Study of Acid DNase in Cow Snout Epidermis by Micro Slab Electrophoresis Using Acidic and Basic Gel Sheet

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Summary: Electrophoretic profiles of acid DNase in cow snout epidermis were studied electrophoretically in relation to the depth of epidermis. Isoelectrophoretic point of acid DNase protein was found to be 4.9 and 8.4. To examine the electrophoretic properties of acidic and basic enzyme proteins, acid DNase was analyzed by micro slab electrophoresis, using 5% (pH 4.3) and 10% (pH 8.8) acrylamide gel sheet, respectively. Acid DNase was detected throughout all three successive layers of epidermis. In the case of acid DNase (basic protein) analysis carried out on the pH 4.3 gel sheet, the additional minor component was found beside major band, in the upper layer of epidermis.

Key words: acid DNase—epidermis—slab electrophoresis—isoelectric point—basic protein

Introduction

It is known that a disappearance of nuclei from the border of the stratum corneum is one of the most characteristic features of the normal keratinization process in the epidermis (Santoianni et al. 1961). Nucleolytic enzymes concerned with DNA degradation are of great significance in the overall biological process of the epidermis. In earlier studies (Mier et al. 1976; Ogura et al. 1978), the activities of the enzymes participating in the degradation of DNA were determined in cow snout epidermis, and their distribution was discussed in relation to the depth of epidermis. Förster et al. (1975) and Ogura et al. (1983) discussed electrophoretic properties of DNase in the epidermis by disc electrophoresis of polyacrylamide gel containing highly polymerized DNA.

To separate enzyme proteins with high sensitivity, micro slab electrophoresis with

vertical sheet of polyacrylamide gel was applied (Been et al. 1972). Prior to slab electrophoresis, the isoelectric point of enzyme protein (DNase) was determined by micro U-shape isoelectric focussing technique. The electrophoretic slab-gel sheet was selected on the basis of the analysis of isoelectric point. To improve a separation of basic and acidic enzyme proteins, the pH 4.3 and 8.8 polyacrylamide slab-gel sheets were prepared using a modification of procedure by Reisfeld et al. (1962). The successive horizontal slices of cow snout epidermis were examined to establish the electrophoretic properties of acid DNase in the epidermis in relation to the depth of epidermis.

Materials and Methods

Cow snouts were obtained from the slaughter house. The snouts were packed

in an ice-cold container and brought back to the laboratory within 1 hour after death. The skin was excised and the subcutaneous tissue removed. The skin was cut into 3-4 cm square pieces, which were then sliced into serial horizontal sections with a cryostat-microtome. The slices obtained from the snout epidermis were separated into three successive layers: upper, middle and lower layer. The individual layers (approximately 0.5 g each) were homogenized in 7 volumes of 0.25M sucrose with a glass homogenizer. The homogenate was centrifuged at $600\times g$ for 10 min. The resulting supernatant was used for enzyme assay.

Micro Isoelectric Focussing

A micro electrophoretic U-shape glass tube (5×340 mm) was used for isoelectric pH (Ip) determination with ampholine (pH 3.5-10) containing sucrose gradient as described by Weller et al. (1968).

The isoelectric focussing was performed at 1.5 mA at 4°C for 15 hours. After electrophoresis, the solution was pushed out gently from the anode side by using peristaltic pump with sucrose solution. The effluent was divided into 0.16 ml fractions. The fractions obtained were used immediately to determine the DNase activity and pH value.

Assay of DNase Activity (acid DNase)

DNase activity was determined by measuring spectrophotometrically the release of low molecular products from highly polymerized DNA, as described by Tabachnick (1964). For the assay of DNase activity, the reaction mixture contained 25 μl of 0.2% salmon DNA-10 mM NaCl solution, 200 μl of 0.1M acetate buffer (pH 5.0) with 5 mM EDTA, and 25 μl of enzyme solution to be assayed. After 30 min incubation at 37°C in Eppendorf micro test-tube, 50 μl of 15% perchloric acid was added to stop the reaction, chilled in an ice-cold water bath for 20 min, and centrifuged at $12,000\times g$

for 5 min. The optical density (OD) of the supernatant was determined at 260 nm by Beckman spectrophotometer ACTA CIII. A DNA blank and a sample blank were incubated simultaneously with each reaction tube and their values subtracted from the reading of each mixture. The enzyme activity (acid DNase activity) was expressed as OD 260 nm at above conditions.

Micro Slab Electrophoresis

The vertical micro-system for electrophoresis used here was designed by Yaegaki et al. (1982) with a modification described by Been et al. (1972). The apparatus consisted of an upper ($40\times 45\times 30$ mm in height) and lower buffer chamber ($30\times 80\times 75$ mm in height). The gel sheet was 33×55 mm in size. For the separation of basic and acidic protein, 5% (pH 4.3) and 10% (pH 8.8) acrylamide gel sheet, respectively, were used. A 100 μl of sample to be analyzed was loaded on the gel sheet. Egg lysozyme and bromphenol blue were used as markers for analysis of basic and acidic protein, respectively. The electrophoretic run with 15% acrylamide gel sheet (pH 4.3) was carried out at 4°C in β -alanine acetic acid buffer (pH 4.5) at 30 mA for 55 min with anode at the upper buffer chamber. The electrophoretic run with 10% acrylamide gel sheet (pH 8.8) was carried out at 4°C in Tris glycine buffer (pH 8.4) at 20 mA for 40 min with cathode at the upper buffer chamber.

After electrophoresis, the removed acrylamide gel sheet was preincubated for 1 h at 4°C in acetate buffer (pH 5.0) with 5 mM EDTA. The gel sheet was placed upon 0.15% agarose sheet containing 0.06% DNA, using a modification of the procedure described by Kim et al. (1982). During incubation with overlapped sheets for 4 hrs at 37°C , DNA in agarose gel was degraded at the location corresponding to the electrophoretic mobility of DNase in the electrophoretic acrylamide sheet. Any remaining high-molecular DNA in

agarose gel sheet was stained with 0.15% gallocyenin-5% chromalaune. After destaining the excess stain with a running tap-water for over night, the position of DNase was identified as the discontinuity in the substrate background of gallocyenin-chromalaune (Förestér et al. 1975). The stained gel sheet was scanned with an Ozumori densitometer model OZ-802, Asaka, Japan, using red filter.

Fig. 1. Isoelectric focussing of acid DNase in cow snout epidermis. A micro electrophoretic U-shape glass tube (5×340mm) was prepared with ampholine (pH 3.5-10.0) containing sucrose gradient. Abscissa indicates the fraction number of effluent (0.16 ml each) from the micro U-shape glass tube after electrophoresis.

Results

Isoelectrophoretic Point

As shown in Fig. 1, the isoelectrophoretic point of acid DNase was found to be 4.9 and 8.4.

Slab Electrophoresis

In the DNA-containing agarose sheet overlapped with electrophoretic slab gel, the location of acid DNase was identified as clear in the dark blue-stained background. The electrophoretic pattern of the staining sheet was shown with transmission peak corresponding to DNase activity as shown in the densitometry tracings of DNA staining gel (Fig. 2 and 3). The acid DNase obtained in each of the three different layers exhibited a distinct major band in both pH 4.3 and 8.8 gel sheets. In addition, minor peaks appeared in pH 4.3 gel sheet, in particular in the sample from the upper layer.

To confirm the migration pattern of protein obtained with the slab electrophoresis, acrylamide gel sheet was stained with amidoblack. Several peaks appeared in the densitogram obtained from the pH 4.3 and 8.8 gel sheets (Fig. 4 and 5). As a marker, egg lysozyme or bromphenol blue (BPB) was mixed with sample to be assayed. These markers (arrow in the Fig. 4 and 5) migrated faster than the proteins. These results show that basic and acidic proteins migrate electrophoretically and can be separated satisfactorily by means of slab electrophoresis. The electrophoretic distance of DNase does not correspond with the electrophoretic mobility of the major parts of epidermal protein.

Fig. 2. Densitometry tracing of DNA-containing 0.15% agarose gel sheet after overlapped with slab electrophoretic 15% acrylamide gel sheet (pH 4.3). The migration distance of the marker (egg lysozyme) is indicated with the arrow. The peak height in densitogram is in relation to DNase activity.

Fig. 3. Densitometry tracing of DNA-containing 0.15% agarose gel sheet after overlapped with slab electrophoretic 10% acrylamide gel sheet (pH 8.8). The migration distance of the marker (bromphenol blue) is indicated with the arrow. The peak height in densitogram is in relation to DNase activity.

Fig. 4. Migration pattern of protein with slab electrophoresis, 15% acrylamide gel sheet (pH 4.3) was stained with amidoblack. The migration distance of the marker (egg lysozyme) is indicated with the arrow.

Fig. 5. Migration pattern of protein with slab electrophoresis; 10% acrylamide gel sheet (pH 8.8) was stained with amidoblack. The migration distance of the marker (bromphenol blue) is indicated with the arrow.

Discussion

The electrophoretic properties of proteins have become a matter of discussion, as it was shown that the basic protein does not migrate readily in commonly used systems of electrophoresis. In the present paper, the slab electrophoresis was carried out with respect to cow snout epidermis, to distinguish between basic and acidic protein. In the case of analysis of basic protein, the pH 4.3 electrophoretic gel

sheet was prepared using a modification of the procedure described by Reisfeld et al. (1962). This technique led to an excellent resolution of mixtures of basic proteins. Yaegaki (1982) has also applied this procedure to analyze the acid DNase of rat submandibular gland and to demonstrate that both acid and basic protein were heterogeneous.

In the previous study (Ogura et al. 1983), DNase of cow snout epidermis was analyzed using commonly used disk elec-

trophoresis (pH 8.8 only). In that study, acid DNase exhibited only a single distinct band throughout all three layers of epidermis. The present analysis of isoelectric point demonstrated that acid DNase protein is heterogeneous (pI : 4.9 and 8.4). In the case of the slab electrophoresis with the pH 4.3 gel sheet, it was shown at present that acid DNase prepared from the upper layer of epidermis shows minor additional bands, beside the major band.

It has been reported that a negligible amount of DNA is present in the normal stratum corneum (Santoianni et al. 1961). The DNA molecule is degraded completely at the border of stratum corneum; hydrolytic enzymes are involved in this catabolism of nucleic acid of the epidermis. The distribution of hydrolytic enzyme activity was reported in relation to the depth of cow snout epidermis by Mier et al. (1976) and Ogura et al. (1978). The majority of acid hydrolases showed a clear peak in the basal and lower spinous cell layers, while acid phosphatase was found mainly in keratinizing zone (Mier et al. 1976). Enzymes participating in the degradation of DNA were found to show the maximal activity in the upper layer (Ogura et al. 1978). Acid DNase activity in cow snout epidermis was 10.5 ± 1.2 , 7.2 ± 1.0 , 5.3 ± 1.2 unit per mg protein respectively, with respect to the depth of epidermis. The highest activity was present in the upper layer of epidermis. In fact, the DNA content was found to decrease progressively with the differentiation of epidermal cells (Ogura et al. 1978). The DNA content per mg protein fell down to a low level in the upper layer, amounting at most to only about one third of DNA content in the lower layer (Ogura et al. 1978). In the present paper, acid DNase was present in all three successive layers of epidermis. In the case of basic protein, it is interesting to note that the additional minor peaks of acid DNase appear in the upper layer in addition to the major peak. The physiological significance of basic protein which

appears in the upper layer is unknown.

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