AN ABSTRACT OF THE THESIS OF

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Title: THE PURIFICATION AND CHARACTERIZATION OF THE FRUCTOSE-1, 6-DIPHOSPHATE ALDOLASE FROM BACILLUS STEAROTHERMOPHILUS (NCA 2184)

Abstract approved: Redacted for privacy
Robert R. Becker

The thermophilic aldolase from Bacillus stearothermophilus (NCA 2184) has been purified to a specific activity of 45.5 μ-moles of fructose-1, 6-diphosphate cleaved per minute per mg of enzyme, representing a 624-fold increase over the crude extract. The preparation appeared to be homogenous by sedimentation velocity ultracentrifugation and by a specific constant activity across the protein peak on Sephadex G-200. However, a trace amount of triose-phosphate isomerase was detectable.

The purified enzyme was determined to be a Type II aldolase on the basis of its metal ion (Mn^{++}) and sulfhydryl requirements, its narrow pH optimum, and its resistance to carboxypeptidase-A treatment and sodium borohydride reduction in the presence of substrate. The K_m (1.1×10^{-4} M) and V_{max} (4650 moles of substrate

cleaved per minute per mole of enzyme) were found to be similar to other Type II aldolases. The molecular weight was found to be about 58,000 by sedimentation equilibrium ultracentrifugation and the uncorrected S_{20} value was found to be 4.25.

Values for the Q_{10} and Arrhenius activation energy were found to be 2.2 and about 16,000 calories per mole, respectively.

The amino acid composition of the aldolase was found to be similar to those reported for other thermophilic proteins, although there are distinct differences. Comparison of this aldolase's hydrophobicity with that of a relatively heat-labile yeast aldolase indicates that the content of hydrophobic amino acids cannot be correlated to the relative thermostability of this enzyme. This suggests that the hydrophobicity parameter is not an adequate explanation for the thermostability of thermophilic proteins.

The Purification and Characterization of the Fructose-1, 6-Diphosphate Aldolase from Bacillus stearothermophilus (NCA 2184)

by

Richard Paul Quinn

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Dean of Graduate School

Date thesis is presented Aug. 30, 1968

Typed by J. Lewis for Richard Paul Quinn

TO MY WIFE:

FOR HER SUPPORT

FOR HER ENCOURAGEMENT

FOR HER TAKING MY PLACE AS A FATHER ON THOSE INNUMERABLE OCCASIONS WHEN I WAS DEPRIVED OF THIS JOY

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THE PURIFICATION AND CHARACTERIZATION OF THE FRUCTOSE-1, 6-DIPHOSPHATE ALDOLASE FROM BACILLUS STEAROTHERMOPHILUS (NCA 2184)

I. INTRODUCTION

The study of the fructose-1, 6-diphosphate aldolase (commonly referred to as aldolase or FDP-aldolase) from Bacillus stearothermophilus has been undertaken with the aim of answering two questions. First, is this enzyme more like those aldolases found in plants and mammals, or more like those aldolases found in microorganisms? Secondly, what, if any, special molecular properties does this protein have which would help to account for the enzyme's thermal stability?

The Presence of Two Types of Aldolases

Rutter (45) has classified aldolases into two distinct categories. He has defined those aldolases which are found normally in plants and the higher animals (including protozoa and some of the algae) as being Type I. Those aldolases which have been found in yeast, bacteria, and the lower animals (including the fungi and the blue-green algae) have been classified as Type II. Some of the properties which are used to distinguish between the two types are: molecular weight, metal ion requirement, sulfhydryl requirement for enzymatic activity, pH optimum range, K_m, and so forth. These differences will be reviewed at greater length in the discussion section of this thesis.

Thompson and Thompson (53) classified the thermophilic aldolase from B. stearothermophilus as Type I on the basis of the inability of added metal ions to stimulate the activity. The inability of several chelating agents to inhibit the activity of this enzyme substantiated this lack of a metal ion requirement. Since the source of this enzyme would suggest that it should be Type II (like those aldolases from yeast and bacteria), it would be interesting to ascertain whether the other characteristic properties of the thermophilic aldolase are also typical of the Type I enzyme. It should be noted that these authors classified the thermophilic aldolase on the basis of the metal ion requirement only, although they reported the ineffectiveness of carboxypeptidase-A treatment to diminish the activity of the enzyme and the inability of the enzyme to use fructose-1-phosphate (F1P) as a substrate (behavior typical of Type II aldolases). Furthermore, the very low S_{20} value reported by Thompson and Thompson for this thermophilic enzyme suggests that this aldolase is significantly smaller than any of the Type I or Type II enzymes which have been studied.

The Molecular Basis of Thermophily

It has been known for at least two centuries that there are certain microorganisms which are able to both survive and grow at very high temperatures (1, 10, 24). The basis of a thermophilic

organism's ability to survive at high temperatures was suggested by Koffler (29, p. 249) to depend upon one or more of the following:

1. The relative heat stability of ordinarily heat-labile molecules,
2. the stabilization of such constituents by protective materials, or
3. the rapid regeneration of heat-damaged molecules.

It has been adequately demonstrated that the activities of certain enzymes from thermophilic organisms are much more heat-stable than the corresponding enzymes from mesophilic organisms (1, 12, 35). This seems to rule out the third possibility, but does not allow one to distinguish between the first two.

Koffler and Gale (29) have shown that cytoplasmic proteins from several thermophilic bacilli strains possess distinct heat stabilities and that this stability is much greater than that shown by the cytoplasmic proteins of several strains of mesophilic bacteria. This work has shown that most, if not all, of the cytoplasmic proteins of these thermophilic bacilli are heat-stable and that this heat stability is a distinctive property of the proteins from each species. Koffler, using the flagella from different strains of thermophilic bacteria (28, 30), has provided further data to support this conclusion.

In order to differentiate between the first and second possibilities for explaining protein thermostability, Campbell (12)

crystallized the α-amylase from the facultative thermophile, Bacillus coagulans. He suggested that previously highly purified thermophilic enzymes may still have had small amounts of protective material associated with them, but that the crystallization procedure which he used should have removed any last traces of protective materials. He concluded that this α-amylase's thermal stability was a characteristic of the protein itself. Thompson reported in his thesis (52) that he had isolated a protective factor for the thermophilic aldolase, but this report has not been confirmed. The fact that other thermophilic enzymes have been purified and have not been found to depend upon a protective substance for their thermostability gives added support to the notion that a thermophilic protein's thermal stability depends only on the composition and the structure of this molecule. The other thermophilic enzymes which have been highly purified or crystallized are: an α-amylase from B. stearothermophilus (33), a protease from Bacillus thermoproteolyticus (39), this aldolase from B. stearothermophilus (53), and a glyceraldehyde-phosphate dehydrogenase from B. stearothermophilus (2).

There are a few proteins which have been isolated from a source other than a thermophilic organism which have been shown to be very heat-stable also. A good example of such a protein is bovine pancreatic ribonuclease-A. One can assume that the heat stability of such enzymes is a by-product of some molecular

structure which allows them to act more efficiently in their natural environment.

Assuming that the thermal stability of proteins from thermophilic organisms is inherent in the structure of the proteins themselves, two structural possibilities have been suggested by Ohta (39, p. 5919) to account for this stability:

1. The enzyme exists as a randomly coiled molecule in the native state, and consequently it no longer loses activity by heating.
2. Thermostability is the result of a rigid structure which is not denatured easily by the change of an external parameter such as temperature or the nature of the solvent.

Campbell concluded that the α-amylase from B. stearothermophilus exists as an essentially random coil (34), although this is not the only interpretation which could have been given to his data. The second possibility would fit just as well. Indeed, it is hard to imagine how a randomly coiled enzyme could maintain enzymatic activity in the light of the recent X-ray data and other research on the mechanisms of action of various enzymes. Ohta (39) concluded that the thermophilic protease was a compact globular molecule on the basis of its observed physical properties. Amelunxen (2) interpreted his data as meaning that the thermophilic glyceraldehyde-

phosphate dehydrogenase is not unfolded, but rather exists in a stable conformation which is very resistant to hydrogen bond breaking substances.

It is of interest to note that all those thermophilic enzymes which have been tested are resistant to effects of concentrated urea and guanidine hydrochloride solutions on their ultraviolet absorption and optical rotation properties.

If one assumes that thermophilic enzymes are globular proteins and that their thermal stability does not depend upon some protective substance, then the amino acid composition of such proteins might reflect to some extent why they are thermostable. Accordingly, Ohta (40) attributed the thermostability of the protease to hydrophobic bonding, as evidenced by the number of hydrophobic amino acids present, and to hydrogen bonding, especially of the tyrosines.

If thermophilic proteins are very compact globular molecules, then a high number of disulfide bonds might be expected to be found in such proteins. A large number of such bonds could help to hold these proteins in a rigid form and thus prevent their thermal denaturation, but this is not the case in at least two thermophilic enzymes. The protease (molecular weight = 37, 500) does not contain any such bonds and the α-amylase (molecular weight = 15, 600) contains only two disulfide bonds. There is no such data on the other

two well-purified thermophilic enzymes.

The tyrosine content may be important in some thermophilic enzymes. There are 29 tyrosyl groups in the protease, and 19 of these groups ionize abnormally, suggesting that they are either involved in hydrogen bonding or are in a hydrophobic environment. Ohta (40) interpreted his data as meaning that the abnormally ionizing tyrosines in his protease were hydrogen bonded. If these hydrogen bonded tyrosines are in a hydrophobic environment, their strength should be even greater than if they were in a fairly polar region of the molecule. The energetics of such bonding have been shown to be dependent upon molecular environment (38). It is therefore possible that such additive effects as this may help to account for the over-all stability of thermophilic enzymes. In the thermophilic α-amylase, there are only three tyrosyl groups (it is not reported whether these ionize abnormally or not) and no tryptophanyl groups, but the molecule has a fairly high proportion of other amino acids which have hydrophobic side chains.

Another general characteristic of thermophilic enzyme structure may be a low content of α-helical regions. This has been noted using optical dispersion measurements for the protease (39), and was suggested for the α-amylase (34) and the glyceraldehyde-phosphate dehydrogenase (2).

One might expect that the apparently low helical content in

these thermophilic enzymes would be reflected in a high proline content for these proteins. Although this may be a characteristic of some thermophilic proteins such as the α-amylase, it is not a general property. The thermophilic protease and glyceraldehyde-phosphate dehydrogenase are normal in this regard when compared to the "average protein" suggested by Smith (49).

As has been suggested above, all of the thermophilic enzymes which have been analyzed seem to be fairly high in those amino acids which are considered to be hydrophobic. Several suggestions have been proposed on how one might "quantitate" the non-polarity of proteins on the basis of their amino acid composition (7, 22, 55). These suggestions will be discussed at length in the discussion section of this thesis.

From the experiments described in this thesis, it is concluded that the FDP-aldolase obtained from B. stearothermophilus (NCA 2184) is a Type II aldolase. In addition, the Arrhenius activation energy for this enzyme (16.1×10^3 calories per mole) was found to be in the usual range ($12\text{-}24 \times 10^3$ calories per mole). Based upon the amino acid composition of the enzyme, the calculated hydrophobicity falls in the range of other thermophilic enzymes. Possible implications in understanding the structural features that account for thermostability are discussed.

II. MATERIALS AND METHODS

Materials

Agarose, 100-200 mesh, was purchased from Bio-Rad Laboratories as Biogel 0.5m.

Aldolase, rabbit muscle; carboxypeptidase-A-DFP, bovine pancreatic; ρ-chloromercuribenzoic acid; D-fructose-1,6-diphosphate, sodium salt; D-fructose-1-phosphate, sodium salt; β-nicotinamide adenine dinucleotide (NAD^+), sodium salt; ρ-nitrotetrazolium blue (NBT); phenazine methosulfate (PMS); and L-tryptophan were purchased from Sigma Chemical Company and were the best grades available. Also obtained from this supplier were Dihydroxyacetone phosphate (DHAP), dimethylketal dimonocyclohexylamine salt, and D, L-glyceraldehyde-3-phosphate (GAP), diethyl acetal monobarium salt. These salts were converted to their free form by first treating them with Dowex 50W x X2 (H^+ form) and then holding them at 37°C for two hours. Following this, the pH was adjusted to near neutrality and the free triose phosphates were either used immediately, or were stored frozen.

Ammonium sulfate, special enzyme grade; guanidine hydrochloride, ultra-pure; and urea, ultra-pure, were obtained from Mann Research Laboratories.

Antifoam C emulsion was purchased from the Dow Corning Corporation.

L(+)-cysteine hydrochloride, monohydrate, reagent grade, was obtained from both Sigma Chemical Company and Matheson, Coleman, and Bell.

Diethylaminoethyl-cellulose (DEAE-cellulose) was purchased from H. Reeve Angel Inc. as Whatman DE-23.

Ethylenediaminetetraacetic acid (EDTA), reagent grade, was purchased from the J. T. Baker Chemical Company.

Glyceraldehyde-phosphate dehydrogenase (GPDH), rabbit muscle, was obtained from both the Sigma Chemical Company and Calbiochem.

N-Z-case peptone (a pancratic digest of casein) was purchased from Sheffield Chemical, a division of National Dairy Products Corporation.

Sephadex G-200 was purchased from Pharmacia Company. This material was in the bead form and had a particle size range of from 40-120 microns.

Trypticase (a tryptic digest of casein) was bought from Baltimore Biological Laboratories.

L-tyrosine was obtained from Calbiochem.

All of the reagents which were used in the disc-gel electrophoresis were purchased from Canalco with the exception of acetic

acid (purchased from Allied Chemical Co.) and tris(hydroxymethyl) aminomethane (TRIS) (purchased from Sigma Chemical Company).

All other chemicals which were used were reagent grade.

Methods

pH Measurements

All pH measurements were made using a Corning pH Meter, Model 12, which was equipped with a Corning Semi-Micro Combination Electrode.

Protein Concentration Measurements

Protein concentrations were estimated during the purification procedure by measuring the absorbance at 280 nm. The purification of the enzyme from nucleic acids was followed by measuring the absorbancy ratio: 280 nm/260 nm. The extinction coefficient of the purified enzyme was found by relating the absorption at 280 nm to the protein content as determined by the biuret method (25).

Aldolase Assay: The Method of Warburg (15)

This was the method of choice for determining the aldolase activity in all of the enzyme preparations. It was used unless otherwise noted.

This procedure involves the use of a coupling enzyme to measure the rate at which the products are formed when aldolase attacks FDP. The products of this reaction are the two triose phosphates, DHAP and GAP, and they are normally readily interconvertible by the action of triose-phosphate isomerase. Since conditions were chosen under which the isomerase would not be active, only the GAP production is measured by using excess amounts of GPDH, which converts GAP into 3-phosphoglyceric acid. This latter conversion also quantitatively converts NAD^+ to NADH. The amount of NADH produced is followed by noting the increase in absorbance at 340 nm.

The activity of the enzyme was determined using a Beckman Model DB spectrophotometer whose cuvette compartment temperature was controlled using a Heto circulating water heating bath. For a final volume of 3.00 ml, the reaction mixture consisted of: 1.0×10^{-2}M FDP (pH 7.6); 1.67×10^{-4}M $MnSO_4$; 1.7×10^{-2}M sodium arsenate; 2.7×10^{-2}M glycine (pH 7.6); 2.0×10^{-2}M cysteine (pH 7.6); and 5.0×10^{-3}M NAD^+. Sufficient amounts of these reagents to give the final concentrations were contained in 2.9 ml in a cuvette. The cuvette was incubated for five minutes to bring it to temperature (43°C, unless otherwise noted), then 0.05 ml of a five mg per ml solution of GPDH in 0.005 M potassium phosphate buffer, pH 7.6, was added to the cuvette followed by 0.05 ml of a

properly diluted enzyme sample, such that the net optical density change was not in excess of 0.5 per minute at 340 nm. The 0.005 M potassium phosphate buffer just mentioned is hereafter referred to as Buffer 1.

One unit is defined as being that amount of enzyme which brings about the conversion of one micromole of FDP per minute to GAP and DHAP.

Aldolase Assay: The Method of Jaganathan

The procedure of Jaganathan was used to determine whether the enzyme could use F1P as a substrate. The method used was the Boyer modification (57) of the hydrazine assay of Jaganathan (26). This assay involves the incubation of the enzyme with substrate in the presence of hydrazine. As the triose phosphates are formed they react quantitatively with the hydrazine to form the simple hydrazones which absorb at 240 nm.

For the assay of the thermophilic aldolase, 2.0 ml of 0.0035 M hydrazine sulfate which is 10^{-4}M in EDTA (pH 7.6) is mixed with 0.05 ml of 0.30 M FDP (pH 7.6) or with 0.50 ml of 0.03 M F1P (pH 7.6), 0.10 ml of 2×10^{-2}M $MnSO_4$, and water to give a volume of 3.00 ml. These are placed in both the sample and reference cuvettes and allowed five minutes to come to temperature (34°C). Then 0.05 ml of an appropriately diluted enzyme sample is added to the sample

cuvette. The increase in the absorbance at 240 nm is followed for five minutes. Activity is defined as the change in optical density per minute per ml of enzyme solution.

Aldolase Assay: The Method of Sibley and Lehninger (48)

This assay involves the incubation of the enzyme in the presence of FDP and hydrazine. As the triose phosphates are formed they react with the hydrazine to give the corresponding hydrazones. The enzymatic reaction is stopped by the addition of trichloroacetic acid, and an aliquot of the deproteinized solution is then treated with base to release the free triose phosphates. The free triose phosphates are then converted to the colored dinitrophenylosazones which are measured at 540 nm.

The assay was performed as described in the reference (48) with the exceptions that 0.1 ml of 0.30 M FDP was substituted for 0.2 ml of 0.05 M FDP and that no buffer was used. However, the final volume was the same in all cases. The pH was always checked before and after the enzyme incubation, and was found to be 7.2.

One unit of activity is defined as being that amount of enzyme which will cleave 4.46×10^{-2} micromoles of FDP per hour at 37°C.

Aldolase Assay: The Method of Dounce, Barnett, and Beyer (19)

This procedure is like that of Sibley and Lehninger in that

initially the simple hydrazones are formed with the triose phosphates produced by the action of the aldolase. The difference is that instead of taking the freed triose phosphates and converting them to the dinitrophenylosazones to measure the amount of product which has been formed, this method converts the freed triose phosphates to acetaldehyde by way of methylglyoxal. The acetaldehyde so produced is then measured by color formation with ρ-hydroxydiphenyl.

The procedure was followed exactly as given in the reference (19). Whether FDP or DHAP was used as substrate or whether or not manganous ion was included in the assay, the final volume was two ml. The color produced by the assay was measured spectrophotometrically at 530 nm.

Assay for Thermophilic Glyceraldehyde-phosphate Dehydrogenase

This assay was conducted in the same manner as the Warburg Method for assaying for aldolase activity (see page 11). For this assay GAP was substituted for FDP and the normal coupling enzyme, rabbit muscle glyceraldehyde-phosphate dehydrogenase, was deleted. The GAP was added to initiate the reaction.

Assay for Thermophilic Triose-phosphate Isomerase

This assay was also conducted in a similar manner to the Warburg Method of assaying for aldolase. In this case, DHAP was

substituted for FDP and Mn^{++} was deleted, since it inhibited the activity of this enzyme.

Procedure for Disc-gel Electrophoresis

Disc-gel electrophoresis experiments were run as suggested by Canalco (14). Reagents were made as suggested (13). Experiments were run using the standard 7-1/2 percent gels (stack at pH 8.3 and run at pH 9.5). All experiments were conducted at room temperature.

III. EXPERIMENTAL AND RESULTS

Growth of the Organism

A sample of Bacillus stearothermophilus (NCA 2184) in soil was provided by Professor Richard Y. Morita, Department of Microbiology, Oregon State University.

The bacteria were initially propagated, using standard techniques, in 2 percent "Trypticase" broth and in the broth media of Table 1. The latter media is based on the media of Welker and Campbell (56). Broth stock cultures were started from the frozen cell paste suspension noted below. The cultures were grown at 55°C and were transferred at least every two days.

Cells were grown in 100 liter batches using an Access-a-Bilt fermentor (Stainless and Steel Products Company, St. Paul, Minnesota) using the media defined in Table 1. Conditions of growth were: temperature, 55 to 60°C; aeration, 100 liters of air per minute; stirring rate, 30 rpm; and antifoam, Dow Corning Antiform A added, as needed. The pH was controlled at 7.0 to 7.5 by the addition of saturated potassium hydroxide, as needed. The inoculum was a three liter broth culture in early log phase grown under the same conditions as noted above in a Microferm Fermentor (New Brunswick Scientific).

Table 1. Growth Media for Bacillus stearothermophilus.

Reagent	Grams per liter
Trypticase	20
N-Z-case	4
K_2HPO_4	2.5
KH_2PO_4	1
NH_4Cl	1
NaCl	1
Sucrose	5
$FeCl_3 \cdot 6H_2O$	5×10^{-3}
$MgCl_2$	5×10^{-3}
$CaCl_2$	5×10^{-3}
Nicotinic acid	1.5×10^{-3}
D, L-valine	0.144

Tap water was used.
The final pH is about 7.5

When the cells had reached late log phase, as determined by the optical density at 525 nm, the fermentor was cooled to about 20°C, and the contents were centrifuged using a Sharples Type AS-14 centrifuge. The yield of cell paste was usually 1000 grams per batch.

The cell paste was suspended in two liters of 0.9 percent saline, using a Waring Blender. This suspension was centrifuged at 14,600 x g for 30 minutes, the supernatant solution discarded, and the cell paste resuspended in one liter of 0.9 percent saline. The washed cells were stored as a frozen suspension at -15°C until used.

Enzyme Purification

Preparation of the Crude Sonicate

The frozen cell suspension containing 250 grams of cell paste, which had been stored at -15°C, was thawed overnight at about 25°C. The suspension was then centrifuged at 18,000 x g for 20 minutes at 25°C. The supernatant fraction was discarded, and the cell paste was resuspended in one liter of Buffer 1, using a Waring Blender. Then, 250 ml aliquots of this suspension were placed in a 400 ml glass beaker on ice and sonicated using a Branson Sonifier (Model S-75) at 6.5 amperes for five minutes.

The sonicated material was centrifuged at 18,800 x g for 20 minutes at 25°C. The supernatant fraction was reserved, the pellet was resuspended in 500 ml of Buffer 1, using a Waring Blender, and the centrifugation procedure was repeated. This supernatant fraction was combined with that from the first centrifugation and the pellet was discarded. The combined supernatant fractions were clarified by centrifugation at 27,000 x g at 25°C for 30 minutes. The supernatant solution (S-1) was either used directly, or stored at 5°C until needed.

pH and Ammonium Sulfate Fractionations

The crude sonicate solution (S-1) was adjusted to 0.1 saturation

with ammonium sulfate, and the rapidly-stirred solution was quickly adjusted to pH 3.6 with 1.47 M phosphoric acid. Then, the thick, creamy suspension was immediately centrifuged at 10,800 x g at 25°C for ten minutes and, as soon as possible, the supernatant fraction was poured off and adjusted to pH 7.6 with 1 M potassium hydroxide. If the pH adjustments were not completed as soon as possible, there was a decrease in the recoverable activity.

This solution was adjusted to 0.50 saturation with ammonium sulfate while the solution was constantly stirred, and the pH was kept at pH 7.6 by the addition of 6 M ammonium hydroxide. Fifteen minutes after the final addition of ammonium sulfate, the suspension was centrifuged at 18,800 x g for 20 minutes at 25°C. The supernatant solution was decanted and adjusted to 0.75 saturation with ammonium sulfate with rapid stirring and constant adjustment of pH, as was done above. Again, fifteen minutes after the final addition of ammonium sulfate, the suspension was centrifuged as above. The supernatant fraction was discarded, and the pellet was taken up in a minimum amount of Buffer 1. This solution (As 75) was either used immediately, or stored at 5°C until needed.

Agarose Gel Chromatography

A 3.5 cm x 105 cm column of Agarose (Biogel A 0.5 m--swollen form) was prepared and equilibrated with Buffer 1. The calculated

V_o of the column was 440 ml. The As 75 sample (routinely 50-60 ml) was layered onto the surface of the column which was then eluted with Buffer 1 at a rate of 25 ml per hour with a hydrostatic head of about 30 cm. Fractions were collected every 30 minutes. A typical plot of the OD at 280 nm and activity versus fraction number can be seen in Figure 1.

Those fractions which possessed high activity, but were lower in protein concentration, were pooled and were either used immediately or were stored at 5°C until needed.

DEAE-cellulose Chromatography

The DEAE-cellulose was conditioned according to the directions of the manufacturer. This included treatment with 0.5 M hydrochloric acid for 30 minutes followed by washing with water until the pH of the exchanger was near neutrality. The cellulose was then treated twice in a similar manner with 0.5 M sodium hydroxide. After the second sodium hydroxide treatment, the exchanger was washed with water until the pH was near 8.5. The material was then suspended in twenty times its volume of Buffer 1, and the pH was adjusted to 7.6 with molar phosphoric acid. After the cellulose had settled, the liquid above it was removed by suction. The exchanger was again resuspended in twenty times its volume of Buffer 1, and the pH was again adjusted to 7.6, as was done above. This process

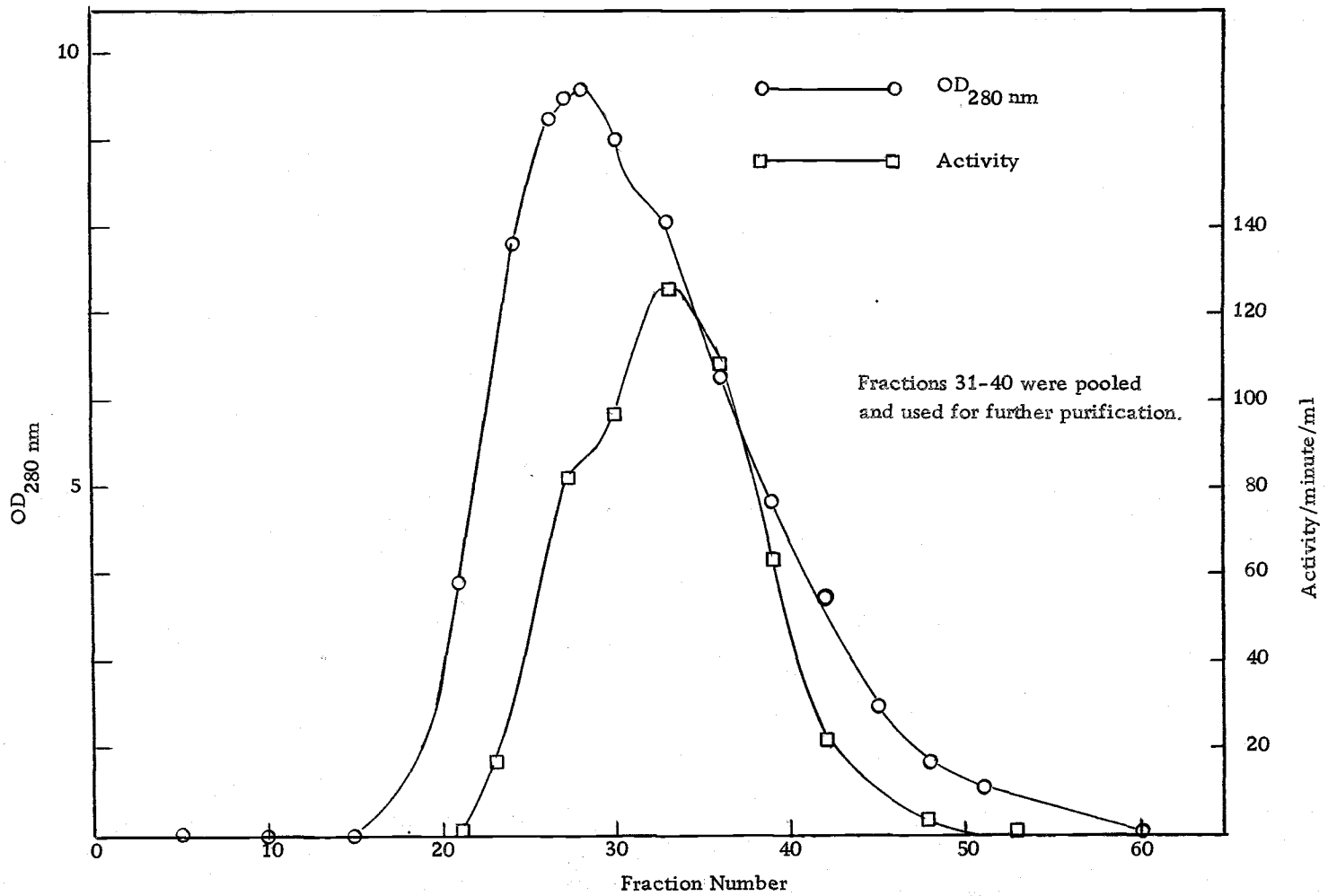

Figure 1. Chromatography of Thermophilic Aldolase on Agarose.

was repeated until the DEAE-cellulose was found to be pH 7.6 without any adjustment.

A 2.5 cm x 90 cm column was poured and was allowed to settle while it was washed with two liters of Buffer 1. The sample which had been obtained by pooling fractions from the agarose chromatography (about 250 ml) was then siphoned onto the column and eluted with a linear potassium phosphate concentration gradient (0.005 M to 0.25 M). The gradient was accompanied by a pH change from 7.6 to 6.0. The total volume of the gradient was eight liters. The flow rate was 100 ml per hour and fractions were collected every twelve minutes. A typical plot of OD at 280 nm, pH, activity, and buffer concentration versus fraction number can be seen in Figure 2.

Those fractions which assayed highest in activity were pooled and concentrated to about 10 percent of the original volume using a Diaflo Concentrator (Model 400) which was equipped with an Amicon UM-1 membrane. This concentration was conducted at 5^{o}C and at 100 psi. Once the volume had been reduced, the sample was further concentrated by taking it to 0.80 saturation with ammonium sulfate at pH 7.6. This suspension was centrifuged at 27,000 x g at 5^{o}C for 20 minutes. The supernatant fraction was discarded and the pellet was taken up in a minimum amount of Buffer 1 and was used immediately for the next step in the purification.

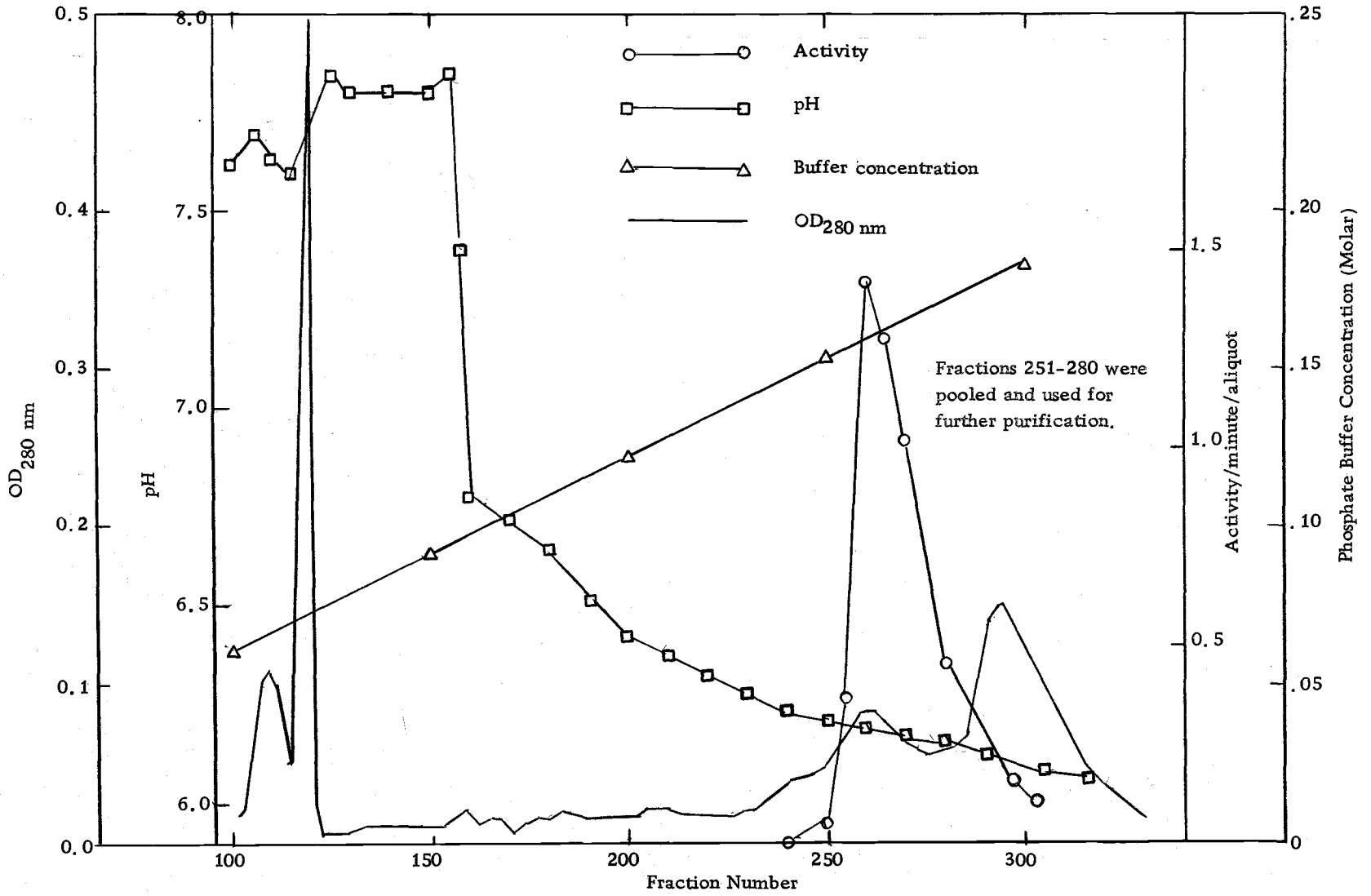

Figure 2. Chromatography of Thermophilic Aldolase on DEAE-cellulose.

Sephadex G-200 Gel Chromatography

Sephadex G-200 was allowed to swell in glass distilled water at room temperature for three days. The supernatant was removed by suction and Buffer 1 was added to the gel at five times the gel volume. This process was repeated three times and then a 2.5 cm x 40 cm column (V_o = 57 ml) was poured making sure that the effective hydrostatic head on the column was between 10 and 20 cm while this was being done. The prepared column was equilibrated by allowing 300 ml of Buffer 1 to pass through it, then the sample which had been concentrated after DEAE-cellulose chromatography was layered onto the top of the column. The volume of sample applied to the column was normally 4-9 ml. Elution was performed at a flow rate of 20 ml per hour using a hydrostatic head of 15 cm, and fractions were collected every twelve minutes. A typical plot of OD at 280 nm, activity, and specific activity versus fraction number can be seen in Figure 3. Those tubes which were highest in specific activity were pooled, and, if they were not to be used immediately, they were concentrated by taking the solution to 0.95 or higher saturation with ammonium sulfate with constant stirring and adjustment of pH to 7.6, as before. The resulting suspension was centrifuged at 27,000 x g for 30 minutes at 5°C, then the supernatant solution was discarded, and the pellet was taken up in a small amount of Buffer 1 and

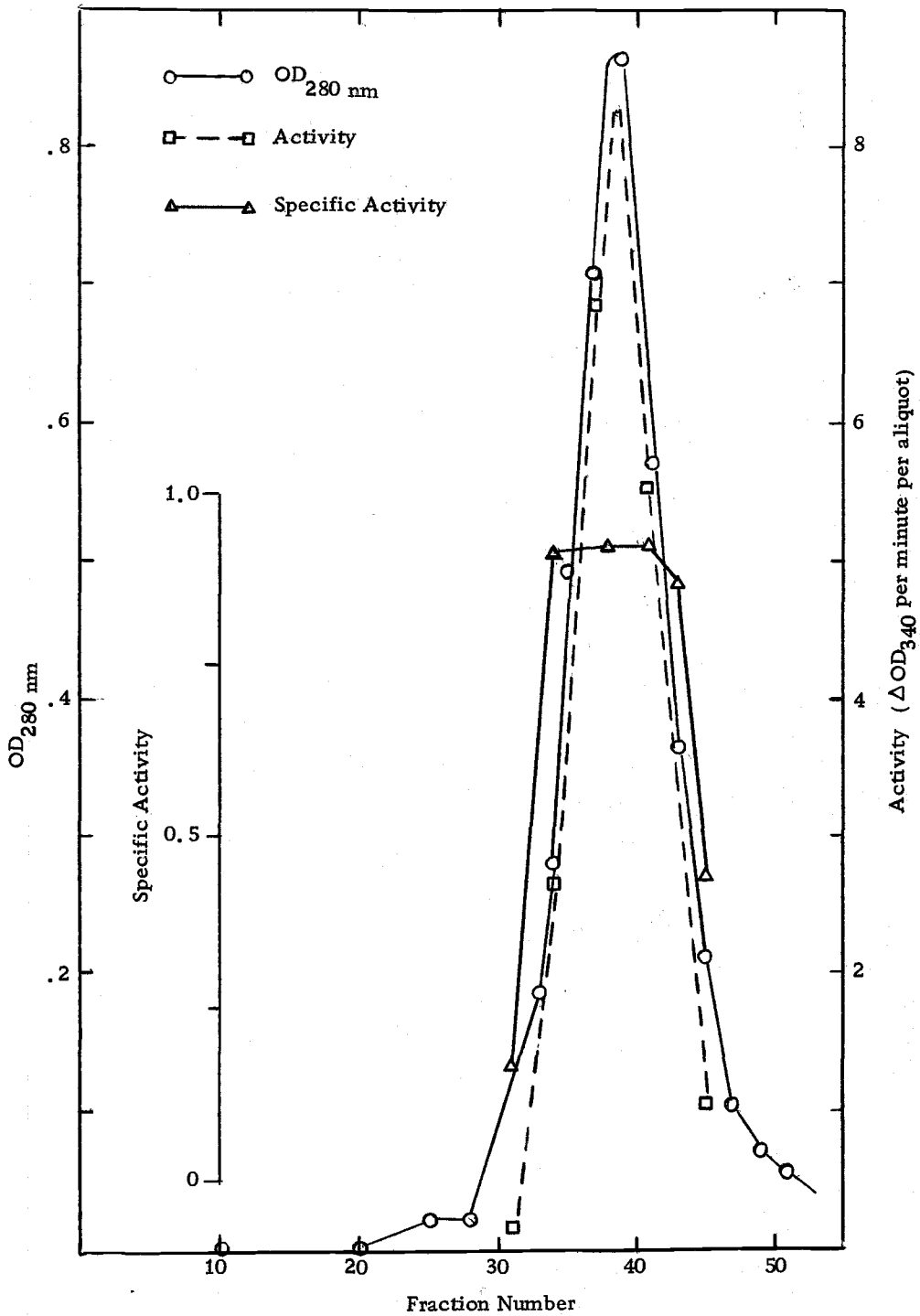

Figure 3. Chromatography of Thermophilic Aldolase on Sephadex G-200.

stored at 5°C until needed. This step seemed to greatly improve the stability of the more purified preparations, which tend to be somewhat unstable as dilute solutions.

It should be noted that the enzyme loses activity upon standing--even in concentrated solutions. If a solution had been stored for any length of time, a good part of the lost activity could be recovered by preincubating the enzyme with cysteine before assaying.

A summary of the purification procedure for the aldolase of B. stearothermophilus can be seen in Table 2.

Table 2. Summary of the Purification Procedure for the Aldolase of Bacillus stearothermophilus.

Fraction	Volume (ml)	Total units	Total OD @ 280 nm	Specific activity	% recovery of activity
1. Crude sonicate (S-1)	1400	6314	86,100	0.073	--
2. 0.50-0.75 saturated ammonium sulfate fraction (As-75)	48.2	5185	4,501	1.152	82
3. Pooled Agarose fraction	169.5	3646	1,984	1.838	58
4. Pooled DEAE-cellulose fractions	345	1276	49	26.04	20
5. Pooled Sephadex G-200 fractions	34.6	533	11.7	45.55	8.4

Criteria of Purity

The most highly purified fractions from Sephadex G-200 chromatography exhibited a specific activity of 45.5 μmoles of FDP cleaved per minute per mg of protein. The plot of activity and optical density @ 280 nm versus fraction number in the final purification step (see Figure 3) suggests that this enzyme is homogenous. Attempts at further purification, using hydroxylapatite column chromatography or rechromatography on DEAE-cellulose, did not increase the specific activity of this preparation. The sedimentation velocity ultracentrifugation experiment (described below) revealed no other component. The results of this analysis can be seen in Figure 4. This experiment was performed within a few days after the Sephadex gel chromatography was completed.

Using their purification procedure, Thompson and Thompson (53) reported a minor component on the descending slope of the sedimentation pattern. This was not observed with this preparation. They also noted that they were able to detect small amounts of triose-phosphate isomerase activity. In experiments designed to reveal contaminating enzymes in this preparation, a thermophilic glyceraldehyde-phosphate dehydrogenase was not detected, but a small amount of the triose-phosphate isomerase was. On the basis of an assumed turnover number of 10^6 for this enzyme, the contamination

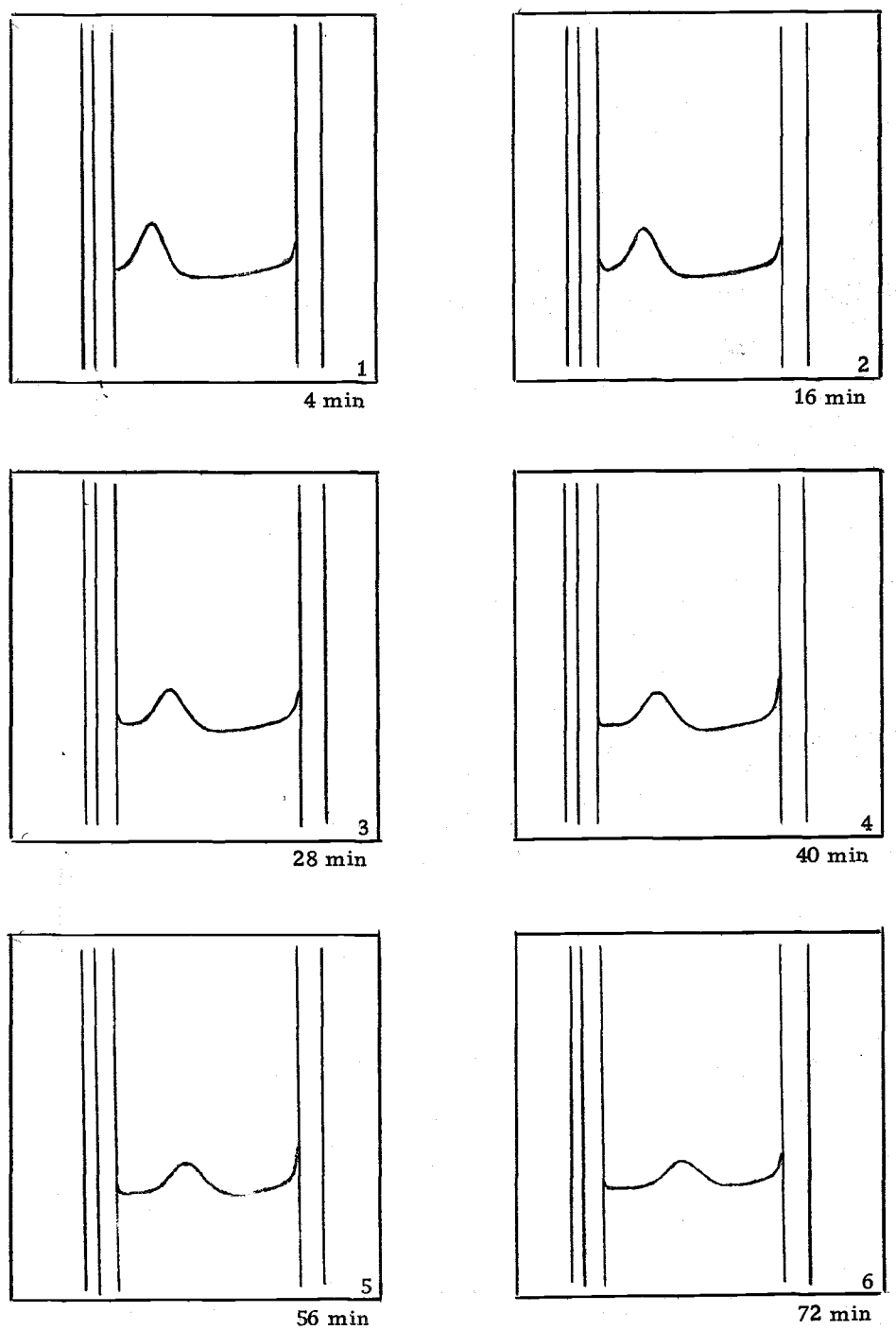

Figure 4. Sedimentation of Purified Thermophilic Aldolase.

due to this enzyme was calculated to be less than 1 percent.

Disc-gel electrophoresis and a sedimentation equilibrium ultracentrifugation experiment (described below) performed about two weeks after the final purification step showed the presence of a minor component in this preparation. The results of this disc-gel experiment and one performed two weeks later on the same sample can be seen in Figure 5. Also in this figure is a gel which was subjected to an activity staining procedure discussed below. This shows that the major band is the active one, and it can be seen that the amount of the second (minor) component has increased over the two week period. The activity of the sample had decreased during this time.

The above observations suggest that the minor component, although inactive under these conditions, has arisen from the major component. Rutter (45) has suggested that the Type II aldolases may be composed of two subunits. The molecular weight of the minor component was calculated to be about half that of the major component in the sedimentation equilibrium experiment. Also, in the disc-gel electrophoresis it can be noted that the minor component has migrated only about half as far as the major component. One explanation for this phenomenon would be that this protein has half of the effective size as the larger one. All of this evidence suggests that the minor component may be a subunit of the enzyme, and the only contaminant in this preparation is a small amount of the triose-phosphate

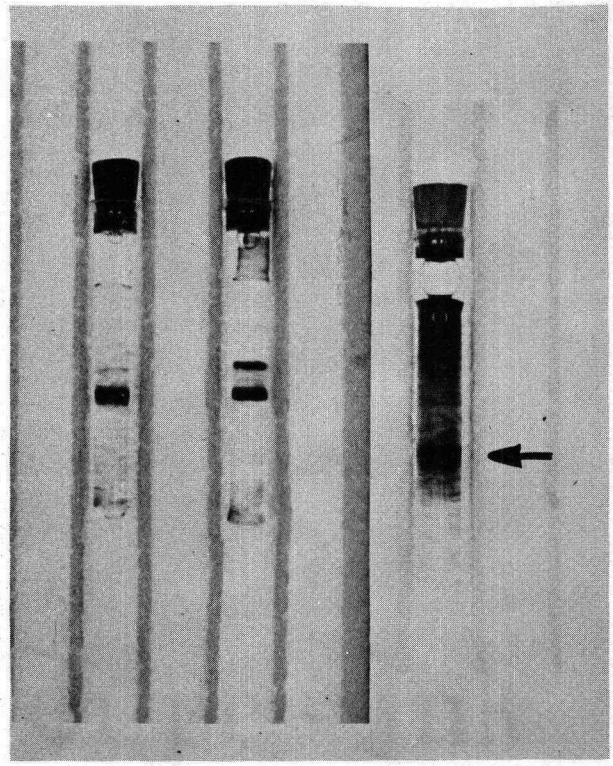

Figure 5. Disc-gel Electrophoresis and Activity Staining of the Purified Aldolase.

A. Disc-gel on which the purified sample was subjected to electrophoresis two weeks after the final purification step. Stained for protein.

B. Disc-gel on which the same sample as in A was subjected to electrophoresis four weeks after the final purification step. Stained for protein.

C. Same as A, except subjected to the activity staining procedure. The arrow indicates the black band obtained with this procedure, corresponding to the lower band in the gels stained for protein. The dark areas at the top of this gel is purple background.

isomerase. Thompson and Thompson (53) reported that whatever steps were taken to remove this enzyme only decreased the aldolase activity. The enzyme, therefore, may be judged to be sufficiently pure for all of the experiments which have been proposed.

Characterization of the Enzyme

Metal Ion Requirement of the Enzyme

The effect of various metal ions on the aldolase activity is seen in Table 3. While Fe^{++}, Cu^{++}, and Mg^{++} were neither activators nor inhibitors, Zn^{++} inhibited the activity, and Mn^{++} activated the enzyme. Co^{++} and some of the heavy metal ions could not be tested, since they interfered with the assay medium itself.

Table 3. The Effect of Various Metal Ions on the Aldolase Activity.

Metal ion added[1]	% of aldolase activity[2]
none	100
Fe^{++}	101
Cu^{++}	100
Mg^{++}	95
Zn^{++}	73
Mn^{++}	172

[1] Metal ion concentration in each case was 3.67×10^{-4} M.

[2] The enzyme sample used for this experiment was purified through the ammonium sulfate fractionation.

The effect of Mn^{++} concentration on the aldolase activity can be seen in Figure 6. It can be seen that the enzyme is saturated at about 2×10^{-4} M Mn^{++}.

The Effect of Mn^{++} on the Aldolase Assay Methods of Sibley and Lehninger and Dounce, Barnett, and Beyer

Since Mn^{++} was shown by Thompson and Thompson (53) to inhibit the aldolase activity using the assay method of Dounce et al. (19), it was decided to compare the effect of Mn^{++} on this enzyme preparation as measured by the method of Warburg and Christian and by the two colorimetric procedures: that of Sibley and Lehninger and that of Dounce, Barnett, and Beyer. The results of the three assays, with and without Mn^{++}, can be seen in Table 4.

Table 4. Enzymatic Activity of the Thermophilic Aldolase as Given by the Various Assay Procedures With and Without Mn^{++}.

Procedure	Relative rate
Warburg and Christian	
(a) no Mn^{++}	100
(b) 6.67×10^{-4} M Mn^{++}	500
Dounce, Barnett, and Beyer	
(a) no Mn^{++}	100
(b) 2.35×10^{-3} M Mn^{++}	60
Sibley and Lehninger	
(a) no Mn^{++}	100
(b) 5.0×10^{-5} M Mn^{++}	34

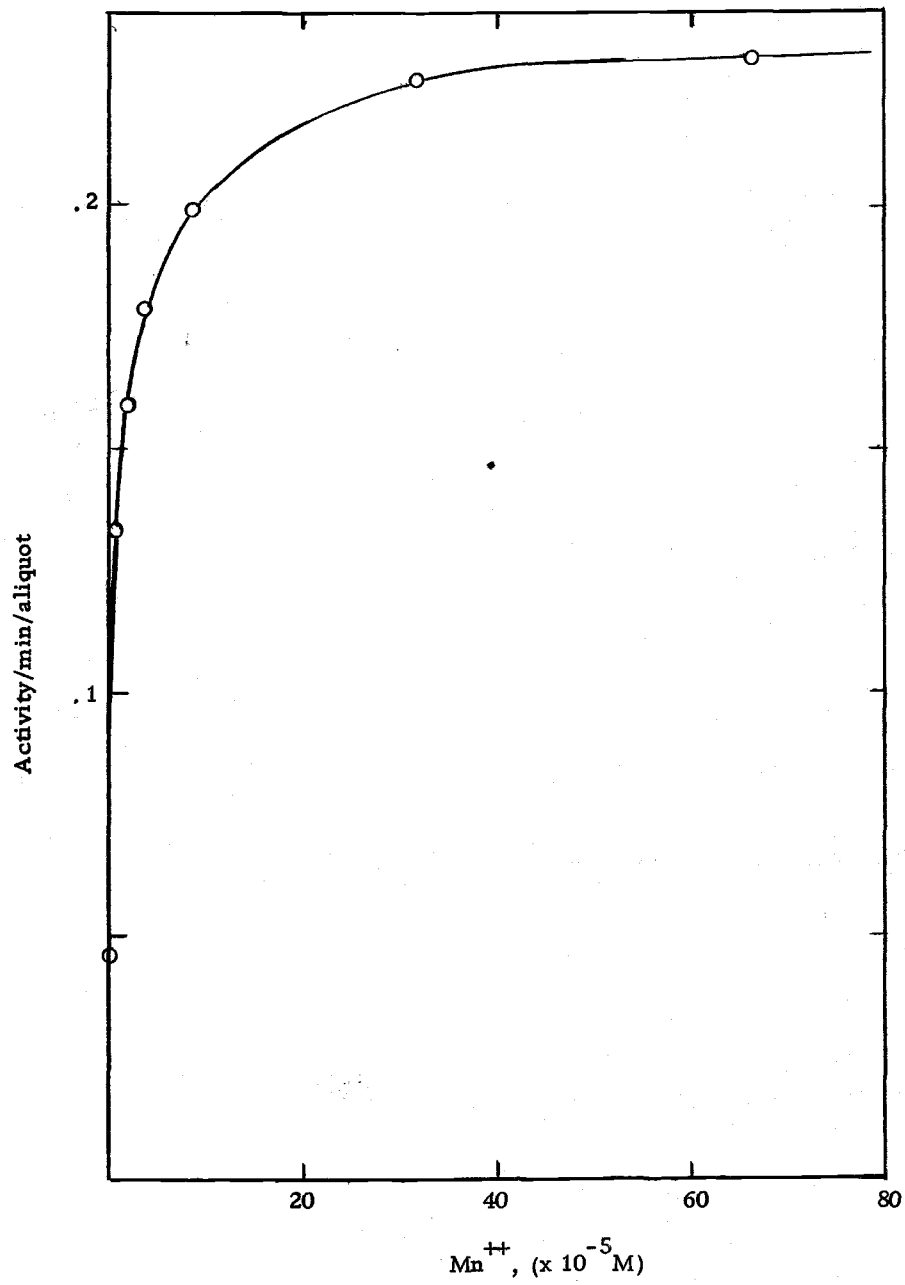

Figure 6. Effect of Mn^{++} Concentration on the Activity of the Thermophilic Aldolase.

In order to explain the apparent contradiction in the metal ion requirement as found by the two colorimetric assay procedures, DHAP (one of the products of the aldolase reaction) was incubated in place of the FDP and enzyme. To see the effect of Mn^{++} on the formation of the phenylosazone in the procedure of Sibley and Lehninger (48), the metal ion was added after the initial incubation. The results of this experiment can be seen in Table 5. To see the effect of Mn^{++} on the entire assay procedure of Dounce, et al. (19), the metal ion was added to the initial incubation mixture. The results of this experiment can be seen in Table 6.

Inhibition by Chelating Agents

The effect of various chelating agents upon the activity of the enzyme is shown in Table 7. The enzyme is sensitive to all three reagents tested, but is most sensitive to EDTA.

The Necessity of Free Sulfhydryl for Maximal Activity

The results of performic acid oxidation of the enzyme, followed by amino acid analysis (see below) show that the enzyme contains cysteine, cystine, or both. To see whether there are any free sulfhydryl groups present in the enzyme, the spectrophotometric titration with ρ-chloromercuribenzoic acid was performed according to the procedure of Boyer (8), as reviewed by Benesch and Benesch

Table 5. The Effect of Mn^{++} on the Color Development of the Assay Method of Sibley and Lehninger.

Mn^{++} concentration	Color yield
0	100
2×10^{-5} M	91
4×10^{-5} M	85
6×10^{-5} M	78

Table 6. The Effect of Mn^{++} on the Assay Method of Dounce, et al.

Mn^{++} concentration	Color yield
0	100
8.2×10^{-5} M	78
2.5×10^{-4} M	63
2.5×10^{-3} M	41

Table 7. The Effect of Various Chelating Agents Upon the Thermophilic Aldolase Activity.

Chelating agent	Molar concentration	Relative rate[1]
EDTA	0	100
	3.24×10^{-5}	71
	1.61×10^{-3}	0
o-phenanthroline	0	100
	3.3×10^{-3}	43
α, α'-dipyridyl	0	100
	5.3×10^{-3}	30

[1] All assays were performed in the presence of 3.3×10^{-4} M manganous ion.

(4). Some titration of sulfhydryl groups was apparent at pH 4.6 in acetate buffer, but quantitative results could not be obtained, since the protein precipitated out before the end of the titration. At pH 7.0 in phosphate buffer, the enzyme was stable during the experiment, but no sulfhydryl groups were titrated.

Upon performing the amperometric titration of the enzyme with silver nitrate by the method of Benesch, et al. (4, 5) using the mercuric oxide: barium hydroxide electrode of Cole, et al. (17) as the reference electrode, no sulfhydryls were detected, even in the presence of six molar urea.

In spite of the preceding results, two pieces of data suggest that this enzyme requires free sulfhydryl groups. (1) The enzyme is much more active in the presence of cysteine in the assay mixture, as seen in Table 8. (2) Aged preparations show an initial lag period when assayed, which can be removed by preincubation of the enzyme with cysteine.

Table 8. The Influence of Cysteine on the Activity of the Enzyme.

mmoles of cysteine added	Relative rate[1]
0	100
4×10^{-3}	610

[1] The enzyme had been stored for 17 days at 5°C after the final purification step.

The pH Optimum

The pH optimum of the enzyme was determined using the normal assay system which was, in addition to the normal constituents, 3.3×10^{-2} M in phosphate buffer. The plot of relative activity versus pH can be seen in Figure 7. From this figure it is seen that the enzyme has a very narrow pH optimum at about pH 7.6.

FDP/F1P Specificity

Using the Jaganathan assay procedure and the same final concentration of FDP as F1P, it was found that at 34°C the enzyme had a rate which was 236 times higher with FDP than with F1P. It was impossible to determine the K_m for F1P under these conditions, because of the very low rates obtained. Therefore, it is not possible to compare this value and that obtained using FDP as a substrate (this comparison is one of the means of distinguishing between the two types of aldolases).

K_m and V_{max} Using FDP as the Substrate

The K_m and V_{max} were calculated using the method of Lineweaver and Burk (32). These data are plotted in Figure 8. At 41.5°C the K_m is 1.11×10^{-4} M and the V_{max} is 4650 moles of FDP cleaved per minute per mole of enzyme.

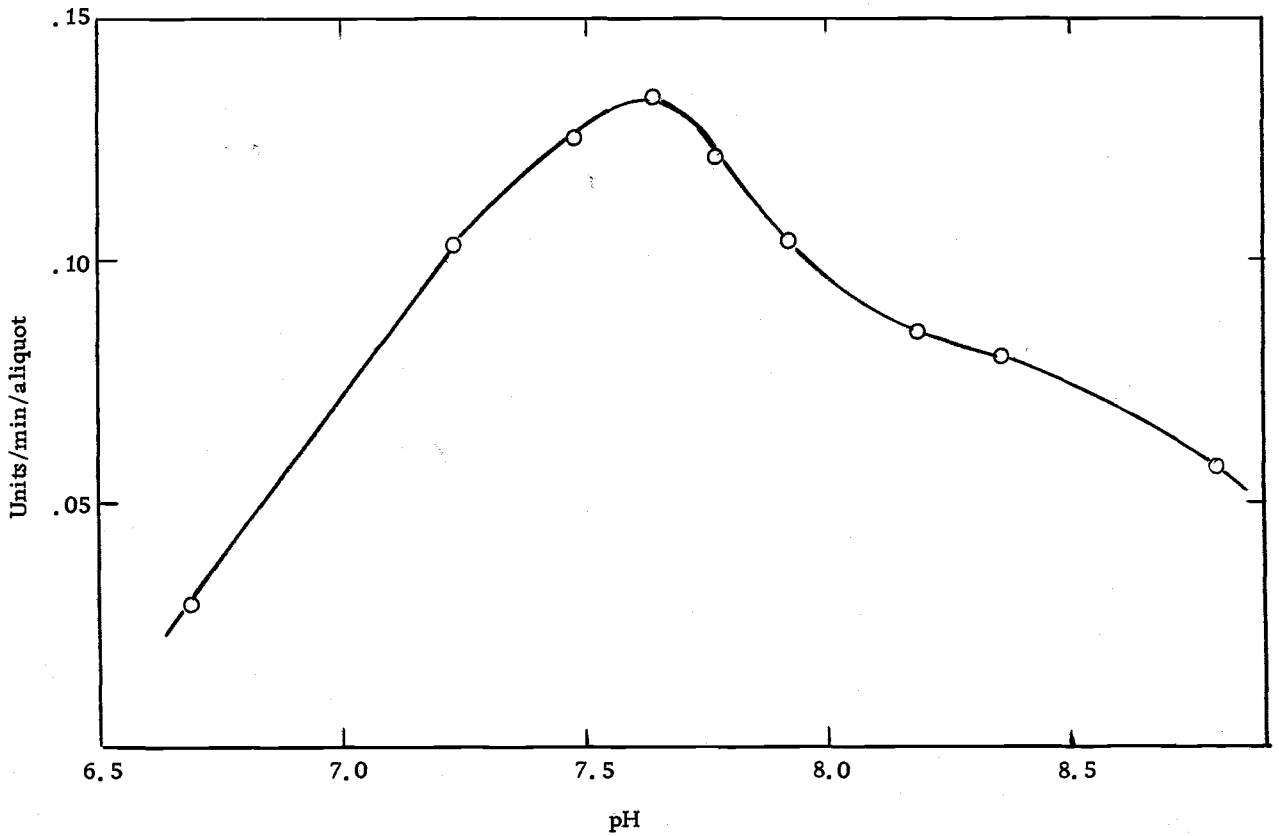

Figure 7. Effect of pH on the Activity of the Thermophilic Aldolase.

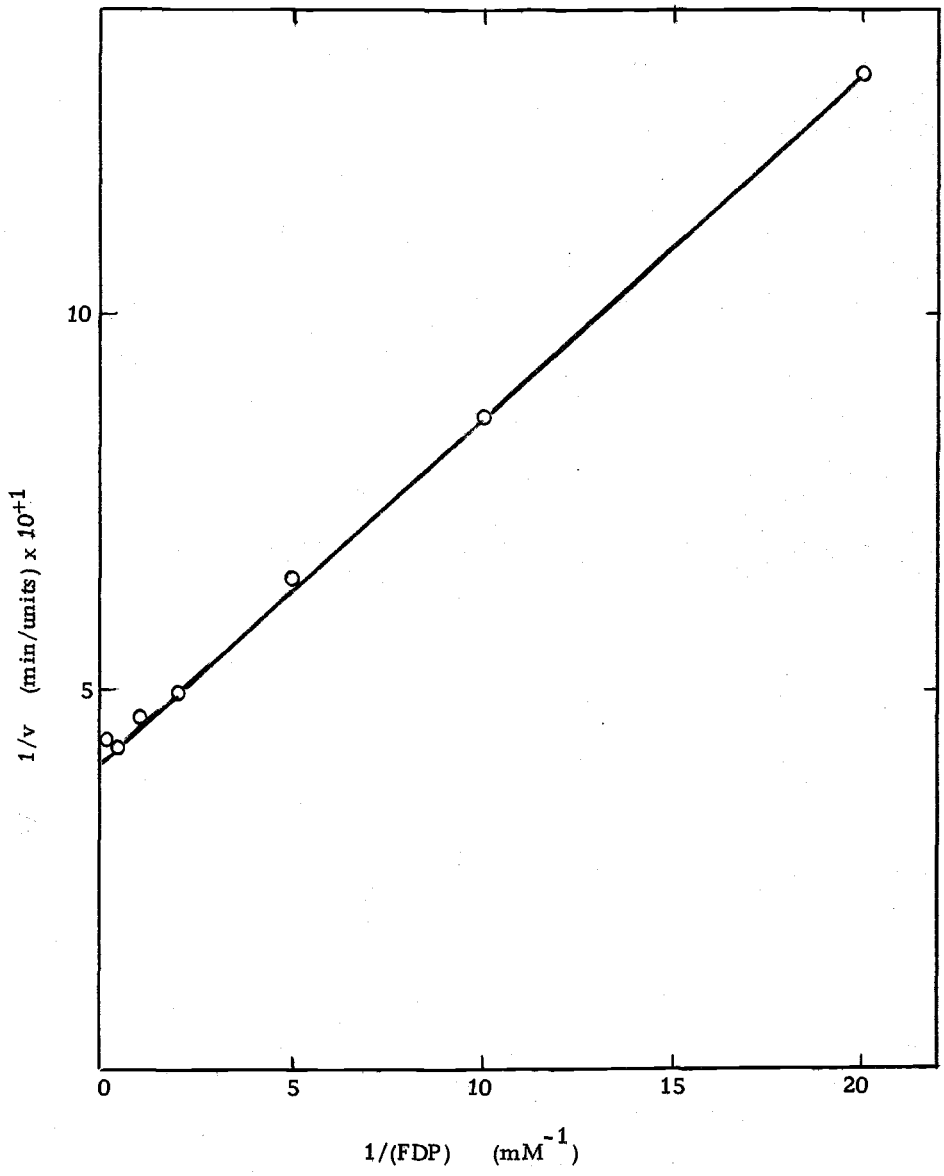

Figure 8. Effect of FDP Concentration on the Activity of the Thermophilic Aldolase.

The Molecular Weight

The molecular weight of the enzyme was determined using two types of ultracentrifugation experiments.

A sedimentation velocity experiment was performed in a Model E Ultracentrifuge (Spinco Division, Beckman Inc.) using a 3.8 cm long pathlength, single sector cell in an An-E rotor. This experiment was performed at 48,000 rpm and at 20°C. The enzyme concentration was 3.08 mg per ml, and the enzyme was in 0.05 M phosphate buffer, pH 7.6. Figure 4 (page 29) shows the pattern obtained using schlieren optics.

The S_{20} value, uncorrected for the viscosity of the solvent, was calculated to be 4.25 S. The plot of $\log_{10}$ versus t (time) which was used to obtain this value, can be seen in Figure 9.

The molecular weight calculated from this S_{20} value and the equation:

$$\text{Mwt.} = \frac{S_{20} RT}{D\ (1 - \bar{v}\rho)}$$

was 56,500 grams per mole. The partial specific volume, $\bar{v}$, was calculated to be 0.738 cc/gm using the method of Cohn and Edsall (16). A diffusion coefficient of 7.0×10^{-7} cm^2/sec and a density of 1.00 were assumed for the molecular weight calculation.

The sedimentation equilibrium experiment was conducted according to Van Holde (54). This was done using a Model E

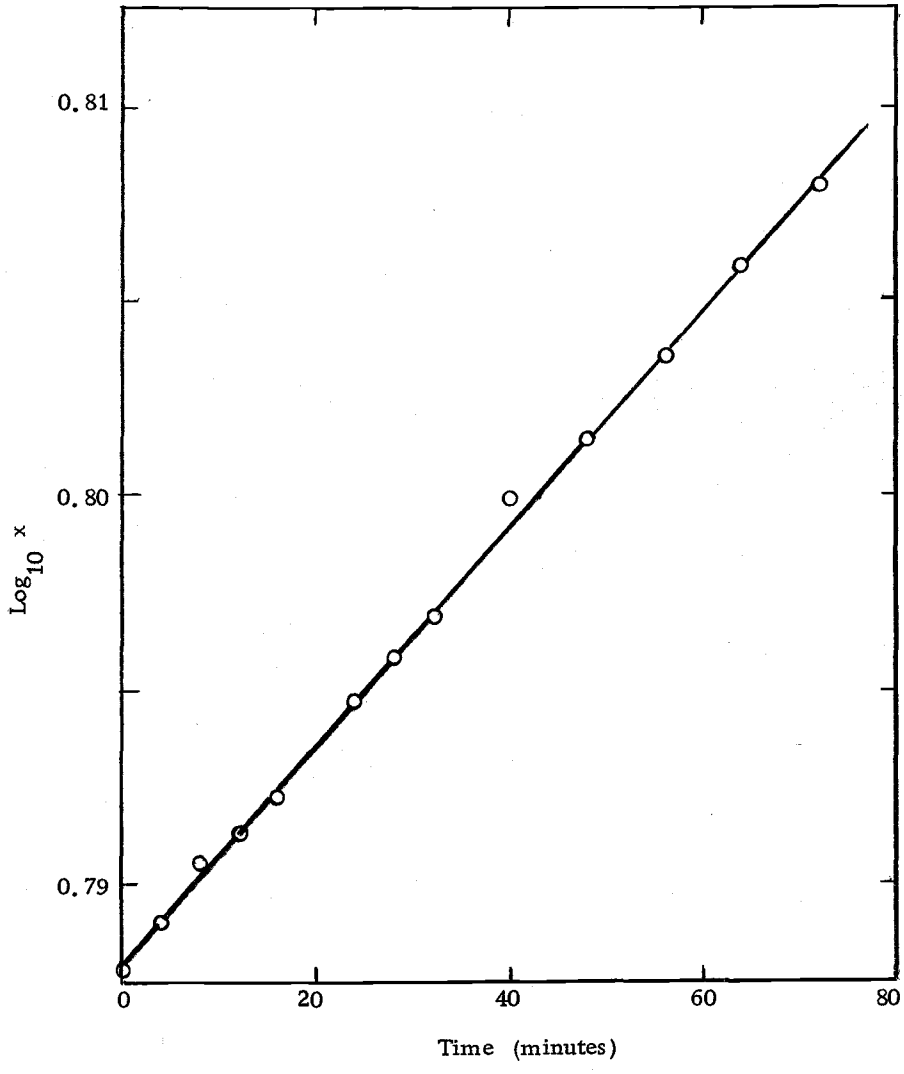

Figure 9. Sedimentation Velocity Ultracentrifugation of Thermophilic Aldolase.

ultracentrifuge equipped with an An-D rotor and interference optics. The rotor speed was 30,000 rpm and the temperature was 20°C. The plot of $\log_{10} j(r)$ versus X^2 resulting from this experiment can be seen in Figure 10. The effect of the smaller (minor) component was subtracted out from the total to give the net effect of the major component. This was done using a CDC 3300 computer and the program ESUB.[1]

Using the partial specific volume noted above and assuming a density of 1.00, the molecular weight of the major component was calculated to be 58,000 grams per mole and the molecular weight of the minor component was calculated to be 26,500 grams/mole.

Effect of Carboxypeptidase-A-DFP Treatment on the Activity of the Enzyme

The procedure followed for this experiment was that of Richards and Rutter (42). No EDTA was used in the buffer. The amount of carboxypeptidase-A-DFP used in both cases was 71 micrograms. The amount of rabbit muscle aldolase, used as a control, was 2.5 mg and the amount of thermophilic aldolase was 1.35 mg. The results of this experiment can be seen in Table 9.

[1]Program of Dr. Walter Hill, Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon.

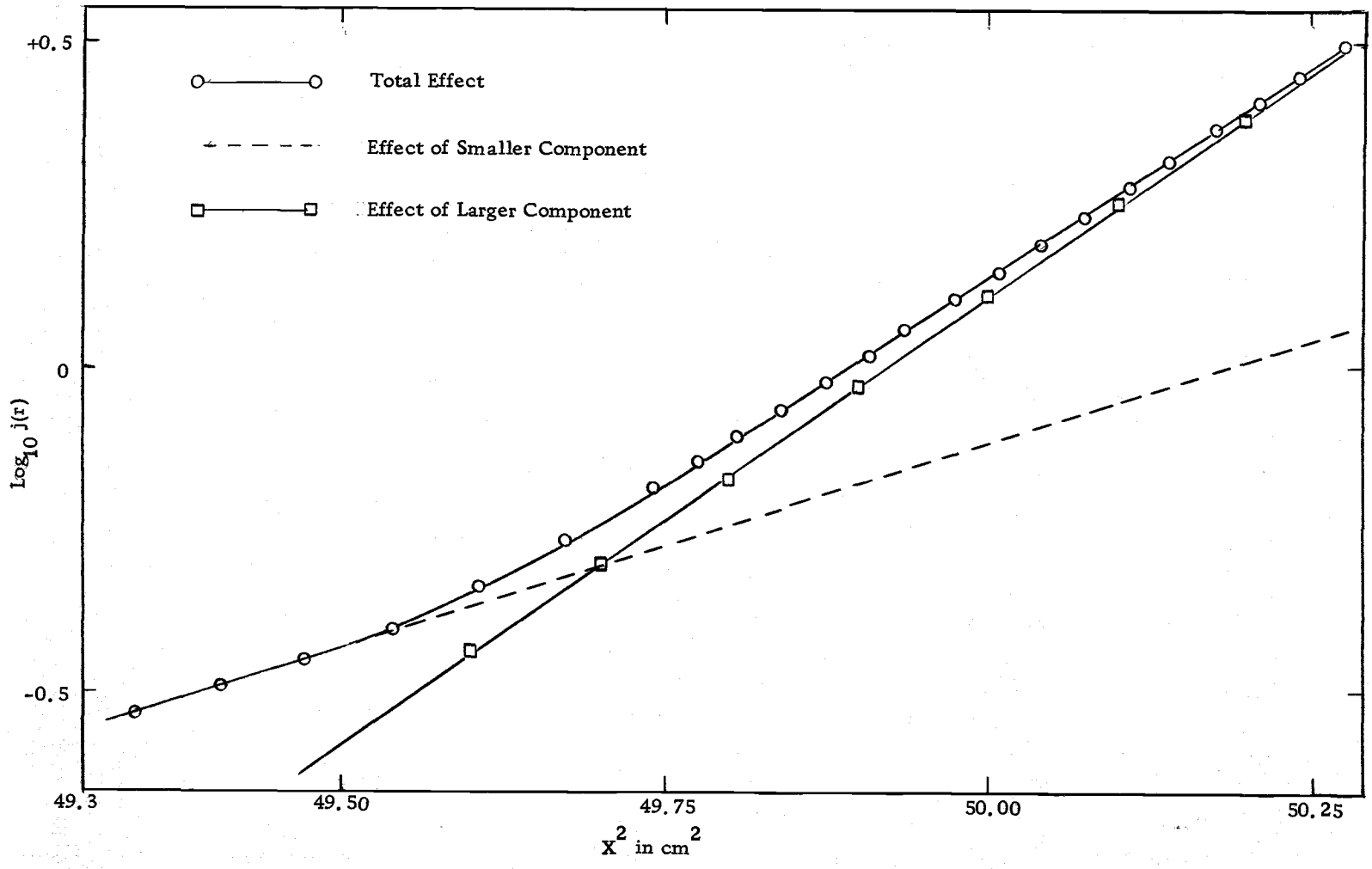

Figure 10. Sedimentation Equilibrium Ultracentrifugation of the Thermophilic Aldolase.

Table 9. The Effect of Carboxypeptidase-A-DFP on the Activities of Rabbit Muscle and Thermophilic Aldolase.

	Activity			
Time	Rabbit muscle	Control[1]	Thermophilic	Control[1]
Initial	4.420	4.420	3.120	3.120
6 min.	0.960	4.420	3.040	3.040
110 min.	0.860	4.360	2.940	3.020

[1] Controls were the same except that 10 percent LiCl was substituted for carboxypeptidase-A-DFP dissolved in 10 percent LiCl.

The Effect of Sodium Borohydride Reduction in the Presence of Substrate on the Activity of the Enzyme

This experiment was performed by incubating approximately 2.5 mg of rabbit muscle aldolase (or approximately 1.5 mg of the thermophilic aldolase) with 1.5×10^{-4} moles of FDP (if used) in a volume of 25 ml at 25°C and pH 7.0. One molar sodium borohydride was added in 0.05 ml increments and the pH was adjusted to 7.0 with 1.7 M acetic acid after each addition. The activity was checked after each four additions. The rabbit muscle aldolase activity was measured using the standard assay, but at 37°C and in the absence of Mn^{++}. The thermophilic aldolase was assayed under the standard conditions. The results of the titration can be seen in Table 10.

The calculated activity ratios (-FDP/+FDP) are 1.79 and 1.03 for the rabbit muscle and thermophilic aldolases, respectively, at this pH.

Table 10. The Effect of Sodium Borohydride Reduction in the Presence of Substrate on the Activity of Rabbit Muscle and Thermophilic Aldolases.

Sodium borohydride added	$\Delta OD_{340\ nm}$ -FDP	$\Delta OD_{340\ nm}$ +FDP
Rabbit muscle aldolase:		
Initial	2.620	2.900
2×10^{-4} moles	2.000	1.560
4×10^{-4} moles	2.000	1.240
Thermophilic aldolase:		
Initial	3.380	3.040
2×10^{-4} moles	3.360	3.248
4×10^{-4} moles	3.480	3.218

Other Studies

Amino Acid Analysis

Amino acid analysis was performed by the method of Moore and Stein (37). The purified protein (1.52 mg in Buffer 1) was transferred to a lyophilization tube (Kontes Glass Co., 5 ml capacity) and after evaporation at reduced pressure, 1 ml of constant boiling HCl was added. The oxygen was displaced by nitrogen, and then the vial was sealed while evacuated. The protein was then hydrolyzed for 18.5 hours in a refluxing toluene bath (110°C). The HCl was removed after hydrolysis by rotary evaporation at reduced pressure, and after rinsing with 1 ml of water and re-evaporation under the same conditions, the sample was taken up in 2.5 ml of

pH 3.48 citrate buffer.

Aliquots of the hydrolysate were chromatographed on a Beckman-Spinco Model 120B Amino Acid Analyzer with an accelerated system (50). The quantity of combined cysteine and cystine was determined by the method of Moore (36). Tryptophan was determined by the method of Edelhoch (21). The results of this analysis can be seen in Table 11.

Table 11. Amino Acid Composition of the Aldolase of Bacillus stearothermophilus.

Amino acid	Calculated no. of residues/molecular weight = 60,000	No. of residues to nearest integer
Alanine	52.02	52
Ammonia	64.80	65
Arginine	20.28	20
Aspartic acid	52.62	53
Cystine/2	6.00	6
Glutamic acid	66.66	67
Glycine	50.52	51
Histidine	11.22	11
Isoleucine	38.64	39
Leucine	35.88	36
Lysine	41.64	42
Methionine	14.10	14
Phenylalanine	16.74	17
Proline	21.78	22
Serine	23.28	23
Threonine	29.34	29
Tryptophan	6.4	6
Tyrosine	16.92	17
Valine	47.76	48

Determination of Q_{10} and Arrhenius Activation Energy

The assay was as usual, but for the higher temperatures a corresponding longer incubation period was allowed for the cuvette to come to temperature before the measurement of activity was initiated. The plot of 1/T(K) versus $\log_{10}$ rate can be seen in Figure 11. The Q_{10} value was calculated from the expression:

$$Q_{10} = \frac{(\log_{10} v_2 - \log_{10} v_1)}{1/T_1 - 1/T_2}$$

where $T_2 = T_1 + 10^{\circ}K$; T in degrees Kelvin.

The value for the Arrhenius activation energy was calculated from the relation:

$$E = -\text{slope} \times 2.303 \times R$$

where R = 1.987 cal/deg/mole

and $$\text{slope} = \frac{d\log_{10} v}{dT/T^2} = \frac{d\log_{10} v}{d(1/T)}$$

where T is the absolute temperature.

The values so obtained are: $Q_{10} = 2.183$ and E = 16,166 cal/mole.

An Activity Stain for the Thermophilic Aldolase

The activity stain of Penhoet, et al. (41) was shown to work properly with rabbit muscle aldolase, but consistently gave a light field with a bleached area at the position where the thermophilic

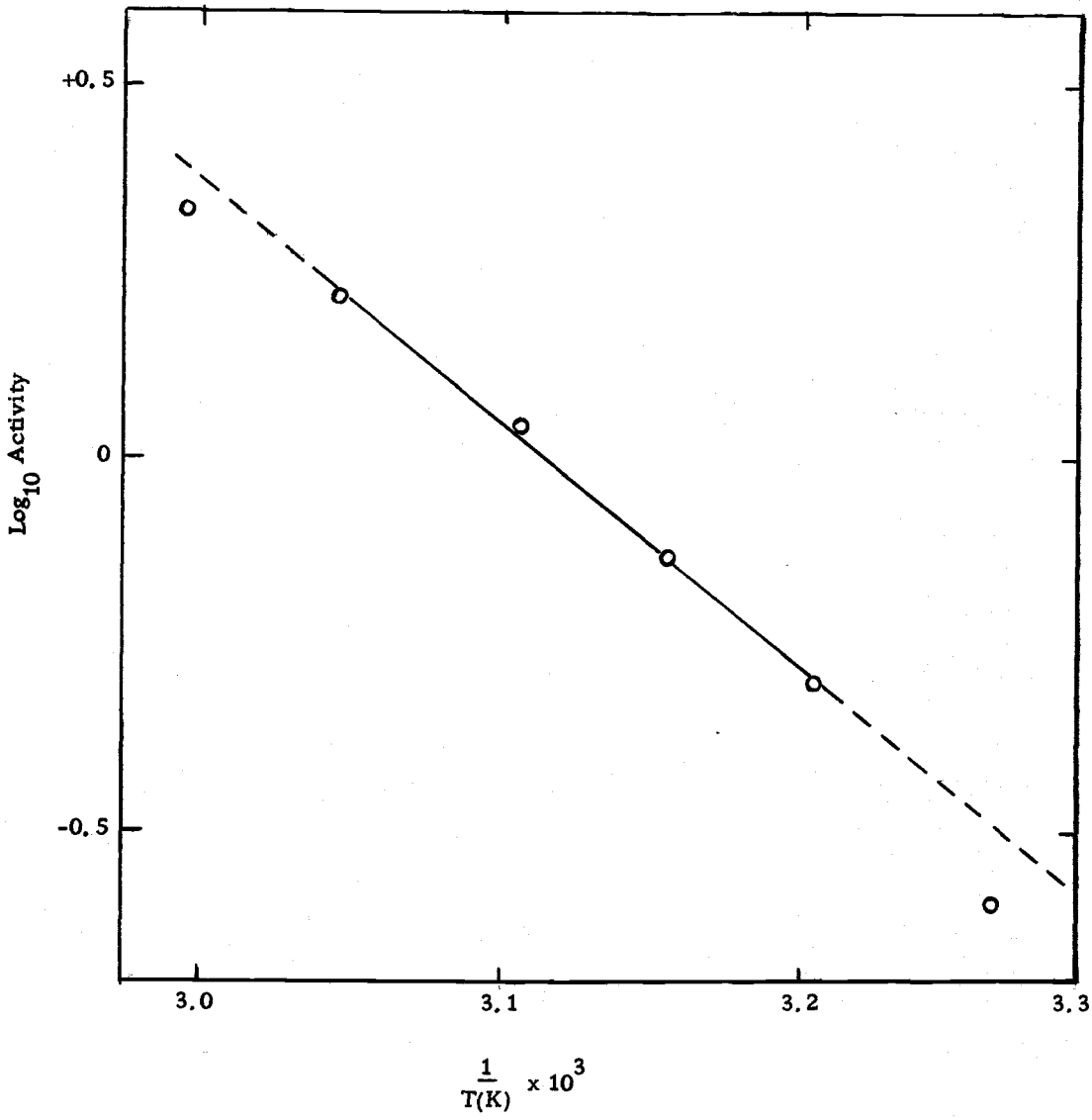

Figure 11. Arrhenius Plot for the Activity of the Thermophilic Aldolase.

aldolase should occur. An activity stain was developed which included manganous ion and was shown to give an adequate stain for the thermophilic enzyme, as well as for the rabbit muscle enzyme. The components and their concentrations can be seen in Table 12. For developing the stain with the thermophilic enzyme, it was found that an incubation of from 20 to 25 minutes at 55 to 60°C in the dark worked adequately.

Table 12. Composition of the Activity Stain for the Thermophilic Aldolase.

Reagent	mg
FDP	10.2
NAD^+	1.0
GPDH	0.5
Manganese sulfate	0.28
Sodium arsenate	55.8
PMS	0.35
NBT	1.0

The final volume was 5 ml.

The last two reagents were added in the dark.

IV. DISCUSSION

Classification of the Thermophilic Aldolase

It was mentioned in the introduction that the aldolases found in plants and the higher animals (Type I) are distinct from those aldolases found in the yeasts, bacteria, and lower animals (Type II). A comparison of the properties of the two types can be seen in Table 13, which shows that the two types are quite distinct in every property listed.

Many organisms have been reported to contain a single FDP-aldolase (either Type I or Type II), but only a few organisms have been determined to possess both types. Rutter (45) reported both types to be present in Euglena glacialis and in Chlamydomonas reinhardti, Russell and Gibbs (43) found both types present in Chlamydomonas mundana, and Antia (3) suggested that both types were present in Anacystis marina. Rutter (45, p. 1255) has suggested one mechanism which would account for this phenomenon:

> We therefore propose that aldolase I arose phylogenetically independently from aldolase II during phylogeny, probably occuring first in a progenitor of Euglena and Chlamydomonas already having a class II aldolase. In the course of evolution (for a reason not yet appreciated) class II aldolase was lost and class I type enzyme was conserved.

The Type I aldolases which have been well characterized are

Table 13. Comparison of the Properties of Type I and Type II Aldolases.

Property	Type I	Type II
Inhibited by chelating agents	no	yes
Metal ion required for full activity	no	yes
SH requirement	no	yes
pH optimum range	c. 2 pH units	c. 0.2 pH units
Activated by potassium ion	no	yes
FDP/F1P specificity	c. 60	c. 2000
K_m	5 to 6 x 10^{-5} M	3 to 15 x 10^{-4} M
V_{max}, moles of FDP cleaved per mole of enzyme per min.	c. 3000	c. 6000
$S_{20,w}$	c. 8	c. 5.5
Molecular weight	140,000	70,000
Effect on enzymatic activity by treatment with carboxypeptidase	inhibited	none
Effect on enzymatic activity upon reduction with sodium borohydride in the presence of FDP	inhibited	none

very constant in their properties and closely parallel the properties of the rabbit muscle enzyme (this enzyme has been chosen by most authors for comparison purposes, since it is the only aldolase which is commercially available). The similarities of these Type I aldolases can be seen in Table 14.

It has already been mentioned that Thompson and Thompson (53) had classified the aldolase from B. stearothermophilus as Type I on the basis of an apparent lack of a metal ion requirement. Consistent with this absence of metal ion activation was the lack of inhibition of the enzyme's activity by several chelating agents (they used 8-hydroxyquinoline and 2,2'-dipyridyl). The present work shows that the thermophilic aldolase does indeed have a metal ion requirement (for Mn^{++}) as shown by the metal ion activation study and the inhibition of the activity of this enzyme by several chelating agents. The reason that Thompson and Thompson did not find this requirement may have been due to an inadequacy of their assay system. Indeed, they found that the aldolase was inhibited by Mn^{++}, as well as by several other metal ions. Using their assay system [the method of Dounce (19)] and an analogous assay system [the method of Sibley and Lehninger (48)], the enzyme has been shown to be inhibited by Mn^{++}. It has also been demonstrated that this metal ion interferes in the color development of both of these assay methods. Dounce (20) reported that rabbit muscle aldolase is inhibited by Cu^{++} using his assay system,

Table 14. Similarity of the Properties of Some Type I Aldolases.

Property[1]	Aldolase source				
	Spinach leaf	Boa constrictor	Drosophilia melanogaster	Spinach chloropla	Rabbit muscle
Inhibited by chelating agents	--	--	no	no	no
pH optimum range	broad	--	broad	broad	broad
K_m (FDP) molar	2×10^{-5}	--	2.7×10^{-5}	6.8×10^{-5}	1.5×10^{-5}
V_{max}, moles of FDP cleaved per minute per mole of enzyme	1584	--	--	3900	3100
Molecular weight	120,000	153,000	125,000	140,000	146,000
FDP/F1P specificity	--	--	10-15	57	60
Enzyme activity inhibited by carboxypeptidase?	yes	--	--	yes	yes
Effect of reduction with sodium borohydride in the presence of FDP	inhibited	--	--	inhibited	inhibited
Reference	23	46	9	11	45

[1] Only those properties listed in Table 13 were used for which there was sufficient data to make a comparison for these enzymes.

Table 15. Similarity of the Properties of Some Type II Aldolases.

Property	Aldolase source			
	Saccharomyces cerevisiae	Clostridium perfringens	Candida utilis	Bacillus stearethermophilus
Inhibited by chelating agents	yes	yes	yes	yes
Activated by a metal ion	yes (Zn^{++})	yes (CO^{++} or Fe^{++})	yes	yes (Mn^{++})
Sulfhydryl requirement	yes	yes	yes	yes
pH optimum range	sharp	sharp	sharp	sharp
Potassium ion activation	yes	yes	yes	--
K_m (FDP) molar	3.7×10^{-4}	3×10^{-4}	8×10^{-5}	1.1×10^{-4}
V_{max}, moles of FDP cleaved per minute per mole of enzyle	6900	5000	5400	4650
S_{20}	5.4S	5.6S	--	4.25S
Molecular weight	70,000	--	67,500	58,000
FDP/F1P specificity	2500	2000	--	--
Enzyme activity inhibited by carboxypeptidase	no	no	--	no
Effect of reduction with sodium borohydride in the presence of FDP	none	none	none	none
References	45	45	31	this work

Table 15. Similarity of the Properties of Some Type II Aldolases.

Property	Aldolase source			
	Saccharomyces cerevisiae	Clostridium perfringens	Candida utilis	Bacillus stearothermophilus
Inhibited by chelating agents	yes	yes	yes	yes
Activated by a metal ion	yes (Zn^{++})	yes Co^{++} or Fe^{++})	yes	yes (Mn^{++})
Sulfhydryl requirement	yes	yes	yes	yes
pH optimum range	sharp	sharp	sharp	sharp
Potassium ion activation	yes	yes	yes	--
K_m (FDP) molar	3.7×10^{-4}	3×10^{-4}	8×10^{-5}	1.1×10^{-4}
V_{max}, moles of FDP cleaved per minute per mole of enzyme	6900	5000	5400	4650
S_{20}	5.4S	5.6S	--	4.25S
Molecular weight	70,000	--	67,500	58,000
FDP/F1P specificity	2500	2000	--	--
Enzyme activity inhibited by carboxypeptidase	no	no	--	no
Effect of reduction with sodium borohydride in the presence of FDP	none	none	none	none
References	45	45	31	this work

In addition to the experiments which were used to type the enzyme, several other experiments were performed. A value of 2.18 was obtained for the Q_{10} of this aldolase and a value of about 16,000 calories per mole was obtained for the Arrhenius activation energy. These values were determined in the range of 36 to 57°C and are quite normal. Also, the amino acid composition of this aldolase has been determined. The significance of the amino acid composition will be discussed below.

The Concept of Hydrophobicity as a Parameter in Thermostability

Several attempts have been made to correlate the properties of protein molecules to their amino acid composition. Bigelow has reviewed these recently (7).

As his parameter for the non-polarity of a protein, Waugh (55) has defined a frequency of non-polar side chains (NPS frequency), which is the number of non-polar residues in a protein divided by the total number of residues of the protein. He found that proteins generally had NPS frequencies varying from 0.21 to 0.47.

Fisher (22) has used as his parameter the polarity ratio (p) of a protein. He defined this ratio as being the ratio of the external volume of the protein molecule (V_e) divided by the internal volume of the molecule (V_i), where only the non-polar amino acids are found

in the internal volume and only the polar residues are found in the external volume.

Bigelow uses the average hydrophobicity (H_{avg}) as his parameter. His calculations are based upon the free energy of transfer of an amino acid residue from an aqueous to a non-polar environment, as determined by Tanford (51). He has found that the range of values obtained for normal globular proteins is from 810 to 1310 calories per residue, with more than half of these falling in the range of from 1000 to 1100 calories per residue.

Bigelow has suggested that the best comparison for the relative hydrophobicity of a particular protein is to compare the average hydrophobicity of this protein to the value obtained for the same protein isolated from a closely related species. Bigelow has done this for the hemoglobins, cytochromes-C, and hemocyanins and has found that the calculated average hydrophobicities, polarity ratios and NPS frequencies are very close to one another.

When comparing the phycocyanins from mesophilic and thermophilic algae [data from Berns, et al. (6)], Bigelow found that the average hydrophobicity and NPS-frequencies are much higher for the thermophilic proteins than for the mesophilic ones. The correlation for the polarity ratio values calculated was very poor. In comparing the average hydrophobicities and NPS frequencies for different α-amylases, Bigelow found that the values of these hydrophobicity

parameters for the thermophilic enzyme (the α-amylase from B. stearothermophilus isolated by Campbell) were much higher than those values calculated for several mesophilic yeasts and bacteria.

Since the average hydrophobicity and NPS frequency seems to be higher for a thermophilic enzyme than for a mesophilic enzyme of the same kind, it was decided to apply this analysis to more recent data on such enzymes. In Table 16 can be seen the values calculated for several bacterial proteases. In this case the same relationship is still present. The same calculations were made for all aldolases whose amino acid compositions have been reported. The calculated values for the hydrophobicity parameters can be seen in Table 17. It can be seen in this table that the average hydrophobicities and NPS frequencies for the aldolases are quite close from one type to another, as well as in the same type group. It should be also noted that the average hydrophobicity for the yeast aldolase is higher than the value for the thermophilic aldolase. It seems, therefore, that the average hydrophobicity and NPS frequency may not be an adequate measure of the relative thermostability of proteins.

Conclusions on the Thermostability of the Thermophilic Aldolase and of Thermophilic Enzymes in General

It was noted in the last section that the calculated average hydrophobicity of the thermophilic aldolase was lower than that of aldolase

Table 16. Hydrophobicity Calculations for Some Bacterial Proteases.

Enzyme source	Average hydrophobicity	NPS frequency	Reference
mesophilic:			
Bacillus subtilis, BPN'	965	0.32	18
Bacillus subtilis, Carlsberg	942	0.30	18
thermophilic:			
Bacillus thermoproteolyticus	1070	0.35	33

Table 17. Hydrophobicity Calculations for Some FDP-aldolases.

Enzyme source	Average hydrophobicity	NPS frequence	Reference
Type I:			
Rabbit muscle	1080	0.32	47
Spinach chloroplast	1043	0.33	11
Boa constrictor	1104	0.34	46
Type II:			
Yeast	1115	0.33	44
B. stearothermophilus	1095	0.33	this work

from yeast, a result which is the opposite of that found in the other thermophilic proteins when they are compared to the corresponding mesophilic proteins [see Bigelow (7) and the preceding section of this thesis]. This suggests that the thermostability of the thermophilic aldolase and possibly other enzymes from thermophilic organisms may be found to depend upon structural factors other than those related generally to the content of hydrophobic amino acid residues.

When comparing the individual amino acid compositions of the thermophilic enzymes for which these data are available, one is unable to make any general correlation besides the fact that the enzymes appear to be slightly higher in the total content of non-polar amino acids (which has been shown not to be a general parameter to be used in relating thermostabilities).

When these individual amino acid compositions are compared to the "average protein" of Smith (49), there are very few places where generalizations can be made. All of these thermophilic enzymes are either normal or slightly low in lysine, phenylalanine, and serine and either normal or slightly high in isoleucine. Each protein is also higher or lower than normal in specific amino acids (such as Campbell's α-amylase being high in proline and the thermophilic protease and phycocyanin being high in tyrosine).

When the thermophilic aldolase is compared on this basis, it is found to be low in proline and slightly high in alanine, glutamic

acid and glycine, as well as being low in leucine, phenylalanine and serine and high in isoleucine (the last four, as was mentioned above, being typical of the other thermophilic proteins as well).

It was mentioned in the introduction that frequency of disulfide bonding did not seem to be a factor in the thermostability of the thermophilic enzymes. The same appears to be true for the thermophilic aldolase. In this enzyme there appears to be two disulfide bonds per molecule at a maximum (there are six half-cystines present and there is evidence for at least one free sulfhydryl group).

One point concerning thermophilic enzymes which has not yet been mentioned is that at least two of these proteins are smaller in size than their mesophilic counterparts. The thermophilic α-amylase has a molecular weight of 15,600 compared to a molecular weight of 48,700 for the α-amylase of Bacillus subtilis and the thermophilic aldolase has a molecular weight of about 58,000 compared to a molecular weight of 82,000 for the yeast aldolase (44). It is as yet unclear whether this observation has any significance.

It will be of interest when sufficient thermophilic aldolase is available to determine the α-helical content and the titration behavior of the tyrosyl groups so that these properties can be compared with the other thermophilic enzymes.

In the introduction it was noted that ribonuclease-A is a very heat-stable enzyme from a non-thermophilic organism. This

compact, globular protein has a temperature optimum for activity near 65°C (27) while having a very low calculated average hydrophobicity of only 870 calories per residue (7).

When ribonuclease-A is compared to the "average protein" of Smith (49) it is found to have a different pattern of amino acid residues than the thermophilic enzymes noted above. It is high in half-cystine, lysine, serine and threonine and is low in arginine, glycine, isoleucine, phenylalanine, and proline. Only the phenylalanine content is typical of the thermophilic enzymes.

Like the thermophilic protease, ribonuclease-A has a fair proportion (half) of its tyrosyl groups which ionize abnormally. It has only a normal complement of this amino acid. Also, like those thermophilic enzymes which have been studied in this regard, ribonuclease-A has a very low α-helical content. Unlike the thermophilic proteins, ribonuclease-A has a fair amount of disulfide bonding (four such bonds in a molecule which has a molecular weight of 13,683). Also, unlike the thermophilic proteins, ribonuclease-A is not resistant to concentrated urea and guanidine hydrochloride solutions. All in all, ribonuclease-A is similar to the thermophilic enzymes in some ways, and since it has been studied so thoroughly, provides a good model for studying the thermostability of one type of thermostable enzyme.

In conclusion, enough experimental evidence is not yet available

to decide whether there are any general structural features for thermophilic enzymes which would account for their thermostability. Many more enzymes will have to be studied and more detailed experiments will have to be performed. At present, it seems doubtful whether any generalizations will be forthcoming. It seems more likely that each thermophilic protein will have its own structural idiosyncrasies. It is entirely feasible that thermophilic enzymes will be found not to depend upon just one major structural factor for their thermostabilities.

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