PROTEIN ISOLATES FROM CHLORELLA ALGAE, TORULA YEASTS, AND HYDROCARBON-ASSIMILATING MICROORGANISMS

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Preparation of protein isolates from the cells of Chlorella, Torula yeasts, and hydrocarbon-assimilating microorganisms is described. Simple pretreatment of the cells with alkali, acid, or some organic solvents enhanced the protein extraction efficiency and made the following purification procedures easier. Bleached cells of Chlorella obtained by growing the algae in a culture medium with high C/N ratio at high temperature were found to release protein more easily than do the normal cells. Structural changes in the cell wall region detected under the electron microscope may be responsible for this. The extracted protein was further purified. Amino acid composition of the protein isolates was determined, and their nutritional values were calculated. Among the essential amino acids the sulfur-containing amino acids were found to be the first limiting amino acid. Supplementing the isolate with methionine resulted in a significant increase in its nutritional value (PER) which became comparable to those of egg albumin and milk casein. The digestibility of the protein isolate from the cells of a hydrocarbon-assimilating yeast, tested in vitro with pepsin, was as high as 80% of that of the reference protein, milk casein, whereas that of the dried cells of the yeast was less than 50%. Viscosity was measured in regard to possible processed forms of the protein isolates. A few methods for disposing of the extraction residues were tested.

The author has published several articles on obtaining protein isolates from microbial cells such as Chlorella algae(1), Torula yeasts(1), a glutamic acid producing bacterium, Corynebacterium glutamicus(1), and hydrocarbon-assimilating microorganisms(2).

The cultivation of microbial cells for food is especially suitable for Japan, where a population of over a hundred million has to live in a small area and where only about 16% of the total area is farm land and only 3% is meadow:

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1 満田久輝
Microbial cells can reproduce much faster than conventional food sources, such as animal and plant; they have high protein content; they can be produced without requiring any of the limited farm land available; and their production can be controlled easily, independent of climate.

There are, however, some problems to be solved before microbial protein can be used as food. In order to enhance nutritive value, the products from microbial cells should be free from indigestibles such as cell walls; free from acute, subacute, and chronic toxicity; free from teratogenic and carcinogenic factors, which may be produced or accumulated in some microorganisms; and of high acceptability, that is, without strong color and unpleasant flavor. The author believes that separation of protein from the other cell materials is essential for meeting these conditions. In addition, it is hoped that the isolated proteins will have better properties for actual food processing.

The generic name MIPRON (from “microbial isolated proteins”) for the protein isolated from microbial cells has recently been proposed(3). This name emphasizes that any microorganism could theoretically be used as a protein food source if the above-mentioned conditions are satisfied and that the proteins must be isolated from other cell materials.

The author has reported on isolation of proteins from microbial cells by freeze-thawing, toluene autolysis, autolysis-butanol(4), urea soaking(1), and alkali extraction(2). These methods can be used independently or in combination with each other or with presoaking the cells in an acid or alkali solution(1). The best method for isolation, however, varies with the type of microorganism, as shown in Table 1.

| Method                  | Microbial   | Protein recovery |
|-------------------------|-------------|------------------|
| Freeze-thawing          | *Chlorella* | 11.3 (%)         |
| Toluene autolysis       | *Chlorella* | 17.5             |
| Autolysis-butanol       | *Chlorella* | 23.6             |
| Urea soaking            | *Chlorella* | 18.4             |
|                         | *Torula*    | 28.2             |
| Alkali extraction       | *HC. A. Y.* | 31.8             |

* Hydrocarbon-assimilating yeast

This paper describes the practical preparation of protein isolates and the development of effective pretreatment, some chemical and physico-chemical properties of the isolates, nutritional evaluation of the isolates, and some trials for disposition of the extraction residues.

**Pretreatment with an acid or alkali**

Treating the cells of *Chlorella* and *Torula* with an acid or an alkali prior to urea soaking resulted in increased protein release into the extract. Presoaking
dried cells of hydrocarbon-assimilating yeast with a concentrated alkali prior to alkali extraction gave more protein isolates than that by simple extraction. After pretreatment, lower concentrations of urea or alkali produced the same extraction efficiency as without pretreatment. The effect of acid pretreatment has been reported for urea extraction of protein from dried cells of Corynebacterium glutamicus(1).

For the combined urea soaking method, the cells were suspended in an alkali or acid solution and heated in boiling water for several minutes prior to the standard extraction procedure. The protein recovery from Chlorella and Torula cells was 18 and 28%, respectively, by urea soaking alone, but after pretreatment with 1 N NaOH at 100°C for 5 and 3 min it was about 50% for both, as shown in Table 2.

Table 2. Effect of alkali pretreatment on protein recovery.

| Method           | Chlorella | Torula | HC. A. Y.* |
|------------------|-----------|--------|------------|
|                  | S        | C      | S          | C      | S        | C      |
| Urea soaking     | 18.4     | 48.9   | 28.2       | 53.5   | -        | -      |
| Alkali extraction| -        | -      | -          | -      | 31.8     | 60.8   |

* Hydrocarbon-assimilating yeast
S: Protein recovery by simple extraction
C: Protein recovery by the extraction method combined with alkali pretreatment

When the dried cells of hydrocarbon-assimilating yeast were presoaked in a volume of 8% NaOH solution 5 times the weight of the cells at 4°C for 2 hr, and then diluted to a final concentration of 2% NaOH and incubated for 2 hr at 35°C, the amount of protein extracted was almost twice as much as that obtained by simple extraction (2% NaOH at 35°C for 2 hr).

**Pretreatment with organic solvents**

Pretreatments with organic solvents were tested for their ability to increase the utility value of microbial cells and the accessibility of urea or alkali to the contents of the cells. Decolorization was performed before the urea soaking of Chlorella cells. A mixed solvent of methanol 4: hexane 3 gave the highest yield of pigments, and in turn highly decolorized cells, as shown in Table 3.

Pretreatment of the cells of hydrocarbon-assimilating yeast with an aqueous alcohol, either ethanol or propanol, at high temperature (70–80°C) could remove lipids, sugars, pigments, and almost all other nonprotein nitrogenous compounds of the cells. It is hoped that treatment with organic solvents would facilitate subsequent procedures and improve the quality of the protein isolates.

**Culturological pretreatment**

Some chemical and culturological treatments of the cells before their harvest
also give profitable results. *Chlorella* cells bleached by high temperatures or strong light intensity or by being grown in a medium with high carbon-to-nitrogen ratio are devoid of the original green color (5). By applying these conditions, we hoped to obtain colorless cells of *Chlorella* and thus eliminate the undesirable effects of pigments on the isolated protein. The bleached cells of *Chlorella ellipsoidea* were prepared under culture conditions where the C/N ratio in the medium was raised to 20:1 from 5:1 of normal conditions, and temperature was raised to 40°C at 48 hr after the inoculation. An additional carbon source (glucose in this case) was supplied to the culture 48, 72, 96, and 120 hr after the start of culture. The algae began to fade at around 50 hr, and the cells had lost all of their greenish tone at the end of culture, as shown in Fig. 1.

The bleached cells thus obtained contained much less nitrogenous substances than do normal cells, as shown in Table 4, but a given volume of culture could produce more cells under bleaching conditions than under normal conditions, as shown in Table 5. The yield of protein per volume of bleached-cell culture was the same as or often larger than normal, as was efficiency in utilization of nitrogen from the medium.

### Table 3. Extraction of Chlorella pigments using conventional solvents. (mg/100 g on dry basis)

| Solvent       | Chlorophyll | Carotene |
|---------------|-------------|----------|
| Acetone       | 473         | 8.65     |
| MeOH          | 614         | 12.41    |
| Acetone: MeOH |             |          |
| 1:1           | 562         | 10.15    |
| 2:1           | 502         | 9.45     |
| 1:1           | 663         | 14.62    |
| MeOH: Hexane  |             |          |
| 1:1           | 801         | 21.24    |
| 2:1           | 894         | 20.05    |
| 4:3           | 1250        | 29.28    |
| 5:3           | 910         | 23.18    |

### Table 4. Protein and carbohydrate contents of Chlorella ellipsoidea cells. (% on dry basis)

|          | Protein^a  | Carbohydrate^b |
|----------|------------|----------------|
| Normal   | 44.96      | 26.28          |
| Bleached | 11.24      | 76.85          |

^a Kjeldahl N × 6.25

^b Determined with anthrone method.
SHIHIRA-ISHIKAWA and HASE (6) reported that “glucose bleaching” of *Chlorella protothecoides* degenerated the lamella structure of chloroplasts, and certain changes in the cell membrane were also observed. The bleached cells of *C. ellipsoidea* in this experiment also exhibited a structural change in the cell wall region, as seen in the electron micrograph of Fig. 2. The outermost electron-translucent layer has disappeared from the bleached cells. This change may account for the ease of extracting protein from the lyophilized bleached cells. After treatment with phosphate buffer, the bleached cells that had been lyophilized were found to release about as much protein as that obtained by the urea soaking method.

![Fig. 1. Left to right: cells grown under normal conditions (carbon-to-nitrogen ratio 5:1 at 25°C); cells grown under high carbon-to-nitrogen ratio (20:1) at 25°C; and bleached cells grown under conditions as stated except that temperature was raised to 40°C 48 hr after the start of culture.](image)

**Table 5.** Cell yield, protein yield, and efficiency in nitrogen utilization of *C. ellipsoidea*.

|                  | Yield (g/liter) | Protein* (A) | Nitrogen content in medium (g/liter) (B) | Efficiencyb (%) |
|------------------|----------------|--------------|-----------------------------------------|-----------------|
|                  | Cells          |              |                                         |                 |
| Normal           | 1.26           | 0.566        | 0.173                                   | 52.6            |
| Bleached         | 5.88           | 0.661        | 0.173                                   | 61.1            |

* Kjeldahl N × 6.25
b (A) × 100/(B) × 6.25
These culture conditions are suitable for producing cells with properties favorable for the present purpose and thus may be one practical way to utilize *Chlorella* as food.

**Fractionation of isolated proteins**

A procedure for isolating protein from microorganism cells is shown in Scheme 1.

About half the extracted crude protein could be precipitated by adjusting pH of the extract to 4.0–4.2. The crude protein content of the lyophilized precipitate was 65–70%, and the preparation retained an odor specific to the dried cells of hydrocarbon-assimilating yeasts previously tested by the author. This protein content is not enough for the preparations to be called “protein isolate,” and the odor is unacceptable for a protein source for human food.

In order to overcome these shortcomings, the alkali extract was subjected to dialysis against water. After about 40 hr dialysis, white precipitates that could be removed by centrifugation were found inside the dialysis bags. The nitrogen content of the precipitate was always very low, and thus loss of protein into this fraction (Ppt-I in Scheme 1) was negligible. This fraction seems to be derived from the cell wall; the hydrolyzate of Ppt-I of yeast contained D-glucose and D-mannose as sugar components, determined by paper chromatography. Ppt-I
must be removed as completely as possible in order to obtain a protein isolate of higher purity at a later stage of isolation. In addition to the characteristic odor described above, some nonprotein compounds could be removed by dialysis.

Dried Cells

- soaked in NaOH soln.

- dialyzed vs. running water

- CaCl₂, 3.3%

- dialyzed vs. running water

- EtOH, 50%

- HCl to pH 4

Scheme 1. Protein isolation from microbial cells.

Table 6. Protein fractionation with ethanol of the extract from hydrocarbon-assimilating yeast.

| EtOH      | Protein recoverya | Protein content |
|-----------|-------------------|-----------------|
| 0-15%     | 0.8%              | 75.0%           |
| 15-20     | 1.8               | 77.0            |
| 20-30     | 19.8              | 73.4            |
| 30-35     | 1.3               | 58.0            |
| 35-50     | 1.2               | 37.0            |
| HCl (to pH 4) to the supernatant fraction of EtOH (50%) | 3.9 | 64.6 |

a Extracted with a volume of 2% NaOH 10 times the weight of the dried cells for 10 hr at 37°C, then dialyzed vs. running tap water for 24 hr.

b Based on the crude protein content of the dried cells.

Alkali extraction would be the most cumbersome step in the whole procedure of isolating proteins, especially for a large-scale preparation, because the high alkalinity of the extract, bulky residues, and strong foaminess of the solution make it impractical to use an ordinary continuous flow type centrifuge. The addition of ethanol to the extraction mixture made it possible to separate the residues by filtration; ethanol concentration up to 50% under these conditions resulted in very little loss of proteins. Ethanol was used successfully by the author in large-scale
preparation of protein isolates for an animal feeding test. Fractional precipitation of protein with ethanol from the dialyzed extract of hydrocarbon-assimilating yeast was tested, and the results are shown in Table 6.

The effects of adding divalent cations to the dialyzed extract of hydrocarbon-assimilating yeast were investigated with the expectation that a cation might precipitate some polysaccharides as hemicellulose (Table 7). Although the amount of insolubles directly produced by the addition of divalent cations was very small, a fairly large amount of precipitation, Ppt-I (Me, D–H2O), occurred on the second dialysis, performed to eliminate excess cations, except with Mg++ which caused no precipitation in the second dialysis. The precipitates are of higher protein content, and more proteins could be obtained by the addition of ethanol or hydrochloric acid to the supernatant solution. Table 7 summarizes the results; among the divalent cations tested, Ca++ was the most suitable for treating food materials.

### Table 7. Protein fractionation of the extracta from hydrocarbon-assimilating yeast with divalent cationsb and dialysis.

| Fraction                              | Protein recoverya | Protein content |
|---------------------------------------|-------------------|-----------------|
| Ppt(Ca, D–H2O)                        | 10.9%             | 87.9%           |
| Ppt(Ca, D–H2O, EtOH–50%)              | 18.1              | 64.5            |
| Ppt(Ba, D–H2O)                        | 7.2               | 72.9            |
| Ppt(Ba, D–H2O, EtOH–50%)              | 22.9              | 66.1            |
| Ppt(Mg, D–H2O)                        | 0.0               | —               |
| Ppt(Mg, D–H2O, EtOH–50%)              | 28.9              | 68.8            |
| Ppt(Cu, D–H2O)                        | 0.5               | 12.2            |
| Ppt(Cu, D–H2O, EtOH–50%)              | 25.3              | 63.9            |

a See Table 6 for the conditions.
b CaCl2, BaCl2, or MgCl2 was added to 3.3%. Fehling solution was used for Cu++ (13.5 ml per 100 ml of the extract).
c Based on the crude protein content of the dried cells.

![Flow diagram of protein isolate preparation](image)

Alkali extraction  Centrifugation  Pump  Gel filtration  Spray drying  Precipitation

Fig. 3. The flow diagram of protein isolate preparation from hydrocarbon-assimilating yeast.

a, westfalia; b, sharps.
Figure 3 shows a flow diagram of protein isolate preparation from hydrocarbon-assimilating yeast.

**Essential amino acid composition**

Essential amino acid patterns of the protein isolates were compared with those of the dried cells. Table 8 shows the ratio of content of each essential amino acid to total essential amino acids, *i.e.*, A/E ratio and compares these ratios to those of whole egg, the reference protein. It is clear from these values that the first limiting amino acid in the cells and protein isolates of hydrocarbon-assimilating yeast is the sulfur-containing amino acid, and the amounts of the rest of the amino acids are close to those of whole egg. The shortage of the sulfur-containing amino acids in the cells and protein isolates of *Chlorella* and *Torula* were comparable with these of hydrocarbon-assimilating yeast. But the protein isolates prepared from those microbial cells are especially abundant in lysine. Thus, if the sulfur-containing amino acids are properly supplemented, those microbial proteins would be of high quality as human food.

![Table 8](image)

| Amino acid | Cells A/E ratio | Isolate A/E ratio |
|------------|-----------------|-------------------|
| Ileu       | 121 mg/g        | (94)              |
| Leu        | 174 (101)       | 195 (113)         |
| Lys        | 176 (141)       | 139 (111)         |
| Aromatic   | 191 (98)        | 220 (113)         |
| S-containing | 64 (60)     | 50 (47)           |
| Thr        | 116 (117)       | 106 (107)         |
| Trp        | 17 (55)         | 30 (97)           |
| Val        | 142 (101)       | 139 (99)          |

*Figures in parentheses represent ratios, in %, of each A/E to the respective A/E of whole egg, the reference protein.*

Digestibility

One of the purposes of isolating protein from the microbial cells was increasing digestibility. Digestibility of the protein isolates was tested by *in vitro* procedures. Figure 4 shows that digestibility by pepsin of hydrocarbon-assimilating yeast is increased significantly by isolation of protein from the other cell materials, reaching the level of milk casein, the reference. Digestibility of the protein isolates from *Chlorella* and *Torula* by pepsin and trypsin was comparable to that of hydrocarbon-assimilating yeast by pepsin.
Animal feeding tests

Animal feeding tests were also carried out in order to assess the nutritional quality of the protein isolates. Table 9 shows that the protein efficiency ratio of the protein isolates from hydrocarbon-assimilating yeast supplemented with methionine compares favorably with the value for egg albumin and casein. A diet of protein isolates supplemented with methionine supports the growth of rats as the egg albumin diet does, whereas the protein isolates themselves are not sufficient. No anomalous symptom was detected in the appearance of animals during or after these feeding tests. Thus, no acute toxic factor was detected. Comparable results were obtained using the protein isolates from Torula yeasts(1).

Physicochemical properties of the isolates:
Viscosity and fiber formation

It is thought necessary to elucidate rheological properties of the protein isolates with every regard to possible processed forms of the microbial isolated proteins. As is well known, a fibrous form of protein, often called spun protein, has been used(8) to give texture to the protein of soybean, wheat, and casein.
These proteinous fibers are formed by extruding an alkali solution of the protein into an acid solution through a nozzle.

The protein isolate from hydrocarbon-assimilating yeast cells was tested for fiber formation. This fraction was difficult to extrude into fibrils under the same conditions in which milk casein, soybean protein isolate, and egg white could be made into fibrous form. However, the addition of a small amount of sodium alginate or an other protein, such as soybean protein isolate, to the protein isolates from hydrocarbon-assimilating yeast made fibril formation possible. The molecules of the microbial isolated proteins seem to be in a state that makes entwine-ment difficult. Whether or not this is related to the amino acid composition of the protein isolate remains to be elucidated.

![Graph](image)

Fig. 5. Concentration dependency of the reduced viscosity of the solution of protein isolates from hydrocarbon-assimilating yeast E (*Candida* sp.).

Viscosity of an alkaline solution of the protein isolate from hydrocarbon-assimilating yeast was measured. Figure 5 shows the concentration dependency of the reduced viscosity, \( \eta_{np}/c \). The intrinsic viscosities, \( [\eta] \), of those preparations were estimated as follows: Ppt-II, 0.163; Ppt-III, 0.124; Ppt-IV, 0.056; Ppt-V, 0.096. The results suggest that each protein fraction has physicochemical properties slightly different from the others.

Based on this observation it might be concluded that the molecules of the microbial protein isolates are in a rather rigid, spherical shape. However, this does not necessarily account for the inability of those isolated proteins to form fibers, because both soybean protein isolates and milk casein, which easily form fibers, exhibit similar properties.
Disposition of the extraction residues

The residues of hydrocarbon-assimilating yeast after alkali extraction are believed to be composed mainly of the cell wall materials, and their approximate composition was found to be as follows: polysaccharides, 70%; crude protein, 19%; ash, 1%; and moisture, 10%. They are insoluble in water, dilute acids, dilute alkalis, and organic solvents. Abandonment of the residues without pretreatment would become a source of severe water pollution. Salvage utilization or, at least, turning them into a chemically inactive form will be one of the most important problems to be solved in utilization of microbial cells for food. A few possible methods of residue disposition have been tested:

Solidification. Kneading with a small amount of calcium hydroxide solidified the pasty extraction residues, and they became hard but brittle after drying. Though the detailed properties of the solid have not been examined yet, solidification would make it easier to handle the residues.

Resinification. (i) Thermosetting resin. Treating the residues with formaldehyde should hydroxymethylate the amino group of the protein remaining in the residues, and an amine-type resin will be produced by the thermal hardening of the hydroxymethylated residues. Most of the polysaccharides probably act as fillers in the resin, but some may participate in cross-linkage formation in the resin.

Resinification with formaldehyde, urea-formaldehyde, and phenol-formaldehyde was tested by conventional procedures, and the molding properties of the resulting resins were examined. Resins produced with either reagent tested were found to be molded fairly easily.

(ii) Thermoplastic resin. Formation of acetate resin was tested under conditions similar to the conventional acetate method in the production of acetyl cellulose. After being treated with the acetylation mixed acids, the residues were dried at 95–105°C; and two acetylated fractions were obtained: one, more acetylated and soluble in acetic acid and in methylene chloride/ethanol (9/1), but insoluble in acetone; the other, less acetylated and insoluble in all three solvents. Both fractions were found to be molded under certain conditions.

Carboxymethylation. The remaining saccharides in the extraction residues were carboxymethylated with monochloroacetic acid and sodium-hydroxide according to the conventional method of preparing sodium carboxymethyl cellulose. The product, a water soluble carboxymethylated preparation, was obtained in relatively high yield (ca. 69%), and the viscosity of its solution was considerably lower than that of a commercial carboxymethyl cellulose preparation.

Though the characteristics of those products have to be examined further and more studies have to be done, the results shown here give hope for disposition of the extraction residues.
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