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THE MECHANISMS OF FLOCCULATION OF FLOCCULENT YEAST DURING THE WORT FERMENTATION

PART I
THE RELATIONSHIP BETWEEN THE BEHAVIOUR OF THE FLOCCULENT YEAST CELL AND ITS CONSTITUENT PROTEINS

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ABSTRACT

It was found that the protein of the flocculent yeast cell was mainly composed of "Zymocasein" with the isoelectric point of pH 4.5, which precipitated when the pH was adjusted to 4.5 with acetic acid, and that "Zymocasein" was a kind of nucleoprotein, and that the yeast cell behaved as an insoluble protein particle of the same property as that of the protein constituting cell. When this property of yeast cell was introduced, it became possible explanation of the phenomenon of yeast flocculation. This phenomenon has presented long baffling problems to investigators in the field of colloid chemistry: for example, the flocculent yeast cells have a maximum flocculating activity in a buffer solution of pH 4.6, in spite of its electrically negative charge. On the contrary, the cells fail to form a flocculence in a buffer solution of pH between 2 and 3 at which the cells become electrically neutral. On the other hand, a phenomenon, of failure of the flocculent yeast cells starved of phosphate to form the flocculence, in spite of electric neutrality on them, found by Eddy et al., could be explained as the cell stabilization attributable to the diminution of "Zymocasein" content. As the sequel, it was discussed that the fundamental substance governing flocculation of yeast cells must be the "Zymocasein" fraction.

Introduction

The problem of yeast flocculation in wort fermentation is the most important matter in the beer-brewing through the bottom fermentation system. Therefore as known in the reviews[1,2,3], the problem of yeast flocculation was studied by many investigators, and many views were proposed. Specially, at the Third Congress of the European Brewery Convention held in Brighton, 1951, this problem was taken up as a theme of pressing question, and studied from various sides by many investigators of various countries. However, no theory was proposed for the complete and systematic explanation of various phenomena of yeast flocculation. The solution of the problem of the mechanism of yeast flocculation was kept attempted thereafter chiefly in a study of the yeast cell wall, by Eddy et al.[4,5], by Masschelein et al.[6,7], and lately was studied by Lycette et al.[8], Mill[9], Brock[10,11] and Kostolanska et al.[12],

too, but concerning the mechanism of it remains as much a riddle as ever.

About ten years ago, the author had studied on the problem of yeast flocculation in wort fermentation, and in the studies, established two hypotheses to explain the problem colloid-chemically. One of the hypotheses was that the flocculent yeast cells behaved like the giant particles of protein with the same property as "Zymocasein" and "Cerevesin" which are the main components of cells, and the other was that the negative charges on yeast cell were attributed to a series of substances related with nucleic acid (which was special component of the cell, and was mainly composed of phosphate) that were present in the cells, but not proteins. As the result, when both hypotheses were introduced into the problem of yeast flocculation, the fact that yeast cells could not form the flocculence at its isoelectric point of between pH 2 and 3, but did flocculate at the far higher pH of 4.6 (though it had the negative charge), may possibly be explained in the electrically charging theories[13,14] on yeast cells. On the other hand, the isoelectric points of wort proteins are supposed to be above pH 6.0 according to St. Johnston's results[15], so that the phenomenon of yeast flocculation during the wort fermentation was explained colloid-chemically as an electric neutralization between the negatively charged yeast cell and the possitively charged wort proteins. In addition, the amounts of the substances related to nucleic acid in yeast cell dynamically change through various internal and external factors intervening during the fermentation process. This is the explanation of the various accelerating and inhibiting factors of yeast flocculation (described in the reports), and of the various phenomena of yeast flocculation brought about during the wort fermentation as pointed out.

The present studies concern to the two hypotheses mentioned above; and the mechanism of adsorption between the yeast cell and wort proteins which were experimentally demonstrated; and the mechanisms of both the yeast flocculation which was observed in fermenting wort (called first "Bruch"), and the essential yeast flocculation (called second "Bruch") which was observed when the yeast fermenting wort was washed with acetate buffer solution of pH 4.6 and suspended in the same buffer solution.

The results of the above experiments indicate that two hypotheses were confirmed to be appropriate; and that the mechanism of the adsorption between the yeast cell and the wort protein was found to be an effect in which wort protein adsorbed on the surface of the yeast cell by simple mutual reaction, which did not affect on the negative charge density on yeast cell without producing electrical neutralization, through the form of tannin-protein complex, accelerated yeast flocculation. Thus, the previous question of wort proteins that stimulated the yeast flocculation was solved. Furthermore, the mechanism of the yeast flocculation during wort fermentation could be reasonably solved colloid-chemically. The present report concerns the first hypothesis, namely, the phenomenon that the yeast cell behaves as an insoluble particle of protein with the same property as the component protein of cells.

In the previous investigation[16], it was recognized that the flocculent yeast cell suspended in the fermenting wort revealed the highest flocculation power at the fixed pH value, namely, at pH 4.6, regardless of resting state or fermenting state. This condition was also found in the buffer solution by many investigators leaving little room for doubt. And this fact was the greatest cause which conducted many investigators who attempted to solve the phenomenon of yeast flocculation colloid-

chemically, to the conclusion that yeast flocculation could not be solved colloid-chemically. However, the author found that the isoelectric points of both yeast proteins ("Zymocasein" and "Cerevesin") were about pH 4.5[17], and that the yeast cells must behave as the particles of protein with the same property as the main constituent protein of cells, through some unknown mechanism. If this consideration is legitimate, pH 4.6 is the isoelectric point for the proteins, and consequently it is natural that the flocculation power of yeast reaches to a maximum, based on its characteristics at the isoelectric point. By assuming this, the phenomenon of yeast flocculation could be elucidated colloid-chemically as described previously. In the following, the experiments and demonstration of the hypotheses are performed.

Experimental Procedure and Results

I *Condition of Tannic-Acid Adsorption on the Whole Yeast Cell and on Its Cell Wall*

The existence of protein reactivity on whole cells and on cell walls was investigated by applying a special reactivity of tannic acid to protein.

(*Experiment*) Brewery yeast cells were broken down by repeatedly applying vibrations with a sonic disintegrator; their cell walls were isolated; after that the raw cell walls were washed with 0.2 % NaOH to remove the intracellular or wort protein adhering on them, and subsequently with water. Thus the yeast cell wall was prepared. Investigation was made on this yeast cell wall preparation and the control of the alkaline-treated whole cell concerning the ability to adsorb the commercial tannic acid. These investigations were also carried out on the yeast cells treated in various ways. The results are shown in Table 1.

Table 1. Condition of Whole Yeast Cells and of Yeast Cell Walls to Adsorb Tannic Acid

Samples	Condition of alkaline treatment	Amount of adsorbed tannic acid	Number of living cell
Cell walls	0.2% NaOH, washed 5 times	7.3 mg/g of dry yeast	
Whole cells (control)	〃 , washed 5 times	71.6 mg/g 〃	0 %
〃	〃 , washed 3 times	71.7 mg/g 〃	0.1%
〃	〃 , washed 1 time	52.6 mg/g 〃	About 50%
〃	0.1% Na_2CO_3, washed 4 times	17.3 mg/g 〃	About 100%

Test yeast: Brewery flocculent yeast. Alkaline washing: Treated at 6°, for 20 min. Treatment of tannic acid: 1 g of the centrifuged samples were suspended in 100 m*l* of solution of 200 p. p. m. of tannic acid, allowed to stand overnight, at 10°. Determination of tannic acid was carried out by Stone and Gray's method[18]. Number of living cell was measured by plating method.

Table 1 showed that the whole cell adsorbed about ten times of tannic acid of the cell wall, and in this case any breakage of the cell wall was not detected on the microscopic examination. Therefore it is clear that tannic acid reacted on the intracellular protein through the cell wall. However, concerning the problem whether tannic acid reacted upon protein at cell surface, an advanced theory[19,20] says that the cell wall is made into a pore structure. In fact, it is recognized to be possible that even the relatively higher molecular substances can pass through the yeast

cell wall[3]. But, on other side, in case the control yeast cells were dead, it is possible that the yeast cell had lost the function of controlling membrane transport, and consequently, the conjecture that the tannic acid reacted upon intracellular protein after passing through the cell wall leaves ample room for doubt.

II *Abilities of the Yeast Cells Cultured in the Synthetic Media and of the Cleaned Brewery Yeast Cells to Adsorb the Cellophane Non-Dialyzable Tannic Acid*

In order to solve the interrogatory point described in the preceding section concerning the activity to adsorb tannic acid by yeast cells, an investigation was conducted using the cellophane non-dialyzable tannic acid to examine the tannic acid-adsorbing abilities of both the living yeast cells without the cleaning treatment, cultured in the synthetic media and the brewery yeast cells cleaned by treatment with a dilute alkaline solution for short durations, to prevent death of the cells as much as possible. Table 2 showed the results of the investigation.

Table 2. The Activities of the Yeasts Cultured in the Synthetic Media and of the Cleaned Brewery Yeasts to Adsorb the Cellophane Non-Dialyzable Tannic Acid

Samples	Procedure cleaned yeast cell	Amount of adsorbed tannic acid
Brewery yeast	With 0.1 % Na_2CO_3, washed 4 times	12.2 mg/g of dry yeast
〃	With 0.1 % NaOH, washed 2 times *	28.9 mg/g 〃
〃	With 0.1 % NaOH, washed 4 times**	29.8 mg/g 〃
Yeast grwon in nitrogen source of $(NH_4)_2SO_4$		19.8 mg/g 〃
Yeast grown in nitrogen source of polypeptone		17.7 mg/g 〃

The flocculent yeast was used for the test. The yeasts cultured in synthetic media were propagated respectively in Wickerham's medium[21] supplied with vitamins and nitrogen sources mentioned in Table 2, at between 6° and 8°. *: One treating time was 10 min., including the centrifuging time. **: All procedures were perfected within 30 min. Each alkaline washing was carried out at 6°. The other conditions were the same as that of Table 1.

As found in Table 2, the yeast cells adsorb a tolerable amount of tannic acid in the living state. Cellophane non-dialyzable tannic acid was used in this experiment so that the adsorption of tannic acid can be accepted as acting on the surface of the yeast cell walls.

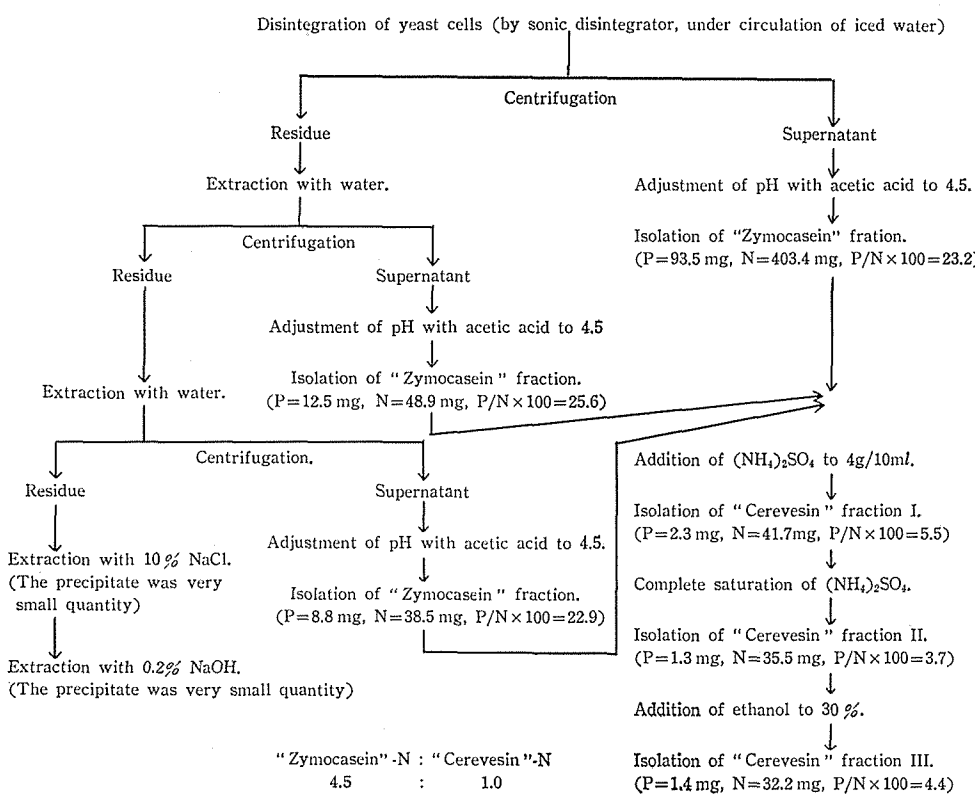
Tannic acid is a substance reacting peculiarly on protein. Considering this view, the yeast cells may be admitted as a kind of particle of protein. The phenomenon of adsorption of tannic acid by yeast cells was found by Chevalier et al.[22] who said that the yeast cell behaved as an insoluble particle dispersed of protein. Besides, isohumulone which revealed the same adsorption ability to nylon as tannic acid was adsorbed[23] by yeast cells: and a phenomenon that the yeast cell, as well as the general proteins, adsorbs copper ion and other metal ions prove this view.

III *Characteristics of Yeast Proteins*

From the experimental results described in the preceding two sections, it was

confirmed that the yeast cell behaved like a protein. In recent years, nevertheless, it was found[24,25,26] that the yeast cell wall, isolated under the mild condition, contains protein in addition to polysaccharides. This finding supports the view that the protein-like behaviour of the yeast cell is carried out by the protein constituting cell wall without a connection with the intracellular proteins. In order to prove this view point the properties of intracellular proteins were investigated. Yeast proteins are recognized[17,27] to be mainly composed of "Zymocasein" which precipitates when the pH of the solution was adjusted to 4.5 with acetic acid and of "Cerevesin" which is yeast albumin that coagulates by being heated, or salts out by being saturated with ammonium sulfate. Both of these are, however, isolated from the dead cells which are dried or plasmolyzed. Their denaturation is doubted. Therefore the possibility of applying these results directly to living cells is doubtful. So, by the procedure shown in Figure 1, various kinds of protein fractions were

Fig. 1. Isolation and Composition of Yeast Proteins



Determinations of nitrogen and phosphorus were performed by microkjeldahl method and Allen's method respectively.

isolated thereby preventing the denaturation as much as possible. As shown in Figure 1, the main constituent of yeast protein is "Zymocasein"; "Cerevesin" is next to that; globulin and glutelin are in very small quantity. "Zymocasein" is very sensitive to pH as well as milkcasein, and its content is in overpowering amounts. Furthermore, of "Cerevesin" fraction 1, the isoelectric point was studied

by means of the Tiselius-type electrophoretic apparatus, and consequently, the isoelectric point of it was recognized to be pH 4.5 as in the literature. Accordingly, it is known that the proteins of flocculent yeast reach an extremely unstable state at pH 4.6. These properties of yeast proteins and the fact that the flocculent yeast cell reveals the maximum flocculation power at pH 4.6 indicate that the flocculent yeast cell behaves as a particle of protein which has the same property as the intracellular protein. This matter is also supported by the following fact, namely, when "Zymocasein" fraction was stored just as it was centrifugally separated, it could no longer form the flocculence by simple adjustment of pH. This is understood as owing to the decomposition of "Zymocasein" fraction into low molecular components by the enzyme adhering or associating with it. Also, the fact is in accord with the phenomenon[16,28] that when the yeast cell was stored it failed to flocculate. The phosphorus content of the "Zymocasein" fraction is not only higher than that[17] of milkcasein, but also than that[29] of "Zymocasein" found in the literatures. Therefore, it is considered to contain nucleic acids, and so, the phosphorus composition of the refined "Zymocasein" fraction was inves-

Table 3. Composition of "Zymocasein" Fraction

Fractions	Phosphorus	Nitrogen
Water-soluble	5.1 mg	15.9 mg
Ethanol-soluble	0.6 mg	1.0 mg
1 N-PCA-soluble	59.8 mg	117.3 mg*
Nucleic-acid form	(56.3 mg)	—
1 N-PCA-insoluble	0.8 mg	62.5 mg
Total	66.3 mg	196.7 mg
Raw protein (N×6.25)		1.229 g
Oily substance in ethanol-soluble compounds		0.381 g
Oily substance/raw protein+oily substance×100		24

Nitrogen, by microkjeldahl method, phosphorus, by Allen's method, phosphorus forming nucleic acid, by ultraviolet absorption method were determined respectively. *: It was made by subducing ethanol-soluble nitrogen and 1 N-PCA-insoluble nitrogen from total nitrogen.

tigated. The result shown in Table 3, of phosphorus of "Zymocasein" fraction 85% is nucleic acid form. "Zmyocasein" fraction was found to be a kind of nucleoprotein and is also considered to contain lipoprotein[30] as it contained oily substances in large amounts. Connected with this matter, Čmelik[31] isolated a nucleoprotein fraction from *Salmonela ballerup* through a method resembling this procedure, and he has recognized that the nucleoprotein fraction has the greatest agglutinogenic effect, but this effect is slightly less than that of intact bacteria, and when the nucleoprotein fraction splits into nucleic acid and protein, the effect falls to a low level.

Discussion

Concerning the connection between "Zymocasein" fraction and the flocculating ability of yeast, Eddy[32] has found that when the yeast cell was grown in a syn-

thetic medium deficient in phosphate ions, it lost at once both its surface charge and its flocculating ability. Eddy et al.[5] have also found that the decrease of electric mobility on the yeast cell starved of phosphorus was attributed to the diminution in the phosphorus content of its cell wall, and they have indicated[32] that the ability of the yeast cell to flocculate could not be elucidated based on the magnitude of the charge density of the yeast cell. The author considered the solution to this problem to be due to the decrease of the "Zymocasein" content of the yeast cell. In this occasion, it is clear that the grown yeast cell is diminished in phosphorus content not only in the cell wall, but also in the cell itself (as indicated from the results obtained by Wiame[33], Fujitani[34] and Nordheim[35]), and yet, Wiame has found that the content of phosphorus, forming nucleic acid in the cell, decreased in parallel with the total phosphorus content of the yeast cell. Consequently, the containing ratio of "Zymocasein" fraction in the cell falls to a low level. This fall of the containing ratio of "Zymocasein" fraction in the cell contributes to the stabilized suspended state of the whole of the yeast proteins. The suspension of the yeast cell is changed naturally into a stability. Accordingly, in this case, it is explained that the charge on the yeast cell does not much influence the flocculation. Rudin[36] has found that when the phosphorus deficient yeast cells held overnight at 18°C, in a medium containing 5 % of glucose and 1.5 % of KH_2PO_4, both the charges on the cells and their electric mobility were restored almost to normal value. In connection with this matter, Wiame[33] has found that when the phosphorus deficient yeast cell was incubated in a glucose solution containing KH_2PO_4, the content of cold acid-soluble metaphosphate and of hot acid-solbule metaphosphate in the cell increase considerably assimilating phosphate in a short time, and furthermore, when the yeast cell was incubated in a medium without phosphate, at the beginning of growth, the content of hot acid-soluble metaphosphate in it decreased considerably and in proportion to the decrease, the nucleic acid content increased. On the other hand, "Zymocasein" swells gradually as the pH moves from 4.0 to lower value. At about pH 2.0, its swelling grade reachs its maximum, and in descending from this point, it begins to dissolve[27]. The properties of "Zymocasein" agree[37] with the phenomenon that the flocculent yeast cell does not form the flocculence between pH 2 and 3, for the rest, it is a established fact that the flocculent yeast is finely flocculated by Ca^{++} ion. This property also accords with the property of nucleoprotein. These matters considered together with the problem that when the flocculent yeast cell or "Zymocasein" fraction was stored its flocculating activity declined (which was mentioned in the preceding section) indicate that the flocculent yeast cells have a function in which they reflect the property of the intracellular proteins on the cell surface through some unknown mechanism. So, it may be assumed that the substance governing the flocculation of the flocculent yeast cell is "Zymocasein" fraction, and in addition, that the activity forming this flocculence is controlled by the content and physical condition of "Zymocasein" fraction.

In recent years, Lycette et al.[8] have discussed the relationship between the flocculating ability of yeast cells and lipids, and have found that when bound lipid or phospholipid was removed from the flocculent yeast cell by treating it with various solvents, its flocculating ability was lost. Mill[9] has also considered an hypothesis that yeast flocculation is attributed to the linkage by the salt bridge of Ca^{++} joined with 2 carboxyl groups on the surface of different cells since the

experimental results showed that the yeast flocculation was promoted by Ca^{++}, and that the yeast flocculation was specially lost by treatment with urea and formamide. It seems very likely that the former is related to the destruction of membrane structure by the removal of the bound lipid and phospholipid and to the change of present state of nucleoprotein brought in its strain. The latter seems to be due to the action of Ca^{++} to precipitate nucleoprotein and the action of urea and formamide to denature nucleoprotein. Thus, both hypotheses support the author's opinion that "Zymocasein" fraction is the fundamental substance on the yeast flocculation.

Summary

1) By investigating the reactivity between tannic acid and protein in yeast cells an experiment was conducted to determine whether the yeast cells have the ability to behave as proteins. In consequence, it was found that the yeast cell behaves like a dispersed insoluble protein.

2) Yeast cells were broken down by the sonic disintegrator (thereby preventing the denaturation as much as possible) and the property of intracellular protein was investigated. As the result, "Zymocasein" fraction was precipitated by an adjustment of the pH to 4.5 with acetic acid as the essential component. The "Zymocasein" amount was about five times the quantity of "Cerevesin"; and very small quantity of globulin and glutelin were found. It was understood by this that the yeast protein reacted very sensitively to pH and was very unstable.

3) About 85 % of phosphorus of "Zymocasein" fraction was nucleic-acid form, and was found to be a kind of nucleoprotein.

4) The behaviours of the flocculent yeast cell to pH and to Ca^{++} are in accord with the properties of "Zymocasein" to those. Therefore, it was recognized that the flocculent yeast cell reflected the property of intracellular protein on its surface through some unknown mechanism.

5) It was concluded that the fundamental substance governing the flocculating ability of the flocculent yeast is "Zymocasein" fraction and the activity to form flocculence is controlled by the content and physical condition of "Zymocasein" fraction.

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