Available online at www.sciencedirect.com
ScienceDirect
International Journal of Pharmaceutics 332 (2007) 196–201
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Note

# An investigation into keratinolytic enzymes to enhance ungual drug delivery

M. Mohorčič [a], A. Torkar [b], J. Friedrich [a], J. Kristl [b], S. Murdan [c,*]

[a] *Laboratory of Biotechnology, National Institute of Chemistry, Hajdrihova 19, SI-1001 Ljubljana, Slovenia*
[b] *Faculty of Pharmacy, University of Ljubljana, Aškerčeva 7, SI-1001 Ljubljana, Slovenia*
[c] *Department of Pharmaceutics, The School of Pharmacy, University of London, Brunswick Square 29/39, London WC1N 1AX, UK*

Received 4 July 2006; received in revised form 21 September 2006; accepted 25 September 2006
Available online 29 September 2006

**Abstract**

The topical therapy of nail diseases is limited by the low permeability of drugs through the nail plate. To increase drug penetration, the integrity of the nail plate must be compromised to a certain extent. We hypothesised that keratinolytic enzymes might decrease the barrier properties of the nail plate by hydrolysing the nail keratins, and thereby enhance ungual drug permeation. To determine enzyme action on nail plates, nail clippings were incubated at 35 °C, in the presence of keratinase at optimal pH for 48 h, after which the nail plates were examined using scanning electron microscopy. It was found that the enzyme acted on the intercellular matrix which holds nail cells together, such that corneocytes on the dorsal surface separated from one another and 'lifted off' the nail plate. In addition, the surface of the corneocytes was corroded. Permeation studies using modified Franz diffusion cells and bovine hoof membranes as a model for the nail plate showed that the enzyme enhanced drug permeation through the hoof membrane. The permeability and partition coefficients, and the drug flux were found to be significantly increased in the presence of the enzyme. We can conclude that the enzyme, via its hydrolytic action on nail plate proteins, could increase ungual drug delivery.


*Keywords:* Nail; Keratinase; Ungual drug delivery; Permeability; Drug permeation

The two most common disorders of the nail unit are onychomycosis (fungal infections of the nail plate and/or bed) and psoriasis. Onychomycosis is normally treated with oral antifungals, while psoriasis necessitates repeated monthly injections of corticosteroids into the nail folds. Ideally, these diseases would be treated topically to eliminate the inherent side effects of the current treatments such as pain, systemic adverse events and drug interactions, and to increase patient compliance (Gupchup and Zatz, 1999; Murdan, 2002). The effectiveness of topical therapy is, however, limited by the very poor permeability of drugs through the nail plate. The latter is a compact structure made up of a large number of layers of dead, keratinised cells and can be divided into two parts: dorsal and ventral, the dorsal cells being flatter and more closely connected to one another than the ventral cells (De Berker and Forslind, 2004). So far, only a few chemicals that can increase drug penetration into the nail plate have been identified. These ungual enhancers include sulphhydryl-containing compounds (Sun et al., 1997, 1999; Van Hoogdalem et al., 1997; Kobayashi et al., 1998; Malhotra and Zatz, 2002), such as *N*-acetyl cysteine, mercaptoethanol, *N*-(2-mercaptopropionyl)glycine. It is thought that these enhancers act by reducing the keratin disulphide bonds which are responsible for the integrity of the nail keratins and hence, for the barrier properties of the nail plate (Sun et al., 1999).

Since the nail plate is composed mainly of keratins (Lynch et al., 1986), we hypothesised that keratinolytic enzymes might also compromise the barrier properties of the nail plate, and thereby act as ungual enhancers, due to their hydrolytic action on the keratins of the nail plate. Keratin filaments and keratinic tissues such as skin stratum corneum and ground nail plate are known to be hydrolysed by keratinase (Friedrich and Kern, 2003; Gradišar et al., 2005) and the release of soluble proteins following incubation of nail plates with keratinase has been shown (Vignardet et al., 1999, 2001). It has also been reported that papain (a protease of plant origin) degraded nail surface and that soaking nail plate in mixtures of papain and salicylic acid increased the permeability of imidazole antimycotics through the nail (Quintanar-Guerrero et al., 1998).

To test the hypothesis that keratinase can damage the nail plate and thereby enhance ungual drug delivery, the effects of keratinase on nail clippings were determined using scanning

* Corresponding author. Tel.: +44 207 753 5810; fax: +44 207 753 5942.
*E-mail addresses:* sudax.murdan@pharmacy.ac.uk, sudax.murdan@ulsop.ac.uk (S. Murdan).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.09.042

electron microscopy and drug permeation profiles, in the presence of keratinase, were determined using modified Franz diffusion cells.

Fresh human nail clippings, obtained from volunteers, were placed in a glass vial containing 1 mL of Tris–HCl buffer at pH 7.5 (enzyme's optimum pH) containing keratinase (from the fungus *Paecilomyces marquandii*, MZKI B-639), at 1 or 10 mg/mL, and were incubated at 35 °C for 48 h in order to observe the effects of keratinase on the nail plate. Since the enzyme activity was known to decrease with time (following incubation in a liquid medium at 35 °C and pH 7.5, the enzyme was found to retain around half of the initial activity after 7 h), an enzyme aliquot was dissolved in the incubation solution every 12 h to retain the initial enzyme activity. Control experiments were conducted by incubating nail clippings in buffer only. At the end of 48 h, the nail clippings were rinsed with distilled water, cut into three portions, sputter gold-coated and observed using scanning electron microscopy (Philips/FEI XL30, Eindhoven, The Netherlands).

Permeation experiments were conducted, using pre-soaked bovine hoof membranes (150–200 μm thick) and metformin hydrochloride as model nail plate and model drug, respectively, to investigate the effect of keratinase on ungual drug permeation. Metformin hydrochloride was selected for its small size; the latter has been found to be the most important physicochemical property which influences ungual drug permeation and is inversely related to permeation (Kobayashi et al., 2004). Bovine hoof membranes were used as a model for the nail plate due to the limited availability of nail plates and the similarities between the two structures: both are keratinic tissues; upon incubation with keratinase, soluble proteins are released from both nail plate and hoof membranes (Vignardet et al., 1999); FT-Raman spectroscopy has revealed that both hoof and nail keratins exist predominantly in an α-helical conformation (Edwards et al., 1998) and Baden and Kubilus (1984) showed similar immunologic properties of hoof and nail fibrous proteins, with respect to cross-reaction between antibodies to soft and hard fibrous keratin. In addition, hoof membranes have been shown to be suitable models for nail plates in transungual permeation experiments (Hemidy et al., 1994) and the relationship between the permeability coefficients of bovine hoof membrane and of human nail plate has been reported to be

$$\log P_N = 3.723 + 1.751 \log P_H$$

where $P_N$ is the permeability coefficient through the nail plate and $P_H$ is the permeability coefficient through the hoof membrane (Mertin and Lippold, 1997). Modified Franz diffusion cells, previously reported by Kierstan et al. (2001) were used. These cells consist of a quartz cuvette as the receptor compartment and a stainless steel cap, which holds the donor phase and enables the hoof membrane to be held securely in place. These diffusion cells enable the continuous measurement of drug concentration in the receptor phase without the need for sampling. The permeation area of the membrane was 2.1 $mm^2$, while the donor phase was 70 μL of Tris buffer, pH 7.5, containing 2 g/L metformin and 30 g/L keratinase. The permeation cells were setup as described by Kierstan et al. (2001) and the diffusion cells were placed on a magnetic plate in an incubator at 35 °C. The absorbance of the receptor phase was read at 231 nm every hour for up to 35 h. The drug concentration in the receptor compartment was calculated from a Beer Lambert plot and the cumulative amount of metformin/area in the receptor cell was plotted against time. To determine permeability, partition and diffusion coefficients, the equation below was used to fit the experimental data (Scheuplein, 1967; Diez-Sales et al., 1996). Because of the lack of data between time points 12 and 21 h, only the readings obtained from time 0 to 12 h were used to fit to the equation below

$$Q(t) = APhC\left[D\frac{t}{h^2} - \frac{1}{6} - \frac{2}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\exp\left(-\frac{Dn^2\pi^2 t}{h^2}\right)\right]$$

where $Q(t)$ is the quantity of drug which passes through the membrane and reaches the receptor phase at a given time, $t$, $A$ the actual diffusional surface area, $P$ the partition coefficient of the permeant between the membrane and the donor vehicle, $h$ the membrane thickness, $D$ the diffusion coefficient of the permeant in the membrane and $C$ is the concentration of the permeant in the donor solution. $P$ and $D$ were calculated through fitting the theoretical equation to individual in vitro permeation data sets using Origin® software. The permeability coefficient, $K_p$, was calculated as $PD/h$. Estimates of lag time were calculated as lag time = $h^2/6D$. Flux was calculated as flux = $K_pC$. Student's $t$-tests were conducted (SPSS 14.0 software, SPSS Inc.) to detect differences, if any, between the permeability, partition and diffusion coefficients, flux and lag time of control and enzyme-assisted permeation profiles. A value of $P < 0.05$ denoted significance in all cases.

Scanning electron microscopy (SEM) revealed that keratinase acted on the intercellular matrix that holds the cells of the nail plate together. Thus, in contrast to the control nail samples where the individual dorsal surface cells cannot be distinguished due to the fact that they were closely bound to one another (Fig. 1), the dorsal corneocytes of the enzyme-treated nail plate were separated from one another (Fig. 2). The enzyme acted in a concentration-dependent manner, such that when

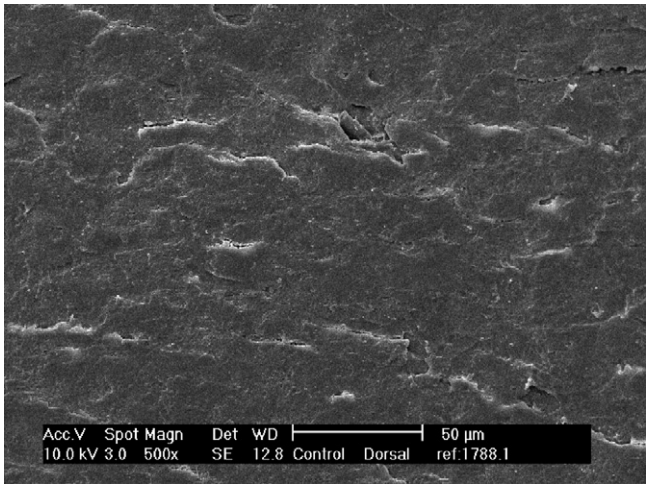


Fig. 1. SEM micrograph of the dorsal side of the nail plate incubated in buffer (control).

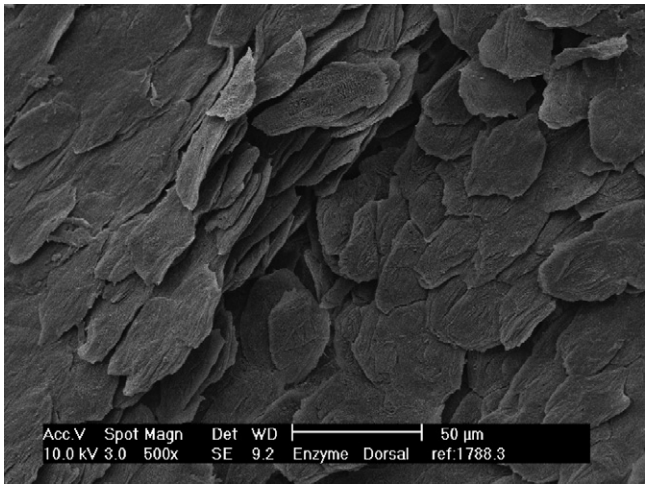


Fig. 2. Upon incubation with keratinase (10 mg/mL), the corneocytes on the dorsal nail surface separate from one another, as keratinase acts on the intercellular matrix.

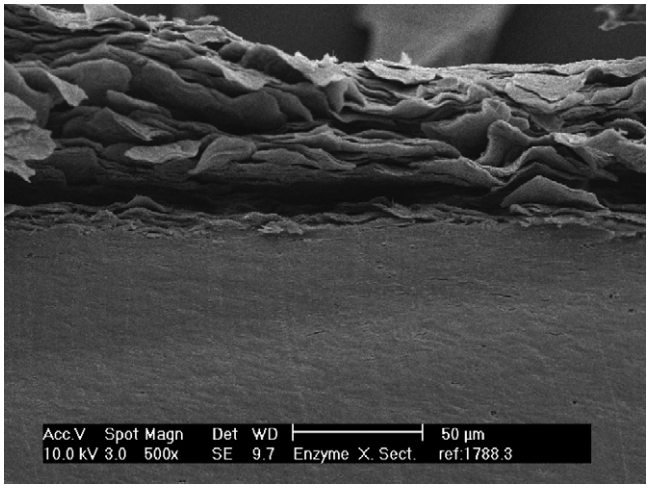


Fig. 4. A cross-section view confirms nail cell separation upon incubation with keratinase.

nail clippings were incubated in a solution containing the lower enzyme concentration of 1 mg/mL (compared to 10 mg/mL in Fig. 2), corneocyte detachment from their neighbours occurred to a lesser extent, as shown in Fig. 3. Very little is known about the intercellular matrix present between nail cells. De Berker and Forslind (2004) have suggested that it is composed of proteins (and/or mucopolysaccharides), which are likely to be in a random coil state. If the intercellular proteins are in a random coil state, this would increase the likelihood of enzyme attack, the random coil state being associated with disorder, which may favour access of the enzyme to its substrate. Separation of the dorsal nail cells from their neighbours was confirmed when the nail plate cross-section was viewed (Fig. 4). The cross-section view also shows that only a small portion of the nail plate (the most exposed part) was severely disrupted by the enzyme. The deeper nail layers were either unaffected or affected to a very small extent. This is probably due to the limited diffusion of the large enzyme molecule (MW ≈ 33,000) into the nail plate.

Limitation of enzyme activity to the exposed part of the nail (in practice, to the dorsal nail surface) may be sufficient for enhanced ungual drug delivery, as the dorsal nail surface is known to be the main barrier to ungual drug permeation and its removal, by filing, has been shown to increase drug flux through the nail plate (Kobayashi et al., 1999).

In addition to acting on the intercellular matrix, keratinase corroded the surface of corneocytes (Fig. 5). Corrosion led to a striated corneocyte surface and it is possible that the ridges represent bundles of keratin fibres, which have been exposed following digestion of the intracellular proteins present among keratin fibres. It is known that in sheep fur, keratin fibres are held together by globular, cystine-rich proteins whose disulphide links act as 'glue' (Fleckman, 1997). A similar arrangement of keratin fibres held by globular, cystine-rich proteins might occur in nail plates, in which case, a preferential action of the keratinase on the globular proteins (rather than on the keratin fibres) would expose the fibres. In general, one can expect degradation of globular proteins (which are water-soluble) to be easier than

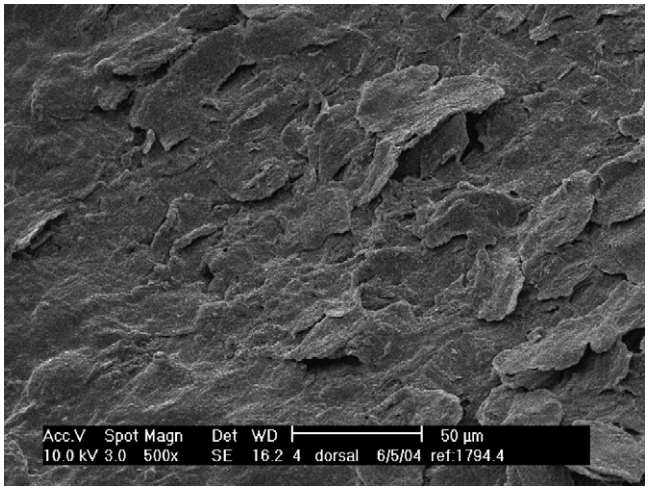


Fig. 3. Upon incubation with a lower concentration of keratinase (1 mg/mL), fewer corneocytes on the dorsal nail surface separate from one another, compared to Fig. 2.

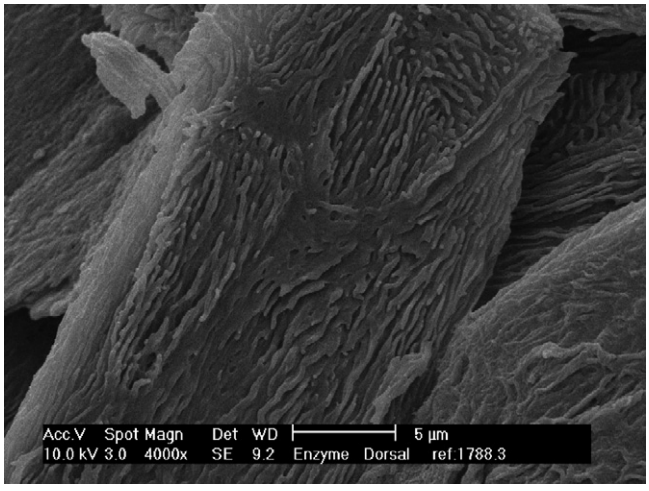


Fig. 5. As well as acting on the intercellular matrix, keratinase corrodes the surface of nail corneocytes.

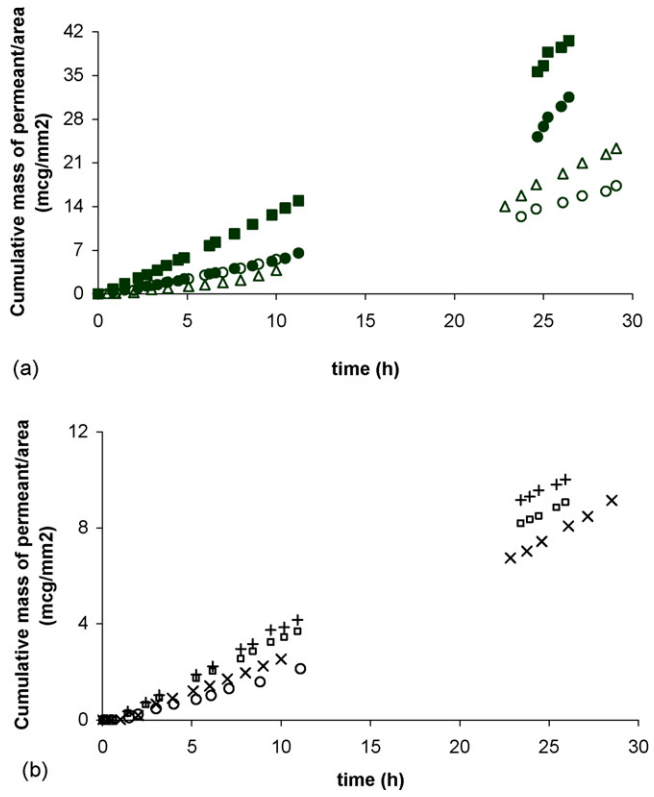


Fig. 6. (a) Cumulative mass (mcg) of permeant/mm$^2$ through enzyme-treated hoof membrane: (■) 140 μm, (●) 170 μm, (△) 190 μm, (◯) 190 μm. (b) Cumulative mass (mcg) of permeant/mm$^2$ through control hoof membrane: (+) 190 μm, (□) 180 μm, (×) 180 μm, (◯) 190 μm.

those of fibrous ones (which are mainly insoluble), as shown by Gradišar et al. (2005) and Friedrich and Kern (2003).

The permeation profiles of the model drug, metformin, in the presence and absence of keratinase are shown in Fig. 6a and b, respectively. After an initial lag, the drug concentration in the receptor phase started to increase, more so in the presence of keratinase, than in its absence. A high variability was observed in the permeation profiles; some of the variability is attributed to the different thicknesses of the membranes used and some to the inherent variability of the biological hoof tissue. Membrane thickness was taken into account in calculations. The flux and the permeability coefficient through the enzyme-exposed bovine hoof membrane were significantly higher than those of the control (*t*-test, $P<0.05$, Table 1). In the presence of keratinase, membrane permeability was more than doubled; this shows that keratinase could have potential as an ungual chemical enhancer. The partition coefficient, but not the diffusion coefficient, was significantly higher (*t*-test, $P<0.05$, Table 1), which indicates that the enzyme-induced increased permeability of the hoof membrane was due to increased partition of the drug into the membrane. Altered drug partition into the hoof membrane indicates a change in the properties of the enzyme-exposed hoof membrane. Such a change could be a result of keratinase-induced release of soluble proteins from hoof membranes, reported by Vignardet et al. (1999). Interestingly, diffusion coefficient did not change in the presence of enzyme (*t*-test, $P>0.05$). Such a lack of change could be due to the fact that the enzyme only attacked the uppermost layers of the exposed hoof membrane, such that diffusion of the drug through the bulk of the membrane was unchanged. This is supported by scanning electron microscopy on cross-sections of hoof membranes (performed at the end of the permeation experiments) which showed no observable difference in the bulk of the hoof membrane between the control membranes and those that were exposed to enzyme (Fig. 7a and b).

The permeation experiments with the enzyme had been conducted for up to 29 h, without the addition of fresh enzyme to the

Table 1
Transport parameters of metformin through bovine hoof membrane, in the presence and absence of enzymes

| | Control | Presence of enzyme |
|---|---|---|
| Diffusion coefficient ($\times10^{-5}$ mm$^2$/min) | 13.2 (2.6) | 13.8 (8.4) |
| Partition coefficient | 1.7 (0.2) | 4.7 (2.8) |
| Permeability coefficient ($\times10^{-3}$ mm/min) | 1.2 (0.3) | 2.9 (1.5) |
| Lag time (min) | 45.1 (8.9) | 55.4 (48.2) |
| Flux ($\times10^{-3}$ μg/mm$^2$ min) | 2.5 (0.6) | 5.7 (2.9) |

Mean (S.D.) are shown, $n=5$–6.

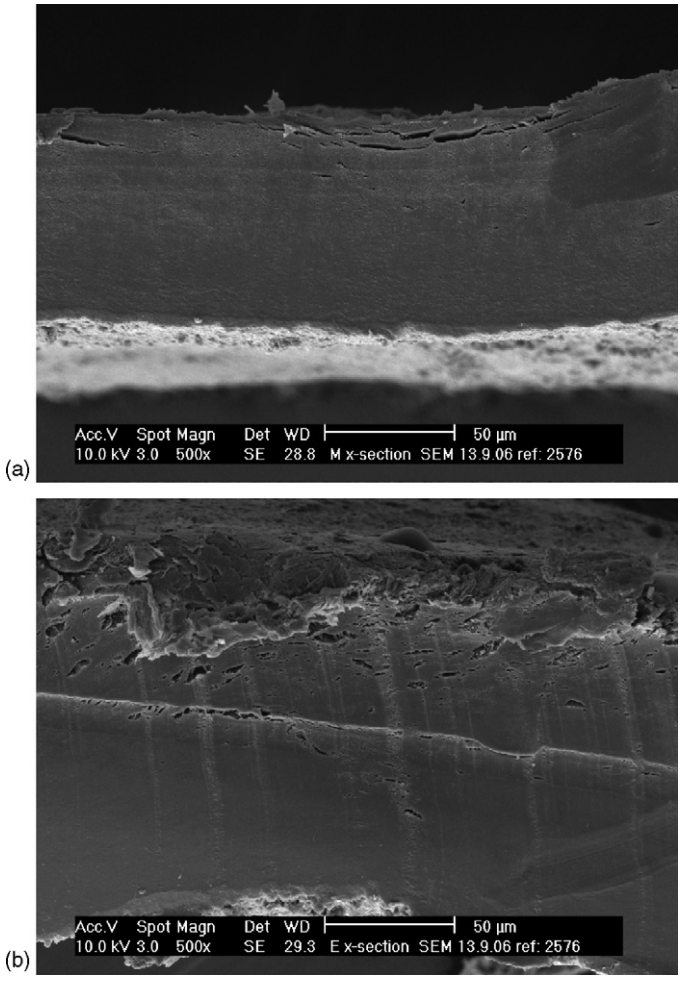


Fig. 7. SEM micrographs of a cross-section of (a) control and (b) keratinase-exposed hoof membrane show no difference in the bulk thickness of the membrane.

donor compartment. Since the enzyme is known to retain only around 50 and 23% of its initial activity after 7 and 24 h incubation in an aqueous medium, respectively (unpublished data), the continued enhanced permeation of the drug in the later stages of the experiment (Fig. 6a) shows that the enzyme-induced damage to the bovine hoof membrane was irreversible for the duration of the experiment. This was expected, as once the hoof proteins had been hydrolysed by the enzyme, with resulting morphological changes, repair of the dead tissue was not possible. The irreversible damage to the hoof membrane and continued enhanced drug permeation indicate that, in practice, it might be sufficient to pre-treat diseased nail plates with an enzyme formulation, for example, using a transdermal-type patch for several hours, after which the patch would be removed and drug application would take place, for example via the application of a drug-containing nail lacquer. Neither the patch nor the nail lacquer is expected to interfere with the patient's normal activities. Application of the enzyme formulation as a pre-treatment for a few hours would resolve the problem of enzyme's loss of activity with time following its application. In future, experiments where the enzyme is applied as a pre-treatment, i.e. before drug application should be conducted to determine the feasibility of changing nail plate permeability before drug application. Such a possibility would obviate the need for co-formulating the drug and the enhancer within one vehicle, which would render formulation easier, in terms of drug and enzyme solubility, stability, pH of vehicles, etc. Experiments must also be conducted using nail plates, to test transferability of hoof membrane results to nail plates treated with enzymes. It must be remembered that the hoof membrane, while used extensively as a model for the nail plate in the ungual research field, is still different to nail plates.

From these studies, we observed that keratinase produced by *P. marquandii* disrupted the nail plate by acting on the intercellular matrix as well as on the nail corneocytes. The keratinase enzyme increased the permeability of bovine hoof membranes, used as model for the nail plate. The results suggest that keratinase enzymes may act as ungual enhancers to increase the permeation of topically applied drugs into the nail plate.

## Acknowledgements

The authors wish to thank Prof. Jonathan Hadgraft (School of Pharmacy, University of London) for critical discussion of the work shown, Prof. J.P. Chaumont (Faculty of Medicine and Pharmacy, Besançon, France) for donating the fungal strain, Nasir Khatab for the preliminary experiments and David McCarthy (School of Pharmacy, University of London) for the SEM analyses. The financial support of the Ministry of Higher Education, Science and Technology of the Republic of Slovenia (program grant P4-0176), the British Council (project grants PSP 2/2003 and BI-GB/04-004), the COST Action D25, the FEMS for the fellowship to M. Mohorčič are also acknowledged.

## References

Baden, H.P., Kubilus, J., 1984. A comparative study of the immunological properties of hoof and nail fibrous proteins. J. Invest. Dermatol. 83, 327–331.

De Berker, D., Forslind, B., 2004. The structure and properties of nails and periungual tissues. In: Forslind, B., Linberg, M., Norlén, L. (Eds.), Skin, Hair, and Nails, Structure and Function. Marcel Dekker, New York, pp. 409–464.

Diez-Sales, O., Watkinson, A.C., Herraez-Dominguez, M., Javaloyes, C., Hadgraft, J., 1996. A mechanistic investigation of the in vitro human skin permeation enhancing effect of Azone®. Int. J. Pharm. 129, 33–40.

Edwards, H.G.M., Hunt, D.E., Sibley, M.G., 1998. FT-Raman spectroscopic study of kertatotic materials: horn, hoof and tortoiseshell. Spectrochim. Acta Part A 54, 745–757.

Fleckman, P., 1997. Basic science of the nail unit. In: Scher, R.K., Daniel, C.R. (Eds.), Nails: Therapy, Diagnosis, Surgery. WB Saunders, Philadelphia, pp. 37–54.

Friedrich, J., Kern, S., 2003. Hydrolysis of native proteins by keratinolytic protease of Doratomyces microsporus. J. Mol. Catal. B: Enzym. 21, 35–37.

Gradišar, H., Friedrich, J., Križaj, I., Jerala, R., 2005. Similarities and specificities of fungal keratinolytic proteases: keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* in comparison to some known proteases. Appl. Environ. Microbiol. 71, 3420–3426.

Gupchup, G.V., Zatz, J.L., 1999. Structual characteristics and permeability properties of the human nail: a review. J. Cosmet. Sci. 50, 363–385.

Hemidy, P.-Y., Makki, S., Muret, P., Chaumont, J.P., Millet, J., 1994. The use of sheep hoof plates for substituting human nails in transungual absorption studies. J. Appl. Cosmetol. 12, 73–84.

Kierstan, K.T.E., Beezer, A.E., Mitchell, J.C., Hadgraft, J., Raghavan, S.L., Davis, A.F., 2001. UV spectrophotometry study of membrane transport processes with a novel diffusion cell. Int. J. Pharm. 229, 87–94.

Kobayashi, Y., Miyamoto, M., Sugibayashi, K., Morimoto, Y., 1998. Enhancing effect of *N*-acetyl-L-cysteine or 2-mercaptoethanol on the in vitro permeation of 5-fluorouracil or tolnaftate through the human nail plate. Chem. Pharm. Bull. 46, 1797–1802.

Kobayashi, Y., Miyamoto, M., Sugibayashi, K., Morimoto, Y., 1999. Drug permeation through the three layers of the human nail plate. J. Pharm. Pharmacol. 51, 271–278.

Kobayashi, Y., Komatsu, T., Sumi, M., Numajiri, S., Miyamoto, M., Kobayashi, D., Sugibayashi, K., Morimoto, Y., 2004. In vitro permeation of several drugs through the human nail plate: relationship between physicochemical properties and nail permeability of drugs. Eur. J. Pharm. Sci. 21, 471–477.

Lynch, M.H., O'Guin, W.M., Hardy, C., Mak, L., Sun, T.T., 1986. Acidic and basic hair/nail "hard" keratins: their co-localisation in upper cortical and cuticle cells of the human hair follicle and their relationship to "soft" keratins. J. Cell Biol. 103, 2593–2606.

Malhotra, G.G., Zatz, J.L., 2002. Investigation of nail permeation enhancement by chemical modification using water as a probe. J. Pharm. Sci. 91, 312–323.

Mertin, D., Lippold, B.C., 1997. In vitro permeability of the human nail and of a keratin membrane from bovine hooves: prediction of the penetration rate of antimycotics through the nail plate and their efficiency. J. Pharm. Pharmacol. 49, 866–872.

Murdan, S., 2002. Drug delivery to the nail following topical application. Int. J. Pharm. 236, 1–26.

Quintanar-Guerrero, D., Ganem-Quintanar, A., Tapia-Olguín, P., Kalia, Y.N., Buri, P., 1998. The effect of keratinolytic agents on the permeability of three imidazole antimycotic drugs through the human nail. Drug Dev. Ind. Pharm. 24, 685–690.

Scheuplein, R.B., 1967. Mechanism of percutaneous absorption. II. Transient diffusion and the relative importance of various routes of skin penetration. J. Invest. Dermatol. 48, 79–88.

Sun, Y., Liu, J.C, Kimbleton, E.S., Wang, J.C.T., December 9, 1997. Antifungal treatment of nails. US Patent 5,696,164.

Sun, Y., Liu, J.C., Wang, J.C.T., De Doncker, P., 1999. Nail Penetration. Focus on topical delivery of antifungal drugs for onychomycosis treatment. In: Bronaugh, R.L., Maibach, H.I. (Eds.), Percutaneous Absorption, Drugs-Cosmetics-Mechanisms-Methodology, 3rd ed. Marcel Dekker Inc., New York, pp. 759–787.

Van Hoogdalem, E.J., van den Hoven, W.E., Terpstra, I.J., van Zijtveld, J., Verschoor, J.S.C., Visser, J.N., 1997. Nail penetration of the antifungal agent oxiconazole after repeated topical application in healthy volunteers, and the effect of acetylcysteine. Eur. J. Pharm. Sci. 5, 119–127.

Vignardet, C., Guillaume, Y.C., Friedrich, J., Millet, J., 1999. A first order experimental design to assess soluble proteins released by a new keratinase from *Doratomyces microsporus* on human substrates. Int. J. Pharm. 191, 95–102.

Vignardet, C., Guillaume, Y.C., Michel, L., Friedrich, J., Millet, J., 2001. Comparison of two hard keratinous substrates submitted to the action of a keratinase using an experimental design. Int. J. Pharm. 224, 115–122.