

R68-148

Purine Ribonucleosidase *g* from *Aspergillus foetidus*

ELWYN T. REESE AND ANNE H. MAGUIRE

Pioneering Research Laboratory, U. S. Army Natick Laboratories, Natick, Massachusetts 01760

Received for publication 9 August 1968

Nucleosidase *g* was prepared by growing *Aspergillus foetidus* on bran, and was purified by passage through a diethylaminoethyl-Sephadex column. The enzyme acted on the purine ribosides (except xanthosine) and on their 5'-phosphates. Action on the latter was a good means for preparing ribose-5-phosphate.

In a preceding paper (6), we described two types of purine ribonucleosidase from fungi. Nucleosidase *a* was produced by several aspergilli and penicillia, and it hydrolyzed adenosine more rapidly than guanosine. Nucleosidase *g* was found only in a commercial fungal cellulase (Cellulase 9X, Miles Laboratories, Inc., Elkhart, Ind.), and hydrolyzed guanosine more rapidly than adenosine. The source of nucleosidase *g* was reported to be an *Aspergillus* of the *niger* type, but in our experiments, no member of this group produced the enzyme. We have now grown the same organisms on wheat bran, and found strains that do produce the desired enzyme (EC 3.2.2.1).

MATERIALS AND METHODS

The methods were essentially those described in the previous paper (6). Wheat bran cultures consisted of bran plus twice its weight of the same salts solution used in the shaker experiments. This produced a spongy mass, on which the aspergilli grew quite well at 29 C. At 4 to 6 days, the bran culture was removed from the flasks, and the enzyme was extracted with water (percolation). Enzyme activity (1 unit) is the amount of enzyme required to produce 1 μ mole of ribose from inosine in 1 min at 40 C. It is equal to 30 of the units described in the previous paper (6).

RESULTS

Screening of cultures for nucleosidase g. According to L. Underkoffler, the Cellulase 9X, in which we found nucleosidase *g*, was derived from a black *Aspergillus*. We, therefore, grew 20 members of the *A. niger* group (representing 13 species) in shake flask experiments on various carbon sources, and tested the solutions for nucleosidase activity. In no case was there more than a trace of activity during an incubation period of 2 weeks.

Ten cultures were then grown on hard wheat bran in an effort to simulate the koji procedure used in the preparation of many commercial enzymes. A layer of wheat bran 1 inch (2.54 cm) deep was placed into each flask. When the culture

had grown to the bottom (4 to 6 days), the contents were removed, and water percolated through the bran culture three times. Since the amounts of reducing sugar in the percolates were high, the solutions were dialyzed before assaying. All cultures produced the nucleosidase (Table 1).

A. foetidus QM 328 was selected for further investigation. Grown on the wheat bran bed, it again produced 1.9 units of inosinase per ml of extract. Grown in shake flasks in wheat bran suspension, the best yield was slightly over 0.1 unit/ml. By using starch in shake flasks, even less enzyme was produced. The incorporation of guanosine (at 0.2%) failed to improve the yield under any of these conditions.

Further attempts to produce nucleosidase *g* in shake flasks failed with these fungi. The materials which induced nucleosidase *a* in the previous investigation (6) did not induce nucleosidase *g* under similar conditions. *A. foetidus* is unable to grow on ribonucleic acid (RNA) from yeast, adenosine, inosine, or adenosine monophosphate (AMP). It can consume guanosine, but no nucleosidase was detectable in these cultures.

We tested the percolates from the wheat bran cultures for enzymes other than nucleosidase *g*. Amylase, cellulase, and β -glucosidase were abundant; β 1,3-glucanase, β 1,6-glucanase, and α -trehalase were present in lesser amounts. These enzymes were also present in cellulase 9X.

Purification of nucleosidase g of A. foetidus. Direct acetone precipitation of the percolates from bran cultures is impossible. The sticky precipitates resist all attempts at drying. Dialysis in cellophane sacs is also impossible because of the presence of cellulase, but the solutions can be dialyzed in animal (gut) membranes (Aronab Prod. Co., San Francisco, Calif.). These dialyzed solutions were either concentrated (rotovap) and lyophilized or applied directly to a column for further purification. The lyophilized preparations retained over 90% of the initial nucleosidase activity.

TABLE 1. Production of nucleosidase *g* by members of the black aspergilli

Organism	Nucleosidase <i>g</i> , ^a
	units/ml
<i>A. foetidus</i> (QM 328)	1.9
<i>A. awamori</i> (QM 8164)	1.3
<i>A. niger</i> (QM 6906)	0.9
<i>A. niger</i> (QM 458)	0.8
<i>A. phoenicis</i> (QM 329)	0.4
<i>A. niger</i> (QM 877)	0.4
<i>A. miyakoensis</i> (QM 3309)	0.1
<i>A. fonsecaeus</i> (QM 330)	0.1
<i>A. violaceo fuscus</i> (QM 335)	0.1

^a The volume of eluant from the bran culture was 6 ml/g of bran.

When the dialyzed preparations were passed through a diethylaminoethyl (DEAE)-Sephadex column, much of the inactive protein and carbohydrate appeared in the early fractions. Nucleosidase *g* appeared at the beginning of the NaCl gradient, just before the appearance of the bulk of the protein and of the other enzymes (Fig. 1). There was a good separation of the nucleosidase from the phosphatase, a separation important for the characterization of the nucleosidase. The extent of purification is summarized in Table 2. Although 48% of the nucleosidase was recovered in the "purified" fraction, less than 1% of the initial amounts of the other enzymes was found.

Properties of the nucleosidase *g*. The points of difference of nucleosidase *g* from nucleosidase *a* were previously described (6). The purpose of these experiments was to determine whether the enzyme from *A. foetidus* is essentially the same as that found in Cellulase 9X.

The purified *A. foetidus* preparation hydrolyzed AMP to adenine and ribose-5-phosphate in the same manner as did Cellulase 9X. Because of a lower phosphatase activity it produced less ribose from the ribose-5-phosphate on prolonged incubation (24 hr).

The relative susceptibility of the purine nucleosides was the same for our preparation and for that of the commercial enzymes (Table 3).

Nucleosidases of three of the other black aspergilli had essentially the same pattern of substrate preferences. AMP was hydrolyzed about as fast as adenosine. Xanthosine was a very poor substrate for nucleosidase *g* preparations of all four of the black aspergilli tested. Xanthosine was a relatively good substrate for nucleosidase *a* (6).

Another property characteristic of nucleosidase *g* was its response to various inhibitors (7).

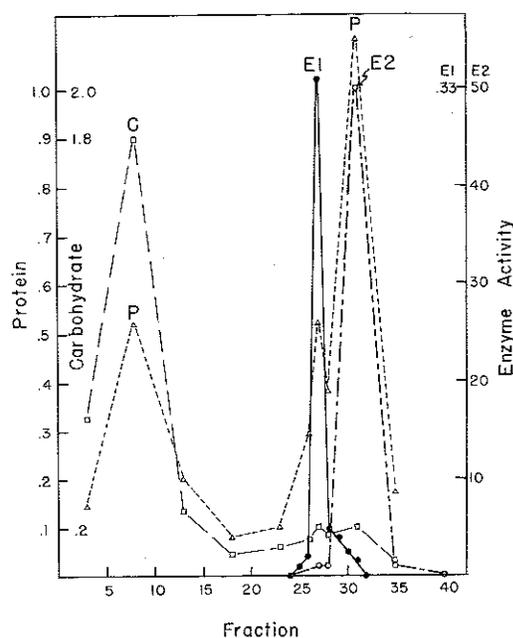


FIG. 1. Purification of nucleosidase *g* on DEAE-Sephadex (100 × 47 mm). Sample: 63 inosinase units of *A. foetidus* in 300 ml water. Elution: 0.02 M phosphate buffer, pH 7.0, in a constant volume mixer (400 ml); 0.02 M phosphate buffer, pH 6.0, plus 0.5 M NaCl in reservoir. Each fraction was 60 ml. C, carbohydrate (milligram per milliliter), by phenol-H₂SO₄ method; P, protein (milligram per milliliter); E1, nucleosidase *g* (units per milliliter); E2, phosphatase (units per milliliter), estimated by the *p*-nitrophenyl-phosphate method.

TABLE 2. Purification of *A. foetidus* enzyme

Property	Dialyzed percolate	Purified fraction ^a
Specific activity (units per milligram of protein).....	0.4	3.8 (9 ×)
Activity in units per milligram of carbohydrate.....	0.36	9.9 (27 ×)
Inosinase-phosphatase ratio.....	0.17	9.5 (56 ×)

^a Values in parentheses are enrichment values for purified fraction as compared to original extract.

Again, our preparations responded in the same manner as did Cellulase 9X (Table 4).

Preparation of ribose-5-phosphate. Since fungal phosphatases that act on 5' nucleotides are inhibited by NaF (4), it is not necessary to remove

TABLE 3. *Substrate specificity of nucleosidase g^a*

Substrate	<i>A. foetidus</i>	Cellulase 9X
	%	%
Inosine.....	100	100
Guanosine.....	33	34
Adenosine.....	15	21
Adenosine 5'-phosphate.....	31	26
Xanthosine.....	0	0

^a Activity relative to that on inosine.

TABLE 4. *Effect of inhibitors on activity of nucleosidase g*

Inhibitor	Inhibitor-substrate ratio for 50% inhibition ^a	
	<i>A. foetidus</i>	Cellulase 9X
Adenosine.....	0.14	0.10
7-β-Adenosine.....	>10.	>10.
5'-Chloroadenosine.....	0.07	0.02
Adenosine monophosphate...	0.30	0.30

^a Ratio of inhibitor to substrate required to give 50% inhibition of hydrolysis. The substrate in these tests was inosine (2 mg/ml of reaction mixture, pH 4.0).

phosphatase from the nucleosidase *g* preparation to prepare ribose-5-phosphate from adenosine 5'-phosphate (or inosine 5'-phosphate). Incorporation of 0.016 N NaF completely suppressed the phosphatase of *A. foetidus* without inhibiting action of the nucleosidase on the glycosidic linkage. Ribose-5-phosphate was the only sugar-containing product of hydrolysis from adenosine 5'-phosphate.

Phosphatases accompanying nucleosidase *a* were similarly inhibited by NaF. In the previous paper (6), our evidence for the inability of nucleosidase *a* to act on AMP was indirect. By repeating the experiments in the presence of 0.003 N NaF, we have now confirmed the earlier conclusion that nucleosidase *a* cannot hydrolyze AMP under conditions in which adenosine is readily hydrolyzed.

DISCUSSION

Nucleosidase *g* was prepared by growing *A. foetidus* (and related black aspergilli) on wheat bran and purified by passage through a DEAE-Sephadex column. The enzyme thus prepared closely resembles that described previously (6) in a commercial cellulase preparation (Cellulase 9X).

Nucleosidases *a* and *g* have several common features. Both are fungal enzymes active only on purine nucleosides. Their optimal pH is low, about 4.0, and they are relatively stable at temperatures up to 40 C.

Nucleosidase *g* differs from nucleosidase *a* in several respects.

The culture for nucleosidase *g* resulted in poor yields, whereas for nucleosidase *a* good yields were produced. Nucleosidase *g* was constitutive, but nucleosidase *a* was induced by RNA, AMP, etc. Nucleosidase *g* was derived from the *A. niger* group, but nucleosidase *a* was derived from several species of *Penicillium* and *Aspergillus* (but not *A. niger*). The order of susceptibility to hydrolysis by nucleosidase *g* was inosine, guanosine, adenosine, and AMP; by nucleosidase *a*, the order was inosine, adenosine, xanthosine, and guanosine. The two nucleosidases differed also in their response to the following inhibitors: 7-β adenosine, guanosine, adenosine, and AMP.

Nucleosidase *g* has two interesting, and potentially useful, properties. First, it cannot hydrolyze xanthosine, and xanthosine is not an inhibitor of its activity. Therefore, it can be used to selectively remove the other purine ribosides from a mixture of ribosides, while leaving xanthosine unaffected. Second, its ability to hydrolyze the nucleoside linkage is not affected by the presence of a phosphate group at C5'. As a result, it can be used to prepare ribose-5-phosphate by hydrolysis of AMP or inosine monophosphate (see also 1, 5).

A glycosidase similar to nucleosidase *g* has been found in *A. oryzae* (4). The latter differs, however, in its specificity, acting on I5'p and G5'p, but not A5'p. Even more specific is the nucleosidase of *Azotobacter vinelandii* which acts only on A5'p (2, 8). None of these hydrolyze purine ribosides having a phosphate at C2' or C3', nor do they act on pyrimidine ribosides. A single example of nucleosidase acting on pyrimidine ribosides containing phosphate at C5' has been reported from *Streptomyces* (3). It is unusual in that it is inactive on uridine or on U3'p, but active on U5'p and deoxy U5'p.

LITERATURE CITED

- Horecker, B. L. 1957. Preparation and analysis of ribose-5-phosphate, p. 188-190. In S. P. Colowick and N. O. Kaplan (ed.), *Methods in enzymology*, vol. 3. Academic Press, Inc., New York.
- Hurwitz, J., L. A. Heppel, and B. L. Horecker. 1957. Enzymatic cleavage of adenylic acid to adenine and ribose-5-phosphate. *J. Biol. Chem.* 226:525.
- Imada, A. 1967. Pyrimidine 5' nucleotide phos-

- phoribo (deoxyribo) hydrolase of *Streptomyces virginiae*. J. Gen. Appl. Microbiol. **13**:267-278.
4. Kuninaka, A. 1959. Ribosidase of *A. oryzae* acting on 6 hydroxy purine ribonucleosides and their 5' monophosphates. Bull. Agr. Chem. Soc. Japan **23**:281-288.
 5. LePage, G. A., and W. W. Umbreit. 1943. Adenosine 3' triphosphate in autotrophic bacteria. J. Biol. Chem. **148**:255-260.
 6. Reese, E. T. 1968. Extracellular purine β -ribosidases from fungi. Can. J. Microbiol. **14**:377-383.
 7. Reese, E. T., L. B. Townsend, and F. W. Parrish. 1968. Inhibition of purine β -ribosidases. Arch. Biochem. Biophys. **125**:175-177.
 8. Yoshino, M., N. Ogasawara, N. Suzuki, and Y. Kotake. 1967. Regulation of AMP nucleosidase in *Azotobacter vinelandii*. Biochim. Biophys. Acta **146**:620-22.