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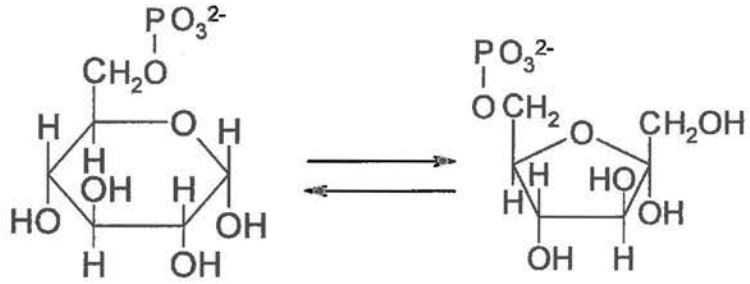
Introduction

Phosphoglucose isomerase (D-glucose-6-phosphate ketoisomerase) is a key enzyme in glycolysis. Glycolysis is the initial pathway in the catabolism of carbohydrates. In this pathway, a molecule of glucose is broken down with a net production of ATP. Phosphoglucose isomerase catalyzes the second step of glycolysis, the interconversion between α-D-glucose-6-phosphate and D-fructose-6-phosphate:



alpha-D-glucose-6-phosphate D-fructose-6-phosphate

This enzyme has been isolated from many sources, including animal muscle, brewer's yeast, spinach, sweet potatoes, and peas (1, 2).

Phosphoglucose isomerase (PGI) originally attracted attention because both it and phosphomannose isomerase can convert the same substrate, fructose, to different products. It was at first unclear whether phosphomannose isomerase catalyzed the interconversion between glucose-6-phosphate and mannose-6-phosphate or between mannose-6-phosphate and fructose-6-phosphate (3). Once the latter was confirmed to be true, it fascinated scientists that these two enzymes could start with the same substrate and yield two different products. Thus there was intense interest to study each of these enzymes and determine their properties.

In order to study a specific protein, such as PGI, the protein of interest must be isolated from the thousands of other proteins in a cell. Several biochemical techniques have been developed to isolate proteins.

One simple way to differentiate between proteins is by utilizing the fact that different proteins have different solubilities in concentrated salt solutions. Many proteins become insoluble at high salt concentrations. This is due to the water that normally solubilizes the proteins being used to hydrate salt ions, thus preventing sufficient hydration of the protein. Different proteins respond differently to specific ionic strengths. Because of this, it is possible to raise the ion concentration to a certain point in order to make some proteins insoluble while leaving others in solution. In such procedures, ammonium sulfate is often used because this salt can yield high ion concentrations without damaging the proteins (4).

Another way to separate proteins with different properties is by centrifugation. Particles spun in a centrifuge are subject to a centrifugal force. The amount of force a particle experiences is related to the mass and size of the particle. Different sized particles will thus experience different centrifugal forces and can be separated. Centrifugation is useful for separating soluble proteins from other cell fragments (4).

Another powerful technique for separating proteins is chromatography. Chromatography involves passing a solution through a medium that exhibits selective adsorption for different solute particles. The rate at which molecules in solution will pass through the medium depends on how strongly they interact with the medium.

Ion-exchange chromatography is used to separate molecules with different electrical charges. To carry out this task, ion-exchange resins are used. These are either polyanions, such as carboxymethyl, or polycations, such as diethylaminoethyl (DEAE). A clear cylinder is packed with the resin, and the solution containing the protein is pored through the column. Proteins that carry the same charge as the ion-exchange resin will be repulsed by the resin and will pass through the column relatively rapidly. Proteins that are neutrally charged will diffuse through

the medium slower than proteins that are the same charge as the medium. Neutral proteins will be inhibited only by the physical barrier the resin presents. Proteins that carry the opposite charge of the ion-exchange resin will be attracted to the resin and will pass through much more slowly. In fact, it is often necessary to use high salt concentrations to remove these particles because of their strong attraction to the medium. Because of this selective attraction for the resin, proteins can be separated and collected as fractions as the solution elutes from the column (4).

Once an enzyme is isolated, an enzyme assay may be performed to measure the enzyme's activity. Photometric methods are the most frequently used of all kinds of enzyme assays. Most of these assays are based on changes in the light absorbed by the solution as the reaction proceeds. An example of this is the change in absorbance of NADP+ when it is reduced by an enzyme at 340nm. NADP+ does not absorb light at 340nm, but its reduced form, NADPH, does. The amount of NADPH produced is detected spectrophotometrically and is a measure of the enzyme's activity (5).

To avoid the necessity of animal sacrifice when isolating this enzyme, many plants have been shown to be reasonable sources for PGI. In addition, the presence of PGI isoenzymes has been detected in many higher plants. Three PGI isoenzymes have been shown to exist in sweet potato, and the presence of two PGI isoenzymes have been discovered in spinach. (1, 2, 6).

One inexpensive and readily available source of PGI is frozen peas. This enzyme has been isolated from peas, but the procedure is long and tedious (7). In addition, the enzyme has not been extensively studied in peas, and it is unreported whether or not the enzyme exists in the isoenzyme form in peas. It is thus of interest to develop a simplified method for isolating this enzyme from peas, which may be accomplished by combining several methods for isolating this

enzyme in other higher plants. Once isolated, it can be determined whether or not there are isoenzymes for PGI in peas. This experiment will attempt to develop a straightforward procedure for isolating PGI from peas and then to detect for the presence of isoenzymes using column chromatography and spectrophotometry.

Materials and Methods

In developing a simpler procedure for purification, steps from many published procedures were combined. For all procedures, samples were kept at 0-4°C. Frozen Flavorite green peas (*Pisum sativum*) were purchased locally and stored at -20°C. Peas were ground with a mortar and pestle in 0.1M Tris·HCl-10mM EDTA, pH 7.5 buffer and then homogenized using a handheld Bamix biomixer in the same buffer. The homogenate was filtered through cheese cloth. Insoluble material was removed by centrifugation for 7 min at 10,800 rpm and solid $(NH_4)_2SO_4$ was added to the supernatant to reach 40% saturation (2, 7). The solution was then centrifuged for 7 min at 10,800 rpm (7). The pellet was dissolved in 0.02M KH_2PO_4 buffer, pH 7.0, containing 7.3mM 2-mercaptoethanol (2). $(NH_4)_2SO_4$ was added to the remainder of the supernatant to reach 60% saturation. This supernatant was then centrifuged for 10 min at 10,800 rpm and the pellet was again dissolved in 0.02M KH_2PO_4 buffer, pH 7.0, containing 7.3mM 2-mercaptoethanol. These two pellet solutions were dialysed against 0.02M KH_2PO_4 buffer, pH 7.0, containing 7.3mM 2-mercaptoethanol three times (3 hr each time) (2). The enzyme assay described below was performed on the crude extract, the sample that had been centrifuged once, and the two samples that had underwent dialysis to determine which of the four samples had the highest specific activity. It was found that the sample that had reached 40% $(NH_4)_2SO_4$ saturation had the highest specific activity at $3.19\times10^{-3}\mu mol\cdot min^{-1}\cdot mg^{-1}$. This sample was then used for all subsequent steps.

A DEAE-cellulose column was prepared with 0.02M KH_2PO_4 buffer, pH 7.0, containing 7.3mM 2-mercaptoethanol. The sample consisting of 10ml of the pea enzyme solution was run through the column, followed by 40ml of the same phosphate buffer, then 50ml of the phosphate buffer containing 0.1M NaCl, and finally 50ml phosphate buffer containing 0.2M NaCl (6). The elute was collected in 60 fractions of about 2ml.

Activity of selected fractions was measured by the change in absorbance at 340nm produced by NADPH at room temperature, with fructose-6-phosphate as the substrate and glucose-6-phosphate dehydrogenase as the indicator enzyme (8). The measurements were made using a Shimadzu UV-2101PC UV-VIS scanning spectrophotometer. All spectrophotometric kinetic analysis was done using a reaction time of 90s and an activity region of 10.00-90.00s. The reaction mixture contained a final volume of 2.055ml; 0.111M Tris buffer, pH 8.00, 1.80ml; 0.0100M NADP+, 0.10ml; 0.333M fructose-6-phosphate, 0.10ml; 1.25 units of glucose-6-phosphate dehydrogenase activity, 5μL; and the enzyme source, 50 μL.

Protein concentration was determined spectrophotometrically by absorbance at 280nm. The entire procedure took approximately 2 months to complete.

The above procedure was repeated a second time with some minor variations. The $(NH_4)_2SO_4$ was added in steps followed by centrifugation to yield samples at 30%, 40%, and 50% saturation. The specific activities of these samples were determined. It was found that the solution made from dissolving the pellet from the centrifugation of the 40% saturated solution in 0.02M KH_2PO_4 buffer, pH 7.0, containing 7.3mM 2-mercaptoethanol had the highest specific activity: 0.186 $\mu mol \cdot min^{-1} \cdot mg^{-1}$. This sample was used in all subsequent procedures. Dialysis in 0.02M KH_2PO_4 buffer, pH 7.0, containing 7.3mM 2-mercaptoethanol was performed after the sample with the highest specific activity was determined. All spectrophotometric enzyme assays

were performed at 30°C using a Neslab EX-111 heater to maintain constant temperature. During DEAE-cellulose column chromatograpy, 18.5 ml of pea extract was poured through the column, and 65 fractions of about 2ml each were collected for analysis. The entire second procedure was completed in six weeks.

Results

The first time the procedure was performed, the main objective was to determine whether or not the procedure extracted any PGI from peas. Only a few of the chromatography fractions collected were measured for protein and enzyme activity. Three peaks of absorption at 280nm (protein concentration) were observed from the fractions eluted from a DEAE-cellulose column when graphed, as shown in Figure 1. The peaks are at tubes 6, 28, and 48. Tube numbers correlate to time of elution, with higher numbered tubes containing samples that eluted later.

Figure 1. Elution of phosphoglucose isomerase of peas from DEAE-cellulose

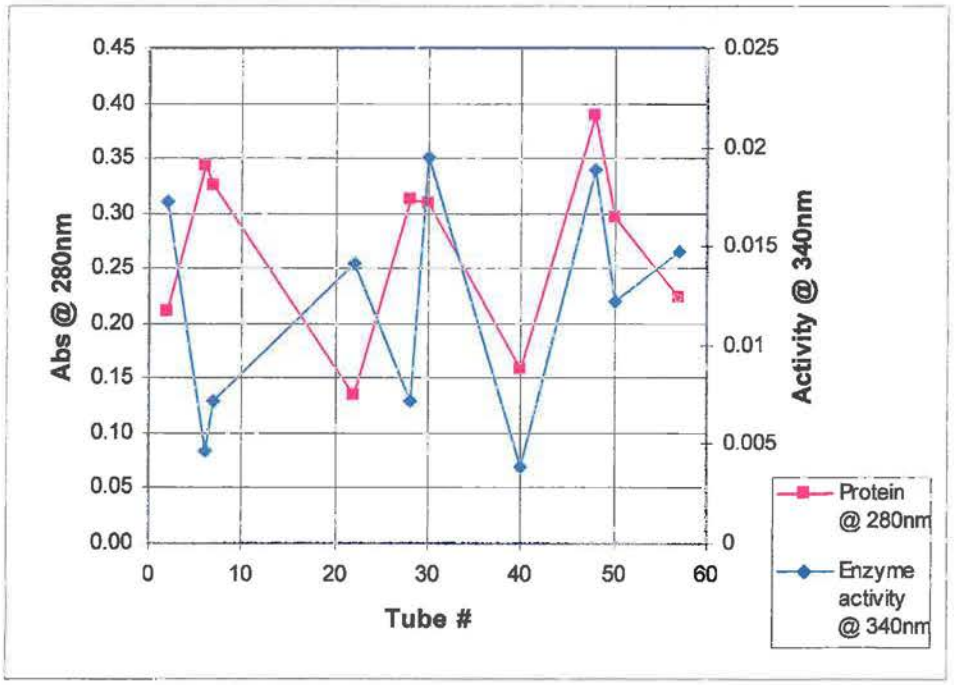
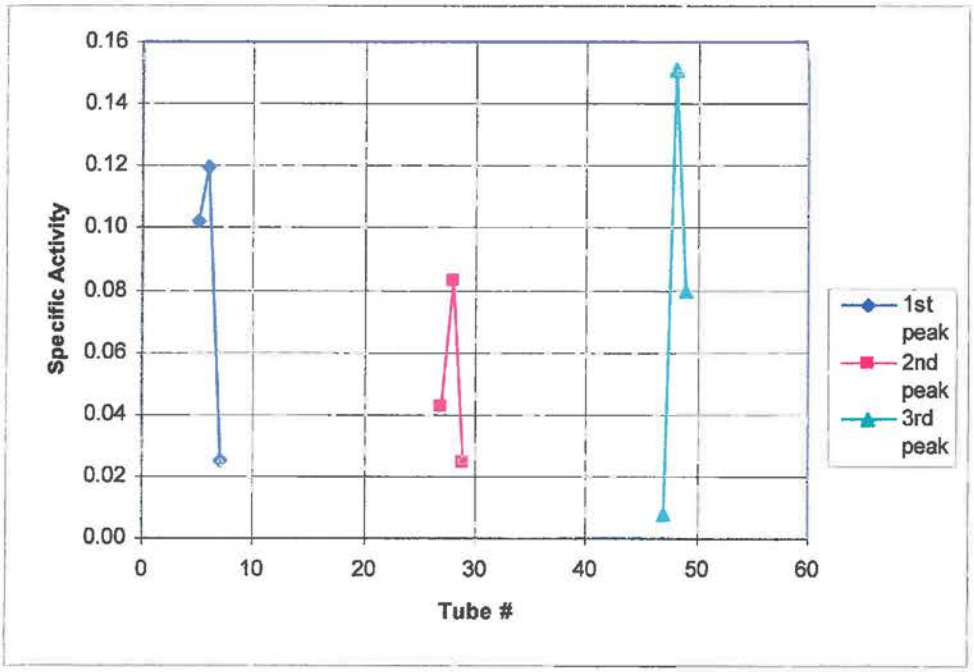


Figure 1 also shows the enzyme activity measured at 340nm. There were not three distinct peaks. Note, however, that there does appear to be two peaks of enzyme activity near two of the peaks of protein concentration.

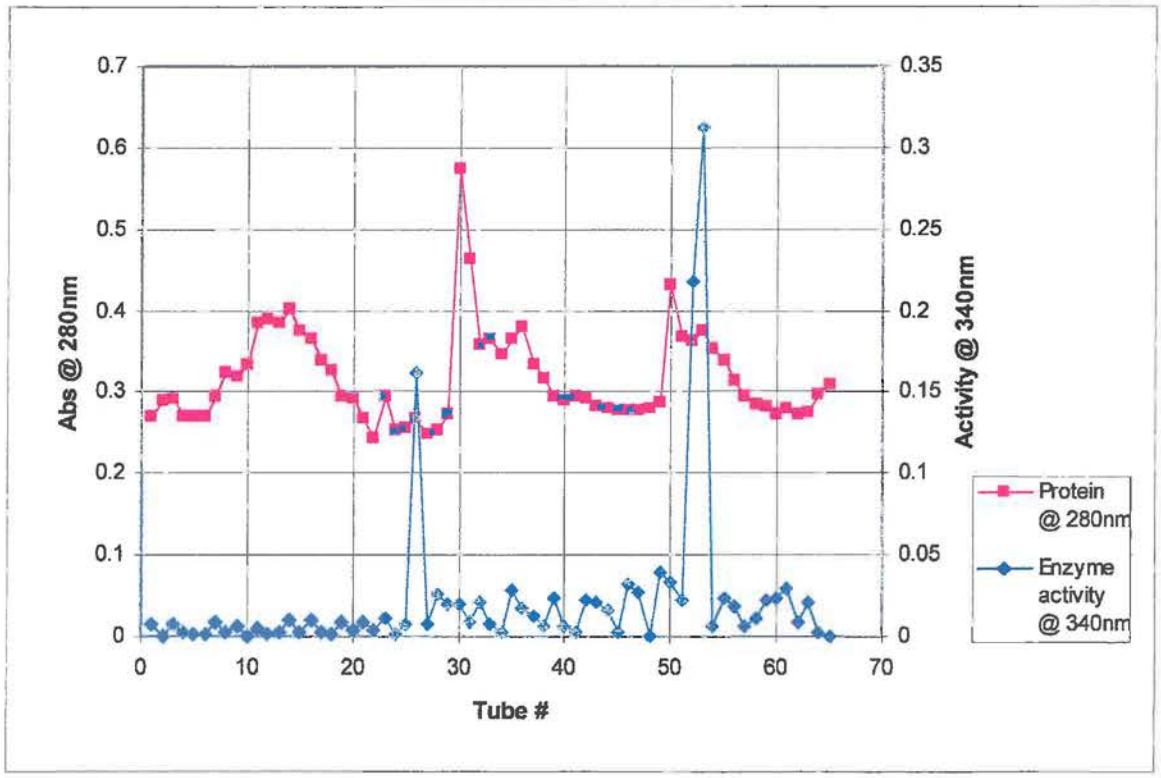
To verify that samples #6, 28, and 48 were peaks of specific activity, the activities of these three peaks was re-measured using the enzyme assay described above. In addition the activities of samples on both sides of these three samples were measured. All samples gave different activity values from those presented previously. The specific activities of all of these samples were calculated. The results are presented in Figure 2. Note that samples 6, 28, and 48 do have higher specific activities than the samples on either side of them.

Figure 2. Specific activities of samples of phosphoglucose isomerase from peas



The second time the procedure was performed, there were again three peaks of absorption at 280nm (protein concentration). In addition, there were two very distinct peaks of enzyme activity in tubes 26 and 53. This can be seen in Figure 3.

Figure 3. Elution of pea phosphoglucose isomerase from DEAE-cellulose



Specific activities were calculated for all the samples in Figure 3. The results can be seen in Figure 4. Note that the specific activity values are very low before the first peak and that the specific activities of the peaks are considerably higher than any other samples. In addition, the peak values in Figure 4 are considerably higher than the peak values of specific activity in Figure 2. The two peak values in Figure 4, the three peak values in Figure 2, and literature values of specific activities for phosphoglucose isomerase from various sources are compared in Table 1.

Figure 4. Observance of specific activity peaks for phosphoglucose isomerase from peas

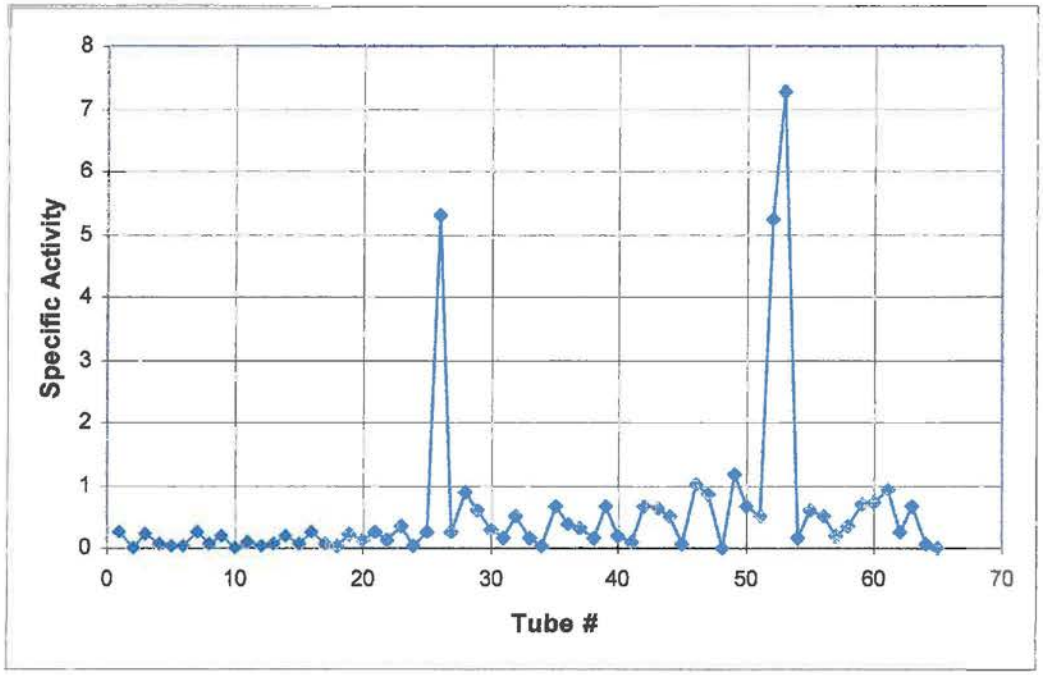


Table 1. Specific activities of phosphoglucose isomerase samples

sample	6	28	48	26	53	peas (7)	spinach leaves (1)
Specific activity ($\mu mol \cdot min^{-1} \cdot mg^{-1}$)	0.12	0.08	0.15	5.30	7.26	1260	1.57

Note: samples 6,28, and 48 are from the first procedure while samples 26 and 53 are from the second procedure.

Discussion

The first objective of this study was to develop a straightforward method for extracting PGI from peas. In this regard, the research seems to have been successful. All samples tested absorbed light at 280nm, indicating the presence of protein. Furthermore, as Figure 3 demonstrates, enzyme activity was detected in several of the samples. Thus this procedure does extract PGI from peas. The procedure developed here excludes zinc acetate and $MgCl_2$ treatments and isopropyl alcohol precipitations performed by other researchers (7) to extract PGI from peas, thus making this procedure simpler. In addition, a previous method (7) used a

DEAE-Sephadex column to elute protein, but did not report the detection of multiple forms of PGI. It would thus seem that the use of a DEAE-cellulose column was a good choice because it allowed the detection of distinct regions of higher protein concentration. However, as is evident in Table 1, Hizukuri *et.al.* (7) report a specific activity higher than that obtained from this study. This indicates that further purification is needed. Future work should include SDS-PAGE gels to determine purity. The efforts to simplify the procedure in this study may have sacrificed the purity of the results.

The second objective of this study was to determine if there are PGI isoenzymes in peas. The first time the procedure was performed, the results suggested that there were three isoenzymes of PGI in peas. Figure 1 shows three peaks of enzyme activity at tubes 6, 28, and 48. In Figure 2, those tubes appear to be peaks of specific activity as well. This suggests the presence of three different types of PGI. However, not all samples collected were measured for specific activity, so these results are inconclusive.

The second time the procedure was performed, the results suggested that there were two PGI isoenzymes in peas. The presence of two distinct peaks of specific activity as shown in Figure 4 suggests the presence of two different types of PGI. Before the first peak, there is negligible appearance of specific activity in any of the samples. In sample 26, the specific activity is suddenly very high then immediately drops, indicating that tube 26 contained PGI. In sample 52, the specific activity jumps, reaching a maximum at tube 53 before immediately dropping to low values. Thus tubes 52 and 53 appear to also contain PGI. However, because the proteins in tubes 26 and 53 did not come out together, they must be different proteins, *i.e.* PGI isoenzymes. Thus there is evidence to suggest two forms of PGI in peas. The slightly higher specific activities after the first peak compared to before the first peak are likely due to traces of

PGI that did not elute with the majority of the enzyme. The data from the second procedure strongly suggests the presence of two PGI isoenzymes in peas.

Table 1 shows that the specific activities from samples obtained by the first procedure (#6, 28, and 48) were significantly smaller than those obtained by the second procedure (#26 and 53). This could be due to the amount of time required to complete each procedure. It is known that phosphoglucose isomerase from higher plants loses activity over time (2, 7). Hizukuri *et.al.* report that the enzyme loses about 50% activity in 5 months at 4°C (7). Because the first procedure required more time to complete than the second procedure, the enzyme extracted during the first procedure had likely lost more of its activity compared to the enzyme extracted from the second procedure. It would thus seem that it is important to analyze enzymes as soon after extraction as possible so that activity is not lost.

Building upon the results of this experiment, further research may be done to study PGI in peas. Using the procedure developed here, the two isoenzymes can be extracted so that their properties may be characterized.

Based upon the results obtained in this study, it is reasonable to believe that peas do contain phosphoglucose isomerase of two slightly different structures. The procedure outlined in this study for extracting PGI from peas appears to be reasonable as it does yield the enzyme, though in a form less pure than previously reported for peas (7). Elution of pea extract from DEAE-cellulose yields distinct peaks in specific activity, suggesting that phosphoglucose isomerase does indeed exist as two isoenzymes in peas. Further research is needed to determine the actual differences between these two enzymes.

Acknowledgements

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SOUTHERN SCHOLARS SENIOR PROJECT

Name: Tyler Dubs Date: 9-22-03 Major: Biochemistry

SENIOR PROJECT

A significant scholarly project, involving research, writing, or special performance, appropriate to the major in question, is ordinarily completed the senior year. The project is expected to be of sufficiently high quality to warrant a grade of A and to justify public presentation.

Under the guidance of a faculty advisor, the Senior Project should be an original work, should use primary sources when applicable, should have a table of contents and works cited page, should give convincing evidence to support a strong thesis, and should use the methods and writing style appropriate to the discipline.

The completed project, to be turned in in duplicate, must be approved by the Honors Committee in consultation with the student's supervising professor three weeks prior to graduation. Please include the advisor's name on the title page. The 2-3 hours of credit for this project is done as directed study or in a research class.

Keeping in mind the above senior project description, please describe in as much detail as you can the project you will undertake. You may attach a separate sheet if you wish:

The project I am undertaking is the isolation and characterization of the enzyme phosphoglucoisomerase from frozen peas. This is an important enzyme in glycolysis. One substrate of this enzyme is fructose 6-phosphate. Fructose 6-phosphate can be converted to glucose 6-phosphate by phosphoglucoisomerase. However, another enzyme, mannose phosphate isomerase, converts fructose 6-phosphate to mannose phosphate. It is thus of interest to understand what causes two similar enzymes that work on the same substrate to create different products. This project will attempt to isolate phosphoglucoisomerase and to characterize this interesting enzyme.

Signature of faculty advisor Rhonda Scott-Ennis Expected date of completion April 2004

Approval to be signed by faculty advisor when completed:

This project has been completed as planned: ✓

This in an "A" project: ✓

This project is worth 2-3 hours of credit: 2 hours credit

Advisor's Final Signature Rhonda Scott-Ennis

Chair, Honors Committee _____ Date Approved: _____

Dear Advisor, please write your final evaluation on the project on the reverse side of this page. Comment on the characteristics that make this "A" quality work.