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LIQUID CHROMATOGRAPHY ASSAY FOR POTATO TUBER NUCLEASE AND RIBONUCLEASE*

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SUMMARY

Isocratic liquid chromatography methods have been developed for assaying the activity of potato tuber nuclease and ribonuclease (RNase). The methods are rapid, accurate and can be used to quantitate the activity of these enzymes on a variety of substrates. Potato nuclease hydrolyzed polynucleotides to yield 5′-ribo- or 5′-deoxyribo-nucleotides. Hydrolysis rates were in the order poly U > poly A > RNA > poly C > poly G > DNA. RNase activity assayed with RNA or nucleotide homopolymers as substrate provided solely cyclic 2′:3′-nucleotides. The hydrolysis rate of poly U exceeded that for other polymers. Further hydrolysis of nucleoside 2′:3′-cyclic monophosphates proceeded at a very slow rate resulting in some 2′- and preferentially 3′-nucleotides of both purine and pyrimidine bases. By applying the acid extraction procedure developed recently for nucleotide analysis of raw and processed potatoes, the presence of 2′- and 3′-nucleotides in processed potatoes could be attributed to the chemical breakdown of cyclic nucleotides.

INTRODUCTION

Plants contain several enzymes which hydrolyze ribonucleic acid (RNA)[1]. These 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 commonly used methods 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,

* The work is dedicated to professor Saul Zalik, Department of Plant Science, on his 65th birthday. He was one of the first to initiate plant nucleic acid research at this University.

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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].

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 activity. 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.

EXPERIMENTAL

Materials

All standard compounds and the following substrates were supplied by Sigma (St. Louis, MO, U.S.A.): 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, U.S.A.). LC-grade water was prepared by reverse osmosis (Milli-RO) and further purified by using a Milli-Q-system (Millipore, Bedford, MA, U.S.A.). All other solvents and chemicals were reagent grade.

LC system

Two different HPLC systems were used, a Beckman Model 110 pump (Beckman Instr., Fullerton, CA, U.S.A.) with a Laboratory Data Control spectromonitor III UV detector and/or a Bio Rad Model 1330 pump (Bio Rad, Richmond, CA, U.S.A.) 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 3380 A integrator. A Whatman Partisil SAX column (25 cm × 4.6 mm I.D.) was used for the separation of nucleotides; the column being protected by a 7 cm × 2.1 mm I.D. guard column containing a pellicular anion exchanger and by a 25 cm × 4.6 mm I.D. pre-injector column (Solvecon) containing silica gel.

LC separation conditions

The mobile phase for the isocratic analysis was 8 m*M* potassium phosphate buffer 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.

Enzyme isolation

Enzymes were isolated from peeled potatoes cv. Pontiac, grown commercially in Southern Alberta. The extraction procedure was a modification of the methods described by 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 m*M* sodium citrate, pH 6.0, containing 2 m*M* cysteine) and were homogenized in a Waring blender. The

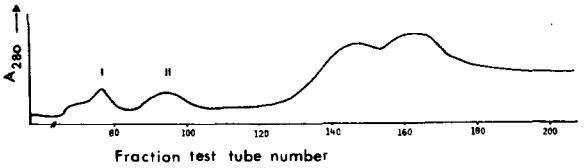


Fig. 1. Separation profile of potato tuber crude enzymes on a Sephadex G-100 column. Fractionation was done by LC-grade water collecting 3 ml/tube. Combined fractions designated as peak I (preparation I) had both nuclease and 3′-nucleotidase activities, while the subsequent fraction, peak II (preparation II) had only RNase activity.

homogenate was filtered under suction through 4-layers of gauze. 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 a RePP Model Freeze Dryer (The Virtis Co., Gardiner, NY, U.S.A.). 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 *g* and 10 ml of the supernatant was loaded on a Sephadex G-100 column, 72 × 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 Fig. 1. The preparations were freeze-dried and stored at 4°C until use. Enzyme preparation I (peak I) contained 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.

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 m*M* potassium phosphate buffer, pH 5.5, for RNase activity. Enzyme solution (200 μl, 1 mg/ml) was pipetted into a 100 × 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 in a water bath. The reaction was terminated by transferring the test tube to an ice bath, adding 200 μl zinc chloride (8 m*M* aqueous solution) and heating the contents in a boiling water bath for 12 min. This ensured complete precipitation of the protein. In blanks ran simultaneously, the enzyme solution was replaced by 200 μl buffer. Next the samples were centrifuged for 5 min at 6000 *g*, the supernatant passed through a 0.45-μm HA-millipore filter, and 50 μl of the clarified solution was injected into the LC system.

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 identified from the chromatograms by their retention times and UV absorbance ratios, 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] which uses bovine serum albumin as a standard. Absorbance readings were taken at 595 nm on a Beckman Model DU-8 spectrophotometer. Specific activities (units/mg protein) were 8.73 units/mg for nuclease (preparation I) and 16.4 (units/mg) for RNase (preparation II).

RESULTS AND DISCUSSION

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 (Fig. 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 GMPs (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 Figs. 3 and 4. The unlabelled peaks in Fig. 4 were due to the nucleotide impurities present in commercial yeast RNA. Not all preparations had these impurities; they could be readily removed by dialysis.

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 screening of assay buffers prior to enzyme isolation. Peeled potatoes (10 g) were 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

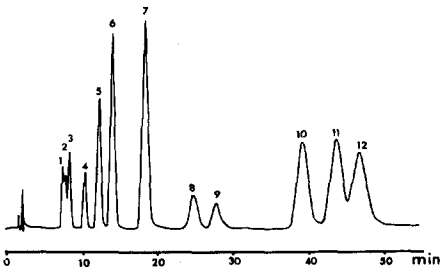
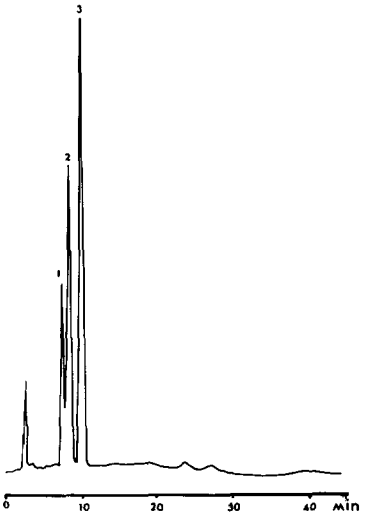


Fig. 2. Liquid chromatogram of nucleotides used as standard. LC conditions: see text. Peak identities: 1 = cyclic 2′:3′-CMP; 2 = cyclic 2′:3′-UMP;3 = cyclic 2′:3′-AMP; 4 = cyclic 2′:3′-GMP; 5 = 5′-CMP; 6 = 5′-UMP; 7 = 5′-AMP; 8 = 2′-AMP; 9 = 3′-AMP; 10 = 5′-GMP; 11 = 3′-GMP and 12 = 2′-GMP. Concentrations used: cyclic nucleotides and 2′- and 3′-AMP 1 μg/ml, others 5 μg/ml.



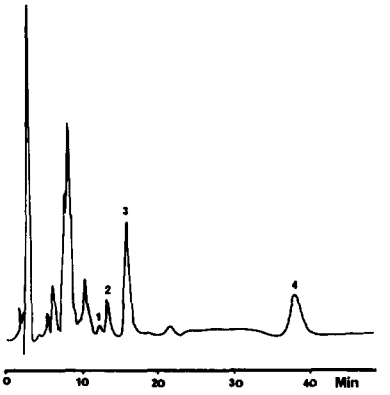


Fig. 3. Chromatogram of RNA solution treated with potato tuber RNase. Assay conditions: a mixture of 2 mg RNA and 200 μg enzyme in 50 m*M* potassium phosphate buffer pH 5.5 was incubated at 52°C for 10 min; LC separation conditions as in Fig. 2. Peak identities: 1 = cyclic 2':3'-CMP + cyclic 2':3'-UMP;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

Fig. 4. Chromatogram of RNA solution treated with potato tuber nuclease. Assay conditions as in Fig. 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.

ring opening of nucleoside 2':3'-cyclic monophosphates produced in RNase catalyzed reaction.

Phosphate buffer in a range of 10 to 500 m*M* was found to inhibit nuclease and promote RNase activity (Table I), while Tris–HCl buffer inhibited RNase and promoted nuclease activity (Table II). Sodium or potassium cations did not significantly affect the enzyme activities. Therefore all subsequent assays were carried out

TABLE I

THE CONTENT (ppm) OF NUCLEOTIDES IN POTATOES

The peeled tubers were slurried in various concentrations of phosphate buffers and then steam-cooked.

Nucleotides	*KH_2PO_4 concentration (pH 6.0) (moles)*					
	0.01	*0.02*	*0.05*	*0.1*	*0.2*	*0.5*
3'-AMP*	7.68	8.21	10.35	8.84	9.49	9.29
2'-GMP	9.88	10.46	12.01	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

* 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 II

BUFFER OPTIMIZATION ASSAY

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

Nucleotide	*Buffer (0.1 M, pH 6.0)*						
	H_2O	*Tris–HCl*	*KH_2PO_4*	*NaH_2PO_4*	*Sodium citrate*	*KCl*	*NaCl*
2'-AMP*	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

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

in Tris–HCl buffer to follow nuclease activity and potassium phosphate buffer for RNase activity.

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.

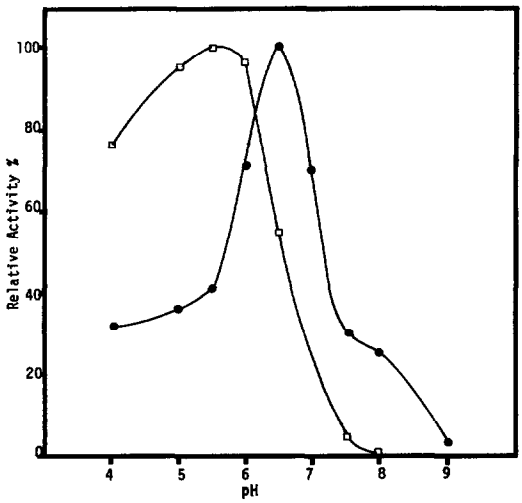


Fig. 5. Effect of pH on potato tuber enzyme activities. RNase (□) and nuclease (●) optimum pH values were 5.5 and 6.5, respectively.

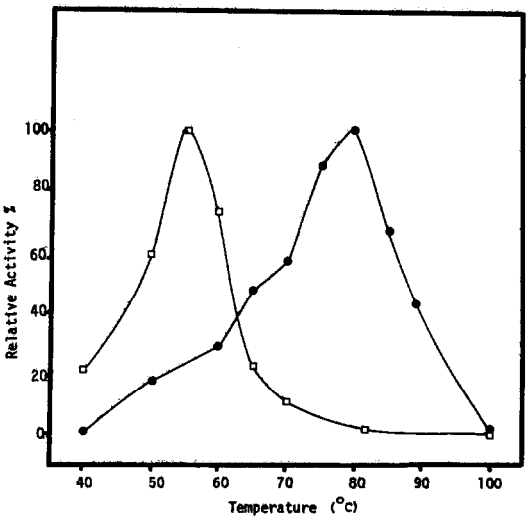


Fig. 6. Effect of temperature on potato tuber enzyme activities. RNase (□) and nuclease (●) optimum temperatures were 52 and 80°C, respectively.

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 (Fig. 5), in agreement with previously reported values[12,16].

The effect of temperature on the activities of nuclease and RNase is shown in Fig. 6. The optimal temperatures found were 80°C for nuclease and 52°C for RNase. Consequently these temperatures were chosen for the enzyme assay.

TABLE III

EFFECT OF SOME ANIONS, CATIONS, GLUTAMATE AND EDTA ON THE ACTIVITIES OF POTATO NUCLEASE AND RNase

*Addition**	*Molarity*	*Relative activity (%) RNA substrate*	
		Nuclease	*RNase*
Blank		100	100
NaH_2PO_4	$1 \cdot 10^{-3}$	91	
$NaHSO_3$	$1 \cdot 10^{-3}$	57	
NaCl	$10 \cdot 10^{-3}$	87	
LiCl	$10 \cdot 10^{-3}$	97	
Sodium glutamate	$10 \cdot 10^{-3}$	128	
$MnCl_2$	$1 \cdot 10^{-3}$	38	
$ZnCl_2$	$1 \cdot 10^{-3}$	0	0
$MgCl_2$	$1 \cdot 10^{-3}$	122	–
EDTA	$1 \cdot 10^{-3}$	17	133
	$1 \cdot 10^{-4}$	50	122

* For nuclease activity addition to 0.1 *M* Tris–HCl buffer pH 6.5, for RNase activity addition to 50 m*M* potassium phosphate buffer pH 5.5.

TABLE IV

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

Enzyme characterization

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 products required an extensive washing for column re-equilibration.

The effect of various ions on nuclease activity was measured in a concentration range of 1 to 10 m*M* (Table III). Chloride ion added as sodium chloride at 10 m*M* (a level usually applied in food processing) had essentially no influence. Monosodium glutamate (MSG), a flavour 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 flavour improvement of food.

As previously reported[16] EDTA was found to be a strong inhibitor of potato nuclease. In the presence of 1 m*M* EDTA the initial activity of the enzyme decreased to 17%. However, EDTA promoted the activity of RNase (Table III). 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 flavour 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) with various polynucleotides used as substrates is presented in Table IV. As measured by the formation of nucleoside-5'-monophosphate the relative hydrolysis rates were: polyU > polyA > RNA > polyC > polyG > DNA. This corroborates the earlier results by Nomura *et al.*[12].

TABLE V

POTATO RNase ACTIVITY TOWARDS VARIOUS POLYNUCLEOTIDES

Substrate	*Relative activity (%)*
Poly A	43
Poly C	60
Poly G	17
Poly U	454
RNA	100

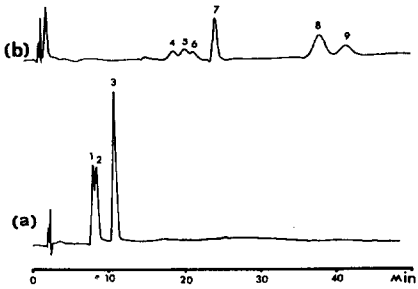


Fig. 7. Chromatograms of a reaction mixture of RNA and potato RNase incubated as in Fig. 3. (a) Reaction mixture without additional treatment; (b) after the reaction mixture was treated with PCA as outlined in procedure for nucleotide extraction from raw and processed potatoes. Peak identities: 1 = cyclic 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 (a) and their disappearance with concomitant production of their 2′- and 3′-nucleotides after PCA treatments (b) is readily revealed.

RNase activity with various polynucleotide substrates is presented in Table V. The relative reaction rates were polyU > RNA > polyC > polyA > polyG. This enzyme, like nuclease, preferentially hydrolyzes polyU. For 10 min incubation at 52°C in 50 m*M* potassium phosphate buffer (pH 5.5), the only reaction products found were cyclic nucleoside 2′:3′-monophosphates (Fig. 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 VI, 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

TABLE VI

THE EXTENT OF 2′- AND 3′-NUCLEOTIDES FORMATION FROM 2′:3′-CYCLIC NUCLEOTIDES AND RNA INCUBATED WITH POTATO RNase AT 37°C, 20 h

ND = not determined.

Substrate	*Nucleotide produced (units)*								*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	–		–		0.012	0.064	–		16	84	1.4
2′:3′-UMP	–		–		–		0.021	0.070	23	77	1.5
RNA	ND	0.007	ND	0.013	Trace	0.031	ND	0.016			

equal to 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 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 increases during cooking RNase was denatured (Fig. 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 occurence of 2'- and 3'-isomers in the extracts, in accordance with a general finding that strong acids or bases hydrolyse cyclic nucleotides[21]. This is supported by chromatograms of substrates incubated by RNase with (Fig. 7b) and without subsequent PCA treatment (Fig. 7a). As seen in Fig. 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 kilodalton[22] and phosphatases with a mol. wt. of about 96 kilodalton[23]. These enzymes were not present in our enzyme preparations since they were unretained on the Sephadex gel column and hence they 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 flavour 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 the three undesirable enzymes while the activity of nuclease would be enhanced to cleave the phosphodiester bond of RNA. Thus, higher amounts of 5'-GMP, the endogenous flavour nucleotide of the processed potato would be generated.

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 flavour-enhancing nucleotide, 5'-GMP. On the other hand, RNase was found to be a heat-labile enzyme which provided only 2':3'-cyclic nucleotides in its first stage of activity, in the second stage of its activity it provided 3'-nucleotides at a very slow reaction rate. Neither of these products is a flavour enhancer.

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

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