AN EVALUATION OF SELECTED TECHNIQUES FOR REDUCTION OF NUCLEIC ACIDS IN PROTEINS OBTAINED FROM *SACCHAROMYCES CEREVISIAE*

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ABSTRACT

AN EVALUATION OF SELECTED TECHNIQUES FOR REDUCTION OF NUCLEIC ACIDS IN PROTEINS OBTAINED FROM SACCHAROMYCES CEREVISIAE

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Methods for decreasing the nucleic acids (NA) of disintegrated yeast cells and their effect on the isolated proteins were investigated. The methods used consisted of:

1. Precipitation of the NA with protamine sulfate, streptomycin, manganous chloride and/or phase-separation. The optimum concentration of protamine sulfate for maximum reduction of NA and minimum loss of protein was determined in which nucleic acids were reduced up to 64 percent with protein loss of only 50 percent. Samples treated with protamine sulfate showed more protein zones on different electrophoretic techniques than any other treatment. Reduction of NA by protamine sulfate caused an increase in total sulfhydryl and available lysine in the proteins. Streptomycin reduced the NA up to 57 percent while protein recovery was 76 percent. $MnCl_2$ caused precipitation of NA up to

55 percent. Protein loss was only 30 percent. Storage of the treated samples at 0°C resulted in additional precipitation of the NA. With polyethylene glycol and dextran, up to 66 percent of the total NA could be separated. The molecular weights of the proteins after removal of NA were measured on gradient SDS-polyacrylamide gel electrophoresis (PAGE). Isoelectric points of most of the proteins were in the range of 4.5 to 7.5. Proteins remaining after these treatments were generally undenatured, since the activities of lactic dehydrogenase and esterase were retained.

2. Separation of proteins from NA by precipitation. This was accomplished by hot sodium chloride or pH adjustment. The protein loss was very significant, but the ratio of proteins to NA was higher than with other methods of separation. Most of the proteins remaining were denatured. Electrophorograms of the proteins exhibited very few zones with different mobility from control.

3. Hydrolysis of NA. Activation of endogenous ribonuclease (RNase) by a heat-shock followed by incubation caused reduction of NA up to 56 percent. Treating yeast cells with bovine RNase lowered the NA to 39 percent. Protein loss was significant in both methods. Heat treatment apparently caused denaturation of the proteins. Proteins were affected more by heat-shock than

incubation. On electrophoresis, the exogenous RNase-treated sample showed fewer zones than the proteins obtained by endogenous RNase. Molecular weight of the proteins in exogenous RNase method were in the range of 14,300 to 318,000 while in endogenous RNase were in the range of 13,000 to 212,000.

Proteins remained after the removal of NA had generally higher levels of essential amino acids than the untreated sample.

4. Removal of NA by affinity chromatography. On a DEAE-cellulose column, most of the proteins were separated from NA. Part of the proteins were eluted along with NA which could be detected on PAGE of the fractions.

Protamine was immobilized on Sepharose and used for separation of NA. Addition of 20 percent dioxane to the elution buffer improved the separation. Removal of NA from a freeze-dried yeast homogenate was more difficult. By a column of immobilized RNA on Sepharose, part of the NA (less than 10 percent) was separated.

Yeast proteinases were investigated in another study. They behave like both chymotrypsin and trypsin. Phenylmethyl sulfonyl fluoride inhibits the activity of these proteinases to a large extent. Soybean trypsin inhibitor at concentrations of about 100 μg per mg yeast protein has strong inhibitory effect on yeast proteinases

while navy bean trypsin inhibitor even at higher concentrations did not have any significant effect. Soybean trypsin inhibitor immobilized on Sepharose removed a significant quantity of the proteinases.

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TABLE OF CONTENTS

Page

LIST OF TABLES vi

LIST OF FIGURES viii

INTRODUCTION 1

LITERATURE REVIEW 3

The Potential Utilization of Single Cell Protein 3
Other Uses of Yeast 5
The Yeast Cell Wall 5
Techniques for Breaking the Cell Wall of Yeasts 6
  Enzymatic Hydrolysis 6
  High Pressure Homogenization 6
  Ultrasonic Vibration 7
  Agitator Mill 7
  Ball-Mill Disintegrator 8
Nucleic Acids in Yeasts 8
Techniques to Lower the Nucleic Acid Content 10
  Removal of Nucleic Acids by $MnCl_2$ . . 10
  Removal of Nucleic Acids by Phase Separation 11
  Removal of Nucleic Acids with Protamine Sulfate 11
  Removal of Nucleic Acids by Streptomycin Sulfate 12
  Lowering the Nucleic Acid Content by Protein Precipitation 13
  Removal of Nucleic Acids by Ribonucleases 14
  Removal of Nucleic Acids by Base Hydrolysis 16
Affinity Techniques in Removing Nucleic Acids 16
Proteinases 18

Page

MATERIALS AND METHODS 22

Chemicals 22
Sample Preparation 22
Removal of Nucleic Acids with $MnCl_2$ 23
Removal of Nucleic Acids by Phase Separation 24
Removal of Nucleic Acids with Streptomycin 25
Removal of Nucleic Acids with Hot NaCl 26
Heat Treatments for Lowering Nucleic Acids 27
Removal of Nucleic Acids by pH Adjustment 28
Removal of Nucleic Acids with Protamine Sulfate 32
Electrophoretic Studies 33
Disc Gel Electrophoresis 33
SDS-Gel Electrophoresis 35
Disc Gel Electrophoresis in Urea 36
Gel Isoelectric Focusing 37
Chemical Analysis 38
Determination of Lysine 38
Sulfhydryl Group Determination 39
Amino Acid Analysis 39
Proteolytic Activity 40
Protein Measurements 40
Lowry Method 49
Biuret Method 41
Bio-Rad Protein Assay 43
Nitrogen 43
Nucleic Acid Measurements 44
Chromatographic Procedures 45
Immobilization Techniques 45
DEAE Column 46
Gel Filtration 46

RESULTS AND DISCUSSION 50

I. Effect of Removing Nucleic Acids on Proteins 50
$MnCl_2$ Precipitation of Nucleic Acids 51
Phase Separation of Nucleic Acids 53
Protamine Sulfate as Nucleic Acid Precipitating Agent 63
pH Adjustment 68

Page

Separation of Yeast Proteins with Sodium Chloride 69
Exogenous Ribonuclease for Reduction of Nucleic Acids 70
Endogenous Ribonuclease for Reducation of Nucleic Acids 73
Effect of Nucleic Acid Removal on Amino Acid Composition 78
II. Removing Nucleic Acids by Affinity Chromatography 81
Diethyl Amino Ehtyl Cellulose (DEAE) . 81
Immobilization of Protamine on Sepharose 86
Immobilization of RNA on Sepharose . . 90
Significance of Affinity Techniques in Removal of Nucleic Acids 93
III. Effect and Control of Yeast Proteinases 94

SUMMARY 115

APPENDIX 117

REFERENCES 121

LIST OF TABLES

Table Page

1. Effect of different treatment on removing nucleic acids and protein recovery . . . 52

2. Effect of different concentrations of protamine sulfate on removal of nucleic acids and total solid and protein recovery 64

3. Effect of removing NA by 2/10 v/v of a protamine solution and proteinase inhibitor on sulfhydryl and disulfide group of yeast proteins 66

4. Effect of removing NA by 2/10 v/v of a protamine sulfate solution and proteinase inhibitor on available lysine in yeast proteins 66

5. Effect of different treatments with or without ribonuclease A on reduction of nucleic acid content in yeast 71

6. Effect of heat shock process used for activation of endogenous ribonuclease in lowering nucleic acid content in yeast 74

7. Amino acid composition of yeast proteins lowered in nucleic acids by different treatments 79

8. Effect of navy bean trypsin inhibitor on yeast proteinases measured by method of Kakade et al. 109

9. Effect of soybean trypsin inhibitor on yeast proteinases measured by method of Kakade et al. 111

Table Page

A.1 Some important chemicals used in this study and their sources 118

A.2 Formulation of 7.5 percent Polyacrylamide Gel Electrophoresis System 120

LIST OF FIGURES

Figure		Page
1.	Flow diagram of preparation of sample . .	23
2.	Flow diagram of removal nucleic acids with $MnCl_2$	24
3.	Flow diagram of removal of nucleic acids by phase separation	25
4.	Flow of Nucleic Acids with Hot NaCl . .	26
5.	Flow diagram of removal of nucleic acids with hot NaCl	27
6.	Flow diagram of removal of nucleic acids by heat shock	29
7.	Flow diagram of removal of nucleic acids by exogenous ribonuclease	30
8.	Flow diagram of removal of nucleic acids by pH adjustment	31
9.	Flow diagram of removal of nucleic acids with protamine sulfate	32
10.	Cyanogen bromide activation of carbohydrate polymers and reaction with enzyme amino group	47
11.	Polyacrylamide gel electrophorogram of yeast proteins stained for lactic dehydrogenase and esterase	54
12.	Polyacrylamide gel electrophorogram of yeast proteins in 7.5-17.5 percent w/v gels after different treatment for reduction of nucleic acids	56

Figure Page

13. Diagram of the SDS electrophoretic patterns of yeast proteins in 7.5-17.5 percent (w/v) gradient gels 58

14. Diagram of the electrophoretic patterns of yeast protein in isoelectric focusing 60

15. Chromatogram of nonsedimentable portion of ca 100 mg of yeast cell homogenate on a 1 x 30 cm column of DEAE-cellulose . . 82

16. Polyacrylamide gel electrophorogram of yeast proteins fractionated on a DEAE column 84

17. Chromatogram of nonsedimentable portion of yeast cell homogenate on a 1 x 15 cm column of Protamine-//-Sepharose 4B . . 88

18. Chromatogram of nonsedimentable portion of yeast cell homogenate on a 1 x 15 cm column of RNA-//-Sepharose 4B 91

19. Diagram of electrophoretic pattern of yeast cell homogenate incubated at 37°C in a water bath shaker for various times 96

20. Diagram of electrophoretic pattern of yeast cell homogenate incubated at 37°C in a water bath shaker for various times 98

21. Action of yeast proteinases on TAME and BTEE measured as change in absorbance at 247 and 256 nm, respectively, according to Walsh and Wilcox 100

22. Densitometric tracing of yeast proteins on gradient SDS-PAG 102

23. Densitometric tracing of yeast proteins on 7.5 percent w/v PAG 104

Figure Page

24. Diagram of the electrophoretic patterns of yeast proteins low in nucleic acid in 7.5-17.5 percent gradient SDS-PAG . . . 106

25. Affinity chromatography of yeast Proteinases on a soybean trypsin inhibitor-//-Sepharose 4B column 112

INTRODUCTION

There is adequate evidence to indicate the need for single cell protein (SCP)* as a supplement to the world protein supply, and research and development on SCP production has been intense for over a decade.

By definition, single cell protein is a generic term used to describe crude or refined proteins originating from unicellular or simple multi-cellular organisms. A process that yields SCP utilizes bacteria, yeast, fungi and algae grown on a variety of substrates ranging from hydrocarbons or oxygenated hydrocarbons such as methane, methanol and gas oil to complex biological media, including carbohydrates, sulfite liquors, molasses and whey.

There are many limitations to the use and acceptance of SCP in foods, of which the resistant cell wall, flavor and nucleic acid content are the most important.

The thick cell wall is a barrier in the utilization of protein and the relatively high nucleic acid content is a limiting factor in the recommended dietary

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*The acronym SCP coined at a conference held at the Massachusetts Institute of Technology to avoid connotation with terms such as "bacterial" or "microbial" proteins.

intake of SCP (whole cell). The total amount of nucleic acid in the diet should be limited (Edozien et al., 1970) because of its effect on uric acid levels in blood and urine.

There are several different ways to lower the nucleic acid content of yeast protein. Major concerns of this research were: (1) to evaluate these techniques and to determine their effect on protein recovery, damage to the proteins, and the effect of nucleic acid removal on the general characteristics and profile of the remaining proteins, (2) to examine the effect of endogenous yeast proteinases on the proteins during isolation and to attempt to inhibit their activity without seriously altering the characteristics of the yeast proteins.

LITERATURE REVIEW

Yeasts have been serving man from prehistoric times starting with the use of spontaneous fermentation of fruits and fruit juices. Later, man used yeasts in leavening bread, brewing wine and beer and in other fermented foods.

Buchner and Buchner in 1897 accidently discovered "Zymase" of yeast, based on the fundamental fermentation postulations of Pasteur. They used a cell free extract of yeast containing sucrose as a preservative. Upon observation of "bubbles and froth" in the cell free alcoholic fermentation, they names the agent responsible "zymase." The commercail production of yeast began in Germany in 1918.

The Potential Utilization of Single Cell Protein

Utilization of single cell protein (SCP) as a food has been considered by authorities concerned with world food supplies and population increase. With the problem of increasing population, there is no doubt that much larger supplies of protein will be needed in the future. Even with improved agricultural technology, the problem of supplying adequate food is not readily achieved. Some industrialized countries such as the Soviet Union and Japan and those in Western Europe depend on the world market for much of their food supply. In 1972, bad weather and drought proved that even North America, which is the major supplier of food to the world, is vulnerable to shortages.

Energy, land and labor are major factors in determining the economic feasibility of providing foods. Production of animal protein requires tremendous amounts of energy, land and labor. In general, for each Kg of animal protein produced 5 Kg plant protein is fed to the livestock (Petimental et al., 1975). Cropland, one of our best resources, is rapidly deteriorating largely as a result of loss of topsoil. Fossil energy is limited, and all these problems are inducements to look for other sources of protein for a growing population.

Single cell proteins have several clear advantages over agriculturally derived proteins:

1. They do not depend upon agricultural land or climatic conditions
2. They have a very rapid mass growing time (20-120 min. for doubling)
3. They have a high protein content compared to high order plants because they do not

have much need for supporting materials such as cellulose

4. Sunlight and surface are not limiting factors for their growth
5. They can be modified genetically for improvement
6. They can be grown on waste materials such as molasses, whey and similar low cost substrates

Other Uses of Yeast

Yeasts are widely used in the food industry for: (1) alcoholic beverages, (2) production of ethanol and glycerol, (3) leavening bread and other baked goods, (4) animal feed, (5) production of lipid materials (Newman et al., 1933), (6) a source of B vitamins, (7) a source of enzymes, and (8) isolation of polysaccharides such as glucans.

The Yeast Cell Wall

The tough yeast wall resists digestion and adds unnecessary bulk to the diet. If the whole cell is ingested, the cell may pass through the digestive tract intact and its protein content may not be available for use. Disruption of the cell wall is therefore important to enable release and recovery of intracellular proteins.

The principal component of the cell wall of Saccharomyces are several types of glucan and a mannan--protein complex. These highly insoluble polysaccharides have predominantly β-1, 3 linkages between the glucose moities, but β-1, 6 linkages have also been found (Phaff, 1977). The cell wall represents about 15 percent of the dry weight of the yeast and is made up of 20-40 percent mannan, 5-10 percent protein, 1 percent chitin and 30-60 percent glucan (Phaff, 1977).

Techniques for Breaking the Cell Wall of Yeasts

Enzymatic Hydrolysis

There are several β-gluconases which are able to break the cell wall. These enzymes may be native to the cell (endogenous) or isolated from other sources (exogenous) such as snail gut or microbes (Phaff, 1977).

High Pressure Homogenization

Industrial type homogenizers which operate at pressures of 25,000 psi or more have been reported to be effective in disrupting the cell wall (Hetherington et al., 1971; Brookman, 1974). The disintegration process may be described by a first-order equation

$$\log [Rm/(Rm-R)] = KNP^{2.9}$$

in which R is the protein released, Rm is the maximum protein available for release (96 mg/g packed yeast), constant K is a function of the suspension temperature, N is the number of passes, and P is the operating pressure (Hetherington et al., 1971). Withworth in 1974 used high pressure homogenization and found that temperatures between 10-30°C had no effect on solubilization of the proteins. Maximum solubilization occurred at very high pressure.

Ultrasonic Vibration

Using a sonifier S-75 (Branson) and a 10 percent (w/v) suspension of *S. cerevisiae* in water, Rosett (1965) found total cell disruption at the end of 10 minutes and increasing alcohol dehydrogenase activity in the supernatant at the end of 25 minutes. The increased dehydrogenase activity was attributed to the release of the enzyme from the cell wall with increased sonification. Hughes (1961) found that disintegration of the cells in an ultrasonic cell was partially due to cavitation. Cell rupture is independent of sonically generated free radicals, and enzyme inactivation (alcohol dehydrogenase) is accelerated by free radicals.

Agitator Mill

Currie et al., (1972) described the disruption of yeasts in a high-speed agitator mill. The mechanism is a first-order rate process. Increased disruption efficiency was obtained at higher agitator speeds, greater loading of bead friction elements and lower rate of upward recycling of yeast suspension through the mill. Cell disruption at optimal loading (5.32 Kg/hr.) was 90 percent.

Ball-Mill Disintegrator

The Ball-Mill disintegrator is applicable on a large scale for batch or continuous operation. Hedenskog et al., (1970) studied the effect of different conditions on protein extracted by this method. A treatment of 10-20 minutes was sufficient to break the majority of the cells. Beads with a diameter of less than 0.2 mm and 1:1 ratio (v/v) of beads to cell suspension gave optimal rupturing. Maximum yield of soluble nitrogen was obtained for yeasts, algae and bacteria by extracting the disintegrated cells with 0.3-0.4 percent sodium hydroxide at pH 11.0-11.5.

Nucleic Acids in Yeasts

Ingestion of large quantities of yeast has been shown to produce elevated uric acid levels in blood and urine (Dirr et al., 1942; Edozien et al., 1970). Yeasts and most SCP have higher nucleic acid contents than conventional foods, probably because of their higher growth rate. After ingestion, the nucleic acids are depolymerized by pancreatic nucleases to nucleotides. Purines are not metabolized further than uric acid in man and higher primates because of lack of the enzyme uricase. Therefore, they appear in urine as uric acid rather than being converted to the more soluble compound "allantoin." Some individuals have a tendency for over production of uric acid which may lead to the precipitation of uric acid in joints or soft tissues and the formation of kidney stone in the urinary tract (Kihlberg, 1972).

The pyrimidines, as well as purines, may cause health problems. For example, it is reported that large amounts of orotic acid have caused fatty liver and liver degeneration (Miller, 1968).

For the above reasons, the daily intake of nucleic acids should not exceed 2g (PAG recommendation, 1975), which is equivalent to an intake 20-30 g of whole yeast (Kihlberg, 1972). Since nucleic acids are ingested in other conventional foods, the amount of SCP recommended in the diet would be practically even lower. Consequently, the high nucleic acid content of microorganisms has been a major limitation to their more widespread use as a food.

Techniques to Lower the Nucleic Acid Content

It is well known that reducing the growth rate of yeast cells will lower the nucleic acid content (Rosett, et al., 1966). This can be achieved by alteration of temperature, diluation rate, composition, and pH of the media (Alroy, et al., 1973). Since fast growth is obligatory for economical production in most processes, the reduction of nucleic acid by this method is not practical. Another disadvantage of this technique is that only a limited reduction in the nucleic acids is achieved.

Techniques of separating nucleic acids from protein which could be applied in the food industry were evaluated in a portion of the research reported in this thesis. The majority of these techniques make use of the fact that nucleic acids are polyanions and consequently, their solubility in the solution can be altered by a variety of reagents including di- and multivalent cations, certain organic cations, strong salts, organic solvents and proteins.

Removal of Nucleic Acids by $MnCl_2$

Korkes et al., in 1951 used manganous chloride to precipitate nucleic acids. Their interest lay in the purification of pyruvate enzymes. They observed that concomitant with the precipitation of nucleic acids there was a loss of proteins. $MnCl_2$ is also used in isolation and purification of proteins (Printz et al., 1967), but is not commonly used to remove nucleic acid.

Removal of Nucleic Acids by Phase Separation

A two-phase system of dextran and polyethylene glycol has been used to precipitate and fractionate nucleic acids as well as proteins (Fried et al., 1971). It is suggested that it acts on the basis of either coprecipitation or of partition between various water: polymer phases. Many investigators explain the process as a complex formation between the macromolecule (protein or nucleic acid) and the ploymer (polyethylene glycol). This process is strongly affected by the electrolyte composition (Albertson, 1965).

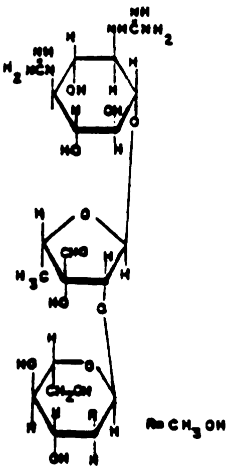
Removal of Nucleic Acids with Protamine Sulfate

Protamines are basic proteins found primarily in the cell nuclei of fish testes. They contain only a few of the amino acids. All of the protamines contain arginine, alanine and serine; most of them contain proline and valine; many contain glycine and isoleucine. Arginine is the dominant amino acid in the protein, comprising up to

91 percent by weight of the total protein. Furthermore, the protamines of different species of fish have different compositions. They have isoelectric points of about 12 (Ando, et al., 1958; Felix, 1960) and molecular weight of less than 17,000. Arginine residues are neutralized by phosophoric acid residues of the nucleic acids, resulting in insolubilization of the nucleic acids and subsequent precipitation.

Removal of Nucleic Acids by Streptomycin Sulfate

Cohn in 1947 reported that nucleic acids could be precipitated by streptomycin. Streptomycin in a positively charged molecule which interacts with the negative charge of phosphate moities of nucleic acids and decreases their solubility.



STREPTOMYCIN A

Moskowitz (1963) compared the effect of streptomycin and dehydrostreptomycin precipitability. He concluded that streptomycin was a more efficient precipitating agent for nucleic acids than dehydrostreptomycin while both more readily precipitate DNA than RNA.

Oxenburgh et al., (1965) used streptomycin to separate nucleic acids from bacterial proteins. He found that precipitation was not affected over the pH range of 6.0 to 8.0 and depended on the salt concentration. As the salt concentration was lowered and the specific resistance rose, the degree of selective precipitation improved. He also pointed out that the higher the amount of streptomycin added, compared with the protein content of the extract, the smaller the loss of protein.

Lowering the Nucleic Acid Content by Protein Precipitation

Hedenskog et al., (1972) studied influence of alkaline protein extraction on the RNA content of protein concentrates. RNA content decreased from 14 percent at pH 7.0 to 8 percent at pH 12. Higher pH values gave a strong decrease of the RNA content, but the yield of protein precipitate also decreased. He noticed that availability of the lysine dropped after extraction at pH 12. The effect of temperature was also evaluated.

He found 80°C much more effective in alkali reduction of RNA than lower temperatures.

Protein low in nucleic acid can also be obtained by precipitation of proteins at low pH. Jayaraman (1973) showed that at pH 3.9 a great majority of nucleic acids could be removed from the proteins in a broken cell suspension.

Robbins (1976) suggested that ultra-high temperature treatment (over 100°C for 10 seconds to 60 minutes) of a ruptured yeast cell suspension was sufficient to separate the majority of proteins from nucleic acids by insolubilization of the proteins. The resultant protein contained 0.5-5 percent RNA compared to the original 12-15 percent.

A hot (80°C), 10 percent (w/v) solution of sodium chloride was reported to deplete yeast of nucleic acids (Kihlberg, 1972). By heat precipitation (80°C) of protein from a suspension of mechanically disintegrated yeast cells at pH 9.5 with NaCl present, a protein fraction was obtained with less than 2 percent nucleic acid (Hedenskog and Mogren, 1973).

Removal of Nucleic Acids by Ribonucleases

Kunitz in 1940 isolated pancreatic ribonuclease and found that it had a molecular weight of 15,000 and isoelectric point of about 8. He pointed out that this heat-stable enzyme was very effective in degrading yeast nucleic acid.

Ne Cas in 1958 studied penetration of ribonuclease into the yeast cell. De Lamater et al., (1959) studied the material which leaked from *B. megaterium* and reported that these materials were primarily digested nucleic acids.

Higuchi and Uemura (1959) suggested that release of the nucleotides in the culture of the yeast cell was inhibited by magnesium and calcium ions and was not necessarily accompanied by death of the cells.

Ribonuclease was found to inhibit the protein synthesis in naked yeast cells up to 100 percent (DeKloet et al., 1961). Allwood et al. in 1968 reported that heat shock induced degradation and leakage of nucleic acids in *S. aureus*. Eichhorn et al., (1969) found optimum concentration of the Mg^{2+} and Ca^{2+} for the activity of bovine pancreatic ribonuclease beyond which the addition of the Mg^{2+} and Ca^{2+} would inhibit enzyme activity.

Castro et al., (1971) suggested that the ribonucleic acid content in yeast could be lowered by addition of bovine pancreatic ribonuclease. The process of penetration of enzyme into the yeast was accelerated by a heat shock (80°C for 30 seconds).

Ohta et al., (1971) demonstrated that endogenous ribonuclease which exhibit increased activity after heat treatment were able to break the nucleic acids to small fragments which could leak from the cell.

Ribonuclease activity is strongly inhibited by purine nucleolides phosphates and only slightly by pyrimidine nucleotide phosphates in yeast (Imada et al., 1972).

Removal of Nucleic Acids by Base Hydrolysis

Yeast ribonucleic acid when incubated at 37°C in 0.5-0.1 N sodium hydroxide was shown to break down into low moleuclar weight dialyzable nucleolides (Daly and Ruiz, 1974; Hedenskog et al., 1970, 1973).

Affinity Techniques in Removing Nucleic Acids

Brown and Watson in 1953 immobilized histones, which are comparable to protamines, on the surface of kieselguhr (celite or diatomaceous earth) and demonstrated ability to absorb DNA. RNA elutes with 0.4 M NaCl solution while DNA is retained. The intact structure of DNA was necessary for effective binding to the immobilized histone.

Larman in 1955 used methylated bovine serum albumin bound to celite in studying DNA. Mandell et al.,

(1960) used the same technique for separation and identification of several natural and derived nucleic acids. He proposed that nucleic acids attach to the column material by salt linkages. He compared his column with that of Brown and Watson and concluded that they were similar in their behavior.

Sueoka and Cheng (1962) described more properties of the column such as recognition of base composition and binding DNA through hydrogen bonds. They were able to fractionate and separate different forms of RNA.

Bautz and Hall in 1962 reported that acetylated phosphocellulose could be used to immobilize DNA. With this column one could isolate RNA by hybrid formation. Bolton and McCarthy (1962) used this technique and studied the process in more detail. Later, they used agar to entrap and immobilize DNA.

Bollum in 1960 used N, N'-diethyl aminoethyl ceullulos (DEAE) to separate nucleic acids from proteins. Peterson and Sober (1962) describe in detail the preparation of the absorbent.

Larson and Mosbach (1971) immobilized pyridine nucleotide (NAD) to Sepharose gel which worked as a coenzyme for lactic dehydrogenase. Weibel et al., (1971) attached NAD to the surface of glass beads by diazo coupling. The insolubilized NAD served as a coenzyme for the yeast alcohol dehydrogenase.

Navarro and Durrand (1977) immobilized yeast cells on porous glass beads and showed that immobilization modified the metabolism of the yeasts. Absorption accelerate metabolism while slowing down the fermentative activity of the cells. Overall conversion of glucose to ethanol increased.

Proteinases

Lenney and Delbec (1967) purified two intracellular yeast proteinases from baker's yeast. They had isoelectric points below 4.8 and molecular weights of 73,000. Metal chelators and alkaline earth metals had no effect on activity or stability suggesting that they were not metallo-enzymes. Both proteases were shown to have pH optima on the acidic side; one had a pH optimum of 3.7 and the other of 6.2. Hata et al., (1967) fractionated three different yeast proteinases which were designated as proteinase A, B and C. Proteinase B and C appeared to be papain-type enzymes which were inhibited by thiol reagents and also to be serine-type enzymes which hydrolyzed peptide esters rapidly and which were inhibited by diisopropyl-phosphofloridate. On the other hand, the enzymatic properties of proteinase A resembled pepsin or acid protease produced by various molds. The optimum pH for these enzymes were between 2-3, 8-9, and 6-8 for proteinase A, B and C, respectively.

Hayashi et al., (1968) reported that proteinases B and C existed in vivo as inactive precursors while A was in an active form. Heat (55-60°C) and extremes in pH activated proteinaso C. Later, Hayashi et al., (1969) isolated and characterized pro-proteinase C. Pro-proteinase C was shown to be a glycoprotein with 8-12 percent true sugar and a molecular weight of 79,200.

Aibara, Hayashi and Hata (1971) further characterized proteinase C and found a molecular weight of about 59,000, 11.9 percent nitrogen and 16.7 percent carbohydrate.

In 1972, Hayashi and Hata used different synthetic peptides and polyamino acids as substrate for proteinase C and showed that the enzyme was not specific in most of the cases. In another paper, they reported (1972) that pro-proteinase C was activated by proteinase A rather than by the denaturing agents mentioned earlier. Maximum activation occurred at pH 3.5 and 0°C. Removal of a small protein of about 19,000 in molecular weight caused activation of pro-proteinase C. Hayashi et al., (1972) used different denaturing agents to observe the extent of activation of pro-proteinase C. Dioxane (30-35 percent v/v) and 4 M urea were found to be very effective. They also found that the enzyme consisted of two subunits.

Tohoyama and Takauwal (1972) looked at proteinse activity in baker's yeast during storage at 30°C. In about ten days, amino nitrogen, peptides and amino acids increased along with the activity of proteinases. An increase in activity of proteinase A and B was not observed. Hayashi, Moore and Stein (1973) isolated and characterized carboxypeptidase Y (formerly called proteinase C) and found it to be a serine type proteinase which could proceed through proline. This was possible due to the -SH group near the serine residue which gave an active site totally different from that in other serine type proteinases such as trypsin, chymotrypsin or subtilisin.

Betz et al., (1974) isolated two proteinase B inhibitors from yeast with molecular weights of 10,000 and specific for proteinase B, but not A and C. Both inhibitors were proteins with isoelectric points of 8.0 and 7.0, stable in the range of pH from 1-10 but easily destroyed by proteinase A.

Saheki and Holzer (1975) suggested that upon incubation of yeast, the proteinases (A, B, C) were activated several times their initial activity. This process was primarily due to degration of proteinase inhibitors rather than from activation of inactive zymogens by limited proteolysis.

Bakalkin et al., (1976) stated that during the exponential phase of growth, the activity of proteinases and the rate of degradation of total cell proteins increased. Phenylmethyl sulfonyl fluoride (serine proteinase inhibitor) and pepstatin (microbial peptide inhibitor of acid proteinases) inhibited proteinases and decreased the rate of protein degradation. The inhibition of proteinases took place during 20-40 minutes of incubation and decreased afterwards.

Afting et al., (1976) studied the effect of different yeast proteinases on yeast phosphofructokinase and found that proteinase B and carboxypeptidase Y did not change the activity of phosphofructokinase during incubation. Incubation with proteinase A resulted in a 40-100 percent activation while continued incubation led to inactivation of the enzyme. The molecular weight of phosphofructokinase in the crude extract was 700,000, without proteinase A inhibitor was 600,000 and after activation was 500,000.

MATERIALS AND METHODS

Chemicals

The chemicals used in this study are listed in Appendix Table A.1. All were reagent grade unless otherwise specified. Deionized distilled water was used in the preparation of all buffers and solutions.

Sample Preparation

Fresh baker's yeast cake was obtained from Anheuser-Busch, Inc., St. Louis, Missouri, and was used fresh or stored at -20°C for later use. A 100 g portion of the yeast was washed three times with Tris-HCl buffer (0.05 M pH 8.5) and suspended in 100 ml cold Tris buffer. The cells were broken in a Bronwill mechanical cell homogenizer (Braun Model MSK), Bronwill Scientific Inc., Rochester, New York, by placing 50 g of 0.5 mm cold glass beads in a 75 ml flask with 20 g of the cell suspension. Liquid CO_2 was used to cool the mixture to 5°C during homogenization. The cells were homogenized at 4,000 agitating cycles per minute for two minutes. More than 98 percent of the cells were ruptured by this treatment. The mixture of beads and broken cells were then centrifuged at 2,000 x g for five minutes to separate the glass beads. The supernatant was centrifuged at 17,000 x g for 20 minuted to remove the cell walls and unbroken cells. The final supernatant was used in this study.

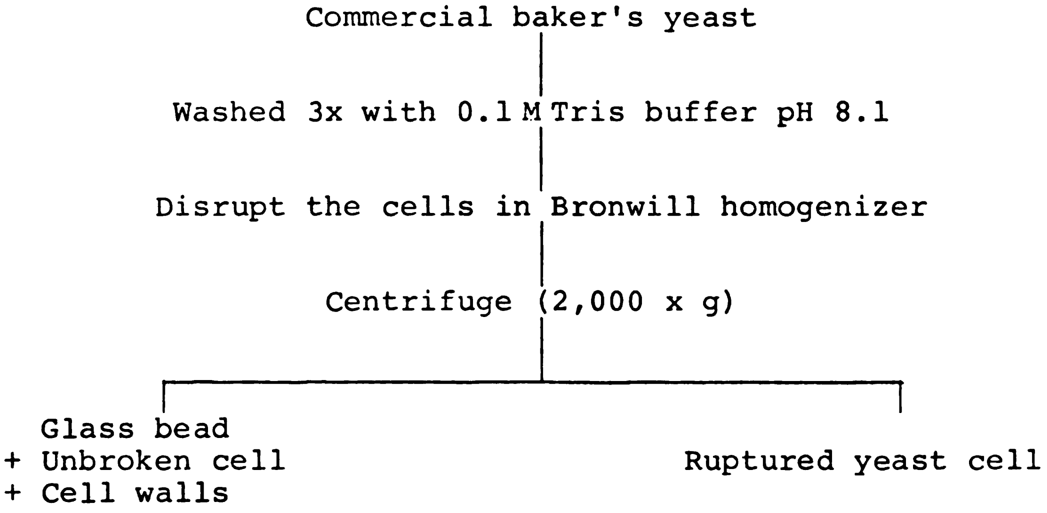


Figure 1.--Flow diagram of preparation of sample.

Removal of Nucleic Acids with $MnCl_2$

To 100 ml of the suspension of broken cells 5 ml of 0.1 M $MnCl_2$ was added. After mixing for 20 minutes, the precipitated nucleic acids were removed by centrifugation (17,000 x g for 20 minutes). This supernatant was used for further experiments.

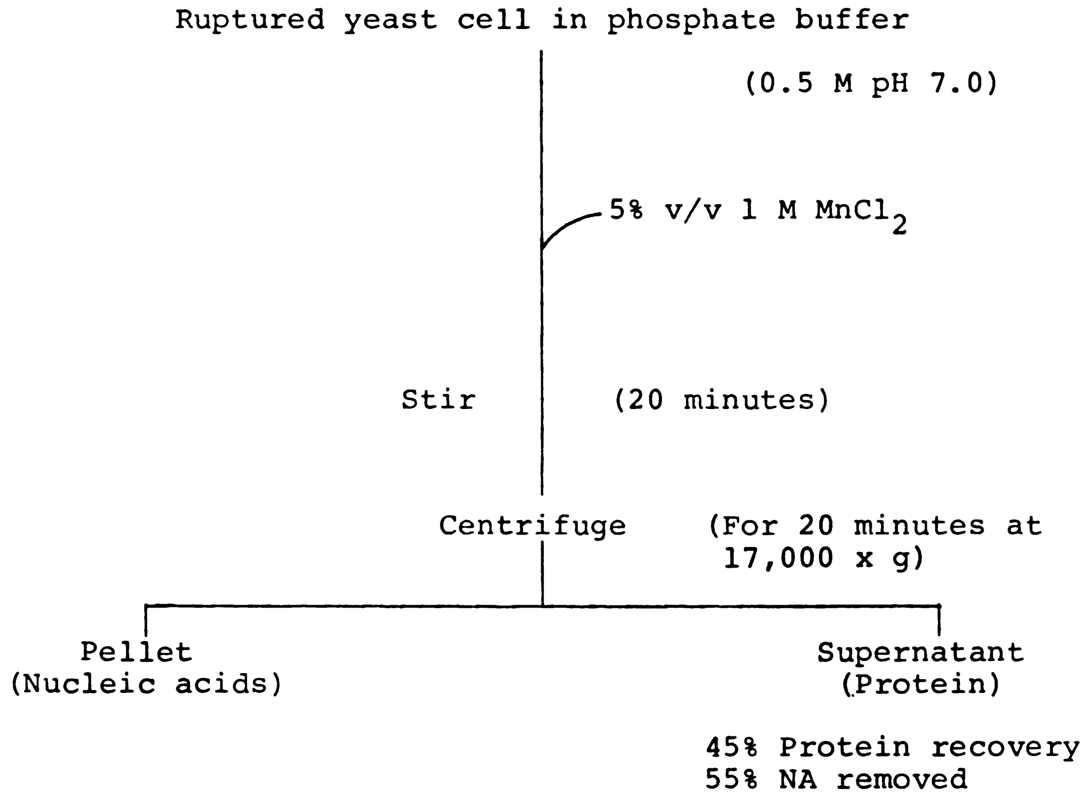


Figure 2.--Flow diagram of removal nucleic acids with MnCl$_2$.

Removal of Nucleic Acids by Phase Separation

To 100 ml of the ruptured yeast cell 11 ml of a 20 percent w/w solution of dextran 500 (dextran of average MW 500,000) was added followed by addition of 30 ml of a 20 percent w/w solution of Polyethylene glycol 6,000 (polyethylene glycol with average MW 6,000). To this mixture sodium chloride was added slowly to yield a solution of 4 M NaCl and the solution stirred for two hours at 4°C. The solution was centrifuged at 1,500 x g for 20 minutes and the supernatant containing reduced nucleic acid content used for further experiments.

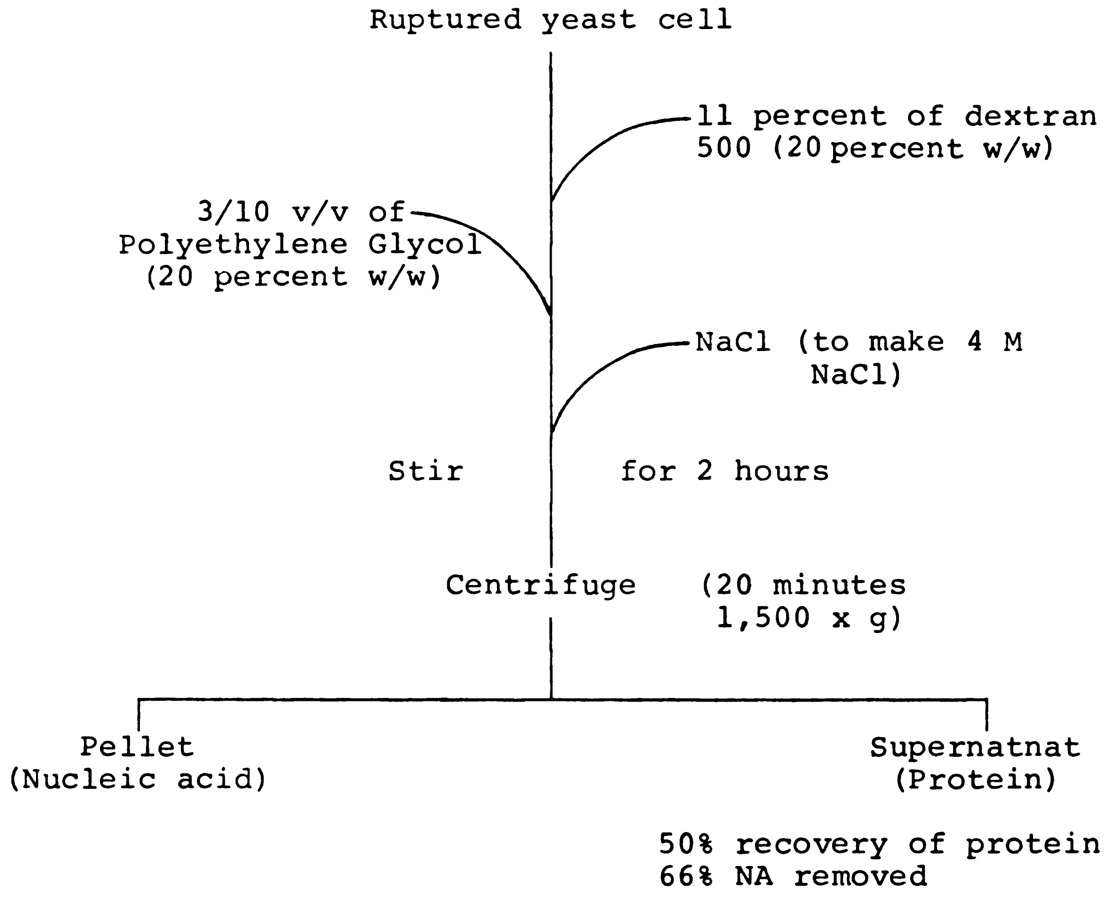


Figure 3.--Flow diagram of removal of nucleic acids by phase separation.

Removal of Nucleic Acids with Streptomycin

To the reptured yeast cell suspension 0.1 M streptomycin (16:1 v/v) was added. After stirring for 10 minutes at 4°C, the mixture was centrifuged at 17,000 x g for 20 minutes and the supernatant collected.

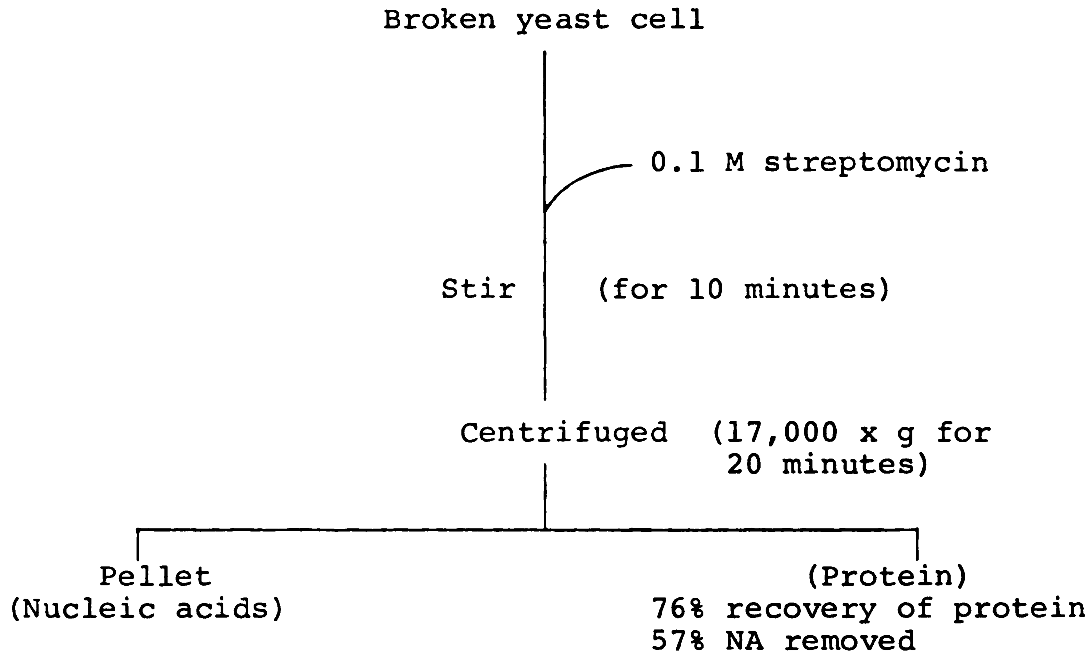
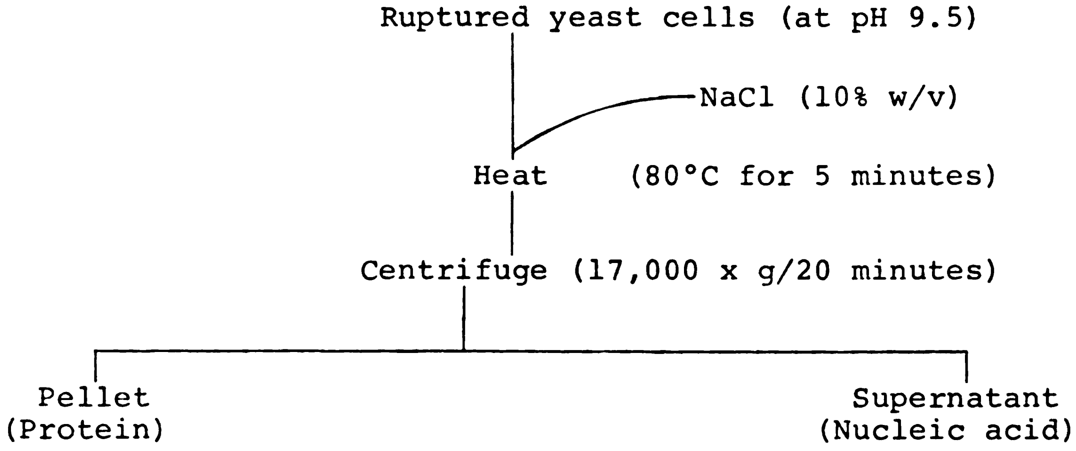


Figure 4.--Flow diagram of removal of nucleic acids with streptomycin.

Removal of Nucleic Acids with Hot NaCl

Ten g of NaCl was added to 90 ml of ruptured yeast cell suspension and the mixture heated to 80°C with agitation. Following centrifugation at 17,000 x g for 20 minutes, the pellet containing the yeast protein was used for further assay.



40% protein recovery
63% NA removed

Figure 5.--Flow diagram of removal of nucleic acids with hot NaCl.

Heat Treatments for Lowering Nucleic Acids

The method of Ohta et al., (1971) was used with slight modification. A stainless steel tube 65 cm in length, of 0.5 mm I.D. and 1.6 mm O.D. immersed in a vigorously stirred water bath was used for the heat treatment. A suspension of whole yeast cells at pH 4 was heat-shocked at 70 ± 2°C for three seconds and cooled in an ice water bath. The heat-shocked cells were incubated at 47°C for one hour, followed by a second incubation at 55°C in a water bath shaker (New Brunswick Scientific, Model G-76, New Brunswick, New Jersey) at

200 rev/min. The suspension was then centrifuged (2,000 x g for 20 minutes), mixed 1:1 with 0.05 M phosphate buffer pH 7.2 and cells were ruptured.

A heat treatment for reducing nucleic acids by exogenous ribonuclease was performed according to Castro et al., (1971). A stainless steel tube 64 cm in length, 2 mm I.D. and 3 mm O.D. immersed in an agitating water bath was employed for the treatment. A suspension of cells (40 mg/ml) was pumped through the tube to attain a treatment of 80 ± 2°C for 30 seconds. The cooled suspension was collected in an ice bath and bovine ribonuclease (1 mg/ml of the suspension) was added. The mixture was incubated at 55°C for one hour; centrifuges at 2,000 x g for 20 minutes and the cells homogenized as described.

Removal of Nucleic Acids by pH Adjustment

Washed yeast cells were suspended in 0.1 M phosphate citrate borate (PCB) buffer pH 7 (Bates, 1970), at 15 g (dry weight) per 100 ml, homogenized and centrifuged at 2,000 x g for 20 minutes. Following adjustment of the pH to 4.0 with 0.1 N HCl, the supernatant was stirred for 10 minutes and centrifuged at 17,000 x g for 20 minutes. The pellet was recovered for further assay.

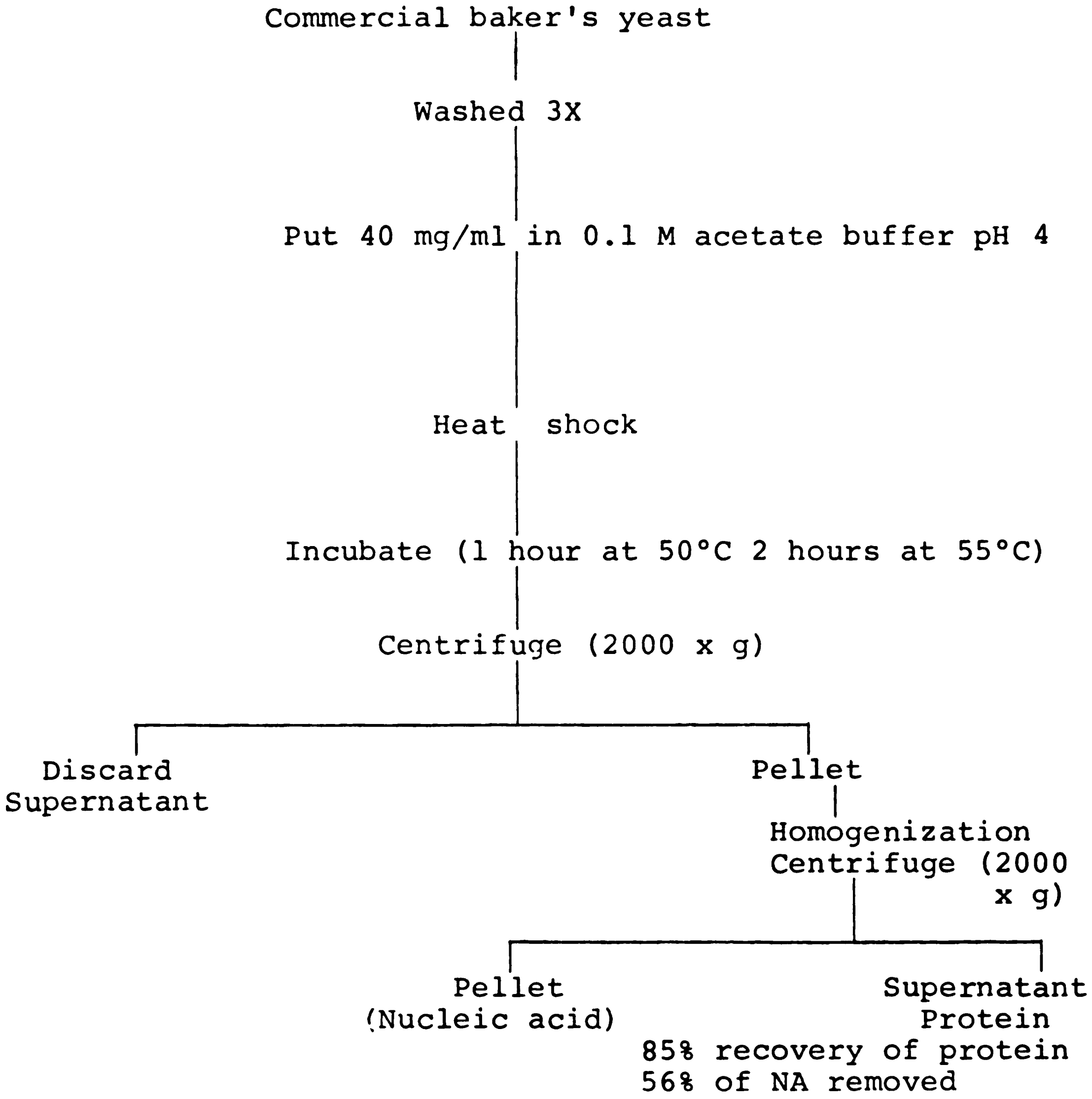


Figure 6.--Flow diagram of removal of nucleic acids by heat shock.

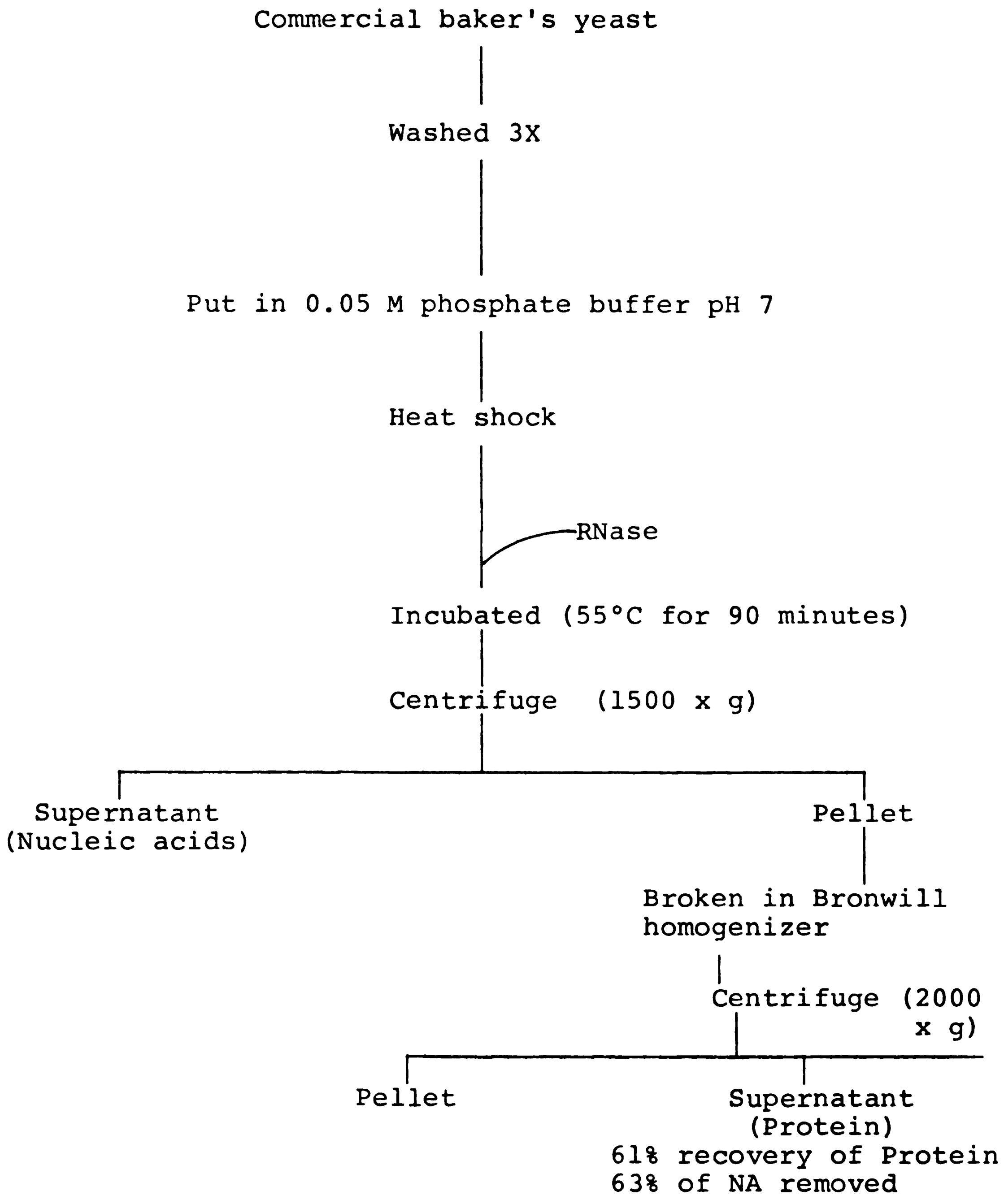


Figure 7.--Flow diagram of removal of nucleic acids by exogenous ribonuclease.

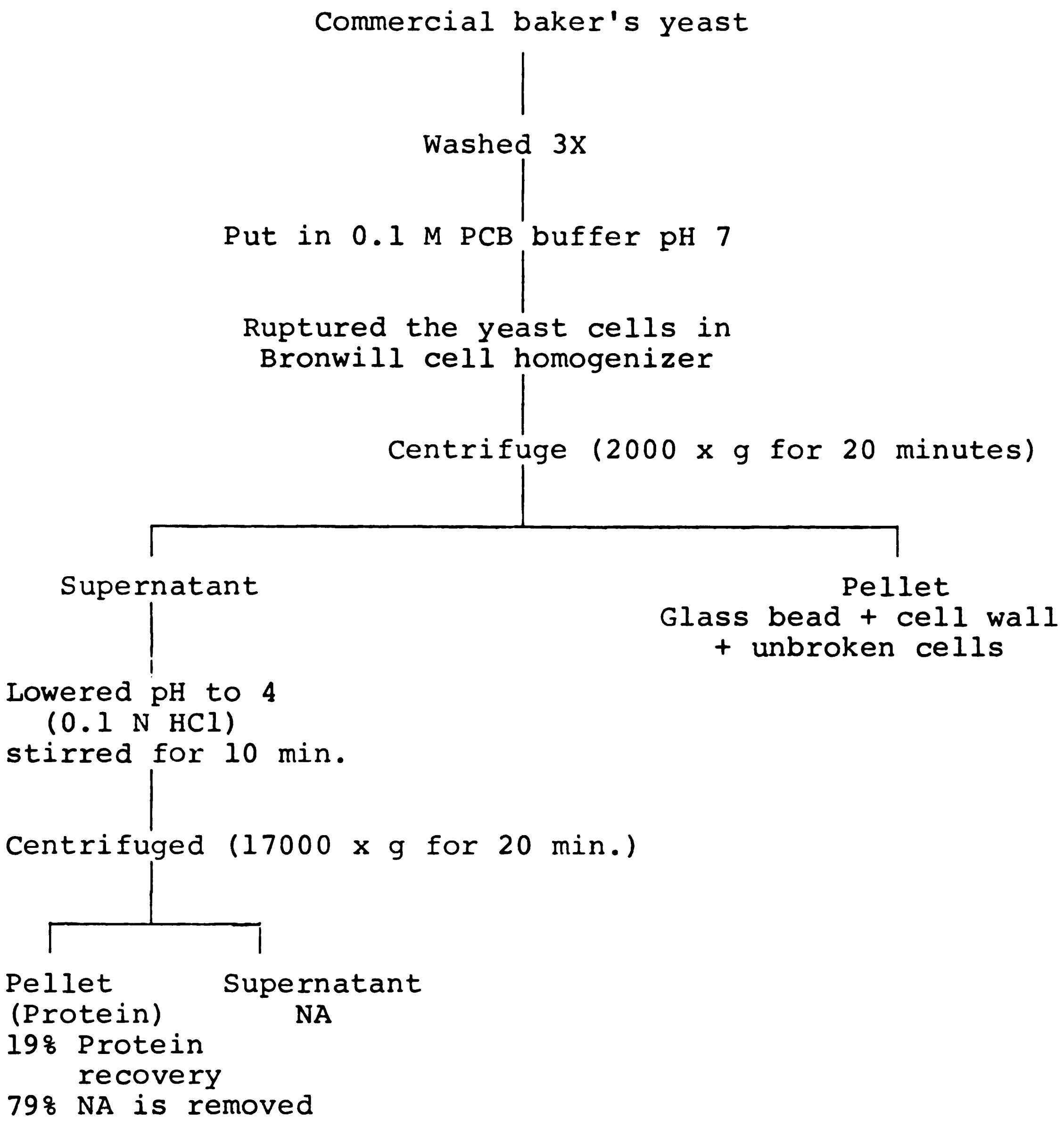


Figure 8.--Flow diagram of removal of nucleic acids by pH adjustment.

Removal of Nucleic Acids with Protamine Sulfate

A 90 ml suspension of ruptured yeast cells was adjusted to pH 6.5 with 0.1 N acetic acid and 10 ml of 2 percent (w/v) protamine sulfate was added. The solution was stirred for 20 minutes at 4°C, centrifuged at 17,000 x g for 20 minutes and the supernatant collected for further assay.

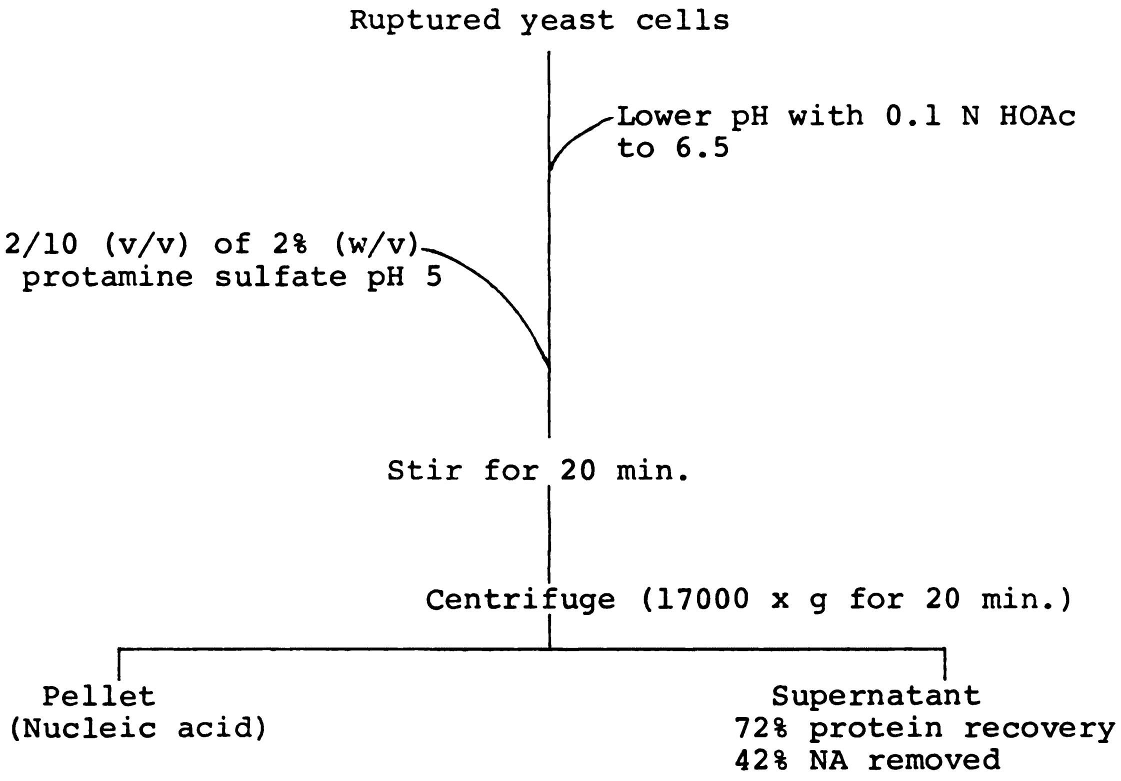


Figure 9.--Flow diagram of removal of nucleic acids with protamine sulfate.

Electrophoretic Studies

All electrophoresis was done using Bio-Rad selected boreglass (75 mm x 5.5 mm I.D.) in a Bio-Rad gel electrophoresis apparatus (Bio-Rad Laboratories, Richmond, California).

Disc Gel Electrophoresis

The yeast proteins were separated using standard techniques of polyacrylamide disc electrophoresis (Maurer, 1971).

Sample preparation. Aliquots of the yeast proteins were prepared either from the freeze-dried samples or freshly prepared yeast protein in Tris-HCl buffer pH 8.5, prior to electrophoresis. One drop of 0.05 percent aqueous solution of bromphenol blue and one drop of glycerin were added prior to application on the gels.

Gel preparation. Glass tubes were washed in sulfuric acid-dichromate solution, rinsed with distilled water and dried. The tubes were stoppered and secured vertically in a rack.

Acrylamide gels were prepared using the formulation of Davis (1964) (see Appendix). The required separating gel (7.5-17.5 percent w/v gradient or other concentrations as indicated) was poured into the tubes to a height of 6.5 cm. A few drops of water were layered on top of the gel to form a flat gel surface and to protect the gel from oxygen. The tubes were left at room temperture to polymerize during a period of 20-30 minutes, and the water layer was then removed by blotting. The stacking gel solution (2.5 percent acrylamide, 0.625 percent Bis) was added on top of the polymerized separating gel to a height 6 mm. A few drops of water were layered on the stacking gel. The stacking gel was photopolymerized using a fluorescent lamp positioned a few cm from the gel. When polymerization was completed, the stacking gel became opaque (15-20 minuted).

Electrophoretic conditions. The glass tubes were then placed in an electrophoresis chamber and buffer was added to the lower and upper chambers. A constant current of 2.5 mA per tube was applied for 3-4 hours or until dye band migrated about 80 percent of the gel length. The gels were removed from the glass tube using a hypodermic syringe and 24 gauge needle.

Staining and destaining. The position of the protein zones was determined by staining the gels according to Malik and Berrie (1972).

Alternatively, the gels were stained for glycoprotein according to Zacharius et al., (1969). The procedure of Gabriel (1971) was followed for dehydrogenase staining. The method of Winters and Cope (1971) was used for esterase assay in the gels and nucleic acids were located in the gels according to Maurer (1971).

Characterization of protein zones. The position of the protein zones were recorded in a Gilford densitometer (Gilford Instruments Lab. Inc., Oberlin, Ohio).

SDS-Gel Electrophoresis

The sodium dodecyl sulfate (SDS) electrophoresis technique was employed suggested by Weber et al., (1972). Protein samples of about 5 mg were put in a tube with 1 ml of 0.01 M sodium phosphate buffer pH 7 containing 2 percent SDS and 2 percent 2-mercaptoethanol. The tubes were transferred to a boiling water bath for two minutes and then cooled to room temperature. To each tube five drops of glycerol and two drops of tracking dye (0.05 percent Bromophenol Blue in water) were added and mixed. 50 μl of this solution was applied directly on the gels. Electrophoresis was conducted at 8 mA per gel until the tracking dye approached the bottom of the gels (about six hours). The gels were removed from the tubes and stained according to the method described by Fairbank et al., (1971) for proteins and for glycoproteins by Zacharius et al., (1969).

Molecular weight for the subunits was estimated from a plot of relative mobility versus log molecular weight for standard proteins. Standard curves used in this study were either from BDH molecular weight markers for SDS polyacrylamide gel electrophoresis (BDH Chemicals, LTD, England) or the following standard proteins: insulin (5733), ribonuclease A (13700), β-lactoglobulin (17500), alcohol dehydrogenase (37000), alkaline phosphatase (100,000), 6-phosphogluconic dehydrogenase (100,000), phosphoglucose isomerase (145,000), glucose oxidase (186,000), and inverlase (270,000). Relative mobilities were evaluated from measurements of the gel column, dye zone and protein zones as follows:

$$\text{Relative mobility} = \frac{\text{distance protein migrated}}{\text{length of gel after destaining}} \times \frac{\text{length of gel before staining}}{\text{distance of dye migration}}$$

Disc Gel Electrophoresis in Urea

The yeast proteins were separated using disc gel electrophoresis in presence of 4 M urea according to Groves et al., (1968). Protein zones were detected according to Malik and Berrie (1972).

Gel Isoelectric Focusing

The method of Wrigley (1971) was used for resolution of the yeast proteins. The stock solutions were: (a) acrylamide 30 g and 1 g N, N′-methylene bisacrylamide dissolved in water to 100 ml (b) amonium persulfate fresh 1 percent solution (0.7 ml was added to 8 ml of final gel solution). The final gel preparation of 8 ml contained 11.25 percent total gel at 0.375 percent cross linkage, 3.75 percent carrier ampholyte (LKB-Produkter, Inc., Bromma, Sweden).

Approximately 1.5 ml of a well mixed, evacuated gel solution was added to a series of vertically held 0.5 x 7.5 cm glass tubes. They were sealed at the bottom with flexible film and fitted into rubber adaptors. The tubes were overlayered and left to polymerize at room temperature. The sample (about 50 μg protein) was incorporated in the gel and at about 5 mm layered on the top of the separating gel and allowed to polymerize. Following removal of the film, the tubes were mounted in numbered grommets of the top vessel of the disc electrophoresis apparatus. The top (cathodic) and bottom (anodic) vessels were filled with aquous solutions of ethanolamine (0.4 percent v/v) and sulfuric acid (0.2 percent) respectively. Focusing was established at constant volt (100 volt for 12 tubes) for eight hours.

After electrolysis, gels were removed with a 24-gauge needle. Selected gels were stained by the Malik-Berrie (1972) method. The unstained gels were cut with a razor blade into 0.5 cm pieces, put in test tubes and crushed with a glass rod. One ml of boiled and cooled deionized distilled water was added to each tube and after one hour, pH was determined.

Chemical Analysis

Determination of Lysine

Available lysine was determined by the method of Kakade and Liener (1969). To 1 ml of solution containing 1 mg protein in 4 percent $NaHCO_3$, pH 8.5, was added 1 ml of a freshly prepared aqueous solution of 0.1 percent, 2, 4, 6-trinitrobenzene sulfonic acid (TNBS). Following incubation at 40°C for two hours, 3 ml concentrated HCl was added. The tubes were capped loosely and the reaction mixture was autoclaved at about 120°C (15 psi) for one hour. After the hydrolyzate had been allowed to cool to room temperature, 5 ml distilled water was added. The contents of each tube were extracted twice with approximately 10 ml ethyl ether in order to remove TNP - N terminal amino acids or peptides as well as picric acid, which is also produced during the course of the reaction. The tubes were placed in hot water for at least 10 minutes in order to remove residual ether. The aqueous solution was read at 346 nm against an appropriate blank. Molar absorptivity of 1.46×10^{4} M^{-1} cm^{-1} was used for calculations.

Sulfhydryl Group Determination

The method of Habeeb (1972) was used in this assay. First, the total protein sulfhydryl. About 0.01-0.04 mole of protein was dissolved in 6 ml of solution containing 2 percent sodium dodecyl sulfate (Pierce Chemical Co.), 0.08 M sodium phosphate buffer, pH 8, and 9.5 mg/ml EDTA. To 3 ml of the solution was added 0.1 ml Ellman's reagent [5,5′ dithiobis (2-nitrobenzoic acid)] DTNB (Aldrich Chemical Company) which consisted of 40 mg DTNB in 10 ml of 0.1 M sodium phosphate buffer, pH 8. The color was developed for 15 minutes and the apparent absorbance read at 410 nm against protein solution in SDS. A reagent blank was subtracted from the apparent absorbance to obtain net absorbance. A molar absorptivity of 13,600 M^{-1} cm^{-1} was used for calculation. Second, the determination of available sulfhydryl. The reaction was performed as above but with no denaturing agents.

Amino Acid Analysis

Amino acid analysis of yeast protein was performed on a Beckman/Spinco 121-C automatic amino acid analyzer (Moore et al., 1958), employing 24 hours acid hydrolysis.

Proteolytic Activity

The modified Kunitz method (1947) was used for measuring proteolytic activity (Kakade et al., 1969). One ml of sample containing 0.01-0.05 mg protein was mixed with one ml of 2 percent casein in Sørensen buffer pH 7.6 (Bates, 1970) and placed in a water bath 35°C for 20 minutes. The reaction was stopped by addition of 3 ml of 5 percent w/v trichloroacetic acid. After centrifugation at 2,000 x g for 10 minutes the absorbancy was read at 260 nm. Effect of yeast proteinases on P-toluenesulfonyl-L-arginine methyl ester (TAME) and N-benzoyl-L-tyrosine ethyl ester (BTEE) was measured according to Walsh and Wilcox (1970).

Protein Measurements

Lowry Method

Protein was measured by the method of Lowry et al., (1951). Lowry solution A (2 percent Na_2CO_3 in 0.1 N NaOH) was mixed with Lowry solution B (0.5 percent $CuSO_4$, $5H_20$ in 1 percent sodium or potassium tartarate) immediately prior to use to give Lowry solution C. Phenol solution was prepared immediately prior to use by dilution (1:1) of Folin and Ciocalteu phenol reagent (Fisher Scientific Co.).

To assay for protein, 5 ml of Lowry solution C was added to 1 ml of appropriately diluted protein solution, mixed and incubated for 10 min at room temperature. The diluted phenol solution (0.5 ml) was then added, mixed rapidly and allowed to stand at room temperature (30 min.) for color development.

Whenever proteins were in Tris-HCl buffer, because of interferring of Tris with the reaction, a modification of the Lowry method was used. To 3 ml appropriately diluted protein solution 50 μL of a 1 percent w/v sodium deoxycholate solution was added and mixed. After 15 minutes incubation at room temperature, 1 ml of a 24 percent trichloroacetic acid solution was added. The mixture was centrifuged at 1,000 x g for one hour. The supernatant was removed carefully and the precipitate was dissolved in 3 ml of Lowry solution C. After 10 minutes 0.6 ml of phenol solution was added and the solutions were rapidly mixed. Absorbance of the solution was measured at 540 nm using water plus all other reagents as a blank. The protein concentration was determined by comparing with a standard curve prepared from crystalline bovine serum albumin (Signal Chemical Co.).

Biuret Method

In some occasions, protein was measured using the biuret method (Layne, 1957). In this case, protein solution (1 ml) was mixed with 4 ml of biuret reagent [1.5 g $CuSO_4$, 5 H_2O and 6 g sodium tartarate in 500 ml water; to this was added under agitation 300 ml 10 percent (w/v) NaOH solution and the total volume made to 1 liter]. The mixture was let to stand for 30 minutes at room temperature and absorbance of the mixture was read at 540 nm using 1 ml of H_2O and 4 ml of biuret reagent as a blank.

In cases where proteins were in Tris-HCl buffer, because of interference of the Tris in the biuret reaction, a modification of biuret was used. Protein sample was put in 3 ml water. To that 50 μL of 1 percent w/v sodium nadeoxycholate was added and mixed. After 15 minutes 1 ml of a 24 percent w/v trichloroacetic acid added. Centrifuged at 1,000 x g for one hour. The top portion was discarded and the precipitate placed in 1 ml of water followed by 4 ml of biuret reagent. Absorbancy of the mixture was measured at 540 nm after 30 minutes against a blank (water and biuret reagent 1:4 ml). A standard curve was prepared using known concentrations of crystalline bovine serum albumin.

For insoluble proteins the biuret method suggested by Strickland (1951) was used. Ten mg of yeast cells was put in 3.3 ml of water, to that, 0.6 ml of 30 percent w/w NaOH was added. The mixture was mixed with 0.1 ml of a 25 percent (w/v) $CuSO_4.5H_{20}$ solution and the precipitate was dispersed with a glass rod, incubated at 100°C water bath for five minutes and centrifuged at 2,000 x g for 15 minutes. Absorbancy was read at 540 nm against a blank.

Bio-Rad Protein Assay

In some cases, Bio-Rad protein assay was used. A standard curve of bovine serum albumin (Bio-Rad protein standard) was compared to unknowns.

Nitrogen

Duplicate nitrogen analysis was performed in a micro-Kjeldahl apparatus. About 20 mg of sample was mixed with 4 ml of digestion mixture consisting of 5.0 g $CuSO_4 \cdot 5 H_2O$ and 5 g SeO_2 in 500 ml of concentrated H_2SO_4. Digestion was carried on for one hour or until solution was clear. The contents of the digestion flasks were allowed to cool for 30 minutes and 1 ml of 30 percent H_2O_2 was added. Digestion was continued for another hour and allowed to cool for 30 minutes. The flasks were then rinsed with 10 ml of deionized water and allowed to cool for additional 30 minutes. The mixture was neutralized with 25 ml of a 40 percent NaOH solution and the released ammonia was steam distilled into 15 ml of a 4 percent boric acid solution containing 5 drops of indicator solution. The indicator solution was made by dissolving 400 mg of bromocresol green and 40 mg of methyl red in 100 ml of 95 percent ethanol. The distillation was continued until a final volume of 75 ml was reached. The ammonia-borate complex was titrated with 0.020 N HCl previously standardized with Tris (hydroxymethyl) aminomethane. A reagent blank was determined and subtracted from the sample values. The average recovery of tryptophan was 97.8 percent. In calculations factor 6.25 was used and percent corrected protein was calculated as follows.

$$\text{Percent corrected protein} = \text{percent crude protein} - \frac{6.25}{6.13} \times \text{percent RNA}$$

Nucleic Acid Measurements

Ribonucleic acids were measured according to Travelyan and Harrison (1956). To 3 ml of sample, 3 ml of 0.5 N HCl was added. The mixture was incubated at 37°C in a water bath shaker for 90-120 minutes, centrifuged at 1,000 x g for 45 minutes and the supernatant was appropriately diluted and read at 260 nm in a Beckman DU spectro photometer against a blank. A standard curve was made from a high quality yeast RNA (Calbiochem) treated along with the sample.

Alternatively nucleic acids were measured by pentose analysis according to Schneider (1957). ATP was used as the standard.

Chromatographic Procedures

Immobilization Techniques

The method of Cuatrecasas and Anfinsen (1971) was used in the immobilization of protamine sulfate and RNA. About 100 ml of Sepharose 4B was washed with 500 ml water in a Buchner funnel and mixed with equal volume of water. Under the hood finely divided solid cyanogen bromide (30 g for 100 ml Sepharose) was added at once to the stirred suspension, and the pH immediately raised to 11 with 5 N NaOH. The pH was maintained at 11 by constant manual titration and temperature was maintained at about 20°C. The reaction is completed in 8-12 minutes as indicated by cessation of base uptake. Ice was then rapidly added to the suspension which was transferred to a Buchner funnel (coarse disk) and washed under suction with cold buffer. The buffer used was 0.1 M $NaHCO_3$, Na_2CO_3 pH 10.5. The volume of wash should be 10-15 times that of the packed Sepharose. The activated Sepharose was put in a beaker and the compound to be coupled was dissolved in another 100 ml of cold buffer (carbonate buffer 0.1 M pH 10.5) and added to the Sepharose. The suspension was mixed rapidly in the cold and held with stirring for 24 hours at 0°C. The pH was checked every 2-3 hours and maintained at 10.5. Again, the slurry was washed with buffer and the volume of the washed solution measured and assayed for the compound used for coupling. Knowing the amount used and the amount unbounded, one can calculate the amount of material bound per volume of Sepharose.

For use, the immobilized compound to Sepharose was packed in a 1 x 10 cm column and equilibrated with the desired buffer.

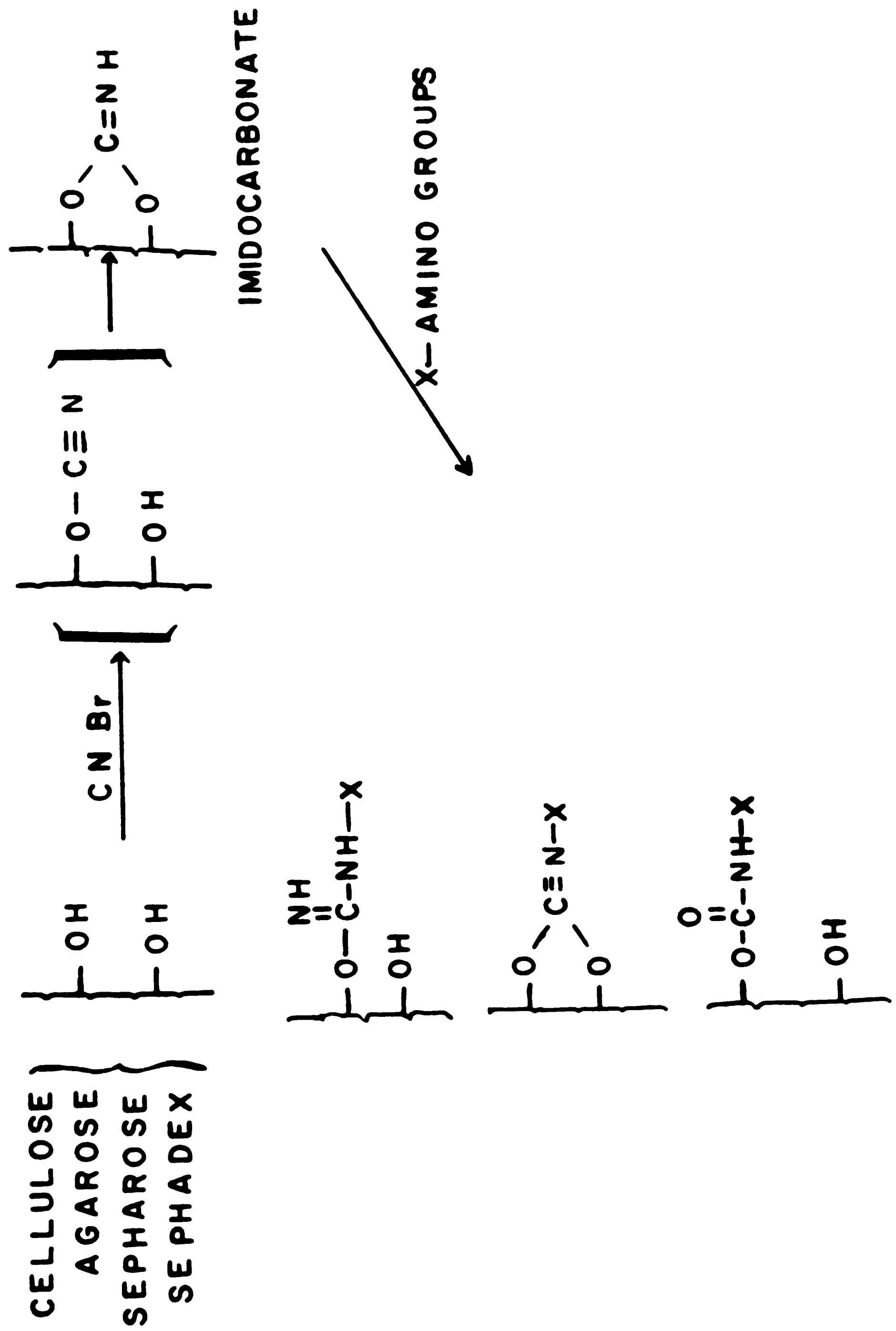
DEAE Column

Bio-Rad Cellex D was used. Fifteen g of Cellex D was suspended in the buffer with gentle stirring and allowed to settle for 15 minutes. The cloudy supernatant was discarded and the Cellex was again washed with 0.25 M NaCl - 0.25 M NaOH solution, twice with water, once with 0.25 M HCl and finally, several times with water. Following equilibration with starting buffer and measurement of pH to insure the pH remained as desired, the material was deaerated and packed in a 1.5 x 30 cm column.

Gel Filtration

Sephacryl S-200 superfine (Pharmacia) was prepared for use by washing with 0.1 M phosphate buffer pH 7.2 on a Buchner funnel, deaerating and packing in a

Figure 10.--Cyanogen bromide activation of carbohydrate polymers and reaction with enzyme amino group.


CELLULOSE
AGAROSE
SEPHAROSE
SEPHADEX
OH
OH
CN Br
O—C≡N
OH
O
O
C=NH
IMIDOCARBONATE
X—AMINO GROUPS
NH
O—C—NH—X
OH
O
O
C≡N—X
O=C—NH—X
O
OH

2.6 x 40 cm column. Before use, the Sephacryl was equilibrated with the desired buffer.

RESULTS AND DISCUSSION

I. Effect of Removing Nucleic Acids on Proteins

Breaking the resistant wall of yeasts increases the amount of protein that can be extracted from the cell. Mechanical disintegrators have an advantage over other procedures in that they result in less protein denaturation. Therefore, in the course of this study a mechanical homogenizer was used to disrupt the cell.

Several different methods were employed and evaluated to obtain yeast protein low in nucleic acids (NA). Since ribonucleic acids (RNA) are the dominant nucleic acid, adequate reduction of RNA content is sufficient for protein to be used for food purposes. Effect of the techniques of removal of NA on the remaining proteins will be evaluated, i.e., protein recovery, extent of denaturation of proteins and changes in amino acid composition of the whole protein fraction. Finally, by using different electrophoretic techniques, the effect of NA removal on the proteins will be evaluated in more detail.

$MnCl_2$ Precipitation of Nucleic Acids

The addition of 0.1 M $MnCl_2$ (5 percent v/v) to the cell homogenate caused precipitation of NA which were readily separated from the protein fraction by centrifugation. A portion of the proteins was also removed. In the experiment done, NA content was lowered from 0.26 percent to 0.12 percent (55 percent removal) and protein was lowered from 2.40 percent to 1.66 percent (30 percent loss, Table 1). Using $MnCl_2$ for the separation of pyruvate enzymes, Korkes et al., (1951) reported a large loss of the enzymes. Mn^{2+} is one of the cations which is able to alter the solubility of NA and results in their precipitation. It is relatively cheap and does not interact with all of the proteinaceous components, i.e., in polyacrylamide gel (Figure 11, 2) esterase were detected while lactic dehydrogenase was not.

If the resultant protein is intended for use as food, the toxic manganese must be removed by dialysis or filtration. Storing the treated sample on ice resulted in still more precipitation of the NA. After five days when the precipitate was removed by centrifugation (17000 x g for 20 minutes), the NA content was reduced to as low as 0.03 percent (from 0.12 percent or 11 percent of the original). However, the protein content was

TABLE 1.--Effect of different treatment on removing nucleic acids and protein recovery

Treatment	% Protein Recovery	% Nucleic Acid Removed	Protein, Nucleic Acid Ratio
Control	100	0.0	9.0
2/10 v/v of 2% protamine sulfate	72	42	12.4
Phase separation	50	66	11.1
$MnCl_2$	70	55	13.9
Streptomycin	76	57	11.3
pH adjustment	19	79	14.5
NaCl precipitation	40	63	22.8
Exogenous RNase	61	63	15.3
Heat shock	75	56	13.0

lowered to 0.42 percent (from 1.66 percent or 17.3 percent of the original).

The polyacrylamide gel electrophoresis (PAGE) pattern of $MnCl_2$ treated yeast protein was very similar to the untreated sample except the protein zones at the lower level of the gel (Figure 12, 5) were not easily detected. The reason could be that the more acidic proteins are more susceptible to precipitation.

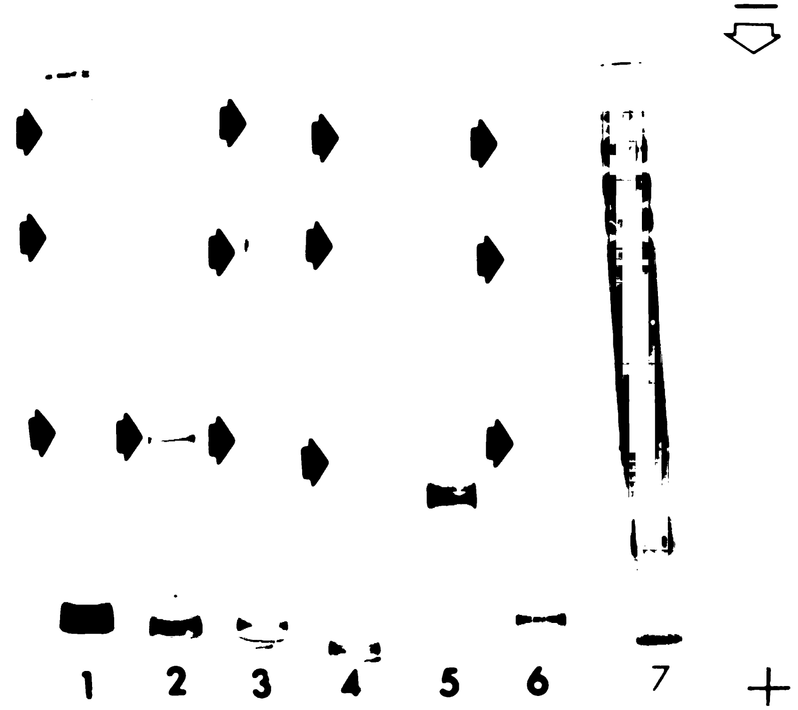
In SDS (sodium dodecyl sulfate)-PAGE, in which the proteins are broken to their constituent subunits and they separate according to their molecular weight, the proteins of the $MnCl_2$ treated sample had molecular weights in the range of 14,000 to 200,000 (Figure 13, 6).

In isoelectric focusing (Figure 14, F), the isoelectric point of the proteins can be compared with that of the control (in this experiment, ampholyte pH range 5-8 was used). Most of the proteins had pI in the range of 4.5-7.5. There could be more proteins detected in the treated sample than in the control, but results were comparable.

Phase Separation of Nucleic Acids

Dextran and polyethylene glycol, when used in appropriate concentrations, will cause precipitation of NA. In this experiment the nucleic acid content in the

Figure 11.--Polyacrylamide gel electrophorogram of yeast proteins stained for lactic dehydrogenase and esterase 1-untreated sample, 2-$MnCl_2$ 3-protamine sulfate + proteinase inhibitor (PMSF) 4-protamine sulfate no proteinase inhibitor, 5-phase separation, 6-streptomycin, 7-protamine sulfate + proteinase inhibitor stained with Coomassie Brilliant Blue G-250. Arrows indicate bands which display either lactic dehydrogenase or esterase enzymatic activity.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+

Figure 12.--Polyacrylamide gel electrophorogram of yeast proteins in 7.5-17.5 percent w/v gels after different treatment for reduction of nucleic acids: 1-control, 2-exogeneous RNase, 3-heat-shock, 4-pH adjustment, 5-$MnCl_2$, 6-protamine sulfate + proteinase inhibitor (PMSF), 7-protamine sulfate no proteinase inhibitor, 8-NaCl, 9-phase separation, 10-streptomycin, 11-commercial spray-dried yeast.

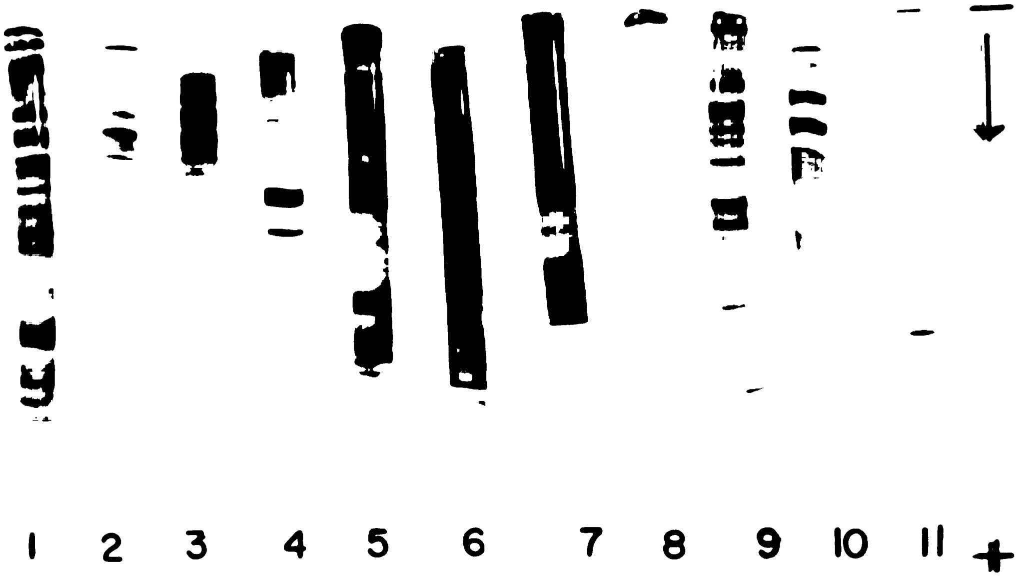
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Figure 13.--Diagram of the SDS electrophoretic patterns of yeast proteins in 7.5-17.5 percent (w/v) gradient gels. Molecular weight of the proteins as determined by BDH molecular weight markers are shown in the left.

1-control, 2-exogenous RNase, 3-heat-shock, 4-NaCl, 5-pH adjustment, 6-$MnCl_2$, 7-protamine sulfate, 8-phase separation, 9-streptomycin.

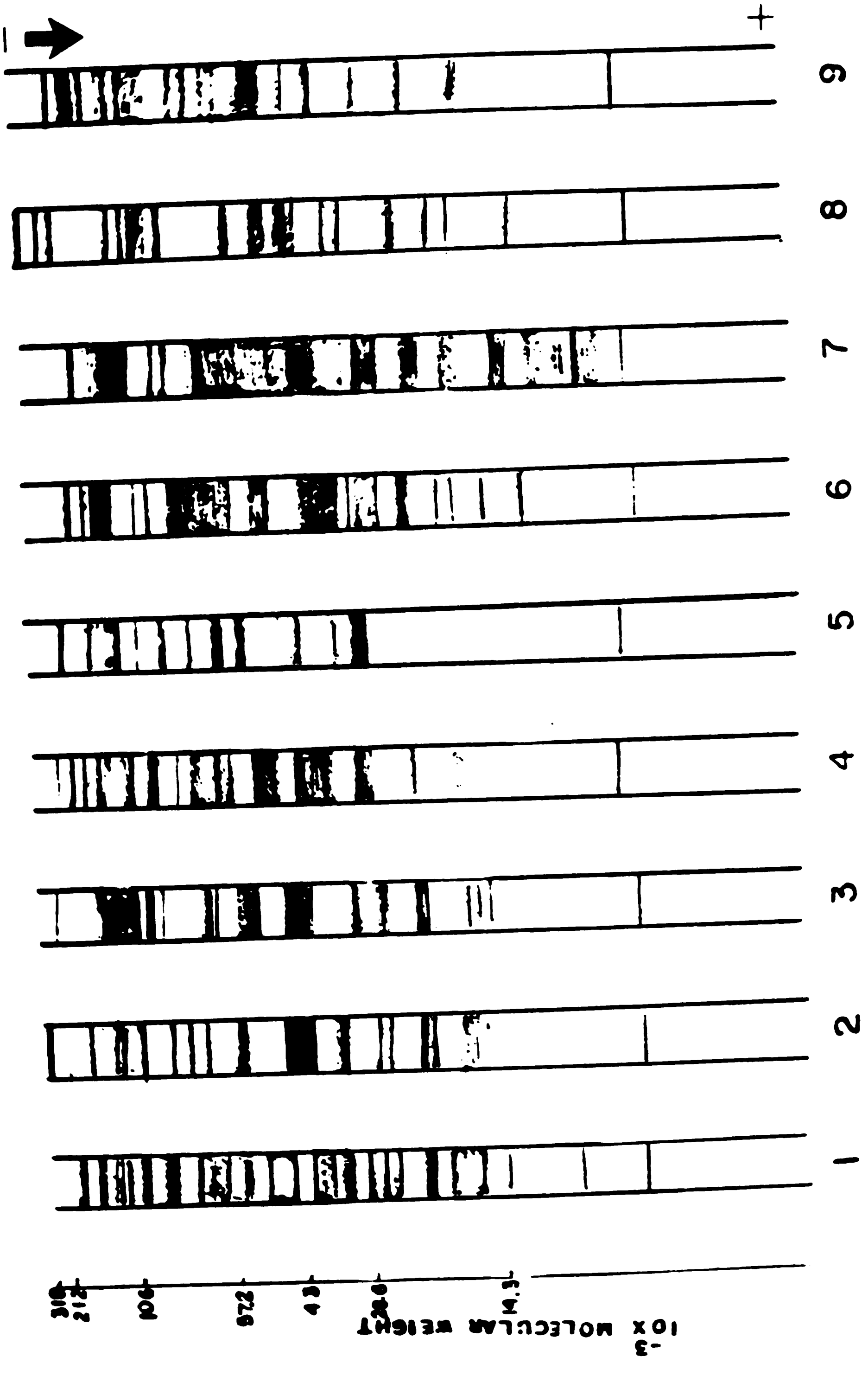
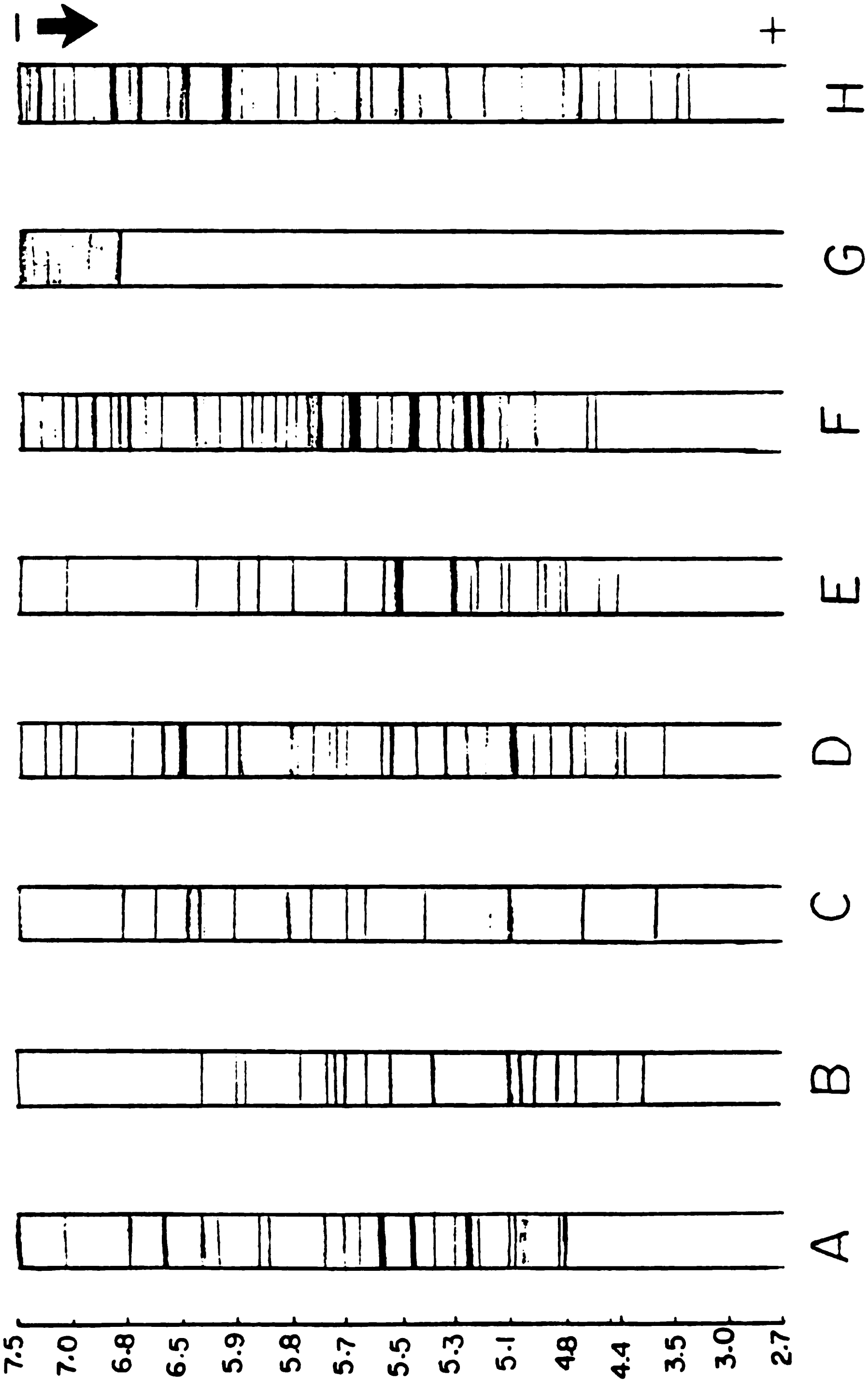

−
+
1
2
3
4
5
6
7
8
9
10^{-3} X MOLECULAR WEIGHT
14.3
29.0
43

Figure 14.--Diagram of the electrophoretic patterns of yeast protein in isoelectric focusing (pH 5-8) A-control, B-exogenous RNase, C-heat-shock, D-streptomycin, E-pH adjustment, F-$MnCl_2$, G-phase separation, H-protamine sulfate. pH gradient formed is shown in the left.


−
+
H
G
F
E
D
C
B
A
7.5
7.0
6.8
6.5
5.9
5.8
5.7
5.5
5.3
5.1
4.8
4.4
3.5
3.0
2.7

homogenized yeast cell was lowered from 0.26 percent to 0.09 percent (66 percent reduction) and protein loss was from 2.4 percent to 1.2 percent (50 percent) (Table 1). The sodium chloride added during this technique is somewhat unattractive in that its subsequent removal would entail additional expense in a commercial process. The cost of polyethylene glycol and dextran is another economic limitation to the method unless they can be recovered. The loss of protein can be attributed to NaCl and complexes formed between proteins and polyethylene glycol. Advantages of this technique are that it does not affect the proteins and thus can be used for isolation of enzymes (Garret et al., 1969). This was not proved when gels were stained for lactic dehydrogenase and esterase (Figure 11, 5). In PAGE, the number of protein zones detected were comparable with that of the control except for a few at the lower level of the gels (Figure 2, 9). In SDS-PAGE (Figure 13, 8), most of the proteins showed different mobility from that of control. This might be from retained traces of polymer attached to the proteins. Proteins which could not get into the gel and remained on the top supports this possibility. In isoelectric focusing, there was a poor resolution (Figure 14, G). Most of the proteins did not move below pI range of 6.9, and this is another evidence for the polymer-protein complex left into the solution mentioned before.

Protamine Sulfate as Nucleic Acid Precipitating Agent

Protamines which are basic proteins (high in arginine) are capable of binding to NA and promote their precipitations. Highly acidic proteins will be removed along with precipitation of NA, and this accounts for protein loss. The more protamine sulfate added to the cell homogenate, the more NA will be removed but with consequent losses in protein, as can be seen in the data presented in Table 2. The optimum concentration of protamine sulfate was obtained when four volumes of a 2 percent w/v protamine sulfate solution (80 mg protamine sulfate) was added to ten volumes of cell homogenate (1.02 g yeast protein). This resulted in a 64 percent reduction of NA and only a 50 percent loss of protein. Higher concentrations of protamine sulfate such 160 mg per 1.02 g yeast protein (8/10 v/v) only reduced the NA 77 percent. In other words, by doubling the protamine sulfate concentration, the NA reduction increased only 13 percent.

Protamines have no adverse effect on the yeast proteins which makes them excellent for purification of enzymes. Lactic dehydrogenase and esterase could be readily detected in gels (Figure 11, 3 and 4).

TABLE 2.--Effect of different concentrations of protamine sulfate on removal of nucleic acids and total solid and protein recovery. Starting cell homogenate contained 13.64 percent total solid, 10.2 percent protein and 1.16 percent nucleic acid

	Percent Total Solid Recovery	Percent Protein Recovery	Percent Nucleic Acid Removal	Protein Nucleic Acid Ratio
No treatment	100	100	0.0	8.8
1/10 (v/v) of Protamine sulfate (2 percent w/v)	97	98	9	10.5
2/10 (v/v) of Protamine sulfate (2 percent w/v)	77	82	19	10.7
4/10 (v/v) of Protamine sulfate (2 percent w/v)	50	48	64	16.2
6/10 (v/v) of Protamine sulfate (2 percent w/v)	47	46	77	19.2
8/10 v/v of Protamine sulfate (2 percent w/v)	46	47	77	21.8

Samples treated with protamine sulfate showed more protein zones (by electrophoresis) than any other treatment, even the control sample exhibited fewer components (Figure 12, 3 and 4) which may be due to the removal of NA which otherwise associate with proteins. In SDS-PAGE, proteins showed molecular weights in the range of less than 10,000 up to 200,000 or more (Figure 13, 7). In isoelectric focusing (Figure 14, H), proteins had isoelectric points ranging from 3.2 to 7.5.

Reduction of NA by protamine sulfate increased total protein sulfhydryl content as shown in Table 3. Available sulfhydryl group has a slight decrease in reduced NA sample. Addition of proteinase inhibitor increases both available and total sulfhydryl group. A similar increase was noted for available lysine (Table 4).

Overall nucleic acid removal with protamine sulfate was quite satisfactory. As protamine sulfate is relatively expensive ($45/100g), recovery and reuse would be important. One way to accomplish this is by immobilization, which will be subsequently discussed.

Streptomycin as a Nucleic Acid Precipitating Agent

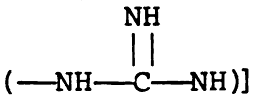
Streptomycin, which is a positively charged molecule [because of guanidino radical  $(\text{—NH—}\overset{\overset{\text{NH}}{\|}}{\text{C}}\text{—NH})$]

TABLE 3.--Effect of removing NA by 2/10 v/v of a protamine solution (2 percent w/v) and proteinase inhibitor (PMSF) on sulfhydryl and disulfide group of yeast proteins

Treatment	SH(μM/g Protein)	SS(μM/g Protein)
Cell homogenate	28.2	39.0
Protamine sulfate treated, no inhibitor	23.0	42.3
Protamine sulfate treated, + inhibitor	33.1	49.7

TABLE 4.--Effect of removing NA by 2/10 v/v of a protamine sulfate solution (2 percent w/v) and proteinase inhibitor (PMSF) on available lysine in yeast proteins

Treatment	g lysine/100 g protein
Cell homogenate	3.3
Protamine sulfate treated, no inhibitor	3.8
Protamine sulfate treated, + inhibitor	4.1

can bind to NA and lower their solubility. In the experiment reported here, 0.1 M streptomycin at ratio of 1:16 to protein, lowered the NA content up to 57 percent while protein recovery was 76 percent and the ratio of protein to NA was raised to about 11.5 (from 9.1).

In PAG, the streptomycin treated sample showed fewer zones at the lower part of the gel (Figure 12, 10). When the gels were stained for lactic dehydrogenase and esterase, the activity of the enzyme could be detected (Figure 11, 6). Apparently, this method of lowering NA does not denature the proteins so that it can be used for isolation of enzymes or where undenatured protein is needed for some functional use. SDS-PAG pattern of streptomycin treated samples (Figure 13, 9) looked much the same as the control except for a few zones which had disappeared at the bottom of the gel. This could be due to reaction of more acidic proteins with streptomycin which is a positively charged compound and as a result precipiation of these proteins along with NA.

In isoelectric focusing (Figure 14, D), the protein zones were quite close and similar to untreated sample. Most of the proteins had isoelectric points in the range of 3.7 to 7.5. Streptomycin is relatively inexpensive but recovery would again be important if it is going to be used in large scale. Streptomycin is not permitted in foods under present regulations, and this also would necessitate quantitative removal and recovery.

pH Adjustment

Jayaraman (1973) suggested that at pH 3.9 most proteins could be separated from nucleic acids. In this research, cells were homogenized in 0.1 M phosphate-citrate-borate buffer pH 7. The proteins were then precipitated by lowering pH of the buffer to 3.9 and removed. Recovery of the proteins in this method was very low (only 19 percent), but 79 percent of the NA were removed (Table 1).

In PAGE, fewer protein zones could be detected and most of the proteins showed had different mobilities (Figure 12, 4). Lactic dehydrogenase and estrase could not be detected in the gels which may be from denaturation or more likely to the poor recovery of protein. SDS-PAG (Figure 13, 5) showed very few protein zones and these were in the molecular weight range of 20,000 to 150,000. Isoelectric focusing (Figure 14, E) showed the proteins to have pI in the range of 4.5 to 6.5.

This method as evaluated indicated very poor protein recovery because of incomplete precipitation at pH 3.9. It has the advantages of being simple, inexpensive and rapid.

Separation of Yeast Proteins with Sodium Chloride

The proteins were precipitated in the presence of 10 percent (w/v) NaCl and the NA remaining in the solution were separated by centrifugation.

The heat employed and the salt are strong enough to cause extensive alteration to the proteins as indicated by loss of enzymatic activity when protein zones separated by electrophoresis were checked for activity. Protein recovery (40 percent) was not satisfactory under the conditions employed, but 63 percent of the NA were removed (Table 1), yielding the highest protein/NA ratio of the methods used.

In PAG (Figure 12, 8), very dew faint zones could be seen. Aggregated proteins which could not get into the gels remained on the top. In SDS-PAG (Figure 13, 4), SDS and mercaptoethanol disaggregates these proteins so more zones were observed. The proteins had molecular weights in the range of 16,000 to about 210,000. In isoelectric focusing, because of aggregation of the proteins, not any zone could be detected while top of the gel was heavily stained.

The problems associated with this technique were poor protein recovery, denaturation of proteins and salt removal. Advantages of the method are speed, low cost and the high protein NA ratio.

Exogenous Ribonuclease for Reduction of Nucleic Acids

Bovine pancreatic ribonuclease A (RNase A), which is commercially available, can be used to hydrolyse ribonucleic acids (RNA). RNase A is an endoribonuclease, i.e., hydrolysis of phosphodiester groups can occur anywhere in the chain. It is specific for single stranded RNA and has absolute specificity for pyrimidine nucleotides.

To the heat-shocked yeast cells, RNase A was added, the mixture incubated, then centrifuged. The cells depleted of NA were then homogenized. The data in Table 5 show the effect and importance of each step in removing NA. Incubation (at 55°C for 60 minutes) helped depleting the cells of NA (three times more NA was leaked out of the cell compared to not incubated sample). Heat-shock is very effective in facilitating penetration of RNase into the cell. It also activates the RNase (Castro et al., 1971). Heat-shock increased reduction of NA five-fold more than not heat-shocked sample as can be seen in Table 5. Addition of RNase helped lower NA, but when compared with heat-shocked and incubated sample (NA breakdown is from endogenous RNase), the difference was not significant.

Protein recovery by this method was 61 percent and NA removal 63 percent and protein NA ratio of 15.3 (Table 1). The main reason for protein loss may well

TABLE 5.--Effect of different treatments with or without ribonuclease A on reduction of nucleic acid content in yeast

Treatment	Percent Protein Recovery	Percent Nucleic Acid Removed	Protein Nucleic Acid Ratio
Control	100	0.0	7.7
Heat shocked + RNase + incubation (55°C)	60	64	15.2
Heat shock + RNase no incubation (left at room temperature)	88	18	9.2
Heat shock no RNase + incubation (55°C)	62	47	11.6
No heat shock + RNase + incubation (55°C)	78	12	8.5

be due to proteolysis of the proteins by endogenous proteinases. Heat-shock activates these proteolytic enzymes and incubation enables them to breakdown the proteins to small peptides and amino acids which can readily leak through the cell wall. In UV measurement of the NA in the supernatant buffer, these amino acids and peptides interfere with the readings, therefore, not all the UV absorbing materials should be credited for NA.

Heat-shock and incubation changed the PAGE profile of the proteins (Figure 12, 2). A thick protein zone was observed on the top of the gels which might be from aggregation of the proteins as a result of the heat treatments. Heat-shock per se affected the proteins more than the incubation when detected by PAGE. Many protein zones were missed in heat-shocked samples and those which remained had different mobility than untreated sample. The damage was less and different in incubated samples. Heat-shock and incubation together substantially altered characteristics of the proteins and lactic dehydrogenase and esterase could not be tested. SDS-PAGE pattern of the proteins were different from that of the control (Figure 13, 2). Proteins of 15,000 in molecular weight or more could be observed with different mobility from untreated sample. In isoelectric focusing, most of the protein zones had pI in the range of 3.6 to 6.0. Part of the proteins which were aggregated and were not able to get into the gel could be seen as a band on the top of the gel (Figure 14, B).

This technique has the disadvantages of high cost of enzyme, loss and denaturation (if of concern) of proteins. The major advantage of this method is the high ratio of protein to NA (15:1).

Endogenous Ribonuclease for Reduction of Nucleic Acids

RNases present in the yeast cell are activated by heat-shock, causing breakdown of the RNA to small fragments which leak from the cell wall during incubation. A heat-shock of 70°C for 1-3 seconds is recommended for this purpose (Ohta et al., 1971).

Table 6 shows effect of heat-shock and incubation in lowering NA. Heat-shock should be followed by incubation to be effective in depleting the cells of NA. Incubation alone caused a reduction of 32.5 percent of NA while a combination of heat-shock and incubation resulted in removal of 52 percent of the nucleic acids.

Protein recovery was approximately 70 percent due to proteolysis which cannot be avoided. The effect of heat-shock on PAGE profile of the proteins was much more apparent than incubation alone. PAGE-pattern of proteins were similar to the exogenous RNase method (Figure 12, 3).

TABLE 6.--Effect of heat shock process used for activation of endogenous ribonuclease in lowering nucleic acid content in yeast

Treatment	Percent Protein Recovery	Percent Nucleic Acid Removal	Protein Nucleic Acidi Ratio
Control	100	0.0	6.8
Heat shock, no incubation	98	2	7.2
Heat shock + incubation	69	52	12.1
No heat shock + incubation	76	32.5	10.9

Protein zones were darker, a few more zones could be detected and fewer proteins were aggregated. Compared to the control, the protein zones at the lower part of the gel were missing (probably due to proteolysis). In SDS-PAGE (Figure 13, 3), some of the protein zones were similar to the exogenous RNase method, but both had proteins with different molecular weight than the untreated sample. Molecular weight of the proteins were in the range of 14,000-110,000. Isoelectric points of the proteins measured by isoelectric focusing were in the range of 3.5-6.8 (Figure 14, C).

This method as in the case of exogenous RNase treatment is slow. Protein loss and heat denaturation of the proteins (lactic dehydrogenase and estrase did not show any activity) are other disadvantages of this technique. As this is an endogenous enzyme treatment, there is no limitation of enzyme cost.

Comparison of the Methods of Removing Nucleic Acids

Each of the methods studied has specific advantages and limitations. On a practical, economic basis, it seems impossible that nucleic acid removal can be attained without concomitant loss of proteins. Some proteins are very closely associated with nucleic acids and thus, tend to be removed with them. Many of the proteins also are acidic behaving somewhat similar to nucleic acids so that in precipitation of nucleic acids part of them will also be removed. Protein loss in some methods such as those which employ enzymatic hydrolysis of the NA is due undoubtedly to undesirable degradation of proteins by proteases which are present.

Obtaining a good yield of undenatured protein low in nucleic acid is difficult. Most methods used in reducing NA cause varying degrees of denaturation of the proteins. Undenatured proteins are needed in cases such as in purifying enzymes, for some texturized food proteins or for uses requiring specific functional properties such as whipping or foaming. Another advantage of using undenatured protein is that one can control the type and extent of processing to obtain a protein which controlled amounts of denaturation for specific purposes. Processes leading to extensive denaturation of proteins may also lower nutritional quality of the proteins, i.e., such as lowering the available lysine or lowering the digestibility of the proteins (Bender, 1972, and Osner and Johnson, 1968). However, many food proteins used purely as food ingredients are denatured and as such denaturation is of lesser consequence.

Techniques mentioned in lowering NA can be categorized in the following classes or manner:

1. Techniques which induce precipitation of the NA such as protamine sulfate, streptomycin, $MnCl_2$ and phase separation. In these techniques, some of proteins which are closely associated with NA and those that are very acidic may be removed along with NA. These methods usually do not denature the proteins. However, on a commercial scale to make them economically feasible, the precipitating agents should be recovered. Extent of NA removed usually depends on how much of these precipitating agents are being used.

2. Techniques in which proteins are precipitated such as NaCl or pH adjustment. Protein loss is significant in these methods. The reason may be different solubility of the proteins at different pH or salt concentrations which makes it impossible to precipitate all of the proteins at a certain pH or ionic strength and therefore, those which are not precipitated will be lost along with NA.

In these techniques, proteins usually are denatured so they would not be suitable for production of undenatured protein low in NA. The NaCl method is more efficient in protein recovery than pH adjustment. Overall, these technqiues are relatively inexpensive.

3. Enzymatic methods. In these methods protein loss is also a problem. One reason for that is proteolysis caused by endogenous proteinases. A heat-shock

process which is required for the activity of ribonucleases can activate these proteinases and causes more protein loss (Hayashi et al., 1968).

Proteins (enzymes) are often sensitive to the heat-shock and heat of incubation (e.g., 55°C for 60 minutes) which is used in these methods. Therefore, these proteins may be denatured, a possible disadvantage for these techniques. In exogenous ribonuclease, high cost of ribonuclease is another drawback unless an immobilized enzyme system is used which still has cost of setting and maintaining the reactor. In the heat-shock process, this would not be a problem.

Effect of Nucleic Acid Removal on Amino Acid Composition

The data in Table 7 illustrates the amino acid (AA) composition of yeast cell homogenate before and after reducing the nucleic acid content. Generally, valine, phenylalanine and methionine (all essential AA) concentrations were improved when NA were reduced. Isoleucine and leucine were present at increased levels in the protein remaining after NA precipitation and in NaCl method, but decreased in other separated proteins.

In general, the AA composition in heat-shock and phase-separated proteins was much different from the others. Essential AA concentrations usually improved

TABLE 7.--Amino acid composition of yeast proteins lowered in nucleic acids by different treatments (g amino acid/100 G corrected protein)

Amino Acid	Control	Protamine Sulfate[1]	Protamine sulfate[2]	Phase Separation	Streptomycin	$McCl_2$	NaCl	pH Adjustment	Exogenous RNase	Heat Shock	Baker Yeast Protein*
Aspartic Acid	12.80	11.72	12.72	10.21	11.99	12.64	12.74	12.86	13.60	12.30	11.41
Threonine	5.01	4.68	4.92	6.47	5.31	5.62	5.59	5.56	5.15	5.66	5.03
Serine	4.76	5.91	4.69	7.56	4.91	5.36	5.28	5.48	6.40	5.43	4.93
Glutamic Acid	10.05	13.03	11.56	11.03	10.89	12.26	11.81	12.64	12.31	12.85	11.52
Proline	3.47	2.93	3.38	3.65	3.29	3.61	3.64	4.44	3.42	4.54	4.32
Glycine	3.95	4.01	3.92	4.92	3.15	4.35	4.44	5.01	4.11	4.06	4.48
Alanine	5.57	5.60	5.76	7.17	6.55	6.40	6.45	6.63	5.41	5.56	6.16
Valine	5.95	6.22	6.18	6.82	6.51	6.49	7.27	6.51	6.14	6.30	5.85
Methionine	1.24	1.50	1.30	1.13	1.64	1.69	1.82	0.98	0.97	1.01	1.92
Cysteine[3]											1.10
Isoleucine	5.00	5.09	5.18	5.33	5.04	5.53	5.10	4.56	4.81	4.60	5.59
Leucine	7.95	8.18	8.04	8.39	9.20	8.87	8.16	7.21	6.92	6.84	8.88
Tryosine	4.52	4.09	4.53	2.69	4.64	3.30	5.00	3.41	4.06	4.15	4.45
Phenylalamine	5.86	6.17	5.78	6.63	7.04	6.76	6.18	6.67	7.95	7.46	5.58
Histidine	2.38	2.47	2.48	2.41	2.58	2.56	2.66	1.97	2.26	1.93	2.86
Lysine	9.43	9.29	9.56	7.45	9.40	10.74	9.5	10.52	9.02	8.14	9.45
Arginine	8.24	5.26	4.36	4.47	3.74	4.92	5.29	4.21	4.12	3.48	5.91
Tryptophan[3]											1.69

*From McCormic, R. D., 1973, Food Product Development, 7:6, 17.

[1] Protamine sulfate treated and proteinase inhibitor (PMSF) added.

[2] Protamine sulfate treated no proteinase inhibitor added.

[3] Was not measured.

when NA were reduced. In NA precipitation techniques, it was expected to see a decrease in aspartic acid and glutamic acid concentrations in the remaining proteins, but this did not occur. Since during acid hydrolysis of proteins aspargine and glutamine have a tendency to lose their amido group (Tower, 1967) and form aspartic acid and glutamic acid, the values for these latter two AA are partly from aspargine and glutamine.

Variation in AA composition is a result of the behavior of different proteins toward the different techniques used to reduce the NA content. For example, some proteins are more susceptible to proteolysis. Therefore, in these methods that proteinases have more chance for activation the susceptible proteins would be in lower concentrations and result in the change in AA composition pattern. The change in AA composition will change quality and properties of that protein system.

Nutritionally, yeast proteins have a good proportion of AA except for sulfur AA (methionine and cysteine). Lysine in yeast protein is higher than most of cereal proteins and yeast protein is therefore an ideal supplement to flour. The other essential AA in the yeast protein are comparable to animal or oil seed proteins (Litchfield and Sachsel, 1965).

II. Removing Nucleic Acids by Affinity Chromatography

Diethyl Amino Ethyl Cellulose (DEAE)

DEAE, which is an anion exchanger, binds stronger to the nucleic acids than the proteins. A pattern of elution of yeast proteins on a DEAE column is shown in Figure 15. NA are eluted from the column at higher ionic strength (last peak). In one run, 13 fractions were collected from the column, concentrated and PAGE was performed on them. The electrophorograms of the fractions are shown in Figure 16. Part of the proteins are very closely associated with nucleic acids and, therefore, are eluted along with them. Also, some of the proteins elute from the column in a range close to the ionic strength that the nucleic acids elute (big peak in Figure 15 and gels numbered 8, 9 and 10 in Figure 16). The latter problem can be overcome by using a longer column, change of buffer system, change of pH and pH gradient elution. In Figure 16, B mercaptoethanol was added to the fractions to see which zones have protein with disulfide bridges. Diffusion and smearing in some of the zones (especially seen in gel number 9) may be taken as an indication of proteins with disulfide bridges.

The removal of nucleic acids by DEAE is a slow process but gentle. Since with this technique there is

Figure 15.--Chromatogram of nonsedimentable portion of ca 100 mg (dry weight) of yeast cell homogenate on a 1 x 30 cm column of DEAE-cellulose. A linear gradient of NaCl (from 0 to 0.4 M in 0.05 M Tris-Hcl pH 8.5) was used. The last large peak was nucleoprotein while the rest were proteins.

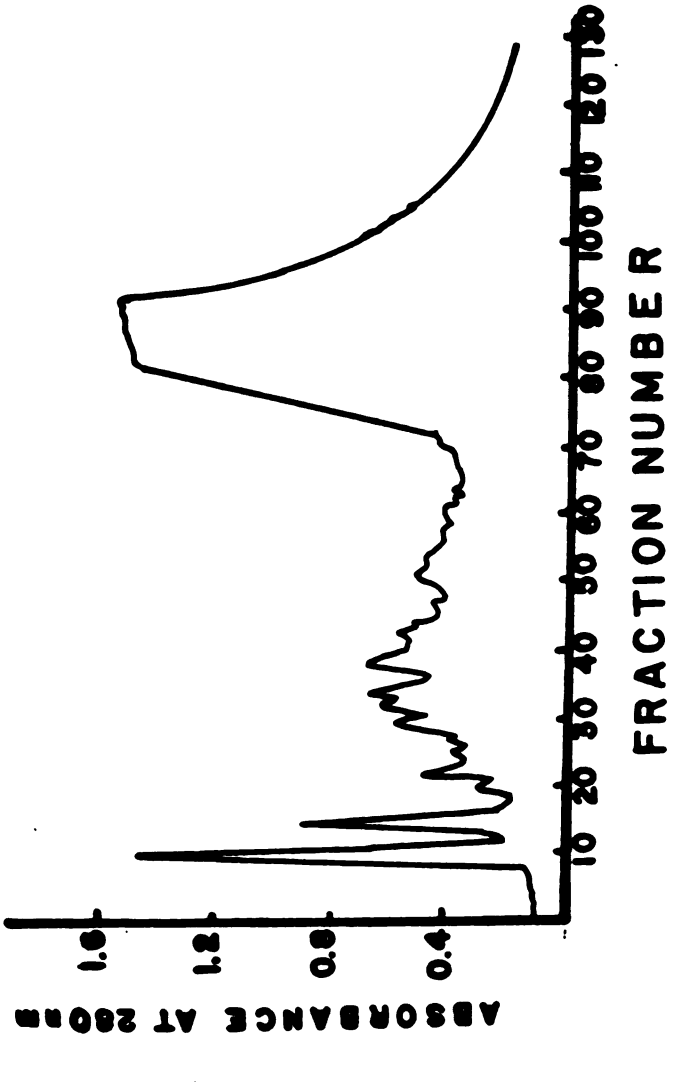
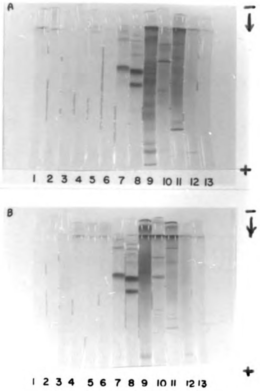

ABSORBANCE AT 280nm
1.6
1.2
0.8
0.4
10
20
30
40
50
60
70
80
90
100
110
120
130
FRACTION NUMBER

Figure 16.--Polyacrylamide gel electrophorogram of yeast proteins fractionated on a DEAE column. A-no mercaptoethanol was added, B-mercaptoethanol was added to the fractions.


A
−
+
1 2 3 4 5 6 7 8 9 10 11 12 13
B
−
+
1 2 3 4 5 6 7 8 9 10 11 12 13

no need for the disruptive treatments associated with many of the other methods for separating nucleic acids. This has made DEAE a classical procedure in the fractionation of enzymes. The initial cost of DEAE and maintenance of the column are the limitations of DEAE on a large scale.

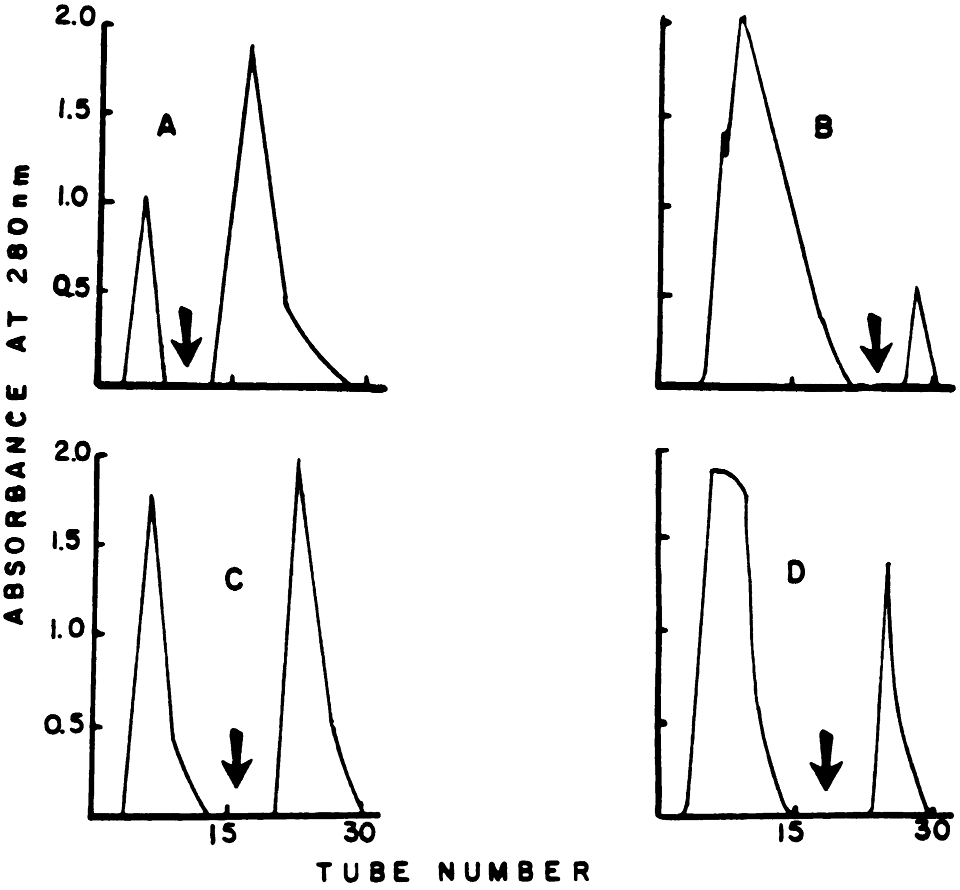
Immobilization of Protamine on Sepharose

Protamines are expensive and one way to make their use more economical is to immobilize them. Sepharose 4B was chosen as the supporting material. The amount of protamine bound to Sepharose was measured by the difference between the amount of protamine taken and the amount which was unbounded. Fourteen mg protamine was bound per ml of Sepharose. Protamine-//-Sepharose 4B was packed in a 1 x 15 cm column and used for these experiments. Figure 17 shows the chromatogram of yeast proteins on the column at different conditions. In Figure 17, A, 2 mg freeze-dried yeast protein in 0.01 M acetate buffer pH 4 was put on the column. The arrow shows change of buffer to 0.1 M carbonate pH 10.5. Analysis of the fractions for protein and NA showed that the ratio of protein to nucleic acid was much greater than the second peak, but overall separation of NA from proteins was not good. Experimental conditions employed for obtaining the data shown in Figure 17, B were the same as those used in obtaining the data in Figure 17, A with the difference that fresh yeast cell homogenate (0.6 ml which contained about 20 mg protein and 1.4 mg NA) was used. The first large peak was from both protein and NA. Most of the proteins were in the first peak. A very small amount of protein could be detected in the second peak while NA were distributed between the two fractions, 2/3 in the first peak and 1/3 in the second. This shows that freeze-drying (and other forms of dehydration also) makes separation of NA from proteins more difficult.

When 12 mg yeast protein with 0.8 mg NA in 0.005 M phosphate buffer pH 7.1 was applied on the same column (Figure 17, C), about 2/3 of the protein and 1/2 of the NA eluted under the first peak and the rest under the second peak.

To improve the NA removal, 20 percent dioxane was added to the phosphate buffer (Figure 17, D). About 4/5 of the proteins and 1/3 of the NA eluted in the first peak. Carbonate buffer (0.1 M pH 10.5) removed the remaining proteins and NA (second peak). Addition of dioxane lowered the polarity of the buffer which helped to get a better separation of NA from the proteins. The problem in this technique as in most of the other cases

Figure 17.--Chromatogram of nonsedimentable portion of yeast cell homogenate on a 1 x 15 cm column of Protamine-//-Sepharose 4B. In A, 2 mg freeze-dried sample was eluted with 0.01 M acetate buffer pH 4. Arrow indicates change of buffer to 0.1 M carbonate pH 10.5. Ratio of protein to nucleic acid was much greater than the second peak. In B, fresh sample was used (20 mg protein and 1.4 mg NA). Other conditions were same as A. Most of the proteins were in the first peak and a very small amount in the second peak. NA were distributed between the two factions, about 2/3 in the first, rest in the second peak were eluted. In C, fresh sample (12 mg protein and 0.8 mg NA) was used. First, buffer was 0.005 M phosphate buffer (pH 7.1). Arrow indicates change of buffer to 0.1 M Carbonate pH 10.5. About 2/3 of the protein and 1/2 of the NA eluted under the first peak and rest under the second peak. In D, conditions were same as C except first buffer had 20 percent dioxane. About 4/5 of proteins and 1/2 of NA eluted in the first peak.


ABSORBANCE AT 280nm
2.0
1.5
1.0
0.5
A
B
C
D
15
30
TUBE NUMBER

is close association of some of the proteins with NA which makes it very difficult to get rid of the NA without losing part of the proteins.

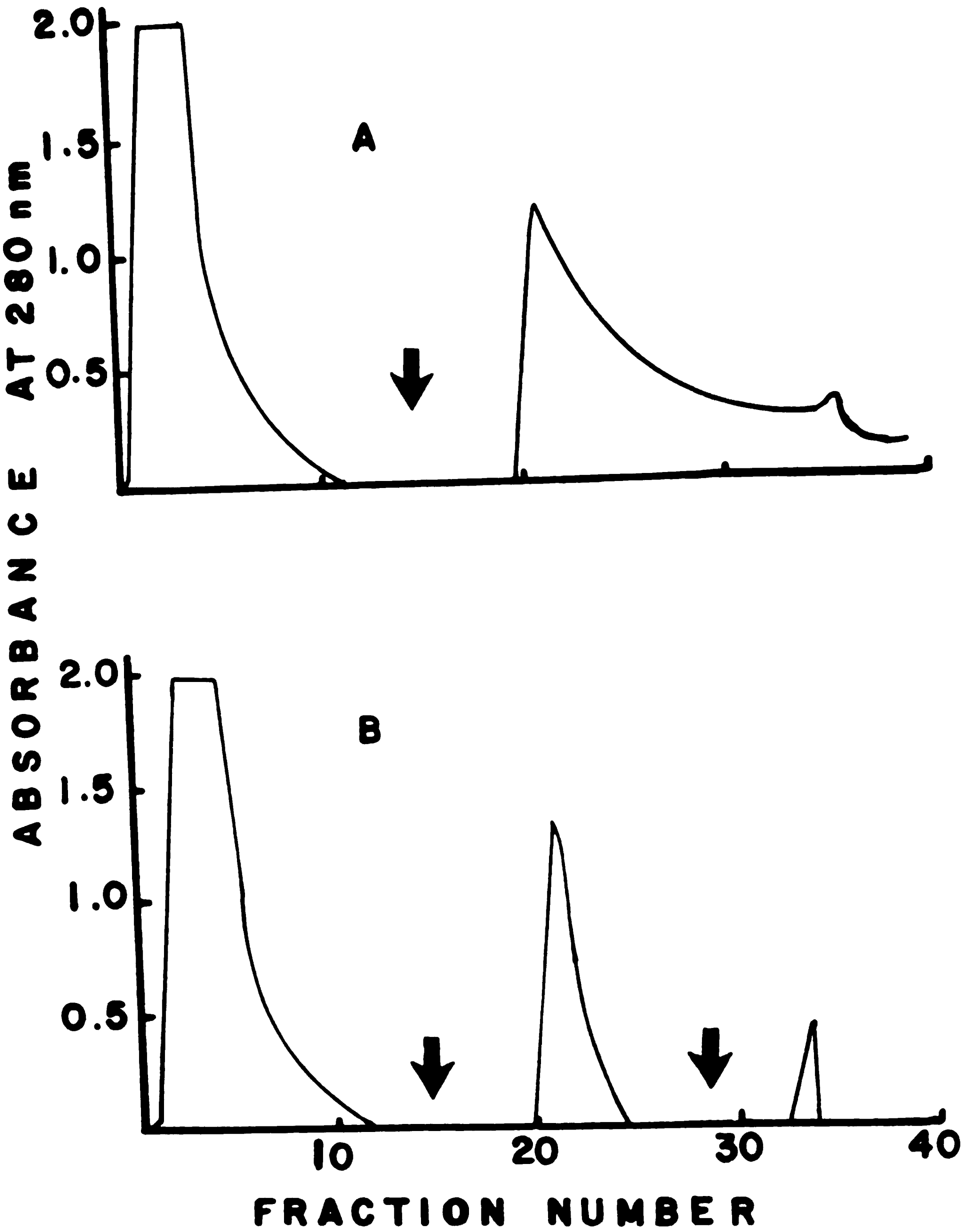
Immobilization of RNA on Sepharose

Cyanogen bromide activated Sepharose was used as a matrix for attachment of RNA. Unbounded RNA was measured in the washed Sepharose after the coupling. In this technique, 120 mg yeast RNA was bound per 1 ml of Sepharose. This amount is much less than protamine bound Sepharose because protamines contain more amino groups.

A column (1 x 15 cm) was packed with the RNA-//-Sepharose and used for the experiment. One ml of yeast protein (24 mg protein and 1.6 mg NA) was applied to the column and eluted with 0.1 M phosphate buffer pH 7.1. Figure 18 shows the chromatographic separation of NA from proteins. In A, the arrow shows change of buffer to 0.1 M accetate buffer pH 3.75. When fractions were assayed for proteins and NA, most of the proteins and 90 percent or more of the NA were found in the fractions collected under the first peak. The amount of protein in the fractions of the second peak was not significant.

Elution of NA from the column by acetate buffer was not satisfactory since they were shown as a tailing band. Therefore, in Figure 18, B buffers were changed.

Figure 18.--Chromatogram of nonsedimentable portion of yeast cell homogenate (24 mg protein and 1.6 mg NA) on a 1 x 15 cm column of RNA-//-Sepharose 4B. In A, first buffer was 0.1 M phosphate pH 7.1. Arrow indicates change of buffer to 0.1 M acetate pH 3.75. In B, first buffer was 0.1 M Tris buffer pH 8.5. First arrow indicates change of buffer to 0.05 M KCE pH 2 and second arrow changing back to starting Tris buffer. More than 90 percent of the NA were found in the fractions collected under the first peak. Amount of protein in the second peak was not significant. In B, the third peak was NA.


ABSORBANCE AT 280 nm
2.0
1.5
1.0
0.5
A
2.0
1.5
1.0
0.5
B
10
20
30
40
FRACTION NUMBER

One-tenth M Tris (pH 8.5) buffer was used first, followed by 0.05 M KCl (pH 2) buffer (shown by arrow). In this case, most of the NA were rapidly eluted off the column, but when changed back to Tris buffer (second arrow to the right) a small peak resulted. When fractions of this small peak were analyzed, more NA were found.

It seemed that part of the tailing band and small peak was caused by removal of NA from Sepharose. This "leakage problem" is also common in immobilization of proteins and can be improved by the use of a variety of water-soluble polyfunctional polymers which contain large numbers of primary amino groups. Coupled polyfunctional polymers to the backbone of agarose matrix, increases chemical stability by multipoint attachment (Parikh et al., 1974).

The principle of removing the NA by this method is mostly pairing and hydorgen bond formation between the nucleic acid bases (affinity of NA toward each other). This method does not seem to be very efficient in removing NA (because of high selectivity of NA), but it is significant in preparation and fractionation of NA for specific experiments such as genetic experiments.

Significance of Affinity Techniques in Removal of Nucleic Acids

In an industrial process for undenatured yeast protein low in nucleic acid, the affinity techniques look promising. Although the initial cost of setting the reactors is high, in the long run it would be economical.

In these methods, the proteins remain mostly in their natural form because no harsh treatment is being used in the process of NA removal. Furthermore, the nucleic acids separated might possibly be recovered for use in flavor enhancers or other purposes.

This research has shown that affinity techniques have possibilities as a viable and efficient procedure for removal of NA from yeast proteins. The research in this thesis has been exploratory in nature. If this is to become a feasible alternative to present-day technology, additional research must be initiated to further examine and clarify the technological aspects.

III. Effect and Control of Yeast Proteinases

Yeast cells contain a variety of proteolytic enzymes. These proteinases pose a serious problem in the preparation and study of other yeast proteins. Part of the protein loss in extraction of proteins from yeast cells results from the action of these enzymes. An example is in lowering NA content by ribonucleases when proteases cause protein degradation during the RNase incubation. Other than protein loss, the effect of these enzymes on the overall characteristics of other yeast proteins is important especially when working with undenatured yeast protein.

Figures 19 and 20 show the extent of proteolysis of yeast proteins incubated at 37°C. The whole PAGE pattern of yeast proteins was changed in less than one hour.

Therefore, it would be desirable to control or remove such enzymes. By removing the proteinases, it is possible to use them under controlled conditions in other areas of food industry or other industries. There are numerous uses for proteinases in food industry such as chill proofing beer; coagulating milk; clarification of wine; reducing bitterness of grapefruit juice; and inhibition of oxidative rancidity in milk and other products (Olson and Richardson, 1974).

Yeast proteinases behave like both chymotrypsin and trypsin. This was shown by incubation of yeast cell homogenates with p-toluenesulfonyl-L-arginine methyl ester (TAME) and N-benzoyl-L-tyrosine ethyl ester (BTEE) which are synthetic substrates specific for trypsin and chymotrypsin respectively (Figure 21). Phenymethyl sulfonyl flouride (PMSF) inhibits the activity of most of the yeast proteinases to a large extent. As can be seen from the electrophorograms in Figures 22, 23 and 24, yeast proteins which contained PMSF (1/20 v/v of a 6 mg/ml PMSF

Figure 19.--Diagram of electrophoretic pattern of yeast cell homogenate incubated at 37°C in a water bath shaker for various times.

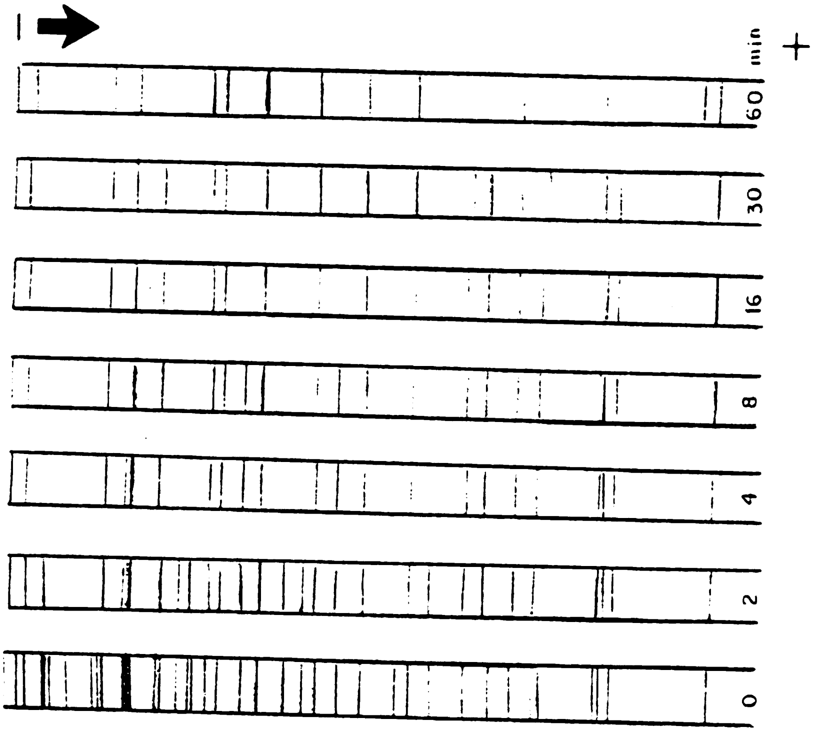

−
+
min
60
30
16
8
4
2
0

Figure 20.--Diagram of electrophoretic pattern of yeast cell homogenate incubated at 37°C in a water bath shaker for various times.

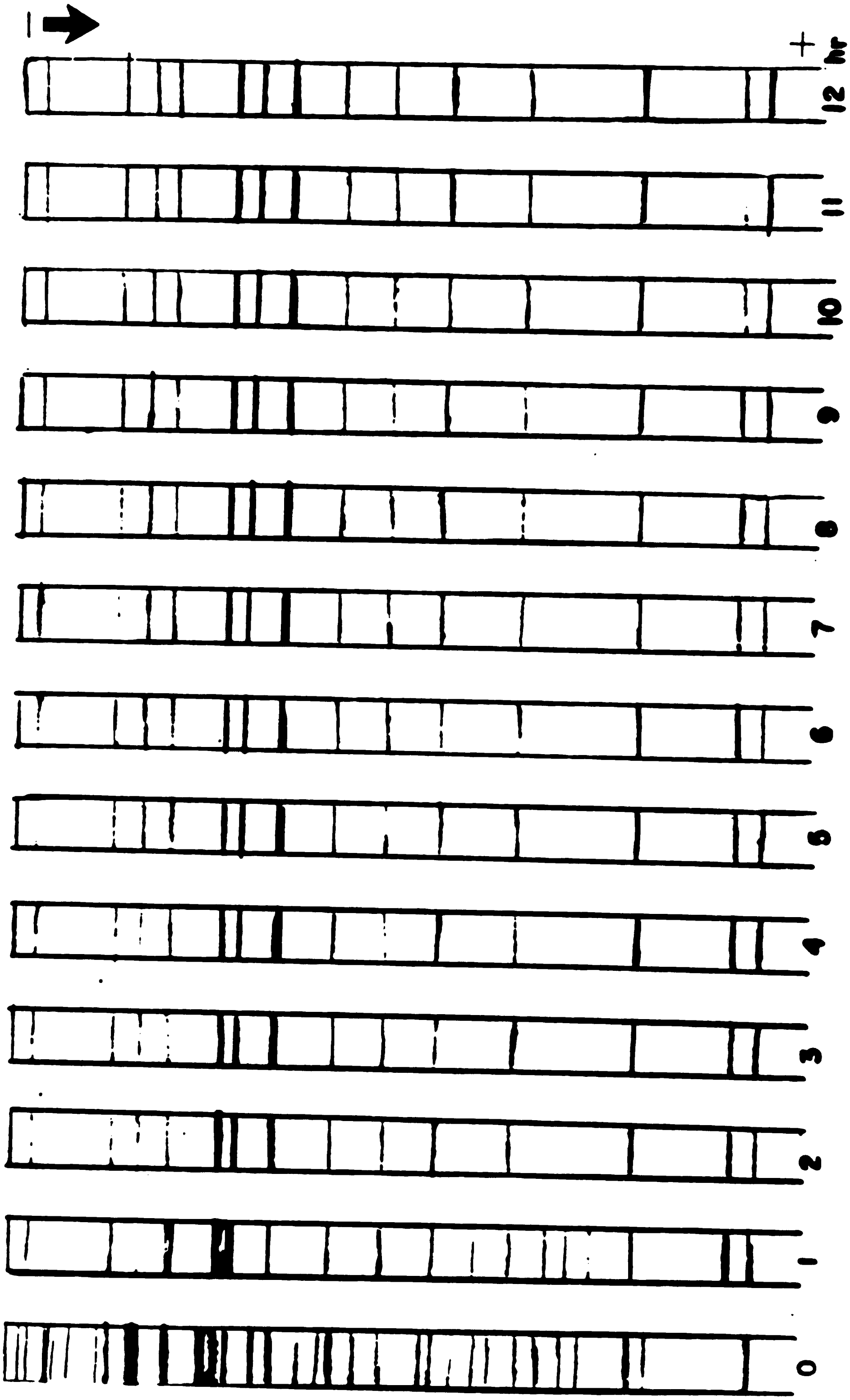

−
+
hr
12
11
10
9
8
7
6
5
4
3
2
1
0

Figure 21.--Action of yeast proteinases on TAME and BTEE (syntheitc substrates specific for trypsin and chymotrypsin enzymes) measured as change in absorbance at 247 and 256 nm, respectively, according to Walsh and Wilcox (1970). One ml of yeast cell homogenate (nonsedimentable) with 67 mg protein was used as stock solution.

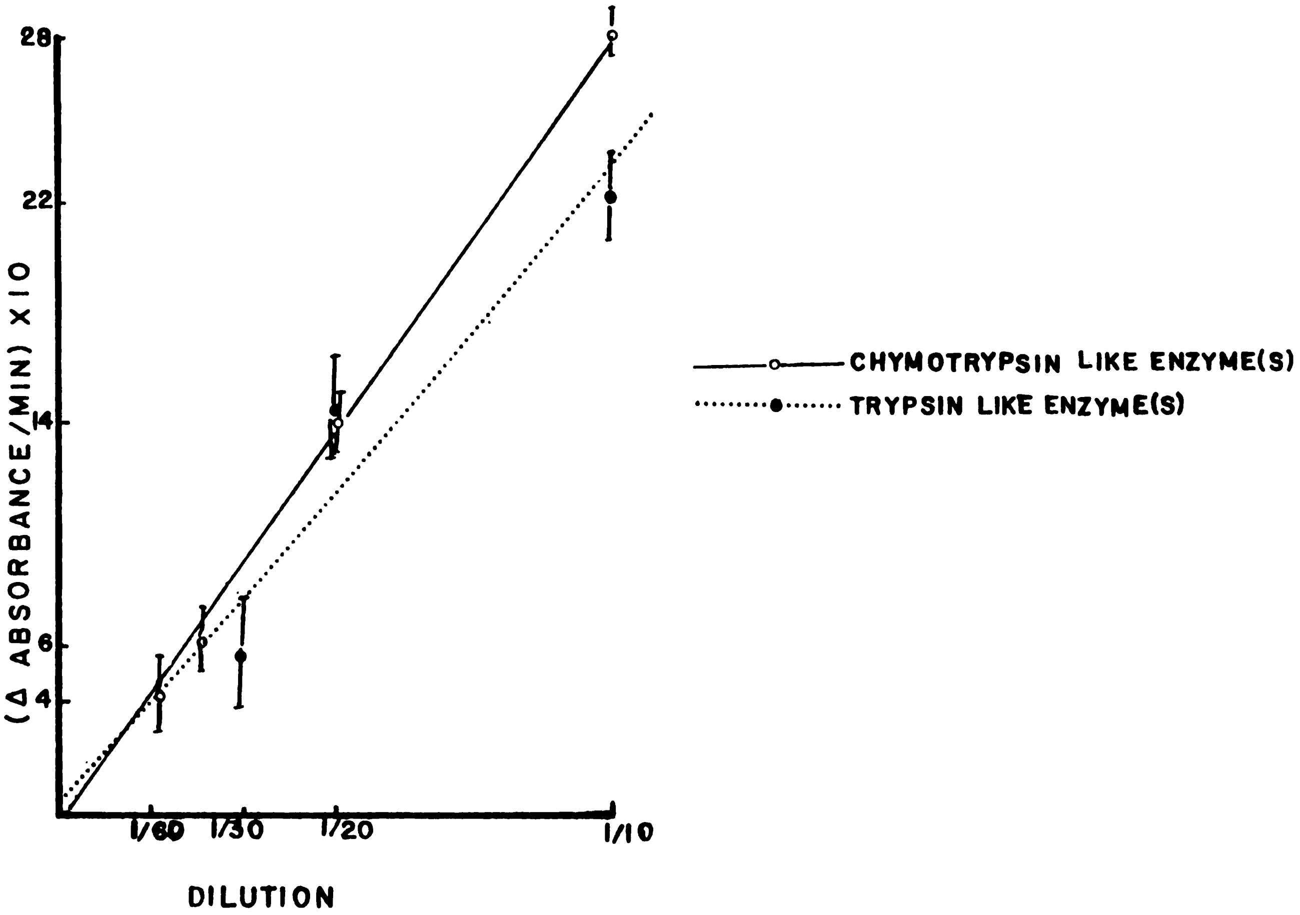
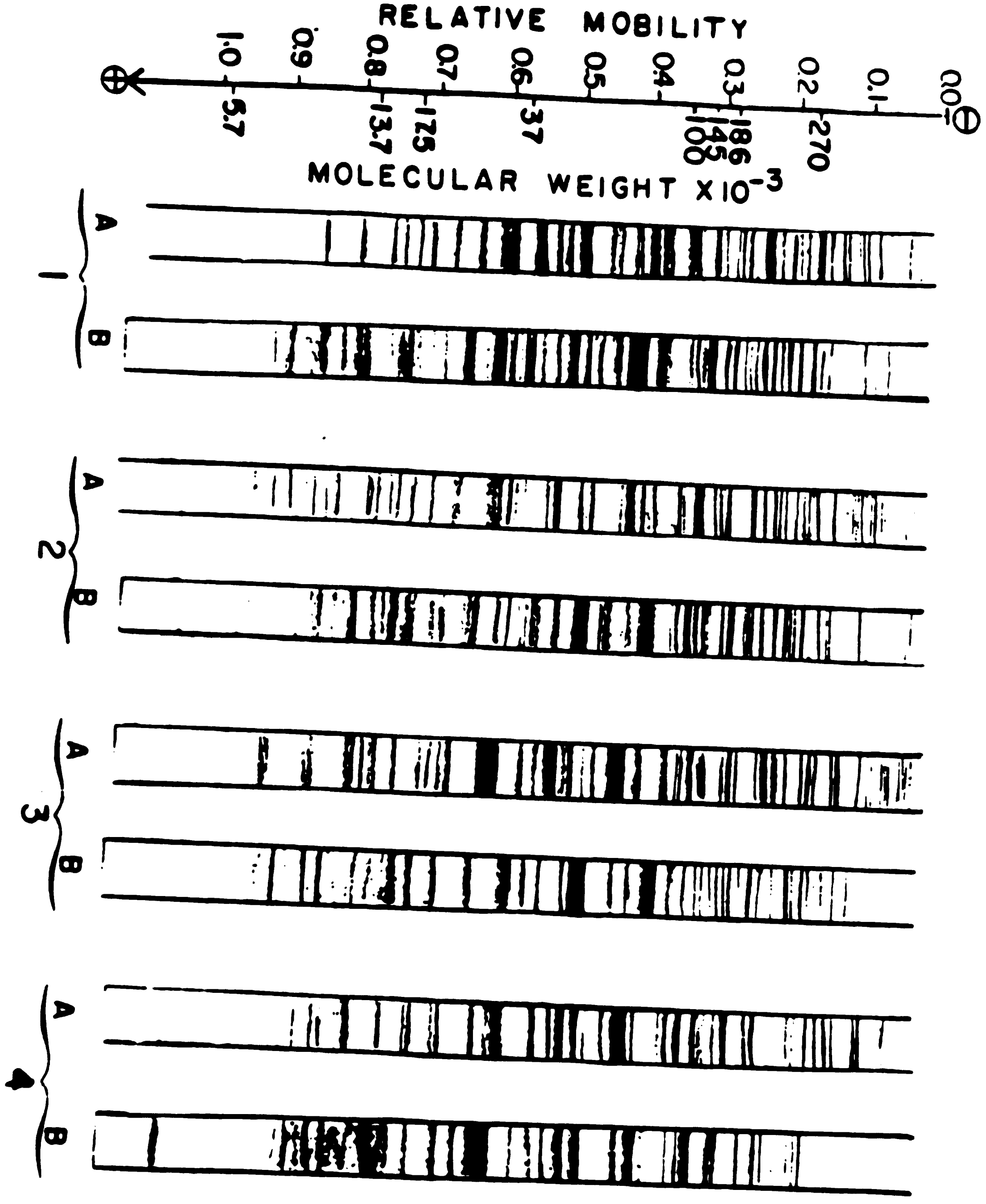

28
22
14
6
4
(Δ ABSORBANCE/MIN) X10^{-2}
1/60
1/30
1/20
1/10
DILUTION
CHYMOTRYPSIN LIKE ENZYME(S)
TRYPSIN LIKE ENZYME(S)

Figure 22.--Densitometric tracing of yeast proteins on gradient SDS-PAG (7.5-17.5 percent w/v).
A is homobenated yeast cell
B is yeast protein low in nucleic acid (treated with 2/10 v/v of a 2 percent protamine sulfate) + proteinase inhibitor (PMSF)
C is same as B but no inhibitor

Figure 23.--Densitometric tracing of yeast proteins on 7.5 percent w/v PAG.
A is homogenated yeast cell + proteinase inhibitor (PMSF)
B is yeast protein low in nucleic acid (treated with 2/10 of a 2 percent protamine sulfate) + PMSF
C is same as B but no inhibitor

Figure 24.--Diagram of the electrophoretic patterns of yeast proteins low in nucleic acid (protamine sulfate treated) in 7.5-17.5 percent (w/v) gradient SDS-PAG. The relationship between relative mobility and molecular weight is shown in the left. 1-untreated, 2-treated with 1/10 v/v, 3-2/10 v/v, 4-4/10 v/v of a 2 percent protamine sulfate in order to precipitate the nucleic acids. A-does not have any proteinase inhibitor B-1/20 v/v of a 6 mg/ml (ethanol) of PMSF was added before treatment.

Note: Protein zones missed due to proteolysis.


RELATIVE MOBILITY
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
270
186
145
100
37
17.5
13.7
5.7
MOLECULAR WEIGHT X10^{-3}
A
B
1
A
B
2
A
B
3
A
B
4

in ethanol), exhibited more zones than the sample without gut PMSF.

The data in Tables 3 and 4 show total disulfide, available sulfhydryl and available lysine were more prevalent in yeast proteins contained PMSF as proteinase inhibitor than in the controls. Sulfonyl halides such as PMSF react with many serine proteinases to sulfonylate the hydroxyl group of a specific serine residue in the active site and thereby inhibit enzymatic activity (Gold, 1967).

Yeasts contain a variety of proteolytic enzymes. Hence, it is quite difficult to eliminate all the proteolytic artifacts by using one inhibitor. Another problem in using the inhibitors is the extreme toxicity of most of these compounds. Therefore, in anther study, effect of some of the naturally occurring antitryptic compounds on these proteinases was evaluated. Navy bean trypsin inhibitor isolated from _Phaseolus_ _vulgaris_ _L_., which is an albumin, even at concentrations of 1:3 to total yeast proteins had no or very small inhibitory effect on yeast proteinases (Table 8). Navy bean trypsin inhibitor interacts strongly with trypsin as well as chymotrypsin (Gomes, 1978).

Soybean trypsin inhibitor (Kunitz) showed strong inhibitory effect on yeast proteinases as can be seen from

TABLE 8.--Effect of navy bean trypsin inhibitor (N.B.T.I.) on yeast proteinases measured by method of Kakade et al., (1969)

Concentration μg N.B.T.I./3.3 mg Protein	Absorbance at 280 nm
0.0	0.380
100	0.380
300	0.360
500	0.380
800	0.375
1100	0.370

the data in Table 9. Percent inhibition was maximum at 300 μg soybean trypsin inhibitor. At higher concentrations of the inhibitor, the inhibition was reduced.

Soybean trypsin inhibitor, which is a globulin, has molecular weight of 21,500. It consists of a single polypeptide chain. At neutral pH, it forms a one-to-one stoichiometric complex with trypsin while appreciable interaction also occurs with chymotrypsin (Steiner and Frattali, 1969).

Because of strong inhibitory effect of soybean trypsin inhibitor on yeast proteinases, immobilized soybean trypsin inhibitor on Sepharose 4B was used to remove the proteases from yeast proteins. When 30 mg of yeast protein was applied on a column (0.5 x 12 cm) packed with soybean trypsin inhibitor-//-Sepharose 4B (Figure 25), more than 50 percent of proteasess were absorbed by the column which were later eluted by 0.1 M acetate buffer (pH 3.0) under the second peak.

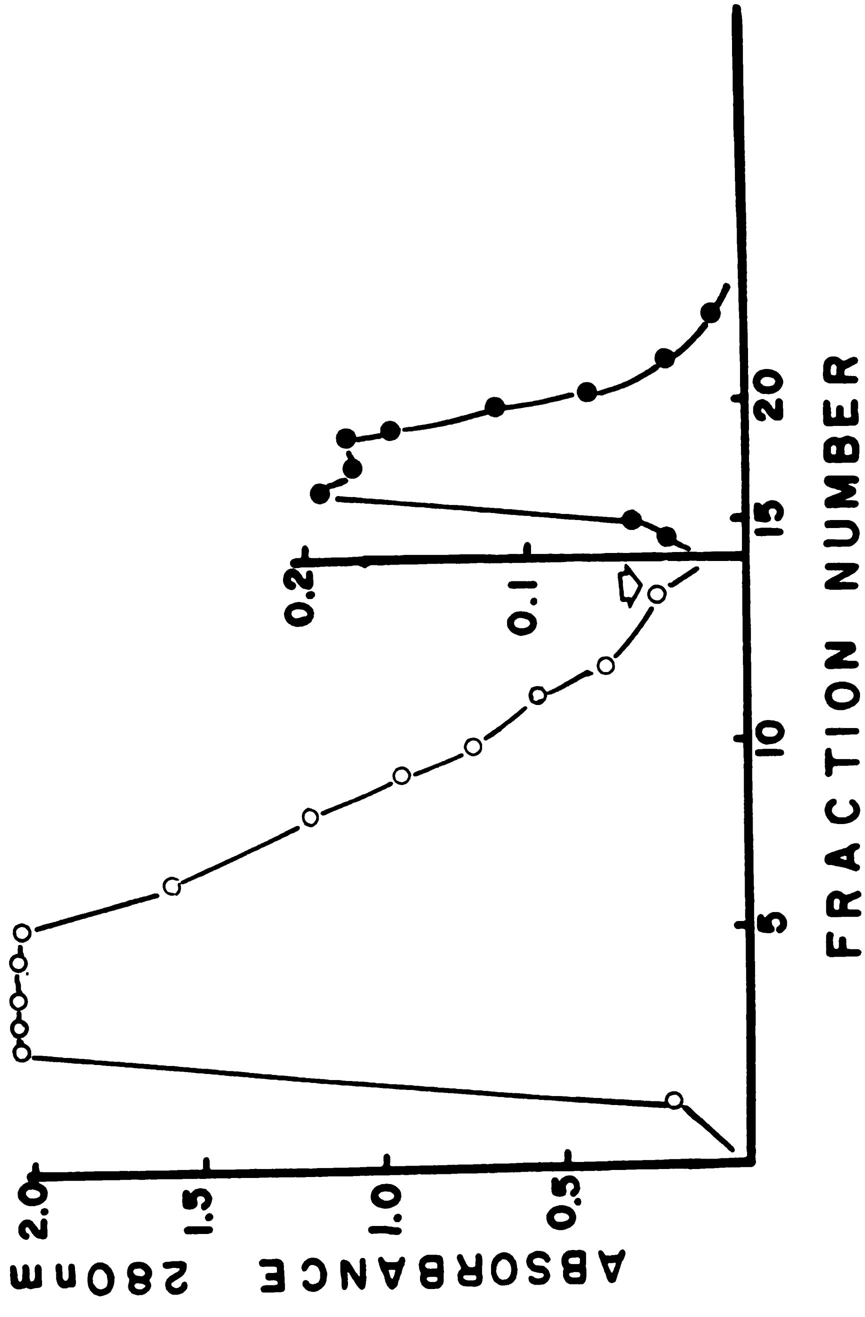
By this method, it is not only possible to minimize the problem of proteolysis, but also prepare purified proteases for other purposes.

There are several other compounds which have affinity toward the proteinases and might be used in immobilized form to remove the reamining proteinases. Aminocaproyl-p-amino-benzamidine which binds to several

TABLE 9.--Effect of soybean trypsin inhibitor (S.B.T.I.) on yeast proteinases measured by method of Kakade et al., 1969

μ g S.B.T.I./3.3 mg Protein	Absorbance/ 280 nm	Percent Inhibition
0.0	0.491	0.0
100	0.115	76
200	0.110	78
300	0.075	85
400	0.073	85
500	0.070	85
800	0.107	78
1000	0.195	60
1500	0.320	35

Figure 25.--Affinity chromatography of yeast Proteinases on a soybean trypsin inhibitor-//-Sepharose 4B column (0.5 x 12 cm). 30 mg yeast protein low in nucleic acid (treated with 4/10 v/v of a 2 percent protamine sulfate) eluted from column by 0.1 M Tris buffer pH 8.0 which contained 50 mM $CaCl_2$. More than 50 percent of proteinases were absorbed to the column and eluted under the second peak.


ABSORBANCE 280nm
2.0
1.5
1.0
0.5
0.2
0.1
5
10
15
20
FRACTION NUMBER

different type of proteinases (Jany et al., 1976) and glycyl-L-tyrosine-azo-benzyl-succinic acid which is specific for carboxypeptidase Y and A (Johansen et al., 1976) are examples of these compounds which can be used.

SUMMARY

Commercial press cake of baker's yeast was washed and disintegrated in a cell homogenizer. A detailed study was made of different techniques to lower the nucleic acid content of the isolated yeast proteins. These techniques can be categorized into three major groups:

1. Precipitation of either proteins or nucleic acids
2. Enzymatic hydrolysis of nucleic acids, and
3. Affinity chromotography

The effect of each process on the separated proteins was studied. Protein yield, amino acid composition, extent of denaturation, electrophoretic characterization and effectiveness of each method in nucleic acid removal were of major interest. Electrophoretic characterization included isoelectric focusing, disc gel electrophoresis and SDS polyacrylamide gel electrophoresis.

Each method for removing nucleic acids has some advantages and some limitations. Protamine sulfate precipitation was found to have no obvious effect on the proteins. Hot NaCl precipitation denatured the majority of the proteins. Enzymatic methods resulted in proteolysis and protein denaturation. These techniques are

comparable as regards to nucleic acid removal. Selection of the method to be used will be determined by economical feasibility, use and fate of the proteins, i.e., if undenatured protein is needed for texturized protein products or commercial enzyme production.

The native proteinases present in yeasts are a serious problem because they result in loss of protein and change in protein characteristics with time. Therefore, in another study the effect, control and removal of these proteinases was examined. Yeast proteinases behave more like chymotrypsin rather than trypsin. Phenylmethyl sulfonyl fluoride can be used to inhibit proteinases in routine laboratory work. Soybean trypsin inhibitor at concentrations of about 100 μg per mg yeast protein has strong inhibitory effect on yeast proteinases while navy bean trypsin inhibitor does not have any significant effect. Soybean trypsin inhibitor was immobilized on Sepharose 4B and was used to remove a significant quantity of the proteinases. Proteinases once separated can be recovered and serve as a source of proteolytic enzymes for appropriate uses in the food industry.

APPENDIX

APPENDIX

TABLE A.1.--Some important chemicals used in this study and their sources

Chemicals	Sources
Acrylamide	Bio-Rad Laboratories Richmond, CA
Alcohol dehydrogenase	Sigma Chemical Co. St. Louis, MO
Alkaline phosphatase	Sigma Chemical Co. St. Louis, MO
N-benzoyl-L-tryrosine ethyl ester	Sigma Chemical Co. St. Louis, MO
Glucose oxidase	Worthington Freehold, NJ
Insulin	Sigma Chemical Co. St. Louis, MO
Invertase	Sigma Chemcial Co. St. Louis, MO
β-Lactoglobulin	Nutritional Bio Chemical Corporation Cleveland, OH
N-N'-methylene-bis-acrylamide	Bio-Rad Laboratories Richmond, CA
Navy bean trypsin inhibitor	Carlos Gomes Dept. of Food Science M.S.U. E. Lansing, MI
Phenyl methyl sulfonyl fluoride	Sigma Chemical Co. St. Louis, MO
6-Phosphogluconic dehydrogenase	Sigma Chemical Co. St. Luois, MO

TABLE A.1.--Continued

Chemicals	Sources
Phosphoglucose isomerase	Sigma Chemical Co. St. Louis, MO
Protamine sulfate	Sigma Chemical Co. St. Louis, MO
RNA	Nutritional Biochemical Corporation Cleveland, OH
SDS	Pierce Rockford, IL
Sepharose	Pharmacia Fine Chemicals Uppsala, Sweden
Soybean trypsin inhibitor-//-Sepharose	Sigma Chemical Co. St. Louis, MO
Streptomycin sulfate	Sigma Chemical Co. St. Louis, MO
TEMED	Bio-Rad Laboratories Richmond, CA
p-toluenesulfonyl-L-arginine methyl ester	Sigma Chemical Co. St. Louis, MO

TABLE A.2.--Formulation of 7.5 percent Polyacrylamide Gel Electrophoresis System

Separating Gel (7.5% Acrylamide, 0.18% BIS, pH 8.9)

Stock solutions	amount /100 ml
a) Tris	36.3 g
N,N,N',N'-Tetramethylethylenediamine (TEMED)	0.23 ml
b) Acrylamide	30 g
N,N'-Methylenebisacrylamide (BIS)	734 mg
c) Ammonium persulfate	140 mg

Working solution - 1 part (a):1 part (b):2 parts (c)

Stacking Gel (2.5% Acrylamide, 0.625% BIS)

Stock solutions	amount/100 ml
a) Tris	5.98 g
TEMED	0.46 ml
1 N HCl to yield pH 6.7	
b) Acrylamide	10 g
BIS	2.5 g
c) Riboflavin	4.0 mg
d) Sucrose	40 g

Working solution - 1 part (a):2 parts (b):1 part (c):
4 parts (d)

Buffer Solution (0.04 M Tris, 0.2 M Glycine, pH 8.3)

	g/l
Tris	3.0
Glycine	14.4

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