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The Heterogeneity of β_2 -Lactoglobulin

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(Received 16 December 1957)

The discovery by Aschaffenburg & Drewry (1955, 1957) that individual cows differed in the β -lactoglobulins that they produce (they have either β_1 - or β_2 -lactoglobulin or a mixture of the two), although going far to explain the heterogeneity of β -lactoglobulin prepared indiscriminately from mixed milk, does not completely account for all the observed indications of heterogeneity.

Thus Ogston & Tombs (1957) found that both β_1 - and β_2 -lactoglobulin gave complex boundaries on electrophoresis, and although most of this heterogeneity could be explained as due to polymerization in solution (Ogston & Tilley, 1955) there remained a possibility that some of the electrophoretic heterogeneity was due to the presence of distinct minor components.

This possibility was investigated for β_1 -lactoglobulin mainly by use of solubility tests (Tombs, 1957), and the presence of about 10% of a minor component was detected. It could be removed by salt fractionation; the purified β_1 -lactoglobulin had a constant-solvent solubility curve, and a variable-solvent solubility curve characteristic of a very nearly homogeneous substance. It still had a complex boundary in electrophoresis, but this could be completely explained as due to polymerization in solution. Some results obtained with β_2 -lactoglobulin by using an approach similar to that already described for β_1 -lactoglobulin are reported here.

EXPERIMENTAL

Experimental details of the preparation of β -lactoglobulins, electrophoresis, determination of concentration and measurements of ultraviolet absorption were the same as in

Ogston & Tombs (1957). Solubility tests were substantially the same as were used for β_1 -lactoglobulin (Tombs, 1957).

Variable-solvent solubility curves

The protein was dissolved in 1.6M-sodium chloride and dialysed against it for at least 24 hr. This solution was then mixed with an equal volume of acetate buffer (pH 4.5; 1.6M-sodium acetate, 2.9M-acetic acid) and samples (1 ml.) were placed in centrifuge tubes. Then 3 ml. of an appropriate ammonium sulphate solution was run in to give final concentrations of 0.2M-sodium chloride, 0.2M-sodium acetate and ammonium sulphate in the range 1.9–2.4M.

After initial experiments in which equilibration took place at 25° (Fig. 2) it was found that cooling was necessary in order to obtain amorphous precipitates; subsequently therefore all solutions were precooled to 4° before mixing, and the tubes were kept in a water bath at 4° during equilibration. The tubes were sealed with silicone-treated rubber bungs. After centrifuging at 4° (10 000g for 20 min.), 2 ml. of the supernatant was removed from each tube and diluted to 3 ml. with water. β_2 -Lactoglobulin has a negative temperature coefficient of solubility in these solvents and dilution was necessary to prevent precipitation occurring when the tubes warmed up to room temperature. Protein concentration in the diluted supernatants was estimated directly by the absorption at 278 m μ in 1 cm. cells.

Constant-solvent solubility curves

β_2 -Lactoglobulin was dissolved in solvent containing 0.8M-sodium chloride, 0.8M-sodium acetate, 1.35M-acetic acid (pH 4.5), and dialysed against the same solvent. Then volumes of the β_2 -lactoglobulin solution not exceeding 1 ml. were carefully measured into centrifuge tubes with an Agla micrometer syringe. Then each sample was made up to 1 ml. with diffusate, and 3 ml. of ammonium sulphate solution was added to give the desired final concentration. The volume of protein solution added to each tube gave a measure of the total amount of protein in the system, and the amount in solution was found by measurement of the absorption of the supernatants at 278 m μ . Tubes were equilibrated at 4°, and supernatants diluted after centrifuging in exactly the same way as for the variable-solvent test.

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Fractionation experiments

About 2 g. of β_2 -lactoglobulin was dissolved in 20 ml. of solvent (0.8M-sodium chloride, 0.8M-sodium acetate, 1.35M-acetic acid; pH 4.5) and 60 ml. of ammonium sulphate solution added to give a final concentration of 2.15M-ammonium sulphate. The mixture was equilibrated at 4° and then the precipitated protein was collected by centrifuging in the no. 30 head of a Spinco model L preparative ultracentrifuge at 29 000 rev./min. for 45 min. (about 50 000 g). The supernatant was then saturated with ammonium sulphate and the precipitated protein was obtained by centrifuging as before. The precipitates were dissolved in the appropriate solvents for further tests.

RESULTS

Electrophoresis

The behaviour of β_2 -lactoglobulin in acetate buffers at pH 4.66 has already been reported (Ogston & Tombs, 1957). Resolution was poor, and a pH value was sought at which resolution might

be better. Fig. 1 shows typical boundaries. All the runs were in acetate buffers (0.1M-sodium acetate, and then acetic acid was added to give the required pH) between pH 4.66 and 5.58. Resolution appeared to be best at pH 5. β_1 -Lactoglobulin gave very similar results in the same buffers.

Solubility experiments. Equilibration at 25° gave solubility curves which appeared satisfactory (Fig. 2), but on examination the precipitates were found to be crystalline. Very fine needle-like crystals were found in the tubes corresponding to part A of the curve, and small hexagonal crystals, together with amorphous material predominated in part B. The initial precipitates were always amorphous but at pH values 4.95, 4.8 and 4.5 at room temperature (15–17°) or at 25° the initial precipitates became partially or wholly crystalline in less than an hour. The crystals sometimes appeared in sheaves similar to those described by Smithies (1954). After 24 hr. the curves had the characteristic sigmoid shape described by Smithies, and the precipitates were completely crystalline. Such curves were useless for analytical purposes.

When the solubility tests were performed at 4°, no crystallinity was ever seen in the precipitates, even after equilibration for several days, on examination at $\times 600$.

β_2 -Lactoglobulin had a negative temperature coefficient of solubility in these solvents, and precipitate appeared when the ultraviolet absorption of the supernatants was measured at room temperature. This difficulty was overcome by diluting the supernatants (see Experimental section), but it was also possible to obtain reproducible curves by allowing the tubes to warm up to room temperature and centrifuging within an hour. Re-equilibration to room temperature appeared to

Fig. 1. Free electrophoresis of β_2 -lactoglobulin. The buffer throughout was 0.1M-sodium acetate and then acetic acid was added to give the required pH. Approximate starting positions are indicated by the vertical lines. All measurements were made after about 150 min. Two starting lines are shown at pH 5.21 for convenience of drawing.

pH	Protein concn. (g./100 ml.)	Current (mA)
4.66	0.96	10
4.74	3.0	10
5.0	1.1	10
5.21	1.25	20
5.34	1.25	20
5.58	0.98	10

Fig. 2. Variable-solvent solubility curve of β_2 -lactoglobulin equilibrated for 6 hr. at 25°, pH 4.5. Needle crystals appeared at A, hexagons at B. Details are given in the Experimental section.

be complete, no crystals could be seen and curves like those in Fig. 3 were obtained at several different protein concentrations, showing the point *T* to be invariant. The dilution technique was thought to be more reliable, however.

Curves obtained at two different protein concentrations are shown in Fig. 3. Several breaks appeared, of which *T* and *Y* appeared to be nearly invariant, though the total solubility at *Y* was higher at the higher protein concentration. The break at *X* could be distinguished only at higher protein concentrations. That at *Z* also appeared to move along the salt axis when protein concentration was changed. These breaks, especially at *X* and *Y*, were never very clearly defined.

A suitable solvent for the constant-solvent test was selected from a consideration of the variable-solvent solubility curve. The plot at 2.12M-ammonium sulphate is shown in Fig. 4. It indi-

Fig. 3. Variable-solvent solubility curve of β_2 -lactoglobulin equilibrated at 4° for 24 hr., pH 4.5. Values on the upper scale of the abscissa refer to the curve at the left (○), those on the lower scale of the abscissa refer to the curve at the right (●). Total protein concentrations (○, ●) were in the ratio 2:1. For *T*, *X*, *Y* and *Z* and experimental details see text.

Fig. 4. Constant-solvent solubility curves of β_2 -lactoglobulin. The solvent for *A* was 2.12M-ammonium sulphate, for *B* 2.17M-ammonium sulphate and in each case the solvent also contained 0.2M-sodium chloride, 0.2M-sodium acetate (pH 4.5). Equilibration was for 24 hr. at 4°. Precipitate first appeared at the points indicated by the arrows.

cated the formation of a solid solution, and no sharp breaks could be distinguished. At a higher ammonium sulphate concentration (2.17M) a precipitate again appeared well before maximum solubility had been reached. It was not possible to determine whether there were any sharp breaks or not.

Fractionation experiments

The precipitate protein and the supernatant protein produced by fractionation with 2.15M-ammonium sulphate were examined by free electrophoresis. At pH 4.66 in acetate buffer ($I=0.1$) at 1° a 2% solution of precipitate protein had a very sharp ascending boundary and a partially resolved descending boundary with 57% of slow component by area analysis. Unfractionated protein under the same conditions had 56% of slow component. A solution of supernatant protein (0.96%) had a skew-descending peak suggesting slightly more fast component than in the unfractionated protein at the same concentration. Resolution was poor at pH 4.66, and acetate buffer ($I=0.1$) pH 5 was substituted. Supernatant protein, precipitated protein and unfractionated β_2 -lactoglobulin were all run in the same batch of buffer, all at 1.5% of total protein. Area analysis of the descending boundaries gave 53% of slow component in the supernatant protein, 57% in the unfractionated and 60% of slow component in the precipitate protein. This difference, although small, was outside the usual error ($\pm 1\%$) for photographs of comparable resolution, and is probably significant.

The precipitate protein was also examined by the constant-solvent solubility test. The precipitate appeared before saturated solubility was reached and a plot made after equilibration for 24 hr. was characteristic of a solid solution. Equilibration for 3 days gave an almost identical plot, and the combined results of both experiments are shown in Fig. 5.

Fig. 5. Constant-solvent solubility curve of the 'precipitate protein' from β_2 -lactoglobulin fractionation. The solvent was as for Fig. 4 (*A*). Curve shows the combined results of two experiments in which equilibration took place at 4° for 1 and 3 days. Precipitate first appeared at the point indicated by the arrow.

DISCUSSION

Because of their lower precision (two extra pipetting stages were introduced) and their more complex nature, the variable-solvent solubility curves of β_2 -lactoglobulin were more difficult to interpret than those of β_1 -lactoglobulin. Nevertheless, there are some points of similarity. The invariant point *T* in Fig. 3, and almost certainly *Y* also, were transition points between the precipitation of one solid form and another. Again, as with β_1 -lactoglobulin, breaks like *X*, and possibly *Z* also, indicated the presence of minor distinct components. If the specific extinction coefficients of all the components were similar, the minor components formed rather less than 10% of the total.

The constant-solvent solubility curves, unlike those obtained with β_1 -lactoglobulin, indicated formation of solid solutions. The solids might have been formed from one component, or they might have been solid solutions of one of the minor components in one or more solids formed by the major component. These solid solutions appeared to be stable and, since it was not possible to distinguish the various possibilities, the constant-solvent solubility curves did not provide clear evidence for or against heterogeneity.

The fractionation experiment, which was designed to leave the suspected minor components in the supernatant, and give a purified major component as the precipitate, supports the variable-solvent solubility experiments in indicating a small degree of heterogeneity. The constant-solvent solubility curve obtained with the precipitate protein emphasizes the ambiguity of this test in this particular set of circumstances.

The crystallization of β_2 -lactoglobulin under conditions where β_1 -lactoglobulin does not and apparently will not crystallize is a minor though striking difference between the proteins, and the fact that different kinds of crystal appear in parts of the solubility curve roughly corresponding to the precipitation of different amorphous solids suggest that the latter may be microcrystalline versions of the large crystals. The existence of transition points in the solubility curves of the β -lactoglobulins points towards a short-range structure of some kind in the solids.

The electrophoresis results reported here and by Ogston & Tombs (1957) make it clear that β_2 -lactoglobulin, like β_1 -lactoglobulin, associates in solution. Below the isoelectric point (pH 5.2) the polymer migrates most rapidly, above it the monomer form. The association process is probably the source of the sharpening of the descending boundary above the isoelectric point. The polymerization

of β_1 -lactoglobulin in solution was found to fit the requirements of Gilbert's (1955) theory fairly well (Tombs, 1957). This theory predicts that at high concentration of protein the proportion of slow component (monomer) from area analysis after electrophoresis will approach zero, provided that there are more than two monomer units in each polymer and that the rate of equilibration is infinitely rapid compared with the rate of resolution.

β_2 -Lactoglobulin does not fit these requirements, since at pH 4.66 it reaches a limiting proportion of 60% of slow component at high protein concentration. The minor components found could not account for even part of the excess of slow component, since they appear to travel as part of the fast boundary in electrophoresis.

It appears that in its association-dissociation behaviour β_2 -lactoglobulin is more complex than β_1 -lactoglobulin. Similarly, it was not possible to gain as clear an understanding of its solubility behaviour, and this again was more complex than that of β_1 -lactoglobulin. On the whole, however, β_2 -lactoglobulin appears to contain rather less minor contaminants than β_1 -lactoglobulin. This may be useful since cows producing only β_2 -lactoglobulin are much commoner than those producing only β_1 -lactoglobulin.

SUMMARY

1. In acetate buffers below pH 5.2 β_2 -lactoglobulin forms a partially resolved descending and a very sharp ascending boundary in electrophoresis. Above pH 5.2 the descending boundary is sharpened and the ascending boundary spread.
2. Application of the variable-solvent solubility test showed two transition points, and two breaks probably due to the presence of distinct minor components, in the solubility curve. The minor components probably did not exceed 10% of the total.
3. The constant-solvent solubility test gave results characteristic of formation of solid solution.
4. Fractionation designed to remove the minor components gave a major fraction with more slow component, and a fraction in which the minor components had been concentrated, with less slow component than the unfractionated protein.
5. The purified β_2 -lactoglobulin gave a constant-solvent solubility curve, characteristic of a solid solution.
6. It is concluded that the major part of the electrophoretic heterogeneity of β_2 -lactoglobulin is due to association in solution, even though theoretical predictions of its extent are not correct.

I should like to thank Dr A. G. Ogston, F.R.S., for his constant interest in this work. The investigation was supported by a grant from the Agricultural Research Council.

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Glycolysis by an Extract from Pea Seeds

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(Received 10 December 1957)

It appears that no complete well-characterized glycolytic system in extracts from plant tissues, capable of transforming hexoses and hexose monophosphates into pyruvic acid or carbon dioxide and ethanol, has been described.

The conversion of carbohydrate into ethanol (Bevers & Gibbs, 1954; James & Ritchie, 1955) and lactate (Barker & el Saifi, 1952) in intact plant tissues provided evidence for a functional glycolytic system. This has been supported by the identification of individual glycolytic enzymes and certain intermediates in extracts from plants (Axelrod & Bevers, 1956). More integrated systems were found by Tanko (1936) and Hanes (1940), who prepared pea-seed extracts capable of converting starch into hexose monophosphates and fructose 1:6-diphosphate (FDP). James, James & Bunting (1941) showed that the production of pyruvic acid by crude barley-leaf saps was increased by the addition of sugars, FDP and 3-phosphoglyceric acid (3-PGA). Stumpf (1950) obtained a pea-seed extract which formed carbon dioxide from FDP in the presence of arsenate by a series of reactions analogous to those of yeast and muscle glycolytic systems. However, phosphoglyceric kinase was absent and the pyruvic kinase yielded adenosine diphosphate (ADP) rather than adenosine triphosphate (ATP).

During investigations on the synthesis of sucrose from glucose 1-phosphate (G 1-P) and fructose by pea-seed extracts, evidence was obtained for the operation of a glycolytic system (Turner, 1954, 1957; Turner & Mapson, 1958). The crude pea extract was partially purified by centrifuging at 20 000 *g*, treatment of the supernatant with ammonium sulphate to 80 % saturation, suspension of the precipitated protein in water and dialysis against phosphate buffer. With hexose and hexose phosphates as substrates and with a yeast nucleo-

tide fraction and Mg^{2+} ions as cofactors, the partially purified pea extract esterified inorganic phosphate and formed FDP and carbon dioxide. For sucrose synthesis the yeast nucleotide fraction could be replaced by a mixture of diphosphopyridine nucleotide (DPN), ATP and uridine monophosphate.

In the present investigation these observations have been extended. The pea extract was capable of quantitatively converting starch, hexoses and hexose phosphates into carbon dioxide and ethanol. The results indicated that a complete glycolytic system of the Embden-Meyerhof-Parnas type was responsible for these transformations. There was no evidence for the operation of alternate pathways of hexose phosphate degradation in the pea-seed extract.

EXPERIMENTAL

Preparation of enzyme extracts. The crude and partially purified pea extracts were prepared as described previously (Turner, 1957) except that the partially purified extract was dialysed against phosphate buffer at pH 6.6 instead of pH 7. The partially purified extract, which contained approx. 45 mg. of protein/ml., showed no decrease in glycolytic activity after 2 weeks at -15° .

Substrates and cofactors. Commercial preparations of glucose and fructose, which gave only one spot when examined on paper chromatograms with butan-1-ol-acetic acid-water (4:1:5, by vol.; Partridge, 1948) as solvent, were used.

ATP, ADP and DPN were obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A., and glucose 6-phosphate (G 6-P) and 3-PGA from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

G 1-P was prepared by an unpublished method of Professor C. S. Hanes and Dr R. Hill. Fructose 6-phosphate (F 6-P) (Neuberg, Lustig & Rothenberg, 1943) and FDP (Neuberg & Lustig, 1942) were also prepared in the laboratory.