Effect of Stages of Lactation on the Concentration of a 90-Kilodalton Heat Shock Protein in Bovine Mammary Tissue

ATSUMI WATANABE,* TORU MIYAMOTO,* NORIO KATOH,†,1 and YUJI TAKAHASHI‡
*Hokkaido Branch Laboratory, National Institute of Animal Health, 4 Hituisjigaoka, Sapporo 062, Japan
‡Laboratory of Biochemistry, National Institute of Animal Health, 3-1-1 Kannondai, Tsukuba, Ibaraki 305, Japan

ABSTRACT

From the normal mammary tissue of a Holstein cow in late lactation, a heat shock protein (90 kDa) was purified by ammonium sulfate fractionation and five-step column chromatography. From 70 g of tissue, 9.5 mg of this heat shock protein were obtained; samples had 98% purity and 19% recovery. The molecular mass of the 90-kDa heat shock protein was estimated to be 86 kDa by SDS-PAGE. Analysis of the amino-terminal amino acid sequence suggested that the protein had been purified as a mixture of two isoforms. The contents of the heat shock protein in cytoplasmic fractions of mammary tissues from Holstein heifers and cows at lactation and involution were measured by quantitative immunoblot analysis using rabbit antiserum raised against the purified heat shock proteins. The contents of the heat shock protein were higher in tissues from lactating cows than in those from heifers and involuting cows. The elevated concentrations of cytoplasmic 90-kDa heat shock protein in lactating tissue suggested that this protein is involved in mammary differentiation and lactation.

(Key words: heat shock protein, mammary tissue, purification, lactation)

Abbreviation key: HSP90 = 90-kDa heat shock protein.

INTRODUCTION

In response to harmful exogenous stimuli, including elevated temperature, transition metals, and AA analogues, the synthesis of a limited number of heat shock proteins, or stress proteins, is induced in a wide variety of cells (12, 21). The 90-kDa heat shock protein (HSP90), one of the major heat shock proteins, exists in relatively high amounts in cells under normal conditions (12) and is highly conserved during evolution (8, 15). This HSP90 has been suggested to be involved in cellular growth and differentiation (12) via the regulation of casein kinase II (11, 14) or steroid hormone receptors (4). In the murine mammary gland, HSP90 is modulated during development and differentiation; the highest concentration of HSP90 is present during lactation (5). Concentration of HSP90 is thought to be elevated by estrogenic stimulation (5).

In various types of cells of nonruminant animals, HSP90 has been shown to be composed of α and β isoforms (8, 15, 20). These two isoforms are induced differently by several stimuli (7) and by several tissue or cell types (1, 19). In cattle, HSP90 has been purified from testis, and the purified protein included only one isoform that corresponded to the human α form (22).

Although HSP90 is a ubiquitous cellular protein and is thought to be important for differentiation of cells, including mammary epithelial cells, its presence and isoform pattern in bovine mammary tissue have not yet been well demonstrated. In the present study, we purified HSP90 from bovine mammary tissue and suggested the presence of two isoforms of HSP90. We produced a specific antiserum to this protein, and, using this anti-HSP90 serum, we evaluated the HSP90 concentrations in healthy mammary tissues of Holstein cows at several developmental stages and in mastitic tissues during involution.

MATERIALS AND METHODS

Mammary Tissues

From a local slaughterhouse, we obtained mammary glands of Holstein heifers (n = 4; 2 yr old) or
cows, including healthy lactating tissues from cows that were 3 to 5yr old (n = 7; samples taken at 5, 30, 60, 115, 180, 186, and 212 d after parturition), involuting tissues from cows that were 3 to 6yr old (n = 4; samples taken at 7 to 30 d from the last suckling), and involuting tissues from cows with mastitis caused by Staphylococcus aureus (n = 5; 3 to 6yr old cows). Mammary samples were quickly frozen and stored at −80°C prior to use.

**Purification of HSP90**

All steps for purification were performed at 4°C. The HSP90 in each fraction was monitored by immunoblot analysis using monoclonal anti-human HSP90 IgG or by measurement of absorbance at 280 nm. Mammary tissue (70 g) minced with a meat grinder was homogenized in 210 ml of an extraction buffer containing 20 mM Tris-HCl (pH 7.5), 2.5 mM ethylene glycol bis(2-aminoethyl)ether)tetraacetate, 10 mM 2-mercaptoethanol, 1 mM adenosine 5’-triphosphate, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml of leupeptin for 30 s using a Polytron homogenizer (Kinematica, Littau, Switzerland) with a PTA-20S shaft at setting 6. The homogenate was centrifuged at 38,000 × g for 40 min. After filtration through glass wool, the supernatant (crude extract) was mixed with the same volume of 2.7 M ammonium sulfate in buffer A [20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 10 mM 2-mercaptoethanol]. After stirring for 1 h, the mixture was centrifuged at 10,000 × g for 30 min. The supernatant was applied to a column (2.2 × 20 cm) packed with Macro-Prep t-butyl HIC support (Bio-Rad Laboratories, Hercules, CA) equilibrated with buffer A plus 1.35 M ammonium sulfate. After washing with 2 column vol of the solution, the bound proteins were eluted with a linear gradient of 1.35 to 0 M ammonium sulfate in buffer A. The fractions that were rich in HSP90 were pooled, dialyzed against buffer A, and applied to a DEAE Bio-Gel A column (1.7 × 11 cm; Bio-Rad Laboratories) equilibrated with buffer A. The absorbed proteins were washed with two column volumes of buffer A, and then were eluted by a linear gradient of 0 to 0.4 M sodium chloride in buffer A. The fractions that were rich in HSP90 were pooled and concentrated by ultrafiltration (Amicon YM10 membrane; Amicon, Beverly, MA). The concentrated proteins were applied to a column (2.6 × 81 cm) packed with Ultrogel AcA34 (Bio Sepra, Marlborough, MA), equilibrated with buffer B [20 mM potassium phosphate (pH 7.5), 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 0.1 M sodium chloride], and eluted with the same buffer solution at a flow rate of 0.35 ml/min. The fractions that were rich in HSP90 were pooled and applied to an HA-Ultrogel column (1.4 × 5.5 cm; Bio Sepra) equilibrated with buffer B. The bound proteins were washed with five column volumes of buffer B, and then were eluted with a linear gradient of 20 to 400 mM potassium phosphate in buffer B. Fractions that were rich in HSP90 were pooled and dialyzed against buffer A plus 0.05 M sodium chloride. The dialysate was applied to a Q Sepharose Fast Flow column (1.4 × 6.0 cm; Pharmacia Biotech, Uppsala, Sweden). After absorbed proteins were washed with buffer A plus 0.05 M sodium chloride, they were eluted by generating a linear gradient of 0.05 to 0.75 M sodium chloride in buffer A. The eluted HSP90 was dialyzed against buffer A with 0.1 M sodium chloride and 20% glycerol and stored at −80°C until use. The HSP90 in the eluate of the Q Sepharose column was further purified by preparative SDS-PAGE (2, 10).

**Amino-Terminal AA Sequencing**

Chromatographically purified HSP90 was subjected to SDS-PAGE. The HSP90 on the polyacrylamide gel was electrically transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, MA), and the band corresponding to HSP90 was cut out. Amino-terminal sequencing of the HSP90 was carried out using a protein sequencer (PSQ-1; Shimadzu Corp., Kyoto, Japan).

**Preparation of Antiserum**

The HSP90 purified by preparative SDS-PAGE was used as an antigen. Approximately 1 mg of the HSP90 in 0.5 ml of Tris-buffered saline [20 mM Tris-HCl (pH 7.4) and 0.15 M sodium chloride] was emulsified with an equal volume of Freund's complete adjuvant (Nakalai Tesque, Kyoto, Japan) and injected intracutaneously into the back of a rabbit. Two weeks later, two consecutive booster injections were given at 2-wk intervals. In each of the booster injections, 0.5 mg of the HSP90 in an emulsion consisting of 0.25 ml of Tris-buffered saline and the same volume of incomplete Freund's adjuvant was given in the same manner as in the first injection. Ten days after the last booster immunization, the rabbit was bled, and the serum was separated. The antiserum was stored at −20°C until use.

**Immunoochemical Detection and Quantification of HSP90**

Protein samples were subjected to SDS-PAGE and electrically transferred to a nitrocellulose membrane

Figure 1. Immunochemical detection of a 90-kDa heat shock protein (HSP90) in bovine mammary tissue using anti-human HSP90 monoclonal IgG. Proteins (15 mg per lane) of mammary crude extract were subjected to SDS-PAGE and immunoblot analyses. Lane 1: acid violet 17 (AV17) staining of the total transferred protein. Proteins were transferred after fixation with acetic acid and water in a 5:95 ratio (vol/vol), destained with ethanol, acetic acid, and water in a 25:10:65 ratio (vol/vol/vol), and visualized with 0.5% (wt/vol) AV17 in acetic acid and water in a 5:95 ratio (vol/vol). Lane 2: Immunoblot analysis of bovine HSP90 by mouse anti-human HSP90 monoclonal IgG. The positions of molecular mass marker proteins are indicated on the left.

Other Methods

Protein concentration was measured by the method of Bradford (3) using bovine gamma globulin (Bio-Rad Laboratories) as the standard. Separation of mammary cytoplasmic and nuclear fractions was carried out according to the methods of Katoh et al. (9). Data were analyzed by one-way ANOVA and Dunnett’s t-test.

RESULTS

Bovine HSP90 was purified from the mammary tissue of cows in late lactation, and HSP90 concentration was monitored by immunoblot analysis using monoclonal anti-human HSP90 IgG (clone 3B6; Affinity BioReagents, Golden, CO) was used as the first antibody at a 100-fold dilution, and donkey anti-mouse IgG antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories) was used as the second antibody.

To screen HSP90 in each fraction during purification, mouse monoclonal anti-human HSP90 IgG (clone 3B6; Affinity BioReagents, Golden, CO) was used as the first antibody at a 100-fold dilution, and donkey anti-mouse IgG antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories) was used as the second antibody.
that bovine mammary tissue contains both \( \alpha \) and \( \beta \) isoforms and also assuming that the sequence of the \( \alpha \) isoform from this tissue was identical to the \( \alpha \) isoform of the bovine testis. The \( \alpha \) and \( \beta \) sequences were presumed to be identical to those of the \( \alpha \) isoform of the bovine testis and highly homologous to the human \( \beta \) isoform, respectively (Table 3), suggesting that HSP90 of the bovine mammary gland had been purified as a mixture of \( \alpha \) and \( \beta \) isoforms.

Results of the immunoblot analysis showed that rabbit antiserum to the electrophoretically purified mammary HSP90 stained the 86-kDa band in the crude extract, as did the mouse monoclonal antibody (Figure 4). Using this antiserum, HSP90 was quantified by immunoblot analysis. A linear calibration curve was obtained ranging from 0.2 to 1.6 \( \mu \)g of HSP90 in 10 \( \mu l \) of SDS-PAGE sample buffer (Figure 5). This quantitative method was applied to measure HSP90 contents in cytoplasmic and nuclear fractions from mammary samples.

The HSP90 accounted for 0.15, 1.25, and 0.54% of cytoplasmic proteins in healthy mammary tissue from cows that were in nulliparous, lactating, and involution stages, respectively (Table 4). The concentration of cytoplasmic HSP90 was higher in lactating tissue \((P < 0.001)\) than that in tissues from nulliparous heifers or cows during involution (Table 4). Although the samples from lactating cows were distributed over a wide range of lactating periods (5 to 212 d from parturition), HSP90 contents were not as different and were maintained at high concentrations (Table 4). The HSP90 content in tissue from cows during involution was higher \((P < 0.05)\) than that in tissue from nulliparous heifers (Table 4). The HSP90 contents of healthy and mastitic tissues from cows during involution varied greatly among tissues. Although mastitic tissues during involution contained a 1.5-fold higher amount of HSP90 than did healthy tissues at the same stage, the difference between these two groups was not significant (Table 4). The HSP90 could not be detected in the nuclear fractions of any of the samples examined.

**DISCUSSION**

In this study, we established a procedure to purify HSP90 from bovine mammary tissue. Identification of HSP90 was confirmed by crossreactivity to anti-human HSP90 monoclonal IgG and conservation of the amino-terminal AA sequence. Purified HSP90 could be used for immunization to produce a specific antiserum to bovine HSP90.

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**TABLE 1. Steps to purify 90-kDa heat shock protein.**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Purity</th>
<th>Recovery</th>
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<tr>
<td>Crude extract</td>
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<td>0.93</td>
<td>100</td>
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<tr>
<td>Ammonium sulfate fractionation</td>
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<td>ND</td>
<td>ND</td>
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<tr>
<td>Macro-Prep t-buty1 HIC support</td>
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<td>DEAE Bio-Gel A</td>
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</tr>
<tr>
<td>Q Sepharose Fast Flow8</td>
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<td>96</td>
<td>19</td>
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1 Determined by the method of Bradford (3).
2 Measured by quantitative immunoblotting (see Figure 5).
3 Calculated from the amounts of total protein and quantitative immunoblotting.
4 Not determined.
5 Bio-Rad Laboratories (Hercules, CA).
6 Measured by densitometry after SDS-PAGE.
7 Bio Sepra (Marlborough, MA).
8 Pharmacia Biotech (Uppsala, Sweden).

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**TABLE 2. Sequence analysis of amino-terminal AA of 90-kDa heat shock protein (HSP90).**

<table>
<thead>
<tr>
<th>AA Derived from phenylthiohydantoin1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
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<th>7</th>
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<td>Thr</td>
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<td>9.2</td>
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<td>20.4</td>
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<td>Phe</td>
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</table>
The results of amino-terminal AA sequencing showed that the HSP90 in the bovine mammary gland may include two isoforms. The alignments of these two isoforms after the fifth AA residues were difficult to specify exactly using only the results of this analysis. Because the amino-terminal sequence of bovine testis HSP90, which is considered to be an α isoform, had been analyzed already (22), we hypothesized that one alignment of mammary HSP90 was identical to that of testis HSP90. In this way, the

Figure 2. Elution profiles of the five-step column chromatography used to purify 90-kDa heat shock proteins (HSP90) from the bovine mammary gland. A) Macro-Prep t-butyl HIC support column chromatography (Bio-Rad Laboratories, Hercules, CA) of mammary tissue extract. The concentration of ammonium sulfate is shown by the broken line. Ten-milliliter fractions were collected. The fractions indicated by the horizontal bar (numbers 21 to 29) were pooled for further purification. B) DEAE Bio-Gel A chromatography (Bio-Rad Laboratories) of the pooled proteins in hydrophobic interaction chromatography (Figure 2A). The concentration of sodium chloride is shown by the broken line. Five-milliliter fractions were collected. Fractions 18 to 28, indicated by the horizontal bar, were pooled. C) Gel filtration on an Ultrogel AcA34 column (Bio Sepra, Marlborough, MA) of the DEAE Bio-Gel A pool. Seven-milliliter fractions were collected, and fractions 23 to 27, indicated by the horizontal bar, were pooled. D) HA-Ultrogel chromatography (Bio Sepra) of the pooled proteins from Ultrogel AcA34 chromatography. The concentration of phosphate is shown by the broken line. Five-milliliter fractions were collected. Fractions 10 to 18, indicated by the horizontal bar, were pooled. E) Q Sepharose Fast Flow chromatography (Pharmacia Biotech, Uppsala, Sweden) of the HA-Ultrogel pool. The concentration of sodium chloride is shown by the broken line. Fraction size was 5 ml. Fractions 29 to 31 were pooled as chromatographically purified HSP90.
alignments of bovine mammary HSP90 isoforms were deduced as described in Table 3.

The concentration of cytoplasmic HSP90 in the mammary gland of heifers was very low. Although tissues of pregnant cows and heifers were not evaluated in this study, synthesis of HSP90 may be largely increased during mammary development during late pregnancy because a high concentration of HSP90 (0.95%) was observed 5 d after parturition. The high concentration of HSP90 was maintained during lactation and then decreased during involution. This change in the concentration of HSP90 seemed to demonstrate that HSP90 has an important role in lactation. In the murine mammary gland, the highest amount of HSP90 mRNA is detected during pregnancy, but the highest HSP90 protein content is observed during lactation (5). Catelli et al. (5) speculated that stability of the HSP90 molecule was increased in the lactating mammary gland because of the decrease of some of the endogenous proteases (16). Consequently, HSP90 would be accumulated in the lactating mammary gland. Cows require much longer periods for both pregnancy and lactation than mice did. To determine the mechanisms for the increase and maintenance of HSP90 concentrations in bovine mammary tissue, examination of the tran-

Figure 3. The SDS-PAGE results of purified 90-kDa heat shock protein (HSP90) from the bovine mammary gland. Approximately 10 μg of chromatographically purified HSP90 before (lane 1) and after (lane 2) preparative SDS-PAGE. Molecular mass marker proteins (M) were also run in the gel. After electrophoresis, proteins were stained with Coomassie brilliant blue R-250. Recovery of HSP90 in the preparative SDS-PAGE was estimated to be 83% by densitometry.

<table>
<thead>
<tr>
<th>Stage</th>
<th>n</th>
<th>Concentration</th>
<th>Range 2 (%) in cytoplasmic proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Nulliparous</td>
<td>4</td>
<td>0.15</td>
<td>0.11</td>
</tr>
<tr>
<td>Lactation</td>
<td>7</td>
<td>1.25</td>
<td>0.18</td>
</tr>
<tr>
<td>Involution</td>
<td>4</td>
<td>0.54</td>
<td>0.22</td>
</tr>
<tr>
<td>Involution (mastitis)</td>
<td>5</td>
<td>0.81</td>
<td>0.31</td>
</tr>
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</table>

1Contents of these tissues are described in Materials and Methods.
2Minimum concentration to maximum concentration.
3Values were higher (P < 0.001) than those for both the nulliparous and involuting groups.
4Values were higher (P < 0.05) than those for the nulliparous group.
5The principal features of the histopathological state of these five tissues were as follows: formation of granulomas in two cases, formation of microabscesses in one case, intraalveolar exudation of granulocytes in two cases, interstitial infiltration of granulocytes in all five cases, focal interstitial accumulation of lymphocytes in four cases, and desquamation of alveolar epithelium in two cases.
scription and the protein concentrations at various developmental stages is required.

Administration of estrogen induces uterine HSP90 in ovariectomized mice, and the HSP90 concentration changes as the concentration of estrogen receptor changes (19). We speculated that changes in mammary HSP90 contents during development and differentiation are mostly caused by estrogenic stimulation. However, the mammary gland is developed and differentiated under the control of multiple hormones, including glucocorticoids and progesterone (6, 13). The effects of these steroid hormones on the expression of HSP90 must be examined because HSP90 forms various complexes, that is, not only an estrogen receptor but also complexes with other cytoplasmic steroid receptors (4, 12).

The HSP90 concentrations of healthy and mastitic tissues at involution varied widely, which might have been because the samples were prepared from cows in various periods of involution and pathological states (Table 4). To improve the criteria used to determine the relevance of mammary HSP90 during mastitis, examination of many samples is required after groups based on mastitic states have been established. In the group from cows with mastitis, there were samples in which HSP90 constituted over 1% of the cytoplasmic proteins. One possibility is that these elevated HSP90 concentrations might have been brought about by the increase in HSP90 concentrations in phagocytes (18), such as macrophages and neutrophils, acting at the sites of inflammation (17).

In this study, we purified HSP90 from bovine mammary tissue. As a result of the quantitative immunoblot analysis using anti-bovine HSP90, mammary HSP90 is suggested as being one of the proteins related to lactation because HSP90 concentrations in the mammary gland increased during lactation.

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REFERENCES