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R-Phycoerythrin as a Fluorescent Time-Temperature Integrator for Monitoring Thermal Processing of Beef Products

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Alicia Orta-Ramirez

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Denise M. Smith
Major professor

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R-PHYCOERYTHRIN AS A FLUORESCENT TIME-TEMPERATURE INTEGRATOR FOR MONITORING THERMAL PROCESSING OF BEEF PRODUCTS

By

Alicia Orta-Ramirez

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ABSTRACT

R-PHYCOERYTHRIN AS A FLUORESCENT TIME-TEMPERATURE INTEGRATOR FOR MONITORING THERMAL PROCESSING OF BEEF PRODUCTS

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Alicia Orta-Ramirez

Time-temperature integrators (TTIs) are a rapid and accurate method to verify thermal processing in foods because they can predict the lethality of a target attribute when the two systems are subjected to the same thermal process. The objective of this study was to characterize and verify the applicability of R-phycoerythrin (R-PE) to be used as the detector component of a TTI for monitoring of thermal processing in beef products.

R-PE, a protein which fluoresces in the visible range, was purified from algal tissues of *Porphyra yezoensis* by precipitation with ammonium sulfate and gel filtration chromatography. The isolated R-PE had a purity greater than 5 as determined by A_{565}/A_{280}. Isothermal experiments were conducted to determine the thermal inactivation parameters of R-PE calculated on the basis of fluorescence loss under different conditions of pH and concentrations of sucrose, NaCl, sodium-dodecyl sulfate (SDS), urea and β-mercaptoethanol (ME). R-PE was most thermostable at pH between 5.0 and 9.0, but became more heat sensitive at pH 4.0 and 10.0. Sucrose and ME had a thermostabilizing effect, while SDS, NaCl and urea decreased thermal stability of R-PE. The variations obtained in the thermal inactivation parameters showed that the kinetics of R-PE can be modified by altering the solution composition.

When R-PE was placed in borate buffer, pH 9.0, the resulting z value (5.99°C) fell

within the range of z values reported for *Salmonella* in ground beef (5.6-6.2°C). *Salmonella* is the target microorganism in thermal processing of beef products.

In non-isothermal experiments, R-PE fluorescence could distinguish between adequate and inadequate heat processes according to USDA requirements for hamburger and roast beef. The results suggested that thermal inactivation kinetics of R-PE do not follow a first-order reaction. A non-linear mathematical model was developed to fit thermal inactivation kinetics of R-PE and *S. senftenberg*. The reaction orders (n) calculated for R-PE and *S. senftenberg* were 0.55 and 1.2, respectively. The activation energies (E_A) were estimated using the Arrhenius model. The calculated E_A for R-PE and *S. senftenberg* were 347.4 and 220.84 kJ $mole^{-1}$, respectively. The lethalities calculated for R-PE and *S. senftenberg* assuming non-linear kinetics were highly predictable (R^2 of 0.98 for R-PE and 0.93 for *S. senftenberg*). Results strongly suggest that, despite the differences in thermal inactivation parameters, the lethality of *Salmonella* can be predicted from R-PE fluorescence.

To tears and laughter,
To sunshine and rain.
To family and friends,
To Lluna, Pruna and Xaloc,
But most of all...

...To James

...For believing in me.

"Stop this day and night with me
and you shall possess the origin of all poems

You shall possess the good of the earth and sun,
(there are millions of suns left)

You shall no longer take things at second or third hand,
nor look through the eyes of the dead,
nor feed on the spectres in books

You shall not look through my eyes either, nor take things from me,

You shall listen to all sides and filter them from your self".

(Walt Whitman, Song of Myself)

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CHAPTER 1: Introduction

Thermal processing is the most common method used for food preparation and preservation. The thermal processing kinetics can be defined in terms of D, z and F values (Singh and Heldman, 1993; Hendrickx et al., 1995). D value is the time in minutes required to decrease a quality attribute by 90% at a constant temperature. The z value is the temperature increase necessary to reduce the D value by 90%. The F value is the time required to achieve a stated reduction in a population of microorganisms or spores. This time is usually expressed as a multiple of the D value. Thermal processes describing changes in a quality attribute such as a microorganism or a protein can also be expressed in terms of chemical kinetics using the Arrhenius equation

$$k = k_0 \exp(-E_A/RT)$$

where k_0 is the pre-exponential factor, E_A is the activation energy of the process, R is the universal gas constant, and T is the temperature. In this context, the D value or thermal reduction time is equivalent to the reaction rate constant, k, and z value or thermal resistance constant can be compared to the activation energy, E_A (Singh and Heldman, 1993). Over a certain range of temperature, both models (D-z and k- E_A) can be applied with the same accuracy (Hendrickx et al., 1995).

Even with reliable thermal processing schedules and equipment, though, there is a need to determine process compliance to ensure food safety. The evaluation of thermal processes in foods can be done using in situ methods, physical-mathematical models or

time-temperature integrators. Time-temperature integrators (TTIs) are devices capable of predicting the time-temperature response of a quality or safety index in a food product. This occurs when the thermal destruction of both TTI and target index are identical ($z_{TTI} = z_{target}$) (Hendrickx et al., 1995; Van Loey et al., 1996).

The United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) has recently changed the thermal processing regulations for meat products to include a lethality standard based on destruction of the pathogenic microorganism *Salmonella* expressed as log reductions. The USDA now requires that manufacturers use a combination of thermal and non-thermal processes to achieve a 6.5-log *Salmonella* reduction in ready-to-eat, cooked beef, roasted beef and cooked corned beef; a 7-log reduction in ready-to-eat poultry products (Federal Register, 1999a). The USDA has proposed a 5-log reduction in cooked uncured meat patties, although this may be increased (Federal Register, 1999a). The development of a TTI with the same z value as *Salmonella* in beef and poultry products could be used to determine the adequacy of any log reduction process.

The main objective of this project was to study the thermal inactivation kinetics of the fluorescent protein R-phycoerythrin and assess its usefulness as a TTI for ensuring the proper cooking of beef products. By manipulation of the thermal inactivation kinetics of R-phycoerythrin, to adjust the z value of the protein to that of *Salmonella* in beef, R-phycoerythrin could be used as the direct sensing component of a new type of TTI to model *Salmonella* destruction in cooked beef products.

CHAPTER 2: Objectives

The overall goal of this research is to develop a time-temperature integrator (TTI) to monitor thermal processing of foods with special emphasis in meat products. The main component of this TTI is a naturally-fluorescent pigment protein called R-phycoerythrin (R-PE).

The specific objectives are:

1. To determine the thermal inactivation kinetics of R-PE as affected by pH and addition of other chemicals (sucrose, sodium-dodecyl sulfate, NaCl, urea and (β-mercaptoethanol);
2. To compare thermal inactivation kinetics of R-PE with experimental heat inactivation data of *Salmonella senftenberg* in beef products;
3. To verify the ability of R-PE to quantify the integrated time-temperature impact during isothermal and non-isothermal heating profiles; and
4. To develop and verify a mathematical expression that fits the thermal inactivation kinetics of *S. senftenberg* in cooked beef products and of R-PE as measured by fluorescence loss.

CHAPTER 3: Significance and justification

Adequate thermal processing is the most important method to eliminate pathogenic bacteria and viruses from meat and poultry products to prevent foodborne diseases. According to Todd (1996), the estimated number of cases resulting from foodborne infections and intoxications were 5.5-6.2 million costing $5.8-8.6 billion. *Salmonella*, *E. coli* O157:H7, *Campylobacter* and *Staphylococcus* are among the most common causative agents of foodborne illnesses in the U.S. Rapid and accurate methods to verify compliance of thermal treatments are needed to ensure safety of cooked meat products. Current USDA methodology for monitoring adequacy of heat treatments is based on determination of the endpoint temperature to which the product has been cooked. Neither of the official tests use the time-temperature integrator concept nor are based on thermal destruction of bacterial pathogens (Doyle and Schoeni, 1984; Goodfellow and Brown, 1978; Line et al., 1991). In addition, these methods are inaccurate, subjective and do not provide enough margin of safety (Smith and Orta-Ramirez, 1995).

This project proposes the development of a TTI for monitoring of thermally processed beef products (for example, roast beef and hamburger patties). Manufacturers and consumers will benefit from improved food quality and safety through the increase in the frequency, accuracy and convenience of thermal process testing. It is envisioned that these TTIs will be devices with a very low cost per unit, ready to be incorporated into the thermal processing procedure, easy to recover, and with a fast and user-friendly readout. Because of the high

convenience, they could be applied to routine monitoring at the retail level (i.e. fast food restaurants, processing plants). More frequent routine testing would yield safer cooked beef products while avoiding overcooking, which in turn results in lower quality foods and higher energy consumption.

We will evaluate the applicability of a protein, R-phycoerythrin (R-PE), to be used as the detecting component of the TTI. By determining the kinetics of thermal inactivation of R-PE and characterizing the factors or conditions that alter such parameters, we can evaluate the modified inactivation kinetics to define the usefulness of the protein to predict destruction of *Salmonella* in beef products.

Although the use of colorimetric-based or protein-based thermal monitoring tests is not totally new, this TTI offers advantages over other alternatives that justify its research and development:

- R-phycoerythrin is a natural pigment-protein derived from an edible algae. This is of interest because of its inherent safety in food applications.
- The algae has abundant pigment (up to 20% of dry weight) which is easily extracted and purified.
- Encapsulation in a defined chemical environment allows known kinetics even in foods of different composition.
- When compared with enzymes, R-phycoerythrin shows a wider extent of applications because of its tolerance over a broader range of chemical conditions.
- The fluorescence loss of R-phycoerythrin is directly and immediately detectable, without the need for additional steps prior to analysis.

CHAPTER 4: Literature review

4.1. THERMAL PROCESSING REGULATIONS

Microbial contamination is considered the most important hazard associated with foodborne illnesses (Todd, 1996; Centers for Disease Control and Prevention, 1998a). From 1988 to 1992, a total of 2,423 foodborne outbreaks were recorded, of which, 79% were caused by bacterial pathogens (Centers for Disease Control and Prevention, 1998a). Inadequate cooking and improper storage and holding temperatures are the most common errors that lead to foodborne outbreaks (Todd, 1996; Centers for Disease Control and Prevention, 1998a). Recent outbreaks of food poisoning due to *Escherichia coli* O157:H7 associated with consumption of underprocessed beef products, have led to a severe scrutiny of the methodology used to ensure safety of cooked meat products. Traditionally, Title 9 of the Code of Federal regulations outlines the requirements for the processing of meat products needed for destruction of pathogenic microorganisms that can cause foodborne disease (USDA-FSIS, 1990). The government regulatory agencies reviewed and updated the current thermal processing regulations after the 1993 outbreak of *E. coli* O157:H7. This outbreak was associated with consumption of undercooked ground beef patties and involved more than 500 people, including four deaths.

In January 1994, the Food and Drug Administration (FDA) published a new Food Code (FDA, 1993) establishing new recommendations for cooking meat products. It was recommended that pork products and comminuted meats be cooked to internal temperatures

of either 63°C (145°F) for 3 min, 66°C (150°F) for 1 min or 68°C (155°F) for 15 sec. Roast beef and corned beef were recommended to be cooked to any of the following time/temperature protocols: 54°C (130°F)/121 min, 56°C (132°F)/77 min, 57°C (134°F)/47 min, 58°C (136°F)/32 min, 59°C (138°F)/19 min, 60°C (140°F)/12 min, 61°C (142°F)/8 min, 62°C (144°F)/5 min or 63°C (145°F)/3 min. Poultry products were recommended to be cooked to 74°C (165°F) or above for 15 sec. In addition, according to USDA-FSIS regulations, official establishments that manufacture fully-cooked patties were required to use one of the following time/temperature protocols: 66.1°C (151°F)/41 sec, 66.7°C (152°F)/32 sec, 67.2°C (153°F)/26 sec, 67.8°C (154°F)/20 sec, 68.3°C (155°F)/16 sec, 68.9°C (156°F)/13 sec, and 69.4°C (157°F) (and up)/10 sec. Also, official establishments that manufacture partially cooked patties were required to heat patties to a minimum internal temperature 60°C (140°F) followed by cooling to a maximum internal temperature of 4°C (40°F) within 2 h (USDA-FSIS, 1993). These schedules were based on destruction of pathogenic microorganisms (FDA, 1993; Goodfellow and Brown, 1978).

In 1996, the FSIS proposed to amend the Federal meat and poultry inspection regulations (Federal Register, 1996). The proposed modifications defined the performance standards that establishments should meet to produce safe products while allowing each processor to use their own specific plant-processing procedures, even if these differed from the ones specified in the current regulations. Three performance standards were proposed by FSIS: lethality, stabilization and handling. The purpose of the lethality standard was to effectively eliminate pathogenic microorganisms that could be present in the product. The reduction of a microbial population can be expressed in terms of decimal reduction time or D value. The D value is defined as the time necessary to achieve a 90% reduction in the

microbial population at a given temperature (Singh and Heldman, 1993).

Traditionally, *Salmonella* was the microorganism of concern in cooked beef products (Goodfellow and Brown, 1978). Although *E. coli* O157:H7 has become of greater concern, especially in meat patties, it is less heat resistant than *Salmonella* (Doyle and Schoeni, 1984; Line et al., 1991; Orta-Ramirez, 1994). In addition, although *Listeria monocytogenes* is more heat resistant than *Salmonella*, its incidence in meat products is usually a result of recontamination. Thus, expected levels of *L. monocytogenes* are much lower than those of *Salmonella*. Therefore, the thermal inactivation of *Salmonella* in meat products is indicative of destruction of other pathogenic microorganisms. For cooked beef, roast beef, cooked corned beef and cooked poultry products, FSIS recommended a lethality resulting in a 7-D reduction in *Salmonella*; for fully cooked uncured meat patties the lethality standard should be a process resulting in a 5-D *Salmonella* reduction. It is important to notice that all time/temperature combinations previously required by USDA for cooked/roast beef, cooked corned beef, poultry products and fully cooked uncured patties, would result in a 7-D or 5-D *Salmonella* reduction, respectively (Federal Register, 1996). Therefore, establishments already employing any of the above mentioned time-temperature schedules would produce cooked beef or poultry products that comply with the proposed lethality standards.

On January 6, 1999, the FSIS formalized the lethality standards (Federal Register, 1999a). It was required that manufacturers use a combination of thermal and non-thermal processes that achieve a 6.5-log *Salmonella* reduction in ready-to-eat, cooked beef, roast beef and cooked corned beef; and a 7-log reduction in ready-to-eat poultry products. Currently, FSIS has not issued a final rule on lethality standards for ready-to-eat meat patties, and, in the meantime, current thermal processing requirements (Federal Register, 1996) will remain in

effect (Federal Register, 1999a).

Although an increase of thermal processing enforcement can help reduce the incidence of foodborne disease, there is still the problem of confirming adequate cooking once the product has been processed. The USDA-FSIS currently employs three different assays to verify proper thermal processing of meat products. The residual Acid Phosphatase test (USDA-FSIS, 1986a) is used for canned hams, picnics and luncheon meats. This assay is based on the residual enzyme activity in water-soluble protein extracts of cooked samples. Positive reactions show a blue color that can be read at 610 nm. One problem with this assay is that it has been demonstrated that there is a large decrease in acid phosphatase activity during frozen storage (Townsend, 1989). This should be taken into consideration when analyzing samples that have been kept under freezing conditions.

The Coagulation test (USDA-FSIS, 1986b) is used for beef and pork products. The method is based on loss of protein solubility during heat processing of the meat product. Protein extracts of cooked samples are heated until cloudiness appears. The temperature at which the turbidity occurs is considered the internal temperature the product reached during cooking. For products cooked to the 63-71°C the assay can differ 8-10°C from the real temperature achieved during processing (Townsend and Blankenship, 1989). This test is thus very subjective and only gives a gross estimation of the thermal process applied.

The Bovine Catalase test (USDA-FSIS, 1989) is used to verify roast beef and cooked beef. The assay is based on the production of foam when catalase reacts with oxygen. Bovine catalase is destroyed in the 60.5 - 61.1°C temperature range. Thus, at temperatures above this range this assay becomes useless. In addition, the visual detection of foam is very subjective.

None of these assays are sensitive and reliable. In addition, the thermal inactivation kinetics of these markers is not known. Moreover, with the introduction of new processing techniques, it would be desirable to identify an indicator whose inactivation depends on time-temperature only (i.e. TTIs) and not on the technique applied. There is urgent need for objective and accurate assays to verify adequate thermal processing.

4.2. *Salmonella*

Salmonella is a microorganism commonly found in the intestinal tract of humans and animals. Although most *Salmonella* are non-host specific, some strains are known to be host-adapted. For example, *S. typhi* and *S. paratyphi* A and C are most typically found in humans; *S. pullorum* and *S. gallinarum* are associated with poultry; *S. typhisuis* and *S. cholerasuis* are found in pigs; *S. dublin*, *S. abortusequi* and *S. abortusovis* are associated with cattle, horses and sheep, respectively (Doyle and Cliver, 1990; Bauer and Hordmansdorfer, 1996).

More than 2000 strains have already been identified. Of those, approximately 150 are associated with disease outbreaks. Although *Salmonella* can be transmitted from person to person, it is believed that the major source is contaminated foods of animal origin.

4.2.1. Characteristics of the microorganism.

Microorganisms of the genus *Salmonella* are part of the Enterobacteriacea family. They are Gram negative, facultative anaerobic, non-spore forming bacilli, generally showing motility. Most strains have shown the presence of flagellae or fimbriae.

There are several biochemical characteristics shared by the members of the genus *Salmonella*. They use citrate as the only source of carbon and rarely ferment lactose or sucrose. Most of them ferment glucose with the subsequent formation of gas.

The *Salmonellae* have been classified based on O or somatic antigen, H or flagellar antigen and Vi or capsular antigen, according to the Kauffman-White scheme. In addition, they can be divided into five subgenera (I to V) based on their biochemical characteristics.

Salmonella can grow at temperatures ranging from 5 to 47°C, with an optimum growth temperature of 37°C. They also can tolerate pHs between 4.0 and 9.0, with an optimum pH range of 6.5-7.5 (Doyle and Cliver, 1990; Bauer and Hormansdorfer, 1996). *Salmonella* are susceptible to freezing and thawing. A reduction of 1-2 log can be achieved with one freeze-thaw cycle (Doyle and Cliver, 1990).

One method to eliminate *Salmonella* from foods is by cooking (CDC, 1998b). This microorganism is mildly resistant to high temperatures. Several factors influence the resistance of *Salmonella* to heat. Some strains are more resistant than others. For example, *S. senftenberg* is unusually heat-resistant. It is considered to be 10-20 times more resistant than the average *Salmonella* spp (Doyle and Cliver, 1990). Another factor is the composition of the food or medium the *Salmonella* is in. For example, the D value at 60°C of *S. typhimurium* in whole eggs was 0.27 min. When 10% sucrose was added to these eggs, the D_{60} value increased to 0.6 min. The water activity and pH of the food or medium also plays an important role in the heat resistance of *Salmonella*. The microorganism is much more resistant to dry than moist heat and shows more heat susceptibility at extreme pHs (Schuman and Sheldon, 1997).

The thermal inactivation of *Salmonella* in foods has been the subject of numerous studies. Schuman and Sheldon (1997) studied the heat destruction of *Salmonella* in egg products. D values ranged from 0.087 min at 62.2°C to 0.28 min at 60°C in egg yolk, and from 1.0 min at 58.3°C to 7.99 min at 55.1°C in egg white. The z values ranged from 3.54 to

4.33°C. Goodfellow and Brown (1978) calculated D values for *Salmonella* spp in ground beef. The D values determined at 51.6, 57.2, and 62.7°C were 61-62, 3.8-4.2, and 0.6-0.7 min, respectively, depending on the recovery method used. The z value was found to be 5.6°C. The results of Goodfellow and Brown were used to design the time-temperature combinations for the cooking of roast beef and hamburger products listed in the USDA thermal processing regulations (USDA-FSIS, 1990).

4.2.2. Clinical presentations.

Salmonellosis is the generic disease caused by bacteria of the genus *Salmonella*. There are three different syndromes associated with this microorganism. The most severe is typhoid fever, which is caused by *S. typhi*. This strain is host-adapted to humans, therefore transmission is due to fecal contamination of water and food. Symptoms include septicemia, high fever, headache, constipation, vomiting and diarrhea (Doyle and Cliver, 1990). Typhoid fever was cause for concern from late 1800s until 1950's in the US and Europe. Due to improvement in sewage disposal, water treatment and implementation of public health measures, this syndrome has become very rare and only occasional outbreaks are seen mostly in developing countries (Tauxe, 1991).

Another type of syndrome associated with *Salmonella* is enteric fever. Symptoms are similar to those of typhoid fever, but less severe. The enteric fever is caused by *S. paratyphi* A, B and C (Doyle and Cliver, 1990).

The most common type of salmonellosis is a gastroenteritis syndrome or food poisoning, caused by all the other types of *Salmonella*. Symptoms include diarrhea, abdominal pain, fever, vomiting, dehydration, chills and headache. The incubation period ranges from 5 to 72 h and the length of the illness is usually from 1 to 4 days (Doyle and

Cliver, 1990; Tauxe, 1991; Eley, 1992; Centers for Disease Control and Prevention, 1998b).

4.2.3. Pathogenesis.

The mode of transmission of *Salmonella* is mostly by ingestion of food contaminated with the microorganism. The most common foods implicated in outbreaks are meat products (especially poultry), eggs, and non-processed milk and dairy products (Eley, 1992; Centers for Disease Control and Prevention, 1998b). After ingestion of contaminated foods, the microorganisms attach to the epithelial cells of the villi in the terminal small intestine and colon. Once attached, they can penetrate the intestinal mucosa and invade the lamina propia and ultimately the lymphatic system. At the lamina propia the microorganisms multiply causing an inflammatory response. In cases of food poisoning, the infection remains localized in the intestinal area, but in cases of typhoid or enteric fever the bacteria progress further producing a systemic infection (Doyle and Cliver, 1990; Eley, 1992; Bauer and Hormansdorfer, 1996). In rare cases, *Salmonella* spp may cause urinary tract infections and septicemia that can lead to meningitis and osteomyelitis (Eley, 1992).

4.2.4. Epidemiology.

Salmonella was first discovered in 1885 by US Surgeon Daniel E. Salmon (Centers for Disease Control and Prevention, 1998b). Currently, over 2300 *Salmonella* strains have been identified. All these strains are pathogenic to human and/or animals, with the degree of virulence depending on strain and susceptibility of the individual infected. Virulence factors associated with *Salmonella* are formation of endotoxins, enterotoxins, cytotoxins, lipopolysaccharides and siderophores, and presence of flagellae and fimbriae (Gast and Beard, 1993; Bauer and Hordmansdorfer, 1996; Centers for Disease Control and Prevention, 1998b).

Since the 1960s *Salmonella* food poisoning has been on the rise. According to the Centers for Disease Control and Prevention (Centers for Disease Control and Prevention, 1998b), there are 40,000 cases of *Salmonella* reported every year. Todd (1996) calculated 40,000 annual cases in the US and 9,000 in Canada. It is also estimated that for each case reported there may be 30-100 cases that go unreported (Tauxe, 1991; Centers for Disease Control and Prevention, 1998b).

Susceptibility to *Salmonella* varies within different groups of the population. Young individuals are generally more susceptible than older ones. Infants of less than one month to six months old show the highest rate of infection, and about 40% of salmonellosis cases occur in children less than 5 years of age (Doyle and Cliver, 1990; Centers for Disease Control and Prevention, 1998b). The incidence of *Salmonella* also increases in individuals over 60 years old and in other immunocompromised groups of the population, especially patients with AIDS. This latter group shows a 20 fold increase in gastroenteritis and septicemia when compared with normal individuals (Tauxe, 1991; Eley, 1992). In addition, there seems to be a seasonal variation in the incidence of salmonellosis, with a maximum peak during summer. This could be attributed to an increase in temperature abuse of foods during these months (Centers for Disease Control and Prevention, 1998b).

4.2.5. Prevention and control.

An extensive review on methods for the detection of *Salmonella* in foods has been published by Blackburn (1993). These include culture techniques, measurements of metabolism (conductance and radiometry), immunoassays and the use of bacteriophages and gene probes. General measures for prevention of salmonellosis include thorough cooking of meats and pasteurization of milk and egg products, avoiding cross-contamination of foods, as

well as meticulous cleaning of kitchen utensils and personal hygiene. It is also important to promote education of food handlers and consumers, and improve hygiene at the farm and slaughter level to prevent contamination (Eley, 1992; Bauer and Hordmansdorfer, 1996; Centers for Disease Control and Prevention, 1998b).

A more novel approach is the use of irradiation to control foodborne pathogens, including *Salmonella*, and to extend shelf-life of food products. In 1992, the USDA approved the use of ionizing radiation for fresh or frozen, uncooked, packaged poultry carcasses and mechanically separated poultry products. The approved dose was a minimum of 1.5 kGy and a maximum 3.0 kGy (USDA-FSIS, 1992). In December 3, 1997, the Food and Drug Administration (FDA) approved irradiation for refrigerated or frozen uncooked meat, meat by-products and certain meat products (Federal Register, 1997). The maximum dose allowed was 4.5 kGy for refrigerated products and 7.0 kGy for frozen products. More recently, FSIS has proposed to amend the FDA final rule in meat and poultry products to make irradiation of poultry more consistent with meat regulations (Federal Register, 1999b). FSIS is proposing to approve irradiation of raw poultry products before packaging, as well as chicken carcasses. It also proposes to remove the minimum dose allowed. The use of irradiation in meat and poultry products can be a safe and effective measure to eliminate *Salmonella* from the food chain when used either as the only method or as a combination with other treatment such as heating or curing (Centers for Disease Control and Prevention, 1998b).

According to Todd (1996), an effective surveillance system at the national level is essential to understand and control foodborne diseases. In the US, government agencies routinely monitor the incidence of salmonellosis. The CDC collaborates with local and State Health Departments to research outbreaks and create control strategies. The FDA inspects

and regulates food products and processing plants. It also promotes the use of safer techniques to handle foods at the retail level. The FDA also regulates the use of antibiotics in animal feeds. The USDA supervises health of animal stocks, egg processing and monitors the quality and safety of slaughtered and processed meat. The US Environmental Protection Agency regulates the safety of the water supply (Centers for Disease Control and Prevention, 1998b).

In Europe, several approaches have been attempted to eliminate *Salmonella* from poultry and animal feeds. However, even with a very comprehensive program they still have an incidence of 3-4%, and reduction of *Salmonella* results in a very expensive product (four to five times the price of poultry in the US) (Doyle and Cliver, 1990; Gast and Beard, 1993). Chander et al. (1997) prepared a vaccine with irradiated *S. typhimurium* and *S. gallinarum*. Immunized chicks developed an immunoresponse and no *Salmonella* was isolated after 35 days, in comparison with control chicks that showed *Salmonella* contamination. The authors concluded that the use of vaccines may be used to raise *Salmonella*-free flocks. However, this vaccine was not tested under field conditions. In addition, the vaccine was administered subcutaneously. This mode of application would not be feasible in routine use. Current investigation includes testing of the vaccine under field conditions and development of a more appropriate method of administration.

4.3. TIME-TEMPERATURE INTEGRATORS

Thermal processing is a commonly used method for the preservation of foods throughout the world. The conditions of a thermal process are typically determined by the food product (composition, properties, consumer acceptability, and destination) and the thermal treatment applied. However, even with modern and sophisticated technology there is

a need to verify that the proper conditions have been achieved for each process. Methods to evaluate the accuracy of thermal processes are classified according to three different approaches: in situ methods, physical-mathematical methods, and time-temperature integrators (Hendrickx et al., 1995; Van Loey et al., 1996).

4.3.1. In situ methods.

In the in situ method, a safety or quality parameter is chosen and its concentration is measured before and after the treatment. Such parameters can be sensory factors, nutritional quality attributes or microbial contaminants. In practice, however, the determination of these parameters, especially after the treatment, can be very difficult, even impossible in some cases, because of the detection limits, analytical techniques and/or sampling requirements. Moreover, these type of methods are often unsuitable for routine monitoring due to their complexity (Hendrickx, 1995).

4.3.2. Physical-mathematical methods.

In the physical-mathematical approach, the impact of the heat treatment on the attribute of interest is estimated based on the time-temperature profile and the known kinetic parameters for such attribute. Limitations of these methods are due to the need for time-temperature data. In some cases, direct recording is not possible due to processing conditions; on the other hand, if the time-temperature history is reconstructed by mathematical modeling, the accuracy of the estimation by model parameters will limit the accuracy of the process evaluation. Conservative estimates used in these models often result in unrealistically severe heat treatment requirements which in turn lead to overprocessing, significant nutrient loss and reduction of quality of the product. In addition, with the introduction of new techniques such as ohmic and microwave heating, and the lack of data

for kinetic models for these techniques, the use of physical-mathematical methods becomes very limited. Sastry (1986) developed a mathematical model for aseptic processing of foods with particulates treated in a scraped surface heat exchanger. The author studied the influence of particle size, residence time distributions and estimated values of convective coefficients. The model was tested under different conditions but because of the lack of experimental data it could not be validated.

4.3.3. Time-temperature integrators (TTIs).

Although the concept of TTIs is not new, they were originally intended to monitor and control shelf life of food products (Taoukis et al., 1991; Singh and Wells, 1985). More recently TTIs have been introduced as an alternative for the monitoring of thermal processes. A TTI is defined as a small device that responds to time-temperature history by undergoing an irreversible and precisely measurable change in a manner that mimics the changes of a target attibute exposed to the same thermal history (Hendrickx et al., 1995). An important advantage of TTIs is that they allow fast and reliable validation of a process without the need for detailed information on the actual time-temperature profile within the product.

A reliable TTI should meet the following characteristics:

- simple and inexpensive in preparation
- easy to recover
- give an accurate and easy detection response
- readily incorporated into the food product without interfering with the heat transfer
- must quantify the process impact on the target attribute

To accomplish the last statement, a TTI must show the same time-temperature

dependent response as that of the target attribute when the temperature is the only rate-determining factor. Mathematically, this can be written as:

$$F_{Target} = F_{TTI} \tag{1}$$

Assuming an nth order TTI system, the rate equation can be expressed as:

$$dC/dt = - k C^n \tag{2}$$

where k is the reaction constant and n the reaction order. For a first order reaction under isothermal conditions, the integrated equation is:

$$ln\ (C_0/C) = kt \tag{3}$$

where C_0 is the initial value of the target attribute and C is the value of a target attribute at a time t. For a non-first order reaction (n^{-1}), the integrated expression can be written as:

$$[1/(n-1)]\ (C^{1-n} - C_0^{1-n}) = kt \tag{4}$$

In many cases, according to the Arrhenius equation the rate constant can be expressed as:

$$k = k_0\ exp\ (-E_A / RT) \tag{5}$$

where k_0 is the pre-exponential factor, E_A is the activation energy, R is the universal gas constant and T is the absolute temperature.

In the general case where the reaction order is different than unity, with the reaction rate constant and independent of the order of reaction, the F value can be written as (Hendrickx et al., 1995):

$$(F_{Tref})_x = \int_0^t \exp \frac{E_A}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right) dt \tag{6}$$

which represents the equivalent heating time at a reference temperature resulting in the same lethality as the time-varying temperature profile. This equation is valid for the target attribute as well, and therefore only when the activation energies of both TTI and target attribute are

the same, equation (1) will be satisfied. In other words, for a system to be used as TTI it must have identical activation energy to that of the attribute of interest. Mathematically this can be written as:

$$E_{ATTI} = E_{ATarget} \tag{7}$$

Since z values are equivalent to activation energies, equation (7) can also be expressed as:

$$Z_{TTI} = Z_{Target} \tag{8}$$

TTIs can be classified according to their working principle (biological, chemical or physical), type of response (single or multi), origin (intrinsic or extrinsic to the food product), application in the food material (dispersed, permeable or isolated) and location in the food (volume average or single point) (Van Loey et al., 1996). Most currently existing TTIs are based on microbiological or enzymatic assays. Microbiological TTIs mainly employ "calibrated" (i.e. they have been validated against a known standard) microorganisms or spores. A thorough review on bacteriological evaluation for thermal process design has been published by Yawger (1978).

Microbial spores can be used to monitor thermal processing using either a survivor curve or an endpoint method. In a survivor curve or "count reduction procedure", the number of microorganisms surviving the heat treatment are related through a calibration curve to obtain a sterilizing value. Usually, ten or more cans are inoculated with 30-50 million spores of a heat-resistant microorganism. A series of thermal treatments of varying time or temperature are applied. The initial population is calculated from a nonprocessed container and survivor counts are obtained from each processed can. The D value for the microorganism is calculated for the series of treatments tested in the assay (Yawger, 1978;

Pflug et al., 1980).

In the endpoint procedure, also called quantal response, several units are subjected to each treatment. After incubation, each unit is tested for either growth or no growth, and the number of survivors is determined from the quantal response. An example of endpoint method is the inoculated pack system (Yawger, 1978). This system consists of a series of cans (usually 100) which are inoculated with a definite number of spores of known resistance. This method is generally used to validate a calculated food sterilization treatment.

The main disadvantages of microbiological TTIs are assay time, because of the microorganism incubation period, and the likelihood of contamination, as well as cost, labor and the need to properly calibrate the spores. In addition, if the initial population of microorganisms is completely destroyed during processing, the actual lethality derived from the heat process cannot be determined. Pflug et al. (1980) studied the performance of *Bacillus stearothermophilus* spores in measuring the sterilization process in cans of food heated in a Steritort process simulator. The spores, packed in a carrier system (plastic rods called biological indicator units or BIUs) and previously calibrated at different temperatures, were placed inside the food cans. After processing, the BIUs were assayed for surviving spores. Cans containing thermocouples were processed in the same fashion. F values were calculated from both the BIUs and thermocouple-equipped cans and compared. The authors found a good agreement between the F values calculated with both methods. However, because the z value of the spores employed in this study (7.8°C) differed from the target z value (10°C), a correction method had to be applied to make both F values comparable. Furthermore, in F values calculated with the spores, an additional correction had to be made to account for heating and cooling lags of the BIUs due to differences in temperature across the BIU wall.

The authors concluded that the accuracy of the method, even with proper calibration, was within 15% of the true F value. Sastry et al. (1988) developed a *B. stearothermophilus* spores-based bioindicator for verification of thermal processes in particulate foods. Individual mushrooms were infused with alginate gels containing a spore suspension, and processed. Although the authors concluded that the bioindicator was potentially useful as an indicator of sterility in continuous aseptic systems, the main problem was that they could not provide conclusive proof of negligible leakage of the spores into the product during the thermal process.

Different enzymes, mainly from microbial origin, have also been proposed as TTIs. Weng et al. (1991a) investigated the thermal behavior of peroxidase that had been modified chemically and physically, and studied its application as a bioindicator for thermal processing. The z value of peroxidase was modified from 26.3°C to 14.1°C by immobilization of the enzyme in glass beads. Furthermore, the z value was lowered to 11.1°C by modification of the environment with organic solvents. In a different study, Weng et al. (1991b) verified the use of the immobilized peroxidase in a dodecane environment as an indicator for pasteurization processes. The peroxidase system had a z value of 10.1°C. When evaluated at processing temperatures between 70 and 80°C, the lethalities of the indicator agreed with those calculated according to the Bigelow's General Method. In a related study, Hendrickx et al. (1992a) studied the thermal denaturation of peroxidase as a function of water activity. Lyophilized horseradish peroxidase was equilibrated over standard salt solutions to achieve a water activity between 0.11 and 0.88. The enzyme was much more thermostable in the dry state than in aqueous solution, and both D and z values changed with water activity. Inactivation temperatures were in the range 140-160°C at low water activities (compared to

70-85°C in aqueous solution). Later, Hendrickx et al. (1992b) studied the application of immobilized peroxidase as an indicator in a can model system under pasteurization conditions. Plastic spheres containing peroxidase were placed in a can and processed at 85°C. The lethalities calculated by integration of the resulting time-temperature profiles coincided very well with those read from the peroxidase system. The authors suggested that the validated bioindicator could be encapsulated in a small vial and used to calculate lethality in the center of the unit.

DeCordt et al. (1992a) studied the application of the enzyme α-amylase as a TTI in thermal processes. The group studied the thermal inactivation kinetics of α-amylase from *Bacillus licheniformis* in the temperature range 90-108°C. They also looked at the influence of immobilization, pH, ionic strength, Ca^{2+}, and concentration of enzyme as effective means to manipulate the thermal stability of the enzyme. The D values at 95°C differed between free and covalently immobilized enzyme. Extrinsic Ca^{2+} conferred stability, but there was a saturation level after which the enzyme was destabilized. The optimum pH to lower thermal inactivation rates was found at pH of 8.5. Also, increasing the concentration of the enzyme resulted in higher thermoresistance. The authors concluded that with manipulation of the environment, the immobilized enzyme showed potential to be used as a TTI for monitoring thermal inactivation of *Clostridium botulinum* spores in food.

In a related paper, DeCordt et al. (1992b) tested biphasic and nth-order models to fit experimental inactivation data of *B. licheniformis* α-amylase immobilized on glass beads. Both isothermal and non-isothermal experiments were used to estimate model parameters (E_A, k and n) using a non-linear regression procedure. The authors calculated an E_A of 293 kJ $mole^{-1}$ for the system, which fell within the range of that of *C. botulinum* (265-340 kJ $mole^{-1}$).

They concluded that the system had potential to be used as a TTI in thermal processes in the temperature range of 96-108°C.

The general effects of polyols and carbohydrates on thermal denaturation kinetics of α-amylase were also studied (DeCordt et al., 1993). The results showed that all polyols (glycerol, mannitol and sorbitol) and carbohydrates (starch and sucrose) tested were powerful thermostabilizers, at least at the temperature range used in the study.

DeCordt et al. (1994) studied the thermostability of α-amylase and peroxidase using differential scanning calorimetry (DSC). DSC peak temperature was used as a measure of protein stability. The thermal inactivation kinetics of α-amylase from two *Bacillus* spp and horseradish peroxidase were determined as a function of the concentration of glycerol, sorbitol and sucrose. *B. amyloliquefaciens* α-amylase was very stable in presence of either polyols and carbohydrate or combination of both, and inactivation temperatures were as high as 127°C. The authors suggested that the use of DSC peak area could be used as a TTI-response.

Maesmans et al. (1994a) conducted a theoretical study of the possibilities of combined mathematical model and TTI to monitor fluid-to-particle convective heat transfer coefficient (CHTC). The authors concluded that although, theoretically, the combined use of both could be indeed suitable to determine the CHTC, in practice, however, there is a need to very carefully examine experimental design considerations. These considerations are specific for each processing condition, TTI employed and type of material, therefore making the combined use of a mathematical model and TTI a rather cumbersome methodology. Also, Maesmans et al. (1994b) evaluated the efficacy of an α-amylase-based TTI to monitor the spatial distribution of thermal processing values in a food model system using a pilot retort.

The TTI in this particular case was used to determine particle-to-particle variation in processing values. One question that was raised was whether the use of two TTIs, characterized by different z values, could determine the "coldest point" at the same position when applied in the same heat process. The authors suggested that results from a TTI should not be extrapolated to another TTI with a different z value without experimental verification. Furthermore, the authors concluded that for an enzyme system to be used as a TTI for the monitoring of heat distribution in foods, it is critical to calibrate the system in terms of the kinetic inactivation parameters (D-z or k-E_A values).

Van Loey et al. (1997a) tested the efficacy of a *B. amyloliquefaciens* α-amylase-based TTI to evaluate in-pack lethalities in a pasteurized food model system. Thermal inactivation of the enzyme was measured as a function of reaction enthalpy using DSC. The reaction enthalpy followed a log-linear reduction at constant temperature. Therefore, a first order reaction was assumed and a z value of 7.6°C was obtained. When tested in a heat processed food model system, the processing values determined from the TTI read-out coincided very well with the actual integrated processing values calculated using the general method, suggesting that this enzyme could be used to monitor pasteurization treatments (up to 90°C). Van Loey et al. (1997b) studied the heat inactivation kinetics of *B. subtilis* α-amylase under steady and non-steady conditions by following enthalpy changes associated with the thermal denaturation of the enzyme. When equilibrated at a water activity of 0.76, the enzyme had a z value of 9.7°C. Because of the similarity to the z value (10°C) of *C. botulinum*, the authors suggested that this enzyme system could be used to monitor the efficacy of sterilization treatments in foods.

Only very few methods based on either chemical or physical indexes are currently

available. The first chemical markers that were studied were compounds in foods for which analytical methods were already established, such as thiamin, pantothenic acid, vitamin C and methylmethionine sulfonium salt (Kim and Taub, 1993). The limitations of most of these methods is the need for additional steps (recovery, post treatment assay) after the thermal process which results in lengthy procedures and fairly technical manipulations (Van Loey et al., 1996). Kinetically, other challenges have to be faced when dealing with chemical TTIs: first, the rate constant of a chemical reaction is usually smaller than that of microbial destruction; secondly, the z values are usually greater (the z value for thiamin is 48°C while the one for *C. botulinum* is 10°C) (Mulley et al., 1975).

According to Kim and Taub (1993), compounds formed during food reactions show more potential to be used as TTIs, since one could determine the gain in concentration of such product as the heat process takes place. However, it is not easy to find such a thermally produced compound that can be easily assayed and whose concentration after the process can be used to verify sterility. Kim and Taub (1993) identified and characterized three chemicals that showed potential to be used as TTIs at aseptic processing temperatures. They reasoned that, since carbohydrates are present in mostly all foods, changes in carbohydrate profile could give an indication of potential markers. To screen for the compounds, they first monitored formation of chemicals by following changes in UV spectra in different food products, including meats, fruits and vegetables. They observed formation of three different compounds (depending on the food product) which they called M-1 (found in heated meats, fruits and vegetables), M-2 (found primarily in heated meats) and M-3 (found in heated fruits and vegetables), and identified (or partially identified) both the markers and their precursors: M-1 (2,3-dihydro-3,5-dihydroxy-6-methyl-(4H)-pyran-4-one) and M-3

(5-hydroxymethylfurfural) are products from the degradation of D-fructose, while M-2 was associated with protein. Once identified, the authors proceeded to study the kinetic parameters of these compounds. They established a linear relationship between M-1 concentration and the sterility index, F, using a non-isothermal process, and developed a model relating microbial destruction to marker formation. They concluded that "preliminary experiments conducted with inoculated samples processed in a commercial facility have indicated that the model is basically valid". However, they acknowledged the need to do more studies using a more controlled time exposure over a wider range of temperatures to validate the model and to calculate the actual precision with which the F value of the thermal process could be determined.

All the methods reviewed above are either very theoretical and need further validation, or they require expensive equipment, tedious calculations and cannot be applied in routine testing. There is a need to develop TTIs that are easily recoverable and give a response that is quickly measured.

Traditionally, the thermal destruction of microorganisms has been assumed to follow first-order kinetics (Kormendy and Kormendy, 1997; Peleg and Cole, 1998). In addition, methods for estimating the safety of commercial thermal processes are also based on this assumption (Peleg and Cole, 1998). Upon closer observation, however, some of the survival curves show a slight curvature. Whiting (1993) explained that, in some cases, the presence of a subpopulation of more heat-resistant bacteria (i.e. inactivation rate is slower than the rest of population) could account for the tailing of the survival curve. Peleg and Cole (1998) fitted previously published survival curves of several pathogenic microorganisms using a mathematical model. They demonstrated that, in all cases, the heat inactivation rate,

previously considered linear, was different from unity. In any case, deviation from linearity may have an impact on calculation of decimal reduction times, which are determined from the linear regression of number of survivors vs time at a given temperature (Singh and Heldman, 1993; Peleg and Cole, 1998). This could result in over- or underestimation of the actual microbial destruction. In the context of food processing, one is more concerned with underestimation, which can lead to foodborne diseases. Similar behavior has been observed in thermal inactivation of certain enzymes. Horseradish peroxidase (Weng et al., 1991a and b; Hendrickx et al., 1992a and b; Saraiva et al., 1996; Garcia et al., 1998), acid phosphatase (Incze et al., 1999), and *Bacillus* spp. α-amylase (DeCordt et al, 1992a and b, 1993, 1994a and b; Van Loey et al., 1995b, 1997a and b), among others, do not follow first-order kinetics. The presence of two or more isoforms of the enzyme showing different thermostability has been used to explain the reason of this behavior. The development of a TTI that mimics the heat destruction of a target attribute (microorganism or enzyme), regardless of the inactivation rate of such attribute, could be used for safe monitoring of thermal processing in foods.

4.4. PHYCOBILIPROTEINS

Phycobiliproteins are photosynthetic antenna pigments that provide the characteristic colors to cyanobacteria, red algae and cryptomonads. They are located on the cytoplasmic face of the thykaloid membrane of the chloroplasts (Glazer, 1989), forming distinctive macromolecular structures called phycobilisomes. Their function is to harvest solar energy in regions of the visible spectrum that show low chlorophyll absorption and then transfer this excitation energy to chlorophyll in the photosynthetic membrane. There are three major phycobiliproteins: phycoerythrin, phycocyanin and allophycocyanin, each one of them

showing specific absorption and fluorescence patterns (Table 4.1) which will vary with species (MacColl and Guard-Friar, 1987).

The phycobiliproteins are naturally pigmented due to the presence of linear tetrapyrrole chromophores, called bilins, which are covalently attached to the apoprotein. These chromophores are not complexed with metal ions and can be easily manipulated by the apoprotein to produce the specific biological characteristics. There are two major

Table 4.1. Absorption and fluorescence characteristics of phycobiliproteins.

Phycobiliprotein	Color	Absorption bands (nm)	Fluorescence Emission bands (nm)
R-phycoerythrin	Red	498-568	570-620
Phycocyanin	Blue	550-630	630-650
Allophycocyanin	Blue	598-680	660-680

chromophores, phycoerythrobilin and phycocyanobilin and at least one of them is present in all phycobiliproteins. These two chromophores are isomeric, with phycocyanobilin having one more double bond than phycoerythrobilin (MacColl and Guard-Friar, 1987). In addition, there are three minor bilins: phycourobilin, criptoviolin and the 697-nm bilin, which usually occur together with one of the major chromophores. The unique spectral properties of each phycobiliprotein depend on the native structure of the polypeptide. Alteration of this native conformation, as by heat or chemical denaturation, will result in partial or complete loss of the optical properties (Ogawa et al., 1991; Creighton, 1993).

R-phycoerythrin (R-PE) contains three different polypeptide chains: α, β, and γ or γ'. At pH near neutrality, these subunits are usually organized as $(\alpha\beta)_6\gamma$ or $(\alpha\beta)_6\gamma'$ complexes (D'Agnolo et al., 1994). Each α and β subunit contains two and three phycoerythrobilins, respectively, while the γ subunit has two phycoerythrobilins and two phycourobilins attached (Glazer, 1981). Thus, each complex contains a total of thirty-four chromophores. O'hEocha (1965) and Fujimori and Pecci (1967) suggested that these chromophores are located in hydrophobic pockets of the protein molecule. Two types of phycoerythrobilin chromophores, donors and acceptors, have been identified. The donors absorb at shorter wavelengths (540-555 nm) exciting the acceptors that absorb and emit at longer wavelengths (565-570 nm) (O'hEocha and O'Carra, 1961; Teale and Dale, 1970). Teale and Dale (1970) speculated that donor and acceptor chromophores are adjacent to each other. Studies of the conformation and configuration of phycocyanobilin, a tetrapyrrole found in phycocyanin, revealed that the preferred conformation of the chromophore in solution is cyclic-helical, while it is extended in the native protein (Rudiger, 1992). Hence, only when the apoprotein is in its native conformation does the tetrapyrrole adopt the extended configuration. Cyclization of the phycocyanobilin results in lower visible and higher near-UV absorbance than an extended conformation (Burke et al., 1972). The spectroscopic properties of R-PE depend on the native structure of the molecule and the interaction among chromophores (O'hEocha and O'Carra, 1961; Teale and Dale, 1970; MacColl and Guard-Friar, 1987). Alterations of the native conformation will result in partial or complete loss of the optical properties (O'hEocha and O'Carra, 1961; MacColl and Guard-Friar, 1987; Ogawa et al., 1991).

Our source of R-PE is the red alga *Porphyra yezoensis*, an edible seaweed that is

consumed in many Asian countries in the form of "nori", a characteristic ingredient of "sushi" (Merrill, 1993). Under neutral conditions, R-PE has a characteristic absorption spectrum showing two maximum peaks at 498 and 565 nm, and a shoulder at about 540 nm. The extinction coefficient has been estimated to be 8.51 mL mg^{-1} cm^{-1} at 565 nm (Merrill, 1985). In fluorescence measurements and under neutral conditions, R-PE shows excitation and emission maxima at 493 and 578 nm, respectively.

4.5. PRINCIPLES OF FLUORESCENCE SPECTROSCOPY

The measurement of fluorescence is a potent and useful technique to obtain information about the structure, dynamics, location and motion of molecules (Lakowicz, 1983; Glazer and Wells, 1998). Fluorescence occurs when a fluorescent molecule (fluorophore) absorbs light (excitation) and subsequently releases it. The emitted light has lower energy than the absorbed light because some of the excitation energy is used in such dynamic processes as vibrations, rotations and collisions within the molecules (Glazer and Wells, 1998). This process is very fast, usually in the range of nanoseconds.

Depending on the type of fluorescence measurement, one can obtain different information on the fluorophore, or the molecule containing such fluorophore. For example, study of the excitation and emission spectra can be used to identify the fluorophore in solution (Lakowicz, 1983). The determination of the fluorescence lifetime can provide information about the fluorophore dynamics in the molecular environment (Glazer and Wells, 1998). Another practical tool is the measurement of fluorescence anisotropy. With the use of polarized light, one can selectively excite fluorophores that show a particular orientation in solution. This will result in a partial polarized fluorescence emission. The extent of polarization can be related to the rotational rate of the fluorophore, and, this, in turn, can be

used to study the hydrodynamic properties of macromolecules (Lakowicz, 1983). The fluorescence efficiency or quantum yield is the ratio of photons emitted to the photons absorbed. The quantum yield is greatly affected by alterations in the microenvironment of the fluorophore and, thus, can be used to monitor changes within the molecules (Lakowicz, 1983).

The main advantages of fluorescence over other colorimetric methods are its high sensitivity and specificity. However, fluorescence is greatly dependent on environmental factors such as temperature, pH, ionic strength, viscosity, etc. Hence, these factors should be carefully monitored and controlled when using fluorescent techniques (Guibault, 1988; Glazer and Wells, 1998).

CHAPTER 5: Effect of pH on the thermal inactivation of R-phycoerythrin from *Porphyra yezoensis*

5.1. ABSTRACT

R-phycoerythrin (R-PE), a protein which fluoresces in the visible range, was purified from fresh and dried cultures of *Porphyra yezoensis* by precipitation with ammonium sulfate and gel permeation chromatography. The isolated R-PE had a purity greater than 5 as determined by A_{565}/A_{280}. The effect of pH on spectral properties and thermal inactivation of R-PE was examined. Fluorescence was stable between pH 5.0 and 9.0 but decreased at pH 4.0 and 10.0. Complete loss of fluorescence was observed at pH 3.0 and below, and pH 11.0 and above. Thermal inactivation parameters calculated on the basis of fluorescence loss were obtained in the pH range 4.0-10.0, at the 60-90°C temperature range. Maximum thermostability was observed in pH 6.0 (D value at 70°C =12,258 min), while R-PE was least thermostable in pH 10.0 (D value at 70°C =1.10 min). The z values ranged from 4.44°C at pH 10.0 to 9.15°C at pH 9.0. These results are of particular significance in the area of protein-based time-temperature integrators for monitoring thermal processes in food products. By modifying the thermal kinetics of R-PE to match that of a target microorganism in a thermal food process, it could be used as a time-temperature integrator to predict destruction of such microorganism during cooking.

5.2. INTRODUCTION

Thermal processing is the most common method used for food preparation and preservation. The conditions of a heat process are usually determined by the type of food

product and the specific thermal treatment applied. Mathematically, the impact of a thermal process can be expressed in terms of D and z values (Singh and Heldman, 1993; Hendrickx et al., 1995). D value, or decimal reduction time, is the time in minutes required to decrease a quality attribute or microbial population by 90% at a constant temperature. The z value is the temperature increase necessary to reduce the D value by 90%. The determination of D and z values for a particular microorganism is very useful in designing a thermal process that targets that specific microorganism (Pflug, 1997). Sterilization processes of canned foods are designed to obtain a 12D reduction in *Clostridium botulinum* (Plug and Odlaug, 1978). The times and temperatures used for milk pasteurization were based on thermal destruction of Mycobacterium tuberculosis (Kay and Graham, 1933). Processing schedules required by the US Department of Agriculture for roast beef and hamburger patties were based on thermal inactivation of *Salmonella* (Goodfellow and Brown, 1978) and *Escherichia coli* O157:H7 (Doyle and Schoeni, 1984; Line et al., 1991), respectively.

Foodborne outbreaks associated with underprocessed food products have prompted the search for methodology to ensure adequate thermal food processing. The evaluation of thermal processes in foods can be done using in situ methods, physical-mathematical models or time-temperature integrators. Time-temperature integrators (TTIs) can be used to predict the time-temperature response of a quality or safety index in a food product. This occurs when the thermal destruction of both TTI and target index are identical ($z_{TTI} = z_{target}$) (Hendrickx et al., 1995; Van Loey et al., 1996).

Most currently existing TTIs are based on microbiological or enzymatic assays. Microbiological TTIs mainly employ vegetative cells or spores from thermophilic bacteria (Yawger, 1978; Pflug et al., 1980; Sastry et al, 1988). The main disadvantages of

microbiological TTIs are the length of assay because of the microorganism incubation period and the likelihood of contamination, as well as cost, labor and the need to properly calibrate the spores.

Different enzymes, mainly from microbial origin, have also been proposed to be used as TTIs. The applicability of these enzymes as TTIs lies on the modification of their inactivation kinetics to match those of specific microorganims targeted in the thermal process (Hendrickx et al., 1995).

R-phycoerythrin (R-PE), one of the major phycobiliproteins, is a protein that fluoresce in the visible range and is found in cyanobacteria, red algae and cryptomonads. The red pigmentation of R-PE is due to the presence of linear tetrapyrrole chromophores, called bilins, which are covalently attached to the apoprotein through one or two thioether bonds (MacColl and Guard-Friar, 1987; Sidler et al., 1989). R-PE contains two different types of chromophores, phycoerythrobilin and phycourobilin (MacColl and Guard-Friar, 1987). The apoprotein is formed by three different polypeptide chains: α, β, and γ or γ'. At pH near neutrality, these subunits are usually organized as $(\alpha\beta)_6\gamma$- or $(\alpha\beta)_6\gamma'$-complexess (D'Agnolo et al., 1994). Each α and β subunit contains two and three phycoerythrobilins, respectively, while the γ subunit has two phycoerythobilins and two phycourobilins (Glazer, 1981). Thus, each complex contains a total of thirty-four chromophores. O'hEocha (1965) and Fujimori and Pecci (1967) suggested that these chromophores are located within hydrophobic regions of the protein molecule. There are two types of phycoerythrobilin chromophores that have been classified as donors and acceptors. Teale and Dale (1970) suggested that there must be a close proximity between donor and acceptor chromophores. The unique spectral properties of R-PE depend on the native structure of the polypeptide and interaction among the different

chromophores (O'hEocha and O'Carra, 1961; Teale and Dale, 1970; MacColl and Guard-Friar, 1987). Alteration of this native conformation, as by heat or chemical denaturation, will result in partial or complete loss of the optical properties (O'hEocha and O'Carra, 1961; MacColl and Guard-Friar, 1987; Ogawa et al., 1991). The amino acid sequence of R-PE subunits in *Porphyridiun cruentum* (Sidler et al., 1989), *Rhodella violacea* (Ficner et al., 1992) and *Aglaothamnion neglectum* (Aptt et al., 1993) has been elucidated. No evidence of disulfide bonds in R-PE molecules has been found.

The main objective of this project was to study the effect of pH on the thermal inactivation parameters of R-PE. By modifying the environmental conditions, the inactivation kinetics of R-PE could be adjusted to match those of target indicators in thermal food processes.

5.3. MATERIALS & METHODS

5.3.1. Source of R-phycoerythrin.

R-phycoerythrin (R-PE) was purified from pure cultures of *Porphyra yezoensis* Ueda sporophyte phase (conchocelis), strain U-51 (inoculum kindly provided by Dr. A. Miura, Tokyo University of Fisheries, Tokyo, Japan). Alternatively, R-PE was also purified from dried whole *P. yezoensis gametophyte thalli* provided by the Tagawa Fisheries Supply Company and the Shin-Futtsu Nori Cooperative, Shin-Futtsu, Japan. The extraction and purification procedure was based on methods in Merrill (1985) and are summarized in Figure 5.1.

5.3.2. Purification of R-phycoerythrin

5.3.2.1. Procedure for fresh algal tissue:

Algal tissues were homogenized with cold 50 mM NaCl, 50 mM phosphate buffer,

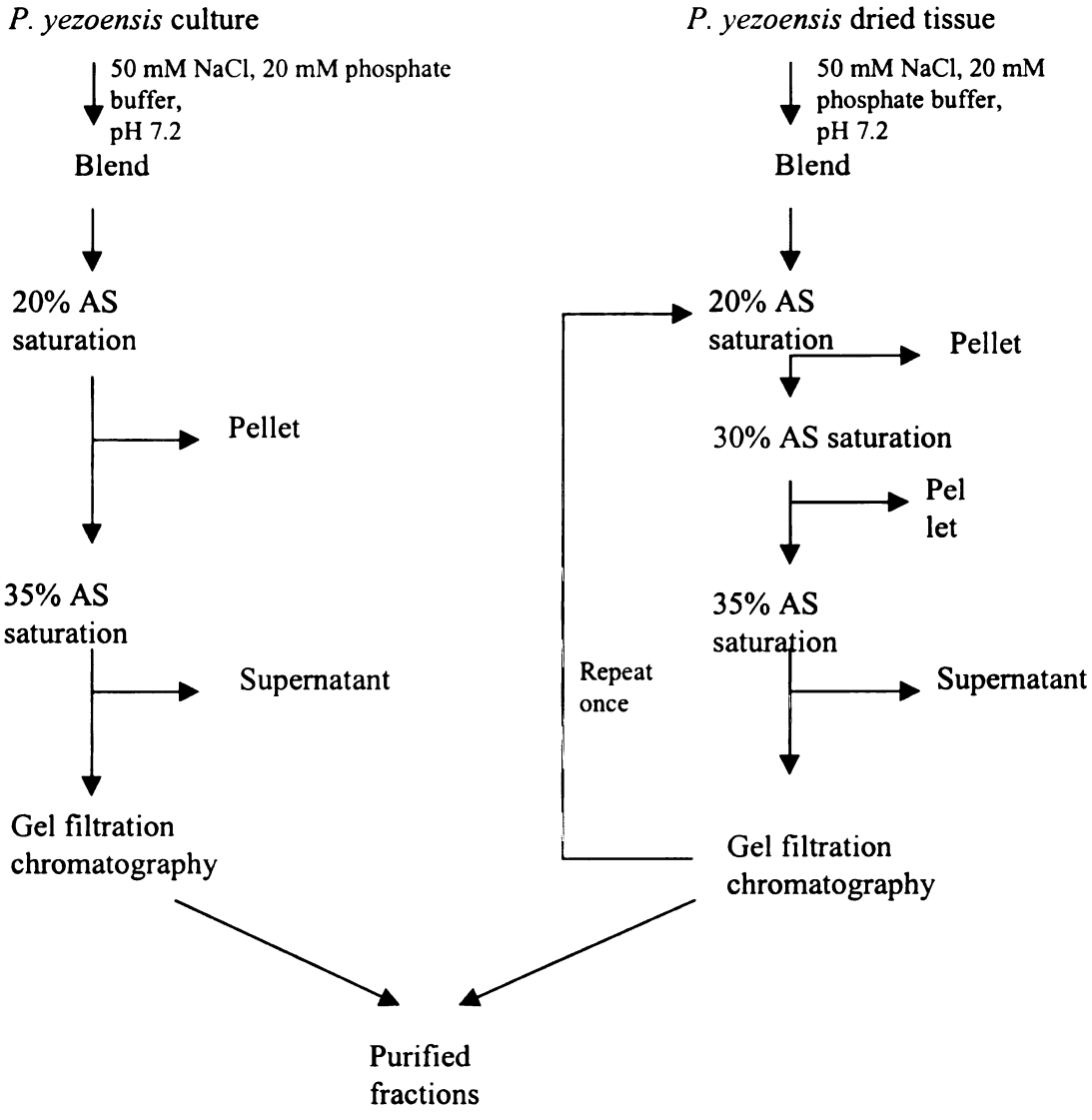


Figure 5.1. Flow diagram for the purification of R-phycoerythrin from *P. yezoensis*. All steps were performed at 4°C.

pH 7.2 (PBS) in a Bead-Beater® (Biospec Products, Bartlesville, OK). The algal suspension was homogenized in four bursts of 30s, followed by cooling periods of 5 min. The resulting homogenate was decanted and centrifuged at 6,000 x g for 15 min at 4°C, to eliminate cell debris. After centrifugation, the extract was brought to 20% saturation with solid ammonium sulfate. After stirring for 1h at 4°C, the suspension was centrifuged at 10,000 x g for 15 min at 4°C. The resulting supernatant was collected and brought to 35% ammonium sulfate saturation. After stirring for 1h at 4°C, the suspension was centrifuged at 10,000 x g for 15 min, at 4°C. The resulting pellet was suspended in 25 mL of PBS. This suspension (10 mL) was loaded onto a 26 x 100 mm Sephacryl S-300 gel filtration chromatography column (Pharmacia, Piscataway, NJ) equilibrated with PBS. Loaded volumes were calculated so as not to exceed 5% of total column bed volume. The column was eluted at a 0.4 mL/min flow rate. Two-milliliter fractions were collected and absorbances at 280 and 565 nm measured. A ratio of $A_{565}/A_{280} > 5$ was established as the standard for purified protein (Gantt, 1969). Fractions showing a $A_{565}/A_{280} > 5$ were pooled, brought to 60% ammonium sulfate saturation and stored at 4°C.

5.3.2.2. Procedure for dry algal tissue:

Dried tissue (5g) was soaked overnight at 4°C in 100 mL of PBS. On the following day, the tissue was extracted using the Bead-Beater® as explained above. The glass beads were rinsed with PBS until a total volume of 1L homogenate was collected. The homogenate was centrifuged at 6,000 x g for 15 min at 4°C, to eliminate cell debris. After centrifugation, the extract was brought to 20% saturation with solid ammonium sulfate. After stirring for 1h at 4°C, the suspension was centrifuged at 10,000 x g for 15 min at 4°C. The same procedure was repeated for 25, 30 and 35% ammonium sulfate saturation. The resulting pellet was

suspended in 90 mL of PBS. The final suspension (15 mL) was loaded onto a 26 x 100 mm Sephacryl S-300 gel filtration chromatography column (Pharmacia) equilibrated with PBS. The column was eluted at a 0.35 mL/min flow rate. Fractions (2 mL) were collected and absorbances at 280 and 565 nm measured. Fractions showing a $A_{565}/A_{280} > 5$ were pooled, brought to 60% ammonium sulfate saturation and stored at 4°C.

5.3.2.3. Purity and yield of extracted R-phycoerythrin

Purity was also confirmed by native polyacrylamide gel electrophoresis (PAGE) (Davis, 1964) using a 4% stacking and a 10% resolving gel. Gels were developed using Coomassie Blue R-250 (Bio-Rad, Hercules, CA).

Absorption spectra of R-PE extracted with each procedure were identical. Fractions were pooled together into what was designated the R-PE stock solution and stored in 60% ammonium sulfate at 4°C. The final solution had a concentration of 0.31 mg/mL and a purity of 5.2 as measured by the ratio A_{565}/A_{280}. Average yield was estimated to be about 1% of the fresh weight.

5.3.3. Determination of molecular weight and isoelectric point.

Sodium dodecyl sulfate (SDS) PAGE (Laemli, 1970) was performed to estimate the molecular weight of the R-PE subunits using a 4% stacking and 14% resolving gel (Mini-Protean II, Bio-Rad, Hercules, CA). Molecular weights were determined by comparing relative mobility of protein bands to those of prestained molecular weight standards (Kaleidoscope Polypeptide Standard ~6.9 - 205 kDa, Cat. No. 161-0324, Bio-Rad) according to the method of Weber and Osborn (1969). Gels were developed using Coomassie Blue R-250 and/or a Silver stain kit (Bio-Rad).

The isoelectric point (pI) of R-PE was determined by isoelectric focusing in a vertical

polyacrylamide minigel system (Model SE250/260, Hoefer, Piscataway, PA) using ampholyte pH range 4.0-7.0 (Sigma Chemical, St. Louis, MO) (Robertson et al., 1987). Isoelectric focusing was performed at constant voltage (200 V) for 2 h. Gels were developed using Coomassie Blue R-250 (Bio-Rad). The pI was determined by comparing relative mobility of protein bands to those of protein standards (pH 3.6-6.6, Sigma) run on the same gel.

5.3.4. Absorbance and fluorescence measurements.

Absorption spectra was determined using a Lambda 20 UV/Vis Spectrometer and the UVWinlab Version 2.0 software (Perkin Elmer Corporation, Norwalk, CT). Fluorescence spectra of R-PE and fluorescence intensity at the emission maximum were determined using a computer enhanced SLM-4000 fluorometer (SLM Instruments, Rochester, NY). A scan of maximum excitation and emission spectra was performed for each solution. For thermal inactivation experiments, excitation was set at 493 nm for all pH conditions, while emission was recorded in the 578 and 581 nm, depending on pH.

5.3.5. Effects of pH on thermal inactivation of R-PE.

To test the effect of pH, R-PE from the stock solution was dialyzed against water at 4°C overnight. The dialyzed R-PE was diluted immediately prior to the experiments in 20 mM buffers containing 50mM NaCl, ranging from pH 3.0 to 12.0, to achieve an absorbance of 0.1 at 565 nm. Buffers employed were: citrate (pH 3.0, 4.0 and 5.0, $pKa_1 = 3.13$, $pKa_2 = 4.45$), phosphate (pH 6.0, 7.0 and 12.0, $pKa_2 = 6.88$, $pKa_3 = 12.38$), tris(hydroxymethyl)amino-methane (TRIS) (pH 8.0 and 9.0, pKa = 8.2), and glycine (pH 10.0, pKa = 9.78). The pH of R-PE in TRIS buffer changed no more than 0.3 units at the temperature used.

The dialyzed R-PE solution was diluted in each buffer to achieve an absorbance of 0.1 at 565 nm, and 2 mL transferred to 10 x 75 mm test tubes (14-961-25, Fisher Scientific, Pittsburgh, PA). The tubes were sealed with Teflon(tape and allowed to equilibrate at 4°C. The test tubes were placed in a wire rack and immersed in a Polystat circulator bath (Model 1268-52, Cole-Parmer Instrument Company, Chicago, IL) connected to a bath programmer (Model 1268-62, Cole-Parmer). Temperatures ranged from 60 to 90°C. The water bath temperature was set 0.2°C above the target temperature to allow R-PE solution to reach target temperature. Temperature in the test tubes was monitored using a thermocouple (Resistance Temperature Detector Probe, Pt 100, 0.15 cm diameter, (1°C accuracy) inserted in a control test tube containing 2 mL of the same solution. The thermocouple was connected to a Solomat MPM 200 Modumeter (Solomat Partners LP, Stanford, CT). Zero time was defined as the time when the R-PE solution reached the target temperature. Preliminary studies were performed to calculate the times and temperature necessary to achieve at least a 1 log reduction in fluorescence. The tubes were removed at predetermined intervals of time and immediately placed in an ice bath, then kept at 4°C until fluorescence was read within 12 h. Preliminary studies were performed to show that no changes of fluorescence occurred during this holding period. Each thermal inactivation study was done in triplicate.

5.3.6. Calculation of D and z values.

Fluorescence counts were converted to logarithms. D values (in minutes) were calculated by linear regression of fluorescence vs time using Microsoft Excel Version 5.0a (Microsoft Corporation, Redmond, WA). Thermal resistance curves were determined by plotting log of D values vs temperature. The z values were determined as the negative reciprocal of the slope of the thermal resistance curve.

Statistical differences among z values were tested using one way analysis of variance (SAS Institute Inc., 1995). Mean z values were compared using Tukey-Kramer HSD test with the mean square error at the 5% level of probability.

5.4. RESULTS & DISCUSSION

5.4.1. Determination of molecular mass and pI.

On SDS-PAGE, R-PE migrated as two bands with molecular masses of 21.2 and 38.3 KDa, corresponding to α and β (resolved as a single band) and γ subunits, respectively. Merrill (1985) reported molecular masses of 20-21 KDa for α and β, and 30 KDa for γ subunits of R-PE in *P. yezoensis*. Glazer and Hixson (1977) reported values of 17.5 KDa for α and β and 30.2 KDa for γ in R-PE from *P. cruentum*. D'Agnolo et al. (1994) calculated a molecular mass of 19-21.5 KDa for α and β, and 30-33 Da for γ subunits of R-PE of *Gracilaria longa*. Slight differences in calculated molecular masses could be due to differences in algal species and/or strains or errors inherent in SDS-PAGE method.

Summation of the individual molecular masses of the R-PE subunits according to the molecular formula $(\alpha\beta)_6\gamma($ (MacColl and Guard-Friar, 1987; D'Agnolo et al., 1994) resulted in a molecular mass of 292 KDa. This value agrees with published values in the literature. The molecular mass of R-PE has been reported to range from 220 to 300 KDa, with variations depending on species (Gantt, 1969; MacColl and Guard-Friar, 1987; D'Agnolo et al., 1994).

On isoelectric focusing, two distinct naturally colored bands appeared with pIs of 4.2 and 4.1, respectively. The presence of two distinct bands, suggested that two R-PE isomers could coexist in the isolated protein. A minor band appeared with a pI of 6.1, which might correspond to subunit γ (Figure 5.2). The pI of R-PE from Rhodella violacea was reported to

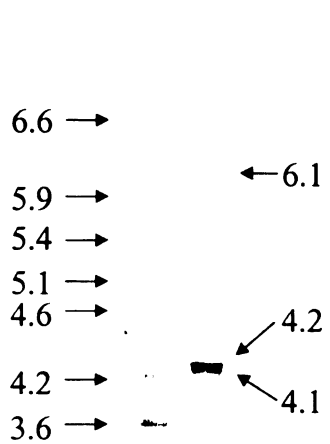


Figure 5.2. Isoelectric focusing of R-phycoerythrin from *P. yezoensis* in a 7.5 % acrylamide gel covering the pH range 4.0 to 7.0. 1) Isoelectric focusing standard, 2) R-phycoerythrin.

be between 4.4 and 4.2 (MacColl and Guard-Friar, 1987). D'Agnolo et al. (1994) reported two pairs of major bands at pI 4.35-4.30 and 4.55-4.50, and a minor component at pI 5.75-5.70 in R-PE from *G. longa*. The authors suggested that the band focused at the more basic pH could correspond to migration of the γ subunit that dissociated from the intact native molecule.

5.4.2. Effect of pH on R-PE spectral properties.

The absorption spectra of purified R-PE in 50mM NaCl, 20 mM phosphate buffer, pH 7.0, showed two peaks at 498 and 565 nm, and a shoulder at about 540 nm (Figure 5.3). Similar spectra has been found for R-PE purified from *Porphyridium cruentum* (Gantt, 1969; Teale and Dale, 1970; Tcheruov et al., 1993), *Rhodella violacea* (MacColl and Guard-Friar, 1987) and *Gracilaria longa* (D'Agnolo et al., 1994).

In fluorescence measurements, while excitation maximum remained the same (493

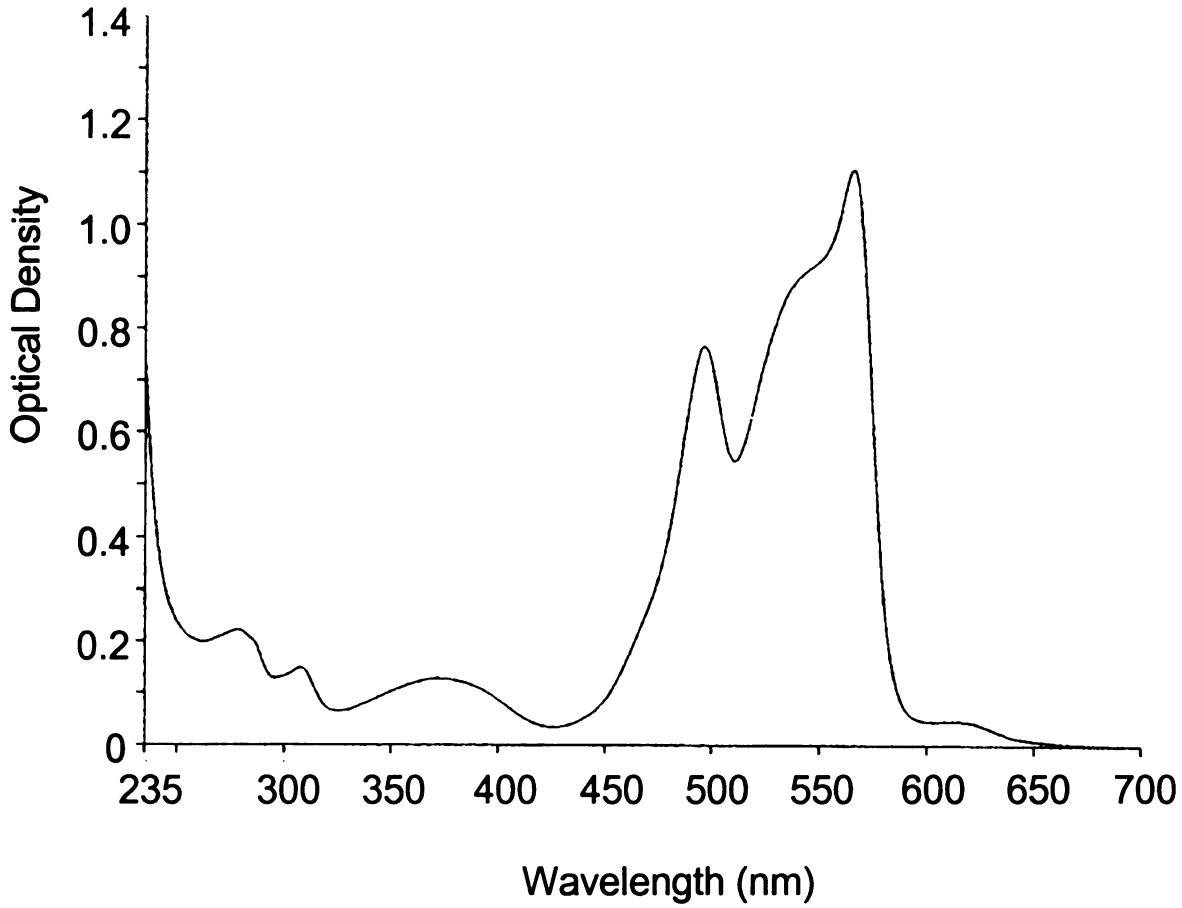


Figure 5.3. Absorption spectrum of R-phycoerythrin from *P. yezoensis* in 50mM NaCl, 20 mM sodium phosphate buffer, pH 7.0.

nm), emission maxima varied slightly with pH. In 50 mM NaCl, 20 mM citrate buffer, pH 4.0, the emission maximum was at 581 nm. In 50 mM NaCl, 20 mM phosphate buffer, pH 7.0, the emission maximum was 578 nm, and in 50 mM NaCl, 20 mM glycine buffer, pH 10.0, it was 579 nm. Excitation and emission maxima did not differ with pH between 5.0 and 9.0.

Fluorescence intensity of R-PE was similar at pH range 5.0 - 9.0, but decreased at pH 4.0 and 10.0. R-PE did not fluoresce at pH's below 4.0 and above 10.0. Moreover, the solution of R-PE under neutral conditions (pH 5.0 - 9.0) showed a bright pink-red color that

shifted to purple at pH's above and below neutrality. The intensity of the purple color increased with very low and very high pH's.

Ogawa et al. (1991) studied the behavior of R-PE in the pH range 3.0 - 11.0 using viscosity and sedimentation measurements and spectroscopic techniques. While they did not observe changes in R-PE absorbance between pH 5.0 and 9.0, all techniques detected changes in protein conformation at pH's below 5.0 and above 9.0. Values of intrinsic viscosity also remained nearly constant between pH 5.0 to 10.0, but increased in more acidic or alkaline regions of pH. The color of the R-PE solution changed from red to purple and the fluorescence disappeared at high and low pH's. The authors concluded that R-PE assumes three forms depending on pH conditions: an aggregated form in acidic solutions near the pI, a globular or compacted form in neutral conditions and partially unfolded form at pH above 9.0.

Phycocyanin, a closely related phycobiliprotein, has been shown to follow reversible association-dissociation of subunits with changes of pH. The pI of phycocyanin is 4.3-4.0 (Hattori et al., 1965). At pH between 5.0 and 7.0, this protein shows an equilibrium of hexamers $(\alpha\beta)_6$, trimers $(\alpha\beta)_3$ and monomers $(\alpha\beta)$ (Hattori et al., 1965; Ogawa et al.,1991). Hattori et al. (1965) reported changes in phycocyanin absorption spectra with pH. The authors concluded that at pH near the pI, the reduction of electrostatic repulsive forces between molecules led to an association of the monomers, which resulted in alteration of the optical properties. MacColl and Guard-Friar (1987) have also described changes in absorption spectra of phycocyanin related to changes in the aggregation pattern of this protein. At low pH, when the protein was denatured, it showed much lower visible absorption. The authors explained that changes in optical properties could be due to alteration

of the conformation of the chromophores resulting from denaturation of the polypeptide. Rudiger (1992) reported that the chromophore phycocyanobilin has a cyclic-helical conformation in solution, while it is extended in the native protein. This suggested that the native structure of the apoprotein allows the chromophore to adopt the linear configuration. Moreover, cyclization of tetrapyrroles resulted in lower visible and higher near-UV absorbance (Burke et al., 1972). Therefore, it can be concluded that the spectral properties of phycocyanin and other phycobiliproteins are influenced by the structure of the protein molecule.

5.4.3. Effect of pH on thermal inactivation of R-PE.

The effects of pH on the thermal inactivation of R-PE were evaluated. The inactivation temperatures selected to obtain a one log reduction in R-PE fluorescence ranged from 60°C to 90°C depending on the pH. Between pH 5.0 and 9.0, thermal inactivation experiments could be performed at temperatures up to 90°C. Thermal inactivation of R-PE at pH's 4.0 and 10.0 could not be performed at temperatures higher than 70°C, due to the substantial loss of fluorescence intensity during the heating lag time. O'hEocha and O'Carra (1961) also noted increasing quenching of fluorescence upon acidification of R-PE solutions. Berns et al. (1963) reported that thermal denaturation of R-PE occurred at lower temperatures at pH 4.7 than at pH 7.0, when measured by fluorescence decay.

The D values were calculated on the basis of fluorescence loss as determined at the maximum excitation peak. It is to be noted that reduction of fluorescence at this band, could result from either loss of fluorescence intensity at this particular point or by shift of the peak to lower or higher regions of the spectra.

Neutral pH's. R-PE was thermostable at pH's between 5.0 and 8.0 where inactivation

temperatures ranged from 70°C to 90°C. D value was defined as the time necessary to reduce R-PE fluorescence by 90% at a given temperature and is indicative of the thermal stability of R-PE at a constant temperature. Thermal inactivation at pH 6.0 resulted in the highest D values at 70°C and 90°C (12258.72 min and 1.43 min, respectively), while the highest D value at 80°C was found at pH 7.0 (461.87 min) (Table 5.1).

Table 5.1. D values for R-phycoerythrin at different pHs.

pH	D values (min)		
4.0	$D_{60} = 303$	$D_{65} = 49$	$D_{70} = 2$
5.0	$D_{70} = 9274$	$D_{80} = 91$	$D_{90} = 0.4$
6.0	$D_{70} = 12259$	$D_{80} = 240$	$D_{90} = 1.4$
7.0	$D_{70} = 2205$	$D_{80} = 462$	$D_{90} = 1.2$
8.0	$D_{70} = 2048$	$D_{80} = 124$	$D_{90} = 1.3$
9.0	$D_{70} = 94$	$D_{80} = 4$	$D_{90} = 0.6$
10.0	$D_{60} = 196$	$D_{65} = 13$	$D_{70} = 1.1$

R-PE has been reported to be highly stable near neutral pH (Gantt, 1969; Ogawa et al., 1991). Ogawa et al. (1991) studied conformational changes of the R-PE molecules and concluded that at pH's between 5.0 and 9.0, the protein has a compact globular shape that confers stability. As the pH increases from the pI, the protein becomes more soluble because of the increase in electrostatic repulsion that reduces aggregation and subsequent precipitation of the molecules (Creighton, 1993). Thermal unfolding of the protein will alter the polypeptide conformation to expose the chromophores that are contained in hydrophobic regions of the protein (O'hEocha, 1965). Unfolding of the protein may cause separation of chromophores that, under native condition, stay in close proximity and interact to produce the specific optical properties of the native protein (Teale and Dale, 1970; Glazer and Hixson, 1977). In addition, denaturation of the protein molecule may result in cyclization of

chromophores with the subsequent loss of absorbance and fluorescence (Burke et al., 1972; Rudiger, 1992).

The z value is the temperature increase necessary to reduce the D value by 90% and is a measure of the temperature dependence of R-PE denaturation. The z values of R-PE increased as pH was increased from 4.58°C at pH 5.0 to 9.15°C at pH 9.0. There was no difference between z values at pH 7.0 and 8.0 ($p > 0.05$). The z value at pH 9.0 (9.15°C) was the highest ($p < 0.05$) among all pH's examined, although the D values were lower at all temperatures when compared with pH's 5.0 to 8.0 (Table 5.2). Results suggest that thermal inactivation of R-PE was less temperature dependent at pH 9.0 at the range of temperatures tested.

Table 5.2. Summary of z values and regression analysis for R-phycoerythrin at different pHs.

pH	z value (°C)	Constant	R squared	X coefficient
4.0	4.59[d]	15.7	0.98	-0.22
5.0	4.58[d]	19.3	0.99	-0.22
6.0	5.09[b]	17.9	0.99	-0.20
7.0	6.13[c]	15.1	0.90	-0.16
8.0	6.25[c]	14.6	0.98	-0.16
9.0	9.15[d]	9.5	0.98	-0.11
10.0	4.44[a]	15.8	0.99	-0.23

Buffers employed were: 20 mM citrate (4.0 and 5.0), 20 mM phosphate (pH 6.0 and 7.0), 20 mM tris(hydroxymethyl)amino-methane (pH 8.0 and 9.0), and 20 mM glycine (pH 10.0). All buffers contained 50 mM NaCl.

Acidic and alkaline pH. At pH 4.0, the selected inactivation temperatures ranged from 60°C to 70°C. At 70°C, the D value (2.01 min) of R-PE at pH 4.0 was several orders of magnitude lower than in neutral pH's, indicating that R-PE was more heat sensitive at pH 4.0

(Table 5.1). At pH 4.0, R-PE is near its isoelectric point (pI = 4.1-4.2) and may form aggregates with reduced solubility (Ogawa et al., 1991).

At pH 10.0, the heating temperatures selected to obtain a one log reduction in R-PE fluorescence ranged from 60°C to 70°C. At 70°C, the D value (2.01 min) was much lower than in neutral pH's. Overall, the D values of R-PE at pH 10.0 were lower than those of pH 4.0 at all temperatures, suggesting that R-PE is more thermostable in acidic than in alkaline conditions (Table 5.1). At pH 10.0, the protein is far from its pI (4.1-4.2) and has a marked negative net charge. Interactions between ionizing groups in the polypeptide are disrupted at this extreme pH, resulting in unfolding of the molecule (Ogawa et al., 199). This partial unfolding results in alteration of the spectroscopical properties of the protein when compared to the neutral solutions. When R-PE is heated at pH 10.0 fluorescence is lost by increasing the exposure of the chromophores to the solution and by decreasing interactions among the chromophores (O'hEocha, 1965; Fujimori and Pecci, 1967; Teale and Dale, 1977).

The z values of R-PE at pH 4.0 and 10.0 were 4.59 and 4.44°C, respectively (Table 5.2). The z values at pH 4.0, 5.0 and 10.0 were similar ($p < 0.05$) even though experiments at pH 5.0 were performed at higher temperatures (70-90°C) than at pH 4.0 or 10.0 (60-70°C). The z values at 4.0, 5.0 and 10.0 were lower ($p > 0.05$) than at pH between 6.0 and 9.0. Results suggest that the thermal inactivation of R-PE was more temperature dependent at acidic and alkaline conditions at the range of temperatures tested.

5.5. CONCLUSIONS

The thermal inactivation behavior of R-PE was successfully modified by altering the solution composition. The protein was very thermostable at pH 5.0 to 9.0 as measured by fluorescence decay at the maximum emission. The z values of R-PE ranged from 4.44°C to

9.15°C depending on pH. Changes in spectroscopical properties were due to changes in the protein conformation that result from chemical (pH) and physical (heat) denaturation. By manipulation of R-PE in a known chemical environment, the z value may be adjusted to approximate the z values of several pathogenic microorganisms used as target indicators in thermal food processes. The encapsulated R-PE could then be used as a TTI to predict destruction of such microorganisms during cooking of food products.

The z values obtained at pH 4.0, 5.0 and 10.0 (4.59, 4.58 and 4.44°C, respectively) should be investigated further for its possible application as TTIs in milk pasteurization, where the target microorganism, *Coxiella burnetti*, has a z value of 4°C (Goff, 1999). The z value of R-PE obtained at pH 6.0 (5.09°C) could be used to monitor destruction of *Escherichia coli* O157:H7 in beef ($z_{E.coli\ O157:H7}$= 5.6°C) (Orta-Ramirez et al., 1995) while at pH 7.0 and 8.0 (6.13 and 6.25°C, respectively) the z value falls within the range of z values reported for *Salmonella* in beef (5.6-6.2°C) (Goodfellow and Brown, 1978; Orta-Ramirez et al., 1995). Additional investigation of the combined effect of pH and additives to further modify the thermal inactivation kinetics of R-PE would be desirable to assess its usefulness in the area of TTIs.

CHAPTER 6: Effects of sucrose, SDS, NaCl, urea and β-mercaptoethanol on the thermal inactivation of R-phycoerythrin

6.1. ABSTRACT

Thermal inactivation kinetics (D and z values) of R-phycoerythrin (R-PE), calculated on the basis of fluorescence loss, were studied under different buffer conditions (pH 4.0, 7.0 and 10.0) and concentrations of sucrose, sodium-dodecyl sulfate (SDS), NaCl, urea and β-mercaptoethanol (ME). Isothermal experiments were performed using capillary tubes to minimize heating lag times. R-PE solutions were heated at temperatures between 40°C and 90°C depending on buffer conditions. The z values ranged from 5.90°C in 50 mM NaCl, 20 mM glycine buffer, pH 10.0, to 37.75°C in 60% sucrose, 50 mM NaCl, 20 mM phosphate buffer, pH 7.0. Thermal inactivation parameters for R-PE were successfully modified by addition of chemicals. Overall, sucrose and ME had a thermostabilizing effect, while SDS, NaCl and urea decreased thermal stability of R-PE.

These results have relevance in the area of protein-based time-temperature integrators to verify compliance with USDA thermal processing of meat products. The z value obtained for R-PE in 50 mM NaCl, 20 mM glycine buffer, pH 10.0 (5.90°C) closely matched the z value reported for *Salmonella* in beef. The development of a TTI with R-PE as the detector component could be used to verify the adequacy of any thermal treatment in beef products.

6.2. INTRODUCTION

Current methodology to assess proper thermal processing in food products can be divided into three categories: in situ methods, mathematical models and time-temperature

integrators (TTIs). A TTI is a device that can predict the thermal history of a cooked product because it shows the same time-temperature response of a target attribute when the temperature is the only rate determining factor. Mathematically, this is accomplished when the z value of the TTI is the same as that of a target attribute ($z_{TTI} = z_{target}$) (Hendrickx et al., 1995; Van Loey et al., 1996). The United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) has recently proposed a modification of the current thermal processing regulations for meat products based on destruction of the pathogenic microorganism *Salmonella* expressed as log reductions. It is required that manufacturers use a combination of thermal and non-thermal processes to achieve a 6.5-log *Salmonella* reduction in ready-to-eat, cooked beef, roasted beef, cooked corned beef. A 5-log reduction in ready-to-eat cooked uncured meat patties has been proposed, although this latter one may be increased (Federal Register, 1996; Federal Register, 1999a). Reported z values of *Salmonella* in beef ranged from 5.6°C to 6.2°C (Goodfellow and Brown, 1978; Orta-Ramirez et al., 1995). The development of a TTI with the same z value as *Salmonella* in beef products could be used to determine the adequacy of any thermal process.

R-phycoerythrin (R-PE), one of the major phycobiliproteins, is a protein found in cyanobacteria, red algae and cryptomonads. The optical properties of R-PE are due to the presence of linear tetrapyrrole chromophores, called bilins, which are covalently attached to the apoprotein through one or two thioether bonds (MacColl and Guard-Friar, 1987; Sidler et al., 1989). R-PE has two different types of chromophores, phycoerythrobilin and phycourobilin (MacColl and Guard-Friar, 1987). The polypeptide chain contains three different subunits, α, β, and γ or γ', usually organized as $(\alpha\beta)_6\gamma$- or $(\alpha\beta)_6\gamma'$-complexess (D'Agnolo et al., 1994). Each α and β subunit contains two and three phycoerythrobilins,

respectively, while γ has two phycoerythobilins and two phycourobilins (Glazer, 1981). Thus, each complex contains a total of thirty-four chromophores. O'hEocha (1965) and Fujimori and Pecci (1967) suggested that these chromophores are located in hydrophobic pockets of the protein molecule. Two types of phycoerythrobilin chromophores, donors and acceptors, have been identified. The donors absorb at shorter wavelengths (565-570 nm) exciting the acceptors that absorb and emit at longer wavelengths (540-555 nm) (O'hEocha and O'Carra, 1961; Teale and Dale, 1970). Teale and Dale (1970) speculated that donor and acceptor chromophores are adjacent to each other. Studies of the conformation and configuration of phycocyanobilin, a tetrapyrrole found in phycocyanin, revealed that the preferred conformation of the chromophore in solution is cyclic-helical, while it is extended in the native protein (Rudiger, 1992). Cyclization of the phycocyanobilin results in lower visible and higher near-UV absorbance than an extended conformation (Burke et al., 1972). The spectroscopic properties of R-PE depend on the native structure of the molecule and the interaction among chromophores (O' hEocha and O' Carra, 1961; Teale and Dale, 1970; MacColl and Guard-Friar, 1987). Modification of this native structure, as by heat or chemical denaturation, will result in partial or complete loss of the optical properties (O'hEocha and O'Carra, 1961; MacColl and Guard-Friar, 1987; Ogawa et al., 1991).

Ogawa et al. (1991) studied the behavior of R-PE in the pH range 3.0-11.0. Although no changes in R-PE between pH 5.0 and 9.0 were observed, there were alterations in protein conformation starting at pHs below 5.0 and above 9.0. The intrinsic viscosity remained nearly constant between pH 5.0 to 10, while it increased in more acidic or alkaline conditions. The authors concluded that R-PE adopts three forms depending on pH conditions:

an aggregated form in acidic solutions, a globular form between pH 5.0 and 9.0 and a partially unfolded form at pH above 9.0.

Thermal inactivation of proteins results in a partial unfolding of the molecule (Klibanov, 1983). The compact structure of a native protein is sustained by a combination of noncovalent interactions between atoms in the molecule, including hydrogen bonds, van der Waals interactions, electrostatic forces, and, most importantly, hydrophobic interactions (Klibanov, 1983; Creighton, 1993). The presence of additives during heating may stabilize or destabilize the native structure of the molecules, thus, affecting the overall thermal inactivation kinetics of the protein.

In previous studies (Chapter 2), the thermal inactivation parameters of R-PE in the pH range 4.0-10.0 were calculated on the basis of fluorescence loss at the maximum emission wavelength. R-PE was very thermostable at pH between 5.0 and 9.0, but fluorescence decreased at pH 4.0 and 10.0. We selected three pHs, 4.0, 7.0 and 10.0, representing acidic, neutral and alkaline conditions, to further investigate the influence of the solution conditions on the thermal inactivation of R-PE. The objective of this study was to determine the inactivation kinetics parameters of the fluorescent protein R-phycoerythrin (R-PE) under different conditions of pH and additives (sucrose, sodium-dodecyl sulfate, NaCl, urea and β-mercaptoethanol). Our goal was to find specific solution conditions that would result in a z value of R-PE close to the reported z value of *Salmonella* in beef.

6.3. MATERIALS AND METHODS

6.3.1. Materials.

R-phycoerythrin (R-PE) was extracted and purified as explained in Chapter 2. Pure R-PE was stored in 60% (w/v) ammonium sulfate and kept at 4°C. Prior to the experiments,

R-PE was dialyzed overnight against distilled deionized water at 4°C, then diluted immediately before use in the solutions described in 6.3.2 to achieve an absorbance of 0.1 at 565 nm. All chemicals were reagent grade or better.

6.3.2. Solution conditions.

The effects of pH and additives on the thermal inactivation kinetics of R-PE were investigated using sucrose (0, 20, 40 and 60% w/v at pH 7.0 and 10.0); sodium dodecyl sulfate (SDS) (0, 0.5, 1 and 2.5% v/v at pH 7.0 and 10.0); NaCl (0, 1, 2 and 4M at pH 7.0, and 0, 0.5, 1 and 2M at pH 10.0); urea (0, 0.1, 0.25 and 0.5M at pH 4.0; 0, 1, 2 and 4M at pH 7.0, and 0, 0.5, 1 and 2M at pH 10.0); and β-mercaptoethanol (ME) (0, 0.5, 1 and 2.5% v/v, at pH 7.0 and 10.0). The buffers employed were: 50 mM NaCl, 20 mM citrate, pH 4.0 (pKa= 4.45), 50 mM NaCl, 20 mM phosphate, pH 7.0 (pKa = 6.88), and 50 mM NaCl, 20 mM glycine, pH 10.0 (pKa = 9.78).

Each compound was dissolved in buffer to achieve predetermined concentrations and pH was adjusted with 1 M HCl or NaOH if necessary. Preliminary studies were conducted to determine concentrations of the additives to achieve fluorescence loss with heat, but to avoid complete denaturation of the protein prior to heating. Control solutions were defined as buffers (pH 4.0, 7.0 or 10.0) containing R-PE to which no additives had been supplemented.

6.3.3. Thermal inactivation.

Capillary tubes (Accu-fill 90 Micropet, Cat. No. 4624, Becton-Dickinson and Co., Parsippany, NJ) were filled with R-PE solution (200 μL) by capillary action. To allow for even head space at both ends, 50 μL were removed using a micropipet (i.e. the R-PE solution was centered between the two sealed ends). One end was sealed using a gas/oxygen flame and the other with Teflon tape. The tubes were placed in a wire rack and immersed in a

Polystat circulator bath (Model 1268-52, Cole-Parmer Instrument Company, Chicago, IL) connected to a bath programmer (Model 1268-62, Cole-Parmer). Temperature in the capillary tubes was monitored using a thermocouple (Resistance Temperature Detector, Pt 100, 0.15 cm diameter, (1°C accuracy) inserted in a control capillary tube containing 150 μL of the same solution. The thermocouple was connected to a Solomat MPM 200 Modumeter (Solomat Partners LP, Stanford, CT). Zero time was defined as the time when the R-PE solution reached the target temperature. The heating lag time in the capillary tubes was 10^{-1} sec.

Temperatures ranged from 40 to 90°C depending on the solution conditions. Preliminary studies were performed to determine the times and temperature necessary to achieve at least a one log reduction in fluorescence. The tubes were removed at the specific intervals of time and immediately placed in an ice-water bath, then kept at 4°C until fluorescence was read within 6 h. Preliminary studies were conducted to show that no changes of fluorescence occurred during this period. Each study was done in triplicate.

6.3.4. Fluorescence measurement.

R-PE fluorescence at the maximum emission wavelength was measured using a CytoFluor II Microwell Fluorescence reader and the CytoFluorII software, Version 2.0c (Biosearch Incorporated, Bedford, MA). Heated and unheated R-PE solutions were placed in 96-well fluorescence reader plates (Cat. No. DG5515, Life Science Products Inc., Denver, CO). Excitation was set at 485/20 nm and emission at 590/35 nm.

6.3.5. Data analysis.

Fluorescence counts were converted to logarithms. D values (in minutes) were calculated by linear regression of fluorescence vs time using Microsoft Excel Version 5.0a

(Microsoft Corporation, Redmond, WA). Thermal resistance curves were determined by plotting log of D values vs temperature. The z values were determined as the negative reciprocal of the slope of the thermal resistance curve.

Statistical differences among z values within treatments were tested using two-factorial analysis of variance (ANOVA) (Systats Version 5.0, Systats, Evanston, IL). Mean z values were compared using Tukey's test with the mean square error at the 5% level of probability.

6.4. RESULTS AND DISCUSSION

The minimum temperature needed to achieve significant fluorescence loss of R-PE was greatly influenced by the solution conditions. Adjustments of heating time and temperature combinations, as well as of concentration of the additives had to be made for each treatment. At pH 4.0, the protein was so unstable that addition of either sucrose, SDS, NaCl or ME resulted in almost complete loss of fluorescence, even at temperatures as low as 40°C.

6.4.1. Effects of sucrose.

At pH 7.0, the heating temperatures to achieve at least a one log reduction in R-PE fluorescence were lower for the control (60°C-80°C) than for the R-PE solutions containing sucrose (70°C-90°C), suggesting that sucrose had a thermostable effect on R-PE (Figure 6.1). The D values of R-PE in the control at pH 7.0 ranged from 2500 min at 60°C to 31.44 min at 80°C (Figure 6.2). In this study, the D value was defined as the time necessary to reduce the fluorescence intensity of R-PE by 90% at a given temperature. The D value is used as an indication of the thermal stability of R-PE at a constant temperature. An increase in the D value of R-PE in sucrose solutions with respect to the control indicates that addition of

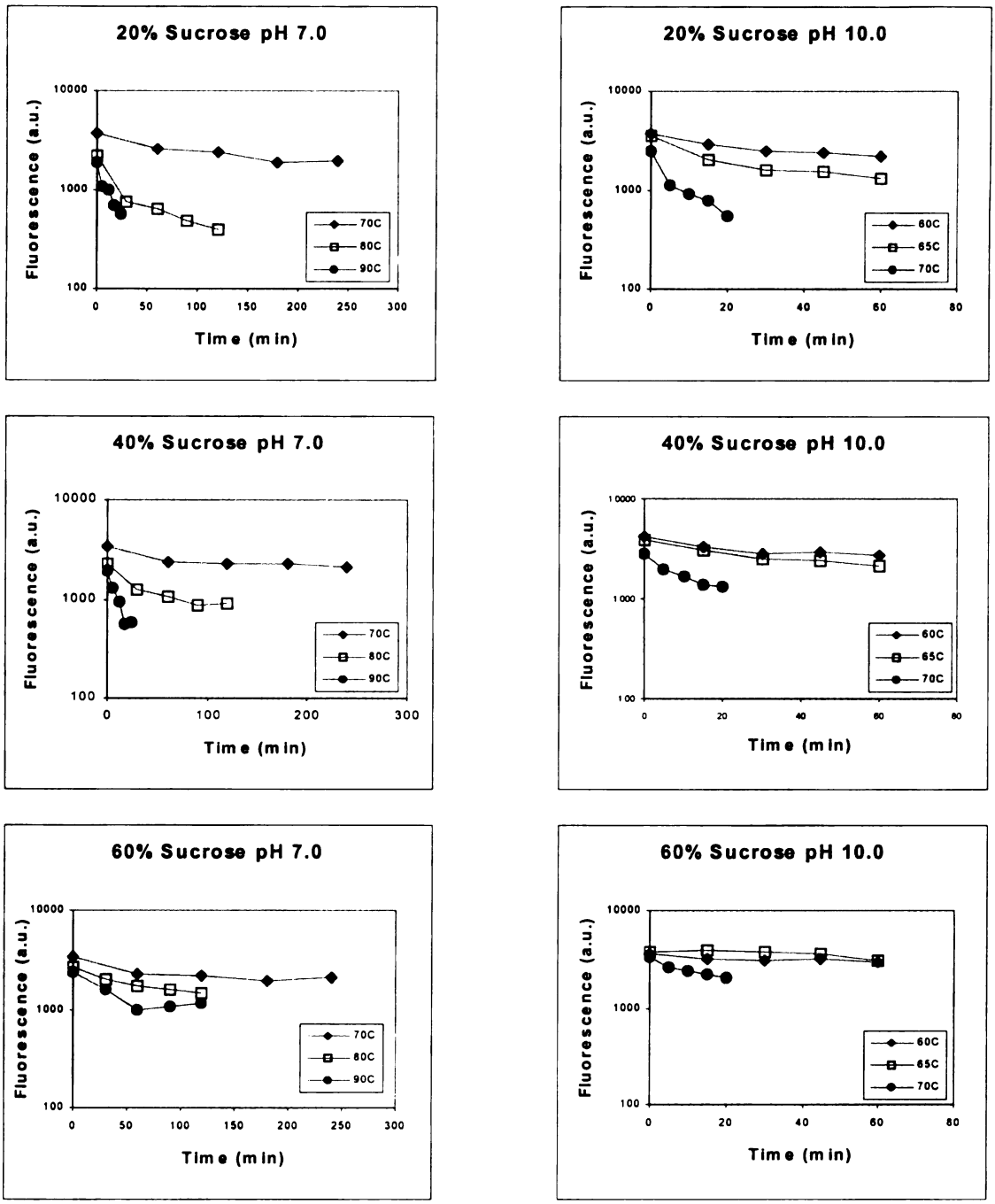
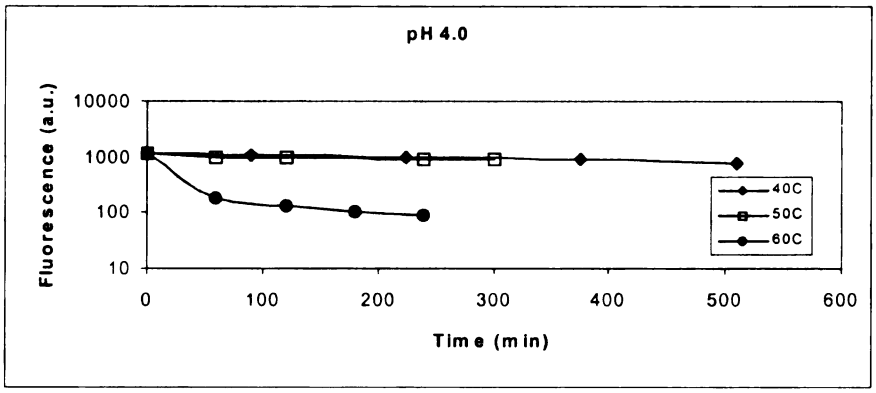
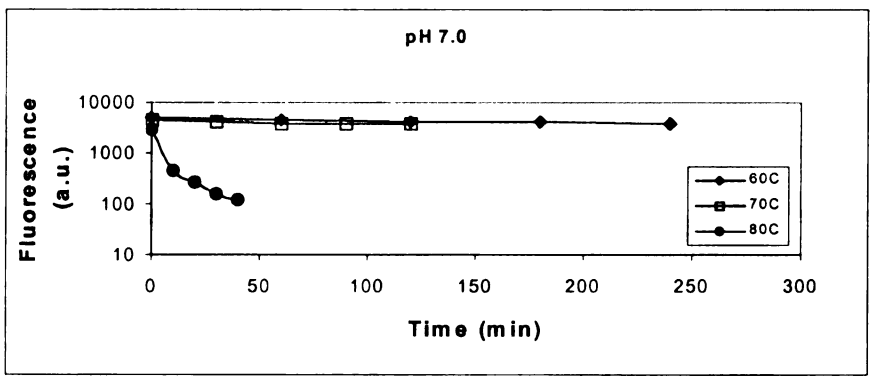


Figure 6.1. Thermal inactivation curves of phycoerythrin in sucrose solutions in 50 mM NaCl, 20 mM phosphate or glycine buffer, pH 7.0 and 10.0, respectively.





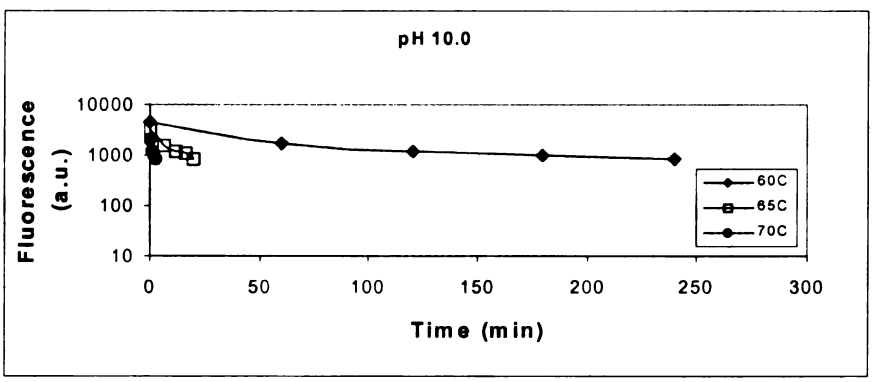


Figure 6.2. Thermal inactivation curves of phycoerythrin in 50 mM, 20 mM citrate buffer, pH 4.0, 50 mM, 20 mM phosphate buffer, pH 7.0 and 50 mM, 20 mM glycine buffer, pH 10.0.

sucrose confers stability towards temperature. When compared at the same temperatures (70°C and 80°C), the D values for R-PE in sucrose solutions at pH 7.0 were much higher than those of the control. When heated at 70°C, the D values of R-PE increased as sucrose concentrations were increased to 40%, but the D value in 60% sucrose was lower than that at 40% sucrose. At 80°C, the D values for R-PE increased with increasing concentrations of sucrose in solution. At 90°C, the D values of R-PE in 20 and 40% sucrose were similar, but increased by almost one log in 60% sucrose (Table 6.1). It can be suggested that, at lower temperatures, sucrose enhances protein stability up to a certain concentration, beyond which sucrose competes with protein for water of solvation. At higher temperatures this was not seen as sucrose was more readily solubilized.

At pH 10.0, the heating temperatures were the same for all R-PE solutions (60-70°C) suggesting that the thermostabilizing effect of sucrose was not as strong as pH 7.0 (Figure 6.1). At pH 10.0, all D values increased as the concentration of sucrose was increased when R-PE was heated at 65°C and 70°C. At 60°C, the D value of R-PE was lower in 20% sucrose than in the control, but then increased with increasing concentrations of sucrose (Table 6.2).

Addition of sucrose to samples of R-PE at pH 7.0 before heating reduced fluorescence intensity by about 17% at 20 and 40% concentration and 20% at 60% sucrose concentration. This suggested that sucrose had a protective effect on R-PE against thermal inactivation but in unheated samples the native conformation was altered in the presence of the sugar. When sucrose was added to R-PE solutions at pH 10.0 before heating, the fluorescence intensity was decreased by 17, 10 and 3% at 20, 40 and 60% sucrose, respectively. Results suggest that at this pH, sucrose had a smaller effect on R-PE native structure than at pH 7.0.

Table 6.1. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in sucrose solutions in 50 mM NaCl, 20 mM phosphate buffer, pH 7.0

Sucrose Conc. (% w/v)	T(°C)	D value (min)	X coefficient	Constant	R squared
0	60	2500	-0.0004	3.68	0.93
	70	124	-0.03	3.65	0.93
	80	31	-0.03	3.19	0.86
20	70	855	-0.001	3.52	0.87
	80	179	-0.006	3.19	0.85
	90	50	-0.02	3.22	0.95
40	70	1408	-0.001	3.48	0.68
	80	313	-0.003	3.26	0.81
	90	44	-0.02	3.25	0.94
60	70	1250	-0.0008	3.47	0.64
	80	493	-0.002	3.38	0.93
	90	369	-0.003	3.29	0.64

Table 6.2. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in sucrose solutions in 50 mM NaCl, 20 mM glycine buffer, pH 10.0

Sucrose Conc. (% w/v)	T(°C)	D value (min)	X coefficient	Constant	R squared
0	60	348	-0.003	3.52	0.85
	65	36	-0.03	3.44	0.93
	70	7.2	-0.14	3.27	0.95
20	60	285	-0.004	3.53	0.90
	65	153	-0.007	3.47	0.86
	70	34	-0.03	3.29	0.90
40	60	370	-0.003	3.58	0.77
	65	240	-0.004	3.56	0.94
	70	62	-0.02	3.41	0.92
60	60	885	-0.001	3.54	0.65
	65	746	-0.001	3.60	0.56
	70	106	-0.002	3.49	0.93

Addition of sugars enhances thermostability of proteins in aqueous solutions because of a "preferential hydration" effect. The sugar molecules interfere with the cohesive force of water at the water-protein interface and are preferentially excluded from the protein's surrounding, thus, favoring the native state over the denatured one (Creighton, 1993; Timasheff and Arakawa, 1997). This effect has been observed in several proteins. DeCordt et al. (1994) used sugars to modify the thermal inactivation kinetics of α-amylase from *Bacillus* spp. Addition of 20, 40 or 60% sucrose resulted in higher thermal stability with respect to the control. These researchers found that, in general, protein stability increased as sucrose concentration was increased from zero to 40%, but addition of 60% sucrose did not confer higher stability. Boye et al. (1996) studied the thermal stability of (-lactoglobulin using differential scanning calorimetry. Addition of either glucose or sucrose (at 50% concentrations) stabilized partly denatured protein, inhibiting its aggregation. Sucrose showed a stronger effect than glucose. Rajeshwara and Prakash (1996) observed that addition of sucrose (at 25 and 50% concentrations) to heat-treated wheat germ increased the thermal stability of the enzyme with respect to the sample in buffer alone.

In our study, the z value was defined as the temperature increase necessary to reduce the D value by 90%. The z value is indicative of the temperature dependence of R-PE denaturation. The z values for R-PE in 20 and 40% sucrose at pH 7.0 (16.12°C and 13.27, respectively) were not different ($p>0.05$) from the control (10.52°C). The z value for the 60% solution (37.75°C) was higher ($p<0.05$) than the other solutions, suggesting that the denaturation of R-PE was less temperature dependent. At pH 10.0, the z values of R-PE in 20, 40 and 60% sucrose (10.92°C, 12.87°C and 10.87°C, respectively) did not differ, but were higher ($p<0.05$) than that of the control (5.90°C) (Table 6.3).

Table 6.3. The z values and regression parameters for thermal inactivation curves of R-phycoerythrin in sucrose solutions in 50 mM NaCl, 20 mM phosphate buffer, pH 7.0 and and 50 mM NaCl, 20 mM glycine buffer, 10.0

pH	Sucrose Conc. (% w/v)	z value (°C)	X coefficient	Constant	R squared
7.0	0	10.5[a]	-0.10	8.98	0.96
	20	16.1[a]	-0.06	7.26	0.99
	40	13.3[a]	-0.08	8.46	0.99
	60	37.8[b]	-0.03	4.90	0.91
10.0	0	5.9[a]	-0.17	12.61	0.99
	20	10.9[b]	-0.09	8.01	0.95
	40	12.9[b]	-0.08	7.30	0.92
	60	10.9[b]	-0.09	8.60	0.81

Values with different superscripts within the same pH are statistically different ($p<0.05$)

Table 6.4. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in sodium-dodecyl sulfate (SDS) solutions in 50 mM NaCl, 20 mM phosphate buffer, pH 7.0

SDS Conc. (% v/v)	T(°C)	D value (min)	X coefficient	Constant	R squared
0	60	2500	-0.0004	3.68	0.93
	70	124	-0.03	3.65	0.93
	80	31	-0.03	3.19	0.86
0.5	50	87	-0.01	3.37	0.98
	55	36	-0.03	3.09	0.98
	60	10	-0.10	3.10	0.95
1.0	50	81	-0.01	3.37	0.99
	55	35	-0.03	2.99	0.93
	60	9.1	-0.11	3.09	0.95
2.5	50	73	-0.01	3.31	0.99
	55	33	-0.03	3.01	0.94
	60	9.1	-0.11	3.00	0.92

In view of the results, R-PE in 50 mM NaCl, 20 mM glycine buffer, pH 10.0 (control) should be investigated further for its applicability as a TTI in thermal processing of beef products. The calculated z value (5.90°C) for R-PE under these conditions fell within the range of *Salmonella* z values (5.6-6.2°C) reported in the literature (Goodfellow and Brown, 1978; Orta-Ramirez, 1995). It is worth noticing that, although not within the scope of this study, the calculated z values for R-PE in 50 mM NaCl, 20 mM phosphate buffer, pH 7.0 (10.52°C), 20% sucrose, pH 10.0 (10.92°C) and 60% sucrose, pH 10.0 (10.87°C), should be investigated further for possible application as a TTI in commercial sterilization processes, where the target microorganism, *Clostridium botulinum*, has a z value of 10°C (Pflug and Odlaugh, 1978).

6.4.2. Effects of SDS.

Addition of SDS to R-PE solutions before heating resulted in loss of fluorescence intensity by 62% at 0.5% concentration and by 75% at 2.5% concentration at pH 10.0. R-PE fluorescence intensity before heating was reduced by 35% at pH 7.0, regardless of SDS concentration, indicating that SDS has a strong denaturant effect even without heating. At pH 7.0 and 10.0, the addition of SDS to R-PE resulted in a lower range of heating temperatures (50-60°C and 40-50°C, respectively) necessary to decrease R-PE fluorescence by a one log, when compared to the controls (60-80°C and 60-70°C, respectively), indicating that SDS had a thermodestabiling effect (Figure 6.3). At pH 7.0 and 10.0, the D values at each temperature were very similar for all concentrations of SDS, but were much lower than the control (Table 6.4, Table 6.5). Results suggest that addition of 0.5% SDS decreased thermal stability of R-PE when compared to the control, and addition of more SDS had little effect at either pH.

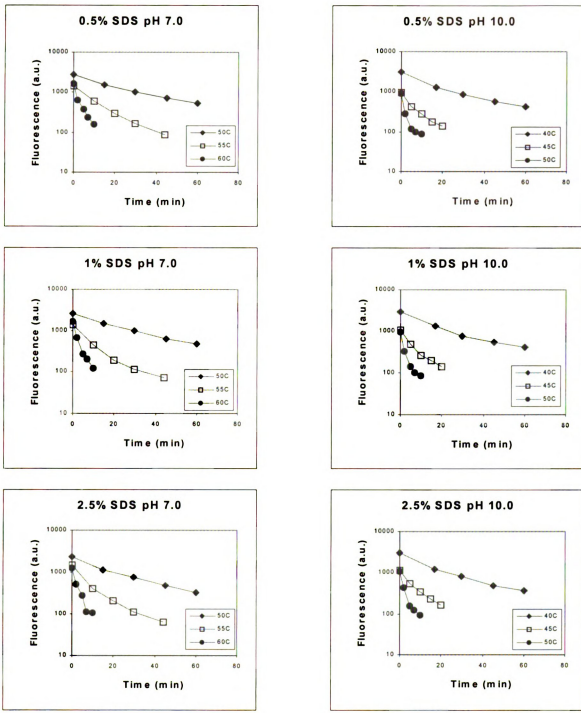


Figure 6.3. Thermal inactivation curves of phycoerythrin in sodium dodecyl sulfate solutions in 50 mM NaCl, 20 mM phosphate or glycine buffer, pH 7.0 and 10.0, respectively.

Table 6.5. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in sodium-dodecyl sulfate (SDS) solutions in 50 mM NaCl, 20 mM glycine buffer, pH 10.0

SDS Conc. (% v/v)	T(°C)	D value (min)	X coefficient	Constant	R squared
0	60	348	-0.003	3.52	0.85
	65	36	-0.03	3.44	0.93
	70	7.2	-0.14	3.27	0.95
0.5	40	70	-0.01	3.41	0.96
	45	24	-0.04	2.90	0.96
	50	10	-0.10	2.75	0.83
1.0	40	71	-0.01	3.39	0.96
	45	24	-0.04	2.94	0.96
	50	10	-0.10	2.80	0.88
2.5	40	66	-0.02	3.41	0.97
	45	24	-0.04	2.99	0.98
	50	9.5	-0.11	2.89	0.91

Table 6.6. The z values and regression parameters for thermal inactivation curves of R-phycoerythrin in sodium-dodecyl sulfate (SDS) solutions in 50 mM NaCl, 20 mM phosphate buffer, pH 7.0 and 50 mM NaCl, 20 mM glycine buffer, 10.0

pH	SDS Conc. (% v/v)	z value (°C)	X coefficient	Constant	R squared
7.0	0	10.5^{a}	-0.10	8.98	0.96
	0.5	14.0^{a}	0.07	7.55	0.96
	1.0	15.9^{a}	-0.06	6.89	0.98
	2.5	21.8^{a}	-0.05	5.48	0.99
10.0	0	5.9^{a}	-0.17	12.61	0.99
	0.5	20.5^{b}	-0.05	5.54	0.96
	1.0	26.1^{c}	-0.04	4.82	0.93
	2.5	33.4^{bc}	-0.03	4.18	0.97

Values with different superscripts within the same pH are statistically different ($p<0.05$)

SDS is a well known protein denaturant used in electrophoretic separations of proteins (Laemli, 1970). As many other detergents, SDS interacts with the nonpolar amino acid residues of the polypeptide chain interfering with the hydrophobic forces that maintain the native structure (Voet and Voet, 1990; Creighton, 1993). The chromophores in R-PE are located within hydrophobic regions of the protein molecule (O'hEocha, 1965; Fujimori and Pecci, 1967). The unique spectral properties of R-PE depend on the native structure of the polypeptide and interaction among the different chromophores (O'hEocha and O'Carra, 1961; Teale and Dale, 1970; MacColl and Guard-Friar, 1987). Alteration of the hydrophobic interactions that maintain the chromophore native environment will result in loss of the spectroscopical properties (i.e. absorbance and fluorescence). In addition, SDS may cause dissociation of subunits which could also result in loss of color and fluorescence. Moreover, denaturation of the molecule can cause cyclization of the chromophores resulting in a decrease of spectral properties (Rudiger, 1992).

Treatment of R-PE with 1% SDS for 3 h at 37°C resulted in loss of fluorescence (Gantt, 1969). The color of the solution changed from pink to purple. Absorbance measurements revealed the disappearance of the peak at 563 nm. The author concluded that addition of SDS caused dissociation of the protein into monomers, which resulted in changes of the optical properties. MacColl and Guard-Friar (1987) reported a decrease in absorption of C-phycocyanin, another phycobiliprotein, upon addition of SDS.

No differences ($p>0.05$) were found in z values when R-PE was heated in 0.5, 1.0 and 2.5% SDS (13.98°C, 15.85°C and 21.79°C, respectively) and the control (10.52°C) at pH 7.0 (Table 6.6), indicating that SDS did not influence the temperature dependence of the inactivation rate.

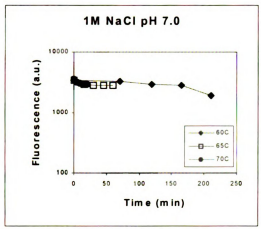
At pH 10.0, the z value of the control (5.90°C) was lower ($p<0.05$) than that for R-PE in SDS solutions. The z value for R-PE in 0.5 (20.52°C) and 1.0% (26.08°C) solutions were different ($p<0.05$) from each other, but not different from 2.5% SDS (33.38°C) (Table 6.6). Large z values indicate that a large increase in temperature is needed to reduce the D values by 90%. The large z values observed when R-PE was heated in SDS solutions indicate that the denaturation of R-PE was not highly temperature dependent.

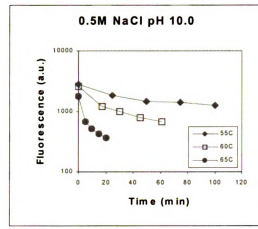
The z values of PE in 2.5 % SDS, pH 7.0 (21.79°C), and 0.5 %, 1.0 and 2.5% SDS, pH 1.0 (20.52, 26.08 and 33.38°C, respectively) should be explored further for its possible application in monitoring destruction of vitamins A (z=23-25°C), B1 (z=22.0-31.3°C) and B6 (z=22.0-31.0°C) during thermal processing of several food products (Holdsworth, 1997).

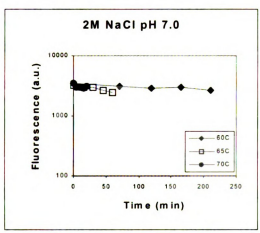
6.4.3. Effects of NaCl.

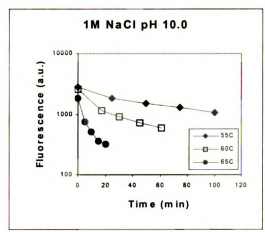
Addition of NaCl to R-PE before heating reduced fluorescence intensity by about 25% at pH 7.0, and by 33% at pH 10.0, regardless of the concentration, suggesting that NaCl had a destabilizing effect on R-PE native structure.

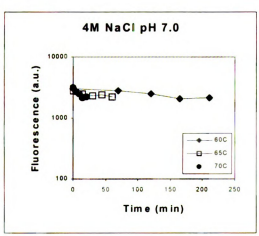
At pH 7.0, the range of heating temperatures used in the inactivation experiments (60°C -70°C) was lower than that of the control (60°C -80°C), suggesting that NaCl had a thermodestabilizing effect on R-PE (Figure 6.4). Preliminary studies had been conducted to determine the maximum temperature at which the R-PE fluorescence would not decrease markedly during the heating lag time. For R-PE in NaCl solutions at pH 7.0 the maximum temperature was 70°C. At higher temperatures, R-PE fluorescence was lost during the heating lag period. However, even after heating for 225 min, at 60, 65 and 70°C, no log reduction in fluorescence was achieved. The D values were then estimated from the fluorescence reduction during this time period. The D values for R-PE at 60°C were lower,











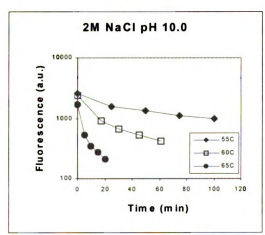


Figure 6.4. Thermal inactivation curves of phycoerythrin in NaCl solutions in 50 mM NaCl, 20 mM phosphate or glycine buffer, pH 7.0 and 10.0, respectively.

but at 70°C were higher than those of the control, for all the NaCl concentrations. No differences in D values for R-PE were observed with increasing concentrations of NaCl (Table 6.7).

At pH 10.0, the range of heating temperatures (55°C -65°C) was also lower than that of the control (60°C -70°C) (Figure 6.4). Overall, the D values at the same temperature decreased with increasing NaCl concentration (Table 6.8). At this pH, the protein was already destabilized by pH, and was more sensitive to presence of ions in the solution. In addition, at this pH, the protein net charge is markedly negative (pI of R-PE = 4.1-4.2). Interactions with the Na^+ in solution may interfere with charge-charge interactions within the protein structure resulting in further unfolding.

Addition of salts to proteins in aqueous solutions can either stabilize or destabilize their native structure (Voet and Voet, 1990; Creighton, 1993; Timasheff and Arakawa, 1997). During thermal denaturation, partial unfolding of the protein occurs resulting in the exposure of amino acid side chains (Klibanov, 1983; Creighton, 1993). This exposure facilitates interactions with the free ions in solution. Folawiyo and Owusu-Apenten (1996) studied the heat stability of cruciferin under different NaCl concentrations. Raising the concentration of NaCl from 0.1 to 1M, had a stabilization effect at temperatures of 70-80°C, but not at temperatures below 50°C. The Tm (temperature at the midpoint of the thermal unfolding transition) also increased linearly as a function of NaCl concentration. The authors concluded that the NaCl stabilization effect could be related to non-specific salt effects by interfering with charge-charge interactions within the native conformation of cruciferin.

At pH 7.0, no differences ($p>0.05$) were found between the z values of control (10.52°C) and any NaCl solutions (15.28, 16.72 and 9.94°C, respectively), suggesting that

Table 6.7. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in NaCl solutions in 20 mM phosphate buffer, pH 7.0

NaCl Conc. (M)	T (°C)	D value (min)	X coefficient	Constant	R squared
0.05	60	2500	-0.0004	3.68	0.93
	70	123	-0.03	3.65	0.93
	80	31	-0.03	3.19	0.86
1.05	60	990	-0.001	3.56	0.74
	65	806	-0.001	3.50	0.73
	70	219	-0.005	3.53	0.90
2.05	60	1818	-0.0006	3.54	0.89
	65	559	-0.002	3.50	0.92
	70	459	-0.002	3.51	0.30
4.05	60	1190	-0.0008	3.49	0.90
	65	725	-0.001	3.43	0.84
	70	117	-0.009	3.49	0.92

Table 6.8. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in NaCl solutions in 20 mM glycine buffer, pH 10.0

NaCl Conc. (M)	T (°C)	D value (min)	X coefficient	Constant	R squared
0.05	60	348	-0.003	3.52	0.85
	65	36	-0.03	3.44	0.93
	70	7.2	-0.14	3.27	0.95
0.55	55	308	-0.003	3.38	0.87
	60	109	-0.009	3.32	0.88
	65	31	-0.03	3.11	0.84
1.05	55	245	-0.004	3.41	0.95
	60	101	-0.01	3.31	0.90
	65	27	-0.04	3.15	0.90
2.05	55	251	-0.004	3.36	0.91
	60	86	-0.01	3.26	0.90
	65	24	-0.04	3.07	0.87

addition of NaCl to R-PE solutions did not affect the temperature dependence of R-PE denaturation. On the other hand, no differences ($p>0.05$) were found in z values of R-PE among the three NaCl solutions (10.09, 10.47 and 9.78°C) at pH 10.0, and they all had greater z values than the control ($p<0.05$) (Table 6.9). The calculated z values for R-PE in NaCl at pH 10.0 could be further investigated for their possible application as a TTI for food sterilization processes (z value of *C. botulinum* = 10°C).

6.4.4. Effects of urea.

Urea was the only additive in which R-PE fluorescence was not completely lost before heating at pH 4.0. Addition of urea to samples before heating reduced fluorescence intensity of R-PE by 17% at pH 4.0, regardless of the concentration. At pH 7.0, addition of urea at 1 and 2 M concentration did not affect R-PE fluorescence intensity before heating, but at 4M concentration resulted in a decrease of R-PE fluorescence by about 25%. When urea was added to unheated samples at pH 10.0 at 0.5 and 1 M concentrations, it did not reduce fluorescence intensity of R-PE. At 2 M concentration, addition of urea at pH 10.0 decreased initial fluorescence intensity of R-PE by about 50%. These observations suggest that the denaturing effect of urea was enhanced by heating at lower concentrations, but at 4 M for pH 7.0 and 2 M for pH 10.0 there was a noticeable effect even before heating. Addition of urea at pH 4.0 allowed for a higher range of heating temperatures (55°C - 65°C) than that for the control (40°C - 60°C), suggesting that urea had a thermostabilizing effect at this pH (Figure 6.5). At 60°C, the D value for R-PE in urea solutions was lower than that of the control. The D values did not change as concentration of urea was increased (Table 6.10). The D values calculated at pH 4.0, however, should be considered carefully. Upon examination of Figure 6.5 it should be noted that values of fluorescence at time zero for pH 4.0 solutions

Table 6.9. The z values and regression parameters for thermal inactivation curves of R-phycoerythrin in NaCl solutions in 20 mM phosphate buffer, pH 7.0 and 50 mM NaCl, 20 mM glycine buffer, pH 10.0

pH	NaCl Conc. (M)	z value (°C)	X coefficient	Constant	R squared
7.0	0.05	10.5[a]	-0.1	8.98	0.96
	1.05	15.3[a]	-0.07	7.00	0.85
	2.05	16.7[a]	-0.06	6.78	0.85
	4.05	9.9[a]	-0.1	9.21	0.90
10.0	0.05	5.9[a]	-0.2	12.61	0.99
	0.55	10.1[b]	-0.1	7.95	0.99
	1.05	10.5[b]	-0.1	7.67	0.99
	2.05	9.8[b]	-0.1	8.04	0.99

Values with different superscripts within the same pH are statistically different ($p<0.05$)

Table 6.10. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in urea solutions in 50 mM NaCl, 20 mM citrate buffer, pH 4.0

Urea Conc. (M)	T (°C)	D value (min)	X coefficient	Constant	R squared
0	40	5000	-0.0002	3.04	0.90
	50	2500	-0.0004	3.04	0.96
	60	270	-0.004	2.70	0.76
0.1	55	222	-0.005	2.60	0.72
	60	40	-0.03	2.98	0.94
	65	7.5	-0.1	2.91	0.99
0.25	55	169	-0.006	2.74	0.96
	60	35	-0.03	3.12	0.94
	65	7.6	-0.1	2.99	0.89
0.50	55	235	-0.004	2.92	0.94
	60	36	-0.03	3.18	0.91
	65	5.9	-0.2	2.81	0.97

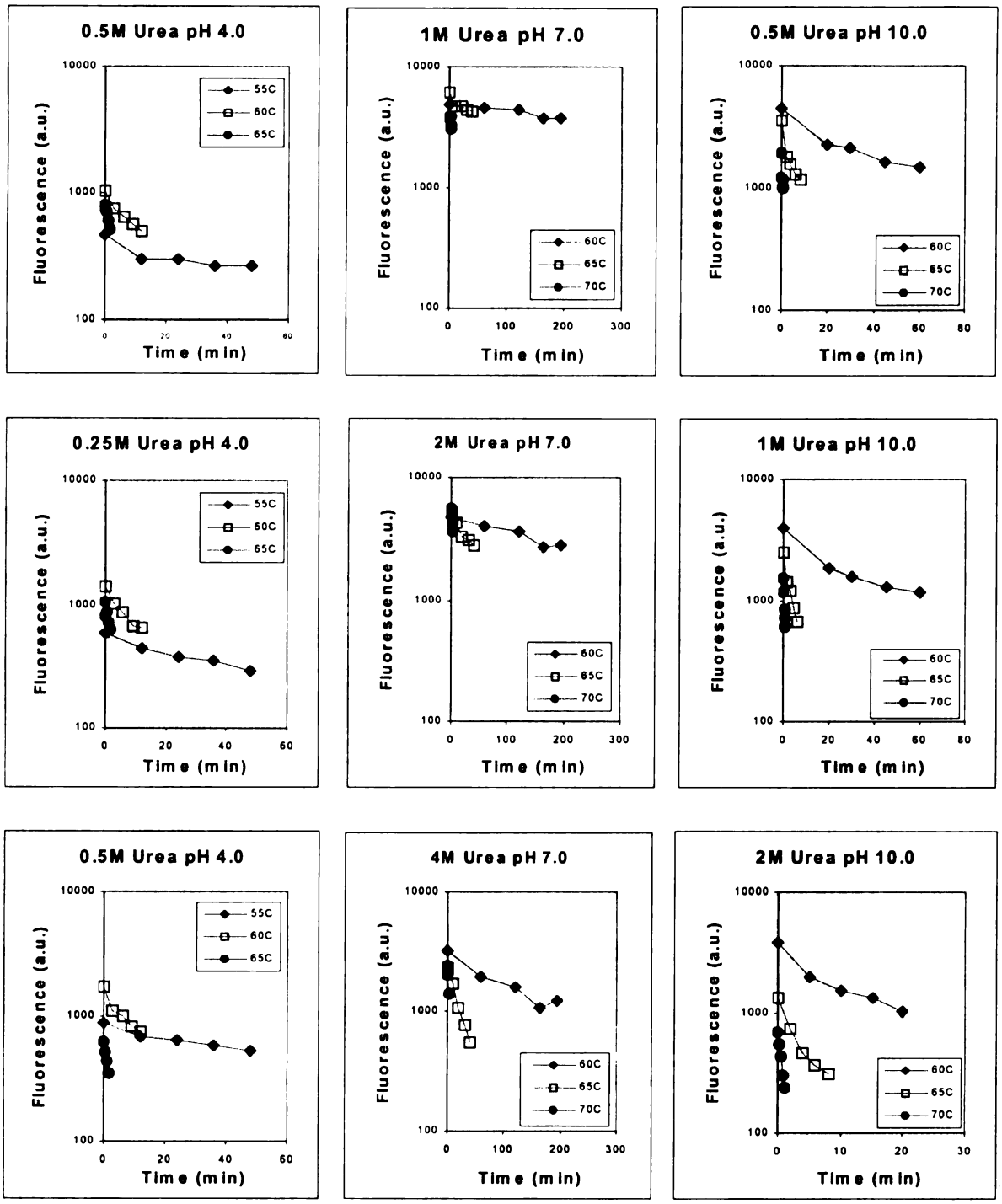


Figure 6.5. Thermal inactivation curves of phycoerythrin in urea solutions in 50 mM NaCl, 20 mM citrate, phosphate or glycine buffer, pH 4.0, 7.0 and 10.0, respectively.

are very low compared to the zero values under other conditions. It can be seen that the protein is undergoing structural changes during the heating lag time resulting in a significant loss of fluorescence. Thus, the calculated D values are overestimating the length of time to reduce the fluorescence by one log.

In contrast to the effect of sucrose, SDS, NaCl and ME on R-PE at pH 4.0, the combination of urea and pH 4.0 did not cause complete loss of R-PE fluorescence before heating. This could be the result of an increase of solubility of the protein in the urea solutions. At pH 4.0, R-PE is at pH near the isoelectric point (pI = 4.1-4.2). Proteins at their pI often have low solubility because the molecule has no net charge, and tend to form aggregates (Voet and Voet, 1990; Creighton, 1993). Addition of urea at pH 4.0 may have help to partially solubilize the protein inhibiting aggregation and allowing the protein to adopt a more native-like conformation.

At pH 7.0, the range of heating temperatures was lower for R-PE in urea solutions (60-70°C) than for the control (60-80°C), indicating that urea had a thermodestabiling effect (Figure 6.5). At all temperatures, the D values for the control were greater than the D values of R-PE in urea solutions at both pH 7.0 and 10.0. In addition, D values of R-PE at pH 7.0 decreased with increasing concentrations of urea at all temperatures (Table 6.11). At pH 10.0,the range of heating temperatures was same for all solutions (60-70°C)(Figure 6.5). All D values for R-PE in the urea solutions were lower when compared with the control at pH 10.0. D values decreased as the concentration of urea was increased (Table 6.12).

When comparing z values, no differences ($p>0.05$) were found among R-PE in urea solutions (6.79, 7.43 and 6.23°C) and control (10.70°C) at pH 4.0, indicating that addition of urea at this pH does not affect the thermal resistance of R-PE. At pH 7.0, the z values of

Table 6.11. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in urea solutions in 50 mM NaCl, 20 mM phosphate buffer, pH 7.0

Urea Conc. (M)	T (°C)	D value (min)	X coefficient	Constant	R squared
0	60	2500	-0.0004	3.68	0.93
	70	124	-0.03	3.65	0.93
	80	31	-0.03	3.19	0.86
1.0	60	1515	-0.0007	3.69	0.92
	65	286	-0.003	3.74	0.77
	70	40	-0.03	3.59	0.74
2.0	60	775	-0.001	3.68	0.94
	65	146	-0.007	3.70	0.94
	70	23	-0.04	3.74	0.99
4.0	60	357	-0.002	3.47	0.92
	65	62	-0.02	3.37	0.99
	70	9.2	-0.1	3.41	0.78

Table 6.12. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in urea solutions in 50 mM NaCl, 20 mM glycine buffer, pH 10.0

Urea Conc. (M)	T (°C)	D value (min)	X coefficient	Constant	R squared
0	60	348	-0.003	3.52	0.85
	65	36	-0.03	3.44	0.93
	70	7.2	-0.1	3.27	0.95
0.5	60	125	-0.008	3.58	0.91
	65	18	-0.05	3.45	0.84
	70	4.0	-0.3	3.21	0.81
1.0	60	114	-0.009	3.53	0.89
	65	11	-0.09	3.35	0.96
	70	2.5	-0.4	3.16	0.98
2.0	60	38	-0.03	3.50	0.91
	65	13	-0.08	3.05	0.94
	70	2.1	-0.5	2.84	0.99

R-PE in solutions containing urea (6.33, 6.56 and 6.30°C) did not differ ($p>0.05$), but were lower ($p<0.05$) than that of the control (10.52°C). These results suggest that urea increases the temperature dependence of R-PE denaturation at pH 7.0 At pH 10.0, the z value of the control (5.90°C) and R-PE in 1 M urea solution (6.02°C) were not different ($p>0.05$). In addition, z values of R-PE in 0.5 (6.66°C) and 2 M (7.98°C) solutions were not different ($p>0.05$), but these latter ones were higher ($p<0.05$) from the former ones, suggesting that addition of 0.5 and 2 M urea decreased the temperature dependence of R-PE denaturation at pH 10.0 (Table 6.13). The z value of R-PE in 1 M urea at pH 10.0 (6.02°C) should be further investigated for its possible application as a TTI to predict destruction of *Salmonella* in beef products ($z_{Salmonella}$ = 5.6-6.2°C)

Urea is a very common protein denaturant. It can reduce the magnitude of hydrophobic bonding by up to one-third causing an increase in protein solubility (Creighton, 1993). Urea also affects the structure of water because of its hydrogen-bonding capabilities (Voet and Voet, 1990; Creighton, 1993). In addition, urea molecules can penetrate the inside of the proteins disrupting the packed interior of the polypeptide (Creighton, 1993). Addition of urea resulted in a decrease of thermal stability of R-PE at pH 7.0 and pH 10.0. By promoting the unfolding of R-PE molecules, the chromophores that, under normal conditions are closely packed in the hydrophobic interior of the molecule, are exposed to the environment resulting in a decrease of fluorescence (O'hEocha, 1965; Fujimori and Pecci, 1967). In addition, denaturation of the protein molecule may promote cyclization of the chromophores, which results in reduced fluorescence intensity (Burke et al., 1972; Rudiger, 1992). Jones and Fujimori (1961) reported reduction in R-PE absorbance at 540 and 565 nm with addition of 6 M urea, although this effect was mild when compared to treatment with

Table 6.13. The z values and regression parameters for thermal inactivation curves of R-phycoerythrin in urea solutions in 50 mM NaCl, 20 mM citrate buffer, 4.0, 50 mM NaCl, 20 mM phosphate buffer, pH 7.0 and 50 mM NaCl, 20 mM glycine buffer, 10.0

pH	Urea Conc. (M)	z value (°C)	X coefficient	Constant	R squared
4.0	0	10.7[a]	-0.1	8.12	0.98
	0.1	6.8[a]	-0.2	10.44	0.99
	0.25	7.4[a]	-0.1	9.62	0.99
	0.5	6.2[a]	-0.2	11.19	0.99
7.0	0	10.5[a]	-0.1	8.98	0.96
	1	6.3[b]	-0.2	12.68	0.99
	2	6.6[b]	-0.2	12.04	0.99
	4	6.3[b]	-0.2	12.09	0.99
10.0	0	5.9[a]	-0.2	12.61	0.99
	0.5	6.7[b]	-0.2	11.07	0.99
	1	6.0[a]	-0.2	11.96	0.98
	2	8.0[b]	-0.1	9.16	0.98

Values with different superscripts within the same pH are statistically different ($p<0.05$)

Table 6.14. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in β-mercaptoethanol (ME) solutions in 50 mM NaCl, 20 mM phosphate buffer, pH 7.0

ME Conc. (% v/v)	T (°C)	D value (min)	X coefficient	Constant	R squared
0	60	2500	-0.0004	3.68	0.93
	70	124	-0.03	3.65	0.93
	80	31.44	-0.03	3.19	0.86
0.5	70	385	-0.003	3.67	0.82
	80	51	-0.02	3.18	0.86
	85	36	-0.03	3.40	0.95
1.0	70	315	-0.003	3.63	0.87
	80	57	-0.02	3.15	0.85
	85	38	-0.03	3.46	0.95
2.5	70	183	-0.005	3.54	0.94
	80	68	-0.01	3.19	0.91
	85	37	-0.03	3.47	0.98

4 M guanidine HCl. O'hEocha and O'Carra (1961) reported almost complete quenching of R-PE fluorescence in the presence of 8 M urea for 24 h at 3-5°C, while phycocyanins lost fluorescence immediately under the same conditions.

6.4.5. Effects of β-mercaptoethanol (ME).

Addition of ME to samples before heating increased the fluorescence intensity of R-PE by 5 and 8% at pH 7.0 and 10.0, respectively, regardless of the concentration, suggesting that ME has a protective effect on the native conformation of R-PE. The range of heating temperatures at pH 7.0 was slightly higher for R-PE in ME-containing solutions (70°C-85°C) than the control (60°C-80°C), suggesting that ME had a thermostabilizing effect on R-PE (Figure 6.6). All D values were higher for the three ME concentrations when compared to those of the control at the same temperature. At 70°C, the D values for R-PE at pH 7.0 decreased with increasing concentration of ME, while at 80°C the opposite relationship was observed. D values were not affected by ME concentration at 85°C (Table 6.14).

At pH 10.0, the heating temperature range for R-PE in solutions containing ME (70-85°C) was higher than the control (60-70°C), indicating that ME had a thermostabilizing effect on R-PE at this pH (Figure 6.6). All D values were higher for R-PE in ME solutions when compared to the control. Overall, the D values did not change as concentration of ME was increased (Table 6.15).

The z value of R-PE at pH 7.0 (10.52°C) was not affected ($p>0.05$) by addition of 0.5 or 1% ME (10.71 and 10.51°C, respectively). The z value of R-PE in 2.5% ME (11.04°C) at

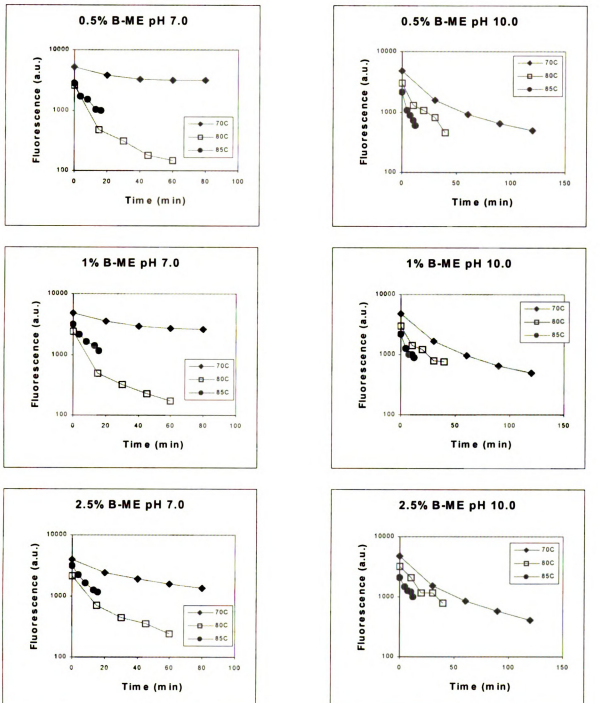


Figure 6.6. Thermal inactivation curves of phycoerythrin in β-mercaptoethanol solutions in 50 mM NaCl, 20 mM phosphate and glycine buffer, pH 7.0 and 10.0, respectively.

Table 6.15. D values and regression parameters for thermal inactivation curves of R-phycoerythrin in β-mercaptoethanol (ME) solutions in 50 mM NaCl, 20 mM glycine buffer, pH 10.0

ME Conc. (% v/v)	T (°C)	D value (min)	X coefficient	Constant	R squared
0	60	348	-0.003	3.52	0.85
	65	36	-0.03	3.44	0.93
	70	7.2	-0.1	3.27	0.95
0.5	70	127	-0.008	3.54	0.91
	80	54	-0.02	3.41	0.94
	85	22	-0.05	3.30	0.98
1.0	70	127	-0.008	3.55	0.93
	80	69	-0.02	3.40	0.89
	85	31	-0.03	3.30	0.94
2.5	70	117	-0.009	3.58	0.93
	80	67	-0.02	3.47	0.94
	85	40	-0.03	3.31	0.98

Table 6.16. The z values and regression parameters for thermal inactivation curves of R-phycoerythrin in β-mercaptoethanol (ME) solutions in 50 mM NaCl, 20 mM phosphate buffer, pH 7.0 and 50 mM NaCl, 20 mM glycine buffer, 10.0

pH	ME Conc. (%)	z value (°C)	X coefficient	Constant	R squared
7.0	0	10.5^{a}	-0.1	8.98	0.96
	0.5	10.7^{a}	-0.1	6.64	0.99
	1.0	10.5^{ab}	-0.1	6.71	0.98
	2.5	11.0^{b}	-0.1	6.43	0.98
10.0	0	5.9^{a}	-0.2	12.61	0.99
	0.5	12.0^{b}	-0.1	5.17	0.99
	1.0	11.6^{bc}	-0.1	5.28	0.99
	2.5	11.8^{c}	-0.1	5.19	0.99

Values with different superscripts within the same pH are statistically different ($p<0.05$)

pH 7.0 was higher ($p<0.05$) than the control and 0.5% ME. At pH 10.0, the z value of the control (5.90°C) was lower ($p<0.05$) than all ME solutions (12, 11.62 and 11.84°C, respectively) (Table 6.16). No differences were found ($p>0.05$) between the z value of R-PE in 1% ME and 0.5% or 2.5% ME at pH 10.0. These results suggest that at pH 7.0 only 2.5% ME concentration affected the temperature dependence of R-PE denaturation, but addition of ME at 0.5% concentration or above increased the thermal resistance of R-PE at pH 10.0. The z values of R-PE in 0.5 and 1% ME at pH 7.0 could be explored further for its possible use as TTIs in the area of sterilization of canned foods ($z_{C.\ botulinum} = 10°C$).

In our experiments, the addition of ME had a marked protective effect on R-PE's fluorescence at both pHs when compared to the control. At this point, no specific conclusions can be made with respect of the mechanism of action for this effect since no evidence of disulfide bonds in the native structure have been observed (Sidler et al., 1989; Ficner et al., 1992). We can speculate that addition of ME may have prevented photobleaching of the chromophores. In addition, ME, being a reducing agent, may protect the protein against oxidation. Jones and Fujimori (1961) reported that the 565 nm absorption peak of R-PE was highly unstable in the presence of 1 % hydrogen peroxide. The authors concluded that the chromophore and apoprotein were susceptible to changes in oxidation-reduction potential.

6.4.6. Implications for food processing.

As mentioned before, a reliable TTI should show the same time-temperature dependent response as that of the target attribute when the temperature is the only rate-determining factor. This happens when the z value (E_A) of both TTI and target attribute are the same (Hendrickx et al., 1995). Therefore, appropriate selection of TTI and target attribute is of utmost importance. Deviations between z values (E_A) of TTI and target

attribute can result in over- or underestimation of the actual lethality of the process. According to Van Loey et al. (1995b), a TTI with a z value larger than the one for the target attribute will result in underestimation of the process lethality if the processing temperatures are above the reference temperature. The process lethality will also be underestimated if the z value of the TTI is smaller than the one for the target and the processing temperature is below the reference temperature. However, if the z value for TTI is smaller than the one for the target attribute, the process lethality will be overestimated when the processing temperature is above the reference temperature. It will also be overestimated if the z value for TTI is larger than the one for the target and the processing temperature is below the reference temperature (since z value and E_A are inversely proportional, the complete opposite will be applied for the Arrhenius model) (Table 6.17). This phenomenon has also been described by Pflug (1997) and Incze et al. (1999). In practice, however, and as seen in certain foodborne outbreaks, overestimation of the process lethality is much more dangerous than underestimation.

Table 6.17. Effects of the holding temperature and z value (E_A) of a time-temperature integrator on the actual processing value reported (Source: Van Loey et al., 1995b)

TDT-model	$z_{TTI}<z_{Target}$	$z_{TTI}>z_{Target}$
$T_H<T_{ref}$	${}^{TTI}F_{Tref}<{}^{Target}F_{Tref}$	${}^{TTI}F_{Tref}>{}^{Target}F_{Tref}$
$T_H > T_{ref}$	${}^{TTI}F_{Tref}>{}^{Target}F_{Tref}$	${}^{TTI}F_{Tref}<{}^{Target}F_{Tref}$
Arrhenius-model	$E_{ATTI}<E_{ATarget}$	$E_{ATTI}>E_{Atarget}$
$T_H<T_{ref}$	${}^{TTI}F_{Tref}>{}^{Target}F_{Tref}$	${}^{TTI}F_{Tref}<{}^{Target}F_{Tref}$
$T_H > T_{ref}$	${}^{TTI}F_{Tref}<{}^{Target}F_{Tref}$	${}^{TTI}F_{Tref}>{}^{Target}F_{Tref}$

TH = holding temperature, Tref = reference temperature, TTIFTref =process lethality calculated from the TTI, TargetFTref =actual process lethality observed in the target attribute, TTIFTref<TargetFTref =underestimation, TTIFTref>TargetFTref =overestimation.

6.5. CONCLUSIONS

The thermal inactivation parameters (D and z values) of R-phycoerythrin as measured by fluorescence loss were modified by addition of sugars or denaturants and compared with control samples where no chemicals had been added. Overall, sucrose and ME had a stabilizing effect, while SDS, NaCl and urea caused protein denaturation. The conformation of the protein in solution played a major part in the denaturation. At pH 4.0, near its pI, R-PE exists in the form of aggregates; at pH 7.0, it has a compact, native conformation, with its chomophores tightly packed in the hydrophobic interior, and at pH 10.0, the protein is in a partially unfolded form (Ogawa et al., 1991). Addition of sucrose, SDS, NaCl or ME at pH 4.0 resulted in complete loss of R-PE fluorescence before heating. Comparatively, at pH 10.0, the protein, being already partially unfolded was more sensitive to addition of the cosolvents than at pH 7.0, where the protein exists in its native conformation.

These results are of particular significance in the area of protein-based time-temperature integrators for monitoring thermal processes in meat products. By modifying the z value of R-PE, it can be adjusted to that of a target microorganism found in meats. Reported z values for *Salmonella*, the target microorganism in cooked beef products, ranged from 5.6°C (Goodfellow and Brown, 1978) to 6.2°C (Orta-Ramirez et al., 1996). The z value obtained for R-PE in 50 mM NaCl, 20 mM glycine buffer, pH 10.0 was 5.90°C, which falls in the middle of this range. The development of a TTI with R-PE as the detector component could be used to monitor thermal processes in beef products.

CHAPTER 7: Validation of R-phycoerythrin as a time-temperature integrator to monitor thermal processes in beef products

7.1. ABSTRACT

Time-temperature integrators (TTIs) can be used to rapidly and accurately verify thermal processing of foods because they respond predictably to thermal history. When they function properly, TTIs predict the same lethality as that of a target microorganism or quality attribute of interest, when the two systems are subjected to the same thermal process. *Salmonella* was identified as the target microorganism in this study. Linear and non-linear models were used to fit experimental heat inactivation data of the protein R-phycoerythrin. D and z values were calculated on the basis of fluorescence loss using linear regression analysis. Kinetic parameters (n, k and E_A) were estimated using non-linear regression analysis. The reaction orders (n) calculated for R-PE and *S. senftenberg* were 0.55 and 1.2, respectively. The calculated E_A for R-PE and *S. senftenberg* were 347.4 and 220.84 kJ $mole^{-1}$, respectively. The lethalities calculated for R-PE and *S. senftenberg* assuming non-linear kinetics were highly predictable (R^2 of 0.98 for R-PE and 0.93 for *S. senftenberg*). Results strongly suggest that lethality of *Salmonella* can be predicted from R-PE fluorescence loss.

7.2. INTRODUCTION

Thermal processing is the most common method used for food preparation and preservation. The principal effects of the heat treatment are: a) modification of the organoleptic properties to give the desirable characteristics that will make the product

acceptable to the consumer; b) inactivation of enzymes and spoilage microorganisms, with the consequent increase in shelf-life of the product; and, c) destruction of pathogenic bacteria to ensure the safety of the cooked product. Mathematically, the impact of a thermal process can be expressed in terms of D and z values (Singh and Heldman, 1993; Hendrickx et al., 1995). The D value, or thermal reduction time, is the time in minutes required to reduce a microbial population by 90% or one log cycle. The z value is defined as the temperature increase necessary to reduce a D value by 90% or 1 log. The determination of the D and z values for a particular microorganism is very useful in designing a thermal process that destroys that specific microorganism. However, even in products that are thermally processed, contamination can be a problem. Inadequate cooking and improper storage and holding temperatures are the most common errors that lead to foodborne outbreaks (Todd, 1989).

Recently, time-temperature integrators (TTIs) have been introduced as an alternative for the monitoring of thermal processes. By definition, a TTI is a small device that responds to time-temperature history by undergoing an irreversible precisely measurable change in a manner that mimics the changes of a target attribute exposed to the same thermal history (Hendrickx et al., 1995). An important advantage of TTIs is that they allow fast and reliable validation of a process without the need for detailed information on the actual time-temperature profile within the product.

The United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) has recently proposed a modification of the current thermal processing regulations for meat products based on destruction of the pathogenic microorganism *Salmonella* expressed as log reduction. The USDA requires that manufacturers use a

combination of thermal and non-thermal processes to achieve a 6.5-log *Salmonella* reduction in ready-to-eat, cooked beef, roasted beef and cooked corned beef and a 7-log reduction in ready-to-eat poultry products. A 5-log reduction in ready-to-eat, cooked uncured meat patties has been proposed, but this may be increased (Federal Register, 1999a). The development of a TTI with the same z value as *Salmonella* in beef and poultry products could be used to determine the adequacy of any log reduction process.

The main objective of this project was to evaluate the applicability of a fluorescent protein, R-phycoerythrin, to be used as a TTI for predicting *Salmonella* destruction in cooked beef products.

7.2.1. Theoretical considerations.

The thermal death time (F value) is the time required to achieve a particular reduction in a target attribute (quality or safety index) value (Hendrickx et al., 1995). The F value can be computed from:

$$F = D_T \log (C_0/C) \qquad (1)$$

where F has units of time, D_T is the decimal reduction time at a given temperature T, and C_0 and C are the initial and final values, respectively, of the target attribute. Thus, C_0 and C may refer to the concentration of a microorganism, activity of an enzyme, or other measurable parameter specific to the target attribute chosen.

A TTI must show the same time-temperature dependent response as that of the target attribute when the temperature is the only rate-determining factor. Mathematically, this can be written as:

$$F_{Target} = F_{TTI} \qquad (2)$$

Assuming an nth order TTI system, the rate equation can be expressed as:

$$dC/dt = - k C^n \quad (3)$$

where k is the reaction constant and n the reaction order. For a first order reaction under isothermal conditions, the equation can be integrated to give:

$$ln (C_0/C) = kt \quad (4)$$

where C_0 is the initial value of the target attribute and C is the value of a target attribute at a time t, and k is the reaction rate constant.

For a non-first order reaction (n^{-1}), the integrated expression can be written as:

$$[1/(n-1)] (C^{1-n} - C_0^{1-n}) = kt \quad (5)$$

In many cases, the rate constant can be expressed by the Arrhenius equation:

$$k = k_0 exp (-E_A / RT) \quad (6)$$

where k_0 is the pre-exponential factor, E_A is the activation energy, R is the universal gas constant and T is the absolute temperature.

In the general case where the reaction order is other than unity, and the reaction rate constant is independent of the order of reaction, the F value for an isothermal process can be written as (Hendrickx et al., 1995):

$$(F_{Tref})_x = \int_0^t \exp \frac{E_A}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right) dt \quad (7)$$

where F_{Tref} represents the equivalent heating time at a reference temperature resulting in the same lethality as the time-varying temperature profile. This equation is valid for the target attribute as well. Therefore equation (2) will only be satisfied when the activation energies of both the TTI and the target attribute are the same. In other words, for a parameter to be used

as a TTI, it must have identical activation energy to that of the attribute of interest. Mathematically this can be written as:

$$E_{ATTI} = E_{ATarget} \quad (8)$$

Since z values are equivalent to activation energies, equation (6) can also be expressed as:

$$Z_{TTI} = Z_{Target} \quad (9)$$

7.3. MATERIALS AND METHODS

7.3.1. Source and preparation of R-phycoerythrin.

R-phycoerythrin (R-PE) was extracted and purified as explained in Chapter 2. Pure R-PE was stored in 60% (w/v) ammonium sulfate and kept at 4°C. Prior to the experiments, R-PE was dialyzed overnight against distilled deionized water at 4°C, then diluted immediately before use in 0.012 M borate buffer, pH 9.0 to achieve an absorbance of 0.1 at 565 nm.

7.3.2. Isothermal experiments.

Isothermal inactivation experiments were conducted as described in Chapter 3. Capillary tubes containing 150 μL of R-PE in 0.012 M borate buffer, pH 9.0 were placed in a wire rack and immersed in a Polystat circulator bath (Model 1268-52, Cole-Parmer Instrument Company, Chicago, IL) connected to a bath programmer (Model 1268-62, Cole-Parmer). Temperature in the capillary tubes was monitored using a thermocouple (Resistance Temperature Detector probe, Pt 100, 0.15 cm diameter, (1°C accuracy) inserted in a control capillary tube containing 150 μL of the same solution. The thermocouple was connected to a Solomat MPM 200 Modumeter (Solomat Partners LP). Zero time was defined

as the time when the R-PE solution reached the target temperature. The heating lag time in the capillary tubes was 10^{-1} sec.

Preliminary studies were performed to calculate the times and temperature necessary to achieve at least a one log reduction in fluorescence. Temperatures ranged from 60°C to 70°C. The tubes were removed at predetermined intervals of time and immediately placed in an ice-water bath, then kept at 4°C until fluorescence was read within 6 h. Preliminary studies were conducted to show that no changes in fluorescence occurred during the holding period. Each experiment was performed in triplicate.

7.3.3. Non-isothermal experiments.

Non-isothermal inactivation experiments were performed in a temperature controlled, programmable thermocycler (GeneAmp PCR System, Model 9600, Perkin Elmer, Norwalk, CT). R-phycoerythrin solutions (125 μL) were placed in 0.2 mL thin-wall microtubes with attached cap (Cat. No PCR-02A, United Scientific Products, San Leandro, CA). Heating protocols representing roast beef and hamburger cooking requirements were performed based on USDA requirements (USDA-FSIS, 1997) to achieve properly cooked, undercooked and overcooked samples (Table 7.1). During the experiments, temperature was increased gradually (Δ = 11°C/min) to the target temperature, followed by a holding period, then decreased at the same rate. Samples were allowed to equilibrate at 25°C inside the thermocycler immediately before and after the heating process. The tubes were cooled in an ice-water bath immediately after removal from the thermocycler.

Predicted fluorescence values corresponding to the heating protocols were calculated from the temperature profiles according to the General Method (Pflug, 1997) using the following expression:

$$Cp = C_0^{\,10\,(-t/Dref)} \qquad (10)$$

where Cp is the predicted fluorescence and C_0 is the initial fluorescence, t is the lethality (in min) calculated for each process, and Dref is the D value at the reference temperature (65°C). Each experiment was performed in triplicate.

The residual fluorescence of the R-PE solutions after heating was analyzed for statistical differences. One way analysis of variance (ANOVA) for the time-temperature combinations was conducted using JMP Version 3.2.2. (SAS Institute Inc., 1995). Mean fluorescence values were compared using Tukey-Kramer HSD test using the mean square error at the 5% level of probability.

7.3.4. Fluorescence measurement.

R-PE fluorescence at the maximum emission was measured using a CytoFluor II Microwell Fluorescence reader and the CytoFluorII software, Version 2.0c (Biosearch Incorporated, Bedford, MA). Heated and unheated R-PE solutions were placed in 96-well fluorescence reader plates (Cat. No. DG5515, Life Science Products Inc., Denver, CO). Excitation was set at 485/20 nm and emission at 590/35 nm.

7.3.5. Experimental heat inactivation data of *S. senftenberg*.

Previously published isothermal inactivation data of *S. senftenberg* (Orta-Ramirez et al., 1995) was used to test the R-PE model. Briefly, sterile ground beef was inoculated with *S. senftenberg* (ATTC 43485) to achieve levels of 10^7 microorganisms/g meat. Isothermal inactivation at 53, 58, 63 and 68°C was performed in the same fashion as above except that

Table 7.1. Time-temperature schedules for non-isothermal heating of R-phycoerythrin in 0.012 M borate buffer, pH 9.0.

Sample No.	Target T (°C)	Holding Time	Heating/ Cooling Lag Time	Cooking Status[a]
1	60.0	0	3 min 10 sec	Undercooked
2	60.0	9 min	3 min 10 sec	Undercooked
3	60.0	12 min	3 min 10 sec	Properly cooked
4	60.0	24 min	3 min 10 sec	Overcooked
5	62.7	0	3 min 25 sec	Undercooked
6	62.7	1.5 min	3 min 25 sec	Undercooked
7	62.7	3 min	3 min 25 sec	Properly cooked
8	62.7	6 min	3 min 25 sec	Overcooked
9	66.1	0	3 min 40 sec	Undercooked
10	66.1	41 sec	3 min 40 sec	Properly cooked
11	66.1	82 sec	3 min 40 sec	Overcooked
12	69.4	0	4 min	Undercooked
13	69.4	10 sec	4 min	Properly cooked
14	69.4	20 sec	4 min	Overcooked
15	69.4	40 sec	4 min	Overcooked

[a]Based on USDA roast beef and hamburger cooking requirements (USDA-FSIS, 1997).

one-gram samples were filled in sterile 10 x 75 thermal death time tubes (14-961-25, Fisher Scientific, Pittsburgh, PA). The tubes were heated to internal target temperatures and held for precalculated intervals of time to achieve at least a one-log reduction in *Salmonella*. Bacterial counts were determined by decimal dilution of the ground beef samples in sterile 0.1% buffered peptone water and plated on Petrifilm(Coliform Count Plates (3M, St. Paul, MN). All samples were incubated at 37°C for 24 h. Plate counts (CFU/mL) were converted to logarithms. Plate counts less than 10 were entered as zeros. Calculation of D and z values was conducted as explained in Section 7.3.6.

7.3.6. Data analysis.

Fluorescence counts were converted to logarithms. D and z values were initially estimated assuming first-order kinetics (Pflug, 1997). D values (in minutes) were calculated by linear regression of fluorescence vs time data, using Microsoft Excel Version 5.0a (Microsoft Corporation, Redmond, WA). Thermal resistance curves were determined by plotting log D values vs temperature. The z value was determined as the negative reciprocal of the slope of the thermal resistance curve.

Kinetic parameters (n and k) were calculated using non-linear regression analysis (Sigma Plot v. 4.01, SPSS Inc., Chicago, IL). First, the D values from the isothermal experiments were used to estimate the rate constant (k) at each temperature according to the relationship:

$$k = 2.303/D \tag{11}$$

The estimated k values for each temperature were used to calculate the reaction order (n) using the following nth-order model, which results from rearranging Equation (5):

$$(C/C_0) = [(n-1)k_e\, t + 1]^{[1/(1-n)]} \tag{12}$$

where C/C_0 is the normalized fluorescent intensity (ratio of fluorescence at time t to fluorescence at time zero), k_e is the rate constant estimated from the D value at the same temperature, and t is time in minutes. The average n obtained from the five temperatures was, in turn, used to re-calculate the rate constant (k) using the model above and solving for k at each temperature. Rate constants were assumed to follow the Arrhenius law:

$$k = k_0\, exp[-E_A/(RT)] \tag{13}$$

where k_0 is the pre-exponential factor, E_A is the activation energy of the process, R is the universal gas constant (8.314 J mole K^{-1}), and T is the temperature.

7.4. RESULTS AND DISCUSSION

7.4.1. Isothermal treatment.

The D values of R-PE in 0.012 M borate buffer, pH 9.0 ranged from 512.82 at 60°C to 10.57 min at 70°C (Table 7.2). A z value of 5.99°C was calculated from the linear regression of log D vs temperature and fell within the range of z values reported for *Salmonella*, the target microorganism in thermal processing of beef products (USDA-FSIS, 1997). Published z values for this microorganism in ground beef ranged from 5.6°C to 6.2°C (Goodfellow and Brown, 1978; Orta-Ramirez et al., 1995). Variations in z values could be due to different bacterial strains, physiological condition of the cells, fat content and pH of the meat, and the method employed for enumeration of survivors. Goodfellow and Brown (1978) used a mixture of *Salmonella* strains and two enumeration methods: Plate count agar overlayed with supplemented XL agar base, and the most probable number technique. The authors did not mention the fat content of the meat employed in their experiments. Orta-Ramirez et al. (1995) used *S. senftenberg* enumerated on Petrifilm Coliform Count Plates. The fat content of the meat in the study was 3.3% and the pH was 6.3.

Table 7.2. Summary of D and z values and regression analysis for R-phycoerythrin in 0.012 M borate buffer, pH 9.0.

T (°C)	D value (min)	X coefficient	Constant	R squared
60	513	-0.002	3.6	0.85
62.5	203	-0.005	3.4	0.85
65	65	-0.02	3.3	0.87
67.5	33	-0.03	3.1	0.87
70	11	-0.1	3.3	0.94
z value	5.99	-0.2	12.7	0.99

Traditionally, the thermal inactivation of microorganisms and proteins has been assumed to follow first-order kinetics (Kormendy and Kormendy, 1997; Peleg and Cole, 1998). In addition, methods for estimating the safety of commercial thermal processes are also based on this assumption (Peleg and Cole, 1998). Even though at lower temperatures regression analysis of R-PE seemed to deviate slightly from linearity, the high square coefficients ($R^2 > 0.85$ for D values at all temperatures and 0.99 for the z value) seemed adequate to assume first-order kinetics to initially and simply fit the inactivation of R-PE. After determining the thermal inactivation parameters, R-PE was verified using non-isothermal heating experiments in an attempt to simulate real thermal treatments used in the processing of beef products.

7.4.2. Non-isothermal treatment.

The non-isothermal experiments were conducted in the 60-69.4°C temperature range using published requirements (USDA-FSIS, 1997). During the experiments, temperature was increased gradually ($\Delta T = 11°C/min$) to the target temperature, followed by a holding period that had been selected to obtain properly cooked, undercooked and overcooked samples based on USDA thermal processing regulations in beef products (Table 7.1).

Residual fluorescence of R-PE decreased for all heat treatments when compared to the unheated protein ($p < 0.01$) (Figure 7.1). In addition, residual fluorescence decreased as holding time was increased at each temperature ($p < 0.01$). In other words, at a given temperature, residual fluorescence of R-PE could be used to distinguish between undercooked, properly cooked and overcooked samples.

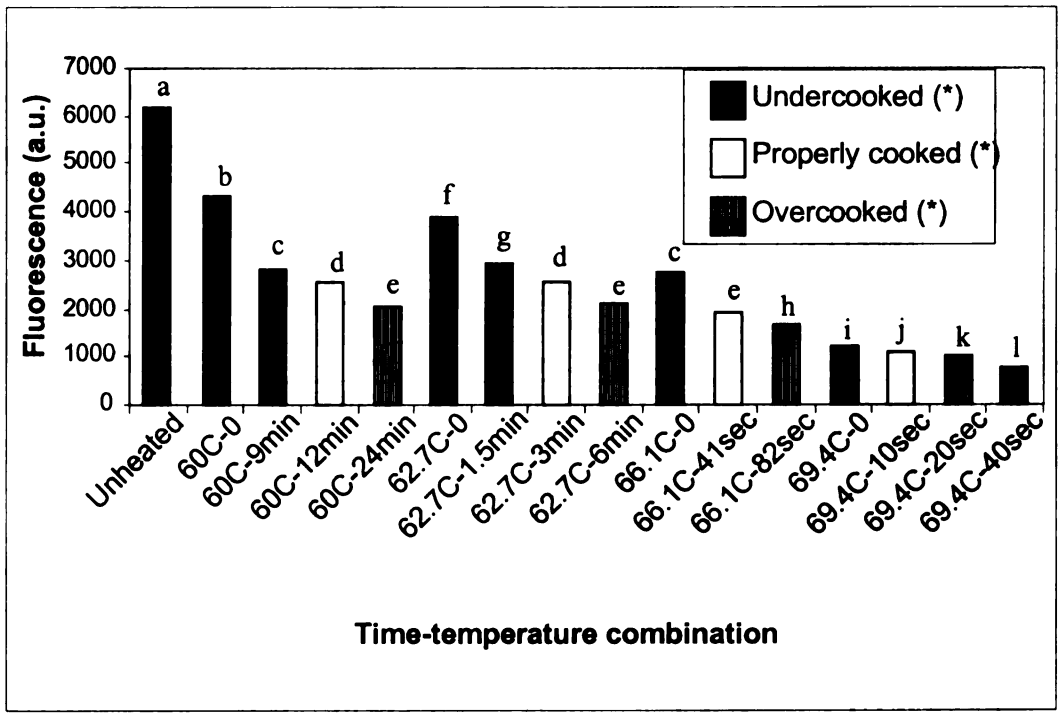


Figure 7.1. Residual fluorescence in arbitrary units (a.u.) of R-phycoerythrin in 0.012 M borate buffer, pH 9.0, after non-isothermal heating

Table 7.3. Residual fluorescence of R-phycoerythrin after thermal processing under USDA cooking regulations.

Temperature (°C)	Holding time	Cooking Status	Fluorescence [1a] (a.u.)
60.0	0	Undercooked	4349±92 [b]
	9 min	Undercooked	2791±90 [c]
	12 min	Properly cooked	2523±88 [d]
	24 min	Overcooked	2031±223 [e]
62.7	0	Undercooked	3889±62 [f]
	1.5 min	Undercooked	2910±81 [g]
	3 min	Properly cooked	2529±30 [d]
	6 min	Overcooked	2085±105 [e]
66.1	0	Undercooked	2723±73 [c]
	41 s	Properly cooked	1929±25 [e]
	82 s	Overcooked	1636±43 [h]
69.4	0	Undercooked	1212±50 [i]
	10 s	Properly cooked	1111±26 [j]
	20 s	Overcooked	1007±47 [k]
	40 s	Overcooked	752±108 [l]

[1]Fluorescence values expressed as mean ± standard deviation
[a]Values with different superscripts are statistically different ($p<0.05$)

Comparing residual fluorescence at equivalent treatments, the residual fluorescence decreased as cooking temperatures increased (Table 7.3). This suggests that, for the safe use of this TTI, the person handling this device would need to know at least the actual temperature of the thermal process. By establishing a cut-off value of residual fluorescence for each temperature, one can know if the product has been undercooked or properly thermal processed. When comparing properly cooked samples, R-PE fluorescence was not different ($p>0.05$) between samples cooked to 60 or 62.7°C ($p<0.05$), but fluorescence was higher than in samples cooked to 66.1 or 69.4°C. In addition, fluorescence was higher ($p<0.05$) in samples properly cooked at 66.1°C than at 69.4°C. Ideally, fluorescence values should have been the same among equivalent processes (i.e. among either undercooked, properly cooked

or overcooked), which, theoretically, result in similar lethalities (Goodfellow and Brown, 1978; USDA-FSIS, 1997). Results suggest that R-PE fluorescence did not respond accurately to the combination of time and temperature.

Predicted fluorescence values corresponding to the heating protocols were calculated from the temperature profiles using the General Method (Pflug, 1997) using 65°C as the reference temperature. Predicted fluorescence values of R-PE were compared to the observed fluorescence values (Figure 7.2). Predicted values were much higher than the observed values, suggesting that, in practice, the heat treatment had a stronger effect on R-PE kinetics than theoretically expected. When observed and predicted fluorescence values were compared, there was weak correspondence ($R^2 < 0.80$), indicating that the non-isothermal inactivation of R-PE did not follow first-order kinetics. When residual fluorescence (expressed as C/C_0, where C is fluorescence at time t and C_0 is initial fluorescence) was compared to the calculated lethality for each of the processes, an exponential relationship was found, which a square coefficient of 0.88 (Figure 7.3). This indicated that fluorescence loss of R-PE upon cooking, could be used as an indicator of thermal process lethality, regardless of its kinetic order. Several enzymes have thermal inactivation kinetics that do not follow first-order, including: horseradish peroxidase (Weng et al., 1991a and b; Hendrickx et al., 1992a and b; Saraiva et al., 1996; Garcia et al., 1998); acid phosphatase (Incze et al., 1999); and *Bacillus* spp α-amylase (DeCordt et al., 1992a and b, 1993, 1994a and b; Van Loey et al., 1995b, 1997a and b), and have been used to monitor process lethality. This non-linear behavior has been explained by the presence of two or more isoforms of the enzyme showing different (Chapter 2) have reported the presence of two or more isoforms of R-PE, as determined on isoelectric focusing, that could account for our findings.

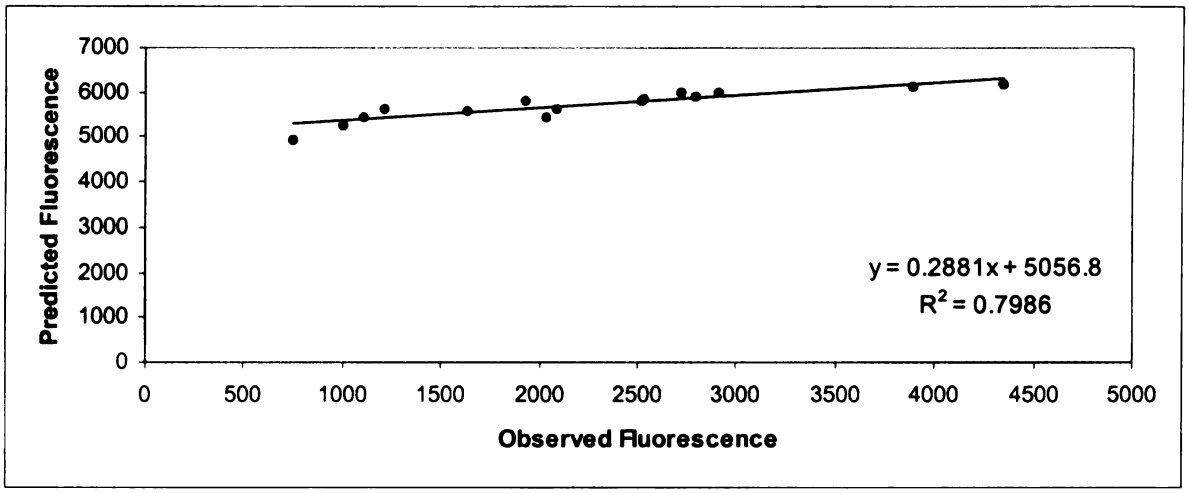


Figure 7.2. Comparison of observed residual fluorescence and predicted fluorescence as calculated by the General Method of R-phycoerythrin in 0.012 M borate buffer, pH 9.0 after non-isothermal experiments.

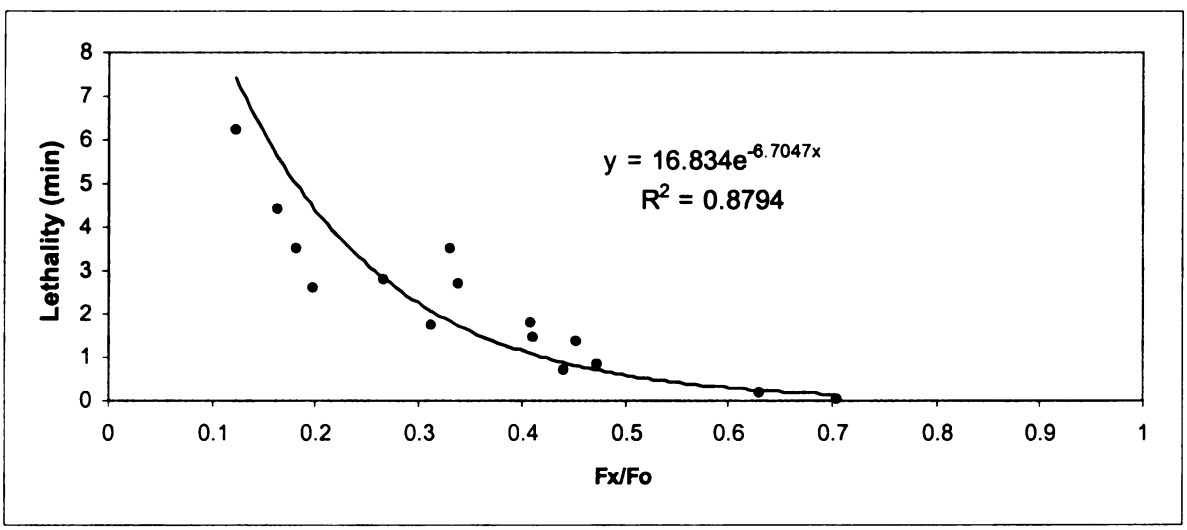


Figure 7.3. Comparison of fluorescence of R-phycoerythrin in 0.012 M borate buffer, pH 9.0, after non-isothermal heating, and experimental lethality of the thermal process.

In view of these results, experimental data of R-PE obtained with isothermal studies was used to develop a non-linear model that could explain the thermal inactivation kinetics of R-PE.

7.4.3. The nth-order model.

The D values obtained in the isothermal experiments of R-PE were used to estimate the reaction constant (k_e) for R-PE in borate buffer, pH 9.0, at 60°C, 62.5°C, 65°C, 67.5°C and 70°C. For each temperature, ke was used to calculate the reaction order (n). The average n obtained from the data sets was, in turn, used to calculate the rate constant (k) at each temperature. The same procedure was applied to experimental data of *S. senftenberg* (Orta-Ramirez et al., 1995) to calculate the rate constant of this microorganism in ground beef. Using the D values obtained in isothermal experiments, we calculated a reaction order of n = 0.55 for R-PE (Table 7.4) while *S. senftenberg* had a reaction order of n = 1.2 (Table 7.5). Peleg and Cole (1998) analyzed previously published survival curves of several microorganisms, including *S. typhimurium*, and demonstrated that they did not follow first-order kinetics, and, in fact, the reaction orders for all cases were substantially different from unity. Van Loey et al. (1995) determined fractional reaction orders for quality factors in green peas and white beans. Calculated rate constants were lower for R-PE (Table 7.6) than for *S. senftenberg* (Table 7.7) at similar temperatures (62.5-63°C and 67.5-68°C), indicating that *S. senftenberg* was more heat sensitive than the protein.

The rate constants (k) for each temperature obtained with the nth-order model were transformed into D values and compared to those obtained assuming first-order kinetics for both R-PE and *S. senftenberg*. Although both D-z and k-E_A models can be applied with the same accuracy over a certain temperature range, the D-z model is more generally adopted in

Table 7.4. Estimation of reaction order (n) for R-phycoerythrin in 0.012 M borate buffer, pH 9.0, using an nth-order model.

T (°C)	n	Std. Error	CV (%)
60	0.52	0.14	27.5
62.5	0.58	0.13	22.8
65	0.45	0.13	29.2
67.5	0.56	0.16	21.5
70	0.61	0.09	14.9

Table 7.5. Estimation of reaction order (n) for *Salmonella senftenberg* in ground beef using an nth-order model.

T (°C)	n	Std. Error	CV (%)
53	0.94	0.08	8.75
58	0.93	0.04	4.33
63	1.38	0.09	6.62
68	1.54	0.06	4.05

Table 7.6. Estimation of reaction constant (k) for R-phycoerythrin in 0.012 M borate buffer, pH 9.0, using an nth-order model.

T (°C)	k	Std. Error	CV (%)
60	0.006	0.0005	8.5
62.5	0.01	0.001	10.0
65	0.04	0.004	8.7
67.5	0.07	0.007	9.7
70	0.2	0.02	6.6

Table 7.7. Estimation of reaction constant (k) for *Salmonella senftenberg* using an nth-order model.

T (°C)	k	Std. Error	CV (%)
53	0.18	0.004	2.4
58	0.30	0.01	3.5
63	0.98	0.07	7.1
68	6.59	0.03	4.5

the area of food science (Hendrickx et al., 1995). The D values for R- PE calculated either by linear or nth-order kinetics were very similar except at 60°C At this temperature, the D value calculated assuming first-order kinetics was 512.8 min while when assuming non-linear kinetics the resulting D value was 388.8 min (Figure 7.4). For *S. senftenberg*, the D values at the 63°C and 68°C were very similar when calculated assuming linear or non-linear kinetics. At 53°C and 58°C the D values calculated assuming first-order (53.5 and 15.2 min, respectively) were noticeably higher than when the D values were calculated assuming non-linear kinetics (12.8 and 7.7 min, respectively) (Figure 7.5).

Overestimation of D values can result in an over- or underestimation of the thermal processing value (F value). The F value is the time required to achieve a stated reduction in a population of microorganisms, and is usually expressed as a multiple of the D value (Singh and Helman, 1993; Hendrickx et al, 1995). Let's assume we want to design a thermal process that results in a 5D reduction in *Salmonella* at 58°C: if we use the D value (15.2 min) calculated using linear regression, 5 x 15.2 min = 76 min, while if we use the D value (7.7 min) obtained using non-linear kinetics, 5 x 7.7 min = 38.5 min, which is half of the previous one. Obviously, this has serious implications for both consumers and meat processors. Underestimation may pose a hazard to the consumer because of the possible foodborne contamination. But, also, overestimation and subsequently, overcooking, may result in loss of quality and nutritional value of the final product, while increasing the cost of production due to unnecessary higher energy consumption.

Isothermal inactivation of *S. senftenberg* in ground beef (Orta-Ramirez et al., 1995) was conducted in thermal death time tubes (10 mm diameter) while for R-PE it was performed in capillary tubes. The advantage of using the latter is that the time required by the

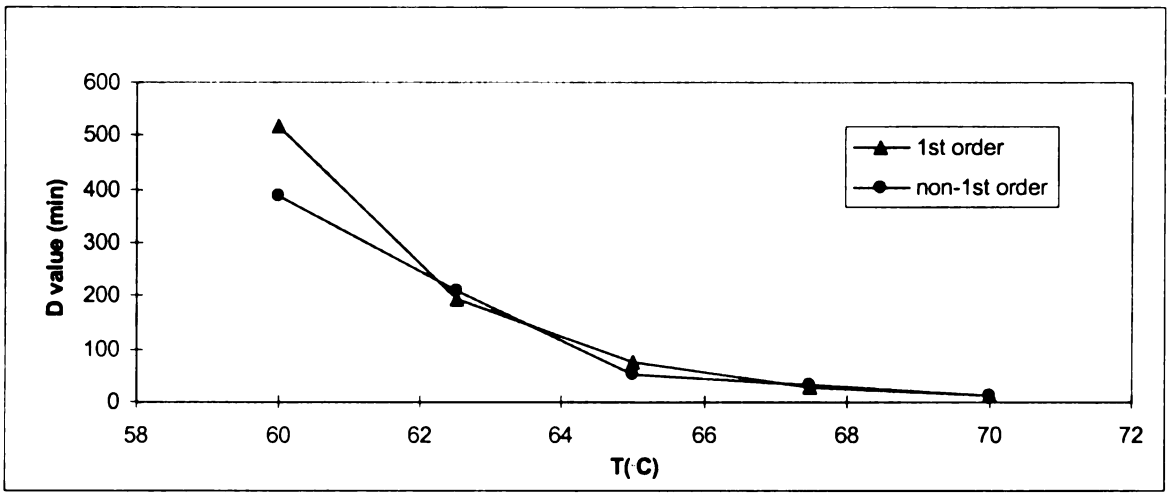


Figure 7.4. Thermal resistance curves of R-phycoerythrin in 0.012 M borate buffer, pH 9.0, calculated using linear (n=1) and non-linear kinetics (n=0.55).

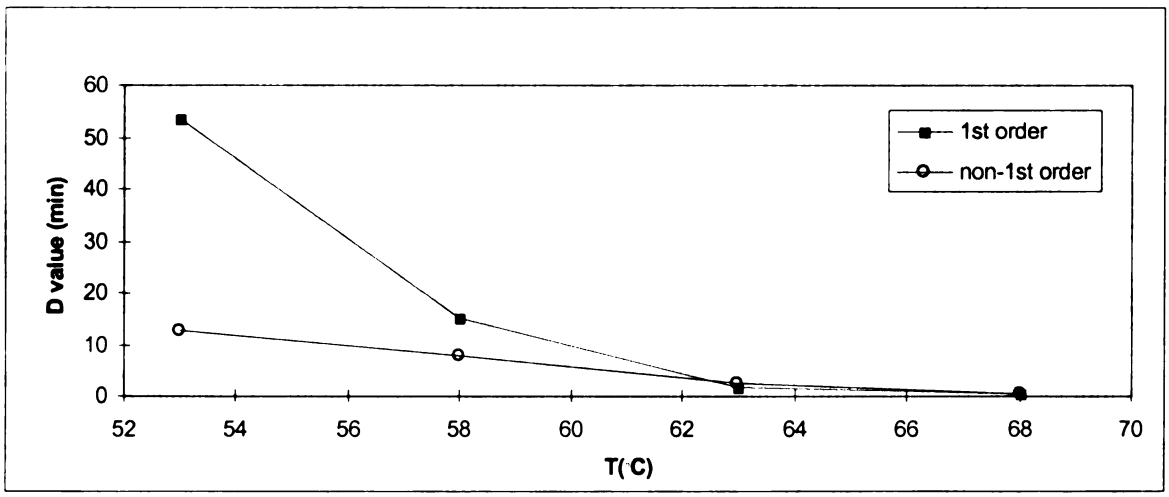


Figure 7.5. Thermal resistance curves of *Salmonella senftenberg* in ground beef calculated using linear (n=1) and non-linear kinetics (n=1.2).

sample to reach the target temperature (heating lag time) can be neglected. In the case of *S. senftenberg*, the use of a meat model system did not allow for the utilization of capillary tubes. This could have interfered with the precision in calculating D values. At the lower temperatures, long heating lag times may result in an actual increase of microbial numbers. At high temperatures, when the microorganism is most sensitive, a considerable destruction of *Salmonella* can occur in the surface of the product while the center has not yet reached the target temperature.

7.4.4. Validation of parameter estimates for the nth-order model to predict thermal inactivation of *S. senftenberg*.

In order to compare the inactivation kinetics of R-PE and *S. senftenberg*, the activation energies (E_A) for both were calculated by a linear regression of ln k vs 1/T. For R-PE we calculated an E_A of 347.41 kJ/mole over the 60-70°C temperature range (Figure 7.6) and for *S. senftenberg* an E_A of 220.84 kJ/mole over the 53-68°C range (Figure 7.7). In both cases, the kinetics were very predictable, showing squared coefficients of 0.93 for *S. senftenberg* and 0.98 for R-PE (Table 7.8). This suggests that, regardless of the discrepancies in model parameters ($E_{Salmonella} \neq E_{PE}$), a mathematical expression could be derived to predict *Salmonella* lethality from R-PE fluorescence loss.

Table 7.8. Kinetic parameters (k_0, E_A and n) of R-phycoerythrin and *Salmonella senftenberg*.

	n	Ln k_0 (min^{-1})	E_A (KJ $mole^{-1}$)	R^2
R-phycoerythrin	0.55	120	347	0.98
S. senftenberg	1.20	80	221	0.93

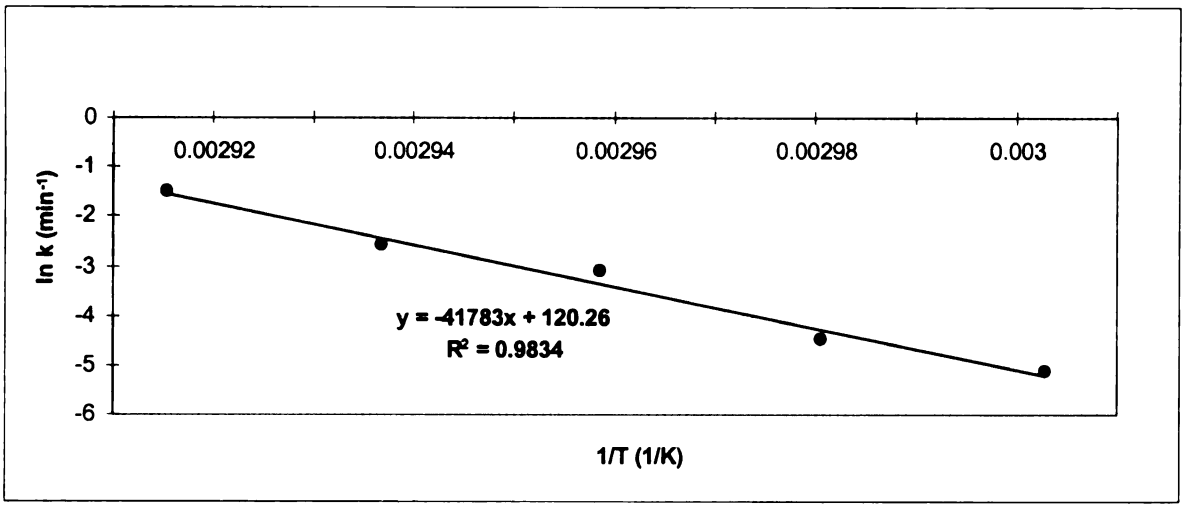


Figure 7.6. Arrhenius plot of R-phycoerythrin in 0.012 M borate buffer, pH 9.0.

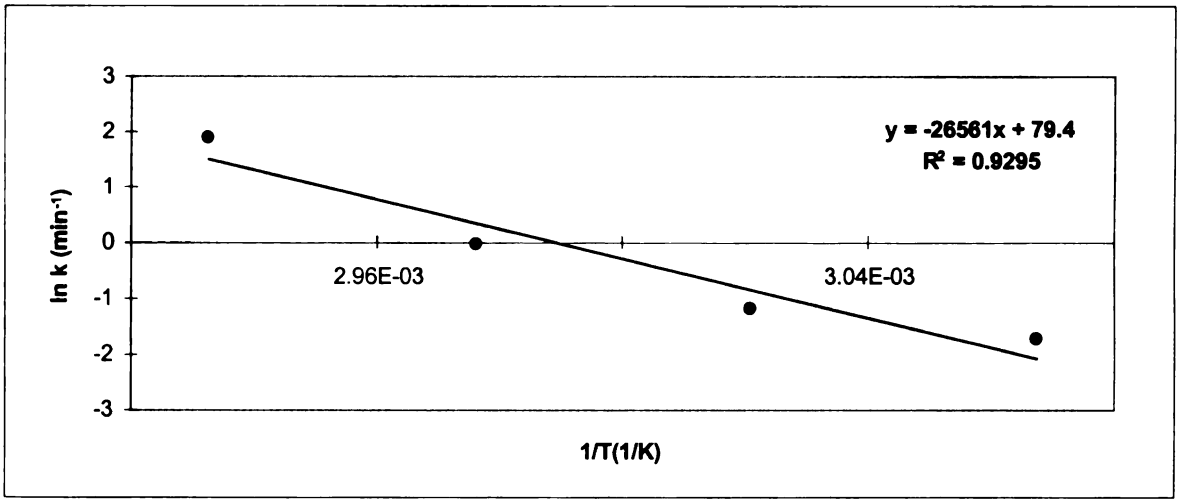


Figure 7.7. Arrhenius plot of *Salmonella senftenberg* in ground beef.

7.5. CONCLUSIONS

In this study, R-phycoerythrin has been proposed to be used as a single component, extrinsic, fluorescence-based TTI for the monitoring of thermal processes in beef products. Assuming first-order kinetics, the z value calculated for R-PE (5.99°C) fell within the range of the z value reported for *Salmonella* in beef products (5.6°C - 6.2°C). Under non-isothermal heating experiments, however, R-PE showed thermal inactivation kinetics that were not first-order. A non-linear model was developed to fit heat inactivation of both, R-PE and *S. senftenberg*. In both cases, lethality was very predictable (R^2 = 0.98 for R-PE and 0.93 for *S. senftenberg*), strongly suggesting that a mathematical relationship can be established between lethality of *S. senftenberg* and fluorescence loss of R-PE. By developing such an expression, R-PE could be used to predict destruction of this microorganism during thermal processing of beef products.

7.6. NOMENCLATURE AND UNITS

C	concentration of a target attribute at time t	
C_0	concentration of a target attribute at time zero	
C_p	predicted concentration of a target attribute	
D	decimal reduction time, time necessary to reduce a quality attribute by 90% at a constant temperature	min
D_{ref}	D value at the reference temperature (65°C in this study)	min
E_A	activation energy of a process	kJ $mole^{-1}$
$E_{ATarget}$	activation energy corresponding to a target attribute	kJ $mole^{-1}$
E_{ATTI}	activation energy corresponding to a time-temperature integrator	kJ $mole^{-1}$
F	processing value	min

$^{Target}F_{Tref}$	processing value as read from the target attribute	min
$^{TTI}F_{Tref}$	processing value as read from the time-temperature integrator	min
k	reaction rate constant	min^{-1}
k_0	pre-exponential factor	min^{-1}
k_e	reaction rate constant estimated from D values of isothermal experiments	min^{-1}
n	reaction order	
R	universal gas constant	J mole K^{-1}
t	time	min
T	temperature (TDT-model)	°C
T	temperature (Arrhenius model)	K
TDT	thermal death time	
T_H	holding temperature	°C
Tref	reference temperature	°C
TTI	time-temperature integrator	
z	thermal resistance constant, temperature increase necessary to reduce the D value by 90%	°C
z_{Target}	z value corresponding to a target attribute	°C
z_{TTI}	z value corresponding to a time-temperature integrator	°C

CHAPTER 8: Conclusions

Adequate thermal processing of meat products destroys pathogenic microorganisms that may be present in the raw meat. In this study, the fluorescent protein R-phycoerythrin (R-PE) was investigated for its application as a time-temperature integrator (TTI) to monitor adequate thermal processing in beef products. R-PE was selected as an extrinsic, single component, fluorescent-based TTI based on the calculated z value of 5.99°C determined when the protein was dissolved in 0.012 M borate buffer, pH 9.0. This z value was found to fall within the range of the reported z value for *Salmonella* in beef (5.6-6.2°C). This microorganism has been identified by the USDA as the target microbe in thermal processing of beef products.

R-PE was isolated from algal tissues of *Porphyra yezoensis* with a high purity ($A_{565}/A_{280}>5$). Isothermal experiments were conducted to determine the thermal inactivation parameters (D and z values) of R-PE under different conditions of pH and additives. Thermal inactivation parameters were calculated on the basis of fluorescence loss at the maximum emission wavelength. The inactivation parameters of this protein were altered by modifying the R-PE solution conditions. R-PE was most heat resistant between pH 5.0 and 9.0, but became more heat sensitive at pH 4.0 and 10.0. Addition of sucrose or β-mercaptoethanol increased the thermostability of R-PE, while sodium-dodecyl sulfate, NaCl and urea had a destabilizing effect, when compared to solutions of R-PE where no chemicals had been added. The wide range of z values calculated (4.44-37.75°C) suggests that R-PE could also

be used as a TTI in other thermal processes, such as sterilization of canned foods or milk pasteurization.

When R-PE was dissolved in 0.012 M borate buffer, pH 9.0, the resulting z value (5.99°C) closely matched that of *Salmonella* in beef (5.6-6.2°C). When R-PE was tested in a buffer system under non-isothermal conditions, changes in R-PE fluorescence could distinguish between adequate and inadequate heat processes as required by the USDA beef processing schedules, but the resulting inactivation kinetics were not first-order.

A non-linear mathematical model was developed to fit the heat inactivation kinetics of R-PE and *Salmonella senftenberg* in ground beef. The reaction orders (n) calculated for R-PE and *S. senftenberg* were 0.55 and 1.2, respectively. The activation energies (E_A) were estimated using the Arrhenius model. The calculated E_A for R-PE and *S. senftenberg* were 347.4 and 220.84 kJ $mole^{-1}$, respectively. Despite the differences in model parameters, the lethalities determined using the non-linear model were highly predictable (R^2=0.98 for for R-PE and 0.93 for *S. senftenberg*), suggesting that a mathematical relationship could be establish between lethality of *Salmonella* and R-PE fluorescence loss. Thus, it was concluded that R-PE could be used as the detector component of a TTI to predict destruction of this microorganism during thermal processing of beef products.

CHAPTER 9: Future research

This study is the first attempt to develop a fluorescence-based time-temperature integrator (TTI) to monitor adequate thermal processing of beef products. The following are recommendations for future research in this area:

Non-isothermal heating experiments were conducted in a buffer model system. Non-isothermal experiments should also be performed in a meat model system to verify that heat transfer within the meat does not affect the rate of R-PE inactivation. The effect of fat content of the meat product on heat transfer should be investigated as well.

It would also be useful to perform non-isothermal experiments of R-PE in *Salmonella*-inoculated beef samples to be able to compare actual destruction of the microorganism and R-PE fluorescence loss during the same heating process.

Experimental inactivation data of *S. senftenberg* was collected during isothermal experiments of ground beef in 10 x 75 mm test tubes. The heating lag time (time necessary for the center of the sample to reach the target temperature) can be considerable at the low temperatures. On the other hand, at high temperatures, when the microorganism is most sensitive, a substantial destruction of *Salmonella* can occur in the outer layers of product while the center has not yet reached the target temperature. Obviously, this would have a great significance in the proper calculation of D and z values and, thus, the actual lethality of *Salmonella* by heat. It would be adequate to calculate the kinetics parameters of *Salmonella*

in such a vessel that allows for instantaneous heating of meat samples to avoid the effect of the heating lag time.

The experiments described here were performed using capillary glass tubes. In a real situation, this would be unacceptable for the obvious safety reasons. Additional research is needed to find a most suitable material to encapsulate R-PE.

With the introduction of new techniques, such as microwave and ohmic heating, additional research is needed to investigate possible application of R-PE as a TTI in these processes.

R-PE belongs to a family of proteins called phycobiliproteins, which also include phycocyanin and allophycocyanin. It would be of interest to test these and other proteins which are fluorescent in the visible range, for their applicability as TTIs in monitoring thermal processes.

Although we studied the use of R-PE in thermal processing of beef products, it would be beneficial to explore the applicability of R-PE as a TTI in other thermal processes of foods. The wide range of z values obtained by modification of solution conditions using pH and additives, suggest that the z value R-PE can be manipulated to monitor adequate commercial sterilization of canned foods (z value for *Clostridium botulinum*= 10°C) and milk pasteurization (z value for *Coxiella burnetti*= 4°C).

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