Partial Purificaton and Properties of β-Amylase Isolated from *Sorghum bicolor* (L.) Moench

Ekong U. Okon and Anthony O. Uwaifo*

β-Amylase was extracted from *Sorghum bicolor* (L.) Moench variety L. 187 and purified by ammonium sulfate fractionation followed by gel filtration on Sephadex G-200. The yield of β-amylase activity in the final purification was 54% of the original activity and specific activity was increased approximately 5-fold. Three components were separated with molecular weights of 20 000, 40 000, and 80 000 based on the selectivity curve for the Sephadex column determined with standard proteins. All components were enzymically active. The enzyme had a pH optimum of 5.0–5.5 and an energy of activation of 57.48 J mol^{-1} between 10 and 40 °C. The calculated V_{max} and K_m values for the enzyme were 7.466×10^{-3} enzyme units and 1.272 g of starch/L, respectively. The results are compared with those reported for other cereals like barley and wheat that are of industrial importance.

β-Amylase (EC 3.2.1.2) is widely distributed in the plant kingdom. Animals are lacking in this enzyme. It hydrolyzes 1,4-α-glucan links in polysaccharides so as to remove successive maltose units from the nonreducing ends of the chains. 1,6-α-Glucan links that occur in branched polysaccharides like amylopectin are not hydrolyzed and act as barriers to the enzyme action. The smallest straight chain polysaccharide molecule readily attacked by β-amylase is maltotetraose. The richest sources of this enzyme, apart from sweet potato, are the cereals, especially wheat and barley. Malting of these cereals develops and activates this enzyme in the grains. Barley malt has a unique use in the brewing industry partly because of its high content of β-amylase. During the mashing stage in the process of beer production, the enzyme acting in concert with other endogenous enzymes brings about the conversion of the mash into sweet wort of which maltose constitutes about 45% of the total carbohydrate content (Harris, 1962). Thus, the study of the occurrence and properties of the β-amylases in cereals is of particular interest and importance to the brewing industry.

Studies on the purification of β-amylases from cereals are relatively few. Wheat and barley β-amylases have been isolated and chracterized by several workers (Tipples and Tkachuk, 1965; Meyer et al., 1953; Danielson and Sandegren, 1947; Danielson, 1948; Tkachuk and Tipples, 1966). The literature on sorghum β-amylase content and its properties is particularly scanty. In fact, it was a commonly accepted view that sorghum malt contained little or no β-amylase activity, and consequently, it was considered unsuitable as a raw material in the classical beer brewing industry. However, contrary to this view, Novellie (1960) reported the isolation of β-amylase from kaffircorn (*Sorghum caffrorum*) malts. No such work has been reported on Nigerian local varieties of this cereal. This work is an attempt to fill that gap.

MATERIALS AND METHODS

Source of Sorghum Grains. *Sorghum bicolor* (L.) Moench variety L. 187 grains were obtained from the Seed Production Unit of the Institute of Agricultural Research, Zaria, Nigeria. The grains were cleaned and found to have satisfactory germinative energy.

Preparation of Malt. Experimental malts were produced from the grains by a modified method described by Meredith et al. (1962). The 24-h-steeped grains were germinated on moistened filter paper in sterile covered Petri dishes at ambient temperature (25–27 °C) for 5 days. The sprouted grains were dried in a forced draught oven at 50 °C for 24 h. The dried polished grains were then ground in a rotating knife mill to pass a 60-mesh screen.

Extraction and Purification Procedures. The procedures followed for the extraction and initial purification of the β-amylase from sorghum are set out in Figure 1. Extraction was by a modified method of Novellie (1960).

Stage IV. A 2-mL aliquot of the dialysate from supernatant III in Figure 1 was carefully loaded manually on the gel filtration column prepared as described below. About 3-mL fractions were collected at 10-min time intervals. The absorbancies of the fractions were taken at 280 and 260 nm with Beckman DU-8 spectrophotometer and plotted against the fraction numbers to obtain an elution profile.

Determination of Protein. Two methods were employed in protein determination. These were the Lowry method (Lowry et al., 1951) and the Warbung–Christian method (Layne, 1957; Copper, 1977). The former was employed for the determination of protein in the fractions produced during the initial stages of purification, while the latter was employed to estimate protein in the effluent fractions from the Sephadex column.

Assay of β-Amylase Activity. β-Amylase activity was measured by a colorimetric method using 3,5-dinitrosalicylic acid reagent according to the method of Noelting and Bernfeld (1948) as modified by Tipples and Tkachuk (1965). One amylase unit was defined as the amount of the enzyme in 1 mL of the enzyme solution that released 1 mg of reducing sugar (maltose) from a 1% starch solution in 1 h at 30 °C and at a pH 5.3. In all the experiments, values were determined in triplicates.

Assay of α-Amylase Activity. This test was included to estimate the contamination of purified β-amylase fractions by α-amylase. The method of Tipples and Tkachuk (1965) was used in the determination.

Determination of pH–Activity Profile. The reaction progress curves for pH–enzyme activity were determined by incubating the enzyme with 1% buffered starch solution at 30 °C and assaying the enzyme activity as described above.

Determination of Energy of Activation. This was determined by measuring the maximum enzyme activity at different temperatures and plotting log V against $1/T$ (Arrhenius plot). The energy of activation (E) was determined from the slope.

Determination of Kinetic Constant. The reaction progress curves for substrate concentration–enzyme ac-

Department of Biochemistry, Microbiology and Oncology Section, College of Medicine, University of Ibadan, Ibadan, Nigeria.

0021-8561/84/1432-0011$01.50/0

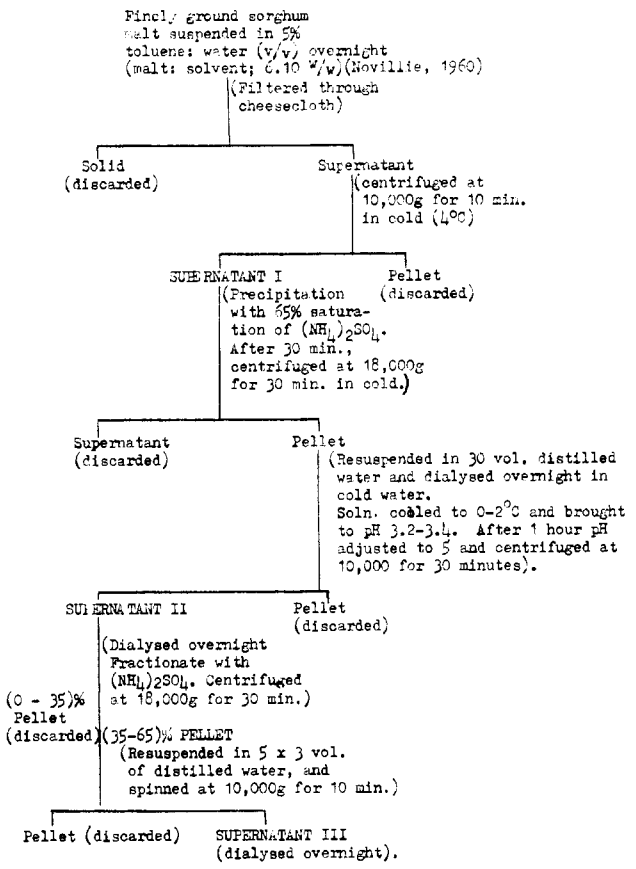

Figure 1. Schematic flow chart describing the major steps in the partial purification of β-amylases from sorghum L. 187.

Table I. $(NH_4)_2SO_4$ Fractionation of Sorghum β-Amylase

$(NH_4)_2SO_4$, %	activity precipitated, units	protein, mg	specific activity, units/mg
0-25	60	9.50	6
25-35	70	11.25	9
35-45[a]	190	13.75	13
45-55[a]	350	17.50	20
55-65[a]	430	19.00	23

[a] Fractions pooled.

tivity were prepared as described by Plummer (1979). From these the activity profiles were obtained. K_m and V_{max} were subsequently determined from the Lineweaver–Burk plot. All experiments were performed in triplicates.

Preparation of Gel Filtration Column. The column was prepared with Sephadex G-200 (Pharmacia) in a 50 × 2.5 cm glass column. Procedures for hydrating the gel and packing the column and determination of void volume were done as described by Cooper (1977) and Rendina (1971). The bed dimension was 35 × 25 cm and it was fitted with flow adapters.

Determination of Molecular Weight. First, the elution volumes of protein standards of known molecular

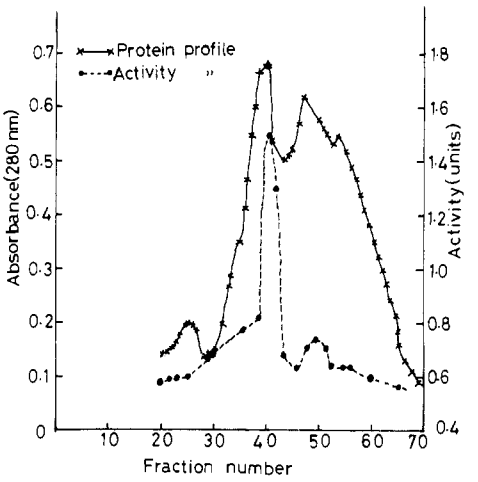

Figure 2. Elution profiles of sorghum β-amylase on Sephadex G-200 (2.5 × 33 cm column). Elution buffer: NaCl solution (0.1 M) buffered with phosphate buffer (0.05 M), pH 7.1. Void volume (V_0) was determined with Blue Dextran 2000 (M_r 2 × 10); V_0 = 18.5; fractions = 50 mL.

weights, namely, cytochrome *c*, Horse heart (Sigma), catalase, bovine liver (Sigma), serum albumin, bovine (BDH Chemicals), and hemoglobin, bovine, were determined on the Sephadex G-200 column from their elution profiles according to the method described by Cooper (1977). A selectivity curve was then prepared by plotting their partition coefficients, K_{av}, against the logarithm of their molecular weights. The partition coefficient of the purified β-amylase was calculated from the previously determined elution volume and interpolated on the curve to obtain its molecular weight.

RESULTS AND DISCUSSION

Typical protein and activity data for the various stages of purification of sorghum β-amylase are given in Tables I and II. The bulk of the enzyme activity was precipitated at 35–65% of the ammonium sulphate saturation as shown in Table I. The specific activity was increased approximately 4-fold in the initial purification (stage III) while it was increased 5-fold in the final purification, stage IV. Also, the yield of β-amylase activity in the final purification was 54% of the original activity. No α-amylase activity was detected in the purified extracts.

Three major peaks were discernible from the gel filtration elution profile shown in Figure 2, but maximum activity of the enzyme was restricted to the first peak (A). The method of gel filtration has been used successfully by many workers for the purification of proteins (Curling, 1970; Andrews, 1974; Ackers, 1975). It has the advantage of being gentle in operation, thus preventing the destruction of labile proteins, and having high resolution and reproducibility. Novellie (1960) purified sorghum β-amylase using the calcium phosphate adsorption technique after ammonium sulfate and alcohol fractionation and got a yield of only 3–4% of the original activity as against 54% we obtained. Thus, the purification method by the gel

Table II. Purification Data of Sorghum β-Amylase

stage/operation	total volume, mL	total protein, mg	total enzyme activity, units	yield, %	enzyme specific activity, units/mg
stage I/salt extraction	75	272.00	1445	100.0	5.3
stage II/acid treatment	47	82.27	1314	90.9	15.9
stage III/$(NH_4)_2SO_4$ fractionation[a]	15	50.25	970	67.1	19.3
stage IV/gel filtration	15	33.70	882	54.1	26.2

[a] Pooled fractions from Table I.

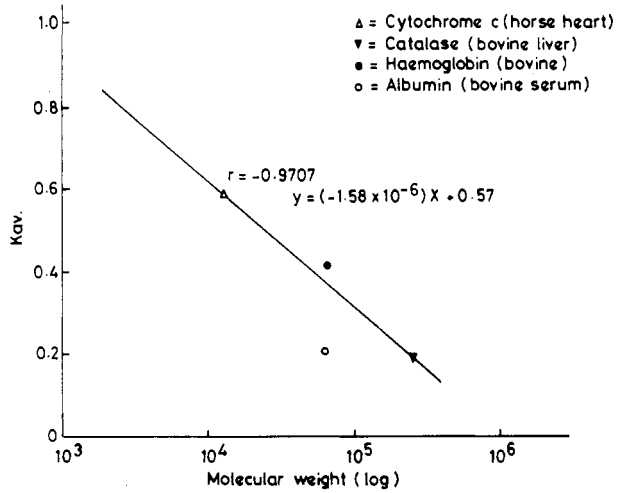

Figure 3. Selectivity curve for the determination of the molecular weight of sorghum β-amylase (Sephadex G-200).

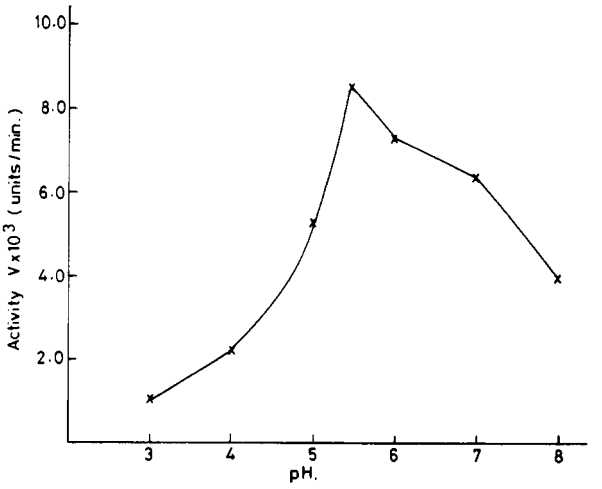

Figure 4. pH-dependent activity profile for sorghum β-amylase.

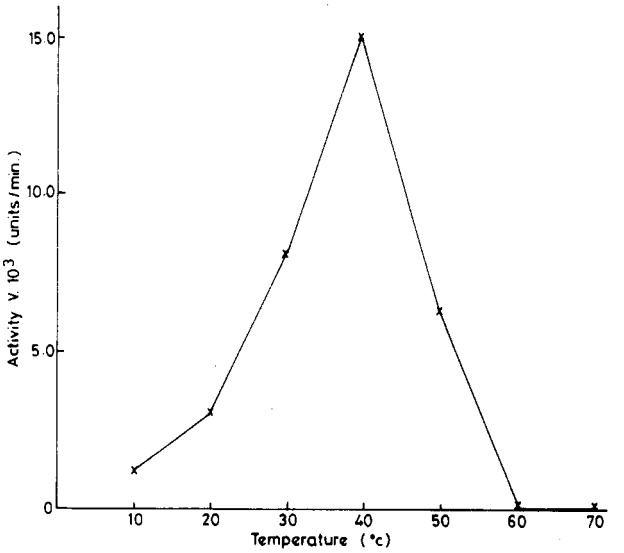

Figure 5. Temperature-dependent activity profile of sorghum β-amylase.

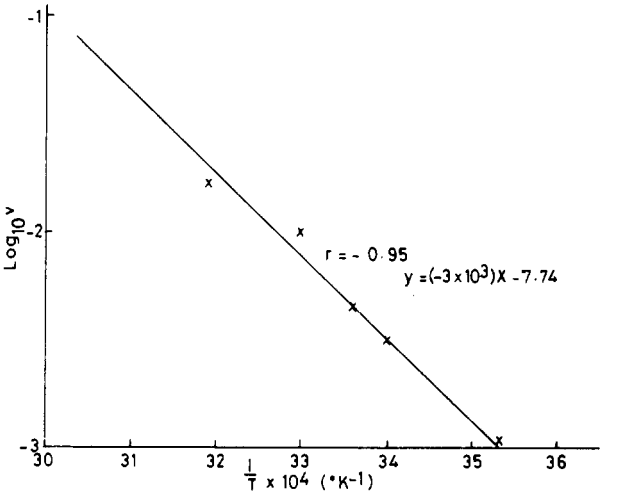

Figure 6. Arrhenius plot for the determination of the energy of activation of sorghum β-amylase.

filtration technique on Sephadex is a more successful method for sorghum β-amylase.

Figure 3 shows the selectivity curve for the protein standards by using Sephadex G-200 for the gel filtration column. From the elution profile for sorghum β-amylase (Figure 2), estimates of three partition coefficients were obtained (0.47, 0.42, and 0.37) that gave molecular weights of 20 000, 40 000 and 80 000, respectively, on the selectivity curve. Molecular weights of this distribution suggest that the 20 000 monomer may be present as dimer and tetramer aggregates in the eultion buffer. However, it is apparent from the elution activity profile (Figure 2) that the M_r 20 000 monomer is probably the functinal unit. Harris (1962) has reported the heterogeneous nature of β-amylase isolated from barley. Examining a highly purified β-amylase from barley malt flour, Ayrapaa and Nihlen (1954) observed that the crystalline enzyme was a mixture of the true enzyme and some closely related and similar albumins that possessed identical physical properties. Cooper and Pollock (1957) similarly observed more than one enzymically active component in purified β-amylase from barley malt. Each of these components was separable from each of the others when mixtures were prepared and submitted to electrophoresis. Moreover, they were distinguishable by ultracentrifugal analysis, which revealed that certain of the fractions were still heterogeneous. It seems, therefore, that the electrophoretically separable zone represents genuine enzymic components of the original mixture. Tkachuk and Tipples (1966) reported the existence of three major and two minor components of wheat β-amylase. All these observations are consistent with our findings on sorghum β-amylase. However, whether the heterogeneity of sorghum β-amylase is due to the synthesis by the cereal grain of various active proteins or is due to the change in the original single component by proteolysis or partial oxidation remains to be investigated. In contrast to the β-amylases of barley malt, the crystalline enzyme from sweet potato was found by Englard and Singer (1950) to be a homogeneous protein of molecular weight 150 000 with an isoelectric point of 4.74–4.79.

The results of this study show that sorghum β-amylase obeys the Michaelis–Menten kinetic laws. The pH range of optimum activity was between 5.0 and 5.5 (Figure 4). Also, the temperature of maximum activity was between 30 and 40 °C (Figure 5). The energy of activation, E, obtained from the Arrhenius plot (Figure 6) was 57.48 J mol^{-1} between 10 and 40 °C. This was close to those of wheat and barley malt β-amylases except that these latter amylases show a break in the graph in the vicinity of 20 °C (Meyer et al., 1953), while that of sorghum β-amylase gave a continuous straight line between 10 and 40 °C.

Also, the V_{max} and K_m values obtained for the sorghum β-amylase were fairly high compared with those obtained by Novellie (1960) for kaffircorn malt. These were 7.466 × 10^{-3} units and 1.272 g of starch/L, respectively, (Figure

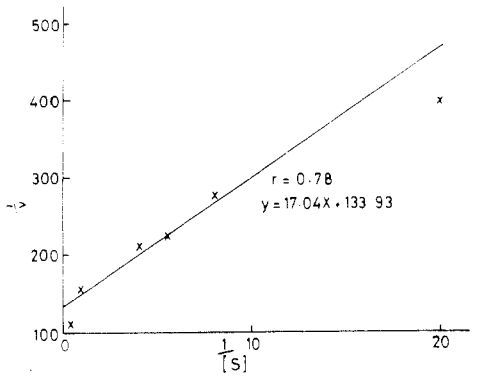

Figure 7. Lineweaver–Burk plot of the activity of sorghum β-amylase.

7). This relatively less affinity of our purified enzyme for the substrate may be an inherent difference in the β-amylases isolated from the two strains of sorghum, *Sorghum bicolor* (Linn) (*S. caffrorum* and *S. bicolor*). These values also compare favorably with those reported for wheat and barley malts (Meyer et al., 1953; Tkachuk and Tipples, 1966).

Registry No. β-Amylase, 9000-91-3; starch, 9005-25-8.

LITERATURE CITED

Ackers, G. K. *Proteins (3rd Ed.)* **1975**, *1* 2.
Andrews, P. *Biochem. J.* **1974**, *91*, 222.
Ayrapaa, T.; Nihlen, H. *Acta Chem. Scand.* **1954**, *8*, 88.
Cooper, A. H.; Pollock, J. R. A. *J. Inst. Brew.* **1957**, *63*, 24.
Cooper, T. G. In "The tools of Biochemistry"; Wiley: New York, 1977; p 399.
Curling, J. M. In "Experiments in Physiology and Biochemistry"; Kerkut, G. A., Ed.; Academic Press: London and New York, 1970; Vol. 3, p 417.
Danielsson, C. E. *Nature (London)* **1948**, *162*, 525.
Danielsson, C. E.; Sandegren, F. *Acta Chem. Scand.* **1947**, *1*, 917.
England, S.; Singer, T. P. *J. Biol. Chem.* **1950**, *187*, 213.
Harris, G. In "Barley and Malt"; Cook, A. H., Ed.; Academic Press: London and New York, 1968; p 624.
Layne, E. *Methods Enzymol.* **1957**, *3*, 447.
Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
Meredith, W. O. S.; Anderson, J. A.; Hudson, L. E. In "Barley and Malt"; Cook, A. H., Ed.; Academic Press: London and New York, 1962; p 247.
Meyer, K. H.; Spahr, P. F.; Fischer, E. H. *Helv. Chim. Acta* **1953**, *36*, 1924.
Noelting, G.; Bernfeld, P. *Helv. Chim. Acta* **1948**, *31*, 286.
Novellie, L. *J. Sci. Food Agric.* **1960**, *11* (8), 457.
Plummer, D. T. In "An introduction to practical biochemistry"; Tata McGraw-Hill: New Delhi, 1979.
Rendina, G. In "Experimental Methods in Modern Biochemistry"; Saunders Co.: Philadelphia, London, and Toronto, 1971; p 184.
Tipples, R. H.; Tkachuk, R. *Cereal Chem.* **1965**, *42* (2), 111.
Tkachuk, R.; Tipples, R. H. *Cereal Chem.* **1966**, *43* (1), 62.

Received for review April 25, 1983. Accepted August 19, 1983.

Enzymatic Determination of Urea and Ammonia in Refrigerated Seafood Products

Wai L. Cheuk and Gunnar Finne*

An enzymatic method for the determination of both urea and ammonia in fresh seafoods was investigated. The method involves the transamination of α-ketoglutarate in the presence of glutamate dehydrogenase and NADH. The consumption of NADH as measured at 340 nm is stoichiometrically equivalent to the amount of ammonia in the test sample. Urea is determined by the same reaction scheme after the addition of urease. When this enzymatic method was used to determine the quality of refrigerated shrimp and crab meat, both ammonia and urea were shown to increase during storage and there was also good correlation between the concentration of both these compounds and traditional spoilage indicators.

Quality changes that occur in fresh seafood products during refrigerated or iced storage are the combined results of both microbiological and tissue enzyme activities. Both give rise to a number of different volatile compounds of which ammonia can be most detrimental. Due to the accumulation during storage, the ammonia concentration has been suggested as an objective index of fresh seafood quality. Ward et al. (1979), using an ammonia-specific electrode, demonstrated the relationship between storage time, total microbial numbers, and ammonia concentration during refrigerated storage of fresh shrimp. Ammonia has also been suggested as an index of quality for crab meat (Burnett, 1965; Steinbrecher, 1973), dogfish, and thornback ray (Vyncke, 1978). Post-mortem ammonia production is especially a problem in elasmobranch species (sharks, dogfish, rays, and skates). These fishes contain high levels of urea both in blood and in tissue, and during iced or refrigerated storage urease-positive microorganisms will rapidly convert urea to ammonia. Both France and Belgium, where dogfish is an important food fish, have stringent quality standards based on the ammonia content of this species.

A number of quantitative methods for the determination of ammonia in seafood products have been developed. Most of these methods rely on microdiffusion, distillation, and/or ion exchange. After being liberated from the tissue by strong alkali, ammonia is trapped in weak acids and measured titrimetrically (Conway and Cooke, 1939) or photometrically by either Nessler's reagent (Seligson and Hirahara, 1957) or the Berthelot reaction (Chaney and Marbach, 1962).

When researching the kinetics of conversion of urea to ammonia in sharks held on ice, we adapted an enzymatic method for the simultaneous determination of urea and ammonia. The method is based on the enzymatic deter-

Animal Science Department, Seafood Technology Section, Texas A&M University, College Station, Texas 77843.

0021-8561/84/1432-0014$01.50/0