Impact of Typical Surfactants on the Collagenolytic and Elastinolytic Activities of Proteases*

by

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Abstract

Most proteases exhibit broad-spectrum activities to all of the protein components of skins, thus, the indiscriminate application of proteases will bring out undue and non-selective loss of skin structural proteins, especially collagen and elastin, and lead to loose and damaged grain. Finding the effective ways to control proteases' activities against collagen and elastin in leather processes is very important. In this work, the influence of typical surfactants on the collagenolytic and elastinolytic activities of frequently used proteases was investigated, and the mechanism was also discussed. The results indicated that the nonionic surfactants had slight effects and anionic surfactants exhibited quite different behaviors on the collagenolytic and elastinolytic activities of selected proteases. Both collagenolytic and elastinolytic activities of trypsin preparations were obviously inhibited by all selected anionic surfactants, especially SDS and SDBS. For the bacteria proteases, their elastinolytic activities were significantly activated and their collagenolytic activities were inhibited to a variable extent by anionic surfactants. Tanners may effectively control the selective action of proteases to collagen and elastin to achieve different requirements through correctly utilizing surfactants.

Introduction

Enzymes, as a kind of high-efficiency biochemical catalysts and clean materials, are generally used in various process stages of leather making, such as soaking, unhairing and bating,[1-4] to reduce environmental pollution and/or improve leather quality.

Proteases are used in the leather processing to mainly break down and remove non-collagenous proteins of skins, in order to open up the collagen fibrous structure, whilst avoid excessive skin structural proteins damage. Collagen and elastin are the most important structural proteins, and they should be selectively broken down to a certain extent depending on the requirements of different leather processing, for example, more extensive decomposition of elastin is needed for garment than for shoe upper leather. Our earlier studies[5] had shown that the specificities of most of proteases to casein and skin structure proteins (collagen and elastin) were significantly different. Even if the caseinolytic activities of different commercial proteases were approximately equivalent, their activities towards collagen and elastin are quite different. Hence, these proteases may result in the entirely different effects on the processed skins, because of their different substrate specificities towards skin proteins. On the other hand, most of commercial proteases exhibit broad-spectrum activities to all of the protein components of skins, which means that the indiscriminate application of proteases will bring out undue and non-selective destruction of collagen and elastin, and lead to loose and damaged grain. So how to correctly choose and use proteases to avoid the inappropriate enzymolysis of skin proteins has been one of the major factors restricting their wider application.

One of the approaches to overcome the side-effect of proteases on skin structure proteins is effectively controlling and balancing their collagenolytic and elastinolytic activities based on correct choosing proteases. Finding the effective ways to make proteases selectively act on collagenolytic or elastinolytic in leather processes is very important. Enzymatic action is easily affected by using conditions, such as pH, temperature, time and so on, but these factors have slight influence on the substrate specificity of proteases toward collagen and elastin. As is known, it's very common to add surfactants to help chemicals penetration and improve processing efficiency in leather processing. They may impact the conformations of substrate, enzyme or the complex of substrate and enzyme molecule,[6,7] and lead to the behavior change of a protease to different substrates.

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In this study, elastin and hide powder stained with active dyestuffs, named elastin-RBB and hide powder-XBR, were used as the substrates to respectively characterize the collagenolytic and elastinolytic activities of proteases. Ten kinds of typical surfactants, including anionic and nonionic, and three kinds of proteases from different sources were selected. The influence of selected surfactants on the collagenolytic and elastinolytic activities of those proteases was investigated in order to identify ways to control the action of proteases and assist tanners to rationally choose and use surfactants in proteases processing.

MATERIALS AND METHODS

Materials

Surfactants (Table I) were purchased from BASF Co., Germany. and SASOL Co., Shanghai. Remazol Brilliant Blue R (RBB) and Reactive Brilliant Blue X-BR (XBR) were purchased from Sigma Chemicals Co., USA. Proteases were purchased from Novozymes, Denmark and Dowell Co., Chengdu, China. All other reagents used in the study were analytical grade.

Methods

Preparation of Elastin-RBB

5g of elastin powder, purified from bovine ligamentum nuchae according to the reference,[8] was soaked in a conical flask with 50ml of distilled water and some glass beads at 60°C for 30 minutes. 50 mL of 0.5%(w/v) an active dyestuff (RBB) solution was then added. After shaking at 60°C for 10 minutes, 10g of sodium sulfate was gradually added in 20 minutes. Then the pH of mixture solution was increased to 11 by successively adding 50mL of 4% (w/v) Na_3PO_4 solution, 20mL of 1.5% Na_2CO_3 (w/v) solution and 4mol/L NaOH solution. After shaking at 60°C for 30 minutes, the mixture was filtered by gauze. 100mL of 5%

TABLE I
The selected surfactants and proteases.

Commercial Name	Chemical Structure	Company
Anionic surfactants		
AES	Sodium alcohol ether sulfate (EO3)	SASOL Co.
SDS	Sodium dodecyl sulfate	SASOL Co.
SDBS	sodium dodecyl benzene sulfonate	SASOL Co.
PS65	sec sodium alkane sulfonate	
ABO	Sodium Dioctylsulfosuccinate	BASF Co.
Nonionic surfactants		
AT80	C_{16}-C_{18}-Fatty alcohol (EO80)	BASF Co.
TO10	C_{13}-Oxo alcohol(EO10)	BASF Co.
TO40	C_{13}-Oxo alcohol(EO40)	BASF Co.
XL90	C_{10}-Guerbet alcohol alkoxylate(EO90)	BASF Co.
XP90	C_{10}-Guerbet alcohol(EO90)	BASF CO.
Proteases		
AX	a neutral bacteria protease	Novozymes Co.
PY	an alkaline bacteria protease	Dowell Co.
DY	a crude pancreatic preparation	Dowell Co.
PTN	a purified trypsin preparation	Novozymes Co.

$(NH_4)_2SO_4$ (m/v) was then added. After shaking for 2 hours at 20°C, the RBB-dyed elastin powder was washed with distilled water until the unfixed dyes were completely washed off. Then the stained elastin powder was dehydrated with acetone, air dried and screened by sieve with 120 meshes, successively.

Preparation of Hide Powder-XBR

A block of middle-layer hide was taken from a piece of fresh cattle hide and cut into small pieces. The hide pieces were degreased and dehydrated with acetone for several times. Then they were milled and screened by sieve with 60 meshes. 5g of hide powder was soaked overnight in a conical flask with 100ml of 0.5%NaCl (w/v) solution. Then the conical flask was shook at 35°C for 2 hours in a rotary shaker. 50ml of 1.5% (w/v) an active dyestuff (XBR) solution was then added. After shaking for 1 hour at 35°C, 50ml of 1% Na_2CO_3 (w/v) solution was added. After shaking for 1 hour at 35°C, the mixture was filtered by gauze. 5%NH_4Cl (liquor ratio 1:20) was then added. After shaking for 2 hour at 35°C, the XBR-dyed hide powder was washed with distilled water until the unfixed dyes were completely washed off. Then the stained hide powder was dehydrated with acetone, air dried and screened by sieve with 120 meshes, successively.

The hide powder was made from the middle layer of fresh cattle hide which was pure collagen component, and it was stained with a kind of low-temperature reactive dyestuff at the temperature below 36°C. Hence, it can be considered that the process does not cause obvious collagen denaturation from intense chemical and thermal treatment. The hide powder-XBR can be used as the substrate to determine protease activity to collagen.

Preparation of Surfactants Solutions

Different quantity of surfactant were dissolved in Na_2CO_3-$NaHCO_3$ buffer solution (0.1M) and the surfactant concentration was adjusted to 0%-0.60% (w/v), respectively. The pH of solutions was adjusted to 8.2 or 9.5 for the determination of collagenolytic or elastinolytic activities of proteases, respectively.

Determination of Collagenolytic and Elastinolytic Activities of Proteases[9]

5±0.1mg of elastin-RBB or hide powder-XBR was accurately weighed and added into a microcentrifuge tube of 1.5ml volume. A protease solution with a certain concentration was mixed with a surfactant solution at the ratio of 1:5 (V/V). The mixing solution was stirred for 30 minutes at 40°C. Then 1.2ml of the mixture solution of protease and surfactant was added into the microcentrifuge tube. It was shook for 30 minutes with the rate of 800 rpm at 40°C. Then it was centrifuged at the rate of 12000 rpm for 5 minutes. The absorbance of the supernatant solution was then read at 596nm (elastin-RBB) or 600nm (hide power-XBR) using a UV spectrophotometer. The protease solution concentration was strictly controlled to insure that substrate quantity was excessive and OD values of the final supernatant solution were kept within a reasonable range.[9,10]

RESULTS

Surfactants can be classified into three types, i.e. anionic surfactants, nonionic surfactants and cationic surfactants,

TABLE II
The activities of proteases towards different substrates.

Proteases	Caseinolytic activities* (U/g)	Collagenolytic activities** (U/g)	Elastinolytic activities*** (U/g)
AX	185383	2052954	56759
PY	371700	1767041	55743
DY	402251	8710466	12681
PTN	92214	4029313	210

*Folin Method. 1 U of caseinolytic activity is the amount of enzyme required to catalyze the hydrolysis of casein to produce 1μg of tyrosine in 1 min at 40°C, pH8.2

**1 U of collagenolytic activity is the amount of enzyme required to catalyze the hydrolysis of 1 μg hide powder-XBR to form soluble substance in 1 min at 40°C, pH8.2

***1 U of elstinolytic activity is the amount of enzyme required to catalyze the hydrolysis of 1 μg elastin-RBB to form soluble substance in 1 min at 40°C, pH9.5

according to the dissociation of polar groups. Cationic surfactants are rarely used in the beamhouse processes. As highly efficient biochemical catalysts, proteases usually are used together with anionic surfactants and nonionic surfactants in leather processing, and their action efficiency to proteins will be influenced by surfactants. So the impact of typical surfactants on the collagenolytic and elastinolytic activities of proteases used in leather processes was investigated.

We investigated the activities of more than ten high-concentration proteases used in leather industry towards elastin-RBB and hide powder-XBR. It was found that most of neutral and alkaline bacteria proteases exhibited both elastinolytic and collagenolytic activities, and the optimum pH of elastinolytic activities was around 9-10. Four proteases from different sources were selected according the ratio of elastinolytic and collagenolytic activity, a neutral bacteria protease (AX), an alkaline bacteria protease (PY), a crude pancreatic preparation (DY) and a purified trypsin (PTN). Their activities toward elastin, collagen and casein are shown in Table II.

Table II indicates that the four selected proteases exhibit different hydrolyses performance in varying protein substrates. DY is a kind of crude pancreatic enzyme, and it has higher elastinolytic activity comparing with PTN, a partially purified trypsin. All of them showed quite high collagenolytic activity at pH8.2 and elastinolytic activity at pH9.5, so the two pHs

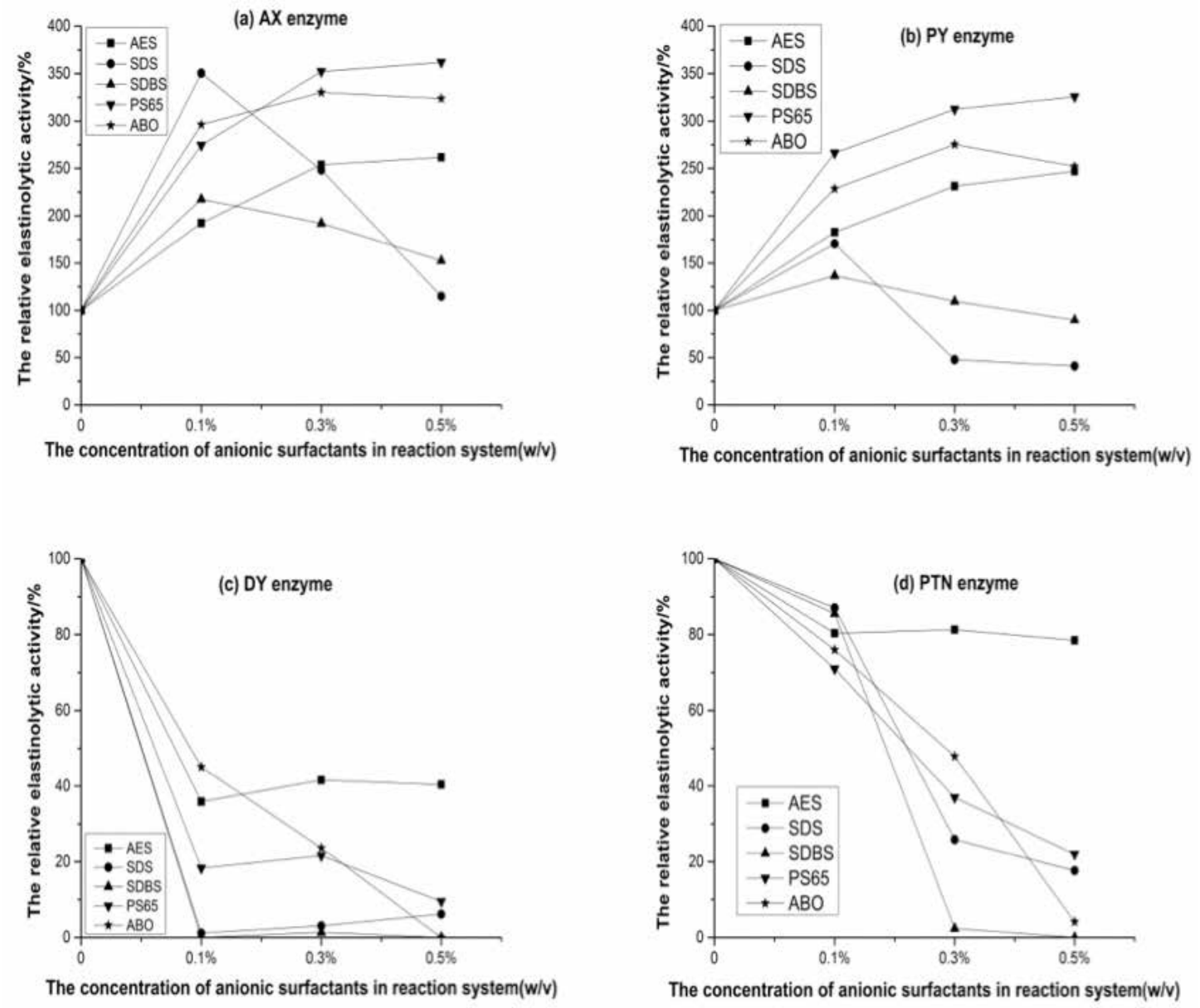

Figure 1. Effects of anionic surfactants at varied concentrations on elastinolytic activities of proteases.

were chosen for determining the influence of surfactants on protease activities against collagen and elastin, respectively.

Effects of Surfactants on Elastinolytic Activities of Proteases

The effects of surfactants, including anionic surfactants and nonionic surfactants, on the elastinolytic activities of proteases from bacteria and animal pancreas were evaluated on basis of using elastin-RBB as the substrate. The results are shown in Figure 1 and Figure 2. The activities of proteases in the buffer without surfactant are regarded as 100% in the figures.

The results show that the effects of anionic and nonionic surfactants on the elastinolytic activities of the selected four proteases are quite different. It can be seen that five typical nonionic surfactants with various hydrophobic chain structure and EO value, including AT80, TO10, TO40, XL90 and XP90, have slight effect on the elastinolytic activities of the four proteases, in contrast to anionic surfactants.

The results also indicate that the elastinolytic activities of two kinds of trypsin preparations, i.e. DY and PTN, are obviously inhibited by all selected anionic surfactants and even totally inactivated by SDBS and SDS when the surfactant concentration is greater than 0.1% or 0.3% (see Figure 1(c) and (d)). But, for the two selected proteases from bacteria, AX and PY, the elastinolytic activities were significantly activated by AES, PS65 (sec sodium alkane sulfonate) and ABO (sodium

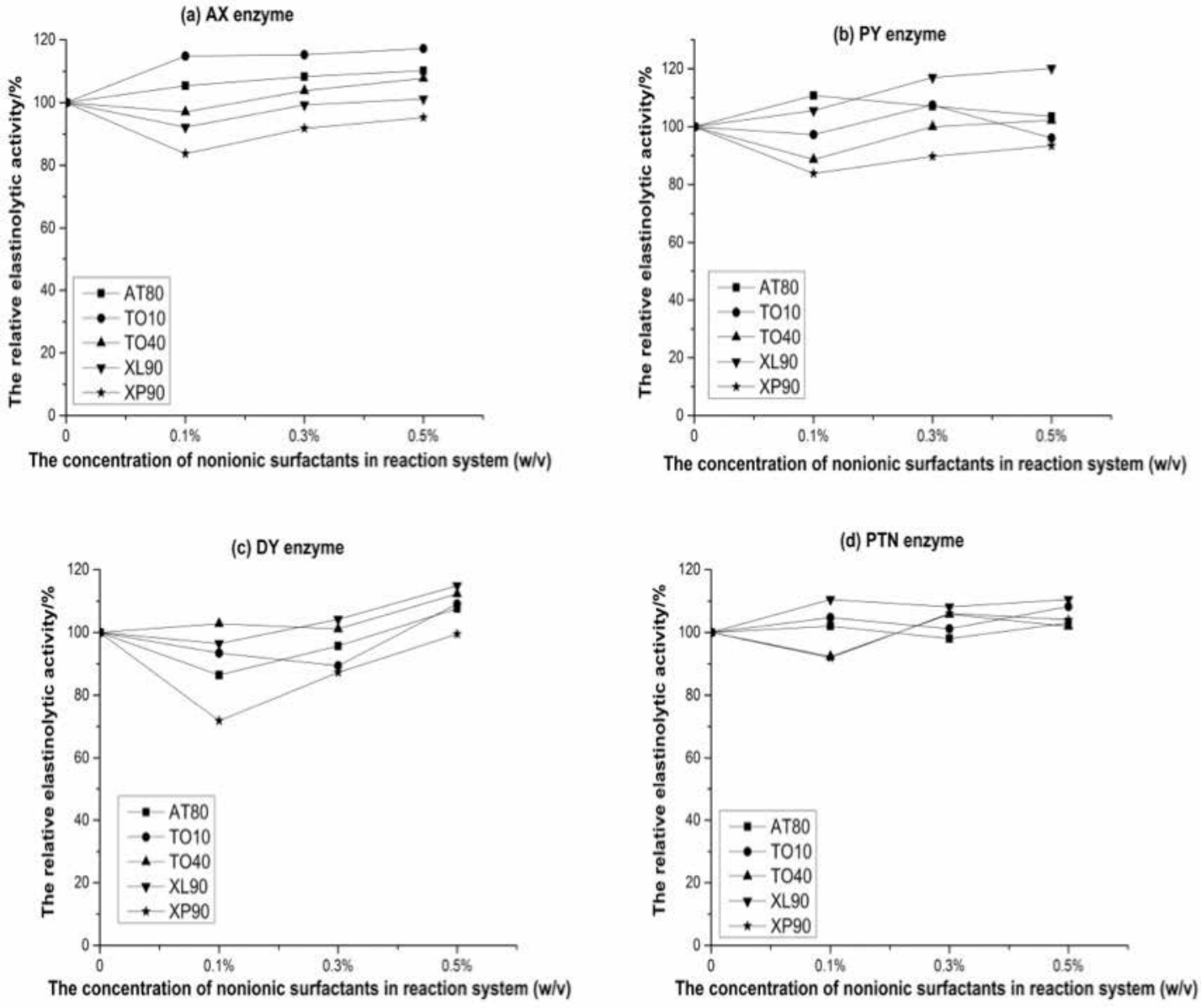

Figure 2. Effects of nonionic surfactants at varied concentrations on elastinolytic activity of proteases.

dioctylsulfosuccinate) with surfactant concentrations increasing. The elastinolytic activities of the two bacteria proteases increase 150%-250% when the surfactant concentration reaches to 0.3%. The elastinolytic activities of the two bacteria proteases are also activated by SDS and SDBS at the surfactant concentration less than 0.1%, and reach to the maximum value at the surfactant concentration of 0.1%. But, when the surfactant concentration is greater than 0.1%, the elastinolytic activities decrease, and even is inhibited for PY.

Effects of Surfactants on Collagenolytic Activities of Proteases

The effects of surfactants, including anionic surfactants and nonionic surfactants, on the collagenolytic activities of the four proteases from bacteria and animal pancreas were also evaluated on basis of using hide powder-XBR as the substrate. The results are shown in Figure 3 and Figure 4. The activities of proteases in the buffer without surfactants are regarded as 100% in the figures. It can be seen that the collagenolytic activities of the selected proteases are slightly activated by the selected nonionic surfactants, and most of the activities increase about 20%.

As for the effect of anionic surfactants on collagenolytic activities, it was another case. The data from Figure 3(c) and Figure 3(d) indicate that the collagenolytic activities of DY and PTN, two kinds of trypsin preparations, are significantly inhibited and even totally inactivated by all the selected anionic surfactants. The collagenolytic activities of AX and PY, two

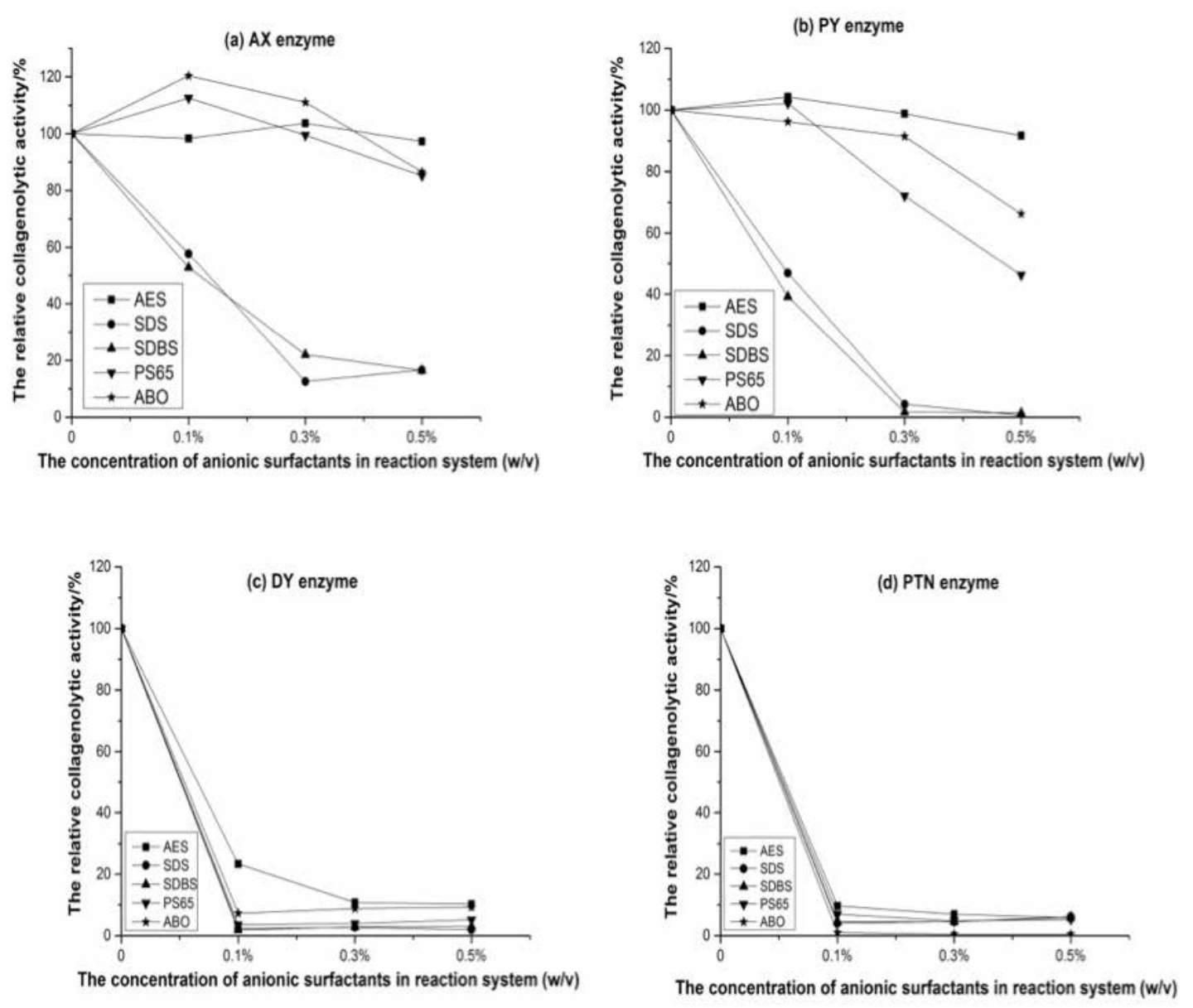

Figure 3. Effects of anionic surfactants at varied concentrations on collagenolytic activities of proteases.

kinds of bacteria proteases, were also obviously inhibited by SDS and SDBS, which is similar to trypsin. However, the collagenolytic activities of AX and PY are less inhibited by AES, PS65 and ABO. Even the collagenolytic activitiy of AX was slightly activated by PS65 and ABO at 0.1% concentration.

DISCUSSION

Effects of Nonionic Surfactants on Collagenolytic and Elastinolytic Activities of Proteases

The above investigation indicates that all of the selected typical nonionic surfactants with various hydrophobic chain structure and EO value have slight effect on both elastinolytic and collagenolytic activities of the four selected proteases, including bacteria preoteases and trypsin preparations.

It can be accepted that nonionic surfactants are weakly bonded to enzyme and substrate protein molecules just through hydrogen-bonding and hydrophobic interactions, which has small influence on the conformations of enzyme and substrate molecules.[11,12] Hence, nonionic surfactants have a little effect on both collagenolytic and elastinolytic activities of proteases.

Effects of Anionic Surfactants on Collagenolytic and Elastinolytic Activities of Trypsin

Anionic surfactants with anionic groups can strongly combine with both enzyme and substrate molecules via ionic bond

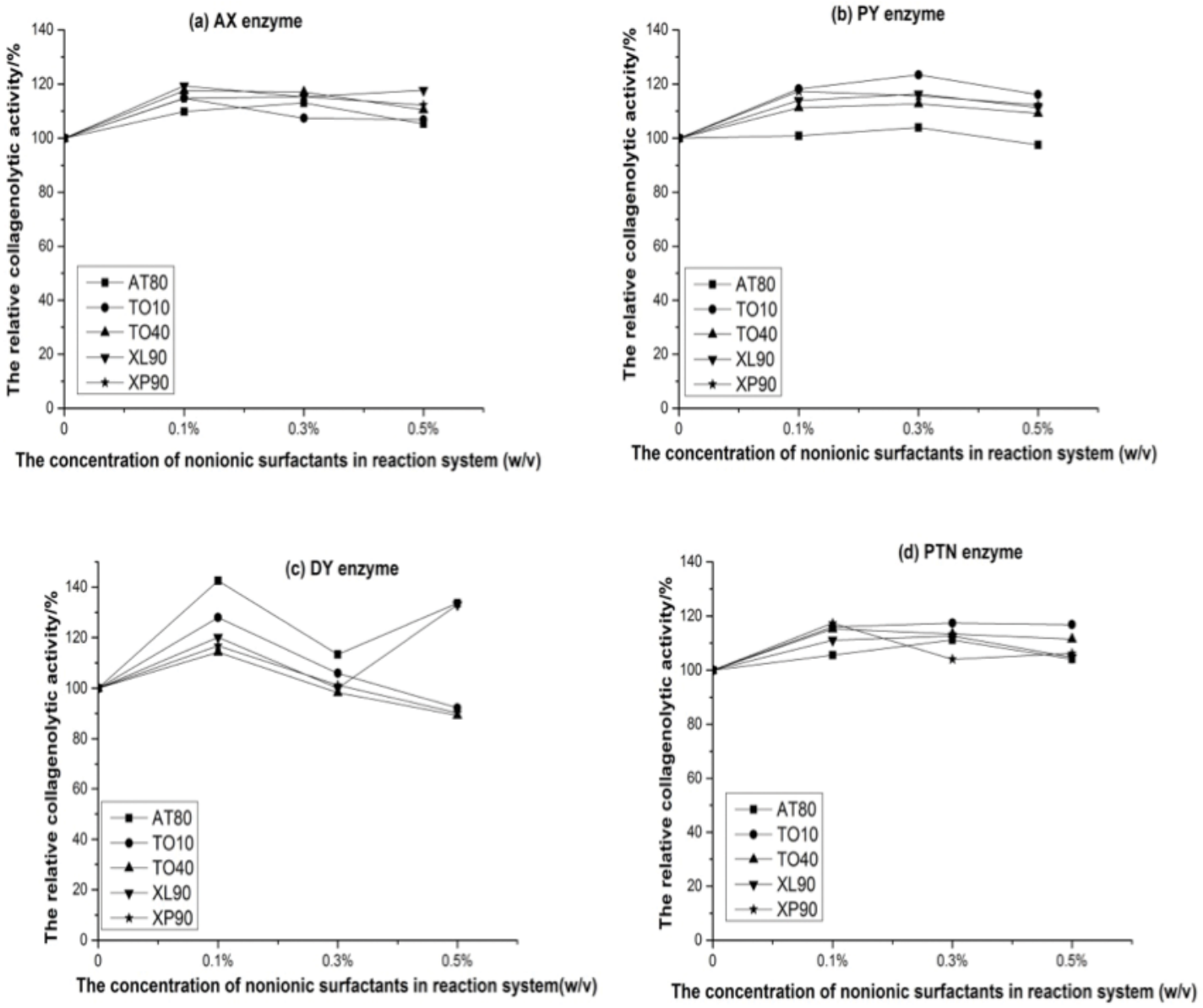

Figure 4. Effects of nonionic surfactants at varied concentrations on collagenolytic activities of proteases.

besides hydrogen-bonding and hydrophobic interaction. The multiple interactions may change the conformations of enzyme and substrate.[13,14] The change may depend on the molecular structures of anionic surfactants, enzymes and substrates, and probably affects the formation of enzyme-substrate complex. Hence there are several possibilities as for the impact of anionic surfactants on protease activities. It can be speculated that the affinity between anionic surfactants and enzymes or substrates will increase with the increasing of the anionic charges of surfactants, and the stereo-hindrance effect of surfactants on enzyme active site contacting with reaction points of protein chain will also be enhanced with the increasing size of hydrophobic groups of surfactant molecules; hence, the affection of anionic surfactants on protease activities will depend on the structures of anionic surfactants, i.e. anionic groups and the molecule size.

Trypsin with strong specificity mainly hydrolyzes the peptide bonds near lysine and arginine residues.[15] Positively charged lysine and arginine residues of proteins can easily combine with negatively charged sulfate and sulfonate groups of surfactants. When collagen and elastin contact with anionic surfactants, lysine and arginine residues of protein substrates will be occupied by anionic surfactants, and the reaction points of trypsin in peptides are hidden by the hydrophobic long chain of surfactants. This may be the main reason that anionic surfactants significantly inhibited the activities of trypsin against both collagen and elastin (see Figure 1(c), (d) and Figure 3 (c), (d)). On the other hand, anionic surfactants will also be bound to the cationic sites on the enzyme surface, the conformation of enzyme active site will be deformed to some extent, and the enzyme activity will be also affected.

Effects of Anionic Surfactants on Collagenolytic Activities of Bacteria Proteases

Unlike trypsin, the specificities of the selected bacteria proteases to peptide bonds are low, and they may randomly hydrolyze peptide bonds of collagen. Hence, the combination of anionic surfactants with substrate proteins at the cationic sites of amino acid residues to prevent enzymes finding suitable reaction sites is not the main factor reducing enzyme activity.

Among the five selected anionic surfactants, only SDS and SDBS significantly inhibit and even inactivate the collagenolytic activities of the two bacteria proteases, AX and PY. In order to further know how SDS and SDBS impact the action of proteases towards collagen substrate, we change the means of adding surfactants. Hide powder-XBR was pre-incubated with SDS or SDBS solution for five minutes, then AX or PY enzyme solution was added. The entirely different results appeared, and were shown in Figure 5.

When SDS and SDBS contacted with substrate at first, they were bound by substrate and exhibited less affection towards the collagenolytic activities of bacteria proteases. On the contrary, when they contacted with proteases at first, they obviously inhibited the collagenolytic activities of proteases. So it can be considered that the influence of anionic surfactants on the conformations of enzyme molecules is the foremost factor affecting enzyme reaction efficiency.

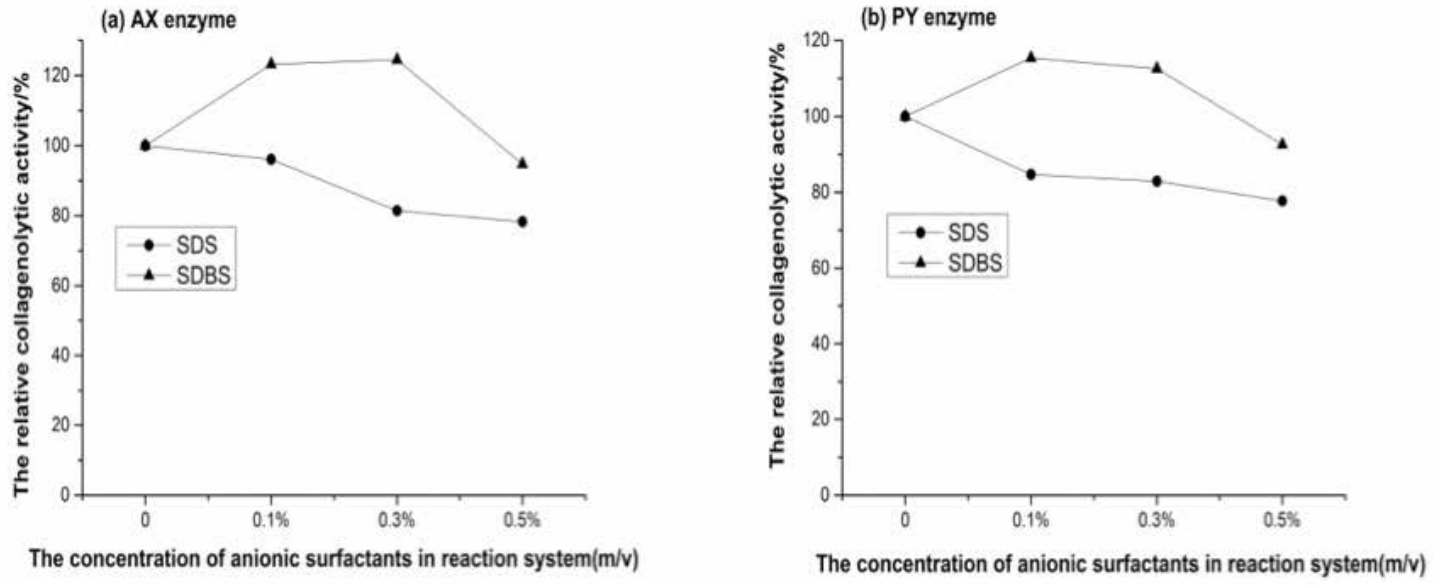

Figure 5. Effects of SDS and SDBS on collagenolytic activities of bacteria proteases with different adding means of surfactant. (Hide powder-XBR was preincubated with SDS or SDBS solutions for five minutes, then AX and PY enzyme was added. The other operating steps were the same as the above.)

SDS is used as a denaturant to break the intramolecular and intermolecular hydrogen bond, unfold and destroy the secondary and tertiary structure of protein molecules in SDS polyacrylamide gel electrophoresis method. SDBS carries a larger hydrophobic group with a benzene ring. When they are bond to the surface of enzyme, they will make enzyme molecule more seriously deforming than AES, PS65 and ABO; hence, the collagenolytic activities of the two bacteria proteases are significantly inhibited and even inactivated by SDS and SDBS, while AES, PS65 and ABO exhibit less affection on collagenolytic activitiy (see Figure 2(a), (b)) even under high surfactant concentration (0.3%).

Effects of Anionic Surfactants on Elastinolytic Activities of Bacteria Proteases

Elastin is mainly composed of nonpolar amino acids, such as glycine, alanine, valine and proline. Kagan et al.[16] thought the anionic surfactants bonded to insoluble elastin formed a substrate complex, which had a higher affinity to enzyme molecule than original elastin, and thus was more susceptible to proteolysis. Because the increased anions of the complex intensifies the binding of enzyme to the elastin and anionic surfactants induce a conformational change in the insoluble elastin, leading to increased accessibility to the enzyme. The viewpoints are supported by the data in Figure 1 (a), (b) which indicate that all the selected anionic surfactants can increase the elastinolytic activities of bacteria proteases at low concentrations.

As mentioned before, SDS and SDBS have more intensive negative charge or larger hydrophobic group than AES, PS65 and ABO, hence the influence of SDS and SDBS on the spatial conformation of enzyme may predominate with the concentration increasing, resulting in the decreased elastinolytic activities under higher surfactant concentration (0.3% and 0.5%).

Most of proteases, especially neutral and alkaline bacteria proteases, exhibit activities to both collagen and elastin. During leather-making processing, especially for soft leather, it is necessary and important to break down elastin in the grain layer of hides whilst avoid excessive collagen damage. That is, we need to increase the elastinolytic activities and decrease the collagenolytic activities of proteases at the same time. Adding suitable anionic surfactants in protease processing, such as SDS, SDBS and so on, may be an effective method.

Conclusions

The effects of typical surfactants on the collagenolytic and elastinolytic activities of proteases used in leather processing were evaluated. The results indicated that the collagenolytic and elastinolytic activities of trypsin were obviously inhibited and even totally inactivated by all selected anionic surfactants. But, for the two selected proteases from bacteria, the elastinolytic activities were significantly activated to 250%-350% by AES, PS65 (sec sodium alkane sulfonate) and ABO (sodium dioctylsulfosuccinate) while the collagenolytic activities were slightly influenced. The elastinolytic activities of bacteria proteases were also activated by SDS and SDBS, and reached to the maximum value at the surfactant concentration of 0.1%. However, the collagenolytic activities of bacteria proteases were apparently inhibited by SDS and SDBS. The nonionic surfactants had slight effect on the collagenolytic and elastinolytic activities of four selected proteases. So correctly utilizing anionic surfactants may be a useful way to effectively control and balance proteases collagenolytic and elastinolytic activities in leather protease processing.

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