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IDENTIFICATION AND CHARACTERISATION OF MANNOPROTEIN EMULSIFIER FROM BAKER'S YEAST

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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November, 1992

ISBN 0-315-87941-6

Mannoprotein emulsifier from yeast

ABSTRACT

The mannoprotein of *Saccharomyces cerevisiae*, baker's yeast, is an emulsifying agent which could be used in foods and cosmetics. This glycoprotein emulsifier can be extracted simply with very good yield by autoclaving yeast cells in neutral buffer. The spent yeast from the beer and wine industries is a suitable raw material for its production. Protein detected by binding of Coomassie blue dye was essential for emulsifying activity. Components of the heat extracted material with greatest emulsifying activity included a high molecular weight fraction (> 200 kDa) which provided viscous and durable emulsions, and a low molecular weight fraction (< 14 kDA) which was very surface active and readily generated nonviscous emulsions. Mannoprotein and the small molecule surfactants lecithin and cetyltrimethylammonium bromide interacted synergistically to increase emulsifying activity at a weight ratio of 100:1 mannoprotein to surfactant. A correction was made to the formula for the Emulsifying Activity Index (Pearce, K.N. and J.E. Kinsella. 1978. J. Agric. Food Chem. **26**:716-723), a measure commonly used for comparing protein emulsifiers.

RESUME

La mannoprotéine de la levure de boulangerie, *Saccharomyces cerevisiae*, est un agent émulsifiant convenable pour les produits alimentaires et les cosmétiques. Cette glycoprotéine peut être extraite de façon simple et ce avec un très bon rendement en autoclavant les levures dans une solution tampon à pH neutre. Les levures rejetées après la fabrication de bière et de vin servent de matières premières adéquates pour la production de cet émulsifiant. Les protéines détectées par coloration au blue de Coomassie sont essentielles à l'activité émulsifiante. Après l'extraction à chaleur, les composants ayant le plus grand pouvoir émulsifiant comprenait une fraction de haut poids moléculaire (> 200 kDa) laquelle donnait des émulsions durables et de haute viscosité et, une fraction de faible poids moléculaire (< 14 kDa) ayant une grande activité comme agent tensioactif et les émulsions générées avaient une très faible viscosité. La mannoprotéine et les petits molécules tensioactives, lécithine et le bromure d'ammonium cetyltrimethylique ont agi synergistiquement pour augmenter l'activité émulsifiante lorsque le ratio poids/poids était de 100:1, mannoprotéine pour 1 agents tensioactifs. Une correction a été apportée à la formule permettant de calculer l'index de l'activité émulsifiante (Pearce, K.N. et J.E. Kinsella. 1978. J. Agric. Food Chem. **26**:716-723).

ACKNOWLEGDEMENTS

I express my sincerest gratitude to Dr. D.G. Cooper, Dr. R.J. Neufeld, of the Department of Chemical Engineering, and to Dr. E.S. Idziak, of the Department of Microbiology, for their attentive supervision, their support and encouragement, and for their collaborative efforts on my behalf.

I also wish to thank Dr. M. Weber, of the Department of Chemical Engineering, for the generous contribution of his expertise and effort which were invaluable in resolving a long standing problem for emulsion researchers.

Thanks also to Dr. F. Archibald, of the Department of Microbiology/PAPRCAN, and Dr. H. Bussey and Charles Boone of the Department of Biology for helpful discussions, advice on experiments and providing experimental materials.

Working as a graduate student in the Department of Chemical Engineering has been an instructive and enjoyable experience, thanks to both students and staff members. The past and present students of Dr. Cooper's lab, and in particular John Rizzi, John Campanelli, Bruce and Juliana Ramsay, Bill McCaffrey, Steve Helle, Peter van Walsum, Mike Zenaitis, Steve Wentworth, and Scot Hughes, offered ideas and comments, assisted with certain experiments, and provided feedback during the project. My office mates, Eric Cheluget and Wenhua Wang, and Denis Poncelet helped in analysis of data. My warmest regards to Jean Dumont of stores, and Anne Prihoda, departmental secretary.

My thanks also to the professors in the Department of Microbiology, and the students, who have contributed greatly to my education in Microbiology, and provoked many stimulating discussions during courses and seminars. My regards and best wishes to Emily Magee and Marlene Parkinson, for keeping me properly registered as a student and informed of seminars and other events. Thanks as well to Mrs. Idziak for rapid courier service, and for answering questions in chemistry.

Large quantities of yeast for experiments were donated by Lallemand Inc., Le Cheval Blanc, and Andrés Wines Ltd. The use of pilot scale equipment at the Centre de recherche et développement alimentaire, Agriculture Canada, in Ste.-Hyacinthe was invaluable. My thanks to Marcel Tanguay and Claude Gagnon for their assistance.

Finally, my love to Marie-Line Desjardins, my wife, who translated the abstract, and Etienne Cameron for their patience and moral support.

CLAIM OF CONTRIBUTIONS TO KNOWLEDGE

1. The mannoprotein of *Saccharomyces cerevisiae*, baker's yeast, is an effective emulsifier which could be used in foods and cosmetics. It can be extracted simply, with high yield by heating yeast cells in neutral buffer, or by treating yeast cells with Zymolyase enzyme. The yield of mannoprotein emulsifier is greater than for any yeast-derived emulsifiers described previously.

2. The spent yeast from beer or wine manufacturers was as good a raw material for production of the emulsifier as fresh baker's yeast. An emulsifier was extracted from yeasts of 13 genera, including *Candida utilis*, which is used for single cell protein.

3. A widely used turbidimetric measure of emulsifying activity, the Emulsifying Activity Index (EAI; Pearce, K.N., and J.E. Kinsella. 1978. J. Agric. Food Chem. 26:716-723) does not provide units of m^2 interfacial area per g emulsifier as claimed. A correction was proposed which provides the desired units and makes the formula theoretically sound.

4. The emulsifying activity in fractions of the heat extracted emulsifier separated with ammonium sulfate was related to protein content measured by binding of Coomassie blue dye.

5. After fractionation of the heat extracted emulsifier by gel filtration, two components with the greatest emulsifying activity were identified, (1) a high molecular weight fraction (> 200 kDa), and (2) a low molecular weight fraction (< 14 kDa). Both components had a high ratio of carbohydrate to protein. Vigorous mixing was required to produce emulsions in the presence of the high molecular weight material; the emulsions were viscous and did not separate after several days. The low molecular weight material was very surface active and readily provided non-viscous emulsions, which separated after several hours by drainage of the external aqueous phase.

6. A synergistic increase in emulsifying activity at neutral pH was observed with a 100:1 weight ratio of yeast mannoprotein and either lecithin or cetyltrimethylammonium bromide. At weight ratios of 10:1 or 1:1 of mannoprotein and other surfactants, various salts or amino acids, emulsifying activity was improved. A synergistic increase in emulsifying activity occurred only at a 1:1 weight ratio of gelatin and lecithin or cetyltrimethylammonium bromide; none occurred with caseinate or whey proteins.

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Candidates have the option, **subject to the approval of their Department**, of including, as part of their thesis, copies of a paper(s) submitted for publication, or the clearly-duplicated text of a published papaer(s), provided that these copies are bound as an integral part of the thesis.
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INTRODUCTION

An emulsion is defined as a homogeneous dispersion of one liquid phase within another. Many foods, beverages and cosmetics are emulsions, for example, milk, salad dressings, ice cream, moisturizing and hand lotions contain droplets of oil within an external aqueous phase. Butter, margarine, cream cheeses, and numerous skin-cleansing lotions are water-in-oil emulsions (Darling and Birkett, 1987; Fox, 1986). Dispersion of one medium in another contributes to texture, appearance, or flavour of the product (Jaynes, 1985). Olive oil may taste greasy on its own but is very acceptable in an emulsified oil and vinegar salad dressing (Dickinson and Stainsby, 1988). Calorific content of a food product and cost of a cosmetic formulation can be reduced by incorporation of more water.

Emulsions are thermodynamically unstable. Making a dispersion of one phase within another greatly increases the area of oil-water interface and proportionally increases the interfacial free energy. The free energy required to increase the area of the oil-water interface by an amount ΔA, or the minimum work required to generate the emulsion is

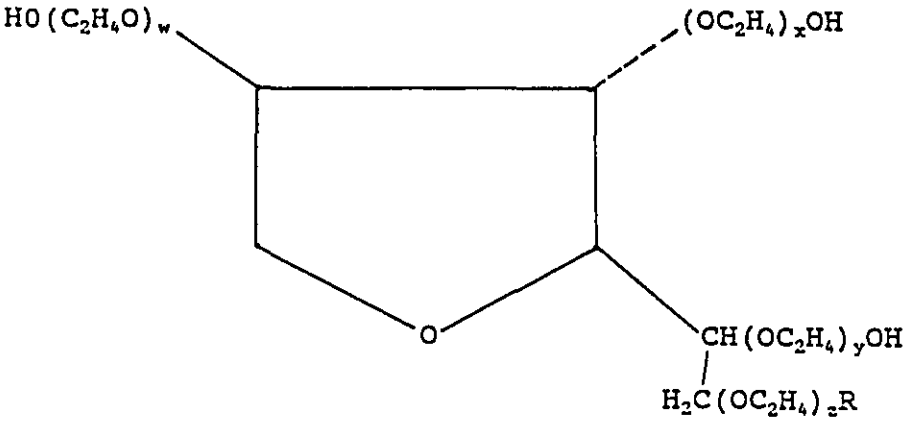
$$W_{min} = \gamma \Delta A$$

where γ is interfacial tension (Zajic and Panchal, 1976; Dickinson, 1992a). The thermodynamic tendency of the system is to minimize free energy by decreasing the interfacial area, so the two phases tend to separate over time (Reddy and Fogler, 1981b). As droplets coalesce, total interfacial area in the

system decreases, since large droplets have proportionally less surface than small droplets.

The rate of phase separation in emulsions can be reduced by adding a third component, an emulsifier. Emulsifying agents enhance two independent processes, (1) formation of new droplets and (2) stabilization of droplets once they are formed (Zajic and Panchal, 1976). Typical emulsifiers have amphiphilic structure and adsorb at the interface between two phases.

The emulsifying agents most commonly used in foods and cosmetics are chemically synthesized. The structures of two synthetic emulsifier types are shown below.



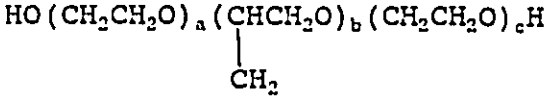
TWEEN 60

$R = (C_{17}H_{35})COO$

TWEEN 80

$R = (C_{17}H_{33})COO$

$(w+x+y+z) = 20$



PLURONIC F68

$(a+c) = 6$

$b = 8$

The hydrophilic functional groups of Tween and Pluronic emulsifiers consist of polyoxyethylene; the hydrophobic functional group is an esterified fatty acid with Tweens (oleate in the case of Tween 80), and polyoxypropylene in the Pluronic emulsifiers. Tweens, also known as polysorbates, are considered as GRAS compounds (generally recognized as safe) by the U.S. Food and Drug Administration. These may be included in foods usually at a concentration not exceeding 0.1 % (wt/wt). Tween 80 (polysorbate 80: poly oxyethylene(20) sorbitan monooleate) is a component of most ice creams. The Pluronic emulsifiers are not approved for foods, but are present in cosmetics, shampoos, *etc.*

Due to increasing consumer demand for "natural" products, emulsifying agents of biological origin are of interest to manufacturers. Various proteins have long been used to stabilize food emulsions, but cost more and are less efficient than the synthetic emulsifiers. The aim of the project was to identify and characterize microbially produced emulsifying agents which could replace the synthetic emulsifiers used in foods or cosmetics. Emulsifiers produced by yeasts were investigated, since yeasts are already used in foods. We considered that an emulsifier from a yeast would be more readily accepted by consumers than one from a bacterium.

The experimental part of the thesis is divided into four chapters, each in the form of a scientic article. Literature

cited is listed at the end of the thesis under the name of the first author.

In the first chapter of experiments (chapter 2), results leading to the identification of an emulsifier, mannoprotein from baker's yeast, are presented. This is followed by chapter 3, in which a method for evaluating emulsifying activity was developed and compared to an existing and very widely used method. The relationship of mannoprotein structure and function as an emulsifier was investigated in chapter 4. Finally in chapter 5, a synergistic interaction between mannoprotein emulsifier and ionic surfactants was described.

CHAPTER 1: LITERATURE REVIEW

1.1 DEFINITIONS AND TERMINOLOGY

In the broadest sense any compound which stabilizes an emulsion is an emulsifier. Zajic and Pancjchal (1976) recognize four types of emulsifiers: (1) electrolytes; (2) solids; (3) surfactants; and (4) macromolecules. Simple inorganic electrolytes such as potassium thiocyanate may temporarily stabilize oil-in-water dispersions by forming an electrical double layer on the aqueous side of the interface. Finely ground solids (*eg.* quartz powder) can also stabilize emulsions (Zajic and Panchal, 1976; Halling, 1981). Neither of these emulsifier types are important for stabilizing emulsions in foods and will not be considered further. Most food emulsions are stabilized by surfactants (amphiphilic compounds of low molecular weight, usually < *ca.* 1000 Da), or macromolecular emulsifiers such as proteins, gums and starches (Dickinson, 1992a).

To differentiate the surfactants from the macromolecular emulsifiers, low molecular weight surfactants will be referred to as such in the thesis. The term "emulsifier" or "emulsifying agent" will be applied exclusively to macromolecules (of molecular weight > 1000 Da.) which stabilize emulsions and possess the following characteristics (a) amphiphilic structure, ie. possess hydrophilic (polar) and

hydrophobic (nonpolar) functional groups,

(b) surface activity, move to the interface between two phases where they reduce interfacial tension by displacing molecules from one or both surfaces,

(c) are soluble in the continuous phase and are available for adsorption around newly formed emulsion droplets,

(d) adsorb quickly around a droplet of the dispersed phase forming a film which reduces tendency of such droplets to collide, coagulate, or coalesce,

(e) are active at low concentration (Zajic and Panchal, 1976).

Examples of macromolecular emulsifiers include proteins, glycoproteins, complex carbohydrates (gums), or various synthetic compounds. Certain macromolecules (starches, hydrophilic gums *eg.* xanthan gum) stabilize emulsions simply by increasing the viscosity of the external aqueous phase to the point where coalescence does not occur because emulsified droplets are effectively immobilized (Tolstoguzov and Braudo, 1985; Dickinson and Stainsby, 1988; Dickinson, 1992b). Viscosifying agents will not be referred to as emulsifiers as they do not fulfil the above conditions.

The interfacial properties of macromolecular emulsifiers and small molecule surfactants are quite different (Tolstoguzov and Braudo, 1985). To differentiate between these two classes of compounds it is useful to distinguish between an <u>efficient</u> surface active agent and an <u>effective</u> surface active agent (Rosen, 1989). Efficiency is a measure of the

concentration of surfactant required to produce a significant reduction in surface tension. It depends on the concentration of the surfactant at the interface, which is related to the entropy of the molecule such that it can move from the bulk of the solution to the interface. Effectiveness is a measure of the minimum value to which a surfactant can lower the surface tension. It depends greatly on cohesiveness of hydrophobic groups in the surfactant, which determines how tightly the molecules can pack at the interface (Rosen, 1989; Zajic and Panchal, 1976; Zajic and Seffens, 1984).

Macromolecular emulsifying agents are efficient surfactants; the low molecular weight amphiphiles tend to be effective surfactants and are not very efficient. As will be described in more detail in section 1.5 (Protein adsorption), low bulk concentrations of a surface active macromolecule result in a high surface concentration and a significant lowering of the surface tension. At low concentrations, surface active macromolecules reduce the surface tension far more than does an equal weight concentration of a small molecule surfactant (Dickinson, 1992a). At high bulk concentrations, compounds such as sodium dodecyl sulfate or lecithin can pack more tightly at the interface than can the macromolecular emulsifiers, and reduce the surface tension to far lower ultimate values (Zajic and Panchal, 1976; Walstra, 1987; Rosen 1989; Dickinson, 1992a).

Very fine emulsions can be generated with small molecule

surfactants, however the emulsions produced are frequently unstable (Zajic and Panchal, 1976; Zajic and Seffens, 1984; Cooper and Paddock, 1983). In some dairy products small molecule surfactants actually destabilize oil-in-water emulsions by displacing proteins from the surface of oil droplets. This permits stiffening of whipped creams and ice creams (Krog, 1991; Darling and Birkett, 1987; Lin and Leeder, 1974). On product labels for chocolate, lecithin is referred to as an emulsifier. Pure lecithin is not an effective emulsifier (Carillo and Kokini, 1988). Low molecular weight surfactants such as lecithin, monoglycerides, stearoyl lactylate *etc.* are included in foods for a variety of purposes (for control of texture, improvement of shelf life, or modification of rheology) only some of which are related to emulsification (Dickinson, 1992a).

1.2 MECHANISMS OF EMULSION BREAKDOWN

Emulsions are unstable and several mechanisms can be identified in their breakdown (Halling, 1981; Reddy and Fogler, 1981b; Tadros and Vincent, 1983; Ostrovsky and Good, 1986). These mechanisms are defined to permit understanding of the role of emulsifying agents in stabilizing emulsions; emulsifiers prevent or reduce the rate of one or more of these processes. Mechanisms of emulsion instability include:

(1) gravitational separation (also known as creaming or sedimentation)

(2) collision

(3) disproportionation (isothermal distillation, Ostwald ripening)

(4) flocculation

(5) coalescence

The first two processes involve flow. Typically the two phases differ in density, so gravity induces (usually) buoyancy of the dispersed phase droplets upwards and the continuous phase downwards. At low oil phase volume (ϕ), the process of creaming is described by the Stokes equation

$$v = 2g \Delta\rho r^2/9n$$

where v is the droplet velocity, g is acceleration due to gravity, $\Delta\rho$ is the density difference between the two phases, r is the droplet radius and n is the viscosity of the continuous phase (Walstra, 1987). For high oil phase volume, $\phi \geq 0.05$, droplet linear velocity is reduced particularly in very fine emulsions (Sherman, 1983). If the continuous phase is non-Newtonian, the Stokes equation is not useful. Creaming can be stopped if the viscoelastic dispersion medium has a sufficiently high yield stress. For emulsion stability, the structure and rheology of the dispersion medium may be far more important than characteristics of the dispersed droplets (Walstra, 1987).

Droplets may undergo collisions due to Brownian motion or to agitation of the whole emulsion. For many systems, the theoretical predictions give a reasonable guide to the rates of these processes. The encounter rate per droplet due to

Brownian motion is

$$\text{Encounter rate} = 4KT\phi/\pi d^3 n$$

where K is Boltzmann's constant, T is temperature and d is droplet diameter (Halling, 1981).

The process of disproportionation depends on diffusion. The driving force is the greater solubility of a substance on the concave side of a curved interface than on the convex side. There is an increased pressure inside spherical droplets (of diameter d) given by the Laplace equation

$$\Delta P = 4\gamma/d \qquad \text{(where } \gamma \text{ is surface tension)}$$

so the pressure and hence the solubility of the disperse phase material is greater for smaller droplets. This creates a driving force for diffusion from small droplets to larger ones and hence the term disproportionation. The rate usually depends on the solubility of the disperse phase in the continuous liquid (Halling, 1981). Disproportionation is not a breakdown mechanism for food oil-in-water emulsions since triglyceride oil is almost insoluble in water. This process may occur in water-in-oil emulsions *eg.* margarine (Walstra, 1987), and may be a factor in the instability of benzene-in-water model emulsions studied by Mita *et al.* (1973, 1974).

Surface properties of emulsified droplets strongly affect the processes of flocculation and coalescence. Droplets brought together by creaming or encounters can either stick together while remaining separated by a thin layer of continuous phase, ie. flocculate, or coalesce. A number of

attactive and repulsive forces (colloidal interactions) are involved when droplets approach closely. Flocculation will occur if the interaction free energy between the particles is negative at some distance of separation. Typically three types of forces are considered; van der Waals attraction, electrostatic repulsion, and steric repulsion. The van der Waals attraction depends on the particle diameter and the Hamaker constant which is affected by the composition of both phases and the surface layer separating them (Walstra, 1987). Electrostatic repulsion depends on the surface potential of the particle, the particle diameter and the ionic strength of the continuous phase. The dielectric constant and temperature may also affect electrostatic repulsion. Protruding flexible macromolecular chains give rise to steric repulsion, which depends mainly on the surface density and length of chains, and on the nature of the solvent. Steric forces may result in attraction between droplets, for example in cases of bridging or cross linking between surface macromolecules. When non-adsorbing macromolecules are present in the continuous phase, depletion flocculation may occur, due to exclusion of emulsified droplets from solvent containing the dissolved macromolecules (Tolstoguzov and Braudo, 1985).

The effects of colloid forces (*ie.* van der Waals attraction, electrostatice repulsion, and steric repulsion) on the interaction energy between approaching surfaces were first analysed in the Deryagin and Landau, Verwey and Overbeek

(DLVO) theory, now much extended and modified. Some general conclusions are:

(1) Electrostatic or steric repulsions can generate a substantial energy barrier which opposes close approach of droplets or lamellae surfaces at separations of a few nm.

(2) When droplets are separated by a greater distance (about 10 nm), a shallow secondary energy minimum can give rise to reversible flocculation. Because of the possibility of flocculation in the secondary minimum, large droplets are usually less stable than small ones.

(3) A high concentration of emulsifier at the surface normally enhances stability. A high average molecular weight and a relatively high bulk concentration of the macromolecular adsorbate generally result in a high surface concentration, and improve emulsion stability.

(4) Electrostatic repulsion is diminished by increasing the ionic strength or by bringing the pH near to the isoelectric point of the adsorbate.

(5) Solvent quality strongly affects the steric repulsion, for example, by altering conformation of the adsorbed macromolecule (Halling, 1981; Walstra, 1987).

Colloidal interactions also affect coalescence rates. There is no generally accepted theory for film stability under quiescent conditions, however the coalescence rate generally increases as drainage proceeds (*ie.* as the solvent between droplets is removed by gravity), or as the oil phase volume

(ϕ) increases (Halling, 1981). Film rupture and coalescence are promoted by a weak repulsion between droplets, a low interfacial tension and a large film diameter (*ie.* large particles)(Darling and Birkett, 1987; Walstra, 1987). Increasing the viscosity of the continuous phase reduces the tendency for close approach of droplets past the DLVO energy barrier and subsequent coalescence. In general, flocculation and coalescence do not occur readily if droplets are less than 10 μm in diameter (Zajic and Panchal, 1976).

1.3 EMULSION FORMATION

Since emulsions are not thermodynamically stable and do not form spontaneously, substantial shearing force must be applied to disperse one phase in another. The most common methods are homogenization, and rapid blending, stirring, or cutting (Jaynes, 1985). During homogenization, two immiscible phases are forced through a narrow aperture under high pressure causing sufficiently high shear to generate small droplets (Walstra, 1983). Blending, shearing, or cutting all provide high shear by forcing a stirrer or blade through a mixture of two phases. Emulsifiers in solution form an interfacial layer, preventing coalescence. The rate at which the emulsifier in solution adsorbs to newly generated interface will then be a factor influencing the minimum droplet size which can be attained (Mangino, 1984).

With increasing time of blending or shearing, the droplet

size falls at first but then approaches a limiting value. This has been observed with all types of emulsifying equipment used, including homogenizers, blenders and sonicators (Halling, 1981; Pearce and Kinsella, 1978; Tornberg and Hermansson, 1977; Tornberg, 1978; Haque and Kinsella, 1988a).

In theory, the minimum diameter (d_{min}) of the droplets produced during emulsifying depends on the energy density E, the interfacial tension γ, and density ρ according to

$$d_{min} \propto E^{-2/5}\gamma^{3/5}\rho^{-1/5} \qquad \text{(Walstra, 1983)}.$$

The importance to the emulsification process of the parameters can be readily deduced from the range of values attainable in practice. The energy density term may vary considerably (from 10^4 W m^{-3} for a paddle stirrer to 10^{12} W m^{-3} for a high pressure homogenizer), but the range of typical interfacial tension values is much smaller (4 to 40 mN m^{-1}). The range of mass density (ρ) is very small (Darling and Birkett, 1987). According to the theory the surface tension term affords only a relatively small degree of control over droplet size, and the energy density (power per unit volume) is the most important factor (Bourgaud *et al.*, 1990).

In practice however, sometimes a high power input is not essential to produce small droplets, depending on the nature of the oil phase, the emulsifying agent or the emulsifying equipment used (Halling, 1981). For example, in the presence of emulsan, gas oil droplets of mean diameter < 1 μm were

produced by mixing on a rotary shaker (150 strokes per min, 2.5 cm stroke length) for 1 h (Rosenberg, 1979a). With the same power and energy consumption, creaming stability (and mean droplet size) of emulsions differed according to the type of emulsifying equipment used (Tornberg, 1978).

1.4 MEASURING EMULSIFIER EFFICIENCY

Emulsifying properties of food proteins are most often tested on simple model systems consisting of an aqueous phase of appropriate pH and ionic strength, emulsifying agent, and an oil phase. Other components of the food product formulation (*eg.* sucrose, hydrocolloids, low molecular weight amphiphiles, *etc.*) are omitted. To facilitate comparisons of emulsifiers, lower protein concentrations and less severe emulsifying conditions are employed than in commercial products (Dickinson, 1992a).

Some of the components omitted from the model system have a significant effect on generating and maintaining stable emulsions. For example the hydrocolloid, xanthan gum is a branched, water soluble polymer included in many foods as a viscosifying agent. In salad oils it reduces the rate of creaming in emulsified salad oils (Hibberd *et al.*, 1987). For long term stability of emulsions, addition of hydrocolloids may be favoured over increasing the concentration of a protein emulsifying agent for economic reasons; hydrocolloids increase viscosity substantially at very low concentrations, and are

very inexpensive compared to food proteins or synthetic emulsifying agents.

Two general methods are most widely used for evaluating emulsifying activity of emulsifiers in model systems. The methods involve measuring (1) the emulsion capacity, or (2) the initial droplet size, or change in droplet size over a short time.

The "emulsifying capacity" of a protein is defined as the maximum amount of oil that can be dispersed in an aqueous phase containing a known amount of the protein under uniform conditions. It is a simple method introduced by Swift *et al.* (1961). An aqueous solution containing the protein is vigorously stirred while oil or melted fat is run in steadily. After a certain volume has been added, the emulsion in the mixer undergoes a sudden change referred to as either inversion or breaking, similar to the endpoint in a titration. The endpoint is detected by a sudden drop in viscosity, which causes a change in stirrer motor sound and a decrease in amperage drawn (Crenwelge *et al.*, 1974), a change in visual appearance of the emulsion especially if an oil-soluble dye is present (Wang and Kinsella, 1976), and a sudden increase in electrical resistance in the emulsion (Webb *et al.*, 1970). The volume of oil added is taken as a measure of emulsifying capacity of the proteins involved. The emulsion capacity is normally expressed as the amount of oil emulsified by unit weight of protein. Inter-laboratory comparisons of emulsifying

capacity are difficult since the values obtained are sensitive to experimental variables such as temperature, the rate of addition of oil, and the stirring speed (Ivey *et al*, 1970; Crenwelge *et al*., 1974). A major criticism of this method is that the parameter measured - the highest oil content just prior to phase inversion - does not necessarily relate directly to the effectiveness of the emulsifier when used under different conditions well away from the threshold of inversion (Dickinson and Stainsby, 1988).

The second general method for evaluating emulsifiers involves measuring droplet size in the emulsion. The most effective emulsifying agents produce either the smallest droplets initially, or emulsions in which rate of coalescence (ie. rate of increase in droplet size) is least, or both. Data is expressed either as average mean droplet diameter, or total interfacial area in the emulsion, calculated using values for the volume-surface mean droplet size and the oil phase volume.

When protein is used as an emulsifier it usually gives rise to very stable emulsions (Tornberg and Ediriweera, 1987). It would be very tedious to follow natural coalescence, so various techniques may be used to accelerate this process. These techniques include heating (Pearce and Kinsella, 1978), freezing (Tornberg and Ediriweera, 1987), centrifugation (Dagorn-Scaviner *et al*., 1987), and agitation (Britten and Giroux, 1991). A general criticism of studies using "accelerated aging" procedures is that not only the rate but

the mechanism of breakdown may be altered, and the results are not necessarily indicative of storage stability (Halling, 1981).

Measurement of droplet size, or total surface area of droplets in emulsions is the method most often used to compare protein emulsifiers. There are a number of different ways to determine droplet size. Prior to measuring droplet size, the emulsion is diluted usually with 0.1 % wt/vol sodium dodecyl sulfate (Pearce and Kinsella, 1978). The detergent displaces macromolecules from the droplet surfaces and inhibits flocculation. A viscosifying agent (*eg.* glycerol or dextran) may also be included (Mita *et al.*, 1973; Campanelli and Cooper, 1989; Cameron *et al.*, 1991). There appears to be no change in droplet sizes upon dilution (Dickinson and Stainsby, 1988).

Optical microscopy is the simplest method for measuring droplet sizes of approximately 1 μm diameter or greater. Since large numbers of droplets have to be counted to obtain a valid size distribution, automated image analysis systems have been developed for use with optical microscopy (Klemaszewski *et al.*, 1989). Coulter counters permit enumeration of droplets as small as 0.6 μm diameter (Walsta and Oortwijn, 1969; Bourgaud *et al.*, 1990). Exclusion of the smallest droplets from size distributions means that the interfacial area will be underestimated. These methods do place more emphasis on the larger droplets in the distribution which may be more

important for long term stability.

Commercial light scattering instruments based on photon correlation spectroscopy are most often used for particle sizing in the range below *ca.* 0.5 μm. By measuring dynamic light scattering at just one angle, the average hydrodynamic diameter of the smallest particles is calculated from the mean Z-average diffusion coefficient. Average particle sizes in polydisperse emulsion samples obtained from dynamic light scattering measured at fixed angles tend to be strongly biased towards the small-particle end of the distribution (Dickinson and Stainsby, 1988). The presence of large droplets which could cause emulsion instability may not be detected. If measurements are taken over a range of scattering angles, more accurate particle size distributions can be produced for polydisperse samples (Frock, 1987). Sample preparation and data analysis for photon correlation spectroscopic methods are more difficult than for Fraunhofer diffraction-based or turbidimetric methods (Gulari *et al.*, 1987).

Instruments based on forward scattering of laser beams (Fraunhofer diffraction) provide size distribution data for micron-sized particles. The scattering measurements are restricted to small angles (forward diffraction) and if particles are much larger (3 to 4 times) than the wavelength of radiation, then the particle properties (real and imaginary refractive indices) are unimportant. Scattering intensities depend only on the diameters of the particles (Dodge, 1984).

Satisfactory data are obtained for particles greater than 1 to 2 μm diameter (Dodge, 1984; Fischbach and Bond, 1984; Gulari *et al.*, 1987).

Photosedimentation, or measuring turbidity changes in a disk centrifuge, is widely used to determine particle size distribution in colloidal suspensions and dispersions (*eg.* for paint pigments), but has been rarely used with food emulsions (Matsumoto and Fukushima, 1974; Kako and Sherman, 1974). Particle size is determined by monitoring sedimentation of the particle in a viscous liquid under a centrifugal force, in a transparent spinning disc. The detector system consists of a lamp and photodiode on opposite sides of the disc. Extinction of transmitted light is recorded as a function of time, and the particle size distribution is derived from the data by using Stokes law and applying optical extinction corrections to convert turbidity to particle concentration (Thomas *et al.*, 1991).

Commercial equipment for measuring particle size distributions by light scattering is very expensive and sample preparation can be problematic (Dickinson and Stainsby, 1988). Dust particles, detergent micelles, *etc.* can cause substantial interferences.

Turbidimetry is the most commonly used method for comparing total interfacial areas or droplet sizes in emulsions since measurements can be obtained with standard laboratory spectrophotometers (Bagchi and Vold, 1975; Pearce

and Kinsella, 1978; Reddy and Fogler, 1981a). For systems within the domain of geometrical optics, ie. absorbance is independent of wavelength over the whole range of visible light (van de Hulst, 1957; Bagchi and Vold, 1975), extinction is primarily due to refraction and reflection of light incident on the particles and diffraction of light passing near the edges of the particles. When none of the light scattered by the turbid sample reaches the photodetector, and for a sample which does not absorb light, the turbidity of the sample is given by

$$T = \frac{2.303\ A}{l}$$

where A is the absorbance and l is pathlength of the cuvette. For a dilute dispersion of spherical particles, which are large compared to the light wavelength,

$$\text{Interfacial area} = 2\ T$$

$$R = \frac{3\ \phi}{2\ T}$$

where ϕ is the volume fraction of the disperse phase, R is the volume-area mean radius of the dispersed particles (Pearce and Kinsella, 1978; Kerker, 1969). For systems satisfying the assumptions, absorbance is proportional to interfacial area of the dispersed droplets. Pearce and Kinsella (1978) used these relationships to define a standard measure of emulsifying activity, the

$$\text{Emulsifying Activity Index (EAI)} = \frac{2\ T}{\phi\ C}$$

where C is weight protein per unit volume of the aqueous phase before the emulsion is formed. The index provides units of interfacial area stabilized per unit weight protein ($m^2.g^{-1}$ emulsifier), and therefore has a rational basis in terms of the thermodynamic tendency of emulsions to minimize interfacial area. With this method, emulsions can be compared simply and rapidly.

In practice, there is substantial error in the estimation of interfacial area due to (a) presence of droplets small relative to the wavelength of light, and (b) a finite angle of acceptance of the photodetector, so that light scattered at low angles as well as transmitted light reaches the photodetector (Pearce and Kinsella, 1978).

More complex equations are available for determining mean particle size (to *ca.* 0.2 μm) in fine emulsions, but their use requires some knowledge of the form and size of the distribution, refractive indices and measurements of turbidity over a range of wavelengths (Walstra, 1965a,b, 1968; Goulden, 1961). In addition, standard laboratory spectrophotometers all have different acceptance angles according to the geometry of the components. In most cases spectrophotometers have to be modified to provide a sufficiently small acceptance angle. This involves increasing the distance between the sample and the photodetector, or placing masks to block out light scattered at low angles (Heller and Tabibian, 1957; Goulden, 1961; Walstra, 1965b). This is rarely done by emulsion

researchers.

Even though there are definite shortcomings with the EAI, it is the method most widely used for comparing emulsifying agents. The values provided are a function of the equipment used to such an extent that interlaboratory comparisons probably are not valid.

1.5 PROTEIN ADSORPTION AT INTERFACES

Most food emulsions are stabilized by an adsorbed layer of protein at the oil-water or air-water interface (Darling and Birkett, 1987). Some information from studies on the thermodynamics of protein adsorption and behaviour of pure protein films at well-defined interfaces may be useful.

Nearly all proteins adsorb at interfaces. The main thermodynamic driving force for protein adsorption at the interface is the removal of non-polar parts of the molecule from the aqueous environment of the bulk solution (Mangino, 1984; Kinsella, 1984). The major factors contributing to the Gibbs free energy of adsorption, roughly in order of importance, are

(1) dehydration of hydrophobic regions of the interface,

(2) changes in protein secondary and tertiary structure,

(3) charge redistribution arising from interaction of electrical fields of the protein and interface,

(4) changes in environment of ions on transference from solution to the protein layer,

(5) changes in degree of protonation (pK values) of amino acid side chains, and

(6) van der Waals interactions between proteins and the interface (Dickinson, 1992a).

Factors 1 and 2 favour adsorption, 3 and 4 oppose adsorption, and 5 and 6 are quantitatively of little significance. The main entropic force is displacement of ordered water molecules from the predominantly hydrophobic interface. A second driving force is associated with unfolding of the protein on adsorption, and depends on configuration of the protein involved (Damodaran and Song, 1988).

The adsorption of proteins is thought to occur in distinct steps: (a) diffusion from the bulk solution to the interface, the rate of which depends on molecular size, (b) penetration of macromolecules into the surface layer, or actual adsorption, and (c) reconformation or rearrangement of adsorbed molecules within the surface layer (Graham and Phillips, 1979a,b,c,; de Feijter and Benjamins, 1987; Damodaran and Song, 1988).

Adsorption isotherms for protein at the air-water and oil-water interface are very steep, and dilution of emulsions with solvent of the continuous phase does not cause appreciable desorption of protein (Walstra, 1987; de Feijter and Benjamins, 1987). Consequently it is frequently assumed that protein adsorption is irreversible, although this is not in fact the case (MacRitchie, 1985; Dickinson *et al.*, 1988b).

The equilibrium constant between the adsorbed and unadsorbed states is proportional to the Boltzman factor, $e^{E/kT}$, where E is the total binding energy. Consider a protein for which a third of the amino acid residues are bound to the interface. Even with a small mean binding energy per amino acid, the energy per molecule is sufficiently large that the Boltzmann factor is overwhelmingly weighted towards the adsorbed state (Stainsby, 1986; Dickinson *et al.*, 1988b; Dickinson, 1992a).

Practical consequences of protein adsorption behaviour for protein-stabilized emulsions are (a) a low bulk concentration of protein results in a high degree of coverage of the interface (*ie.* macromolecular emulsifiers are efficient surface active agents; de Feijter and Benjamins, 1987), and (b) when a mixture of two types of surface active molecules are present (*eg.* more than one protein, or a protein and a small molecule surfactant), the component which reduces the surface tension most will predominate at the interface (Robson and Dalgleish, 1987; Courthaudon *et al.*, 1991a,b; Dickinson and Tanai, 1992). If present at sufficient concentration, small molecule surfactants such as those present in natural triglyceride oils readily displace proteins from the oil-water interface (Walstra, 1987; Darling and Birkett, 1987; Krog, 1991). Small molecule amphiphiles have greater affinity for the non polar phase and at high bulk phase concentration can pack more effectively at the fluid interface than conformationally-constrained proteins (Stainsby, 1986).

A difference between a surface saturated with protein versus one containing small-molecule surfactants is that only a fraction of the available surface area is covered by unfolded protein, compared with nearly 100 % for the surfactant (Dickinson, 1992a). Topological constraints imposed by the peptide-linked backbone, and presence of hydrophilic as well as hydrophobic amino acids in any protein limit coverage of the interface (Stainsby, 1986).

After a protein has adsorbed at an interface, changes of molecular configuration occur. Depending on the protein involved, equilibrium interfacial tension may be reached only after some time (de Feijter and Benjamins, 1987; Dickinson *et al.*, 1987, 1988a,b; Damodaran and Song, 1988). The consequences for making an emulsion are that an extended period of blending or multiple passes through a homogenizer may be required to obtain maximum emulsifying activity of a protein emulsifier. Parts of the protein may penetrate the lipid phase. Proteins such as ß-casein which have little secondary structure and no intramolecular disulfide bonds denature at interfaces far more readily than globular proteins such as lysozyme or bovine serum albumin. Much of the secondary and some of the tertiary structure of globular proteins is retained on adsorption (Graham and Phillips, 1979a,b,c; de Feijter and Benjamins, 1987; Dickinson, 1992a). The total interfacial area in emulsions stabilized with bovine serum albumin was greater when disulfide bonds had been

cleaved (Klemaszewski *et al.*, 1990). Proteins such as ovalbumin and whey proteins can denature at interfaces to such an extent that they become insoluble; repeated adsorption and desorption as occurs during prolonged emulsification may result in aggregation and a reduction in their ability to stabilize emulsions (Pearce and Kinsella, 1978).

The extent of surface denaturation of proteins at oil-water interfaces also varies depending on protein concentration. At low bulk concentrations, ß-casein unfolded so that little tertiary or secondary structure remained, while at higher concentrations, native and unfolded molecules coexisted at the surface, and multi-layers adsorption occurred (Graham and Phillips, 1979c; de Feijter and Benjamins, 1987). The result is that stable emulsions can be obtained even with a very low concentration of a protein emulsifier.

1.6 CHARACTERISTICS OF PROTEIN EMULSIFIERS

While nearly all proteins adsorb at oil-water or air-water interfaces, some are clearly better emulsifying agents than others. Proteins which emulsify efficiently are soluble and possess sufficient hydrophobicity. Proteins have minimum solubility at their isoelectric pH (Scopes, 1982); when the net charge on the protein is zero, the protein adopts its most compact configuration, and interaction with the solvent, water, is a minimum (Halling, 1981; Mangino, 1984). Emulsifying activity of proteins is typically minimum at the

isoelectric point (Mita *et al.*, 1973, 1974; Crenwelge *et al.*, 1974; Aoki *et al.*, 1980; Haque and Kinsella, 1988b; Das and Kinsella, 1989; Halling, 1981). Undissolved protein appears to make little or no contribution to emulsification (Halling, 1981). Solubility and emulsifying activity of a number of proteins varied in similar fashion with changes in pH (Crenwelge *et al.*, 1974). At pH values far from the isoelectric point, emulsifying activity was as much as fourfold greater than at the isoelectric point (Crenwelge *et al.*, 1974; Das and Kinsella, 1989).

The isoelectric points of most proteins are between slightly acidic to neutral pH values, which is also the pH of most emulsified foods. The potential emulsifying activity is therefore not realized in most food products. Research has been directed towards improving protein solubility in mildly acidic to neutral conditions by chemical or enzymatic modification. One approach is covalent attachment of charged molecules to protein emulsifiers. Succinylation, phosphorylation, acetylation, or attachment of acidic amino acids to proteins improved the solubility and emulsifying activity (Franzen and Kinsella, 1976; Kinsella and Shetty, 1979; Waniska *et al.*, 1981; Huang and Kinsella, 1987; Kinsella and Whitehead, 1988; Gueguen *et al.*, 1990). Chemical glycosylation increased solubility and altered interfacial properties of certain casein fractions near their isoelectric points (Closs *et al.*, 1990; LeMeste *et al.*, 1990; Cayot *et*

al., 1991). Glycosylated caseins adsorbed more rapidly at the interface and reduced surface tension more than unmodified caseins (Closs *et al.*, 1990). Attachment of carbohydrate polymers (Kato *et al.*, 1988, 1989; Dickinson and Semenova, 1992; Nakamura *et al.*, 1992) improved emulsifying properties of a variety of globular proteins. The improvement in emulsifying activity of glycosylated proteins appeared to be a result of increased water binding by the emulsifier, and increased steric repulsion between emulsified droplets (Stainsby, 1986; Dickinson and Semenova, 1992).

In addition to being soluble, efficient protein emulsifiers must also be sufficiently hydrophobic (Nakai, 1983; Das and Kinsella, 1990; Mine *et al.*, 1991; Klemaszewski *et al.*, 1992). A balance between hydrophilic and lipophilic portions of the protein is required for optimum emulsifying properties (Aoki, *et al.*, 1981; Chobert *et al.*, 1987; Akita and Nakai, 1990). A significant correlation was obtained between protein surface hydrophobicity (determined fluorimetrically, by binding of a hydrophobic probe, *cis*-parinaric acid), interfacial activity, and emulsifying capacity for a large number of proteins (Kato and Nakai, 1980; Nakai *et al.*, 1980). The surface hydrophobicity also correlated positively with emulsion capacity of proteins which were partially denatured by heating. With extensive heat denaturation, however, a decrease in protein solubility limited the emulsifying activity of these proteins (Voutsinas

et al., 1983; Kato *et al.*, 1986).

Certain proteins contain covalently bound lipids (*eg.* lipoprotein in egg yolk) which anchor the macromolecule at the oil-water interface and enhances the emulsifying capacity by reducing the interfacial tension substantially (Stainsby, 1986; Carillo and Kokini, 1988). Covalent attachment of lipophilic moieties such as hydrophobic amino acids (Arai and Watanabe, 1987; Chobert *et al.*, 1987), fatty acids (Haque and Kito, 1983a,b; Akita and Nakai, 1990), alkyl chains (Magdassi and Stawsky, 1989) improved the emulsifying activity of a variety of soluble proteins.

1.7 POLYSACCHARIDE EMULSIFYING AGENTS

Gum arabic and emulsan are macromolecular emulsifying agents consisting mainly of polysaccharide, and a small amount of protein, which differ in emulsifying properties and applications from those of the protein emulsifiers.

Gum arabic is derived from plants of the genus *Acacia*, and is used to stabilize flavour oil emulsions in soft drinks. It consists of a mixture of highly branched arabinogalactan heteropolymers with *ca.* 2 % covalently linked protein (Dickinson and Euston, 1991). The protein is associated mainly with a high molecular weight fraction representing approximately 20 to 30 % of the total gum (Randall *et al.*, 1988; Dickinson *et al.*, 1991). The protein rich fractions adsorb at the oil-water interface and are mainly responsible

for the emulsifying and stabilizing properties of the natural gum (Dickinson *et al.*, 1988c, 1991).

The interfacial tension between an aqueous solution of gum arabic and an oil phase decreases rapidly in the first few minutes of contact, then decreases more gradually until equilibrium is attained after 3 to 4 days (Dickinson *et al.*, 1988c). With gum arabic, a great deal of work was required to produce an emulsion which, once set up, was very stable (Zajic and Panchal, 1976).

Emulsan is a polysaccharide-based emulsifying agent used commercially for cleaning oil-contaminated vessels and storage tanks. Aqueous emulsions of heavy crude oil stabilized with emulsan have lower viscosity which greatly facilitates pipeline transport of the hydrocarbon (Fiechter, 1992). Emulsan is produced by the bacterium *Acinetobacter calcoaceticus* RAG-1 when grown on hydrocarbon substrates (Rosenberg *et al.*, 1979a; Zuckerberg *et al.*, 1979; Pines and Gutnick, 1986). Purified emulsan is an anionic heteropolysaccharide of average molecular weight *ca.* 10^6 Da, and contains galactosamine, galactosamine uronic acid, and an unidentified hexosamine. The amphipathic properties of emulsan are due in part to fatty acids joined to the polysaccharide backbone by ester and amide linkages (Belsky *et al.*, 1979; Zuckerberg *et al.*, 1979).

Emulsifying activity of emulsan in sea water was near maximal between pH 5 and 9. Above pH 6, emulsifier activity

was dependent on divalent cations. The loss of emulsifying activity in acidic conditions corresponded to protonation of the carboxyl groups (pK 3.05) in the polymer (Zuckerberg *et al.*, 1979).

Emulsan produced stable emulsions with gas oil or any hydrocarbon mixture containing both aliphatic and aromatic compounds. Pure aliphatic (hexadecane), aromatic (methylnaphthalene), or cyclic (cyclohexane) hydrocarbons were not emulsified (Rosenberg *et al.*, 1979b). Average droplet sizes of hyrocarbon-in-water emulsions were less than 1 μm diameter; upon standing several days, the emulsions separated by creaming rather than by coalescence (Rosenberg, 1986). Water binding by the carbohydrate portion, electrostatic repulsion, and steric factors account for the properties of emulsions made with emulsan.

Increased yields of emulsan were obtained with mutant strains of *Acinetobacter calcoaceticus* selected by their resistance to cetyltrimethyl-ammonium bromide (Shabtai and Gutnick, 1986).

1.8 EMULSIFYING AGENTS FROM YEASTS

Yeasts were chosen as a potential source of food grade bioemulsifier for several reasons. Yeasts which produce emulsifiers have been described in the literature. Due to their rapid growth rates and simple nutritional requirements, yeasts can be produced inexpensively. Most consumers associate

yeasts with edibles such as bread, beer and wine. Consumer acceptance of an emulsifying agent from a yeast might be obtained more readily than for an emulsifier produced by a bacterium.

Yeasts known to produce emulsifying agents are listed in Table 1.1. As with the emulsifier emulsan which is produced by the oil-degrading bacterium *Acinetobacter calcoaceticus* (Rosenberg *et al.*, 1979a), emulsifiers were first detected in hydrocarbon-utilizing yeasts (Iguchi *et al.*, 1969; Kappeli and Fiechter, 1977; Kappeli *et al.*, 1978; Pareilleux, 1979; Roy *et al.*, 1979; Cirigliano and Carman, 1984, 1985; Singh *et al*, 1990). Typically the emulsifiers are produced only in the presence of the hydrocarbon substrate (Table 1.1), and facilitate its assimilation. Addition of emulsifier to glucose-grown (emulsifier negative) *Candida lipolytica* permitted growth on hydrocarbons without a lag phase (Pareilleux, 1979).

Most of the emulsifying agents from hydrocarbon-grown yeasts were isolated from the culture broth (Table 1.1). The emulsifier of *Candida tropicalis* was attached to the cell surface and was released by treatment with the protease, Pronase (Kappeli and Fiechter, 1977). The emulsifier of *Torulopsis petrophilum* was attached to the cells in growing cultures but was released from stationary phase cells (Cooper and Paddock, 1983).

For use as an emulsifier in foods, there are several

Table 1.1. Characteristics of known emulsifiers from yeasts

Species	Reference	Yield	Induced by		Location[a]		Composition[b]
		(g/g cell dry wt)	alkanes	glucose	culture broth	cell-bound	
Candida petrophilum	Iguchi et al., 1969		+	-	+		peptide/fatty acid
Candida tropicalis	Kappeli et al., 1978	3.8%	+	-		+	mannan/FA 95.7%/ 4.3%
Candida lipolytica	Pareilleux, 1979		+	-	+		CHO/protein/FA 44%/ 27%/ 3%
Endomycopsis lipolytica	Roy et al., 1979	4.0%	+	-			protein
Torulopsis petrophilum	Cooper & Paddock, 1983	0.8%	-	+	85%	15%	protein
Candida lipolytica	Cirigliano & Carman, 1985	1.9%[c]	+	-	+		CHO/protein 83%/ 17%
Candida tropicalis	Singh et al., 1990		+	+	+	+	

[a] Origin of the emulsifier studied; from the culture broth or attached to the cells, or both.

[b] CHO, carbohydrate; FA, fatty acid

[c] Cell dry weight was not reported, so the yield of cells was calculated from the initial concentration of alkane in the growth medium, assuming complete utilization and a conversion efficiency of 1.0 (Reed, 1982).

problems with emulsifiers from yeasts grown on hydrocarbons (1) low yields of emulsifier and the requirement to grow yeast specifically for emulsifier production would make the process costly,

(2) hydrocarbons are expensive compared to carbohydrate as substrates for yeast growth,

(3) all traces of the hydrocarbon would have to be removed prior to use of the emulsifier in foods.

1.9 STRUCTURE AND COMPOSITION OF YEAST CELL WALLS

The emulsifiers from *Candida tropicalis* (Kappeli and Fiechter, 1977; Kappeli *et al.*, 1978) and *Torulopsis petrophilum* (Cooper and Paddock, 1983) were attached to the surface of the yeast cells. Knowledge of the structure and composition of yeast cell walls could be useful for isolating these emulsifiers. The cell walls of most yeasts are believed to be organized in roughly similar fashion (Ballou, 1976). The cell envelope of *Saccharomyces cerevisiae* will be described briefly as it has been studied in greatest detail.

The cell walls of *S. cerevisiae* consist mainly of glucan and mannoproteins, in approximately equal amounts (Cabib *et al.*, 1982). The glucan is a branched, meshlike polymer of glucose subunits joined by ß-1,3 and ß-1,6 linkages. Mannoproteins are extracellular proteins to which mannose polymers are covalently attached by N-linkage to asparagine or O-linkage to serine and threonine (Ballou, 1982). A small

amount of another carbohydrate polymer, chitin, is present mainly in the bud scars.

Immunochemical and histological data from previous studies provide evidence for a layer of mannoprotein at the surface of the yeast cell wall (Ballou, 1976; Zlotnick *et al.*, 1984). Antibodies to mannoproteins adhere to the surface of yeast cells (Ballou, 1976). Transmission electron micrographs of the yeast cell wall demonstrate an electron-dense zone at the cell surface (Zlotnick *et al.*, 1984). This layer was removed by treatment with a protease contained in Zymolyase (a yeast-lytic enzyme preparation), and also with reducing agent (ß-mercaptoethanol), or detergent. Mannoproteins at the cell surface are therefore believed to be attached by disulfide bridges or by hydrophobic interactions (Kidby and Davies, 1970; Ballou, 1982; Zlotnick *et al.*, 1984). One of the roles of the mannoprotein surface layer may be to provide the yeast cell with a hydrophilic exterior to maintain contact with dissolved nutrients in the medium. The hydrophobic portions of mannoproteins anchor the molecules to the cell wall (Cabib *et al.*, 1982).

The extracellular compartment of yeast cells contains at least 30 individual mannoproteins (Sentandreu *et al.*, 1984), located in the cell wall or the periplasm. The functions of at least two thirds of these mannoproteins are not known (Lehle and Tanner, 1987; Kukuruzinska *et al.*, 1987; Reid, 1991). Mannoproteins with known function include cell mating factors

and agglutinins, and extracellular enzymes such as invertase, acid phosphatase, chitinases, and a variety of glucanases and glucan-synthetic enzymes (Sentandreu *et al.*, 1984; Lehle and Tanner, 1987). A structural role has been ascribed to mannoproteins for which no specific function is known.

The DNA sequences of three genes encoding cell wall mannoproteins have been determined recently. These mannoproteins contain between *ca.* 10 to 60 % carbohydrate and each possesses a hydrophobic region. A chitinase (M_r 130 kDa) required for separation of growing *S. cerevisiae* cells contained four domains: a signal sequence (amino acids 1-20); a catalytic domain (amino acids 21-327); a serine/threonine rich region (amino acids 328-480); and a carboxy terminal domain (amino acids 481-562) with high binding affinity for chitin (Kuranda and Robbins, 1991). The surface of insoluble chitin fibers is essentially hydrophobic. The secreted chitinase was quantitatively adsorbed from culture supernatants by addition of insoluble chitin. This interaction was stable in high salts (5 M NaCl), over a wide range of pH (3-10), and to many denaturants including 8 M urea (Kuranda and Robbins, 1991), and therefore has the characteristics of a hydrophobic interaction. The predicted molecular weight from the amino acid sequence accounted for 46 % of the weight of the isolated glycoprotein, the remainder of the mass being contributed by O-linked oligosaccharides attached to serine and threonine (Kuranda and Robbins, 1991).

An exo ß-1,3 glucanase (29 kDa) was solubilized by heating isolated cell walls at 90°C and purified by high pressure liquid chromatography (Klebl and Tanner, 1989). The purified glycoprotein showed lectin-like binding to insoluble hydrophobic polymers ß-1,3 glucan and chitin. It was estimated that more than 90 % of the glycoprotein originally present in the yeast cell walls was lost during purification due to binding to cell wall components and a strong tendency to self aggregate (Klebl and Tanner, 1989). Two potential N-glycosylation sites are contained in the predicted amino acid sequence. After treatment of the purified glycoprotein with endo-N-acetylglucosaminidase, the molecular weight was 26 kDa.

The *kre1* gene encodes a protein of 313 amino acids (predicted molecular weight 32 kDa) which is highly glycosylated since the molecular weight determined by gel electrophoresis is 80 kDa (Boone *et al.*, 1990). Disruption of the gene results in reduction of content of ß-1,6 glucan in the cell wall. The protein contains 25 % threonine and 15 % serine residues. The 21 carboxy terminal amino acids of the glycoprotein form a hydrophobic sequence which may serve as a membrane spanning domain or provide a signal for attachment of a glycosyl-phosphatidyl inositol membrane anchor (Boone *et al.*, 1990; Conzelmann *et al.*, 1988).

1.10 OBJECTIVES

The objectives of this study are

(1) to identify an emulsifying agent produced by a yeast, which could replace the synthetic emulsifying agents used in foods and cosmetics.

(2) to develop standard methods for evaluating emulsifier activity, and use these to characterize the emulsifier selected.

(3) to investigate structure and function of the emulsifier and identify the most active components, to permit optimization of emulsifier yield and/or activity.

CHAPTER 2: IDENTIFICATION OF MANNOPROTEIN FROM BAKER'S YEAST AS AN EMULSIFIER.

Published as:

Cameron, D.R., D.G. Cooper, and R.J. Neufeld. 1988. The mannoprotein of *Saccharomyces cerevisiae* is an effective bioemulsifier. Appl. Environ. Microbiol. **54**:1420-1425.

PREFACE

In preliminary experiments, several yeast strains were identified which produced emulsifiers when grown on glucose. Some of the emulsifiers from these strains were attached to the cells, or were released progressively in older cultures, similar to the emulsifier from *Torulopsis petrophilum* (Cooper and Paddock, 1983). A study by Kappeli and Fiechter (1977) describes a method to release cell-bound emulsifier from alkane-grown *Candida tropicalis*, by treating the cells with the protease Pronase.

We tested the ß-1,3 glucanase enzyme, Zymolyase, for releasing emulsifier bound to yeast cell surfaces. This enzyme is capable of lysing cells of many yeast species, by degrading the glucan in cell walls (Kaneko et al., 1973). Whole cells of *Saccharomyces cerevisiae*, which did not emulsify whatsoever, were included as a control for the enzyme digestion. Emulsifier was released from all of the emulsifying strains, and surprisingly from *S. cerevisiae* as well.

A second method of releasing cell wall components from *S. cerevisiae* (autoclaving in neutral buffer; Peat et al, 1961) also released emulsifier. This second method would be simpler than enzyme digestion for production of emulsifier on a large scale, and would have the advantage of sterilizing the product at the same time. Obtaining the emulsifier from a raw material already available (baker's yeast) would eliminate the need for

a growth step in the production scheme. Approval of an emulsifier from *S. cerevisiae* for use in foods should be obtained readily. The spent yeast from beer and wine production were also suitable for producing the emulsifier. This could eliminate or reduce a waste disposal problem for these industries.

2.1 ABSTRACT

The mannoprotein which is a major component of the cell wall of *Saccharomyces cerevisiae* is an effective bioemulsifier. Mannoprotein emulsifier was extracted in high yield from whole cells of fresh baker's yeast by two methods, autoclaving in neutral citrate buffer and by digestion with Zymolase, a ß-1,3 glucanase. Heat-extracted emulsifier was purified by ultrafiltration and contained approximately 44% carbohydrate (mannose) and 17% protein. Treatment of the emulsifier with protease eliminated emulsification. Kerosene-in-water emulsions were stabilized over a broad range of conditions, from pH 2 to 11, with up to 5% sodium chloride or up to 50% ethanol in the aqueous phase. In the presence of a low concentration of various solutes emulsions were stable to three cycles of freezing and thawing. An emulsifying agent was extracted from each species or strain of yeast tested, including 13 species of genera other than *Saccharomyces*. Spent yeast from the manufacture of beer and wine was demonstrated to be a possible source for large scale production of this bioemulsifier.

2.2 INTRODUCTION

Despite their typically high production cost, emulsifiers derived from natural sources may have certain advantages over the chemically synthesized emulsifying agents. Due to the increasingly anti-synthetic or "organic" preferences of North American consumers, bioemulsifiers may eventually become cost effective for various applications.

All of the known bioemulsifiers from yeasts are produced by yeasts growing on water-immiscible substrates such as alkanes or oils. These yeasts include *Candida petrophilum* (Iguchi *et al.*, 1969), *Candida tropicalis* (Kappeli and Fiechter, 1977; Kappeli *et al.*, 1978), *Torulopsis petrophilum* (Cooper and Paddock, 1983), and *Yarrowia lipolytica* (also known as *Candida lipolytica* and *Endomycopsis lipolytica* (Cirigliano and Carman, 1984, 1985; Pareilleux, 1979; Roy *et al.*, 1979)). The emulsifying agents were produced only in the presence of the water-immiscible substrates and appear to facilitate their metabolism (Pareilleux, 1979).

The bioemulsifiers were extracellular or bound to the cell surface and all contained carbohydrate and peptide material. Various methods have been used to isolate the bioemulsifiers. These include enzymic digestion (Kappeli and Fiechter, 1977; 1978), foam fractionation (Pareilleux, 1979), and repeated extractions with chloroform/methanol (Cirigliano and Carman, 1984; 1985). The low yields obtained, the

requirement for hydrocarbon substrates and the complex procedures for preparation make commercial development of the known yeast-derived bioemulsifiers unlikely.

We undertook this study to increase the yield of yeast-derived bioemulsifiers. We describe a novel bioemulsifier which can be extracted simply and with very good yield from *Saccharomyces cerevisiae*, baker's yeast. This bioemulsifier was identified as mannoprotein, a major component of the yeast cell wall. Emulsification characteristics of mannoprotein were determined. Emulsifying agents were extracted from a variety of yeasts including the spent yeast from production of beer and wine, from yeasts used for single cell protein, and from 13 yeasts of diverse genera.

2.3 MATERIALS AND METHODS

Chemicals and enzymes. All chemicals used were of reagent grade. Zymolyase 20T was from Miles Laboratories (Toronto, Ontario); Pronase, from Boehringer Mannheim Co. (Montreal, Quebec). Mineral oil (USP grade 70) was donated by Witco (Montreal). Kerosene and corn oil were purchased in retail stores.

Yeast strains and growth media. Fresh baker's yeast (*S. cerevisiae*) was obtained from Lallemand Inc. (Montreal). Spent yeast was obtained by settling after the primary fermentation from small batches of beer and wine inoculated with

commercially available yeast strains. Other yeast species were kindly provided by Mr. R. Latta of the Culture Collection of the National Research Council of Canada (Ottawa, Ontario). *Candida utilis* Y900 was from the collection of Weston Research Centre, Toronto. Pure cultures of all yeast strains were maintained at 4°C on slopes of YPD medium (0.5% yeast extract, 1% peptone, 1% dextrose and 1.5% agar). Yeasts were grown in YPD liquid medium for 2 days on a New Brunswick G25 incubator-shaker with rotary shaking (150 $rev.min^{-1}$) at 28°C. For screening of emulsifier production by intact cells, strains were grown similarly in YCD medium in which 1% casamino acids replaced the peptone in YPG.

Extraction and purification of emulsifier. The method of Peat (*et al.*, 1961) for the extraction of mannan was modified to extract emulsifier from yeasts. A reducing agent, potassium metabisulfite, was included in the heat-extraction buffer as it increased the emulsifier yield. Fresh baker's yeast or spent yeast was washed twice in distilled water and suspended at 20% (wet wt/vol) in 0.1 M potassium citrate and 0.02 M potassium metabisulfite. The pH of the mixture was adjusted to 7.0. After autoclaving 3 h (121°C) the mixture was centrifuged at 5000 xg for 10 min at ambient temperature. For crude preparations of emulsifier, three volumes of 95% ethanol containing 1% (vol/vol) acetic acid were added to the supernatant. The solution was cooled and kept at 4°C for 16 h. The resultant precipitate was collected by centrifugation

(10,000 xg). The crude emulsifier was air-dried and stored at room temperature. Crude emulsifiers from other yeast species were prepared similarly.

For large scale preparations of purified emulsifier the supernatant (after autoclaving) was concentrated by passage through a hollow fibre ultrafiltration unit (Romicon; Woburn, Mass.) with a molecular weight exclusion limit of 10,000. Subsequently, distilled water (5 x the volume of permeate) was passed through the filter, then the sample was concentrated to approximately one tenth the original volume. The ultrafiltered emulsifier (referred to henceforth as the "purified emulsifier") was stored in liquid form at 4°C or was precipitated with ethanol and dried as described previously.

Assay of emulsification activity. Emulsification was evaluated as described previously (Akit *et al.*, 1981; Cooper and Paddock, 1983): material to be tested was dissolved in 4 ml of distilled water in a 13 x 122 mm test tube, 6 ml of kerosene (or another water-immiscible liquid) was added and the tube contents were mixed to homogeneity on a Vortex-genie (Fisher) at the maximum setting. After 1 h the proportion of kerosene emulsified was determined (*ie.* (the total volume of kerosene minus the volume of kerosene not emulsified) divided by the total volume of kerosene). This parameter was known as the % of the kerosene phase emulsified. The content of kerosene in the emulsion was calculated by dividing the volume of kerosene in the emulsion phase by the total volume of the

emulsion. In the absence of an emulsifying agent, emulsions generated by vigorous mixing separated completely within one hour.

Screening for production of emulsifier by intact cells was done by mixing 2 ml of YCD broth culture with 2 ml of water and 6 ml of kerosene and vortexing as before. Unlike YPD medium, uninoculated YCD medium did not emulsify kerosene.

Enzyme digestions. For the Zymolyase treatment, fresh yeast (Lallemand) or yeasts grown freshly on YPD medium were washed twice in distilled water. The cells were suspended at a concentration of 6 mg (wet weight) of yeast per ml of buffer, 67 mM phosphate (pH 7.5), 20 mM 2-mercaptoethanol, 1mM phenylmethylsulfonyl fluoride. Zymolyase 20T was dissolved in the same buffer and was added to a final concentration of 1 to 2 $U.ml^{-1}$ (Kitamura *et al.*, 1974). The mixture was incubated 3 h at 25°C with gentle shaking. Insoluble material was then removed by centrifugation (10 min at 5,000 x *g*). Crude emulsifier was precipitated from the supernatant with ethanol, collected, and dried as described previously.

For protease treatment, emulsifier (1.4 $mg.ml^{-1}$) was dissolved in 50 mM Tris-HCl, pH 7.5, and a few drops of toluene were added. Pronase was added to a final concentration of 30 $\mu g.ml^{-1}$ and the mixture was incubated 24 h at 30°C. This material was tested for emulsification as before.

Analytical methods. Protein was determined by the dye-binding assay (Bradford, 1976) with bovine serum albumin

as the standard. Soluble protein was also measured by absorbance at 280 nm in a Coleman 124 double beam spectrophotometer. Carbohydrates were determined with the phenol-sulfuric acid procedure (Gerhardt, 1981) with glucose as the standard. The sugar components of acid-hydrolyzed emulsifier (0.35 g emulsifier, 90 ml 0.1 N H_2SO_4; 121°C, 1 h) were identified by HPLC at Weston Research Centre (Toronto, Ontario).

Surface tensions of solutions of purified emulsifier in distilled water were measured with an autotensiomat (Fisher Scientific Co., Pittsburgh, Pa.)(Akit *et al.*, 1981).

Properties of the emulsions. Stabilization of emulsions by emulsifier from baker's yeast was evaluated over a range of chemical and physical conditions. Purified emulsifier was dissolved in distilled water and the pH was adjusted to various pH values between pH 2 and 11 with HCl or KOH. After adding kerosene, tubes were vortexed and the emulsion parameters were measured after 1 h. The influence of various salt and ethanol concentrations was tested by adding 0, 0.5, 1.0, 2.5 and 5% (wt/vol) sodium chloride and 0, 10, 25 and 50% (vol/vol) ethanol to the aqueous phase containing emulsifier (pH 7).

For evaluation of stability, emulsions containing 0.14% (wt/vol) emulsifier in distilled water and kerosene were incubated at 4°C and room temperature for an extended period. In addition, emulsions were subjected to three cycles of

heating (40°C, 16 h) and cooling (room temperature, 8 h). Finally the ability of emulsions to withstand three cycles of freezing (-18°C, 16 h) and thawing (23°C, 8 h) was tested in the presence of solutes including potassium citrate, glucose, potassium chloride and calcium chloride.

Emulsions were made with purified emulsifier from baker's yeast and a variety of hydrocarbons and organic solvents, and the emulsion parameters were measured.

2.4 RESULTS

Isolation and identification of the emulsifier. Two extraction methods were used to obtain an emulsifying agent from cells of *S. cerevisiae*, baker's yeast. In the first method washed yeast cells were autoclaved in neutral buffer. An emulsifying agent was released into the aqueous medium and was concentrated by ultrafiltration. The emulsifier was precipitated by three volumes of ethanol and 1% acetic acid.

The ultrafiltered emulsifier from baker's yeast contained approximately 44% carbohydrate and 17% protein (Table 2.1). Analysis of acid-hydrolysed purified emulsifier by HPLC demonstrated that mannose was the major component of the carbohydrate fraction. No additional sugars were detected. Treatment of the purified emulsifier with the proteolytic enzyme Pronase eliminated its ability to emulsify.

Crude emulsifier was extracted from washed spent beer and wine yeast by the same method used for baker's yeast. The

Table 2.1. Yield and characterization of emulsifier extracted from commercial strains of *S. cerevisiae*.

Source	% Yield of emulsifier (g/100 g wet wt cells)	% Protein content[a]	% Carbohydrate content[b]	Emulsions with a 0.16% (wt/vol) solution	
				% Kerosene emulsified	% Kerosene in emulsion
Crude emulsifier from baker's yeast	17.8	5.4	17	97	75
Purified emulsifier from baker's yeast	8.0	17	44	98	78
Crude emulsifier extracted from baker's yeast by Zymolyase	16.7	16.6	38	92	74
Crude emulsifier from spent wine yeast	19.4	4.5	20.3	97	73
Crude emulsifier from spent beer yeast	17.8	2.3	19.2	95	76

[a] Measured by the dye binding assay (Bradford, 1976) with bovine serum albumin as standard, and expressed as g protein per g (dry wt) emulsifier.

[b] Measured by the phenol test (Gerhart, 1981) with glucose as standard, and expressed as g carbohydrate per g (dry wt) emulsifier.

yield, protein and carbohydrate contents, and ability to emulsify were comparable to the crude emulsifier from baker's yeast (Table 2.1).

Treatment of cells of baker's yeast with Zymolyase also released an emulsifying agent (Figure 2.1). Lysis of the yeast cell walls by Zymolyase was monitored by the decrease in OD_{660} of the digestion mixture. As lysis proceeded the concentration of soluble protein (A_{280}) and emulsifying capacity of the digestion mixture supernatant increased (Figure 2.1). Treatment of the digestion supernatant at 180 min with Pronase enzyme completely eliminated its ability to emulsify. The composition of emulsifier extracted by Zymolyase treatment was similar to that of the purified heat-extracted emulsifier (Table 2.1).

Properties of the emulsions. The purified emulsifier was tested for stabilization of emulsions under a range of chemical and physical conditions which might be encountered in various applications.

The relation between fraction of the kerosene phase emulsified and concentration of purified emulsifier (Figure 2.2) provides a basis for comparison of emulsification capacity under different physical and chemical conditions. Using kerosene and distilled water as the two initial phases, a maximum of 97% of the kerosene phase was emulsified with 0.08% (wt/vol) or more of the purified emulsifier.

To facilitate detection of possible detrimental effects

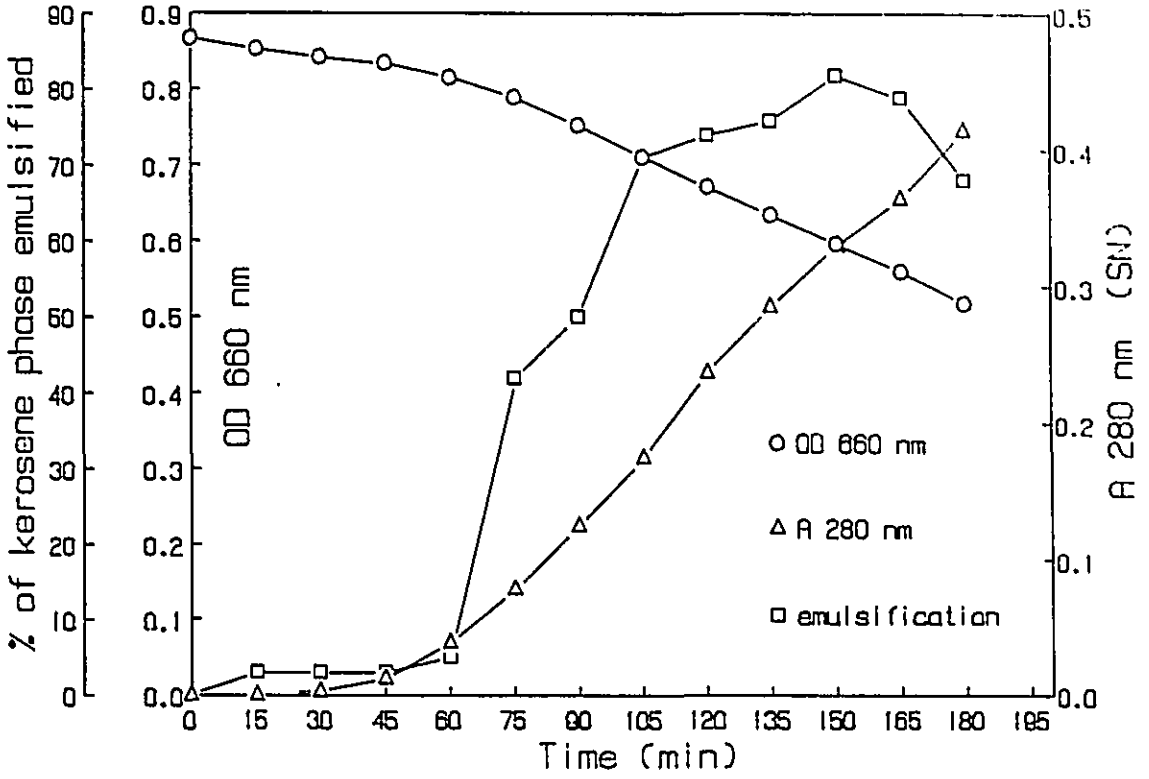


Figure 2.1. Release of emulsifier and soluble protein by Zymolyase digestion of *S. cerevisiae*, baker's yeast. Treatment of the digestion supernatant (180 min) with the protease, Pronase, eliminated emulsification.

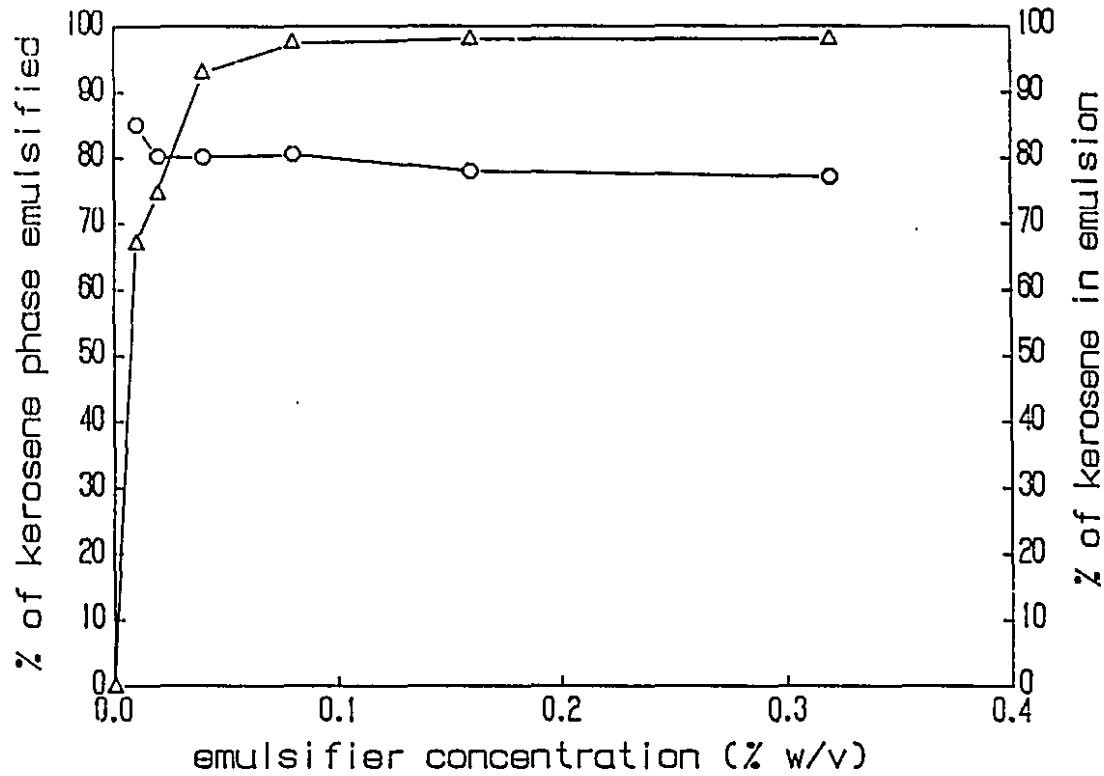


Figure 2.2. Relation of fraction of the kerosene phase emulsififed (△), and the percentage of kerosene (by volume) in the emulsion (o) to concentration of purified emulsifier.

of pH, sodium chloride, or ethanol on emulsification emulsions were made with 0.05% (wt/vol) purified emulsifier (Table 2.2). For pH values between 2 and 11 the pH of the aqueous phase had little effect on the amount of the kerosene phase emulsified (Table 2.2). Below pH 4, the emulsifying agent was insoluble but nonetheless stabilized emulsions. In the presence of 0.5% to 5.0% (wt/vol) sodium chloride in the aqueous phase, stable emulsions were formed. Emulsions made in the presence of salt contained a smaller proportion of the oil phase than those without (Table 2.2). Stable emulsions were formed in the presence of up to 50% (vol/vol) ethanol in the aqueous phase and the usual amount of kerosene (Table 2.2).

Physical treatments known to reduce emulsion stability were tested on emulsions containing 0.14% (wt/vol) purified emulsifier. Three cycles of freezing at -18°C for 16 h and thawing at 23°C for 8 h broke the emulsions (Table 2.3). Addition of 30 mM tripotassium citrate stabilized the emulsions to freezing and thawing (Table 2.3). In addition, 5 mM calcium chloride or 10 mM glucose, sodium chloride or potassium chloride prevented breakage of emulsions containing 0.14% (wt/vol) purified emulsifier during 3 cycles of freezing and thawing (data not shown).

Emulsions were not disrupted by three cycles of heating to 40°C (16 h) then cooling to 23°C (8 h). During storage at 4°C, the % of the kerosene phase emulsified did not change over a three month period (Table 2.3). Emulsion stability

Table 2.2. Effect of pH, sodium chloride and ethanol on emulsions with 0.05% (wt/vol) purified emulsifier.

Parameter	% Kerosene phase emulsified	% Kerosene in the emulsion
pH		
2	91	79
3	95	73
4	95	74
5	97	77
6	97	82
7	97	84
8	95	83
9	97	80
10	95	83
11	97	86
% NaCl[a]		
0	95	83
0.5	97	73
1.0	95	76
2.5	97	73
5.0	97	72
% Ethanol[a]		
0	97	80
5	97	84
10	97	86
25	97	77
50	97	82

[a] The aqueous phase was at pH 7.

Table 2.3. Stability of emulsions to three cycles of freezing and thawing and to storage at room temperature and at 4 C in the presence of tripotassium citrate[a].

Citrate conc (mM)	% Kerosene phase emulsified			
	Initial	After 3 freeze-thaw cycles	After 144 h at room temp	After 3 months at 4 C
0	91	15	24	96
2.5	96	8	21	98
5	96	5	38	98
10	96	38	49	98
20	96	89	56	96
30	96	96	64	96
60	96	94	79	96
100	96	94	93	96
250	98	98	100	98

[a] Emulsions were made with 0.14% (wt/vol) purified emulsifier.

during storage at room temperature was improved by the addition of citrate (Table 2.3).

The emulsifier extracted from baker's yeast emulsified all of the oils, hydrocarbons, and organic solvents tested (Table 2.4). Although chloroform appeared to be emulsified, the water content in this emulsion was very low. Emulsions with most other non-aqueous liquids tested contained between 10% to 30% water (Table 2.4).

The emulsions generated by the emulsifying agents extracted from yeast were of the oil-in-water variety. The surface tension of a 0.04% (wt/vol) solution of purified heat-extracted emulsifier was 61 $mN.m^{-1}$; a 0.4% solution had surface tension of 55.5 $mN.m^{-1}$. The surface tension of distilled water was 74 $mN.m^{-1}$.

Extraction of emulsifier from diverse yeast species. Broth cultures of 8 of the 14 yeast strains emulsified kerosene (Table 2.5). Yeasts which produced cell bound or extracellular emulsifiers when grown on glucose included *C. tropicalis, C. utilis, D. naardenensis, G. penicillatum, H. anomala, K. fragilis, M. lunata* and *P. tannophilus*. An emulsifying agent was obtained from all of the yeasts tested, by using the heat extraction procedure (Table 2.5). The yields of crude emulsifier were from 2.9 g/g wet cell weight for *T. ernobii* to 17.8 g/g wet cell weight for *S. cerevisiae*. Protein was detected in all of the crude emulsifiers. The emulsifiers from *D. naardenensis* and *M. lunata* contained approximately

Table 2.4. Oils, alkanes and organic solvents emulsified with various concentrations of purified emulsifier.

	% Nonaqueous phase emulsified with emulsifier concn (%[wt/vol]) of:		
Nonaqueous phase	0.04	0.2	0.4
Kerosene	89	97	97
Vegetable oil	60	82	90
Mineral oil	75	78	100
Pentane	8	80	88
Octane	75	89	100
Hexadecane	83	100	100
Ethyl acetate	0	93	93
Chloroform	100	100	100
Dichloromethane	100	100	100
Toluene	84	98	100
Xylene	90	100	100
	% Nonaqueous phase in emulsions with emulsifier concn (%[wt/vol]) of:		
Nonaqueous phase	0.04	0.2	0.4
Kerosene	81	80	80
Vegetable oil	45	60	71
Mineral oil	63	51	62
Pentane		91	91
Octane	90	80	82
Hexadecane	86	74	69
Ethyl acetate		83	81
Chloroform	100	100	100
Dichloromethane	100	90	90
Toluene	83	77	74
Xylene	87	74	74

Table 2.5. Heat extraction and characterization of emulsifier from various yeast species.

Yeast species[a]	Emulsification by whole broth	% Yield of emulsifier (g/100 g wet wt cells)	% Protein content	Emulsification of a 0.08% (wt/vol) solution	
				% Kerosene phase emulsified	% Kerosene in emulsion
Candida tropicalis NRCC 2774	++	8.5	6.1	64	77
Candida utilis Y900	+	8.7	7.2	68	93
Debaromyces marama NRCC 2621	-	7.6	5.6	57	78
Dekkera naardenensis NRCC 2565	+	7	18.8	78	73
Geotrichum penicillatum NRCC 2623	++	14.5	2.7	64	83
Hansenula anomala NRCC 2505	++	8.3	7.9	65	80
Kluveromyces fragilis NRCC 2475	+	10	7.6	51	73
Lipomyces starkeyi NRCC 2519	-	11.6	8.2	64	74
Metschnikowia lunata NRCC 2545	++	14.6	13.8	59	79
Pachysolen tannophilus NRCC 2507	+	9	6.5	49	75
Pichia abadieae NRCC 2644	-	18	6.6	24	60
Saccharomyces cerevisiae	-	17.8	5.4	86	77
Torulopsis ernobii NRCC 2663	-	2.9	8.4	27	83
Zygosaccharomyces fermentati NRCC 2743	-	14.8	7	59	76

[a] NRCC numbers indicate strain reference number at the Culture Collection of the National Research Council of Canada

three times as much protein as that from *S. cerevisiae*, however emulsified less kerosene per unit weight (Table 2.5).

2.5 DISCUSSION

Two procedures which solubilize mannoproteins from the cell wall of *S. cerevisiae* (Peat *et al.*, 1961; Valentin *et al.*, 1984) have been shown to simultaneously release an emulsifying agent. After purification the emulsifying agent had a molecular weight greater than 10,000 and major components mannose, and protein which was necessary for its action as an emulsifier. Clearly the compound is mannoprotein. The emulsifying activity of mannoprotein has not been recognized previously.

The structure of mannoproteins has been described in the literature. Two general classes of mannoproteins are identified: mannoproteins with a structural role contain approximately 90% mannose and 5 to 10% protein (Ballou and Raschke, 1974). Structural mannoproteins are interspersed within a network of glucan to form the outer layer of the cell wall of *S. cerevisiae* (Sentandreu *et al.*, 1984). The second class of mannoproteins, the mannan enzymes, contain 50 to 70% mannose and the remainder protein (Ballou and Raschke, 1974). The mannan enzymes include invertase, glucosidase, melibiase, phosphatase and proteases (Ballou 1976), and appear to be periplasmic since they do not sediment with cell wall fragments after mechanical breakage of cells (Sentandreu *et*

al., 1984).

The structural mannoproteins are among the most abundant macromolecules of the yeast cell; they comprise 12 to 14% of the cell dry weight (Nakajima and Ballou, 1974; Okubo *et al.*, 1978; Trevelyan and Harrison, 1952). From the high yield of purified emulsifier (8% of the weight of fresh baker's yeast) it is likely that this consists mostly of structural mannoproteins. The content of mannose in the purified emulsifier was 72% (calculated as the content of carbohydrate divided by the total of the contents of carbohydrate plus protein; Ballou and Rashke, 1974) which is slightly higher than that of the mannan enzymes. A portion of the carbohydrate may have been lost after fragmentation due to high shearing forces generated during ultrafiltration.

The heat-extraction procedure solubilizes the structural mannoproteins in the outer layer of the cell wall (Peat *et al.*, 1961). Treatment with Zymolyase degrades the glucan component of the cell wall and would therefore be expected to release both the structural mannoproteins as well as the mannan enzymes from the periplasm. This interpretation is supported by the higher proportion of protein in the Zymolase-extracted emulsifier compared to that in the crude heat-extracted emulsifiers (Table 2.1).

Mannoproteins consist of mannose polymers covalently attached to a protein backbone (Ballou, 1976). The structure of mannoprotein suggests its mode of action as an emulsifying

agent. The ability of various proteins to stabilize oil-in-water emulsions has long been known (Jaynes, 1985). Since protease treatment abolished emulsification, the protein component of mannoprotein was essential for its emulsification properties. The presence of hydrophilic mannose polymers covalently attached to the protein backbone provide mannoprotein with the amphiphilic structure common to surface active agents and many effective emulsifiers (Cooper, 1986; Cooper and Paddock, 1983).

Due to the wide range of conditions under which mannoprotein emulsifier was effective, it is expected that this bioemulsifier could be used in a variety of applications. Ability of emulsions to withstand freezing and thawing is a particularly useful property.

Comparison of mannoprotein to known bioemulsifiers from yeast. The mannoprotein emulsifier of *S. cerevisiae* has chemical composition similar to the bioemulsifiers produced by alkane-grown yeasts. Purified liposan emulsifier from *Candida lipolytica* is a glycoprotein which contains 83% carbohydrate and 17% protein (Cirigliano and Carman, 1985). Its major component has a molecular weight of 27,600. The emulsifiers from *Candida petrophilum* and *Endomycopsis lipolytica* also contain carbohydrate and protein (Iguchi *et al.*, 1969; Roy *et al.*, 1979). Treatment of the latter emulsifier with Pronase destroyed its ability to emulsify. These emulsifiers may also be cell wall glycoproteins and it may be possible to increase

their yield by use of the extraction procedures described here.

Many yeast species have cell walls with structures like that of *S. cerevisiae*, and contain glycoproteins with a structural role similar to the mannoprotein in *S. cerevisiae* (Ballou and Raschke, 1984; Kaneko *et al.*, 1973). The heat extraction method was used to extract cell wall glycoproteins capable of emulsifying from every one of 13 yeast species tested in addition to *S. cerevisiae*. None of these yeasts was grown on hydrocarbon substrates.

As an emulsifying agent, mannoprotein from *S. cerevisiae* may present certain advantages. The difficulty of removing residual hydrocarbons from bioemulsifiers from alkane-grown yeasts would preclude use in certain applications. Since *S. cerevisiae* is edible and is used in the manufacture of food and beverage products, it is expected that mannoprotein bioemulsifier would be nontoxic.

The yield of mannoprotein emulsifier was far greater than previously known bioemulsifiers from yeasts. The yield of mannoprotein emulsifier was approximately 8% of the wet weight of yeast biomass. The yield of liposan from a 300 ml broth culture of *Candida lipolytica* was 50 mg (Cirigliano and Carman, 1985); from an equivalent culture (containing 15 g cells dry weight of *S. cerevisiae*), the yield of mannoprotein emulsifier would be approximately 1.2 g. The yields of bioemulsifiers from *Candida tropicalis*, *C. petrophilum*, and

Endomycopsis lipolytica were also a very small proportion of the yeast biomass (Iguchi *et al.*, 1969; Kappeli and Fiechter, 1978; Roy *et al.*, 1979).

Mannoprotein may be extracted in high yield from yeast cells, ultrafiltered to remove low molecular weight solutes, and dried by simple procedures. Both the heat-extraction and purification by ultrafiltration are amenable to large-scale operations. The spent yeast produced as a byproduct in the brewing and wine industries could provide a source of raw material for mass production of mannoprotein emulsifier. This would eliminate the need to grow up the yeast specifically for the production of emulsifier, as with the bioemulsifiers from other yeasts. Currently spent yeast has low value, and is dried for use as a protein supplement in animal feed or is treated as high BOD waste. In addition it may be possible to extract glycoprotein bioemulsifier as a high value product from fodder yeasts used subsequently for single cell protein.

2.6 ACKNOWLEDGEMENTS

This work was supported by the Weston Research Centre (Toronto, Ontario) in conjunction with a PILP Biotechnology Contribution Arrangement (# CA910-4-0015/B/23). Helpful discussions with J. Fein, M. Beavan and R. Lawford of the Weston Research Centre are gratefully acknowledged.

CHAPTER 3: STANDARDIZATION OF A METHOD FOR MEASURING EMULSIFIER ACTIVITY

Published as:

Cameron, D.R., M.E. Weber, E.S. Idziak, R.J. Neufeld, and D.G. Cooper. 1991. Determination of interfacial areas in emulsions using turbidimetric and droplet size data: Correction of the formula for the Emulsifying Activity Index. J. Agric. Food Chem. 39:655-659.

PREFACE

The assay used for measuring emulsifying activity in Chapter 2 was developed by Akit *et al.* (1981) for evaluating microbially-produced bioemulsifiers. It involves vortexing 4 ml of an aqueous phase containing emulsifier with 6 ml of alkane, and measuring the proportion of the alkane phase present as an emulsion after a given time.

Since foods or cosmetics are the targetted applications for mannoprotein emulsifier, it was considered desirable to measure its emulsifying activity by a standard assay used for food emulsifiers. As described in Chapter 1, the assay developed by Pearce and Kinsella (1978) appeared to be such a standard assay. It is simple to perform and is widely used for comparing protein emulsifiers. A major advantage of this assay over other emulsion assays is that the Emulsifying Activity Index (EAI) determined by this method has a rational basis in terms of the action of emulsifiers; the index provides (or claims to provide) units of m^2 interfacial area stabilized per g emulsifier.

The recent acquisition of a Malvern particle sizer provided the opportunity to verify the accuracy of Pearce and Kinsella's turbidimetric method for measuring interfacial areas. To our knowledge this has not been done previously.

There was a large discrepancy between EAI values derived by turbidimetry and using the particle sizer. After considering my data, Dr. M.E. Weber (Department of Chemical

Engineering, McGill U.) derived the formula for the EAI from first principles and showed that the index as originally proposed does not provide the units claimed. A correction was proposed which provides the desired units and makes the index theoretically sound. The experiments which pointed out the discrepancy between surface areas measured using the two methods, Dr. Weber's derivation of the formula for the EAI, and comparisons of data using the two methods are presented in this chapter.

Small emulsifier samples (10 mg or less) were sufficient for the emulsion assay used in the previous chapter (Akit *et al.*, 1981). Measurement of the EAI required a larger sample of emulsifier (40 mg per assay). The EAI method was used for data in Chapter 3, and in Fig. 5.1, and the % Emulsion Height method was used elsewhere, when the supply of emulsifier was limited.

3.1 ABSTRACT

Derivation of the Emulsifying Activity Index (EAI; Pearce, K.N., and J.E. Kinsella, 1978. J. Agric. Food Chem. **26**:716-723) from first principles demonstrates that a simple correction must be made. The original EAI does not provide units of interfacial area per g emulsifier as claimed: its value varies with the oil volume fraction. The correction provides the index with the desired units of m^2 interfacial area per g emulsifier. The interfacial areas of three suspensions of monodisperse latex beads were determined in four different ways: by turbidimetry using the original and corrected formulae for EAI; by calculation from the droplet size distribution measured by laser diffraction; and by calculation using bead diameters supplied by the manufacturer. For three bead sizes covering the range of droplet sizes in typical emulsions the interfacial area value based on the distribution of particle sizes was closest to the actual value. The interfacial area calculated by original formula for EAI was least accurate. The correction to the EAI formula improved correspondence of interfacial area measured turbidimetrically to the actual value and eliminated its dependence on the oil volume fraction in the emulsion.

3.2 INTRODUCTION

Emulsifiers are essential components of foods which contain two immiscible phases, one of which is dispersed as droplets within the other. The emulsifying agent reduces the rate of separation of the two phases to an acceptable level. The emulsifier properties of protein ingredients in foods are one of their most important functional properties. In practice, synthetic emulsifiers (e.g. polysorbates) are frequently incorporated in foods since these can be custom designed to provide particular characteristics and consequently are more effective emulsifiers than most of the protein preparations available.

Viscosifying agents (e.g. xanthan gum) are also common additives which improve emulsion stability in foods. These compounds have little surface activity but stabilize emulsions by increasing the viscosity of the continuous phase such that collisions between droplets of the dispersed phase are less frequent and phase separation takes longer to occur.

Emulsions are thermodynamically unstable due to the high free energy of the interface between the two phases. Emulsions can break by a variety of processes which include creaming, flocculation, coalescence and oiling-off (Becher, 1965). Equilibrium is reached when the area of contact between the two phases is at a minimum. Emulsifiers slow the rate of phase separation. Emulsifiers often have an amphiphilic molecular structure and concentrate at the interface between two phases.

Intuitively, an attractive method of evaluating emulsifiers would directly measure the area of contact between the two phases. Such a method was devised by Pearce and Kinsella (1978) and has been widely used.

According to the Mie theory of light scattering, the turbidity of a dilute suspension of spherical particles is related to its interfacial area (Kerker, 1969; Van de Hulst, 1957). Pearce and Kinsella (1978) used this relationship as the basis of the Emulsifying Activity Index (EAI). They defined EAI = $2T/\phi c$, where turbidity, T = 2.303 (Absorbance at 500 nm)/(optical path length), ϕ = the oil volume fraction in the emulsion, and c = the mass of emulsifier per unit volume of the aqueous phase before the emulsion is formed. They claimed the EAI has units of m^2 of interfacial area per g emulsifier, while in fact this is not so: the EAI values depend on ϕ. Their derivation of the formula was not presented.

We determined interfacial area in a dilute emulsion by a second method, by laser diffraction using a Malvern particle sizer. The interfacial area was computed from the droplet size distribution and phase concentration, and an index with the units claimed to be provided by the EAI was calculated. Wide divergence in the values obtained by the two methods led us to derive the formula for EAI from first principles. A simple correction to the formula of Pearce and Kinsella (1978) provides a corrected EAI which has units of m^2 interfacial

area per g emulsifier and thereby makes the index theoretically sound. Data illustrating the effect of the correction is provided for model oil-in-water emulsions and for suspensions of monodisperse latex beads covering the range of droplet sizes in typical food emulsions.

3.3 THEORY

For a polydisperse suspension where:

τ = optical transmittance

D = particle diameter

$$\tau = \exp\left[-\frac{3}{2}\,\phi\,\ell\,\frac{K}{D_{32}}\right]$$ (Dobbins and Jizmagian, 1965)

ϕ = volume fraction of the dispersed phase

ℓ = optical path length

K = mean scattering coefficient

D_{32} = volume-surface mean diameter

The terms surface area and interfacial area of particles are equivalent.

Both K and D_{32} are defined in terms of the particle size distribution function N(D):

$$D_{32} = \frac{\int_0^{Dmax} N(D)*D^3*dD}{\int_0^{Dmax} N(D)*D^2*dD} = \frac{\text{volume of particles}}{\text{area of particles}} * 6$$

$$K = \frac{\int_0^{Dmax} K(D,m) * N(D) * D^3 * dD}{\int_0^{Dmax} N(D) * D^2 * dD}$$

where D_{max} is the largest particle in the suspension.

For calculation of the total surface area of a given mass of particulate material the D_{32} is a useful quantity. If the polydispersion is replaced by a monodispersion possessing the same volume-to-surface ratio, then the diameter of the particles composing the monodispersion equals the D_{32} of the polydispersion.

The scattering coefficient K of a dielectric sphere is defined as the ratio of the scattering cross section to the geometrical cross section. K is a function of m and D where m is the ratio of the refractive index of the suspended phase to that of the suspending medium. If K is plotted against the phase shift parameter

$$\rho_{32} = 2(m-1)\pi \frac{D_{32}}{\lambda}$$

where λ = wavelength of light in the suspending medium, the value of K reaches a maximum at $\rho_{32} \approx 4$ and K approaches 2 at ρ_{32} values greater than approximately 20 (Dobbins and Jizmagian, 1965; Kerker, 1969). In the present study where m = 1.20 for polyvinyltoluene or styrene/divinylbenzene latex beads in water or m = 1.10 for corn oil in water emulsions, the maximum value of K is approximately 3.1 (Van de Hulst, 1957).

Specific turbidity,

$$T = \frac{1}{\ell} \ln (1/\tau)$$

substituting for τ

$$T = \frac{3}{2} \phi \frac{K}{D_{32}}$$

but

$$\frac{\text{volume of particles}}{\text{surface area of particles}} = \frac{D_{32}}{6}$$

Hence

$$T = \frac{3}{2} \phi K \frac{\text{surface area particles}}{6 * \text{volume of particles}}$$

$$\frac{4T}{K} = \phi * \frac{\text{surface area of particles}}{\text{volume of particles}}$$

If, as in Pearce and Kinsella (1978)

$$c = \frac{\text{mass emulsifier}}{\text{aqueous phase volume}}$$

$$\frac{4T}{K\phi c} = \frac{\text{surface area of particles}}{\text{mass emulsifier}} * \frac{\text{aqueous phase volume}}{\text{volume of particles}}$$

Note that

$$\frac{\text{aqueous phase volume}}{\text{volume of particles}} = \frac{1-\phi}{\phi}$$

Hence

$$\frac{4T}{K\phi c} = \frac{\text{surface area of particles}}{\text{mass emulsifier}} * \frac{1-\phi}{\phi}$$

Let $EAI = \frac{4T}{K\phi c}$ note that if K = 2 then there is agreement with Pearce and Kinsella (1978).

Then $$\frac{\text{surface area of particles}}{\text{mass emulsifier}} = EAI * \frac{\phi}{1-\phi}$$

Hence the units of the EAI as originally defined by Pearce and Kinsella (1978) are actually

$$\frac{\text{interfacial area of particles}}{\text{mass emulsifier}} * \frac{\text{aqueous volume}}{\text{volume of particles}}$$

and not $\frac{\text{interfacial area of particles}}{\text{mass emulsifier}}$ as claimed.

Going back to

$$\frac{4T}{K} = \phi * \frac{\text{surface area of particles}}{\text{volume of particles}}$$

since

$$\phi = \frac{\text{volume of particles}}{\text{total volume}}$$

then

$$\frac{4T}{K} = \frac{\text{surface area of particles}}{\text{total volume}}$$

Let

$$c' = \frac{\text{mass emulsifier}}{\text{total volume}} \quad \{ = c(1-\phi) \}$$

then

$$\frac{4T}{Kc'} = \frac{\text{surface area of particles}}{\text{mass emulsifier}}$$

Now assuming that $K = 2$, a theoretically more sound index would be

$$\frac{2T}{c(1-\phi)} = \frac{\text{interfacial area of particles}}{\text{mass emulsifier}}$$

This index will be referred to subsequently as the corrected EAI. It has the units m^2 interfacial area per g emulsifier.

The following experiments demonstrate the effect of the correction when interfacial area of a model emulsion is calculated. The values obtained by the original and corrected EAI are compared to that determined from a distribution of particle sizes obtained by laser diffraction using a Malvern particle sizer. The accuracy of the three measures for evaluating interfacial area was determined on a defined

system, i.e. a monodisperse suspension of latex beads of known size.

3.4 MATERIALS and METHODS

Reagents. Tween 60 (polyoxyethylene 20 sorbitan monostearate) from Atkemix (Montreal, Que.) was used as the test emulsifier. A commercial brand of corn oil free of additives was used. Reagent grade dextran (average mol. wt. 487,000) and NP-40 were from Sigma (St. Louis, MO). Latex beads were from Seradyn (Indianapolis, IN). Distilled water (Milli-Q; Millipore, Mississauga, Ont.) was used throughout.

Preparation of emulsions. Emulsifying agent was dissolved in water. This solution was adjusted to pH 7 with NaOH or HCl and was made up to the desired volume. This was added to a measured volume of corn oil in an Oster blender and the mixture was homogenized 2 min at the highest speed setting.

Measurement of turbidity and droplet size distribution. Immediately after blending, 0.1 ml samples of the emulsions were removed and added to 39.9 ml of diluent which contained 1% (wt/vol) dextran, 0.1% (vol/vol) NP-40, pH 7.0. Dextran was included in the diluent to increase viscosity and reduce the rate of flotation of oil droplets, and the detergent was used to prevent flocculation and coalescence of oil droplets. Unlike sodium dodecyl sulfate, the nonionic detergent NP-40 produced a minimal degree of low angle scattering of laser light in the particle sizer. The turbidity and particle size

distributions of the diluted samples did not change over 1 h.

Stock solutions of latex beads were diluted in distilled water and mixed vigorously before measuring turbidity and particle size distributions.

Optical density readings were taken using an LKB Biochrom Ultrospec II spectrophotometer set at 500 nm, using quartz cuvettes with a light path of 1 cm. Samples were diluted (with diluent described earlier) to yield ODs of less than 0.4. Readings were taken on triplicate samples. Turbidity was calculated by the formula $T= 2.303\ OD_{500}$ * dilution/.01 m light path.

The droplet size distribution was measured on the same samples using a Malvern 2600C Droplet and Particle Sizer (Malvern, England), using a cuvette with a 14.3 mm light path and the 63 mm lens. Illumination is provided by an HeNe laser (633 nm). The sample was diluted to the appropriate concentration as indicated by the instrument software.

Measurement of dispersed phase volume. The oil volume fractions (ϕ) of these oil-in-water emulsions were measured by drying triplicate emulsion samples (usually 1 ml) to constant weight at 110°C. The concentration of latex beads in stock solutions provided by the manufacturer (nominally 10% solids) was measured by drying triplicate 10 ml samples after diluting the stock solution 1:100 with distilled water. Weight concentrations were converted to volumetric concentrations by dividing by density: 0.92 g/ml for corn oil (Merck Index);

1.027 g/ml for polyvinyltoluene and 1.05 g/ml for 95% styrene/5% divinylbenzene (Seradyn product data sheet).

Emulsion stability. Stability of emulsions over time was evaluated using a method similar to that of Pearce and Kinsella (1978), and Jackman et al. (1989). The emulsion tested consisted of 37.5 ml of 0.133% (wt/vol) Tween 60 mixed with 12.5 ml corn oil. The emulsion was poured into a burette and samples were taken at timed intervals from the bottom by opening the valve at the base.

3.5 RESULTS

In the presence of 1% dextran and 0.1% NP-40, oil droplets did not adhere to surfaces of the cuvette or sample containers. Turbidity was directly proportional to the concentration of the dispersed phase up to an optical density of 0.4. By scanning over the range from 320 to 900 nm, the optical density of the diluted emulsion was independent of wavelength (data not shown).

An emulsion was made with an oil volume fraction of 0.4 and was then serially diluted to provide ϕ = 0.2, 0.1 and 0.05. The value of interfacial area per gram emulsifier, which was expected to be identical for all dilutions, was calculated by the EAI, the corrected EAI and by computation from the droplet size distribution. The values are plotted in Fig. 3.1. Values obtained with the corrected EAI and the particle sizer correspond although there is a constant difference. Values

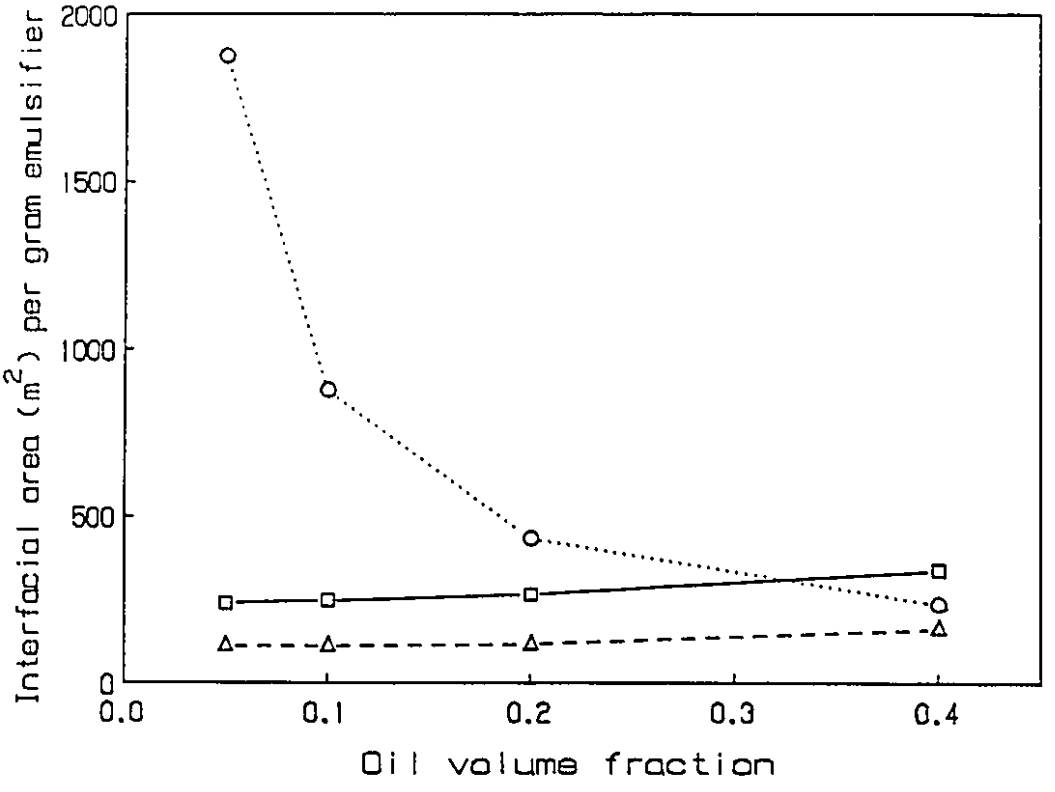


Figure 3.1. Comparison of values of interfacial area per gram emulsifier calculated using the original (O) and corrected (Δ) Emulsifying Activity Index and using data from the particle sizer (□). One emulsion was serially diluted with water to provide various oil volume fractions. Values were calculated as, for the particle sizer, (specific surface area * ϕ)/c(1-ϕ), EAI = 2T/ϕc, and the corrected EAI = 2T/c(1-ϕ). For calculation purposes c was expressed in $g.m^{-3}$ of the original aqueous phase.

obtained with the original EAI diverge from the other two increasingly at low oil volume fractions.

To compare the accuracy of the three methods, suspensions of monodisperse latex beads of known size were used. The data are presented in Table 3.1. For each of the three bead sizes the mean diameter measured with the particle sizer was larger than specified by the manufacturer. The measured standard deviations of the mean diameters were also larger than those provided by the supplier.

The surface areas of latex beads per ml of suspension measured by the particle sizer were 87%, 94% and 93% of the values calculated for suspensions of beads 2.02, 10.2 and 19.6 μm in diameter, respectively. The corresponding surface areas per ml of suspension calculated by the corrected EAI were 71%, 45% and 48% of those obtained from the known bead sizes and concentrations. The surface areas per ml calculated from the original EAI were far from correct, i.e. 674%, 383% and 383% larger than the predicted values.

Stability of a model emulsion was monitored over time. Samples were taken from the lower part of the emulsion at timed intervals and OD_{500}, ϕ, and particle size distribution were determined. The data are shown in Table 3.2. In this emulsion, the OD_{500} of the lower phase remained roughly constant even though the oil volume fraction decreased from 25.3 to 10.3%. Values for interfacial area per ml calculated by the corrected EAI remained approximately constant.

Table 3.1. Comparison of nominal and experimentally determined diameters and surface areas of three sizes of latex beads.

Nominal D (SD)[a] (μm)	Measured D (SD) using droplet sizer (μm)	Oil volume fraction ϕ	OD_{500}[b]	Surface area ($m^2.mL^{-1}$ of suspension)			
				Nominal[c]	Measured using		
					Droplet sizer[d]	EAI[e]	Corrected EAI[f]
2.02 (0.014)	2.29 (0.24)	0.1024	0.118	0.304	0.265	1.91	0.217
10.2 (0.31)	10.73 (1.25)	0.1048	0.150	0.0616	0.0578	0.2360	0.0276
19.6 (0.59)	19.77 (5.3)	0.1127	0.182	0.0345	0.0321	0.1320	0.0167

a Values for D, sd from the supplier. The standard deviation for 10.2 μm diameter beads was determined microscopically.

b Dilution factors for measuring OD_{500} were 4,000, 400, 200 for beads 2.02, 10.2, and 19.6 μm respectively.

c Nominal surface area per ml stock solution = no. beads/ml * πD^2

d Surface area/ml = specific surface area (m^2/ml of dispersed phase) * ϕ

e Surface area/ml = $\frac{2T(1-\phi)}{\phi}$ = EAI * $c(1-\phi)$, where T = $\frac{2.303\ OD_{500} * \text{dilution}}{1\text{ cm light path}}$

f Surface area/ml = 2T

Table 3.2. Stability over time of an emulsion made with 0.133% Tween 60 and initial oil volume fraction ϕ = 0.25.

Time (min)	Oil volume fraction	OD_{500}[a]	D_{32}[b] (μm)	Interfacial Area (m^2/ml)[c] calculated using		
				EAI	corrected EAI	droplet sizer
0	.253	.309	6.5	.336	.114	.238
1	.246	.336	6.5	.379	.124	.230
3	.213	.335	6.2	.456	.123	.210
10	.168	.334	5.6	.610	.123	.185
20	.142	.353	4.9	.786	.130	.178
40	.122	.359	4.2	.952	.132	.182
60	.115	.344	4.2	.975	.127	.168
90	.111	.341	3.9	1.01	.126	.175
120	.103	.341	3.9	1.09	.126	.166

a All samples diluted 1:800.

b Volume-surface mean diameter.

c Calculated as in Table 1.

According to the EAI in its original form, the interfacial area per ml increased approximately threefold during two hours. Data from the particle sizer demonstrate that the volume-surface mean diameter decreased from 6.5 to 3.9 μm and that the interfacial area per ml also decreased. Changes in the volumetric distribution of droplet sizes for this emulsion are shown in Fig. 3.2.

3.6 DISCUSSION

The development of the Emulsifying Activity Index by Pearce and Kinsella (1978) was a useful advance in the science of comparing and evaluating emulsifiers and has been widely used. The index has physical units which are easily interpreted in terms of the dynamics of emulsions. As these authors noted, the values obtained are strongly dependent on the apparatus used to make and measure the emulsion; nevertheless comparison of different emulsifiers in any given laboratory is possible.

As shown by this work the correction made to the original formula increases accuracy of the EAI for determining interfacial area in emulsions based on turbidimetric measurements. The improvement will be particularly noticeable when comparing emulsions which contain different oil volume fractions.

As noted by Pearce and Kinsella (1978) and Dickinson and Stainsby (1988), and as demonstrated here using latex beads,

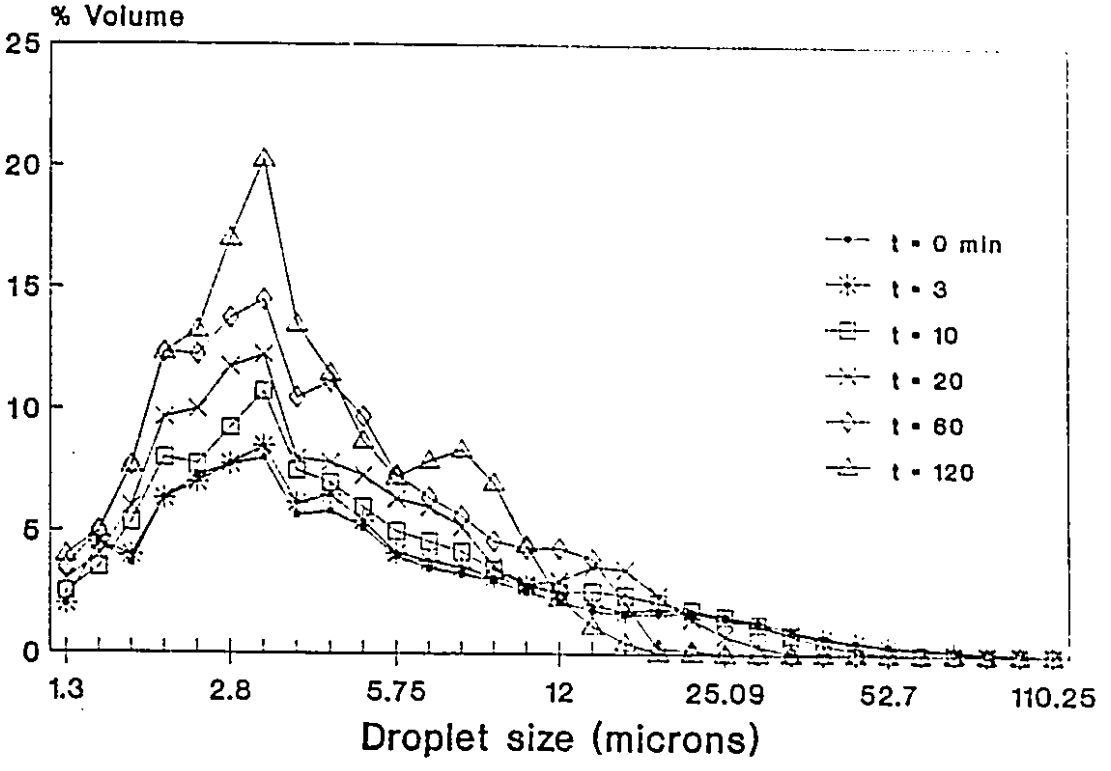


Figure 3.2. Change in volumetric distribution of droplet sizes over time in samples from the lower part of an emulsion made with 0.133% Tween 60 and 0.25 oil volume fraction.

the absolute values for interfacial area calculated from turbidity as measured with typical unmodified laboratory spectrophotometers are not highly accurate. Ways to improve their precision have been described in detail elsewhere (Walstra, 1965a,b; Heller and Tabibian, 1957).

The principal sources of error may be ascribed to two factors (a) the design of the spectrophotometer and (b) the dependence of the value of the scattering coefficent on particle size. Typical laboratory spectrophotometers are more suitable for measuring absorbance than for light scattering. Due to the short distance between sample and photodetector, light scattered at low angles may be received by the detector as transmitted light. For droplets large relative to the wavelength of light a large proportion of the total light scattered is diffracted into a small cone in the forward direction (Lothian and Chappel, 1951). Although the theoretical value of the scattering coefficient is based on an acceptance angle of 0°, even after modification most spectrophotometers provide a solid angle of 1° or more and a correction factor must be used to provide a realistic value of K (Walstra, 1965a).

In addition, the value of the scattering coefficient varies with droplet diameter. For a polydispersion the value of K depends primarily on the volume-surface mean diameter and is only weakly dependent on the shape of the droplet size distribution function (Dobbins and Jizmagian, 1965). For corn

oil in water emulsions (m = 1.1) and for optical densities measured at 500 nm (in water λ = .374 μm), K has a maximum theoretical value of approximately 3.1 for oil droplets 2.4 μm in diameter (at ρ = 4). Its value approaches 2 for particles 11.9 μm in diameter (at ρ = 20) (Van de Hulst, 1957). However with a short distance between the sample and photodetector and consequently a large acceptance angle, some of the scattered light reaches the detector and the value of the apparent K will be smaller (Sinclair, 1947; Walstra, 1965a).

Because the value of the scattering coefficient varies with droplet diameter and may differ from the assumed value of 2, the values for interfacial area calculated from turbidity measurements will not necessarily be correct. When two or more emulsifiers are compared using a given system the difference between the actual and the assumed value of K will tend to underestimate interfacial areas. Any detected differences in the corrected EAI values will therefore be genuine.

Despite its high initial cost the droplet sizer provides certain advantages for comparing emulsifying agents and emulsions. This machine measures droplet sizes based on Fraunhofer (low angle) diffraction of laser light by particles. The lower size limit for particles to be accurately measured is 1.2 μm. In a typical determination laser light is diffracted by several hundred thousand individual particles. The intensity of light scattered at low angles is measured by 32 concentric photodetector elements. The expected pattern of

light diffracted by a theoretical distribution of particles is compared to the observed intensities, and the best fit is chosen by computer. The primary data are based on a volumetric distribution but this may be readily converted to surface, diameter or numerical distributions using the software accompanying the instrument.

Experiments with suspensions of monodisperse latex beads (Table 3.1) show good correspondence to actual bead sizes measured by electron microscopy by the manufacturer. The three sizes of beads were chosen to cover the range of droplet sizes in the model emulsions produced here. The somewhat higher values for mean diameters according to the particle sizer may have been due to occasional clumping of two or more beads. This would also broaden the particle size distribution as was observed in the standard deviation of particle diameters from the mean. Accordingly, the specific surface area of the dispersed phase was slightly smaller than that predicted.

For measurement of emulsion stability over time, the particle sizer presents distinct advantages over turbidimetry. As creaming occurs, the mean droplet size decreases to particles which have a negligible upward velocity (described by Stokes equation), and the theoretical value of the scattering coefficient changes. Hence the accuracy of values of interfacial area derived by turbidimetry will change likewise. The particle sizer is highly accurate throughout its specified range.

In conclusion, the original formula for the Emulsifying Activity Index is incorrect. Interfacial areas are greatly overestimated in emulsions with a low oil volume fraction. The correction made here removes the false dependence of the EAI on the oil volume fraction and provides a corrected EAI which is theoretically sound. Interfacial areas in suspensions of latex beads and model emulsions measured by turbidimetry and particle sizing support the correction. Particle sizing was most accurate, but use of the corrected EAI nevertheless permits valid comparisons of emulsifiers to be made with standard laboratory equipment.

3.7 ACKNOWLEDGMENT

Financial support from the government of Quebec through the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche is gratefully acknowledged.

CHAPTER 4: IDENTIFICATION OF THE ACTIVE COMPONENTS OF MANNOPROTEIN EMULSIFIER

To be submitted for publication as:

Cameron, D.R., E.S. Idziak, R.J. Neufeld, and D.G. Cooper. 1992. Identification of the active components of mannoprotein emulsifier.

PREFACE

Autoclaving yeast cells in neutral citrate buffer extracts mannoproteins which remain in solution after removal of cell debris by centrifugation and cooling. Previous experiments demonstrated that protein is an essential component since treatment of the extract with the protease Pronase eliminated emulsifying activity (Ch. 2, Figure 2.1). The aim of the following experiments was to determine whether all mannoproteins in the extract emulsify with equal efficiency and whether mannoproteins extracted from a series of mutant strains of *S. cerevisiae* defective in N-linked glycosylation of secreted proteins would have similar emulsifying activity.

4.1 ABSTRACT

To identify components with the greatest emulsifying activity mannoproteins extracted from *Saccharomyces cerevisiae* were fractionated using ammonium sulfate and gel filtration. The emulsifying activity of fractions precipitated with ammonium sulfate correlated with their protein content. Two components with the greatest emulsifying activity were identified by gel filtration. The high molecular weight component (> 200 kDa) contained a large proportion of carbohydrate and produced viscous and durable emulsions. This component was soluble in saturated ammonium sulfate solutions and migrated as a discrete band during SDS-polyacrylamide gel electrophoresis. It may correspond to the high molecular weight cell wall mannoprotein identified by Frevert and Ballou (Biochemistry **1985**, *24*, 753-759). The low molecular weight component (< 14 kDa) had the greatest emulsifier activity per mg protein, was very surface active, and produced nonviscous emulsions which separated on aging. Mannoproteins with the lowest ratio of carbohydrate to protein had the lowest emulsifying activity. Mannoproteins were extracted from five mutant *mnn* strains of *S. cerevisiae* with specific defects in the structure of the N-linked glycosyl moieties which are attached to secreted proteins. These materials emulsified as well as that from the parental strain. Optimization of a process for producing mannoprotein emulsifier should be directed towards extracting a maximum of soluble protein,

particularly the high and the low molecular weight components which have the greatest emulsifying activities.

4.2 INTRODUCTION

The mannoprotein from baker's yeast is a potent bioemulsifier suitable for use in foods (Cameron *et al.*, 1988). Mannoproteins can be extracted with high yield by autoclaving yeast in neutral citrate buffer (Peat *et al.*, 1961). Mannoproteins are glycoproteins containing mannose covalently linked to peptides and account for roughly 50 % of the weight of the yeast cell wall (Cabib *et al.*, 1982). The mannoproteins include structural components of the wall and extracellular enzymes (Ballou, 1982). Since as many as 30 to 40 glycoproteins may be present in the yeast cell wall (Pastor *et al.*, 1984; Sentandreu *et al.*, 1984), it was of interest to determine whether one particular mannoprotein had greater emulsifying activity than other mannoproteins in the extracted material.

Two methods have been used in the past to purify yeast mannoproteins. The first method involves precipitation as a copper complex with Fehling's solution (Peat *et al.*, 1961). The alkaline conditions required may hydrolyse glycosyl-serine, glycosyl-threonine linkages, phosphodiester bonds, some peptide and disulfide bonds and acyl ester linkages of the mannoproteins (Ballou, 1976). Hydrolysis of covalent bonds in mannoproteins could affect emulsifying activity. The second

method involves precipitation as a complex with borate and the surfactant cetyltrimethylammonium bromide (cetavlon) at pH 8.8 (Lloyd, 1970; Ballou, 1990). We found that cetavlon and mannoprotein emulsify synergistically, and even trace amounts of cetavlon have a large effect. This interaction is the subject of a separate study. The two methods described above may render the mannoproteins unsuitable for testing emulsifying activity. In this study we chose to fractionate heat-extracted mannoproteins by two different methods, by solubility in the presence of ammonium sulfate and by gel filtration. The various fractions were then assayed for emulsifying activity.

Yeast cell wall mannoproteins contain as much as 50 to 90 percent covalently attached carbohydrate (Fig. 4.1; Ballou, 1982). Covalently attached carbohydrates may affect the emulsifying activity of food proteins (Cayot *et al.*, 1991; Closs *et al.*, 1990; Dickinson and Semenova, 1992; Kato *et al.*, 1989). The influence of variation in the N-linked carbohydrate substituents on the emulsifying activity of mannoproteins was investigated by comparing the activities of mannoproteins extracted from mutants with known defects in glycosylation of secreted proteins.

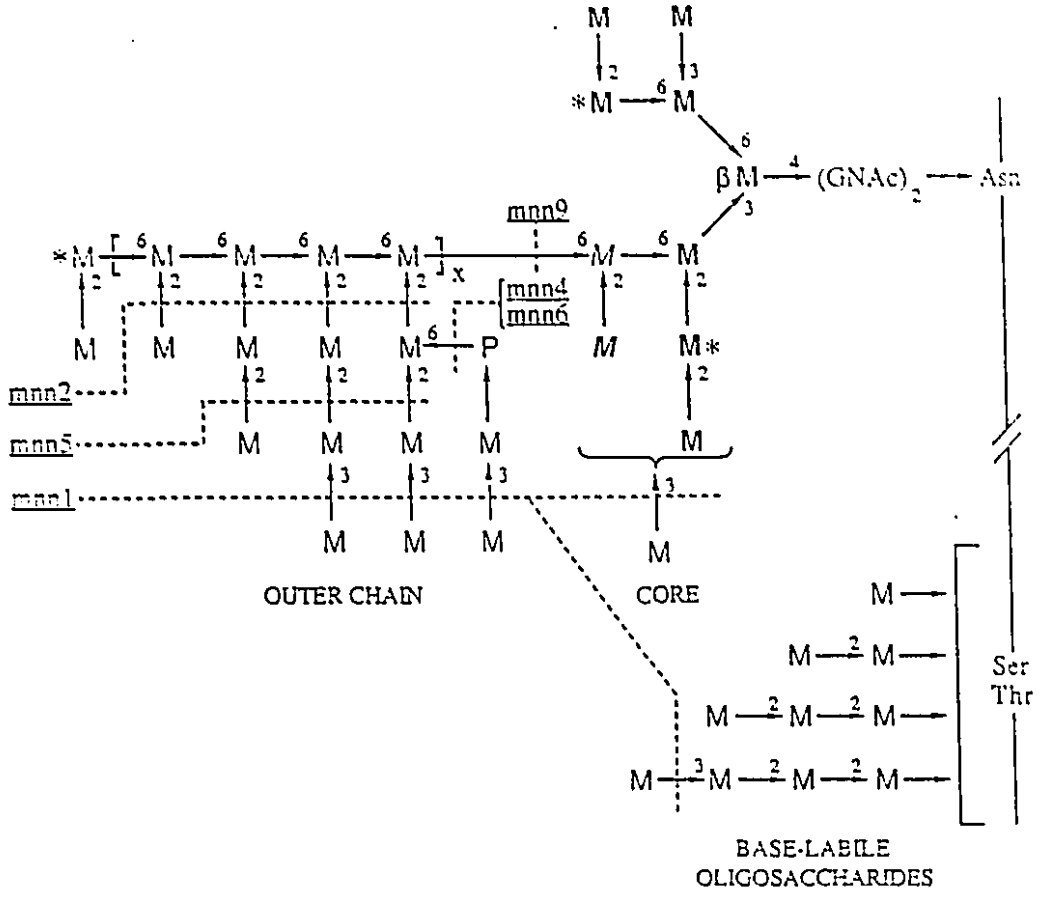


Figure 4.1. Structure of glycoproteins in wild type and mannoprotein *mnn* mutant strains of *S. cerevisiae* with regard to the carbohydrate and phosphate substituents. In the wild type strain x is approximately 10, * indicates additional sites of phosphorylation (Ballou, 1990).

4.3 MATERIALS AND METHODS

Mannoprotein samples. A commercial strain of *S. cerevisiae* (fresh baker's yeast; Lallemand Inc., Montreal) was suspended in 20 mM citrate at *ca.* 5% solids. The pH was adjusted to 7.0 with NaOH or HCl and the suspension was autoclaved for 3 h at 121°C. Cell debris was removed by centrifugation at 5000 x *g* for 10 min at ambient temperature. Three volumes of 95% ethanol containing 1% acetic acid were added to the supernatant. After cooling to 4°C, crude emulsifier was collected by centrifugation for 5 min at 10000 x *g*. The precipitate was suspended in distilled water, the pH was adjusted to 7.0 and was either dialysed or ultrafiltered. Samples were dialysed using Spectrapor 2 membranes (12 to 14 kDa nominal exclusion limit; Fisher, Montreal) for 24 h at 4°C against several changes of distilled water. A Millipore Pellicon system equipped with filter cassettes (10 kDa nominal molecular weight cutoff limit) was used for ultrafiltration; the retentate was washed with five volumes of distilled water while maintaining a constant volume. After dialysis or ultrafiltration mannoprotein samples were frozen (-18°C) or were lyophilized and stored at room temperature.

Protein content was determined by binding of Coomassie blue dye (Bradford, 1976) with bovine serum albumin (BSA) as the standard; total carbohydrate, by the phenol-sulfuric acid method (Dubois *et al.*, 1956) with mannose as the standard; and total phosphate, by the method of Ames (1966) with sodium

phosphate as the standard.

Emulsifying activity was measured as described previously (Cameron *et al.*, 1988), with 4 ml of an aqueous solution, pH 7.0, typically containing 10 mg of emulsifier, and 6 ml of trimethyl pentane in a 13 x 122 mm test tube. This provided a standard emulsifier concentration of 0.1 % (wt/vol). After inserting a rubber stopper the tube contents were mixed to homogeneity on a Vortex-genie (Fisher) at the maximum setting, and vortexing was continued for a further 2 min. The mixture was then left standing for 1 h and the height of alkane not in the emulsion was recorded. The % Emulsion Height was then calculated as follows.

$$\frac{\text{(initial height of trimethyl pentane) - (height of trimethyl pentane not emulsified)}}{\text{(initial height of trimethyl pentane)}} \times 100$$

Fractionation with ammonium sulfate. A sample (3 g) of dialysed mannoprotein was dissolved in 100 ml of 0.5 mM EDTA pH 7.0 and solid ammonium sulfate was added (Scopes, 1982). At 25, 50, 75, and 100% saturation the mixtures were equilibrated for 1 h at room temperature and the precipitates collected by centrifugation at 1000 x *g* for 10 min. Methanol (3 volumes) was added to recover material soluble in the 100 % saturated ammonium sulfate solution. Precipitates were suspended in distilled water, dialysed as described previously, and lyophilized. Approximately 80% of the original sample was

recovered after fractionation, dialysis and lyophilization.

The effect of each fraction on surface tension of an aqueous system (8 ml), pH 7.0, containing 20 mg of test material, in a 40 ml pyrex beaker was determined using a Fisher autotensiomat. Interfacial tension was determined after adding 12 ml trimethyl pentane and equilibrating for 5 min. The surface tension of pure water was 72 $mN.m^{-1}$ and interfacial tension at the water trimethyl pentane interface was 45.5 $mN.m^{-1}$.

Samples from each fraction were analysed by SDS-polyacrylamide gel electrophoresis (Lugtenberg *et al.*, 1975; Frevert and Ballou, 1985), using 3% acrylamide monomer concentration in the stacking gel, 5% in the separating gel. Solutions (5 $mg.ml^{-1}$) in sample buffer were boiled 2 min, and 150 μg (dry wt) of material were loaded per well. Gels were run 2 h at constant current density and were stained either for protein using Coomassie brilliant blue (Lugtenberg *et al.*, 1975) or for carbohydrate using the periodic acid-Schiff stain (Fairbanks *et al.*, 1971).

Gel filtration. Beads of Sephadex G200 were hydrated and poured into a glass column 2.54 cm (d) x 50 cm (h). The column buffer (10 mM HEPES, pH 7.2, 50 mM NaCl) was fed into the column by gravity, maintaining a constant 16 cm head. After equilibration, a sample of ultrafiltered, heat extracted emulsifier (120 mg in 5 ml of buffer) was layered onto the column. The eluate was monitored for absorbance at 280 nm

using an LKB 2138 UVicord S coupled to a chart recorder. Fractions (100 drops) were collected in selected test tubes of uniform weight, with an LKB Redirack. After the run was complete, tubes were weighed to determine fraction volumes, and protein and carbohydrate contents of each fraction were measured. Molecular weight standards blue dextran (2000 kDa), BSA (66 kDa), and lysozyme (14 kDa) were used to calibrate the column.

Emulsifying activity of each fraction was determined by mixing as described earlier, a 1 ml aliquot made up to 4 ml with distilled water and 6 ml trimethyl pentane. When more than 90% of the alkane was emulsified, emulsion activity measurements were repeated with smaller aliquots.

Mannoprotein mutant strains. Mannoprotein *mnn* mutant strains (Ballou, 1990) and the parental strain X2180-1A of *S. cerevisiae* were provided by H. Bussey (Dept. of Biology, McGill). Strains were maintained on YPG agar slants (0.5% (wt/vol) yeast extract, 1% (wt/vol) peptone, 1% (wt/vol) glucose) at 4°C. For each strain, isolated colonies were inoculated into five 500 ml Erlenmeyer flasks containing 200 ml sterile YPG broth. After incubating for 72 h at 29°C with vigorous shaking (250 $rev.min^{-1}$) on a New Brunswick G25 incubator-shaker, cells were harvested by centrifugation (5000 x *g*, 5 min) and washed twice with distilled water. Emulsifier was extracted and collected as described earlier. The quantity of the dialysed heat extracted material from the parental

strain required to emulsify 75% of the alkane phase was determined, and its content of protein was measured. Dialysed heat extract from each of the *mnn* mutant strains, containing the same amount of protein (which provided a 75% Emulsion Height for the parental strain) was assayed for emulsifying activity.

Statistical analysis. Standard procedures were followed for linear regression and single-factor analysis of variance for the data (Campbell, 1974).

4.4 RESULTS AND DISCUSSION

Heterogeneous mannoproteins are solubilized by autoclaving yeast cells for 3 h in neutral buffer (Cabib *et al.*, 1982). Small quantities of nucleic acids and carbohydrate polymers (eg. glycogen, glucan fragments) may also be present in the heat extract (Lloyd, 1970; Okubo *et al.*, 1978, 1981). Low molecular weight solutes are eliminated by dialysis or ultrafiltration. To identify the components with greatest emulsifier activity, the heat extracted material was fractionated by two different methods.

Precipitation with ammonium sulfate. The heat-extracted dialysed emulsifier was divided into five fractions according to solubility with increasing concentrations of ammonium sulfate. The yields and biochemical components of the five fractions obtained are shown in Table 4.1. Protein, carbohydrate and phosphate were detected in all fractions.

TABLE 4.1. Yield and composition of fractions of mannoprotein emulsifier extracted from *S. cerevisiae*

Fraction no.	% Saturation with ammonium sulfate	Yield[a] (%)	Percent of material in fraction as[b]		
			Protein	Carbohydrate	Phosphate
I	25	18.7	34.2	14.7	2.6
II	50	33.9	14.2	13.0	12.8
III	75	12.0	7.2	14.1	11.4
IV	100	4.8	5.8	16.6	10.7
V	>100	19.7	4.5	54.8	2.8

[a] From starting material.

[b] Standards for protein, carbohydrate and phosphate assays were BSA, mannose and $-PO_4H_2$, respectively.

Fraction I contained the most protein, with the amount decreasing progressively in Fractions II to V. Fractions I to IV contained approximately equal amounts of carbohydrate (13 to 16.6%), while Fraction V which was soluble at 100% saturation with ammonium sulfate contained 54.8% carbohydrate.

The amount of phosphate in the dialysed heat extracted emulsifier (7.3%) was higher than that found in mannoprotein in another study (1.3%; Okubo *et al.*, 1981). Differences in the extraction procedure, yeast strain, or culture conditions could account for this variation. Differences in the phosphate content in Fractions I to V (Table 4.1) were not related to variation in % Emulsion Heights (Fig. 4.2). There are insufficient data to determine the importance of phosphate for emulsifying activity.

The % Emulsion Heights provided by 0.1 % (wt/vol) material from each fraction are shown in Fig. 4.2. The results indicate a correlation between % Emulsion Height and protein content of the fraction ($P < 0.01$), represented by the equation $Y = 2.18X + 24.6$ with a correlation coefficient $r = 0.978$. Fraction I with the highest protein content (Table 4.1) had the greatest emulsifying activity (Fig. 4.2), and is clearly the most efficient emulsifier per unit dry weight.

The fact that the regression line did not pass through the origin may indicate that a minimum quantity of protein is necessary to produce an emulsion. Another possibility is that not all the protein present is detected by the

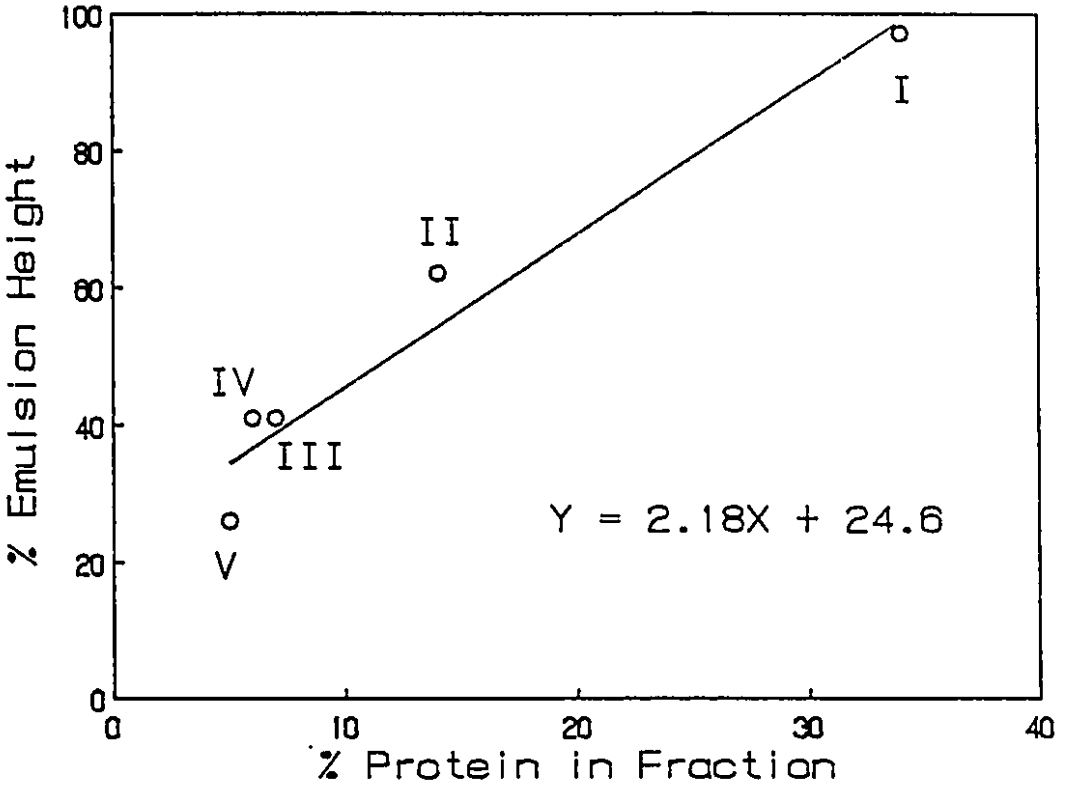


Figure 4.2. Relationship between % Emulsion Height and Coomassie blue-protein content with 0.1% (wt/vol) material from Fractions I to V. Emulsion Height varied significantly with protein content of the fraction ($P < 0.01$).

semiquantitative dye binding assay used (Sedmak and Grossberg, 1977). Different proteins are known to react differently with Coomassie blue dye, for example bovine serum albumin gives more than twice the absorbance at 595 nm than ovalbumin or IgG, per unit dry weight (BioRad, 1984). The color intensity difference is probably related to the content of arginine, lysine, and histidine (Compton and Jones, 1985; Kratzer *et al.*, 1990). Furthermore it has been shown that protein concentration is underestimated after protein (*eg.* casein) is autoclaved in the presence of reducing sugar (lactose, glucose) due to reduced intensity of staining (by 86 and 92%, respectively) with the dye (Kratzer *et al.*, 1990). Even in the absence of reducing sugar, dye binding by casein was reduced by 5 to 30% after autoclaving for 60 min. The minimum size of peptide capable of interacting with the dye is somewhere between penta- and nonapeptides (Tal *et al.*, 1980). Perhaps measuring Kjeldahl nitrogen would provide a more accurate estimate of the protein content of the emulsifier, provided that nucleic acids were not present.

Emulsifying activity of proteins has been related to their ability to reduce surface tension and interfacial tension (Nakai *et al.*, 1980; Kato *et al.*, 1981; Elizalde *et al.*, 1991). All fractions contained surface active material (Table 4.2). At the concentrations used in the emulsion assays, surface tension of the aqueous phase was reduced to between 53 and 58 $mN.m^{-1}$ with no significant difference noted

TABLE 4.2. Effect of various ammonium sulfate fractions of mannoproteins on surface and interfacial tensions[a]

Fraction no.	Surface tension[b] ($mN.m^{-1}$)	Interfacial tension ($mN.m^{-1}$)
I	53.6 ± 0.1 A	17.8 ± 0.3 A
II	57.8 ± 0.3 B	18.7 ± 0.4 AB
III	56.1 ± 0.6 B	19.4 ± 0.1 B
IV	55.3 ± 0.2 B	19.9 ± 0.1 B
V	53.9 ± 0.7 A	18.2 ± 0.3 A

[a] Surface tension was measured on samples containing 8 ml distilled water and 20 mg of the emulsifier; interfacial tension was measured after adding 12 ml trimethyl pentane and equilibrating for 5 min.

[b] Mean value ± standard deviation ($n \geq 3$). Values in the same column with different letters are significantly different by single factor analysis of variance ($P < 0.001$).

for systems containing the best (Fraction I) and worst (Fraction V) emulsifier fractions (Table 4.2).

The amphiphilic character of Fraction V is remarkable; it could not be precipitated with ammonium sulfate and therefore must be very hydrophilic, yet it was very effective in reducing surface and interfacial tensions.

Material from each of the fractions was analyzed by polyacrylamide gel electrophoresis (data not shown). As in previous studies (Ballou, 1976) mannan-proteins did not resolve well. Lanes for Fractions I and II contained heterogeneous material which stained both for protein and carbohydrate, of molecular weights between *ca.* 14 to 66 kDa and *ca.* 28 to 94 kDa, respectively. Samples from Fractions III and IV contained heterogeneous material of higher molecular weight which stained very faintly for protein and carbohydrate.

A discrete band of very high molecular weight material (>200 kDa) which just entered the separating gel was present in the lane containing Fraction V. The band stained intensely for carbohydrate but not for protein. A small amount of heterogeneous carbohydrate-containing material of slightly lower molecular weight was also present. According to its source, electrophoretic behaviour, and staining properties, the material from Fraction V in the discrete band resembles the high molecular weight cell wall mannoprotein identified by Frevert and Ballou (1985). That mannoprotein was extracted

from yeast with either ß-glucanase or 1% sodium dodecyl sulfate and was purified by DEAE-Sephadex and HTP-hydroxylapatite chromatography (Frevert and Ballou, 1985). Gel electrophoresis revealed a discrete band of apparent molecular weight of 180 kDa which stained for carbohydrate but not protein. By gel filtration on Ultragel ACA 34, the cell wall mannoprotein had apparent molecular weight of 192 kDa. It contained 88% carbohydrate, and 12% protein determined with the Lowry procedure (Frevert and Ballou, 1985). If the major component of Fraction V is indeed the cell wall mannoprotein identified by Frevert and Ballou (1985), then the information that it is soluble in saturated ammonium sulfate solution may be useful for future rapid bulk preparations.

Gel filtration. The gel filtration profiles of carbohydrate and protein and the A_{280} of heat-extracted, ultrafiltered emulsifier from *S. cerevisiae* (Fig. 4.3A) were similar to those for yeast mannoproteins extracted either with ethylenediamine (Al-Bassam *et al.*, 1986) or with hot citrate buffer (Okubo *et al.*, 1981). Peak concentrations of carbohydrate, protein and material absorbing at 280 nm eluted in fractions 9 - 11, in the void volume (Fig. 4.3A). This material was excluded from pores in the gel beads, and would be expected to have molecular weight greater than 200 kDa.

A broad peak in protein concentration eluted in fractions 19 -26, corresponding to molecular weights between 66 and 14 kDa (Fig. 4.3A). A small quantity of carbohydrate was also

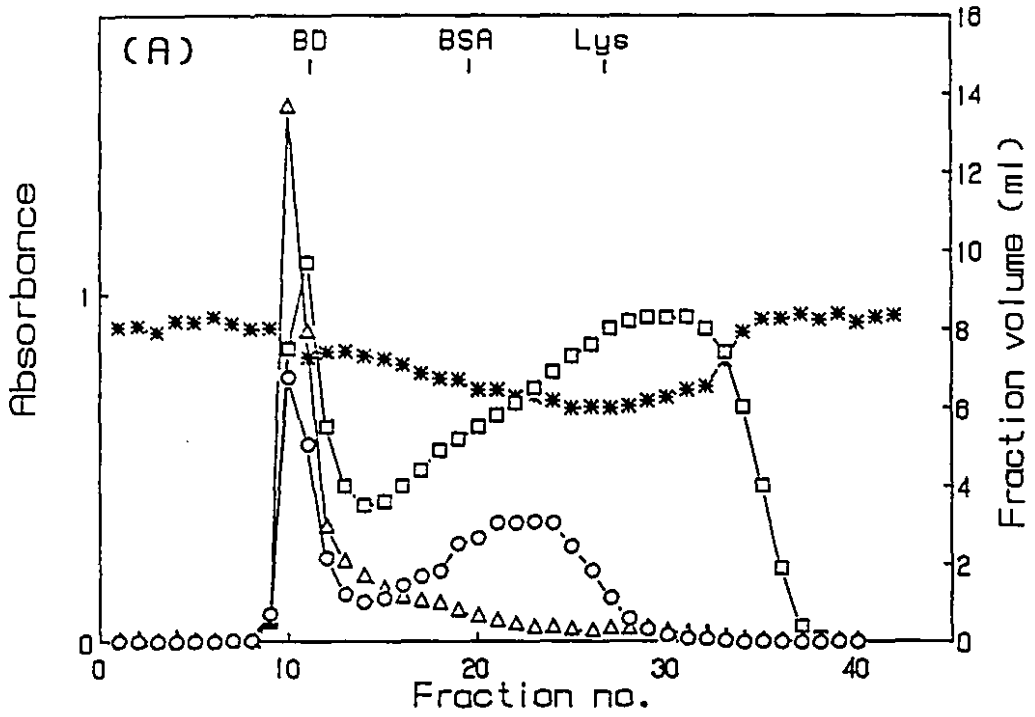


Figure 4.3A. Fractionation of heat-extracted, ultrafiltered emulsifier by gel filtration with Sephadex G200. Absorbance due to carbohydrate at 490 nm (△), protein, at 595 nm (○), absorbance at 280 nm (□), and fraction volume (*) are shown. The positions of elution of molecular weight markers blue dextran (2000 kDa), bovine serum albumin (66 kDa) and lysozyme (14 kDa) are indicated.

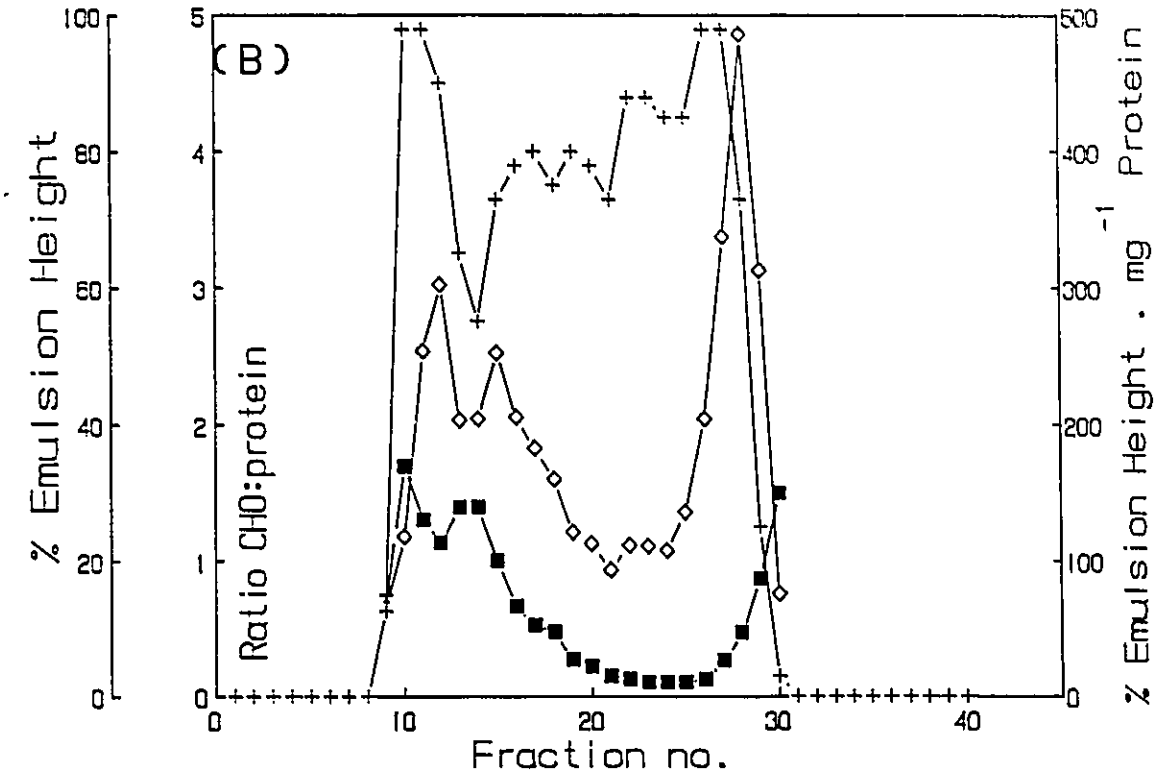


Figure 4.3B. Emulsifying activity of each fraction using a 1 ml sample in the standard emulsion assay (+), % Emulsion Height per mg protein (◊), and ratio of carbohydrate to protein in each fraction (■).

present in these fractions. Fractions 27 - 30 contained little carbohydrate and protein, and absorbed strongly at 280 nm. This material had apparent molecular weight of less than 14 kDa.

Emulsions were generated with samples from all fractions in which Coomassie protein was detected (Figs. 4.3A,B). It was not possible to quantify emulsifier activity per unit dry weight as the dry weight per fraction was too small. Instead, the % Emulsion Height per mg Coomassie protein was calculated for each fraction where stable emulsions were formed. Maxima were recorded for fractions 11, 12 and fractions 27, 28 (Fig. 4.3B).

The ratio of carbohydrate to protein was high in fractions with the greatest emulsifying activity and was lowest in fractions where emulsifying activity was least. The carbohydrate content therefore appears to affect emulsifying activity of mannoproteins. In other studies, covalent attachment of polymeric carbohydrates substantially improved the emulsifying activity of globular proteins (Kato *et al.*, 1988, 1989; Dickinson and Semenova, 1992).

Each fraction contained 100 drops of liquid eluting from the column. Although a constant pressure was maintained on the buffer fed into the column, droplet volume varied probably due to differences in surface tension. The volume provided by 100 droplets varied between 8 ml for column buffer alone and approximately 6 ml for fraction 27 (Fig. 4.3A). Based on the

principle that surface tension varies with the droplet weight (Adamson, 1976), the materials in fractions 27, 28 were the most active in reducing surface tension (Fig. 4.3A). Material in fractions 30 - 35 absorbed strongly at 280 nm, reduced surface tension, but contained no Coomassie protein and was inactive as an emulsifier. It appears therefore that protein capable of binding Coomassie blue dye is essential for emulsifier activity.

A qualitative difference was noted between emulsions made with the high molecular weight (fractions 11, 12) and low molecular weight materials (fractions 27, 28). Very intensive vortexing was required to mix the aqueous and alkane phases to homogeneity with high molecular weight material. The resulting emulsions were viscous, and did not change in appearance after several days. Emulsions containing low molecular weight material (fractions 26 - 28) were generated almost immediately on mixing, and flowed easily but separated after several hours. After one day, a filamentous structure containing very little water was all that remained of the emulsion in the alkane phase.

The material eluted in gel filtration fractions 11, 12 (molecular weight > 200 kDa) contained a high proportion of carbohydrate and is similar to ammonium sulfate Fraction V. The high content of carbohydrate in this material differentiates it from the protein emulsifiers (eg. caseinates, whey proteins, soy proteins) which contain little

carbohydrate.

The high molecular weight emulsifier might be better compared to gum arabic, a naturally-occurring protein-polysaccharide hybrid. Gum arabic consists mostly of polysaccharide and contains highly branched arabinogalactan heteropolymers with approximately 2% attached protein (Dickinson and Euston, 1991). The protein is mainly associated with a high molecular weight fraction representing 20 to 30% of the total gum (Randall *et al.*, 1988). This fraction forms a skin-like layer at the surface of water and is mainly responsible for the emulsifying and stabilizing properties of the natural gum (Dickinson *et al.*, 1991; Randall *et al.*, 1988). As with the high molecular weight emulsifier extracted from yeast, much work was required initially to establish an emulsion in the presence of gum arabic, which, once set up, was very stable (Zajic and Panchal, 1976).

The material which eluted in gel filtration fractions 27, 28 had the greatest emulsifying activity per mg protein of any material in the heat extracted mannoprotein, and had an apparent molecular weight of 14 kDa or slightly less. It contained a small amount of both carbohydrate and protein, absorbed strongly at 280 nm and was the most surface active of all fractions. The principal mannoproteins released from yeast cell walls by detergent or Zymolase digestion had molecular weights greater than 19.5 kDa (Valentin *et al.*, 1984). The low molecular weight emulsifier may have resulted from the

fragmentation of larger mannoproteins during the heat extraction or ultrafiltration steps.

Fragmentation induced by physical, chemical or enzymatic treatment has been used to increase the emulsifying activity, emulsion stability, or foaming capacity of food proteins (Kuehler and Stine, 1974; Adler-Nissen and Olson, 1979; Jost *et al.*, 1982; Haque, 1991; Turgeon *et al.*, 1992). After limited proteolysis, the normally condensed configuration of globular proteins in aqueous solutions cannot be maintained, and hydrophobic regions previously maintained in the interior of the protein become exposed to the surrounding medium. Fragmentation, by whatever means, has to be limited since a peptide length of about 20 residues is generally thought to be the minimum required to provide good emulsifying and interfacial properties (Lee *et al.*, 1987; Turgeon *et al.*, 1991). Excessive proteolysis produced amino acid mixtures and short peptides which did not emulsify (Turgeon *et al.*, 1992; Haque, 1991).

As in the studies on emulsifying activity of proteolytic fragments of food proteins, a minimum molecular weight appeared necessary for emulsifying activity of mannoprotein fragments. The material which eluted last from the gel filtration column (fractions 30 - 34; molecular weight < *ca.* 10 kDa) absorbed strongly at 280 nm, was surface active since fraction volumes were reduced, but did not emulsify. Trace quantities of carbohydrate and no Commassie blue protein were

detected in these fractions (Figs. 4.3A,B).

The two components of the heat extracted material with greatest activity produced very different emulsions. The high molecular weight component produced viscous emulsions after very intensive mixing of the aqueous and oil phases, while the low molecular weight component was very surface active and rapidly produced nonviscous emulsions which were less stable. Whether it is possible to alter the emulsion characteristics of the unfractionated heat extracted material by altering the proportions of its individual components remains to be determined. Mixtures of emulsifying agents are often used in commercial products to provide emulsions with desired properties (Lee *et al.*, 1987; Shimizu *et al.*, 1986; Zajic and Panchal, 1976).

Mannoprotein mutants. To investigate the effect of the mannose and phosphate substituents of mannoproteins on emulsification, emulsifier material was extracted from a series of mannoprotein *mnn* mutant strains derived from *S. cerevisiae* X2180-1A. Extracellular mannoproteins from these otherwise isogenic strains differ only in the structure of attached complex carbohydrates (Fig. 4.1). The *mnn1* strains lack the terminal α-1,3 linked mannose units on both the O-linked and N-linked glycosyl substituents. Only the structure of the N-linked carbohydrate, about 100 sugar units long in wild type strains, is affected in the other mutants. The *mnn2* strain produces an unbranched α-1,6 linked outer chain of

mannose units. The level of phosphorylation is decreased 90% in *mnn4* and *mnn6* mutants (Ballou, 1990). In *mnn9* mutants, the N-linked carbohydrate substituent consists of a core without any part of the outer chain (Fig. 4.1).

Heat extracted, dialysed material containing a standard amount of protein (equivalent to 1 mg BSA) from the parental and each of the mutant strains was tested for emulsifier activity. Similar % Emulsion Heights were obtained for all strains (Table 4.3). Thus, variations in mannose and phosphate substituents on mannoproteins from these strains did not influence emulsifying activity.

In the most extreme of the glycosylation-defective mutants, *mnn9*, mannoproteins contain truncated N-linked carbohydrates consisting of 12 mannose and 2 N-acetylglucosamine subunits, as well as O-linked mannose oligomers (Fig. 4.1). The high molecular weight cell wall mannoprotein from an *mnn9* strain contained 88 % carbohydrate, and 12 % protein as measured by the Lowry procedure (Frevert and Ballou, 1985). For this mannoprotein, 39 % of the carbohydrate was N-linked since it was released after treatment with endoglucosaminidase H. The remaining carbohydrate appeared to be O-linked to serine or threonine (Frevert and Ballou, 1985). The ratio of total carbohydrate to protein for the *mnn9* mannoprotein is higher than that of any of the mannoproteins in Fig. 4.3B. Therefore even though the N- and O-linked glycosyl moieties are truncated in the

TABLE 4.3. Emulsifying activity of heat extracted emulsifier (1 mg protein) from *S. cerevisiae* mannoprotein mutant strains

Relevant genotype	Strain	% Emulsion Height[a]
parental strain	X2180-1A	73.7 ± 2.1
mnn1	LB1-220	72.6 ± 7.4
mnn2-1	LB1-3B	71.8 ± 2.1
mnn1,mnn4	LB16B-10A	75.2 ± 1.2
mnn6	LB1425-1B	77.7 ± 4.8
mnn9	LB2134-3B	75.6 ± 1.3

[a] Mean value ± standard deviation (n = 3).

mannoproteins from the mutant strains, the reduction in total carbohydrate content was insufficient to affect emulsifying activity. Additional experiments, in which a greater amount of carbohydrate is eliminated, may be required to determine the relative importance of the N- and O-linked carbohydrate substituents of yeast mannoproteins in emulsification.

To summarize, the protein in the heat extracted material from yeast is necessary for emulsifying activity. In addition it appears that the amount of carbohydrate also affects emulsifying activity since mannoproteins with a high ratio of carbohydrate to protein had greater emulsifying activity than those with a low ratio. Quantitative and qualitative differences in emulsifying activity were noted for isolated components of the heat extracted mannoproteins. Process optimization for producing yeast mannoprotein on a large scale should be directed towards firstly, extracting a maximum of soluble protein from the cells, and secondly, obtaining the desired balance between the high molecular weight and the low molecular weight glycoproteins which appear to be the most efficient emulsifiers. The former provide viscous emulsions while the latter are very surface active and emulsify very readily but do not produce durable emulsions on their own.

4.5 ACKNOWLEDGMENTS

Financial support for this work from the Fonds pour la formation de chercheurs et l'aide à la recherche of the governement of Quebec is gratefully acknowledged. We thank H. Bussey for supplying mannoprotein mutant strains and summer students M. Zenaitis, S. Wentworth and S. Hughes who initiated some of the experiments.

CHAPTER 5: SYNERGISTIC INTERACTION OF MANNOPROTEIN AND CETYLTRIMETHYLAMMONIUM BROMIDE OR LECITHIN IN EMULSIONS

To be submitted for publication as:

Cameron, D.R., E.S. Idziak, R.J. Neufeld, and D.G. Cooper. Synergistic interaction of yeast mannoprotein and cetyltrimethylammonium bromide or lecithin in emulsions.

PREFACE

Heterogeneous cell wall mannoproteins are extracted by autoclaving yeast cells in neutral buffer (Cabib *et al.*, 1982). To identify components with greatest emulsifying activity it was necessary to fractionate material in the heat extract using standard biochemical methods (chapter 4). The method most often used to isolate mannoproteins from the heat extract is by fractional precipitation as a complex with borate and the cationic surfactant, cetyltrimethylammonium bromide (CTAB) (Lloyd, 1970; Ballou, 1990). Even though most of the detergent is removed from precipitated mannoproteins by washing with acetone and dialysing against water, remaining trace amounts could conceivably affect emulsifying activity. The effect of trace quantities of CTAB on emulsifying activity was measured by adding increasing amounts to a solution containing mannoprotein. A remarkable synergistic interaction was detected in emulsions containing a weight ratio of 1:100 CTAB:yeast mannoprotein. Since food emulsions (ice cream, cream liqueurs, *etc.*) contain both protein emulsifiers and low molecular weight surfactants, any interaction between these components would be of interest. This phenomenon was investigated by the experiments described in this chapter. Needless to say, the use of CTAB was ruled out for isolating mannoproteins and measuring emulsion properties of components of the heat extracted emulsifier.

5.1 ABSTRACT

Mannoprotein is a potential food grade emulsifier which can be extracted with high yield from yeast. As with many food proteins, the emulsifying activity of mannoprotein was lowest in acidic conditions, and coincided with its minimum in solubility. In the presence of a variety of surfactants, salts, and amino acids, the emulsifying activity of mannoprotein increased more than four-fold. The strongest interaction was with surfactants bearing a positively charged group, such as the cationic detergent cetyltrimethylammonium bromide (CTAB) or the zwitterionic phospholipid lecithin. A weight ratio of 1:100 CTAB or lecithin:mannoprotein produced the greatest increase in emulsifying activity. The emulsifying activity in the presence of both components was far greater than for each one by itself hence increased synergistically. Data on surface and interfacial tensions for mixtures of CTAB and mannoprotein support the conclusion that a molecular interaction occurs. Salt did not reduce the emulsifying activity of a mixture of mannoprotein and CTAB. Emulsifying activity was decreased slightly at highest surfactant concentrations. Emulsifying activity of caseinate or whey protein concentrate did not increase in the presence of lecithin, however a high concentration of lecithin increased emulsifying activity of gelatin. The emulsifying activity in neutral and slightly acidic conditions of food proteins other than mannoprotein might be improved by this method.

5.2 INTRODUCTION

Emulsifying activity is one of the most important functional properties of proteins in foods (Kinsella, 1976). Proteins are involved in stabilizing oil-in-water type emulsions in ice cream, milk, cream liqueurs, salad oils, gravies and many other foods (Dickinson and Stainsby, 1988). The emulsifying properties of proteins are generally studied using model systems. Here an aqueous phase containing an emulsifier and an oil phase are homogenized together. In model systems, the efficiency of protein emulsifiers varies considerably with the pH of the aqueous phase (Halling, 1981). For many of the proteins tested, emulsifying activity is far greater in alkaline conditions than at pH values from neutral to slightly acidic, common to many food products. For example, the specific surface area of oil droplets in an oil-water emulsion stabilized by ß-lactoglobulin was 0.63 $m^2.ml^{-1}$ at pH 5, and 3.8 $m^2.ml^{-1}$ at pH 9.7 (Das and Kinsella, 1989). The oil phase volume at inversion of an emulsion with cottonseed protein increased from 22% at pH 4 to 89% at pH 8 (Crenwelge *et al.*, 1974). Emulsifier activity is often related to protein solubility, and undissolved protein makes little or no contribution to emulsification (Halling, 1981).

Proteins are least soluble at their isoelectric point (Scopes, 1982). At this pH, the net charge on the protein is zero, electrostatic repulsion between different parts of the protein is minimized, and the protein adopts a compact

configuration (Halling, 1981). Hydrophobic regions remain in the interior of condensed, aggregated proteins at or near their isoelectric point and consequently are not available for stabilizing the interface between the aqueous and oil phases (Mangino, 1984). Consequently there may be considerable unused "potential" emulsifying capacity in proteins where the isoelectric pH is near the pH of the emulsified food in which they are used. Any method to put this unused emulsifying capacity into service would be of great interest.

One approach has been to attach charged or hydrophilic substituents onto the proteins to increase their solubility. Hydrophilic groups used include organic acids (succinylation; Kinsella and Shetty, 1979) amino acids (Chobert *et al.*, 1987), simple sugars (Closs *et al.*, 1990; Cayot *et al.*, 1991), and polysaccharides (Kato *et al.*, 1989; Dickinson and Semenova, 1992). In most cases, some improvement in emulsifying properties occurs at a particular ratio of hydrophile to protein, but not with all proteins. Problems with this approach are that highly reactive compounds not permitted in foods may be required for covalent coupling, and that certain functional groups on the protein (*eg.* lysine, for coupling via Maillard reactions) may be eliminated.

Another method used to improve the emulsifying activity of proteins is partial hydrolysis with proteases (Kuehler and Stine, 1974; Turgeon *et al.*, 1991). Limited hydrolysis of the protein alters the protein structure, exposing peptide

sequences from the interior of the protein to the solution, and permitting hydrophobic regions to associate with the emulsion interface. Both hydrophilic and hydrophobic clusters of amino acids were present on surface active peptides generated by proteolysis (Turgeon *et al.*, 1991, 1992). Excessive hydrolysis produced amino acids and small peptides mixtures which did not emulsify.

Mannoprotein emulsifier contains a mixture of glycoproteins extracted from the yeast cell wall (Cameron *et al.*, 1988; Cameron *et al.*, submitted). Mannoproteins possess the amphiphilic structure common to many emulsifying agents; attached carbohydrate substituents are believed to provide hydrophilic functional groups and the protein supplies a hydrophobic functional group on the molecule (Cameron *et al.*, submitted). Yeast mannoprotein and trace quantities of a cationic surfactant or lecithin interact synergistically, increasing emulsifying activity more than four-fold in a model system at neutral pH.

5.3 MATERIALS & METHODS

Materials. The surfactants used included cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) from Sigma (St. Louis, MO), Tween 60 (polysorbate 60: polyoxyethylene (20) sorbitan monostearate) and Span 60 (sorbitan monostearate) from Atkemix (Montreal, Que.), and vegetable lecithin (> 95%, U.S. Biochemicals, Cleveland, OH).

Protein emulsifiers included sodium caseinate and whey protein concentrate (both from Universal Foods, Montreal) and commercial gelatin (Knox, Toronto). Samples of sodium caseinate and whey protein concentrate were dialysed 24 h at 4°C using Spectrapor 2 dialysis tubing (exclusion limit 12 to 14 kDa) against several changes of distilled water, and were lyophilized. Gelatin was used without further treatment after being dissolved by heating briefly in a boiling water bath.

Mannoprotein emulsifier was extracted by autoclaving a suspension (5% solids) of fresh baker's yeast (Lallemand, Inc., Montreal) in 20 mM citrate, pH 7 for 3 h at 121°C (Cameron *et al.*, 1988). Cell debris was removed by centrifugation (5,000 x *g*). Emulsifier was precipitated by adding 3 vol. ethanol containing 1% acetic acid, and collected by centrifugation (10,000 x *g*, 5 min). The precipitate was suspended in distilled water, and the pH was adjusted to 7.0 with concentrated NaOH. The solution was ultrafiltered using a Millipore Pellicon system with filter cassettes having a molecular weight exclusion limit of 10,000 Da. The retentate volume was maintained constant by adding distilled water continuously to replace permeate until 5 times the retentate volume had been added. The final retentate was then lyophilized.

The water-immiscible phase in emulsions was corn oil (free of additives) or reagent grade trimethyl pentane.

Emulsion assays. Two methods were used for assaying emulsifying activity. In the first method, 30 ml water containing 40 mg emulsifier, pH 7.0, and 10 ml corn oil were blended 2 min in an Oster blender at maximum speed (Cameron *et al.*, 1991). To determine oil content, duplicate 1 ml samples of the emulsion were dried (100°C, 16 h) in tared weighing pans. To measure interfacial area of the oil droplets in the emulsion, samples (50 μl) were added to 39.95 ml of water containing 0.1 % (vol/vol) of NP-40 nonionic detergent, 1 % (wt/vol) dextran (486 kDa avg. molecular weight), pH 7.0. The droplet size distribution on triplicate samples was measured using a Malvern 2600 particle sizer (Malvern, U.K.). The Emulsion Activity Index (Pearce and Kinsella, 1978), with units of m^2 interfacial area . g^{-1} emulsifier, was calculated as described previously (Cameron *et al.*, 1991).

For the second emulsion assay (Akit *et al.*, 1981), 4 ml of water, pH 7, containing emulsifier and 6 ml of trimethyl pentane were vortexed to homogeneity and mixed an additional 2 min at maximum speed on a Vortex-Genie (Fisher) in a stoppered glass test tube (13 x 122 mm). After 1 h the % Emulsion Height was calculated as

$$\frac{\text{(initial height of trimethyl pentane) - (height of trimethyl pentane not emulsified)}}{\text{(initial height of trimethyl pentane)}} \times 100$$

Values reported are means of triplicate assays.

Effect of pH on Solubility. Solutions containing mannoprotein (final concentration of 0.1 % wt/vol) were

adjusted to the desired pH value with HCl or NaOH. After centrifugation (10,000 x *g*, 10 min), an aliquot (0.3 ml) of the supernatant was diluted 10-fold with buffer (0.1 M HEPES, pH 7.2) in a quartz cuvette and A_{280} was measured. The percent solubility was calculated as the ratio of A_{280} after centrifugation divided by A_{280} before centrifugation.

Chemical analyses. Carbon, hydrogen, and nitrogen composition of the mannoprotein emulsifier was determined using a model 240XA Analyzer (Control Equipment Corp., Lowell, MA). The hexose sugar content was measured using the phenol-sulfuric acid assay (Dubois *et al* ., 1956) with mannose as standard. Phosphate content was determined using the method of Ames (1966) with sodium phosphate as standard.

5.4 RESULTS

The emulsifier extracted from baker's yeast by heating in neutral buffer contained protein (78%, calculated as 6.25 x % N), carbohydrate (18.5%) and phosphate (3.5%) as major components.

As with numerous protein emulsifiers, the emulsifying activity measured as the Emulsifying Activity Index (EAI; Pearce and Kinsella, 1978), varied with the pH of the aqueous phase (Fig. 5.1). Minimum activity was recorded at pH 4.0, where the solubility was least.

Although the emulsifying activity at pH 7.0 is about one half that at pH 11 (Fig. 5.1), further tests were carried out

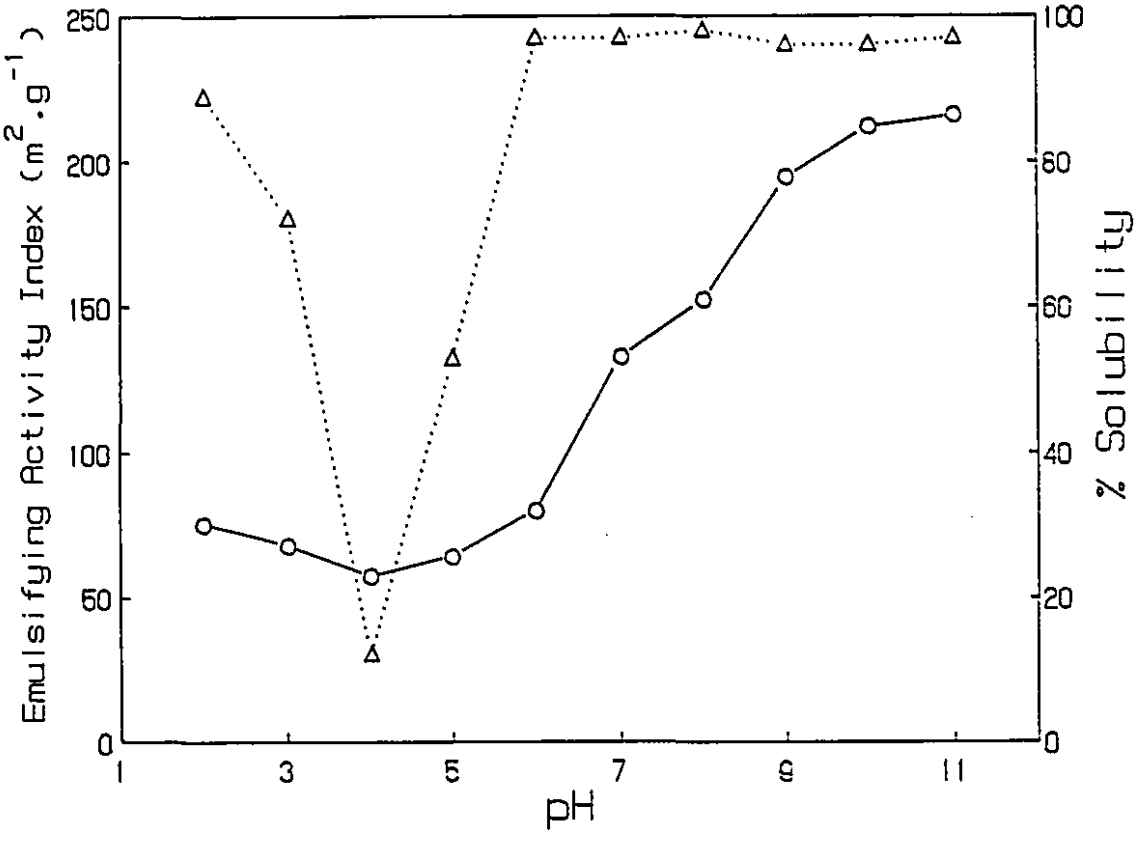
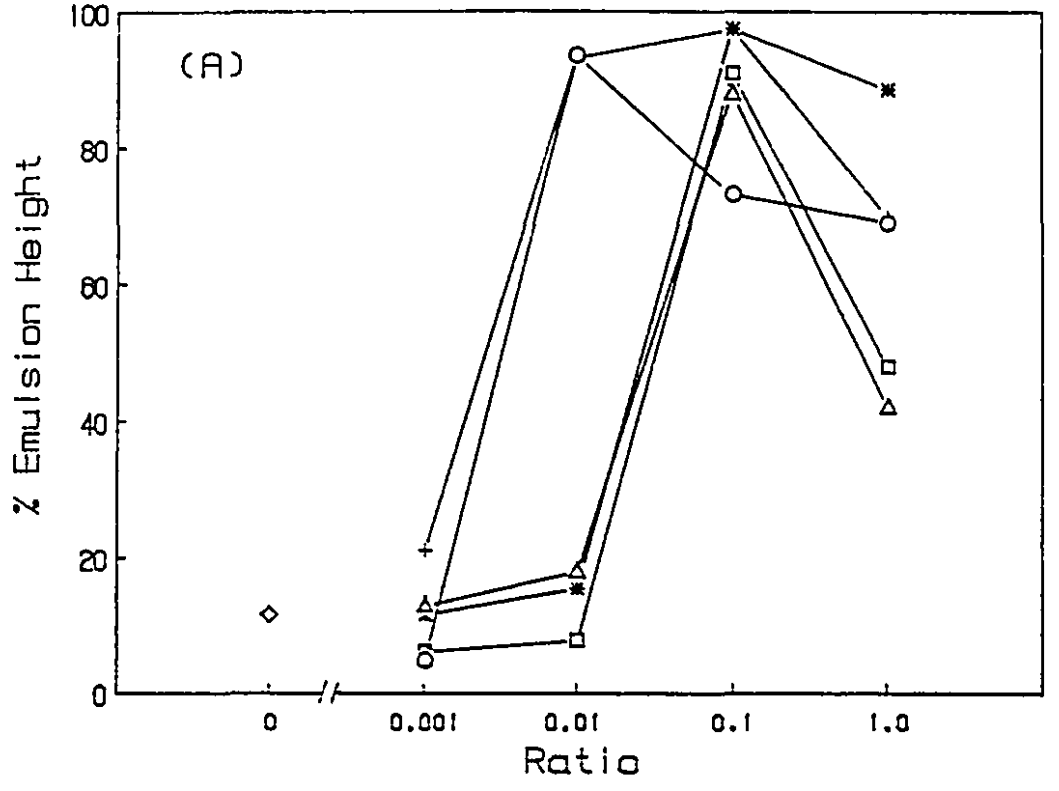


Figure 5.1 Effect of pH on Emulsifying Activity Index (○ ; with units of m^2 interfacial area per g emulsifier) and % solubility (△) of yeast mannoprotein. Emulsions contained 25 % (vol/vol) corn oil, and 0.1 % (wt/vol) yeast mannoprotein.

at pH 7.0 to more reflect the situation in a majority of food systems. A variety of compounds were added to a standard quantity of mannoprotein (0.015 % wt/vol), which emulsified approximately 10% of the alkane phase (Fig. 5.2A). Concentrations of the added surfactants, salts, or amino acids were chosen to provide weight ratios between 1:1000 and 1:1 of surfactant, or other substance, to the constant quantity of mannoprotein. Identical concentrations of these compounds were tested for emulsifying activity in the absence of mannoprotein, where stable emulsions were obtained only with the highest concentrations of the surfactants (Fig. 5.2B). Salts and amino acids alone did not emulsify (data not shown).

Emulsifying activity greater than that obtained with either of the single components was observed with mixtures of mannoprotein with various surfactants, simple salts or amino acids, suggesting a synergistic interaction. What is remarkable is that addition of an extremely small quantity of either lecithin or CTAB to solutions containing mannoprotein produced a great increase in emulsifying activity. Mannoprotein and 1/100 the weight of either lecithin or CTAB emulsified over 90% of the alkane phase; in contrast, mannoprotein alone emulsified only 10% of the alkane phase (Fig. 5.2A,B). Even with 1/1000 parts of lecithin, a slight increase in emulsifying activity was observed. At pH 7.0, CTAB is positively charged, lecithin (phosphatidyl choline) is neutral and zwitterionic. Whereas at weight ratios of 1:100


(A)
% Emulsion Height
100
80
60
40
20
0
0
0.001
0.01
0.1
1.0
Ratio

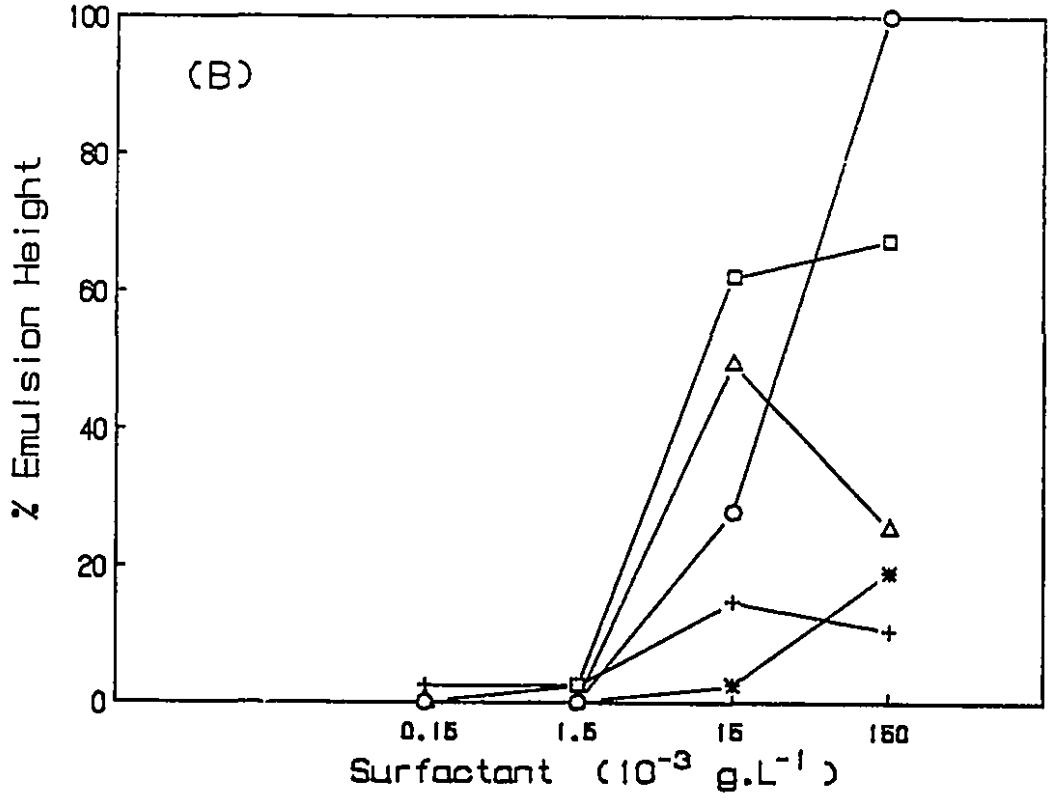

(B)
% Emulsion Height
100
80
60
40
20
0
0.15
1.5
15
150
Surfactant (10⁻³ g.L⁻¹)

Figure 5.2 Effect of surfactants on emulsions containing 60 % (vol/vol) trimethyl pentane and 0.015 % (wt/vol) yeast mannoprotein, pH 7.0. In **(A)**, the % Emulsion Height is plotted as a function of the surfactant:mannoprotein weight ratio, with increasing surfactant and a fixed concentration of mannoprotein. At ratio 0, the % Emulsion Height provided by 0.015 % (wt/vol) mannoprotein in the absence of surfactants is indicated (◊). In **(B)**, the % Emulsion Heights were provided by identical surfactant concentrations as in **(A)**, in the absence of mannoprotein. The surfactants included the cationic cetyltrimethylammonium bromide (○; CTAB), the anionic sodium dodecyl sulfate (△), the non-ionic water-soluble Tween 60 (□), the non-ionic oil-soluble Span 60 (*), and the zwitterionic lecithin (+).

only CTAB and lecithin increased emulsifying activity, at weight ratios of 1:10 all of the surfactants (ionic and nonionic) interacted synergistically with mannoprotein and increased the emulsifying activity. An increase in the ratio of all the surfactant: mannoprotein to 1:1, resulted in a decrease in emulsifying activity (Fig. 5.2A). At the highest concentration of Tween 60 and CTAB, the emulsifying activity of the surfactant alone was greater than that in the presence of mannoprotein (Fig. 5.2A,B).

The surface and interfacial tensions were measured on solutions containing CTAB of identical concentration to those assayed for emulsifying activity (Fig. 5.3). CTAB alone, at the three lowest concentrations, reduced the surface tension of water only slightly (Fig. 5.3). With the highest concentration of CTAB, which is above the critical micelle concentration (cmc) of 9.2×10^{-4} M (Rosen, 1989), the surface tension of water was reduced substantially. Assuming that little CTAB dissolves in the alkane phase, the cmc in this system (in which 60 % of the volume of the liquid phase is trimethyl pentane) is calculated to be 3.68×10^{-4} M (134 $mg.L^{-1}$ of emulsion, indicated by the arrow in Fig. 5.3).

With mannoprotein alone in the solution, the surface tension was 62 $mN.m^{-1}$ (Fig. 5.3). Addition of increasing amounts of CTAB to solutions containing mannoprotein resulted in a progressive decrease in surface tensions. Values for interfacial tensions between the aqueous and the alkane phases

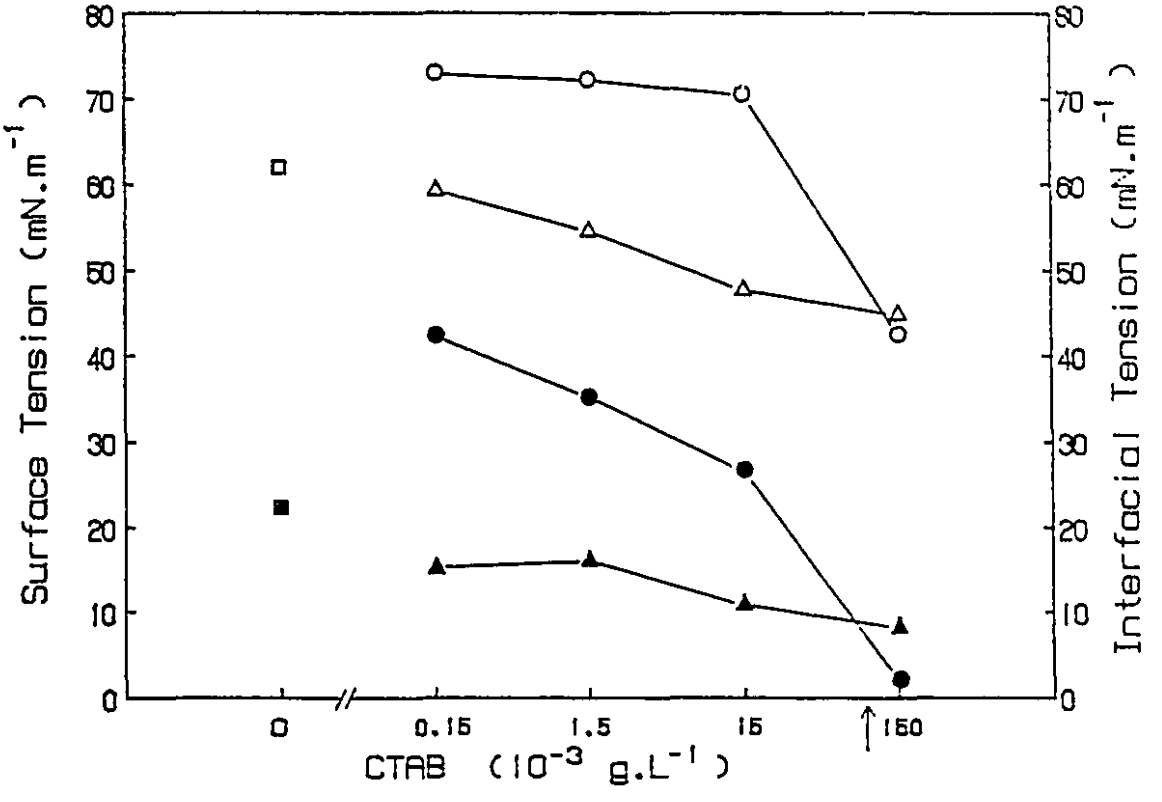


Figure 5.3 Surface tension (open symbols) and interfacial tension (closed symbols) with increasing concentration of CTAB in the presence (△,▲) and absence (○, ●) of 0.015 % (wt/vol) mannoprotein. The surface tension (□) and interfacial tension (■) of mannoprotein solution in the absence of CTAB is indicated. The arrow marks the critical micelle concentration of CTAB (134 mg.L^{-1} ; Rosen, 1989).

varied in a similar fashion to those for surface tensions (Fig. 5.3).

At weight ratios of 1:10 or 1:1 of salts to mannoprotein an increase in emulsifying activity occurred with sodium chloride, sodium phosphate, and sodium citrate (Fig. 5.4). Emulsifying activity increased only at a 1:1 ratio with sodium acetate.

Increasing concentrations of NaCl did not reduce the emulsifying activity in the presence of both mannoprotein and 1/100 of CTAB (Fig. 5.5). Emulsifying activity of a system containing mannoprotein, 1/1000 parts CTAB, and increasing concentrations of sodium chloride, was similar to that with mannoprotein and sodium chloride alone (Fig. 5.4, 5.5). Thus the synergistic increase in emulsifying activity is maintained in mixtures containing mannoprotein, sodium chloride and cationic surfactant.

An increase in emulsifying activity occurred with mannoprotein plus a 1:10 weight ratio of the amino acid cysteine (Fig. 5.6). With equal weight concentrations of amino acid and mannoprotein, the emulsifying activity of mannoprotein increased with glutamate (anionic), asparagine (cationic), and cysteine (neutral). At all concentrations tested, methionine, tryptophan, and leucine (neutral, nonpolar), histidine (neutral, polar) and glycine (neutral) had little effect on the emulsifying activity of mannoprotein (Fig. 5.6 footnote).

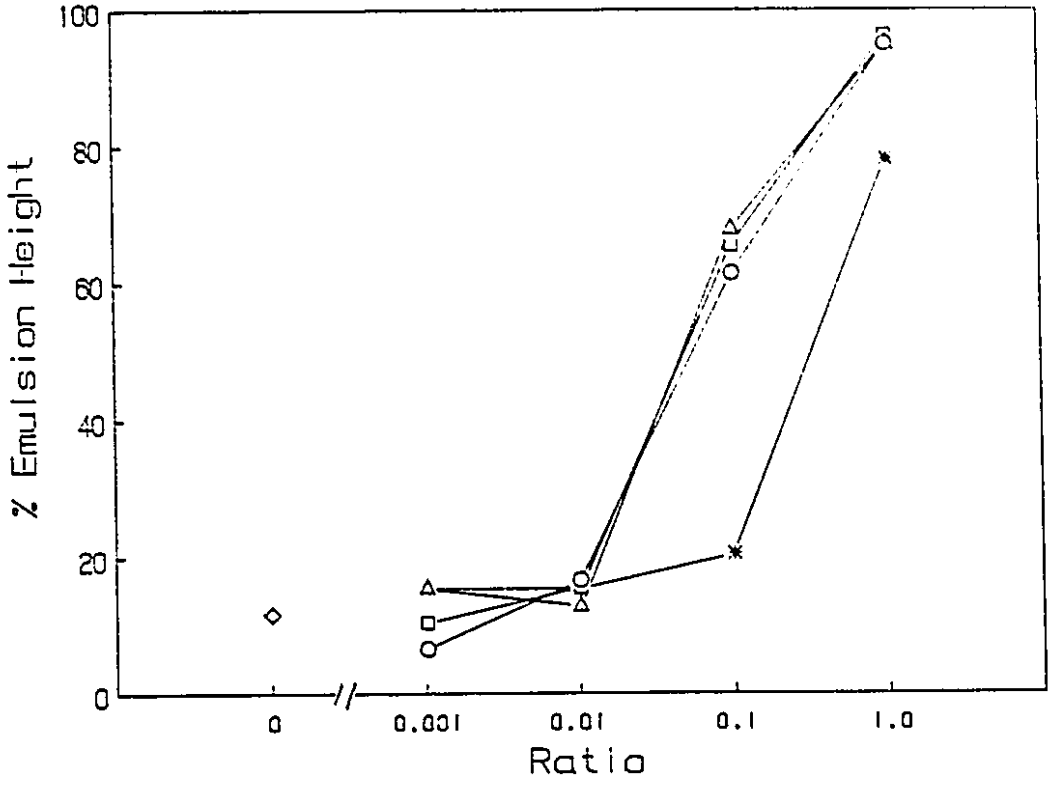


Figure 5.4 Effect of sodium salts in emulsions containing 60 % (vol/vol) trimethyl pentane and constant concentration (0.015 % wt/vol) mannoprotein. The % Emulsion Height is plotted as a function of salt:mannoprotein weight ratio, for sodium chloride (O), sodium phosphate (Δ), sodium citrate (□), and sodium acetate (*). The % Emulsion Height provided by mannoprotein in the absence of salts is indicated (◊). In the absence of mannoprotein, salts at the same weight concentrations as for surfactants in Fig 5.2B did not emulsify.

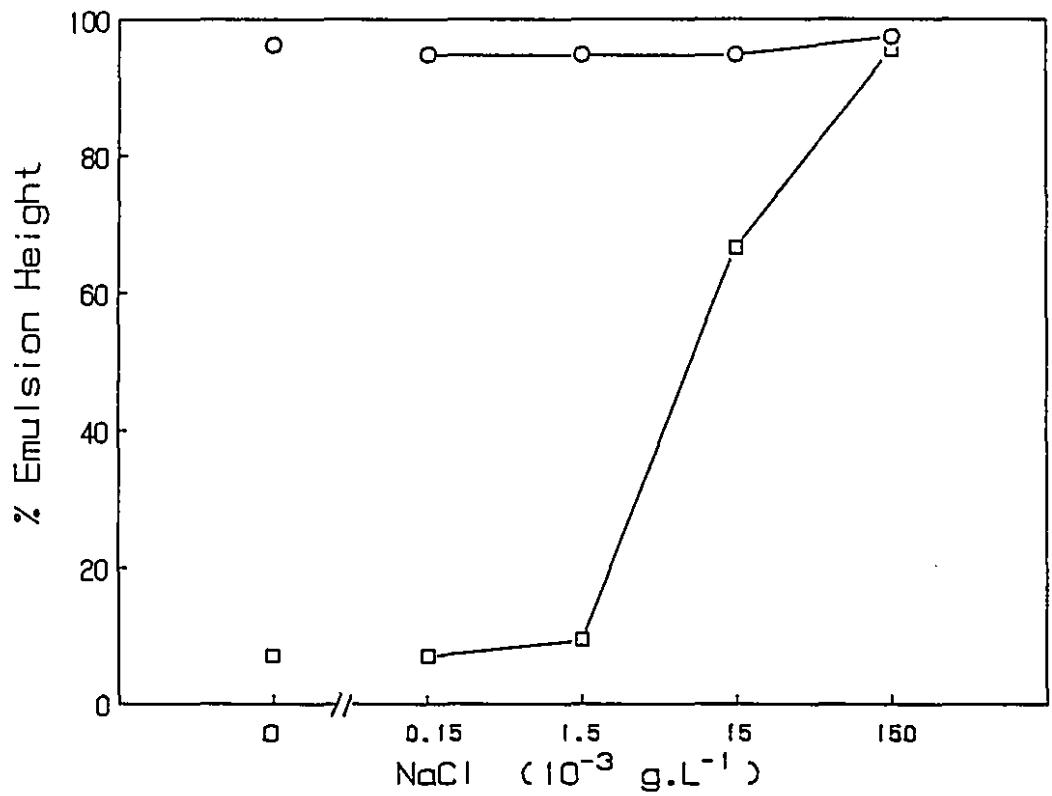


Figure 5.5 Effect of sodium chloride on emulsions containing CTAB and 0.015 % (wt/vol) mannoprotein at a weight ratio of 1:100 (O), or 1:1000 (□). The % Emulsion Height in the absence of sodium chloride is indicated.

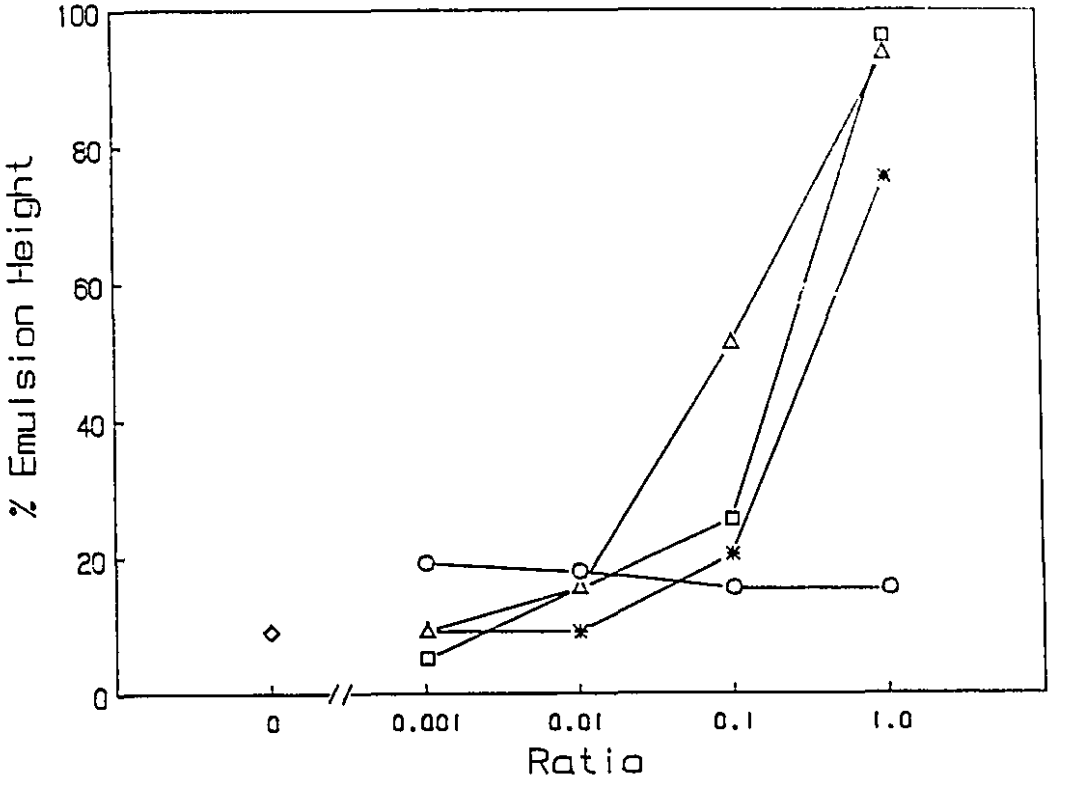


Figure 5.6 Effect of amino acids in emulsions containing 60 % (vol/vol) trimethyl pentane and constant concentration (0.015 % wt/vol) mannoprotein. The % Emulsion Height is plotted as a function of amino acid:mannoprotein weight ratio, with the same weight concentrations as in Figure 5.2B, for mannoprotein alone (◊), methionine (○), cysteine (△), glutamate (□), and asparagine (*). Tryptophan, histidine, leucine and glycine provided results similar to those with methionine. Amino acids did not emulsify in the absence of mannoprotein.

Lesser amounts of three commonly used proteins provided similar emulsions to that obtained with 0.015 % mannoprotein. The concentrations of caseinate, gelatin, and whey protein required to emulsify approximately 10% of the alkane phase were respectively 1/3, 1/3, and 1/6 that of mannoprotein. Lecithin was added at weight ratios from 1:1000 to 1:1 to solutions containing each of the proteins (Fig. 5.7). In these systems, a synergistic increase in emulsifying activity was noted only with a 1:1 weight ratio of lecithin and gelatin. Data for addition of CTAB were similar, and are not shown.

5.5 DISCUSSION

As with many protein emulsifiers, the emulsifying activity of mannoprotein in the pH range of most foods (neutral to slightly acidic) was only a fraction of that at alkaline pH. The minimum in emulsifying activity of mannoprotein coincided with its solubility minimum (Fig. 5.1). The choice of centrifugation speed for measuring protein solubility is somewhat arbitrary (Kinsella, 1984; Schein, 1990). Use of a faster centrifugation speed would have resulted in generally lower solubility values, and probably a closer correspondence between the curves for solubility and emulsifying activity over the entire pH range.

The point of minimum solubility of the mannoprotein is assumed to be the pH where its net electrical charge is a minimum (Scopes, 1982). Electrical repulsion between adjacent

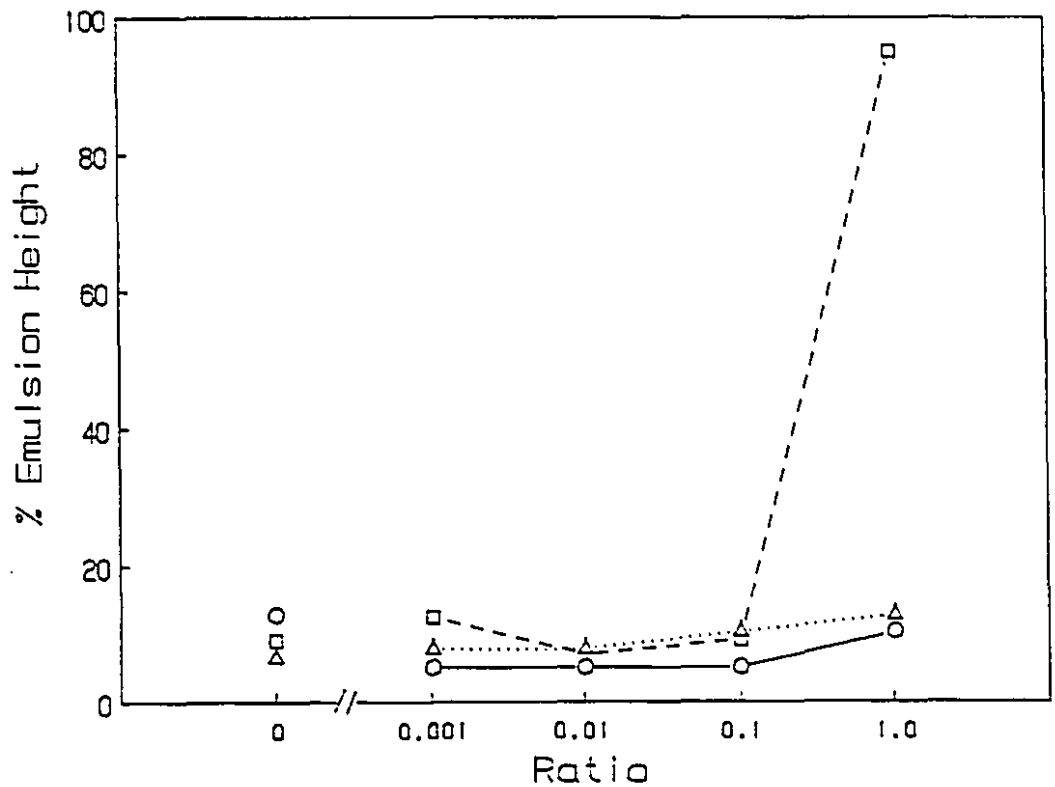


Figure 5.7 Effect of lecithin in emulsions containing 0.005 % (wt/vol) caseinate (○), 0.0025 % (wt/vol) whey protein concentrate (△), or 0.005 % (wt/vol) gelatin (□). The ratio indicated on the x axis is the weight ratio of lecithin:protein. The % Emulsion Height provided by each protein in the absence of lecithin is also shown.

portions of the mannoprotein would be minimal and a condensed, compact configuration is expected at the isoelectric pH (Halling, 1981). At pH values above or below this point, the net charge on mannoprotein increases. This increases repulsion between adjacent portions of the molecule and favours denaturation or unfolding. The greater the extent of unfolding at oil-water interface, the greater the interfacial area occupied by mannoprotein, measured as emulsifying activity. Therefore it follows that for a number of proteins an increase in emulsifying activity is observed at pH values far removed from the isoelectric point (Mita *et al.*, 1973, 1974; Crenwelge *et al.*, 1974; Halling, 1981; Das and Kinsella, 1989).

A method to improve the emulsifying activity of mannoprotein at neutral pH has been identified in this study. Addition of a small quantity of various surfactants, common salts, or amino acids increased the emulsifying acitivity of mannoprotein by at least a factor of four (Figs. 5.2, 5.4, 5.6). Surfactants bearing a positively charged group were most effective; mannoprotein plus 1/100 the weight of either lecithin or CTAB increased emulsifying activity eight-fold.

Improvement of the emulsifying properties of globular proteins by sonicating with lecithin has been reported previously (Hirotsuka *et al.*, 1984; Mizutani and Nakamura, 1988; Nakamura *et al.*, 1988). Emulsifying activity of blood globin and wheat gluten increased by up to three- to four-fold, depending on pH and lecithin concentration (Nakamura *et*

al., 1988; Mizutani and Nakamura, 1988). However a far greater proportion of lecithin was required; the weight ratio of lecithin to protein utilized was between 2:1 and 10:1. No results were reported for lower ratios. By contrast, in the present study, the most effective ratio was 1:100 lecithin to mannoprotein.

The changes in emulsifying activities with variations in pH suggest that electrostatic interactions are involved in maintaining protein configuration which in turn affects emulsifying activity. At pH 7.0, addition of electrolytes increased the emulsifying activity of mannoprotein (Fig. 5.4, 5.6), probably by neutralizing charge interactions between adjacent portions of the molecule and favouring denaturation.

In the system for measuring surface and interfacial tensions (Fig. 5.3), the surface area was only 5 cm^2, whereas that in an emulsion approaches 2.7 m^2. This value was calculated using data in Fig. 5.1, at pH 7.0. The values for surface tension and interfacial tension (Fig. 5.3) are therefore not directly applicable to emulsified systems.

The only use intended for the surface and interfacial tension data is to provide evidence for an interaction between mannoprotein and the cationic surfactant CTAB. At pH values above the point of its minimum solubility, mannoprotein bears a net negative charge due to carboxylate groups on acidic amino acids in the protein and phosphate monoesters and diesters in the carbohydrate substituents (Ballou, 1990).

Addition of CTAB at the three lowest concentrations, scarcely affected the surface tension of water but substantially decreased the surface tension of a solution containing mannoprotein (Fig. 5.3). The decrease in surface tension is likely due to an increased concentration of a mannoprotein-CTAB complex at the surface.

The binding of ionic surfactants by charged water soluble polymers has been studied in detail (Goddard and Hannan, 1976; Malikova *et al.*, 1984; Hayakawa *et al.*, 1983; Prud'homme and Long, 1983). According to the surface tension data, mannoprotein binds CTAB at more than 2 orders of magnitude of concentration below its critical micelle concentration (Fig. 5.3). The first binding of surfactant to polyionic polymer was by electrostatic interaction, and also occurred at very low free surfactant concentrations (Malikova *et al.*, 1984). Electrostatic binding of a charged surfactant by a polyionic polymer reduces the net charge and simultaneously increases its hydrophobicity. Both of these events increase the affinity of the complex for the oil-water interface (Goddard and Hannan, 1976; Prud'homme and Long, 1983).

Proteins also bind detergent ions in successive stages, involving increasing numbers of bound ions (Tanford, 1968). The number of dodecyl sulfate ions initially bound by bovine serum albumin corresponded to the number of positive charges on the protein (Tal *et al.*, 1980). At higher concentrations, binding of ionic surfactant induces conformational changes and

denaturation of the protein, exposing additional detergent binding sites (Jones and Brass, 1991; Su and Jirgensons, 1977). Denaturation of the mannoprotein upon binding a small quantity of surfactant exposes hydrophobic regions which preferentially associate with the alkane phase, and would be expected to increase the emulsifying activity.

At high detergent concentrations additional surfactant binds to the surfactant-polymer complex by hydrophobic interaction ("tail to tail" adsorption). Binding of the second layer of ionic surfactant increases the net charge on the complex and it therefore becomes more soluble (Goddard and Hannan, 1976). Desorption of the mannoprotein-surfactant complex from the alkane-water interface likely occurs with high surfactant concentrations. This would be accompanied by a decrease in emulsifying activity if the complex is replaced at the interface by a surfactant with little emulsifying activity by itself (*eg.* lecithin or Span 60; Fig. 5.2A,B). Hence the decrease in emulsifying activity observed with mannoprotein and a high concentration of surfactant can be explained.

ß-Casein was displaced from the surface of emulsified droplets of tetradecane by lecithin at lecithin: ß-casein weight ratios of 0.5 or greater (Courthaudon *et al.*, 1991a). Nonionic oil-soluble surfactants octaethylene glycol dodecyl ether and glycerol monostearate also displaced ß-casein from the interface in emulsions with soya oil or tetradecane

(Courthaudon *et al.*, 1991b; Dickinson and Tanai, 1992).

The emulsifying activity of whey proteins and caseinate did not increase in the presence of lecithin or CTAB (Fig. 5.7). Very small quantities of these proteins were required initially to emulsify approximately 10% of the alkane phase, hence they may be already maximally unfolded at the initial conditions of the assay. ß-Casein, a component of caseinate, denatured rapidly at the air-water interface (Graham and Phillips, 1979a); its structure is described as flexible, disordered coil with only 10% helical content. Addition of surfactant increased emulsifying activity of gelatin, only at the highest surfactant concentration.

In summary, a mechanism for the synergistic increase in emulsifying activity has been proposed which is consistent with the data. Mannoprotein is negatively charged at neutral pH and interacts with a trace quantity of surfactant bearing a positively charged group. This interaction increases the affinity of the complex for the interface and greatly increases emulsifying activity. At high surfactant concentrations, binding of additional surfactant by hydrophobic interactions increases solubility of the complex, causes desorption from the interface, and results in a decrease in emulsifying activity.

If the results from the model system used here can be extrapolated to actual foods, addition of a small quantity of lecithin would improve the prospects for using mannoprotein as

an emulsifier in consumer products. Whether other proteins can be found with similar emulsifying behaviour to mannoprotein will be determined in future experiments. According to results from this study, a precise ratio of charged surfactant to protein may be required to provide optimal emulsifying activity.

5.6 ACKNOWLEDGEMENTS

This study was supported by the Fonds pour la formation de chercheurs et l'aide à la recherche of the Quebec government.

SUMMARY AND CONCLUSIONS

Mannoprotein from baker's yeast is an emulsifying agent with suitable properties for use in foods (Cameron *et al.*, 1988). It can be extracted simply, with high yield by heating yeast cells in neutral buffer, or by treating yeast cells with Zymolyase enzyme. Mannoprotein emulsifier can be extracted with equally good yield from low cost substrates such as spent yeast from beer or wine production.

A correction was made to the formula for the Emulsifying Activity Index (Pearce and Kinsella, 1978), so that the index provides the units desired, m^2 interfacial area per g emulsifier. This index has been widely used for comparing activity of protein emulsifiers.

The heat extracted emulsifier contains heterogeneous mannoproteins all of which are capable of stabilizing emulsions. The emulsifying activity in fractions of the heat extracted emulsifier separated with ammonium sulfate was related to protein content measured by binding of Coomassie blue dye. After fractionation of the heat extracted emulsifier by gel filtration, two components with the greatest emulsifying activity were identified. Both contained a high ratio of carbohydrate to protein. Vigorous mixing was required to produce emulsions with the high molecular weight component; the emulsions were viscous and did not separate after several days. The low molecular weight component was very surface

active and readily provided non-viscous emulsions which separated after several hours. Mannoproteins with the lowest ratio of carbohydrate to protein were the least active emulsifiers. Mannoproteins from mutant strains of *S. cerevisiae* with defects in the structure of the N-linked and O-linked carbohydrate had similar emulsifying activity to mannoproteins extracted from the parental strain.

The mannoproteins in the heat extract possess the characteristics of effective protein emulsifiers, namely solubility and sufficient hydrophobicity. The method of preparation of this emulsifier is selective for soluble materials; insoluble materials and cell debris are removed by centrifugation after the heating step. The component of the heat extracted emulsifier separated by gel filtration which had greatest emulsifying activity also had greatest surface activity. Fraction V of the emulsifier was soluble in 100 % saturated ammonium sulfate, and was one of two fractions with greatest surface activity. All of the ammonium sulfate fractions (at a concentration of 0.1% wt/vol) reduced the surface tension of water to below 58 $mN.m^{-1}$ (Table 4.2). Mannoproteins therefore have amphiphilic properties, in common with other effective emulsifying agents.

A synergistic increase in emulsifying activity at neutral pH occurred with a 100:1 weight ratio of yeast mannoprotein and either lecithin or cetyltrimethylammonium bromide. Emulsifying activity was also increased in the presence of

mannoprotein plus a variety of other surfactants, salts, or amino acids, at weight ratios of 10:1 or 1:1. Improvement of the emulsifying activity of mannoprotein in the presence of a small quantity of another substance may increase the chances for this emulsifier to be used commercially.

The behaviour of mannoprotein emulsifier in the simple model systems used here may be different from that in actual foods or cosmetics. Whether mannoprotein can be produced economically on a large scale and whether it will be a useful emulsifier for actual consumer products is still to be determined.

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