CERTAIN CHEMICAL AND NUTRITIONAL ASPECTS OF SOYBEAN TEMPE

Dissertation for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
SLAMET SUDARMADJI
1975

This is to certify that the

thesis entitled

CERTAIN CHEMICAL AND NUTRITIONAL ASPECTS
OF SOYBEAN TEMPE

presented by

Slamet Sudarmadji

has been accepted towards fulfillment
of the requirements for

PhD degree in Food Science &
Human Nutrition

Major professor

Date July 8, 1975

O-7639

ABSTRACT

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By

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Tempe was prepared from soybeans and a pure culture of Rhizopus oligosporus. Scanning and transmittance electron micrographs of tempe showed some disruptions of the soybean cell walls and penetration of the mycelium into two layers of cotyledon cells.

Based on the liberation of free fatty acids, microbial growth and organoleptic changes, the tempe fermentation may be differentiated into three phases: (I) Rapid phase (0-30 hr.), (II) Transition phase (30-60 hr.), and (III) Deterioration phase (beyond 60 hr. of fermentation). It is believed that primarily the contaminating sporeforming bacteria were responsible for the deterioration of tempe.

Frying of tempe in coconut oil resulted in a migration of some free fatty acids, but not glycerides, from tempe into the frying oil.

Based on the TBA test and organoleptic evaluation, fried tempe, stored for 1.5 months, at room temperature, in bags containing air, did not deteriorate.

The phytic acid content of soybeans decreased from 1.4% to 1.0% as a result of the tempe fermentation. A strong phytase activity was detected and measured in the tempe and the tempe mold. The pH optimum of this phytase was 5.6 and the Michaelis constant was 0.28×10^{-3}M.

There were no statistically significant differences in the Protein Efficiency Ratio values (AOAC procedure) between fried soybeans, fried tempe and fried, sesame-supplemented tempe at the 10% protein level in the diet.

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Food Science and Human Nutrition

1975

ACKNOWLEDGMENTS

The author very sincerely wishes to express his deepest appreciation to Dr. Pericles Markakis for the excellent guidance, encouragement and invaluable assistance throughout the doctoral program toward the completion of this dissertation.

The same appreciation is extended to Dr. Georg A. Borgstrom, Dr. E. S. Beneke, Dr. T. I. Hedrick, and Dr. F. R. Peabody for their assistance and suggestions.

Special thanks are due to Dr. K. Stevenson and Mrs. Marguerite Dynnik for their help in some microbial aspects of this study, and to Mrs. June P. Mack and Mrs. A. Ackerson for their assistance in electron microscopy.

Last but not least the author wishes to express his gratitude to the Government of Indonesia, the Midwest Universities Consortium for International Activities (MUCIA Inc.) and Gajah Mada University for providing the leave of absence and the financial support for this program. The Department of Food Science and Human Nutrition at Michigan State University has provided ample facilities and excellent cooperation for the whole program.

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INTRODUCTION

In recent decades the explosive growth of world population, the accelerating depletion of natural resources, and the severe food scarcity in large areas of the world have placed new emphasis on the production, storage, transportation, processing, preservation and distribution of foods, as well as on the study of their nutritional qualities.

According to the Food and Nutrition Board, National Academy of Sciences, National Research Council, the recommended daily dietary allowance for adult American male is 2800 Cal. and 65 g of protein. For small bodied South East Asian people, working manual labor in 25°C climate, FAO has computed at between 1625-1821 Cal. per person per day, and minimum crude protein requirement is 24.5 g (Latham et al., 1972; Clark, 1972). Meanwhile for a slightly bigger East Asian adult man (body weight 55 kg), joint FAO/WHO expert groups computed at 2530 Cal./day (FAO/US HEW, 1972). For women the caloric requirements are about 25% less.

The Food Consumption Table compiled by FAO (1971) from 132 countries, has revealed that Ireland and New Zealand are two countries with the highest average calorie and protein consumption in the world, 3455 and 3454 Cal. and 91.5 and 109.6 g protein per capita per day, respectively. On the contrary, the Indonesian people consumed the least food in the world with only 1760 Cal. and 38.4 g protein per person per day, which stand at the precarious border of hunger even by the South East Asian standards. Furthermore, Table 1 shows that most of the foods consumed by the Indonesians originate from plant materials.

Table 1.--Calorie, protein and fat consumption per capita per day in Indonesia (FAO, 1971).

	Calorie (total)	Protein (g)	Fat (g)
Vegetable	1714 (97.4%)	33.2 (86.5%)	20.4 (89.5%)
Animal	46 (2.6%)	5.2 (13.5%)	2.4 (10.5%)
Total	1760 (100%)	38.4 (100%)	22.8 (100%)

For comparison, Table 2 shows the calorie, protein and fat consumption in some selected countries. The protein and calorie malnutrition may be critical to child development. Besides the well known kwashiorkor and marasmus syndrome, protein deficiency more importantly will affect the brain development especially in fetal and early life of a child. Besides zinc and vitamin B6,

Table 2.--Per capita daily consumption of calorie, protein and fat in selected countries* (FAO, 1971).

Country	Cal/day	Protein (g) per day	Fat (g) per day
Afghanistan	2057	65.4	26.0
Albania	2366	71.3	39.1
Argentina	2885	90.2	105.6
Australia	3130	90.9	129.2
Bolivia	1765	45.8	33.9
Brazil	2541	63.9	51.3
Burma	2011	44.1	30.5
Canada	3142	94.5	141.4
China	2045	58.2	31.2
Columbia	2192	50.1	47.3
Congo	2160	39.8	37.5
Cuba	2501	62.8	43.7
France	3108	98.2	136.6
Germany (East)	3040	76.4	129.0
Germany (West)	2927	80.1	133.8
Greece	2901	97.7	94.8
Honduras	1930	48.6	37.6
Iceland	2899	98.8	131.6
India	1964	50.6	28.2
Indonesia	1760	38.4	22.8
Iran	2029	55.2	39.0
Ireland	3455	91.5	135.8
Japan	2416	72.4	41.3
Malaysia	2200	49.4	40.8
Mexico	2624	66.5	58.2
Mongolia	2538	92.9	81.7
New Zealand	3454	109.6	154.0
Pakistan	1995	49.6	28.6
Philippines	1911	43.8	31.4
Saudi Arabia	2082	56.2	29.9
Singapore	2443	63.3	48.9
Taiwan	2379	58.7	45.2
Thailand	2226	50.9	27.3
UK	3233	88.6	141.9
USA	3156	93.7	148.0
USSR	3182	92.2	74.5

*Figures do not include beverages.

proteins act as the backbone of lipid incorporation in the brain, and therefore a protein deficit will cause a longer lasting deleterious effect on the brain than a transient deficit in essential fatty acids and calorie.

The critical period for brain development in man begins with the second half of fetal life and ends about 18 months after birth. Apathy of the children is one of the most constant signs of protein-calorie malnutrition; the loss of curiosity and the lack of desire for exploration, a progressive withdrawal from the environment are other symptoms of acute protein deficiency (Cheek, 1968; Muralt, 1972; O'Neal et al., 1970; Anonymous, 1972).

The provision of adequate proteins and nutritious foods for the people is of prime importance for the present and future generations. Besides the promising source of food from the ocean, it is obvious that foods from plant origins play a very important role in the diet of Indonesians as well as the peoples from other developing countries, because of their immediate availability and traditional acceptance. One of the most promising plant foods is soybeans due to its acceptability (especially in the Orient) and the source of good quality proteins. The protein, fat, carbohydrate and ash contents of soybeans are presented in Table 3.

Protein analysis of soybeans (Table 6, p. 24) shows the composition of amino acids which contains all the essential amino acids, i.e., Lysine, leucine, isoleucine,

Table 3.--Soybean composition, % on moisture-free basis (Cowan, 1969).

Assayed	Protein (Nx6.25)	Fat	Carbohydrate	Ash
Whole bean	40	21	34	4.9
Cotyledon	43	23	29	5.0
Hull	8.8	1.0	86	4.3

methionine, phenylalanine, threonine, tryptophan and valine. Methionine is present in relatively low concentration.

A plant protein may be utilized by humans best when it is consumed directly without converting it into animal protein. Through conversion, soybeans yield about 43 lbs of edible animal protein per acre, while through direct consumption they yield about 600 lbs of protein per acre (Spaeth, 1974).

Through the advancement of soybean technology, it is feasible now to convert soybeans into many kinds of products which have higher acceptability as food than the unprocessed soybeans. Many soybean base products are already available in the market and others are being developed. The Figure 1 shows the varieties of soybean base products.

The Association of South East Asian Nations (ASEAN) on their meeting in Jakarta has recognized the importance of soybeans as food and recommended "further studies to improve soybean processing techniques to overcome protein

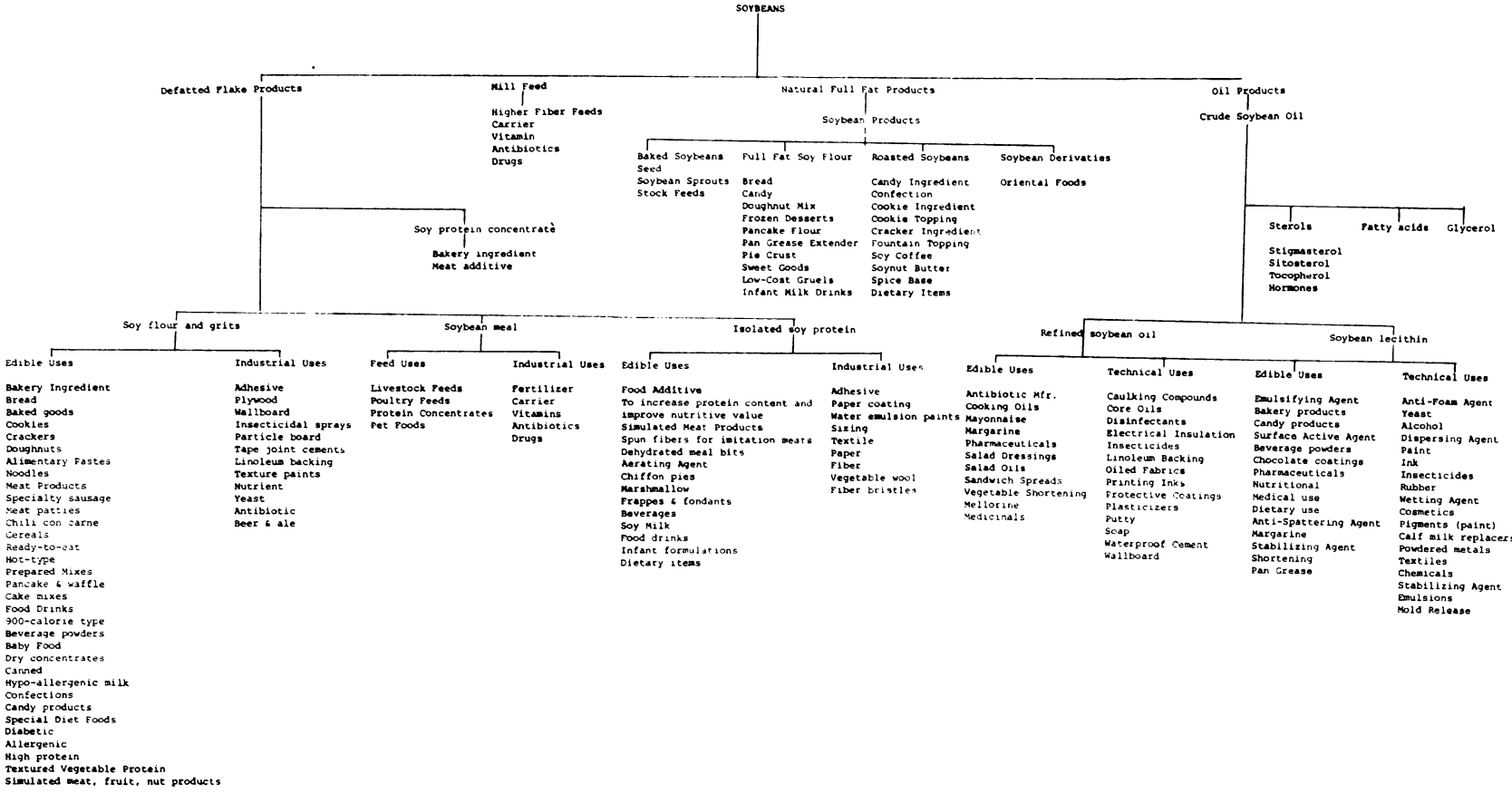


Figure 1. Products derived from soybeans (Scott and Aldrich, 1970).

deficiency problems" (The Strait Times of Singapore, May 10, 1974). Soybeans are widely cultivated in South East Asia and already play an important part of people diet for centuries. The largest soybeans producer among the ASEAN members is Indonesia, which is also the fifth largest soybean producer in the world after the USA, China, Brazil, and the USSR (United Nations, 1974, Table 4).

Some of the new soybean products such as textured soy protein, simulated meat etc., require a high degree of technological know-how and big capital inputs which are lacking in most developing countries at the present time. Therefore traditional soybean fermentation processes which require only simple equipment and can be done at the village level, are the most feasible answer. Sufu, Hamanatto, Shoyu, Miso and Tofu are soybean products of China and Japan, while Tempe and Kecap are products of Indonesia which owe their popularity to the simplicity in processing and better acceptability as foods and condiments than the plain unfermented soybeans.

Tempe has been one of the most important traditional foods in Indonesia for centuries. However, the traditional village-type of tempe fermentation process lends itself to some improvements, such as in the area of sanitary conditions, the use of a desirable and purified mold culture stocks and finding a way for simple preservation methods for a delayed consumption time. The implementation of those improvement programs will require a tedious education

Table 4.--Soybeans production; in thousand metric tons (United Nations, 1974).

Country	1966	1967	1968	1969	1970	1971	1972
WORLD	39,084	40,709	43,997	45,180	46,525	48,680	53,289
Argentina	18	21	22	32	27	59	78
Australia	1	1	1	1	6	9	26
Brazil	595	716	654	1,057	1,509	2,218	3,666
Canada	245	220	246	209	283	280	375
China	10,970	11,100	10,670	10,920	11,580	11,680	11,500
Colombia	52	80	101	100	96	83	80
Hungary	1	0.0	0.0	0.0	0.0	0.0	0.0
Indonesia	417	416	420	389	498	475	515
Japan	199	190	168	136	126	122	127
Khmer Rep.	7	8	4	9	4	2	3
Korea, Dem. People's Rep.	215	225	240	225	228	230	235
Korea, Republic of	161	201	245	229	232	222	224
Mexico	95	106	275	266	280	250	366
Nigeria	52	60	54	62	58	63	63
Paraguay	20	18	14	22	30	74	128
Romania	20	41	47	51	91	165	186
South Africa	3	4	4	7	3	3	4
Thailand	38	53	45	61	50	54	56
Turkey	5	6	9	11	12	11	13
USSR	586	543	528	434	604	535	580
United States	25,270	26,575	30,127	30,839	30,675	32,006	34,916
Viet-Nam, Dem. Rep. of	14	17	17	18	19	19	19
Viet-Nam, Rep. of	8	6	7	6	7	8	7
Yugoslavia	11	9	3	5	5	4	6

efforts in health and nutrition for the people, a favorable economic condition to acquire an improved new technique and most be supported by research and experiments in chemistry and preservation technology. One alternative for improvement is a centralized tempe fermentation plant, by setting up modern and scientifically controlled plants in some key locations. At this time, however, the extent of the impacts of this endeavor must be carefully studied to the employment and the livelihood of many small traditional tempe producers.

This present study on tempe is only one small part of the never-ending assignments of scientific quest and better understanding of our food for more efficient utilization of this limited resource. On his essay review, Hudson (1974) wrote that lipid is the first group of the major naturally occurring bulk organic compounds, which yields the battery of elegant techniques, especially gas chromatography, providing an analysis with a more precise result in a few hours.

Lipids are the key to many biological processes, whether they are chloroplasts or erythrocytes, or the structure of brain and nervous sytems. In the preservation of foods, lipids play an important role also. The development of off flavor during storage or processing of foods has been attributed to the degradation of lipids, either catalyzed by enzymes or initiated by air oxydation.

It is now recognized that oils and fats play a far more important role than merely as sources of energy.

Nutrition aspects besides the aesthetic, pleasing properties and wholesomeness are of prime importance in foods and should serve as the main objective directly or indirectly in food research. In this study, the tempe fermentation is explored, especially on its effect to the lipids, protein and phytic acid. Phytic acid has drawn much attention in the last few years because of its metal binding properties capable of inducing trace element deficiencies in human and animals.

The tempe fermentation has been proven to improve the nutritional value of soybeans. Hopefully, this present study will contribute to the understanding of tempe fermentation and thence the appreciation of this ancient and remarkable fermentation practice.

REVIEW OF LITERATURE

Tempe (tempeh) is an Indonesian food generally made from soybeans fermented with Rhizopus oligosporus Saito and other Rhizopus species. It is a cake-like solid mass of soybeans held together by mold mycelia, whitish in color and never eaten raw but usually cooked as soups, deep fried in oil or fried as chips.

This fermented soybean food is eaten by millions of people in Indonesia and Surinam, and some tempe is manufactured in Holland. Hesseltine (1965) reported that tempe is highly acceptable to Americans and Europeans.

For cheap substitution of soybeans or simply as another variation of tempe, copra cake (bungkil, bongkrek) can be used solely or mixed with soybeans. The fermented product is known as tempe bongkrek. Other types of tempe such as tempe koro made from Phaseolus beans, tempe gembus made from soybean curd (tofu) whey are also popular.

Another product similar to tempe called oncom is made from pressed peanut cake (as peanut oil by-product) and fermented with Neurospora sp., has a yellowish pink color rather than whitish as in tempe (Steinkraus et al., 1965a).

Hesseltine et al. (1967) successfully introduced new fermented tempe-type products prepared from wheat, oat, rye, barley, rice and a combination of rice or wheat with soybeans. The new products possess a very pleasant odor, a desirable color and a very acceptable mild taste.

Village-type preparation of tempe

Dry soybeans are soaked overnight in tap water and then placed in a bamboo basket at the edge of a stream so as to float the seed coats away while the beans are trod on with the feet. The dehulled beans are then boiled without pressure in excess water for half an hour. The beans are removed from the cooking water thoroughly drained and cooled.

After cooling and draining, the beans are inoculated with spores of Rhizopus strains. The inoculated beans are tightly packed and wrapped in banana leaves about 1 cm thick, in various shapes and sizes. The banana leaves are believed to contribute to a desirable flavor. Some forms of tempe are fermented in bamboo cylinders.

The Rhizopus inoculum is taken from pieces of a previous fermentation cakes or from the wrapper, and also commercially available as dry spore preparations on dry Hibiscus tiliaceus leaves.

Mass production of tempe

Martinelli et al. (1964) reported a method to make tempe rapidly in large amounts by pure culture fermentation

in shallow wooden and metal trays with perforated bottoms and covers. Excellent tempe was also made in perforated plastic bags and tubes.

For plant-type mass production of tempe, Steinkraus et al. (1965b) described a pilot-plant process for the production of dehydrated tempe. The hydrated beans were passed through a burr mill and the hulls were removed by floatation with water or through gravity separator. The hydration was accomplished by soaking the dry beans in water acidified with lactic or acetic acid to inhibit bacterial growth overnight at room temperature. The beans were precooked at 100°C in water, and then drained and cooled. As the temperature fell to 35-38°C, they were inoculated with lyophilized tempe mold, three grams per kilogram of precooked beans. The inoculated beans were mixed for five minutes in a bowl with a Hobart mixer. About 3 kg of inoculated beans were spread on a 35 x 81 x 1.3 cm stainless steel tray with 3 mm mesh bottom, covered with waxed paper to decrease moisture loss and were incubated at 35-38°C in 75-85% relative humidity for 15-18 hours. Wang et al. (1975) successfully mass-produced Rhizopus oligosporus spores on rice, combination of rice: wheat bran or wheat: wheat bran, then freeze-dried and ground into fine powder for tempe inoculation.

Tempe mold

Village-style fermentation of tempe is usually done with impure cultures of molds.

Stahel (1946), Van Veen and Schaefer (1950) and Steikraus et al. (1960) stated that Rhizopus oryzae Went and Geerligs is the mold chiefly responsible for tempe fermentation. Later Hesseltine (1965) isolated 40 strains of Rhizopus from tempe which could produce an acceptable product when soybeans were inoculated with these strains (Table 5).

Table 5.--Strains of Rhizopus species which made acceptable tempe (Hesseltine, 1965).

Name	No. of Strains
Rhizopus oligosporus Saito	25
R. stolonifer (Ehren) Vuill	4
R. arrhizus Fischer	3
R. oryzae Went and Geerligs	3
R. formosaensis Nakazawa	3
R. achlamydosporus Takeda	2
Total	40

Hesseltine concluded that obviously R. oligosporus is the principal species used in Indonesia for making tempe.

Boedijn (1958) described R. oligosporus as a mold which can always be isolated from tempe cakes, bungkil (cattle cakes) and fermenting tobacco.

General description of Rhizopus (Frazier, 1957).

Systematical classification:

DIVISION: Thallophyta

SUBDIVISION: Eumycetes (true fungi)

CLASS: Phycomycetes (nonseptate)

SUBCLASS: Zygomycetes (sexual spores are zygospores)

ORDER: Mucorales

GENUS: Rhizopus

Genus Rhizopus has distinguishing characteristics (Figure 2):

1. nonseptate
2. has stolons and rhizoids, often darkening in age
3. sporangiophores arises at the nodes where rhizoids also are formed
4. sporangia are large and usually black
5. hemispherical columella and cup-shaped apophysis (base to the sporangium)
6. abundant, cottony mycellium
7. no sporangioles

R. oligosporus can use materials such as xylose, glucose, galactose, trehalose, cellobiose and soluble starch, soybean oil, but not raffinose. It can use asparagine and ammonium sulfate as sources of nitrogen. It also produces a protease and lipase (Wagenknecht et al., 1961 and Hesseltine, 1965).

Oxygen is essential to the growth of the mold. Steinkraus et al. (1960) and Martinelli et al. (1964) have

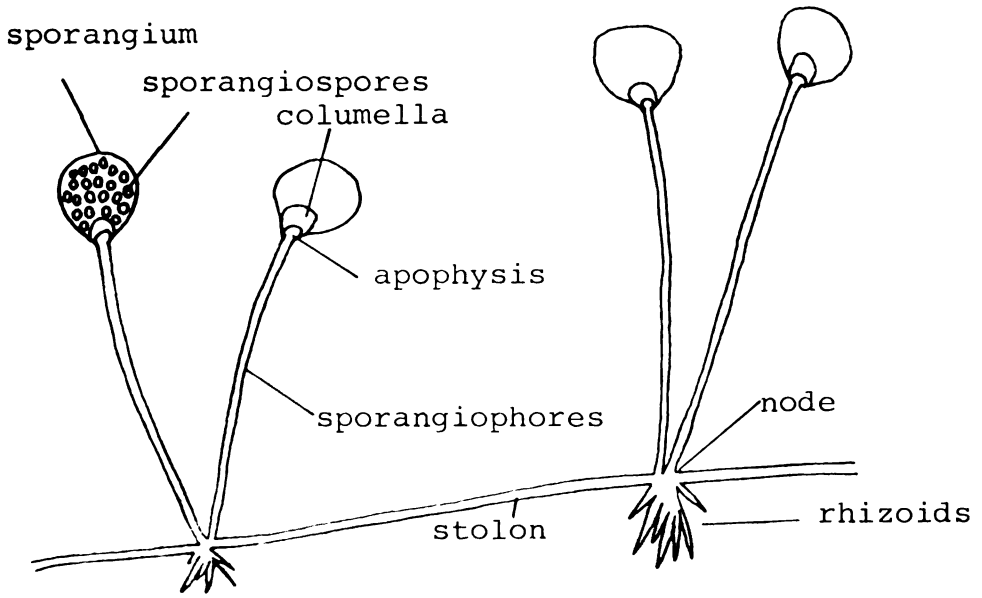


Figure 2. Rnizopus mold (Frazier, 1957).

demonstrated that when the layer of fermenting beans was thicker than 2 inches (5 cm) mold growth became less rapid and less heavy in the center than it was in the thinner layer. To make a good tempe they recommended that the thickness should not be more than 3 cm.

To get the right amount of aeration, perforated metal trays or plastic bags can beused. However, if the amount of aeration is in excess, the soybeans at the surface will dry out before mold starts to develop, and produce undesirable black spores (Martinelli et al., 1964).

The tempe mold grew well at 31-37°C and requires 22-24 hrs. to full fermentation. At temperatures below 30° and above 44°C soybeans fail to ferment properly. The length of time to full fermentation depends on the temperature of incubation. At 25°C it requires 80 hrs. and at 28°C requires 26 hrs.

If the pH falls below 3.5 the growth is slow.

Mold growth inhibition

The important step in making tempe is to boil the dehulled soybeans in excess water and then drain and cool prior to inoculation. If dehulling and draining are not applied the mold does not grow well, a lot of spores are produced and the tempe does not have a pleasant flavor (Hesseltine, 1965 and Steinkraus et al., 1960).

Hesseltine, et al. (1963) observed that an inhibiting substance exists in all 16 varieties of soybeans tested. This inhibitor must be soluble in water and heat stable. Another possibility is a growth promoting substance for bacteria might remain in the soybeans and in turn restricts the growth of fermenting mold. Addition of tetracycline to the beans and lack of bacterial evidence from samples grown on nutrient agar, proves that the latter possibility is unlikely.

Antibacterial compound

A substance having bacterial inhibiting activity has been isolated by Wang et al. (1969) from tempe. This substance, probably consisting of four or five components, inhibits the growth of some Gram positive and Gram negative bacteria. This antibacterial substance is produced by R. oligosporus and behaves like many antibiotics which at low level stimulate bacterial growth and at higher level inhibit their growth.

The antibacterial activity of crude tempe extracts is rapidly destroyed at room temperature. The isolated product, on the other hand, is fairly stable to boiling and in the pH range of 2 to 7. This substance perhaps contributes to the resistance of Indonesian people to diseases.

Nutritional aspects of tempe fermentation

The plain soybeans are almost unpalatable to the Indonesians, and consumed only in small amount as snacks, soybean milk (saridele) and bean curd (tofu); soybean sprouts are also used. Some factors such as the difficulty to digest in soybeans insufficiently cooked (antitryptic factors), their undesirable beany flavor and flatulence which may accompany their ingestion make plain soybeans unpopular as food. The high phytic acid content in raw soybeans may also have adverse nutritional effects, by making certain minerals unavailable to the body. However, when soybeans are made into tempe, they are easy to digest, have a pleasant odor, and even they are suitable for people suffering from dysentry and nutritional edema (Van Veen and Schaefer, 1950). In 1974, the procedure to improve soybean flavor was patented in Japan by immersing beans in a liquid containing a ribonuclease from Rhizopus (Toyo Spinning Co., Japanese Patent, 1974). There must be some desirable changes to the soybeans during tempe fermentation by Rhizopus mold.

György (1961) studied the tempe nutritionally as compared to soybean flour and skim milk. After a ten week feeding period of rats, identical weight gain and equal protein efficiency were noted for tempe, soybean flour and skim milk at the 20% protein level. In contrast at the 10% protein level the PER of plain soybeans was smaller than that of skim milk, whereas tempe was as good as skim milk.

The superiority in nutritive value of tempe over the control plain soybeans is significant only at low protein intakes. Some rats receiving diet containing 10% protein, all from soybean flour, died of severe massive hemmorrhagic necrosis of the liver (severe vitamine E deficiency), and the rest of the rats receiving the same ration suffered from cirrhosis of the liver or acute necrotizing nephrosis. Rats with tempe as well as with skim milk diet both at 20% and 10% protein have shown at autopsy normal livers and kidneys.

The prevention of rancidity in tempe and the prevention of vitamine E deficiency symptoms in rats receiving tempe can be traced to the presence of an "antioxidant." It has been reported that soyflour developed strong rancidity early after exposure to air at room temperature, in contrast with tempe under identical conditions remain free from organoleptic rancidity even after 1.5 years.

In 1964, György et al. isolated an active concentrate extracted from tempe with alcohol or ether showing

a definite protection in hemolysis test, but did not correspond to α-tocopherol or to F84 which was isolated from yeast.

Steinkraus et al. (1961) also noted that the superiority of tempe PER is damaged through drying at 150°F.

Loss of solids and protein in dehulling, soaking, washing and cooking of soybeans did not reduce the nutritive value of tempe as already demonstrated by Smith et al. (1964). They further proved using rats as experimental animals that methionine supplementation of tempe significantly increased rate of growth and protein efficiency values.

About one-third of the thiamin of soybeans was used up by R. oryzae was reported by Roelofsen and Talens (1964) but riboflavin, niacin, and vitamin B6 increased considerably to 5-6 times higher than the unfermented soybeans. Vitamin A was reported about the same as in raw soybeans (Jansen and Donath, 1924; Murata, 1965).

A nutritional improvement project for children in developing countries using tempe as a food source has been initiated by FAO. A very promising large scale tempe feeding experiment has been reported in Southern Rhodesia (Autret and Van Veen, 1955).

Changes in carbohydrates and soluble solids

Soluble solids, soluble nitrogen, fiber, nitrogen-free extract (NFE) and ammonia are increasing during tempe

fermentation. The temperature in the fermenting bean mass is rising above the incubator temperature, reaching maximum after 30 hours and then declines.

Steinkraus et al. (1960, 1961) observed that soluble solids rise from about 13% to nearly 28%. Soluble nitrogen rises from about 0.5% to nearly 2%, while total nitrogen remains about 7.5%. The pH also rises during the fermentation (Fig. 3).

Van Buren et al., (1972) reported an increase in fiber, nitrogen free extract (NFE) and ammonia. They also suggested that the increased water-soluble NFE, in contrast to the decreasing 66% ethanol-soluble, NFE, is due to water soluble pectic and hemicellulose-type material solubilized by mold enzymes and apparently responsible for the softening effect of the mold on the cooked soybeans. Non enzymatic browning during fermentation and drying was also reported.

Steinkraus et al. (1960) also made cytological studies on tempe and concluced that the cells of beans cooked in water and the tempe made from them remain intact after blending in a Waring blender while soybeans cooked in steam for 90 minutes contained relatively fewer intact cells after similar blending.

Changes in protein

The ammonia content rises during the course of tempe fermentation suggesting deamination of amino acids.

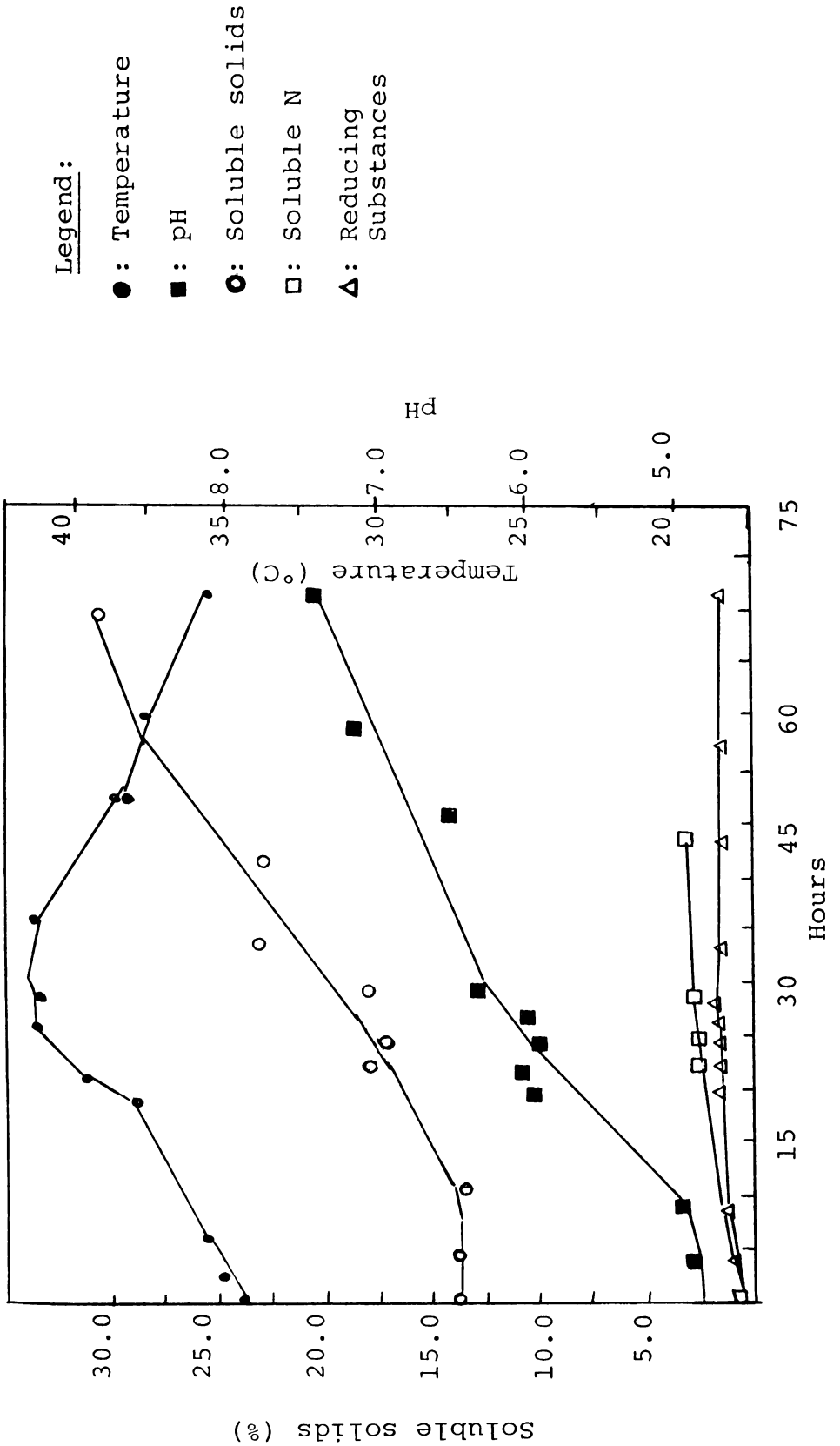


Figure 3. Changes occurring during the tempe fermentation (Steinkraus et al., 1961).

Experiments by Van Buren et al. (1972) showed the increasing water soluble protein during the active mold growth. The disappearance of crude protein, soluble in 66% ethanol, shows that the mold utilizes amino acids and low molecular weight peptides, and probably the intermediate-size protein breakdown products contribute to the water soluble protein. After growth ceased there was an accumulation of low molecular weight nitrogen compounds. It was also noted that the solubilization of protein was accomplished without the development of bitter materials as in other cases of enzymatic proteolysis of soy protein.

Murata (1965) concluded from his chemical analyses of tempe that the protein, amino acids, moisture, crude fat, ash and fiber contents are not much different from those of unfermented soybeans. The amino acid composition of soybeans and tempe appears in Table 6.

Changes in lipids

Wagenknecht et al. (1961) and Van Buren et al. (1972) investigated the changes in soybean lipids during tempe fermentation using R. oryzae mold. One of their conclusions was that the concentration of total ether-extractable lipids rose slightly at the time of most active mold growth (20-30 hrs) and then diminished. The acidity increased, but at the same time the pH also showed a steady increase throughout the fermentation time. This is presumably due to liberation of ammonia or other basic

Table 6.--Amino acid composition of tempe and unfermented soybeans (by HITACHI amino acid analyzer) (Murata, 1965).

Amino Acids	Unfermented Soybeans	(mg/gN) Tempe
Aspartic acid	744	756
Threonine	278	282
Serine	270	268
Glutamic acid	1049	1001
Proline	342	309
Glycine	292	275
Alanine	250	228
Cystine (M)	113	121
Valine	328	345
Methionine (M)	77	81
Isoleucine	338	356
Leucine	525	565
Tyrosine	171	161
Phenylalanine	302	302
Tryptophan (M)	67	87
Lysine	392	410
Histidine	160	167
(NH3)	140	169
Arginine	491	440
Nitrogen %	7.85	8.06

Note: (M) = microbiological assay using Leuconostoc mesenteroides.

end products of protein. The chemical characteristics of the oils of soybeans and tempe are presented in Table 7.

Table 7.--Chemical characteristics of oils from tempe and unfermented soybeans (Murata, 1965).

	Unfermented Soybeans	Tempe
Acid value	1.02	50.59
Refractive index n_D^{25}	1.4730	1.4711
Iodine value	128.5	126.1
Saponification value	191.1	189.2
Composition of fatty acids:		
$C_{16:0}$	10.4%	10.9%
$C_{18:0}$	5.1%	4.9%
$C_{18:1}$	26.8%	28.1%
$C_{18:2}$	50.0%	49.4%
$C_{18:3}$	7.8%	6.8%

The lipase activity of R. oryzae is also increasing during fermentation time. About one-third of neutral fat of soybeans is hydrolyzed by the fungal lipase, but there is no subsequent utilization of the liberated fatty acids. The fatty acid content of tempe during fermentation is shown in Table 8.

Steinkraus et al. (1961) and György (1961) detected and isolated an antioxidant produced during the tempe fermentation. They determined the peroxide values of dried, pulverized and stored soybeans ranged from 18.3 to 201.9,

Table 8.--Distribution of free fatty acids during tempe fermentation (Wagenknecht et al., 1961).

	Mg/100 g tempe					
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Cooked soybeans neutral fat	. .	. .	. .	. .	. .	
free fatty acids	41	31	127	0	0	199
24 hour tempe	420	175	713	2510	293	4111
30 hour tempe	771	202	802	2543	204	4522
48 hour tempe	665	202	1359	4138	304	6668
69 hour tempe	863	367	1671	5032	302	8235
	Mol percent					
Cooked soybeans neutral fat	10.86	3.90	18.11	61.00	6.13	
free fatty acids	22.24	15.21	62.53	0	0	
24 hour tempe	19.81	3.75	15.39	54.62	6.43	
30 hour tempe	18.23	4.31	17.24	55.08	4.45	
48 hour tempe	10.83	2.96	20.07	61.57	4.56	
69 hour tempe	11.37	4.36	19.98	60.62	3.67	

	Total free fatty acids g/100 g tempe	Percent free fatty acids of total ether extract
Cooked soybeans	0.26	1.09
24 hour tempe	3.59	13.87
30 hour tempe	4.77	18.93
48 hour tempe	6.93	30.00
69 hour tempe	8.19	35.11

while the peroxide values of tempe under identical conditions ranged from 0 to 1.1.

György et al. (1964) isolated by paper chromatography a compound (R_f=0.92) which proved to prevent the hemolysis of red blood cells of a vitamin E deficient rat with dialuric acid, as well as giving a positive ferric chloric reaction and Emmerie-Engel test.

This compound was subsequently identified as 6,7,4' trihydroxyisoflavone, which has λmax of 260 and 327 nm, and also is a normal constituent of unfermented soybeans. This antioxidant can be liberated from raw soybeans by hydrochloric acid hydrolysis. The hypothesis was that the liberation of trihydroxyisoflavone will protect the biologically effective vitamine E presents in soybeans. The effect of trihydroxyisoflavone itself in vitamine E deficient animal is still unknown.

Toxic materials produced in tempe "bongkrek"

Soybean tempe or locally called tempe kedele is never reported as the cause of any kind of food poisoning.

However, if the soybeans are mixed with coconut cake (copra cake) or prepared entirely from copra (for a cheaper, low quality tempe), the product bongkrek may occasionally become poisonous. These bongkrek poisons are very potent toxins. People die or become seriously ill after a consumption of the poisonous bongkrek even in small quantities (Van Veen and Mertens, 1934; Van Veen, 1935, 1936).

The occasional toxicity of bongkrek is attributed to Pseudomonas cocovenenans, an aerobic bacterium growing as a contaminant during the bongkrek fermentation.

Two primary substances, namely toxoflavin and bongkrekic acid, are responsible for the bongkrek toxicity (Figures 4 and 5).

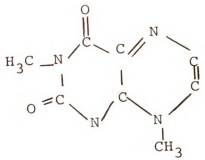


Figure 4. Toxoflavin (Van Damme et al., 1960).

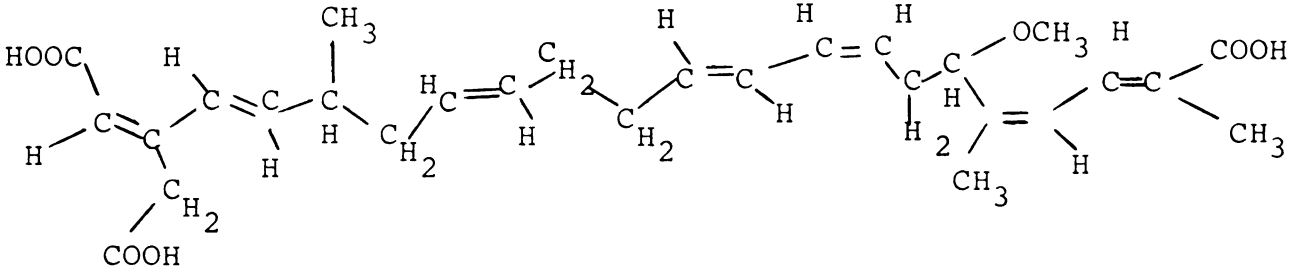


Figure 5. Bongkrekic acid (De Bruijn et al., 1973).

MATERIALS AND METHODS

Tempe preparation

The tempe was prepared by a method similar to that practiced in Indonesian villages and described by Hesseltine (1965).

The soybeans variety Harosoy 63 were supplied by the Michigan Foundation Seed Association Inc., East Lansing. The dry soybeans were soaked overnight in tap water at room temperature and the skin was removed by hand in running water. The soaking was proved to be essential by the fact that fermentation was about 100% successful, while unsoaked soybeans failed to ferment properly in 95% of the cases.

The peeled soaked soybeans were boiled in a steam jacketed kettle for 30 minutes, cooled and drained prior to inoculation with Rhizopus oligosporus NRRL 2710, which has been kindly supplied by the Northern Regional Research Laboratory, Peoria, Illinois. One agar slant culture of mold suspended in about 3 ml of sterilized distilled water was sufficient to inoculate 300 g of cooked soybeans.

The well mixed inoculated soybeans were then packed tightly into a stainless steel form, 25 x 10 x 3 cm, and wrapped with Dow Handiwrap plastic sheet perforated

with pinholes for air supply. For a smaller sample, the soybeans can be fermented in plastic bags.

The tempe was fermented at 30-31°C in an incubator. The relative humidity was maintained at about 45% by placing water in the incubator.

Samples for analyses were sliced from the fermenting tempe cake as desired.

Sample preparation for electron microscopy

Scanning electron microsocopy (SEM)

Sliced, dried tempe about 1/4 cm. thick and 1 cm. in diameter was mounted on a special metal setting and gold coated.

Raw tempe, similar in size to the dried one, was dipped in a 1% solution of O_sO_4 in phosphate buffer for two hours. It was then washed in solutions of increasing ethanol concentration, 25%, 50%, 75%, 85%, 95%, and twice at 100%, for half an hour in each solution. It was then dried in a Bomar SPC-900 EX critical point dryer and gold coated.

Transmittance electron microscopy (TEM)

Soybeans and tempe samples were cut into small strips about 0.5 x 0.5 x 2 mm. and placed in 5% glutaraldehyde fixative in 0.1 M Sorensen buffer, and they were left in this solution for about 4 hours, and then washed 3 times (about 1 hour) with 0.1 M Sorensen buffer solution.

Subsequently they were fixed in 1% buffered OsO4 solution, embedded in Spurr's resin, sectioned in Porter-Blum MT2 ultramicrotome, stained in uranylacetate and lead-citrate, mounted and examined in a Philips 300 electron microscope.

Extraction of lipids

For comparison, two methods of lipid extraction were used.

Method I

About 100 g tempe was sliced thinly and dried in a 29 inch vacuum oven at 70 C for 5 hours. The vacuum-dried tempe was then ground into a fine powder (20 mesh).

Fifteen grams of ground tempe was wrapped in Whatman filter paper and extracted with petroleum ether in a Soxhlet apparatus for 24 hours. The ether extract was filtered and washed with diethylether, dried under nitrogen, weighed and stored under nitrogen at 0°C for further analysis. Free fatty acids were separated according to Mattick and Lee (1959). After drying under nitrogen over sodium sulfate, the glycerides and free fatty acids were weighed. After appropriate esterification of the glycerides and the free fatty acids, each sample was injected in a Perkin Elmer 900 chromatograph, and SP 1000 liquid phase on Chromosorb was the packing material of the column. The temperature of the column was set at 200 C.

Method II

About five grams tempe was blended in a Waring blender for 2 minutes with 100 ml Chloroform : Methanol = 2:1 (v/v), according to the method of Folch et al. (1957).

The finely blended homogenate was filtered through Buchner vacuum filter on Whatman No. 1 filter paper. The lipid solution was then dried in a flash evaporator at 40°C. After most of the chloroform methanol solvent was evaporated, the free fatty acids were separated from the glycerides, as in Method I.

The glycerides and free fatty acids were then dried in a vacuum desiccator overnight and weighed. After esterification, each sample was injected in a Perkin Elmer 900 chromatograph, containing a DEGS column and programmed at 100-200°C.

Separation of free fatty acids from glycerides

The free fatty acids were extracted by the method of Mattick and Lee (1959). A mixture of standard free fatty acids (Applied Science Laboratories Inc.) was treated similarly. Figure 6 shows the steps in this extraction method.

Diazomethane preparation and esterification of fatty acids

Diazomethane was prepared in ether on the ice bath to a bright intensive yellow color as shown at Figure 7.

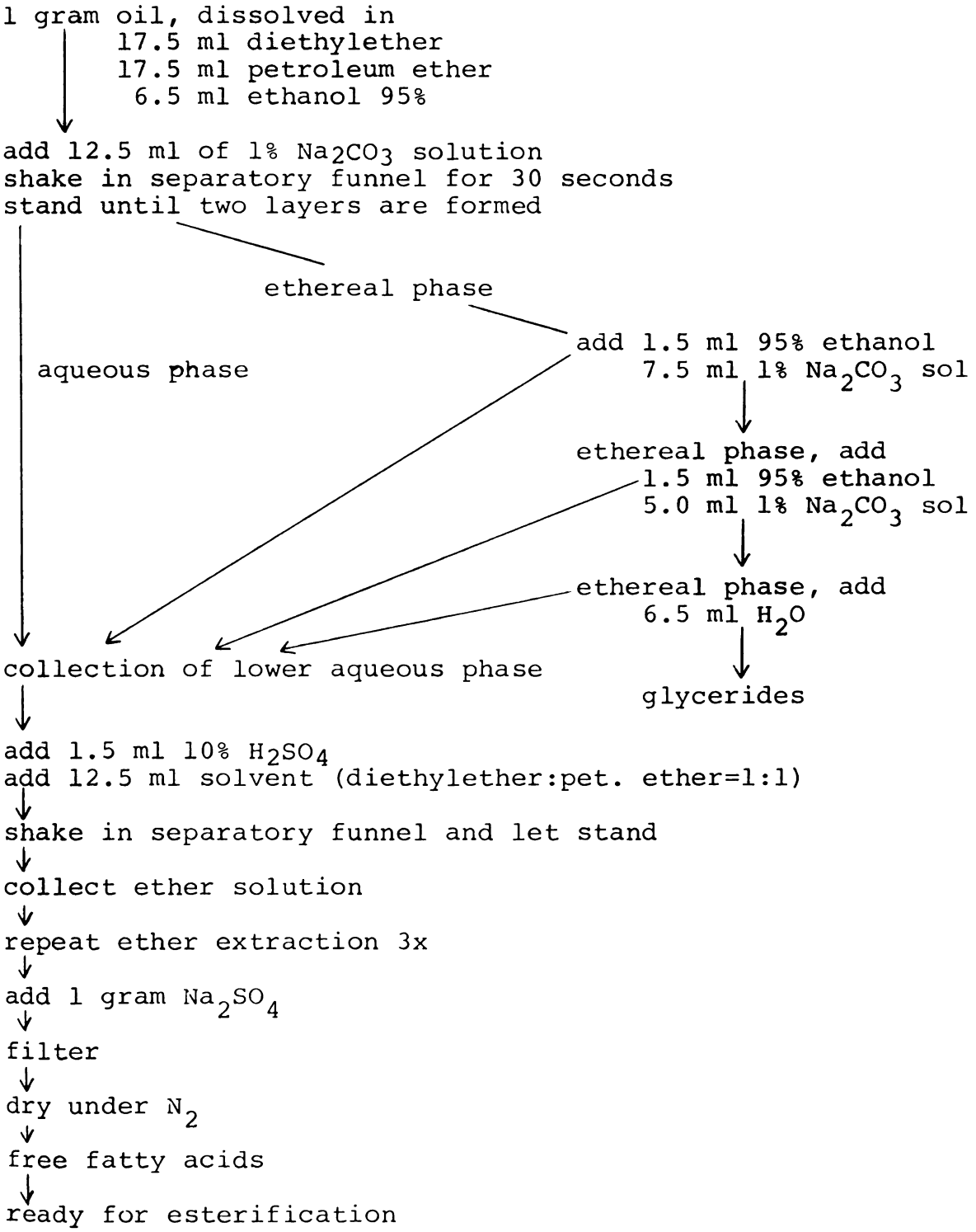


Figure 6. Free fatty acid extraction (Mattick and Lee, 1959).

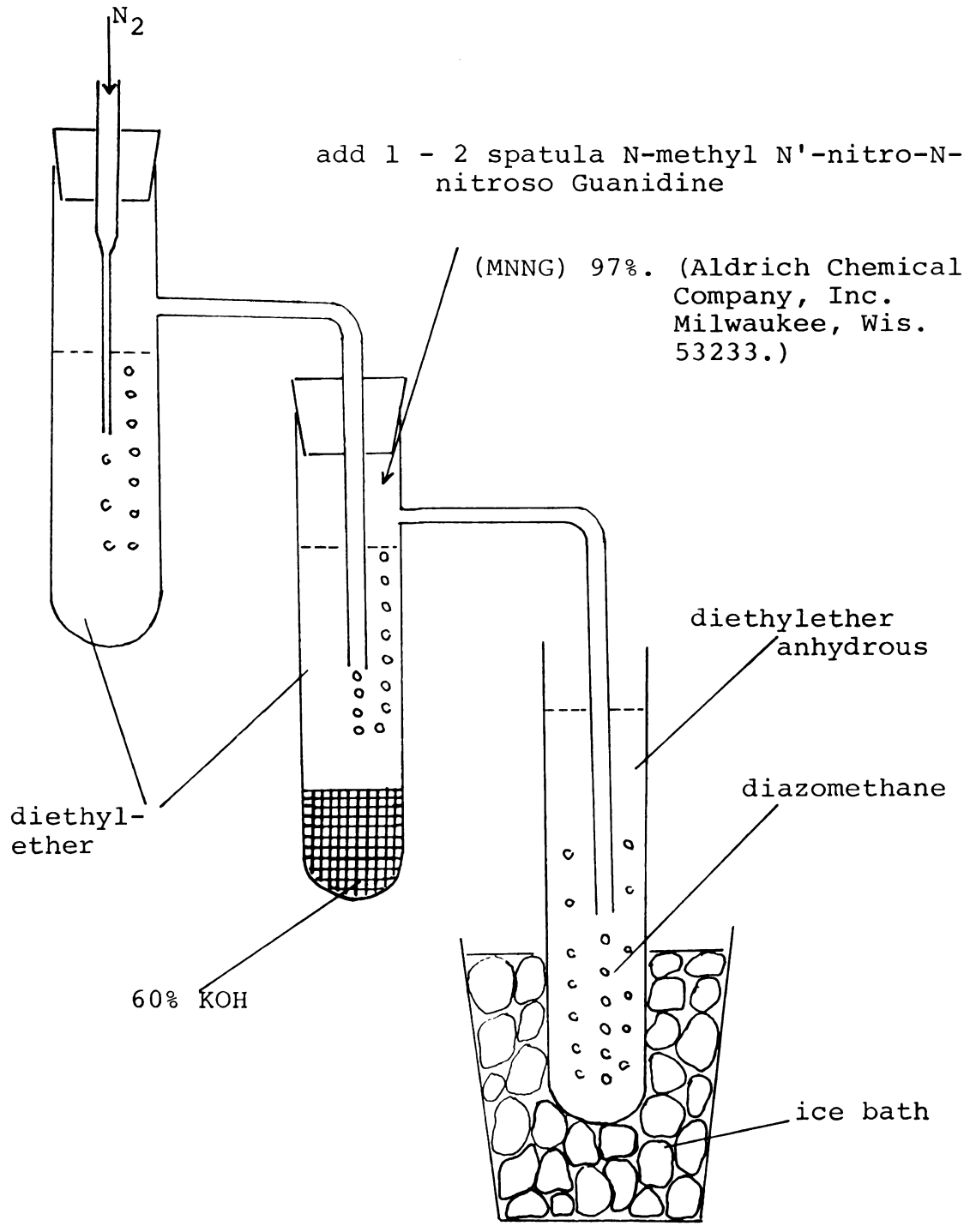


Figure 7. Diazomethane preparation (Schlenk & Gellerman, 1960).

About 2 ml of diazomethane solution was added to the fatty acid sample, and a few ml of 10% methanol solution in diethyl ether were added to speed up the reaction. Yellow color will persist when bubbling stops in about 15 mins. at room temperature (25°C) if a sufficient amount of diazomethane was added. When the bubbling stops, blow the excess diazomethane and ether under N_2, then dissolve the fatty acid methyl ester in diethyl ether to a desired volume (10 ml). A few µl of the ester solution was ready for injection in the Gas Chromatograph.

Transesterification of glycerides

The glycerides remaining after the extraction of the free fatty acids were transesterified using the method of Mason and Waller (1964a) and Mason et al. (1964b). Approximately 50 mg of glycerides were added to a reagent consisting of 3.5 ml dry benzene, 0.25 ml 2,2 dimethoxypropane (DMP) and 1.25 ml methanolic-HCl in a 15 ml screw-cap vessel. After mixing to allow an even reaction, the mixture was left overnight, or at least for 6 hours, at room temperature (22°C) to ensure a complete transesterification. The entire sample was neutralized in 30 min. with one gram of neutralizing agent consisting of sodium bicarbonate, sodium carbonate and sodium sulfate anhydrous mixed in the proportions 2:1:2. This neutralizing agent was dried overnight before use at 110°C.

A few µl of this solution was injected into a gas chromatograph for fatty acid ester analysis. Pure glycerol was treated similarly and served as a standard elution peak as isopropylidineglycerol (Mason et al., 1964a).

Deep frying of tempe

The tempe was cut to pieces 2.5 x 7 x 0.5 cm, weighed and deep-fried for 3-4 minutes in 150 ml coconut oil in a 1000 ml glass beaker on a hot plate. The temperature of the oil was 180-210°C. The fried tempe was drained at room temperature, weighed and the oil was extracted and analyzed.

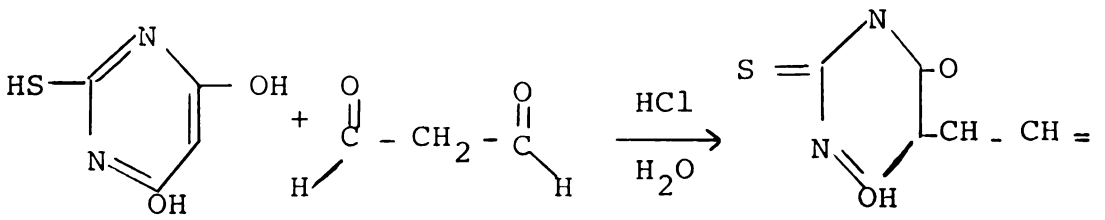
Total plate count

To follow the growth of bacterial population in each step of tempe preparation and fermentation, total plate count procedure was used.

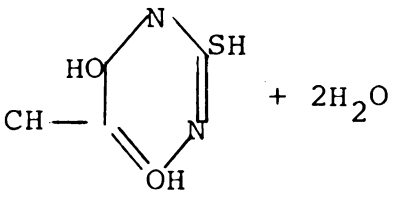
50 g sample was blended in a sterile blender with 450 ml of 0.1% peptone dilution blank for 1 minute. One ml of sample suspension was transferred into 99 ml dilution blank, and repeated until the desired dilution was obtained (about 10^{-5}-10^{7}). One ml of the diluted sample was placed in a sterile petri dish, mixed with warm Bacto Plate Count Agar, and incubated at 30°C for 48 hours. To prevent the growth of mold from tempe, 100 ppm Actidione (Upjohn Company) was added to the agar medium. The bacterial count was computed per gram of sample.

TBA method for determination of oxidative stability

Proposed TBA reaction (Sinnhuber et al., 1958a; Dahle et al., 1962)



TBA Malonaldehyde TBA chromogen



Patton et al. (1951) identified the active color-producing compound in TBA (2 thiobarbituric acid) reaction with rancid oil was malonaldehyde. Sinnhuber et al. (1957, 1958a, 1958b) suggested that two molecules of TBA condensed with one of malonaldehyde to produce the color substance. Some of the advantages of TBA test compared to other tests for rancidity are as follows:

1. The test can be performed on the whole food rather than on extracted fat.
2. Therefore the test can be expected to measure oxidation products of protein-bound lipids and phospholipids which would not be extracted by ordinary fat solvents.

3. The TBA test correlates better to the organoleptic rancidity testing (correlation coefficient, r_s = 0.89) than the peroxide or carbonyl tests.

One disadvantage is that malonaldehyde appears to contribute a relatively small fraction of the total odor complex of rancid sample, and by itself does not taste or smell bad (Tarladgis et al., 1960; Sidwell et al., 1954).

The test procedure involved the heating of the food sample with a strong acid solution. This step appears to be essential for the liberation of malonaldehyde from some precursors as well as for the condensation of malonaldehyde with TBA. Tarladgis et al. (1960) developed the distillation method for TBA test. They investigated the factors that might affect the maximum liberation of malonaldehyde:

1. pH of the material to be distilled
2. effect of time of heating during distillation
3. amount of distillate collected

And they have concluded that maximum optical density was obtained at pH 1.5 for both standard solution of 1,1,3,3-tetraethoxypropane (TEP) which yields malonaldehyde on acid hydrolysis and cooked meat sample. The longer the time of heating during distillation will provide the increased color development on meat sample, but decreased on standard TEP. It was thought that fat oxidation may occur in meat samples during the prolonged distillation time. In the TEP standard the greatest amount of malonaldehyde was obtained when the distillate was collected in the shortest time

possible (i.e., the highest heating available). From 100 ml of water blended sample, the maximum optical density was obtained when 50 ml of distillate was collected.

The heating time of the distillate and TBA reagent mixture was 35 minutes in a boiling water bath. Distillate can be held over 24 hours at room temperature with very little change. However, after the reaction of the distillate with TBA, the optical density will increase about 10%, if the solution is held for 24 hours.

Reagents

TBA Reagent: 0.02 M 2-thiobarbituric acid in 90% glacial acetic acid. Bring into solution by warming slightly in a boiling water bath.

HCl Solution: 1 part of concentrated HCl to 2 parts of distilled water (approx. 4N).

Procedure

Blend a 3 g carefully weighed sample, with 50 ml distilled water in Waring Blender for 2 minutes. Transfer the mixture to a distillation flask (1000 ml) quantitatively by washing with an additional 48.5 ml distilled water. Add 1.5 ml of HCl solution to bring the pH to 1.5. Add anti-foam agent and few boiling chips, assemble distillation apparatus with the highest heating available. In approximately 10 minutes, 50 ml of distillate was collected. Mix the distillate, filter, and pipette 5 ml into 50 ml glass stoppered flask and add 5 ml of TBA reagent. Mix the content and immerse in a boiling water bath for 35 minutes.

A distilled water-TBA reagent blank should be prepared and treated like the samples.

After heating, cool in tap water for 10 minutes, transfer a portion to a cuvette and read the optical density of the sample against the blank at the wavelength of 528 nm. The optical density is used as an arbitrary unit to compare the stages of rancidity of the samples.

Sensory interpretation of TBA test value

To make the values of the TBA test meaningful, the following sensory test was used for relating rancid flavor to TBA values.

About 2 lbs. of dried soybeans were soaked overnight, peeled by hand and then boiled for 30 minutes. The boiled soybeans were blended in a Waring blender in about 2 liters of distilled water. The slurry was dried in oven at 200°F overnight on shallow pans. The dried mass was then ground in Arthur Thomas mill, 20 mesh screen. This soybean powder was used as a base for sensory evaluation.

Rancid flavoring was prepared from diluted linoleic acid in ethanol by bubbling oxygen overnight at 100°C. The ethanol was then evaporated.

Samples were prepared by mixing about 25 g soybean flour base with rancid linoleic acid in quantities from 0 to 1 ml in individual screw capped jars.

The samples were presented to a test panel consisting of ten members. The instruction sheets, standard

rancid linoleic acid and soybean flour base were provided as reference.

The values were tabulated, using arbitrary values as follows:

- no rancidity = 0
- weak rancidity = 1
- strong rancidity = 2

Fractions of those numbers were used if a member's decision was in between those three categories.

Phytic acid determination

The phytic acid contents of soybeans and tempe were determined by using a combination of the methods of Makower (1970) and Wheeler and Ferrel (1971).

1. Weigh accurately 2 g finely ground dry sample (Cyclone Sample Mill, about 40 mesh) into a 125 ml Erlenmeyer flask.
2. Extract with 40 ml of 3% trichloroacetic acid (TCA, CCl_3COOH) for 45 minutes under mechanical shaking.
3. Centrifuge the suspension at 12,000 x G for 10 minutes.
4. Transfer 10 ml aliquot of the supernatant into a centrifuge tube.
5. Add 5 ml $FeCl_3$ solution (containing 2 mg. ferric ion per ml in 3% TCA), to the aliquot by blowing rapidly from the pipet.

6. Heat the tube and content in a boiling water bath for 1 hr. If the supernatant is not clear after 30 minutes add 1 or 2 drops of 3% sodium sulfate in 3% TCA and continue heating.
7. Centrifuge for 10-15 minutes at 12,000 x G and carefully decant the clear supernatant.
8. Wash precipitate twice by dispersing well in 20 ml 3% TCA, heating in a boiling water bath 5 to 10 minutes, and centrifuging.
9. Repeat wash once with H_2O.
10. Disperse precipitate in 5 ml H_2O and add 5 ml 0.6 N NaOH.
11. Heat in boiling water for 45 minutes to coagulate $Fe(OH)_3$.
12. Centrifuge for 10-15 minutes at 12,000 x G and decant carefully.
13. Wash precipitate with H_2O, recentrifuge and decant.
14. Precipitate was dissolved in 5 ml 0.5 N HCl with heating in boiling water for 10-15 min. until clear yellow color of $FeCl_3$ was obtained.
15. Transfer to 100 ml volumetric flask and make up to volume with 0.1 N HCl.
16. Fe analysis

    Transfer 1 ml of solution from step 15 to a 25 ml volumetric flask, add 1 ml 10% hydroxylamine (NH_2OH-HCl) solution, rotate flask and let stand few minutes. Add 9.5 ml 2M NaOAc solution and

1 ml O-phenanthroline solution (0.1 g/100 ml). Dilute with H_2O to volume and mix. Let stand at least 5 min. and read at 510 nm against a water blank.

Phosphorus determination
(Murphy and Riley method, 1962)

Reagent A:

Dissolve 12 g of ammonium molybdate in 250 ml distilled H_2O. In 100 ml distilled H_2O dissolve 0.2908 g of antimony potassium tartrate. Add both of dissolved reagents to 1000 ml of 5N H_2SO_4 (148 ml concentrated H_2SO_4 in 1 liter), mix thoroughly and make to 2000 ml. Store in Pyrex glass bottle in a dark and cool compartment.

Reagent B:

Dissolve 1.056 g of ascorbic acid in 200 ml of reagent A and mix. This reagent should be prepared as required as it does not keep for more than 24 hours.

Pipette aliquot to be tested (0.5 ml) into 5 ml volumetric flask. Add 0.8 ml reagent B, bring to volume with distilled H_2O. Read at 700 nm against a reagent blank.

Protein evaluation methods

Protein evaluation is most accurately carried out by feeding tests using the same animal species for which the protein is intended as a regular feed. Since this

seldom can be accomplished (especially for man), more convenient methods were developed using standard animal species (in vivo tests) and also purely chemical tests (in vitro tests).

No single protein test is absolute since there are variations between tests. These variations arise from differences in strains or varieties, from variations in raw material sources and from changes induced by processing. Some of the frequently used standard animal tests are the following:

1. The Protein Efficiency Ratio (PER) is the gain in weight of a growing animal divided by its protein intake. Disadvantages of PER test are (a) PER is not a true efficiency ratio because not all the protein is used for growth, only that consumed above maintenance, and (b) varies with the acceptability of the feed.
2. The Biological Value (BV) is determined by nitrogen balance and is defined by the ratio of nitrogen retained/nitrogen absorbed.
3. The Net Protein Utilization (NPU) is the product of the coefficient of digestibility and the BV, and therefore represents the proportion of food nitrogen retained (nitrogen retained/nitrogen intake).

The in vitro tests were designed to save time, space and expense, some of which are described below:

1. The Chemical Score is determined by comparing the protein amino acid composition with that of a reference protein (whole egg protein). The chemical score is equal to the greatest percentage deficit in an essential amino acid of the protein being evaluated.
2. Protein Score (FAO/WHO procedure) is the ratio between the % limiting essential amino acid to the % corresponding amino acid of the reference pattern.

$$\% \text{ essential amino acid} = \frac{\text{essential amino acid}}{\text{total essential amino acid}} \times 100\%$$

3. Chemical methods for example: Urease Inactivation, Protein solubility test, Enzymatic and Microbiological tests.

Procedure for the protein efficiency ratio test

In this present study of protein tempe evaluation the PER test was used because of its simplicity and popularity. This method was developed by Osborne et al. (1917). There were four feed samples:

1. High Protein Casein (Teklad Test Diets Company).
2. Tempe, prepared as described in the beginning of this chapter, sliced, fried, most of the oil then extracted with hexane, and ground.
3. Soybeans, prepared as the tempe but without fermentation.

4. Sesame seed supplemented tempe. Nine part of boiled soybeans were mixed with one part of ground sesame seed, fermented and treated as the tempe. The diets were prepared according to the AOAC method (Horwitz, 1970).

The nitrogen content of the food was determined by the Micro Kjeldahl method, and the oil content by the Goldfisch diethyl ether extraction method. Protein = 6.25 x N.

All feeds were standardized to meet the following composition:

Protein	10%
Oil	8%
Salt mixture USP	5%
Vitamin mix	1%
Cellulose	1%
Corn starch to make	100%

Casein: High Protein Casein (Teklad Test Diets Company, 2826 Latham Dr., Madison, WI 53713).

Oil: Pure Corn Oil (Miesel Company, Detroit and Cleveland).

Salt: Salt Mixture, USP XVII (catalog no. 170890, Teklad Test Diets).

Vitamin: Vitamin Mix AOAC, Teklad Test Diets, cat. no. 40055.

Cellulose: Non nutritive Fiber, cat. no. 160390, Teklad Test Diets.

Corn Starch: Staley Company, Oak Brook, Ill.

Experimental animal

Twenty-four Spargue-Dawley male rats, were supplied by the Spartan ResearchAnimal Inc., Haslett, Michigan. Age: 21 days, weight: 51 g average. Acclimation period: 4 days on standard casein diet. At the end of acclimation period (beginning of test period), six rats were assigned into one group. Weight of rats in each group: 362.5 g, average weight of rat 60.4 g.

Assay period

The rats were kept in individual cages and both diet and water were provided ad libitum. Body weight and food intake for each rat were recorded every 3 days. The experiment was terminated 28 days from the beginning of the assay period.

Calculation

Calculate average 28 day weight gain and protein intake per rat for each group. Calculate Protein Efficiency Ratio (weight gain/protein intake) for each group. Report quality sample ratio/casein ratio x 100%.

RESULTS AND DISCUSSIONS

Electron Micrographs of soybeans and tempe

From the scanning electron micrograph (Figure 8) the pallisade-like cells of boiled soybean cotyledon measured about 25 x 100μ. These large cells contain mostly the protein bodies (aleuron grains) and the spherosomes.

The protein bodies (PB, Figure 9) which measure about 2 to 20μ contain proteins, mostly glycinin, but also RNA, phytic acid and phospholipids. Neutral lipids are not an appreciable part of the protein bodies.

The protein bodies are storage particles which disintegrate on germination (Tombs, 1961, Wolf, 1970). The oil is located in the smaller structures called spherosomes (S, Figure 9) which are interspersed between protein bodies and are 0.2 to 0.5μ in diameter.

After soaking overnight, the protein bodies and the spherosomes were still confined in the cells and no indications of breaking of the cell walls, Figure 9. However, after boiling the soaked bean for 30 minutes, the protein bodies and the spherosomes were located in the cell wall areas (Figure 10). The disruption of the cell wall was

probably essential to facilitate the fast growth of the tempe mold. The photomicrographs of tempe (Figures 11A through e) show various stages of cell disruptions, from cell completely still intact, cell wall partially broken and some already completely lost their cell contents.

The scanning electron micrographs (Figure 12 and 13) show that the Rhizopus mold did not penetrate the soybeans more than two cell layers. Therefore, enzymes must be excreted by the mold deep into the bean mass to facilitate the chemical changes during tempe fermentation. An example of such excretion of enzyme is shown in Table 36.

Chromatography of standard fatty acids

Method I: Chromatographic profile of standard fatty acids mixture on SP 1000 column is presented in Figure 14. The conditions were set as the following:

Column:
length: 6 feet
diam. : 1/8"

Sample: 1% solution standard ffa in diethyl ether (Applied Science Laboratories Inc., Ann Arbor)
Size: 3.8 µl

Liquid phase: SP1000
wt : 10%
Support : Chromosorb W/AW
mesh : 100/120
Carrier gas : N_2
rotameter : 2.5
inlet press : 50
Chart speed : 30"/hr
Detector : FID
Flow rate H_2: 24
air : 50

50μ

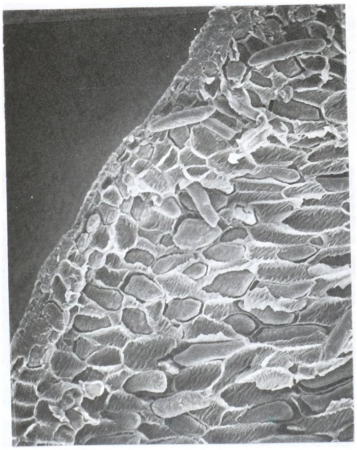


Figure 8. Scanning electron micrograph of boiled soybeans (magnification 200x).

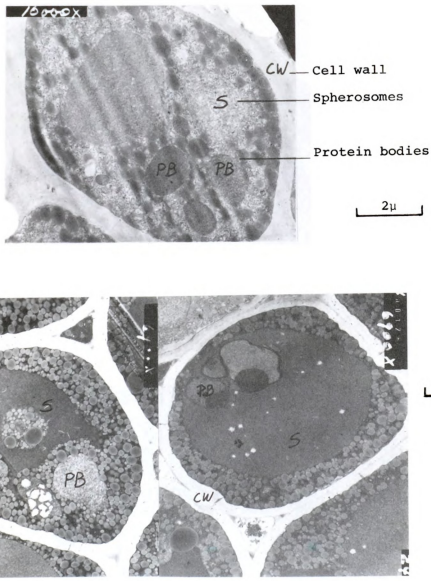
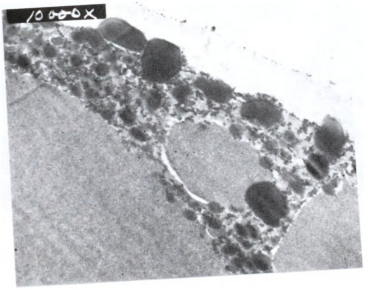


Figure 9. Transmittance electron micrograph of soaked soybeans.



2μ

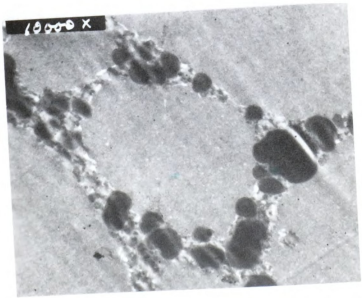
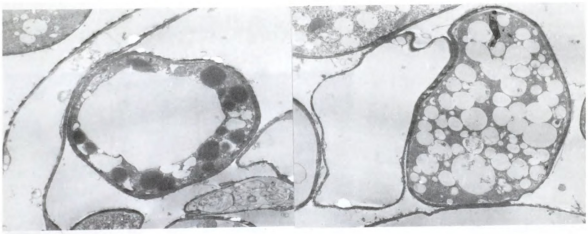


Figure 10. Transmittance electron micrograph of boiled soybeans (magnification 10,000 x).



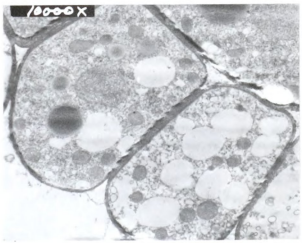
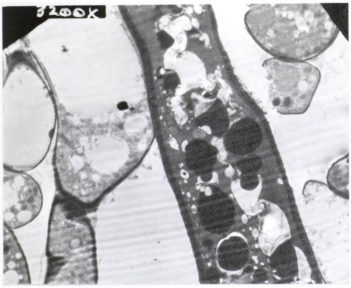
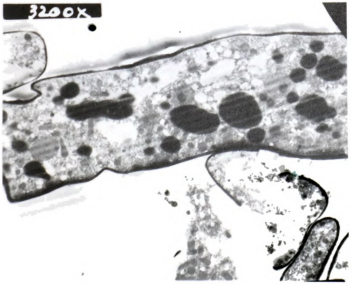


Figure 11. Transmittance electron micrographs of tempe.

Magnification 3,200 x

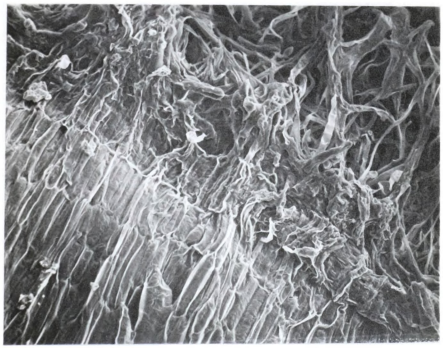


6.25μ



6.25μ

Magnification 160 x



50μ

Figure 12. Scanning electron micrograph of dried tempe.

50μ

Magnification 200 x

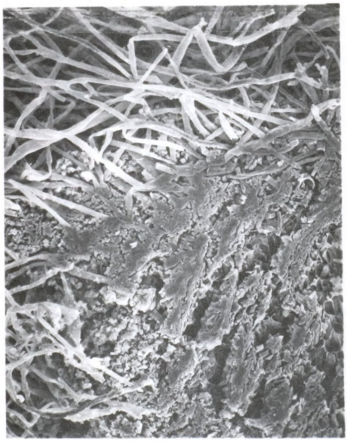


Figure 13. Scanning electron micrograph of soybean tempe.

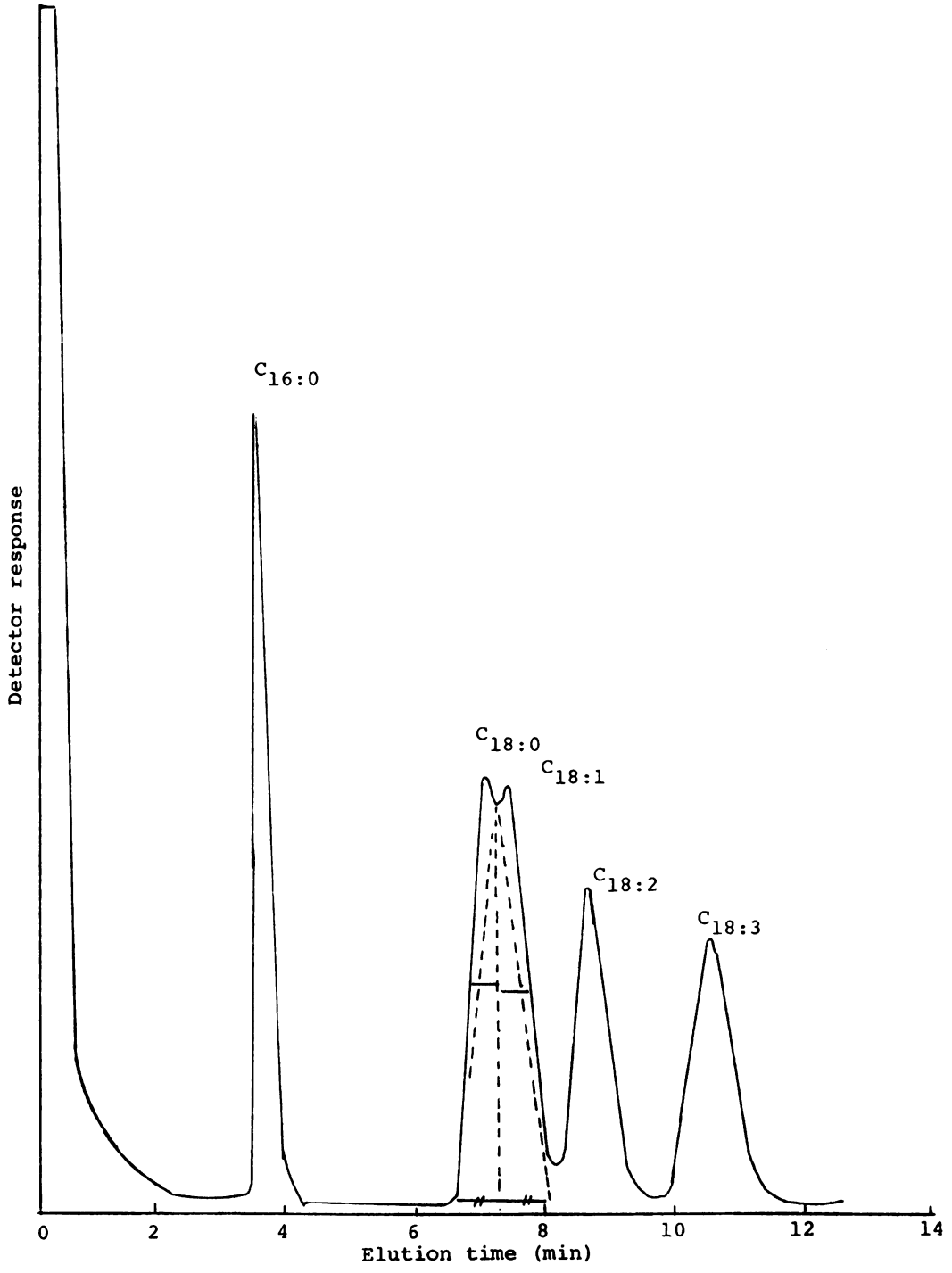


Figure 14. The chromatographic profile of standard fatty acid mixture on a 10% SP 1000 column.

Temperature

detect: 240°C

inject: 240°C

column: 200°C

Range : 10x

Attenuation : 128x

Standard free fatty acids, 100 mg extracted as in procedure, esterified and diluted to 10 ml in diethyl ether. Sample injected: 3.8 µl = 38 µg. Atten: 128X.

The height (h) and width (w, determined at half height) of each peak were measured and from the values of h x w, the proportion of each peak which represented the individual fatty acid was determined.

The results are as the followings:

Peaks	height cm	width cm	h x w	%	µg
$C_{16:0}$	15.7	0.30	4.71	20.9	7.9
$C_{18:0}$	8.4	0.55	4.62	20.5	7.8
$C_{18:1}$	8.3	0.55	4.56	20.2	7.7
$C_{18:2}$	6.4	0.70	4.48	19.9	7.6
$C_{18:3}$	5.2	0.80	4.16	18.9	7.0
			22.53		38.0

Response Factor

$C_{16:0}$: 7.9/4.71 = 1.68

$C_{18:0}$: 7.8/4.62 = 1.69

$C_{18:1}$: 7.7/4.56 = 1.69

$C_{18:2}$: 7.6/4.48 = 1.69

$C_{18:3}$: 7.0/4.16 = 1.68

Because of the poor separation between stearic and oleic acids ($C_{18:0}$ and $C_{18:1}$), the height and width of these two peaks were determined as in Figure 14.

The proportion of fatty acids determined by this present method was very closely in agreement with the reference given by the company which supplied the standard fatty acid mixture.

Method II: Chromatographic profile of standard fatty acid mixture on DEGS-PS column is presented in Figure 15.

The conditions were set as the following:

Column:
- length : 6 ft.
- diam. : 1/8"

Sample: standard free fatty acids, extracted, and dissolved in diethyl ether (1% solution)

Temperature:
- detector: 240°C
- injector: 240°C
- column :
  - init: 100
  - final: 200
  - rate: 10°/min

Liquid phase : DEGS-PS
- wt. : 10%

Support : Supelcoport
- mesh : 80/100

Carrier gas : N_2

Rotameter : 2.5
- inlet press: 50

Chart speed : 30"/h4

Detector : FID

Flow rate H_2 : 24
- air : 50

Range : 10x

Attenuation : 128x

The separation between stearic and oleic acids was much better on the DEGS-PS column than on the previous SP 1000 column (Figure 15 and 14).

The proportion of fatty acids in Method II was determined as in Method I, and similar results were obtained. Standard free fatty acid mixture, 100 mg was extracted as in the procedure esterified and diluted to 10 ml diethyl ether (1% solution).

Sample injected : 2.2 µl = 22 µ gram.

Percentage calculation of each peak.

Peaks	height	width	h x w	%	µ gram
$C_{16:0}$	23.5	0.10	2.35	19.76	4.35
$C_{18:0}$	14.0	0.18	2.45	20.61	4.53
$C_{18:1}$	11.8	0.20	2.36	19.85	4.37
$C_{18:2}$	9.45	0.25	2.36	19.85	4.37
$C_{18:3}$	7.40	0.32	2.37	19.93	4.38
				100.00	22.00

Response factor:

$C_{16:0}$: 4.35/2.35 = 1.85

$C_{18:0}$: 4.53/2.45 = 1.85

$C_{18:1}$: 4.37/2.36 = 1.85

$C_{18:2}$: 4.37/2.36 = 1.85

$C_{18:3}$: 4.38/2.37 = 1.85

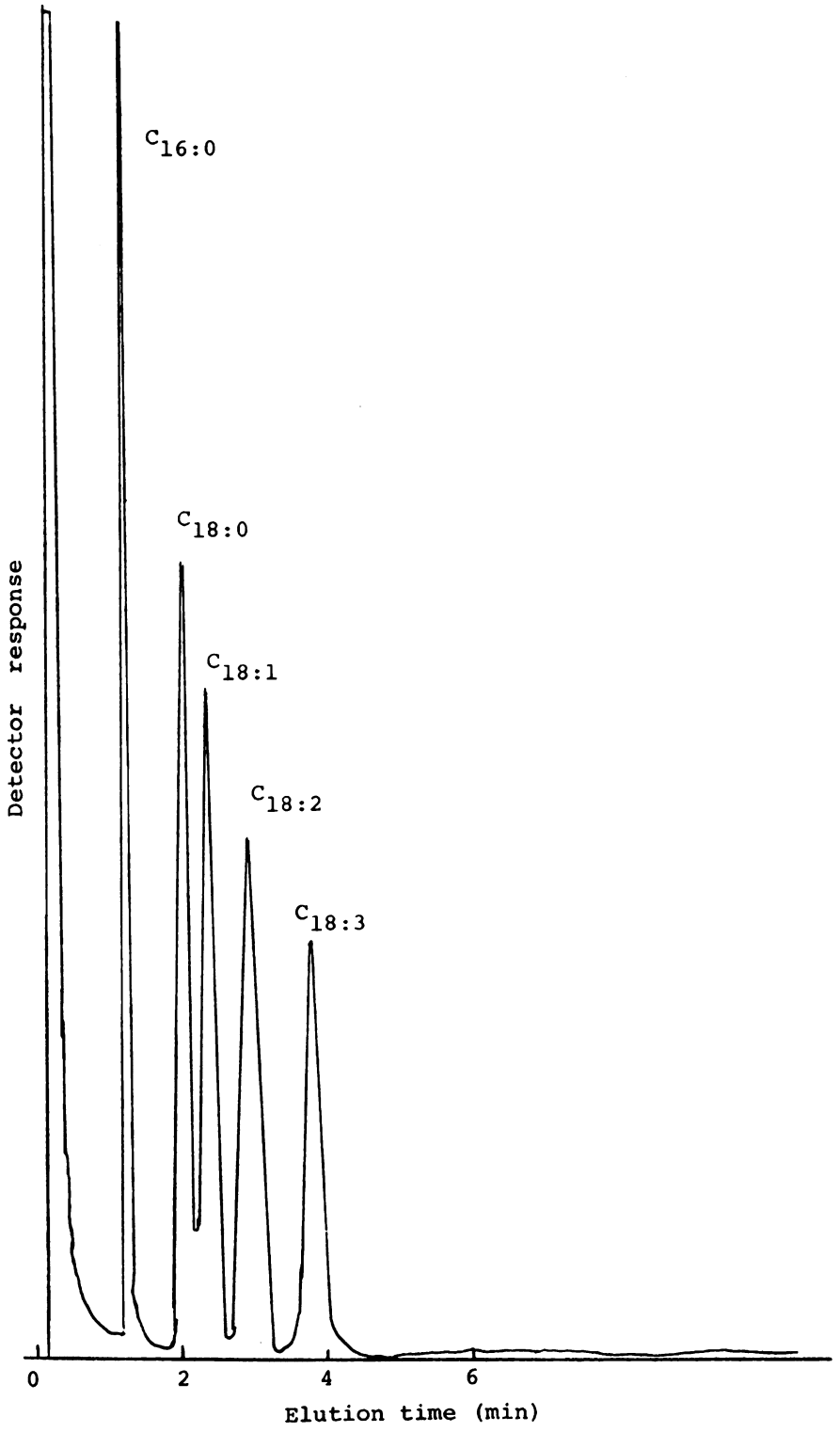


Figure 15. The chromatographic profile of standard fatty acid mixture on a 10% DEGS-PS column.

Changes in fatty acid profile of tempe during fermentation

The crude oil, free fatty acids and water contents of tempe during fermentation are presented in Tables 9, 10a and 10b.

Table 9.--Water and crude oil contents of soybeans and tempe.

Sample	Water (%)	Crude oil (% dry basis)
Boiled soybeans	67.0	31.87
18 hr. tempe	61.8	26.48
24 hr. tempe	60.8	22.15
30 hr. tempe	62.8	23.40
40 hr. tempe	63.6	23.54
50 hr. tempe	63.2	25.41
70 hr. tempe	63.8	25.50
90 hr. tempe	63.6	22.38
112 hr. tempe	65.4	19.02

From the two fatty acid analysis methods, obviously Method II was better than Method I in response sensitivity as well as the separation between fatty acids (Figure 14, 15 and Tables 10a and 10b). Based on the observations of Murata (1965), Wagenknecht et al. (1961) and Steinkraus and co-workers (1960, 1961) that the amino acids composition changed only insignificantly and carbohydrates only changed in their solubility properties, therefore, the most

Table 10a.--Free fatty acids in soybeans and tempe, method I (mg in 100 g moisture free sample).

Sample	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Raw soybeans	3.1	0.2	2.7	10.9	1.0	17.9
Boiled soybean	0.3	0.0	0.2	1.3	0.1	1.9
18 hr. tempe	187.5	37.0	371.8	641.2	119.7	1357.2
24 hr. tempe	322.3	74.3	754.9	1455.7	193.9	2801.1
30 hr. tempe	432.6	134.6	1346.0	1961.1	271.0	4145.3
65 hr. tempe	529.9	151.3	1512.5	1904.6	250.5	4348.8
72 hr. tempe	788.4	104.3	2427.8	3054.7	389.3	6764.5
96 hr. tempe	1078.3	277.2	2906.1	3335.6	494.6	8091.8

Table 10b.--Free fatty acids in soybeans and tempe, method II (mg in 100 g moisture free sample).

Sample	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Raw soybean	12.98	2.10	10.49	31.48	5.56	62.61
Boiled soybean	trace	trace	trace	trace	trace	. .
18 hr. tempe	333.77	125.65	541.88	831.15	202.88	2,035.33
24 hr. tempe	433.67	232.60	988.88	1374.54	213.11	3,242.80
30 hr. tempe	725.89	257.89	1485.75	2057.14	302.70	4,829.35
40 hr. tempe	578.63	247.25	1449.45	1857.86	286.76	4,419.95
50 hr. tempe	463.48	234.02	1150.38	1787.39	265.65	3,900.92
70 hr. tempe	890.50	352.60	2284.70	3412.43	505.36	7,445.59
90 hr. tempe	1107.58	598.63	3344.40	4878.57	749.01	10,678.19
112 hr. tempe	901.45	531.16	2359.02	3988.44	451.50	8,231.57

possible simple criteria of microbial or physiological activity of tempe fermentation was to observe the changes of free fatty acid being liberated and also the temperature.

Tables 10a and 10b show that free fatty acids increased steadily during fermentation beginning from zero time to about 30 hours. After 30 hours the free fatty acid content of tempe remained essentially constant up to 50-65 hours. Beyond that time the free fatty acids increased again and deterioration signs appeared, i.e., loss of previously pleasant taste, ammonia smell, darkening of color, stickiness and general collapse of tempe structure. The free fatty acid continued to accumulate until the 90th hour; thereafter the tempe started drying and the free fatty acid content started to drop. Therefore essentially there were three phases in tempe fermentation:

Phase I (0 - 30 hr.) : Rapid Phase

Phase II (30- 60 hr.) : Transition Phase

Phase III (beyond phase II) : Deterioration Phase

Based on the data of Tables 10a and 10b, Figure 16 was drawn which shows the three phases of tempe fermentation.

The contaminating organisms which survive from boiling process, mainly sporeforming Bacilli, multiply rapidly from 0 time to 19 hrs. with a generating time (g) of about 1.6 hrs. and then decline somewhat to a new exponential growth rate with a generating time of about 6-7 hrs. (Figure 17). The contaminating Bacilli are shown in the transmittance electron micrographs, Figure 18, from

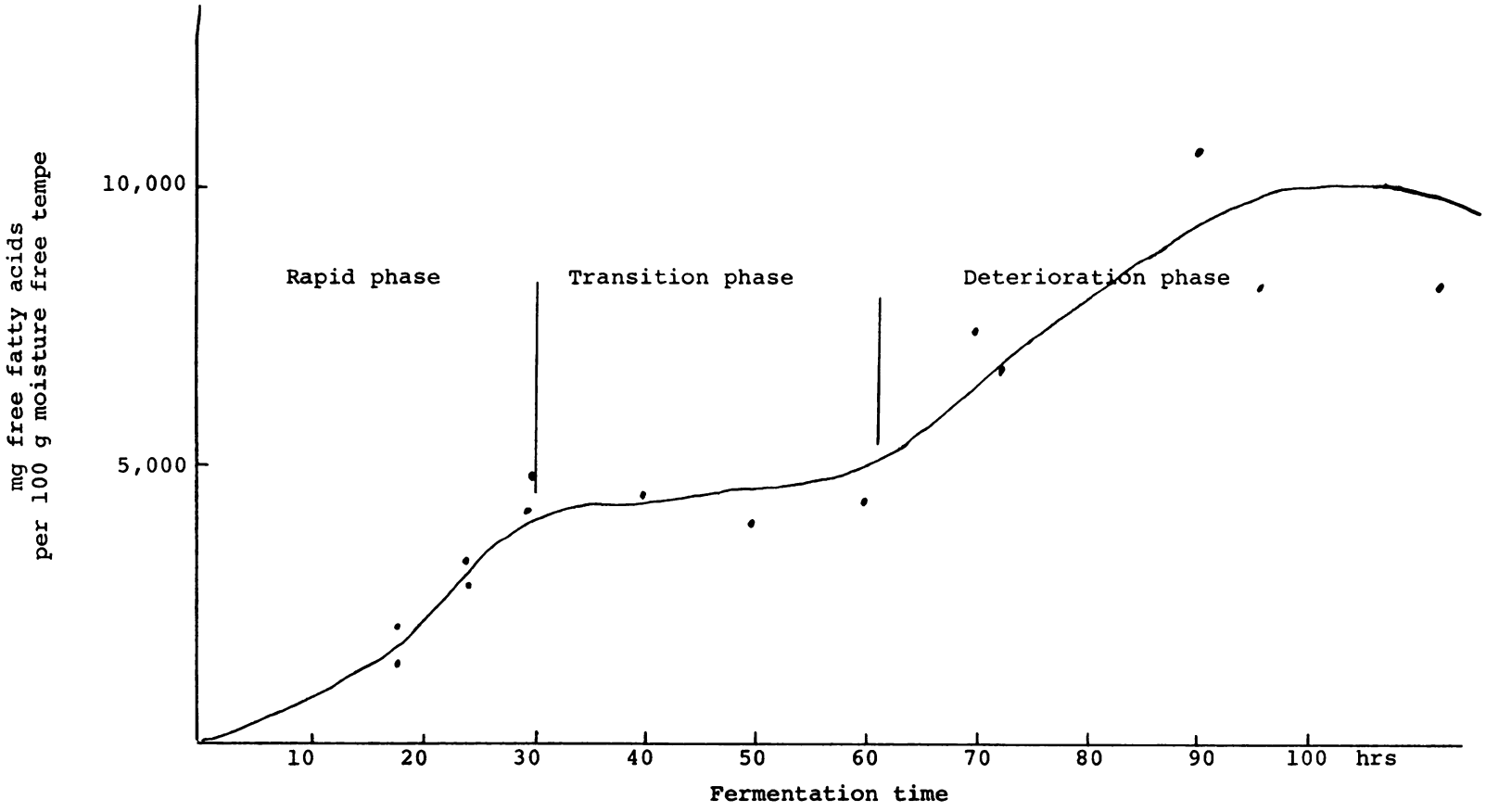


Figure 16. Total free fatty acids produced during fermentation (data from Tables 10a and 10b).

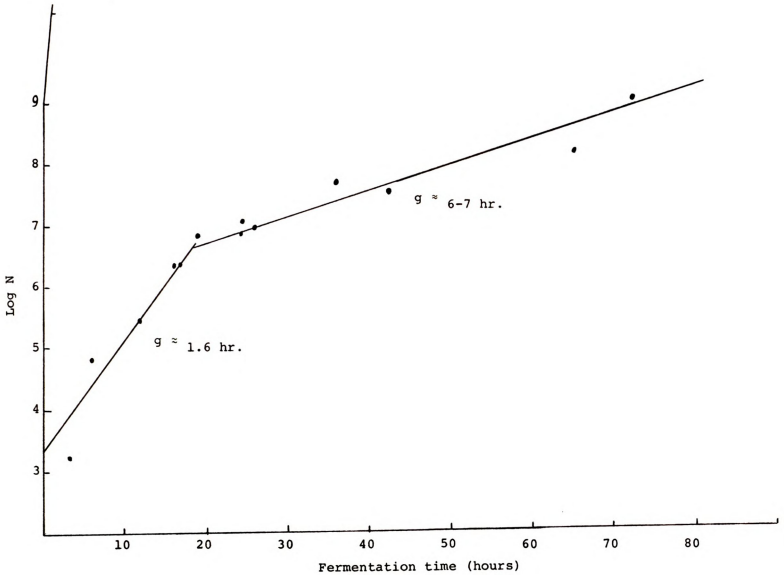


Figure 17. Total bacterial count in tempe during fermentation (total count/g tempe).

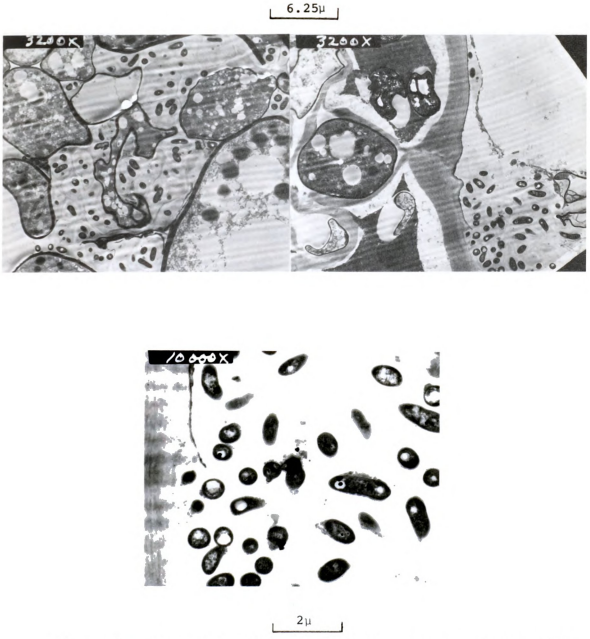


Figure 18. Transmittance electron micrograph of contaminating bacteria in tempe.

a 48 hr. tempe. Afterwards, repeated efforts to photograph this microorganism from tempe were unsuccessful. Most of the Bacilli were identified later as B. licheniformis and the rest were B. cereus (Stevenson, 1975).

The fast growth rate of Bacilli coincided with the rapid phase of tempe fermentation (Figure 16 and 17) and with the slow phase of temperature increase (Figure 19), up to the 19th hour. The second half of the rapid phase from the 19th hour to the 30th hour, the temperature rose rapidly and at this time the Bacilli entered their new slower growth phase.

The increase of temperature itself which never exceeded 45 C (Figure 19) was unlikely to cause the retardation of the growth of the thermoresistant spore-formers. One possibility cause of the slowering growth rate of the sporeformers was antibacterial compounds produced by the tempe mold especially during its peak of fermentation activity as already suggested by Wang et al. (1969).

Up to the 30th hour of fermentation, the tempe mold was likely the dominant organism in splitting the soybean lipids to produce free fatty acids (Figure 16). At this time, the temperature of tempe started to fall (Figure 19), showing the declining activity of the mold; the content in free fatty acids leveled off and even decreased for some of them.

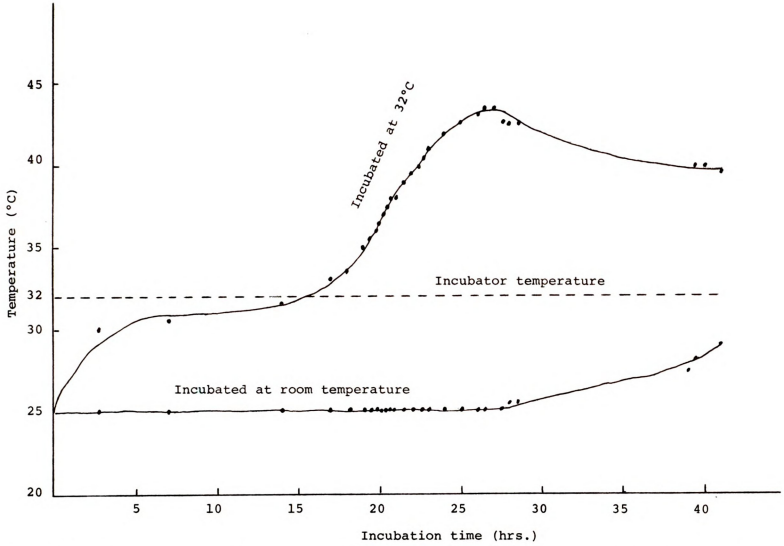


Figure 19. The temperature of tempe incubated at room temperature and in an incubator at 32°C.

At the rapid and transition phases of tempe fermentation, apparently the Bacilli utilized the readily available nutrients produced by the mold. During the transition phase, both the dying mold and the Bacilli utilized the readily available nutrients remaining from the rapid phase. But after the transition phase was over, the Bacilli took over as the dominating organism in tempe, with a new outburst of fatty acid liberation. At this period, tempe deterioration commenced (deterioration phase).

Tempe at the early stage of the deterioration period is still consumable, in small amount, as a flavoring, or in a special recipe in Indonesia.

The general trend of tempe metabolism activity in these present experiments was basically in agreement with the result of Wagenknecht et al. (1961) which showed a stagnation trend of free fatty acid production at 25-30 hour of fermentation.

To show the general trend of fatty acid composition changes during fermentation in the glyceride and free fatty acid portion of tempe, relative chromatographic analyses were made, and the results are presented in Tables 11a and 11b and summarized in Figure 20.

The soybean oil fatty acid composition, as compiled by Herb and Martin (1970) from many oil analyses, is presented in Table 12.

The results of the fatty acid analyses of soybean oils conducted in this work as presented in Tables 11a

Table 11a.--Percent distribution of fatty acids among the free fatty acids (ffa) and the glycerides (gly) of tempe (from SP1000 column).

Sample	Method I					
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Soybeans						
ffa	18.33	1.43	15.27	58.86	6.11	100.0
gly	10.71	2.70	29.74	50.51	6.34	100.0
Boiled soybeans						
ffa	17.67	1.06	10.60	67.14	3.53	100.0
gly	10.04	3.29	24.65	54.63	7.39	100.0
18 hr. tempe						
ffa	13.81	2.73	27.40	47.24	8.82	100.0
gly	10.93	2.63	29.15	49.19	8.10	100.0
24 hr. tempe						
ffa	11.51	2.65	26.95	51.95	6.92	100.0
gly	10.50	2.53	27.40	51.80	7.77	100.0
30 hr. tempe						
ffa	10.44	3.25	32.47	47.30	6.54	100.0
gly	10.16	2.71	29.80	50.11	7.22	100.0
65 hr. tempe						
ffa	12.18	3.48	34.78	43.80	5.76	100.0
gly	12.18	1.98	32.94	45.00	7.90	100.0
72 hr. tempe						
ffa	11.65	1.54	35.89	45.16	5.76	100.0
gly	8.17	2.11	33.73	49.79	6.20	100.0
96 hr. tempe						
ffa	13.33	3.43	35.91	41.22	6.11	100.0
gly	9.70	2.24	33.65	48.59	5.82	100.0

Table 11b.--Percent distribution of fatty acids among the free fatty acids (ffa) and the glycerides (gly) of tempe (from DEGS-PS column).

Sample	Method II					
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Raw soybeans						
ffa	20.90	3.35	16.72	50.17	8.86	100.0
gly	12.64	4.16	24.98	50.25	7.97	100.0
Boiled soybeans						
ffa	30.56	5.56	22.22	33.33	8.33	100.0
gly	11.92	4.29	24.79	49.82	9.18	100.0
18 hr. tempe						
ffa	16.40	6.18	26.64	40.79	9.99	100.0
gly	10.30	3.69	25.66	51.51	8.84	100.0
24 hr. tempe						
ffa	13.35	7.19	30.48	42.38	6.60	100.0
gly	8.09	3.43	32.47	49.39	6.62	100.0
30 hr. tempe						
ffa	15.03	5.33	30.77	42.60	6.27	100.0
gly	9.65	4.68	27.52	50.92	7.23	100.0
40 hr. tempe						
ffa	13.08	5.61	32.78	42.06	6.47	100.0
gly	8.78	3.38	30.49	49.66	7.69	100.0
50 hr. tempe						
ffa	11.88	6.02	29.49	45.81	6.80	100.0
gly	8.61	4.06	33.08	46.82	7.43	100.0
70 hr. tempe						
ffa	11.95	4.73	30.69	45.84	6.79	100.0
gly	8.23	4.41	29.58	50.24	7.54	100.0
90 hr. tempe						
ffa	10.37	5.61	31.31	45.69	7.02	100.0
gly	9.37	4.21	30.02	49.71	6.69	100.0
112 hr. tempe						
ffa	10.96	6.46	28.65	48.45	5.48	100.0
gly	7.39	5.97	29.54	49.72	7.38	100.0

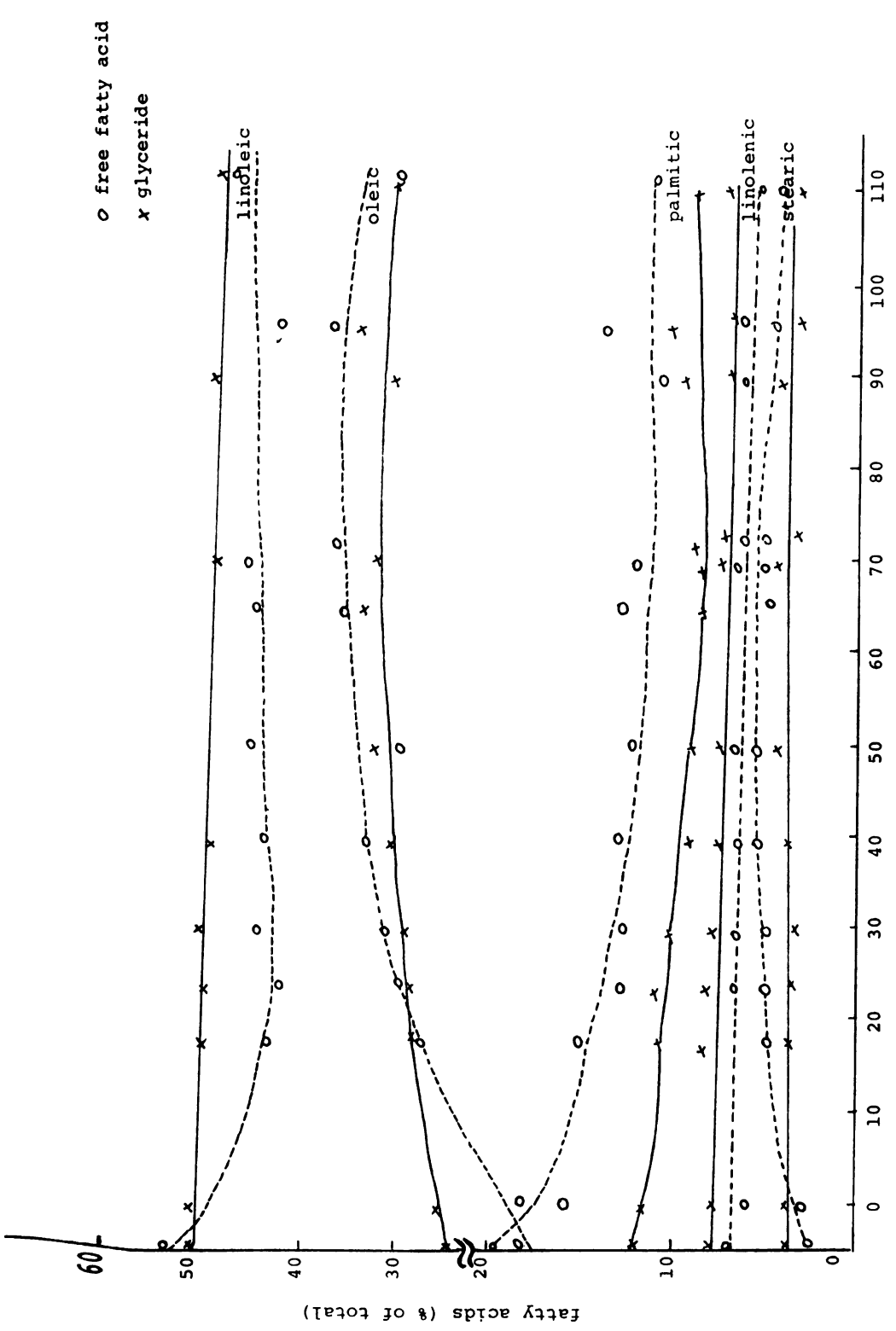


Figure 20. Fatty acid composition (in %) of free fatty acid and glyceride portions of tempe during fermentation (data from Tables 11a and 11b).

Table 12.--Fatty acid composition of soybean oil (Herb and Martin, 1970).

	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
Mean values in %	0.09	9.27	4.25	47.65	35.78	2.97

and 11b, showed a higher proportion in linolenic and linoleic acids but lower proportion in oleic and stearic acids compared to the values of Herb and Martin.

The values corresponding to the two methods used (Tables 11a and 11b) were reasonably close. The data from those tables were summarized in Figure 20.

Figure 20 shows the relative composition of fatty acids present as free fatty acids and glycerides in tempe during the course of fermentation. Oleic acid was liberated from the soybeans relatively more than other fatty acids. The percentage of palmitic acid, both in the free fatty acid and the glyceride portions shows a decreasing trend. This phenomenon was probably caused either by palmitic acid utilization by the mold or contaminating sporeformers or lost by other means.

Linoleic and linolenic acids were liberated in a smaller degree than the other fatty acids and their percentages in the glyceride portion remained constant. This preservation of linoleic and linolenic in their glyceride forms probably contributes to the relative stability of tempe in storage, besides the antioxidant liberated during

the fermentation process. Linolenic acid especially in its free form, is known as the main cause of soybean instability during storage. The soybean oil is known as the most unstable of all vegetable oils due to its content of linoleic and linolenic acids. Soybean oil is more sensitive than other oils to iron, copper and some extent of heating and aging (Cowan et al., 1970; Dutton et al., 1951; Antunes, 1971).

Changes in the fatty acid composition of tempe after frying

Tempe slices about 2.5 x 7 x 0.5 cm (weigh about 12-14 g) were fried in coconut oil at temperatures of 180 to 210°C for 3-4 minutes, and then cooled at room temperature in a vacuum desiccator for several hours. The oil was extracted according to the method of Folch et al. (1957). Free fatty acids were extracted as described in the procedure. The analyses of fatty acids were carried out with GLC for both free fatty acid and glyceride fractions.

In general, frying resulted in a decrease of free fatty acids, as shown in Table 13. Palmitic, stearic, oleic and linoleic decreased in all frying conditions, but linolenic increased after frying at 210°C for 3 minutes and at 190°C for 4 minutes. Heating and exchange of fatty acids between tempe and frying oil were considered as possible causes of the change in free fatty acids during frying. To investigate the heat factor similar sizes of

Table 13.--Free fatty acids content of tempe before and after frying in coconut oil at 180°-210°C.

Sample	mg of fatty acid per 100 g moisture free tempe					
	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Raw tempe	631.10	337.21	1396.79	1764.16	351.84	4129.26
Fried at 210°C for 3 minutes	355.96	267.47	917.84	1211.71	438.89	3191.87
Fried at 190°C for 4 minutes	379.10	260.87	853.81	1197.57	419.53	3110.88
Fried at 180°C for 4 minutes	185.81	152.02	552.84	573.83	253.37	1717.87

tempe were heated in oven at 200°C for 20 minutes, and the results are shown in Tables 14 and 15.

Heat increased the free fatty acids in tempe, especially linolenic acid (Table 14).

Table 14.--Free fatty acids content of tempe before and after heating in an oven at 200°C for 20 minutes (g in 100 g sample dry basis).

Sample	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Raw tempe	0.579	0.289	1.423	1.663	0.226	4.181
Heated tempe	0.671	0.371	1.628	2.168	0.310	5.096
Increased (%)	15%	10%	14%	30%	37%	22%

Table 15.--Fatty acid composition of free fatty acid and glyceride portions of tempe, before and after heating in an oven at 200°C for 20 minutes (in percent of total free fatty acid or glyceride).

	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Raw tempe						
--free fatty acid	13.85	6.93	34.04	39.78	5.40	100.0
--glyceride	8.44	3.24	30.62	50.24	7.46	100.0
Heated tempe						
--free fatty acid	13.17	6.24	31.94	42.55	6.10	100.0
--glyceride	9.43	3.48	33.27	48.26	5.56	100.0

Linoleic and linolenic acids were liberated more, in relative terms, than the other fatty acids during heating. The percentages of linoleic and linolenic acids in the free fatty acid fraction increased from 39.78% and 5.10% to 42.55% and 6.10%, respectively. The consequence of these increases of free linoleic and linolenic acid was that the percentage of both acids decreased in the glyceride fraction from 50.24% and 7.46% to 48.26% and 5.56%, respectively after heating.

To test the seepage of free fatty acids from tempe to the frying oil, ten grams of frying oil which has been used several times for frying tempe was analyzed. The free fatty acid composition extracted from frying oil is shown in Table 16. For reference, the fatty acid composition of fresh coconut oil was analyzed and appears in Table 17.

From those two tables, it is obvious that fatty acid characteristic to coconut oil, caproic, caprylic,

Table 16.--Free fatty acids found in coconut frying oil. The frying oil has been used for frying tempe several times (2 hours). There were no free fatty acids in the unheated oil (mg in 100 gram oil).

$C_{6:3}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
0.0	0.59	0.50	8.17	4.34	6.63	4.64	7.31	9.04	0.74	41.96 mg
(0.0)	(1.41)	(1.19)	(19.47)	(10.34)	(15.80)	(11.06)	(17.42)	(21.55)	(1.76)	(100) %

Table 17.--Composition of fatty acid present in the glycerides in fresh coconut oil (in percent of total fatty acid).

$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
0.75	6.68	5.31	46.05	18.42	9.21	3.83	7.50	2.25	0.0	(100) %

capric, lauric and myristic proportionately are much lower in free fatty acid portion of the frying oil, compared to their percentage in the fresh oil. On the other hand, the fatty acids characteristic to the soybean oil, palmitic, stearic oleic, linoleic and linolenic, proportionately were much higher in the free fatty acid portion of the coconut frying oil. This evidence led to a conclusion that free fatty acids seeped from tempe into the frying oil.

The glyceride portion of coconut oil was also analyzed. Table 18 shows the fatty acid composition of coconut oil before and after frying. The compositions of fatty acids from those two oil samples were relatively unchanged.

For reference, the mean value of Herb and Martin (1970) is presented in Table 19. In their publication, Herb and Martin compiled the results of fatty acid analyses of oils (same sample provided) from more than 25 laboratories in the USA, Canada, and Sweden. Their conclusion was that the coconut oil was the most difficult to analyze (based on their standard deviation) compared to other oils analyzed. The result of the present study of fatty acid composition in coconut oil closely agrees with the values of Herb and Martin. The analysis of fatty acids in the glyceride portion of tempe before and after frying is presented in Table 20.

The coconut frying oil apparently was absorbed by the tempe during frying as anticipated. The fatty acid

Table 18.--Fatty acid composition of the glyceride portion of coconut oil, before and after frying of tempe.

$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
					%				
Before frying									
0.75	6.68	5.31	46.05	18.42	9.21	3.83	7.50	2.25	0.0
After frying (1 hour)									
0.69	6.51	5.35	46.84	18.69	9.23	3.98	6.92	1.79	0.0
0.51	5.96	5.24	48.08	17.68	10.25	3.43	6.91	1.94	0.0

Table 19.--Fatty acid composition of coconut oil (Herb and Martin, 1970).

$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$
					%				
0.66	7.80	6.38	47.28	16.61	8.74	2.70	6.71	2.12	0.0

Table 20.--Fatty acid composition of the glyceride portion of tempe, before and after frying in coconut oil (% of fatty acid).

Sample	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	Total
Raw tempe	0.0	0.0	0.0	0.0	0.0	8.95	3.39	29.69	51.02	6.95	100
Fried tempe at 210°C for 3 minutes	0.0	3.41	3.44	30.25	11.09	9.12	3.88	16.01	19.39	3.41	100
Fried tempe at 190°C for 4 minutes	0.0	3.35	3.51	29.94	11.84	8.84	3.45	15.58	20.21	3.28	100
Fried tempe at 180°C for 4 minutes	0.0	3.34	3.28	29.82	11.12	8.88	3.74	16.50	20.35	2.97	100

content of glyceride portion of fried tempe was a result of equilibrium of the two components, i.e., the soybean oil and the coconut oil. The fatty acids characteristic of coconut oil were found in fried tempe i.e., caprylic, capric, lauric and myristic acids. Two common fatty acids of tempe and coconut oil, the palmitic and stearic, their percentages were relatively unchanged from the raw and fried tempe. On the other hand, the percentage of fatty acids characteristic to the tempe oil; oleic, linoleic and linolenic acids were reduced appreciably.

Table 18 shows that the fatty acid profile of the glyceride portion of the coconut frying oil was unchanged compared to the fresh oil control. These data suggested that no glyceride migration had occurred from the tempe to the frying oil.

Rancidity test of tempe using the TBA method

Rancid oil was distilled in water, and the distillate was reacted with TBA reagent (details were described in the Materials and Methods) and the absorbance was read in different wavelengths using a Beckman DU spectrophotometer. The result is shown in Table 21. The wavelength which produced maximum light absorption was between 528-530 nm, which is the same with the wavelength determined by Tarladgis et al. (1960) and Sidwell et al. (1954).

Table 21.--Absorbance reading of rancid oil sample in different wavelengths.

Wavelength (nm)	Absorbance
518	0.70
520	0.78
522	0.84
524	0.88
526	0.92
528*	0.94
530*	0.94
532	0.92
534	0.86
536	0.78
538	0.69
540	0.59

* Maximum light absorption.

The correlation between the TBA value (as absorbance) and rancidity, was determined by a ten-member panel. The result is presented in Table 22. Three panel members failed to detect any rancidity in sample 3, while only one failed to detect any rancidity in sample 4. All ten members recognized rancidity (by smelling) in samples 5 and 6.

For practical purposes, it was therefore suggested to use the absorbance value of 0.300 as borderline indicator between weak, not readily detected rancid smell, and readily detected pronounced rancid smell in soybean flour. To compare the stability of soybeans and tempe to rancidity

Table 22.--Sensory test and TBA value.

Sample no.	Mean sensory value*	TBA value (absorbance)
1	0.0	0.160
2	0.4	0.180
3	0.8	0.235
4	0.9	0.270
5	1.7	0.300
6	1.9	0.320

*Mean value from ten panel members: 0 = no rancidity; 1 = weak rancidity; 2 = strong rancidity.

stored at room temperature (25°C), several soybean and tempe samples were prepared and stored in plastic bags, as summarized in Table 23. The samples were stored at room temperature in plastic bags not under vacuum.

The TBA values of the samples were determined and are presented in Table 24. The reports of Sidwell et al. (1954) and Rhee and Watts (1966) indicated that the TBA test correlates better with the stability of an oil than do the peroxide or carbonyl tests. They showed that soybean oil had a higher TBA value than cottonseed oil even when they had approximately the same peroxide or carbonyl values. They concluded that the TBA test correlates better than the other tests with the unacceptability of oil flavor, and they suggested an association of the TBA value with the linoleic and linolenic acids content of a fat.

Table 23.--Soybeans and tempe samples.

Sample no.	Treatment
1	Soybeans soaked, peeled, vacuum dried and ground
2	Soybeans soaked, peeled, boiled 30 min. vacuum dried and ground
3	Soybeans soaked, peeled, boiled 30 min. vacuum dried and fried in coconut oil for 1 min. at 200°C
4	Tempe, vacuum dried and ground
5	Tempe, freeze dried, and ground
6	Tempe, dried and fried in coconut oil at 200°C

Table 24.--The TBA value of samples described in the Table 23.

Sample no. and time storage		TBA value as absorbance
1 soybeans	1 month	> 2.00
2 soybeans boiled	1 month	0.165
3 soybeans boiled, fried	1 month	0.360
4 tempe, dried	2½ months (3½ months)	0.165(0.170)
5 tempe, freeze dried	2½ months	0.125
6 tempe, freid	1½ month	0.115
7 raw untreated soybeans (ground)		0.450
8 fresh soaked, boiled, ground and dried soybeans		0.235

The fried soybeans showed a higher TBA value than the fried tempe after storage of more than one month (Samples 3 and 6). This observation closely agreed with the study conducted by György et al. (1964) on the antioxidant trihydroxyisoflavone which was liberated during tempe fermentation from raw soybeans. This liberated antioxidant was possibly responsible for the protection of tempe from oxidation.

In tempe, dried or fried (samples 4 and 6, Table 24) and stored at room temperature up to 2.5 months, showed relatively low TBA values. The tempe had no distinct rancid or beany flavor. On the other hand, using the TBA value of 0.300 as the border value for rancidity, the fried soybeans stored for one month (sample 3) was then categorized as rancid.

The dried soybeans (sample 1) also had a much higher TBA value than the boiled soybeans and tempe after storage. Freshly boiled and dried soybeans showed a lower TBA value than the freshly ground unboiled soybeans (samples 7 and 8). In this case, boiling and drying probably reduced the "beany flavor" which also reduced the TBA value. The boiling perhaps inactivated the lipoxygenase in soybeans and prevented the soybeans from rancidity during storage.

Rackis et al. (1970) investigated the flavor constituents of soybeans and soybean flakes and concluded that the TBA-reactive substances presumably were protein-bound

and associated with the non-lipid fractions. They also reported that there were no clear correlations between the TBA value and the organoleptic evaluation. In this present study, the TBA test appeared not specific to the degree of fat oxidation or rancidity only, but also showed a positive correlation with raw soybean flavor. The raw soybeans showed a strong beany flavor in its distillate, but not rancid odor, and possessed a high TBA value of 0.450. This phenomenon had also been reported by Sessa et al. (1969).

The TBA test of rancidity in soybeans and soybean products was therefore not a specific test because of the complication provided by the beany flavors.

Phytic acid content in soybeans and tempe

The following samples were prepared:

1. Soybeans, raw, broken and skinned.
2. Soybeans soaked overnight (300 g soybeans in 800 ml tap water) peeled and dried in vacuum at 87°C.
3. Soybeans soaked, boiled (30 minutes) and dried under vacuum at 87°C.
4. Tempe (fermented 30 hours), sliced and dried under vacuum at 87°C.

All the samples were ground through the Cyclone Sample Mill into a fine powder. The phytic acid content was calculated from the absorbance reading as follows:

$$\text{Mg. of physic acid per 100 g moisture free sample} = \frac{A(510)}{0.783} - 0.007 \times \text{dilution factor} \times 2.9546$$

Each sample was run in duplicate. The phytic acid contents of the samples are presented in Table 25.

Table 25.--Phytic acid content of soybeans and tempe.

Sample		Phytic acid (% moisture free)	
		Sample I	Sample II
1. soybeans, raw		1.40%	1.42%
		1.42%	1.38%
	average	1.41%	1.40%
2. soybeans, soaked		1.39%	1.49%
		1.35%	1.49%
	average	1.37%	1.49%
3. soybeans, boiled		1.07%	1.39%
		1.02%	1.42%
	average	1.05%	1.40%
4. tempe		0.78%	1.11%
		0.86%	1.11%
	average	0.82%	1.11%

The presence of phytates (Figure 21) in foods may lead to deficiencies of iron, magnesium, zinc, calcium and possibly other elements in man and other non-ruminant animals (Berlyne et al., 1973; McBean and Speckman, 1974; Reinhold et al., 1973; Ranhotra, 1972; Ranhotra et al., 1974a).

In ruminants the presence of active intestinal phytase makes phytate interference of mineral adsorption a much less serious problem (Anonymous, 1967; Pileggi et al.,

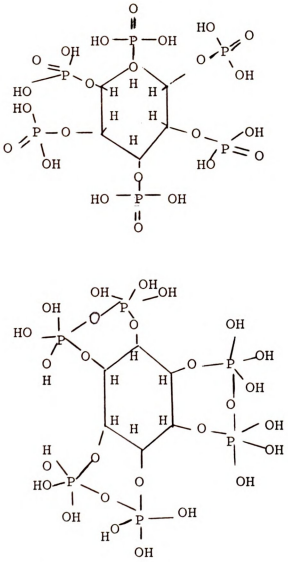


Figure 21. Inositol hexaphosphate (phytic acid). Two possible structures.

1955; Maddaiah et al., 1963; Nelson et al., 1971; Ranhotra et al., 1974a).

Some phytate hydrolysis occurs in man probably due to microbial phytases in the gut or to nonenzymatic cleavage (Nicolaysen and Njaa, 1951; Hegsted et al., 1954; Subrahmanyan et al., 1955).

Because of the susceptibility of man to mineral deficiencies due to the ingestion of phytic acid, the reduction of phytic acid content of foods or balancing the diet with high mineral content is nutritionally advantageous. Therefore, the reduction of the phytic acid content during the fermentation of tempe can be considered as one contribution to the superiority of tempe as food compared to the unfermented soybeans.

In the soaking water (300 gr soybean soaked overnight in 800 ml tap water) there was 1.63 - 1.56 ppm phytic acid. This phytic acid probably came from the skin and the germ where the germination process is the most active. In the cotyledon the reduction of phytic acid during soaking and boiling was not significant. Mollgaard et al. (1946) indicated that most oilseeds, including soybeans, contain no phytase. Even if there was a weak phytase activity in soybeans, the boiling which preceeded the fermentation would inactivate it. The possibility of phytase activity from soybeans in tempe was zero. Therefore the phytase activity in tempe fermentation must originate in the Rhizopus mold.

The phytates yield inositol upon complete hydrolysis. Inositol has nutritional properties as an anti-spectacled eye factor, antialopecia factor and growth factor in rats as well as a growth factor for microorganisms. Since some phytate was hydrolysed during tempe fermentation, inositol may be formed and provided additional advantages to tempe as a food.

Phosphorus determination

The formula to calculate the amount of inorganic phosphorus, based in Murphy and Riley's work (1962), is:

$$\text{Phosphorus } (\mu g) = \left(\frac{A_{700}}{0.016} - 0.213\right) \times \frac{30.974}{200} \times \text{dilution factor}$$

The inorganic phosphate contents in boiled soybeans and tempe were determined (in duplicate) and the results are presented in Table 26.

Inorganic phosphorus balance calculation

1 Phytic Acid $\longrightarrow$ 6 P + 1 inositol

MW Phytic Acid : 660 MW P : 31

I Calculation was based on average value of:

boiled soybeans	phytic acid	: 1.05%
	inorganic P	: 15.4 mg/100 g.
tempe	phytic acid	: 0.82%
	inorganic P	: 82.2 mg/100 g

In 100 g, the loss of phytic acid from boiling to fermented tempe is

1050 mg - 820 mg = 230 mg

Table 26.--Inorganic phosphate content of boiled soybeans and tempe (as μ P per g moisture free sample).

Item		P	
		Sample I	Sample II
Boiled soybean		122.63	142.63
		186.74	219.00
	average	154.68	180.82
Tempe		882.40	872.01
		821.90	1020.85
	average	822.15	946.43

Inorganic P gain = 82.2 mg - 15.4 mg = 66.8 mg.

Theoretical gain of inorganic P (assuming only from phytic acid):

$$\frac{230}{660} \times 6 \times 31 = 65.1 \text{ mg}$$

II Phytic Acid content of boiled soybean : 1.40%

Phytic Acid content of tempe : 1.11%

Inorganic phosphorus of boiled soybean: 18.0 mg/100 g

Inorganic phosphorus of tempe : 94.6 mg/100 g

In 100 g moisture free sample, the phytic acid loss during tempe fermentation was 1400 - 1110 mg = 290 mg or equivalent to 290/660 x 6 x 31 = 81.65 mg P. The actual P gain was 94.6 - 18.0 mg = 76.6 mg. According to these calculations the increase in P during the tempe fermentation may be explained on the basis of phytic acid hydrolysis.

Ranhotra et al. (1974b) investigated the total phosphorus and phytic acid phosphorus in several soyflour products, and concluded that all soybean flours tested were high in total P (about 600 mg/100 g fullfat soyflour) with most of it in the form of phytic acid.

The phosphorous balance calculation was made on the assumption that inorganic P gain during fermentation was derived from the hydrolysis of phytic acid. In calculations from two experiments of soybeans before and after fermentation, the actual increase in P was considerably close to the theoretical value from the phytic acid loss. The discrepancies of theoretical and actual values were due to analytical error, other sources of P rather than phytic acid, and also the possibility of chances of partial cleavage from the six P atoms present in one molecule of phytic acid.

Investigation of phytase activity in tempe

Crude enzyme extraction

1. Blend 25 g tempe (fermented 30 hours) with 150 ml 2% solution of $CaCl_2$ for about 4 min. intermittently.
2. Centrifuge the slurry at 12,000 x G for 15 minutes.
3. Filter through Whatman No. 1 in vacuum to remove traces of fat.
4. Fill in dialysis casing and dialyze in maleate buffer, pH 6.5 for about 30 hrs.

5. Centrifuge at 12,000 x G for 10 minutes.
6. Check the supernatant and precipitate for enzyme activity.

Substrate preparation

2.5×10^{-3} M Na-phytate solution was prepared to give 1×10^{-3} M in 5 ml final solution of enzyme essay.
M.W. Na-phytate = 923.8
Dissolve 1.15 g of Na-phytate in distilled water, bring the pH to 6.0 with dilute HCl and the volume to 500 ml with water.

Buffer preparation

Prepared according to Gomori (1955).

Phytase essay

The activity of the crude enzyme extract was assayed as shown in Table 27. Enzyme-substrate solutions were placed in test tubes with teflon caps and incubated in a water bath at 40°C.

At the end of the incubation period, 1 ml of solution was added to 1 ml solution of 20% TCA to precipitate all the protein. Centrifugation followed for 10 minutes. To 0.5 ml of supernatant add 1.6 ml Murphy and Riley Reagent B, bring to volume in 10 ml volumetric flask and let stand for over 10 minutes. Read absorbance at 700 nm against mixture of reagent and water blank. Calculate the phosphorous content using the standard formula

Table 27.--Enzyme activity assay in buffered substrate solution.

Enzyme solution (ml)		Substrate (ml)	H_2O (ml)	Buffer* (ml)
Supernatant				
1	0.1	2.0	0.9	2.0
2	0.5	2.0	0.5	2.0
3	1.0	2.0	0.0	2.0
4	2.0	1.0	1.0	2.0
5	2.0	0.0	1.0	2.0
Precipitate**				
6	1.0	2.0	0.0	2.0

*Acetate buffer 0.2 M pH 5.6.
**Precipitate diluted in 10 ml water.

for phosphorous. The phytase activity results are presented in Table 28.

Table 28 shows, solution No. 3 provided the most efficient enzyme activity in 2.5 hr. incubation time. The activity decreased at 24 hr. incubation. The precipitate of crude enzyme after dialysis also had a phytase activity, but the absolute quantity compared to the solution was small (tube No. 6, table above).

The effect of pH on tempe phytase activity

The enzyme was extracted from tempe and precipitated with ammonium sulfate as described below:

Table 28.--Total P liberated in µg, and enzyme activity as µg P produced/ml enzyme/hour.

Enzyme-Substrate Solution No.	2.5 hr. incubation at 40°C		24 hr. incubation at 40°C	
	Total P	Activity	Total P	Activity
1	0.00	0.00	8.96	3.73
2	9.67	7.74	37.74	1.57
3	21.92	8.77	54.83	2.29
4	22.63	4.53	92.90	1.94
5	5.44	1.09	10.24	0.21
6	13.21	5.28	31.60	1.32

1. Blend 100 g tempe in 300 ml $CaCl_2$ 2% solution for about 5 mins.
2. Centrifuge the slurry at 12,000 G for 15 min.
3. Separate the supernatant and filter through Whatman 44.
4. Add 200 g of $(NH_4)_2SO_4$ to the filtrate.
5. Centrifuge at 12,000 G for 15 min. to precipitate protein.
6. Dissolve precipitate in 100 ml maleate buffer (pH 5.6).
7. Dialyze the solution in a cold room for 36 hours.
8. Centrifuge the solution at 12,000 G for 15 min. and discard the precipitate.
9. Store the supernatant containing enzymes at 35°F.

Phytase assay

The following enzyme-substrate solution was prepared:

enzyme sol.	substrate (Na-phytate 2.5×10^{-3} M)	buffer
1 ml	2 ml	2 ml

The buffered enzyme-substrate solutions (pH 3.9 to 6.9) were incubated in 40°C water bath for 3 hours. The enzyme activity was determined as µg P/ml enzyme/hour and the result is tabulated in Table 29.

Table 29.--Effect of pH on tempe phytase activity in acetate and maleate buffers.

pH*	Activity (µg P/ml enzyme/hr)	% of max. activity
3.9	2.98	50.68
4.2	2.65	45.07
4.6	1.04	17.69
4.8	1.85	31.46
4.9	3.30	56.12
5.0	4.11	69.90
5.3	5.33	90.57
5.6	5.88	100.00
6.0	5.24	89.12
6.6	3.62	61.56
6.9	2.82	47.96

*pH 3.9 - 5.3 : acetate buffer

pH 5.6 - 6.9 : maleate buffer

Table 29 shows that tempe phytase had a maximum activity at pH 5.6. The experiment was repeated by using a single buffer system of 0.1 M citrate throughout the entire pH range of 2.9 to 6.0. The total phosphate production is presented in Table 30 and the phytase activity in Table 31. Table 31 led to the conclusion that tempe phytase had a maximum activity at pH 5.6.

Figure 22 was drawn based on the data in the Tables 29 and 31; this figure clearly shows the maximum phytase activity at the pH of 5.6 regardless of the buffer system used.

The partially purified tempe phytase in this present study apparently was not very different in pH activity from that of the phytase of germinated dwarf beans, Phaseolus vulgaris, which had an optimum pH of 5.2 as reported by Gibbins and Norris (1962).

On the other hand, the phytase extracted from animal small intestines had a higher pH optimum. Works of Bitar and Reinhold (1972) showed the pH optima for phytases extracted from the mucosae of the small intestines of rats, chickens, calves and human to be 7.0 - 7.5, 8.3, 8.6 and 7.4, respectively.

Determination of Vmax and Km of tempe phytase

The arrangement of buffered enzyme-substrate solutions are shown on Table 32. The final substrate concentrations were from 0 to 1.5×10^{-3} M. The

Table 30.--Total production of P in μg per 5 ml reaction mixture (10^{-3} M phytate, 1 ml phytase extract in citrate buffers of various pH).

pH	1 hr	2 hr	3 hr
2.9	0	0	5.887
3.1	0	0	4.947
3.4	11.851	29.251	39.747
3.7	16.084	42.889	59.499
3.8	19.187	44.300	59.499
4.0	19.376	46.651	58.559
4.2	20.316	46.651	56.677
4.4	23.608	46.651	57.618
4.6	25.312	46.651	54.796
4.8	27.370	51.354	59.499
5.0	30.850	55.116	72.667
5.2	38.657	61.700	74.548
5.3	45.523	64.522	92.419
5.4	52.107	64.522	114.992
5.6	62.641	81.452	130.982
5.8	61.701	72.046	125.339
6.0	63.582	72.516	119.695

Table 31.--Tempe phytase activity at different pH* levels and its percent of maximum activity.

pH	Activity (µg P/ml enzyme/hr)	% of max. activity
2.9	1.962	4.00
3.1	1.649	3.36
3.4	13.242	27.02
3.7	19.121	39.02
3.8	20.390	41.60
4.0	20.740	42.32
4.2	20.845	42.53
4.4	22.047	44.99
4.6	22.301	45.50
4.8	24.293	49.57
5.0	27.543	56.20
5.2	31.452	64.18
5.3	36.197	73.86
5.4	40.900	83.45
5.6	49.009	100.00
5.8	46.501	94.88
6.0	46.579	95.04

*0.1 M citrate buffer.

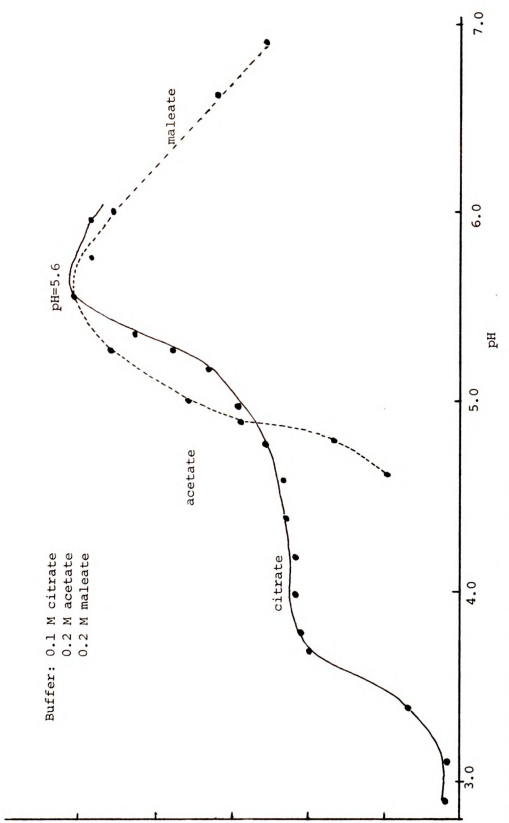


Figure 22. pH optimum curve for tempe phytase.

Table 32.--Buffered enzyme-substrate solutions.

Tube no.	Substrate 2.5×10^{-3}M Na-phytate (ml)	Phytase (ml)	Buffer Citrate pH 5.6 (ml)	Water (ml)	Substrate final conc.
1	0.0	1.0	2.0	2.0	0.00
2	0.2	1.0	2.0	1.8	0.10×10^{-3}M
3	0.5	1.0	2.0	1.5	0.25×10^{-3}M
4	1.0	1.0	2.0	1.0	0.50×10^{-3}M
5	1.5	1.0	2.0	0.5	0.75×10^{-3}M
6	2.0	1.0	2.0	0.0	1.00×10^{-3}M
7	3.0	1.0	1.0	0.0	1.50×10^{-3}M

enzyme-substrate solutions were incubated in test tubes in a 45°C water bath, and P production was determined at 0, 10, 20, 30, 40, 50, and 60 minutes. The results are shown in Table 33.

From the data of Table 33, the initial velocities of the phytate-phytase reaction at various substrate concentrations were calculated using linear regression analysis and with the help of a Wang 600 desk calculator. These results are shown in Table 34. From Table 34, Figure 23 was drawn to show the correlation between substrate concentration, S, and velocity of reaction, V. To determine the Vmax and Km, a Lineweaver-Burk plot was prepared (Figure 24). The 1/V and 1/S values are shown in Table 35.

Table 33.--Effect of substrate (phytate) concentration on the P release by tempe phytase in µg/5 ml reaction mixture.

time (min)	substrate concentration 10^{-3}M						
	0.00	0.10	0.25	0.50	0.75	1.00	1.50
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	5.16	7.33	8.64	8.93	9.87	11.13
20	0.00	8.37	13.54	16.84	17.96	20.50	22.52
30	0.00	12.70	20.63	28.22	28.88	33.39	36.06
40	0.00	16.27	28.21	38.09	42.42	46.74	52.33
50	0.00	17.49	33.11	49.76	50.79	55.49	63.34
60	0.00	18.53	37.81	52.59	58.51	63.11	66.63

Table 34.--Effect of substrate concentration on the initial velocity of phytate-phytase reaction.

	substrate concentration, 10^{-3}M						
	0.00	0.10	0.25	0.50	0.75	1.00	1.50
Velocity (µg P/10 min.)	0.00	3.77	6.62	9.38	10.28	10.98	11.89
Correlation coef. (r)	. .	0.998	0.998	0.992	0.997	0.996	0.989

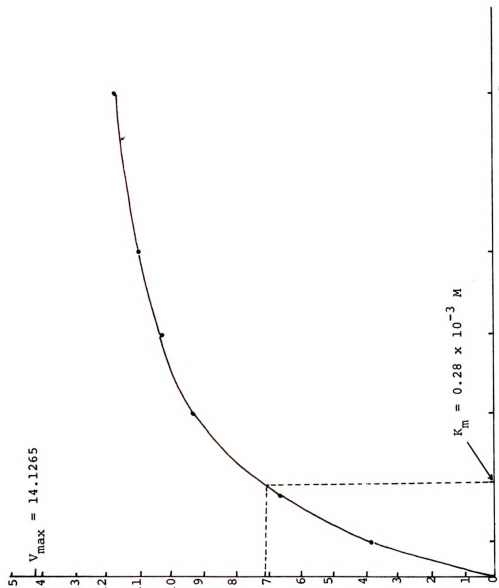


Figure 23. Effect of phytate concentrations on tempe phytase activity.

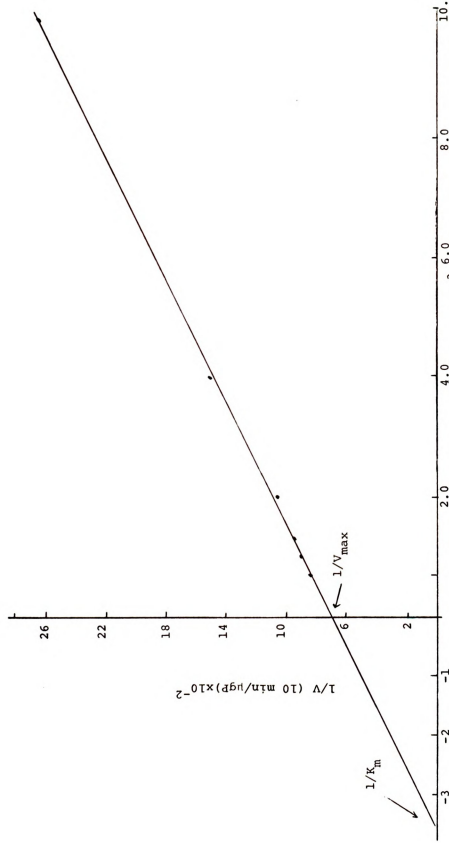


Figure 24. Lineweaver-Burk plot for the tempe phytase-phytate reaction.

Table 35.--Lineweaver-Burk plot of the phytate-phytase reaction. Values of 1/S and 1/V.

S	V	1/S	1/V
10^{-3}M	µg P/10 min	10^{3}/M	10 min/µg P
0.10	3.77	10.00	0.2655
0.25	6.62	4.00	0.1510
0.50	9.38	2.00	0.1066
0.75	10.28	1.33	0.0972
1.00	10.98	1.00	0.0910
1.50	11.89	0.67	0.0841

The intercept with ordinate is $1/V_{max}$ and the intercept with the abcisa is $1/K_m$, and thence the V_{max} and K_m can be determined.

By regression analysis the following linear equation was obtained.

$$1/V = 0.0195 \times {}^{1}/S + 0.0708$$

The intercept with ordinate is 0.0708, which is also $1/V_{max}$, and therefore

$$V_{max} = 1/ (0.0708) = 14.1265 \text{ µg P/10 min.}$$

The intercept with abcisa is $- 3.55 \times 10^{3}$/M which is also $- 1/K_m$, and

$$K_m = 0.28 \times 10^{-3}M$$

The work of Gibbins and Norris (1962) indicated the inhibition effect of substrate (Na-phytate) of 2×10^{-3}M to the phytase activity of germinated dwarf bean, Phaseolus vulgaris. The Michaelis constant for this particular phytase was reported as 0.15×10^{-3}M, a value not very different from that for the tempe phytase.

Phytase activity in the tempe mold, Rhizopus oligosporus

The Rhizopus grown on solid Potato Dextrose Agar (PDA) in two days at 30°C, was harvested and ground in the Polytron grinder in 2% $CaCl_2$ solution and the enzyme extraction was proceeded as in tempe enzyme extraction method.

The enzyme activity was tested in buffer systems (citrate and maleate) of pH 2.9 - 6.3, with 1.0×10^{-3}M Na-phytate as substrate; no activity was detected.

Further studies were needed to elucidate the nature of activation or inhibition of this strong tempe phytase. The immediate practical questions is whether the production of phytase is activated by the presence of phytic acid in the substrate or whether any bacterial contaminants contribute to the production of phytase in tempe.

The possibility of phytase activity naturally present in soybeans seems remote since after soaking overnight the beans was boiled for 30 minutes, and also a work of Mollgaard et al. (1946) indicated that most oilseeds including soybeans contain no naturally present phytase.

To investigate the possibility of substrate (Na-phytate) induction and bacterial contaminants interaction to the production of phytase, the following experiments were carried out:

1. Effect of phytate.

4.9 g PDA powder was mixed with 100 maleate buffer pH 6.3, stirred and heated. Then add 1.57 g of Na-phytate (acidified to pH 6.0 with 0.1N HCl) to the mixture (about 1.5% phytate) and was autoclaved in test tubes, at 250°F for 15 mins. Prepare as standard agar slants.

2. Sterile culture of Rhizopus in PDA-soybeans agar.

Dissolve 5 g PDA in 100 ml water and add 5 g boiled soybeans, blended in a Waring blender. Then heated on hot plate, autoclaved in test tubes at 250°F for 15 minutes. Prepare as standard agar slants.

Inoculate both groups of media aseptically with R. oligosporus, and incubated at 30°C for 2 days.

Growth of Rhizopus on media

Rhizopus grew poorly on phytate medium, growth only on the agar surface, very thin, stunted and produced a lot of spores. On the other hand, Rhizopus grew luxuriantly on soybean medium, thick and whitish in color due to lack of spores and filled the spaces in test tubes. The mold grew on unsupplemented PDA less luxuriantly than on PDA-soybean, but much more mycelia were produced than on the PDA-phytate medium. After the mycelia were harvested, the

enzyme was extracted from the mycelia by grinding in a Polytron grinder with 2% $CaCl_2$ solution. The phytase activity was assayed in maleate buffer pH 5.6 at 45°C with 1.0×10^{-3}M Na-phytate.

Phytase activity

The phytase activity of R. oligosporus is tabulated in Table 36. No phytase activity was detected in the PDA-phytate medium. On the other hand, in the mycelia harvested from PDA-soybeans medium a mild phytase activity was detected. After the mycelia were removed, including the rhizoid portion on the surface of medium, the clear medium was washed with water twice. Extract the agar medium with enzyme extraction procedure and phytase activity was assayed. A weak enzyme activity was detected in this medium, showing that the phytase was also excreted by the mold into the medium. From the above experiments of phytase activity of mold grown on PDA-soybeans and the lack of activity on PDA-phytate medium, it may be deduced that phytate did not induce the production of phytase in the tempe mold; apparently the protein in soybeans was involved in the production of the phytase. To test the validity of this hypothesis, PDA-peptone and PDA-peptone-phytate media were prepared.

PDA-peptone-phytate medium

5 g PDA + 1 g peptone + 1.5 g Na-phytate (which was acidified to pH 6.0 with 0.1 N HCl), and were made into

Table 36.--Phytase activities of Rhizopus oligosporus NRRL 2710 grown on PDA, PDA-phytate, PDA-soybeans, PDA-peptone-phytate, PDA-peptone and PDA-coconut liquid and of the mycelia-free medium.

	Enzyme sol. (ml)	Substrate (Na-phytate, ml)	Buffer pH 5.6 (maleate, ml)	Phytase activity (µg P/10 min/ml)
Mycelia on				
PDA	1	2	2	0.0
PDA-phytate	1	2	2	0.0
PDA-soybeans	1	2	2	3.23
PDA-peptone-phytate	1	2	2	3.95
PDA-peptone	1	2	2	21.94
PDA-coconut liquid	1	2	2	1.21
Medium (mycelia free)				
PDA-Soybeans	1	2	2	1.21

100 ml with deionized water. Agar slants were prepared from this medium.

PDA-peptone medium

5 g PDA + 1 g peptone, the volume was brought to 100 ml with H_2O, sterilized and prepared as agar slants.

In addition, a PDA-coconut liquid medium was also prepared to see whether this coconut liquid endosperm had the capacity to induce the production of phytase in Rhizopus.

PDA-coconut liquid medium

Free coconut liquid was collected from a coconut purchased at a local store, and filtered. The pH was 5.6. Five grams of PDA was added to 100 ml coconut liquid, which was then sterilized and converted to agar slants.

Rhizopus was inoculated on the media and incubated in 30°C for two days. The mold grew very well on PDA-peptone, and PDA-peptone-phytate, similar as on the PDA-soybeans medium. The mold grew less well on PDA-coconut liquid, and on the unsupplemented PDA medium. The phytase activities of mycelia grown on those media were assayed.

Table 36 shows that phytase activity was the highest in the mycelia harvested from PDA-peptone medium. The activity was essentially similar from both PDA-soybeans and PDA-peptone-phytate, which suggested that the phytate naturally present in soybeans and Na-phytate artificially supplied did not promote the growth of mycelia. On the other hand, peptone promoted strongly both the growth of Rhizopus and the phytase activity of the mold. There was a seemingly positive correlation between the growth of mycelium and the phytase activity in the mold. Further studies are needed to quantitate this correlation using equivalent amount of mycelia. The coconut-liquid only supported minimally the production of phytase in the tempe mold.

The protein efficiency ratio values of soybeans, tempe and sesame-supplemented tempe

Feed preparation:

Feed code : 0

Type : Standard Casein diet

Protein source : High Protein Case in (Cat. no. 160030, Tekland Test Diets Company)

Analysis : Protein 87.0%

Fat 1.2%

Feed preparation:

Feed to be prepared: 5000 g

	g	
Casein	574.7	(10% protein)
Corn oil added (400g-6.9g)	393.1	(8%)
Salt	250.0	(5%)
Vitamin	50.0	(1%)
Cellulose	50.0	(1%)
Corn starch	3682.2	
Total	5000.0	

Feed code : I

Type : Fresh fried tempe

Protein source: Soybean tempe (fermentation 30 hrs), fried in coconut oil at 200°C. for 3 minutes. Most of the oil was extracted with hexane in Waring blender and filtered. Tempe flour was dried in hood for overnight.

Analysis : Protein 55.65 % (Micro Kjeldahl AOAC)

Oil 8.5 % (Goldfisch method)

Feed preparation:

	g
Tempe flour	753 (419 g protein = 10%)
Corn oil added (335.2 g - 64.0 g)	271.2 (8%)
Salt	209.5 (5%)
Vitamin	41.9 (1%)
Cellulose	41.9 (1%)
Corn starch	2872.5
Total	4190.0

Feed code : II

Type : Fried boiled soybean

Protein source : soaked and boiled soybeans prepared as for tempe but not fermented. The soybeans then fried in coconut oil at 200°C for 3 minutes. Most of the oil was then extracted with hexane as in feed I. Dried and ground into flour.

Analysis : Protein 56.8%

Oil 11.0%

Feed preparation:

	g
Soybean flour	800.0 (454.4 g protein = 10%)
Corn oil added (363.5-88.0 g)	275.5 (8%)

Salt	227.2 (5%)
Vitamin	45.4 (1%)
Cellulose	45.4 (1%)
Corn starch	3150.5
Total	4544.0

Feed code : III

Type : Sesame supplemented tempe

Protein source : Soaked boiled soybeans prepared as feed I. Staley sesame was lightly ground in mortar to break the seeds. Protein analysis of boiled soybean was 17.2%, and sesame was also 17.2%. Nine part of boiled soybeans were mixed with one part of sesame (by weight). The soybeans-sesame mixture was inoculated with tempe mold and fermented for 30 hrs. Then fried in coconut oil at 200°C for 3 minutes. Extracted with hexane in Waring blender, dried and ground into flour.

Analysis : Protein 57.21%

Oil 10.16%

Feed preparation:

	g
Sesame tempe flour	725.0 (414.77 g protein = 10%)
Corn oil added (331.8-73.8)	258.0 (8%)

Salt	207.5 (5%)
Vitamin	41.5 (1%)
Cellulose	41.5 (1%)
Corn starch	2874.2
Total	4147.7

Test animal: 24 Sprague-Dawley male rats, supplied by the Spartan Research Animals, Inc., Haslett, Michigan 48840. Age: 21 days, weight 46-58 g. Acclimation period: 4 days with casein diet. Standard Diet (Feed Code O) and drinking water ad libitum.

After the acclimation period, the rats were assigned into four groups with six rats in each group. Each group of rats received one of four feed preparations. The average weight of rats in each group during 28 days of feeding experiment are summarized in Figure 25, and weight gains in each group of rats related with their age are shown in Figure 26. The rats in the casein diet gained more weight than the other three groups.

The weight gains of all groups of rats dipped into a minimum when they reached 40-45 days of age. The Protein Efficiency Ratios were calculated for each protein and their variations were statistically analyzed.

The PER of the proteins of casein, fried soybeans, fried tempe and fried sesame-supplemented tempe, in the 10% protein feed blends, for 28 days using male Sprague-Dawley rats was presented in Table 37.

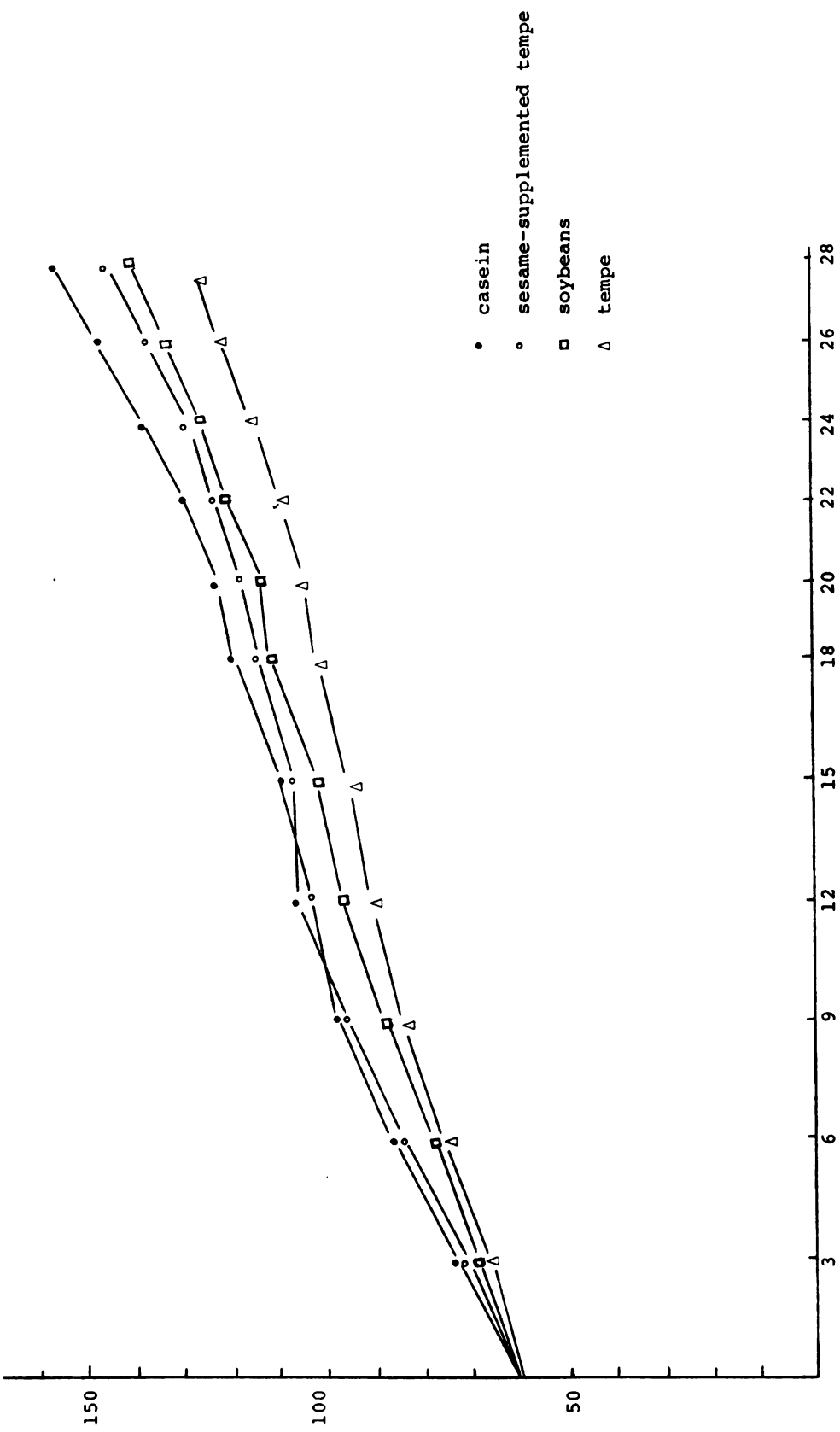


Figure 25. Average weight of rats in each of four groups fed casein, tempe, soybeans and sesame-supplemented tempe basal diets at 10% total protein in the diet during 28 days.

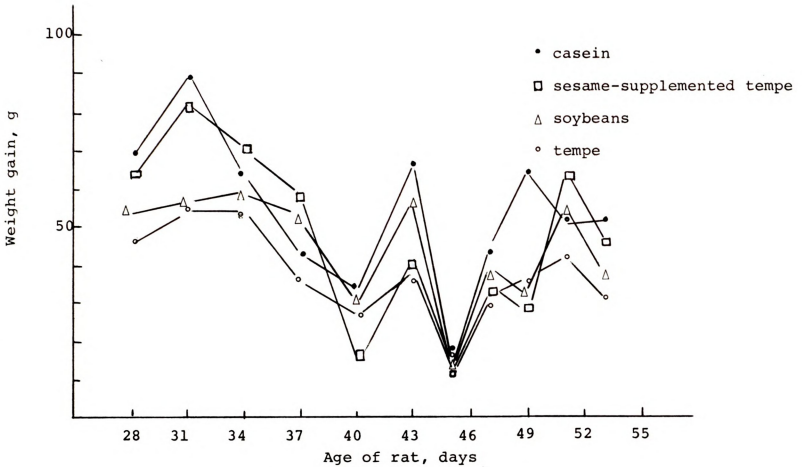


Figure 26. Weight gain (g) of rat groups (6 rats/group), fed different diets, at 10% total protein in the diet.

Table 37.--The PER values of casein, soybeans, tempe and sesame-supplemented tempe (values are mean of 6 male rats in each group; level of protein: 10%).

Source of protein	Protein intake (grams)	Gain in weight (grams)	PER (28 days)	% Casein PER	For casein PER = 2.50
Casein	28.93	98.00	3.39	100%	2.50
Soybeans (fried)	30.00	81.67	2.70	79.6	1.99
Tempe (fried)	26.92	68.17	2.53	74.9	1.87
Sesame-supplemented tempe (fried)	30.65	85.00	2.77	82.0	2.04

One-way analysis of variance and Tukey's test was used for statistical analysis.

Total SS:

$$SS_Y = \sum\sum_{}^{46} Y_{ij} - (Y..^2/24)$$

$$= 197.04 - 193.77 = 3.27$$

Treatment SS:

$$SS_T = (\sum_{i=1}^{4} Y_{i.}^2/6) - (Y..^2/24)$$

$$= 196.33 - 193.77 = 2.56$$

Error SS:

$$SS_E = SS_Y - SS_T = 3.27 - 2.56 = 0.71$$

Source of variation	df	SS	MS
Treatment	3	2.56	$SS_T/3 = 2.56/3 = 0.85$
Error	20	0.71	$SS_E/20 = 0.71/20 = 0.035$

$F_{0.05,\ 3.20} = 3.1$ (table)

Actual $F = \frac{MS_T}{MS_E} = 0.85/0.035 = 23.94 >> 3.1$, there is significant treatment mean difference,

Specific test (Tukey's):

$$F = (q_{0.05,\ 4,20})\ (\sqrt{MS_E/6})$$
$$= 3.958 \times 0.0768$$
$$= 0.3040$$

The F_{values} of PER : casein and soybeans = 3.39 - 2.70 = 0.69

casein and tempe = 3.39 - 2.53 = 0.86

casein and sesame supplemented tempe = 3.39 - 2.77 = 0.62

Because those F_{values} are bigger than 0.3040, so that there are significant differences between casein and all soybeans, tempe and sesame-supplemented tempe proteins.

The F_{values} of PER : soybeans and tempe = 2.70 - 2.53 = 0.16

soybeans and sesame - tempe = 2.70 - 2.77 = 0.07

tempe and sesame - tempe = 2.77 - 2.53 = 0.24

The F_{values} of soybeans, tempe and sesame-supplemented tempe are smaller than 0.3040, therefore no significant difference exists in the protein quality of soybeans, tempe and sesame-supplemented tempe.

The protein quality of tempe, sesame supplemented tempe and soybeans was statistically the same. The PER value of sesame-supplemented tempe was slightly higher than the unsupplemented tempe, but not significantly at the 5% statistical level. Figure 25 shows the rats receiving the sesame-supplemented tempe gained more weight compared to the unsupplemented tempe and the plain soybeans. This extra weight gain was due to extra consumption of feed and does not contribute to the improvement of sesame-supplemented tempe PER.

Fried tempe protein was not significantly different, from the fried unfermented soybean protein. This finding was in agreement with the statement of György's (1961) that the heating of tempe at 66°C lowered the PER value in comparison with unfermented soybean flour. In the present experiment (Table 37), frying tempe at 200°C for 3 minutes resulted in a PER slightly lower than that of fried unfermented soybeans. The data from Chang and Murray (1949) also showed that autoclaving soybean curd lowered the PER to 1.59 from the PER of autoclaved soybeans of 1.73. Apparently, the curdling and the tempe fermentation processes modify the protein structure so that heat has more detrimental effect on the PER of the protein than on the plain soybean protein.

Intact proteins are digested and absorbed in human subjects rapidly enough to be metabolized along with the free amino acids consumed at the same meal. In planning

dietaries, the administration of amino acids whether in the form of foods or crystalline amino acids can be applied satisfactorily with no significant difference (Clark, 1965). Consequently, the report by Van Buren et al. (1972) that at the end of tempe fermentation there was an accumulation of low molecular weight nitrogen compounds and solubilization of proteins, should not be expected in the improvement of protein quality in soybeans before and after fermentation. Furthermore, Murata (1965) had proved that protein content and amino acid profile of tempe and unfermented soybeans were essentially the same.

György (1961) showed a significant PER improvement of lyophilized tempe over the unfermented soy flour using a 10-week feeding period in rats at the 10% protein level. However, drying tempe at 150°F (66°C) has resulted in similar PER value with similarly treated unfermented soy flour. Smith et al. (1964) also reported a slight decreasing PER value of tempe at 14% protein level from 2.42 to 2.35 after autoclaving the tempe for 40 minutes at atmospheric pressure. Processing has some detrimental effect on amino acid availability, especially if the intakes were minimal. For example, 9% of the lysine in the wheat flour was destroyed or became unavailable during baking. Amino acids in foods high in carbohydrates are particularly susceptible to damage by heat (Clark, 1965). Results of Chang and Murray (1949) also suggested that autoclaved soybeans and unautoclaved soybean-curd have the

same PER value of 1.73. However, by autoclaving soybean-curd (110°C for 30 minutes) the PER dropped to 1.59. Apparently soybean-curd protein was more sensitive to heat than the unprocessed soybeans.

The biological values of many proteins are considerably changed by heat processing in the absence of discernible destruction of amino acids. The possible reasons for this change in biological value are as follows:

1. The digestibility of protein may be depressed.
2. The application of heat to a protein may promote certain combinations between terminal groupings that are resistant to proteolytic action (Sheffner, 1967).

In this study, however, the fried tempe as well as the fried soybeans control were investigated, since dried raw tempe is never consumed in Indonesia.

The supplementation of sesame seed in soybean tempe

Numerous attempts have been made to improve the quality of protein sources for man be judicious supplementation with essential amino acids or by combining foods in a manner to achieve mutual supplementation. The objective of these supplementation efforts was to improve the balance or proportions among the essential amino acids by overcoming a deficit without causing any amino acid to become either limiting or excessive.

Table 38 shows the amino acid compositions of tempe, the FAO reference pattern, egg, human milk, cow's milk, sesame seed and the pattern of amino acid requirements of human beings.

The reference pattern of amino acids was designed to evaluate the quality of protein foods, singly or in combination; it was developed in 1957 by the Committee on Protein Requirements of the Food and Agricultural Organization (FAO, 1957). The assumption was made that the proportions of individual amino acids in this pattern, were optimal.

It has been demonstrated that efficient synthesis of tissue proteins occurs only when all essential amino acids are supplied simultaneously, and in proper proportions (Cannon et al., 1947; Geiger, 1947; Schaeffer and Geiger, 1947).

Chang and Murray (1949) supplemented sesame seed to soybeans (dried autoclaved soybean: sesame - 10:4) and to soybean-curd (dried curd: sesame meal = 10:6). The PER of autoclaved soybean diet was 1.73 and increased to 2.17 when supplemented with sesame meal. The PER of standard whole milk powder was 2.30.

Kies and Fox (1971) evaluated the protein nutritional value of TVP (textured vegetable protein), 1% DL-methionine enriched TVP and beef in human adults. From their experiments they concluded that at 8.8 g N intake level, TVP, methionine-fortified TVP and ground beef were of equal

Table 38.--The amino acid composition of an FAO pattern and of eggs, human milk, cow's milk, tempe and sesame. The amino acid requirements of man are also listed.[1]

	FAO[3] Pattern	Egg[3]	Human[3] Milk	Cow's[3] Milk	Tempe[4]	Sesame[5] seed	Pattern of amino acid requirements[2]				
							Infant		Child	Adults	
							0-1/2y	1/2-1Y		Female	Male
Arginine	. .	6.6	4.1	3.7	7.0	11.4	. .	. .	. .	. .	. .
Histidine	. .	2.4	2.2	2.7	2.7	2.7	2.4	. .	. .	. .	. .
Lysine	4.2	6.6	6.6	7.9	6.6	3.1	7.7	7.5	10.7	5.1	5.1
Leucine	4.8	8.8	9.1	10.0	9.0	7.8	10.9	10.9	8.0	6.1	7.0
Isoleucine	4.2	6.6	5.5	6.5	5.7	4.7	6.6	9.2	5.3	4.6	4.5
Methionine	2.2	3.1	2.3	2.5	1.3	3.3	4.8	3.3	. .	3.5	1.3
Cystine	. .	2.3	2.0	0.9	1.9	1.0	. .	. .	. .	2.1	5.1
Total S-acids	4.2	5.4	4.3	3.4	. .	. .	6.2	. .	4.8	5.6	6.4
Phenylalanine	2.8	5.8	4.4	4.9	4.8	5.4	6.6	6.5	4.8	2.2	1.9
Tyrosine	2.8	5.0	5.5	5.1	2.6	3.5	. .	. .	. .	9.1	7.0
Total aromatic	5.6	10.8	9.9	10.0	. .	. .	. .	. .	. .	11.3	8.9
Threonine	2.8	5.0	4.5	4.7	4.5	4.7	4.4	6.3	6.1	3.0	3.2
Tryptophan	1.4	1.7	1.6	1.4	1.4	1.4	1.6	1.6	1.6	1.6	1.6
Valine	4.2	7.4	6.3	7.0	5.5	5.7	6.7	7.6	5.9	6.6	5.1

[1]Expressed as g/16 g N or g/100 g protein.
[2]National Academy of Science, 1963.
[3]Clark, 1965.
[4]Murata, 1965.
[5]FAO/USHEW, 1972.

nutritional value, with a nitrogen balance of + 0.74, + 0.78 and + 0.72 respectively. On the other hand, at the 4.8 g daily N intake, ground beef was seemingly superior to both methionine - fortified TVP and the unenriched TVP. At 4.8 g N intake, methionine fortification at 1% level resulted in improvement in protein value but not to the extent of being equal to beef. At this low level N intake, the N balance of beef, methionine-enriched TVP and unenriched TVP were -0.30, -0.45, and -0.70, respectively.

Additional data from the same authors suggested that methionine supplemented TVP improved the PER value for growing rats, as shown in the respective PER values for TVP, methionine-enriched TVP and beef of 2.12, 2.82, and 2.37.

Experimental results of the present study (Table 37) showed no improvement in the PER of fried, sesame-supplemented tempe over fried non-supplemented tempe.

This non-significant improvement of sesame-supplemented tempe may be due to insufficient amount of sesame, as 9 parts of soy protein was supplemented with only 1 part of sesame protein. Another possibility is the fact that the deficiency of methionine in soybeans itself is not very great as in other beans, and the supplementation effect is minimal.

At 14% protein level, Smith et al. (1964) reported a PER improvement in freeze dried soybean-tempe supplemented

with 0.3% methionine. The PER value of methionine-supplemented tempe was 3.09 compared to 2.48 for unsupplemented soybean tempe.

The report of PER improvement from sesame-supplemented soybeans and soybean curd by Chang and Murray (1949) was probably due to a larger proportion of sesame used (10:4 and 10:6). Also they did not fry the soybean-sesame mixture in their experiment.

Kies and Fox (1971) reported that fortification of TVP with methionine had an insignificant effect on the N balance of adult males. The same authors, however, reported an increase in the PER of TVP supplement by methionine in rats and an improvement in the N balance of adolescent boys (Korslund et al., 1973).

Russell et al. (1946) investigated the PER values of various legumes and the effect of methionine supplementation. For cooked and dried soybeans, the PER increased from 2.1 for basal diet to 2.8 for 0.1% methionine supplemented soybeans. The PER remained at 2.6 even though the methionine supplementation was increased to 0.6%. They noted that soybeans provided sufficient methionine to support growth in the basal diet, and the supplementation with methionine did not result in increases of PER as high as those observed with other legumes.

SUMMARY AND CONCLUSIONS

Tempe was prepared from soybeans and a pure culture of Rhizopus oligosporus. Electron micrographs of soybeans and tempe showed that boiling of soybeans resulted in the exit of protein bodies and spherosomes from the cell through the disrupted cell walls. The cell disruption apparently facilitates the tempe mold to grow. The mold penetrated only about two cell layers of soybean cotyledons.

Based on the liberation of free fatty acids, microbial growth and organoleptic changes, the tempe fermentation may be differentiated into three phases: (1) rapid phase (0-30 hr), (2) transition phase, and (3) deterioration phase (beyond 60 hr of fermentation). It is believed that the contaminating, sporeforming Bacilli were mainly responsible for the deterioration of tempe.

During tempe fermentation, oleic acid was liberated from glycerides more than any other fatty acid in the soybeans.

In general, frying of tempe decreased its content in free fatty acids, except linolenic acid. This decrease was due to migration of free fatty acids from the tempe to

the frying oil. Linolenic acid also migrated, but an almost equal quantity of it was liberated from the tempe glycerides, and therefore its concentration in the tempe slightly increased after frying. No migration of glycerides from tempe to frying oil was detected.

The TBA test was not specific for rancidity determination in soybeans since fresh, raw soybeans with a strong beany flavor, but not rancid flavor, had a high TBA value. Tempe, as well as boiled soybeans had lower TBA values than plain soybeans after storage at room temperature for at least one month.

The phytic acid content of soybeans decreased from about 1.4% to 1.0% after tempe fermentation. The phosphate liberated was equivalent to the loss of phytic acid.

There was a strong phytase activity in tempe. The phytase was produced by the tempe mold, R. oligosporus. Natural or added phytates did not promote the phytase synthesis by the Rhizopus; on the contrary it had an inhibiting effect. The tempe phytase had an optimum activity (sodium-phytate as substrate) at pH 5.6, and a Michaelis constant (K_m) of 0.28×10^{-3}M.

The Protein Efficiency Ratio values of fried tempe (2.53) sesame-supplemented tempe (2.77) and plain soybeans (2.70) were not statistically different at the 10% total protein content of the diet.

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APPENDICES

APPENDIX A

FATTY ACIDS

APPENDIX A

FATTY ACIDS

Carbon atoms and double bonds	Systematic name	Common name
$C_{4:0}$	Butanoic	Butyric
$C_{6:0}$	Hexanoic	Caproic
$C_{8:0}$	Octanoic	Caprylic
$C_{10:0}$	Decanoic	Capric
$C_{10:1}$	4 Decenoic	Obtusilic
$C_{10:1}$	9 Decenoic	Caproleic
$C_{12:0}$	Dodecanoic	Lauric
$C_{12:1}$	4 Dodecenoic	Linderic
$C_{12:1}$	9 Dodecenoic	Lauroleic
$C_{14:0}$	Tetradecanoic	Myristic
$C_{14:1}$	4 Tetradecenoic	Tsuzuic
$C_{14:1}$	5 Tetradecenoic	Physteric
$C_{14:1}$	9 Tetradecenoic	Myristoleic
$C_{16:0}$	Hexadecanoic	Palmitic
$C_{16:1}$	9 Hexadecenoic	Palmitoleic
$C_{18:0}$	Octadecanoic	Stearic
$C_{18:1}$	6 Octadecenoic	Petroselinic

Carbon atoms and double bonds	Systematic name	Common name
$C_{18:1}$	9 Octadecenoic	Oleic
$C_{18:1}$	11 trans-Octadecenoic	Vaccenic
$C_{18:2}$	cis-cis-9,12, Octadecadienoic	Linoleic
$C_{18:3}$	cis-cis-cis-9,12,15 Octadecatrienoic	Linolenic
$C_{18:3}$	cis-trans-trans- 9,11,13, Octadecatrienoic	Eleostearic
$C_{18:3}$	trans-trans-trans 9,11,13, Octadecatrienoic	Eleostearic
$C_{18:4}$	9,11,13,15, Octadecatetraenoic	Parinaric
$C_{20:0}$	Eicosanoic	Arachidic
$C_{20:1}$	9, Eicosenoic	Gadoleic
$C_{20:1}$	11, Eicosenoic	--
$C_{20:4}$	5,8,11,14 Eicostatetraenoic	Arachidonic
$C_{22:0}$	Docosanoic	Behenic
$C_{22:1}$	11 Docosenoic	Cetoleic
$C_{22:1}$	13 Docosenoic	Eurcic
$C_{22:5}$	4,8,12,15,19 Docosapentaenoic	Clupanodonic
$C_{24:0}$	Tetracosanoic	Lignoceric
$C_{24:1}$	15 Tetracosenoic	Selacholeic
$C_{26:1}$	17 Hexacosenoic	Ximenic
$C_{30:1}$	21 Triacontenoic	Lumequeic

APPENDIX B

TEMPERATURE OF TEMPE DURING FERMENTATION AT 25°C AND 32°C

APPENDIX B

TEMPERATURE OF TEMPE DURING FERMENTATION

AT 25°C AND 32°C

Time (hrs)	At 25°C	At 32°C
0	25.0	25.0
2-3/4	25.0	30.0
7	25.0	30.5
14	25.0	31.5
17	25.0	33.0
18	25.0	33.5
19	25.0	35.0
19.5	25.0	35.5
19.75	25.0	36.0
20	25.0	36.5
20.25	25.0	37.0
20.5	25.0	37.5
20.75	25.0	38.0
21	25.0	38.0
21.5	25.0	39.0
22	25.0	40.0
22.5	25.0	40.0
22.75	25.0	40.5
23	25.0	41.0
24	25.0	42.0
25	25.0	42.5
26	25.0	43.0
26.5	25.0	43.5
27	25.0	43.5

Time (hrs)	At 25°C	At 32°C
27.5	25.0	42.5
28	25.5	42.5
28.5	25.5	42.5
39.5	28.0	40.0
40.0	28.5	40.0
41.0	29.0	39.5

APPENDIX C

SENSORY RANCIDITY TEST

APPENDIX C

SENSORY RANCIDITY TEST

Please indicate the rancidity of the following numbered soybean samples, by smelling an open jar, one at a time.

If the sample is rancid, please indicate the magnitude of its rancidity.

Sample No.	Rancidity		
	None	Weak	Strong
_____	____	____	____
_____	____	____	____
_____	____	____	____
_____	____	____	____
_____	____	____	____
_____	____	____	____
_____	____	____	____

APPENDIX D

ANIMAL FEEDING LOG

APPENDIX D

ANIMAL FEEDING LOG

Init. date ..... Animal Feeding Log Observation date .....

Animal no.	Initial Feed + Container	Spent Feed + Container	Wasted Feed	Feed Consumed	Animal Weight: At Init. date	Animal Weight: At obser. date	Gain
Feed:.....							
...	...	...	...	....		...	
...	...	...	...	...		...	
...	...	...	...	...		...	
...	...	...	...	...		...	
Feed:.....							
...	...	...	...	...		...	
...	...	...	...	...		...	
...	...	...	...	...		...	
...	...	...	...	...		...	

APPENDIX E

STANDARD CURVE FOR Fe

APPENDIX E

STANDARD CURVE FOR Fe