BIOCHEMICAL AND IMMUNOLOGICAL STUDIES ON PURINE NUCLEOSIDE PHOSPHORYLASE ISOLATED FROM COW SNOUT EPIDERMIS

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Purine nucleoside phosphrylase (purine nucleoside : orthophosphate ribosyl transferase PNPase, EC 2. 4. 2. 1) was purified 237-fold in a specific activity from cow snout epidermis. Inosine was highly utilized as substrate among the purine nucleosides tested. The molecular weight of the enzyme was estimated at about 80,000 by the use of Sephadex G-200 gelfiltration. The Michaelis constant for inosine was 0.04 mM.

There was no immunological cross-reactivity between antiserum of cow snout epidermal PNPase and calf spleen PNPase or extracts of cow liver, intestinal epithelium, kidney.

INTRODUCTION

One of the physiological functions of the epidermis is a production of the horny layer. Histologically, no cellular constituents including nuclei are present in the horny layer. During normal keratinization, nucleoproteins are first broken down to DNA and RNA. Further catabolism of nucleic acids probably proceeds along the same pathways as in other mammalian tissues. The endogenous levels of DNA are to be under the control of both synthetic and catabolic enzymes in the epidermis. DNA-nucleolytic enzymes which effect this degradation may be of great significance in the overall biological control processes in the epidermis. Since work

from our laboratory has shown that the DNA-nucleolytic enzymes are purified from cow snout epidermis, their biological characteristics in the cellular differentiation have been discussed. During past studies (Miyagawa and Ogura, 1971; Hidaka, 1976). It became apparent that there were a number of enzymes present in the epidermis which were capable of degrading DNA into nucleoside.

However, biochemical characteristics of purine nucleoside phosphorylase in the epidermis remain to be examined using a homogenous enzyme preparation.

The present work describes purification and characterization of purine nucleoside phosphorylase (PNPase) in

Abbreviation used

PNPase : purine nucleoside phosphorylase

cow snout epidermis.

MATERIALS AND METHODS

1. *Materials*

Cow snout epidermis was used as a source of PNPase preparation. The cow snout was obtained in the slaughter house. Crystalline PNPase from calf spleen and xanthine oxidase were purchased from Boehringer Mannheim. Inosine, adenosine, hypoxanthine, uric acid were obtained from Wako Co. Ltd. All other chemicals were of the highest purity commercially available.

2. *Preparation of crude extracts*

The skin was excised from cow snout and the subcutaneous tissue was removed. The epidermis was sliced by a laboratory cryostat-microtome (Yamatokoki, Japan Model 1118) with the cutting blade adjusted to 10 micron thickness. The slices obtained from whole epidermis were pooled, and homogenized in 10 vol. of distilled water with a glass homogenizer, and centrifuged at 20,000 $\times$ g for 15 min; the supernatant fluid was used for enzyme assays, as an epidermal homogenate in this paper.

3. *Assay of purine nucleoside phosphorylase*

Assay utilized in this paper is the coupled xanthine oxidase method of Kalckar (1947), which is based on the measurement of the increase in absorbance at 292 nm due to the formation of uric acid.

The optical absorption at 292 nm of reaction mixture was determined with a Beckman model DB spectrophotometer equipped with a Beckman linear-log recorder.

The reaction mixture for the stan-

dard assay contained the following components; 0.5 μ mole of inosine; 0.02 unit of xanthine oxidase with 50 μ mole of potassium phosphate buffer (pH 7.5) and the enzyme solution to be assayed in a final volume of 1 ml enzyme solution. The mixture of all components except enzyme solution, was preincubated at 37° for about 1 minute to remove any trace of hypoxanthine or xanthine introduced as a contaminant in the inosine after a preincubation, the reaction was started by the addition of enzyme solution to be assayed. The concentration of formed uric acid was calculated from the reading taking at an A 292 nm value.

One unit of PNPase was defined as that amount of enzyme which gives an optical density increase of 12.5 per min. (1 μ mole of uric acid formation) at 292 nm under above standard assay conditions.

For the assay of substrate specificity, a modification of the Park-Johnson method was employed (1949). The reaction mixture (total volume 0.5 ml) contained 50 mM Tris-HCl buffer (pH 7.8) and 10 mM disodium arsenate for phosphate. The formation of ribose from the purine ribonucleoside (or deoxyribose from the purine deoxyribonucleoside) was determined spectrophotometrically with a sensitive reducing sugar assay method.

4. *Assay of adenosine deaminase*

The activity was measured spectrophotometrically using adenosine as substrate at pH 7.5. In the addition to an excess of nucleoside phosphorylase and xanthine oxidase, the rate of the increase in absorbance at 292 nm, resulting from the conversion of adenosine to uric acid, yields a measure of adenosine desminase activity (Hopkinson and Harris, 1969). The standard assay mix-

ture contained 0.5 mM adenosine, 0.05 M sodium phosphate buffer (pH 7.5), 0.02 unit of xanthine oxidase, 0.025 unit of nucleoside phosphorylase and 50 μ l of enzyme solution in a total volume of 1.05 ml were incubated.

5. Assay of xanthine oxidase

The absorbance of the reaction mixture at 292 nm upon the aerobic oxidation of hypoxanthine to uric acid was determined by the method of Kalckar (1947). For the purpose of enzyme assay, 1 M glycine buffer (pH 8.8), 2.58 mM hypoxanthine, and enzyme solution (diluted with 0.07 % bovine albumin solution to make a concentration of enzyme is to be 50-335 unit) in the final volume of 3.0 ml were incubated for 30 minutes. Incubation mixture was measured spectrophotometrically.

6. Measurement of protein concentration

Protein concentrations were measured by the biuret procedure (Gornall et al., 1949) or by the method of Lowry et al. (1951).

7. Polyacrylamide gel electrophoresis

The method of Ornstein and Davis (1964) was used at 4°C. Approximately 10 μ g of the purified PNPase was applied to a 7 % polyacrylamide gel column. The current was regulated at 5 mA. per gel tube. The analytical gels were run in duplicate, with bromphenol blue as an anionic marker. The gels were stained with amidoschwarz black.

8. Preparation of antibody

PNPase purified from cow snout epidermis was mixed with an equal volume of Freund's complete adjuvant.

Immunizing injections (500 μ g of protein) were made in the foot-pads of guinea pigs. Two weeks later, guinea pigs were given with simple booster injection intraperitoneally with 500 μ g of the immunogen 2 weeks after initial immunization. One month after the last booster injection, blood was collected. In the separate experiment, calf spleen PNPase (purchased from Böehringer Mannheim) was used as reference.

9. Ouchterlony double diffusion analysis

Gel diffusion precipitation was carried out as described by Ouchterlony (1962). The immunodiffusion media containing 1 % agarose, 0.1 % sodium azide, and phosphate buffered saline, was poured on a slide glass to make a depth of 1 mm. Four-millimeter diameter wells were punched with a template resulting in a pattern of wells with center-to-center distances of 8 mm. After filling the wells with the appropriate proteins, the plates were incubated at 37° for at least 12 hours.

RESULTS

1. Enzyme activity in the epidermal homogenate

Enzyme activity was proportional to the amount of enzyme used (Fig. 1) and to time of incubation (Fig. 2).

Purine nucleoside phosphorylase activity in cow snout epidermal homogenate was assayed with inosine and guanosine as substrate respectively. PNPase activity with inosine as substrate is approximately 2 times higher than that with guanosine as substrate. In this paper, PNPase is particularly to be discussed for inosine as substrate. Table 1 shows the specific activities of

PNPase and the related enzymes with purine base metabolism in various bovine tissue extracts; liver, kidney, intestinal epithelium and snout epidermis. As compared with other tissues, the snout epidermis contains relatively high activity of enzymes except for xanthine oxidase.

To study the distribution of PNPase activity in relation to depth of epidermis, a quantitative determination was made on successive slices of epidermis. According to the process of epidermal preparation, usually 60 slices ($10\ \mu$ thickness) could be obtained with a laboratory cryostat-microtome.

Fig. 1 Effect of enzyme concentration on PNPase activity.

Fig. 2 Time course of PNPase activity.

TABLE 1
Comparison of purine nucleoside phosphorylase activity
in the bovine various tissues

	Epidermis	Liver	Kidney	Intestinal Epithelium
Purine nucleoside phosphorylase ($\times 10^{-3}$) (with inosine as substrate)	$416.2 \pm 21.8(3)$	$86.4 \pm 4.03(3)$	$129.7 \pm 8.15(3)$	$195.1 \pm 7.82(3)$
Adenosine deaminase ($\times 10^{-3}$)	$60.8 \pm 5.79(3)$	$36.1 \pm 2.34(3)$	$11.5 \pm 1.11(3)$	$29.4 \pm 0.31(3)$
Xanthine oxidase ($\times 10^{-3}$)	$0.110 \pm 0.005(3)$	$0.441 \pm 0.042(3)$	$0.084 \pm 0.024(3)$	$0.079 \pm 0.016(3)$

Enzyme activity: unit per mg protein
(): number of samples determined

TABLE 2
*Distribution of enzyme activity of purine metabolism
 in successive layers of cow snout epidermis*

	Upper layer	Middle layer	Lower layer
Purine nucleoside phosphorylase ($\times 10^{-3}$) (with inosine as substrate)	372.1 $\pm$ 66.4 (3)	327.1 $\pm$ 3.10 (3)	258.3 $\pm$ 19.0 (3)
Adenosine deaminase ($\times 10^{-3}$)	131.2 $\pm$ 3.71 (3)	100.1 $\pm$ 6.68 (3)	67.7 $\pm$ 1.25 (3)
Xanthine oxidase ($\times 10^{-3}$)	0.139 $\pm$ 0.006 (3)	0.131 $\pm$ 0.031 (3)	0.131 $\pm$ 0.01 (3)

Enzyme activity: unit per mg protein
 (): number of samples determined

The epidermis was divided into three successive layers with about 20 consecutive slices respectively (upper, middle and lower epidermis).

Table 2 shows the distribution of enzyme activity of PNPase, adenosine deaminase and xanthine oxidase in the three successive layers. The specific activities of these enzymes are found to be high in the upper layer of epidermis, except for xanthine oxidase.

2. Purification of PNPase from cow snout epidermis

All steps were carried out at 3-5°, unless otherwise stated.

Step 1: Preparation of extract

The epidermis (20 g wet weight) was homogenized in 10 vol. of distilled water, followed by centrifugation at 20,000 $\times$ g for 15 minutes at 0°. The resulting supernatant could be stored for lengthy periods in a deep freezer without a significant loss of activity.

Step 2: Ammonium sulfate fractionation

The above supernatant solution was brought to 40 % saturation by adding a solid ammonium sulfate (23.11g/100 ml of eluate). After the 30 min in room

temperature, the precipitate was removed by centrifugation at 9,000 $\times$ g for 30 min. To the supernatant fluid a solid ammonium sulfate (12.31g/100 ml of supernatant fluid) was added to bring the concentration to 60 % saturation. After 60 minutes, the precipitate was collected by centrifugation at 9,000 $\times$ g for 40 min, and dissolved again in 15 ml of 0.1 M Tris-acetate buffer, pH 7.5 (loss of the total activity was 27.6 %). The solution obtained from the ammonium sulfate fractionation was dialyzed overnight against several changes of 0.03 M Tris-acetate buffer, pH 7.5.

Step 3: DEAE-cellulose column chromatography

The dialyzed solution was loaded on a DEAE-cellulose column (acetate form, 2.5 $\times$ 12 cm) which had been previously equilibrated with 0.03 M Tris-acetate buffer, pH 7.5. Fifty ml of the same buffer, was used to eluate the first protein peak, and then a linear gradient was produced with Tris-acetate buffer (pH 7.5) varying from 0.03 to 0.34 of molar concentration. The eluate was collected in the test-tubes with 10 ml each. The optical density of eluate was read at 280 nm. The result obtained in this procedure is shown in Figure 3.

The three main protein peaks were obtained, elution of 95 % of the enzyme activity emerged between 60 and 100 of

tube number. The fractions demonstrating PNPase activity were pooled, and was hereafter referred to as the

Fig. 3 DEAE-cellulose column chromatogram of cow snout epidermal PNPase obtained in step 2 of the purification procedure.

TABLE 3
*Purification of purine nucleoside phosphorylase
from cow snout epidermis*

Fraction	Total volume (ml)	Total activity (units)	Specific activity (units/mg)	Protein (mg/ml)	Recovery (%)	Purification (-fold)
1 Homogenate	200	77	0.016	23.8	100	1
2 20,000× g centrifugation	148	76	0.130	4.1	99	8
3 Ammonium sulfate fractionation (40~60%)	20	55	0.360	7.6	71	22
4 DEAE-cellulose (acetate) column	310	46	3.809	0.039	60	237

partially purified PNPase in the present paper. The summary of the purification processes is given in Table 3.

In the preparation of antibody, the resulting material was further concentrated by membrane filter (Amicon ; UM 05 9 cm).

3. Acrylamide gel electrophoresis of purified PNPase

The partially purified PNPase was applied on the polyacrylamide gel electrophoresis. The pattern obtained from the enzyme preparation is shown in Fig. 4. Only one band was detected. Enzyme preparation appears to be homogenous in polyacrylamide gel electrophoresis.

Fig. 4 Polyacrylamide gel electrophoresis of purified PNPase from cow snout epidermis (anode : top and cathode : bottom).

4. Properties of the enzyme

pH optimum

The pH activity curve of the partially purified PNPase is shown in Fig. 5. The pH optimum was 7.5. (Tris-KH₂PO₄ buffer and adjusting to the desired pH with either KOH or HCl).

Fig. 5 The effect of pH on purified enzyme.

Substrate specificity

The enzyme activity of partially purified PNPase was determined by measuring the liberation of ribose from nucleoside with inosine, guanosine and adenosine as substrate respectively. The substrate specificity was shown in Table 4 as the relative activity for inosine as substrate. Cow snout PNPase scatalyzed inosine and guanosine to the purine base, but does not react with adenosine. The K_m value for inosine was estimated to be 0.04 mM, based on the method of Lineweaver-Burk plot (Fig. 6).

TABLE 4

Comparison of substrate specificity of PNPase from cow snout epidermis. Enzyme activity was measured by determining the amount of reducing sugar formed from the arsenolysis of each substrate in 10 min. Inosine arsenolysis was assigned as value of 100; other substrate activities were given as a relative value that of inosine

Substrate	Relative activity
Inosine	100 %
Guanosine	45
Adenosine	0

Fig. 6 The effect of concentration of inosine on the velocity of PNPase activity.

Fig. 7 Effect of aging for 3 months on the enzyme activity of partially purified PNPase.

Effects of p-chloromercuribenzoate

The partially purified PNPase was incubated for 30 minutes at 37° with various concentrations of p-chloromercuribenzoate (PCMB). As shown in Table 5, PNPase was inhibited by PCMB in high concentration of 10^{-2} M in a final concentration.

Effect of metals

No significant effect of the Mg^{2+} or EDTA to the enzyme activity was detected (Table 6).

Stability of enzyme

The purified enzyme could be stored as a suspension in 80 % saturated ammonium sulfate at 0° without a significant loss of activity. As shown in Fig. 7, 33% of the original activity was

TABLE 5

Effect of p-chloromercuribenzoate on the activity of Partially purified PNPase

p-chloromercuribenzoate	Enzyme activity
M	%
10^{-2}	56
10^{-1}	100
none	100

TABLE 6

Effect of metals on the activity of partially purified PNPase

Metals	Enzyme activity (unit)
None	416.2 ± 21.8
0.1 mM Mg^{2+}	416.2
1 mM Mg^{2+}	422.5
10 mM Mg^{2+}	407.9
0.1 mM EDTA	427.8
1 mM EDTA	439.8
10 mM EDTA	438.1

lost by allowing the partially purified enzyme to stand in 0.1 M Tris-acetate buffer (pH 7.5) for 3 months at -20°C , whereas at 4°C more than 55 % loss of activity was found.

Effect of temperature

The effect of temperature on the partially purified PNPase is shown in Fig. 8. The activity of enzyme was found to have a broad optimum between 30° and 50° (40°). The enzyme shows a significant loss of activity above 50°C .

Fig. 8 Effect of temperature on the PNPase activity.

5. Estimation of molecular weight

The estimation of the molecular weight was performed according to the

Fig. 9 Estimation of molecular weight of cow snout epidermal PNPase.

method of Andrews (1964) with a sephadex G-200 column (1.9×50 cm) equilibrated with a 0.1 M Tris-acetate buffer (pH 8.0) containing 1×10^{-4} M dithiothreitol. The following marker proteins were mixed with partially purified PNPase in 1.5 ml of 0.1 M.

Tris-acetate (pH 7.5) containing 10^{-3} M dithiothreitol, pyruvate kinase (PYK ; mol wt 230,000) ; lactate dehydrogenase (LDH ; mol wt 120,000) and bovine serum albumin (mol wt 67,000).

The mixed samples were fractionated by a sephadex G-200 column. The fraction tube numbers showing the highest absorbance at $280 \text{ m}\mu$ in the each separated protein were plotted against the logarithm of the known molecular weights of the proteins. The molecular weight of the cow snout epidermal PNPase was estimated at about 80,000 (Fig. 9).

6. Immunologic properties

Guinea pig antiserum to purified enzyme was tested by the Ouchterlony double immunodiffusion technique (1962) against the purified PNPase from cow snout epidermis and the calf spleen PNPase purchased from Boehringer

Mannheim. An identical single precipitin band was obtained in all of the preparations. The guinea pig antiserum to PNPase prepared from cow snout epidermis and from calf spleen was used to identify with cow snout epidermis, spleen, liver, kidney and intestinal epithelial homogenate.

Fig. 10 Photograph of immunodiffusion plates.

All wells were filled with 20 μ l of the indicated protein preparations.

A : the center well contained cow snout epidermal PNPase antiserum. wells 1 and 3 contained calf spleen PNPase, wells 2 and 4 contained cow snout epidermal PNPase.

B : the center well contained cow snout epidermal PNPase antiserum. wells 1 and 3 contained cow snout epidermal PNPase, well 2 contained crude extracts of cow intestinal epithelium, well 4 contained calf spleen PNPase.

C : the center well contained cow snout epidermal PNPase antiserum. wells 1 and 3 contained cow snout epidermal PNPase, and well 2 contained crude extracts of cow liver, well 4 contained crude extracts of cow kidney.

D : the center well contained calf spleen PNPase antiserum. wells 1 and 3 contained calf spleen PNPase and wells 2 and 4 contained cow snout epidermal PNPase.

As shown in Fig. 8 A, B, C, the antiserum to cow snout epidermal PNPase cause a precipitation with cow snout epidermal PNPase, but did not precipitate with liver, kidney, intestinal epithelial extract and calf spleen PNPase.

The antiserum to calf spleen PNPase cause a cross-react with calf spleen PNPase, but does not react with others (Fig. 10).

An attempt was made to find the influence of antiserum on the activity of cow snout epidermal PNPase.

The purified PNPase was incubated with 100 μ l of antiserum for 15 min. at 37°. There was an elevation of 48 % in PNPase activity after incubation.

DISCUSSION

Levene et al. (1911) early studied the enzymatic degradation of purine nucleosides. In 1945, Kalckar made the important discovery, in which ribose 1-phosphate was found to be one of the products of the PNPase reaction. PNPase was found to catalyze purine nucleotide to purine base and ribose phosphate by transphosphorylation as follows.

PNPase is known to catalyze guanosine to guanine, not to convert adenosine to free purine base. Adenosine is deaminated to inosine by adenosine deaminase, and finally, guanine and hypoxanthine are oxidized to uric acid by xanthine oxidase.

In human skin, the presence of PNPase has been demonstrated by Hershey and Mendell (1954). Then, the further metabolism of the purine nucleoside in the epidermis was established to proceed according to the enzymes similar to those described in the

other tissues.

In the present work PNPase was purified by ammonium sulfate fractionation and DEAE-cellulose chromatography.

Result of acrylamide gel electrophoresis indicated homogenous preparations. Yield of enzyme was 60 % after 237-fold purification. As compared with other tissues, the snout epidermis was found to contain relatively high activity of PNPase.

PNPase in the epidermis appears to play an important role in the metabolism of nucleic acid. Uric acid has been known to be a final product of purine base in the mammalian tissue. Although an almost complete degradation of nucleoprotein occurs in the horny layer, the presence of uric acid has not been found at all in human epidermis (De Bersaques, 1967 ; Schwarz, 1963). Cow snout epidermal PNPase activity was also highest in the upper layer. The unique location of PNPase activity suggests that the enzyme may play a role in the latter stages of keratinization.

In the present work, xanthine oxidase exhibit a low activity in cow snout epidermis. It is interesting that PNPase related with a production of purine base is found high in activity. However, the present study can not dissolve the problem how PNPase plays a biological role in connection with uric acid metabolism in the epidermis. Concerning the distribution of enzymes of cow snout epidermis, the data reported by Mier et al. (1976) showed that acid phosphatase was found mainly in the upper keratinizing zone. In our laboratory, the extremely high activity of nuclease was found in the upper layer (Ogura et al, 1977 ; Hidaka, 1976 ; Miyagawa and Ogura, 1971). In the present paper, PNPase was also high in the upper layer of cow snout epidermis.

As shown in the results of Ouchterlony (Fig. 10), the enzyme protein of PNPase is immunologically different from that of liver, intestinal epithelium, kidney and spleen. The findings in the present paper suggest that PNPase in the epidermis shows an organ specificity.

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