Ribonuclease II: A Glycoprotein with Differential Distribution in Tissues of two Nonacclimated Cultivars of Winter Barley

Gregory James Bunkers

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RIBONUCLEASE II: A GLYCOPROTEIN WITH DIfferential DISTRIBUTION IN TISSUES OF TWO NONACCLIMATED CULTIVARS OF WINTER BARLEY

By

Gregory James Bunkers

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A thesis submitted in partial fulfillment of the requirements for the degree Master of Science Major in Biology

South Dakota State University 1985
RIBONUCLEASE II: A GLYCOPROTEIN WITH
DIFFERENTIAL DISTRIBUTION IN TISSUES
OF TWO NONACCLIMATED CULTIVARS
OF WINTER BARLEY

This thesis is approved as a creditable and independent investi-
gation by a candidate for the degree, Master of Science, and is
acceptable for meeting the thesis requirements for this degree. Accep-
tance of this thesis does not imply that the conclusions reached by the
candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGEMENTS

I wish to express my deep appreciation to Dr. Don Kenefick for his guidance, encouragement and continual patience during this study. I also thank Elaine Hall for her help with the lab chores.

Special thanks to my parents and family. Thanks to my friends, all of them special, for their help.

Special thanks and acknowledgement is given to my friend Veloy for her encouragement and steadfast patience throughout this study.

Appreciation is expressed to Dr. David Hildebrand and the Chemistry Department for financial assistance which made this study possible.
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INTRODUCTION

Observations have been that a relationship exists between the levels of winter hardiness and the differences in the RNA-degrading enzymes of winter barley, *Hordeum vulgare* L. (10, 11, 12). In pursuit of more information on these enzymes, Wong (33) purified a ribonuclease (RNase) from barley seedlings. The purified enzymes from each of two barley cultivars showed different substrate specificities when evaluated by dinucleoside assays. Wong's purification procedure, though effective in purifying RNase, was lengthy and enabled purification of only small amounts of RNase. Hawthorne et al. (7) using RNase obtained by Wong's method of purification, showed that a RNase from two cultivars of winter barley possess similar antigenic determinant sites. He did not however conclusively show that the RNase from these two sources were physiochemically identical. More definitive molecular information was needed about the enzyme.

The purpose of this research was to develop a more efficient procedure that would enable purification of adequate RNase necessary to further characterize the physical, chemical, and catalytic properties of the enzymes involved. Two cultivars of winter barley were selected because of previous observations showing differences in RNase amounts and types which was related to freeze survival potential (10, 11, 12).

Plant RNases from many species and cultivars of plants have been studied (2, 20, 31). Reddi (20) proposed a classification system for plant ribonucleases that was later extended by Wilson (29). All enzymes that cleave a polynucleotide can be identified as either a nuclease or
a phosphodiesterase. Wilson classified plant nucleases into three main
groups: (a) RNase I, (b) RNase II, (c) nuclease I. RNase is used to
describe only those enzymes that are capable of hydrolyzing RNA. Both
RNase I and RNase II split RNA by a transfer reaction to form 2'\(3')\)-
cyclic nucleotides. However, RNase I hydrolyzes only the purine cyclic-
nucleotides to produce a 3' nucleotide final product, whereas RNase II
hydrolyzes both purine and pyrimidine cyclic nucleotides to 3' nucleo-
tide final products. RNase I has an optimal pH of 5.0 to 6.0 whereas
the RNase II optimum is pH 6.0 to 7.0 (32). Enzymes that can hydrolyze
DNA and RNA are classed as sugar non-specific enzymes or nucleases.

Partially purified RNase preparations have been obtained from
various plant tissues, including roots, seed, shoots and leaves (2). Efforts to obtain pure RNases from plant tissues have been generally
unsuccessful; however, there are a few examples of reliable purifi-
cation. Maize endosperm RNase I has been purified 4,600-fold by Wilson
(27), barley leaf RNase I was purified 29,000-fold by Lantero and
Klosterman (15), and mung bean nuclease I was purified 45,000-fold by
Kowalski et al. (13). Purification factors in these reports are of
higher magnitude than those usually reported.

The presence of isozymes unique to cellular locations in the
tissue often lead to variability in reported properties of the plant
nucleases (32). Most studies reported involve crude and vigorous homo-
genizing techniques with no subsequent identification of the subcellular
particles and associated enzymes. Non-specific binding of the enzymes
and the variability found with different homogenizing media make it
difficult to draw any conclusions about cellular function and
localization of the various RNases.

Conventional biochemical techniques, such as differential salt precipitation, column chromatography and gel electrophoresis have been used for the separation and purification of plant nucleases. Wilson (27, 28) obtained preparations of RNase I, RNase II and nuclease I from the roots of corn seedlings. The three enzymes were isolated from homogenates by adsorption chromatography on a carboxymethyl-cellulose column. A citrate buffer of pH 6.0 was used to elute and separate RNase I, RNase II and nuclease I. Wilson characterized these enzymes on the basis of molecular weight, pH optimum, ionic requirement and electrophoretic mobility.

Lantero and Klosterman (15) extracted RNase from barley leaf tissue. They used a procedure consisting of ammonium sulfate fractionation, chromatography on Bio-Gel P-30, Cellex-P, and Cellex-0, and then finally preparative gel electrophoresis. They reported two RNase activity peaks. One was contaminated by DNase and phosphomonoesterase activity and was discarded. The second peak, which was relatively free of nucleases and phosphoesterases, had the properties of a RNase I type enzyme.

Oleson and Sasakuma (17) purified S1 nuclease from Aspergillus oryzae 1600-fold with a yield of 32%. The purification procedure included ammonium sulfate fractionation, chromatography on DEAE-cellulose, Concanavalin A-Sepharose, Sephadex G-100 gel filtration and finally phenyl-Sepharose. The S1 nuclease was reported to be a glycoprotein with an associated nucleotidase activity.

Clapham (5) obtained a 2500-fold purification of nuclease I
enzyme, from Tradescantia leaves, with a 50% yield in three steps. The procedure consisted of ammonium sulfate fractionation, 70 C heat treatment followed by Concanavalin A-Sepharose chromatography. He reported the nuclease to be a glycoprotein.

Cuatrecasus et al. (6) reviewed the use of affinity chromatography for selective enzyme purification. In affinity chromatography, the enzyme to be purified is passed through a column matrix having a covalently attached specific competitive inhibitor of the enzyme bound to it. Such inhibitors compete with the substrate for the active site of the enzyme but, once bound, cannot be catalyzed by the enzyme. Since substrate is not present in the column buffers, the covalently attached inhibitor acts as a ligand to bind the enzyme. All proteins without specific affinity for the matrix bound inhibitor pass directly through the column. The bound enzyme can then be eluted in pure form by changing such parameters as salt concentration or pH, or by addition of a competitive inhibitor as an eluant (6). Jervis used 2',(3') guanosine monophosphate, a potent inhibitor of tobacco RNases, attached to Sepharose to purify tobacco RNase I (9). He obtained a 5850-fold purification from partially purified tobacco RNase preparations.

Janski and Oleson (8) reported that nicotinamide adenine dinucleotide phosphate (NADP), immobilized on agarose, was an effective affinity adsorbant for many nucleases. The nucleases they tested included tobacco extracellular nuclease, mung bean nuclease, staphylococcal nuclease, pancreatic RNase and barley leaf RNase.

Wong (33) achieved a 140-fold purification of barley shoot RNase without the use of affinity chromatography as a final step. Hawthorne
et al. (7) used serological techniques, to show this RNase fraction to be homogeneous.

It was surmised that the low specific activity obtained by Wong might be explained by the presence of large amounts of inactive enzyme which could be removed by an affinity procedure. A purification procedure was adopted in this report that used four different properties of proteins for enzyme isolation. The four properties exploited were: a) differential solubility, b) net charge, c) molecular weight and d) active site specificity. Techniques used include ammonium sulfate fractionation, ion-exchange chromatography, gel filtration and affinity chromatography, respectively.
MATERIALS AND METHODS

Plant Materials. Seed from two winter barley cultivars, *Hordeum vulgare* L. cv. "Dicktoo" and "Tennesee Winter", were germinated in 200 ml of 0.1 mM CaCl₂. Adequate seed were started to produce 275 g of shoot tissue from each cultivar after four days germination. The seed were spread uniformly on the blotter paper in a plastic tray which was then covered with opaque plastic. Seed was allowed to germinate for four days in a constant temperature chamber at 25°C. The germination time used for Dicktoo seed was two to three hours less than that for Tennesee Winter. This germination procedure produced shoot tissue of uniform length for both cultivars.

The purification schedule (Figure 1) which follows was designed for handling 275 g of harvested tissue in steps 1 and 2 of purification. Two samples from step 2 were pooled for use in steps 3 to 7. All steps were performed at 2 to 4°C unless otherwise stated.

Step 1. Preparation of Soluble Protein Extract. Ten trays of 4-day seedlings were prechilled in a cold room for at least 10 to 15 minutes before harvest. Harvested shoots were divided into four lots, wrapped in a double layer of aluminum foil and frozen in powdered dry ice. The frozen shoots were pulverized using a prechilled pestle and mortar. The pulverized tissue was transferred to a beaker and mixed with 100 g of insoluble polyvinylpyrrolidone (PVP) and 50 ml of extraction medium. PVP was purified by boiling in 10% hydrochloric acid for at least 30 minutes, then washed with glass distilled water and finally equilibrated with tissue extraction medium before use. Extraction medium consisted of 250 mM sucrose, 600 mM ammonium sulfate, 20 mM
magnesium acetate, 5 mM 2-mercaptoethanol and 50 mM MES buffer, pH 6.5. In order to facilitate thorough tissue extraction, the slurry of pulverized tissue, PVP and extraction medium was mixed in a beaker after being warmed to 0°C. The slurry was filtered through a single layer of Miracloth fitted to a Buchner funnel. Rinsing the slurry allowed about 425 ml of filtrate to be collected for preparative centrifugation. The filtered sample was centrifuged for three hours at 143,000g (0°C) in a Model 35 rotor of a Spinco L-2 centrifuge.

**Step 2. Ammonium Sulfate Fractionation.** The supernatant fraction (415 ml) was mixed with solid (NH₄)₂SO₄ to 40% saturation. After 30 minutes, the suspension was centrifuged at 22,000g for 15 minutes and the precipitate was discarded. Solid (NH₄)₂SO₄ was again added to the supernatant to 80% saturation, and after three hours the suspension was centrifuged a second time. The supernatant was discarded, and the pellet dissolved with 20 ml of 50 mM Tris buffer, pH 7.4.

**Step 3. Combining Samples.** Two 80% (NH₄)₂SO₄ precipitates, each dissolved in 20 ml of buffer, were combined and enough 100% saturated (NH₄)₂SO₄ solution was added to make a 40% (NH₄)₂SO₄ suspension. After 30 minutes, the suspension was centrifuged at 22,000g for 15 minutes and the precipitate was discarded. Solid (NH₄)₂SO₄ was added to the supernatant to 75% saturation, and after three hours the suspension was again centrifuged. The supernatant was discarded and the precipitate dissolved with 20 ml of 50 mM Tris buffer, pH 7.4.

**Step 4. Desalting by Column Chromatography.** The dissolved precipitate was desalted on a column of P-6 DG (Bio-Rad). The bed dimensions were 2x35 cm having a total volume of 110 ml. A 50 mM
Tris-HCl buffer, pH 7.4 was used to equilibrate the column and also to elute the protein peak. The protein peak was collected by measuring effluent absorbance at 280 nm following the void volume.

Step 5. Ion-Exchange Chromatography. A 2.5x3 cm bed of DEAE-Sepharose (Sigma) was used to bind RNase from the extracts of two 275 g batches of tissue. The column was equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 0.01% Triton X-100. After the P-6 DG desalted fraction was layered onto the DEAE-Sepharose column, 250 ml of equilibration buffer was used to wash the column content. This buffer wash was followed by approximately 1500 ml of equilibration buffer containing 20 mM KCl. Elution of the protein peak, having ribonuclease activity, was accomplished with the equilibrating buffer containing 500 mM KCl. The elution step was terminated when the absorbance of the effluent dropped below 0.20 A<sub>280</sub>. About 35 ml were collected from the column containing the RNase fraction. This fraction was prepared for the next step by desalting on a 2x35 cm bed of P-6 DG equilibrated with 50 mM Tris-HCl buffer, pH 7.4. The desalted fraction was shell-frozen in a flask and lyophilized.

Step 6. Bio-Gel P-100 Gel Filtration Chromatography. A 2.6 cm diameter column was filled to 94 cm with Bio-Gel P-100 equilibrated with a buffer containing 50 mM Tris-HCl, pH 7.4, 125 mM sucrose, and 100 mM KCl. The lyophilized sample from DEAE-Sepharose chromatography (Step 5) was hydrated in 2 to 3 ml of the equilibrating buffer and layered onto the column. The flow rate was approximately 8 ml per hour and fractions of 2.5 ml were collected. Fractions containing two distinct peaks of ribonuclease activity were pooled separately and the samples
were desalted and lyophilized as previously described (Step 5), thereby preparing the sample for the next step.

**Step 7. Affinity chromatography.** A 1.25 x 1.5 cm bed of Agarose 5'-(p-aminophenyl phosphoryl) uridine 2'(3') phosphate (APUP-agarose) purchased from Miles-Yeda Ltd., was equilibrated with 20 mM KAc buffer, pH 5.0 containing 0.01% Triton X-100. A lyophilized sample from gel filtration (Step 6) was dissolved in 5 ml of equilibrating buffer and layered onto the column. The column was washed with approximately 25 bed volumes of equilibrating buffer. Elution of the purified ribonuclease activity was accomplished with the equilibrating buffer containing 500 mM KCl.

**Concanavalin A - Sepharose Column Chromatography.** Concanavalin A - Sepharose (Con A), obtained from Sigma, was poured to a 4 cm height in a 2.5 cm dia. column and was equilibrated with 50 mM Tris-HCl buffer, pH 7.4 containing 10 mM CaCl₂ and 0.01% Triton X-100. The column was washed with equilibrating buffer until the A₂₈₀ of the effluent was 0.10 or less compared to a buffer blank. Effluent fractions collected during the initial application of the sample contained RNase activity that was not bound by the Con A. The RNase activity retained by the column was eluted with the equilibrating buffer containing 500 mM α-methyl-D-mannoside. Thus, two fractions of RNase were obtained by this step.

**SDS - Acrylamide Electrophoresis.** The discontinuous SDS buffer system described by Laemmli (14) was followed with some modifications. Gel slab dimensions were .75 x 160 x 180 mm. The separating gel contained 10.0% acrylamide (Eastman), 0.26% bisacrylamide (Eastman), 0.1%
SDS, 375 mM Tris-HCl, pH 8.8, 0.033% ammonium persulfate, 0.033% (V/V) TEMED (Eastman). The stacking gel contained 4.0% acrylamide, 0.1% bisacrylamide, 0.1% SDS, 125 mM Tris-HCl, pH 6.8, 0.008% ammonium persulfate, 0.005% TEMED. Samples, containing 20 to 40 ug of protein, were mixed 1:1 volume ratio with sample buffer containing 125 mM Tris-HCl, pH 6.8, 4.0% SDS, 10.0% 2-mercaptoethanol, 0.01% bromphenol blue and 40% sucrose. Samples were heated for two minutes at 100°C, 25 to 75 ul aliquots were applied to the gels. Electrode buffer containing 19 mM glycine, 25 mM Tris-HCl, pH 8.5, and 0.1% SDS was used in both electrode chambers. Electrophoresis was conducted at 40 mA with variable voltage until the bromphenol blue tracking dye reached the bottom of the gel. Duration of electrophoresis was about 2.5 to 3 hours.

Protein was silver stained following the method of Morrissey (16). Gels were prefixed in a shallow pyrex dish in a 50% methanol and 10% acetic acid solution for 30 minutes. The gels were then fixed for 30 minutes in 10% glutaraldehyde and soaked in a large volume of glass distilled water overnight. The next day, gels were soaked in 5 ug/ml dithiothreitol for 30 minutes and then treated with a 0.1% silver nitrate solution for 30 minutes. After silver nitrate treatment, the gels were rinsed once rapidly with a small amount of glass distilled water and then twice rapidly with a small amount of developer. Gels were soaked in developer containing 3% sodium carbonate and 0.0185% formaldehyde until the desired level of staining was attained. Staining was stopped by adding 2.3 M citric acid.

Protein Determination. Protein was determined by the method of Bradford (4). For this analysis, Bio-Rad reagent was used with bovine
serum albumin as the standard. In some cases, monitoring column ef-
fluents at 280 nm was also used as an approximate measure of protein
distribution.

**Ribonuclease Assay.** Ribonuclease activity was measured using
the procedure described by Tuve and Anfinsen (24), with modification.
The reaction mixture consisted of 50 mM potassium acetate, pH 5.5, 160
mM KCl, 4 mM EDTA, 4 mg of yeast RNA (Crestfield preparation), and the
enzyme solution. The final volume of the reaction mixture was 2.5 ml.
Two milligrams of yeast RNA were used per tube in routine assays of the
effluent fractions from column chromatography. The reaction mixture was
incubated for 30 minutes at 37 C in a water bath. After incubation,
the reaction mixture was cooled immediately to 0 C and the reaction was
stopped by the addition of 0.5 ml of 25% perchloric acid containing
0.75% uranyl acetate. The tubes were centrifuged for 10 minutes at
2,200g (0 C). A 20-fold dilution of 0.2 ml of the enzyme reaction was
made using glass-distilled water. The absorbance of the diluted samples
was read at 260 nm.

Wilson (31) defined a standard unit of enzyme as that amount of
RNase which releases one A260 unit of acid-soluble nucleotide/ minute in
a volume of 1 ml. Since the standard assay volume + precipitant = 3.0
ml and the A260 unit is defined for 1 ml, this is equivalent to a dilu-
tion factor of 3. The calculations used were modified to reflect this
dilution:

\[
\text{units/ml/min} = \frac{A_{260} \times (3.0 \text{ ml} ÷ 1.0 \text{ ml}) \times \text{(dilution factor)}}{\text{ml of enzyme} \times \text{minutes}}
\]
Nuclease Assay  Nuclease activity was also measured under the same assay conditions as for ribonuclease, except that DNA was the substrate and 60 mM MgCl₂ was added to the reaction mixture. Salmon sperm DNA was boiled for 20 minutes and cooled rapidly on ice to denature. After the assay, followed by centrifugation, the supernatant was diluted 20-fold and the absorbance was read at 260 nm.

Phosphomonoesterase Assay  The activity of phosphomonoesterase was measured with the method described by Wong (33). The reaction mixture consisted of 0.4 ml of 125 mM potassium acetate buffer pH 5.0, 0.5 mM p-nitropheny1 phosphate (pNPP) and enzyme solution to provide a final volume of 1 ml. An incubation period of 30 minutes at 37°C was used for the assay. The reaction was stopped by cooling the reaction mixture to 0°C and adding 0.4 ml of 300 mM sodium hydroxide. Absorbance of the resulting solution was read at 420 nm as a measure of enzyme activity. The pNPP is catalyzed by several enzymes (17). Therefore, the convention was adopted of using "pNPPase" to describe the collective activity of these enzymes.
RESULTS AND DISCUSSION

A RNase was purified from two cultivars of winter barley, *Hordeum vulgare* L. cv. "Dicktoo" and "Tennessee Winter"; these two cultivars represent the extremes in freezing survival potential when properly cold acclimated. However, the seedlings used in this study were not cold acclimated, therefore differences found in RNase characteristics are genotypically inherent in these cultivars and not induced by low temperature. "Hardy" and "non-hardy" cultivar will be used hereafter as conventional descriptions when referring to Dicktoo, a cultivar ranked among the most freeze resistant in *Hordeum* and Tennessee Winter one which is most susceptible to freezing. These conventions emphasize potential hardiness levels in the two cultivars. By contrast the major attention given by others has been on the physiological development of this potential. The research described here focused on freeze selection effects apart from those due to acclimation.

Purification of RNase. The purification procedure was developed from modification of methods used by several others (15, 26, 33). Approximately 60% of the total RNase activity was precipitated from the crude tissue extract between 40 and 80% (NH₄)₂SO₄ saturation. RNase yield was a compromise between recovery of enzyme and the degree of contaminant removal. This (NH₄)₂SO₄ fractionation resulted in a 40% loss of enzyme activity, but a 3-fold increase in specific activity (Tables 1 and 2). The salting-out procedure also permitted a volume reduction of the crude extract, allowing for more convenience in the liquid chromatography procedures that followed in purification.

DEAE - Sepharose was selected for ion-exchange chromatography
instead of the Sephadex matrix used by Wong (33), because of the high amount of non-specific binding associated with the Sephadex (Kenefick, unpublished data).

Binding studies determined that over a pH range of 6.0 to 8.0, pH 7.4 allowed for the maximum binding of the RNase activity to the column. Some RNase activity was not absorbed by the column; this non-absorbed fraction typically contained less than 3% of the total activity and was discarded. A large volume of buffer was required to wash the column of residual pNPPase activity before the absorbed RNase activity was eluted with 500 mM KCl.

The first attempts at DEAE-Sephrose chromatography gave low yields of RNase activity. Due to the length of time the enzyme was required to be on the column through the washing procedures, it was thought that the enzyme may be irreversibly bound to DEAE or became inactive. However, activity decay curves in the absence of DEAE, showed the enzyme to be stable over 18 hours both at 4°C and at 37°C. Full retention of enzyme activity under these conditions indicated that the problem was not decay in catalytic function or due to protease degradation. The second possibility investigated was that of an irreversible adsorption of the enzyme to the matrix and/or the glass column. Suelter and DeLuca (23) reported that Triton X-100, a non-ionic detergent, was effective in preventing non-specific adsorption of proteins. By including Triton X-100 in the DEAE-Sephrose elution buffer, enzyme recovery was increased about 2-fold for each cultivar (Table 3).

Gel filtration on Bio-Gel P-100 resulted in the identification of three peaks of RNase activity for each cultivar (Figure 2 and 3).
The first peak eluted, fractions 10 to 20, typically contained less than 5% of the total RNase activity and was heavily contaminated with extraneous protein, pNPPase activity and was yellowish in color typical of the applied sample. This first peak was discarded. The second gel filtration peak (GF-PII) in fractions 30 to 45, was relatively free of extraneous protein indicated by a lower 280 nm, but was primarily contaminated by pNPPase activity. The third gel filtration peak (GF-PIII) eluted in fractions 60 to 70 for the hardy cultivar and 50 to 65 for the non-hardy cultivar also had no detectible contaminating proteins (280 nm) and contained low levels of pNPPase activity.

A comparison between the elution profiles for the two cultivars shows a unique difference in the distribution of RNase activity between GF-PII and GF-PIII (Table 4). The non-hardy cultivar showed almost equal distribution between the two peaks, GF-PII contained 27% of the total activity applied to the column and the GF-PIII contained 35%. The GF-PII for the hardy cultivar contained 67% of the total activity and GF-PIII contained only 16%. Wong (33) and Hawthorne et al. (7) also found three peaks of RNase in preparations from these two cultivars after P-100 gel filtration, despite using vastly different approaches to purification prior to this step as reported here.

The final step in the purification procedure was affinity chromatography. Affinity chromatography of RNase has been used successfully by several investigators (8, 9, 26) to obtain purified enzyme in high yield. Prior to this final stage in purification, the concentration of contaminating protein was low, but considerable pNPPase activity was still present (Figures 2 and 3).
Three affinity resins were tested for their ability to separate the pNPPase activity from the RNase activity: (a) cAMP-agarose, (b) NADP-agarose and (c) APUP-agarose. Yang (34) reported using cAMP-agarose to purify three phosphatases to a high specific activity. Several attempts were made to bind the pNPPase activity to the cAMP-agarose column with the expectation that pure RNase activity would be present in the column effluent. I found that both the RNase and pNPPase had a strong affinity for the cAMP-agarose and could not be easily separated by this method. This might be an expected result because Reddi (20) suggested that cyclic nucleotides are substrates of RNase.

NADP-agarose did allow separation of GF-PIII RNase from the pNPPase, but the recovery of pure RNase was low. APUP-agarose was the affinity resin of choice, because it allowed for good separation of the pNPPase and RNase, along with a high yield of RNase.

Figure 4 shows representative GF-PIII elution profiles on APUP-agarose for both cultivars. Small amounts of contaminating protein were removed from the RNase, and presumably inactive forms of the enzyme as well. RNase of both cultivars was effectively purified by this procedure (Tables 1 and 2). The fractions were designated RNase-PIII\textsubscript{H} and RNase-PIII\textsubscript{NH}.

The identification of the purified RNase-PIII was based upon Wilson's criteria (29). The RNase-PIII activity from both cultivars was not inhibited by EDTA as opposed to that reported for nuclease I. Both RNase-PIII\textsubscript{H} and RNase-PIII\textsubscript{NH} corresponded in electrophoretic mobility to RNase I from corn roots (28, 29, 30, 33). Wilson (32) reports
a range of pH 5.0 to 6.0 for optimum RNase I activity. The pH optimum for both RNase-PIII\textsubscript{NH} and RNase-PIII\textsubscript{H} was found to be pH 5.4 (Figure 10). On the basis of this data I have determined RNase-PIII\textsubscript{H} and RNase-PIII\textsubscript{NH} to be RNase I.

The GF-P\textsubscript{II} fraction of both cultivars contained high amounts of pNPPase. An attempt was made to remove the pNPPase before gel filtration. The data in Table 2 suggests that perhaps hydrophobic interactions contributed to low RNase recovery because Triton X-100 improved enzyme yield. The large amount of buffer required to wash the residual pNPPase activity off the column also suggested hydrophobic interactions may have affected separation.

The high ionic strength used for our extraction of RNase likely removed some membrane proteins (21), including phosphatases. Phosphatases are associated with cell membranes as glycoproteins (1) and pNPP serves as a synthetic substrate for these enzymes (17). Therefore, Con A affinity chromatography was attempted as a means to remove from GF-P\textsubscript{II} some of these extracted glycoproteins, including those having pNPPase activity. Con A did bind some pNPPase activity, but it also retained a sizeable proportion of the total RNase recovered from high salt extracted tissue. Attention was therefore directed to the possibility of this plant RNase also being a glycoprotein.

There was a significant difference between the amount of RNase bound by Con A for the two cultivars (Table 5). The differential distribution of RNase in the two cultivars shown by gel filtration (Table 4) and also their division into a bound and unbound fractions by Con A (Table 5), suggested that these two procedures were separating
two enzymes by different molecular properties that could be useful in purification. Such a possibility could be demonstrated by inserting Con A chromatography into the procedure prior to gel filtration.

Figure 5 shows a gel filtration elution profile of the RNase fraction from the non-hardy cultivar that was previously bound by Con A. The second prominent peak of RNase activity, fractions 35 to 45, was in approximately the same position as GF-P_{II} of a protein preparation not subjected to Con A chromatography (Figure 2). Figure 5 also shows a trailing peak of RNase. It typically contained one tenth the amount of RNase found in the second peak. Subsequent studies showed it to be GF-P_{III} RNase that could be removed by thorough rinsing of the Con A column.

Figure 6 shows the gel filtration elution profile of the RNase fraction from the non-hardy cultivar that was not bound by Con A. There was only one RNase peak eluted from the gel filtration column when this sample was chromatographed. The position of the peak corresponds with the position of GF-P_{III} not previously subjected to Con A chromatography (Figure 2). RNase corresponding to GF-P_{II} was not present in this gel filtration effluent, indicating that only the GF-P_{II} fraction was totally bound by the Con A. Figures 7 and 8 show similar results for P-100 gel filtration of RNase fractions from the hardy cultivar. The Con A bound and non-bound RNase fractions of the hardy cultivar corresponded to GF-P_{II} and GF-P_{III}, respectively. The amount of RNase activity found in the single peaks of Figures 7 (GF-P_{II}) and 8 (GF-P_{III}) relate well to the amount of GF-P_{II} and GF-P_{III} present after gel filtration as reported in Table 4. This data conclusively shows that
GF-PII was bound by Con A indicating that it is a glycoprotein.

Con A chromatography provided new information about the GF-PII RNase in plants, however, it did not meet the original objective of separating the pNPPase from this fraction. Attempts to separate the pNPPase from the GF-PII by Con A or other methods were not successful. GF-PII was not retained by the NADP-agarose in contrast to GF-PIII. GF-PII was bound to APUP-agarose, however, it was impossible to remove it from the column using 500 mM KCl, 30% ethylene glycol or 20 mM Tris-HCl pH 7.8. Other approaches will be required to further purify GF-PII.

The GF-PII and RNase-PIII fractions were assayed with DNA as a substrate to determine DNase and/or nuclease activity. These fractions were shown to be RNA specific and were not inhibited by EDTA (Table 6). GF-PII fractions showed slight residual DNase and/or nuclease activity but this could be expected with the impurities still remaining in the fraction.

The pH optimum for GF-PII RNase was found to be pH 6.2 (Figure 10). Wilson (29) reports a range of pH 6.0 to 7.0 for optimum RNase II activity. On the basis of the pH data, and the absence of contaminating nuclease, I have identified GF-PII as RNase II. Affinity of the GF-PII fractions for Con A and their elution from the immobilized lectin by α-methyl-D-mannoside presents new information that suggests barley RNase II is a glycoprotein.

Electrophoresis on SDS-acrylamide gels was used as a criteria for purity. Figure 9 shows a gel silver-stained for protein. The lanes containing GF-PII (lanes d and f) show several proteins still present in
the preparation. GF-PIII (RNase fraction prior to affinity chromatography in lanes e and g) show some contaminating proteins still present at this stage in purification. The final preparation of RNase-PIII (lane h) shows only a single band of stained protein.

In previous studies on barley shoot tissue, Wong (33) concluded that the non-hardy cultivar contained much higher RNase activity than did the hardy cultivar. This conclusion was based on activity measurements of only purified RNase I. Hawthorne et al. (7) applied serological techniques to characterize RNase I from barley shoots, using Wong's method to obtain pure RNase. Antibody against GF-PIII (RNase I) was developed in rabbits. Hawthorne et al. (7) demonstrated with rocket immunoelectrophoresis that crude extracts from the hardy cultivar contained as much RNase as did crude extracts from the nonhardy cultivar. This serological procedure detects molecular forms of RNase and is not dependent on activity measurement. Subsequent unpublished results (Hawthorne) showed that both GF-PII and GF-PIII were cross-reactive with anti-GF-PIII, indicating that in crude extracts both forms of RNase were measured by serology. Though RNase amounts were the same, catalytic rate still appeared to be lower for RNase from the hardy cultivar (7). Because of the carbohydrate moiety, it may be possible however, that GF-PII is difficult to recover from the tissue extract and could be lost without proper precautions in the purification steps, leading to lower recovery from the hardy tissue.

Table 3 shows that the effect of Triton X-100 addition during ion exchange resulted in equal amounts of RNase recovered for each cultivar. However, in the absence of Triton X-100, the yield of enzyme
was lower from the hardy cultivar, indicating greater difficulty in the recovery of the glycoprotein RNase II. Thus, detection of RNase from plant tissue may be influenced by the type of RNase present (Table 4), and measurement at its optimum pH (Table 10).

The purification method developed for this study allowed us to isolate both RNase I and RNase II. Activity measurements indicate that the non-hardy cultivar has, as a percent of total activity, a higher amount of RNase I compared to RNase II (Table 7). The hardy cultivar has the reverse situation, that of low amounts of RNase I and high amounts of RNase II. This differential enzyme distribution is a fixed trait which is not dependent upon cold acclimation.

These results lead to the speculation that the hardy cultivar has more RNase associated with membranes and less free enzyme in the cytoplasm than does the non-hardy cultivar. Some alternative explanations for this differential distribution of RNase might be:

A. In barley a dynamic equilibrium may exist between RNase I and RNase II that controls the amount of RNase soluble in the cytoplasm. Hawthorne (unpublished data) showed that GF-PII (RNase II) and GF-PIII (RNase I) had the same antigenic sites. Since the carbohydrate component does not influence cross-reactivity (18), one can conclude that RNase I and RNase II in barley have the same amino acid composition and likely come from the same DNA sequence. Plummer and Hirs (19) showed that pancreatic RNase A and RNase B (a glycoenzyme) have essentially the same sequence of amino acids and the same catalytic properties. Results reported here suggest that RNase may be in an interchangeable pool in plant cells similar to the RNase A and RNase B
found in rat liver cells. Perhaps RNase functions as some cellular signal, similar to that found in animal cells by Baynes and Wold (3).

B. Increased amounts of glycoproteins in membranes of hardy cultivars may serve some functional purpose such as increased stability or to facilitate transport in membranes. Following a lethal frost, there is often a loss in the semipermeability properties of the plasma membrane in plants. This has led to the belief that the plasma membrane is the primary site of freezing injury (35). It has been demonstrated that glycoproteins in plasma membrane are responsive to cold adaptation of rye (25).

C. Perhaps the hardy cultivar has smaller cells, or higher content of membranes per cell, and thus more membrane per unit of tissue. The hardy cultivar may therefore contain more RNase II bound by membranes.

Any of these possibilities could explain the distribution of RNase as presented in this report. These results maybe significant if it can be assumed that the RNase variance is a result of freeze selection. This possibility is supported by previous evidence presented on winter barley (10, 11, 12).

There is mounting evidence to indicate that membranes are intimately involved in freezing injury (25). Yoshida (35) has demonstrated that membrane lipid unsaturation which causes fluidity is not related to the development of cold hardiness. He presented evidence that changes in membrane protein may have a role in freeze survival. Glycoprotein changes during cold acclimation may suggest an alteration in function (25). Plasma membranes undergo a phase transition related to freezing
injury; as cold hardiness increases the phase transition temperatures are shifted downward. This transition appears to depend not on the lipids, but primarily on membrane proteins (35), which have the effect of preventing lipid chains from crystallizing (22). Further information on protein changes in plasma membranes during cold acclimation is still required.

This report presents evidence that RNase II is a glycoprotein in winter barley and appears to vary in proportion to the intensity of freeze selection. The variation that exists is inherent to the cultivar and not induced by low temperature. These findings should aid in understanding the function of these enzymes in winter barley cultivars and establishing their roles in relation to freeze survival potential.

Results in this paper present a purification method that can be used for the isolation of at least two fractions of RNase from barley shoot tissue. One fraction, RNase P-III from both the hardy and non-hardy cultivar, was identified as being RNase I type enzyme. RNase I was purified 2680-fold with a yield of 7% from the non-hardy cultivar and 1772-fold with 3% yield from the hardy cultivar (Tables 1 and 2). This report also presents the first evidence to our knowledge for a RNase from plants that is a glycoprotein. GF-PIII was identified, by the use of Con A, as a glycoprotein. The complete purification of GF-PIII has yet to be accomplished.
Literature Cited


Table 1. Purification of Barley RNase from the non-hardy cultivar.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Enzyme Units*</th>
<th>Total Protein* (mg)</th>
<th>Specific Activity**</th>
<th>Enzyme Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Centrifuge Supernatant</td>
<td>415</td>
<td>1538</td>
<td>582</td>
<td>2.7</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. 40-80% ((NH_4)_2SO_4) Cut</td>
<td>20</td>
<td>956</td>
<td>121</td>
<td>7.9</td>
<td>62</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two ((NH_4)_2SO_4) Cuts Were Pooled</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 40-75% ((NH_4)_2SO_4) Cut</td>
<td>20</td>
<td>1824</td>
<td>216</td>
<td>8.4</td>
<td>59</td>
<td>3</td>
</tr>
<tr>
<td>4. Desalted Step 3 Sample</td>
<td>29</td>
<td>1833</td>
<td>219</td>
<td>8.4</td>
<td>59</td>
<td>3</td>
</tr>
<tr>
<td>5. Ion-exchange Eluant</td>
<td>52</td>
<td>1071</td>
<td>66</td>
<td>16.2</td>
<td>35</td>
<td>6</td>
</tr>
<tr>
<td>6. Gel Filtration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) GF-PII (RNase II)</td>
<td>31</td>
<td>205</td>
<td>10</td>
<td>20.5</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>b) GF-PIII (RNase I)</td>
<td>42</td>
<td>370</td>
<td>4</td>
<td>88.0</td>
<td>12</td>
<td>33</td>
</tr>
<tr>
<td>7. Affinity Chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNase-PIII_{NH} (RNase I)</td>
<td>37</td>
<td>202</td>
<td>0.028</td>
<td>7232.0</td>
<td>7</td>
<td>2680</td>
</tr>
</tbody>
</table>

* See Materials and Methods for definition of an enzyme unit and protein determination.

** Enzyme units/mg protein.
Table 2. Purification of Barley RNase from the hardy cultivar.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Enzyme Units*</th>
<th>Total Protein* (mg)</th>
<th>Specific Activity**</th>
<th>Enzyme Recovery (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Centrifuge Supernatant</td>
<td>415</td>
<td>1066</td>
<td>426</td>
<td>2.5</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. 40-80% (NH₄)₂SO₄ Cut</td>
<td>20</td>
<td>616</td>
<td>92</td>
<td>6.7</td>
<td>58</td>
<td>3</td>
</tr>
<tr>
<td>Two (NH₄)₂SO₄ Cuts Were Pooled</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. 40-75% (NH₄)₂SO₄ Cut</td>
<td>20</td>
<td>1080</td>
<td>158</td>
<td>6.8</td>
<td>50</td>
<td>3</td>
</tr>
<tr>
<td>4. Desalted Step 3 Sample</td>
<td>31</td>
<td>1284</td>
<td>186</td>
<td>6.8</td>
<td>58</td>
<td>3</td>
</tr>
<tr>
<td>5. Ion-exchange Eluant</td>
<td>36</td>
<td>716</td>
<td>61</td>
<td>11.7</td>
<td>34</td>
<td>5</td>
</tr>
<tr>
<td>6. Gel Filtration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) GF-PII (RNase II)</td>
<td>31</td>
<td>459</td>
<td>10</td>
<td>45.9</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>b) GF-PIII (RNase I)</td>
<td>21</td>
<td>103</td>
<td>1</td>
<td>103.0</td>
<td>5</td>
<td>41</td>
</tr>
<tr>
<td>7. Affinity Chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNase-PIIIH (RNase I)</td>
<td>32</td>
<td>62</td>
<td>0.014</td>
<td>4430.0</td>
<td>3</td>
<td>1772</td>
</tr>
</tbody>
</table>

* See Materials and Methods for definition of an enzyme unit and protein determination.

** Enzyme units/mg protein.
Table 3. A comparison of the effect of Triton X-100 on RNase recovery during DEAE-Sepharose ion exchange of protein extracts* from each winter barley cultivar

<table>
<thead>
<tr>
<th></th>
<th>Hardy +Triton</th>
<th>Hardy -Triton</th>
<th>Non-Hardy +Triton</th>
<th>Non-Hardy -Triton</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase units** applied to the column</td>
<td>1624</td>
<td>1230</td>
<td>1909</td>
<td>1714</td>
</tr>
<tr>
<td>RNase units recovered by 500 mM KCl elution</td>
<td>1050</td>
<td>358</td>
<td>1230</td>
<td>625</td>
</tr>
<tr>
<td>Percent recovery</td>
<td>65%</td>
<td>29%</td>
<td>64%</td>
<td>36%</td>
</tr>
</tbody>
</table>

* The soluble protein fraction used was obtained from a 40 to 80% (NH₄)₂SO₄ saturation cut of a 4-day shoot tissue extraction. About 225 mg of protein was applied to a 2.5 by 3.0 cm bed of DEAE Sepharose and washed with 1500 ml of buffer containing (+) or without (-) Triton X-100 before 500 mM KCl elution.

** See Materials and Methods for definition of an enzyme unit.
Table 4. A comparison of RNase activity distribution between GF-PII and GF-PIII from gel filtration of each winter barley cultivar extract.

<table>
<thead>
<tr>
<th></th>
<th>Hardy</th>
<th>Non-Hardy</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase units* applied to the gel filtration bed**</td>
<td>716</td>
<td>1230</td>
</tr>
<tr>
<td>RNase contained in GF-PII fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Units of activity</td>
<td>459</td>
<td>338</td>
</tr>
<tr>
<td>b) % of total activity</td>
<td>64%</td>
<td>27%</td>
</tr>
<tr>
<td>RNase contained in GF-PIII fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Units of activity</td>
<td>103</td>
<td>428</td>
</tr>
<tr>
<td>b) % of total activity</td>
<td>14%</td>
<td>35%</td>
</tr>
</tbody>
</table>

* See Materials and Methods for definition of an enzyme unit.

** The soluble protein fraction used was the 500 mM KCl eluant from a DEAE-Sepharose column. The 500 mM KCl eluant was desalted and lyophilized and redissolved before application to the column.
Table 5. A comparison of the amount of RNase bound by Concanavalin A - Sepharose from protein extracts* of two winter barley cultivars.

<table>
<thead>
<tr>
<th>RNase units** applied to the column</th>
<th>Hardy</th>
<th>Non-Hardy</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase not bound by the Con-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Units of activity</td>
<td>198</td>
<td>476</td>
</tr>
<tr>
<td>b) % of total activity</td>
<td>16%</td>
<td>34%</td>
</tr>
<tr>
<td>RNase bound by the Con-A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Units of activity</td>
<td>525</td>
<td>361</td>
</tr>
<tr>
<td>b) % of total activity</td>
<td>44%</td>
<td>26%</td>
</tr>
</tbody>
</table>

* The soluble protein fraction used was obtained from a 40 to 80% (NH₄)₂SO₄ saturation cut of a 4-day shoot tissue extraction. About 225 mg of protein was applied to a 2.5 by 3.0 cm bed of Con A - Sepharose. The bound enzyme was eluted with 500 mM α -methyl -D- mannoside.

** See Materials and Methods for definition of an enzyme unit.
Table 6. Nuclease and ribonuclease determination in the GF-P_{II} and GF-P_{III} fractions from both cultivars of winter barley. Assays preformed with (+) and without (-) EDTA.

<table>
<thead>
<tr>
<th></th>
<th>RNase Assay $A_{260}$</th>
<th>Nuclease* Assay $A_{260}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+EDTA</td>
<td>-EDTA</td>
</tr>
<tr>
<td>GF-P_{II} (non-hardy)</td>
<td>.26 .29</td>
<td>.30 .31</td>
</tr>
<tr>
<td>GF-P_{III} (non-hardy)</td>
<td>.55 .56</td>
<td>.56 .54</td>
</tr>
<tr>
<td>GF-P_{II} (hardy)</td>
<td>.53 .55</td>
<td>.60 .61</td>
</tr>
<tr>
<td>GF-P_{III} (hardy)</td>
<td>.42 .43</td>
<td>.40 .42</td>
</tr>
<tr>
<td></td>
<td>Bl .04</td>
<td>B1 .04</td>
</tr>
</tbody>
</table>

* Same as RNase assay except DNA was substituted for RNA, with addition of 60 mM MgCl$_2$. 
Table 7. A comparison of GF-PIII (RNase I) and GF-PII (RNase II) activity present in two cultivars of winter barley after gel filtration.

<table>
<thead>
<tr>
<th></th>
<th>RNase I</th>
<th>RNase II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-hardy cultivar</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) units of activity*</td>
<td>428</td>
<td>338</td>
</tr>
<tr>
<td>b) % of total activity</td>
<td>56%</td>
<td>44%</td>
</tr>
<tr>
<td><strong>Hardy cultivar</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) units of activity*</td>
<td>103</td>
<td>459</td>
</tr>
<tr>
<td>b) % of total activity</td>
<td>18%</td>
<td>82%</td>
</tr>
</tbody>
</table>

* See Materials and Methods for definition of an enzyme unit.
4-day shoot tissue 275g 275g
Frozen, pulverized and mixed with buffer and PVP
Miracloth filtration

Step 1. Centrifugation (3 hr. at 143,000g)

Step 2. 40-80% (NH₄)₂SO₄ Precipitation
Dissolve in 20 ml of 50 mM Tris-pH 7.4
80% (NH₄)₂SO₄ precipitates pooled

Step 3. 40-75% (NH₄)₂SO₄ precipitation
Dissolve in 20 ml of 50 mM Tris-pH 7.4
(NH₄)₂SO₄ Precipitated RNase fraction

Step 4. Desalting (P-6 DG)
Desalted RNase Fraction

Step 5. Ion-exchange (DEAE-Sepharose)
RNase Fraction (500 mM KCl)

Step 6. Gel Filtration (P-100)
GF-PII
GF-PIII

Step 7. Affinity Chromatography (APUP-agarose)
RNase-PIII

Figure 1. Purification schedule for plant RNase.
Figure 2. Bio-Gel P-100 gel filtration of the DEAE-Sepharose fraction from the non-hardy cultivar. Approximately 900 units of enzyme in 2 ml were applied to the column. Gel bed dimension: 2.6 x 94 cm; elution buffer: 50 mM Tris-HCl, pH 7.4, with 125 mM sucrose and 100 mM KCl; flow rate: 7 ml/hr. Protein was estimated at 280 nm absorbance, the RNase assay products were measured at 260 nm and the pNPPase assay products were measured at 420 nm.
Figure 3. Bio-Gel P-100 gel filtration of the DEAE-Sepharose fraction from the hardy cultivar. Approximately 800 units of enzyme in 3 ml were applied to the column. Gel bed dimension: 2.6 x 94 cm; elution buffer: 50 mM Tris-HCl, pH 7.4, with 125 mM sucrose and 100 mM KCl; flow rate: 7 ml/hr. Protein was estimated at 280 nm absorbance, the RNase assay products were measured at 260 nm and the pNPPase assay products were measured at 420 nm.
Figure 4. Uridine - 2'\( (3') \)-phosphate-agarose affinity chromatography elution profiles of GF-PIII from the non-hardy cultivar (left panel) and the hardy cultivar (right panel). Approximately 350 units of enzyme in 10 ml were applied to the column. Gel bed dimension: 1.25 x 1.5 cm; equilibrating buffer: 20 mM KAc - pH 5.0, with 0.01% Triton X-100; elution buffer: 20 mM KAc - pH 5.0, with 0.01% Triton X-100 and 500 mM KCl; flow rate: 15 ml/hr. The arrows show the fractions where 100 mM KCl was added to the wash buffer and where 500 mM KCl was used to elute the RNase. The Triton X-100 used in the buffer accounts for the high background absorbance at 280.
Figure 5. Bio-Gel P-100 gel filtration of the protein fraction from the non-hardy cultivar and bound by Con A. Approximately 500 units of enzyme in 3 ml were applied to the column. The RNase assay products were measured at 260 nm and the pNPPase assay products were measured at 420 nm.
Figure 6. Bio-Gel P-100 gel filtration of the protein fraction from the non-hardy cultivar and not bound by Con A. Approximately 350 units of enzyme in 3 ml were applied to the column. The RNase assay products were measured at 260 nm and the pNPPase assay products were measured at 420 nm.
Figure 7. Bio-Gel P-100 gel filtration of the protein fraction from the hardy cultivar and bound by Con A. Approximately 500 units of enzyme in 3 ml were applied to the column. Protein was estimated at 280 nm absorbance, the RNase assay products were measured at 260 nm and the pNPPase assay products were measured at 420 nm.
Figure 8. Bio-Gel P-100 gel filtration of the protein fraction from the hardy cultivar and not bound by Con A. Approximately 200 units of enzyme in 3 ml were applied to the column. Protein was estimated at 280 nm absorbance, the RNase assay products were measured at 260 nm and the pNPPase assay product were measured at 420 nm.
Figure 9. SDS-acrylamide gel, silver stained for protein. A diagrammatic trace from a photograph of the acrylamide gel (3/5 actual size).

a) Mixture of Cytochrome C (MW 12,400), Glyceraldehyde-3-phosphate dehydrogenase (MW 36,000), Egg Albumin (MW 45,000), BSA (MW 66,000)
b) Pancreatic RNase A
c) Pancreatic RNase B
d) GF-PIIH
e) GF-PIIIH
f) GF-PIINH
g) GF-PIIIINH
h) RNase-PIIIINH
Figure 10. A comparison of the pH optimum for GF-P II (RNase II) and RNase-PIII (RNase I). A 50 mM KAc buffer was used for pH 4.6, 5.0 and 5.4; 50 mM Mes buffer was used for pH 5.8, 6.2, 6.6 and 7.0.