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α-ISOAMYLASES OF RYE SEEDS

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Key Word Index—*Secale cereale*; *Hordeum vulgare*; *Triticum vulgare*; Gramineae; α-amylase; stability; temperature.

Abstract—Rye seeds contained 5 α-type amylases. Three behaved like typical α-amylases and were called Aα-amylases. Two showed chemical activity like α-amylase, but behaved differently physically and were called Bα-amylases. The latter were partly inactivated at pH 3·3 and at 70°. They were more resistant to EDTA than were the Aα-amylases. Barley and wheat seeds contained amylases behaving like Bα-amylases. Aleurone layers contained relatively large amounts of Aα-amylases. Relative amounts of Aα-and Bα-amylases depended on temperature during germination. Bα-amylases remained active for a longer time after germination than Aα-amylases.

INTRODUCTION

GERMINATED grains contain several enzymes which degrade starch. Usually, α- and β-amylase (E.C. 3.2.1.1. and 3.2.1.2.) are present in considerable quantities. A separate determination of both types of enzyme is difficult. In most experiments the method of Ohlsson and Uddenberg[1] has been used in which one type of enzyme is inactivated when the other type may be determined. α-Amylase is inactivated in 20 min at pH 3·3 and β-amylase in 15 min at 70°. These authors obtained reasonable results with wheat and barley but the method could not be used for rye. Another way to determine α-amylases is by the method of Briggs,[2] in which β-limit dextrin is used as the substrate.

To obtain information on various starch degrading enzymes of rye, amylases were separated by agar electrophoresis and their properties were investigated. Several α-amylases were found and their relative amounts changed during germination.

RESULTS

Rye was germinated for 6 days at 25° or 35 days at 5°. All preparations contained 5 enzymes which degraded starch to products which could not be stained with I_2–KI; they could be divided on the basis of electrophoresis into 2 groups: 2 fast and 3 slow moving enzymes (Table 1). The 3 slowest moving enzymes behaved like α-amylases. A high activity remained after heating to 70° for 15 min. Activity of α-amylases depends on the presence of Ca^{2+}. Rowsell and Goad[3] inactivated α-amylases by dialysis overnight against 10^{-2} M EDTA. In our experiments the 3 slowest moving enzymes were inactivated by such a treatment. The stability of the 2 fastest moving enzymes was different from that of the 3 slowest moving ones. These 2 enzymes were partly inactivated in 15 min at 70° and at pH

[1] OHLSSON, E. and UDDENBERG, C. L. (1933) *Z. Phys. Chem.* **221**, 165.
[2] BRIGGS, D. E. (1967) *J. Inst. Brewing* **73**, 361.
[3] ROWSELL, E. V. and GOAD, L. J. (1964) *Proc. Biochem. Soc.* **90**, 11.

3·3. They were inactivated completely if 4×10^{-2} M EDTA was added to a homogenate of seeds 30 min before electrophoresis was started. If a homogenate was dialysed overnight against 10^{-2} M EDTA and tested on β-limit dextrin for remaining activity, both enzymes showed considerable activity. If starch was used as substrate, activity of the fastest moving of the 2 enzymes was greatly reduced.

TABLE 1. AMYLASES OF WINTER SEEDS OF RYE GERMINATED FOR 4 WEEKS AT 5°

Band No.	Control	70°	pH 3·3	10^{-2} M EDTA Starch	10^{-2} M EDTA β-limit Dextrin	4×10^{-2} M EDTA Starch	4×10^{-2} M EDTA β-limit Dextrin
Aα-amylases							
5	+	+	−	−	−	−	−
6	+	+	−	−	−	−	−
7	+	+	−	−	−	−	−
Bα-amylases							
9	+	×	×	+	+	−	−
10	+	×	×	×	+	−	−

Homogenates were kept for 15 min at 70° or pH 3·3; 4×10^{-2} M EDTA was added 30 min before electrophoresis was started; homogenates were dialysed against 10^{-2} M EDTA overnight. +, enzyme activity present; −, no enzyme activity, ×, enzyme activity reduced.

The 3 slow moving enzymes were called Aα-amylases and the 2 fast moving enzymes Bα-amylases. The 2 groups of enzymes behaved differently in the digestion of starch. Table 2 shows that activity of the Aα-amylases was small. After digestion for 18 hr, the products

TABLE 2. DIGESTION OF STARCH AND PRODUCTION OF MALTOSE BY THE 3 SLOW-MOVING AMYLASES (Aα) AND THE 2 FAST-MOVING ENZYMES (Bα) OF RYE SEEDS, IN THE PRESENCE AND ABSENCE OF BOVINE SERUM ALBUMIN (BSA)

	Starch*	Maltose*
−BSA		
Aα	0·23	0·16
Bα	1·84	1·10
+BSA		
Aα	6 00	2·73
Bα	2·22	1·56

The enzymes were obtained from agar slices cut following electrophoresis and eluted overnight in acetate buffer (0·001 M pH 5·5, 0 001 M Ca^{2+}) with or without 0 25 % BSA. * mg/hr at 30°.

of digestion stained purple with I_2–KI. Bα-amylases showed a higher activity. After digestion for 18 hr the products no longer stained purple with I_2–KI. When bovine serum albumin

(BSA) was added to the elution buffer, activity of the Aα-amylases was high. BSA had little influence on the activity if Bα-amylases and their stability may be greater than that of Aα-amylases. If the digestion period was extended until all starch was digested (after about 24 hr) both groups of enzymes produced 22·5 mg maltose from 25 mg starch. The final products were identified by GLC.[5] Small amounts of glucose and of maltotrioses were found but the main product was maltose. It can be concluded, therefore, that all 5 enzymes are α-type amylases.

Seeds were separated into the scutellum, the endosperm and the aleurone layer. All 5 enzymes were present in the endosperm. In the aleurone layers Bα-amylases were predominant. The scutellum contained a small amount of each of the 5 enzymes. This may possibly be due to contamination by endosperm. Bα-Amylases were found occasionally in the germination medium. Aleurone layers contained an extra enzyme which was located between Aα- and Bα- amylases on electrophoresis. It was relatively stable towards EDTA, but was inactivated at 70°. Scutellum, aleurone layers, and endosperm contained another fast moving enzyme in small quantities which showed as a narrow upward curved band beneath enzyme 10. The enzyme was inactivated at 70° and was stable towards EDTA.

TABLE 3. AMYLASES SEEN ON ELECTROPHORESIS OF EXTRACTS FROM GERMINATED SEEDS OF WHEAT, BARLEY AND RYE BEFORE (CONTROL) AND AFTER TREATMENT WITH 70° FOR 15 min OR 10^{-2} M EDTA FOR 30 min

Band No.	Wheat			Barley			Rye		
	Control	70°	EDTA	Control	70°	EDTA	Control	70°	EDTA
1	−			+	−	+	−		
2	−			+	−	+	−		
Starting point									
3	+	+	−	+	+	+	−		
4	+	+	−	+	+	+	−		
5	+	+	−	+	+	−	+	+	−
6	+	+	+	+	+	−	+	+	−
7	+	+	+	−			+	+	−
8	+	−	+	−			+	−	+
9	+	−	+	+	−	+	+	+	+
10	+	−	+	−			+	+	+
11	+	?	+	−			+		

Activities of isoamylases of barley (*Hordeum vulgare*, L. cv. Pirkka) and of wheat (*Triticum vulgare*, L. cv. Jufy) were compared simultaneously by electrophoresis. The seeds were germinated for 4 days at 25° (Table 3). All grains contained Aα-type amylases: bands 3, 4 and 5 in wheat, bands 5 and 6 in barley, and bands 5, 6 and 7 in rye. Bα- amylases were found in all seeds: bands 6 and 7 in wheat, bands 3 and 4 in barley, and bands 9 and 10 in rye. Bands 8–11 in wheat and bands 1 and 2 in barley behaved like β-amylases. Bands 3–7 in wheat had a purple background, as if β-amylase was present. No β-amylases were seen in germinated seeds of rye. They may be present in very small amounts. Ungerminated seeds of rye contained 2 β-amylases which moved faster than did Bα-amylases.

An investigation was made to determine whether Aα- and Bα-amylases are present in the same relative quantities in rye seeds germinated at different temperatures. Usually, enzyme band 6 showed the greatest activity and this enzyme was used as reference. After 21–35 days from germination at 5° enzyme 5 was present in greater quantities than enzyme 7. In these seeds the relative activities of enzymes 9 and 10 were high, band 10 being almost

[4] By courtesy of Dr. B. TUNING.

as great as band 6 and larger than band 9. Total amylase activity (Table 4), however, was highest on the 28th day after germination. The decrease in total amylase activity between the 28th and 35th day after germination was accompanied by a relative decrease in Aα-amylases. These enzymes may be less stable *in vivo* than Bα-amylases. No difference was found between seeds germinated before or after dry storage.

TABLE 4. AMYLASE ACTIVITY IN SPRING SEEDS OF RYE GERMINATED AT 5° BEFORE STORAGE AND AT 25° BEFORE AND AFTER STORAGE FOR 1 YEAR AT 20°

Germinated at 5°		Germinated at 25°		
No of days germinated	Before storage	No. of days germinated	Before storage	After storage
7	10·5	1	8 8	9·4
14	27·0	3	23·2	24·7
21	32·5	4	24·0	39·3
28	36·9	6	24·8	35·4
35	20·9	7	14 2	22·2

Activity expressed to mg starch digested per seed in 4 min at 30°.

When seeds were germinated at 25° shortly after harvest (Table 5) activity of enzyme 5 was small. The activity increased with time after germination. Enzyme 7 was present in greater quantities than enzyme 5; the amount, however, decreased with time after germination. On the 7th day after germination, a similar relatively high activity of enzymes 9 and 10 was found, as in seeds germinated at 5°. Seeds germinated at 25° after dry storage for 12 months at 20° showed an increase in the activity of enzyme 5.

TABLE 5 AMYLASES IN RYE SEEDS SEVERAL DAYS AFTER GERMINATION AT 25°. SEEDS WERE GERMINATED AFTER DRY STORAGE FOR 3 OR 12 MONTHS AT 20°

Band No.	4th day	6th day	7th day
3 months Aα-amylases			
5	−	+	+
6	+ + + +	+ + + +	+ + + +
7	+ +	+	+
Bα-amylases			
9	+ +	+ +	+ + +
10	+ + + +	+ + +	+ + + +
12 months Aα-amylases			
5	+	+ +	+ + +
6	+ + + +	+ + + +	+ + + +
7	+ +	+	+
Bα-amylases			
9	+ +	+ +	+ + −
10	+ + + +	+ + + +	+ + + + +

− Enzyme activity was very small.

Comparison of seeds germinated at 5 or 25° shows that the activity of enzyme 5 and 9 is relatively high at 5°.

DISCUSSION

The 2 types of amylases found in rye behaved differently not only *in vitro*, but also *in vivo*. One group, consisting of 3 isoenzymes, behaved like well known α-amylases *in vitro*, and were called Aα-amylases. The other group consisted of 2 isoenzymes, which were designated as Bα-amylases. Their physical stability more closely resembled β-amylase. The 2 types of amylases were also found in barley and wheat in agreement with Frydenberg and Nielsen[5] and Olered and Jonsson.[6] Aα- and Bα-amylases of various grains did not move with the same speed or in the same order on electrophoresis. Manners and Marshall[7] isolated 2 amylase fractions from rye by means of continuous electrophoresis. The enzymes of their fractions A and B closely resemble our Aα- and Bα-amylases. These authors suggested that the enzyme of fraction B depends less on Ca^{2+} for stability and activity than the enzyme of fraction A. Our results tend to the same conclusion.

When it was tested on starch as substrate, one of the Bα amylases was inactivated by EDTA. If β limit dextrin was used, however, the enzyme remained active. It may be that the band contains one enzyme which behaves differently towards 2 substrates as suggested for peroxidases of tomato,[8] or the band contains 2 enzymes which behave differently with both substrates.

In vivo Aα-amylases followed more closely the total amylase activity of homogenates of seeds than did the Bα-amylases. When towards the end of an experiment total amylase activity began to decrease, a relatively high activity of Bα-amylases was found. Bα-Amylases seem to be less susceptible to degradation by proteases than Aα-amylases.

One of the Aα-amylases and one of the Bα-amylases was present in relatively large quantities in seeds germinated at 5°. The relative amounts of 2 of the Aα-amylases were changed by dry storage in seeds subsequently germinated at 25°. It may be that induction of the various isoenzymes follows different patterns. The experiments did not indicate that the various isoenzymes were synthesized in different parts of a seed. The enzymes may be located in different parts of a cell, and may be more or less susceptible to degradation by proteases.

EXPERIMENTAL

Rye seeds (*Secale cereale*, L. cv. Petkuser) were sterilized by shaking for 5 min in 0·1 M $HgCl_2$. After washing, the seeds were treated with 0·5 per cent hydroxylamine for 30 min. This was followed by another rinse in sterile H_2O. Seeds were germinated in dist. H_2O for some days at 25° or some weeks at 5°. A sample of 50 seeds was collected. After removal of shoots and roots, seeds were homogenized in dist. H_2O. The pH was about 5 5. Amylases were detected by the method of Frydenberg and Nielsen.[5] Slides were covered with 2 ml 0·7% agarose. Electrophoresis was carried out in a 0 018 M phosphate buffer at pH 7·0 with 60 V per slide at 3 mA. After electrophoresis for 2 hr each slide was covered with a slide containing agar with 1% starch After 15 min at 40° the original slides were stained with I_2–KI solution (0·2% I_2 in 2·0% KI). White and purple bands marked locations of α- and β-amylases respectively. In experiments in which the products of enzyme activity were examined, the enzymes were separated by agar electrophoresis and covered with slides containing starch for 15 min at 30°. The 3 slow-moving enzymes as well as the 2 fast-moving ones were combined and eluted overnight with acetate buffer of pH 5·5 containing 0·001 M Ca^{2+}, with or without 0·25% bovine serum albumin. Starch was added and amylase activity measured as digestion of starch by the method of Paleg[9] and as production of maltose by the method of Neolting.[10]

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