Journal of Chromatography, 391 (1987) 257–265
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 268

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF POTATO TUBER 3'-NUCLEOTIDASE

T. T. NGUYEN*, M. M. PALCIC and D. HADZIYEV
Department of Food Science, University of Alberta, Edmonton, Alberta, T6G 2P5 (Canada)
(First received September 10th, 1986; revised manuscript received November 11th, 1986)

SUMMARY

A rapid and reproducible high-performance liquid chromatographic method is presented for assaying the activity of 3'-nucleotidase isolated from potato tubers. Nucleotide substrates were incubated with the enzyme and the activity was determined by measuring the concentration of nucleosides as reaction products. The relative rate of substrate hydrolysis was 3'-AMP > 3'-UMP > 3'-GMP > 3'-CMP > 3'-dAMP. The optimal enzyme temperature was 75°C and the optimal pH 6.5 when 3'-AMP was used as a substrate. Enzyme inhibition was observed in the presence of EDTA, phosphate anion, zinc chloride and the flavor-enhancing nucleotide 5'-GMP. This assay could also be used for the simultaneous determination of amino- or keto nucleotides and deoxynucleotides as substrates.

INTRODUCTION

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

In our studies 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 paper, 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 latter, the only substrates and products measured were pyrimidine-5'-nucleotides and their deoxy analogues. 3'-Nucleotidase exhibits broad substrate specificity, acting preferentially on the purine nucleotide 3'-AMP and on pyrimidine nucleotides[9]. The HPLC method reported here is rapid and accurate and has been used for the characterization of potato 3'-nucleotidase properties, including substrate specificity, pH and temperature optima, and for some potential inhibitor evaluations.

0021-9673/87/$03.50

EXPERIMENTAL

Chromatographic equipment

The HPLC system consisted of a Model 1330 pump (Bio-Rad Labs., Richmond, CA, U.S.A.), a Bio-Rad Model 1305 variabele-wavelength detector and a 50-μl fixed volume Rheodyne sample injector loop. A Whatman Partisil SCX column (Whatman, Clifton, NJ, U.S.A.), 250 × 4.6 mm I.D., was used for the separation of nucleosides; this column was used in conjunction with a 70 × 2.1 mm I.D. guard column containing a pellicular cation exchanger and a 250 × 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, U.S.A.).

HPLC separation conditions

The mobile phase was 10 m*M* potassium phosphate buffer (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.

Materials

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

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 m*M* sodium citrate buffer (pH 6.0) containing 2 m*M* cysteine, homogenate filtration and starch gravity sedimentation, followed by chromatography of the clarified supernatant on a Sephadex G-100 column[5].

Enzyme assay conditions

Assay mixtures contained 200 μg of 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 × 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 8 m*M* 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 was 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 m*M* sodium acetate buffer) injected before and after sample separations.

One unit of enzyme was defined as the amount forming 1 nmol of adenosine per minute. Protein concentrations were determined with the Bio-Rad assay kit based on Bradford's method[11], using bovine serum albumin as a standard. The specific activity of 3'-nucelotidase 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.) and casting discontinuous gels consisting of a resolving and a stacking gel 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) kdaltons were supplied by Sigma.

RESULTS AND DISCUSSION

HPLC separation

Reversed-phase HPLC is widely used in the analysis of nucleosides and nucleobases[13–17]. However, their separation by ion-exchange methods still shows its power when cation-exchange columns are applied. In previous work[18], a Whatman SCX column and 10 m*M* phosphate buffer (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 to the assay of 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 I and the linear responses and detection limits for standard nucleosides are given in Table II. A typical chromatogram of an incubation mixture of 3'-AMP and 3'-nucleotidase is shown in Fig. 1.

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 (Fig. 2), in contrast to pH 8 as reported by Nomura *et al.*[9]. The optimal temperature for a 10 min incubation time was between 70 and 75°C (Fig. 3),

TABLE I

RETENTION TIMES OF NUCLEOTIDE SUBSTRATES AND CORRESPONDING NUCLEOSIDE PRODUCTS OF 3'-NUCLEOTIDASE-CATALYZED HYDROLYSIS

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

TABLE II

LINEARITY OF PEAK-HEIGHT MEASUREMENTS FOR NUCLEOSIDES IN THE CONCENTRATION RANGE 0.050–10.0 μg/ml

Type	*Nucleoside*	*Correlation coefficient*	*Regression equation**	*Detection limit (ppb)*
Ribonucleosides	Adenosine	0.9999	$y = 910x - 43$	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
	Thymidlne	0.9999	$y = 1264x - 12$	10

* x = Concentration of standard solution (μg/ml); y = peak height.

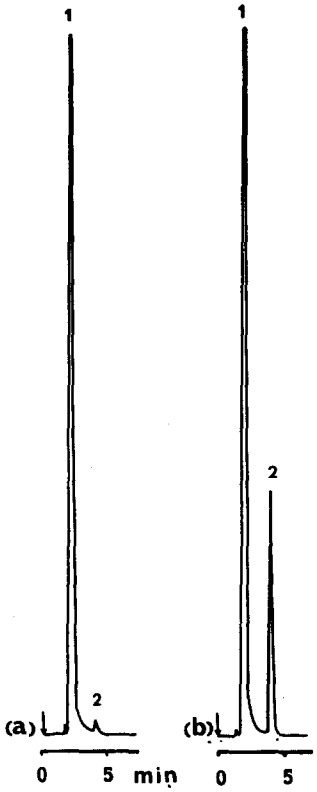

Fig. 1. Chromatograms of the incubation solutions of 3'-AMP as substrate (a) without (blank) and (b) with potato tuber 3'-nucleotidase. Peaks: 1 = 3'-AMP; 2 = adenosine.

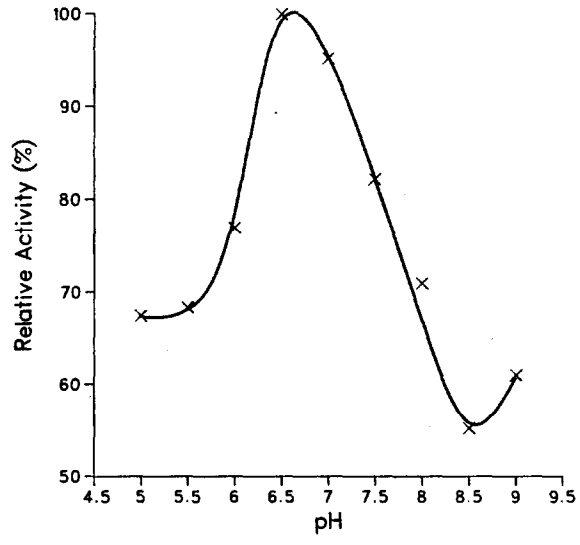

Fig. 2. Effect of pH on tuber 3'-nucleotidase activity. Buffer: 0.1 *M* sodium acetate.

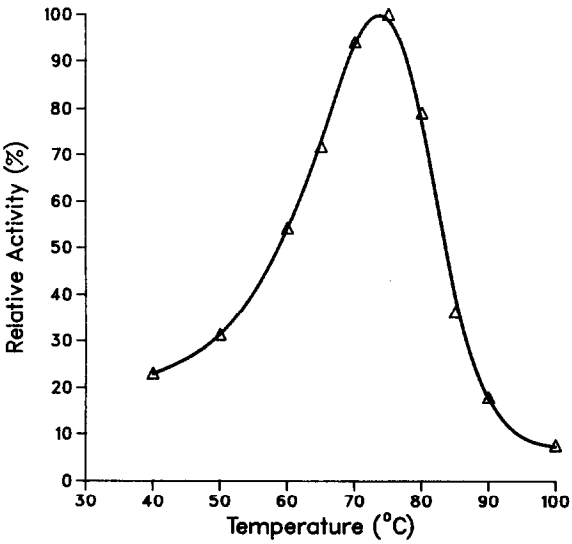

Fig. 3. Effect of temperature on tuber 3'-nucleotidase activity. The incubation mixture contained 3'-AMP as a substrate in 0.1 *M* sodium acetate buffer (pH 6.5). After termination of the reaction the adenosine yield was determined.

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 (Fig. 4). The Michaelis–Menten constant (K_m) for 3'-AMP under our conditions was 0.5 μ*M* compared with 0.72 μ*M* (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 were used, which is 2.6 times the K_m of 3'-AMP. Higher concentrations of substrate caused tailing of the nucleotide peaks and overlapping with the product nucleosides, causing difficulties in quantitation.

Substrate specificity of 3'-nucleotidase

Substrate specificity data for this enzyme are summarized in Table III. 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, whereas 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, corroborating the data reported by Nomura *et al.*[9]. The rate of hydrolysis of deoxyadenosine-3'-monophosphate (3'-dAMP) was much lower than that of 3'-AMP (Table III), suggesting that the enzyme's active site requires the presence of a 2'-hydroxy group. The 3'-nucleotidase activity was associated with enzyme preparation 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 kdaltons. Nomura *et al.*[9] attempted

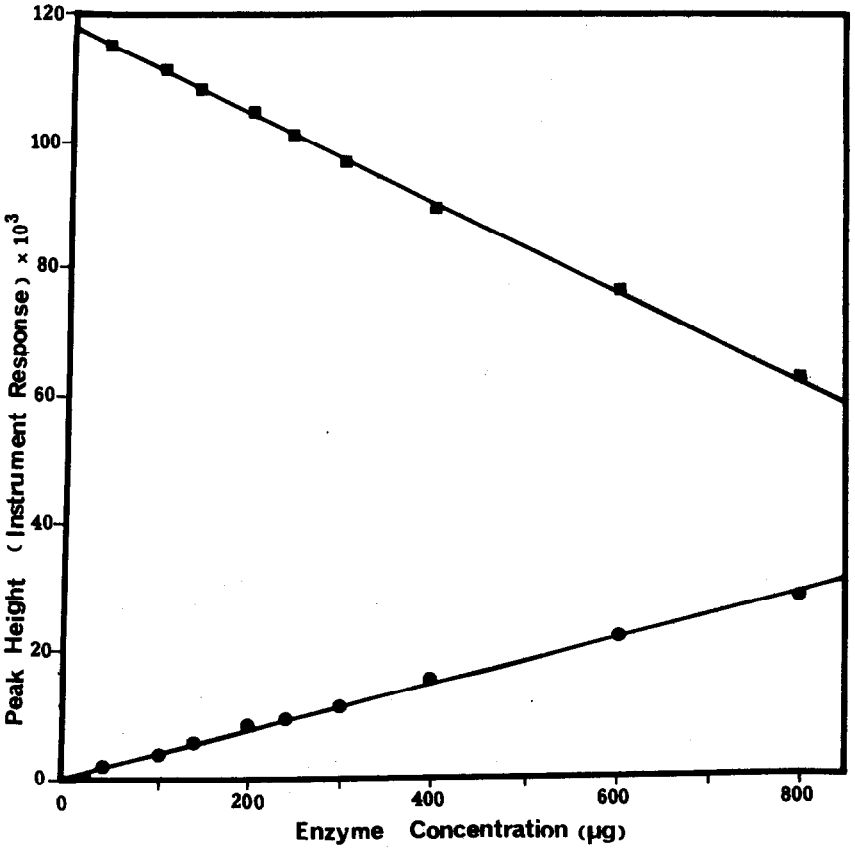

Fig. 4. Linearity in the relationship between the substrate concentration (3'-AMP) and concomitant increase in the yield of 3'-nucleotidase reaction product (adenosine). ■ = 3'-AMP; ● = adenosine.

TABLE III

3'-NUCLEOTIDASE-CATALYZED HYDROLYSIS OF VARIOUS NUCLEOTIDES

Calculation is based on the amount of nucleoside in nanomoles formed during incubation for 10 min at 70°C. The result is expressed as a percentage of the amount of adenosine formed in the enzyme-catalyzed reaction with 3'-AMP as substrate.

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

TABLE IV

EFFECT OF SOME ANIONS, CATIONS, GLUTAMATE AND EDTA ON THE ACTIVITY OF POTATO 3'-NUCLEOTIDASE

Addition to incubation mixture	*Concentration (mM)*	*Relative activity versus 3'-AMP substrate (%)*
Control		100
NaH_2PO_4	10	53
NaH_2PO_4	100	17
$NaHSO_3$	10	151
NaCl	10	98
LiCL	10	100
Sodium glutamate	10	115
$MnCl_2$	1	90
$ZnCl_2$	1	10
$MgCl_2$	1	106
EDTA	1	25
	0.1	62

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 results 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].

Inhibitor evaluations

The effects of some ions and additives used in food processing were tested at concentrations from 0.1 to 100 m*M* (Table IV). Chloride ion added as NaCl (10 m*M*) had no influence, whereas monosodium glutamate and sulfite promoted 3'-nucleotidase activity. The chelator EDTA was found to be a strong inhibitor. At a concentration of 1 m*M* it decreased the activity of nucleotidase to 25% of a control. Phosphate ion and zinc chloride were also inhibitory, but magnesium chloride had little (6%) effect on enzyme activity. Similarly, manganese chloride had a 10% inhibitory effect whereas 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 (Fig. 5).

Multiple substrate assay

As the nucleosides of the oxo and amino groups and also ribo- and deoxyribonucleosides were well resolved from one another (Table I), a multiple-substrate assay could be performed. Fig. 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.

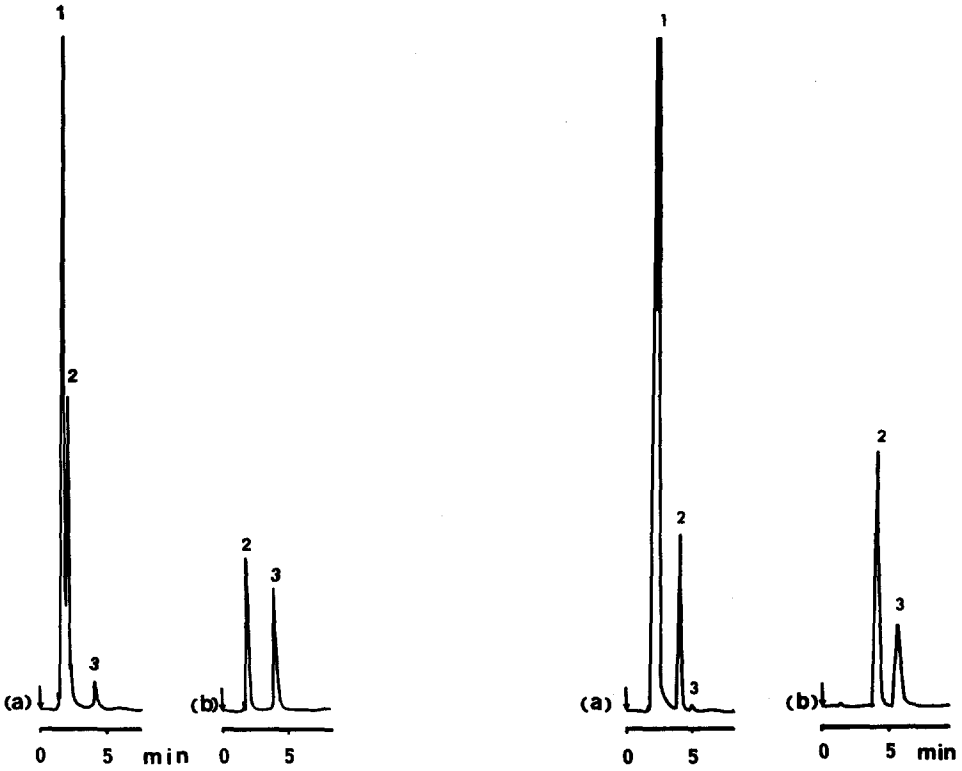

Fig. 5. Inhibition of 3′-nucleotidase activity with 5′-GMP. Incubation mixture of tuber 3′-nucleotidase (200 μg) contained (a) 3′-AMP (50 μg) and 5′-GMP (600 μg) and (b) only 3′-AMP (50 μg). Peaks: 1 = 5′-GMP; 2 = 3′-AMP; 3 = adenosine.

Fig. 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 m*M* sodium acetate buffer. Peaks: 1 = 3′-AMP + 5′-dAMP; 2 = adenosine; 3 = deoxyadenosine.

CONCLUSIONS

An HPLC method using an ion-exchange Partisil SCX column has been developed for assaying potato tuber 3′-nucleotidase activity. This method is accurate and rapid. The 3′-nucleotidase was associated with tuber nuclease activity in the isolated and purified enzyme preparations and was found to be relatively stable to heat and specific for the 3′-phosphomonoester bond. The accuracy and precision of the method were demonstrated by five fully repeated analyses of the activity on 3′-AMP which provided 12.95 ± 0.03 enzyme units. The method presented can also be used for the simultaneous assay of purine or pyrimidine ribo- and deoxyribonucleotides as 3′-nucleotidase substrates.

REFERENCES

1 M. Zakaria and P. R. Brown, *J. Chromatogr.*, 226 (1981) 267.
2 P. R. Brown (Editor), *HPLC in Nucleic Acid Research —Methods and Application*, Marcel Dekker, New York, 1984.
3 R. C. Simpson and P. R. Brown, *J. Chromatogr.*, 379 (1986) 269.
4 T. Robinson (Editor), *The Organic Constituents of Higher Plants —Their Chemistry and Interrelationships*, Cordus Press, North Amherst, MA, 3rd ed., 1975, pp. 258–277.
5 T. T. Nguyen, M. M. Palcic and D. Hadziyev, *J. Chromatogr.*, 388 (1987) 189.
6 R. J. L. Allen, *Biochem. J.*, 34 (1940) 856.
7 T. Sakai, S. Yanagihara and K. Ushio, *J. Chromatogr.*, 239 (1982) 717.
8 L. Cook, M. Schafer-Mitchell, C. Angle and S. Stohs, *J. Chromatogr.*, 339 (1985) 293.
9 A. Nomura, M. Suno and Y. Mizuno, *J. Biochem. (Tokyo)*, 70 (1971) 993.
10 E. Dumelin and J. Solms, *Potato Res.*, 19 (1976) 215.
11 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
12 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
13 C. W. Gehrke, K. C. Kuo, G. E. Davis, R. D. Suits, T. P. Waalkes and E. Borek, *J. Chromatogr.*, 150 (1978) 455.
14 R. A. Hartwick, S. P. Assenza and P. R. Brown, *J. Chromatogr.*, 186 (1979) 647.
15 R. Boulieu, C. Bory and C. Gonnet, *J. Chromatogr.*, 339 (1985) 380.
16 A. M. Krstulovic, R. A. Hartwick and P. R. Brown, *Clin. Chim. Acta*, 97 (1979) 159.
17 G. A. Taylor, P. J. Dady and K. R. Harrap, *J. Chromatogr.*, 183 (1980) 421.
18 T. T. Nguyen, *MSc Thesis*, University of Alberta, Edmonton, 1984.
19 M. Suno, A. Nomura and Y. Mizuno, *J. Biochem. (Tokyo)*, 72 (1973) 1291.
20 G. N. Wilkinson, *Biochem. J.*, 80 (1960) 324.
21 A. J. Mikulski and M. Laskowski, Sr., *J. Biol. Chem.*, 245 (1970) 5026.
22 C. M. Wilson, *Annu. Rev. Plant Physiol.*, 26 (1975) 187.