加熱処理による蔬菜中の 5'-Nucleotides の変化

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CHANGE OF 5'-NUCLEOTIDES IN VEGETABLES DURING HEAT PROCESSING

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SUMMARY

Influence of mild cooking in water on the changes in nucleotide composition of some vegetables such as Shii-take, French mushrooms and asparagus were investigated.

Several kinds of 5'-nucleotides in the vegetables increased rapidly with the decreasing of ribonucleic acid (RNA) or adenosine triphosphate (ATP). Fractions containing ribonuclease (RNase) phosphodiesterase (PDase) or phosphomonoesterase (PMase) were isolated from the fresh vegetables by means of DEAE-cellulose columns and their activities were examined in relation to the changes in 5'-nucleotides. In the consequence, it seems reasonable that RNA of Shii-take is decomposed by its own RNase and four kinds of 5'-nucleotides are accumulated. ATP of French mushrooms or asparagus is degraded and 5'-adenosine monophosphate (5'-AMP) is formed by the action of PMase.

INTRODUCTION

Remarkable progress has recently been made on the studies of flavor enhancing abilities of 5'-nucleotides in foods. (Kuninaka, 1960; Fujita *et al*, 1961a; Honjo *et al*, 1963; Shimazono, 1964). Nucleic acid derivatives, particularly 5'-nucleotides involved in vegetables and mushrooms were analysed with ion exchange chromatography by many investigators. (Bergkvist, 1958 a,b; Mori *et al*, 1960; Nakajima *et al*, 1961; Fujita *et al*, 1961b; Brown, 1962; Gregoire *et al*, 1963; Hashida *et al*, 1963; Hashida *et al*, 1964). Shimazono (1964) suggested that the nucleotide distribution patterns in vegetables and mushrooms were different from those in meat, poultry and fish. 5'-AMP and 5'-uridine monophosphate (5'-UMP) have been found existing mainly in vegetables and mushrooms. Moreover, in case of Shii-take (*Lentinus edodes*, one of edible mushrooms popularly used in Japan), remarkable amounts of 5'-guanosine monophosphate (5'-GMP), 5'-AMP, 5'-UMP and 5'-cytidine monophosphate (5'-CMP) were found in

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the boiled extract (Nakajima *et al*, 1961; Mouri *et al*, 1965). It is recognized that 5'-GMP, as well as 5'-inosine monophosphate (5'-IMP), is a potential flavor enhancer.

By the way, the heat treatment is a important processing stage in food manufactures. There has been hardly found any report relating to the change of 5'-nucleotides in vegetables during heat processing.

In our preliminay experiments it was found that the levels of free 5'-nucleotides of some mushrooms and vegetables became higher during heating in water (around 50 to 70°C). Free nucleotides were contained in appreciable amount in the original raw samples, and moreover the increase of 5'-nucleotides was remarkable during the heat treatment. We thought that the change in the amount of 5'-nucleotides during heating in water was caused by the enzyme actions upon RNA or nucleoside polyhosphate. Our experiments were aimed to clarify these points.

This paper presents the data on the decomposition of RNA and the accumulation of four kinds of 5'-nucleotide in Shii-take, and also on the degradation of ATP and the formation of 5'-AMP in asparagus and French mushrooms during heating in water.

MATERIALS AND METHODS

Sodium salts of 5'-IMP, 5'-GMP, 5'-AMP, 5'-CMP and 5'-UMP and the enzymes 5'-nucleotidase and 3'-nucleotidase were supplied through the courtesy of Takeda Chemical Industries, Ltd., Osaka, Japan. Adenosine diphosphate (ADP), ATP, Yeast RNA and other substances related to nucleic acid were obtained from commercial source.

French mushrooms (*Psalliota bisporus*) and white asparagus were grown for experimental purposes in Toyo Food Institute, Kawanishi-shi, Japan. Fresh Shii-take (*Lentinus edodes*) was commercially purchased.

Preparation of vegetable extracts Fresh vegetables such as French mushrooms, Shii-take, and asparagus were cut into fine slices and extracted with hot water or cold perchloric acid. Two procedures were adopted for the preparation. a) Raw materials were homogenized and extracted with cold 10%-perchloric acid and then twice with cold 5%-perchloric acid. The extract was neutralized with 5N-KOH, centrifuged, and the supernatant was submitted to chromatographical estimation. The figures obtained may represent the free nucleotides present in the raw materials. b) Finely sliced materials were put into flasks containing water (about 80g for 10g material) previously kept at various temperatures, and the heating was continued for scheduled period. Immediately after the elapse of given period of heating, the content was filtered through absorbent cotton. The filtrate and the cold perchloric acid-extract of the residue were applied on a column for chromatography.

Nucleic acid estimation Homogenates of the vegetables or mushrooms were fractionated by Schmidt-Thannhauser's procedure, and the phosphorus contents of total-P, acid soluble-P, RNA-P, and deoxyribonucleic acid (DNA)-P fractions were assayed by Fiske-Subbarow's method.

Nucleotide estimation Vegetable extracts were fractionated on Dowex 1×8 column and the individual nucleotide was determined according to the procedure proposed by Bergkvist *et al.* (1954) and modified by Nakajima *et al* (1961). The total amount of 5'-nucleotides or 3'-nucleotides was determined from the phosphoric acid liberated by 5'- or 3'-nucleotidase, respectively, according to the method by Nakajima *et al* (1963).

Estimation of enzyme activities Activities of RNase and PDase were determined according to the method of Suhara *et al* (1964). PMase activity was measured by using p-nitrophenyl phosphoric acid as the substrate instead of bis-p-nitrophenyl phosphoric acid in PDase determination. An amount of protein was measured by the absorption at 280 mμ or by the method of Folin-Ciocalteu.

RESULTS AND DISCUSSION

Shii-take *(Lentinus edodes) Increase of 5'-nucleotides during heating in water* Finely sliced samples of fresh Shii-take were heated with water for 5 minutes at various

Table 1 Effect of temperatures on the formation of nucleotides in Shii-take (*Lentinus edodes*) during heating in water

Heating	5'-Nucleotide μmol/g dry wt.	3'-Nucleotide μmol/g dry wt.
Cold perchloric acid-extraction	1.97	0
30°C, 5 min.	1.47	0
50°C, 〃	4.77	0
60°C, 〃	16.60	0
70°C, 〃	13.90	0
80°C, 〃	9.80	0
100°C, 〃	4.16	0

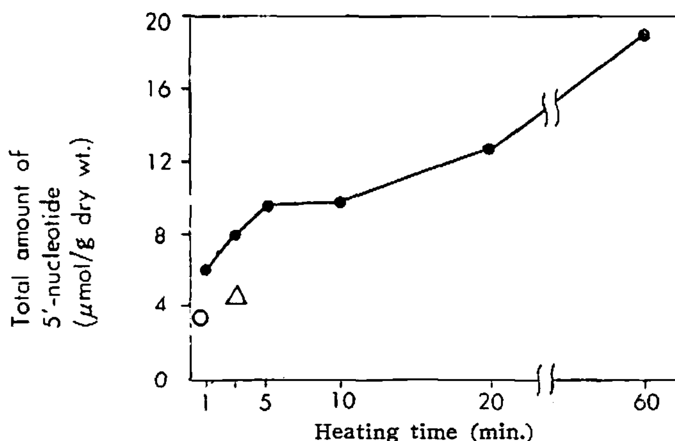


Fig. 1 Formation of 5'-nucleotides during heating extraction of Shii-take (*Lentinus edodes*) at 70°C.

○: Free 5'-nucleotides extracted with perchloric acid (cold)
△: 5'-Nucleotides extracted with water at 100°C for 3 min.

temperatures. As shown in Table 1, 5'-nucleotides were extracted in the highest amounts at 60°C and 70°C. The figure for 5'-nucleotides indicates the total amount of four kinds of 5'-nucleotide. Greater amount of 5'-nucleotides was extracted with water at 60°C or 70°C than with water at 100°C or with cold perchloric acid. 5'-nucleotides extracted with cold perchloric acid may be considered as free nucleotides originally present in the raw material, therefore, an amount of 5'-nucleotides thus obtained in excess of the free 5'-nucleotides shows their increase of 5'-nucleotides by and during the heating process. Fig. 1 shows the increase of extracted 5'-nucleotides during heating at 70°C over a period of 30 min.

On the other hand, the amonts of RNA-phosphorus were measured before and after heating. Fresh Shii-take was sliced and heated in water gradually from room temperature up to 100°C. After keeping at 100°C for 3 min., it was cooled. The solid and the extracted liquid were collected separately and subjected to Schmidt-Thannhauser's procedure. As shown in Table 2, phosphorus of RNA fraction in fresh Shii-take was decreased nearly by half, accompanying the decrease of acid-soluble fraction. As a result, four kinds of 5'-nucleotides, i.e. 5'-AMP, 5'-GMP, 5'-CMP and 5'-UMP were accumulated in the boiled extract as given in Fig. 2. Thus it may be possible to speculate that RNA of fresh Shii-take was decomposed during heating process and resulted in the increase of four kinds of 5'-nucleotides constituting the RNA. The

Table 2 Content of phosphorus in some fractions of fresh and boiled Shii-take

Sample	Phosphorus μmol/g dry wt. material			
	Total phosphorus	Acid-soluble fraction	RNA fraction	DNA fraction
Fresh	113	74	16	2.9
Boiled (in solid)	87	30	9	1.4
〃 (in liquid)	40			

Composition of eluting solutions

① distilled water, including sample solution	⑥ {0.1N-HCOOH, 0.1N-HCOONa
	⑦ {0.1N-HCOOH, 0.3N-HCOONa
② 0.005N-HCOOH	⑧ {0.1N-HCOOH, 0.6N-HCOONa
③ 0.02N-HCOOH	
④ 0.1N-HCOOH	⑨ {0.2N-HCOOH, 0.8N-HCOONa
⑤ {0.1N-HCOOH, 0.05N-HCOONa	⑩ {0.2N-HCOOH, 1.0N-HCOONa

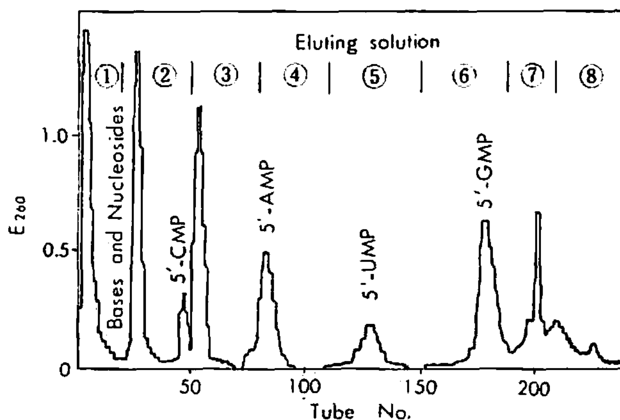


Fig. 2 Chromatogram of boiled extract of Shii-take (*Lentinus edodes*).

probability of enzymatic break-down of RNA was examined in the following way.

Fractionation of enzyme One part fresh Shii-take was homogenized and extracted with five parts of distilled water at 5°C for 2 hrs. 2.5 l of crude extract was saturated with ammonium sulfate. The precipitate was separated by centrifugation (18500xG), and dissolved in 0.01 M Na-acetate buffer (pH 6.0) and dialysed against water for 1 day. The dialysed solution containing enzymes was loaded on a column (3×25 cm) of DEAE-cellulose equilibrated with 0.01 M acetate buffer. A gradient elution was carried out with pH 6.0 acetate buffer concentrations from 0.01 M to 0.5M. The effluent was collected in 10 ml fractions and the enzyme activities and the protein contents were mesured as described in Materials and Methods. The chromatogram thereof is shown in Fig. 3. The RNase activity was found in three elution peaks, two major and one minor.

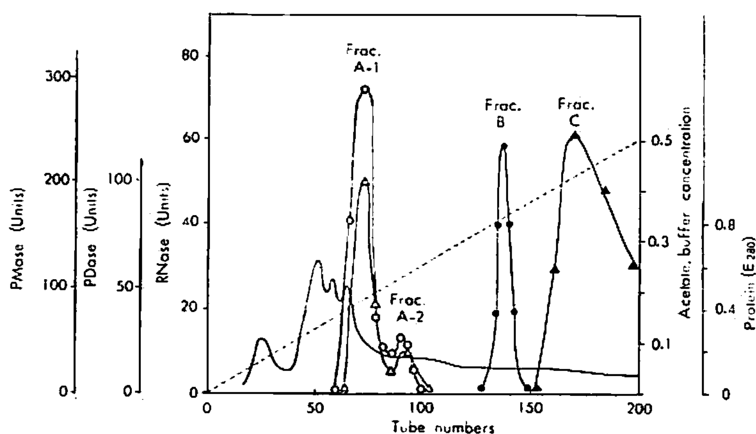


Fig. 3 Chromatography of RNase and PMase of Shii-take on a DEAE-cellulose column.

—○—○— RNase (pH 4.5) —△—△— PDase
—●—●— RNase (pH 7.5) —▲—▲— PMase
———— Protein (E 280)

Table 3 Purification of RNase of Shii-take

Purification step	RNase (units)	Protein (mg)	Specific activity (units/mg Protein)
Filtrate of extract	250,000	2,070	121
Ammonium sulfate	90,000	174.7	515
DEAE-Cellulose No. 1			
Peak A-1	35,047	30.8	1168
Peak A-2	2,652	5.6	468
Peak B	3,167	3.4	932
DEAE-Cellulose No. 2			
Peak A-1	12,507	1.3	8851
Peak A-2	1,250	0.1	8333
Peak B	2,120	0.4	5170

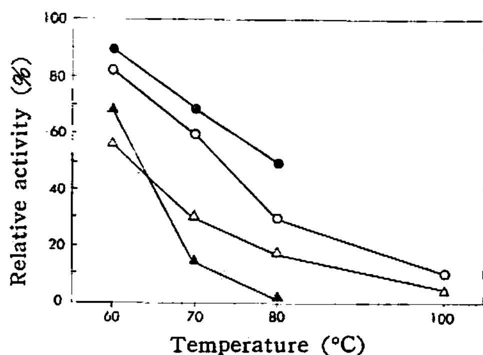
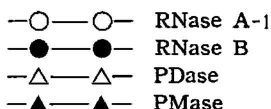


Fig. 4 Heat-stability of enzymatic activies.



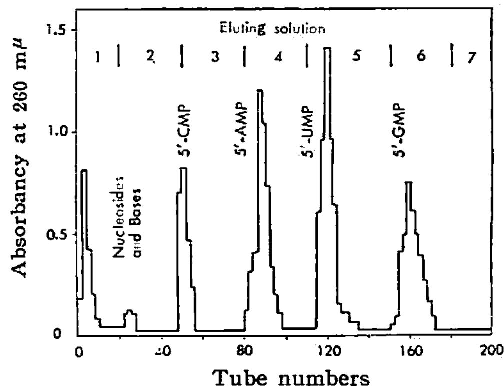


Fig. 5 Chromatogram of accumulated 5′-nucleotide through the degradation of yeast RNA with RNase A-1.

One of two major peaks had an optimal pH of 4.5 for RNase and the other that of 7.5. The former was designated tentatively as RNase A-1 and the latter as RNase B. The fraction involving RNase A-1 had a PDase activity at pH 7.5. PMase activity was observed in the other effluent separate from RNase. It was designated as PMase C.

As shown in Table 3, purification of RNases A-1 and B by rechromatography with DEAE-cellulose yielded 8- and 50-fold active preparations, respectively. The optimal condition for the RNase activity of fraction A-1 was found to be at 50 to 70°C and in the pH range 4.0 to 5.0 in acetate buffer, and that of fraction B to be at 60 to 70°C in the pH range 7.0 to 8.0 in tris-aminomethane-HCl buffer. Optimal PMase activity of fraction C occurred around pH 4.5 in acetate buffer, while inactive in alkaline solution. RNases A-1 and B were rather heat-stable while PMase C became unstable at 70°C as shown in Fig. 4. It is noteworthy that RNases A-1 and B are still active even around pH 6.0.

Digestion of RNA Yeast RNA was digested with RNase A-1 in acetate buffer (pH 4.5) or with RNase B in Tris-HCl buffer (pH 7.5), at 37°C for 22 hrs. The reaction mixtures were extracted with cold perchloric acid, and the extract was purified with active carbon. The ethanol-ammonia effluent was concentrated in vacuo and applied on Dowex 1×8 column. A result on digestion with RNase A-1 is shown in Fig. 5. Accumulation of four kinds of 5′-nucleotides i.e. 5′-CMP, 5′-AMP, 5′-UMP and 5′-GMP was observed clearly.

Considerations on the formation of 5′-nucleotides It seems reasonable to consider that in the case of heating (at tempereature 50 to 70°C, around pH 6.0), RNases A-1 and B contained in Shii-take decompose Shii-take's RNA and form a mixture of four

kinds of 5′-nucleotides in the same manner as in autolysis. On the contrary, the activity of PMase C fraction is rather poor and considerably inactivated by heat, therefore, the 5′-nucleotides above mentioned are not decomposed and remain in a remarkable amount. When finely sliced fresh Shii-take was incubated in Tris buffer at pH 8.0, remarkable amount of 5′-nucleotide mixture was found probably because of the decomposition by RNase B and accumulation of 5′-nucleotide due to no effect of PMase C.

French mushrooms *Increase of 5′-AMP during heating in water* French mushrooms was extracted with cold perchloric acid. The nucleotides in perchloric acid extracts were separated and analysed by means of a Dowex 1×8 column. The fractions comprising the various peaks were pooled and identified. Fig. 6 shows the asborption

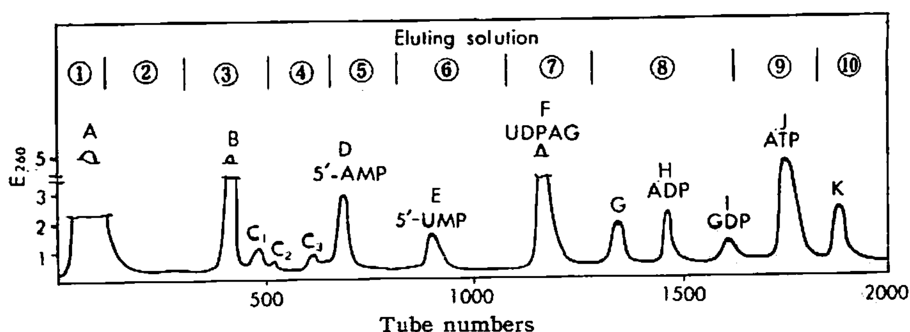


Fig. 6 Chromatogram of perchloric acid-extract of fresh mushroom (*P. bisporus.*) (a large scale column)

chromatogram at 260 mμ of the effluent fractions.

The peaks were identified as follows:

A and B: mixture of bases and nucleosides; D: 5′-AMP; E: 5′-UMP;
F: UDPAG; H: ADP; I: GDP; J: ATP

The result shows the presence of ADP and ATP in pretty noticeable amount.

The change of 5′-nucleotides and related nucleoside polyphosphates during heating in water was investigated in the following way. Sliced mushrooms were heated at various temperatures for the period of 5 min. As shown in Table 4, the total amount of 5′-nucleotide was the highest at 50 and 70°C. The amount of 5′-nucleotide extracted

Table 4 Effect of temperatures on the formation of nucleotides in French mushrooms during heating in water

Boiling condition	5′-Nucleotide (μmol/g dry wt.)	3′-Nucleotide (μmol/g dry wt.)
Cold perchloric acid-extraction	2.86	0
30°C, 5 min.	1.94	0
50°C, 〃	3.48	0
70°C, 〃	4.08	0
100°C, 〃	3.34	0

with heated water at 50 or 70°C was higher than that of cold perchloric acid or boiled water at 100°C. No. 3'-nucleotide was found. During the heating of sliced mushrooms at 70°C, the amount of 5'-nucleotide increased gradually during a 60 min. period as shown in Fig. 7. Fresh mushrooms was sliced and heated in water gradually up to 100°C. After keeping at 100°C for 3 min, it was cooled. The solid and the extracted liquid were collected separately and analysed by column chromatography. A typical chromatogram of the acid-soluble adenine nucleotides of mushrooms after heating in water was compared with that obtained from an equivalent sample of fresh mushrooms. As a result, both the decrease of ADP and ATP and the increase of 5'-AMP were observed as shown in Table 5. The increase of other 5'-nucleotides except 5'-AMP was negligible.

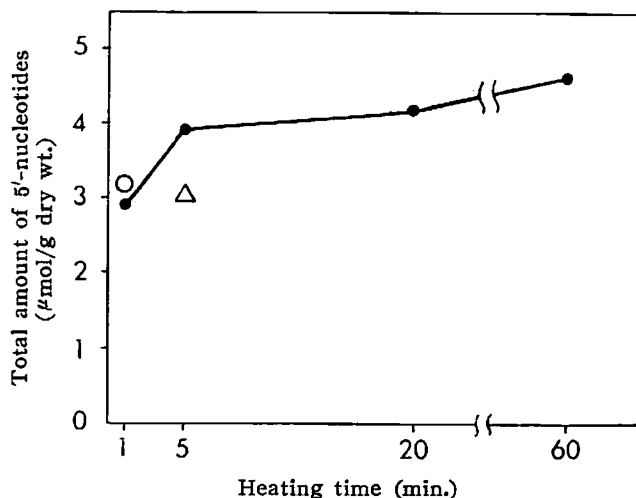


Fig. 7 Formation of 5'-nucleotides during heating extraction of mushrooms at 70°C.

○: Free 5'-nucleotides extracted with cold perchloric acid

△: 5'-nucleotides extracted with water at 100°C, for 5 min.

Enzymatic considerations As the observed increase in 5'-AMP appeared to be brought about by the enzymatic breakdown of related derivatives, enzymatic experiments were done with crude extract of mushrooms homogenates. Enzymes contained in mushrooms which degrade nucleic acid relating substances were isolated using a DEAE-cellulose column, in the same way as Shii-take. Purification of each fraction of RNase, PDase, and PMase by rechromatography with DEAE-cellulose yielded 30-, 50-, and 20-fold active preparations, respectively. RNase fraction had an optimum temperature of 60°C and pH range of 7.5 to 8.0. An optimum condition for PDase fraction was temperature of 45°C and and pH range of 7.5 to 8.0. PMase fraction was most active at 40°C and pH range of 4.0 to 5.0.

Table 5 Changes of 5'-AMP, ADP, and ATP in French mushrooms during heating

Sample	5'-AMP (μmol/g dry wt.)	ADP (μmol/g dry wt.)	ATP (μmol/g dry wt.)
Fresh	0.32	3.84	6.6
Boiled (in solid)	0.36	0.46	0.38
(in liquid)	2.33	2.40	4.62

PMase fraction from mushrooms was incubated with authentic ATP at pH 6.0, 37°C, for 60 min. After the reaction, the product was chromatographed on Dowex 1×8 resin. Both the disappearance of ATP and the formation of ADP and 5′-AMP were observed as shown in Fig. 8. It seems reasonable to consider that during the mild heating of mushrooms in water with a PH of around 6.0, 5′-AMP is formed from ATP by the action of PMase containing fraction.

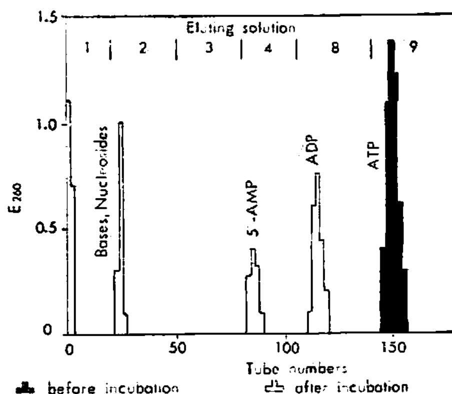


Fig. 8 Chromatogram of 5′-AMP and related substances formed through the degradation of authentic ATP with mushrooms' crude enzyme extract.

French mushrooms contains appreciable amounts of 5′-AMP and 5′-UMP but not measurable amount of 5′-GMP, however. Shii-take contains remarkable amounts of 5′-GMP, 5′-AMP, 5′-CMP and 5′-UMP. The acid range-enzyme activities of French mushrooms are different from those of Shii-take, and the fact may account for the difference of the distribution pattern of 5′-nucleotides between both.

Asparagus *Increase of 5′-AMP during heating in water* Sliced pieces of asparagus were put into water and heated at various temperatures for 5 min. As shown in Table 6, the amount of total 5′-nucleotides was found highest in the range of 50 to 70°C. Comparing the figures of cold perchloric acid extraction with those of heating at 50 to 70°C,

Table 6 Effect of temperatures on the formation of nucleotides in asparagus during heating in water

Heating	5′-Nucleotide μmol/g dry wt.	3′-Nucleotide μmol/g dry wt.
Cold perchloric acid-extraction	0.25	0.05
37°C, 5 min.	0.24	0.22
50°C, 〃	0.86	0.14
60°C, 〃	0.93	0.14
70°C, 〃	0.72	0.25
80°C, 〃	0.51	0.28
100°C, 〃	0.42	0.21

Table 7 Changes of 5′-AMP, ADP, and ATP in asparagus during heating

Sample	5′-AMP (μmol/g dry wt.)	ADP (μmol/g dry wt.)	ATP (μmol/g dry wt.)
Fresh	0.78	0.69	0.94
Boiled (in solid)	0.37	0.38	0.28
(in liquid)	0.74	trace	0.20

Table 8 Formation of nucleotides in the autolysates of asparagus at various pH

Buffer solution	pH	5′-Nucleotide (μmol/g dry wt.)	3′-Nucleotide (μmol/g dry wt.)
Acetate	4.0	0.83	0.90
	5.0	0.80	0.83
	6.0	0.83	0.83
Tris-aminomethane-HCl	7.0	2.29	0.75
	8.0	9.62	0.80
	9.0	12.1	

the increase of the latter was noticed. Chromatographical analysis proved that 5′-AMP represented the increase of 5′-nucleotide mixture. Table 7 shows, the degradation of ATP and ADP as well as the formation of 5′-AMP and its related nucleosides and bases during heating in water.

Asparagus homogenates were incubated at various pH in acetate and Tris-HCl buffers at 50°C for 2 hrs. As shown in Table 8, remarkable amount of 5′-nucleotide was accumulated in the range of pH 8 to 9 in Tris-HCl buffer. The supernatant was loaded on a Dowex 1×8 column and the distribution of individual 5′-nucleotide was measured. Each kind of 5′-nucleotides, i.e. 5′-GMP, 5′-AMP, 5′-UMP, and 5′-CMP was found in a fairly large amount. While in sliced asparagus, heated in water around pH 6, the breakdown of RNA was hardly found, and there was no more increase of 5′-nucleotide.

Enzymatic considerations Sliced pieces of white asparagus were homogenized in distilled water, and the crude extract was obtained with centrifugation. Enzymes related to the degradation of nucleic acid were isolated from the crude extract in the same way as Shii-take. Purification of each fraction of RNase, PDase, and PMase by rechromatography with DEAE-cellulose yielded 100-, 60-, and 80-fold active preparations, respectively. RNase fraction had an optimum temperature of 70°C and pH range of 7.0 to 8.0. An optimum condition for PDase fraction was temperature of 50°C and pH of around 5.0. PMase fraction was most active at 50°C and pH 5.0. PDase and PMase were unstable at 80°C and 60°C, respectively, but RNase was rather stable at 80°C. PMase fraction degraded ATP which was added exogenously and formed ADP and 5′-AMP successively.

It may be reasonable to infer from the results of enzymatic study that when asparagus homogenate is incubated in acid range, the accumulation of 5′-nucleotides is hardly found, while. in alkaline range, a remarkable amount of four kinds of 5′-nucleotides is accumulated by the enzymatic decomposition of asparagus RNA. When sliced asparagus is heated mildly in water (around pH 6.0), only 5′-AMP increases by the degradation of ATP, but the other 5′-nucleotides are not formed because the RNase is not active in the acid range.

Conclusion Shimazono (1964) divided the distribution patterns of free nucleotides

in vegetables and mushrooms into two types namely 'plant type' and 'autolysate type'. The former was speculated to be derived from uridine derivatives and ATP, and the latter to be derived from RNA. We may summarize our results and conclude that the increase of 5'-nucleotides during heating at 50-70°C is due to the accerelation of the 5'-nucleotide formation by heat in the same way as suggested by Shimazono in non-heated state. In the case of Shii-take, four kinds of 5'-nucleotides may be formed by the breakdown of intracellular RNA by its own RNase in an autolysis. In the case of French mushrooms and asparagus, 5'-AMP may be derived from the degradation of intracellular ATP in the patten belonging to the plant type.

要　　旨

5'-グアニル酸（5'-GMP）5'-アデニル酸（5'-AMP）など 5'-ヌクレオチド類は食品の重要な呈味成分であり，5'-GMP はきのこ類，5'-AMP は蔬菜類，きのこ類にかなり含まれていることが知られている．本報はシイタケ，マッシュルーム，アスパラガスなど数種の蔬菜類，きのこ類の加熱工程でのヌクレオチド組成の変化とその原因について検討したものである．食品中のヌクレオチドは冷時過塩素酸で抽出したものについて，5'-ヌクレオチダーゼを用うる酵素法，または Dowex 1×8 によるカラムクロマトグラフィによって定量した．核酸の分解に関連する Ribonuclease (RNase)，Phosphodiesterase (PDase)，Phosphomonoesterase (PMase) の諸活性は須原氏らの方法によって測定した．

煮出し，すなわち原料を水の中で穏和な条件で加熱しながら抽出すると，原料のリボ核酸あるいはアデノシン-3-燐酸（ATP）の減少に伴なって 5'-ヌクレオチドの増加が認められた．これが生の蔬菜類に含まれている核酸分解酵素系の作用に由来するのではないかとの観点から，シイタケなどの酵素系 RNase, PDase, PMase などを DEAE-セルロースカラムで分離し，その性質をしらべた．その結果シイタケでは，その RNase によってリボ核酸が分解されて，シイタケの旨味といわれる 5'-GMP など 4 種の 5'-ヌクレオチドを生成すること，マッシュルームやアスパラガスでは，それらの PMase 画分によって ATP が分解されて，5'-AMP を生成することがみとめられた．

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