*International Journal of Pharmaceutics*, 44 (1988) 125–130
Elsevier



IJP 01491

# Esterase activity in snake skin

Ba Thu Nghiem and Takeru Higuchi

*Department of Pharmaceutical Chemistry, School of Pharmacy, The University of Kansas, Lawrence, KS 66045 (U.S.A.)*

(Received 23 July 1987)
(Modified version received 15 September 1987)
(Accepted 19 November 1987)

*Key words*: Snake skin; Model membrane; Esterase activity

## Summary

The relative importance of esterase activity in snake skin was studied. It was found that: (1) an aqueous snake skin extract which was prepared from the clean dry dorsal side of the snake skin hydrolyzed *p*-nitrophenyl acetate significantly at pH = 7.2 and 32 °C. (2) Pretreatment of the snake skin at 40 °C has no effect on the esterase activity of snake skin extract. After treatment at 80 °C for 15 min, however, over 90% of the activity is lost. (3) The esterase enzyme is still active for at least 6 months under dry storage conditions at room temperature or at −20 °C showing little change from recently shed snake skin. It appears that sheds of snake skin have, at least qualitatively, similar properties with respect to esterase enzymes which may still be active in the mass of dead cells of the skin stratum corneum as suggested by others.

## Introduction

In a previous study it was suggested that shed snake skin may be a useful model membrane for the in vitro study of transdermal delivery of drugs (Ibuki, 1985). Shed snake skin was shown to be similar to human stratum corneum in terms of thickness, composition of constituents and structure and to exhibit similar transport resistance (Ibuki, 1985). Basically, the composition of constituents of both the shed snake skin and human stratum corneum are keratinized proteins and lipids. It has been demonstrated that the shed snake skin lipid matrix significantly resembles that of the human skin in terms of its compositional features and with respect to its permiability to water (Roberts and Lillywhite, 1980; 1983). The present study concerns the evaluation of possible esterase activity within the snake skin and the passive metabolism which may take place in the dead cells of the stratum corneum.

It has been suggested that many intracellular enzymes are still active in the mass of dead cells of the horny layer (Schaefer et al., 1982). Passive enzymatic hydrolysis of esters has been assumed to occur in the human stratum corneum (Whitefield, 1970; Raab and Sieber, 1974a and b). It has not been demonstrated, however, that enzymatic hydrolysis actually occurs in the epidermis and dermis (Schaefer et al., 1982; Steigleder et al., 1959a; and b; Kammeran et al., 1975). Whether hydrolytic enzymatic activity actually exists in the horny layer or is limited to the viable tissue has not been determined. In particular, esterase activity in the stratum corneum has not been clearly

*Correspondence*: B.T. Nghiem, Solvay Veterinary, Inc., Building 259 Oakdale Campus, Technology Innovation Center, University of Iowa, Iowa City, IA 52242, U.S.A.

demonstrated using in vitro techniques because the human stratum corneum is difficult to obtain. Snake skin which is periodically shed in the form of a larger natural intact horny sheet (Banerjee and Mittal, 1978) appears to be useful for the in vitro study of esterase activity in this mass of dead cells. Therefore, the purposes of the present study are to determine: (a) whether the non-living cells of the snake skin contain intracellular esterases which are still active; (b) if snake skin contains enzymes which are capable of metabolizing drug substances; (c) what is the approximate in vitro value of the Michaelis–Menten constant, $K_m$, at experimental biological pH and temperature conditions if such activity is present; (d) to what extent heat affects the snake skin esterase activity; (e) to what extent storage conditions influence the snake skin esterase activity.

## Materials and Methods

*Reagents and solvents*

The following reagents and solvents were used: *p*-nitrophenol, *p*-nitrophenyl acetate both from Sigma Chemical Co., and reagent grade *t*-butyl alcohol, sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, all from Fisher Scientific Co.

*Phosphate buffer*

A 0.10 M, pH = 7.0 phosphate buffer was used in the hydrolysis rate studies. A 0.0050 M, pH = 7.2 isotonic phosphate buffer was used to homogenize the shed snake skin.

*In vitro determination of skin esterase activity*

*Snake skin.* Adult *Elaphe obsoleta obsoleta* (black rat snake) sheds which had been stored at room temperature for 5–6 months were used.

*Snake skin extract preparation.* The dorsal side of shed snake skin was washed with deionized water and allowed to dry at room temperature for 24 h. 2.7 g of the clean dry snake skin was homogenized for 10 min with 100 ml of 0.0050 M, pH = 7.2 isotonic phosphate buffer using an Osterizer blender equipped with a miniblend container. The homogenate was filtered through a buchner funnel. The filtrate was then centrifuged at 3500 rpm (25 °C) for 15 min. The supernatant solution was divided into separate 1.5 ml portions and recentrifuged for 2 min with an Eppendorf 5414 centrifuge. The resulting supernatant solution was stored frozen in a freezer at −20 °C. Portions of the extract were thawed and used as needed over a 2 day period.

*Hydrolytic rate study.* 0.40 ml of the snake skin extract and varying amounts of *p*-nitrophenyl acetate in *t*-butyl alcohol ($8.278 \times 10^{-3}$ M and $8.278 \times 10^{-4}$ M) were added to 0.10 M, pH = 7.0 phosphate buffer to give a final volume of 3.0 ml of clear solution containing the equivalent of the extract from 3.6 mg of clean dry snake skin/ml. The concentration of the hydrolytic product, *p*-nitrophenol, was measured spectrophotometrically at 400 nm. The non-enzymatic and enzymatic hydrolytic studies were performed at 32 °C on a Perkin Elmer Lambda 5 UV/Vis Spectrophotometer equipped with a thermostatted cell holder.

*Influence of heat on snake skin esterase activity study*

Freshly shed snake skin specimens were immediately stored at −20 °C and kept for 5-6 months before use in this study. The snake skin extract was prepared as described above except that 4.9 g of the clean dry snake skin was homogenized with 300 ml of phosphate buffer. The resulting supernatant was divided into 4 portions. The first portion was kept at room temperature. The second portion was heated at 40 °C for 15 min. The third portion was heated at 40 °C for 30 min. The last portion was heated at 80 °C for 15 min. The rate of hydrolysis was studied as described above except that 0.50 ml of the supernatant was mixed with the phosphate buffer to give a clear final solution which contained the equivalent of the extract from 2.7 mg of clean dry snake skin/ml.

## Results and Discussion

*Esterase activity in snake skin*

A typical rate of hydrolysis of *p*-nitrophenyl acetate in 0.10 M, pH = 7.0 phosphate buffer at 32 °C in the absence and presence of snake skin extract corresponding to 3.6 mg of clean dry snake skin/ml of the final solution is shown in Fig. 1.

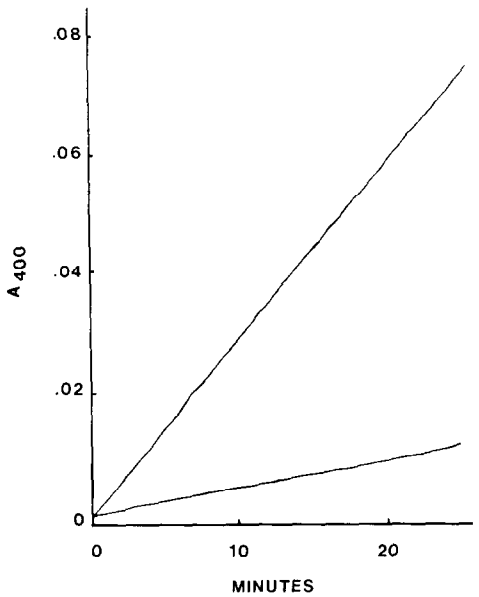


Fig. 1. Change in absorbance at 400 nm as a function of time for a solution containing initially $6.68 \times 10^{-5}$ M *p*-nitrophenyl acetate in the absence (bottom line) or presence (top line) of aqueous snake skin extract corresponding to 3.6 mg of clean dry snake skin/ml of the final solution at pH = 7.0 and 32° C.

All the reactions studied appeared to follow pseudo-zero-order kinetics for the 25 min initial time period. The rate of enzymatic hydrolytic cleavage of *p*-nitrophenyl acetate was obtained by subtracting the initial rate of non-enzymatic hydrolysis of the same concentration of *p*-nitrophenyl acetate in the absence of the snake skin extract.

A plot of the initial enzymatic rate vs *p*-nitrophenyl acetate concentration is shown in Fig. 2. The data correspond to a system which contains 3.6 mg of clean dry snake skin/ml of the final solution at 32° C. The plot strongly suggests some sort of an enzyme–substrate complex formation at high concentration of *p*-nitrophenyl acetate.

The reciprocal plot of the above data appears to follow the Lineweaver–Burke relationship at the lower concentrations of *p*-nitrophenyl acetate, as is evident from Fig. 3. The apparent Michaelis Menten constant, $K_m$, calculated from the plot corresponds to 0.047 mM and the apparent maximum velocity, $V_{max}$, corresponds to $1.28 \times 10^{-10}$ mol/min/mg of clean dry snake skin. These re-

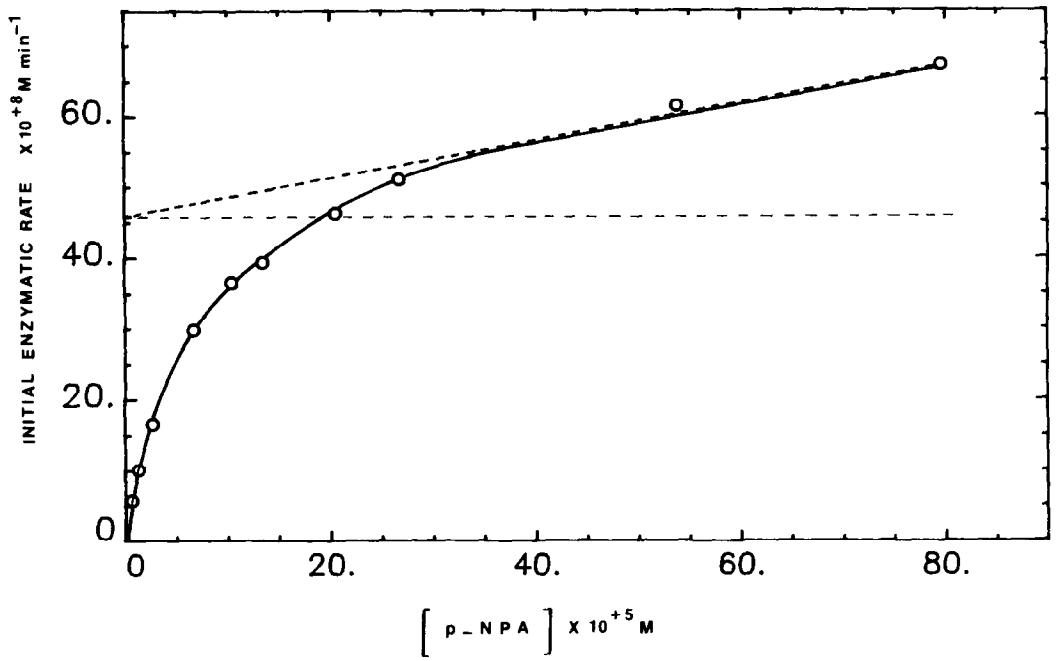


Fig. 2. Observed initial catalyzed hydrolytic rate of *p*-nitrophenyl acetate as a function of initial concentration at pH = 7.0 and 32° C. The aqueous snake skin extract corresponded to that obtained from 3.6 mg of clean dry snake skin/ml of the final solution. The upper dotted line corresponds to the non-saturable linearly dependent reaction. The lower dotted line corresponds to the apparent maximum velocity of the observed initial enzymatic rate.

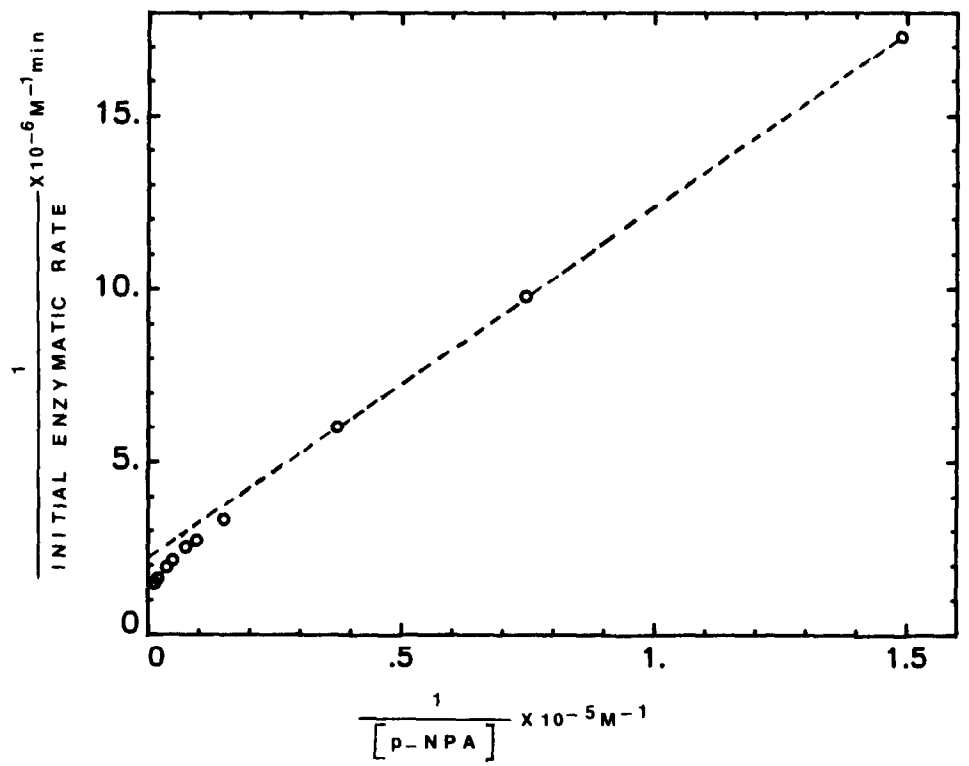


Fig. 3. Reciprocal plot of observed initial enzymatic rate of *p*-nitrophenyl acetate vs its concentration at pH = 7.0 and 32° C. The aqueous snake skin extract corresponded to that obtained from 3.6 mg/ml of the final solution.

sults appear to suggest the possibility that either a single or a few similar enzymatic species are largely responsible for the esterase activity in the skin. It would appear unlikely that a gross mixture of enzymes would yield such a simple plot. The deviation from the line at higher substrate concentration (Fig. 3) appears to be due to the contribution from a lower catalytic activity which is not easily saturable. This non-saturable linear dependent reaction is shown as the upper dotted line in Fig. 2. This contribution corresponds to the difference between two dotted lines in Fig. 2. The lower dotted line corresponds to the apparent maximum velocity of the observed initial enzymatic rate.

*The influence of heat on esterase activity in the snake skin*

The enzymatic nature of the hydrolysis reaction by the snake skin extract is further substantiated by results of experiments in which the snake skin extract had undergone a heat treatment at 40° C for 15 min or 30 min and 80° C for 15 min. The extract used in these studies differed from that of the preceding section in that it was prepared from freshly shed skin which had been stored at −20° C. The results (Table 1) show that the pretreatment at 40° C has no effect on the esterase activity of the snake skin extract. After treatment at 80° C for 15 min, however, over 90% of the activity is lost, a loss attributable to denaturation of the esterase enzyme in the snake skin extract.

*The influence of storage condition on snake skin esterase activity*

Comparison of the rate data shown in Table 1 for extracts obtained from shed skin stored at −20° C with corresponding results for that prepared from shed skins stored for 5–6 months at room temperature suggests that storage conditions

TABLE 1

*Observed initial catalyzed hydrolytic rate of p-nitrophenyl acetate (p-NPA) in the presence of non-treated aqueous snake skin extract (Study A) and heat-pretreated aqueous snake skin extract at 40°C for 15 min (Study B), 30 min (Study C) and 80°C for 15 min (Study D)*

| [*p*-NPA] ($M \times 10^{-5}$) | Observed initial enzymatic rate ($M \times 10^{-9}$/min) | | | |
|---|---|---|---|---|
| | Study A | Study B | Study C | Study D |
| 1.43 | 61.2 | 66.1 | 65.3 | 2.4 |
| 1.78 | 70.3 | 68.4 | 70.1 | 2.8 |
| 2.12 | 79.2 | 87.2 | 89.0 | 3.0 |
| 2.80 | 93.1 | 91.3 | 93.1 | 3.2 |
| 9.05 | 233.2 | 243.0 | 223.0 | 9.8 |

The rates were determined in aqueous buffer (0.1 M phosphate buffer, pH = 7.0) at 32°C for a system containing equivalent aqueous extract corresponding to that obtained from 2.7 mg of clean dry snake skin/ml of the final solution.

had little effect on the residual enzymatic activity. The data for extracts kept both at room temperature and 40°C correspond to an apparent Michaelis–Menten constant of 0.043 mM and to an apparent maximum velocity of $8.89 \times 10^{-11}$ mol/ml/mg of clean dry snake skin. These compare with values of apparent $K_m = 0.047$ mM and apparent $V_{max} = 1.28 \times 10^{-10}$ mol/min/mg of clean dry snake skin for sheds stored for 5–6 months at room temperature. These results, therefore, strongly suggest that the esterase enzyme is still active for at least up to 6 months under dry storage conditions at room temperature or at −20°C.

The relative significance of the esterase activity on the snake skin can be roughly estimated on the following basis. The $V_{max}$ values obtained for the snake skin aqueous extract in two studies were $1.28 \times 10^{-10}$ mol/min/mg and $8.89 \times 10^{-11}$ mol/min/mg. Since there were approximately 2.1 mg of dry snake skin/cm$^2$, a square cm of the snake skin is capable of hydrolyzing $2.69 \times 10^{-10}$ mol/min/cm$^2$ and $1.87 \times 10^{-10}$ mol/min/cm$^2$ at maximal rate in the two studies. This would suggest an activity sufficient to cleave a substantial portion of applied hydrolytically sensitive esters during their passage across the skin. The activity is such that it would require about 50 min to cleave 5 μg of a hydrolytically sensitive ester (MW = 500) similar to *p*-nitrophenyl acetate from an applied amount of 5 mg of 0.1% ointment over 1 cm$^2$ of snake skin.

The activity of esterase enzymes in the snake skin on a number of ester prodrugs of acetaminophen was also studied in our laboratory. This in vitro percutaneous absorption study indicates that the ester prodrugs of acetaminophen, which are stable in the receptor buffer at the pH studied, appear to be hydrolyzed by esterase enzymes in the snake skin (Higuchi and Konishi, 1986).

## Conclusion

These results further show the possible utility of shed snake skin as a model membrane for the in vitro evaluation of transdermal drug delivery systems. The model membrane not only simulates the barrier property of skin as suggested by Ibuki but also appears to have, at least qualitatively, similar properties of esterase enzymes which may be still active in the mass of dead cells of the horny layer of skin.

## Acknowledgements

The authors acknowledge the support of this work by a grant from the Kansas Advance Technology Commission of the State of Kansas and from IPRX, Inc., of Lawrence, KS.

## References

Banerjee, T.K. and Mittal, A.K., Histochemistry of snake skin. In Spearman, R.I.C. and Riley, P.A. (Eds.), *The Skin of Vertebrates*, Academic, London, 1978, pp. 23–32.

Higuchi, T. and Konishi, R., In vitro testing and transdermal delivery. In *Therapeutic Research, Proceeding of The Second Transdermal Therapeutic System Symposium*, 38 (1986) 82–90.

Ibuki, R., *Use of Shed Snake Skin as Model Membrane for Percutaneous Absorption Studies. Behavior of Several Penetration Enhancers in the System*, Ph.D. Thesis, The University of Kansas, 1985.

Kammerau, B., Zesch, A. and Schaefer, H., Absolute concentrations of dithranol and triacetyldithranol in the skin layers after local treatment: in vivo investigations with four different types of pharmaceutical vehicles. *J. Invest. Dermatol*, 64 (195) 145–149.

Raab, W. and Siber, H., Glucose-6-phosphat-dehydrogenase-Aktivitat durch Glucocorticoide. *Arch. Dermatol. Forsch.*, 249 (1974a) 357–366.

Raab, W. and Siber, H., Die Hemmung der Glucose-6-phosphatdehydrogenase-Activitat durch Glucocorticoide. *Arch. Dermatol. Forsch.* 249 (1974b) 357–366.

Roberts, J.B. and Lillywhite, H.B., Lipid barrier to water exchange in reptile epidermis. *Science*, 207 (1980) 1077–1079.

Roberts, J.B. and Lillywhite, H.B., Lipids and the permeability of epidermis from snake. *J. Exp. Zool.*, 228 (1983) 1–9.

Schaefer, H. et al., *Skin Permeability*, Springer, New York, 1982, p. 582–583.

Steigleder, G.K. and Elschner, H., Die Fähigkeit der Hautoberfläche zur Esterspaltung. *Arch. Klin. Exp. Dermatol.*, 208 (1959a) 489–501.

Steigleder, G.K., Die Fähigkeit der Hautoberfläche zur Esterspaltung und Esterbildung. *Arch. Klin. Exp. Derm.*, 209 (1959b) 313–326.

Whitefield, M., The treatment of psoriasis with triacetoxyanthracene. *Arzneim. Forsch.*, 20 (1970) 137–139.