A C T A A L I M E N T A R I A P O L O N I C A

Vol. XV (XXXIX), No. 1 1989

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MECHANISM OF PROTEOLYSIS AND COAGULATION OF MILK FORTIFIED WITH ALCOHOL-PRECIPITATED WHEY PROTEINS. PART I. PROTEOLYSIS

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Key words: rennin effect on milk, peptides, glycopeptides, alcohol milk proteins

Rennin proteolysis of milk proteins was investigated in milk fortified with alcohol-precipitated whey proteins concentrate. A mechanism of interactions between alcohol-denatured whey proteins and micellar casein is proposed on the basis of results of starch-gel electrophoresis, the amounts of released peptides and glycopeptides, and the determined alcohol and heat stability.

INTRODUCTION

There are many ways of recovering proteins from whey. The method of precipitating whey proteins with ethanol is interesting in that it enables a wide-ranging utilization of whey. The precipitating agent, alcohol in this case, may be produced from whey following its deproteinization, and may be reused many times. Moreover, ethand is easily removed from the protein concentrate and its trace remains pose no health hazard. Earlier studies indicate that whey proteins exposed to ethanol undergo denaturation, forming aggregates, mainly through acid amino acids radicals [12]. Simultaneously, the hydrophobic regions of particles become exposed, which makes posible interaction with casein. Experiments with milk fortified with coagulating enzymes recovered from whey together with whey proteins by alcohol precipitation demonstrated that nearly all the added proteins pass on to the curd [13]. The available literature lacks detailed data on the mechanism of proteolysis of milk fortified with other concetrates of whey proteins. It was suspected that alcohol-denatured proteins will modify the course of casein proteolysis, similary as it happens in the case of the formation of complexes of heat-denaturated β-lactoglobulin with κ-casein.

MATERIAL AND METHODS

The preparations of alcohol-precipitated whey proteins (S_A) and of untreated whey proteins (S_o) were obtained by a previously described method [12], Skim

milk with no additions, skim milk with the S_A preparation in doses ranging from 0.1 to 0.4% (W/V), and skim milk with the S_o preparation in analigous concentrations, were subjected to the heat stability test described by Davies and White [8]. Simultaneously, methanol stability was determined in identical samples by the White-Davies method [27].

Skim milk with no additions (M), skim milk with 0.4% whey proteins untreated with alcohol (B), and skim milk with 0.4% alcohol-precipitated whey proteins (A), where incubated at 305 K and pH 6.6 and 5.5 with an amount of crystalline rennin causing coagulation of skim milk during 0.5 h. Incubation lasted 0.5, 12 and 24 h, and samples were analysed immediately after this time. Milk proteolysis was characterized on the basis of the amounts of nonprotein nitrogen compounds soluble in 12% TCA (glycopeptides) according to the method of Alais [6], and the content of nitrogen compounds soluble in 2% TCA (total released peptides) by the method of Wilson and Wheelock [29]. Nitrogen content in the investigated substrates and in the isolated peptides and glycopeptides was determined by Kjeldahl's method [19]. The samples were also subjected to electrophorersis in starch-urea gel with pH 8.6 according to Schmitd's method [24]. The quantitative composition of the individual fractions was determined densitometrically (560 nm).

RESULTS AND DISCUSSION

Both additions — of alcohol-precipitated and undenatured whey proteins — reduced milk stability toward to methanol and the milk's thermal stability. A 0.1% addition of the protein concentrates caused the greatest stability decrease. Greater amounts of the concentrates (0.2-0.4%) did little to further reduce the system's sensitivity to methanol and drastic thermal conditions (Figs.

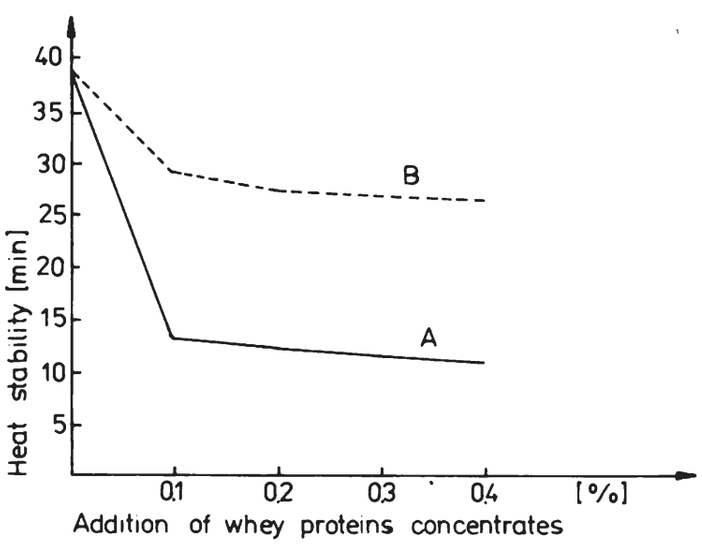

Fig. 1. Effect of whey proteins concentrate addition to milk on milk heat stability; A — skim milk fortified with alcohol-precipitated whey proteins concentrate, B — skim milk fortified with untreated whey proteins

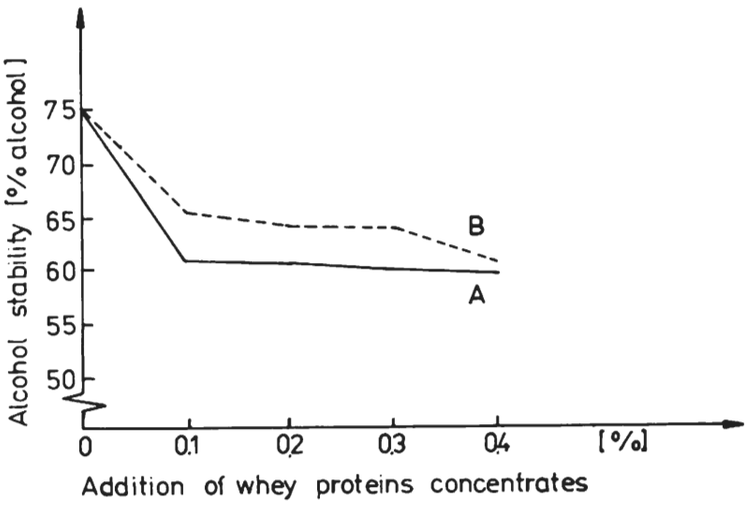

Fig. 2. Effect of whey proteins concentrate addition to milk on milk alcohol stability; A — skim milk fortified with alcohol-precipitated whey proteins concentrate, B — skim milk fortified with untreated whey proteins

1 and 2). Milk fortified with the S_A preparation displayed a thermal stability considerably lower (by about 50%) than that of milk fortified with the S_o lyophilizate. In contrast, the differences in alcohol-induced instability changes were in both cases small (less than 10%). These differences are due to unidentical mechanisms of micellar system destabilization brought about by methyl alcohol and elevated temperature.

The milk stability decrease accompanying increasing concentrations of whey proteins was most probably caused by charge changes on the casein micelle surface due to whey proteins depositing there. The effect of charge changes brought about by 0.1% additions of the protein concentrates turned out to be greater than the effect of the shielding of casein from calcium by complexes formed on micelle surfaces. Further increases of the proteins concentration in milk lead to charge change with a simultaneous restriction of calcium access to casein. The poorer stability of milk fortified with the S_A preparation, compared with the stability of milk with an S_o addition, was due to the fact that the alcohol-precipitated protein concentrate interacts with casein more readily, its hydrophobic regions being exposed.

The fortification of milk with pH 6.6 with a 0.54% addition of alcohol-percipitated whey proteins concentrate led to a severe drop in peptides release from casein (by about 43%) in the enzymatic phase of milk coagulation (0.5 h incubation). During secondary proteolysis (12 and 24 h of incubation) this inhibition relaxed somewhat, and more peptides soluble in 2% TCA were released. However, the amounts continued to be lower than in unfortified milk and in milk with undenatured whey proteins lyophilizate (Fig. 3).

The addition of undenatured whey proteins to milk with pH 6.6 decreased by about 17% the total amount of released peptides (compared to the figure for unfortified milk) in the specific phase of proteolysis by rennin. In the secondary proteolysis phase this inhibitory effect vanished altogether, and the amount of peptides released from casein was similar to that in milk (Fig. 3).

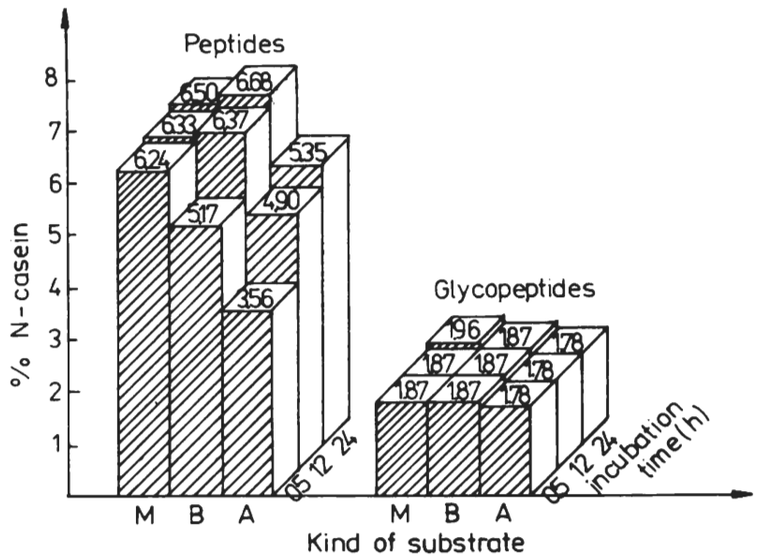

Fig. 3. Effect of substrate kind and incubation time on the degree of peptides and glycopeptides release by rennin at pH 6.6; M — skim milk, B — skim milk fortified with 0.4% untreated whey proteins, A — skim milk fortified with 0.4% alcohol-precipitated whey proteins

The profound changes in the proteins' internal structure caused by alcohol together with the exposing of hydrophobic regions [12] created conditions for the interaction of these proteins with casein and, consequently, for the reduction of the rate and magnitude of peptides release. However, this effect did not turn out to be permanent, since in the secondary proteolysis phase rennin was less inhibitory: after 24 h the amount of released peptides was already a more 18% lower than in unfortified milk. The fact that inhibition decreases with time suggests that there occur changes in the character of interaction between the alcohol-denatured whey proteins and the paracasein particles in the gel undergoing syneresis. Alterations in the gel structure most probably led to increased access to the initially shielded κ-casein particles.

At pH 5.5 there was a general increase in amounts of peptides and glycopeptides released from casein in all three substrates (Fig. 4). This was no doubt because the milk's pH was closer to the pH optimum for rennin, which ranges from 3.5 to 3.7, depending on the substrate [23, 25]. Also observed was an inhibition of peptides release by rennin in the milk with whey proteins additions (more severe in substrate A than in substrate B). In general, however, the inhibition of peptides release was lower than at pH 6.6. In the general phase of casein proteolysis in unfortified milk and milk containing nondenaturated whey proteins, the proteolysis rate increase was higher than at pH 6.6.

The electrophoretic picture of the casein fraction was quite complementary, both quantitatively and qualitatively, to results concerning the release by rennin of peptides and glycopeptides from micellar casein in all three milk media (Fig. 5, Tables 1 and 2). The only casein form hydrolysed by rennin during incubation of up to 24 h was κ-casein (Fig. 5). Some authors suggest that in the course of secondary proteolysis of casein by rennin, also α_s-casein, and even β-casein may disintegrate [15, 16, 22]. However, these authors studied proteolysis in model systems in which reconstituted milk behaves similarly, but never identically as

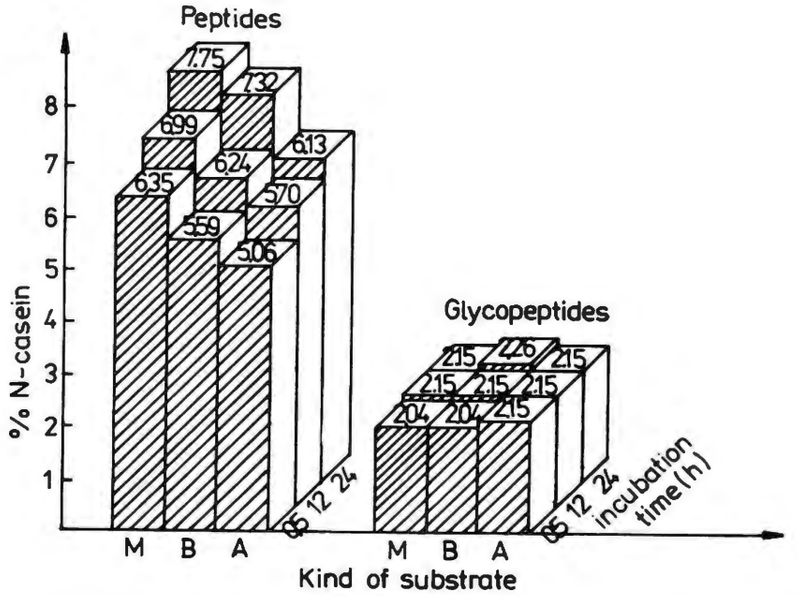

Fig. 4. Effect of substrate kind and incubation time on the degree of peptides and glycopeptides release by rennin at pH 5.5. See Fig. 3 for explanations

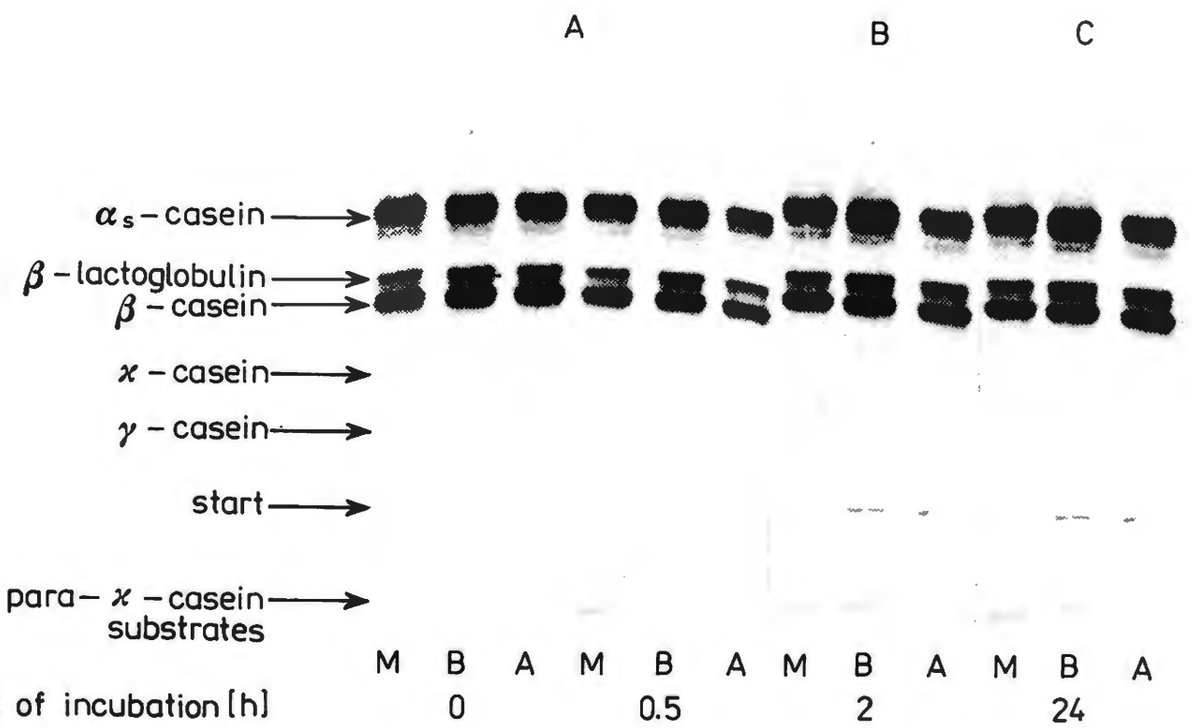

Fig. 5. Starch-gel electrophoresis of milk proteins incubated with rennin at pH 6.6. See Fig. 3 for explanations

natural milk. Even slight changes of internal structure, unavoidable in preparing the components of reconstituted milk, could have facilitated the enzyme's access to casein forms other than κ-casein. The densitometric analysis of electrophoretically separated casein fractions confirmed the obtained figures for total peptides released (Tables 1 and 2).

The amount of glycopeptides released from casein at pH 5.5 was slightly higher than at pH 6.6; in all the studied substrates, regardless of the pH, this amount was practically constant in time (Figs. 3 and 4). It turns out that the separation of peptides together with carbohydrate radicals takes place only in the

Table 1. Content of milk proteins in substrates incubated with rennin at pH 6.6; M — skim milk, B — skim milk fortified with 0.4% whey proteins; A — skim milk fortified with 0.4% alcohol precipitated whey proteins concentrate

Incubation time h	Substrates	Caseins				Para-casein %	β-Lacto-globulin %
		-γ %*)	-κ %	-β %	-α_s %		
0	M	3.9	17.1	30.8	48.2	0	10.1
	B	2.1	16.8	31.4	49.7	0	20.4
	A	3.0	17.7	33.0	50.3	0	17.2
0.5	M	1.4	6.8	28.6	51.2	8.1	11.7
	B	2.8	7.9	32.1	47.8	6.9	21.2
	A	2.9	10.1	30.6	49.2	4.9	16.9
12	M	3.1	7.3	28.6	50.8	9.8	10.6
	B	2.7	7.0	31.2	48.9	10.2	19.8
	A	3.3	9.8	29.7	51.2	6.5	16.4
24	M	3.2	7.4	31.4	50.3	11.3	9.8
	B	2.9	7.6	30.7	51.1	10.8	18.7
	A	3.6	9.9	29.8	49.8	6.1	15.9

*) Percentage content of caseins forms, para-κ-casein and β-lactoglobulin was expressed according to total casein in milk

Table 2. Content of milk proteins in substrates incubated with rennin at pH 5.5; M — skim milk, B — skim milk fortified with 0.4% whey proteins; A — skim milk fortified with 0.4% alcohol prevcipitated whey proteins concentrate

Incubation time h	Substrates	Caseins				Para-casein %	β-Lacto-globulin %
		-γ %*)	-κ %	-β %	-α_s %		
0	M	2.3	16.5	29.6	51.8	0	9.0
	B	2.7	16.6	27.7	52.2	0	21.6
	A	2.4	17.6	31.2	48.8	0	14.9
0.5	M	1.9	6.7	27.6	50.1	9.4	11.1
	B	2.2	9.3	30.6	48.6	7.1	19.9
	A	2.7	10.8	29.4	51.3	6.2	16.3
12	M	2.3	6.9	30.4	49.7	10.5	10.8
	B	2.1	7.2	29.7	51.3	9.8	20.4
	A	2.6	9.2	31.3	48.9	6.4	16.5
24	M	2.7	7.0	29.6	49.6	11.0	10.7
	B	2.3	6.8	30.2	51.0	10.6	19.3
	A	2.0	8.9	29.5	48.6	6.7	15.8

*) Percentage content of caseins forms, para-κ-casein and α-lactoglobulin was expressed according to total casein in milk.

enzymatic phase of milk coagulation, and that aggregates of alcohol-denatured whey proteins do not inhibit glycopeptides separation. Hence the conclusion that the whey proteins modified by alcohol from complexes mainly with the carbohydrate-free κ-casein. This would suggest that the interactions in this complex are different than those between thermally aggregated β-lactoglobulin and κ-casein, since the heat-denatured whey proteins combine with κ-casein containing carbohydrates and inhibit the release of glycopeptides by coagulating enzymes in the enzymatic phase of coagulation [7, 9, 10, 26]. On the other hand, if the interactions between alcohol-denatured whey proteins are of hydrophobic nature, it seems logical that such aggregates have more affinity to κ-casein without carbohydrates, since they lack the strong hydrophylic region that is found in κ-casein containing sugars.

Another aspect of the problem in hand is the distribution of κ-casein with and without carbohydrates in the casein micelle. The casein micelle structure is currently believed to be submicellar [21]. The views about the position of κ-casein in the micelle are widely divergent. The majority of recently obtained results indicate that κ-casein fractions occur both inside and on the surface of micelles [4, 10, 11, 14, 17]. According to Carroll and Farrell [2], in large micelles κ-casein is found mainly in the outher regions, whereas in the smaller micelles it occurs in their entire volume. Assuming that the differences in hydrophobic character between macro-peptides and glycomacropeptides radical have no great effect on interactions with alcohol-denaturated whey proteins, and that the interactions are mainly with the hydrophobic part of κ-paracasein, it may be suggested, basing on the results obtained in this study that κ-casein with carbohydrates is found inside micelles, while that without sugars concentrates on the micelles' surface. It is known that the casein micelle has a porous structure and that enzymes may penetrate it. Whey protein aggregates, on the other hand, are so large that, most probably, they cannot pass through to the micelle's interior. Such a model of distribution of κ-casein with and without sugars would provide a rational explanation of phenomena taking place during casein proteolysis by rennin enzymes in milk fortified with concentrates of alcohol-precipitated whey proteins. This suggestion contradicts results obtained by Damicz and Dziuba [5, 6, 10, 14]. However, Creamer [3] proposes a similar pattern of distribution of κ-casein with and without sugars in the casein micelle. It seems that the results obtained so far do not suffice to resolve the problem univocally. The issue thus remains open.

The complexes formed by heat-denatured β-lactoglobulin and κ-casein are more stable, and cause more severe inhibition of the action of rennin [5-7, 9, 10, 20, 26, 28] than interactions between aggregates of alcohol-precipitated whey proteins and κ-casein. The extent of these effects is also different in both cases. The heat-denatur ed whey proteins reduce the rate of glycopeptides release [7] while alcohol-aggregated proteins do not affect the rate of proteolysis of κ-casein containing carbohydrates, and, consequently, so not limit the access of rennin to the κ-casein fractions with sugars. Dziuba [10] demonstrated earlier that the

complex of heat-aggregated β-lactoglobulin and κ-casein is formed mainly as a result of hydrophobic interactions. Studies of alcohol-induced denaturation changes in whey proteins [12] also admit the possibility that the bonding between these proteins and casein is of hydrophobic nature. In denaturing proteins, alcohol exposes residues of nonpolar amino acids [12]. Despite being generally described as hydrophobic, the interactions between aggregated whey proteins and κ-casein must in both cases differ slightly as to character and stability, mainly because of the different character of proteins aggregation. Thermal aggregations are more stable and immune to the medium thanks to their disulphidic bonds [29]. Alcohol-induced aggregation involves mainly acid amino acid residues. A significant role ought to be played here by hydrogen and ion bonds, weak and sensitive to changes in the medium. This is indeed confirmed by the fact that alcohol-precipitated whey proteins are less inhibitory to rennin at pH 5.5 (Fig. 4). It is possible that they act on micellar casein by hindering but not limiting the access of rennin to casein.

CONCLUSION

1. The fortification of milk with alcohol-aggregated whey proteins leads to their interaction with casein through exposed hydrophobic regions.
2. The interaction between alcohol-denatured whey proteins and κ-casein reduces by about 40% the rate of casein proteolysis by rennin in the enzymatic phase of milk coagulation. In the secondary phase of proteolysis, the inhibitory effect is partly reduced.
3. The alcohol-precipitated whey proteins interact mainly with κ-casein lacking carbohydrate residues.
4. The proposed mechanism of the effect of alcohol-denatured whey proteins on micellar casein indicates the possibility of removing the inhibition of proteolysis, and suggests that κ-casein with and without carbohydrates occupies different positions within the casein micelle.

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Manuscript received: August 1987
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MECHANIZM PROTEOLIZY I KOAGULACJI MLEKA WZBOGACONEGO KONCENTRATEM BIAŁEK SERWATKOWYCH WYTRĄCONYCH ALKOHOLEM. I. PROTEOLIZA

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S t r e s z c z e n i e

Przeprowadzono badania proteolizy przez podpuszczkę mleka wzbogaconego koncentratem białek serwatkowych wytrąconych alkoholem. Stwierdzono, że białka serwatkowe zdenaturowane alkoholem wchodzą w interakcje z kazeiną przez wyeksponowane regiony hydrofobowe. W wyniku tych oddziaływań zmniejszyła się ok. 40% szybkość proteolizy kazeiny przez podpuszczkę w enzymatycznej fazie ścinania mleka. We wtórnej fazie proteolizy efekt hamowania został częściowo zniesiony. Jedyną formą kazeiny, która w tych warunkach uległa proteolizie była kazeina κ. Koncentrat białek serwatkowych wytrąconych alkoholem wchodził w interakcję głównie z kazeiną κ pozbawioną reszt węglowodanowych i nie powodował hamowania uwalniania glikopeptydów. Mogłoby to sugerować, że kazeina κ bez węglowodanów znajduje się na powierzchni, a glikokazeina κ w całej objętości micel kazeinowych, choć sugestia ta nie jest zgodna z niektórymi badaniami innych autorów.

Właściwości koncentratów białek serwtkowych wytrąconych alkoholem oraz jego zachowanie się w układzie białek koagulującego enzymatycznie mleka, pozwalają postulować możliwość wzbogacenia mleka serowarskiego tym preparatem białkowym.