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A novel type of putrescine (diamine)-acetylating enzyme from the nematode *Ascaris suum*

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A cytosolic enzyme catalysing the acetylation of the diamines putrescine, cadaverine, 1,3-diaminopropane and 1,6-diaminohexane has been partially purified from reproductive tissue of the intestinal parasitic nematode *Ascaris suum*. The enzyme formed *N*-acetylated derivatives of the above diamines when incubated in the presence of acetyl-CoA. The Michaelis constants (K_m) for the above diamines were 0.25 mM, 0.1 mM, 1.25 mM and 0.4 mM respectively, and the apparent K_m for acetyl-CoA was 7.7 μM. *sym*-Norspermidine was also acetylated by this enzyme preparation, and, at a much lower rate, the enzyme acted on *sym*-norspermine. The common polyamines, spermidine and spermine, and histones were not substrates. Purification steps involved a freezing-and-thawing procedure to release enzyme activity from unknown inhibitors, DEAE-cellulose chromatography and affinity chromatography on cadaverine–Sepharose, from which the enzyme was eluted by increasing ionic strength. The enzyme exhibited an apparent M_r of about 38000–40000, and it consisted of at least two subunits, of which the catalytic one had an M_r of about 13000. The partially purified enzyme showed no deacetylase activity, and its activity was competively inhibited by the product *N*-acetylputrescine, but not by CoA. The name putrescine *N*-acetyltransferase is suggested for this enzyme, which may have an important function in the degradation of diamines of lower eukaryotes.

INTRODUCTION

The polyamine metabolism of prokaryotes and higher eukaryotes has attracted intense attention during the last decades, especially with regard to enzymes involved in this pathway. Although polyamine function is not fully understood, knowledge of their biochemistry is comprehensive (Pegg & McCann, 1982; Tabor & Tabor, 1984, 1985; Pegg, 1986). The diamine putrescine, derived from ornithine by decarboxylation, is known as the precursor in the biosynthesis of the polyamines spermidine and spermine. Putrescine, however, is also a product of polyamine catabolism, arising from *N*-acetylation of spermine and spermidine and subsequent oxidation of these *N*-acetyl derivatives. In this way the putrescine moiety is retained in the cell; a putrescine/polyamine cycle has been proposed for mammals (Seiler *et al.*, 1981). However, putrescine is found in eukaryotic cells only in minute amounts, whereas spermidine and spermine represent the main polyamines present in cells in millimolar concentration. Putrescine may be degraded in two ways. One route, catalysed by diamine oxidase, leads to γ-aminobutyric acid, which is channelled into the tricarboxylic acid cycle. The other route involves *N*-acetylation, yielding *N*-acetylputrescine. The *N*-acetyltransferase and oxidase therefore compete for the same substrate if not separated from each other by cell compartmentation. *N*-Acetylation of putrescine has been described, with the highest activities found in nuclear and microsomal preparations from rat organs (Seiler & Al-Therib, 1974). Properties of a purified nuclear *N*-acetyltransferase showing high activities for histones, diamines and polyamines were demonstrated (Libby, 1978). *N*-Acetylputrescine has been reported as an excretion product from the polyamine metabolism of filarial parasites (Wittich *et al.*, 1987). The present paper describes the partial purification and some properties of a putrescine-acetylating enzyme responsible for the degradation of this diamine in the nematode *Ascaris suum*. Properties of the enzyme described here distinguish it from any enzyme described so far in microbial, plant and mammalian polyamine metabolism.

EXPERIMENTAL

Materials

[1-^{14}C]Acetyl-CoA (55 Ci/mol) was purchased from Amersham–Buchler, Braunschweig, Germany. 3,3′-Diaminodipropylamine (*sym*-norspermidine) and *NN*′-bis-(3-aminopropyl)-1,3-diaminopropane (*sym*-norspermine) were from Aldrich, Steinheim, Germany. Solvents and Brij 35 were from Merck, Darmstadt, Germany. Activated CH-Sepharose 4B and Sephacryl S-200 (superfine grade) were from Pharmacia, Freiburg, Germany, and DE52 DEAE-cellulose and P81 phosphocellulose paper were from Whatman, Maidstone, Kent, U.K. All other chemicals were from Sigma, Deisenhofen, Germany, or from origins of highest purity commercially available.

Parasites

Adult female *Ascaris suum* were collected at a local slaughterhouse and maintained in RPMI 1640 medium until dissection. Ovary and uterus were excised from worms and transferred into ice-cold phosphate-buffered saline [20 mM-KH_2PO_4 (pH 7.2)/140 mM-NaCl] containing 0.1 mM-phenylmethanesulphonyl fluoride (PMSF). All biological material was twice washed with

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride.

the above saline and stored at −79 °C until further preparation.

Preparation of crude extract

All procedures were performed at 0–4 °C. Reproductive tissue was homogenized by hand with a glass homogenizer in 3 vol. of buffer A [50 mM-Tris/HCl, pH 8.8, containing 0.02% (w/v) Brij 35 and 0.1 mM-PMSF]. Homogenates were centrifuged at 20000 *g* for 15 min, and the resulting supernatant was re-centrifuged at 100000 *g* for 1 h. To recover the enzyme activity from this preparation (crude extract I) the supernatant was frozen at −20 °C, then slowly thawed and maintained at 0 °C for several hours. The flocky precipitate arising during this procedure was removed by centrifugation for 10 min at 20000 *g*. The supernatant was then dialysed against 2 × 50 vol. of buffer A overnight, subsequently cleared by centrifugation at 20000 *g* for 10 min (giving 'crude extract II') and used for further purification steps.

Enzyme purification

The crude extract II was applied to a column (2.5 cm i.d. × 10 cm) of DEAE-cellulose (Whatman DE52) previously equilibrated with buffer A at a flow rate of 25 ml/h. The column was washed with 5 column volumes of buffer A and eluted with a linear gradient of 0.0–0.4 M-NaCl in buffer A (total volume 400 ml). The gradient profile was established by using a LKB 11300 Ultrograd programmer. Fractions of volume 5 ml were collected. Fractions containing enzyme activity were pooled and dialysed against 2 × 50 vol. of buffer B (50 mM-Tris/HCl, pH 7.8, containing 0.02% Brij 35 and 0.1 mM-PMSF). The dialysed preparation was loaded on a column of cadaverine–Sepharose (1 cm i.d. × 10 cm) at a flow rate of 5 ml/h, washed with 10 column volumes of buffer B and eluted with a linear gradient of buffer B containing 0.0–0.4 M-NaCl (total volume 80 ml). Fractions containing enzyme activity were pooled and extensively dialysed against buffer B to remove NaCl.

Preparation of cadaverine–Sepharose

Cadaverine (10-fold excess) was linked to activated CH-Sepharose 4B (Pharmacia) as described by the manufacturer. The gel was stored in buffer B containing 0.8 M-NaCl and 0.005% (w/v) NaN_3 at 4 °C until use.

Acetyltransferase assay

Enzyme activity was measured as described by Libby (1978), with modification. The assay mixture contained, in a final vol. of 100 μl: 5 mM-putrescine or the substrate mentioned, 50 nCi of [1-^{14}C]acetyl-CoA (9.5 μM) and enzyme preparation in buffer B. The reaction was started by addition of acetyl-CoA and proceeded for 5 min at 37 °C. The assay was terminated by the addition of 20 μl of 1 M-hydroxylamine hydrochloride and heating for 5 min at 95 °C. After cooling to room temperature, samples were centrifuged in an Eppendorf microfuge for 2 min at 10000 *g*. A 100 μl portion of the supernatant was applied to Whatman P81 phosphocellulose paper squares (3.5 cm × 3.5 cm) and allowed to dry. Subsequently paper squares were individually batch-washed in Petri dishes with 2 × 8 ml of distilled water for 20 min on a rotary shaker at 90 rev./min. Paper squares were transferred to a sintered-glass filter and washed with 5 × 5 ml of distilled water and then with 3 × 5 ml of ethanol. After drying, paper squares were assayed for radioactivity in 5 ml of Packard Pico-Fluor 30 liquid-scintillation cocktail. Counting efficiency was 80–82% in the Packard Tri-Carb 2000 CA liquid-scintillation counter. Acetylation of histones was done under conditions described by Libby (1978), Belikoff *et al.* (1980) and Wiktorowicz & Bonner (1982), with type II-AS, II-A and III-S histones (from Sigma). Histone concentration was 0.1–0.5 mg/ml. Enzyme activity is expressed in units (incorporation of 1 nmol of acetyl residue into substrate per min).

Identification of *N*-acetylputrescine

T.l.c. was used for identification of *N*-acetylputrescine extracted from the reaction mixture containing 5 mM-putrescine, 100 μM-acetyl-CoA and an appropriate amount of enzyme in 1 ml of buffer B. After incubation for 30 min at 37 °C, the reaction was stopped by adding 100 μl of 2 M-$HClO_4$ and chilling on ice. After centrifugation at 10000 *g* for 2 min, the supernatant was removed, saturated with solid Na_2CO_3, and the derivative was formed with dansyl chloride (2 mg in 1 ml of acetone) overnight at room temperature. Excess reagent was removed with proline for a further 1 h. Samples were extracted with 5 ml of diethyl ether. The ether layer was removed and evaporated to dryness. The residue was dissolved in methanol and spotted on to Merck silica-gel G plates, which were developed first in ethyl acetate and then twice in chloroform/tetrachloromethane/methanol (14:6:1, by vol.) as described by Seiler & Knödgen (1979). Additionally, the reaction products were identified by using a modification of the gradient h.p.l.c. system described by Wittich *et al.* (1987). Solvent A consisted of 75% octanesulphonic acid (20 mM, pH 3.5) and 25% acetonitrile, and solvent B was made of 20% octanesulphonic acid and 80% acetonitrile. The gradient program was as follows (proportions of solvent B): 0 min, 20%; 7 min, 25%; 9 min, 65%; 17 min, 66%; 19 min, 90%; 22 min, 100%; 33 min, 100%; 36 min, 20%; 40 min, 20%. Radiolabelled compounds were monitored by using the LB 506D h.p.l.c. detector from Berthold, Wildbad, Germany, equipped with an yttrium-glass detector flow cell producing an 80% counting efficiency for ^{14}C.

Test for deacetylating-enzyme activity

[1-^{14}C]Acetyl-labelled acetylputrescine was prepared by using the above assay mixture scaled up 20-fold. Reaction proceeded over 1 h. The product formed was extracted from alkaline solution and purified in the above t.l.c. system. Desorbed and concentrated labelled *N*-acetylputrescine was incubated in the presence of enzyme preparation in buffer A. Alteration in *N*-acetylputrescine concentration and potential putrescine formation were monitored by h.p.l.c.

Estimation of M_r

The apparent M_r of the enzyme was determined by gel-filtration chromatography on a Pharmacia Sephacryl S-200 superfine column (2.6 cm × 97 cm) at a flow rate of 12.5 ml/h, with 100 mM-Tris/HCl buffer, pH 7.8, containing 0.1 M- or 0.5 M-NaCl, 0.1 mM-PMSF and NaN_3 (0.005%) as the eluent. The column was calibrated previously by the use of standard proteins (catalase, M_r = 240000; bovine serum albumin, M_r = 67000; ovalbumin, M_r = 45000; myoglobin, M_r = 17800; and cytochrome

c, M_r = 12300). Additionally, the size of the enzyme was determined by h.p.l.c. on a DuPont Zorbax GF-250 gel-filtration column with potassium phosphate buffer (200 mM, pH 7.0). The system consisted of a Kontron 420 h.p.l.c. pump, a Rheodyne 7125 injector and a Kontron 720 LD micro-spectrophotometer equipped with a model 21 recorder.

Protein determination

Protein concentration was determined as described by Bradford (1976), with bovine serum albumin as standard.

RESULTS

Purification of putrescine *N*-acetyltransferase

Reproductive tissue from about 30 nematodes was removed and prepared as described in the Experimental section. Enzyme activity became detectable after the freezing-and-thawing procedure in the 100000 *g* supernatant. The enzyme preparation was loaded directly on a DEAE-cellulose column and eluted between 0.12 M- and 0.3 M-NaCl. Fractions containing enzyme activity were pooled, and after dialysis the enzyme solution was applied to cadaverine–Sepharose, from which it was eluted by a linear gradient of NaCl (Fig. 1), since affinity elution with cadaverine resulted in a broad peak and poor recovery of the enzyme activity. The entire enrichment procedure, resulting in an overall purification of about 1000-fold, is summarized in Table 1. The acetyltransferase did not bind to spermine, spermidine or norspermine coupled to Sepharose. Putrescine–Sepharose alternatively used as affinity matrix was less efficient than cadaverine–Sepharose; a repeated chromatography on putrescine–Sepharose with elution by NaCl gradient and subsequently by 10 mM-putrescine did not give any advantage. The enzyme preparation was far from homogeneity, as judged from SDS/polyacrylamide-gel electrophoresis (results not shown).

Estimation of the M_r of the enzyme

By chromatography on an analytical Zorbax GF-250 gel-permeation column, an approximate M_r size of about 38000–40000 was found. Gel filtration on Sephacryl S-200 was suitable to demonstrate the M_r of an obvious

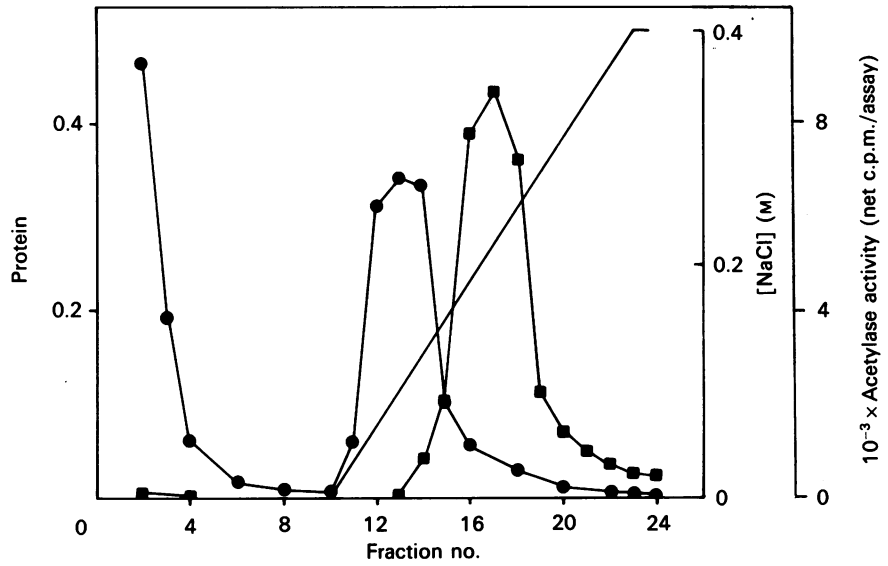

Fig. 1. Purification of putrescine *N*-acetyltransferase by chromatography on cadaverine–Sepharose

The pooled fractions of the DEAE-cellulose step were dialysed against buffer B and applied to affinity chromatography. Enzyme activity (■) was eluted with a linear gradient of NaCl (——) in buffer B. ●, Protein (A_{595}, Bradford micro assay).

Table 1. Purification of putrescine *N*-acetyltransferase from *A. suum*

One enzyme unit represents the incorporation of 1 nmol of acetyl group into substrate/min at 37 °C under standard assay conditions.

Step	Protein (mg)	Total activity (units)	Sp. activity (units/mg)	Purification factor
Crude extract I	207.00	< 1.0	< 0.01	–
Crude extract II	184.00	7.28	0.42	1
DEAE-cellulose fraction	44.60	800.60	18.07	43
Cadaverine–Sepharose fraction	1.12	483.17	431.4	1027

Table 2. Substrate specificity of putrescine *N*-acetyltransferase from *A. suum*

Relative rate of acetylation of various substrates (concn. 5 mM) and apparent K_m values (concn. of acetyl-CoA was 9.4 μM) are shown. Assays for histone *N*-acetyltransferase activity contained 0.5 mg of different histone types/ml: n.d., not determined.

Compound tested	Relative rate of reaction (%)	Apparent K_m (mM)
1,4-Diaminobutane (putrescine)	100	0.25
1,3-Diaminopropane	116	1.25
1,5-Diaminopentane (cadaverine)	39	0.10
1,6-Diaminohexane	50	0.40
Spermidine	3	> 5
Spermine	4	> 5
sym-Norspermidine	59	> 5
sym-Norspermine	11	> 5
N-Acetylputrescine, *N*-acetylcadaverine	2	n.d.
N^1-Acetylspermidine	< 1	n.d.
N-Acetylspermine	1	n.d.
Histones	< 1	n.d.

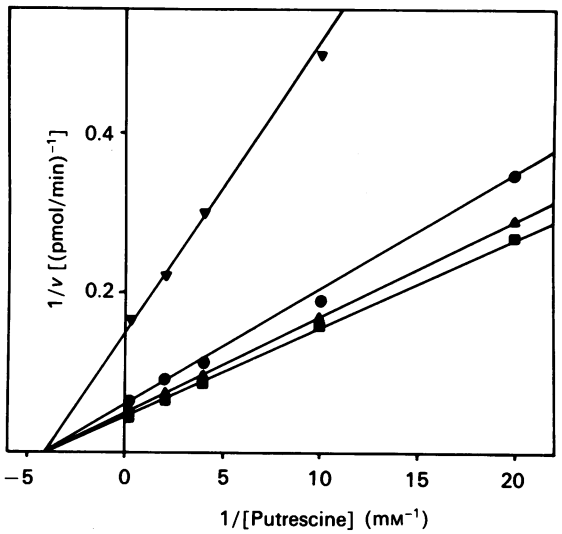

Fig. 2. Determination of K_m for putrescine of putrescine *N*-acetyltransferase from *A. suum*

Standard assay conditions were employed at various concentrations of acetyl-CoA (■, 18.9 μM; ▲, 9.4 μM; ●, 4.7 μM; ▼, 1.9 μM), and reciprocal of initial velocity (v) was plotted against reciprocal of variable putrescine concentration.

subunit of the native enzyme. Precipitation of crude extract II preparation with $(NH_4)_2SO_4$ (80% saturation), subsequently followed by chromatography of the precipitated fraction on Sephacryl S-200 in the presence of 0.5 M-NaCl in buffer B, resulted in the separation of a catalytically active subunit of the enzyme, with an M_r of about 13000.

Optimum of pH and temperature

High conversion rates were found in the range pH 7.6–8.0 in Tris/HCl buffer, and maximal activity was between pH 7.7 and 7.9. Enzyme activity was linear over at least 5 min at 30 °C, but was considerably decreased at above 42 °C, so that standard assays were performed at pH 7.8 and 37 °C over a period of 5 min.

Stability of the enzyme

The inclusion of PMSF and Brij 35 was essential to prevent rapid loss of activity of the enzyme preparation. Crude extract II preparations from *A. suum* were stable for about 3–4 months without significant loss of activity when stored at −22 °C. However, the enzyme eluted from the cadaverine–Sepharose column lost about 10–16% of its activity per month when stored at −22 °C, and about 5–9% per day at 4 °C. At 37 °C the half-life of the enzyme was greater than 1 h, and at 56 °C it was less than 0.5 min.

Properties of the enzyme

As shown in Table 2, the partially purified enzyme revealed high activities for the diamines listed. Spermidine, spermine and histones were poor substrates for *N*-acetylation by acetyl-CoA, as were the acetylated diamines and polyamines. However, *sym*-norspermidine and *sym*-norspermine were acetylated at a considerable rate. When putrescine was taken as substrate, only *N*-acetylputrescine could be identified as the product of the acetylase reaction, by comparing its properties on t.l.c. and h.p.l.c. with commercially available polyamines. Additionally, *N*-acetyl-1,3-diaminopropane, *N*-acetyl-1,5-diaminopentane and *N*-acetyl-1,6-diaminohexane were identified as the probable reaction products from acetylation of non-labelled 1,3-diaminopropane, 1,5-diaminopentane and 1,6-diaminohexane, respectively, by using [1-^{14}C]acetyl-CoA. The apparent K_m value was determined to be 250 μM for putrescine as substrate. Fig. 2 shows Michaelis–Menten plots with variable concentrations of putrescine at various fixed concentrations of acetyl-CoA (1.9–18.9 μM), yielding a series of lines that intersected to the left of the ordinate. The apparent K_m value for acetyl-CoA was found to be 7.7 μM at saturating putrescine concentration (5 mM). One product of reaction, CoA, was not inhibitory even at 50 μM and in the presence of 1.9 μM-acetyl-CoA. The other product, *N*-acetylputrescine, was slightly inhibitory and exhibited a K_i value of 1.7 mM. The type of inhibition was found to be competitive.

DISCUSSION

The purification procedure summarized in Table 1 reveals an approx. 1000-fold enrichment of a putrescine *N*-acetyltransferase when crude extract II is compared with pooled fractions eluted from the affinity-chromatography step. However, there remain considerable discrepancies concerning total activity found in crude extract II preparation and at the DEAE-cellulose step, giving evidence of incomplete removal of inhibitory compounds and thus preventing correct calculation of enrichment factors. The character of the inhibitory material which could be removed by the established freezing-and-thawing procedure is still unknown, and requires further investigation.

The enzyme isolated from *A. suum* is clearly different from mammalian histone acetylases, which also acetylate

putrescine and polyamines and were first described by Libby (1978, 1980). The acetylase from calf liver exhibited high $V_{max.}$ values for histones, spermidine, spermine and norspermidine, whereas the diamines 1,3-diaminopropane, 1,4-diaminobutane (putrescine), 1,5-diaminopentane (cadaverine) and 1,6-diaminohexane were less active as substrates. The enzyme preparation from rat liver demonstrated comparable activity for histones and polyamines, less activity for diamines and none for norspermidine and 1,3-diaminopropane. Acetylation of polyamines by a chromatin-bound enzyme was demonstrated by Blankenship & Walle (1977). $V_{max.}$ values of 47, 33 and 16 pmol/min per mg for putrescine, spermidine and spermine, and K_m values for these compounds of 2.6, 0.5 and 1 mM, were reported for rat liver. Belikoff *et al.* (1980) described a histone *N*-acetylase in cell nuclei of calf thymus exhibiting an M_r of about 70000; the K_m for histones of about 20 μM correlated with a K_m for acetyl-CoA of 0.5–1.0 μM. Wiktorowicz & Bonner (1982) partially purified a rat liver nuclear histone acetyltransferase of M_r 96000 and with a pH optimum at 7.5. However, as the above-cited preparations were far from homogeneous, it is questionable if acetylation of diamines, polyamines and histones in nuclear and/or microsomal extracts is catalysed by one or by more enzymes. A cytosolic and inducible spermidine/spermine *N*-acetyltransferase from mammalian origin was described by Pegg and collaborators (Matsui *et al.*, 1981; Della Ragione & Pegg, 1982, 1983). This enzyme (M_r 115000, consisting of two subunits of M_r 60000) shows high conversion rates for spermidine, spermine and N^1-acetylspermine. Rates for *sym*-norspermidine and *sym*-norspermine were about 50% higher than that for spermidine; correspondingly, their K_m values were much lower than those of naturally occurring polyamines. Little activity was found for 1,3-diaminopropane, and other diamines were completely inactive, just as histones. This enzyme shows high selectivity for the aminopropyl moiety of polyamines; aminobutyl residues of diamines and polyamines were not acetylated.

The putrescine *N*-acetyltransferase of *A. suum* is clearly distinguished from other acetyltransferases by the M_r of 38000–40000. A subunit of M_r 13000, possibly derived from dissociation of the polymeric enzyme into catalytic or catalytic and regulatory subunits, was found after pretreatment and chromatography at high salt concentration. Enzymically active subunits were not detected during the purification steps on DEAE-cellulose and affinity chromatography on diamine–Sepharose or when the partially purified enzyme preparation was applied to gel-permeation chromatography.

The enzyme studied in the present work shows high conversion rates, correspondingly low K_m values for the four diamines tested and appreciable rates for the turnover of *sym*-norspermidine and, to a much lesser extent, *sym*-norspermine. These data suggest that the putrescine *N*-acetyltransferase from *A. suum* prefers substrates such as diamines and polyamines consisting of propylamine residues. Substrate specificities, and the finding that the nematode enzyme is neither inhibited by CoA nor bound to Affi-Gel Blue, distinguish the enzyme clearly from nuclear and microsomal enzyme preparations of vertebrates as well as from soluble acetylase of rat liver (Seiler & Al-Therib, 1974; Della Ragione & Pegg, 1983; Della Ragione *et al.*, 1983). In addition, application of the extraction and enrichment procedures described in the present paper to rat liver, spleen and kidney, to dog liver and to pig brain produced only negligible amounts of enzyme activity from these animals and organs.

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