

STUDIES ON THE PROTEOLYTIC BACTERIA OF MILK

V. ACTION OF PROTEOLYTIC BACTERIA ON MILK SERUM

WILLIAM C. FRAZIER AND PHILIP RUPP

Research Laboratories, Bureau of Dairy Industry, United States Department of Agriculture

Received for publication October 11, 1930

In previous papers of this series (Frazier and Rupp, 1928), the action of proteolytic bacteria of milk on casein and on skim milk has been reported. In order to make the study of proteolysis in milk more complete a study was made of the action of these organisms on milk from which the casein and lactoglobulin had been removed and in which lactalbumin was the only remaining protein. A number of the proteolytic bacteria were found to cause an increase in ammonia and in amino-nitrogen according to the formol titration method in milk, but were unable to split casein in any of the media used. This led to the supposition that lactalbumin might have been decomposed. In this work the organisms were grown in a milk serum, made by the removal of all casein and lactoglobulin from milk. This milk serum contained lactalbumin as its only protein and small amounts of non-protein nitrogen.

No references have been found to the action of bacteria on milk serum, although Franssen (1929) recommends its use in culture media. The only reference to the action of bacteria on lactalbumin is a preliminary report by Supplee (1917) on the action of milk bacteria on some of the nitrogenous constituents of milk. He obtained results which suggested that lactalbumin might have been decomposed. He grew organisms in milk for twenty-four hours at 30°C. and then analyzed for casein, albumin and other fractions. He sometimes found an increase over the control in the albumin fraction and concluded that the increase was due to proteoses from the casein. In a few cases, however,

he found a decrease in the albumin fraction and concluded that some species of bacteria could attack lactalbumin more readily than casein. Among the organisms which caused a decrease in the albumin were: *Mic. albidus*, *Ps. liquefaciens*, *Bact. bulgaricum*, *B. coli-communis* and *Bact. aerogenes*.

METHODS

The milk serum used in these experiments was obtained by ultrafiltration of skim milk through a collodion membrane. In this way all of the casein, and probably all of the lactoglobulin, were removed. The serum was sterilized by filtration through a Berkefeld filter candle. All flasks of serum were incubated for several days at 30°C. to test for sterility.

Tests on the milk serum showed the absence of casein. If traces of casein had remained in the serum after ultrafiltration, they would have been removed by the Berkefeld filter. All tests for lactoglobulin in the milk serum were either clearly negative or doubtful. Negative results were obtained by half-saturation of the serum with ammonium sulphate, by saturation with sodium chloride with slight acidification, and by the method of Howe (1922) for the precipitation of globulin from milk serum. Howe found that 21.5 per cent of sodium sulphate would precipitate the euglobulin and two pseudo-globulins and leave the albumin in solution. No trace of a precipitate was obtained with 21.5 per cent sodium sulphate in the milk serum used in these experiments.

Analyses of the serum for total nitrogen showed between 0.06 and 0.066 per cent in different tests, or, expressed as lactalbumin, between 0.38 and 0.422 per cent. The Berkefeld candle removed very little nitrogen. According to Denis and Minot (1919) the average amount of non-protein nitrogen in milk is about 21 mgm. in 100 cc., of which 9 to 10 mgm. is urea and the remainder chiefly amino-acid, creatine and creatinine nitrogen. Bleyer and Kallmann (1924) found about the same amount of urea. Analyses of the milk serum used in these experiments showed about 25 to 28 mgm. of non-protein nitrogen per 100 cc. of serum or 23 to 26 mgm. per 100 cc. of milk.

The milk serum cultures of the proteolytic bacteria were ana-

lyzed for total, protein, non-protein, amino and ammonia nitrogen. Protein (lactalbumin) nitrogen was estimated by difference between total nitrogen and non-protein nitrogen. Cultures of the organisms in skim milk were analyzed for casein nitrogen as well as total, non-protein, amino and ammonia nitrogen.

Total nitrogen was determined by the Kjeldahl method: 3.0 grams of milk or milk serum were used with 5.0 grams of sodium sulphate, 0.1 gram of copper sulphate and 15 cc. of concentrated sulphuric acid.

Casein nitrogen in the milk was determined by precipitation with 10 per cent acetic acid and analysis of the precipitate for nitrogen. Precipitation with a saturated solution of alum was also used (Van Slyke and Hart, 1902). Alum is supposed to precipitate the caseoses also.

In the determination of non-protein nitrogen 10 cc. of milk or milk serum were treated with 70 cc. of water, 10 cc. of 10 per cent sodium tungstate solution and 10 cc. of sulphuric acid (Folin and Wu, 1919). The filtrate was analyzed for nitrogen.

Ammonia nitrogen was determined by the Folin aeration method (Hawk, 1923) in 50 cc. of the non-protein nitrogen filtrate.

Amino nitrogen was determined on 5 cc. of the ammonia-free filtrate by the Folin (1922) colorimetric method and also by the Sörenson formol titration method on 10 cc. of the milk or milk serum in 35 cc. of water. This formol titration figure includes ammonia, and therefore the amount of ammonia nitrogen previously found was subtracted to obtain the amino nitrogen. Titratable acidity was found by titration with N/10 sodium hydroxide to a pink color with phenolphthalein.

RESULTS

Tables 1 and 2 show the results of analyses of cultures of proteolytic bacteria in milk serum after incubation for ten days at 30°C. For the purpose of comparison, analyses of cultures of *S. lactis*, *L. bulgaricus*, *A. aerogenes*, and *L. casei* in milk serum have been included in table 1. The figures given in the tables were obtained by subtracting the control, and gains over the

control are indicated by positive quantities and losses by negative quantities. Ability of the organisms to ferment urea has also been included in the tables.

Most of the cocci (table 1) show a comparatively small decrease in protein nitrogen with a correspondingly small increase in non-

TABLE 1
Action of proteolytic cocci on milk serum (also four lactic acid bacteria)

ORGANISM	pH	TITRAT- ABLE ACID- ITY*	NON- PRO- TEIN NITRO- GEN†	PRO- TEIN NITRO- GEN	FREE AMMO- NIA	AMINO- NITRO- GEN (FOLIN)	AMINO NITRO- GEN (SOREN- SON)	UREA FERMENTA- TION
		cc.	mgm.	mgm.	mgm.	mgm.	mgm.	
Control.....	6.7	0.0	0.0	0.0	0.0	0.0	0.0	
<i>M. citreus</i>	6.0	+7.8	+4.5	-4.5	+1.5	+1.5	-0.5	-
<i>M. perflavus</i>	6.5	-3.3	+2.4	-2.4	+11.7	+1.0	-2.9	+
<i>M. varians</i>	6.3	+6.9	+2.8	-2.8	+12.3	+1.0	-0.7	+
<i>M. casei</i> (yellow).....	6.2	+6.0	+5.6	-5.6	+1.5	+2.5	-1.5	-
<i>M. percitreus</i>	6.7	+1.0	-14.7	+14.7	+2.9	+1.0	-0.4	+
<i>M. cereus</i>	6.7	-3.3	+2.2	-2.2	+1.8	+1.0	+2.2	-
<i>M. subflavescens</i>	6.8	-1.0	+7.8	-7.8	+8.1	0.0	+1.2	-
<i>M. luteus</i>	6.4	+1.8	+20.3	-20.3	+12.7	+2.0	+0.5	+
P 147.....	6.1	-1.9	+7.4	-7.4	+7.4	0.0	0.0	(acid)
<i>M. casei</i> (white).....	5.9	+9.8	+2.3	-2.3	+1.0	+2.0	+0.1	-
<i>Staph. albus</i>	5.8	+11.7	+2.6	-2.6	+0.5	+2.0	+1.8	-
<i>M. freudenreichii</i>	6.0	+5.6	+0.4	-1.1	+13.0	+2.0	-1.7	+
<i>M. ureae</i>	8.0	-7.4	-3.6	+3.6	+16.1	0.0	-3.7	+
P 204.....	6.1	+8.0	-2.4	+2.4	-0.3	0.0	+1.4	-
P 269.....	4.7	+1.5	-12.0	+12.0	+13.4	-2.0	0.0	+
<i>Str. liquefaciens</i>	4.8	+12.8	+0.7	-2.1	+0.3	+1.0	0.0	-
<i>Str. bovis</i>	5.6	+9.1	+0.7	-0.7	+8.0	+1.0	+5.3	-
<i>Str. lactis</i>	4.3	+24.3	+6.8	-6.8	-1.1	+4.0	+0.1	-
<i>L. bulgaricus</i>	3.5	+11.4	+7.4	-7.4	+1.5	+2.0	-1.1	-
<i>A. aerogenes</i>	4.9	+19.6	-5.6	+5.6	+6.5	+2.0	-1.1	+
<i>L. casei</i>	3.4	+13.4	+6.2	-6.2	+4.1	+4.0	-3.0	-

* Increase or decrease in acidity expressed as cubic centimeters of N/10 NaOH per 100 cc. of medium.

† All nitrogen figures expressed as increase or decrease in milligrams per 100 cc. of medium.

protein nitrogen. This would indicate a small amount of decomposition of lactalbumin. With bacteria like *M. citreus* and *M. casei*, most of this gain in non-protein nitrogen can be accounted

for as ammonia and amino-nitrogen. Such cocci as *M. perflavus* and *M. varians*, however, produce a much larger amount of

TABLE 2
Action of proteolytic rods on milk serum

ORGANISM	pH	TITRAT- ABLE ACID- ITY*	NON- PRO- TEIN NITRO- GEN†	PRO- TEIN NITRO- GEN	FREE AMMO- NIA	AMINO- NITRO- GEN (FOLIN)	AMINO- NITRO- GEN (SOREN- SON)	UREA FERMENTA- TION
		cc.	mgm.	mgm.	mgm.	mgm.	mgm.	
Control.....	6.7	0.0	0.0	0.0	0.0	0.0	0.0	
<i>Flavobacterium</i>								
<i>synzanthum</i>	8.1	-2.2	+13.7	-13.7	+25.3	+6.0	+12.2	-
<i>Flavobacterium lactis</i>	8.2	-10.6	+13.1	-13.1	+18.6	-0.5	-3.4	-
<i>Flavobacterium</i>								
<i>tremelloides</i>	6.3	+3.8	+10.4	-10.4	+6.0	+1.5	-0.4	-
<i>Serratia marcescens</i>	8.1	-5.2	+2.3	-2.3	+6.4	-1.0	0.0	+
P 263 (<i>Serratia</i>).....	8.2	-11.9	-5.7	+5.7	+9.0	0.0	-2.7	+
<i>Achromobacter</i>								
<i>coadunatum</i>	5.5	+8.2	-4.6	+4.2	+0.2	+1.0	-0.2	+(acid)
<i>Achromobacter</i>								
<i>liquefaciens</i>	7.0	-5.0	+11.7	-11.7	+9.7	0.0	-2.3	-
<i>Achromobacter</i>								
<i>delicatulum</i>	7.7	+6.2	-13.1	+13.1	+2.7	0.0	-0.6	+(acid)
<i>Alcaligenes bookeri</i>	8.2	-8.9	+8.7	-9.9	+10.0	-1.5	-2.0	-
<i>Proteus vulgaris</i>	7.1	-1.1	-10.6	+10.6	+2.4	-2.0	-1.7	+(acid)
P 107 (<i>Escherichia</i>).....	5.0	+32.5	-5.1	+5.2	+13.1	0.0	0.0	+
<i>B. albolactis</i>	5.9	+13.8	+4.7	-4.7	+0.4	+3.5	-1.0	-
<i>B. cereus</i> "A".....	8.4	-13.0	+16.9	-16.9	+7.1	+1.7	-4.1	-
<i>B. cereus</i> "B".....	8.0	-12.2	+11.1	-11.1	+6.9	+1.0	-4.6	-
<i>B. vulgatus</i>	8.4	-11.4	+17.3	-17.3	+6.2	+4.0	-4.5	-
<i>B. subtilis</i>	8.0	-9.9	+13.9	-13.9	+8.0	0.0	+0.6	+
<i>B. simplex</i>	7.7	-0.2	+15.9	-15.9	+16.8	+2.0	0.0	+
<i>B. mesentericus</i>	6.8	-2.0	-1.0	+1.0	+1.8	0.0	0.0	-
<i>B. cohaerens</i>	6.7	-4.4	+4.9	-4.9	+0.6	-1.0	+3.0	-
<i>B. tumescens</i>	8.4	-17.9	+4.1	-4.1	+10.7	-2.0	+0.5	+
<i>B. megatherium</i>	8.4	-15.1	+18.7	-18.7	+7.3	0.0	-1.1	-
<i>B. ruminatus</i>	7.2	+0.7	+12.2	-12.2	+12.9	+1.5	+5.2	+
<i>B. macerans</i>	5.8	+7.8	+10.9	-10.9	+0.6	+2.0	+3.6	-
P 67.....	8.6	-13.9	-7.8	+7.8	+5.1	-2.0	0.0	+
P 285.....	6.4	+3.1	-6.4	+6.4	+2.7	0.0	0.0	-

* See footnote to table 1.

† See footnote to table 1.

ammonia nitrogen than can be accounted for by loss of protein nitrogen. These organisms are able to break down urea, and the

amount of ammonia found corresponds closely to the amount which would be formed from 10 to 12 mgm. of urea nitrogen in the milk serum (Denis and Minot, 1919).

It is difficult to interpret the results obtained with *M. percitreus*, *M. ureae*, P 204 and P 269, where a large increase in protein nitrogen was obtained with the analytical methods used and a decrease in non-protein nitrogen. *M. percitreus* is apparently able to decompose lactalbumin with an increase in amino-nitrogen. In one analysis there was an increase of as much as 6.2 mgm. of amino-nitrogen per 100 cc. of milk serum. This organism is also very actively caseolytic. *M. ureae* and P 269, like *M. percitreus*, are able to decompose urea, but unlike *M. percitreus* cause a considerable increase in ammonia nitrogen. Their action in this way resembles that of the urea fermenters *perflavus* and *M. varians*.

The Gram-negative rods (table 2), like the cocci, may be divided into those which decrease protein nitrogen and increase non-protein nitrogen and those which accomplish the opposite result. Organisms like the *Flavobacteria*, *Achromobacter liquefaciens* and *Alcaligenes bookeri* are obviously able to decompose the lactalbumin in milk serum. *Achromobacter coadunatum* would undoubtedly be able to break down lactalbumin in the absence of fermentable sugar as it did casein (Paper III of this series).

All of the Gram-positive, spore-forming rods except *B. mesentericus* decomposed the lactalbumin. In most cases there was a marked increase in ammonia. It will be observed that, even with these actively proteolytic bacteria, there was not a large gain in amino-nitrogen.

In agreement with the results of Supplee (1917) *L. bulgaricus* in table 1 is apparently shown to break down lactalbumin to some extent although *A. aerogenes* did not do so in these experiments. Both *L. casei* and *S. lactis* also increased the non-protein nitrogen and decreased the protein nitrogen.

In order to compare the action of the proteolytic bacteria on milk and on milk serum (milk minus casein and lactoglobulin) table 3 has been given in addition to tables 1 and 2. Table 3 shows the results of quantitative analyses of ten-day milk cultures for the

various nitrogen fractions. It will be observed that a number of the cocci, which break down large amounts of casein in milk, do not decrease the amount of protein in milk serum to a great

TABLE 3
Action of proteolytic bacteria on milk (also two lactic acid bacteria)

ORGANISM	pH	TITRAT- ABLE ACID- ITY*	CASEIN NITRO- GEN (ACETIC)	CASEIN NITRO- GEN (ALUM)	NON- PROTEIN NITRO- GEN	FREE AMMO- NIA	AMINO- NITRO- GEN (FOLIN)	AMINO- NITRO- GEN (SOREN- SON)
		cc.	mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
Control.....	6.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
<i>M. citreus</i>	5.6	+26.7	-209.4	-199.2	+69.3	+0.7	+6.0	+5.1
<i>M. perflavus</i>	5.9	+29.4	-162.3	-175.3	+110.0	+14.3	0.0	+2.2
<i>M. varians</i>	5.4	+36.0	-270.1	-254.2	+65.8	+12.3	0.0	+1.2
<i>M. casei</i> (yellow).....	5.7	+35.4	-265.1	-253.9	+115.2	+2.4	0.0	+10.5
<i>M. percitreus</i>	5.3	+24.8	-158.4	-160.8	+50.1	+5.2	+2.0	+1.1
<i>M. cereus</i>	6.5	-0.9	-10.0	+1.9	+6.7	+0.2	+2.0	-1.4
<i>M. luteus</i>	5.4	+28.4	-399.6	-383.0	+245.8	+7.2	+10.0	+13.6
<i>M. casei</i> (white).....	5.7	+31.9	-65.7	-88.7	+35.0	+1.1	+2.0	+1.7
<i>Staph. albus</i>	5.8	+35.8	-230.0	-221.3	+68.6	+2.7	-4.0	+7.3
<i>M. freudenreichii</i>	5.6	+35.3	-38.6	-37.1	+36.0	+10.1	+0.9	+2.1
<i>Str. liquefaciens</i>	4.8	+98.3	-302.9	-311.8	+180.4	+1.7	+6.0	+8.2
<i>Str. bovis</i>	5.6	+25.1	-292.0	-293.3	+90.0	+1.4	-2.0	+7.6
<i>Flavobacter synzanthum</i>	7.0	+26.4	-415.9	-411.7	+359.0	+42.0	+90.0	+45.1
<i>Achromobacter</i> <i>coadunatum</i>	5.8	+38.5	-54.5	-58.8	+10.1	+0.4	0.0	-0.5
<i>Achromobacter</i> <i>liquefaciens</i>	6.8	+15.7	-177.9	-176.2	+146.8	+20.9	+26.0	+17.5
<i>Alcaligenes bookeri</i>	8.2	+12.9	-372.4	-358.9	+343.7	+43.0	+110.0	+86.6
<i>Proteus vulgaris</i>	6.8	+25.3	-265.3	-260.4	+218.6	+26.1	+30.0	+22.6
<i>B. albolactis</i>	5.3	+68.7	-245.3	-243.9	+232.5	+8.6	+13.5	+12.1
<i>B. cereus</i> "A".....	7.3	+19.4	-333.2	-319.7	+300.1	+27.2	+26.0	+15.0
<i>B. cereus</i> "B".....	7.3	+10.2	-364.7	-344.3	+333.7	+45.0	+39.0	+31.4
<i>B. vulgatus</i>	6.9	+34.9	-427.4	-425.2	+370.9	+18.5	+18.0	+13.8
<i>B. subtilis</i>	7.4	+22.3	-425.8	-385.3	+409.4	+6.5	+24.0	+17.2
<i>B. simplex</i>	6.5	+9.2	-185.4	-174.6	+101.8	+4.2	+1.0	+7.1
<i>B. cohaerens</i>	6.8	+11.8	-259.4	-218.7	+130.5	+7.3	+11.0	+10.5
<i>L. bulgaricus</i>	3.5	+203.8	-11.7	-19.7	+9.8	-0.3	-2.0	-3.5
<i>L. casei</i>	3.5	+203.1	-22.6	-28.2	+15.4	+0.7	0.0	-2.4

* See footnote to table 1.

extent. The final pH values attained in milk and in milk serum are almost the same, but the more heavily buffered milk allowed

more growth, greater fermentation of lactose and probably greater action on the proteins before the limiting pH was reached.

The bromine test for free tryptophane as described in the second paper of this series (Frazier and Rupp, 1928) was found to indicate casein decomposition in milk, but was always negative in milk serum cultures, although lactalbumin, like casein, contains tryptophane.

The determination of amino-nitrogen did not prove useful in the measurement or detection of proteolysis of either casein or lactalbumin in milk or milk serum. With most cultures the results with either the Folin colorimetric or the Sörensen titration method were too close to the limit of error definitely to indicate protein decomposition. Yet a marked decrease in protein nitrogen in the milk serum and a decrease in casein nitrogen in the milk definitely showed proteolysis. Neither is an increase in ammonia an indication of protein decomposition in milk or milk serum, for it has been pointed out that urea-fermenting organisms, like *M. perflavus* or *M. varians* which break down lactalbumin little, if at all, cause more of an increase in ammonia in milk serum than an actively proteolytic organism like *B. vulgatus*. These two micrococci produce a similar increase in ammonia in milk, although they form little ammonia in a casein medium. If they were judged by their ammonia production in milk or milk serum they would be called actively proteolytic when, as a matter of fact, they break down casein or lactalbumin in milk only slightly.

The bromine test for free tryptophane has been found the most satisfactory test for small amounts of casein decomposition. The breaking down of lactalbumin is best detected by analysis for protein nitrogen.

SUMMARY

Ten-day cultures of proteolytic bacteria grown in milk at 30°C. have been quantitatively analyzed before and after incubation for total, casein, non-protein, amino and ammonia nitrogen and ten-day cultures in milk serum have been analyzed before and after incubation for total, protein, non-protein, amino and ammonia nitrogen. Data given are on changes in analytical values.

Most of the organisms which decomposed casein were also able to break down the lactalbumin in milk serum. Some of the cocci did not decompose as great a proportion of the lactalbumin in milk serum as they did of casein in milk. Bacteria like *M. perflavus* and *M. varians* in milk serum caused a considerable increase in ammonia nitrogen which was apparently due to the splitting of urea by these organisms.

For the qualitative detection of caseolysis the bromine test for free tryptophane was satisfactory, but it did not indicate decomposition of lactalbumin.

Neither amino-nitrogen nor ammonia determinations served as a good indication of proteolysis in milk or milk serum, although they proved useful with synthetic media.

Quantitative determinations of casein nitrogen in milk and of protein nitrogen in milk serum served as the best measure of protein decomposition and the best indication of proteolysis.

S. lactis, *L. bulgaricus*, and *L. casei* were apparently able to decompose lactalbumin in milk serum.

Milk serum sterilized by filtration is a good culture medium for many of the common bacteria.

The writers wish to express their thanks to E. F. Deysher of these laboratories for his assistance in the preparation of the milk serum by ultrafiltration.

REFERENCES

- BLEYER, B., AND KALLMANN, O. 1924 *Biochem. Z.*, **153**, 459-486.
DENIS, W., AND MINOT, A. S. 1919 *Jour. Biol. Chem.*, **37**, 353-366.
FOLIN, O. 1922 *Jour. Biol. Chem.*, **51**, 377-391.
FOLIN, O., AND WU, H. 1919 *Jour. Biol. Chem.*, **38**, 81-110.
FRANSEN, R. 1929 *Zent. Bakt.*, I, Orig., **114**, 153-157.
FRAZIER, W. C., AND RUPP, P. 1928 *Jour. Bacteriol.*, **16**, 65-78.
FRAZIER, W. C., AND RUPP, P. 1928 *Jour. Bacteriol.*, **16**, 187-196.
HAWK, P. B. 1923 *Practical Physiological Chemistry*. P. Blakiston's Son and Co., Philadelphia, p. 528.
HOWE, P. E. 1922 *Jour. Biol. Chem.*, **52**, 51-68.
SUPPLEE, G. C. 1917 *Jour. Dairy Sci.*, **1**, 313-319.
VAN SLYKE, L. L., AND HART, E. B. 1902 *N. Y. Agric. Expt. Sta. (Geneva) Bul.* 215.