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Title: INVESTIGATION OF METHODS FOR DETERMINATION AND PREVENTION OF PROTEIN INSTABILITY IN WINES.

Abstract APPROVED: _____
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The protein content and the degree of protein instability in 20 selected 1978 and 1979 Oregon and Washington white varietal table wines were determined. Each corresponding wine was made from grapes grown on the same location. The 1978 wines contained an average of 153.2 mg/L of protein as determined by the Diemaier and Maier procedure and required the equivalent of 4.9 lbs of bentonite/1000 gal (58.8 g/hl) for stabilization. The 1979 wines contained 177.6 mg/L of protein and required the equivalent of 3.6 lbs of bentonite/1000 gal (43.2 g/hl) for stabilization. A few wines required up to 12 lbs of bentonite/1000 gal (144 g/hl).

The prevention of protein instability in wines by bentonite substitutes including protease enzymes was investigated. Wine protein fractions responsible for causing the instability in Chenin blanc, White riesling and Gewurztraminer were isolated and purified by dialysis, concentration at low temperatures and gel filtration by Sephadex G-25. These fractions were further characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis and isoelectric focusing.

For the determination of non-dialyzable protein in white wines, the Bradford procedure using Coomassie Blue G-250 was more rapid and more reliable than both the Diemaier and Maier and the Kjeldahl methods.

Baykisol-30 (a silica sol), gelatin, PVPP and XAD-4 were investigated as bentonite substitutes for protein stabilizing wines. They had no significant impact on the protein stability of selected wines.

A commercial protease (Rohm protease S) was ineffective as a substitute for bentonite in protein stabilizing wines, presumably because of its low activity at wine pH. However, it was possible to produce protein stable wines from grape juices treated with Rohm experimental protease EL57-79 (50 mg/L) at 45-50°C for 12 hours.

Four fractions were obtained from Sephadex G-25 gel chromatography of control wines. The first fraction could be removed by treatment with either protease EL57-79 or bentonite. It was determined that this fraction contained mainly nitrogen, including protein nitrogen.

Chenin blanc proteins had MW of 23,000-71,000 and pIs of 5.2-5.9; its heat unstable proteins had MW of 23,000-24,000 and pIs of 5.2-5.5. White riesling proteins had MW of 22,000-38,000 and pIs of 5.2-5.9; its head unstable proteins had MW of 22,000-24,000 and pIs of 5.5-5.8. The MW of Gewurztraminer proteins ranged from 16,500-90,000 and their pIs were between 4.8-8.0; its heat unstable proteins had MW of 16,000-19,000 and pIs of 6.6, 7.1 and 8.0.

In general, the proteins responsible for the instability in Chenin blanc, White riesling and Gewurztraminer tended to have lower MW and higher pIs than their more stable counterparts.

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INTRODUCTION

Clarity is one of the major characteristics that influence consumer acceptance of wine. Among the substances contained in white wines, proteins are of special concern to the winemaker. Under favorable conditions, wine proteins can associate with phenolics and metal ions to form insoluble complexes which may come out of solution, When this occurs and a visible haze or amorphous deposit appears, the wine is classified as protein unstable and is, therefore, unacceptable for sale (Moretti and Berg, 1965). The occurence of haze suggests that the wine requires additional treatment.

There are methods currently available to prevent protein instability in wines. The most commonly used methods include: the application of heat and bentonite fining. However, these methods do not always yield the desired results (Datounachvilli, 1970). For instance, heat treatment of wine is undesirable because it may cause damage to its aroma (Ferenczy, 1966; Singleton, 1974). Therefore, it is seldom applied to premium quality wines. Bentonite fining is the international method of choice for stabilizing wines. Unfortunately, it has disadvantages such as extraction of color, the costly loss of wine in the lees (Singleton, 1974), its earthy flavor which can be imparted to wine, if used in excessive amounts (Sheat and Discombe, 1976) and gross alterations in wine constituents including reduction in volatiles which contribute to the taste and aroma of wines (Hartmaier, 1979; Ough et al., 1979).

In addition, bentonite is difficult to rehydrate and may sometimes be used improperly. Consequently, there is international interest in the discovery of suitable substitutes to bentonite fining (Singleton, 1974).

In a review on protein instability Singleton (1974) recommended that further research be conducted on the problem of protein instability, particularly that another look be given to the use of enzymes in wines. The use of enzyme preparations to hydrolyze proteins would eliminate the problem of overfining and some of the problems inherent with the use of bentonite (Datounachvilli, 1970; Rankine and Pocock, 1971).

Furthermore, even though considerable research has been conducted on wine proteins, current methods of protein determination in wines are either time consuming or unreliable (Amerine and Ough, 1980). This led to the recommendation that several methods be used on the same wine and that results be compared (Cordonnier, 1966).

To date, little is known about why some proteins are stable in wines, whereas others are not. Therefore, more information is needed on which wine proteins fractions are troublesome.

Although there is an on-going program at Oregon State University for screening Pacific Northwest (PNW) wines for their protein instability (Watson et al., 1980), there is no information in the literature on the protein content, the use of bentonite substitutes, and the characterization of protein fractions present in wines produced in the PNW (Idaho, Oregon, Washington) region of the United States of America. Therefore, it seemed desirable that an investigation should be carried out on these wines to clarify the situation.

This investigation was carried out to:

1. Screen selected Oregon and Washington wines for their protein concentration and the degree of their protein instability.

2. Compare three of the methods of detecting protein instability (50°C and 80°C heat and cold tests and the Bentotest).

3. Find an objective method to measure wine "protein" haze.

4. Find an alternative method for protein determination in wine that would be more rapid and reliable than current methods.

5. Test the feasibility of using two enzymes, one commercial, protease S, and one experimental, protease EL57-79 from Rohm Gmbh, Darmstadt, West Germany, to prevent protein instability in wines.

6. To characterize the activity of the above enzymes.

7. To isolate and characterize the individual protein fractions which are responsible for the protein instability in wines.

LITERATURE REVIEW

Introduction to Protein Instability in Wines

Why the Concern About Protein Instability?

Clarity is one of the chief characteristics of wines that the consumer appreciates. Therefore, it is imperative that wines remain brilliant. Among the numerous substances contained in wine, nitrogenous compounds, particularly proteins are of major concern to the winemaker since they can become insoluble forming hazes and precipitates. The appearance of a haze or deposit indicates that the wine is defective and, therefore, unacceptable for sale. When this occurs during processing it causes delays, but when it occurs at the market place it causes economic losses.

Beverages Susceptible to Protein Instability

It is generally recognized that only beverages with low polyphenol content (i.e. white wines and non-alcoholic white and red grape juices) or beverages over-fined with gelatin are subject to proteinaceous haze formation (Rentschler, 1969; Singleton, 1974). Red wines contain large amounts of phenolics which tend to insolubilize protein and eliminate them in the lees during vinification (Ribereau-Gayon et al, 1976). This review will limit itself to the problem of protein instability in white table wines.

It has been noted that proteins act as deposition centers for many cations. The proteins can bind to ferric phosphate leading to sediments

or suspensions. In addition to proteins and metals, peptides and phenolics also play a role in the formation of haze.

Despite the vast amount of literature on protein instability in wines, the actual protein level at which a wine may be considered stable is not known (Anelli, 1977). The particular protein fractions which tend to precipitate out are also not known. There are conflicting reports in the literature on this subject (Moretti and Berg, 1965; Bayly and Berg, 1967). It has been suggested that it is not the total amount of protein in wines, but only a part of the protein mixture or the ratio of various protein fractions that determines the stability of a wine. According to Danilatos and Sotiropoulos (1968), wines which contain less than 10 mg/L of protein may be considered stable. However, wines with higher concentrations of protein have also been reported to be stable (Singleton, 1974). Therefore, protein instability remains a potential problem to the winemaker since there are no qualitative tests for the detection of unstable proteins.

Description of Wine Proteins

Wine proteins are globular proteins, mainly albumins (Hennig, 1970). Many of the albumins are water soluble and form colloids; some are soluble in salt solutions and others are completely insoluble. In wine, the only albumins present are those which, in colloidal solution, cannot be precipitated by a 10% (v/v) solution of alcohol (Hennig, 1970).

There is a considerable variation in the MW of wine proteins; they have been reported to range anywhere from MW of 8,000-150,000

(Moretti and Berg, 1965; Tarantolo, 1971; Feuillat and Bergeret, 1972; Somers and Ziemelis, 1973a; Singleton, 1974).

Origin and Concentration of Proteins in Wines

Origin of Wine Proteins

Bayly and Berg (1967) carried out an extensive study of protein fractions in varietal white grape juices and wines. They concluded that the critical proteins originate in the grape and that the fermentation process apparently does not contribute any protein to the medium. The same conclusion was reached by Feuillat and Bergeret (1972) who found the same protein fractions in corresponding musts and wines.

In contrast, Moretti and Berg (1965) reported that wines generally have more protein than the juices from which they are made. They proposed that the additional protein is released by the yeast during fermentation. However, it has been shown that it is only when wine spends a long time on its fermentation lees that there is a significant contribution to wine proteins from the yeast (Poux et al., 1964; Tercelj, 1965; Sheat and Discombe, 1976).

Concentration of Proteins in Wines

The amount of protein in grape juices and wines is very variable and depends on several factors. Some of these factors are grape variety, nutritional status, conditions of maturation, time of harvest, season and region (Tarantola, 1971). For example, more protein is found in wines from warmer seasons (Tarantola, 1971; Thoukis, 1974; Sheat and Discombe, 1976). Also, rotten grapes which have lost a great deal of

their sugar, yield a wine with an abnormally high nitrogen content (Genevois and Ribereau-Gayon, 1936). But, in general, the level of protein in wine is low; it has been estimated to be 3.0-14.7 mg/L (Anelli, 1977), 7-23 mg/L (Hennig, 1970), and 180 mg/L (Rentchler, 1969). However, much higher values are not uncommon (Singleton, 1974).

Composition of Wine "Protein" Haze

The material that constitutes "protein" haze has been isolated and analysed. The residue obtained after precipitation with acids and drying is composed of nitrogen including protein nitrogen, phenolics, polysaccharides and minerals. In general, the percentage of the above components is variable. Ribereau-Gayon et al. (1976) reported that "protein" haze contained 5-12% of nitrogen (of which 50-80% is protein), 1-15% of ash, the remainder being divided between adsorbed phenolics (2-5%) and polysaccharides (12-14%) which, upon hydrolysis, yield reducing sugars. Higher proportions of the protein component (40-50%) have been reported by Moretti and Berg (1965) and Usseglio-Tomasset (1978).

Factors Affecting the Stability of Wine Proteins

The phenomenon of protein precipitation from wine is complex and is affected by several factors, both extrinsic and intrinsic.

Extrinsic Factors

Among the extrinsic factors, temperature and added gelatin are the most important. In handling wines, one must be careful in controlling

the temperature. If bottled wines are subjected to a sudden increase in temperature, above 25°C for example (as may happen during transportation) followed by cooling down to refrigeration temperature, the proteins can floculate out as a haze or precipitate.

Gelatin is sometimes used as a fining agent in the wine industry. However, it is possible to "overfine" wines with added gelatin, such that the wine is less protein stable owing to residual gelatin in the wine (Singleton, 1974).

Intrinsic Factors

The nature of wine proteins, pH and isoelectric point (pI), and the type and concentration of phenolics are among the intrinsic factors that affect the stability of proteins in wine. Excessive total concentration of protein is not the usual source of protein instability in wine, as unstable wines have been found to have low protein content; conversely wines with fairly high protein content have been found to be stable under practical conditions (Singleton, 1974; Ribereau-Gayon, 1976).

Wine phenolics contribute to "protein" haze, in that they may oxidize and polymerize into tannins which are capable of addition reactions with the amines and sulfhydric groups of proteins (Singleton, 1974).

The pH and pI of a wine can affect its stability. It has been shown that wines with a pH close to 3.0 are more likely to be heat stable than wines of higher pH value (Moretti and Berg, 1965; Bayly and Berg, 1967). However, it should be recognized that a high pH wine is not necessarily protein unstable (i.e. some wines with pH values ranging from

3.33-3.75 have been found to be protein stable) (Moretti and Berg, 1965).

The pI of proteins from various wines have been reported to fall in the range of 3.3-3.7 and are very close to the pH of the wines. In general, the wine protein which precipitates first is likely to be the largest, most denatured, and nearest to its pI. Its precipitation may be induced by metals or oxidized phenolics (Singleton, 1974).

Mechanisms of Haze Formation in Wine

The various colloids of wine react to form "protein" haze. Two mechanisms have been proposed to account for these reactions. The first mechanism is based on electrical charge. In wine, proteins exist in solution as positively charged particles of colloidal size whereas tannins are negatively charged. It is proposed that the positive charge of the proteins is neutralized by the negative charge of the tannins, resulting in the formation of a complex which floculates and forms a haze (Ferenczy, 1966; Somers and Ziemelis, 1973a; Thoukis, 1974). The second mechanism involves reactions between molecules such as the interaction between phenolic hydroxyl groups and basic amino acids, and residues of proteins in salt linkages. It is proposed that hydropholic interactions between the aromatic ring structures of phenolics and hydrophobic regions of proteins are also involved (Loomis, 1974).

Protein Stability Tests

To prevent commercial rejection of wines, the winemaker used empirical tests to determine the amount of fining agent or agents that are required to protein stabilize each wine. The tests most often employed include: the heat and cold tests, the trichloroacetic acid (TCA) test, the bentotest or the Microkjeldahl test.

Heat and Cold Tests

The heat and cold tests are probably most comparable to the eventual effects of storage on bottled wine. The main disadvantage is that they tend to be slow. A test for protein stability recommended by Berg and Akiyoshi (1965) for Californian wines consists of holding wines at elevated temperatures (usually 50°C) for 48 hrs; then examining the wine for haze in a strong beam of parallel light. This is followed by storage at room temperature for 24 hrs, then by storage at low temperature for 48 hrs and another examination of the wine for up to 24 hours. The test takes six days to complete. The wine is classified as unstable if a visible haze or amorphous deposit appears as a result of the treatment. A nephelometer can also be used to measure the haze in place of visual observation.

The heat test is based on the assumption that storage at elevated temperatures for two days will correlate with behavior at ambiant temperatures for a longer period. Past experience shows this assumption to be generally true (Moretti and Berg, 1967).

Variations of the heat test are available, which require less time to complete. For example, larger California wineries heat wine for 2 days at 49°C followed by storage at room temperature for 24 hrs (Berg and Akiyoshi, 1961), whereas Australian wineries use an 80°C test which involves heating the wine for six hrs followed by cooling in a refrigerator at 4°C overnight (Pockock and Rankine, 1973). In a comparison between the 50°C and the 80°C heat tests on Australian wines, the latter was found to be less sensitive than the former (Pocock and Rankine, 1973). Other combinations of time and temperature have been reported, but none has been found to be as satisfactory as the 50°C or the 80°C (Pocock and Rankine, 1973).

Trichloroacetic Acid (TCA) Test

The TCA test consists of adding one ml of a 55% TCA solution to 10 ml of wine, followed by heating (2 min) in boiling water and a reaction period of 15 min at room temperature. However, the amount of haze formed does not always correlate with protein stability as measured by the heat and cold tests. The presence of unstable substances other than protein apparently does not affect the test (Berg and Akiyoshi, 1961).

Bentotest

The bentotest reagent consists of a solution of phosphomolybdic acid in HCl. It precipitates the protein in the wine by neutralizing the charge on the protein molecules and also aggregates them by cross-linking with the heavy molybdenum ion. In general, the bentotest tends to be more sensitive than the heat test and may lead to overfining (Pocock and Rankine, 1973).

Microkjeldahl Test

This test consists of determining the protein content of a filtered wine according to the procedure devised by Kean (1956). It involves the determination of total nitrogen by Microkjeldahl following vacuum concentration of the wine and precipitation of the protein with 25% TCA. Wines are considered stable if their protein content is below a certain value. Although described as satisfactory, this test is seldom used because it is time consuming and the amount of protein seldom correlates with stability (Berg and Akiyoshi, 1961).

Methods of Haze Prevention in Wines

Theoretically, there are four main ways that wines can be rendered protein stable. They include: heat treatment (e.g. bentonite) and treatment by protease. The latter method is not commercial practice at this time due to the lack of adequate proteases.

Heat Treatment

Heat treatment of a juice or wine can produce practical stability to protein haze while some protein still remains in solution. This is because unstable wine proteins are ordinarily rendered insoluble by higher temperature; unstable wine proteins have been found to coagulate between 60-75°C (Singleton, 1974). At this temperature the globular protein molecule unfolds, becomes more extended, denatured until it precipitates out. The temperature and the holding time of heat treatment vary. Flash pasteurization of the juice is usually carried out at

87°C for 2 minutes. It removes unstable wine proteins while also achieving biological stabilization and is preferable to heating the wine itself.

Other forms of thermal treatment of wines have been reported (Ferenczy, 1966). They include: (1) holding the wine at 30°C for several months; (2) 40°C for 30 min; or (3) 70°C for 30 minutes.

They are aimed at modifying the proteins enough to cause their precipitation prior to bottling the wine. Unfortunately, heating any wine for a substantial length of time is not recommended since it causes damage to its flavor, not to mention the potential problem of microbial spoilage. In general, heat treatment is not a widely used means of achieving protein stability in quality wines since it adversely modifies wine flavor.

Tannin-Gelatin Fining

Tannin and gelatin (15-120 mg/L each) were the most common fining agents in California in the pre-Prohibition era (Amerine et al., 1979). Gelatin micelles are positively charged while tannin particles are usually negatively charged. When they are mixed, they neutralize each other, causing their rapid precipitation and co-precipitation of wine proteins. The reaction is affected by temperature, pH, aeration, metals and previous treatment of the wine. The danger of overfining which causes persistent cloudiness is the reason that wines are seldom fined by this method now (Amerine et al., 1979).

Bentonite Fining

Bentonite fining is the international method of choice for fining wines. Bentonite is a naturally occuring, hydrated aluminum silicate clay consisting of montmorillonite and minerals (i.e. Mg, Fe, Ca, Na, etc.). It is odorless, hygroscoric and has a slight earthy taste (Sheat and Discombe, 1976). In solution, it exists as small negatively charged plates approximately 1 nm thick by 500 nm wide. When bentonite is soaked in water, the plates separate more or less completely to form a homogenous colloidal suspension which presents an enormous surface area. It is a selective absorbant for proteins which, according to Singleton (1974), causes the progressive removal of both stable and unstable proteins, but tends to remove the more heat unstable proteins first. The mechanism of its action involves electrostatic precipitation by neutralizing positively charged particles, either by proton transfer or cation exchange (i.e. absorption of proteins by binding them through their amino groups or absorption of uncharged molecules by hydrogen bonding (Singleton, 1967a; Amerine et al., 1979).

As normally used (1-12 lbs/1000 gal of wine), bentonite removes 20-60% protein but sometimes removes all of it (Singleton, 1974). It can be added to the wine after it is fermented, or to the grape juice prior to fermentation. According to Jakob (1975), the advantages of bentonite fining include:

(1) Low cost.

(2) The removal of certain toxic side products of microbial proceedings (e.g. histamine) and biogenic amines.

(3) Elimination of soluble metabolites caused by the use of systematic fungicides.

(4) Removal of protein, based on reliable test methods which can be carried out on wine.

The negative effects of bentonite fining include:

(1) The possible release of calcium, sodium, aluminum and other trace metals. For instance the release of calcium by calcium bentonite can cause the formation of calcium tartrate precipitates.

(2) Loss of color (i.e. excessive brightening of the wine) (Ough et al., 1969).

(3) Gross alterations in wine constituents, including reduction in volatiles which contribute to the taste and aroma of the wine (Ough et al., 1969).

(4) Loss of wine due to bentonite lees.

It is clear then that, although bentonite fining is convenient, it is not the recommended method for all wines (Somers and Ziemelis, 1973b; Jakob, 1975).

Enzyme Treatment

Although attempts to add proteases directly to wines and juices were made almost 50 years ago, more serious studies were only undertaken during the last two decades (Tzakov, 1970). The literature on the subject is scattered and findings are inconclusive (Boehringer and Jaeger, 1953; Rentschler, 1969; Datounachvili, 1970). There are several reasons for this situation: (1) studies conducted were not precise; and (2)

many of the traditional proteases (papain, pepsin, trypsin) are not suitable for use in wine; they require high temperatures that are deleterious to wine quality, and they do not have their pH optimum in the range of 3-4 which is ideal for wines.

Therefore, further studies using different and more highly purified proteases are needed so that the specificity of their action is better defined and exploited (Dravert, 1967; Rentschler, 1969; Singleton, 1974). If suitable proteases were discovered, it is probable that their use in enology would become common practice. Several reasons account for this optimism:

(1) proteases are naturally present in grapes, although in minute quantities (Cordonnier and Dugal, 1968; Cordonnier, 1970; Tzakov, 1970; Marteau, 1972; Feuillat et al., 1980).

(2) Proteolytic enzymes capable of hydrolyzing protein at must pH (3-4) have been reported, although they were not pure enough to be characterized (Marteau, 1972).

(3) Proteases have been successfully used in the beer industry for seven decades to prevent "chill haze", which is in many aspects similar to "protein" haze.

Other Methods

Ultrafiltration, casein and silica sols have been assayed as haze prevention tools. According to Wucherpfenning (1978) it should be possible to prevent protein haze formation in wines with the use of ultrafiltration membranes that retain substances of a MW larger than 10,000.

Such membranes also remove condensed polyphenols which are believed to complex with proteins to form haze (Wucherpfennig, 1978).

A water-soluble modified casein (Na or K caseinate has been added to wines for fining purposes (Amerine et al., 1979). The acidity of the wine is believed to neutralize the alkali of the caseinate causing a curd-like precipitate which physically removes protein as it settles. However, casein does not compare well with bentonite as a fining agent (O'Neal and Cruess, 1950).

Silicates have been used as fining agents in the wine and juice industry in Europe. They work by decreasing protein and phenolics; they have been found to be superior to tannin-gelatin in terms of lees, clarity achieved and speed of sedimentation (Amerine et al., 1979).

Protein Determination Methods

Although the nitrogenous constituents of wines and musts have been the subject of many investigations over the years, current methods of measuring proteins in wines are inadequate (Potty, 1969; Somers and Ziemelis, 1973a). It is not uncommon to find variations of several orders of magnitude in the protein content of the same wine determined by different procedures. Several reasons account for this including:

(1) protein exists in wine in very small amounts.

(2) Many of the analytical methods are empirical and sometimes arbitrary in nature.

(3) Approximately one-half of the protein is bound to a minor quantity of phenolics, which interfere with colorimetric protein determination (Potty, 1969; Loomis, 1974).

(4) Many of the methods are based upon precipitation of the protein fraction by ethanol, TCA, ammonium sulfate, etc., followed by testing with Kjeldahl or colorimetric protein determination by the biuret reaction (Somers and Ziemelis, 1973a).

Thus the reliability of these analyses depends upon the complete precipitation of the protein material which is difficult to achieve (Koch and Sajak, 1959; Bayly and Berg, 1967; Somers and Ziemelis, 1973b). The more important methods that are currently being used to determine protein in wine include: the Kjeldahl, the Voit, the Diemaier and Maier procedure and gel filtration.

Kjeldahl Method

Most early investigations on wine protein were carried out by determining nitrogen by the Kjeldahl procedure on precipitated samples and multiplying by 6.25. Unfortunately, values obtained this way are probably too high and not representative of protein since grapes and wines contain, in addition to proteins, a large amount of nitrogenous compounds which are precipitated along with the proteins (Ferenczy, 1966; Bayly and Berg, 1967).

Voit Method

This method was developed in an attempt to overcome some of the disadvantages of the Kjeldahl method (Koch and Sajak, 1959). It involves using phosphomolybdic acid to precipitate the large nitrogenous compounds followed by the use of 95% alcohol to wash the precipitate and selectively remove the proteins, which are then measured by Kjeldahl.

However, there is concern that the designation of the nitrogen flocculable by alcohol as protein nitrogen is arbitrary (Cordonnier, 1966).

Diemaier and Maier Method

The modified Diemaier and Maier procedure as described by Bayly and Berg (1967) utilizes both phosphomolybdic acid and 96% alcohol to precipitate the proteins, followed by their determination by the Biuret procedure according to Lowry. It is the method recommended by the Office International de la Vigne et du Vin (OIV). Unfortunately, it has been found that precipitated peptides also react with the Biuret reagent resulting in artificially high protein values (Somers and Ziemelis, 1973a).

Gel Filtration Method

This method, proposed by Somers and Ziemelis (1973a) is based upon the separation of the protein fractions by Sephadex G-25, followed by analysis by UV absorption. It proved that a linear relationship exists between peak height and protein content in a finished wine. One disadvantage of this method is its time consuming nature.

Other Methods

Many other methods have been reported, some of them related to those discussed above. A good discussion of the various wine protein methods is reported by Amerine and Ough (1980). It is concluded that existing methods for determining proteins in wines all have deficiencies and that accurate data is difficult to obtain (Cordonnier, 1966; Ferenczy, 1966; Kichkovsky and Mekhouzla, 1967; Amerine and Ough, 1980).

Purification of Wine Proteins

It is difficult to obtain a good separation of wine proteins because they tend to be associated with other macromolecules of a polyphenolic or pectinic nature (Feuillat and Bergeret, 1972). Early studies on wine proteins met with limited success due to the lack of purification procedures that would separate proteins from association molecules without denaturing them. A mere precipitation by such reagents as TCA, ammonium sulfate and alcohol were not good enough (Kichkovsky and Mekhouzla, 1967). Furthermore, dialysis alone was unable to remove interfering molecules such as polyphenols and nucleic acids (Bayly and Berg, 1967; Somers and Ziemelis, 1973a).

Satisfactory analysis of wine proteins requires that the initial separation of proteins from lower MW substances be more efficient than has proved to be possible with any denaturant or complexing agent. It has been reported that only a combination of dialysis, gel filtration and low temperature concentration can lead to protein fractions that are sufficiently purified to be analyzed by electrophoresis (Feuillat and Bergeret, 1972).

The Problem of Beer "Chill-Haze"

Description of Beer "Chill-Haze"

In the early 1900's, the U.S. beer industry had problems with bottled beer; the beer would develop a haze, especially on chilling, which greatly lessened its appeal (Wallerstein, 1961). It was sus-

pected and later confirmed that protein was causing the haze, along with phenolics and metals.

There exists a strong analogy between wine "protein" haze and beer "chill-haze". "Chill-haze" like "protein" haze is composed of protein (up to 60%), iron and copper (2-4%), carbohydrates (2-4%) and tannins (20-30%) (Singleton, 1967). The MW of the proteins in "chill-haze" has been estimated to range from 10,000-100,000. The MW of wine proteins is in the same range (Singleton, 1974). In "chill-haze" as in "protein" haze the protein serves only as a "support" on which tannins and phenolics become attached causing co-precipitation.

Use of Fining Agents in Beer

Synthetic compounds such as polyvinyl pyrollidone (PVP) and nylon have been used in beer to prevent "chill-haze". They work fine, but their use is limited because of the artificial "label" associated with such additives; particularly as some residues may remain in the beer after treatment (McFarlane, 1961; Singleton, 1967).

The Use of Proteases to Prevent Beer "Chill-Haze"

The addition of proteases to beer was first proposed by Wallerstein (1961) in 1910 and it led to the successful solution of the "chill-haze" problem as soon as suitable enzymes were developed. The technique has contributed more to the successful production of packaged beer than any other technical development (Hebert *et al.*, 1978). The enzyme most often used in beer is papain.

The enzyme (papain) is added to the beer during pasteurization which follows bottling. The papain becomes active and the proteins which cloud the beer when chilled are so modified by the proteolysis that the resulting beer remains clear and brilliant, no longer being sensitive to cold (Wallerstein, 1961).

The history of the successful application of proteases to the solution of the problem of "chill-haze" in beer, therefore, would serve as a valuable blueprint in our effort to find and apply suitable proteases for the clarification of white table wines.

Dye Release Method for Protease Assays

A simple colorimetric method to detect proteolytic enzymes in fluids was developed by Rinderknecht et al. (1968) and modified by Little et al. (1979). It is based on the release of Remazobrilliant blue dye covalently bound to an insoluble protein substrate (hide powder) by the action of protease on peptide bonds. This Hide Powder Azure (HPA) test is similar to that used in the beer industry to measure residual protease activity following pasteurization (Hebert et al., 1978). The HPA method will be used to characterize the two proteases used in this study.

MATERIALS AND METHODS

Grapes and Wines

Grapes used in this study were produced in 1978, 1979, and 1980 seasons in Oregon and Washington. Three varieties were studied in detail. They were: from Oregon, Chenin blanc 877 (Hyland Vineyards, Sheridan); White riesling 946 (a blend of grapes from Sokol Blosser Winery and Vineyards, Dundee, Lewis Brown Horticulture Farm, Corvallis, and the Southern Oregon Experiment Station in Medford); from Washington, Gewurztraminer 80 (Sagemoore Farms).

Grapes were processed at the Oregon State University (OSU) Winery, except Gewurztraminer 80 which was processed at the Sokol Blosser Winery. After routine determination of their sugar content (% solids) pH and titratable acidity, they were crushed with a Garolla stemmer-crusher, with approximately 50 ppm sulfur dioxide (SO_2) added to the juice which was stored frozen at -40°F in glass jugs. When needed, the juice was thawed at 53°F, and the SO_2 of the settled juice was adjusted to 25-30 ppm. The wines that were screened for protein instability were produced by the OSU Experimental Winery.

Protease Enzymes

Source of the Proteases

The two enzymes used in this study were supplied by Rohm Gmbh, Darmstadt, West Germany, via their U.S. subsidiary Fermco Biochemics, Inc., Elk Grove Village, Illinois. They were: (1) Fungal Protease S (commercially available); and Fungal Proteinase EL57-79 (from Aspergillus sp.)

The latter was an experimental protease selected by Rohm for its potential application in wines. Upon receipt, both proteases were stored in a refrigerator (ca. 4°C) until needed.

Protease Treatment of Juice

The required amount of enzyme was weighed and dispersed in a minimal amount of distilled water in a beaker. A glass rod was used to stir and dissolve the enzyme. When a given juice sample did not require the application of heat, the dissolved enzyme was added to the juice, followed by mixing and storage at 34°F for up to 24 hrs before the addition of yeast to commence fermentation. When heating was required, the juice was heated in a water bath maintained at the desired temperature and the enzyme was added with constant stirring. The mix was held for the specified period of time before the addition of yeast.

Protease Treatment of Wine

The required amount of enzyme was added to the wine with constant stirring and the wine was held at the desired temperature for the specified length of time. The free SO_2 was adjusted to about 30 ppm and the wine was bottled and stored at 53°F until evaluated.

Fermentation

A yeast starter was prepared by rehydrating active dry wine yeast, Champagne Strain (Universal Foods Corporation, Milwaukee, Wisconsin), in 1:1 grape juice-water at 30-35°C. The rehydrated culture (2%) was incubated at room temperature for up to three hours.

The settled juice or enzyme treated juice was racked into one gal jugs and inoculated with 2% yeast starter. The containers were sealed with fermentation locks or cotton plugs. All wines were fermented to dryness at 71°F, racked twice, their free SO_2 adjusted to 30 ppm and they were bottled and stored at 53°F until needed.

Effect of Protease on Fermentation

Fermentation Rate

Small scale fermentations were carried out to determine whether heat treatment or protease treatment had any effect on the rate of fermentation and the wine chemistry. One hundred ml. of each juice was transferred into a 250 flask, two ml of yeast inoculum was added and a fermentation lock was installed; the weight of each flask was determined at time zero, and then every six hrs during the first four days, and thereafter every 12 hrs until fermentation was complete. Each trial was conducted in triplicate and the average mean mass loss was plotted against time and the slope of the different curves compared.

Chemical Composition of Wines

The content of each of the flasks was analysed for chemical changes and changes in color. Color was measured by determining the absorbance at 420 nm. The recommended procedures of Amerine and Ough (1980) were used for the determination of pH, titratable acidity (TA), alcohol, soluble solids (refractive index), total and free SO_2 and total phenols.

Wine Fining with Bentonite Substitute Fining

Baykisol-30

A 10% solution of Baykisol-30 (a pure solution of colloidal silicic acid) obtained from Erbslok and Co. (Geisenheim/Rhein, West Germany) was prepared and 3.7-37 ml of this solution was added per gal of wine and mixed in with agitation. The silicate was allowed to react for up to two days at 53°F. The clarified wine was then racked off its lees and stored in bottles at 53°F until needed.

Gelatin

A 1% solution of gelatin was prepared and added to wine separately or with the Baykisol-30. The final concentration of gelatin was 12.5-100 mg/L.

Baykisol-30 was added as above. The wine was allowed to clarify and it was then processed as above.

Proteases

Twenty-five to 200 mg of proteases S and EL57-79 were added to the juices and wines as described in the sections on enzyme treatment of juice and wine.

PVPP and XAD-4

PVPP and XAD-4 were added as described below (page 29).

Protein Stability Tests

Heat and Cold Stability Tests

A Volclay bentonite solution (30 g/L) was heated to incipient boiling three times, cooled down and stored in a 4°C refrigerator until needed. One ml of this solution, added to 250 ml of wine, was equivalent to adding 1 lb of bentonite to 1000 gallons of wine. Each sample of wine was fined with 0-5 lbs of bentonite and stored at room temperature for at least 48 hours. The wine was then racked off the lees and filtered through Whatman paper (No. 5) and Millipore membrane (0.45 μ).

Filtered samples were transferred into 65 ml bottles leaving a 10% headspace, and subjected to protein stability tests. The short (80°C) heat test and the Bentotest were compared to the long 50°C test for 24 wines produced in 1978 and 1979 by the OSU Experimental Winery.

Bentotest

One ml of Bentotest solution (Dr. Jakob, Fritz Merkel, Geisenheim Rheim, West Germany) was added to 10 ml of filtered wine in test tubes. The test tubes were stoppered, and the contents mixed and observed for haze which forms immediately if any unstable proteins are present. A precipitate/flocculate, if any, appears within one hour. The intensity of haze or precipitate was recorded.

Use of Hunter Color/Difference Meter to Measure Wine "Protein" Haze

After the heat and cold test was completed, the tested samples were mixed for homogeneicity then poured into a plastic spectrophotometer

cell (one cm pathlength). The cell was placed in a Model D25P-2 Hunter Color/Difference Meter (Hunter Associates Lab., Inc., Fairfax, Virginia) and haze value measured according to manufacturer's instructions.

Protein Determination Methods

The protein content of wines and juices was determined by the Diemaier and Maier, the Bradford and a modified Kjeldahl procedures as follows.

Modified Diemaier and Maier Method

The Diemaier and Maier protein method as described by Bayly and Berg (1967) was used with slight modifications: 20 ml of phosphomolybdic acid solution were added to 20 ml of wine in 50 ml centrifuge tubes and stored at room temperature for 24 hrs instead of 15 hours.

Bradford (Bio Rad) Method

The protein reagent described by Bradford (1976) was purchased from Bio Rad Laboratories (Richmond, California) and five-fold dilutions prepared. Five ml of the diluted reagent were added to test tubes containing 100 ul of wine (100 ul of water in the control). The content of the tube was mixed using a lab bench top mixer and the absorbance was measured at 595 nm using a one cm glass cell and a Perkin-Elmer 550 Spectrophotometer (Perkin-Elmer Corporation, Coleman Instrument Division, Oak Brook, Illinois).

Modified Kjeldahl Method

The Kjeldahl method used was a modification of that described by Amerine (1980). Proteins were precipitated and washed as in the Diemaier procedure above. The precipitate was transferred with water into a 100 ml Kjeldahl digestion flask. Five ml of concentrated sulfuric acid and two g of catalyst were added to the flask, along with two boiling chips, and the digestion was conducted with moderate heat for up to six hours. The digested sample was suspended in a minimum amount of water and redistilled using saturated NaOH and the recovered NH_3 was titrated with a standard 0.1 N HCL solution.

All three methods were performed on unmodified samples of white wines, then on the same wines after dialysis and treatment with PVPP and/or XAD-4. Selected red varietal wines were also analysed to determine the effect of their high phenol content on the procedures.

Removal of Wine Phenolics with PVPP and XAD-4

The polymer polyvinylnyrrolidone (insoluble PVP) and the hydrophobic synthetic resin Amberlite XAD-4 were used separately and as a mixture to remove phenols. The PVPP was treated as described by Loomis and Bataille (1966) and Loomis (1974), and XAD-4 was pretreated as described by Loomis et al. (1979). The PVPP and/or XAD-4 (0-4.5 g of each) were mixed with 50 ml of wine and stored in a covered beaker in a refrigerator. The following day the wine was centrifuged at 16,000 rpm for 15 min and the supernatant was decanted and analysed for protein and total phenols. As optimum removal of phenols occurred when one part of

XAD-4 was added to 29 parts of wine (w/v), the same ratio was used in subsequent trials.

Purification of Wine Proteins

To obtain protein extracts suitable for electrophoresis, juices and wines were submitted to dialysis, low temperature concentration, gel filtration and a second concentration as follows.

Dialysis

One hundred ml of wine or juice was filtered through Whatman paper (No. 5) layered with diatomaceous earth and then through millipore membrane. Up to 1% of sodium sorbate was added to juices to prevent mold growth. The samples were then dialyzed against a Tris-glycine buffer (pH 9.2) for 60 hours. The buffer was changed after 12 and 36 hours. The dialysis tubing was a 1¼" cellulose membrane (Arthur H. Thomas Company, Philadelphia, Pennsylvania) with a MW cut off of ca. 8,000.

Vacuum Concentration

Dialyzed juice or wine was placed in a 250 ml round-bottom flasks and concentrated with a rotary evaporator (Buchi Laboratories, Flawil, Switzerland) to about 1/20 of its original volume. The temperature of the water bath was maintained at 32-35°C and the temperature of the concentrate was never allowed to exceed 20°C. Following gel filtration, it was determined that the protein was in the first eight fractions (40 ml), which were pooled and concentrated to about 5 ml. Approximately

0.025% of sodium azide was added to the concentrated sample which was stored at 4°C if used within one week or freeze dried if longer storage periods were required.

Gel Filtration

Gel filtration was performed using a 1.4 cm X 50 cm chromatography column (Pharmacia Fine Chemicals, a Division of Pharmacia, Inc., Piscataway, New Jersey) packed with G-25 sephadex gel (Pharmacia). The gel was pre-soaked in a 3% acetic acid buffer (pH 3.6). Approximately five ml of the dialyzed and concentrated sample were applied on the gel with a sample applicator. The flow rate of the buffer was maintained at one ml per min with a peristatic pump (Fluid Metering, Inc., Oyster Bay, New York) and the effluent was collected in test tubes with a fraction collector (Model 272) and a volumeter (Model 400) both from Instrumentation Specialties, Inc., Lincoln, Nebraska. The system was set to deliver five ml per tube. The absorbance of each fraction was measured at 280 nm.

Electrophoresis

Various juices and wines (control, heat-treated, enzyme-treated, fined with increasing concentrations of bentonite) were treated as described above, and subjected to electrophoresis.

Polyacrylamide Gel Electrophoresis

The number of protein fractions in each juice and wine sample was determined by polyacrylamide gel electrophoresis (PAGE). The procedure was that of Davis (1964) with the modifications described by Halim and

Montgomery (1978) and Smith (1979), except that a 12.5% gel was used instead of a 7% gel. The voltage power supply was an EC-Model 454 (EC Apparatus Corporation, Philadelphia, Pennsylvania).

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The relative MW of the different protein fractions present in the juices and wines was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The method employed was a modification of that described by Laemmli (1970), discs being used instead of slabs. A 12.5% running gel and a 4% stacking gel were found to provide the best resolution. The staining solution and the destaining solution were prepared as described by Smith (1979).

Low MW protein standards consisting of a mixture of proteins of MW 10,000-100,000 were obtained from Bio Rad Laboratories, Richmond, California, and diluted 20 fold with the sample buffer prepared according to Laemmli (1970). Gels were prepared in glass tubing 0.5 cm I.D. and 12 cm in length. Both the standard and the samples were mixed with sample buffer (one to one) in test tubes and heated in a boiling water bath for at least two min prior to their application on the gels. Fifty ul of each sample containing up to 25 ug of protein was applied to the gel, and electrophoresis was conducted at 150 volts (8 ma/tube) for 30 min, then the current was increased to 225 volts until the dye reached one cm from the bottom of the discs.

Following electrophoresis the gels were put in test tubes filled with staining solution and held for up to 10 hrs, then destained as in PAGE. The MW of the fractions was determined from a standard curve

obtained by plotting log MW of protein standard against their relative mobility.

Isoelectric Focusing

To further characterize the protein fractions in the various wines, their pIs were determined by isoelectric focusing. The latter was performed in a Desaga/Brinkmann Double Chamber Thin Layer Isoelectric Focusing (Brinkmann Instruments, Inc., Cantiague Road, Westbury, New York). The power was regulated by a No. 3-1155 voltage and Current Regulated Power Supply (Buchler Instruments, Division of Searle Analytical, Inc., Fort Lee, New Jersey).

One percent agarose gel in water was prepared according to Anonymous (1980) and while still warm (65-75°C) between 2-3% of the following ampholites were added to it: Pharmalyte^{tm} pH 3-10 and pH 6.5-9 (Pharmacia) and Ampholine^{r} pH 4-6 (LKB Produkter, Bromma, Sweden). The gel was poured on a 13 x 13 cm gel bond film (FMC Marine Colloids, Division of Bio Products, Rockland, Maryland) set on a warm TLC plate. A glass rod was used to spread the gel on the film. The gel was allowed to harden at room temperature for 15 min then put in the chamber and "cured" for one hr at 4°C.

Fifty mg of the freeze-dried wines were mixed with 0.5 ml of water in test tubes and stored on ice. The gel was prefocused at 150 volts for 15 min, then the current was increased to 200 volts and 10 ul of each sample was loaded (in 6-8 drops) one cm apart on the gel. The voltage was increased every 5-10 min up to a maximum of 900 volts. The run was completed in 1.5 hours.

The fixing, staining and destaining solutions were prepared according to Winter et al. (1978). The fixing solution was poured on the gel and held for 30 minutes. The gel was then washed with destaining solution to wash off any remaining ampholites. Staining (with Coumassie Blue) was performed for one hr, after which the gel was washed with distilled water, covered with destaining solution and stored overnight. After removing the gel from the destaining solution, it was washed with water and dried with an air dryer. The isoelectric points were determined by plotting the distance travelled by the protein fractions against pH.

Characterization of Protease Enzymes

Preliminary Protease Assay

Studies concerning the properties of the proteases were based upon the Hide Powder Azure (HPA) procedure described in the literature review. HPA was obtained from Calbiochem, Bering Corporation, La Jolla, California. One tenth molar citrate buffer solutions with pH ranging from 3-6 in increments of 0.2 pH unit were prepared. A citrate buffer (pH 3.5) was also prepared. Conditions under which the assay conditions were linear (with respect to both time and enzyme concentration) were determined by measuring absorbance against time as described below. Initial velocity was determined from the linear portion of the curve within 30 min of iniation of the reaction. One unit of enzyme activity was determined as the amount of enzyme which caused a change in absorbance of 0.01 per minute.

Effect of pH on the Activity of Proteases S and EL57-79

Nine ml of citrate buffer (pH 3-6) were added to 16 25 ml erlenmeyers. One ml of one mg/ml enzyme solution and 10 mg of HPA were added to each erlenmeyer before incubation at 45°C for one hr in an environmental incubator shaker (New Brunswick Scientific Company, New Brunswick, New Jersey). The shaking speed was maintained at 180 rpm. The blue solution that resulted was filtered through Whatman paper No. 4 and read at 595 nm on a Bausch and Lomb Spectronic 20, (Bausch and Lomb, Inc., Rochester, New York). The reference was citrate buffer (pH 3.5).

Effect of Temperature on the Activity of Protease EL57-79

One-half ml of a one mg/ml enzyme solution was added to 49.5 ml of citrate buffer contained in each of eight 300 ml sidearm flasks (Bellco, Vineland, New Jersey). Fifty mg of HPA were sealed in Miracloth bags, (Chicopee Mills, Inc., Millton, New Jersey), and prepared according to the procedure described by Little et al. (1979). The HPA bags were placed in the flasks containing the enzyme and each was incubated at 10, 20, 30, 40, 50, 60, 70 or 80°C and the absorbance was measured at 595 nm at time 0 and every 5 min for 60 minutes. An activity curve was derived by plotting (ΔA_{595}/min).

Effect of Concentration on the Activity of Protease EL57-79

One-tenth to one ml of a 5 mg/ml enzyme solution was added to citrate buffer (pH 3.5) in a sidearm flask to make a final volume of 50 ml. The enzyme concentration in these flasks was 10-100 mg/l. HPA was

added as above. Each flask was incubated at 50°C for up to 60 minutes. The absorbance was measured as previously and the activity as a function of enzyme concentration was determined.

Heat Stability of Protease EL57-79

Solutions of protease EL57-79 were prepared as above and stored in beakers at 10, 20, 30, 40, 50, 60, 70 and 80°C. After two and 12 hrs, aliquots of the incubated enzymes were removed and their activity was determined as described previously. Stability curves were derived by plotting residual activity against temperature.

Effect of Sulfur Dioxide on the Activity of Protease EL57-79

A 1% solution of SO_2 was prepared by dissolving 17.7 g of sodium metabisulfite (Mallinckrodt Chemical Works, St. Louis, Missouri) in 1 L of H_2O.

Ten, 25, 50, 100 and 200 ppm of SO_2 was added to sidearm flasks containing 0.5 ml of one mg/ml enzyme solution. Citrate buffer (pH 3.5) was added to make a final volume of 50 ml. Each flask was incubated at 50°C and the protease activity as a function of SO_2 concentration was determined.

Effect of Alcohol on the Activity of Protease EL57-79

To study the effect of alcohol on the activity of protease EL57-79, 0, 5, 10, 15, 20 and 25% alcohol was added to sidearm flasks along with 0.5 ml enzyme solution (1 mg/ml) and citrate buffer (pH 3.5) to a final volume of 50 ml. All flasks were incubated at 50°C and an activity curve was derived as a function of alcohol content.

RESULTS AND DISCUSSION

In this report the term wine will refer to white varietal table wine produced from grapes grown in Oregon and/or Washington, unless otherwise indicated; the terms stable and unstable will be used to designate wines containing proteins that are stable or unstable to heat; heat test will refer to heat and cold stability test.

Survey of Selected 1978 and 1979 Oregon and Washington Wines

A total of 20 Oregon and Washington white varietal table wines (10 varieties each from the 1978 and 1979 vintages) were screened for protein stability. Their pH, alcohol and protein contents were also determined. These wines were made from grapes grown on the same locations. The data obtained is summarized in Tables 1 and 2. They indicate that the 1978 and 1979 wines required an average of 4.9 and 3.6 lbs of bentonite/1000 gal respectively to become protein stable. It is noteworthy that some of the wines required up to 12 lbs of bentonite/1000 gallons. It is common knowledge that when such high amounts of bentonite are added to a wine, it adversely affects its appearance and its sensory quality (Ough et al, 1969; Somers and Ziemelis, 1973; Jakob, 1975; Hartmier, 1979). Therefore, it is apparent that, in some cases, protein instability is a concern in wines made from grapes harvested in Oregon and Washington. Also, since a few of the wines were very highly unstable, there is a need for a substitute for bentonite which would have a less harsh effect on the very highly unstable wines.

Table 1. Survey of 1978 Oregon and Washington White Varietal Table Wines.

Wine Code	Wine Variety	pH	Percent Alcohol	Protein* Concentration (mg/L)	Bentonite** (lb/1000 gal)
806	Gewurztraminer	3.71	12.1	205	12
828	Flora	3.48	13.4	229	6
833	Welsh riesling	3.14	11.0	111	2
835	French Colombard	2.90	12.8	168	1
852	Sauvignon blanc	3.90	12.4	200	12
856	Semillon	3.75	10.5	152	9
860	White riesling	3.11	11.7	112	2
865	Sylvaner	3.30	10.7	88	2
867	Chardonay	3.11	12.6	103	1
877	Chenin blanc	3.04	11.6	164	2
Average		3.34	11.8	153.2	4.9

* Determined by the Diemaier and Maier procedure.

** Amount of bentonite that was added to render each wine protein stable (1 lb/1000 gal. is equivalent to 12g/hl).

Table 2. Survey of 1979 Oregon and Washington White Varietal Table Wines.

Wine Code	Wine Variety	pH	Percent Alcohol	Protein* Content (mg/L)	Bentonite** (lb/1000 gal)
910	Gewurztraminer	3.56	12.4	390	12
915	Sylvaner	3.29	14.0	90	5
918	Flora	3.24	13.4	210	4
921	Semillon	3.23	12.8	96	3
945	White riesling	2.82	8.0	246	2
954	Sauvignon blanc	3.19	12.2	152	4
959	French colombard	3.07	11.2	122	1
961	Chardonay	3.17	11.2	134	1
966	Welsh riesling	3.14	8.0	192	2
967	Chenin blanc	3.03	9.0	144	2
Average		3.17	11.2	177.6	3.6

* Determined by the Diemaier and Maier procedure.

** Amount of bentonite that was added to render each wine protein stable (1 lb/1000 gal. is equivalent to 12 g/hl).

Furthermore, it is apparent from this data that there is no definite relationship between protein content and bentonite demand. For example, 1979 wines contained an average of 177.6 m g/L of protein as determined by the Diemaier and Maier procedure and required an average of 3.6 lbs of bentonite/1000 gal, whereas, 1978 wines contained a lower amount of protein (153.2 mg/L) but required an additional 1.3 lbs of bentonite/1000 gal to become stable. This is consistent with information available in the literature (Anelli, 1977). Furthermore, both tables show that the more highly unstable wines are the ones with higher than average pH, whereas, those wines with pH closer to 3 tended to be more stable. This finding is also compatible with that reported elsewhere (Moretti and Berg, 1965; Bayly and Berg, 1967).

Comparison Between Bentotest, the 50°C and the 80°C Heat and Cold Tests

In order to determine what tests are better suited and least time consuming for the determination of protein instability in PNW wines, a comparison was made between the long (50°C) and short (80°C) heat tests and the Bentotest. The three tests were performed on 20 randomly selected wines made in the OSU Experimental Winery. The Bentotest was more sensitive than either of the heat tests (Table 3). Using the Bentotest, the 20 wines required on the average of 2 lbs of bentonite/1000 gal more than the heat tests indicated. The 80°C heat test compared favorably with the 50°C test. According to both tests the 20 wines required the same level of bentonite treatment (4.1 and 4.3 lb/1000 gal). Because the 80°C heat test requires only 24 hrs to complete

Table 3. Comparison between Bentotest, 80°C and 50°C Heat Tests.

Wine* Code	Variety	Bentotest[a]	80°C[a]	50°C[a]
DAH 8	Oregon Gewurztraminer	2	0	0
DAH 9	Oregon Gewurztraminer	5	5	5
DAH 10	Washington Gewurztraminer	3	2	2
DAH 11	Washington Gewurztraminer	6	6	5
DAH 15	Oregon riesling	0	0	0
DAH 16	Oregon riesling	5	2	2
DAH 18	Washington riesling	0	1	1
DAH 19	Washington riesling	7	4	4
828	Flora	6	6	6
833	Welsh riesling	2	2	2
845	Semillon	6	2	2
852	Sauvignon blanc	20	12	12
856	Semillon	16	9	9
861	Washington riesling	7	2	2
869	White riesling	3	3	2
876	French colombard	4	1	1
877	Chenin blanc	2	2	2
878	French colombard	6	4	2
880	Pinot gris	16	14	14
881	Melon	16	9	9
Average	---	6.2	4.3	4.1

[a]Represents the amount of bentonite (in pounds) added to 1,000 gallons of wines to insure protein stability.

* Wines are from the 1978 vintage.

whereas the 50°C test requires up to six days, and because the Bentotest is very sensitive and can lead to overfining of the wines (Rankine and Pocock, 1971), it was concluded that the 80°C heat test provided a dependable and accurate way to predict protein stability. Thus, all succeeding protein stability tests were conducted at 80°C.

An Objective Method for the Measurement of Wine "Protein" Haze

Visual observation of the haze formed when unstable wines are subjected to the heat/cold tests or Bentotest is routine in the industry. This is very subjective methodology. Various instruments, among them the Spectrophotometer and the Hunter Color/Difference Meter were assayed for possible use in measuring "protein" haze. Only the Hunter meter was found to be adequate. The results obtained when the Hunter Color/Difference Meter was used to measure haze in three selected white varietal wines (treated with 0-5 lbs of bentonite/1000 gal) are shown on Table 4 and Figure 1. The latter is a graphical representation of the former. It shows the effect of increasing bentonite concentration on wine Hunter haze values. It is apparent that Hunter haze values decreased with increasing bentonite concentration; however, the decrease was not linear. Although these results were taken for three varietal wines, similar results were obtained with other varieties. This led to the conclusion that a Hunter haze value of 1.5 or less was indicative of a stable wine.

A Comparison of Three Protein Determination Methods

The Bradford (Bio-Rad) method of protein determination (Bradford,

Table 4. Determination of the Amount of Wine "Protein" Haze by Hunter Color/Difference Meter.

Sample and Bentonite added (lb/1000 gal)	Stability** by 80°C heat test	Stability** by Bentotest	Hunter Haze Value
Sylvaner (915)			
Control*	U+++	U+++	40
1	U+++	U+++	24
2	U+++	U+++	13.7
3	U++	U+++	7.5
4	S	U++	1.9
5	S	S	.7
distilled H_2O	----	----	.3
Gewurztraminer (80)			
Control[a]	U+++	U+++	37
1	U+++	U+++	15.0
2	U+++	U+++	10
3	U++	U+++	7.4
4	U+	U++	3.9
5	S	U+	2.0
6	S	S	1.0
Chenin blanc (877)			
Control[a]	U++	U+++	6.0
1	U+	U++	2.3
2	S	U+	1.2
3	S	S	.7
4	S	S	.6

* control (no bentonite added).

** S, stable; U, unstable. Amount of haze indicated by light (+) to strong (+++).

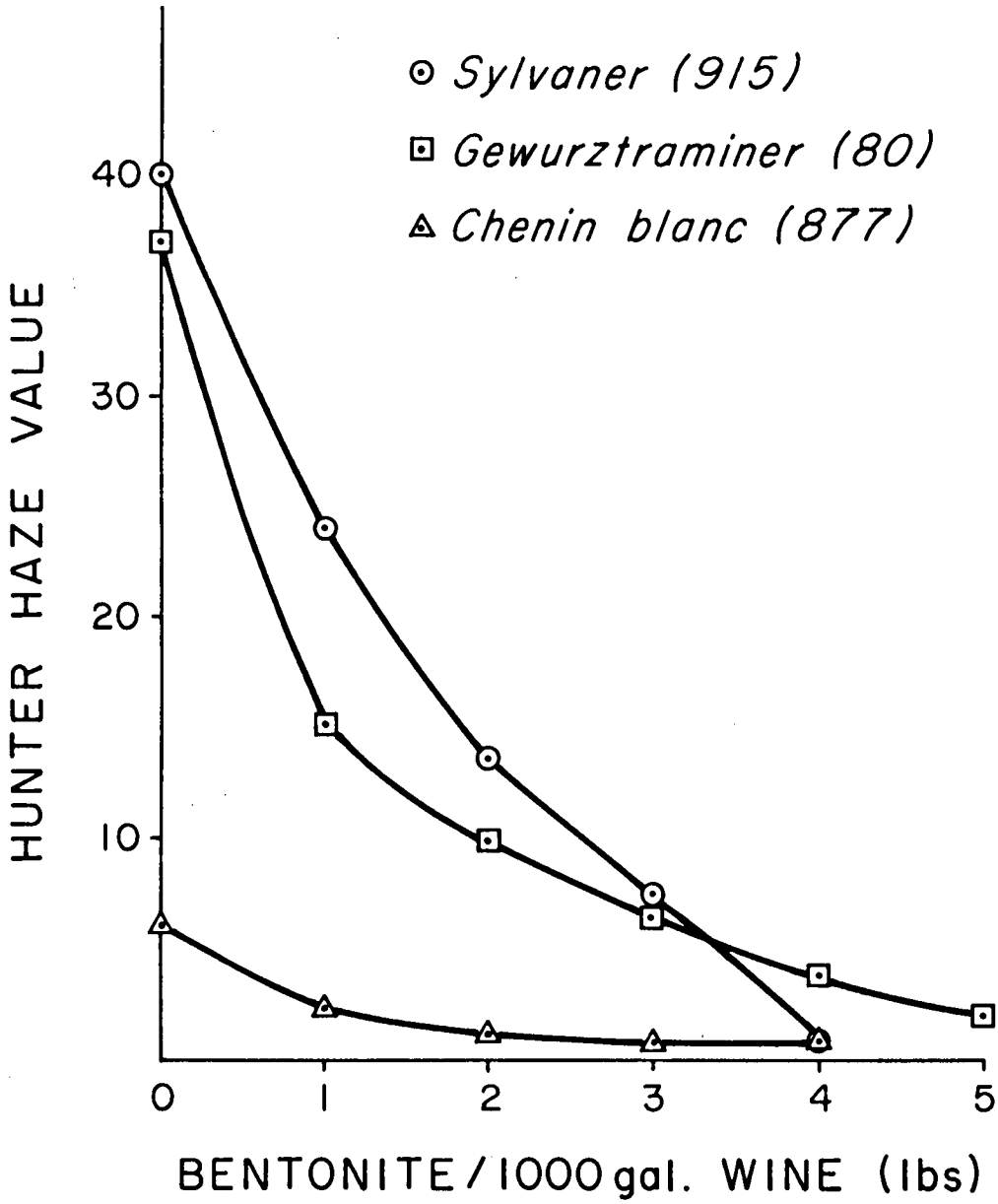

Figure 1. Effect of bentonite concentration* on wine Hunter Haze value.

* 1 lb/1000 gal. is equivalent to 12g/hl

1976) has been reported to be the best available method for protein determination in media which contain a high level of phenolics (Robinson, 1979). Wine is such a medium. Although the Bradford method had not been used to measure wine protein, it was used in this study. Wine protein values obtained with the Bradford method were compared to those obtained with two of the most commonly used methods, the Diemaier and Maier (D and M) and the Kjeldahl.

White Varietal Table Wines

Table 5 shows the results obtained when the three methods were applied to selected white varietal wines. There are large differences between the values obtained by the three methods. The D and M gave the highest values. This was not unexpected since the method is not selective for proteins. Amino acids and other non-dialyzable nitrogen compounds (e.g. low MW polypeptides) are known to contribute to protein values by this method (Somers and Ziemelis, 1973b). For example, using this method, Bayly and Berg (1967) determined that a model wine contained 500 mg/L of protein; it was later determined that the same wine contained no non-dialyzable material (i.e. no protein). Therefore, the D and M procedure is likely to give artifically high values. On the average, values obtained with the Kjeldahl (46.4 mg/L) were closer to those obtained with the Bradford method (38.4 mg/L) than with those obtained with the D and M procedure (158.7 mg/L). To determine the effect of non-proteinaceous materials on the values obtained by the three methods, the tests were conducted on the same white wines after they had been dialyzed for 60 hrs (Table 6). Dialysis caused a reduc-

Table 5. Protein Content of Selected White Varietal Table Wines Determined by the Bradford, Diemaier and Maier, and Kjeldahl Procedures.

Wine No. Code	Total Phenols (mg/L)	Protein Content* by Bradford (mg/L)	Protein Content* by Diemaier and Maier (mg/L)	Protein Content* by Kjeldahl (mg/L)
904	243	10.4 ± 0.7	56.0 ± 5.6	68.7 ± 6.2
931	168	76.0 ± 1.0	397.0 ± 18.4	23.3 ± 8.1
946	251	39.6 ± 0.7	142.4 ± 5.6	18.4 ± 2.0
959	127	20.3 ± 0.5	122.2 ± 9.2	50.0 ± 7.0
961	186	50.7 ± 0.0	134.1 ± 4.2	70.1 ± 10.2
964	184	30.0 ± 1.4	96.3 ± 6.3	24.0 ± 5.8
877	225	42.0 ± 3.1	163.0 ± 11.0	170.2 ± 14.5
Average	---	38.4	158.7	46.4

*Average of three determinations ± standard deviation

Table 6. Protein Content of Selected Dialysed White Varietal Table Wines Determined by the Bradford, Diemaier and Maier, and Kjeldahl Procedures.

Wine No. Code	Total Phenols (mg/L)	Protein Content* by Bradford (mg/L)	Protein Content* by Diemaier and Maier (mg/L)	Protein Content* by Kjeldahl (mg/L)
904	14.6	10.4 ± 0.0	40.3 ± 7.0	30.1 ± 1.8
931	16.2	88.1 ± 0.7	256.1 ± 12.7	33.0 ± 2.8
946	11.6	44.0 ± 0.7	98.2 ± 9.9	7.0 ± 1.1
959	17.3	18.2 ± 0.7	74.0 ± 7.7	22.2 ± 3.3
961	12.1	52.1 ± 1.4	92.1 ± 4.2	25.1 ± 1.4
964	11.6	28.2 ± 2.1	70.4 ± 7.1	15.1 ± 3.7
877	20.0	38.0 ± 3.5	104.0 ± 9.9	75.0 ± 5.6
Average	----	39.9	105.0	29.6

*Average of three determinations ± standard deviation

tion of about one-third in the values obtained by the Kjeldahl and the D and M methods (29.6 and 104.9 mg/L respectively). The value obtained with the Bradford method remained essentially the same (39.9 mg/L and 38.4 mg/L). It is proposed that dialysis caused a loss in the amount of peptides and small polypeptides that were contributing to the high values obtained with the Diemaier and Maier method. Those polypeptides could also have contributed to the Kjeldahl values. The Bradford procedure has been reported to be specific for proteins (Bradford, 1976). Dialysis did not affect protein values obtained by the Bradford procedure. Therefore, dialyzable wine compounds were not contributing to the protein content of wine as determined by the Bradford method. It was concluded that for the determination of non-dialyzable protein in white wines, the Bradford procedure is reliable and reproducible. Its use is therefore recommended. Also, it has the advantage of simplicity and quickness which are lacking in both the Kjeldahl and the D and M methods.

Red Varietal Table Wines

The possible interference of high phenolics concentrations on protein determination by the three methods discussed above was investigated. The tests were performed on selected red varietal wines (Table 7). It is apparent that the large amounts of phenolics contained in most red wines do interfere with both the Bradford and the D and M procedures. Protein values obtained by these two methods were misleadingly high, resulting from the absorbance measurement of phenolics rather than proteins. It is well established that high levels of

Table 7. Comparison of the Bradford, Diemaier and Maier, and Kjeldahl Procedures for the Determination of Proteins in Selected Red Varietal Table Wines*.

Wine Code	Wine Variety	Protein** Content (mg/L) by Bradford Method	Protein** Content (mg/L) by Diemaier and Maier Method	Protein** Content (mg/L) by Kjeldahl Method	Total Phenols (mg/L)
925	Malbec	>500	>500	11.5 ± 1.8	1574
928	Pinot noir	>500	106.5 ± 10.0	19.2 ± 3.1	1059
929	Meunier	328.8 ± 43.8	262.2 ± 19.8	50.3 ± 4.3	612
934	Cabernet Sauvignon	>500	>500	9.5 ± 3.1	1288
936	Pinot Francais	>500	120.4 ± 17.7	26.7 ± 2.1	572
939	Gamay teinturier	>500	>500	1.6 ± 0.2	904
947	Zinfandel	>500	>500	4.0 ± 0.5	1030
953	Pinot noir	480.7 ± 24.1	84.4 ± 19.8	12.5 ± 0.7	790

* Wines are from the 1979 vintage.

** Average of three determinations ± standard deviation

phenolics interfere with protein determination (Robinson, 1976). This does not detract from the usefulness of the Bradford method since the amount of protein in red wines is usually insignificant and of little concern to the winemaker (Ribereau-Gayon, 1976; Ough and Anelli, 1979).

Effect of Bentonite Substitutes on Wine Protein Stability

Baykisol-30, Gelatin, PVPP and XAD-4

It has been reported that silica sols, which are negatively charged can co-precipitate wine proteins which are positively charged (Gutcho, 1976). Therefore, if a wine were fined with silica sol, it could become stable. This possibility was investigated. When 3.7 to 18.5 ml/gal of Baykisol-30 was added to protein unstable wines, no significant improvement in protein stability resulted. The concentrations that were used are those recommended for use in beer (Gutcho, 1976). However, when considerably higher concentrations (37 ml/gal) were added, there was a small decrease of approximately one lb in the amount of bentonite required to protein stabilize 1000 gal of wine.

When Baykisol-30 (3.7 to 37 ml/gal) was used in combination with a 1% solution of gelatin in amounts corresponding to 50 to 400 mg/gal, not only was there no improvement in protein stability, but, at higher levels of gelatin, the residual gelatin contributed to the protein instability. Therefore, both of these two approaches were abandoned.

The use of PVPP and XAD-4 at levels used in the beer industry (Gutcho, 1976) did reduce the amount of total phenols by up to 50%, but they had no effect on protein stability as determined by the 80°C heat/cold test. Furthermore, the "artificial" label of these two ad-

ditives would be difficult to sell to a public that is more and more concerned about "natural" products. This approach was also abandoned.

Proteases S and EL57-79

Wines were treated with 25 to 200 mg/L of commercial protease S and experimental protease EL57-79 (P-EL). Both of these proteases had no significant effect on protein stability when they were added to wines and stored at either 59°F (14°C) or 71°F (22°C) for up to two weeks. However, a significant improvement in stability was attained when P-EL was added to wines at 115°F (46°C) and 140°F (60°C) and incubated for up to 24 hours. This agrees with the findings of Boehringer and Jaeger (1953) who found that increasing the temperature of a wine before adding enzyme increased its energy of activation. Under the same conditions, commercial protease S had only a slight effect on protein stability. This may be due to the fact that the pH optimum of protease S is greater than the pH of wine (refer to section on effect of pH on activity). Due to the deleterious effects of high temperatures on the aroma of wines, it was more desirable that the proteases should be added to the juices prior to fermentation instead of to the wines. Because the fermentation process does not add unstable proteins to wine (Boehringer and Jaeger, 1953; Bayly and Berg, 1967; Ough and Anelli, 1979), if one can heat stabilize a juice with proteases at high temperature, the resulting wine should remain stable. If this is the case, the overall quality of the wine would suffer less.

In an attempt to find the optimum conditions for the application of the protease to the juices, various combinations of protease con-

centration, time and temperature were tried. Table 8 reports the effects of protease concentration on the protein stability of grape juice. The highest stability was achieved at 50 mg/L. Higher enzyme concentrations seemed to contribute to the "instability", presumably due to residual enzyme protein. Table 9 compares the Hunter haze values obtained when a Gewurztraminer juice was heated with and without P-EL (50 mg/L) at 46°C for 0-24 hrs and then fermented. This data proves that heating grape juice without protease for up to 24 hrs does not render the wine made from this juice protein stable. This is in agreement with the reports of Boehringer and Jaeger (1953) and Trecelj (1966) who found that heating grape juice or wine at 50°C had no impact on nitrogenous compounds. However, heating the juice in the presence of P-EL for 6 hrs resulted in a drop in bentonite demand from 5 lbs/ 1000 gal to 1 lb/1000 gallon. When the enzyme was allowed to react for 12 hrs, the wine obtained after fementation of the juice was protein stable (i.e. had a haze value of 1.5). Evidently, enzyme action was complete after 12 hrs, since no further change in Hunter haze value (i.e. in protein stability occurred between 12-24 hours. Similar results were obtained when the same experiments were conducted with the two other varieties (Chenin blanc and White riesling).

It was concluded that it would be possible to produce protein stable wines from juices treated with P-EL (50 mg/L) at 45-50°C for 12 hours.

Table 8. Effect of Protease EL57-79 Concentrations on the Protein Stability of 1980 Gewurztraminer Juice.*

Enzyme Concentration	Bentonite Concentration (lb/1000 gal)	Heat Stability**	Hunter Haze Value
0 mg/L	0	U+++	13.5
	1	U+++	12.9
	2	U++	8.5
	3	U+	4.0
	4	U+	3.8
25 mg/L	0	U+++	12.5
	1	U++	7.0
	2	U++	6.5
	3	U+	4.0
	4	U+	3.5
50 mg/L	0	U+	5.6
	1	S	1.5
	2	S	1.2
	3	S	1.0
	4	S	.8
75 mg/L	0	U+++	11.7
	1	U++	8.6
	2	U+	6.3
	3	U+	4.4
	4	S	2.1
100 mg/L	0	U+++	10.9
	1	U+++	9.6
	2	U++	7.9
	3	U+	7.3
	4	U+	6.9
150 mg/L	0	U+++	15.2
	1	U+++	15.1
	2	U+++	14.3
	3	U+++	11.5
	4	U++	5.1
200 mg/L	0	U+++	20.5
	1	U+++	16.3
	2	U+++	16.3
	3	U+++	16.1
	4	U+++	12.2

* Incubated at 46°C for 12 hours.

** Determined by the 80°C heat/cold test (6 hours at 80°C, 12 hours at 4°C) U, unstable; S, stable. Amount of haze indicated by light (+) to strong (+++).

Table 9. Effect of Protease EL 57-79 Treatment* of Grape Juice** on the Protein Stability of Corresponding Wines.

CONTROL (NO ENZYME)			ENZYME ADDED		
Incubation Time (hrs)	Bentonite***	Hunter Haze Value	Incubation Time (hrs)	Bentonite***	Hunter Haze Value
0	0	14.8	0	0	19.8
	1	7.4		1	10.7
	2	3.9		2	5.1
	3	3.0		3	1.5
	4	2.0		4	1.1
	5	1.1		5	.7
2	0	17.4	2	0	14.7
	1	7.0		1	5.5
	2	3.5		2	2.8
	3	1.6		3	1.4
	4	1.3		4	1.3
	5	.7		5	1.1
6	0	12.8	6	0	8.8
	1	4.7		1	3.2
	2	1.8		2	1.2
	3	1.0		3	.8
	4	1.0		4	.7
	5	.7		5	.7
12	0	9.4	12	0	1.5
	1	2.5		1	1.2
	2	2.0		2	1.0
	3	1.0		3	.7
	4	.7		4	.7
	5	.7		5	.7
24	0	12.7	24	0	1.5
	1	8.7		1	1.5
	2	1.3		2	1.0
	3	1.7		3	.7
	4	1.1		4	.7
	5			5	.7

* 50 mg/L of protease, incubation at 46°C.

** 1980 Gewurztraminer juice.

*** Amount of bentonite added to wines (in lb/1000 gal.).

Characterization of Proteases S and EL57-79

Effect of pH on the Activity of Proteases S and EL57-79

P-EL was most active between pH 2 and 6, with its greatest activity occurring between pH 3 and 5. The optimum activity occurred at pH 3.5 (Figure 2). Thus P-EL belongs to the class of acid proteases. Furthermore, pH 3.0-3.7 is of special interest since most wines have their pH in that range (Singleton, 1974). This suggests that this enzyme is well suited for use in juices and wines, notwithstanding interferences from other wine components. The effect of pH on the activity of commercial protease S was also determined (Figure 3). It appears that protease S was most active between pH 5.5 and 8 with a pH optimum around pH 6.5, with gelatin as substrate. Similar pH activity range and optimum were obtained with hemoglobin and casein as substrates (Anonymous, 1978). Figure 3 clearly shows that, at the normal pH of wine (3-3.5), protease S was only 30-35% active. Furthermore, as mentioned earlier, when wines were treated with protease S their stability was not affected significantly. It was concluded that protease S was not effective for the treatment of grape juices and wines to prevent protein instability. Therefore, all further studies were conducted with P-EL only.

Heat Stability of Protease EL57-79

Since the treatment of juices and wines by enzymes requires an incubation period, and since the possibility of autodigestion exists with proteases, the influence of time and temperature on the retention

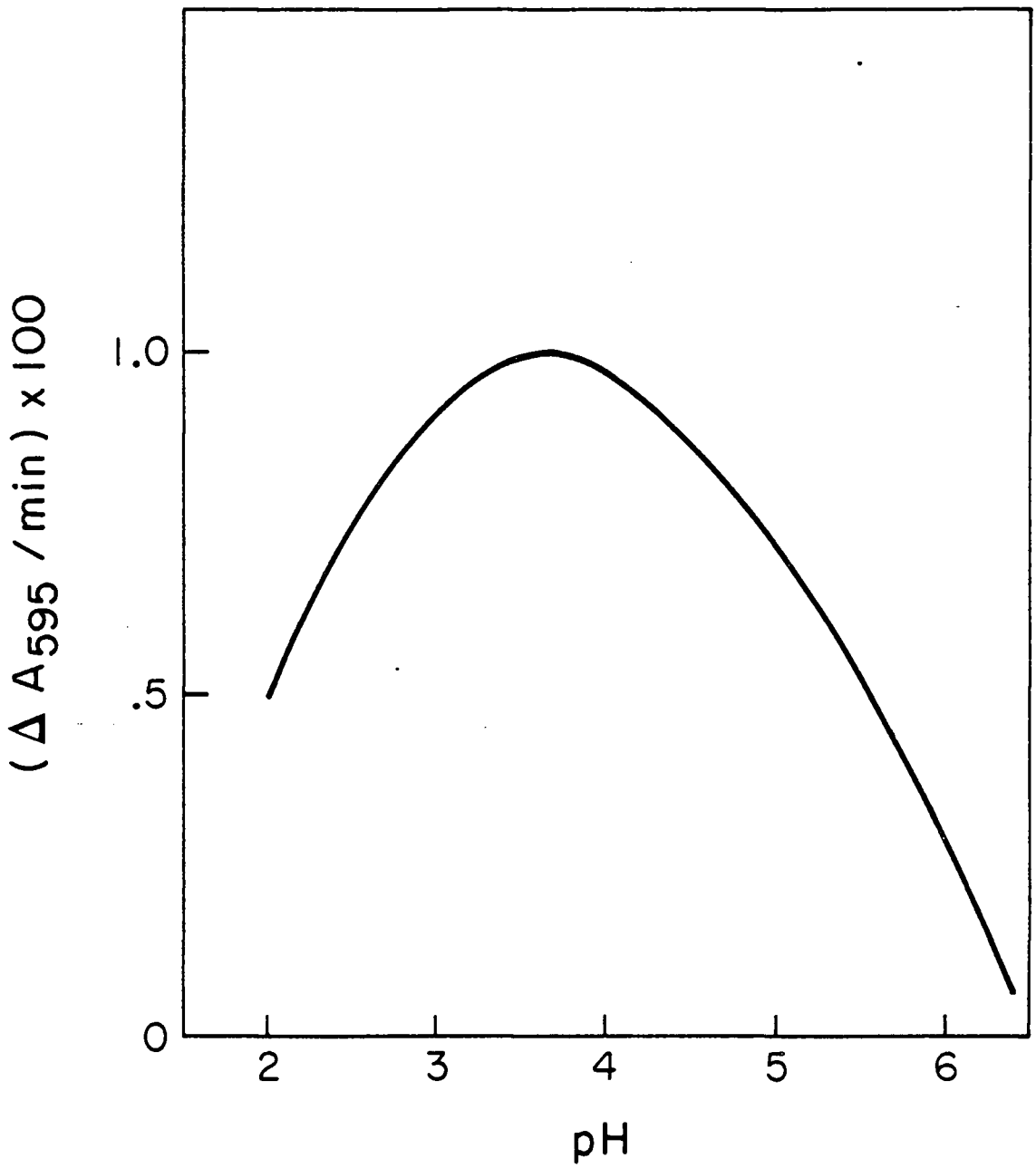

Figure 2. Effect of pH on the activity of Protease EL57-79.

One ml of the protease solution (one mg/ml) was added to nine ml of 0.1M citrate buffer and 10 mg of Hide Powder Azure. Incubation was conducted at 45°C. The absorbance was measured every 5 min. for up to 60 minutes.

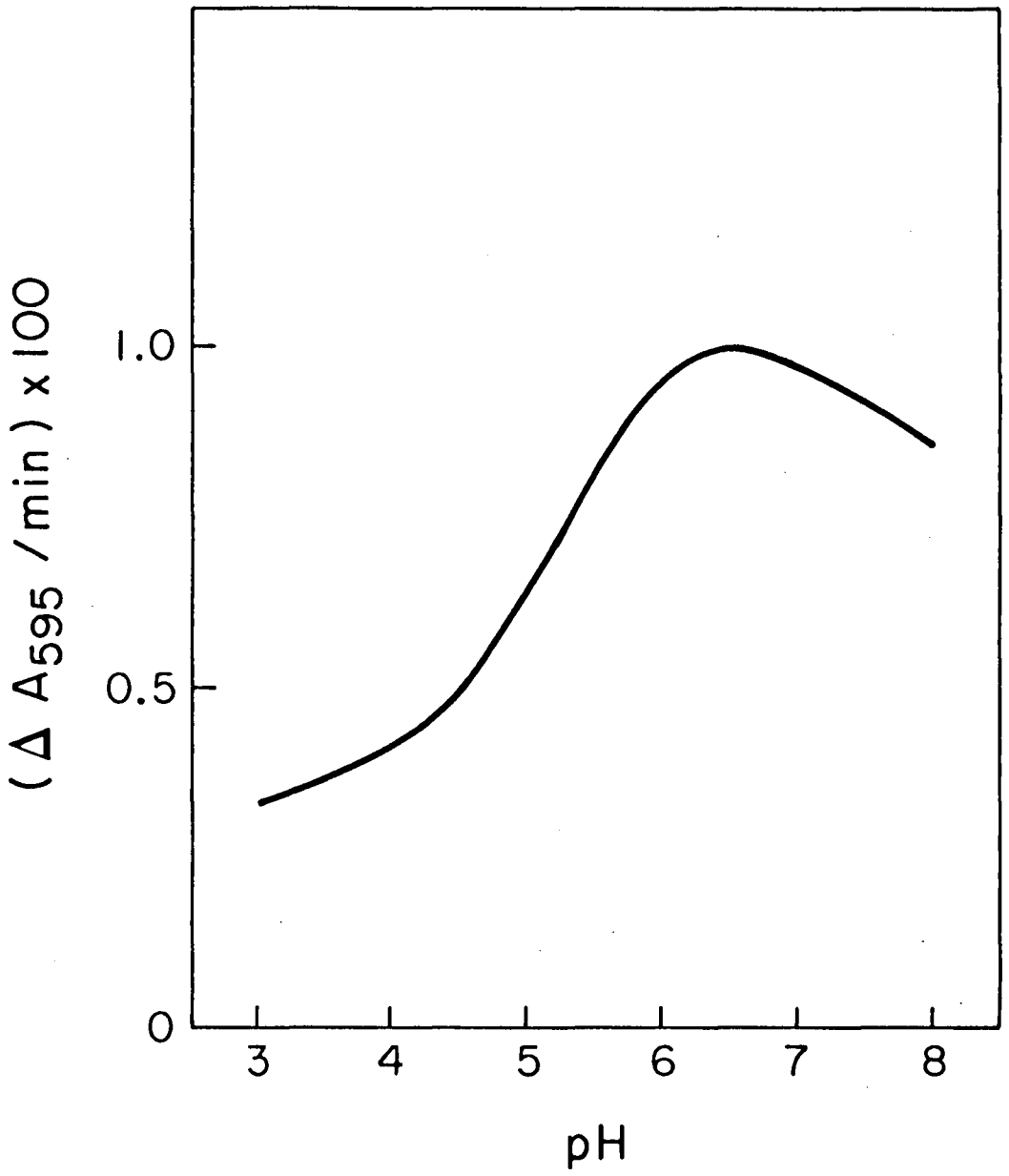

Figure 3. Effect of pH on the activity of commercial protease S. One ml of the protease solution (one mg/ml) was added to nine ml of 0.1M citrate buffer and 10 mg of Hide Powder Azure. Incubation was conducted at 45°C. The absorbance was measured every 5 min. for up to 60 minutes.

of activity was investigated. P-EL was stored at temperatures ranging from 0-80°C for 2 and 12 hrs and its residual activity was measured (Figure 4). Between 0-10°C P-EL retained almost 100% of its activity. At 30°C P-EL retained 80% of its activity after 12 hrs and only 60% after 2 hours. At 60°C the protease lost approximately 50% of its activity after 2 hrs and up to 80% after 12 hours. At 70°C, P-EL lost 95% of its activity after 2 hrs and was totally inactive at 80°C.

Effect of Temperature on the Activity of Protease EL57-79

To determine the optimum temperature of P-EL, its relative activity was determined at 10-80°C (Figure 5). P-EL was essentially inactive between 0-15°C. The same conclusion was reached when wine protein was used as substrate (i.e. wines held at 14°C and treated with the P-EL were as unstable as control wines). Figure 6 also shows that P-EL was most active between 35°C and 60°C, with the highest activity occurring at 50°C. The latter was the optimum temperature with gelatin (in HPA) as substrate. Using wine protein as substrate, it was determined that P-EL was most active between 45-55°C. Forty-six degrees centigrade was chosen as incubation temperature for further treatments of juices and wines with P-EL.

Effect of Enzyme Concentration on the Activity of Protease EL57-79

Figure 6 reports the influence of protease concentration on the activity of P-EL. It is apparent that P-EL was most active when used at 50 mg/L. Above 50 mg/L, there was a gradual decrease in its activity. It is probable that, at concentrations above 50 mg/L,

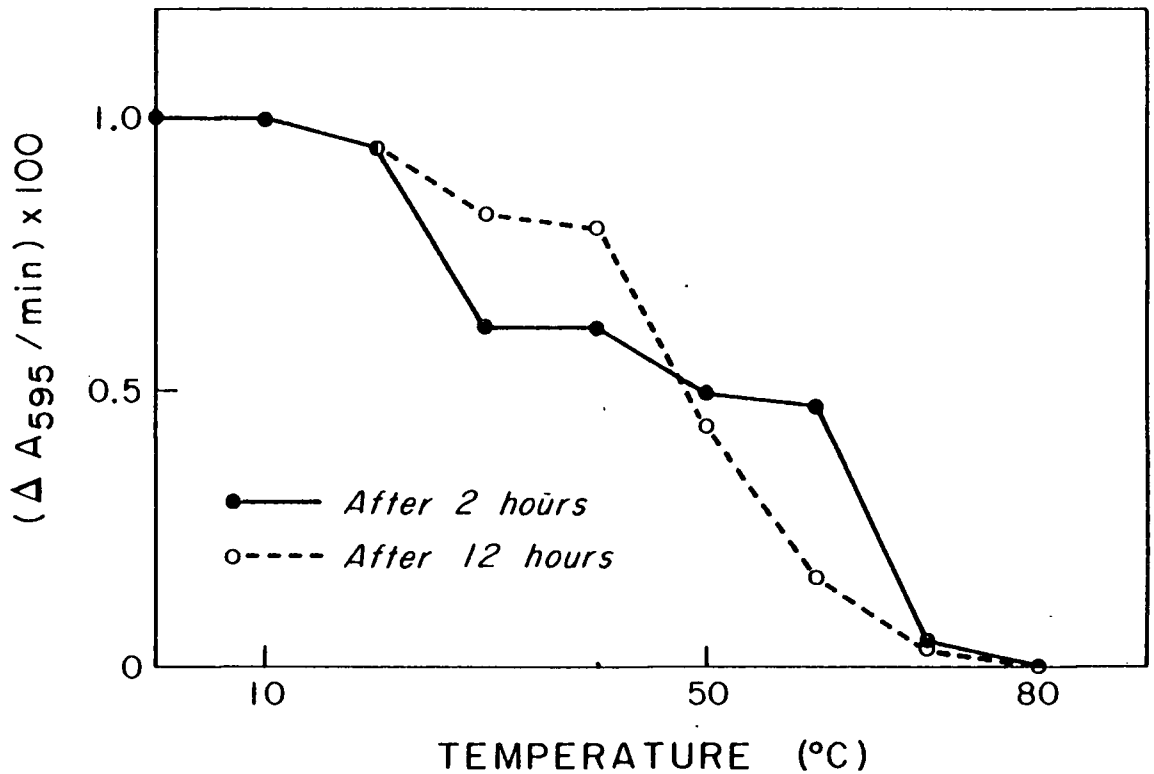

Figure 4. Heat stability of protease EL57-79 at pH 3.5.

Solutions containing five mg/ml of protease EL57-79 in 0.1M citrate buffer (pH 3.5) were held at 0-80°C. After two and twelve hours, samples were removed and their activity on Hide Powder Azure was determined at pH 3.5.

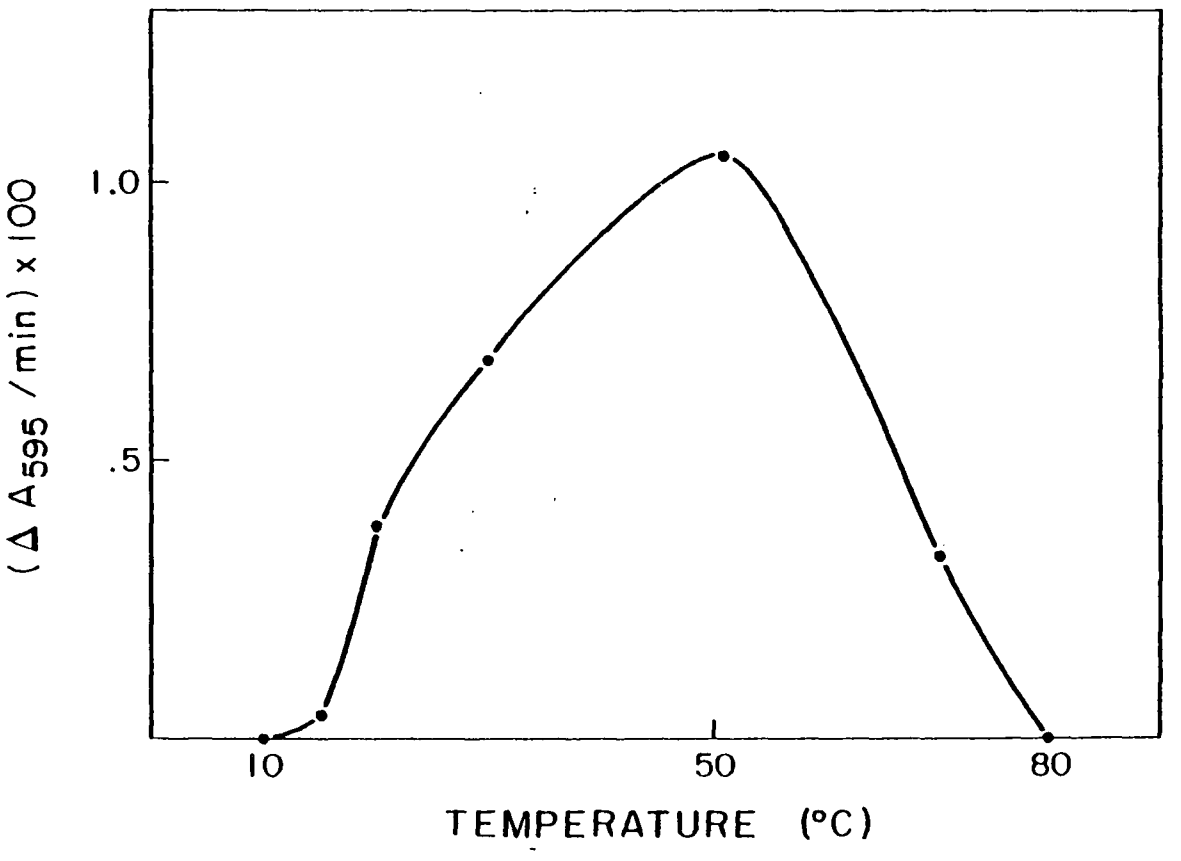

Figure 5. Effect of temperature on the activity of protease EL57-79.

0.5 ml of protease EL (one mg/ml) was added to flasks containing 49.5 ml of 0.1 M citrate buffer (pH 3.5) and 50 mg Hide Powder Azure. Incubation was at 10-80°C for 60 minutes.

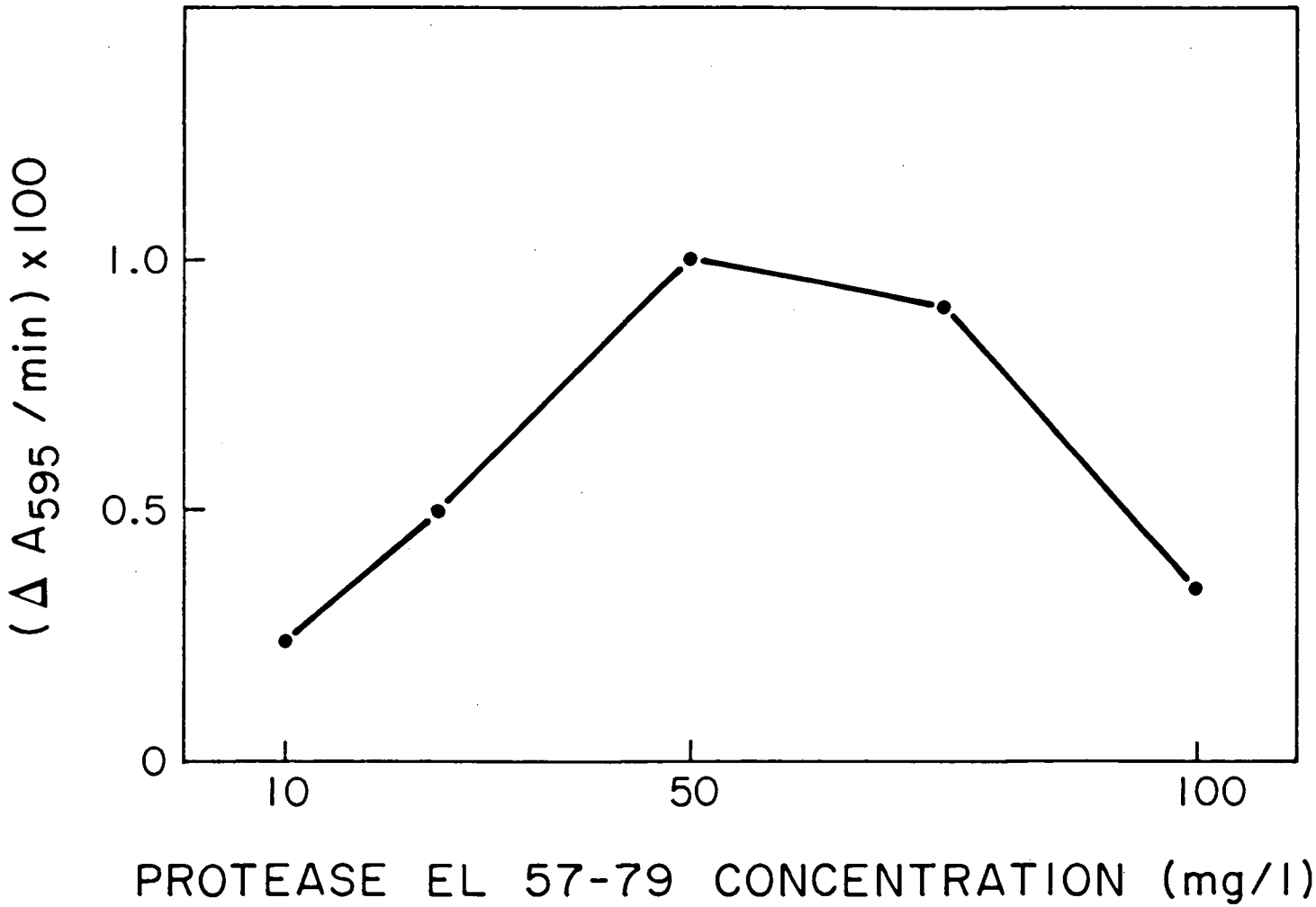

Figure 6. Effect of concentration on the activity of protease EL57-79.

0.1 to one ml of enzyme solution (five mg/ml) was added to 50 ml of 0.1 M citrate buffer (pH 3.5) and 50 mg Hide Powder Azure. Incubation was conducted at 50°C for 30 minutes.

autodigestion was occurring. It should be noted that when P-EL was applied to the grape juice itself, there was a decrease in the protein stability, probably due to autodigestion of the enzyme (Table 8). Therefore, when applying P-EL or any other protease to juices or wines an optimum concentration of the enzyme must be determined.

Effect of Sulfur Dioxide on the Activity of Protease EL57-79

Sulfur dioxide (SO_2) is a necessary component of white winemaking operation. SO_2 has been reported to both inhibit and activate proteases (Vos and Gray, 1979). Therefore, the activity of P-EL in the presence of SO_2 was determined (Figure 7). The SO_2 (25-200 ppm) did not decrease the activity of P-EL. in fact, SO_2 seemed to increase the activity of P-EL as has been reported for other proteases (Vos and Gray, 1979). Therefore, it is recommended that the enzyme addition to a juice should immediately follow the addition of SO_2, followed by holding at 45-50°C. The SO_2 would play a triple role, providing protection against oxidative browning, acting as an antimicrobial agent and increasing the activity of proteases.

Effect of Alcohol on the Activity of Protease EL57-79

Table wines contain up to 14% alcohol. The effectiveness of any enzyme added to wine is dependent on the ability of the former to tolerate the alcohol. The effect of 5-25% alcohol (v/v) on the activity of P-EL was investigated. The results are shown in Figure 8. Five to 13% alcohol did not have any detrimental effect on the activity of P-EL. At 14% alcohol, P-EL retained 90% of its activity. There was a signifi-

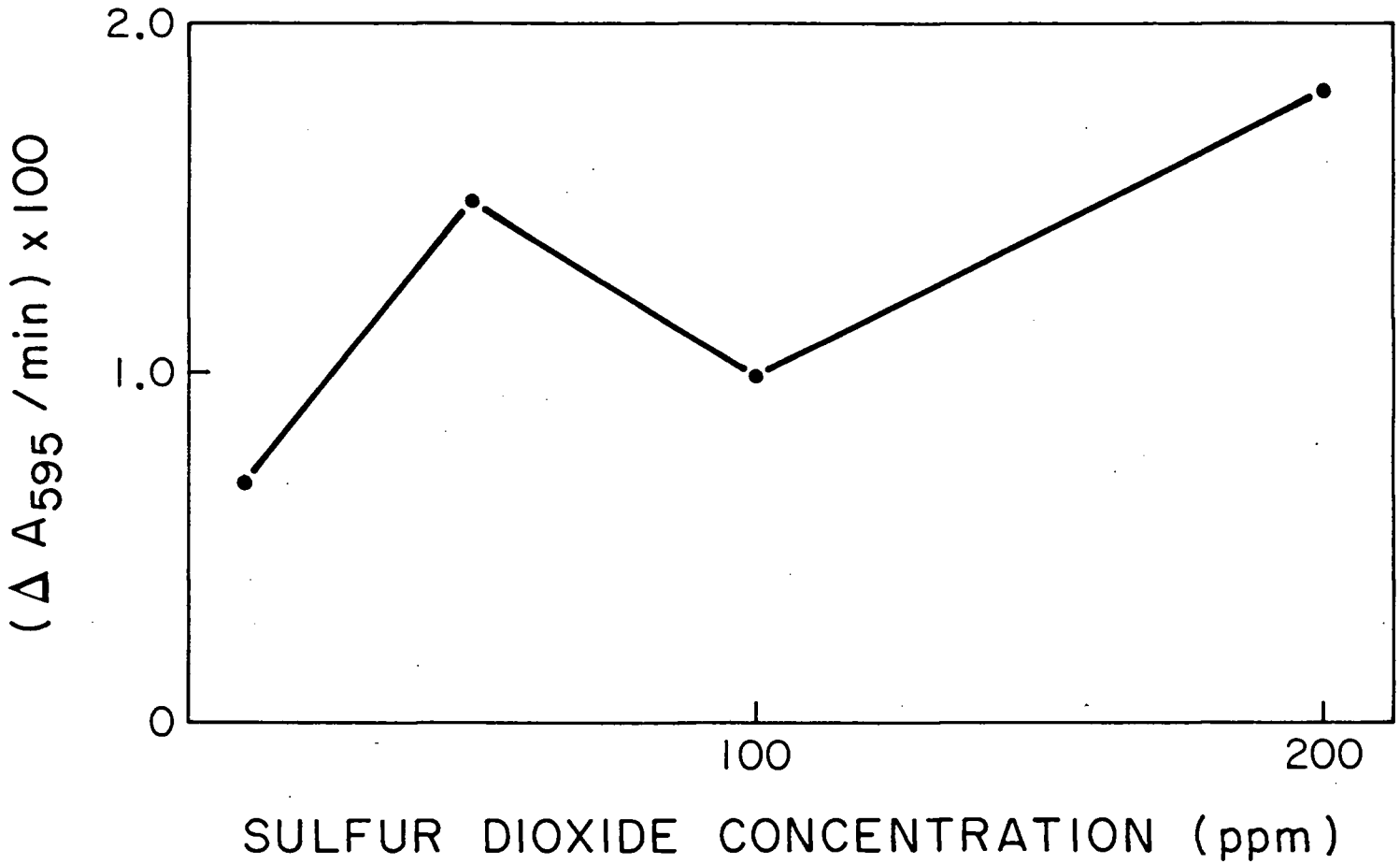

Figure 7. Effect of sulfur dioxide on the activity of protease EL57-79.

0.5 ml of protease EL57-79 solution (one mg/ml) was added to flasks containing 0.1 M citrate buffer (pH 3.5) and 50 mg Hide Powder Azure. SO_2 was added and each flask was incubated at 50°C for 60 minutes.

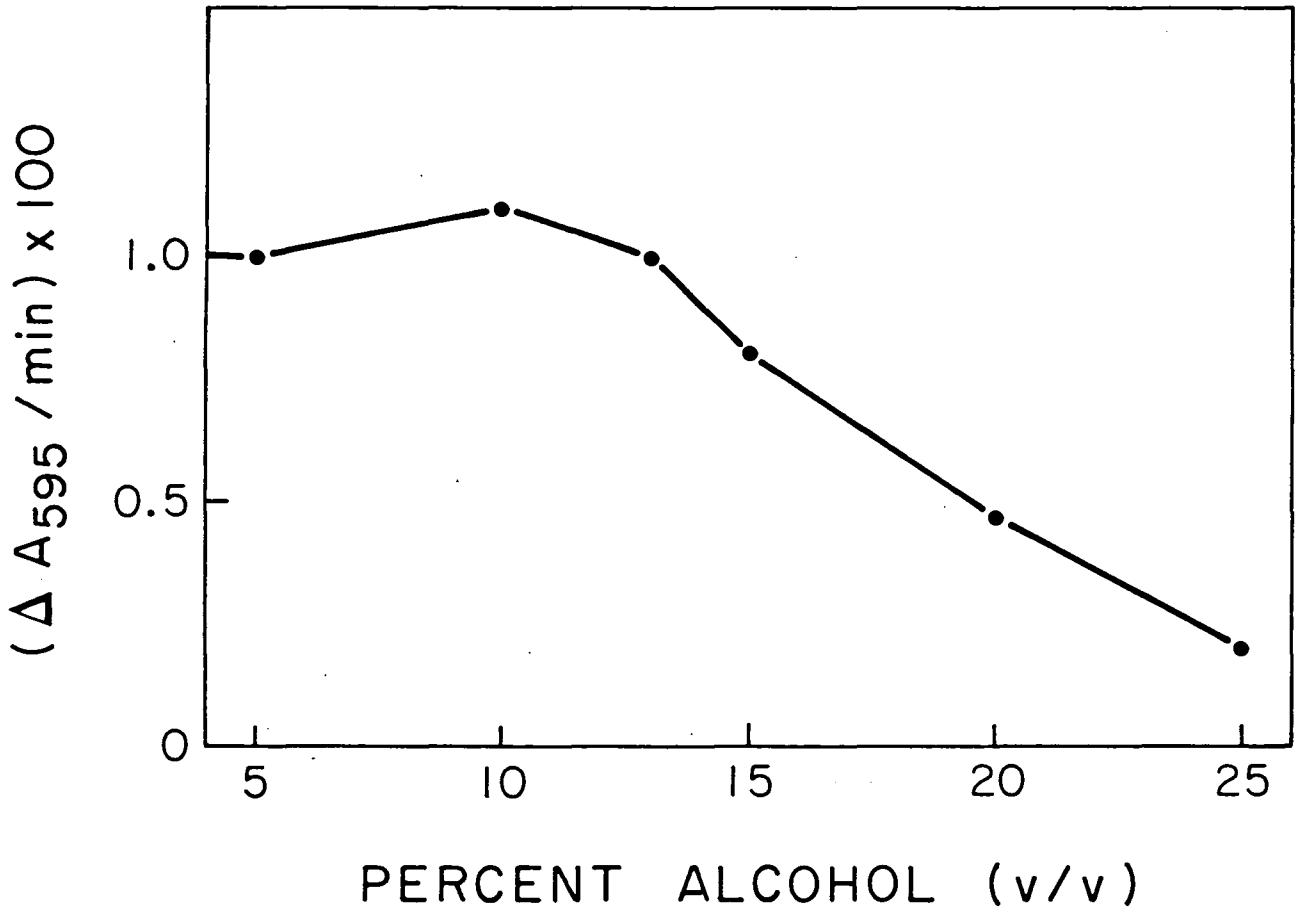

Figure 8. Effect of alcohol on the activity of protease EL57-79.

0.5 ml of protease EL57-79 solution (one mg/ml) was added to flasks containing 0.1 M citrate buffer (pH 3.5) and 50 mg Hide Powder Azure. Alcohol was added and each flask was incubated at 50°C for 60 minutes.

cant decrease in the activity of P-EL at above 15% alcohol. Because, by law, wines must contain 14% or less alcohol (Kunkee and Goswell, 1977) it was concluded that alcohol was not a limiting factor in the application of P-EL to prevent protein instability in wines.

Effect of Protease EL57-79 on Fermentation

Fermentation Rate

The possible effect on fermentation of heating a grape juice or treating the latter with protease EL 57-79 was investigated. Proteases have been reported to influence fermentation when added to grape juice (Vos and Gray, 1979). There was no effect on fermentation rate when the juice was heated without protease or heated in the presence of P-EL (Figure 9).

Chemical Composition of Wines

There was no noticeable difference in the chemical composition of treated and control wines (Table 10). This was a contrast to a report by Datounachvili et al. (1979) who found that the addition of a protease to grape juice resulted in significant biochemical changes in wine composition. This difference was probably due to the fact that these authors added bentonite and gelatin to their wines following treatment with protease and fermentation.

In addition, no hydrogen sulfide was detected by sensory evaluation as has been reported in some instances when proteases have been added to wines (Vos and Gray, 1979).

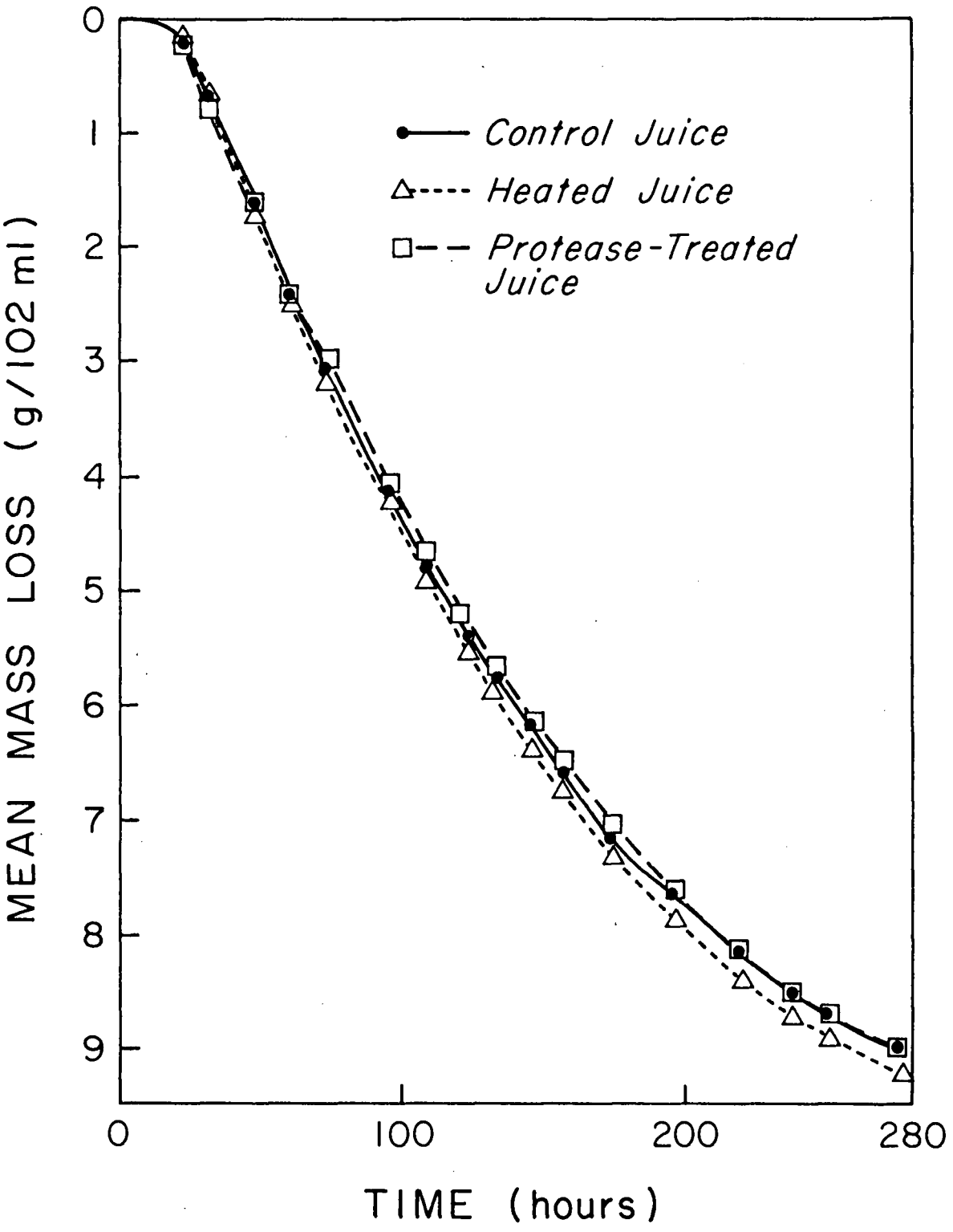

Figure 9. Effect of heat or protease treatment on the fermentation rate.

Table 10. Chemical Composition of Wines Made from Heat or Protease Treated Grape Juice.

Sample	Treatment Applied	pH	Percent Alcohol (v/v)	Titratable Acidity*	Volatile Acidity**	Total Phenols (mg/L)	Color (A_{420})
Chenin blanc							
877-1	Control	3.04	10.7	.987	.062	139	.080
877-4	Wine from heated juice	3.04	11.2	1.050	.050	149	.082
877-5	Wine from Enzyme-treated juice	3.06	11.2	1.046	.051	138	.087
White riesling							
946-6	Control	3.14	10.95	.731	.035	247	.099
946-d	Wine from heated juice	3.11	11.20	.725	.035	252	.090
946-e	Wine from enzyme-treated juice	3.14	11.08	.730	.036	252	.092
Gewurztraminer							
80-F	Control	3.46	10.5	.543	.054	250	.091
80-I	Wine from heated juice	3.45	10.7	.540	.055	252	.087
80-D	Wine from enzyme-treated juice	3.43	10.5	.545	.052	247	.090

* g/100 ml as tartaric acid.

** g/100 ml as acetic acid.

UV Profile of a Control and a Protease-Treated Wine

Figure 10 shows a typical UV absorption profile of a control wine (A) and of the same wine treated with P-EL (B) after they were subjected to gel filtration on Sephadex G-25. Although the figure was obtained with Chenin blanc, similar profiles were obtained with other varieties of white wines.

Figure 10(A) shows four fractions. Fraction one was determined to consist mainly of nitrogenous compounds including proteins. Very little or no phenolics were present in fraction one, as determined by the negative response obtained by a qualitative test for phenolics (Fuleki and Francis, 1968). Fraction one, when present, always eluted with the first 50 ml from the gel. It contained protein as determined by the Bradford procedure. There was little or no protein in fractions 2, 3 and 4 as determined by the same method.

It is noteworthy that after the wine was treated with enzyme, the relative amount of fraction one was substantially reduced to a point where it could not be detected by a spectrophotometer (Figure 10B), whereas fractions 2, 3 and 4 remained unchanged. This finding seemed to indicate that the addition of protease enzyme to the wine resulted in the modification of the protein(s) in fraction one into smaller molecules that were no longer excluded by Sephadex G-25. It should be pointed out that, after treatment of the wine with bentonite, the same profile as in Figure 10B was obtained. This would indicate that proteases may be as effective as bentonite in their protein removing (modifying) capacity.

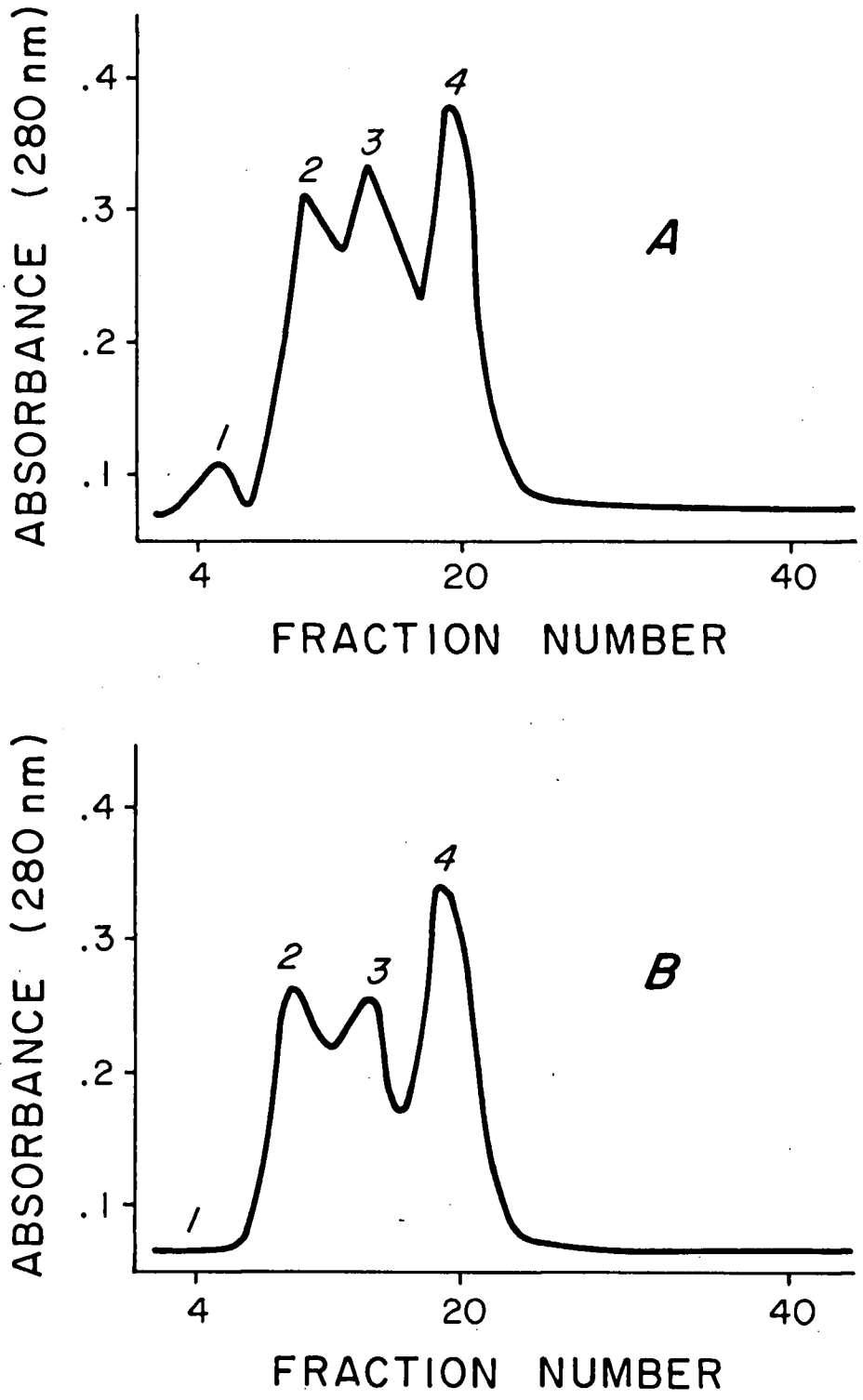

Figure 10. UV profiles of a control (A) and a protease-treated (B) wine.

Electrophoretic Analysis of Juice and Wine Proteins

Chenin blanc (877)

PAGE determined the presence of four protein bands in Chenin blanc (877). When subjected to SDS-PAGE, the same wine showed five fractions. Their MW belonged to four categories: 23,000-24,000; 26,000-27,500, 32,000-32,500; 37,000-39,000 and 66,000-71,000. These results are summarized in Figure 11 and Table 11. It is noteworthy that the same number of fractions found in the juice were also found in the control wine. Evidently, the fermentation process did not add to the protein fractions. This confirms the finding of Feuillat and Bergeret, 1974 and Sheat and Discombe (1976). Table 11 was interpreted as follows: in heated juice (877-7), the 32,000-32,500 fraction has disappeared. This suggests that they were heat labile. The wine that was made from the above juice (877-4) also had the same 32,000-32,500 fraction missing. Since both 877-4 and 877-7 remained protein unstable, it was concluded that the fraction with a MW of 32,000-32,500 probably was not responsible for causing instability in Chenin blanc. The 66,000-71,000 fraction(s) was present in all samples, both stable and unstable. Therefore, it was concluded that the fraction(s) with MW of 66,000-71,000 was not causing the instability. The wine made from protease-treated Chenin blanc juice (877-5) was protein stable. The main difference between the latter and the other samples was the conspicious absence of two bands of MW 23,000-24,000 and 26,000-27,500. These two fractions were, therefore, believed to contribute to the instability. The wine treated with P-EL (877-9) contained the 26,000 fraction but not the 23,000 one.

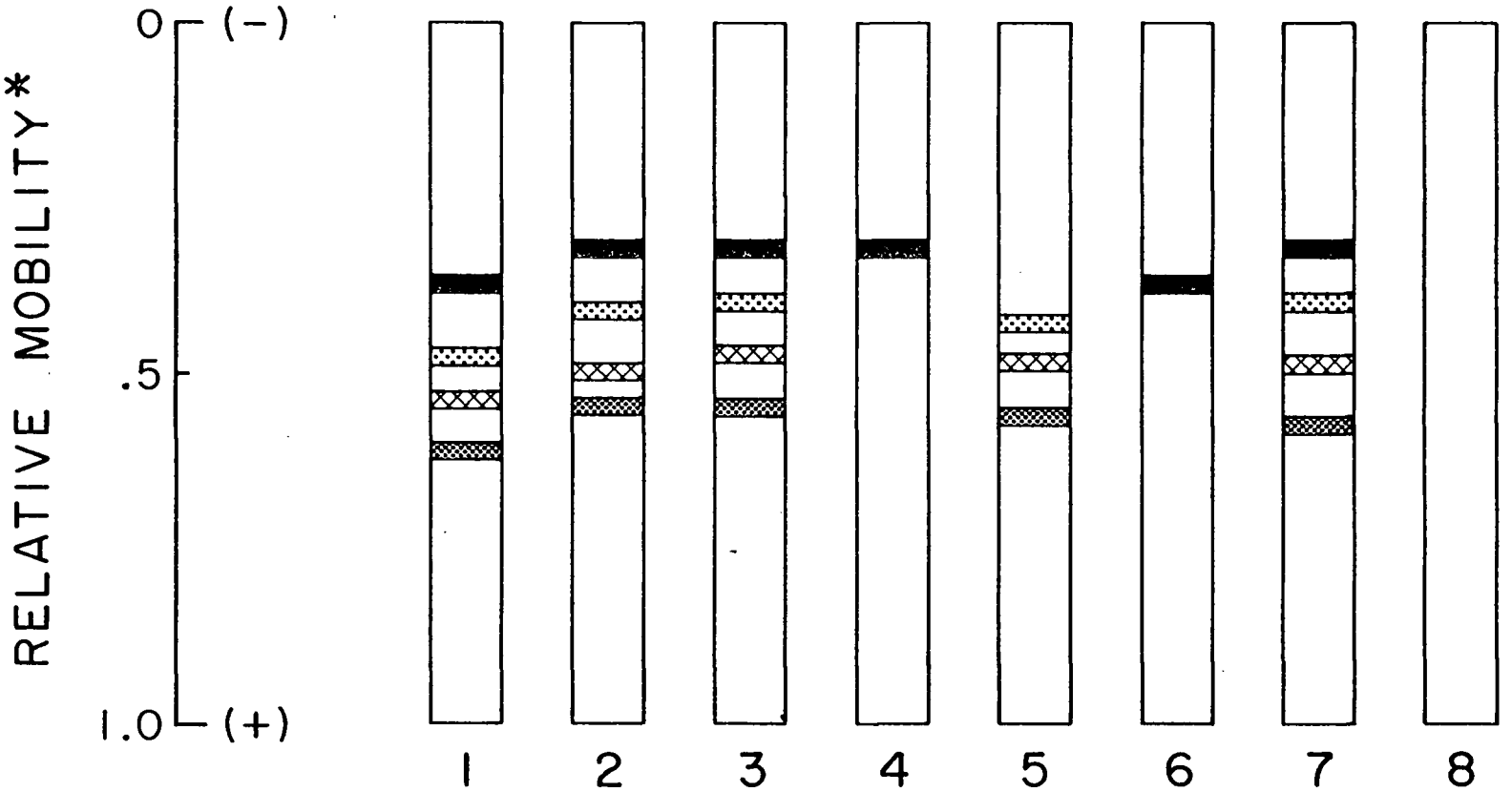

Figure 11. Electrophoretic patterns of Chenin blanc (877).

1. Control Juice (877-0)
2. Control Wine (877-1)
3. Wine from Heated Juice (877-4)
4. Wine from Protease** Treated Juice (877-5)
5. Heated Juice (877-7)
6. Wine Treated with Protease** (877-9)
7. Heated Wine (877-10)
8. Wine Treated with Bentonite (877-2#)

* Relative mobility based on migration of bromophenol blue marker dye.

** Protease EL57-79.

Table 11. Effect of Heat, Protease or Bentonite Treatment on the PROTEIN FRACTIONS* of Chenin Blanc (877) Juices and Wines.

SAMPLE	877-0	877-1	877-4	877-5	877-7	877-9	877-10	877-2#
TREATMENT APPLIED	CONTROL JUICE	CONTROL WINE	WINE MADE FROM HEATED JUICE	WINE FROM ENZYME** TREATED JUICE	HEATED JUICE	WINE TREATED WITH ENZYME** AND HEATED	HEATED WINE	877-1 FINED WITH BENTONITE (2 lb/1000gal)
PROTEIN*** STABILITY	U	U	U	S	U	S	U	S
NUMBER OF FRACTIONS (PAGE)	4	4	4	1	3	1	4	0
MW OF PROTEIN FRACTIONS	71,000	66,000	65,000	65,300	66,000	65,000	63,500 58,500	----
	37,000	39,000	----	----	----	----	----	----
	32,000	32,500	----	----	----	----	35,000	----
	27,000	27,500	27,500	----	26,000	26,000	29,500	----
	24,000	23,500	23,500	----	23,000	----	26,000	----

* Separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

** Protease EL57-79.

*** Determined by the 80°C heat/cold test (6 hours at 80°C overnight) U, unstable; S, stable.

Considering that 877-9 was protein stable, it was concluded that the 26,000 fraction(s) was heat stable and that the fraction of MW 23,000-24,000 was the major protein responsible for causing the instability in Chenin blanc (877).

White riesling (946)

PAGE determined that there were up to five fractions in White riesling (946) wines and juices (Figure 12). As with Chenin blanc (877) the same number of fractions present in the juice were also present in the wine. SDS-PAGE showed that the five protein fractions had MW of 22,000-25,000 and 34,000-38,000 (Table 12). The same two MW fractions were present in all white riesling (946) samples except the wine treated with P-EL (946-C) and that fined with bentonite (946-2#) which showed no fractions with either PAGE or SDS-PAGE and the wine made from P-EL-treated juice (946-E) which had only the 37,500 fraction. This led to the conclusion that the protein fraction(s) with a MW of 22,000-25,000 was responsible for causing the instability in White riesling (946), since all samples in which it was present were protein unstable, whereas the samples in which it was absent were protein stable.

Gewurztraminer (80)

Figure 13 and Table 13 summarize the results of the PAGE and SDS-PAGE obtained with Gewurztraminer (80). Both the juice and wine had a maximum of seven bands with their MW ranging from 16,500-90,000. It is noteworthy that this particular sample which had a higher concentration

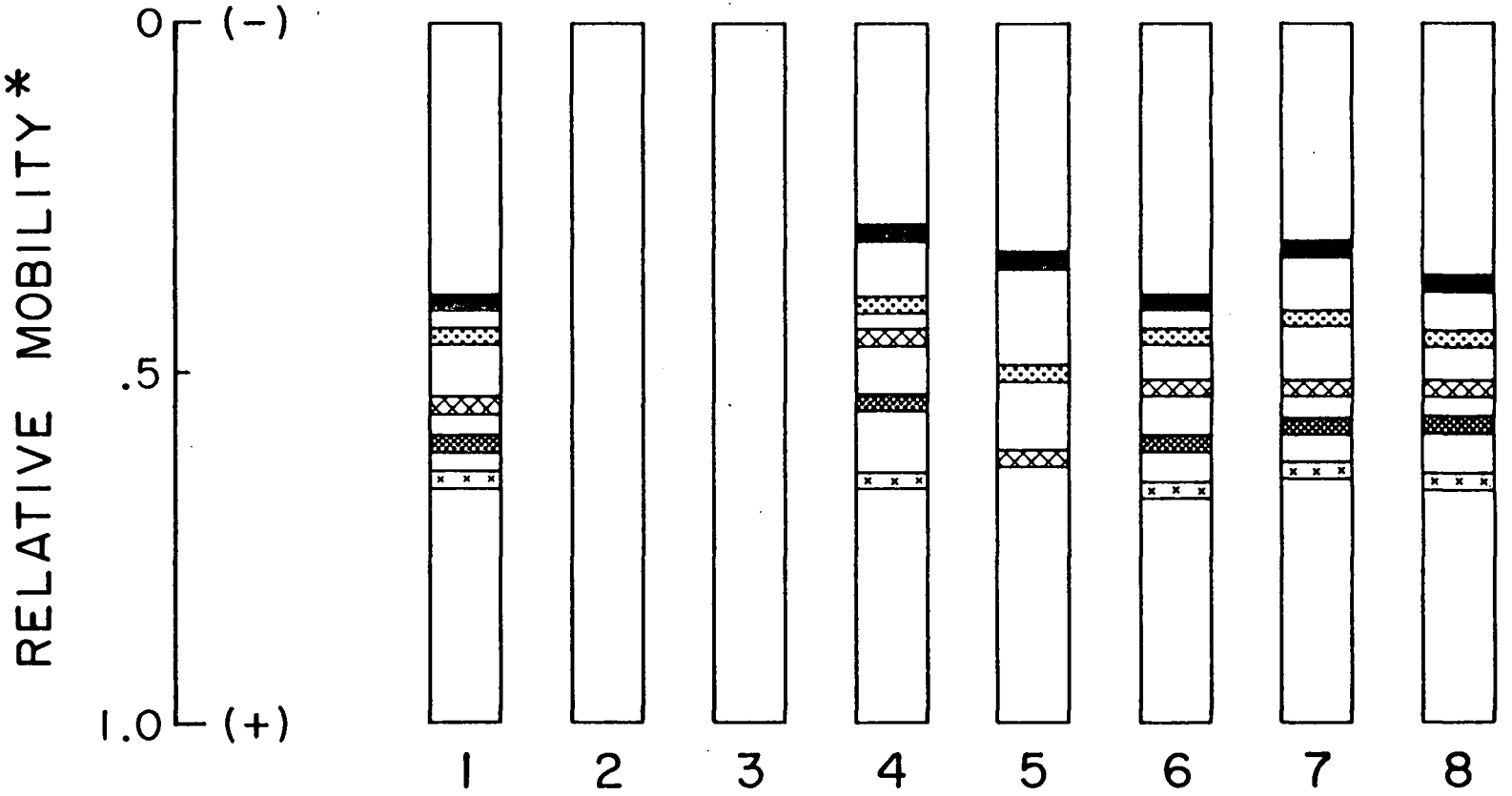

Figure 12. Electrophoretic patterns of White riesling (946).

1. Control Wine (946-6)
2. Wine Treated with Bentonite (946-2#)
3. Wine Treated with Protease** (946-C)
4. Wine Made from Heated Juice (946-D)
5. Wine from Protease** Treated Juice (946-E)
6. Control Juice (946-G)
7. Heated Juice (946-H)
8. Heated Wine (946-I)

* Relative mobility based on migration of Bromophenol blue marker dye.

** Protease EL57-79.

Table 12. Effect of Heat, Protease or Bentonite Treatments on the PROTEIN FRACTIONS* of White Riesling (946) Juices and Wines.

SAMPLE	946-6	946-2#	946-C	946-d	946-e	946-g	946-h
TREATMENT APPLIED	CONTROL WINE	946-6 FINED WITH BENTONITE (2 lb/1000 gal)	WINE TREATED WITH ENZYME** AND HEATED	WINE MADE FROM HEATED JUICE	WINE FROM ENZYME** AND HEAT TREATED JUICE	CONTROL JUICE	HEATED JUICE
PROTEIN*** STABILITY	U	S	S	U	S	U	U
NUMBER OF FRACTIONS (PAGE)	5	0	0	5	3	5	5
MW OF PROTEIN FRACTIONS	35,000 22,500	---- ----	---- ----	34,000 25,000	37,000 ----	38,000 24,000	35,000 22,000

* Separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

** Protease EL57-79.

*** Determined by the 80°C heat/cold test (6 hours at 80°C then 4°C overnight) U, unstable; S, stable).

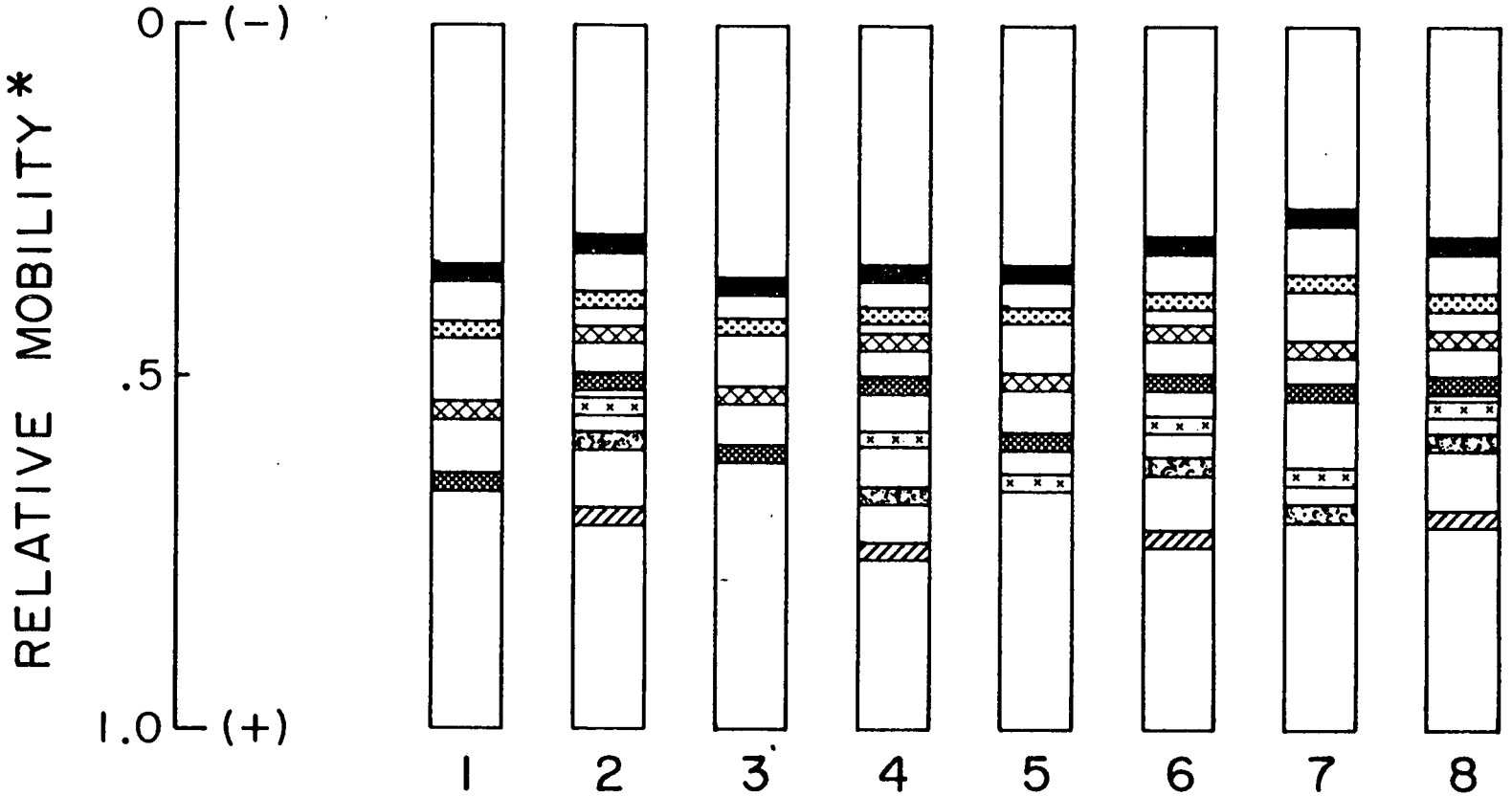

Figure 13. Electrophoretic patterns of Gewurztraminer 80.

1. Wine from Protease** Treated Juice (80-D)
2. Control Wine (80-F)
3. Wine Treated with Bentonite (80-F-4)
4. Wine from Heated Juice (80-I)
5. Wine Treated with Protease** (80-EL)
6. Control Juice (80-M)
7. Protease EL57-79
8. Heated Juice (80-K)

* Relative mobility based on migration of Bromophenol blue marker dye.

** Protease EL57-79.

Table 13. Effect of Heat, Protease or Bentonite Treatment on the PROTEIN FRACTIONS* of Gewurztraminer (80) Juices and Wines.

SAMPLE	80-0	80-F	80-F-4	80-I	80-L	80-M	80-K	Protease EL57-79
TREATMENT APPLIED	WINE MADE FROM ENZYME** TREATED JUICE	CONTROL WINE	80-F fined with bentonite (4 lb/1000 gal)	WINE MADE FROM HEATED JUICE	WINE TREATED WITH ENZYME**	CONTROL JUICE	HEATED JUICE	----
PROTEIN*** STABILITY	S	U	S	U	S	U	U	----
NUMBER OF FRACTIONS (PAGE)	4	7	4	7	5	7	7	6
MW OF PROTEIN FRACTIONS	87,000	90,000				88,500	87,000	90,000
								78,000
	63,500	58,500		61,500	60,000	60,000	60,000	73,000
	54,000			53,000	51,500		55,000	65,000
	44,000	32,500	36,500	37,000	36,500	35.000	42,000	52,500
	32,500							
	30,000		30,000					45,000
	23,000	26,500	26,000	27,500	26,500	26,000	32,500	
		22,500		23,000	22,500	25,000		31,000
		19,000		21,000		20,000	30,000	26,000
		16,500				18,500	22,500	

* Separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

** Protease EL57-79.

*** Determined by the 80°C heat/cold test (6 hours at 80°C then 4°C overnight) U, unstable; S, stable.

of protein than either the Chenin blanc or the White riesling, required more bentonite to become stable (4 lbs/1000 gal), also had a higher number of protein fractions.

A conspicious feature of Table 13 is the presence of the 16,500-18,500 and the 20,000-23,000 fractions in the protein unstable samples (80-F, 80-I and 80-M). Furthermore, the three stable samples (80-D, 80-F-4# and 80-1) contained the 20,500 but not the 16,000 and 19,000 fractions. By a process of elimination it was concluded that the protein fractions of MW 16,000-19,000 were responsible for causing the instability in Gewurztraminer (80) juice and wine.

In the latter sample, as in Chenin blanc (877) and in White riesling (946), the lowest MW fractions were responsible for causing the protein instability. One explanation is that, when polymerized phenolics or tannins mix with the larger MW protein fractions, the resulting copolymers are too "heavy" and precipitate out during normal vinification, whereas the copolymers formed with the low MW protein fractions are not "heavy" enough to be eliminated, thus they remain in the wine and become unstable in time, after bottling, when conditions are more favorable for their precipitation. Another important factor is the isoelectric points of the wine proteins which determine when the individual proteins remain soluble or precipitate out of solution. Furthermore, it may be that smaller polypeptides or protein are more easily denatured by alcohol produced during fermentation and are more readily attacked by proteases. Indeed, their secondary and/or tertiairy structures may be more easily disrupted and require less energy to denature than those of larger more complex proteins.

Effect of Bentonite Concentration on Wine Protein Fractions

Table 14 shows the effect of increasing bentonite concentrations on the various fractions of proteins in wines. In all three varieties studied, as bentonite was added it tended to remove the larger MW fractions of protein first. This could be due to a size factor since the larger MW proteins have more surface area and can come in contact with more of the bentonite micelles. Upon removing these fractions the wine did not become protein stable. Only after the lower MW protein fractions were removed by the addition of more bentonite did the wines become protein stable. In particular, after the addition of 3 lbs/1000 gal of bentonite to Gewurztraminer (80) the 90,000 and 60,000 MW fractions were eliminated, but the wine was still protein unstable. After four lbs of bentonite/1000 gal the 19,000 and 23,000 fractions were also removed, and the wine became protein stable. Therefore, it was concluded that either one or both of these fractions were causing the instability in Gewurztraminer (80).

These findings are very significant because the same results were obtained after treatment of the wines with P-EL57-79; the latter eliminated the large MW protein fractions first. Therefore, it is proposed that there exists a similarity in the way bentonite and proteases stabilize wine proteins.

In summary, when bentonite or protease EL57-79 were added to juices or wines made from Chenin blanc (877), White riesling (946) and Gewurztraminer (80) they first removed or modified the larger proteins, then they did the same to the lower MW proteins which were responsible for causing the instability. This is in contrast to previous reports in the

Table 14. Effect of Increasing Bentonite Concentrations on the Protein Fractions* of Chenin blanc, White Riesling and Gewurztraminer.

A. CHENIN BLANC 877

Treatment	Control	0.5#	1.0#	1.5#	2.0#
Protein** Stability	U	U	S	S	S
Number of Fractions (PAGE)	5	4	2	0	0
MW of Fractions	66,000	66,000	66,000	----	----
	39,000	40,000	----	----	----
	32,500	31,000	31,000	----	----
	27,500	----	----	----	----
	22,500	22,500	----	----	----

B. WHITE RIESLING 946

Treatment	Control	0.5#	1.0#	1.5#	2.0#
Protein** Stability	U	U	S	S	S
Number of Fractions (PAGE)	3	1			
MW of Fractions	35,000	----	----	----	----
	26,500	----	----	----	----
	21,200	20,500	----	----	----

C. GEWURZTRAMINER 80

Treatment	Control	3#	4#	5#	6#
Protein** Stability	U	U	S	S	S
Number of Fractions (PAGE)	7	6	4	4	3
MW of Fractions	90,000	----	----	----	----
	58,500	----	----	----	----
	----	36,500	36,500	36,000	----
	32,500	31,000	30,600	31,500	31,500
	26,500	26,500	26,000	25,800	26,200
	22,500	23,000	----	----	----
	19,500	19,000	----	----	----
	16,500	----	----	----	----

* Separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis.

** Determined by the 80°C heat/cold test (6 hours at 80°C then 4°C overnight) U, unstable; S, stable.

literature which state that the higher MW fractions are responsible for instability and that bentonite tends to remove the more unstable proteins in priority (Singleton, 1974). It is possible that this discrepancy was due to varietal differences.

Isoelectric Focusing of Wine Proteins

The isoelectric points (pIs) of the proteins in Chenin blanc (877), White riesling (946) and Gewurztraminer (80) were determined (Table 15).

There were more pIs than the number of protein fractions determined by PAGE and SDS-PAGE. One explanation is that two or more proteins had the same MW, but not the same pIs. The pIs ranged from 4.8-8.0 with most of them being between 5.2-5.9. This was in contrast to reports in the literature which claim that the pIs of proteins from wines fall in the range of 3.3-3.7 and are very close to the pH of the wines (Singleton, 1974). Isoelectric points of 3.89-8.22 have been reported for grape juice proteins, but those with the highest pIs are believed to be eliminated during wine processing (Singleton, 1974).

Chenin blanc (877) proteins had pIs of 5.2-5.9, only the fraction with a pI of 5.8 was eliminated by heat treatment (877-4). P-EL removed all protein fractions from Chenin blanc except that which has a pI of 5.9 (877-5), whereas bentonite fining removed all fractions. This is in agreement with results obtained by electrophoresis (Figure 12). The protein fraction with a pI of 5.9 was present in all samples, both stable and unstable. Therefore, it was probably not responsible for the instability. SDS-PAGE determined that the protein with a MW of 65,000-71,000 was present in all Chenin blanc samples (Table 11). In

particular, wine 877-5 showed only one fraction with a MW of 65,300 and its isoelectric focusing showed one pI at 5.9 (Table 15). Therefore, it was concluded that the protein fraction with a MW 65,300 had a pI of 5.9 and was not "unstable". The unstable fraction(s) could be any of those with pIs of 5.2-5.9.

The protein fractions in White riesling (946) had MW of 32,500 and 35,000 (Table 12). Isoelectric focusing showed that these fractions had eight pIs ranging from 5.2-5.9. White riesling 946-2#, which was protein stable as a result of bentonite fining, showed no protein fractions with isoelectric focusing (Table 15) as with electrophoresis (Table 14). The White riesling wine which was made from the enzyme-treated juice (946-E) was also protein stable and its three protein fractions had pIs of 5.2, 5.3 and 5.4. All proteins with a pI higher than 5.4 had been eliminated, except for that with pI 5.9, which was present in all samples, stable and unstable. Therefore, it was concluded that the unstable fractions had pIs of 5.5-5.8, whereas the stable fractions had pIs of 5.2-5.4.

Gewurztraminer (80) protein fractions had pIs ranging from 4.8-8.0. Those with pIs of 4.8-5.6 were present in one or more of the stable wines (80-D and 80-F-4#) as shown in Table 15. Those protein fractions with pIs of 6.6, 7.1, 7.6 and 8.0 were present in the unstable samples (80-F and 80-I) but were missing in the stable samples. It was concluded that either one or all of these fractions were unstable. So, as in White riesling, those proteins with the highest pIs were more prone to causing the instability. This is compatible with information available in the literature (Singleton, 1974). Isoelectric

Table. I5. Isoelectric Points of the Protein Fractions in Chenin Blanc, White Riesling and Gewurztraminer Wines.

SAMPLE	Chenin blanc 877				White Riesling 946				Gewurztraminer 80			
TREATMENT APPLIED	CONTRDL WINE	WINE MADE FRDM HEATED JUICE	WINE FROM ENZYME* TREATED JUICE	CONTROL WINE TREATED WITH BENTONITE	CONTRDL WINE	WINE MADE FROM HEATED JUICE	WINE FROM ENZYME* TREATED JUICE	CONTROL WINE TREATED WITH BENTONITE	CONTROL WINE	WINE MAOE FRDM HEATED JUICE	WINE FROM ENZYME* TREATED JUICE	CONTROL WINE TREATED WITH BENTONITE
PROTEIN** STABILITY	U	U	S	S	U	U	S	S	U	U	S	S
ISOELECTRIC POINT	----	----	----	NO PROTEIN	----	----	----	NO PROTEIN	4.8	----	4.8	----
	5.2	5.2	---		5.2	5.2	5.2		5.2	5.2	5.2	5.2
	5.3	5.3	---		5.3	5.3	5.3		5.3	5.3	5.3	5.3
	5.4	5.4	---		5.4	5.4	5.4		5.4	5.4	5.4	5.4
	5.5	5.5	---		5.5	5.5	---		5.5	5.5	---	5.5
	---	---	---		5.6	5.6	---		5.6	5.6	5.6	---
	---	---	---		5.7	5.7	---		---	---	---	---
	5.8	---	---		5.8	5.8	---		5.8	---	---	5.8
	5.9	5.9	5.9		5.9	5.9	5.9		5.9	5.9	5.9	5.9
	---	---	---		---	---	---		6.6	6.6	---	---
	---	---	---		---	---	---		7.I	---	---	---
	---	---	---		---	---	---		7.6	7.6	---	---
	---	---	---		---	---	---		8.0	---	---	---

* Protease EL57-79

** Determined by the 80°C heat/cold test (6 hours at 80°C then 4°C overnight) U, unstable; S, stable.

focusing also confirmed that heat treatment (up to 50°C), when applied without enzyme, did not have much effect on wine protein fractions (Table 15).

In general, the protein fractions in the varieties studied, Chenin blanc, White riesling and Gewurztraminer had their pIs in the range of 4.8-8.0 with the majority of the pIs between 5.0-6.0, and they had MW of 16,000-90,000. Those protein fractions responsible for the instability, tended to have lower MW and higher pIs than the more heat stable ones.

SUMMARY

Selected 1978 and 1979 Oregon and Washington white varietal wines were screened for protein content and protein stability. Three varieties, chenin blanc, White riesling and Gewurztraminer were studied in detail. The potential use of commercial protease S and experimental protease EL57-79, PVPP, XAD-4 and silica sols as bentonite substitutes to protein stabilize wines was investigated.

Individual protein fractions present in the control (unstable) wines and the wines stabilized with proteases or bentonite were studied by electrophoresis and isoelectric focusing.

The results and conclusions may be summarized as follows:

1. 1978 wines contained an average of 153.2 mg/L of protein and required 4.9 lbs of bentonite/1000 gal. for stabilization; 1979 contained 177.6 mg/L of protein and required 3.6 lbs of bentonite/1000 gal. for stabilization. A few of the wines required up to 12 lbs of bentonite/1000 gal. There was protein instability in wines made from Oregon and Washington grapes.

2. The shorter (one day) 80°C heat test was more convenient and it compared favorably with the longer (six days) heat test, when used to predict protein instability.

3. It was possible to use the Hunter Color/Difference Meter to measure protein haze and correlate the results to practical protein stability.

4. The Bradford procedure for the determination of protein was a rapid and reliable method to determine non-dialyzable proteins in white table wines.

5. PVPP, XAD-4 and Silica sols did not significantly improve the protein stability of wines to which they were added.

6. Commercial protease S was ineffective as a substitute for bentonite in protein stabilizing wines, due to their low activity at wine pH.

7. It was possible to produce protein stable wines using expermental protease EL57-79 on grape juices or wines at temperatures of 45-50°C.

8. Experimental protease EL57-79 had the most activity between pH 3 and 5 and had a pH optimum of 3.5 and a temperature optimum of 50°C with gelatin as substrate. It was inactive between 0°C and 15°C. Its activity was not impaired by up to 13% alcohol and up to 200 ppm sulfur dioxide.

9. The application of protease to the juice did not have any effect on the fermentation rate and on the color and the chemical composition of the resulting wines.

10. Four fractions were obtained from chromatography of control wine on Sephadex G-25. The first fraction consisting mainly of protein was almost totally removed after treatment of the wine with either protease or bentonite.

11. Chenin blanc (877) contained proteins with MW ranging from 23,000 to 71,000 and PIs ranging from 5.2 to 5.9. Those protein(s) with MW of 23,000-24,000 and PIs of 5.2-5.5 were unstable.

12. White riesling (946) had proteins with MW of 22,000-38,000 and PIs of 5.2 to 5.9; the unstable ones had MW of 22,000-24,000 and PIs of 5.5-5.8.

13. Gewurztraminer (80) proteins had MW of 16,000-90,000 and PIs of 4.8-8.0. The unstable proteins had MW of 16,000-19,000 and PIs of 6.6, 7.1 and 8.0.

14. There was a similarity in the ways bentonite and protease EL57-79 stabilize wines. Both tended to eliminate (or modify) the large MW proteins first, before acting on the lower MW proteins which were more unstable.

15. The application of a protease enzyme such as experimental protease EL57-79 as a bentonite substitute for protein stabilizing wines is a promising technology deserving further evaluation.

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