Immunochemical Characterization of Casein from Rabbit Mammary Gland

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1. Excellent precipitating antibodies to rabbit recombined casein polypeptides were obtained in a sheep after 8 weeks of immunization with rabbit recombined polypeptides coupled to Sepharose–albumin. 2. The antiserum was assessed for specificity by several immunochemical techniques and was monospecific when tested against acid-precipitated casein, recombined casein and extracts of lactating rabbit mammary tissue. 3. A specific anti-casein immunoglobulin fraction was prepared by immunoabsorption of the antiserum by using Sepharose-recombined casein as immunoabsorbent. 4. The specific anti-casein immunoglobulin was used to prepare a Sepharose–anti-casein immunoabsorbent for the isolation of casein from extracts of rabbit mammary tissue.

The poor immune response to casein injected into rabbits and sheep has been noted by many investigators. Feldman (1974) reported that antibodies to mouse casein (administered in 7M-urea) could precipitate only 62% of the rennin-precipitable [³H]casein in a direct radioimmunoassay. Terry *et al.* (1975) used a very prolonged immunization schedule with rennin-precipitated casein, administered initially in urea, to raise an antiserum which was subsequently used in an indirect radioimmunoassay with ¹²⁵I-labelled casein. Antiserum to acid-precipitated rabbit casein, raised in sheep after several months of bi-weekly injections of the antigen, precipitated less than half of the [³H]casein at equivalence (Houdebine & Gaye, 1975). Rosen *et al.* (1975) reported that it was essential to remove potential contaminating antigens from casein to lessen the possibility of the production of a polyspecific antiserum containing weak-affinity antibodies to casein, particularly when the antiserum is to be used for the development of a definitive and specific immunoprecipitation assay. Weakly precipitating antisera to acid-precipitated guinea-pig casein were raised in rabbits by Craig *et al.* (1976), but proved adequate when used in a double-antibody technique.

It was thus decided to design methods to overcome problems related to the antigenicity of casein, the purity of the subsequent antiserum and the isolation of casein from tissue extracts. The present paper describes (i) the production of an excellent precipitating antiserum to rabbit casein by a simple procedure after a short immunization schedule (8 weeks) with purified casein (Al-Sarraj *et al.*, 1978), (ii) the characterization and subsequent purification of the antiserum with Sepharose-recombined casein as immunoabsorbent, and (iii) the isolation of casein from a tissue extract of lactating rabbit mammary gland

with Sepharose-anti-casein IgG as immunoabsorbent.

Materials and Methods

Animals

The sheep used in these studies was housed at the Joint Animal Breeding Unit, University of Nottingham School of Agriculture, Sutton Bonington, Leics., U.K. New Zealand White rabbits were obtained from the same source.

Materials

Triton X-100 was obtained from Lennig Chemicals, Croydon CB9 3NB, U.K. Agarose and barbitone were purchased from BDH Chemicals, Poole, Dorset, U.K. Glycine (A.R.) and barbitone sodium were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K. Minicon concentrators type B15 were obtained from Amicon, High Wycombe, Bucks., U.K. [³²P]P_i was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All the other materials used have been described previously (Walker *et al.*, 1976).

Preparation of antisera

Antiserum to rabbit casein was raised in sheep by injection of a Sepharose–albumin–casein conjugate.

Synthesis of Sepharose–albumin–casein antigen. Casein was isolated from skim milk of lactating rabbits (day 15 of lactation) by isoelectric precipitation at pH4.6, and the individual polypeptides (i.e. 1, 2, 3, 4a and 4b) were purified as described by Al-Sarraj *et al.* (1978). Equal amounts (by weight) of each polypeptide were then recombined to give the 'recombined casein fraction'. Bovine serum albumin

Abbreviation used: IgG, immunoglobulin G.

(fatty acid-poor, 50mg) was covalently attached to Sepharose 2B (8ml) by means of CNBr under conditions recommended for moderate activation by Porath *et al.* (1973). This yielded 5mg of bovine serum albumin bound/ml of Sepharose. Recombined casein (41 mg) was then incubated overnight at room temperature with the Sepharose-albumin in the presence of glutaraldehyde (final concentration 5%, v/v). The preparation was washed extensively with 20mM-sodium phosphate buffer, pH 7.0, containing 0.15M-NaCl and yielded on analysis 3.38mg of recombined casein/ml of Sepharose-albumin.

Raising of antiserum. The antigen was injected at four subcutaneous and four intramuscular sites at 14-day intervals for 8 weeks (i.e. a total of 32 injections). Each injection contained 0.5ml of antigen solution and 0.5ml of Freund's complete adjuvant. A total of 35.8mg of recombined casein and 40mg of bovine serum albumin was injected into the sheep over the 2 months. At 1 week after the final injection the animal was bled by jugular cannulation and the blood was processed to give the immunoglobulin fraction as described by Speake *et al.* (1975).

An excellent antiserum to conjugated acid-precipitated casein was also raised in preliminary experiments in a different sheep (results not shown) and gave qualitatively similar results to those described in this paper.

Assessment of antiserum specificity

Immunodiffusion. Immunodiffusion was carried out in 1% (w/v) agarose gel containing 20mM-sodium phosphate buffer, pH 7.0, and 0.15M-NaCl. Diffusion was allowed to proceed for 24h at 25°C in a moist atmosphere.

Immunoelectrophoresis. Crossed-rocket immunoelectrophoresis was carried out as described by Weeke (1973). The antiserum was tested against recombined casein and tissue extracts from mammary glands of lactating rabbits. The gels were then stained for protein with Coomassie Blue as described by Weeke (1973).

Radioautograms of immunoelectrophoresis gels containing ^{32}P -labelled casein were prepared after staining for protein. The dried gel was placed in contact with Kodak X-ray film KD5UT (Kodak, Kirkby, Liverpool, U.K.) and sandwiched between two glass plates held together with spring clips. The assembly was left in the dark at 20°C for 8 days, developed with Ilford PQ universal developer (Ilford Ltd., Ilford, Essex) and fixed with Kodak FX-40 X-ray fixer.

Preparation of ^{32}P -labelled tissue extract and ^{32}P -labelled recombined casein from the mammary glands of a 15-day lactating rabbit

$^{32}\text{P}[\text{P}_i]$ (5mCi) saturated with Gentian Violet in the phosphate/saline buffer defined above (2ml) was

injected intraductally (four or five sites) into the mammary glands of a lactating rabbit. After 4h the animal was killed and some Gentian Violet-stained tissue was removed and homogenized at 4°C (in an MSE Topdrive homogenizer) in water. The rest of the stained tissue was placed in 20mM- KH_2PO_4 buffer, pH 7.0, and processed to give the ^{32}P -labelled recombined casein as described by Al-Sarraj *et al.* (1978).

Tissue extracts are defined as the supernatant fractions obtained by centrifugation ($6 \times 10^6 \text{g-min}$) of tissue homogenates prepared in water.

Immunoprecipitation studies

Increasing volumes (i.e. 0, 200, 400, 600, 800 and 1000 μl) of control (preimmunization) serum (30mg of protein/ml), or antiserum against recombined casein (35mg of protein/ml), were mixed with a solution of ^{32}P -labelled recombined casein (12 μg) in 20mM-sodium phosphate buffer, pH 7.0, containing 0.15M-NaCl (20 μl). The volume of each incubation was adjusted to 1ml with the same buffer, and the mixture was incubated at 37°C for 30min and then at 4°C for 48h. The precipitates were collected by centrifugation at 1000g for 30min at room temperature and washed twice with 20mM-sodium phosphate buffer, pH 7.0, containing 0.15M-NaCl (1ml).

The final precipitates were dissolved in 0.1M-NaOH (0.1ml) and protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (fatty acid-free) as standard. A sample (1ml) of the final colorimetric solution was then used for the measurement of radioactivity.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and urea

Electrophoresis on polyacrylamide in the presence of sodium dodecyl sulphate and urea was carried out in duplicate gels as described previously (Al-Sarraj *et al.*, 1978). After electrophoresis one gel from each pair was stained for protein and the other was frozen at -20°C, sliced into 2mm lengths and solubilized by incubating with 0.5ml of H_2O_2 (50vol.) overnight at 60°C. Scintillation fluid (5ml) (White *et al.*, 1971) was added to each of the dissolved gel slices and the radioactivity measured as described below.

Adsorption of antiserum by Sepharose-recombined casein immunoabsorbent

Adsorption of antiserum was performed with Sepharose-recombined casein as immunoabsorbent. Recombined casein (13mg) was coupled to Sepharose 2B (15ml) by CNBr activation exactly as described for Sepharose-albumin above (0.48mg of recombined casein was bound/ml of Sepharose).

Immunoabsorption was carried out by incubating 40 ml of antiserum (44 mg of protein/ml) with the immunoabsorbent (15 ml) overnight at 4°C. The mixture was then poured into a sintered-glass funnel and the first washings were collected and shown to be free of antibodies by immunodiffusion. The immunoabsorbent was washed extensively with 20 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl, and the anti-casein IgG was then eluted with 0.2 M-glycine/HCl buffer, pH 2.2 (20 ml). The glycine/HCl eluate was neutralized with 5 M-Tris and dialysed overnight at 4°C against 20 mM-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl. Denatured protein was collected by centrifugation at 1000g_{av} for 20 min at 4°C. The supernatant fraction (21 ml) was tested for anti-casein IgG by several immunochemical techniques.

Binding of casein to Sepharose-anti-casein immunoabsorbent

Sepharose-anti-casein IgG was prepared by incubating anti-casein IgG (15 ml, 0.5 mg of protein/ml) with CNBr-activated Sepharose 2B (15 ml) overnight at 4°C. This procedure resulted in the binding of 0.33 mg of anti-casein IgG/ml of Sepharose and is termed 'antiserum-Sepharose'.

Control serum (60 µl, 30 mg of protein/ml) was incubated similarly with CNBr-activated Sepharose 2B (3 ml) and yielded a 'control-serum-Sepharose', where 0.5 mg of control serum IgG was linked/ml of Sepharose. The immunoabsorbents were poured into plastic syringe barrels and used as described by Walker *et al.* (1976). Samples of ³²P-labelled tissue extract in water and ³²P-labelled recombinant casein in water were both applied to 'antiserum-Sepharose' and 'control-serum-Sepharose' columns at room temperature. The samples were left in contact with the columns for 4 h and then washed with water (20 ml). Fractions (0.5 ml) were collected and assayed for radioactivity. The columns were finally eluted with 8 M-urea, pH 7.0 (20 ml), containing 2% (w/v) sodium dodecyl sulphate. The eluates were dialysed against water overnight at room temperature, and concentrated with a Minicon concentrator. Samples of the concentrate were analysed by polyacrylamide-gel electrophoresis in the presence of urea and sodium dodecyl sulphate. The gel was cut into slices and radioactivity measured as described above. Sometimes the protein in the eluate was measured by the method of Lowry *et al.* (1951).

Results

Assessment of antiserum specificity

(i) Immunodiffusion studies of antiserum raised against rabbit recombinant casein (unadsorbed and

adsorbed) by means of Ouchterlony (1949) double-diffusion analysis (Fig. 1) indicated: (a) the presence of antibodies to acid-precipitated casein, recombinant casein and casein in tissue extract from mammary gland of a lactating rabbit (Fig. 1a); (b) the lack of antibodies to albumin (Fig. 1a); (c) that the tissue extract obtained from lactating rabbit mammary glands contained only one antigen, which showed complete reaction of identity with acid-precipitated casein and recombinant casein (Fig. 1b); (d) that the antiserum was monospecific by this analytical technique, since a single precipitin line was obtained for each antigen preparation; (e) that control serum did not react with the antigen, since no precipitin lines were seen with this serum (Fig. 1a).

(ii) Crossed-rocket immunoelectrophoresis and radioautographic studies with ³²P-labelled tissue extract from mammary glands of lactating rabbits and ³²P-labelled recombinant casein are shown in Fig. 2. Only one antigen-antibody system with unadsorbed and adsorbed antisera is seen. All protein-staining rockets (Figs. 2A and 2B) were present on the radioautograph of the gel (Figs. 2C and 2D), indicating that all the rocket(s) contained [³²P]phosphoproteins.

(iii) Immunoprecipitation of ³²P-labelled recombinant casein (12 µg, 2527 c.p.m.) with unadsorbed antiserum (Fig. 3) showed that 400 µl of unadsorbed antiserum precipitated 12 µg of recombinant casein at equivalence. Casein was not precipitated by pre-immunization serum.

Polyacrylamide-gel electrophoresis

Analysis of ³²P-labelled recombinant casein (12 µg, 2527 c.p.m.) and of the washed ³²P-labelled total casein immunoprecipitate (taken at equivalence) by polyacrylamide-gel electrophoresis in the presence of urea and sodium dodecyl sulphate showed only one radioactive peak from each sample, and this corresponded to the *R_F* of casein in the duplicate polyacrylamide gels stained for protein (Fig. 4). A total of 1820 c.p.m. (i.e. 73% of the radioactivity loaded on the gel) was present in the peak for recombinant casein and 73% of this radioactivity was recovered in subunits of ³²P-labelled recombinant casein from the gel of the immunoprecipitate (Fig. 4b).

Adsorption of antiserum

Antiserum to recombinant casein was adsorbed with a Sepharose-recombinant-casein immunoabsorbent. This procedure yielded 15 mg of anti-casein IgG from the 1760 mg of protein in the original unadsorbed antiserum and represents an overall yield of about 0.8%. The adsorbed antiserum was tested by immunodiffusion (Fig. 1), crossed-rocket immunoelectrophoresis (Fig. 2) and immunoprecipitation

Fig. 1. *Ouchterlony double-diffusion analysis of casein purified from mammary gland of lactating rabbit*
Immunodiffusion was carried out for 24 h at room temperature in a moist atmosphere by the procedure of Weeke (1973) as described in the Materials and Methods section. (a) Wells 1, 2, 3 and 4 contained acid-precipitated casein, recombined casein, tissue extract from mammary gland of lactating rabbit and albumin respectively. The sets of opposing wells (5) contained unadsorbed antiserum. (b) The central well contained adsorbed antiserum and the outer wells contained acid-precipitated casein (1 and 4), tissue extract (2 and 5) and recombined casein (3 and 6).

Fig. 2. Crossed-rocket immunoelectrophoresis of ^{32}P -labelled recombinant casein and ^{32}P -labelled tissue extract from mammary gland of lactating rabbit

Immunoelectrophoresis was carried out in 1% (w/v) agarose gels as described by Weeke (1973). Figs. 2(A) and 2(B) show Coomassie Blue staining of the gels of ^{32}P -labelled recombinant casein (Fig. 2a) and ^{32}P -labelled tissue extract (Fig. 2c) tested against unadsorbed antiserum (A) and adsorbed antiserum (B). Figs. 2(C) and 2(D) show radioautograms of Figs. 2(A) and 2(B) tested against unadsorbed antiserum (C) and adsorbed antiserum (D). For details see the text and the Materials and Methods section.

Fig. 3. Immunoprecipitation of ^{32}P -labelled casein. Increasing volumes of antiserum (●) and control (preimmunization) serum (■) were added to a fixed amount of ^{32}P -labelled recombinant casein ($12\mu\text{g}$). For details see the Materials and Methods section.

(results not shown). It was identical immunochemically with the unadsorbed antiserum.

Characterization of the Sepharose-anti-casein immunoadsorbent

The Sepharose-anti-casein IgG immunoadsorbent bound $7.5\text{--}10\mu\text{g}$ of recombinant casein/ml and all the adsorbed casein could be eluted with 8M -urea containing sodium dodecyl sulphate (2%, w/v). The immuno-

adsorbent also bound ^{32}P -labelled casein from a water extract of lactating mammary gland and the protein which was eluted with urea/sodium dodecyl sulphate gave a single radioactive peak coincident with casein on polyacrylamide-gel electrophoresis in the presence of urea and sodium dodecyl sulphate (Fig. 5). No binding of recombinant casein to control serum-Sepharose occurred, since no protein was detected in the urea/sodium dodecyl sulphate eluate from the control column.

Discussion

The results presented in this paper demonstrate that, contrary to previous studies by Feldman (1974) and Terry *et al.* (1975) on mouse casein, by Houdebine & Gaye (1975, 1976) on rabbit and ewe casein, by Rosen *et al.* (1975) on rat casein and by Craig *et al.* (1976) on guinea-pig casein, excellent precipitating antibodies to rabbit casein can be raised in 8 weeks by immunization of a sheep with a Sepharose-albumin-recombined casein complex.

The generation of a monospecific antiserum to casein is dependent on prior purification of the individual casein components (Rosen *et al.*, 1975), and purification methods which depend on isoelectric precipitation alone or which utilize the rennin/ Ca^{2+} precipitation technique may result in casein preparations that are contaminated with whey or other non-

Fig. 4. Polyacrylamide-gel electrophoresis of ^{32}P -labelled recombined casein and immunoprecipitated ^{32}P -labelled total casein

Samples ($12\mu\text{g}$) of ^{32}P -labelled recombined casein (Fig. 4a) and washed ^{32}P -labelled casein immunoprecipitate (Fig. 4b) were analysed. For details see the Materials and Methods section. The position of the radioactive peak on each gel corresponds to the R_F of casein.

Fig. 5. Polyacrylamide-gel electrophoresis of ^{32}P -labelled casein immunisolated from a water extract of lactating mammary gland

^{32}P -labelled tissue extract ($50\mu\text{l}$, 2527c.p.m.) was applied to the Sepharose-anti-casein IgG column (11ml) and left in contact with the column for 4h at room temperature. ^{32}P -labelled casein was eluted and processed for radioactivity as described in the Materials and Methods section.

antibodies and make accurate immunological quantification of casein difficult. Walker *et al.* (1976) have suggested that use of only one technique is insufficient when assessing the specificity of an antiserum. The antiserum used in the present study was raised against recombined casein polypeptides which had been previously purified by DEAE-cellulose chromatography (Al-Sarraj *et al.*, 1978). Double-diffusion analysis of unadsorbed and adsorbed antisera by the method of Ouchterlony (1949) (Figs. 1a and 1b) gave reactions of identity with single precipitin lines against acid-precipitated casein, recombined casein, casein in a water extract of lactating rabbit mammary gland and also with each individual casein polypeptide after only 24h of immunodiffusion. Protein staining and radioautography of crossed-rocket immunoelectrophoretograms of water extracts from ^{32}P -labelled lactating rabbit mammary gland and ^{32}P -labelled recombined casein (Fig. 2) also demonstrated the presence of a single antigen-antibody system which contained phosphoprotein, by using both adsorbed and unadsorbed antisera. In contrast with this, antiserum to mouse casein which had been adsorbed with mouse serum gave at least three precipitin lines against tissue extracts from lactating mouse mammary gland, mouse milk and even the immunizing antigen mouse casein after 4 days of immunodiffusion (Terry *et al.*, 1975). Immunodiffusion analysis of rennin-precipitated rat casein with rat casein antiserum raised in rabbits (Feldman & Ceriani, 1970) revealed a single precipitin line, whereas classical immunoelectrophoretic analysis gave several precipitation arcs.

Radioimmunoprecipitation analysis performed with the unadsorbed antiserum (Fig. 3) showed that 73% of the recombined casein was precipitated by the antiserum. This calculation is based on the radioactivity in casein subunits resolved by electrophoresis of the immunoprecipitate on polyacrylamide gel in the presence of urea and sodium dodecyl sulphate. The percentage of casein immunoprecipitated at equivalence is considerably more than that which has been previously described for rabbit casein [50% (Houdebine & Gaye, 1976)], mouse casein [61.7% (Feldman, 1974) and 40% (Terry *et al.*, 1975)], and guinea-pig casein [32% (Craig *et al.*, 1976)]. These latter values were derived from the radioactivity in the immunoprecipitate divided by the radioactivity in the added antigen preparation at equivalence. If the antigen is impure then such calculations will yield low values for the percentage precipitation. An accurate estimate of the percentage immunoprecipitation of casein can only be derived from analysis of the immunoprecipitated antigen and antigen preparation by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate and urea.

The assessments of antiserum specificity (Figs. 1 and 2) showed that the unadsorbed and adsorbed

phosphoproteins (Tan *et al.*, 1972; Feldman & Hohmann, 1971). Immunization with such casein preparations may then give rise to contaminating

antisera were qualitatively identical. However, adsorption of the antiserum (with Sepharose-recombined casein) was carried out routinely to obtain the specific immunoglobulin fraction for casein, and this specific immunoglobulin was used to prepare a Sepharose-anti-casein immunoabsorbent for the isolation of casein from tissue extracts. The use of such a specific immunoglobulin for the immunoabsorbent might be expected to decrease considerably the degree of non-specific adsorption. Casein in tissue extracts bound quantitatively to the immunoabsorbent (results not shown) and was eluted completely with 8M-urea containing sodium dodecyl sulphate (2%, w/v; Fig. 5). No other protein-staining bands were seen on the gels of the urea eluates, suggesting that non-specific protein binding to the immunoabsorbent was low.

The use of immunoabsorbents for the isolation of antigens from tissue extracts and subsequent analysis by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate has a great advantage over immunoprecipitation in, for instance, studies on protein turnover. Whereas various proportions of immunoglobulins are contained in immunoprecipitates, the material eluted from an immunoabsorbent is predominantly antigen and therefore in this case much more antigen can be loaded on to polyacrylamide gels. For example, only 1.4% of the protein in the immunoprecipitates to rabbit casein is casein (K. Al-Sarraj, D. A. White & R. J. Mayer, unpublished work). Such a problem becomes acute in studies on protein turnover, where the specific radioactivity of labelled amino acid in the isolated antigen may be very low.

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