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Lipoxygenase activity and protein solubility in extracts from soybeans treated with heat and ethanol

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**Lipoxygenase activity and protein solubility in
extracts from soybeans treated with
heat and ethanol**

by

Manoochehr Borhan

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

Major: Food Technology

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1979

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INTRODUCTION

The world need for protein is increasing. In many foods proteins perform roles besides their nutritional function. Nutritionally balanced food may be prepared, but acceptance of new food products depends on an acceptable flavor.

Soybeans are an excellent protein source from the point of view of availability and nutrition, Liener (1972). Soy products should be more attractive in the future as animal protein becomes more expensive and soy products are improved in flavor and functionality.

One of the primary limitations on food uses of soy protein is the green-beany flavor that normally is associated with soybean products. Degradation products of enzymic and nonenzymic oxidation of lipids have been blamed for most of the flavor in soy products. Lipoxygenase and unsaturated triglycerides are probably the major factors in off-flavor formation, Sessa and Rackis (1977), particularly in products such as soymilk. Some researchers do not consider lipoxygenase important in soybean off-flavor, however, inactivation of lipoxygenase improves the flavor of soy products.

Heating is probably the simplest technique for inactivating lipoxygenase and other undesirable biologically active factors in soybean.

Heating may be applied by dry heating, steaming, immersion cooking, hot grinding, and extrusion cooking.

Another approach to improving soy protein flavor is the extraction of residual lipids and their decomposed products. This may be done by ethanol washing or azeotrope-extraction of soy protein. Ethanol washing causes a considerable improvement in flavor and color of the product.

Any treatment to inactivate lipoxygenase or to remove flavor constituents causes denaturation of soy protein. It is desirable to find a method to prevent off-flavor formation, to remove inherent flavor compounds, if any, and to minimize protein denaturation.

Ethanol soaking of intact soybeans has recently been shown by Eldridge et al. (1977) to inactivate lipoxygenase in situ and to improve the flavor of the resulting product.

The major theme of the present work was to examine combined effects of temperature and ethanol on lipoxygenase activity and nitrogen solubility index (NSI) of soybeans. The effect of chemicals added to the ethanolic soaking solution on lipoxygenase activity and NSI of soybean were also tested.

Ethanol soaking may be the method of choice in soybean processing. It is hypothesized that ethanol-heat soaking of soybeans inactivates lipoxygenase before exposure to atmospheric oxygen of the disrupted soybeans. Ethanol

soaking may also be useful in elimination by extraction of some undesirable constituents present in soybeans.

LITERATURE REVIEW

Origin of Soybean Off-Flavor

The green-beany flavor of soybeans has been recognized as a major defect to their utilization as food. This flavor is not present in the intact raw whole soybean but occurs immediately after disruption of the soybean by the action of lipoxygenases in the presence of water and oxygen, Wilkens et al. (1967); Nelson et al. (1971). Wolf (1975) has emphasized that the off-flavor compounds in soy proteins originate enzymatically or by autoxidation of the lipid components. Several other workers proposed that formation of green-beany flavor is the result of enzymatic action, since the flavor appears only in the unblanched product, Eldridge et al. (1963), Kon et al. (1970), Cowan et al. (1973), Snyder (1973), Kalbrener et al. (1974), Grosch et al. (1976), Sessa and Rackis (1977), and Eldridge et al. (1977).

Lipoxygenase (E.C. 1.13.11.12), linoleate:oxygen oxidoreductase, catalyses oxidation of methylene interrupted polyunsaturated fatty acids and has been found in many vegetables, legumes and cereal grains. The greatest lipoxygenase activity is in soybeans, Tappel (1961). Presumably lipoxygenase acting on linoleic and linolenic acid in partially processed soybeans is responsible for green-beany flavor, Rackis et al. (1970).

Privett et al. (1955) provided evidence that the principal initial products of lipoxygenase catalysis are optically active, cis-trans conjugated, monomeric hydroperoxides. They confirmed the enzymatic origin, as opposed to autoxidation, of the hydroperoxides. Gardner and Weisleder (1972) confirmed the structure of the isomerized double bond in the hydroperoxides from linoleic and linolenic acids. Hydroperoxide degradation products, formed through a complex series of reactions, produce the typical predominant green-beany flavor, Kalbrener et al. (1974), Grosch et al., (1976), and Sessa and Rackis (1977).

In contrast to the concept of enzymatic origin of soybean off-flavor, Rackis et al. (1970) suggested that the beany, bitter, green and other flavor characteristics of raw, full-fat, and defatted flakes may preexist in the whole soybeans. Rackis et al. (1972) found little correlation between lipoxygenase activity and the presence of the green-beany flavor in maturing soybeans. They suggested that objectionable flavor can also be formed by autoxidation under certain processing conditions, such as wet grinding of raw full-fat soybeans.

Flavor Compounds

Teeter et al. (1955) fractionated the alcoholic extract of defatted soybean flour in an attempt to isolate beany and bitter compounds. The beany volatiles were collected and identified as ketones, carboxylic acids, esters, phenols, and some other neutral compounds. Fujimaki et al. (1965)

studied aliphatic carbonyl compounds in soybeans. In defatted soybean flour, they isolated by distillation methanal, ethanal, n-hexanal, 2-propanone, 2-pentanone, 2-heptanone, 2-heptenal and 2,4-decadienal, and from raw soybean they isolated ethanal, n-hexanal, and 2-propanone. They found four nonvolatile carbonyl compounds in defatted soybean flour. The cause of the flavor problem was attributed to autoxidation of residual fat during the defatting process and/or during storage. n-Hexanal was considered to be one of the main components of the green-beany flavor in raw soybean and to a lesser degree in the defatted soybean flour.

Arai et al. (1966) studied acidic and basic fractions of flavor components in raw soybean. The acidic fraction contained nine volatile fatty acids with sour, bitter and astringent flavors. The basic fraction exhibited a dried fishery-product odor. Arai et al. (1967) also studied volatile neutral compounds from ground soybeans. Among the alcohols that they identified, isopentanol, n-hexanol and n-heptanol were considered to be important in the green-beany flavor of soybean.

Mattick and Hand (1969) found that the green-beany flavor which develops in soybeans is attributable to a volatile component isolated and identified as ethyl vinyl ketone. This agrees with earlier results of Hill and Hammond (1965) which attribute the flavor of early autoxidation of soybean oil to mixture of pentanal and ethyl vinyl ketone.

Sessa et al. (1969) found that in both full-fat and defatted flakes, n-hexanal, acetaldehyde and acetone represented the major volatile carbonyl compounds. Lipid degradation occurs during preparation of full-fat and defatted flakes, and removal of volatile carbonyl compound from defatted flakes does not change the characteristic flavor. Therefore, they concluded, the odor and flavor associated with volatile carbonyl compounds contribute little to the over-all soybean flavor.

Badenhop and Wilkens (1969) studied the effect of soaking soybeans on the flavor of the resulting milk. They found that the compound 1-octen-3-ol was enzymatically formed during the preliminary soaking step in the processing of soymilk and that the odor of this compound is musty and earthy. The rate of 1-octen-3-ol formation reaches a maximum within 6-hr soaking at 50°C. The optimum pH for 1-octen-3-ol formation was between 6 and 7.

Wilkens and Lin (1970) identified 41 compounds from volatile components of whole-fat soymilk. Hexanal was the major volatile component contributing to the disagreeable aroma of soymilk. The majority of the identified compounds possessed undesirable flavor, and several of them could be described as beany. Wilkens and Lin (1970) suggested that the typical green-beany flavor may be due to a mixture of many compounds.

Angelo et al. (1972) found hexanal to be the principal secondary reaction product formed by the enzymatic oxidation of linoleic acid by soybean lipoxygenase. Kalbrener et al. (1974) prepared hydroperoxides of linoleic and linolenic acid using soybean lipoxygenase. A trained taste panel characterized flavors associated with the hydroperoxides. Linoleic acid hydroperoxide was described as grassy-beany, musty-stale, and bitter, and linolenic acid hydroperoxide was described as grassy-beany, bitter and astringent.

Grosch and Laskawy (1975) found that lipoxygenases-2 and -3 form significantly more carbonyl compounds than lipoxygenase-1 when incubated with linoleic or linolenic acid. Lipoxygenase-1 forms primarily 2-trans-hexenal whereas lipoxygenases-2 and -3 form primarily propanal.

Grosch et al. (1976) suggested that formation of volatile carbonyl compounds takes place very slowly and flavor compounds only arise to a very small extent directly from lipoxygenase activity. However, the formation of hydroperoxides leads to volatile carbonyl compounds through heating or through reaction with traces of heavy metals or ascorbic acid.

Bitter taste of soybean products has also been the subject of many investigations. Nash et al. (1967) extracted soybean proteins with 86% (v./v.) ethanol. Phosphatidyl choline, phosphatidyl ethanolamine,

saponins, sitosterol, glycosides, and genistein were identified from the alcohol-extractable materials. Fujimaki et al. (1968) isolated seven diffusible bitter peptides from a peptic hydrolyzate of soybean protein. These diffusible peptides were estimated to be less than 15,000 in molecular weight. Rackis et al. (1972) observed a correlation between lipoxygenase activity and bitterness in maturing soybeans. They assumed that the formation of bitter material was caused by the action of peroxidase enzyme on hydroperoxides formed by lipoxygenase. Kalbrener et al. (1974) reported that bitterness can arise from autoxidation of unsaturated fatty acids or their methyl esters. Baur et al. (1977) oxidized linoleic acid with a soybean protein fraction containing lipoxygenase and peroxidase activities. They identified 9, 12, 13-trihydroxyoctadec-trans-10-enoic acid and 9, 10, 13-trihydroxyoctadec-trans-11-enoic acid as the main bitter substances in the mixture. They suggested the necessity of at least three oxygen functions in the alkyl chain for the appearance of a bitter taste in fatty acids.

Sessa et al. (1974) claimed that bitter taste is not associated with triglyceride degradation products, but comes from oxidation of phospholipids. They isolated an intensely bitter fraction containing a modified phosphatidyl choline as the major constituent from autoxidized defatted soybean flakes. Sessa and Rackis (1977) reported that oxidized phosphatidyl

choline does not have a bitter taste and that the interaction of bitter tasting nonvolatile constituents with proteins and amino acids causes loss of the bitter effect because they are no longer available to the bitter taste site on the tongue.

Several lines of study have been conducted to recognize off-flavor factors and their origin in soybeans. Different compounds are found contributing to the soybean off-flavor. Such compounds are aldehydes, ketones, alcohols, acids, and esters. Flavor compounds may originate from enzymatic and nonenzymatic oxidation of unsaturated lipids and/or may inherently preexist in soybean. Enzymatic decomposition of fatty acid hydroperoxides follows various pathways. Major nonenzymatic decomposition of hydroperoxides occurs by complex reactions, Gardner (1975). These reactions cause formation of a wide variety of compounds. Although many of these compounds have been found to possess some flavor characteristics of soybean, none of them could be singled out as the only responsible factor for the whole spectrum of off-flavor in soybean products.

Lipids and lipoxygenase have commonly been suggested as the major source of off-flavor in soybeans. However, a comprehensive elucidation of the problem is lacking and it is the subject of controversy.

Flavor Improvement

Rackis et al. (1972) concluded, based on their belief of preexistence of objectionable flavor in immature soybeans, that development of processes for the preparation of bland soy protein product should include:

- a. Effective removal of preexisting flavor
- b. Prevention or removal, or both, of derived flavor constituents.

Similarly, Wolf (1975) suggested two general methods for correcting the flavor problem:

- a. Removal of the volatile compounds
- b. Modification of processes to prevent formation of volatile constituents.

The latter approach is most feasible for preparation of soymilk. Since generation of off-flavors results from reaction of lipoxygenase with unsaturated fatty acids, inactivation of lipoxygenase is a key step in the preparation of good flavored soy protein products, Mustakas et al. (1969).

Lipoxygenase Inactivation

Heat has been used in most of the processes to inactivate lipoxygenase and to eliminate other undesirable biological factors.

Farkas and Goldblith (1962) studied the kinetics of thermal inactivation of a purified preparation of lipoxygenase as a function of pH at 65°C.

They found a relation between the rate of inactivation of lipoxygenase and pH, demonstrated by the linear relation between the logarithm of the residual activity and time of heating. At pH 4-5, the rate of inactivation was greatest, at pH 5-7 it was lowest, and at pH 7-9 it was almost as rapid as at pH 4-5.

Albrecht et al. (1966) investigated atmospheric steaming and immersion cooking of soybeans to obtain an optimum process. The effect of moisture and particle size on NSI after 30 min atmospheric steaming was studied. No NSI value greater than 50% was obtained, and soybeans steamed at higher moisture had lower NSI values than the beans with 8% moisture during the early steaming period. Regardless of initial moisture, 2-hr steaming of all the beans gave a NSI of about 15%. Albrecht and coworkers also found that immersion cooking of soaked beans for 2 min completes destruction of urease activity and gives a NSI level of 40%. To destroy trypsin inhibitor activity, 5 min immersion cooking of soaked beans was required.

Moser et al. (1967) observed that 3-min steaming of full-fat soy flour significantly decreased the flavor. Increasing the time of steaming to 10 min decreased the beany flavors still more. However, 20-min steaming or more was no more effective than 10 min.

Wilkens et al. (1967) proposed a method of producing soybean milk free of rancid off-flavor by use of a high-temperature, rapid hydration

grinding process to inactivate lipoxygenase and retain protein extractability. They suggested that the off-flavors of soymilk were not present in the dry soybean but were formed during processing, and that grinding the beans in boiling water prevented the formation of the strong beany flavors. They attributed this result to the rapid heat-inactivation of the lipoxygenase in the soybean and concluded that the contribution of nonenzymatic fat oxidation to off-flavor under these conditions was insignificant.

Mustakas et al. (1969) evaluated various heat treatments for effectiveness of lipoxygenase inactivation. They found dry heat at 100°C, steaming, or both inactivates lipoxygenase and gives full-fat soy flour with good flavor. Direct steaming in which the temperature reached 85°C in 25 min destroys 99% of lipoxygenase. The NSI by this process was about 28%. These workers were also able to inactivate lipoxygenase in 6-8 min of direct continuous dry heating with a final temperature of 105°C.

Mustakas et al. (1970) developed extrusion cooking for production of full-fat soy flour. Temperature, moisture, and retention time affected product quality. A short cooking time in the extruder minimizes nutritional damage. They optimized the extruder conditions to give good flavor, oxidative

stability and destruction of growth inhibitors. Bookwalter et al. (1971) studied properties of extrusion-cooked soybeans. Protein quality of the product was good as evidenced by a protein efficiency ratio (PER) of about 2. The highest NSI value attained by this procedure was 50% with conditions of 15% moisture and extrusion at 135° C for the retention time of 1.25 min. Jansen et al. (1978) evaluated the nutritional value of a full-fat soy flour made by a dry-roasting process that utilized a bed of hot agitated salt. They found that roasting at 234° C for 15 sec gave a PER of 2.3. This process destroyed 75-90% of trypsin inhibitor activity.

Nelson et al. (1971) developed methods of blanching whole soybeans to inactivate lipoxigenase and prevent off-flavor development.

Treatment can be done in three ways:

1. Soaking beans for 4 hr followed by blanching at 99° C,
2. Blanching dry beans directly for 20 min in boiling tap water, and
3. Soaking and blanching in 0.5% sodium bicarbonate for food applications which require tender beans.

The proper combination of time and temperature should be chosen with respect to the soybean variety and to the ultimate use of the blanched beans.

Snyder (1973) found that only a 2-min boiling of presoaked soybeans was sufficient to eliminate objectionable green-beany flavor, and 3 min of heating dry soybeans at 100° C provided the same inhibition. These

blanching procedures, however, were not sufficient to destroy growth inhibition factors.

Pringle (1974) suggests that NSI can be used to evaluate heat processing for inactivation of antinutritive factors, for optimizing nutritive value of full fat soy flour and for removal of bitter, beany flavor. He considered a NSI of 15-20% as indicative of efficient heating.

Baker and Mustakas (1973) concluded that lipoxygenase is very sensitive to heat inactivation. They achieved total inactivation of lipoxygenase in 15 min immersion cooking at 83° C. Hutton and Foxcroft (1975) subjected the whole soybean to radiant heat for various times and temperatures. They observed that a temperature of 200-225° C was required for reducing urease and trypsin inhibitor activities. The NSI dropped to a low level of about 13% when 95 sec heating at 225° C was applied. This heating was sufficient to reduce trypsin inhibitor activity about seven fold.

Johnson and Snyder (1978) studied the time required for inhibiting lipoxygenase and off-flavor development by heating steeped soybeans at different temperatures and times and tasting for disappearance of green-beany flavor. They found 2 1/2-hr heating at 60° C eliminated the production of green-beany off-flavor in soaked soybeans. However, some hard shell soybeans were not fully hydrated, and, in such beans, the

lipoxygenase is not destroyed by 2-min heating at 100° C suggested by Snyder (1973).

Johnson and Snyder (1978) investigated possible reasons for the poor yield of solids and protein when soybeans were heated before disruption for preparation of soymilk. They found that applying no heat, or heating soybeans during the grinding process for preparation of soymilk gives about 0.9 g of soymilk solids for every gram of solids in the slurry. Whereas heating the soybeans at 100° C for 30 min before grinding gives only 0.5 g of solids in soymilk for each gram of solids in the slurry. They considered poor recovery of solids in this process to result from fixed protein bodies and their subsequent removal by centrifugation in preparation of soymilk. Their photomicrographs showed heat fixed intact protein bodies in preparations which had been heated before cell disruption. These fixed protein bodies were not observed in soymilks prepared without heating or by heating during grinding.

Several methods other than heat have been used to inactivate lipoxygenase and to eliminate the off-flavor problem of soybean protein products. Fujimaki et al. (1968) studied the effect of proteolytic enzymes on the flavor of soybean protein. Digestion products showed a decrease in beany flavor in the early stages of proteolysis.

Anderson and Warner (1976) noticed that removal of an acid sensitive fraction from soy protein significantly improved flavor scores;

the most noticeable change was a decrease in the intensity of grassy-beany flavor. The acid-sensitive fraction binds the grassy-beany, bitter, astringent soy flavor components.

Kon et al. (1970) studied the effect of pH adjustment on lipoxygenase-induced off-flavor. Grinding of soybeans at pH values below 3.83 controlled the oxidized off-flavor of raw soybeans. A similar experiment was conducted by Baker and Mustakas (1973). They found that the addition of acid or base to the cooking water accelerated the initial inactivation of urease and lipoxygenase. The addition of 1% NaOH or HCl to the cooking water caused about 50% inactivation of lipoxygenase activity in 15 min at 49° C. Cooking at 49° C for 60 min with caustic solution was more effective in reducing lipoxygenase than acidic cooking which gave residual activity of 4%. Furthermore, they showed that lipoxygenase is readily destroyed by 1% alkaline solutions of NH_4OH , NaHCO_3 , and NaOH after 15 min at 74° C.

Badenhop and Hackler (1970) studied the effect of soaking soybeans in NaOH solution on various aspects of soymilk quality. They observed that as the pH of the soymilk increased from 6.55 (water soak) to 9.18 (soaking in 0.097 N NaOH) the PER decreased from 2.41 to 1.7. Taste panels preferred the flavor of soymilk of pH 7.37 (soybeans soaked in 0.048 N NaOH) over samples with higher or lower pH. Addition of sodium

hydroxide to the soak water increases the rate of hydration of the beans.

Badenhop and Hackler concluded that soaking soybeans in a sodium hydroxide solution of 0.05 N is a desirable pretreatment to a high temperature grinding process for preparation of soymilk.

Khaleque et al. (1970) demonstrated that soaking soybeans in dilute solutions of sodium carbonate and sodium hydroxide, as a pretreatment in the production of soymilk, markedly reduced the beany flavor when compared with milk prepared by water soaking. They concluded that the lipoxygenase contribution to the beany flavor is relatively small and that there are more significant factors than lipoxygenase activity that are responsible for beany flavor. However, Bourne et al. (1976) explained the observation of Khaleque and coworkers as due to insufficient temperature to inactivate the lipoxygenase. Bourne and coworkers studied the effect of alkali addition in flavoring bland-flavored soymilk made by the boiling water grinding process. They hypothesized that the sodium ion concentration rather than pH is the key factor in the flavor of soymilk.

Although some workers do not think lipoxygenase is important for flavor formation in soybeans, inactivation of lipoxygenase improves soybean flavor.

Heating is the simplest means to inactivate lipoxygenase and other biologically active factors. However, disruption of the soybean prior

to heating causes lipoxygenase catalyzed lipid oxidation especially in the presence of moisture. Dry heating of the intact beans is not favorable because the high temperature required to heat up the inside of the bean causes overheating of outer sections of the cotyledon. Steaming and immersion cooking, on the other hand, are usually done at elevated temperatures which entail poor control over protein denaturation. Heating hydrated soybean at intermediate temperatures causes some undesirable chemical and bacteriological changes in the bean. Extrusion cooking is an efficient heating technique which provides NSI values as high as 50% and improves the nutritional quality of the product.

Functional Properties

High temperatures and moisture can cause denaturation and insolubilization of the functional proteins of soybeans.

Kinsella (1976) considered functional properties as most important in determining the uses of protein for the development of new food products by industry. Many of the important functional properties of protein relate to water-protein interaction, i.e. solubility, viscosity, gelation, foaming, and emulsification. Correlations have been obtained between the functional properties of soy flour and NSI in various applications, Johnson (1970). NSI is being used increasingly as a guide to protein functionality. Good solubility

can markedly expand potential applications of proteins, and solubility is perhaps the most practical index of the extent of denaturation.

Denaturation may be defined in general terms as irreversible modification of secondary, tertiary or quaternary structure of the protein molecule that does not break covalent bonds. Therefore a change in protein structure is usually associated with changes in physical-chemical and functional properties, Wu and Inglett (1974).

Sosulski and Garratt (1976) evaluated functional properties of ten legume flours. They tested for nitrogen solubility, water and fat absorption, emulsification, whippability, viscosity, and gelation. Unheated soy flour with 85% NSI value showed water absorption of 214%, 138% fat absorption and 82% fat emulsification capacities, highest among all the legume flours tested.

Alcohol Treatment

Various alcohols are useful for treating soybean products to denature soy proteins and to extract flavor compounds such as residual lipids and their oxidized products. Beckel et al. (1948) studied the effects of solvents on soybean oil extraction and on properties of the oil, meal, and protein. They suggested that ethanol extraction improved the color of oil, meal, and protein and functioned as a debittering agent for the soybean meal.

Beckel et al. (1949) developed a new soybean product, Gelsoy, that had a bland taste, whipping, and gelling properties. Gelsoy was a water-soluble product made from soybean flakes which had been washed with ethanol.

Mustakas et al. (1961) investigated the conditions for removing the beany and bitter flavor from defatted soybean meal with ethanol. Effective debittering was obtained with 95% ethanol, and in the presence of alcohols, soybean protein sensitivity to denaturation increased when temperature, moisture, and exposure time increased. Mann and Briggs (1950) studied electrophoretically the effect of solvent and heat treatment on soybean proteins and found that ethanol extraction of the meal reduced the water extractability of all the proteins, but the effect was most pronounced on the globulins. They suggested that heat is more important in reducing protein solubility of soybean meal than alcohol, and the effect of heat is dependent upon the amount of water present. An analogous explanation for alcohol denaturation of the soybean protein was used by Smith et al. (1951). However, they found that water is less effective as a denaturant than the pure organic solvent, and ethanol-water solutions at 40-60% concentrations are the most effective denaturants. These workers also studied the effect of time and temperature on denaturation at various alcohol concentrations and demonstrated that denaturation proceeds

very rapidly at room or higher temperatures for all alcohol concentrations between 10 and 90%.

Roberts and Briggs (1963) studied characteristics of the soybean globulins in response to denaturation by ethanol. They reported 80% denaturation of soybean globulins by 50% ethanol in 15 min and the 7 S component is very rapidly denatured when the wet curd is brought into contact with ethanol-water mixture of 20% or greater concentrations of ethanol. However, the rates of denaturation of the 11 S and 15 S components are slow and the 2 S is not denatured at all.

Eldridge et al. (1963) studied alcohol washing of soybean protein to remove a phospholipid-like material. The washed protein had improved color and flavor, produced stable low density foam and whipped similar to egg white. They indicated that increased foam stability is the result of removing an alcohol-soluble foam inhibitor. Wolf et al. (1963) studied the effect of alcohol on the physical properties of soybean proteins. By means of optical rotation, viscosity, and ultracentrifugal measurements, they indicated that the proteins which retain their solubility are undenatured, i.e. loss of protein solubility is caused by denaturation rather than by removal of phospholipids by alcohols. Removal of phospholipids did decrease foam inhibition.

Fukushima (1969) suggested that denaturation of soybean protein by organic solvents should be of interest for some aspects of soybean utilization because the soybean protein molecules contain some hydrophobic regions which are not expected to be altered by water. He studied the denaturation ability of about 30 kinds of organic solvents on soybean proteins and found that the denaturing ability of organic solvents depends on their hydrophobicities and the degree of dilution by water. The water-solvent mixture has a denaturing ability that cannot be attained by the individual components.

Wang (1969) showed a considerable decrease in the amount of the major nucleotides in mature-soybean meal when it was washed with 80% ethanol. Alcohol washing or autoclaving apparently disrupts pigment — nucleotide complexes, which permits a more complete extraction of nucleotides from meals.

Eldridge et al. (1971) evaluated the effect of hexane-ethanol azeotrope-extraction on the flavor of soybean flakes and isolates and on protein solubility of the flakes. A 2% dispersion of sodium proteinates from the azeotrope-extracted flakes possessed no beany flavor. Defatted flakes from hexane:ethanol (80:18 v/v) azeotropic-extraction gave a NSI value of about 70%. This value decreased considerably to about 25-30% when hexane:methanol (75:25 v/v) was used for extraction. They concluded

that hexane:ethanol (80:18 v/v) azeotropic extraction does not markedly denature the proteins.

Maga and Johnson (1972) observed a higher efficiency of a polar solvent (hexane:ethanol) extraction in removing residual lipid material than for a nonpolar solvent (petroleum ether). Products extracted by the polar-solvent procedure were less beany and bitter than those extracted with nonpolar solvent. Steinkraus (1973) believed that residual bound fat is responsible for much of the off-flavor or beaniness and that alcohol treatment results in a loosening of the bond between bound fat, principally phospholipids, and soybean protein. Subsequently, chloroform can be used to extract undesirable flavor-bearing lipids. He prepared organoleptically bland soybean meal by extracting ground, untreated, unprocessed soybean with 95% ethanol for 2 hr at 60–65°C followed by 1:1 v/v chloroform:ethanol extraction for 22 hr. The meal was completely defatted and debittered, and free of undesirable mouth-coating factor. Rackis et al. (1975) found that a combination of hexane-ethanol extraction and steaming improves the flavor and nutritive value of defatted soy flakes however, NSI decreased extensively to the level of 31%. This process destroyed over 99% of the lipoxygenase activity, but the azeotrope-extraction alone did not inactivate trypsin inhibitors. They suggested that proper heat treatment is needed

for maximum use of the essential nutrients in soy protein, while disagreeable flavor must be eliminated without destruction of functional and nutritional qualities of the protein.

Eldridge et al. (1977) found soaking soybeans in ethanol at room temperature decreases the grassy, beany, and bitter flavor characteristics of treated soybean products. The improvement in flavor seemed to parallel the inactivation of lipoxygenase. They provided more support to the idea that the flavor compounds in soy products were generated by lipoxygenase action when the seed structure was disrupted. Ethanol soaking of intact soybeans inactivates lipoxygenase in situ. They also assumed that volatile flavor compounds, if present in the intact beans, may have been lost during removal of alcohols by evaporation. The highest flavor scores were obtained by soaking soybeans in a 40–60% v/v concentration of aqueous ethyl alcohol; nitrogen solubility and lipoxygenase activity were reduced extensively.

Ethanol treatment of soy proteins has been practiced by either washing soy proteins by concentrated ethanol or an azeotrope-extraction of lipids. Ethanol treatment causes denaturation of soy proteins, especially in the presence of water. These methods do not prevent early flavor formation by lipoxygenase. The lipoxygenase remains active during seed disruption. Flavor compounds can be formed by polyunsaturated

triglycerides and lipoxygenase after exposure to atmospheric oxygen. These flavor compounds may become adsorbed onto soy protein and be nonremovable by ethanol washing.

Ethanol soaking of intact soybean serves to meet two purposes: to inactivate lipoxygenase and to remove naturally occurring flavor constituents in the bean.

MATERIALS AND METHODS

Materials

Reagent grade chemicals were used in all experiments. Petroleum ether (Skelly Solve-B with boiling point of 60-70°C) was used for extraction of full-fat soy meal. Various concentrations of ethanol for soaking solutions were dilutions of 95% ethanol.

Deionized distilled water, obtained from a commercial still, passed through a Barnstead standard mixed bed ion exchange resin and stored in a polyethylene tank, was used in all experiments.

Seed grade soybeans of Amsoy 71 variety were stored at 4°C until used.

Linoleic acid (99% pure), purchased from Sigma Chemical Company, was used to prepare the substrate for lipoxygenase assay by mixing 140 mg of linoleic acid with 5 ml 0.1 M sodium hydroxide solution to give a stock solution of 0.1 M sodium linoleate. The linoleic acid was added to the reaction mixture with a microliter syringe to give a final substrate concentration of 3.5×10^{-4} M. Emulsifiers, such as Tween 20, were not used in those preparations. The substrate stock solution was stored frozen under nitrogen in the dark.

Methods

Sample preparations

Treatment of soybeans Soybeans were soaked in various concentrations of ethanol at a 1:4, bean:ethanol ratio for periods of time at desired temperatures. After soaking, soybeans were drained and rinsed with tap water followed by an additional rinse with distilled water. Spreading soybeans on paper towel removed exterior water. Then the soybeans were transferred into paper cups and frozen at -29°C overnight. Frozen soybeans were dried in a VirTis cabinet model freeze-drier for 36–48 hr to a moisture level of about 5%.

Fat extraction Forty g of freeze-dried soybeans were ground in a Wiley mill to pass a 20-mesh screen. Resulting full-fat meals were defatted by magnetically stirring in 100 ml of Skelly Solve-B for 30 min and allowing the slurry to stand for 10 min followed by decanting the top layer of solvent. Extraction was repeated by an additional 70 ml solvent and stirring for 15 min. The slurries were filtered through Whatman No. 1 filter papers and washed twice with 50 ml of solvent. Defatted soy meals were allowed to dry at room temperature.

Lipoxygenase preparations

Crude soybean extract (CSE) was prepared by two different meal: water proportions.

A 1:10, meal:water extraction was prepared by putting 2 g of 20-mesh defatted soy meal (DSM) into 20 ml of deionized distilled water. The slurry was stirred on a magnetic stirrer for 30 min at room temperature, and then was passed through four layers of cheese cloth to remove large particles. The filtrate was centrifuged for 15 min at 12,000 x g and the supernatant, CSE, was used as the source of lipoxygenase.

A 1:50, meal:water extraction was used as the major preparation for lipoxygenase activity assay and for determination of nitrogen solubility index (NSI) throughout the study. Defatted soy flour (120 mesh - 500 mg) was put into a 50 ml baker and 25 ml of distilled water was added. The slurry was stirred magnetically for 2 hr at room temperature. The slurry was then passed through four layers of cheese cloth into a 50-ml centrifuge tube and centrifuged at 1086 x g for 15 min. The supernatant, CSE, was used for lipoxygenase assay.

Lipoxygenase assay

An oxygen uptake method, Zimmerman and Snyder (1974), was employed to measure lipoxygenase activity of preparations by using a Yellow Spring Instrument Co. (YSI) Model 53 oxygen monitor attached to a Sargent Welch SRG recorder. The YSI instrument has a Clark-type oxygen sensor isolated from the reaction environment by a thin, gas-permeable, Teflon membrane. The reaction mixture was kept out of

contact with air by a tight fitting plunger which occupied all the space in the reaction chamber above the reaction mixture. The reaction chamber was in a temperature controlled bath. The reaction mixture was stirred magnetically, and the concentration of oxygen in the buffer solution was assumed to be the same as that of air saturated water at the same temperature ($240 \mu\text{M}$ at 25°C). This concentration corresponded to 100% of saturation on the recorder. Following the addition of buffer and sodium linoleate into the reaction chamber and reaching a constant oxygen concentration, appropriate volumes of CSE ($10\text{--}100 \mu\text{l}$) depending on lipoxygenase activity, were injected into the reaction mixture. The rate of oxygen consumption was calculated as micromoles per min per ml of CSE.

Nitrogen Solubility Index (NSI)

NSI is a measure of the soluble nitrogen in soybean products. It is determined by the ratio of water soluble nitrogen to the total nitrogen multiplied by 100.

$$\text{NSI} = \frac{\text{water soluble nitrogen}}{\text{total nitrogen}} \times 100$$

A modified procedure of AACC method No. 46-23, revised (1969), was employed for extraction of soluble nitrogen from defatted soy meal of treated soybeans, as explained for lipoxygenase preparation, (1:50 CSE).

Protein determination

Five ml of CSE were pipeted into each 100-ml Kjeldahl flask and were heated to dryness in a 100°C convection oven. A modified micro-Kjeldahl method (38.012 A.O.A.C., 1970) was employed to determine the protein content of DSM and CSE. The conversion factor of 6.25 was used to calculate the percentage of protein. Samples were digested with 2 ml of concentrated sulfuric acid on a Lab. Con. Co. digestion apparatus and using 0.2 g of copper selenite as the catalyst along with 0.3 g of potassium sulfate to raise the boiling point of the acid. Gentle heating was applied for 45-60 min. Washing the flask neck with concentrate sulfuric acid was done after the first 30 min of digestion. After cooling the digested samples to room temperature, samples were diluted by addition of about 10 ml deionized, distilled water. The mixture was transferred to a Lab. Con. Co. distillation apparatus and a solution of 40% (w/v) sodium hydroxide was added to release the ammonia. The ammonia was trapped in 10 ml of 4% boric acid solution by 3 min distillation and titrated with 0.1 N HCl by using Tashiro's indicator. Tashiro's indicator was prepared by dissolving 0.25 g methylene blue and 0.375 g methyl red in 300 ml of 95% ethanol.

Soluble solids determination

Total soluble constituents of soybeans leached into 200 ml of soaking solutions during 24 hr were determined gravimetrically by evaporating on a steam bath to reduce the volume to about 50 ml. The resultant syrup-like solution was then transferred into aluminum drying pan and freeze dried. The freeze dried sample was put in an 80° C vacuum oven for 2 hr and cooled in a desiccator before weighing.

Determination of trypsin inhibitor

We used the procedure of Kakade et al. (1974) for determination of trypsin inhibitor activity. A 1:50 extraction of DSM in 0.01 N NaOH at pH 9 was prepared at room temperature by stirring for one hr. The suspension was diluted to 1:10,000 with distilled water. One ml of diluted suspension was used for trypsin inhibitor determination.

Sugar determination

The colorimetric method of Dubois et al. (1956) for determination of sugars was used to measure the sugar content of ethanolic soaking solutions. In this method, an orange-yellow color was developed when sugars were treated with phenol and concentrated sulfuric acid. The amount of sugar was determined by reference to a sucrose standard curve under the experimental conditions.

Phosphorus determination

The method of Morrison (1964) was used for determination of phosphorus content of soluble constituents of soaking solutions. Principal steps of the procedure are wet oxidation of organic matter, liberating phosphoric acid, formation of phosphomolybdic acid complex which is quantitatively reduced to give a blue color, and measurement of the absorbance by spectrophotometry. The amount of phosphorus was calculated by using a phosphorus standard curve constructed under experimental conditions.

Determination of peroxidase activity

A modified procedure of Vetter et al. (1956), used by Rackis et al. (1972), was used to assay peroxidase activity of defatted soy flour preparations. The method involves the colorimetric determination of the color formed when the enzyme oxidizes orthophenylenediamine in the presence of hydrogen peroxide. Extractions of 1:50, DSM:phosphate-citrate buffer pH 6.5, were prepared by stirring the mixtures for 2 hr. The extracts were diluted to 1:2,500 with the same buffer, and 1 ml of each diluted extract was used for determination of peroxidase activity. Peroxidase activity in DSM is calculated as absorbance unit per g protein.

Organoleptic test

Presence or absence of beany flavor was judged by four people who had already experienced typical green-beany flavor of raw soybeans.

The determinations in this study were done in duplicate and reported values are averages.

RESULTS AND DISCUSSION

Effect of Heat on Crude Preparations of Lipoxygenase

Christopher et al. (1970) differentiated heat stabilities of purified lipoxygenase isoenzymes 1 and 2. They found a survival half-time of 25 min at 69° C for lipoxygenase-1, while lipoxygenase-2 was much less stable, having a half-time of 0.7 min under the same conditions. The heat stabilities of lipoxygenases in unpurified systems such as our CSE preparations or in their natural location in the intact soybean are also of practical interest. Fig. 1 shows heat inactivation of lipoxygenase in 1:10 CSE after heating at 69° C for various times. I found a half-time of 15 min for lipoxygenase-1 in CSE. The calculation for lipoxygenase-2 half-time was done by subtraction of lipoxygenase-1 activity at pH 6.8 from the total lipoxygenase activity at pH 6.8. The parallel sections of the curves of inactivation were considered to be activity of lipoxygenase-1 at pH 6.8 and 9.0, because lipoxygenase-1 is active at both pHs. The calculated half-time value for lipoxygenase-2 was 0.8 min in CSE at 69° C. This experiment indicates a similar stability to heat of lipoxygenases 1 and 2 in CSE as the purified lipoxygenases reported by Christopher et al. (1970). I found in my previous work (Borhan, 1974) that crude preparations of lipoxygenase are more stable than purified preparations to physical treatments such as shaking and dilution.

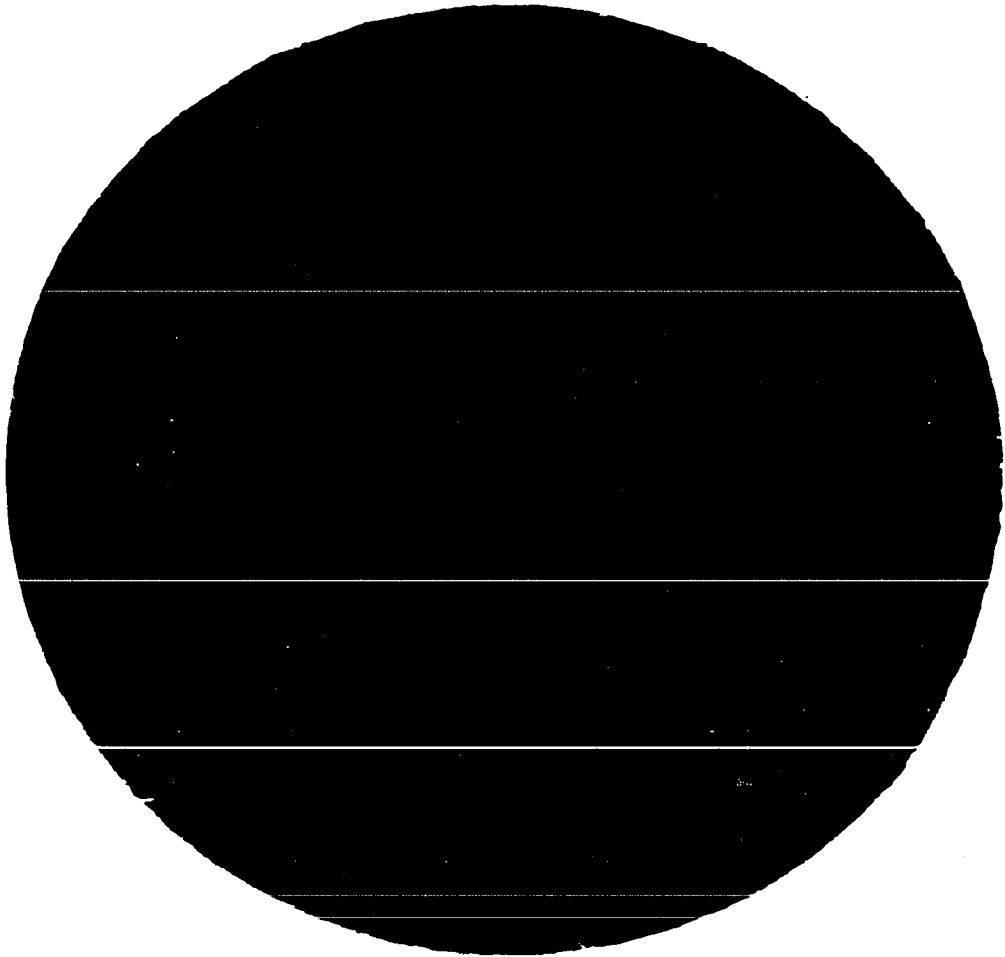


Figure 1. Inactivation of 1:10 CSE lipoxygenases by heating at 69°.

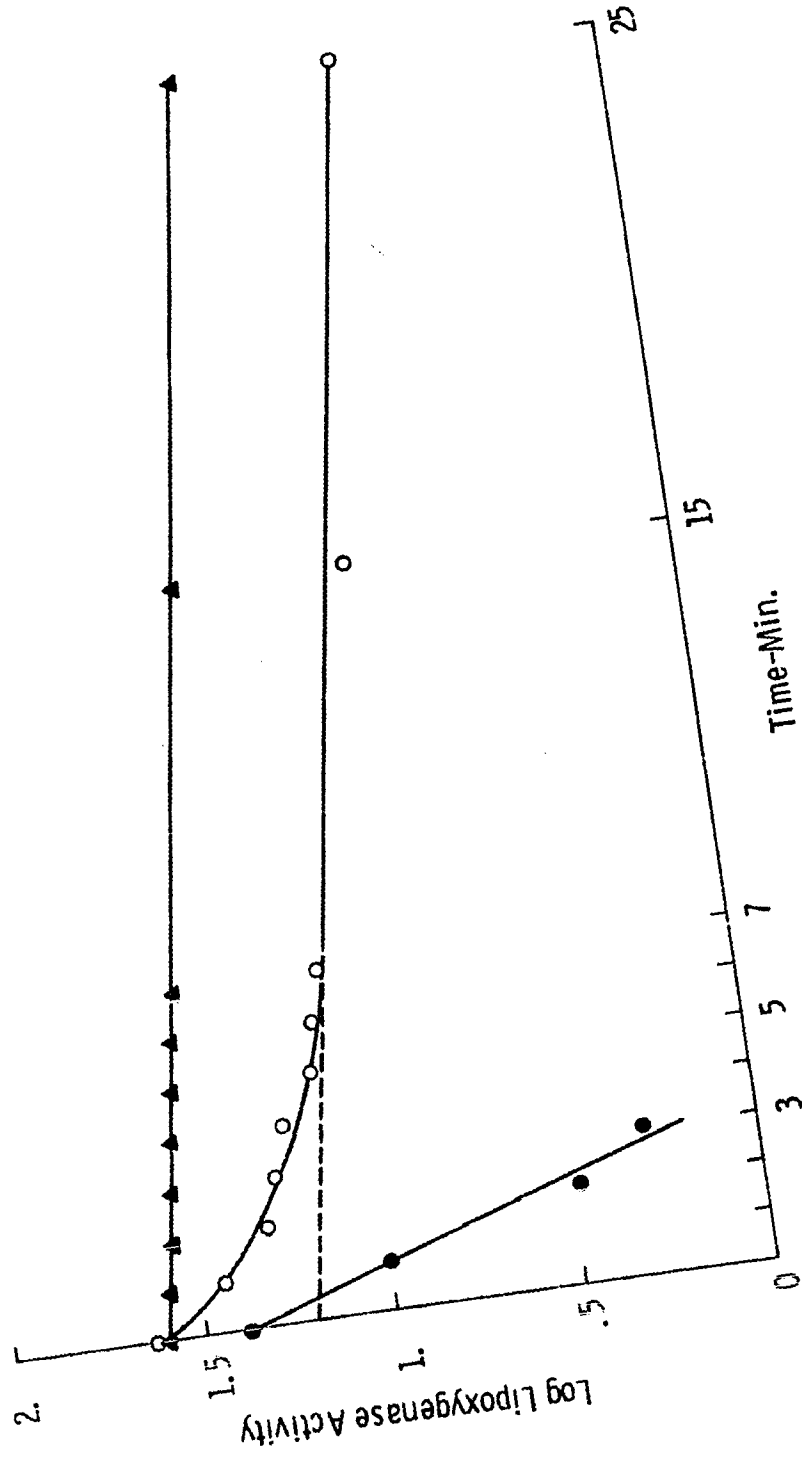
▲—▲ Assayed at pH 9, lipoxygenase-1

○—○ Assayed at pH 6.8, lipoxygenases 1, 2

●—● Lipoxygenase-2

The reaction was in 0.2 M Tris buffer with 0.35 mM sodium linoleate 25°C. Lipoxygenase activity was expressed in μ mol O₂ min⁻¹ ml⁻¹ CSE.

- pH 6.8
- ▲ pH 9.0
- Lipoxygenase 2



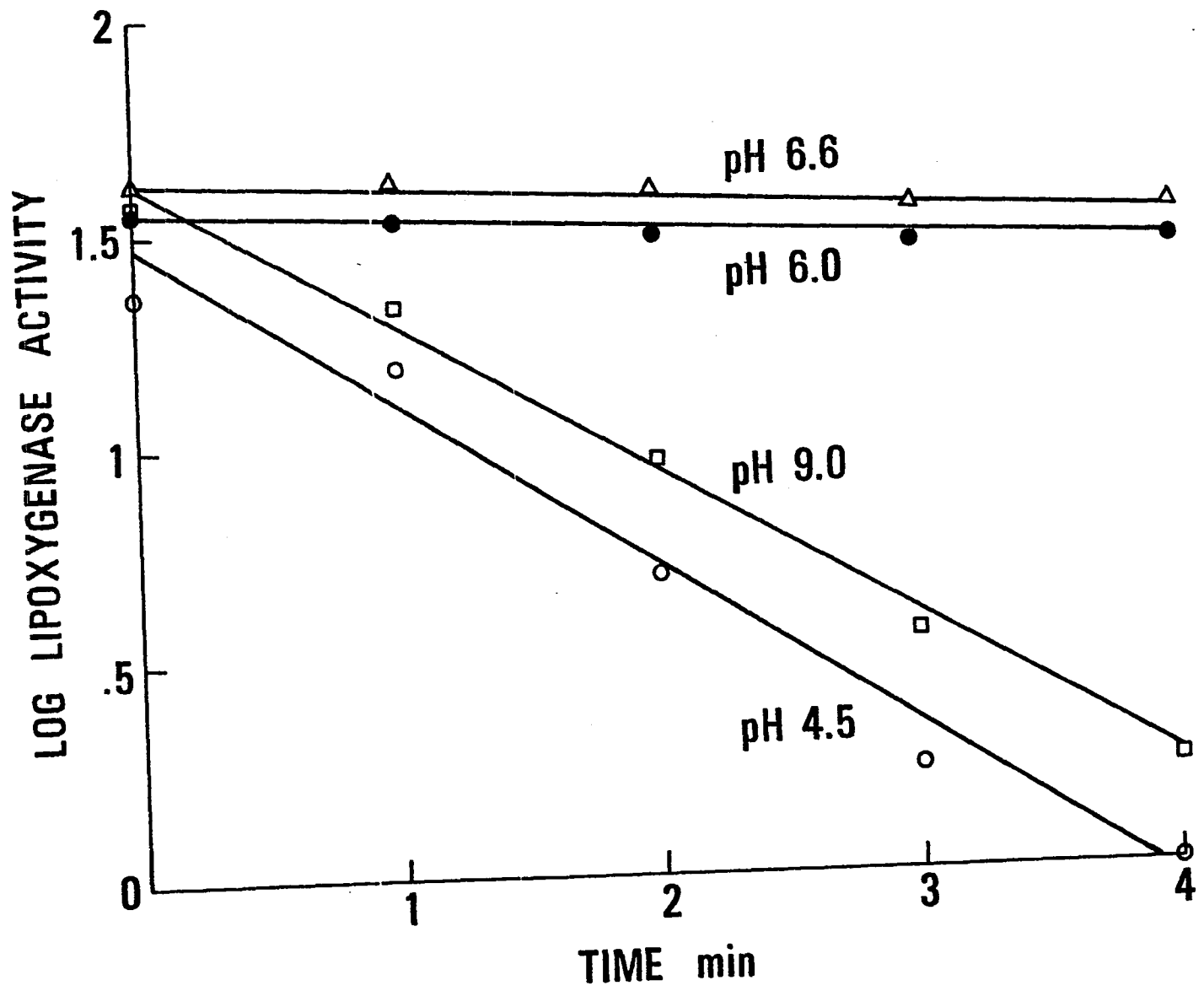
Effect of pH on Heat Inactivation
of CSE Lipoxygenase-1

Thermal inactivation of CSE lipoxygenase-1 as a function of pH was studied by extracting and heating CSE at various pH values. Heating was done at 69° C, and lipoxygenase activity was assayed at pH 9.0, corresponding to the optimum pH of lipoxygenase-1. Slopes of heat inactivation of CSE lipoxygenase-1, shown in Fig. 2, indicate the existence of three pH regions. Heating at pH 4.5 and pH 9.0 caused a rapid inactivation of lipoxygenase, whereas heating CSE at pH 6.0 and 6.6 decreased lipoxygenase activity at a much slower rate. This result confirms the observation of Farkas and Goldblith (1962) concerning heat inactivation of purified preparation of lipoxygenase as a function of pH. Three pH regions, they reported, were between pH 4-5, with a sharp inactivation by heating, and between 5-7 with a rather constant, higher residual activity, and between pH 7-9 with a relatively quick loss in residual activity.

I observed that the half-time of 15 min for lipoxygenase-1 at pH 6.6 heating at 69° C decreased to about 0.9 and 1 min for preparations extracted and heated at pH 4.5 and 9.0, respectively.

Figure 2. Heat inactivation of lipoxygenase-1 in CSE at various pH's of extraction followed by heating at 69° C.

Lipoxygenase activity was assayed at pH 9.0 and expressed in $\mu \text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1} \text{ CSE}$.



Effect of Ethanol on Lipoxygenase

Table 1 shows the effect of different concentrations of ethanol on lipoxygenase activity of soybeans soaked for 24 hr at 4°C. This experiment was to examine the effect of ethanol on deactivation of soybean lipoxygenase with no heating applied. The green-beany flavor existed along with lipoxygenase activity, and complete destruction of enzyme was not achieved by any concentration of ethanol tested. The range of the most effective concentration was 30–50% ethanol. Lack of lipoxygenase

Table 1. Inactivation of soybean lipoxygenase due to soaking in various concentrations of ethanol for 24 hr at 4°C

Ethanol Concentration % v/v	Soybean Weight Increase	Beany Flavor	Lipoxygenase Activity $\mu \text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$	
			pH 6.8	pH 9.0
0	110%	+	75	56
5	104%	+	53	47
10	90%	+	25	33
30	68%	+	2	3
50	49%	+	5	8
80	8%	+	48	45

inactivation by 80% ethanol is due to poor diffusibility of ethanol into the intact beans as is evident from the small weight increase. As ethanol concentration increased, solvent uptake by beans decreased.

Eldridge et al. (1977) also observed that lipoxygenase was greatly inactivated by intermediate concentrations of ethyl alcohol. Soaking at 25° C gave better organoleptic responses than -4° C. This can be ascribed to the presence of residual lipoxygenase activity in soybeans soaked at -4° C.

Furthermore, I found that the inactivation of lipoxygenase by ethanol treatment at 4° C is reversible upon removal of ethanol. Table 2 shows that removal of ethanol by an additional 24 hr soaking in water reactivates lipoxygenase. The reactivation was not complete, particularly in soybeans which were soaked in 30% and 50% ethanol. This incompleteness of reversibility is because of the presence in CSE of lipoxygenase-2, which is more sensitive to denaturation than lipoxygenase-1 (data in Table 2 at pH 6.8). Mitsuda et al. (1967) found that denaturation of purified crystalline lipoxygenase (lipoxygenase-1), Theorell et al. (1947) preparation, by alcohols is reversible, and the reversibility of the inhibition was almost complete. Hence, the irreversible inhibition of lipoxygenase by ethanol is probably due to lipoxygenase-2.

Table 2. Reactivation of soybean lipoxygenase due to resoaking soybeans in water for 24 hr at 4°C

1st 24 hr in ethanol % v/v	Beany Flavor	Lipoxygenase Activity $\mu\text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$	
		pH 6.8	pH 9.0
0	+	70	56
5	+	59	53
10	+	58	51
30	+	32	46
50	+	20	29
80	+	50	52

Irreversible Inactivation of Lipoxygenase When

Heat and Ethanol Treatment are Combined

Table 3 shows the results of combining heat and ethanol on lipoxygenase activity and beany flavor. Soaking soybeans in 50% ethanol at 45°C for 24 hr caused irreversible inactivation of the enzyme. Further soaking in water did not reactivate lipoxygenase, and no beany flavor was detected in the samples.

Table 3. Irreversible inactivation of lipoxygenase when heat and ethanol treatment are combined

1st 24 hr soak in ethanol % v/v 45° C	2nd 24 hr soak in	3rd 24 hr soak in	Beany Flavor	Lipoxygenase Activity pH 9.0
50			—	0.0
50	water		—	0.0
50	water	water	—	0.0

Effect of Presoaking on Inactivation of

Lipoxygenase by Concentrated Ethanol

Previously, I observed that concentrated ethanol failed to inactivate lipoxygenase in ethanol-soaked soybeans, (80% ethanol in Table 1), and even warm (45° C) concentrated ethanol failed to diffuse into the bean and to inactivate lipoxygenase (Table 4). Presoaking soybeans in water prior to ethanol soaking facilitates diffusion of 95% ethanol into the bean and causes irreversible deactivation of lipoxygenase in 24 hr at 4° C (Table 5). Removal of ethanol by resoaking soybeans in water did not reactivate lipoxygenase in 24 hr but measurable reactivation was present after 48 hr.

Table 4. Lack of lipoxygenase inactivation by soaking soybeans in concentrated ethanol at 45°C

1st 24 hr soak in	2nd 24 hr soak in	Beany Flavor	Lipoxygenase Activity $\mu \text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$	
			pH 6.8	pH 9.0
95% Ethanol		+	33	27
95% Ethanol	water	+	32	29

Table 5. Inactivation of soybean lipoxygenase by concentrated ethanol at 4°C due to presoaking in water

1st 24 hr soak in	2nd 24 hr soak in	3rd 24 hr soak in	4th 24 hr soak in	Lipoxygenase Activity pH 9.0
water	95% ethanol			0.0
water	95% ethanol	water		0.0
water	95% ethanol	water	water	0.7

Combined Effect of Heating and Ethanol

The effects on green-beany flavor and on lipoxygenase activity were studied by following combined treatment of soybeans with low to intermediate concentrations of ethanol and mild heating of soaking solutions at 20, 30, and 40° C. Results in Fig. 3 indicate that as temperature decreases greater ethanol concentration is required to destroy lipoxygenase. A direct relationship exists between lipoxygenase activity and presence of beany flavor. When lipoxygenase activity is destroyed beany flavor disappears. This relationship supports the idea of lipoxygenase causing green-beany flavor, as claimed by: Wilkens et al. (1967), Mattick and Hand (1969), Kalbrener et al. (1974), Wolf (1975), Grosch et al. (1976), and Eldridge et al. (1977).

In the same experiment, protein solubility in the 1:10 CSE was measured. Fig. 4 shows the higher the temperature and the ethanol concentration, within the range of experimental conditions, the lower the solubility of protein in the water extract. The relationship is not linear.

To find optimum conditions of temperature and ethanol concentration for total destruction of lipoxygenase and for maximum protein solubility, the previous experiment was extended to a wider range of temperatures and ethanol concentrations. The 1:50 CSE preparation was used as the

Figure 3. Effect of a combination of various ethanol concentrations and temperatures on lipoxygenase activity (a) and flavor (b) of soybeans soaked for 24 hr.

Lipoxygenase activity was assayed at pH 9.0 and expressed in $\mu \text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1} \text{ CSE}$.

LIPOXYGENASE ACTIVITY

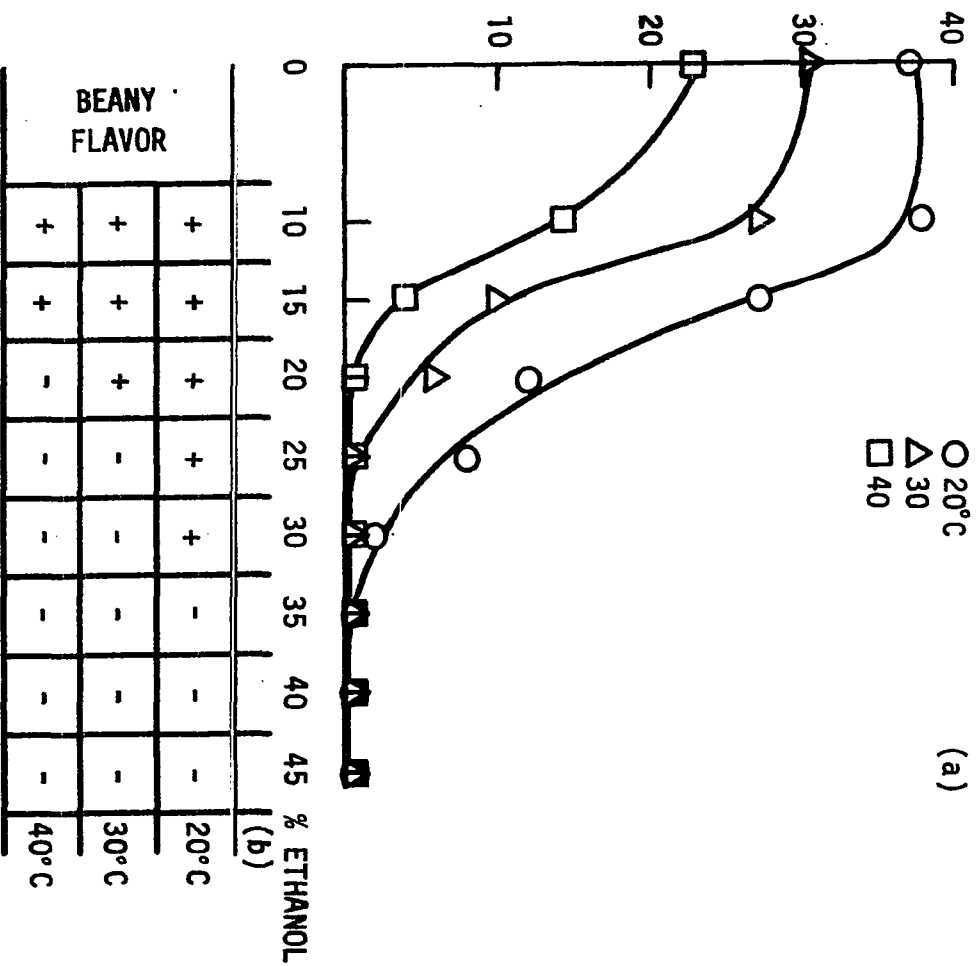
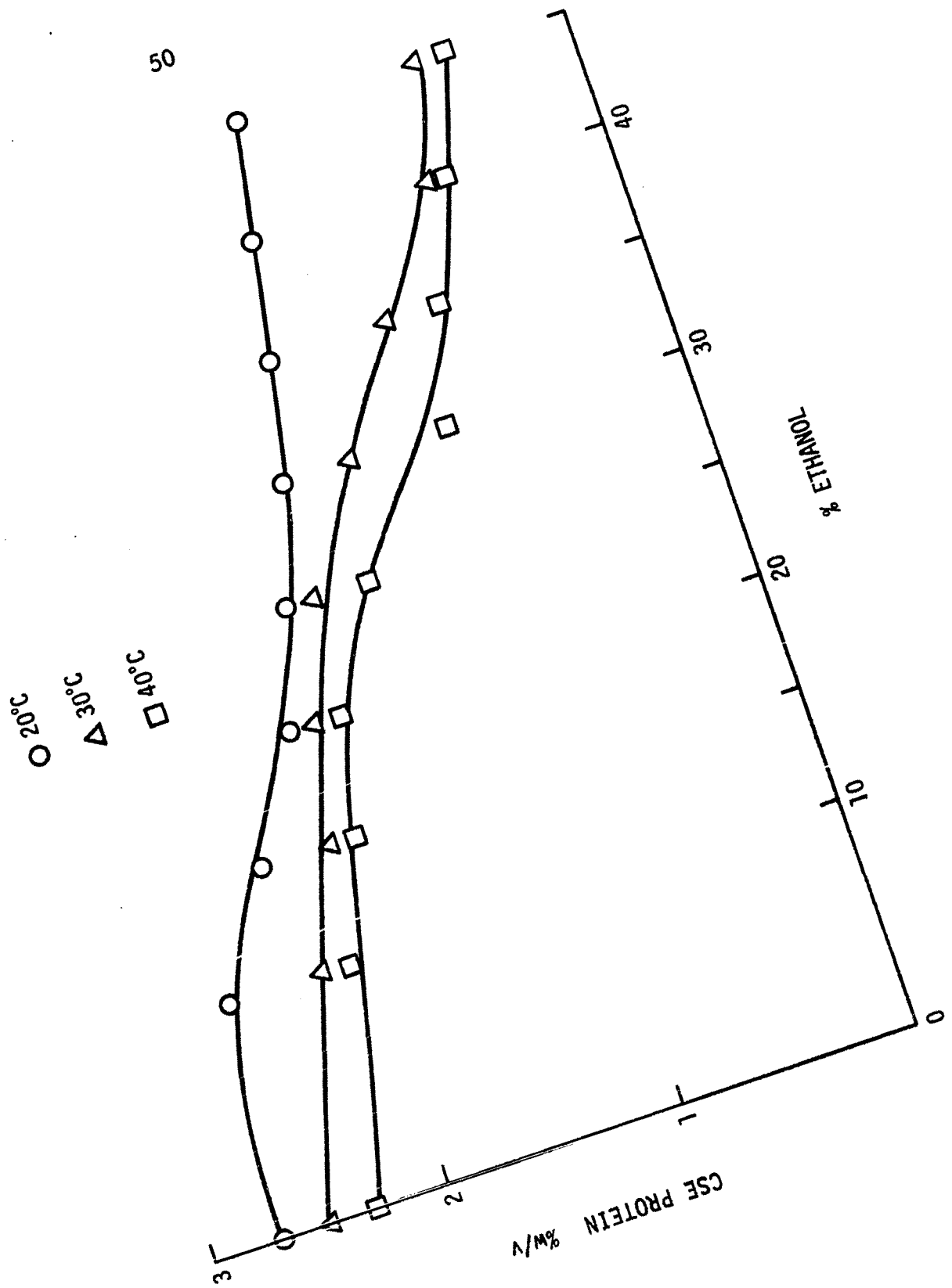


Figure 4. Protein content of 1:10, DSM:water, extract as related to temperature and ethanol concentration of soaking solution used to soak soybeans for 24 hr.



lipoxygenase source and inactivation of lipoxygenase was considered complete when 99% or more of the original activity present in the raw soybean was eliminated.

Fig. 5 shows the lipoxygenase activities of defatted soy meals of heat-ethanol treated beans. The lipoxygenase activities of soybeans treated with 0% ethanol indicate that as temperature of soaking increases the need for an additive effect of ethanol tends to be diminished. However, total inactivation of lipoxygenase was not achieved without the effect of ethanol within the range of temperatures used in this experiment. Soaking soybeans in water at 60° C for 24 hr destroyed most of the lipoxygenase activity, but very minute residual activity is sufficient to cause off-flavor of the resulting product over a long time, Wolf (1975). Johnson and Snyder (1978) obtained an immediate flavor improvement following 2 1/2 hr soaking of soybeans at 60° C, but the incomplete destruction of lipoxygenase may not be detectable by tasting immediately after treatment. Combining heating with ethanol treatment facilitates inactivation of lipoxygenase. Moser et al. (1967) found a higher flavor score for full-fat flour from combined treatment by ethanol and steaming for 20 min compared to the flavor scores of soy flour treated by ethanol or steaming alone.

The NSIs of defatted soy meals from heat-ethanol treated soybeans are shown in Fig. 6. Protein denaturation is considerable in those soybeans

Figure 5. Inactivation of lipoxygenase due to 24 hr soaking of soybeans at various temperatures and ethanol concentrations.

Lipoxygenase activity was assayed at pH 9.0 and expressed in $\mu\text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$ CSE.

LIPOXYGENASE ACTIVITY

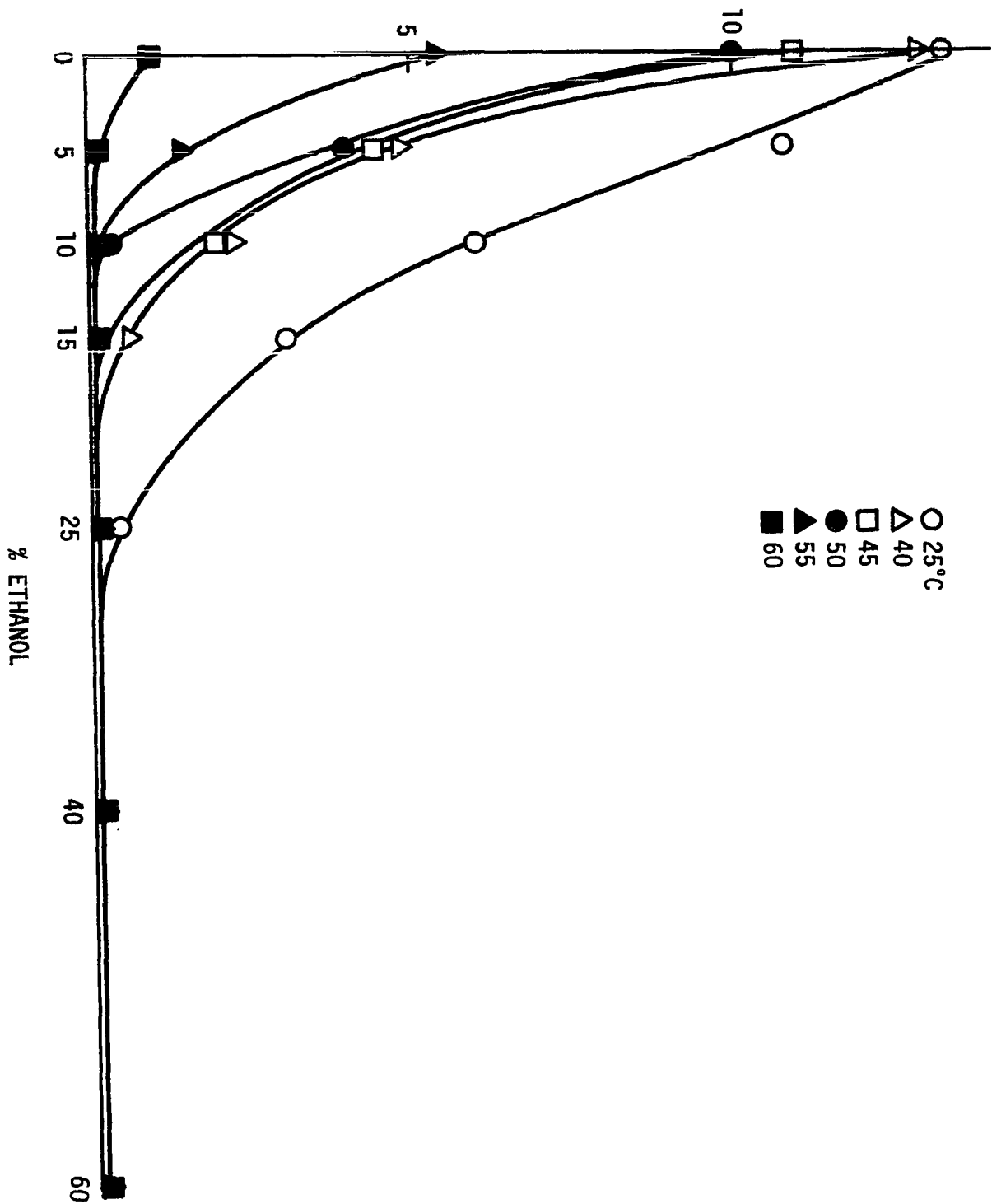


Figure 6. Changes in NSI values of DSM due to soaking soybeans at various temperatures and ethanol concentrations for 24 hr.

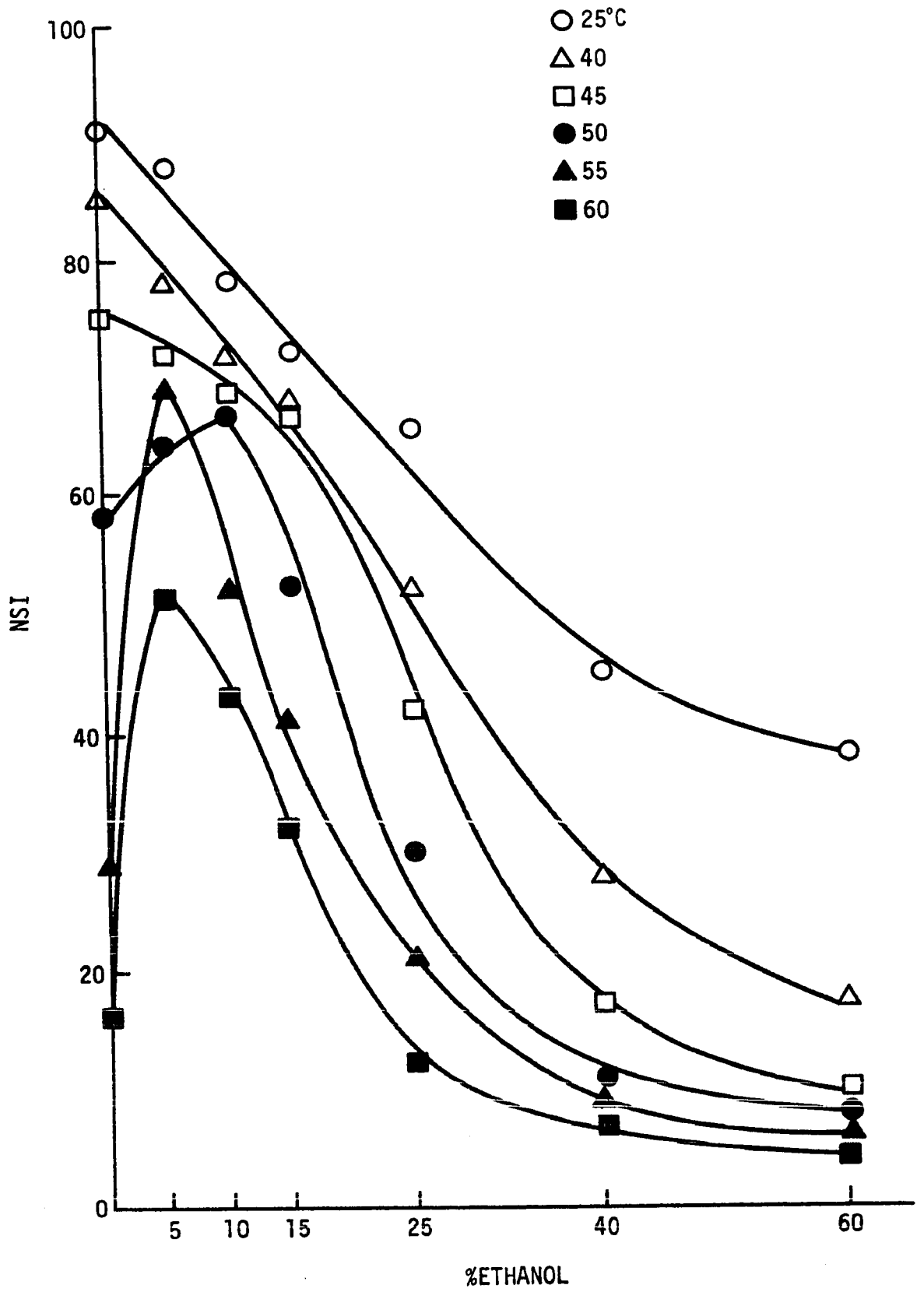


Table 6. Soaking conditions of temperature and ethanol concentration which inactivate lipoxygenase of soybeans in 24 hr soaking

Temperature	Ethanol %	NSI
25° C	40	43
40° C	25	52
45° C	15	67
50° C	15	52
55° C	10	52
60° C	5	51

soaked in ethanol concentrations of 25% and above at temperatures of 40° C and higher. One obvious point is that extremes of ethanol concentration and temperature are not useful because changes are either very slow or drastically sharp and uncontrollable. But a combination of lower ethanol concentrations and intermediate temperatures seems to give relatively high NSI with complete lipoxygenase inactivation.

NSI values of soybeans soaked in water at 50, 55, and 60° C decreased unexpectedly compared to the NSI of beans at 5% or 10% ethanol at the same temperatures. This decrease is probably due to the growth of bacteria which reduced pH and caused greater heat denaturation,

whereas inclusion of 5% ethanol in the soaking solution inhibits bacterial growth, and denaturation was limited to the effect of heat and ethanol, but not lower pHs.

Data in Table 6 represent a summary of soaking temperature and ethanol concentration combinations, obtained from Fig. 5 and 6, that inactivate lipoxygenase completely in 24 hr soaking. Soaking at 45°C and 15% ethanol for 24 hr gave the highest NSI value of 67 percent. This temperature-ethanol combination was chosen for further experiments to study properties of the resulting DSM and soaking solutions.

I found the minimum soaking time required to inactivate lipoxygenase in soybeans at 45°C and 15% ethanol is 12 hr. Fig. 7 shows that decreasing the soaking time from 24 to 12 hr improves NSI about 1-2%.

A 1:3 w/v, soybean:soaking solution ratio is sufficient to reduce lipoxygenase activity to less than 1% of original activity by soaking in 15% ethanol at 45°C for 12 hr, Table 7. Ku (1972) showed in experiments on cooking soybeans in water that hydration was directly proportional to the soybean:water ratio. The least water present, 1:3 within their experimental conditions, resulted in the most water absorbed because the solids loss was lowest. Nevertheless, I used a 1:4 ratio throughout the study to prevent any possible insufficiency of soaking solution.

Figure 7. Changes with time of lipoxygenase activity and NSI of soybeans soaked at 45°C with 15% ethanol.

Lipoxygenase activity was assayed at pH 9.0 and expressed in $\mu\text{mol O}_2 \text{min}^{-1} \text{ml}^{-1}$ CSE.

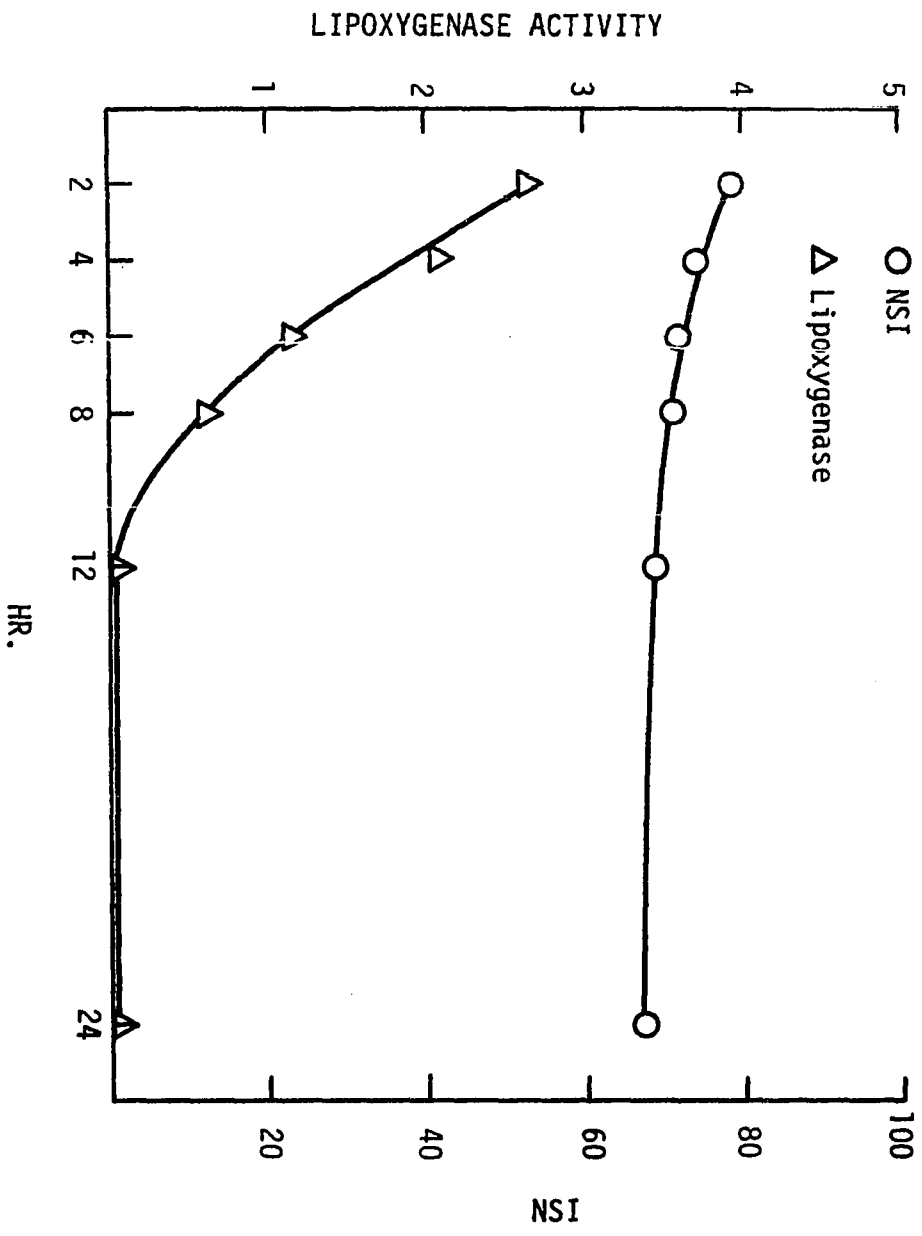


Table 7. Effect of bean-to-ethanolic soak solution ratio on lipoxygenase activity and NSI

Bean:Solvent Ratio w/v	NSI	Lipoxygenase Activity pH 9.0
1:1	70	0.39
1:2	70	0.03
1:3	69	0.03
1:4	68	0.00
1:5	68	0.00

Effect of Chemicals

Soaking solution pH

The effect of extraction pH on lipoxygenase heat-inactivation of crude soybean extract was shown in Fig. 2. Baker and Mustakas (1973) observed that adding acid or base to the cooking water accelerated the heat inactivation of lipoxygenase. The results of addition of acid or base to ethanolic soaking solutions on lipoxygenase activity and NSI are shown in Figs. 8 and 9. Soybeans were soaked in 15% (Fig. 8) and 10% (Fig. 9) ethanol at 45°C for 12 hr. As was expected, 15% ethanol

Figure 8. Changes in lipoxygenase activity, NSI, and pH due to addition of hydrochloric acid or sodium hydroxide to the soybean soaking solutions.

Soybeans were soaked in 15% ethanol for 12 hr at 45° C.
Lipoxygenase activity was assayed at pH 9.0 and expressed in $\mu \text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1} \text{ CSE}$.

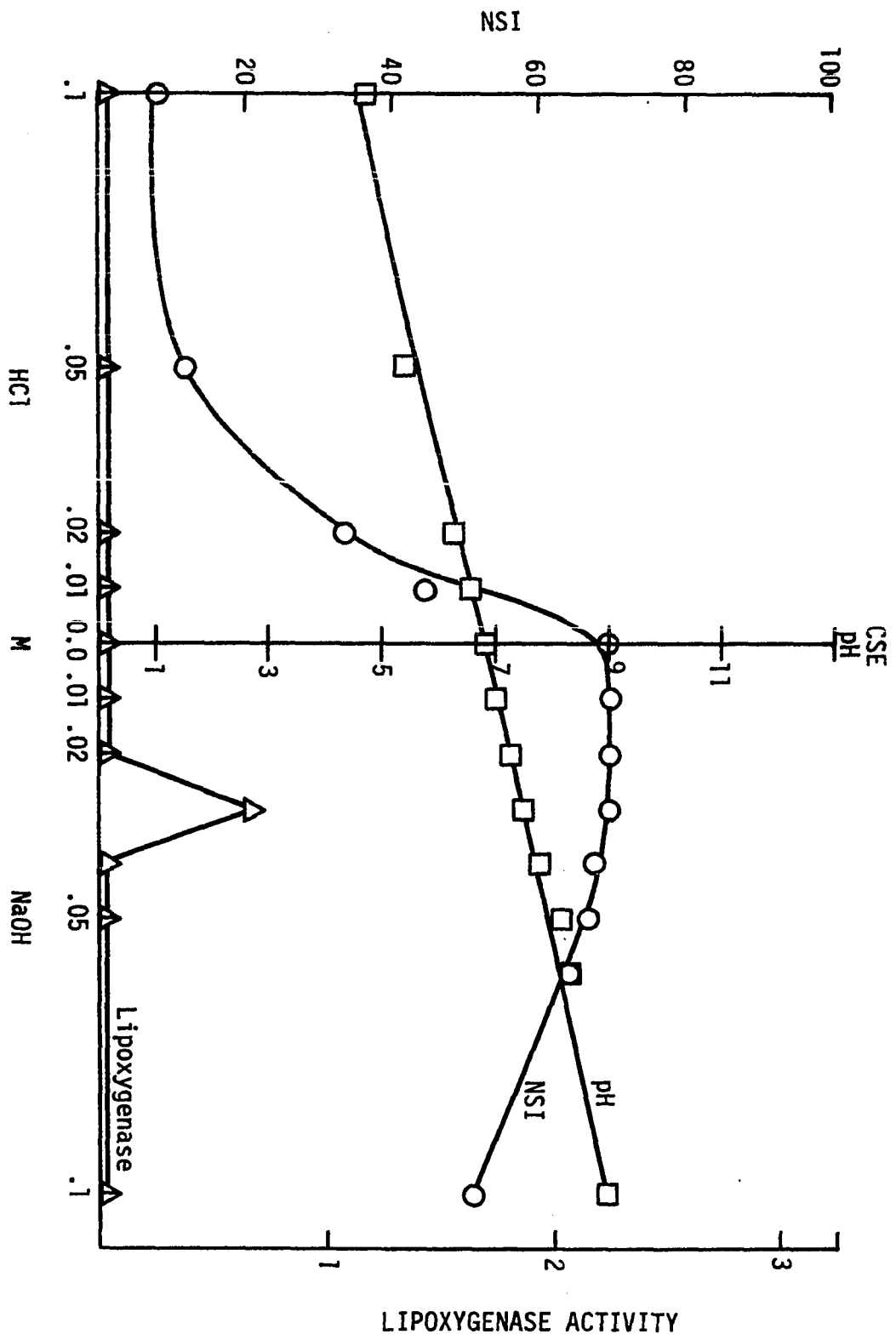
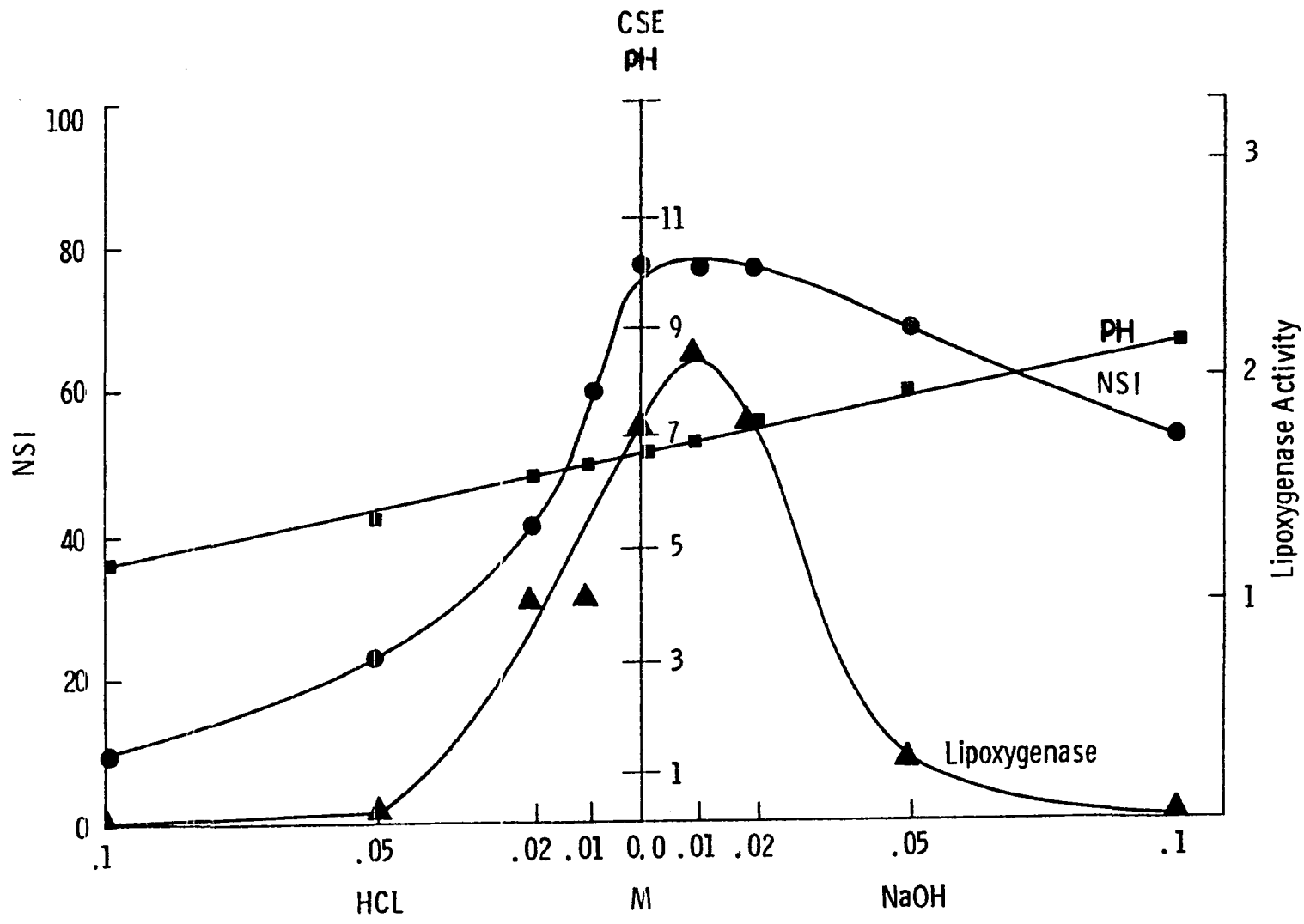


Figure 9. Changes in lipoxygenase activity, NSI, and pH due to addition of hydrochloric acid or sodium hydroxide to the soybean soaking solutions.

Soybeans were soaked in 10% ethanol for 12 hr at 45°C. Lipoxygenase activity was assayed at pH 9.0 and expressed in $\mu \text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$ CSE.



soaking is sufficient to eliminate lipoxygenase activity. Minor residual activity in the sample soaked in 0.3 M NaOH ethanolic solution may be due to hard shell beans which resist soaking. The NSI as a function of pH unexpectedly is different in the presence of ethanol. Wolf (1972) has shown in aqueous solutions a continuous increase in soy protein solubility as the extraction pH increases. NSI of alkaline-ethanol soaked soybeans tend to decrease as molar concentrations of sodium hydroxide increase above 0.03 M. This behavior may be interpreted as a synergistic effect of lower and higher pH's on heat-ethanol denaturation. Similar observations were made by Khaleque et al. (1970) who found that soaking soybeans in sodium hydroxide solution reduced the yield of soluble protein compared to the beans soaked in water. They postulated that sodium hydroxide causes protein swelling or interaction which inhibited diffusion from the cotyledon during preparation of soymilk.

Addition of 0.1 M NaOH to 10% ethanolic soak is needed to cause complete destruction of lipoxygenase after 12 hr soaking at 45°C. NSI is about the same as with 15% ethanol treatment. This means that at 0.1 M NaOH, the pH-heat denaturation eliminates the effect of the ethanol concentration difference between 10 and 15% on NSI.

EDTA

Restrepo et al. (1973) found that lipoxygenase-2 was activated by calcium ions. I investigated the removal of divalent cations by addition of EDTA to ethanolic soaking solutions of soybeans to see if lipoxygenase would be inactivated in intact beans. Fig. 10 shows the effect of various concentrations of EDTA (in 10% ethanolic soak solutions) on lipoxygenase activity, pH, and NSI of the resulting DSM. An overall decrease in pH, lipoxygenase activity and NSI occurs and is more pronounced as the molar concentration of EDTA increased. The effect of EDTA seems to be a lowering of pH which enhanced denaturation of soy proteins and lipoxygenase, rather than inactivation due to calcium removal.

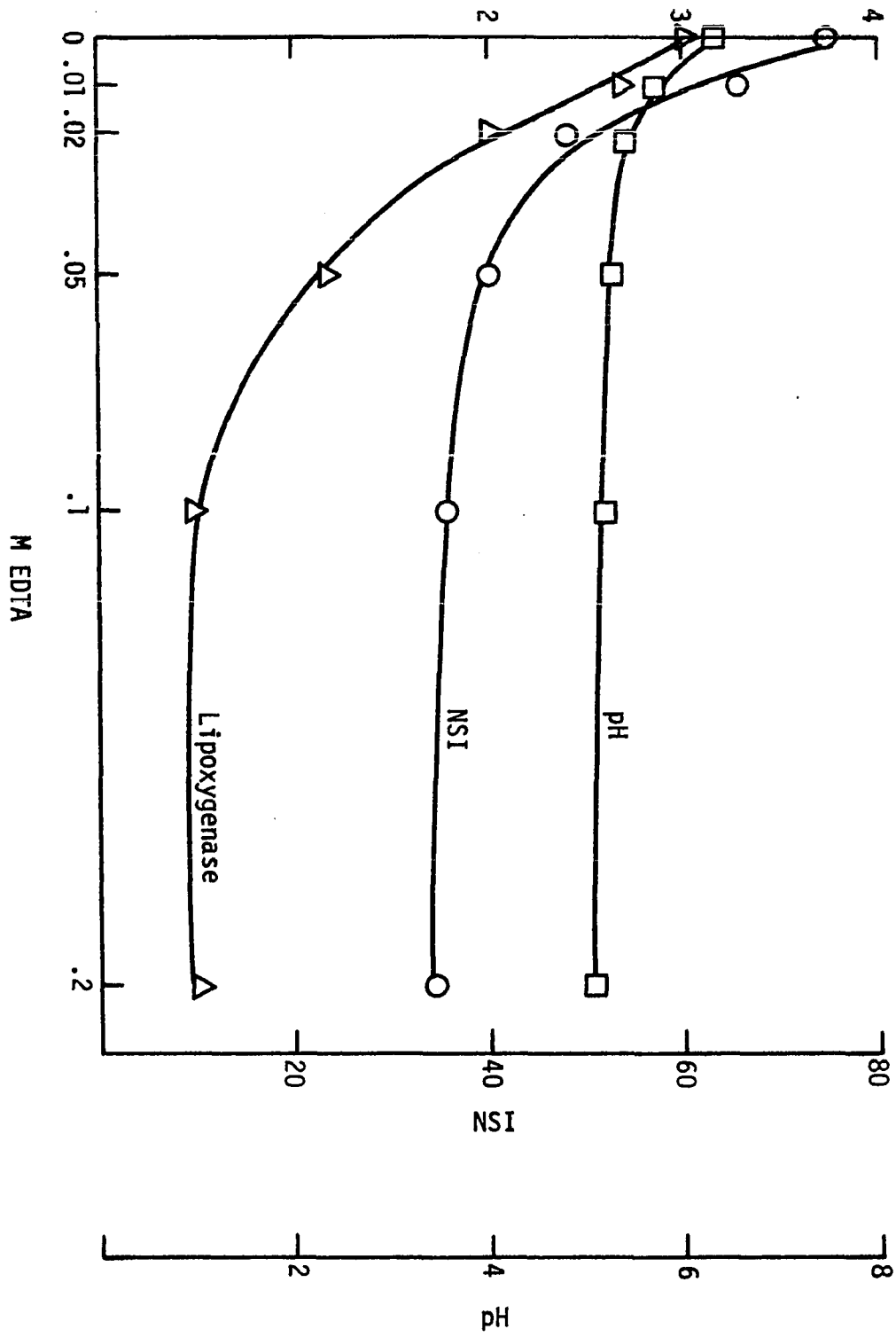
Sodium dithionite

Yasumoto et al. (1970) found nordihydroguaiaretic acid (NDGA), a phenolic reducing agent, is a potent irreversible inactivator of lipoxygenase at pH 9. However, they showed that NDGA is a competitive inhibitor at pH 7. Roza and Francke (1973) reported lipoxygenase inhibition by a combination of L (+) cysteine and o-phenanthroline under aerobic conditions. They questioned whether the inactivation was the direct result of cysteine oxidation and/or removal of the iron. In previous

Figure 10. Effect of addition of EDTA to soaking solutions on the pH and on lipoxygenase activity and NSI of soaked soybeans.

Conditions were 10% ethanol 45°C and 12 hr soaking. Lipoxygenase was assayed at pH 9.0 and expressed in $\mu\text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1}$ CSE.

LIPOXYGENASE ACTIVITY



work (Borhan, 1974), I found that sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$), a strong reducing agent, caused inhibition of purified lipoxygenase. This inhibition could be prevented by the addition of heated CSE to the lipoxygenase preparation. I concluded that the loss of activity does not take place upon reduction of lipoxygenase but only after the reducing activity is used up.

Fig. 11 shows a protective effect of sodium dithionite upon lipoxygenase activity of intact soybeans soaked in 10% ethanol (45°C , 12 hr) containing 0.01 to 0.5 M sodium dithionite. The pH of the soaking solution was unchanged and NSI showed a decreasing trend. Increasing the molar concentration of sodium dithionite in soaking solutions caused an increase in lipoxygenase activity of soaked beans. If inactivation of lipoxygenase is a function of O_2 availability, this behavior is explainable because less O_2 would be present at the higher concentrations of sodium dithionite. The lipoxygenase protection by sodium dithionite may also be due to the effect of ionic strength of soaking solutions as will be shown in the next experiment.

Sodium bicarbonate, sodium carbonate, sodium chloride

Fig. 12 shows the effect of sodium bicarbonate on lipoxygenase inactivation when added to 10% ethanolic soaking solution (45°C , 12 hr).

Figure 11. Changes in lipoxygenase activity and NSI of DSM due to addition of various amount of sodium dithionite to ethanolic soaking solution of soybeans.

Conditions were 45°C, 10% ethanol and 12 hr soaking. Lipoxygenase activity was assayed at pH 9.0 and expressed in $\mu\text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1} \text{ CSE}$.

LIPOXYGENASE ACTIVITY

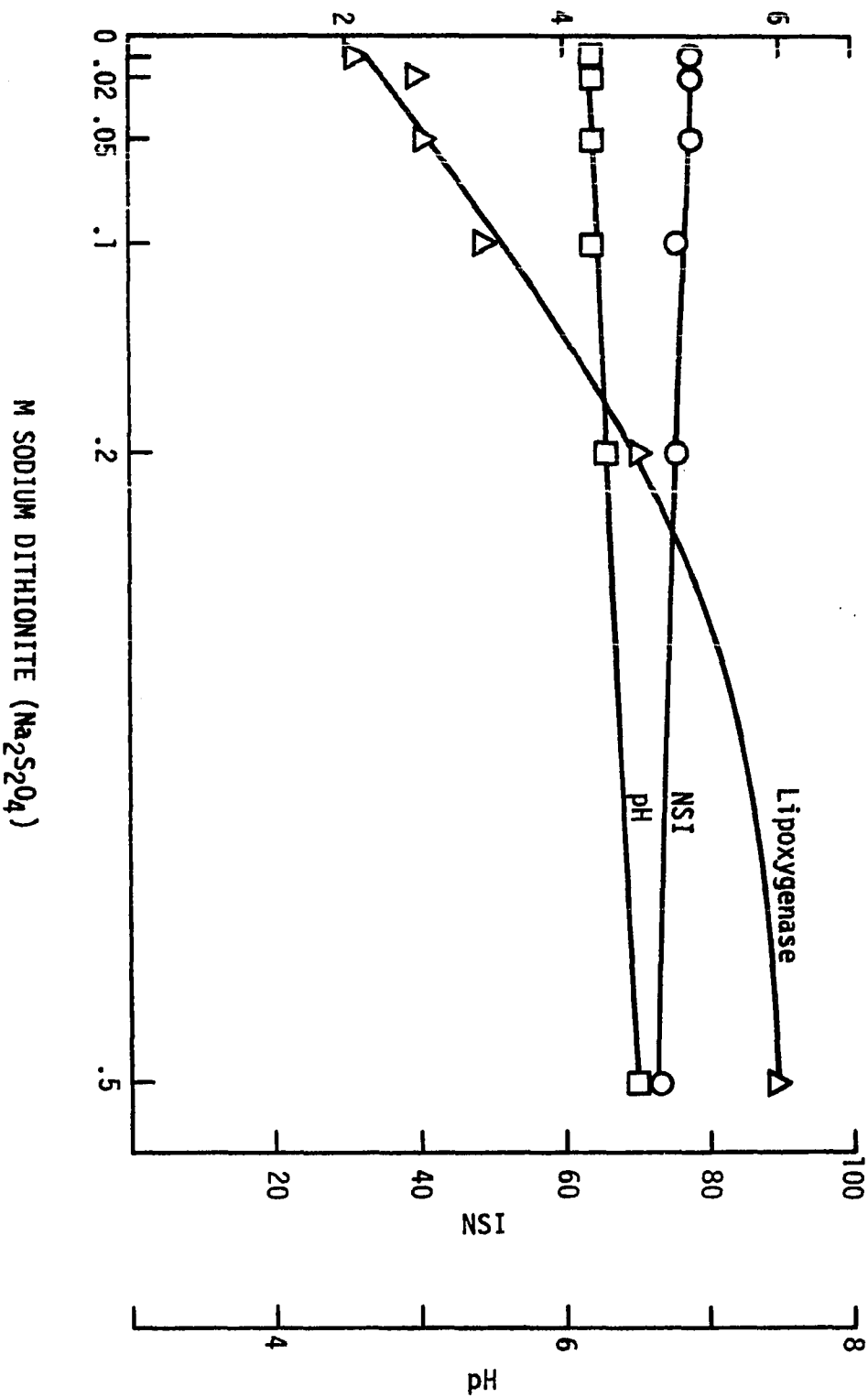
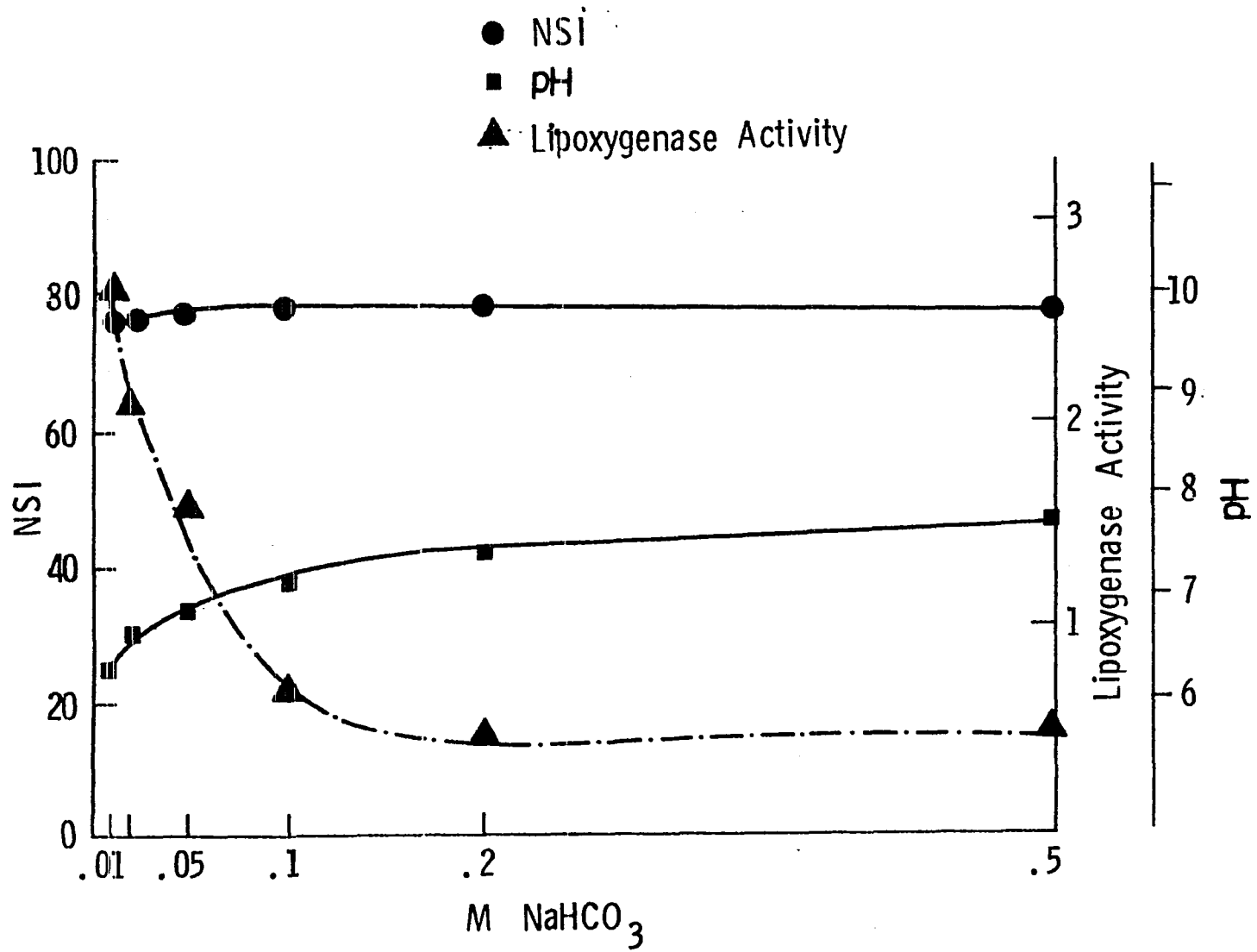


Figure 12. Effect of sodium bicarbonate on lipoxygenase activity of soybeans soaked in 10% ethanol at 45°C for 12 hr.

Lipoxygenase activity was assayed at pH 9.0 and expressed in $\mu\text{mol O}_2 \text{min}^{-1} \text{ml}^{-1} \text{CSE}$.



Though lipoxygenase was not completely inhibited by any concentration of NaHCO_3 , increasing its concentration decreased lipoxygenase activity. This inactivation is because of the pH increase. Nelson et al. (1976) observed a flavor improvement in soymilk prepared from soybeans which were soaked in 0.5% NaHCO_3 overnight followed by 30 min blanching. Enhanced lipoxygenase inactivation caused by addition of NaHCO_3 to the soaking solution may explain the Nelson et al. (1976) finding, although 30 min blanching (100°C) of soaked soybeans is sufficient treatment to destroy lipoxygenase. The effect of NaHCO_3 can better be shown by the results of Baker and Mustakas (1973) when lipoxygenase was readily inactivated by 1% alkaline solutions of NH_4OH , NaHCO_3 , or NaOH for 15 min at 75°C . Such a time and temperature are not sufficient to destroy lipoxygenase without alkaline additives. Inclusion of 1% NaOH in cook water by these workers decreased the temperature required to inactivate lipoxygenase in 15 min from 83°C to 75°C . Khaleque et al. (1970) found no significant differences between beany flavor of soymilks prepared from 0.5 M NaHCO_3 soaked soybeans for 24 hr at room temperature and water soaked ones. This observation is expected, since 0.5 M NaHCO_3 alone is insufficient to inactivate lipoxygenase.

Fig. 13 shows the combined effects of heat, ethanol, and sodium carbonate on NSI and lipoxygenase activity of DSM. The added Na_2CO_3 caused inactivation of lipoxygenase to less than 1% of the original activity at 0.1 M and pH 9.8. At this concentration, NSI starts to decrease. Nevertheless, this combined treatment gave an NSI of 75%, the highest that could be achieved with the condition of lipoxygenase inactivation. The greater effect of Na_2CO_3 compared to NaHCO_3 on lipoxygenase inactivation is probably due to a greater pH change caused by Na_2CO_3 . Khaleque et al. (1970) found that presoaking soybeans in 0.4 M sodium carbonate improved flavor and yield of protein in resulting soymilk as compared with presoaking in water or in sodium hydroxide. They did not think lipoxygenase was important in beany-flavor formation, because they were not able to detect a significant difference in intensity of beany flavor in soymilks prepared by using either cold or hot grinding procedures, even though lipoxygenase activity was not measurable after grinding at the higher temperature. Bourne et al. (1976) attributed this observation to insufficient heat for instantaneous and complete inactivation of lipoxygenase.

I found that increasing the ionic strength of ethanol soaking solutions by addition of NaCl protected lipoxygenase against inactivation (Fig. 14). In contrast with lipoxygenase activity, pH

Figure 13. Effect of adding Na_2CO_3 to soaking solution on lipoxygenase activity, NSI and pH.

Soaking conditions were 10% ethanol, 45° C and 12 hr.
Lipoxygenase activity was assayed at pH 9.0 and expressed in $\mu\text{ mol O}_2\text{ min}^{-1}\text{ ml}^{-1}\text{ CSE}$.

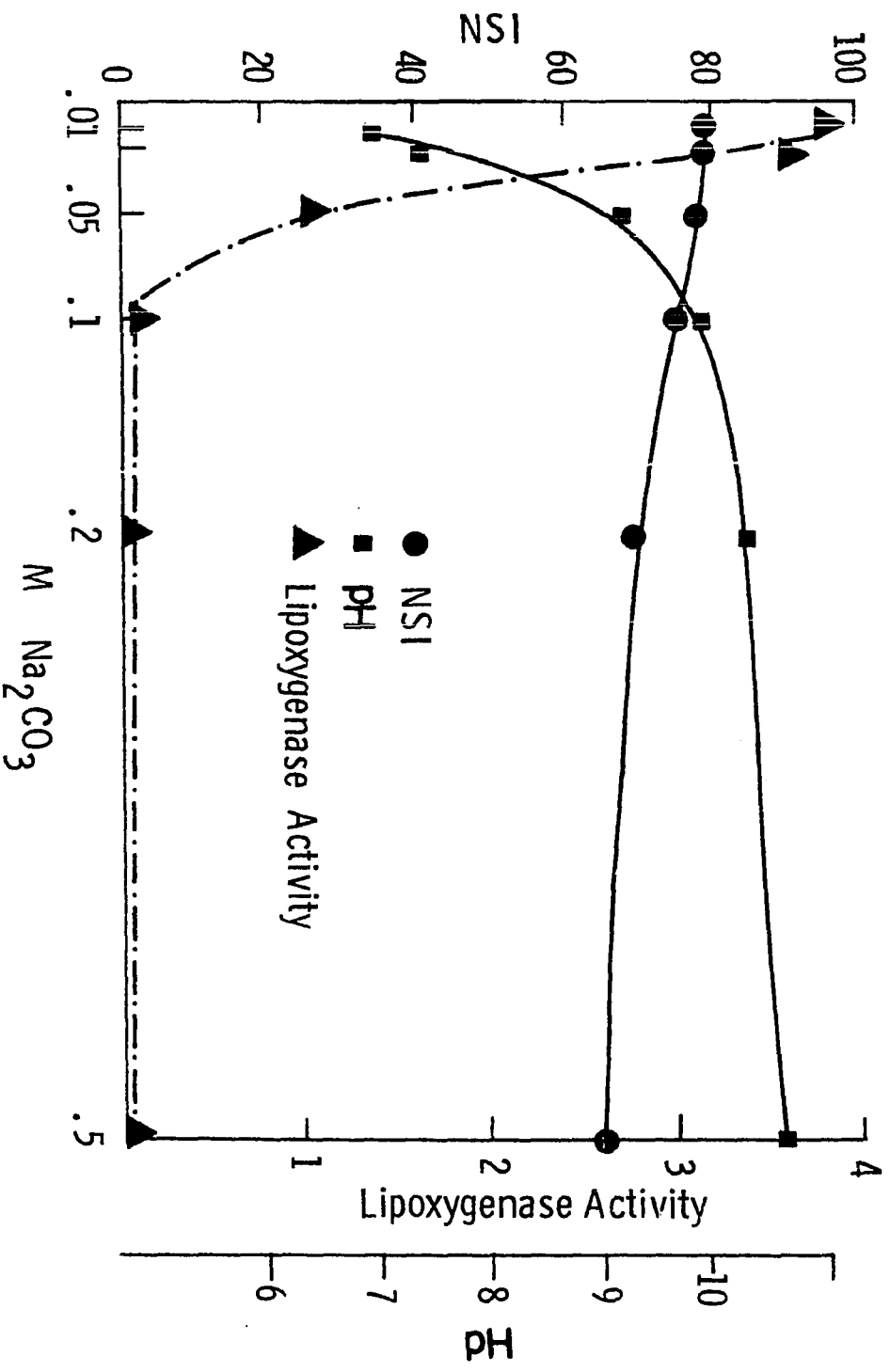
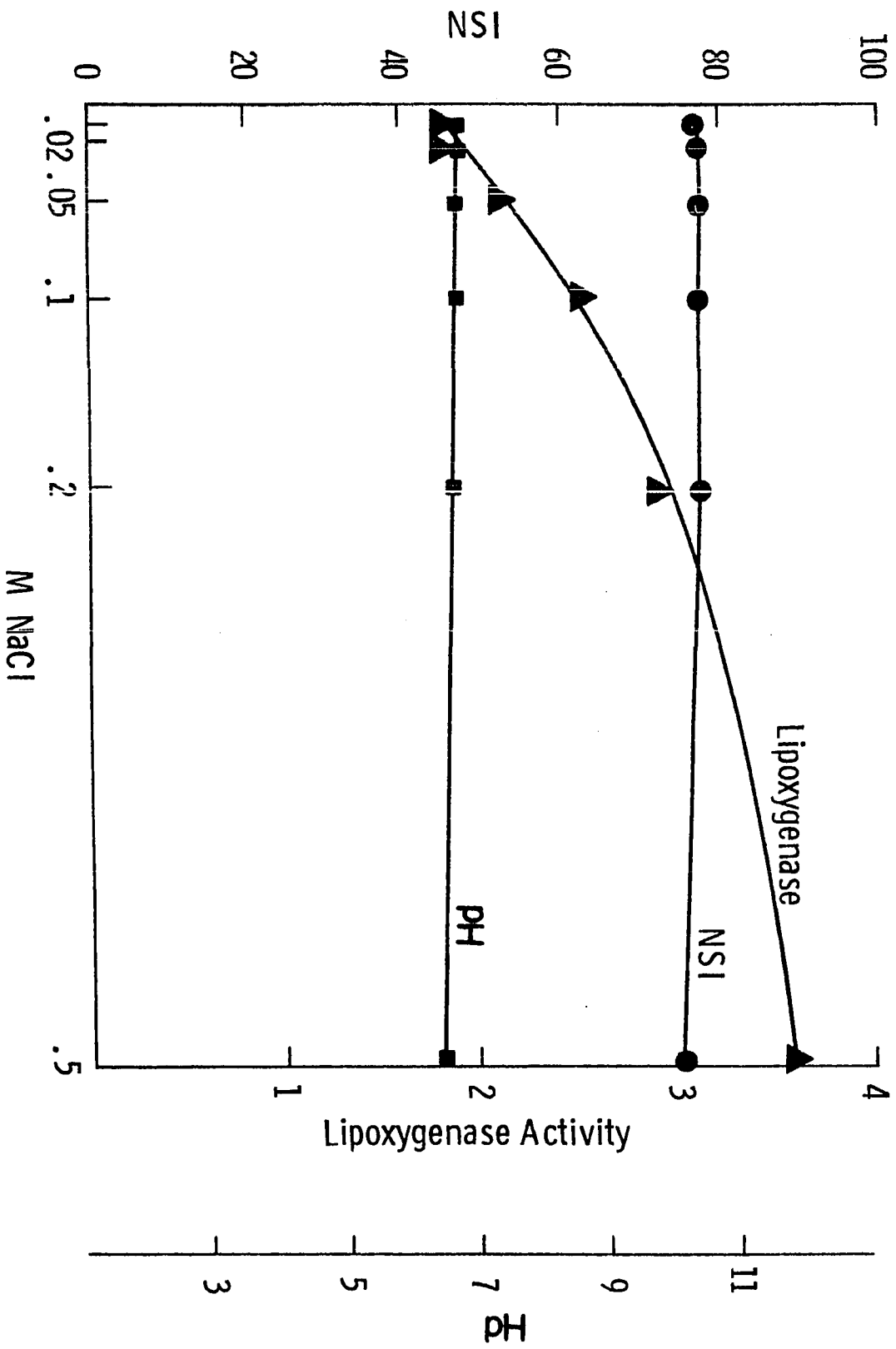


Figure 14. Changes in lipoxygenase activity and NSI of DSM due to soaking soybeans at 45°C, 10% ethanol for 12 hr in various concentrations of sodium chloride.

Lipoxygenase activity was assayed at pH 9.0 and expressed in $\mu \text{ mol O}_2 \text{ min}^{-1} \text{ ml}^{-1} \text{ CSE}$.



of the soaking solution and NSI values decreased slightly with increasing concentration of sodium chloride. This observation may also explain the protective effect of sodium dithionite in the previous experiment.

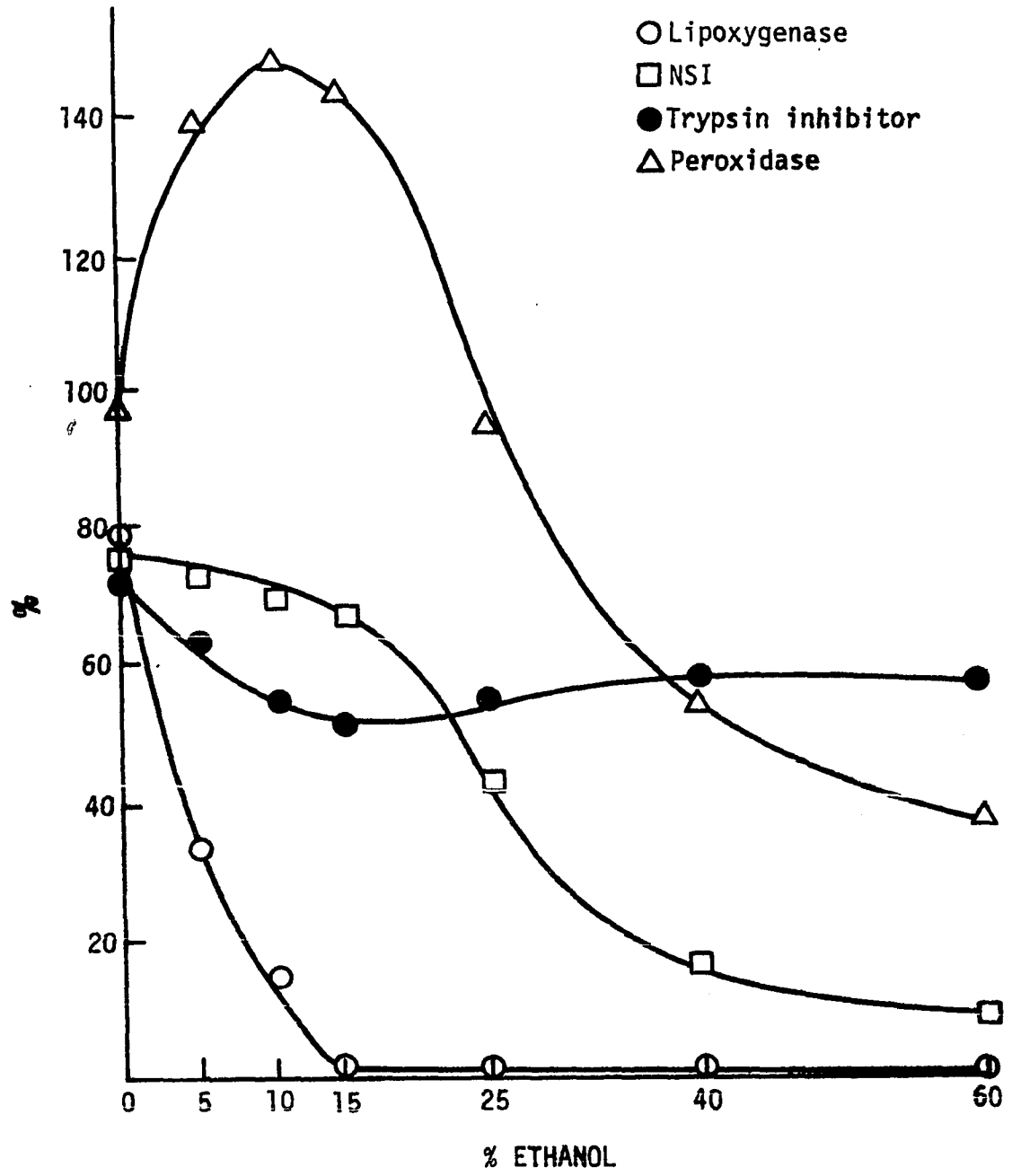
Properties of Treatment Products

Defatted soy meal

The results of heat-ethanol treatment on biologically active components of DSM are shown in Fig. 15. Lipoxygenase was the most labile among all tested. Ethanol treatment (15%) at 45° C caused a maximum of 50% destruction of trypsin inhibitor, but greater ethanol concentrations failed to inactivate trypsin inhibitor to a greater extent. This agrees with the finding of Eldridge et al. (1977) that only partial inactivation of trypsin inhibitor results from soaking soybeans in different concentrations of ethanol at 25° C for 24 hr. The stability of trypsin inhibitor to aqueous ethanol is not surprising since Kunitz (1946) used 80% ethanol for crystallization of this protein.

Data in Fig. 15 indicate a much greater stability for peroxidase than lipoxygenase toward heat-ethanol treatment. Gardner et al. (1969) suggested a greater heat stability of peroxidase than for most other enzymes. Rackis et al. (1972) considered peroxidase a very active enzyme capable

Figure 15. Changes in percentage of NSI, trypsin inhibitor activity, lipoxygenase activity and peroxidase activity of DSM due to soaking soybeans in various concentrations of ethanol at 45° C for 24 hr.



of attacking lipohydroperoxides which can be produced even in the absence of lipoxygenase activity. They postulated the possibility of bitterness formation through formation of peroxides followed by reaction with antioxidant aided by peroxidase enzyme, and subsequent polymerization of the radicals that form. Rackis et al. (1975) blamed peroxidase as the enzyme which causes flavor formation. And because it is relatively stable, measurement of peroxidase activity might be used in establishment of process conditions for production of bland soy products. Sessa and Rackis (1977) reported that peroxidase enzymes are very sensitive to 50% ethanol treatment. I observed that soaking soybeans in 5 to 25% ethanol at 45°C for 24 hr increased peroxidase activity of the resulting defatted soy meals. This result was confirmed by two subsequent observations. Rackis et al. (1975) also showed an increase in peroxidase activity of soy flakes when a hexane-ethanol azeotrope-extraction was conducted for 6 hr. I found that ethanol concentrations above 25% decreased peroxidase activity rapidly to a level of about 40% of the original activity at 60% ethanol.

Soaking solution

Data in Table 8 show soluble substances leached from 100 g soybeans into various water-ethanol mixtures at 45°C during 24 hr soaking. Ethanol concentrations of 5 to 15 percent caused the greatest loss of total

Table 8. Substances leached into the soaking solution from 100 g soybeans in 24 hr soaking at 45° C

Soak Ethanol % v/v	pH	Total g	Ash g	N x 6.25 g	Sugars as Sucrose g	Total P mg	Chloroform Extracted μ g P
0	5.9	10	1.9	1.9	5.1	137	33
5	6.1	12	1.9	2.5	6.2	155	41
10	6.2	12	1.8	2.5	6.3	117	62
15	6.2	12	1.7	2.1	6.2	101	47
25	6.4	11	1.5	1.7	6.1	73	45
40	6.6	10	1.3	0.8	6.0	32	45
60	6.8	9	1.0	0.6	5.6	27	37

solubles, about 12 percent. Proteins, minerals, and sugars were predominant constituents contributing to total solubles. At this range of ethanol concentrations, both easy diffusion and solubilizing characteristics exist of water for water soluble materials and of ethanol for ethanol soluble materials. However, higher concentrations of ethanol restricted solubilities of proteins and minerals.

Lo et al. (1968) studied the composition of soak water and the material removed from soybeans during soaking. They found that soaking Harasoy soybeans in water (1:3 w/v) for 24 hr at 1°C resulted in a 5% loss of solids. This loss of solids increased to 10% by 72 hr. Approximately one-half of the nitrogen was nonprotein nitrogen. Changes occurred during soaking because the decrease in total protein was accompanied by an increase in nonprotein nitrogen. Nelson et al. (1976) found that 11% of the total solids in the raw bean leached into the soaking solution during 10 hr soaking in 0.5% NaHCO₃ at room temperature. The nitrogen loss into soaking and blanching solution was mainly nonprotein constituents. My results show a maximum calculated protein loss of about 2.5% in 10% ethanol. Ethanol soaking for shorter periods than 24 hr may be preventing the metabolic changes and total protein loss as well.

Ku (1972) found that about 55% of the soluble carbohydrates in soybeans leached into cooking water during 60 min cooking in 1:5

bean-to-water ratio. This value was about 30% when the bean-to-water ratio was 1:3. Maximum removal (59%) was achieved with a 1:10 bean:water ratio after 60 min cooking.

My results show a loss of about 60–65% of soluble sugars, calculated as sucrose, after 24 hr in a 1:4 soybean:soaking solution (45° C).

Oligosaccharides accounted for most of the loss, since a qualitative thin layer chromatograph showed that the major constituents of soybean soluble sugars in soaking solution had R_f values the same as sucrose, raffinose, and stachyose standards. Removal of oligosaccharides by ethanol soaking is advantageous because water-soluble, low molecular weight carbohydrates have been found as the flatus producing factor of soybeans. Human and dog experiments indicate that soybean flatulence is caused by anaerobic fermentation of carbohydrates in the ileum and colon, with egestion of high concentrations of CO_2 and H_2 , Rackis et al. (1970).

The phosphorus content of soluble substances leached into the 5 to 10% ethanolic soaking solution was about 15–25% of the total phosphorus content of dry mature soybeans. Removal of soybean phosphorus by soaking is nutritionally useful because the principal source of phosphorus in soybeans is phytate, Smith and Circle (1972). Phytic acid in its natural form as a phytate-mineral-protein complex decreases the nutritional availability of zinc, manganese, copper, molybdenum,

calcium, magnesium, and iron, Rackis (1974).

The chloroform extractable phosphorus in the soak solution was minimal. This phosphorus is of phospholipid origin, and phospholipids may be responsible for the bitterness characteristic of soy products.

Minimum Times Required for Processing Soybeans

With Heat and Ethanol

To have a guideline for inactivation of soybean lipoxygenase by ethanol soaking of soybeans, the interactions of temperature, ethanol concentration, and time need to be known. This knowledge can be used to design an economical process depending upon the various costs.

Results of experiments throughout this study showed that the proper temperature for soaking ranges from slightly above room temperature up to about 70° C, because mild heating provides a slower rate and better control over protein denaturation by ethanol-heat treatment. This can also be related to the findings of Catsimpoolas et al. (1969) who found that glycinin, 11 S fraction, is stable to heating up to 50° C and very little change occurs between 50 to 70° C, but a significant and sudden loss of solubility occurs when the protein is heated at temperatures above 70° C.

Therefore, combinations of three temperatures, 40, 50, and

60°C, and four ethanol concentrations, 15, 30, 45 and 60%, were chosen for soaking. The third independent variable, soaking time, was varied so that complete inactivation of lipoxygenase was achieved in minimal time. The result shown in Fig. 16 is a surface constructed by plotting the data in three dimensions. The surface represents a part of a larger surface in which every individual point is a particular combination of temperature, ethanol concentration and time, which causes complete inactivation of soybean lipoxygenase and gives maximum NSI value. In the space above the surface protein denaturation is in excess of that needed for lipoxygenase destruction, therefore, NSI are lower. In contrast, the space below the surface represents combinations of the variables which are not sufficiently destructive to lipoxygenase activity. The NSI of defatted soy meals obtained from soybeans soaked under experimental conditions shown in Fig. 16 are given in Table 9. These data suggest that higher temperatures-lower concentrations of ethanol and shorter times are preferable soaking conditions. But any combination may fit qualifications of particular processes when all variables are translated to a common unit, cost, for evaluation of the choices.

Statistical analysis of experimental data related lipoxygenase activity and NSI to independent variables of temperature, ethanol concentration, and time by deriving the following equations:

Figure 16. The surface for total inactivation of soybean lipoxygenase by soaking at various temperatures, ethanol concentrations and times.

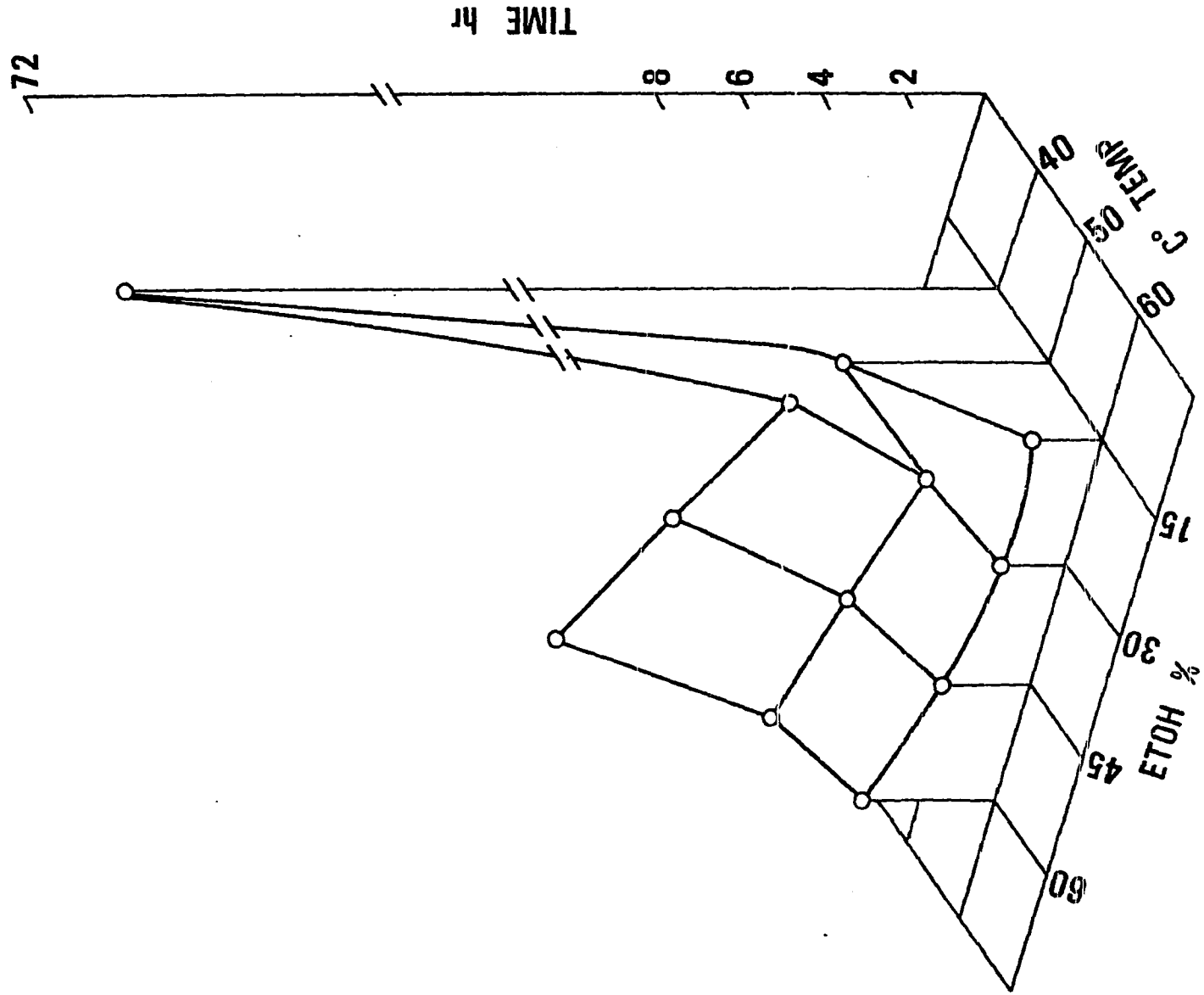


Table 9. Changes of NSI under conditions for lipoxygenase destruction

Temperature °C	Ethanol % v/v	Time hr	NSI %
40	15	72	72
40	30	4	61
40	45	6	41
40	60	8	39
50	15	5	62
50	30	2	52
50	45	3	34
50	60	4	39
60	15	1.5	54
60	30	1.5	33
60	45	2	29
60	60	3	27

1. $Y = 5.262 - 0.061 W - 0.146 X + 0.640 Z + 0.001 X^2 + 0.001 WX - 0.015 WZ - 0.004 XZ$
2. $NSI = 1.284 - 0.025 X + 0.055 Z - 0.0001 W^2 + 0.0003 X^2 - 0.0011 WZ - 0.0008 XZ$

Where:

W = temperature °C,

X = ethanol %,

Y = lipoxygenase activity and

Z = time hr.

Equation 1 accounts for 56% variability in lipoxygenase activity and equation 2 accounts for 98% of NSI variability. The reason for relatively incomplete accounting for lipoxygenase variability by independent variables is the design of the experiment. Independent variables were chosen so that lipoxygenase activity would be close to zero.

These formulas can be used for estimation of lipoxygenase activity and NSI of treated soybeans by inserting into the equations values for each of the independent variables. The range of the independent variables is limited to 40 to 60° C and 15 to 60% ethanol.

SUMMARY AND CONCLUSIONS

Information gathered in this study indicates a greater heat stability for lipoxygenase-1 than lipoxygenase-2 in crude preparations. Survival half-time values for these two isoenzymes were similar to values of purified forms found by Christopher et al. (1970). I found that pH adjustment of CSE during extraction decreases lipoxygenase-1 stability toward heating at 69° C. A 15 to 17 fold reduction in half-time occurred by adjustment of CSE pH from 6.6 to 9.0 and 4.5, respectively.

Ethanol soaking of soybeans in the cold reversibly inhibits lipoxygenase activity. The extent of reactivation, by removing ethanol, is greater for lipoxygenase-1 than for lipoxygenase-2. Ethanol concentrations between 30-50% have the strongest inhibitory effect. Combination of ethanol and heat treatment caused irreversible inactivation of lipoxygenase in soaked soybeans. I found that soaking soybeans in ethanol concentrations higher than 80% did not inhibit lipoxygenase activity, even in the presence of some heating. This was due to lack of diffusability of highly concentrated ethanol into intact whole soybeans. In contrast, presoaking soybeans in water facilitated diffusion of concentrated ethanol into the beans and caused inactivation of lipoxygenase.

A direct relationship between presence of lipoxygenase activity and presence of typical green-beany flavor was observed. An inverse

relationship between combined temperature and ethanol concentration of soaking solution and protein concentration or lipoxygenase activity of CSE was evident. Among various combinations of temperature-ethanol concentration tested, conditions of 45° C, 15% ethanol, 24 hr soaking gave the highest nitrogen solubility index (NSI), about 67 percent. At this condition, the lipoxygenase activity of soaked soybeans was totally inhibited. NSI values were reduced drastically by soaking soybeans in ethanol concentrations greater than 25% up to 60% at temperatures higher than 40° C. This agrees with the results of Eldridge et al. (1977).

I observed that addition of acid or base to ethanolic soaking solution increases lipoxygenase inhibition and decreases the NSI of treated samples. In spite of the report of Wolf (1972), increasing pH, in the presence of ethanol at 45° C, is not accompanied by an increase in the NSI value of defatted soy meal. I attribute this phenomenon to a sensitizing effect of pH on heat-ethanol denaturation. Inclusion of EDTA in the soaking solution reduced both NSI and lipoxygenase activity. This is concluded as the result of lowering pH rather than removal of divalent cation, Ca^{++} , which is known to be a lipoxygenase-2 activator. The addition of sodium dithionite, a reducing agent, to soaking solution at various concentrations protected lipoxygenase from ethanol-heat inactivation, reduced NSI slightly, and maintained the pH of the soaking solution almost unchanged. The protective effect of sodium dithionite on

lipoxygenase is through either O_2 elimination, if lipoxygenase inactivation occurs in the presence of oxygen, or effect of ionic strength.

I studied the effect of sodium ion concentration in soaking solution on lipoxygenase activity and NSI by adding three different sodium salts. Sodium bicarbonate at various concentrations reduced the lipoxygenase activity of ethanol-soaked soybeans to a low level. Sodium carbonate at 0.1 M concentration completely eliminated lipoxygenase activity of soaked soybeans in the presence of 10% ethanol at 45° C for 12 hr. In contrast, sodium chloride showed a protective effect on lipoxygenase activity against ethanol-heat inhibition. The effect of different sodium salts can be attributed to their degree of contribution to pH changes because sodium carbonate considerably increased the pH. This change was rather small by addition of sodium bicarbonate, whereas the addition of sodium chloride caused a negligible change in pH of the soaking solution. Sodium ion concentration per se, regardless of its source, did not inhibit lipoxygenase, but rather protected it.

Defatted soy meal from soaked soybeans at 45° C, 15% ethanol for 24 hr, showed a 50% inhibition of soybean trypsin inhibitor. No greater inhibition was achieved by either higher or lower concentrations of ethanol. At 45° C, peroxidase showed a strong stability up to 25% ethanol. Higher ethanol concentrations reduced peroxidase activity. At 60% ethanol only 40% activity remained.

Proximate analysis of soluble material leached from soybeans into ethanolic soaking solution showed a loss of about 5% nitrogen, 15% phosphorus, and 65% of oligosaccharides based on original content of dry soybeans. Removal of oligosaccharides is a beneficial process, since they are responsible for flatulence. Also, phosphorus removal is advantageous because the main source of phosphorus in soybean is phytates which are nutritionally undesirable due to zinc binding.

A systematic study of soaking variables (temperature, ethanol percentage, and time) to evaluate corresponding dependent variables of NSI and lipoxygenase activity led to construction of a three dimensional surface. This surface represents soaking conditions for lipoxygenase inhibition at minimum soaking time.

Statistical analysis of the data provided two equations relating lipoxygenase activity and NSI, dependent variables, to temperature, ethanol concentration, and time of soaking. The equations are useful to estimate lipoxygenase activity and NSI of soybeans treated by a particular ethanol soaking process, within the limits of this experiment.

Lipoxygenase inactivation is a major step to improve the flavor property of soy protein products. Complete inactivation of the more stable isoenzyme, lipoxygenase-1, optimally active at pH 9.0, indicates total inactivation of lipoxygenases.

Combinations of lower concentrations of ethanol and intermediate temperatures are irreversibly inhibitory to lipoxygenase activity of soaked soybeans. Introducing several denaturing factors or synergizers to the soaking solution of soybeans causes stronger denaturing effect. Therefore, the intensity of each factor in combination needed for inhibition is much milder than when acting individually. Such a moderate, cooperative action provides easier control, finer adjustment, and selective denaturation of soybean lipoxygenase.

Ethanol soaking of soybeans may be useful since it provides an aqueous medium for inclusion of other factors to be combined. Ethanol soaking of soybeans meets the purposes of in situ inactivation of lipoxygenase and other biological changes. It also washes out undesirable constituents in soybeans. Whether the flavor problem is caused by lipoxygenase action or is inherent in soybeans, or both, ethanol soaking treatment seems useful.

Ethanol soaking of soybeans may be commercially feasible if further processing can be accomplished by use of soaked beans. Such a process is the production of soymilk, where expensive removal of absorbed water is not required. Additional heating would complete elimination of undesirable biologically active factors which were not totally destroyed by ethanol soaking.

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