Special Issue

A small variance in the antigenicity but not function of recombinant β -lactoglobulin purified from the culture supernatant of transformed yeast cells

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Received July 1996

Key words: antigenicity, β -lactoglobulin, monoclonal antibody, recombinant protein

Abstract

We purified recombinant bovine β -lactoglobulin (r β -LG) from the culture supernatant of transformed yeast and investigated whether r β -LG maintained the functional ability and antigenicity of native β -LG. Immunostaining following gel electrophoresis and reversed-phase high-performance liquid chromatography confirmed that r β -LG was purified homogeneously. r β -LG showed almost the same retinol-binding ability as native β -LG purified from bovine milk. However, affinities of two anti- β -LG monoclonal antibodies (mAbs) to r β -LG were different from those to native β -LG, although three other mAbs bound these two proteins equally. Since our panel of five mAbs has been previously shown to be able to detect structural changes occurring in β -LG, this variance in antigenicity can be attributed to conformational differences between r β -LG and native β -LG. Then, we studied which step in the production and purification procedure was responsible for altering the antigenicity of r β -LG. Bovine milk native β -LG was added to several steps in this procedure and purified in the same manner as r β -LG. The results suggested that incubation in the yeast culture had adverse effects on maintaining the antigenicity of this recombinant protein. We conclude from these results that even if no difference between the native and recombinant proteins can be detected by functional analysis, some subtle conformational change which can be distinguished by mAbs may be incorporated into the recombinant protein during its production and ultimately cause a different immune reaction *in vivo*.

Abbreviations: β -LG, β -lactoglobulin; r β -LG, recombinant β -LG; PBS, phosphate-buffered saline; PBS-Tween, PBS containing 0.05% Tween 20; ELISA, enzyme-linked immunosorbent assay

Introduction

There is now growing demand for applying biologically active proteins to pharmaceutical use, and for the most part these proteins have been produced by the transformed bacteria or mammalian cells carrying heterologous genes. Although a recombinant protein should be able to completely substitute its prototype native protein for use as a pharmaceutical agent, a recombinant protein does not always seem to have the same structure and function as the original native protein. It has been reported that when recombinant

proteins are used in clinical trials, patients sometimes show severe anaphylactic reactions (Garía *et al.*, 1993), suggesting that the native and recombinant proteins are recognized as different antigens *in vivo* by the immune system due to their structural discrepancies. Considering that these subtle differences between the native and recombinant proteins may cause unexpected results, detailed functional and structural comparisons between native and recombinant proteins are required.

Bovine milk β -lactoglobulin (β -LG) is known as a member protein of the lipocalin superfamily which is characterized on the basis of the results of the binding

abilities to low-molecular-weight hydrophobic molecules and its particular three-dimensional structure of β -barrel. These lipocalin proteins including β -LG may be applicable for developing the drug delivery system by a protein molecule. Protein engineering technique based upon the recombinant DNA technology is helpful for improving and modifying the ligand-binding activities of these proteins. We addressed the question whether recombinant β -LG (r β -LG) has completely the same biological function of ligand-binding ability and antigenicity as those of the native form of this protein. Using our set of monoclonal antibody (mAb) specific for β -LG, we have been analyzing conformational differences in several forms of the protein. In this study, we took advantage of availability of those mAbs as well as the expression system of yeast.

Batt *et al.* have cloned a truncated form of bovine β -LG cDNA and established the expression system in *E. coli* using the full length cDNA ligated with a synthesized 5'-end oligonucleotides (Jamieson *et al.*, 1987). They obtained r β -LG after solubilizing the protein which had accumulated inside the bacteria with guanidine-HCl (Batt *et al.*, 1990). However, we previously reported that after β -LG was denatured in the presence of 6 M guanidine-HCl, β -LG could not be completely refolded by any of the proven renaturation treatments (Hattori *et al.*, 1993), suggesting that r β -LG produced in *E. coli* might not have the native conformation. We then cloned the full-length β -LG cDNA from bovine mammary gland and developed an expression system where yeast can secrete r β -LG into the culture supernatant (Totsuka *et al.*, 1990). We report here that we have established a method to purify secreted r β -LG from the supernatant of transformed yeast cells, and investigated whether r β -LG is completely the same as native β -LG by analyzing both their retinol-bindings and reactivities with anti- β -LG mAbs. In addition to clarifying the difference between native and recombinant proteins, we have also discussed the cause and effects of those differences.

Materials and methods

Native and RCM β -LG

Fresh milk from a Holstein cow with an AA genotype for β -LG was supplied by the dairy farm at Utsunomiya University (Tochigi, Japan). Crude β -LG was prepared by the method established by Aschaffenberg and Drewry (Aschaffenberg *et al.*, 1957), and puri-

fied by DEAE-Sephacel (Pharmacia, Uppsala, Sweden) ion-exchange chromatography with a linear ionic strength gradient from 0 to 0.5 M NaCl concentration in 0.05 M imidazole-HCl buffer (pH 6.7). A single band was obtained for native β -LG by polyacrylamide gel electrophoresis (PAGE) performed both under denaturing and nondenaturing conditions. Reduced and carboxymethylated (RCM) β -LG, which is a partially, but largely denatured form of β -LG, was prepared by applying a carboxymethylation treatment on reduced β -LG according to the method established by McKenzie (McKenzie *et al.*, 1972). Concentration of the β -LG solution was determined from its absorbance at 280 nm, using an absorbance coefficient of $\epsilon_{1\%}^{1\text{cm}} = 9.6$ (Townend *et al.*, 1964).

*Transformation of *Saccharomyces cerevisiae* with the β -LG-expression plasmid*

Bovine β -LG cDNA isolation and expression in yeast were described previously (Totsuka *et al.*, 1990). The β -LG-expression plasmid designated pYBSS1, which was derived from pYG100 containing the promoter and the terminator of the yeast glyceraldehyde-3-phosphate dehydrogenase (Kumagai *et al.*, 1989), was introduced into *Saccharomyces cerevisiae* strain AH22 (Mata, *leu2-3, leu2-112, his4-519, can1*) by the lithium acetate method (Ito *et al.*, 1983). To select for Leu⁺ transformants, the cells were grown in a modified synthetic minimal medium (Ichikawa *et al.*, 1989) containing a 0.67% yeast nitrogen base without amino acids (Difco, Detroit, MI, USA), 10% glucose, 0.5% ammonium sulfate, 0.1% K₂HPO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.002% FeSO₄, and 40 μ g/ml of *L*-histidine.

Purification of r β -LG from the culture supernatant of yeast

Transformed yeast cells were cultured at 30°C for 4 days with vigorous aeration. The culture supernatant (10 liters) was concentrated to 500 ml by ultrafiltration (Millipore, Bedford, MA, U.S.A.), and 8-fold diafiltrated with 0.05 M imidazole-HCl buffer (pH 6.7). This solution (500 ml) was then applied to a DEAE-Sephacel column (2.5 cm x 15 cm) equilibrated with 0.05 M imidazole-HCl buffer (pH 6.7), and eluted with a linear ionic strength gradient from 0 to 0.5 M NaCl in a starting buffer. The fraction containing β -LG detected by sandwich enzyme-linked immunosorbent assay (sandwich ELISA) was dialyzed against deion-

ized water and lyophilized. This lyophilizate was dissolved in phosphate buffered saline (PBS; 0.11 M phosphate, 0.04 M NaCl, pH 7.2, 0.02% NaN₃) and applied to size-exclusion HPLC, using a TSKgel G3000SW_{XL} column (7.5 mm x 30 cm, Tosoh, Tokyo, Japan) equilibrated with PBS. The fractions containing β -LG were dialyzed, lyophilized and stored at -20°C.

In order to clarify the influences of the production and purification procedure of r β -LG on its antigenicity, we added bovine milk native β -LG at a step on the way of this procedure, purified in the same manner as r β -LG and evaluated their structural alteration by competitive ELISA.

Western blotting

After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, proteins in the gels were electroblotted onto an Immobilon PVDF membrane (Millipore) using Milliblot-SDE (Millipore) as described in the instruction's manual. The transferred membrane was blocked with 1% bovine serum albumin in PBS-Tween (0.05% Tween-20 in PBS), and incubated with 5 μ g of mAb 21B3 against β -LG. The bound mAb 21B3 was detected with the blotting detection kit for mouse antibodies (Amersham, Buckinghamshire, UK) as described by the manufacturer.

HPLC

The purity of r β -LG was confirmed by reverse-phased HPLC with a Vydac C18 protein & peptide column (0.46 cm x 25 cm, Separations Group, Hesperia, CA, U.S.A.). The elution was performed with a linear gradient from 5 to 67% acetonitrile in 0.1% trifluoroacetic acid (TFA).

Retinol-binding study

Fluorescence titration experiments were performed with a Hitachi (Tokyo, Japan) 650-10S fluorescence spectrophotometer. Saturable binding assays of all-*trans*-retinol (Sigma, St. Louis, MO, U.S.A.) to β -LGs were performed by a modification of the titration method established by Fugate and Song (Fugate *et al.*, 1980). PBS containing 600 pmol of β -LG (1 ml) was placed in a cuvette, 1- μ l aliquots containing 25 pmol of all-*trans*-retinol were then mixed and incubated in the dark for 1 min. The fluorescence was determined by using an excitation wavelength of 342 nm and an emission wavelength of 475 nm, and the relative fluores-

Table 1. Properties of anti- β -LG monoclonal antibodies

mAb	Binding specificity	Peptide region important for binding to mAb
61B4	native β -LG >> RCM β -LG	¹²⁵ Thr- ¹³⁵ Lys
62A6	native β -LG >> RCM β -LG	close to ¹²⁵ Thr- ¹³⁵ Lys
61C1	native β -LG > RCM β -LG	close to ¹⁵ Val- ²⁹ Ile
21B3	native β -LG << RCM β -LG	¹⁵ Val- ²⁹ Ile
31A4	native β -LG << RCM β -LG	⁸ Lys- ¹⁹ Trp

Figure 1. Size-exclusion chromatography using a TSKgel G3000SW_{XL} column of an ion-exchange chromatography fraction containing r β -LG. r β -LG in profile was detected by sandwich ELISA as described in MATERIALS AND METHODS. The top arrows in a figure indicate the retention times for bovine serum albumin (MW: 66000), ovalbumin (MW: 45000), α -lactalbumin (MW: 14200), horse cytochrome c (MW: 12300) and tryptophane (MW: 204). These proteins, except for tryptophane, were used as molecular weight markers. (A); native β -LG, (B); r β -LG.

cence intensity was calculated by fixing the saturated fluorescence intensity at 1.0.

mAbs and ELISA

Anti- β -LG mAbs from the 21B3, 31A4, 61B4, 61C1 and 62A6 B cell hybridoma clones were prepared and purified as described previously (Kaminogawa *et al.*, 1987; Kaminogawa *et al.*, 1989). The epitopes and the relative binding specificities of these anti- β -LG mAbs are described in Table 1.

β -LG in each fraction was detected by sandwich ELISA (Totsuka *et al.*, 1990). β -LGs in the fractions were captured by mAb 62A6 coated onto a microtitration plate (Maxisorp, Nunc, Roskilde,

Denmark), and detected by incubating with alkaline phosphatase (ALP; type VII-S, Sigma) -labeled mAb 61C1 and subsequently reacting with a substrate of 0.1% *p*-nitrophenylphosphate, disodium salt in 1 M diethanolamine-HCl buffer (pH 9.8) containing 0.01% MgCl₂ and 0.02% NaN₃. The concentration of correctly folded β -LG in the solution was determined from a dose-response curve drawn by plotting the ELISA value (405 nm) vs. logarithm of the concentration of native β -LG. Conjugation of mAb 61C1 with ALP was described elsewhere (Blake *et al.*, 1984).

Competitive ELISA was performed to investigate whether native β -LG and r β -LG had different binding affinities with these mAbs. The microtitration plates were coated with bovine milk β -LG in PBS (100 μ l at 0.01%) and incubated at room temperature for 2 h. After removing the antigen and washing the plate three times with PBS-Tween, the wells were blocked with 125 μ l of 1% ovalbumin in PBS. Following the washing of the plate, solutions containing mAbs and varying concentrations of competitor β -LGs were then added. The competitive bindings of mAbs to the coated native β -LG in the presence of the above competitors were detected with ALP-labeled goat anti-mouse immunoglobulin (Cappel, Cochranville, PA) (Kaminogawa *et al.*, 1989). The substrate and its subsequent reaction were the same as those for sandwich ELISA.

Results and discussion

Purification of r β -LG secreted into the culture supernatant of transformed yeast cells

We purified r β -LG secreted into the culture supernatant of transformed yeast cells using a two-step procedure, ion-exchange and size-exclusion chromatography, as described in MATERIALS AND METHODS. The size-exclusion HPLC profile showed that r β -LG exists as a dimer, and is similar to the native β -LG purified from bovine milk (Figure 1). Estimated molecular weights for r β -LG and native β -LG were calculated to be 35,100 and 34,300, respectively (Figure 1), roughly corresponding to twice the molecular weight of 18,300 for the monomer, suggesting that r β -LG seems to maintain the dimer-forming ability similar to native protein at physiological conditions.

The purity of r β -LG was examined by Coomassie Brilliant Blue (CBB) staining, immunostaining after SDS-PAGE, and reversed-phase HPLC using an octadecyl silica column. The CBB staining showed

that the eluate after ion-exchange chromatography contained several high molecular weight proteins besides β -LG (Figure 2A, lane 1). However, r β -LG fractionated by size-exclusion chromatography showed a single band (Figure 2A, lane 2) which corresponds to native β -LG as shown by immunostaining (Figure 2B, lanes 1 and 3). The purity of r β -LG obtained by this two-step procedure was also confirmed by reversed-phase HPLC. r β -LG had a single peak with the same retention time as that of native β -LG (Figure 3A and B). The yield for each purification step was determined by sandwich ELISA (Table 2), the data indicating that the yield was reasonable.

Functional ability of r β -LG

Although the physiological function of β -LG has yet to be identified, β -LG belongs to the lipocalin superfamily, in which the member proteins share the ability to bind to small hydrophobic molecules (Pervaiz *et al.*, 1987; Zimmermann, 1988; Sivaprasadarao *et al.*, 1993; McAlpine *et al.*, 1990), and retinol has been reported to bind to β -LG (Fugate *et al.*, 1980). Therefore, we examined the binding ability of retinol to these β -LGs in order to determine the functionality of these β -LG species.

All-*trans*-retinol is a weakly fluorescing compound in solution, but fluorescence is markedly enhanced upon binding to β -LG (Futterman *et al.*, 1972). This property enables us to monitor the binding of all-*trans*-retinol to β -LG by measuring the fluorescence intensity. The concentration-dependent binding curves for all-*trans*-retinol to native and r β -LG show that saturation points for these two proteins are at about the same concentration of all-*trans*-retinol (Figure 4), indicating that the binding abilities and binding modes of retinol to r β -LG was almost the same as that to the native protein itself. We can further demonstrate from this functional assay that the yet unspecified retinol-binding site (Papiz *et al.*, 1986; Monaco *et al.*, 1987; Cho *et al.*, 1994; Katakura *et al.*, 1994) and the structure surrounding retinol in r β -LG were the same as those in native β -LG.

Antigenicity of r β -LG

Binding ability of mAb is affected by structural alteration of the epitope regions and mAb has been being used as a tool for detecting conformational change of protein. Our panel of anti- β -LG mAbs used in this study has been also shown to be able to probe for subtle

Figure 2. SDS-PAGE (A) and Western blotting (B) analyses of $r\beta$ -LG. (A) Lane 1, the fraction containing $r\beta$ -LG obtained from ion-exchange chromatography; lane 2, $r\beta$ -LG (0.1 μ g); lane 3, native β -LG (0.1 μ g). SDS polyacrylamide gel containing 15% acrylamide was used as running gel and electrophoresis was performed under reducing condition as described by Laemmli (Laemmli, 1970). This gel was stained with Coomassie Brilliant Blue. (B) Lane 1, native β -LG (10 ng); lane 2, lyophilizate of the culture supernatant of yeast transformed with pYG100 (10 ng); lane 3, $r\beta$ -LG (10 ng). The membrane was developed using anti- β -LG mAb 21B3 as described in MATERIALS AND METHODS.

and regional structural changes at the epitope regions of β -LG (Kaminogawa *et al.*, 1989). We could determine whether the structure of specific regions in β -LG molecule which underwent heat- or chemical-induced denaturation was more similar to that in the native or RCM β -LG (Hattori *et al.*, 1993; Kaminogawa *et al.*, 1989; Katakura *et al.*, 1994). When binding ability was tested with two mAbs (21B3 and 31A4), $r\beta$ -LG was shown to have an altered antigenicity as compared to native β -LG (Figures 5D and E). This alteration can be regarded to occur only at limited region(s), since three other mAbs (61C1, 62A6 and 61B4) showed no difference in binding ability between the two proteins in question (Figures 5A, B and C). However, the different binding affinities observed by this assay suggest that $r\beta$ -LG and native β -LG was recognized as different molecules by those antibodies. These imply that $r\beta$ -LG may induce a different immune response in vivo that would not be elicited by native β -LG.

Previously we have shown that particular peptides from β -LG can bind to some of anti- β -LG mAbs. Larger binding abilities of mAbs 21B3 and 31A4 to $r\beta$ -LG than those to native β -LG can be considered to be corresponding to structural perturbation in the regions of epitopes for these mAbs, since both mAbs

21B3 and 31A4 have been shown to bind to denatured β -LG more strongly than to native one. In this study, we can assume that $r\beta$ -LG is partially denatured in the regions containing ^{15}Val - ^{29}Ile and ^8Lys - ^{19}Trp , which are the peptide regions important for binding to mAbs 21B3 and 31A4, respectively (Figures 5D and E, and Table 1), while the epitope regions for mAb 61B4 (the region containing ^{125}Thr - ^{135}Lys , Table 1), 61C1 and 62A6 are maintained as in the native structure (Figures 5A, B and C). Although in the native protein mAbs 61C1 and 21B3 recognize conformationally close regions, and mAb 21B3 binds to the recombinant protein with greater affinity than to the native protein, mAb 61C1 binds to the recombinant protein with the same affinity as to the native protein (Figures 5A and D). These results suggest that although $r\beta$ -LG has a different structure from the native protein, this structural alteration is subtle and regional. Considering these with the previous finding that the structure of the region containing ^8Lys - ^{29}Ile was more difficult to refold and more fragile to denaturation treatment than other regions in the β -LG molecule (Kaminogawa *et al.*, 1987; Cho *et al.*, 1994), we can conclude that this structural alteration in the $r\beta$ -LG molecule is similar to other β -LG molecules having undergone heat- or

Figure 3. Reversed-phase HPLC profiles of $r\beta$ -LG. Purities of these β -LGs were confirmed by analytical RP-HPLC on a Vydac C18 protein & peptide column (0.46 cm x 25 cm), conducted on a Shimadzu (Kyoto, Japan) chromatography system consisting of a LC-6A gradient pump, an SPD-6A UV spectrophotometric detector and software. These β -LGs were chromatographed by using a linear gradient from 5 to 67 % acetonitrile in 60 min at a flow rate of 1 ml/min. β -LGs were detected by absorbance at 280 nm. (A); native β -LG and (B); $r\beta$ -LG.

Figure 4. Ability of native and $r\beta$ -LG to enhance all-*trans*-retinol fluorescence. All-*trans*-retinol dissolved in ethanol was added to a 1 ml solution containing 600 pmol of β -LG. Fluorescence was determined by using an excitation wavelength of 342 nm and an emission wavelength of 475 nm. $\circ$: native β -LG and $\bullet$: $r\beta$ -LG. Fluorescence intensity of free retinol in *N*-acetyl-*L*-tryptophanamide solution is denoted by $\triangle$. X axis denotes retinol concentration in pmol and the Y axis demonstrates relative fluorescence intensity.

chemical-induced denaturation. These results suggest that when producing recombinant protein in a strictly native form, it is important to elucidate whether the local structure with conformational fragility is maintained as a native protein.

Effect of the production and purification conditions on the antigenicity of $r\beta$ -LG

In order to clarify which step in this production and purification procedure is responsible for alteration of the antigenicity of $r\beta$ -LG, we added bovine milk native β -LGs at each step on the way of this procedure and evaluated their structural alteration by competitive ELISA. Firstly, native β -LG (10 mg) was added to 10 liters of the mock-transformed yeast culture, cultured with a vigorous agitation for 4 days at 30°C and purified in the same manner as $r\beta$ -LG (control β -LG-1). Secondly, after completion of culture of the mock-transformed yeast cells for 4 days at 30°C, native β -LG (10 mg) was added to this spent culture and purified in the same manner as $r\beta$ -LG (control β -LG-2). Finally, native β -LG was dissolved in imidazole buffer at 2 mg/l which is almost the same concentration as that for purifying $r\beta$ -LG just prior to applying this solution to ion-exchange chromatography, and purified similarly (control β -LG-3). We then applied these three control β -LGs to competitive ELISA.

The binding abilities of mAbs to these three control β -LGs were assessed by the equilibrium constant (K_{AS}) calculated by the method of Hogg *et al.* (Hogg *et al.*, 1987) based on the competitive and noncompetitive ELISA results (Figure 6). The results indicated that control β -LG-1 and -2 bound to all our mAbs similarly as $r\beta$ -LG, while control β -LG-3 had the same binding ability as native β -LG. These suggest that control β -LG-1 and -2, not but control β -LG-3, were partially denatured similarly to $r\beta$ -LG. The conclusion is that this two-step purification of the anion-exchange and size-exclusion chromatography and the final lyophilization has no effect on altering the antigenicity of $r\beta$ -LG. However, dissolving β -LG at a low protein concentration in the transformed yeast culture and/or its supernatant, but not aeration during this culture, was found to induce the antigenic alteration in the β -LG molecule. This conclusion is derived from that control β -LG-1 underwent the vigorous incubation with aeration before diafiltration, but that control β -LG-2 having the same binding ability of control β -LG-1 to mAbs was just dissolved in the yeast culture without the long-time incubation. Considering our

Figure 5. Binding curves of mAbs to β -LGs. Competitive ELISA was performed as described in MATERIALS AND METHODS. B and B_0 are absorbances at 405 nm determined at the last step of the competitive ELISA in the presence and absence of the competitor antigen, respectively. The competitive antigen used was either native β -LG (○), RCM β -LG (△), or r β -LG (●). X axis denotes the concentration of the competitive antigen and the Y axis demonstrates B/B_0 .

observation that the pH value of the spent culture medium of yeast cells fell to 3~4, the lowered pH may be responsible for the antigenic alteration. These results suggest that environmental differences to which native and r β -LGs are exposed during their production may affect antigenicity. However, we cannot perfectly deny the possibility that yeast produced partially denatured β -LG.

Since β -LG is known to be able to bind a small hydrophobic molecule(s) as well as retinol, some mole-

cules in the yeast culture supernatants may bind to β -LG during its culture. This interaction might have affected the binding abilities of mAbs 21B3 and 31A4, leading to alter the antigenicity. If this was true, then the binding affinity of the two mAbs should have been reduced by the predicted structural hindrance induced by this ligand binding. However, we show that the binding abilities of mAbs 21B3 and 31A4 to r β -LG were in fact greater than to native β -LG, and that mAb 61C1, which binds to native β -LG at the epitope close

Figure 6. Equilibrium constants (K_{AS}) of β -LGs with mAbs. $\circ$: native β -LG, $\bullet$: RCM β -LG, $\square$: r β -LG, $\diamond$: control β -LG-1, $\triangle$: control β -LG-2, $\blacktriangle$: control β -LG-3. K_{AS} values were calculated from the results from competitive ELISA according to the procedure of Hogg et al. (Hogg et al., 1987).

to that of mAb 21B3, bound equally to native and r β -LG. These results suggest that the increase of binding affinities of mAbs 21B3 and 31A4 are not due to a random binding of impurities in the yeast culture supernatants. Thus, the change in binding affinities of these mAbs can be attributed to a structural change at certain locations of β -LG during its incubation with the transformed yeast. In any case, when producing a recombinant protein using genetic engineering techniques and applying the product for pharmaceutical use, we must pay the close attention to the culture condition, since recombinant protein produced and purified differently from the native protein may have altered antigenicity and may thus induce a quite different immune response in vivo.

Concluding remarks

The results in this report show that r β -LG possess the same retinol-binding ability as native β -LG obtained from bovine milk, although the antigenicity of r β -LG was altered. Thus the biological function of ligand-binding is tolerant to a minor change, even when antigen-antibody interaction can discriminate that minor change between native and partially altered proteins. Our results imply that production and purification of human recombinant protein pharmaceuticals using relative biological activity as an index does not necessarily ensure the acquisition of nonimmunogenic protein in humans, and that even a small change in the recombinant protein's molecular structure may induce improper immune responses in vivo. Binding assays using mAbs whose epitopes cover all surface regions can be useful for assessing a change in antigenicity.

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