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Enzyme-Linked Immunosorbent Assay Development for Advanced Glycation End Products and Brochocin-C

by

Siu Kwong Alan Kwok

A thesis submitted to
the Faculty of Graduate Studies and Research in partial fulfillment of
the requirements for the degree of Master of Science

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall 1997

University of Alberta

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Enzyme-Linked Immunosorbent Assay Development for Advanced Glycation End Products and Brochocin-C** submitted by **Siu Kwong Alan Kwok** in partial fulfillment of the requirements for the degree of **Master of Science** in **Food Science and Technology**.

Dr. Lynn M. McMullen (Supervisor)

Dr. Peter Sporns (Supervisor)

Dr. Walter T. Dixon

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Date: October 2, 1997

ABSTRACT

Advanced glycation end products (AGEs) are important components to detect in diabetic patients. Polyclonal antiserum against both physiological and non-physiological AGEs was generated. Following a competitive enzyme-linked immunosorbent assay (ELISA) with the antiserum, the absorbance maximum could be reduced 50% using 89.6 ppm glucose glycated ovalbumin. The antiserum seemed to recognize AGE crosslinks that were cleaved by *N*-phenacylthiazolium bromide, however, simple glycated proteins were not recognized.

Attempts to develop an ELISA procedure for brochocin-C, an anti-botulinal bacteriocin, were not successful. Large losses of brochocin-C using standard purification procedures were traced to butanol extraction and subsequent size exclusion chromatography due to extensive agglomeration of brochocin-C. Polyclonal antiserum developed against brochocin-C conjugate failed to provide useful antibodies for ELISA development due to the lack of sufficient purified brochocin-C. Polyclonal antiserum against carboxy-terminal synthetic peptide of brochocin-C weakly recognized the brochocin-C conjugate, but polyclonal antiserum against amino-terminal peptide did not.

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LIST OF ABBREVIATIONS

2616 ..... 7-Residue N-Terminal Synthetic Peptide

2617 ..... 7-Residue C-Terminal Synthetic Peptide

5D4 ..... Antiserum Developed by using G-LPH

5K7 ..... Antiserum Developed by using 2616 Synthetic Peptide

5K8 ..... Antiserum Developed by using 2617 Synthetic Peptide

7A4 ..... Antiserum Developed by using broc-BSA

7A5 ..... Antiserum Developed by using broc-BSA

a ..... Upper Asymptote of Competitive Enzyme Immunoassay Standard Curve (i. e. Bo)

AGEs ..... Advanced Glycation End Products

a. i. ..... Arbitrary Intensity

ATCC ..... American Type Culture Collection

AU ..... Activity Unit

b ..... Slope of Standard Curve at the Midpoint between "a" and "d" values

B ..... Response (Absorbance) of Sample

B/Bo ..... Relative Response (Absorbance Ratio) to Bo

Bo ..... Highest Background Absorbance Standard

broc-BSA ..... Brochocin-C Conjugated to Bovine Serum Albumin Using Glutaraldehyde

BSA ..... Bovine Serum Albumin

c ..... Inflection point of Competitive Enzyme Immunoassay Standard Curve (i.e. I_{50} value)

CAA ..... Casamino Acids

$(CD_3)_2SO$ ..... Deuterated Dimethyl Sulfoxide

cid-ELISA ..... Competitive Indirect Enzyme-Linked Immunosorbent Assay

CML ..... Carboxymethyl-Lysine

d ..... Lower Asymptote of Competitive Enzyme Immunoassay Standard Curve

Da ..... Dalton

DMSO ..... Dimethyl Sulfoxide

EIA ..... Enzyme Immunoassay

ELISA ..... Enzyme-Linked Immunosorbent Assay

FFI ..... Furoyl-Furanyl Imidazole

FPLC ..... Fast Performance Liquid Chromatography

G-LPH ..... Non-physiologically Glycated *Limulus polyphemus* Hemolymph with D-Glucose

glutar. BSA ..... Glutaraldehyde Treated Bovine Serum Albumin Control

G-OVA ..... Non-physiologically Glycated Ovalbumin with D-Glucose

H ..... Heavy

HRP ..... Horseradish Peroxidase

I_{50} ..... Analyte Concentration Required to Reduce Bo by 50% (i.e. "c" value)

Ig ..... Immunoglobulin

KLH ..... Keyhole Limpet Hemocyanin

L ..... Light

LAB ..... Lactic Acid Bacteria

LPH ..... *Limulus polyphemus* Hemolymph

MAb ..... Monoclonal Antibody

MALDI ..... (or MALDI-TOFMS) Matrix-Assisted, Laser Desorption/Ionization Time of Flight Mass Spectrometry

MW ..... Molecular Weight

m/z ..... Mass per Charge

ncid-ELISA ..... Non-Competitive Indirect Enzyme-Linked Immunosorbent Assay

NMR ..... Nuclear Magnetic Resonance

OVA ..... Ovalbumin

PBS ..... Phosphate-Buffered Saline

PBST ..... Phosphate-Buffered Saline containing Tween 20

ppm ..... Parts per Million

ppt ..... Parts per thousand

PTB ..... *N*-Phenacylthiazolium Bromide

RH ..... Relative Humidity

SDS-PAGE ..... Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

TFA ..... Trifluoroacetic Acid

TMB ..... 3,3'-5,5'-Tetramethylbenzidine

UAL ..... University of Alberta Food Microbiology Culture Collection

x ..... Concentration of Analyte

y ..... Response (i.e. Absorbance Units)

1. INTRODUCTION

1.1 GLYCATED PROTEINS

1.1.1 The Maillard reaction in general

Brown pigment formation in a heat-treated glucose and lysine solution was first demonstrated by Maillard (1912). Pigment formation is due to a sugar-amine non-enzymatic browning reaction, which came to be known as the Maillard reaction (Hodge, 1953). This is distinct from the sugar-sugar caramelisation reaction occurring when heating a pure sugar solution (Heyns and Klier, 1968). The two amino groups on lysine and the carbonyl group on glucose are involved in the Maillard reaction (Hodge, 1953). After the initial sugar-amine reaction, the reacted sugar molecule undergoes the same sugar fragmentation reaction as occurs in caramelisation but at a lower temperature (Houminer, 1973).

Generally, the Maillard reaction can occur between the free amino group on any protein, peptide, amino acid or amine molecule and the carbonyl group on any reducing sugar, ketone or aldehyde molecule. The Maillard reaction can be temporally divided into three levels: early Maillard reactions, advanced Maillard reactions and final Maillard reactions.

The early Maillard reactions are started with a simple condensation reaction between the carbonyl and the free amino groups of a reducing sugar and a protein, respectively, to form a Schiff base. An Amadori rearrangement takes place to change the Schiff base into an Amadori product (Figure 1). The reaction to this point is reversible with the Amadori product being relatively more stable than the Schiff base. In food systems, initial compounds formed in this reaction level remain colorless and contribute no flavor. However, these initial compounds greatly decrease the nutritive value of food (Hurrell and Carpenter, 1974).

The advanced Maillard reactions are composed of numerous chemical pathways, including enolization, Strecker degradation, Amadori product

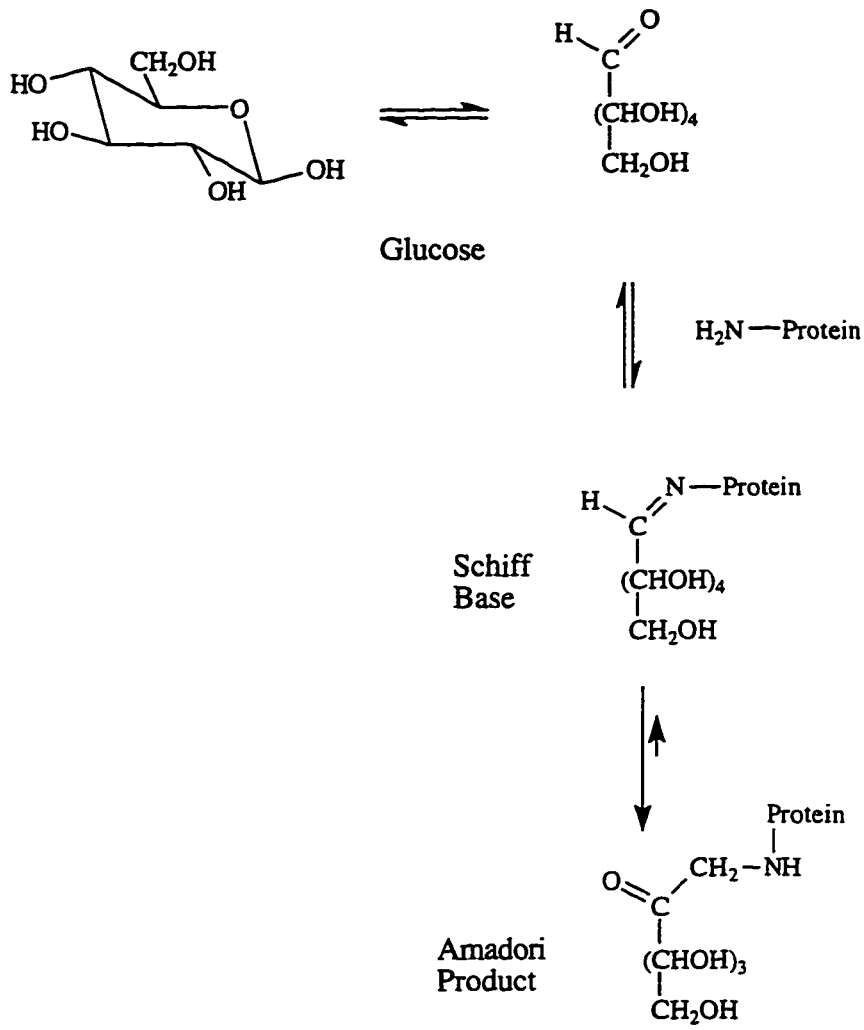
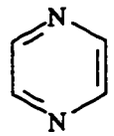


Figure 1. Early Maillard reactions between glucose and protein (Hodge, 1953).

substitution and Schiff base transamination (Hodge, 1953; Burton and McWeeny, 1964; Høltermand, 1966). A large variety of heterocyclic compounds are formed when approaching the end of this phase of the reaction. Figure 2 shows some of the heterocyclic compounds formed. These can be *N*-heterocyclic, including pyrazines, pyrroles, pyrrolines, oxazolines and thiazoles, and *O*-heterocyclic, such as maltol and isomaltol (Hodge *et al.*, 1972; Rizzi, 1969; Tonsbeek *et al.*, 1971). These heterocyclic compounds contribute to food flavors.

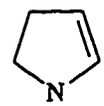
During the final Maillard reactions, the formation of pigmented polymers with varied degrees of solubility takes place. Polymerization is caused by the presence of reactive substances (e.g., unsaturated carbonyl compounds and furfural) resulting from advanced Maillard reactions (Reynolds, 1965). Chemistry of this polymerization step is not yet well understood; however, the polymers formed are somewhat inert chemically and biologically and cause toughening in stored foods (Labuza *et al.*, 1977).

The rate of the Maillard reaction is strongly dependent on the reaction conditions. The rate of amino-nitrogen loss can be increased 40,000 times with an increase in reaction temperature from 0 to 80°C in a casein-glucose mixture (Lea and Hannan, 1949). ε-Amino groups of lysine react at similar rates in an albumin-glucose system at 37°C for 30 days and the same system at 121°C for 15 minutes (Hurrell and Carpenter, 1974). Therefore, the Maillard reaction increases with increasing temperature and/or incubation time. Moisture level in the reaction system also plays an important role in the Maillard reaction. Water is the necessary medium for the initial reaction to be carried out, but too much moisture also inhibits the subsequent dehydrations involved in Maillard reactions (Wolfrom and Rooney, 1953). Maximum pigment formation and lysine ε-amino group reaction occurs at moisture levels of 30% and 15 to 18%, respectively (Wolfrom and Rooney, 1953; Lea and Hannan, 1949). Alkaline pH favors

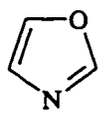


pyrazine

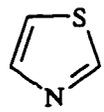
pyrrole



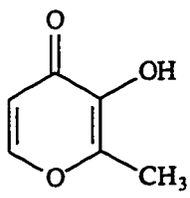
2-pyrroline



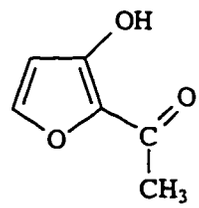
oxazoline



thiazole



maltol



isomaltol

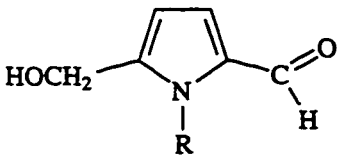
Figure 2. Examples of heterocyclic compounds.

the Maillard reaction (Lea and Hannan, 1949; Underwood *et al.*, 1959) with an enhanced reaction effect from the buffer capacities of phosphate and citrate (Saunders and Jervis, 1966). Furthermore, reducing sugar reactivity increases as the number of sugar carbon atoms decreases because aldopentoses are more susceptible to Maillard reaction than aldohexoses while reducing disaccharides are even less reactive (Spark, 1969). However, non-reducing sugar, such as sucrose, may also become reactive in Maillard reaction after hydrolysis under acidic conditions (Hurrell and Carpenter, 1977).

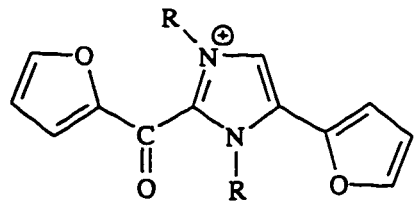
1.1.2 The Maillard reaction *in vivo*

The Maillard reaction *in vivo* was first discovered by the synthesis of the Amadori product of serotonin (Mester and Mester, 1975) and was further confirmed with the identification of Amadori-type sugar derivatives of hemoglobin found at high levels in diabetic patients who have elevated blood glucose concentrations (Koenig *et al.*, 1976). Since then, extensive research has contributed to the understanding of the nature of Maillard reaction under physiological conditions (Njoroge and Monnier, 1989; Ledl and Schliecher, 1990; Bucala and Cerami, 1992).

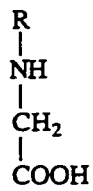
The presence of glucose in human blood continuously supplies the reducing sugar to react with body proteins in a physiological Maillard reaction pathway, which is essentially the same as in Figure 1 (p. 2). All compounds subsequently derived after Amadori product formation are called advanced glycation end products (AGEs). Some AGEs have been isolated from *in vitro* systems, such as pyrraline, furoyl-furanyl imidazole (FFI), carboxymethyl-lysine (CML), 1-alkyl-2-formyl-3,4-glycosyl-pyrrole (AFGP) and crossline (Pongor *et al.*, 1989; Ahmed *et al.*, 1988; Farmar *et al.*, 1988; Njoroge *et al.*, 1987; Nakamura *et al.*, 1992); and from *in vivo*, such as pentosidine (Sell and Monnier, 1984). Figure 3 shows some examples of the AGE structures. AGEs are characterized by fluorescence,



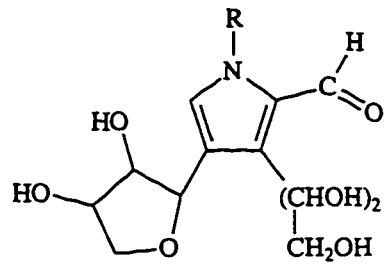
Pyrraline



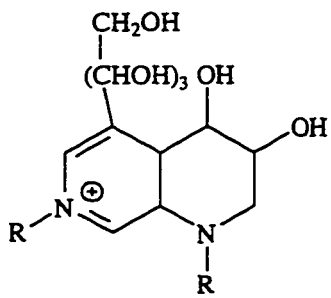
Furoyl-furanyl imidazole (FFI)



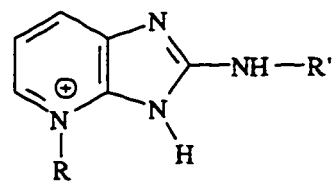
Carboxymethyl-lysine (CML)



1-alkyl-2-formyl-3,4-
glycosyl-pyrrole (AFGP)



Crossline



Pentosidine

Figure 3. Examples of advanced glycation end products.

R = lysine side chain of protein

R' = arginine side chain of protein

brown color and crosslinking ability with other macromolecules, especially long-lived proteins (Lee and Cerami, 1990).

Formation of AGEs is a concern as a contributing factor in complications of diabetes (Makita *et al.*, 1991; Sell and Monnier, 1990), aging (Lee and Cerami, 1990) and Alzheimer's disease (Vitek *et al.*, 1994). AGEs may contribute to the pathogenesis of atherosclerosis, diabetes-related blindness and kidney failure, joint stiffening, deoxyribonucleic acid (DNA) damage and Alzheimer's disease amyloidosis (Lee and Cerami, 1990; Ziyadeh, 1993; Vitek *et al.*, 1994). However, AGEs are recognized as foreign compounds by the human body and can slowly be removed by phagocytosis (Radoff *et al.*, 1988). Pharmaceutical inhibitors of AGE formation which have been investigated include aminoguanidine (Brownlee *et al.*, 1986); ibuprofen, glutathione, ®Aspirin (Ajiboye and Harding, 1989); ®Diclophenac (van Boekel *et al.*, 1991) and Tenilsetam (Münch *et al.*, 1994). Of these, aminoguanidine has received most of the attention for its inhibitory effect of AGE formation, such as reducing AGE accumulation in collagen and in kidney glomerular basement membrane; postponing the beginning of diabetes-related vasodilatory abnormalities, retinal vascular lesions and nephropathy; and lessening neuropathology in streptozotocin-induced diabetic rats (Oxlund and Andreassen, 1992; Ellis and Good, 1991; Huijberts *et al.*, 1993; Hammes *et al.*, 1994; Itakura *et al.*, 1991; Yagihashi *et al.*, 1992). Aminoguanidine reacts with the AGE dicarbonyl group, which then cannot crosslink with other macromolecules (Chen and Cerami, 1993).

1.1.3 Detection of AGEs

AGE detection can be based on the fluorescent properties of AGEs (Monnier and Cerami, 1981). After the excitation at 370 nm, fluorescence emission wavelength is typically at 440 nm due to the presence of

heterocyclic compounds. However, the exact quantitation of AGEs is difficult to achieve due to the lack of proper means to represent the whole diverse AGE family. Candiano *et al.* (1986) have suggested an alternative method for quantitation involving the formation of a chromophore after reacting AGEs with diazonium salts and subsequently measuring absorbance at 490 nm wavelength.

Recently, the use of immunoassays has been incorporated into many AGE investigations, including research on FFI (Chang *et al.*, 1985), pyrraline (Miyata and Monnier, 1992), lipoprotein AGEs (Doucet *et al.*, 1994), CML (Reddy *et al.*, 1995), pentosidine (Miyata *et al.*, 1996), ribonuclease AGEs (Khalifah *et al.*, 1996), AGE crosslinks (Vasan *et al.*, 1996) and imidazolones (Niwa *et al.*, 1997). However, the antibodies produced may recognize only a limited number of AGEs and leave others undetected.

The need for wide spectrum method for the detection of AGEs was the bases for the present study. This broad-spectrum detection method was sought through the development of a polyclonal antiserum against a mixture of AGEs generated under vigorous non-physiological conditions.

1.2 BROCHOCIN-C

1.2.1 Bacteriocins in general

Lactic acid bacteria (LAB) are a group of non-sporeforming gram-positive bacteria that produce lactic acid during carbohydrate metabolism (Kandler, 1983). Traditionally they have been used as starter cultures in foods for preservation and flavor contribution. The metabolites produced during their growth act as antimicrobial substances to inhibit the growth of other bacteria. LAB not only produce lactic and acetic acids, which lower the pH of the food, but also other inhibitory substances such as hydrogen peroxide and bacteriocins.

Bacteriocins are antimicrobial peptides which inhibit the growth of other, usually closely related, bacteria (Klaenhammer, 1993). LAB bacteriocins can be classified into four classes (Klaenhammer, 1993). Class I bacteriocins are small membrane active peptides with a molecular weight less than 5 kDa. Class II bacteriocins are heat stable membrane active peptides with a molecular weight less than 10 kDa. Class III contains heat sensitive protein bacteriocins with a molecular weight greater than 30 kDa. Lastly, class IV are complex bacteriocins with components of a protein plus a lipid or carbohydrate. Most LAB bacteriocins are sensitive to alkaline pH (8.0 or above).

1.2.2 LAB bacteriocin as food preservatives

There is a promising future for the use of LAB bacteriocins as natural food preservatives because of the demand to replace chemical preservatives in foods (Lloyd and Drake, 1975). Some LAB bacteriocins have inhibitory activity against food spoilage microorganisms and foodborne pathogens such as *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Listeria monocytogenes* and *Staphylococcus aureus*. Several class II bacteriocins have been shown to be effective antimicrobials in meat systems; for example, pediocin has been used to

inhibit the growth of *Listeria monocytogenes* in fresh meats (Motlagh *et al.*, 1992; Nielsen *et al.*, 1990) and processed meats (Berry *et al.*, 1991; Degnan and Luchansky, 1992); sakacin A can control the growth of *L. monocytogenes* in a pasteurized ground meat product and raw meat sausage (Shillinger *et al.*, 1991).

Many bacteriocins are heat stable and can be used as antimicrobials in heat-treated foods. It is possible that with bacteriocins the need for extreme heating could be reduced, which would improve the nutritional and sensory qualities of the products.

1.2.3 Brochocin-C

Brochocin-C is a bacteriocin produced by *Brochothrix campestris* ATCC 43754 (Siragusa and Nettles Cutter, 1993). *B. campestris* was isolated from soil and grass (Talon *et al.*, 1988) and has not yet reported to be found in foods, yet the bacteriocin it produces inhibits the growth of microflora of meat.

Poon (1995) studied the biochemical and genetic properties of brochocin-C. Brochocin-C is a very hydrophobic peptide with 59 amino acid residues (Figure 4) and has a molecular weight of 5241.21 ± 1.22 Da. The inhibitory spectrum of brochocin-C includes a wide range of gram-positive bacteria. The crude form of brochocin-C is stable up to 121 °C for 15 minutes and is stable between pH 2 to 9. It is proteinaceous in nature and can be inactivated by proteolytic enzymes.

1.2.4 Detection method for brochocin-C

Brochocin-C was detected by using a spot-on-lawn assay (Ahn and Stiles, 1990). The assay depends on the inhibitory action of brochocin-C against the growth of an indicator organism, UAL 8. This method requires an overnight incubation in 10% carbon dioxide and 90% nitrogen to allow a clear inhibition zone to be visible. Bioassays for bacteriocin detection are

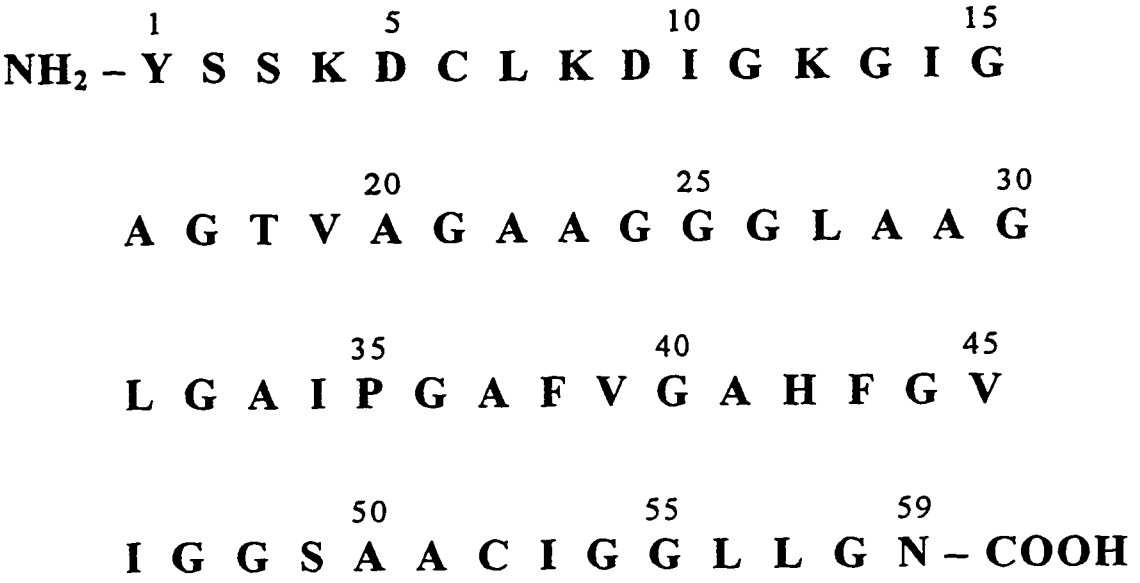


Figure 4. Amino acid sequence of brochocin-C (Poon, 1995).

time consuming and often the result is dependent on the selection of a sensitive indicator organism. Detection of bacteriocins in foods requires extensive extraction and purification which can be an extremely lengthy process. If bacteriocins are to be used as antimicrobials in foods, more sensitive and quantitative methods of detection need to be developed.

1.2.5 Antibodies to brochocin-C

Antibodies for bacteriocin detection have been developed against nisin (Falahee *et al.*, 1990; Stringer *et al.*, 1995; Suárez *et al.*, 1996a; Suárez *et al.*, 1996b) and pediocin (Bhunia, 1994; Bhunia and Johnson, 1992; Bhunia *et al.*, 1990). However, some of the antibodies developed did not have satisfactory titers. When the bacteriocin is injected into an animal as an immunogen by itself, its size is usually too small to elicit a good immune response. Also, the high hydrophobicity of the bacteriocin may prevent proper antibody interaction by agglomeration, which may obstruct the binding epitopes.

Though there are problems to be overcome when developing antibodies against bacteriocins, an immunoassay for detection of brochocin-C would be valuable since there is no convenient and accurate method of analysis. Detection of brochocin-C in a complex system, such as meat, can be used to indicate that preservation is due to the presence of brochocin-C, not other factors (e.g., pH effect). An immunoassay might be a good choice of technique for brochocin-C detection in a complex system because antibodies are usually very specific against one antigen. In addition, brochocin-C specific antibodies developed for an immunoassay might also be usefully applied in brochocin-C purification.

1.3 ENZYME-LINKED IMMUNOSORBENT ASSAY

An enzyme immunoassay (EIA) is an assay based on the non-covalent interaction between an antigen and an antibody to form an immune complex (antigen-antibody) which can then be measured by an enzyme-labeled antigen or antibody (Morris *et al.*, 1988). The use of an enzymes as a detection system is popular because EIAs can replace most applications of radioimmunoassays which despite their usefulness have potential hazard with radioisotopes and require expensive equipment for detection (Rittenburg, 1990). An enzyme-linked immunosorbent assay (ELISA) is an EIA in which an immunoreactant, either the antigen or the antibody, is coated on a solid phase. This facilitates the separation of the labeled and unlabeled immunoreactants from each other before measuring the extent of the immune reaction (Deshpande, 1996). ELISA offers an economical and convenient method for highly sensitive detection of low concentration compounds (Rittenburg, 1990). Important elements of the ELISA system, including the antibody, the antigen, the enzyme label and the assay format, are discussed in the following sections.

1.3.1 Antibody

An antibody is a glycosylated protein, called immunoglobulin (Ig), produced by the animal's immune system in response to the challenge of foreign molecule (Harlow and Lane, 1988). In mammalian systems, Igs can be classified into five groups, IgG, IgM, IgA, IgD and IgE. IgG is the basic Ig in mammalian serum for ELISA application (Tijssen, 1985).

The major components of an IgG molecule include two identical heavy (50 to 75 kDa) and light (~22.5 kDa) polypeptide chains linked together by disulfide bridges to form an Y-shaped structure (Figure 5). The first 110 amino acid residues of the N-terminals of the H and L chains are highly variable and form the antigen binding site, also called the paratope. The rest of the antibody structure is conserved within the animal

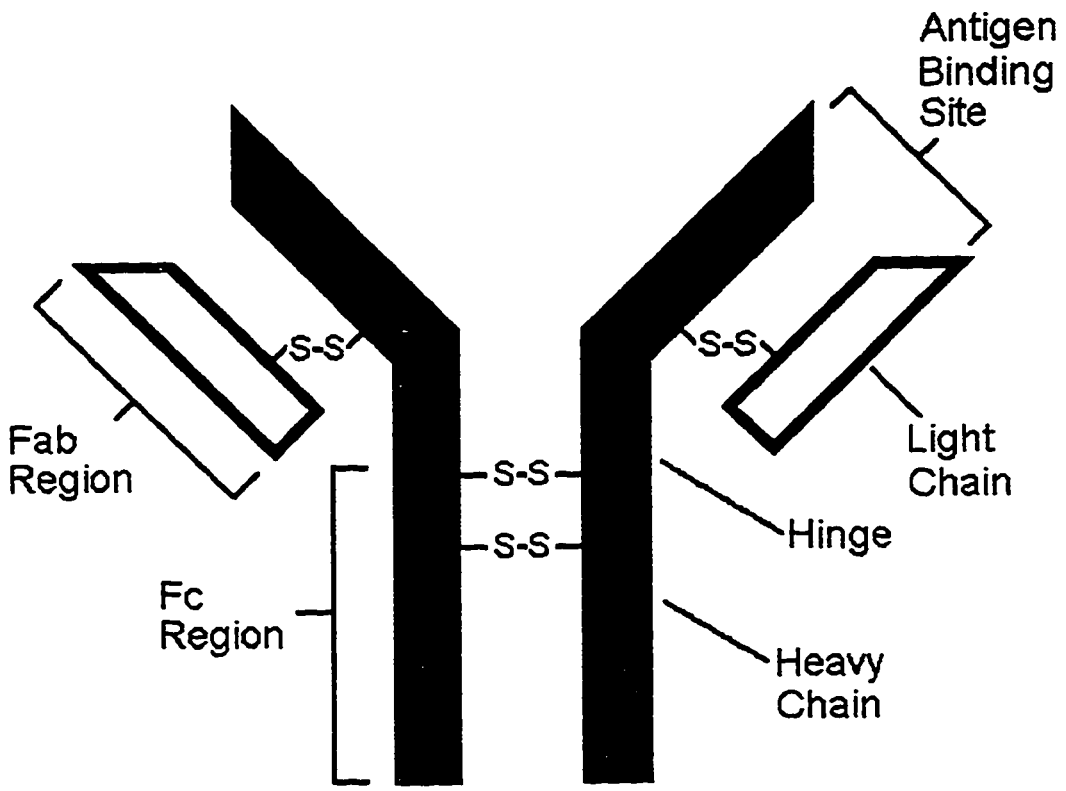


Figure 5. IgG antibody basic structure.

Fab = Fraction having the antigen binding site

Fc = Fraction that crystallizes

-S-S- = disulfide bridge

species that produced the antibody (Butler, 1980; Coleman *et al.*, 1989; Nisonoff, 1982). The antigen binding site is a pouch-shaped region which will accommodate a region on an antigen, called the epitope, about 3 to 7 glucose molecules (Nisonoff, 1982) or 5 to 7 amino acids (Gazzaz *et al.*, 1992). The high specificity of a paratope enables the antibody to distinguish between two different epitopes (Coleman *et al.*, 1989). The binding between paratope and epitope involves several kinds of non-covalent interactions such as electrostatic attractions, hydrophobic interactions, hydrogen bonds and van der Waals forces (Coleman *et al.*, 1989; Nisonoff, 1982). However, these non-covalent interactions can be disturbed or broken by high salt concentration or extreme pH (Gazzaz *et al.*, 1992).

Both polyclonal and monoclonal antibodies are commonly used in ELISA (Lee and Morgan, 1993). Monoclonal antibodies (MAbs) are developed usually using mouse lymphocytes by fusion of B-lymphocytes (B-cells), from an appropriately stimulated animal, and myeloma cells to produce a hybridoma cell line which produces antibodies specified to a single epitope (Campbell, 1984). The genetic properties of B-cells, to produce antibodies, and myeloma cells, to grow continuously in cell culture, are combined (Tijssen, 1985), so that a permanent antibody supply is established. However, the MAb technique is time-consuming and expensive; MAbs may be unstable even to a mild matrix changes such as salt concentration and acidity; sensitivity of MAbs is limited as compared to polyclonal antibodies; and MAbs, recognizing a single epitope, can only distinguish a small part of the total antigenic repertoire of the immunogen (Deshpande, 1996).

On the other hand, polyclonal antibodies are developed from blood serum by challenging an animal's immune system with an immunogen that is foreign to the animal species. Common animals used for polyclonal antibody syntheses are rabbits, sheep and goats (Goding, 1986).

Advantages of polyclonal antibody development are: convenience; low cost (Campbell, 1984); often high antibody affinity (Stanker and Beier, 1996); and the ability to recognize a large part of the total antigenic profile of an immunogen (Harlow and Lane, 1988). This is due to the presence of antibody subpopulations that interact with more than one epitope on the antigen (Campbell, 1984). Although the supply of an individual polyclonal antiserum is limited to the number of appropriately stimulated animals, it is a suitable choice of technique for this study in that a broad spectrum of detection is needed for the multiple Maillard reaction products or various antibody binding sites on bacteriocin.

1.3.2 Antigen

An antigen is a substance that interacts with an antibody. An immunogen is an antigen molecule that can induce an immune response. The ability of the immunogen to induce such a response is called immunogenicity (Harlow and Lane, 1988). The difference between an antigen and an immunogen is that all immunogens are antigens, but not all antigens are immunogens. It is necessary for an animal's body to eliminate any foreign and potentially hazardous substance by an immune mechanism in order to maintain health.

Antigens with a molecular weight greater than 100 kDa are usually immunogenic. Immunogens commonly used include proteins, lipids, carbohydrates and nucleic acids (Coleman *et al.*, 1989; Nisonoff, 1982). Large antigen molecules may carry many epitopes (or antigenic determinants), which are about 5 to 7 amino acids in size, with a density of one epitope per 40 to 80 amino acids for proteins. However, compounds having a molecular weight less than 1000 Da are often not immunogenic by themselves (Gazzaz *et al.*, 1992).

Haptens are low molecular weight compounds (which contain at least one epitope for antibody binding) which can become immunogenic by

conjugation to carrier molecules, usually proteins such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) and ovalbumin (Tijssen, 1985; Harlow and Lane, 1988). A synthetic peptide as small as 6 amino acids in size (or sometimes even smaller) can also be used as a hapten to produce antibodies that recognize the parent protein (Harlow and Lane, 1988). Functional groups, such as amino, carboxyl, phenolate, imidazolyl, guanidinyl, and sulfhydryl groups, available on the hapten may be used for conjugation to the carrier compound (Deshpande, 1996). Coupling reagents, including glutaraldehyde, carbodiimides, bis-diazobenzidine and *m*-maleimido-benzoyl-*N*-hydroxysuccinimide ester, can be used to carry out hapten-carrier protein conjugations (Harlow and Lane, 1988). Optimum hapten densities to produce a good immunological response may be between 3 and 25 (Roe, 1991) or 8 and 25 (Erlanger, 1980; Tijssen, 1985) molecules of hapten per molecule of carrier.

Antibodies resulting from immunization of a hapten-carrier conjugate often preserve the binding specificity of the unconjugated hapten (Coleman *et al.*, 1989) among the specificities which develop to other parts of the whole hapten-carrier conjugate. Different carriers for immunization and detection, if available, are used in hapten conjugations to screen only the antibodies of interest (Tijssen, 1985).

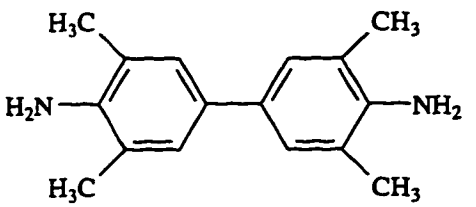
1.3.3 Enzyme label

The major objective of an EIA development is to quantitate a low concentration of a compound. Enzyme labels on antibodies or antigens facilitate spectrophotometric quantitations in EIAs. Common properties of enzymes used for labeling include low cost, high turnover rate, stability, ease of purification, ease of conjugation, and ease of end product assessments (Deshpande, 1996). An enzyme label can have a turnover rate of changing 10^6 substrate molecules into product per enzyme molecule per minute or higher (Rittenburg, 1990). Typical enzyme labels include

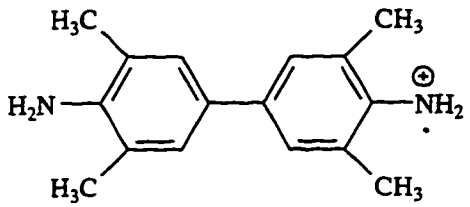
horseradish peroxidase (HRP), β-D-galactosidase and alkaline phosphatase (Porstmann and Porstmann, 1988). HRP is the most common enzyme label used (Gosling, 1990). It is a 40 kDa glycoprotein, with eight neutral carbohydrate residues, extracted from the horseradish *Armoracia rusticana*. The small size and the carbohydrate side chains of HRP help the molecule to stay in solution with less non-specific binding to its surroundings (i.e. background) than other enzymes (Deshpande, 1996). Isozyme C is the dominant form of HRP used in EIAs (Tijssen, 1985). Three common aqueous soluble substrates, requiring hydrogen peroxide (H_2O_2), for HRP are 3,3',5,5'-tetramethylbenzidine (TMB), *o*-phenylenediamine and 2,2'-azino-di(3-ethyl-benzthiazoline) sulfonic acid, of which TMB is the most popular because of its ability to produce high absorbances and low background readings (Deshpande, 1996). A reaction mechanism of HRP with TMB in the presence of H_2O_2 to produce 3,3',5,5'-tetramethyl-1,1'-diphenoquinone-4,4'-diimonium ion (a colored end product) is shown in Figure 6. Conjugation between the amino group of HRP and the thiol group of an antibody or antigen can be carried out by using the heterobifunctional reagents, *N*-succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate and *N*-succinimidyl 6-maleimidohexanoate, to give a 1:1 coupling ratio (Deshpande, 1996). According to the detection format it is either the primary antibody (which binds to the antigen) or the secondary antibody (which binds specifically to the Ig type of the primary antibody) which is used for enzyme coupling.

1.3.4 Solid-phase ELISA systems

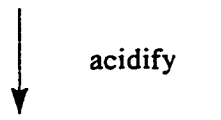
Solid-phase systems offer several advantages over the liquid-phase system, such as efficient analysis, low non-specific interaction and easy immunoreactant separation. ELISA is an EIA in which the immunoreactant, an antigen or an antibody, is adsorbed on a solid-phase

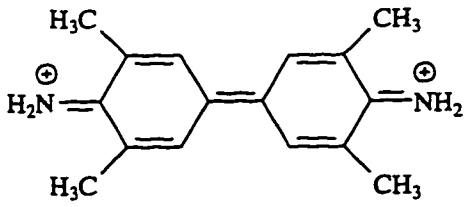


TMB (colorless)



TMB radical cation (blue)
λmax = 370 nm and 650 nm





3,3'-5,5'-tetramethyl-1,1'-diphenoquinone-4,4'-diimonium ion (yellow) λmax = 450 nm

Figure 6. TMB color development (Porstmann and Porstmann, 1988).

surface prior to the analysis, so that the immunoreactant becomes insoluble throughout the assay (Deshpande, 1996). The solid-phase can be made of various materials, such as polystyrene, polyvinyl, nylon, silica, glass, cellulose, polyacrylamide, sepharose and agarose (Campbell, 1984; Rittenburg, 1990), in different formats, including microtiter plate, tube, bead and membrane (Deshpande, 1996). The polystyrene or polyvinyl 96-well microtiter plate is the most commonly used solid-phase format (Campbell, 1984).

The protein immunoreactant is adsorbed onto the plastic surface of the well by non-covalent general hydrophobic attractions (Tijssen, 1985). The amount of protein adsorbed is related to the concentration and diffusion coefficient of coating substrate, the ratio of coating area to coating solution volume, and the incubation temperature and time (Clark and Engvall, 1980). Although multiple protein layers may be formed under extreme conditions, the amount of protein available for binding remains unchanged (Cantarero *et al.*, 1980).

The unoccupied protein-plastic binding sites are usually blocked with a non-specific irrelevant protein to eliminate further non-specific binding of the immunoreactants (Roe, 1991). Blocking agents, such as BSA, nonfat dried milk and gelatin, have the ability to fill unoccupied binding sites, the lack of interference with the coated reactant, the inability to participate in further immunoreactions and the undetectability in absorbance measurement (Deshpande, 1996). Non-ionic type detergents (e.g., Tween 20 and Triton X-100), which decrease the non-specific interaction potential of soluble immunoreactants, are applied in incubation stages (excluding the coating and the blocking) and washing steps (starting from the step after blocking) of ELISA to prevent any subsequent non-specific binding to the solid-phase (Clark and Engvall, 1980; Deshpande, 1996).

1.3.5 ELISA formats

There are various ELISA detection formats. Figures 7, 8, 9 and 10 present examples of several formats. The indirect ELISA format utilizes a secondary antibody, such as the HRP-conjugated goat anti-rabbit IgG antibody, which leaves the primary antibody-antigen binding kinetics uninterrupted and allows effective usage of the primary antibody. The unbound immunoreactants are washed away between the stages. Color development (or absorbance) is inversely proportional to the quantity of antigen, in the solution sample, analyzed in every competitive ELISA format; hence, the absorbance increases when the amount of soluble antigen decreases. The competitive indirect ELISA (cid-ELISA) and the non-competitive indirect ELISA (ncid-ELISA) formats (Figures 7 and 8, respectively) were used in this study.

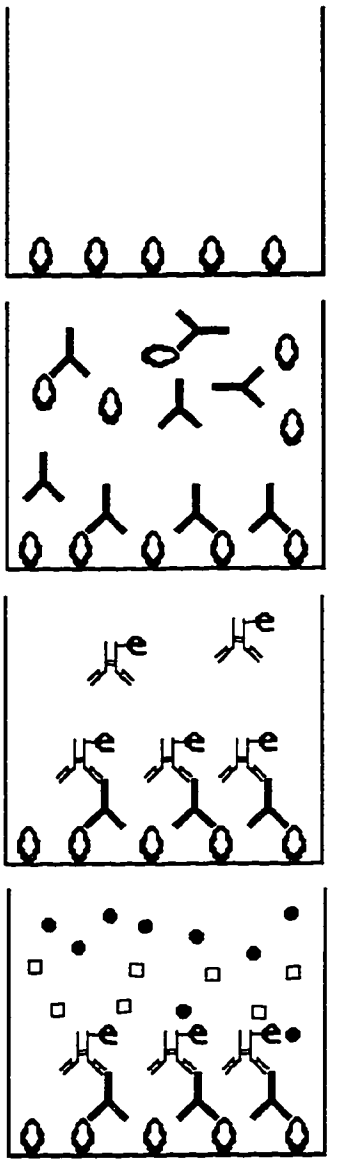
Figure 7. Competitive indirect ELISA (cid-ELISA).

1/ Coating plate with antigen

2/ Adding primary antibodies and free antigen for competition

3/ Adding enzyme-labeled secondary antibodies

4/ Adding enzyme substrate and developing color



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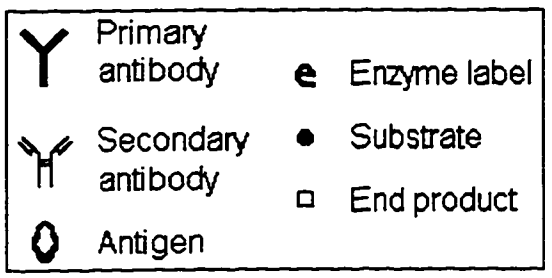
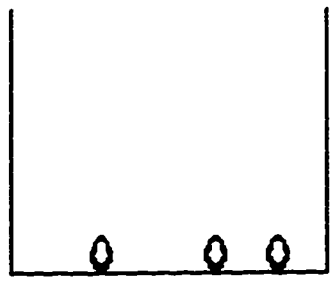
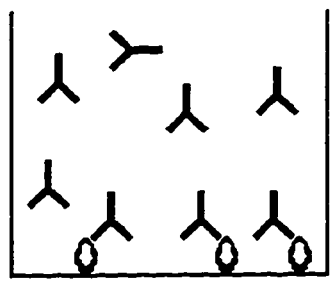


Figure 8. Non-competitive indirect ELISA (ncid-ELISA).

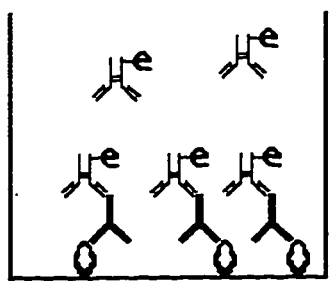
1/ Coating plate with antigen



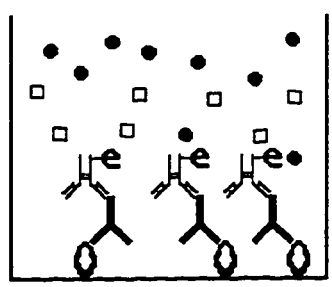
2/ Adding primary antibodies for antigen recognition



3/ Adding enzyme-labeled secondary antibodies



4/ Adding enzyme substrate and developing color



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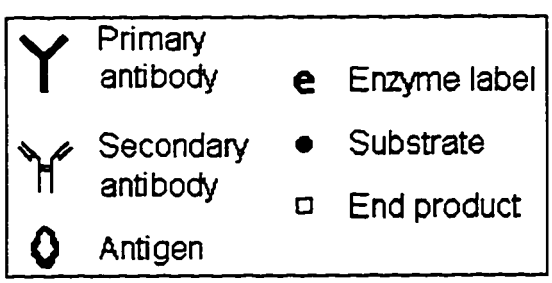
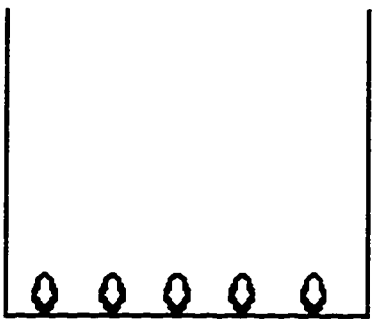
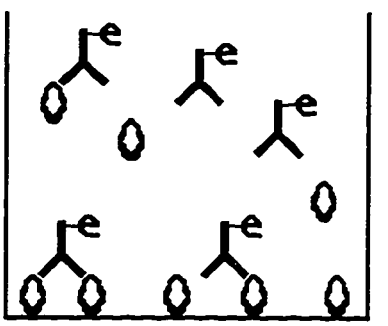


Figure 9. Competitive direct ELISA using enzyme-labeled antibody.

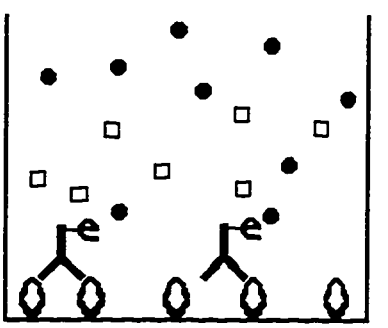
1/ Coating plate with antigen



2/ Adding free antigen and enzyme-labeled antibodies for competition



3/ Adding enzyme substrate and developing color



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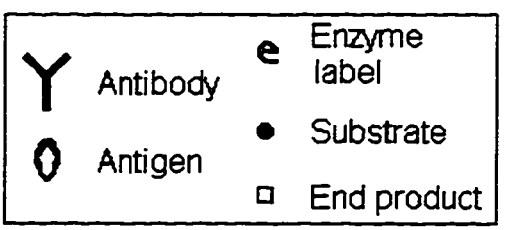
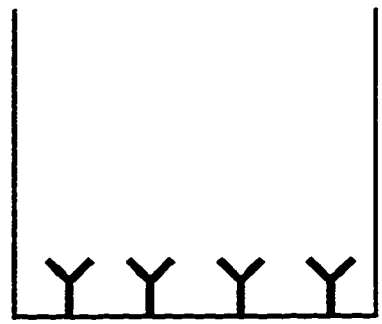
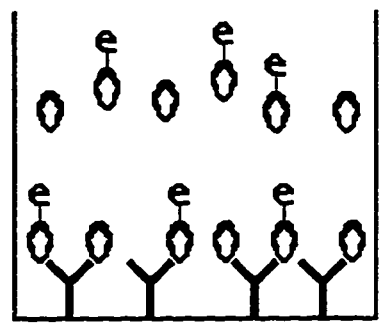


Figure 10. Competitive direct ELISA using enzyme-labeled antigen.

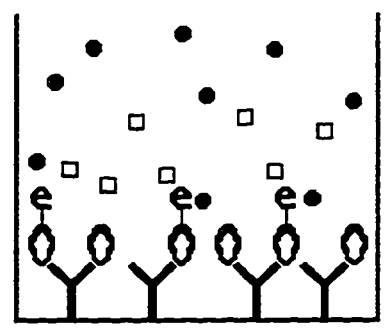
1/ Coating plate with antibodies



2/ Adding free and enzyme-labeled antigens for competition



3/ Adding enzyme substrate and developing color



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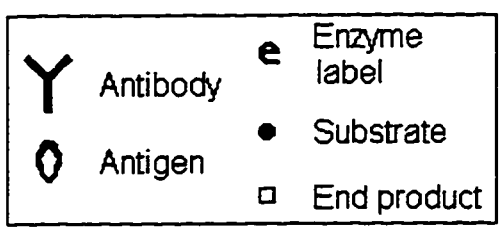

2. EXPERIMENTAL

2.1 INSTRUMENTATION

ELISA analyses were carried out in Immulon 2, 96-well, flat bottom microtiter plates from Dynatech Laboratories, Inc. (Chantilly, VA) with Linbro nonsterile acetate plate sealers from ICN Biomedicals, Inc. (Costa Mesa, CA) to prevent moisture loss. Microtiter plate optical densities were measured by a THERMOmax microplate reader with a SOFTmax version 2.32 for Macintosh software from Molecular Devices Corp. (Menlo Park, CA).

Water was purified through a Milli-Q system from Millipore Corp. (Milford, MA) before use. Samples were dialyzed through Spectra/Por 2 molecularporous membrane tubing (12-14,000 molecular weight cutoff) from Spectrum Medical Industries, Inc. (Los Angeles, CA). Macrosep 10K filter units (10,000 molecular weight cutoff) were purchased from Pall Filtron Corp. (Northborough, MA). Centrifugation was performed in a J2-21 centrifuge (Beckman Instruments, Inc., Palo Alto, CA). Solvents were removed by using a Büchi Rotavapor RE 121 (Fisher Scientific, Ottawa, ON). A Virtis 5L freeze-drier from The Virtis Company, Inc. (Gardiner, NY) was used for lyophilization.

Sephadex G-50 fine gel (for size exclusion column) and fast performance liquid chromatography (FPLC) equipment, which included a Superose 12 HR 10/30 gel filtration column, a High Precision Pump P-500, a V-7 Valve, a Single Path Monitor UV-1 with both control and optical units, and a Two-Channel Recorder REC-482, were purchased from Pharmacia Biotech Inc. (Baie d'Urfé, QC) for protein purifications. Mini-PROTEAN II Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA) was used for gel electrophoresis.

Nuclear magnetic resonance (NMR) and elemental analyses were performed by Chemistry Services at the University of Alberta. NMR

spectra were measured with a Bruker WH-400 instrument. Matrix-assisted, laser desorption/ionization time of flight mass spectrometry (MALDI or MALDI-TOFMS) analyses were performed by V. Fursey of the Mass Spectrometry Division in the Bruker Analytical Systems, Inc. (Billerica, MA). The matrix used was 3,5-dimethoxy-4-hydroxycinnamic acid for all samples analyzed in this study. Spectrophotometric analyses were carried out in a HP 8452A Diode-Array Spectrophotometer from Hewlett-Packard (Canada) Ltd. (Mississauga, ON).

Bacterial culture growth was carried out with pH maintained by a Chemcadet pH controller (Cole-Parmer, Chicago, IL). The 2M NaOH added for pH control was sterilized through a Millex-GS sterile 0.22 μm filter unit (Millipore Corp., Milford, MA).

2.2 MATERIALS

Tween 20, ovalbumin (OVA), bovine serum albumin (BSA), *Limulus polyphemus* hemolymph (LPH), Teleostean gelatin, protease (type XIV), glycine, acrylamide, bisacrylamide, Temed, 3,3',5,5'-tetramethylbenzidine (TMB) dihydrochloride, trifluoroacetic acid (TFA), ammonium persulfate and di-ammonium citrate were purchased from Sigma Chemical Co. (St. Louis, MO). 2-Bromoacetophenone and thiazole were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). D-Glucose, glycerol, di-potassium phosphate and manganous sulfate were obtained from BDH Inc. (Toronto, ON). Glutaraldehyde (25% solution), dimethyl sulfoxide (DMSO), D-fructose, α-lactose and sucrose were obtained from Fisher Scientific (Edmonton, AB). Casamino acids, Freund's complete adjuvant and Freund's incomplete adjuvant were purchased from Difco Laboratories (Detroit, MI). Urea peroxide and peroxidase-conjugated goat anti-rabbit antibodies were obtained from Calbiochem Co. (San Diego, CA). Yeast extract was purchased from Becton Dickinson and Company (Cockeysville, MD); Tween 80 from Anachemia Canada Inc. (Ville St. Pierre, QC); and

magnesium sulfate from J. T. Baker Inc. (Phillipsburg, NJ). Tricine was purchased from ICN Pharmaceuticals Inc. (Costa Mesa, CA); Tris from Life Technologies Inc. (Gaithersburg, MD); sodium dodecyl sulfate from Caledon Laboratories Ltd. (Georgetown, ON); low-range protein molecular weight markers from Promega Corp. (Madison, WI); Coomassie Blue stain from Bio-Rad (Hercules, CA).

Bacterial cultures used for brochocin-C production was *Brochothrix campestris* [American Type Culture Collection (ATCC) 43754]; and the indicator organism used in bioassays was *Carnobacterium piscicola* [University of Alberta Food Microbiology culture collection (UAL) 8].

2.3 REAGENTS

Phosphate-buffered saline (PBS) solution was prepared by dissolving NaCl (9.0 g), disodium hydrogen phosphate (1.108 g), and potassium dihydrogen phosphate (0.3 g) in 1 L of water and adjusting the pH to 7.3 with 6 N NaOH. To prepare PBST, Tween 20 (1.0 g) was added before the pH was adjusted. Citrate buffer solution (0.1 M) was prepared by dissolving citric acid monohydrate (21.0 g) and sodium citrate (29.4 g) in 2 L of water and adjusting the pH to 4.0 with 1 N HCl.

Casamino acids (CAA) medium for the growth of *Brochothrix campestris* ATCC 43754 was prepared by dissolving casamino acids (15 g), yeast extract (5 g), D-glucose (25 g), di-potassium phosphate (2 g), Tween 80 (1 mL), di-ammonium citrate (2 g), magnesium sulfate (0.1 g) and manganous sulfate (0.05 g) in 1 L of water, then autoclaving for 15 min.

2.4 GLYCATED PROTEINS

2.4.1 Non-physiological advanced glycation end products (AGEs)

Maillard reaction conditions of Matsuda *et al.* (1992) were applied. D-glucose (50 mg) and LPH (50 mg) were dissolved in 5 mL of water, then the pH adjusted to between 8.0 and 8.2 with 0.1 M NaOH solution. The

mixture was lyophilized, and kept for 4 days at 53 °C and 67% RH, which was maintained with a saturated cupric chloride solution. After the reaction, the sample was dialyzed, lyophilized and stored at −20 °C until analyses. Another sample was prepared in the same manner with OVA (descriptions in Table 2, p. 43). The non-physiologically glycated LPH (G-LPH) was used as an immunogen to produce antisera (5D4) in rabbits.

2.4.2 Separation of crosslinked proteins

Aged G-OVA (descriptions in Table 2, p. 43) from the above reaction with different degrees of crosslinking were separated using FPLC. A pH 7 phosphate buffer (0.05 M) with 0.15 M NaCl was used as an eluent after degassing. 100 µL of sample (25 mg/mL) was injected at a flow rate of 25 mL per hour. Fractions A and B (Figure 11) were collected for ELISA analyses.

2.4.3 Simple glycated proteins

The procedure from Yaylayan *et al.* (1992) was used to prepare some simple glycated proteins. A solution of BSA (0.1 mg/mL) and 0.22 M glucose in PBS was sterilized through a 0.2 µm sterile filter. Chloroform (0.2 mL) was added to each 50 mL sugar-protein solution for preservation. The samples were incubated at 37 °C for 5 and 12 days (which produced BSA linked with 5 and 10 glucose molecules, respectively) and dialyzed before analyses.

2.4.4 Physiological AGEs

A modified procedure from Grandhee and Monnier (1991) was used to prepare physiological AGEs. A D-glucose solution (5 mg/mL) was prepared by dissolving the sugar in PBS, and a BSA solution (5 mg/mL) was prepared in the same manner. A sugar-protein solution was prepared by combining 5 mL each of the sugar and protein solutions, the solution

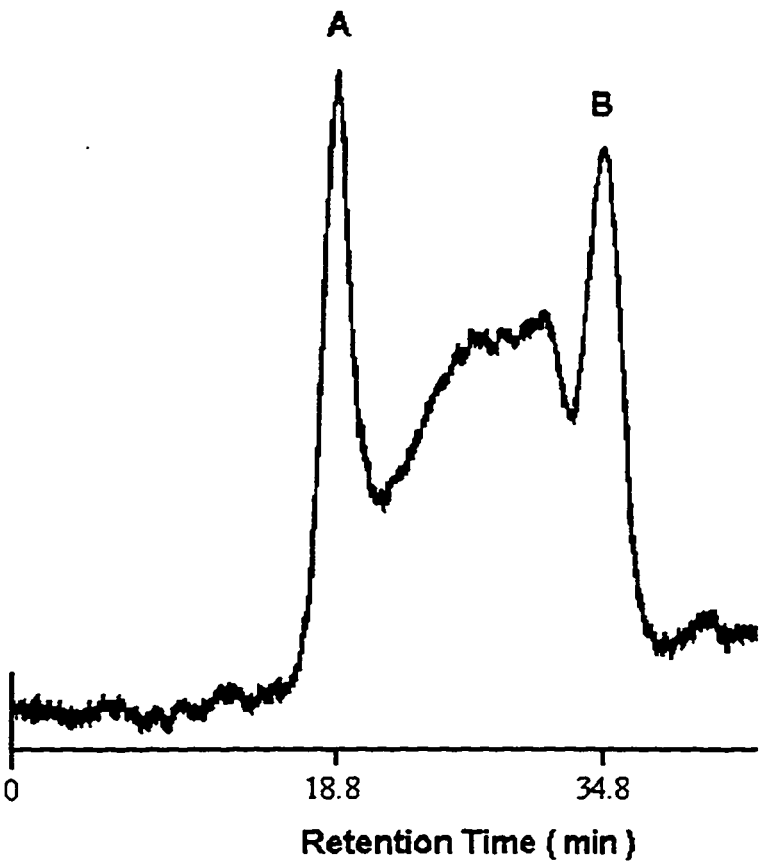

Figure 11. FPLC separation chromatograph.

Fractions A and B were used as competition samples in an ELISA. Native OVA was eluted 36.3 min after injection.

was filtered through a 0.2 μm sterile filter. The sugar-protein solution was stored at 37 °C for various time intervals. After a known period of time, a sample was dialyzed and lyophilized before analyses. Different combinations of sugar-protein solutions were also prepared with combinations of D-fructose, α-lactose and OVA.

2.4.5 Preparation of *N*-phenacylthiazolium bromide (PTB)

A glycation crosslink cleaving agent (PTB) was synthesized according to Vasan *et al.* (1996). Thiazole (0.214 mL) and 2-bromoacetophenone (0.6021 g) were dissolved in 3.0 mL of dried ethanol and refluxed for 2 hours. The product was recrystallized from 90% aqueous ethanol as a white powder (0.116 g, 13.6%): melting point, 233-233.5 °C (literature melting point, 223-223.5 °C; Ukai *et al.*, 1943); ^{1}H nuclear magnetic resonance (NMR) [400 MHz, $(CD_3)_2SO$] δ 10.15 (multiplet, 1H, –N=C*H*–S–), 8.47 (multiplet, 1H, 4 Hz, –N–C*H*=C–), 8.38 (multiplet, 1H, 4 Hz, –C=C*H*–S–), 8.04 (multiplet, 2H, 8 Hz, aromatic *H*), 7.78 (multiplet, 1H, 8 Hz, aromatic *H*), 7.65 (multiplet, 2H, 8 Hz, aromatic *H*), 6.40 (singlet, 2H, –C–CH_2–N–); moisture residue δ 3.35 (singlet, 15H, H_2O). Crosslink cleaving reaction pathway of PTB is shown in Figure 12.

In the PTB crosslink cleaving experiment, it should be noticed that following the binding of goat anti-rabbit antibodies, F-OVA, BF28 and OF28 (descriptions in Table 2, p. 43) were stored at 4 °C with 200 μL of PBST for 12 h (which was different from the normal procedure of taking a measurement immediately). Their absorbances were then read 1 h 15 min after the addition of TMB solution. The color development time was 5 times longer than the 15 min color development of the aged and the fresh G-OVAs. However, the two groups have their own sets of controls for analyses.

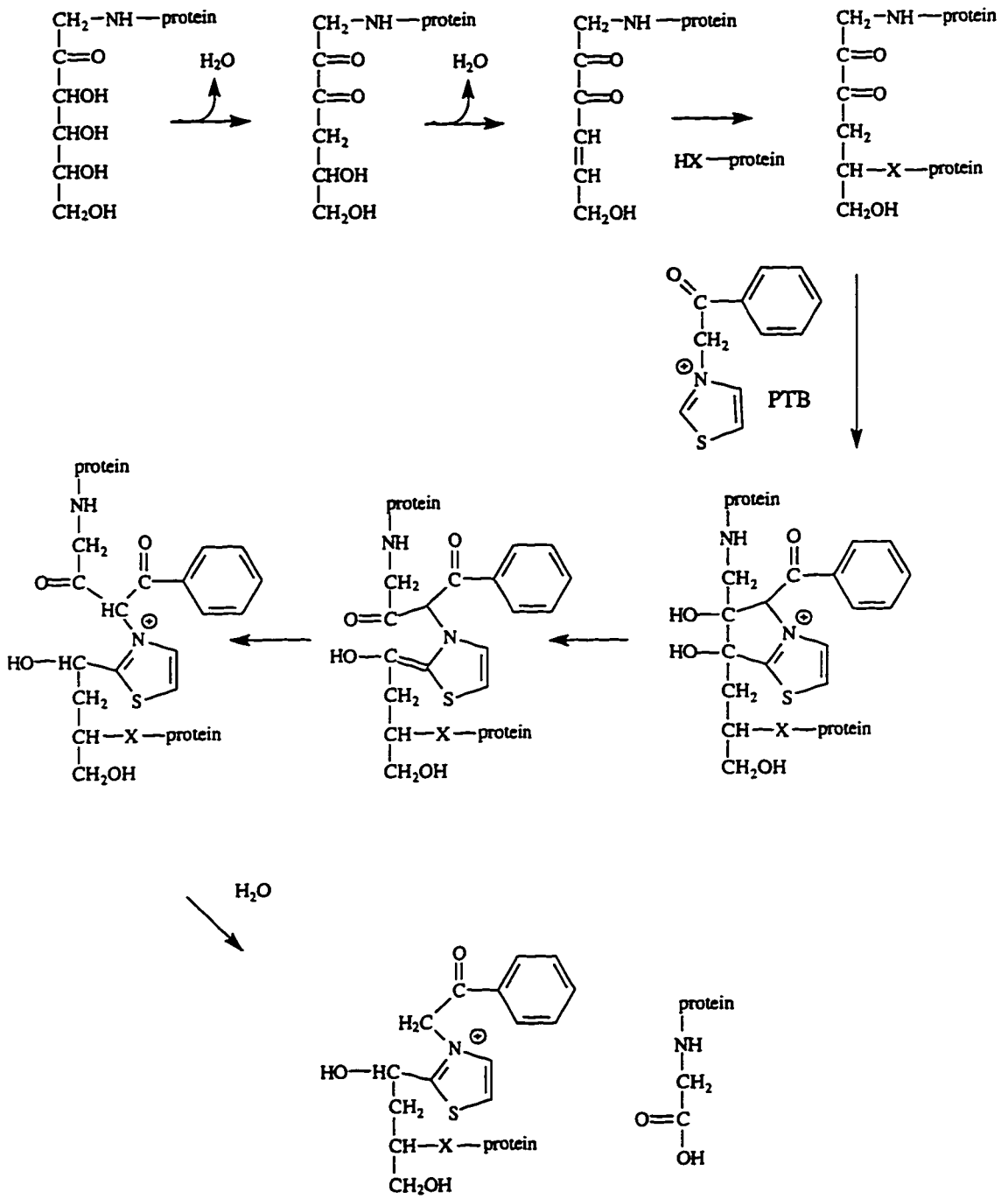

Figure 12. PTB crosslink cleaving reaction (Vasan *et al.*, 1996).

2.5 BROCHOCIN-C

2.5.1 Production and purification of brochocin-C

A 24 h culture of *Brochothrix campestris* ATCC 43754 was inoculated at 2% (v/v) into 3 L of sterile room temperature CAA medium (Hastings *et al.*, 1991; Poon, 1995) with 2.5% (w/v) glucose. After 18 hours growth at a constant pH of 6.7 the cells were removed by centrifugation at 10,000 rpm/2 °C for 20 minutes. The clear culture broth was boiled for 10 minutes to inactivate any proteases and then cooled. The broth was extracted with n-butanol (broth:n-butanol = 11:3) by stirring vigorously overnight. The n-butanol layer was separated by centrifugation at 10,000 rpm/2 °C for 10 minutes. The n-butanol extract was vacuum evaporated at 35 °C with continuous water dilution until all the n-butanol was removed. The aqueous extract (about 90 mL) was mixed with 1 L of cold acetone (-70 °C) and stored at 4 °C for 24 hours. The precipitate was obtained after centrifugation at 12,000 rpm/2 °C for 20 minutes. The precipitate was dissolved in 10 mL of 0.1% (v/v) aqueous TFA.

After the cold acetone precipitation, Macrosep 10K filter units were used for brochocin-C purification. Two filter units were pre-washed each with 10 mL of 0.1% (v/v) TFA by centrifugally filtering some TFA washing solution at 6000 rpm/10 °C for 20 min. Brochocin-C, collected from three batches of cold acetone precipitations, was loaded in the filter units with 12.7 mL per unit, then centrifuged at 6000 rpm/10 °C for 1 hour. The residual volumes were washed each with 10 mL of 0.1% TFA in the same conditions for 90 min. All fractions of greater than 10 kDa, less than 10 kDa and less than 10 kDa (wash) were collected and stored at –20 °C until analyses.

However, both the less than 10 kDa and the less than 10 kDa wash fractions exhibited no antimicrobial activity in a spot-on-lawn assay, but the greater than 10 kDa fraction retained antimicrobial activity (Figure 13).

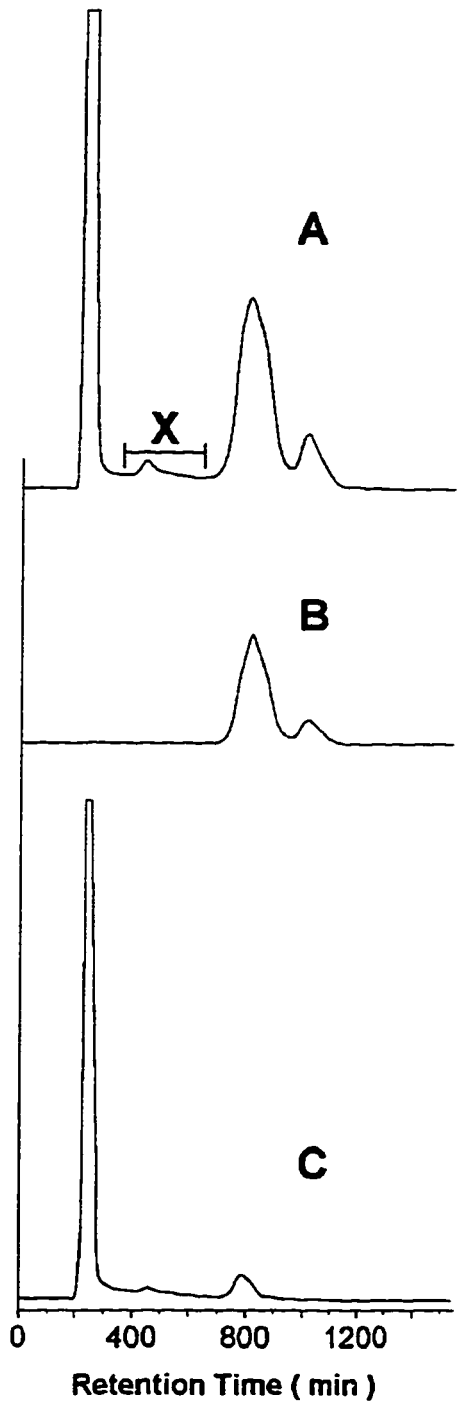

Figure 13. Sephadex G-50 size exclusion column chromatographs.

Crude brochocin-C from cold acetone precipitation was further purified by a 10 kDa Macrosep filter unit. A was the separation with protein MW greater than 10 kDa; B was the less than 10 kDa protein filtrate; C was the re-load of the first peak (i. e. the fraction between 210 min and 360 min) of A. Region X in chromatograph A was the brochocin-C fraction used for SDS-PAGE analyses and conjugation to a carrier protein.

Thus, the greater than 10 kDa fraction was loaded to a Sephadex G-50 column (length: 104 cm; diameter: 3 cm) for purification. The column was eluded with 0.1% (v/v) TFA at a flow rate of 0.6 mL/min. The collected fractions were tested for antimicrobial activity.

2.5.2 Spot-on-lawn activity analysis for brochocin-C

The activity of brochocin-C was determined by the spot-on-lawn assay (Ahn and Stiles, 1990). On an APT plate, 10 μL of a 1:1 doubling dilution of sample with sterile water was spotted and overlaid with soft APT agar containing a 1% (v/v) inoculum of UAL 8. The plate was placed in a gas jar filled with 10% carbon dioxide and 90% nitrogen and stored overnight at room temperature. The activity was quantified by an arbitrary activity unit (AU) defined as the reciprocal of the highest dilution that still indicated a clear growth inhibition zone on the plate.

i.e. AU per 10 μL = 2^n , where n = the highest possible number of dilution with activity

2.5.3 Protein assay for brochocin-C

A protease (type XIV) was used to inactivate the inhibition activity of the produced brochocin-C for confirming its proteinaceous nature. It was similar to the spot-on-lawn assay with 10 μL of protease solution (Sambrook *et al.*, 1989) spotted next to a dried spot of brochocin-C. An eclipsed moon shape of inhibition zone was formed as the brochocin-C was digested by the protease.

2.5.4 Tricine SDS-PAGE analysis of brochocin-C

Composition analysis for various stages of brochocin-C purification was carried out in a Tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system (Schägger and von Jagow, 1987). A

49.5% T, 3% C, where T was denoted as the total percentage concentration (w/v) of both acrylamide and bisacrylamide, and C as the percentage concentration (w/v) of bisacrylamide relative to T, polyacrylamide gels in 3 M Tris-HCl were used. The voltage for electrophoresis was supplied at 30 V for 1 hour, and then, 90 V for 2 hours. The gels were fixed in 50% ethanol, 10% acetic acid for 30 minutes with swirling at room temperature. The gels were either stained with Coomassie Blue or overlaid for antimicrobial activity assay (Barefoot and Klaenhammer, 1983).

2.5.5 Conjugation of brochocin-C to carrier proteins

Brochocin-C was conjugated to BSA and to OVA using glutaraldehyde (Harlow and Lane, 1988; Reichlin, 1980). The collected fractions with antimicrobial activity after the G-50 column were concentrated and combined, so that the concentration of brochocin-C in TFA solution was about 1.39 mg/mL. This approximation of brochocin-C content was based on Table 1. A brochocin-C solution of 800 µL was adjusted with NaOH to about pH 7, then PBS was added for a total volume of 2 mL. BSA (3.12 mg) was added to the brochocin-C solution, so that the ratio of brochocin-C to BSA was about 4.5 to 1. The conjugation was carried at room temperature by slowly adding 2 mL of 0.2% (w/v) glutaraldehyde in PBS to the brochocin-C solution with stirring. The mixture was allowed to react for 20 hours, then stopped by adding 1 mL of 1 M glycine in PBS solution. The mixture was dialyzed with 4 changes of 4 L water at 4 °C overnight, then lyophilized. A control BSA sample was also produced in a similar way except there was no brochocin-C in the TFA solution before neutralization.

Table 1. Recovery of brochocin-C from purification.

Purification step	Volume (mL)	Activity (AU/mL)	Total activity (AU)	Protein conc. (mg/mL)	Special activity (AU/mg)	% recovery
supernatant	3000	3200	9.6×10^{6}	1.197	2.67×10^{3}	100
butanol extraction	100	51,200	5.12×10^{6}	3.155	1.62×10^{4}	53
acetone precipitation	20	204,800	4.096×10^{6}	ND	ND	43
Sephadex G-50 size exclusion	8	51,200	4.096×10^{5}	0.085	6.02×10^{5}	4.3

ND = not determined

The table is adapted from Poon (1995).

2.5.6 Brochocin-C peptide fragment conjugates from Alberta Peptide Institute (API)

The peptide syntheses and conjugations were carried out by Alberta Peptide Institute (Department of Biochemistry, University of Alberta, Edmonton, Canada). The amino acid sequence of brochocin-C (Figure 4, p. 11) was determined by Poon (1995). Seven residues from the N-terminal and the C-terminal were used for peptide syntheses, except that the original cysteine at the sixth residue from the N-terminal was switched to alanine. Ornithine-benzoyl benzoic acid [Orn(Bb)] was used for the N-terminal peptide (2616: NH_2-Y S S K D A L Orn(Bb)-COOH) conjugation to the carrier proteins; and benzoyl benzoic acid (Bb) was used for the C-terminal peptide (2617: BbNle-I G G L L G N-COOH) conjugation. The carrier proteins were bovine serum albumin (BSA) and keyhole limpet hemocyanin (KLH). The synthetic peptides were purified by high performance liquid chromatography (HPLC), and their composition was confirmed by amino acid analysis and mass spectrometry. The average peptide:carrier conjugation ratios were determined as 2616-KLH and 2616-BSA were both 5:1; 2617-KLH and 2617-BSA were 27:1 and 29:1, respectively.

2.6 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

2.6.1 Immunization of rabbits

Two rabbits, Flemish Giant × French Lop Ear, were immunized with 1.0 mg (about 0.1-0.2 mg for glutaraldehyde conjugated brochocin-C immunogen) of conjugate in 2 mL of sterile PBS/Freund's complete adjuvant (1:1) for prime injection. Each rabbit was injected with 1.5 mL of the conjugate-adjuvant mixture. First boost was made after 4 weeks, in a similar manner but using Freund's incomplete adjuvant. Test-bleed samples (2-3 mL each) were taken 10 days after the boost injection. The blood samples were allowed to clot at 4 °C and centrifuged (10,000 rpm for 10 minutes) in microtainer tubes to remove blood cells. The antisera were collected and stored at -20 °C for antibody titer test later.

2.6.2 Checker board ELISA

Antibody titer was determined by performing a modified version of the checker board ELISA procedure of Plhak and Sporns (1992). Coating conjugate was dissolved at concentrations from 1 to 10 ppm in PBS. Each well of a microtiter plate was filled with 200 µL (or less) of one of the above coating conjugate solutions. The plate with a plastic sealer was stored overnight at 4 °C. The solution was removed from the plate and each well was washed 3 times with 200 µL PBS and coated with 200 µL of 1% (w/v) BSA (or Teleostean gelatin) in PBS. This solution was left for 1 h at room temperature. The wells were emptied and washed with PBST as before.

Antiserum was diluted 1/100 with 0.05% (w/v) BSA in PBST. Serial dilutions (1:10) from 1/100 to 1/10,000,000 and a blank of 0.05% (w/v) BSA in PBST were prepared. Aliquots (100 µL) of water were then added to each well of the coated microtiter plate, immediately followed by 100 µL aliquots of diluted antiserum or the 0.05% (w/v) BSA in PBST blank

solution such that each row of the microtiter plate contained a different antiserum dilution. The plate was incubated for 2 h at room temperature with a plastic sealer. The wells were emptied and washed with PBST. Goat anti-rabbit antibody-peroxidase conjugate was diluted 1/3000 with 0.05% (w/v) BSA in PBST and added to each well in 200 µL quantities. This was incubated for 2 h at room temperature with a plastic sealer, then washed with PBST. An enzyme substrate solution was prepared by combining 200 µL TMB (10 mg/mL), which was dissolved in DMSO, and 20 mL urea peroxide (1 mg/mL), which was dissolved in citrate buffer. The enzyme substrate solution (200 µL) was added to each well and absorbance (A_{450nm} - A_{650nm}) measured after 15 min at room temperature (Thomson, 1994). The titer was defined as a combination of minimum rabbit antiserum and coating conjugate dilutions that gave an absorbance reading that was at least 3 times greater than variation in background absorbances (with no serum).

The absorbances were analyzed using the Microsoft Excel, version 5.0a, solver from Microsoft Corp. (Mississauga, ON). Best fit analysis curves of absorbance versus log analyte concentration were plotted using the sigmoidal equation:

$$y = (a - d) / [1 + (x / c)^{b}] + d$$

where x = concentration of analyte, y = response (i.e. absorbance units), a = asymptote at low values of x-axis, d = asymptote at high values of the x-axis, c = x value corresponding to the midpoint between a and d values (i.e. I_{50} value), and b = slope of curve at the midpoint between a and d values.

2.6.3 Non-competitive indirect ELISA (ncid-ELISA)

After finding the combination of minimum rabbit serum and coating

conjugate dilutions, a similar procedure as the checker board ELISA was applied. During the coating step (overnight at 4 °C), the wells were coated with either the coating conjugate or samples for analyses at known concentrations (Figure 8, p. 23). The remaining steps of the ELISA were the same as described for the checker broad ELISA.

2.6.4 Competitive indirect ELISA (cid-ELISA)

This was similar to the ncid-ELISA; however, the wells were coated only with the coating conjugate. The sample (100 µL) for analysis was incubated together with 100 µL of the rabbit serum for 2 hours at room temperature (Figure 7, p. 22). Other steps were unchanged.

3. RESULTS AND DISCUSSION

3.1 ADVANCED GLYCATION END PRODUCTS (AGEs)

The objective in the development of an ELISA for glycated proteins was to maximize the diversity of AGEs that could be detected by antibodies. Hence, the method of Matsuda *et al.* (1992) was used to generate wide varieties of glycated proteins, ranging from simple glycated proteins to crosslinked AGEs (vigorous non-physiological conditions), for rabbit immunization. (Descriptions of sample codes used in section 3.1 are summarized in Table 2.)

3.1.1 MALDI analysis

Comparison of the two MALDI spectra of OVA and aged G-OVA (Figures 14 and 15, respectively) confirmed that there was significant glycation of OVA (average mass increase of OVA from 44268 to 49490). The molecular weight different was 5222, which is roughly equal to 32 glucose molecules. The increase in mass could not totally be due to the glucose attachments because mixtures of numerous compounds would have been formed in Maillard reactions, including the crosslinkings between OVA and many other AGEs formed. Moreover, there seemed to be no significant unreacted OVA present. This was further confirmed using FPLC (section 3.1.3).

3.1.2 Anti-AGEs antibodies sensitivity and specificity against non-physiological AGEs

The antibodies in antiserum 5D4 developed against G-LPH were sensitive and specific against similar kinds of glycated protein antigens, fresh G-OVA, aged G-OVA and F-OVA (Figure 16). The I_{50} value of the aged G-OVA curve (5.48 ppm) is less than that of the fresh G-OVA (89.6 ppm). The results suggested that the aged G-OVA was more competitive

Table 2. Descriptions of codes used in section 3.1.

Code	Description
OVA	native ovalbumin
BSA	native bovine serum albumin
LPH	native *Limulus polyphemus* hemolymph
fresh G-OVA	non-physiologically glycated OVA with glucose (Matsuda *et al.*, 1992)
aged G-OVA	same as fresh G-OVA except the product was stored at room temperature for about a year
G-LPH	same as fresh G-OVA except LPH was used instead of OVA
F-OVA	same as fresh G-OVA except fructose was used instead of glucose
L-OVA	same as fresh G-OVA except lactose was used instead of glucose
S-OVA	same as fresh G-OVA except sucrose was used instead of glucose
OVA control	same as fresh G-OVA, but no glucose was used
OG	physiologically (in PBS solution; pH 7.3; 37 °C) glycated OVA with glucose for various reaction times (Grandhee and Monnier, 1991)
OF	same as OG except fructose was used instead of glucose
OF28	OF glycated for 28 weeks
OL	same as OG except lactose was used instead of glucose
BG	same as OG except BSA was used instead of OVA
BF	same as BG except fructose was used instead of glucose
BF28	BF glycated for 28 weeks
BL	same as BG except lactose was used instead of glucose

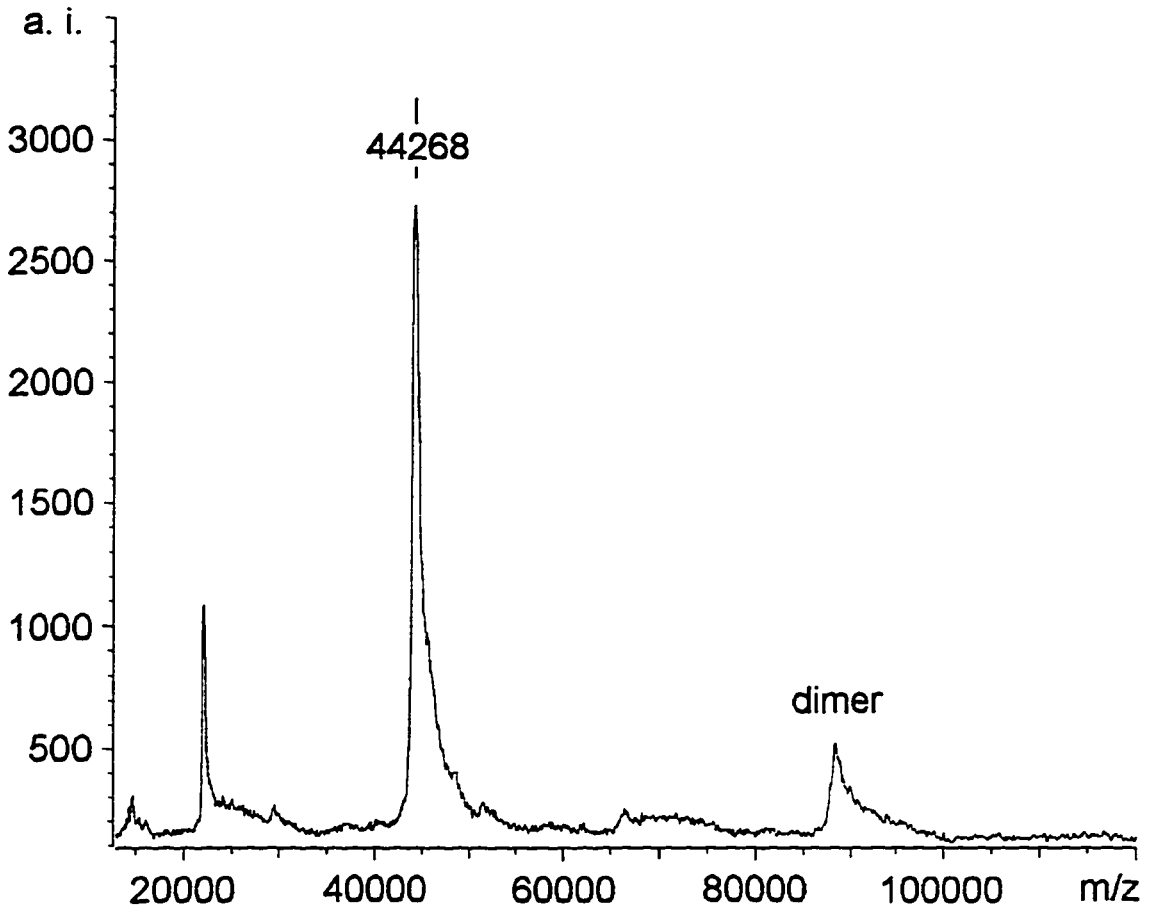

Figure 14. MALDI spectrum of OVA.

a. i. = arbitrary intensity

m/z = mass per charge

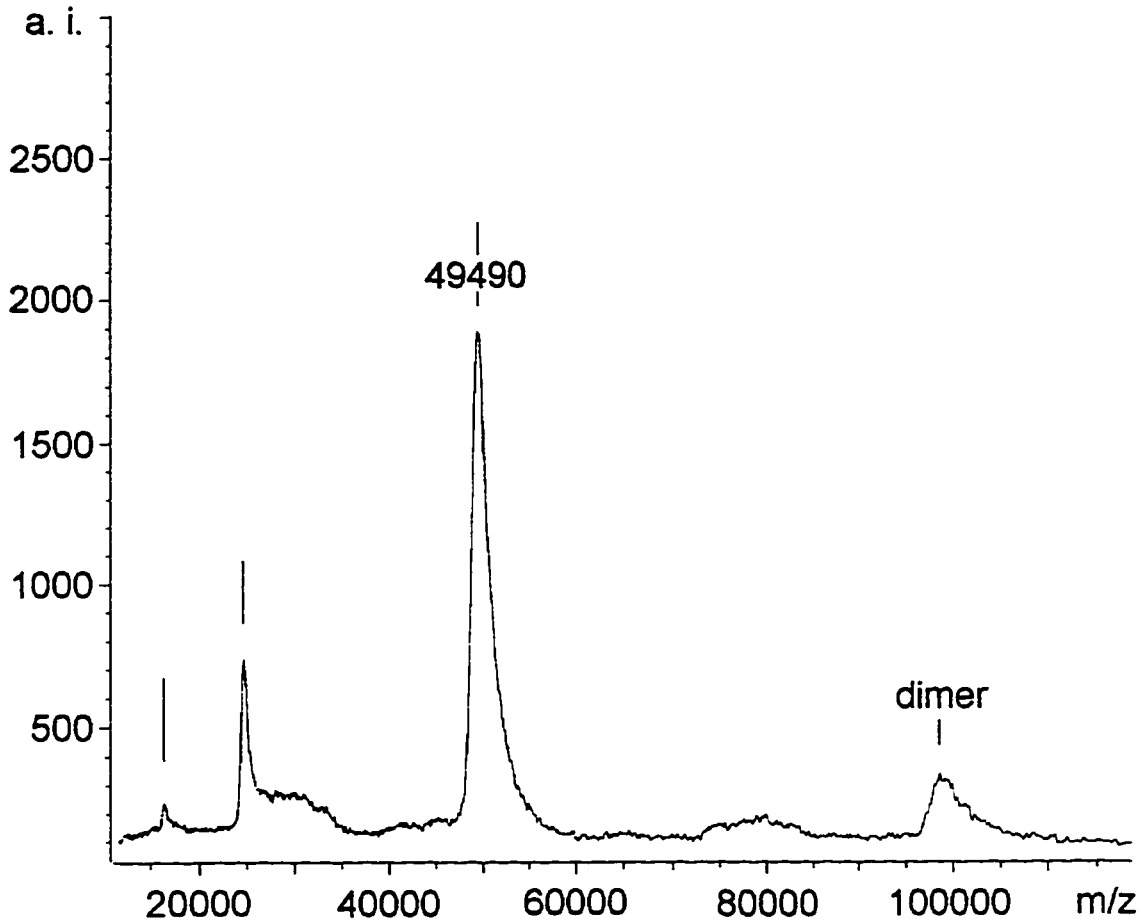

Figure 15. MALDI spectrum of aged G-OVA.

a. i. = arbitrary intensity

m/z = mass per charge

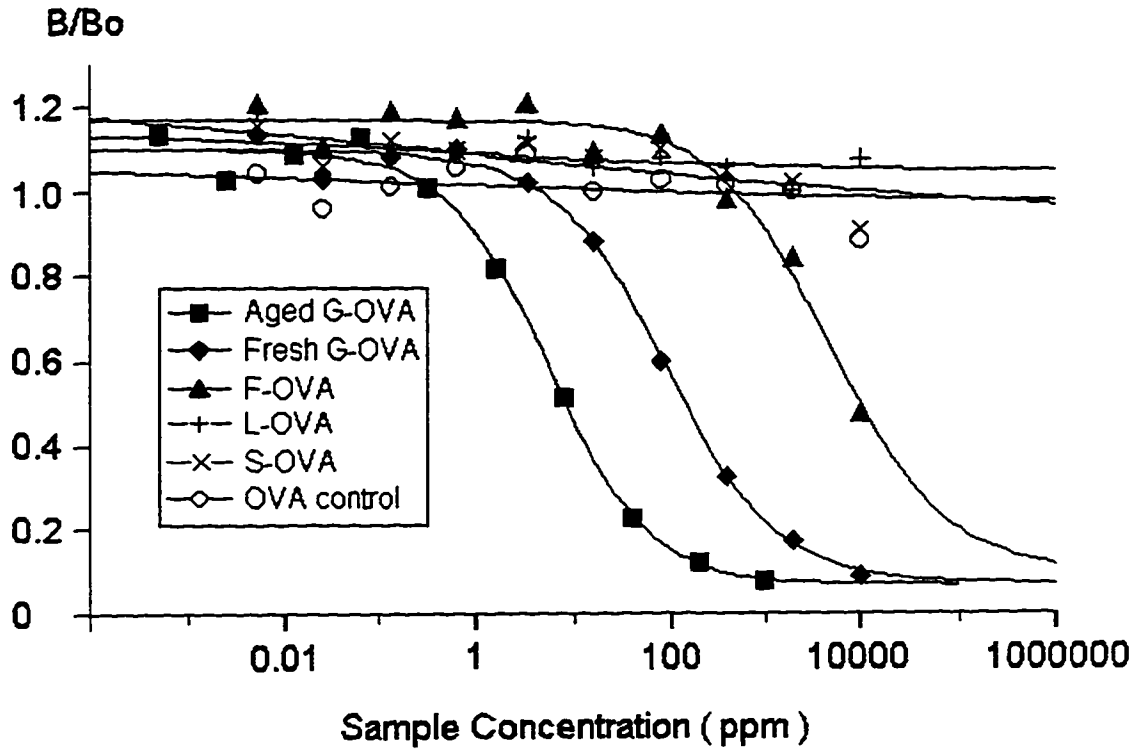

Figure 16. The cid-ELISAs of non-physiologically glycated OVAs.

Curve parameter of aged G-OVA (■): "a"=1.10, "b"=0.857, "c"=5.48 and "d"=0.0684; the correlation coefficient (r) was 0.997. Curve parameters of fresh G-OVA (◆): "a"=1.10, "b"=0.757, "c"=89.6 and "d"=0.0712; the correlation coefficient (r) was 0.997. Curve parameter of F-OVA (▲): "a"=1.17, "b"=0.732, "c"=4870 and "d"=0.0917; the correlation coefficient (r) was 0.980. There was no significant recognition of L-OVA (+), S-OVA (×) and OVA control (O). Each well was coated with 200 μL of 10 ppm fresh G-OVA; antiserum 5D4 dilution was 1:100,000.

than the fresh G-OVA. The F-OVA, which showed an I_{50} value of 4870 ppm, was a weak competitor in comparison with both the aged and the fresh G-OVAs. However, L-OVA, S-OVA and OVA control exhibited no binding to the antibodies.

3.1.3 Anti-AGEs antibodies and the crosslinked AGEs

The crosslinked AGEs were separated using FPLC, a gel permeation column, which fractionated proteins according to molecular weight differences (Figure 11, p. 30). High molecular weight proteins were eluted faster than lower molecular weight proteins. The early and the late peak fractions, A (with high molecular weight) and B (with low molecular weight), were analyzed with a cid-ELISA (Figure 17). The B/Bo value, which inversely indicates the degree of competition for antibodies, was significantly lower for fraction A than fraction B. Therefore the antibodies had a higher binding affinity to the modified proteins in fraction A than the proteins in fraction B. Since both fractions A and B (18.8 and 34.8 min, respectively) were eluted faster than the native OVA (36.3 min), the assumption was made that fraction A contained most of the crosslinked OVA because of its higher mass. Furthermore, glycated protein molecules can continue to crosslink with each others even without the presence of reducing sugar (Eble *et al.*, 1983). Figure 16 (p. 46) shows the antiserum 5D4 was more sensitive against the aged G-OVA than the fresh G-OVA. Hence, the antibodies in antiserum 5D4 were more sensitive and specific against AGE crosslinks.

3.1.4 Comparison between physiological and non-physiological conditions for preparing AGEs

Physiologically and non-physiologically glycated proteins were tested for recognition by the developed antiserum 5D4 (Figures 18 and 19). Protein samples of OF, BG, BF, BL (physiologically glycated for 28 weeks)

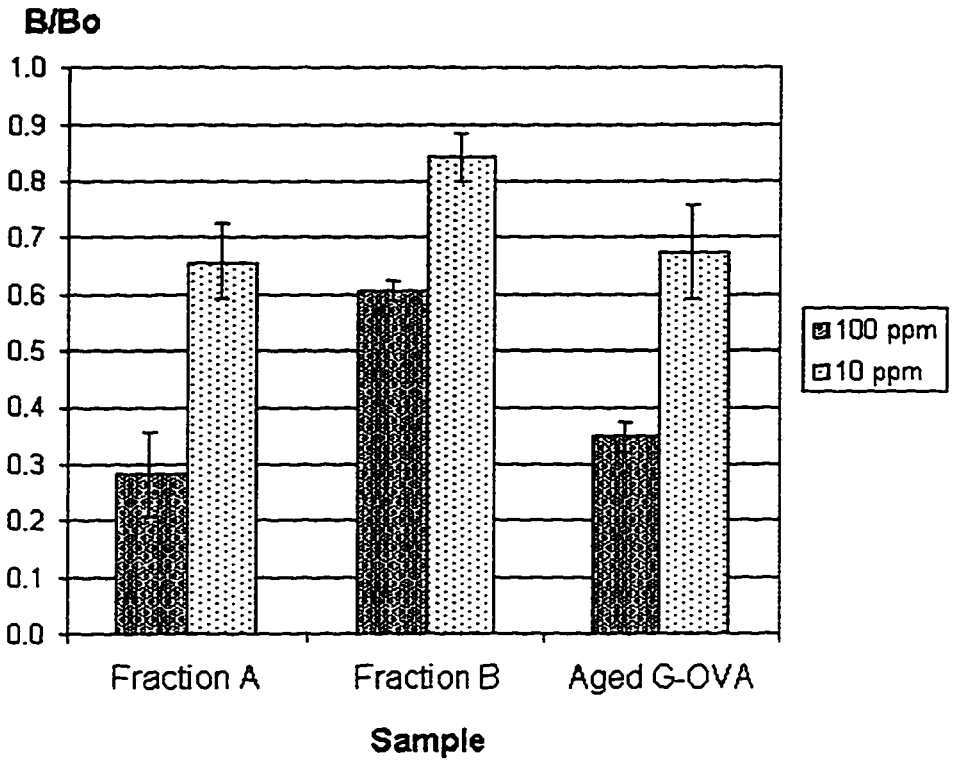

Figure 17. Comparison between FPLC fractions and aged G-OVA.

Each column represents the mean B/Bo value of 3 wells; error bars indicate the standard deviation of the mean. Each well was coated with 200 µL of 10 ppm fresh G-OVA; antiserum 5D4 dilution was 1:100,000.

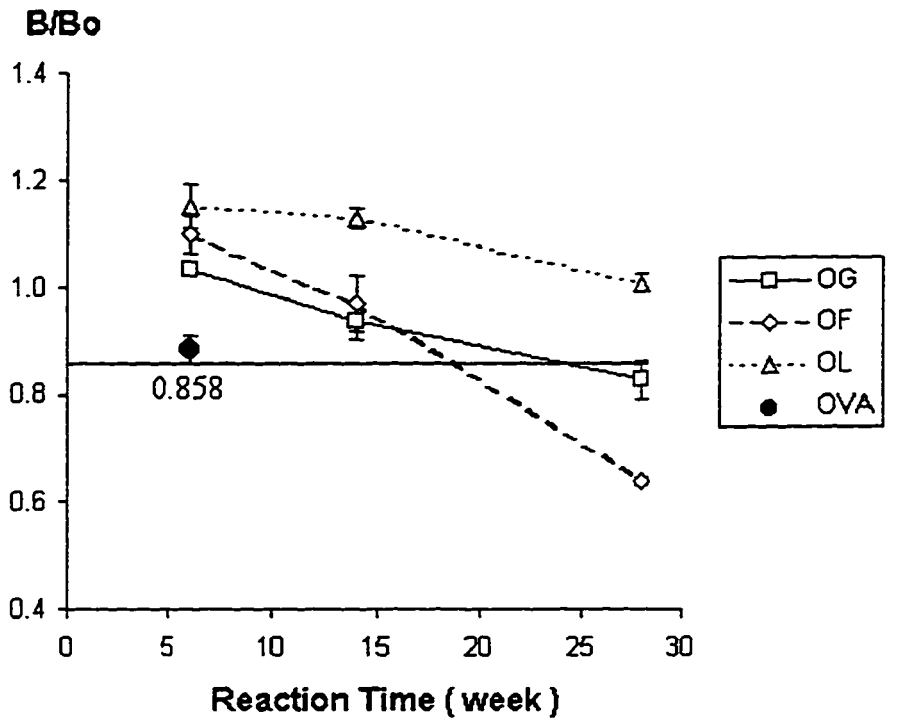

Figure 18. Recognition of physiologically glycated OVAs with different reducing sugars.

Each point represents the mean B/Bo value of 6 wells; error bars indicate the standard deviation of the mean. Each well was coated with 200 μL of 10 ppm fresh G-OVA; antiserum 5D4 dilution was 1:100,000; competition sample concentrations were 10,000 ppm.

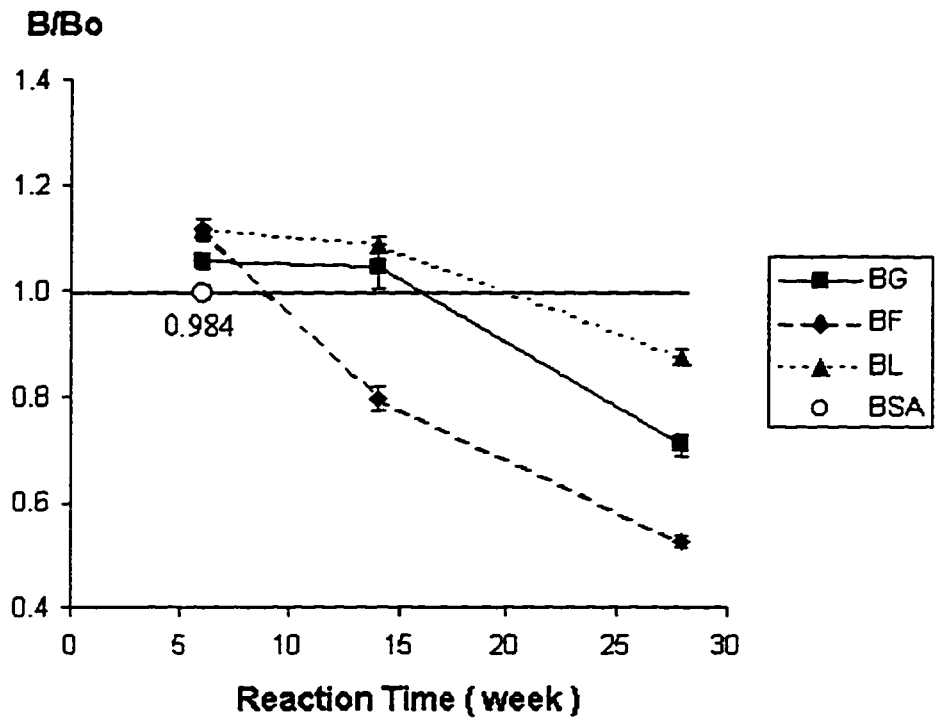

Figure 19. Recognition of physiologically glycated BSAs with different reducing sugars.

Each point represents the mean B/Bo value of 6 wells; error bars indicate the standard deviation of the mean. Each well was coated with 200 µL of 10 ppm fresh G-OVA; antiserum 5D4 dilution was 1:100,000; competition sample concentrations were 10,000 ppm.

and BF (physiologically glycated for 14 weeks) were recognized by the antiserum 5D4 when compared with their controls (i.e. OVA and BSA) at the same concentration level in cid-ELISAs. The similarities of AGE structures, regardless of the type of reacting proteins, were investigated (Horiuchi *et al.*, 1991). In this study, similarities of AGE structures, which were recognized by the antiserum 5D4, were present even though the reaction conditions (i.e. physiological and non-physiological) and the reacting reducing sugars were different. However, the findings in this study appear to contradict Münch *et al.* (1994) who suggested that the glycation pH and temperature should be adjusted to physiological conditions (i.e. pH 7.4 and 37 °C, respectively) in order to yield physiological AGEs. In this study, the antibodies, which were developed against a non-physiologically glycated protein, were shown to have an overlapping sensitivity and specificity against both the physiological and the non-physiological AGEs.

3.1.5 Cleavage of crosslinks in AGEs by *N*-phenacylthiazolium bromide (PTB)

After reacting the AGE samples with PTB, a known crosslink cleaving agent (Vasan *et al.*, 1996), the competitiveness of the AGE samples decreased as shown by the increase in their B/Bo values when compared with their controls at time zero (Figure 20). The results indicate that the degree and type of crosslinking likely varied between samples since the results of reaction with PTB were different.

3.1.6 Simple glycated proteins

The antibodies were tested in cid-ELISAs against other substances (Table 3), but no significant recognition was found for any compounds in Table 3 as the B/Bo values were not significantly lower than 1. However, most of the B/Bo values were greater than 1. This increase in B/Bo value,

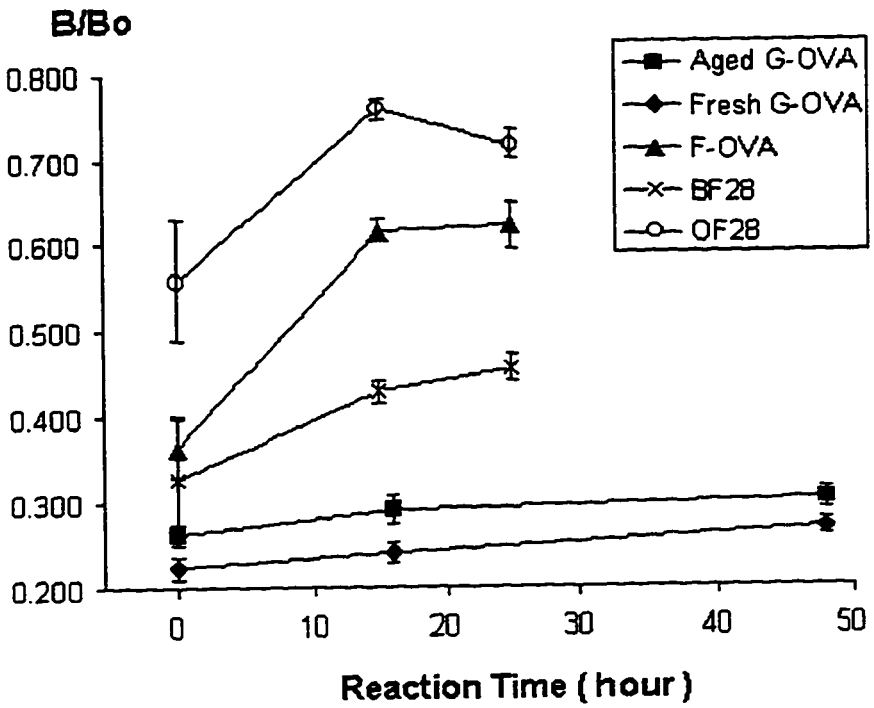

Figure 20. ***N*-phenacylthiazolium bromide (PTB) crosslink cleaving cid-ELISAs.**

Each point represents the mean B/Bo value of 4 wells; error bars indicate the standard deviation of the mean. Initial concentrations for aged G-OVA (■) and fresh G-OVA (◆) were 40 ppm and 1000 ppm respectively; F-OVA (▲), BF28 (X) and OF28 (O) were at 10,000 ppm; PTB concentration was at 3.0 mM. Each well was coated with 200 mL of 10 ppm fresh G-OVA; antiserum 5D4 dilution was 1:100,000.

Table 3. Results of samples without recognition by antiserum 5D4 in cid-ELISAs.

Sample	B/Bo
Glucose	
100 ppm	1.17 ± 0.04
1000 ppm	0.955 ± 0.189
Simple glycated BSA	
with 5 glucose molecules attached	0.910 ± 0.162
(blank control)	(0.983 ± 0.109)
with 10 glucose molecules attached	0.962 ± 0.195
(blank control)	(1.00 ± 0.20)
Glycated human serum albumin (with 1-3 moles hexose per mole albumin)	
1 ppm	1.01 ± 0.03
10 ppm	1.01 ± 0.11
100 ppm	1.11 ± 0.03
1000 ppm	2.11 ± 0.43
Human serum albumin	
1 ppm	0.919 ± 0.05
10 ppm	1.24 ± 0.10
100 ppm	1.16 ± 0.03
1000 ppm	1.73 ± 0.18

Table 3 (continued)

Glycated human albumin (with 1-5 moles hexose per mole albumin)	
1 ppm	1.06 ± 0.06
100 ppm	1.09 ± 0.06
Human albumin	
1 ppm	1.02 ± 0.02
100 ppm	0.989 ± 0.100
Glycated bovine albumin (with 1-2 moles hexose per mole albumin)	
1 ppm	1.01 ± 0.05
100 ppm	1.00 ± 0.09
Bovine albumin	
1 ppm	1.05 ± 0.04
100 ppm	1.07 ± 0.09
Embryo samples from McGill University	
Control	1.33 ± 0.06
G1 [18.75]	1.20 ± 0.07
Tox [100]	1.39 ± 0.04
Tox [1000]	1.38 ± 0.04
Tox [5000]	1.32 ± 0.04
T2 [100]	1.37 ± 0.13
T2 [1000]	1.29 ± 0.04
T2 [5000]	1.27 ± 0.08

Table 3 (continued)

T12 [100]	2.11 ± 0.16
T12 [1000]	1.77 ± 0.28
T12 [5000]	2.05 ± 0.40

Simple glycated BSAs were prepared by the method of Yaylayan *et al.* (1992).

Glycated human serum albumin, human serum albumin, glycated human albumin, human albumin, glycated bovine albumin and bovine albumin were standards obtained from Sigma Chemical Co. (St. Louis, MO) and shipped from McGill University.

The embryo samples were supplied by Dr. Yaylayan of McGill University. Stock solutions of the technical toxaphene mixture (Ultra Scientific, North Kingstown, RI) and the toxaphene congeners, 2-exo,3-endo,5-exo,6-endo,8,8,10,10-octachlorobornane (T2) and 2-exo,3-endo,5-exo,6-endo,8,8,9,10,10-nonachlorobornane (T12) which were obtained from Promochem (Wesel, Germany), were prepared by dissolving in DMSO and each was combined with male rat serum. Control samples were early stage rat embryo culture solutions containing 0.01% (v/v) DMSO. G1 [18.75] samples were control samples containing 18.75 mM of D-glucose (Sigma Chemical Co., St. Louis, MO). Tox [100], Tox [1000] and Tox [5000] were G1 [18.75] samples containing 100 ng/mL, 1000 ng/mL and 5000 ng/mL of the technical toxaphene mixture, respectively. T2 [100], T2 [1000] and T2 [5000] were G1 [18.75] containing 100 ng/mL, 1000 ng/mL and 5000 ng/mL of the T2 congener, respectively. T12 [100], T12 [1000] and T12 [5000] were G1 [18.75] containing 100 ng/mL, 1000 ng/mL and 5000 ng/mL of the T12 congener, respectively.

which indicates enhanced binding of antibodies (no competition) to the G-OVA coating on the ELISA plate, was likely due to changes in competition conditions by the large amount of proteins (up to 1 ppt) examined. All of these samples (Table 3) indicated that the polyclonal antibodies that we prepared had no recognition for simple glycated proteins because their B/Bo values were staying around 1.

3.2 BROCHOCIN-C

Descriptions of sample codes used in section 3.2 are summarized in Table 4.

3.2.1 Purification of brochocin-C

During the purification steps, the amount of pure brochocin-C obtained was very low (Table 1, p. 37). A large amount of antimicrobial activity was retained in the interphase solids formed during the butanol extraction (Figure 21) which accounts for about 50% loss of the total brochocin-C production in Table 1 (p. 37). Active brochocin-C could only be determined with the spot-on-lawn technique (Ahn and Stiles, 1990). The brochocin-C activity given in Table 1 (p. 37) may have large errors due to the fact that serial dilutions of 1:1 were performed to quantitate the activity. Therefore error in activity estimation could be as large as 50%.

In Figure 13 (p. 34), antimicrobial activity of brochocin-C was found in the 210-825 min eluted fractions in chromatograph A, and the 240-300 and 405-555 min eluted fractions in chromatograph C, but none in chromatograph B. The failure of the Macrosep 10K filter unit as a tool for brochocin-C isolation might be due to the hydrophobic interaction between brochocin-C and other high molecular weight proteins, such that the 5241 Da brochocin-C could not pass through the 10 kDa cutoff filter membrane.

Although Poon (1995) further purified the brochocin-C with reverse-phase HPLC after the Sephadex G-50 size exclusion step, this resulted in at least another 50% greater loss of brochocin-C (Table 1, p. 37). Furthermore gel examinations (Figures 22 and 23) of the purified brochocin-C product from the Sephadex G-50 column (fractions eluted between 345 and 645 min; region X of chromatograph A in Figure 13, p. 34) indicated only one Coomassie Blue stained band (Figure 22, lane E) which also displayed the antimicrobial activity expected of brochocin-C (Figure 23, lane E). Although the Coomassie Blue stained brochocin-C

Table 4. Descriptions of codes used in section 3.2.

Code	Description
BSA	native bovine serum albumin
OVA	native ovalbumin
broc-BSA	BSA treated with glutaraldehyde and brochocin-C (Reichlin, 1980; Briand *et al.*, 1985)
broc-OVA	same as broc-BSA except OVA was used instead of BSA
glutar. BSA	BSA treated with only glutaraldehyde
glutar. OVA	same as glutar. BSA except OVA was used instead of BSA
2616-BSA	brochocin-C N-terminal synthetic peptide linked BSA
2617-BSA	brochocin-C C-terminal synthetic peptide linked BSA

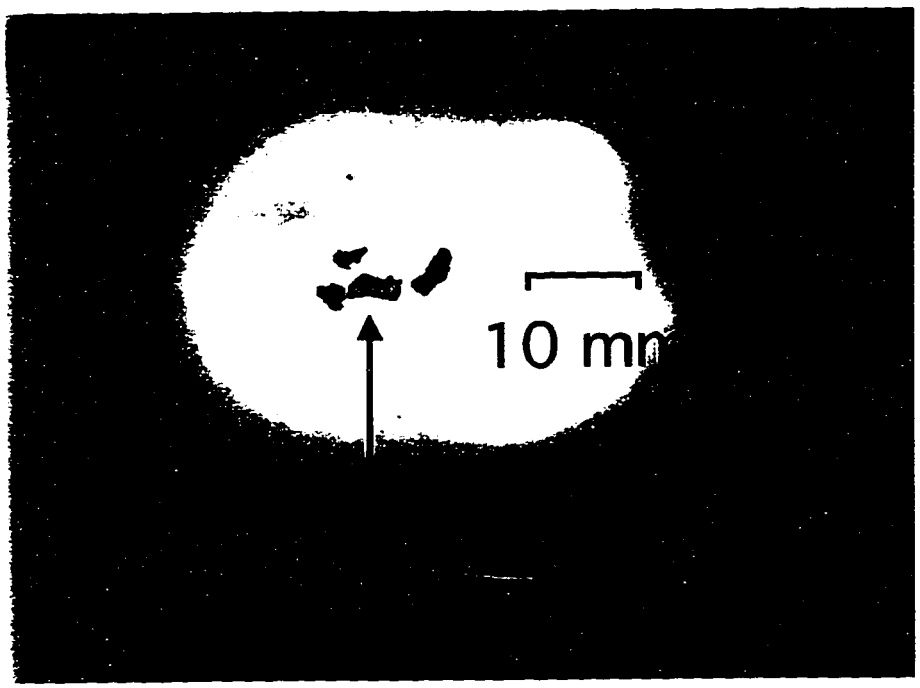

Figure 21. Spot-on-lawn assay of brochocin-C inhibition activity retained in the interphase solids formed between the aqueous and the butanol layers during extraction (section 2.5.1).

The interphase solids were washed with ethanol before analysis. A clear growth inhibition zone of the spot-on-lawn assay was usually 10 mm in diameter. Large amount of brochocin-C activity was retained in the interphase solids because the inhibition zone was at least 30 mm in diameter.

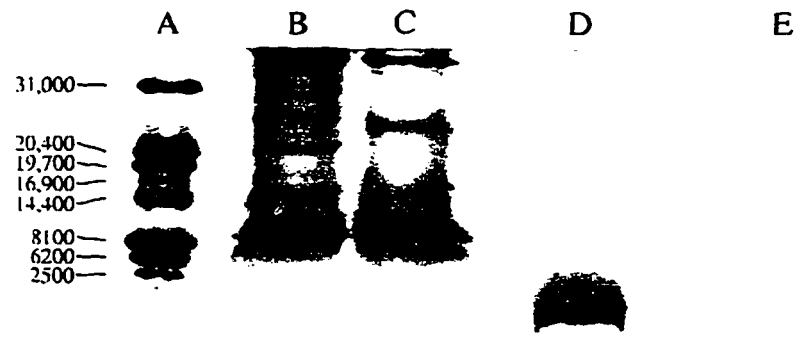

Figure 22. Coomassie Blue stained SDS-PAGE gel.

A = protein molecular weight markers (Da)

B = culture broth

C = culture broth after one butanol extraction

D = cold acetone precipitate dissolved in 0.1% TFA

E = Sephadex G-50 column purified brochocin-C

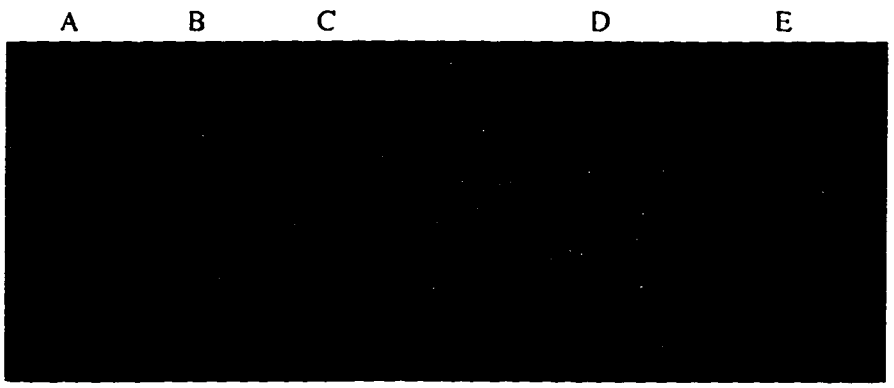

Figure 23. Brochocin-C inhibition activity of SDS-PAGE separated bands.

A = protein molecular weight markers (Da)

B = culture broth

C = culture broth after one butanol extraction

D = cold acetone precipitate dissolved in 0.1% TFA

E = Sephadex G-50 column purified brochocin-C

band and its microbial growth inhibition zone are not correlated to the protein molecular weight markers (where brochocin-C molecular weight = 5241 Da), this phenomenon is also common for leucocin, a bacteriocin produced by *Leuconostoc gelidum* (Hastings *et al.*, 1991). Therefore it was decided to use brochocin-C purified after the Sephadex G-50 column for preparation of the protein conjugate.

3.2.2 Brochocin-C conjugation to BSA using glutaraldehyde (broc-BSA)

The glutaraldehyde method was used for conjugation of brochocin-C as it could produce a stable conjugation link between the brochocin-C and the carrier BSA (Reichlin, 1980; Briand *et al.*, 1985) in one step without significant loss of purified brochocin-C. To assess the results of conjugation three MALDI spectra were evaluated, BSA, glutar. BSA and broc-BSA (Figures 24, 25 and 26, respectively). While the degree of brochocin-C substitution was not as high as desired, there was a definite increase in the average mass from glutaraldehyde treated BSA (MW 76379 Da) to broc-BSA (MW 78403 Da). Since the mass of brochocin-C is 5241 Da, this would correspond to a peptide to protein carrier substitution of about 0.5 brochocin-C per BSA molecule.

3.2.3 Antibodies developed against broc-BSA

When antisera against broc-BSA, 7A4 and 7A5, were examined in a ncid-ELISA, the results were disappointing (Figure 27). Although antibody titers increased as expected, there was no significant difference in binding between broc-BSA and a control of glutar. BSA. However, there was a trend for the absorbances of the broc-BSA to be higher than that of the glutar. BSA control. The results suggested that some weak binding antibodies against brochocin-C might be present in the antisera.

Antibodies against other bacteriocins, nisin (Falahee *et al.*, 1990)

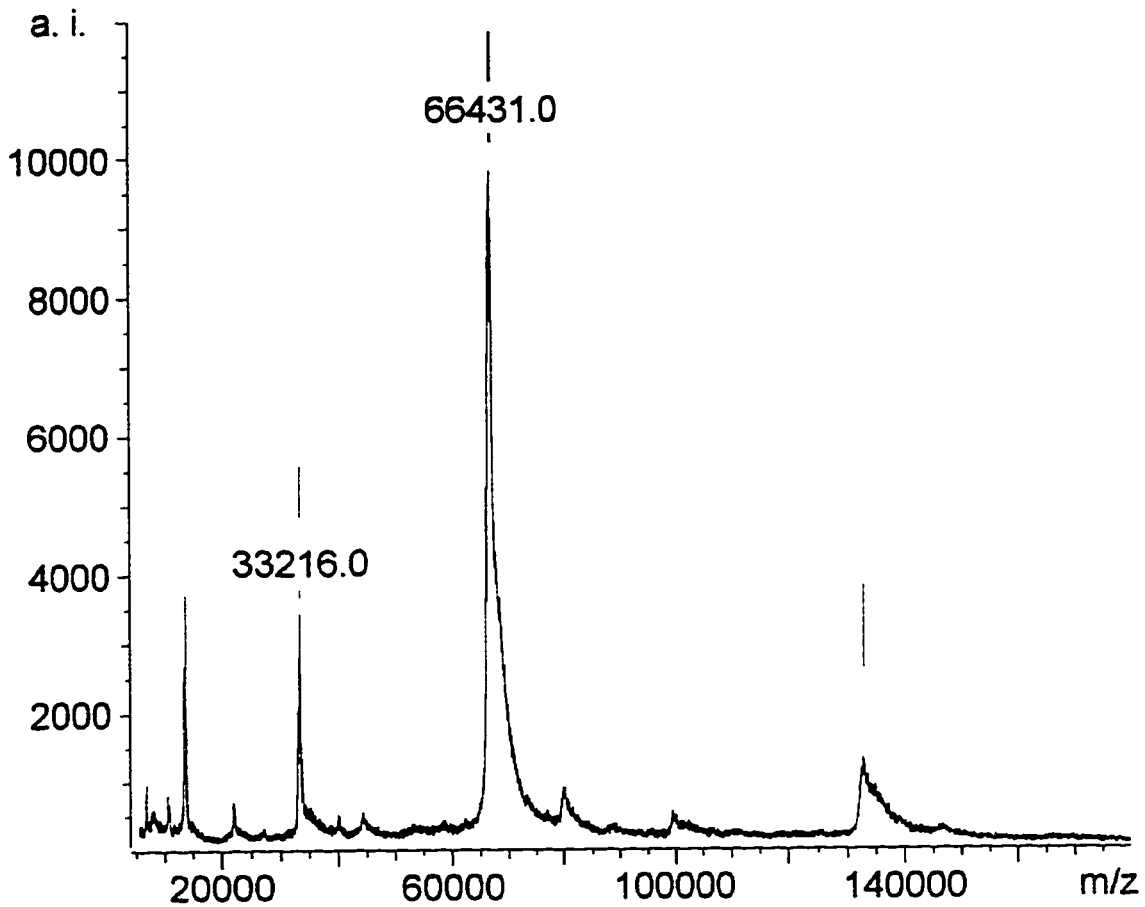

Figure 24. MALDI spectrum of BSA.

a. i. = arbitrary intensity

m/z = mass per charge

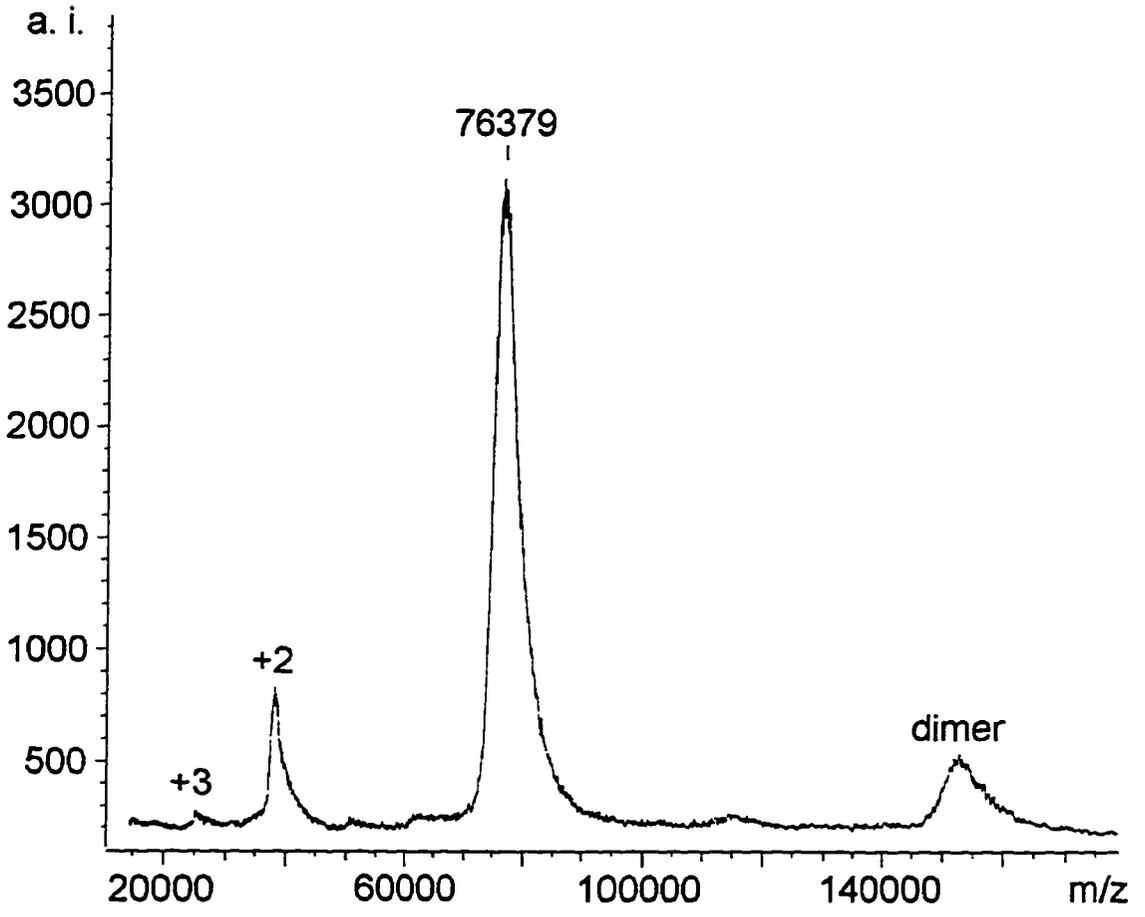

Figure 25. MALDI spectrum of glutar. BSA.

a. i. = arbitrary intensity

m/z = mass per charge

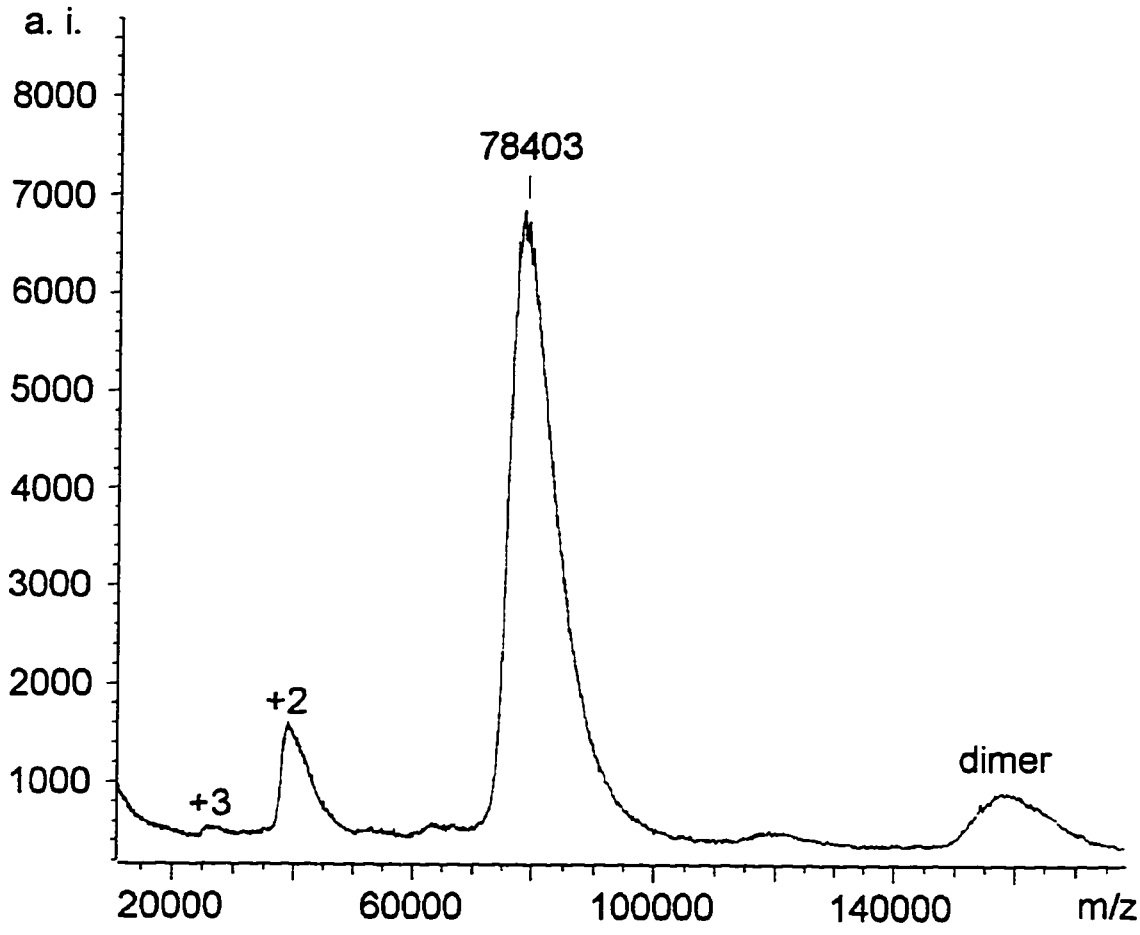

Figure 26. MALDI spectrum of broc-BSA.

a. i. = arbitrary intensity

m/z = mass per charge

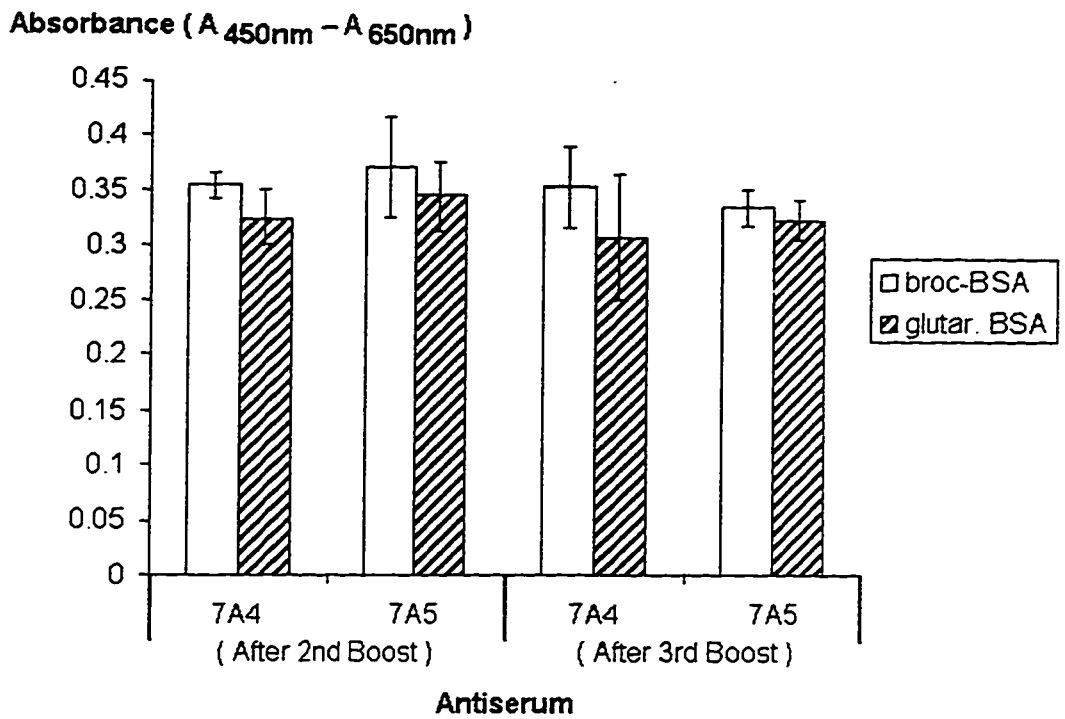

Figure 27. The ncid-ELISA for glutaraldehyde conjugated brochocin-C-BSA (broc-BSA) and glutaraldehyde treated BSA control (glutar. BSA).

Columns for antisera after the third boost represent the mean absorbance of 6 wells after background correction; the second boost of 4 wells; error bars indicate the standard deviation of the mean. Mean absorbances of BSA and OVA for antiserum 7A4 were 0.083 ± 0.028 and –0.005 ± 0.005; antiserum 7A5 were 0.059 ± 0.016 and 0.003 ± 0.001 respectively. Each well was coated with 100 µL of the protein conjugate at 10 ppm; antisera dilutions were all at 1:100,000.

and nisin A (Suárez *et al.*, 1996a; Suárez *et al.*, 1996b) have been generated; but there are also many unsuccessful or unsatisfactory results recorded in the literature, pediocin AcH (Bhunia *et al.*, 1990), pediocin RS2 (Bhunia, 1994) and nisin A (Stringer *et al.*, 1995). The reasons for failure in developing antibodies might have been due to the non-immunogenic nature in some of bacteriocins if injected alone (Bhunia *et al.*, 1990) and the choices of their carrier proteins. In the case of brochocin-C, the weak antibodies might be a result of insufficient brochocin-C groups on the carrier BSA.

3.2.4 Antibodies developed against synthetic peptides

Antibodies against the brochocin-C N-terminal peptide (2616) and C-terminal peptide (2617) were developed by injection of KLH conjugated with the desired peptides. Figure 28 shows the antibodies binding to respective peptide BSA conjugates. By comparing the antibody binding between the two antisera, the antiserum 5K7, which was developed against the N-terminal peptide (2616) KLH conjugate, was more sensitive against the same peptide linked to BSA carrier than that of the antiserum 5K8, which was developed against the C-terminal peptide (2617) KLH conjugate.

In a ncid-ELISA format, antibodies in antiserum 5K7 did not recognize the broc-BSA coating conjugate. It should be noted that all free amine groups, one N-terminal and three lysines (i.e. residues 1, 4, 8 and 12), in brochocin-C are located close to the N-terminal of the brochocin-C. Therefore, the N-terminal portion of the brochocin-C peptide might not be available for antibody binding because of the block of a glutaraldehyde crosslink which bonded two amine groups between brochocin-C and the carrier BSA. Antibodies in antiserum 5K8, which was developed against the synthetic peptide (2617) of the 7 residues from the amino acid sequence of the C-terminal of brochocin-C, were somewhat sensitive to the

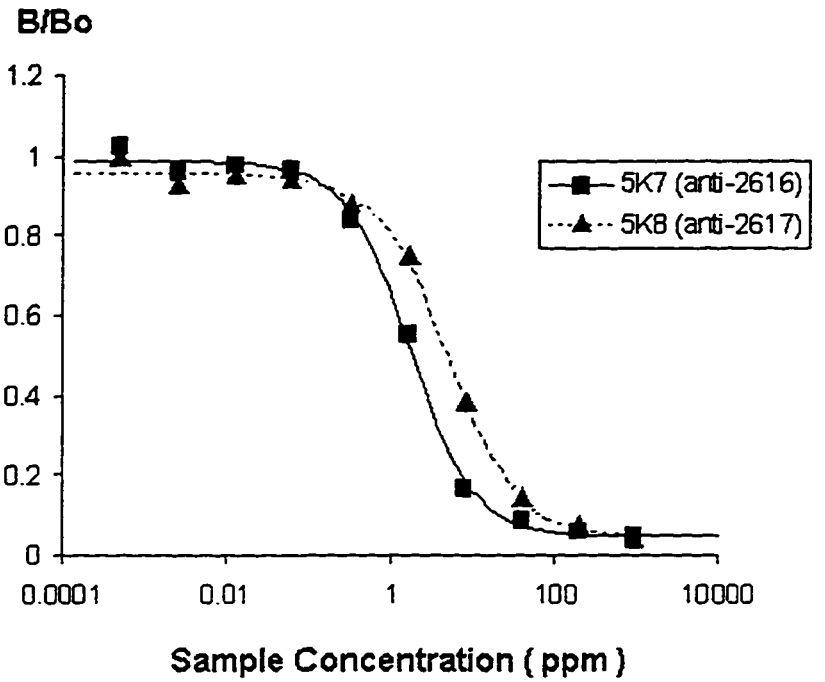

Figure 28. The cid-ELISAs of synthetic peptide linked protein conjugates.

Curve parameter of antiserum 5K7 (■): "a"=0.986, "b"=1.13, "c"=1.70, "d"=0.0492 and the correlation coefficient (r) was 0.998; antiserum 5K8 (▲): "a"=0.956, "b"=0.998, "c"=4.84, "d"=0.0461 and the correlation coefficient (r) was 0.999. For antiserum 5K7, each well was coated with 200 µL of 1 ppm 2616-BSA; dilution was 1:1,000,000. For antiserum 5K8, each well was coated with 200 µL of 10 ppm 2617-BSA; dilution was 1:10,000.

broc-BSA (Figure 29). It is possible that the antibodies in antiserum 5K7 did not recognize the brochocin-C on the broc-BSA conjugate because of the blocking of the antibody binding sites after the glutaraldehyde conjugation.

The ELISA detection format used in this study was different from that proposed by Martínez *et al.* (1997) who did not use a cid-ELISA format to detect the bacteriocin of interest as in this study. They used a competitive direct ELISA with enzyme-labeled antigen format (i.e. Figure 10, p. 25) which coated the antiserum (or ascites fluid), containing bacteriocin-induced antibodies, to the plate (Suárez *et al.*, 1996a). However, similar results were found in that the antibodies were specific and sensitive against their induction peptides, but did not recognize the bacteriocins from which the peptides were derived.

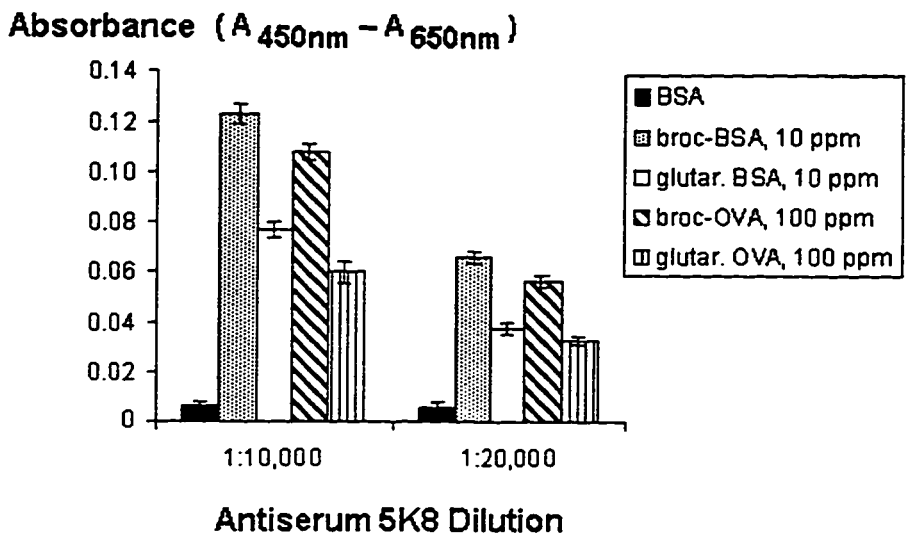

Figure 29. The ncid-ELISA for brochocin-C linked BSA conjugate recognition by antiserum 5K8.

4. CONCLUSION

4.1 GLYCATED PROTEINS

Polyclonal antiserum 5D4 contained antibodies that recognized AGEs generated from different reactants under both physiological and non-physiological conditions, but to a different extent. In this study, the antiserum 5D4 was found to be more specific against the crosslinked AGEs than the non-crosslinked AGEs in a cid-ELISA. Hence, the high molecular weight crosslinked AGE portion, which was separated by using FPLC, competed against the antiserum 5D4 antibodies better than the low molecular weight non-crosslinked AGE portion. The crosslinked AGEs were further investigated by applying PTB, a crosslink cleaving agent. The PTB experiment showed a drastic decrease in the competitiveness of physiological AGEs, but only a moderate decrease in the competitiveness of non-physiological AGEs (except the F-OVA), against the antiserum 5D4. Thus, there is likely more than one type of crosslink existing in AGEs. Furthermore, the antiserum 5D4 failed to recognize any simple glycated proteins. Simple glycated proteins might already present in abundance in the animal before immunization, and are therefore tolerated by the immune system.

The polyclonal antiserum 5D4 antibodies may be used for affinity columns to isolate the antigenic AGE population, which can then be used to further study AGE chemistry. Furthermore, the quantity of an individual AGE in a sample can be estimated with the use of a known AGE as detection standard; hence, the degree of antibody binding against the sample is directly related to the amount of the antigenic AGE.

4.2 BROCHOCIN-C

Brochocin-C loss during purification was mainly due to the stickiness of brochocin-C to other macromolecules. It is shown by the large portion of antimicrobial activity retained in the interphase solids from butanol extraction step, the unsuccessful separation of brochocin-C through a Macrosep 10 kDa filtration membrane, and the antimicrobial activity in the void volume eluted from a Sephadex G-50 column. More recently, other research in our laboratories using the Mini Prep Cell from Bio-Rad Laboratories (Hercules, CA) indicates that brochocin-C can be isolated from the butanol extraction interphase solids.

Although the results shown in this study were not completely successful (only the anti-C-terminal antiserum 5K8 exhibited some recognition against brochocin-C conjugated on BSA carrier), the use of synthetic peptides from the amino acid sequence of brochocin-C to develop specific antibodies remains attractive. This is justified by the difficulties of obtaining a sufficient amount of native purified brochocin-C for hapten-carrier conjugation.

In future research a binding antibody against brochocin-C might be developed with the injection of glutaraldehyde-conjugated broc-BSA into rabbits. A stronger binding affinity may be achieved by injection of an immunogen with increased number of conjugated brochocin-C molecules (comparing with the 0.5 brochocin-C molecule per BSA used in this study). The use of MAb technique may also be tried in the future study because purity of immunogen is not as great problem for MAb development (Deshpande, 1996). Once a specific brochocin-C antiserum is generated, an affinity column for brochocin-C separation may be constructed to develop an efficient purification process, too.

It should be noticed that the term "brochocin-C" used in this study is used in the same way as by Poon (1995). However, recent studies showed that brochocin-C is a bacteriocin composed of two components, necessary

for antimicrobial activity (Poon *et al.*, 1997). The amino acid sequence of brochocin-C (i.e. Figure 4, p. 11) reported in this study is actually that of brochocin-A (McCormick, unpublished data). It is now known that the presence of both brochocin-A and brochocin-B are required for detection of antimicrobial activity. Thus, the brochocin-C used for antibody production in this study was a mixture of brochocin-A and brochocin-B. To develop an ELISA for the detection of brochocin production in foods, antibodies for both of these peptides will be necessary.

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