The effects were assessed of high hydrostatic pressure on the activity and structure of rabbit skeletal muscle proteasome. The pressure effects on the activity were measured by the amount of fluorometric products released from synthetic substrates under pressure and from fluorescein isothiocyanate (FITC)-labeled casein after releasing the pressure. The effects on the structure were measured by fluorescence spectroscopy under pressure, and by circular dichroism (CD) spectroscopy and surface hydrophobicity after releasing the pressure. The optimal pressure for the hydrolyzing activity of synthetic peptides was 50 MPa. The degradation of FITC-labeled casein increased linearly with increasing pressure applied up to 200 MPa, and then markedly decreased up to at 400 MPa.

The changes in the tertiary structure detected by fluorometric measurement were irreversible, whereas the changes in the secondary structure were small compared with those by heat treatment. The pressure-induced activation of proteasome therefore seems to have been due to a little unfolding of the active sites of proteasome.

Key words: proteasome; high hydrostatic pressure; fluorescein isothiocyanate (FITC)-labeled casein; circular dichroism (CD) spectra; surface hydrophobicity

Proteasome or multicatalytic proteinase complex (MCP) was first isolated from bovine pituitaries. This enzyme is a high-molecular-mass intracellular proteinase (20S proteasome; 700 kDa) which has a complex subunit composition and multicatalytic proteolytic activities with different specificity.2–5

Proteasomes have been classified into two isoforms with apparent sedimentation coefficients of 20S and 26S proteasome (1,600 kDa), respectively. 20S proteasome is considered to be the core unit for the proteinase activity of 26S proteasome, and other components are assumed to be subunits that make the proteinase activity of 26S proteasome ATP-dependent. It has also been shown that ATP was essential for maintaining the 26S complex, and that a depletion of ATP caused rapid dissociation of the 26S complex into 20S proteasome and other components.6 However, under the post-mortem condition without ATP, it is necessary to investigate the properties of 20S proteasome.

20S proteasome has a cylinder-shaped structure arranged as four axially stacked heptameric rings composed exclusively of either α-subunits (two outer rings, α1–α7) or β-subunits (two inner rings, β1–β7), respectively (α7β7β7α7). The multiple catalytic sites of this proteolytic complex are exclusively associated with the β-subunits because of the particular structural topology of the particle, and they are assumed to be all sequestered within the hollow cavity of the cylinder.7 This proteasome has two unique enzymological properties as a protease: multiple peptidase activities, and a latent form. According to Mykles and Harie,8 the proteasome has at least five activities — peptidylglutamyl peptide hydrolase (PGPH), trypsin-like, chymotrypsin-like, branched-chain amino acid-preferring, and small neutral amino acid-preferring — within the single proteasome complex. Furthermore, the three major activities (PGPH, trypsin-like and chymotrypsin-like) have been respectively assigned to the three active subunits, β1, β2 and β5, based on mutational and crystal structural analysis studies.9,10 The proteasome isolated from tissues in a latent form can be activated by various chemicals and such treatments as polylysine,11,12 SDS11,13–15 and fatty acids,13) heat15–18) and high hydrostatic pressure.15,19 These results indicate that proteasome activity is stimulated by a mild denaturing treatment. However, it has not yet been made clear

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why the proteasome is activated, especially by a high hydrostatic pressure treatment.

High hydrostatic pressurization is one of the new technologies for tendering or accelerating of meat aging which is a very important process for the meat industry. The availability of intramuscular proteinases during meat aging has been well investigated. In particular, cathepsin and calpain have been assumed to participate in proteolysis during meat aging.\(^{20-22}\)

Several studies have been made on the effects of high hydrostatic pressurization to treat intramuscular proteinase. Homma \textit{et al.} have reported that the pressure-induced increase in proteolytic level in the muscle was due to a release of cathepsins from lysosomes.\(^{23}\) In respect of calpain systems, Suzuki \textit{et al.} have provided direct evidence for the pressure-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum by using electron micrographs of the pyroantimonate-fixed fiber bundles prepared from pressurized muscle tissue.\(^{24}\) Ca\(^{2+}\) dispersion into myofibrils may cause an increase in activated calpain. Homma \textit{et al.} have reported that the calpain level was retained in muscle pressurized up to 200 MPa, whereas the calpastatin level was lowered by pressurization. The total calpain activity in pressurized muscle therefore appears to be increased by a pressure treatment, and this may result in tenderization of the meat.\(^{25,26}\)

The activation of proteasome induced by high pressure may be involved in the pressure-induced modification or breakdown of muscle proteins.\(^{15,19}\) However, it is not yet clear why proteasome is activated by high hydrostatic pressure. This paper therefore describes the effects of a high hydrostatic pressure treatment on the structure and activities of rabbit skeletal muscle proteasome in comparison with those of a heat treatment.

\textbf{Materials and Methods}

\textit{Purification of proteasome.} Proteasome was purified from rabbit skeletal muscle by the method of Otsuka \textit{et al.}\(^{15}\) Briefly, a rabbit was anaesthetized with sodium pentobarbital (2.5 ml) and d-tubocurarine chloride pentahydrate (0.5 ml) just before exsanguination. The back and leg muscles were immediately excised and subjected to extraction. The crude proteasome that had been extracted was purified by chromatography in a DEAE-Sepacel column. The fractions with activity were subsequently concentrated and further purified in a phenyl Sepharose HP column. Those fractions with activity were finally concentrated and rechromatographed once again in the phenyl Sepharose HP column. The active fraction from the second chromatography in the phenyl Sepharose HP column is designated as purified proteasome.

\textit{Pressurization.} Pressurization was carried out with the method described by Homma \textit{et al.}\(^{27}\) Each purified proteasome solution was vacuum-sealed in a polyethylene bag, and this bag was transferred to a larger polyethylene bag. The space between the two bags was filled with water. Each double bag was then placed in a pressure vessel with water and subjected to 0.1–400 MPa at about 10 °C for 10 min with a cold isostatic press (CIP) from Nikkiso Co., Ltd., Tokyo, Japan.

\textit{Heat treatment.} The purified proteasome was heated at 20–80 °C for 30 min. After chilling on ice for 10 min, the sample was subjected to the subsequent analyses.

\textit{In situ measurement of proteasome activities under high pressure.} The proteasome activities were assayed by fluorometric measurement of the amount of 7-amino-4-methylcoumarin (\textendashNH-Mec; AMC) released from synthetic substrates. The substrates used were Suc-Leu-Leu-Val-Tyr-NH-Mec (LLVY; chymotrypsin-like activity) and Ala-Ala-Phe-NH-Mec (AAF; chymotrypsin-like activity) purchased from Sigma (St. Louis, MO, U.S.A.), and Boc-Leu-sec-Thy-NH-Mec (LSTR; trypsin-like activity) purchased from the Peptide Institute (Osaka, Japan), these being selected to assay distinct proteolytic activities of the proteasome. All methylcoumarylamide substrates were stored as 1 mM solutions in N,N-dimethylformamide at −20°C, and then diluted with water to a 20-μl solution prior to use.

An enzyme solution (300 μl) was mixed with 900 μl of 100 mM Tris–HCl (pH 8.0) and 300 μl of 6 mM DTT. After preincubating at 37°C for 5 min, 300 μl of a substrate was added to the reaction mixture.

The activities under high pressure (0.1–300 MPa, 0–30 min) were measured in a high-pressure vessel with a Hitachi F-2000 spectrofluorimeter at 480 nm, with excitation at 370 nm. This device consisted of a temperature-controlled high pressure vessel equipped with sapphire windows and a TP-500 high-pressure hydrostatic pump capable of elevating the pressure to 400 MPa (Teramecs Co., Kyoto, Japan). The vessel was positioned in the light beam of the spectrofluorimeter, and a quartz cuvette containing the sample solution was placed inside the vessel.

\textit{Determination of the FITC-labeled casein hydrolyzing activities under high pressure.} The mixture of the proteasome and FITC-labeled casein in 100 mM Tris–HCl and 1 mM DTT (pH 8.0, 2.0 ml) was pressurized to 0.1–400 MPa at 37°C for 90 min. After releasing the pressure, the products released from the FITC-labeled casein were measured by modified method of that presented by Lonergam \textit{et al.}\(^{28}\) A 600-μl amount of the reaction mixture was added to 600 μl of 5% trichloroacetic acid (TCA), and subsequently to 300 μl of 50 mg/ml of a casein buffer (50 mM Na\(_2\)CO\(_3\) and 150 mM NaCl at pH 9.5) to be coprecipitated with the remaining FITC-labeled casein. The TCA-insoluble protein was removed by centrifugation at 10,000 × g for 20 min. The resulting supernatant (1.0 ml) was again centrifuged under the
same conditions to clarify the supernatant. The supernatant (500 μl) was diluted to 2.5 ml with 500 mM Na₂HPO₄ (pH 8.5), before the fluorescence was measured at 515 nm, with excitation at 490 nm.

**Measurement of the fluorescence spectra and center of spectral mass of proteasome under high pressure.** A proteasome solution (0.03 mg/ml) in 40 mM Tris–HCl, 10 mM EDTA, 10 mM 2-mercaptoethanol and 100 mM NaCl (pH 7.5) was subjected to a high pressure of 0.1–400 MPa for 10 min. Changes in the fluorescence spectrum of the proteasome were measured with a Hitachi F-2000 spectrofluorometer fitted with a high-pressure vessel. The fluorescence spectra of the proteasome were recorded under pressure between 300–420 nm, with excitation at 280 nm.

Changes in the center of spectral mass \((\langle \nu \rangle)\) were calculated according to the method of Ruan et al.\(^{29}\)

\[
\langle \nu \rangle = \Sigma v_i \times F_i / \Sigma F_i
\]

where \(v_i\) is the wavenumber and \(F_i\) the fluorescence intensity at \(v_i\).

**Measurement of the secondary structure of proteasome.** The circular dichroism (CD) spectra of pressurized or heated proteasome solutions (0.1 mg/ml) in 40 mM Tris–HCl, 10 mM EDTA, 10 mM 2-mercaptoethanol and 100 mM NaCl (pH 7.5) were recorded by a Jasco J-725 spectropolarimeter at 20°C. The mean residual ellipticity \([\theta]\) is expressed in degrees⋅cm\(^2\)/d·mol. \([\theta]\) was calculated by using a molecular weight of 700,000 and 7,000 residues. The secondary structure of proteasome was analyzed with the program of Yang et al.\(^{30}\)

**Measurement of the surface hydrophobicity of proteasome.** The surface hydrophobicity of pressurized or heated proteasome solutions (0.05 mg/ml, in 40 mM Tris–HCl, 10 mM EDTA, 10 mM 2-mercaptoethanol and 100 mM NaCl (pH 7.5)) was measured according to the method of Boyer et al.\(^{31}\)

Either 12.5 μl of 1 mM cis-parinaric acid (cPA) or 8 μl of 5 mM 8-anilino-1-naphthalene-sulphonic acid (ANS) was added to 2.5 ml of the proteasome solution. The fluorescence intensity of the cPA-protein conjugates was measured at 410 nm with excitation at 325 nm. The excitation and emission wavelengths were 380 and 475 nm, respectively, for the ANSA-protein conjugates.

**Results and Discussion**

**Effects of high pressure on the synthetic peptides and FITC-labelled casein hydrolyzing activities**

Changes in the LLVY, LSTR and AAF hydrolyzing activities under high pressure are shown in Fig. 1A–C. During the 30-minute reaction, the degradation of synthetic peptides increased linearly with the applied pressure up to 150 MPa, and was greater than that of the unpressurized sample (0.1 MPa). A pressure of 200 MPa or more resulted in no progress in the degradation of LLVY or AAF after the first reaction of 2 min (Fig. 1A and C). However, LSTR degradation increased linearly at 200 MPa, and no progress in degradation was apparent after a first reaction for 3 min at 300 MPa (Fig. 1B). As shown in Fig. 1A–C, the optimal pressure for the hydrolyzing activity of the synthetic peptide used in this experiment was 50 MPa. Otsuka et al.\(^{15}\) have found that the optimal pressure for inducing the highest synthetic peptide hydrolyzing activity in the pressurized proteasome was about 100 MPa. This difference may have been due to different analytical methods: the former was measured in situ under high pressure, and the latter was measured only after the pressure had been released. The increase in proteasome activity was perhaps due to an increase in the interaction between the substrate and active site of the proteasome that had been unfolded by the high-pressure treatment.

The \(K_m\) and \(V_{max}\) values of proteasome for the synthetic peptides under high pressure are shown in Table 1. The calculated \(K_m\) and \(V_{max}\) values for LLVY and AAF were maximal at 50 MPa, the \(K_m\) and \(V_{max}\) values then continuing to decrease up to 200 MPa or higher. However, the \(K_m\) and \(V_{max}\) values for LSTR were not influenced by the pressure treatment. In the present experiment, it seems that the difference in the change of \(K_m\) and \(V_{max}\) values for LLVY/AAF vs. those for LSTR was caused by the difference in active subunits of proteasome. It is known that the chymotrypsin-like (LLVY, AAF) and trypsin-like (LSTR) activities of proteasome are respectively derived from active subunits β5 and β2. Our results suggest that the β5 subunits were more susceptible to pressure than the β2 subunits, resulting in increased LLVY and AAF hydrolyzing activity under a relatively low pressure.

The effects of pressurization on the casein-degrading activity are shown in Fig. 2. During the 90-minute reaction, the degradation of FITC-labeled casein increased linearly with increasing pressure applied up to 200 MPa. When 400 MPa was applied, the degradation of casein during the first 30 min of the reaction was higher than at 0.1 MPa (control)–150 MPa. However, after 30 min of the reaction, the progress of degradation was slower than that of the proteasome subjected to lower pressures, and finally after 90 min of the reaction, the degradation was less than that of proteasome pressurized at 50–200 MPa. This activation of caseinolytic activity was probably due to an increase in the interaction between the substrate and active site of the proteasome unfolded by the high-pressure treatment.

The difference in optimal pressure for the hydrolyzing activities of the synthetic peptides and FITC-labeled casein may have been derived from two factors: the difference in the active site of proteasome for the substrate, and the higher susceptibility of denatured protein to the proteasome than that of the non-denatured proteins. In the case of casein, it seems that the
Fig. 1. Effect on the Hydrolyzing Activities of Synthetic Peptides.
A, LLVY hydrolysis; B, LSTR hydrolysis; C, AAF hydrolysis. ○, 0.1 MPa (Control); ●, 50 MPa; △, 100 MPa; ▲, 150 MPa; □, 200 MPa; ■, 300 MPa. Excitation wavelength = 370 nm. Emission wavelength = 480 nm.
high-pressure treatment activated the proteasome activities in parallel with casein denaturation. Therefore, the caseinolytic activity of the proteasome was increased. However, when excessive pressure was applied, the proteasome was denatured and inactivated. Gardrat et al. 19) have reported that the caseinolytic activity progressively increased up to 150 MPa, and was inactivated at 450 MPa. These differences in caseinolytic activity may have been caused by differences in the proteasome preparation, purification method and reaction method.

Changes in the fluorescence spectra and center of spectral mass of proteasome under high pressure

Changes in the fluorescence spectra are shown in Fig. 3. The red shift and decrease in fluorescence of the proteasome gradually progressed with increasing high pressure applied. This indicates that the environmental polarity of the tryptophan residues became stronger due to a pressure-induced change in the tertiary structure. The center of mass (\( \langle v \rangle \)) calculated from Fig. 3. is shown in Fig. 4. The \( \langle v \rangle \) value decreased gradually with increasing pressure applied. Furthermore, the \( \langle v \rangle \) values during decompression were lower than those at the same pressures during compression. In other words, hysteresis in the recovery of the fluorescence parameters was not apparent during decompression. The \( \langle v \rangle \) values therefore suggest that high-pressure-induced structural change in proteasome was irreversible (a minute structural change led to activation of the proteasome).

Changes in the secondary structure of proteasome

Changes in the secondary structure of proteasome induced by high pressure and heat treatment are shown in Tables 2 and 3, respectively. The \( \alpha \)-helix content of the proteasome was lower at 400 MPa, although the change was not significant. During heat treatment of the proteasome, the \( \alpha \)-helix content gradually decreased with increasing temperature. The decrease in \( \alpha \)-helix content was especially significant at 70 \( ^\circ \)C and 80 \( ^\circ \)C. The change obtained in this experiment in the secondary structure of the proteasome does not correlate with the results of Otsuka et al. 15) Otsuka et al. reported that the synthetic peptide hydrolyzing activity increased with increasing applied pressure up to 150 MPa, and then decreased at 200 MPa or higher. They suggested that the activation of proteasome induced by high pressure might be one cause of the pressure-induced modification of the proteasome structure. However, from our results, such pressure-induced modification of the proteasome structure was not apparent. On the other hand, Otsuka et al. have reported that the inactivation of proteasome was caused by excessive pressurization or heat treatment. The results of our experiment suggest that the reduction

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max} ) (pmol/min/ml)</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max} ) (pmol/min/ml)</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max} ) (pmol/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>30.63</td>
<td>397.08</td>
<td>16.97</td>
<td>139.63</td>
<td>12.36</td>
<td>173.13</td>
</tr>
<tr>
<td>50</td>
<td>41.95</td>
<td>685.32</td>
<td>17.18</td>
<td>195.49</td>
<td>16.06</td>
<td>270.36</td>
</tr>
<tr>
<td>100</td>
<td>34.09</td>
<td>509.25</td>
<td>17.14</td>
<td>167.19</td>
<td>15.33</td>
<td>209.10</td>
</tr>
<tr>
<td>150</td>
<td>31.14</td>
<td>453.80</td>
<td>17.13</td>
<td>160.84</td>
<td>11.41</td>
<td>161.19</td>
</tr>
<tr>
<td>200</td>
<td>8.02</td>
<td>67.95</td>
<td>15.01</td>
<td>117.65</td>
<td>5.01</td>
<td>54.05</td>
</tr>
<tr>
<td>300</td>
<td>7.89</td>
<td>61.98</td>
<td>10.20</td>
<td>83.60</td>
<td>3.23</td>
<td>27.18</td>
</tr>
</tbody>
</table>
Fig. 3. Fluorescence Spectra and Center of Spectral Mass of Proteasome under High Pressure. 
A, 50 MPa; B, 100 MPa; C, 200 MPa; D, 400 MPa. —, before pressurization; —, under pressure; —, after pressurization. Excitation wavelength = 280 nm. Emission wavelength range = 280–420 nm.

Table 2. Secondary Structure of Pressure-Treated Proteasome

<table>
<thead>
<tr>
<th>Pressure (MPa)</th>
<th>Helix</th>
<th>Beta</th>
<th>Turn</th>
<th>Random</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>48.07 ± 1.65</td>
<td>19.43 ± 5.25</td>
<td>8.57 ± 6.19</td>
<td>23.97 ± 0.58</td>
<td>100.0</td>
</tr>
<tr>
<td>50</td>
<td>49.07 ± 0.78</td>
<td>17.13 ± 8.29</td>
<td>10.83 ± 8.18</td>
<td>22.93 ± 2.29</td>
<td>100.0</td>
</tr>
<tr>
<td>100</td>
<td>46.65 ± 1.63</td>
<td>22.05 ± 1.06</td>
<td>6.25 ± 4.17</td>
<td>25.15 ± 1.48</td>
<td>100.0</td>
</tr>
<tr>
<td>200</td>
<td>47.13 ± 5.40</td>
<td>20.23 ± 2.91</td>
<td>7.30 ± 3.40</td>
<td>25.33 ± 1.15</td>
<td>100.0</td>
</tr>
<tr>
<td>300</td>
<td>46.03 ± 5.09</td>
<td>21.33 ± 2.97</td>
<td>8.30 ± 4.76</td>
<td>24.33 ± 1.36</td>
<td>100.0</td>
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<tr>
<td>400</td>
<td>42.53 ± 7.42</td>
<td>22.93 ± 1.48</td>
<td>7.20 ± 4.44</td>
<td>27.33 ± 5.05</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Each value is expressed as the mean ± standard deviation (n = 4); *p < 0.05.

Fig. 4. Pressure Dependence of the Center of Spectral Mass of Proteasome. 
○, compression; ●, decompression.
in the amount of α-helix in proteasome at 400 MPa or at 70 °C and 80 °C led to inactivation of the proteasome.

Measurement of the surface hydrophobicity of proteasome

The changes in surface hydrophobicity measured by cPA and ANSA are shown in Figs. 5 and 6, respectively. No significant change in aliphatic hydrophobicity was apparent in the pressurized proteasome. However, an increase in aliphatic hydrophobicity was observed in the proteasome heated at temperatures over 60 °C, especially at 80 °C. Significant changes in the aromatic hydrophobicity were observed in both the pressurized and heated proteasome. The aromatic hydrophobicity markedly increased at above 70 °C and above 300 MPa. It seems that the increase in aliphatic and aromatic hydrophobicity of the pressurized proteasome had almost no effect on the proteasome activity. Otsuka et al. have reported that when the proteasome was heated at 60 °C for 20 min and pressurized to 100–150 MPa, its activity increased. However, when the proteasome was heated at 70 °C or higher and pressurized to 200 MPa or higher, the activity gradually decreased.15) Our results indicate that when the proteasome was heated at 50–60 °C, increases in the surface aliphatic and aromatic hydrophobicity led to an increase in the proteasome activity. Above 70 °C, however, this increase in the aliphatic and aromatic hydrophobicity caused denaturation of the proteasome.

The results of this study demonstrate that high hydrostatic pressurization led to proteasome activation and irreversible change (excessive structural change leading inactivation of the proteasome) in the tertiary structure as detected from fluorometric measurements, whereas the change in secondary structure was slight compared with that by heat treatment. Therefore, pressure-induced activation of the proteasome seems to have been due to a small amount of unfolding of the active sites of proteasome. Further experiments will be required to clarify this result.

We can presently conclude that proteasome may play a role in high-pressure-induced meat tenderization or the acceleration of meat aging, in addition to better-known cathepsin and calpain.

Table 3. Secondary Structure of Heat-Treated Proteasome

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
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<th>Beta</th>
<th>Turn</th>
<th>Random</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>47.27 ± 2.99</td>
<td>19.90 ± 6.29</td>
<td>10.40 ± 10.82</td>
<td>22.43 ± 4.65</td>
<td>100.0</td>
</tr>
<tr>
<td>40</td>
<td>46.83 ± 4.69</td>
<td>19.38 ± 2.15</td>
<td>8.45 ± 3.76</td>
<td>24.58 ± 2.83</td>
<td>100.0</td>
</tr>
<tr>
<td>50</td>
<td>45.98 ± 5.82</td>
<td>20.03 ± 4.29</td>
<td>9.85 ± 4.89</td>
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<td>60</td>
<td>46.98 ± 5.95</td>
<td>18.30 ± 8.18</td>
<td>9.00 ± 5.76</td>
<td>25.73 ± 3.11</td>
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<tr>
<td>70</td>
<td>38.38 ± 5.91*</td>
<td>26.28 ± 10.02</td>
<td>7.45 ± 7.44</td>
<td>27.85 ± 3.72</td>
<td>100.0</td>
</tr>
<tr>
<td>80</td>
<td>31.75 ± 14.87*</td>
<td>8.90 ± 8.00</td>
<td>19.15 ± 8.89</td>
<td>40.18 ± 14.13*</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Each value is expressed as the mean ± standard deviation (n = 4); *p < 0.05.

Fig. 5. Effect of Heat and Pressure Treatments on the Aliphatic Hydrophobicity of Proteasome.

○, pressure treatment; ●, heat treatment. Excitation wavelength = 325 nm. Emission wavelength = 410 nm. Each value is expressed as the mean ± standard deviation (n = 6); *p < 0.01.
Acknowledgment

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References


