Expression and Characterization of Bovine Lactoperoxidase by Recombinant Baculovirus

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Lactoperoxidase (LPO) is a heme-containing oxidation-reduction enzyme present in milk. In this study, the gene encoding bovine lactoperoxidase (bLPO) was inserted into a baculovirus transfer vector, and a recombinant virus expressing bLPO was isolated. A bLPO-related recombinant baculovirus-expressed protein of 78 kDa was detected using anti-bLPO antibodies. After digestion with N-glycosidase F, the molecular weight of the recombinant bLPO (rbLPO) decreased. In addition, rbLPO reacted with lectin, indicating that the protein was glycosylated. The rbLPO activity and heme content in the culture supernatants increased upon addition of δ-aminolevulinic acid, which is a heme precursor. Differences in the δ-aminolevulinic acid-dependent circular dichroism spectrum and rbLPO pepsin hydrolysis were observed. These results suggest that the secondary structure and structural stability of rbLPO depends on the heme environment. Our data suggest that this bLPO expression system is useful for studying structure, catalytic mechanisms, and biological function.

Key words: lactoperoxidase; baculovirus; glycosylation; heme; peroxidase activity

Lactoperoxidase (LPO) is a heme-containing oxidation-reduction enzyme present in milk and saliva, and is part of an antimicrobial system that converts thiocyanate to hypothiocyanate in a hydrogen peroxide-dependent reaction. The molecular mass of LPO is approximately 78 kDa and the carbohydrate moiety accounts for about 10% of the total.1,2) LPO, myeloperoxidase (MPO), eosinophil peroxidase (EPO), and thyroid peroxidase (TPO) belong to the homologous mammalian peroxidase family and share 50–70% identity. Greater identity is observed among the active-site-related residues. These peroxidases use hydrogen peroxide to catalyze the oxidation of halides and pseudohalides, such as thiols,5) phenols,6) catechol amines,7,8) steroid hormones,9) halides, and nitrite.10,11) We previously reported that recombinant bLPO (rbLPO) may be produced using Chinese hamster ovary (CHO) cells.12) Recently, much attention has been paid to the easy-to-handle baculovirus expression system because of its high productivity and ability to incorporate most posttranslational modifications of heterologous proteins.13) As a result, the expressed proteins were usually correctly folded and glycosylated, and they retained their biological activities.14,15) To advance investigations of structure-function relationships, this study describes the expression, purification, and biological activities of rbLPO produced in baculovirus-infected insect cells. Furthermore, we examine the expression, glycosylation, enzyme activity, heme content, conformation, and structural stability of rbLPO using Spodoptera frugiperda (Sf9) and Tricoplusia ni (High5) cells.

Materials and Methods

Virus and Cells. Autographa californica nuclear polyhedrosis virus (AcNPV) and recombinant viruses were grown in Spodoptera frugiperda (Sf9) or Tricoplusia ni (High5) cells in TC-100 insect medium (Life Technologies Japan, Tokyo) supplemented with 10% fetal bovine serum (FBS) and 0.26% Bacto tryptose broth (Difco Laboratories, Detroit, MI).

Construction of recombinant baculovirus that expresses bLPO. The open reading frame of bLPO was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) from bovine mammary gland cDNA using a pair of primers, 5'−ATAGAC-GGTATAAAAAAGCCGG-3' and 5'−CTGAGTCT-ACCTGAAAATGGCATCG-3'. The length of the PCR products covered the full length of bLPO (2,295

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Expression and Characterization of Recombinant Milk Protein

2255

bp. The sequence analysis was done using an Applied Biosystems sequencer (Foster City, CA). The PCR products were blunted by T4 DNA polymerase and ligated with the baculovirus transfer vector pBacPAK8 (Clontech Laboratories, Inc., Palo Alto, CA), which was cut with SmaI and then blunted. The plasmid (pBacPAK8/bLPO) was transfected into Sf9 cells containing a mixture of AcNPV DNA using lipofectin reagent (Life Technologies, Japan) according to the method of Xuan et al. After 4 d of incubation at 27°C, the culture supernatants containing recombinant virus were harvested and isolated by plaque purification. Following three cycles of polyhedrin negative plaque purification, a recombinant baculovirus was selected, and designated AcbLPO.

**Immunofluorescence test (IFAT).** Sf9 cells were infected with AcbLPO (10 plaque-forming units (PFU)/cell, 4 d) and tested by an indirect IFAT. The infected-Sf9 cells were fixed with acetone and incubated with mouse anti-bLPO monoclonal antibody (anti-bLPO mAb) or rabbit anti-bLPO polyclonal antibody (anti-bLPO Ab), and the cells were stained with fluorescein-conjugated goat anti-mouse antibody (Southern Biotechnology Associates Inc., Birmingham, AL) or fluorescein-conjugated sheep anti-rabbit antibody (Waco Pure Chemical, Osaka). The cells were observed using fluorescence microscopy.

**Western blot analysis.** Sf9 or High5 cells infected with AcbLPO at a multiplicity of 10 PFU/cell were harvested and washed twice with phosphate-buffered saline (PBS). The supernatants were filtered through a 0.22-μm filter (Millipore Corp., Bedford, MA) and then blunted. The plasmid (pBacPAK8/bLPO) was transfected into Sf9 cells containing a mixture of AcNPV DNA using lipofectin reagent (Life Technologies, Japan) according to the method of Xuan et al. After 4 d of incubation at 27°C, the culture supernatants containing recombinant baculovirus were harvested and isolated by plaque purification. Following three cycles of polyhedrin negative plaque purification, a recombinant baculovirus was selected, and designated AcbLPO.

Purification of rbLPO. After Sf9 or High5 cells infected with AcbLPO were incubated in protein-free Sf-900 medium (Life Technologies, Japan) for 4 d with or without 250 μM δ-aminolevulinic acid, the culture supernatants were purified by using a HiTrap-SP HP column (Amersham Bioscience Corp., Piscataway, NJ) equilibrated with 50 mM phosphate buffer (pH 6.4) containing 0.1 M NaCl (binding buffer). The culture supernatants were put onto the column, the column was washed with 10 ml of binding buffer, and rbLPO was eluted stepwise in 5-ml increments using 0.2 M and 0.4 M NaCl in the binding buffer. After purification of rbLPO, these samples were concentrated and desalted using an Ultrafree-MC Centrifugal Filter Unit (Millipore Corp.).

**Deglycosylation of rbLPO.** Recombinant bLPO was deglycosylated using N-glycosidase F, O-glycosidase, and neuraminidase, following the manufacturer’s recommendations (Roche Diagnostics GmbH, Mannheim, Germany).

**Lectin assay.** Recombinant bLPO and native bLPO were transferred onto PVDF membranes (Osmonics Inc.) after electrophoresis. Binding of lectin to protein bands was made visible by reaction with 0.3 mg/ml diaminobenzidine and 0.003% H2O2. Carbohydrate analysis was done using the peroxidase lectin kit (Hohnen Co., Ltd., Tokyo) containing six types of lectins, including concanavalin A (Con A), caster bean (RCA120) lectin, lentil lectin (LCA), wheat germ (WGA) lectin, peanut lectin (PNA), and lectin from Phaseolus vulgaris (PHA-E).

N-terminal amino acid sequence analysis. N-terminal amino acid sequences were analyzed using a gas-phase sequencer Procise 492 from Perkin Elmer (MA).

**Measurement of rbLPO activity.** Enzyme activity was assayed using 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) a substrate. ABTS assays were done in 0.1 mM acetate buffer at final concentrations of 1.0 mM ABTS and 0.1 mM hydrogen peroxide. Reactions (3.0 ml final volume) were started by the addition of hydrogen peroxide and kept at 25°C in 4.5-ml polystyrene cuvettes set in a spectrophotometer. Formation of the ABTS peroxidation product was monitored by observing the increase in absorbance at 412 nm, and the initial rate calculated on the basis of the first 1 min of the reaction.

**Spectral measurement.** The rbLPO absorption spectrum was measured on a spectrophotometer. The ratio of absorptions at A413 nm/A280 nm was calculated to estimate the purity of rbLPO.

**Circular dichroism spectra.** Circular dichroism (CD) recordings were made on a JASCO Model J-725 dichrograph (Japan Spectroscopic Co., Ltd., Tokyo). The data are expressed in terms of molar ellipticity, [θ] in degrees × cm² × dmol⁻¹. We used
0.05 M sodium phosphate buffer (pH 6.2) containing 0.05 M NaCl as the solvent. All recordings were done at room temperature. Estimation of the $\alpha$-helix, $\beta$-structure, $\beta$-turn, and unordered structure content was done using the reference CD data of Yang et al. 19)

**Proteolytic digestion.** Pepsin digestion was done for 8 h at pH 2.5, which was attained by the addition of 1 M HCl to the rbLPO solution. The reaction was stopped by heating the reaction mixture at 80°C for 15 min. Thereafter, the hydrolysis was adjusted to pH 7.0, and any precipitate formed was removed by centrifugation. Porcine pepsin (Sigma Chemical Co.) was used and the enzyme/substrate ratio was 1/20.

**Reverse-phase HPLC.** Reverse-phase (RP) HPLC was done using CAPCELL PAK C8 SG300 (4.6 mm i.d. × 15 cm, Shiseido, Tokyo) columns. For elution, a mixture of eluents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) was used, using a linear or convex gradient of A:B from 100:0 to 40:60 for 30 to 40 min at a flow rate of 1 ml/min. The absorbance of the eluate was monitored at 230 nm.

**Results**

**Expression of rbLPO in baculovirus-infected Sf9 or High5 cells**

Linearized baculovirus and pBacPAK8/bLPO were allowed to transfect Sf9 cells, and virus-containing medium of the transfected Sf9 cells was collected and analyzed by IFAT or Western blotting in the presence of rbLPO. An rbLPO-expressing clone was selected by the plaque-assay technique. AcbLPO-infected cells were examined by IFAT and reacted with both anti-bLPO mAb (Fig. 1B) and anti-bLPO Ab (Fig. 1C). The rbLPO-positive clone was amplified to $2 \times 10^8$ PFU/ml medium and the high-titer working stock was then used for the preparative expression of rbLPO in Sf9 or High5 cells. As shown in Fig. 2A, B, rabbit anti-bLPO Ab reacted with a 78-kDa protein in cells (lane 3) and culture supernatants (lane 5) from AcbLPO-infected cells, and with a 78-kDa protein in native bLPO (Sigma Chemical Co., St. Louis, MO) (lane 4). No band was detected in the Sf9 or High5 cells (lane 1) and Ac green fluorescence protein (GFP)-infected cells (lane 2). A similar pattern of immunoreactivity was observed against anti-bLPO mAb (Data not shown).

**Purification of rbLPO**

Recombinant bLPO was purified from the culture supernatants obtained 4 d after infection by means of an HiTrap-SP HT column. After the culture supernatants were put onto the column, the column was washed with binding buffer. After eluting with...
2257

Expression and Characterization of Recombinant Milk Protein

0.2 M NaCl, the recombinant proteins were eluted with 0.4 M NaCl. SDS-PAGE showed that the molecular weight of rbLPO was lower than that of native bLPO (Fig. 2C), and the molecular weight of rbLPO was not affected by the addition of δ-aminolevulinic acid.

Carbohydrate analysis of rbLPO

After the bLPO and rbLPO obtained from the supernatants of SF9 or High5 cells were treated with N-glycosidase F, the proteins migrated at a slower rate during SDS-PAGE analysis (Fig. 3 lane 4, 6). However, O-glycosidase and neuramidase treatment did not affect the migration of rbLPO or bLPO (data not shown). These results indicated that both rbLPO and bLPO were N-linked glycosylated but not O-linked glycosylated.

The carbohydrate structure of rbLPO and native bLPO was studied using six lectins (Table 1). Distinctly stained bands were observed in response to Con A, LCA, PHA-E4, PNA, and WGA, but not PHA-E4 and RCA-120, on rbLPO. On the other hand, distinctly stained bands were observed in response to Con A, LCA, PHA-E4, RCA-120, and WGA, but not PNA, on native bLPO. These results indicated that the carbohydrate structure of between rbLPO and native bLPO was different.

N-terminal amino acid residue of rbLPO

The N-terminal amino acid residue of the rbLPO isolated from insect cells was ^10DTTLTNVTDP^110. Likewise, a separate study using CHO cells also identified the N-terminal amino acid of rbLPO as Asp-101. The numbers applied to these amino acid residues are based on the nucleotide sequence of bLPO cDNA obtained by Dull et al. These results indicate that the preprotein was correctly processed during synthesis and secretion from insect cells.

Characterization of rbLPO

Table 2 shows the levels of peroxidase activity detected for rbLPO. The maximum level of activity was observed in the culture medium supplemented with a heme precursor, δ-aminolevulinic acid, 4 d post infection with the recombinant virus.

Purified-rbLPO showed an absorption spectrum at 412 nm indicative of the presence of heme, similar to that of bLPO upon the addition of δ-aminolevulinic acid (Fig. 4). These results indicate that during the course of expression, the heme molecule synthesized in insect cells mainly from δ-aminolevulinic acid was properly incorporated into the individual recombinant peroxidases as a prosthetic group. The absorbance ratios of rbLPO from SF9 and rbLPO from High5 cells at 412 nm and 280 nm were identical (0.62) in the presence of δ-aminolevulinic acid. These observations suggest that incorporation of heme into the recombinant peroxidases was somewhat lower.

Table 1. Lectin Reactivity of rbLPO and Native bLPO

<table>
<thead>
<tr>
<th>Lectin</th>
<th>ConA</th>
<th>LCA</th>
<th>PHA-E4</th>
<th>PNA</th>
<th>RCA120</th>
<th>WGA</th>
</tr>
</thead>
<tbody>
<tr>
<td>rbLPO</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Hi5</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Native bLPO</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*+*, stained; *-, not stained.
Table 2. Purification of rbLPO from Insect Cell Culture Supernatants

<table>
<thead>
<tr>
<th></th>
<th>rbLPO (Total activity)</th>
<th>Protein (mg/L)</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sf9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-</td>
<td>16.53</td>
<td>0.077</td>
<td>214.68</td>
</tr>
<tr>
<td>d+</td>
<td>124.39</td>
<td>0.066</td>
<td>1873.31</td>
</tr>
<tr>
<td>Hi5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-</td>
<td>36.94</td>
<td>0.160</td>
<td>230.87</td>
</tr>
<tr>
<td>d+</td>
<td>438.67</td>
<td>0.123</td>
<td>3566.45</td>
</tr>
</tbody>
</table>

* 10^{-3} (mol Product/60 sec.
** Without of δ-aminolevulinic acid.
*** With added δ-aminolevulinic acid.

Note: Proteins in 50 ml of insect cell culture supernatants were fractionated on a HiTrap SP column. Peroxidase activity of rbLPO was measured using rbLPO purified by a HiTrap SP HP column.

Table 3. Secondary Structure of rbLPO and Native bLPO

<table>
<thead>
<tr>
<th></th>
<th>Native bLPO (%)</th>
<th>rbLPO (%)</th>
<th>Reported Values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Helix</td>
<td>20</td>
<td>20</td>
<td>15, 23, 17, 17.8, 18.6</td>
</tr>
<tr>
<td>β-Structure</td>
<td>50</td>
<td>45</td>
<td>66, 54.2, 50.1, 31.3</td>
</tr>
<tr>
<td>Unordered</td>
<td>30</td>
<td>45</td>
<td>12, 28, 31.3</td>
</tr>
</tbody>
</table>

* The addition of δ-aminolevulinic acid.
** No addition of δ-aminolevulinic acid.
† Reported value by Sievers.
‡ Maguire and Dunford.
§ Watanabe et al.

The stability of rbLPO in response to pepsin hydrolysis

The stability of rbLPO in response to pepsin hydrolysis was analyzed by reverse-phase HPLC with or without δ-aminolevulinic acid. The chromatographic profiles of native bLPO and rbLPO treated with δ-aminolevulinic acid were similar. However, the chromatographic profile of rbLPO without δ-aminolevulinic acid was many more peaks.

Discussion

A number of mammalian and plant heme peroxidases, bLPO and horseradish peroxidases have been expressed using baculovirus-insect cell systems with High5 cells, while at the same time retaining their catalytic activity. Recombinant bLPO has also been produced using a mammalian system. Recombinant human MPO, expressed by a baculovirus insect cell system using Sf9 cells, has been reported to show no peroxidases activity, and has not been reported to produce bLPO using Sf9 cells. In this study, rbLPO expressed in both Sf9 and High5 cells was found to be catalytically active. These rbLPO have an advantage to resolve the fine structure of catalytic sites and heme binding construction, for conformational analysis and molecular stability analysis or to obtain single crystal for X-ray analysis, etc.

Recombinant bLPO produced in insect cells migrated at 78 kDa in Western blot analyses (Fig. 2A, B lane 3). There is no difference in the molecular masses between intracellular rbLPO and native bLPO. However, the molecular masses of culture supernatants in Western blot analyses and rbLPO purified by HiTrap-SP HT column were less than that of native bLPO (Fig. 2A, B lane 5; Fig. 2C lane 1, 2). These size differences may be due to differences in glycosylation level and differences in processing level between insect cells and mammary glands. The partial domain of the rbLPO may un-
Expression and Characterization of Recombinant Milk Protein

dero proteolysis in insect cells. After treatment of rbLPO with peptide-N-glycosidase F, higher mobility was observed by SDS-PAGE (Fig. 3 lane 4, 6). Furthermore, the carbohydrate structure analysis of purified rbLPO and native bLPO found different reactivity with PHA-E4, PNA, and RCA120 (Table 1). These results suggest that rbLPO is modified by a complex sugar. These enzymes also reacted with PNA, revealing terminal galactose residues linked α(1–4) to N-acetylglucosamine residues. However, the effects of lectin on rLPO did not differ between Sf9 and High5 cells.

The N-terminal amino acid residue of rbLPO is Asp-101 according to the cDNA sequence data. However, Watanabe et al. found that each preparation of bLPO showed a different N-terminal amino acid residue. These heterogeneous observations may result from differences in the disk-electrophoresis and ion-exchange chromatography methods used for analysis. In this study, we also identified Asp-101 as the N-terminal amino acid residue in native bLPO. Most of the bLPO extracted from milk showed Asp-101 as the N-terminal amino acid residue. Therefore, preprotein rbLPO was correctly processed during synthesis and secretion from insect cells as well as mammary glands.

The specific peroxidase activity of rbLPO was also measured, using ABTS as a substrate, and the results showed that the specific activity of rbLPO without δ-aminolevulinic acid was lower than that of rbLPO with δ-aminolevulinic acid (Table 2). The ratio of distinct absorption at Abs413 nm/AbS280 nm was lower for rbLPO from Sf9 or High5 cells than that observed for native bLPO (Fig. 4). These lower values were also observed for rbLPO produced in insect cells without δ-aminolevulinic acid. Thus, the proportion of the protein having a bound heme moiety is low. From the ratio of absorbance at 280 nm to that at 413 nm, we found that about 60% of the rbLPO contains a covalently bound heme moiety. These results suggest that the specific activity of rbLPO depends on the heme environment.

CD measurements were done to study the conformation of the native rbLPO and bLPO (Fig. 5). The far-UV or amide region (190 to 250 nm) is dominated by contributions from peptide bonds, thereby reflecting the secondary structure. The gross patterns for each bLPO showed a trough at 207–208 nm, which indicates that these proteins are rich in β-sheet structure. Native bLPO and rbLPO had identical conformational features upon addition of δ-aminolevulinic acid (Table 3). These structural profiles are consistent with other reported findings. However, the gross pattern of rbLPO without δ-aminolevulinic acid had stark differences. These results suggest that the secondary structure of rbLPO depends on the heme environment.

The stability of rbLPO in pepsin hydrolyses was examined by measuring the absorbance at 230 nm, which should be influenced by the heme environment (Fig. 6). The heme-dependent changes in the RP-HPLC profiles of pepsin hydrolyses containing rbLPO were affected by the presence of δ-aminolevulinic acid. This may indicate that the heme moiety alters the rbLPO, and suggests the possibility that heme contributes to the structural stability of rbLPO in peptide hydrolyses.

In conclusion, the rbLPO purified from Sf9 and High5 cells had properties similar to bLPO, in terms of its reactivity with anti-bLPO antibodies, N-terminal amino acid residues and its absorption spectrum. However, the carbohydrate structure and the heme

Fig. 6. Reverse-phase HPLC Profiles of Pepsin Hydrolyses of Native bLPO and rbLPO.
Column, CAPCELL PAK C8 SG300 (4.6 mm i.d. × 15 cm); flow rate, 1.0 ml/min; elution mode, 0–50% convex gradient of 0.1% TFA in water and 0.1% TFA in acetonitrile; detection wavelength, 230 nm (A) native bLPO. (B) rbLPO purified from high5 cells with added δ-aminolevulinic acid. (C) rbLPO purified from high5 cells without δ-aminolevulinic acid.
content of these rbLPO differed from those of bLPO. Furthermore, the protein production and specific activities of rbLPO purified from High5 cells were higher than those from Sf9 cells with or without \( \delta \)-aminolevulinic acid. Generally, High5 cells may have a greater capacity to express functional mammalian peroxidases than Sf9 cells. Our method using the baculovirus-insect cell system will likely be useful to rapidly create mutant proteins in studies of the relationship between structure and function of bLPO. Further studies using rbLPO will be necessary to fully elucidate the functional mechanisms of LPO and its heme structure-function relationships.

Acknowledgments

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Expression and Characterization of Recombinant Milk Protein


