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<https://doi.org/10.5109/22727>

出版情報 : 九州大学大学院農学研究院紀要. 13 (3), pp.361-368, 1965-05. Kyushu University
バージョン :
権利関係 :

A deoxyribonuclease I from rice bran

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Our knowledges about deoxyribonucleases of plant origin are rather scanty as compared with those of animal and microbial origins?") The present work aims at the purification and characterization of a deoxyribonuclease from rice bran. Special efforts were made in this experiment to eliminate the contamination of phosphatases, which are known^{3,4)} to exist abundantly in rice bran, since these were feared to interfere invariably with the identification of the deoxyribonuclease digests of DNA by further splitting off the terminal phosphate bonds.

Materials and methods

The substrate used for deoxyribonuclease determination is a preparation from calf thymus by the method of Kay et al.⁴⁾ Sodium ribonucleate of yeast purchased from N.B.C. was purified⁶⁾ and used as a substrate for ribonuclease. Calcium bis-paranitrophenylphosphate was synthesized from commercially available triphenylphosphate (Katayama) by the method of Laskowski et al.⁷⁾ and was used as a substrate for phosphodiesterase. Sodium paranitrophenylphosphate, used in phosphomonoesterase assay, is a commercial product (Katayama) of a guaranteed reagent grade.

Determination of deoxyribonuclease activity—This was finally established after examination and comparison of several prevailing procedures⁸⁻¹²⁾ adopting a principle of so-called "acid-soluble UV absorption measurement". The procedure in its entirety is as follows. Half a milliliter of the substrate solution (2 mg DNA/ml 0.01 M NaCl) was incubated with an appropriate amount of enzyme in 1 ml of 0.1 M ammonium acetate buffer of pH 5.5 for 60 minutes at 37°C. The reaction was terminated by cooling the mixture in an ice-water bath and precipitating the undigested DNA by adding 1 ml of cold N

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perchloric acid. After 10 minutes' settling the suspension was centrifuged for 5 minutes at 3000 r.p.m. The supernatant was diluted 10 fold with water and read at $260\text{ m}\mu$. Appropriate blanks, usually enzyme- and substrate-, were run at the same time and subtracted from the complete system. One unit of deoxyribonuclease activity is defined to be an amount of enzyme that produces 1.0 acid-soluble optical density/1 cm light path at $260\text{ m}\mu$ in 1 ml of the reaction mixture under the standard condition stated above. In other words, the enzyme unit can be routinely calculated from the optical density value by simply multiplying by 20 times. Here 2 times multiplication is for dilution caused by addition of the same volume of perchloric acid, and 10 times for dilution for reading. This unit also shows how much of the substrate DNA added was rendered acid-soluble, if one approximates 1 mg of DNA to 20 optical density units at $260\text{ m}\mu$. Potency of the enzyme was expressed in terms of deoxyribonuclease units/mg protein/ml reaction mixture. One optical density /1 cm light path at $280\text{ m}\mu$ is assumed to have a protein concentration of 1 mg/1 ml.

Determination of phosphomono- and phosphodiesterase activities— These enzymes were estimated essentially according to the method of Bjork¹³ except: that buffer solutions were replaced by ammonium acetate of pH 5.5. The molarity of NaOH, added for termination of reaction and coloration of free paranitrophenol, was also increased from 0.05 to 0.2^{14} in the present experiment.

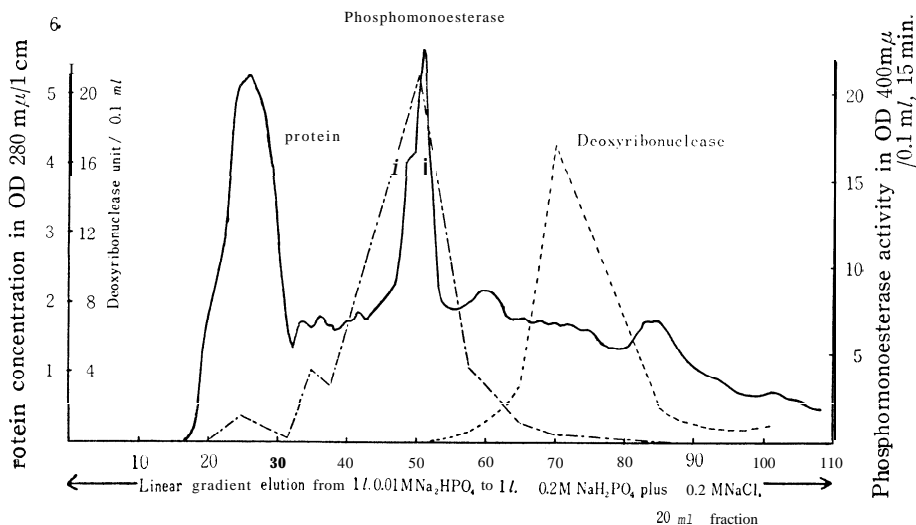


Fig. 1. DEAE-cellulose column chromatography (5 × 30 cm) of acetone fraction.

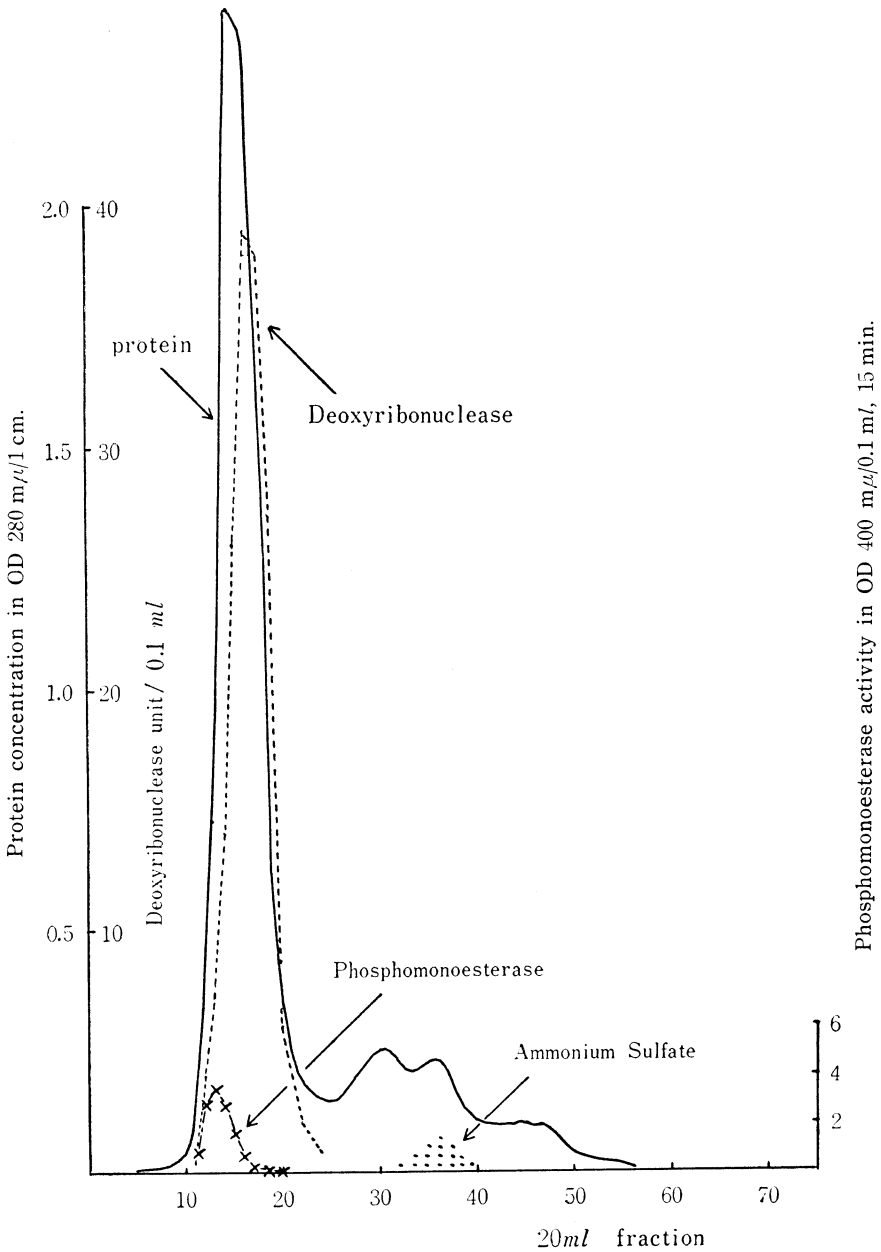


Fig. 2. Gel filtration through Sephadex G-75 (5×35 cm, in 0.1 M acetate, pH 6.5).

Purification of deoxyribonuclease, with special consideration of the elimination of phosphatases

All the steps were processed at 0-5°C. Initial ammonium sulfate and acetone steps were found suited for concentration of deoxyribonuclease activity. Chromatographies on DEAE- and CM-cellulose columns as well as gel filtration through Sephadex G-75 at later steps proved effective for the separation of deoxyribonuclease and phosphomonoesterase. The complete removal of the last trace of the contaminant, however, could not be attained so far. CM-cellulose column step was usually accompanied by a considerable loss. The results are shown in Fig. 1, 2, and 3, and are summarized in Table 1.

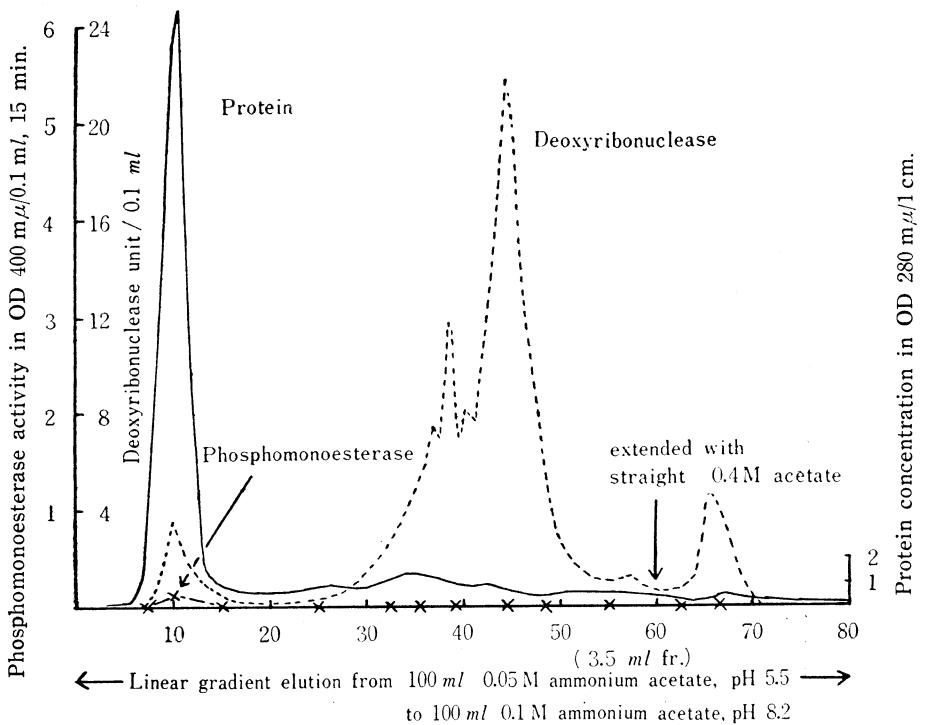


Fig. 3. CM-cellulose column chromatography (0.8 × 40 cm) of Sephadex filtrate.

Table 1. Summary of purification procedure.

Step	Volume	OD 280	Total protein	Potency	Total activity	Recovery
Crude extract from 1 kg of rice bran	4610 ^{ml}	284	1310000	0.069	90390	100 %
Ammonium sulfate Ppt., 0.35-0.60 saturation	1215	49.8	60500	1.46	88400	98
Acetone Ppt., 33-55%, at pH 7.5	94	60.1	5650	9.3	52600	58
DEAE-cellulose fr., Tube No. 65-85	382	1.52	581	52.5	30500	33
Sephadex G-75 fr., Tube No. 15-20	107	1.00	107	260	27100	31
CM cellulose fr., Tube No. 42	3.6	0.246	0.89	566		
43	"	0.218	0.79	737		
44	"	0.202	0.73	1120		
45	"	0.182	0.66	712		
46	"	0.170	0.61	570		

Table 2. Selective inhibition of phosphomonoesterase.

Relative activity of	Deoxyribonuclease	Phosphomonoesterase
In the presence of:		
None (Control)	100	100
0.01 M EDTA	69	114
0.001 M "	96	116
0.01 M MgCl ₂	78	114
0.001 M "	96	111
0.01 M CaCl ₂	83	108
0.001 M "	98	116
0.01 M NaF*	100	5
0.001 M " *	101	25
0.01 M Na arsenate*	100	26
0.001 M " *	100	41
0.01 M phosphate	59	11
0.001 M "	82	24

* Overnight incubation

Properties of deoxyribonuclease and contaminating phosphomonoesterase

Optimal acidity was found to be around pH 5.5 both for deoxyribonuclease and for phosphomonoesterase. Both were shown to require no special ions for full activity. These observations at first seemed discouraging in thinking of using the nuclease as a tool for the structural studies of DNA, because of the reason mentioned in the introductory remarks. Fortunately, however, the sensitivities of the two enzymes against fluoride ion were found quite dissimilar. As is shown in Table 2, the deoxyribonuclease was not affected at all whereas phosphomonoesterase was inhibited by 95 % at 0.01 M fluoride concentration even in an overnight incubation. This difference was made use of in the digestion experiment designed for specificity study.

Specificity of rice bran deoxyribonuclease

Three hundreds and twenty four mg of thymus DNA in 12 ml of water was incubated with 9.4 ml of CM-cellulose eluates (Tube No. 43, 44, and 45) in three portions, at pH 5.5 and in the presence of 0.01 M fluoride, for 48 hours at 37°C. The mixture was neutralized to pH 7.5 by concentrated ammonia and deproteinized by Sevag's¹⁵⁾ method. The material, after freed from the solvent odour by suction in a desiccator, was filled up to 25 ml in a measuring flask. An 8 ml fraction was pipetted out, diluted with 500 ml of water, and made alkaline to pH 10.5 by concentrated ammonia. It was in turn loaded on a column (1 × 9 cm) of Dowex 1 acetate, and fractionally eluted according to the method of Sinsheimer.¹⁶⁾ The pattern is profiled in Fig. 4. A break-thru peak, emerging with a buffer front and so oc-

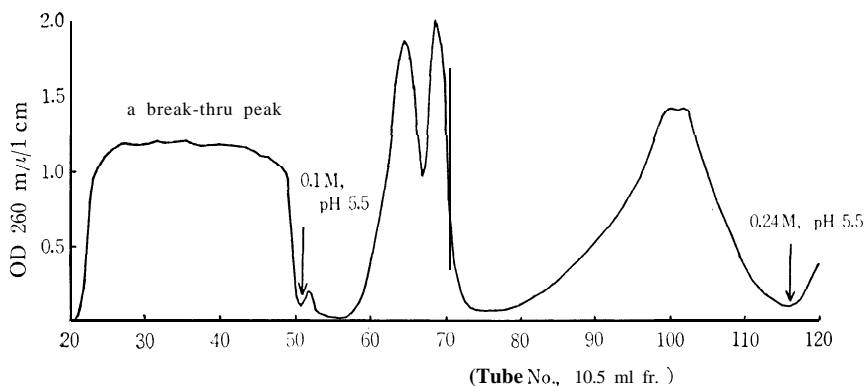


Fig. 4. Initial fractionation of deoxyribonuclease digest on Dowex-I acetate (1 × 9 cm).

copying the place expected for free bases and nucleosides, was first examined, for it was unbelievable in view of the fact that only 0.3 % of the total phosphorus was liberated as inorganic. It turned out as follows. It remained on a starting line when subjected to paperchromatography with 86 % n-butanol as a solvent, indicating it is phosphorylated. Bases and nucleosides would move.“) It was observed undiffusible through Visking cellophane tubing bag and insoluble in 0.5 N perchloric acid. It was also found that the material could not be retained on a charcoal column. Bases, nucleosides, and nucleotides of low molecular weights would be strongly held. Sedimentation constant measured by Spinco Ultracentrifuge Model E was 6.7. It looked homogeneous in the field. It yielded adenine, guanine, cytosine, and thymine on hydrolysis by 60 % perchloric acid at 100°C. It was thus settled that the material is of a polynucleotide nature too large in size to penetrate into a net-work of Dowex resin with 2 % cross linkage.

The mononucleotide fraction, eluted with 0.1 M ammonium acetate at pH 5.5, was rechromatographed on a column (1 × 20 cm) of Dowex 1 formate using a gradient elution system of Canellakis and Mantsavinou.¹⁸⁾ Three separate peaks were obtained. They showed the spectra characteristic of deoxymononucleotides of cytosine, thymine, and adenine in order of elution. Each of them was desalted by charcoal method and incubated separately with crude venom of “ Habu ” (*Trimeresurus flavobilides*) and with the purified venom phosphodiesterase in the presence of 0.01 M Mg at pH 9 for 4 hours at 37°C. Specificities and application of these venom enzymes have been established.¹⁹⁾ Each fraction yielded quantitatively the corresponding nucleoside by the action of crude venom, showing that they are phosphorylated at 5'-position. All of them were found resistant against the phosphodiesterase action, indicating that they are not dinucleoside monophosphates, which should, if present, occupy the same place as mononucleotides under the elution condition used. Identification of three 5'-deoxymononucleotides was thus attained, excluding the possibility of dinucleoside monophosphates. Deoxyguanylic acid could not be detected.

Discussion

This rice bran deoxyribonuclease is peculiar in the fact that it is a 5'-phosphate former in spite of its acting at acidic pH and requiring no Mg ion. Anyway it will be designated as deoxyribonuclease I. The next thing worthy to mention is that the enzyme leaves a fairly large amount of higher oligonucleotides besides mononucleotides. This means an endonucleolytic action with a limited specificity. It is clear that either 5'-phosphodiester linkage or 3'-phosphate linkage of deoxy-

guanosine residue is resistant from the result of mononucleotides analysis. At present it is impossible to tell which the case is. It will be clarified by examination of the terminal residues of di- and oligonucleotides in the digests.

Summary

A method of purification of a deoxyribonuclease I from rice bran was devised. Though it is not the final, the enzyme preparation is purified about 16,000 fold with respect to deoxyribonuclease activity, and the contamination of phosphatase is reduced to a trace. The pH optimum is 5.5. No metal ion is required. Deoxyguanylic acid alone was missing among 5'-mononucleotides identified in the digestion products. The enzyme action appears endonucleolytic.

Acknowledgment-1 am especially indebted to Prof. S. Akune for his unrestrained generosity throughout this experiment. It was supported in part by a grant from the Ministry of Education to him.

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