

## Comparative Immunogenic Properties of Native and Crystalline $\beta$ -Lactoglobulins Prepared by Two Methods

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### Abstract

$\beta$ -Lactoglobulin ( $\beta$ -Lg) was the dominant sensitizing protein in bovine skim-milk and milk whey protein. Significant differences in the anaphylactogenic sensitizing capacities in guinea pigs were detected among samples of crystalline  $\beta$ -Lg prepared by the same method in different laboratories, as well as among crystalline  $\beta$ -Lg samples prepared by different methods in the same laboratory. These differences could not be related to differences in their precipitin reactions, heterogeneity, carbohydrate content or genetic variants. These observations suggest that something other than the procedure for isolating crystalline  $\beta$ -Lg may be responsible for the differences in immunogenic potency.

Native  $\beta$ -Lg was 2 to 4 times more immunogenic than crystalline  $\beta$ -Lg prepared by the Palmer method, and 12 to 26 times more potent than  $\beta$ -Lg prepared by the Larson and Jenness method.

### Introduction

In comparing the immunogenic activity of potential allergens or antigens in a complex such as cow's milk, it is essential to isolate the individual components in as near the native state as possible. Stanworth (21) has postulated that extraction and isolation of allergenic substances may unmask additional antigenic determinants that play no part in spontaneous sensitization by the native material. It seems equally likely that isolation procedures may alter or disrupt antigenic or structural features that may be responsible for sensitization. A previous report from this laboratory (4) has shown that the sensitizing properties of antigens are more susceptible to degradation by chemical and physical processes than other immunogenic properties, such as capacity to elicit

skin responses, or to induce anaphylactic shock in sensitized guinea pigs.

The purpose of this paper is to demonstrate: a) the relative immunogenicities of several purified milk proteins, b) that  $\beta$ -lactoglobulin ( $\beta$ -Lg) was the dominant immunogen in skimmilk, c) significant differences in immunogenic potency of native and crystalline  $\beta$ -Lg, and d) significant differences in immunogenicity of preparations of crystalline  $\beta$ -Lg isolated by the two methods as well as differences in potency of different preparations isolated by the same method in different laboratories. Deutsch (7) has previously reported marked differences in the immunogenicity of crystalline  $\beta$ -Lg prepared by different methods.

### Materials and Methods

$\beta$ -Lg specimens, P-1 and P-2, were isolated from pooled milk by the Palmer method (17) as described by McMeekin et al. (15). Sample P-3 was prepared by McMeekin (unpublished) from a portion of P-2 by fractional crystallization at pH 4.8 instead of the usual pH 5.2.  $\beta$ -Lg samples LJ-1 and LJ-2 were prepared in two remote laboratories by the method of Larson and Jenness (12). All  $\beta$ -Lg samples were recrystallized at least 4 times and stored dry at 5 C.

$\alpha$ -Lactalbumin ( $\alpha$ -La) was isolated and crystallized 4 times by the method of Gordon and Ziegler (8).  $\alpha$ -Casein fraction ( $\alpha$ -Cf) was prepared by the Warner method (22) as modified by McMeekin et al. (16).  $\beta$ -Casein ( $\beta$ -Cn) was prepared from the filtrate of the  $\alpha$ -casein separation by the method of Warner (22). The whey protein was obtained by centrifuging fresh skimmilk at  $44,330 \times g$  for 120 min. The calcium caseinate pellets were discarded and the supernatant containing the whey proteins was lyophilized (6). Bovine serum albumin (BSA) (Armour Laboratories, Lot P67704)<sup>2</sup> and bovine gamma globulin (BGG) (Cailard Schlesinger Corp., Lot A3371)<sup>2</sup> were commercial preparations.

<sup>2</sup> Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

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*Rabbit antisera to  $\beta$ -Lg.*  $\beta$ -Lg antiserum, Pool 1, was obtained by pooling the sera from only 1 of 5 New Zealand Albino rabbits that had been immunized with a multiple course of injections with  $\beta$ -Lg (LJ-1). The first course of immunization consisted of 4 intravenous injections per week from the first through the fourth week with 2 mg  $\beta$ -Lg in saline. The second course consisted of 4 subcutaneous injections per week with 2 mg of alum-precipitated  $\beta$ -Lg from the 7th through the 10th week. The 3rd, 4th, and 5th courses were the same as the 2nd, and were injected during the 12th, 14 to 15th, and 16 to 17th week, respectively. The 6th course of immunization consisted of 4 intravenous injections per week of 10 mg of  $\beta$ -Lg in saline during the 19th and 20th week.

Rabbits were bled within 5 to 9 days following the final injection of each course. Only 1 of 5 rabbits produced significant quantities of precipitin. Accordingly, Pool 1 consisted of a pool of sera from the second to final bleeding of the one rabbit. This antiserum contained about .11 mg antibody-nitrogen (AbN) per milliliter.

For production of  $\beta$ -Lg antiserum, Pool 2, Freund's complete adjuvant was prepared by emulsifying 1 ml of a saline solution containing 9.25 mg of  $\beta$ -Lg (LJ-1) with 1 ml of Freund's incomplete adjuvant (Difco)<sup>2</sup> to which had been added 2.5 mg of killed tubercle bacilli. One-tenth milliliter of the emulsified antigen was injected into each of the footpads of 2 rabbits. On Day 28, both rabbits were injected intravenously with 10 mg of  $\beta$ -Lg in saline, and the rabbits were bled on Day 35. A booster dose of 3.3 mg of  $\beta$ -Lg in saline was then injected intravenously and the rabbits were bled out one week later. The pooled antiserum contained about .5 mg AbN per milliliter.

Both antiserum pools were preserved by the addition of .01% Merthiolate and stored at -20 C until used.

*Carbohydrate analyses.* Neutral sugar analyses of the  $\beta$ -Lg samples were conducted by an unpublished modification<sup>3</sup> of the method of Lee et al. (14). The proteins were hydrolyzed in sealed tubes in N sulfuric acid for 5

hr at 100 C before analyses for carbohydrates. Only small amounts of carbohydrates were in the samples; accordingly, the peaks were small and the amounts reported are only approximations.

*Precipitin determinations.* Quantitative precipitin determinations were by the method of Heidelberger and Kendall (10) as described by Kabat and Mayer (11). The analyses were performed by adding 1.0 of undiluted (Pool 1) or 1:5 diluted (Pool 2) antiserum to each of a series of tubes containing increasing amounts of  $\beta$ -Lg in 1 ml of buffered saline. The contents were immediately mixed and stored 1 hr at 37 C and then 5 days at 5 C. The precipitates were centrifuged at 2 C and washed twice with cold saline. Nitrogen in the precipitates was determined by the micro-Kjeldahl method. Results are recorded as AbN precipitated per milliliter of serum.

*In vitro anaphylaxis.* Sensitivity induced in guinea pigs with different dosages of skimmilk was determined by the Schultz-Dale technic (5). Two groups of 10 virgin female guinea pigs, weighing 200 to 250 g each were sensitized by single intraperitoneal injections of skimmilk. Animals in the first group were sensitized at a dosage of 125  $\mu$ g nitrogen (N), and animals in the second group were sensitized with 500  $\mu$ g N. Following incubation of 3 to 4 weeks, the animals were killed and the uterine muscles were excised. Each horn of the uterus was cut transversely into 2 strips and mounted in separate 50 ml Dale baths. The uterine strips were then examined for sensitivity to the 4 principal milk proteins;  $\alpha$ -Cf,  $\beta$ -Cn,  $\alpha$ -La,  $\beta$ -Lg, and to skimmilk. Sensitivity to  $\beta$ -Lg was determined by challenge at a dosage of 1  $\mu$ g N. Sensitivity to the other proteins, including skimmilk, was tested by challenge with 10  $\mu$ g N.

Animals sensitized with whey protein were similarly tested for sensitivity to the major milk proteins, including in addition, BSA and BGG.

*Determination of median sensitizing doses.* Immunogenic potency of the  $\beta$ -Lg preparations were compared by determining the median sensitizing dose ( $SD_{50}$ ) of each preparation. Guinea pigs weighing 280 to 320 g were injected intraperitoneally at 5 to 8 dosage-levels of antigen designed to cover the range from an occasional fatal sensitization to nearly uniform fatal sensitivity.<sup>4</sup> Following an incuba-

<sup>3</sup>The authors are indebted to M. L. Groves, Eastern Marketing and Nutrition Research Division, U.S. Department of Agriculture, Philadelphia, for determination of neutral sugars by the Lee et al. method (14) as modified by M. L. Groves and D. M. Freidman.

<sup>4</sup>Albino guinea pigs of both sexes were progeny of a random-bred, closed colony that has been maintained in this Laboratory since 1937 (3).

TABLE 1. In vitro anaphylactic reactions in uterine tissues from guinea pigs sensitized with skimmilk and whey proteins.

Sensitizing dose	Anaphylactic responses to <sup>a</sup> :					
	$\alpha$ -Cf	$\beta$ -Cn	$\alpha$ -La	$\beta$ -Lg	BSA	BGG
			Reaction ratios <sup>b</sup>			
125 $\mu$ g N Skimmilk	0/10	0/10	0/10	6/10	...	...
500 $\mu$ g N Skimmilk	0/10	4/10	1/10	10/10	...	...
95 $\mu$ g N Whey protein	1/6 <sup>c</sup>	0/6	0/6	6/6	1/5	1/5 <sup>c</sup>

<sup>a</sup> Challenge doses were 1  $\mu$ g N in a 50-ml bath for  $\beta$ -Lg and 10  $\mu$ g N for the other proteins.

<sup>b</sup> Reaction ratios =  $\frac{\text{Number of animals reacting}}{\text{Number of animals tested}}$ .

<sup>c</sup> These were very slight or doubtful reactions indicative of a barely perceptible sensitivity.

tion of 21 days, each animal was challenged with an excess of homologous protein contained in .5 ml of saline. Details of the procedure and the method of calculating the SD<sub>50</sub> have appeared in previous reports (3).

### Results

The relative immunogenic activities of the principal proteins in raw skimmilk and the whey protein fraction as determined by in vitro anaphylaxis are in Table 1. Tissues from 6 to 10 animals sensitized at a dosage of 125  $\mu$ g N of skimmilk reacted to 1  $\mu$ g N of  $\beta$ -Lg, and to 10  $\mu$ g N of skimmilk (not shown). None of them responded to 10  $\mu$ g doses of  $\alpha$ -Cf,  $\beta$ -Cn, or  $\alpha$ -La. In every case in which sensitivity to skimmilk was observed, the tissue was also sensitive to  $\beta$ -Lg. Moreover, prior reaction with, and desensitization to  $\beta$ -Lg always desensitized the tissue to subsequent reaction to skimmilk. Uterine muscle from all 10 animals sensitized at 500  $\mu$ g N were sensitive to  $\beta$ -Lg, 4 were sensitive to  $\beta$ -Cn and one was sensitive to  $\alpha$ -La. None of the uterine

strips reacted to  $\alpha$ -Cf.

Similar experiments were conducted with uterine muscles from guinea pigs sensitized at a dosage of 95  $\mu$ g N of the whey protein. A preliminary experiment indicated that at this dosage the uterine muscles reacted only to  $\beta$ -Lg. Accordingly, 5 more animals were sensitized at this dosage and the uterine strips were tested for sensitivity to the 4 major milk proteins, and in addition, to BSA and BGG. Uterine strips from all 6 animals were sensitive to the 1  $\mu$ g N dose of  $\beta$ -Lg. Tissues from 1 of 6 animals showed a barely perceptible reaction to  $\alpha$ -Cf, and 1 of 5 showed about the same degree of sensitivity to BGG at the 10  $\mu$ g N dosage. In addition, the tissue of 1 of 5 animals showed a definite response to BSA.

These results show that when guinea pigs are sensitized with one intraperitoneal injection at a dosage not exceeding 125  $\mu$ g N as skimmilk or 95  $\mu$ g N of milk whey protein, the animals develop anaphylactic sensitivity to the  $\beta$ -Lg fraction and only an occasional animal develops sensitivity to other milk proteins. Accordingly, this observation provided a means

TABLE 2. Data for calculation of median sensitizing doses of  $\beta$ -Lg prepared by two methods.<sup>a,b</sup>

Sample	Sensitizing dosages in micrograms <sup>c</sup>								
	12.5	25	50	100	200	400	800	1,600	3,200
	Mortality ratios <sup>d</sup>								
P-1 <sup>e</sup>	1/18	6/18	4/18	9/18	14/15	14/15			
P-2			2/10	4/10	7/10	6/10	9/10		
P-3			2/10	6/10	7/10	6/10	10/10		
LJ-1 <sup>e</sup>	0/5	0/6	0/20	6/20	7/20	8/20	13/20	9/10	
LJ-2				0/10	2/10	2/10	3/10	7/10	9/10

<sup>a</sup> Incubation was 21 days.

<sup>b</sup> Challenge dose was 1 mg of  $\beta$ -Lg in 0.5 ml saline.

<sup>c</sup> Air-dried solid basis.

<sup>d</sup> Mortality ratio upon subsequent challenge.

<sup>e</sup> Data for P-1 and LJ-1 are composites of two closely agreeing assays (See text).

TABLE 3. Data for calculation of medium sensitizing dose of skimmilk and milk whey proteins<sup>a,b</sup>.

Skimmilk		Milk whey	
Sensitizing dose (N basis)	Mortality ratio <sup>c</sup>	Sensitizing dose (Dry basis)	Mortality ratio <sup>c</sup>
( $\mu\text{g}$ )		( $\mu\text{g}$ )	
15.63	1/10	12.5	0/10
31.25	3/10	25	1/10
62.5	5/10	50	4/10
125	8/10	100	9/10
		200	7/10
		400 <sup>d</sup>	10/10

<sup>a</sup> Incubation was 21 days.

<sup>b</sup> Challenge dose was 1 mg N of skimmilk and .5 mg whey protein, respectively.

<sup>c</sup> Mortality ratio upon challenge with homologous antigen.

<sup>d</sup> This highest dosage-level is equivalent to only 47.3  $\mu\text{g}$  N, or about half that used for the in vitro tests in Table 1.

for determining the  $SD_{50}$  of  $\beta$ -Lg in its native state.

*Sensitizing potency of milk proteins.* Data collected for determinations of the  $SD_{50}$  for the  $\beta$ -Lg samples, skimmilk and whey protein are in Table 2 and 3, respectively. These data were used to construct dosage-response curves as previously described (3). Agreement between the observations and the fitted lines, as determined by the  $\chi^2$  test were satisfactory in every case, and the slopes of the dosage-mortality curves were essentially parallel. This permitted a direct comparison of the calculated  $SD_{50}$ s for the native  $\beta$ -Lg and the crystalline proteins. The results are in Table 4. The  $SD_{50}$ s of the  $\beta$ -Lg specimen prepared by the P-method ranged from  $10 \pm 2$  to  $22 \pm 7 \mu\text{g}$  N. While the difference between the high and low  $SD_{50}$ s for these preparations is

TABLE 4. Calculated median sensitizing doses for  $\beta$ -lactoglobulins, skimmilk, and milk whey proteins.

Preparation	Median sensitizing doses	
	Solid basis	N basis
	( $\mu\text{g}$ )	( $\mu\text{g}$ )
$\beta$ -Lg, P-1	$72 \pm 12^a$	$10 \pm 2^a$
$\beta$ -Lg, P-2	$150 \pm 40$	$22 \pm 7$
$\beta$ -Lg, P-3	$120 \pm 40$	$17 \pm 5$
$\beta$ -Lg, LJ-1	$410 \pm 80$	$58 \pm 12$
$\beta$ -Lg, LJ-2	$940 \pm 210$	$130 \pm 30$
Skimmilk		$57 \pm 13$
Whey protein	$68 \pm 12$	$8 \pm 1.5$

<sup>a</sup> Average standard errors.

barely significant ( $P = .03$ ), the 3 results together are of the same order of magnitude ( $P = .07$ ). In contrast, the  $SD_{50}$ s of the LJ samples were  $58 \pm 12$  and  $130 \pm 30 \mu\text{g}$  N, respectively. Thus the immunogenic potency of the P samples were from 3 to 13 times greater than those of the LJ samples. This is a highly significant difference ( $P = .001$ ).

Reproducibility of the method for determining the  $SD_{50}$  of protein antigens was demonstrated from the data collected for Samples P-1 and LJ-1. The data in Table 2 for these samples are composites of two individual assays for each specimen. The calculated  $SD_{50}$  for the first and second assays for P-1 were  $11 \pm 4$  and  $10 \pm 2 \mu\text{g}$  N, respectively. The separate assays for Samples LJ-1 were  $72 \pm 25$ , and  $48 \pm 13 \mu\text{g}$  N. Thus, both repeat assays yielded results within the expected experimental error of the method ( $P = .69$  and  $.37$ ).

The  $SD_{50}$ s of the total proteins in skimmilk and the whey protein fraction were  $57 \pm 13$  and  $8 \pm 1.5 \mu\text{g}$  N. These dosages are significantly lower than the effective sensitizing doses of the other milk proteins. Accordingly, these results may be used to determine the  $SD_{50}$  of native  $\beta$ -Lg. Thus, since  $\beta$ -Lg accounts for about 7 to 12% of the total proteins of skimmilk, and about 45 to 55% of the proteins of the whey fraction (9), it may be calculated that the  $SD_{50}$  of skimmilk contains about  $5 \mu\text{g}$  N as  $\beta$ -Lg, and the  $SD_{50}$  of the whey fraction contains about  $4 \mu\text{g}$  N as  $\beta$ -Lg. Therefore, it may be assumed that the  $SD_{50}$  of native  $\beta$ -Lg is about 4 to  $5 \mu\text{g}$  N, or about  $30 \mu\text{g}$  of protein.

*Quantitative precipitin tests.* In their reactions with two  $\beta$ -Lg antisera, all 5 preparations yielded precipitin curves typical of essentially homogeneous proteins reacting with homologous antisera. Clear-cut equivalence zones were observed when these proteins reacted with from 5 to  $30 \mu\text{g}$  of antigen-nitrogen (AgN) added for Pool 1, and 10 to  $100 \mu\text{g}$  AgN added per milliliter for Pool 2. Maximal AbN/ml precipitated from Pool 1 averaged .12 mg/ml for the P-samples (range: .11 to .13), and .11 mg/ml for the LJ-samples (.10 to .11). Maximal AbN/ml precipitated from Pool 2 averaged .52 for the P-samples (.50 to .54), and .53 mg/ml for the LJ-samples (.53 and .53). The AbN/AgN ratios for Pools 1 and 2 were 7 and 13. Plots of the AbN/AgN ratios in the precipitates versus AgN added per milliliter of serum gave straight-line relationships

with each  $\beta$ -Lg preparation. Accordingly, the precipitin analyses showed that all of the  $\beta$ -Lg samples were essentially homogeneous<sup>5</sup>, and equal in precipitating capacities.

**Carbohydrate analyses.** The neutral sugars, calculated as glucose, ranged from .06% through .12% to .18% for P-2, P-1, and P-3 samples, respectively, and .03% and .6% for LJ-1 and LJ-2. No mannose, fucose or galactose was detected in any of the samples<sup>3</sup>.

**Genetic variants.** The  $\beta$ -Lg preparations in this investigation were isolated from pooled milk before information of the genetic variants was available. These samples were all composed of about equal proportions of  $\beta$ -Lg-A and  $\beta$ -Lg-B.

### Discussion

Investigations of the quantitative anaphylactogenic properties of  $\beta$ -lactoglobulins prepared by two commonly used methods have revealed significant differences in sensitizing capacities of the product. Thus, crystalline  $\beta$ -Lg prepared by the Palmer method (17) were from 3 to 13 times more potent in their capacities to sensitize guinea pigs than  $\beta$ -Lg prepared by the Larson and Jenness method (12). Additionally, there were significant differences in the sensitizing capacities of  $\beta$ -Lgs prepared by the same method. Thus, sample P-2 was only half as potent as P-1 ( $P = .03$ ), and there was about the same differences in potencies of LJ-1 and LJ-2 ( $P = .008$ ). The results of these assays were shown to be reproducible, and did not change with age of the samples. The  $SD_{50}$  for sample P-1 had not changed when the assay was repeated after storage at 5 C for 8 years. In contrast, native  $\beta$ -Lg was 2 to 4 times more immunogenic than crystalline  $\beta$ -Lg prepared by the Palmer method, and 12 to 26 times more potent than  $\beta$ -Lg prepared by the Larson and Jenness method.

These differences in immunogenic potency could not be related to inhomogeneity of the proteins. All 5 preparations were homogeneous as determined by the quantitative precipitin technic, and all were equal in their capacity to precipitate antibody from rabbit anti- $\beta$ -Lg

sera. In addition,  $\beta$ -Lg gave a single line of precipitate by the Ouchterlony double-diffusion method against homologous antisera, and by immunoelectrophoresis. These results confirm previous findings (1, 9, 13, 18, 20). However, a slight amount of  $\alpha$ -lactalbumin was detected by disc-electrophoresis in one of the samples, and this was confirmed by passive hemagglutination. Presumably, the amount of  $\alpha$ -La in the  $\beta$ -Lg preparation was too small to induce antibody formation in either of the two anti- $\beta$ -Lg rabbit sera. This would account for the failure to detect the contamination by serological methods.

The fact that significant differences in immunogenic potencies were observed in  $\beta$ -Lgs prepared by the same method, and in the same laboratory (i.e. Samples P-1 and P-2), suggest that something other than the procedure for isolating the crystalline protein may be responsible for these differences. Bleumink and Young (2) reported that the allergenic activity of native  $\beta$ -Lg is markedly enhanced when N-glycosidic sugar-protein linkages are incorporated into the protein molecule.  $\beta$ -Lactoglobulins prepared in their laboratory were stated to contain from .1 to 1.1% carbohydrate (2). In contrast, our  $\beta$ -Lg samples contained only .03 to .18%. The latter value is equivalent to only .4 moles of glucose per 36,000g of  $\beta$ -Lg. Moreover, there was no correlation between the sensitizing capacities of the proteins and their carbohydrate content. This would appear to rule out any possible effect of carbohydrate on the immunogenic properties of the  $\beta$ -Lg samples.

Other possible factors considered were the genetic variant composition of the  $\beta$ -Lg specimen. Previous work by other investigators has shown that the antigenic specificity of the variants  $\beta$ -Lg-A,  $\beta$ -Lg-B,  $\beta$ -Lg-C, and  $\beta$ -Lg-D are indistinguishable by the quantitative precipitin technic, Ouchterlony double-diffusion, and immunoelectrophoresis (1, 9, 13, 18, 20), but the variants could be distinguished by complement fixation methods (18). Since our samples were all composed of about equal proportions of  $\beta$ -Lg-A and  $\beta$ -Lg-B, it is not probable that differences in the genetic variant composition of our samples could explain the differences observed in their sensitizing capacities. Nevertheless, since our method appears to detect subtle differences in structural features not detectable by the usual serological methods, it would be of interest to compare the anaphylactogenic sensitizing properties of the genetic variants.

<sup>5</sup> In confirmation, no contaminants could be detected in  $\beta$ -Lg (LJ-1) by immunodiffusion or by immunoelectrophoresis. However, on disc electrophoresis a faint band occupied the  $\alpha$ -La region. The slight contamination of  $\beta$ -Lg with  $\alpha$ -La was confirmed by passive hemagglutination tests. These analyses were conducted by Mrs. Carol S. Pierce, formerly of this Laboratory.

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