ISOLATION AND CHARACTERIZATION OF TWO IMMUNOLOGICALLY DISTINCT FORMS OF α -AMYLASE AND A β -AMYLASE FROM SEEDS OF GERMINATED SORGHUM BICOLOR (L.) MOENCH

by

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Keywords: Affinity chromatography, hydrophobic interaction, isoelectric focusing, multiple molecular forms, enzyme inhibition, monospecific antibodies

Two immunologically distinct forms of α -amylase named α -1 and α -2 and a β -amylase were isolated from germinated sorghum by affinity-, hydroxylapatite- and hydrophobic interaction chromatography. The two α -amylases showed similar molecular weights of 41,500 - 42,700 as determined by electrophoresis and gel permeation chromatography. The two enzymes have similar amino acid compositions except for differences in Ser, Val, Ile, and Cys. They have slightly different isoelectric points of 4.65 for α -1 (major form) and 5.1 for α -2. α -1 and α -2 amylases contain 0.7% and 5.7% carbohydrate by weight respectively and they exhibit different kinetic properties and substrate specificities.

Crossed immunoelectrophoresis and immunodiffusion of sorghum α -1 amylase against monospecific anti-barley α -2 amylase showed immunological identity between barley malt α -2 and sorghum malt α -1 amylase. Non-identity was seen between sorghum α -2 and the barley malt amylase. Sorghum α -1 and α -2 amylase show partial immunological identity. α -1 amylase could only be detected in trace amounts in ungerminated sorghum.

Abbreviations:

CHpA = cycloheptaamylose; EDTA = Ethylenediamine-tetraacetate-disodium salt; IEF = isoelectric focusing; IgG = immunoglobulin G; = SDS-PAGE sodium dodecyl-sulfate polyacrylamide gel electrophoresis.

1. INTRODUCTION

Multiple forms of amylase have been detected in a number of cereals (5, 12, 19, 25, 30) and several studies have suggested that these forms are the result of ontogenic and/or tissue specific differential gene expression (8, 27). Genetic and biochemical studies (12, 19) of amylases in barley have established the existence of two forms of α -amylase, now generally referred to as α -1 (green) and α -2 (malt) amylase. Similar work with maize revealed the existence of two α -isozymes controlled by co-dominant alleles (5).

Studies on the histological development of amylases during cereal germination have shown that β -amylase is synthesized during seed development (14). The de novo synthesis of α -amylase by barley aleurone layers is well documented (6) and work on this system has elucidated the level of molecular control of protein synthesis by gibberellic and abscisic acids (21). Recent work has also shown that the scutellum is important in hydrolase production during the early stages of germination in rice (24) and barley (13).

Active α -amylases produced during the germination of sorghum are both soluble and insoluble. An early study by NOVELLIE (23) suggested that insoluble sorghum α -amylase can be released during extraction by desorption from the surface of an insoluble protein. A later study of DAIBER (7) showed that soluble α -amylase is inhibited by endogenous polyphenols during extraction.

The present study was undertaken to 1) determine if multiple forms of α -amylase exist in malted sorghum, 2) to characterize the possibly existing forms and 3) to produce monospecific rabbit antibodies against amylases present in germinated sorghum for subsequent immunohistological studies.

2. MATERIALS AND METHODS

2.1. Plant material

Seeds of *Sorghum bicolor* (L.) Moench (cv. Dabar) a white, low-tannin, testa-less Sudanese variety were kindly supplied by Dr. S. BADI, Food Research Center, Khartoum, Sudan. Sorghum green malt was prepared by steeping seed for 24 hours at 30 °C with two 1 hour aerations, followed by a five day germination in a rotating-

drum laboratory unit at 28 °C-30 °C and 80-90% relative humidity.

2.2. Chemicals

Sephadex G-75 superfine and LH-20, epoxy-activated Sepharose 6B, Octyl-Sepharose CL-4B, DEAE-Sephadex A50, Phadebas blue starch tablets and low molecular weight size standards were from Pharmacia, Uppsala, Sweden. Biogel P100 and Biogel HTP were from Biorad Labs. Richmond, California, USA. Cycloheptaamylose (Schardinger β -cyclodextrin, CHpA) and oyster glycogen were from Sigma Chemical Co., St. Louis, Missouri, USA. Carrier ampholytes 4-6 were obtained from LKB, Bromma, Sweden. β -limit dextrin was from BDH Chemicals, Poole, England. Soluble starch and TLC silica gel 60 aluminum sheets were from Merck, Darmstadt, Germany. Pullulan was a kind gift from lic. techn. B. ENEVOLDSEN, Carlsberg Research Laboratory, Copenhagen, Denmark. Monospecific anti-barley α -2 amylase and anti-barley β -amylase were a gift of Dr. G. GIBBONS, Carlsberg Research Laboratory. All purchased chemicals were of reagent grade and were used without further purification.

2.3. Electrophoretic techniques

Immunoelectrophoresis and isoelectric focusing in polyacrylamide gel (IEF-PAGE) were run as previously described (1 and 34) with 1mM- CaCl_2 incorporated in the gels. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed after NEVILLE (22) using a 12.5% gel. Total malt extracts for SDS-PAGE were precipitated with 5% trichloroacetic acid for 30 min on ice prior to reduction. Serine protease activity was inhibited by including 10mM-EDTA or 500 μM -phenyl-methyl-sulfonyl chloride or n - α -p tosyl-L-Lysine in the extraction buffer.

2.4. Measurement of amylase activity

Amylolytic activity was localized in immuno- and IEF-gels as described by HEJGAARD (14) using 2% β -limit dextrin in 50 mM-Na-acetate buffer, pH 5.5, 1 mM- CaCl_2 for specific detection of α -amylase.

Amylolytic activity in chromatographic column fractions was routinely monitored with Phadebas blue starch tablets.

Activity measurements for kinetic analysis were performed using either the dinitro-salicylic acid reducing sugar test (33) or with the neocuproin reagent described by DYGERT et al. (10).

Hydrolysis products released by the enzymes from 1% soluble starch were examined on thin layer silica gel plates developed once in butanol-formic acid-water (33:50:17) after KOLLER AND NEUKON (17), sprayed with 5% H_2SO_4 in ethanol and developed by heating at 110 °C for 10 min.

2.5. Amino acid analysis and carbohydrate content of purified proteins

Amino acid compositions were determined for duplicate 40 µg samples hydrolyzed for 24, 48, and 72 hours, respectively. Values for serine and threonine were extrapolated to 0 hour, while those for valine and isoleucine were the average of 48 and 72 hours. Cysteine was determined as cysteic acid after HIRS (15) and tryptophan from the tyrosine/tryptophan ratio by the method of BENZE and SCHMID (2). All hydrolysates were analyzed on a Durrum D-500 amino acid analyzer.

Carbohydrate was determined by the method of DUBOIS et al. (9) using a glucose standard.

2.6. Antibody production

Antibodies were prepared against partly purified sorghum green malt immunogens as well as against purified β -amylase and two forms of α -amylase. Total immunogens were extracted from 20 g lyophilized sorghum malt (5 day germination) in 100 ml 0.2M-Na-acetate, pH 5.5, 80 mM- β -mercaptoethanol, 1 mM- CaCl_2 , for 1 hour at 4 °C. The extract was centrifuged twice at 25,000 x g for 15 min and filtered twice through two layers of miracloth and glass wool. The cleared, brownish supernatant was applied to a 2.5 x 20 cm bed of LH-20 Sephadex equilibrated in 0.2M-Na-acetate, pH 5.5, 1mM- CaCl_2 , and eluted at approximately 200 ml·h⁻¹ in the equilibration buffer. Colored substances were strongly absorbed at the top of the gel. Eluted proteins were monitored at 280nm, the peaks pooled, extensively dialyzed against distilled water and lyophilized. Preparation of amylases for antigen production is described in section 2.8.

Rabbits were immunized and tapped by Dakopatts, Copenhagen, Denmark using the im-

munization schedule of INGILD & HARBOE (1). The final immunization concentration of antigen was 2mg·ml⁻¹ for the total-soluble green malt antigens and 150 µg·ml⁻¹ for the purified enzymes. Rabbits were injected with 1 ml of the antigen/adjuvans mixture in three dorsal subcutaneous depots of 0.33 ml.

The IgG fraction was isolated from rabbit sera by $(\text{NH}_4)_2\text{SO}_4$ precipitation as described by AXELSEN et al. (1). Purified antibody was tested by immunodiffusion and crossed immunoelectrophoresis against the appropriate antigens and stored at 4 °C in 15mM- NaN_3 .

2.7. Molecular weight determination

Molecular weights of purified proteins were estimated by SDS-PAGE and by gel filtration on Sephadex G75-superfine and Biogel P100. Gel filtration on a 2.5 x 90cm column of Sephadex was performed at pH 6.5 in Na-acetate buffers of various ionic strengths. Chromatography on a 1.6 x 90cm column of Biogel was performed in 0.2M-Tris-HCl, pH 7.2, 0.2 M-NaCl, 1mM- CaCl_2 at a flow rate of 3-4 ml·h⁻¹. The columns were calibrated using the following molecular size standards: ferritin (0.5 mg·ml⁻¹) bovine serum albumin (7 mg·ml⁻¹), ovalbumin (7 mg·ml⁻¹), soya trypsin inhibitor (5 mg·ml⁻¹) and bovine ribonuclease (10 mg·ml⁻¹).

2.8. Isolation of amylases

Amylases were extracted from 100 g finely ground lyophilized sorghum green malt with 400ml of 0.2M-Na-acetate buffer, pH 5.5, 80mM- β -mercaptoethanol and 1% insoluble PVP by stirring for 1 hour at 4 °C. Following ethanol and glycogen precipitation according to SCHRAMM and LOYTER (28), the precipitate was resuspended in 50 mM-Na-acetate and incubated at 37 °C for 1 hour to digest the glycogen. Insoluble material was removed by centrifugation at 40,000 x g for 15 min.

Isolation and purification of amylases was carried out according to the flow chart in Fig. 1. All solutions used in chromatography contained 1mM- CaCl_2 and all columns were run at 4 °C. Column elution profiles were monitored for protein at 280nm, for α -amylase activity with Phadebas blue starch tablets and by fused-rocket immunoelectrophoresis against anti-total sorghum

Figure 1. Sorghum α - and β -amylase isolation flow scheme.

malt antigens. Purity of column fractions was checked by crossed immunoelectrophoresis and SDS-PAGE.

2.8.1. Ion-exchange chromatography

The resuspended glycogen precipitate was chromatographed on a 2.5 x 25cm column of DEAE-Sephadex A50 equilibrated with Tris-HCl, pH 7.2. Elution was performed with a linear gradient of NaCl (0 - 0.5M) at a flow rate of 15ml $\cdot$ h⁻¹.

2.8.2. Affinity chromatography

CHpA-Sepharose 6B affinity chromatography was performed as described by SILVANOVITCH and HILL (29) using 50mM-Na-acetate elution buffer, pH 5.5, 1 mM-CaCl₂, at a flow rate of 35 ml $\cdot$ h⁻¹. Full release of the enzymes from the affinity gel was obtained with 8 mg $\cdot$ ml⁻¹ CHpA in the elution buffer.

2.8.3. Hydroxylapatite chromatography

The bound protein top from the CHpA affinity column (A-2) was concentrated in an Amicon concentrator fitted with a PM 10 membrane and the buffer changed to 10 mM-phosphate buffer, pH 6.8. The solution was applied to a 2.5 x 30cm column of Biogel HTP equilibrated in the phosphate buffer. Elution was performed with a linear

gradient of phosphate buffer (10 - 150 mM) at a flow rate of 35 ml $\cdot$ h⁻¹.

2.8.4. Hydrophobic interaction chromatography

Unbound protein from the affinity column (A-1) was concentrated and the buffer exchanged to 10 mM-phosphate buffer, pH 6.8. The solution was 25% saturated with (NH₄)₂SO₄ at 4 °C, allowed to stand at least 1 hour at 4 °C and then centrifuged at 20,000 x g for 15 min. The supernatant was chromatographed on a 1.5 x 20 cm column of Octyl-Sepharose CL-4B as described by Pharmacia (16) using a decreasing linear gradient of (NH₄)₂SO₄ (25 - 0%) and an increasing linear gradient of ethyleneglycol (0 - 50%).

3. RESULTS AND DISCUSSION

3.1. Demonstration of two immunologically distinct forms of α -amylase

Crossed immunoelectrophoresis of total sorghum malt extracts versus anti-total sorghum malt antigens revealed three amylolytic peaks among a total of 15-20 protein peaks (Figures 2a and b). These enzymes are β -amylase and two α -amylases which have been named α -1 (most anodic) and α -2 amylase solely according to their mobility in the alkaline gel (pH 8.6). α -1 (major malt α -amylase) and β -amylase are readily visi-

Figure 2a. Total sorghum malt (5 day) antigens (15 µl, 10 mg·ml⁻¹ vs. 200 µl anti-total malt rabbit IgG fractionation.

Figure 2b. Amylogram of the gel shown in Figure 2a incubated and developed after (14).

Figure 3. Fused rocket immunoelectrophoretogram of DEAE-Sephadex column fractions vs. 250 µl anti-total malt rabbit IgG.

Note: β-amylase top is diffuse due to low titre in serum used.

ble in 1-dimensional gels but the activity of α-2 amylase is generally too low to be seen.

The same amylolytic enzyme pattern is seen in the elution profile of a glycogen precipitate chromatographed on DEAE-Sephadex (Figure 3).

Figure 4. Fused rocket immunoelectrophoretogram of CHpA-Sepharose affinity column fractions vs. 150 µl anti-total malt rabbit IgG.

The amylases elute from the ion-exchanger in order of their isoelectric points (see below).

α-1 and α-2 are separated from β-amylase by affinity chromatography on CHpA-Sepharose (Figure 4). It was not possible to differentially elute one of the bound α-amylases using gradients of NaCl or β-limit dextrin. Ouchterlony immunodiffusion and crossed immunoelectrophoresis showed that the α-amylases present in the unbound affinity top (A-1) are immunologically identical to the enzymes binding to the column. Subsequent runs with less enzyme concentration of approximately 1.1 mg protein·ml⁻¹ gel.

The two α-enzymes are separated by DEAE-Sephadex chromatography but chromatography

Figure 5. Fused rocket immunoelectrophoretogram of hydroxylapatite column fractions vs. 300 µl anti-total malt rabbit IgG.

Figure 6. IEF-PAGE (pH 4-6) of hydroxylapatite column fractions.

Lane 1, HA-1 (40 μ g, α -2). Lanes 2 and 3, HA-2 & 3 (10 μ g, α -1).

on hydroxylapatite separated α -2 from α -1 and resolved the α -1 component into two separate tops referred to as HA-2 and HA-3 (Figure 5). It should be noted that peaks HA-2 and HA-3 are immunologically identical and that the HA-2 peak furthermore appears to be a double peak. This pattern of separation was reproducible and was not due to overloading; chromatography of an identical preparation on a larger column produced the same separation. Although the mechanism of the interaction between hydroxylapatite and enzymes is unknown, $\text{Ca}_3(\text{PO}_4)_2$ may be particularly well suited for the separation of Ca^{+2} dependent enzymes such as α -amylase.

Isoelectric focusing (Figure 6) of the three peaks from the hydroxylapatite column revealed up to 3 bands (1 major, 2 minor) at pI 5.1 for α -2 amylase (HA-1) and always 3 closely spaced bands at pI 4.65 for α -1 amylase (HA-2&3). The outermost two bands of the α -2 group are electrophoretic artefacts; refocusing the major α -2 band (pI 5.1) results in the accumulation of broad bands of activity extending to the two outer bands. Refocusing the 3 bands of the α -1 group does not indicate artefact formation and their banding pattern is reproducible in IEF-PAGE gels containing 0.5% β -limit dextrin. That these

three bands may represent in vivo multiple molecular forms of α -amylase (α -1_a, α -1_b, α -1_c) is supported by differences in the isoelectric focusing pattern of α -1 amylase separated into fractions HA-2 and HA-3 (Figure 6).

It is concluded from these results that sorghum malt contains two immunologically distinct forms of α -amylase named α -1 (α -1_a, α -1_b, α -1_c) and α -2 amylase which are separable chromatographically and which have different isoelectric points. The chromatographic results are in agreement with BOTES et al. (4) who postulated the presence of 4 α -amylase isozymes in sorghum malt from an activity/absorption profile of purified α -amylase chromatographed on hydroxylapatite. In Table I it can be seen that α -2 accounts for up to 25% by weight but only for some 2% of the saccharifying α -amylase activity in the sorghum variety used in this study. Speculation on the in vivo significance of the three forms of α -1 amylase must await further study. The reader is referred to (11) for a discussion of isozyme classification.

Adequate separation of β -amylase from the α -amylases for mono-specific antibody production could not be achieved using ion-exchange columns. The differential precipitation of sorghum α -amylase from β -amylase reported by BOTES et al. (4) combined with the suspected hydrophobicity of the α -amylases seen in gel filtration experiments (section 3.2.1.) suggested the use of hydrophobic interaction chromatography for

Figure 7. Fused rocket immunoelectrophoretogram of Octyl-Sepharose column fractions vs. 125 μ l anti-total malt rabbit IgG.

Table I.

Purification and yields of sorghum α -amylases isolated by affinity and hydroxylapatite chromatography

	Phabedas total units	Total mg protein	Spec. Act. units/mg protein	% yield units	% yield protein
Crude extract	5.4×10^8	18×10^3	3.0×10^4	100.0	100.0
Glycogen ppt.	6.4×10^7	81.25	7.8×10^5	11.8	0.45
A-1 (unbound)	4.0×10^7	53.0	7.5×10^5	7.4	0.29
A-2 (bound)	2.3×10^7	26.5	8.8×10^5	4.2	0.15
HA-1 (α -2)	3.6×10^5	2.6	1.4×10^5	—	0.01
HA-2 (α -1)	3.0×10^7	10.3	2.9×10^6	5.5	0.06

separating β - from α -amylase. 25% $(\text{NH}_4)_2\text{SO}_4$ saturation of fraction HA-1 resulted in precipitation of 20-30% of the α -amylase activity and 2 of the non-amylolytic proteins present in the glycogen precipitate. The 25% $(\text{NH}_4)_2\text{SO}_4$ supernatant gave three protein peaks when chromatographed on Octyl-Sepharose (Figure 7). OS-1 is a non-amylolytic protein, OS-2 is β -amylase and OS-3 contains both α -1 and α -2 amylase. The retarded elution of the α -amylases indicates that they are relatively hydrophobic, a property which may partly explain the low amylolytic power of sorghum malt extracts compared with extracts of barley malt.

3.2. Physical and chemical characterization of amylases

Physical and chemical properties of α -1 and α -2 amylase are summarized in Table II.

3.2.1. Molecular weight determination

The molecular weights of β - and α -amylases were calculated from SDS-PAGE and by gel filtration. SDS-PAGE yielded values of 53,000 for β -amylase, 41,500 for α -1 and 41,500-42,700 for α -2 (Figure 8). α -2 amylase runs as a diffuse band on all gels examined, a peculiarity which may be due to its higher carbohydrate content

Figure 8. SDS-PAGE of total malt extracts and pooled column fractions. Lanes 1-4, 0,1,3,5 day malt extracts.

Lane 5, glycogen precipitate. Lane 6, CHpA-Sepharose fraction A-2 (bound). Lane 7, hydroxylapatite fraction HA-2 (10 μg α -1-amylase) and lane 8, fraction HA-1 (40 μg α -2-amylase). Lane 9, DEAE-Sephadex fraction DE-2 (15 μg partially purified β -amylase). Lane 10, molecular size standards (Daltons).

Table II.

Comparison of properties of sorghum α -amylases

	sorghum α -1	sorghum α -2
pH optima	5.0-6.5	4.5-6.5
K_m (10^{-4} g $\cdot$ starch $\cdot$ ml $^{-1}$)	3.6	0.7
V_{max} (μmole maltose $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$ enzyme at 25 $^{\circ}\text{C}$)	1460	0.01
Q_{10} (20-30 $^{\circ}\text{C}$)	2.2	1.3
Q_{10} (30-40 $^{\circ}\text{C}$)	1.6	1.2
K_m (10^{-4} g blue starch $\cdot$ ml $^{-1}$)	68.6	4.25
K_i (10^{-4} g CHpA $\cdot$ ml $^{-1}$)	5 ¹⁾	4.3-6.7 ²⁾
1 $^{\circ}$ soluble starch hydrolysis products molecular weight (Daltons)	G_2, G_3, G_1 ³⁾	DP 6-8 ⁴⁾ , G_2
% (w/w) carbohydrate	0.7	5.7
P_i	4.65	5.1

1) competitive 2) non-competitive 3) G = glucose residue 4) DP = degree of polymerization

(section 3.2.2). Although the band at 20,000 in lane 8 was not reproducible, it should be noted that RODAWAY (26) described the co-purification with barley α -2 amylase of a dimer of 43,000 (monomer weight = 21,500) called band-2 protein. Given the low activity of α -2 amylase, the possibility cannot be excluded that α -2 preparations contain a co-purified α -amylase inhibitor with a subunit weight of approximately 20,000.

The electrophoretic results are in agreement with molecular weights obtained by molecular exclusion chromatography. Gel filtration on Sephadex G75 superfine in 50 mM-Na-acetate, pH 6.5, and on Biogel P-100 in 0.2M-TRIS-HCl, pH 7.2 gave a molecular weight of approximately 52,500 for β -amylase. In contrast, α -1 and α -2 were retarded on these columns, both eluting as two broad peaks at 30,000 and 8,000 from the Sephadex gel. At higher ionic strength (0.2M-Na-acetate, 0.3M-NaCl) the two α -amylases elute from the Sephadex gel at 36,000-38,000. Biogel P-100 chromatography in 0.2M-TRIS and 0.4 M-NaCl showed a major top at 39,000-42,000 and a minor top at 50,000 (Figure 9). Although BOTES et al. (4) obtained a weight of approx-

imately 48,000 for sorghum α -amylase in ultracentrifuge studies, values between 40,000 and 45,000 are most commonly reported in recent investigations of cereal α -amylases (26, 30, 31.). The higher value of 48,000 may be due either to contamination of the α -amylase preparation with β -amylase or to a slight underestimation of the partial specific volume of α -amylase employed in the ultracentrifuge determination. To insure that the lower molecular weight obtained in the present study was not due to degradation of α -amylase by proteases during extraction, total malt extracts were made with buffers containing EDTA, and the serine proteinase inhibitors phenyl-methyl-sulfonyl chloride and *n*- α -p tosyl-L-lysine. SDS-PAGE of these extracts and of their glycogen precipitates produced patterns identical to those of normal Na-acetate extracts.

3.2.2. Amino acid analysis and carbohydrate content

Results of the amino acid analysis for α -1 and α -2 are given in Table III along with values extrapolated from BOTES et al. (4) for sorghum malt α -amylase and for wheat and barley α -amylase (26, 31). It is seen that α -1 and α -2 from sorghum have similar compositions except for differences in Ser, Val, Ile, and Cys. The comparative composition data for sorghum, barley and wheat, considered together with the partial immunological identity between the sorghum α -1 and barley α -2 amylases (section 3.3), indicates that there is extensive amino acid homology between the germination α -amylases of sorghum, barley and wheat. The major difference between the sorghum and barley α -amylases is a higher number of Ile and Leu and a lower number of His, Lys, and Arg residues in the former. This explains the lower isoelectric point found for sorghum α -amylase (pI 4.65 for α -1) versus that for barley α -2 amylase and malted wheat α -amylase (pI 6.1, see 19, 31).

Using the phenol- H_2SO_4 reagent of DUBOIS et al. (9), α -1 and α -2 showed 0.7% and 5.7% (w/w) carbohydrate respectively.

3.2.3. Kinetics and action patterns of α -amylases

The kinetic properties of the α -amylases are summarized in Table II. The values for α -1 are similar to those reported from other cereals (30).

Figure 9. Fused rocket immunoelectrophoretogram of Biogel P-100 column fractions vs. 250 μ l anti-total malt IgG.

Material used in this run is combined DEAE-Sephadex fractions DE-2 & 3. Low α -2 content is due to incomplete separation of α -2 from β -amylase in ion-exchange chromatography.

The much lower K_m and V_{max} values for α -2 compared with α -1 are in keeping with the much lower specific activity of α -2 preparations and with the differences in the action patterns of the two enzymes on soluble starch.

Lineweaver-Burke plots showed that α -1 is competitively inhibited by CHpA at concentrations of 1 and 4 $\text{mg} \cdot \text{ml}^{-1}$ with a K_i of $0.05 \cdot 10^{-4} \text{ g} \cdot \text{ml}^{-1}$. Higher concentrations of CHpA result in a loss of activity without a loss in specific activity which is presumably due to precipitation of the enzyme. α -2 is also inhibited by CHpA but the pattern of inhibition approximates that of a noncompetitive inhibitor with a K_i of $0.043\text{--}0.070 \times 10^{-4} \text{ g} \cdot \text{ml}^{-1}$.

These results for α -1 are in agreement with a

recent study of CHpA inhibition of Triticale α -amylase (32) which reported from equilibrium dialysis experiments that the CHpA-enzyme interaction must occur at a site other than the active site. The same study suggested that α -amylase can accommodate a number of CHpA molecules and that this may cause aggregation and precipitation as is seen with glycogen complexes (18). It is well documented (3) that cyclodextrins form inclusion complexes with hydrophobic molecules in aqueous solution. The partially hydrophobic character of sorghum α -amylase seen in the present study suggests that part of the inhibitory action of CHpA may be due to hydrophobic interactions with the enzyme.

The action pattern of the α -amylases on soluble starch is shown in Figure 10. The primary hydrolysis products of α -1 amylase are maltose, maltotriose and glucose while that of α -2 are oligosaccharides of DP 6-8 and some maltose and maltotriose. In the incubations shown, the amount of enzyme is very high and 10 x as much α -2 by weight is used than α -1. In similar experiments with equal numbers of Phadebas units, the same differences are seen, i.e. α -2 produces quan-

Table III

Amino acid compositions of sorghum, barley, & wheat α -amylases (number of amino acid residues per protein molecule)

	sorghum α -1	sorghum α -2	sorghum ¹⁾ (4)	barley (26)	wheat ²⁾ (26,31)
Asp	50.0	47.3	54.2	49.1	41.5
Thr	15.2	13.8	16.5	16.0	20.4
Ser	13.9	20.3	15.4	13.9	20.4
Glu	33.1	30.4	30.5	26.5	41.0
Pro	18.2	17.5	18.4	19.6	26.1
Met	5.2	5.3	4.2	6.5	6.2
Gly	46.1	45.0	48.7	46.2	42.0
Ala	32.3	35.0	36.9	33.2	33.0
Val	21.6	26.9	21.6	22.3	24.8
Ile	21.9	15.4	23.6	17.2	19.9
Leu	28.7	26.5	27.7	25.3	27.4
Tyr	15.1	16.1	14.6	14.3	12.7
Phe	16.5	14.2	16.7	16.8	14.3
His	14.2	14.7	14.4	16.2	12.8
Lys	17.3	17.9	16.1	22.2	18.8
Arg	13.6	12.4	14.7	16.7	17.3
$\frac{1}{2}$ Cys	3.7 ³⁾	5.6 ³⁾	3.4 ⁴⁾	- ⁵⁾	4.4 ³⁾
Trp	9.6 ⁶⁾	11.3 ⁶⁾	-	-	11.0
Total	376.2	375.6	377.6	362.0	391.8

¹⁾ determined from % w/w values adjusted for α -amylase of 41,500 molecular weight, Trp content = 4.1% from this study ²⁾ molecular weight given as 43,300 ³⁾ as cysteic acid after (14) ⁴⁾ as $\frac{1}{2}$ Cys CySH ⁵⁾ not determined ⁶⁾ from spectrophotometric determination of Trp/Tyr ratio after (2)

Figure 10. Thin layer chromatogram showing the products of the hydrolysis of soluble starch by sorghum α -amylases and β -amylase.

Lanes 1-3, 10 μg G_1 , G_2 , G_3 . Lanes 4-6, 2,10,45 min. incubation of 20 μg 1% soluble starch with 25 μg α -2-amylase (fraction HA-1). Lane 7, 10 μg starch. Lanes 8-10, 2,10,45 min. incubation of 20 μg starch with 2.5 μg α -1-amylase (fraction HA-2). Lane 11, 20 μg starch. Lanes 12-14, 2,10,45 min. incubation of 20 μg starch with 25 μg β -amylase (fraction OS-2). Lanes 15-17, 20 μg G_3 , G_2 , G_1 .

titatively more of the DP 6-8 oligosaccharides than α -1. Similar incubations with pullulan as substrate showed that neither of the sorghum α -amylases could hydrolyze $\alpha(1\rightarrow6)$ glucosidic linkages. The production of high concentrations of DP 6-8 oligosaccharides previously reported as a peculiarity of sorghum α -amylase (4) is therefore attributable to the α -2 form of the enzyme. During prolonged incubations (up to 48 hours) with high concentrations of α -2 or with equal mixtures of α -1 and α -2, the concentration of the DP 6-8 oligosaccharides decreases until only G_1 , G_2 , and G_3 remain. It is not known whether the DP 6-8 oligosaccharides are inhibitory to the action of sorghum α -amylase.

These physico-chemical data suggest that α -1 and α -2 are either post-translationally modified forms of the same polypeptide or that they are isozymes coded for by different genes. The possibility that α -2 preparations contain a co-purified protein of approximately 42,000 daltons (subunit weight approximately 20,000) is being investigated by chromatography and electrophoresis under strongly denaturing conditions.

3.3. Immunological comparison of sorghum and barley germination amylases

Crossed immunoelectrophoresis of sorghum malt extracts showed that α -1 is present in trace amounts in ungerminated sorghum and that both α -1 and α -2 amylase increase during malting. Ouchterlony immunodiffusion of the enzymes against monospecific anti- α -1 and anti- α -2 amylase showed that there is partial identity between α -1 and α -2 and confirmed that α -2 precipitins are amylolytic.

Immunodiffusion and crossed immuno-

electrophoresis of sorghum α -1 amylase against anti-total barley malt immunogens and monospecific anti-barley α -2 amylase showed immunological identity between these two major malt enzymes (Figure 11). No similar reaction was seen for sorghum α -2 amylase. It is concluded that barley α -2 amylase displays extensive structural homology with sorghum α -1 amylase, while the sorghum α -2 form of the enzyme does not appear to have an immunological counterpart in barley malt.

4. CONCLUDING REMARKS

This communication presents evidence to show that barley α -2 and sorghum α -1 malt amylases have similar molecular weights, amino acid compositions, kinetic properties and immunological determinants. In addition to the major α -1 amylase, sorghum malt contains an immunologically distinct form of α -amylase called α -2 amylase. Preliminary kinetic analysis indicates that the very low saccharifying activity of α -2 amylase is due to a different substrate specificity than that of the major α -1 form of the enzyme.

ACKNOWLEDGEMENTS

I wish to express my gratitude to Fil. Dr. LARS MUNCK and Dr. GREGORY GIBBONS who greatly influenced this work through guidance and discussion. I especially thank Dr. IB JONASSEN and Professor BENT FOLTMANN for their suggestions during the isolation experiments. The work of Civ. Ing. LARS HALLGREN and the technical assistance of BODIL STILLING, and BODIL CORNELIUSSEN with the amino acid analyses and of ANNETTE HANSEN for preparing the figures is gratefully acknowledged.

Figure 11. Ouchterlony double diffusion of sorghum α -1 and α -2 amylases against monospecific anti-barley α -2, anti-sorghum α -1, and anti-sorghum α -2 amylases. 15 μ l of each antiserum vs. 6 μ g antigen.

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