ADP-ribosyltransferase from *Helix pomatia*

Purification and characterization

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ADP-ribosyltransferases from several higher eukaryotes have been purified and characterized, but little is known about ADP-ribosyltransferases in lower eukaryotes. We have purified an ADP-ribosyltransferase (EC 2.4.2.30) from *Helix pomatia*. The enzyme has an apparent K_m of 26.7 μM . Optimal conditions for the enzyme reaction are 17.5 °C and pH 8. The time course is linear during the first 10 min of the reaction. The enzyme is capable of poly-ADP-ribosylation. The most highly purified preparation shows one major band at an M_r of 75000 on electrophoresis in an SDS/polyacrylamide gel, with minor bands at M_r 115000 and 155000. Re-activation of SDS/polyacrylamide gels *in situ* shows the 75000- M_r band to be enzymically active and additional active bands with M_r values of 115000, 90000 and 87000 respectively. The 115000- M_r and 75000- M_r bands cross-react with a polyclonal affinity-purified antiserum against human ADP-ribosyltransferase. Like enzymes from higher eukaryotes, the activity from *Helix pomatia* is inhibited by thymidine, theophylline, theobromine nicotinamide, 3-methoxybenzamide and 3-aminobenzamide, and is dependent on histone and DNA.

INTRODUCTION

ADP-ribosyltransferases (EC 2.4.2.30) are enzymes that modify proteins covalently by attaching one or several ADP-ribosyl residues, using NAD^+ . ADP-ribosylation is involved in many biological processes, such as DNA synthesis and cellular proliferation [1,2] as well as differentiation and development [1]. Inhibition of ADP-ribosyltransferases enhances the toxicity of alkylating agents whereby the ligation step of DNA repair could be affected [3,4].

ADP-ribosyltransferases from higher eukaryotes have been purified and characterized [2]. Much less is known about the enzymes in lower eukaryotes. Considering the importance of ADP-ribosylation, it seemed worthwhile to purify and characterize an ADP-ribosyltransferase from an animal of the lower branches of the phylogenetic tree and to compare it with the enzymes of higher organisms. Applying an affinity-chromatographic procedure, which we had developed previously [5], we isolated an ADP-ribosyltransferase from the mollusc *Helix pomatia*. This paper presents to our knowledge the first purification and characterization of an ADP-ribosyltransferase from an invertebrate eukaryote.

MATERIALS AND METHODS

Materials

Phosphocellulose P11 was purchased from Whatman. 3-Aminobenzamide-Sepharose was prepared as described previously [5]. Hydroxyapatite was synthesized according to the method of Bernardi [6]. Glass-fibre and nitrocellulose filters were from Schleicher und Schüll.

[adenine-2,8- ^3H]NAD $^+$ and [adenylate- ^{32}P]NAD $^+$ were from New England Nuclear. Unlabelled NAD $^+$, calf thymus DNA, histone type V-S from calf thymus, thymidine, caffeine, theophylline, theobromine, 3-aminobenzamide, pepstatin A, phenylmethanesulphonyl fluoride, Tween 20, 3'-AMP, 5'-AMP, ADP-ribose, phosphorylase *b* from rabbit muscle, bovine serum albumin and phosphodiesterase I from *Crotalus atrox* venom type VII were from Sigma Chemical Co.; 3-methoxybenzamide was from Aldrich Chemical Co.; RNAase inhibitor and human ADP-ribosyltransferase were prepared in our laboratory; other chemicals used were of analytical grade. The ultrafinity EP column was purchased from Beckman. Alkaline-phosphatase-labelled anti-(rabbit IgG) antibody was from Promega Biotec. Blue Sepharose was from Pharmacia. Plates for t.l.c. were Polygram CEL 300 UV 254 from Macherey-Nagel.

Electrophoresis

Polyacrylamide (10%) slab gels were used according to the method of Davis [7] as modified by Laemmli [8]. Gels were stained with silver according to published methods [9,10]: Gels were fixed with 50% (v/v) methanol/12% (v/v) acetic acid for 20 min, washed with 10% (v/v) ethanol/5% (v/v) acetic acid for three periods of 10 min, oxidized with $\text{K}_2\text{Cr}_2\text{O}_7$ (1 g/l) in 3.2 mM- HNO_3 for 15 min, washed with distilled water until colourless, and finally placed into a 0.2% AgNO_3 solution for 45 min. They were developed for up to 15 min with 0.28 M- K_2CO_3 containing 0.5 ml of 35% formalin/l. Development was stopped by addition of 10% (v/v) acetic acid containing 5% (v/v) glycerol.

Abbreviation used: 3-aminobenzamide-Sepharose, O-[15-[3-(aminocarbonyl)phenyl-1-imino-11,14-dioxo-2,6,10,15-tetra-azapentadecyl]]-Sepharose.

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Protein determination

Protein concentrations were determined by the Coomassie Blue dye-binding assay in accordance with Bradford [11], with bovine serum albumin as standard.

Enzyme assay

ADP-ribosyltransferase activity was assayed essentially as described previously [5] but at 15 °C for 10 min. A 10 µl portion of enzyme solution was added to 90 µl of reaction mixture {0.1 M-Tris/HCl buffer, pH 8, containing 1 mM-dithiothreitol, 22 µg of activated calf thymus DNA/ml, 22 µg of histone/ml, 33 µCi of [³H]NAD⁺ (25 Ci/mmol)/ml and 10 µM-NAD⁺}. The reaction was stopped by addition of 100 µl of 40% (w/v) trichloroacetic acid, and 160 µl samples from each tube were spotted on to glass-fibre filter discs. The filters were washed with 5% trichloroacetic acid followed by methanol/diethyl ether (1:1, v/v) and dried at 100 °C. Filter-bound radioactivity was measured in a liquid-scintillation counter.

Enzyme purification

ADP-ribosyltransferase was purified according to published methods [5], with some minor modifications. The animals were dissected, and feet and livers were put immediately into liquid N₂ and then stored at -80 °C until used. All procedures were carried out between 0 and 4 °C.

Extraction. A 100 g batch of frozen livers was placed into 500 ml of ADPRT buffer [0.1 M-Tris/HCl buffer containing 17% (v/v) glycerol, 25 mM-K₂S₂O₈, 12 mM-2-mercaptoethanol, 5 mM-EDTA, 50 mM-KH₂PO₄ and 1 µM-pepstatin A, pH adjusted to 7.2 at room temperature] containing 1 mM-phenylmethanesulphonyl fluoride and 0.5 M-KCl and left for 15 min at room temperature (buffer temperature does not rise above 0 °C). The tissue was homogenized in an Omnimix homogenizer for four periods of 15 s, mixed with 100 g of DE 52 DEAE-cellulose and stirred for 15 min. The homogenate was then centrifuged at 14000 g for 5 min.

Hydroxyapatite chromatography. The crude extract was filtered through cheesecloth and then shaken with 100 g wet wt. of hydroxyapatite (equilibrated in ADPRT buffer containing 0.5 M-KCl) for 60 min. The suspension was centrifuged at 1600 g for 5 min, the supernatant was discarded and the hydroxyapatite pellet was resuspended in 800 ml of ADPRT buffer containing 1 mM-phenylmethanesulphonyl fluoride. This suspension was again centrifuged at 1600 g for 5 min, and the hydroxyapatite was resuspended in 160 ml of ADPRT buffer (but with KH₂PO₄ at 0.5 M) containing 1 mM-phenylmethanesulphonyl fluoride. The suspension was shaken for 30 min and then centrifuged as before.

Phosphocellulose chromatography. A 40 g wet wt. portion of phosphocellulose (equilibrated in ADPRT buffer) was suspended in the hydroxyapatite eluate, which had been diluted 5-fold with ADPRT buffer, and this suspension was agitated for 60 min and then centrifuged at 1600 g for 5 min. The phosphocellulose pellet was washed out of the tube with 20 ml of ADPRT buffer and packed into a 2.5 cm-diameter column. Then a linear gradient of 0–2 M-KCl in ADPRT buffer was applied.

Affinity chromatography on 3-aminobenzamide-Sepharose. Active fractions from the phosphocellulose chromatography were applied directly to the affinity column (2.5 cm × 8 cm). The column was washed with ADPRT buffer containing 0.3 M-KCl and then eluted with ADPRT buffer containing 0.3 M-KCl and 1 mM-3-methoxybenzamide. The eluate was allowed to drop directly on top of a small hydroxyapatite column (0.7 cm × 0.5 cm), which was then washed with ADPRT buffer containing 0.3 M-KCl and eluted with ADPRT buffer with KH₂PO₄ at 0.5 M.

Re-activation of ADP-ribosyltransferase *in situ*

This was done in accordance with Scovassi *et al.* [12]. The samples were run on an SDS/10%-polyacrylamide gel (8 cm, 0.75 mm thick; 120 V for 1.5 h) containing 100 µg of calf thymus DNA/ml. After electrophoresis SDS was removed and ADP-ribosyltransferase was re-activated. The gel was incubated with reaction mixture containing 10 µCi of [³²P]NAD⁺ (1000 Ci/mmol)/ml, washed and dried as described above. Active bands were detected by autoradiography with the use of an intensifying screen.

Immunoblotting

A 3 ml portion of crude antiserum against human ADP-ribosyltransferase in buffer A (0.5 M-KCl/0.15 M-NaCl/10 mM-Na₂HPO₄, adjusted to pH 7.0 with acetic acid) was applied to an ultraaffinity EP column (50 mm × 4.6 mm) to which 3 mg of human ADP-ribosyltransferase had been bound as recommended by the supplier. Elution with a 2 ml pulse of buffer B (as buffer A, but adjusted to pH 3.4 with acetic acid) yielded 1.4 mg of affinity-purified antibodies. Proteins were separated according to their *M_r* on an SDS/10%-polyacrylamide gel (8 cm, 0.75 mm thick; 200 V for 1 h) and transferred to nitrocellulose in a transblot chamber [blot buffer: 10% (v/v) methanol/0.05% SDS/25 mM-Tris/187 mM-glycine, pH 8.3] by applying 30 V for 3 h at room temperature. The nitrocellulose membranes were then soaked in blocking solution [20 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NaCl and 2% (w/v) commercial skim-milk powder; the solution was heated to 90 °C, cooled and filtered before use] for 1 h and incubated with 2.8 µg of affinity-purified anti-ADP-ribosyltransferase antibodies/ml in blocking solution overnight. To remove unspecifically bound antibodies, the nitrocellulose sheet was washed four times with blocking solution. Antibodies were detected with alkaline-phosphatase-labelled anti-(rabbit IgG) antibodies. The sheet was incubated for 3 h with a 1:7500 dilution of anti-antibodies in blocking buffer, washed three times with blocking buffer and twice with alkaline phosphatase buffer (0.1 M-Tris/HCl buffer, pH 9.5, containing 100 mM-NaCl and 5 mM-MgCl₂) and stained with 5 ml of alkaline phosphatase buffer containing 16.5 µl of 5-bromo-4-chloroindol-3-yl phosphate solution (50 mg/ml) and 33 µl of Nitro Blue Tetrazolium solution (50 mg/ml). After an appropriate time the staining reaction was stopped with water.

pH-dependence

After affinity chromatography 200 µl (36 µg) of the enzyme solution was spun through a Sephadex G-50 column (1 cm × 3.5 cm) equilibrated in 17% (v/v) glycerol/12 mM-2-mercaptoethanol and then tested at

Table 1. Purification of ADP-ribosyltransferase from *Helix pomatia*

Step	Total protein (mg)	Specific activity (nmol/min per mg)	Purification factor (fold)	Recovery (%)
Crude extract	6750	0.011	1	100
Hydroxyapatite	240	0.17	15	55
Phosphocellulose	11.4	1.3	120	20
3-Aminobenzamide-Sephacrose	0.054	54.5	4950	4

different pH values by adjusting the reaction mixture with Tris to pH 6, 7, 7.5, 8, 8.5, 9 and 9.6. The assay was carried out at 15 °C for 10 min.

Temperature optimum

Portions [10 µl (0.18 µg)] of enzyme solution were each added to 90 µl of the prewarmed/precooled reaction mixture. Reaction time was 5 min. Temperatures tested were 0, 5, 10, 12.5, 15, 17.5, 20, 25, 30, 35, 40 and 50 °C. The reaction was stopped by adding 100 µl of 40% (w/v) trichloroacetic acid.

Time course

Enzyme and reaction mixture were adjusted separately to 15 °C and then mixed. Samples (100 µl) were taken at 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50 and 60 min and pipetted on to 100 µl of 40% (w/v) trichloroacetic acid.

Enzyme kinetics

Portions [10 µl (0.18 µg)] of enzyme solution and 10 µl of NAD⁺ of different concentrations (end concentrations in assay: 50, 25, 17, 13 and 10 µM) were added to a reaction mixture containing all components except unlabelled NAD⁺. Reaction time was 10 min at 15 °C.

Inhibitors

Thymidine, caffeine and nicotinamide were dissolved in water, theophylline in 0.1 M-Tris/HCl buffer, pH 8, and theobromine in 20 mM-NaOH. 3-Methoxybenzamide and 3-aminobenzamide were diluted from a methanolic stock solution. Portions (10 µl) of each inhibitor solution (10-fold concentrated) were added to 80 µl of reaction mixture, and 10 µl of enzyme solution was added afterwards. Reaction time was 10 min at 15 °C. For control 10 µl of water without inhibitor was added to some tubes; 1% methanol was taken as control for 3-methoxybenzamide and 3-aminobenzamide.

DNA and histone requirements

Portions (10 µl) of enzyme with or without 10 µl of calf thymus DNA (in water, 0.2 mg/ml) with or without 10 µl of histone or bovine serum albumin (in water, 0.2 mg/ml) were added to 70 µl of reaction mixture. Reaction time was 10 min at 15 °C.

Product analysis

A 100 µl portion of affinity-purified ADP-ribosyltransferase was added to 400 µl of reaction mix (see enzyme assay) with a lowered NAD⁺ concentration of 1 µM and 5 µl of [³²P]NAD⁺ (667 Ci/mmol; 3.55 mCi/

Fig. 1. SDS/polyacrylamide-gel electrophoresis of ADP-ribosyltransferase

Electrophoresis was in a 10% polyacrylamide gel (14 cm, 1.2 mm thick; 250 V for 3 h). Lane 1, 1.5 µg of human ADP-ribosyltransferase; lane 2, 0.7 µg of human ADP-ribosyltransferase fragmented by incubation at room temperature for 8 h; lane 3, 0.8 µg ADP-ribosyltransferase purified from *Helix pomatia* liver with phenylmethanesulphonyl fluoride and K₂S₂O₈ as proteinase inhibitors; lane 4, 0.8 µg of ADP-ribosyltransferase purified from *Helix pomatia* liver with phenylmethanesulphonyl fluoride, K₂S₂O₈, pepstatin A and EDTA as proteinase inhibitors; lane 5, 1.8 µg of ADP-ribosyltransferase purified from *Helix pomatia* foot, with the same inhibitors as in lane 4. *M_r* markers were ADP-ribosyltransferase (human placenta), *M_r* 116000, phosphorylase b (rabbit muscle), *M_r* 97000, bovine serum albumin, *M_r* 67000, and RNAase inhibitor (human placenta), *M_r* 52000.

ml) instead of [³H]NAD⁺ and incubated for 30 min at 15 °C, and then the reaction was stopped by addition of 500 µl of 40% (w/v) trichloroacetic acid and left at 0 °C for 2 h. After centrifugation the pellet was washed three times with 1 ml of 10% (w/v) trichloroacetic acid

Fig. 2. Re-activation of ADP-ribosyltransferase *in situ*

Proteins were separated by electrophoresis in an SDS/10% polyacrylamide gel, re-activated and incubated with [32 P]NAD $^{+}$ in accordance with Scovassi *et al.* [12]. Active bands were located by autoradiography. Lane 1, freshly prepared extract from human placenta (86 μ g); lane 2, purified human ADP-ribosyltransferase fragmented by freezing and thawing (0.3 μ g); lane 3, as lane 2 but three times frozen and thawed (0.3 μ g); lanes 4–6, purification steps of *Helix pomatia* liver ADP-ribosyltransferase, hydroxyapatite eluate (18 μ g), phosphocellulose pool (18 μ g) and affinity-chromatography-purified preparation (0.3 μ g) respectively. M_r values refer to human ADP-ribosyltransferase and its fragments.

containing 1 mM-NAD $^{+}$ and finally with 1 ml of methanol/diethyl ether (1:1, v/v), and the pellet was dried in a vacuum desiccator and then dissolved in TM buffer (10 mM-Tris/HCl buffer, pH 8.0, containing 10 mM-MgCl $_2$). A portion (10 μ l) of this solution was digested for 8 h at 37 $^{\circ}$ C with 1 unit of phosphodiesterase I, which had been purified with the use of Blue-Sepharose by the method of Oka *et al.* [13]. The product of the digestion was analysed by t.l.c. on cellulose sheets with the solvent system 0.1 M-NaH $_2$ PO $_4$ /(NH $_4$) $_2$ SO $_4$ /propan-1-ol (50:30:1, v/w/v) at room temperature for 4.5 h [14]. The thin-layer sheet was cut into strips and radioactivity was determined in a liquid-scintillation counter.

RESULTS AND DISCUSSION

ADP-ribosyltransferase activity from *Helix pomatia* was purified by an affinity-chromatography procedure as described previously [5]. The procedure resulted in a 4000–5000-fold-enriched enzyme preparation. Data for a representative purification are shown in Table 1. The enzyme exhibited similar elution patterns as ADP-ribosyltransferases from human, horse, rat and chicken tissue [5]. It bound to hydroxyapatite from a solution containing 0.5 M-KCl and 50 mM-KH $_2$ PO $_4$, was removed from phosphocellulose with 0.3 M-KCl and after binding to 3-aminobenzamide-Sepharose it could be eluted with 1 mM-3-methoxybenzamide.

Fig. 3. Immunological relationship of ADP-ribosyltransferases from man and *Helix pomatia*

Proteins were separated by electrophoresis in an SDS/10% polyacrylamide gel, which was blotted to nitrocellulose membrane and incubated with affinity-purified polyclonal antibodies against human ADP-ribosyltransferase. Antibodies bound were detected by alkaline-phosphatase-labelled second antibodies. Lane 1, 300 ng of human ADP-ribosyltransferase fragmented by freezing and thawing; lane 2, 2 μ g of histone H1; lane 3, 2 μ g of bovine serum albumin; lane 4, 1.2 μ g of ADP-ribosyltransferase from *Helix pomatia* liver; lane 5, 2.2 μ g of ADP-ribosyltransferase from *Helix pomatia* foot.

The final enzyme preparation showed a major band at M_r 75000 on electrophoresis in an SDS/polyacrylamide gel (Fig. 1), with faint bands appearing at M_r 115000 and 155000. It should be noted that the same purification conditions that yielded homogeneous ADP-ribosyltransferase from human tissue with an M_r of 116000 [5] even without extensive use of proteinase inhibitors (Fig. 1, lane 1) resulted with *Helix pomatia* mainly in a 75000- M_r protein. It seems to be unlikely that proteolytic degradation during the enzyme preparation could be responsible for the appearance of the 75000- M_r band in the *Helix pomatia* preparation, since addition of proteinase inhibitors did not significantly alter the intensity of this band in an SDS/polyacrylamide gel (Fig. 1, lanes 3, 4 and 5) and additionally the purification procedure applied is the fastest one known for ADP-ribosyltransferases, lasting only about 8–10 h from homogenization to the affinity-purified preparation.

Since in *Helix pomatia* there is a very close association of liver and gut, this might be a cause of some proteolysis. To test this, we purified the enzyme also from the foot part of *Helix pomatia*. This purification gave the same gel pattern (Fig. 1, lane 5). Preparation of human ADP-ribosyltransferase sometimes also gives a protein band at M_r 66000, but this band is weak even when only K $_2$ S $_2$ O $_8$ is used for proteinase inhibition (Fig. 1, lane 2).

Re-activation of the ADP-ribosyltransferase activity *in situ* in SDS/polyacrylamide gels [12] shows that the major activity resides in the 75000- M_r band. Minor activities appear at M_r 90000, 95000 and 115000, the last-mentioned only after the affinity-chromatography

Table 2. DNA- and histone-dependence of ADP-ribosyltransferase from *Helix pomatia*

Reaction conditions were as described in the Materials and methods section; DNA and/or histone were added separately where indicated. Results are given as means $\pm$ S.D. for six measurements.

DNA (20 μ g/ml)	Histone (20 μ g/ml)	Bovine serum albumin (20 μ g/ml)	Activity (% of maximum)
+	+	—	100
—	—	—	4 $\pm$ 1.7
—	—	+	2 $\pm$ 1.9
—	+	—	5 $\pm$ 1.5
+	—	—	8 $\pm$ 2.1
+	—	+	9 $\pm$ 4.6

Table 3. Inhibitors of ADP-ribosyltransferase from *Helix pomatia*

See the Materials and methods section for reaction conditions. Results are given as means $\pm$ S.D. for the numbers of measurements given in parentheses.

Inhibitor	Concentration (mM)	Activity (% of control)
Control	—	100
Thymidine	1	30 $\pm$ 8.6 (9)
Caffeine	1	77 $\pm$ 6.5 (9)
Theophylline	1	28 $\pm$ 7.7 (9)
Theobromine	1	24 $\pm$ 7.9 (9)
Nicotinamide	1	9 $\pm$ 1.7 (9)
Methanol	300	101 $\pm$ 6.8 (9)
3-Methoxybenzamide	1	2 $\pm$ 2.7 (7)
3-Aminobenzamide	1	2 $\pm$ 0.7 (5)

step (Fig. 2). In preparations of the mammalian enzyme a single 116000- M_r activity is present, and degradation products at M_r 66000 and 85000 appear only after prolonged storage at room temperature or repeated freezing and thawing.

Since the activity from *Helix pomatia* behaves very similarly to the enzyme from human during the course of purification, we tested its immunological relationship by using an affinity-purified antibody against the human enzyme. In an immunoblot analysis both the 75000- M_r and the 115000- M_r bands weakly react with the affinity-purified antiserum (Fig. 3).

The activity from *Helix pomatia* is dependent on DNA and histone (Table 2), DNA or histone alone being nearly ineffective in stimulation; histone cannot be replaced by bovine serum albumin. The pH optimum is 8 (six measurements) and the optimum temperature was determined to 17.5 $\pm$ 2.5 $^{\circ}$ C (nine measurements). The enzyme reaction is linear for 10 min and levels off after about 40 min. This might be due to inactivation by auto-ADP-ribosylation according to the properties of the enzymes from higher eukaryotes [15,16]. The apparent K_m of the enzyme preparation is 26.7 $\pm$ 4.7 μ M-NAD⁺ (mean $\pm$ S.E.M., 15 determinations) with a V_{max} of

Fig. 4. Product analysis of ADP-ribosyltransferase reaction

The ³²P-labelled reaction products were digested with snake-venom phosphodiesterase and identified by t.l.c. as described in the Materials and methods section. The peaks correspond to 5'-AMP and 2'-(5'-phosphoribosyl)-5'-AMP (PR-AMP). R_F values of markers: 3'-AMP, 0.20; 5'-AMP, 0.32; ADP-ribose, 0.41.

109 $\pm$ 14 nmol/min per mg (mean $\pm$ S.E.M., 15 determinations). The enzyme is sensitive to most of the common ADP-ribosyltransferase inhibitors (Table 3). It may be noted that caffeine has almost no effect on activity, whereas two other methylxanthines, theophylline and theobromine, are inhibitory.

The enzyme activity is capable of poly-ADP-ribosylation, since we found 5'-AMP and 2'-(5'-phosphoribosyl)-5'-AMP in t.l.c. after phosphodiesterase digestion of the reaction product (Fig. 4). From the ratio of these two products an average chain length of 1.3 was calculated. This is approximately the same value as that reported for high-mobility-group proteins and histone H1 previously [14].

In summary, the ADP-ribosylating activity from *Helix pomatia* is similar to enzymes from higher eukaryotes in terms of the parameters K_m , pH optimum, time course, inhibitors, DNA and histone requirements [5] and its capability of poly-ADP-ribosylation. It shows immunological cross-reactivity with an antiserum against human ADP-ribosyltransferase. These data and the fact that an activity with the same M_r as that of the human enzyme was found in the purified preparation suggest that ADP-ribosyltransferases are conserved throughout evolution. The enrichment factor in our preparation is similar to the human enzyme, where only a 116000- M_r band appears, and addition of proteinase inhibitors does not alter the protein pattern of the purified preparations. This indicates that the 75000- M_r protein is the mature form of the ADP-ribosyltransferase of *Helix pomatia*.

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