α-AMYLASE FROM TRITICALE 6A190: ISOLATION AND CHARACTERIZATION

A Thesis

Submitted to the Faculty

of

Graduate Studies

The University of Manitoba

by

Mikola Peter Silvanovich

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

Department of Plant Science

October, 1977

THE UNIVERSITY OF MANITOBA LIBRARIES

α-AMYLASE FROM TRITICALE 6A190: ISOLATION AND CHARACTERIZATION.

by

Mikola Peter Silvanovich

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

DOCTOR OF PHILOSOPHY

© 1977

Permission has been granted to the LIBRARY OF THE UNIVERSITY OF MANITOBA to lend or sell copies of this dissertation, to the NATIONAL LIBRARY OF CANADA to microfilm this dissertation and to lend or sell copies of the film, and UNIVERSITY MICROFILMS to publish an abstract of this dissertation.

The author reserves other publication rights, and neither the dissertation nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

ACKNOWLEDGMENTS

The author would like to express his sincerest thanks to:

Dr. Robert D. Hill for his guidance and helpful suggestions during the course of this work and especially in the preparation of this manuscript;

Dr. V. Bendelow of the Canadian Department of Agriculture for malting the triticale used in this work;

Dr. R. Tkachuk of the Grain Research Laboratory, Canadian Grain Commission, for access to his manuscript entitled "Competitive affinity chromatography of wheat α-amylase" prior to publication;

Mr. B. Marchylo for performing isoelectric focussing.

The financial support of the National Research Council is gratefully acknowledged.

TABLE OF CONTENTS

	Page
LIST OF TABLES	vii
LIST OF FIGURES	viii
ABSTRACT	x
INTRODUCTION	1
LITERATURE REVIEW	3
Amylases	3
Amylases of Mature and Immature Grain	5
Amylases of Germinated Seed	7
Synthesis and Morphological Distribution of α-Amylase	9
Basic Common Biophysical Properties of Cereal α-Amylases	11
Substrate and Mode of Action of α-Amylases	13
1. Substrates	13
2. Specificity and Action Patterns	14
Purification of α-Amylases	23
1. Conventional Methods	23
2. Affinity Methods	23
3. Cyclodextrins and their Interactions with α-Amylases	25
Assay Methods	28
MATERIALS AND METHODS	31
Materials	31
Determination of α-Amylase Activity	31
Protein Determination	33

Page

Disc Gel Electrophoresis 33

Molecular Weight Determination 33

Zymograms 33

Isolation of α-Amylases - Procedure used to Obtain Pure α-Amylases 34

Extraction 34

Heat Treatment 34

Glycogen Precipitation 34

Affinity Chromatography 35

Ion-Exchange on Carboxymethyl Cellulose 36

Other Chromatographic Experiments 36

Effect of Various Agents on the Release of α-Amylase from Affinity Chromatography Columns 36

Chromatography with Concanavalin A-Sepharose 38

Affinity Chromatography with Dextrin-Sepharose 38

Ion-Exchange Chromatography on DEAE Cellulose 38

Other Methods 39

pH Optimum 39

pH Stability 39

Michaelis Constants 39

Calcium Requirement 40

Ammonium Sulfate Precipitation 40

Isoelectric Focussing 40

RESULTS AND DISCUSSION 41

A. The characterization of α-amylases of triticale and its parents by disc gel electrophoresis 41

		Page
	Electrophoretic Data	41
B.	Isolation of α-amylases	45
	Preparation for chromatography	45
	Glycogen precipitation	45
	Storage methods	47
	Ammonium sulfate precipitation	51
C.	Chromatography	51
	Introduction	51
	Affinity Chromatography	54
	Ion-exchange chromatography	59
	Concanavalin A-Sepharose	63
	Chromatography with AH-Sepharose 4B-Dextrin	66
	Chromatography with diethylaminoethyl cellulose	67
	Effects of agents other than cyclohepta-amylose on the release of α-amylase from the affinity chromatography column	75
D.	Characterization	81
	pH Optimum	81
	pH Stability	81
	Michaelis constants	88
	Activation energy	91
	Temperature stability	91
	Calcium requirement	94
	Determination of molecular weight by SDS gel electrophoresis	99
	Isoelectric Focussing	99

Page

Effect of cyclohepta-amylose on α-amylase activity 102

GENERAL DISCUSSION AND CONCLUSIONS 108

BIBLIOGRAPHY 111

LIST OF TABLES

Table		Page
1.	Comparison of the Properties of α- and β-Amylases	4
2.	Existence of Multiple Forms of α-Amylase in some Common Cereals	8
3.	Breakdown Products of Plant α-Amylase Action on Short Oligosaccharides	17
4.	Physical Properties of the Cycloamyloses	27
5.	Purification of α-Amylase by Glycogen Precipitation using the Method of Schramm and Loyter (1966)	46
6.	A Comparison of Various Storage Methods on the Stability of α-Amylase in Crude Extracts of Triticale 6A190	50
7.	Stability of Glycogen Precipitates and Ammonium Sulfate Precipitates of Malted Triticale 6A190 α-Amylase Stored at 4°C	52
8.	Purification of α-Amylase from Triticale 6A190 by Glycogen Precipitation and Cyclohepta-amylose Epoxy Sepharose 6B Affinity Chromatography	53
9.	α-Amylase Recovered from Cyclohepta-amylose Affinity Chromatography Column by Elution with Various Media	76

LIST OF FIGURES

Figure		Page
1.	Susceptibility of Certain α-Glucosidic Bonds of Starch to Plant α-Amylase Degradation	19
2.	Patterns of α-Amylase Activity as Obtained by Zymograms of Polyacrylamide Gel Electrophoresis of Extracts of Triticale 6A190, Prolific Rye, and Stewart Wheat during Different Stages of Growth	43
3.	The Effect of Varying Glycogen Concentration on the Precipitation of α-Amylase	48
4.	Affinity Chromatography of Glycogen Precipitated α-Amylase	55
5.	Protein and α-Amylase Activity Patterns of Disc Electrophoresis Gels of Crude Extract, Resuspended Glycogen Precipitate, and Affinity Chromatography Purified α-Amylase	57
6.	Ion-exchange Chromatography of Triticale α-Amylase on Carboxymethyl Cellulose	61
7.	Chromatography of Resuspended Glycogen Precipitate on Concanavalin A-Sepharose	64
8.	Chromatography of Resuspended Glycogen Precipitate on β-Limit Dextrin-AH-Sepharose	68
9.	Zymograms and Protein Staining Patterns of Disc Electrophoresis Gels of β-Limit Dextrin-AH-Sepharose 4B Affinity Chromatography Column	70
10.	Ion-exchange Chromatography of Malted Triticale α-Amylase α-II at pH 7.5.	73
11.	Isozymes Released from Affinity Chromatography Column by Various Methods	77
12.	Effect of pH on the Activity of Malted Triticale α-Amylase α-I	82
13.	Effect of pH on the Activity of Malted Triticale α-Amylase α-II	84

Figure		Page
14.	pH Stability of Triticale α-Amylases α-I and α-II with and without β-Limit Dextrin	86
15.	Determination of Michaelis-Menten Constant, K_m, for Triticale α-Amylases α-I and α-II	89
16.	Arrhenius Plot for Malted Triticale α-Amylases α-I and α-II	92
17.	Stability of Triticale α-Amylases α-I and α-II at 45, 60 and 70°C	95
18.	Effect of Calcium-ion Concentration on Triticale α-Amylase Activity	97
19.	Isoelectric Focussing of Triticale α-Amylase	100
20.	Inhibition of Triticale α-Amylase Activity by Cyclohepta-amylose	103
21.	Effect of Cyclohepta-amylose on the Linearity of Absorption by Iodine β-Limit Dextrin Solutions at Various Levels of Digestion by α-Amylase	106

ABSTRACT

Silvanovich, Mikola Peter. Ph.D., The University of Manitoba, October, 1977. α-Amylase from Triticale 6A190: Isolation and Characterization. Major Professor: Dr. Robert D. Hill.

The α-amylases in malted triticale 6A190 (X Triticosecale Wittmack) were isolated and characterized. Triticale 6A190 suffers from seed-shrivelling at maturity resulting in low test weight. The α-amylases present in the kernel were characterized to determine whether or not they displayed unusual properties which may have accounted for seed shrivelling. No unusual properties were found when compared to known data for other cereals. The isozymes were characterized in terms of pH optimum and stability, K_m, V_{max}, activation energy, temperature stability, calcium requirement, molecular weight, isoelectric point, and inhibition by cyclohepta-amylose.

The procedure used to isolate purified α-amylases consisted of extraction of malted triticale, glycogen precipitation, affinity chromatography on cyclohepta-amylose covalently bonded to Epoxy-Sepharose 6B, and separation on carboxymethyl cellulose. The isozymes were separated into two kinds with a purification in the order of 150 to 200-fold, with 25-30% yield. Several other chromatographic procedures were investigated.

INTRODUCTION

Triticale (X Triticosecale Wittmack) is a synthetic species developed by the combination of Triticum (wheat) and Secale (rye) genomes. (Larter et al., 1970; Hulse and Spurgeon, 1974). One of the most persistent problems in some lines of triticale has been poor kernel characteristics. The seed does not develop fully, but rather, becomes shrivelled at maturity resulting in low test weight. Hill et al. (1974) found that α-amylase activity increased at maturity in shrivelled lines (e.g. cultivar 6A190) but not in non-shrivelled lines (e.g. cultivar 6A250). This suggested that precocious germination may be occurring in shrivelled varieties of triticale, and that the resulting increase in α-amylase activity resulted in breakdown of starch in the kernel leading to shrinkage and partial collapse of the endosperm. Dedio et al. (1974) showed that, in fact, such a phenomenon was occurring. It is not yet certain that a cause-effect relationship exists between seed shrivelling and α-amylase activity. There may be a number of biological and physiological causes for seed shrivelling in triticale, and the complex genetic factors (Scoles and Kaltsikes, 1974; Kaltsikes and Roupakias, 1975) involved in such hybrids increase the likelihood of abnormal kernel development. Triticale 6A190 displays aberrant nuclei in the developing endosperm (Kaltsikes et al., 1975), and these aberrations may lead to kernel-shrivelling.

This investigation of the α-amylase from triticale was undertaken to: (a) see if any properties of the enzyme could account for kernel shrivelling; (b) compare the properties with those of other cereal α-amylases.

LITERATURE REVIEW

Amylases

Amylase catalyzes the hydrolysis of α, 1 → 4 linked glucose polymer by the transfer of a glucosyl residue to water. Amylases are referred to as α- or β-amylases or as endo- or exo-amylases. Alpha-amylases (α, 1 → 4 glucan 4-glucanohydrolase, E.C. 3.2.1.1.) are so-named because their products of hydrolysis possess α-configuration. Alpha-amylases will cleave large linear substrate molecules at most internal bonds, whereas β-amylases, being exoamylases, cleave alternate linkages from the non-reducing end of a substrate. Beta-amylases (α, 1 → 4 glucanmaltohydrolase, E.C. 3.2.1.2) are so-named because they produce maltose in the β-configuration (Thoma et al., 1971, and references therein). Table 1 compares α- and β-amylases. The characteristics of β-amylases will be discussed no further as they have a vast body of literature in their own right and α-amylases are the topic of this review.

Alpha-amylases seem to be found in all types of living organisms, but only those of plants will be discussed here. All plant amylases share some basic characteristics, among them a molecular weight of about 45,000, and an association with Ca^{2+} - having 1 gram atom of calcium per mole of enzyme (Northcote, 1975). Multiple forms of α-amylase seem to be quite common among cereals, having been found in barley (Frydenberg and Nielsen, 1965), wheat (Kruger and Tkachuk,

TABLE 1. Comparison of the properties of α- and β-amylases[a]

Characteristic	α-amylase	β-amylase
Cleavage Point	α, 1 → 4 glucosidic bond Cleave $C_1 - O'_4$ bond	α, 1 → 4 glucosidic bond Cleave $C_1 - O'_4$ bond
Configuration of New Reducing Unit	α	β
Mechanism of Attach	endo	exo
End Products	oligo saccharide mixture	maltose
Action at Branch Points	can bypass	cannot bypass
Origin	plant and animal	plant
Stability at pH 3.6*	low	high
Stability to 70°C for 15 min*	high	low

[a]Modified after Thoma et al., 1971

*Plant α-amylases

1969), rye (Wagenaar and Lugtenborg, 1973), and triticale (Lee and Unrau, 1969). Whether or not all of the isozymes observed are real or artefactual has yet to be determined.

Amylases of Mature and Immature Grain

It was in 1936 that Chrazaszcz and Janicki discovered that the α-amylase activity in the growing kernels of barley, wheat, oats, and rye was a dynamic system - continually undergoing change. In 1946, while doing photomicrographic studies of wheat starch, Sandstedt observed that starch in the pericarp was being digested while the kernel grew and practically disappeared by the time the kernel reached maximum length. His later work showed the enzyme to be α-amylase (Sandstedt and Beckord, 1946), and also indicated that during the growth of the kernel α-amylase activity rose to a peak and then declined. More recent findings have shown similar results for various cereals.

In 1964 Stewart concluded that there was α-amylase activity in sound mature wheat, and Olered and Jönsson (1970) confirmed this fact in an extensive work, as did Kruger (1972a). The level of α-amylase activity found in mature kernels is low and quite variable. One reason for this appears to be "reactivation" of α-amylase when mature grain is dampened (Olered and Jönsson, 1970) even though no visible germination occurs.

The electrophoretic studies of α-amylase in wheat done by Olered and Jönsson (1970) were the first in a series of electrophoretic-enzymatic studies of various cereals to take place in the next six years.

They detected two forms of α-amylase which they called "green" α-amylase and "malt" α-amylase. The former is characteristic of mature or growing seed while the latter is characteristic of germinating or malted seed except that "green" α-amylase is also found in such seed. Olered and Jönsson proposed that normal ripening consisted of a continuous but reversible inactivation of green α-amylase. The moisture equilibrium of the seed was believed to be responsible for the degree of activity found, with dehydration being responsible for inactivation.

MacGregor et al. (1971a) studying barley, confirmed this by measuring the actual activity and finding that activity increased very rapidly from ear emergence to 11 days after anthesis and then declined rapidly to one-tenth of its maximal value after 28 days, remaining constant until maturity. Duffus and Rosie (1973), also studying barley, found maximal α-amylase activity 20 to 30 days post-anthesis with a decline to about one-fifth maximum by the fortieth day. Kruger (1972a) observed the same phenomenon in hard red spring wheat, using electrophoresis. Meredith and Jenkins (1973b) studied wheat, barley, and oats and found that all three cereals went through a growth pattern characterized by a peak of green α-amylase activity followed by a decline toward maturity resulting either in total disappearance or very low levels of activity at maturity.

There is one exception to these observations, and it is found in certain lines of triticale. Klassen et al. (1971) noted that triticale 6A190, a shrivelled line, had a decrease in α-amylase activity in the early stages of growth, but as maturity approached this activity rose again, increasing toward maturity. The level of

activity in mature 6A190 was 13 times as high as in 6A250 a non-shrivelled line. In general, mature seed contains little if any detectable α-amylase activity. (Certain lines of triticale being the exception). These low levels of activity have discouraged research, and as a result the most work has been carried out using germinated or malted seed, with some being done on immature "green" kernels.

Amylases of Germinated Seed

It is with germinated seed that the majority of research on α-amylases has been done, due to the higher levels of α-amylase activity present. (The terms "germinated" and "malted" will be used interchangeably here even though there is some indication that malting and germination do not produce quite the same results - Meredith and Jenkins, 1973a).

In the subsequent review it is mainly the results obtained with germinated or malted seed that will be discussed. As it may be apparent by now, more than one type of α-amylase has been found in cereals. The major division has been made on the basis of electrophoretic mobility and coincides with the two groups - "green" and "malt" as described by Olered and Jönsson (1970). The number of isozymes within each group varies from cereal to cereal, and can vary with any given cereal depending on the analytical method(s) used.

Table 2 is a summary of the data obtained from various laboratories. The disagreements between different sets of data

TABLE 2. Existence of Multiple Forms of α-Amylase in Some Common Cereals

Source	Least Number of Forms	References	Methods
Immature Barley	1	MacGregor et al (1974)	column chromatography
Malted Barley	2	MacGregor et al (1971)	column chromatography
Immature Wheat (Hard Red Spring)	3	Kruger (1972a)	electrophoresis
Immature Wheat	3	Marchylo et al (1976)	electrophoresis and chromatography
Malted Wheat	4	Kruger and Tkachuk (1969)	chromatography
Germinated Wheat	7	Kruger (1972b)	electrophoresis
Germinated Rye	5	Wagenaar and Lugtenborg (1973)	electrophoresis
Malted Rye	2	Manners and Marshall (1972)	electrophoresis
Germinated Triticale	3	Lee and Unrau (1969)	electrophoresis and chromatography

reflect the limitations of the techniques used. There is some doubt as to whether or not all of the "isozymes" seen are real or artefactual. It is fairly certain that the green and malt forms are not artefactual, but this cannot be said with certainty about the isozymes present within each group.

Synthesis and Morphological Distribution of α-Amylase

For some time it was not known whether α-amylase arose in germinating seed by *de novo* synthesis or by reactivation of pre-existing α-amylase. In 1960, Paleg discovered that the gibberellic acid, a plant hormone known to affect many biological systems in plants, would increase the level of soluble amylolytic activity in barley endosperm. It was not much longer until Varner (1964) was able to show that barley α-amylase was synthesised *de novo* in the barley aleurone layer in response to gibberellic acid. This was determined by following the incorporation of C^{14}-labelled phenylalanine into α-amylase. Later work by Chrispeels and Varner (1967) and Filner and Varner (1967) confirmed *de novo* synthesis using other techniques. Daussant and Renard (1972), studied α-amylase in germinating wheat using immunochemical absorption techniques and found that 98% of activity detected was in the form of newly synthesised protein. In current studies isolated barley aleurone layers are most commonly used. Perhaps the most striking recent work has appeared from Jacobsen's laboratory. Using cytochemical and immunofluorescent techniques (Jacobsen and Knox, 1973), it has been shown that the gibberellic acid-induced synthesis of α-amylase is

localized in the aleurone grains - the grain membranes in particular. In a follow-up on the earlier work of Ho and Varner (1974), it was shown that gibberellic acid enhances the level of mRNA that codes for α-amylase. The mRNA was isolated and cell-free synthesis of α-amylase accomplished. (Higgins et al.,1976).

Initial work seemed to indicate α-amylase produced in response to gibberellic acid stimulus was soluble in nature (Jones, 1972) but more recent work (Gibson and Paleg, 1972; Firn, 1975) shows that it can be lysosomal in nature (Gibson and Paleg, 1972; Firn, 1975).

Although the site of synthesis of α-amylase in germinating seed was determined some time ago (Varner, 1964), the morphological distribution of α-amylase in mature seed was a source of contention for some time longer. In 1946, Sandstedt observed the breakdown of starch granules in the pericarp of growing wheat, but in 1971 both Bilderback and Stoddart concluded that α-amylase in barley exists predominantly in the aleurone layer. This contradiction was not resolved until recently when MacGregor et al.(1972) using barley, Kruger (1972) using wheat and Meredith and Jenkins (1973b) using barley, showed that the pericarp is the location of α-amylase in mature grain. In 1974, Dedio et al.looked at the morphological distribution of α-amylase in triticale, wheat, and rye. They too found the highest levels in pericarp. However, there was one exception to that finding. Triticale 6A190, a shrivelled line, displayed α-amylase activity in the aleurone and endosperm in the later stages of kernel development, whereas levels in the pericarp decreased.