Quantitative Analysis of FITC-trypsin Distribution in Goatskin Matrix

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Abstract

The application of enzyme for leather making has attracted much attention in recent years. Therefore, the enzyme diffusion mechanism deserves to be investigated and it is helpful to enzyme application in leather industry. In this study, a novel method basing fluorescence detection technology was developed to achieve quantitative detection of trypsin distribution into goatskin matrix in the model of one-way and turbulent diffusion. In one-way diffusion, trypsin diffusion from the flesh side was faster than that from the grain side. As for turbulent diffusion assay, the trypsin diffusions in grain and flesh layer were directly influenced by the position of goatskin matrix such as back and belly, which could lead to different fluorescence intensity distribution. In addition, the modeling equations, which were fitted with fluorescence intensities, confirmed the quantitation feature in trypsin diffusion process. These results indicated that the method was competent for quantitative detection of enzyme spatial distribution in goatskin matrix. And it would provide the basis foundation for the development of researches in enzyme mass transfer kinetics.

Introduction

Enzyme, which is considered as high efficiency and eco-friendliness, has attracted much attention in preliminary steps of leather production including soaking,[1] unhairing,[2, 3] degreasing[4, 5] and especially bating.[6] The enzyme diffusion occurred in skin matrix has a directly influence on the application effect of enzyme. As known, skin is mainly composed by collagen fibers with triple-helix structure, thus it provides a biological matrix for enzyme action. According to research reported, the enzymatic reactions in leather processing is a mass transfer-controlled process.[7] Therefore, it is significant to investigate enzyme mass transfer to further understand the behavior of enzyme, which is helpful to the potential application of enzyme. However, few researches have been conducted involving the mass transfer behavior of enzyme in leather-making process due to lack of effective quantitative methods.

Fluorescence detection is an effective technique for detecting biomolecules, and it is currently widely used in gene profiling,[8] proteomics,[9] drug discovery,[10] disease diagnostics,[11] and protein monitoring.[12] It possess high spatial and temporal resolution for labeling and tracing proteins, which based on differentiate fluorescently labeled protein from unlabeled protein, as well as its inherent great sensitivity.[13] Fluorescein 5(6)-isothiocyanate (FITC) was capable of attaching the fluorescein chromophore to amino groups of proteins, and it could emit a visible yellow-green light after absorbing blue or ultraviolet light.[14] Thus the FITC could be considered as a feasible label material for fluorescence detection of enzyme against skin. Recently, how enzyme transported in skin matrix was studied originally using fluorescence tracer technology by Zeng.[15, 16] It plays a vital role on researches of enzyme mass transfer kinetic, and the visualized and quasi-quantitative analysis of enzyme transportation were performed. However, the quantitative analysis of the enzyme spatial distribution in skin was preliminary and needed to a more in-depth study.

Trypsin played an important role in leather production especially in bating process. In this study, it presented a novel method for the quantitative determination of the trypsin which distributed in skin matrix, and the mass-transfer process was observed and analyzed. By studying the mass transfer characteristics in one-way and turbulent diffusion, enzyme distribution in skin matrix was investigated. This research would provide an improved tool for monitoring of enzyme content not only in skin but also in other collagen materials.

Experimental

Materials

Fluorescein 5(6)-isothiocyanate, Sephadex G-25 and trypsin (270000 U/g, from bovine pancreas) were obtained from Sigma-Aldrich (Shanghai, China). Amicon Ultra-15 Centrifugal Filter Units (10 kDa, Millipore) was purchased from Merck Millipore Ltd. (Shanghai, China). Salted Hankou goatskins of commercial

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scale level (Approximately 1 kg for every hide) were donated by Shandong JunCheng Leather Co. Ltd. All the chemicals used for fluorescence detection were of analytical grade.

Pretreatment of Goatskin

Salted goatskins were soaked, fleshed and dehaired following Vedaraman's method. [17] And then, ammonium sulfate was titrated into the water to adjust the pH to 7.5 ± 0.5. The thickness of these delimed goatskin were approximately 2.5 mm thick and they were stored at -20°C for subsequent experiments.

Preparation of Fluorescent Trypsin

Fluorescent trypsin was prepared using a method of Wu, S *et al* [18] with slight modification. In brief, 2 g trypsin and 20 mg FTIC were dissolved in 40 mL and 10 mL NH_3HCO_3 buffer (0.025 M, pH 8.2 ± 0.5), respectively. Then the dissolved solutions were mixed in a 100 mL beaker and stirred at 4°C for 14 h in dark. The mixture was further concentrated using 10K Centrifugal Filter Units at 3000 g for 30 min for 2 times and purified with a Sephadex column. At last, the labelled trypsin (FITC-trypsin) was collected in eluate and stored in the dark at -20°C. Activity of purified FITC-trypsin used for further experiment was 76.3 U/mL measured by Lowry's method. [19] In order to further determine the linear relationship between FITC-trypsin and fluorescence intensity, the FITC-trypsin solution was stepwise-diluted to prepare standard curve in the range between 150 U/mL to 5 U/mL with mentioned sodium carbonate buffer. Then fluorescence intensity at the maximum emission wavelength of each emission spectra was plotted. In our study, the concentration of FITC-trypsin (76.3 U/mL) used in experiments was much higher than the traditional concentration of trypsin used in bating of skins. The purpose of the designed high concentration was to make the effective experimental phenomena more obvious.

Experimental Apparatuses

In tanning process, one-way mass transport of enzyme appeared in stationary condition for example enzymatic unhairing by painting,[20] and turbulent diffusion was usually occurred under mechanical forces such as soaking, bating, and unhairing by liming.[21] Therefore, two experimental apparatuses were designed for simulating enzyme transport with different pathway. Furthermore, the apparatus used to observe one-way diffusion pathway (ODP) was placed in a stationary environment, while another for turbulent diffusion pathway (TDP) was stirred to study non-stationary diffusion.

Sampling

In order to observe enzyme diffusion behavior, the skin matrix was treated with trypsin using ODP and TDP apparatus and samples in longitudinal sections were collected and imaged. For one-way diffusion pathway assay, the goatskin was sampled at 40 mm in diameter with four parallel samples and tested using ODP apparatus. After 15 min, the horizontal slices of two samples were imaged respectively. Fluorescence intensity in sodium carbonate buffer (0.5 M, pH 8.2 ± 0.5) was detected every 10 min and the total time was 7 h. In the meantime, to study the turbulent diffusion pathway, the procedure was performed in a tanning drum (GSD 450, Wuxi Xinda Light Industrial Machinery Co. Ltd., China) using the TDP apparatus (agitated at 6 rpm in 25 ± 2°C) which could provide intensive mechanical stirring. Owing to that different portion of the pelt had different interwoven fibrous structure especially on back and belly skin, which may lead to the difference of mass transfer characteristic. Thus, two pieces of test sample (30×70 mm) were cut off from the back and belly areas of goatskin pieces were observed. A 5×5 mm square was subsampled for slices at 5 min and 15 min from the two pieces of pelt respectively. Then the subsamples were sectioned horizontally into 25 μm slices on a freezing microtome (CM1950 type, Leica Company, Germany).

Fluorescence Microscopy

The collected samples were sectioned vertically at 25 μm by the freezing microtome (CM1950 type, Leica Company, Germany), the fluorescence intensity distribution was observed using a Zeiss Axio Scope A1 fluorescence microscopy (Carl Zeiss, Germany). Filters were visualized under blue light for FITC fluorescence (excitation filter: 420-485 nm), and images were recorded with a CCD camera.

Quantitative Determination

For quantitative analysis, the slices sectioned horizontally from the subsamples were collected continuously every 3 layers and the fluorescence intensity was recorded with a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies, America) at 25 ± 2°C. The photomultiplier tube (PMT) voltage was 600 V with excitation and emission slits at 5 nm. Slices spectra were recorded with an excitation wavelength of 495 nm as well as emission wavelength of 525 nm. The non-dimensional concentration of enzyme was represented by fluorescence intensity.

Design of Formula for quantitative analysis

The skin matrix can be regarded as a series of equal-thickness layers from surface to central position. In the case of enzyme mass transport, every layer would provide resistance to the penetration of enzyme molecules. Therefore, the enzyme concentration would exhibit a decrease tendency with enzyme molecule transporting in skin matrix. When assuming the transfer resistances of different layer were same, the enzyme concentration could be modelled as exponential distribution from the surface towards the center. The distribution curves of enzyme concentration were fitted by ExpDecay1 regression using ORIGIN software. The constant corresponding to transport resistance was denoted κ. The highest enzyme concentration appeared at the surface layer of skin matrix and it was denoted. Besides, the lowest enzyme concentration in skin matrix was directly influence by time thereby it was denoted.

Furthermore, the diffusion distance was denoted which staring from surface. Based on these set value, the formula using for quantitative analysis was obtained as below, and represented the enzyme concentration at diffusion distance.

$$c = c_t + c_0 e^{-\frac{l}{\kappa}} \quad (1)$$

Results and Discussion

Experimental Apparatuses

In order to investigate the pathway of enzyme penetration, the apparatus had been designed to study one-way and turbulent diffusion. To one-way diffusion analysis, the designed apparatus was shown in Figure 1(a). The goatskin sample of 40 mm diameter should be firmly fixed between two silicone barrels (30 mm outer diameter, 20 mm inner diameter and 60 mm height). The barrel above was filled with FITC-trypsin solution (76.3 U/mL) and the barrel below was filled with 0.5 M sodium carbonate buffer (pH 8.2 ± 0.5). In the meantime, the fluorescent trypsin solution was added into the barrel above. By this apparatus, the trypsin could penetrate through the goatskin from only one side under stationary condition. Thereby, the one-way diffusion was successfully proceeded. The fluorescence intensity detection would be performed by extraction of the buffer in bottom using a microsyringe. On the other hand, the designed apparatus (Figure 1(b)) used for turbulent diffusion process mainly composed by a plastic bottle (60 mm diameter, 85 mm height) and a glass rod. After 30×70 mm goatskin sample was put into the bottle, fluorescent trypsin (76.3 U/mL) was added by 1.2 liquid ratio and the bottle was stirred by leather-producing machines to provide mechanical action at 6 revolutions per minute at 25°C. Thus, the goatskin was treated under non-stationary condition while trypsin would uniformly dispersed in all sides of goatskin.

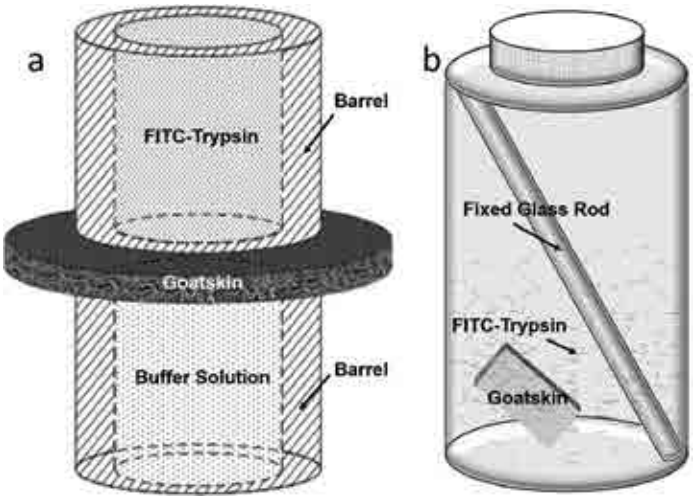

Figure 1. Designed apparatus for enzyme diffusion evaluation (a) in one-way diffusion and (b) turbulent diffusion.

Observation of Distribution of FITC-Trypsin

In fluorescence microscopy analysis, a similar diffusion behavior was observed to the existing results. [16] From Figure 2, the diffusion phenomenon of FITC-trypsin in skin can be summarized as the process that the enzyme concentration gradually decays from the skin surface towards the inside.

Quantitative determination

From Figure 3(a), it was seen that the optimum emission of fluorescent trypsin was at 525 nm, which should be selected for further analysis because the wavelength light has better penetration through biological tissues and weaker autofluorescence.[22] Furthermore, a steadily maximum emission wavelength and a good linear relationship (R^2=0.99) between fluorescent trypsin concentration and fluorescence intensity was shown in Figure 3(b). Fluorescent trypsin concentration could be calculated according to the linear equation thereby the quantitative determination of trypsin in goatskin could be carried out easily and effectively.

One-way Diffusion Analysis

The dermis of the goatskin could be further divided into papillary and reticular dermis, and they were named as grain layer and flesh layer in tannery, respectively. It was known that the fibers composing of grain layer were thin and loose, while the fibers in flesh layer possessed greater density. [3] Besides, proteoglycan decorin mainly existed in grain layer, and a certain amount of elastic proteins were contained in flesh layer.[24] Owing to these differences, the one-way diffusion, which the enzyme penetrated from the grain surface to flesh surface or reverse process, had a significance to be investigated. The fluorescence intensity in different depths of goatskin were observed at 15 min in Figure 4(a). Due to the flesh surface had a denser fiber-structure, it resulted slower diffusion of enzyme. Therefore, the

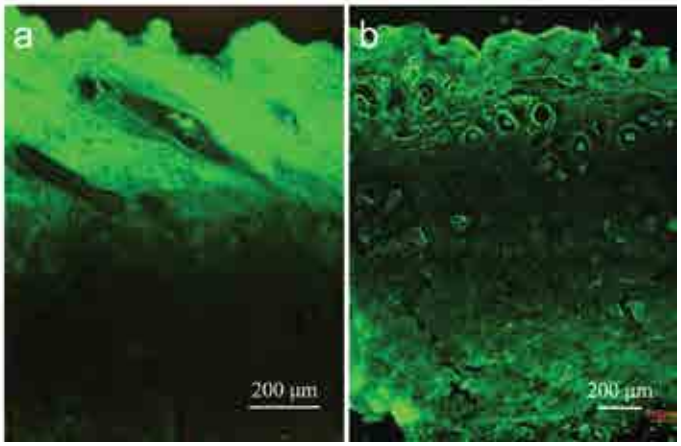

Figure 2. Fluorescence microscopy observation of goatskin treated with FITC-trypsin. (a) one-way diffusion, (b) turbulent diffusion.

fluorescence intensity was higher when the trypsin penetrated from the flesh layer. Meantime, with the increase of penetration time, the fluorescence intensity of buffer which the trypsin passed from grain layer was greater than that from flesh layer as shown in Figure 4(b). It was explained that trypsin had a stronger reaction with the interstitial protein in flesh layer than that of grain layer.

After the trypsin concentration at different portions of goatskin in Figure 4(a) were fitted with equation (1), the formulas of one-way diffusion process could be obtained as below:

$$c = 50 + 1000e^{-\frac{l}{259}} \quad (2)$$

$$c = 50 + 1000e^{-\frac{l}{330}} \quad (3)$$

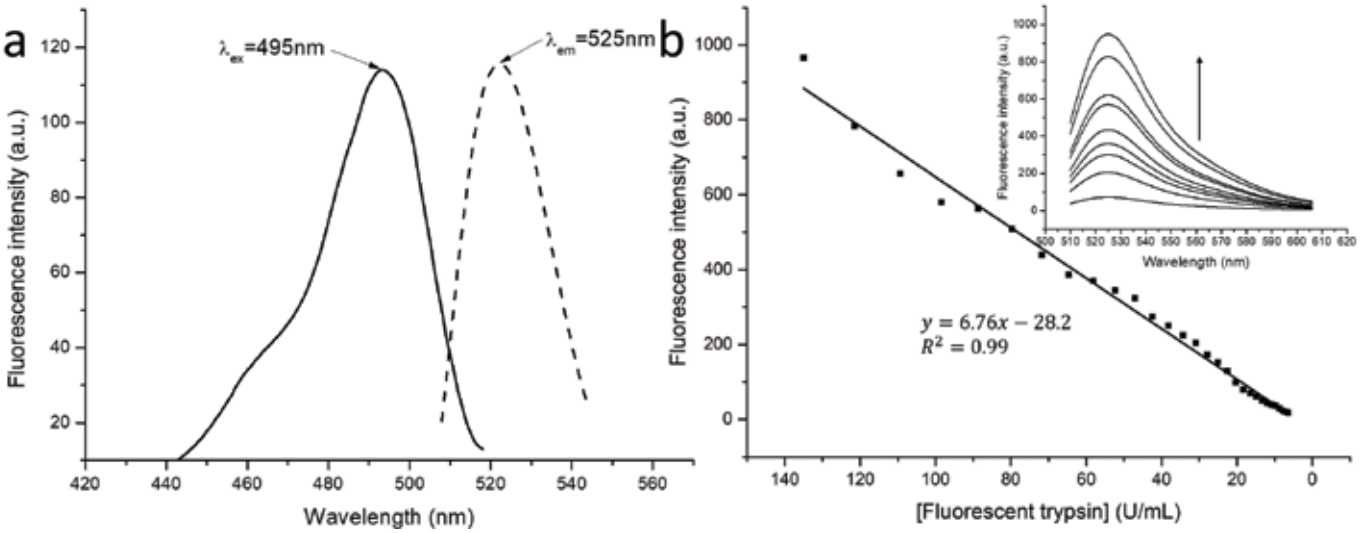

Figure 3. (a) The selection of wavelength used for analyzing fluorescence intensity; (b) The standard curve of [fluorescent trypsin] and fluorescence intensity. The inset showed the fluorescence emission spectra of fluorescent trypsin diluted in different multiples. The arrows indicate the signal changes with increasing in [fluorescent trypsin].

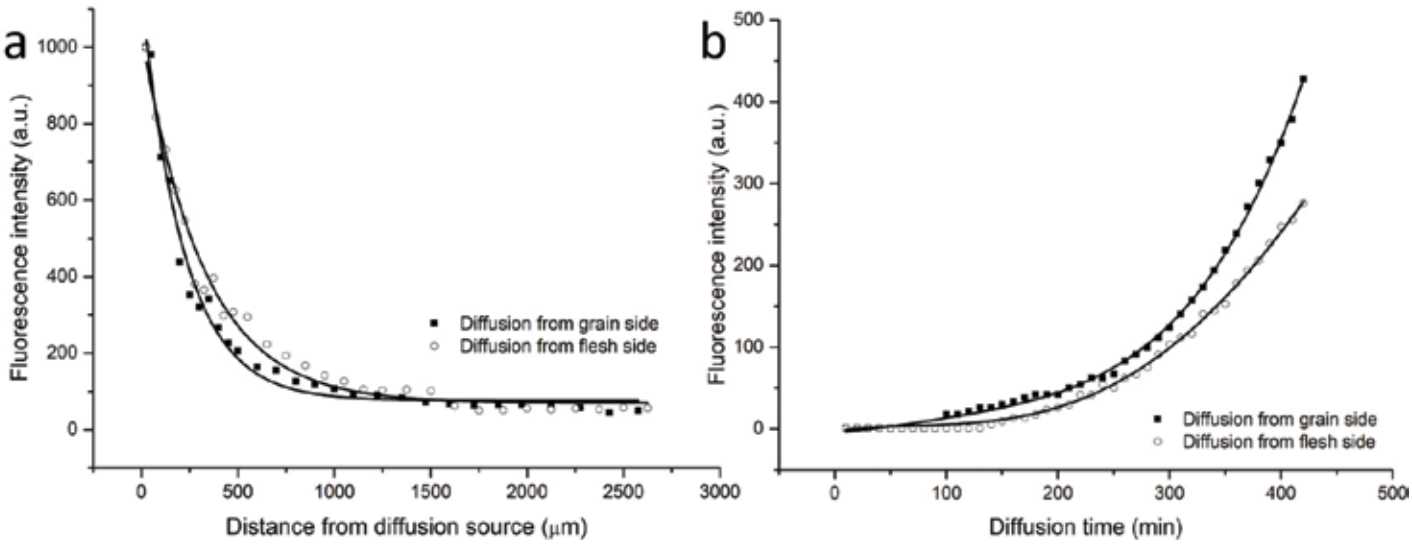

Figure 4. (a) Distribution of FITC-trypsin in goatskin matrix processed for 15 min. (b) Dependence of fluorescence intensity of buffer solution on diffusion time.

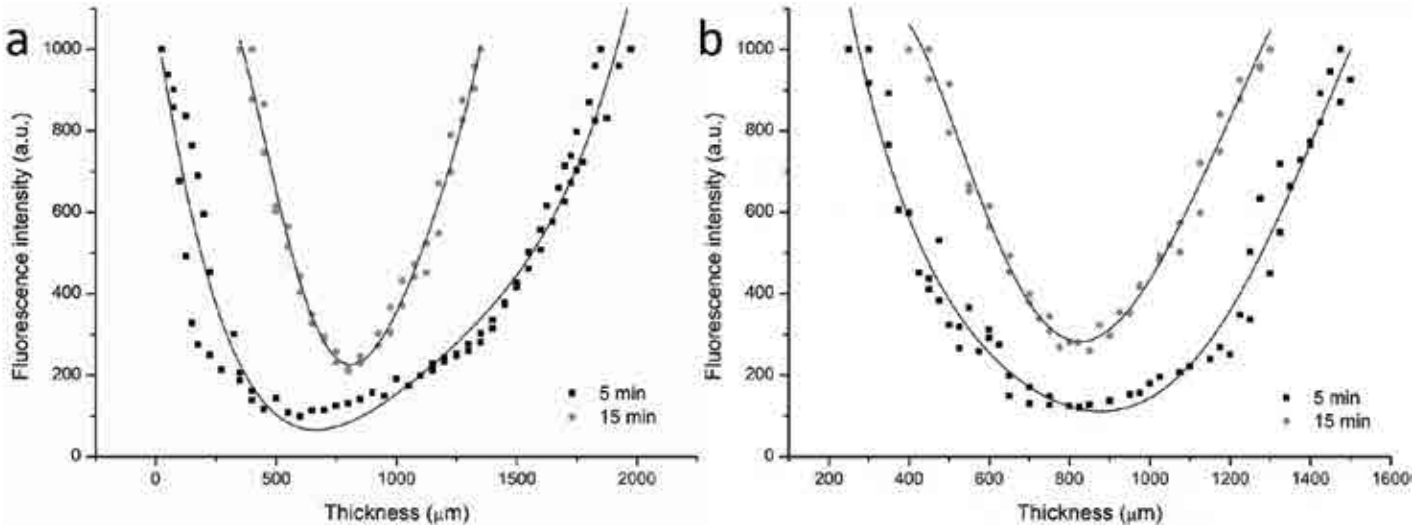

Figure 5. Fluorescence intensity in different portions of goatskin with turbulent diffusion. (a) Back area; (b) Belly area.

Equation (2) and equation (3) represented that enzyme was transported from grain surface and flesh surface respectively. After fitting, the κ constant in equation (2) was 259 while it was 330 in equation (3). It indicated the mass transfer resistance was stronger in grain surface the flesh surface, which could be explained that the grain layer had a denser fiber-structure.

Turbulent Diffusion Analysis

As mentioned previously, the structure and composition of grain layer and flesh layer were different. It resulted the skin-portion differences such as back area and belly area. The back area consisted of a larger-scale flesh layer structure, which endowed the back area thicker, denser and stronger properties than belly area of goatskin.[25, 26] In turbulent diffusion pathway, the trypsin permeated into goatskin from all sides simultaneously. Thus, the diversity of goatskin portion could affect the penetration, and the back and belly areas were selected as the representative samples to be investigated.

In order to determine the trypsin concentrations in goatskin with turbulent diffusion, samples were obtained from grain surface to flesh surface at different thickness with three parallel measurements. From Figure 5, it was found that symmetry of fluorescence intensity of back area was more significant, which was decided by tighter collagen-fiber bundles and higher percentage of flesh layer in back area. Besides, with the increasing of time from 5 min to 15 min, the fluorescence intensity of back area and belly area increased. Meantime, fluorescence intensity of trypsin would be more similar at the same time when the position of sample was closer to the center of the sample of different goatskin portions. Therefore, the central sample was more suitable for quantitative determination of turbulent diffusion.

In turbulent diffusion, the enzyme penetration was performed from both grain side and flesh side. From Figure 5, it was found that the lowest fluorescence intensity did not appeared in the central of skin matrix. Therefore, an offset of the X axis was introduced to the independent variable in equation (1) and equation (4) was shown as below.

$$c = c_t + c_0 e^{-\frac{l-b}{\kappa}} \quad (4)$$

Afterwards, the fluorescence intensity in Figure 5 were fitted with equation (4). Equation (5) and equation (7) represented trypsin was transported in grain layer at 5 min and 15 min respectively, while the trypsin dispersed in flesh layer at 5 min and 15 min were fitted and equation (6) and equation (8) were obtained.

$$c = 100 + 1000e^{-\frac{l-19}{164}} \quad (5)$$

$$c = 100 + 1000e^{-\frac{l-204}{492}} \quad (6)$$

$$c = 215 + 1000e^{-\frac{l-320}{182}} \quad (7)$$

$$c = 215 + 1000e^{-\frac{l-331}{238}} \quad (8)$$

It could be seen from the parameter in equation (5) was calculated as 19. It was lower than that in equation (6), which indicated enzyme diffusion distance in grain layer was higher than that in flesh layer at 5 min. This result was attributed to denser collagen-fiber bundles and higher percentage of flesh layer in back area. Meantime, similar offsets were noted in equation (7) and equation (8). It suggested trypsin diffusion distances were similar in grain and flesh layer at 15 min. Besides,

the fitting κ constant at 15 min in equation (6) and (8) was both higher than that in equation (5) and equation (7). This suggested that enzyme diffusion in flesh layer was faster than that in grain layer. Based on the results above, in order to quantify the precise content of trypsin in skin matrix by fluorescent tracer method, fluorescence intensity was successfully fitted by equations. It was expected to provide a competent and efficient method for studying enzyme transport behavior in skin matrix with the advantages of quantification and high sensitivity.

Conclusions

The detection of the enzyme concentration in the skin is of great importance for guiding the tanning processes. A quantitative research method of enzyme mass transfer in skin matrix was offered, which combined fluorescence labeling and tissue sectioning technique. Results of the one-way diffusion pathway assay and turbulent diffusion pathway assay showed a successful quantitative detection of enzyme at different depths of the skin matrix. In addition, modeling equation result suggested a fitted feature of the experimental data obtained by the developed method. Considering its simple testing equipment and relevant measuring results, the proposed method may be a robust research technique for enzyme diffusion assay in skin matrix. Potentially, the method is except to supply a theoretical basis to the further research in the field of enzyme mass transfer kinetics and the guidance for enzyme using in the tannery.

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