with particular reference to the germinated cereal α-amylases. Most studies have been carried out on the malted cereal α-amylases and in the following sections malted and germinated cereal α-amylases will be considered synonymous. It should be noted that recent work by Meredith and Jenkins (1973a) gives some indication that differences may exist between sprouted wheat and malted or laboratory germinated wheat.

(i) pH effects: In the case of germinated cereal α-amylase, the pH optimumis centered around pH 5.5 For example, pH optima of 5.5 and 5.5 - 5.6 have been reported for malted barley(Greenwood and MacGregor, 1965) and malted wheat (Tkachuk and Kruger, 1974), respectively.

With respect to pH stability, the enzyme is stable between pH 5.0 - 8.0 but very unstable at low pH's and is irreversibly inactivated at pH's below 4.0 (Greenwood and MacGregor, 1965).

(ii) Temperature stability: As with other enzymes, the stability of germinated cereal α-amylases is temperature dependant. In general, cereal α-amylases are stable at room temperature or below over long periods of time (Allen and Spradlin, 1974). Cereal α-amylases are also fairly stable at high temperatures over short periods of time. For example, malted wheat α-amylase (Tkachuk and Kruger, 1974) retained 50% of its activity after 15 min. at 70°C. This stability is

often used to advantage in purification procedures since many proteins become denatured and fall out of solution at these elevated temperatures. Most notably, β-amylase is quickly denatured at 70°C temperatures and can thus be removed from the α-amylase still in solution.

(iii) Calcium stabilization: The stabilization of germinated cereal α-amylase by calcium is one property which has held the interest of many researchers. Early work by Hollenbeck and Blish (1941) demonstrated that the calcium ion protected malted wheat α-amylase from inactivation by heating. Similarly, Kneen et al (1943) illustrated this protective effect of calcium with malted barley as well as malted wheat α-amylase. Initially, the role of the calcium ion was not certain, but some workers (Redfern, 1950) felt that the α-amylase molecule actually contained calcium. Vallee et al (1959) confirmed this with four crystalline α-amylases (human, porcine, bacterial and fungal) and this is now known to be a general property of all α-amylases (Fischer and Stein, 1960). Stein et al (1964) found that the strength of calcium binding depended on the source of the α-amylase as follows: plant < mammalian < bacterial < fungal. Thus the germinated cereal α-amylases do not bind calcium as strongly as the α-amylases from the latter three sources,

The requirement for calcium is easily displayed by the addition of calcium chelating agents such as Ethylenediaminetetra-acetic acid (EDTA). Schwimmer and Balls (1949b) found with malted barley α-amylase that the addition of such a reagent resulted in loss in activity. Greenwood and Milne (1968b) found that this loss in activity for malted barley α-amylase and in general for plant α-amylases was reversible on addition of excess calcium.

Calcium also stabilizes malted cereal α-amylases against proteo-

lytic attack. Greenwood and MacGregor (1965) found that malted barley α-amylase was not susceptible to proteolytic attack by trypsin unless EDTA was added. On addition of EDTA, α-amylase activity was irreversibly lost in the presence of trypsin. This is in agreement with the results obtained by Stein and Fischer (1958) for other α-amylases.

The exact role played by calcium is not known but a suggested explanation is that the calcium ion confers a tight structural rigidity to the α-amylase molecule by forming a tight, intramolecular metal chelate structure (Hsui et al, 1964).

(iv) Glycogen complex formation: The purification of many malted cereal α-amylases such as malted wheat (Kruger and Tkachuk, 1969) and malted rye (Manners and Marshall, 1972), has been facilitated by the discovery that this enzyme forms an insoluble complex with glycogen (Schwimmer and Balls, 1949a; Loyter and Schramm, 1962). This property, unique to all α-amylases, results from a complex formation between the enzyme and a glycogen limit dextrin (Levitzki and Schramm, 1963). The insolubility of the complex has been attributed to the dextrin and the enzyme each possessing two binding sites so that an insoluble lattice structure containing many molecules of both could be formed (Levitzki and Schramm, 1963; Loyter and Schramm, 1966). Indications are that this complex is analogous to an antibody - antigen system (Levitzki et al, 1964).

4. Multiple forms of germinated cereal α-amylase: As with many other germinated cereal enzymes such as β-amylase, peroxidase, esterase, etc., α-amylase is composed of multiple forms termed isozymes. Early research with germinated barley α-amylase indicated that only one molecular species was present (Grabbar and Daussant, 1964). Subsequent research by Frydenberg and Nielsen (1965) and MacGregor and Meredith (1971) employing electrophoresis and Daussant et al (1974) using immunochemical techniques indicated that at least two molecular species were present. Similarly with malted wheat, two electrophoretically different sets of α-amylases were present (Olered and Jönsson, 1970; Alexandrescu and Mihailescu, 1970; Kruger, 1972b). Kruger (1972b) using a more refined technique was able to further separate these two sets into individual isozymes. The more mobile set on basic gel electrophoresis was composed of three isozymes, while the less mobile set was composed of four and sometimes five isozymes.

Other cereals that have been studied are germinated oats (Smith and Bennett, 1974) and malted rye (Manners and Marshall, 1972). In both cases, multiple forms of α-amylase were present.

Most of the studies carried out on germinated cereal α-amylase isozymes have involved examination of the activity distribution after some form of gel electrophoresis. Little work has been directed towards separation of the isozymes on a preparative scale, but some preparative work has been carried out on malted wheat α-amylase isozymes (Kruger and Tkachuk, 1969), malted barley (MacGregor et al, 1971) and malted rye (Manners and Marshall, 1972).

Tkachuk and Kruger (1974) have also carried out a physical characterization of the malted wheat isozymes. Small differences in isozyme

Table II

Amino Acid Compositions of Malted Wheat α-Amylases
(Moles per g)[a,b]

	Isozyme Components				
	I	II	III	IV	Average
Tryptophan[c]	255	297	n.d.	213	255
Lysine	441	415	471	410	434
Histidine	276	292	327	290	296
Ammonia	795	853	824	789	815
Arginine	461	401	396	342	400
Aspartic Acid	939	923	1010	965	959
Threonine	464	451	495	472	471
Serine	469	477	467	475	472
Glutamic Acid	943	970	986	893	948
Proline	589	586	596	642	603
Glycine	972	959	1025	931	972
Alanine	795	714	798	743	763
Cystine[d]	44	68	61	49	51
Valine	582	584	590	535	573
Methionine	117	157	115	182	143
Isoleucine	436	464	492	446	460
Leucine	599	621	694	617	633
Tyrosine	272	280	316	302	293
Phenylalanine	289	309	359	367	331
Cysteine	0	0	0	0	0
Actual Experimental amine acid nitrogen recovery, %	83	89	94	92	

a. Calculated to 100% amino acid residue weight recoveries, e.g., values in column 1 were multiplied by 100/83.

b. Threonine, serine, proline, valine, and isoleucine recoveries multiplied by 105, 109, 105, 108 and 107% to correct for incomplete hydrolysis and decomposition during hydrolysis.

c. Determined by analyzing barium hydroxide hydrolysates.

d. Determined as cysteic acid.

From Tkachuk and Kruger, 1974.

properties were evident, such as in energy of activation and pH optima, but the major difference was a result of charge differences between the isozymes. These charge differences resulted from basic differences in the amino acid composition of the isozymes, as shown in Table II. It may be seen that the arginine lysine, glutamic acid and aspartic acid contents are significantly different between the four α-amylase isozymes, resulting in differences in charge between the isozymes.

It should be noted that the possibility does exist that the isozymes are an artifact of the isolation procedures although indications are that this is not so. For example, studies on porcine pancreas α-amylase isozymes (Rowe et al, 1968) showed that the α-amylase isozymes were definitely not artifacts due to separation procedures or association phenomena and this is probably the case with the cereal α-amylase isozymes.

5. Mode of attack on starch by the germinated cereal α-amylase: Cereals synthesize starch as a reserve polysaccharide and store it in the endosperm of the seed in the form of a granule. Upon germination of the seed, the starch is broken down by carbohydrate-degrading enzymes to smaller products used by the growing plant. Before studying the α-amylolytic breakdown of starch, it is necessary to have an understanding of its chemical and morphological structure.

Starch consists of two components, amylose and amylopectin, which form a paracrystalline network constituting a starch granule. Amylose is basically an unbranched linear polymer of α-D-glucose residues, joined by α-1,4 glucosidic linkages. On the other hand, amylopectin is a branched polymer composed of short linear chains of 20 - 24 glucose residues joined by α-1, 4 linkages with α-1, 6 linkages forming the branch points between linear portions (Kneen et al, 1941; Marshall, 1972). The starch granules located in the endosperm of mature barley or wheat kernels are composed of two distinct types, these being large and small granules. Mature barley starch has a definite bimodal distribution of granules whereas mature wheat does not (Palmer, 1972; Kulp, 1973). It should be noted that although the small granules make up 80% of the total number, the large granules make up 90% of the total starch weight (Stamberg, 1939).

As well as considering the mode of attack of the α-amylase on the amylose and amylopectin, the mode of attack on the whole granule must also be studied. The attack on the whole starch granule and the effect on its gross morphology will be discussed first followed by the attack on amylose and amylopectin with particular reference to action pattern.

(i) Mode of attack on the starch granule: In early studies, researchers did not know which hydrolytic enzyme actually attacked the starch granule i.e. whether it was α-amylase, β-amylase or some other carbohydrate metabolizing enzyme. A special raw starch degrading factor was postulated by Blish et al (1937), but Sandstedt (1954) showed that α-amylase was the hydrolytic enzyme responsible. Walker and Hope (1963) conclusively showed that a raw starch degrading factor was unnecessary. Recent studies by Dunn (1974) have confirmed Sandstedt's work and he has formulated the following simple model for starch granule breakdown: a) initial attack by α-amylase on the starch granule in vivo b) subsequent degradation by other starch degrading enzymes (β-amylase, limit dextrinase, and α-glucosidase) of the dextrins released into solution by the amylase attack on the granule itself.

The rate of attack of the germinated cereal α-amylases appears dependent upon the size of the starch granule. In the case of germinating wheat it has been established through scanning electron microscopy that α-amylase attack in the early stages of germination is upon the large starch granules (Dronzek et al, 1972). As germination proceeds, the small granules are subsequently attacked.

Studies by Evers and McDermott (1970) and Dronzek et al (1972) indicate that there is a preferential attack by germinated wheat α-amylase at the surface in the equatorial groove of the starch granule. Evers and MacDermott (1970) also discovered that the attack on the surface of the starch granule resulted in the formation of pits. Subsequently, a channel was hydrolyzed passing radially through a number of shells in the lamellar structure of the starch granule.

Evers and MacDermott (1970), after viewing these modifications

of the starch granule by α-amylolysis, concluded that the pattern of susceptibility of the starch granules was most probably a property of the individual granules. In a later paper, Evers et al (1971) postulated that the preferential attack on various parts of the starch granule could result from the germinated α-amylase being basically restricted to attacking α-1, 4 linkages interior to the starch chains. This could limit the attack to only those areas where a suitable molecular arrangement made hydrolysis possible.

In viewing these findings, it becomes apparent that the relationship between starch granules and germinated cereal α-amylase is not analagous to a simple enzyme substrate interaction. In fact, a complicated system is present with a great many unknowns still left uncovered.

(ii) Action pattern of germinated cereal α-amylase: The α-amylolysis of starch is characterized by an initial rapid loss in iodine colour and viscosity (Bernfeld, 1951). This phase has been called the dextrinization phase and is overlapped by the saccharification phase, where the reducing power slowly increases (Hopkins, 1946). In the dextrinization phase, the amylopectin or amylose is broken down into smaller dextrins or short chains plus maltose and glucose. In the second phase, the much slower breakdown of these small sugars take place. It should be realized that these apparent stages are not due to separate reactions but are merely the result of the differences in affinity of the enzyme for large and small substrate molecules (Greenwood et al, 1965c). If the incubation period is very long, the final products of α-amylolysis would be glucose, maltose and sometimes maltotriose (Bernfeld, 1951; Roberts and Whelan, 1951). On the other hand, if the incubation time is relatively short, larger products will be obtained which are characteristic of the α-amylase in question, i.e., cereal, bacterial, fungal, etc., α-amylase.

Fig. 1. Bonds in α-1,4-glucans attacked non-randomly by α-amylases.

⬡ = a glucose residue.

⬢ = a reducing glucose residue.

\- = an -1,4 bond.

a. Bonds which are Resistant to α-amylolysis.

A and B are resistant to attack by cereal bean, porcine pancreatic and B. subtilis α-amylases.

C and D and E are resistant to attack by cereal, bean and B. subtilis α-amylases.

F is resistant to attack by cereal, porcine pancreatic and B. subtilis α-amylases.

b. Bonds which are Preferentially Attacked by α-Amylases.

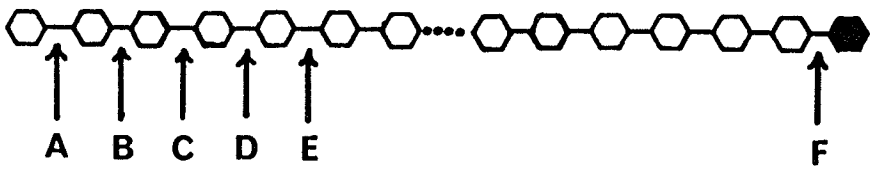
G is labile to bean and B. subtilis α-amylases.

H is labile to cereal, bean and porcine pancreatic α-amylases.

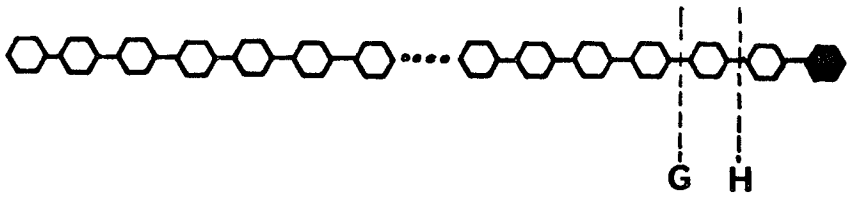
From Greenwood and Milne (1968b).

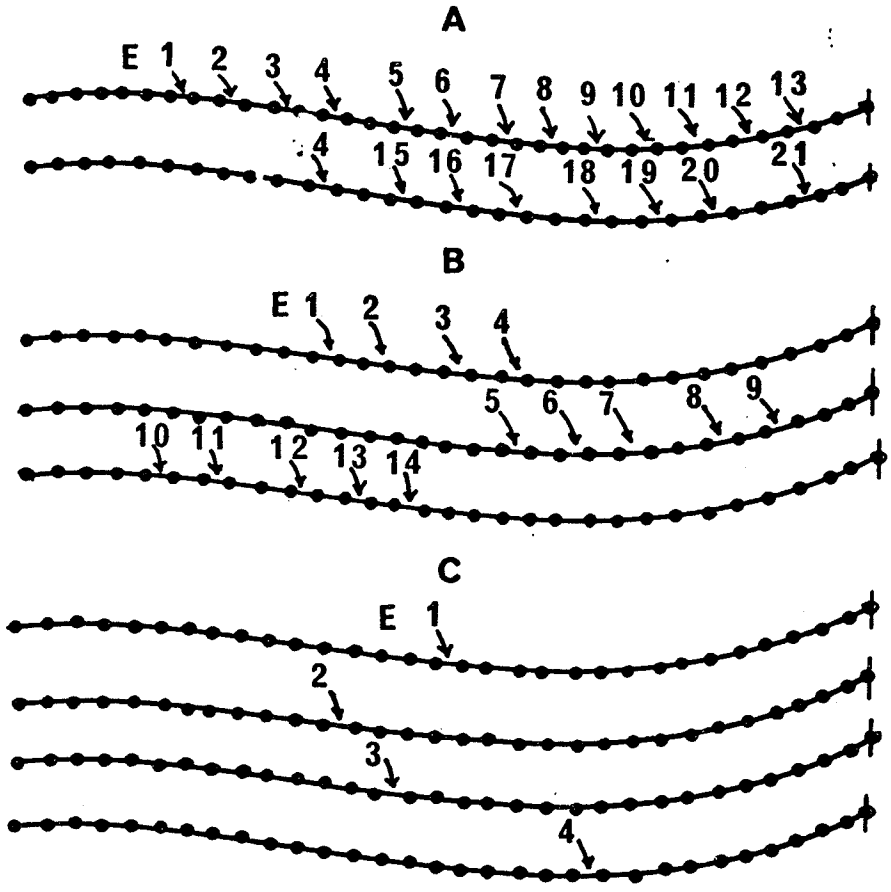
Fig. 2. Types of attack patterns for endoamylases: (A) single chain, (B) multiple attack, and (C) multichain. Each case illustrates the action of a single enzyme molecule. The arrows represent the catalytic hydrolysis of a glycosidic bond; (●) amylosemolecule made up of glucosyl units linked α-1,4; (◆) reducing hemiacetal end group. The numbers refer to the sequence of hydrolytic events by the enzyme. The oligosaccharide product specificity in cases (A) and (B) is arbitrarily assumed to be maltose and maltotriose. The authors have assumed a definite polarity of action toward the reducing end. The actual direction of the action is now known to be toward the non-reducing end for pancreatic α-amylase. For illustrative purposes the amylose molecule is pictured as a long "string"; however, it undoubtedly possesses a certain amount of secondary helical structure that is not illustrated. From Robyt and French (1967).

a


A
B
C
D
E
F

b


G
H


A
E 1 2 3 4 5 6 7 8 9 10 11 12 13
4 15 16 17 18 19 20 21
B
E 1 2 3 4
5 6 7 8 9
10 11 12 13 14
C
E 1
2
3
4

Basically there are two models which describe the action pattern of α-amylase with respect to the breakdown of amylose. As either model could be valid in the case of cereal α-amylases, both will be discussed:

a. Preferred attack:

The preferred attack hypothesis states that the action of α-amylase on amylose is random in the initial stages of hydrolysis, but becomes non-random in the latter stages due to resistant bonds in the substrate.

Originally it was believed that all α-amylases attacked their substrates in a random manner yielding the same products (Meyer and Bernfeld, 1941). Bird and Hopkins (1954) showed that this view was erroneous. In fact they felt that, although the attack on a large substrate molecule was basically random, as the substrate size decreased the attack became non-random due to resistant bonds near chain ends. These resistant bonds were found to be different for malt barley as compared to salivary or bacterial α-amylases. Greenwood and Co-workers (Greenwood and MacGregor, 1965; Greenwood et al, 1965a,b,c,d; Greenwood and Milne, 1968 a,b,c.) carried out further studies expanding upon the model of Bird and Hopkins. A pictorial representation of the resistant and susceptible bonds in a linear amylose molecule is shown in Fig. 1.

b. Repetitive attack:

The repetitive attack hypothesis was initially introduced to explain the action of the exo enzyme β-amylase (French, 1961). This has since been expanded by Robyt and French (1967, 1970) to cover the endoenzyme α-amylase. This hypothesis states that once the α-amylase binds with the substrate and hydrolyzes a bond, it does not dissociate away but carries out the hydrolysis of more bonds during the encounter.

This is often called the multiple attack and is intermediate between single chain and multichain attack (random attack, Fig. 2). Multiple attack was shown to take place with porcine pancreas α-amylase (Robyt and French, 1970) but Banks et al (1970, 1971) did not find any evidence for the hypothesis with malted rye or human salivary α-amylase.

These models have been derived from studies involving the action of α-amylase on amylose. In the case of the more complex amylopectin, Manners and Marshall (1971) found that the α-1, 6 linkages conferred resistance to adjacent α-1, 4 linkages. Greenwood and Milne (1968b) found that cereal α-amylases acted randomly on the amylopectin except for those bonds near a substrate chain end, a branch point, or those regions of the amylopectin made inaccessible due to steric hindrance.

C. Immature Cereal α-Amylase

To this date the α-amylase present in immature cereal seeds has not been studied as intensively as the α-amylase found in germinated cereal seeds. Recent work by various researchers, however, has yielded some knowledge as to the level of this enzyme in the immature seed, its morphological distribution and a few of its properties. Immature cereal α-amylases will be discussed in light of this new knowledge.

1. Changes in activity levels of immature cereal α-amylase: The developing cereal grain is not a stagnant system, but a dynamic one, undergoing continuous changes in its chemical and physical make up. Chrazaszcz and Janicki (1936) found that α-amylase was a part of this chemical change in wheat, barley, oats and rye. They observed that the α-amylase activity in these immature cereal kernels appeared shortly after blossoming and then gradually disappeared with ripening. Sandstedt (1946) realized that a carbohydrate degrading enzyme was present in the immature wheat kernel, since the pericarp starch, deposited a few days after pollination, was enzymatically digested during kernel development. This enzyme was subsequently shown to be α-amylase and was found to increase in the first week of growth followed by a slow decrease during the ripening of the wheat seed (Sandstedt and Beckford, 1946). Schwimmer (1947), on the other hand, found that on a wheat kernel basis the α-amylase content was relatively constant, although on a dry weight basis the activity decreased rapidly. Recently Olered and Jönsson (1970) also found that the α-amylase of immature wheat (which they called "green α-amylase") slowly disappeared with ripening. This confirmed the previous findings of Sandstedt and Beckford (1946). In addition, they found that the decreasing α-amylase

activity could be regenerated to an extent by increases in the moisture distribution or equilibrium of the ripening seed. The suggested explanation for this was that the "green α-amylase" was inactivated by dehydration of the kernel during the ripening process.

Similar results have been obtained with the developing barley kernel. MacGregor et al (1971) found that the α-amylase activity increased rapidly from emergence to eleven days post emergence and then declined sharply to one tenth of the maximum level after twenty-eight days. This low level was subsequently maintained until maturity. Similar results were obtained by Duffus (1969) and Duffus and Rosie (1973) who found that the maximum activity was reached twenty to thirty days after anthesis. Studies by LaBerge et al (1971) indicated that there was no great varietal differences with respect to the changes in α-amylase activity in barley.

One point of note is that, in contrast to the synthesis of the germinated cereal α-amylases, the synthesis of the immature α-amylases of wheat (Olered and Jönsson, 1970) and barley (Bilderback 1971, MacGregor, 1972) is not induced by gibberellic acid. This conflicts with the report of Duffus (1969) who found that chlorcholine chloride, a gibberellic acid synthesis inhibitor, inhibited α-amylase synthesis in immature barley. A possible explanation for the first result was put forward by MacGregor et al (1972), who suggested that the non-effect of gibberellic acid could be due to different systems being present for the synthesis of immature and germinated α-amylases. The only other feasible explanation would be that an excess of gibberellic acid is already present in the seed. The reason for the effect of chlorcholine chloride remains unknown.

2. Morphological distribution: Sandstedt and Beckford (1946) found that α-amylase activity in immature wheat was present in the pericarp and not in the endosperm. These findings have since been verified by Kruger (1972a), who used an electrophoretic technique to show that the major portion of the α-amylase was present in the pericarp. Only small amounts were found in the seed coat and endosperm with none present in the embryo. Banks et al (1972) obtained a similar result with the α-amylase being present primarily in the pericarp-testa, and only a negligible amount present in the endosperm-germ. Meredith and Jenkins (1973b) dissected immature wheat grains into white pericarp and green inner part and found double the activity in the pericarp.

With respect to the α-amylases of immature barley, differences of opinion exist as to the location in the kernel. Duffus (1969) and Bilderback (1971) found that the endosperm as well as the aleurone contained considerable amounts of activity. This conflicts with the results of Stoddart (1971) who found that the α-amylase was almost completely contained in the aleurone. On the other hand, MacGregor et al (1972) found that the α-amylase activity was confined to the pericarp with very little being present in the endosperm. These discrepancies probably arose as a result of dissection techniques and consequently it is difficult to ascertain where the α-amylase is actually situated in the immature barley. It would appear most likely that the α-amylase of the immature barley kernel is present in the outer layers of the kernel, as is the case with wheat.

A reasonable assumption for the presence of the α-amylase in the outer layers of the immature cereal kernel would be to hydrolyze the

pericarp starch to provide some of the energy and sugar requirements of the growing seed (Kruger 1972a; MacGregor et al 1972).

3. Immature cereal α-amylase isozymes: Electrophoretic work by many researchers had indicated that α-amylase isozymes are present in immature wheat (Olered and Jönsson, 1970; Kruger 1972a; Iliev, 1974). Kruger (1972a) found three electrophoretically mobile isozymes present in immature wheat. Olered and Jönsson (1970) also found several isozymes present but the resolution of their electrophoretic technique was not sufficient to give an exact number. Kruger (1972a) found that these three isozymes were present in all varieties of Canadian (HRS) wheat studied. The isozymes were not preferentially separated from each other into the various anatomical tissues of the whole seed and were found in the same proportions.

Kruger (1972a) also noted that the 3 α-amylase isozymes of immature wheat were electrophoretically identical on basic polyacrylamidegel electrophoresis to a set of three isozymes present in germinated wheat and were possibly related. It was suggested that the immature α-amylase isozymes were regenerated to yield the three isozymes found in the germinated seed. Some evidence to support this assumption was the discovery by Daussant and Renard (1972) that 2% of the activity of germinated cereals could be attributed to immature α-amylases.

Olered and Jönsson (1970) found that the isozymes of immature wheat differed to some degree with the α-amylase of malt. The "green α-amylases" were found to be slightly more heat sensitive, with this sensitivity becoming greater during ripening.

A simpler isozyme system is present in immature barley as both Stoddart (1971) and MacGregor et al (1974) found only 1 isozyme of

α-amylase to be present in the immature kernel. MacGregor et al (1974) also found the immature α-amylase isozyme to be electrophoretically identical to a distinct isozyme of malt barley. On the other hand, Bilderback (1971) found that an immature Himalayan 2-row cultivar apparently contained four α-amylase isozymes. There is the possibility that this was just an isolated case and different from the norm. As with immature wheat α-amylase, Stoddart (1971) also found that the α-amylase isozymes of the developmental stages differed from those present in the germinating phase.

MacGregor et al (1974) have continued this work by purifying and partially characterizing the α-amylase isozyme present in immature barley.

α-Amylase In Breadmaking

Alpha-amylase has found a great deal of importance in the breadmaking industry, as it has a large influence on the quality of bread obtained from a flour. To realize the importance of this enzyme in the breadmaking process, it is first necessary to understand the role that it plays.

In breadmaking the α-amylase in dough breaks down the starch to dextrins and fermentable sugars. In the fermentation stage only the damaged starch granules are available for attack whereas in the gelatinization period,before the α-amylase is inactivated, both the damaged and undamaged starch granules are attacked (Farrand, 1964). α-Amylase in conjunction with β-amylase can affect dough in 3 ways, these being: i) formation of fermentable sugars ii) removal of the damaged starch fraction and iii) the formation of dextrins (Bloksma, 1972).

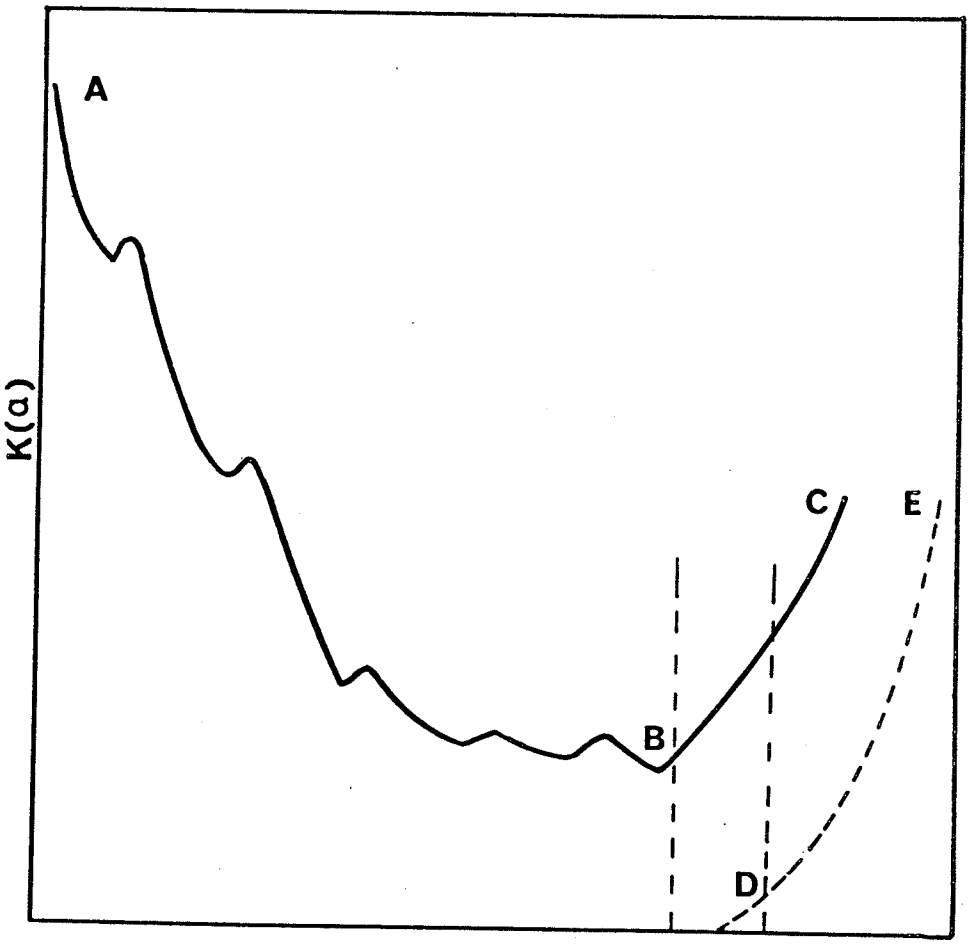
In the first case sufficient fermentable sugars must be present so that the yeast will produce enough carbon dioxide to yield a loaf of proper size, grain and texture (Geddes, 1946). The removal of the starch damaged fraction, in most cases, is not of major importance due to the presence of only a few % damaged starch granules in normal flour (Johnson and Miller, 1953). Some flours, of course, do have a high content of damaged starch especially if they are put through a breadmaking process such as the Chorleywood process. In cases such as these, the α-amylase content will become more important. In the gelatinization period, the presence of α-amylase is extremely important since the enzyme is highly active and quickly breaks down the starch to dextrins. Starch is more highly hydrated than dextrins and as a result, excessive liquefaction and dextrinization will occur if too much

Fig. 3. Diagram showing the variations in α-amylase activity (K (α)) in unripe wheat grains during ripening and the appearance of α-amylase from malting.

AB, Kernel development until full ripeness. Variations in α-amylase activity caused by changes in moisture distribution; BC, 'primary' α-amylases are reactivated by delayed drying; DE, synthesis of α-amylase during malting.

The period between B and D is characterised by a high α-amylase activity and low falling numbers without the appearance of visible sprouts.

From Olered and Jönsson (1970).


A
K(a)
C
E
B
D

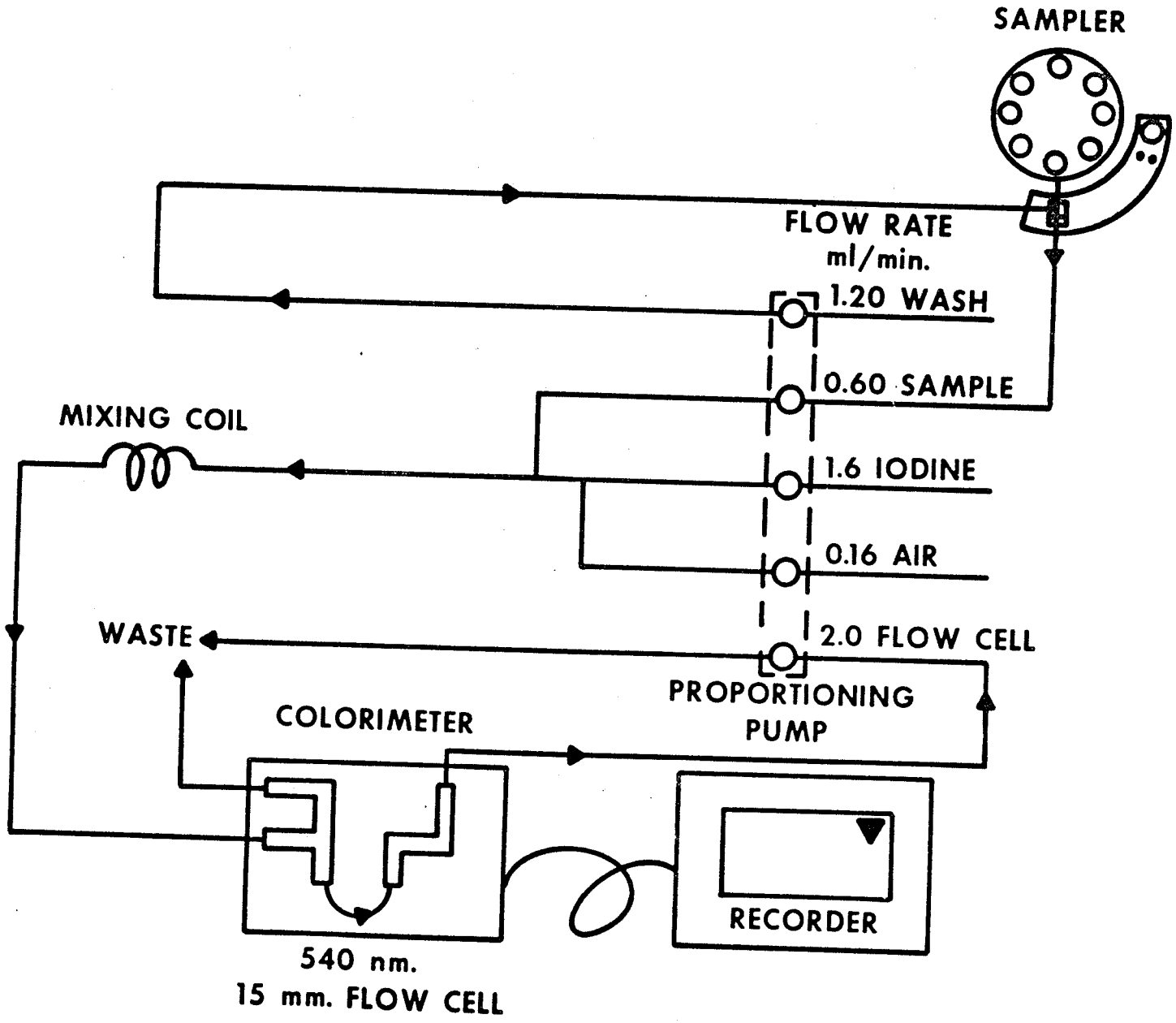
α-amylase is present. This will yield a loaf of poor quality with a wet and sticky crumb (Geddes, 1953; Becktel et al, 1964; Bloksma, 1971).

It is evident that α-amylase is extremely important in bread-making, as it can have a major influence on gassing power, loaf volume, texture, crumb and even crust colour. Minimization of high levels of this enzyme in wheat crops has been, therefore, the concern of cereal technologists around the world.

In many cases, this excess α-amylase results from sprouted wheat. Tipples et al (1966) carried out baking tests with a heavily sprouted wheat and found that it resulted in deterioration due to large holes in the loaf. They also pointed out that many commercial grades of wheat allow a certain amount of sprouted wheat. Thus, since this wheat contains high α-amylase, minimal amounts may cause deleterious effects in the baking quality of the flour.

Olered and Jönsson (1970) suggested that excess α-amylase may actually come from a source other than sprouted wheat. As mentioned in a previous section, they found that "green α-amylase" may be re-activated during the latter stages of maturation. In fact, even when a kernel appears mature these "green α-amylase" may be present. Fig. 3 shows that in the period B-D, just before sprouting, the "green α-amylase" may be much more active than the germinated α-amylase. Thus, wheat containing reactivated "green α-amylase" or immature kernels could have a detrimental effect on baking quality similar to that obtained with sprouted wheat.

Fig. 4. Technicon AutoAnalyzer Flow-sheet for semi-automation of α-amylase, with β-limit dextrin as substrate.


SAMPLER
FLOW RATE
ml/min.
1.20 WASH
0.60 SAMPLE
MIXING COIL
1.6 IODINE
0.16 AIR
WASTE
2.0 FLOW CELL
PROPORTIONING
PUMP
COLORIMETER
RECORDER
540 nm.
15 mm. FLOW CELL

MATERIALS AND METHODS

Immature Neepawa, a Canadian HRS wheat, was obtained at approximately 20 days after anthesis. The heads and part of the stems were freeze dried and stored until usage. Before extractions the freeze dried wheat was ground in a Wiley Mill (equipped with a 1mm. mesh sieve).

α-Amylase Activity

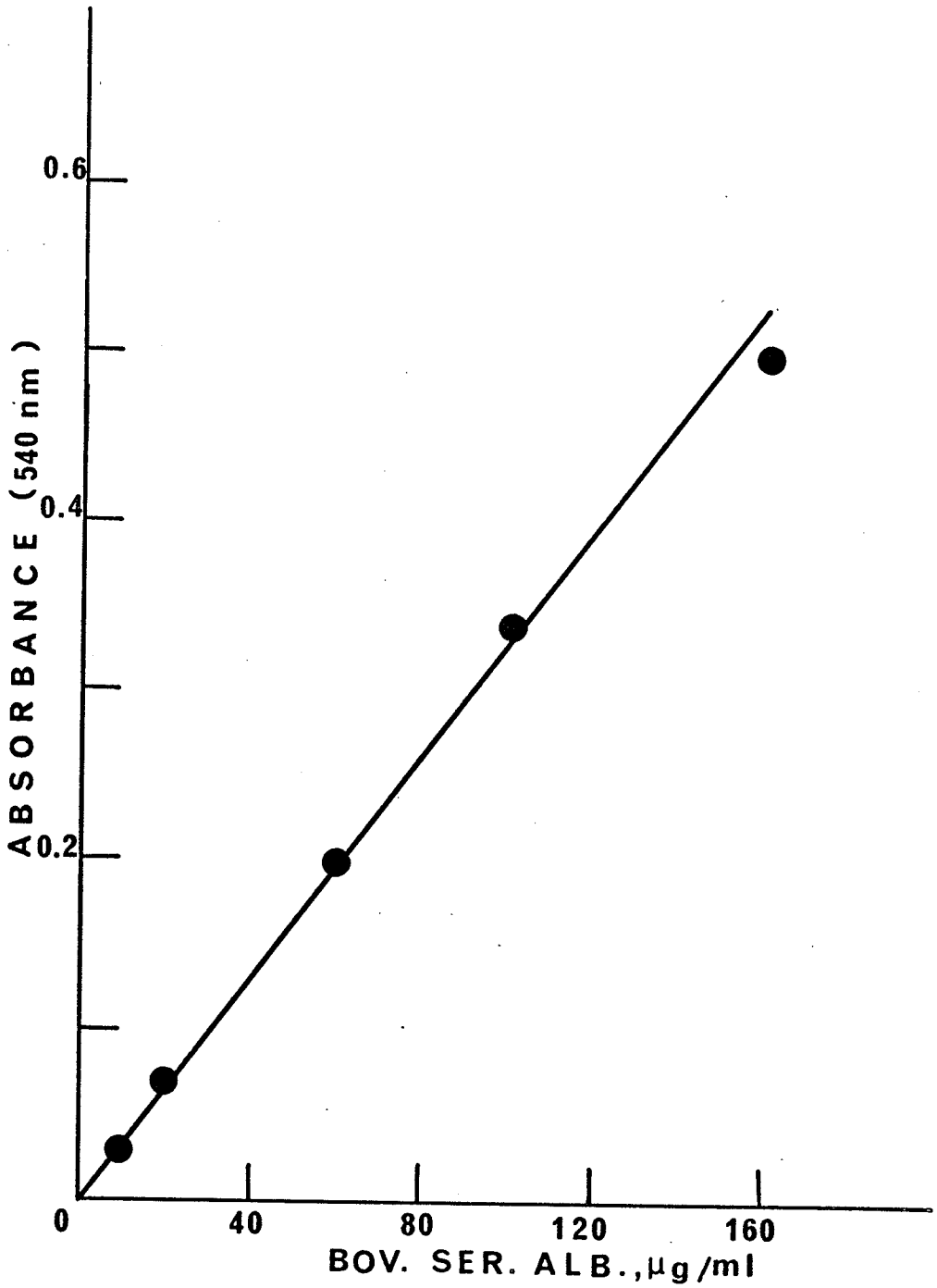
Routine determinations of α-amylase activity were carried out using a semi-automated iodine β-limit dextrin assay developed from the manual method of Briggs (1961), as modified by MacGregor et al (1971). The basic Technicon AutoAnalyzer (Technicon Corp., Chauncey, N.Y.) was used for semi-automating the assay. The system consisted of a Sampler II; Proportioning pump II; colorimeter with a 540 mm. filter and a recorder using absorbance paper. The flow diagram is listed in Fig. 4. The substrate used was a 0.08% solution of β-limit dextrin in 0.2M. sodium acetate buffer, pH 5.5 and 0.001M calcium chloride. The β-limit dextrin was prepared from waxy maize starch as described by Kruger and Marchylo (1972). The assay involved incubating 2 ml of enzyme solution with 2 ml. of β-limit dextrin for 10 to 20 minutes (depending on the activity of the sample). This digest was cooled to about 5°C, then placed in a sample cup and sampled at 0.6 ml/min.

The α-amylase activity was calculated using the following equation, which is a modification of the equation used by Olered (1967):

$$k(\alpha) = \frac{1}{t} \times f \times (\log E_{xo} - \log E_{x})$$

Where t is the time of incubation in minutes, f is the dilution factor required to bring the absorbance in the range of 0.2 - 0.8 OD, E_{xo} is

Fig. 5. Calibration curve for the determination of protein by the automated Folin phenol assay, using Bovine Serum Albumin as the standard protein.


0.6
0.4
0.2
0
ABSORBANCE (540 nm)
40
80
120
160
BOV. SER. ALB., µg/ml

the absorbance on the colorimeter of the β-limit dextrin iodine complex at t = 0 and E_x is the absorbance after t minutes of incubation with the enzyme.

Protein Determination

Protein determinations were routinely carried out using an automated Folin phenol protein assay developed from the manual procedure of Lowry et al (1951). A linear calibration curve (Fig. 5) was obtained using bovine serum albumin (Calbiochem, Los Angeles, Calif.) standards from 10-160 g/ml.

Polyacrylamide Slab Electrophoresis

An Ortec Model 4200 slab electrophoresis system was used with an Ortec model 4100 pulsed constant power supply (Ortec Inc., Oak Ridge, Tenn.). The Ornstein and Davis (1962) basic and acidic gel systems were used. The small pore acrylamide gel was prepared in a 9 by 10 by 0.3 cm I.D. casting cell to a depth of 6.5 cm. The large pore was then placed on top of the small pore and a 12 sample teflon well former was inserted. The sample wells were then loaded with 0.125 ml. of sample solution containing equal parts of α-amylase solution and 50% sucrose in water. Electrophoresis was carried out at 325V. with the initial pulse rate being set at 75 pps. At 5 minute intervals the pulse rate was raised to 150 pps, 225 pps, and finally 300 pps.

Detection of α-Amylase Isozymes

In order to determine the position of the α-amylase isozymes in the gel following electrophoresis, the slab method of Doane (1967) as modified by MacGregor et al (1974) was employed. In this case, 0.1g of starch was used in the preparation of the detection plates. The

gels were placed between two starch plates (prepared by the above method) and were allowed to incubate in a covered tray containing a small amount of 0.2M acetate buffer, pH 5.5 containing 0.001M $CaCl_2$ at 35°C for 30 - 40 min., depending on the activity of the sample. The plates were then developed in a 0.2% potassium iodide and 0.02% iodine solution. Colorless bands against a blue background indicated the position of the α-amylase isozymes.

Isolation of the α-Amylase Isozymes

1. Extraction of crude α-amylase: The extraction was carried out in a 128 oz jar with a 2.4 l of 0.2% $CaCl_2$ solution being added to 300 g. of ground freeze-dried immature Neepawa wheat. The jar was sealed and placed on a shaker for a period of 4 hr. at 4°C. The resultant suspension was then centrifuged at 4°C and 12,000 x g for 10 minutes.

2. Heat treatment: A heat treatment step was carried out to remove β-amylase plus other contaminating heat labile proteins. 200 ml. of the crude extract was placed in a 95° water bath and, with stirring, was brought to 70°C. The solution was then transferred to a 70°C constant temperature water bath. The total time for the heat treatment was 15 min. The extract was then quickly placed in an ice bath and cooled to 24° - 30°C., followed by centrifugation at 4°C for 5 min. at 12,000 x g.

3. Acetone fractionation: The heat treated extract was maintained at 4°C and acetone at -10°C was slowly added with stirring to a concentration of 15% (v/v). The temperature of the extract was then lowered to -5°C. and maintained there with an ice salt bath. Acetone at -10°C was again added with stirring until its concentration reached

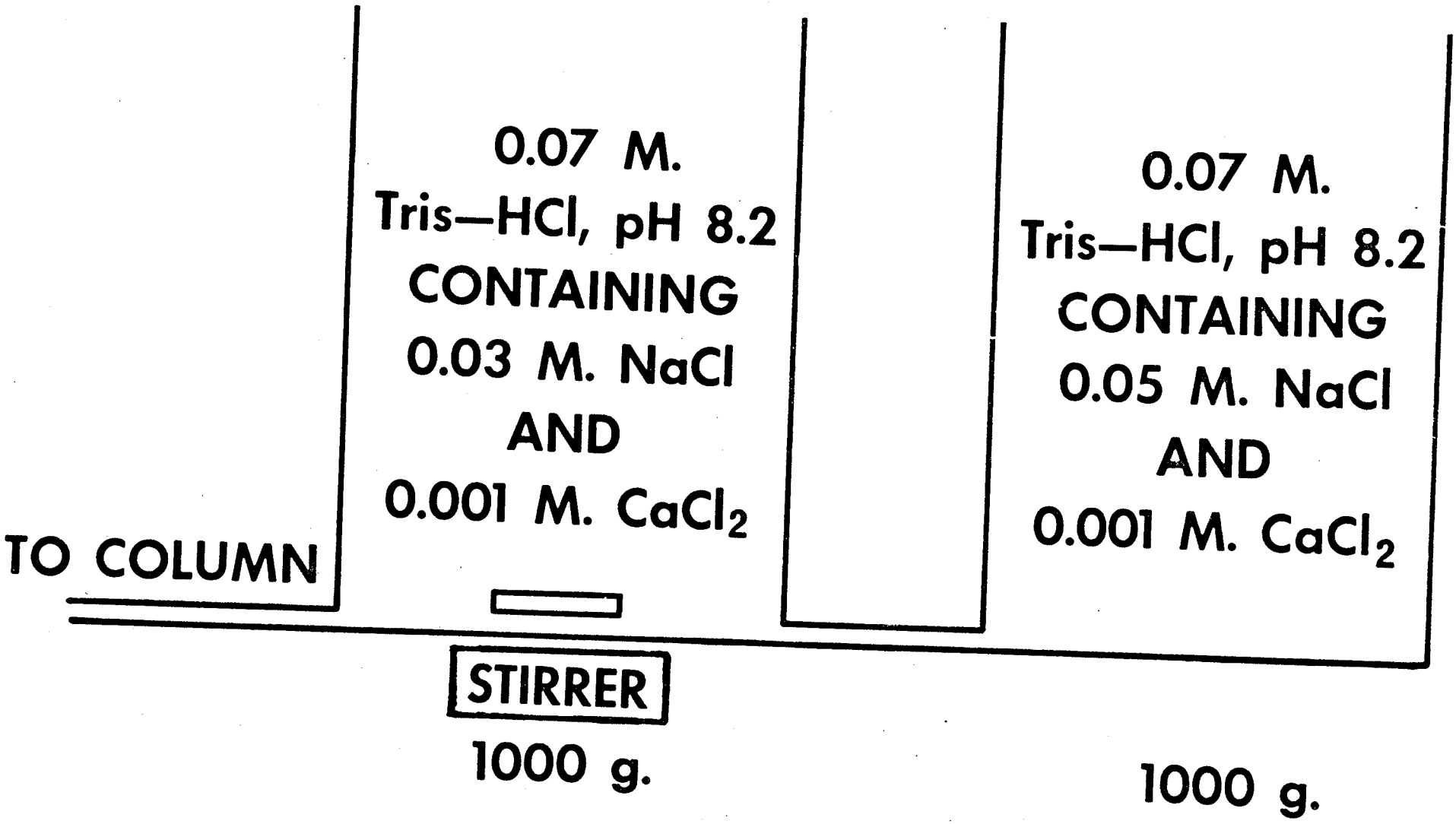
25% (v/v). This mixture was allowed to stand for 20 min., followed by centrifugation at 12,000 g for 5 min. at -5°C. The precipitate was discarded and acetone at -10°C was added to the supernatant to a concentration of 54% (v/v). This was centrifuged at -5°C and 12,000 x g for 5 min. The precipitated protein containing the α-amylase was dissolved overnight in 75 ml. of 0.07M. Tris-HCl pH 8.2 containing 0.001M $CaCl_2$. The undissolved protein was then removed by centrifugation at 12,000 x g and 4°C for 5 minutes.

4. Glycogen complex: Commercial rabbit liver glycogen (Sigma Chemical Co., St. Louis, Mo.) contained considerable protein, so a further purification was carried out as described by Loyter and Schramm (1962) using Amberlite Monobed Resin MB-3 (Analytical grade). The final purified glycogen was freeze dried and stored at 8°C.

The glycogen complexing procedure used was as described by Schramm and Loyter (1962) and as modified by Kruger and Tkachuk (1969) with a few further modifications.

Cold ethanol at 4°C was slowly added to the extract over a period of an hour with stirring to a 40% (v/v) concentration. This was followed by centrifugation at 12,000 x g and 4°C for 10 min. to remove the small amount of precipitate formed. To the resultant solution, 6 mg. of purified glycogen dissolved in 0.1ml distilled water slowly added dropwise. The solution was left stirring for 10 min. followed by centrifugation for 5 min. at 1900 x g and 4°C. The supernatant was then allowed to drain away and the precipitate was dissolved in cold 0.07M Tris-HCl pH 8.2 containing 0.001M $CaCl_2$ and 0.03M NaCl. Two more glycogen-complex precipitations were carried out using 25 mg. portions of glycogen in 0.4 ml water. The complexes were then combined in a

Fig. 6. Linear gradient employed in the separation of immature wheat α-amylase isozymes by ion-exchange chromatography on DEAE-cellulose.


0.07 M.
Tris—HCl, pH 8.2
CONTAINING
0.03 M. NaCl
AND
0.001 M. $CaCl_2$
0.07 M.
Tris—HCl, pH 8.2
CONTAINING
0.05 M. NaCl
AND
0.001 M. $CaCl_2$
TO COLUMN
STIRRER
1000 g.
1000 g.

total volume of 10 - 15 ml. and were allowed to sit at room temperature for 2 hours followed by overnight storage at 8°C. It was found necessary to carry out a second glycogen complex, as described above, to give a sufficiently pure α-amylase fraction. This second glycogen complex was dissolved in 2-5 ml of the aforementioned Tris-HCl buffer.

Chromatography

Ion exchange chromatography was used to separate the 3 α-amylase isozymes and effect a final purification. A 0.9 cm. by 59 cm. column was packed with micro-granular N,N-Diethylaminoethyl cellulose (Whatman DE-32). Before the column was packed, the cellulose was conditioned by successive washes with 0.5N HCl, 0.5N NaOH, followed by several washes with 0.07M Tris-HCl buffer, pH 8.2 containing 0.03N NaCl and 0.001M $CaCl_2$. Successive decantations were used to remove the fines. After the column was packed, degassed 0.07M Tris-HCl pH 8.2 containing 0.03N NaCl and 0.001M $CaCl_2$ was pumped through the column overnight. A linear gradient was employed in the chromatographic run as shown in Fig 6, with the limit buffers being degassed. The gradient was run through the column at 1.0 ml/min and the effluent was collected in 15 ml. aliquots. Protein monitoring was carried out using an LKB Uvicord Ultraviolet analyzer.

Reducing Sugar Assay (Neocuproin)

In order to determine some of the properties of the purified α-amylase isozymes, an automated reducing sugar assay was developed from the manual method of Dygert et al (1965). This method was used to measure the reducing sugars liberated by the hydrolysis of the substrate by the α-amylase isozymes; thus giving a measure of the α-amylase activity.

Fig. 7. Technicon AutoAnalyzer flow sheet for measurement of reducing sugars.

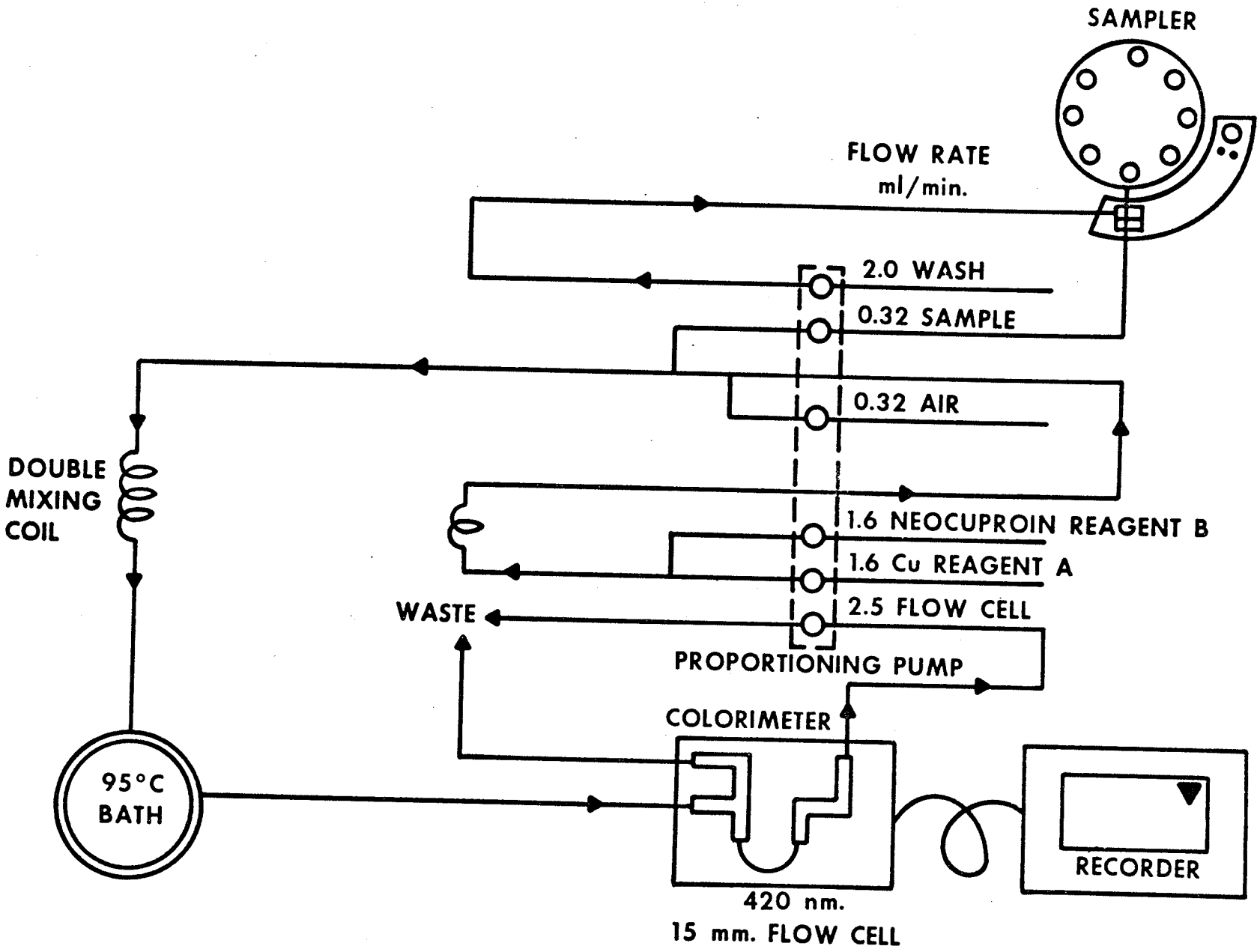
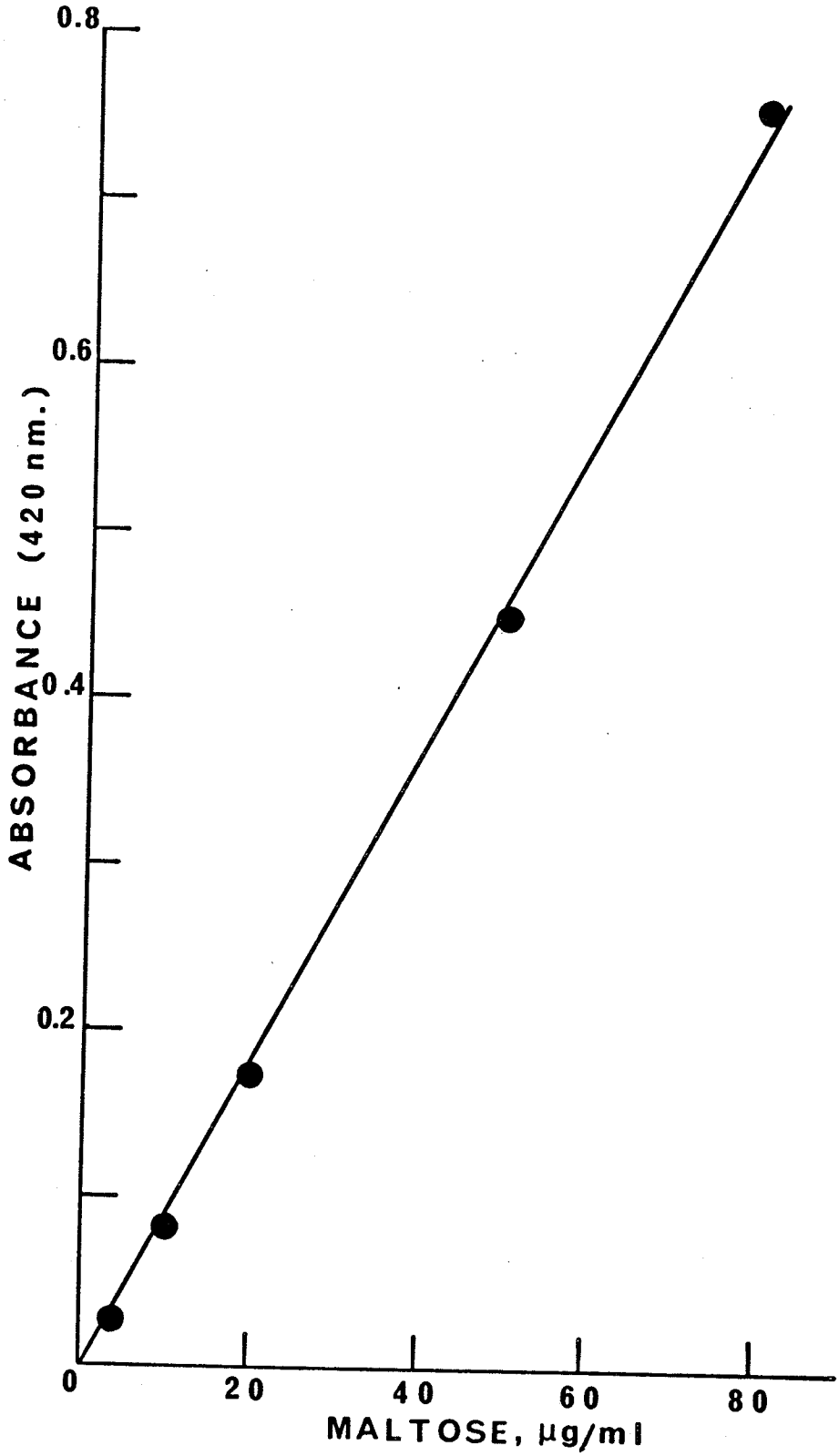

SAMPLER
FLOW RATE
ml/min.
2.0 WASH
0.32 SAMPLE
0.32 AIR
DOUBLE
MIXING
COIL
1.6 NEOCUPROIN REAGENT B
1.6 Cu REAGENT A
WASTE
2.5 FLOW CELL
PROPORTIONING PUMP
COLORIMETER
95°C
BATH
RECORDER
420 nm.
15 mm. FLOW CELL

Fig. 8 Calibration curve for the determination of reducing sugar by the automated neocuproin assay, using maltose as the standard reducing sugar.


0.8
0.6
0.4
0.2
0
ABSORBANCE (420 nm.)
20
40
60
80
MALTOSE, µg/ml

The basic Technicon AutoAnalyzer (Technicon Corp., Chauncey, N.Y.) was used to automate the procedure. The flow diagram for the system is shown in fig. 7. The system consisted of a Sampler II; Proportioning pump II; 95°C water bath with a standard 20 ft. glass coil; colorimeter with a 420 nm. filter and linear recorder. To prepare reagent A, 40 g. anhydrous sodium carbonate was first dissolved in 600 ml of distilled H_2O, followed by 16 g of glycine. 0.450 g of copper sulfate ($CuSO_4 \cdot 5H_2O$) was then dissolved and the solution was brought to 1 l. Reagent B consisted of 0.12% neocuproin HCl (BDH, Poole, Eng.) and was stored in a dark bottle. A linear calibration curve was obtained using maltose standards ranging from 4 - 80 g/ml. (Fig. 8).

Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE)

The molecular weight of the α-amylase isozymes was determined following the procedure of Shapiro, Vinuela and Maizel (1967), as explained by Weber and Osborn (1969) with the strong reducing conditions of Robyt et al (1971) being employed. The protein solutions were prepared by incubation in 0.01M sodium phosphate buffer, pH 8.5 with 1.5mM Dithiothreitol as the reducing agent. Slab gels were used instead of disc gels with an Ortec power system (Ortec Inc., Oak Ridge). Electrophoresis was carried out at a pulse rate of 225 pps. at 325. V. The standard proteins used for calibration of the gel were: ovalbumin; aldolase (Pharmacia, Uppsala); bovine serum albumin; pepsin (Calbiochem, Los Angeles, Calif.); myoglobin, cytochrome C (Schwarz Mann, Orangeburg, N.Y.); chymotrypsinogen A, (Worthington, Freehold, New Jersey). Staining of the gels was carried out with Coomassie Blue over

a period of 18 hours.

Gel Filtration

A Biogel P-100 column, 2.0 cm. by 47 cm., was prepared and equilibrated by pumping 0.1M Tris-HCl pH 7.0 buffer containing 0.001 M $CaCl_2$ through the column overnight. Apoferritin (Calbiochem, San Diego, Calif.) was used to determine the void volume. The standard proteins used for calibrating the column were: bovine serum albumin, ovalbumin, myoglobin and cytochrome C (obtained from the sources stated above).

Isoelectric Focusing

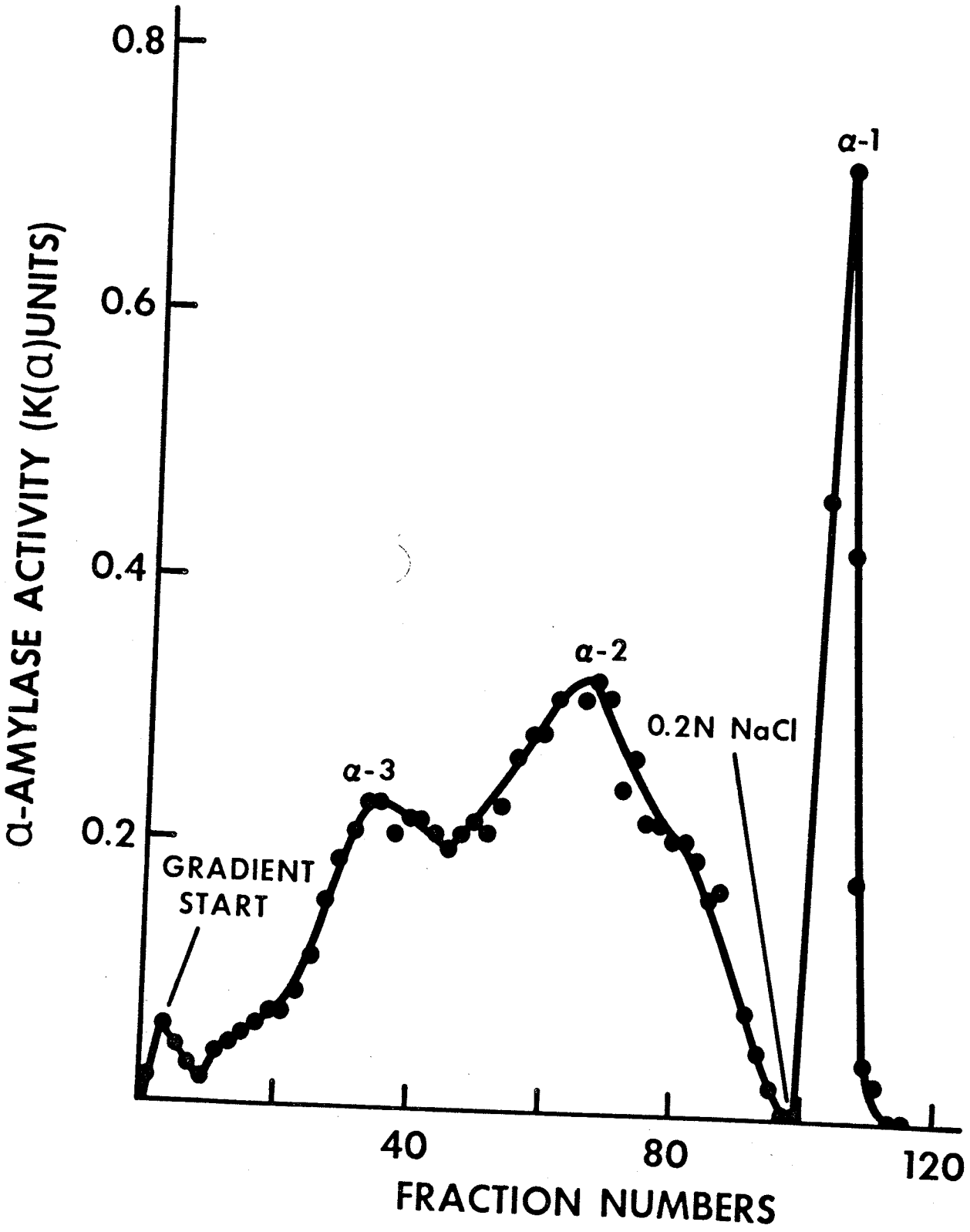
Electrofocusing was carried out as described by Vesterberg and Svenson (1966), with a 110 ml. column, ampholytes, power supply and gradient mixer purchased from LKB products. Electrofocusing was carried out at 300 V. and 5°C for 72 hours. A pH 4 - 6 gradient was employed. Following electrofocusing 2.1 ml fractions were collected at 5 - 8°C and the pH of each fraction was determined at the same temperature using a Fischer Accumet Model 521 Digital pH/ion meter. The activity of the fractions was determined by the Iodine β - limit dextrin semi-automated assay.

Table III

Purification of α-Amylase From Immature Wheat

Stage	Volume ml.	α-Amylase Concentration k(α)/ml	Total α-Amylase k(α)	Protein mg/ml	Specific Activity k(α)/mg protein	Yield %	Purification
Initial Extract	1,050	2.76	2,898	6.87	0.40	100	1.00
Heat-treated Extract	1,000	2.44	2,440	4.54	0.54	84.2	1.35
24-54% Acetone Fraction	72	17.98	1,294	3.53	5.09	44.7	12.72
Glycogen-complex I	10.4	77.22	803	0.40	193.05	27.7	482.63
Glycogen-complex II	5.0	106.51	533	0.14	760.8	18.4	1902.00

Fig. 9. DEAE-cellulose ion-exchange chromatography of immature wheat α-amylase purified to the glycogen complex stage. α-Amylase activity in terms of K(α)/ml.


0.8
0.6
0.4
0.2
α-AMYLASE ACTIVITY (K(α)UNITS)
α-1
α-2
α-3
0.2N NaCl
GRADIENT START
40
80
120
FRACTION NUMBERS

RESULTS

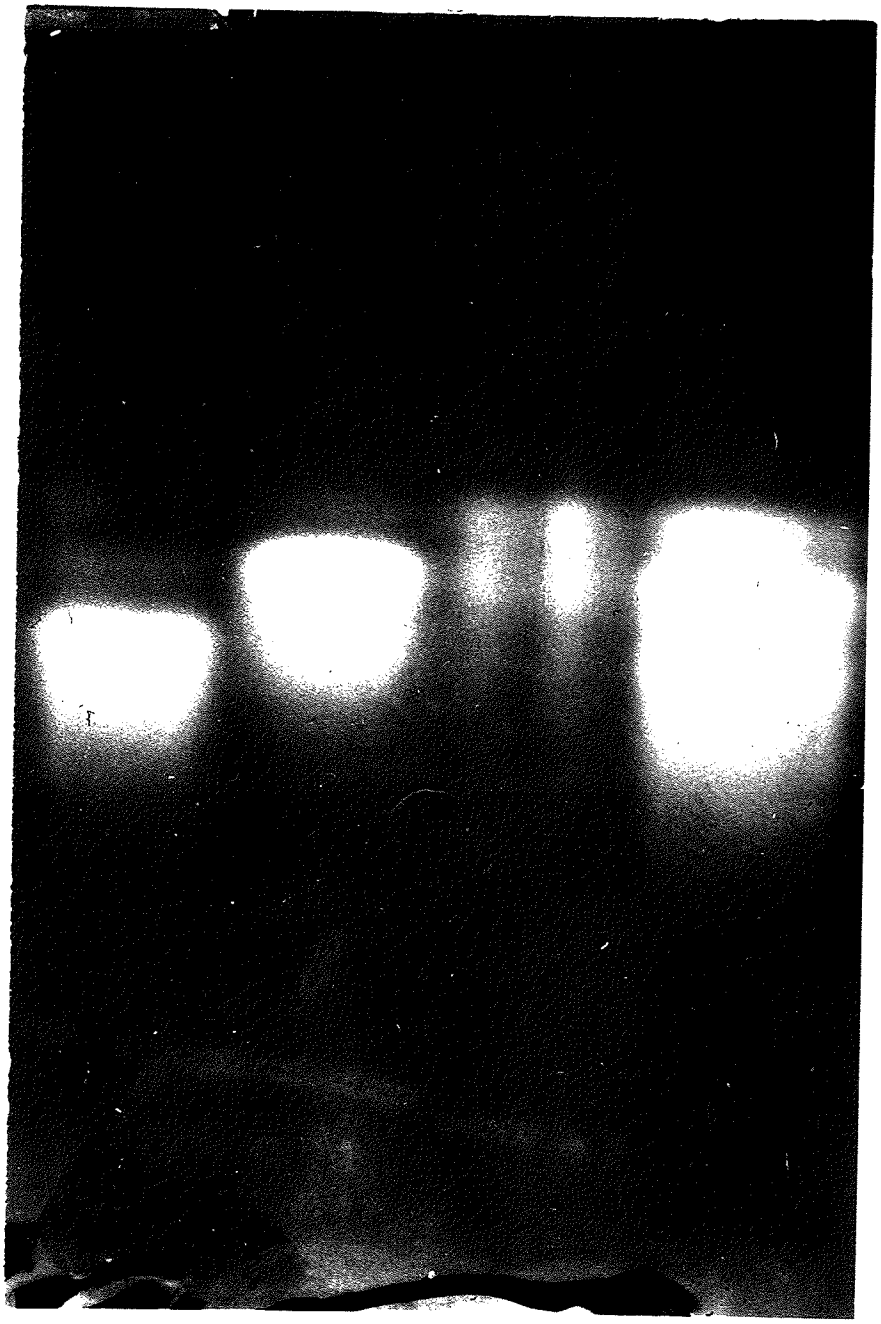
A. Isolation

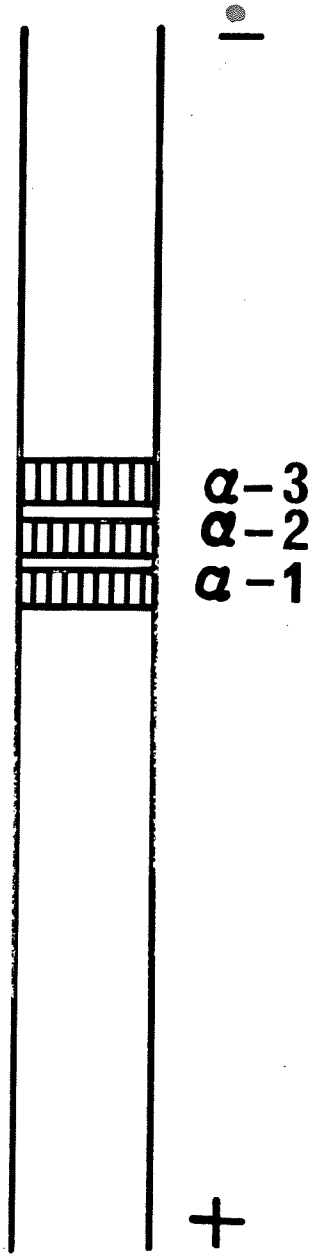
The three α-amylase isozymes of the immature HRS wheat Neepawa were successfully isolated. The isolation involved extraction of the α-amylase, heat-treatment for 15 min. at 70°C, acetone fractionation from 25% to 54%, complexing with glycogen and finally ion-exchange chromatography on microgranular DEAE-cellulose. Table III illustrates the protein and enzymic recoveries for a typical purification.

At the glycogen complex stage II, approximately 18% of the α-amylase was recovered with a 1900 fold increase in specific activity. It would appear that the immature α-amylase isozymes are more heat labile than those isolated by Kruger and Tkachuk (1969) from malted wheat. This is apparent when one considers the heat treatment step in Table III where a 16% loss in activity was incurred, as compared to an 8.5% loss with the malted wheat.

Initial attempts to purify and separate the α-amylase isozymes were made on microgranular Carboxymethyl cellulose (Whatman CM-32) but it was found that no clear cut separation of the 3 isozymes could be achieved. Chromatography with N,N-Diethylaminoethyl cellulose (Whatman DE-32), using the gradient described in Fig. 6, gave the chromatogram as shown in Fig. 9. Good resolution of the 3 isozymes was obtained using this gradient, although it was found that α-2, and α-3 overlapped.Whereas α-1 was separated from the other isozymes, it still remained on the column after the gradient elution. As a result 0.2N NaCl was passed through the column to elute α-1. Due to the small amounts of protein involved, no protein trace of the α-amylase isozymes could be detected on the U.V. monitor. Small amounts of

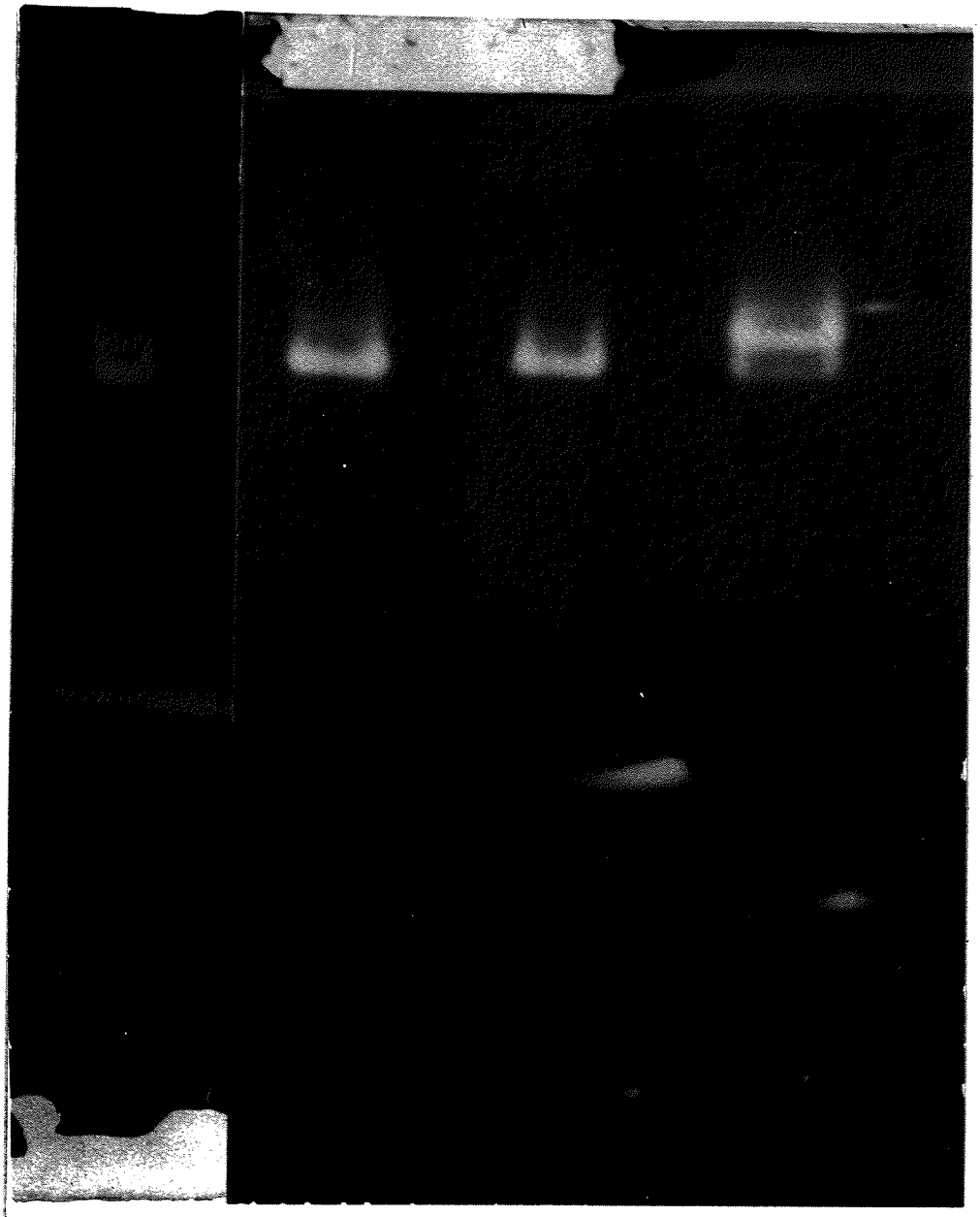
Fig. 10. Electrophoretic behavior on polyacrylamide-slabs at pH 8.9 of α-amylase purified to the second glycogen complex stage and component α-1, α-2 and α-3 from DEAE-cellulose ion-exchange chromatography. Detection of isozymes was by incubation against a starch plate.

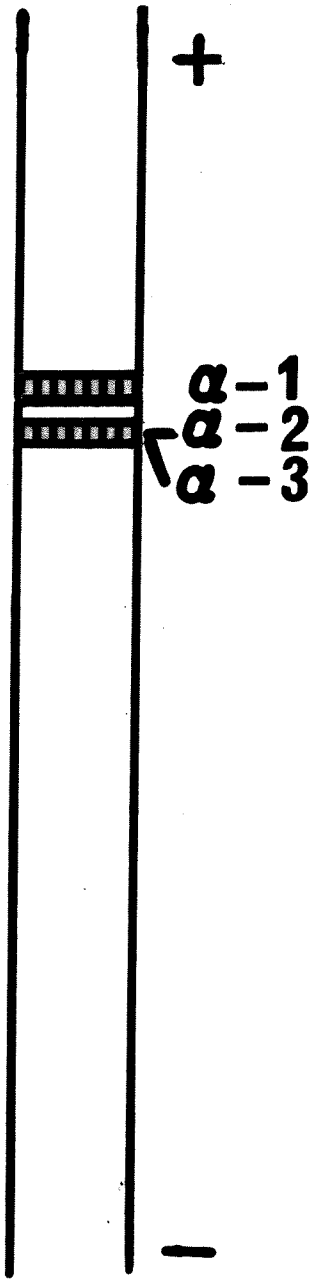




α-1 α-2 α-3 GLYCOGEN COMPLEX

Fig. 11. Electrophoretic behavior on polyacrylamide-slabs at pH 4.75 of α-amylase purified to the second glycogen complex stage and components α-1, α-2 and α-3 from DEAE-cellulose ion-exchange chromatography. Detection of isozymes was by incubation against a starch plate.





GLYCOGEN
COMPLEX α-3 α-2 α-1

protein contaminants did come off in the through peak, as shown on the U.V. monitor, but no contaminants were detected in the effluent containing the α-amylase isozymes.

Gel electrophoresis was carried out on concentrated central portions of each peak. Since large volumes were involved, the central portions were initially concentrated using a Model 202 Amicon Concentration Cell. This concentration cell brought the effluent volume down to about 20 ml which was then further concentrated by the Amicon model 8-MC micro-ultrafiltration system. Both systems used Diaflo UM-10 ultrafiltration membranes (Amicon Corp., Lexington, Mass.). Electrophoresis was carried out on both acid and basic gels as previously described with the resultant starch plates shown in Fig. 10 and 11. The basic gel electrophoresis showed that a separation was effected. No contaminating β-amylase was present on the starch plates. The acid gel showed only 2 bands of activity with α-2 and α-3 migrating the same distance. A Coomassie Blue protein stain was obtained for glycogen complex II on acid gel with the only protein bands present being those attributable to the α-amylase. This was a good indication of the purity of the glycogen complex II. Unfortunately insufficient protein was present to give a protein stain for the concentrated portions of the individual isozyme peaks. A protein stain also was attempted for glycogen complex II on basic gel. In this instance it was found that the protein quickly diffused giving no sharp protein bands.

The isozyme first eluted was found to be the electrophoretically slowest isozyme on basic gel and following the nomenclature of Kruger (1972a) was designated α-3. Peak 2 was electrophoretically the second

slowest component and corresponded to α-2 while peak 3, the most mobile on basic gel electrophoresis, was designated α-1.

Fig. 9 indicated that, with respect to activity, the major α-amylase isozyme present was the α-2 component.

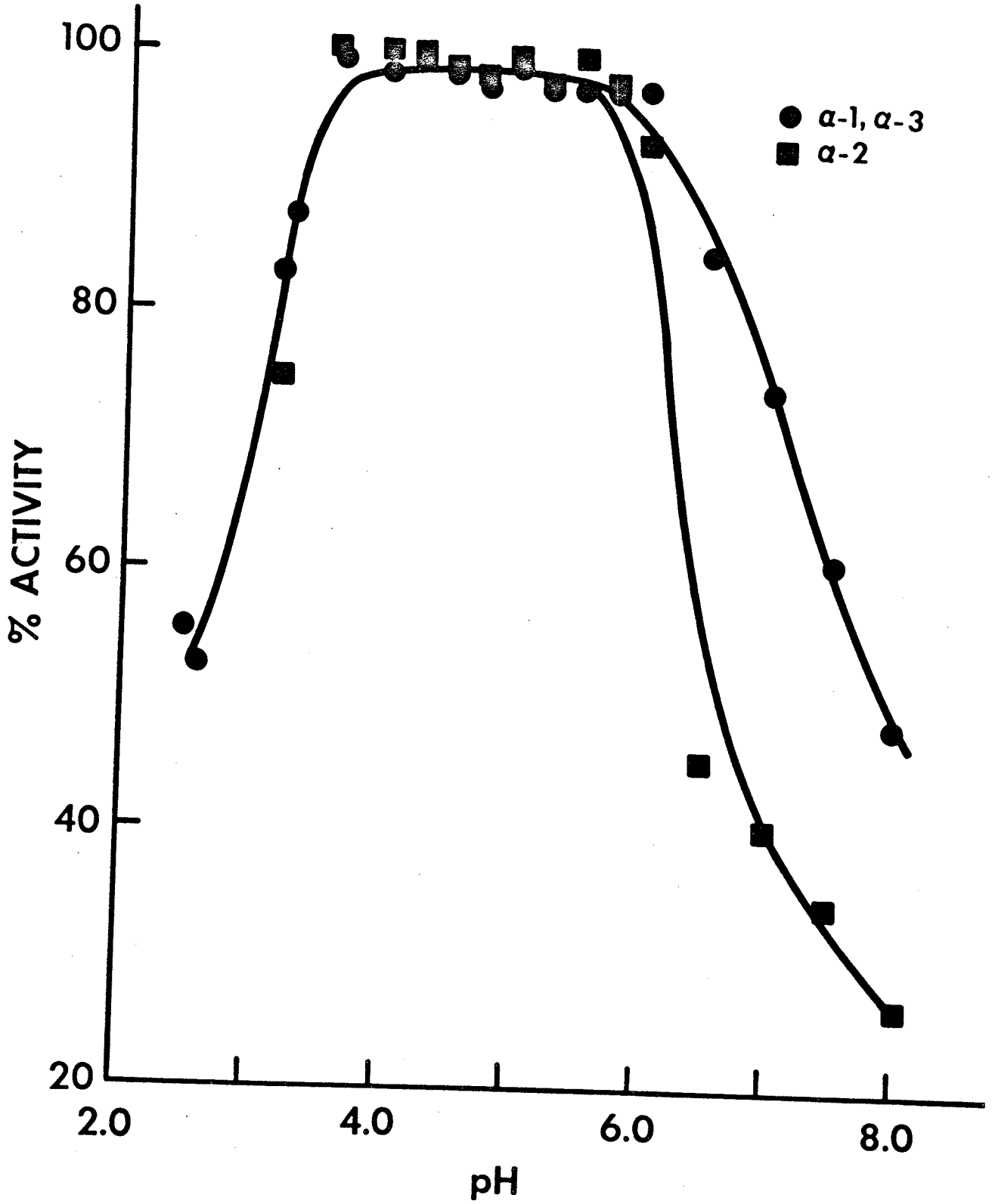
B. Characterization

Enzymatic properties

Unless otherwise stated, the determination of enzyme activities was carried out by the automated reducing sugar assay. Reduced starch was required as the substrate in order to obtain a low substrate blank with the enzyme assay. This was prepared from lintnerized starch as described by Strumyer and Romano (1967) with some modification. The starch-borohydride mixture was left stirring overnight and was then centrifuged at 10,000 g and 4 C for 10 min. 0.2 ml of acetone was added per 10 ml of supernatant to remove any excess sodium borohydride. The supernatant was then freeze dried and ground into a powder.

pH optimum: The pH buffers used were barbitol-sodium acetate buffers containing 0.001 M $CaCl_2$, as described by Michaelis (1931). For each pH, 0.1 ml of enzyme (dialyzed against 0.001M $CaCl_2$) was added to 2 ml of buffer plus 2 ml of reduced starch in distilled water (brought to pH 7). The pH of each solution was measured before the addition of the enzyme. In the case of the α-2 component, the final starch concentration was 0.25% while for the α-1 and α-3 components 1.0% reduced starch was used. In the case of the α-2 component, a time course study of the reaction mixture was used with the slope of the resultant curve giving the α-amylase activity. The activities for the α-1 and α-3 isozymes were determined at each pH by incubating the enzyme and substrate for 5 minutes. The reaction was

Fig. 12. Effect of pH on the activity of immature wheat α-amylase isozymes.


100
80
60
40
20
% ACTIVITY
2.0
4.0
6.0
8.0
pH
α-1, α-3
α-2

stopped at the end of this time period by the addition of 0.2 ml 5N HCl. Blanks were run at each pH studied.

The pH optimum for the α-2 component was also determined using the iodine β - limit dextrin assay. In this case 0.1 ml enzyme was added to 4 ml of buffered 0.065% β-limit dextrin and incubated for 5 minutes. The reaction was stopped with the addition of 0.2 ml 5N HCl. The activity profile obtained by this method was identical to that of the other assay. For all three α-amylase isozymes a very broad pH optima was obtained, going from pH 3.6 - 5.75 as shown in Fig. 12.

A linearity check was carried out at pH 5.5 at substrate concentrations of 0.25% and 1.0% with the α-2 component. It was found that the formation of reducing sugars was a linear function of time for the 13 min. time period studied. This indicated that the substrate was in excess.

pH stability: The pH stability of α-2 component was determined at pH 3.0, 4.5, 5.75, 7.0 and 8.0 using the barbitol-acetate buffer system previously described. 0.1 ml of enzyme solution was incubated in 3.8 ml of buffer for periods of 0, 2, 5, 10 and 30 min. Upon completion of the incubation, 0.2 ml of 5% reduced starch was added and the enzyme substrate mixture was continuously sampled using the reducing sugar assay. The slope of the curve of absorbance vs. time yielded a measure of activity. By comparison of the activities at $t = 0$, to the activities of the enzyme after the various incubation times, it was found that in all cases except at pH 3.0, the α-2 isozyme was stable for the 30 min. time period. At pH 3.0 the enzyme was inactivated after 2.0 min incubation.

Fig. 13. Plot of decrease in iodine reduced β-limit dextrin color versus increase in reducing power for immature wheat isozyme α-2, and heat treated, germinated Manitou at pH values of 4.0, 5.5 and 9.0.

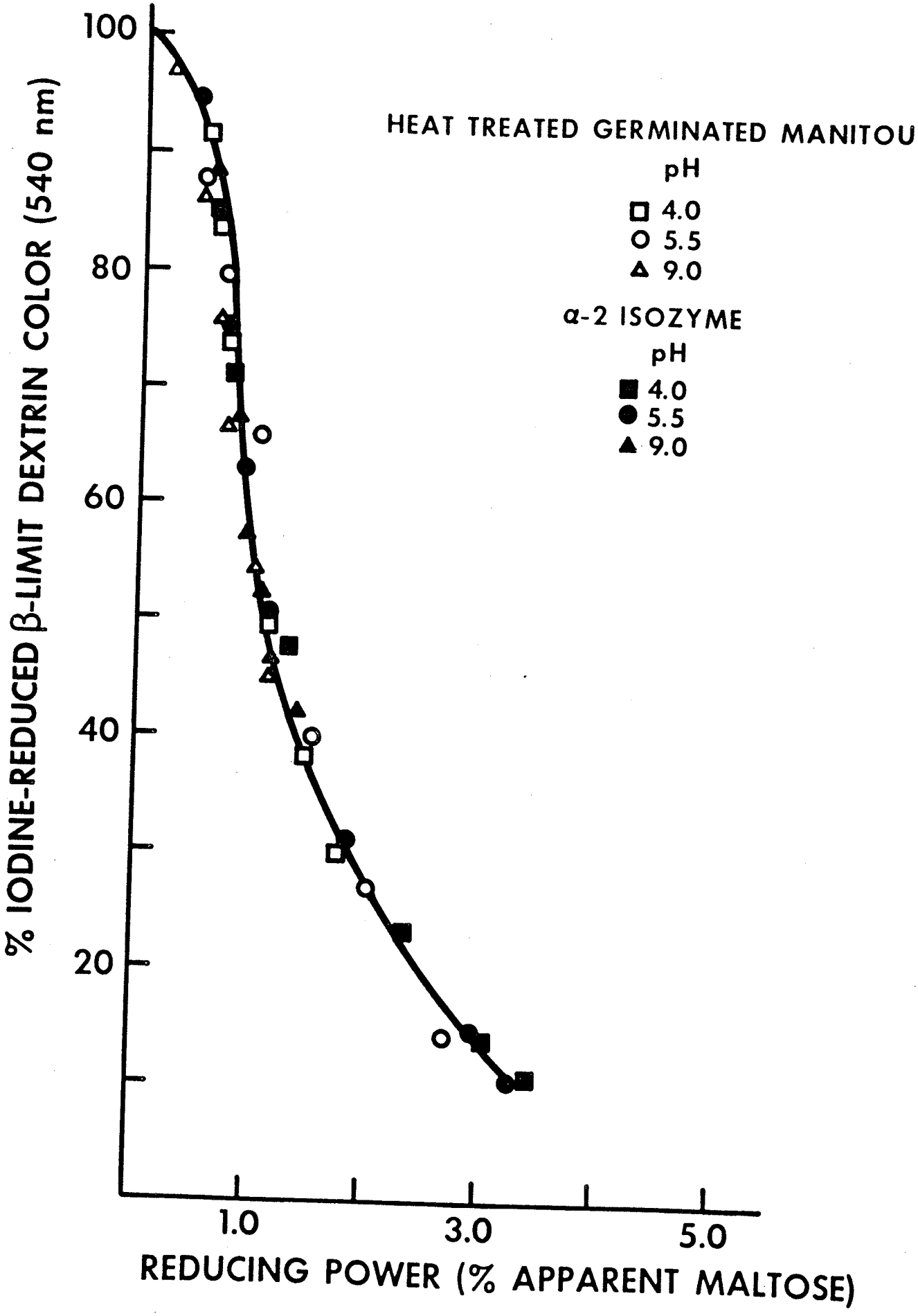

HEAT TREATED GERMINATED MANITOU
pH
4.0
5.5
9.0
α-2 ISOZYME
pH
4.0
5.5
9.0
% IODINE-REDUCED β-LIMIT DEXTRIN COLOR (540 nm)
100
80
60
40
20
1.0
3.0
5.0
REDUCING POWER (% APPARENT MALTOSE)

Fig. 14. Determination of Michaelis-Menten constants, Km for immature wheat α-amylases.

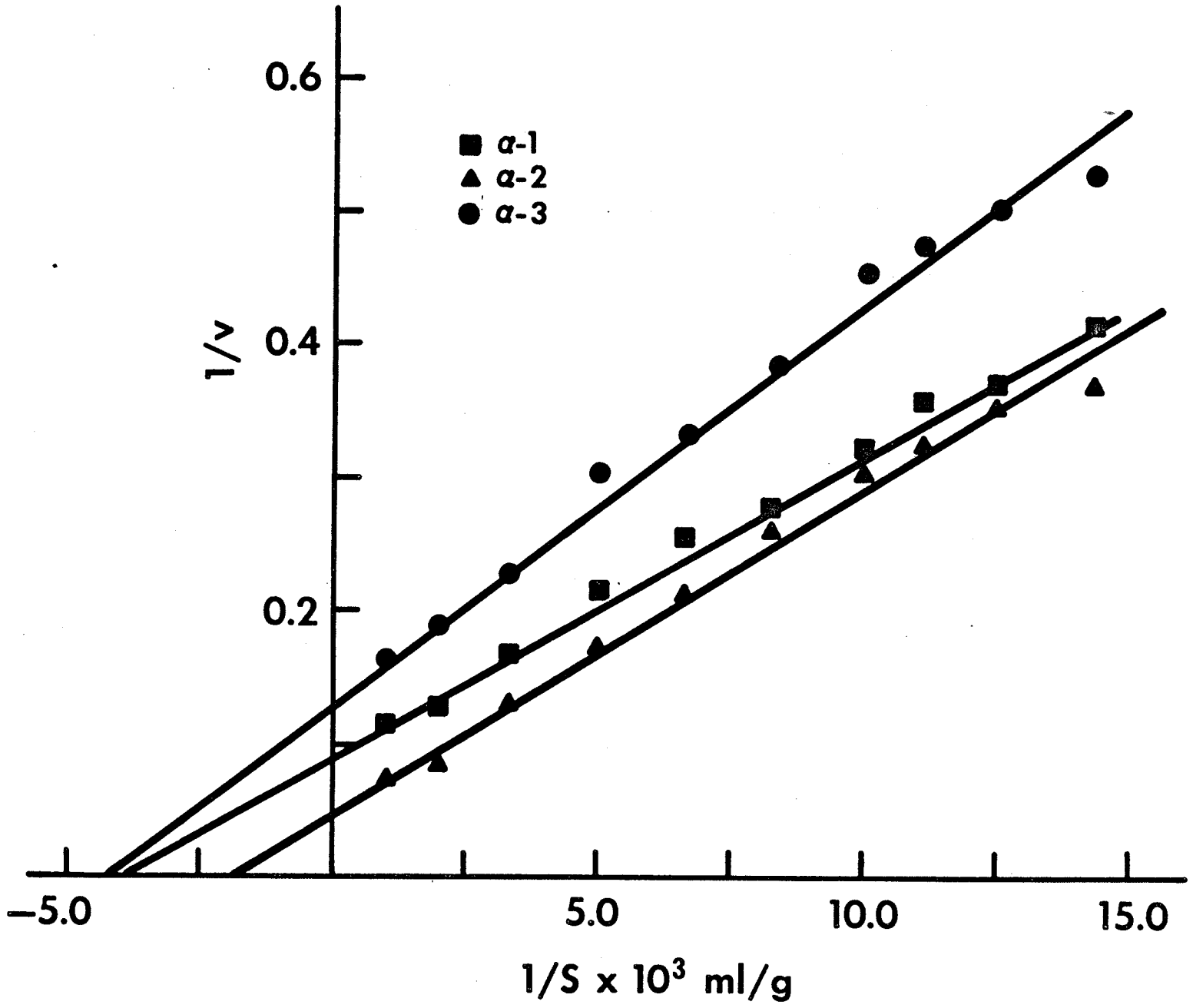
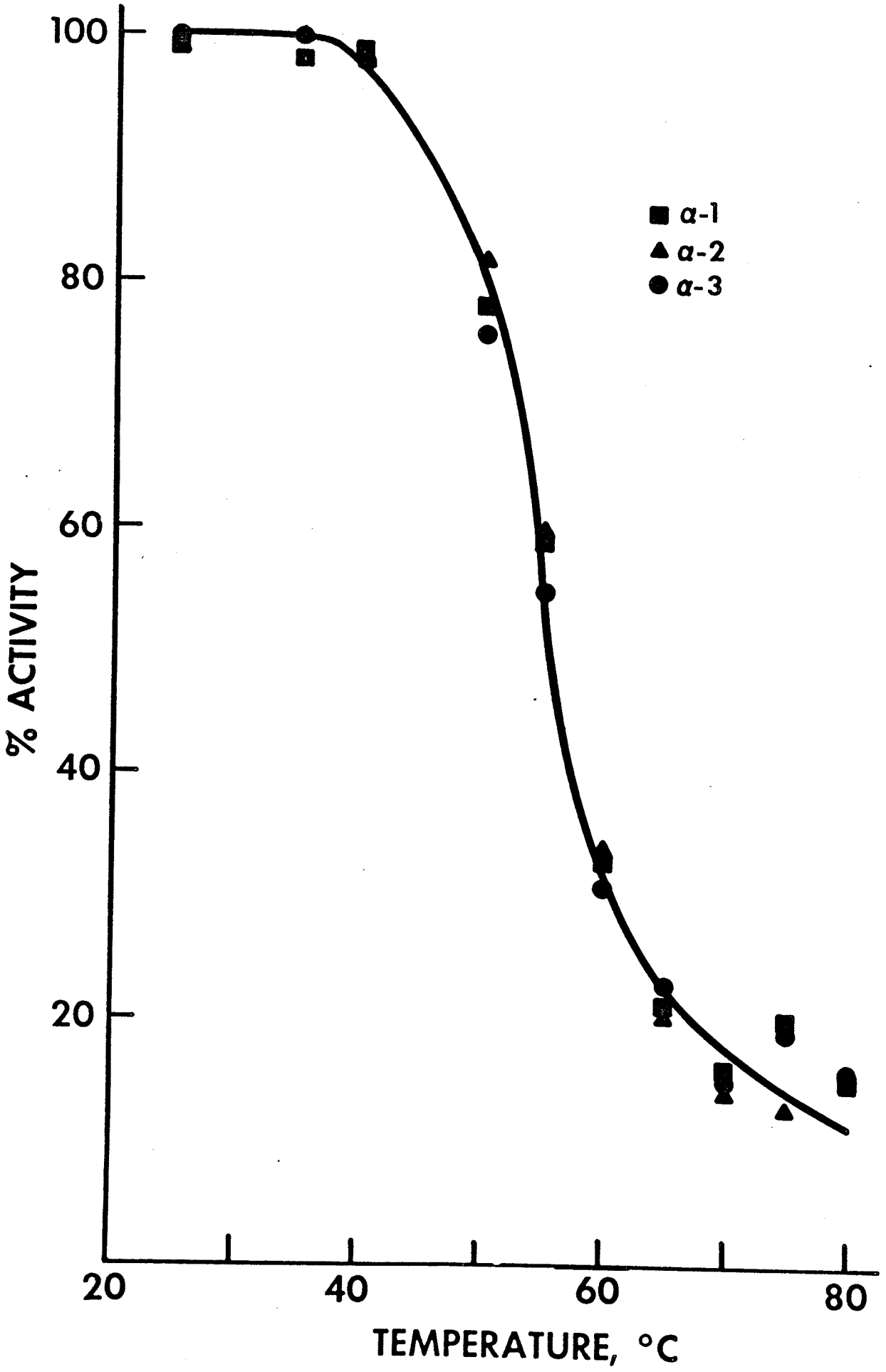

0.6
0.4
0.2
1/v
α-1
α-2
α-3
−5.0
5.0
10.0
15.0
1/S x 10³ ml/g

Fig. 15. Effect of temperature on immature wheat α-amylase isozymes.


100
80
60
40
20
% ACTIVITY
■ α-1
▲ α-2
● α-3
20
40
60
80
TEMPERATURE, °C

Change in action pattern with pH: To ascertain whether there was a change in action pattern with pH, a plot of percentage of the original absorption value of the iodine β-limit dextrin complex vs. reducing power was determined at 3 pH's. The semi-automated iodine β-limit dextrin assay and the automated reducing sugar assay were used to obtain this plot. The substrate used was 0.036% reduced β-limit dextrin. This substrate was used as it could be employed by both assays. The study was carried out on the major α-2 isozyme and a heat-treated germinated Manitou extract at pH values of 4.0, 5.5 and 9.0 using Michaelis barbitol-acetate buffers. A heat-treated germinated Manitou extract was used since there would be no contaminating β-amylase. The reactions were carried out for time periods of up to 1200 sec. with the reaction being stopped by the addition of 0.2 ml 5N HCl. at each time period, aliquots were withdrawn and reducing power and iodine dextrin color determined. Blanks were run in all cases. The total apparent maltose was determined by complete degradation of the substrate with amyloglucosidase.

The action pattern did not change with pH for either of the α-amylases studied, as shown in Fig. 13.

Effect of substrate concentration on activity: The substrate used was reduced starch buffered with 0.2M sodium acetate, pH 5.5 containing 0.001M $CaCl_2$. The reaction was carried out at 35°C with 0.1 ml enzyme solution being added to 4 ml buffered substrate and incubated for 5 min. After 5 min. the reaction was stopped by the addition of 0.2 ml 5N HCl. A blank was run at each substrate concentration.

Fig. 16. SDS polyacrylamide gel electrophoresis of immature wheat α-amylase purified to the second glycogen complex stage. BSA, OVA, ALD, PEP, CHYMOA, MYO and CYTC are abbreviations for standard protein markers bovine serum albumin, ovalbumin, aldolase, pepsin, chymotrypsinogen A, myoglobin and cytochrome with molecular weights of 68,000, 43,000, 40,000, 35,000, 25,700, 17,200 and 12,400 respectively.

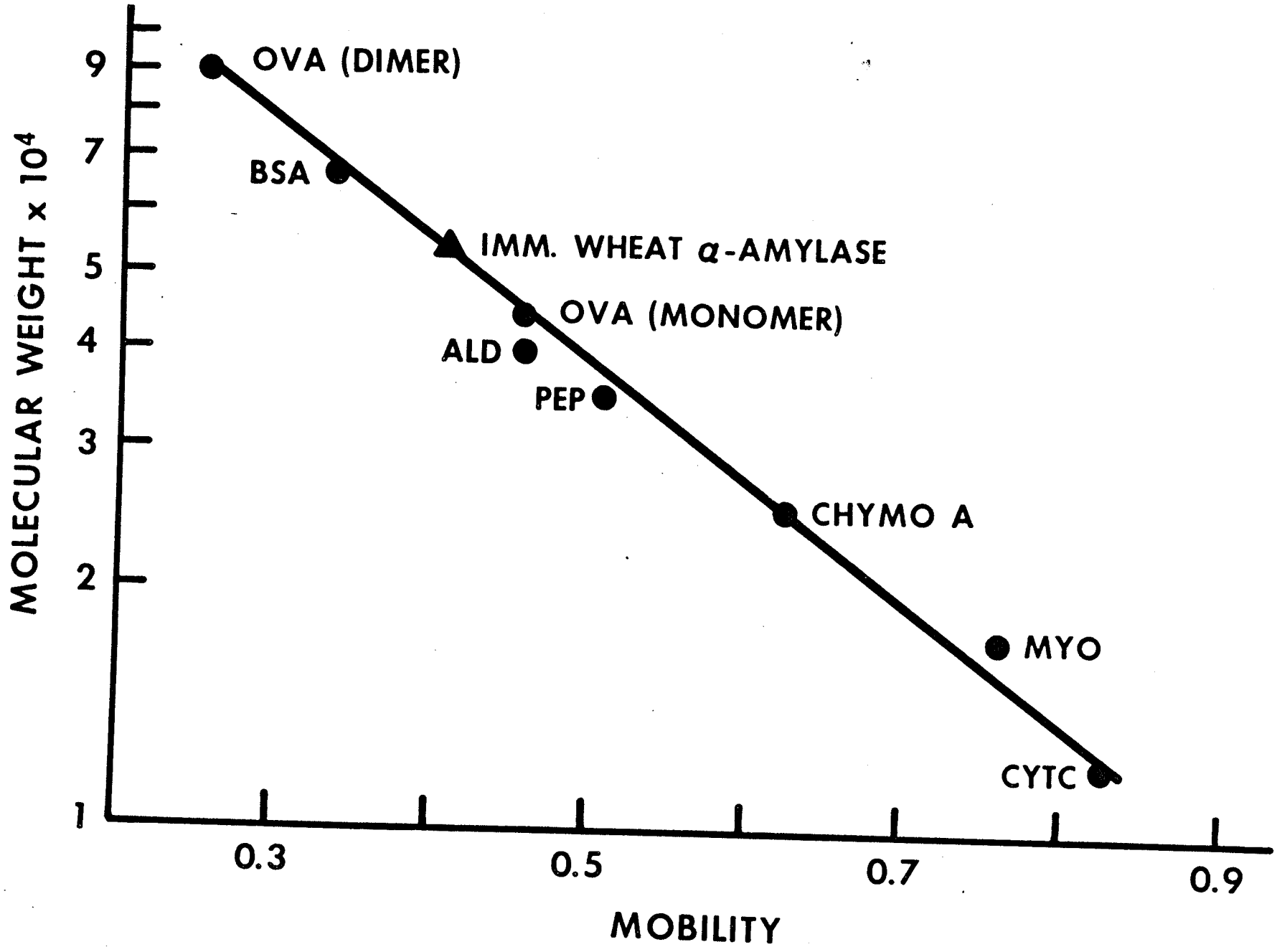
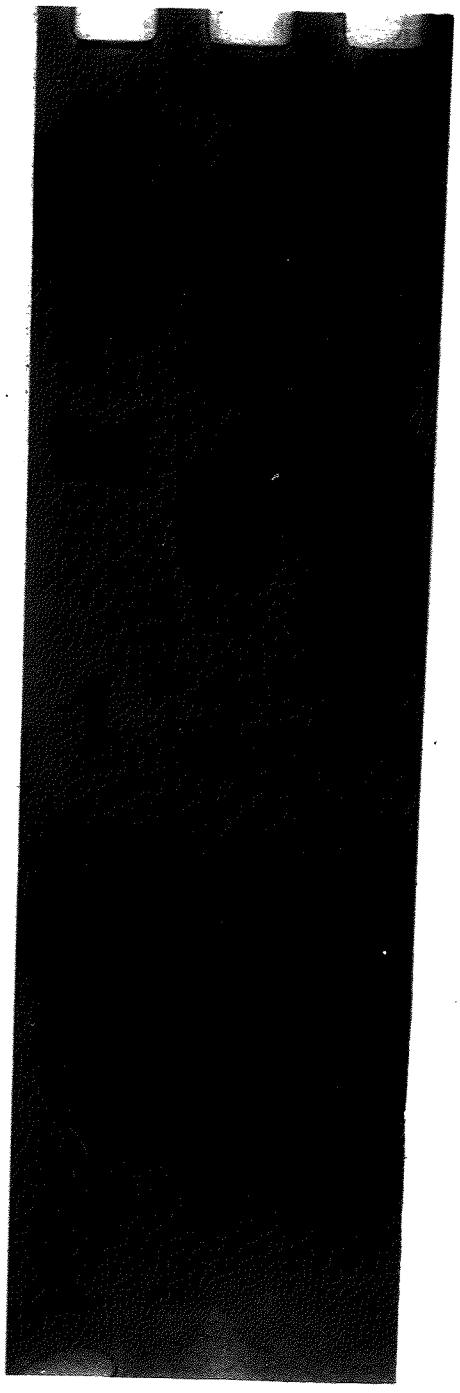

MOLECULAR WEIGHT x 10^4
9
7
5
4
3
2
1
OVA (DIMER)
BSA
IMM. WHEAT α-AMYLASE
OVA (MONOMER)
ALD
PEP
CHYMO A
MYO
CYTC
0.3
0.5
0.7
0.9
MOBILITY

Fig. 17. SDS polyacrylamide gel electrophoresis of immature wheat α-amylase and two reference proteins. 1. immature wheat α-amylase. 2. aldolase. 3. ovalbumin.



The apparent Michaelis constant was determined by plotting 1/v vs. 1/S in the graphical representation of Lineweaver- Burk as shown in Fig. 14. The reciprocal of the intercept of the plotted line with the abscissaindicated that the Km of the 3 isozymes was similar: α-1, 2.50 x 10^{-4}g/ml; α-2, 5.33 x 10^{-4}g/ml; α-3, 2.35 x 10^{-4}g/ml.

Thermal stability: The thermal stability of the isozymes was determined at pH 5.5 in 0.2 M acetate buffer containing 0.001 M $CaCl_2$, for a duration of 30 minutes at 25°, 35°, 40°, 50°, 60°, 70°, 75° and 80°C. The activity was assayed using 1.0% reduced starch as substrate. As can be seen in Fig. 15, all the isozymes behaved in a similar fashion.

Effect of sulfhydryl (SH) reagents: Inhibition of the α-amylase isozymes by 1 x 10^{-4}solutions of iodoacetic acid, N-ethylmaleimide, mercuric chloride and 5 x 10^{-4}M mercuric chloride was determined by incubating 1 ml of the enzyme in 4 ml of the above solutions which contained 1% reduced starch. The reaction was carried out at 35°C for 15 min. and was stopped by the addition of 0.3 ml 5 N HCl. Controls containing no inhibitor were also run.

It was found that, within experimental error, the iodo-acetic acid and N-ethylmaleimide gave no inhibition. In the case of both mercuric chloride solutions there was loss in activity with α-1 inhibited 90%, α-2, 96% and α-3, 89%.

Physical properties

Determination of molecular weight:

i. SDS gel electrophoresis: Pure glycogen complex II was submitted to electrophoresis and it was found that the 3 isozymes did not separate into distinct isozyme bands. This indicated that the 3

Fig. 18. Molecular seive chromatography of immature wheat α-amylase purified to the second glycogen complex stage. BSA, OVA, MYO and CYTC are abbreviations for standard protein markers bovine serum albumin, ovalbumin myoglobin and cytochrome C with molecular weights of 68,000, 43,000, 17,200 and 12,400 respectively.

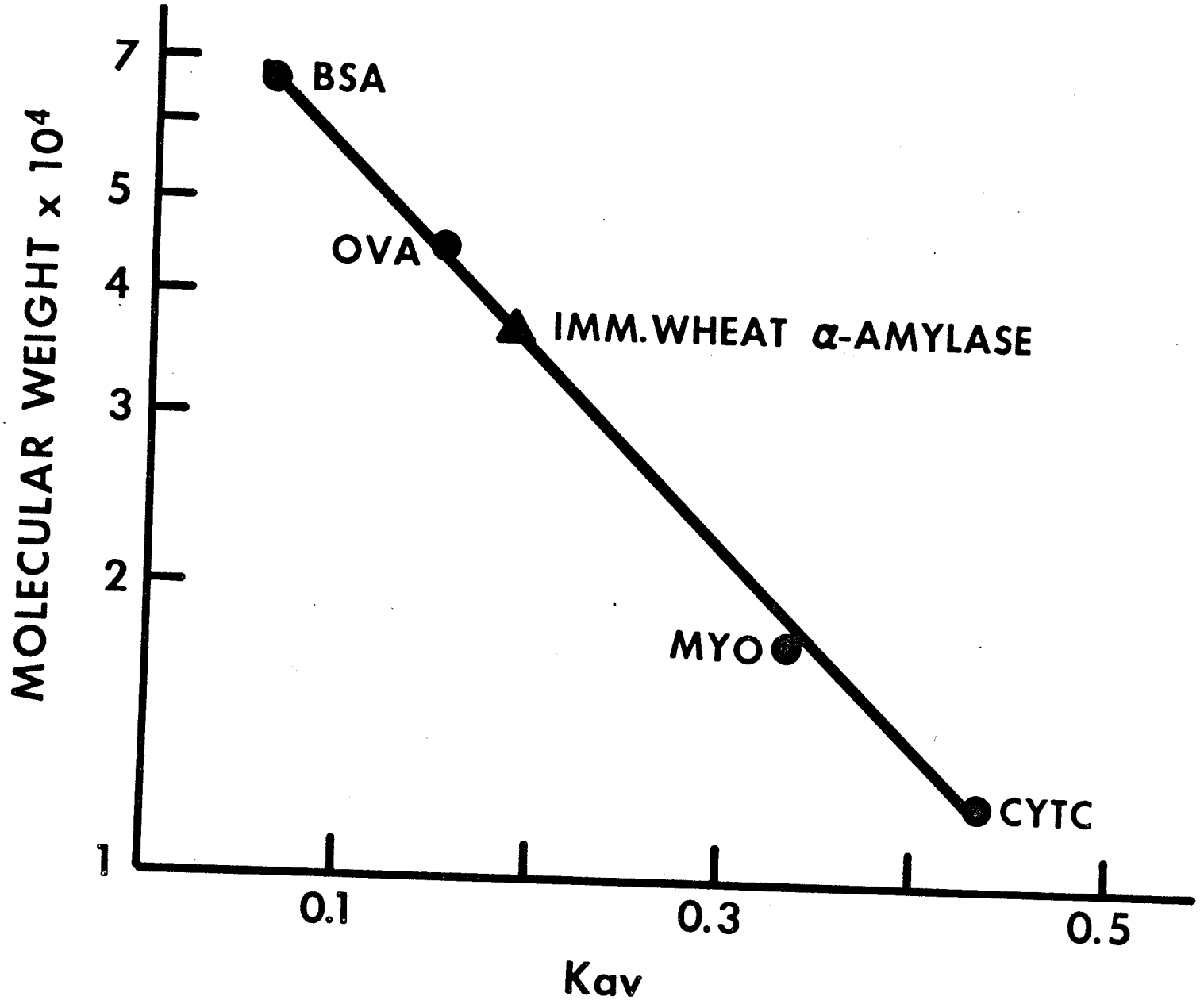

MOLECULAR WEIGHT x 10^4
7
5
4
3
2
1
BSA
OVA
IMM.WHEAT α-AMYLASE
MYO
CYTC
0.1
0.3
0.5
Kav

Fig. 19. Arrhenius plot for immature wheat α-amylases.

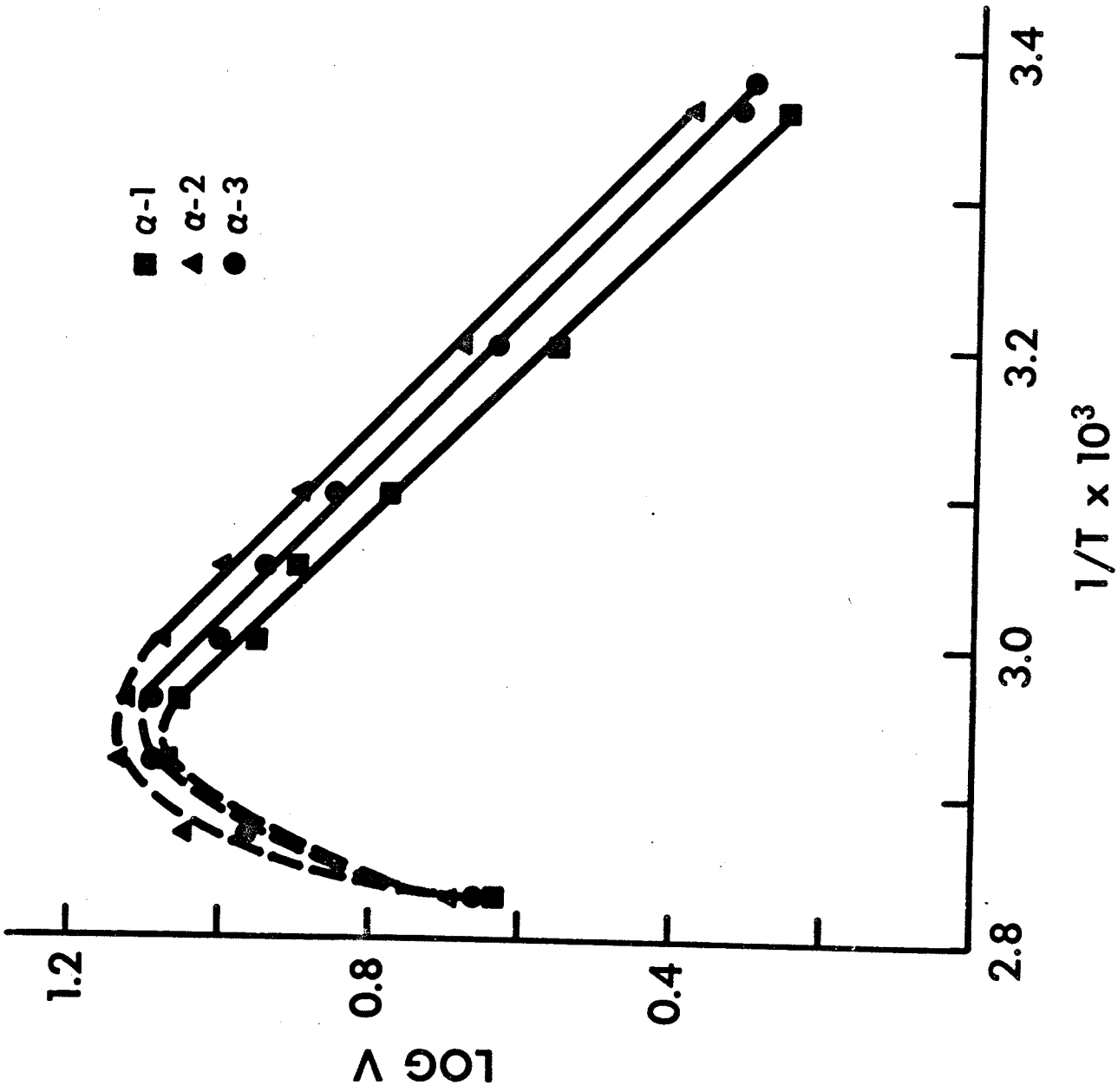
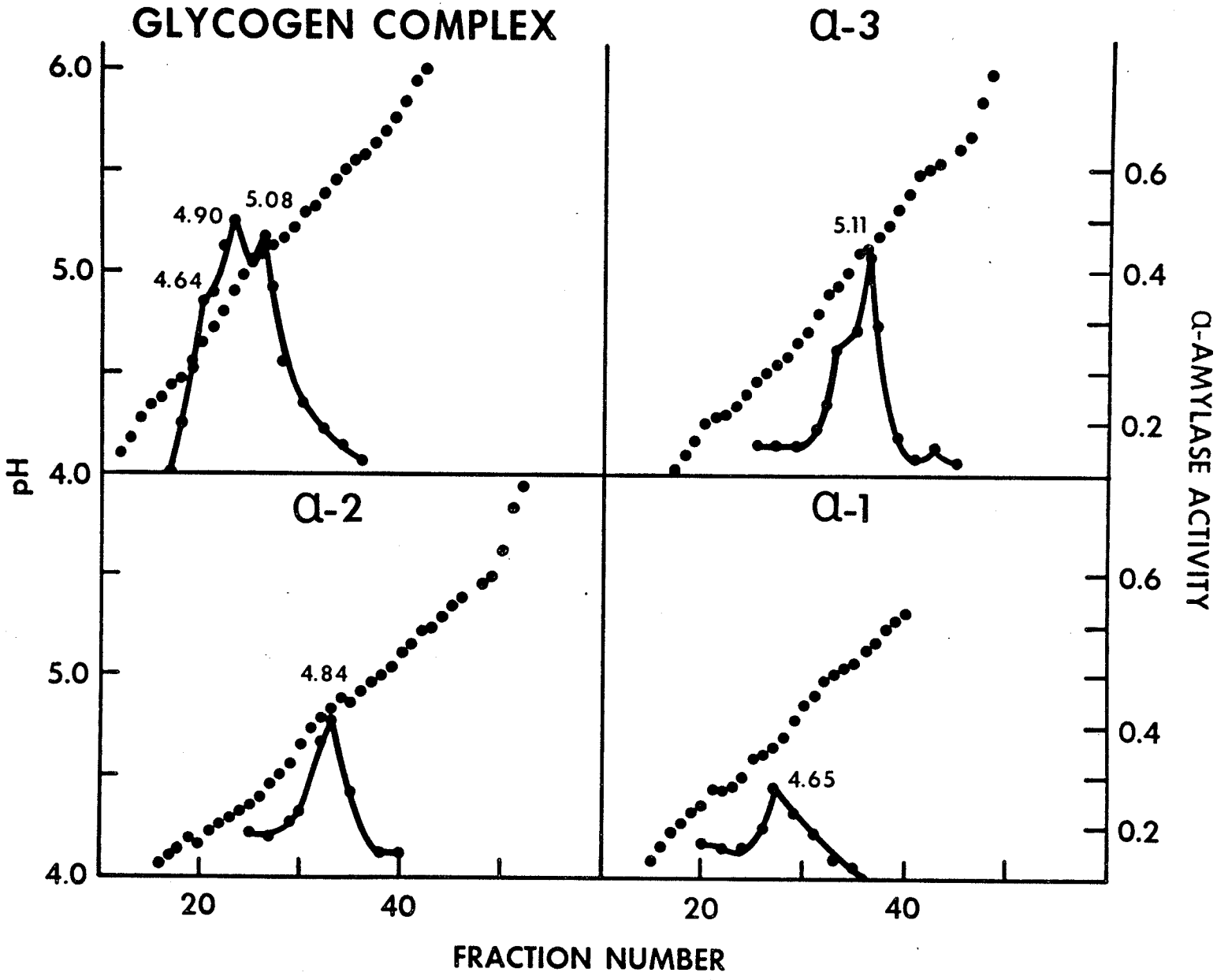

α-1
α-2
α-3
LOG V
1.2
0.8
0.4
2.8
3.0
3.2
3.4
1/T x 10³

Fig. 20. Isoelectric focusing of immature wheat α-amylase purified to the second glycogen complex stage and isozymes α-1, α-2 and α-3. Solid line = α-amylase activity; unjoined circles = pH.


GLYCOGEN COMPLEX
α-3
α-2
α-1
6.0
5.0
4.0
pH
4.90
5.08
4.64
5.11
4.84
4.65
0.6
0.4
0.2
α-AMYLASE ACTIVITY
20
40
FRACTION NUMBER

isozymes were the same molecular weight i.e. 52,000 - 54,000 g/mole (Fig 16). No other protein bands showed up on the Coomassie blue stained gel giving another indication of the high purity of the glycogen complex II (Fig. 17).

ii. Gel filtration: The glycogen complex II solution was dialyzed overnight against eluting buffer to remove breakdown sugars. The glycogen complex II fraction was then concentrated to approximately 0.75 ml by an Amicon 8-MC micro-ultrafiltration system, using a UM-10 Diaflo Ultra Filtration membrane (Amicon Corp., Lexington, Mass.). The concentrate was subsequently applied to the Biogel P-100 column and 2 ml fractions were collected at a flow rate of 11.8 ml/hr.

Little enzyme protein was present and, as a result, a very small peak was obtained on the U.V. protein monitor. In order to determine the volume, an activity profile was prepared using the semi-automated iodine β-limit dextrin α-amylase assay to determine the activity of the fractions. The elution volume of the isozymes was 57.5 ml which corresponded to a K_{AV} of 0.189 and a molecular weight of 36,000 - 38,000 g/mole (Fig. 18). These results were anomalously low so further studies were attempted as shown in appendix A.

Activation energy: The effect of temperature on the hydrolysis rate of a 1.0% reduced starch solution buffered at pH 5.5 with 0.2M acetate containing 0.001M $CaCl_2$ was determined for the three α-amylase isozymes over the temperature range 25 - 80°C. The activation energies for α-1, α-2 and α-3, calculated from the Arrhenius plot shown in Fig. 19, were found to be 9.66, 9.04 and 8.82 Kcal/mole, respectively.

Isoelectric focusing: Isoelectric focusing was carried out as described in Materials and Methods. The pH of each fraction as well as the α-amylase activity was plotted against the fraction number (Fig.20). pI values for α-1, α-2 and α-3 were 4.65, 4.84 and 5.11 respectively.

DISCUSSION

In examining the purification of immature wheat α-amylase (Table III), it can be seen that a second glycogen complex was required to give a reasonable purification. Although glycogen complex formation is specific for α-amylase (Levitzki and Schramm, 1963), it was found that the first complex contained contaminating proteins. These were probably present as a result of protein occlusion during the precipitation. This contamination necessitated the second glycogen complex formation. A low overall yield was obtained although it was slightly higher than in the purification of immature barley α-amylase (MacGregor et al, 1974). This low yield came as a result of losses during each of the purification steps. The largest loss was incurred during the acetone fractionation, as was the case in the malted wheat α-amylase purification (Kruger and Tkachuk, 1969). This loss probably resulted from the denaturing effect of the organic solvent acetone, even though the extract was cooled and stirred so as to minimize the loss. Losses were incurred also in the heat treatment and glycogen complex stage. In the first case, this was most probably due to the α-amylase being heat labile. At the glycogen complex stage, enzymes are probably lost during the addition of ethanol and because of limited solubility of the glycogen-enzyme complex.

The final yield of protein was very low not only because of losses but also because only small amounts of α-amylase are present in the immature wheat. Olered and Jönsson (1970) felt that immature wheat may have as little as 1000 times less activity than germinated

wheat. MacGregor et al (1974) have suggested that with Conquest barley, the immature α-amylase activity is 50 to 60 times less than the α-amylase activity of the corresponding malt barley. Since such small amounts of protein were obtained in this study, certain physical properties such as amino acid composition were unobtainable.

DEAE-cellulose chromatography, using a linear gradient with limit buffers as shown in Fig. 6, was successful in separating the isozymes. Basic gel electrophoresis of concentrated central portions of the isozyme peaks, followed by isozyme detection on starch slabs (Fig. 10) indicated that a separation had indeed taken place with limited overlap. Also,the zymogram showed no bands present other than the 3 α-amylase isozymes. This indicated that β-amylase had been removed in the purification. Purity of the α-amylase was indicated by the absence of any protein bands other than those associated with the α-amylase isozymes on both basic and acid gel electrophoresis. SDS gel electrophoresis also gave an indication of the purity of glycogen complex II. As shown in Fig. 17, no protein band other than that a single band at MW 52,000 was present.

As may be seen in Fig. 11 there was no separation between the α-2 and α-3 isozymes on acid gel electrophoresis. This probably resulted from the acid gel being run at pH 4.75 which is very close to the pI's of the 3 isozymes. Thus,the isozymes would not be highly charged and would not move very far during the duration of electrophoresis. As a result,α-2 and α-3, although differing in charge, did not have time to separate in the gel.

A broad pH optima of 3.6 - 5.75 was obtained for all three isozymes. The activity curves shown in Fig. 12 were identical for the α-1 and α-3 components while α-2 deviated only slightly from these on the basic side. In the case of some α-amylases previously studied, i.e., malted wheat (Tkachuk and Kruger 1974) and immature barley (MacGregor et al, 1974), the pH activity curves were much sharper with the pH optima being about pH 5.5. The difference between these α-amylases and the immature wheat α-amylase isozymes appears to lie in the acid region where the immature wheat α-amylase is more stable. This acid stability is also evident in the case of the immature barley α-amylase studied by MacGregor et al (1974) where approximately 50% of the enzyme activity remained at pH 3.5. Conversely, with malted wheat α-amylase isozymes (Tkachuk and Kruger, 1974), there was only 15 - 25% enzyme activity remaining at pH 3.5. There is the possibility that this acid stability may be a general characteristic of the immature cereal α-amylases.

The chemical reason for the broad pH optima could be the result of the active site containing two amino acids of widely separated pKa's. Physiologically speaking,the reason may be a direct result of the quickly changing conditions in the growing kernel. The reason for the presence of the immature α-amylases is probably to degrade the pericarp starch (MacGregor et al, 1972; Kruger 1972b). This degradation takes place over a period of time during which many protein and other seed components are being synthesized. Thus the seed composition is quickly changing and possibly, as a result of this, the pH of the pericarp may be rapidly changing. Thus, the α-amylase

isozymes would have to be active over a wide pH range in order to facilitate the rapid breakdown of the pericarp starch.

The pH stability checks at pH 4.5, 5.75, 7.0 and 8.0 indicated that over a 30 minute time period the α-2 component was stable. At pH 3.0 the enzymic activity was lost after 2 minutes. Due to the similarity of the 3 isozymes, the α-1 and α-3 components probably behave much the same as α-2.

It is of note that in the pH optimum study at pH 3.0, the enzymes retained approximately 70% of their activity as contrasted to the pH stability check, where α-2 was inactivated after 2 minutes at pH 3.0. These results actually show the stabilizing effect of the substrate. In the stability check the enzyme was first added to a pH 3.0 buffered solution containing no substrate whereas in the pH optima study the enzyme was added to buffered substrate. This showed that the substrate conferred extra stability to the enzyme which in turn could have given rise to the broad pH activity curves. This has been found to be the case with other α-amylases such as Taka amylase, where substrate prevented loss in activity under unfavourable conditions (Hanrahan and Caldwell, 1953).

It had been found that differences in action pattern (for α-amylases) could be discerned by plotting blue value vs. reducing power (as percent maltose), (Robyt and French, 1967; Banks et al 1970, 1971). Robyt and French (1967) also found that changes in pH could result in a change in the action pattern of an α-amylase. This change could be seen by making a plot of blue value vs. reducing power at each pH. Amylose was used in these studies, but Greenwood et al (1965b)

did obtain a plot for broad bean α-amylase with amylopectin β-limit dextrin as substrate.

No difference in action pattern with reduced amylopectin β-limit dextrin could be detected between the α-2 component and the heat treated α-amylase from germinated Manitou. Also, no discernable change in action pattern was perceived with either of the above α-amylases as shown in Fig. 13.

In this figure we also see that the iodine complex absorption value decreases rapidly with only a small increase in reducing power. According to Robyt and French (1967), this indicates a large degree of multichain attack (random attack). This is in agreement with the observations of Greenwood and Milne (1968b) who felt that the attack on amylopectin would basically be random due to the large number of α-1,6 glucosidic linkages. There is the possibility of a small degree of multiple attack in the linear portions of the reduced amylopectin β-limit dextrin but the increase in reducing power appears to be too slow to give credence to this assumption. Since this study was carried out only on the α-2 isozyme, it is difficult to say whether the α-1 and α-3 isozymes would behave similarly. In the case of the porcine pancreas α-amylase system, Banks et al (1971) found the isozymes to behave the same with respect to action pattern. Most probably, this is also the case with the immature wheat isozymes.

One puzzling fact, related to the random action of the α-2 isozyme, is the pH stability of the isozyme in the presence of substrate. Inherent in the concept of multichain (random) attack (Fig.13) is the dissociation of the enzyme from the substrate following the cleavage of an α-1, 4 linkage. The enzyme is then able to bind to

the next substrate molecule. Thus, after a cleavage, the enzyme molecule must spend a certain time in solution without being bound to a substrate molecule. If this is the case, then the enzyme should be susceptible to denaturation by acid, as indicated by pH stability studies (in the absence of substrates) which revealed a 100% loss in activity within 2 minutes. Such is not the case, for as noted, the enzyme is stable even at pH 3.5. A suitable explanation is not available for this behaviour and further studies are necessary to clarify these observations.

The three isozymes were found to have similar Michaelis-Menten constants (Fig. 14) with reduced starch as substrate. The Km's for α-1, α-2 and α-3 were 2.5×10^{-4} g/ml, 5.33×10^{-4} and 2.35×10^{-4} g/ml and indicated that the α-amylase isozymes have a very high affinity for this substrate. A comparable result of 2.0×10^{-4} g/ml (Swain and Dekker, 1966) has been recorded for the α-amylase purified from germinating peas with starch as the substrate.

The thermostability of the isozymes was found to be the same, as illustrated in Fig. 15. No loss in activity was recorded in 30 minutes until approximately 37°C, above which the activity dropped off rapidly. The immature α-amylase isozymes appear to be more heat labile than those of malted wheat (Tkachuk and Kruger, 1974) which lost less than 10% activity after heating for the same period. On the other hand, they are comparable to immature barley α-amylase which became rapidly inactivated above 40°C (MacGregor et al, 1974). It should be realized that this thermostability is for the pure enzyme. The thermostability of an impure α-amylase, such as that found in

Table IV

Summary of α-Amylase Properties

	Immature Wheat α-Amylase			Malted Wheat α-Amylase Components (Tkachuk and Kruger, 1974)				Immature Barley α-Amylase (MacGregor, et al, 1974)
	α-1	α-2	α-3	I	II	III	IV	
pH optimum	3.6 - 5.75	3.6 - 5.75	3.6 - 5.75	5.5	5.7	5.5	5.5	5.5
Km, g/ml	2.50×10^{-4}	5.33×10^{-4}	2.35×10^{-4}	n.d.	n.d.	n.d.	n.d.	n.d.
Molecular weight								
SDS gel electrophoresis		52,000 - 54,000			41,500 - 42,500			46,000
Biogel P-100		36,000 - 38,000			23,000			
Activation energy kcal.per mole	9.66	9.04	8.82	8.47	7.01	9.28	7.01	9.5
Isoelectric point, pI	4.65	4.84	5.11	6.16	6.20	6.05	6.17	4.83

a crude extract, will be much higher due to stabilization by other proteins. This is why the original heat treatment at 70°C did not inactivate the α-amylase. It is also for this reason that Meredith and Jenkins (1973) recently found wheat pericarp α-amylase to be stable to high temperatures.

In the study of the effect of temperature on the hydrolysis rate of reduced starch by the 3 α-amylase isozymes, the Arrhenius plot gave a linear relationship from 25° - 60°C. This yielded energy of activations (Ea) as noted in Table IV (the values obtained for malted wheat α-amylases (Tkachuk and Kruger 1974) and immature barley α-amylase (MacGregor et al, 1974) included for comparison). It would appear that the Ea for the immature wheat isozymes are comparable to those of α-amylases from other cereal sources.

Scrutiny of the Arrhenius plots (Fig. 19) seems to indicate that the isozymes are stable up to about 60°C. These results seem to be in conflict with the previous thermostability check, which showed the isozymes to be thermolabile. This apparent conflict of results can be explained by looking at the experimental methods employed. In the thermostability check, the α-amylase is incubated in pH 5.5 acetate buffer. On the other hand, in the Ea study the enzyme is added to buffered substrate and then incubated. Thus, we have another instance of stability being conferred to the α-amylase isozymes by their substrate.

This thermal stability in the presence of substrate could be of importance in breadmaking. During baking, the immature α-amylase

present in the dough has an excess of substrate present to stabilize it. The enzyme could remain active therefore, to a fairly high temperature and cause extensive starch degradation. Thus, if there was a preponderance of immature kernels present in a wheat crop, the resultant flour could be of poor breadmaking quality.

Mercuric chloride was found to be the only sulfhydryl reagent which had an inhibitory effect on the α-amylase isozymes with 89% - 96% inhibition. This inhibition was probably due to a heavy metal binding effect. This is in agreement with Greenwood and Milne (1968b) who stated that heavy metal ions such as mercury were inhibitory. The mercury assumedly binds to the α-amylase in such a way as to cause a change in protein conformation leading to loss in activity.

The molecular weight of the immature α-amylase was obtained in two ways, i.e. sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and gel filtration. The first method gave a value of 52,000 - 54,000 g/mole (Fig. 16) for the molecular weight of all three isozymes. Fig. 17 clearly shows that all three isozymes migrate the same distance indicating that the molecular weight of the 3 isozymes could be considered to be identical. As can be seen in Table IV, the molecular weight of the immature wheat α-amylase was slightly higher than that of malted wheat α-amylase isozymes and immature barley α-amylase. Fischer and Stein (1960) felt that in general the molecular weight of α-amylases is about 50,000 which is very close to that of the immature α-amylase considering the limits of the method.

The second technique of gel filtration on Biogel P-100 yielded a molecular weight of 36,000 - 38,000 g/mole (Fig. 18). No separation of the isozymes was detected by this method,as was the case with SDS PAGE. This result is exceptionally low in light of the value of 52,000 - 54,000 obtained by SDS PAGE. Low results by gel filtration have also been obtained in the case of malted wheat α-amylase (Tkachuk and Kruger, 1974), α-amylase of various plant origins (Greenwood and Milne, 1968b) and immature barley (Stoddart, 1971). Tkachuk and Kruger (1974) attributed their low results to some degree of enzyme substrate interaction with Sephadex, but in the case of biogel this seems unlikely. The possibility does exist for this anomolous behaviour to occur as a result of α-amylase having a very tight compact structure, as proposed by Fischer and Stein (1960). This could cause the α-amylase to behave like a protein of a much lower molecular weight. Further studies in Appendix A were carried out in an attempt to find the cause of this anomolous behaviour with little successs. One of the major differences between the α-1, α-2 and α-3 isozymes was their behaviour during basic gel electrophoresis and ion exchange chromatography on DEAE-cellulose. These differences can readily be explained by looking at the isoelectric points of each isozyme. α-1,which had the lowest pI of 4.65,was found to be the most electrophoretically mobile on basic gel and the last to elute on DEAE-cellulose chromatography. This is reasonable since at the basic pH's of electrophoresis and chromatography, α-1 would be the most highly charged. Thus, in a potential field, it would migrate the farthest; and would bind the

strongest to the DEAE-cellulose. α-2 and α-3 with pI's of 4.84 and 5.11 are successively less charged and this would similarily explain their behaviour.

As can be seen in Table IV, the immature α-amylase isozymes are about one pI unit lower than the α-amylases of malted wheat. As suggested by Kruger (1972b), the immature α-amylase isozymes may be identical to a set of 3 isozymes present in malted wheat. If this is the case,then they would have the same pI's, which explains why there is such a wide gap between the 4-5 malted wheat α-amylase isozymes (pI's shown in Table IV) and the faster set of 3 immature amylases (also present in malted wheat).

Worthy of mention is the partial separation of the 3 immature α-amylase isozymes during electrofocusing of the glycogen complex II fraction (Fig. 20). If a shallower pH gradient could be employed, the possibility does exist for the use of isoelectric focusing in the purification and separation of the isozymes instead of ion exchange chromatography.

From these studies of the immature α-amylase isozymes, it is apparent that they are similar with respect to their properties. Most probably there are only slight differences in their primary structure which would account for differences in their isoelectric points and electrophoretic and chromatographic properties. There does seem, however, to be a substantial variation in properties between the immature and malted wheat isozymes. These differences are not surprising since in one case the α-amylases must carry out their

hydrolysis under germinating or malted seed conditions while in the other the hydrolysis takes place in the immature seeds.

The properties of immature α-amylase, such as its high thermostability, suggest that this enzyme could be quite important in the breadmaking process. High levels of the enzyme may have a deleterious effect on the quality of a wheat giving rise to a decrease in its market value. Thus, in future studies, the effects of the immature α-amylases with respect to rheological and baking properties must be assessed for various wheat varieties. It would be profitable also to determine if the consistent presence of excess α-amylase in certain wheat varieties such as Cypress results from the presence of immature α-amylases. By learning these facts it may be possible at a future date to more easily predict the quality of wheat obtainable from a specific variety.

CONTRIBUTIONS TO KNOWLEDGE

1. The α-amylase of an immature HRS wheat can be isolated by acetone fractionation of a heat treated wheat extract, followed by complexing with glycogen.
2. The isolated immature α-amylase was composed of three isozymes which could be separated by gradient elution on a DEAE-cellulose column.
3. The α-2 isozyme component was the major component with respect to activity.
4. The pH optimum of the three isozymes was very broad ranging from 3.60 - 5.75.
5. The shapes of the pH optima curves indicated that the three isozymes were very similar although the α-2 component was slightly more base sensitive.
6. The α-2 component was found to have good pH stability with no loss in activity after 30 minutes incubation at pH 4.5, 5.75, 7.0 and 8.0. Incubation at pH 3.0 inactivated the protein within 2 minutes.
7. The presence of substrate increased the pH stability of the enzyme substantially.
8. The action pattern of the α-amylase of germinated Manitou was the same as that of the α-2 isozyme and neither varied with a chance in pH.
9. The Km's of the isozymes were found to be α-1, 2.50×10^{-4} g/ml; α-2, 5.33×10^{-4} g/ml; α-3, 2.35×10^{-4} g/ml indicating a high

affinity for the reduced starch used as substrate.

10. The thermal stabilities of the 3 isozymes were found to be the same. The isozymes were stable for 30 minutes in pH 5.5 acetate buffer up to about 37°C, above which temperature they were rapidly inactivated.

11. The activation energies of the three components were found to be: α-1, 9.66 Kcal/mole; α-2, 9.04 Kcal/mole; α-3, 8.82 Kcal/mole.

12. The Arrhenius plot was found to be linear from 25°C to at least 60°C. This indicated that reduced starch as substrate greatly increased the thermostability of the enzyme.

13. Mercuric chloride was found to inhibit α-1, 90%; α-2,96%; α-3, 89%,while N-ethylmaleimide and iodoacetic acid gave no inhibition. This indicated that SH groups were not essential for catalytic activity. The mercuric chloride inhibition was probably a result of non-specific binding to the protein.

14. The molecular weight of the immature α-amylase was found to be 52,000-54,000 g/mole by SDS PAGE.

15. The molecular weight as determined by gel filtration on biogel P-100 was 36,000-38,000 g/mole. This anomalous behavior suggests that immature wheat α-amylase may have a tight, compact protein structure.

16. The isozymes could not be separated by either SDS PAGE or gel filtration and indicated that all had the same molecular weight.

17. The isoelectric points of the three isozymes were found to be: α-1, 4.65; α-2, 4.84; α-3, 5.11.
18. The isozymes were found to be partially separable by isoelectric focusing over a pH 4 - 6 gradient.
19. In general the 3 immature α-amylase isozymes could be considered as having almost identical properties.

APPENDIX

Appendix A

Behaviour Of Purified Immature HRS Wheat α-Amylase on Biogel P-100

INTRODUCTION

Anomalously low values for the molecular weight of cereal α-amylases, as determined by gel filtration, have been reported by several enzymologists (Greenwood and Milne, 1968b; Manners and Marshall, 1972; Tkachuk and Kruger, 1974). This author also obtained a low value of 37,000 g/mole for immature wheat α-amylase while employing a Biogel P-100 column. This differed markedly from the value of 52,000 to 54,000 g/mole obtained by SDS PAGE. The object of the following study was to attempt a clarification of the above anomolous results.

MATERIALS AND METHODS

Purification

Immature HRS wheat α-amylase was purified to the glycogen complex II stage as previously described in Materials and Methods of the main text.

Gel Filtration

The α-amylase from glycogen complex II was chromatographed on a Biogel P-100 column and the molecular weight was determined as described in Results of the main text using the calibration curve shown in Fig. 18.

The α-amylase fractions obtained from the first gel filtration were pooled and then dialyzed overnight against the starting buffer

used in DEAE-cellulose chromatography i.e. 0.08M Tris-HCl pH 8.2, being 10^{-3}M in $CaCl_2$ and 0.03M in NaCl. After dialysis, the enzyme solution was loaded on the DEAE-cellulose column as described in Materials and Methods of the main text. Starting buffer was passed through the column for a period of 2 hours. The α-amylase was then eluted off the column with starting buffer containing 0.2M NaCl. The α-amylase was collected and concentrated to a 10 - 15 ml volume using an Amicon System with a diaflo UM-10 ultrafiltration membrane. This solution was then dialyzed overnight against the eluent buffer used in gel filtration. After dialysis, the enzyme solution was further concentrated to 0.75 mls. and chromatographed on the Biogel P-100 column. The molecular weight was then determined using the calibration curve in Fig. 18.

RESULTS AND DISCUSSION

The K_{AV} of the dialyzed α-amylase was found to be 0.189 which corresponded to a molecular weight of 36,000 - 38,000 g/mole. The α-amylase off the DEAE-cellulose column had a K_{AV} of 0.337 and a M.W. of 18,000 - 19,000 g/mole. These same results were obtained on two occasions and indicated that after the α-amylase ran through the DEAE-cellulose column, the apparent molecular weight of the enzyme was halved. The reason for chromatographing the α-amylase on DEAE-cellulose was due to the reports of several workers that there were α-amylase resistant regions in the glycogen molecule. Heller and Schramm (1954) first noticed that macro limit dextrins were present. Subsequently, Brammer et al (1972) showed that when porcine pancreatic α-amylase acted on shellfish glycogen α-amylase

limit dextrin, about 23 macrodextrin regions of about M.W. of 6000 were present for 10^6 Daltons of original glycogen. These dextrins were bound extremely tightly to purified α-amylase preparations and were not removed by simple dialysis (Malacinski and Rutter, 1969).

It was hoped that any such macrodextrin units bound to the enzyme would be removed by binding the α-amylase to DEAE-cellulose and eluting the dextrin with starting buffer. This was found to be the case as reducing power was found in the through peak of the chromatographic effluent.

The removal of this macrodextrin resulted in the molecular weight of the α-amylase being cut in half. This behaviour is very hard to rationalize. It could simply mean that the α-amylase bound more than one macrodextrin. Unfortunately, this would mean that the M.W. of the α-amylase is about 18,000 - 19,000 g/mole which is exceptionally low. The molecular weight obtained by SDS PAGE was 52,000 - 54,000 g/mole and is probably the correct value since the SDS would cause the protein molecule to open up, giving a true value. In order to relate these two values, one would have to say that during gel filtration, the α-amylase acts like a low molecular protein. The only way the α-amylase could act this way is if it had a very compact structure. This has been suggested for some other α-amylases (Vallee et al, 1959; Greenwood and Milne 1968a), but it is hard to visualize such a compact structure that would give an apparent M.W. of 18,000 - 19,000.

The other possibility that presents itself is that α-amylase is composed of 3 subunits of M.W. 18,000. This is unlikely,however, since SDS PAGE does not give any indication of a subunit structure.

Further studies must be carried out to ascertain the reason for this anomalous behaviour.

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