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THE UNIVERSITY OF ALBERTA

FORMATION OF FLAVOR NUCLEOTIDES IN VEGETABLE PROCESSING

by

THANH THI NGUYEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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Department of Food Science.

We, the undersigned, Drs. D. Hadziyev, M. Palcic and P. Sporns, all from the Department of Food Science, Faculty of Agriculture and Forestry, University of Alberta, provide consent that the following papers:

1. T.T. Nguyen, P. Sporns and D. Hadziyev, Simultaneous Liquid Chromatographic Determination of Ribonucleoside-5'-Monophosphates and Their Isomers in Potato Tuber, J. Chromatogr. 363(2):361-371 (1986).
2. T.T. Nguyen, M.M. Palcic and D. Hadziyev, Liquid Chromatography Assay of Potato Tuber Nuclease and Ribonuclease, J. Chromatogr. 388(1):189-199 (1987).
3. T.T. Nguyen, M.M. Palcic and D. Hadziyev, HPLC Assay for Potato 3'-Nucleotidase, J. Chromatogr. 39(1):257-265 (1987).
4. T.T. Nguyen, M.M. Palcic and D. Hadziyev, Characterization of Cell-Wall Bound Nuclease and Ribonuclease from Potato Tubers, Agric. Biol. Chem. (in press).
5. T.T. Nguyen, M.M. Palcic and D. Hadziyev, Rapid Enzymatic-HPLC Determination of the Content of Major Nucleotides in Vegetables, Agric. Biol. Chem. (in press).
6. T.T. Nguyen, M.M. Palcic and D. Hadziyev, Enzymatic Formation of Nucleotides and the Flavor Enhancer 5'-GMP During Vegetable Processing. Bioflavor '87 International Conference. Wurzburg, FRG. September 29-30, 1987. Walter de Gruyter, New York (in press).

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December 4, 1987.

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Supervisor

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Date Nov. 26, 1987

Dedicated to my diligent and poor countrymen who have provided me with great motivation and inspiration to pursue my scientific career.

ABSTRACT

Isocratic HPLC methods using Whatman Partisil 10 SAX and SCX columns have been developed for determination of flavor-enhancing nucleotides (5'-GMP and 5'-IMP) and other nucleoside monophosphates and their corresponding nucleosides. The procedure used in analysis of nucleotides consisted of sample extraction with cold perchloric acid (PCA), and removal of starch by addition of methanol and centrifugation. Prior to injection into an HPLC system, the PCA was removed from the extract by treatment with tri-*n*-octylamine-Freon 113 solution. With a strong anion exchange column (SAX) and a mobile phase of 3% methanol in 8 mM KH_2PO_4 (solution pH 4.15), the nucleoside-5'-monophosphates and the 2'- and 3'-isomers of GMP were well resolved.

The method was applied for analysis of the free nucleotide content of vegetables, for assaying the activity of vegetable enzymes involved in formation and degradation of nucleotides, and for determining nucleotide constituents of vegetable RNA. Thirteen vegetables were studied, including representatives of flowerhead, fruity, leafy, seed, stem/shoot and tuberous vegetables, and mushrooms. Studies on characterization of vegetable nuclease and ribonuclease (RNase) were carried out by preparation of crude enzyme powders, of which potato crude enzymes from both cytoplasm and cell wall were further purified. Purification of cytoplasmic enzyme was achieved by applying the crude enzyme solution on a gel filtration column, and cell

wall enzyme was fractionated using ammonium sulfate, gel filtration column and ion-exchange chromatography. The activities of both crude vegetable and purified potato nucleases exhibited high (65-75°C) temperature optima, whereas RNases showed lower optimal temperatures (50-60°C). The nuclease and 3'-nucleotidase activity resided in the same protein of both cytoplasmic and cell wall enzymes isolated from potato tubers. Purified cell wall nuclease showed its preference in hydrolysis of RNA for formation of purine nucleotides. The free nucleotide content of raw vegetables was low, but the level increased markedly in cooked vegetables due to enzymatic hydrolysis of endogenous RNA. Differences in the levels of 5'-nucleotides were observed in steamed vegetables. Of two flavor nucleotides, 5'-IMP and 5'-GMP, only 5'-GMP was found in vegetables. The 5'-GMP derived from endogenous RNA during steam cooking ranged from 0% (cabbage) to 44% (shiitake mushroom) of total nucleotides.

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1. INTRODUCTION

High performance liquid chromatography (HPLC) has been available to analytical researchers since the beginning of the 1970's [1,2]. Separation methods for nucleic acid constituents were then developed rapidly with this powerful technique [3-5].

This study was designed to develop a series of HPLC methods for the analysis of nucleic acid related substances. The methods developed were applied in assaying the enzyme activities involved in formation and degradation of nucleotide monophosphates. The major objective was to maximize the amount of natural flavor nucleotides, especially guanosine 5'-monophosphate (5'-GMP), in processing of vegetables.

Several papers have been published during the course of this study. This thesis is presented in the "paper format", following guidelines of the Faculty of Graduate Studies and Research, University of Alberta. Since published papers have to be somewhat "condensed versions", this chapter provides the detailed background information for the subjects involved.

1.1 Nucleic Acids and their Constituents

Nucleic acids and their related enzymes are of immense biological importance in all living cells. There are two groups of nucleic acids, differentiated by the carbohydrate present. Those which contain β-D-ribose are generally known

as ribonucleic acids (RNA's), while those containing β D-2 deoxyribose are deoxyribonucleic acids (DNA's). Both RNA and DNA are polymers composed of monomeric units called nucleotides, thus a nucleic acid is also a polynucleotide. Nucleic acids are long chain, high molecular weight polymers. A short chain polymer is referred to as an oligonucleotide (Greek oligo, "few") [6].

At one time, it was assumed that RNA was the characteristic nucleic acid of plants and DNA that of animals. It is now clear that plants and animals each have both types of nucleic acids. RNA is found principally in the cytoplasm, although small amounts are found in the nucleus, nucleoli and chromosomes. DNA is found predominantly in the nucleus. The earlier supposition resulted from the fact that animal cells used in experiments had been ones with predominant nuclei and little cytoplasm, while the reverse was true of plant cells [7].

Both RNA and DNA contain four different heterocyclic bases which are the derivatives of pyrimidine and purine compounds possessing aromatic character. Purine may itself be regarded as a derivative of pyrimidine by fusing together a pyrimidine and an imidazole ring. Two purine bases, adenine and guanine, and the pyrimidine cytosine are common to both RNA and DNA, whereas uracil and thymine are the respective second pyrimidines in RNA and DNA. Chemical structures of pyrimidine, purine and the major base components of nucleic acids are shown in Figure 1.1.

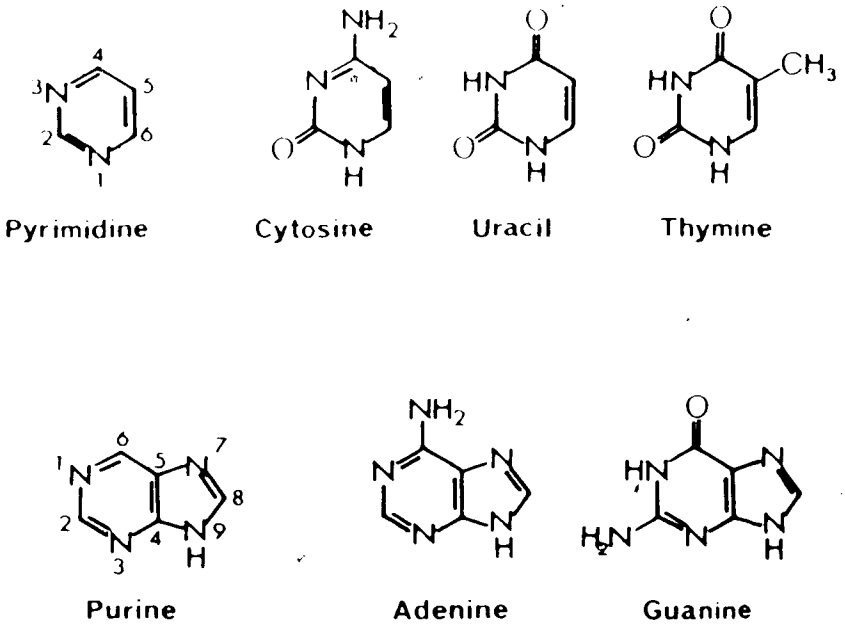


Figure 1.1. Chemical structures of pyrimidine, purine and the major base constituents of nucleic acids.

Table 1.1: Some of the Important Minor Bases in RNA

1-Methyladenine	Dihydrouracil
2-Methyladenine	5-Hydroxyuracil
6-Methyladenine	5-Carboxymethyluracil
6,6-Dimethyladenine	5-Methyluracil (thymine)
6-Isopentenyladenine	5-Hydroxymethyluracil
2-Methylthio-6-isopentenyladenine	2-Thiouracil
6-Hydroxymethylbutenyladenine	3-Methyluracil
6-Hydroxymethylbutenyl-2-methylthioadenine	5-Methylamino-2-thiouracil
1-Methylguanine	5-Methyl-2-thiouracil
2-Methylguanine	5-Uracil-5-hydroxyacetic acid
2,2-Dimethylguanine	3-Methylcytosine
7-Methylguanine	4-Methylcytosine
2,2,7-Trimethylguanine	5-Methylcytosine
Hypoxanthine	5-Hydroxymethylcytosine
1-Methylhypoxanthine	2-Thiocytosine
Xanthine	4-Acetylcytosine
6-Aminoacyladenine	

Source: ref. 8.

In addition to the common bases mentioned above, a large number of pyrimidine derivatives called minor bases occur in small amounts in some nucleic acids (Table 1.1). Minor bases are especially important in transfer RNA's, which may contain up to 10% of these components [8].

All pyrimidine and purine bases of nucleic acids are colorless, crystalline compounds. They are slightly soluble in water, but are readily soluble in either dilute acid or alkali. They are generally insoluble in organic solvents. The strongly characteristic ultraviolet absorption of nucleic acid constituents in the region of 250 to 280 nm is very useful for the detection of pyrimidine and purine containing compounds using UV detectors in liquid chromatography [9].

A nucleoside is composed of one nitrogenous heterocyclic base and one pentose, a D-ribose for ribonucleoside and a D-2-deoxyribose for deoxyribonucleoside. The pentose is joined to the base by a β-N-glycosyl bond between carbon atom 1 of the pentose and nitrogen atom 1 of the pyrimidine base or nitrogen atom 9 of the purine base. The numbering systems for the pyrimidine and purine follow the conventions for the bases. The carbon atoms of the carbohydrate are numbered with primes to distinguish them from the atoms of the heterocyclic base.

A nucleotide is a combination of a nucleoside and one or more molecules of phosphoric acid. The attachment of the phosphate group(s) is of considerable importance. It can be

an ester linkage with carbon atoms 2', 3' or 5' in ribose or carbon atoms 3' or 5' in deoxyribose, giving 2'-, 3'- and 5'-ribonucleotides, and 3'- and 5'-deoxyribonucleotides. A nucleotide is named in sequence by a combination of the name of its corresponding nucleoside, the position of the attachment of phosphate group to the pentose, and the number of phosphate groups. Figure 1.2 illustrates the structure of a base, its nucleoside and nucleotide.

The 5'-ribonucleotides can be distinguished from their 2'- and 3'-isomers and from the deoxynucleotides by the fact that they are susceptible to periodate oxidation owing to the presence of the cis-diol group [10,11], as shown in Figure 1.3.

All common ribonucleotides and deoxyribonucleotides occur in natural biological systems as 5'-phosphate esters [12], including 5'-mono-, di- and triphosphates. In addition to the 5'-nucleotides, some cyclic nucleotides have been found in cells. Among these nucleotides, well known as enzymatic intermediates, are nucleoside 2':3'-cyclic monophosphates (2':3'-cyclic nucleotides). These cyclic nucleotides and ribonucleoside-2'- and -3'-monophosphates are the products of ribonuclease catalyzing the degradation of RNA [6]. These low molecular weight compounds are called "acid soluble nucleotides" as they are soluble in cold dilute solutions of perchloric acid (PCA) and trichloroacetic acid (TCA) commonly used to kill and extract cells. Polynucleotides containing more than about ten monomers are

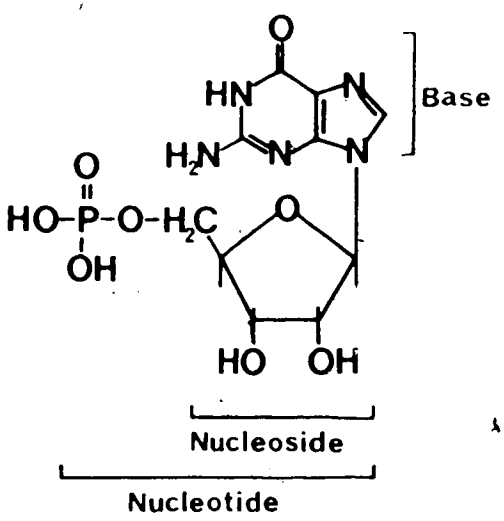


Figure 1.2. Structure of guanosine-5'-monophosphate (5'-GMP), showing the base and nucleoside components of the nucleotide.

Table 1.2: The pKa Values of Nucleic Acid Constituents

Type	Compound	pKa_1	pKa_2	pKa_3	pKa_4
Bases:					
	Adenine	4.15	9.80		
	Cytosine	4.60	12.16		
	Guanine	3.30	9.20	12.3	
	Thymine	9.82			
	Uracil	9.45			
Nucleosides:					
	Adenosine	3.5	12.5		
	Cytidine	4.2	12.5		
	Guanosine	1.6	9.2	12.3	
	Uridine	9.2	12.5		
	Deoxyadenosine	3.8			
	Deoxycytidine	4.3	>13.0		
	Deoxyguanosine	2.40	9.40		
	Thymidine	9.8	>13.0		
Nucleotides:					
	Adenosine-2'-phosphate		3.81	6.17	
	Adenosine-3'phosphate		3.6 - 3.7	5.92	
	Adenosine-5'-phosphate	0.9	3.7	6.2 - 6.4	
	Cytidine-3'-phosphate		4.3	6.04	
	Cytidine-5'-phosphate	0.8	4.2	6.0	
	Guanosine-2'-phosphate		2.21		9.56
	Guanosine-3'-phosphate		2.3	9.42	
	Guanosine-5'-phosphate	0.7	2.4	6.0	9.3
	Uridine-3'-phosphate		5.88	9.43	
	Uridine-5'-phosphate	1.0	5.9	9.4	
	Deoxyguanosine-5'-phosphate		2.9	6.4	9.7
	Thymidine-5'-phosphate			6.5	10.0

Source: refs. 14 and 15.

insoluble in cold diluted PCA and TCA [13].

The nucleotides, nucleosides and bases are strongly acidic, neutral and weakly basic compounds, respectively. These distinct chemical properties of the nucleic acid constituents are of importance in chemical analysis using ion-exchange chromatography. Ionization constants of the nucleic acid constituents are summarized in Table 1.2 [14,15].

The two types of nucleic acids, RNA and DNA, share a number of chemical and physical properties. In both the successive nucleotide units are covalently linked by phosphodiester bridges formed between the 5'-phosphoryl group of one nucleotide and the 3'-hydroxyl group of the adjacent nucleotide (Figure 1.4). The presentation of polynucleotide chains by complete formula is clumsy, thus shorthand designations of the chains are in common use. As shown in Figure 1.4, the base is identified by a single-letter code (N in general, or specifically A, C, G, T, U). One convention utilizes a vertical line and a diagonal slash to represent the sugar unit, with phosphodiester bonds being represented by the letter P between the 3'- and 5'-slashes. A simpler representation uses pN to symbolize 5'-monophosphonucleotide and Np to represent 3'-compound. A polynucleotide is then written as a succession of pN notations, reading left to right from the 5'- to the 3'-terminus [8,16]. For cyclic nucleotides, the representation may utilize the symbol cyclic-P or >P to indicate a

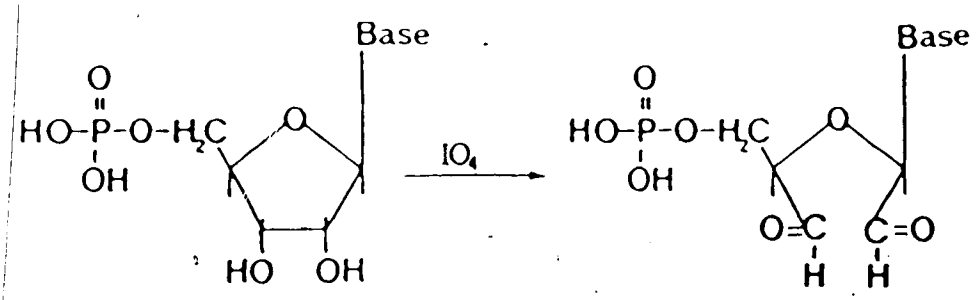


Figure 1.3. Periodate oxidation of a ribonucleotide.

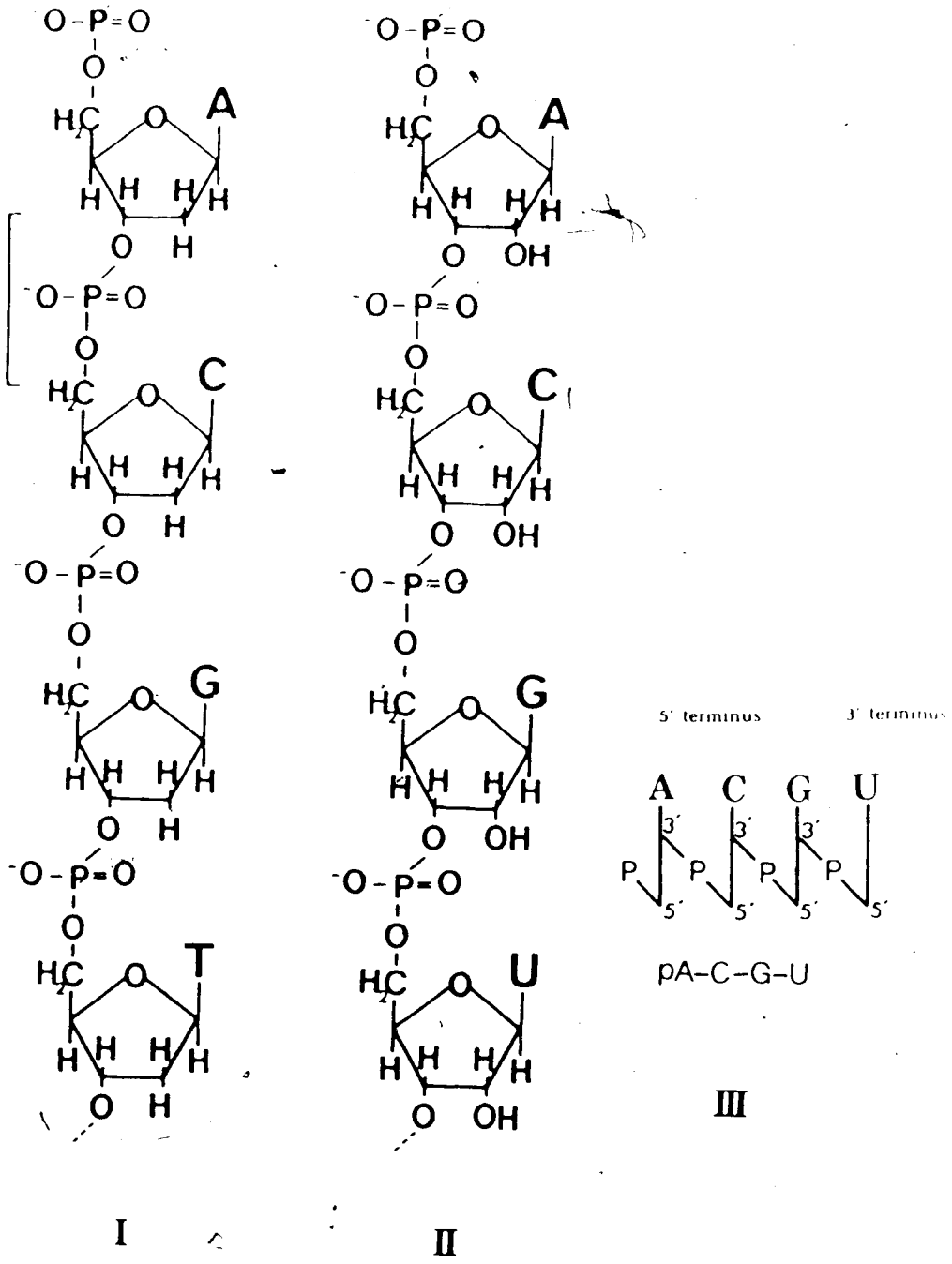


Figure 1.4. A section of the polynucleotide chain of DNA (I), RNA (II) and shorthand designation of RNA (III).

2':3'-phosphoryl group. Thus, G-cyclic-P or G>P denotes guanosine-2':3'-cyclic monophosphate (2':3'-GMP) [8].

In all living cells RNA is generally found to have a much lower molecular weight than DNA. The three main types of RNA are ribosomal RNA (rRNA), transfer RNA (tRNA) and messenger RNA (mRNA). Each occurs in multiple molecular forms, resulting in different molecular weights. Transfer RNA's have molecular weights as low as 23 kdal, and ribosomal RNA's are of higher molecular weight (up to 10^3 kdal), accounting for 80% of the total RNA's [8].

1.2 Degradation of Nucleic Acids and Nucleotides

1.2.1 Chemical hydrolysis of nucleic acids

Acid hydrolysis of RNA or DNA yields its constituent bases, phosphoric acid and ribose or deoxyribose.

The behavior of RNA in alkaline solution differs markedly from that of DNA. Alkaline digestion of RNA (e.g. 0.1 M NaOH, 25°C) yields a mixture of 2'- and 3'-ribonucleoside phosphates. The reaction occurs successively via formation of the intermediate 2':3'-cyclic nucleotides (Figure 1.5). DNA is stable in basic solution under the conditions that hydrolyze RNA since DNA lacks the 2'-hydroxyl group required for intramolecular catalysis [17].

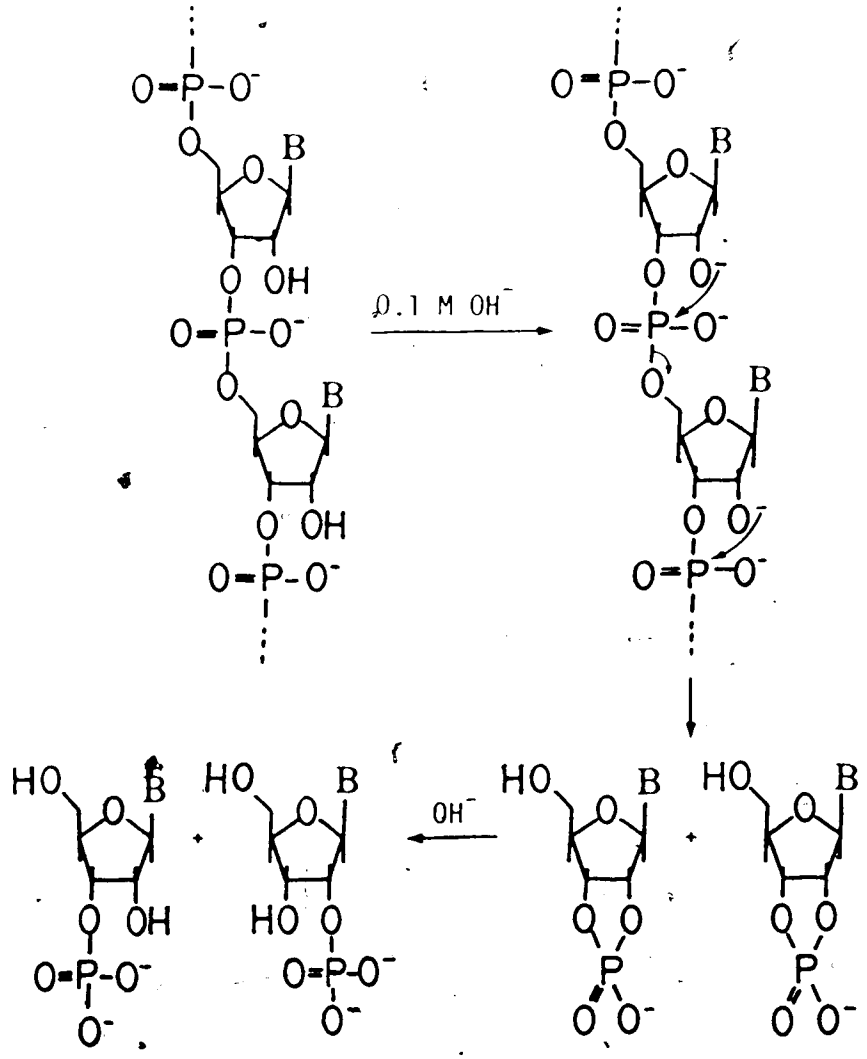


Figure 1.5. Alkaline digestion of RNA, showing the formation of intermediate cyclic nucleotides and their breakdown to a mixture of 2'- and 3'-ribonucleotides [17].

1.2.2 Enzymatic hydrolysis of nucleic acids

The sugar moieties in the primary structures of RNA and DNA are the major difference between these two classes of nucleic acids. On this basis, several enzymes which act specifically on RNA or DNA can be distinguished.

The definition of a "nuclease" is an enzyme which cleaves a phosphodiester bond of a polynucleotide chain in both RNA and DNA (i.e. no preference for the nature of sugar moiety). A "ribonuclease" (RNase) is an enzyme that hydrolyzes specifically RNA, and a "deoxyribonuclease" (DNase) is one that acts exclusively on DNA. In a strict sense, a phosphodiesterase is any enzyme which splits a bond involving diesterified phosphate, thereby including RNases and DNases. However, a phosphodiesterase is not specific in hydrolysis of only nucleic acids, i.e. it does not cleave only a nucleotidyl bond [18-22].

Like other depolymerizing enzymes, nucleic acid hydrolyzing enzymes can be divided into two groups based on the point of attack. Those that split the polymer at the terminal bonds, either 3'- or 5'-terminus, are known as exo-enzymes. The endo-enzymes attack interphosphodiester bonds of nucleic acids.

The 3'-5'-phosphodiester bonds of nucleic acids can be cleaved to yield 3'-nucleotides [22] or 5'-nucleotides [23], depending upon the character of the specific enzyme. Those enzymes which hydrolyze the 3'-linkage are referred to as 5'-nucleotide formers, whereas those that split the

5' linkage are called 3'-nucleotide formers. The general enzymatic reaction of these two types of nucleases is summarized in Figure 1.6.

The mechanism of RNase hydrolysis of RNA has been well studied [24,25]. In general, the reaction occurs by initial depolymerization which involves a nucleophilic attack of the oxygen of the 2' hydroxyl group of one nucleotide on the electrophilic phosphorus atom of the adjacent nucleotide. As a result, intermediate 2':3'-cyclic phosphate esters are formed. The formation of this intermediate is similar to the first step in alkaline hydrolysis [17] (see section 1.2.1). The opening of the 2':3'-cyclic phosphate esters is the second step of the same RNase catalyzing reaction, giving 3'-ribonucleotides (Figure 1.7). Some RNase's can not perform the ring opening step, or they do so at a very slow rate, such that the 2':3'-cyclic phosphate accumulates [21,26]. The enzymatic reaction as shown in step I, Figure 1.7 is a transfer of a phosphoryl group, not a hydrolytic reaction. Thus, the RNases possessing this ability are referred to as nucleotidyl-transferases and their specific name depends on preference for either purine or pyrimidine base [21,26-28].

Three types of RNase have been classified [21]:

(1) Ribonucleate nucleotido-2'-transferase (cyclizing) [EC 2.7.7.17] has no specificity for the base. This type of RNase forms all four 2':3'-cyclic nucleotides.

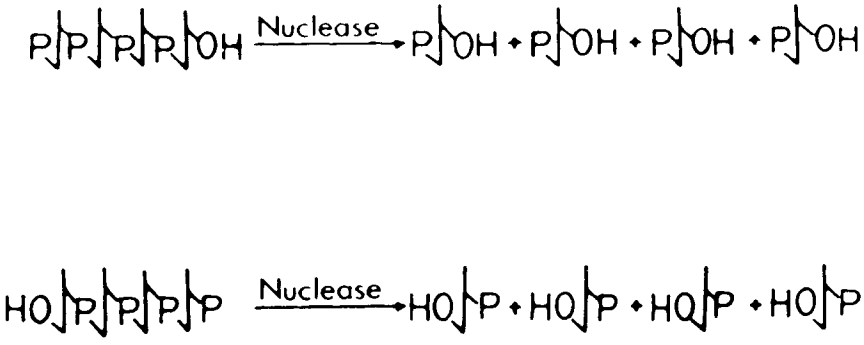


Figure 1.6. Nonspecific nucleases that hydrolyze both DNA and RNA.

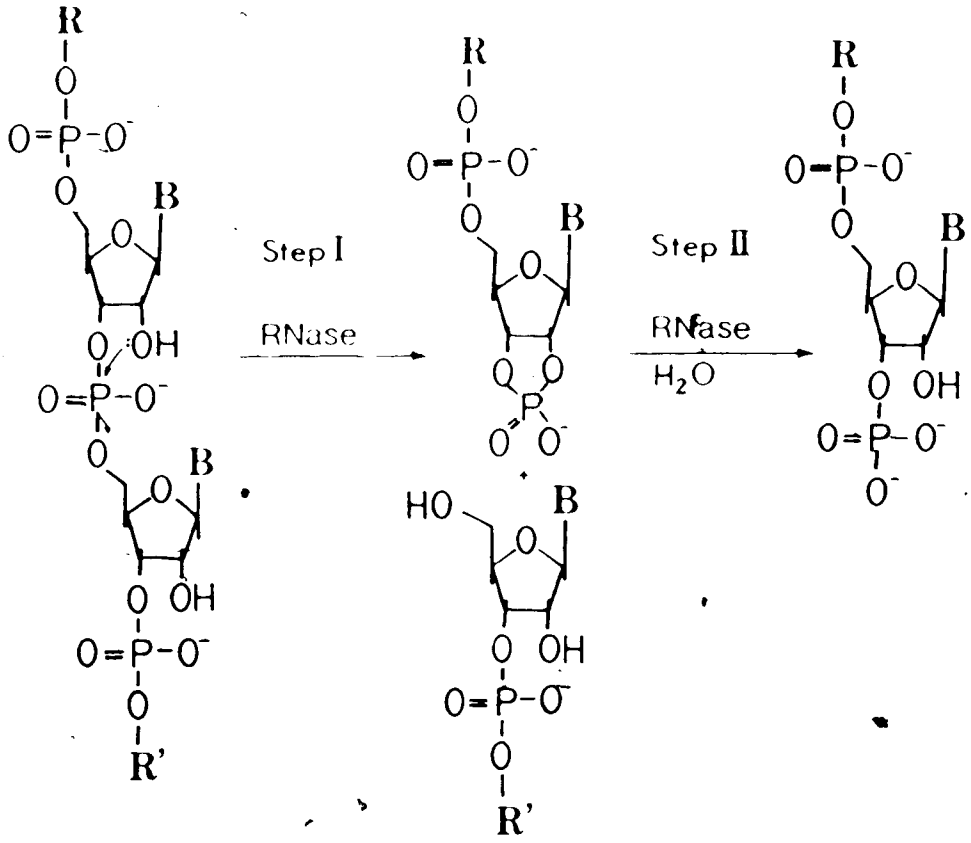


Figure 1.7. Hydrolysis of RNA catalyzed by RNase.

(2) Ribonucleate pyrimidine nucleotido-2'-transferase (cyclizing) [EC 2.7.7.16] specifically attacks at the 5' linkage near the pyrimidine base, forming intermediate 2':3' cyclic pyrimidine nucleotides.

(3) Ribonucleate guanine nucleotido-2'-transferase (cyclizing) [EC 2.7.7.26] splits the P-O bonds by formation of 2':3'-cyclic intermediates with only guanine.

The plant RNases, in general, show a preference for phosphodiester bonds involving the guanine base [28]. Among the many RNases identified, it is likely that pancreatic RNase is the most studied [25]. Enzymes related to plants, especially vegetable nucleic acids, are not as extensively studied for their properties and structures. There are relatively few examples in the literature in which there is in-depth study of vegetable nucleases and/or ribonucleases. However, some critical reviews on plant nucleases have appeared [20,29,30]. Properties of plant nucleic acid hydrolyzing enzymes are summarized in Table 1.3.

1.2.3 Enzymatic hydrolysis of nucleotides

Phosphoric monoester hydrolases are enzymes found widely in biological systems [18]. They are generally subclassed into three groups, phosphatases [31-35] and 3'- and 5'-nucleotidases [36,37]. Phosphatase is a non-specific phosphomonoesterase that hydrolyzes all phosphomonoester bonds, whereas 3'- and 5'-nucleotidases are phosphatases specific for hydrolysis of a phosphoric bond in

Table 1.3: The Major Characteristics of Plant Phosphodiesterases

		RNase I	RNase II	Nuclease I	Exonuclease I
1.	Substrate	RNA; R > p	RNA; N > p DNA > native DNA;	RNA > denatured Np	DNA > RNA; Nitrophenyl-pT, Nitrophenyl-pN, DNPP
2.	Products Secondary products	N > p Rp	N > p Np	pN; 3'-OH terminals	pN
3.	Mode of actions	Endonuclease	Endonuclease	Endonuclease	Exonuclease
4.	Base specificity	G > A = U > C	G > A = U > C	A > U(T), G, C	None
5.	Molecular weight	19,700-24,000	17,000-21,000	31,000-39,000	> 100,000
6.	pH Optimum	5.0-6.0	6.0-7.0	5.0-6.5	7.0-9.0
7.	Intracellular location	Soluble	Microsomes	Particles, membranes?	?
8.	EDTA sensitivity	Low	Low,	High, Low	High
9.	Enzyme nomenclature classification	2.7.7.17 (1965) 3.1.4.23 (1972) 3.1.27.1 (1978)	2.7.7.17 (1965) 3.1.4.23 (1972)	3.1.4.9 (1972) 3.1.30.1 (1978)	3.1.4.1 (1978) or 3.1.15.1 (1978)

N > p (a 2':3'-cyclic monophosphate nucleotide); Np (a 3'-monophosphate nucleotide); pN (a 5'-monophosphate nucleotide); R (unspecified purine nucleoside); DNPP (dinitrophenyl phosphate); nitrophenyl-pT (p-nitrophenyl-5'-thymidylic acid).

Source: ref. 29.

3'-nucleotides and 5'-nucleotides, respectively [36]. Hence, the phosphatases and 5'-nucleotidases are considered undesirable phosphomonoesterases with regard to flavor nucleotides (see section 1.3). Products of phosphomonoesterases are the nucleosides and phosphoric acids shown in Figure 1.8.

1.3 Flavor Nucleotides

During the 1950's, several discoveries on characteristic flavor activity of purine 5'-nucleotides led to such compounds becoming important in the food industry [38]. It was found that, among the three isomers of inosine monophosphate (IMP), only 5'-IMP possessed flavor activity. As early as 1913 the major flavoring substance of dried bonito extract was thought to be the histidine salt of IMP [39]. Almost fifty years later, however, histidine was proved not to be necessary for the flavor action of 5'-IMP. The absolute requirement for a nucleotide to have flavor activity is that it must be a 5'-purine ribonucleotide with a hydroxyl (or keto, its tautomeric form) group at the 6-position [40,41]. Guanosine-5'-monophosphate (5'-GMP) and xanthosine-5'-monophosphate (5'-XMP) were also recognized as compounds possessing similar activity to that of 5'-IMP. Meanwhile, 5'-GMP was found to be the major savory (flavor enhancing) constituent of Japanese mushroom (shiitake) [40]. Adenosine-5'-monophosphate (5'-AMP) contains a 6-amino group, resulting in reduction of flavor activity. Thus,

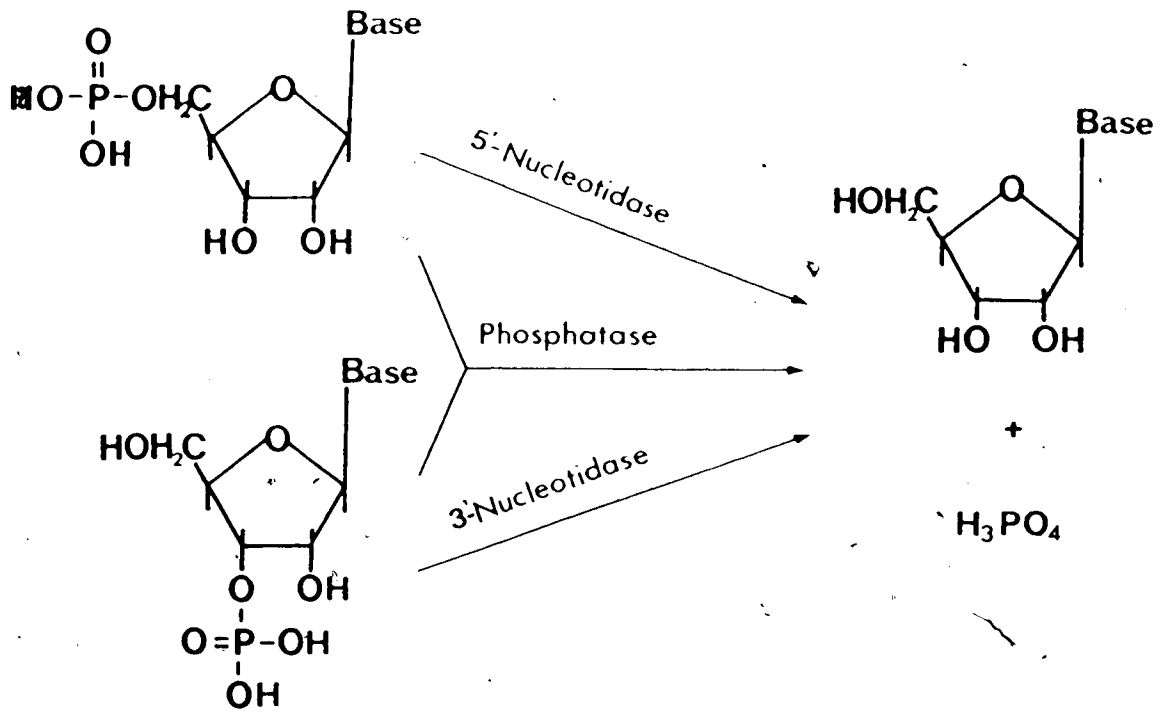


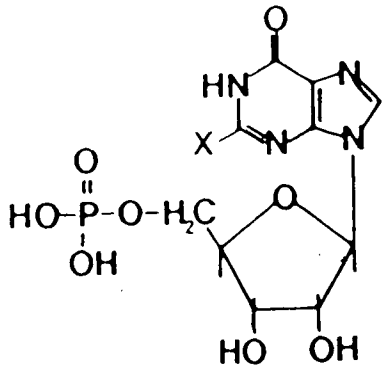
Figure 1.8. Hydrolysis of nucleotides catalyzed by phosphomonoesterases.

5'-AMP was less active in flavor-enhancement when compared with 5'-XMP, 5'-IMP and 5'-GMP.

The latter three 6-hydroxy-purine 5'-ribonucleotide monophosphates are known as the "flavor nucleotides" [42]. They differ chemically by the substitution at position 2 with OH, H and NH_2, respectively (Figure 1.9). Among the three flavor nucleotides, 5'-XMP has not been produced commercially because its flavor activity is less than that of 5'-IMP and 5'-GMP. The flavor activity of 5'-GMP is 2.3-4 times greater than that of 5'-IMP [42].

Due to the synergistic taste effect of the flavor nucleotides with monosodium glutamate (MSG), 5'-IMP and 5'-GMP have been manufactured and applied to food as flavor enhancers throughout the world. The flavor nucleotides are recognized as GRAS ("generally recognized as safe") substances, and were approved by Japanese Food Additive Petitions and the US Food and Drug Administration in the early 1960's. Molecular theory of flavor nucleotides was reviewed by Kuninaka [42], and their toxicological aspects were discussed by Kojima [43].

Both 5'-IMP and 5'-GMP occur naturally in foods [44]. The 5'-IMP is usually rich in animal foods, especially in animal muscle. Furthermore, 5'-IMP is derived mainly from the reaction of deaminase catalyzed hydrolysis of 5'-AMP [45,46]. Several sea foods are rich in AMP rather than IMP, probably because of a lack of AMP deaminase [44,47].



GMP : X = NH_2

IMP : X = H

XMP : X = OH

Figure 1.9. Chemical structures of the flavor nucleotides.

Table 1.4: Ribonucleotide Content of Vegetables — Extraction using Boiling Water

Vegetable	Nucleotide (mg/ 100g)				
	5'-AMP	5'-CMP	5'-GMP	5'-IMP	5'-UMP
Green asparagus	3.8	1.9	Trace	0.0	1.9
White asparagus	2.4	1.3	Trace	0.0	2.6
Green onion	0.9	0.0	0.0	0.0	0.4
Lettuce	0.9	0.0	Trace	Trace	0.9
Tomato	10.4	0.5	0.0	0.0	2.2
Green bean	1.8	Trace	0.0	0.0	1.3
Cucumber	0.5	Trace	0.0	0.0	0.6
White radish	1.3	Trace	0.0	Trace	1.4
Onion (round)	0.8	Trace	0.0	Trace	0.5
Bamboo sprout	1.1	0.5	0.0	0.0	1.3
Bean sprout	2.3	0.0	0.0	0.0	0.7
Green bell pepper	4.0	—	0.0	0.0	—
Egg plant	2.6	—	0.0	0.0	—
Spinach	4.4	—	0.0	0.0	—
Chinese cabbage	0.6	—	0.0	0.0	—
Sweet potato	2.7	—	0.0	0.0	—
Green pea (dried)	15.5	—	0.5	0.5	1.8
Japanese "shiitake" (mushroom *Lentinus edodes*) [fresh]	54.9	29.4	70.1	0.0	37.6
Shiitake (dried)	106.9	55.6	146.7	0.0	111.2
White mushroom	11.3	Trace	Trace	0.0	6.4
Potato tuber (cooked) [a]	3.0	—	2.1	—	2.1

Source: refs. 48 and [a] 51

Vegetables have not been found to contain 5'-IMP (Table 1.4). Nevertheless, 5'-GMP, along with the other three major nucleotide constituents of RNA, have been found in some vegetables which were extracted using the boiling-water extraction method [48]. Experiments to follow the change of nucleotides in asparagus and Japanese shiitake dried mushroom during cooking were carried out by Teramoto *et al.* [49]. The rate of increase of 5'-nucleotides was reported to be maximized at certain temperatures, then decreasing quickly when the temperature increased continuously (Table 1.5). This led to suggestions that the nucleotides found were the products of enzymatic degradation of intracellular RNA [44,49-51]. The specific enzymes involved in production of vegetable flavor nucleotides, however, have not been studied intensively.

The potato is an important agricultural product of Canada and its province of Alberta. The net income per year of potatoes averages $200,000,000 in Canada [52] and $27,000,000 in Alberta [53]. Optimization of natural flavor nucleotides in potatoes through understanding of the enzyme characteristics involved in their formation was the major objective of this research. Potato tubers were studied in depth for their nucleotide contents and the properties of both cytoplasmic and cell wall bound enzymes involved in formation/degradation of nucleotides. The nucleotide contents of potato under various processing conditions were also studied.

Table 1.5: Effect of Temperature on the Formation of Nucleotides in Vegetables during Heating in Water - Extraction using Cold Perchloric Acid

Heating	Total 5'-nucleotides (μ mol/g dry basis)		Total 3'-nucleotides (μ mol/g dry basis)	
	Shiitake mushroom	Asparagus	Shiitake mushroom	Asparagus
30°C , 5 min	1.47	–	0	–
50°C , "	4.77	0.86	0	0.14
60°C , "	16.60	0.93	0	0.14
70°C , "	13.90	0.72	0	0.25
80°C , "	9.80	0.51	0	0.28
100°C , "	4.16	0.42	0	0.21
Fresh (not heated)	1.97	0.25	0	0.05

Source: ref. 49.

Furthermore, other common vegetables, including bean sprouts, green beans, green bell peppers, broccoli, cauliflower, cabbage, asparagus, sweet potatoes, radishes, Japanese mushrooms and white and oyster mushrooms, were studied for nucleotide contents and temperature effects on their nuclease and RNase activities. The data provided a general overview of vegetable nucleotides and the enzymes involved in their formation. To meet these objectives, analytical methods have been established and are described in the following chapters.

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2. SIMULTANEOUS LIQUID CHROMATOGRAPHIC DETERMINATION OF RIBONUCLEOSIDE-5'-MONOPHOSPHATES AND THEIR ISOMERS IN POTATO TUBERS'

2.1 Introduction

Ribonucleoside-5'-monophosphates are found in most foods of animal and plant origin [1]. Among the nucleotides degraded from ribonucleic acid (RNA), guanosine-5'-monophosphate (5'-GMP) is found to be a powerful flavor enhancer, while adenosine-5'-monophosphate (5'-AMP) with its 6-amino group, is a lesser flavor potentiator [2]. However, it is well known that, in the presence of a deaminase, 5'-AMP is readily converted to inosine-5'-monophosphate (5'-IMP), which is a strong flavor enhancer. In vegetables 5'-GMP and 5'-IMP are present in lesser quantity than other nucleotides such as cytidine-5'-monophosphate (5'-CMP), uridine-5'-monophosphate (5'-UMP) or 5'-AMP [1]. Solms and Wyler [3] reported that the ribonucleoside-5'-monophosphates present in cooked potatoes are due to degradation of RNA during tuber cooking. The methods so far used for detection and quantitation of nucleotides in food are prone to interference and are time consuming [4-7].

With the advent of liquid chromatography (LC) excellent new methods were developed for nucleotide determination. Several methods have been described for separation of a mixture of nucleotides using various LC systems [8-12], but

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most of these separations were accomplished on "model systems" consisting mostly of a mixture of pure compounds. Several attempts have been made to develop an LC method for nucleotide determination in food [13-16] and for biological research [9,10,17-19]. Recently, a review of the use of LC in nucleic acid research was given by Brown [20].

Separation by gradient elution is the commonly used LC method. A high concentration of salt buffer is often needed in the gradient elution method, which can shorten the analytical column life and increase the analysis time because of the need to reequilibrate the column to the initial conditions.

The extraction of nucleotides prior to LC analysis has been discussed by several authors [8,13,19]. Perchloric acid (PCA) or trichloroacetic acid are usually applied to precipitate proteins. Subsequently, the acids are removed by neutralization with potassium hydroxide or by an amine.

This chapter describes a modified extraction method using PCA to extract nucleotides from raw and steam-cooked potatoes, followed by removal of soluble starch, protein, and PCA by applying methanol and an octylamine-Freon solution. In addition, an isocratic LC method for the simultaneous analysis of ribonucleoside-5'-monophosphates and 2'- and 3'-isomers of AMP and GMP in potatoes is presented.

2.2 Materials and Methods

2.2.1 Apparatus and chemicals

2.2.1.1 LC system

The LC system consisted of a Beckman Model 110A pump, a 50 μL Rheodyne loop injector, a Data Control Spectromonitor III (Model 1204A, with the UV detector set to 254 nm), a Hewlett-Packard Model 3388A integrator, a Whatman 25 cm x 4.6 mm i.d. Partisil SAX column protected by a 7 cm x 2.1 mm i.d. guard column containing pellicular anion exchanger and by an additional 25 cm x 4.6 mm i.d. pre-injector column (Solvecon) containing silica gel.

LC-grade water was prepared by reverse osmosis (Milli-RO) and purified additionally by Milli-Q system (Millipore, Bedford, MA). Methanol and potassium dihydrogen phosphate were LC grade. Tri-*n*-octylamine (98%) was supplied by Aldrich (Milwaukee, WI) and Freon 113 by Terochem (Edmonton, AB). A solution of 0.5 M tri-*n*-octylamine in Freon 113 was freshly prepared prior to the extraction step. All other common solvents or chemicals used were of analytical grade.

2.2.1.2 Standards

All nucleotides used as standards were obtained from Sigma (St. Louis, MO). Standard compounds were dissolved in water as a stock solution (1 mg/mL). Lower concentrations were freshly prepared by dilution using methanol-water (1:2,

v/v) as solvent.

2.2.1.3 LC separation conditions

The mobile phase used was 3% methanol in 0.008 M phosphate solution adjusted to pH 4.15 with phosphoric acid. The solution was prepared fresh daily and, after degassing, was used for LC analysis at ambient temperature with a flow rate of 1.5 mL/min.

2.2.2 Sample preparation

2.2.2.1 Potatoes

Russet Burbank, Norgold Russet, Norland, Pontiac, Kennebec and Shepody cultivars grown in Southern and Central Alberta were used.

The tubers were stored at 4°C and prior to analysis were reconditioned at 20°C for one week. They were kindly provided by Dr. W. Andrew (Department of Plant Science, University of Alberta, Edmonton), the Edmonton Potato Growers Association and I&S Produce Ltd., a local food processor.

2.2.2.2 Nucleotide extraction

Peeled potato tubers were diced (0.2 x 0.2 cm) and mixed thoroughly to provide a uniform sample. To about 10 g of raw potato sample was added 10 mL of pre-cooled (4°C) 0.5 M PCA in order to prevent browning and other enzymatic reactions. Steam-cooked potatoes were prepared under pressure at 100°C for 30 min. The heating was stopped

abruptly by immersing the glass beaker with sample in an ice bath. All samples were prepared at least in duplicate.

The raw or cooked potatoes were transferred to a 50 mL container of a Sorvall-Omni mixer. Pre-cooled 0.5 M PCA solution of about five times the volume of the sample was then added, followed by homogenization for 30-60 s with the container cooled in an ice bath. The total weight of the slurry obtained was recorded. An aliquot of close to 20 g was transferred to a 50 mL polypropylene centrifuge tube which was then cooled in ice and 10 mL of pre-cooled methanol was added. The resulting PCA-potato-methanol slurry was mixed vigorously and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was collected. The residue was re-extracted with 10 mL 0.5 M PCA and 5 mL methanol, again centrifuged, and the supernatants combined. The volume was then made up to 50 mL at room temperature.

2.2.2.3 Treatment of the acid-methanol extract

The procedure adopted to remove PCA from the aqueous nucleotide samples was that developed by Khym [21] as later applied by Chen *et al.* [8] and Riss *et al.* [19]. In our laboratory, it was used by Currie *et al.* [13] for nucleotide extraction from meat samples. A 0.5 M tri-*n*-octylamine-Freon 113 solution (solution A) was used to remove PCA from the aqueous nucleotide extract.

Supernatant and solution A in equal volumes were thoroughly mixed in a screw cap test tube for 2 min using a Vortex mixer. The content was then centrifuged at 800 x g to

promote layer separation. Three layers were clearly separated, with the top water layer having a pH of 5.0 and containing all the nucleotides. An aliquot of this layer was then injected into the LC column.

2.2.2.4 Calculation

The concentration of potato nucleotides was calculated using either peak area or peak height of the effluent as determined at 254 nm. The calculation is based on both wet and dry potato bases as follows:

$$A = \frac{B}{C} \quad D \quad \frac{(50\ G)}{(F\ H)}$$

where A is the potato nucleotide content in μg/g; B the peak area or height of sample; C the peak area or height of standard; D the nucleotide concentration in μg/mL in the standard solution; F the weight (g) of the PCA-potato slurry aliquot; G the total weight (g) of PCA-potato slurry; and H the weight (g) of potato sample on fresh or dry basis.

In the above calculation no volume correction was required for the PCA extraction with tri-*n*-octylamine-Freon 113 since the aqueous volume containing the nucleotides did not change during extraction. This was confirmed experimentally with standards and is shown in the recovery data of Table 2.6.

2.3 Results and Discussion

As shown in Figure 2.1 and Tables 2.1-2.3, isocratic LC elution separates the four major ribonucleoside-5'-monophosphates of RNA in addition to 5'-IMP and 2'- and 3'-isomers of AMP and GMP. The complete procedure, which included the extraction step, required only 75 minutes. The possible interfering compounds, such as deoxyribonucleoside-5'- and 3'-monophosphates, and cyclic phosphate esters (2':3'- and 3':5'-ribonucleoside esters), were injected into the LC and found to have different retention times (Tables 2.1-2.3). The corresponding nucleosides and free bases were eluted right after the solvent peak. However, as can be seen in Tables 2.1-2.3, 2'- and 3'-CMP had very similar retention times to 2'-AMP. Also, the retention times of these three compounds were close to that of 3'-UMP, such that an accurate quantitation of 2'-AMP was not possible. Attempts were made to separate these compounds, including changes in potassium phosphate solution molarity, pH, and the methanol to solution ratio, but without success.

The use of 0.01 M phosphate solution of pH 4.0, containing 10% methanol, brought about an overlap of 3'- and 5'-GMP isomers. In the same mobile phase system, but without methanol, 5'-CMP and 5'-UMP remained unresolved. The effects on resolution of the compounds assayed of changing solution molarities from 0.007 M to 0.02 M, ratios of methanol to solution from 1.5 to 15% and pH from 3.60 to 4.50 were systematically examined. The optimum phosphate solution

Table 2.1: Retention Times of Ribonucleotides recorded by the Outlined Liquid Chromatography Procedure

Nucleotide	Retention time (min)
5'-CMP	11.4
5'-UMP	14.3
5'-AMP	17.8
5'-IMP	21.5
2'-UMP	22.1
3'-UMP	23.3
2'-AMP	23.9
3'-CMP	24.0
2'-CMP	24.4
3'-AMP	26.5
5'-GMP	35.8
3'-GMP	39.8
2'-GMP	43.2

In this and the following Tables, the separation conditions are as shown in Fig. 2.1.

Table 2.2: Retention Times of Deoxynucleotides recorded by the Outlined Liquid Chromatography Procedure

Deoxynucleotide	Retention time (min)
5'-dCMP	10.1
5'-TMP	13.1
3'-dCMP	15.5
3'-TMP	16.7
5'-dAMP	19.6
3'-dAMP	19.9
3'-dGMP	26.0
5'-dGMP	35.9

Table 2.3: Retention Times of Cyclic Nucleotides recorded by the Outlined Liquid Chromatography Procedure

Nucleotide	Retention time (min)
2':3' UMP	8.3
2':3'-CMP	8.4
3':5' UMP	8.5
3':5' CMP	8.5
2':3'-AMP	8.9
3':5'-AMP	9.2
2':3' GMP	10.5
3':5'-GMP	11.0

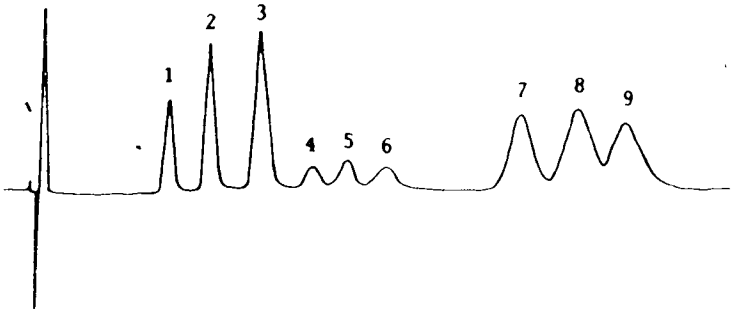

Figure 2.1. Liquid chromatogram of nucleotide standard solution. Peak identities: 1 = 5'-CMP (3 μg/mL); 2 = 5'-UMP (3 μg/ml); 3 = 5'-AMP (3 μg/mL; 4 = 5'-IMP (0.6 μg/mL); 5 = 2'-AMP (0.6 μg/mL); 6 = 3'-AMP (0.6 μg/mL); 7 = 5'-GMP (3 μg/mL); 8 = 3'-GMP (3 μg/mL); and 9 = 2'-GMP (3 μg/mL. Solvent system: 0.008 M phosphate solution (pH 4.15) containing 3% methanol. Flow rate: 1.5 mL/min.

molarity was 0.008 M at pH 4.15. Changes in mobile phase pH resulted in several unresolved peaks (see Figures 2.2 and 2.3). The optimum ratio of methanol to solution was 3% (v/v). The nucleotides could not be well resolved if methanol was omitted.

Further confirmation of the assignment of nucleotide isomers found in potatoes was achieved by periodate oxidation. Addition of an excess of sodium periodate to the sample extract, followed by injection into the LC column, removes ribonucleoside 3'-phosphates, the *cis*-diol sugars of which are susceptible to oxidation, while 2'- and 3'-ribonucleotide peaks are retained (Figure 2.4).

A linear response of peak areas or peak heights at 254 nm was obtained for all nucleotides (Table 2.4). The detection limits given are for peaks for which the height was at least twice that of the noise level. Table 2.5 presents the data for potato nucleotides when measured at three wavelengths. The absorbance ratios at these wavelengths and particularly those of 250/260 and 280/260 nm were equal to those obtained for pure nucleotides except for 2'-AMP. As already stated, this peak was partly co-eluted with 2'- and 3'-CMP and 3'-UMP.

The high content of starch in potato parenchyma tissue was gelatinized during steam cooking. Such gelatinized, and partly leached-out and solubilized starch interfered with the extraction procedure. Several extraction methods were examined to maximize the efficiency of nucleotide recovery.

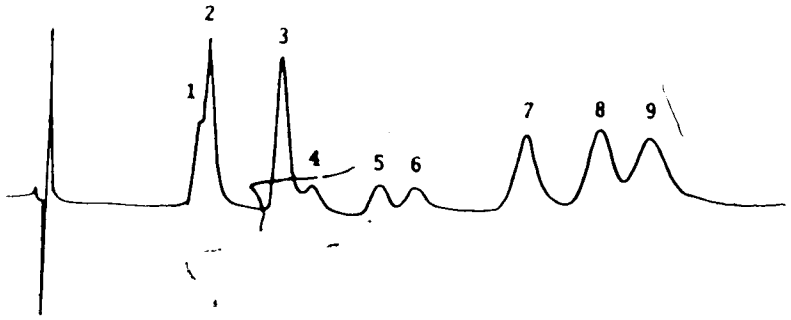

Figure 2.2. Liquid chromatogram of nucleotide standard solution. Peak identities: 1 = 5'-CMP; 2 = 5'-UMP; 3 = 5'-AMP; 4 = 5'-IMP; 5 = 2'-AMP; 6 = 3'-AMP; 7 = 5'-GMP; 8 = 3'-GMP; and 9 = 2'-GMP. Solvent system: 0.008 M phosphate solution (pH 4.40) containing 3% methanol. Flow rate: 1.5 mL/min.

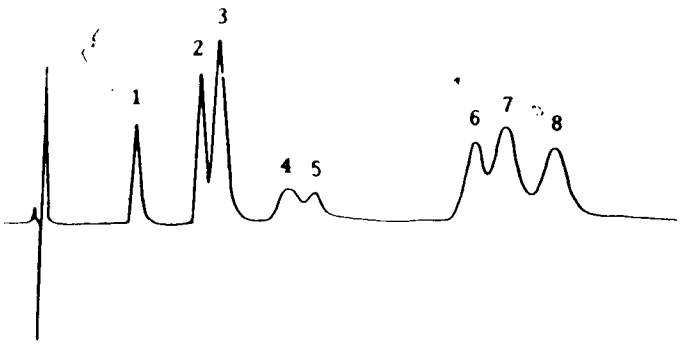

Figure 2.3. Liquid chromatogram of nucleotide standard solution. Peak identities: 1 = 5'-CMP; 2 = 5'-UMP; 3 = 5'-AMP; 4 = 5'-IMP + 2'-AMP; 5 = 3'-AMP; 6 = 5'-GMP; 7 = 3'-GMP; and 8 = 2'-GMP. Solvent system: 0.008 M phosphate solution (pH 3.85) containing 3% methanol. Flow rate: 1.5 mL/min.

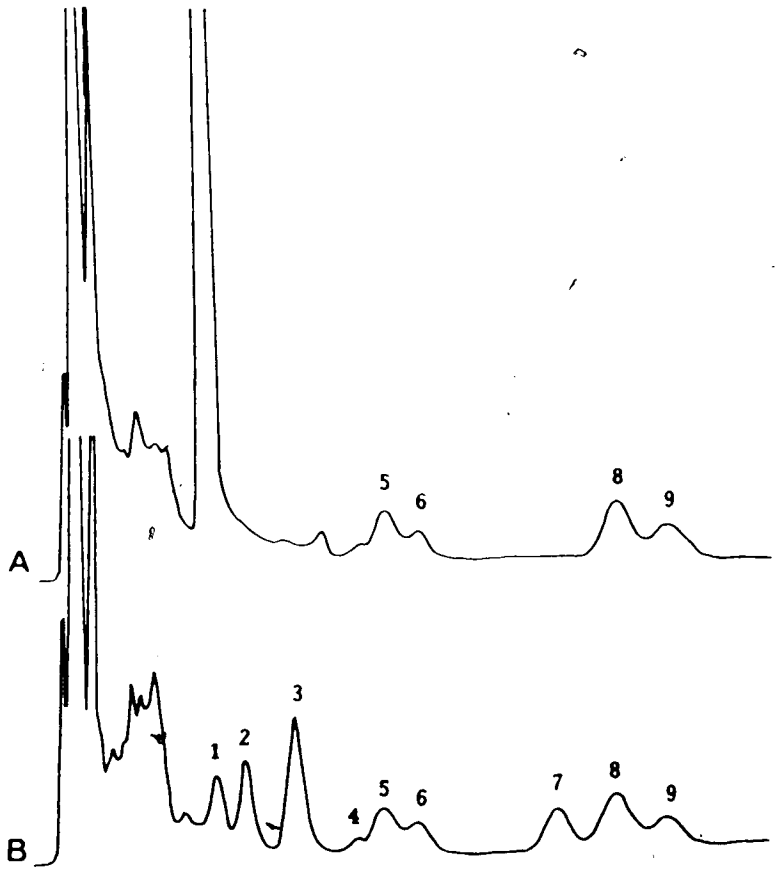

Figure 2.4. Liquid chromatogram of nucleotides extracted from steam-cooked potato cv. Pontiac. (B) Potato extract prior to and (A) after oxidation with periodate. Peak identities: 1 = 5'-CMP; 2 = 5'-UMP; 3 = 5'-AMP; 4 = unidentified; 5 = 2'-AMP; 6 = 3'-AMP; 7 = 5'-GMP; 8 = 3'-GMP; and 9 = 2'-GMP.

Table 2.4: Nucleotides: Linearity of Peak Area and Height Measurements (at a Concentration Range of 0.5 [a] to 5.0 ppm)

Nucleotide	Correlation coefficient			
	Peak area		Peak height	
	Regression equation [b]	Correlation coefficient	Regression equation [b]	Correlation coefficient
2'-AMP	y = 22.4x + 0.03	0.9999	Y = 7.45x – 0.29	0.9998
3'-AMP	y = 17.5x – 0.72	0.9999	Y = 5.61x – 0.40	0.9999
5'-AMP	y = 17.2x + 0.01	0.9999	Y = 7.58x – 0.11	0.9998
5'-CMP	y = 9.34x + 0.30	0.9999	Y = 4.90x + 0.33	0.9998
2'-GMP	y = 19.6x – 4.45	0.9991	Y = 3.14x – 0.13	0.9985
3'-GMP	y = 13.7x + 2.39	0.9993	Y = 2.88x + 0.29	0.9998
5'-GMP	y = 14.3x + 1.24	0.9999	Y = 3.22x – 0.16	0.9993
5'-IMP	y = 14.6x + 0.40	0.9999	Y = 5.67x – 0.13	0.9996
5'-UMP	y = 14.0x – 0.18	0.9999	Y = 7.55x + 0.15	0.9999

Detection limits are 100 ppb, except for 2'-, 3'- and 5'-GMP's, where detection limits are 200 ppb.

[a] A level of 0.5 ppm was the lowest limit which provided reproducible results.

[b] x = Concentration of standard solution (µg/mL)
y = Peak area (mm^2); Y = peak height (mm).

Table 2.5: Nucleotides (ppm) present in Steam-Cooked Potato cv. Shepody measured at Various Wavelengths

Nucleotide	Wavelength (nm)		
	254	264	274
2'-AMP	7.9	8.4	12.6
3'-AMP	6.5	6.5	6.5
5'-AMP	16.6	17.0	16.8
5'-CMP	19.9	19.6	20.3
2'-GMP	7.8	8.0	7.8
3'-GMP	13.5	13.7	13.9
5'-GMP	20.1	20.6	20.4
5'-UMP	20.6	20.1	20.6

A modification of the extraction procedure developed by Khym [21] proved to be suitable. Khym's procedure used an amine-Freon solution to remove PCA from the sample extract. However, with cooked potato samples, removal of starch was necessary prior to PCA removal. Thus the modified extraction method developed involved the addition of methanol to the homogenized potato-PCA slurry. Methanol addition removed the solubilized starch from the aqueous nucleotide phase and, after centrifugation, provided a clear and transparent supernatant. If methanol was omitted, the supernatant was opaque, as was the nucleotide extract obtained after amine-Freon treatment.

The possibility of interferences by potato tuber constituents other than starch was also investigated.

The contents of reducing sugars, glucose and fructose, and of sucrose in tubers were found at harvest time to be 0.80±0.14 and 3.35±0.14 g/100 g dry matter, respectively. The tubers stored for three months had an increased content of reducing sugars, an average of 1.28±0.17, while the sucrose content decreased to 0.60±0.10 g/100 g dry matter [22]. These sugars were extracted along with the nucleotides and were eluted at the solvent front.

The major organic acids of potato were oxalic, citric, malic, fumaric and pyroglutamic acids. Citric acid was predominant, and its content was three- to four-times higher than malic acid, the second major acid in the tuber [23]. These acids were mostly eluted at the solvent front and the

absorbance above 220 nm was low, while at 254 nm it was non-existent. Pyroglutamic acid was eluted at a retention time (t_R) of 9.7 min, and was detected only at levels well above those found in tubers.

The major phenolic acids present in potatoes were chlorogenic and caffeic acids, while other phenolic acids were present in traces. The high absorbing chlorogenic acid was eluted at (t_R) = 13.3 min and did not interfere with the determination of ribonucleotides; however, in deoxy-nucleotide separation it co-eluted with the peak of 5'-TMP.

The major free amino acids of potato, extracted along with the nucleotides, were aspartic and glutamic acids. These acidic amino acids were present in raw tubers at respective levels of 28.5 and 20.3 mg/g dry matter, while in steam-cooked samples the amounts were lowered to 23.0 and 18.5 mg/g dry matter, respectively [24]. In our HPLC system, these acids were eluted at (t_R) = 9.3 min (aspartic) and 6.5 min (glutamic), and their detection limit at 254 nm was 10 mg/mL. This limit is much higher than the average 0.4 mg/mL of acids found in potato extract. Thus, these amino acids did not affect the determination of nucleotides.

The neutral and polar aromatic amino acids, such as tyrosine, phenylalanine and tryptophan, had high absorbances at 254 nm, however, along with other neutral and basic amino acids they were not retained in the strong anion-exchange column and eluted at the solvent front.

Table 2.6: Nucleotide Recoveries from Steam-Cooked Potatoes, cv. Pontiac

Nucleotide	Added (μg)	Content originally present in potato (μg)	Total found (μg)	Recovery [a] (%)
2'-GMP	100	59.5	157.3	98
	150	58.7	211.1	101
	200	59.0	258.3	100
3'-GMP	100	91.9	192.0	100
	150	90.8	245.2	103
	200	91.2	294.8	102
5'-GMP	100	74.6	172.7	98
	150	73.6	229.9	104
	200	74.0	280.5	103
5'-UMP	100	68.1	171.5	103
	150	67.2	223.6	104
	200	67.6	276.0	104

[a] Recovery % = (found - content originally present) / (amount added) x 100.

Table 2.7: Precision Analysis in a Steam-Cooked Potato, cv. Kennebec

Nucleotide	Content in ppm on fresh weight basis					
	Replicate					
	1	2	3	4	Mean	SD
3'-AMP	7.0	7.0	7.1	6.9	7.0	0.07
5'-AMP	7.1	7.4	7.0	7.4	7.2	0.18
5'-CMP	9.2	9.0	9.2	9.4	9.2	0.13
2'-GMP	14.9	14.6	14.6	14.8	14.7	0.17
3'-GMP	27.2	26.9	26.8	27.1	27.0	0.17
5'-GMP	5.0	5.0	5.1	5.1	5.0	0.05
2'-UMP	2.0	2.1	2.0	2.1	2.1	0.07
5'-UMP	10.5	10.3	10.8	10.6	10.6	0.20

Recovery data and the precision of analysis results using steam-cooked potatoes are illustrated by some data given in Tables 2.6 and 2.7. Both analyses were performed on portions of a single tuber carried through the entire extraction procedure for each analysis. They provided a proof that no nucleotide was lost by starch adsorption and/or interaction with potato proteins.

Since heat treatment at acidic conditions may bring about isomerization of 2'- and 3'-isomers into an equimolar mixture of these isomers for both purine and pyrimidine ribonucleotides [25,26] and thus provide a source of error, pure 2'-AMP and 2'-GMP, and 3'-AMP and 3'-GMP standard solutions were subjected to the same heat treatment as potatoes. Their standard solutions were boiled for 30 min in 0.05 M phosphate buffer (pH 6.0, close to the pH of potatoes). After this treatment, the usual extraction procedure was followed and the heat-treated standard solutions were examined by LC. These data revealed that no isomer interconversion occurred.

The contents of nucleotides in five commercially-grown potato cultivars are presented in Tables 2.8 and 2.9. In all cultivars, after steam cooking, the amount of 5'-GMP was much higher than that found in raw tubers. Along with 5'-GMP, its 2'- and 3'-isomers were also detected in the cooked potatoes. In all cultivars 5'-IMP could not be detected. The 2'- and 3'-isomers were present in cooked but not in raw potatoes, indicating thermal and/or enzymatic

Table 2.8: Nucleotide Content (ppm/dry matter) found in some Raw Potato Cultivars, grown in Southern and Central Alberta

Nucleotide	Potato cultivar [a]				
	Kennebec	Norgold Russet	Norland	Pontiac	Russet Burbank
5'-AMP	85.0	97.2	76.2	15.0	12.0
5'-GMP	17.2	14.3	16.0	nd [b]	nd
5'-UMP	68.6	193.6	31.2	45.6	150.6

[a] Moisture contents % were as follows: Kennebec, 80; Norgold Russet, 80; Norland, 81; Pontiac, 81 and Russet Burbank, 78.

[b] nd: Not detected due to below the detection limit.

Table 2.9 Nucleotide Content (ppm/dry matter) found in some Steam Cooked Potato Cultivars, grown in Southern and Central Alberta

Nucleotide	Potato cultivar				
	Kennebec	Norgold Russet	Norland	Pontiac	Russet Burbank
3' AMP	32.5	18.9	8.5	27.0	13.4
5' AMP	48.0	74.5	80.5	94.0	86.1
5' CMP	39.0	115.9	74.9	67.8	86.5
2' GMP	87.3	46.7	37.7	52.3	26.6
3' GMP	112.5	97.4	71.1	103.0	110.9
5' GMP	51.1	65.5	67.6	92.5	82.3
5' UMP	94.4	141.5	98.8	92.8	140.6

breakdown of the tuber RNA. Further study of this breakdown is in progress.

2.4 Conclusion

The procedure used in this study provides a rapid and accurate analysis for nucleotides in raw and cooked potatoes. An isocratic LC method was developed which involves simultaneous determination of ribonucleoside-5'-monophosphates and the 2' and 3'-isomers of AMP and GMP.

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3. LIQUID CHROMATOGRAPHIC ASSAY FOR POTATO TUBER NUCLEASE AND RIBONUCLEASE[a]

3.1 Introduction

Plants contain several enzymes which hydrolyzed ribonucleic acid (RNA) [1]. They are either ribonucleases, which are specific for RNA as a substrate, or nucleases, which exhibit no specificity for the pentose moiety. The action of these enzymes on the endogenous plant RNA yields a variety of nucleotide products, one of which is the flavor potentiator 5'-GMP [2,3].

The methods commonly used for assaying nucleases and RNases are the spectrophotometric determination of acid-soluble hydrolysis products, the quantitation of released inorganic phosphate, or the direct spectrophotometric assay with RNA or synthetic 2':3'-cyclic monophosphates as enzyme substrates [1,4]. These methods are time consuming, prone to interference, and are not suitable for the simultaneous assay of multiple enzyme activities [1,5].

Liquid chromatography (LC) has shown its versatility in nucleic acid research and several enzymes acting on nucleic acids have been studied using high performance liquid chromatography (HPLC) [5]. These include 5'-nucleotidase [6,7], adenosine deaminase [8], purine nucleoside phosphorylase [9], and nicotinate phosphoribosyltransferase [10].

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[a] Published in J. Chromatogr. 388 (1987) 189-199.

This report describes rapid and accurate HPLC methods for the assay of potato tuber nuclease and RNase. These assays are based on measuring the formation of nucleoside 2':3'-cyclic monophosphate products for RNase activity and 5'-ribo- or 5'-deoxyribonucleotides for nuclease acitivity. These assays have been used for extensive characterization of potato tuber nuclease and RNase in order to obtain information for the optimized production of the flavor enhancer 5'-GMP in commercial potato processing.

3.2 Experimental

3.2.1 Materials

All standard compounds and the following substrates were supplied by Sigma (St. Louis, MO): yeast RNA, calf thymus DNA, sodium salts of adenosine, cytidine, guanosine and uridine, 2':3'-cyclic monophosphates, potassium salts of 5'-isomers of polyadenylic, polycytidylic, polyguanylic and polyuridylic acid. HPLC-grade methanol and potassium phosphate were supplied by Fisher Scientific (Fair Lawn, NJ). LC-grade water was prepared by reverse osmosis (Milli-RO) and further purified by using a Milli-Q system (Millipore, Bedford, MA). All other solvents and chemicals were reagent grade.

3.2.2 LC system

Two HPLC systems were used, a Beckman Model 110 pump (Beckman Instr., Fullerton, CA) with a Laboratory Data Control spectromonitor III UV detector, and/or a Bio-Rad Model 1330 pump (Bio-Rad, Richmond, CA) with a Bio-Rad Model 1305 variable wavelength detector. Both systems were equipped with 50 µL Rheodyne loop injectors. The detectors were set at 254 nm unless otherwise stated. Quantitation was performed electronically with a Hewlett-Packard 3388A integrator. A Whatman Partisil SAX column (25 cm x 4.6 mm i.d.) was used for the separation of nucleotides; the column being protected by a 7 cm x 2.1 mm i.d. guard column containing a pellicular anion exchanger and by a 25 cm x 4.6 mm i.d. pre-injector column (Solvecon) containing silica gel.

3.2.3 LC separation conditions

The mobile phase for the isocratic analysis was 8 mM potassium phosphate solution of pH 4.15, containing 3% (v/v) methanol [3]. Isocratic mode at ambient temperature was employed; the flow rate was 1.5 mL/min.

3.2.4 Enzyme isolation

Enzymes were isolated from peeled potatoes cv. Pontiac, grown commercially in Southern Alberta. The extraction procedure was a modification of the methods of Dumelin and Solms [11] and Nomura *et al.* [12]. Peeled potato tubers (300

g) were treated with four volumes of precooled (4°C) buffer (50 mM sodium citrate, pH 6.0, containing 2 mM cysteine) and were homogenized in a Waring blender. The homogenate was filtered under suction through 4 layers of cheese cloth. Gravity sedimentation of the starch granules was accomplished by storing the filtrate at 4°C for 12 h. The clear supernatant was collected by decantation and freeze-dried at -5°C in an RePP Model Freeze Dryer (The Virtis Co., Gardiner, NY). This provided a pale-yellow powder of crude enzymes. For further purification, 3 g of the powder was dissolved in 20 mL LC water, centrifuged at 10,000 x g and 10 mL of the supernatant was loaded on a Sephadex G-100 column, 72 x 2.5 cm i.d. (Pharmacia, Uppsala, Sweden), which was pre-equilibrated with LC water. Fractionation was achieved by using LC water with the effluent being monitored at 280 nm. Fractions of 3 mL/tube were collected with a flow rate of 5 mL/h. All these purification steps were performed at 4°C. Fractions 68-84 and 85-108 were combined as preparations I and II, respectively, as illustrated by the separation profile (peaks I and II) shown in Figure 3.1. The preparations were freeze-dried and stored at 4°C until use. Enzyme preparation I (peak I) exhibited both nuclease and 3'-nucleotidase activity, while preparation II (peak II) possessed an RNase (nucleotido-2'-transferase) cyclizing activity. These enzyme preparations were used in all assays given in this study.

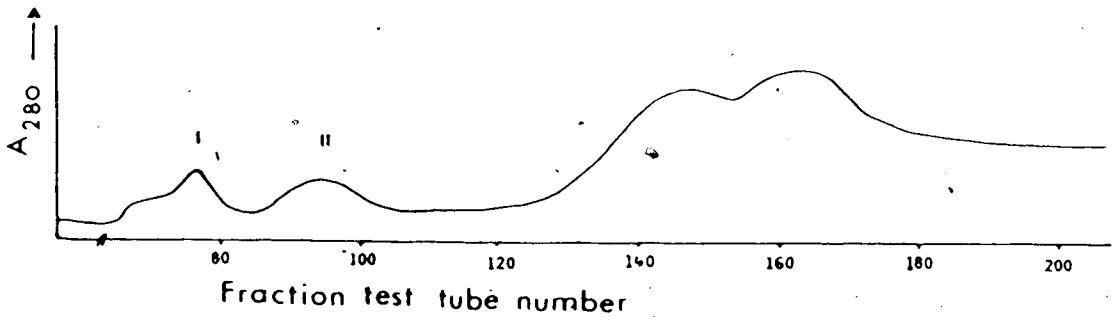

Figure 3.1. Separation profile of potato tuber crude enzymes on a Sephadex G-100 column.

3.2.5 Enzymatic reaction assay

After a prior optimization study of the reaction conditions, the following buffers were employed: 0.1 M Tris-HCl, pH 6.5, for nuclease, and 50 mM potassium phosphate, pH 5.5, for RNase activity. Enzyme solution (200 µL, 1 mg/mL) was pipetted into a 100 x 10 mm test tube containing 1 mL buffer. This was followed by 200 µL of the substrate solution (2 mg/mL). The tube was sealed, mixed by vortexing and incubated at 80°C (nuclease) and 52°C (RNase). The reaction was terminated by transferring the test tube to an ice bath, adding 200 µL of 8 mM zinc chloride and heating the contents in a boiling water bath for 12 min. This ensured complete precipitation of the protein. After the samples were centrifuged for 5 min at 6,000 x g and the supernatant was passed through a 0.45 µm HA-Millipore filter, 50 µL of the clarified solution was injected into the LC system. In blanks run simultaneously, the enzyme solution was replaced by 200 µL buffer.

Guanosine-5'-monophosphate (5'GMP) and guanosine-2':3'-cyclic monophosphate (2':3'-GMP) were selected as markers for the end products of the reaction of nuclease and RNase, respectively. The markers were located on the chromatograms based on their retention times, and were quantitated by comparison of the peak heights with standards injected before and after the sample.

One unit of enzyme activity was defined as the amount of enzyme forming one nanomole of product per minute under

the assay conditions described. Protein concentrations were estimated with Bio Rad protein assay, which is based on a method described by Bradford [13] that uses bovine serum albumin as a standard. Absorbance readings were taken at 595 nm on a Beckman Model DU-8 spectrophotometer.

3.3 Results and Discussion

3.3.1 LC separation

The LC separation of nucleotides described previously [3,14] was found to be suitable for the enzymatic assay of nuclease and RNase, since the products of nuclease activity (ribonucleoside-5'-monophosphates) were well resolved from the products of RNase (nucleotido-2'-transferase) activity (cyclic 2':3'-nucleotides) (Figure 3.2). Also, all three isomers of a given base (2'-, 3'- and 5'-nucleotides) and the corresponding cyclic form are well resolved from one another, which allowed monitoring of the action of RNase on specific substrates such as yeast RNA, polyA, polyC, polyG and polyU. Quantitation of GMP's (5'-GMP for nuclease and 2':3'-GMP for RNase) provided an insight into the competitive reactions, which occur in the presence of the same substrate (RNA). The liquid chromatograms of the products of RNA incubated separately with nuclease (enzyme preparation I) and RNase (preparation II) are shown in Figures 3.3 and 3.4. The unlabelled peaks in Figure 3.4 were due to the impurities present in commercial yeast RNA. Not

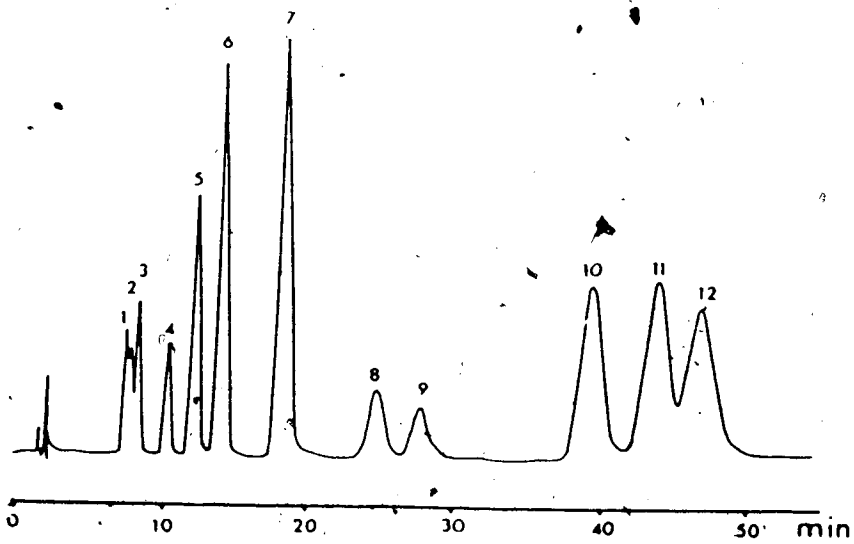

Figure 3.2. Liquid chromatogram of nucleotides used as standard. LC conditions: see text. Peak identities: 1 = cyclic 2':3'-CMP; 2 = cyclic 2':3'-UMP; 3 = cyclic 2':3'-AMP; 4 = cyclic 2':3'-GMP; 5 = 5'-CMP; 6 = 5'-UMP; 7 = 5'-AMP; 8 = 2'-AMP; 9 = 3'-AMP; 10 = 5'-GMP; 11 = 3'-GMP; and 12 = 2'-GMP. Concentration used: cyclic nucleotides and 2'- and 3'-AMP 1 μg/mL; others 5 μg/mL.

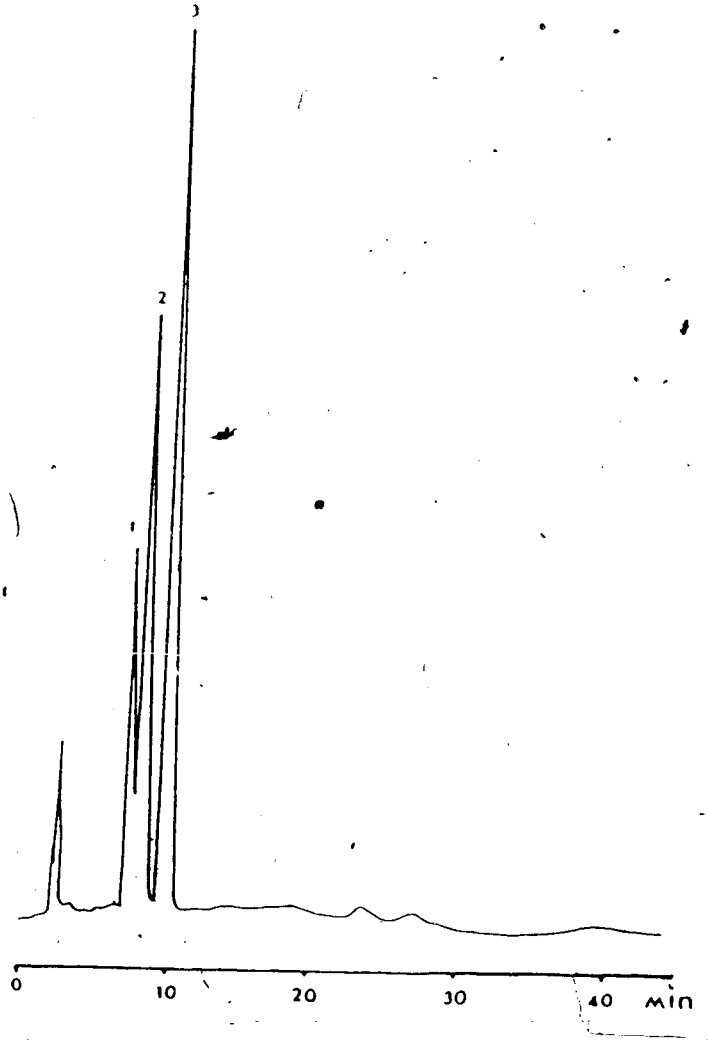

Figure 3.3. Chromatogram of RNA solution treated with potato tuber RNase. Assay conditions: a mixture of 2 mg RNA and 200 μg enzyme in 50 mM potassium phosphate buffer pH 5.5 was incubated at 52°C for 10 min. LC separation conditions as in Figure 3.2. Peak identities: 1 = cyclic 2':3'-CMP + cyclic 2':3'-UMP; 2 = cyclic 2':3'-AMP; and 3 = cyclic 2':3'-GMP.

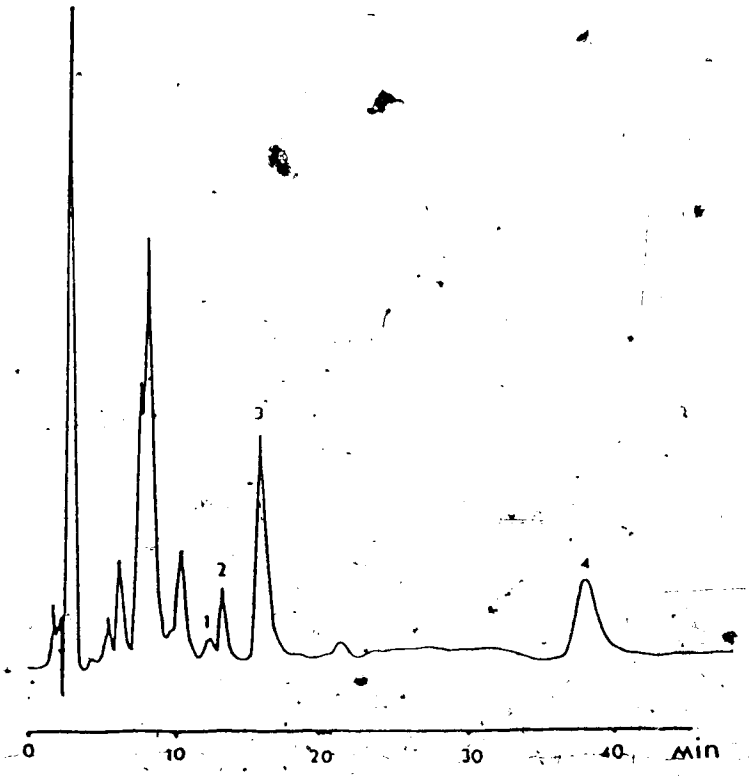

Figure 3.4. Chromatogram of RNA solution treated with potato tuber nuclease. Assay conditions as in Figure 3.3 but with 0.1 M Tris-HCl buffer pH 6.5 incubated at 80°C. Peak identities: 1 = 5'-CMP; 2 = 5'-UMP; 3 = 5'-AMP; and 4 = 5'-GMP.

all preparations had these impurities; they could be readily removed by dialysis.

3.3.2 Preliminary buffer optimization assays

It has been shown that, while the levels of nucleotides in raw potatoes are low, homogenization and heating result in enhanced nucleotide production which can be correlated with nuclease and RNase activity [11,15]. This provided a basis for the rapid selection of the most suitable buffers for the two enzymes prior to HPLC assay. Peeled potato (10 g) was homogenized with three volumes of buffer or salt solutions and steamed for 30 min. Following perchloric acid (PCA) extraction, LC analysis was used to monitor 5' nucleotides for quantitation of nuclease activity, and 2'- and 3'-nucleoside monophosphates for RNase activity. The latter products result from the acid-catalyzed ring opening of nucleoside 2':3'-cyclic monophosphates produced in RNase-catalyzed reaction.

Phosphate buffer in a range of 10 to 500 mM was found to inhibit nuclease and promote RNase activity (Table 3.1), while Tris-HCl at the same molarity and pH when compared to phosphate buffer inhibited RNase and promoted nuclease activity (Table 3.2). Sodium or potassium cations did not significantly affect the enzyme activities. Therefore, all subsequent assays were carried out in Tris-HCl buffer to follow nuclease activity and potassium phosphate buffer for RNase activity.

Table 3.1: Content (ppm) of Nucleotides in Potatoes [a]

Nucleotide	KH_2PO_4 / K_2HPO_4 Concentration (pH 6.0), moles					
	0.01	0.02	0.05	0.1	0.2	0.5
3' AMP [b]	7.68	8.21	10.35	8.84	9.49	9.29
2' GMP	9.88	10.46	12.61	11.40	11.91	11.73
3' GMP	18.83	19.60	22.99	21.01	22.11	19.11
5' AMP	7.53	8.67	6.30	5.10	3.59	1.20
5' CMP	2.10	2.31	2.20	1.10	0.00	0.00
5' GMP	4.83	5.39	4.46	3.08	1.87	0.50
5' UMP	2.33	2.46	2.66	2.09	1.65	1.51

[a] The peeled tubers were slurried in various concentrations of phosphate buffers and then steam cooked

[b] The 2'- and 3'-isomers are the end-products of the ring opening of the corresponding 2':3'-cyclic nucleotides during PCA extraction [3].

Table 3.2: Buffer Optimization Assay [a]

Nucleotide	H_2O (control)	Solution (0.1 M, pH 6.0) Tris-HCl	Potassium phosphate	Sodium phosphate	Sodium citrate	KCl	NaCl
2'-AMP [b]	2.45	2.83	4.20	4.33		2.46	2.77
3'-AMP	6.49	7.76	8.21	9.12	8.79	7.54	7.73
2'-GMP	6.26	7.68	9.65	10.35	6.94	7.01	7.94
3'-GMP	11.96	14.76	16.86	18.88	13.65	14.23	14.91
5'-AMP	15.95	12.77	6.14	6.21	11.21	14.76	16.11
5'-CMP	6.23	7.93	1.02	0.75	2.48	7.79	8.47
5'-GMP	12.10	9.61	2.91	3.17	6.16	11.22	12.04
5'-UMP	6.74	6.52	1.71	1.47	3.65	7.52	7.38

[a] Nucleotide levels (ppm) are related to RNase and nuclease activities. The peeled potato tubers were slurried in salt solutions and then steam cooked.

[b] The 2'- and 3'-isomers are the end-products of the ring opening of the corresponding 2':3'-cyclic nucleotides during PCA extraction [3].

3.3.3 LC optimization assay

The activity of nuclease and RNase was linear in the range of 10 to 500 μg of enzyme using RNA as a substrate. Therefore, 200 μg (0.2 mL of 1 mg/mL enzyme solution) was selected for all incubation assays. For this concentration product formation was proportional with time up to 30 min of incubation. For convenience a 10-min reaction time was used.

The optimum pH range for RNase was 4.5-6.0. Hence, pH 5.5 was chosen for assaying this enzyme. The optimum pH for nuclease was 6.5 (Figure 3.5), in agreement with previously reported values [12].

The effect of temperature on the activities of nuclease and RNase is shown in Figure 3.6. The optimal temperatures found were 80°C for nuclease and 52°C for RNase. Consequently, these temperatures were chosen for the enzyme assay.

3.3.4 Enzyme characterization

Specific activities (units/mg protein) were 8.73 for nuclease (preparation I) and 16.4 for RNase (preparation II). The Michaelis-Menten constants (K_m) were estimated under the assay conditions outlined above. A program based on the statistical method of Wilkinson [17] was used for data analysis. The K_m values for nuclease and RNase were 39.9±4.8 (μg, yeast RNA) and 119.0±0.3 (μg, yeast RNA), respectively. Substrate at a level of 400 μg was used since at higher concentrations the formation of oligonucleotide

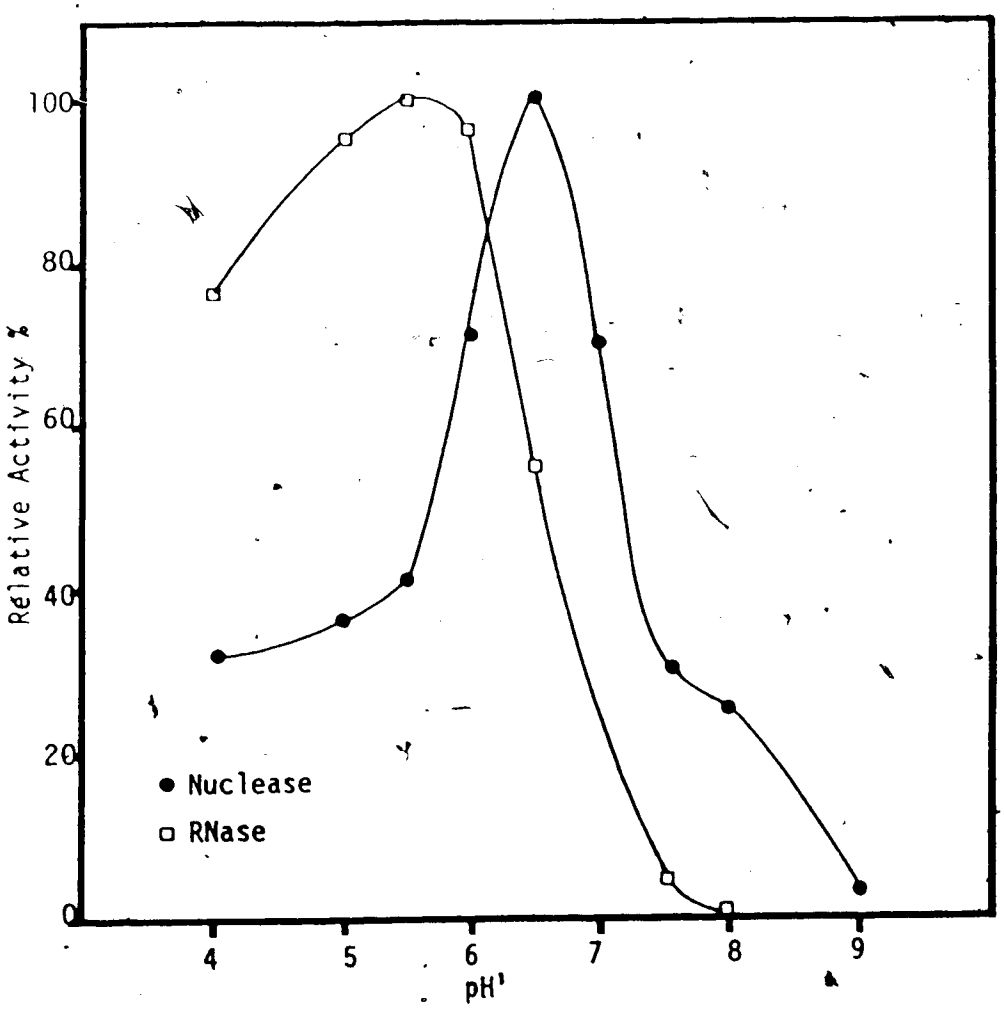

Figure 3.5. Effect of pH on potato tuber enzyme activities.

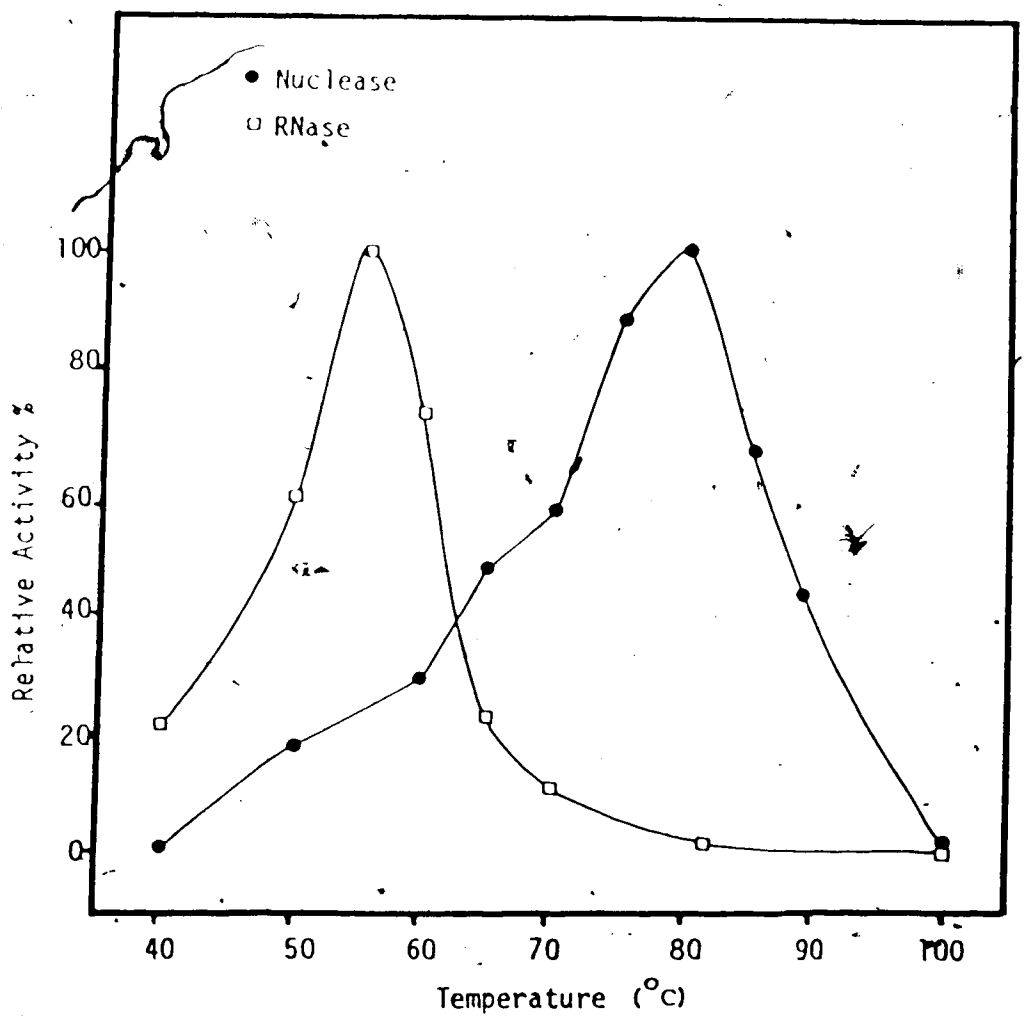

Figure 3.6. Effect of temperature on potato tuber enzyme activities.

products required extensive washing for column re-equilibration.

The effect of various ions on nuclease activity was measured in a concentration range of 1 to 10 mM (Table 3.3). Chloride ion added as sodium chloride at 10 mM (a level usually applied in food processing) decreased the nuclease activity by 13%. Monosodium glutamate (MSG), a flavor enhancer used extensively in food industry, was found to be a good promoter for nuclease. In its presence the enzymes produced more 5'-GMP. Due to a synergistic taste effect between 5'-GMP and MSG, there would be a large flavor improvement of food.

As previously reported [16], EDTA was found to be a strong inhibitor of potato nuclease. The initial activity of the enzyme decreased to 17% in the presence of 1 mM EDTA. However, EDTA promoted the activity of RNase (Table 3.3). Since both nuclease and RNase are present in potato tubers and since both utilize the same substrate (RNA), but since nuclease forms the 5'-nucleotide flavor enhancer, this suggested that caution should be exercised by the food industry in the use of chelating agents, in order to prevent the loss of nuclease activity.

The nuclease activity (enzyme preparation I) using various polynucleotides as substrates is presented in Table 3.4. As measured by the formation of nucleoside-5'-monophosphate, the relative hydrolysis rates were: polyU > polyA > RNA > polyC > polyG > DNA. This corroborates the earlier

Table 3.3 Effect of some Anions, Cations, Glutamate and EDTA on the Activities of Potato Nuclease and RNase

Additions [a]	Molarity	Relative Activity (%) [b]	
		Nuclease	RNase
Blank		100	100
NaH_2PO_4	1×10^{-3}	91	
$NaHSO_3$	1×10^{-3}	57	
NaCl	10×10^{-3}	87	
LiCl	10×10^{-3}	97	
Na-Glutamate	10×10^{-3}	128	
$MnCl_2$	1×10^{-3}	38	
$ZnCl_2$	1×10^{-3}	0	0
$MgCl_2$	1×10^{-3}	122	—
EDTA	1×10^{-3}	17	133
	1×10^{-4}	50	122

[a] For nuclease activity, addition to 0.1 M Tris-HCl buffer pH 6.5; for RNase activity, addition to 50 mM potassium-phosphate buffer pH 5.5, using yeast RNA as a substrate.

[b] A mean of two replicates which did not differ by more than 2%.

Table 3.4: Action of Potato Nuclease on Various Homo- and Native Polynucleotides

Substrate	Relative activity (%)
Poly A	129
Poly C	65
Poly G	12
Poly U	310
Native DNA	9
RNA	100

[a] A mean of two replicates which did not differ by more than 2%.

results by Nomura *et al.* [12].

RNase activity with various polynucleotide substrates is presented in Table 3.5. The relative reaction rates were polyU > RNA > polyC > polyA > polyG. This enzyme, like nuclease, preferentially hydrolyzes polyU. After 10 min incubation at 52°C in 50 mM phosphate buffer (pH 5.5), the only reaction products found were cyclic nucleoside 2':3'-monophosphates (Figure 3.7a). This is in accord with the generally accepted reaction mechanism for RNase [4,18-20], which suggests that initial depolymerization of RNA occurs by nucleophilic attack of the 2'-OH group on the adjacent 3'-phosphoryl group, resulting in 2':3'-cyclic phosphates. These cyclic intermediates are then slowly hydrolyzed to the corresponding 3'-nucleotides.

However, as shown in Table 3.6, after prolonged incubation at 37°C, both 2'- and 3'-nucleotides were formed in non-stoichiometric amounts from all cyclic substrates. After 20 h, between 0.7% (2':3'-CMP) and 1.8% (2':3'-AMP) hydrolysis occurred. Under identical conditions the hydrolysis of blanks without enzyme was negligible. The RNase-catalyzed hydrolysis rates for cyclic nucleotides as substrates were 2':3'-AMP > 2':3'-UMP > 2':3'-GMP > 2':3'-CMP. Hydrolysis product ratios were 16% of 2'- and 84% of 3'-isomer for AMP and GMP. Cyclic UMP provided 23% and 77%, respectively. We attribute the small amounts of the 2'-isomers to the action of residual phosphodiesterase in tuber RNase preparation [11], since pure RNase forms only

Table 3.5: Potato RNase Activity towards Various Polynucleotides

Substrate	Relative activity (%) [a]
Poly. A	43
Poly C	60
Poly G	17
Poly U	454
RNA	100

[a] A mean of two replicates which did not differ by more than 2%.

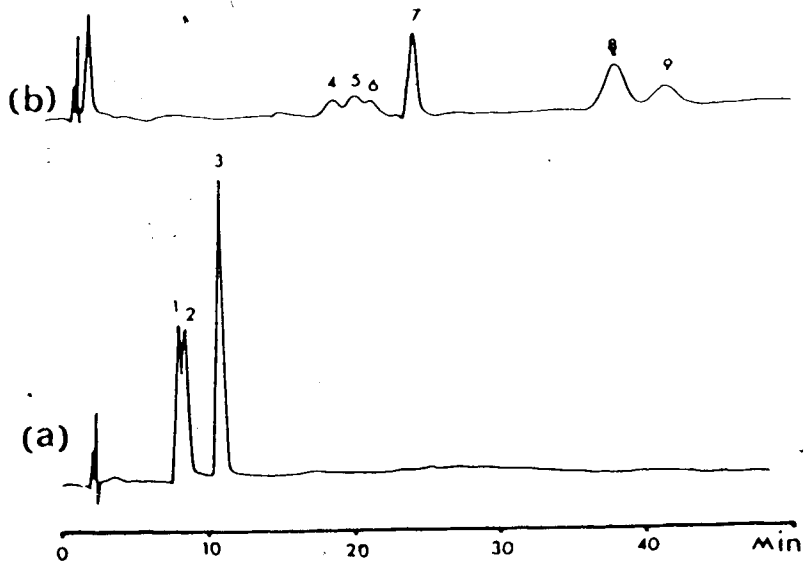

Figure 3.7. Chromatograms of a reaction mixture of RNA and potato RNase incubated as in Figure 3.3. (a) Reaction mixture without additional treatment; (b) after the reaction mixture was treated with PCA. Peak identities: 1 = cyclic 2':3'-CMP + cyclic 2':3'-UMP; 2 = cyclic 2':3'-AMP; 3 = cyclic 2':3'-GMP; 4-7 = 2'- and 3'-nucleotides of adenine, cytosine and uracil; 8 = 3'-GMP; and 9 = 2'-GMP. The presence of cyclic nucleotides prior to PCA treatment (a) and their disappearance with concomitant production of their 2'- and 3'-nucleotides after PCA treatment (b) are readily revealed.

Table 3.6: Extent of 2'- and 3'-Nucleotides Formation from 2':3'-Cyclic Nucleotides and RNA incubated with Potato RNase at 37°C, 20 hr

Substrate	Nucleotide produced (units) [a]								Formation of isomer (%)		Substrate hydrolysed (%)
	AMP		CMP		GMP		UMP				
	2'	3'	2'	3'	2'	3'	2'	3'	2'	3'	
2':3'-AMP	0.016	0.082	—		—		—		16	84	1.8
2':3'-CMP	—		0.045		—		—		—		0.7
2':3'-GMP	—		—		0.12	0.064	—		16	84	1.4
2':3'-UMP	—		—		—		0.021	0.070	23	77	1.5
RNA	nd [b]	0.007	nd	0.013	Trace	0.031	nd	0.016			

[a] The data are means of two replicates which did not differ by more than 2%.

[b] nd: Not determined.

3' nucleoside phosphates.

In previous studies we reported that cooked potatoes contain 2' and 3' nucleotides and it was assumed that their presence was due to enzymatic breakdown of potato tuber RNA [3,14]. These products arise from the cleavage of cyclic 2':3' nucleotides formed by RNase. However, as the temperature of the tuber increased during cooking, RNase was denatured (Figure 3.6) and thus could not catalyze the next reaction stage (ring cleavage). Thus we concluded that PCA used in the extraction step of the tubers is primarily responsible for the occurrence of 2' and 3'-isomers in the extracts, in accordance with a general finding that strong acids or bases hydrolyze cyclic nucleotides [21]. This is supported by chromatograms of substrates incubated by RNase with (Figure 3.7b) and without (Figure 3.7a) subsequent PCA treatment. As seen in Figure 3.7b, the formation of isomers is accompanied by a concomitant disappearance of the peaks for cyclic nucleotides.

It is known that other enzymes are present in potato tuber which are involved in the breakdown of 5'-nucleotides such as 5'-nucleotidase with a mol. wt. of 50 kdal [22] and phosphatases with a mol. wt. of about 96 kdal [23]. These enzymes were not present in our enzyme preparations since they were unretained on the Sephadex gel column and hence were removed in the purification step. However, during potato processing, the 5'-nucleotides produced by nuclease might be hydrolyzed by these enzymes into nucleosides which

are not flavor potentiators. Nevertheless, the advantages of potato processing at a temperature close to 80°C should be maintained, since both 5' nucleotidase and phosphatases are, like RNase, heat-labile enzymes [11,22]. Therefore, rapid heat transfer to tuber cells would inactivate all three undesirable enzymes while the activity of nuclease would be enhanced to leave the phosphodiester bond of RNA. Thus, higher amounts of 5'-GMP, the endogenous flavor nucleotide of processed potato, would be generated.

3.4 Conclusion

The determination of the activities of potato RNase (A) and nuclease (B) by LC has a number of advantages over conventional methods, including the simultaneous assay of both activities. Enzyme product analysis can be done accurately within 12 and 45 min for A and B, respectively.

Nuclease and RNase activities were determined by measuring 5'-GMP and cyclic 2':3'-GMP, as enzymatic reaction end products. The former enzyme was found to be important for potato processing since it generated the flavor-enhancing nucleotide, 5'-GMP. On the other hand, RNase had a much lower optimal temperature activity, providing only 2':3'-cyclic nucleotides in its first stage of activity; in the second stage it provided 3'-nucleotides at a very slow reaction rate. Neither of these products is a flavor enhancer.

The results of this work support the processing of potatoes at higher temperatures (80°C) to enhance the formation of flavor potentiator, 5'-GMP, by nuclease-catalyzed hydrolysis of tuber RNA. Such temperatures promote nuclease activity and reduce the activity of the 5' nucleotidase, phosphatases [11] and RNase, as proved by this study.

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4. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF POTATO TUBER 3'-NUCLEOTIDASE[1]

4.1 Introduction

The separation of the constituents of nucleic acids by high performance liquid chromatography (HPLC) in biomedical research has been reviewed extensively [1,2,3]. However, Robinson [4] emphasized that generalizations derived from animal and/or microorganism assay may not be extended to higher plants such as fruits and vegetables.

In our study on the enzymatic cleavage of nucleic acids in vegetables, we have recently developed an HPLC method for assaying the activities of potato nuclease and RNase [5]. In this report, we describe an HPLC method for quantitating potato tuber 3'-nucleotidase activity. This enzyme catalyzes the hydrolysis of nucleoside-3'-monophosphates and is commonly assayed by measuring liberated inorganic phosphate [6]. Methods using isocratic reversed-phase HPLC have been reported for the related 5'-nucleotidase enzyme in human erythrocytes and blood plasma [7,8]. However, in the analysis of blood plasma, the only substrates and products measured were pyrimidine-5'-nucleotides and their deoxy-analogs. 3'-Nucleotidase exhibits broad substrate specificity, acting preferentially on the purine nucleotide 3'-AMP and on pyrimidine nucleotides [9]. The HPLC method reported in this study is rapid, accurate and has been used

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[1] Published in J. Chromatogr. 391 (1987) 257-265.

for the characterization of potato 3'-nucleotidase properties, including substrate specificity, pH and temperature optima, and for some potential inhibitor evaluations.

4.2 Experimental

4.2.1 Chromatographic equipment

The HPLC system consisted of a Model 1330 pump (Bio-Rad, Richmond, CA), a Bio-Rad Model 1305 variable-wavelength detector and a 50 μL fixed volume Rheodyne sample injector loop. A Whatman Partisil SCX column (Whatman, Clifton, NJ), 250 x 4.6 mm i.d. was used for the separation of nucleosides; this column was used in conjunction with a 70 x 2.1 mm i.d. guard column containing a pellicular cation exchanger and a 250 x 4.6 mm i.d. pre-injector silica gel column (Whatman, Solvecon). The absorbance of nucleosides was monitored at 254 nm and peak quantitation was performed with a Hewlett-Packard 3388A integrator (Hewlett-Packard, Avondale, PA).

4.2.2 HPLC separation conditions

The mobile phase was 10 mM potassium phosphate solution, pH 3.60, filtered through a 0.45 μm Millipore membrane and degassed. Isocratic elution at ambient temperature was carried out at a flow rate of 1.5 mL/min.

4.2.3 Materials

All standards and substrates were obtained from Sigma (St. Louis, MO), and HPLC-grade sodium acetate and potassium dihydrogen phosphate from Fisher Scientific (Fair Lawn, NJ). Water for HPLC was purified by reverse osmosis (Mill-RO) and further purified by using a Milli-Q System (Millipore Co., Bedford, MA). All other chemicals were analytical-reagent grade.

4.2.4 Enzyme purification

Partially purified 3'-nucleotidase was prepared by a modification of literature methods [9,10] from peeled potato tubers, cv. Pontiac, grown commercially in Southern Alberta. Isolation steps included diced tuber homogenization in 50 mM sodium citrate buffer (pH 6.0) containing 2 mM cysteine, homogenate filtration and starch gravity sedimentation, followed by chromatography of the clarified supernatant on a Sephadex G-100 column [5].

4.2.5 Enzyme assay conditions

Assay mixtures contained 200 µg enzyme and 500 µg of substrate in a total volume of 1.4 ml 0.1 M sodium acetate buffer (pH 6.5) in 100 x 10 mm test tubes. Samples were incubated at 70°C and the reaction was terminated by transferring the tubes to an ice bath, adding 200 µL of 8mM zinc chloride solution and heating in a boiling-water bath for 12 min. In blanks run simultaneously, the enzyme

solution was replaced with buffer. All samples and blanks were then diluted 5-fold with HPLC-grade water, filtered through a 0.45 μm Millipore membrane and an aliquot injected into the HPLC system. Adenosine was used as a marker for the end-product of the reaction of 3'-AMP and 3'-nucleotidase. The corresponding nucleosides of other substrates were used as markers to follow the action of 3'-nucleotidase on various nucleotides. Markers were identified by retention times and were quantitated by comparison with peak heights of standards (diluted in 20 mM sodium acetate buffer) injected before and after sample separations.

One unit of enzyme was defined as the amount forming 1 nmole of adenosine per minute. Protein concentrations were estimated with the Bio-Rad assay kit based on Bradford's method [11], using bovine serum albumin as a standard. The specific activity of potato tuber 3'-nucleotidase was 100 units/mg protein. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight determinations were carried out using the protein II slab cell (Bio-Rad Labs.), casting discontinuous gels consisting of resolving and stacking gels and applying the discontinuous buffer system of Laemmli [12]. Protein standard markers covering a molecular weight range of 14.3 (lysozyme) to 45.0 (albumin) kdal were supplied by Sigma.

4.3 Results and Discussion

4.3.1 HPLC separation

Reversed-phase HPLC is widely used in the analysis of nucleosides and nucleobases [13-17]. However, assay of nucleotidase activity by ion-exchange methods still shows its power when cation-exchange columns are applied. In the previous work [18], a Whatman SCX column and 10 mM potassium phosphate solution of pH 3.60 were used for the HPLC separation of 5'-GMP, 5'-IMP and their corresponding nucleosides and bases. This system was successfully applied for assaying the activity of 3'-nucleotidase. All isomers of ribo- and deoxyribonucleotides were well separated from their corresponding nucleosides. The retention times of nucleotide substrates and their corresponding nucleoside products are shown in Table 4.1 and the linear relationships and detection limits for standard nucleosides are given in Table 4.2. A typical chromatogram of an incubation mixture of 3'-AMP and 3'-nucleotidase is shown in Figure 4.1. The precision of the method was demonstrated by five fully repeated analyses of the activity on 3'-AMP, which provided 12.95±0.03 enzyme units.

Enzyme-catalyzed product formation was linear up to 30 min incubation time, and a 10 min reaction time was chosen for convenience. The optimal pH for 3'-nucleotidase was 6.5 (Figure 4.2), in contrast to pH 8 as reported by Nomura *et al.* [9]. The optimal temperature for a 10 min incubation

Table 4.1: Retention Times of Nucleotide Substrates and corresponding Nucleoside Products of 3'-Nucleotidase-Catalyzed Hydrolysis

Type		Retention time (min)	
	Nucleotide [a]	Substrate	Product (nucleoside)
Aminonucleotides	2'-AMP	2.12	4.30
	3'-AMP	2.10	4.30
	5'-AMP	2.12	4.30
	3'-dAMP	2.15	5.36
	5'-dAMP	2.33	5.36
	2'-CMP	2.30	4.92
	3'-CMP	2.24	4.92
	5'-CMP	2.21	4.92
	5'-dCMP	2.37	6.68
Oxonucleotides	2'-GMP	1.64	2.47
	3'-GMP	1.63	2.47
	5'-GMP	1.65	2.47
	5'-dGMP	1.71	2.79
	2'-UMP	1.61	2.29
	3'-UMP	1.60	2.29
	5'-UMP	1.60	2.29
	5'-TMP	1.63	2.61

[a] The above table also provides data for 2'- and 5'-nucleotides since they might be useful for further studies of other potato tuber phosphomonoesterases (2'- and 5'-nucleotidases as well as phosphatases).

Table 4.2: Linearity of Peak Height Measurements for Nucleosides in the Concentration Range of 0.050-10.0 µg/mL

Type	Nucleoside	Correlation coefficient	Regression equation	Detection limit (ppb)
Ribonucleosides	Adenosine	0.9999	y = 910x - 43 [a]	20
	Cytidine	0.9999	y = 445x	20
	Guanosine	0.9997	y = 927x + 72	10
	Uridine	0.9999	y = 1316x + 41	10
Deoxyribonucleosides	Deoxyadenosine	0.9999	y = 662x - 27	20
	Deoxycytidine	0.9999	y = 330x - 14	20
	Deoxyguanosine	0.9997	y = 1168x + 129	10
	Thymidine	0.9999	y = 1264x - 12	10

[a] x = Concentration of standard solution (µg/mL)
y = peak height

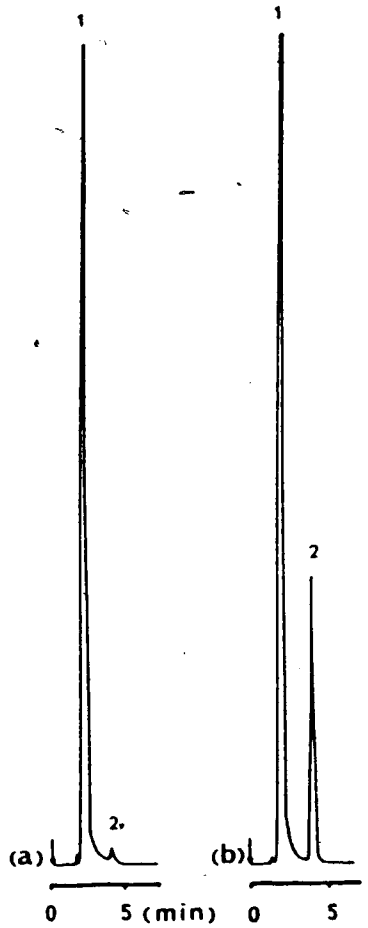

Figure 4.1. Chromatograms of the incubation solutions of 3'-AMP as substrate (a) without (blank) and (b) with potato tuber 3'-nucleotidase. Peaks: 1 = 3'-AMP; 2 = adenosine.

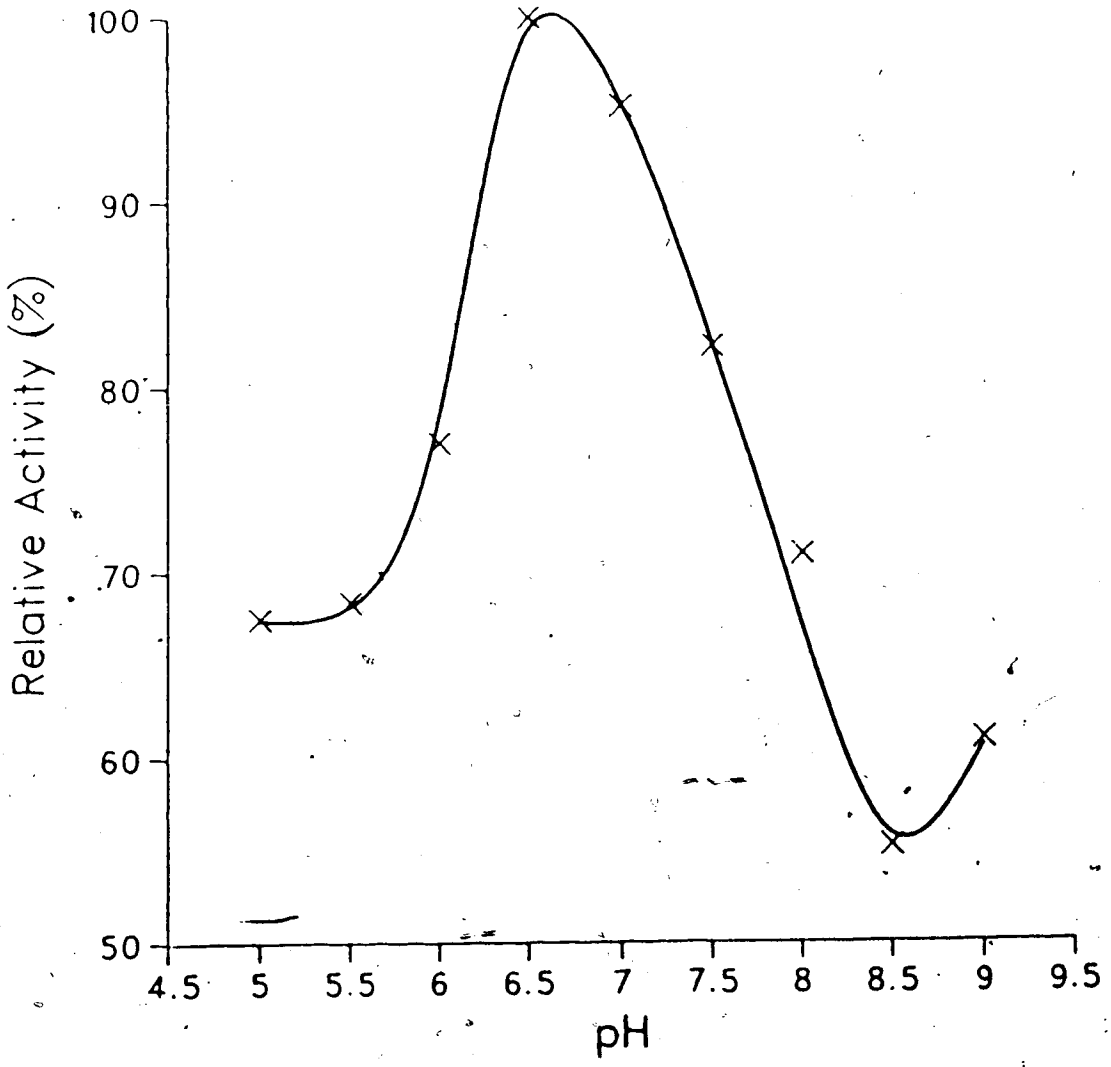

Figure 4.2. Effect of pH on tuber 3'-nucleotidase activity.

time was between 70 75°C (Figure 4.3), and 70°C was used for all subsequent characterizations. A decrease in the substrate 3' AMP concentration and a concomitant increase in adenosine levels as the reaction end product was linear for an enzyme concentration range of 0 800 µg (Figure 4.4). The Michaelis Menten constant (K_m) for 3'-AMP under our conditions was 0.5 µM compared with 0.72 µM (37°C, pH 7.6, Tris HCl) reported by Suno *et al.* [19]. A program based on the statistical method of Wilkinson [20] was applied for data analysis. For all other nucleotides, 500 µg of substrate was used, which was 2.6 times the K_m of potato tuber 3'-nucleotidase when 3' AMP was used as a substrate. Higher concentrations of substrate caused tailing of the nucleotide peaks and overlapping with the product nucleosides, causing difficulties in quantitation.

4.3.2 Substrate specificity of 3'-nucleotidase

Substrate specificity data for this enzyme are summarized in Table 4.3. As can be seen from the results, the enzyme was essentially free of 5'-nucleotidase activity. The enzyme lacked phosphomonoesterase activity on pyrimidine 2'-nucleotides, while it slowly hydrolyzed the phosphomonoester linkage at the 2'-position of purine nucleotides. Effective hydrolysis of nucleoside-3'-monophosphates of all four bases demonstrated the enzyme's action specificity as a 3'-nucleotidase. The relative rates of hydrolysis of 3'-ribonucleotides were 3'-AMP > 3'-UMP > 3'-GMP > 3'-CMP,

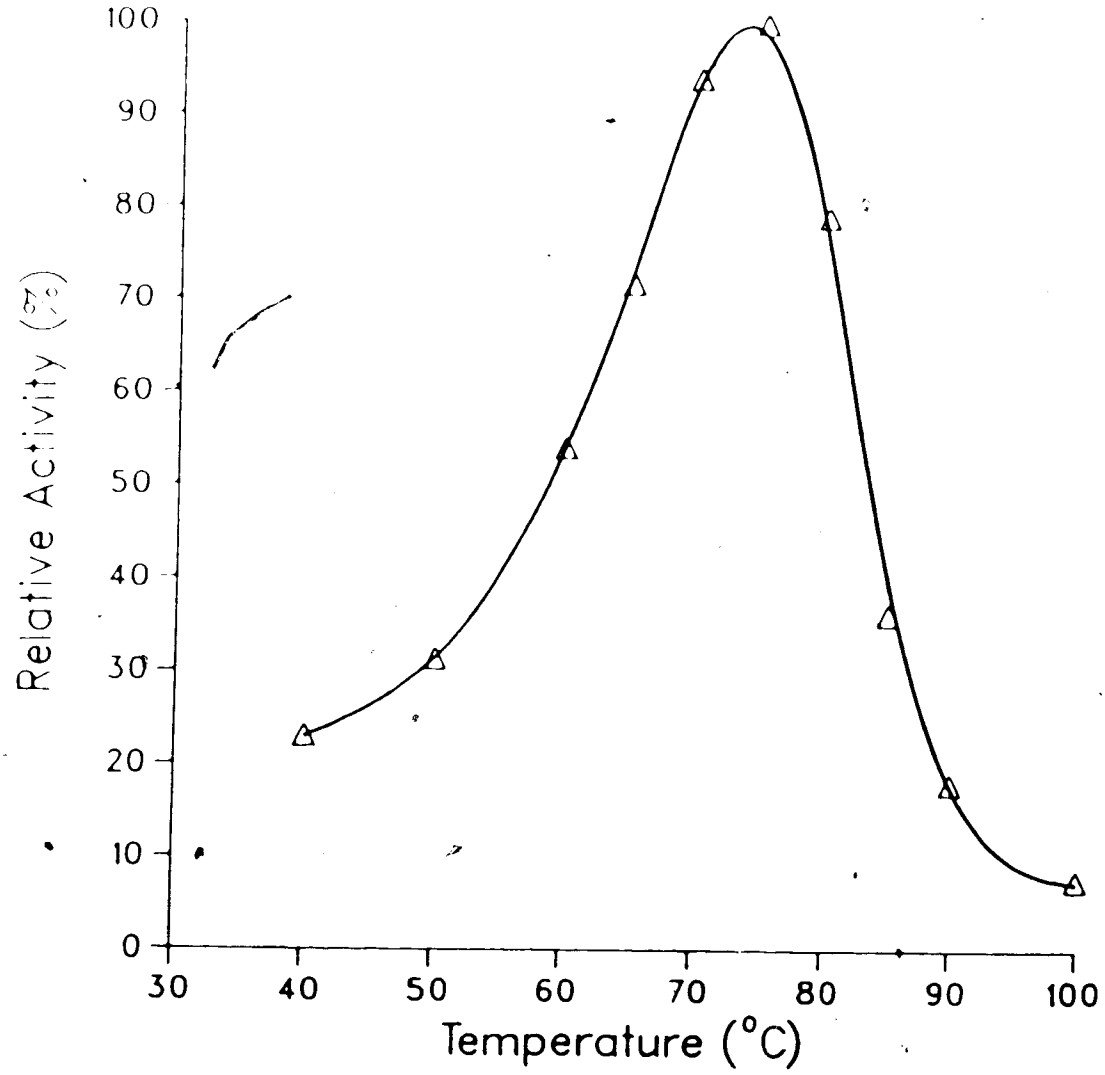

Figure 4.3. Effect of temperature on tuber 3'-nucleotidase activity.

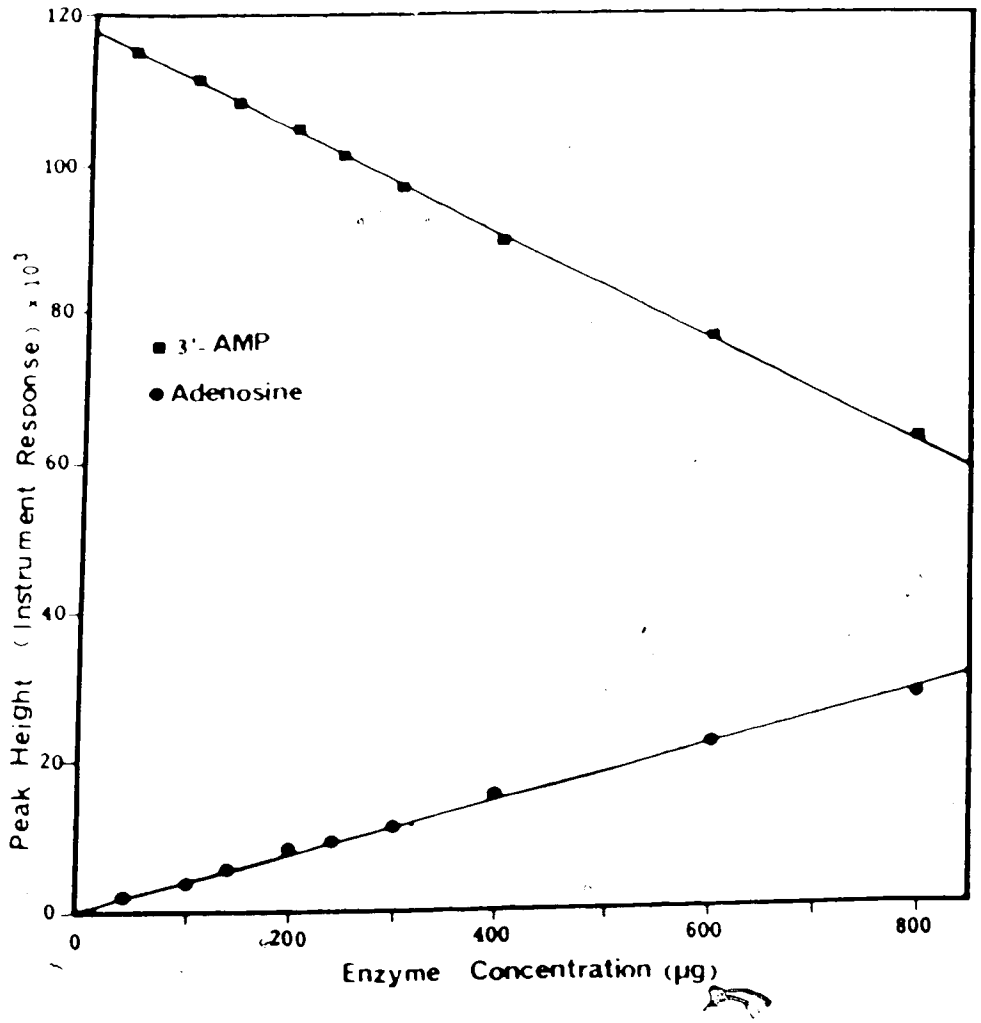

Figure 4.4. Linearity in the relationship between the substrate concentration (3'-AMP) and concomitant increase in the yield of 3'-nucleotidase reaction product (adenosine). ■ = 3'-AMP; ● = adenosine.

Table 4.3: Action of Potato Tuber 3' Nucleotidase on Various Nucleotides

Substrate		Relative activity[a]
Type	Nucleotide	(%)
Ribonucleotides	2'-AMP	3.1
	2'-CMP	0.0
	2'-GMP	5.3
	2'-UMP	1.1
	3'-AMP	100.0
	3'-CMP	23.5
	3'-GMP	42.3
	3'-UMP	54.2
	5'-AMP	3.1
	5'-CMP	0.7
	5'-GMP	1.7
	5'-UMP	1.3
Deoxyribonucleotides	3'-dAMP	13.3
	5'-dAMP	6.8
	5'-dCMP	3.3
	5'-dGMP	2.4
	5'-dTMP	1.4

[a] Calculation is based on the corresponding amount of nucleoside in nanomoles formed during incubation for 10 min at 70°C. The result is expressed as percentage of the amount of adenosine formed in the enzyme-catalyzed reaction with 3'-AMP as substrate. The data are means of two replicates which did not differ by more than 2%.

corroborating the data reported by Nomura *et al.* [9]. The hydrolysis rate of deoxyadenosine-3'-monophosphate (3'-dAMP) was much lower than that of 3'-AMP (Table 4.3), suggesting that the enzyme's active site requires the presence of a 2'-hydroxy group. The potato tuber 3' nucleotidase activity was associated with tuber nuclease activity. The two activities could not be separated by Sephadex column fractionation or by SDS-PAGE. Both enzyme activities resided within a single band of a molecular weight close to 34.2 kdal. Nomura *et al.* [9] attempted to separate the two activities by ion-exchange chromatography on phosphocellulose, DEAE-cellulose and DEAE-Sephadex at pH 5 and 9. Nevertheless, the enzyme activities were always co-eluted. These attempts suggest that both enzyme activities reside within the same protein molecule. The enzyme specificity from potato tuber is not an exception. The best characterized nuclease from mung bean sprouts has an equally active 3'-nucleotidase activity [21,22].

4.3.3 Effects of some ions and food additives

The effects on potato 3'-nucleotidase activity of some ions and additives used in food processing were tested from 0.1 to 100 mM (Table 4.4). Chloride ion added as NaCl (10 mM) had almost no influence, while glutamate and sulfite promoted 3'-nucleotidase activity. The chelator EDTA was found to be a strong inhibitor. At a concentration of 1 mM it decreased the activity of nucleotidase to 25% of the

Table 4.4: Effect of some Anions, Cations, Glutamate and EDTA on the Activity of Potato 3'-Nucleotidase

Additive	Concentration (mM)	Relative activity versus 3'-AMP substrate (%) [a]
Control		100
NaH_2PO_4	10	53
NaH_2PO_4	100	17
$NaHSO_3$	10	151
NaCl	10	98
LiCl	10	100
Na-glutamate	10	115
$MnCl_2$	1	90
$ZnCl_2$	1	10
$MgCl_2$	1	106
EDTA	1	25
	0.1	62

[a] The data are means of two replicates which did not differ by more than 2%.

control. Phosphate ion and zinc chloride were also inhibitory, but 1 mM magnesium chloride had little (6%) effect on enzyme activity. Similarly, 1 mM manganese chloride had a 10% inhibitory effect, whereas 10 mM lithium chloride had no effect. The flavor-enhancing nucleotide 5'-GMP inhibited 3'-nucleotidase activity; for incubations containing 50 μg of 3'-AMP as substrate, the activity decreased by 23% in the presence of 600 μg of 5'-GMP (Figure 4.5).

4.3.4 Multiple-substrate assay

As the nucleosides of the oxo and amino groups and also ribo- and deoxyribonucleosides were well resolved from one another (Table 4.1), a multiple-substrate assay could be performed. Figure 4.6 demonstrates an example of a two-substrate assay in which equimolar concentrations of 3'-AMP and 5'-dAMP were included in the incubation mixture.

4.4 Conclusions

An HPLC method using an ion-exchange Partisil SCX column was developed for assaying potato tuber 3'-nucleotidase activity. This method was accurate and rapid, The 3'-nucleotidase was associated with tuber nuclease activity in the isolated and purified enzyme preparations (see Chapter 3) and was found to be specific for the 3'-phosphomonoester bond. The method presented can also be used for the simultaneous assay of purine or

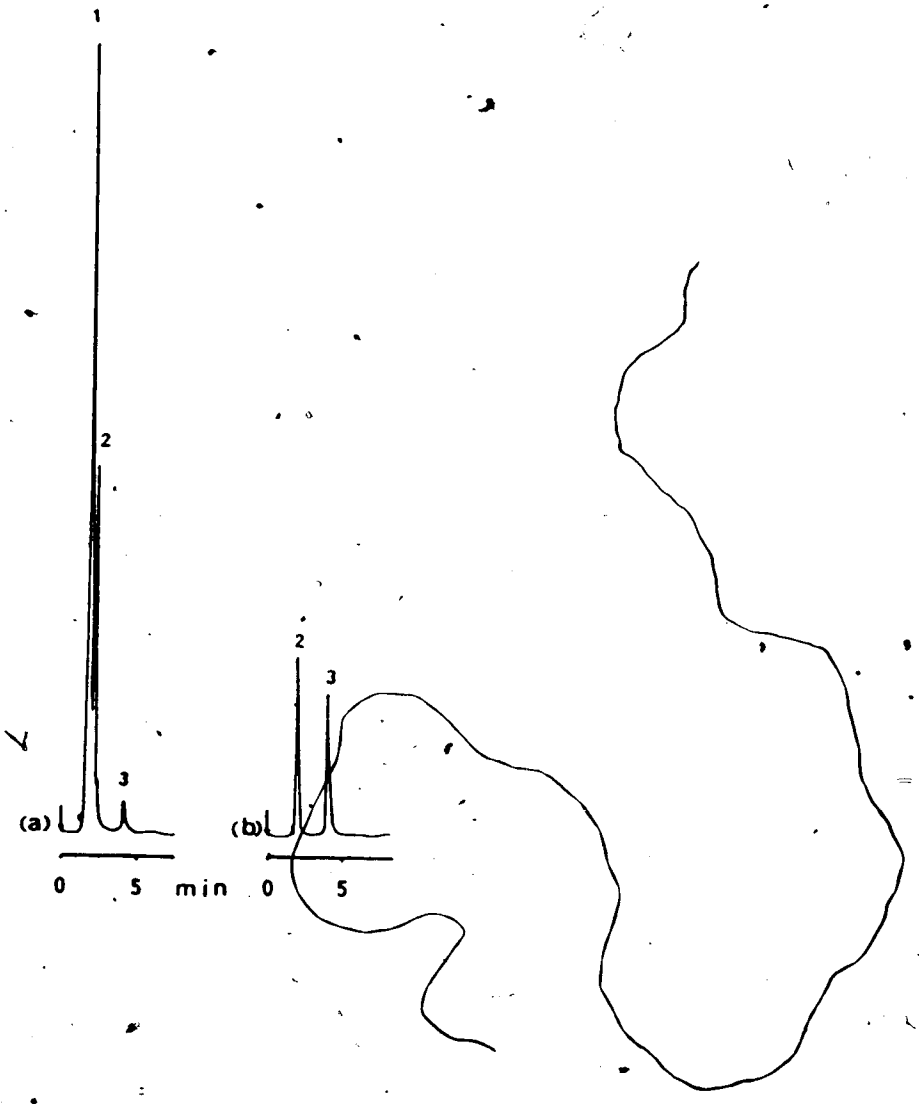

Figure 4.5. Inhibition of 3'-nucleotidase activity with 5'-GMP. Incubation mixture of tuber 3'-nucleotidase (200 µg) contained (a) 3'-AMP (50 µg) and 5'-GMP (600 µg) and (b) only 3'-AMP (50 µg). Peaks: 1 = 5'-GMP; 2 = 3'-AMP; 3 = adenosine.

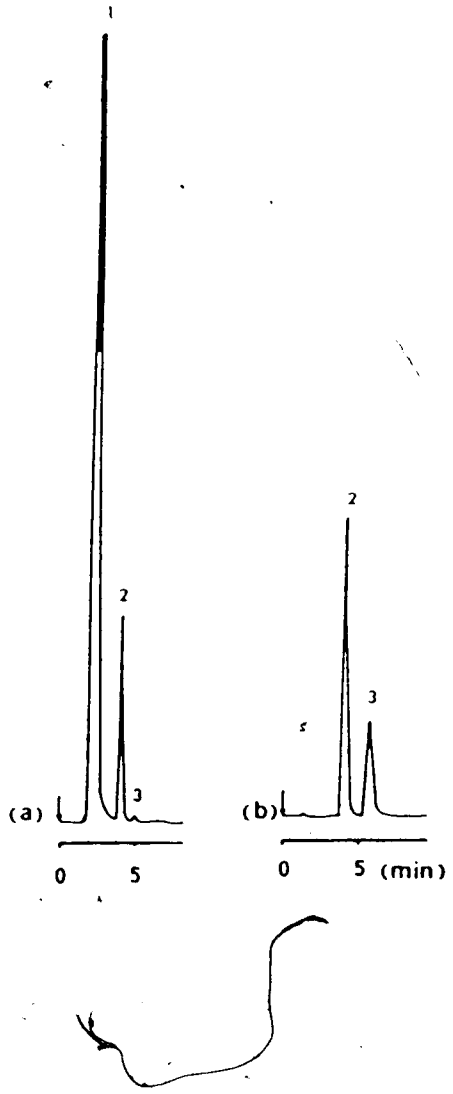

Figure 4.6. Chromatograms of an incubation mixture of (a) two substrates, 3'-AMP and 5'-dAMP (both 500 μg) with potato tuber 3'-nucleotidase, and (b) standard solution of adenosine (8 μg/mL) and deoxyadenosine (4 μg/mL) in 20 mM sodium acetate buffer. Peaks: 1 = 3'-AMP + 5'-dAMP; 2 = adenosine; 3 = deoxyadenosine.

pyrimidine ribo- and deoxyribonucleotides as 3'-nucleotidase substrates.

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5. CHARACTERIZATION OF CELL WALL BOUND NUCLEASE AND RIBONUCLEASE FROM POTATO TUBER[4]

5.1 Introduction

The cell wall is a distinct plant component which contains a variety of enzymes [1,2] as well as RNA [3]. Several hydrolases have been identified in plant cell walls, including invertases, glucanases, pectin methylesterase and galacturonases[4]. The pectic enzymes have been the most intensively studied, since they are responsible for changes in vegetable texture during blanching in food processing [5,6].

The only potato tuber cell wall enzymes that have been characterized are phosphatases [7] and pectin methylesterase [6,8]. The existence of cell wall bound enzymes involved in formation of nucleotides has not been proven. To provide better knowledge about these enzymes and their possible differences with previously studied cytoplasmic enzymes (see Chapters 3 and 4), in this chapter the isolation and characterization of cell wall bound nuclease and RNase of potato tuber are presented.

5.2 Materials and Methods

All standard compounds and the following substrates were obtained from Sigma Chemical Co. (St. Louis, MO): yeast tRNA, calf thymus DNA, potassium salts of polyadenylic

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[4] Agric. Biol. Chem. (in press).

(polyA; Mol. weight 0.2≈1.0 Mdal), polycytidylic (polyC; Mol. weight 0.2≈1.0 Mdal), polyguanylic (polyG; Mol. weight 0.14≈0.40 Mdal), and polyuridylic (polyU; Mol. weight ≅0.1 Mdal) acids. HPLC-grade methanol and potassium dihydrogen phosphate were from Fisher Scientific (Fair Lawn, NJ). Ammonium sulfate, special enzyme grade, was from Schwarz-Mann (Cambridge, MA). Sephadex G-200 and DEAE-Sephadex A-50 were from Pharmacia Fine Chemicals (Uppsala, Sweden). A protein assay kit, with bovine serum albumin as a standard, was from Bio-Rad Labs. (Richmond, CA). HPLC-grade water used in all experiments was prepared by reverse osmosis (Milli-RO) and further purified by using a Milli-Q system (Millipore, Bedford, MA).

5.2.1 Enzyme isolation and purification

All isolation and purification steps were carried out at 4°C unless otherwise stated. Glassware was autoclaved for 30 min at 121°C prior to use, to inactivate any contaminating RNases.

5.2.2 Cell wall isolation

Potato cell walls were isolated by a combination of the methods described by Moledina *et al.* [6], Sugawara *et al.* [7] and Harris [10]. Peeled potato tubers, cv. Pontiac, 5 kg, were blended with 25 L cold 0.1% isoascorbic acid using a Waring blender. The potato slurry was washed thoroughly with tap water, in a bag made up of four layers of cheese

cloth, until free of starch as determined by the absence of starch-iodine blue complex and as monitored by polarized light microscopic examination. To ensure that all of the cells were broken, the residue was re-homogenized with two volumes of 0.1% isoascorbic acid using a Sorvall Omni-mixer, washed with distilled water, and filtered through a Buchner funnel containing two layers of 5 μm mesh nylon. The yield of wet cell walls from 5 kg of potato tubers was 311 g, with a moisture content of 82.4%, determined using an AOAC method [11].

5.2.3 Enzyme solubilization and ammonium sulfate fractionation

The wet cell walls were suspended overnight in 6 L of 1 M NaCl. The ionically-bound enzymes were released and filtered from cell walls using a Buchner funnel as described above (see section 5.2.2). The residue was washed several times with a total volume of 1 L of 1 M NaCl. The filtrates were pooled, and solid ammonium sulfate was added with gentle stirring to 30% saturation. The suspension was left to settle overnight. The precipitate removed by centrifugation (10,000 x g, 20 min) and the supernatant was brought to 80% saturation by the addition of solid ammonium sulfate. After standing overnight, the yellowish-white precipitate was collected by centrifugation for 20 min at 10,000 x g, dissolved in 100 mL of 1 M NaCl and dialysed against 4 L of Milli-Q water with one change. The precipitate that formed

was removed by centrifugation at 17,000 x g for 20 min and the supernatant was freeze dried at 5°C. The yield of crude enzyme powder was 1.60 g.

5.2.4 Gel filtration

The lyophilized crude enzyme powder, 1.60 g, was dissolved in 16 mL of 10 mM Tris-HCl buffer, pH 7.1, centrifuged at 10,000 x g for 15 min, and the supernatant applied to a 2.5 x 100 cm column of Sephadex G-200 which was equilibrated with the same buffer. The column was eluted at a flow rate of 0.1 mL/min, and 1.5 mL fractions were collected.

5.2.5 DEAE-Sephadex A-50 (I)

Fractions 231-320 from the Sephadex G 200 column were pooled and dialysed against 2 x 4 L of 10 mM Tris-acetate buffer pH 7.5, and concentrated to 118 mL in an ultra-filtration cell equipped with a PM-10 membrane. The concentrated solution was applied to a 2.5 x 20 cm column of DEAE-Sephadex A-50 equilibrated with 10 mM Tris-acetate buffer, pH 7.5. The column was eluted in a stepwise fashion, using 180 mL buffer containing 0.1 M NaCl, then increasing salt in 0.1 M increments up to 0.5 M, at a flow rate of 0.45 mL/min; 10 mL fractions were collected.

5.2.6 DEAE-Sephadex A-50 (II)

Fractions 49 54, which eluted at 0.2 M NaCl from the first DEAE Sephadex A-50 column, were pooled, concentrated by ultrafiltration to about 6 mL and 1.5 mL of this enzyme solution was rechromatographed on a 2.5 x 20 cm DEAE-Sephadex A-50 column which was equilibrated with 10 mM Tris acetate buffer, pH 7.5, containing 0.15 M NaCl. Elution was carried out isocratically with the same buffer solution, at a flow rate of 0.45 mL/min, and 5 mL fractions were collected.

5.2.7 Standard enzyme assay

Nuclease activity was determined by a modification of the previously described method [9]. Incubation mixtures (0.8 mL, containing 0.3 mL enzyme solution and 0.5 mL of yeast RNA solution, 0.8 mg/mL Tris-HCl buffer of 0.1 M, pH 7.5), were incubated at 70°C for 30 min. Reaction was terminated by immersing the mixture in an ice bath and adding 0.16 mL of 3 M perchloric acid (PCA). The unhydrolysed RNA and protein which precipitated were removed by centrifugation at 7,000 x g for 10 min. The supernatant solution, which contained the nucleotide reaction products, was pipetted into a centrifuge tube, mixed with an equal volume of 0.5 M Freon-octylamine and subjected to HPLC analysis on a Whatman Partisil 10 SAX column as previously described [9,12].

Mixtures of nucleotide standards of known concentration were extracted in duplicate using PCA and Freon-octylamine in the same manner as employed for the nuclease assay. The standard extracts were injected into the HPLC system and the recovery was estimated by comparison with the standard solutions injected directly into the HPLC system. Peak heights were linear for nucleotides in the concentration range of 0.5 to 5 μg/mL in this HPLC system [12]. The nucleotides produced after enzyme incubations were quantitated by comparison of their peak heights with the average of peak heights of standards injected before and after the sample. One unit of nuclease activity is defined as the amount of enzyme producing one nanomole of 5'-GMP per minute from yeast RNA.

For RNase assays, incubation mixtures (0.7 mL; containing 0.3 mL of enzyme solution and 0.4 mL of yeast RNA, 1 mg/mL, dissolved in 0.1 M potassium phosphate buffer, pH 5.50) were incubated at 65°C for 30 min. Reaction was terminated by adding 0.1 mL of 8 mM zinc chloride solution and boiling the incubation mixture for 15 min. RNase activity was determined by monitoring 2':3'-cyclic GMP formation by HPLC with a Partisil 10 SAX column as described previously [9]. One unit of RNase activity is defined as the amount of enzyme producing one nanomole of 2':3'-cyclic GMP per minute.

Protein concentrations were estimated using a Bio-Rad protein assay which was based on the Bradford method [13],

using bovine serum albumin as a standard. Specific activity of enzymes was expressed as units per milligram of protein.

5.2.8 Substrate specificity

Calf thymus DNA, yeast RNA, polyA, polyC, polyG and polyU (400 μg) were used as substrates under the standard assay conditions. The nuclease substrate specificities were established by measuring the formation of 5'-dGMP from DNA, 5'-GMP from RNA and the corresponding 5'-nucleotides from the homopolymers. The 2':3'-cyclic GMP from RNA and the corresponding 2':3'-cyclic nucleotides from the homopolymers were measured to follow the action of RNase on these substrates.

5.2.9 Kinetic studies

Steady-state kinetic studies for hydrolysis of yeast RNA by cell wall nuclease were determined by quantitative analysis of the 5'-GMP formed for various concentrations of yeast RNA. Measurements for RNase were carried out using two substrates, yeast RNA and polyA. In this case, 2':3'-cyclic GMP and 2':3'-cyclic AMP were quantitated as the RNase reaction end products. The Michaelis-Menten parameters V_{max} and K_m were estimated using a computer program based on the method described by Wilkinson [14].

5.2.10 Polyacrylamide gel electrophoresis

SDS-PAG electrophoresis was carried out using an SE-200 small slab cell (Hoefer Scientific Instruments, San Francisco, CA), casting discontinuous gels consisting of a stacking and resolving gel and applying the discontinuous buffer system of Laemmli [15]. Protein standard markers covering a molecular weight range of 14.2 (α-lactalbumin, bovine milk) to 45.0 (egg albumin) kdal were supplied by Sigma.

5.3 Results

5.3.1 Effects of extraction procedure on nucleotide recovery

Table 5.1 demonstrates quantitative recovery for all nucleotides examined under the same conditions of extraction and HPLC analysis used in the enzyme assays. This ensures that the nucleotides produced by the nuclease were not lost in the extraction step. The use of PCA exhibits some advantages in HPLC assay of enzyme activity. First, the reaction was stopped by addition of PCA. Second, the undigested polynucleotide and protein were precipitated and removed. Only acid-soluble nucleotides were extracted and injected into the HPLC. This enables the HPLC column lifetime to be prolonged as these negatively-charged long-chain polymers are not retained.

Table 5.1: Recovery of Nucleotides using Freon-Octylamine Extraction (Average of two Extracts)

Nucleotide	Amount added (µg/ml)	Amount Found (µg/ml)	Recovery (%)
2'-AMP	0.4	0.39	98
2'-GMP	1.0	1.01	101
3'-AMP	0.4	0.38	96
3'-GMP	2.0	1.94	97
5'-AMP	2.0	1.94	97
5'-CMP	2.0	1.96	98
5'-GMP	2.0	1.92	96
5'-UMP	2.0	2.02	101

5.3.2 Enzyme purification

The elution profile of the Sephadex G-200 column is shown in Figure 5.1. Different fractions were pooled and assayed for nuclease and RNase activity. In all fractions, nuclease and cyclizing RNase activities, as well as nucleotidase and nucleosidase activities, can be estimated by measuring the various end products, i.e. nucleotides, nucleosides or bases appearing on the HPLC chromatograms from SAX (nucleotides) or SCX (nucleosides and bases) columns. Some of these results are presented in Tables 5.2 and 5.3. Further purification and characterization of enzymes in fractions 231-320 (Figure 5.1) was carried out for comparision with the low molecular weight cytoplasmic enzymes described previously [9].

The DEAE-Sephadex A-50 chromatographic separation of the pooled fractions from the G-200 column gave four peaks (Figure 5.2). HPLC assay of the enzyme fractions showed low, non-base specific nuclease and cyclizing RNase activity in peak I. There was negligible nuclease and RNase activity in peaks II and III. The 5'-nucleotide-forming nuclease with preferential specificity for purine bases was found in peak IV, along with RNase activity. Several chromatographic techniques were attempted to separate the nuclease and RNase activities, including gel filtration (Sephadex G-75) and cation-exchange chromatography (phosphocellulose and Sephadex CM-50). These did not effectively separate the enzymes. Peak IV was therefore rechromatographed on a DEAE-

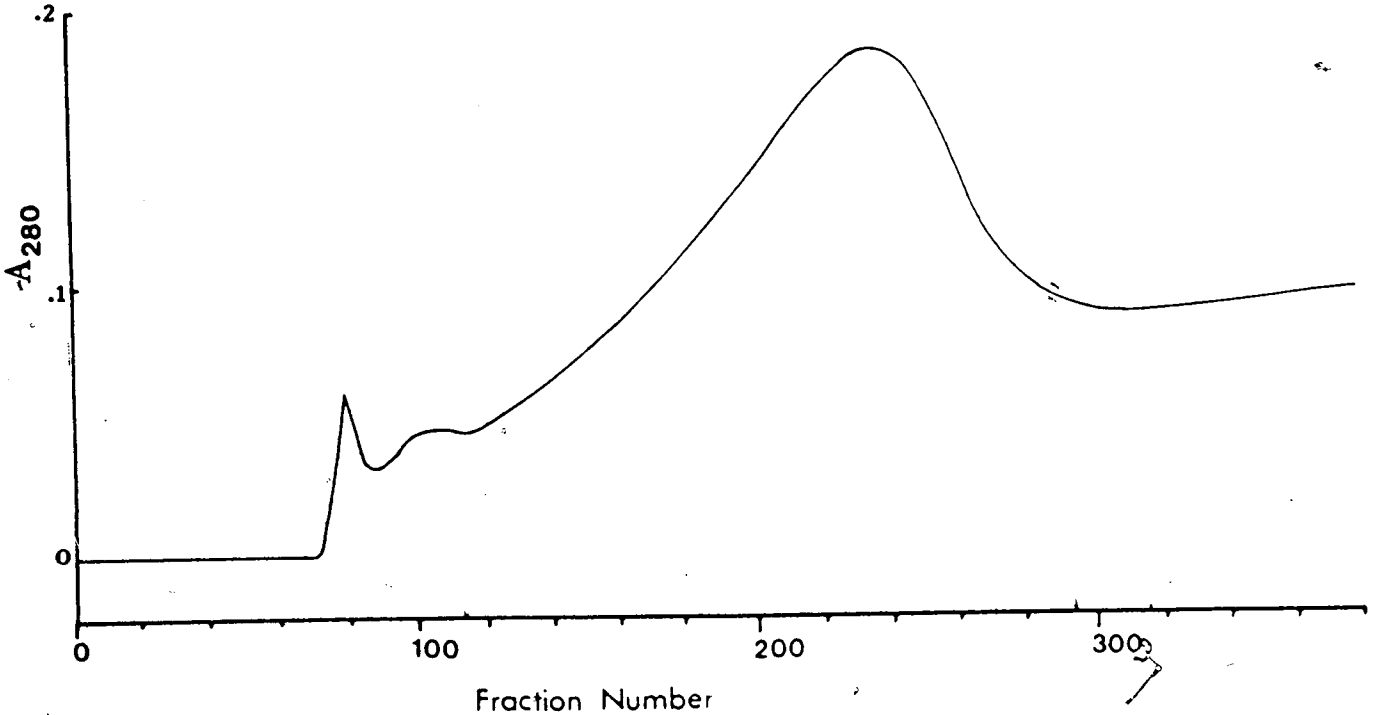

Figure 5.1. Elution profile of cell wall bound enzymes from a Sephadex G-200 column. The column (100 x 2.5 cm) was eluted with 10 mM Tris-HCl buffer (pH 7.1) with a flow rate of 0.1 mL/min; volume collected was 1.5 mL/fraction.

Table 5.2: Enzyme Activities as shown by End Products (Nucleotides, nmoles) detected by HPLC System [a]

Compound	Unheated fractions						Heated fractions					
	75	105	140	190	230	255	75	105	140	190	230	255
Nucleotide:												
5'-AMP	1.89	2.13	3.48	19.8	2.28	1.44	9.92	8.36	3.96	10.2	13.4	2.76
5'-CMP	0.46	1.67	4.19	20.8	5.22	3.48	nd [b]	1.39	0.54	2.45	2.63	3.56
5'-GMP	1.98	2.30	3.89	27.6	1.66	1.26	9.07	7.63	3.89	9.14	11.4	3.06
5'-UMP	0.87	1.68	2.57	16.9	2.05	0.76	3.26	3.06	1.24	3.33	4.30	1.21
3'-AMP	nd	2.59	2.61	0.36	1.67	5.31	nd	0.03	nd	nd	2.30	5.27
2'-GMP	nd	nd	0.10	2.96	1.02	nd	nd	nd	nd	nd	nd	nd
3'-GMP	0.80	3.67	10.0	1.36	10.3	13.0	nd	nd	nd	nd	nd	11.3
2':3'-cyclic Nucleotide:												
AMP	4.09	4.16	11.4	14.9	88.2	60.6	6.13	5.16	4.19	10.5	11.9	40.3
GMP	0.96	0.75	2.36	9.77	46.8	33.8	nd	nd	nd	nd	1.24	17.9

[a] From fractions collected on Sephadex G-200 column, a 0.5mL aliquot was transferred to a test tube and heated in a water bath at 73°C for 30 min. This provided heat-treated enzyme for comparision of the activities with an unheated sample.
For enzyme activity assay, 1.0mL of yeast RNA (0.4 mg/mL in 0.1M tris-HCl buffer pH 6.50) was added to 0.5mL of heated / unheated enzyme solution and then incubated at 52°C for 10 min.

[b] nd: Not detected.

Table 5.3: Enzyme Activities as shown by End Products (Nucleosides and Bases, nmoles) detected by HPLC System [a]

Compounds	Unheated fractions						Heated fractions					
	75	105	140	190	230	255	75	105	140	190	230	255
Nucleoside:												
Adenosine	2.27	1.26	12.9	21.8	6.38	1.63	2.09	2.08	0.29	2.12	2.52	2.56
Cytidine	0.58	0.56	3.16	5.05	2.53	0.89	0.73	1.25	0.22	0.89	0.76	0.89
Guanosine	12.7	nd [b]	81.7	183	70.8	43.4	15.6	13.9	10.8	19.0	28.1	53.2
Uridine	30.9	nd	97.4	133	168	145	59.4	53.9	25.5	64.9	102	123
Base:												
Adenine	nd	0.09	10.3	17.0	0.88	0.17	nd	nd	nd	nd	nd	nd
Cytosine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Guanine	22.9	23.4	108	163	95.2	55.1	41.5	38.8	20.9	45.7	53.0	59.7
Uracil	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

[a] See Table 5.2 for enzyme assay procedure.

[b] nd: Not detected.

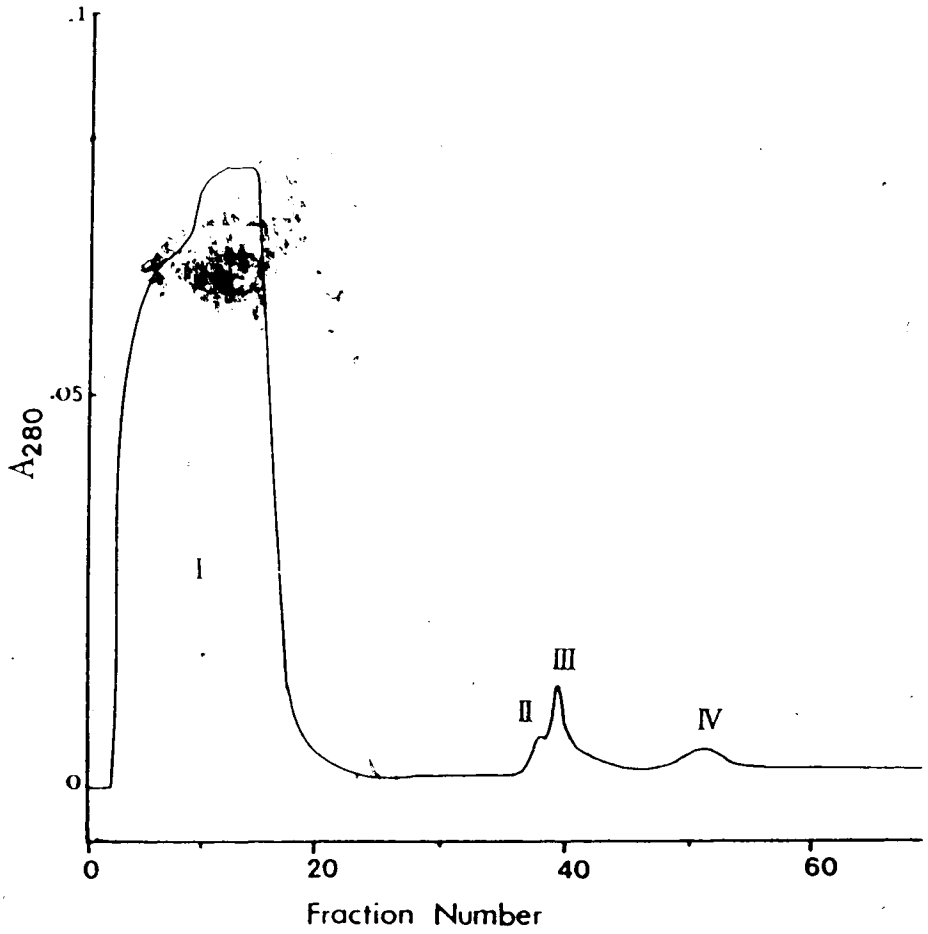

Figure 5.2. DEAE-Sephadex A-50 column chromatography of pooled fractions 231-320 collected from a preliminary purification on a Sephadex G-200 column. Enzyme solution loaded on the column was separated by using isocratic mode and step-wise gradient starting with 10 mM Tris-acetate buffer (pH 7.5); increments of 0.1 M NaCl were applied at every 180 mL up to 0.5 M NaCl. The elution was monitored at 280 nm at a flow rate of 0.45 mL/min. A volume of 10 mL per fraction was collected.

Sephadex A-50 column. The nuclease activity eluted predominantly in fractions 24-29 and the RNase activity started in fraction 30 (Figures 5.3 and 5.4). Fractions 24-29 were pooled and used for subsequent nuclease characterizations, while fractions 37-50 were pooled and used for RNase characterizations. The results of the overall purification steps for nuclease enzyme are summarized in Table 5.4.

5.3.3 Properties of cell wall nucleases

5.3.3.1 Molecular weight

SDS-PAGE of pooled fractions 49-54 (peak IV) of the first DEAE-Sephadex A-50 column showed two protein bands, corresponding to the nuclease and RNase enzymes. The molecular weights of these proteins were estimated to be 37.6 and 36.3 kdal (Figure 5.5). The lower molecular weight protein band corresponded to that of the RNase band of pooled fractions 37-50 of the second DEAE-Sephadex A-50 column which was concentrated and run in the same gel system.

5.3.3.2 Effect of pH and temperature

The nuclease exhibits a broad pH optimum, from 6.5 to 7.5. The pH optimum of the RNase was 4.5 (Figure 5.6). The temperature activity curves of the cell wall enzymes are shown in Figure 5.7. The temperature optimum of the RNase was 65°C, that for nuclease was 70°C. Although, at these

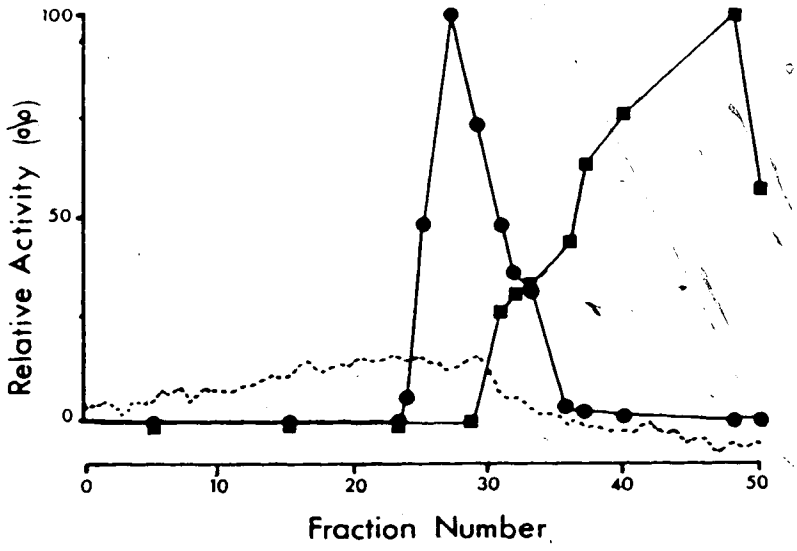

Figure 5.3. Additional DEAE-Sephadex A-50 column chromatography of pooled fractions 49-54 collected after purification on DEAE-Sephadex A-50 column (I). The enzymes were separated isocratically using 0.15 M NaCl in 10 mM Tris-acetate buffer (pH 7.5) monitored at 280 nm with a flow rate of 0.45 mL/min, collecting 5 mL/fraction. (•), nuclease activity; (■), RNase activity, and (...), A_{280}.

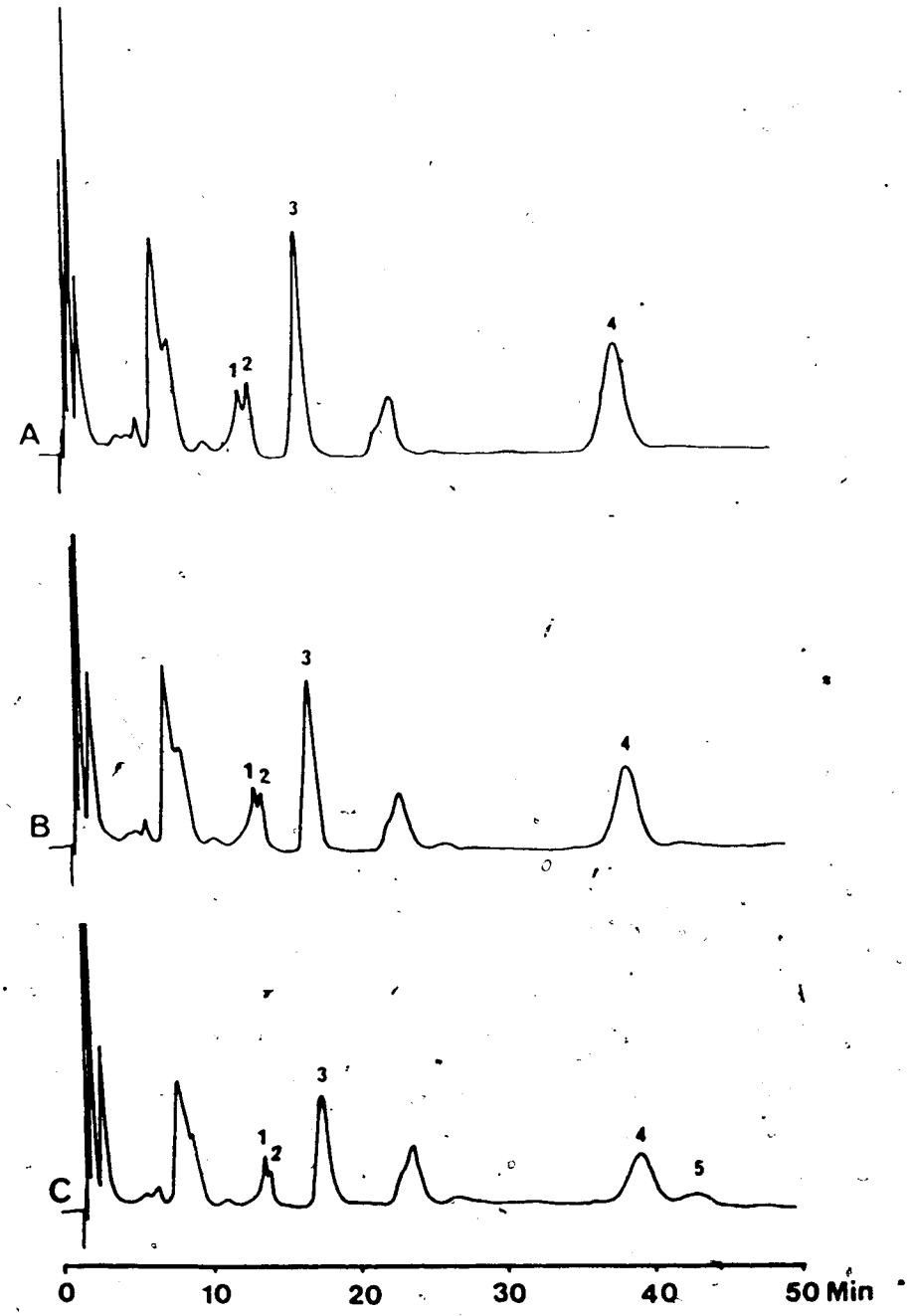

Figure 5.4. Liquid chromatograms of assay solution with the enzymes recovered from fractions 27 (A), 29 (B) and 31 (C) after the second purification on a DEAE-Sephadex A-50 column. Peak identification: 1 = 5'-CMP; 2 = 5'-UMP; 3 = 5'-AMP; 4 = 5'-GMP; and 5 = 3'-GMP. The purity of nuclease activity in fractions 24-29 is demonstrated by the absence of 3'- and 2'-GMP on A and B chromatograms.

Table 5.4 Purification of Cell Wall Bound Potato Tuber Nuclease

Step	Total protein (mg)	Specific activity (units / mg protein)	Total units
Crude extract	82.27		a
Ammonium sulfate, 80% sat	73.41		a
Sephadex G-200	24.97	2.40	60
DEAE-Sephadex A 50 (I)	0.106	399	42
DEAE-Sephadex A 50 (II)	0.008	3290	26

[a] Nuclease units could not be accurately estimated since these fractions contained phosphomonoesterases, nucleosidases and even oxo-nucleases (i.e. high molecular weight nucleases, around 70 - 100 kdal).

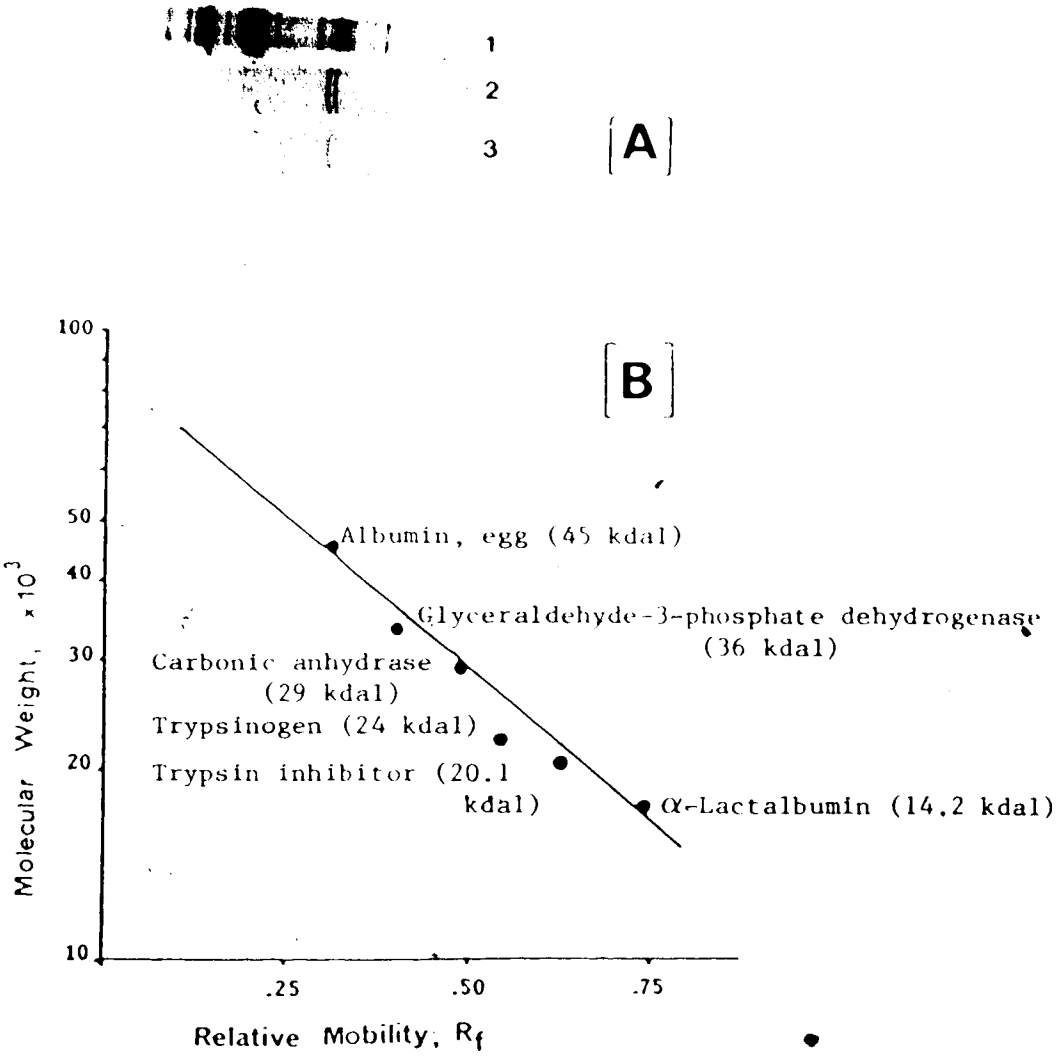

Figure 5.5. SDS-PAGE used for determination of the purity and molecular weight of cell wall bound nuclease and RNase. A: gel separation band of pooled fractions of 1) 231-320 Sephadex G-200 column; 2) 49-54 from DEAE-Sephadex A-50 column I and 3) 37-50 from DEAE-Sephadex A-50 column II. B: standard curve (semilog plot of relative mobility vs molecular weight of protein markers) used for determination of molecular weights of nuclease and RNase. Protein standards were run simultaneously with cell wall bound enzymes.

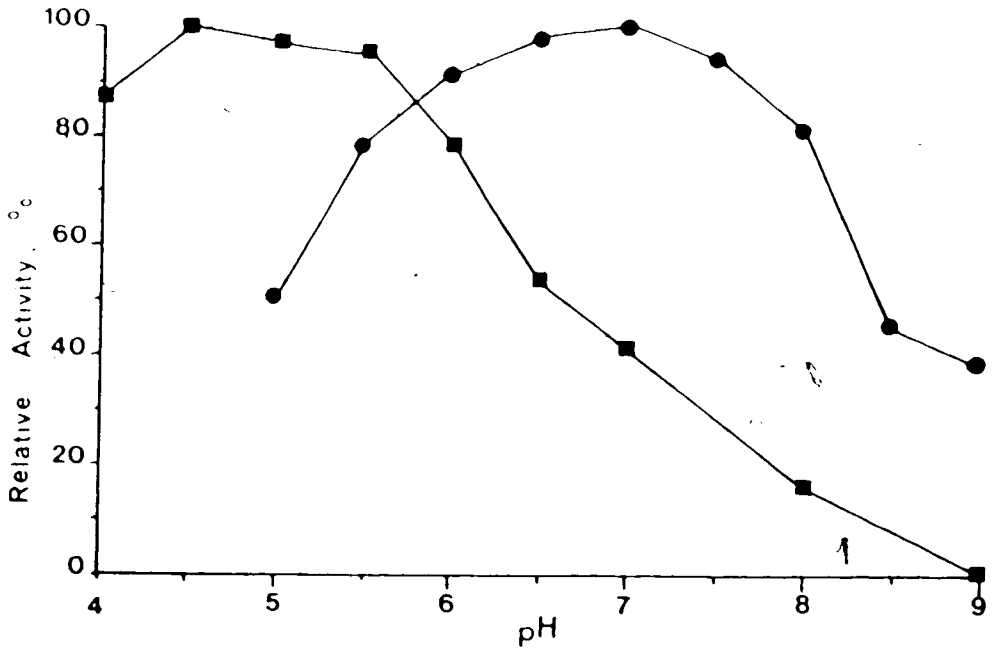

Figure 5.6. Effect of pH on the activity of cell wall bound nuclease (•) and RNase (■). The buffer solutions used were: 0.1 M sodium acetate, pH 4.0-6.5 for nuclease and 0.1 M potassium phosphate, pH 4.0-6.5 for RNase activity assay, respectively. In the range of pH 7.0-9.0, both enzymes were assayed in 0.1 M Tris-HCl buffer.

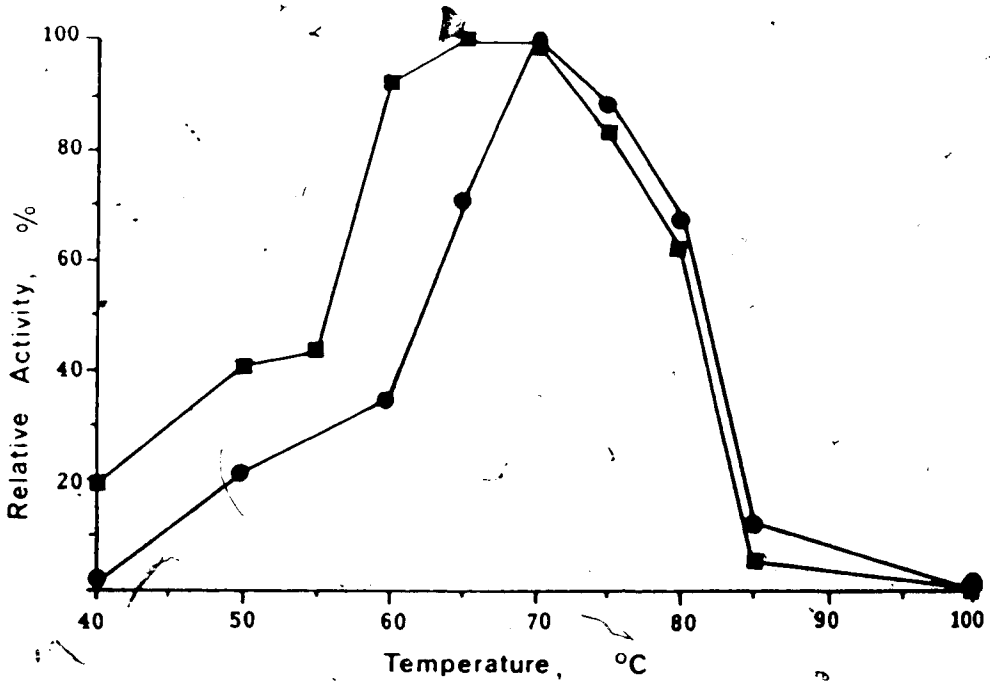

Figure 5.7. Effect of temperature on the activity of cell wall bound nuclease (•) and RNase (■).

temperatures, the enzymes might exist as an equilibrium between the native and reversibly/irreversibly denatured forms, a lower temperature was not used for routine analysis. As exemplified in Figure 5.7, at 40°C there is a five-fold decrease in RNase activity, whereas nuclease activity is close to zero. The choice of using high optimal temperatures was favored since they are close to those of a blanching step used in potato processing.

5.3.3.3 Inhibitors

The effects of a variety of food additives and heavy metal ions are presented in Table 5.5. Zn^{++} and Cu^{++} are strong inhibitors of both the nuclease and RNase, while Mg^{++} and glutamate at 10 mM did not show significant effect on either enzyme. Their effect on enzyme activities at higher concentrations warrants further study. Citrate, phosphate, Mn^{++} and EDTA inhibited the nuclease. However, the RNase activity was stimulated strongly by phosphate, Mn^{++} and EDTA, while citrate did not show a significant effect on this enzyme. Of the other food additives, pyrophosphate completely destroyed the nuclease activity, while 45% of the RNase activity was lost in the presence of the same amount of this anion; nitrate and sulfite did not affect the nuclease activity but both inhibited RNase.

5.3.3.4 Substrate specificity

The purine ribonucleoside-5'-monophosphates 5'-AMP and 5'-GMP are the major products of the cell wall nuclease

Table 5.5: Effect of Various Ions and EDTA on Nuclease and RNase Activity [a]

Additive	Concentration (mM)	Relative Activity (%)	
		Nuclease	RNase
Control (none)	—	100	100
Na-glutamate	10	82	103
Na-citrate	10	21	95
$NaNO_3$	10	104	74
$NaHSO_3$	10	95	39
NaH_2PO_4	10	51	120
Na-Pyrophosphate	10	0	55
$CuSO_4$	1	0	0
$ZnCl_2$	1	0	0
$MnCl_2$	1	20	116
$MgCl_2$	1	92	108
EDTA	1	17	153

[a] The results are means of two replicates which did not differ by more than 2%.

Table 5.6: Action of Cell Wall Nuclease on Various Native and Homo-polynucleotides

Substrate	Product formed (5'-nucleotide)	Yield [a]		Relative activity (%) [b]
		nmole	%	
RNA	AMP	3.55	0.35	
	CMP	2.95	0.27	
	GMP	4.14	0.42	100
	UMP	1.22	0.11	
DNA	dAMP	0.99	0.09	
	dGMP	0.69	0.07	17
Poly A	AMP	2.76	0.27	67
Poly C	CMP	1.85	0.17	45
Poly G	GMP	0.95	0.10	23
Poly U	UMP	9.00	0.83	217

[a] The results are means of two replicates which did not differ by more than 2%.

[b] Percent based on the weight of the substrate used and the weight of nucleotide formed under the standard assay conditions.

hydrolysis of yeast RNA (Table 5.6). The percentage of the individual mononucleotides shown in Table 5.6 was calculated based on the amount of each nucleotide formed in the incubation mixture per initial weight of the polynucleotide used as a substrate. The cell wall nuclease hydrolysed DNA at a much slower rate than RNA. The rate of liberation of mononucleotides from their corresponding homopolymers is, in decreasing order, polyU, polyA, polyC and polyG. We attribute the difference in homopolymer hydrolysis rates relative to RNA to differences in the chain lengths of these substrates. It has been reported that the shorter the chain length of the homopolymer, the faster the rate of hydrolysis [16].

Results for cell wall bound RNase (Table 5.7) revealed that the rate of cyclic 2':3'-GMP formation is close to that of cell wall bound nuclease. The liberation of the mononucleotides from their homopolymers is, in decreasing order, polyU, polyA, polyC, and polyG, i.e. the same as for nuclease, but in molar yields 3-times less, except for polyU, which had a yield 3-times higher than for nuclease enzyme.

5.4 Discussion

Several different cell wall enzymes that degrade RNA could be detected by our HPLC assay method. The 5'-GMP-forming enzyme is the most important in flavor nucleotide research since 5'-GMP exhibits flavor-enhancing activity,

Table 5.7: Action of Cell Wall RNase on Various Native and Homo-polynucleotides

Substrate	Product formed (2':3'-cyclic nucleotide)	Yield [a]		Relative Activity (%) [b]
		nmole	%	
RNA	GMP	4.05	0.39	100
Poly A	AMP	1.62	0.15	40
Poly C	CMP	0.42	0.04	10
Poly G	GMP	0.30	0.03	7
Poly U	UMP	29.64	2.59	732

[a] The results were means of duplicate measurements which did not differ by more than 2%.

[b] Percent based on the weight of the substrate used and the weight of nucleotide formed under the standard assay conditions.

particularly synergistically with monosodium glutamate [17]. We have previously isolated and characterized a 5'-nucleotide-forming nuclease and an RNase which forms 2':3'-cyclic nucleotides from the cytoplasm of potato tubers [9]. In the present study, we have isolated and characterized a corresponding nuclease and RNase from the cell walls of potato tubers. In food processing it is desirable to optimize the activity of the nuclease and minimize that of RNase which competes for endogenous RNA.

The cell wall nuclease was found to be accompanied by 3'-nucleotidase activity (see Chapter 6). This has been confirmed for cytoplasmic nucleases in potato tubers [9,18], tea leaves [19], pea seeds [20] and other plant materials [21,22]. Both Zn^{++} and EDTA are strong inhibitors of the cytoplasmic [9] and cell wall enzymes.

Both cytoplasmic and cell wall bound enzymes exhibited optimal activity at a neutral pH, however, the cell wall enzyme showed a broad pH-activity curve (Figure 5.6). The temperature optimum of cell wall nuclease was lower than that of cytoplasmic nuclease (70°C and 80°C, respectively). The cell wall enzyme differed from the potato tuber cytoplasmic nuclease with respect to its molecular weight, 37.7 kdal (cell wall) and 34.2 kdal (cytoplasmic) (see section 4.3.2), and also substrate specificity. The Michaelis-Menten constants, K_m, of the cell wall and cytoplasmic nucleases were 60 µg and 40 µg, respectively, when yeast RNA was used as a substrate.

In a homogeneous form, without any contaminating RNase activity (fractions 24-29 of DEAE-Sephadex A-50 II), the nuclease activity was lost rapidly during storage at 4°C in Tris-acetate buffer pH 7.5. Under these conditions, the activity was lost after one week of storage.

The cell wall RNase is an acidic enzyme with pH optimum of 4.5, while that of cytoplasmic RNase is 5.5 [9]. The former exhibited a broad temperature optimum and its optimal temperature was higher than that of cytoplasmic enzyme, 65°C (cell wall) and 55°C (cytoplasmic). The molecular weights of the RNases were 36.2 kdal (cell wall) and 19.2 kdal (cytoplasmic). When yeast RNA was used as a substrate, the K_m's of cell wall and cytoplasmic RNase were 242 and 119 μg, respectively. Both RNases were promoted by EDTA and phosphate anion.

As mentioned above, the nuclease enzyme reported is just one of several classes of nucleases isolated from potato tuber cell walls. Since there are several common properties of plant nucleases from different sources [21], the properties of the cell wall nuclease are similar not only to the potato tuber cytoplasmic enzyme but also to all of the other cytoplasmic plant nucleases studied to date [9,18-22]. The differences in pH and temperature optima and substrate specificity as well as inhibition by a number of cations and/or anions suggest that the cell wall bound nuclease and the cytoplasmic nuclease [9] are two distinct enzyme species.

Thus, it can be assumed that cell wall nuclease is as important as cytoplasmic enzyme for production of 5'-GMP in potato processing. Leaching of 5'-GMP to the water during blanching (commonly used in manufacture of French fries), the occurrence and extent of which is supplemented by data in the Appendix (Chapter 10, Tables 10.6 and 10.7), partially results from the action of cell wall nuclease hydrolysing cell wall RNA.

5.5 Conclusion

Two different enzymes, a nuclease and a ribonuclease (RNase) have been isolated from the cell walls of potato tubers. For the nuclease, a purification of 1370 fold was achieved by fractionation with ammonium sulfate and chromatography on Sephadex G-200 and DEAE-Sephadex columns. The purified nuclease hydrolysed both RNA and DNA, yielding primarily purine 5'-nucleotides. The ribonuclease did not cleave DNA and the hydrolysis of RNA produced 2':3'-cyclic nucleotides as products. Both enzymes were completely inhibited by Cu^{++} and Zn^{++}, while EDTA was an inhibitor and promotor of the nuclease and RNase, respectively. The cell wall enzymes were different from the corresponding cytoplasmic enzymes previously characterized (Chapters 3 and 4) with respect to pH and temperature optima and molecular weights.

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6. DIFFERENCES BETWEEN CYTOPLASMIC AND CELL WALL BOUND 3'-NUCLEOTIDASE OF POTATO TUBERS'

6.1 Introduction

Enzymes which specifically hydrolyze the phosphoryl group of a 3' nucleotide are referred to as 3'-nucleotidases. These enzymes exist predominantly in the plant kingdom, and several have been identified from a variety of plants [1]. The 3'-nucleotidase activity has been found to be associated with proteins of plant nuclease [2] and 2':3' cyclic phosphate diesterase of microorganisms [3].

The earlier studies [4,5] on characterization of cytoplasmic nuclease isolated from potato tubers corroborated these reports. Since cell wall is a distinct plant component, the nucleases bound to cell wall of potato tubers have been isolated and studied for their properties in order to optimize the vegetable processing conditions [6]. The isolated cell wall nuclease also exhibits 3'-nucleotidase activity. This character differentiates it from that of cytoplasmic 3'-nucleotidase and is described in this chapter.

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[1]Presented at 30th Annual Conference of the Canadian Institute of Food Science and Technology (Hamilton, 1987): Biotechnology.

6.2 Materials and Methods

The isolation and purification of cytoplasmic and cell wall bound 3' nucleotidases essentially followed the procedure described in Chapters 3 and 5. Peeled potato tubers cv. Pontiac grown in Southern Alberta were used as the enzyme source. Two procedures were applied for preparation of enzymes. Cytoplasmic 3'-nucleotidase was prepared by homogenization, filtration and starch removal by gravity sedimentation, followed by gel filtration of the clarified supernatant on a Sephadex G-100 column using purified water as eluent [4] (Figure 6.1). The cell wall bound 3'-nucleotidase preparation included cell wall isolation by repeated blending and removal of starch by extensive washing, followed by filtration. The residue, free of starch and unbound protein, was suspended in 1 M NaCl overnight and filtered. The filtrate containing cell wall bound enzymes was fractionated by precipitation with 30-80% ammonium sulfate, followed by chromatography on a Sephadex G-200 column, ion-exchange chromatography on a DEAE-Sephadex A-50 column and, finally, rechromatography on a DEAE-Sephadex A-50 column [6]. The isolation and purification procedures are summarized in Figures 6.2 and 6.3.

The enzyme assay mixture (1.4 mL) consisted of 1 mL of 0.1 M sodium acetate buffer pH 6.50, 3'-AMP (0.2 mL of 2.5 mg/mL solution) and enzyme solution (cytoplasmic enzyme, 0.2 mL of a solution of 1 mg/mL in sodium acetate buffer 0.1 M, pH 6.50; cell wall bound enzyme, 0.2 mL of pooled fractions

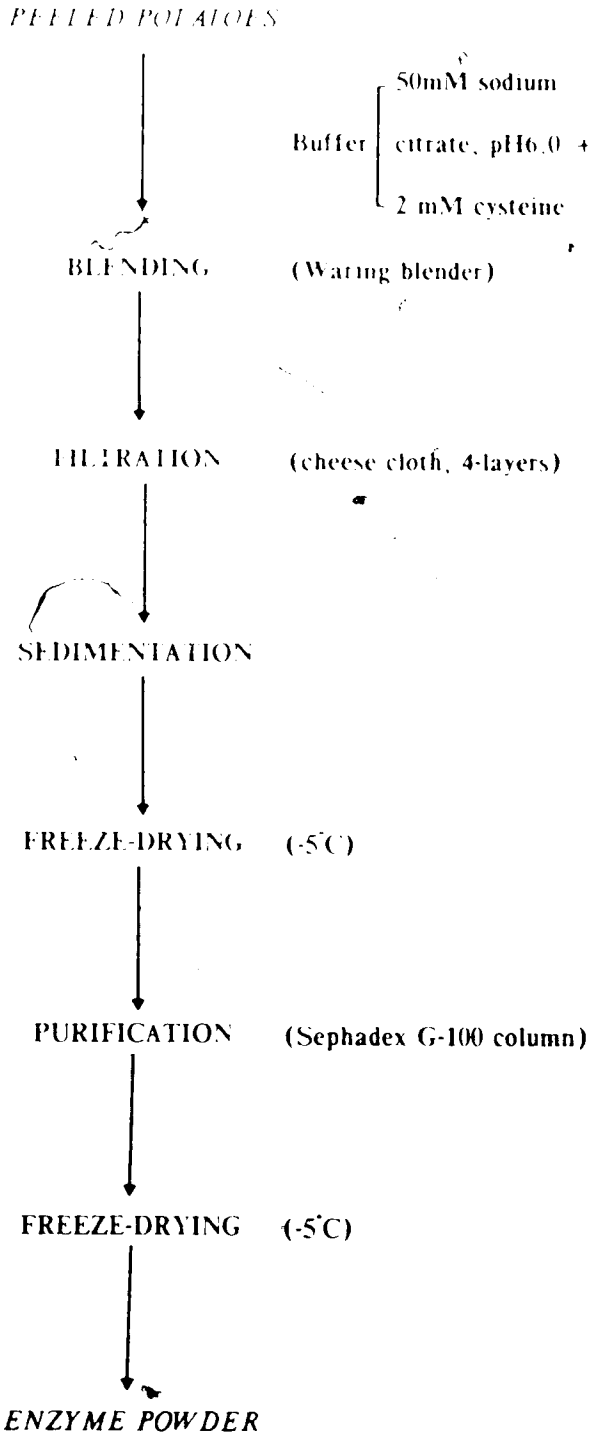

Figure 6.1. Flow diagram of isolation of cytoplasmic enzymes from potato tubers.

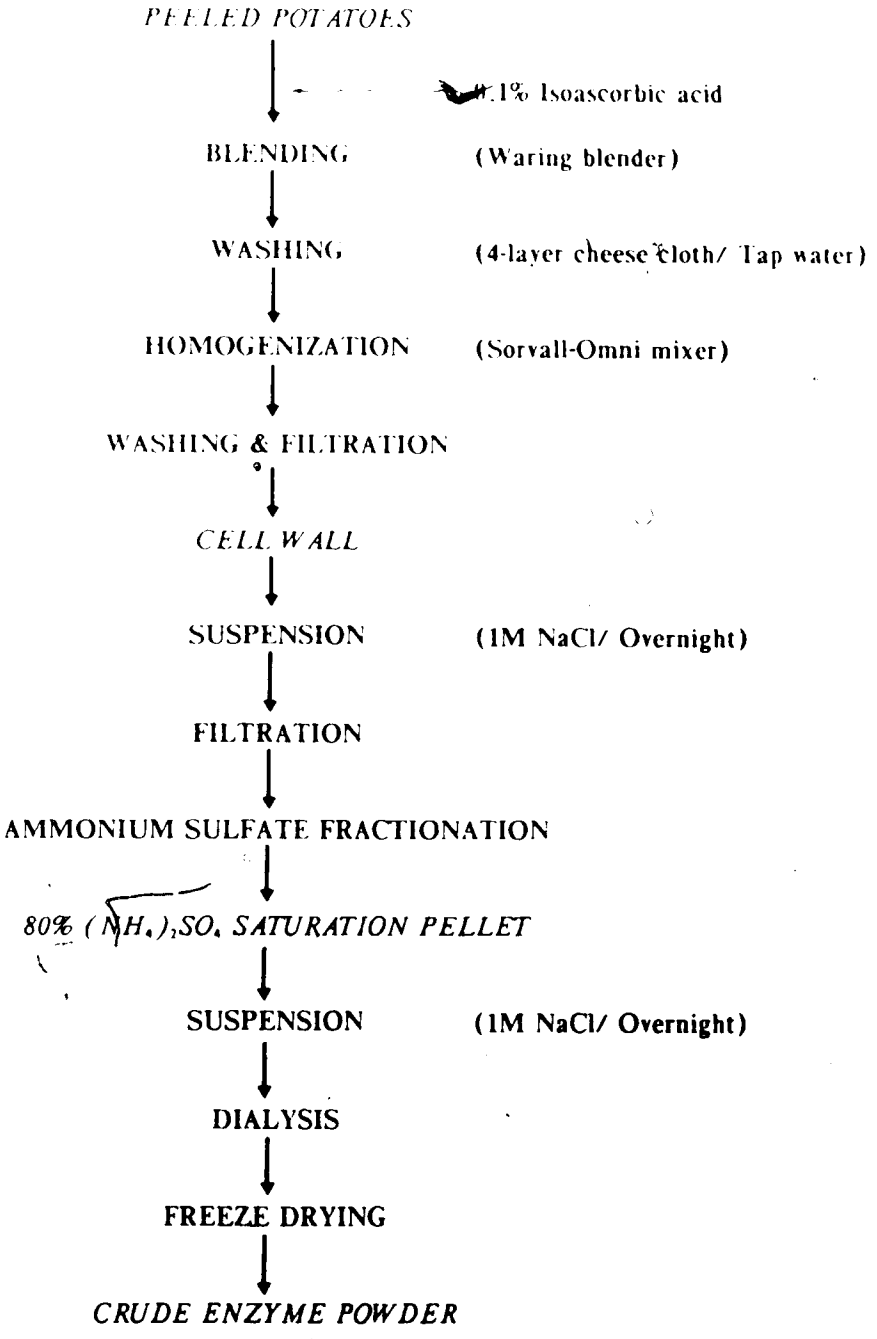

Figure 6.2. Flow diagram of isolation of cell-wall enzymes from potato tubers.

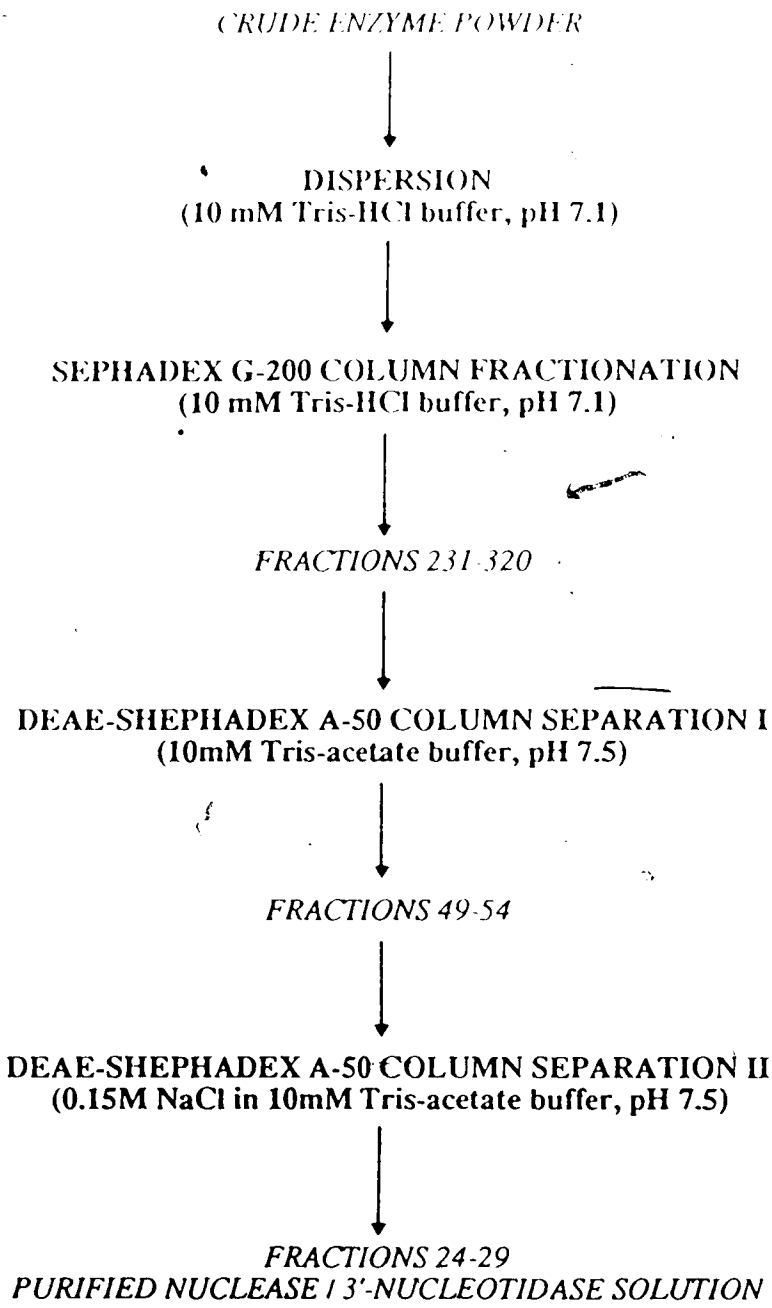

Figure 6.3. Flow diagram of purification of cell wall crude enzymes from potato tubers.

24-29 from DEAE-Sephadex A-50 column II). Following incubation at 70°C for 10 min, the reaction was terminated by boiling the mixture for 12 min. Then the mixture was diluted, centrifuged at 5,000 x g for 10 min, and an aliquot of 20-50 μL clear supernatant injected into the HPLC system. One enzyme unit was defined as an amount of 3'-nucleotidase producing one nanomole of adenosine from 3'-AMP per minute.

Assays of 3'-nucleotidases were carried out by using an isocratic HPLC method [5] in which a Whatman SCX column and 10 mM potassium dihydrogen phosphate solution adjusted to pH 3.60 by addition of phosphoric acid were used in separation of the substrate (3'-AMP) and the product (adenosine). The optimal conditions for 3'-nucleotidase assay were reported previously [5] and the procedure is summarized diagrammatically in Figure 6.4. Nucleotide substrates and nucleoside products were monitored by UV detection at 254 nm and quantitated electronically using a digital integrator.

6.3 Results and Discussion

Figure 6.5 illustrates a typical chromatogram of incubated enzyme mixture.

The optimal pH and temperature for 3'-nucleotidase activities are shown in Figures 6.6 and 6.7, respectively. Both cytoplasmic and cell wall bound 3'-nucleotidases had the same pH optimum (6.50). Both enzymes exhibited high optimal temperature activity (65-75°C), although the cytoplasmic 3'-nucleotidase possessed slightly higher

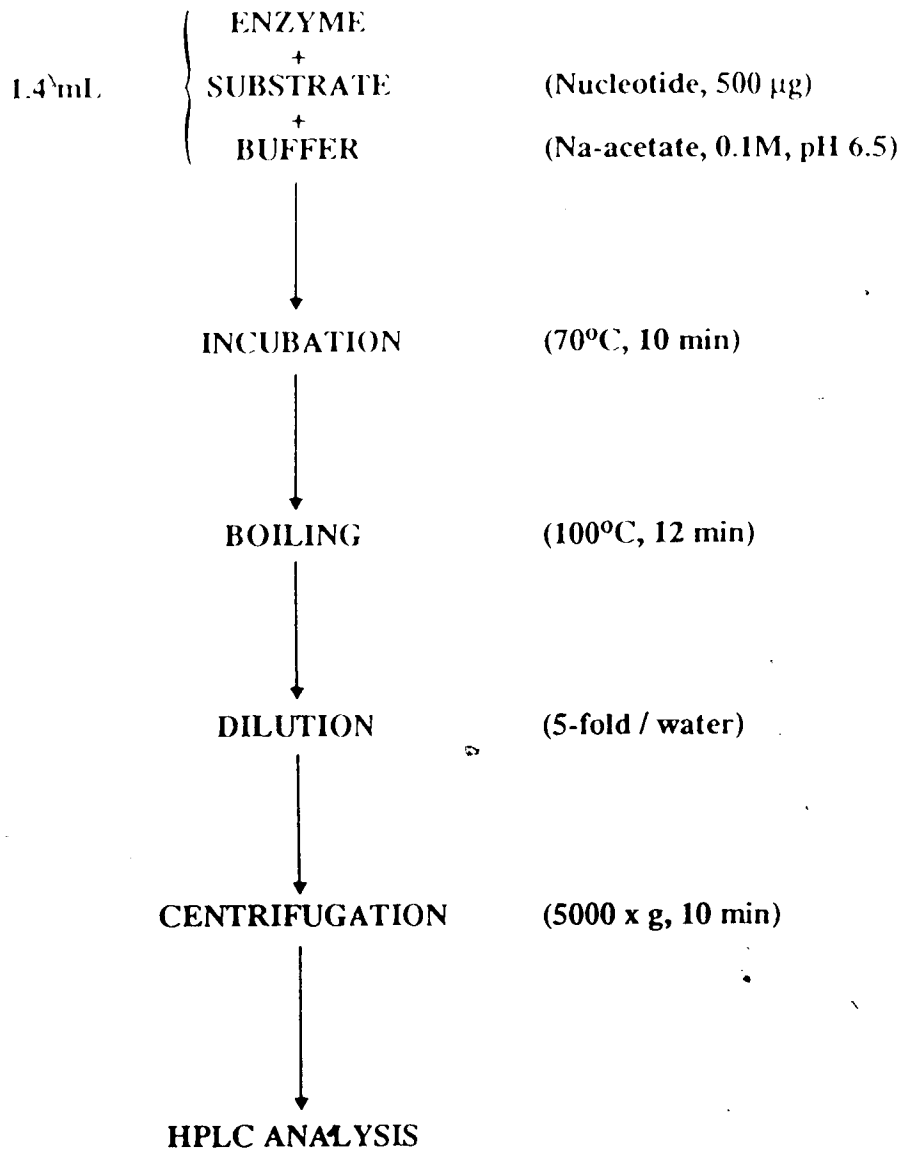

Figure 6.4. Optimal conditions for assay of 3'-nucleotidases.

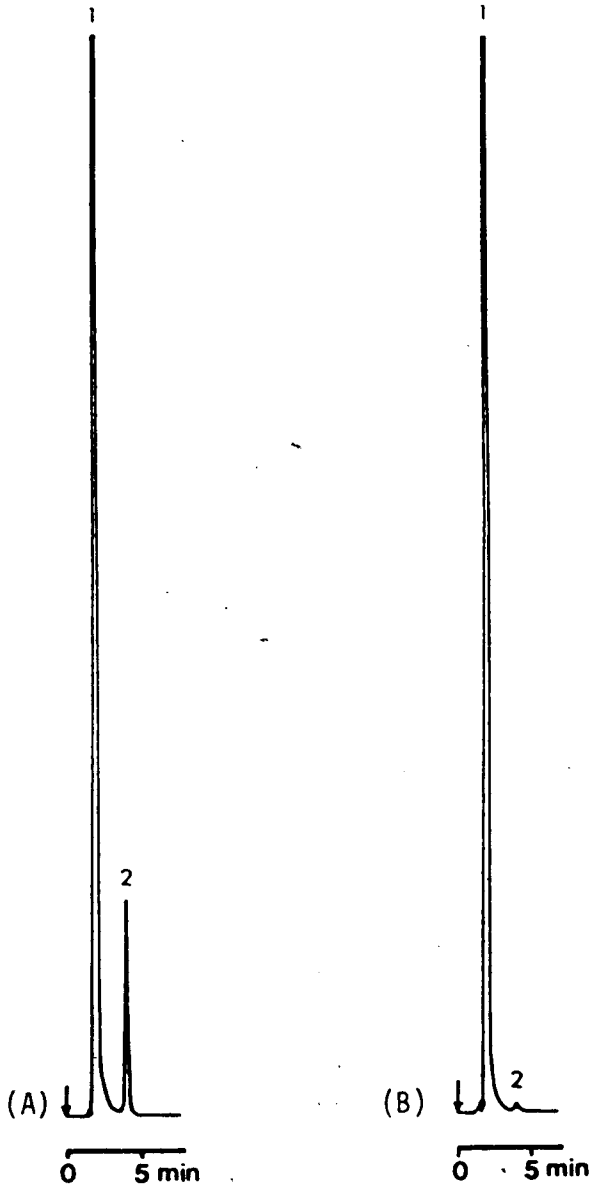

Figure 6.5. Typical chromatograms of the cell wall 3'-nucleotidase, the assay mixture (A) and blank (B). Peak designation: 1 = 3'-AMP; 2 = adenosine.

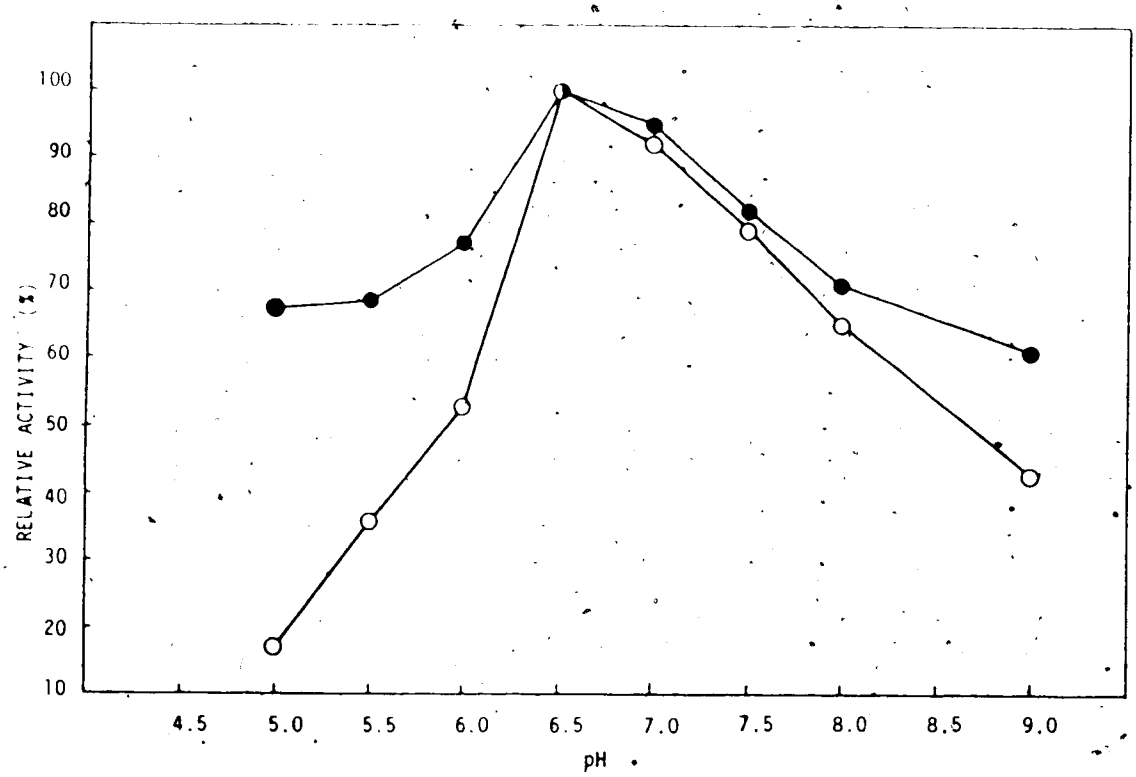

Figure 6.6. The pH activity curves of cytoplasmic (•) and cell wall (○) 3'-nucleotidases from potato tubers. Buffers used were: 0.1 M sodium acetate (pH 5.0-6.5); and 0.1 M Tris-HCl (pH 7.0-9.0).

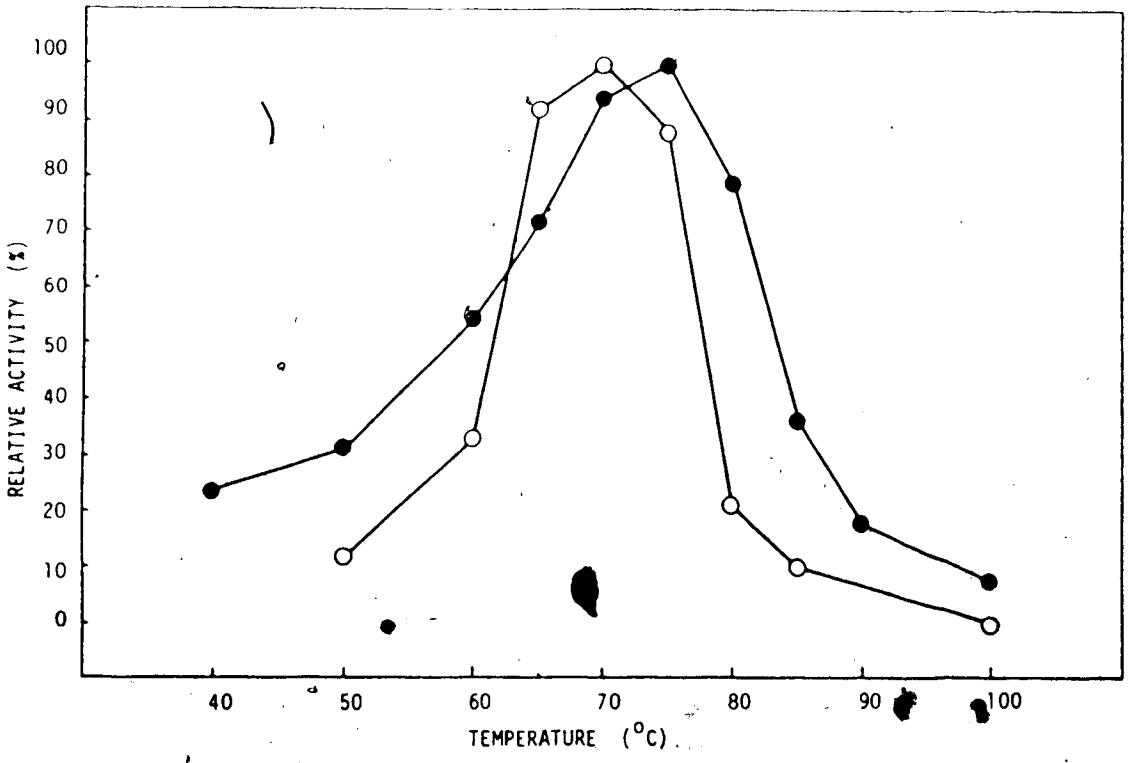

Figure 6.7. The temperature activity curves of cytoplasmic (•) and cell wall (○) 3'-nucleotidases from potato tubers.

temperature activity than the enzyme bound to cell wall.

The effects of some common food additives, metal ions and a chelating agent (EDTA) are shown in Table 6.1. Inhibitors of cell wall 3'-nucleotidase were citrate, sulfite, phosphate and pyrophosphate anions, whereas sulfite enhanced the activity of cytoplasmic enzyme. The flavor enhancer monosodium glutamate promoted both enzyme activities. Glutamate, at a level of 10 mM, increased the cytoplasmic and cellwall 3'-nucleotidase activities to 115 and 125% of control values, respectively. Manganese cation was an effective inhibitor of the cell wall enzyme. In the presence of 1 mM $MnCl_2$, 40% activity was lost, whereas at the same concentration the cytoplasmic enzyme lost only 10% activity. Magnesium ion strongly enhanced the activity of cell wall 3'-nucleotidase. Thus, with 1 mM $MgCl_2$ its activity increased to 173% of the control value. The chelating agent EDTA, as well as heavy metal ions such as zinc and copper, were strong inhibitors of both cytoplasmic and cell wall 3'-nucleotidases.

Of the 17 nucleotides tested as substrates, 3'-AMP was hydrolyzed most rapidly by both enzymes (Table 6.2). The relative rate of hydrolysis of 3'-ribonucleotides by the cytoplasmic enzyme was 3'-AMP > 3'-UMP > 3'-GMP > 3'-CMP, and that of the cell wall bound enzyme 3'-AMP > 3'-GMP > 3'-UMP > 3'-CMP. The cell wall bound 3'-nucleotidase exhibited specificity for purine nucleotides. It was devoid of activity for 2'- and 5'-ribonucleotides. On the other

Table 6.1: Effect of Some Anions, Cations, and EDTA on the Activity of Potato Tuber 3'-Nucleotidases

Additive	Concentration (mM)	Relative activity (%)[a]	
		Cytoplasmic	Cell wall bound
Na-Citrate	10	—	44
Na-Nitrate	10	—	116
$NaHSO_3$	10	151	50
NaH_2PO_4	10	53	69
Na-Pyrophosphate	10	—	1
Na-Glutamate	10	115	125
NaCl	10	98	—
LiCl	10	100	—
$MnCl_2$	1	90	60
$MgCl_2$	1	106	173
$ZnCl_2$	1	10	3
$CuSO_4$	1	—	3
EDTA	1	25	30
Control[b]	—	100	100

[a] The data are averages of two determinations which did not differ by more than 2%.

[b] Incubation mixture, 1.4 ml in all tests, containing 500µg of 3'-AMP as substrate, purified enzyme solution, assay compound, all in 100 mM Na-acetate / acetic acid solution pH 6.50. The result is expressed as a percentage of the amount of adenosine formed in the control assay, which consisted of enzyme substrate and buffer alone.

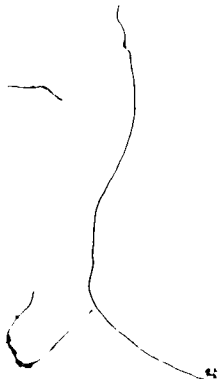

Table 6.2: Action of Potato Tuber 3'-Nucleotidases on Various Nucleotides

Substrate		Relative activity % [a]	
Type	Nucleotide	Cytoplasmic	Cell wall bound
Ribonucleotides	2'-AMP	3.1	0.0
	2'-CMP	0.0	0.0
	2'-GMP	5.3	0.0
	2'-UMP	1.1	0.0
	3'-AMP	100.0	100.0
	3'-CMP	23.5	3.0
	3'-GMP	42.3	32.7
	3'-UMP	54.2	25.2
	5'-AMP	3.1	0.0
	5'-CMP	0.7	0.0
	5'-GMP	1.7	0.0
	5'-UMP	1.3	0.0
Deoxyribonucleotides	3'-dAMP	13.3	—
	5'-dAMP	6.8	—
	5'-dCMP	3.3	—
	5'-dGMP	2.4	—
	5'-dTMP	1.4	—

[a] Calculation is based on the amount of the corresponding nucleoside, in nanomoles, formed from 500 µg of a given nucleotide at 70°C for 10 min, while the result is expressed as percentage of the amount of adenosine formed in the enzyme-catalyzed reaction with 3'-AMP as a substrate. The data are averages of two determinations which did not differ by more than 2%.

hand, the cytoplasmic enzyme showed activities on 2'- and 5'-ribonucleotides and 3'- and 5'-deoxyribonucleotides. This might be attributed to other phosphomonoesterases present in our cytoplasmic enzyme preparation.

Both the cytoplasmic and cell wall bound potato tuber 3'-nucleotidase activities have been associated with the nuclease activity, namely the ability to hydrolyze RNA and DNA [4,6]. It was suggested that the two activities (3'-nucleotidase and nuclease) reside in the same protein [7] as was found in many other plant nucleases [2,8,9]. Their molecular weights were 33.4 kdal (cytoplasmic enzyme) [4,7] and 37.6 kdal (cell wall enzyme) [6].

6.4 Conclusion

Extensive characterization of cytoplasmic and cell wall bound 3'-nucleotidases isolated from potato tubers (*cv.* Pontiac) have been carried out by applying a novel HPLC technique. Enzymes from both sources possessed the same pH optimum (6.5), while the temperature optima of cytoplasmic and cell wall bound 3'-nucleotidases were 75 and 70°C, respectively. Additional inhibition and substrate specificity studies and the difference in molecular weight strongly suggested that 3'-nucleotidase present in potato tuber actually consists of two distinct enzyme species.

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7. RAPID ENZYMATIC-HPLC DETERMINATION OF THE CONTENT OF MAJOR NUCLEOTIDES IN VEGETABLES*

7.1 Introduction

Flowery, fruity, leafy or tuberous vegetables, such as broccoli, cabbage, cauliflower or sweet potatoes, in the raw state contain a very low level of free nucleotides. This level increased markedly after blanching and/or steam cooking due to enzymatic breakdown of the endogenous tissue RNA [1]. Several enzymes have been found to be involved in the degradation of RNA in plant tissue in general, forming purine and pyrimidine nucleotides [2,3].

The importance of the presence of nucleotides in vegetables is two-fold. Firstly, 5'-guanosine monophosphate (5'-GMP) is a flavor enhancer which surpasses monosodium glutamate in potency and, secondly, low purine base content in vegetables is a dietary recommendation for metabolic disturbances such as gout [4-6]. It has been suggested that the total consumption of nucleic acid, from all dietary sources, should not exceed 4 g/day [7].

Determination of free purine bases and nucleotides and those bound in nucleic acid, in general, and in some low purine content vegetables and other food stuffs, in particular, is difficult and the available data are conflicting and inconsistent [5]. Despite their relevance to food flavor and human nutrition, data on the content of

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*Agric. Biol. Chem. (in press).

free- and nucleic acid incorporated nucleotides, the latter being the major precursors of free nucleotides in processed vegetables, are scanty.

Data on vegetable nucleotides (see Chapter 8) have shown that free nucleotide contents of raw vegetables are much less than found in cooked vegetables. This chapter reports a rapid method for determination of the four major nucleotides incorporated in RNA. White potato was chosen for the study since its high starch content makes this vegetable the most cumbersome to analyze.

7.2 Materials and Methods

7.2.1 Reagents

Lyophilized pure nuclease P1 (nucleate 5'-nucleotidohydrolase 3'-phosphohydrolase, EC. 3.1.30.1) from *Penicillium citrinum* [8,9] was provided by Yamasa Shoyu Co. Ltd. (Choshi, Chiba, Japan). Standard nucleotides and baker's yeast RNA were from Sigma Chem. Co. (St. Louis, MO). Yeast RNA was also prepared by applying the procedure of Crestfield *et al.* [10]. HPLC-grade potassium dihydrogen phosphate and methanol were from Fisher Scientific (Fair Lawn, NJ). Tri-*n*-octylamine (98% purity) was from Aldrich Chem. Co. (Milwaukee, WI) and Freon 113 from Terochem Labs. Ltd. (Edmonton, Alta). A fresh solution of 0.5 M tri-*n*-octylamine in Freon 113 was prepared before use. HPLC-grade water was prepared by reverse osmosis (Milli-RO)

and purified additionally by Milli-Q system (Millipore, Bedford, MA). All other reagents were of analytical grade.

7.2.2 HPLC system and separation conditions

The HPLC system consisted of a Bio-Rad Model 1330 pump (Bio Rad, Richmond, CA), a 20 μL Rheodyne loop injector, a Whatman 250 x 4.6 mm i.d. Partisil 10 SAX anionic column protected by a 70 x 2.1 mm i.d. guard column containing pellicular anion exchanger, packed by us, and by a 250 x 4.6 mm i.d. pre-injector column (Whatman Solvecon) containing silica gel, with a Bio-Rad Model 1305 variable wavelength detector set at 254 nm and a Hewlett Packard Model 3388A integrator.

The optimized HPLC separation conditions were as described in Chapter 2, with 3% methanol in 8 mM potassium dihydrogen phosphate solution (adjusted to pH 4.15 by phosphoric acid) as an eluent. Isocratic elution of nucleotides was carried out at room temperature at an eluent flow rate of 1.5 mL/min.

7.2.3 Vegetable

Potato tubers cv. Shepody (sp. gr. 1.081; average starch content 71.6%) were used throughout this study. They were grown commercially under irrigation in Southern Alberta and were provided by I & S Produce Co. Ltd. (Edmonton, Alta). Potato starch gelatinization onset was at 56°C and end 65°C, and the size of the majority of the grains was

either below 38 μm (52%) or between 38-53 μm (42%). The amylose content was 21.4%. Starch swelling power was 86 (at 65°C) and 102 (at 70°C), while its solubility was 18% (at 65°C) and 21% (at 70°C).

7.2.4 Isolation of tuber nucleic acid

Nucleic acid isolation followed the procedure outlined by Hadziyev *et al.* [12], with some modifications. Peeled and diced tubers (50 g) were suspended in 40 mL ice-cold 0.1 M Tris-HCl buffer of pH 9.0 containing 6 mL 22% sodium dodecyl sulfate and 4 mL 80 mM zinc chloride solution. The suspension was homogenized with the aid of sea sand in a mortar with 68 mL ice-cold 80% (w/w) aqueous phenol. The slurry was transferred to an erlenmeyer, shaken by a Lab-Line orbit shaker at room temperature for 1 hr, and then centrifuged in thick-wall glass centrifuge tubes at 17,000 x g for 20 min. The upper layer was collected, and was then reextracted with an equal volume of aqueous phenol and 4 mL of 80 mM zinc chloride solution. After standing for 30 min, the aqueous layer containing nucleic acid was collected. Nucleic acid was precipitated at 4°C overnight after addition of 0.1 volume of 10% sodium chloride and 2.5 volumes of 98% ethanol. The precipitated nucleic acid was recovered by low speed centrifugation, discarding the supernatant, washing the precipitate with ethanol, and drying the precipitate in a stream of nitrogen, followed by its storage in a desiccator under phosphorus pentoxide. The yield of

this crude nucleic acid was 5 mg/g fresh potato tuber.

7.2.5 Preparation of potato nucleotides

The nucleotide preparations were obtained at least in duplicate by four different procedures using nuclease P1 enzyme or alkaline hydrolysis, as follows:

(a) Enzymatic digestion of isolated nucleic acid

Nucleic acid powder, about 10 mg, was added to a test tube containing 2.0 mL 100 mM sodium acetate buffer of pH 5.30, 2 mL 8 mM zinc chloride and 200 μg nuclease P1 enzyme. A blank was run in the same way, but without nuclease P1. Both sample and blank were incubated at 60°C for 1 h. The reaction was stopped by immersing the tubes in an ice bath and by addition of an equal volume of 1.0 M perchloric acid (PCA). After centrifugation at 5,000 x g for 10 min, a 2 mL aliquot of the supernatant was collected and mixed gently with 2 mL of 0.5 M Freon-octylamine solution. This mixture was separated into three distint layers by low-speed centrifugation at 800 x g for 10 min. The top layer, containing nucleotides, was collected and used for HPLC analysis.

(b) Enzymatic digestion <u>in situ</u> of potato tuber tissue

Abdut 3 g of diced potato tuber was weighed into a 50 mL centrifuge tube, then 7 mL of 100 mM sodium acetate buffer pH 5.30 and 0.2 mL 80 mM zinc chloride solution were added. The mixture was slurried for 2 min using a Polytron homogenizer. The homogenate was steam cooked for 30 min. The sample homogenate, with its starch now fully gelatinized,

was cooled, dispersed in 8 mL of the sodium acetate buffer, and the mixture then incubated with nuclease P1 enzyme (200 μg) at 60°C for 1 h. The hydrolysate in the tube was immersed in an ice bath. Acid-soluble nucleotides were extracted by addition of 15 mL 1.5 M PCA, followed by 15 mL methanol to precipitate the solubilized and interfering starch [11]. A low-speed centrifugation (800 x g, 10 min) followed. This extraction step was repeated and the combined supernatants were then made up to 100 mL and treated with Freon-octylamine as mentioned above.

(c) Alkaline hydrolysis of isolated nucleic acid

Nucleic acid powder, about 10 mg, was dissolved in 5 mL 0.5 M sodium hydroxide and incubated at 37°C for 18 h with continuous shaking using a Lab-Line orbit shaker. The hydrolysate was neutralized using 60% PCA, made up to a final concentration of 0.5 M PCA, then the volume was adjusted to 10 mL prior to centrifugation. The supernatant, containing the nucleotides, was collected and additionally extracted by Freon-octylamine solution and analyzed by HPLC as described above.

(d) Alkaline digestion <u>in situ</u> of potato tuber tissue

About 5 g of diced potatoes in a 50 mL centrifuge tube were slurried, using the Polytron homogenizer, with 10 mL of 0.5 M sodium hydroxide in ethanol. Alkaline hydrolysis followed under conditions outlined in procedure (c) above. After neutralization, the tissue suspension was adjusted to 0.75 M PCA, and then methanol was added to give a final

concentration of 0.5 M PCA. Centrifugation at 5,000 x g was used to recover the nucleotides in the supernatant. After the removal of PCA by Freon-octylamine, the sample was analysed by HPLC as described above (see section 7.2.2).

7.2.6 Preparation of standard by alkaline hydrolysis

The incubation test tube contained a mixture of known amounts of 2'- and 3'-isomers of AMP, CMP, GMP and UMP in 0.5 M sodium hydroxide. Incubation and extraction steps prior to HPLC analysis were the same as in procedure (c). This solution was used as a standard for quantitation of nucleotides obtained by alkaline hydrolysis by procedures (c) and (d).

7.2.7 Baker's yeast nucleotide preparation

Baker's yeast RNA was chosen as a model system and subjected to hydrolysis. The RNA was dissolved at a concentration of 5.0 mg/mL in 0.1 M sodium acetate buffer of pH 5.30. The P1 nuclease hydrolysate was prepared in six replicates. Incubation and nucleotide extraction steps followed by HPLC analysis were the same as in procedure (a).

7.3 Results and Discussion

To verify the reliability and accuracy of enzymatic and alkaline hydrolysis procedures, yeast RNA was used as a model substrate and its nucleotide composition assayed. A typical elution pattern of its four major ribonucleotides in

Table 7.1: Nucleotide Composition of Commercial Baker's Yeast RNA

Mole %					Method	Ref.
AMP	CMP	GMP	UMP	Pu/Py [a]		
21	24	33	22	1.2	HPLC	P [b]
25	20	28	27	1.1	Conv. [c]	10
22	23	34	22	1.2	Conv.	13
19	24	34	23	1.1	Conv.	14

[a] Pu : purine
Py : pyrimidine.

[b] P : the present study.

[c] Conv. : Conventional method.

an enzymatic hydrolysis is shown in Figure 7.1. Nucleotide identification was based on comparison with retention times obtained by running a standard nucleotide mixture subjected to the same incubation and extractions steps as yeast RNA. Optimized conditions for resolution as applied here were discussed previously [11]. Table 7.1 shows the nucleotide composition of baker's yeast RNA in mole percent. Yeast RNA used in this experiment was purified by the procedure of Crestfield *et al.* [10] and, as shown by HPLC runs, it was identical to the common commercially available yeast RNA used widely in the literature [13,14]. As seen from Table 7.1, its nucleotide composition, although obtained by different conditions of hydrolysis and separation of its nucleotides, was consistent with our results when nuclease P1 hydrolysis, followed by HPLC separation, was applied.

The highly purified nuclease P1 used in this study has been proven to have a high capacity to hydrolyse transfer RNA and both light and heavy ribosomal RNA's under the assay conditions applied, i.e. 0.1 M acetate buffer of pH 5.30 and an incubation temperature of 60°C [9].

Individual and total contents of four major nucleotides obtained by enzymatic hydrolysis of yeast RNA are presented in Table 7.2. The sum of four major nucleotides was 72±0.8% of the initial RNA weight. The remaining 28% should be attributed to contaminating impurities, the presence of minor nucleotides in the RNA chain, and the tenaciously bound protein and moisture.

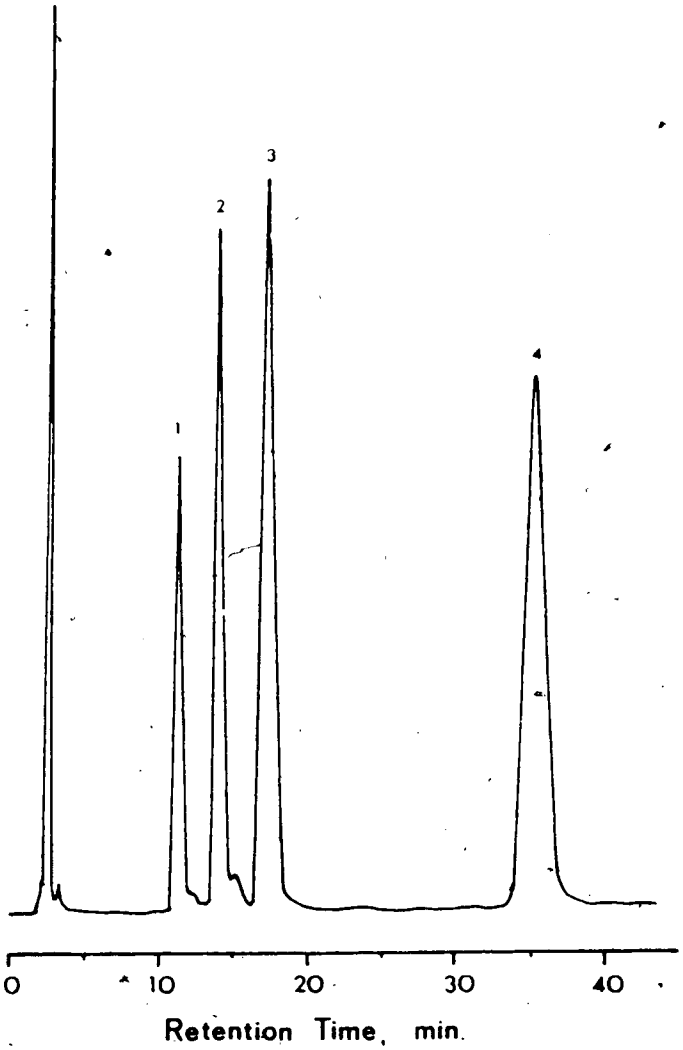

Figure 7.1. Liquid chromatogram of yeast RNA nuclease P1 hydolysate. Peak identification was based on retention times of ribonucleotide standards. Peak identities: 1) 5'-CMP; 2) 5'-UMP; 3) 5'-AMP; and 4) 5'-GMP.

Table 7.2: Precision Analysis of Nuclease P1 Hydrolysis of Baker's Yeast RNA

5'- Ribonucleotide	Amount (μg)							Coefficient of variation (%)
	Replicate						Mean	
	1	2	3	4	5	6		
AMP	780.6	766.9	760.6	774.5	786.2	793.2	777.0 ± 12.1	1.55
CMP	791.3	783.8	773.0	784.8	796.7	795.3	787.5 ± 8.84	1.12
GMP	1270	1246	1255	1261	1286	1289	1268 ± 17.2	1.36
UMP	735.4	752.2	748.7	747.4	748.7	759.1	750.3 ± 8.77	1.17
Total nucleotides	3577	3549	3537	3568	3628	3637	3593 ± 41.2	
% [a]	71.5	71.0	70.7	71.3	72.5	72.7	71.6 ± 0.81	

[a] Calculation was based on 5mg yeast RNA as an initial material. The final percentage does not take into account the contribution of water in the hydrolysis of the phosphodiester bond. This would be approximately 5%, and percentages would be reduced accordingly.

Table 7.3: Precision Analysis of Nuclease P1 Hydrolysis of Potato Tuber Nucleic Acids

5'- Ribonucleotide	Amount (µg)						Coefficient of variation (%)
	Replicate					Mean	
	1	2	3	4	5		
AMP	122.96	123.24	118.66	121.79	124.21	122.17 ± 2.14	1.75
CMP	122.97	121.89	120.60	124.91	124.37	122.95 ± 1.77	1.44
GMP	183.52	185.12	176.76	184.28	185.22	182.98 ± 3.54	1.93
UMP	96.86	97.79	93.58	94.75	97.87	96.17 ± 1.92	1.99
Total nucleotides	526.31	528.04	518.06	525.73	531.67	525.96 ± 4.98	
% [a]	5.26	5.28	5.18	5.26	5.32	5.26 ± 0.05	

[a] Calculation was based on 10mg isolated crude potato tuber nucleic acids. The final percentage does not take into account the contribution of water in the hydrolysis of the phosphodiester bond. This would be approximately 5%, and percentages would be reduced accordingly.

As shown in Tables 7.2 and 7.3, coefficient of variations for nucleotide determinations are in the range of 1.12-1.99%, which indicates that this analysis method is precise.

The low recovery of nucleotides from isolated tuber RNA is not unexpected. Besides the minor nucleotides not measured, the tuber RNA preparation contained DNA, as shown by the presence of deoxyribonucleotide peaks in Figures 7.2B and Figure 7.3. Starch was a major contaminant. Nucleic acid extraction using phenol followed by ethanol precipitation could not avoid coprecipitation of a large portion of solubilized starch molecules, some of which would be present as a phenol-starch clathrate. This was readily verified by iodine blue staining of the RNA preparation, and also by the release of a phenol odor after RNA powder was rehydrated.

A large portion of the starch contamination from enzymatic or alkaline hydrolysate of tuber tissue after nucleotide extraction with PCA, was removed by addition of methanol. This step was required since PCA, in a range of 1-5 M, was a powerful acid for starch solubilization in homogenized plant tissue [15].

The enzymatic method of hydrolysis, as applied here, determines the exact content of four 5'-ribonucleotides present in tuberous vegetable endogenous RNA. The HPLC conditions used permitted separation of both classes of nucleotides: ribo- and deoxyribonucleotides. The nuclease P1 enzyme has also been used in analysis of total nucleotides

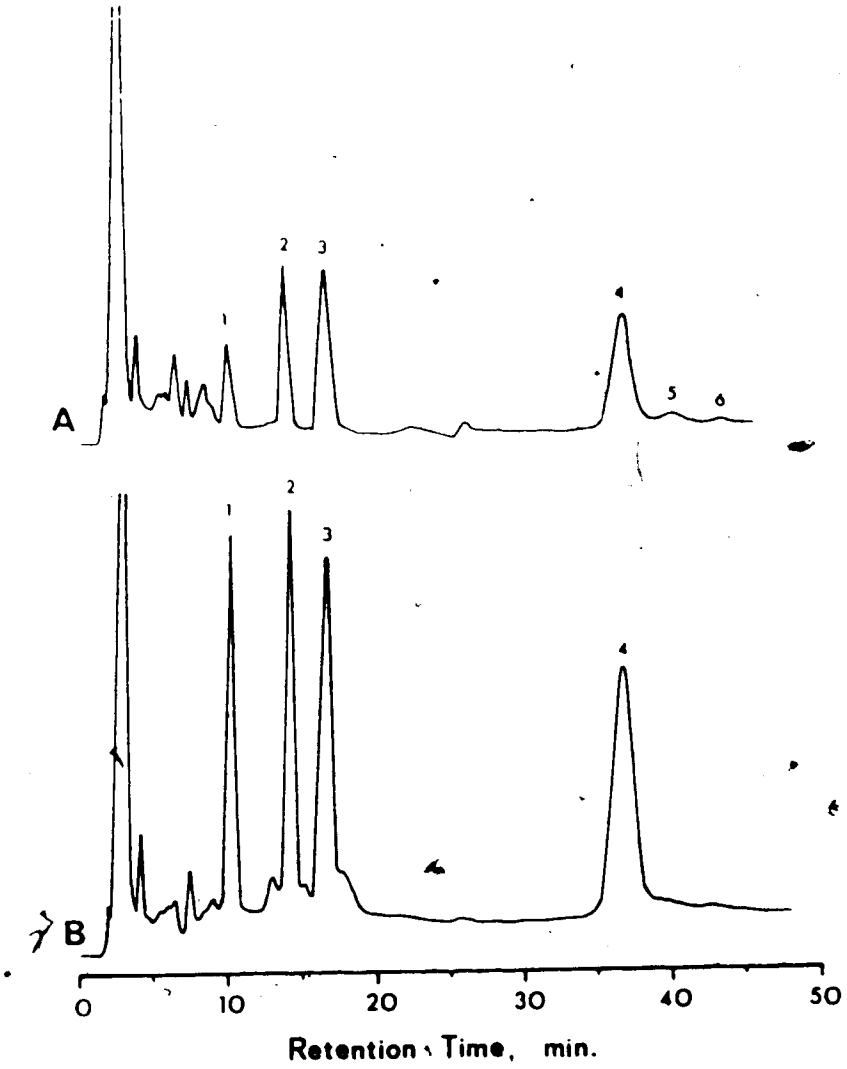

Figure 7.2. Liquid chromatogram of nucleotide extracts of steam-cooked (A) and P1 nuclease hydrolysate of the steam-cooked potato homogenate (B). Comparison of the chromatograms shows the inactivation of tuber RNase by zinc ion and the ability of nuclease P1 enzyme to hydrolyse *in situ* the tuber RNA. Peak identities: 1) 5'-CMP; 2) 5'-UMP; 3) 5'-AMP; 4) 5'-GMP; 5) 3'-GMP; and 6) 2'-GMP. Small peaks corresponding to deoxyribonucleotides are not designated.

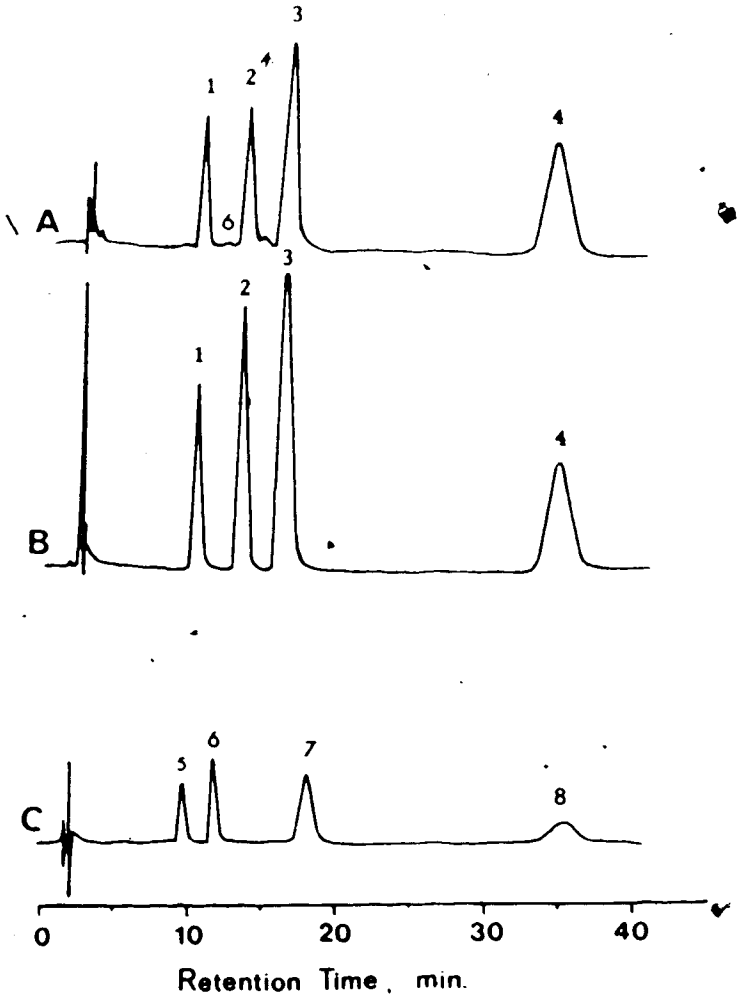

Figure 7.3. Liquid chromatograms of isolated tuber RNA hydrolysate by nuclease P1 enzyme (A), a standard mixture of 5'-ribonucleotides (B), and a standard mixture of 5'-deoxyribonucleotides (C). Peak designations: 1) 5'-CMP; 2) 5'-UMP; 3) 5'-AMP; 4) 5'-GMP; 5) 5'-dCMP; 6) 5'-dUMP; 7) 5'-dAMP; and 8) 5'-dGMP. In the case of coelution of ribo- and deoxyribonucleotides, quantitation was by peak height or area difference after removal of ribonucleotides from assay solution by oxidation using potassium periodate [11].

present in other vegetables, e.g. flowerhead, fruity, leafy, rooty, seed or stem/shoot vegetables or mushrooms [1].

Liquid chromatograms of the extracts of steam-cooked potato tuber, before and after addition of nuclease P1 enzyme, are shown in Figures 7.2A and 7.2B, respectively. As demonstrated by us, zinc ion is a strong inhibitor of potato nuclease and ribonuclease [16], but has no effect on the activity of nuclease P1. The latter finding was also reported by Fujimoto *et al.* [9]. Hence, it was useful to employ zinc chloride for inhibition of vegetable RNA enzymes during sample preparation (slicing, dicing and homogenization) and it was not necessary to remove the metal ion prior to subsequent hydrolysis by nuclease P1, or for HPLC analysis. Heat treatment of vegetable samples prior to nuclease P1 digestion was required to denature enzymes in plant tissue and to prevent proteolytic degradation of the P1 enzyme, and protect the nucleotides released from further decomposition into nucleosides and free bases. In addition, heat enhanced the detachment of proteins from nucleic acid and its exposure to digestion by nuclease.

The nucleotide composition of potato tuber RNA is presented in Table 7.4. The values for the four major nucleotides, calculated as μmoles/100 g fresh potato and mole %, and obtained from a nuclease P1 hydrolysis *in situ* of RNA in tuber tissue and from previously isolated RNA, show some discrepancies. The content of pyrimidine nucleotides, expressed as μmoles/fresh weight tissue, is

Table 7.4: Nucleotide Composition of Potato RNA obtained by Various Methods of Analysis

RNA degradation method	Ribonucleoside monophosphate								Ratio Pu/Py
	µmoles / 100g fresh potato				mole %				
	AMP	CMP	GMP	UMP	AMP	CMP	GMP	UMP	
Enzymatic / Potato tuber	13.4±2.16	21.2±1.69	21.2±0.42	13.4±2.16	19	31	31	19	1.0
Enzymatic / Isolated tuber RNA	17.7±0.31	19.1±0.27	25.3±0.49	14.9±0.30	23	25	31	21	1.2
Alkaline / Potato tuber	nd [a]	nd	23.9±0.03 [b]	nd	—	—	—	—	—
Alkaline / Isolated tuber RNA	nd	nd	24.5±0.01	nd	—	—	—	—	—

[a] Not detected due to co-elution of 2'- and 3'-nucleotides.

[b] Sum of 2'- and 3'-GMP.

essentially the same regardless of the form of RNA being hydrolyzed, but this is not so for the content of purine nucleotides. They are consistently lower in content when determined in tuber tissue *in situ*. This finding is not unexpected since zinc ion cannot inactivate tuber nucleotidases (phosphomonoesterases) or nucleosidases. Nor can the slow heat transfer during vegetable heat treatment eliminate their effect, a suggestion supported by the finding that plant tissue phosphomonoesterases prefer purine rather than pyrimidine nucleotides [17].

In the conventional and some other more recent methods [18-21], nucleotides and/or bases are determined from acid and/or alkaline hydrolysate of RNA. In both cases the neutralization results in a large amount of salt, which interferes with HPLC separation. To assess and compare the merits of alkaline vs enzymatic hydrolysis, both tuber tissue RNA *in situ* and isolated tuber RNA were subjected to alkaline digestion by 0.5 M sodium hydroxide. The hydrolysate was neutralized with PCA, and nucleotides extracted and analyzed by HPLC. As seen in Figure 7.4 and by the results compiled in Table 7.4, an alkaline hydrolysate of RNA is not suitable for HPLC analysis. All six 2'- and 3'-isomers of AMP, CMP and UMP in the neutralized solution were co-eluted in one peak; only 2'- and 3'-GMP were well separated and individually quantifiable. This is due to the excess salt formed in the neutralization step and thus present in the injection solution. The salt competed with

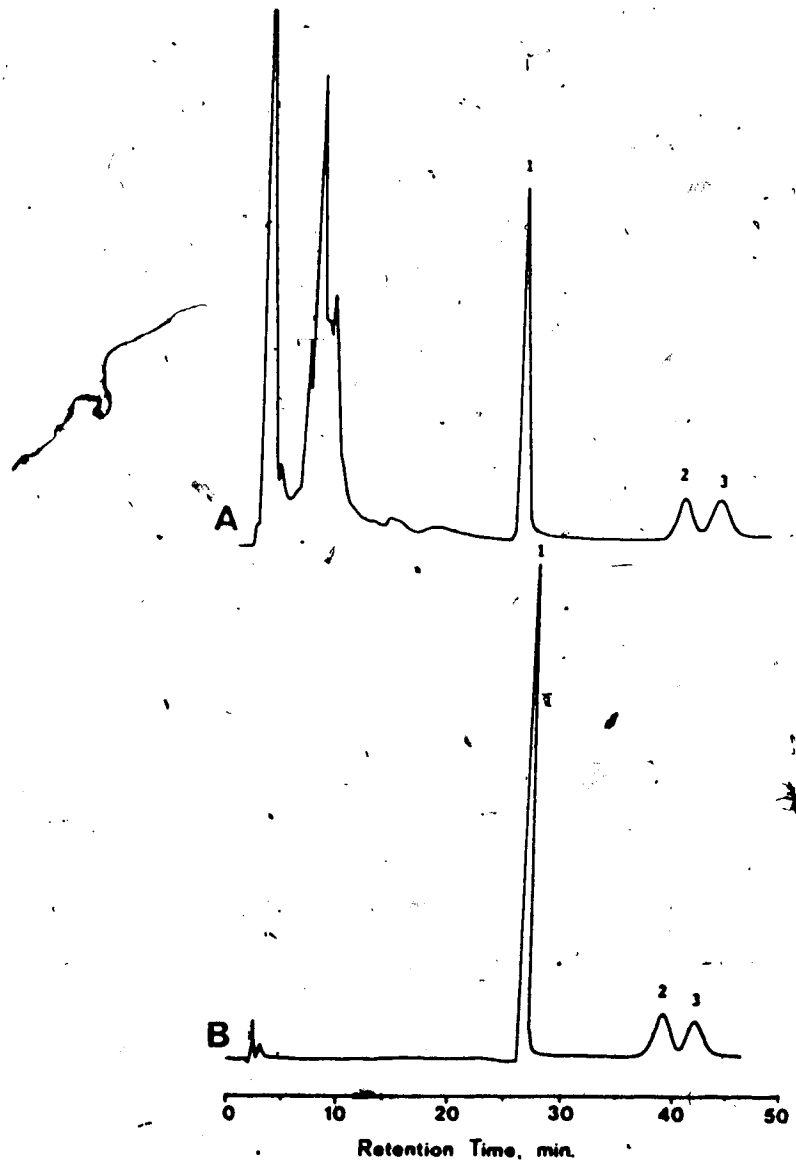

Figure 7.4. Liquid chromatograms of the alkaline hydrolysate of potato tuber RNA *in situ* (A), and nucleotide standard solution (B) containing the 2'- and 3'-isomers of AMP, CMP, GMP and UMP. Peak designations: 1) mixture of 2'- and 3'-isomers of AMP, CMP and UMP; 2) 3'-GMP; and 3) 2'-GMP.

these six nucleotides for the anion exchange sites of the column during the initial 25 min of elution. The elution of 2'- and 3'-GMP occurred at a retention time greater than 40 min when the excess salt had already eluted. Nevertheless, the sum of 2'- and 3'-GMP quantitated after alkaline hydrolysis of both forms of RNA was still sufficient to corroborate the results provided by the enzymatic hydrolysate method using nuclease P1 (Table 7.4).

7.4 Conclusion

Determination of the content of the major constituent ribonucleotides of vegetable tissue endogenous RNA by HPLC analysis after nuclease P1 enzyme hydrolysis provided a simple, rapid and reliable method of nucleotide analysis. Total ribonucleotide content and the corresponding RNA nucleotide composition could be determined without the need for prior isolation of nucleic acid. Enzymatic and not alkaline hydrolysis of RNA of the homogenized plant tissue *in situ* is the method of choice.

7.5 Acknowledgement

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8. ENZYMATIC FORMATION OF THE FLAVOR ENHANCER 5'-GMP AND OTHER NUCLEOTIDES DURING VEGETABLE PROCESSING'

8.1 Introduction

During the processing of vegetables, endogenous RNA is hydrolysed by nucleases and ribonucleases, producing a variety of nucleotides. These include nucleoside-5'-monophosphates, nucleoside-3'-monophosphates, and nucleoside-2':3'-cyclic monophosphates. Of all of these products, only guanosine-5'-monophosphate (5'-GMP) exhibits flavor-enhancing activity [1].

Recently, we have developed a rapid HPLC method for the simultaneous determination of 5'-ribonucleotides, 5'-deoxyribonucleotides, and their corresponding 2'- and 3'-isomers [2]. This method has been used to measure changes in the levels of nucleotides in potato tubers through several processes [3] and to characterize potato tuber nuclease and ribonuclease enzymes [4]. In this report, we employ this HPLC method to determine the nucleotide content of selected raw vegetables, representing flowerhead, fruity, leafy, seed, stem/shoot, and tuberous vegetables, and mushrooms, before and after simulated commercial steam cooking preceeded by an 8-10 min blanching step. In addition, 5'-GMP-forming nucleases of these vegetables were characterized.

' Presented at International Conference, Bioflavor '87. Würzburg, West Germany. (Sept. 29-30, 1987).

8.2 Materials and Methods

Vegetables were supplied by local markets, and represented the following classes: flowerhead (broccoli, *Brassica oleracea*); fruity (green bell pepper, *Capsicum annuum*); leafy (common cabbage, *Brassica* convar. *capita*, f. *alba*); rooty (radish, *Raphanus sativus*, var. *sativus*; sweet potato, *Impomoea batatas*); seed (green bean, *Phaseolus vulgaris*; bean sprout, *Phaseolus aureus*); stem/shoot (asparagus, *Asparagus officinalis*); tuberous (white potato, *Solanum tuberosum*); and mushrooms (shiitake, *Lenzite edodes*; snow white, *Agaricus bisporus*; oyster, *Pleurotus florida*).

The edible part of each vegetable was washed and thinly sliced to give a homogeneous sample. All of the raw, blanched and steam-cooked vegetables were extracted with 3 vol. cold 0.75 M perchloric acid (PCA), slurried with a Polytron homogenizer, and diluted with methanol to 0.5 M PCA. Removal of PCA was achieved by extracting with 0.5 M Freon-octylamine prior to analysis [2, see section 2.2.1.3]. Isocratic HPLC separation of nucleotides was carried out using a strong anionic Whatman Partisil 10 SAX column and 3% methanol in 8 mM KH_2PO_4 as a mobile phase, as previously described [2]. The eluted nucleotides were monitored with a UV detector at 254 nm and quantitated by peak height comparison of unknowns with standard compounds.

Crude enzyme powders were prepared by homogenization of vegetables with 50 mM sodium citrate buffer, pH 6.0, containing 2 mM cysteine, centrifugation and freeze-drying.

The total activities of RNase and nuclease in the crude enzyme powder were estimated using a previously reported HPLC procedure [4]. Enzyme powders (400 μg) were incubated with 2 mg yeast RNA in Tris-HCl buffer (0.1 M, pH 6.50) for 30 min. Reaction was terminated with the addition of 3 M PCA to a final concentration of 0.5 M PCA; samples were centrifuged to remove precipitate and extracted with 0.5 M Freon-octylamine. Isocratic HPLC separation of RNA hydrolysis products, 2'- and 3'-GMP for ribonuclease and 5'-GMP for nuclease, was carried out as described in section 2.2.1.

Total 5'-GMP, both free and incorporated in endogenous RNA, of each vegetable was determined by HPLC analysis of a P1 nuclease hydrolysate of the homogenized vegetable (see Chapter 7).

8.3 Results and Discussion

The nucleotide content of raw and steam-cooked vegetables is given in Tables 8.1 and 8.2. In general, raw vegetable samples contain low or no detectable levels of free nucleotides. Following steam treatment, the levels of nucleotides increased; the largest change was observed for shiitake mushrooms (Table 8.2). Other studies [1,5-7] report higher levels of nucleotides in raw vegetables than we observed. Since, in these studies, nucleotides were extracted by boiling water [8-13], we attribute the higher levels of nucleotides to the activation of endogenous

Table 8.1: Ribonucleotide Content in Raw Vegetables [a]

Vegetable	Nucleotide (nmoles/g) [b]					
	5'-AMP	5'-CMP	2'-GMP	3'-GMP	5'-GMP	5'-UMP
Asparagus	48	3	nd [c]	nd	6	84
Bean (green)	22	nd	nd	nd	nd	70
Bean sprout	16	nd	nd	nd	4	30
Bell pepper (green)	2	nd	nd	nd	nd	19
Broccoli	9	nd	nd	47	nd	32
Cabbage	3	nd	nd	nd	nd	36
Cauliflower	8	nd	nd	nd	99	37
Mushroom (shiitake)	nd	nd	nd	nd	nd	nd
Mushroom (white)	21	30	nd	nd	7	44
Potato (sweet)	6	100	nd	nd	nd	34
Potato (white, cv. Shepody)	78	7	nd	nd	nd	15
Radish (red skin)	4	40	nd	nd	nd	35

[a] Moisture content (%): Asparagus (94); Green beans (89); Bean sprout (92); Green bell pepper (94); Broccoli (90); Cabbage (93); Cauliflower (93); Mushroom [dried shiitake] (9); Mushroom [white] (93); Sweet potato (78); White potato (80); Radish (95).

[b] The data presented are an average of two replicates.

[c] nd: Not detectable, i.e. below the detection limit (100 ppb for 5'-AMP, 5'-CMP, 5'-UMP and 200 ppb for GMP's).

Table 8.2: Ribonucleotide Content in Blanched and Steamed Vegetables [a]

Vegetable	Nucleotide (nmoles/g) [b]					
	5'-AMP	5'-CMP	2'-GMP	3'-GMP	5'-GMP	5'-UMP
Asparagus	28	7	106	157	12	72
Beans (green)	130	81	19	49	105	141
Bean sprout	87	80	13	31	39	73
Bell pepper (green)	59	17	3	12	43	47
Broccoli	43	27	47	81	35	64
Cabbage	13	219	8	12	nd	40
Cauliflower	24	29	nd	12	83	55
Mushroom (shiitake)	2090	1390	nd	nd	3410	1700
Mushroom (white)	338	116	40	71	122	127
Potato (sweet)	127	66	35	63	31	106
Potato (white, cv. Shepody)	30	50	19	33	22	40
Radish (red skin)	22	60	nd	16	5	46

[a] Moisture content: see Table 8.1.

[b] The data presented are an average of two replicates.

nucleases and ribonucleases and their attack on endogenous vegetable RNA. As found by us, steam cooking preceeded by a blanching step as well as boiling water extraction activates the RNA-hydrolysing enzymes, i.e. heat-induced degradation of RNA does not occur. That this degradation does not occur was proved by heating the slurried leafy or tuberous vegetables in the presence of $ZnCl_2$, a powerful inhibitor of RNA-hydrolysing enzymes. In the presence of zinc ion at a heating temperature up to 100°C and heating time even longer than 30 min, the content of nucleotides of the blanched and/or cooked vegetables was the same as that of raw vegetables. The use of PCA to inactivate these enzymes before RNA can be hydrolysed, as applied by us, allows more accurate estimation of free nucleotides present in raw vegetables.

The most important enzymes involved in the formation and degradation of 5'-nucleotides are nucleases, ribonucleases and phosphomonoesterases. The level of these enzymes, as well as the enzyme base specificity, vary in the vegetables surveyed. Thus, the amount of 5'-ribonucleotides and, hence, of the flavor nucleotide 5'-GMP differs among vegetables and is dependent on both the active enzymes and the amount of vegetable RNA. Total 5'-ribonucleotides (free and bound) in each vegetable were estimated by a P1 nuclease digest, and are shown in Table 8.3. The ratio of free 5'-GMP to total 5'-GMP in steamed vegetables is given in Table 8.4. Blanching and steaming of mushrooms (shiitake), green beans

and green bell peppers results in the highest formation of 5'-GMP from RNA, with respective levels of 44, 31 and 29%.

Nucleases, which exhibit no specificity for the sugar moiety, were found in all vegetables; these enzymes hydrolyse both RNA and DNA (Table 8.5). Both nucleases and RNases act on RNA as a substrate, however, the products of nuclease reactions are 5'-nucleotides and those from RNase are cyclic nucleotides which are further hydrolysed to a mixture of 2'- and 3'-nucleotides during an acid extraction step [4]. Our HPLC method can be used to measure the amount of 5'-GMP and the sum of 2'- and 3'-GMP, which reflect the activities of nucleases and RNases, respectively. Of the eight vegetables surveyed, the ribonucleases exhibited much higher activity than nucleases when yeast RNA was used as a substrate (Figures 8.1-8.4).

The dependence of the activities of the vegetable nucleases and ribonucleases on temperature is shown in Figures 8.1-8.4. Under the assay conditions employed with pH 6.50 buffer, close to vegetable pH, the temperature optima of RNases were found to be around 50-60°C. At the peak of the temperature activity curves, the ratio of nuclease activities compared to those of RNases ranged from 0.6 (oyster mushroom) to 61% (white potato). Nucleases possessed higher temperature optima, around 65-75°C. In general, except in the case of mushrooms, the activity of RNases decreased rapidly as temperature was elevated. At the peak of nuclease activity curves the ratio of nuclease/RNase

Table 8.3: Major Ribonucleotide Constituents of the Vegetables Studied

Vegetable	Nucleotide (nmoles / g) [a]				Molar ratios	
	Purine base		Pyrimidine base			
	AMP	GMP	CMP	UMP	GMP/CMP	AMP/UMP
Asparagus	531	697	610	497	1.14	1.07
Bean (green)	272	342	339	337	1.01	0.81
Bean sprout	307	419	425	329	0.99	0.93
Bell pepper (green)	121	98	146	109	1.49	1.11
Broccoli	659	1059	1226	802	1.16	0.82
Cabbage	187	218	300	190	1.38	0.98
Cauliflower	772	847	1217	733	1.44	1.05
Mushroom (shiitake)	6646	6790	7746	6568	1.14	1.01
Mushroom (white)	1138	1207	1120	1258	0.93	0.90
Potato (sweet)	194	280	216	225	0.77	0.86
Potato (white, cv. Shepody)	153	204	179	147	0.88	1.04
Radish (red skin)	95	142	98	103	0.69	0.92

[a] The data are averages of two replicates.

Table 8.4: Ratio of 5'-GMP formed during Steaming to Vegetable Total (free + RNA incorporated) GMP

Vegetables	5'-GMP / Total GMP (%)
Asparagus	1.7
Bean (green)	30.7
Bean sprout	9.3
Bell pepper (green)	29.4
Broccoli	2.8
Cabbage	0.0
Cauliflower	6.8
Mushroom (shiitake)	44.0
Mushroom (white)	10.9
Potato (sweet)	14.3
Potato (white, cv. Shepody or Pontiac)	12.3
Radish (red skin)	5.1

Table 8.5: Action of Vegetable Nucleases on RNA and DNA [a]

Enzyme Source	5'-Nucleotides formed (nmoles)							
	AMP	CMP	GMP	UMP	dAMP	dCMP	dGMP	dUMP
Asparagus	2.04	2.18	0.93	0.43	1.36	0.76	1.71	2.11
Bean (green)	2.81	2.29	2.48	0.81	1.51	nd	nd	1.78
Broccoli	2.30	5.66	2.16	1.00	1.23	nd	nd	1.52
Bell pepper (green)	1.23	2.07	0.96	0.30	0.99	nd	nd	1.80
Cauliflower	1.00	1.31	1.28	0.13	0.76	nd	nd	1.51
Mushroom (oyster)	1.05	0.41	0.39	0.78	0.55	0.98	nd	0.78
Mushroom (white)	1.17	1.12	1.77	0.78	1.24	2.63	0.24	2.24
Potato (white, cv. Pontiac)	9.48	7.35	10.22	2.88	1.38	nd	1.18	1.63

[a] Vegetable crude enzyme powders (200 µg) were incubated with 400 µg yeast RNA or calf Thymus DNA in 0.1M Tris-HCl buffer, pH 7.0 for 30 min at 70°C. The term "nuclease" is fully justified only if the enzyme is able to hydrolyze both DNA and RNA as its substrate [17].

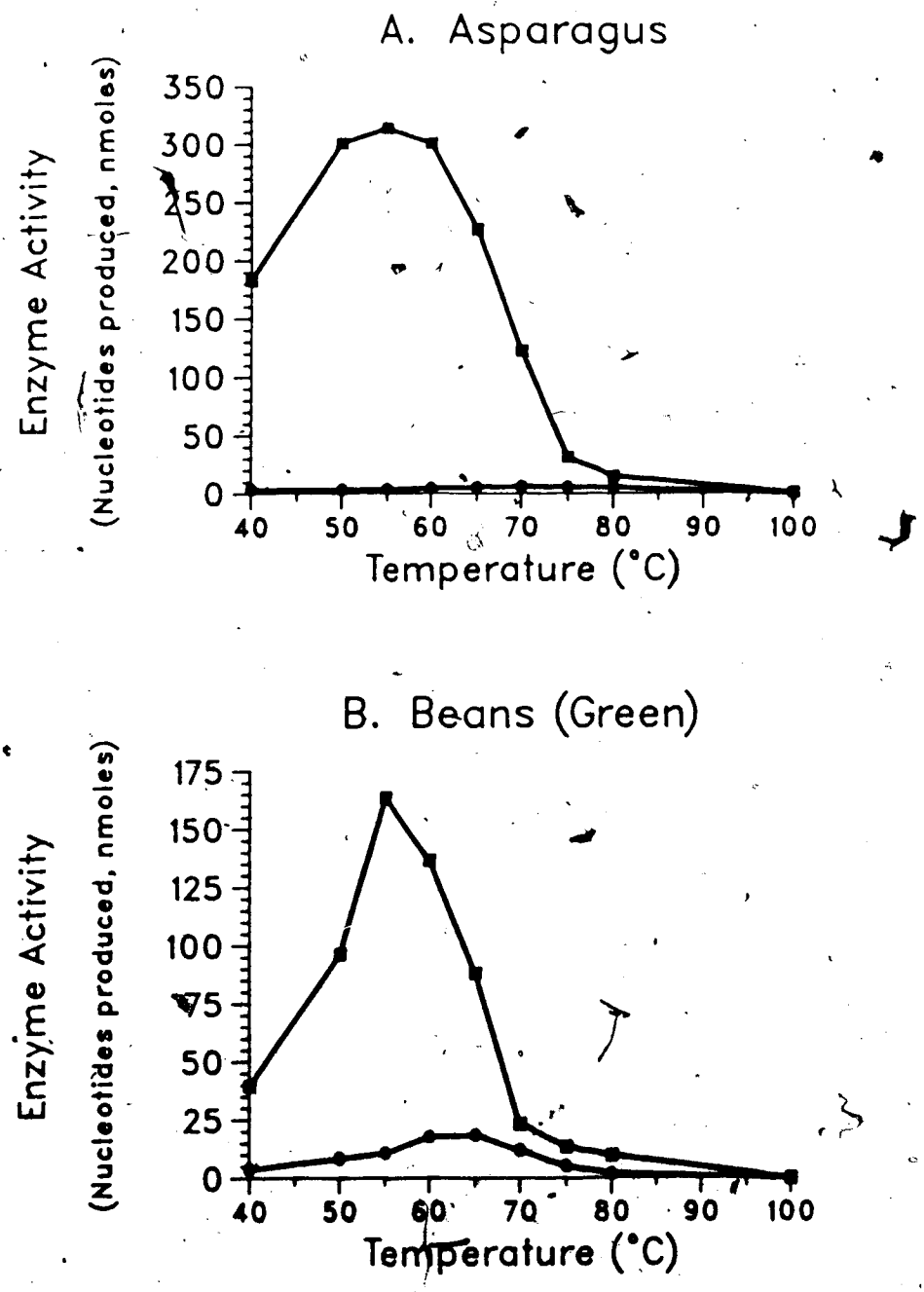

Figure 8.1. Effect of temperature on activities of (■) RNases and (●) nucleases from asparagus (A) and green beans (B). Crude enzyme incubated with yeast RNA in Tris-HCl buffer (0.1 M, pH 6.50) for 30 min at various temperatures.

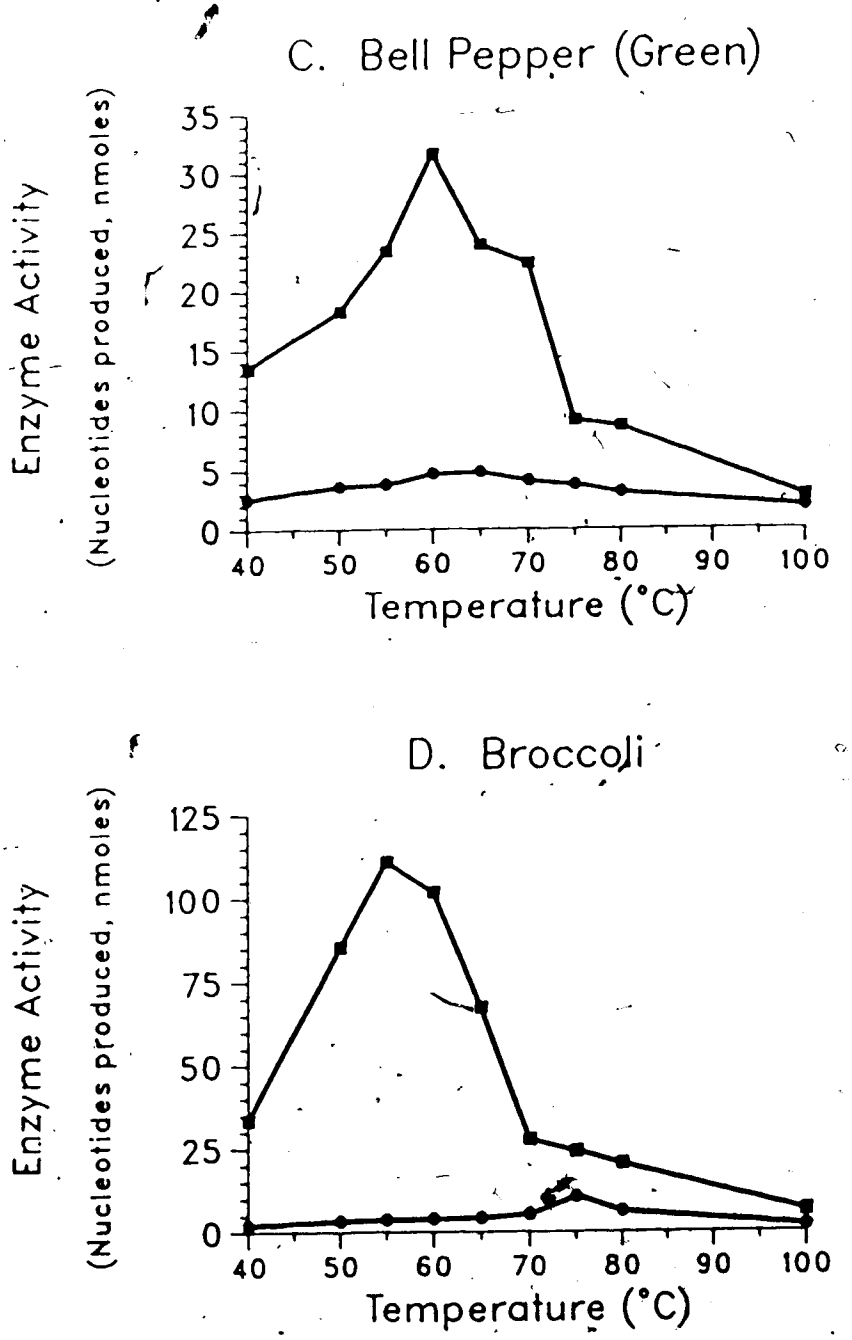

Figure 8.2. Effect of temperature on activities of (■) RNases and (●) nucleases from green bell pepper (C) and broccoli (D). Crude enzyme incubated with yeast RNA in Tris-HCl buffer (0.1 M, pH 6.50) for 30 min at various temperatures.

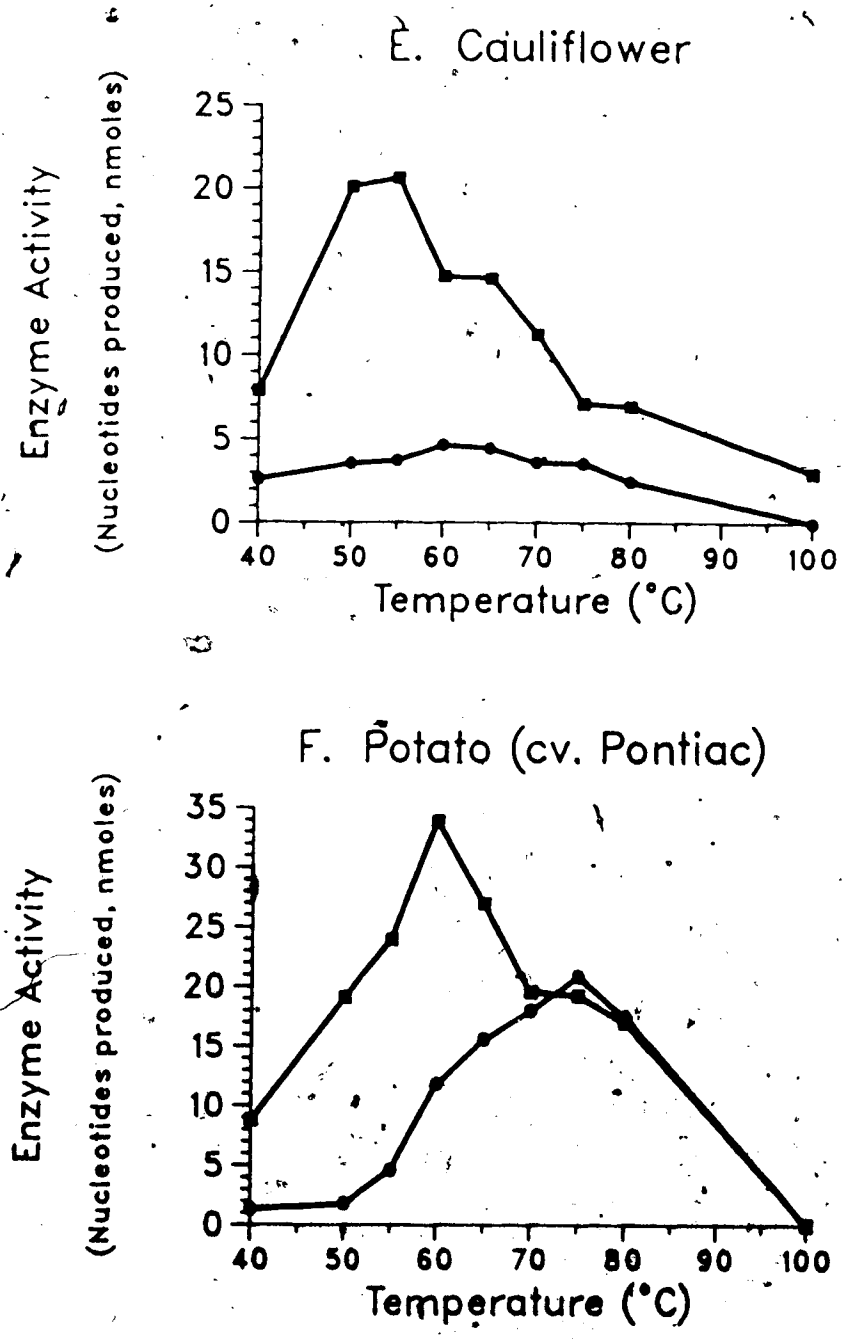

Figure 8.3. Effect of temperature on activities of (■) RNases and (●) nucleases from cauliflower (E) and potato (F). Crude enzyme incubated with yeast RNA in Tris-HCl buffer (0.1 M, pH 6.50) for 30 min at various temperatures.

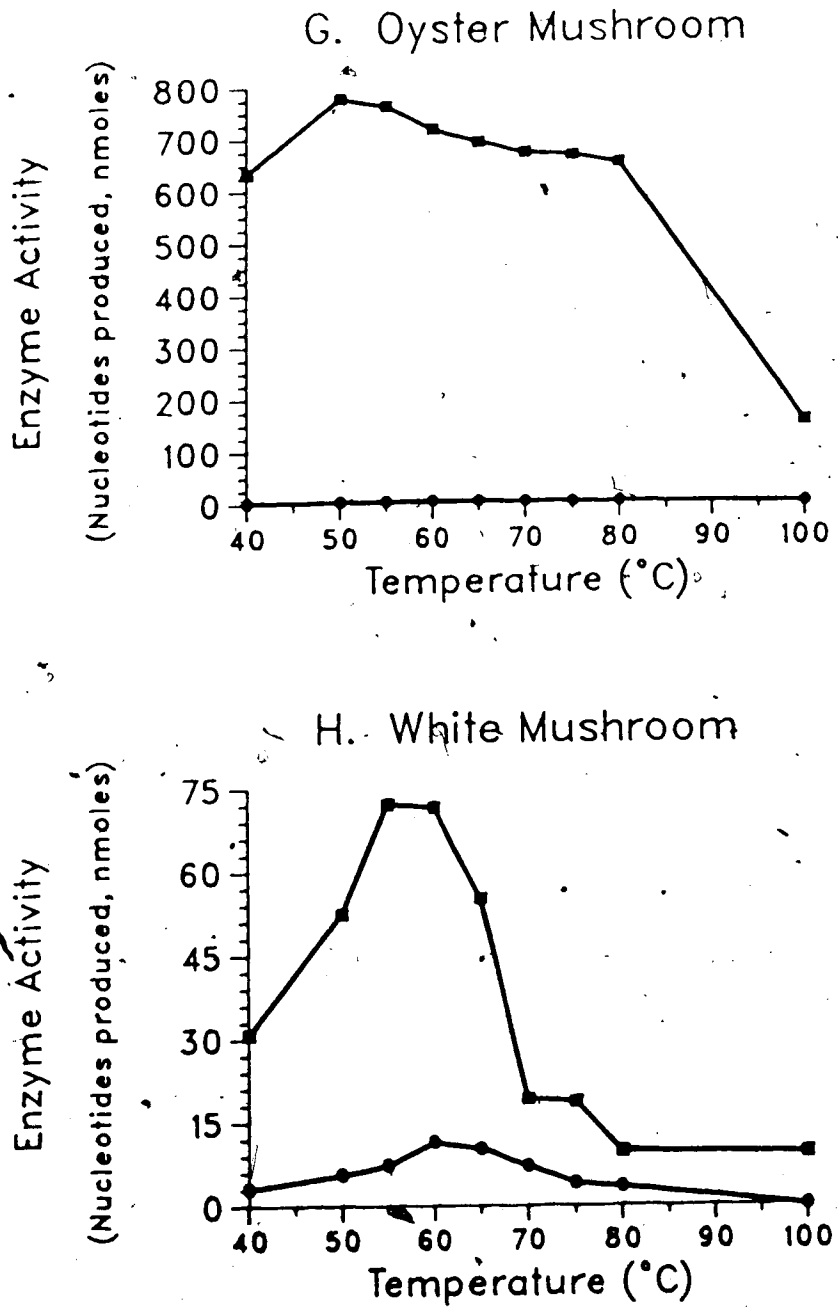

Figure 8.4. Effect of temperature on activities of (■) RNases and (●) nucleases from oyster mushroom (G) and white mushroom (H). Crude enzyme incubated with yeast RNA in Tris-HCl buffer (0.1 M, pH 6.50) for 30 min at various temperatures.

activities increased and ranged from 0.7 (oyster mushroom) to 108% (white potato).

In vegetable processing, natural flavor nucleotide production will be maximized if heat transfer is enhanced so that the internal vegetable temperature is raised as quickly as possible to about 70°C, and the temperature maintained at, but not higher than, 70-75°C. Under these conditions, the undesirable RNase and phosphomonoesterase enzymes are rapidly inactivated. The 5'-nucleotides produced by nuclease will not be further degraded by phosphomonoesterases, since the latter enzymes are heat labile [14-16].

8.4 Conclusion

HPLC was used for the quantitative analysis of vegetable nucleotides and to assay the activities of the nuclease enzymes which catalyze formation of 5'-GMP. This flavor nucleotide was found in all classes of blanched and steam-cooked vegetables. The difference in the levels of 5'-GMP obtained from various vegetables was attributed to differences in the properties of the native enzymes as well as the amount of endogenous RNA present in each vegetable. Temperatures of 65-75°C are suggested for the blanching step of vegetable processing in order to maximize the formation of the flavor nucleotide 5'-GMP.

8.5 References

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9. DISCUSSION AND CONCLUSIONS

Analysis of nucleic acid constituents and assay of the enzymes related to nucleic acids using conventional methods can be tedious, laborious and time-consuming. Thus, it is not surprising that relatively little data have been obtained from this field in food research. It is apparent that there is a need for efficient (i.e. rapid, reliable and accurate) methods for fundamental studies of the substances involved in food nucleic acids.

9.1 Nucleotide Analysis by the Isocratic HPLC Method

The isocratic HPLC method developed for nucleotide analysis has several advantages over the ion-pair and/or gradient elution modes which have been commonly used for research into nucleic acid components. This method has the advantages of convenience, simplicity and reproducibility of chromatographic data. The samples can be injected successively without the need for column re-equilibration as with a gradient elution mode. The life of the column with an isocratic HPLC method is much longer compared with other HPLC methods, thus making it an economically more attractive procedure. The mobile phase, containing methanol modifier in a low concentration phosphate solution, enables separation of nucleotide constituents of both RNA and DNA, and permits separation in a reasonable time.

In addition, the good resolution of 5'-GMP and its 2'- and 3'-isomers shows the importance of this HPLC method for

measurement of 5'-GMP without interference from its isomers. When acid extraction is used for nucleotide analysis of cooked foods or for RNase assay using RNA as a substrate, as done in this study, all three GMP isomers are usually present in the extract.

The method is also valuable in determining the extent of nuclease/RNase purity during enzyme isolation and purification. The purity of 5'-GMP forming nuclease without interference of RNase can be readily revealed by incubation of the enzyme with RNA in phosphate buffer of about pH 5 at optimal temperature for RNase (and not for nuclease) activity, followed by HPLC analysis. If the RNase is present in the enzyme preparation, the HPLC chromatogram will show peaks of cyclic or 2'- and 3'-GMP. It is equally valuable for determining the purity of cyclizing or 3'-nucleotide-forming RNases, i.e. the presence of contaminating nuclease.

Nuclease and RNase purities can be detected using the same HPLC procedure, and the amount of enzyme preparation required can be as little as 0.1 μg, qualifying the method as an efficient and rapid test for examining these activities during purification steps.

Good resolution of four major nucleotide constituents of RNA could be achieved by this HPLC method. Since numerous base-specific nucleases are available commercially, purity of such nuclease preparations could be controlled by this HPLC procedure. Measurement of the activity of a base-specific nuclease would be simple. Incubation of the

specific enzyme with RNA would be required, followed by quantifying the corresponding nucleotide.

9.2 Strong Cation Exchange HPLC Method

The second HPLC method developed uses a strong cation exchange column (Whatman, Partisil 10 SCX) for analysis of nucleosides and bases. It permits assaying many characteristic phosphomonoesterases present in food systems. The nucleoside of the corresponding nucleotide can be quantitatively measured as the end product of the phosphomonoesterase-catalyzed hydrolysis of a nucleotide.

9.3 Quantitation of Total Ribonucleotides by HPLC

The third method developed combines the application of the HPLC method for analysis of nucleotide composition and *in situ* enzymatic hydrolysis of RNA. The method permits quantitation of total ribonucleotides (i.e. sum of free and bound ribonucleotides of RNA) in vegetables. This is helpful in understanding the patterns of free 5'-nucleotide liberated from the polymer during processing of various vegetables.

A spectrum of ribonucleotides and their related enzymes, was found in vegetable systems. This provided information on both free and bound nucleotides and the flavor nucleotides (5'-IMP and 5'-GMP), as well as the characteristic nucleic acid enzymes involved.

Vegetables, like other biological materials, have their unique physiological properties, represented by their enzymes and nucleic acid systems. In all the vegetables studied, 5'-IMP was not found, even though its detection limit was 100 ppb under the HPLC conditions used. However, free 5'-GMP was found and its content varied amongst the cooked vegetables examined. Although the total GMP mole % in vegetables was the highest of the four major nucleotides, the ratio of free to bound 5'-GMP's measured in blanched and/or cooked vegetables was as high as 44% (Japanese dried mushroom) and as low as 0% (cabbage). The great variation in 5'-GMP contents from vegetable to vegetable can be attributed to the large difference in the quantities of bound GMP's and the individual enzyme characteristicss of the vegetables. Many types of enzymes (see Chapter 1) could participate in multiple enzymatic reactions which could occur intracellularly in the vegetables during processing. The free 5'-GMP quantitated in cooked vegetables was the sole end product remaining after successive enzymatic reactions, starting from the hydrolysis of the phosphodiester bond of RNA that possesses a guanine base residue.

As discussed above, the HPLC method allows measurement of the activity of base-specific 5'-nucleotide-forming enzymes as well as cyclic- and 3'-nucleotide-forming enzymes. However, this study concentrated on the flavor nucleotide (5'-GMP). The activity of "nuclease" was represented by the amount of 5'-GMP released enzymatically

from RNA. The "ribonuclease (RNase)" activity was defined as the amount of 2':3'-cyclic GMP or the sum of 2'- and 3'-GMP's formed.

The 5'-GMP producing enzymes in most vegetable sources had a high optimal temperature range, 70-80°C, while that of RNases was 50-60°C. Therefore, in vegetable processing, the content of natural flavor nucleotides would be maximized if heat transfer was introduced to the vegetables in such a way that the internal temperature was raised as quickly as possible to about 70°C, then maintained near that level but not higher than 80°C. In this manner, the undesirable enzymes (RNases and phosphomonoesterases) are destroyed rapidly. As a result, the substrate is better retained for the 5'-nucleotide-forming enzymes, and 5'-GMP is stable without the attack of phosphomonoesterases, which are generally heat-labile enzymes [1-3].

Continuing study of purification and characterization of vegetable enzymes will provide further understanding of hydrolysis of vegetable nucleic acids by specific enzymes, and of degradation of nucleotides by vegetable phosphomonoesterases. This should provide an interesting basis for further research.

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10. APPENDIX

10.1 Nucleotide Formation in Potato Tuber as affected by Steaming Time

The nucleotide content of raw potato tubers was negligible. During heating, various nucleotides were formed. Time of heating was related to the amount of nucleotides found in cooked potato tubers. This was demonstrated experimentally by steaming potato cubes for various lengths of time. Samples were analyzed for nucleotide content using HPLC (see sample preparation procedure in Chapter 2). The formation of 5'-nucleotides was highest after steaming for 30 min (Table 10.1). Therefore, in all experiments where steaming was necessary, the sample was steamed for 30 min.

10.2 Nucleotide Content as affected by Potato Tuber Anatomy

Duplicate samples were prepared. Potato tuber was cut longitudinally into halves. The anatomic sections of one half were selected separately, as shown in Figure 10.1. The other half was used in its entirety. After steaming for 30 min at 100°C, nucleotide extraction and analysis were as described in Chapter 2. The results obtained from two cultivars, Norgold Russet and Kennebec, are shown in Tables 10.2 and 10.3, respectively.

Table 10.1: Effect of Steaming Time on the Formation of Nucleotides in Potato Tubers (cv. Russet Burbank)

Time (min)	5'-AMP	5'-CMP	5'-UMP	5'-GMP	3'-GMP	2'-GMP
0	1.71	nd	nd	nd	nd	nd
6	15.59	8.29	13.61	9.97	11.76	5.99
10	16.52	15.27	18.84	12.11	13.58	7.51
15	19.37	18.25	25.77	12.48	14.31	7.62
20	22.30	18.56	28.26	12.26	14.59	9.36
25	25.62	25.77	29.04	14.78	15.17	10.81
30	28.12	27.69	34.44	16.78	16.43	11.24

Amount expressed in ppm per fresh basis.

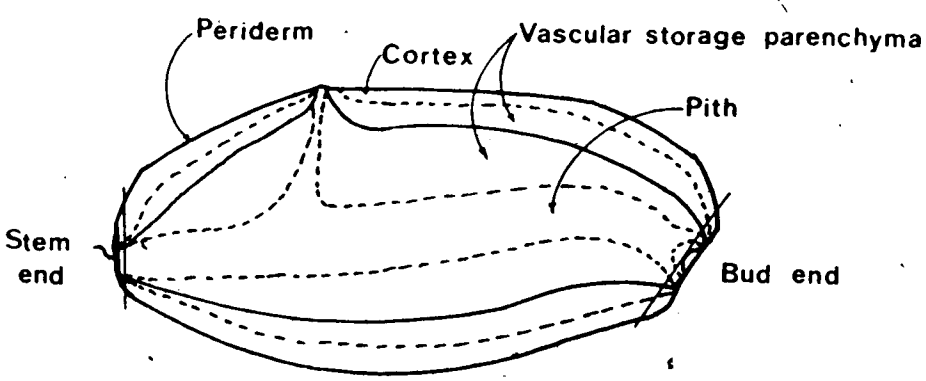


Figure 10.1. Longitudinal section of a Russet Burbank potato, showing principal structural features.

Table 10.2: Nucleotide (ppm) Distribution in the Anatomical Regions of Potato Tuber (cv. Norgold Russet)

Sample [a]	5'-AMP	5'-CMP	5'-UMP	5'-GMP	3'-GMP	2'-GMP
Whole tuber	16.27	45.62	23.45	14.33	14.99	7.46
Stem end	11.31	37.06	20.31	13.36	14.71	8.07
Bud end	15.20	64.39	19.56	15.53	25.47	12.90
Periderm + part of cortex	143.37	1031.67	Trace	42.46	16.91	13.28
Cortex	19.10	112.72	42.63	20.44	26.05	14.24
Vascular storage parenchyma	15.94	22.36	16.80	11.22	11.13	5.90
Pith	22.14	21.93	18.62	13.12	11.67	6.18

[a] Dry matter content (%): whole tuber and stem and bud ends, 24.2 ± 0.84; periderm + part of the adhering cortex, 20.1 ± 1.05; cortex (included the peel and the adjacent 5 mm thick layer of the tuber), 22.2 ± 1.00; vascular storage parenchyma + outer phloem, 26.9 ± 0.35; medulla (pith), 16.2 ± 0.94.

Table 10.3: Nucleotide (ppm) Distribution in the Anatomical Regions of Potato Tuber (cv. Kennebec)

Sample [a]	5'-AMP	5'-CMP	5'-UMP	5'-GMP	3'-GMP	2'-GMP
Whole tuber	9.58	17.18	11.49	8.90	27.28	16.19
Stem end	9.07	20,54	15.28	11.25	30.96	18.56
Bud end	4.88	24.94	11.80	9.22	31.33	16.97
Periderm	65.91	350.66	nd	14.55	17.96	13.39
Cortex	17.76	28.36	12.73	7.63	33.11	19.99
Vascular Storage Parenchyma	16.33	16.77	9.82	8.58	24.80	14.02
Pith	10.18	20.21	14.64	12.12	21.83	12.58

[a] Dry matter content: similar to data given in Table 10.2.

10.3 Changes of Nucleotides during Storage

Two cultivars, Russet Burbank and Shepody, grown in southern Alberta, were harvested in September 1985. The tubers weighed about 200 g each and were stored at 4°C. Analysis of nucleotide content was performed each two weeks over a six month period. Four tubers were sampled each time, allowing them to sit at room temperature for 24 h prior to use. The nucleotide content of each steamed tuber was extracted in duplicate, and HPLC analysis was as described in Chapter 2. These results are presented in Tables 10.4 and 10.5.

10.4 Formation and Leaching of GMP's during Preheating of French Fries

The potato tubers (cv. Russet Burbank) were sliced into 1 cm x 1 cm x 4 cm pieces as in the usual preparation of French fries. The slices were blanched in hot water for various times at various temperatures. Nucleotides in the aqueous phase and the residue were extracted separately and analysed using HPLC as described in Chapter 2. The data presented in Tables 10.6 and 10.7 are the results of the activity of a multiple enzyme system.

The RNase optimum activity was at 50-65°C, whereas nuclease was at 70-80°C. In potato slices the heat transfer is slow. As exemplified by Figure 10.2, after immersing the slice into water heated to 70°C, the core of the slice reaches 70°C only after 8 min.

Table 10.4: Nucleotide Content of Potato Tuber, cv. Russet Burbank during Storage

Time (weeks)	5'-AMP	5'-CMP	5'-UMP	5'-GMP	3'-GMP	2'-GMP
0	9.5 ± 1.2	12.4 ± 2.3	11.6 ± 1.4	17.3 ± 1.2	15.0 ± 1.8	8.6 ± 0.5
2	14.3 ± 1.3	16.1 ± 1.4	13.8 ± 1.1	16.5 ± 2.3	18.8 ± 3.2	12.2 ± 1.1
4	15.9 ± 1.5	20.8 ± 2.3	12.5 ± 0.9	14.2 ± 0.6	14.5 ± 0.4	7.6 ± 0.4
6	16.9 ± 2.2	14.5 ± 0.7	13.4 ± 0.2	15.8 ± 1.7	17.3 ± 3.8	8.7 ± 2.4
8	18.3 ± 0.4	14.8 ± 1.0	12.7 ± 0.4	15.4 ± 0.5	19.7 ± 2.3	10.2 ± 0.6
10	15.8 ± 1.4	15.5 ± 1.4	16.2 ± 1.9	13.4 ± 1.1	17.0 ± 1.2	9.7 ± 0.3
12	23.2 ± 1.6	20.6 ± 1.0	12.9 ± 1.0	15.0 ± 1.1	15.0 ± 0.6	9.5 ± 0.9
15	13.4 ± 1.8	13.0 ± 1.2	17.4 ± 1.2	11.4 ± 1.2	19.3 ± 1.8	12.7 ± 1.2
17	13.2 ± 1.6	—	19.1 ± 1.2	14.5 ± 1.4	25.9 ± 2.3	15.9 ± 1.6
24	7.1 ± 0.6	—	10.7 ± 0.1	7.4 ± 0.8	13.5 ± 1.8	7.4 ± 1.0

Amount expressed in ppm (fresh basis). At harvest time, the moisture content of potato tuber was 77.8 ± 1.2%.

Table 10.5: Nucleotide Content of Potato Tuber, cv. Shepody during Storage

Time (weeks)	5'-AMP	5'-CMP	5'-UMP	5'-GMP	3'-GMP	2'-GMP
0	23.4 ± 3.2	10.4 ± 1.2	19.5 ± 2.1	22.9 ± 1.6	21.8 ± 2.8	13.1 ± 0.6
2	24.4 ± 1.5	30.2 ± 2.8	18.9 ± 1.1	24.2 ± 0.8	19.3 ± 1.4	10.6 ± 1.8
4	37.1 ± 3.8	28.6 ± 2.9	24.6 ± 1.8	29.5 ± 1.7	20.4 ± 3.8	11.5 ± 2.3
6	31.0 ± 1.8	18.1 ± 0.4	17.8 ± 0.1	25.3 ± 1.7	21.5 ± 0.6	11.9 ± 1.0
8	26.9 ± 0.8	21.4 ± 2.3	20.2 ± 1.9	24.7 ± 2.1	21.2 ± 1.8	11.0 ± 1.3
10	27.1 ± 0.9	20.7 ± 0.7	19.5 ± 1.1	23.3 ± 0.4	19.9 ± 0.1	11.3 ± 0.1
12	28.3 ± 4.7	26.7 ± 2.5	22.0 ± 1.6	22.5 ± 2.8	19.5 ± 1.0	11.6 ± 0.8
17	27.8 ± 0.9	24.4 ± 0.5	22.3 ± 0.9	25.6 ± 0.4	13.6 ± 0.8	6.2 ± 1.3
19	26.6 ± 0.7	19.9 ± 1.3	20.4 ± 0.5	20.1 ± 1.1	13.5 ± 0.9	7.8 ± 0.6
26	30.7 ± 1.6	20.4 ± 1.5	23.7 ± 1.6	21.3 ± 0.5	18.8 ± 0.5	10.2 ± 0.7

Amount expressed in ppm (fresh basis). At harvest time, the moisture content of potato tuber was 77.4 ± 1.7 %.

Table 10.6: GMP's (ppm) remaining in Potato Slices (cut for French Fries) after Blanching for Various Times at Various Temperatures

Temp. (°C)	GMP's	Time (min)				
		10	15	20	25	30
65	5'-	0.00	0.00	0.00	0.57	—
	3'-	7.44	4.54	0.94	0.00	—
	2'-	3.00	2.31	1.07	0.32	—
70	5'-	3.22	5.22	3.31	4.60	7.28
	3'-	4.35	1.80	1.52	0.71	0.50
	2'-	2.42	2.58	0.65	0.65	1.09
75	5'-	2.67	4.23	5.76	4.26	4.07
	3'-	7.33	4.14	1.71	1.22	1.66
	2'-	3.15	1.99	1.20	1.62	1.59

Table 10.7: GMP's (ppm) leached to the Aqueous Phase after Blanching of Potato Slices (for French Fries) for Various Times at Various Temperatures

Temp. (°C)	GMP's	Time (min)				
		10	15	20	25	30
65	5'-	0.00	0.60	0.56	0.86	—
	3'-	2.20	1.81	0.99	0.71	—
	2'-	1.12	1.17	0.72	0.69	—
70	5'-	1.34	3.45	2.78	4.16	6.05
	3'-	1.76	1.14	0.94	1.35	0.82
	2'-	1.20	0.63	0.65	0.82	0.93
75	5'-	1.07	2.65	2.96	2.60	2.65
	3'-	2.53	1.87	1.38	1.41	1.47
	2'-	1.16	0.99	0.95	1.04	0.81

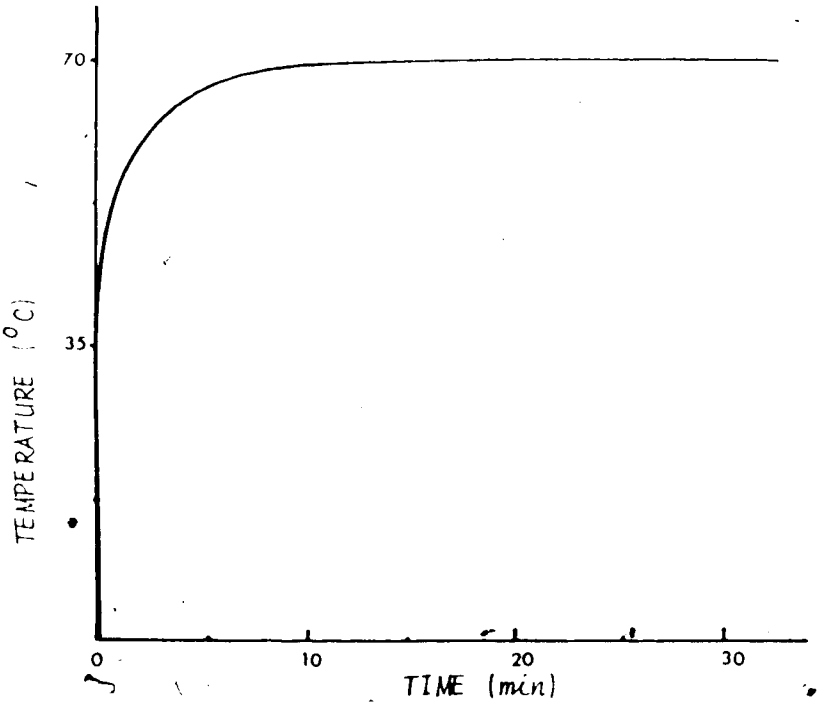


Figure 10.2. Internal temperature of slices cut for French fries vs time of blanching in hot water (70°C). A thermocouple was placed in the center of the slice to follow the increase of its internal temperature during blanching.

The same trend in the content of nucleotides retained in slices and of nucleotides leached into water might also indicate an enzyme leaching. The possibility of nucleotide hydrolysis at the potato pH of 6.3 is excluded. These finding warrant further study.

10.5 Effect of Size and Maturity on the Nucleotide Content of Potato Tubers

The potato tubers (cv. Norland) examined were grown at the University of Alberta Farm in Edmonton. The tubers were harvested from the same potato hill (plant) and were analyzed for nucleotide content versus tuber size. The nucleotide contents were determined for tubers harvested from various hills 82, 97 and 118 days after planting. Samples were steamed for 30 min at 100°C. Nucleotide extraction and analysis was performed by the method described in Chapter 2. The results obtained are presented in Tables 10.8 and 10.9.

10.6 Effect of Sodium Chloride on the Activity of Nuclease and RNase of Potato Tubers

Potato tubers (cv. Pontiac) were cut into small cubes and mixed to give a homogeneous sample. The potato cubes were cooked for 30 min by various heating regimens, including boiling starting with cold water, boiling water, boiling 1% sodium chloride solution, and steaming at 100°C. The nucleotides were extracted and analyzed by HPLC as

Table 10.8: Nucleotide Content of Potato Tubers cv. Norland of Various Sizes obtained from the same Hill

Tuber size (g)	5'-AMP	5'-CMP	5'-UMP	5'-GMP	3'-GMP	2'-GMP
5	43.25	18.73	26.48	13.92	23.46	12.59
50	33.25	21.00	12.34	13.62	33.73	19.12
80	24.10	17.20	18.42	12.25	20.18	14.13
120	21.42	14.98	16.77	10.56	17.60	8.53
170	22.27	18.19	20.70	11.23	15.13	9.30

Harvested at maturity of 118 days. Amount expressed in ppm (fresh basis). Average of three tubers examined.

Table 10.9: Nucleotide Content of Potato Tubers cv. Norland of the same Size (about 100 g) but after different Growth Periods and obtained from Various Hills

Tuber maturity (days)	5'-AMP	5'-CMP	5'-UMP	5'-GMP	3'-GMP	2'-GMP
82	17.80	26.78	19.87	8.51	28.48	13.70
97	21.41	20.31	19.40	10.55	19.94	12.82
118	21.42	14.98	16.77	10.56	17.60	8.53

Amount expressed in ppm (fresh basis). Average of three tubers examined.

described in Chapter 2. The results are presented in Table 10.10.

10.7 Steaming Time vs Temperature Changes in Sample steamed in a Test Tube

The steam-cooked samples were usually heated in a test tube using a blanch-steamer. Data presented in Figure 10.3 and Table 10.11 show the correlation between heat transfer and steaming time.

10.8 Kinetic Studies of Potato Tuber Enzymes

For these studies, standard assay conditions outlined in previous chapters were applied, and the statistical method of Wilkinson [1] was used for the computer-generated plots. Figures 10.4 and 10.5 show the results of steady-state kinetic studies for cytoplasmic and cell wall 3'-nucleotidases, respectively. Figure 10.6 presents the kinetic data for cell wall nuclease. Two substrates, yeast RNA and polyA, were used for kinetic studies. As shown in Figures 10.7 and 10.8, respectively, the K_m value of RNase when polyA was the substrate was less than the K_m when yeast RNA was the substrate. Thus, polyA would be a better candidate as substrate for RNase assay. Moreover, in the enzyme assay using PCA extraction, use of polyA as substrate yields cyclic 2':3'-AMP, along with its breakdown products, 2'- and 3'-AMP. These isomers and the parent compound are well resolved in the HPLC system, and the separation period

Table 10.10: GMP Yield (ppm) obtained from Potato Tubers cv. Russet Burbank after Various Heating Regimens

Sample	2'-GMP	3'-GMP	5'-GMP
Raw	nd [a]	nd	nd
Steamed	14.16	22.05	17.80
Boiled / boiling water	nd	3.66	18.40
Boiled / cold water	6.19	7.46	18.88
Boiled / boiling 1% NaCl solution	nd	2.33	22.61

[a] nd: Not detected.

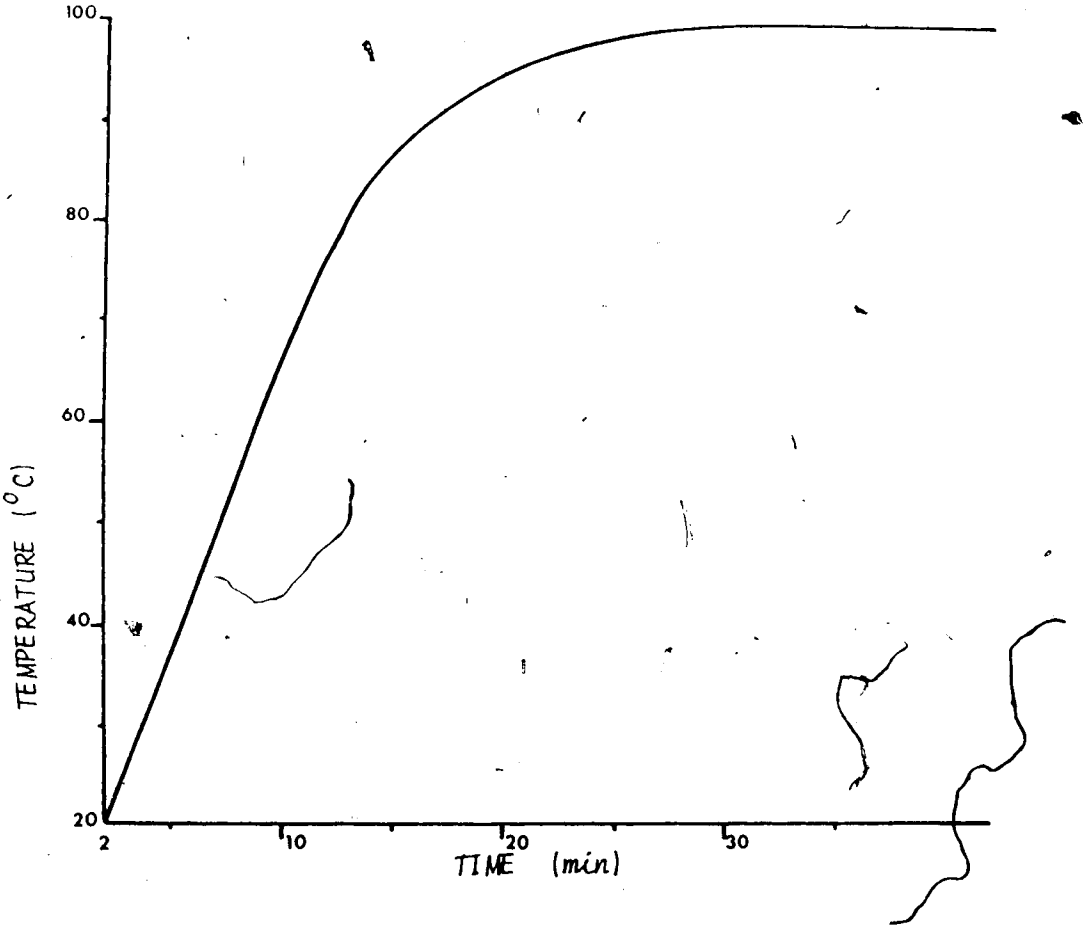


Figure 10.3. Changes of temperature inside a potato slurry in a test tube vs time of steaming at 100°C. A thermocouple was placed in the center of the test tube containing 50 mL of potato slurry, with initial temperature of 4°C. The changes of temperature were recorded by an electronic data logger.

Table 10.11: Changes of Temperature vs Time of Steaming at 100°C
(Ref. to Fig. 10.3)

Time (min)	Temperature (°C)	Time (min)	Temperature(°C)
0:00	4.0	10:00	75:0
2:00	20.0	11:00	80.0
2:42	25.0	12:20	85.0
3:27	30.0	13:45	88.0
4:06	35.0	14:15	90.0
4:48	40.0	15:15	92.0
5:28	45.0	17:00	95.0
6:08	50.0	18:40	96.0
6:24	52.0	19:40	97.0
6:52	55.0	20:00	97.5
7:32	60.0	20:40	98.0
8:20	65.0	22:40	98.5
9:08	70.0	25:00	98.5
9:28	72.0	26:00 - 30:00	99.0

is short, only 27 min.

10.9 Reference

1. Wilkinson, G.N. Biochem. J. 81 (1961) 324.

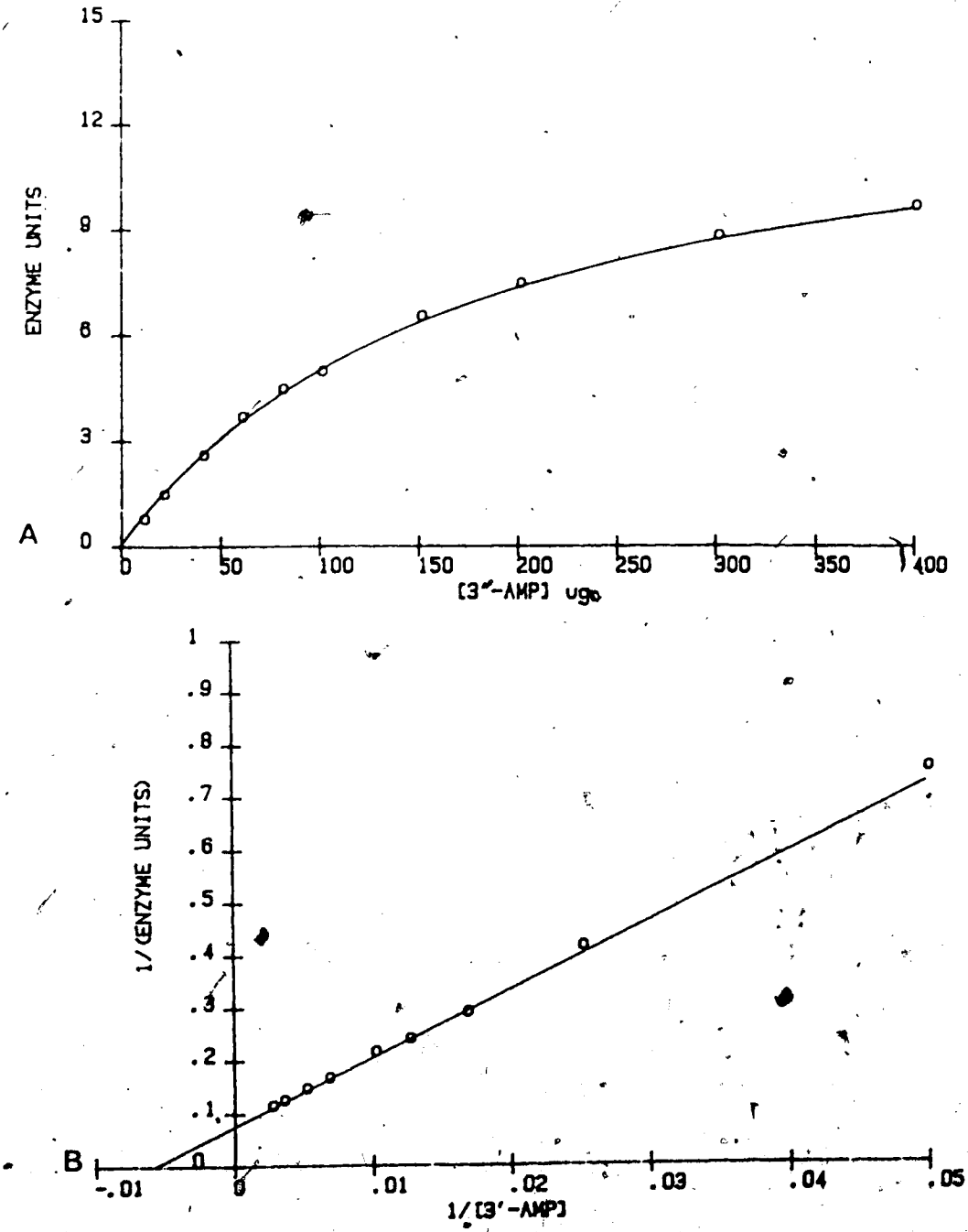


Figure 10.4. Michaelis-Menten (A) and Lineweaver-Burk (B) plots of kinetic data of cytoplasmic 3'-nucleotidase from potato tubers. V_{max} = 13.8±0.2 and K_m = 0.52±0.01 μmoles 3'-AMP.

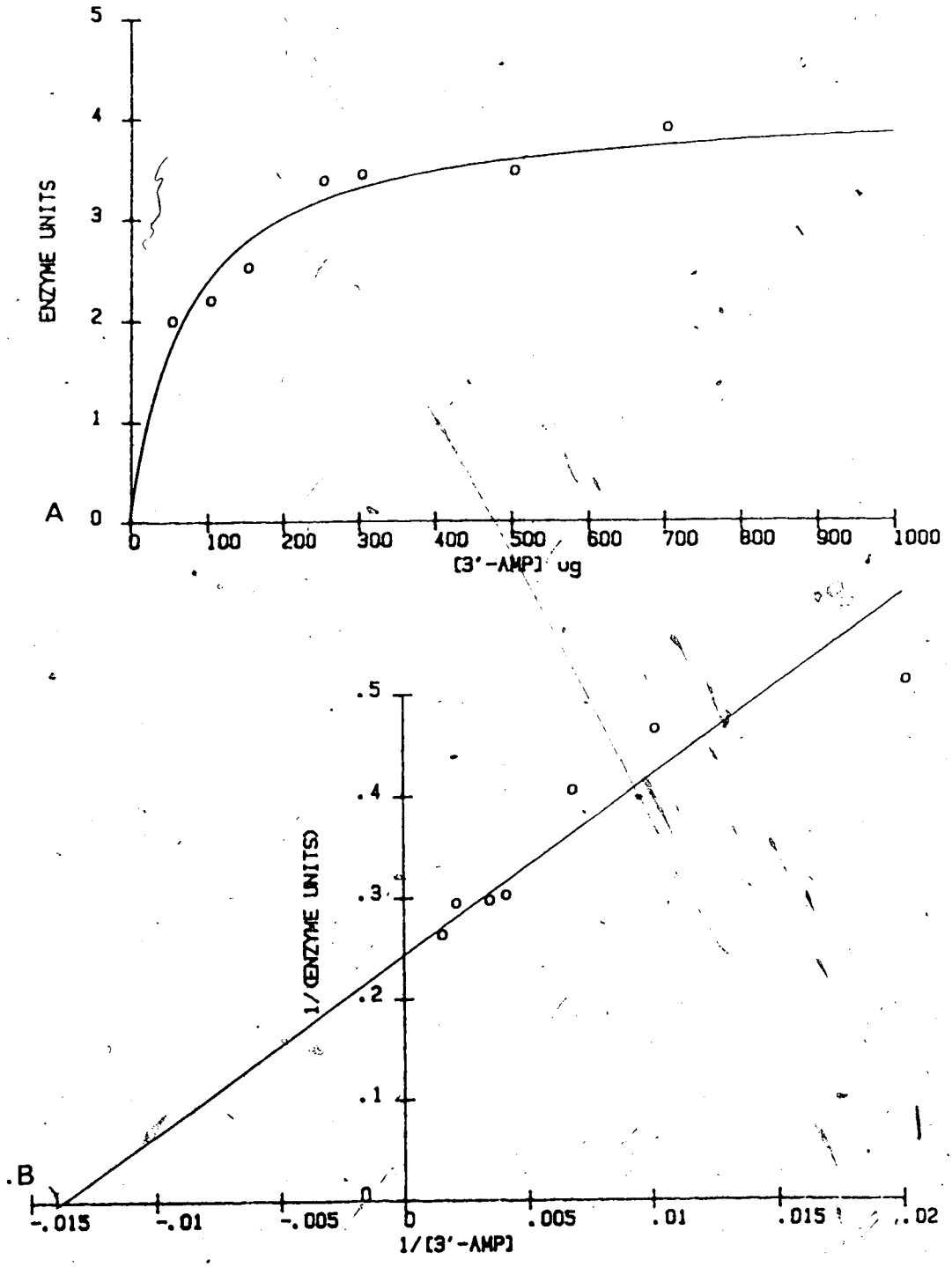
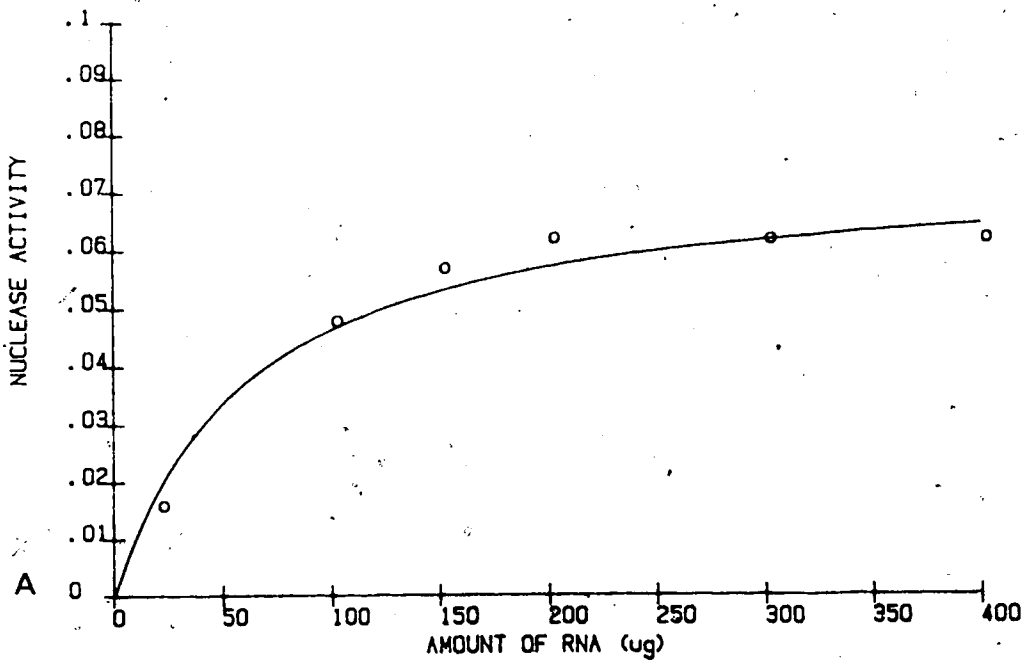


Figure 10.5. Michaelis-Menten (A) and Lineweaver-Burk (B) plots of kinetic data of cell wall 3'-nucleotidase from potato tubers. V_{max} = 4.1±0.2 and K_m = 0.21±0.04 μmoles 3'-AMP.



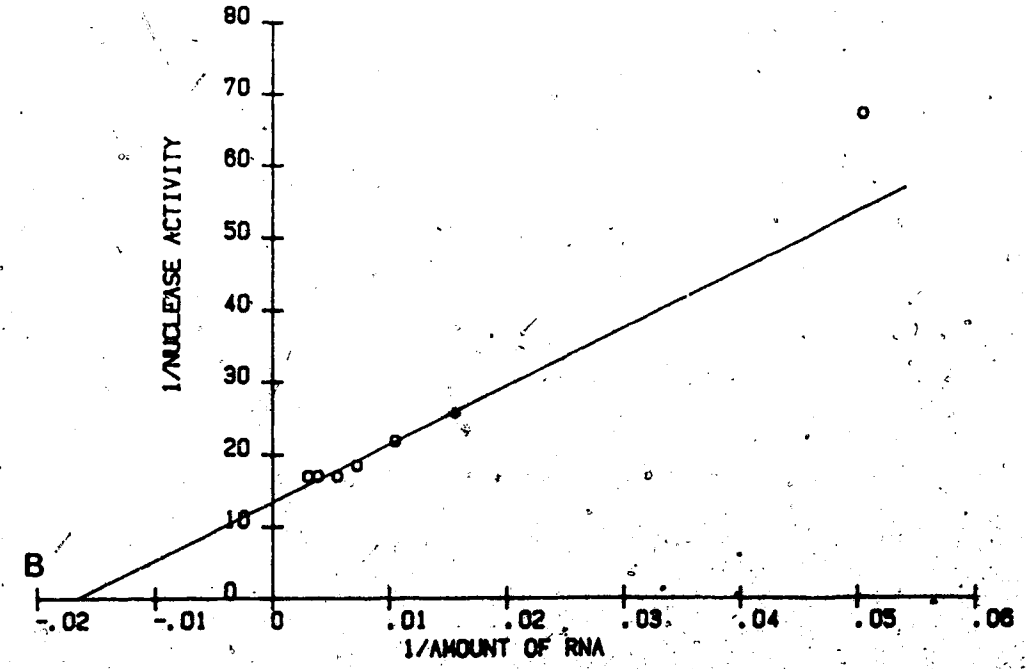


Figure 10.6. Michaelis-Menten (A) and Lineweaver-Burk (B) plots of kinetic data of cell wall nuclease from potato tubers. V_{max} = 0.074±0.004 and K_m = 82.1±19.0 µg yeast RNA.

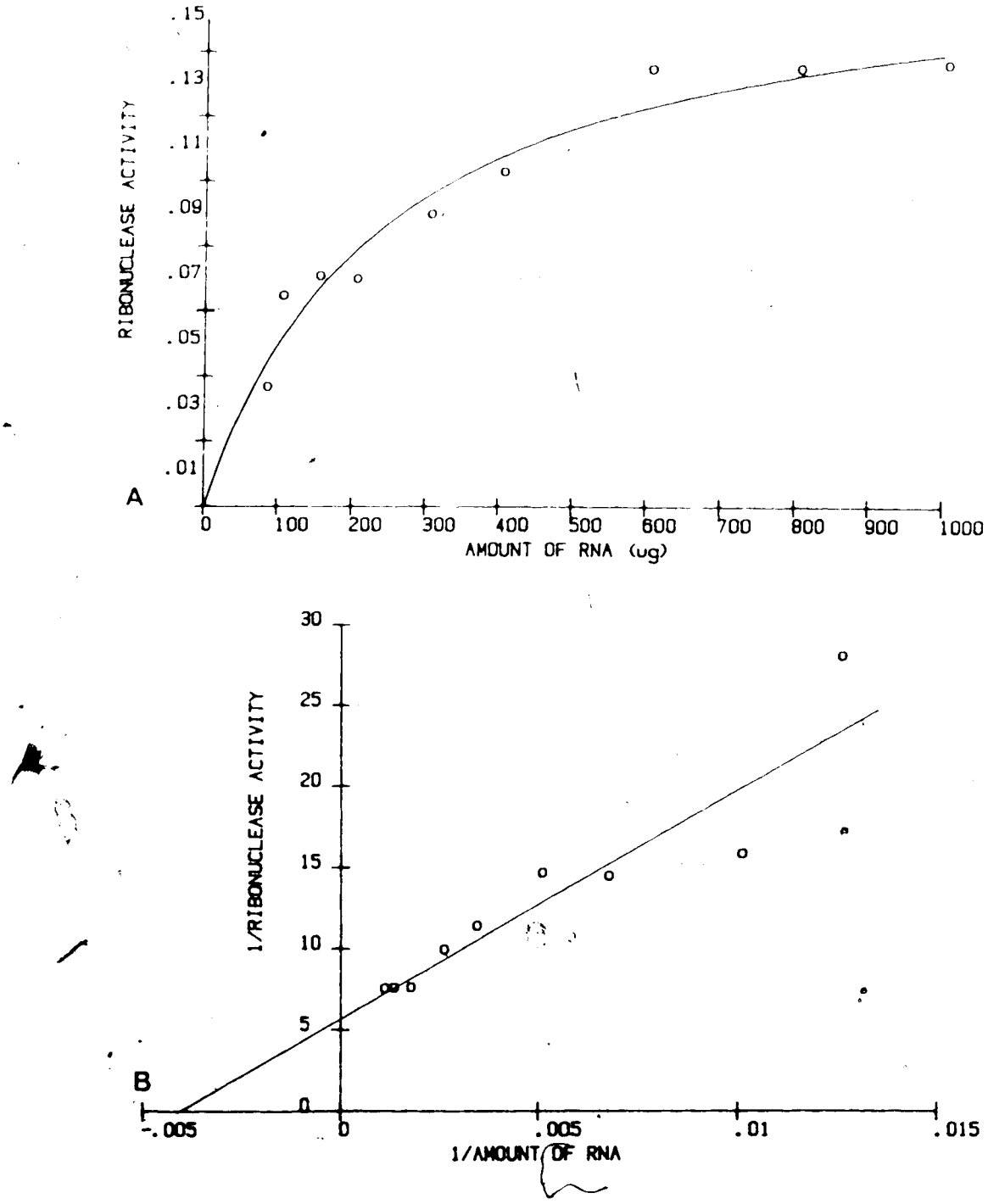


Figure 10.7. Michaelis-Menten (A) and Lineweaver-Burk (B) plots of kinetic data of cell wall ribonuclease from potato tubers. V_{max} = 0.173±0.011 and K_m = 242.5±39.7 µg yeast RNA.

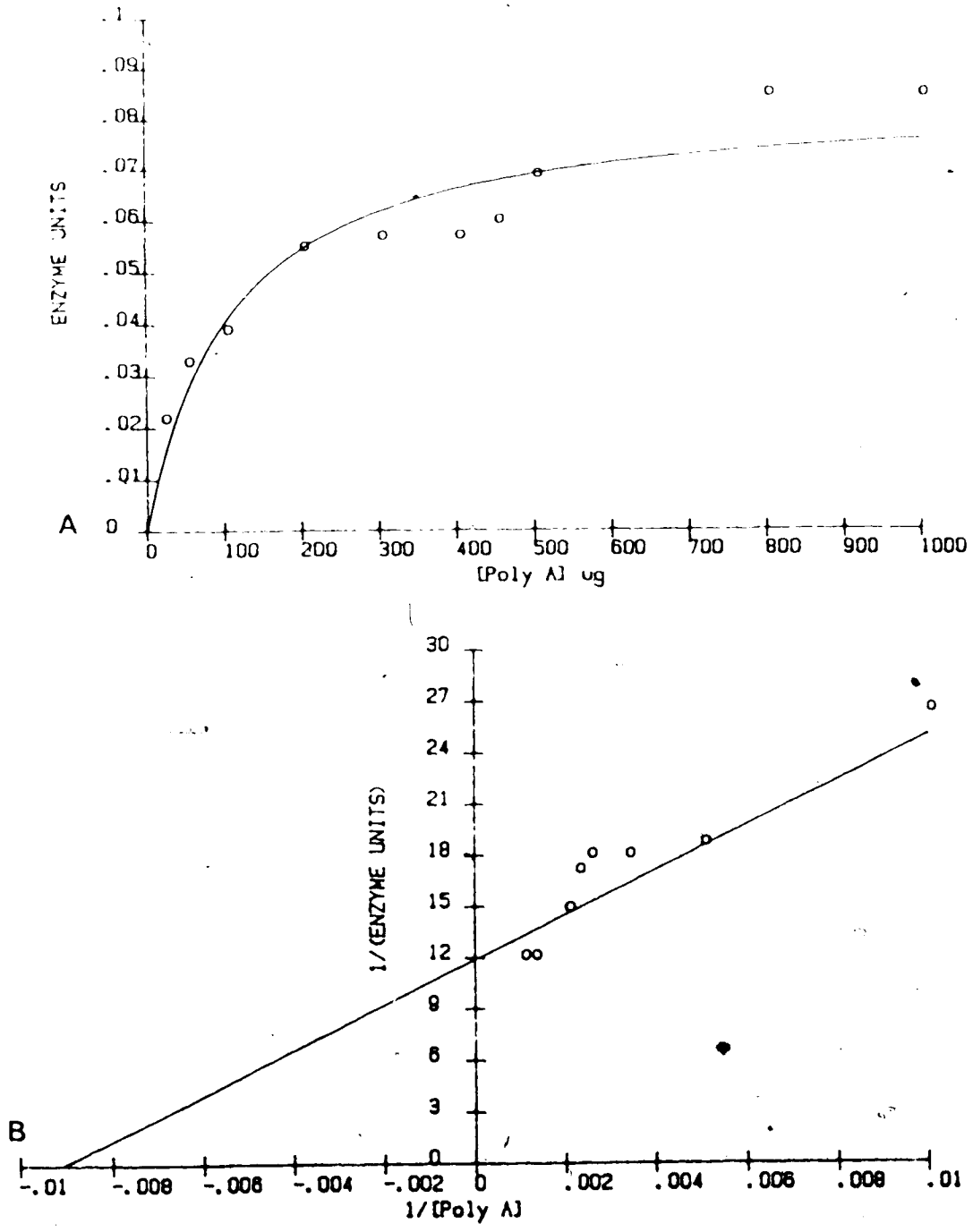


Figure 10.8. Michaelis-Menten (A) and Lineweaver-Burk (B) plots of the kinetic data of cell wall ribonuclease from potato tubers. V_{max} = 0.084±0.006 and K_m = 110.0±29.8 µg polyA.