CROSS-LINKING OF SOY PROTEIN ISOLATE USING MICROBIAL TRANSGLUTAMINASE FOLLOWED BY RIBOSE-INDUCED MAILLARD REACTION

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by

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LIST OF SYMBOLS / ABBREVIATION

Symbol/Abbreviation	Caption
a*	redness
AABA	L-α-amino-n-butyric acid
AGEs	advanced glycation end products
ANOVA	one-way analysis of variance
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
ARPs	Amadori Rearrangement Products
AS	acidic subunit
ATP	adenosine triphosphate
a_w	water activity
b*	blueness
BS	basic subunits
BSA	bovine serum albumin
Ca^{2+}	calcium
Cu^{2+}	copper
D[3,2]	surface area mean diameter
D[4,3]	volume mean diameter
D_2O	deuterium oxide
DHA	docosahexaenoic acid
DOGDIC	lysine arginine dimer
E_A	asymptotic residual modulus
EPA	eicosapentaenoic acid
FESEM	field emission scanning electron microscope

FOC	fish oil content
FTIR	Fourier transform infrared spectroscopy
G'	storage modulus
GI	glycemic index
GODIC	glyoxal lysine arginine dimer
GOLD	glyoxal lysine dimer
GRAS	generally recognized as safe
GTGase	guinea pig liver transglutaminase
H^+	hydrogen ion
HCl	hydrochloric acid
HI	hydrolysis index
IR	infrared
IVPD	in-vitro protein digestibility
K_1, K_2	viscoelasticity
L*	lightness
Li^+	lithium
MEY	microencapsulation yield
MODIC	methylglyoxal lysine arginine dimer
MOLD	methylglyoxal lysine dimer
MTGase	microbial transglutaminase
NaOH	sodium hydroxide
OD	optical density
p-A.V.	p-anisidine value
Pb^{2+}	plumbum
pI	isoelectric point
PUFA	n-3 polyunsaturated fatty acids
RH	relative humidity

SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SPI	soy protein isolate
SSA	specific surface area
TAN	total adenine nucleotide
TCA	trichloroacetic acid
TGase	transglutaminase
TNBS	trinitrobenzenesulfonic acid
TPN	total parental nutrition
UHT	ultra high temperature
WHC	water holding capacity
WPI	whey protein isolate
Zn^{2+}	zinc
β-ME	β-mercaptoethanol

LIST OF APPENDICES

LIST OF PUBLICATIONS & SEMINARS

Seminar & Exhibition

1 Gan, C.Y., Cheng, L.H., & Easa, A.M. (2006). Soy protein isolate modification via cross-linking using microbial transglutaminase and ribose. 5th Food Science & Technology Seminar. Terengganu, Malaysia, 2006. Poster presentation. 3rd place.

2 Gan, C.Y., Cheng, L.H., Phuah, E.T., Chin, P.N., & Easa, A.M. CXP: Densely Cross-linked protein for controlled-release of pharmaceuticals and nutraceuticals. *19th International Invention, Innovation & Technology Exhibition (ITEX 2008)*. Bronze.

Publications

1 Gan, C.Y., Cheng, L.H., & Easa, A.M. (2008). Assessment of Maillard reaction and cross-linking in transglutaminase-cross-linked powdered soy protein isolate gel. *Food Research International*. Communicating.

2 Gan, C.Y., Cheng, L.H., & Easa, A.M. (2008). Effects of Ribose Addition on Physicochemical Properties and Microstructures of Microbial Transglutaminase Cross-linked-Soy Protein Isolate Gels. *Food Research International*. **41**: 600-605.

3 Gan, C.Y., Cheng, L.H., & Easa, A.M. (2008). The impact of protein cross-linking treatments on functional properties, *in-vitro* digestibility and amino acid composition of soy protein isolate gels. *Food Chemistry*. Communicating.

4 Gan, C.Y., Ong, W.H., Wong, L.M., & Easa, A.M. (2008). Effects of ribose, microbial transglutaminase and soy protein isolate on physical properties and *in-vitro* starch hydrolysis of yellow noodles. *LWT-Food Science and Technology*. *Article in Press*, doi: 10.1016/j.lwt.2008.05.004.

5 Gan, C.Y., Cheng, L.H., & Easa, A.M. (2008). Evaluation of Microbial Transglutaminase and Ribose Cross-linked Soy Protein Isolate-based Microcapsules Containing Fish Oil. *Innovative Food Science and Emerging Technologies*. *Article in Press*, doi: 10.1016/j.ifset.2008.04.004.

6 Gan, C.Y., Cheng, L.H., Phuah, E.T., Chin, P.N., AlKharkhi, A.F.M. & Easa, A.M. (2008). Combined cross-linking treatments of bovine serum albumin gel beadlets for controlled- delivery of caffeine. *Food Hydrocolloids.* Accepted with minor revision.

7 Gan, C.Y., AlKharkhi, A.F.M. & Easa, A.M. (2008). Using response surface methodology to optimize process parameters and cross-linking agents for production of combined-crosslinked bovine serum albumin gels. *International Journal of Biological Macromolecules*.

HUBUNG-SILANG ISOLAT PROTEIN SOYA MENGGUNAKAN TRANSGLUTAMINASE MIKROBIAL DIIKUTI OLEH TINDAKBALAS MAILLARD ARUHAN-RIBOSA

ABSTRAK

Tesis ini menjelaskan tentang penggunaan pengolahan hubung-silang gabungan untuk mengubahsuaikan sifat-sifat berfungsi isolat protein soya (SPI). Ampaian SPI telah dihubung-silang dengan menggunakan transglutaminase mikrobial (MTGase) pada suhu 40 °C untuk 5 atau 24 jam, diikuti dengan pengeringan sejuk-beku ke bentuk serbuk. SPI terhubung-silang MTGase kemudian dipanaskan dengan 2% (v/w) larutan ribosa pada suhu 95 atau 100 °C untuk menghasilkan jel SPI terhubung-silang gabungan. Hubung-silang protein telah dibuktikan dengan menggunakan teknik elektroforesis (SDS-PAGE), ujian keterlarutan dalam pelarut-pelarut pemecah, teknik mikroskopik penskanan elektron pemancaran medan (FESEM) dan penilaian sifat-sifat mekanikal dengan menggunakan alat analisa tekstur. Jel terhubung-silang gabungan menunjukkan kehilangan semua garisan fraksi dalam profil SDS-PAGE menandakan kesemua fraksi SPI telah dihubung-silang, dan jel-jel ini adalah rendah keterlarutan, tinggi dalam sifat-sifat mekanikal (kekuatan jel termampat, kelikat-kenyalan dan kekerasan) dan mempamerkan struktur rangkaian yang lebih padat berbanding jel-jel lain yang dihasilkan daripada pengolahan hubung-silang tunggal dengan MTGase atau ribosa. Keputusan ini mengesahkan kewujudan ikatan ε-(γ-glutamyl)lysine dan hubung-silang Maillard di dalam jel-jel terhubung-silang gabungan. Jel-jel dihubung-silang gabungan adalah lebih rendah dari segi pemerangan Maillard dan hubung-silang Maillard, dan menunjukkan retensi asid amino yang tinggi berbanding jel SPI yang diolah tunggal dengan ribosa. Oleh kerana hubung-silang MTGase telah

menggunakan suatu kuantiti lisina dan glutamina, jumlah asid amino ini untuk tindakbalas Maillard telah berkurangan. Dalam fasa aplikasi, pengolahan hubung-silang gabungan telah diuji dalam mi kuning yang ditambah SPI, mikrokapsul SPI dan "beadlet" jel albumin serum bovin (BSA) untuk memperbaiki sifat-sifat indeks glisemik (GI), memperlahankan pembebasan in-vitro minyak ikan tinggi ω-3 dan kafeina masing-masing. Mi kuning yang ditambah SPI menggunakan pengolahan hubung-silang gabungan adalah lebih kuat dari segi tekstur and lebih rendah dalam GI secara signifikan ($p<0.05$) berbanding mi lain terhasil daripada pengolahan tunggal. Pengolahan hubung-silang dalam mikrokapsul SPI telah menunjukkan penambahbaikan pada pembebasan-terkawal minyak ikan berbanding sampel kawalan, namun profil pembebasan adalah sama seperti mikrokapsul diolah tunggal dengan ribosa. Hayat simpanan minyak dalam mikrokapsul telah dipanjangkan sama dengan mikrokapsul diolah tunggal yang mengandungi ribosa. Kejadian ini mungkin disebabkan oleh pembebasan produk-produk tindakbalas Maillard yang bersifat anti-pengoksidaan semasa pemanasan dan penyimpanan dan kadar penembusan gas melalui kapsul yang perlahan. "Beadlet" jel BSA terhasil menggunakan pengolahan hubung-silang gabungan telah memberikan tindakan tertangguh dalam pembebasan kafeina dengan berkesan. Morfologi "beadlet" yang didapati melalui FESEM mencadangkan bahawa pembaikan kelakuan pembebasan ini adalah disebabkan pembentukan rangkaian yang padat and pemegangan kafeina dalam rangkaian beadlet yang menyekat pembauran kafeina dan juga menghalang protein daripada membengkak. Kesimpulannya, thesis ini telah menunjukkan bahawa hubung-silang Maillard aruhan-ribosa dapat dibentuk dalam rangkaian protein dihubung-silang MTGase. Pengolahan hubung-silang gabungan berpotensi diaplikasikan dalam produk-produk makanan dan nutraseutikal.

CROSS-LINKING OF SOY PROTEIN ISOLATE USING MICROBIAL TRANSGLUTAMINASE FOLLOWED BY RIBOSE-INDUCED MAILLARD REACTION

ABSTRACT

This thesis describes the use of combined cross-linking treatment techniques to modify functional properties of soy protein isolate (SPI). SPI suspensions were cross-linked with microbial transglutaminase (MTGase) at 40 °C for 5 or 24 hrs, followed by lyophilization of the suspensions into powders. MTGase pre-crosslinked SPI was then subjected to a heating treatment with solution containing 2% (w/v) ribose at 95 or 100 °C to produce combined cross-linked SPI gel. Cross-linking of protein was monitored using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), solubility studies in disruptive solvents, field emission scanning electron microscopic (FESEM) technique and evaluation of mechanical properties of the gels using texture analyzer. Combined cross-linked gels showed disappearance of all the bands in SDS-PAGE profile indicating all protein fractions of SPI were cross-linked and these gels were lower in solubility, higher in gel mechanical properties (i.e. compressive gel strength, viscoelasticity and solidity) and exhibited a denser network structure than those produced using single cross-linking treatments with MTGase or ribose. These results confirmed the occurrence of ε-(γ-glutamyl)lysine bonds and Maillard cross-linking in the combined cross-linked gels. The combined cross-linked gels were lower in the extent of Maillard browning and Maillard cross-linking, and had higher retention of amino acids compared to that of single treated SPI gel with ribose. As MTGase cross-linking consumed a quantity of lysine and glutamine, less of these amino acids were available for the Maillard reaction to occur. In the application phase of the

study, the combined cross-linking treatment was tested in SPI incorporated-yellow noodles, SPI microcapsules and bovine serum albumin (BSA) gel beadlets to improve glycemic index (GI) properties, sustain the in-vitro release of high ω-3 fish oil and caffeine respectively. SPI incorporated-yellow noodles produced using the combined cross-linking treatment were significantly ($p<0.05$) stronger in texture and lower in GI than those produced using single treatment. The combined cross-linking treatment of SPI microcapsules showed an improved sustained-release of fish oil compared to the control, but the profile of release was similar to that of single treated microcapsules with ribose. The shelf-life of the oil in the microcapsules was extended in combined cross-linked as well as in the single treated microcapsule containing ribose. This may be due to the release of anti-oxidative Maillard reaction products during heating and storage and a slower rate of gas permeability through the capsules. BSA gel beadlets produced using combined cross-linking treatment had effectively provided a delay action in releasing caffeine. The morphology of the beadlets obtained via FESEM suggested that this improved release behaviour was mainly due to the denser network formed and the holding of the caffeine within the beadlet's network that restricted the diffusion of the caffeine as well as preventing protein from further swelling. In conclusion, this thesis showed that ribose-induced Maillard cross-linking could be formed within the MTGase pre-crosslinked protein network. The combined cross-linking treatment may find useful applications in food and nutraceutical products.

CHAPTER 1
INTRODUCTION

1.1 Background and Rationale

Modification of soy protein for functionality improvements has been carried out via physical means such as heat treatment (Renkema & van Vliet, 2002) and application of pressure (Torrezan *et al*., 2007) or via chemical means such as acidification (Tay *et al*., 2005), addition of salts (Puppo & Añón, 1999) and by the Maillard reaction induced cross-linkings (Md Yasir *et al*., 2007b). However, one of the most popular methods of protein modification in industry involves the application of transglutaminase enzyme (Md Yasir *et al*., 2007a; Tang, 2007). Microbial transglutaminase (MTGase; protein-glutamine: amine γ-glutamyltransferase, E.C. 2.3.2.13) functions by catalyzing an acyl-transfer reaction between the γ-carboxyamide group of peptide-bound glutamine residues (acyl donors) and variety of primary amines (acyl acceptors), including the ε-amino group of lysine residues to form an ε-(γ-glutamyl)lysine bond (Motoki & Seguro, 1998). This treatment has been used in meat products (Trespalacios & Pla, 2007), fish products (Jongjareonrak *et al*., 2006), dairy products (Lorenzen, 2007), legume products (Tang *et al*., 2007) and wheat products (Caballero *et al*., 2007) to enhance their textural and functional properties.

To further enhance the functionalities of cross-linked protein it is possible for technologists to use a combination of cross-linking treatments. The "Maillard cross-link" that is induced during heating a protein and reducing sugars via the Maillard reaction, has been shown to produce cross-linked protein and improve protein gels and food texture (Gerrard *et al*., 2002; Hill & Easa, 1998; Md Yasir *et*

al., 2007b; Oliver *et al*., 2006). Walsh *et al*. (2003) and Cabodevila *et al*. (1994) suggested that 7S and 11S globulins of SPI have different susceptibility in MTGase cross-linking and Maillard reaction. Therefore, it is hypothesized that polymerization of SPI protein fractions can be maximized via this combined cross-linking treatment to improve the protein gelling capacity and thus form a densed network that could enhance the mechanical and other functional properties of SPI gels.

A major drawback of the Maillard reaction and "Maillard cross-links" has been associated with the anti-nutritional properties such as indigestibility of isopeptide bonds and bioavailability of lysine, caused by destruction of amino acids, structural changes and inhibition of digestive enzyme activity (Friedman, 1996b). In contrast to "Maillard cross-links", ε-(γ-glutamyl)lysine moiety could be more accessible during digestion (Seguro *et al*., 1995, 1996a, 1996b), and was able to almost completely replace L-lysine in animal feeding studies (Waibel & Carpenter, 1972). Therefore attempts to control the destructive effects of the Maillard reaction by monitoring the Maillard reactions parameters/factors (e.g. temperature, water activity, pressure, pH and concentration of reactants) (Hill *et al*., 1996), incorporation of flavonoids (Schamberger & Labuza, 2007), supercritical carbon dioxide treatment (Casal *et al*., 2006), deglycation methods employing bacterial enzyme, fructosyl-*N*-alkyl oxidase (EC 1.5.3) (Gerhardinger *et al*., 1995) and modification of amino groups by acetylation (Friedman, 1996a) are beneficial for the food processors.

The techniques that control the extent of the Maillard reaction and its subsequent cross-linking have not been commercially viable due to the complexity of the methods. Hence, the use of MTGase to control the Maillard reaction and "Maillard cross-links" is suggested. MTGase will be introduced into soy protein to

initiate the ε-(γ-glutamyl)lysine bonds. Ribose, an emerging nutraceutical ingredient (Hellsten *et al*., 2004), which is also known for its high reactivity in terms of reacting with protein via the Maillard reaction (Ashoor & Zent, 1984) and capability to cross-link proteins (Graham, 1996), is then added to the MTGase pre-crosslinked SPI and the mixtures are heated to induce the Maillard reaction and "Maillard cross-links". Other than the potential "Maillard cross-link", ribose may also produce other changes that is related to the Maillard reaction, such as charge modification and pH adjustment (Easa, 1996; Yaylayan, 1997). Since the formation of ε-(γ-glutamyl)lysine bonds catalyzed by MTGase may cause the loss of lysine and glutamine, the Maillard-derived browning and its subsequent cross-links could be restricted. Thus, the MTGase pre-incubation will preserve the nutritional value of soy protein by protecting the amino acids, particularly lysine residues against the damaging effects of the Maillard reaction.

The techniques of protein modification described in this thesis are directed to the applications in either food or nutraceutical systems that use protein as matrix, in order to enhance the physical properties or to monitor the controlled-release of drugs from food-grade matrix. It will be appreciated that this combined cross-linking treatment would render the protein more feasible to these applications by modifying the protein properties that suit to the product requirement.

1.2 Objectives

The main objective of this study is to develop a cross-linking treatment involving MTGase pre-incubation of SPI followed by ribose-induced Maillard reaction. The resultant gel product of combined cross-linking treatment can be applied in food and nutraceutical systems in order to suit to the functional requirements of the product. The measurable objectives of this study are listed as follows:

1. To show the effect of MTGase pre-incubation on Maillard reaction by retaining amino acids from destruction effects of the Maillard reaction.

2. To explore the feasibility of introducing ribose into MTGase cross-linked SPI.

3. To produce and to evaluate the physicochemical properties of SPI gels treated via combined cross-linked treatment.

4. To assess the applicability of the combined cross-linking treatment in 3 different food and nutraceutical systems: (i) SPI-incorporated yellow noodle, (ii) SPI microcapsules containing fish oil and (iii) caffeine-encapsulated gel beadlets.

1.3 Thesis Outline

The covalent cross-linking treatments of soy protein isolate using microbial transglutaminase incubation followed by ribose-induced Maillard reaction for food and nutraceutical applications is presented in this thesis. The main body of this dissertation consists of a general introduction and background, literature reviews, material and methods, results and discussion, general conclusions as well as recommendations for future study.

CHAPTER ONE is a general introduction on the background of this project in which the current situations and challenges encountered by food industry regarding the modifications protein. It also presented the proposed method to solve the problems with detailed background that supports the application of combined cross-linking treatment in modification of SPI. Besides, the rationales and the objectives of this study are briefly discussed.

This project deals with combination of both MTGase incubation and the Maillard reaction to induce covalent cross-links in SPI, the model system. The general literature review of SPI and the modification methods (i.e. transglutaminase and the Maillard reaction) is illustrated in CHAPTER TWO.

CHAPTER THREE lists down all the applied materials and methodology for every single assay conducted throughout the whole study.

In CHAPTER FOUR, the experimental results with discussions are presented. Basically, this chapter is divided into two major parts with six sections, in which the first part of this chapter involves fundamental studies: (section 1) the preliminary study on the effects of MTGase incubation on SPIs, (section 2) the evaluation on the effects of ribose-induced Maillard reaction and the effects of MTGase pre-incubation on ribose-induced Maillard reaction, (section 3) the

elucidation on the gelation of covalent cross-linked SPIs, (section 4) the evaluation on the physico-chemical properties of covalent cross-linked SPI gels and (section 5) the evaluation on the digestibility of the covalent cross-linked SPI gels. Whereas, the second part involves the applications of combined cross-linking treatment in food and nutraceutical systems (section 6). Each sub-section describes and summarizes the results and the statistical analysis was used to evaluate the result. Note that bovine serum albumin (BSA) was used in one of the application evaluations. This is because BSA is widely accepted in pharmaceutical industry that the overall distribution, metabolism and efficacy of many drugs can be altered based on their affinity to BSA. Also, the potential of using a different source of protein in applying the combined cross-linking treatment could also be explored.

The last chapter (CHAPTER FIVE) consists of overall conclusions on the whole study and recommendations for the future study of this combined cross-linking treatment developed.

CHAPTER 2
LITERATURE REVIEWS

In this chapter, literature reviews is divided into three sections. Sections 2.1 will review the components of soy protein isolate followed by the interactions between the subunits of the protein as well as the gelling mechanisms. Two major modification treatments, i.e. microbial transglutaminase and Maillard reaction, will be reviewed in following sections (2.2 and 2.3, respectively). These sections include the factors and mechanisms of the modifications as well as the effects on the protein matrix.

2.1 Soy Protein Isolate (SPI)

A variety of soy protein, for instances soy flours, soy concentrates and soy isolates, possessing a range of functionalities such as gelling, emulsifying, and foaming capacity (Utsumi *et al*., 2002) has been widely used in food industry. Soy flours are used in a wide range of foods, particularly in bakery products and cereals whereas concentrates (70% protein), because of their improved flavour, colour, and higher protein content, it can be used in a greater quantities in many of the same foods, especially when higher level of protein (nutrition, functionality) are required. Soy protein isolate (SPI), which is of particular interest in this project, are prepared commercially with minimum heat-treatment, contain approximately 90-95% pure protein on dry basis (Kinsella, 1975; Wolf & Cowan, 1971) and has been used in comminuted meats and dairy foods where emulsifying, thickening and gelling properties are of prime importance. The general amino acid composition of SPI is shown in Table 2.1.

Table 2.1 Amino acid composition of soy protein isolate.

Amino Acids	Percentage
Essential	
Lysine	6.1
Methionine	1.1
Cystine	1.0
Tryptophan	1.4
Threonine	3.7
Isoleucine	4.9
Leucine	7.7
Phenylalanine	5.4
Valine	4.8
Non-essential	
Arginine	7.8
Histidine	2.5
Tyrosine	3.7
Serine	5.5
Glutamic acid	20.5
Aspartic acid	11.9
Glycine	4.0
Alanine	3.9
Proline	5.3

(adapted from: Wolf & Cowan, 1971)

2.1.1 Structures/components of soy protein

Approximately 85-95% of the soybean storage proteins are globulin, i.e. those proteins insoluble in water near their isoelectric points (pH 4.2-4.6), but soluble in dilute salt solutions or at neutral pH and above (Kinsella, 1975; Wolf & Cowan, 1971). Soybean globulins are generally classified on the basis of their sedimentation coefficients. Four major fractions are reported and designated as 2, 7, 11 and 15S (Table 2.2). Both 11S globulin (glycinin) and 7S globulin (β-conglycinin) are considered the major fractions of SPI with different compositions/subunits, structures and functionalities (Kilara & Sharkasi, 1985). The subunits of β-conglycinin-rich (lane a) and glycinin-rich (lane b) SPIs are illustrated in SDS-PAGE profiles (Plate. 2.1).

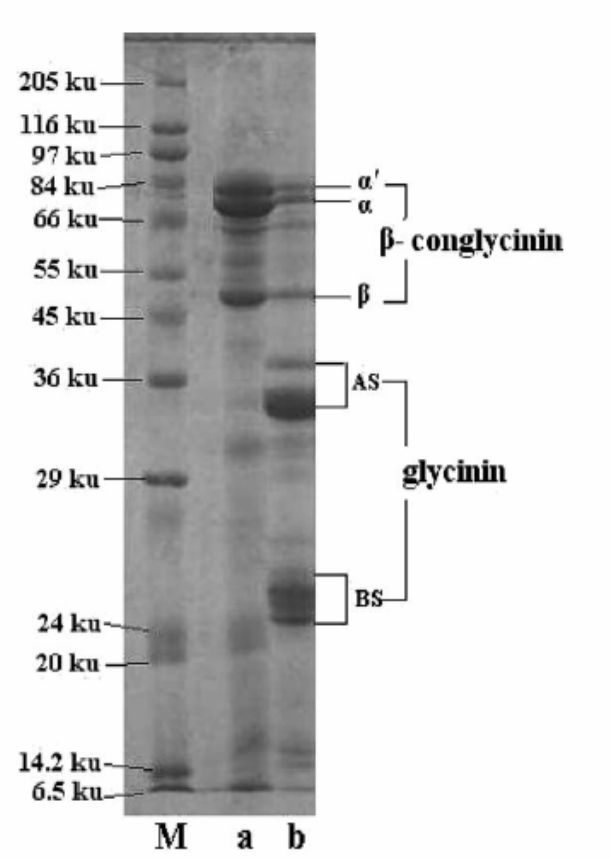

Plate 2.1 The SDS-PAGE patterns of glycinin-rich and β-conglycinin-rich SPIs. The lanes a and b indicate the β-conglycinin-rich and glycinin-rich SPIs, respectively. Lane M indicates the standard protein markers. (adapted from: Tang *et al*., 2006b)

Table 2.2 Approximate amounts and components of ultracentrifuge fractions of water extractable soybean proteins.

Fraction	Percent of Total	Components	Molecular Weight
2S	22	Trypsin inhibitors	8,000 - 21,500
		Cytochrome *c*	12,000
7S	37	Hemagglutinins	110,000
		Lipoxygenases	102,000
		α-Amylase	61,700
		7S Globulin	180,000 - 210,000
11S	31	11S Globulin	350,000
15S	11	-	600,000

(adapted from: Wolf & Cowan, 1971)

2.1.1.1 The 7S (β-conglycinin) component

β-Conglycinin, a major 7S protein, exists in monomeric (7S) and dimeric (9S) forms at 0.5 and 0.1 ionic strength, respectively. The original 7S fraction is a glycoprotein and contains the carbohydrates as one unit attached to the aspartic acid residue at the N-terminal end of the molecule. The carbohydrate moiety consists of 38 mannose and 12 glucosamine residues per molecule of protein. The molecular weight of the 7S form is in the range 150,000 to 175,000 and that of the 9S form is 370,000. The 7S form is composed of three subunits (α, α' and β) which interact to produce six isomeric forms (B_1 to B_6), shown in Fig. 2.1 with varying properties (Table 2.3 and 2.4) and compositions (Table 2.5).

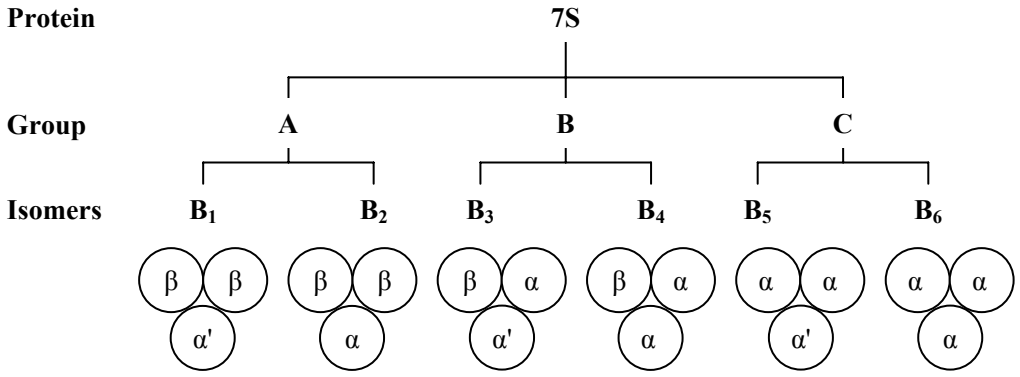

Figure 2.1 Subunits of 7S protein from soybean in each of six isomers. (adapted from: Kilara & Sharkasi,1985)

Table 2.3 Physicochemical properties of β-conglycinin (7S) component.

Characteristic	7S (monomer)	9S (dimer)	7S Isomers			
			Group A	Group B	Group C	β_3
Molecular weight						
From sedimentation.						
Stokes radius	175,000					137,000
From subunit size			141,000	156,000	171,000	
From sedimentation-diffusion	150,000	370,000				
N-terminal	Val, Leu	Val, Leu	Val (1)	Val (2)	Val (3)	-
Amino acid			Leu (2)	Leu (1)	-	Leu (3)
Carbohydrate	Mannose, glucosamine					

(adapted from: Kilara & Sharkasi,1985)

Table 2.4 Physicochemical properties of the subunits of β-conglycinin (7S) component.

	Subunits			
	α	α'	β	γ
Molecular weight				
Electrophoresis				
Urea/acetate (10% acrylamide gel)	68,000	68,000	42,000	-
SDS (10% acrylamide gel)	59,000	58,000	44,000	44,000
Urea/SDS (9% acrylamide gel)	57,000	58,000	46,000	46,000
Gel filtration	57,000	57,000	42,000	-
Carbohydrate (moles)				
Mannose (%)	3.88	3.81	2.46	4.53
Glucosamine (%)	1.27	1.22	0.84	1.25
Isoelectric point	4.90	5.18	5.66 - 6.00	-

(adapted from: Kilara & Sharkasi,1985)

Table 2.5 Amino acid composition of β-conglycinin (7S) component.

Amino acid	7S Isomers (mole %)			
	Group A	Group B	Group C	β_3 Isomer
Asp	12.40	12.53	12.35	12.40
Thr	2.27	1.98	1.98	2.90
Ser	8.22	5.76	6.79	8.70
Glu	17.57	20.54	23.07	18.60
Pro	4.57	5.94	7.72	4.90
Gly	4.30	6.17	6.48	6.60
Ala	7.59	4.57	4.85	5.20
Val	5.67	5.42	3.48	5.30
Leu	10.31	8.98	7.12	9.30
Ile	4.74	6.07	6.05	5.20
Tyr	1.58	1.89	2.85	1.90
Phe	6.02	5.76	4.41	5.30
His	2.29	2.26	1.31	1.90
Lys	5.68	6.00	5.85	5.60
Arg	6.19	6.12	5.67	6.00
Met	-	-	-	0.13

(adapted from: Kilara & Sharkasi,1985)

2.1.1.2 The 11S (glycinin) component

The 11S globulin (glycinin) is made up of 12 subunits, 6 acidic and 6 basic, and has a molecular weight of 302,000 to 375,000, which are packed into hexagons placed one over the other to form a hollow oblate cylinder. Table 2.6 and 2.7 showed the physicochemical properties and the amino acid composition of glycinin component, respectively.

Table 2.6 Physicochemical properties of glycinin (11S) component.

Molecular weight	
Gel filtration	302,000 ± 33,000
Sedimentation equilibrium	317,000 ± 15,000
Sedimentation diffusion	322,000 ± 15,000
From subunit size	326,000 ± 35,000
Gel electrophoresis	350,000 ± 35,000
Number of subunits	12 (6 Acidic [A], and 6 Basic [B])
Intermediary subunits	
Urea or SDS treated	A_1B_3, A_2B_3, A_3B_1, A_3B_2, $2A_4B_4$
(Urea or SDS) + β-ME	A_1A_2, $2A_3$, $2A_4$, B_1, B_2, $2B_3$, $2B_4$
Molecular weight of acidic subunits	
A_1, A_2, A_4, A_5	38,000
A_3	45,000
Molecular weight of basic subunits	
B_1, B_2, B_3, B_4	~21,000
N-terminal amino acids	
Acidic: A_1 Phe; A_2 Leu; A_3 Ile; A_4 Ile	Basic: $B_1 – B_4$ Gly
Size	
Electron microscopic	100 × 100 × 70 Å
X-ray scattering	110 × 110 × 75 Å
Isoelectric point	
Acidic: $A_1 = 5.15$; $A_2 = 5.40$; $A_3 = 4.75$	Basic: $B_1 = 8.0$; $B_2 = 8.25$; $B_3 = 8.50$

(adapted from: Kilara & Sharkasi,1985)

Table 2.7 Amino acid composition of glycinin (11S) component.

Amino Acid	Acidic subunits				Basic subunits			
	A_1	A_2	A_3	A_4	B_1	B_2	B_3	B_4
Asp	36.8	42.1	45.5	50.8	25.5	24.3	19.2	20.7
Thr	12.0	12.3	15.5	11.8	8.1	9.1	6.2	5.4
Ser	18.3	16.4	27.1	23.5	13.5	12.4	12.1	12.4
Glu	85.3	86.4	91.6	92.6	22.5	22.7	24.8	21.0
Pro	24.0	21.3	33.9	27.3	10.5	10.8	10.2	9.1
Gly	31.0	29.9	29.5	22.4	11.1	10.4	13.4	16.1
Ala	14.4	18.1	10.9	6.2	15.6	14.3	12.4	11.2
Val	11.9	15.3	17.4	12.1	11.4	10.8	17.0	19.2
Leu	20.1	20.0	21.8	14.0	17.9	17.4	18.1	18.1
Ile	17.6	15.3	12.2	10.4	9.2	9.8	7.0	7.3
Try	7.3	6.6	5.6	4.4	2.8	2.5	5.8	8.4
Phe	12.2	12.3	12.0	7.7	8.6	9.1	6.0	5.7
His	6.0	2.6	14.1	9.5	2.1	2.7	4.8	4.2
Lys	21.2	14.9	14.8	18.8	5.9	5.9	7.0	6.5
Arg	18.1	22.7	22.2	28.4	8.9	9.9	10.9	12.5
Met	3.6	5.8	2.4	1.4	2.3	2.7	0	1.3
Cys	4.5	4.3	3.6	0.7	1.7	1.5	0.2	1.5

(adapted from: Kilara & Sharkasi,1985)

2.1.1.3 7S and 11S globulins: the mixed system

Lawrence *et al*. (1994) demonstrated that the entire region of 11S globulin can be mapped onto 7S sequences in a way that preserves the structure of the 7S globulins. They observed 30 residues that are globally conserved or conservatively exchanged across the 7S and 11S globulins. These globally conserved residues correspond predominantly in the 7S structure to residues forming part of the inter-monomer packing or to residues in the inter-strand loops. Considering the presence of an intra-disulfide bond at the N-terminus of the 11S acidic polypeptide and insertion of the hypervariable region in the 11S acidic polypeptide, the authors suggested that the 11S N- and C-terminal halves are paired oppositely to 7S modules. Further, the 11S globulin sequence was suggested can be aligned like the 7S

globulins, which likely consist of two 7S-like trimers, indicating that the 11S globulin would also exhibit 32 symmetry.

In the mixed system such as SPI, the gelation behaviour is influenced by the interaction of the individual components. Therefore, the interaction among major soy protein components, i.e., glycinin and β-conglycinin, during gelation has been studied. These will be reviewed in details in later section.

2.1.2 Gelation

In general, gel produced by heating protein solutions involved a two-step process according to Ferry (1948) and the gelation of globular protein has been widely reviewed by Clark & Lee-Tuffnell (1986), Clark (1992), Clark *et al*. (2001), Doi (1993), and Gosal & Ross-Murphy (2000). Figure 2.2 illustrates a model representing the possible aggregation steps in a typical heat-set globular protein. Both dimmer and monomer to denatured monomer equilibrium are shown. At pH values well below the isoelectric point fibrils are formed simply from aggregated monomers (A). Under other conditions, (B), a pre-aggregate is formed, which in turn leads to a more particulate gel (Gosal & Ross-Murphy, 2000). Another general mechanism of thermal gelation of globular proteins, which involve reversible gelation on cooling after denaturation, had also been proposed by Damodaran (1988), shown in Fig. 2.3.

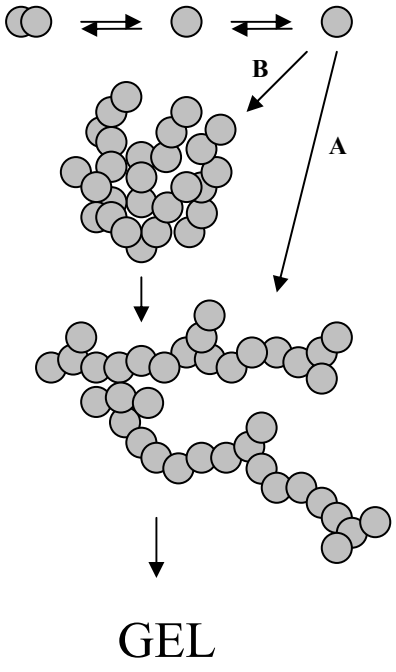

Figure 2.2 Formation of heat set gels. (adapted from: Gosal & Ross-Murphy, 2000)

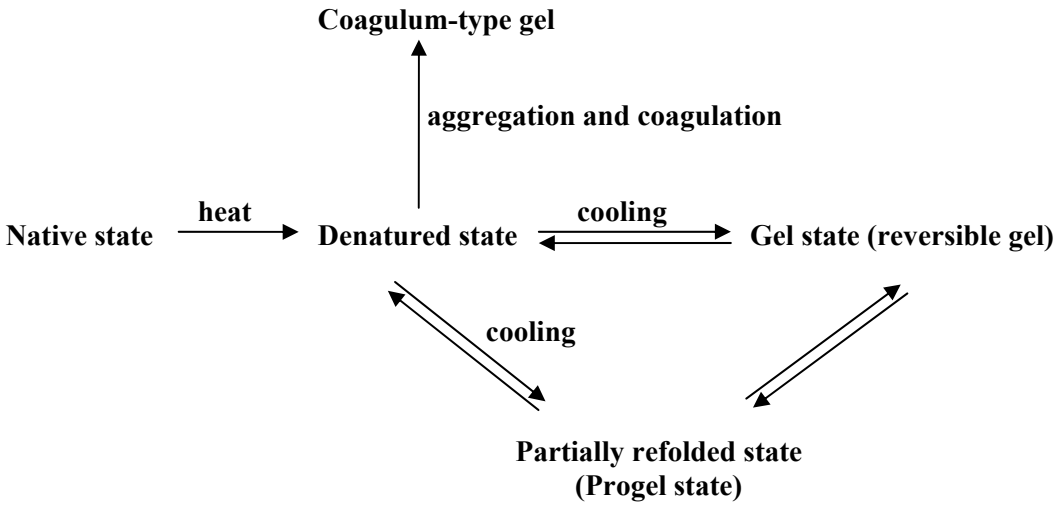

Figure 2.3 Mechanism of thermal gelation of globular proteins. (adapted from: Damodaran, 1988)

However, a more widely accepted model for gelation of soy protein (Fig. 2.4) had been suggested by Catsimpoolas & Meyer (1970). The authors have reported the two state models for soy proteins as follows: The first step is the loss of secondary and tertiary structure by heating (denaturation) which is irreversible ('progel state'). The actual formation of the gel association, which occurs on cooling of the protein suspension, depends on a controlled aggregation of the protein subunits such that solution is trapped in the three-dimensional network; this step is reversible.

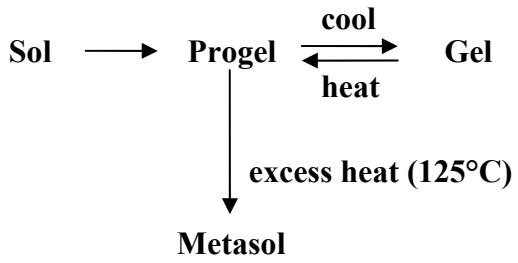

Figure 2.4 Gelation of soybean globulins. (adapted from: Catsimpoolas & Meyer, 1970)

Of all models mentioned above, the mechanisms and conformations involved could be summarized as in Table 2.8.

Table 2.8 Mechanisms and conformations involved in structure formation of protein.

Protein-solvent	Protein-protein
Mechanism(s)	Mechanism(s)
Dissociation	Association
Denaturation	Precipitation
Solubilization	Coagulation
Swelling	Flocculation
	Aggregation
Conformation(s)	Conformation(s)
Coil	Helix
	Native structure
	Three-dimensional structure

(adapted from: Hermansson, 1986a)

2.1.2.1 SPI gelation (mixed system)

Glycinin and β-conglycinin exhibited different structures and gel properties. The gelling properties of both of these globulins have been investigated individually (Utsumi *et al*., 1997). Interactions between glycinin and β-conglycinin are of particular interest in this section since SPI is a mixed system of both globulins.

Babajimopoulous *et al*., (1983) demonstrated that soy protein isolate exhibited better gelling properties at 80 °C than either of the constituent protein fractions. This reflects the interaction between the subunits of the constituent glycinin and β-conglycinin during heating. Nakamura *et al*. (1986) also found interaction of both globulins in the mixed system during heat-induced gelation at 100 °C under high ionic strength (0.5). However, the results showed that the gelation of glycinin was suppressed by β-conglycinin. For instance, although the lowest protein concentration for the formation of self-supporting gel for glycinin and β-conglycinin were 2.5 and 7.5%, respectively, for the mixed system at a 1:1 ratio of the two globulins, the lowest concentration was 7.5%. Furthermore, the gel hardness of a

mixed system was between that of glycinin and β-conglycinin at most protein concentrations. The difference between these two studies may be attributable to the different heating conditions employed and the presence or absence of reducing agents.

The interaction between glycinin and β-conglycinin was also demonstrated in commercial SPI. It was reported that the SPI which has a higher proportion of β subunit and basic polypeptides in water soluble fraction, exhibited good gel-forming ability (Arrese *et al*., 1991). This indicates the importance of the presence of a soluble form of β subunit of β-conglycinin and basic polypeptides of glycinin in SPI for gelation. These subunits may interact electrostatically and produce macroaggregates that lead to gel formation of SPI upon heating (Utsumi *et al*., 1984). Further, it was reported that the soluble macromolecule complexes formed upon heating of soy isolates were composed mostly of basic subunits of 11S associated with β subunits of 7S, mostly via electrostatic interaction. Association of basic subunits via disulfide bonds also occurred (Utsumi *et al*., 1984). It was also suggested that subunit A_3 plays an important role in increasing the hardness of the 11S globulin gels and coincide with the observation that A_4 is liberated during the formation of the soluble aggregate (transient intermediate) during heating of 11S (Nakamura *et al*., 1984)

Investigations into the gel properties of mixed system reveal information on contributions of two globulins to the physical properties of the SPI gels (Kang *et al*., 1991). In the mixed systems prepared by mixing the acid-precipitated proteins and β-conglycinin, hardness and unfracturability of the gels increase remarkably with heating temperature above 93 °C. The elasticity of the gels decreases gradually with an increase in the heating temperature (80-100 °C). The mixed system with a

glycinin: β-conglycinin ratio of 2.41 exhibits higher gel hardness at heating temperature above 93 °C than those of with a ratio of 0.88. Unfracturability of the gels is higher in the mixed system having a higher ratio than those having a lower ratio over the heating temperature range 80-100 °C whereas gel elasticity is higher in the mixed system with lower glycinin content. In the mixed system, the gel properties are thus changeable depending on the glycinin: β-conglycinin ratio and heating temperature. Although complex interactions occur in the mixed system, some insight is still obtainable with regard to the specific contribution of each globulin fraction to gel properties. Glycinin is apparently related to hardness and unfracturability of gels. β-conglycinin largely contributes to the elasticity of the gels. In addition, it has been shown that the basic polypeptides of glycinin preferentially associate with the β-subunit of β-conglycinin via electrostatic interaction, and that glycinin and β-conglycinin interact non-covalently with each other to form composite aggregates during gel formation (Damodaran & Kinsella, 1982; German *et al*., 1982; Kinsella, 1979; Nakamura *et al*., 1986). These interactions and their extents are likely to be influenced by glycinin: β-conglycinin ratio (Damodaran & Kinsella, 1982), and they may play a role in the manifestation of gel properties in the mixed system.

2.1.2.2 Forces involved in gelation

Structure formation normally involves mechanisms depending on chain-solvent as well as chain-chain interactions. It was suggested that the network structure might be formed via hydrogen bonding, hydrophobic association, ionic interactions and electrostatic cross-links, and also through some sulphydryl-

disulphide linkages of unfolded polypeptides (Catsimpoolas *et al*., 1970; Catsimpoolas & Meyer, 1970; Utsumi & Kinsella, 1985). Non-covalent bonding is more favorable in the direct cooling process, covalent bonding more favorable in the heating process.

In the mixed system, disulfide bonding and various non-covalent bonds and interactions between the subunits of glycinin and β-conglycinin are involved in determining the properties of the gel. Among these molecular forces, disulfide bonds play an important role in gelation. Evidence for this role comes from the effects of β-mercaptoethanol, which cleaves disulfide bonds, on the formation of gel network and gel hardness (Kinsella, 1979; Mori *et al*., 1982; Mori *et al*., 1986). Also, correlations have been shown between disulfide bond formation and gel firmness from direct determination of sulfhydryl and disulfide bond contents in SPI gels (Shimada & Cheftel, 1988). The free sulfhydryl groups present in the unheated SPI play an important role in the formation of a firm gel. The sulfhydryl groups are present in α and α' subunits of β-conglycinin and acidic and basic polypeptides of glycinin. These can either undergo oxidation and/or catalyze the SH–S-S interchange reaction. It is likely that the sulfhydryl group content of SPI varies widely depending on the procedures used for its preparation. Thus, variations in SH content can also cause variations in the properties of SPI gels.

A more recent study by Renkema *et al*. (2002) discussed about the heat-induced gel formation of 10% SPI suspension at pH 7 and low salt concentration. Gel stiffness, measured as the elastic modulus, *G'*, increased with proportion of denatured glycinin, which varied by changing the heating temperature (Renkema & van Vliet, 2002; Wongprecha *et al*., 2000). Pre-heating of SPI and glycinin and β-conglycinin fractions well above the denaturation temperature drastically decreased gel formation

and gel properties. (Nagano *et al*., 2000). This indicates that the aggregation stage following denaturation strongly affects the resulting *G'*.

During prolonged heating at pH 7 of SPI suspension at 90 °C a further increase in *G'* was observed, which has been explained by the occurrence of rearrangements in network structure and probably also some further incorporation of protein in network (Renkema & van Vliet, 2002). At 90 °C the gel exhibits a rather viscous characteristic at low frequencies, which is assumed to promote rearrangement.

Other researches on the rheological and mechanical properties of soy protein gels have also been studied in different conditions (Chronakis, 1996; Kang *et al*., 1991; Puppo & Añón, 1999; Renkema & van Vliet, 2004; Renkema, 2004; Renkema *et al*., 2001, 2000).

Other functional properties of SPI such as solubility, emulsifying and foaming capacity have been reviewed elsewhere (Kilara & Sharkasi, 1985; Kinsella, 1979; Utsumi *et al*., 1997; Wolf & Cowan, 1971).

2.1.3 Modification of SPI

Owing to the gelation of SPI that requires high protein concentration (Grindberg*et al*., 1992), modification of soy protein for functionality improvements have been carried out via physical means such as heat treatment (Renkema & van Vliet, 2002) and application of pressure (Molina *et al*., 2002; Torrezan *et al*., 2007) or via chemical means such as acidification (Tay *et al*., 2005), addition of salts (Puppo & Añón, 1999) and by the Maillard reaction induced cross-linkings (Cabodevila *et al*., 1994; Md Yasir *et al*., 2007b). Enzymatic modifications

(transglutaminase) based on polymerization also provide a broad potential for designing functionality for specific applications (Md Yasir *et al*., 2007a; Tang *et al*., 2006b; Tang, 2007).

In addition, to further enhance the functionalities of protein it is possible for technologists to combine two or more of the modification treatments. For example, chymotrypsin/acid pre-digestion or heat pre-treatment was performed prior to transglutaminase cross-linking or polysaccharide conjugation was carried out in order to improve gelation of protein and other functional properties (Babiker, 2000; Babiker *et al*., 1996; Walsh *et al*., 2003; Hassan *et al*., 2006; Tang, 2007).

Combination of two cross-linking treatments using transglutaminase incubation followed by heating with ribose to induce Maillard cross-linking in order to enhance physical properties of SPI gels (e.g. textural properties, colour and water holding capacity) has never been conducted. The cross-linking between amino acids of the soy protein by microbial transglutaminase (MTGase) and the Maillard reaction are of particular interest due to the differences in susceptibility of soy protein fractions in both of these treatments (Cabodevila *et al*., 1994; Walsh *et al*., 2003). The details of these modifications will be reviewed in later sections (2.2 and 2.3).

2.2 Microbial Transglutaminase (MTGase)

Transglutaminase (TGase) was first introduced by Clarke *et al*. (1959), which is widely distributed in various living organisms, fulfilling a great variety of biological functions (Griffin *et al*., 2002; Lorand & Graham, 2003), and responsible for the transamidating activity of guinea pig liver. Such enzymes, represent protein-glutamine γ-glutamyltransferase (enzyme class [EC] 2.3.2.13), have been found in animal tissues and body fluids (Folk, 1980), fish (Worratao & Yongsawatdigul, 2005), plants (Icekson & Apelbaum, 1987) and microorganisms (Yan *et al*., 2005; Zheng *et al*., 2002).

Guinea pig liver transglutaminase (GTGase) was the only TGase commercially available until the late 1980s. Owing to the extensive purification procedure, the market price is high, hence the potential for industrial applications as a texture enhancer was affected (Motoki & Kumazawa 2000; Zhu *et al*., 1995, 1999). In addition, calcium ion (Ca^{2+}) was required for its activation, which leads to protein precipitation in some food systems containing casein, soy bean globulin or myosin (Seguro *et al*., 1996b). On the other hand, Factor XIII, a TGase isolated from blood, is also rarely used in the food industry due to its detrimental red pigmentation and thrombin was required for its activation (Motoki & Kumazawa 2000; Yokoyama *et al*., 2004).

Therefore, a number of efforts were made to obtain TGase by genetic manipulation of various microorganisms such as *Escherichia coli* (Ikura *et al*., 1990; Yokoyama *et al*., 2004) but none of these enzymes have been commercialized because of the lack of public acceptability of additives used for, e.g. texture enhancement in a particular food systems (Motoki & Kumazawa, 2000; Yokoyama *et al*., 2004) until TGase from *Streptoverticillium* S-8112 was found by Ando *et al*.,