MODELING TECHNOLOGICAL CONDITIONS FOR BREAKDOWN OF WASTE SHEEP WOOL

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ABSTRACT

The contribution deals with hydrolytic processing of sheep wool in two stages (alkaline pre-processing and enzymatic hydrolysis) into keratin hydrolysate. Employed proteolytic enzymes were Esperase 6.0 T and Everlase 6.0 T. Modeling technological conditions for breakdown proceeded applying the method of factor tests of 2³ type, in which factors under study were additions of Ca(OH)₂ (3-9 %, w/w), temperatures in first processing stage (40-80 °C) and temperatures in second processing stage (40-60 °C) influencing quantity of broken down wool (determined through gravimetric analysis). When using enzyme Esperase 6.0 T quantities of broken down wool were 8.4-59.2 % wool; with enzyme Everlase 6.0 T 4.7-39.6 % wool (depending on conditions of hydrolysis). Results were statistically processed and statistic significance of factors under study was analyzed. Results of sheep wool breakdown presented graphically in contour charts indicate the influence of two most significant factors with a selected level of third factor and show the possibility to model breakdown conditions in such manner that keratin hydrolysate of required properties is obtained.

Keywords: Enzyme hydrolysis; Keratin hydrolysate; Organic waste; Sheep wool; Waste treatment

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INTRODUCTION

Wool is one of many natural fibers belonging to the group of proteins called α-keratins. Keratins are rich in amino acids containing sulphur (cysteine, cystine, methionine) causing their high chemical resistance (insolubility) and mechanical resilience. The secondary structure of α-keratins is a helix¹ ².

The annual world production of raw sheep wool, according to newest data of the British Wool Marketing Board (2010), is approximately 1.2 million metric tons. The greatest wool producer is Australia (approx 400,000 metric tons of wool annually); other major producers are New Zealand and China. Approximately 90 % produced wool is consumed by the textile industry to produce fibers. The entire quantity of keratin wastes – sheep wool, wastes of poultry farms (feathers), also wastes from slaughterhouses, meat processing units and tanneries (hair, fur, bristles, hoofs, horns, cloven hoofs, etc) produced in the whole world is estimated at more than 5 million metric tons annually. That is quite a quantity of bio-polymeric material of protein (keratin) character that has so far not been adequately exploited³.

One of the methods for processing keratin wastes is production of keratin hydrolysates. Keratin waste first has to be degreased through extraction by organic solvents (petroleum ether, chloroform, petrol and others) or through application of lipolytic enzymes. Hydrolysis of keratin may be performed by means of oxidizing and reducing agents, for example, by 2-mercaptoethanol, urea, hydrogen peroxide, thioglycolic acid, sodium hydrogen sulphite⁴ ⁵.

Katoh et al. utilized sodium dodecyl sulphate to break down wool, immersing wool in a reducing solution and next heating the mixture to 100 °C with further shaking for 30 min. On cooling the mix, dissolved keratin hydrolysate was obtained by filtration and subsequent dialysis. Powder was produced by thickening the solution on a vacuum evaporator with following spray drying⁶. Other conditions for
reduction breakdown (with dithiothreitole and guanidine hydrochloride) were applied by Yang et al. The mixture of wool and reducing agents was heated to 50 °C and incubated for 15 hours in a protective nitrogen atmosphere. Dissolved keratin was then obtained through dialysis.

Alkaline hydrolysis of keratin wastes utilises strong solutions of hydroxides. Coward-Kelly et al. processed poultry feathers and cow hair into keratin hydrolysates with calcium hydroxide in a temperature-controlled autoclave with stirrer. Hydrolysis temperature was 50-150 °C, duration ranged from 0-300 min, quantity of processed dry material was 18-80 g/L and quantity of employed lime was 0.0-0.4 g per gram dry material. The quantity of keratin that passed into solution was 90 % after 3 hours of hydrolysis at 150 °C. Keratin hydrolysates obtained in this manner can be employed as a feed supplement for ruminants. Acid hydrolysis of keratin wastes may be performed, for example, by means of acetic acid or peroxoacetic acid. Employing 6 mol/L HCl at 80 °C enables to break down up to 86 % keratin; nevertheless, drastic breakdown conditions are a disadvantage, which shows negatively in the quality of keratin hydrolysate.

In recent years, keratin breakdown has utilised enzymatic hydrolysis. Nature has huge quantities of bacteria producing enzymes able to break down keratin under mild conditions (temperatures usually 25-60 °C and pH≈8 depending on kind of used enzyme or bacteria). Most used bacteria are from the Bacillus and Streptomyces genera. Kida et al processed ground horns and hoofs of cows (particle size up to 250 µm) by enzymatic hydrolysis (enzymes Bacillus subtilis, B. species, B. licheniformis, Aspergillus mellaeus and an enzyme from pig pancreas). Forty mg ground material and 7 mL phosphate buffer (pH 8.3) were put in a reactor and the mixture was mixed for 10 min at 50 °C. Eight mg enzyme was then added to the material and mixing continued for another hour at 50 °C. Factors under study for hydrolysis efficiency were kind and concentration of used enzyme, temperature and reaction time.

Dalev carried out breakdown of chicken feathers through a combined two-stage enzymatic-alkaline hydrolysis. He first mixed 40 kg feathers with 30 L of 0.3 M NaOH for 30 min at 80 °C. On adjusting pH to 8.0-8.3 (by adding HCl 10 %), he added 100 g proteinase B72 from Bacillus subtilis to the mixture. The material was hydrolysed under constant stirring for another 2 hours at 55 °C. When hydrolysis finished, enzyme was inactivated by lowering pH to 7 (adding HCl 10 %) and heating the solution to 95 °C for 30 min. Efficiency of hydrolysis was about 50 %. Dried keratin hydrolysate possesses a high content of proteins (795 g/kg) and is suitable as a feed for farm animals.

The object of this paper is processing of sheep wool by a two-stage alkaline-enzymatic hydrolysis into a keratin hydrolysate and studying influence of selected technological conditions – added Ca(OH)₂, temperatures in first and second hydrolysis stage – on sheep wool breakdown efficiency. Work also focused on comparing breakdown efficiency when using two proteolytic enzymes – Esperase 6.0 T and Everlase 6.0 T (supplied by Novozymes A/S Bagsvaerd, Denmark). The positive influence of enzyme Esperase 6.0 T in hydrolysis of waste collagen materials was reported by Kupec et al.

EXPERIMENTAL

Materials
Raw sheep wool was supplied from slaughterhouses in the Czech Republic; first analyses were performed by standard methods. Composition of raw sheep wool was as follows: dry matter content = 91.6 %; in dry matter: nitrogen content = 12.2 %, fat content = 8.2 %, sulfur content = 2.5 %, ash content = 2.3 %. Raw sheep wool was first washed several times in a sufficient excess of warm water; the water was mechanically squeezed out and wool was dried in a drier for 48 hours at 50 °C. Wool was then degreased by means of enzyme Lipex 100 T (Novozymes, Denmark) through the procedure as follows. Wool was mixed with water in ratio 1:50, pH level was adjusted to 8±0.2 with added NaOH solution (1 % conc.), 1 % enzyme was added (related to mass of dry wool, w/w) and degreasing proceeded in an incubator for 24 hours at 40±1 °C; during the first 12 hours of degreasing, the contents were shortly mixed in 1-hour intervals. After degreasing, the mix was filtered through a sieve; wool was washed with adequate water and dried in a hot-air drier to constant mass at 103±2 °C. Dried wool was finally ground to a particle size of 1 mm and prior to breakdown experiments proper was kept in a desiccator (over dried silica gel) at room temperature.
Apparatus and equipment

Heater plate Schott Ceran 93020 (Germany), magnetic stirrer with temperature control Ika ETS-D4 fuzzy (Germany), drier WTB Binder E/B 28 (Germany), incubator WTC Binder B53 (Germany), knifing mill Fritsch Pulverisette 19 (Germany), electronic balances KERN 770/GS/GJ and 440-47 (Germany), rotary vacuum evaporator Laborota 4000 (Heidolph Instruments, Germany), pH-meter WTW pH526 (Germany), mineralization apparatus Hach Digesdahl (USA), muffle furnace Nabertherm L 9/S 27 (Germany), Parnas-Wagner distillation apparatus, Soxhlet extraction apparatus, silicone-rubber coated plate 270x210 mm (Tescoma, the Czech Republic), filter paper Filpap KA-1 of 9-cm diameter (the Czech Republic).

Chemicals

Powdery proteinases (produced by submersion fermentation of genetically modified micro-organism Bacillus) Esperase 6.0 T (declared activity 6.0 KNPU/g) and Everlase 6.0 T (declared activity 6.0 EPU/g), powdery lipase (produced by submersion fermentation of genetically modified strain Aspergillus) Lipex 100 T (declared activity 100 KLU /g) were supplied by Novozymes A/S Bagsvaerd, Denmark. Ca(OH)$_2$ and HCl, (analytical grade) were supplied by Petr Lukes, the Czech Republic.

Concept of tests for sheep wool breakdown

Processing of sheep wool into keratin hydrolysate utilised a two-stage breakdown that had proved successful in producing keratin hydrolysate from chicken feathers$^{20}$. The principle of this processing consists in incubating keratin material in an alkaline environment (pH in the first stage $\approx 11.5$), which effects its swelling, and having disulphide and peptide bonds split in the second stage through action of a convenient enzyme (after adapting pH). Conditions of hydrolytic processing had to be adapted to enable applying the mentioned two-stage technology to break down sheep wool. Based on preliminary tests, it was decided to realise the modelling of technological conditions for sheep wool breakdown by the method of statistic factor tests and factor tests of $2^3$ type (3 studied factors on two levels – minimal and maximal) with 2 center points were employed to this purpose. The examined factors were:

- **Factor A**: added quantity of Ca(OH)$_2$ (related to weighed-in quantity of dried sheep wool, w/w): bottom limit 3 %, upper limit 9 %
- **Factor B**: temperature in first processing stage: bottom limit 40±0.5 $^\circ$C, upper limit 80±0.5 $^\circ$C
- **Factor C**: temperature in second processing stage: bottom limit 40±0.5 $^\circ$C, upper limit 60±0.5 $^\circ$C

Evaluation of measured data was carried out in statistic program Statgraphics 6.0 (Manugistic, Inc., USA).

Working method for sheep wool breakdown

Processing of degreased and dried (103 $^\circ$C) sheep wool into keratin hydrolysate (see diagram in Fig. 1) proceeded according to working procedure as follows. In the first processing stage, wool (10 g) was weighed into an Erlenmayer flask and 0.3 g Ca(OH)$_2$ (corresponding to an addition of 3 % per weighed-in wool – bottom limit of factor A) was weighed in, or 0.9 g Ca(OH)$_2$ (corresponding to an addition of 9 % per weighed-in wool – upper limit of Factor A). Distilled water (150 mL) was added, pre-heated to temperature planned according to factor B (40 $^\circ$C at bottom limit, or 80 $^\circ$C at upper limit), the flask was closed and vigorously shaken for 60 sec after which care was taken to have all wool wetted without adhering to flask walls. A cylinder-shaped small stirrer (8x40 mm) was inserted in flask, which was then closed and its contents were stirred for 6 hours at 650 rpm on magnetic stirrer at planned temperature (Factor B). The cylinder-shaped stirrer was then removed and the mixture was incubated in closed flask for 18 hours at the same temperature. When the first processing stage was finished, pH level of the mixture was adjusted to 9.0±0.1 by adding HCl solution (5 %, 1.5-2.5 mL). Before second processing stage, temperature of the mixture in flask was checked and adapted to temperature planned in accordance with factor C (40 $^\circ$C at bottom limit or 60 $^\circ$C at upper limit). Proteolytic enzyme (Esperase 6.0 T, or Everlase 6.0 T) was then added (5 % w/w, i.e. 0.50 g) to the mixture, the cylinder-shaped small stirrer was inserted, flask was closed and contents were stirred 6 hours at 650 rpm on a magnetic stirrer at planned temperature (Factor C). The cylinder-shaped stirrer was then removed and the mixture was incubated in closed flask for 18 hours at the same temperature. After second processing stage, the remaining non-decomposed wool fraction was separated from keratin hydrolysate by filtering through
polyamide cloth (pore diameter 150 µm) folded twenty fold. Non-decomposed wool fraction was finally washed with 40 mL distilled water (washing water was added to keratin hydrolysate), then dried in a drier at 103±2 °C to constant mass, and on cooling in desiccator (filled with dried silica gel), the quantity of non-decomposed wool was determined through gravimetry. Immediately after filtration, the solution of keratin hydrolysate was heated to 85±2 °C at which it was kept for 10 min in order to inactivate the enzyme used in hydrolysis. Keratin hydrolysate was then thickened on vacuum evaporator to about half of its volume at 65±2 °C, after that cast on silicone plate and thus dried for about 16 hours at 103±2 °C; film of keratin hydrolysate obtained in this manner was finally ground to powder.

RESULTS AND DISCUSSION

A survey of factor tests organization and summarized results of sheep wool breakdown by two-stage alkaline-enzymatic hydrolysis using two different enzymes (Esperase 6.0 T and Everlase 6.0 T) are presented in Table 1.

Table-1: Results of sheep wool breakdown through two-stage alkaline-enzymatic hydrolysis with use of enzymes Esperase 6.0 T and Everlase 6.0 T.

<table>
<thead>
<tr>
<th>Run</th>
<th>Factor A: Added quantity of Ca(OH)₂ (%)</th>
<th>Factor B: Temperature in 1st processing stage (°C)</th>
<th>Factor C: Temperature in 2nd processing stage (°C)</th>
<th>Decomposed wool (%) a</th>
<th>Hydrolysis using enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>40</td>
<td>40</td>
<td>8.4</td>
<td>Esperase 6.0 T</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>40</td>
<td>60</td>
<td>15.3</td>
<td>Esperase 6.0 T</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>80</td>
<td>40</td>
<td>9.8</td>
<td>Everlase 6.0 T</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>80</td>
<td>60</td>
<td>22.4</td>
<td>Everlase 6.0 T</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>60</td>
<td>50</td>
<td>19.7</td>
<td>Everlase 6.0 T</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>60</td>
<td>60</td>
<td>19.2</td>
<td>Everlase 6.0 T</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>40</td>
<td>40</td>
<td>14.9</td>
<td>Everlase 6.0 T</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>40</td>
<td>60</td>
<td>24.2</td>
<td>Everlase 6.0 T</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>80</td>
<td>40</td>
<td>42.9</td>
<td>Everlase 6.0 T</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>80</td>
<td>60</td>
<td>59.2</td>
<td>Everlase 6.0 T</td>
</tr>
</tbody>
</table>

a. related to weighed-in quantity of dried sheep wool

Quantity of decomposed wool (monitored by 3-factor tests) is described by means of the linear equation as follows:

\[
y = k + A.b_1 + B.b_2 + C.b_3 + AB.b_{12} + AC.b_{13} + BC.b_{23}
\]  

Where, A, B, C…factors under observation during hydrolysis (A = added quantity of Ca(OH)₂, B = temperature in first processing stage, C = temperature in second processing stage); AB, AC, BC…interaction elements; k, b₁, b₂, b₃, b₁₂, b₁₃, b₂₃…regression coefficients.

When breaking down wool with enzyme Esperase 6.0 T, the equation is as follows: \(y = 19.5857 – 4.52917A – 0.63125B – 0.065C + 0.113542AB + 0.0254167AC + 0.0079375BC\). High correlation factor \(R^2 = 0.9807\) indicates that 98.07 % variable factors (A, B, C) to observed response (quantity of decomposed wool) are described by the presented linear equation. When breaking down wool with enzyme Everlase 6.0 T, the equation is as follows: \(y = 21.48 – 2.775A – 0.555B – 0.25C + 0.0804167AB + 0.004167AC + 0.0085BC\). High correlation factor \(R^2 = 0.9723\) confirms again excellent applicability of this model.

Statistical significance of particular factors for monitored quantities was evaluated by standard procedure applying the Fisher test of significance at a confidence level of 95 % in which the critical F-value is 10.1321. Results of evaluation are listed in Table 2.
Table -2: Results of analysis of scatter and of Fischer test of statistical significance of examined factors on amount of decomposed wool.

<table>
<thead>
<tr>
<th>Factors under study</th>
<th>Hydrolysis of wool using enzyme</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Esperase 6.0 T</td>
<td>Everlase 6.0 T</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sum of Squares</td>
<td>F-value</td>
<td>Sum of Squares</td>
</tr>
<tr>
<td>Factor A: added quantity of Ca(OH)$_2$</td>
<td>909.51</td>
<td>62.88$^a$</td>
<td>367.21</td>
</tr>
<tr>
<td>Factor B: temperature in 1$^{st}$ processing stage</td>
<td>639.03</td>
<td>44.18$^a$</td>
<td>397.62</td>
</tr>
<tr>
<td>Factor C: temperature in 2$^{nd}$ processing stage</td>
<td>254.25</td>
<td>17.58$^a$</td>
<td>64.98</td>
</tr>
<tr>
<td>Interaction AB</td>
<td>371.28</td>
<td>25.67$^a$</td>
<td>186.25</td>
</tr>
<tr>
<td>AC</td>
<td>4.65</td>
<td>0.32</td>
<td>0.13</td>
</tr>
<tr>
<td>BC</td>
<td>20.16</td>
<td>1.39</td>
<td>23.12</td>
</tr>
<tr>
<td>Total error</td>
<td>43.39</td>
<td></td>
<td>29.65</td>
</tr>
<tr>
<td>Total error (corr.)</td>
<td>2242.27</td>
<td></td>
<td>1068.96</td>
</tr>
</tbody>
</table>

F$_{crit}^{95\%}(1;3) = 10.13$; $^a$ statistically significant fa

Breakdown of sheep wool by enzyme Esperase 6.0 T

As results of analyses by the Fisher test indicate (Table 2), all three factors under observation (A, B, C) within selected limits are statistically significant; the same was recorded with factors interaction AB. A summarized presentation of the influence exerted by studied factors on quantity of broken down wool employed contour charts worked out in the Statgraphics program. Fig. 2 indicates influence of two statistically most significant factors – factor A (addition of calcium hydroxide) and factor B (temperature in first processing stage) – on quantity of decomposed wool at minimum and maximum level of factor C (40 and 60$^\circ$C, temperature in second processing stage). It is obvious in Fig. 2a (temperature in second processing stage = 40$^\circ$C) that at minimum levels of factors A, B – 3% addition of Ca(OH)$_2$ and temperature 40$^\circ$C in first processing stage – the quantity of decomposed wool is very low (about 8%). When higher levels of factors A, B are mutually combined, quantity of decomposed wool increases and when levels of both studied factors are at their maximum – 9% addition of Ca(OH)$_2$ and temperature 80$^\circ$C in first processing stage – the quantity of broken down wool is roughly fivefold greater (about 39%) than at minimum levels of these factors. Increasing temperature to 60$^\circ$C in the second processing stage produces higher hydrolysis efficiency – see Fig. 2b, here factors A and B were at their minimum with 15% decomposed wool, and at their maximum they achieved 57% decomposed wool. Contour graphs in Fig. 2 present quite a wide possibility to model conditions for breaking down sheep wool in such manner that maximum process efficiency is achieved and thus related quality of prepared keratin hydrolysates. In case keratin hydrolysate of low ash content is desired, lower additions of Ca(OH)$_2$ (Factor A) have to be adopted which, however, shows up in lower efficiency of the whole hydrolytic process. Higher hydrolysis efficiency may be attained in this case by elevated temperatures in the first processing stage (Factor B) and second processing stage (Factor C).

Breakdown of sheep wool by enzyme Everlase 6.0 T

As results of analyses by the Fisher test indicate (Table 2), factors A and B in selected limits are statistically significant; the same was recorded with factors interaction AB. Fig. 3 presents a contour chart indicating influence of factor A (addition of calcium hydroxide) and factor B (temperature in first processing stage) on quantity of decomposed wool at minimum and maximum level of factor C (40 and 60$^\circ$C, temperature in second processing stage). It is obvious in Fig. 3a (temperature in second processing stage = 40$^\circ$C) that at minimum levels of factors A, B – 3% addition of Ca(OH)$_2$ and temperature 40$^\circ$C in first processing stage – the quantity of decomposed wool was only about 5%. At maximum levels of both studied factors – 9% addition of Ca(OH)$_2$ and temperature 80$^\circ$C in first processing stage – the quantity of broken down wool is about 5.6 times greater (28%). Increasing temperature to 60$^\circ$C in the second processing stage produced higher hydrolysis efficiency – at maximum levels of factors A and B it was as much as 37% decomposed wool (Fig. 3b). Nevertheless, general efficiency of hydrolysis with application...
of enzyme Everlase 6.0 T is lower than with enzyme Esperase 6.0 T (see breakdown results in Table 1 and compare Figs 2 and 3).

![Diagram](image)

**Note:** w/w – related to weighed-in quantity of dried wool

**Fig. 1:** Scheme of processing sheep wool into keratin hydrolysate.

**Evaluating breakdown of waste sheep wool**

Processing sheep wool by two-stage alkaline-enzymatic hydrolysis into keratin hydrolysate is marked by quite high breakdown efficiency. When testing two selected proteolytic enzymes, quantity of decomposed wool (depending on hydrolysis conditions) was 8.4-59.2% (with enzyme Esperase 6.0 T) or 4.7-39.6% wool (enzyme Everlase 6.0 T). For confirming the advantage of good efficiency exhibited by the mentioned two-stage wool breakdown, additional tests were run on decomposition of sheep wool. In these, wool was processed merely in the first stage of breakdown technology, conditions corresponding to minimum levels of factors A and B – added 3% Ca(OH)\(_2\) at 40 °C (i.e. Run 1), medium levels of factors A and B-addition of 6% Ca(OH)\(_2\) at 60 °C (meaning Run 5 or 6) and maximum levels of factors A and B-addition of 9% Ca(OH)\(_2\) at 80 °C (Run 10).

It was found that processing wool merely in the first stage of breakdown technology achieves half efficiency of breakdown (as compared to the two-stage process when Esperase 6.0 T was applied) –
quantity of decomposed wool was only 4 % with minimum levels of factors A and B, 8.9 % wool with medium levels of factors A and B, and 30.0 % wool with maximum levels of A and B.

At present, our research activity is focused on studying properties of keratin hydrolysates (composition, distribution of molecular masses, etc) which are indispensable for intended employment of keratin hydrolysates in practice (for example, as packaging materials or encapsulates in agriculture). A step taken further will be calculating economic costs of the whole hydrolytic process, which will be essential for possibly implementing technology in practice.

Fig.-2: Influence of added quantity of Ca(OH)$_2$ and temperature in first processing stage on quantity of wool decomposed during hydrolysis with enzyme Esperase 6.0 T: (a) temperature in second processing stage = 40 $^\circ$C; (b) temperature in second processing stage = 60 $^\circ$C.

Fig.-3:  Influence of added quantity of Ca(OH)$_2$ and temperature in first processing stage on quantity of wool decomposed during hydrolysis with enzyme Everlase 6.0 T: (a) temperature in second processing stage = 40 $^\circ$C; (b) temperature in second processing stage = 60 $^\circ$C.
CONCLUSION
Modeling technological conditions for hydrolytic breakdown of sheep wool in two stages (alkaline pre-processing and alkaline hydrolysis) proceeded utilising factor tests of $2^3$ types. Research studied the influence of 3 selected factors in hydrolysis on quantity of decomposed wool: addition of Ca(OH)$_2$, temperature in first processing stage and temperature in second processing stage. Statistic significance of studied factors was evaluated by F-test. Graphical presentation of results by means of contour graphs indicates potential to model conditions during breakdown in such manner that a product (keratin hydrolysate) of required properties (ash content, molecular mass) is obtained. The difference was also confirmed between wool breakdown efficiency when using proteolytic enzymes Esperase 6.0 T and Everlase 6.0 T.

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