The purification of fumarylacetoacetate hydrolase.

Holly Kaiyuan Hsiang

University of Windsor

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THE PURIFICATION OF
FUMARYLACETOACETATE
HYDROLASE

BY
HOLLY KAIYUAN HSIANG

A Thesis
Submitted to the Faculty of Graduate Studies through the
Department of Chemistry in Partial Fulfillment
of the Requirements for the Degree of
Master of Science at the
University of Windsor

Windsor, Ontario
1971
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360633

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ABSTRACT

A method for the 180-fold purification of fumarylacetoacetate hydrolase (EC 3.7.1.2.) from beef liver is described. This hydrolase enzyme cleaves a carbon carbon bond in fumarylacetoacetate, an intermediate in the metabolic pathway for the degradation of tyrosine and phenylalanine. An assay procedure based on the ability of the enzyme to hydrolyze 2,4-diketo acids is presented.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. D.E. Schmidt Jr., for his patient encouragement and invaluable guidance throughout this research work.

I also wish to express my appreciation and thanks to Dr. W.P. Aston, and Dr. L.R. Sabina for their helpful criticisms. I am grateful to Miss S. Sim, for her preparation of acetopyruvic acid and to Mr. C.V. Braun for his assistance in preparing this manuscript. Finally, I wish to thank my fellow graduate students for their encouragement and assistance.

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td><strong>EXPERIMENTAL</strong></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><strong>A. MATERIALS</strong></td>
<td>3</td>
</tr>
<tr>
<td></td>
<td><strong>B. METHODS</strong></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1) Preparation of Sephadex G-25 Gel</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2) Preparation of DEAE- and TEAE-cellulose</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>3) Preparation of Bio-Gel P-200</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>4) Spectrophotometric Method for Enzyme Assay</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>5) Determination of Protein Concentration</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>6) Calculations</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>7) Preparation of Crude Enzyme Paste</td>
<td>6</td>
</tr>
<tr>
<td>Chapter</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>8) Enzyme Fractionation Methods</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>9) Vertical Polyacrylamide Gel Electrophoresis</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>10) Pyruvate Determination</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>a) Standard Curve for Pyruvic Acid</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>b) Determination of Pyruvic Acid in Sample</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>c) Kinetic Study of Aceto-pyruvic Acid</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>d) Identification of Pyruvic Acid Produced</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>3 RESULTS</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>A. PURIFICATION</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>B. FORMATION OF PYRUVIC ACID</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>4 DISCUSSION</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>5 SUMMARY</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td>BIBLIOGRAPHY</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>VITA AUCTORIS</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>diethylamino ethyl-cellulose</td>
<td></td>
</tr>
<tr>
<td>DTE</td>
<td>dithioerythritol</td>
<td></td>
</tr>
<tr>
<td>FAAH</td>
<td>fumarylacetoacetate hydrolase</td>
<td></td>
</tr>
<tr>
<td>O.D.</td>
<td>optical density</td>
<td></td>
</tr>
<tr>
<td>TEAE-cellulose</td>
<td>triethylamino ethyl-cellulose</td>
<td></td>
</tr>
<tr>
<td>THAM</td>
<td>tris(hydroxymethyl)aminomethane</td>
<td></td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.</td>
<td>The Protein Profile from TEAE-cellulose Chromatography</td>
<td>18</td>
</tr>
<tr>
<td>2.</td>
<td>A Protein Profile from Bio-Gel P-200 Chromatography</td>
<td>20</td>
</tr>
<tr>
<td>3.</td>
<td>The Comparison of Reaction Rate between Substrate Decomposition by FAAH and Product Formation</td>
<td>22</td>
</tr>
<tr>
<td>4.</td>
<td>Standard Curve of the 2,4-dinitrophenylhydrazone Pyruvic Acid Derivative in the Concentration Range of 1x10^-9 moles/ml</td>
<td>26</td>
</tr>
<tr>
<td>5.</td>
<td>Standard Curve of the 2,4-dinitrophenylhydrazone Pyruvic Acid Derivative in the Concentration Range of 1x10^-9 moles/ml to 6x10^-8 moles/ml</td>
<td>28</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Purification of Fumarylacetoacetate Hydrolase</td>
<td>16</td>
</tr>
<tr>
<td>II</td>
<td>Hydrolysis of Acetopyruvic Acid by Fumarylacetoacetate Hydrolase</td>
<td>23</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

A method of purification of triacetic acid-hydrolyzing enzyme was described in 1949 by Connors and Stotz (1). The purity of the enzyme from fresh beef liver was increased 100-fold by a combination of alcohol and acetone fractionations and a heat denaturation step. They have also studied the specificity of this enzyme. The enzyme effectively hydrolyzed 2,4-diketo acids and 3,5-diketo acids. A proposed reaction sequence of this enzyme catalysis was shown to be

\[
\text{CH}_3\text{CCH}_2\text{CCH}_2\text{CO}_2\text{H} \rightarrow \text{CH}_3\text{CCH}_2\text{CO}_2\text{H} + \text{CH}_3\text{CO}_2\text{H}
\]

triacetic acid    acetoacetic acid    acetic acid

In the previous year, Mäister and Greenstein (2) indicated that an enzyme, which they called acylypyruvase, hydrolyzes 2,4-diketo acids into pyruvic acid and the corresponding fatty acid. This acylypyruvase is plentiful in animal liver and kidney. The hydrolysis reaction is as follows:

\[
\text{R-CCH}_2\text{CCO}_2\text{H} \rightarrow \text{R-CO}_2\text{H} + \text{CH}_3\text{CCO}_2\text{H}
\]

pyruvic acid

Further work (3,4,5,6) on these two enzymes indicated
that they were identical and were probably fumarylacetoacetate hydrolase (FAAH) (EC 3.7.1.2) which hydrolyzes fumarylacetoacetate acetic acid to fumaric acid and an acetoacetic acid.

\[
\begin{align*}
\text{fumarylacetoacetic acid} & \quad \text{fumaric acid} \\
\text{H} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{CO}_2\text{H} & \quad \text{H} & \quad \text{C} & \quad \text{C} & \quad \text{CO}_2\text{H} & \quad \text{CH}_3\text{CCH}_2\text{CO}_2\text{H} \\
\text{HO}_2\text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{C} & \quad \text{CO}_2\text{H} & \quad \text{H} & \quad \text{C} & \quad \text{C} & \quad \text{CO}_2\text{H} & \quad \text{H} & \quad \text{O} \\
\text{H} & \quad \text{H} & \quad \text{H}_2 & \quad \text{H}_2 & \quad \text{FAAH} & \quad \text{H} & \quad \text{H} & \quad \text{H} & \quad \text{H} & \quad \text{H} & \quad \text{CH}_3\text{CCH}_2\text{CO}_2\text{H} \\

\end{align*}
\]

Some new methods for a greater purification of FAAH are presented in this paper and an assay method for FAAH activity is described using acetopyruvic acid instead of its natural substrate. The purification of this enzyme was undertaken so that the mechanism for the rather unique enzymatic cleavage of a carbon-carbon bond by hydrolysis could be studied.
CHAPTER 2

EXPERIMENTAL

A. MATERIALS

The following materials were obtained commercially:
tris(hydroxymethyl)aminomethane, disodium hydrogen phosphate,
sodium chloride, ammonium persulfate (Fisher Scientific Co.);
ammonium sulfate (Mallinckrodt Chemical Works. Ltd.);
dithioerythritol (Division Travenol Lab. Inc.); pyruvic acid
(99%), concentrated hydrochloric acid (grade A) (Matheson,
Coleman and Bell Co.); 2,4-dinitrophenylhydrazine, acryl-
amide, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylenediamine (Eastman Organic Chemicals Inc.);
concentrated phosphoric acid (Baker and Adamson Scientific Co.);
Sephadex G-25 gel (Pharmacia, Sweden); diethylamino ethyl cellulose,
triethylamino ethyl cellulose (Sigma Chemical Co.); Bio-
Gel P-200 (Bio-Rad Lab.). All solutions were made up in
deionized water.

THAM was acidified with concentrated hydrochloric acid
grade A. Disodium hydrogen phosphate was acidified by con-
centrated phosphoric acid. All pH values were measured on
a Radiometer pH meter Model 26 (Radiometer Copenhagen, Den-
mark).

Acetopyruvic acid was synthesized in this laboratory
by Miss Susan Sim (7).
B. METHODS

1) Preparation of Sephadex G-25 gel

Ten grams of Sephadex G-25 gel (medium) was allowed to swell in 500 ml of 0.025M THAM buffer, pH 8.5, containing $1 \times 10^{-4}$ M DTE. The suspension was allowed to stand 4 hours and degassed before being poured into a glass column with a scintillated glass filter on the bottom (inside diameter 2.1 cm). The length of resin bed was 22 cm. Two liters of the above buffer was run through the column before use.

2) Preparation of DEAE- and TEAE-Cellulose

The method of Sere and Kosicki (8) was followed with one additional step to prepare the resins for use. As a final step of their method two liters of deionized water with $1 \times 10^{-4}$ M DTE and one liter of 0.02M THAM buffer, pH 8.5, containing $1 \times 10^{-4}$ M DTE were used to wash the column until the effluent had the same ionic strength as measured by conductivity bridge Model RC-16B1 (Industrial Instruments Inc., New Jersey). After the cellulose column (2.1 cm in diameter, 22 cm in resin length) was used for a protein separation, the same series of cleaning steps were performed for the next packing.

3) Preparation of Bio-Gel P-200

Twenty grams of dry Bio-Gel P-200 (50-100 mesh) was allowed to swell in two liters of 0.5M THAM buffer, pH 8.5, containing $1 \times 10^{-4}$ M DTE overnight. The fully hydrated gel was packed into a K25/45 column (Pharmacia, Sweden) fitted with the appropriate flow adaptors. The inside diameter of column was 2.5 cm, the height of gel was 40 cm.
4) **Spectrophotometric Method for Enzyme Assay**

The buffer used was 0.025\text{M} sodium phosphate, pH 7.3. Acetopyruvic acid $1.22 \times 10^{-3}$M dissolved in the sodium phosphate buffer was used as a substrate. The substrate solution was stored at $4^\circ$C.

Into a 3 ml cuvette were pipetted 2.6 ml of sodium phosphate buffer and 0.3 ml of acetopyruvic acid solution. The final concentration of acetopyruvic acid was $1.22 \times 10^{-4}$ M. To this solution was added 0.05 ml of enzyme solution and the disappearance of substrate recorded at 295 nm for 10 to 15 minutes. Three milliliters of buffer was used as a blank. All enzyme activity determinations were performed on a Beckman spectrophotometer Model DU with a Gilford recorder.

5) **Determination of Protein Concentration**

Into a one milliliter cuvette was pipetted an appropriate amount of the enzyme solution and this was diluted to one milliliter with sodium phosphate buffer. The optical density readings were recorded at 260 nm and 280 nm. Sodium phosphate (0.025M, pH 7.3) was used as a blank. The concentration of protein in milligrams per milliliter was determined using the nomograph of Warburg and Christian (9).

6) **Calculations**

The unit of enzyme activity is defined as:

$$\frac{\Delta \text{O.D.}}{300 \text{ sec.}} = \frac{\text{total volume of enzyme solution}}{\text{volume of enzyme solution used in assay}}$$

The specific activity is expressed as the ratio of enzyme activity to protein content in milligrams per milliliter as determined by the method of Warburg and Christian.
(9).

After each fractionation step the measurements of recovery and extent of purification are determined.

Thus the recovery equals:

\[
\frac{\text{total activity after fractionation}}{\text{total original activity}} \times 100\%
\]

and the fold purification equals:

\[
\frac{\text{total specific activity after fractionation}}{\text{total original specific activity}}
\]

7) **Preparation of Crude Enzyme Paste**

A fresh beef liver was brought directly from a slaughter-house in an ice container to a cold room (4°C). The liver was cut into small cubes and the white connective tissue discarded. All cubes were passed through a meat grinder. Each 200 gm portion of ground liver was then mixed with 400 ml of ice-cold 0.85% sodium chloride solution containing 1x10^{-4} M DTE, in a Waring Blender, and homogenized for two minutes. The mixed homogenates from approximately 10 lb. of tissue (18 runs) were allowed to stand 3 to 5 hours. The total volume was about nine liters. To 800 ml of homogenate were added 890 ml of ice-cold 95% ethyl alcohol, with stirring, to obtain a final concentration of 50% alcohol. The mixture was allowed to stand overnight. It was then centrifuged at 16,300x g for 5 minutes and the solid material discarded. Total volume was fifteen liters.

To 1000 ml of the clear yellow or red solution were mixed 800 ml of ice-cold 95% ethyl alcohol to produce an alcohol concentration of 70%. The solution was allowed
to stand overnight while the enzyme precipitated. The supernatant fluid was carefully poured out and the precipitated protein was packed by centrifugation at 16,300 x g for 5 minutes. Ten pounds of fresh beef liver gave approximately 120 gm of precipitated protein. This crude enzyme paste could be stored at -20°C for periods as long as one month without appreciable loss of activity.

8) Enzyme Fractionation Methods

To 24 gm of enzyme paste were added 40 ml of 0.025M sodium phosphate buffer, pH 7.3, containing 1x10⁻⁴ M DTE. The suspension was stirred for 30 minutes. The supernatant was recovered by centrifugation at 16,300 x g for ten minutes. A second extraction of the centrifuged precipitate was performed with 10 ml of buffer. The two portions were combined and solid ammonium sulfate (20 gm) was dissolved in the clear extract to a final concentration of 40% (w/v). The mixture was allowed to stand for 4 hours and centrifuged.

The precipitated protein was extracted with stirring for 30 minutes with 7 ml of 0.02 M THAM buffer, pH 8.5, containing 1x10⁻⁴ M DTE. After centrifugation at 16,300 x g for 5 minutes the precipitated material was discarded and the supernatant chromatographed on Sephadex G-25. The column was developed with 0.02 M THAM, pH 8.5, containing 1x10⁻⁴ M DTE at the flow speed of 100 ml per hour and ten milliliter fractions were collected. Enzyme activity was usually eluted in the third and fourth fractions.

The fluid containing active enzyme was placed onto
a column of TEAE-cellulose. Ten milliliter fractions were collected at a flow speed of 60 ml per hour. Two hundred milliliters of 0.02 M THAM buffer, pH 8.5, containing $1 \times 10^{-4}$ M DTE were run through the column. After the 200 ml effluent was obtained, an exponential gradient was started with 300 ml of THAM buffer ($1 \times 10^{-4}$ M DTE) (0.2 M, pH 8.5) and 100 ml of THAM buffer ($1 \times 10^{-4}$ M DTE) (0.02 M, pH 8.5). Enzyme activity was assayed for in each third fraction and those fractions between fractions 31 to 35 which were observed to contain protein. Specific activity and units were calculated. All fractions having high specific activity, usually between fractions 31 and 35, were mixed and the protein precipitated by the addition of sufficient ammonium sulfate to give a final concentration of 40% (w/v). After standing overnight, the white precipitate was compacted by centrifugation. To the small amount of this precipitate was added 5 ml of 0.2 M THAM buffer, pH 8.5, containing $1 \times 10^{-4}$ M DTE. After stirring for five to ten minutes, the suspension was centrifuged and the supernatant was used for the Bio-Gel P-200 separation.

The clear supernatant was made to 5% (w/v) sucrose and pumped onto the bottom of a Pharmacia K25/45 column (10) packed with Bio-Gel P-200. The column was developed by an upward flow at a rate of 40 ml per hour. Five milliliter of degassed 0.5M THAM buffer, pH 8.5, containing $1 \times 10^{-4}$ M DTE with 0.5 gm sucrose dissolved (10% sucrose solution) in the buffer was pumped onto column following sample applica-
tion. Another 500 ml degassed 0.5 M THAM buffer, pH 8.5, containing 1x10^{-4} M DTE was then used to elute the protein. Ten milliliter fractions were collected. The protein was usually eluted in the tenth to twelfth fractions. Enzyme assays were performed on every fraction. To the combined enzymatically active fractions was added ammonium sulfate to a final concentration of 40% of saturation. The cloudy solution was allowed to stand overnight. The small amount of precipitate was compacted by centrifugation and dissolved in 3 ml of 0.5 M THAM buffer, pH 8.5, containing 1x10^{-4} M DTE.

9) Vertical Polyacrylamide Gel Electrophoresis

A stock of solution of 30% acrylamide in 0.05 M THAM at pH 7.4 was prepared by dissolving 0.3 gm/ml acrylamide, 0.002 gm/ml N,N'-methylenebidacrylamide and 0.0046 ml/ml N,N,N',N'-tetramethylenediamine in the buffer.

Immediately prior to use the stock solution was diluted with the THAM buffer to give a concentration of 7.5% acrylamide. Ammonium persulphate (400\times 0.4 gm/ml) was added with stirring to a final concentration 1.0x10^{-3}gm/ml (11).

Samples were subjected to electrophoresis at pH 9.0 and 300 volts and 4-6 milliamps per cm for 4-5 hours (12). The protein components were detected by staining the gel with Amido Black (13) and subsequent destaining with a mixture of methanol, acetic acid and water (5 : 1 : 5 by volume).
10) Pyruvate Determination

a) Standard Curve for Pyruvic Acid

Pure pyruvic acid was prepared by three redistillations of 99% pyruvic acid in vacuo, the fraction boiling at 52°C - 55°C/13 mmHg, being collected. The clear solution was cooled to 4°C. One milliliter of pure pyruvic acid solution was carefully pipetted into a weighing bottle (1 ml = 1.2524 gm) and then dissolved in 250 ml of ice-cold deionized water. The acid solution was then neutralized with 1 N sodium hydroxide. Care was taken not to allow the temperature to rise above 4°C. The final concentration was 5.69 x 10^{-5} moles/ml. Two milliliters of this solution was diluted to 100 ml with ice-cold deionized water to reach a final concentration of 1.138 x 10^{-6} moles/ml. Into 7 ten milliliter volumetric flasks, aliquots of 0.1 ml, 0.3 ml, 0.5 ml, 0.7 ml, 1 ml, 1.2 ml and 1.5 ml were carefully pipetted from the pyruvic acid solution of 1.138 x 10^{-6} moles/ml concentration. Ice-cold 0.025 M sodium phosphate buffer, pH 7.3, was used for dilution to 10 ml. All solutions were kept at 0°C. The concentrations were 1.14 x 10^{-8}, 3.41 x 10^{-8}, 5.69 x 10^{-8}, 7.97 x 10^{-8}, 1.14 x 10^{-7}, 1.36 x 10^{-7}, 1.71 x 10^{-7} moles/ml.

For further dilution, 0.3 ml and 0.6 ml were pipetted from the flask of 1.14 x 10^{-7} moles/ml concentration and diluted to 10 ml with cold sodium phosphate buffer. The final concentration were 3.41 x 10^{-9} and 6.83 x 10^{-9} moles/ml.

Duplicate 2 ml portions of the above nine different concentrations of pyruvic acid were pipetted into
eighteen clean tubes and 1 ml of 0.1% 2,4-dinitrophenylhydrazine solution (prepared in 2 N hydrochloric acid solution) was added to each tube. A pale yellow color developed in at least 10 minutes. The solutions were allowed to stand for 30 minutes. A portion of 4 ml of 1 N sodium hydroxide was added, with mixing, into each tube. A red color developed immediately, which was stable for longer than 90 minutes. The pyruvate was determined spectrophotometrically using the tungsten lamp of the Beckman spectrophotometer DU model at 450 nm. Three milliliters of 0.025 M sodium phosphate buffer, pH 7.3, was used as blank. The optical density readings were recorded and plotted against the concentration of pyruvic acid.

b) Determination of Pyruvic Acid in Sample

Into sixteen clean test tubes were added 3 ml solutions of acetopyruvic acid under the same conditions as used in the enzyme assay. The reaction was begun by adding 0.05 ml of enzyme solution and quenched by adding 0.5 ml of concentrated hydrochloric acid at the appropriate time. The reactions were quenched after periods of 3, 5, 10, 15, 25, 35 and 45 minutes. After the concentrated hydrochloric acid was added, the tube was placed into boiling water for one hour to remove interfering ketoacids (14). The solution was neutralized by adding 6 ml of 40% sodium hydroxide and allowed to cool. A portion of 2 ml of sodium phosphate buffer was added for further dilution. The amount of pyruvic acid in the samples was then determined.
by measuring the optical density at 450 nm and using the standard curve previously obtained.

c) Kinetic Study of Acetopyruvic Acid

The same enzyme solution used for the determination of pyruvic acid in (b) was also used for this experiment. The same concentration of acetopyruvic acid as for a normal enzyme assay was also used. Three milliliters of 0.025 M sodium phosphate buffer, pH 7.3, was used as a blank. A full scale deflection of an optical density of 1.00 was set on the spectrophotometer. As soon as the hydrolysis reaction started, the recorder connected to spectrophotometer was switched on and run for one hour. The decrease in optical density at 295 nm divided by the extinction coefficient of acetopyruvic acid, \( \epsilon = 6400 \) (15), gives the decrease in acetopyruvic acid which should be equivalent to the formation of pyruvic acid.

d) Identification of Pyruvic Acid Produced

An aliquot (0.6 ml) of purified enzyme solution was added to 10 ml of 0.2 M THAM buffer, pH 8.5, containing \( 1 \times 10^{-4} \) M DTE and 10 mg acetopyruvic acid. The reaction mixture was incubated at room temperature for 24 hours. Then the freshly prepared 2,4-dinitrophenylhydrazine solution (16) was added and the mixture was allowed to stand for 15 minutes at room temperature. After the yellow solution was heated for approximately 10 minutes on a steam bath yellow crystals formed. These crystals had a melting point of 214°C-217°C and were identified as 2,4-dinitrophenylhydrazone.
derivative of pyruvic acid (2).
CHAPTER 3

RESULTS

A. PURIFICATION

Fumarylacetoacetate hydrolase was assayed after each step. The specific activity of the 0.85% sodium chloride extract was 0.07 units/mg. The purity increased 10-fold and 5-fold after protein precipitations from the 50% and 70% alcohol respectively. Ammonium sulfate precipitation increased the activity about 48-fold. Sephadex G-25 chromatography was necessary to adjust the enzyme solution to the correct ionic strength for chromatography on TEAE-cellulose. TEAE-cellulose chromatography caused an approximate 116-fold purification and also removed the yellow color which was present during all the previous steps. Chromatography on TEAE-cellulose gave a 1.9% yield of active protein. The second ammonium sulfate precipitation increased the activity about 179-fold. Bio-Gel P-200 chromatography did not increase the enzyme activity but was included to ascertain whether the protein was homogeneous by molecular sieve chromatography. The overall yield of active protein using this method was 1%. See Table I for the data pertaining to the purification at each step.

From TEAE-cellulose two protein peaks were obtained as determined by measuring the optical density at 280 nm.
(Figure 1). The first peak showed no enzyme activity at all by the spectrophotometric assay. The second peak contained the specific protein for hydrolyzing the substrate acetopyrubic acid. Both peaks are sharp, and are well separated. The specific activity of each fraction containing enzymatic activity was evaluated.

The active protein emerged from the Bio-Gel P-200 as a single symmetrical peak. In relation to Blue Dextran 2000 (molecular weight around $2 \times 10^6$) the enzyme was retarded during chromatography. The Blue Dextran was eluted from the column in fractions 5-8, and emerging activity was excluded in fractions 9-13 (Figure 2). A recovery of almost 100% of the active protein applied to the column was obtained and the specific activity during this step was virtually unchanged.

B. FORMATION OF PYRUVIC ACID

Using purified enzyme, the rate of hydrolysis of acetopyrubic acid was equal to the rate of pyruvic acid production (Figure 3, Table II). Maximum hydrolysis occurs in the first 25 minutes with an initial acetopyrubic acid concentration of $1.22 \times 10^{-7}$ moles/ml. The standard curve of pyruvate is presented in Figure 4. At high concentrations of pyruvic acid the curve was no longer linear. The part of the line under the concentration of $6.0 \times 10^{-8}$ moles/ml was expanded and is shown in Figure 5. All determinations of the pyruvic acid concentration in the actual enzymatic reaction (Method 10b)
<table>
<thead>
<tr>
<th>Fractionation Steps</th>
<th>Enzyme Activity</th>
<th>Yield %</th>
<th>Specific Activity</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) extraction by sodium chloride</td>
<td>28,000</td>
<td>100</td>
<td>0.075</td>
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</tr>
<tr>
<td>2) 50% ethanol precipitation</td>
<td>10,000</td>
<td>35.7</td>
<td>0.8</td>
<td>10.6</td>
</tr>
<tr>
<td>3) 70% ethanol precipitation</td>
<td>1,000</td>
<td>3.57</td>
<td>0.4</td>
<td>5.3</td>
</tr>
<tr>
<td>4) ammonium sulfate precipitation</td>
<td>815</td>
<td>2.9</td>
<td>3.6</td>
<td>48</td>
</tr>
<tr>
<td>5) Sephadex G-25 gel chromatography</td>
<td>685</td>
<td>2.45</td>
<td>3.6</td>
<td>48</td>
</tr>
<tr>
<td>6) TEAE-cellulose chromatography</td>
<td>525</td>
<td>1.88</td>
<td>8.7</td>
<td>116</td>
</tr>
<tr>
<td>7) ammonium sulfate precipitation</td>
<td>310</td>
<td>1.11</td>
<td>13.4</td>
<td>178.6</td>
</tr>
<tr>
<td>8) Bio-Gel P-200 chromatography</td>
<td>308</td>
<td>1.10</td>
<td>13.4</td>
<td>178.6</td>
</tr>
<tr>
<td>9) ammonium sulfate precipitation</td>
<td>300</td>
<td>1.07</td>
<td>13.5</td>
<td>180</td>
</tr>
</tbody>
</table>
Fig. 1. The protein profile from TEAE-cellulose chromatography.

The data was obtained with 1 ml of sample taken from each third 10 ml fraction. One milliliter of 0.025 M sodium phosphate buffer (pH 7.3) as a blank.

0 : Optical Density at 280 nm.
● : Specific Activity.
Figure 1

VOLUME of EFFLUENT $\times 10^{-1}$ milliliter

OPTICAL DENSITY at 280 nm $\times 10$

SPECIFIC ACTIVITY

units/mg
Fig. 2. A protein profile from Bio-Gel P-200 chromato-
tography.

The data was obtained with 1 ml of sample out of each 10 ml
fraction and using 0.025 M sodium phosphate buffer (pH 7.3)
as a blank.

0 : Optical Density at 280 nm.
• : Specific Activity.
Fig. 3. The comparison of reaction rate between substrate decomposition by FAAH and product formation. The substrate is acetopyruvic acid, product is pyruvic acid. o: disappearance of acetopyruvic acid. ●: appearance of pyruvic acid.
Figure 3

[Graph showing the relationship between concentration (10^-8 moles/ml) and incubation time (minutes). The graph plots data points over time, with a trend line indicating a possible exponential increase.]
Hydrolysis of Acetopyruvic Acid by Fumarylacetoacetate Hydrolase

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>The Amount of Acetopyruvic Acid Hydrolyzed (10^-8 moles/ml)</th>
<th>The Amount of Pyruvic Acid Produced (10^-8 moles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6</td>
<td>2.39</td>
</tr>
<tr>
<td>3</td>
<td>2.31</td>
<td>4.65</td>
</tr>
<tr>
<td>5</td>
<td>3.2</td>
<td>6.95</td>
</tr>
<tr>
<td>10</td>
<td>5.35</td>
<td>8.89</td>
</tr>
<tr>
<td>15</td>
<td>6.95</td>
<td>10.1</td>
</tr>
<tr>
<td>25</td>
<td>8.89</td>
<td>11.02</td>
</tr>
<tr>
<td>35</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>11.02</td>
<td></td>
</tr>
</tbody>
</table>
were determined by using the straight portion of this curve. The formation of pyruvic acid was identified by isolation as the 2,4-dinitrophenylhydrazone pyruvic acid derivative which was recrystallized from ethyl acetate. The melting point of the isolated compound was 214°C - 217°C. The reported melting point of the 2,4-dinitrophenylhydrazone derivative of pyruvic acid is 214°C (2).
Fig. 4. Standard curve of the 2,4-dinitrophenylhydrazone pyruvic acid derivative in the concentration range of $1 \times 10^{-9}$ moles/ml to $1.6 \times 10^{-7}$ moles/ml. The data was obtained with 2 ml of pyruvic acid, 1 ml of 2,4-dinitrophenylhydrazine (0.1%) and 4 ml of 1 N sodium hydroxide solution.
Fig. 5. Standard curve of the 2,4-dinitrophenylhydrazone pyruvic acid derivative in the concentration range of 1 x 10^{-9} moles/ml to 6.0 x 10^{-8} moles/ml.
The data was obtained with 2 ml of pyruvic acid solution, 1 ml of 0.1% 2,4-dinitrophenylhydrazine and 4 ml of 1 N sodium hydroxide solution.
CHAPTER 4

DISCUSSION

Connors and Stotz (1) established that the triacetate acid hydrolyzing enzyme was stable between pH 6.0-8.5. All procedures were carried out within this pH range.

The method presented in this thesis utilized a combination of alcohol precipitation, salt precipitation and chromatographic separations on TEAE-cellulose and Bio-Gel P-200. It was initially observed that the heat denaturation step adopted by Connors and Stotz (1) which followed extraction of the enzyme paste by 70% ethanol did not improve the purity of the active protein. It was found that a combination of salt precipitation and ion exchange chromatography gave better results.

In addition to chromatography on TEAE-cellulose and Bio-Gel P-200 fractionations on calcium phosphate, Sephadex G-100, G-150 and G-200 and DEAE-cellulose were attempted. It was found that the enzyme could not be recovered in an active form from a column of calcium phosphate (2.5 x 20 cm) at either pH 6.0 or 7.0 using sodium phosphate buffers, each containing $1 \times 10^{-4}$ M DTE, of concentration 0.02 M, 0.025 M, 0.1 M and 0.5 M. DEAE-cellulose caused nearly the same extent of purification of FAAH as TEAE-cellulose did.
Molecular sieve chromatography was utilized in an attempt to further purify the active protein obtained from TEAE-cellulose ion exchange chromatography. Bio-Gel P-200 was used in preference to Sephadex G-100, G-150 or G-200. It was found that no active protein could be eluted from columns (2.5 x 20 cm) of Sephadex G-100, G-150 or G-200 using a 0.1 M THAM buffer, pH 8.5, containing 1 x 10^{-4} M DTE. Whereas using the Bio-Gel P-200 almost 100% recovery of active protein was obtained.

The purified protein migrated as a single band on polyacrylamide gel electrophoresis (7.5%). As the active protein also chromatographed as a single peak on Bio-Gel P-200, it is presumed that the FAAH prepared is essentially homogeneous.

In the enzyme assay acetopyruvic acid was used as a substrate due to its ready availability (2). Although the natural substrate for FAAH is fumarylacetacetic acid, this compound was extremely difficult to obtain from biological material (6). In the study of enzymatic hydrolysis of 2,4-diketo acids (2), the pH optima for hydrolysis of acetopyruvic acid are in the range 7.2 to 7.6. Therefore, the enzyme assay was performed at pH 7.3.

A specific and sensitive micro-estimation of pyruvic acid was adopted (17). Other keto acids present in the mixture were removed by the method of Elgant and Nelson (14). A solution of 0.1% 2,4-dinitrophenylhydrazine has been used to form a derivative with pyruvic acid which could be determined spectrophotometrically. A yellow color developed on the formation of the 2,4-dinitrophenylhydrazone derivative.
of pyruvic acid which changes to red color in basic solution. The pyruvic acid derivative has maximum spectroscopic sensitivity at a wavelength of 450 nm. When the concentration of pyruvic acid is higher than $6 \times 10^{-8}$ moles/ml, the optical density readings do not obey the Beer-Lambert Law. This fact was also demonstrated by Meister and Greenstein (2).

It is necessary to prepare carefully the concentration of acetopyruvic acid within the range of $1 \times 10^{-9}$ moles/ml to $6 \times 10^{-8}$ moles/ml, so that the amounts of pyruvic acid produced would be read directly from the linear portion of the standard curve. Since the rate of acetopyruvic acid disappearance was equivalent to the rate of appearance of pyruvic acid is shown in Figure 3, the following enzymatic reaction presumably occurs:

$$\text{acetopyruvic acid} \rightarrow \text{acetic acid} + \text{pyruvic acid}$$

A similar reaction scheme was proposed by Krebs and Johnson (18) for acylpyruvase catalysis. The amount of acetic acid was not analyzed in this experiment. The production of pyruvic acid was isolated and identified as crystals of its 2,4-dinitrophenylhydrazone derivative with a m.p. of 214°C (2).

The purified protein could be classified as an acylpyruvase and is most probably fumarylacetoacetate hydrolase (EC 3.7.1.2). Further identification of the properties
and mechanism of FAAH are being studied.
A serial method for purification of FAAH from beef liver has been illustrated. Alcohol fractionation, ammonium sulfate fraction and TEAE-cellulose increase the purity of protein about 180 times over the crude extraction. The yield of protein is around 1% compared to the 0.85% sodium chloride extraction. Acetopyruvic acid has been studied as a substrate for enzyme activity. Pyruvic acid determinations indicate that the enzyme splits acetopyruvic acid into two molecules, one of which is pyruvic acid and the other presumably acetic acid. The formation of pyruvic acid has been demonstrated by the isolation and identification of a 2,4-dinitrophenylhydrazone derivative.

\[
\begin{align*}
\text{CH}_2\text{CCH}_2\text{CO}_2\text{H} & \rightarrow \text{CH}_3\text{CO}_2\text{H} + \text{CH}_3\text{CO}_2\text{H} \\
\text{acetopyruvic acid} & \rightarrow \text{acetic acid} + \text{pyruvic acid}
\end{align*}
\]
BIBLIOGRAPHY


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