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Proteolytic enzyme conjugated to SC-glucan as an enzymatic transdermal drug penetration enhancer

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The objective of this study was to investigate the effect of papain, a proteolytic enzyme, on the percutaneous absorption of drugs. To guarantee the enzyme stability during the skin penetration, papain was modified by the conjugation to SC-glucan. The enhancing activity of drug penetration was evaluated using antipyrine and indomethacin as hydrophilic and hydrophobic model drugs, respectively. The SC-glucan-papain conjugate was found to be very effective for facilitating the percutaneous absorption of antipyrine. Microscopic observations showed that the thickness of stratum corneum and viable epidermis was increased by the treatment of the SC-glucan-papain conjugate. Moreover, it induced phase separation, lacuna formation, and lamellar disruption within the stratum corneum interstices. These structural changes by the SC-glucan-papain conjugate are likely to be induced from hydrolysis of extensive crosslinking of corneocyte envelopes and intracellular proteins. However, the SC-glucan-papain conjugate showed no skin irritation according to the Draize test, which may be due to the difficulty of the SC-glucan-papain conjugate in penetrating into the skin.

1. Introduction

Transdermal drug delivery has attracted considerable interest due to many advantages, such as avoidance of first-pass gastrointestinal and hepatic metabolism of drugs and drug efficacy enhancement resulting from controlled delivery for long periods without frequent dosing. However, drugs administered percutaneously have to pass through the stratum corneum, which is the outermost skin layer efficiently restricting molecular transport between the external environment and the interior of the mammalian body. Generally, the organization of the stratum corneum is described as 'bricks and mortar' with the bricks representing the corneocytes and the mortar representing the intercellular lipid [1]. According to the model, diffusion of the drug is possible through two domains: a protein-rich region corresponding to the corneocytes and a lipid-rich region providing the mortar [2–6]. The transcellular pathway refers to the passage through hydrophilic and discontinuous protein-rich domains of the stratum corneum. The intercellular pathway consists of penetration through the continuous intercellular lipid domain of the stratum corneum. It has been suggested that transdermal drug penetration can be increased by modifying polar, non-polar, and a combination of the both physicochemical pathways [7–8].

To this purpose, a large number of chemical enhancers have been investigated for increasing transdermal penetration of drugs. For example, fatty acids, surfactants, and solvents can act as a penetration enhancers by altering the structure of the lipid layers in the stratum corneum [9–13]. However, these small molecular weight enhancers are likely to be absorbed in the skin, often resulting in unfavorable skin irritations [14–16].

In order to avoid these side effects, polymeric penetration enhancers hardly absorbed through the skin have been suggested as an alternative [17, 18]. In this sense, proteolytic enzymes are also expected to be less irritant compared with other permeation enhancers having a low molecular weight, because they are too large to permeate the viable epidermis and their action is highly specific toward proteins. However, the main drawback in employing proteases as penetration enhancers is their intrinsic instability in formulations. When they are exposed to oils or surfactants, often abundant in dermatological formulations including cosmetics, their conformation is susceptible to be disrupted. Thus, it is necessary to improve the stability of the enzyme for practical uses. In our previous study, the stability of papain, a proteolytic enzyme, in a cosmetic formulation was shown to be dramatically improved by conjugating the enzyme to SC-glucan, a high molecular weight polysaccharide produced by *Schizophyllum commune* [19].

In this study, the SC-glucan-papain conjugate (see Fig. 1) was prepared and examined as a transdermal drug penetration enhancer. Stratum corneum proteins are well known

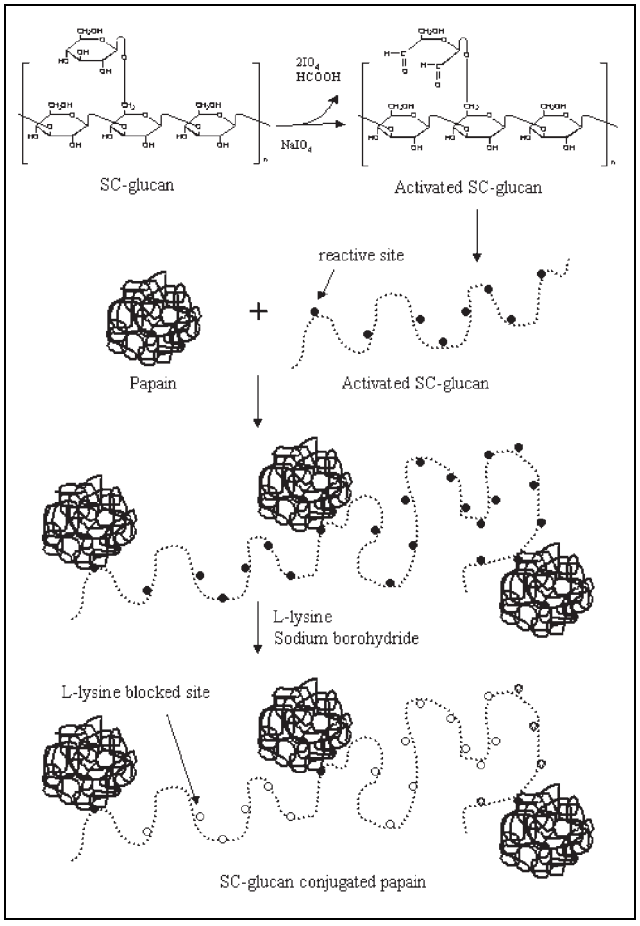
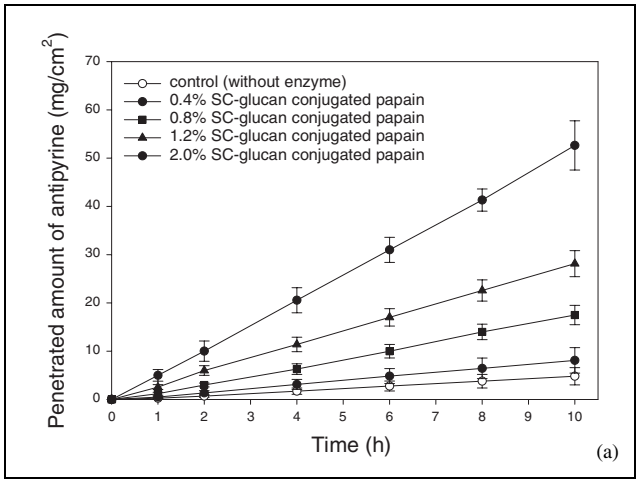


Fig. 1: Preparation of SC-glucan conjugated papain



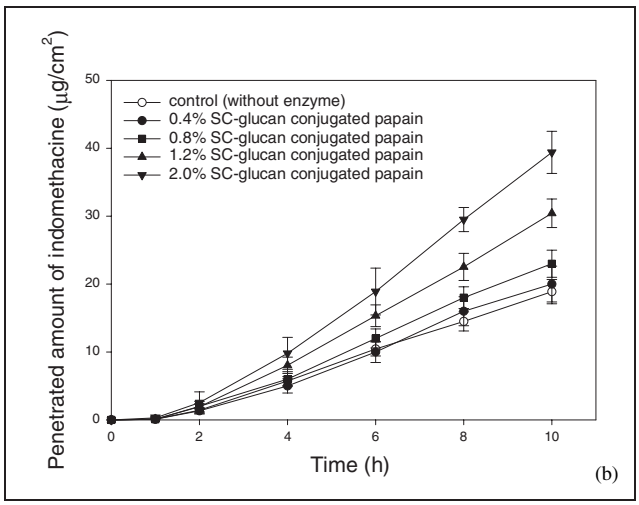


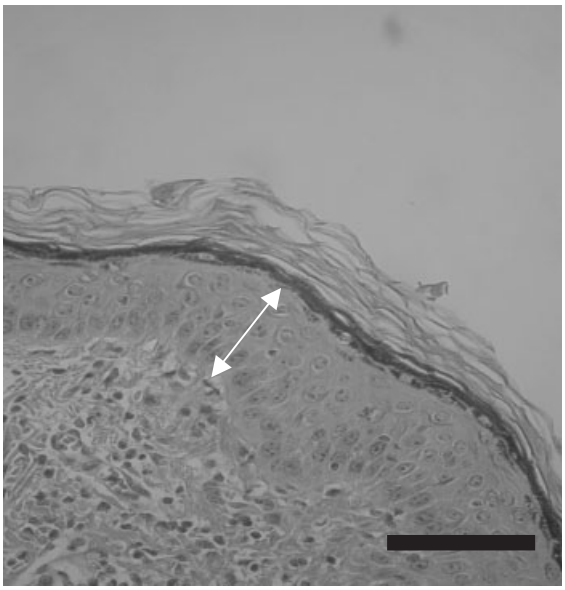
Fig. 2: Cumulative amount of antipyrine (a) and indomethacin (b) penetrated through hairless guinea pig skin with and without the SC-glucan-papain conjugate pre-treatment. Each point represents the mean ± standard deviation value of at least five experiments

for their insolubility, which is owing to their extensive crosslinking of both corneocyte envelopes and intracellular proteins [5, 6]. Conformational changes of the stratum corneum proteins and concomitant reduction of the crosslinking density have been expected to enhance drug diffusivity through the stratum corneum by opening up aqueous channels for drug transport [6, 8]. To the best of our knowledge, this is the first scientific report investigating a proteolytic enzyme as a transdermal penetration enhancer. The enhancing properties of drug penetration were evaluated using a Franz-type diffusion cell, and the structural changes of skin induced by the SC-glucan-papain conjugate were observed using a transmission electron microscopy. In addition, the Draize test was carried out in order to examine whether the SC-glucan-papain conjugate induces the primary skin irritation or not.

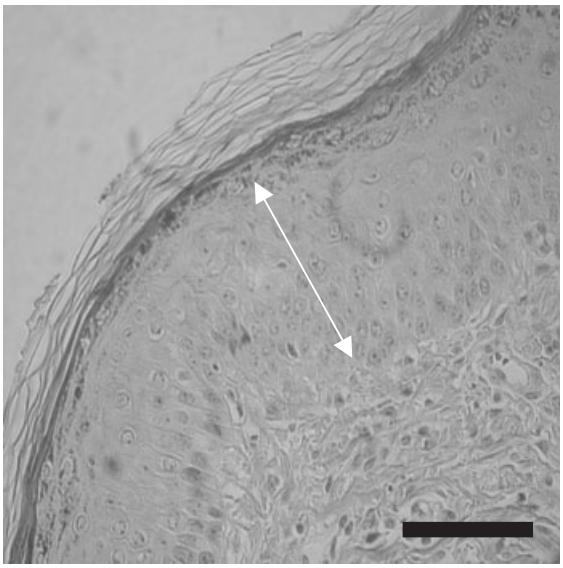
2. Investigations, results and discussion

The cumulative penetration profiles of antipyrine (a) and indomethacin (b) through the abdominal skin of hairless guinea pig are presented in Fig. 2. The transdermal penetration of antipyrine was clearly accelerated by the 1 h pre-treatment of the SC-glucan-papain conjugate. The cumulative amount of antipyrine during 10 h penetration increased about eleven-fold compared of control, when the skin was pre-treated with 2.0% of the SC-glucan-papain conjugate (Fig. 2a). On the other hand, the SC-glucan-papain conjugate was much less effective for enhancing the transdermal penetration of indomethacin (Fig. 2b). The result shows that enhancement of drug penetration by SC-glucan-papain conjugate dramatically differs with the choice of the drug. This result can be clearly represented by the permeability coefficients for antipyrine and indomethacin calculated as $P = dQ/C_v Adt$ from Eq. (1) [20]. The values of the permeability coefficient with different concentrations of the SC-glucan-papain conjugate were determined from the slope of the steady state of the penetration profiles of the drugs in Fig. 3. The permeability coefficient of antipyrine augmented by about 10.8 fold and that of indomethacin by about 2.0 fold with the 1 h pretreatment of 2.0% (w/v) solution of the SC-glucan-papain conjugate, as summarized in Table 1.

In vivo histological alterations in the SC-glucan-papain conjugate-treated skin were examined using optical microscopic observations with hematoxylin and eosin (H&E) staining, as shown in Fig. 3. A normal pattern of the stratum corneum was also noted as compared with the buffer-treated control. The viable epidermal layer of hairless guinea pig was thickened when treated by the SC-glucan-papain conjugate. It is well known that the epidermal thickening can result from a disruption of the barrier function of the stratum corneum [21–23]. However, this does not mean that the SC-glucan-papain conjugate have to penetrate the skin. Considering the high molecular weight of the SC-glucan-papain conjugate (M_v is more than ca. 2×10^6), it is much more reasonable to speculate that external stresses on the stratum corneum affect the proliferation and/or differentiation of epidermal cells. Many studies



(a)



(b)

Fig. 3: Light microscopy of a vertically sectioned hairless guinea pig skin tissue following hematoxylin and eosin (H&E) staining. Dorsal skin of a live hairless guinea pig was treated at every 24 h for 48 h without (a) and with (b) SC-glucan conjugated papain. Scale bar = 100 μm

demonstrate that the acceleration of cell proliferation and the concomitant thickening of epidermis could be induced if only the upper stratum corneum is damaged. Barthel et al. [23] showed that a mild mechanical irritation (tape-stripping) just affecting the stratum corneum has a major stimulatory effect on the cell kinetics of proliferative keratinocytes in the basal layer of the epidermis, indicating the existence of a powerful regulatory mechanism. Hatta et al. [22] also reported that epidermal cell proliferation was significantly increased in the normal human skin after tape stripping. Furthermore, stratum corneum tryptic and chymotryptic enzymes (SCTE and SCCE) in normal epidermis are involved in the desquamation process [24–25], which means that they could accelerate the desquamation process and potentially weaken the barrier function of stratum corneum. Therefore, the thickening of the stratum corneum and viable epidermis can be explained by the conformational changes of stratum corneum proteins, which were induced by hydrolytic activity of the SC-glucan-papain conjugate, rather than the penetration of the SC-glucan-papain conjugate.

Table 1: Permeability coefficients and enhancement factors for antipyrine and indomethacin

Drug	C[a]	P[b] ($cm \cdot h^{-1}$)	E[c]
Antipyrine	0%	2.45×10^{-3}	–
	0.4%	4.15×10^{-3}	1.69
	0.8%	8.90×10^{-3}	3.65
	1.2%	1.41×10^{-2}	5.86
	2.0%	2.64×10^{-2}	10.95
Indomethacin	0%	1.09×10^{-4}	–
	0.4%	1.21×10^{-4}	1.06
	0.8%	1.35×10^{-4}	1.22
	1.2%	1.79×10^{-4}	1.61
	2.0%	2.31×10^{-4}	2.09

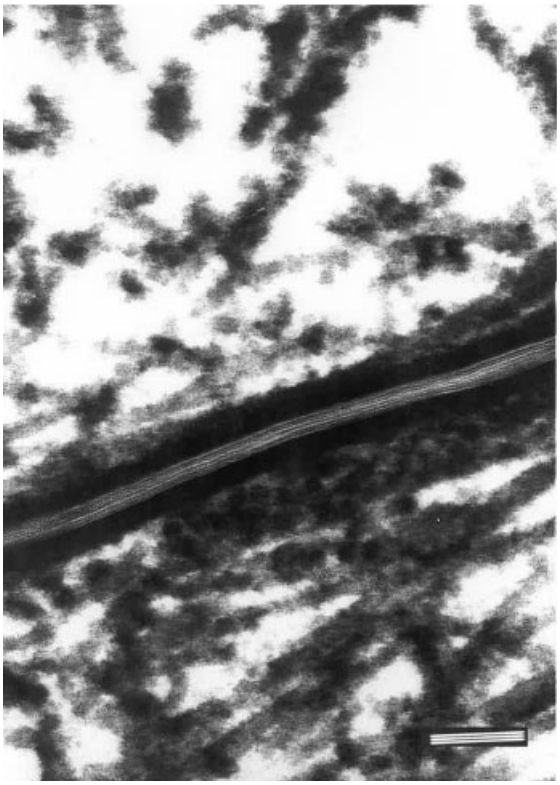
[a] Concentration of the SC-glucan-papain conjugate pre-treated
[b] Permeability coefficient, which was calculated graphically from Fig. 2.
[c] Enhancement factor, which was evaluated as the ratio of the penetrated drug quantity for 10 h with the pre-treatment of the SC-glucan-papain conjugate to that without the pre-treatment.

To gain further insight into the mechanisms by which the SC-glucan-papain conjugate affects transdermal drug permeability, hairless guinea pig skin was treated by the SC-glucan-papain conjugate and structural changes of the stratum corneum were observed by electron microscopy. Ruthenium tetroxide (RuO_4) stains stratum corneum lipids and delineates the intercellular domains and lipid lamellar structure. Fig. 4a shows that the intercellular lamellar membrane structure appeared normal in the skin without the treatment of SC-glucan conjugated papain (control). On the other hand, slight separation of intercellular lamella (arrows, Fig. 4b) and lacunar dilatation (asterisk, Fig. 4c) were observed, when the skin was treated with the SC-glucan-papain conjugate. The SC-glucan-papain conjugate seems to induce phase separation, lacuna formation, and lamellar disruption within the stratum corneum interstices. These phenomena were also observed in hairless mouse skin by co-applications of iontophoresis and oleic acid [26]. It was suggested that phase separation of intercellular lamellar and dilation of lacuna formation could be the important processes responsible for the enhanced penetration of hydrophilic drugs through skin. It should be also noted that water transport across the stratum corneum follows a lipid environment, and that the rate of transport is intimately correlated with the organization of the stratum corneum lipids, as clearly demonstrated by Potts and Francoeur [27]. Thus, it can be inferred that the SC-glucan-papain conjugate induces structural changes of the intercellular domains in the stratum corneum, and, in turn, these structural changes might be responsible for the enhanced transdermal penetration of hydrophilic drugs. This hypothesis means that hydrophilic drugs might mainly diffuse across the stratum corneum via an intercellular lipoidal pathway [28], although the relevance of these observations for the quantitative contribu-

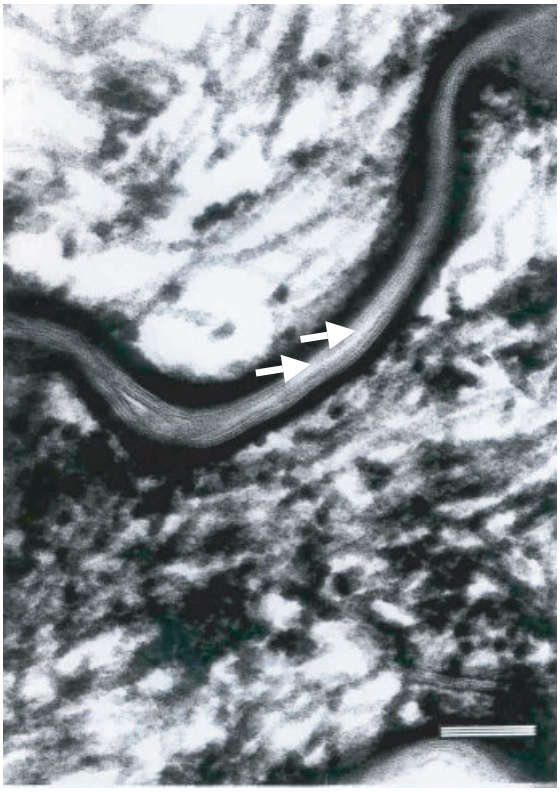
Table 2: Primary skin irritation on rabbit dorsal skin after treating with 2% (w/v) of the SC-glucan-papain conjugate dissolved in 0.5 ml of water for 4 h

Type of response	Time after removal of dressing				PII*
	1 h	24 h	48 h	72 h	
Erythema	0	0	0	0	0
Edema	0	0	0	0	

* PII: primary irritation index



(a)



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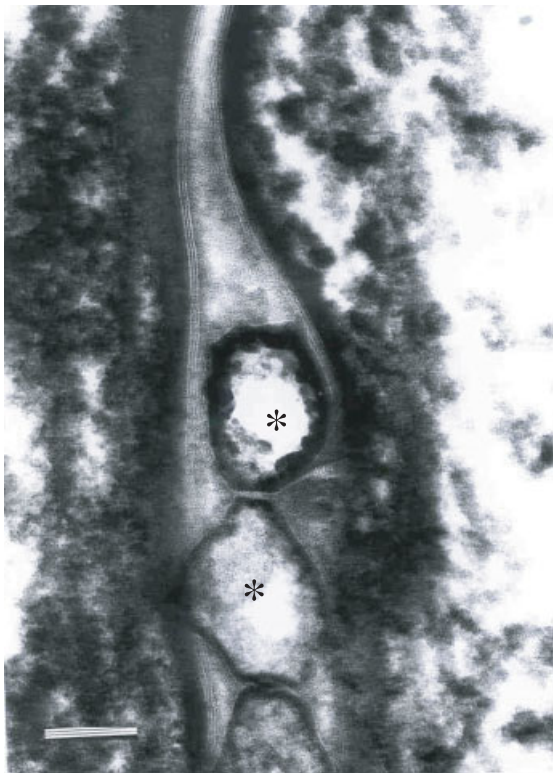

(c)

Fig. 4: Electron micrographs showing ultrastructures of the stratum corneum in hairless guinea pig dorsal skin: control skin (a) and the SC-glucan-papain conjugate-treated skin (b and c). Scale bar = 100 nm

tion to the enhanced skin penetration of hydrophilic drugs still remains to be further investigated.

The Draize test was carried out on the SC-glucan-papain conjugate in order to estimate skin irritation, as shown in Table 2. The primary irritation index was zero. This indicated that the SC-glucan-papain conjugate obviously did not irritate the skin. Moreover, as reported previously, no sign of skin irritation was observed after a month topical application of a cosmetic lotion containing 1.0% of the SC-glucan-papain conjugate, while only 1.0% lactic acid caused clearly visible signs of skin irritation after 5-day application [19]. It would be due to the difficulty of the SC-glucan-papain conjugate in penetrating into the skin owing to its large molecular weight.

In conclusion, topical applications of the SC-glucan-papain conjugate, a stabilized proteolytic enzyme, were investigated for enhancement of skin penetration of drugs. It has been demonstrated that the SC-glucan-papain conjugate could significantly enhance the transdermal penetration of antipyrine (a hydrophilic model drug). This enhancing activity of the SC-glucan-papain conjugate was attributed to the structural changes of the stratum corneum, as a result of conformational changes of stratum corneum proteins. Moreover, the SC-glucan-papain conjugate induced phase separation, lacuna formation, and lamellar disruption within the stratum corneum interstices. This implies that the hydrophilic drug transport can be stimulated by the structural alternations of the intercellular space by the SC-glucan-papain conjugate. Now, we expect that the SC-glucan-papain conjugate can be potentially utilized as a transdermal enhancer to promote the therapeutic efficacy of drugs owing to its safety and high enhancing property. Further investigations based on the SC-glucan-papain conjugate are in progress to address the fundamental aspects of skin penetration enhancing mechanisms.

Table 3: Physicochemical properties of antipyrine and indomethacin

	Antipyrine	Indomethacin
Molecular weight (g/mol)	188.23	357.80
Water solubility (mg/l)	$5.19 \cdot 10^4$	0.937
Log P (octanol-water)	0.38	4.27

3. Experimental

3.1. Chemicals

SC-Glucan, a soluble β-1,3-glucan with β-1,-branches produced by *Schizophyllum commune* (thus named SC), having a M_v of ca. 2×10^6, was supplied as a gift from Amore Pacific corp. (Seoul, Korea). Papain (EC 3.4.22.2, molecular weight 23,000 Da) was obtained from Jain Irrigation systems Ltd. (India). Obtained papain was dialyzed against deionized distilled water to eliminate impurities, and then freshly freeze-dried for use in this study. Fluorescein isothiocyanate (FITC, isomer I), antipyrine, and indomethacin were purchased from Sigma, and sodium periodate and sodium borohydride from Aldrich. All other reagents were of analytical grade.

3.2. Coupling reaction of papain to SC-glucan

SC-glucan conjugated papain was prepared by direct coupling reaction of papain to SC-glucan, as described previously [19]. SC-glucan was dissolved in deionized distilled water at a concentration of 0.4% (w/v) and then reacted with 10 mM sodium periodate under vigorous agitation for 1 h at room temperature [29]. The oxidized SC-glucan solution was dialyzed against excess 50 mM phosphate buffer (pH 6.5) overnight and then mixed with a papain solution (2%, w/v). The mixture was gently stirred for 4 h at room temperature and stored at 4 °C overnight. After the coupling reaction, 10 mM sodium borohydride and 5 mM L-lysine were added. The final solution of the SC-glucan-papain conjugate was obtained after washing and concentration by ultrafilteration with MW 100,000 cut-off membrane. The preparation procedure of the SC-glucan-papain conjugate is described in Fig. 1.

3.3. In vitro skin permeation study

In vitro skin permeation studies were conducted using a Franz-type diffusion cell (Model FCDS-1200C, Fine Scientific, Seoul, Korea). Female hairless guinea pigs (strain IAF/HA-hrBR, 8-week-old) were used as an animal model for human skin since they have a relatively thick epidermis and do not require shaving [21]. The abdominal skin was excised and then divided to mount on the diffusion cells (0.9 cm in diameter). The receptor compartment (5 ml) was filled with 50 mM phosphate buffered saline (PBS, pH 7.4, 40 mM NaCl). Before the drug penetration experiment, an aqueous solution containing the SC-glucan-papain conjugate in 50 mM PBS (pH 7.4, 40 mM NaCl and 30 mM cysteine) was put into the donor compartment and stirred for 1 h at 32 °C. Antipyrine and indomethacin were used as a model drug (Fig. 2). After removing the solution in the donor compartment, 0.2 ml of saturated drug solution (2%, w/v) in 50 mM PBS (pH 7.4, 40 mM NaCl) was loaded in the donor compartment and incubated at 32 °C. The amount of drugs permeated through the skin was analyzed at a predetermined time with a C_{18} reversed-phase column (Nova-Pak® C18, 3.9 × 150 mm, Waters) by HPLC composed of HP 1100 series (Hewlett-Packard). The flow rate of the eluents was 1.0 ml/min. For the determination of antipyrine, the mobile phase was the 75/25 (v/v) mixture of acetonitrile and deionized distilled water. For the analysis of indomethacin, the mobile phase was the 6/4 (v/v) mixture of acetonitrile/45 mM KH_2PO_4 aqueous solution adjusted at pH 3.0 with phosphoric acid. The eluate was monitored by UV absorption measurement at 254 nm for antipyrine and at 240 nm for indomethacin.

3.4. Data analysis

The penetration enhancing effect by the SC-glucan-papain conjugate can be expressed in terms of a permeability coefficient. The drug penetration rate dQ/dt at the steady state is described by Fick's law:

$$\frac{dQ}{dt} = \frac{KDC_vA}{L} = PC_vA \quad (1)$$

where K is a partition coefficient between the stratum corneum and the vehicle, C_v is the concentration of the drug dissolved in the donor phase, D is a diffusion coefficient, A is the effective area of the drug penetration, L is the thickness of the barrier, and P (= KD/L) is a permeability coefficient. The cumulative amount of drugs penetrated per unit skin surface area was plotted against time, and the slope of the linear portion of the plot was estimated as the steady-state flux (dQ/A dt). Statistical comparisons were made with a two-tailed Student's t test. The level of significance was taken as $p < 0.05$.

3.5. Electron microscopy

SC-glucan conjugated papain (2% w/v, 1 ml) in 50 mM PBS (pH 7.4) containing 30 mM cysteine was applied to the dorsal skin (1.5×1.5 cm^2) of a live hairless guinea pig. All other experimental conditions were the same with those of the CLSM analysis. At 6 h after the application, biopsies were taken and minced to < 0.05 mm^3, fixed in modified Karnovsky's fixative overnight, washed with 0.1 M cacodylate buffer, and then postfixed in 0.25% ruthenium tetroxide (RuO_4) in 0.1 M cacodylate buffer for 45 min in the dark at room temperature. After rinsing in a buffer solution, the samples were dehydrated in graded ethanol solutions and embedded in an Epon-epoxy mixture. Ultrathin 60–80 nm sections were viewed under a transmission electron microscope (TEM, H-500, Hitachi, Japan) after further contrasting with uranyl acetate and lead citrate.

3.6. Primary skin irritation

The primary skin irritation was evaluated by the Draize test using six New Zealand albino rabbit weighting 2.0–2.5 kg (Samtako Bio Korea). 0.5 ml of 2.0% (w/v) aqueous solution of the SC-glucan-papain conjugate was applied to intact and abraded skin sites for 4 h using an occlusive patch. The skin was examined for the presence of erythema (redness) and edema (swelling), and graded with a visual scoring system as follows: no visible reactions (0), mild erythema or endema (1), obvious erythema or edema (2), medium erythema or edema (3), and intense erythema or edema and mild incrustation (4). Primary irritation index (PII), a combined average value of the erythema values and the edema values at 1, 24, 48, and 72 h, was considered indicative of a positive irritant.

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