β-Lactoglobulins A and B

I. CHROMATOGRAPHIC SEPARATION AND AMINO ACID COMPOSITION*

KARL A. PIEZ,† EARL W. DAVIE,‡ J. E. FOLK,† AND JULES A. GLADNER§

From the National Institutes of Health, Public Health Service, United States Department of Health, Education and Welfare, Bethesda, Maryland and Western Reserve University, Cleveland, Ohio

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It was shown by Aschaffenburg and Drewry (2) that bovine β-lactoglobulin occurs in two different forms apparently under the control of allelic genes. One cow may produce both together or either one alone. It is known that the two phenotypic proteins differ in isolectric point (2-4) and that β-lactoglobulin A has two more carboxyl groups than β-lactoglobulin B per mole-
cule of 36,000 molecular weight (4). Timasheff, Townend, and coworkers, in a series of papers (5-10), have reviewed the early work and have cleared up much of the confusion that existed concerning the identity and number of molecular species present in mixed preparations. They demonstrated that β-lactoglobulin A forms heavy aggregates whereas β-lactoglobulin B does not (6, 7, 9) and that the 36,000 molecular weight species dissociates at low pH into two subunits that appear to be identical (8, 10).

As part of a study to characterize further the two proteins, we report here the fractionation of mixed β-lactoglobulin by chromatography on diethylaminoethyl cellulose and demonstrate by amino acid analysis of the two major components, corresponding to β-lactoglobulin A and β-lactoglobulin B, that two amino acid substitutions are present. A preliminary report of these results has appeared (1). Gordon, Basch, and Kalan (11) have independently obtained similar evidence.

EXPERIMENTAL PROCEDURE

Chromatographic Separation of β-Lactoglobulin—The preparations used in this study were three times crystallized commercial products (Pentex, Inc.), containing approximately equal amounts of β-lactoglobulin A and β-lactoglobulin B. Before chromatography, the protein was dissolved at a concentration of 50 mg per ml in 0.05 M sodium phosphate, pH 5.8, and dialyzed against the same buffer for 18 hours at 4°. For 200 mg samples, a 1.8-× 10-cm column was used; for 1 g samples, a 1.8- × 35-cm column was used. The adsorbent was reagent grade DEAE Selectacel, type 40, with a capacity of 1.05 meq per g (Brown Company). Before use, the adsorbent was washed with 0.10 M hydrochloric acid, 0.10 M sodium hydroxide, and then equilibrated with 0.05 M sodium phosphate buffer, pH 5.8. For elution of the smaller column, a linear gradient was established by placing 350 ml of 0.05 M sodium phosphate, pH 5.8, in the mixing chamber and 350 ml of 0.05 M sodium chloride-0.05 M sodium phosphate, pH 5.8, in the adjacent reservoir. Five milliliter fractions were collected at a rate of 8 ml per hour at 5°. For the larger column, the gradient was formed from 450 ml of 0.02 M sodium chloride-0.05 M sodium phosphate, pH 5.8, in the mixing chamber and 450 ml of 0.11 M sodium chloride-0.05 M sodium phosphate, pH 5.8, in the adjacent reservoir. When necessary, elution was continued with the higher strength buffer to complete elution of the second peak. Five milliliter fractions were collected at a rate of 12 ml per hour at 5°. The major fractions were combined, dialyzed at 4° against several changes of distilled water, and lyophilized. For preparative purposes, all samples were chromatographed a second time to insure complete separation. The protein concentrations were determined by absorbancy measurements at 280 μμ with an absorbancy index of 9.7 for a 1.0% solution at neutral pH (12).

Electrophoresis and Ultracentrifugation—A Spinco paper electrophoresis unit was used with barbital buffer, pH 8.6, Γ/2 = 0.10 (3). Moving boundary electrophoresis was performed in a Spinco model H instrument with an 11-ml cell and acetate buffer, pH 5.3, Γ/2 = 0.10 (5). The sedimentation behavior of the proteins was examined in a Spinco model E ultracentrifuge.

Amino Acid Analysis—Samples of β-lactoglobulin A and β-lactoglobulin B weighing approximately 7 mg were hydrolyzed at 106° in 4 ml of constant boiling HCl for 24, 48, or 96 hours in glass ampules which were flushed with nitrogen and sealed. The hydrolysates were taken to dryness once under vacuum with gentle heat, and the residues were dissolved in water and diluted to 5 ml. One-fifth aliquots were analyzed on the automatic amino acid analyzer described by Piez and Morris (13), which is a modification of the instrument designed by Spackman, Stein, and Moore (14). Each of the six hydrolysates was analyzed in duplicate, and the averages were calculated. Standard amino acid solutions were analyzed before and after the samples to check the performance of the instrument. The small amounts of methionine sulfoxide (1 to 3% of the methionine), cysteic acid (<1% of the cystine), and alloisoleucine (1 to 2% of the isoleu-
cine) found were added to the values obtained for the parent compound. Free sulfhydryl groups were determined on separate samples of the native proteins dissolved in 0.1 M sodium dodecyl sulfate by the N-ethylmaleimide reaction of Alexander (15) as described by Cole, Stein, and Moore (16). The reaction was complete in 200 minutes with β-lactoglobulin B and 100 minutes

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† National Institute of Dental Research, National Institutes of Health.
§ National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.
with $\beta$-lactoglobulin A. Tyrosine and tryptophan values were calculated from the ultraviolet absorption spectra (17).

**RESULTS**

A typical chromatographic fractionation of crystalline $\beta$-lactoglobulin prepared from pooled milk is shown in Fig. 1. The two major peaks contained approximately equal quantities of protein and together accounted for about 85% of the original protein. The individual peaks were identified as $\beta$-lactoglobulin A and $\beta$-lactoglobulin B by electrophoresis and sedimentation. It has been established (6) that $\beta$-lactoglobulin A is the fast moving component when subjected to paper electrophoresis and that, unlike $\beta$-lactoglobulin B, it forms aggregates at pH 4.65 which can be observed in the ultracentrifuge. The minor peaks shown in Fig. 1 varied both in amount and position with the particular batch of $\beta$-lactoglobulin. Some preparations showed minor components of less than 5% while others showed minor components of nearly 20% of the total protein. When the ascending portion of Peak B or the descending portion of Peak A was rechromatographed, each showed single peaks in the same position and with the same shape as found with the original mixture indicating that each peak represented a single component. Symmetrical peaks of the individual proteins were found only when the sample size was greatly reduced. When dialyzed against distilled water, $\beta$-lactoglobulin A readily crystallized as long needles. Conditions for crystallization of $\beta$-lactoglobulin B were not investigated.

The electrophoretic patterns of the two proteins at a pH close to their isoelectric points were essentially identical to those published by Timasheff and Townend (5) for samples prepared from the milk of cows homozygous for $\alpha$-lactoglobulin A or $\beta$-lactoglobulin B. The $\beta$-lactoglobulin A peak displayed a slight skewness in the forward direction on the descending limb; the $\beta$-lactoglobulin B peak showed the presence of a second component after prolonged electrophoresis (e.g. 1080 minutes at 5.5 volts per cm). It was observed that the mobility of $\beta$-lactoglobulin B changed as the second peak separated which may indicate degradation rather than true heterogeneity.

The complete amino acid data are presented in Table I. The values obtained by ion exchange chromatography (the first six columns) represent averages of two determinations. To obviate differences resulting from errors in sample size, minor adjustments were made in the data in the following manner. First, as a base which could be used for all hydrolysates, the total number of micromoles of those amino acids that did not change with time of hydrolysis was determined (partial totals, Table I). All the values in each hydrolysate were then multiplied by an appropriate factor to make each partial total equal to the average. This was done separately for $\beta$-lactoglobulin A and $\beta$-lactoglobulin B. The factors were all between 0.99 and 1.02. Corrections were then made for hydrolytic losses or incomplete hydrolysis as described below and the three hydrolysates were averaged. It was then assumed, as was later shown to be correct by the internal consistency of the data, that $\beta$-lactoglobulin A and $\beta$-lactoglobulin B contained the same total number of amino acid residues per protein equivalent and the values in use set ($\beta$-lactoglobulin B) were multiplied by a factor (1.030) to make the total number of micromoles in both proteins identical. This factor was also applied to the data in the individual hydrolysates of $\beta$-lactoglobulin B so that comparisons could be made across the columns in Table I. The data were finally converted to micromoles in approximately 1 mg of protein (1.002 mg for $\beta$-lactoglobulin A and 0.997 mg for $\beta$-lactoglobulin B) calculated for 100% recovery. It was necessary for the weights to be not quite equal since it was desired to maintain the same total micromoles and, as was later shown (see below), the proteins have slightly different molecular weights because of the differences in amino acid composition. The fact that all of the weight can be accounted for as amino acids has been well documented previously (18, 19). The recoveries, corrected for water content, obtained by us were 94% ($\beta$-lactoglobulin B) and 97% ($\beta$-lactoglobulin A), the differences from 100% probably being ascribable to incomplete removal of buffer salts.

These corrections are all within expected experimental variation. They do not change the data in the sense that the values presented are not significantly different from the values observed, but they permit a more precise comparison since, in the present instance, the errors introduced in measuring sample size are greater than the precision inherent in the analytical method.

Columns 7 and 8 of Table I present averages of those amino acids which did not change with time and corrected values for the other amino acids. Threonine showed an average decrease of 3.0% per 24 hours; serine decreased an average of 6.3% per 24 hours. The 24-hour values for these two amino acids were therefore increased by these amounts. Valine and isoleucine continued to increase through 96 hours of hydrolysis. The 96-hour values were assumed to represent essentially complete liberation. In the case of isoleucine, the 24- and 48-hour values agreed almost exactly. Therefore, the average of the 96-hour values was assigned to both proteins. Methionine and tyrosine required corrections for hydrolytic destruction of 2.5 and 1%, respectively. Ammonia increased slightly more between 24 and 48 hours than can be accounted for by destruction of serine and threonine, and the rate of increase decreased between 48 and 96 hours. The correction which should be applied to ammonia was therefore somewhat uncertain. This was done arbitrarily by subtracting the average change between 24 and 48 hours from the 24-hour value. The concentrations of cysteine, determined by reaction with N-ethylmaleimide (16), and tryptophan, calculated from the ultraviolet absorption spectra (17), are included in Table I. The cysteine content is in agreement with previously determined values (20, 21). The value for cysteine obtained by

![Fig. 1. Fractionation of 210 mg of $\beta$-lactoglobulin on a 1.8 × 10-cm DEAE column with the use of a linear gradient between 0.05 M phosphate, pH 5.8, and 0.05 M phosphate-0.08 M sodium chloride, pH 5.8. See text for details.](http://www.jbc.org/content/236/11/2913/F1)
ion exchange chromatography presumably does not include significant amounts of cysteine since no attempt was made to oxidize the sulfhydryl compound to the disulfide and all operations before chromatography were conducted under nitrogen, in a vacuum, or in the cold.

Column 9 shows the differences between $\beta$-lactoglobulin A and $\beta$-lactoglobulin B. Aspartic acid, glycine, alanine, and valine differed significantly. On the assumption that these differences represent substitutions of single residues per protein equivalent, the difference (average, 0.055) is the micromole equivalent to one residue in the weight of protein represented, which is about 1 mg. more remarkable than the differences considering the relative amount of protein.

The minor differences which occur can be readily ascribed to the differences in Column 9 can become considerably larger than the statistical error. The partial totals refer to the values of 1.7 and 2.3 calculated from the data of Brand et al. (18) and Spies and Chambers (22), respectively.

**DISCUSSION**

The amino acid data in Table I are in good agreement with the earlier data on a mixture of $\beta$-lactoglobulins obtained by Brand et al. who used chemical and microbiological methods, and Stein and Moore (19), who used starch column chromatography. The minor differences which occur can be readily ascribed to the expected kind of analytical error. The similarities are perhaps more remarkable than the differences considering the relative difficulty of the earlier methods.

Our results provide a clear picture of the chemical relationship between the two genetic forms of $\beta$-lactoglobulin. $\beta$-Lactoglobulin A contains one more residue each of aspartic acid and valine and one less residue each of glycine and alanine than are found in $\beta$-lactoglobulin B. This demonstration by amino acid analysis of a whole protein of single residue substitutions indicates

### Table I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$\beta$-Lactoglobulin A, hours</th>
<th>$\beta$-Lactoglobulin B, hours</th>
<th>Corrected</th>
<th>Residues protein equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hydrolyzed</td>
<td>hydrolyzed</td>
<td></td>
<td>$\mu$moles/0.055</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>48</td>
<td>96</td>
<td>21</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.8655</td>
<td>0.8603</td>
<td>0.8577</td>
<td>0.8655</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.431</td>
<td>0.420</td>
<td>0.388</td>
<td>0.431</td>
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<tr>
<td>Serine</td>
<td>0.357</td>
<td>0.326</td>
<td>0.277</td>
<td>0.350</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>1.379</td>
<td>1.356</td>
<td>1.333</td>
<td>1.371</td>
</tr>
<tr>
<td>Proline</td>
<td>0.450</td>
<td>0.446</td>
<td>0.453</td>
<td>0.456</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.1669</td>
<td>0.1658</td>
<td>0.1682</td>
<td>0.2165</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.7654</td>
<td>0.7616</td>
<td>0.708</td>
<td>0.8213</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>0.209</td>
<td>0.209</td>
<td>0.218</td>
<td>0.223</td>
</tr>
<tr>
<td>Valine</td>
<td>0.525</td>
<td>0.540</td>
<td>0.464</td>
<td>0.471</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.201</td>
<td>0.192</td>
<td>0.188</td>
<td>0.200</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.483</td>
<td>0.520</td>
<td>0.539</td>
<td>0.484</td>
</tr>
<tr>
<td>Leucine</td>
<td>1.200</td>
<td>1.210</td>
<td>1.205</td>
<td>1.200</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.194</td>
<td>0.193</td>
<td>0.187</td>
<td>0.194</td>
</tr>
<tr>
<td>Phenytoinamine</td>
<td>0.210</td>
<td>0.215</td>
<td>0.213</td>
<td>0.219</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.823</td>
<td>0.830</td>
<td>0.810</td>
<td>0.810</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.099</td>
<td>0.108</td>
<td>0.102</td>
<td>0.097</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.157</td>
<td>0.158</td>
<td>0.152</td>
<td>0.158</td>
</tr>
<tr>
<td>Amide N</td>
<td>(0.772)</td>
<td>(0.817)</td>
<td>(0.873)</td>
<td>(0.790)</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.085</td>
<td>0.089</td>
<td>0.102</td>
<td>0.077</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.198</td>
<td>0.199</td>
<td>0.195</td>
<td>0.198</td>
</tr>
<tr>
<td>Total</td>
<td>1.002</td>
<td>0.997</td>
<td>102</td>
<td>102</td>
</tr>
</tbody>
</table>

* Adjusted values are presented which are very nearly equal to the observed micromoles per mg; see text. Some of the values in this table are given to four figures. The last figure is not significant. However, since these values are used to obtain averages which are later subtracted from one another, it is necessary to retain this number of figures to avoid rounding-off errors. If this is not done, the error introduced into the differences in Column 9 can become considerably larger than the statistical error.

* Those amino acids the concentration of which did not change with time of hydrolysis are italicized. The partial totals refer to the values of 1.7 and 2.3 calculated from the data of Brand et al. (18) and Spies and Chambers (22), respectively.

* Determined on separate samples by reaction with N-ethylmaleimide (16).

* Determined on separate samples by ultraviolet spectroscopy (17).

* Values of 3.7 and 3.7 were calculated from the ultraviolet spectra (17).
that the method should be applicable in many instances to the
search for genetic variants among proteins with minimal mole-
cular weights below about 20,000 which can be obtained in pure
form. Further, the method can detect genetic substitutions in-
volving uncharged amino acids that more readily escape detection
due to the electrophoretic methods generally used. This is an im-
portant feature of total amino acid analysis since there is no a
priori reason to expect genetic differences involving charged resi-
dues to be more common than those involving uncharged resi-
dues. More of the former type have so far been reported but
perhaps only because the electrophoretic techniques have been
more widely used. Another example of a situation in which
both types of substitution occur is found in the case of a chem-
ically induced mutant of tobacco mosaic virus in which it has
been shown by amino acid analysis that three substitutions are
present of which two involve neutral amino acids only (23).

It is pertinent to examine the data in Table I more closely to
determine the precision with which our conclusions are made.
Standard errors can be calculated for the differences between the
amounts of those amino acids involved in the genetic substitu-
tions. Each average is based on six values (two analyses of
each of three hydrolysates) except valine where only the 96-hour
hydrolysates were used. Valine, therefore, is omitted from the
calculations. The following values are obtained: aspartic acid,
0.0543 ± 0.0026; glycine, 0.0637 ± 0.0008; alanine, 0.0568 ±
0.0020. It should be noted that these errors are probably a
measure of the true accuracy since they are differences between
numbers subject to the same systematic errors which therefore
would cancel out. The unweighted average is 0.0546 ± 0.0012.
Since the units of this figure are microequivalents of a single
residue per mg, the inverse $X^{10}$ is the equivalent weight.
With the exact weights represented, 1.002 mg for $\beta$-lactoglobulin
A and 0.997 mg for $\beta$-lactoglobulin B (Table I), the minimal
molecular weights by this method are 18,364 ± 400 for $\beta$-la-
globulin A and 18,270 ± 400 for $\beta$-lactoglobulin B. The aver-
age difference weighted according to the inverse of the square of
the errors is 0.0540 ± 0.0007 and the molecular weights calcu-
lated from this value are 18,560 ± 230 and 18,470 ± 230.
These weights are all close to one-half the accepted value for mixed
$\beta$-lactoglobulin as determined by several physical methods (see
(8)) and support the evidence that the molecule consists of two
identical subunits (8, 10). Two identical chains are also indi-
cated by the presence of two identical amino terminal and car-
boxyl terminal groups per 38,000 molecular weight (12, 24, 25).

The minimal molecular weights calculated from the number of
residues rounded off to the nearest integer are 18,364 for $\beta$-lac-
globulin A and 18,278 for $\beta$-lactoglobulin B. In the determi-
nation of the absolute number of residues, in contrast to the dif-
ferences, systematic errors may be present. Particularly for those
amino acids present in large quantities or amino acids that are
subject to greater analytical variation, errors of one residue may
occur. These molecular weights may therefore not be exact and
can be confirmed or corrected only by a knowledge of the com-
plete amino acid sequence. However, the close agreement of
these values with the ones calculated from the stoichiometry of
the difference between the two proteins allows considerable con-
fidence to be placed in the method of calculation and in the re-

results.

When either $\beta$-lactoglobulin A or $\beta$-lactoglobulin B are isolated
from individual cows homozygous for lactoglobulins A or B,
they were heterogeneous in electrophoresis experiments (8, 9).

In the case of $\beta$-lactoglobulin A, this is due at least in part to
molecular association (6, 7) although the presence of a second
molecular species has been indicated (9). With $\beta$-lactoglobulin
B, the apparent electrophoretic heterogeneity has not been fully
explained but may result from degradation as noted above.
Heterogeneity has also been reported by Tombs (20) in solubility
studies in which minor components were observed in both pro-
teins, accounting for about 10% of each. Polia et al. (27) have
isolated a $\beta$-lactoglobulin which seems to be different from both
$\beta$-lactoglobulin A and $\beta$-lactoglobulin B (5). The variable minor
components found in the column chromatograms may explain in
part these various reports of heterogeneity. At least, we can con-
clude that the samples analyzed by us could not have con-
tained appreciable amounts of genetically different species.
Otherwise, it would not have been possible to observe whole
residue substitutions. For each of the four amino acids, the
differences are exactly one residue with an error of 5% or less,
as discussed above.

The presence of two more aspartic acid residues per molecule
(36,000 molecular weight) of $\beta$-lactoglobulin A than $\beta$-la-
toglobulin B, while the amide content is the same for both forms,
explains the observations that the titration curves show two
additional carboxyl groups for $\beta$-lactoglobulin A (4) and that the
isoelectric points of the two proteins differ (2-4). It is also
likely that the different aggregation properties are related to the
difference in charge. According to present concepts, it seems
unlikely that hydrophobic bonds (28) involving the additional
valine side chain of $\beta$-lactoglobulin A can be a factor since aggre-
gation is maximal at low temperatures (7) where entropy-sta-

bilized bonds are least stable (28). However, the question of the
effect of the extra valine cannot be answered with any certainty
without having available for study $\beta$-lactoglobulin containing
only the one substitution.

SUMMARY

Commercial preparations of $\beta$-lactoglobulin were fractionated
by chromatography on diethylaminomethyl-cellulose to yield the
genetically different proteins $\beta$-lactoglobulin A and $\beta$-lactog-
lobulin B in high purity. Complete amino acid analysis of the
native proteins established that $\beta$-lactoglobulin A has one more
residue each of aspartic acid (16 versus 15) and valine (10 versus
9) and one less residue each of glycine (3 versus 4) and alanine
(14 versus 15) than $\beta$-lactoglobulin B in a subunit of 18,300
molecular weight.

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