ISOLATION AND PURIFICATION OF A RIBONUCLEASE FROM WHEAT SPROUT[1]

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Enzymes from plant sources which depolymerize ribonucleic acid (RNA) have been reported by many laboratories (Holden, 1955; Frisch-Niggemeyer, 1957; Shuster, 1957; Shuster, 1959; Reddi, 1958; Markham, 1956; Anfisen, 1954; Sung, 1962; Tuve, 1960). Although none has been prepared as an entirely pure protein, some of these have been highly purified (Holden, 1955; Frisch-Niggemeyer, 1957; Shuster, 1957; Shuster, 1959; Sung, 1962; Tuve, 1960). The most inspiring examples are mung bean nuclease (Sung 1962) and spinach ribonuclease (Tuve, 1960). The activities of these two enzymes have been raised to 2500-fold purification.

The main purpose of the present work was to obtain a pure ribonuclease from higher plants so that a comparison on its properties and mode of action might be made with enzymes of the same category from other sources. Although the author was unable to achieve such an objective, efforts were made to isolate a ribonuclease from wheat sprout with a some 80-fold purification reached and its properties characterized. Furthermore, a reproducible procedure for purification was established.

Materials and methods

Wheat sprout was supplied by a local candy factory in Sing-Juang, Taipei Shiann, Taiwan. Occasionally, it was prepared in the laboratory by soaking wheat seeds in water for 24 hours first, then spread it in a shallow glass tray with frequent spraying of water. After 2-3 days, the germinated seeds were ready for extraction.

Yeast RNA obtained from British Drug House Ltd. was used for both activity assays and products identification without further purification.

Calcium phosphate gel was prepared following Keilin and Hartree's procedure (1955).

Enzyme assay—While polynucleotides and larger oligonucleotides are in-

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soluble in MacFadyen reagent (MacFadyen, 1934), mono-and dinucleotides may be safely assumed to be soluble. Accordingly, assay of activity of wheat sprout ribonuclease was carried out by measuring the increase in absorption at 260mμ. The reaction mixture contained 5 mg of RNA (0.5 ml), 0.4 ml of 0.05 M citrate buffer, pH 5.2, after equilibrated at 37°C for 5 minutes, suitable amount of enzyme solution was added. The mixture was incubated at 37°C for 15 minutes, followed by addition of 1 ml. of MacFadyen reagent and the incubation continued for another 15 minutes. The mixture was cooled in a referigerator for 10–15 minutes and then centrifuged. Aliquots of the supernatnat were taken and their optical density at 260 mμ was measured in a Beckman model DU spectrophotometer. Values observed were corrected by a zero time blank incubated with enzyme. All determinations were worked out in duplicates or triplicates.

Expression of enzyme unit and specific activity—The amout of wheat sprout ribonuclease required to cause an increase of 1.00 in absorption at 260 mμ per minute was defined as 1 unit of activity. Specific activity was expressed as units of activity per mg of protein.

Measurement of protein—Protein content was invariably estimated by applying Warburg and Christian's (1954) method. Data obtained from these measurements were occasionally checked by the method of Lowry (1951) and found reasonably agreed.

All pH determinations were carried out with a Coleman Metrion pH meter, model 28, at room temperature (24–26°C).

Isolation of Enzyme

Step I; Preparation of crude extract—One Kg. of wheat sprout about 2–4 cm long, together with the seed and its roots was homogenized in a Waring blender, with a total volume of 2000 ml of cold distilled water added in successive batches. The homogenate was sqeezed through a cheesecloth and the cloudy extract was centrifuged in an International refrigerated centrifuge to remove starch and cell debris. The light brown colored supernatant served as crude extract of wheat sprout ribonuclase.

Step II: First ammonium sulfate fractionation—Unless otherwise indicated, all additions of ammonium sulfate in the purification procedure followed the table prepared by Dixon (1953). To the crude extract of wheat sprout, solid ammonium sulfate was added to attain a 45% saturation. The cloudy mixture was set in a refrigerator overnight and the precipitrate was removed by centrifugation (3000 rpm, 30 min.) and discarded. To the clear supernatant, solid ammonium sulfate was added to attain 70 percent saturation. After a settling time of 1 hour in the refrigerator, the precipitate was collected by centrifugation and dissloved in 0.02 M phosphate buffer, pH 5.8, the volume was made up to about one-

twentieth of the crude extract. The enzyme solution was then dialyzed against 5 litters of the same buffer for 6 hours.

Step III. Second ammonium sulfate fractionation—Solid ammonium sulfate was added to the dialysate from step II to a saturation of 40 percent, The precipitate was removed and discarded as before. To the supernatant, ammonium sulfate was saturated to 70 percent. The precipitate collected from centrifugation was taken up in a minimum volume of the same buffer. After dialysis for 16 hours against 6 liters of the buffer, the dialysate was stored in a deep freezer (—20°C) until step IV began.

Step IV: Alcohol precipitation—The protein concentration of the enzyme preparation from step III was adjusted to 30 mg per milliter with 0.02 M phosphate buffer, pH 5.8. The solution was cooled in an ice bath and 95 percent ethanol (counted as 100%), precooled to —20°C, was added to 5 percent saturation. The precipitate formed was centrifuged at —2°C and discarded. An additional portion of ethanol was added to attain a saturation of 30 percent. The precipitate was collected by centrifugation at —10°C and taken up in 0.05 M ammonium acetate buffer, pH 6.0, and dialyzed against the same buffer for 4 hours and stored in a deep freezer (—20°).

Step V: Calcium phosphate adsorption—The concentration of protein of the enzyme solution from step IV was first adjusted to 10–11 mg per milliter. In this step, fractional adsorption technique (Oliver, 1961) was applied. Elution was accomplished with 25% ammonium sulfate solution, pH 7.2. To the eluate, solid ammonium sulfate was added to attain 80 % saturation. The precipitate formed was taken up in the smallest volume of 0.05 M phophate buffer, pH 5.8 and dialyzed for 4 hours against 4 liters of the same buffer.

Unless otherwise indicated, all operations were performed at 4–5°C

Results and Discussion

1. *Purification*—Table 1 is a summary of the purification procedure of the enzyme.

Table 1. *Summary of purification*

Step	Fraction	Total protein mg.	Total activity units	Specific activity unit/mg. protein	Yield %
1	crude extract from 1kg wheat sprout	40,000	20,000	0.5	100
2	1st ammonium sulfate fractionation	4,200	8,440	2.02	42.1
3	2nd ammonium sulfate fractionation	800	5,120	6.4	25.6
4	Alcohol precipitation	232	4,150	17.9	20.7
5	Calcium phosphate gel adsorption	22.1	870	39.3	4.35

Heating at 60°C for 5 minutes at pH 4 could reduce the protein content to one-third. However, no purification was attained because of concomitant loss in activity. Attempts to purify the enzyme by applying carboxylmethyl cellulose chromatography and methanol precipitation led to the same result as heating.

DEAE—cellulose adsorbed all the protein in the enzyme preparation to such a degree that elution was simply impossible even in very drastic changes of both pH and molarity of the eluent.

Nevertheless, further purification is possible if suitable conditions are found out. The following is a description of the factors which may affect the purification procedure.

(1) No activity loss could be detected for intact wheat sprout kept below —20°C for 5 months. In contrast, repeated freezing and thawing seemed to result in better yield.

(2) Extraction with citrate buffer, distilled water or Tris buffer was exactly the same. No changes in activity of the extracts were observed.

(3) In ammonium sulfate fractionation, the yield was rather too low compared with results of other investigators applying the same method (Sung, 1962; Tuve, 1960). Perhaps better result may be obtained if the fraction-cutting is broader, say from 35 to 75% saturation. However, it may also be doubtful if the same degree of purification (13 folds) could be reached when broader fractions are chosen.

(4) In both steps IV and V, initial concentration of protein as well as ionic strength is of great importance. When the protein content was low (10 mg/ml), the most active portion often existed between 33% to 60% saturation of ethanol, this portion shifted to a fraction between 5% and 30% if the initial protein concentration was greater than 30 mg/ml. Recovery of activity seemed to be better in the latter case.

In calcium phosphate gel adsorption, an ionic strength of 0.005 to 0.01 M is advisable. However, purification in this step is often poorly reproducible. Out of four experiments applying the same method, the highest purification reached 5 folds. For the integrity of data, this set of values was excluded from Table 1.

2. Properties of wheat sprout ribonuclease—

(1) Optimum pH

The partially purified enzyme showed a fairly sharp optimum pH at 5.2 (Fig. 1), this value is the same as that of the ribonuclease found in the endosperm of two-week old wheat seedlings (Matsushita, 1959) and also compares with the reported values of 5.5 for pea leaf ribonuclease (Holden, 1955), 5.1 for tobacco leaf ribonuclease (Frisch-Niggemeyer, 1957), 4.5 for rye grass ribonuclease (Shuster, 1957), 5.0 for mung bean nuclease (Sung, 1962) and 5–6 for spinach ribonu-

clease (Tuve, 1960).

Preference to buffer systems approximately took the following order; phosphate, citrate-phosphate, citrate, acetate, Tris. Because of stability and buffering ranges of the systems, citrate buffer was chosen through out the experiment for assays of activities.

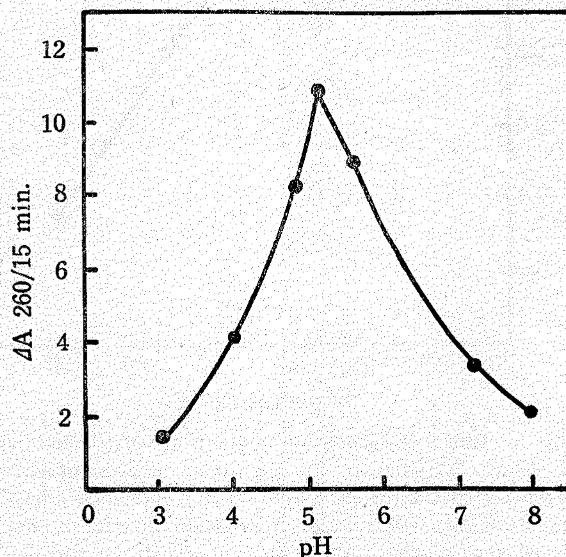

Fig. 1. Effect of pH on the activity of wheat sprout ribonuclease. Each tube contained 5 mg of RNA (0.5 ml.), 0.7 unit of enzyme solution, 0.4 ml. of 0.05 M buffer. Buffer used in the pH range of 3.1 to 5.3 was citrate, in the range 5.7 to 8.0 was phosphate.

(2) Effect of temperature—

Fig. 2 shows that the incubation temperature at which the enzyme is most active is about 52°C.

(3) Standard curve of enzyme concentration—

An investigation of the dependence of reaction rate on enzyme concentration yielded the results presented in Fig. 3. For the activity assays through out the purification procedure, aliquots were chosen to give observed A260 values within this linear portion of the curve. It is seen that amount of the enzyme between 0.08 to 2.9 units are suitable for the estimations of enzyme activity.

(4) Time curve—

The time curve is presented in Fig. 4. It is seen that the course of the enzymatic reaction is linear within the first 20 minutes. Accordingly, an incubation time of 15 minutes was chosen for all determinations of enzyme activity. This was done to assure that in the presence of excess substrate, a zero order of reaction was followed for the assays of enzyme activity.

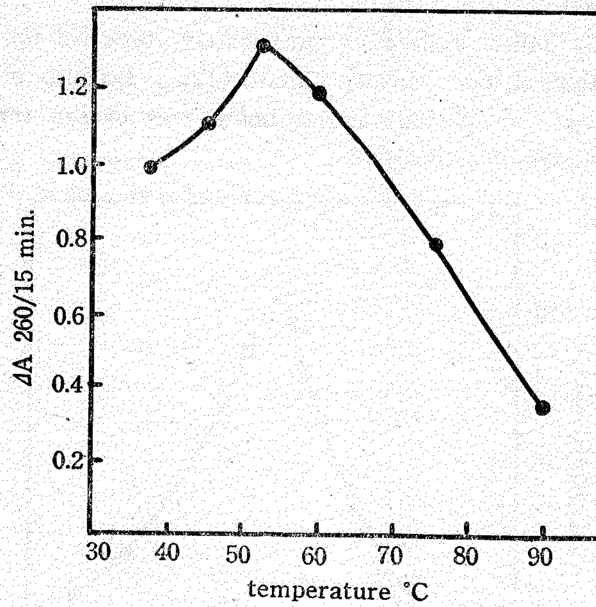

Fig. 2. Effect of incubation temperature on the activity of wheat sprout ribonuclease. For each determination of activity, enzyme solution was preincubated at the indicated temperature for 2 minutes. After which, appropriate amount of enzyme was introduced to the tube containing substrate and buffer. The remaining steps followed the routine procedure.

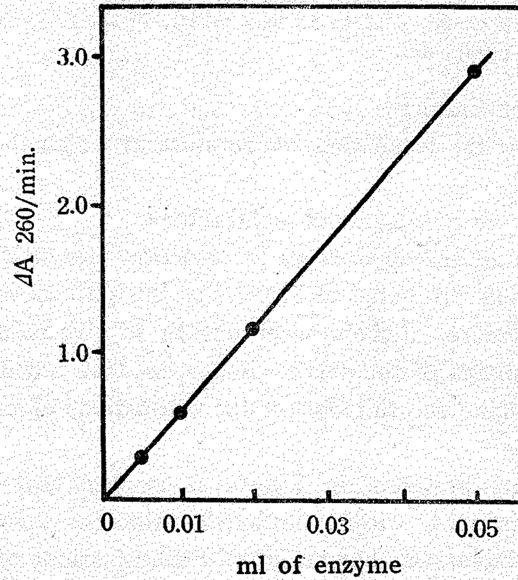

Fig. 3. Plot of enzyme amount against increase in absorption at 260 mμ in routine assay procedure.

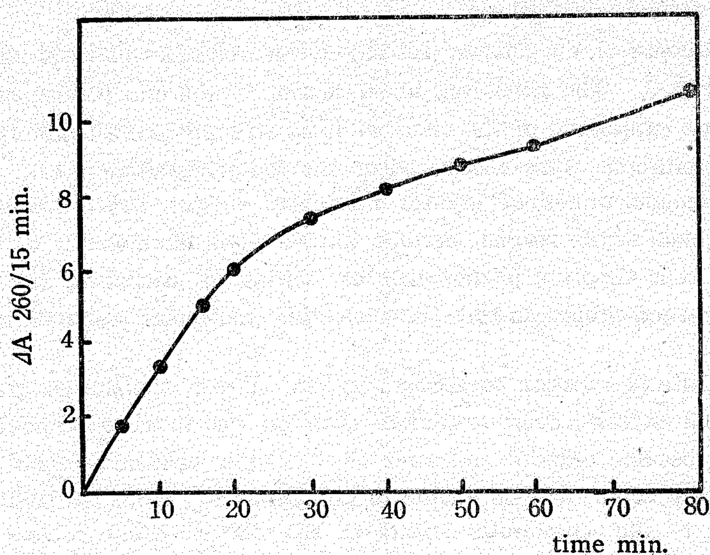

Fig. 4. Time curve: Each determination was carried out exactly as the routine procedure, except for the indicated incubation time.

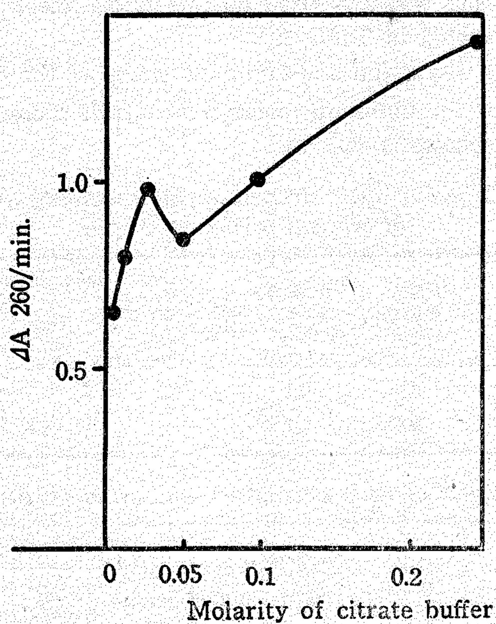

Fig. 5. Effect of ionic strength on the activity of wheat sprout ribonuclease. Except for the indicated molarity of citrate buffer (initial concentration), others followed the routine procedure for activity assay.

(5) Effect of ionic strength—

The response of the enzyme activity toward changes of ionic strength was shown in Fig. 5. The ascending linear section of the end portion of the curve is not a true expression of the effect of ionic strength but a result from incomplete precipitation. This was based on the following assumptions:

A. Oligonucleotides of greater molecular weight which are normally insoluble in MacFadyen reagent become soluble when the molarity of buffer used is higher. It is simply a phenomenon of "salting in" caused by the interaction between charged groups in the oligonucleotide molecules and inorganic ions of the buffer.

B. Citrate is a strong chelating reagent. When it exists in greater ionic strength, the effective concentration of uranium ions in the MacFadyen reagent will surely become reduced and some of the larger oligonucleotides formed by the enzymatic action will be unable to precipitate out from the solution.

Owing to the combining effects of the above stated facts, incomplete precipitation will reasonably be resulted in greater molarity of the buffer used.

Further evidence was obtained from an experiment carried out in the manner shown in Table 2.

Since the higher molarity buffer was added after the incubation time, the difference in A260/15 min. is doubtless due to incomplete precipitation.

Similar experiment was also tried on the initial linear portion of the curve. The data were presented in Table 3.

From the above discussion and from the shape of the curve in Fig. 5, it is quite clear now that the optimum molarity of citrate buffer for the activity of wheat sprout ribonuclease is 0.025 M.

Table 2. *Effect of ionic strength at the ascending linear section of the end portion of Fig. 5.*

Tube No.	0.05 M citrate buffer, pH 5.2 (ml.)	RNA 10 mg/ml. (ml.)	Enzyme sol'n (ml.)	After 15 min. incubation			A260*/15 min.
				0.25 M citrate buffer pH 5.2 (ml.)	H_2O (ml.)	MacFadyen reagent (ml.)	
1	0.4	0.5	0.1	0.5		1.5	27.48
2	0.4	0.5	0.1		0.5	1.5	14.28

Table 3. *Effect of ionic strength at the initial portion of Fig. 5.*

Tube No.	H_2O (ml.)	RNA (ml.)	Enzyme sol'n (ml.)	After 15 min. incubation			A260*/15 min.
				0.01 M citrate buffer pH 5.2 (ml.)	H_2O (ml.)	MacFadyen reagent (ml.)	
1	0.4	0.5	0.1	0.5		1.5	11.0
2	0.4	0.5	0.1		0.5	1,5	11.0

* These values were corrected from separate substrate-enzyme controls.

(6) Stability—

This enzyme was quite unstable. The partially purified enzyme lost 25% of its activity upon storing in a refrigerator (5°–10°C) for 24 hours and 50% of its activity would be lost after 48 hours. However, this loss in activity became more slowly once it dropped to half of its original value.

Prolonged dialysis often accompanied with, although not always, loss of activity.

(7) Activator and inhibitor—

The ribonuclease found in two-week old wheat seedlings (Matsushita, 1959) which also had an optimum pH at 5.2 was activated by EDTA in a concentration of 10^{-3} M. On the other hand, a ribonuclease in the mushroom (Chen, 1962) showed no activity at all when citrate buffer was used. The wheat sprout ribonuclease reported in the present paper was quite different from these two cases. It was neither activated nor inhibited by EDTA or citrate.

(8) Specificity—

This enzyme neither showed activity toward native nor heat-denatured DNA. However, it was persistently contaminated with a phosphatase.

3. *Identification of end products*—

Identification of the end products of the enzymatic hydrolysate was based on the following principles:

(1) At lower pH (3.5), owing to the difference in net negative charges, mononucleotides of all four different bases are separable by paper electrophoresis into distinct bands (Smith, 1955).

(2) The fact that sugar residues with cis glycol groups are capable of forming complexes with borate enables the electrophoretic separation of mononucleoside—5′—phosphates from their 2′ and 3′—isomers (Jaenicke and Vollbrechtshausen, 1955). The former ones are strongly affected by borate while those of the latter categories are not.

(3) By using Markham and Smith's solvent 3 (saturated ammonium sulfate, 79%; water, 19%; and isopropanol, 2%; v/v/v), the 2′ and 3′—isomers of purine nucleotides are well separated by paper chromatography (Markham and Smith, 1951).

The reaction mixture was prepared as usual. In addition, a few drops of sodium fluoride solution was added to exclude the effect of phosphatase present in the enzyme solution. This mixture was covered with a few drops of toluene and set at room temperature for 24 hours. After addition of MacFadyen reagent, the supernatant was treated with Dowex 50 W × 12 to remove excess uranium ions. The clear solution thus obtained was now ready for use in the identification process. Vacuum evaporation may be applied if higher concentration is desired. Since RNA is knwn to be easily undergoing spontaneous degradation,

corrections were made by controls prepared in the same way except without enzyme.

Picric acid was used as a reference marker in each electrophoretic running. Caffaine was also applied to correct the endoosmotic flow. Spots or bands were located under an ultraviolet lamp.

Table 4. shows the results obtained in paper electrophoresis in 0.1 M ammonium formate buffer, pH 3.5, field strength 40v/cm.

Table 4. *Mobilities relative to picric acid ($R_{picrate}$) in 0.1 M ammonium formate buffer pH 3.5 at 40v/cm.*

Authentic sample	$R_{picrate}$	Authentic sample	$R_{picrate}$	Enzyme digest	$R_{picrate}$
5′—CMP	0.14	2′3′*—CMP	0.14	band I	0.13
5′—AMP	0.30	2′3′ —AMP	0.31	band II	0.29
5′—GMP	0.60	2′3′ —GMP	0.62	band III	0.59
5′—UMP	0.78	2′3′ —UMP	0.80	band IV	0.76

Four bands were observed as shown in the table. These were eluted with distilled water and the first two drops of each eluate were collected and used in another running of electrophoresis in 0.05 M borax buffer, pH 9.2, field strength 40v/cm. The results are shown in table 5.

Table 5. *Relative mobilities of the eluates of the four bands in 0.05 M borax buffer, pH 9.2, at 40v/cm.*

Authentic sample	$R_{picrate}$	Authentic sample	$R_{picrate}$	Enzyme digest	$R_{picrate}$
5′—AMP	1.22	2′3′*—AMP	1.05	band II	1.05
5′—CMP	1.25	2′3′ —CMP	1.10	band I	1.10
5′—GMP	1.29	2′3′ —GMP	1.17	band III	1.17
5′—UMP	1.33	2′3′ —UMP	1.25	band IV	1.25

*2 and 3′ — isomer mixture

From the two tables it is quite apparent that the end products in the hydrolysate are all mononucleotides. Furthermore, from table 5, the 5′—isomers may well be excluded. The question now lies on whether they are the 2′—isomers, 3′—isomers or both. To solve this, bands II and III were cut off and eluted as before. The collected eluates were further identified by paper chromatography using Markham and Smith's solvent system. The chromatogram obtained as shown in table 6 indicates that bands II and III in table 4 are actually mixtures of 2′ and 3′—purine mononucleotides.

The 2′ and 3′—pyrimidine mononucleotides are, as a matter of fact, very difficult to separate. Although electrophoretic separation of these isomers has

been reported (Crestfield and Allen, 1955), the auther has not been able to achieve the same purpose under the reported conditions. The end products of the enzymatic hydrolysate identified are thus 2′—AMP, 3′—AMP, 2′—GMP, 3′—GMP 2′ or 3′—CMP or both, 2′ or 3′—UMP or both. No other products are detectable.

In view of the products identified, the action of the enzyme is rather obscure. However, the following two possibilities may be included: a, the ribonuclease is a nonspecific one. b, the enzyme preparation is a mixture of two or more ribonucleases of different specificities.

Table 6. *Chromatogram of bands II and III*

Sample	R_f	Sample	K_f
3′—AMP	0.21	band II_1	0.21
2′—AMP	0.31	band II_2	0.31
3′—GMP	0.47	band III_1	0.47
2′—GMP	0.58	band III_2	0.58

Summary

A ribonuclease from 2–3 days old wheat sprout (3–4 cm) was partially purified, its properties characterized and its end products identified. With an optimum pH at 5.2 and the optimal concentration of buffer 0.025 M, this enzyme is comparable to a number of plant ribonuleases from other sources (Sung, 1962; Tuve, 1960; Matsushita, 1959). Factors related to the purification procedure were also discussed.

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小麥芽中核酸分解酵素之分離及淨製

李 建 中

植物中核酸分解酵素之分離，近年來已屢見不鮮；作者自二至三公分長之小麥芽中分離得一種核酸分解酵素經淨製約八十倍，其反應之最適 pH 及緩衝液濃度等性質，與已見諸多

種文獻之其他植物中核酸分解酵素，甚相類似，但其分解產物之能溶於 MacFadyen 試劑者均爲單核苷酸類 (mononucleotides), 且 2′ 及 3′ 二種異構物均能檢出，是爲與自其他植物中所分離之核酸分解酵素之相異處。

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