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Comparative Biochemistry and Physiology Part A 130 (2001) 835–847

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A zymogel enhances the self-cleaning abilities of the skin of the pilot whale (*Globicephala melas*)

C. Baum[a,b], W. Meyer[b,*], D. Roessner[c], D. Siebers[a], L.-G. Fleischer[d]

[a] *Alfred Wegener Institute Foundation for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany*
[b] *Anatomical Institute, Department of Histology and Embryology, School of Veterinary Medicine, Bischofsholer Damm 15, D-30173 Hannover, Germany*
[c] *Wyatt Technology Deutschland GmbH, In der Steubach 9, D-57614 Woldert, Germany*
[d] *Institute of Food Technology and Center of Biotechnology, Technical University of Berlin, Amrumer Str. 32, D-13353 Berlin, Germany*

Received 30 March 2001; received in revised form 24 July 2001; accepted 30 July 2001

Abstract

Enzyme activity in the stratum corneum of the pilot whale *Globicephala melas* was investigated employing colorimetric enzyme screening assays combined with NATIVE PAGE, size exclusion chromatography (SEC) and histochemical staining procedures. Applying different substrates, several enzymes were detected. The histochemical demonstration of some selected hydrolytic enzymes enriched in the stratum corneum showed high extracellular accumulation. As demonstrated by size exclusion chromatography, high molar mass aggregates were built up from a glycoprotein-rich 20–30-kD fraction. Using NATIVE PAGE experiments under non-reducing conditions, a selection of five degrading enzymes was recovered within the above-reported aggregates. Activity of extracellular aggregate-attached enzymes in the superficial layer of the stratum corneum exhibited no remarkable decrease potentially resulting from self-degradation. We thus conclude that due to their enclosure within the microenvironment of aggregates, a zymogel is formed and autolysis of the stratum corneum is reduced. With respect to the skin surface, the zymogel with hydrolytic activities covering major parts of it enhances the self-cleaning abilities of the skin of the pilot whale based on physical pre-requisites by hydrolyzing adhesive glycoconjugates of settling biofouling organisms considered as primary steps in fouling.

Keywords: Stratum corneum; Enzymes; Gel formation; Epidermis; *Globicephala melas*; Dolphins; Skin protection; Self-cleaning abilities

* Corresponding author. Tel.: +49-511-865-7215; fax: +59-511-856-7683.
E-mail address: wilfried.meyer@tiho-hannover.de (W. Meyer).

1095-6433/01/$ - see front matter
PII: S1095-6433(01)00445-7

1. Introduction

Contaminations of the external surfaces of plants and animals by inorganic and organic molecules, particles and micro-organisms are stress factors, which may impair the functional surface properties in several ways. They may, for example, impede sensory perception, metabolism and exchange, adhesion or motion. In order to avoid or minimize these impediments, various self-cleaning capabilities have evolved as related to a permanent air or water contact.

Considering such capabilities in dolphins, low critical surface tensions of the skin which reduce its wettability were reported by Gucinski et al. (1984) and Gucinski (1986). In addition, the continuous epidermis renewal as manifested by the desquamation of superficial cell layers of the stratum corneum, seems to play a role in the avoidance of attachment of micro- and macrofouling organisms (Brown et al., 1983; Hicks et al., 1985). In contrast, findings from St. Aubin et al. (1990) and Smith et al. (1992) imply that the self-cleaning abilities are reduced in dolphin species with stronger discontinuous seasonal desquamation. It is also known that under permanent mechanical stress, for example, in digging grey whales, the self-cleaning properties of the epidermis are reduced. In addition, blooms of algae or the presence of oils or detergents contained in the ambient water may represent high risks limiting the self-cleaning abilities of the cetacean skin.

Our interest focuses on dolphins which visually exhibit remarkably clean epidermal surfaces. With emphasis on the related surface morphology, we investigated the skin of the pilot whale *Globicephala melas* using cryo-scanning electron microscopic techniques in combination with trypsin-digestion and dynamic contact angle measurements (Baum et al., 2000; Baum et al., unpublished results). Our findings have so far shown, that epidermal self-cleaning in this species is ensured by co-operating physical skin properties, based in particular on the macromolecular surface coating of the corneocytes. As demonstrated after mild freeze-drying under electron optical control, this coating forms a biogel of alternating hydrophilic and hydrophobic sectors. The biogel is embedded within a relief of nanoridges originating from the desmosomal junction system of the plasmalemma of the epidermal cells (Baum et al., 2000; Baum et al., unpublished results, see also Geraci et al., 1986). In dolphins, such nanostructures smooth the skin surface into an average nanorough plain surface exhibiting no particular microniches for settling organisms and adhering particles to hide within. Therefore, the removal of contaminants is facilitated during jumping of the dolphin, when organisms and particles are challenged to the turbulent water or the air–liquid interfaces of air-bubbles reaching the smooth surface (Baum et al., 2001, in preparation).

Following the above-reported physical skin properties, the present approach was designed to study the self-cleaning mechanisms with emphasis on the chemical nature of the delphinid epidermis. In this connection, our interest concentrated on the distribution of enzymes in the stratum corneum, that skin layer representing the actual interphase between the organism and its environment, and the primary site for contaminants. It is known that in fish, hydrolytic and glycolytic enzymes, in particular *N*-acetylhexosaminidases, in the external mucoid surface behave antibacterial and antifungal (e.g. Nakagawa et al., 1987; Alexander and Ingram, 1992). We performed experiments to detect possible enzyme activities in the epidermis of dolphins. In the aquatic environment, a lot of contaminants originate from the pool of glycoconjugates present in the water column. These glycoconjugates are released from algal species and — due to their adhesive properties — self-assemble freely and adhere on charged surfaces (Chin et al., 1998; Passow, 2000). Therefore, our analysis had to include polysaccharide-hydrolyzing enzymes also.

In addition, this study was directed to the comparison of enzyme activity of the stratum corneum of marine and terrestrial mammals, since the terrestrial ancestors of cetaceans adapted to the marine environment in several ways including, e.g. changes in keratosis (Spearman, 1972). In the skin of terrestrial mammals, lysosomal enzymes catalyze intracellular digestion of proteins, whereas cytoplasmatic enzymes metabolize proteins and glycoconjugates during keratinization, or activate pro-enzymes (Meyer and Neurand, 1976, 1977; Fräki and Hopsu-Havu, 1975; Fräki et al., 1983; Nemanic et al., 1983; Lundstrom and Egelrud, 1988; Brysk et al., 1994; Boderke et al., 1997; Selvanayagam and Lei, 1998; Watkinson, 1999; Egelrud, 2000; Pierad et al., 2000). Accord-

ing to the proven role of exo- and endo-peptidases and acid phosphatases in the strength of hydrolytic digestion of macromolecules in terrestrial mammals (Rutherford and Pawlowski, 1974; Mier and van den Hurk, 1975a,b,c; Meyer and Neurand, 1976, 1977; Fräki et al., 1983; Nemanic et al., 1983; Lundstrom and Egelrud, 1988; Suzuki et al., 1996) we were specifically interested in the presence of such enzymes in the dolphin, expecting some similarities between the non-hairy skin of dolphins and sparsely-haired skin-types in terrestrial mammals. For example, in elephants (Luck and Wright, 1964), hippopotamus (Spearman, 1970) or in domesticated mammals (Meyer, 1986) correlations have been reported between accumulated peptidases and acid phosphatases indicating a long-term activity of enzymes. In sparsely-haired skin-types the high protein content of the corneocytes coincides with intense keratinization, a process of stiffening the cells, since macromolecules are transformed into high molar mass aggregates instead of being degraded into monomers (Meyer, 1986).

2. Materials and methods

2.1. Sample preparation

Samples of stratum corneum squames of approximately 180 long-finned pilot whales (obtained legally from four grinds, see Bloch et al., 1993) *Globicephala melas* of both sexes and different ages were manually collected on the Faroe Islands in 1999 by means of rotating movements of a stainless steel brush. Care was taken to avoid portions of the underlaying stratum spinosum. Pooled samples of stratum corneum were stored in liquid nitrogen until use. The abraded deep-frozen flaky samples were taken out of their tubes, transferred into Eppendorf cups, where they warmed up to room temperature during centrifugation (Labofuge, Hettich) at 3000 U/min for 10 min. The Eppendorf cups were percolated with a small hole at the bottom and inserted in plastic tubes, which collected most of the skin material during centrifugation, but excluded the flaky stratum corneum not passing the hole. Analyses were performed from samples of the supernatant. We collected approximately 1-ml centrifugate per 50 g stratum corneum squames.

2.2. Enzyme assays

The centrifugate was diluted to a final protein concentration of 1 mg ml^{-1} with isotonic NaCl (0.9%) and sterile-filtered (Millipore, 0.2 μm). With respect to the storage of stratum corneum samples in liquid nitrogen for months and the sterile filtration procedure, we excluded enzyme activities of non-stratum-corneum origin in our measurements. Since micro-organisms have not been detected in histological slices of the stratum corneum (Fig. 2A–D) nor on the skin surface (Baum et al., 2000), we also excluded that extracellular microbial enzymes were measured in our assays. According to the manual, the test, if applied to detect microbial enzyme activities, needs an initial density of at least 10^6 cells ml^{-1}.

Samples were then pipetted into the cavities of diagnostic microplates (Merlin Diagnostika) filled with different chromogenic enzyme subtrates (MIRCONAUT-RPO, MIRCONAUT-REN and MIRCONAUT-RC). The reactions liberated *p*-nitrophenol or an azo-dye which was coupled to *p*-amino-zimtaldehyde following incubation. Absorbance of *p*-nitrophenol at 405 nm or the azo-dye at 585 nm was measured instantly after loading of the samples and 4 and 24 h later in a Multiwell-Reader Photometer (1420 Victor, Wallace).

2.3. NATIVE PAGE

The centrifugate was analyzed by NATIVE PAGE after Laemmli (1970) in the Mini-Protean-II (Biorad) at 200 V. A linear 8–18% gradient gel (30% T) was used in combination with a 4% stacking gel. Lanes were loaded with 20-μl undiluted samples (approx. 6 mg ml^{-1} protein, Bradfort) or samples diluted with equal volumes of sample buffer. The gels were calibrated with a mixture of three proteins of known molecular weight (Sigma). Subsequently, gels were blotted onto PVDF-membranes (Biorad) in the Mini-Protean-II-Blotmodule at 70 V for 70 min. Blot-membranes were divided into two pieces. One part was stained with brilliant blue, and the other part was incubated with the naphthylamide derivatives (1 mg ml^{-1}) of L-alanine and L-hydroxy-proline, the 5-brom-4-chlor-3-indolyl-derivatives of acetate, phosphate, *N*-acetyl-β-D-glucosamine and *N*-acetyl-β-D-galactosamine and ni-

troblue-tetrazolium for 10 min. All chemicals were purchased from Sigma.

In order to estimate the pH optima of the enzymes selected, we pre-incubated the blotmembranes with the substrates at two different pH values (pH 6.3 and pH 8). It was tested that within the pre-incubation time of 10 min, hydrolyzed substrates did not diffuse from the sites of enzyme activity. After coupling the hydrolyzed substrates with tetranitro tetrazolium (0.1 mg ml^{-1}) the colored violett tetrazolium salt indicated the sites of substrate hydrolyzation. All blot membranes were scanned, and the densitometric evaluation was performed with the program SigmaGel (Jandel Scientific).

2.4. Enzyme histochemistry

Two batches of fresh and intact skin samples were cut into blocks of approximately 0.5 cm^3. One of them was fixed in 4% neutrally buffered formalin for 2 h, and the blocks were then rinsed in PBS (phosphate buffered saline, pH 7.4). Both batches were stored in liquid nitrogen. Sections of 7–15-μm thickness were cut from the frozen blocks with a cryo-microtome (Bright 5030) at −18°C. The detection of enzymes was performed by the nitroblue-tetrazolium methods according to the procedures described in Stoward and Pearse (1992). In short, slices were incubated for 10–30 min with 3-bromo-5-chloro-indolyl-conjugates of galactose and β-*N*-acetylhexosamino sugars, phosphate and acetate and also with L-aminoacid derivatives of naphtylamide (Sigma). Substrates cleaved by the enzymes of the slice were visualized by coupling with tetranitro tetrazolium blue (Sigma). A third batch of fresh stratum corneum samples stored in liquid nitrogen was used to transfer impresses of the skin surface to blot membranes, which were analysed for enzyme activity (see NATIVE PAGE).

2.5. Size exclusion chromatography

Twenty microlitres of the centrifuged samples of stratum corneum diluted with isotonic NaCl (1:10) were injected into a BIOSIL-250 column (Biorad), and the UV signals (280 nm) of the eluted fractions were recorded with a spectrophotometer (SpectroMonitor D, Milton Roy). Flow rate of isotonic NaCl was 0.1 ml/min. Thereafter, the same fractions were measured online with the DAWN DSP multi angle laser light scattering detector (Wyatt Technology) to determine molar masses absolute, followed by the RI-detector Optilab 903 (Wyatt Technology). Scattering angles between 34.8 and 110.7° were selected for the extrapolation of scattering the angle to zero. Absolute molar masses were calculated using Berry equation $(K\,c/R)^{0.5}$ implemented in the Astra menue (Wyatt Technology). The data of the extrapolated light scattering signals were evaluated by measurements of concentrations using the Optilab 903 differential refractive index detector. Samples were injected every hour over a time span of 12 h.

2.6. Determination of molar mass

Light scattering is one of a few methods available for the determination of absolute molar mass (Wyatt, 1993).

Values for the molar mass M and the radius of gyration $(\langle R_G^2 \rangle^{1/2})$ at each slice across the distribution were calculated using the usual light scattering equations:

$$\frac{K \cdot c}{R_\vartheta} = \frac{1}{M_w \cdot P_{(\vartheta)}} + 2A_2 \cdot c \tag{1}$$

where

$$K = \frac{4\pi^2 n_0^2}{N_A \lambda_0^4} \cdot \left(\frac{dn}{dc}\right)^2 \tag{2}$$

$$R_\vartheta = \frac{r^2 \cdot I_\vartheta}{I_0 \cdot V} \tag{3}$$

$$P_{(\vartheta)}^{-1} = 1 + \frac{\langle R_G^2 \rangle_z \cdot q^2}{3} + \ldots \tag{4}$$

$$q = \frac{4\pi n_0}{\lambda_0} \cdot \sin\left(\frac{\vartheta}{2}\right) \tag{5}$$

This equation incorporates the light scattering constant for vertically polarized incident light K, which contains the refractive index increment dn/dc, the concentration c, the Rayleigh ratio R_ϑ at the scattering angle ϑ, the weight-average molar mass M_w and the second virial coefficient A_2. The Rayleigh ratio R_ϑ is used to obtain a parameter for the scattered light intensity that is

Table 1
Substrates are cleaved by the enzymes included in the centrifuged stratum corneum of the pilot whale (*Globicephala melas*)

Substrate class (activity of)	Substrates	pH range
L-aminoacids and peptides (peptidases)	*Alanine*, arginine, aspartate, glutamine, glycine, glycylproline, glycyltryptophane, histidine, *hydroxyproline*, leucine, lysine, phenylalanine proline, tripeptide, tryptamine, tyrosine, valine	8
Glycosides and aminosugars (glycosidases)	*N*-acetyl-β-D-galactose, *N-acetyl*-β-D-*galactosamine*, *N*-acetyl-β-D-glucosamine α-glucose, β-glucose, β-*galactose*	6
Esters (esterases)	Diphosphoesters, 2-desoxythymidine-5′-phosphate, *Acetate*	8
Phospholipids (lipases)	Phospholipids	

Underlined substrates were selected for NATIVE-PAGE and histochemistry.

independent of the measuring conditions. The function P_ϑ describes the angular dependency of the scattered light intensity. For small angles it is only dependent upon the radius of gyration and not on factors such as molecular conformation or branching. Irrespective of the fractionation method, one must assume that in a fractionation experiment on polymers with a unique relationship between molar mass and radius of gyration and elution volume, the distribution within a given element of the elution volume is so negligibly small that the weight-average molar mass and the *z*-average radius of gyration become equivalent to the values of a monodisperse sample.

3. Results

3.1. Microwell plate enzyme assays and NATIVE PAGE

The sterile-filtered and diluted centrifugates of the stratum corneum samples incubated in the microwell plates with different chromogenic enzyme subtrates exhibited activities of various hydrolytic enzymes: peptidases; esterases; phosphatases; phosholipases; and glycosidases including *N*-acetyl-hexosaminidases (Table 1).

Of the enzyme classes detected in the microwell plates several (those underlined in Table 1) were selected for further tests using NATIVE-PAGE.

In lanes 1 and 2 of Fig. 1 original samples of stratum corneum were separated and visualized with brilliant blue. Several major bands of native proteins occurred in the molecular mass range of approximately 7–100 kD. In comparison, lane 3 shows the separation of proteins from the same blot incubated with the substrate for unspecific esterases. The developing reaction products are visible as one major band in the molecular mass range of 700–1000 kD. Remarkably, also all other enzymes selected were recovered in this comparatively high molar mass range (700–1000 kD), a finding indicative of enzyme aggregates. No bands were detected of non-aggregated active enzymes with lower molar masses. Using this method, non-active enzymes will not be stained. Neither prolonged incubations nor higher substrate concentrations resulted in additional bands.

Equal and high sample amounts were loaded to the three lanes (1, 2 and 3) in order to obtain sufficient material for enzyme assays. We suppose that the concentration of active enzymes in the centrifugate of each stratum corneum sample is not high enough for the recovery of non-aggregated molecules. Bands representing molar masses between approximately 100 and 1000 kD are most probably contained in lanes 1 and 2, however, are less or not visible because of the high background protein load.

Pre-incubation series at two different pH values (Table 1) used to indicate the pH dependency showed that at pH 6.3, *N*-acetyl-β-D-glucosamine, *N*-acetyl-β-D-galactosamine and phosphate were hydrolyzed most intensively, whereas at pH 8.0 (close to pH of seawater), derivatives of acetate, alanine and hydroxyproline were hydrolyzed more

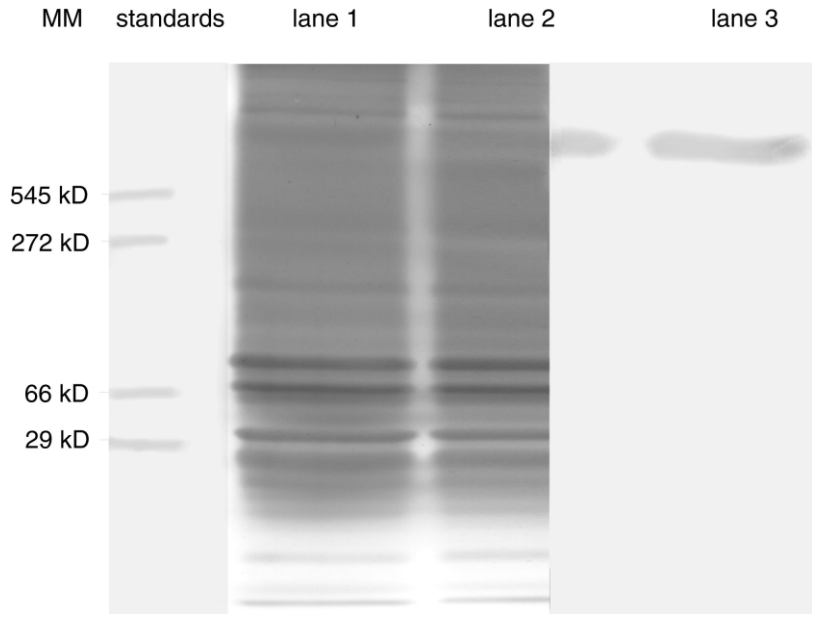

Fig. 1. NATIVE-PAGE of the stratum corneum centrifugate of the skin of the pilot whale *Globicephala melas* (lane 1). Unspecific esterase reactions occurred in the high molar mass segment ~ 700–1000 kD (lanes 2 and 3).

strongly compared to pH 6.3, the pH of the stratum corneum centrifugate.

Enzyme activity was recovered in impresses of the fresh frozen skin surfaces (data not shown).

3.2. Enzyme histochemistry

Slices of the epidermis (7–15 μm) of the pilot whale prepared for enzyme histochemistry showed the accumulation of enzymes within this skin part. The five enzymes selected for the histochemical demonstration also found in the crude mixture of exo-and endo-enzymes after centrifugation were identical to the enzymes previously selected for NATIVE PAGE (Table 2). Acid phosphatase, unspecific esterases, L-alanylaminoarylpeptidase, α-glycosidase and *N*-acetyl-hexosaminidase were present in the epidermis, particularly enriched in the transition zone (uppermost layer of the stratum spinosum and the stratum corneum) (Table 2). Only the alkaline phosphatase was found to be endothelial in the blood capillaries of the

Table 2
Distribution of enzymes of the formol-fixed skin of the pilot whale (*Glodicephala melas*)

Strata of the skin	Enzymes					
	ALP	ACP	UE	LAP	α-G	NAH
Epidermal strata						
Str. corneum	−	+++	++	+	+/++	+/++
Upper Str. spinosum	−	−	+	++/+++	++	++/+++
Lower Str. spinosum	−	−	++	+/++	+	+
Str. basale	−	++	+++	+/++	+	+
Dermal strata						
Str. papillare	+++ (endothelial)	++ (paraendothelial)	+ (diffuse)	−	−	−

Abbreviations: ALP, alkaline phosphatase; ACP, acid phosphatase; UE, unspecific esterase; LAP, L-alanylaminoarylpeptidase; α-glycosidase; NAH, *N*-acetyl-hexoaminidase; reaction: −, non; ++, moderate; and +++, strong.

dermal stratum papillare and not in the epidermis. In samples fixed in formalin prior to the cyro-conservation, visual inspection showed a decreasing gradient of enzyme concentration from the inner towards the superficial layers of the stratum corneum (Fig. 2A–C) with the reaction products being localized mostly extracellularly.

In contrast, in the non-fixed samples enzymes converted the substrates in all layers of the stratum corneum comparatively homogeneously (Fig. 2D). It is a certain disadvantage of non-fixed samples that the deposits of the dye could not be differentiated as clearly at particular sites as was possible in fixed samples. On the other hand, fixation may influence the enzyme activities and thus the amounts of detectable products.

Fig. 2A shows the distribution of *N*-acetyl-hexosaminidases in the uppermost stratum spinosum and in the stratum corneum of the formalin-fixed epidermis. Prominent sites of activity were found in the transition zone. In the stratum corneum deposits of the dye were recovered along the intercellular space. Such a distribution was also found in formalin-fixed samples stained for the aminoaryl peptidases (see Fig. 2B, slice incubated with L-alanyl-naphtylamide) and α-glycosidase (Fig. 2C). Fig. 2D shows the presence of acid phophatases in the stratum corneum of the non-fixed skin.

3.3. Size exclusion chromatography

Using SEC we separated approximately seven major molar mass fractions (using detector of refractive index) of the stratum corneum centrifugate. Fig. 3 shows the ultraviolet signal at 280 nm for proteins (dash-dotted line) and the refractive index signal as a mass indicator (solid line). Comparing the UV with the RI signals, shows that the protein concentration (UV) of the stratum corneum centrifugate is approximately 1.5 orders of magnitude lower than that of the total mass of components detected by the RI signal (Fig. 3) except in the low molecular mass range eluting at approximately 12 ml. In this molar mass range (4–6 kD), the UV signals are not corresponding with protein content (the signal is higher than the mass of the fraction detected by RI), but indicate autofluorescence or additional absorption phenomena. Since the stratum corneum centrifugate contains substances with different extinction coefficients, we used the RI signal only to determine the absolute molar masses (see Section 2).

Calculated from the intensity of the scattered light, the higher absolute molar masses of the fractions separated (Fig. 3, circles) were eluted at the beginning of the experiment (5–6-ml elution volume, Fig. 3), whereas within increasing elution volume the molar masses decreased. It is noteworthy that the samples of stratum corneum contained remarkably high molar masses exceeding 10^7 g mol^{-1} and also molar masses below 10^3 g mol^{-1}. Comparing the data of Figs. 1 and 3, the aggregated unspecific esterases shown in lane 3 of Fig. 1 would be expected to elute at approximately 6-ml elution volume (Fig. 3). Furthermore, as a measure of the molecular volume, the root mean radii moments analyzed from the light scattering data (not shown) displayed no decrease with decreasing molar masses, a finding suggesting that during size exclusion chromatography, larger aggregates of the centrifuged stratum corneum material continuously eluted in small amounts. We therefore calculated the absolute molar masses from the top of the peaks, i.e. at a position where the concentration and the corresponding molar mass of the bypassing aggregates are of minor importance compared to the eluted fraction.

Injecting aliquots of the same sample in hourly intervals, different stages of polymerization were observed as strong changes in the RI signals (Fig. 4) suggesting a shift towards increased molar masses. Within 1 h, the peak with a molar mass of approximately 20–30 kD eluting at 10.1 ml (t_0) started to decrease — compare dotted with dashed-dotted line (1 h) — until reaching the background level (solid line, 8.5 h) (Fig. 4). Within the same period, the peaks eluting between 7.5 and 8.5 ml (molar mass of approx. 50–70 kD) exhibited increased concentrations. This is also true for the peak at 6 ml with molar masses ranging from 500 to more than 1000 kD. The peak at 7.0 ml decreased to background level after 8.5 h and a new peak at 7.3 ml (90–120 kD) arose from the background. The increased molar masses approximately observed correspond to multiples of the decreased molar masses eluted at 10.1 ml.

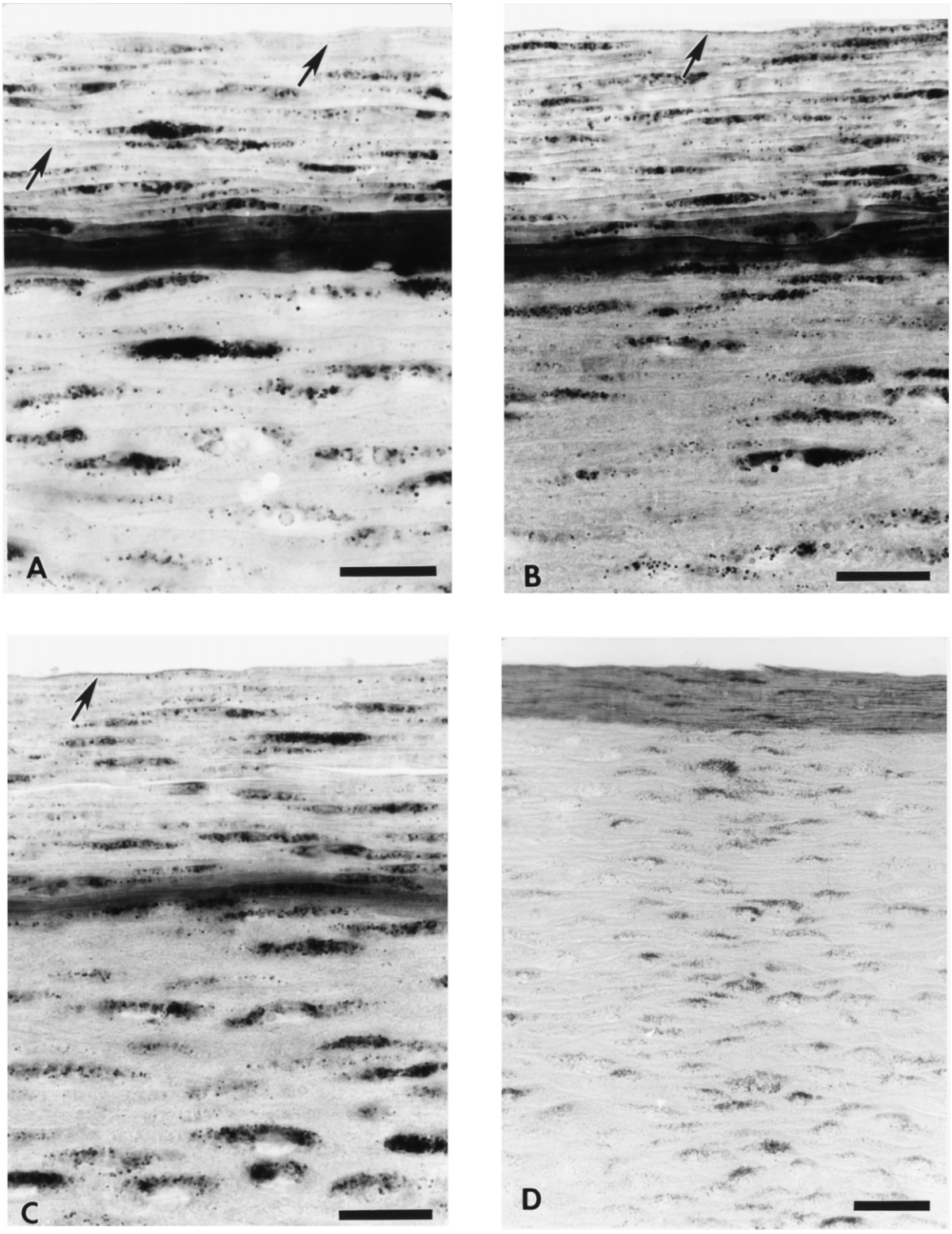

Fig. 2. Histochemically detected *N*-acetyl-glucosaminidase (A), glycosidase (B), alanyl-aminoarylpeptidase (C) and acid phosphatase (D). (A–C) Enzyme activity was enriched in the transition zone between stratum spinosum and stratum corneum (dark area) and intercellular space (arrows). Horizontal bar 20 μm (A–C) and 100 μm (D). (A–C) Formalin-fixed sample, (D) non-fixed sample.

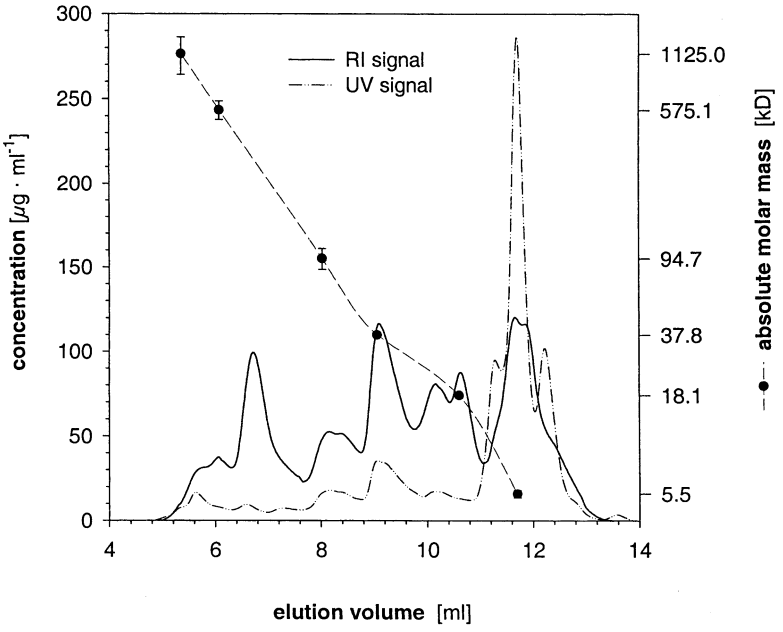

Fig. 3. Size exclusion chromatography of the centrifuged stratum corneum of the pilot whale.

4. Discussion

4.1. Microwell plate enzyme assays and NATIVE PAGE

Using sterile-filtration of the centrifuged samples, we excluded the presence of micro-organisms and could thus employ screening assays designed for microbiobal testing. With the aid of diagnostic microplates, various enzymes were demonstrated in the stratum corneum of *Globicephala melas* for the first time in marine mammals. The broad

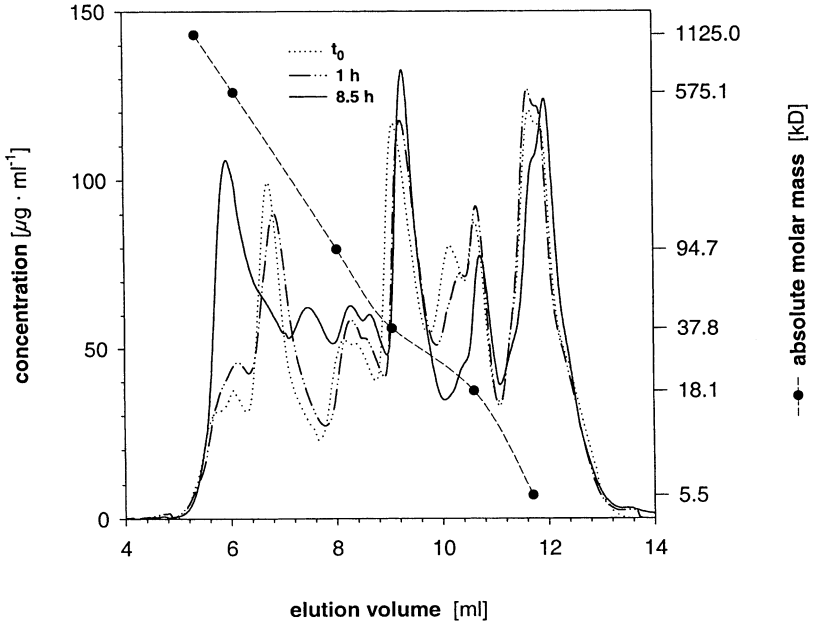

Fig. 4. Size exclusion chromatography of the centrifuged stratum corneum of the pilot whale. Comparison of RI signals at 0, 1 and 8.5 h after sample centrifugation.

spectrum of hydrolyzing enzymes (peptidases, esterases, phosphatases, phosho-lipases and glycosidases including *N*-acetyl-hexosaminidases) emphasizes active cellular metabolism in this skin layer. This finding is confirmed by the histochemical demonstration of hydrolytic enzymes particularly enriched in the transition zone between stratum spinosum and stratum corneum (see below). Particularly, the presence of peptidases together with acid phosphatase is similiar to the skin of sparsely-haired mammals, which still show the metabolically active cells in the stratum corneum conjunctum (Meyer, 1986). In this connection, it has been shown by Meyer (1986) that the presence of acid phosphatase in the stratum corneum conjunctum of terrestrial mammals is indicative of slow and weak degradation of intercellular connections and cellular membranes remaining in the degradative process as macromolecular aggregates rather than monomers. Correspondingly, this enzyme detected in the skin of the pilot whale is considered as a marker of the slow aggregate formation.

Employing radiolabeling experiments in dolphins, Brown et al. (1983) and Hicks et al. (1985) found long periods of migration of epidermal cells from the basal layer to the superfical layer of the stratum corneum extending from approximately 60 to 72 days. These results confirm, from our above-mentioned conclusion derived from enzyme analysis, that the degradation of the stratum corneum (as the final cell migration step) is a time-consuming process. As calculated by Brown et al. (1983) and Hicks et al. (1985), renewal of the superficial epidermal cell layer in the investigated species exhibiting approximately 10–12 cell layers of the stratum corneum was approximately 12 times a day indicating that the complete stratum corneum would be renewed daily. Our experiments indicate a slower renewal rate.

Remarkably, all enzymes selected for tests using NATIVE-PAGE developed reaction products visible as one major band in the molecular mass range of 700–1000 kD. These comparatively high molar masses are indicative of formation of aggregates including enzymes within the centrifuged samples. Here, we report that activity remains in the enzymes even when these are components of aggregates. Such aggregates may form a microenvironment inhibiting the enzymes to digest cellular macromolecules including other enzymes and thus, perhaps slowing down the degradative process. This seems to be the means of transporting the hydrolytic activity to the superficial cell layer. Here, hydrolysis is needed to counteract the establishment of a primary organic film representing the first step in the process of biofouling (Baum et al., 2000). The transitory attachment of the hydrolytic enzymes to aggregates may protect the intact cellular metabolism during cell migration to the skin surface.

With respect to the slow degradation of macromolecules and membranes, further investigations are planned to study the relation between the enzyme-containing aggregates and lipids (Menon et al., 1986). While the aggregates are zymogel forming hydrophilic compartments, the lipids represent strictly hydrophobic elements. In the corneocytes of cetaceans, intracellular lipids are rarely enclosed in membranes, but mostly stored in droplets and also lamellar bodies (containing membrane-enclosed lipids) are not extensively found in the intercellular space (Elias et al., 1987; Pfeiffer and Jones, 1993). It was observed by Baum et al. (2000) that the skin surface of the pilot whale exhibits alternating hydrophilic and hydrophobic sectors located within a relief of nanoridges which are considered as the most prominent skin characteristics. These sectors should originate from the above-described cellular separation into a hydrophilic and a hydrophobic phase.

The hydrolytic enzymes can be separated into two major pH optimum ranges (approx. 6 and 8). With regard to the pH of the seawater (buffered, approx. 8), the esterases and petidases (Table 1) are active at the skin surface exposed to this medium. The glycosidases are highly active at a pH ~ 6. Adhering microorganisms and polysaccharides will decrease the ambient sea water pH down to 6–7 due to the neutral or acidic surface charges of their chemical groups around their surface (Dagostino et al., 1991; Vandevivere and Kirchman, 1993; Beveridge et al., 1997). We thus conclude, that the dolphin skin has developed a mode of defense against microorganisms including their acidic excretion products and polysaccharides by taking advantage of the environment for a high activity of hydrolytic enzymes created by the biofoulers in the pilot whale skin. Comparable reports have been published on the enzymatic defense against bacteria and fungi in fish mucus (Nakagawa et al., 1987; Alexander and Ingram, 1992).

4.2. Enzyme histochemistry

Acid phosphatase, unspecific esterases, L-alanylaminoarylpeptidase, α-glycosidase and *n*-acetyl-hexosaminidase were present in the epidermis. It is noteworthy that they were enriched extracellularly in the uppermost layer of the stratum spinosum and the stratum corneum. In terrestrial mammals, two of these enzymes, α-glycosidase and *N*-acetyl-hexosaminidase, were cytochemically demonstrated in lysosomes, laminar bodies and in the cytoplasm of stratum corneum cells (Igbal and Gerson, 1971; Mier and van den Hurk, 1975b, 1976). In the pilot whale both enzymes were accumulated preferentially in the extracellular space, a proper localization of the enzyme containing gel playing a role in the self-cleaning process.

4.3. Size exclusion chromatography

Using size exclusion chromatography we found, that the protein content of the centrifugate was 1 order of magnitude lower than the total mass detected by RI. This finding shows that the stratum corneum centrifugate also contains high amounts of non-proteinaceous substances, among them, glycoconjugates and lipids (Menon et al., 1986). We also detected in the molar mass region of approximately 4–6 kD molecules with high UV absorption potentially represented by orange-red pheomelanins, water-soluble polymerized derivatives of cysteine and tyrosin. Fractions separated displayed a broad molar mass range exceeding 10^7 g mol^{-1} and also molar masses below 10^3 g mol^{-1}. When injecting aliquots of the same sample in hourly intervals, different stages of aggregation were observed as strong changes in the RI signals. A shift towards increased molar masses corresponds approximately to multiples of the decreased molar masses eluting at 10.1 ml (20–30 kD). We anticipated this aggregation from the presence of the jelly hydrophilic sectors covering the skin surface of the pilot whale (Baum et al., 2000).

5. Resumé

In respect to the results of rheological experiments (Baum et al., 2001), the stratum corneum centrifugate shows gel properties characteristic of covalently cross-linked gels. The gel includes lipidic microcavities with high energy dissipation performance, which indicate phase separation (Baum et al., 2001). In addition, as obvious from the contact angle measurements, the gelled stratum corneum centrifugate exhibits both hydrophilic and hydrophobic properties interpreted as amphiphilic in nature (Baum et al., 2001, unpublished), thus linking with each other the hydrophobic and the hydrophilic sectors seen at the skin surface of the pilot whale (Baum et al., 2000). Since the skin surface is smoothed into an average nanorough plain surface by this gel coating (Baum et al., 2000; Baum et al., in preparation), biofoulers and adhering particles are more easily challenged to high shear and air–liquid interfaces of air-bubbles during jumping due to the lack of micro-niches and hiding places.

In this study, we found aggregate-attached enzymes, forming a zymogel, which play the crucial role in reducing the attachment of adhesives and biofouling organisms chemically. This chemical mode of defense in co-operation with the above-reported physical properties is considered effective against biofouling of the dolphin skin.

Acknowledgements

We thank Dr D. Bloch, Museum of Natural History, Faroe Islands, and Mrs J. Zachariassen, Mrs M. Mortensen and Dr H.P. Joensen, University of the Faroe Islands, for their help in specimen collection from legal harvest and for kindly providing the laboratory facilities on the Faroe Islands. We also gratefully acknowledge the gift of the microplates from Dr M. Molitor, Merlin Gesellschaft für mikrobiologische Diagnostika mbH, Germany, and Dr D. Schlote, Chemical Institute of the School of Verterinary Medicine Hannover, Germany, for providing the multiwell photometer. This study was supported by a grant of the Deutsche Forschungsgemeinschaft (ME 1755/1–1).

References

Alexander, J.B., Ingram, G.A., 1992. Non-cellular non-specific defense mechanisms of fish. Annu. Rev. Fish. Dis. 2, 249–279.

Baum, C., Stelzer, R., Meyer, W., Siebers, D., Fleischer, L.-G., 2000. A cryo-scanning electron microscopy study of the skin surface of the pilot whale *Globicephala melas*. Aqua. Mammals 26, 7–16.

Baum, C., Fleischer, L.G., Meyer, W., Siebers, D., Stelzer, R., 2001. Gelation kinetics of a gel collected from the corneocytes of the pilot whale (*Globicephala melas*). Zoology 104 (4), 53.

Bloch, D., Desportes, G., Mouritsen, R., Skaaning, S., Stefansson, E., 1993. An introduction to studies of the ecology and status of the long-finned pilot whale (*Globicephala melas*) off the Faroe Islands, 1986–1988. Rep. Int. Whal. Commn. 14, 1–32. (Special Issue)

Beveridge, T.J., Makin, S.A., Kadurugamuva, J.L., Li, Z., 1997. Interactions between biofilm and the environment. FEMS Microbiol. Rev. 20, 291–303.

Boderke, P., Merkle, H.P., Cullander, C., Ponec, M., Bodde, H.E., 1997. Localization of aminopeptidase activity in freshly excised human skin: direct visualization by confocal laser scanning microscopy. J. Invest. Dermatol. 108, 83–86.

Brown, W.R., Geraci, J.R., Hicks, B.D., St. Aubin, D.J., Schroeder, J.P., 1983. Epidermal cell proliferation in the bottlenose dolphin *Tursiops truncatus*. Can. J. Zool. 61, 1587–1590.

Brysk, M.M., Bell, T., Brysk, H., Selvanayagam, P., Rajaraman, S., 1994. Enzymatic activity of desquamin. Exp. Cell Res. 214, 22–26.

Chin, W.-C., Orellana, M.V., Verdugo, P., 1998. Spontaneous assembly of marine dissolved organic matter into polymer gels. Nature 391, 568–572.

Dagostino, L., Goodman, A.E., Marshall, K.C., 1991. Physiological responses induced in bacteria adhering to surfaces. Biofouling 4, 113–119.

Elias, P.M., Menon, G.K., Grayson, S., Brown, B.E., Rehfeld, S.J., 1987. Avian sebokeratinocytes and marine mammals lipokeratinocytes: structural, lipid biochemical, and functional considerations. Am. J. Anat. 180, 161–177.

Egelrud, T., 2000. Desquamation in the stratum corneum. Acta Dermatol. Venereol. Suppl. 208, 44–45.

Fräki, J.E., Hopsu-Havu, V.K., 1975. Human skin proteases. Arch. Dermatol. Res. 253, 261–276.

Fräki, J.E., Lazarus, G.S., Hopsu-Havu, V.K., 1983. Protein catabolism in the skin. In: Goldsmith, L.A. (Ed.), Biochemistry and Physiology of the Skin, Chapt. 15, 1. Oxford University Press, New York, pp. 338–362.

Geraci, J.R., St. Aubin, D.J., Hicks, B.D., 1986. The epidermis of odontocetes: a view from within. In: Bryden, M.M., Harrrison, R. (Eds.), Research on Dolphins, Part 1. Clarendon Press, Oxford, pp. 3–21.

Gucinski, H., 1986. Correlation of biophysical surface characteristics with hydrodynamic properties of adhesive biofilms. State University of New York at Buffalo, dissertation.

Gucinski, H., Baier, R.E., Meyer, A.E., Fornalik, M.S., King, R.W., 1984. Surface microlayer properties affecting drag phenomena in seawater. In: Proceedings of the 6th Marine Corrosion Congress, Athens, Greece, pp. 1–24.

Hicks, D.B., St. Aubin, D.J., Geraci, J.R., Brown, W.R., 1985. Epidermal growth in the bottlenose dolphin, *Tursiops truncatus*. J. Invest. Dermatol. 85, 60–63.

Igbal, M., Gerson, S., 1971. Biochemical features of oral epithelium. In: Squier, C.A., Meyer, J. (Eds.), Current Concepts of the Histology of Oral Mucosa. Springfield, Thomas, pp. 129–142.

Laemmli, U.K., 1970. Cleavage of structural proteins during assembly of the head of the bacteriophage T4. Nature 227, 680–685.

Luck, G.P., Wright, P.G., 1964. Aspects of the anatomy and physiology of the skin of hippopotamus (*H. amphibius*). Quart. J. Exp. Physiol. 49, 1–14.

Lundstrom, A., Egelrud, T., 1988. Cell shedding from human plantar skin in vitro: evidence of 1st dependence on endogenous proteolysis. J. Invest. Dermatol. 91, 340–343.

Menon, G.K., Grayson, S., Brown, B.E., Elias, P.M., 1986. Lipokeratinocytes of the epidermis of a cetacean (*Phocoena phocoena*). Cell Tissue Res. 244, 385–394.

Meyer, W., 1986. Die Haut des Schweines (The Porcine Integument). Schlütersche Verlagsanstalt, Hannover.

Meyer, W., Neurand, K., 1976. Distribution of enzymes in the skin of the domestic pig. Lab. Anim. 10, 237–247.

Meyer, W., Neurand, K., 1977. The distribution of enzymes in the epidermis of the domestic cat. Arch. Dermatol. Res. 260, 29–38.

Mier, P.D., van den Hurk, J.J.M.A., 1975a. Lysosomal hydrolases of the epidermis. 2. Ester hydrolases. Br. J. Dermatol. 92, 391—398.

Mier, P.D., van den Hurk, J.J.M.A., 1975b. Lysosomal hydrolases of the epidermis. 1. Glycosidases. Br. J. Dermatol. 93, 1–10.

Mier, P.D., van den Hurk, J.J.M.A., 1975c. Lysosomal hydrolases of the epidermis. 3. Peptide hydrolases. Br. J. Dermatol. 93, 509–517.

Mier, P.D., van den Hurk, J.J.M.A., 1976. Lysosomal hydrolases of the epidermis. 4. Overall profile in comparison with dermis and other tissues. Br. J. Dermatol. 94, 443–446.

Nakagawa, H., Enomoto, N., Asakawa, M., 1987. Occurence of glycosidases and enzymatic properties of *N*-acetylhexoaminidases in the external mucous material of various fishes. Nippon Suisan Gakkaishi Bull. Jpn. Soc. Sci. Fish. 53, 1039–1054.

Nemanic, M.K., Whitehead, J.S., Elias, P.M., 1983. Alterations in membrane sugars during epidermal

differentiation: visualization with lectins and role of glycosidases. J. Histochem. Cytochem. 31, 887–897.
Passow, U., 2000. Formation of transparent exopolymer particles, TEP, from dissolved precursor material. Mar. Ecol. Progr. Ser. 192, 1–11.
Pfeiffer, C.J., Jones, F.M., 1993. Epidermal lipid in several cetacean species: ultrastructural observation. Anat. Embryol. 188, 209–218.
Pierad, G.E., Goffin, V., Hermanns-Le, T., Piera-Franchimont, C., 2000. Corneocyte desquamation (review). Int. J. Mol. Med. 6, 217–221.
Rutherford, T., Pawlowski, A., 1974. Acid phosphatase staining of the stratum corneum as a marker of damage by low irritable compounds. Br. J. Dermatol. 91, 503–506.
Selvanayagam, P., Lei, G., 1998. Desquamin is an epidermal ribonuclease. J. Cell. Biochem. 68, 74–82.
Spearman, R.I.C., 1970. The epidermis and its keratinization in the African elephant (*Loxodonta africana*). Zool. Afri. 5, 327–338.
Spearman, R.I.C., 1972. The epidermal stratum corneum of the whale. J. Anat. 113, 373–381.
Smith, T.G., St. Aubin, D.J., Hammill, M.O., 1992. Rubbing behavior of belugas, *Delphinapterus leucas*, in a high Arctic estuary. Can. J. Zool. 70, 2405–2409.
St. Aubin, D.J., Smith, T.G., Geraci, J.R., 1990. Seasonal epidermal molt in beluga whales, *Delphinapterus leucas*. Can. J. Zool. 68, 359–367.
Stoward, P.J., Pearse, A.G.E., 1992. Histochemistry: Theoretical and Applied, vol 3: Enzyme Histochemistry. Churchill Livingstone, Edinburgh, London.
Suzuki, Y., Nomura, J., Koyama, J., Horii, I., 1996. The role of proteases in stratum corneum: involvement in stratum corneum desquamation. Arch. Dermatol. Res. 286, 249–253.
Vandevivere, P., Kirchman, D.L., 1993. Attachment stimulates exopolysaccharides synthesis by a bacterium. Appl. Environ. Microbiol. 59, 3280–3286.
Watkinson, A., 1999. Stratum corneum thiol protease (SCTP): a novel cysteine protease of late epidermal differentiation. Arch. Dermatol. Res. 291, 260–268.
Wyatt, P.J., 1993. Lightscattering and the absolute characterization of macromolecules. Analyt. Chim. Acta 272, 1–40.