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Introduction

A large number of works have been done on the action of surface active agents in releasing cellular materials of bacteria in relation to their bactericidal effects. Of the substances released amino acids, phosphate and 260mμ-absorbing materials such as adenine, guanine, uracil and adenosine have been reported[1,2,3].

During the course of an investigation of the bactericidal action of surface active agents, it was observed that the addition of bactericidal surfactants to intact cell suspension of microorganisms results in the release of nicotinamide adenine dinucleotide (NAD).

This paper deals with a method for the extraction of NAD from bacteria and yeasts by surfactants, and the extraction of NAD by various treatments is compared.

Materials and Methods

Microorganisms. All the strains except *Candida krusei* (isolated from soil and identified) were supplied by the Institute of Applied Microbiology, University of Tokyo.

Media and growth conditions for each microorganisms are listed in Table 1. Cells

Table 1. Microorganisms and their cultural conditions

Strains	Media	Culture*
Saccharomyces cerevisiae WH-5-2	Koji-extract	Shaking
Candida krusei	Synthetic medium**	〃
Lactobacillus plantarum 11	Koji-extract containing 1% peptone (with 2% $CaCO_3$)	Stationary
Lactobacillus sake O12	〃	〃
Lactobacillus acidophilus 506	〃	〃
Pediococcus soyae PS-27	〃 +10% NaCl (without $CaCO_3$)	〃
Staphylococcus aureus IAM-0092	Glucose, 2%; peptone, 0.3%; dried yeast extract, 0.3%; NaCl, 0.5%; K_2HPO_4, 0.1% (pH 5.5).	Shaking
Micrococcus luteus IAM-1097	〃	〃
Escherichia coli IAM-1016	〃	〃
Aerobacter aerogenes IAM-1019	〃	〃

* Saking culture: The medium of 60ml was dispensed in 300-ml Erlenmeyer flask and it was sterilized. After inoculation, the medium was incubated at 30° for 2 days on a shaker.

Statinary culture: The medium of 600ml in 1-liter Erlenmeyer flask was incubated at 30° for 2 days under static condition.

** Synthetic medium: glucose, 10%; urea, 1%, $(NH_4)H_2PO_4$, 0.1%; KCl, 0.05%, K_2HPO_4, 0.1%, $MgSO_4 \cdot 7H_2O$, 0.05%; $FeSO_4 \cdot 7H_2O$, 0.001% (pH 5.8).

were harvested from the liquid media by centrifugation, washed three times with distilled water, and resuspended in distilled water.

Dry-weight determination. The dry-weight of the washed cell suspensions were determined directly, by drying 1 ml of suspensions to constant weight at 105°. Moreover, dry-weight of yeast cells was calculated from a previously prepared standard curve relating dry-weight to optical density obtained by use of a Hitachi EPO-B colorimeter and a 430 mμ filter.

Surface active agents. The cationic surfactant cetyltrimethyl ammonium bromide (CTAB) and anionics sodium lauryl sulfate (LAS) were used, unless otherwise stated.

Procedures for the extraction of NAD by surfactants. Extraction of NAD was carried out by adding known amounts of surfactants to cell suspensions, allowing them (total volume; 5 ml) to stand for an adequate period, removing the cells by centrifugation. The supernatants thus obtained were designed as "extract". NAD and the absorbancy at 260 mμ of the extracts were measured, and compared with those amounts extracted by holding the same cells suspended in distilled water at 98° for 5 minutes (referred to as "boiling extract").

Determination of NAD. NAD was determined with alcohol dehydrogenase according to the method of Bonnichsen[4] with slight modifications. Namely, 1.0 ml of sample solution, 5.0 ml of pH 8.0, M/20 phosphate buffer containing M/200 semicarbazid and 2.4 % ethanol, and 0.5 ml of alcohol dehydrogenase solution were mixed and the increase in optical density at 340 mμ was measured by use of Hitachi EPU-II A spectrophotometer. The alcohol dehydrogenase solution was prepared by dissolving 1.0 mg of purified alcohol dehydrogenase, which was obtained by the method of Racker[5] from commercial baker's yeast, in 2.0 ml of pH 8.0, M/15 phosphate buffer containing 0.1 % bovine albumin. The value of 6.22×10^6 cm^2/mole[6] was used as the extinction coefficient of reduced NAD at 340 mμ. If the procedures for the extraction were carried out at a cell concentration of A mg dried cells per ml and then B mg of NAD was contained in 1 ml of the extract odtained, the amount of NAD extracted from 1 g dried cells was calculated according to the following equation:

$$B\times\frac{1{,}000}{A}\ \text{mg}=\text{amount of NAD extracted per 1 g dried cells}$$

For a calculation of a ratio of 260 mμ-absorption given by NAD to the total 260 mμ-absorbancy of the extract, the value of 18.0×10^6 cm^2/mole[7] was used as the extinction coefficient of oxidized NAD at 260 mμ.

Measurement of 260 mμ-absorbancy of the extract. Light absorbancy at the wave length of 260 mμ of the extract was measured. The absorbancy at 260 mμ of the extract was expressed as log I_0/I (light path 1 cm) calculated for 10 mg dried cells per ml.

Results

I. Extraction of NAD from yeasts

(1). Effect of the amounts of CTAB and LAS added on the extraction of NAD. Washed cells of *Sacch. cerevisiae* were suspended in distilled water. The suspensions (pH 4.5) were incubated with various amounts of CTAB or LAS for 90 minutes at 4°, then extracts were obtained by centrifugation. The relationship between the amounts of

NAD in the extracts and surfactants added is shown in Table 2. The addition of 52 μg of CTAB per mg dried cells showed the extraction of the amount of NAD comparable to the amount extracted with boiling. NAD also could be extracted by LAS, but LAS was less sensitive, ca 100 μg or more of LAS per mg dried cells being required for an efficient extraction of NAD. Hereafter, CTAB was added at the level around 60 μg per mg dried cells in the procedures for extraction.

Table 2. Effect of the amount of CTAB or LAS on the extraction of NAD from *Sacch. cerevisiae.*

Surfactants added μg/mg dried cells \ Extract	260mμ-absorbancy log I_0/I (10mg dried cells/ml)	NAC mg/g dried cells	Ratio of 260mμ-absorption given by NAD to total 260mμ-absorbaacy of the extract %
CTAB 26	1.57	1.2	20.7
〃 39	2.25	1.5	18.6
〃 52	2.61	1.8	18.7
〃 104	2.72	1.9	19.1
LAS 26	0.99	0.2	6.6
〃 52	1.63	1.2	19.9
〃 104	3.19	1.6	13.3
Boiling extraction	3.46	2.0	16.0

Extraction was carried out at the cell concentration of 34.8mg dried cells per ml and at 4° for 90 minutes (pH 4.5).

(2). Effect of temperature on the extraction of NAD. Cells of *Sacch. cerevisiae* suspended in distilled water were treated wite CTAB corresponding to 58 μg per mg dried cells at 4, 15 and 35°. NAD and the absorbancy at 260 mμ of the extract were measured over periods of 30, 60 and 120 minutes, respectively (Figure 1). The amount of 260 mμ-

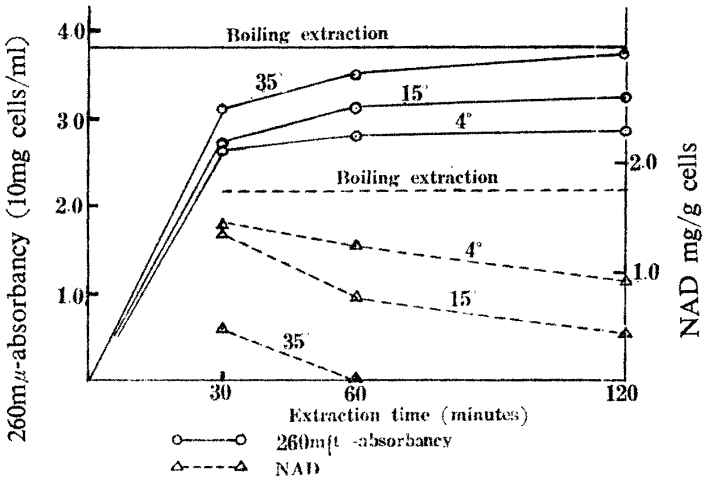

Fig. 1. Effect of temperature on the extraction of NAD from *Sacch. cerevisiae.*
Cell concentration on the extraction procedures: 34 mg dried cells/ml.
CTAB added: 58μg/mg dried cells.

absorbancy of the extract was increased by raising the temperature. Conversely, the amount of NAD extracted was decreased; and NAD was not contained in the extract prepared at 35° for 60 minutes. This result is thought to be caused by the decomposition of NAD during its extraction. Thus, a rapid destruction of NAD during the treatment of the cells of *Sacch. cerevisiae* suspended in distilled water with CTAB at 35° was observed. On the other hand, from the cells which were preincubated without CTAB at 35° for 60 minutes, NAD comparable to the amount extracted from the original intact cells was obained with boiling or CTAB-treatment (Table 3).

Table 3. Decomposition of NAD during CTAB-treatment of *Sacch. cerevisiae.*

Conditions of extraction					Extract	
Cells used	Addition of CTAB 60μg/mg cells	Temp.	pH	Time min.	260mμ-absorbancy (10mg cells/ml)	NAD mg/g cells
Intact cells	+	4°	4.5	60	2.93	1.6
〃	+	35°	〃	〃	4.29	0.2
〃	—	〃	〃	〃	0.44	0
〃	Boiling extraction				3.69	1.5
Preincubated cells*	+	4°	3.5	45	2.70	1.4
〃	Boiling extraction				3.83	1.3

* After preincubation of intact cells at 35° for 60 minutes without CTAB, extraction procedures were carried out.
Cell concentration on the extraction procedures: 23.4mg dried cells/ml.

(3). Effect of pH on the extraction of NAD. To investigate the effect of pH on the decomposition of NAD during its extraction, washed cells of *Sacch. cerevisiae* were suspended in pH 3.5, M/20 acetate buffer or pH 8.0, M/20 phosphate buffer, and incubated with CTAB at room temperature, 20°. As shown in Table 4, NAD was rapidly decomposed

Table 4. Effect of pH on the extraction of NAD by CTAB-treatment from *Sacch. cerevisiae.*

Conditions of extraction			Extract		
Temp.	pH	Time min.	260mμ-absorbancy (10mg cells/ml)	NAD mg/g cells	Ratio of 260mμ-absorption given by NAD to total 260mμ-absorbancy of the extract %
20°	3.5	45	2.70	1.8	18.3
〃	〃	90	2.79	1.7	16.1
〃	4.5	45	2.42	1.3	15.0
〃	〃	90	3.01	1.0	9.2
〃	8.0	45	3.00	0	—
〃	〃	90	3.36	0	—
Boiling extraction			3.05	1.5	13.2

Cell concentration on the extraction probedures: 58mg dried cells/ml.
CTAB added: 60μg/mg dried cells.

Table 5. Extraction of NAD by CTAB-treatment from *Candida Krusei.*

Conditions of extraction			Extrract	
Temp.	pH	Time min.	260mμ-absorbancy (10mg cells/ml)	NAD mg/g cells
4°	5.0	45	4.57	2.0
〃	〃	90	4.71	1.3
20	〃	45	4.72	0.5
〃	〃	90	4.90	0.1
35	〃	45	5.45	0.1
〃	〃	90	6.15	0
20	3.5	45	4.93	2.2
〃	〃	90	5.12	1.9
Boilling extraction			6.72	2.0

Cell concentration on the extraction procedures: 67mg dried cells/ml.
CTAB added: 60μg/mg dried cells.

at pH 8.0. On the other hand, at pH 3.5 NAD was scarcely decomposed at all, and a larger amount of NAD was extracted than with boiling.

Similar results were obtained with the cells of *Candida krusei* (Table 5).

II. Extraction of NAD from *Lactobacillus plantarum.*

Washed cells of *L. plantarum* suspended in distilled water were treated with CTAB (60 μg per mg dried cells) for 60 minutes. The amounts of NAD extracted by CTAB-treatment at 4, 20 and 35° were almost equal, ca 7 mg per g dried cells, and larger than the amount extracted by boiling (Table 6). It is obvious that NAD does not decompose during extraction by CTAB-treatment even at 35°, in contrast with yeast cells.

Table 6. Extraction of NAD by CTAB-treatment from *Lactobacillus plantarum.*

Conditions of extraction			Extract		
Temp.	pH	Time min.	260mμ-absorbancy (10mg cells/ml)	NAD mg/g cells	Ratio of 260mμ-absorption given by NAD to total 260mμ-absorbancy of the extract %
4°	4.2	60	7.98	7.1	24.1
20	〃	〃	8.62	7.2	22.6
35	〃	〃	9.24	7.2	21.1
Boiling extraction			12.7	6.5	13.9

Cell concentration on the extraction procedures: 15.2mg dried cells/ml.
CTAB added: 60μg/mg dried cells.

III. Extraction of NAD from various bacterial cells.

Extraction of NAD from various bacterial cells by treatment with CTAB or LAS was studied. Several examples were shown in Table 7. Extraction of NAD was observed in all organisms used, and CTAB was more effective, in general, than LAS for extraction.

Larger amount of surfactants seems to be required for an efficient extraction of NAD from Gram-negative bacteria than from Gram-positive bacteria. It was noteworthy that 260 mμ-absorbancy given by NAD extracted accounts for approximately 50 % of total 260 mμ-absorbancy of the extract in *L. acidophilus*.

Table 7. Extraction of NAD from various bacteria

Strains and cell concentration on the extraction procedures	Surfactants added μg/mg cells	Extract		
		260mμ-absorbancy (10mg cells per ml)	NAD mg per g cells	Ratio of 260mμ-absorption given by NAD to total 260mμ-absorbancy of the extract %
L. plantarum 21.2mg/ml	CTAB 47	7.14	5.7	21.6
	LAS 47	3.23	0.7	5.9
	Boiling ext.	10.30	5.0	13.2
L. sake 11.4mg/ml	CTAB 52	7.15	6.5	24.6
	LAS 52	4.55	2.9	17.3
	Boiling ext.	10.10	6.2	16.6
L. acidophilus 12.0mg/ml	CTAB 51	3.92	7.2	50.0
	LAS 51	3.88	5.4	37.7
	Boiling ext.	4.38	7.8	48.3
P. soyae 7.7mg/ml	CTAB 72	6.80	6.2	24.7
	LAS 72	6.43	5.7	24.0
	Boiling ext.	7.58	5.1	18.2
S. aureus 9.4mg/ml	CTAB 64	5.07	2.3	12.3
	LAS 64	3.57	trace	—
	Boiling ext.	7.63	2.3	7.1
M. luteus 22.2mg/ml	CTAB 18	0.74	0.4	14.6
	CTAB 45	3.69	3.9	28.6
	LAS 45	1.25	0.9	19.5
	LAS 90	3.30	2.2	18.1
	Boiling ext.	4.52	4.0	24.0
E. coli 28.0mg/ml	CTAB 30	0.48	trace	—
	CTAB 83	2.80	1.6	15.5
	LAS 83	1.15	0.8	18.3
	Boiling ext.	9.59	3.1	8.7
A. aerogenes 12.3mg/ml	CTAB 47	1.89	0.6	8.6
	CTAB 70	2.25	1.1	13.2
	LAS 47	0.59	0.8	36.7
	Boiling ext.	19.20	3.3	4.7

Treatments with surfactants were carried out at 4° for 90 minutes.

IV. Extraction of NAD by various treatments from *L. acidophilus* and *C. krusei*.

Extraction of NAD by the treatment with various surfactants and by lyophilization or acetone-treatment was compared by using the cells of *L. acidophilus*, which have comparatively high content of NAD. Lyophilized or acetone-treated cells were suspended in distilled water. After allowing them to stand for 60 minutes at 4°, extracts were obtained by centrifugation. As shown in Table 8, NAD was not extracted by nonionic surfactants (N-1 and N-2), whereas, cationics (CTAB and CPC), anionics (LAS and LBS) and

amphoteri ionics (AIC), all of which are bactericidal[8], showed an activity for extraction of NAD. In a procedure similar to *L. acidophilus*, release of NAD from lyophilized cells of *C. krusei* was observed, although at a rate of only 40 % of that by CTAB-treatment or boiling.

Table 8. Extraction of NAD by various treatment

Strains and cell concentration on the extraction procedures	Surfactants added or treatments	Extract	
		260mμ-absorbancy (10mg cells/ml)	NAD mg/g cells
L. acidophilus 20mg/ml	N-1 60μg/mg cells	0.20	—
	N-2 〃	0.71	—
	CTAB 40 〃	3.76	7.7
	CPC 〃	3.60	7.2
	LAS 〃	4.39	7.6
	LBS 〃	4.23	7.7
	AIC 〃	1.14	2.1
	Acetone-treatment	2.57	6.0
	Lyophilization	3.41	6.1
	Boiling extraction	4.91	6.8
C. krusei 56mg/ml	CTAB 60μg/mg cells	4.77	2.0
	CPC 〃	4.45	1.9
	LAS 〃	2.10	0.7
	Lyophilization	3.86	0.8
	Boiling extraction	7.19	2.2

Treatments with surfactants were carried out at 4°, pH 4.5 for 60 minutes.
N-1 : polyoxyethylene sorbitan monostearate
N-2 : polyoxyethylene alkyl phenol ether
CPC: cetyl pyridinium chloride
LBS : sodium lauryl benzene sulfonate
AIC : alkyl imidazole carbonate
Acetone-treatment, lyophilization: see text for details.

Discussion

A method for the extraction of NAD from yeast with acidic ether-ethanol has been proposed by Sumner, Krishnan and Sisler[9]. However, NAD of organisms is generally extracted by boiling water. During boiling extraction, NAD may be decomposed by heat[10,11] and also by the action of intracellular enzymes. By the treatment of yeast with CTAB, NAD was rapidly extracted and its decomposition could be repressed by treating at a lower temperature and pH 3.5; a larger amount of NAD was extracted by CTAB-treatment than by boiling extraction. Moreover, as reported in the following paper[12] it is possible to obtain cytochrome c and enzymes such as alcohol dehydrogenase and catalase from the cell debris, from which NAD has been extracted by CTAB-treatment.

This extraction of NAD by ionic surfactants seems to be universal for microorganisms. However, the amount of NAD extracted from Gram-negative bacteria by surfactants was comparatively small. It has been reported by Gilby and Few[13] and Salton[14] that

surfactants combined with cellular phospholipid. It has been described that the cell walls of Gram-negative bacteria contained an appreciable amount of lipid, in contrast to a very small amount in the wall of Gram-positive bacteria by Salton[15]. Therefore, the differences in efficiency of CTAB on extraction of NAD between Gram-positive and Gram-negative bacteria may be attributable to differences in lipid contents in the cells of bacteria. Comparatively high content of NAD in the lactic acid bacteria was observed confirming the report of Takebe and Kitahara[16].

Sutton, Arnow and Lampen[17] nave reported that the inhibition of fermentation of yeast cells by nystatin was ascribable to the leakage of salts and cofactors essential for a glycolytic system such as NAD and adenosine triphosphate. Herrera, Peterson, Cooper and Peppler[18] have reported that the release of NAD from cells should be a reason for the decrease of fermentation by drying the yeast cells. Release of NAD from cells by an alteration in their permeability seems to be a general phenomenon. Treatment of yeast cells suspended in distilled water with CTAB at 35° resulted in rapid decomposition of NAD. On the other hand, when incubated without CTAB at 35° for 60 minutes any appreciable decomposition of NAD did not occur. From these findings it is suggested that permeability alterations by CTAB followed by a disarrangement of the structure of cytoplasm and its autolysis. This is probably one of the mechanisms for the inhibiting action on the metabolism of microorganisms and bactericidal action of ionic surface active agents.

Sammary

Treatment of cell-suspension with cetyltrimethyl ammonium bromide (CTAB) (60 μg per mg dried cells) resulted in the extraction of nicotinamide adenine dinucleotide (NAD). About 2 mg of NAD was extracted from 1 g of dried cells of *Saccharomyces cerevisiae* or *Candida krusei* by the treatment at pH 3.5, 20° for 45 minutes. *Lactobacillus plantarum* solubilized 7.2 mg of NAD from 1 g dried cells by the similar treatment without any appreciable decomposition of NAD even at higher temperature in contrast with yeast cells.

By CTAB-treatment the extraction of NAD from various bacteria: *Lactobacillus acidophilus*, *Lactobacillus saké*, *Pediococcus soyae*, *Staphylococcus aureus*, *Micrococcus luteus*, *Escherichia coli* and *Aerobacter aerogenes*; was also observed.

The solubilization of NAD from *L. acidophilus* by various treatment: treatment with various surface active agents, acetone-treatment and lyophilization; was compared. The mechanism of the bactericidal action of ionic surfactants is discussed.

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