The Solubilization of Unheated Cattle Achilles Tendon with Actinidin under Neutral and Acidic Conditions

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Received June 10, 2003; Accepted October 17, 2003

The solubilization of cattle achilles tendon with actinidin was investigated under neutral and acidic conditions. 1.43 to 1.92 and 0.97 to 3.19% of collagen were solubilized by treating the cattle achilles tendon with actinidin at 20˚C at pH 6.0 and 3.3, respectively. Furthermore, SDS-polyacrylamide gel electrophoresis of reaction mixtures with actinidin demonstrated that actinidin degraded the tendon into collagen subunit chain, β- and α-chain and peptide fragments of various sizes at 20˚C at pH 6.0 and 3.3. These results indicated that actinidin could solubilize the insoluble collagen in unheated cattle achilles tendon at 20˚C at pH 6.0 and 3.3 and that a large proportion of the resulting peptide fragments by actinidin seemed to be actinidin digests against elastin with a small contribution of hydroxyproline.

Keywords: unheated cattle achilles tendon, actinidin, solubilization, neutral and acidic conditions

Tenderness is an important attribute of meat that affects its acceptability. High hydrostatic pressurization is one of the techniques for tenderizing meat (Suzuki et al., 1993; Cheftel & Culioli, 1997). Wada et al. (2002) showed that the kiwi fruit protease (crude actinidin) treatment with 0–500 MPa pressure could tenderize adult cattle semitendinosus muscle and degrade insoluble collagen in connective tissue into collagen subunit chain, α-chain. However, the relationships between the pressure-induced changes and kiwi fruit protease treatment had not been clear. Plant thiol proteinases, such as ficin, papain and bromelain, have been used in tenderizing meat. The action of the proteolytic enzymes is preferentially against connective tissue fibres. Actinidin is one of the plant thiol proteinases, and is present in fruits of the kiwi (Kamphuis et al., 1985). Ohyama et al. (1997) showed that when type I collagen from rat skin tendon was treated with crude actinidin at 27˚C for 60 min at pH 3, 6 or 9, insoluble collagen was converted to collagen subunit chain, α-chain at pH 3, but not converted at pH 6 or 9. Dominant constituents of connective tissue are collagen in collagen fibre and elastin in elastic fibre, and tenderness variations due to connective tissue are related to collagen content, perimysial fibre diameter and cross-linking (Foged & Laick, 1986). But the plant thiol proteinases do not attack native collagen, they act on the collagen as it is denatured by heat during cooking (Lawrie, 1998) and it has been generally accepted that the collagen in cattle connective tissue is liable to denature to temperatures above 25˚C or below 0˚C.

In the present study, the composition and arrangement of intramuscular connective tissue of cattle semitendinosus muscle was estimated to be similar to those of connective tissue in the cattle achilles tendon, so the tendon was used as an alternate for cattle semitendinosus muscle. Unheated cattle achilles tendon was treated with actinidin at 20˚C under neutral and acidic conditions, and then the solubilization product of the tendon was characterized by determination of the hydroxyproline content and SDS-PAGE analysis of the actinidin digests.

Materials and Methods

Material and actinidin  Cattle achilles tendon was excised from a 6-year-old cattle within 1 day postmortem. The tendon was cleaned of adhering fat and muscle, cut into small pieces, and thoroughly washed in 0.05 M Tris-HCl (pH 6.8) and distilled water. The purified fibre of tendon was centrifuged at 4˚C at 13,000×g for 15 min, and used as substrate (Steven, 1967). All these steps were performed at 4˚C. A preliminary extraction of tendons to remove soluble collagen and non-collagenous protein was not carried out, because they constitute only a negligible proportion of the total protein in the achilles tendon (Wada et al., 2000; Einbinder & Schubert, 1951). Enzyme, actinidin (EC 3.4.22.14) was purchased from Bio Pur AG and used without further purification. The collagen model peptide, DNP-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH, was purchased from the Peptide Institute Corp., and also used without further purification.

Actinidin digests  The effects of pH on the solubilization of cattle achilles tendon with actinidin were investigated under neutral and acidic conditions. Under a neutral condition, the buffer used was 0.1 M sodium phosphate buffer/0.5 M NaCl (pH 6.0) (A). Under an acidic condition, two different pH buffers were used, 0.2 mM sodium formate/20 mM EDTA/10 mM cysteine/0.35% acetic acid (pH 3.3) (B); and 0.5 M acetic acid (pH 2.7–2.9) (C), because the high activities of kiwi fruit protease are shown in low pH regions (Sugiyama et al., 1997). The cattle achilles tendon was treated with actinidin at 20˚C for 0, 4, 8, 16 and 24 h, respectively, in each of the following mediums, A, B or C. Zero point zero one milligram of actinidin was added to each reaction vessel containing 0.2 mg of the tendon samples in 1 ml of the three different solutions. The reaction mixtures were stirred at 20˚C for 0, 4, 8, 16, and 24 h, respectively, after which they were heated at 90˚C for 5 min to stop the enzymatic reaction by the
method of Takagi et al. (1992). The control experiment was carried out at 20°C for 24 h in the same reaction mixture in the absence of actinidin in each medium and heated at 90°C for 5 min.

SDS-polyacrylamide gel electrophoresis The effects of actinidin on cattle achilles tendon proteins were investigated using SDS-polyacrylamide gel electrophoresis. The reaction mixtures after the enzyme inactivation were then centrifuged at 13,000×g at 10°C for 15 min; thereafter 50 μl of 0.1 M Tris-HCl/1% SDS (sodium dodecyl sulfate) (pH 6.8), 2 μl of 2-mercaptoethanol, and 3 μl of 0.1% bromophenol blue were added to 50 μl of each supernatant sample, and the mixture was heated at 90°C for 10 min. Ten microliters aliquot samples were treated by SDS-PAGE with a 10% acrylamide separating gel, and the extent of actinidin digest was assayed by comparing electrophoretic patterns of sample with actinidin with patterns from the control sample (no actinidin) in accordance with the method of Laemmli (1970). Gels were stained with Coomassie Brilliant Blue (CBB).

Determination of hydroxyproline The hydrolysis of collagen and elastin by actinidin was determined according to the method of Nishihara et al. (1965). The reaction mixtures after the enzyme inactivation were ultracentrifuged at 59,000×g at 10°C for 30 min and then 1 ml of the supernatant was lyophilized. One milliliter of 6 M HCl was added to the lyophilized sample, and it was hydrolyzed at 110°C for 24 h. The hydroxyproline in the sample was quantitatively determined by the method of Woessner (1961). The hydroxyproline content was multiplied by 7.25 (Palka, 1999) to yield the solubilized collagen weight. The solubilization percent was calculated on the basis of starting material of tendon.

Digestion of collagen model peptide with actinidin The digestion of DNP-peptide with actinidin was analyzed by protein sequencer. The amino acid sequence of collagen model peptide, DNP-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH, is identical to that of amino acid residues, 774–780, of bovine collagen at α I (I) chain (Fietzek & Kuhn, 1976). Duplicate samples containing a total of 0.1 mg of collagen model peptide were dissolved in 100 μl of 0.5 M acetic acid containing 0.01 mg of actinidin and digested at 30°C for 60 min or 24 h. The PTH (phenyl thiophydantoin)-amino acids in the reaction mixtures were assayed by using a Shimadzu PPSQ-23, gas-phase protein sequencer (Shimadzu Corp.) (Ueda et al., 1997).

Results and Discussion

Actinidin-solubilized collagen The solubilization of the cattle achilles tendon was measured by another method. Hydroxyproline content was measured to determine the actinidin-solubilized collagen in the reaction mixtures after the enzyme inactivation (medium A, B or C). Collagen content was calculated assuming about 13% hydroxyproline in collagen by weight and ignoring the small contribution of hydroxyproline from elastin (Senior et al., 1975). Table 1 shows how much collagen solubilized by treating with actinidin in medium A, B or C at 20°C for 4, 8, 16 and 24 h. Under a neutral condition (medium A), the actinidin-solubilized collagen cattle achilles tendon was at the level of 1.43 to 1.92%. Under an acidic condition, the actinidin-solubilized collagen was at the level of 0.97 to 3.19% (medium B) and 0.10 to 0.26% (medium C), respectively. This result indicated that actinidin could digest the tendon at 20°C at pH 3.3 and 6.0, but digest little of it at 20°C at pH 2.7–2.9. Since acid soluble collagen in the tendon was estimated to be below<0.1% (Wada et al., 2000), the actinidin-solubilized collagen seemed to be the actinidin digests of the insoluble collagen in the tendon.

SDS-PAGE analysis To examine the mode of cattle achilles tendon degradation by actinidin, we examined its electrophoretic patterns. The electrophoretic patterns of control samples, which were carried out at 20°C for 24 h in the same reaction mixture in the absence of actinidin and heated at 90°C for 5 min, were not detected (data not shown). The result indicated that the control samples did not contain collagen subunit chain, β- and α-chain. Figure 1 shows that the resulting gel patterns were detected in the reaction mixtures after the enzyme inactivation (medium A, B or C) after 8 h at 20°C. Under a neutral condition (medium A), collagen subunit chain, β- and α-chain and peptide frag-

Table 1. Effect of actinidin on cattle achilles tendon.

<table>
<thead>
<tr>
<th>Medium</th>
<th>0 h</th>
<th>4 h</th>
<th>8 h</th>
<th>16 h</th>
<th>24 h</th>
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<tr>
<td>Medium A</td>
<td>0</td>
<td>1.92</td>
<td>1.43</td>
<td>1.92</td>
<td>1.85</td>
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<tr>
<td>Medium B</td>
<td>0</td>
<td>0.97</td>
<td>1.27</td>
<td>3.19</td>
<td>3.17</td>
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<tr>
<td>Medium C</td>
<td>0</td>
<td>0.14</td>
<td>0.22</td>
<td>0.10</td>
<td>0.26</td>
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Cattle achilles tendon was treated with actinidin at 20°C for 0, 4, 8, 16 and 24 h, respectively, in medium A, B or C.

Values: means of two measurements.

Medium A: 0.1 M sodium phosphate buffer/0.5 M NaCl (pH 6.0).
Medium B: 0.2 mM sodium formate/20 mM EDTA/10 mM cysteine/0.35% acetic acid (pH 3.3).
Medium C: 0.5 M acetic acid (pH 2.7–2.9).
ments of various sizes were detected (lane b). Under an acidic condition, collagen subunit chain, β- and α-chain and many variously sized peptide fragments were clearly detected in the reaction mixture with actinidin (medium B) (lane c), but few were detected in the reaction mixture with actinidin (medium C) (lane d). This result indicated that actinidin could degrade the insoluble collagen in the cattle achilles tendon into collagen subunit chain, β- and α-chain at 20°C at pH 3.3 and 6.0, but degrade it little at 20°C at pH 2.7–2.9 and that a large proportion of peptide fragments of various sizes at 20°C under neutral and acidic conditions seemed to be elasin digests in the tendon by actinidin, because it has been reported that papain and bromelain have a high hydrolitic activity for elastin under a neutral condition (Takagi et al., 1992) and papain also has more hydrolitic activity against elastin than collagen (Senior & Bielefeld, 1972).

Collagen model-peptide digests Collagen model-peptide has generally been employed as substrate for the assay of peptidase activity of collagenase preparation, the DNP-peptide fragments released were determined by measuring the absorbances at 365 nm, and activities were calculated as change in absorbance of various sized peptide fragments at 20°C under neutral and acidic conditions, collagen subunit chain, α-chain at 20°C at pH 3.3 and 6.0, but degrade it little at 20°C at pH 2.7–2.9 and that a large proportion of peptide fragments of various sizes at 20°C under neutral and acidic conditions seemed to be elasin digests in the tendon by actinidin, because it has been reported that papain and bromelain have a high hydrolitic activity for elastin under a neutral condition (Takagi et al., 1992) and papain also has more hydrolitic activity against elastin than collagen (Senior & Bielefeld, 1972).

<table>
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<tr>
<th>PTH amino acid</th>
<th>1</th>
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<th>3–2</th>
<th>4–3</th>
<th>5–4</th>
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<td>0</td>
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<td>—</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.6</td>
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<tr>
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<tr>
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<td>—</td>
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<td>1.1</td>
<td>0</td>
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A total of 0.1 mg of collagen model peptide was dissolved in 100 μl of 0.5 m acetic acid containing 0.01 mg of actinidin and digested at 30°C for 60 min or 24 h.

Table 2. Edman degradation assay of DNP-peptide digests with actinidin.

References