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Isolation of Inhibitory Factor in Raw Milk Whey Active Against Propionibacteria[1]

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Preparative isolation of the active component(s) in skim milk whey inhibitory for propionibacteria was made by using $(NH_4)_2SO_4$ salt fractionation. The crude preparation was further purified by Sephadex G-100 column separation. Disc-gel electrophoresis of the active peak from the Sephadex elution pattern (peak I) showed that this fraction contained almost all of the immune globulin in the column sample. The biologically inactive peaks did not contain any immune globulin. Starch-gel electrophoresis of the active peak revealed the presence of three separate immune globulin fractions. A correlation was also observed between hemolytic reaction of propionibacterial strains and relative resistance to whey inhibition. The investigation showed that one of the immune globulins of milk, pseudoglobulin, was mainly responsible for the suppressive activity of whey.

Several naturally occurring microbial inhibitors have been reported in raw milk. These include lactenins (2), lactoperoxidase (21), heat-stable, nondialyzable principle(s) active against thermophilic spore-formers (3), casein-associated, iron-chelating factor inhibitory for *Bacillus stearothermophilus* (1), dialyzable thiocyanate fractions (14), agglutinins active against strains of group *N* streptococci (6), whey-associated factor(s) inhibitory against propionibacteria (20), and other nonspecific inhibitors (7, 14).

Vedamuthu et al. (20) found that the inhibitory principle(s) in raw milk whey active against propionibacteria suppressed the growth of six of the eight *Propionibacterium* species tested. They also found that the factor(s) was nondialyzable, labile to heating in a boiling-water bath for 10 min, and affected by treatment with the strongly proteolytic enzyme, Pronase (Calbiochem, Los Angeles, Calif.). They suggested that the inhibitory agent(s) could be similar to or closely related to the immune globulins of milk.

This paper describes investigations related to the partial isolation and identification of the suppressive agent in raw milk whey active against propionibacteria.

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MATERIALS AND METHODS

Culture. The test culture used was *Propionibacterium shermanii* 1298 (Sahli) from the culture collection at the Department of Food Technology, Iowa State University.

Media. The culture was routinely propagated by weekly transfers in sodium-lactate broth (13) and incubating for 24 hr at 32 C. Between transfers, the culture was held at 5 C.

For bioassays, the test microorganism was grown as a lawn in semisolid agar over a solid agar base. The composition of the medium is given in our previous report (20).

Demonstration of inhibition. The disc-assay technique was used (20). Fractions tested for suppressive activity were sterilized by filtration through a membrane filter of 0.22-μm pore size (Millipore Corp., Bedford, Mass.).

Preparation of whey and whey-protein fractionation. The schematic procedure used for the preparative isolation of the active fraction from skim milk acid-whey is shown in Fig. 1. The skim milk was obtained by separation of raw whole milk at 5 C in a continuous centrifugal separator. The salt-fractionation procedure was the method recommended by Smith (17) to isolate the immune globulins of milk, euglobulin, and pseudoglobulin.

Lyophilization. The active component(s) obtained from the preparative salt fractionation of whey was made in bulk and lyophilized in a flat stainless-steel pan. The dry flakes were stored in a tightly closed bottle and held in a desiccator containing anhydrous $CaCl_2$ at 5 C.

Lyophilization of other materials in this investigation was done similarly.

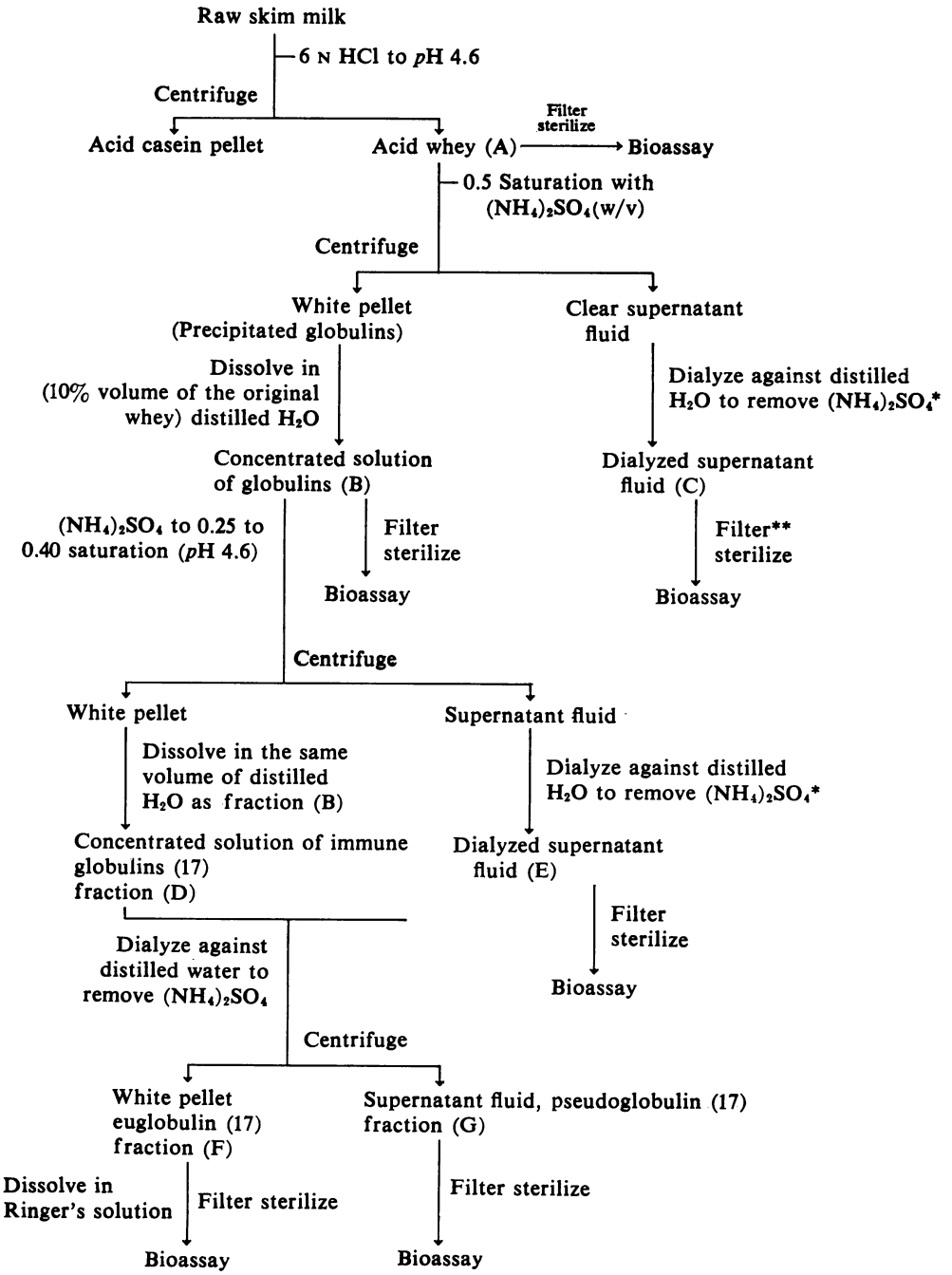

FIG. 1. *Fractionation procedure used for preliminary isolation of the active fraction in acid whey from raw skim milk inhibitory against propionibacteria. *SO_4 was tested in dialysate with 1:1 mixture of 5% $BaCl_2$ and 5% CH_3COOH. **About 2 ml of each fraction was used for bioassay after filter sterilization.*

Protein determination. The protein content of the lyophilized active fraction obtained by salt precipitation of the whey was determined by the method of Lowry et al. (11) after reconstituting in the eluant used for further purification of the fraction on a Sephadex G-100 column.

Sephadex gel separation. Further separation of the active fraction was achieved on a Sephadex gel column. Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) swollen in the eluant (0.005 M NaCl) for 5 days with intermittent removal of the "fines" was poured into an unjacketed glass column measuring 2.5 by 45 cm (V. V. Garcia, M.S. thesis, Iowa State University, 1969). The column was equilibrated at room temperature for 48 hr with the eluant. The sample consisted of 5.0 ml solution of the lyophilized active fraction made up in the eluant to give between 10 and 40 mg of protein per ml. This was designated as the column sample.

The flow rate was 0.75 ml/min, and 10-ml portions were collected by a LKB Radi Rac Fraction Collector (type 3403B). The individual fractions were continuously monitored at 253 nm through an LKB Uvicord (type 4701A) unit, and the response was transcribed on an LKB Recorder (type 6520A). All of these instruments are manufactured by LKB-Produkter AB, Stockholm-Bromma, Sweden.

The void volume of the column was determined by using Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.).

Fractions comprising individual peaks obtained from several consecutive Sephadex gel separations were correspondingly pooled together, lyophilized, reconstituted in the minimum volume of double-distilled water, filter sterilized and checked for inhibitory activity.

Disc-gel electrophoresis. Portions of the filter-sterilized solutions, representing individual peaks from the Sephadex column separation, were examined by disc-gel electrophoresis. The procedure of Davis (5) was used.

Starch-gel electrophoresis. Because greater resolution of milk immune globulins is possible by starch-gel electrophoresis, the starch-gel electrophoretic technique of Smithies (18) with minor modifications was used to separate the proteins in whole whey, the column sample, and the biologically active peak I from Sephadex column separation.

RESULTS AND DISCUSSION

Tests for biological activity of the various fractions (Fig. 1) revealed that the inhibitory substance(s) was associated with fractions A, B, D, and G. A very slight suppressive activity was detected in fraction E, probably caused by a very small, unrecovered residual of fraction G, which exhibited the greatest inhibition of the test strain. According to this salt fractionation scheme, the active fraction G would represent the pseudoglobulin of milk. This would correspond to the immunoglobulin (Ig) fractions IgG and "secretory IgA" under the newly proposed system of nomenclature for milk proteins (15). Once it was determined that fraction G is the active ingredient, fraction G was prepared in bulk and lyophilized to obtain a uniform sample for further work. The inhibitory activity of the fraction was unaffected by lyophilization.

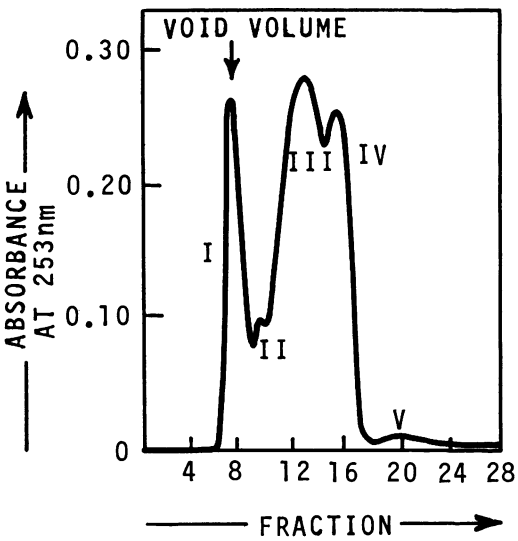

FIG. 2. *Sephadex G-100 column elution pattern of the crude lyophilized preparation of the inhibitory fraction from whey (fraction G).*

The separation pattern of the reconstituted, lyophilized fraction G on Sephadex G-100 column is shown in Fig. 2. Four distinct peaks were obtained consistently. The second peak was very small (sometimes appearing as a shoulder between peaks I and III) and appeared only when a very concentrated sample was applied to the column (protein concentration $\geq$ 30 mg/ml). A fifth peak in the form of a very low, flat plateau appeared in some of our preparations. The elution pattern was similar for several different lyophilized samples prepared from separate lots of raw skim milk.

Uusi-Rauva et al. (19) obtained a very similar elution pattern with whey prepared from colostrum, in which the immune globulin content is about 85 to 90% of the total protein (17), on a Sephadex G-100 column (5 by 90 cm) by using 0.01 M phosphate buffer (*p*H 7.0) containing 0.25 M NaCl. The first four peaks in our separation closely correspond to the first four peaks in their elution pattern. The elution volumes at which these peaks appeared, however, were different. This difference was probably because of the larger void volume for their longer and wider column. They identified the first two peaks as

immune globulins by the disc-gel electrophoretic behavior of these fractions. The fifth peak in their elution pattern was more prominent than ours. As mentioned, this peak appeared only in some of our preparations as a low, flat plateau. In our column samples, the fraction(s) represented by this peak probably was either present in very minute concentration or was lost during preliminary fractionation and extensive dialysis used for the preparative isolation of the biologically active protein component.

Bioassay of peaks I, III, and IV (representing composites of several different separations from a single bulk sample) revealed that most of the inhibitory function was confined to peak I. A very slight activity was observed with peak III, which could probably be attributed to the overlapping between these peaks. No assays were possible with peaks II and V because of the very low concentration of protein and limited and nonuniform occurrence of these components in the elution pattern (insufficiency of material to obtain authentic results).

Figure 3 shows the disc-gel electrophoretic separation of acid whey from skim milk (A), column sample (B), peak I (C), peak III (D), and peak IV (E). As compared with the whey, the column sample shows a very prominent immunoglobulin band. This indicates that these protein components were concentrated during the preparative fractionation to obtain the active component G (Fig. 1). The disc-gel electrophoretic pattern of peak I shows that almost all of the immunoglobulin from the column sample is present in this fraction. The other peaks examined did not show the immunoglobulin band. These observations indicate that the inhibitory activity of raw milk whey against propionibacteria is associated with the immunoglobulin portion and, presumably, with the pseudoglobulin rather than the euglobulin fraction.

Figure 4 shows the disc-gel electrophoretic patterns of acid whey from skim milk and column sample (fraction G) before and after heat treatment in a boiling-water bath for 10 min. The suppressive activity of acid whey was destroyed by this treatment (20). The lack of inhibitory activity in the heated whey seems to coincide with the loss of the immunoglobulin band in the electrophoretic pattern obtained for this preparation.

Starch-gel electrophoresis revealed three immunoglobulin fractions with different migration patterns in the active peak I from Sephadex separation. An increased concentration of the immunoglobulins in the column sample and peak I (as compared with whole whey) was also seen in these preparations.

In an earlier report, Vedamuthu et al. (E. R. Vedamuthu et al., J. Dairy Sci. **51**:927., 1968) mentioned that several samples of bovine blood serum from healthy animals showed inhibitory activity against *P. shermanii* 1298 similar to that of raw milk whey by the disc-assay technique. The immunoglobulins of bovine milk are similar to and definitely derived from the immunoglobulins of bovine blood (15). Reiter and Oram (14) recently suggested that nonspecific antibodies could be induced in ruminants by plant tissue and rumen microorganisms, which may, in part, contribute to the over-all antibacterial activity of raw milk. A few species of propionibacteria form a part of the large heterogenous

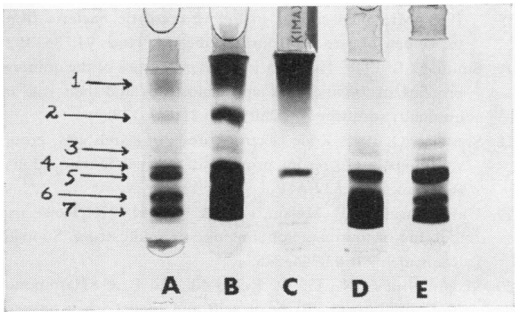

FIG. 3. *Disc-gel electrophoretic separation of acid whey from raw skim milk (A), fraction G-column sample (B), peak I (C), peak III (D), and peak IV (E). (1) Immunoglobulin; (2) proteose peptone (minor); (3) bovine serum albumin; (4) proteose peptone (major); (5) α-lactalbumin; (6) β-lactoglobulin B; and (7) β-lactoglobulin A (according to references 4, 8).*

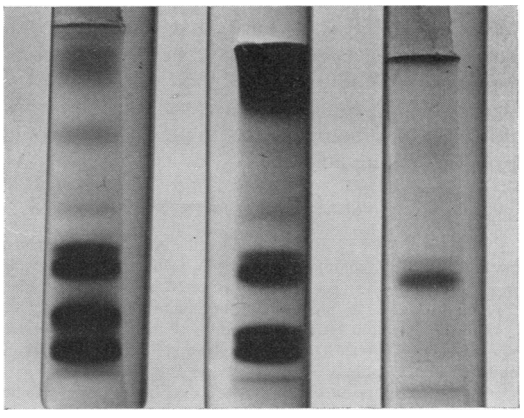

FIG. 4. *Disc-gel electrophoretic separation of acid whey from raw skim milk (left), fraction G-column sample (middle), and fraction G-column sample after heating in a boiling-water bath for 10 min (right).*

flora of bovine rumen (16). A recent study (L. O. Skogen, M.S. thesis, Iowa State University, Ames., 1970) revealed that the intensely red-pigmented *P. rubrum* strains, unaffected by the whey filtrate (20), produced copious amounts of capsular and slime material. Of the three *P. thoenii* tested in our previous study (20), none was susceptible to whey inhibition; however, two of these strains failed to exhibit any capsule or slime formation. Because of this anomaly, no definite correlation could be made between slime or capsule production and induction of or susceptibility to specific or nonspecific antibodies in milk whey possibly derived from the cow's blood stream.

We recently examined the blood hemolytic reactions of eight species of the genus *Propionibacterium* by using human, bovine, equine, rabbit, sheep, and porcine blood. Definite and rapid β-hemolytic reactions of the blood were obtained only with the darkly pigmented species *P. rubrum* and *P. thoenii*. Very slight or delayed β-hemolysis (needed 6 days at 32 C to see the reaction) of either the horse or the rabbit blood, or both, was obtained with certain other strains. In all instances, the strongly or weakly hemolytic strains were pigmented and were either totally resistant to or relatively less susceptible (turbid zone of inhibition) to the suppressive activity of raw milk whey (20). The exact mechanism for this correlation is unknown.

Jain and Jasper (10) found that the bactericidal activity of milk against *Aerobacter aerogenes* coincided with the increase in the relative percentages of blood serum albumin and immunoglobulins in the whey. Similar observations were made by Emmons et al. (6) for lactic streptococcal agglutinins in milk and blood of the lactating cow. It is probable that a similar correlation exists between the titer for the propionibacterial antibodies in the blood stream and the milk whey in the bovine system. Further work utilizing immunological techniques (9, 12) would provide definite answers.

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