## PURIFICATION OF GLUTAMATE OXALACETATE TRANSAMINASE FROM BOVINE MUSCLE

### THEORY

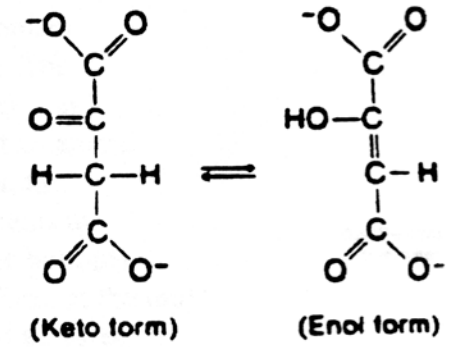
The detailed study of enzyme mechanisms requires use of purified if not homogeneous enzymes. This experiment presents some procedures generally useful in enzyme purification and methods employed for determining if a procedure is usable. The purification of any new enzyme is a separate problem in itself, but some of the general principles used in this experiment are often applicable to other enzymes.

One of the most important requirements of an enzymological study, both in purification and in studying mechanisms, is an enzyme assay that is rapid and accurate. This experiment employs one of the most common methods used in enzyme assay, measurement of a change in the absorbance of an assay mixture as a reaction proceeds.

Glutamate oxalacetate transaminase (GOT) catalyzes the reaction of Equation 1:

(1) L-aspartate + $\alpha$-ketogluterate $\leftrightarrow$ oxalacetate + L-glutamate

If you have access to a spectrophotometer capable of measuring absorbance at 256 nm, you can follow the formation of oxalacetate directly because oxalacetate contains a doubly activated methylene group that participates in the following equilibrium:




At basic pH values favored by GOT (e.g., pH 8.3) this keto-enol equilibrium is displaced in favor of the enol, which absorbs maximally at 256 nm (in the UV range), providing a direct assay for oxalacetate formation. In contrast to direct assay, this experiment determines oxalacetate indirectly through use of a second or indicator reaction catalyzed by malic dehydrogenase (MDH, Equation 10-2).

(2) oxalacetate + NADH + $H^+$ $\leftrightarrow$ L-malate + $NAD^+$

Valid use of such indicator reactions requires that the indicator reaction not be rate limiting. The indicator reaction of this experiment, or that reaction catalyzed by malic dehydrogenase, employs saturating levels (or great excess) of NADH and MDH in order to avoid being rate limiting. Thus, the rate of NADH oxidation, as measured by decrease in absorbance at 340 nm (in the visible range) represents the rate of oxalacetate production in the GOT catalyzed reaction.

The glutamate dehydrogenase (GDH) reaction (Equation 10-3) can interfere with this coupled assay.

(3) $\text{NADH} + \alpha\text{-ketogluterate} + \text{NH}_4^+ \leftrightarrow \text{glutamate} + \text{NAD}^+$

All of the substrates for GDH are added in the assay except ammonia, but ammonia may be present in crude extracts and is added (as ammonium sulfate) in some steps. Because GOT activity is dependent on aspartate, you can determine the extent of interference from GDH in a simple control experiment in which you omit aspartate from the assay mixture. Interference by GDH should not be a serious problem in this purification procedure because GDH activity is primarily found in the mitochondria which are removed during the first step of the fractionation.

The following reaction shows the mechanism of transamination as it is believed to occur. The pyridoxal phosphate bound to the enzyme acts as an amino group carrier in the conversion of an amino acid to an α-keto acid (Figure 10- 1). Thus, the enzyme oscillates between a form that contains an amino group (the pyridoxamine form, at the bottom) and the form that does not (the pyridoxal form, at the top). The pyridoxal form is believed to occur not as a free aldehyde, but rather linked as an aldimine (Schiff's base) to the ε-amino group of a specific lysine residue in the. protein chain. The addition of α-ketoglutarate to GOT causes the enzyme to be present largely as the pyridoxal form, which is most stable to heat. The shift of GOT to the pyridoxal form by excess α-ketoglutarate is the result of reaction with any pyridoxamine form present to release glutamate and pyridoxal enzyme (see Figure 10-1). The substrate analog, maleate, binds to the pyridoxal form of the enzyme to yield a complex. An excess of maleate, a "dead-end" inhibitor, can therefore by used to stabilize GOT in the presence of α-ketoglutarate to make it extremely heat stable. Thus, an important step in the purification of GOT is heat denaturation of other proteins and the resultant precipitation while transaminase is stabilized.

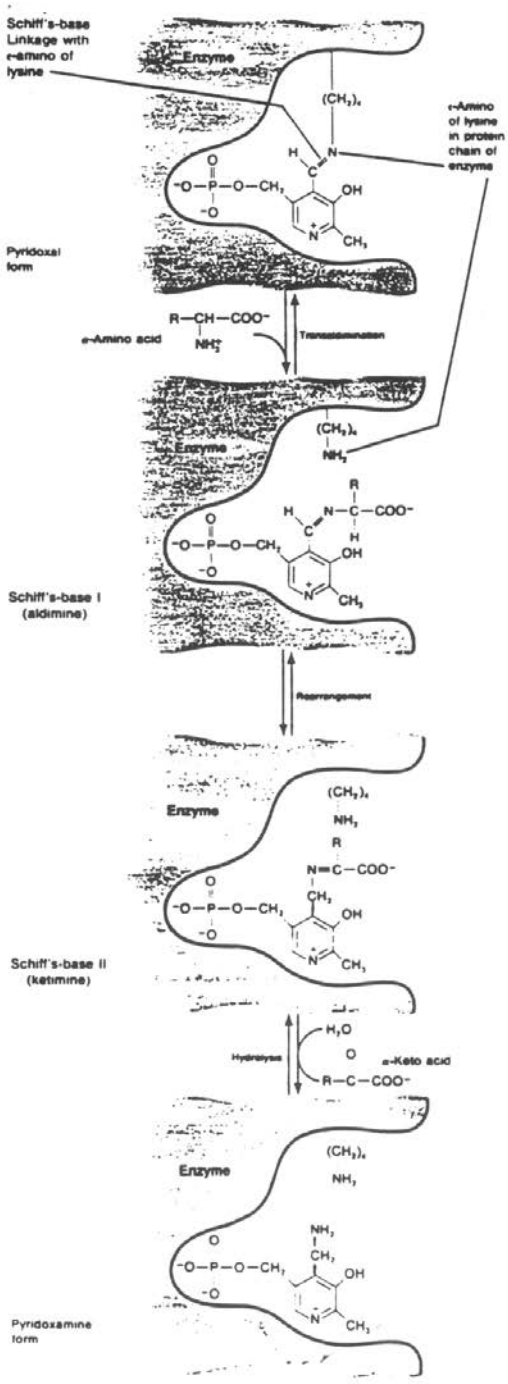



**FIGURE 10-1**
Mechanism of transamination catalyzed by glutamate oxaiacetate transaminase.

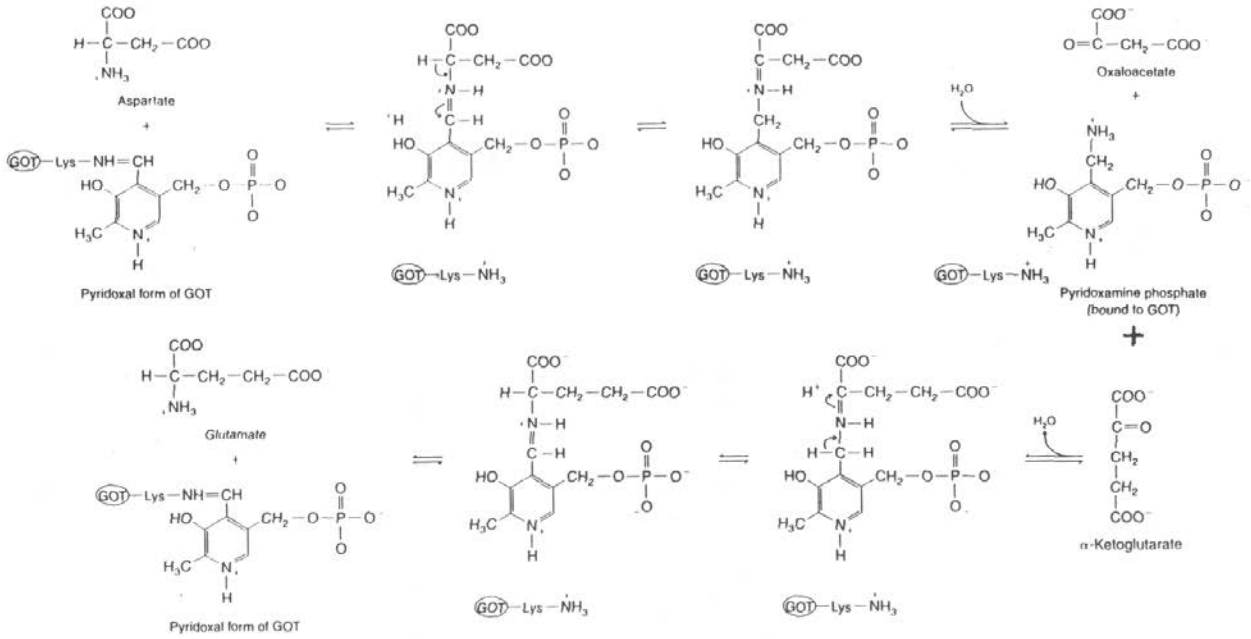



**FIGURE 2.** Complete mechanism of transamination by GOT.

## MATERIALS

0.05 M potassium maleate buffer (pH 6.0) containing 5 mM EDTA
0.04 M α-ketoglutarate (adjusted to pH 6.0 with sodium hydroxide)
Ammonium sulfate (reagent grade)
0.03 M sodium acetate buffer (pH 5.4)
0.03 M sodium acetate buffer (pH 5.0)
0.10 M potassium phosphate buffer (pH 7.5)
Standard protein solutions (1 mg lysozyme per ml)
(dissolve lysozyme in 0.03 M sodium acetate, pH 5.0)
Bovine Serum Albumin in 0.1 M potassium phosphate buffer (pH 7.5)
2 N Folin-Ciocalteu (Folin phenol) reagent
2% $Na_2CO_3$ in 0.1 N NaOH
1.0% $CuS0_4 \bullet 5H_2O$
2% sodium/potassium tartrate
Centrifuge tubes and bottles
Cheesecloth
Lean chopped steak (fresh)
Waring blender
0.1 M potassium phosphate-0.12 M aspartate assay buffer (pH 7.5)
10 mM NADH in 0.1 M potassium phosphate (pH 7.5), prepared fresh daily and stored on ice
*(hint: each vial of NADH contains 2 mg or 2.56 μmoles of NADH)*
Malate dehydrogenase (MDH), 200 units/ml in 0.1 M potassium phosphate buffer (pH 7.5), store on ice
0.1 M α-ketoglutarate (pH 7.5)

**PURIFICATION OF MUSCLE TRANSAMINASES**

*(Key to success: keep all enzyme-containing fractions as cold as possible.)*

Add 75 ml of cold (2-4$^{o}$C) 0.05 M potassium maleate-5 mM EDTA buffer (pH 6.0) to a 50 ml portion of lean chopped steak in a large Waring blender. Homogenize the tissue for 30 seconds at high speed. Record the total volume of the homogenate (should be approximately 120 ml) and save 5 ml of this crude homogenate for protein concentration and enzyme activity determinations. Centrifuge the 5 ml aliquot at 7300g (set the RCF to this on the Sorvall RC6+) for 10 min and store the supernatant (labeled Fraction I) *on ice* until later use for enzyme assays and protein determinations. This supernatant from the 5 ml portion will be used for quantifying the amount of protein and enzyme activity in the 70 ml referred as Fraction I.

Place a 70 ml (also referred to as Fraction I) sample of the above crude homogenate in a water bath at 80°-85°C. Swirl the homogenate gently to ensure even heating and follow the actual temperature of the homogenate closely by inserting a thermometer directly into the homogenate. When the temperature reaches 60$^{o}$C, add 15 ml of 0.04 M $\alpha$-ketoglutarate (pH 6.0) and continue heating until the temperature reaches 73-75$^{o}$C. Maintain the extract at approximately 75$^{o}$C (not above) for 20 min. The total time of heating above 60$^{o}$C should not exceed 40 min. After heating, cool the homogenate by swirling in an ice bath (Each protein fraction should always be kept on ice from this stage forward for the remainder of the isolation and analysis). When the homogenate is cool (about 5$^{o}$C), filter it through a single layer of cheesecloth. Squeeze out the liquid gently and discard the denatured protein. Centrifuge the filtrate at 7300g for 10 min (note: mitochondria will spin down in the pellet so most of the side reaction due to endogenous glutamate dehydrogenase will be removed from the supernatant). Pour off the supernatant fluid, record its volume, and place it on ice. This is Fraction II.

Continue by saving 5 ml of Fraction II for subsequent enzyme assays and protein determinations (keep very cold). Measure the volume of remaining Fraction II and dilute it with an equal volume of 0.05 M potassium maleate-5 mM EDTA buffer, pH 6.0. Proceed with ammonium sulfate fractionation of diluted Fraction II. The idea is to first add enough $(NH_4)_2SO_4$ to precipitate the most protein without precipitating a significant portion (e.g. less than 10%) of the transaminase enzyme activity. Secondly, take the supernatant from the first $(NH_4)_2SO_4$ step and add incremental amounts of $(NH_4)_2SO_4$ so that the second precipitate will contain most of the transaminase activity (e.g. more than 90%), but no more protein than necessary. Ideally, a pilot study should be carried out to ascertain the appropriate concentrations of $(NH_4)_2SO_4$ to be used for steps 1 and 2; however, lack of time prohibits a pilot study, and we will choose 50% and 65% final concentrations of $(NH_4)_2SO_4$ respectively.

**TABLE 1.** Milligrams of solid $(NH_4)_2SO_4$ to add to 1ml of solution to achieve desired saturation at $0^oC$.

| Initial % Saturation $(NH_4)_2SO_4$ | Final concentration of $(NH_4)_2SO_4$ in solution | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *20* | *25* | *30* | *35* | *40* | *45* | *50* | *55* | *60* | *65* | *70* | *75* | *80* | *85* | *90* | *95* | *100* |
| 0 | 106 | 134 | 164 | 194 | 226 | 258 | 291 | 326 | 361 | 398 | 436 | 476 | 516 | 559 | 603 | 630 | 697 |
| 5 | 79 | 108 | 137 | 166 | 197 | 229 | 262 | 296 | 331 | 368 | 405 | 444 | 494 | 526 | 570 | 615 | 662 |
| 10 | 53 | 81 | 109 | 139 | 169 | 200 | 233 | 266 | 301 | 337 | 374 | 412 | 452 | 493 | 536 | 581 | 627 |
| 15 | 26 | 54 | 82 | 111 | 141 | 172 | 204 | 237 | 271 | 316 | 343 | 381 | 420 | 460 | 503 | 547 | 559 |
| 20 | 0 | 25 | 55 | 83 | 113 | 143 | 175 | 207 | 241 | 276 | 312 | 349 | 387 | 427 | 469 | 512 | 557 |
| 25 | | 0 | 27 | 56 | 84 | 115 | 146 | 179 | 211 | 245 | 280 | 317 | 365 | 395 | 436 | 488 | 522 |
| 30 | | | 0 | 28 | 56 | 86 | 117 | 148 | 181 | 214 | 249 | 285 | 323 | 362 | 402 | 445 | 488 |
| 35 | | | | 0 | 28 | 57 | 87 | 118 | 151 | 194 | 218 | 254 | 291 | 329 | 369 | 410 | 453 |
| 40 | | | | | 0 | 29 | 58 | 89 | 120 | 153 | 182 | 212 | 258 | 296 | 335 | 376 | 418 |
| 45 | | | | | | 0 | 29 | 59 | 90 | 123 | 156 | 190 | 226 | 263 | 302 | 342 | 383 |
| 50 | | | | | | | 0 | 30 | 60 | 92 | 125 | 159 | 194 | 230 | 268 | 308 | 348 |
| 55 | | | | | | | | 0 | 30 | 61 | 93 | 127 | 161 | 197 | 235 | 273 | 313 |
| 60 | | | | | | | | | 0 | 31 | 62 | 95 | 129 | 164 | 201 | 239 | 279 |
| 65 | | | | | | | | | | 0 | 31 | 63 | 97 | 132 | 163 | 205 | 244 |
| 70 | | | | | | | | | | | 0 | 32 | 65 | 99 | 134 | 171 | 209 |
| 75 | | | | | | | | | | | | 0 | 32 | 66 | 101 | 137 | 174 |
| 80 | | | | | | | | | | | | | 0 | 33 | 67 | 103 | 139 |
| 85 | | | | | | | | | | | | | | 0 | 34 | 68 | 105 |
| 90 | | | | | | | | | | | | | | | 0 | 34 | 70 |
| 95 | | | | | | | | | | | | | | | | 0 | 35 |
| 100 | | | | | | | | | | | | | | | | | 0 |

Use Table 1 to determine the grams of $(NH_4)_2SO_4$ needed to go from an initial concentration of zero percent $(NH_4)_2SO_4$ to a 50% final concentration of $(NH_4)_2SO_4$. Mass this amount of $(NH_4)_2SO_4$ and *slowly* add it to diluted Fraction II while stirring gently with a magnetic stir bar. A good way to do this is to place the beaker with the magnetic stir bar inside directly in an ice bucket or larger beaker with ice, and place the whole combination on top of the magnetic stir plate. To avoid any pockets of high $(NH_4)_2SO_4$ concentrations that may prematurely precipitate out the GOT, do not add more than a few grams of $(NH_4)_2SO_4$ at a time and then be sure all the solid is dissolved before adding more. After all the appropriate amount is dissolved, allow the mixture to remain at $0^oC$ for 20 min to assure maximum precipitation. Remove the precipitated protein by centrifugation at 11,400g for 10 min. Pour off and *save* the supernatant. Dissolve the precipitate in 2 ml of cold 0.03 M acetate buffer, pH 5.4. Label this is fraction AS-I. This is a suitable stopping point. GOT will be stable in $(NH_4)_2SO_4$ in the refrigerator for more than a week. To the supernatant fluid add additional solid $(NH_4)_2SO_4$ to go from 50% saturation to a final concentration of 65% $(NH_4)_2SO_4$ saturation. Again add the salt slowly with stirring and after all is dissolved, allow the mixture to remain at $0^oC$ for 20 min. Collect the precipitated protein by centrifugation as before and dissolve it in 2 ml of 0.03 M acetate buffer, pH 5.0. Label this fraction AS-II and label the final supernatant fraction AS-III. Record the volume of AS-III. Store each AS fraction on ice for enzyme assays and protein determinations. (Note: AS-III will not be used for protein determinations, since the Folin-Ciocalteu procedure is inaccurate in the presence of high concentrations of $NH_4^+$ ions. If time permits, AS-III could first be desalted by dialysis and then subjected to a protein determination.)

**ASSAY FOR TRANSAMINASE ACTIVITY**

You can measure the transaminase activity of GOT using the spectrophotometer. GOT uses pyridoxal phosphate as a coenzyme and catalyses the reaction shown in equation 1. The assay is based on the use of a coupled enzyme system. The system involves the oxidation of NADH to $NAD^+$ (this is what is detected spectrophotometrically) by malate dehydrogenase (MDH) using the oxalacetate produced by GOT as a substrate (see equation 2).

You will be making initial velocity measurements in order to determine enzyme activities. You will do this by measuring changes in absorbance as a function of time using a notebook computer and the Spectro Pro software to record the time-dependent data. In brief, collecting 1 data point per second for 60 seconds should be an adequate data set for initial velocity determination.

| Component Number | Reagent | Volume |
|---|---|---|
| 1 | 0.1 M potassium phosphate-0.12 M aspartate (buffer-substrate) solution, pH 7.5. *(zero the spectrometer @ 340 nm)* | 2.57 ml |
| 2 | 10 mM NADH in 0. 1 M phosphate buffer, pH 7.5 *(check for an A340 nm reading between 0.7 and 0.9)* | 0.03 ml |
| 3 | Malate dehydrogenase (MDH) solution (200 units per ml) | 0.10 ml |
| 4 | GOT purification fraction *(may require dilution)* | 0.10 ml |
| 5 | 0.1 M α-ketoglutarate | 0.20 ml |
| | Total Volume | 3.00 ml |

The protocol above should be used to assay GOT in a 3 ml reaction volume. NADH solutions are not always predictable, so you ought to add reagent #1 first to a cuvette and use this to calibrate a Spectronic 20D to zero absorbance at 340 nm. Now thoroughly mix (cover cuvette with parafilm and invert) into the cuvette reagent #2 and check for an A-340 reading between 0.7 and 0.9. If the reading is too low, add an appropriate extra volume of reagent #2. If it is too high, discard the cuvette's contents and set up a new one adding appropriately less reagent #2 this time. Now add reagent #3 and your diluted enzyme (e.g., diluted AS-II or diluted Fraction I). Mix contents thoroughly and insert the cuvette into the spectrophotometer. Since you have not yet added substrate for GOT, there should be no reaction visible as a loss of absorbance at 340 nm. This step is not the GOT assay, but it is a test for any endogenous substrate of dehydrogenase. These should not be problems, and the absorbance should be constant (30 seconds is more than enough to identify any endogenous activity).

Initiate GOT activity by adding 0.2 ml of 0.1 M α-ketoglutarate (reagent #5) to the assay mixture in the cuvette. (Thoroughly and rapidly mix the cuvette solution). You will see one of three kinetic traces on your screen.

- A flat, horizontal tracing in the 0.7 to 0.9 absorbance range meaning that you are seeing no detectable GOT activity.
- A flat, horizontal tracing at very low absorbance meaning that the reaction reached equilibrium in the dead time of your experiment—a problem of too much enzyme. If that occurs, you need to use a more dilute enzyme sample.
- A diagonal tracing starting out straight but eventually perhaps tailing off as the reaction proceeds toward equilibrium.

The third profile is necessary in order to measure the initial velocity ($\Delta A_{340}$/min). The initial velocity is the slope of the linear portion of your plot, prior to roll over due to substrate depletion.

Don't forget that you need to obtain a reasonable initial velocity ($\Delta A_{340}$/min) for GOT activity in Fraction I, Fraction II, AS-I, AS-II, and AS-III respectively. You have no choice but to start by guessing at a reasonable dilution of each fraction to use in an assay. Try diluting 0.1 ml of a fraction with 0.9 ml of 0.1 M phosphate buffer (pH 7.5) as a start, i.e., a 1: 10 dilution. If inappropriate (i.e., flat horizontal lines on the recorder), then adjust the dilution in a reasonable manner and assay again. Write dilutions and any other relevant comments in your notebook beside each plot or you will be hopelessly lost in writing a report on this lab (*hint: you shouldn't expect every fraction to show GOT activity. Prepare a flow chart tracing the location of GOT through each step of the purification and include it in your lab report*).

**PROTEIN DETERMINATIONS (FOLIN-CIOCALTEU METHOD)**

Someone in your lab group should begin the protein determinations in the early part of the afternoon. Set up 0.1 ml and 0.2 ml samples of 20-fold dilutions (e.g., 0.1 ml diluted to 2 ml with distilled $H_2O$) of Fraction I, AS-I, and AS-II respectively. Make a 5-fold dilution of Fraction II and set up 0.1 ml and 0.2 ml samples. Do not bother with AS-III (explained earlier). Bring all sample volumes up to 0.3 ml with distilled $H_2O$. Also set up a blank containing 0.3 ml of $H_2O$ and a series of protein standards having 0.05, 0.1, 0.2 and 0.3 mg of lysozyme respectively (stock is 1 mg/ml) in final volumes of 0.3 ml.

Separately prepare l00 ml of fresh alkaline copper reagent by mixing, in order, 1 ml of 1.0% $CuSO_4 \bullet 5H_2O$, 1 ml of 2% sodium/potassium tartrate, and 98 ml of 2% $Na_2CO_3$ in 0.1 N NaOH. Add with immediate mixing 6 ml of the alkaline copper reagent to each tube (blank, standards, and samples) and let stand for 10 min at room temperature. Then add 0.3 ml of Folin-Ciocalteu (Folin phenol) reagent (with mixing) to each tube and allow each tube to stand for 30 more minutes. Measure the absorbance of each sample at 600 nm. Use the absorbance data for the lysozyme samples to construct a standard curve and determine the protein concentration (in units of mg protein/ml) of each original fraction (don't forget to account for dilutions and aliquot sizes).

## ANALYSIS OF RESULTS

From your records of volume, protein concentrations, and enzyme activities, calculate the specific activity and total activity of each fraction. The following table might be helpful. You can define one unit as equal to 1 μmole/min of NADH disappearance where the molar extinction coefficient of NADH at 340 nm is 6.22 x $10^3$ $M^{-1}cm^{-1}$. The latter is used to convert $\Delta A_{340}$/min values to Δμmol NADH/min (recall that absorbance = extinction coefficient * path length * concentration, A=ε*l*c). It is assumed that there is 1:1 stoichiometry between NADH and substrates in these reactions. Evaluate each step for purification and yield. Account for any significant losses or increases in total activity. *Did each step proceed as expected? Explain.*

In the table below, volumes are the total fraction volumes recorded during the purification. Protein concentration is determined for each fraction (except AS-III) by the Folin-Ciocalteu method using the lysozyme standard curve. Activity measurements are derived from assay for transaminase activity for each fraction and the extinction coefficient of NADH. Specific activity, in units of units/mg) is the quotient of activity and protein concentration. Total activity is the product of the activity and the volume for each fraction. Percent yield is the percentage of GOT activity that after each purification step. The percent yield will drop with each purification step as enzyme is lost or destroyed during manipulation. Fold purification is based on the increase in specific activity with each purification step. The fold purification should increase with each purification step as proteins other than GOT are removed and the specific activity increases.

| Fraction | Volume (ml) | Protein Concentration (mg/ml) | Activity (units/ml) | Specific Activity (units/mg) | Total Activity (units) | Percent yield | Fold Purification |
|---|---|---|---|---|---|---|---|
| Fraction I | | | | | | 100 % | 1 X |
| Fraction II | | | | | | | |
| AS-I | | | | | | ------ | ------ |
| AS-II | | | | | | | |
| AS-III | | ------ | | ------ | | ------ | ------ |

## DISCUSSION QUESTIONS

1. Explain the phenomena that account for "salting out" or precipitation of proteins by ammonium sulfate. Can the converse ("salting in") occur? *Hint: most enzymes for molecular biology are sold and stored in reasonable concentrations of NaCl.* Include in your answer an abbreviation of the Hofmeister series relating to the questions above.
2. Develop an alternate purification protocol for bovine GOT that does not use $(NH_4)_2SO_4$ precipitation but instead utilizes an ion-exchange chromatography protocol. The electronic laboratory resources include the FASTA sequence of cytoplasmic glutamate oxalacetate transaminase from the cow (*Bos taurus*). Please be

sure to include the type (anion or cation) of exchange resin and the pH at which you will perform the separation. You do not have to include any information about extraction or desalting.

3. During attempts to purify a protein, how does one know that a preparation is pure or homogeneous? Stated another way, are there other easily achieved measures of protein purity beyond the measurements of specific activity that you made? What do you do if your protein of interest doesn't have an enzyme activity?
4. Determination of the level of GOT activity in the blood serum (sGOT) of human patients has proven very useful in clinical diagnosis of various diseases that lead to extensive tissue damage and cell breakdown. What are some examples? Comment.

## REFERENCES


Clark, J.M., and Switzer, R.L. 1977. **Experimental Biochemistry, 2nd edition**. W .H. Freeman and Company, San Francisco.

Sizer, I.W., and Jenkins, W.T. 1962. Glutamic Aspartic Transaminase from Pig Ventricles. Preparation and Assay of Enzymes. **Methods in Enzymology**. Vol. V.

# Appendix / Reagents and Materials

**(Note: Do not make up the solutions as described below. Before you begin, you need to determine the appropriate amounts of each solution needed and make up only about twice as much in case a procedure needs to be repeated)**

1,000 ml 0.05 M Potassium maleate buffer (pH 6.0): Dissolve 5.8 g maleic acid and 1.86 g $Na_2EDTA \cdot 2H_2O$ in 800 ml $H_2O$, and adjust the pH to 6.0 with concentrated (2 N or stronger) KOH. Dilute to 1,000 ml. Store at 1°-3° C. If this buffer is to be stored for longer than a week, you can add a drop of $CHCl_3$) or toluene to prevent bacterial growth.

600 ml 0.04 M α-Ketoglutarate: Dissolve 3.5 g of α-ketoglutaric acid in 520 ml $H_2O$, adjust the pH to 6.0 with NAOH (1.0 N or stronger), and dilute to 600 ml. Prepare this reagent *just before use;* store at 1°-3° C.

500 g Ammonium sulfate, solid

2,000 ml 0.03 M Sodium acetate buffer (pH 5.4): Dilute 3.44 ml of glacial acetic acid to 1.8 l with $H_2O$, adjust the pH to 5.4 with NaOH (1 N or stronger), and dilute to 2,000 ml. Store at 1°-3° C. If this buffer is to be stored for longer than a week, add a few drops of $CHCl_3$ or toluene. This can also used for preparing the lysozyme solutions.

500 ml Folin-Ciocalteu (phenol) reagent, 2 N. (This is not the phenol reagent used in the DNA experiment.)

1,000 ml 2% $Na_2C0_3$ in 0.1 N NaOH (wt/vol).

100 ml 2% Sodium/potasium tartrate (wt/vol).

100 ml 1% $CuSO_4 \cdot 5H_2O$ (wt/vol).

600 ml Protein standard (1 mg/ml): (1 mg lysozyme per ml)
(dissolve lysozyme in 0.03 M acetate, pH 5.0) Store at 1°-3° C.

1,000 ml 0.10 M Potassium phosphate buffer (pH 7.5): Dissolve 14.11g of anhydrous $K_2HPO_4$, and 2.18g anhydrous $KH_2PO_4$ in 800 ml $H_2O$, dilute to 1,000 ml. Store at 1°-3° C.

3,000 ml 0.10 M Potassium phosphate-0.12 M aspartate assay buffer (pH 7.5): Dissolve 52.2g anhydrous $K_2HP0_4$ and 48.0 g L-aspartic acid in 2,400 ml $H_2O$, adjust pH to 7.5 with dilute KOH, and dilute to 3,000 ml. Store cold. If this buffer is to be stored for longer than one week, add a few drops of $CHCl_3$ or toluene.

600 ml 0.10 M α-Ketoglutarate (pH 7.5): Dissolve 8.76g of α-ketoglutaric acid in 480 ml $H_2O$, adjust to pH 7.5 with NaOH and dilute to 600 ml. Prepare this reagent *just before use* and keep at 1°-3° C.

10 ml Malate dehydrogenase (MDH): Dilute commercially available enzyme (2000-4000 units/mg) in 0.1 M potassium phosphate buffer (pH 7.5) preparation, in which 1 **mg/ml bovine serum albumin** has been dissolved, to a final concentration of about 200 units of MDH/ml. Prepare just before experiment and keep cold. Stable 4-5 days at 4° C. To avoid waste, it may be useful to have an assistant dispense this reagent.

4,000 ml 0.03 M Sodium acetate buffer (pH 5.0)
(Note: In leu of making up this solution, you may substitute the 0.03 M Sodium acetate pH 5.4 solution listed above.)
Dilute 6.88 ml of glacial acetic acid to 3.8 liters with $H_2O$, and adjust the pH to 5.0 with NaOH (1N or stronger). Do *not* overtitrate or back titrate with acid. It is essential that this buffer not contain excess salt. Dilute to 4,000 ml. Store at 1°-3° C. If this buffer is to be stored for longer than a week add a few drops of $CHCl_3$ or toluene.

200 ml 0.08 M Sodium acetate buffer (pH 5.0):
Dilute 0.92 ml of glacial acidic acid to 180 ml with $H_2O$, adjust the pH to 5.0 with NaOH (1 N or stronger) and dilute to 200 ml. Store at 1°-3° C. If this buffer is to be stored for longer than a week, add a few drops of $CHCl_3$ or toluene.

1 Pig heart
1 Meat grinder
1 or 2 Waring blenders
Cheesecloth
75° C Water bath (first day of experiment)
1 or 2 high speed centrifuges with 50 ml tubes and 300 ml bottles or cans
1 or 2 Magnetic stirrers and stirring bars